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[Continued on next page]

(54) Title: BACTERIA ENGINEERED TO TREAT NONALCOHOLIC STEATOHEPATITIS (NASH)

Fig. 1A

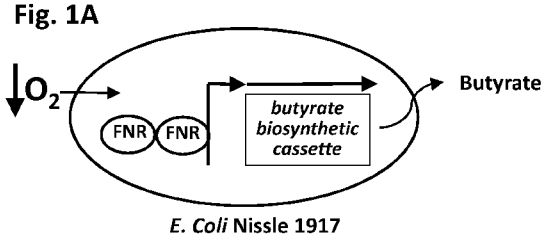


Fig. 1B

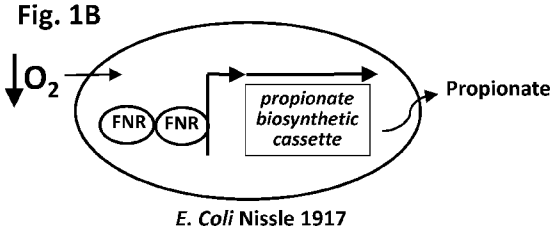
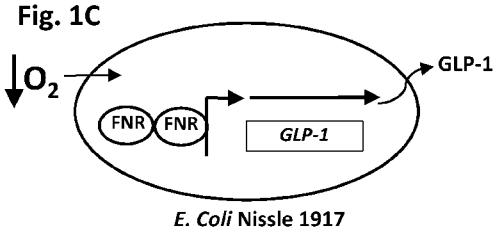


Fig. 1C



(57) Abstract: The present disclosure provides engineered bacterial cells comprising a heterologous gene encoding a propionate biosynthesis gene cassette; a butyrate biosynthesis gene cassette; GLP-1; a propionate biosynthesis gene cassette and a butyrate biosynthesis gene cassette; a propionate biosynthesis gene cassette and GLP-1; a butyrate biosynthesis gene cassette and GLP-1; or a propionate biosynthesis gene cassette, a butyrate biosynthesis gene cassette, and GLP- 1. In another aspect, the engineered bacterial cells further comprise a bacterial kill switch. The disclosure further provides pharmaceutical compositions comprising the engineered bacteria, and methods for treating liver disease, such as nonalcoholic steatohepatitis (NASH), using the pharmaceutical compositions of the disclosure.

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BACTERIA ENGINEERED TO TREAT NONALCOHOLIC STEATOHEPATITIS (NASH)

Related Applications

[01] The present application claims the benefit of priority to International Application No. PCT/US2016/020530, filed March 2, 2016; International Application No. PCT/US2016/050836, filed September 8, 2016; U.S. Patent Application No. 15/260,319, filed September 8, 2016; International Application No. PCT/US2017/016603, filed February 3, 2017; International Application No. PCT/US2016/032565, filed May 13, 2016; U.S. Provisional Patent Application No. 62/347,576, filed June 8, 2016; U.S. Provisional Patent Application No. 62/348,620, filed June 10, 2016; International Application No. PCT/US2016/039444, filed June 24, 2016; International Application No. PCT/US2016/069052, filed December 28, 2016; U.S. Provisional Patent Application No. 62/347,508, filed June 8, 2016; U.S. Provisional Patent Application No. 62/354,682, filed June 24, 2016; U.S. Provisional Patent Application No. 62/362,954, filed July 15, 2016; U.S. Provisional Patent Application No. 62/385,235, filed September 8, 2016; U.S. Provisional Patent Application No. 62/423,170, filed November 16, 2016; U.S. Provisional Patent Application No. 62/439,871, filed December 28, 2016; International Application No. PCT/US2017/016609, filed February 3, 2017; U.S. Provisional Patent Application No. 62/336,012, filed May 13, 2016; U.S. Provisional Patent Application No. 62/362,863, filed July 15, 2016; International Application No. PCT/US2017/012946, filed January 11, 2017; U.S. Provisional Patent Application No. 62/335,780, filed May 13, 2016, U.S. Provisional Patent Application No. 62/345,242, filed June 3, 2016; U.S. Provisional Patent Application No. 62/293,695, filed on February 10, 2016; U.S. Provisional Patent Application No. 62/347,554, filed on June 8, 2016; U.S. Provisional Patent Application No. 62/348,416, filed on June 10, 2016; U.S. Provisional Patent Application No. 62/354,681, filed on June 24, 2016 and International Application No. PCT/US2017/013074, filed January 11, 2017, the contents of which are hereby incorporated by reference herein in their entirety.

Background

[02] Non-alcoholic fatty liver disease (NAFLD) describes a range of conditions caused by a build-up of fat within liver cells. The first stage of NAFLD is simple fatty liver –

also called hepatic steatosis, which often does not cause severe symptoms in the liver; however hepatic steatosis in some patients can progress to more severe forms of NAFLD.

[03] Non-alcoholic steatohepatitis (NASH) is a severe form of NAFLD, where excess fat accumulation in the liver results in chronic inflammation and damage. NASH affects approximately 3-5% of the population in America, especially in those identified as obese. NASH is characterized by such abnormalities as advanced lipotoxic metabolites, pro-inflammatory substrate, fibrosis, and increased hepatic lipid deposition. If left untreated, NASH can lead to cirrhosis, liver failure, and hepatocellular carcinoma (HCC).

[04] Although patients diagnosed with alcoholic steatohepatitis demonstrate similar symptoms and liver damage, NASH develops in individuals who do not consume alcohol, and the underlying causes of NASH are unknown. Possible factors include insulin resistance, cytokine imbalance (specifically, an increase in the tumor necrosis factor-alpha (TNF-α)/adiponectin ratio), and oxidative stress resulting from mitochondrial abnormalities. Furthermore, methionine and choline deficiency have been described in *in vivo* animal models to cause increased hepatocellular injury and weight loss in addition to inflammation, oxidative stress, and fibrosis. See, for example, Caballero, *et al., J. Bio. Chem.*, 285(24):18528-18536 (2010).

[05] Currently, there is no accepted approach to treating NASH. Therapy generally involves treating known risk factors such as correction of obesity through diet and exercise, treating hyperglycemia through diet and insulin, avoiding alcohol consumption, and avoiding unnecessary medication. In animal models, administration of butyrate has been shown to reduce hepatic steatosis, inflammation, and fat deposition (see, for example, Jin *et al.,* British J. Nutrition, 114(11):1745-1755, 2015 and Endo *et al.,* PLoS One, 8(5):e63388, 2013). Colonic propionate delivery has also been shown to reduce intrahepatocellular lipid content in NASH patients, including improvements in weight gain and intra-abdominal fat deposition (see, for example, Chambers *et al.,* Gut, gutjnl-2014), and GLP-1 administration has been shown to reduce the degree of lipotoxic metabolites and pro-inflammatory substrates, both of which have been shown to speed NASH development, as well as reduce hepatic lipid deposition (see, for example, Bernsmeier *et al.,* PLoS One, 9(1):e87488, 2014 and Armstrong *et al.,* J. Hepatol., 2015). Studies have also suggested that rapid weight loss through bariatric surgery (*e.g.* gastric bypass) is effective in decreasing steatosis, hepatic inflammation, and fibrosis. Other treatments have involved using anti-diabetic medications such as metformin, rosiglitazone, and pioglitazone. Though inconclusive, the studies suggest that the medications stimulate insulin sensitivity in NASH patients, thus alleviating liver damage. In

cases were NASH has resulted in advanced cirrhosis, the only treatment is a liver transplant. Regardless, no current treatments are wholly determinative or reliable for treating NASH. Therefore, a need exists for improved therapies and treatments of NASH.

Summary

[06] The present disclosure provides engineered bacterial cells, pharmaceutical compositions thereof, and methods of modulating and treating disorders associated with nonalcoholic steatohepatitis (NASH). In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more enzymes present in a biosynthetic pathway for producing a short chain fatty acid, *e.g.*, butyrate, propionate, and/or acetate. In some embodiments, the engineered bacteria comprise one or more gene cassette(s) encoding a biosynthetic pathway for producing a short chain fatty acid, *e.g.*, butyrate, propionate, and/or acetate. Thus, in some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more GLP-1 peptides. In some embodiments, the engineered bacteria comprise genetic circuitry for reducing bile salt. For example, in some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s) and/or gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise one or more gene cassettes which modulate typtophan levels, *e.g.*, in the serum and/or in the gut. For example, in some embodiments, tryptophan catabolized into one or more of its metabolites, collectively called kynurenine and indole metabolites herein.. In some embodiments, the engineered bacteria comprise one or more gene cassettes which modulate kynurenine levels, *e.g.*, in the serum and/or in the gut. For example, Figs 2A-2G In certain embodiments, the engineered bacteria comprise one or more gene cassettes as described herein, which modulate levels of downstream indole tryptophan metabolites described herein, including, but not limited to those listed in **Table 12** and elsewhere herein, in the patient, *e.g.*, in the serum and/or in the gut.

[07] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which modulate the TRP/KYN ratio in the patient,

e.g., in the serum and/or in the gut. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of tryptophan to one or more indole tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of tryptophan to one or more kynurenine downstream metabolites described herein, *e.g.*, in **Fig. 29**. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of kynurenine to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of kynurenine to one or more downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios between two downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios between one or more tryptophan metabolites, including, but not limited to those listed in **Table 13** and elsewhere herein.

[08] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase typtophan levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase kynurenine levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase levels of downstream kynurenine metabolites described herein in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase levels of downstream tryptophan metabolites described herein,including, but not limited to those listed in **Table 12** and elsewhere herein, in the patient, *e.g.*, in the serum and/or in the gut.

[09] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase the TRP/KYN ratio in the patient, *e.g.*, in the serum and/or in the gut. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios of tryptophan to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes

which increase the ratios of tryptophan to one or more kynurenine downstream metabolites described herein, *e.g.*, in **Fig. 29**. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios of kynurenine to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios of kynurenine to one or more downstream kynurenine metabolites, including, but not limited to those listed in Table 13 and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios between two downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios between one or more tryptophan metabolites, including, but not limited to those listed in Table 13 and elsewhere herein.

[010] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease typtophan levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease kynurenine levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease levels of downstream kynurenine metabolites described herein in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease levels of downstream tryptophan metabolites described herein, including, but not limited to those listed in **Table 12,** and elsewhere herein, in the patient, *e.g.*, in the serum and/or in the gut.

[011] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease the TRP/KYN ratio in the patient, *e.g.*, in the serum and/or in the gut. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios of tryptophan to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios of tryptophan to one or more kynurenine downstream metabolites described herein, *e.g.*, in **Fig. 16**. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios of kynurenine to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which

decrease the ratios of kynurenine to one or more downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios between two downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios between one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise a gene cassette which modulates serotonin and or melatonin levels. In some embodiments, the genetically engineered bacteria comprise a gene cassette which increases serotonin and or melatonin levels. In some embodiments, the genetically engineered bacteria comprise a gene cassette which decreases serotonin and or melatonin levels. In some embodiments, the genetically engineered bacteria comprise a gene cassette which modulates the tryptophan to serotonin and or melatonin ratios. In some embodiments, the genetically engineered bacteria comprise a gene cassette which increases the tryptophan to serotonin and or melatonin ratios. In some embodiments, the genetically engineered bacteria comprise a gene cassette which decreases the tryptophan to serotonin and or melatonin ratios

[012] In some embodiments, the engineered bacteria comprise gene sequence(s) and/or gene cassette(s) selected from one or more of the following: gene cassette(s) encoding a biosynthetic pathway for producing a short chain fatty acid, *e.g.*, one or more propionate biosynthesis gene cassette(s), one or more butyrate biosynthesis gene cassette(s), one or more acetate biosynthetic cassettes; gene sequence(s) encoding one or more GLP-1 polypeptides; gene sequence(s) encoding one or more bile salt hydrolase polypeptides; gene sequence(s) encoding one or more bile salt transporters; gene sequence(s) for the production and/or catabolism of tryptophan and its metabolites; and combinations thereof. Thus, in some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.* acetate biosynthetic cassette(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for

producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthetic cassette(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), and at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.* acetate biosynthetic cassette(s).

[013] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*,

propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more GLP-1 polypeptide(s).

[014] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s).

[015] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), for producing GLP-1, and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s).

[016] In some embodiments, the bacteria comprise gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more bile salt transporter(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more bile salt transporter(s).

[017] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), and gene sequence(s) encoding GLP-1 and a transporter.

[018] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). For example, in some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s).

[019] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), gene sequence(s) encoding GLP-1, gene sequences encoding a transporter and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). .

[020] In some embodiments, the engineered bacteria comprise at least one gene sequence(s) encoding GLP-1 and one or more bile salt hydrolase polypeptide(s).

[021] In some embodiments, the engineered bacteria comprise at least one gene sequence(s) encoding GLP-1 and a transporter.

[022] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and at least one gene cassette for the production or catabolism of tryptophan and/or one of its

metabolites. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In any of these embodiments, the genetically engineered bacteria may further comprise one or more cassettes for the consumption of ammonia. Suitable gene sequences and circuits for the consumption of ammonia are described in pending International Patent Application PCT/US2015/64140 (published as WO/2016/090343) and International Patent Application PCT/US2016/34200, the contents of which is herein incorporated by reference in its entirety.

[023] In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan

metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In some embodiments, the engineered bacteria comprise at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid.

[024] In some embodiments, the engineered bacteria further compriseoptional genetic circuitry designed to ensure the safety and non-colonization of a subject that is administered the engineered bacteria, such as, for example, one or more auxotrophies, kill switches, and combinations thereof. These engineered bacteria are safe and well tolerated and augment the innate activities of the subject's microbiome to achieve a therapeutic effect.

[025] In some embodiments, a bacterial cell disclosed herein has been genetically engineered to comprise one or more synthetic circuits selected from a propionate biosynthesis gene cassette, a butyrate biosynthesis gene cassette, GLP-1 sequence, and combinations thereof, and is capable of processing propionate, butyrate, and/or GLP-1 in low-oxygen environments, *e.g.,* the gut. Thus, the genetically engineered bacterial cells and pharmaceutical compositions comprising the bacterial cells disclosed herein may be used to treat and/or prevent liver disease, such as nonalcoholic steatohepatitis (NASH). Specifically, butyrate expression functions to improve hepatic inflammation and intestinal barrier function. Propionate expression functions to reduce intrahepatocellular lipid content, and GLP-1 expression functions to decrease lipotoxicity in NASH patients.

[026] In one aspect, disclosed herein is a pharmaceutical composition comprising an engineered bacterial cell, wherein the engineered bacterial cell comprises a heterologous propionate gene cassette; a heterologous butyrate gene cassette; or a heterologous GLP-1 gene operably linked to a first inducible promoter, and a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition further comprises a heterologous gene

encoding a substance that is toxic to the bacterial cell that is operably linked to a second inducible promoter, wherein expression of the substance that is toxic to the bacterial cell is delayed in time as compared to the expression of the heterologous propionate gene cassette; the heterologous butyrate gene cassette; or the heterologous GLP-1 gene.

[027] In one aspect, disclosed herein is a pharmaceutical composition comprising an engineered bacterial cell, wherein the engineered bacterial cell comprises a heterologous propionate gene cassette; a heterologous butyrate gene cassette; or a heterologous GLP-1 gene operably linked to a first inducible promoter, a heterologous gene encoding a substance that is toxic to the bacterial cell that is operably linked to a second inducible promoter, and a pharmaceutically acceptable carrier, wherein expression of the substance that is toxic to the bacterial cell is delayed in time as compared to the expression of the heterologous propionate gene cassette; the heterologous butyrate gene cassette; or the heterologous GLP-1 gene.

[028] In one embodiment, the pharmaceutical composition comprises a heterologous propionate gene cassette and a heterologous butyrate gene cassette. In one embodiment, the pharmaceutical composition comprises a heterologous propionate gene cassette and a heterologous GLP-1 gene. In one embodiment, the pharmaceutical composition comprises a heterologous butyrate gene cassette and a heterologous GLP-1 gene. In one embodiment, the pharmaceutical composition comprises a heterologous propionate gene cassette, a heterologous butyrate gene cassette, and a heterologous GLP-1 gene.

[029] In one embodiment, the heterologous propionate gene cassette is from *Clostridium propionicum, Megasphaera elsdenii*, or *Prevotella ruminicola*. In one embodiment, the heterologous propionate gene cassette comprises *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC* genes. In one embodiment, the heterologous propionate gene cassette comprises $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, and *lpd* genes.

[030] In one embodiment, the heterologous butyrate gene cassette is from *Clostridium, Peptoclostridium, Fusobacterium, Butyrivibrio, Eubacterium,* or *Treponema*. In one embodiment, the heterologous butyrate gene cassette comprises *bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt,* and *buk* genes.

[031] In one embodiment, the heterologous GLP-1 gene is from *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus acidophilus*, *Lactobacillus reuteri, Lactobacillus brevis*, *Lactobacillus gasseri*, *Bifidobacterium longum, Bacillus subtilis, Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus, Streptomyces lividans,* or *Homo sapiens*. In one embodiment, the heterologous GLP-1

gene has a sequence with at least 90% identity to a nucleic acid sequence encoding SEQ ID NO:40. In one embodiment, the heterologous GLP-1 gene comprises a nucleic acid sequence encoding SEQ ID NO:40. In one embodiment, the heterologous GLP-1 gene consists of a nucleic acid sequence encoding SEQ ID NO:40.

[032] In one embodiment, the engineered bacterial cell is not capable of colonizing the gut of a mammal.

[033] In one embodiment, the heterologous propionate gene cassette, the heterologous butyrate gene cassette, and/or the heterologous GLP-1 gene is located on a plasmid in the bacterial cell. In one embodiment, the heterologous propionate gene cassette, the heterologous butyrate gene cassette, and/or the heterologous GLP-1 gene is located on a chromosome in the bacterial cell.

[034] In one embodiment, the first inducible promoter and the second inducible promoter are separate copies of the same inducible promoter. In one embodiment, the first inducible promoter and the second inducible promoter are different promoters.

[035] In one embodiment, the first inducible promoter is not operably linked with the heterologous propionate gene cassette, the heterologous butyrate gene cassette, or the heterologous GLP-1 gene in nature.

[036] In one embodiment, the second inducible promoter is not operably linked with the heterologous gene encoding the substance that is toxic to the bacterial cell in nature.

[037] In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each directly induced by environmental conditions. In another embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each indirectly induced by environmental conditions.

[038] In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each directly or indirectly induced by environmental conditions specific to the small intestine of a mammal. In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each directly or indirectly induced by low-oxygen or anaerobic conditions. In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each an FNR responsive promoter. In one embodiment, the FNR responsive promoter is a promoter selected from the group consisting of a promoter comprising SEQ ID NO:1, a promoter comprising SEQ ID NO:2, a promoter comprising

SEQ ID NO:3, a promoter comprising SEQ ID NO:4, a promoter comprising SEQ ID NO:5, a promoter comprising SEQ ID NO:6, a promoter comprising SEQ ID NO:7, a promoter comprising SEQ ID NO:8, a promoter comprising SEQ ID NO:9, a promoter comprising SEQ ID NO:10, a promoter comprising SEQ ID NO:11, and a promoter comprising SEQ ID NO:12.

[039] In one embodiment, the engineered bacterial cell is an engineered probiotic bacterial cell.

[040] In one embodiment, the engineered bacterial cell is a member of a genus selected from the group consisting of *Bacteroides*, *Bifidobacterium, Clostridium, Escherichia, Lactobacillus* and *Lactococcus*. In one embodiment, the engineered bacterial cell is of the genus *Escherichia*. In one embodiment, the engineered bacterial cell is of the species *Escherichia coli* strain *Nissle*.

[041] In one embodiment, the engineered bacterial cell is an auxotroph in a gene that is complemented when the engineered bacterial cell is present in a mammalian gut. In one embodiment, the mammalian gut is a human gut. In one embodiment, the engineered bacterial cell is an auxotroph in diaminopimelic acid or an enzyme in the thymine biosynthetic pathway.

[042] In one embodiment, the second inducible promoter is directly or indirectly induced by an environmental condition not naturally present in the mammalian gut.

[043] In another aspect, disclosed herein is a method for treating nonalcoholic steatohepatitis (NASH) in a subject, the method comprising administering a pharmaceutical composition comprising a programmed engineered bacterial cell to the subject, wherein the programmed engineered bacterial cell expresses: a heterologous propionate gene cassette; a heterologous butyrate gene cassette; a heterologous GLP-1 gene; a heterologous propionate gene cassette and a heterologous butyrate gene cassette; a heterologous propionate gene cassette and a heterologous GLP-1 gene; a heterologous butyrate gene cassette and a heterologous GLP-1 gene; or a heterologous propionate gene cassette, a heterologous butyrate gene cassette, and a heterologous GLP-1 gene; in response to an exogenous environmental condition in the subject, thereby treating nonalcoholic steatohepatitis (NASH) in the subject.

[044] In one embodiment, the programmed engineered bacterial cell further comprises a heterologous gene encoding a substance that is toxic to the bacterial cell that is operably linked to a second inducible promoter, wherein expression of the substance that is toxic to the bacterial cell is delayed in time as compared to the expression of the heterologous

propionate gene cassette; the heterologous butyrate gene cassette; or the heterologous GLP-1 gene.

[045] In one embodiment, the heterologous propionate gene cassette is from *Clostridium propionicum*, *Megasphaera elsdenii*, or *Prevotella ruminicola*. In one embodiment, the heterologous propionate gene cassette comprises *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC* genes. In one embodiment, the heterologous propionate gene cassette comprises hrA^{fbr}, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, and *lpd* genes.

[046] In one embodiment, the heterologous butyrate gene cassette is from *Clostridium, Peptoclostridium, Fusobacterium, Butyrivibrio, Eubacterium,* or *Treponema.* In one embodiment, the heterologous butyrate gene cassette comprises *bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt,* and *buk* genes.

[047] In one embodiment, the heterologous GLP-1 gene is at least 90% identical to a nucleic acid sequence encoding SEQ ID NO:40. In one embodiment, the heterologous GLP-1 gene comprises a nucleic acid sequence encoding SEQ ID NO:40.

[048] In one embodiment, the engineered bacterial cell is not capable of colonizing the gut of the subject. In one embodiment, the engineered bacterial cell does not colonize the gut of the subject.

[049] In one embodiment, the heterologous propionate gene cassette, the heterologous butyrate gene cassette, and/or the heterologous GLP-1 gene is located on a plasmid in the bacterial cell. In another embodiment, the heterologous propionate gene cassette, the heterologous butyrate gene cassette, and/or the heterologous GLP-1 gene is located on a chromosome in the bacterial cell.

[050] In one embodiment, the first inducible promoter and the second inducible promoter are separate copies of the same inducible promoter. In one embodiment, the first inducible promoter and the second inducible promoter are different promoters.

[051] In one embodiment, the first inducible promoter is not operably linked with the heterologous propionate gene cassette, the heterologous butyrate gene cassette, or the heterologous GLP-1 gene in nature. In one embodiment, the second inducible promoter is not operably linked with the heterologous gene encoding the substance that is toxic to the bacterial cell in nature.

[052] In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each directly induced by environmental conditions. In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter,

are each indirectly induced by environmental conditions. In one embodiment, the environmental conditions are environmental conditions specific to the small intestine of a mammal. In one embodiment, the environmental conditions specific to the small intestine of a mammal are low-oxygen or anaerobic conditions.

[053] In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each an FNR responsive promoter. In one embodiment, the FNR responsive promoter is a promoter selected from the group consisting of a promoter comprising SEQ ID NO:1, a promoter comprising SEQ ID NO:2, a promoter comprising SEQ ID NO:3, a promoter comprising SEQ ID NO:4, a promoter comprising SEQ ID NO:5, a promoter comprising SEQ ID NO:6, a promoter comprising SEQ ID NO:7, a promoter comprising SEQ ID NO:8, a promoter comprising SEQ ID NO:9, a promoter comprising SEQ ID NO:10, a promoter comprising SEQ ID NO:11, and a promoter comprising SEQ ID NO:12.

[054] In one embodiment, the engineered bacterial cell is an engineered probiotic bacterial cell.

[055] In one embodiment, the engineered bacterial cell is a member of a genus selected from the group consisting of *Bacteroides*, *Bifidobacterium, Clostridium, Escherichia, Lactobacillus* and *Lactococcus*. In one embodiment, the engineered bacterial cell is of the genus *Escherichia*. In one embodiment, the engineered bacterial cell is of the species *Escherichia coli* strain *Nissle*.

[056] In one embodiment, the engineered bacterial cell is an auxotroph in a gene that is complemented when the engineered bacterial cell is present in a mammalian gut. In one embodiment, the mammalian gut is a human gut. In one embodiment, the engineered bacterial cell is an auxotroph in diaminopimelic acid or an enzyme in the thymine biosynthetic pathway.

[057] In one embodiment, the second inducible promoter is directly or indirectly induced by an environmental condition not naturally present in the mammalian gut.

[058] In one aspect, disclosed herein is a method for treating nonalcoholic steatohepatitis (NASH) in a subject, the method comprising administering a pharmaceutical composition described herein to the subject, thereby treating nonalcoholic steatohepatitis (NASH) in the subject. In one embodiment, the pharmaceutical composition is administered orally. In one embodiment, the subject is fed a meal within one hour of administering the pharmaceutical composition. In one embodiment, the subject is fed a meal concurrently with administering the pharmaceutical composition.

[059] In another aspect, disclosed herein is an engineered bacterial cell comprising a heterologous propionate gene cassette, a heterologous butyrate gene cassette, and/or a heterologous GLP-1 gene operably linked to a first inducible promoter, wherein the first inducible promoter is a fumarate and nitrate reductase (FNR) responsive promoter or a propionate promoter.

[060] In one embodiment, the engineered bacterial cell further comprises a heterologous gene encoding a substance that is toxic to the bacterial cell that is operably linked to a second inducible promoter, wherein expression of the substance that is toxic to the bacterial cell is delayed in time as compared to the expression of the heterologous propionate gene cassette; the heterologous butyrate gene cassette; or the heterologous GLP-1 gene.

[061] In one embodiment, the heterologous propionate gene cassette is from *Clostridium propionicum*, *Megasphaera elsdenii*, or *Prevotella ruminicola*. In one embodiment, the heterologous propionate gene cassette comprises *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC* genes. In one embodiment, the heterologous propionate gene cassette comprises $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, and *lpd* genes.

[062] In one embodiment, the heterologous butyrate gene cassette comprises *bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt,* and *buk* genes.

[063] In one embodiment, the heterologous GLP-1 gene has at least 90% identity to a nucleic acid sequence encoding SEQ ID NO:40. In one embodiment, the heterologous GLP-1 gene comprises a nucleic acid sequence encoding SEQ ID NO:40.

[064] In one embodiment, the engineered bacterial cell is not capable of colonizing the gut of a mammal. In one embodiment, the mammal is a human.

[065] In one embodiment, the heterologous propionate gene cassette, the heterologous butyrate gene cassette, and/or the heterologous GLP-1 gene is located on a plasmid in the bacterial cell. In another embodiment, the heterologous propionate gene cassette, the heterologous butyrate gene cassette, and/or the heterologous GLP-1 gene is located on a chromosome in the bacterial cell.

[066] In one embodiment, the first inducible promoter and the second inducible promoter are separate copies of the same inducible promoter. In one embodiment, the first inducible promoter and the second inducible promoter are different promoters.

[067] In one embodiment, the first inducible promoter is not operably linked with the heterologous propionate gene cassette, the heterologous butyrate gene cassette, or the heterologous GLP-1 gene in nature. In one embodiment, the second inducible promoter is

not operably linked with the heterologous gene encoding the substance that is toxic to the bacterial cell in nature.

[068] In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each directly induced by environmental conditions. In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each indirectly induced by environmental conditions. In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each directly or indirectly induced by environmental conditions specific to the small intestine of a mammal. In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each directly or indirectly induced by low-oxygen or anaerobic conditions.

[069] In one embodiment, the first inducible promoter, the second inducible promoter, or the first inducible promoter and the second inducible promoter, are each an FNR responsive promoter. In one embodiment, the FNR responsive promoter is a promoter selected from the group consisting of a promoter comprising SEQ ID NO:1, a promoter comprising SEQ ID NO:2, a promoter comprising SEQ ID NO:3, a promoter comprising SEQ ID NO:4, a promoter comprising SEQ ID NO:5, a promoter comprising SEQ ID NO:6, a promoter comprising SEQ ID NO:7, a promoter comprising SEQ ID NO:8, a promoter comprising SEQ ID NO:9, a promoter comprising SEQ ID NO:10, a promoter comprising SEQ ID NO:11, and a promoter comprising SEQ ID NO:12.

[070] In one embodiment, the engineered bacterial cell is an engineered probiotic bacterial cell.

[071] In one embodiment, the engineered bacterial cell is a member of a genus selected from the group consisting of *Bacteroides*, *Bifidobacterium, Clostridium, Escherichia, Lactobacillus* and *Lactococcus*. In one embodiment, the engineered bacterial cell is of the genus *Escherichia*. In one embodiment, the engineered bacterial cell is of the species *Escherichia coli* strain *Nissle*.

[072] In one embodiment, the engineered bacterial cell is an auxotroph in a gene that is complemented when the engineered bacterial cell is present in a mammalian gut. In one embodiment, the mammalian gut is a human gut. In one embodiment, the engineered bacterial cell is an auxotroph in diaminopimelic acid or an enzyme in the thymine biosynthetic pathway.

[073] In one embodiment, the second inducible promoter is directly or indirectly induced by an environmental condition not naturally present in the mammalian gut.

[074] In one aspect, disclosed herein is a pharmaceutical composition comprising an engineered bacterial cell and a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition is formulated for oral administration.

[075] In one aspect, disclosed herein is a method for treating nonalcoholic steatohepatitis (NASH) in a subject, the method comprising administering a pharmaceutical composition described herein to the subject, thereby treating nonalcoholic steatohepatitis (NASH) in the subject. In one embodiment, the pharmaceutical composition is administered orally. In one embodiment, the subject is fed a meal within one hour of administering the pharmaceutical composition. In one embodiment, the subject is fed a meal concurrently with administering the pharmaceutical composition. In one embodiment, the subject is fed a meal before administering the pharmaceutical composition. In another embodiment, the subject is fed a meal after administering the pharmaceutical composition.

Brief Description of the Drawings

[076] **Figs. 1A, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, and 1J** depict schematics of exemplary circuits described herein, which are inducible under anaerobic or low oxygen conditions, *e.g.*, a butyrate circuit (**Fig. 1A),** a propionate circuit (**Fig. 1B**), and a GLP-1 gene that is inducible under anaerobic or low oxygen conditions (**Fig. 1C**). **Fig. 1D** depicts a schematic of a bile salt hydrolase enzyme and/or bile salt transporter construct(s) inducible under anaerobic or low oxygen conditions. The recombinant bacterial cell may further comprise an auxotrophic mutation, a secretion system, *e.g.*, leaky membrane system or type III secretion system, and/or a kill switch, as further described herein. The recombinant bacterial cell may further optionally comprise an auxotrophy. **Figs. 1E, 1F, and 1G** depict exemplary dual circuits described herein, including, for example, a propionate circuit and a butyrate circuit that are inducible under anaerobic or low oxygen conditions (**Fig. 1E**); a propionate circuit and a GLP-1 gene that are inducible under anaerobic or low oxygen conditions (**Fig. 1F**); and a butyrate circuit and GLP-1 gene that are inducible under anaerobic or low oxygen conditions (**Fig. 1G**). **Fig. 1H, 1I and 1J** depict schematics of exemplary combination circuits described herein. Circuits include, for example, a propionate circuit, a butyrate circuit, and a GLP-1 gene that are inducible under anaerobic or low oxygen conditions (**Fig. 1H**); a bile salt hydrolase and/or bile salt importer circuit, a butyrate circuit,

and a GLP-1 gene that are inducible under anaerobic or low oxygen conditions (**Fig. 1I**); a bile salt hydrolase a circuit, a butyrate circuit, and a GLP-1 gene that are inducible under anaerobic or low oxygen conditions (**Fig. 1J**). Optionally, the engineered bacterium shown in any of **Figs. 1A, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, and 1J** may also have an auxotrophy, *e.g.*, in one example, the *thyA* gene can be been mutated in the *E. coli Nissle* genome, so thymidine must be supplied in the culture medium to support growth.

[077] **Figs. 2A, 2B, 2C, 2D, 2E, 2F, and 2G** depict schematics of exemplary circuits described herein, which are inducible under anaerobic or low oxygen conditions. **Fig. 2A** depicts a schematic showing an exemplary Kynurenine Degradation Circuit. Kynurenine is imported into the cell through expression of the aroP, tnaB or mtr transporter. Kynureninase is expressed to metabolize Kynurenine to Anthranilic acid in the cell. Both the transporter and kynureninase genes are optionally expressed from an inducible promoter, *e.g.*, a FNR-inducible promoter. **Fig. 2B** depicts a schematic showing an exemplary Kynurenine Synthesis Circuit. Kynurenine and or Tryptophan is imported into the cell through expression of the aroP, tnaB or mtr transporter. Kynurenine biosynthetic cassette is expressed to produce Kynurenine. Both the transporter and Kynurenine biosynthetic cassette genes are optionally expressed from an inducible promoter, *e.g.*, a FNR-inducible promoter. **Fig. 2C** depicts a schematic showing an exemplary Kynurenine Synthesis Circuit. Kynurenine and or Tryptophan is imported into the cell through expression of the aroP, tnaB or mtr transporter. Tryptophan is synthesized and then Kynurenine is synthesized from the synthesized tryptophan or from tryptophan imported into the cell. Both the transporter and kynureninase biosynthetic genes are optionally expressed from an inducible promoter, *e.g.*, a FNR-inducible promoter. **Fig. 2D** depicts a schematic of an *E. coli* that is genetically engineered to produce kynurenine, butyrate, and tryptophan (which can be converted to kynurenine or exported), under the control of a FNR-responsive promoter and further comprising a secretion system as known in the art or described herein. Export mechanism for kynurenine and/or tryptophan is also expressed or provided. **Fig. 2E** depicts a schematic of an *E. coli* that is genetically engineered to produce kynurenine, butyrate, and tryptophan (which can be converted to kynurenine or exported), under the control of a FNR-responsive promoter and further comprising a secretion system as known in the art or described herein. A tryptophan transporter for import of tryptophan also expressed. Export mechanism for kynurenine is also expressed or provided. **Fig. 2F** depicts a schematic of an *E. coli* that is genetically engineered to produce butyrate, tryptophan metabolites, and tryptophan (which can be converted to bioactive tryptophan metabolites or exported), under the control of a FNR-responsive

promoter and further comprising a secretion system as known in the art or described herein. Export mechanism for tryptophan and/or tryptophan metabolites is also expressed or provided. **Fig. 2G** depicts a schematic of an *E. coli* that is genetically engineered to produce butyrate, and propionate, kynurenine and/or other tryptophan metabolites, and GLP-1, under the control of a FNR-responsive promoter and further comprising a secretion system, *e.g.*, for GLP-1 secretion as known in the art or described herein. Export mechanism for kynurenine/or tryptophan metabolites is also expressed or provided. Optionally, the engineered bacterium shown in any of **Figs. 2A, 2B, 2C, 2D, 2E, 2F, and 2G** may also have an auxotrophy, *e.g.*, in one example, the *thyA* gene can be been mutated in the *E. coli Nissle* genome, so thymidine must be supplied in the culture medium to support growth.

[078] **Figs. 3A, 3B, 3C, and 3D** depict the pathway and a schematic of different butyrate producing circuits. **Fig3A** depicts a metabolic pathway for butyrate production **Figs. 3B** and **3C** depict two schematics of two different butyrate producing circuits (found in SYN-UCD503 and SYN-UCD504), both under the control of a tetracycline inducible promoter. **Fig. 3D** depicts a schematic of a third butyrate gene cassette (found in SYN-UCD505) under the control of a tetracycline inducible promoter. SYN-UCD503 comprises a bdc2 butyrate cassette under control of tet promoter on a plasmid. A "bdc2 cassette" or "bdc2 butyrate cassette" refres to a butyrate producing cassette that comprises at least the following genes: bcd2, etfB3, etfA3, hbd, crt2, pbt, and buk genes. SYN-UCD504 comprises a ter butyrate cassette (ter gene replaces the bcd2, etfB3, and etfA3 genes) under control of tet promoter on a plasmid. A "ter cassette" or "ter butyrate cassette" refers to a butyrate producing cassete that comprises at least the following genes: ter, thiA1, hbd, crt2, pbt, buk. SYN-UCD505 comprises a tesB butyrate cassette (ter gene is present and tesB gene replaces the pbt gene and the buk gene) under control of tet promoter on a plasmid. A "tes or tesB cassette or "tes or tesB butyrate cassette" refers to a butyrate producing cassette that comprises at least ter, thiA1, hbd, crt2, and tesB genes. An alternative butyrate cassette of the disclosure comprises at least bcd2, etfB3, etfA3, thiA1, hbd, crt2, and tesB genes. In some embodiments, the tes or tesB cassette is under control of an inducible promoter other than tetracycline. Exemplary inducible promoters which may control the expression of the tesB cassette include oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), promoters induced by molecules or metabolites indicative of liver damage (*e.g.*, bilirubin), promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (*e.g.*, can be exogenously added) in the gut, *e.g.*, arabinose and tetracycline.

[079] **Figs. 4A, 4B, 4C, 4D, 4E, and 4F** depict schematics of the gene organization of exemplary bacteria of the disclosure. **Figs. 4A** and **4B** depict the gene organization of an exemplary engineered bacterium of the invention and its induction of butyrate production under low-oxygen conditions. **Fig. 4A** depicts relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the butyrate biosynthesis enzymes (*bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt,* and *buk*; white boxes) is expressed. **Fig. 4B** depicts increased butyrate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate. **Figs. 4C** and **4D** depict the gene organization of an exemplary recombinant bacterium of the invention and its derepression in the presence of nitric oxide (NO). In **Fig. 4C**, in the absence of NO, the NsrR transcription factor (gray circle, "NsrR") binds to and represses a corresponding regulatory region. Therefore, none of the butyrate biosynthesis enzymes (bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt, buk; white boxes) is expressed. In **Fig. 4D**, in the presence of NO, the NsrR transcription factor interacts with NO, and no longer binds to or represses the regulatory sequence. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

Figs. 4E and 4F depict the gene organization of an exemplary recombinant bacterium of the invention and its induction in the presence of H2O2. In **Fig. 4E**, in the absence of H2O2, the OxyR transcription factor (gray circle, "OxyR") binds to, but does not induce, the oxyS promoter. Therefore, none of the butyrate biosynthesis enzymes (bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt, buk; white boxes) is expressed. In **Fig. 4F**, in the presence of H2O2, the OxyR transcription factor interacts with H2O2 and is then capable of inducing the oxyS promoter. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

[080] **Figs. 5A, 5B, 5C, 5D, 5E, and 5F** depict schematics of the gene organization of exemplary bacteria of the disclosure. **Figs. 5A** and **5B** depict the gene organization of another exemplary engineered bacterium of the invention and its induction of butyrate production under low-oxygen conditions using a different butyrate circuit from that shown in **Fig. 4**. **Fig. 5A** depicts relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the butyrate

biosynthesis enzymes (*ter, thiA1, hbd, crt2, pbt,* and *buk*; white boxes) is expressed. **Fig. 5B** depicts increased butyrate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate. **Figs. 5C** and **5D** depict the gene organization of another exemplary recombinant bacterium of the invention and its derepression in the presence of NO. In **Fig. 5C**, in the absence of NO, the NsrR transcription factor (gray circle, "NsrR") binds to and represses a corresponding regulatory region. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2, pbt, buk;* white boxes) is expressed. In **Fig. 5D**, in the presence of NO, the NsrR transcription factor interacts with NO, and no longer binds to or represses the regulatory sequence. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate. **Figs. 5E** and **5F** depict the gene organization of another exemplary recombinant bacterium of the invention and its induction in the presence of H_2O_2. In **Fig. 5E**, in the absence of H_2O_2, the OxyR transcription factor (gray circle, "OxyR") binds to, but does not induce, the *oxyS* promoter. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2, pbt, buk;* white boxes) is expressed. In **Fig. 5F**, in the presence of H_2O_2, the OxyR transcription factor interacts with H_2O_2 and is then capable of inducing the *oxyS* promoter. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

[081] **Figs. 6A, 6B, 6C, 6D, 6E, and 6F** depict schematics of the gene organization of exemplary bacteria of the disclosure. **Figs. 6A** and **6B** depict the gene organization of an exemplary recombinant bacterium of the invention and its induction under low-oxygen conditions. **Fig. 6A** depicts relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2,* and *tesB*; white boxes) is expressed. **Fig. 6B** depicts increased butyrate production under low-oxygen conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate. **Figs. 6C** and **6D** depict the gene organization of another exemplary recombinant bacterium of the invention and its derepression in the presence of NO. In **Fig. 6C**, in the absence of NO, the NsrR transcription factor (gray circle, "NsrR") binds to and represses a corresponding regulatory region. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd,*

crt2, tesB; white boxes) is expressed. In **Fig. 6D**, in the presence of NO, the NsrR transcription factor interacts with NO, and no longer binds to or represses the regulatory sequence. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate. **Figs. 6E** and **6F** depict the gene organization of another exemplary recombinant bacterium of the invention and its induction in the presence of H_2O_2. In **Fig. 6E**, in the absence of H_2O_2, the OxyR transcription factor (gray circle, "OxyR") binds to, but does not induce, the *oxyS* promoter. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2, tesB;* white boxes) is expressed. In **Figs. 6F**, in the presence of H_2O_2, the OxyR transcription factor interacts with H_2O_2 and is then capable of inducing the *oxyS* promoter. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

[082] **Figs. 7A, 7B, and 7C** depict schematics of the gene organization of exemplary bacteria of the disclosure for inducible propionate production. **Fig. 7A** depicts relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*pct, lcdA, lcdB, lcdC, etfA, acrB, acrC*; white boxes) is expressed. **Fig. 7B** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the propionate biosynthesis enzymes, which leads to the production of propionate. **Fig. 7C** depicts an exemplary propionate biosynthesis gene cassette. In other embodiments, propionate production is induced by NO or H_2O_2 as depicted and described for the butyrate cassette(s) in the preceding **Fig. 4C-4F, 5C-5F, 6C-6F**.

[083] **Figs. 8A, 8B, and 8C** depict schematics of the gene organization of exemplary bacteria of the disclosure for inducible propionate production. **Fig. 8A** depicts relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*thrA, thrB, thrC, ilvA, aceE, aceF, lpd*; white boxes) is expressed. **Fig. 8B** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the propionate biosynthesis enzymes, which leads to the production of propionate. **Fig. 8C** depicts an exemplary propionate biosynthesis gene cassette. In other embodiments,

propionate production is induced by NO or H_2O_2 as depicted and described for the butyrate cassette(s) in the preceding **Fig. 4C-4F, 5C-5F, 6C-6F**.

[084] **Figs. 8D, 8E, and 8F** depict schematics of the gene organization of exemplary bacteria of the disclosure for inducible propionate production. **Fig. 8D** depicts relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*thrA, thrB, thrC, ilvA, aceE, aceF, lpd, tesB*; white boxes) is expressed. **Fig. 8E** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the propionate biosynthesis enzymes, which leads to the production of propionate. **Fig. 8F** depicts an exemplary propionate biosynthesis gene cassette. In other embodiments, propionate production is induced by NO or H_2O_2 as depicted and described for the butyrate cassette(s) in the preceding **Fig. 4C-4F, 5C-5F, 6C-6F**.

[085] **Fig. 9A, 9B, and 9C** depict schematics of the sleeping beauty pathway and the gene organization of an exemplary bacterium of the disclosure. **Fig. 9A** depicts a schematic of a genetically engineered sleeping beauty metabolic pathway from *E. coli* for propionate production. The SBM pathway is cyclical and composed of a series of biochemical conversions forming propionate as a fermentative product while regenerating the starting molecule of succinyl-CoA. **Figs. 9A** and **9B** depict schematics of the gene organization of another exemplary engineered bacterium of the invention and its induction of propionate production under low-oxygen conditions. **Fig. 9A** depicts relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*sbm, ygfD, ygfG, ygfH*; white boxes) is expressed. **Fig. 9B** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the propionate biosynthesis enzymes, which leads to the production of propionate. In other embodiments, propionate production is induced by NO or H_2O_2 as depicted and described for the butyrate cassette(s) in the preceding **Fig. 4C-4F, 5C-5F, 6C-6F**.

[086] **Fig. 10** depicts bile salt metabolism. Bile salts are synthesized from cholesterol in the liver and stored in the gallbladder. After release into the duodenum, microbial bile salt hydrolase activity in the small intestine deconjugates the glycine or taurine

molecules to produce primary bile acids (also known as unconjugated bile acids). Most bile acids are reabsorbed into the enterohepatic portal system, but some enter the large intestine where they are further metabolized by microbial 7α-dehydroxylase to produce secondary bile acids. Excess bile acids are also lost in the stool (200 mg – 600 mg per day).

[087] **Fig. 11** depicts the structure of bile salts and the location at which bile salt hydrolase enzymes deconjugate the bile salts. BSH activity has been detected in *Lactobacillus spp, Bifidobacterium spp, Enterococcus spp, Clostridium spp, and Bacteroides spp*. BSH positive bacteria are gram positive with the exception of two Bacteroides strains. BSH in has been detected in pathogenic bacteria, *e.g., Listeria monocytogenes* and *Enterococcus feacalis*. *E. coli* does not demonstrate BSH actvity nor contain bsh homolog in genome

[088] **Fig. 12** depicts the state of one non-limiting embodiment of the bile salt hydrolase enzyme construct under inducing conditions. Expression of the bile salt hydrolase enzyme and a bile salt transporter are both induced by the FNR promoter in the absence of oxygen. The *thyA* gene has been mutated in the *E. coli Nissle* genome, so thymidine must be supplied in the culture medium to support growth. The recombinant bacterial cell may further comprise an auxotrophic mutation, a type III secretion system, and/or a kill switch, as further described herein.

[089] **Fig. 13** depicts a schematic of tryptophan metabolism in humans. The abbreviations for the enzymes are as follows: 3-HAO: 3-hydroxyl-anthranilate 3,4-dioxidase; AAAD: aromatic –amino acid decarboxylase; ACMSD, alpha-amino-beta-carboxymuconate-epsilon-semialdehyde decarboxylase; HIOMT, hydroxyl-O-methyltransferase; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine amino transferases I-III; KMO: kynurenine 3-monooxygenase; KYNU, kynureninase; NAT, N-acetyltransferase; TDO, tryptophan 2,3-dioxygenase; TPH, tryptophan hydroxylase; QPRT, quinolinic acid phosphoribosyl transferase. In certain embodiments of the disclosure, the genetically engineered bacteria comprise gene cassettes comprising one or more of the tryptophan metabolism enzymes depicted in **Fig. 13**, or bacterial functional homologs thereof. In certain embodiments of the disclosure, the genetically engineered bacteria comprise gene cassettes which produce one or more of the tryptophan metabolites depicted in **Fig. 13**. In certain embodiments, the one or more cassettes are on a plasmid; in other embodiments, the cassettes are integrated into the genome. In certain embodiments the one or more cassettes are under the control of inducible promoters which are induced under low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver

damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[090] **Fig. 14** depicts a schematic of molecular mechanisms of action of indole and its metabolites on host physiology and disease. Tryptophan catabolized by bacteria to yield indole and other indole metabolites, *e.g.*, Indole-3-propionate (IPA) and Indole-3-aldehyde (I3A), in the gut lumen. IPA acts on intestinal cells via pregnane X receptors (PXR) to maintain mucosal homeostasis and barrier function. I3A acts on the aryl hydrocarbon receptor (AhR) found on intestinal immune cells and promotes IL-22 production. Activation of AhR plays a crucial role in gut immunity, such as in maintaining the epithelial barrier function and promoting immune tolerance to promote microbial commensalism while protecting against pathogenic infections. Indole has a number of roles, such as a signaling molecule to intestinal L cells to produce glucagon-like protein 1 (GLP-1) or as a ligand for AhR (Zhang et al. Genome Med. 2016; 8: 46).

[091] **Fig. 15** depicts a schematic of a bacterial tryptophan catabolism machinery, which is genetically and functionally homologous to IDO1 enzymatic activity, as described in Vujkovic-Cvijin et al., Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism; Sci Transl Med. 2013 July 10; 5(193): 193ra91, the contents of which is herein incorporated by reference in its entirety. In certain embodiments of the disclosure, the genetically engineered bacteria comprise gene cassettes comprising one or more of the bacterial tryptophan metabolism enzymes depicted in **Fig. 39**. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes which produce one or more of the metabolites depicted in **Fig. 39,** including but not limited to, kynurenine, indole-3-aldehyde, indole-3-acetic acid, and/or indole-3 acetaldehyde. In certain embodiments, the one or more cassettes are on a plasmid; in other embodiments, the cassettes are integrated into the genome. In certain embodiments the one or more cassettes are under the control of inducible promoters which are induced under low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In certain embodiments, the tryptophan metabolism enzymes, which are encoded by the genetically engineered bacteria are derived from genera within phyla, which include, but are not limited to, proteobacteria, actinobacteria, firmicutes, bacteroidetes, chloroflexi, cyanobacteria, an euryarchaeota (*e.g.*, as described in Vujkovic-Cvijin et al.).

[092] **Fig. 16** depicts a schematic of the trypophan metabolic pathway. Host and microbiota metabolites with AhR agonistic activity are in in diamond and circled, respectively (see, *e.g.*, Lamas et al., CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands; Nature Medicine 22, 598–605 (2016). In certain embodiments of the disclosure, the genetically engineered bacteria comprise gene cassettes comprising one or more of the bacterial tryptophan metabolism enzymes which catalyze the reactions shown in **Fig. 16**. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes which produce one or more of the metabolites depicted in **Fig. 16,** including but not limited to, kynurenine, indole-3-aldehyde, indole-3-acetic acid, and/or indole-3 acetaldehyde. In certain embodiments, the one or more cassettes are on a plasmid; in other embodiments, the cassettes are integrated into the genome. In certain embodiments the one or more cassettes are under the control of inducible promoters which are induced under low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[093] **Fig. 17A** and **Fig. 17B** depict diagrams of bacterial tryptophan metabolism pathways. **Fig. 17A** depicts a schematic of the bacterial tryptophan metabolism, as described, *e.g.*, in Enzymes are numbered as follows 1) Trp 2,3 dioxygenase (EC 1.13.11.11); 2) kynurenine formidase (EC 3.5.1.49); 3) kynureninase (EC 3.7.1.3); 4) tryptophanase (EC 4.1.99.1); 5) Trp aminotransferase (EC 2.6.1.27); 6) indole lactate dehydrogenase (EC1.1.1.110); 7) Trp decarboxylase (EC 4.1.1.28); 8) tryptamine oxidase (EC 1.4.3.4); 9) Trp side chain oxidase (EC 4.1.1.43); 10) indole acetaldehyde dehydrogenase (EC 1.2.1.3); 11) indole acetic acid oxidase; 13) Trp 2-monooxygenase (EC 1.13.12.3); and 14) indole acetamide hydrolase (EC 3.5.1.0). The dotted lines (———) indicate a spontaneous reaction. **Fig. 17B** Depicts a schematic of tryptophan derived pathways. Known AHR agonists are with asterisk. Abbreviations are as follows. Trp: Tryptophan; TrA: Tryptamine; IAAld: Indole-3-acetaldehyde; IAA: Indole-3-acetic acid; FICZ: 6-formylindolo(3,2-b)carbazole; IPyA: Indole-3-pyruvic acid; IAM: Indole-3-acetamine; IAOx: Indole-3-acetaldoxime; IAN: Indole-3-acetonitrile; N-formyl Kyn: N-formylkynurenine;; Kyn:Kynurenine; KynA: Kynurenic acid; I3C: Indole-3-carbinol; IAld: Indole-3-aldehyde; DIM: 3,3'-Diindolylmethane; ICZ: Indolo(3,2-b)carbazole. Enzymes are numbered as follows: 1. EC 1.13.11.11 (Tdo2, Bna2), EC 1.13.11.11 (Ido1); 2. EC 4.1.1.28 (Tdc); 3. EC 1.4.3.22, EC 1.4.3.4 (TynA); 4. EC 1.2.1.3

(lad1), EC 1.2.3.7 (Aao1); 5. EC 3.5.1.9 (Afmid Bna3); 6. EC 2.6.1.7 (Cclb1, Cclb2, Aadat, Got2); 7. EC 1.4.99.1 (TnaA); 8. EC 1.14.13.125 (CYP79B2, CYP79B3); 9. EC 1.4.3.2 (StaO), EC 2.6.1.27 (Aro9, aspC), EC 2.6.1.99 (Taa1), EC 1.4.1.19 (TrpDH); 10. EC 1.13.12.3 (laaM); 11. EC 4.1.1.74 (IpdC); 12. EC 1.14.13.168 (Yuc2); 13. EC 3.5.1.4 (IaaH); 14. EC 3.5.5.1. (Nit1); 15. EC 4.2.1.84 (Nit1); 16. EC 4.99.1.6 (CYP71A13); 17. EC 3.2.1.147 (Pen2). In certain embodiments of the disclosure, the genetically engineered bacteria comprise gene cassettes comprising one or more of the bacterial tryptophan metabolism enzymes depicted in **Fig. 17**. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes which produce one or more of the metabolites depicted in **Fig. 17**. In certain embodiments, the one or more cassettes are on a plasmid; in other embodiments, the cassettes are integrated into the genome. In certain embodiments the one or more cassettes are under the control of inducible promoters which are induced under low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[094] **Fig. 18** depicts schematic of the *E. coli* tryptophan synthesis pathway, including genes, enzymes, and reactions involved. The seven genes, or genetic segments, seven enzymes, or enzyme domains, and seven reactions, involved in tryptophan formation are shown. Only one of the reactions is reversible. The products of four other pathways contribute carbon and/or nitrogen during tryptophan formation. Two of the tryptophan pathway enzymes often function as polypeptide complexes: anthranilate synthase, consisting of the TrpG and TrpE polypeptides, and tryptophan synthase, consisting of the TrpB and TrpA polypeptides.

[095] **Fig. 19.** shows a schematic depicting an exemplary Tryptophan circuit. Tryptophan is produced from the Chorismate precursor through expression of the trpE, trpG-D, trpC-F, trpB and trpA genes. Optional knockout of the tryptophan Repressor trpR is also depicted. Optional production of the Chorismate precursor through expression of aroG/F/H and aroB, aroD, aroE, aroK and aroC genes is also shown. All of these genes are optionally expressed from an inducible promoter, *e.g.*, a FNR-inducible promoter. The bacteria may also include an auxotrophy, *e.g.*, deletion of thyA (Δ thyA; thymidine dependence). The bacteria may also include gene sequence(s) for yddG to express YddG to assist in the exportation of tryptophan. Non limiting example of a bacterial strain is listed.

[096] **Figs. 20A-20D** depicts schematics of exemplary embodiments of the disclosure, in which the genetically engineered bacteria comprise circuits for the production of tryptophan. Any of the gene(s), gene sequence(s) and/or gene circuit(s) or cassette(s) are optionally expressed from an inducible promoter. In certain embodiments the one or more cassettes are under the control of constitutive promoters. Exemplary inducible promoters which may control the expression of the gene(s), gene sequence(s) and/or gene circuit(s) or cassette(s) include oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (*e.g.*, can be exogenously added) in the gut, *e.g.*, arabinose and tetracycline. The bacteria may also include an auxotrophy, *e.g.*, deletion of thyA (Δ thyA; thymidine dependence). **FIG. 20A** shows a schematic depicting an exemplary Tryptophan circuit. Tryptophan is produced from its precursor, chorismate, through expression of the trpE, trpG-D (also referred to as trpD), trpC-F (also referred to as trpC), trpB and trpA genes. Optional knockout of the tryptophan repressor trpR is also depicted. Optional production of chorismate through expression of aroG/F/H and aroB, aroD, aroE, aroK and aroC genes is also shown. The bacteria may optionally also include gene sequence(s) for the expression of YddG, which functions as a tryptophan exporter. The bacteria may optionally also comprise one or more gene sequence(s) depicted or described in **FIG. 20B**, and/or **FIG. 20C**, and/or **FIG. 20D. FIG. 20B** depicts a tryptophan producing strain, in which tryptophan is produced from the chorismate precursor through expression of the trpE, trpG-D, trpC-F, trpB and trpA genes. AroG and TrpE are replaced with feedback resistant versions to improve tryptophan production. Optionally, bacteria may comprise any of the transporters and/or additional tryptophan circuits depicted in **FIG. 20A** and/or described in the description of **FIG. 20A**. The bacteria may optionally also comprise one or more gene sequence(s) depicted or described in **FIG. 20C**, and/or **FIG. 20D.** Optionally, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced. **FIG. 20C** depicts a tryptophan producing strain, in which tryptophan is produced from the chorismate precursor through expression of the trpE, trpG-D, trpC-F, trpB and trpA genes. AroG and TrpE are replaced with feedback resistant versions to improve tryptophan production. The strain further comprises either a wild type or a feedback resistant SerA gene. *Escherichia coli* serA-encoded 3-phosphoglycerate (3PG) dehydrogenase catalyzes the first step of the major phosphorylated pathway of L-serine (Ser) biosynthesis. This step is an oxidation of 3PG to 3-phosphohydroxypyruvate (3PHP) with the

concomitant reduction of NAD1 to NADH. *E. coli* uses one serine for each tryptophan produced. As a result, by expressing serA, tryptophan production is improved. Optionally, bacteria may comprise any of the transporters and/or additional tryptophan circuits depicted in **FIG. 20A** and/or described in the description of **FIG. 20A**. The bacteria may optionally also comprise one or more gene sequence(s) depicted or described in **FIG. 20B**, and/or **FIG. 20D.** Optionally, Trp Repressor and/or the tnaA gene are deleted to further increase levels of tryptophan produced. The bacteria may optionally also include gene sequence(s) for the expression of YddG, which functions as a tryptophan exporter. **FIG. 20D** depicts a non-limiting example of a tryptophan producing strain, in which tryptophan is produced from the chorismate precursor through expression of the trpE, trpG-D, trpC-F, trpB and trpA genes. AroG and TrpE are replaced with feedback resistant versions to improve tryptophan production. The strain further optionally comprises either a wild type or a feedback resistant SerA gene. Optionally, bacteria may comprise any of the transporters and/or additional tryptophan circuits depicted in **FIG. 20A** and/or described in the description of **FIG. 20A**. The bacteria may optionally also comprise one or more gene sequence(s) depicted or described in **FIG. 20B**, and/or **FIG. 20C.** Optionally, Trp Repressor and/or the tnaA gene are deleted to further increase levels of tryptophan produced. The bacteria may optionally also include gene sequence(s) for the expression of YddG, which functions as a tryptophan exporter. Optionally, the bacteria may also comprise a deletion in PheA, which prevents conversion of chorismate into phenylalanine and thereby promotes the production of anthranilate and tryptophan.

[097] **Figs. 21A-21H** depict schematics of non-limiting examples of embodiments of the disclosure. In all embodiments, optionally gene(s) which encode exporters may also be included. **FIG. 21A** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce tryptamine from tryptophan. In certain embodiments the one or more cassettes are under the control of inducible promoters. In certain embodiments the one or more cassettes are under the control of constitutive promoters. The bacteria may comprise any of the transporters and/or tryptophan circuits depicted and described in **FIG. 20A** and/or and/or **FIG. 20B,** and/or **FIG. 20C,** and/or **FIG. 20D** for the production of tryptophan. Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit for Tryptophan decarboxylase, *e.g.*, from *Catharanthus roseus*, which converts tryptophan to tryptamine, *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. **FIG. 21B** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce indole-3-acetaldehyde and

FICZ from tryptophan. The bacteria may comprise any of the transporters and/or tryptophan circuits depicted and described in **FIG. 20A** and/or **FIG. 20B,** and/or **FIG. 20C,** and/or **FIG. 20D** for the production of tryptophan. Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit for aro9 (L-tryptophan aminotransferase, *e.g.*, from S. cerevisae) or aspC (aspartate aminotransferase, *e.g.*, from *E. coli*, or taa1 (L-tryptophan-pyruvate aminotransferase, *e.g.*, from Arabidopsis thaliana) or staO (L-tryptophan oxidase, *e.g.*, from streptomyces sp. TP-A0274) or trpDH (Tryptophan dehydrogenase, *e.g.*, from Nostoc punctiforme NIES-2108) and ipdC (Indole-3-pyruvate decarboxylase, *e.g.*, from Enterobacter cloacae) which together produce indole-3-acetaldehyde and FICZ from tryptophan, *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. **FIG. 21C** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce indole-3-acetaldehyde and FICZ from tryptophan. The bacteria may comprise any of the transporters and/or tryptophan circuits depicted and described in **FIG. 20A** and/or and/or **FIG. 20B,** and/or **FIG. 20C,** and/or **FIG. 20D** for the production of tryptophan. Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising tdc (Tryptophan decarboxylase, *e.g.*, from Catharanthus roseus and/or Clostridium sporogenes), and tynA (Monoamine oxidase, *e.g.*, from *E. coli*), which converts tryptophan to indole-3-acetaldehyde and FICZ, *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. **FIG. 21D** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce indole-3-acetonitrile from tryptophan. The bacteria may comprise any of the transporters and/or tryptophan circuits depicted and described in **FIG. 20A** and/or and/or **FIG. 20B,** and/or **FIG. 20C,** and/or **FIG. 20D** for the production of tryptophan. Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit for cyp79B2, (tryptophan N-monooxygenase, *e.g.*, from Arabidopsis thaliana) or cyp79B3 (tryptophan N-monooxygenase, *e.g.*, from Arabidopsis thaliana), which together convert tryptophan to indole-3-acetonitrile, *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. **FIG. 21E** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce kynurenine from tryptophan. The bacteria may comprise any of the transporters and/or tryptophan circuits depicted and described in **FIG. 20A** and/or and/or **FIG. 20B,** and/or **FIG. 20C,** and/or **FIG. 20D** for the production of tryptophan. Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising IDO1(indoleamine 2,3-

dioxygenase, *e.g.*, from homo sapiens or TDO2 (tryptophan 2,3-dioxygenase, *e.g.*, from homo sapiens) or BNA2 (indoleamine 2,3-dioxygenase, *e.g.*, from S. cerevisiae) and Afmid: Kynurenine formamidase, *e.g.*, from mouse) or BNA3 (kynurenine--oxoglutarate transaminase, *e.g.*, from S. cerevisae) which together convert tryptophan to kynurenine, *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. **FIG. 21F** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce kynureninic acid from tryptophan. The bacteria may comprise any of the transporters and/or tryptophan circuits depicted and described in **FIG. 20A** and/or and/or **FIG. 20B,** and/or **FIG. 20C,** and/or **FIG. 20D** for the production of tryptophan. Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising IDO1(indoleamine 2,3-dioxygenase, *e.g.*, from homo sapiens or TDO2 (tryptophan 2,3-dioxygenase, *e.g.*, from homo sapiens) or BNA2 (indoleamine 2,3-dioxygenase, *e.g.*, from S. cerevisiae) and Afmid: Kynurenine formamidase, *e.g.*, from mouse) or BNA3 (kynurenine--oxoglutarate transaminase, *e.g.*, from S. cerevisae) and GOT2 (Aspartate aminotransferase, mitochondrial, *e.g.*,from homo sapiens or AADAT (Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial, *e.g.*, from homo sapiens), or CCLB1 (Kynurenine--oxoglutarate transaminase 1, *e.g.*, from homo sapiens) or CCLB2 (kynurenine--oxoglutarate transaminase 3, *e.g.*, from homo sapiens, which together produce kynureninic acid from tryptophan, under the control of an inducible promoter, *e.g.*, an FNR promoter. **FIG. 21G** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce indole from tryptophan. The bacteria may comprise any of the transporters and/or tryptophan circuits depicted and described in **FIG. 20A** and/or and/or **FIG. 20B,** and/or **FIG. 20C,** and/or **FIG. 20D** for the production of tryptophan. Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit for tnaA (tryptophanase, *e.g.*, from *E. coli*), which converts tryptophan to indole, *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. **FIG. 21H** depicts one embodiment of the disclosure, in which the genetically engineered bacteria produce indole-3-carbinol, indole-3-aldehyde, 3,3' diindolylmethane (DIM), indolo(3,2-b) carbazole (ICZ) from indole glucosinolate taken up through the diet. The genetically engineered bacteria comprise a circuit comprising pne2 (myrosinase, *e.g.*, from Arabidopsis thaliana) under the control of an inducible promoter, *e.g.* an FNR promoter. The engineered bacterium shown in any of **FIG. 21A, FIG. 21B, FIG. 21D, FIG. 21D, FIG. 21E, FIG. 21F, FIG. 21G and FIG. 21H** may also have an auxotrophy, *e.g.*, in one example,

the *thyA* gene can be been mutated in the *E. coli Nissle* genome, so thymidine must be supplied in the culture medium to support growth.

[098] **Figs. 22A-22F** depict schematics of exemplary embodiments of the disclosure, in which the genetically engineered bacteria convert tryptophan into indole-3-acetic acid. In certain embodiments, the one or more cassettes are under the control of inducible promoters. In certain embodiments, the one or more cassettes are under the control of constitutive promoters. In **FIG. 22A**, the optional circuits for tryptophan production are as depicted and described in **FIG. 20A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D.** Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising aro9 (L-tryptophan aminotransferase, *e.g.*, from S. cerevisae) or aspC (aspartate aminotransferase, *e.g.*, from *E. coli*, or taa1 (L-tryptophan-pyruvate aminotransferase, *e.g.*, from Arabidopsis thaliana) or staO (L-tryptophan oxidase, *e.g.*, from streptomyces sp. TP-A0274) or trpDH (Tryptophan dehydrogenase, *e.g.*, from Nostoc punctiforme NIES-2108) and ipdC (Indole-3-pyruvate decarboxylase, *e.g.*, from Enterobacter cloacae) and iad1 (Indole-3-acetaldehyde dehydrogenase, *e.g.*, from Ustilago maydis) or AAO1 (Indole-3-acetaldehyde oxidase, *e.g.*, from Arabidopsis thaliana) which together produce indole-3-acetic acid from tryptophan, *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. In **FIG. 22B** the optional circuits for tryptophan production are as depicted and described in **FIG. 20A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D.** Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising tdc (Tryptophan decarboxylase, *e.g.*,from Catharanthus roseus and/or Clostridium sporogenes) ot tynA (Monoamine oxidase, *e.g.*, from *E. coli*) and or iad1 (Indole-3-acetaldehyde dehydrogenase, *e.g.*, from Ustilago maydis) or AAO1 (Indole-3-acetaldehyde oxidase, *e.g.*, from Arabidopsis thaliana), *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. In **FIG. 22C** the optional circuits for tryptophan production are as depicted and described in **FIG. 20A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D.** Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising aro9 (L-tryptophan aminotransferase, *e.g.*, from S. cerevisae) or aspC (aspartate aminotransferase, *e.g.*, from *E. coli*, or taa1 (L-tryptophan-pyruvate aminotransferase, *e.g.*, from Arabidopsis thaliana) or staO (L-tryptophan oxidase, *e.g.*, from streptomyces sp. TP-A0274) or trpDH

(Tryptophan dehydrogenase, *e.g.*, from Nostoc punctiforme NIES-2108) and yuc2 (indole-3-pyruvate monoxygenase, *e.g.*, from Arabidopsis thaliana) *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. In **FIG. 22D** the optional circuits for tryptophan production are as depicted and described in **FIG. 20A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D.** Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising IaaM (Tryptophan 2-monooxygenase *e.g.*, from Pseudomonas savastanoi) and iaaH (Indoleacetamide hydrolase, *e.g.*, from Pseudomonas savastanoi), *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. In **FIG. 22E** the optional circuits for tryptophan production are as depicted and described in **FIG. 20A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D.** Alternatively, optionally, tryptophan can be imported through a transporter. In addition, the genetically engineered bacteria comprise a circuit comprising cyp79B2 (tryptophan N-monooxygenase, *e.g.*, from Arabidopsis thaliana) or cyp79B3 (tryptophan N-monooxygenase, *e.g.*, from Arabidopsis thaliana and cyp71a13 (indoleacetaldoxime dehydratase, *e.g.*, from Arabidopis thaliana) and nit1 (Nitrilase, *e.g.*, from Arabidopsis thaliana) and iaaH (Indoleacetamide hydrolase, *e.g.*, from Pseudomonas savastanoi), *e.g.*, under the control of an inducible promoter *e.g.*, an FNR promoter. the engineered bacterium shown in any of **FIG. 22A, FIG. 22B, FIG. 22C, FIG. 22D, and FIG. 22E** may also have an auxotrophy, *e.g.*, in one example, the *thyA* gene can be been mutated in the *E. coli Nissle* genome, so thymidine must be supplied in the culture medium to support growth. In **FIG. 22F** the optional circuits for tryptophan production are as depicted and described in **FIG. 20A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D.** Alternatively, optionally, tryptophan can be imported through a transporter. Additionally, the strain comprises trpDH (Tryptophan dehydrogenase, *e.g.*, from Nostoc punctiforme NIES-2108) and ipdC (Indole-3-pyruvate decarboxylase, *e.g.*, from Enterobacter cloacae) which together produce indole-3-acetaldehyde and FICZ though an (indol-3yl)pyruvate intermediate, and iad1 (Indole-3-acetaldehyde dehydrogenase, *e.g.*, from Ustilago maydis), which converts indole-3-acetaldehyde into indole-3-acetate.

[099] **Fig. 23A, Fig. 23B, and Fig. 23C** depict schematics of exemplary embodiments of the disclosure, in which the genetically engineered bacteria comprise circuits for the production of tryptophan, tryptamine, indole acetic acid, and indole propionic acid. Any of the gene(s), gene sequence(s) and/or gene circuit(s) or cassette(s) are optionally

expressed from an inducible promoter. In certain embodiments, the one or more cassettes are under the control of constitutive promoters. Exemplary inducible promoters which may control the expression of the gene(s), gene sequence(s) and/or gene circuit(s) or cassette(s) include oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (*e.g.*, can be exogenously added) in the gut, *e.g.*, arabinose and tetracycline. The bacteria may also include an auxotrophy, *e.g.*, deletion of thyA (Δ thyA; thymidine dependence). **FIG. 23A** a depicts non-limiting example of a tryptamine producing strain. Tryptophan is optionally produced from chorismate precursor, and the strain optionally comprises circuits as depicted and/or described in **FIG. 20A** and/or **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D**. Additionally, the strain comprises tdc (tryptophan decarboxylase, *e.g.*, from *Catharanthus roseus* and/or Clostridium sporogenes), which converts tryptophan into tryptamine. **FIG. 23B** depicts a non-limiting example of an indole-3-acetate producing strain. Tryptophan is optionally produced from chorismate precursor, and the strain optionally comprises circuits as depicted and/or described in **FIG. 20A** and/or **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D**. Additionally, the strain comprises trpDH (Tryptophan dehydrogenase, *e.g.*, from *Nostoc punctiforme* NIES-2108) and ipdC (Indole-3-pyruvate decarboxylase, *e.g.*, from Enterobacter cloacae) which together produce indole-3-acetaldehyde and FICZ though an (indol-3yl)pyruvate intermediate, and iad1 (Indole-3-acetaldehyde dehydrogenase, *e.g.*, from Ustilago maydis), which converts indole-3-acetaldehyde into indole-3-acetate. **FIG. 23C** depicts a non-limiting example of an indole-3-propionate-producing strain. Tryptophan is optionally produced from chorismate precursor, and the strain optionally comprises circuits as depicted and/or described in **FIG. 20A** and/or **FIG. 20B** and/or **FIG. 20C** and/or **FIG. 20D**. Additionally, the strain comprises a circuit as described in **FIG. 28**, comprising trpDH (Tryptophan dehydrogenase, *e.g.*, from Nostoc punctiforme NIES-2108, which produces (indol-3yl)pyruvate from tryptophan), fldA (indole-3-propionyl-CoA:indole-3-lactate CoA transferase, *e.g.*, from Clostridium sporogenes, which converts converts indole-3-lactate and indol-3-propionyl-CoA to indole-3-propionic acid and indole-3-lactate-CoA), fldB and fldC (indole-3-lactate dehydratase *e.g.*, from Clostridium sporogenes, which converts indole-3-lactate-CoA to indole-3-acrylyl-CoA) fldD and/or AcuI: (indole-3-acrylyl-CoA reductase, *e.g.*, from Clostridium sporogenes and/or acrylyl-CoA reductase, *e.g.*, from Rhodobacter sphaeroides, which convert indole-3-acrylyl-CoA to indole-3-propionyl-CoA). The circuits

further comprise *fldH1 and/or fldH2 (*indole-3-lactate dehydrogenase 1 and/or 2, *e.g.*, from Clostridium sporogenes), which converts (indol-3-yl)pyruvate into indole-3-lactate).

[0100] **Fig. 24A and Fig. 24B** depict schematics showing exemplary engineering strategies which can be employed for tryptophan production. **FIG. 24A** depicts a schematic showing intermediates in tryptophan biosynthesis and the gene products catalyzing the production of these intermediates. Phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) are used to generate 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP). DHAP is catabolized to chorismate and then anthranilate, which is converted to tryptophan (Trp) by the tryptophan operon. Alternatively, chorismate can be used in the synthesis of tyrosine (Tyr) and/or phenylalanine (Phe). In the serine biosynthesis pathway, D-3-phosphoglycerate is converted to serine, which can also be a source for tryptophan biosynthesis. AroG, AroF, AroH: DAHP synthase catalyzes an aldol reaction between phosphoenolpyruvate and D-erythrose 4-phosphate to generate 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP). There are three isozymes of DAHP synthase, each specifically feedback regulated by tyrosine (AroF), phenylalanine (AroG) or tryptophan(AroH). AroB: Dehydroquinate synthase (DHQ synthase) is involved in the second step of the chorismate pathway, which leads to the biosynthesis of aromatic amino acids. DHQ synthase catalyzes the cyclization of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) to dehydroquinate (DHQ). AroD: 3-Dehydroquinate dehydratase (DHQ dehydratase) is involved in the 3rd step of the chorismate pathway, which leads to the biosynthesis of aromatic amino acids. DHQ dehydratase catalyzes the conversion of DHQ to 3-dehydroshikimate and introduces the first double bond of the aromatic ring. AroE, YdiB: *E. coli* expresses two shikimate dehydrogenase paralogs, AroE and YdiB. Shikimate dehydrogenase is involved in the 4th step of the chorismate pathway, which leads to the biosynthesis of aromatic amino acids. This enzyme converts 3-dehydroshikimate to shikimate by catalyzing the NADPH linked reduction of 3-dehydro-shikimate. AroL/AroK: Shikimate kinase is involved in the fifth step of the chorismate pathway, which leads to the biosynthesis of aromatic amino acids. Shikimate kinase catalyzes the formation of shikimate 3-phosphate from shikimate and ATP. There are two shikimate kinase enzymes, I (AroK) and II (AroL). AroA: 3-Phosphoshikimate-1-carboxyvinyltransferase (EPSP synthase) is involved in the 6th step of the chorismate pathway, which leads to the biosynthesis of aromatic amino acids. EPSP synthase catalyzes the transfer of the enolpyruvoyl moiety from phosphoenolpyruvate to the hydroxyl group of carbon 5 of shikimate 3-phosphate with the elimination of phosphate to produce 5-enolpyruvoyl shikimate 3-phosphate (EPSP). AroC: Chorismate synthase (AroC)

is involved in the 7th and last step of the chorismate pathway, which leads to the biosynthesis of aromatic amino acids. This enzyme catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate into chorismate, which is the branch point compound that serves as the starting substrate for the three terminal pathways of aromatic amino acid biosynthesis. This reaction introduces a second double bond into the aromatic ring system. TrpEDCAB (E coli trp operon): TrpE (anthranilate synthase) converts chorismate and L-glutamine into anthranilate, pyruvate and L-glutamate. Anthranilate phosphoribosyl transferase (TrpD) catalyzes the second step in the pathway of tryptophan biosynthesis. TrpD catalyzes a phosphoribosyltransferase reaction that generates N-(5'-phosphoribosyl)-anthranilate. The phosphoribosyl transferase and anthranilate synthase contributing portions of TrpD are present in different portions of the protein. Bifunctional phosphoribosylanthranilate isomerase / indole-3-glycerol phosphate synthase (TrpC) carries out the third and fourth steps in the tryptophan biosynthesis pathway. The phosphoribosylanthranilate isomerase activity of TrpC catalyzes the Amadori rearrangement of its substrate into carboxyphenylaminodeoxyribulose phosphate. The indole-glycerol phosphate synthase activity of TrpC catalyzes the ring closure of this product to yield indole-3-glycerol phosphate. The TrpA polypeptide (TSase α) functions as the α subunit of the tetrameric (α2-β2) tryptophan synthase complex. The TrpB polypeptide functions as the β subunit of the complex, which catalyzes the synthesis of L-tryptophan from indole and L-serine, also termed the β reaction. TnaA: Tryptophanase or tryptophan indole-lyase (TnaA) is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the cleavage of L-tryptophan to indole, pyruvate and NH4+. PheA: Bifunctional chorismate mutase / prephenate dehydratase (PheA) carries out the shared first step in the parallel biosynthetic pathways for the aromatic amino acids tyrosine and phenylalanine, as well as the second step in phenylalanine biosynthesis. TyrA: Bifunctional chorismate mutase / prephenate dehydrogenase (TyrA) carries out the shared first step in the parallel biosynthetic pathways for the aromatic amino acids tyrosine and phenylalanine, as well as the second step in tyrosine biosynthesis. TyrB, ilvE, AspC: Tyrosine aminotransferase (TyrB), also known as aromatic-amino acid aminotransferase, is a broad-specificity enzyme that catalyzes the final step in tyrosine, leucine, and phenylalanine biosynthesis. TyrB catalyzes the transamination of 2-ketoisocaproate, p-hydroxyphenylpyruvate, and phenylpyruvate to yield leucine, tyrosine, and phenylalanine, respectively. TyrB overlaps with the catalytic activities of branched-chain amino-acid aminotransferase (IlvE), which also produces leucine, and aspartate aminotransferase, PLP-dependent (AspC), which also produces phenylalanine. SerA: D-3-

phosphoglycerate dehydrogenase catalyzes the first committed step in the biosynthesis of L-serine. SerC: The *serC*-encoded enzyme, phosphoserine/phosphohydroxythreonine aminotransferase, functions in the biosythesis of both serine and pyridoxine, by using different substrates. Pyridoxal 5'-phosphate is a cofactor for both enzyme activities. SerB: Phosphoserine phosphatase catalyzes the last step in serine biosynthesis. Steps which are negatively regulated by the Trp Repressor (2), Tyr Repressor (1), or tyrosine (3), phenylalanine (4), or tryptophan (4) or positively regulated by trptophan (6) are indicated. **FIG. 24B** depicts a schematic showing exemplary engineering strategies which can improve tryptophan production. Each of these exemplary strategies can be used alone or two or more strategies can be combined to increase tryptophan production. Intervention points are in bold, italics and underlined. In one embodiment of the disclosure, bacteria are engineered to express a feedback resistant from of AroG (AroGfbr). In one embodiment, bacteria are engineered to express AroL. In one embodiment, bacteria are engineered to comprise one or more copies of a feedback resistant form of TrpE (TrpEfbr). In one embodiment, bacteria are engineered to comprise one or more additional copies of the Trp operon, *e.g.*, TrpE, *e.g.* TrpEfbr, and/or TrpD, and/or TrpC, and/or TrpA, and/or TrpB. In one embodiment, endogenous TnaA is knocked out through mutation(s) and/or deletion(s). In one embodiment, bacteria are engineered to comprise one or more additional copies of SerA. In one embodiment, bacteria are engineered to comprise one or more additional copies of YddG, a tryptophan exporter. In one embodiment, endogenous PheA is knocked out through mutation(s) and/or deletion(s). In one embodiment, two or more of the strategies depicted in the schematic of **FIG. 24B** are engineered into a bacterial strain. Alternatively, other gene products in this pathway may be mutated or overexpressed.

[0101] **Fig. 25A**, **Fig. 25B**, and **Fig. 25C** depict bar graphs showing tryptophan production by various engineered bacterial strains. **FIG.25A** depicts a bar graph showing tryptophan production by various tryptophan producing strains. The data show expressing a feedback resistant form of AroG ($AroG^{fbr}$) is necessary to get tryptophan production. Additionally, using a feedback resistant trpE ($trpE^{fbr}$) has a positive effect on tryptophan production. **FIG. 25B** shows tryptophan production from a strain comprising a tet-$trpE^{fbr}$DCBA, tet-$aroG^{fbr}$ construct, comparing glucose and glucuronate as carbon sources in the presence and absence of oxygen. It takes *E. coli* two molecules of phosphoenolpyruvate (PEP) to produce one molecule of tryptophan. When glucose is used as the carbon source, 50% of all available PEP is used to import glucose into the cell through the PTS system (Phosphotransferase system). Tryptophan production is improved by using a non-PTS sugar

(glucuronate) aerobically. The data also show the positive effect of deleting tnaA (only at early time point aerobically). **FIG. 25C** depicts a bar graph showing improved tryptophan production by engineered strain comprising *ΔtrpRΔtnaA, tet-trpE^{fbr}DCBA, tet-aroG^{fbr}* through the addition of serine.

[0102] **FIG. 26** depicts a bar graph showing a comparison in tryptophan production in strains SYN2126, SYN2323, SYN2339, SYN2473, and SYN2476. SYN2126 ΔtrpRΔtnaA. ΔtrpRΔtnaA, tet-aroGfbr. SYN2339 comprises ΔtrpRΔtnaA, tet-aroGfbr, tet-trpEfbrDCBA. SYN2473 comprises ΔtrpRΔtnaA, tet-aroGfbr-serA, tet-trpEfbrDCBA. SYN2476 comprises ΔtrpRΔtnaA, tet-trpEfbrDCBA. Results indicate that expressing aroG is not sufficient nor necessary under these conditions to get Trp production and that expressing serA is beneficial for tryptophan production.

[0103] **FIG. 27** depicts a schematic of an indole-3-propionic acid (IPA) synthesis circuit. IPA produced by the gut microbiota has a significant positive effect on barrier integrity. IPA does not signal through AhR, but rather through a different receptor (PXR) (Venkatesh *et al.*, *Symbiotic Bacterial Metabolites Regulate Gastrointestinal Bardrier Function via the Xenobiotic Sensor PXR and Toll-like Receptor* 4; Immunity 41, 296–310, August 21, 2014). In some embodiments, IPA can be produced in a synthetic circuit by expressing two enzymes, a tryptophan ammonia lyase and an indole-3-acrylate reductase (*e.g.*, Tryptophan ammonia lyase (WAL) (*e.g.*, from *Rubrivivax benzoatilyticus*) and indole-3-acrylate reductase (*e.g.*, from *Clostridum botulinum*). Tryptophan ammonia lyase converts tryptophan to indole-3-acrylic acid, and indole-3-acrylate reductase converts indole-3-acrylic acid into IPA. Without wishing to be bound by theory, no oxygen is needed for this reaction, allowing it to proceed under low or no oxygen conditions, *e.g.*, as those found in the mammalian gut. In some embodiments, the genetically engineered bacteria further comprise one or more circuits for the production of tryptophan, *e.g.*, as shown in **FIGS. 20 (A-D)** and **FIG. 24** and as described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced.

[0104] **Fig. 28** depicts a schematic of indole-3-propionic acid (IPA), indole acetic acid (IAA), and tryptamine synthesis(TrA) circuits. Enzymes are as follows : 1. TrpDH: tryptophan dehydrogenase, *e.g.*, from from Nostoc punctiforme NIES-2108; FldH1/FldH2: indole-3-lactate dehydrogenase, *e.g.*, from Clostridium sporogenes; FldA: indole-3-

propionyl-CoA:indole-3-lactate CoA transferase, *e.g.*, from Clostridium sporogenes; FldBC: indole-3-lactate dehydratase, *e.g.*, from Clostridium sporogenes; FldD: indole-3-acrylyl-CoA reductase, *e.g.*, from Clostridium sporogenes; AcuI: acrylyl-CoA reductase, *e.g.*, from Rhodobacter sphaeroides. lpdC: Indole-3-pyruvate decarboxylase, *e.g.*, from Enterobacter cloacae; lad1: Indole-3-acetaldehyde dehydrogenase, *e.g.*, from Ustilago maydis; Tdc: Tryptophan decarboxylase, *e.g.*, from Catharanthus roseus or from Clostridium sporogenes.

[0105] Tryptophan dehydrogenase (EC 1.4.1.19) is an enzyme that catalyzes the reversible chemical reaction converting L-tryptophan, NAD(P) and water to (indol-3-yl)pyruvate (IPyA), NH3, NAD(P)H and H+. Indole-3-lactate dehydrogenase ((EC 1.1.1.110, *e.g.*, Clostridium sporogenes or Lactobacillus casei) converts (indol-3yl)pyruvate (IpyA) and NADH and H+ to indole-3-lactate (ILA) and NAD+. Indole-3-propionyl-CoA:indole-3-lactate CoA transferase (FldA) converts indole-3-lactate (ILA) and indol-3-propionyl-CoA to indole-3-propionic acid (IPA) and indole-3-lactate-CoA. Indole-3-acrylyl-CoA reductase (FldD) and acrylyl-CoA reductase (AcuI) convert indole-3-acrylyl-CoA to indole-3-propionyl-CoA. Indole-3-lactate dehydratase (FldBC) converts indole-3-lactate-CoA to indole-3-acrylyl-CoA. Indole-3-pyruvate decarboxylase (lpdC:) converts Indole-3-pyruvic acid (IPyA) into Indole-3-acetaldehyde (IAAld) lad1: Indole-3-acetaldehyde dehydrogenase coverts Indole-3-acetaldehyde (IAAld) into Indole-3-acetic acid (IAA) Tdc: Tryptophan decarboxylase converts tryptophan (Trp) into tryptamine (TrA). In some embodiments, the genetically engineered bacteria further comprise one or more circuits for the production of tryptophan, *e.g.*, as shown in **FIGS. 20 (A-D)** and **FIG. 24** and as described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced.

[0106] **Fig. 29** depicts a bar graph showing tryptophan and indole acetic acid production for strains SYN2126, SYN2339 and SYN2342. SYN2126: comprises ΔtrpR and ΔtnaA (ΔtrpRΔtnaA). SYN2339 comprises circuitry for the production of tryptophan (ΔtrpRΔtnaA, tetR-Ptet-trpEfbrDCBA (pSC101), tetR-Ptet-aroGfbr (p15A)). SYN2342 comprises the same tryptophan production circuitry as the parental strain SYN2339, and additionally comprises ipdC-iad1 incorporated at the end of the second construct (ΔtrpRΔtnaA, tetR-Ptet-trpEfbrDCBA (pSC101), tetR-Ptet-aroGfbr-trpDH-ipdC-iad1 (p15A)). SYN2126 produced no tryptophan, SYN2339 produces increasing tryptophan over the time points measured, and SYN2342 converts all trypophan it produces into IAA.

[0107] **Fig. 30** depicts a bar graph showing tryptophan and tryptamine production for strains SYN2339, SYN2340, and SYN2794. SYN2339 is used as a control which can produce tryptophan but cannot convert it to tryptamine and comprises ΔtrpRΔtnaA, tetR-Ptet-trpEfbrDCBA (pSC101), tetR-Ptet-aroGfbr (p15A). SYN2340 comprises ΔtrpRΔtnaA, tetR-Ptet-trpEfbrDCBA (pSC101), tetR-Ptet-aroGfbr-tdcCr (p15A). SYN2794 comprises ΔtrpRΔtnaA, tetR-Ptet-trpEfbrDCBA (pSC101), tetR-Ptet-aroGfbr-tdcCs (p15A). Results indicate that TdcCs from Clostridium sporogenes is more efficient the TdcCr from Catharanthus roseus in tryptamine production and converts all the tryptophan produced into tryptamine.

[0108] **Fig. 31A** and **Fig. 31B** depict bar graphs showing butyrate production of butyrate producing strains of the disclosure. **Fig. 31A** shows butyrate production in strains pLOGIC031 and pLOGIC046 in the presence and absence of oxygen, in which there is no significant difference in butyrate production. Enhanced butyrate production was shown in Nissle in low copy plasmid expressing pLOGIC046 which contain a deletion of the final two genes (ptb-buk) and their replacement with the endogenous *E. coli* tesB gene (a thioesterase that cleaves off the butyrate portion from butyryl CoA). Overnight cultures of cells were diluted 1:100 in Lb and grown for 1.5 hours until early log phase was reached at which point anhydrous tet was added at a final concentration of 100ng/ml to induce plasmid expression. After 2 hours induction, cells were washed and resuspended in M9 minimal media containing 0.5% glucose at OD600=0.5. Samples were removed at indicated times and cells spun down. The supernatant was tested for butyrate production using LC-MS. **Fig. 31B** shows butyrate production in strains comprising a tet–butyrate cassette having ter substitution (pLOGIC046) or the tesB substitution (ptb-buk deletion), demonstrating that the tesB substituted strain has greater butyrate production.

[0109] **Fig. 32** depicts a graph of butyrate production using different butyrate-producing circuits comprising a nuoB gene deletion. Strains depicted are BW25113 comprising a bcd-butyrate cassette, with or without a nuoB deletion, and BW25113 comprising a ter-butyrate cassette, with or without a nuoB deletion. Strains with deletion are labeled with nuoB. The NuoB gene deletion results in greater levels of butyrate production as compared to a wild-type parent control in butyrate producing strains. NuoB is a main protein complex involved in the oxidation of NADH during respiratory growth. In some embodiments, preventing the coupling of NADH oxidation to electron transport increases the amount of NADH being used to support butyrate production.

[0110] **Fig. 33A** depicts a schematic of a butyrate producing circuit under the control of an FNR promoter.

[0111] **Fig. 33B** depicts a bar graph of anaerobic induction of butyrate production. FNR-responsive promoters were fused to butyrate cassettes containing either the bcd or ter circuits. Transformed cells were grown in LB to early log and placed in anaerobic chamber for 4 hours to induce expression of butyrate genes. Cells were washed and resuspended in minimal media w/ 0.5% glucose and incubated microaerobically to monitor butyrate production over time. SYN-UCD501 led to significant butyrate production under anaerobic conditions.

[0112] **Fig. 33C** depicts SYN-UCD501 in the presence and absence of glucose and oxygen *in vitro*. SYN-UCD501 comprises pSC101 PydfZ-ter butyrate plasmid; SYN-UCD500 comprises pSC101 PydfZ-bcd butyrate plasmid; SYN-UCD506 comprises pSC101 nirB-bcd butyrate plasmid.

[0113] **Fig. 33D** depicts levels of mouse lipocalin 2 and calprotectin quantified by ELISA using the fecal samples in an *in vivo* model of HE. SYN-UCD501 reduces inflammation and/or protects gut barrier function as compared to wild type Nissle control.

[0114] **Fig. 34A and Fig. 34B** depicts bar graphs showing *in vitro* arginine **(Fig. 34A)** and butyrate **(Fig. 34B)** production for (1) butyrate producing strain; (2) arginine producing strain (ammonia consuming strain), and (3) strain that produces butyrate and also consumes ammonia. SYN-UCD501 (butyrate producing strain comprising Logic156 (pSC101 PydfZ-ter butyrate plasmid; amp resistance)), and SYN-UCD305 (arginine producing/ammonia consuming strain comprising ΔArgR, PfnrS- ArgAfbr integrated into the chromosome at the malEK locus, and ΔThyA, with no antibiotic resistance), and SYN-UCD601 (butyrate producing and arginine producing/ammonia consuming strain comprising ΔArgR, PfnrS- ArgAfbr integrated into the chromosome at the malEK locus, ΔThyA, and Logic156 (pSC101 PydfZ-ter butyrate plasmid; amp resistance)). The data show that SYN-UCD601 is able to produce similar levels of arginine as SYN-UCD305 and similar levels of butyrate as SYN-UCD501 *in vitro*.

[0115] **Fig. 35** depicts butyrate production using SYN001 + tet (control wild-type Nissle comprising no plasmid), SYN067 + tet (Nissle comprising the pLOGIC031 ATC-inducible butyrate plasmid), and SYN080 + tet (Nissle comprising the pLOGIC046 ATC-inducible butyrate plasmid).

[0116] **Fig. 36** depicts butyrate production by genetically engineered Nissle comprising the pLogic031-nsrR-norB-butyrate construct (SYN-UCD507) or the pLogic046-

nsrR-norB-butyrate construct (SYN-UCD508), which produce more butyrate as compared to wild-type Nissle.

[0117] **Fig. 37** depicts a scatter graph of butyrate concentrations in the feces of mice gavaged with either H2O, 100 mM butyrate in H20, streptomycin resistant Nissle control or SYN501 comprising a PydfZ-ter ->pbt-buk butyrate plasmid. Significantly greater levels of butyrate were detected in the feces of the mice gavaged with SYN501 as compared mice gavaged with the Nissle control or those given water only. Levels are close to 2 mM and higher than the levels seen in the mice fed with H20 (+) 200 mM butyrate.

[0118] **Fig. 38A** depicts a bar graph showing butyrate concentrations produced *in vitro* by strains comprising chromsolmally integrated butyrate copies as compared to plasmid copies. Integrated butyrate strains, SYN1001 and SYN1002 (both integrated at the agaI/rsml locus) gave comparable butyrate production to the plasmid strain SYN501.

[0119] **Fig.38B** and **Fig. 38C** depict bar graphs showing the effect of the supernatants from the engineered butyrate-producing strain, SYN1001, on alkaline phosphatase activity in HT-29 cells represented in bar (**Fig. 38B**) and nonlinear fit (**Fig. 38C**) graphical formats.

[0120] **Fig. 39** depicts a bar graph comparing butyrate concentrations produced *in vitro* by the butyrate cassette plasmid strain SYN501 as compared to Clostridia butyricum MIYARISAN (a Japanese probiotic strain), Clostridium tyrobutyricum VPI 5392 (Type Strain), and Clostridium butyricum NCTC 7423 (Type Strain) under aerobic and anaerobic conditions at the indicated timepoints. The Nissle strain comprising the butyrate cassette produces butyrate levels comparable to *Clostridium spp.* in RCM media.

[0121] **Fig. 40A and 40B** depicts a propionate production strategy of the disclosure. **Fig. 40A** depicts a schematic of a construct comprising the sleeping beauty mutase operon from *E. coli* under the control of a heterologous FnrS promoter. **Fig. 40B** depicts a bar graph of propionate concentrations produced *in vitro* by the wild type E coli BW25113 strain and a BW25113 strain which comprises the endogenous SBM operon under the control of the FnrS promoter, as depicted in the schematic in **Fig. 40A**.

[0122] **Fig. 41A- 41F** depict graphs comparing mice fed a choline deficient, L-amino acid defined, high-fat diet (CDAHFD) for nine days to mice on normal chow with respect to weight and various markers of hepatic inflammation and fibrosis. **Fig. 41A** depicts a graph showing changes in body weight over the 9 day time course in CDAHFD fed mice and mice fed a normal chow. **Fig. 41B** depicts a bar graph showing serum MCP-1 levels in day nine in CDAHFD fed mice and mice fed a normal chow, as determined by ELISA. **Fig. 41C-41F** depict fold changes in expression of Col1A1 (Collagen Type I Alpha 1; **Fig. 41C**), Col3A1

(Collagen Type III Alpha 1; **Fig. 41D**), Col4A1 (Collagen Type IV Alpha 1; **Fig. 41E**), and ACTA2 (Actin, Alpha 2, Smooth Muscle, Aorta; **Fig. 41F**) as determined by qRT-PCR.

[0123] **Fig. 42** depicts a map of integration sites within the *E. coli* Nissle chromosome. These sites indicate regions where circuit components may be inserted into the chromosome without interfering with essential gene expression. Backslashes (/) are used to show that the insertion will occur between divergently or convergently expressed genes. Insertions within biosynthetic genes, such as *thyA*, can be useful for creating nutrient auxotrophies. In some embodiments, an individual circuit component is inserted into more than one of the indicated sites.

[0124] **Fig. 43** depicts three bacterial strains which constitutively express red fluorescent protein (RFP). In strains 1-3, the *rfp* gene was inserted into different sites in the bacterial chromosome, and resulted in varying degrees of brightness under fluorescent light. Unmodified *E. coli* Nissle (strain 4) is non-fluorescent.

[0125] **Fig. 44** depicts an exemplary schematic of the *E. coli* 1917 Nissle chromosome comprising multiple mechanisms of action (MoAs).

[0126] **Figs. 45A, 45B, 45C, 45D, 45E, 45F, 45G, 45H, and 45I** depict schematics of bacterial chromosomes, for example the *E. coli* Nissle 1917 Chromosome. For example, **Fig. 45A, 45B, and 45C** each depict schematics of an engineered bacterium comprising one circuit, a circuit for butyrate production (**Fig. 45A**), a circuit for propionate production (**Fig. 45B**), and a circuit for GLP-1 expression (**Fig. 45C**). **Fig. 45D, 45E, 4545F and 45G** each depict a schematic of an engineered bacterium comprising two circuits, a circuit for BSH expression and a circuit for expression of a bile salt importer (**Fig.45D**), a circuit for butryate production and a circuit for GLP-1 expression (**Fig.45E**), a circuit for butryate production and a circuit for propionate production (**Fig.45F**), a circuit for propionate production and a circuit for GLP-1 expression (**Fig.45G**). **Fig. 45H** depicts a schematic of an engineered bacterium comprising three circuits, including a circuit for butyrate production, propionate production, and GLP-1 expression. **Fig. 45I** depicts a schematic of an engineered bacterium comprising four circuits, including a circuit for butyrate production, GLP-1 expression, BSH expression, and expression of a bile salt importer.

[0127] **Fig. 46** depicts β-galactosidase levels in samples comprising bacteria harboring a low-copy plasmid expressing *lacZ* from an FNR-responsive promoter selected from the exemplary FNR promoters shown in **Tables 52-56** (Pfnr1-5). Different FNR-responsive promoters were used to create a library of anaerobic-inducible reporters with a variety of expression levels and dynamic ranges. These promoters included strong ribosome

binding sites. Bacterial cultures were grown in either aerobic ($+O_2$) or anaerobic conditions ($-O_2$). Samples were removed at 4 hrs and the promoter activity based on β-galactosidase levels was analyzed by performing standard β-galactosidase colorimetric assays.

[0128] **Fig. 47A** depicts a schematic representation of the *lacZ* gene under the control of an exemplary FNR promoter (P_{fnrS}). *LacZ* encodes the β-galactosidase enzyme and is a common reporter gene in bacteria. **Fig. 47B** depicts a bar graph of FNR promoter activity as a function of β-galactosidase activity in SYN340. SYN340, an engineered bacterial strain harboring a low-copy *fnrS-lacZ* fusion gene, was grown in the presence or absence of oxygen. Values for standard β-galactosidase colorimetric assays are expressed in Miller units (Miller, 1972). These data suggest that the *fnrS* promoter begins to drive high-level gene expression within 1 hr under anaerobic conditions. **Fig. 47C** depicts a line graph of the growth of bacterial cell cultures expressing *lacZ* over time, both in the presence and absence of oxygen.

[0129] **FIG. 48A** depicts a "Oxygen bypass switch" useful for aerobic pre-induction of a strain comprising one or proteins of interest (POI), *e.g.*, one or more metabolic and/or satiety effector(s) (POI1) and /or immune modulator and/or one or more transporter(s)/importer(s) and/or exporter(s) (POI2) under the control of a low oxygen FNR promoter *in vitro* in a culture vessel (*e.g.*, flask, fermenter or other vessel, *e.g.*, used during with cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture). In some embodiments, it is desirable to pre-load a strain with active payload(s) prior to administration. This can be done by pre-inducing the expression of these enzymes as the strains are propagated, (*e.g.*, in flasks, fermenters or other appropriate vesicles) and are prepared for *in vivo* administration. In some embodiments, strains are induced under anaerobic and/or low oxygen conditions, *e.g.* to induce FNR promoter activity and drive expression of one or more proteins of interest. In some embodiments, it is desirable to prepare, pre-load and pre-induce the strains under aerobic or microaerobic conditions with one or more proteins of interest. This allows more efficient growth and, in some cases, reduces the build-up of toxic metabolites.

[0130] FNRS24Y is a mutated form of FNR which is more resistant to inactivation by oxygen, and therefore can activate FNR promoters under aerobic conditions (see *e.g.*, Jervis AJ, The O2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe-4S] to [2Fe-2S] conversion, Proc Natl Acad Sci U S A. 2009 Mar 24;106(12):4659-64, the contents of which is herein incorporated by reference in its entirety). The O2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the

kinetics of [4Fe-4S] to [2Fe-2S] conversion, Proc Natl Acad Sci U S A. 2009 Mar 24;106(12):4659-64, the contents of which is herein incorporated by reference in its entirety). In this oxygen bypass system, FNRS24Y is induced by addition of arabinose and then drives the expression of one or more POIs by binding and activating the FNR promoter under aerobic conditions. Thus, strains can be grown, produced or manufactured efficiently under aerobic conditions, while being effectively pre-induced and pre-loaded, as the system takes advantage of the strong FNR promoter resulting in of high levels of expression of one or more POIs. This system does not interfere with or compromise *in vivo* activation, since the mutated FNRS24Y is no longer expressed in the absence of arabinose, and wild type FNR then binds to the FNR promoter and drives expression of the POIs *in vivo*.

[0131] In some embodiments, a LacI promoter and IPTG induction are used in this system (in lieu of Para and arabinose induction). In some embodiments, a rhamnose inducible promoter is used in this system. In some embodiments, a temperature sensitive promoter is used to drive expression of FNRS24Y.

[0132] **FIG 48B** depicts a strategy to allow the expression of one or more POI(s) under aerobic conditions through the arabinose inducible expression of FNRS24Y. By using a ribosome binding site optimization strategy, the levels of FnrS24Y expression can be fine-tuned, *e.g.*, under optimal inducing conditions (adequate amounts of arabinose for full induction). Fine-tuning is accomplished by selection of an appropriate RBS with the appropriate translation initiation rate. Bioinformatics tools for optimization of RBS are known in the art.

[0133] **FIG. 48C** depicts a strategy to fine-tune the expression of a Para-POI construct by using a ribosome binding site optimization strategy. Bioinformatics tools for optimization of RBS are known in the art. In one strategy, arabinose controlled POI genes can be integrated into the chromosome to provide for efficient aerobic growth and pre-induction of the strain (*e.g.*, in flasks, fermenters or other appropriate vesicles), while integrated versions of PfnrS-POI constructs are maintained to allow for strong *in vivo* induction.

[0134] **FIG. 49** depicts the gene organization of an exemplary construct, comprising a cloned protein of interest (POI) gene under the control of a Tet promoter sequence and a Tet repressor gene.

[0135] **FIG. 50** depicts the gene organization of an exemplary construct comprising LacI in reverse orientation, and a IPTG inducible promoter driving the expression of a protein of interest (POI, *e.g.*, one or more metabolic effector(s) described herein). In some

embodiments, this construct is useful for pre-induction and pre-loading of a therapeutic strain prior to *in vivo* administration under aerobic conditions and in the presence of inducer, *e.g.*, IPTG. In some embodiments, this construct is used alone. In some embodiments, the construct is used in combination with other constitutive or inducible POI constructs, *e.g.*, low oxygen, arabinose or IPTG inducible constructs. In some embodiments, the construct is used in combination with a low-oxygen inducible construct which is active in an *in vivo* setting.

[0136] **FIG. 51** depicts a construct comprising FNRS24Y driven by the arabinose inducible promoter and araC in reverse direction.

[0137] **FIG. 52A, FIG. 52B, and FIG. 52C** depict schematics of non-limiting examples of constructs expressing a protein of interest (POI). **FIG. 52A** depicts a schematic of a non-limiting example of the organization of a construct for POI expression under the control a lambda CI inducible promoter. The construct also provides the coding sequence of a mutant of CI, CI857, which is a temperature sensitive mutant of CI. The temperature sensitive CI repressor mutant, CI857, binds tightly at 30 degrees C but is unable to bind (repress) at temperatures of 37 C and above. In some embodiments, the construct comprises SEQ ID NO: 101. In some embodiments, this construct is used alone. In some embodiments, the temperature sensitive construct is used in combination with other constitutive or inducible POI constructs, *e.g.*, low oxygen, arabinose, rhamnose, or IPTG inducible constructs. In some embodiments, the construct allows pre-induction and pre-loading of one or more POIs prior to *in vivo* administration. In some embodiments, the construct provides *in vivo* activity. In some embodiments, the construct is located on a plasmid, *e.g.*, a low copy or a high copy plasmid. In some embodiments, the construct is located on a plasmid component of a biosafety system. In some embodiments, the construct is integrated into the bacterial chromosome at one or more locations. In some embodiments, the construct is used in combination with other POI constructs, which can either be provided on a plasmid or is integrated into the bacterial chromosome at one or more locations. In some embodiments, a temperature sensitive system can be used to set up a conditional auxotrophy. In a a strain comprising deltaThyA or deltaDapA, a dapA or thyA gene can be introduced into the strain under the control of a thermoregulated promoter system. The strain can grow in the absence of Thy and Dap only at the permissive temperature, *e.g.*, 37 C (and not lower).

[0138] **FIG. 52B** depicts a schematic of a non-limiting example of the organization of a construct for POI expression under the control of a rhamnose inducible promoter. For the application of the rhamnose expression system it is not necessary to express the regulatory proteins in larger quantities, because the amounts expressed from the chromosome are

sufficient to activate transcription even on multi-copy plasmids. Therefore, only the rhaP BAD promoter is cloned upstream of the gene that is to be expressed. In some embodiments, this construct is used alone. In some embodiments, the rhamnose inducible construct is used in combination with other constitutive or inducible POI constructs, *e.g.*, low oxygen, arabinose, temperature sensitive, or IPTG inducible constructs. In some embodiments, the construct allows pre-induction and pre-loading of one or more POIs prior to *in vivo* administration. In a non-limiting example, the construct is useful for pre-induction and is combined with low-oxygen inducible constructs. In some embodiments, the construct is located on a plasmid, *e.g.*, a low copy or a high copy plasmid. In some embodiments, the construct is located on a plasmid component of a biosafety system. In some embodiments, the construct is integrated into the bacterial chromosome at one or more locations.

[0139] **FIG. 52C** depicts a schematic of a non-limiting example of the organization of a construct for POI expression under the control of an arabinose inducible promoter. The arabinose inducible POI construct comprises AraC (in reverse orientation), a region comprising an Arabinose inducible promoter, and the POI gene. In some embodiments, this construct is used alone. In some embodiments, the rhamnose inducible construct is used in combination with other constitutive or inducible POI constructs, *e.g.*, low oxygen, arabinose, temperature sensitive, or IPTG inducible constructs. In some embodiments, the construct allows pre-induction and pre-loading of one or more POI(s) prior to *in vivo* administration. In a non-limiting example, the construct is useful for pre-induction and is combined with low-oxygen inducible constructs. In some embodiments, the construct is located on a plasmid, *e.g.*, a low copy or a high copy plasmid. In some embodiments, the construct is located on a plasmid component of a biosafety system. In some embodiments, the construct is integrated into the bacterial chromosome at one or more locations.

[0140] **FIG. 53A** depicts a schematic of the gene organization of a PssB promoter. The ssB gene product protects ssDNA from degradation; SSB interacts directly with numerous enzymes of DNA metabolism and is believed to have a central role in organizing the nucleoprotein complexes and processes involved in DNA replication (and replication restart), recombination and repair. The PssB promoter was cloned in front of a LacZ reporter and beta-galactosidase activity was measured.

[0141] **FIG. 53B** depicts a line graph showing the reporter gene activity for the PssB promoter under aerobic and anaerobic conditions. Briefly, cells were grown aerobically overnight, then diluted 1:100 and split into two different tubes. One tube was placed in the anaerobic chamber, and the other was kept in aerobic conditions for the length of the

experiment. At specific times, the cells were analyzed for promoter induction. The Pssb promoter is active under aerobic conditions, and shuts off under anaerobic conditions. This promoter can be used to express a gene of interest under aerobic conditions. This promoter can also be used to tightly control the expression of a gene product such that it is only expressed under anaerobic and/or low oxygen conditions. In this case, the oxygen induced PssB promoter induces the expression of a repressor, which represses the expression of a gene of interest. Thus, the gene of interest is only expressed in the absence of the repressor, *i.e.*, under anaerobic and/or low oxygen conditions. This strategy has the advantage of an additional level of control for improved fine-tuning and tighter control. In one non-limiting example, this strategy can be used to control expression of thyA and/or dapA, *e.g.*, to make a conditional auxotroph. The chromosomal copy of dapA or ThyA is knocked out. Under anaerobic and/or low oxygen conditions, dapA or thyA -as the case may be- are expressed, and the strain can grow in the absence of dap or thymidine. Under aerobic conditions, dapA or thyA expression is shut off, and the strain cannot grow in the absence of dap or thymidine. Such a strategy can, for example be employed to allow survival of bacteria under anaerobic and/or low oxygen conditions, *e.g.*, the gut, but prevent survival under aerobic conditions (biosafety switch).

[0142] **Fig. 54A, 54B, and 54C** depict ATC (**Fig. 54A**) or nitric oxide-inducible (**Fig. 54B**) reporter constructs. These constructs, when induced by their cognate inducer, lead to expression of GFP. Nissle cells harboring plasmids with either the control, ATC-inducible P_{tet}-GFP reporter construct or the nitric oxide inducible P_{nsrR}-GFP reporter construct induced across a range of concentrations. Promoter activity is expressed as relative florescence units. **Fig. 54C** depicts a schematic of the constructs.

[0143] **Fig. 55** depicts a dot blot of bacteria harboring a plasmid expressing NsrR under control of a constitutive promoter and the reporter gene *gfp* (green fluorescent protein) under control of an NsrR-inducible promoter. DSS-treated mice serve as exemplary models for HE. As in HE subjects, the guts of mice are damaged by supplementing drinking water with 2-3% dextran sodium sulfate (DSS). Chemiluminescent is shown for NsrR-regulated promoters induced in DSS-treated mice.

[0144] **Fig. 56** depicts a schematic of another non-limiting embodiment of the disclosure, wherein the expression of a heterologous gene is activated by an exogenous environmental signal, *e.g.*, low-oxygen conditions. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to

bind to and activate the araBAD promoter, which induces expression of TetR (tet repressor) and an anti-toxin. The anti-toxin builds up in the recombinant bacterial cell, while TetR prevents expression of a toxin (which is under the control of a promoter having a TetR binding site). However, when arabinose is not present, both the anti-toxin and TetR are not expressed. Since TetR is not present to repress expression of the toxin, the toxin is expressed and kills the cell. **Fig. 56** also depicts another non-limiting embodiment of the disclosure, wherein the expression of an essential gene not found in the recombinant bacteria is activated by an exogenous environmental signal. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription of the essential gene under the control of the araBAD promoter and the bacterial cell cannot survive. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the araBAD promoter, which induces expression of the essential gene and maintains viability of the bacterial cell.

[0145] **Fig. 57A, 57B, and 57C** depict schematics of non-limiting examples of the disclosure. **Fig. 57A** depicts a schematic of a non-limiting embodiment of the disclosure, where an anti-toxin is expressed from a constitutive promoter, and expression of a heterologous gene is activated by an exogenous environmental signal. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the araBAD promoter, which induces expression of TetR, thus preventing expression of a toxin. However, when arabinose is not present, TetR is not expressed, and the toxin is expressed, eventually overcoming the anti-toxin and killing the cell. The constitutive promoter regulating expression of the anti-toxin should be a weaker promoter than the promoter driving expression of the toxin. The araC gene is under the control of a constitutive promoter in this circuit. **Fig. 57B** depicts a schematic of a repression-based kill switch in which the AraC transcription factor is activated in the presence of arabinose and induces expression of TetR and an anti-toxin. TetR prevents the expression of the toxin. When arabinose is removed, TetR and the anti-toxin do not get made and the toxin is produced which kills the cell. **Fig. 57C** depicts another non-limiting embodiment of the disclosure, wherein the expression of a heterologous gene is activated by an exogenous environmental signal. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the araBAD promoter, which induces expression of TetR (tet repressor) and an anti-toxin.

The anti-toxin builds up in the recombinant bacterial cell, while TetR prevents expression of a toxin (which is under the control of a promoter having a TetR binding site). However, when arabinose is not present, both the anti-toxin and TetR are not expressed. Since TetR is not present to repress expression of the toxin, the toxin is expressed and kills the cell. The araC gene is under the control of a constitutive promoter in this circuit.

[0146] **Fig. 58** depicts a schematic of one non-limiting embodiment of the disclosure, where an exogenous environmental condition, *e.g.*, low-oxygen conditions, or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, and the natural kinetics of the recombinase create a time delay in expression of the toxin, allowing the heterologous gene to be fully expressed. Once the toxin is expressed, it kills the cell.

[0147] **Fig. 59** depicts a schematic of another non-limiting embodiment of the disclosure, where an exogenous environmental condition, *e.g.*, low-oxygen conditions, or one or more environmental signals activates expression of a heterologous gene, an anti-toxin, and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, but the presence of the accumulated anti-toxin suppresses the activity of the toxin. Once the exogenous environmental condition or cue(s) is no longer present, expression of the anti-toxin is turned off. The toxin is constitutively expressed, continues to accumulate, and kills the bacterial cell.

[0148] **Fig. 60** depicts aschematic of one non-limiting embodiment of the disclosure, in which the genetically engineered bacteria produces equal amount of a Hok toxin and a short-lived Sok anti-toxin. When the cell loses the plasmid, the anti-toxin decays, and the cell dies. In the upper panel, the cell produces equal amounts of toxin and anti-toxin and is stable. In the center panel, the cell loses the plasmid and anti-toxin begins to decay. In the lower panel, the anti-toxin decays completely, and the cell dies.

[0149] **Fig. 61** depicts a schematic of another non-limiting embodiment of the disclosure, where an exogenous environmental condition, *e.g.*, low-oxygen conditions, or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips at least one excision enzyme into an activated conformation. The at least one excision enzyme then excises one or more essential genes, leading to senescence, and eventual cell death. The natural kinetics of the recombinase and excision genes cause a time delay, the

kinetics of which can be altered and optimized depending on the number and choice of essential genes to be excised, allowing cell death to occur within a matter of hours or days. The presence of multiple nested recombinases can be used to further control the timing of cell death.

[0150] **Fig. 62** depicts a schematic of another non-limiting embodiment of the disclosure, where an exogenous environmental condition, *e.g.*, low-oxygen conditions, or one or more environmental signals activates expression of a heterologous gene, an anti-toxin, and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, but the presence of the accumulated anti-toxin suppresses the activity of the toxin. Once the exogenous environmental condition or cue(s) is no longer present, expression of the anti-toxin is turned off. The toxin is constitutively expressed, continues to accumulate, and kills the bacterial cell.

[0151] **Fig. 63** depicts a schematic of a secretion system based on the flagellar type III secretion in which an incomplete flagellum is used to secrete a therapeutic peptide of interest (star) by recombinantly fusing the peptide to an N-terminal flagellar secretion signal of a native flagellar component so that the intracellularly expressed chimeric peptide can be mobilized across the inner and outer membranes into the surrounding host environment.

[0152] **Fig. 64** depicts a schematic of a type V secretion system for the extracellular production of recombinant proteins in which a therapeutic peptide (star) can be fused to an N-terminal secretion signal, a linker and the beta-domain of an autotransporter. In this system, the N-terminal signal sequence directs the protein to the SecA-YEG machinery which moves the protein across the inner membrane into the periplasm, followed by subsequent cleavage of the signal sequence. The beta-domain is recruited to the Bam complex where the beta-domain is folded and inserted into the outer membrane as a beta-barrel structure. The therapeutic peptide is then thread through the hollow pore of the beta-barrel structure ahead of the linker sequence. The therapeutic peptide is freed from the linker system by an autocatalytic cleavage or by targeting of a membrane-associated peptidase (scissors) to a complementary protease cut site in the linker.

[0153] **Fig. 65** depicts a schematic of a type I secretion system, which translocates a passenger peptide directly from the cytoplasm to the extracellular space using HlyB (an ATP-binding cassette transporter); HlyD (a membrane fusion protein); and TolC (an outer membrane protein) which form a channel through both the inner and outer membranes. The

secretion signal-containing C-terminal portion of HlyA is fused to the C-terminal portion of a therapeutic peptide (star) to mediate secretion of this peptide.

[0154] **Fig. 66** depicts a schematic of the outer and inner membranes of a gram-negative bacterium, and several deletion targets for generating a leaky or destabilized outer membrane, thereby facilitating the translocation of a therapeutic polypeptides to the extracellular space, *e.g.*, therapeutic polypeptides of eukaryotic origin containing disulphide bonds. Deactivating mutations of one or more genes encoding a protein that tethers the outer membrane to the peptidoglycan skeleton, *e.g.*, lpp, ompC, ompA, ompF, tolA, tolB, pal, and/or one or more genes encoding a periplasmic protease, *e.g.*, degS, degP, nlpI, generates a leaky phenotype. Combinations of mutations may synergistically enhance the leaky phenotype.

[0155] **Fig. 67** depicts a modified type 3 secretion system (T3SS) to allow the bacteria to inject secreted therapeutic proteins into the gut lumen. An inducible promoter (small arrow, top), *e.g.* a FNR-inducible promoter, drives expression of the T3 secretion system gene cassette (3 large arrows, top) that produces the apparatus that secretes tagged peptides out of the cell. An inducible promoter (small arrow, bottom), *e.g.* a FNR-inducible promoter, drives expression of a regulatory factor, *e.g.* T7 polymerase, that then activates the expression of the tagged therapeutic peptide (hexagons).

[0156] **Fig. 68** depicts the use of GeneGuards as an engineered safety component. All engineered DNA is present on a plasmid which can be conditionally destroyed. *See, e.g.*, Wright et al., "GeneGuard: A Modular Plasmid System Designed for Biosafety," ACS Synthetic Biology (2015) 4: 307-316.

[0157] **Figs. 69A-69D** depict schematics of non-limiting examples of the gene organization of plasmids, which function as a component of a biosafety system (**Fig. 69A** and **Fig. 69B**), which also contains a chromosomal component (shown in **Fig. 69C** and **Fig. 69D**). The bosafety plasmid system vector comprises Kid Toxin and R6K minimal ori, dapA (**Fig. 69A**) and thyA (**Fig. 69B**) and promoter elements driving expression of these components. In some embodiments, bla is knocked out and replaced with one or more constructs described herein, in which a first protein of interest (POI1) and/or a second protein of interest, *e.g.*, a transporter (POI2), and/or a third protein of interest (POI3) are expressed from an inducible or constitutive promoter. **Fig. 69C** and **Fig. 69D** depict schematics of the gene organization of the chromosomal component of a biosafety system. **Fig. 69C** depicts a construct comprising low copy Rep (Pi) and Kis antitoxin, in which transcription of Pi (Rep), which is required for the replication of the plasmid component of the system, is driven by a low copy

RBS containing promoter. **Fig. 69D** depicts a construct comprising a medium-copy Rep (Pi) and Kis antitoxin, in which transcription of Pi (Rep), which is required for the replication of the plasmid component of the system, is driven by a medium copy RBS containing promoter. If the plasmid containing the functional DapA is used (as shown in **Fig. 69A**), then the chromosomal constructs shown in **Fig. 69C** and **Fig. 69D** are knocked into the DapA locus. If the plasmid containing the functional ThyA is used (as shown in **Fig. 69B**), then the chromosomal constructs shown in **Fig. 69C** and **Fig. 69D** are knocked into the ThyA locus. In this system, the bacteria comprising the chromosomal construct and a knocked out dapA or thyA gene can grow in the absence of dap or thymidine only in the presence of the plasmid.

[0158] **Fig. 70** depicts a graph of Nissle residence *in vivo*. Streptomycin-resistant Nissle was administered to mice via oral gavage without antibiotic pre-treatment. Fecal pellets from six total mice were monitored post-administration to determine the amount of administered Nissle still residing within the mouse gastrointestinal tract. The bars represent the number of bacteria administered to the mice. The line represents the number of Nissle recovered from the fecal samples each day for 10 consecutive days.

[0159] **Fig. 71** depicts a bar graph of residence over time for streptomycin resistant Nissle in various compartments of the intestinal tract at 1, 4, 8, 12, 24, and 30 hours post gavage. Mice were treated with approximately 109 CFU, and at each timepoint, animals (n=4) were euthanized, and intestine, cecum, and colon were removed. The small intestine was cut into three sections, and the large intestine and colon each into two sections. Intestinal effluents gathered and CFUs in each compartment were determined by serial dilution plating.

[0160] **Fig. 72A** depicts a graph showing bacterial cell growth of a Nissle thyA auxotroph strain (thyA knock-out) in various concentrations of thymidine. A chloramphenicol-resistant Nissle thyA auxotroph strain was grown overnight in LB + 10mM thymidine at 37C. The next day, cells were diluted 1:100 in 1 mL LB + 10mM thymidine, and incubated at 37C for 4 hours. The cells were then diluted 1:100 in 1 mL LB + varying concentrations of thymidine in triplicate in a 96-well plate. The plate is incubated at 37C with shaking, and the OD600 is measured every 5 minutes for 720 minutes. This data shows that Nissle thyA auxotroph does not grow in environments lacking thymidine.

[0161] **Fig. 72B** depicts a bar graph of Nissle residence *in vivo* of wildtype Nissle versus Nissle thyA auxotroph (thyA knock-out). Streptomycin- resistant Nissle (wildtype or thyA auxotroph) was administered to mice via oral gavage without antibiotic pre- treatment. Fecal pellets from 6 total mice were monitored post-administration to determine the amount of administered Nissle still residing within the mouse gastrointestinal tract. Each bar

represents the number of Nissle recovered from the fecal samples each day for 7 consecutive days. There were no bacteria recovered in fecal samples from mice gavaged with Nissle thyA auxotroph bacteria after day 3. This data shows that the Nissle thyA auxotroph does not persist *in vivo* in mice.

[0162] **Fig. 73** depicts the *prpR* propionate-responsive inducible promoter. The sequence for one propionate-responsive promoter is also disclosed herein as **SEQ ID NO: 584**.

[0163] **Fig. 74** depicts a schematic of a wild-type *clbA* construct and a *clbA* knock-out construct.

[0164] **Figs. 75A-75E** depict a schematic of non-limiting manufacturing processes for upstream and downstream production of the genetically engineered bacteria of the present disclosure. **Fig. 75A** depicts the parameters for starter culture 1 (SC1): loop full – glycerol stock, duration overnight, temperature 37° C, shaking at 250 rpm. **Fig. 75B** depicts the parameters for starter culture 2 (SC2): 1/100 dilution from SC1, duration 1.5 hours, temperature 37° C, shaking at 250 rpm. **Fig. 75C** depicts the parameters for the production bioreactor: inoculum – SC2, temperature 37° C, pH set point 7.00, pH dead band 0.05, dissolved oxygen set point 50%, dissolved oxygen cascade agitation/gas FLO, agitation limits 300-1200 rpm, gas FLO limits 0.5-20 standard liters per minute, duration 24 hours. **Fig. 75D** depicts the parameters for harvest: centrifugation at speed 4000 rpm and duration 30 minutes, wash 1X 10% glycerol/PBS, centrifugation, re-suspension 10% glycerol/PBS. **Fig. 75E** depicts the parameters for vial fill/storage: 1-2 mL aliquots, -80° C.

[0165] **Fig. 76** depicts a simple, robust, and rapid platform for generating and characterizing synthetic biotics, comprising steps 1 through 10. Step 1 comprises designing one orm more disease pathway(s); Step 2 comprises identifying one or more target metabolite(s); Step 3 comprises designing on e or more gene circuit(s); Step 4 comprises building the synthetic biotic; Step 5 comprises activating the one or more circuit(s) *in vitro*; Step 6 comprises characterizing circuit activation kinetics; Step 7 comprises optimizing *in vitro* productivity to the disease threshold; Step 8 comprises testing the optimized circuit(s) in animal disease model(s); Step 9 comprises assimilating into the microbiome; Step 10 comprises developing understanding of the *in vivo* PK and dosing regimen.

[0166] **FIG. 77A, FIG. 77B**, and **FIG. 77C** depict schematics of the gene organization of exemplary circuits of the disclosure for the expression of therapeutic polypeptides, *e.g.*, metabolic and/or satiety effector and/or immune modulator polypeptides described herein, which are secreted using components of the flagellar type III secretion

system. A therapeutic polypeptide of interest, is assembled behind a fliC-5'UTR, and is driven by the native fliC and/or fliD promoter (**FIG. 77A** and **FIG. 77B**) or a tet-inducible promoter (**FIG. 77C**). In alternate embodiments, an inducible promoter such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), and promoters induced by a metabolite that may or may not be naturally present (*e.g.*, can be exogenously added) in the gut, *e.g.*, arabinose can be used. In certain embodiments the one or more cassettes are under the control of constitutive promoters. The therapeutic polypeptide of interest is either expressed from a plasmid (*e.g.*, a medium copy plasmid) or integrated into fliC loci (thereby deleting all or a portion of fliC and/or fliD). Optionally, an N terminal part of FliC is included in the construct, as shown in **FIG. 77B** and **FIG. 77C**.

[0167] **FIG. 78A and FIG. 78B** depict schematics of the gene organization of exemplary circuits of the disclosure for the expression of therapeutic polypeptides, *e.g.*, metabolic and/or satiety effector and/or immune modulator polypeptides described herein, which are secreted via a diffusible outer membrane (DOM) system. The therapeutic polypeptide of interest is fused to a prototypical N-terminal Sec-dependent secretion signal or Tat-dependent secretion signal, which is is cleaved upon secretion into the periplasmic space. Exemplary secretion tags include sec-dependent PhoA, OmpF, OmpA, cvaC, and Tat-dependent tags (TorA, FdnG, DmsA). In certain embodiments, the genetically engineered bacteria comprise deletions in one or more of lpp, pal, tolA, and/or nlpI. Optionally, periplasmic proteases are also deleted, including, but not limited to, degP and ompT, *e.g.*, to increase stability of the polypeptide in the periplasm. A FRT-KanR-FRT cassette is used for downstream integration. Expression is driven by a tet promoter (**FIG. 78A**) or an inducible promoter, such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter, **FIG. 78B**), and promoters induced by a metabolite that may or may not be naturally present (*e.g.*, can be exogenously added) in the gut, *e.g.*, arabinose. In certain embodiments the one or more cassettes are under the control of constitutive promoters.

[0168] **Fig. 79** depicts a schematic of a polypeptide of interest displayed on the surface of the bacterium. A non-limiting example of such a therapeutic protein is a scFv. The polypeptide is expressed as a fusion protein, which comprises a outer membrane anchor from another protein, which was developed as part of a display system. Non-limiting examples of such anchors are described herein and include LppOmpA, NGIgAsig-NGIgAP, InaQ, Intimin, Invasin, pelB-PAL, and blcA/BAN. In a nonlimiting example a bacterial strain which has one or more diffusible outer membrane phenotype ("leaky membrane") mutation, *e.g.*, as described herein.

[0169] **Fig. 80** depicts a schematic of a construct comprising GLP-1 (1-37) under the control of the FliC promoter and 5'UTR containing the N-terminal flagellar secretion signal for secretion.

[0170] **FIG. 81A, FIG. 81B, FIG. 81C**, and **FIG. 81D** depict schematics of the organization of exemplary GLP-1 secretion constructs with phoA (**FIG. 81A** and **FIG. 81B**) or OmpA (**FIG. 81C** and **FIG. 81D**) secretion tags. Three different RBS binding sites, 20K (**FIG. 81A** and **FIG. 81C**), 100K (**FIG. 81B**), and 67K (**FIG. 81D**) with varying strength (20<67<100) are used. In some embodiments, the Tet inducible promoter and the TetR sequence is replaced by a different inducible promoter system or a constitutive promoter in these constructs. In some embodiments, the background of the strain which contains these constructs and from which GLP-1 is secreted comprises a deletion or mutation in lpp. **FIG. 81A** depicts a schematic of a GLP-1 secretion construct which is expressed by the genetically engineered bacteria and comprises TetR-pTet-20K RBS -PhoA-Glp1. **FIG. 81B** depicts a schematic of a GLP-1 secretion construct which is expressed by the genetically engineered bacteria and comprises TetR-pTet-100K RBS -PhoA-Glp1. **FIG. 81C** depicts a schematic of a GLP-1 secretion construct which is expressed by the genetically engineered bacteria and comprises TetR-pTet-20K RBS -OmpF-Glp1. **FIG. 81D** depicts a schematic of a GLP-1 secretion construct which is expressed by the genetically engineered bacteria and comprisesTetR-pTet-67K RBS -OmpF-Glp1.

[0171] **FIG. 82A and FIG. 82B** depict schematics of the genetically engineered strains SYN2627 (comprising TetR-pTet-20K RBS -PhoA-Glp1) and SYN2643 (comprising TetR-pTet-20K RBS -PhoA-Glp1). Both strains comprise a deletion or mutation in lpp.

[0172] **FIG. 82C** depicts a bar graph showing the intracellular and secreted levels of GLP-1 as detected by ELISA assay for strains SYN2627 and SYN2643.

[0173] **FIG. 83A** and **FIG. 83B** depict line graphs of ELISA results. **FIG. 83A** depicts a line graph, showing an phopho-STAT3 (Tyr705) ELISA conducted on extracts from serum-starved Colo205 cells treated with supernatants from engineered bacteria comprising a PAL deletion and an integrated construct encoding hIL-22 with a phoA secretion tag. The data demonstrate that hIL-22 secreted from the engineered bacteria is functionally active. **FIG. 83B** depicts a line graph, showing an phopho-STAT3 (Tyr705) ELISA showing a antibody completion assay. Extracts from Colo205 cells were treated with the bacterial supernatants from the IL-22 overexpressing strain preincubated with increasing concentrations of neutralizing anti-IL-22 antibody. The data demonstrated that phospho-Stat3 signal induced by the secreted hIL-22 is competed away by the hIL-22 antibody MAB7821.

[0174] **FIG. 83C** depicts a line graph showing SYN3001 (PhoA-IL-22 in pal mutant chassi), but not SYN3000 (pal mutant chassi) supernatant induces STAT3 activation.

[0175] **FIG. 83D** depicts a line graph showing that anti IL-22 neutralizing antibody inhibits SYN3001-induced STAT3 activation (n=3).

[0176] **FIG. 84A and FIG. 84B** depict line graphs showing acetate production over a 6 hour time course post-induction in 0.5% glucose MOPS (pH6.8) (**FIG. 84A**) and in 0.5% glucuronic acid MOPS (pH6.3) (**FIG. 84B**). Acetate production of an engineered *E. coli* Nissle strain comprising a deletion in the endenous ldh gene (SYN2001) was compared with streptomycin resistant Nissle (SYN94).

[0177] **FIG. 84C** and **FIG. 84D** depict bar graphs showing acetate and butyrate production in 0.5% glucose MOPS (pH6.8) (**FIG. 84C**) and acetate and butyrate production in 0.5% glucuronic acid MOPS (pH6.3) (**FIG. 84D**). Deletions in endogenous adhE (Aldehyde-alcohol dehydrogenase) and ldh (lactate dehydrogenase) were introduced into Nissle strains with either integrated FNRS ter-tesB or FNRS-ter-pbt-buk butyrate cassettes. SYN2006 comprises a FNRS ter-tesB cassette integrated at the HA1/2 locus and a deletion in the endogenous adhE gene. SYN2007 comprises a FNRS ter-tesB cassette integrated at the HA1/2 locus and a deletion in the endogenous ldhA gene. SYN2008 comprises a FNRS-ter-pbt-buk butyrate cassette and a deletion in the endogenous adhE gene. SYN2003 comprises a FNRS-ter-pbt-buk butyrate cassette and a deletion in the endogenous ldhA gene.

[0178] **FIG. 84E** depicts a bar graph showing acetate and butyrate production at the indicated time points post induction in 0.5% glucose MOPS (pH6.8). A strain comprising a FNRS-ter-tesB butyrate cassette integrated at the HA1/2 locus of the chromosome (SYN1004) was compared with a strain comprising the same integrated cassette and additionally a deletion in the endogenous frd gene (SYN2005).

[0179] **FIG. 84F** depicts a bar graph showing acetate and butyrate production at 18 hours in 0.5% glucose MOPS (pH6.8), comparing three strains engineered to produce short chain fatty acids. SYN2001 comprises a deletion in the endenous ldh gene; SYN2002 comprises a FNRS-ter-tesB butyrate cassette integrated at the HA1/2 locus and deletions in the endogenous adhE and pta genes. SYN2003 comprises FNRS-ter-pbt-buk butyrate cassette integrated at the HA1/2 locus and a deletion in the endogenous ldhA gene.

[0180] **FIG. 84G and FIG. 84H** depict line graphs showing the effect of supernatants from the engineered acetate-producing strain, SYN2001, on LPS-induced IFN□ secretion in primary human PBMC cells from donor 1 (D1) (**FIG. 84G**) and donor 2 (D2) (**FIG. 84H**).

[0181] **FIG. 85** depicts a schematic illustrating a strategy for increasing butyrate and acetate production in engineered bacteria. Aerobic metabolism through the citric acid cycle (TCA cycle) (crossed out) is inactive in the anaerobic environment of the colon. *E. coli* makes high levels of acetate as an end production of fermentation. To improve acetate production, while still maintaining highlevels of butyrate production, targeted deletion can be introduced to prevent the production of unnecessary metabolic fermentative byproducts (thereby simultaneously increasing butyrate and acetate production). Non-limiting examples of competing routes (shown in in rounded boxes) are frdA (converts phosphoenolpyruvate to succinate), ldhA (converts pyruvate to lactate) and adhE (converts Acetyl-CoA to Ethanol). Deletions of interest therefore include deletion of adhE, ldh, and frd. Thus, in certain embodiments, the genetically engineered bacteria further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0182] **FIG. 86A** and **FIG. 86B** depict bar graphs showing Acetate/Butyrate production in 0.5% glucose MOPS (pH6.8) (**FIG. 86A**) and Acetate/Butyrate production in 0.5% glucuronic acid MOPS (pH6.3) (**FIG. 86B**). Deletions in endogenous adhE (Aldehyde-alcohol dehydrogenase) and ldh (lactate dehydrogenase) were introduced into Nissle strains with either integrated FNRS ter-tesB or FNRS-ter-pbt-buk butyrate cassettes.

[0183] **FIG. 87** depicts a schematic of an exemplary propionate biosynthesis gene cassette.

[0184] **FIG. 88** depicts exemplary circuit designs for the recombinant bacteria of the disclosure. In some embodiments, two bile salt hydrolase (BSH) genes from *Lactobacillus salivarius* (BSH1 and BSH2) are under the control of an aTc-inducible promoter in a single operon. In some embodiments, two bile salt hydrolase (BSH) genes (BSH1 and BSH2) are each under the control of an aTc-inducible promote for individual expression. In some embodiments, the BSH1 and BSH2 genes encode the same bile salt hydrolase enzyme. In other embodiments, the BSH1 and BSH2 genes encode different bile salt hydrolase enzymes.

Detailed Description

[0185] As mentioned above, non-alcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver abnormalities which range from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH is a severe form of NAFLD, where excess fat accumulation in the liver results in chronic inflammation and damage. Nonalcoholic fatty liver disease is a component of metabolic syndrome and a spectrum of liver disorders ranging from simple

steatosis to nonalcoholic steatohepatitis (NASH). Simple liver steatosis is defined as a benign form of NAFLD with minimal risk of progression, in contrast to NASH, which tends to progress to cirrhosis in up to 20% of patients and can subsequently lead to liver failure or hepatocellular carcinoma. NASH affects approximately 3-5% of the population in America, especially in those identified as obese. NASH is characterized by such abnormalities as advanced lipotoxic metabolites, pro-inflammatory substrate, fibrosis (*e.g.*, in which collagen deposition is manifested in a particular perivenular and/or pericellular pattern) , and increased hepatic lipid deposition. If left untreated, NASH can lead to cirrhosis, liver failure, and hepatocellular carcinoma.

[0186] Although patients diagnosed with alcoholic steatohepatitis demonstrate similar symptoms and liver damage, NASH develops in individuals who do not consume alcohol, and the underlying causes of NASH are unknown. Hepatic steatosis occurs when the amount of imported and synthesized lipids exceeds the export or catabolism in hepatocytes. An excess intake of fat or carbohydrate is the main cause of hepatic steatosis. NAFLD patients exhibit signs of liver inflammation and increased hepatic lipid accumulation. In addition, the development of NAFLD in obese individuals is closely associated with insulin resistance and other metabolic disorders and thus might be of clinical relevance). Therfore, possible causative factors include insulin resistance, cytokine imbalance (specifically, an increase in the tumor necrosis factor-alpha (TNF-α)/adiponectin ratio), and oxidative stress resulting from mitochondrial abnormalities.

[0187] Although the majority of patients can be effectively diagnosed with NAFLD using noninvasive tests, liver biopsy is needed for the accurate assessment of the graduation of steatosis, necroinflammatory changes, and fibrosis and allows NASH and steatosis to be distinguished (Sanches et al., Nonalcoholic Steatohepatitis: A Search for Factual Animal Models BioMed Research International Volume 2015). At least two grading systems for NASH have been developed, which take into consideration the severity of hepatic steatosis, portal and lobular inflammation, and collagen deposition.

[0188] In a first grading system described in Sanches et al and references therein, hepatic steatosis is scored as follows: Grade 0 (minimal or no steatosis (<5% of hepatocytes affected); grade 1 (mild steatosis (5 to 32% of hepatocytes affected)); grade 2 (moderate to severe steatosis (33 to 66% of hepatocytes affected)); grade 3 (severe steatosis (>66% of hepatocytes affected)).The portal and lobular inflammation is also scored as follows: grade 0 (minimal or no inflammation);grade 1 (mild); grade 2 (moderate to severe); grade 3 (severe). The collagen deposition is scored as follows: Grade 0 (minimal or no evidence of fibrosis);

grade 1 (mild fibrosis); grade 2 (moderate to severe fibrosis); grade 3 (severe fibrosis). The SAF system is a second way of grading NASH, which also consistes of a semiquantitative score of steatosis (S), inflammatory activity (A), and fibrosis (F) Bedossa et al., Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients; Hepatology, vol. 56, no. 5, pp. 1751–1759, 2012). In one embodiment, genetically engineered bacteria may be useful in some embodiments to improve the grade of hepatic steatosis, portal and lobular inflammation, and collagen deposition.

[0189] Currently, there is no accepted approach to treating NASH. Therapy generally involves treating known risk factors such as correction of obesity through diet and exercise, treating hyperglycemia through diet and insulin, avoiding alcohol consumption, and avoiding unnecessary medication.

[0190] Studies have also suggested that rapid weight loss through bariatric surgery (*e.g.* gastric bypass) is effective in decreasing steatosis, hepatic inflammation, and fibrosis. Other treatments have involved using anti-diabetic medications such as metformin, rosiglitazone, and pioglitazone. Though inconclusive, the studies suggest that the medications stimulate insulin sensitivity in NASH patients, thus alleviating liver damage. In cases were NASH has resulted in advanced cirrhosis, the only treatment is a liver transplant. Regardless, no current treatments are wholly determinative or reliable for treating NASH. Therefore, a need exists for improved therapies and treatments of NASH.

[0191] As such, one strategy in the treatment, prevention, and/or management of NASH may include approaches to help promote the feeling of satiety in the patient, *e.g.* through the administration of a satiety effector, examples of which can be found infra.

[0192] Another strategy in the treatment, prevention, and/or management of NASH may include approaches to reduce liver triglyceride content, *e.g.*, by increasing fatty acid oxidation, decreasing lipogenesis, and improving hepatic glucose metabolism.

[0193] For example, glucagon-like peptide 1 (an incretin secreted by L-cells in the small intestine in response to food intake) and GLP-1 analogs have been used to stimulate insulin secretion in the treatment of type-two diabetes and non-alcoholic steatohepatitis (NASH).

[0194] Research in the identification of new approaches in the the treatment, prevention, and/or management of NASH has focused on improved understanding of the disease pathogenesis. The development of NASH has been considered a “two hit” process. Insulin resistance (with increased lipogenesis), found in obesity and type II diabetes, has been considered the most important factor in the development of hepatic steatosis (the “first hit”).

The "second hit" results in necroinflammatory activity and fibrosis. Because the "second hit can be caused by a number of different mechanisms, including increased gut permeability, and resulting gut-derived endotoxins (*e.g.*, LPS) in the liver, mitochondrial dysfunction, oxidative stress, and/or or proinflammatory cytokines (TNF-alpha, interleukins, *e.g.*, IL-1,IL-6, and IL-8), more recently, a concept of multiple hits has been developed. Moreover, inflammation can occasionally precede steatosis and patients with NASH can present without much steatosis, suggesting that inflammation can occur first, indicating that many of these hits can occur in different orders or in parallel at the same time. Contributing factors to these hits include intestinal dysbiosis, dietary factors, changes to intestinal permeability, as well as endoplasmic reticulum stress and activation of additional signalling pathways. In addition, a patatin-like phospholipase 3 (PNPLA3) gene polymorphism also plays a key role in the development of NASH. Although fatty liver is usually non-progressive, it can progress in patients harboring the risk allele of the PNPLA3 gene.

[0195] Evidence is increasing that the gut and liver have multiple levels of associated interdependence, and disturbance of the gut–liver axis has been implicated in a number of conditions linked to obesity, including NAFLD and NASH. The liver has both an arterial and venous blood supply, with the majority of hepatic blood flow coming from the gut via the portal vein. In NASH the liver is exposed to potentially harmful substances derived from the gut (thought increased gut permeability and reduced intestinal integrity), including translocated bacteria, LPS and endotoxins, food antigens, as well as secreted cytokines. Tight junction proteins, such as zonula occludens, normally seal the junction between intestinal endothelial cells at their apical aspect and thus have a vital role in preventing translocation of harmful substances from the gut into the portal system. In NAFLD/NASH, these tight junctions are disrupted, increasing mucosal permeability and exposing both the gut mucosal cells and the liver to potentially pro-inflammatory bacterial products. Translocated microbial products might contribute to the pathogenesis of fatty liver disease by several mechanisms, including stimulating pro-inflammatory and profibrotic pathways via a range of cytokines.

[0196] As such, one strategy in the treatment, prevention, and/or management of NASH may include approaches to help maintain and/or reestablish gut barrier function, *e.g.* through the prevention, treatment and/or management of inflammatory events at the root of increased permeability, *e.g.* through the administration of anti-inflammatory effectors.

[0197] For example, leading metabolites that play gut-protective roles are short chain fatty acids, *e.g.* acetate, butyrate and propionate, and those derived from tryptophan metabolism. These metabolites have been shown to play a major role in the prevention of

inflammatory disease. As such one approach in the treatment, prevention, and/or management of gut barrier health may be to provide a treatment which contains one or more of such metabolites.

[0198] For example, butyrate and other SCFA, *e.g.*, derived from the microbiota, are known to promote maintaining intestinal integrity (*e.g.*, as reviewed in Thorburn et al., Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases; Immunity Volume 40, Issue 6, 19 June 2014, Pages 833–842). (A) SCFA-induced promotion of mucus by gut epithelial cells, possibly through signaling through metabolite sensing GPCRs; (B) SCFA-induced secretion of IgA by B cells; (C) SCFA-induced promotion of tissue repair and wound healing; (D) SCFA-induced promotion of Treg cell development in the gut in a process that presumably facilitates immunological tolerance; (E) SCFA- mediated enhancement of epithelial integrity in a process dependent on inflammasome activation (*e.g.*, via NALP3) and IL-18 production; and (F) anti-inflammatory effects, inhibition of inflammatory cytokine production (*e.g.*, TNF, Il-6, and IFN-gamma), and inhibition of NF-κB. Many of these actions of SCFAs in gut homeostatis can be ascribed to GPR43 and GPR109A, which are expressed by the colonic epithelium, by inflammatory leukocytes (*e.g.* neutrophils and marcophages) and by Treg cells. These receptors signal through G proteins, coupled to MAPK, PI3K and mTOR, as well as a separate arrestin- pathway, leading to NFkappa B inhibition. Other effects can be ascribed to SCFA-mediated HDAC inhibition, *e.g.* butyrate, which may regulate macrophage function and promote TReg cells.

[0199] In animal models, administration of butyrate has been shown to reduce hepatic steatosis, inflammation, and fat deposition (see, for example, Jin *et al.,* British J. Nutrition, 114(11):1745-1755, 2015 and Endo *et al.,* PLoS One, 8(5):e63388, 2013). Colonic propionate delivery has also been shown to reduce intrahepatocellular lipid content in NASH patients, including improvements in weight gain and intra-abdominal fat deposition (see, for example, Chambers *et al.,* Gut, gutjnl-2014), and GLP-1 administration has been shown to reduce the degree of lipotoxic metabolites and pro-inflammatory substrates, both of which have been shown to speed NASH development, as well as reduce hepatic lipid deposition (see, for example, Bernsmeier *et al.,* PLoS One, 9(1):e87488, 2014 and Armstrong *et al.,* J. Hepatol., 2015).

[0200] In addition, a number of trptophan metabolites, including kynurenine and kynurenic acid, as well as several indoles, such as indole-3 aldehhyde, and several other indole metabolites (which can be derived from microbiota or the diet) described infra, have been shown ot be essential for gut homeostais and promote gut-barrier health. These

metabolites bind to aryl hydrocarbon receptor (Ahr). After agonist binding, AhR translocates to the nucleus, where it forms a heterodimer with AhR nuclear translocator (ARNT). AhR-dependent gene expression includes genes involved in the production of mediators important for gut homeostasis; these mediators include IL-22, antimicrobicidal factors, increased Th17 cell activity, and the maintenance of intraepithelial lymphocytes and RORγt+ innate lymphoid cells.

[0201] Tryptophan can also be transported across the epithelium by transport machinery comprising angiotensin I converting enzyme 2 (Ace2). Tryptophan is degraded to kynurenine, another AhR agonist, by the immune-regulatory enzyme indoleamine 2,3-dioxygenase (IDO), which is linked to suppression of T cell responses, promotion of Treg cells, and immune tolerance. Moreover, a number of tryptophan metabolites, including kynurenic acid and niacin, agonize metabolite-sensing GPCRs, such as GPR35 and GPR109A and thus multiple elements of tryptophan catabolism facilitate gut homeostasis.

[0202] In addition, some indole metabolites, *e.g.*, indole 3-propionic acid (IPA), may exert their effect an acitvating ligand of Pregnane X receptor (PXR), which is thought to play a key role as an essential regulator of intestinal barrier function, through downregulation of TLR4 signaling (Venkatesh et al., 2014 Symbiotic Bacterial Metabolites Regulate Gastrointestinal Barrier Function via the Xenobiotic Sensor PXR and Toll-like Receptor 4; Immunity 41, 296–310, August 21, 2014). As a result, indole levels may through the activation of PXR regulate and balance the levels of TLR4 expression to promote homeostasis and gut barrier health.

[0203] The role of bile acids in the pathogenesis of NAFLD and NASH has been extensively studied (Leung et al., The Role Of The Gut Microbiota In NAFLD; Nature Reviews | Gastroenterology & Hepatology). For example, in one study study, manipulation of the gut microbiota changed intestinal bile acid composition leading to intestinal antagonism of FRX, the master regulator of bile acid metabolism. This FXR antagonism reduced ceramide synthesis and de novo lipogenesis in the liver (Jiang, C. *et al.* Intestinal farnesoid X receptor signaling promotes nonalcoholic fatty liver disease. *J. Clin. Invest.* 125, 386–402 (2015)).

[0204] In some embodiments, the genetically engineered bacteria are useful for the prevention, treatment, and/or management of NAFLD and/or NASH. In some embodiments, the genetically engineered bacteria comprise circuits which reduce inflammation. In some embodiments the circuits stimulate insulin secretion and/or promote satiety.

[0205] In some embodiments, the genetically engineered bacteria comprise one or more gene cassettes for the production of short-chain fatty acids, *e.g.*, butyrate and/or propionate, and/or acetate. In some embodiments, the genetically engineered bacteria comprise one or more gene cassettes for the production of GLP-1. In some embodiments, the genetically engineered bacteria comprise one or more gene cassettes for the production of short-chain fatty acids, *e.g.*, butyrate and/or propionate for the treatment of NAFLD and/or NASH. In some embodiments, the genetically engineered bacteria comprise one or more gene cassettes for the increase of bile salt catabolism, including but not limited to bile salt hydrolase or bile salt transporter producing cassettes.

[0206] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which modulate typtophan levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which modulate kynurenine levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which modulate levels of downstream kynurenine metabolites described herein in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which modulate levels of downstream indole tryptophan metabolites described herein, including, but not limited to those listed in **Table 12** and elsewhere herein, in the patient, *e.g.*, in the serum and/or in the gut.

[0207] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which modulate the TRP/KYN ratio in the patient, *e.g.*, in the serum and/or in the gut. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of tryptophan to one or more indole tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of tryptophan to one or more kynurenine downstream metabolites described herein, *e.g.*, in **FIG. 17**. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of kynurenine to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios of kynurenine to one or more downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which

modulate the ratios between two downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which modulate the ratios between one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein.

[0208] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase typtophan levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase kynurenine levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase levels of downstream kynurenine metabolites described herein in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase levels of downstream tryptophan metabolites described herein, including, but not limited to those listed in **Table 12** and elsewhere herein, in the patient, *e.g.*, in the serum and/or in the gut.

[0209] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which increase the TRP/KYN ratio in the patient, *e.g.*, in the serum and/or in the gut. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios of tryptophan to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios of tryptophan to one or more kynurenine downstream metabolites described herein, *e.g.*, in **FIG. 16**. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios of kynurenine to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios of kynurenine to one or more downstream kynurenine metabolites, including, but not limited to those listed in Table 13 and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios between two downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which increase the ratios between one or more tryptophan metabolites, including, but not limited to those listed in Table 13 and elsewhere herein.

[0210] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease typtophan levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease kynurenine levels in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease levels of downstream kynurenine metabolites described herein in the patient, *e.g.*, in the serum and/or in the gut. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease levels of downstream tryptophan metabolites described herein, including, but not limited to those listed in **Table 12,** and elsewhere herein, in the patient, *e.g.*, in the serum and/or in the gut.

[0211] In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes as described herein, which decrease the TRP/KYN ratio in the patient, *e.g.*, in the serum and/or in the gut. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios of tryptophan to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios of tryptophan to one or more kynurenine downstream metabolites described herein, *e.g.*, in **FIG. 16**. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios of kynurenine to one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios of kynurenine to one or more downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios between two downstream kynurenine metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein. In some embodiments, the genetically engineered bacteria comprise gene cassettes which decrease the ratios between one or more tryptophan metabolites, including, but not limited to those listed in **Table 12** and elsewhere herein.

[0212] In some embodiments, the genetically engineered bacteria comprise a gene cassette which modulates serotonin and or melatonin levels. In some embodiments, the genetically engineered bacteria comprise a gene cassette which increases serotonin and or melatonin levels. In some embodiments, the genetically engineered bacteria comprise a gene cassette which decreases serotonin and or melatonin levels. In some embodiments, the

genetically engineered bacteria comprise a gene cassette which modulates the tryptophan to serotonin and or melatonin ratios. In some embodiments, the genetically engineered bacteria comprise a gene cassette which increases the tryptophan to serotonin and or melatonin ratios. In some embodiments, the genetically engineered bacteria comprise a gene cassette which decreases the tryptophan to serotonin and or melatonin ratios.

[0213] In some embodiments,the genetically engineered bacteria comprise a gene cassette which comprises a heterologous gene encoding a bile salt hydrolase (BSH) enzyme and is capable of processing and reducing levels of bile salts in low-oxygen environments, *e.g.*, the gut. Thus, the genetically engineered bacterial cells and pharmaceutical compositions comprising the genes, gene cassettes disclosed herein may be used to convert excess bile salts into non-toxic molecules in order to treat and/or prevent disorders associated with bile salts, such as cardiovascular disease, metabolic disease, cirrhosis, cancer, liver disease, and C. difficile infection.

[0214] In certain embodiments, one or more of these circuits may be combined for the treatment of NASH and/or NAFLD. In a non-limiting example, butyrate producing, GLP-1 secreting , and tryptophan pathway modulating cassettes may be expressed in combination by the genetically engineered bacteria for the treatment of NASH and/or NAFLD.

[0215] The present disclosure provides engineered bacterial cells, pharmaceutical compositions thereof, and methods of modulating and treating nonalcoholic steatohepatitis (NASH). Specifically, the engineered bacteria disclosed herein have been constructed to comprise genetic circuits composed of, for example, one or more butyrate cassette(s), one or more propionate cassette(s), and/or one or more GLP-1 nucleic acid sequence(s), to treat the disease, as well as other optional circuitry to ensure the safety and non-colonization of the subject that is administered the engineered bacteria, such as auxotrophies, kill switches, *etc.* These engineered bacteria are safe and well tolerated and augment the innate activities of the subject's microbiome to achieve a therapeutic effect.

[0216] In some embodiments, a bacterial cell disclosed herein has been genetically engineered to comprise one or more biosynthetic circuits selected from a propionate gene cassette; a butyrate gene cassette; a GLP-1 gene, and combinations thereof, and is capable of producing propionate, butyrate, and/or GLP-1 in low-oxygen or anaerobic environments, *e.g.*, the gut. Thus, the genetically engineered bacterial cells and pharmaceutical compositions comprising the bacterial cells disclosed herein may be used to produce propionate, butyrate, and/or GLP-1, in order to treat and/or prevent liver disease, such as nonalcoholic steatohepatitis (NASH).

[0217] In order that the disclosure may be more readily understood, certain terms are first defined. These definitions should be read in light of the remainder of the disclosure and as understood by a person of ordinary skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. Additional definitions are set forth throughout the detailed description.

[0218] As used herein, the term "engineered bacterial cell" or "engineered bacteria" refers to a bacterial cell or bacteria that have been genetically modified from their native state. For instance, an engineered bacterial cell may have nucleotide insertions, nucleotide deletions, nucleotide rearrangements, and nucleotide modifications introduced into their DNA. These genetic modifications may be present in the chromosome of the bacteria or bacterial cell, or on a plasmid in the bacteria or bacterial cell. Engineered bacterial cells disclosed herein may comprise exogenous nucleotide sequences on plasmids. Alternatively, engineered bacterial cells may comprise exogenous nucleotide sequences stably incorporated into their chromosome.

[0219] A "programmed bacterial cell" or "programmed engineered bacterial cell" is an engineered bacterial cell that has been genetically modified from its native state to perform a specific function. In certain embodiments, the programmed or engineered bacterial cell has been modified to express one or more proteins, for example, one or more proteins that have a therapeutic activity or serve a therapeutic purpose. The programmed or engineered bacterial cell may additionally have the ability to stop growing or to destroy itself once the protein(s) of interest have been expressed.

[0220] As used herein, the term "gene" refers to a nucleic acid fragment that encodes a protein or fragment thereof, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. In one embodiment, a "gene" does not include regulatory sequences preceding and following the coding sequence. A "native gene" refers to a gene as found in nature, optionally with its own regulatory sequences preceding and following the coding sequence. A "chimeric gene" refers to any gene that is not a native gene, optionally comprising regulatory sequences preceding and following the coding sequence, wherein the coding sequences and/or the regulatory sequences, in whole or in part, are not found together in nature. Thus, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory and coding sequences that are derived from the same source, but arranged differently than is found in nature.

[0221] As used herein, a "heterologous" gene or "heterologous sequence" refers to a nucleotide sequence that is not normally found in a given cell in nature. As used herein, a heterologous sequence encompasses a nucleic acid sequence that is exogenously introduced into a given cell. "Heterologous gene" includes a native gene, or fragment thereof, that has been introduced into the host cell in a form that is different from the corresponding native gene. For example, a heterologous gene may include a native coding sequence that is a portion of a chimeric gene to include a native coding sequence that is a portion of a chimeric gene to include non-native regulatory regions that is reintroduced into the host cell. A heterologous gene may also include a native gene, or fragment thereof, introduced into a non-native host cell. Thus, a heterologous gene may be foreign or native to the recipient cell; a nucleic acid sequence that is naturally found in a given cell but expresses an unnatural amount of the nucleic acid and/or the polypeptide which it encodes; and/or two or more nucleic acid sequences that are not found in the same relationship to each other in nature. As used herein, the term "endogenous gene" refers to a native gene in its natural location in the genome of an organism. As used herein, the term "transgene" refers to a gene that has been introduced into the host organism, *e.g.,* host bacterial cell, genome.

[0222] As used herein, the term "low oxygen" is meant to refer to a level, amount, or concentration of oxygen (O_2) that is lower than the level, amount, or concentration of oxygen that is present in the atmosphere (*e.g.*, <21% O_2; <160 torr O_2). Thus, the term "low oxygen condition or conditions" or "low oxygen environment" refers to conditions or environments containing lower levels of oxygen than are present in the atmosphere. In some embodiments, the term "low oxygen" is meant to refer to the level, amount, or concentration of oxygen (O_2) found in a mammalian gut, *e.g.*, lumen, stomach, small intestine, duodenum, jejunum, ileum, large intestine, cecum, colon, distal sigmoid colon, rectum, and anal canal.

[0223] In some embodiments, the term "low oxygen" is meant to refer to a level, amount, or concentration of O_2 that is 0-60 mmHg O_2 (0-60 torr O_2) (*e.g.*, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45,46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, and 60 mmHg O_2), including any and all incremental fraction(s) thereof (*e.g.*, 0.2 mmHg, 0.5 mmHg O_2, 0.75 mmHg O_2, 1.25 mmHg O_2, 2.175 mmHg O_2, 3.45 mmHg O_2, 3.75 mmHg O_2, 4.5 mmHg O_2, 6.8 mmHg O_2, 11.35 mmHg O2, 46.3 mmHg O_2, 58.75 mmHg, etc., which exemplary fractions are listed here for illustrative purposes and not meant to be limiting in any way). In some embodiments, "low oxygen" refers to about 60 mmHg O_2 or less (*e.g.*, 0 to about 60 mmHg O_2). The term "low oxygen" may also refer to a range

of O_2 levels, amounts, or concentrations between 0-60 mmHg O_2 (inclusive), *e.g.*, 0-5 mmHg O_2, < 1.5 mmHg O_2, 6-10 mmHg, < 8 mmHg, 47-60 mmHg, etc. which listed exemplary ranges are listed here for illustrative purposes and not meant to be limiting in any way. See, for example, Albenberg et al., Gastroenterology, 147(5): 1055-1063 (2014); Bergofsky et al., J Clin. Invest., 41(11): 1971- 1980 (1962); Crompton et al., J Exp. Biol., 43: 473-478 (1965); He et al., PNAS (USA), 96: 4586-4591 (1999); McKeown, Br. J. Radiol., 87:20130676 (2014) (doi: 10.1259/brj.20130676), each of which discusses the oxygen levels found in the mammalian gut of various species and each of which are incorportated by reference herewith in their entireties.

[0224] In some embodiments, the term "low oxygen" is meant to refer to the level, amount, or concentration of oxygen (O_2) found in a mammalian organ or tissue other than the gut, *e.g.*, urogenital tract, tumor tissue, etc. in which oxygen is present at a reduced level, *e.g.*, at a hypoxic or anoxic level. In some embodiments, "low oxygen" is meant to refer to the level, amount, or concentration of oxygen (O_2) present in partially aerobic, semi aerobic, microaerobic, nanoaerobic, microoxic, hypoxic, anoxic, and/or anaerobic conditions. For example, **Table A** summarizes the amount of oxygen present in various organs and tissues. In some embodiments, the level, amount, or concentration of oxygen (O_2) is expressed as the amount of dissolved oxygen ("DO") which refers to the level of free, non-compound oxygen (O_2) present in liquids and is typically reported in milligrams per liter (mg/L), parts per million (ppm; 1mg/L = 1 ppm), or in micromoles (umole) (1 umole O_2 = 0.022391 mg/L O_2). Fondriest Environmental, Inc., "Dissolved Oxygen", Fundamentals of Environmental Measurements, 19 Nov 2013, <www.fondriest.com/environmental-measurements/parameters/water-quality/dissolved- oxygen/>.

[0225] In some embodiments, the term "low oxygen" is meant to refer to a level, amount, or concentration of oxygen (O_2) that is about 6.0 mg/L DO or less, *e.g.*, 6.0 mg/L, 5.0 mg/L, 4.0 mg/L, 3.0 mg/L, 2.0 mg/L, 1.0 mg/L, or 0 mg/L, and any fraction therein, *e.g.*, 3.25 mg/L, 2.5 mg/L, 1.75 mg/L, 1.5 mg/L, 1.25 mg/L, 0.9 mg/L, 0.8 mg/L, 0.7 mg/L, 0.6 mg/L, 0.5 mg/L, 0.4 mg/L, 0.3 mg/L, 0.2 mg/L and 0.1 mg/L DO, which exemplary fractions are listed here for illustrative purposes and not meant to be limiting in any way. The level of oxygen in a liquid or solution may also be reported as a percentage of air saturation or as a percentage of oxygen saturation (the ratio of the concentration of dissolved oxygen (O_2) in the solution to the maximum amount of oxygen that will dissolve in the solution at a certain temperature, pressure, and salinity under stable equilibrium). Well-aerated solutions (*e.g.*, solutions subjected to mixing and/or stirring) without oxygen producers or consumers are

100% air saturated. In some embodiments, the term "low oxygen" is meant to refer to 40% air saturation or less, *e.g.*, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, and 0% air saturation, including any and all incremental fraction(s) thereof (*e.g.*, 30.25%, 22.70%, 15.5%, 7.7%, 5.0%, 2.8%, 2.0%, 1.65%, 1.0%, 0.9%, 0.8%, 0.75%, 0.68%, 0.5%. 0.44%, 0.3%, 0.25%, 0.2%, 0.1%, 0.08%, 0.075%, 0.058%, 0.04%. 0.032%, 0.025%, 0.01%, etc.) and any range of air saturation levels between 0-40%, inclusive (*e.g.*, 0-5%, 0.05 – 0.1%, 0.1-0.2%, 0.1-0.5%, 0.5 – 2.0%, 0-10%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, etc.). The exemplary fractions and ranges listed here are for illustrative purposes and not meant to be limiting in any way. In some embodiments, the term "low oxygen" is meant to refer to 9% O_2 saturation or less, *e.g.*, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0%, O_2 saturation, including any and all incremental fraction(s) thereof (*e.g.*, 6.5%, 5.0%, 2.2%, 1.7%, 1.4%, 0.9%, 0.8%, 0.75%, 0.68%, 0.5%. 0.44%, 0.3%, 0.25%, 0.2%, 0.1%, 0.08%, 0.075%, 0.058%, 0.04%. 0.032%, 0.025%, 0.01%, etc.) and any range of O_2 saturation levels between 0-9%, inclusive (*e.g.*, 0-5%, 0.05 – 0.1%, 0.1-0.2%, 0.1-0.5%, 0.5 – 2.0%, 0-8%, 5-7%, 0.3-4.2% O_2, etc.). The exemplary fractions and ranges listed here are for illustrative purposes and not meant to be limiting in any way.

Table A.

Compartment	Oxygen Tension
stomach	~60 torr (*e.g.*, 58 +/- 15 torr)
duodenum and first part of jejunum	~30 torr (*e.g.*, 32 +/- 8 torr); ~20% oxygen in ambient air
Ileum (mid- small intestine)	~10 torr; ~6% oxygen in ambient air (*e.g.*, 11 +/- 3 torr)
Distal sigmoid colon	~ 3 torr (*e.g.*, 3 +/- 1 torr)
colon	<2torr
Lumen of cecum	<1 torr
tumor	<32 torr (most tumors are <15 torr)

[0226] As used herein, a "non-native" nucleic acid sequence refers to a nucleic acid sequence not normally present in a microorganism, e.g., an extra copy of an endogenous sequence, or a heterologous sequence such as a sequence from a different species, strain, or substrain of bacteria or virus, or a sequence that is modified and/or mutated as compared to the unmodified sequence from bacteria or virus of the same subtype. In some embodiments, the non-native nucleic acid sequence is a synthetic, non-naturally occurring sequence (see,

e.g., Purcell et al., 2013). The non-native nucleic acid sequence may be a regulatory region, a promoter, a gene, and/or one or more genes in gene cassette. In some embodiments, "non-native" refers to two or more nucleic acid sequences that are not found in the same relationship to each other in nature. The non-native nucleic acid sequence may be present on a plasmid or chromosome. In some embodiments, the genetically engineered microorganism of the disclosure comprises a gene that is operably linked to a promoter that is not associated with said gene in nature. For example, in some embodiments, the genetically engineered bacteria disclosed herein comprise a gene that is operably linked to a directly or indirectly inducible promoter that is not associated with said gene in nature, *e.g.,* an FNR responsive promoter (or other promoter disclosed herein) operably linked to an anti-inflammatory or gut barrier enhancer molecule. In some embodiments, the genetically engineered virus of the disclosure comprises a gene that is operably linked to a directly or indirectly inducible promoter that is not associated with said gene in nature, e.g., a promoter operably linked to a gene encoding an anti-inflammatory or gut barrier enhancer molecule.

[0227] As used herein, the term "coding region" refers to a nucleotide sequence that codes for a specific amino acid sequence. The term "regulatory sequence" refers to a nucleotide sequence located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influences the transcription, RNA processing, RNA stability, or translation of the associated coding sequence. Examples of regulatory sequences include, but are not limited to, promoters, translation leader sequences, effector binding sites, and stem-loop structures. In one embodiment, the regulatory sequence comprises a promoter, *e.g.,* an FNR responsive promoter.

[0228] As used herein, a "gene cassette" or "operon" encoding a biosynthetic pathway refers to the two or more genes that are required to produce a molecule, *e.g.,* propionate or butyrate. In addition to encoding a set of genes capable of producing said molecule, the gene cassette or operon may also comprise additional transcription and translation elements, *e.g.,* a ribosome binding site.

[0229] A "butyrogenic gene cassette," "butyrate biosynthesis gene cassette," "butyrate gene cassette" and "butyrate operon" are used interchangeably to refer to a set of genes capable of producing butyrate in a biosynthetic pathway. Unmodified bacteria that are capable of producing butyrate via an endogenous butyrate biosynthesis pathway include, but are not limited to, Clostridium, Peptoclostridium, Fusobacterium, Butyrivibrio, Eubacterium, and Treponema. The genetically engineered bacteria of the invention may comprise butyrate biosynthesis genes from a different species, strain, or substrain of bacteria, or a combination

of butyrate biosynthesis genes from different species, strains, and/or substrains of bacteria. A butyrogenic gene cassette may comprise, for example, the eight genes of the butyrate production pathway from Peptoclostridium difficile (also called Clostridium difficile): bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt, and buk, which encode butyryl-CoA dehydrogenase subunit, electron transfer flavoprotein subunit beta, electron transfer flavoprotein subunit alpha, acetyl-CoA C-acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, phosphate butyryltransferase, and butyrate kinase, respectively (Aboulnaga et al., 2013). One or more of the butyrate biosynthesis genes may be functionally replaced or modified, *e.g.*, codon optimized. Peptoclostridium difficile strain 630 and strain 1296 are both capable of producing butyrate, but comprise different nucleic acid sequences for etfA3, thiA1, hbd, crt2, pbt, and buk. A butyrogenic gene cassette may comprise bcd2, etfB3, etfA3, and thiA1 from Peptoclostridium difficile strain 630, and hbd, crt2, pbt, and buk from Peptoclostridium difficile strain 1296. Alternatively, a single gene from Treponema denticola (ter, encoding trans-2-enoynl-CoA reductase) is capable of functionally replacing all three of the bcd2, etfB3, and etfA3 genes from Peptoclostridium difficile. Thus, a butyrogenic gene cassette may comprise thiA1, hbd, crt2, pbt, and buk from Peptoclostridium difficile and ter from Treponema denticola. The butyrogenic gene cassette may comprise genes for the aerobic biosynthesis of butyrate and/or genes for the anaerobic or microaerobic biosynthesis of butyrate. In another example of a butyrate gene cassette, the *pbt* and *buk* genes are replaced with *tesB* (*e.g.*, from E coli). Thus a butyrogenic gene cassette may comprise ter, thiA1, hbd, crt2, and tesB.

[0230] As used herein, the term "butyrate biosynthesis gene" refers to a gene present in a butyrate gene cassette and which performs an enzymatic function in the production of butyrate in a butyrate biosynthetic pathway.

[0231] A "propionate gene cassette" or "propionate operon" refers to a set of genes capable of producing propionate in a biosynthetic pathway. Unmodified bacteria that are capable of producing propionate via an endogenous propionate biosynthesis pathway include, but are not limited to, Clostridium propionicum, Megasphaera elsdenii, and Prevotella ruminicola. The genetically engineered bacteria of the invention may comprise propionate biosynthesis genes from a different species, strain, or substrain of bacteria, or a combination of propionate biosynthesis genes from different species, strains, and/or substrains of bacteria. In some embodiments, the propionate gene cassette comprises acrylate pathway propionate biosynthesis genes, *e.g.*, *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*, which encode propionate CoA-transferase, lactoyl-CoA dehydratase A, lactoyl-CoA dehydratase B, lactoyl-CoA

dehydratase C, electron transfer flavoprotein subunit A, acryloyl-CoA reductase B, and acryloyl-CoA reductase C, respectively (Hetzel et al., 2003, Selmer et al., 2002, and Kandasamy 2012 Engineering *Escherichia coli* with acrylate pathway genes for propionic acid synthesis and its impact on mixed-acid fermentation). This operon catalyses the reduction of lactate to propionate. Dehydration of (*R*)-lactoyl-CoA leads to the production of the intermediate acryloyl-CoA by lactoyl-CoA dehydratase (LcdABC). Acrolyl-CoA is converted to propionyl-CoA by acrolyl-CoA reductase (EtfA, AcrBC). In some embodiments, the rate limiting step catalyzed by the enzymes encoded by *etfA*, *acrB* and *acrC*, are replaced by the *acuI* gene from *R. sphaeroides*. This gene product catalyzes the NADPH-dependent acrylyl-CoA reduction to produce propionyl-CoA (Acrylyl-Coenzyme A Reductase, an Enzyme Involved in the Assimilation of 3-Hydroxypropionate by Rhodobacter sphaeroides; Asao 2013). Thus the propionate cassette comprises *pct*, *lcdA*, *lcdB*, *lcdC*, and *acuI*. In another embodiment, the homolog of AcuI in *E coli*, YhdH is used (see.*e.g.*, Structure of *Escherichia coli* YhdH, a putative quinone oxidoreductase. Sulzenbacher 2004). This the propionate cassette comprises *pct*, *lcdA*, *lcdB*, lcdC, and yhdH. In alternate embodiments, the propionate gene cassette comprises pyruvate pathway propionate biosynthesis genes (see, *e.g.*, Tseng et al., 2012), *e.g.*, thrAfbr, thrB, thrC, ilvAfbr, aceE, aceF, and lpd, which encode homoserine dehydrogenase 1, homoserine kinase, L-threonine synthase, L-threonine dehydratase, pyruvate dehydrogenase, dihydrolipoamide acetyltrasferase, and dihydrolipoyl dehydrogenase, respectively. In some embodiments, the propionate gene cassette further comprises tesB, which encodes acyl-CoA thioesterase.

[0232] In another example of a propionate gene cassette comprises the genes of the Sleeping Beauty Mutase operon, *e.g.*, from *E. coli* (sbm, ygfD, ygfG, ygfH). Recently, this pathway has been considered and utilized for the high yield industrial production of propionate from glycerol (Akawi et al., Engineering *Escherichia coli* for high-level production of propionate; J Ind Microbiol Biotechnol (2015) 42:1057–1072, the contents of which is herein incorporated by reference in its entirety). In addition, as described herein, it has been found that this pathway is also suitable for production of proprionate from glucose, *e.g.* by the genetically engineered bacteria of the disclosure. The SBM pathway is cyclical and composed of a series of biochemical conversions forming propionate as a fermentative product while regenerating the starting molecule of succinyl-CoA. Sbm (methylmalonyl-CoA mutase) converts succinyl CoA to L-methylmalonylCoA, YgfD is a Sbm-interacting protein kinase with GTPase activity, ygfG (methylmalonylCoA decarboxylase) converts L-

methylmalonylCoA into PropionylCoA, and ygfH (propionyl-CoA/succinylCoA transferase) converts propionylCoA into propionate and succinate into succinylCoA (Sleeping beauty mutase (sbm) is expressed and interacts with ygfd in *Escherichia coli*; Froese 2009). This pathway is very similar to the oxidative propionate pathway of Propionibacteria, which also converts succinate to propionate. Succinyl-CoA is converted to R-methylmalonyl-CoA by methymalonyl-CoA mutase (mutAB). This is in turn converted to S-methylmalonyl-CoA via methymalonyl-CoA epimerase (GI:18042134). There are three genes which encode methylmalonyl-CoA carboxytransferase (mmdA, PFREUD_18870, bccp) which converts methylmalonyl-CoA to propionyl-CoA.

[0233] The propionate gene cassette may comprise genes for the aerobic biosynthesis of propionate and/or genes for the anaerobic or microaerobic biosynthesis of propionate. One or more of the propionate biosynthesis genes may be functionally replaced or modified, *e.g.*, codon optimized.

[0234] An "acetate gene cassette" or "acetate operon" refers to a set of genes capable of producing acetate in a biosynthetic pathway. Bacteria "synthesize acetate from a number of carbon and energy sources," including a variety of substrates such as cellulose, lignin, and inorganic gases, and utilize different biosynthetic mechanisms and genes, which are known in the art (Ragsdale et al., 2008). The genetically engineered bacteria of the invention may comprise acetate biosynthesis genes from a different species, strain, or substrain of bacteria, or a combination of acetate biosynthesis genes from different species, strains, and/or substrains of bacteria. *Escherichia coli* are capable of consuming glucose and oxygen to produce acetate and carbon dioxide during aerobic growth (Kleman *et al.*, 1994). Several bacteria, such as *Acetitomaculum, Acetoanaerobium, Acetohalobium, Acetonema, Balutia, Butyribacterium, Clostridium, Moorella, Oxobacter, Sporomusa,* and *Thermoacetogenium,* are acetogenic anaerobes that are capable of converting CO or $CO_2 + H_2$ into acetate, *e.g.*, using the Wood-Ljungdahl pathway (Schiel-Bengelsdorf et al, 2012). Genes in the Wood-Ljungdahl pathway for various bacterial species are known in the art. The acetate gene cassette may comprise genes for the aerobic biosynthesis of acetate and/or genes for the anaerobic or microaerobic biosynthesis of acetate. One or more of the acetate biosynthesis genes may be functionally replaced or modified, e.g., codon optimized.

[0235] Each gene or gene cassette may be present on a plasmid or bacterial chromosome. In addition, multiple copies of any gene, gene cassette, or regulatory region may be present in the bacterium, wherein one or more copies of the gene, gene cassette, or regulatory region may be mutated or otherwise altered as described herein. In some

embodiments, the genetically engineered bacteria are engineered to comprise multiple copies of the same gene, gene cassette, or regulatory region in order to enhance copy number or to comprise multiple different components of a gene cassette performing multiple different functions.

[0236] As used herein, the term "modulate" and its cognates means to alter, regulate, or adjust positively or negatively a molecular or physiological readout, outcome, or process, to effect a change in said readout, outcome, or process as compared to a normal, average, wild-type, or baseline measurement. Thus, for example, “modulate” or “modulation” includes up-regulation and down-regulation. A non-limiting example of modulating a readout, outcome, or process is effecting a change or alteration in the normal or baseline functioning, activity, expression, or secretion of a biomolecule (*e.g.* a protein, enzyme, cytokine, growth factor, hormone, metabolite, short chain fatty acid, or other compound). Another non-limiting example of modulating a readout, outcome, or process is effecting a change in the amount or level of a biomolecule of interest, *e.g.* in the serum and/or the gut lumen. In another non-limiting example, modulating a readout, outcome, or process relates to a phenotypic change or alteration in one or more disease symptoms. Thus, “modulate” is used to refer to an increase, decrease, masking, altering, overriding or restoring the normal functioning, activity, or levels of a readout, outcome or process (e.g, biomolecule of interest, and/or molecular or physiological process, and/or a phenotypic change in one or more disease symptoms).

[0237] Treating the diseases described herein may encompass increasing levels of propionate, increasing levels of butyrate, and increasing GLP-1, and/or modulating levels of tryptophan and/or its metabolites (*e.g.*, kynurenine), and does not necessarily encompass the elimination of the underlying disease.

[0238] As used herein the term “codon-optimized” refers to the modification of codons in the gene or coding regions of a nucleic acid molecule to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the nucleic acid molecule. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of the host organism.

[0239] Each gene or gene cassette may be present on a plasmid or bacterial chromosome. In addition, multiple copies of any gene, gene cassette, or regulatory region may be present in the bacterium, wherein one or more copies of the gene, gene cassette, or regulatory region may be mutated or otherwise altered as described herein. In some embodiments, the genetically engineered bacteria are engineered to comprise multiple copies

of the same gene, gene cassette, or regulatory region in order to enhance copy number or to comprise multiple different components of a gene cassette performing multiple different functions.

[0240] Each gene or gene cassette may be operably linked to a promoter that is induced under low-oxygen conditions. "Operably linked" refers a nucleic acid sequence, e.g., a gene or gene cassette for producing an anti-inflammatory or gut barrier enhancer molecule, that is joined to a regulatory region sequence in a manner which allows expression of the nucleic acid sequence, e.g., acts in cis. A regulatory region "Operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. A regulatory element is operably linked with a coding sequence when it is capable of affecting the expression of the gene coding sequence, regardless of the distance between the regulatory element and the coding sequence. More specifically, operably linked refers to a nucleic acid sequence, *e.g.,* a gene encoding an anti-inflammatory or gut barrier enhancer molecule, that is joined to a regulatory sequence in a manner which allows expression of the nucleic acid sequence, *e.g.,* the gene encoding the anti-inflammatory or gut barrier enhancer molecule. In other words, the regulatory sequence acts in cis. In one embodiment, a gene may be "directly linked" to a regulatory sequence in a manner which allows expression of the gene. In another embodiment, a gene may be "indirectly linked" to a regulatory sequence in a manner which allows expression of the gene. In one embodiment, two or more genes may be directly or indirectly linked to a regulatory sequence in a manner which allows expression of the two or more genes.
A regulatory region or sequence is a nucleic acid that can direct transcription of a gene of interest and may comprise promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, promoter control elements, protein binding sequences, 5′ and 3′ untranslated regions, transcriptional start sites, termination sequences, polyadenylation sequences, and introns.

[0241] A "promoter" as used herein, refers to a nucleotide sequence that is capable of controlling the expression of a coding sequence or gene. Promoters are generally located 5' of the sequence that they regulate. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from promoters found in nature, and/or comprise synthetic nucleotide segments. Those skilled in the art will readily ascertain that different promoters may regulate expression of a coding sequence or gene in response to a particular stimulus, *e.g.,* in a cell- or tissue-specific manner, in response to different environmental or physiological conditions, or in response to specific compounds.

Prokaryotic promoters are typically classified into two classes: inducible and constitutive. A "constitutive promoter" refers to a promoter that allows for continual transcription of the coding sequence or gene under its control.

[0242] "Constitutive promoter" refers to a promoter that is capable of facilitating continuous transcription of a coding sequence or gene under its control and/or to which it is operably linked. Constitutive promoters and variants are well known in the art and include, but are not limited to, BBa_J23100, a constitutive *Escherichia coli* σ^S promoter (*e.g.*, an osmY promoter (International Genetically Engineered Machine (iGEM) Registry of Standard Biological Parts Name BBa_J45992; BBa_J45993)), a constitutive *Escherichia coli* σ^{32} promoter (*e.g.*, htpG heat shock promoter (BBa_J45504)), a constitutive *Escherichia coli* σ^{70} promoter (*e.g.*, lacq promoter (BBa_J54200; BBa_J56015), *E. coli* CreABCD phosphate sensing operon promoter (BBa_J64951), GlnRS promoter (BBa_K088007), lacZ promoter (BBa_K119000; BBa_K119001); M13K07 gene I promoter (BBa_M13101); M13K07 gene II promoter (BBa_M13102), M13K07 gene III promoter (BBa_M13103), M13K07 gene IV promoter (BBa_M13104), M13K07 gene V promoter (BBa_M13105), M13K07 gene VI promoter (BBa_M13106), M13K07 gene VIII promoter (BBa_M13108), M13110 (BBa_M13110)), a constitutive *Bacillus subtilis* σ^A promoter (*e.g.*, promoter veg (BBa_K143013), promoter 43 (BBa_K143013), P_{liaG} (BBa_K823000), P_{lepA} (BBa_K823002), P_{veg} (BBa_K823003)), a constitutive *Bacillus subtilis* σ^B promoter (*e.g.*, promoter ctc (BBa_K143010), promoter gsiB (BBa_K143011)), a *Salmonella* promoter (*e.g.*, Pspv2 from *Salmonella* (BBa_K112706), Pspv from *Salmonella* (BBa_K112707)), a bacteriophage T7 promoter (*e.g.*, T7 promoter (BBa_I712074; BBa_I719005; BBa_J34814; BBa_J64997; BBa_K113010; BBa_K113011; BBa_K113012; BBa_R0085; BBa_R0180; BBa_R0181; BBa_R0182; BBa_R0183; BBa_Z0251; BBa_Z0252; BBa_Z0253)), and a bacteriophage SP6 promoter (*e.g.*, SP6 promoter (BBa_J64998)).

[0243] An "inducible promoter" refers to a regulatory region that is operably linked to one or more genes, wherein expression of the gene(s) is increased in the presence of an inducer of said regulatory region. An "inducible promoter" refers to a promoter that initiates increased levels of transcription of the coding sequence or gene under its control in response to a stimulus or an exogenous environmental condition. A "directly inducible promoter" refers to a regulatory region, wherein the regulatory region is operably linked to a gene encoding a protein or polypeptide, where, in the presence of an inducer of said regulatory region, the protein or polypeptide is expressed. An "indirectly inducible promoter" refers to a regulatory system comprising two or more regulatory regions, for example, a first regulatory

region that is operably linked to a first gene encoding a first protein, polypeptide, or factor, e.g., a transcriptional regulator, which is capable of regulating a second regulatory region that is operably linked to a second gene, the second regulatory region may be activated or repressed, thereby activating or repressing expression of the second gene. Both a directly inducible promoter and an indirectly inducible promoter are encompassed by "inducible promoter." Exemplary inducible promoters described herein include oxygen level-dependent promoters (e.g., FNR-inducible promoter), promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose and tetracycline. Examples of inducible promoters include, but are not limited to, an FNR responsive promoter, a ParaC promoter, a ParaBAD promoter, and a PTetR promoter, each of which are described in more detail herein. Examples of other inducible promoters are provided herein below.

[0244] As used herein, "stably maintained" or "stable" bacterium is used to refer to a bacterial host cell carrying non-native genetic material, *e.g.,* a gene or gene cassette(s), that is incorporated into the host genome or propagated on a self-replicating extra-chromosomal plasmid, such that the non-native genetic material is retained, expressed, and propagated. The stable bacterium is capable of survival and/or growth *in vitro*, *e.g.,* in medium, and/or *in vivo*, *e.g.,* in the gut. For example, the stable bacterium may be a genetically engineered bacterium comprising a gene or gene cassette, in which the plasmid or chromosome carrying the gene or gene cassette is stably maintained in the bacterium, such that the gene or gene cassette can be expressed in the bacterium, and the bacterium is capable of survival and/or growth *in vitro* and/or *in vivo*. In some embodiments, copy number affects the stability of expression of the non-native genetic material. In some embodiments, copy number affects the level of expression of the non-native genetic material.

[0245] As used herein, the term "expression" refers to the transcription and stable accumulation of sense (mRNA) or anti-sense RNA derived from a nucleic acid, and/or to translation of an mRNA into a polypeptide.

[0246] As used herein, the term "plasmid" or "vector" refers to an extrachromosomal nucleic acid, e.g., DNA, construct that is not integrated into a bacterial cell's genome. Plasmids are usually circular and capable of autonomous replication. Plasmids may be low-copy, medium-copy, or high-copy, as is well known in the art. Plasmids may optionally comprise a selectable marker, such as an antibiotic resistance gene, which helps select for bacterial cells containing the plasmid and which ensures that the plasmid is retained in the

bacterial cell. A plasmid disclosed herein may comprise a nucleic acid sequence encoding a heterologous gene, e.g., a gene encoding an anti-inflammatory or gut barrier enhancer molecule. A plasmid may comprise a nucleic acid sequence encoding a heterologous gene or gene cassette.

[0247] As used herein, the term "transform" or "transformation" refers to the transfer of a nucleic acid fragment into a host bacterial cell, resulting in genetically-stable inheritance. Host bacterial cells comprising the transformed nucleic acid fragment are referred to as "recombinant" or "transgenic" or "transformed" organisms.

[0248] The term "genetic modification," as used herein, refers to any genetic change. Exemplary genetic modifications include those that increase, decrease, or abolish the expression of a gene, including, for example, modifications of native chromosomal or extrachromosomal genetic material. Exemplary genetic modifications also include the introduction of at least one plasmid, modification, mutation, base deletion, base addition, and/or codon modification of chromosomal or extrachromosomal genetic sequence(s), gene over-expression, gene amplification, gene suppression, promoter modification or substitution, gene addition (either single or multi-copy), antisense expression or suppression, or any other change to the genetic elements of a host cell, whether the change produces a change in phenotype or not. Genetic modification can include the introduction of a plasmid, *e.g.,* a plasmid comprising a gene or gene cassette operably linked to a promoter, into a bacterial cell. Genetic modification can also involve a targeted replacement in the chromosome, *e.g.,* to replace a native gene promoter with an inducible promoter, regulated promoter, strong promoter, or constitutive promoter. Genetic modification can also involve gene amplification, *e.g.,* introduction of at least one additional copy of a native gene into the chromosome of the cell. Alternatively, chromosomal genetic modification can involve a genetic mutation.

[0249] As used herein, the term "genetic mutation" refers to a change or changes in a nucleotide sequence of a gene or related regulatory region that alters the nucleotide sequence as compared to its native or wild-type sequence. Mutations include, for example, substitutions, additions, and deletions, in whole or in part, within the wild-type sequence. Such substitutions, additions, or deletions can be single nucleotide changes (*e.g.,* one or more point mutations), or can be two or more nucleotide changes, which may result in substantial changes to the sequence. Mutations can occur within the coding region of the gene as well as within the non-coding and regulatory sequence of the gene. The term "genetic mutation" is intended to include silent and conservative mutations within a coding region as well as

changes which alter the amino acid sequence of the polypeptide encoded by the gene. A genetic mutation in a gene coding sequence may, for example, increase, decrease, or otherwise alter the activity (*e.g.,* enzymatic activity) of the gene's polypeptide product. A genetic mutation in a regulatory sequence may increase, decrease, or otherwise alter the expression of sequences operably linked to the altered regulatory sequence.

[0250] As used herein, the term "transporter" is meant to refer to a mechanism, e.g., protein, proteins, or protein complex, for importing a molecule, e.g., amino acid, peptide (di-peptide, tri-peptide, polypeptide, etc), toxin, metabolite, substrate, as well as other biomolecules into the microorganism from the extracellular milieu.

[0251] As used herein, the phrase "exogenous environmental condition" or "exogenous environment signal" refers to settings, circumstances, stimuli, or biological molecules under which a promoter described herein is directly or indirectly induced. The phrase "exogenous environmental conditions" is meant to refer to the environmental conditions external to the engineered micororganism, but endogenous or native to the host subject environment. Thus, "exogenous" and "endogenous" may be used interchangeably to refer to environmental conditions in which the environmental conditions are endogenous to a mammalian body, but external or exogenous to an intact microorganism cell. In some embodiments, the exogenous environmental conditions are specific to the gut of a mammal. In some embodiments, the exogenous environmental conditions are specific to the upper gastrointestinal tract of a mammal. In some embodiments, the exogenous environmental conditions are specific to the lower gastrointestinal tract of a mammal. In some embodiments, the exogenous environmental conditions are specific to the small intestine of a mammal. In some embodiments, the exogenous environmental conditions are low-oxygen, microaerobic, or anaerobic conditions, such as the environment of the mammalian gut. In some embodiments, exogenous environmental conditions are molecules or metabolites that are specific to the mammalian gut, e.g., propionate. In some embodiments, the exogenous environmental condition is a tissue-specific or disease-specific metabolite or molecule(s). In some embodiments, the exogenous environmental condition is specific to an inflammatory disease. In some embodiments, the exogenous environmental condition is a low-pH environment. In some embodiments, the genetically engineered microorganism of the disclosure comprises a pH-dependent promoter. In some embodiments, the genetically engineered microorganism of the diclosure comprise an oxygen level-dependent promoter. In some aspects, bacteria have evolved transcription factors that are capable of sensing oxygen levels. Different signaling pathways may be triggered by different oxygen levels and occur

with different kinetics. An "oxygen level-dependent promoter" or "oxygen level-dependent regulatory region" refers to a nucleic acid sequence to which one or more oxygen level-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression.

[0252] Examples of oxygen level-dependent transcription factors include, but are not limited to, FNR (fumarate and nitrate reductase), ANR, and DNR. Corresponding FNR-responsive promoters, ANR (anaerobic nitrate respiration)-responsive promoters, and DNR (dissimilatory nitrate respiration regulator)-responsive promoters are known in the art (see, e.g., Castiglione et al., 2009; Eiglmeier et al., 1989; Galimand et al., 1991; Hasegawa et al., 1998; Hoeren et al., 1993; Salmon et al., 2003), and non-limiting examples are shown in **Table B**.

[0253] In a non-limiting example, a promoter (PfnrS) was derived from the E. coli Nissle fumarate and nitrate reductase gene S (fnrS) that is known to be highly expressed under conditions of low or no environmental oxygen (Durand and Storz, 2010; Boysen et al, 2010). The PfnrS promoter is activated under anaerobic conditions by the global transcriptional regulator FNR that is naturally found in Nissle. Under anaerobic conditions, FNR forms a dimer and binds to specific sequences in the promoters of specific genes under its control, thereby activating their expression. However, under aerobic conditions, oxygen reacts with iron-sulfur clusters in FNR dimers and converts them to an inactive form. In this way, the PfnrS inducible promoter is adopted to modulate the expression of proteins or RNA. PfnrS is used interchangeably in this application as FNRS, fnrs, FNR, P-FNRS promoter and other such related designations to indicate the promoter PfnrS.

Table B. Examples of transcription factors and responsive genes and regulatory regions

Transcription Factor	**Examples of responsive genes, promoters, and/or regulatory regions:**
FNR	*nirB, ydfZ, pdhR, focA, ndH, hlyE, narK, narX, narG, yfiD, tdcD*
ANR	*arcDABC*
DNR	*norb, norC*

[0254] As used herein, a "tunable regulatory region" refers to a nucleic acid sequence under direct or indirect control of a transcription factor and which is capable of activating, repressing, derepressing, or otherwise controlling gene expression relative to levels of an inducer. In some embodiments, the tunable regulatory region comprises a promoter sequence. The inducer may be RNS, or other inducer described herein, and the tunable regulatory region may be a RNS-responsive regulatory region or other responsive regulatory region described herein. The tunable regulatory region may be operatively linked to a gene sequence(s) or gene cassette for the production of one or more payloads, e.g., a butyrogenic or other gene cassette or gene sequence(s). For example, in one specific embodiment, the tunable regulatory region is a RNS-derepressible regulatory region, and when RNS is present, a RNS-sensing transcription factor no longer binds to and/or represses the regulatory region, thereby permitting expression of the operatively linked gene or gene cassette. In this instance, the tunable regulatory region derepresses gene or gene cassette expression relative to RNS levels. Each gene or gene cassette may be operatively linked to a tunable regulatory region that is directly or indirectly controlled by a transcription factor that is capable of sensing at least one RNS.

[0255] In some embodiments, the exogenous environmental conditions are the presence or absence of reactive oxygen species (ROS). In other embodiments, the exogenous environmental conditions are the presence or absence of reactive nitrogen species (RNS). In some embodiments, exogenous environmental conditions are biological molecules that are involved in the inflammatory response, for example, molecules present in an inflammatory disorder of the gut. In some embodiments, the exogenous environmental conditions or signals exist naturally or are naturally absent in the environment in which the recombinant bacterial cell resides. In some embodiments, the exogenous environmental conditions or signals are artificially created, for example, by the creation or removal of biological conditions and/or the administration or removal of biological molecules.

[0256] In some embodiments, the exogenous environmental condition(s) and/or signal(s) stimulates the activity of an inducible promoter. In some embodiments, the exogenous environmental condition(s) and/or signal(s) that serves to activate the inducible promoter is not naturally present within the gut of a mammal. In some embodiments, the inducible promoter is stimulated by a molecule or metabolite that is administered in combination with the pharmaceutical composition of the disclosure, for example, tetracycline, arabinose, or any biological molecule that serves to activate an inducible promoter. In some embodiments, the exogenous environmental condition(s) and/or signal(s)

is added to culture media comprising an engineered bacterial cell of the disclosure. In some embodiments, the exogenous environmental condition that serves to activate the inducible promoter is naturally present within the gut of a mammal (for example, low oxygen or anaerobic conditions, or biological molecules involved in an inflammatory response). In some embodiments, the loss of exposure to an exogenous environmental condition (for example, *in vivo*) inhibits the activity of an inducible promoter, as the exogenous environmental condition is not present to induce the promoter (for example, an aerobic environment outside the gut).

[0257] "Gut" refers to the organs, glands, tracts, and systems that are responsible for the transfer and digestion of food, absorption of nutrients, and excretion of waste. In humans, the gut comprises the gastrointestinal (GI) tract, which starts at the mouth and ends at the anus, and additionally comprises the esophagus, stomach, small intestine, and large intestine. The gut also comprises accessory organs and glands, such as the spleen, liver, gallbladder, and pancreas. The upper gastrointestinal tract comprises the esophagus, stomach, and duodenum of the small intestine. The lower gastrointestinal tract comprises the remainder of the small intestine, *i.e.,* the jejunum and ileum, and all of the large intestine, *i.e.,* the cecum, colon, rectum, and anal canal. Bacteria can be found throughout the gut, *e.g.,* in the gastrointestinal tract, and particularly in the intestines.

[0258] "Microorganism" refers to an organism or microbe of microscopic, submicroscopic, or ultramicroscopic size that typically consists of a single cell. Examples of microrganisms include bacteria, viruses, parasites, fungi, certain algae, and protozoa. In some aspects, the microorganism is engineered ("engineered microorganism") to produce one or more therapeutic molecules. In certain aspects, the microorganism is engineered to import and/or catabolize certain toxic metabolites, substrates, or other compounds from its environment, *e.g.*, the gut. In certain aspects, the microorganism is engineered to synthesize certain beneficial metabolites, molecules, or other compounds (synthetic or naturally occurring) and release them into its environment. In certain embodiments, the engineered microorganism is an engineered bacterium. In certain embodiments, the engineered microorganism is an engineered virus.

[0259] "Non-pathogenic bacteria" refer to bacteria that are not capable of causing disease or harmful responses in a host. In some embodiments, non-pathogenic bacteria are commensal bacteria. Examples of non-pathogenic bacteria include, but are not limited to *Bacillus, Bacteroides, Bifidobacterium*, *Brevibacteria, Clostridium, Enterococcus, Escherichia coli, Lactobacillus, Lactococcus, Saccharomyces,* and *Staphylococcus, e.g.*,

Bacillus coagulans, *Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium bifidum*, *Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium butyricum, Enterococcus faecium*, *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei*, *Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis,* and *Saccharomyces boulardii* (Sonnenborn *et al.,* 2009; Dinleyici *et al.*, 2014; U.S. Patent No. 6,835,376; U.S. Patent No. 6,203,797; U.S. Patent No. 5,589,168; U.S. Patent No. 7,731,976). Naturally pathogenic bacteria may be genetically engineered to provide reduce or eliminate pathogenicity.

[0260] "Probiotic" is used to refer to live, non-pathogenic microorganisms, *e.g.*, bacteria, which can confer health benefits to a host organism that contains an appropriate amount of the microorganism. In some embodiments, the host organism is a mammal. In some embodiments, the host organism is a human. Some species, strains, and/or subtypes of non-pathogenic bacteria are currently recognized as probiotic bacteria. Examples of probiotic bacteria include, but are not limited to, *Bifidobacteria*, *Escherichia coli*, *Lactobacillus,* and *Saccharomyces e.g., Bifidobacterium bifidum, Enterococcus faecium, Escherichia coli* strain Nissle, *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Saccharomyces boulardii* (Dinleyici *et al.,* 2014; U.S. Patent No. 5,589,168; U.S. Patent No. 6,203,797; U.S. Patent 6,835,376). The probiotic may be a variant or a mutant strain of bacterium (Arthur *et al.,* 2012; Cuevas-Ramos *et al.,* 2010; Olier *et al.,* 2012; Nougayrede *et al.,* 2006). Non-pathogenic bacteria may be genetically engineered to enhance or improve desired biological properties, *e.g.,* survivability. Non-pathogenic bacteria may be genetically engineered to provide probiotic properties. Probiotic bacteria may be genetically engineered to enhance or improve probiotic properties.

[0261] As used herein, the term "modulate" and its cognates means to alter, regulate, or adjust positively or negatively a molecular or physiological readout, outcome, or process, to effect a change in said readout, outcome, or process as compared to a normal, average, wild-type, or baseline measurement. Thus, for example, "modulate" or "modulation" includes up-regulation and down-regulation. A non-limiting example of modulating a readout, outcome, or process is effecting a change or alteration in the normal or baseline functioning, activity, expression, or secretion of a biomolecule (e.g. a protein, enzyme, cytokine, growth factor, hormone, metabolite, short chain fatty acid, or other compound). Another non-limiting example of modulating a readout, outcome, or process is effecting a change in the amount or level of a biomolecule of interest, e.g. in the serum and/or the gut lumen. In another non-

limiting example, modulating a readout, outcome, or process relates to a phenotypic change or alteration in one or more disease symptoms. Thus, "modulate" is used to refer to an increase, decrease, masking, altering, overriding or restoring the normal functioning, activity, or levels of a readout, outcome or process (e.g, biomolecule of interest, and/or molecular or physiological process, and/or a phenotypic change in one or more disease symptoms).

[0262] As used herein, the term "auxotroph" or "auxotrophic" refers to an organism that requires a specific factor, *e.g.,* an amino acid, a sugar, or other nutrient, to support its growth. An "auxotrophic modification" is a genetic modification that causes the organism to die in the absence of an exogenously added nutrient essential for survival or growth because it is unable to produce said nutrient. As used herein, the term "essential gene" refers to a gene which is necessary to for cell growth and/or survival. Essential genes are described in more detail *infra* and include, but are not limited to, DNA synthesis genes (such as *thyA*), cell wall synthesis genes (such as *dapA*), and amino acid genes (such as *serA* and *metA*).

[0263] As used herein, the terms "modulate" and "treat" a disease and their cognates refer to an amelioration of a disease, disorder, and/or condition, or at least one discernible symptom thereof. In another embodiment, "modulate" and "treat" refer to an amelioration of at least one measurable physical parameter, not necessarily discernible by the patient. In another embodiment, "modulate" and "treat" refer to inhibiting the progression of a disease, disorder, and/or condition, either physically (e.g., stabilization of a discernible symptom), physiologically (e.g., stabilization of a physical parameter), or both. In another embodiment, "modulate" and "treat" refer to slowing the progression or reversing the progression of a disease, disorder, and/or condition. As used herein, "prevent" and its cognates refer to delaying the onset or reducing the risk of acquiring a given disease, disorder and/or condition or a symptom associated with such disease, disorder, and/or condition.

[0264] Those in need of treatment may include individuals already having a particular medical disease, as well as those at risk of having, or who may ultimately acquire the disease. The need for treatment is assessed, for example, by the presence of one or more risk factors associated with the development of a disease, the presence or progression of a disease, or likely receptiveness to treatment of a subject having the disease. Liver disease, *e.g.,* nonalcoholic steatohepatitis (NASH), may be caused by inborn genetic mutations for which there are no known cures. Diseases can also be secondary to other conditions, *e.g.,* an intestinal disorder or a bacterial infection. Treating nonalcoholic steatohepatitis (NASH) may encompass increasing levels of propionate, increasing levels of butyrate, and increasing GLP-1, and does not necessarily encompass the elimination of the underlying disease.

[0265] As used herein a "pharmaceutical composition" refers to a preparation of bacterial cells with other components such as a physiologically suitable carrier and/or excipient.

[0266] The phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be used interchangeably refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered bacterial compound. An adjuvant is included under these phrases.

[0267] The term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples include, but are not limited to, calcium bicarbonate, sodium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20.

[0268] The terms “therapeutically effective dose” and “therapeutically effective amount” are used to refer to an amount of a compound that results in prevention, delay of onset of symptoms, or amelioration of symptoms of a disease, *e.g.,* nonalcoholic steatohepatitis (NASH). A therapeutically effective amount may, for example, be sufficient to treat, prevent, reduce the severity, delay the onset, and/or reduce the risk of occurrence of one or more symptoms of NASH. Methods for diagnosing liver diseases, such as nonalcoholic steatohepatitis (NASH), are known in the art (see, for example, U.S. 2015/0290154 and U.S. 2015/0247149, the entire contents of each of which are expressly incorporated herein by reference). A therapeutically effective amount, as well as a therapeutically effective frequency of administration, can be determined by methods known in the art and discussed below.

[0269] As used herein, the term "bacteriostatic” or "cytostatic” refers to a molecule or protein which is capable of arresting, retarding, or inhibiting the growth, division, multiplication or replication of engineered bacterial cell of the disclosure.

[0270] As used herein, the term “bactericidal” refers to a molecule or protein which is capable of killing the engineered bacterial cell of the disclosure.

[0271] As used herein, the term “toxin” refers to a protein, enzyme, or polypeptide fragment thereof, or other molecule which is capable of arresting, retarding, or inhibiting the growth, division, multiplication or replication of the engineered bacterial cell of the disclosure, or which is capable of killing the engineered bacterial cell of the disclosure. The term “toxin” is intended to include bacteriostatic proteins and bactericidal proteins. The term

"toxin" is intended to include, but not limited to, lytic proteins, bacteriocins (*e.g.*, microcins and colicins), gyrase inhibitors, polymerase inhibitors, transcription inhibitors, translation inhibitors, DNases, and RNases. The term "anti-toxin" or "antitoxin," as used herein, refers to a protein or enzyme which is capable of inhibiting the activity of a toxin. The term anti-toxin is intended to include, but not limited to, immunity modulators, and inhibitors of toxin expression. Examples of toxins and antitoxins are known in the art and described in more detail *infra*.

[0272] As used herein, "payload" refers to one or more molecules of interest to be produced by a genetically engineered microorganism, such as a bacteria or a virus. In some embodiments, the payload is a therapeutic payload, e.g. and antiinflammatory or gut barrier enhancer molecule, e.g. butyrate, acetate, propionate, GLP-2, IL-10, IL-22, IL-2, other interleukins, and/or tryptophan and/or one or more of its metabolites. In some embodiments, the payload is a regulatory molecule, e.g., a transcriptional regulator such as FNR. In some embodiments, the payload comprises a regulatory element, such as a promoter or a repressor. In some embodiments, the payload comprises an inducible promoter, such as from FNRS. In some embodiments the payload comprises a repressor element, such as a kill switch. In some embodiments the payload comprises an antibiotic resistance gene or genes. In some embodiments, the payload is encoded by a gene, multiple genes, gene cassette, or an operon. In alternate embodiments, the payload is produced by a biosynthetic or biochemical pathway, wherein the biosynthetic or biochemical pathway may optionally be endogenous to the microorganism. In alternate embodiments, the payload is produced by a biosynthetic or biochemical pathway, wherein the biosynthetic or biochemical pathway is not endogenous to the microorganism. In some embodiments, the genetically engineered microorganism comprises two or more payloads.

[0273] As used herein, the term "conventional treatment" or "conventional therapy" refers to treatment or therapy that is currently accepted, considered current standard of care, and/or used by most healthcare professionals for treating a disease or disorder associated with BCAA. It is different from alternative or complementary therapies, which are not as widely used.

[0274] As used herein, the term "polypeptide" includes "polypeptide" as well as "polypeptides," and refers to a molecule composed of amino acid monomers linearly linked by amide bonds (i.e., peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, "peptides," "dipeptides," "tripeptides, "oligopeptides," "protein," "amino acid chain," or any

other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including but not limited to glycosylation, acetylation, phosphorylation, amidation, derivatization, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide may be derived from a natural biological source or produced by recombinant technology. In other embodiments, the polypeptide is produced by the genetically engineered bacteria or virus of the current invention. A polypeptide of the invention may be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides may have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides, which do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, are referred to as unfolded. The term "peptide" or "polypeptide" may refer to an amino acid sequence that corresponds to a protein or a portion of a protein or may refer to an amino acid sequence that corresponds with non-protein sequence, e.g., a sequence selected from a regulatory peptide sequence, leader peptide sequence, signal peptide sequence, linker peptide sequence, and other peptide sequence.

[0275] An "isolated" polypeptide or a fragment, variant, or derivative thereof refers to a polypeptide that is not in its natural milieu. No particular level of purification is required. Recombinantly produced polypeptides and proteins expressed in host cells, including but not limited to bacterial or mammalian cells, are considered isolated for purposed of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique. Recombinant peptides, polypeptides or proteins refer to peptides, polypeptides or proteins produced by recombinant DNA techniques, i.e. produced from cells, microbial or mammalian, transformed by an exogenous recombinant DNA expression construct encoding the polypeptide. Proteins or peptides expressed in most bacterial cultures will typically be free of glycan. Fragments, derivatives, analogs or variants of the foregoing polypeptides, and any combination thereof are also included as polypeptides. The terms "fragment," "variant," "derivative" and "analog" include polypeptides having an amino acid sequence sufficiently similar to the amino acid sequence of the original peptide and include any polypeptides, which retain at least one or more properties of the corresponding original polypeptide. Fragments of polypeptides of the

present invention include proteolytic fragments, as well as deletion fragments. Fragments also include specific antibody or bioactive fragments or immunologically active fragments derived from any polypeptides described herein. Variants may occur naturally or be non-naturally occurring. Non-naturally occurring variants may be produced using mutagenesis methods known in the art. Variant polypeptides may comprise conservative or non-conservative amino acid substitutions, deletions or additions.

[0276] Polypeptides also include fusion proteins. As used herein, the term "variant" includes a fusion protein, which comprises a sequence of the original peptide or sufficiently similar to the original peptide. As used herein, the term "fusion protein" refers to a chimeric protein comprising amino acid sequences of two or more different proteins. Typically, fusion proteins result from well known in vitro recombination techniques. Fusion proteins may have a similar structural function (but not necessarily to the same extent), and/or similar regulatory function (but not necessarily to the same extent), and/or similar biochemical function (but not necessarily to the same extent) and/or immunological activity (but not necessarily to the same extent) as the individual original proteins which are the components of the fusion proteins."Derivatives" include but are not limited to peptides, which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. "Similarity" between two peptides is determined by comparing the amino acid sequence of one peptide to the sequence of a second peptide. An amino acid of one peptide is similar to the corresponding amino acid of a second peptide if it is identical or a conservative amino acid substitution. Conservative substitutions include those described in Dayhoff, M. O., ed., The Atlas of Protein Sequence and Structure 5, National Biomedical Research Foundation, Washington, D.C. (1978), and in Argos, EMBO J. 8 (1989), 779-785. For example, amino acids belonging to one of the following groups represent conservative changes or substitutions: -Ala, Pro, Gly, Gln, Asn, Ser, Thr; -Cys, Ser, Tyr, Thr; -Val, Ile, Leu, Met, Ala, Phe; -Lys, Arg, His; -Phe, Tyr, Trp, His; and -Asp, Glu.

[0277] n antibody generally refers to a polypeptide of the immunoglobulin family or a polypeptide comprising fragments of an immunoglobulin that is capable of noncovalently, reversibly, and in a specific manner binding a corresponding antigen. An exemplary antibody structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD), connected through a disulfide bond. The recognized immunoglobulin genes include the κ, λ, α, γ, δ, ε, and μ constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either κ or λ. Heavy chains are classified

as γ, μ, α, δ, or ε, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these regions of light and heavy chains respectively.

[0278] As used herein, the term "antibody" or "antibodies"is meant to encompasses all variations of antibody and fragments thereof that possess one or more particular binding specificities. Thus, the term "antibody" or "antibodies" is meant to include full length antibodies, chimeric antibodies, humanized antibodies, single chain antibodies (ScFv, camelids), Fab, Fab', multimeric versions of these fragments (e.g., F(ab')2), single domain antibodies (sdAB, VHH frаments), heavy chain antibodies (HCAb), nanobodies, diabodies, and minibodies. Antibodies can have more than one binding specificity, e.g., be bispecific. The term "antibody" is also meant to include so-called antibody mimetics. Antibody mimetics refers to small molecules, e.g., 3-30 kDa, which can be single amino acid chain molecules, which can specifically bind antigens but do not have an antibody-related structure. Antibody mimetics, include, but are not limited to, Affibody molecules (Z domain of Protein A), Affilins (Gamma-B crystalline), Ubiquitin, Affimers (Cystatin), Affitins (Sac7d (from Sulfolobus acidocaldarius), Alphabodies (Triple helix coiled coil), Anticalins (Lipocalins), Avimers (domains of various membrane receptors), DARPins (Ankyrin repeat motif), Fynomers (SH3 domain of Fyn), Kunitz domain peptides Kunitz domains of various protease inhibitors), Ecallantide (Kalbitor), and Monobodies. In certain aspects, the term "antibody" or "antibodies" is meant to refer to a single chain antibody(ies), single domain antibody(ies), and camelid antibody(ies). Utility of antibodies in the treatment of cancer and additional antibodies can for example be found in Scott et al., Antibody Therapy for Cancer, Nature Reviews Cancer April 2012 Volume 12, incorporated by reference in its entirety.

[0279] A "single-chain antibody" or "single-chain antibodies" typically refers to a peptide comprising a heavy chain of an immunoglobulin, a light chain of an immunoglobulin, and optionally a linker or bond, such as a disulfide bond. The single-chain antibody lacks the constant Fc region found in traditional antibodies. In some embodiments, the single-chain antibody is a naturally occurring single-chain antibody, e.g., a camelid antibody. In some embodiments, the single-chain antibody is a synthetic, engineered, or modified single-chain antibody. In some embodiments, the single-chain antibody is capable of retaining substantially the same antigen specificity as compared to the original immunoglobulin despite the addition of a linker and the removal of the constant regions. In some aspects, the single

chain antibody can be a "scFv antibody", which refers to a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins (without any constant regions), optionally connected with a short linker peptide of ten to about 25 amino acids, as described, for example, in U.S. Patent No. 4,946,778, the contents of which is herein incorporated by reference in its entirety. The Fv fragment is the smallest fragment that holds a binding site of an antibody, which binding site may, in some aspects, maintain the specificity of the original antibody. Techniques for the production of single chain antibodies are described in U.S. Patent No. 4,946,778. The Vh and VL sequences of the scFv can be connected via the N-terminus of the VH connecting to the C-terminus of the VL or via the C-terminus of the VH connecting to the N-terminus of the VL. ScFv fragments are independent folding entities that can be fused indistinctively on either end to other epitope tags or protein domains. Linkers of varying length can be used to link the Vh and VL sequences, which the linkers can be glycine rich (provides flexibility) and serine or threonine rich (increases solubility). Short linkers may prevent association of the two domains and can result in multimers (diabodies, tribodies, etc.). Long linkers may result in proteolysis or weak domain association (described in Voelkel et al el., 2011). Linkers of length between 15 and 20 amino acids or 18 and 20 amino acids are most often used. Additional non-limiting examples of linkers, including other flexible linkers are described in Chen et al., 2013 (Adv Drug Deliv Rev. 2013 Oct 15; 65(10): 1357–1369. Fusion Protein Linkers: Property, Design and Functionality), the contents of which is herein incorporated by reference in its entirety. Flexible linkers are also rich in small or polar amino acids such as Glycine and Serine, but can contain additional amino acids such as Threonine and Alanine to maintain flexibility, as well as polar amino acids such as Lysine and Glutamate to improve solubility. Exemplary linkers include, but are not limited to, (Gly-Gly-Gly-Gly-Ser)n, KESGSVSSEQLAQFRSLD and EGKSSGSGSESKST, (Gly)8, and Gly and Ser rich flexible linker, GSAGSAAGSGEF. "Single chain antibodies" as used herein also include single-domain antibodies, which include camelid antibodies and other heavy chain antibodies, light chain antibodies, including nanobodies and single domains VH or VL domains derived from human, mouse or other species. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, fish, shark, goat, rabbit, and bovine. Single domain antibodies include domain antigen-binding units which have a camelid scaffold, derived from camels, llamas, or alpacas. Camelids produce functional antibodies devoid of light chains. The heavy chain variable (VH) domain folds autonomously and functions independently as an antigen-binding unit. Its binding surface involves only three CDRs as compared to the six

CDRs in classical antigen-binding molecules (Fabs) or single chain variable fragments (scFvs). Camelid antibodies are capable of attaining binding affinities comparable to those of conventional antibodies. Camelid scaffold-based antibodies can be produced using methods well known in the art. Cartilaginous fishes also have heavy-chain antibodies (IgNAR, 'immunoglobulin new antigen receptor'), from which single-domain antibodies called VNAR fragments can be obtained. Alternatively, the dimeric variable domains from IgG from humans or mice can be split into monomers. Nanobodies are single chain antibodies derived from light chains. The term "single chain antibody" also refers to antibody mimetics.

[0280] In some embodiments, the antibodies expressed by the engineered microorganisms are bispecfic. In certain embodiments, a bispecific antibody molecule comprises a scFv, or fragment thereof, have binding specificity for a first epitope and a scFv, or fragment thereof, have binding specificity for a second epitope. Antigen-binding fragments or antibody portions include bivalent scFv (diabody), bispecific scFv antibodies where the antibody molecule recognizes two different epitopes, single binding domains (dAbs), and minibodies. Monomeric single-chain diabodies (scDb) are readily assembled in bacterial and mammalian cells and show improved stability under physiological conditions (Voelkel et al., 2001 and references therein; Protein Eng. (2001) 14 (10): 815-823 (describes optimized linker sequences for the expression of monomeric and dimeric bispecific single-chain diabodies).

[0281] As used herein, the term "sufficiently similar" means a first amino acid sequence that contains a sufficient or minimum number of identical or equivalent amino acid residues relative to a second amino acid sequence such that the first and second amino acid sequences have a common structural domain and/or common functional activity. For example, amino acid sequences that comprise a common structural domain that is at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100%, identical are defined herein as sufficiently similar. Preferably, variants will be sufficiently similar to the amino acid sequence of the peptides of the invention. Such variants generally retain the functional activity of the peptides of the present invention. Variants include peptides that differ in amino acid sequence from the native and wt peptide, respectively, by way of one or more amino acid deletion(s), addition(s), and/or

substitution(s). These may be naturally occurring variants as well as artificially designed ones.

[0282] As used herein the term "linker", "linker peptide" or "peptide linkers" or "linker" refers to synthetic or non-native or non-naturally-occurring amino acid sequences that connect or link two polypeptide sequences, e.g., that link two polypeptide domains. As used herein the term "synthetic" refers to amino acid sequences that are not naturally occurring. Exemplary linkers are described herein. Additional exemplary linkers are provided in US 20140079701, the contents of which are herein incorporated by reference in its entirety.

[0283] As used herein the term "codon-optimized" refers to the modification of codons in the gene or coding regions of a nucleic acid molecule to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the nucleic acid molecule. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of the host organism. A "codon-optimized sequence" refers to a sequence, which was modified from an existing coding sequence, or designed, for example, to improve translation in an expression host cell or organism of a transcript RNA molecule transcribed from the coding sequence, or to improve transcription of a coding sequence. Codon optimization includes, but is not limited to, processes including selecting codons for the coding sequence to suit the codon preference of the expression host organism. Many organisms display a bias or preference for use of particular codons to code for insertion of a particular amino acid in a growing polypeptide chain. Codon preference or codon bias, differences in codon usage between organisms, is allowed by the degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, inter alia, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

[0284] As used herein, the terms "secretion system" or "secretion protein" refers to a native or non-native secretion mechanism capable of secreting or exporting a biomolecule, e.g., polypeptide from the microbial, e.g., bacterial cytoplasm. The secretion system may comprise a single protein or may comprise two or more proteins assembled in a complex e.g.,HlyBD. Non-limiting examples of secretion systems for gram negative bacteria include

the modified type III flagellar, type I (e.g., hemolysin secretion system), type II, type IV, type V, type VI, and type VII secretion systems, resistance-nodulation-division (RND) multi-drug efflux pumps, various single membrane secretion systems. Non-liming examples of secretion systems for gram positive bacteria include Sec and TAT secretion systems. In some embodiments, the polypeptide to be secreted include a "secretion tag" of either RNA or peptide origin to direct the polypeptide to specific secretion systems. In some embodiments, the secretion system is able to remove this tag before secreting the polyppetide from the engineered bacteria. For example, in Type V auto-secretion-mediated secretion the N-terminal peptide secretion tag is removed upon translocation of the "passenger" peptide from the cytoplasm into the periplasmic compartment by the native Sec system. Further, once the auto-secretor is translocated across the outer membrane the C-terminal secretion tag can be removed by either an autocatalytic or protease-catalyzed e.g., OmpT cleavage thereby releasing the antinflammatory or barrier enhancer molecule(s) into the extracellular milieu. In some embodiments, the secretion system involves the generation of a "leaky" or de-stabilized outer membrane, which may be accomplished by deleting or mutagenizing genes responsible for tethering the outer membrane to the rigid peptidoglycan skeleton, including for example, lpp, ompC, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl. Lpp functions as the primary 'staple' of the bacterial cell wall to the peptidoglycan. TolA-PAL and OmpA complexes function similarly to Lpp and are other deletion targets to generate a leaky phenotype. Additionally, leaky phenotypes have been observed when periplasmic proteases, such as degS, degP or nlpI, are deactivated. Thus, in some embodiments, the engineered bacteria have one or more deleted or mutated membrane genes, e.g., selected from lpp, ompA, ompA, ompF, tolA, tolB, and pal genes. In some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes, e.g., selected from degS, degP, and nlpl. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from lpp, ompA, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl genes.

[0285] The articles "a" and "an," as used herein, should be understood to mean "at least one," unless clearly indicated to the contrary.

[0286] The phrase "and/or," when used between elements in a list, is intended to mean either (1) that only a single listed element is present, or (2) that more than one element of the list is present. For example, "A, B, and/or C" indicates that the selection may be A alone; B alone; C alone; A and B; A and C; B and C; or A, B, and C. The phrase "and/or" may be used interchangeably with "at least one of" or "one or more of" the elements in a list.

[0287] Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Bacterial Strains

[0288] The disclosure provides a bacterial cell that comprises a heterologous propionate gene cassette; a heterologous butyrate gene cassette; a heterologous GLP-1 gene; a heterologous propionate gene cassette and a heterologous butyrate gene cassette; a heterologous propionate gene cassette and a heterologous GLP-1 gene; a heterologous butyrate gene cassette and a heterologous GLP-1 gene; or a heterologous propionate gene cassette, a heterologous butyrate gene cassette, and a heterologous GLP-1 gene. In any of these embodiments, the bacterial cell may contain more than one copy of the respective gene cassette(s) and/or gene(s). In some embodiments, the bacterial cell is a non-pathogenic bacterial cell. In some embodiments, the bacterial cell is a commensal bacterial cell. In some embodiments, the bacterial cell is a probiotic bacterial cell.

[0289] In certain embodiments, the bacterial cell is selected from the group consisting of a Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Clostridium butyricum, Clostridium scindens, Escherichia coli, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus reuteri, Lactococcus lactis, and Oxalobacter formigenes bacterial cell. In one embodiment, the bacterial cell is a Bacteroides fragilis bacterial cell. In one embodiment, the bacterial cell is a Bacteroides thetaiotaomicron bacterial cell. In one embodiment, the bacterial cell is a Bacteroides subtilis bacterial cell. In one embodiment, the bacterial cell is a Bifidobacterium animalis bacterial cell. In one embodiment, the bacterial cell is a Bifidobacterium bifidum bacterial cell. In one embodiment, the bacterial cell is a Bifidobacterium infantis bacterial cell. In one embodiment, the bacterial cell is a Bifidobacterium lactis bacterial cell. In one embodiment, the bacterial cell is a Clostridium butyricum bacterial cell. In one embodiment, the bacterial cell is a Clostridium scindens bacterial cell. In one embodiment, the bacterial cell is an Escherichia coli bacterial cell. In one embodiment, the bacterial cell is a Lactobacillus acidophilus bacterial cell. In one embodiment, the bacterial cell is a Lactobacillus plantarum bacterial cell. In one embodiment, the bacterial cell is a Lactobacillus reuteri bacterial cell. In one embodiment, the bacterial cell is a Lactococcus lactis bacterial cell. In one

embodiment, the bacterial cell is a Oxalobacter formigenes bacterial cell. In another embodiment, the bacterial cell does not include Oxalobacter formigenes.

[0290] In one embodiment, the bacterial cell is a Gram positive bacterial cell. In another embodiment, the bacterial cell is a Gram negative bacterial cell.

[0291] In some embodiments, the bacterial cell is *Escherichia coli* strain Nissle 1917 (*E. coli* Nissle), a Gram-negative bacterium of the *Enterobacteriaceae* family that has evolved into one of the best characterized probiotics (Ukena *et al.*, 2007). The strain is characterized by its complete harmlessness (Schultz, 2008), and has GRAS (generally recognized as safe) status (Reister *et al.*, 2014, emphasis added). Genomic sequencing confirmed that *E. coli* Nissle lacks prominent virulence factors (*e.g., E. coli* α-hemolysin, P-fimbrial adhesins) (Schultz, 2008), and *E. coli* Nissle does not carry pathogenic adhesion factors and does not produce any enterotoxins or cytotoxins, it is not invasive, not uropathogenic (Sonnenborn *et al.*, 2009). As early as in 1917, *E. coli* Nissle was packaged into medicinal capsules, called Mutaflor, for therapeutic use. It is commonly accepted that *E. coli* Nissle's therapeutic efficacy and safety have convincingly been proven (Ukena *et al.*, 2007). In a recent study in non-human primates, Nissle was well tolerated by female cynomolgus monkeys after 28 days of daily NG dose administration at doses up to 1 x 1012 CFU/animal. No Nissle related mortality occurred and no Nissle related effects were identified upon clinical observation, body weight, and clinical pathology assessment (see, *e.g.*, PCT/US16/34200).

[0292] In one embodiment, the engineered bacterial cell does not colonize the subject having NASH.

[0293] One of ordinary skill in the art would appreciate that the genetic modifications disclosed herein may be adapted for other species, strains, and subtypes of bacteria. Furthermore, genes from one or more different species can be introduced into one another, *e.g.,* a gene from *Lactobacillus plantarum* or *Methanobrevibacter smithii 3142* can be expressed in *Escherichia coli.*

[0294] In some embodiments, the bacterial cell is a genetically engineered bacterial cell. In another embodiment, the bacterial cell is an engineered bacterial cell. In some embodiments, the disclosure comprises a colony of bacterial cells.

[0295] In another aspect, the disclosure provides an engineered bacterial culture which comprises bacterial cells.

[0296] In some embodiments of the above described genetically engineered bacteria, the gene or gene cassette(s) are present on a plasmid in the bacterium and operatively linked

on the plasmid to the promoter that is induced under low-oxygen or anaerobic conditions. In other embodiments, the gene or gene cassette(s) is present in the bacterial chromosome and is operatively linked in the chromosome to the promoter that is induced under low-oxygen or anaerobic conditions.

[0297] In some embodiments, the genetically engineered bacteria is an auxotroph or a conditional auxotroph. In one embodiment, the genetically engineered bacteria is an auxotroph selected from a *cysE, glnA, ilvD, leuB, lysA, serA, metA, glyA, hisB, ilvA, pheA, proA, thrC, trpC, tyrA, thyA, uraA, dapA, dapB, dapD, dapE, dapF, flhD, metB, metC, proAB, and thi1* auxotroph. In some embodiments, the engineered bacteria have more than one auxotrophy, for example, they may be a Δ*thyA* and Δ*dapA auxotroph.*

[0298] In some embodiments, the genetically engineered bacteria further comprise a kill-switch circuit, such as any of the kill-switch circuits provided herein. For example, in some embodiments, the genetically engineered bacteria further comprise one or more genes encoding one or more recombinase(s) under the control of an inducible promoter, and an inverted toxin sequence. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding one or more recombinase(s) under the control of an inducible promoter and one or more inverted excision genes, wherein the excision gene(s) encode an enzyme that deletes an essential gene. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding a toxin under the control of an promoter having a TetR repressor binding site and a gene encoding the TetR under the control of an inducible promoter that is induced by arabinose, such as P_{araBAD}. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin.

[0299] In some embodiments, the genetically engineered bacteria is an auxotroph and further comprises a kill-switch circuit, such as any of the kill-switch circuits described herein.

[0300] In some embodiments of the above described genetically engineered bacteria, the gene or gene cassette(s) are present on a plasmid in the bacterium and operatively linked on the plasmid to the promoter that is induced under low-oxygen or anaerobic conditions. In other embodiments, the gene or gene cassette(s) are present in the bacterial chromosome and is operatively linked in the chromosome to the promoter that is induced under low-oxygen or anaerobic conditions.

Metabolic and Anti-inflammatory Effector Molecules

[0301] The genetically engineered bacteria of the invention comprise a gene encoding a non-native metabolic and/or satiety effector molecule, or a gene cassette encoding a biosynthetic pathway capable of producing a metabolic and/or satiety effector molecule. In some embodiments, the metabolic and/or satiety effector molecule is selected from the group consisting of n-acyl-phophatidylethanolamines (NAPEs), n-acyl-ethanolamines (NAEs), ghrelin receptor antagonists, peptide YY3-36, cholecystokinin (CCK) family molecules, CCK58, CCK33, CCK22, CCK8, bombesin family molecules, bombesin, gastrin releasing peptide (GRP), neuromedin B (P), glucagon, GLP-1, GLP-2, apolipoprotein A-IV, amylin, somatostatin, enterostatin, oxyntomodulin, pancreatic peptide, short-chain fatty acids, butyrate, propionate, acetate, serotonin receptor agonists, nicotinamide adenine dinucleotide (NAD), nicotinamide mononucleotide (NMN), nucleotide riboside (NR), nicotinamide, and nicotinic acid (NA). A molecule may be primarily a metabolic effector, or primarily a satiety effector. Alternatively, a molecule may be both a metabolic and satiety effector.

[0302] In some embodiments, the genetically engineered bacteria of the invention comprise one or more gene(s) or gene cassette(s) which are capable of producing an effector, which can modulate the inflammatory status. Non-limiting examples include short shain fatty acides, and tryptophan and its metabolites, as described herein.

[0303] The effect of the genetically engineered bacteria on the inflammatory status can be measured by methods known in the art, *e.g.*, plasma can be drawn before and after administraton of the genetically engineered bacteria. The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and plasma viscosity (PV) blood tests are commonly used to detect this increase n inflammation. In some embodiments the genetically engineered bacteria modulate, *e.g.* decrease or increase, levels of inflammatory markers, eg. C-reactive protein (CRP).

[0304] In some embodiments, the genetically engineered bacteria modulate, *e.g.* decrease, levels of inflammatory growth factors and cytokines, *e.g.*, IL-1β, IL-6, and/or TNF-α and proinflammatory signaling, *e.g.* NF-kappaB signaling. In some embodiments the genetically engineered bacteria modulate, *e.g.* increase, levels of anti- inflammatory growth factors and cytokines, *e.g.*, IL4, IL-10, IL-13, IFN-alpha and/or transforming growth factor-beta.

[0305] In some embodiments, the genetically engineered bacteria produce effectors, which bind to and stimulate the aromatic hydrocarbon receptor. In some embodiments the genetically engineered bacteria stimulate AHR signaling in immune cell types, including T

cells, B cells, NK cells, macrophages, and dendritic cells (DCs), and/or in epithelial cells. In some embodiments, the genetically engineered bacteria modulate, *e.g.*, increase the levels of IL-22, *e.g.*, through stimulation of AHR.

[0306] In some emobodiments, the genetically engineered bacteria may reduce gut permeability. In some embodiments, the the genetically engineered bacteria may reduce the amounts of LPS and in the circulation, which are increase in metabolic disease, *e.g.*, in NASH..

[0307] In some embodiments, the genetically engineered bacteria may alter glucose metabolism, appetite, weight, and/or blood pressure in a mammal. In some embodiments, the genetically engineered bacteria may reduce liver triglyceride content. In some embodiments, the geneticially engineered bacteria may comprise one or more gene(s) and/or gene cassette(s), which, when expressed, produce metabolites or polypeptides which function to increase fatty acid oxidation, and/or decreasing lipogenesis and/or and improve hepatic glucose metabolism.

[0308] In some embodiments, the geneticially engineered bacteria may comprise one or more gene(s) and/or gene cassette(s), which, when expressed, produce metabolites or polypeptides which function to stimulate glucose-dependent insulin secretion in pancreatic β-cells.

[0309] In some embodiments, the geneticially engineered bacteria may comprise one or more gene(s) and/or gene cassette(s), which, when expressed, produce bile salt, which, when expressed, produce metabolites or polypeptides which function to convert excess bile salts into non-toxic molecules in order to treat and/or prevent disorders associated with bile salts, such as cardiovascular disease, metabolic disease, cirrhosis, cancer, liver disease, and C. difficile infection. .

[0310] The gene or gene cassette for producing the metabolic and/or satiety effector molecule and/or modulator of inflammation may be expressed under the control of a constitutive promoter, a promoter that is induced by exogenous environmental conditions, a promoter that is induced by exogenous environmental conditions, molecules, or metabolites specific to the gut of a mammal, and/or a promoter that is induced by low-oxygen or anaerobic conditions, such as the environment of the mammalian gut.

[0311] The gene or gene cassette for producing the metabolic and/or satiety effector and/or modulator of inflammation may be expressed on a high-copy plasmid, a low-copy plasmid, or a chromosome. In some embodiments, expression from the plasmid may be useful for increasing expression of the metabolic and/or satiety effector molecule. In some

embodiments, expression from the chromosome may be useful for increasing stability of expression of the metabolic and/or satiety effector molecule. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is integrated into the bacterial chromosome at one or more integration sites in the genetically engineered bacteria. For example, one or more copies of the propionate biosynthesis gene cassette may be integrated into the bacterial chromosome. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is expressed from a plasmid in the genetically engineered bacteria. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is inserted into the bacterial genome at one or more of the following insertion sites in *E. coli* Nissle: malE/K, araC/BAD, lacZ, thyA, malP/T. Any suitable insertion site may be used (see, *e.g.* **FIG. 42**). The insertion site may be anywhere in the genome, *e.g.*, in a gene required for survival and/or growth, such as thyA (to create an auxotroph); in an active area of the genome, such as near the site of genome replication; and/or in between divergent promoters in order to reduce the risk of unintended transcription, such as between AraB and AraC of the arabinose operon. In some embodiments, the genetically engineered bacteria of the invention are capable of expressing a metabolic and/or satiety effector molecule that is encoded by a single gene, *e.g.*, the molecule is GLP-1 and encoded by the GLP-1 gene.

[0312] One of skill in the art would appreciate that additional genes and gene cassettes capable of producing metabolic and/or satiety effector molecules and/or modulator of inflammation are known in the art and may be expressed by the genetically engineered bacteria of the invention. In some embodiments, the gene or gene cassette for producing a therapeutic molecule also comprises additional transcription and translation elements, *e.g.*, a ribosome binding site, to enhance expression of the therapeutic molecule.

[0313] In some embodiments, the genetically engineered bacteria produce two or more metabolic and/or satiety effector molecules and/or modulator of inflammation. In certain embodiments, the two or more molecules behave synergistically to ameliorate metabolic and/or liver disease, e.g., NASH and NAFLD. In some embodiments, the genetically engineered bacteria express at least one metabolic effector molecule and at least one satiety effector molecule and at least one modulator of inflammation.

Short Chain Fatty Acids

[0314] Short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate, are metabolites formed by gut microbiota from complex dietary carbohydrates. Butyrate and acetate were reported to protect against diet-induced obesity without causing hypophagia,

while propionate was shown to reduce food intake. In rodent models of genetic or diet-induced obesity, supplementation of butyrate in diet, and oral administration of acetate was shown to suppress weight gain independent of food intake suppression; Propionate was reported to inhibit food intake in humans (see, *e.g.*, Lin et al., Butyrate and Propionate Protect against Diet-Induced Obesity and Regulate Gut Hormones via Free Fatty Acid Receptor 3-Independent Mechanisms, and refernces therein). Therefore, the production of SCFAs is likely efficacious in the treatment of metabolic syndrome and related disorders, and/or diabetes type2, and/or obesity.

[0315] SCFAs represent a major constituent of the luminal contents of the colon. Among SCFAs butyrate is believed to play an important role for epithelial homeostasis. Acetate and propionate have anti-inflammatory properties, which are comparable to those of butyrate (Tedelind et al., World J Gastroenterol. 2007 May 28; 13(20): 2826–2832. Acetate and propionate, similar to butyrate, inhibit TNFα-mediated activation of the NF-κB pathway. These findings suggest that propionate and acetate, in addition to butyrate, could be efficacious in the treatment of inflammatory conditions.

[0316] As such, strategy in the treatment, prevention, and/or management of NASH may include approaches to help maintain and/or reestablish gut barrier function, e.g. through the prevention, treatment and/or management of inflammatory events at the root of increased permeability, e.g. through the administration of anti-inflammatory effectors and /or gut barrier effectors.

[0317] For example, leading metabolites that play gut-protective roles are short chain fatty acids, e.g. acetate, butyrate and propionate, and those derived from tryptophan metabolism. These metabolites have been shown to play a major role in the prevention of inflammatory disease. As such one approach in the treatment, prevention, and/or management of gut barrier health may be to provide a treatment which contains one or more of such metabolites.

[0318] For example, butyrate and other SCFA, e.g., derived from the microbiota, are known to promote maintaining intestinal integrity (e.g., as reviewed in Thorburn et al., Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases; Immunity Volume 40, Issue 6, 19 June 2014, Pages 833–842). (A) SCFA-induced promotion of mucus by gut epithelial cells, possibly through signaling through metabolite sensing GPCRs; (B) SCFA-induced secretion of IgA by B cells; (C) SCFA-induced promotion of tissue repair and wound healing; (D) SCFA-induced promotion of Treg cell development in the gut in a process that presumably facilitates immunological tolerance; (E) SCFA- mediated enhancement of

epithelial integrity in a process dependent on inflammasome activation (e.g., via NALP3) and IL-18 production; and (F) anti-inflammatory effects, inhibition of inflammatory cytokine production (e.g., TNF, Il-6, and IFN-gamma), and inhibition of NF-κB. Many of these actions of SCFAs in gut homeostatis can be ascribed to GPR43 and GPR109A, which are expressed by the colonic epithelium, by inflammatory leukocytes (e.g. neutrophils and marcophages) and by Treg cells. These receptors signal through G proteins, coupled to MAPK, PI3K and mTOR, as well as a separate arrestin- pathway, leading to NFkappa B inhibition. Other effects can be ascribed to SCFA-mediated HDAC inhibition, e.g. butyrate, which may regulate macrophage function and promote TReg cells.

[0319] In addition, a number of tryptophan metabolites, including kynurenine and kynurenic acid, as well as several indoles, such as indole-3 aldehyde, indole-3 propionic acid, and several other indole metabolites (which can be derived from microbiota or the diet) described infra, have been shown to be essential for gut homeostais and promote gut-barrier health. These metabolites bind to aryl hydrocarbon receptor (Ahr). After agonist binding, AhR translocates to the nucleus, where it forms a heterodimer with AhR nuclear translocator (ARNT). AhR-dependent gene expression includes genes involved in the production of mediators important for gut homeostasis; these mediators include IL-22, antimicrobicidal factors, increased Th17 cell activity, and the maintenance of intraepithelial lymphocytes and RORγt+ innate lymphoid cells.

[0320] Tryptophan can also be transported across the epithelium by transport machinery comprising angiotensin I converting enzyme 2 (Ace2). Tryptophan is degraded to kynurenine, another AhR agonist, by the immune-regulatory enzyme indoleamine 2,3-dioxygenase (IDO), which is linked to suppression of T cell responses, promotion of Treg cells, and immune tolerance. Moreover, a number of tryptophan metabolites, including kynurenic acid and niacin, agonize metabolite-sensing GPCRs, such as GPR35 and GPR109A and thus multiple elements of tryptophan catabolism facilitate gut homeostasis.

[0321] In addition, some indole metabolites, e.g., indole 3-propionic acid (IPA), may exert their effect an acitvating ligand of Pregnane X receptor (PXR), which is thought to play a key role as an essential regulator of intestinal barrier function, through downregulation of TLR4 signaling (Venkatesh et al., 2014 Symbiotic Bacterial Metabolites Regulate Gastrointestinal Barrier Function via the Xenobiotic Sensor PXR and Toll-like Receptor 4; Immunity 41, 296–310, August 21, 2014). As a result, indole levels may through the activation of PXR regulate and balance the levels of TLR4 expression to promote homeostasis and gut barrier health.

[0322] Thus, in some embodiments, the genetically engineered bacteria of the disclosure produce one or more short chain fatty acids and/or one or more tryprophan metabolites.

Acetate

[0323] In some embodiments, the genetically engineered bacteria of the invention comprise an acetate gene cassette and are capable of producing acetate. The genetically engineered bacteria may include any suitable set of acetate biosynthesis genes. In other embodiments, the bacteria comprise an endogenous acetate biosynthetic gene or gene cassette and naturally produce acetate. Unmodified bacteria comprising acetate biosynthesis genes are known in the art and are capable of consuming various substrates to produce acetate under aerobic and/or anaerobic conditions (*see, e.g.,* Ragsdale, 2008), and these endogenous acetate biosynthesis pathways may be a source of genes for the genetically engineered bacteria of the invention. In some embodiments, the genetically engineered bacteria of the invention comprise acetate biosynthesis genes from a different species, strain, or substrain of bacteria. In some embodiments, the native acetate biosynthesis genes in the genetically engineered bacteria are enhanced. In some embodiments, the genetically engineered bacteria comprise aerobic acetate biosynthesis genes, e.g., from *Escherichia coli*. In some embodiments, the genetically engineered bacteria comprise anaerobic acetate biosynthesis genes, e.g., from *Acetitomaculum, Acetoanaerobium, Acetohalobium, Acetonema, Balutia, Butyribacterium, Clostridium, Moorella, Oxobacter, Sporomusa,* and/or *Thermoacetogenium*. The genetically engineered bacteria may comprise genes for aerobic acetate biosynthesis or genes for anaerobic or microaerobic acetate biosynthesis. In some embodiments, the genetically engineered bacteria comprise both aerobic and anaerobic or microaerobic acetate biosynthesis genes. In some embodiments, the genetically engineered bacteria comprise a combination of acetate biosynthesis genes from different species, strains, and/or substrains of bacteria, and are capable of producing acetate. In some embodiments, one or more of the acetate biosynthesis genes is functionally replaced, modified, and/or mutated in order to enhance stability and/or acetate production. In some embodiments, the genetically engineered bacteria are capable of expressing the acetate biosynthesis cassette and producing acetate under inducing conditions. In some embodiments, the genetically engineered bacteria are capable of producing an alternate short-chain fatty acid.

[0316] In E. coli Nissle, acetate is generated as an end product of fermentation. In E coli, glucose fermentation occurs in two steps, (1) the glycolysis reactions and (2) the NADH recycling reactions, i.e. these reactions re-oxidize the NAD+ generated during the

fermentation process. E. coli employs the "mixed acid" fermentation pathway (see, e.g., **FIG 25**). Through the "mixed acid" pathway, E coli generates several alternative end products and in variable amounts (e.g., lactate, acetate, formate, succinate, ethanol, carbon dioxide, and hydrogen) though various arms of the fermentation pathway, e.g., as shown in **FIG. 25**. Without wishing to be bound by theory, prevention or reduction of flux through one or more metabolic arm(s) generating metabolites other than acetate, e.g. through mutation, deletion and/or inhibition of one or more gene(s) encoding key enzymes in these metabolic arms, results in an increase in production of acetate for NAD recycling. As disclosed herein, e.g., in Example 20, deletions in gene(s) encoding such enzymes increase acetate production. Such enzymes include fumarate reductase (encoded by the frd genes), lactate dehydrogenase (encoded by the ldh gene), and aldehyde-alcohol dehydrogenase (encoded by the adhE gene).

[0317] LdhA is a soluble NAD-linked lactate dehydrogenase (LDH) that is specific for the production of D-lactate and is a homotetramer and shows positive homotropic cooperativity under higher pH conditions. E. coli carrying ldhA mutations show no observable growth defect and can still ferment sugars to a variety of products other than lactate.

[0318] In some embodiments, the genetically engineered bacteria producing acetate comprise a mutation and/or deletion in the endogenous ldhA gene, thereby reducing or eliminating the activity of ldhA.

[0319] AdhE is a homopolymeric protein with three catalytic functions: alcohol dehydrogenase, coenzyme A-dependent acetaldehyde dehydrogenase, and pyruvate formate-lyase deactivase. During fermentation, AdhE has catalyzes two steps towards the generation of ethanol: (1) the reduction of acetyl-CoA to acetaldehyde and (2) the reduction of acetaldehyde to to ethanol. Deletion of adhE has been employed to enhance production of certain metabolites inducstrially, including succinate, D-lactate, and polyhydroxyalkanoates (Singh et al, Manipulating redox and ATP balancing for improved production of succinate in E. coli.; Metab Eng. 2011 Jan;13(1):76-81; Zhou et al., Evaluation of genetic manipulation strategies on D-lactate production by Escherichia coli, Curr Microbiol. 2011 Mar;62(3):981-9; Jian et al., Production of polyhydroxyalkanoates by Escherichia coli mutants with defected mixed acid fermentation pathways, Appl Microbiol Biotechnol. 2010 Aug;87(6):2247-56).

[0320] In some embodiments, the genetically engineered bacteria producing acetate comprise a mutation and/or deletion in the endogenous adhE gene thereby reducing or eliminating the activity of AdhE.

[0321] The fumarate reductase enzyme complex, encoded by the frdABCD operon, allows Escherichia coli to utilize fumarate as a terminal electron acceptor for anaerobic oxidative phosphorylation. FrdA is one of two catalytic subunits in the four subunit fumarate reductase complex. FrdB is the second catalytic subunit of the complex. FrdC and FrdD are two integral membrane protein components of the fumarate reductase complex. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous frdA gene, thereby reducing or eliminating the activity of FrdA.

[0322] In some embodiments, the genetically engineered bacteria producing acetate comprise a mutation and/or deletion in the endogenous FrdA gene. In some embodiments, the genetically engineered bacteria producing acetate comprise a mutation and/or deletion in the endogenous FrdB, FrdC, and/or FrdD gene(s thereby reducing or eliminating the activity of FrdB, FrdC, and/or FrdD.

[0323] In some embodiments, the genetically engineered bacteria producing acetate comprise a mutation and/or deletion in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous ldhA and rdA genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes.

[0324] In some embodiments, the genetically engineered bacteria comprising one or more of these mutations also comprise a butyrate cassette.

[0325] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-

fold, or fifty-fold, more acetate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0326] In certain situations, the need may arise to prevent and/or reduce acetate production by of an engineered or naturally occurring strain, e.g., E. coli Nissle. Without wishing to be bound by theory, one or more mutations and/or deletions in one or more gene(s) encoding one or more enzyme(s) which function in the acetate producing metabolic arm of fermentation should reduce and/or prevent production of acetate.

[0327] Phosphate acetyltransferase (Pta) catalyzes the reversible conversion between acetyl-CoA and acetylphosphate, a step in the metabolism of acetate (Campos-Bermudez et al., Functional dissection of Escherichia coli phosphotransacetylase structural domains and analysis of key compounds involved in activity regulation; FEBS J. 2010 Apr;277(8):1957-66). Both pyruvate and phosphoenolpyruvate activate the enzyme in the direction of acetylphosphate synthesis and inhibit the enzyme in the direction of acetyl-CoA synthesis. The acetate formation from acetyl-CoA I pathway has been the target of metabolic engineering to reduce the flux to acetate and increase the production of commercially desired end products (see, e.g., Singh, et al., Manipulating redox and ATP balancing for improved production of succinate in E. coli; Metab Eng. 2011 Jan;13(1):76-81). A pta mutant does not grow on acetate as the sole source of carbon (Brown et al., The enzymic interconversion of acetate and acetyl-coenzyme A in Escherichia coli; J Gen Microbiol. 1977 Oct;102(2):327-36).

[0328] In some embodiments, the genetically engineered bacteria produce lower amounts of acetate than the amounts produced by the wild type bacterium under the same conditions.

[0329] In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta and ldhA genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise a mutation

and/or deletion in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0330] In some embodiments, the genetically engineered bacteria further comprise one or more gene cassettes for the production of butyrate.

[0331] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% less acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold less acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, less acetate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0332] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of acetate is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with NASH, such as hyperammonemia or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with hyperammonemia or liver damage, e.g., as seen in NASH.

[0333] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more

chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0334] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides and/or comprising one or more mutations or deletions in endogenous genes for the production of acetate is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of acetate is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of acetate is modified and/or mutated, e.g., to enhance stability, or increase acetate production.

[0335] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of acetate may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of acetate are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0336] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences described herein for the consumption of ammonia. Suitable gene sequences and circuits for the consumption of ammonia are described in Pending International Patent Application PCT/US2015/64140

(published as WO/2016/090343) and Pending International Patent Application PCT/US2016/34200, the contents of each of which is herein incorporated by reference in its entirety.

[0337] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0338] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0339] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production or catabolism of tryptophan and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of acetate further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

Propionate

[0340] In alternate embodiments, the genetically engineered bacteria of the invention are capable of producing a metabolic and/or satiety effector molecule, *e.g.*, propionate, that is synthesized by a biosynthetic pathway requiring multiple genes and/or enzymes.

[0341] In some embodiments, the genetically engineered bacteria of the invention comprise a propionate gene cassette and are capable of producing propionate under particular exogenous environmental conditions. The bacterial cells described herein comprise a propionate gene cassette and are capable of producing propionate in order to treat liver disease, such as NASH. In one embodiment, the propionate gene cassette increases the level of propionate in the cell or in the subject as compared to the level of propionate in the cell or in the subject prior to expression of the propionate gene cassette.

[0342] The genetically engineered bacteria may express any suitable set of propionate biosynthesis genes (*see, e.g.,* **Table 2**). Unmodified bacteria that are capable of producing propionate via an endogenous propionate biosynthesis pathway include, but are not limited to, *Clostridium propionicum*, *Megasphaera elsdenii*, and *Prevotella ruminicola*. In some embodiments, the genetically engineered bacteria of the invention comprise propionate biosynthesis genes from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise the genes *pct, lcd,* and *acr* from *Clostridium propionicum.* In some embodiments, the genetically engineered bacteria comprise acrylate pathway genes for propionate biosynthesis, *e.g.*, *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*. In some embodiments, the rate limiting step catalyzed by the Acr enzyme, is replaced by the AcuI from R. sphaeroides, which catalyzes the NADPH-dependent acrylyl-CoA reduction to produce propionyl-CoA. Thus the propionate cassette comprises pct, lcdA, lcdB, lcdC, and acuI. In another embodiment, the homolog of AcuI in E coli, yhdH is used. This the propionate cassette comprises pct, lcdA, lcdB, lcdC, and yhdH. In alternate embodiments, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, *e.g.*, $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, and *lpd,* and optionally further comprise *tesB*. In another embodiment, the propionate gene cassette comprises the genes of the Sleepting Beauty Mutase operon, *e.g.*, from *E. coli* (sbm, ygfD, ygfG, ygfH). The SBM pathway is cyclical and composed of a series of biochemical conversions forming propionate as a fermentative product while regenerating the starting molecule of succinyl-CoA. Sbm converts succinyl CoA to L-methylmalonylCoA, ygfG converts L-methylmalonylCoA into PropionylCoA, and ygfH converts propionylCoA into propionate and succinate into succinylCoA.

[0343] This pathway is very similar to the oxidative propionate pathway of Propionibacteria, which also converts succinate to propionate. Succinyl-CoA is converted to R-methylmalonyl-CoA by methymalonyl-CoA mutase (mutAB). This is in turn converted to S-methylmalonyl-CoA via methymalonyl-CoA epimerase (GI:18042134). There are three genes which encode methylmalonyl-CoA carboxytransferase (mmdA, PFREUD_18870, bccp) which converts methylmalonyl-CoA to propionyl-CoA.

[0344] The genes may be codon-optimized, and translational and transcriptional elements may be added. **Tables 1-3** lists the nucleic acid sequences of exemplary genes in the propionate biosynthesis gene cassette. **Table 4** lists the polypeptide sequences expressed by exemplary propionate biosynthesis genes.

Table 1. Propionate Cassette Sequences (Acrylate Pathway)

Gene sequence	Description
pct **SEQ ID NO: 1**	ATGCGCAAAGTGCCGATTATCACGGCTGACGAGGCCGCAAAAC TGATCAAGGACGGCGACACCGTGACAACTAGCGGCTTTGTGGGT AACGCGATCCCTGAGGCCCTTGACCGTGCAGTCGAAAAGCGTTT CCTGGAAACGGGCGAACCGAAGAACATTACTTATGTATATTGCG GCAGTCAGGGCAATCGCGACGGTCGTGGCGCAGAACATTTCGC GCATGAAGGCCTGCTGAAACGTTATATCGCTGGCCATTGGGCGA CCGTCCCGGCGTTAGGGAAAATGGCCATGGAGAATAAAATGGA GGCCTACAATGTCTCTCAGGGCGCCTTGTGTCATCTCTTTCGCGA TATTGCGAGCCATAAACCGGGTGTGTTCACGAAAGTAGGAATCG GCACCTTCATTGATCCACGTAACGGTGGTGGGAAGGTCAACGAT ATTACCAAGGAAGATATCGTAGAACTGGTGGAAATTAAAGGGC AGGAATACCTGTTTTATCCGGCGTTCCCGATCCATGTCGCGCTG ATTCGTGGCACCTATGCGGACGAGAGTGGTAACATCACCTTTGA AAAAGAGGTAGCGCCTTTGGAAGGGACTTCTGTCTGTCAAGCGG TGAAGAACTCGGGTGGCATTGTCGTGGTTCAGGTTGAGCGTGTC GTCAAAGCAGGCACGCTGGATCCGCGCCATGTGAAAGTTCCGG GTATCTATGTAGATTACGTAGTCGTCGCGGATCCGGAGGACCAT CAACAGTCCCTTGACTGCGAATATGATCCTGCCCTTAGTGGAGA GCACCGTCGTCCGGAGGTGGTGGGTGAACCACTGCCTTTATCCG CGAAGAAAGTCATCGGCCGCCGTGGCGCGATTGAGCTCGAGAA AGACGTTGCAGTGAACCTTGGGGTAGGTGCACCTGAGTATGTGG CCTCCGTGGCCGATGAAGAAGGCATTGTGGATTTTATGACTCTC ACAGCGGAGTCCGGCGCTATCGGTGGCGTTCCAGCCGGCGGTGT TCGCTTTGGGGCGAGCTACAATGCTGACGCCTTGATCGACCAGG GCTACCAATTTGATTATTACGACGGTGGGGGTCTGGATCTTTGTT ACCTGGGTTTAGCTGAATGCGACGAAAAGGGTAATATCAATGTT AGCCGCTTCGGTCCTCGTATCGCTGGGTGCGGCGGATTCATTAA CATTACCCAAAACACGCCGAAAGTCTTCTTTTGTGGGACCTTTA CAGCCGGGGGGCTGAAAGTGAAAATTGAAGATGGTAAGGTGAT TATCGTTCAGGAAGGGAAACAGAAGAAATTCCTTAAGGCAGTG GAGCAAATCACCTTTAATGGAGACGTGGCCTTAGCGAACAAGC

Gene sequence	Description
	AACAAGTTACCTACATCACGGAGCGTTGCGTCTTCCTCCTCAAA GAAGACGGTTTACACCTTTCGGAAATCGCGCCAGGCATCGATCT GCAGACCCAGATTTTGGATGTTATGGACTTTGCCCCGATCATTG ATCGTGACGCAAACGGGCAGATTAAACTGATGGACGCGGCGTT ATTCGCAGAAGGGCTGATGGGCTTGAAAGAAATGAAGTCTTAA
lcdA **SEQ ID NO: 2**	ATGAGCTTAACCCAAGGCATGAAAGCTAAACAACTGTTAGCAT ACTTTCAGGGTAAAGCCGATCAGGATGCACGTGAAGCGAAAGC CCGCGGTGAGCTGGTCTGCTGGTCGGCGTCAGTCGCGCCGCCGG AATTTTGCGTAACAATGGGCATTGCCATGATCTACCCGGAGACT CATGCAGCGGGCATCGGTGCCCGCAAAGGTGCGATGGACATGC TGGAAGTTGCGGACCGCAAAGGCTACAACGTGGATTGTTGTTCC TACGGCCGTGTAAATATGGGTTACATGGAATGTTTAAAAGAAGC CGCCATCACGGGCGTCAAGCCGGAAGTTTTGGTTAATTCCCCTG CTGCTGACGTTCCGCTTCCCGATTTGGTGATTACGTGTAATAATA TCTGTAACACGCTGCTGAAATGGTACGAAAACTTAGCAGCAGA ACTCGATATTCCTTGCATCGTGATCGACGTACCGTTTAATCATAC CATGCCGATTCCGGAATATGCCAAGGCCTACATCGCGGACCAGT TCCGCAATGCAATTTCTCAGCTGGAAGTTATTTGTGGCCGTCCGT TCGATTGGAAGAAATTTAAGGAGGTCAAAGATCAGACCCAGCG TAGCGTATACCACTGGAACCGCATTGCCGAGATGGCGAAATAC AAGCCTAGCCCGCTGAACGGCTTCGATCTGTTCAATTACATGGC GTTAATCGTGGCGTGCCGCAGCCTGGATTATGCAGAAATTACCT TTAAAGCGTTCGCGGACGAATTAGAAGAGAATTTGAAGGCGGG TATCTACGCCTTTAAAGGTGCGGAAAAAACGCGCTTTCAATGGG AAGGTATCGCGGTGTGGCCACATTTAGGTCACACGTTTAAATCT ATGAAGAATCTGAATTCGATTATGACCGGTACGGCATACCCCGC CCTTTGGGACCTGCACTATGACGCTAACGACGAATCTATGCACT CTATGGCTGAAGCGTACACCCGTATTTATATTAATACTTGTCTGC AGAACAAAGTAGAGGTCCTGCTTGGGATCATGGAAAAAGGCCA GGTGGATGGTACCGTATATCATCTGAATCGCAGCTGCAAACTGA TGAGTTTCCTGAACGTGGAAACGGCTGAAATTATTAAAGAGAA GAACGGTCTTCCTTACGTCTCCATTGATGGCGATCAGACCGATC CTCGCGTTTTTTCTCCGGCCCAGTTTGATACCCGTGTTCAGGCCC TGGTTGAGATGATGGAGGCCAATATGGCGGCAGCGGAATAA
lcdB **SEQ ID NO: 3**	ATGTCACGCGTGGAGGCAATCCTGTCGCAGCTGAAAGATGTCGC CGCGAATCCGAAAAAAGCCATGGATGACTATAAAGCTGAAACA GGTAAGGGCGCGGTTGGTATCATGCCGATCTACAGCCCCGAAG AAATGGTACACGCCGCTGGCTATTTGCCGATGGGAATCTGGGGC GCCCAGGGCAAAACGATTAGTAAAGCGCGCACCTATCTGCCTGC TTTTGCCTGCAGCGTAATGCAGCAGGTTATGGAATTACAGTGCG AGGGCGCGTATGATGACCTGTCCGCAGTTATTTTTAGCGTACCG TGCGACACTCTCAAATGTCTTAGCCAGAAATGGAAAGGTACGTC CCCAGTGATTGTATTTACGCATCCGCAGAACCGCGGATTAGAAG CGGCGAACCAATTCTTGGTTACCGAGTATGAACTGGTAAAAGCA

Gene sequence	Description
	CAACTGGAATCAGTTCTGGGTGTGAAAATTTCAAACGCCGCCCT GGAAAATTCGATTGCAATTTATAACGAGAATCGTGCCGTGATGC GTGAGTTCGTGAAAGTGGCAGCGGACTATCCTCAAGTCATTGAC GCAGTGAGCCGCCACGCGGTTTTTAAAGCGCGCCAGTTTATGCT TAAGGAAAAACATACCGCACTTGTGAAAGAACTGATCGCTGAG ATTAAAGCAACGCCAGTCCAGCCGTGGGACGGAAAAAAGGTTG TAGTGACGGGCATTCTGTTGGAACCGAATGAGTTATTAGATATC TTTAATGAGTTTAAGATCGCGATTGTTGATGATGATTTAGCGCA GGAAAGCCGTCAGATCCGTGTTGACGTTCTGGACGGAGAAGGC GGACCGCTCTACCGTATGGCTAAAGCGTGGCAGCAAATGTATGG CTGCTCGCTGGCAACCGACACCAAGAAGGGTCGCGGCCGTATGT TAATTAACAAAACGATTCAGACCGGTGCGGACGCTATCGTAGTT GCAATGATGAAGTTTTGCGACCCAGAAGAATGGGATTATCCGGT AATGTACCGTGAATTTGAAGAAAAAGGGGTCAAATCACTTATG ATTGAGGTGGATCAGGAAGTATCGTCTTTCGAACAGATTAAAAC CCGTCTGCAGTCATTCGTCGAAATGCTTTAA
lcdC **SEQ ID NO: 4**	ATGTATACCTTGGGGATTGATGTCGGTTCTGCCTCTAGTAAAGC GGTGATTCTGAAAGATGGAAAAGATATTGTCGCTGCCGAGGTTG TCCAAGTCGGTACCGGCTCCTCGGGTCCCCAACGCGCACTGGAC AAAGCCTTTGAAGTCTCTGGCTTAAAAAAGGAAGACATCAGCTA CACAGTAGCTACGGGCTATGGGCGCTTCAATTTTAGCGACGCGG ATAAACAGATTTCGGAAATTAGCTGTCATGCCAAAGGCATTTAT TTCTTAGTACCAACTGCGCGCACTATTATTGACATTGGCGGCCA AGATGCGAAAGCCATCCGCCTGGACGACAAGGGGGGTATTAAG CAATTCTTCATGAATGATAAATGCGCGGCGGGCACGGGGCGTTT CCTGGAAGTCATGGCTCGCGTACTTGAAACCACCCTGGATGAAA TGGCTGAACTGGATGAACAGGCGACTGACACCGCTCCCATTTCA AGCACCTGCACGGTTTTCGCCGAAAGCGAAGTAATTAGCCAATT GAGCAATGGTGTCTCACGCAACAACATCATTAAAGGTGTCCATC TGAGCGTTGCGTCACGTGCGTGTGGTCTGGCGTATCGCGGCGGT TTGGAGAAAGATGTTGTTATGACAGGTGGCGTGGCAAAAAATG CAGGGGTGGTGCGCGCGGTGGCGGGCGTTCTGAAGACCGATGT TATCGTTGCTCCGAATCCTCAGACGACCGGTGCACTGGGGGCAG CGCTGTATGCTTATGAGGCCGCCCAGAAGAAGTA
etfA **SEQ ID NO: 5**	ATGGCCTTCAATAGCGCAGATATTAATTCTTTCCGCGATATTTGG GTGTTTTGTGAACAGCGTGAGGGCAAACTGATTAACACCGATTT CGAATTAATTAGCGAAGGTCGTAAACTGGCTGACGAACGCGGA AGCAAACTGGTTGGAATTTTGCTGGGGCACGAAGTTGAAGAAA TCGCAAAAGAATTAGGCGGCTATGGTGCGGACAAGGTAATTGT GTGCGATCATCCGGAACTTAAATTTTACACTACGGATGCTTATG CCAAAGTTTTATGTGACGTCGTGATGGAAGAGAAACCGGAGGT AATTTTGATCGGTGCCACCAACATTGGCCGTGATCTCGGACCGC GTTGTGCTGCACGCTTGCACACGGGGCTGACGGCTGATTGCACG CACCTGGATATTGATATGAATAAATATGTGGACTTTCTTAGCAC CAGTAGCACCTTGGATATCTCGTCGATGACTTTCCCTATGGAAG ATACAAACCTTAAAATGACGCGCCCTGCATTTGGCGGACATCTG ATGGCAACGATCATTTGTCCACGCTTCCGTCCCTGTATGAGCAC AGTGCGCCCCGGAGTGATGAAGAAAGCGGAGTTCTCGCAGGAG

Gene sequence	Description
	ATGGCGCAAGCATGTCAAGTAGTGACCCGTCACGTAAATTTGTC GGATGAAGACCTTAAAACTAAAGTAATTAATATCGTGAAGGAA ACGAAAAAGATTGTGGATCTGATCGGCGCAGAAATTATTGTGTC AGTTGGTCGTGGTATCTCGAAAGATGTCCAAGGTGGAATTGCAC TGGCTGAAAAACTTGCGGACGCATTTGGTAACGGTGTCGTGGGC GGCTCGCGCGCAGTGATTGATTCCGGCTGGTTACCTGCGGATCA TCAGGTTGGACAAACCGGTAAGACCGTGCACCCGAAAGTCTAC GTGGCGCTGGGTATTAGTGGGGCTATCCAGCATAAGGCTGGGAT GCAAGACTCTGAACTGATCATTGCCGTCAACAAAGACGAAACG GCGCCTATCTTCGACTGCGCCGATTATGGCATCACCGGTGATTT ATTTAAAATCGTACCGATGATGATCGACGCGATCAAAGAGGGT AAAAACGCATGA
acrB **SEQ ID NO: 6**	ATGCGCATCTATGTGTGTGTGAAACAAGTCCCAGATACGAGCGG CAAGGTGGCCGTTAACCCTGATGGGACCCTTAACCGTGCCTCAA TGGCAGCGATTATTAACCCGGACGATATGTCCGCGATCGAACAG GCATTAAAACTGAAAGATGAAACCGGATGCCAGGTTACGGCGC TTACGATGGGTCCTCCTCCTGCCGAGGGCATGTTGCGCGAAATT ATTGCAATGGGGGCCGACGATGGTGTGCTGATTTCGGCCCGTGA ATTTGGGGGGTCCGATACCTTCGCAACCAGTCAAATTATTAGCG CGGCAATCCATAAATTAGGCTTAAGCAATGAAGACATGATCTTT TGCGGTCGTCAGGCCATTGACGGTGATACGGCCCAAGTCGGCCC TCAAATTGCCGAAAAACTGAGCATCCCACAGGTAACCTATGGCG CAGGAATCAAAAAATCTGGTGATTTAGTGCTGGTGAAGCGTATG TTGGAGGATGGTTATATGATGATCGAAGTCGAAACTCCATGTCT GATTACCTGCATTCAGGATAAAGCGGTAAAACCACGTTACATGA CTCTCAACGGTATTATGGAATGCTACTCCAAGCCGCTCCTCGTTC TCGATTACGAAGCACTGAAAGATGAACCGCTGATCGAACTTGAT ACCATTGGGCTTAAAGGCTCCCCGACGAATATCTTTAAATCGTT TACGCCGCCTCAGAAAGGCGTTGGTGTCATGCTCCAAGGCACCG ATAAGGAAAAAGTCGAGGATCTGGTGGATAAGCTGATGCAGAA ACATGTCATCTAA
acrC **SEQ ID NO: 7**	ATGTTCTTACTGAAGATTAAAAAAGAACGTATGAAACGCATGG ACTTTAGTTTAACGCGTGAACAGGAGATGTTAAAAAAACTGGCG CGTCAGTTTGCTGAGATCGAGCTGGAACCGGTGGCCGAAGAGA TTGATCGTGAGCACGTTTTTCCTGCAGAAAACTTTAAGAAGATG GCGGAAATTGGCTTAACCGGCATTGGTATCCCGAAAGAATTTGG TGGCTCCGGTGGAGGCACCCTGGAGAAGGTCATTGCCGTGTCAG AATTCGGCAAAAAGTGTATGGCCTCAGCTTCCATTTTAAGCATT CATCTTATCGCGCCGCAGGCAATCTACAAATATGGGACCAAAGA ACAGAAAGAGACGTACCTGCCGCGTCTTACCAAAGGTGGTGAA CTGGGCGCCTTTGCGCTGACAGAACCAAACGCCGGAAGCGATG CCGGCGCGGTAAAAACGACCGCGATTCTGGACAGCCAGACAAA CGAGTACGTGCTGAATGGCACCAAATGCTTTATCAGCGGGGGCG GGCGCGCGGGTGTTCTTGTAATTTTTGCGCTTACTGAACCGAAA AAAGGTCTGAAAGGGATGAGCGCGATTATCGTGGAGAAAGGGA CCCCGGGCTTCAGCATCGGCAAGGTGGAGAGCAAGATGGGGAT CGCAGGTTCGGAAACCGCGGAACTTATCTTCGAAGATTGTCGCG TTCCGGCTGCCAACCTTTTAGGTAAAGAAGGCAAAGGCTTTAAA

Gene sequence	Description
	ATTGCTATGGAAGCCCTGGATGGCGCCCGTATTGGCGTGGGCGC TCAAGCAATCGGAATTGCCGAGGGGGCGATCGACCTGAGTGTG AAGTACGTTCACGAGCGCATTCAATTTGGTAAACCGATCGCGAA TCTGCAGGGAATTCAATGGTATATCGCGGATATGGCGACCAAAA CCGCCGCGGCACGCGCACTTGTTGAGTTTGCAGCGTATCTTGAA GACGCGGGTAAACCGTTCACAAAGGAATCTGCTATGTGCAAGCT GAACGCCTCCGAAAACGCGCGTTTTGTGACAAATTTAGCTCTGC AGATTCACGGGGGTTACGGTTATATGAAAGATTATCCGTTAGAG CGTATGTATCGCGATGCTAAGATTACGGAAATTTACGAGGGGAC ATCAGAAATCCATAAGGTGGTGATTGCGCGTGAAGTAATGAAA CGCTAA
thrA^{fbr} **SEQ ID NO: 8**	ATGCGAGTGTTGAAGTTCGGCGGTACATCAGTGGCAAATGCAG AACGTTTTCTGCGTGTTGCCGATATTCTGGAAAGCAATGCCAGG CAGGGGCAGGTGGCCACCGTCCTCTCTGCCCCCGCCAAAATCAC CAACCACCTGGTGGCGATGATTGAAAAAACCATTAGCGGCCAG GATGCTTTACCCAATATCAGCGATGCCGAACGTATTTTTGCCGA ACTTTTGACGGGACTCGCCGCCGCCCAGCCGGGGTTCCCGCTGG CGCAATTGAAAACTTTCGTCGATCAGGAATTTGCCCAAATAAAA CATGTCCTGCATGGCATTAGTTTGTTGGGGCAGTGCCCGGATAG CATCAACGCTGCGCTGATTTGCCGTGGCGAGAAAATGTCGATCG CCATTATGGCCGGCGTATTAGAAGCGCGCGGTCACAACGTTACT GTTATCGATCCGGTCGAAAAACTGCTGGCAGTGGGGCATTACCT CGAATCTACCGTCGATATTGCTGAGTCCACCCGCCGTATTGCGG CAAGCCGCATTCCGGCTGATCACATGGTGCTGATGGCAGGTTTC ACCGCCGGTAATGAAAAAGGCGAACTGGTGGTGCTTGGACGCA ACGGTTCCGACTACTCTGCTGCGGTGCTGGCTGCCTGTTTACGC GCCGATTGTTGCGAGATTTGGACGGACGTTGACGGGGTCTATAC CTGCGACCCGCGTCAGGTGCCCGATGCGAGGTTGTTGAAGTCGA TGTCCTACCAGGAAGCGATGGAGCTTTCCTACTTCGGCGCTAAA GTTCTTCACCCCCGCACCATTACCCCCATCGCCCAGTTCCAGATC CCTTGCCTGATTAAAAATACCGGAAATCCTCAAGCACCAGGTAC GCTCATTGGTGCCAGCCGTGATGAAGACGAATTACCGGTCAAGG GCATTTCCAATCTGAATAACATGGCAATGTTCAGCGTTTCTGGT CCGGGGATGAAAGGGATGGTCGGCATGGCGGCGCGCGTCTTTG CAGCGATGTCACGCGCCCGTATTTCCGTGGTGCTGATTACGCAA TCATCTTCCGAATACAGCATCAGTTTCTGCGTTCCACAAAGCGA CTGTGTGCGAGCTGAACGGGCAATGCAGGAAGAGTTCTACCTG GAACTGAAAGAAGGCTTACTGGAGCCGCTGGCAGTGACGGAAC GGCTGGCCATTATCTCGGTGGTAGGTGATGGTATGCGCACCTTG CGTGGGATCTCGGCGAAATTCTTTGCCGCACTGGCCCGCGCCAA TATCAACATTGTCGCCATTGCTCAGAGATCTTCTGAACGCTCAA TCTCTGTCGTGGTAAATAACGATGATGCGACCACTGGCGTGCGC GTTACTCATCAGATGCTGTTCAATACCGATCAGGTTATCGAAGT GTTTGTGATTGGCGTCGGTGGCGTTGGCGGTGCGCTGCTGGAGC AACTGAAGCGTCAGCAAAGCTGGCTGAAGAATAAACATATCGA CTTACGTGTCTGCGGTGTTGCCAACTCGAAGGCTCTGCTCACCA ATGTACATGGCCTTAATCTGGAAAACTGGCAGGAAGAACTGGC GCAAGCCAAAGAGCCGTTTAATCTCGGGCGCTTAATTCGCCTCG

Gene sequence	Description
	TGAAAGAATATCATCTGCTGAACCCGGTCATTGTTGACTGCACT TCCAGCCAGGCAGTGGCGGATCAATATGCCGACTTCCTGCGCGA AGGTTTCCACGTTGTCACGCCGAACAAAAAGGCCAACACCTCGT CGATGGATTACTACCATCAGTTGCGTTATGCGGCGGAAAAATCG CGGCGTAAATTCCTCTATGACACCAACGTTGGGGCTGGATTACC GGTTATTGAGAACCTGCAAAATCTGCTCAATGCAGGTGATGAAT TGATGAAGTTCTCCGGCATTCTTTCTGGTTCGCTTTCTTATATCTT CGGCAAGTTAGACGAAGGCATGAGTTTCTCCGAGGCGACCACG CTGGCGCGGGAAATGGGTTATACCGAACCGGACCCGCGAGATG ATCTTTCTGGTATGGATGTGGCGCGTAAACTATTGATTCTCGCTC GTGAAACGGGACGTGAACTGGAGCTGGCGGATATTGAAATTGA ACCTGTGCTGCCCGCAGAGTTTAACGCCGAGGGTGATGTTGCCG CTTTTATGGCGAATCTGTCACAACTCGACGATCTCTTTGCCGCGC GCGTGGCGAAGGCCCGTGATGAAGGAAAAGTTTTGCGCTATGTT GGCAATATTGATGAAGATGGCGTCTGCCGCGTGAAGATTGCCGA AGTGGATGGTAATGATCCGCTGTTCAAAGTGAAAAATGGCGAA AACGCCCTGGCCTTCTATAGCCACTATTATCAGCCGCTGCCGTT GGTACTGCGCGGATATGGTGCGGGCAATGACGTTACAGCTGCCG GTGTCTTTGCTGATCTGCTACGTACCCTCTCATGGAAGTTAGGA GTCTGA
thrB **SEQ ID NO: 9**	ATGGTTAAAGTTTATGCCCCGGCTTCCAGTGCCAATATGAGCGT CGGGTTTGATGTGCTCGGGGCGGCGGTGACACCTGTTGATGGTG CATTGCTCGGAGATGTAGTCACGGTTGAGGCGGCAGAGACATTC AGTCTCAACAACCTCGGACGCTTTGCCGATAAGCTGCCGTCAGA ACCACGGGAAAATATCGTTTATCAGTGCTGGGAGCGTTTTTGCC AGGAACTGGGTAAGCAAATTCCAGTGGCGATGACCCTGGAAAA GAATATGCCGATCGGTTCGGGCTTAGGCTCCAGTGCCTGTTCGG TGGTCGCGGCGCTGATGGCGATGAATGAACACTGCGGCAAGCC GCTTAATGACACTCGTTTGCTGGCTTTGATGGGCGAGCTGGAAG GCCGTATCTCCGGCAGCATTCATTACGACAACGTGGCACCGTGT TTTCTCGGTGGTATGCAGTTGATGATCGAAGAAAACGACATCAT CAGCCAGCAAGTGCCAGGGTTTGATGAGTGGCTGTGGGTGCTGG CGTATCCGGGGATTAAAGTCTCGACGGCAGAAGCCAGGGCTATT TTACCGGCGCAGTATCGCCGCCAGGATTGCATTGCGCACGGGCG ACATCTGGCAGGCTTCATTCACGCCTGCTATTCCCGTCAGCCTG AGCTTGCCGCGAAGCTGATGAAAGATGTTATCGCTGAACCCTAC CGTGAACGGTTACTGCCAGGCTTCCGGCAGGCGCGGCAGGCGG TCGCGGAAATCGGCGCGGTAGCGAGCGGTATCTCCGGCTCCGGC CCGACCTTGTTCGCTCTGTGTGACAAGCCGGAAACCGCCCAGCG CGTTGCCGACTGGTTGGGTAAGAACTACCTGCAAAATCAGGAA GGTTTTGTTCATATTTGCCGGCTGGATACGGCGGGCGCACGAGT ACTGGAAAACTAA

Gene sequence	Description
thrC **SEQ ID NO: 10**	ATGAAACTCTACAATCTGAAAGATCACAACGAGCAGGTCAGCTT TGCGCAAGCCGTAACCCAGGGGTTGGGCAAAAATCAGGGGCTG TTTTTTCCGCACGACCTGCCGGAATTCAGCCTGACTGAAATTGA TGAGATGCTGAAGCTGGATTTTGTCACCCGCAGTGCGAAGATCC TCTCGGCGTTTATTGGTGATGAAATCCCACAGGAAATCCTGGAA GAGCGCGTGCGCGCGGCGTTTGCCTTCCCGGCTCCGGTCGCCAA TGTTGAAAGCGATGTCGGTTGTCTGGAATTGTTCCACGGGCCAA CGCTGGCATTTAAAGATTTCGGCGGTCGCTTTATGGCACAAATG CTGACCCATATTGCGGGTGATAAGCCAGTGACCATTCTGACCGC GACCTCCGGTGATACCGGAGCGGCAGTGGCTCATGCTTTCTACG GTTTACCGAATGTGAAAGTGGTTATCCTCTATCCACGAGGCAAA ATCAGTCCACTGCAAGAAAAACTGTTCTGTACATTGGGCGGCAA TATCGAAACTGTTGCCATCGACGGCGATTTCGATGCCTGTCAGG CGCTGGTGAAGCAGGCGTTTGATGATGAAGAACTGAAAGTGGC GCTAGGGTTAAACTCGGCTAACTCGATTAACATCAGCCGTTTGC TGGCGCAGATTTGCTACTACTTTGAAGCTGTTGCGCAGCTGCCG CAGGAGACGCGCAACCAGCTGGTTGTCTCGGTGCCAAGCGGAA ACTTCGGCGATTTGACGGCGGGTCTGCTGGCGAAGTCACTCGGT CTGCCGGTGAAACGTTTTATTGCTGCGACCAACGTGAACGATAC CGTGCCACGTTTCCTGCACGACGGTCAGTGGTCACCCAAAGCGA CTCAGGCGACGTTATCCAACGCGATGGACGTGAGTCAGCCGAA CAACTGGCCGCGTGTGGAAGAGTTGTTCCGCCGCAAAATCTGGC AACTGAAAGAGCTGGGTTATGCAGCCGTGGATGATGAAACCAC GCAACAGACAATGCGTGAGTTAAAAGAACTGGGCTACACTTCG GAGCCGCACGCTGCCGTAGCTTATCGTGCGCTGCGTGATCAGTT GAATCCAGGCGAATATGGCTTGTTCCTCGGCACCGCGCATCCGG CGAAATTTAAAGAGAGCGTGGAAGCGATTCTCGGTGAAACGTT GGATCTGCCAAAAGAGCTGGCAGAACGTGCTGATTTACCCTTGC TTTCACATAATCTGCCCGCCGATTTTGCTGCGTTGCGTAAATTGA TGATGAATCATCAGTAA
ilvA^{fbr} **SEQ ID NO: 11**	ATGAGTGAAACATACGTGTCTGAGAAAAGTCCAGGAGTGATGG CTAGCGGAGCGGAGCTGATTCGTGCCGCCGACATTCAAACGGC GCAGGCACGAATTTCCTCCGTCATTGCACCAACTCCATTGCAGT ATTGCCCTCGTCTTTCTGAGGAAACCGGAGCGGAAATCTACCTT AAGCGTGAGGATCTGCAGGATGTTCGTTCCTACAAGATCCGCGG TGCGCTGAACTCTGGAGCGCAGCTCACCCAAGAGCAGCGCGAT GCAGGTATCGTTGCCGCATCTGCAGGTAACCATGCCCAGGGCGT GGCCTATGTGTGCAAGTCCTTGGGCGTTCAGGGACGCATCTATG TTCCTGTGCAGACTCCAAAGCAAAAGCGTGACCGCATCATGGTT CACGGCGGAGAGTTTGTCTCCTTGGTGGTCACTGGCAATAACTT CGACGAAGCATCGGCTGCAGCGCATGAAGATGCAGAGCGCACC GGCGCAACGCTGATCGAGCCTTTCGATGCTCGCAACACCGTCAT CGGTCAGGGCACCGTGGCTGCTGAGATCTTGTCGCAGCTGACTT CCATGGGCAAGAGTGCAGATCACGTGATGGTTCCAGTCGGCGGT GGCGGACTTCTTGCAGGTGTGGTCAGCTACATGGCTGATATGGC ACCTCGCACTGCGATCGTTGGTATCGAACCAGCGGGAGCAGCAT CCATGCAGGCTGCATTGCACAATGGTGGACCAATCACTTTGGAG ACTGTTGATCCCTTTGTGGACGGCGCAGCAGTCAAACGTGTCGG

Gene sequence	Description
	AGATCTCAACTACACCATCGTGGAGAAGAACCAGGGTCGCGTG CACATGATGAGCGCGACCGAGGGCGCTGTGTGTACTGAGATGCT CGATCTTTACCAAAACGAAGGCATCATCGCGGAGCCTGCTGGCG CGCTGTCTATCGCTGGGTTGAAGGAAATGTCCTTTGCACCTGGT TCTGCAGTGGTGTGCATCATCTCTGGTGGCAACAACGATGTGCT GCGTTATGCGGAAATCGCTGAGCGCTCCTTGGTGCACCGCGGTT TGAAGCACTACTTCTTGGTGAACTTCCCGCAAAAGCCTGGTCAG TTGCGTCACTTCCTGGAAGATATCCTGGGACCGGATGATGACAT CACGCTGTTTGAGTACCTCAAGCGCAACAACCGTGAGACCGGTA CTGCGTTGGTGGGTATTCACTTGAGTGAAGCATCAGGATTGGAT TCTTTGCTGGAACGTATGGAGGAATCGGCAATTGATTCCCGTCG CCTCGAGCCGGGCACCCCTGAGTACGAATACTTGACCTAA
aceE **SEQ ID NO: 12**	ATGTCAGAACGTTTCCCAAATGACGTGGATCCGATCGAAACTCG CGACTGGCTCCAGGCGATCGAATCGGTCATCCGTGAAGAAGGT GTTGAGCGTGCTCAGTATCTGATCGACCAACTGCTTGCTGAAGC CCGCAAAGGCGGTGTAAACGTAGCCGCAGGCACAGGTATCAGC AACTACATCAACACCATCCCCGTTGAAGAACAACCGGAGTATCC GGGTAATCTGGAACTGGAACGCCGTATTCGTTCAGCTATCCGCT GGAACGCCATCATGACGGTGCTGCGTGCGTCGAAAAAAGACCT CGAACTGGGCGGCCATATGGCGTCCTTCCAGTCTTCCGCAACCA TTTATGATGTGTGCTTTAACCACTTCTTCCGTGCACGCAACGAGC AGGATGGCGGCGACCTGGTTTACTTCCAGGGCCACATCTCCCCG GGCGTGTACGCTCGTGCTTTCCTGGAAGGTCGTCTGACTCAGGA GCAGCTGGATAACTTCCGTCAGGAAGTTCACGGCAATGGCCTCT CTTCCTATCCGCACCCGAAACTGATGCCGGAATTCTGGCAGTTC CCGACCGTATCTATGGGTCTGGGTCCGATTGGTGCTATTTACCA GGCTAAATTCCTGAAATATCTGGAACACCGTGGCCTGAAAGATA CCTCTAAACAAACCGTTTACGCGTTCCTCGGTGACGGTGAAATG GACGAACCGGAATCCAAAGGTGCGATCACCATCGCTACCCGTG AAAAACTGGATAACCTGGTCTTCGTTATCAACTGTAACCTGCAG CGTCTTGACGGCCCGGTCACCGGTAACGGCAAGATCATCAACGA ACTGGAAGGCATCTTCGAAGGTGCTGGCTGGAACGTGATCAAA GTGATGTGGGGTAGCCGTTGGGATGAACTGCTGCGTAAGGATAC CAGCGGTAAACTGATCCAGCTGATGAACGAAACCGTTGACGGC GACTACCAGACCTTCAAATCGAAAGATGGTGCGTACGTTCGTGA ACACTTCTTCGGTAAATATCCTGAAACCGCAGCACTGGTTGCAG ACTGGACTGACGAGCAGATCTGGGCACTGAACCGTGGTGGTCA CGATCCGAAGAAAATCTACGCTGCATTCAAGAAAGCGCAGGAA ACCAAAGGCAAAGCGACAGTAATCCTTGCTCATACCATTAAAG GTTACGGCATGGGCGACGCGGCTGAAGGTAAAAACATCGCGCA CCAGGTTAAGAAAATGAACATGGACGGTGTGCGTCATATCCGC GACCGTTTCAATGTGCCGGTGTCTGATGCAGATATCGAAAAACT GCCGTACATCACCTTCCCGGAAGGTTCTGAAGAGCATACCTATC TGCACGCTCAGCGTCAGAAACTGCACGGTTATCTGCCAAGCCGT CAGCCGAACTTCACCGAGAAGCTTGAGCTGCCGAGCCTGCAAG ACTTCGGCGCGCTGTTGGAAGAGCAGAGCAAAGAGATCTCTAC CACTATCGCTTTCGTTCGTGCTCTGAACGTGATGCTGAAGAACA AGTCGATCAAAGATCGTCTGGTACCGATCATCGCCGACGAAGCG

Gene sequence	Description
	CGTACTTTCGGTATGGAAGGTCTGTTCCGTCAGATTGGTATTTAC AGCCCGAACGGTCAGCAGTACACCCCGCAGGACCGCGAGCAGG TTGCTTACTATAAAGAAGACGAGAAAGGTCAGATTCTGCAGGA AGGGATCAACGAGCTGGGCGCAGGTTGTTCCTGGCTGGCAGCG GCGACCTCTTACAGCACCAACAATCTGCCGATGATCCCGTTCTA CATCTATTACTCGATGTTCGGCTTCCAGCGTATTGGCGATCTGTG CTGGGCGGCTGGCGACCAGCAAGCGCGTGGCTTCCTGATCGGCG GTACTTCCGGTCGTACCACCCTGAACGGCGAAGGTCTGCAGCAC GAAGATGGTCACAGCCACATTCAGTCGCTGACTATCCCGAACTG TATCTCTTACGACCCGGCTTACGCTTACGAAGTTGCTGTCATCAT GCATGACGGTCTGGAGCGTATGTACGGTGAAAAACAAGAGAAC GTTTACTACTACATCACTACGCTGAACGAAAACTACCACATGCC GGCAATGCCGGAAGGTGCTGAGGAAGGTATCCGTAAAGGTATC TACAAACTCGAAACTATTGAAGGTAGCAAAGGTAAAGTTCAGC TGCTCGGCTCCGGTTCTATCCTGCGTCACGTCCGTGAAGCAGCT GAGATCCTGGCGAAAGATTACGGCGTAGGTTCTGACGTTTATAG CGTGACCTCCTTCACCGAGCTGGCGCGTGATGGTCAGGATTGTG AACGCTGGAACATGCTGCACCCGCTGGAAACTCCGCGCGTTCCG TATATCGCTCAGGTGATGAACGACGCTCCGGCAGTGGCATCTAC CGACTATATGAAACTGTTCGCTGAGCAGGTCCGTACTTACGTAC CGGCTGACGACTACCGCGTACTGGGTACTGATGGCTTCGGTCGT TCCGACAGCCGTGAGAACCTGCGTCACCACTTCGAAGTTGATGC TTCTTATGTCGTGGTTGCGGCGCTGGGCGAACTGGCTAAACGTG GCGAAATCGATAAGAAAGTGGTTGCTGACGCAATCGCCAAATT CAACATCGATGCAGATAAAGTTAACCCGCGTCTGGCGTAA
aceF **SEQ ID NO: 13**	ATGGCTATCGAAATCAAAGTACCGGACATCGGGGCTGATGAAG TTGAAATCACCGAGATCCTGGTCAAAGTGGGCGACAAAGTTGA AGCCGAACAGTCGCTGATCACCGTAGAAGGCGACAAAGCCTCT ATGGAAGTTCCGTCTCCGCAGGCGGGTATCGTTAAAGAGATCAA AGTCTCTGTTGGCGATAAAACCCAGACCGGCGCACTGATTATGA TTTTCGATTCCGCCGACGGTGCAGCAGACGCTGCACCTGCTCAG GCAGAAGAGAAGAAAGAAGCAGCTCCGGCAGCAGCACCAGCG GCTGCGGCGGCAAAAGACGTTAACGTTCCGGATATCGGCAGCG ACGAAGTTGAAGTGACCGAAATCCTGGTGAAAGTTGGCGATAA AGTTGAAGCTGAACAGTCGCTGATCACCGTAGAAGGCGACAAG GCTTCTATGGAAGTTCCGGCTCCGTTTGCTGGCACCGTGAAAGA GATCAAAGTGAACGTGGGTGACAAAGTGTCTACCGGCTCGCTG ATTATGGTCTTCGAAGTCGCGGGTGAAGCAGGCGCGGCAGCTCC GGCCGCTAAACAGGAAGCAGCTCCGGCAGCGGCCCCTGCACCA GCGGCTGGCGTGAAAGAAGTTAACGTTCCGGATATCGGCGGTG ACGAAGTTGAAGTGACTGAAGTGATGGTGAAAGTGGGCGACAA AGTTGCCGCTGAACAGTCACTGATCACCGTAGAAGGCGACAAA GCTTCTATGGAAGTTCCGGCGCCGTTTGCAGGCGTCGTGAAGGA ACTGAAAGTCAACGTTGGCGATAAAGTGAAAACTGGCTCGCTG ATTATGATCTTCGAAGTTGAAGGCGCAGCGCCTGCGGCAGCTCC TGCGAAACAGGAAGCGGCAGCGCCGGCACCGGCAGCAAAAGCT GAAGCCCCGGCAGCAGCACCAGCTGCGAAAGCGGAAGGCAAAT CTGAATTTGCTGAAAACGACGCTTATGTTCACGCGACTCCGCTG

Gene sequence	Description
	ATCCGCCGTCTGGCACGCGAGTTTGGTGTTAACCTTGCGAAAGT GAAGGGCACTGGCCGTAAAGGTCGTATCCTGCGCGAAGACGTT CAGGCTTACGTGAAAGAAGCTATCAAACGTGCAGAAGCAGCTC CGGCAGCGACTGGCGGTGGTATCCCTGGCATGCTGCCGTGGCCG AAGGTGGACTTCAGCAAGTTTGGTGAAATCGAAGAAGTGGAAC TGGGCCGCATCCAGAAAATCTCTGGTGCGAACCTGAGCCGTAAC TGGGTAATGATCCCGCATGTTACTCACTTCGACAAAACCGATAT CACCGAGTTGGAAGCGTTCCGTAAACAGCAGAACGAAGAAGCG GCGAAACGTAAGCTGGATGTGAAGATCACCCCGGTTGTCTTCAT CATGAAAGCCGTTGCTGCAGCTCTTGAGCAGATGCCTCGCTTCA ATAGTTCGCTGTCGGAAGACGGTCAGCGTCTGACCCTGAAGAAA TACATCAACATCGGTGTGGCGGTGGATACCCCGAACGGTCTGGT TGTTCCGGTATTCAAAGACGTCAACAAGAAAGGCATCATCGAGC TGTCTCGCGAGCTGATGACTATTTCTAAGAAAGCGCGTGACGGT AAGCTGACTGCGGGCGAAATGCAGGGCGGTTGCTTCACCATCTC CAGCATCGGCGGCCTGGGTACTACCCACTTCGCGCCGATTGTGA ACGCGCCGGAAGTGGCTATCCTCGGCGTTTCCAAGTCCGCGATG GAGCCGGTGTGGAATGGTAAAGAGTTCGTGCCGCGTCTGATGCT GCCGATTTCTCTCTCCTTCGACCACCGCGTGATCGACGGTGCTG ATGGTGCCCGTTTCATTACCATCATTAACAACACGCTGTCTGAC ATTCGCCGTCTGGTGATGTAA
lpd **SEQ ID NO: 14**	ATGAGTACTGAAATCAAAACTCAGGTCGTGGTACTTGGGGCAG GCCCCGCAGGTTACTCCGCTGCCTTCCGTTGCGCTGATTTAGGTC TGGAAACCGTAATCGTAGAACGTTACAACACCCTTGGCGGTGTT TGCCTGAACGTCGGCTGTATCCCTTCTAAAGCACTGCTGCACGT AGCAAAAGTTATCGAAGAAGCCAAAGCGCTGGCTGAACACGGT ATCGTCTTCGGCGAACCGAAAACCGATATCGACAAGATTCGTAC CTGGAAAGAGAAAGTGATCAATCAGCTGACCGGTGGTCTGGCT GGTATGGCGAAAGGCCGCAAAGTCAAAGTGGTCAACGGTCTGG GTAAATTCACCGGGGCTAACACCCTGGAAGTTGAAGGTGAGAA CGGCAAAACCGTGATCAACTTCGACAACGCGATCATTGCAGCG GGTTCTCGCCCGATCCAACTGCCGTTTATTCCGCATGAAGATCC GCGTATCTGGGACTCCACTGACGCGCTGGAACTGAAAGAAGTA CCAGAACGCCTGCTGGTAATGGGTGGCGGTATCATCGGTCTGGA AATGGGCACCGTTTACCACGCGCTGGGTTCACAGATTGACGTGG TTGAAATGTTCGACCAGGTTATCCCGGCAGCTGACAAAGACATC GTTAAAGTCTTCACCAAGCGTATCAGCAAGAAATTCAACCTGAT GCTGGAAACCAAAGTTACCGCCGTTGAAGCGAAAGAAGACGGC ATTTATGTGACGATGGAAGGCAAAAAAGCACCCGCTGAACCGC AGCGTTACGACGCCGTGCTGGTAGCGATTGGTCGTGTGCCGAAC GGTAAAAACCTCGACGCAGGCAAAGCAGGCGTGGAAGTTGACG ACCGTGGTTTCATCCGCGTTGACAAACAGCTGCGTACCAACGTA CCGCACATCTTTGCTATCGGCGATATCGTCGGTCAACCGATGCT GGCACACAAAGGTGTTCACGAAGGTCACGTTGCCGCTGAAGTTA TCGCCGGTAAGAAACACTACTTCGATCCGAAAGTTATCCCGTCC ATCGCCTATACCAAACCAGAAGTTGCATGGGTGGGTCTGACTGA GAAAGAAGCGAAAGAGAAAGGCATCAGCTATGAAACCGCCACC TTCCCGTGGGCTGCTTCTGGTCGTGCTATCGCTTCCGACTGCGCA

Gene sequence	Description
	GACGGTATGACCAAGCTGATTTTCGACAAAGAATCTCACCGTGT GATCGGTGGTGCGATTGTCGGTACTAACGGCGGCGAGCTGCTGG GTGAAATCGGCCTGGCAATCGAAATGGGTTGTGATGCTGAAGA CATCGCACTGACCATCCACGCGCACCCGACTCTGCACGAGTCTG TGGGCCTGGCGGCAGAAGTGTTCGAAGGTAGCATTACCGACCTG CCGAACCCGAAAGCGAAGAAGAAGTAA
tesB **SEQ ID NO: 15**	ATGAGTCAGGCGCTAAAAAATTTACTGACATTGTTAAATCTGGA AAAAATTGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTTA GGTTTACGCCAGGTGTTTGGCGGCCAGGTCGTGGGTCAGGCCTT GTATGCTGCAAAAGAGACCGTCCCTGAAGAGCGGCTGGTACATT CGTTTCACAGCTACTTTCTTCGCCCTGGCGATAGTAAGAAGCCG ATTATTTATGATGTCGAAACGCTGCGTGACGGTAACAGCTTCAG CGCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCGATTTTTT ATATGACTGCCTCTTTCCAGGCACCAGAAGCGGGTTTCGAACAT CAAAAAACAATGCCGTCCGCGCCAGCGCCTGATGGCCTCCCTTC GGAAACGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCA GTGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCG TCCGGTGGAGTTTCATAACCCACTGAAAGGTCACGTCGCAGAAC CACATCGTCAGGTGTGGATCCGCGCAAATGGTAGCGTGCCGGAT GACCTGCGCGTTCATCAGTATCTGCTCGGTTACGCTTCTGATCTT AACTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCT CGAACCGGGGATTCAGATTGCCACCATTGACCATTCCATGTGGT TCCATCGCCCGTTTAATTTGAATGAATGGCTGCTGTATAGCGTG GAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGA GTTTTATACCCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGG AAGGGGTGATGCGTAATCACAATTAA
acuI **SEQ ID NO: 16**	ATGCGTGCGGTACTGATCGAGAAGTCCGATGATACACAGTCCGT CTCTGTCACCGAACTGGCTGAAGATCAACTGCCGGAAGGCGAC GTTTTGGTAGATGTTGCTTATTCAACACTGAACTACAAAGACGC CCTGGCAATTACCGGTAAAGCCCCCGTCGTTCGTCGTTTTCCGAT GGTACCTGGAATCGACTTTACGGGTACCGTGGCCCAGTCTTCCC ACGCCGACTTCAAGCCAGGTGATCGCGTAATCCTGAATGGTTGG GGTGTGGGGGAAAAACATTGGGGCGGTTTAGCGGAGCGCGCTC GCGTGCGCGGAGACTGGCTTGTTCCCTTGCCAGCCCCCCTGGAC TTACGCCAAGCGGCCATGATCGGTACAGCAGGATACACGGCGA TGTTGTGCGTTCTGGCGCTTGAACGTCACGGAGTGGTGCCGGGT AATGGGGAAATCGTGGTGTCCGGTGCAGCAGGCGGCGTCGGCT CCGTTGCGACGACCCTTCTTGCCGCTAAGGGCTATGAGGTAGCG GCAGTGACTGGACGTGCGTCCGAAGCAGAATATCTGCGCGGTTT GGGGGCGGCGAGCGTAATTGATCGTAACGAATTAACGGGGAAG GTACGCCCGCTGGGTCAGGAGCGTTGGGCTGGCGGGATTGACGT GGCGGGATCAACCGTGCTTGCGAACATGCTTTCTATGATGAAGT ATCGCGGGGTAGTCGCTGCGTGTGGCCTGGCCGCGGGCATGGAT CTGCCCGCGTCTGTCGCGCCCTTTATTCTTCGTGGGATGACGCTG GCAGGGGTGGATAGCGTTATGTGCCCAAAGACAGATCGTTTAGC

Gene sequence	Description
	AGCGTGGGCCCGTTTGGCGTCAGATCTTGACCCTGCCAAGCTGG AGGAGATGACTACAGAGTTGCCGTTTAGTGAAGTAATCGAGAC AGCACCCAAATTCTTGGACGGGACGGTTCGTGGCCGCATTGTTA TCCCCGTAACGCCCTAA

Table 2. Propionate Cassette Sequences Sleeping Beauty Operon

Sbm **SEQ ID NO: 17**	ATGTCTAACGTGCAGGAGTGGCAACAGCTTGCCAACAAGGAA TTGAGCCGTCGGGAGAAAACTGTCGACTCGCTGGTTCATCAAA CCGCGGAAGGGATCGCCATCAAGCCGCTGTATACCGAAGCCG ATCTCGATAATCTGGAGGTGACAGGTACCCTTCCTGGTTTGCC GCCCTACGTTCGTGGCCCGCGTGCCACTATGTATACCGCCCAA CCGTGGACCATCCGTCAGTATGCTGGTTTTTCAACAGCAAAAG AGTCCAACGCTTTTTATCGCCGTAACCTGGCCGCCGGGCAAAA AGGTCTTTCCGTTGCGTTTGACCTTGCCACCCACCGTGGCTAC GACTCCGATAACCCGCGCGTGGCGGGCGACGTCGGCAAAGCG GGCGTCGCTATCGACACCGTGGAAGATATGAAAGTCCTGTTCG ACCAGATCCCGCTGGATAAAATGTCGGTTTCGATGACCATGAA TGGCGCAGTGCTACCAGTACTGGCGTTTTATATCGTCGCCGCA GAAGAGCAAGGTGTTACACCTGATAAACTGACCGGCACCATT CAAAACGATATTCTCAAAGAGTACCTCTGCCGCAACACCTATA TTTACCCACCAAAACCGTCAATGCGCATTATCGCCGACATCAT CGCCTGGTGTTCCGGCAACATGCCGCGATTTAATACCATCAGT ATCAGCGGTTACCACATGGGTGAAGCGGGTGCCAACTGCGTG CAGCAGGTAGCATTTACGCTCGCTGATGGGATTGAGTACATCA AAGCAGCAATCTCTGCCGGACTGAAAATTGATGACTTCGCTCC TCGCCTGTCGTTCTTCTTCGGCATCGGCATGGATCTGTTTATGA ACGTCGCCATGTTGCGTGCGGCACGTTATTTATGGAGCGAAGC GGTCAGTGGATTTGGCGCACAGGACCCGAAATCACTGGCGCT GCGTACCCACTGCCAGACCTCAGGCTGGAGCCTGACTGAACA GGATCCGTATAACAACGTTATCCGCACCACCATTGAAGCGCTG GCTGCGACGCTGGGCGGTACTCAGTCACTGCATACCAACGCCT TTGACGAAGCGCTTGGTTTGCCTACCGATTTCTCAGCACGCAT TGCCCGCAACACCCAGATCATCATCCAGGAAGAATCAGAACT CTGCCGCACCGTCGATCCACTGGCCGGATCCTATTACATTGAG TCGCTGACCGATCAAATCGTCAAACAAGCCAGAGCTATTATCC AACAGATCGACGAAGCCGGTGGCATGGCGAAAGCGATCGAAG CAGGTCTGCCAAAACGAATGATCGAAGAGGCCTCAGCGCGCG AACAGTCGCTGATCGACCAGGGCAAGCGTGTCATCGTTGGTGT CAACAAGTACAAACTGGATCACGAAGACGAAACCGATGTACT TGAGATCGACAACGTGATGGTGCGTAACGAGCAAATTGCTTC GCTGGAACGCATTCGCGCCACCCGTGATGATGCCGCCGTAACC GCCGCGTTGAACGCCCTGACTCACGCCGCACAGCATAACGAA AACCTGCTGGCTGCCGCTGTTAATGCCGCTCGCGTTCGCGCCA CCCTGGGTGAAATTTCCGATGCGCTGGAAGTCGCTTTCGACCG TTATCTGGTGCCAAGCCAGTGTGTTACCGGCGTGATTGCGCAA AGCTATCATCAGTCTGAGAAATCGGCCTCCGAGTTCGATGCCA TTGTTGCGCAAACGGAGCAGTTCCTTGCCGACAATGGTCGTCG

	CCCGCGCATTCTGATCGCTAAGATGGGCCAGGATGGACACGA TCGCGGCGCGAAAGTGATCGCCAGCGCCTATTCCGATCTCGGT TTCGACGTAGATTTAAGCCCGATGTTCTCTACACCTGAAGAGA TCGCCCGCCTGGCCGTAGAAAACGACGTTCACGTAGTGGGCG CATCCTCACTGGCTGCCGGTCATAAAACGCTGATCCCGGAACT GGTCGAAGCGCTGAAAAAATGGGGACGCGAAGATATCTGCGT GGTCGCGGGTGGCGTCATTCCGCCGCAGGATTACGCCTTCCTG CAAGAGCGCGGCGTGGCGGCGATTTATGGTCCAGGTACACCT ATGCTCGACAGTGTGCGCGACGTACTGAATCTGATAAGCCAGC ATCATGATTAA
ygfD **SEQ ID NO: 18**	ATGATTAATGAAGCCACGCTGGCAGAAAGTATTCGCCGCTTAC GTCAGGGTGAGCGTGCCACACTCGCCCAGGCCATGACGCTGG TGGAAAGCCGTCACCCGCGTCATCAGGCACTAAGTACGCAGC TGCTTGATGCCATTATGCCGTACTGCGGTAACACCCTGCGACT GGGCGTTACCGGCACCCCCGGCGCGGGGAAAAGTACCTTTCTT GAGGCCTTTGGCATGTTGTTGATTCGAGAGGGATTAAAGGTCG CGGTTATTGCGGTCGATCCCAGCAGCCCGGTCACTGGCGGTAG CATTCTCGGGGATAAAACCCGCATGAATGACCTGGCGCGTGCC GAAGCGGCGTTTATTCGCCCGGTACCATCCTCCGGTCATCTGG GCGGTGCCAGTCAGCGAGCGCGGGAATTAATGCTGTTATGCG AAGCAGCGGGTTATGACGTAGTGATTGTCGAAACGGTTGGCG TCGGGCAGTCGGAAACAGAAGTCGCCCGCATGGTGGACTGTT TTATCTCGTTGCAAATTGCCGGTGGCGGCGATGATCTGCAGGG CATTAAAAAAGGGCTGATGGAAGTGGCTGATCTGATCGTTATC AACAAAGACGATGGCGATAACCATACCAATGTCGCCATTGCC CGGCATATGTACGAGAGTGCCCTGCATATTCTGCGACGTAAAT ACGACGAATGGCAGCCACGGGTTCTGACTTGTAGCGCACTGG AAAAACGTGGAATCGATGAGATCTGGCACGCCATCATCGACT TCAAAACCGCGCTAACTGCCAGTGGTCGTTTACAACAAGTGCG GCAACAACAATCGGTGGAATGGCTGCGTAAGCAGACCGAAGA AGAAGTACTGAATCACCTGTTCGCGAATGAAGATTTCGATCGC TATTACCGCCAGACGCTTTTAGCGGTCAAAAACAATACGCTCT CACCGCGCACCGGCCTGCGGCAGCTCAGTGAATTTATCCAGAC GCAATATTTTGATTAA
ygfG **SEQ ID NO: 19**	ATGTCTTATCAGTATGTTAACGTTGTCACTATCAACAAAGTGG CGGTCATTGAGTTTAACTATGGCCGAAAACTTAATGCCTTAAG TAAAGTCTTTATTGATGATCTTATGCAGGCGTTAAGCGATCTC AACCGGCCGGAAATTCGCTGTATCATTTTGCGCGCACCGAGTG GATCCAAAGTCTTCTCCGCAGGTCACGATATTCACGAACTGCC GTCTGGCGGTCGCGATCCGCTCTCCTATGATGATCCATTGCGT CAAATCACCCGCATGATCCAAAAATTCCCGAAACCGATCATTT CGATGGTGGAAGGTAGTGTTTGGGGTGGCGCATTTGAAATGAT CATGAGTTCCGATCTGATCATCGCCGCCAGTACCTCAACCTTC TCAATGACGCCTGTAAACCTCGGCGTCCCGTATAACCTGGTCG GCATTCACAACCTGACCCGCGACGCGGGCTTCCACATTGTCAA AGAGCTGATTTTTACCGCTTCGCCAATCACCGCCCAGCGCGCG CTGGCTGTCGGCATCCTCAACCATGTTGTGGAAGTGGAAGAAC TGGAAGATTTCACCTTACAAATGGCGCACCACATCTCTGAGAA AGCGCCGTTAGCCATTGCCGTTATCAAAGAAGAGCTGCGTGTA CTGGGCGAAGCACACACCATGAACTCCGATGAATTTGAACGT

	ATTCAGGGGATGCGCCGCGCGGTGTATGACAGCGAAGATTAC CAGGAAGGGATGAACGCTTTCCTCGAAAAACGTAAACCTAAT TTCGTTGGTCATTAA
ygfH **SEQ ID NO: 20**	ATGGAAACTCAGTGGACAAGGATGACCGCCAATGAAGCGGCA GAAATTATCCAGCATAACGACATGGTGGCATTTAGCGGCTTTA CCCCGGCGGGTTCGCCGAAAGCCCTACCCACCGCGATTGCCCG CAGAGCTAACGAACAGCATGAGGCCAAAAAGCCGTATCAAAT TCGCCTTCTGACGGGTGCGTCAATCAGCGCCGCCGCTGACGAT GTACTTTCTGACGCCGATGCTGTTTCCTGGCGTGCGCCATATC AAACATCGTCCGGTTTACGTAAAAAGATCAATCAGGGCGCGG TGAGTTTCGTTGACCTGCATTTGAGCGAAGTGGCGCAAATGGT CAATTACGGTTTCTTCGGCGACATTGATGTTGCCGTCATTGAA GCATCGGCACTGGCACCGGATGGTCGAGTCTGGTTAACCAGC GGGATCGGTAATGCGCCGACCTGGCTGCTGCGGGCGAAGAAA GTGATCATTGAACTCAATCACTATCACGATCCGCGCGTTGCAG AACTGGCGGATATTGTGATTCCTGGCGCGCCACCGCGGCGCAA TAGCGTGTCGATCTTCCATGCAATGGATCGCGTCGGTACCCGC TATGTGCAAATCGATCCGAAAAAGATTGTCGCCGTCGTGGAA ACCAACTTGCCCGACGCCGGTAATATGCTGGATAAGCAAAAT CCCATGTGCCAGCAGATTGCCGATAACGTGGTCACGTTCTTAT TGCAGGAAATGGCGCATGGGCGTATTCCGCCGGAATTTCTGCC GCTGCAAAGTGGCGTGGGCAATATCAATAATGCGGTAATGGC GCGTCTGGGGGGAAAACCCGGTAATTCCTCCGTTTATGATGTAT TCGGAAGTGCTACAGGAATCGGTGGTGCATTTACTGGAAACC GGCAAAATCAGCGGGGCCAGCGCCTCCAGCCTGACAATCTCG GCCGATTCCCTGCGCAAGATTTACGACAATATGGATTACTTTG CCAGCCGCATTGTGTTGCGTCCGCAGGAGATTTCCAATAACCC GGAAATCATCCGTCGTCTGGGCGTCATCGCTCTGAACGTCGGC CTGGAGTTTGATATTTACGGGCATGCCAACTCAACACACGTAG CCGGGGTCGATCTGATGAACGGCATCGGCGGCAGCGGTGATT TTGAACGCAACGCGTATCTGTCGATCTTTATGGCCCCGTCGAT TGCTAAAGAAGGCAAGATCTCAACCGTCGTGCCAATGTGCAG CCATGTTGATCACAGCGAACACAGCGTCAAAGTGATCATCACC GAACAAGGGATCGCCGATCTGCGCGGTCTTTCCCCGCTTCAAC GCGCCCGCACTATCATTGATAATTGTGCACATCCTATGTATCG GGATTATCTGCATCGCTATCTGGAAAATGCGCCTGGCGGACAT ATTCACCACGATCTTAGCCACGTCTTCGACTTACACCGTAATTT AATTGCAACCGGCTCGATGCTGGGTTAA

Table 3. Sequences of Propionate Cassette from Propioni Bacteria

Description	**Sequence**
mutA SEQ ID NO: 21	ATGAGCAGCACGGATCAGGGGACCAACCCCGCCGACACTGAC GACCTCACTCCCACCACACTCAGTCTGGCCGGGGATTTCCCCA AGGCCACTGAGGAGCAGTGGGAGCGCGAAGTTGAGAAGGTAT TCAACCGTGGTCGTCCACCGGAGAAGCAGCTGACCTTCGCCGA GTGTCTGAAGCGCCTGACGGTTCACACCGTCGATGGCATCGAC ATCGTGCCGATGTACCGTCCGAAGGACGCGCCGAAGAAGCTG GGTTACCCCGGCGTCACCCCCTTCACCCGCGGCACCACGGTGC

	GCAACGGTGACATGGATGCCTGGGACGTGCGCGCCCTGCACG AGGATCCCGACGAGAAGTTCACCCGCAAGGCGATCCTTGAAG ACCTGGAGCGTGGCGTCACCTCCCTGTTGTTGCGCGTTGATCC CGACGCGATCGCACCCGAGCACCTCGACGAGGTCCTCTCCGAC GTCCTGCTGGAAATGACCAAGGTGGAGGTCTTCAGCCGCTACG ACCAGGGTGCCGCCGCCGAGGCCTTGATGGGCGTCTACGAGC GCTCCGACAAGCCGGCGAAGGACCTGGCCCTGAACCTGGGCC TGGATCCCATCGGCTTCGCGGCCCTGCAGGGCACCGAGCCGG ATCTGACCGTGCTCGGTGACTGGGTGCGCCGCCTGGCGAAGTT CTCACCGGACTCGCGCGCCGTCACGATCGACGCGAACGTCTAC CACAACGCCGGTGCCGGCGACGTGGCAGAGCTCGCTTGGGCA CTGGCCACCGGCGCGGAGTACGTGCGCGCCCTGGTCGAACAG GGCTTCAACGCCACAGAGGCCTTCGACACGATCAACTTCCGTG TCACCGCCACCCACGACCAGTTCCTCACGATCGCCCGTCTTCG CGCCCTGCGCGAGGCATGGGCCCGCATCGGCGAGGTCTTTGGC GTGGACGAGGACAAGCGCGGCGCTCGCCAGAATGCGATCACC AGTTGGCGTGAGCTCACCCGCGAAGACCCCTATGTCAACATCC TTCGCGGTTCGATTGCCACCTTCTCCGCCTCCGTTGGCGGGGC CGAGTCGATCACGACGCTGCCCTTCACCCAGGCCCTCGGCCTG CCGGAGGACGACTTCCCGCTGCGCATCGCGCGCAACACGGGC ATCGTGCTCGCCGAAGAGGTGAACATCGGCCGCGTCAACGAC CCGGCCGGTGGCTCCTACTACGTCGAGTCGCTCACTCGCACCC TGGCCGACGCTGCCTGGAAGGAATTCCAGGAGGTCGAGAAGC TCGGTGGCATGTCGAAGGCGGTCATGACCGAGCACGTCACCA AGGTGCTCGACGCCTGCAATGCCGAGCGCGCCAAGCGCCTGG CCAACCGCAAGCAGCCGATCACCGCGGTCAGCGAGTTCCCGA TGATCGGGGCCCGCAGCATCGAGACCAAGCCGTTCCCAACCG CTCCGGCGCGCAAGGGCCTGGCCTGGCATCGCGATTCCGAGGT GTTCGAGCAGCTGATGGATCGCTCCACCAGCGTCTCCGAGCGC CCCAAGGTGTTCCTTGCCTGCCTGGGCACCCGTCGCGACTTCG GTGGCCGCGAGGGCTTCTCCAGCCCGGTATGGCACATCGCCGG TATCGACACCCCGCAGGTCGAAGGCGGCACCACCGCCGAGAT CGTCGAGGCGTTCAAGAAGTCGGGCGCCCAGGTGGCCGATCT CTGCTCGTCCGCCAAGATCTACGCGCAGCAGGGACTTGAGGTT GCCAAGGCGCTCAAGGCCGCCGGCGCGAAGGCCCTGTATCTG TCGGGCGCCTTCAAGGAGTTCGGCGATGACGCCGCCGAGGCC GAGAAGCTGATCGACGGACGCCTGTACATGGGCATGGATGTC GTCGACACCCTGTCCTCCACCCTTGATATCTTGGGAGTCGCGA AGTGA
mutB SEQ ID NO: 22	GTGAGCACTCTGCCCCGTTTTGATTCAGTTGACCTGGGCAATG CCCCGGTTCCTGCTGATGCCGCACAGCGCTTCGAGGAGTTGGC CGCCAAGGCCGGCACCGAAGAGGCGTGGGAGACGGCTGAGCA GATTCCGGTTGGCACCCTGTTCAACGAAGACGTCTACAAGGAC ATGGACTGGCTGGACACCTACGCCGGTATCCCGCCGTTCGTCC ACGGCCCATATGCAACCATGTACGCGTTCCGTCCCTGGACGAT TCGCCAGTACGCCGGCTTCTCCACGGCCAAGGAGTCCAACGCC TTCTACCGCCGCAACCTTGCGGCGGGCCAGAAGGGCCTGTCGG TTGCCTTCGACCTGCCCACCCACCGCGGCTACGACTCGGACAA TCCCCGCGTCGCCGGTGACGTCGGCATGGCCGGGGTGGCCATC

	GACTCCATCTATGACATGCGCGAGCTGTTCGCCGGCATTCCGC TGGACCAGATGAGCGTGTCGATGACCATGAACGGCGCCGTGC TGCCGATCCTGGCCCTCTATGTGGTGACCGCCGAGGAGCAGGG CGTCAAGCCCGAGCAGCTCGCCGGGACGATCCAGAACGACAT CCTCAAGGAGTTCATGGTTCGTAACACCTATATCTACCCGCCG CAGCCGAGTATGCGAATCATCTCCGAGATCTTCGCCTACACGA GTGCCAATATGCCGAAGTGGAATTCGATTTCCATTTCCGGCTA CCACATGCAGGAAGCCGGCGCCACGGCCGACATCGAGATGGC CTACACCCTGGCCGACGGTGTCGACTACATCCGCGCCGGCGAG TCGGTGGGCCTCAATGTCGACCAGTTCGCGCCGCGTCTGTCCT TCTTCTGGGGCATCGGCATGAACTTCTTCATGGAGGTTGCCAA GCTGCGTGCCGCACGTATGTTGTGGGCCAAGCTGGTGCATCAG TTCGGGCCGAAGAATCCGAAGTCGATGAGCCTGCGCACCCAC TCGCAGACCTCCGGTTGGTCGCTGACCGCCCAGGACGTCTACA ACAACGTCGTGCGTACCTGCATCGAGGCCATGGCCGCCACCCA GGGCCATACCCAGTCGCTGCACACGAACTCGCTCGACGAGGC CATTGCCCTACCGACCGATTTCAGCGCCCGCATCGCCCGTAAC ACCCAGCTGTTCCTGCAGCAGGAATCGGGCACGACGCGCGTG ATCGACCCGTGGAGCGGCTCGGCATACGTCGAGGAGCTCACC TGGGACCTGGCCCGCAAGGCATGGGGCCACATCCAGGAGGTC GAGAAGGTCGGCGGCATGGCCAAGGCCATCGAAAAGGGCATC CCCAAGATGCGCATTGAGGAAGCCGCCGCCCGCACCCAGGCA CGCATCGACTCCGGCCGTCAGCCGCTGATCGGCGTGAACAAGT ACCGCCTGGAGCACGAGCCGCCGCTCGATGTGCTCAAGGTTG ACAACTCCACGGTGCTCGCCGAGCAGAAGGCCAAGCTGGTCA AGCTGCGCGCCGAGCGCGATCCCGAGAAGGTCAAGGCCGCCC TCGACAAGATCACCTGGGCTGCCGCCAACCCCGACGACAAGG ATCCGGATCGCAACCTGCTGAAGCTGTGCATCGACGCTGGCCG CGCCATGGCGACGGTCGGCGAGATGAGCGACGCGCTCGAGAA GGTCTTCGGACGCTACACCGCCCAGATTCGCACCATCTCCGGT GTGTACTCGAAGGAAGTGAAGAACACGCCTGAGGTTGAGGAA GCACGCGAGCTCGTTGAGGAATTCGAGCAGGCCGAGGGCCGT CGTCCTCGCATCCTGCTGGCCAAGATGGGCCAGGACGGTCACG ACCGTGGCCAGAAGGTCATCGCCACCGCCTATGCCGACCTCGG TTTCGACGTCGACGTGGGCCCGCTGTTCCAGACCCCGGAGGAG ACCGCACGTCAGGCCGTCGAGGCCGATGTGCACGTGGTGGGC GTTTCGTCGCTCGCCGGCGGGCATCTGACGCTGGTTCCGGCCC TGCGCAAGGAGCTGGACAAGCTCGGACGTCCCGACATCCTCA TCACCGTGGGCGGCGTGATCCCTGAGCAGGACTTCGACGAGCT GCGTAAGGACGGCGCCGTGGAGATCTACACCCCCGGCACCGT CATTCCGGAGTCGGCGATCTCGCTGGTCAAGAAACTGCGGGCT TCGCTCGATGCCTAG
GI:18042134 SEQ ID NO: 23	ATGAGTAATGAGGATCTTTTCATCTGTATCGATCACGTGGCAT ATGCGTGCCCCGACGCCGACGAGGCTTCCAAGTACTACCAGG AGACCTTCGGCTGGCATGAGCTCCACCGCGAGGAGAACCCGG AGCAGGGAGTCGTCGAGATCATGATGGCCCCGGCTGCGAAGC TGACCGAGCACATGACCCAGGTTCAGGTCATGGCCCCGCTCAA CGACGAGTCGACCGTTGCCAAGTGGCTTGCCAAGCACAATGG TCGCGCCGGACTGCACCACATGGCATGGCGTGTCGATGACATC

	GACGCCGTCAGCGCCACCCTGCGCGAGCGCGGCGTGCAGCTG CTGTATGACGAGCCCAAGCTCGGCACCGGCGGCAACCGCATC AACTTCATGCATCCCAAGTCGGGCAAGGGCGTGCTCATCGAGC TCACCCAGTACCCGAAGAACTGA
mmdA SEQ ID NO: 24	ATGGCTGAAAACAACAATTTGAAGCTCGCCAGCACCATGGAA GGTCGCGTGGAGCAGCTCGCAGAGCAGCGCCAGGTGATCGAA GCCGGTGGCGGCGAACGTCGCGTCGAGAAGCAACATTCCCAG GGTAAGCAGACCGCTCGTGAGCGCCTGAACAACCTGCTCGAT CCCCATTCGTTCGACGAGGTCGGCGCTTTCCGCAAGCACCGCA CCACGTTGTTCGGCATGGACAAGGCCGTCGTCCCGGCAGATGG CGTGGTCACCGGCCGTGGCACCATCCTTGGTCGTCCCGTGCAC GCCGCGTCCCAGGACTTCACGGTCATGGGTGGTTCGGCTGGCG AGACGCAGTCCACGAAGGTCGTCGAGACGATGGAACAGGCGC TGCTCACCGGCACGCCCTTCCTGTTCTTCTACGATTCGGGCGG CGCCCGGATCCAGGAGGGCATCGACTCGCTGAGCGGTTACGG CAAGATGTTCTTCGCCAACGTGAAGCTGTCGGGCGTCGTGCCG CAGATCGCCATCATTGCCGGCCCCTGTGCCGGTGGCGCCTCGT ATTCGCCGGCACTGACTGACTTCATCATCATGACCAAGAAGGC CCATATGTTCATCACGGGCCCCCAGGTCATCAAGTCGGTCACC GGCGAGGATGTCACCGCTGACGAACTCGGTGGCGCTGAGGCC CATATGGCCATCTCGGGCAATATCCACTTCGTGGCCGAGGACG ACGACGCCGCGGAGCTCATTGCCAAGAAGCTGCTGAGCTTCCT TCCGCAGAACAACACTGAGGAAGCATCCTTCGTCAACCCGAA CAATGACGTCAGCCCCAATACCGAGCTGCGCGACATCGTTCCG ATTGACGGCAAGAAGGGCTATGACGTGCGCGATGTCATTGCC AAGATCGTCGACTGGGGTGACTACCTCGAGGTCAAGGCCGGC TATGCCACCAACCTCGTGACCGCCTTCGCCCGGGTCAATGGTC GTTCGGTGGGCATCGTGGCCAATCAGCCGTCGGTGATGTCGGG TTGCCTCGACATCAACGCCTCTGACAAGGCCGCCGAATTCGTG AATTTCTGCGATTCGTTCAACATCCCGCTGGTGCAGCTGGTCG ACGTGCCGGGCTTCCTGCCCGGCGTGCAGCAGGAGTACGGCG GCATCATTCGCCATGGCGCGAAGATGCTGTACGCCTACTCCGA GGCCACCGTGCCGAAGATCACCGTGGTGCTCCGCAAGGCCTA CGGCGGCTCCTACCTGGCCATGTGCAACCGTGACCTTGGTGCC GACGCCGTGTACGCCTGGCCCAGCGCCGAGATTGCGGTGATG GGCGCCGAGGGTGCGGCAAATGTGATCTTCCGCAAGGAGATC AAGGCTGCCGACGATCCCGACGCCATGCGCGCCGAGAAGATC GAGGAGTACCAGAACGCGTTCAACACGCCGTACGTGGCCGCC GCCCGCGGTCAGGTCGACGACGTGATTGACCCGGCTGATACCC GTCGAAAGATTGCTTCCGCCCTGGAGATGTACGCCACCAAGCG TCAGACCCGCCCGGCGAAGAAGCATGGAAACTTCCCCTGCTG A
PFREUD_18870 SEQ ID NO: 25	ATGAGTCCGCGAGAAATTGAGGTTTCCGAGCCGCGCGAGGTT GGTATCACCGAGCTCGTGCTGCGCGATGCCCATCAGAGCCTGA TGGCCACACGAATGGCAATGGAAGACATGGTCGGCGCCTGTG CAGACATTGATGCTGCCGGGTACTGGTCAGTGGAGTGTTGGGG

	TGGTGCCACGTATGACTCGTGTATCCGCTTCCTCAACGAGGAT CCTTGGGAGCGTCTGCGCACGTTCCGCAAGCTGATGCCCAACA GCCGTCTCCAGATGCTGCTGCGTGGCCAGAACCTGCTGGGTTA CCGCCACTACAACGACGAGGTCGTCGATCGCTTCGTCGACAAG TCCGCTGAGAACGGCATGGACGTGTTCCGTGTCTTCGACGCCA TGAATGATCCCCGCAACATGGCGCACGCCATGGCTGCCGTCAA GAAGGCCGGCAAGCACGCGCAGGGCACCATTTGCTACACGAT CAGCCCGGTCCACACCGTTGAGGGCTATGTCAAGCTTGCTGGT CAGCTGCTCGACATGGGTGCTGATTCCATCGCCCTGAAGGACA TGGCCGCCCTGCTCAAGCCGCAGCCGGCCTACGACATCATCAA GGCCATCAAGGACACCTACGGCCAGAAGACGCAGATCAACCT GCACTGCCACTCCACCACGGGTGTCACCGAGGTCTCCCTCATG AAGGCCATCGAGGCCGGCGTCGACGTCGTCGACACCGCCATC TCGTCCATGTCGCTCGGCCCGGGCCACAACCCCACCGAGTCGG TTGCCGAGATGCTCGAGGGCACCGGGTACACCACCAACCTTG ACTACGATCGCCTGCACAAGATCCGCGATCACTTCAAGGCCAT CCGCCCGAAGTACAAGAAGTTCGAGTCGAAGACGCTTGTCGA CACCTCGATCTTCAAGTCGCAGATCCCCGGCGGCATGCTCTCC AACATGGAGTCGCAGCTGCGCGCCCAGGGCGCCGAGGACAAG ATGGACGAGGTCATGGCAGAGGTGCCGCGCGTCCGCAAGGCC GCCGGCTTCCCGCCCCTGGTCACCCCGTCCAGCCAGATCGTCG GCACGCAGGCCGTGTTCAACGTGATGATGGGCGAGTACAAGA GGATGACCGGCGAGTTCGCCGACATCATGCTCGGCTACTACGG CGCCAGCCCGGCCGATCGCGATCCGAAGGTGGTCAAGTTGGC CGAGGAGCAGTCCGGCAAGAAGCCGATCACCCAGCGCCCGGC CGATCTGCTGCCCCCCGAGTGGGAGGAGCAGTCCAAGGAGGC CGCGGCCCTCAAGGGCTTCAACGGCACCGACGAGGACGTGCT CACCTATGCACTGTTCCCGCAGGTCGCTCCGGTCTTCTTCGAG CATCGCGCCGAGGGCCCGCACAGCGTGGCTCTCACCGATGCCC AGCTGAAGGCCGAGGCCGAGGGCGACGAGAAGTCGCTCGCCG TGGCCGGTCCCGTCACCTACAACGTGAACGTGGGCGGAACCG TCCGCGAAGTCACCGTTCAGCAGGCGTGA
Bccp SEQ ID NO: 26	ATGAAACTGAAGGTAACAGTCAACGGCACTGCGTATGACGTT GACGTTGACGTCGACAAGTCACACGAAAACCCGATGGGCACC ATCCTGTTCGGCGGCGGCACCGGCGGCGCGCCGGCACCGCGC GCAGCAGGTGGCGCAGGCGCCGGTAAGGCCGGAGAGGGCGA GATTCCCGCTCCGCTGGCCGGCACCGTCTCCAAGATCCTCGTG AAGGAGGGTGACACGGTCAAGGCTGGTCAGACCGTGCTCGTT CTCGAGGCCATGAAGATGGAGACCGAGATCAACGCTCCCACC GACGGCAAGGTCGAGAAGGTCCTTGTCAAGGAGCGTGACGCC GTGCAGGGCGGTCAGGGTCTCATCAAGATCGGCTGA

[0345] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequence(s) of **Table 3** (**SEQ ID NO: 21**- **SEQ ID NO: 26**) or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same

polypeptide as one or more nucleic acid s sequence(s) of **Table 3** (**SEQ ID NO: 21- SEQ ID NO: 26**) or a functional fragment thereof. In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of one or more nucleic acid sequence(s) of **Table 3** (**SEQ ID NO: 21- SEQ ID NO: 26**) or a functional fragment thereof, or a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as one or more nucleic acid sequence(s) of **Table 3** (**SEQ ID NO: 21- SEQ ID NO: 26**) or a functional fragment thereof.

[0346] **Table 4** lists exemplary polypeptide sequences, which may be encoded by the propionate production gene(s) or cattette(s) of the genetically engineered bacteria.

Table 4 Polypeptide Sequences for Propionate Synthesis

Pct **SEQ ID NO: 27**	MRKVPIITADEAAKLIKDGDTVTTSGFVGNAIPEALDRAVEKRFLETGE PKNITYVYCGSQGNRDGRGAEHFAHEGLLKRYIAGHWATVPALGKM AMENKMEAYNVSQGALCHLFRDIASHKPGVFTKVGIGTFIDPRNGGG KVNDITKEDIVELVEIKGQEYLFYPAFPIHVALIRGTYADESGNITFEKE VAPLEGTSVCQAVKNSGGIVVVQVERVVKAGTLDPRHVKVPGIYVDY VVVADPEDHQQSLDCEYDPALSGEHRRPEVVGEPLPLSAKKVIGRRGA IELEKDVAVNLGVGAPEYVASVADEEGIVDFMTLTAESGAIGGVPAGG VRFGASYNADALIDQGYQFDYYDGGGLDLCYLGLAECDEKGNINVSR FGPRIAGCGGFINITQNTPKVFFCGTFTAGGLKVKIEDGKVIIVQEGKQK KFLKAVEQITFNGDVALANKQQVTYITERCVFLLKEDGLHLSEIAPGID LQTQILDVMDFAPIIDRDANGQIKLMDAALFAEGLMGLKEMKS*
lcdA **SEQ ID NO: 28**	MSLTQGMKAKQLLAYFQGKADQDAREAKARGELVCWSASVAPPEFC VTMGIAMIYPETHAAGIGARKGAMDMLEVADRKGYNVDCCSYGRVN MGYMECLKEAAITGVKPEVLVNSPAADVPLPDLVITCNNICNTLLKWY ENLAAELDIPCIVIDVPFNHTMPIPEYAKAYIADQFRNAISQLEVICGRPF DWKKFKEVKDQTQRSVYHWNRIAEMAKYKPSPLNGFDLFNYMALIV ACRSLDYAEITFKAFADELEENLKAGIYAFKGAEKTRFQWEGIAVWPH LGHTFKSMKNLNSIMTGTAYPALWDLHYDANDESMHSMAEAYTRIYI NTCLQNKVEVLLGIMEKGQVDGTVYHLNRSCKLMSFLNVETAEIIKEK NGLPYVSIDGDQTDPRVFSPAQFDTRVQALVEMMEANMAAAE*
lcdB **SEQ ID NO: 29**	MSRVEAILSQLKDVAANPKKAMDDYKAETGKGAVGIMPIYSPEEMVH AAGYLPMGIWGAQGKTISKARTYLPAFACSVMQQVMELQCEGAYDD LSAVIFSVPCDTLKCLSQKWKGTSPVIVFTHPQNRGLEAANQFLVTEYE LVKAQLESVLGVKISNAALENSIAIYNENRAVMREFVKVAADYPQVID AVSRHAVFKARQFMLKEKHTALVKELIAEIKATPVQPWDGKKVVVTG ILLEPNELLDIFNEFKIAIVDDDLAQESRQIRVDVLDGEGGPLYRMAKA WQQMYGCSLATDTKKGRGRMLINKTIQTGADAIVVAMMKFCDPEEW DYPVMYREFEEKGVKSLMIEVDQEVSSFEQIKTRLQSFVEML*
lcdC **SEQ ID NO: 30**	MYTLGIDVGSASSKAVILKDGKDIVAAEVVQVGTGSSGPQRALDKAFEV SGLKKEDISYTVATGYGRFNFSDADKQISEISCHAKGIYFLVPTARTIIDIG GQDAKAIRLDDKGGIKQFFMNDKCAAGTGRFLEVMARVLETTLDEMAE LDEQATDTAPISSTCTVFAESEVISQLSNGVSRNNIIKGVHLSVASRACGL AYRGGLEKDVVMTGGVAKNAGVVRAVAGVLKTDVIVAPNPQTTGALG

	AALYAYEAAQKKX
etfA **SEQ ID NO: 31**	MAFNSADINSFRDIWVFCEQREGKLINTDFELISEGRKLADERGSKLVG ILLGHEVEEIAKELGGYGADKVIVCDHPELKFYTTDAYAKVLCDVVME EKPEVILIGATNIGRDLGPRCAARLHTGLTADCTHLDIDMNKYVDFLST SSTLDISSMTFPMEDTNLKMTRPAFGGHLMATIICPRFRPCMSTVRPGV MKKAEFSQEMAQACQVVTRHVNLSDEDLKTKVINIVKETKKIVDLIGA EIIVSVGRGISKDVQGGIALAEKLADAFGNGVVGGSRAVIDSGWLPAD HQVGQTGKTVHPKVYVALGISGAIQHKAGMQDSELIIAVNKDETAPIF DCADYGITGDLFKIVPMMIDAIKEGKNA*
acrB **SEQ ID NO: 32**	MRIYVCVKQVPDTSGKVAVNPDGTLNRASMAAIINPDDMSAIEQALKL KDETGCQVTALTMGPPPAEGMLREIIAMGADDGVLISAREFGGSDTFA TSQIISAAIHKLGLSNEDMIFCGRQAIDGDTAQVGPQIAEKLSIPQVTYG AGIKKSGDLVLVKRMLEDGYMMIEVETPCLITCIQDKAVKPRYMTLN GIMECYSKPLLVLDYEALKDEPLIELDTIGLKGSPTNIFKSFTPPQKGVG VMLQGTDKEKVEDLVDKLMQKHVI*
acrC **SEQ ID NO: 33**	MFLLKIKKERMKRMDFSLTREQEMLKKLARQFAEIELEPVAEEIDREH VFPAENFKKMAEIGLTGIGIPKEFGGSGGGTLEKVIAVSEFGKKCMASA SILSIHLIAPQAIYKYGTKEQKETYLPRLTKGGELGAFALTEPNAGSDAG AVKTTAILDSQTNEYVLNGTKCFISGGGRAGVLVIFALTEPKKGLKGM SAIIVEKGTPGFSIGKVESKMGIAGSETAELIFEDCRVPAANLLGKEGKG FKIAMEALDGARIGVGAQAIGIAEGAIDLSVKYVHERIQFGKPIANLQGI QWYIADMATKTAAARALVEFAAYLEDAGKPFTKESAMCKLNASENA RFVTNLALQIHGGYGYMKDYPLERMYRDAKITEIYEGTSEIHKVVIAR EVMKR*
thrAfbr **SEQ ID NO: 34**	MRVLKFGGTSVANAERFLRVADILESNARQGQVATVLSAPAKITNHLV AMIEKTISGQDALPNISDAERIFAELLTGLAAAQPGFPLAQLKTFVDQEF AQIKHVLHGISLLGQCPDSINAALICRGEKMSIAIMAGVLEARGHNVTV IDPVEKLLAVGHYLESTVDIAESTRRIAASRIPADHMVLMAGFTAGNEK GELVVLGRNGSDYSAAVLAACLRADCCEIWTDVDGVYTCDPRQVPD ARLLKSMSYQEAMELSYFGAKVLHPRTITPIAQFQIPCLIKNTGNPQAP GTLIGASRDEDELPVKGISNLNNMAMFSVSGPGMKGMVGMAARVFA AMSRARISVVLITQSSSEYSISFCVPQSDCVRAERAMQEEFYLELKEGLL EPLAVTERLAIISVVGDGMRTLRGISAKFFAALARANINIVAIAQRSSER SISVVVNNDDATTGVRVTHQMLFNTDQVIEVFVIGVGGVGGALLEQL KRQQSWLKNKHIDLRVCGVANSKALLTNVHGLNLENWQEELAQAKE PFNLGRLIRLVKEYHLLNPVIVDCTSSQAVADQYADFLREGFHVVTPN KKANTSSMDYYHQLRYAAEKSRRKFLYDTNVGAGLPVIENLQNLLNA GDELMKFSGILSGSLSYIFGKLDEGMSFSEATTLAREMGYTEPDPRDDL SGMDVARKLLILARETGRELELADIEIEPVLPAEFNAEGDVAAFMANLS QLDDLFAARVAKARDEGKVLRYVGNIDEDGVCRVKIAEVDGNDPLFK VKNGENALAFYSHYYQPLPLVLRGYGAGNDVTAAGVFADLLRTLSW KLGV*
thrB **SEQ ID NO: 35**	MVKVYAPASSANMSVGFDVLGAAVTPVDGALLGDVVTVEAAETFSL NNLGRFADKLPSEPRENIVYQCWERFCQELGKQIPVAMTLEKNMPIGS GLGSSACSVVAALMAMNEHCGKPLNDTRLLALMGELEGRISGSIHYD NVAPCFLGGMQLMIEENDIISQQVPGFDEWLWVLAYPGIKVSTAEARA ILPAQYRRQDCIAHGRHLAGFIHACYSRQPELAAKLMKDVIAEPYRER LLPGFRQARQAVAEIGAVASGISGSGPTLFALCDKPETAQRVADWLGK NYLQNQEGFVHICRLDTAGARVLEN*
thrC	MKLYNLKDHNEQVSFAQAVTQGLGKNQGLFFPHDLPEFSLTEIDEML

SEQ ID NO: 36	KLDFVTRSAKILSAFIGDEIPQEILEERVRAAFAFPAPVANVESDVGCLE LFHGPTLAFKDFGGRFMAQMLTHIAGDKPVTILTATSGDTGAAVAHAF YGLPNVKVVILYPRGKISPLQEKLFCTLGGNIETVAIDGDFDACQALVK QAFDDEELKVALGLNSANSINISRLLAQICYYFEAVAQLPQETRNQLVV SVPSGNFGDLTAGLLAKSLGLPVKRFIAATNVNDTVPRFLHDGQWSPK ATQATLSNAMDVSQPNNWPRVEELFRRKIWQLKELGYAAVDDETTQ QTMRELKELGYTSEPHAAVAYRALRDQLNPGEYGLFLGTAHPAKFKE SVEAILGETLDLPKELAERADLPLLSHNLPADFAALRKLMMNHQ*
ilvA^{fbr} **SEQ ID NO: 37**	MSETYVSEKSPGVMASGAELIRAADIQTAQARISSVIAPTPLQYCPRLSE ETGAEIYLKREDLQDVRSYKIRGALNSGAQLTQEQRDAGIVAASAGNH AQGVAYVCKSLGVQGRIYVPVQTPKQKRDRIMVHGGEFVSLVVTGNN FDEASAAAHEDAERTGATLIEPFDARNTVIGQGTVAAEILSQLTSMGKS ADHVMVPVGGGGLLAGVVSYMADMAPRTAIVGIEPAGAASMQAALH NGGPITLETVDPFVDGAAVKRVGDLNYTIVEKNQGRVHMMSATEGAV CTEMLDLYQNEGIIAEPAGALSIAGLKEMSFAPGSAVVCIISGGNNDVL RYAEIAERSLVHRGLKHYFLVNFPQKPGQLRHFLEDILGPDDDITLFEY LKRNNRETGTALVGIHLSEASGLDSLLERMEESAIDSRRLEPGTPEYEY LT*
aceE **SEQ ID NO: 38**	MSERFPNDVDPIETRDWLQAIESVIREEGVERAQYLIDQLLAEARKGGV NVAAGTGISNYINTIPVEEQPEYPGNLELERRIRSAIRWNAIMTVLRASK KDLELGGHMASFQSSATIYDVCFNHFFRARNEQDGGDLVYFQGHISPG VYARAFLEGRLTQEQLDNFRQEVHGNGLSSYPHPKLMPEFWQFPTVS MGLGPIGAIYQAKFLKYLEHRGLKDTSKQTVYAFLGDGEMDEPESKG AITIATREKLDNLVFVINCNLQRLDGPVTGNGKIINELEGIFEGAGWNVI KVMWGSRWDELLRKDTSGKLIQLMNETVDGDYQTFKSKDGAYVREH FFGKYPETAALVADWTDEQIWALNRGGHDPKKIYAAFKKAQETKGK ATVILAHTIKGYGMGDAAEGKNIAHQVKKMNMDGVRHIRDRFNVPVS DADIEKLPYITFPEGSEEHTYLHAQRQKLHGYLPSRQPNFTEKLELPSLQ DFGALLEEQSKEISTTIAFVRALNVMLKNKSIKDRLVPIIADEARTFGME GLFRQIGIYSPNGQQYTPQDREQVAYYKEDEKGQILQEGINELGAGCS WLAAATSYSTNNLPMIPFYIYYSMFGFQRIGDLCWAAGDQQARGFLIG GTSGRTTLNGEGLQHEDGHSHIQSLTIPNCISYDPAYAYEVAVIMHDGL ERMYGEKQENVYYYITTLNENYHMPAMPEGAEEGIRKGIYKLETIEGS KGKVQLLGSGSILRHVREAAEILAKDYGVGSDVYSVTSFTELARDGQD CERWNMLHPLETPRVPYIAQVMNDAPAVASTDYMKLFAEQVRTYVP ADDYRVLGTDGFGRSDSRENLRHHFEVDASYVVVAALGELAKRGEID KKVVADAIAKFNIDADKVNPRLA*
aceF **SEQ ID NO: 39**	MAIEIKVPDIGADEVEITEILVKVGDKVEAEQSLITVEGDKASMEVPSPQ AGIVKEIKVSVGDKTQTGALIMIFDSADGAADAAPAQAEEKKEAAPAA APAAAAAKDVNVPDIGSDEVEVTEILVKVGDKVEAEQSLITVEGDKAS MEVPAPFAGTVKEIKVNVGDKVSTGSLIMVFEVAGEAGAAAPAAKQE AAPAAAPAPAAGVKEVNVPDIGGDEVEVTEVMVKVGDKVAAEQSLIT VEGDKASMEVPAPFAGVVKELKVNVGDKVKTGSLIMIFEVEGAAPAA APAKQEAAAPAPAAKAEAPAAAPAAKAEGKSEFAENDAYVHATPLIR RLAREFGVNLAKVKGTGRKGRILREDVQAYVKEAIKRAEAAPAATGG GIPGMLPWPKVDFSKFGEIEEVELGRIQKISGANLSRNWVMIPHVTHFD KTDITELEAFRKQQNEEAAKRKLDVKITPVVFIMKAVAAALEQMPRFN SSLSEDGQRLTLKKYINIGVAVDTPNGLVVPVFKDVNKKGIIELSRELM TISKKARDGKLTAGEMQGGCFTISSIGGLGTTHFAPIVNAPEVAILGVSK SAMEPVWNGKEFVPRLMLPISLSFDHRVIDGADGARFITIINNTLSDIRR

	LVM*
lpd **SEQ ID NO: 40**	MSTEIKTQVVVLGAGPAGYSAAFRCADLGLETVIVERYNTLGGVCLN VGCIPSKALLHVAKVIEEAKALAEHGIVFGEPKTDIDKIRTWKEKVINQ LTGGLAGMAKGRKVKVVNGLGKFTGANTLEVEGENGKTVINFDNAII AAGSRPIQLPFIPHEDPRIWDSTDALELKEVPERLLVMGGGIIGLEMGTV YHALGSQIDVVEMFDQVIPAADKDIVKVFTKRISKKFNLMLETKVTAV EAKEDGIYVTMEGKKAPAEPQRYDAVLVAIGRVPNGKNLDAGKAGV EVDDRGFIRVDKQLRTNVPHIFAIGDIVGQPMLAHKGVHEGHVAAEVI AGKKHYFDPKVIPSIAYTKPEVAWVGLTEKEAKEKGISYETATFPWAA SGRAIASDCADGMTKLIFDKESHRVIGGAIVGTNGGELLGEIGLAIEMG CDAEDIALTIHAHPTLHESVGLAAEVFEGSITDLPNPKAKKK*
tesB **SEQ ID NO: 41**	MSQALKNLLTLLNLEKIEEGLFRGQSEDLGLRQVFGGQVVGQALYAA KETVPEERLVHSFHSYFLRPGDSKKPIIYDVETLRDGNSFSARRVAAIQ NGKPIFYMTASFQAPEAGFEHQKTMPSAPAPDGLPSETQIAQSLAHLLP PVLKDKFICDRPLEVRPVEFHNPLKGHVAEPHRQVWIRANGSVPDDLR VHQYLLGYASDLNFLPVALQPHGIGFLEPGIQIATIDHSMWFHRPFNLN EWLLYSVESTSASSARGFVRGEFYTQDGVLVASTVQEGVMRNHN*
acuI **SEQ ID NO: 42**	MRAVLIEKSDDTQSVSVTELAEDQLPEGDVLVDVAYSTLNYKDALAIT GKAPVVRRFPMVPGIDFTGTVAQSSHADFKPGDRVILNGWGVGEKHW GGLAERARVRGDWLVPLPAPLDLRQAAMIGTAGYTAMLCVLALERH GVVPGNGEIVVSGAAGGVGSVATTLLAAKGYEVAAVTGRASEAEYLR GLGAASVIDRNELTGKVRPLGQERWAGGIDVAGSTVLANMLSMMKY RGVVAACGLAAGMDLPASVAPFILRGMTLAGVDSVMCPKTDRLAAW ARLASDLDPAKLEEMTTELPFSEVIETAPKFLDGTVRGRIVIPVTP*
Sbm **SEQ ID NO: 43**	MSNVQEWQQLANKELSRREKTVDSLVHQTAEGIAIKPLYTEADLDNL EVTGTLPGLPPYVRGPRATMYTAQPWTIRQYAGFSTAKESNAFYRRNL AAGQKGLSVAFDLATHRGYDSDNPRVAGDVGKAGVAIDTVEDMKVL FDQIPLDKMSVSMTMNGAVLPVLAFYIVAAEEQGVTPDKLTGTIQNDI LKEYLCRNTYIYPPKPSMRIIADIIAWCSGNMPRFNTISISGYHMGEAGA NCVQQVAFTLADGIEYIKAAISAGLKIDDFAPRLSFFFGIGMDLFMNVA MLRAARYLWSEAVSGFGAQDPKSLALRTHCQTSGWSLTEQDPYNNVI RTTIEALAATLGGTQSLHTNAFDEALGLPTDFSARIARNTQIIIQEESELC RTVDPLAGSYYIESLTDQIVKQARAIIQQIDEAGGMAKAIEAGLPKRMI EEASAREQSLIDQGKRVIVGVNKYKLDHEDETDVLEIDNVMVRNEQIA SLERIRATRDDAAVTAALNALTHAAQHNENLLAAAVNAARVRATLGE ISDALEVAFDRYLVPSQCVTGVIAQSYHQSEKSASEFDAIVAQTEQFLA DNGRRPRILIAKMGQDGHDRGAKVIASAYSDLGFDVDLSPMFSTPEEIA RLAVENDVHVVGASSLAAGHKTLIPELVEALKKWGREDICVVAGGVIP PQDYAFLQERGVAAIYGPGTPMLDSVRDVLNLISQHHD*
ygfD **SEQ ID NO: 44**	MINEATLAESIRRLRQGERATLAQAMTLVESRHPRHQALSTQLLDAIM PYCGNTLRLGVTGTPGAGKSTFLEAFGMLLIREGLKVAVIAVDPSSPVT GGSILGDKTRMNDLARAEAAFIRPVPSSGHLGGASQRARELMLLCEAA GYDVVIVETVGVGQSETEVARMVDCFISLQIAGGGDDLQGIKKGLME VADLIVINKDDGDNHTNVAIARHMYESALHILRRKYDEWQPRVLTCS ALEKRGIDEIWHAIIDFKTALTASGRLQQVRQQQSVEWLRKQTEEEVL NHLFANEDFDRYYRQTLLAVKNNTLSPRTGLRQLSEFIQTQYFD*
ygfG **SEQ ID NO: 45**	MSYQYVNVVTINKVAVIEFNYGRKLNALSKVFIDDLMQALSDLNRPEI RCIILRAPSGSKVFSAGHDIHELPSGGRDPLSYDDPLRQITRMIQKFPKPI ISMVEGSVWGGAFEMIMSSDLIIAASTSTFSMTPVNLGVPYNLVGIHNL TRDAGFHIVKELIFTASPITAQRALAVGILNHVVEVEELEDFTLQMAHH

	ISEKAPLAIAVIKEELRVLGEAHTMNSDEFERIQGMRRAVYDSEDYQEG MNAFLEKRKPNFVGH*
ygfH **SEQ ID NO: 46**	METQWTRMTANEAAEIIQHNDMVAFSGFTPAGSPKALPTAIARRANEQ HEAKKPYQIRLLTGASISAAADDVLSDADAVSWRAPYQTSSGLRKKINQ GAVSFVDLHLSEVAQMVNYGFFGDIDVAVIEASALAPDGRVWLTSGIGN APTWLLRAKKVIIELNHYHDPRVAELADIVIPGAPPRRNSVSIFHAMDRV GTRYVQIDPKKIVAVVETNLPDAGNMLDKQNPMCQQIADNVVTFLLQE MAHGRIPPEFLPLQSGVGNINNAVMARLGENPVIPPFMMYSEVLQESVV HLLETGKISGASASSLTISADSLRKIYDNMDYFASRIVLRPQEISNNPEIIR RLGVIALNVGLEFDIYGHANSTHVAGVDLMNGIGGSGDFERNAYLSIFM APSIAKEGKISTVVPMCSHVDHSEHSVKVIITEQGIADLRGLSPLQRARTII DNCAHPMYRDYLHRYLENAPGGHIHHDLSHVFDLHRNLIATGSMLG*
mutA SEQ ID NO: 47	MSSTDQGTNPADTDDLTPTTLSLAGDFPKATEEQWEREVEKVFNRGRPP EKQLTFAECLKRLTVHTVDGIDIVPMYRPKDAPKKLGYPGVTPFTRGTT VRNGDMDAWDVRALHEDPDEKFTRKAILEDLERGVTSLLLRVDPDAIA PEHLDEVLSDVLLEMTKVEVFSRYDQGAAAEALMGVYERSDKPAKDLA LNLGLDPIGFAALQGTEPDLTVLGDWVRRLAKFSPDSRAVTIDANVYHN AGAGDVAELAWALATGAEYVRALVEQGFNATEAFDTINFRVTATHDQF LTIARLRALREAWARIGEVFGVDEDKRGARQNAITSWRELTREDPYVNI LRGSIATFSASVGGAESITTLPFTQALGLPEDDFPLRIARNTGIVLAEEVNI GRVNDPAGGSYYVESLTRTLADAAWKEFQEVEKLGGMSKAVMTEHVT KVLDACNAERAKRLANRKQPITAVSEFPMIGARSIETKPFPTAPARKGLA WHRDSEVFEQLMDRSTSVSERPKVFLACLGTRRDFGGREGFSSPVWHIA GIDTPQVEGGTTAEIVEAFKKSGAQVADLCSSAKIYAQQGLEVAKALKA AGAKALYLSGAFKEFGDDAAEAEKLIDGRLYMGMDVVDTLSSTLDILG VAK
mutB SEQ ID NO: 48	VSTLPRFDSVDLGNAPVPADAAQRFEELAAKAGTEEAWETAEQIPVGTL FNEDVYKDMDWLDTYAGIPPFVHGPYATMYAFRPWTIRQYAGFSTAKE SNAFYRRNLAAGQKGLSVAFDLPTHRGYDSDNPRVAGDVGMAGVAIDS IYDMRELFAGIPLDQMSVSMTMNGAVLPILALYVVTAEEQGVKPEQLA GTIQNDILKEFMVRNTYIYPPQPSMRIISEIFAYTSANMPKWNSISISGYH MQEAGATADIEMAYTLADGVDYIRAGESVGLNVDQFAPRLSFFWGIGM NFFMEVAKLRAARMLWAKLVHQFGPKNPKSMSLRTHSQTSGWSLTAQ DVYNNVVRTCIEAMAATQGHTQSLHTNSLDEAIALPTDFSARIARNTQL FLQQESGTTRVIDPWSGSAYVEELTWDLARKAWGHIQEVEKVGGMAK AIEKGIPKMRIEEAAARTQARIDSGRQPLIGVNKYRLEHEPPLDVLKVDN STVLAEQKAKLVKLRAERDPEKVKAALDKITWAAANPDDKDPDRNLLK LCIDAGRAMATVGEMSDALEKVFGRYTAQIRTISGVYSKEVKNTPEVEE ARELVEEFEQAEGRRPRILLAKMGQDGHDRGQKVIATAYADLGFDVDV GPLFQTPEETARQAVEADVHVVGVSSLAGGHLTLVPALRKELDKLGRP DILITVGGVIPEQDFDELRKDGAVEIYTPGTVIPESAISLVKKLRASLDA
GI:18042134 SEQ ID	MSNEDLFICIDHVAYACPDADEASKYYQETFGWHELHREENPEQGVVEI MMAPAAKLTEHMTQVQVMAPLNDESTVAKWLAKHNGRAGLHHMAW RVDDIDAVSATLRERGVQLLYDEPKLGTGGNRINFMHPKSGKGVLIELT

NO: 49	QYPKN
mmdA SEQ ID NO: 50	MAENNNLKLASTMEGRVEQLAEQRQVIEAGGGERRVEKQHSQGKQTA RERLNNLLDPHSFDEVGAFRKHRTTLFGMDKAVVPADGVVTGRGTILG RPVHAASQDFTVMGGSAGETQSTKVVETMEQALLTGTPFLFFYDSGGA RIQEGIDSLSGYGKMFFANVKLSGVVPQIAIIAGPCAGGASYSPALTDFII MTKKAHMFITGPQVIKSVTGEDVTADELGGAEAHMAISGNIHFVAEDD DAAELIAKKLLSFLPQNNTEEASFVNPNNDVSPNTELRDIVPIDGKKGYD VRDVIAKIVDWGDYLEVKAGYATNLVTAFARVNGRSVGIVANQPSVMS GCLDINASDKAAEFVNFCDSFNIPLVQLVDVPGFLPGVQQEYGGIIRHGA KMLYAYSEATVPKITVVLRKAYGGSYLAMCNRDLGADAVYAWPSAEI AVMGAEGAANVIFRKEIKAADDPDAMRAEKIEEYQNAFNTPYVAAARG QVDDVIDPADTRRKIASALEMYATKRQTRPAKKHGNFPC
PFREUD_18870 SEQ ID NO: 51	MSPREIEVSEPREVGITELVLRDAHQSLMATRMAMEDMVGACADIDAA GYWSVECWGGATYDSCIRFLNEDPWERLRTFRKLMPNSRLQMLLRGQN LLGYRHYNDEVVDRFVDKSAENGMDVFRVFDAMNDPRNMAHAMAAV KKAGKHAQGTICYTISPVHTVEGYVKLAGQLLDMGADSIALKDMAALL KPQPAYDIIKAIKDTYGQKTQINLHCHSTTGVTEVSLMKAIEAGVDVVD TAISSMSLGPGHNPTESVAEMLEGTGYTTNLDYDRLHKIRDHFKAIRPKY KKFESKTLVDTSIFKSQIPGGMLSNMESQLRAQGAEDKMDEVMAEVPR VRKAAGFPPLVTPSSQIVGTQAVFNVMMGEYKRMTGEFADIMLGYYGA SPADRDPKVVKLAEEQSGKKPITQRPADLLPPEWEEQSKEAAALKGFNG TDEDVLTYALFPQVAPVFFEHRAEGPHSVALTDAQLKAEAEGDEKSLAV AGPVTYNVNVGGTVREVTVQQA
Bccp SEQ ID NO: 52	MKLKVTVNGTAYDVDVDVDKSHENPMGTILFGGGTGGAPAPRAAGGA GAGKAGEGEIPAPLAGTVSKILVKEGDTVKAGQTVLVLEAMKMETEIN APTDGKVEKVLVKERDAVQGGQGLIKIG

[0347] In some embodiments, the genetically engineered bacteria encode one or more polypeptide sequences of **Table 4** (**SEQ ID NO: 27-SEQ ID NO: 52**) or a functional fragment or variant thereof. In some embodiments, genetically engineered bacteria comprise a polypeptide sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the polypeptide sequence of one or more polypeptide sequence of **Table 4** (**SEQ ID NO: 27-SEQ ID NO: 52**) or a functional fragment thereof.

[0348] In one embodiment, the bacterial cell comprises a heterologous propionate gene cassette. In some embodiments, the disclosure provides a bacterial cell that comprises a heterologous propionate gene cassette operably linked to a first promoter. In one embodiment, the first promoter is an inducible promoter. In one embodiment, the bacterial cell comprises a propionate gene cassette from a different organism, *e.g.*, a different species

of bacteria. In another embodiment, the bacterial cell comprises more than one copy of a native gene encoding a propionate gene cassette. In yet another embodiment, the bacterial cell comprises at least one native gene encoding a propionate gene cassette, as well as at least one copy of a propionate gene cassette from a different organism, *e.g.,* a different species of bacteria. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of a gene encoding a propionate gene cassette. In one embodiment, the bacterial cell comprises multiple copies of a gene or genes encoding a propionate gene cassette.

[0349] Multiple distinct propionate gene cassettes are known in the art. In some embodiments, a propionate gene cassette is encoded by a gene cassette derived from a bacterial species. In some embodiments, a propionate gene cassette is encoded by a gene cassette derived from a non-bacterial species. In some embodiments, a propionate gene cassette is encoded by a gene derived from a eukaryotic species, *e.g.,* a fungi. In one embodiment, the gene encoding the propionate gene cassette is derived from an organism of the genus or species that includes, but is not limited to, *Clostridium propionicum*, *Megasphaera elsdenii*, or *Prevotella ruminicola*.

[0350] In one embodiment, the propionate gene cassette has been codon-optimized for use in the engineered bacterial cell. In one embodiment, the propionate gene cassette has been codon-optimized for use in *Escherichia coli*. In another embodiment, the propionate gene cassette has been codon-optimized for use in *Lactococcus*. When the propionate gene cassette is expressed in the engineered bacterial cells, the bacterial cells produce more propionate than unmodified bacteria of the same bacterial subtype under the same conditions (*e.g.,* culture or environmental conditions). Thus, the genetically engineered bacteria comprising a heterologous propionate gene cassette may be used to generate propionate to treat liver disease, such as nonalcoholic steatohepatitis (NASH).

[0351] The present disclosure further comprises genes encoding functional fragments of propionate biosynthesis enzymes or functional variants of a propionate biosynthesis enzyme. As used herein, the term "functional fragment thereof" or "functional variant thereof" relates to an element having qualitative biological activity in common with the wild-type enzyme from which the fragment or variant was derived. For example, a functional fragment or a functional variant of a mutated propionate biosynthesis enzyme is one which retains essentially the same ability to synthesize propionate as the propionate biosynthesis enzyme from which the functional fragment or functional variant was derived. For example a polypeptide having propionate biosynthesis enzyme activity may be truncated at the N-terminus or C-terminus, and the retention of propionate biosynthesis enzyme activity assessed

using assays known to those of skill in the art, including the exemplary assays provided herein. In one embodiment, the engineered bacterial cell comprises a heterologous gene encoding a propionate biosynthesis enzyme functional variant. In another embodiment, the engineered bacterial cell comprises a heterologous gene encoding a propionate biosynthesis enzyme functional fragment.

[0352] As used herein, the term "percent (%) sequence identity" or "percent (%) identity," also including "homology," is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference sequences after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman, 1981, Ads App. Math. 2, 482, by means of the local homology algorithm of Neddleman and Wunsch, 1970, J. Mol. Biol. 48, 443, by means of the similarity search method of Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85, 2444, or by means of computer programs which use these algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

[0353] The present disclosure encompasses propionate biosynthesis enzymes comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein. Amino acid sequences that are substantially the same as the sequences described herein include sequences comprising conservative amino acid substitutions, as well as amino acid deletions and/or insertions. A conservative amino acid substitution refers to the replacement of a first amino acid by a second amino acid that has chemical and/or physical properties (*e.g.,* charge, structure, polarity, hydrophobicity/hydrophilicity) that are similar to those of the first amino acid. Conservative substitutions include replacement of one amino acid by another within the following groups: lysine (K), arginine (R) and histidine (H); aspartate (D) and glutamate (E); asparagine (N), glutamine (Q), serine (S), threonine (T), tyrosine (Y), K, R, H, D and E; alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), tryptophan (W), methionine (M), cysteine (C) and glycine (G); F, W and Y; C, S and T. Similarly contemplated is replacing a basic amino acid with another basic amino acid (*e.g.,* replacement among Lys, Arg, His), replacing an acidic amino acid with another acidic amino acid (*e.g.,* replacement

among Asp and Glu), replacing a neutral amino acid with another neutral amino acid (*e.g.*, replacement among Ala, Gly, Ser, Met, Thr, Leu, Ile, Asn, Gln, Phe, Cys, Pro, Trp, Tyr, Val).

[0354] In some embodiments, a propionate biosynthesis enzyme is mutagenized; mutants exhibiting increased activity are selected; and the mutagenized gene encoding the propionate biosynthesis enzyme is isolated and inserted into the bacterial cell of the disclosure. The gene comprising the modifications described herein may be present on a plasmid or chromosome.

[0355] In one embodiment, the propionate biosynthesis gene cassette is from *Clostridium* spp. In one embodiment, the *Clostridium* spp. is *Clostridium propionicum*. In another embodiment, the propionate biosynthesis gene cassette is from a *Megasphaera* spp. In one embodiment, the *Megasphaera* spp. is *Megasphaera elsdenii*. In another embodiment, the propionate biosynthesis gene cassette is from *Prevotella* spp. In one embodiment, the *Prevotella* spp. is *Prevotella ruminicola*. Other propionate biosynthesis gene cassettes are well-known to one of ordinary skill in the art.

[0356] In some embodiments, the genetically engineered bacteria comprise the genes *pct, lcd,* and *acr* from *Clostridium propionicum.* In some embodiments, the genetically engineered bacteria comprise acrylate pathway genes for propionate biosynthesis, *e.g., pct, lcdA, lcdB, lcdC, etfA, acrB,* and *acrC*. In alternate embodiments, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, *e.g., thrA*^{fbr}, *thrB, thrC, ilvA*^{fbr}, *aceE, aceF,* and *lpd,* and optionally further comprise *tesB*. The genes may be codon-optimized, and translational and transcriptional elements may be added.

[0357] In one embodiment, the *pct* gene has at least about 80% identity with **SEQ ID NO: 1**. In another embodiment, the *pct* gene has at least about 85% identity with **SEQ ID NO: 1**. In one embodiment, the *pct* gene has at least about 90% identity with **SEQ ID NO: 1**. In one embodiment, the *pct* gene has at least about 95% identity with **SEQ ID NO: 1**. In another embodiment, the *pct* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 1**. Accordingly, in one embodiment, the *pct* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 1**. In another embodiment, the *pct* gene comprises the sequence of **SEQ ID NO: 1**. In yet another embodiment the *pct* gene consists of the sequence of **SEQ ID NO: 1**.

[0358] In one embodiment, the *lcdA* gene has at least about 80% identity with **SEQ ID NO: 2**. In another embodiment, the *lcdA* gene has at least about 85% identity with **SEQ ID NO: 2**. In one embodiment, the *lcdA* gene has at least about 90% identity with **SEQ ID**

NO: 2. In one embodiment, the *lcdA* gene has at least about 95% identity with **SEQ ID NO: 2**. In another embodiment, the *lcdA* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 2**. Accordingly, in one embodiment, the *lcdA* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 2**. In another embodiment, the *lcdA* gene comprises the sequence of **SEQ ID NO: 2**. In yet another embodiment the *lcdA* gene consists of the sequence of **SEQ ID NO: 2**.

[0359] In one embodiment, the *lcdB* gene has at least about 80% identity with **SEQ ID NO: 3**. In another embodiment, the *lcdB* gene has at least about 85% identity with **SEQ ID NO: 3**. In one embodiment, the *lcdB* gene has at least about 90% identity with **SEQ ID NO: 3**. In one embodiment, the *lcdB* gene has at least about 95% identity with **SEQ ID NO: 3**. In another embodiment, the *lcdB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 3**. Accordingly, in one embodiment, the *lcdB* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 3**. In another embodiment, the *lcdB* gene comprises the sequence of **SEQ ID NO: 3**. In yet another embodiment the *lcdB* gene consists of the sequence of **SEQ ID NO: 3**.

[0360] In one embodiment, the *lcdC* gene has at least about 80% identity with **SEQ ID NO: 4**. In another embodiment, the *lcdC* gene has at least about 85% identity with **SEQ ID NO: 4**. In one embodiment, the *lcdC* gene has at least about 90% identity with **SEQ ID NO: 4**. In one embodiment, the *lcdC* gene has at least about 95% identity with **SEQ ID NO: 4**. In another embodiment, the *lcdC* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 4**. Accordingly, in one embodiment, the *lcdA* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 4**. In another embodiment, the *lcdC* gene comprises the sequence of **SEQ ID NO: 4**. In yet another embodiment the *lcdC* gene consists of the sequence of **SEQ ID NO: 4**.

[0361] In one embodiment, the *etfA* gene has at least about 80% identity with **SEQ ID NO: 5**. In another embodiment, the *etfA* gene has at least about 85% identity with **SEQ ID NO: 5**. In one embodiment, the *etfA* gene has at least about 90% identity with **SEQ ID NO: 5**. In one embodiment, the *etfA* gene has at least about 95% identity with **SEQ ID NO: 5**. In another embodiment, the *etfA* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 5**. Accordingly, in one embodiment, the *etfA* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,

97%, 98%, or 99% identity with **SEQ ID NO: 5**. In another embodiment, the *etfA* gene comprises the sequence of **SEQ ID NO: 5**. In yet another embodiment the *etfA* gene consists of the sequence of **SEQ ID NO: 5**.

[0362] In one embodiment, the *acrB* gene has at least about 80% identity with **SEQ ID NO: 6**. In another embodiment, the *acrB* gene has at least about 85% identity with **SEQ ID NO: 6**. In one embodiment, the *acrB* gene has at least about 90% identity with **SEQ ID NO: 6**. In one embodiment, the *acrB* gene has at least about 95% identity with **SEQ ID NO: 6**. In another embodiment, the *acrB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 6**. Accordingly, in one embodiment, the *acrB* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 6**. In another embodiment, the *acrB* gene comprises the sequence of **SEQ ID NO: 6**. In yet another embodiment the *acrB* gene consists of the sequence of **SEQ ID NO: 6**.

[0363] In one embodiment, the *acrC* gene has at least about 80% identity with **SEQ ID NO: 7**. In another embodiment, the *acrC* gene has at least about 85% identity with **SEQ ID NO: 7**. In one embodiment, the *acrC* gene has at least about 90% identity with **SEQ ID NO: 7**. In one embodiment, the *acrC* gene has at least about 95% identity with **SEQ ID NO: 7**. In another embodiment, the *acrC* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 7**. Accordingly, in one embodiment, the *acrC* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 7**. In another embodiment, the *acrC* gene comprises the sequence of **SEQ ID NO: 7**. In yet another embodiment the *acrC* gene consists of the sequence of **SEQ ID NO: 7**.

[0364] In one embodiment, the $thrA^{fbr}$ gene has at least about 80% identity with **SEQ ID NO: 8**. In another embodiment, the $thrA^{fbr}$ gene has at least about 85% identity with **SEQ ID NO: 8**. In one embodiment, the $thrA^{fbr}$ gene has at least about 90% identity with **SEQ ID NO: 8**. In one embodiment, the $thrA^{fbr}$ gene has at least about 95% identity with **SEQ ID NO: 8**. In another embodiment, the $thrA^{fbr}$ gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 8**. Accordingly, in one embodiment, the $thrA^{fbr}$ gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 8**. In another embodiment, the $thrA^{fbr}$ gene comprises the sequence of **SEQ ID NO: 8**. In yet another embodiment the $thrA^{fbr}$ gene consists of the sequence of **SEQ ID NO: 8**.

[0365] In one embodiment, the *thrB* gene has at least about 80% identity with **SEQ ID NO: 9**. In another embodiment, the *thrB* gene has at least about 85% identity with **SEQ ID NO: 9**. In one embodiment, the *thrB* gene has at least about 90% identity with **SEQ ID NO: 9**. In one embodiment, the *thrB* gene has at least about 95% identity with **SEQ ID NO: 9**. In another embodiment, the *thrB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 9**. Accordingly, in one embodiment, the *thrB* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 9**. In another embodiment, the *thrB* gene comprises the sequence of **SEQ ID NO: 9**. In yet another embodiment the *thrB* gene consists of the sequence of **SEQ ID NO: 9**.

[0366] In one embodiment, the *thrC* gene has at least about 80% identity with **SEQ ID NO: 10**. In another embodiment, the *thrC* gene has at least about 85% identity with **SEQ ID NO: 10**. In one embodiment, the *thrC* gene has at least about 90% identity with **SEQ ID NO: 10**. In one embodiment, the *thrC* gene has at least about 95% identity with **SEQ ID NO: 10**. In another embodiment, the *thrC* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 10**. Accordingly, in one embodiment, the *thrC* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 10**. In another embodiment, the *thrC* gene comprises the sequence of **SEQ ID NO: 10**. In yet another embodiment the *thrC* gene consists of the sequence of **SEQ ID NO: 10**.

[0367] In one embodiment, the $ilvA^{fbr}$ gene has at least about 80% identity with **SEQ ID NO: 11**. In another embodiment, the $ilvA^{fbr}$ gene has at least about 85% identity with **SEQ ID NO: 11**. In one embodiment, the $ilvA^{fbr}$ gene has at least about 90% identity with **SEQ ID NO: 11**. In one embodiment, the $ilvA^{fbr}$ gene has at least about 95% identity with **SEQ ID NO: 11**. In another embodiment, the $ilvA^{fbr}$ gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 11**. Accordingly, in one embodiment, the $ilvA^{fbr}$ gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 11**. In another embodiment, the $ilvA^{fbr}$ gene comprises the sequence of **SEQ ID NO: 11**. In yet another embodiment the $ilvA^{fbr}$ gene consists of the sequence of **SEQ ID NO: 11**.

[0368] In one embodiment, the *aceE* gene has at least about 80% identity with **SEQ ID NO: 12**. In another embodiment, the *aceE* gene has at least about 85% identity with **SEQ ID NO: 12**. In one embodiment, the *aceE* gene has at least about 90% identity with **SEQ ID NO: 12**. In one embodiment, the *aceE* gene has at least about 95% identity with **SEQ ID**

NO: 12. In another embodiment, the *aceE* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 12**. Accordingly, in one embodiment, the *aceE* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 12**. In another embodiment, the *aceE* gene comprises the sequence of **SEQ ID NO: 12**. In yet another embodiment the *aceE* gene consists of the sequence of **SEQ ID NO: 12**.

[0369] In one embodiment, the *aceF* gene has at least about 80% identity with **SEQ ID NO: 13**. In another embodiment, the *aceF* gene has at least about 85% identity with **SEQ ID NO: 13**. In one embodiment, the *aceF* gene has at least about 90% identity with **SEQ ID NO: 13**. In one embodiment, the *aceF* gene has at least about 95% identity with **SEQ ID NO: 13**. In another embodiment, the *aceF* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 13**. Accordingly, in one embodiment, the *aceF* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 13**. In another embodiment, the *aceF* gene comprises the sequence of **SEQ ID NO: 13**. In yet another embodiment the *aceF* gene consists of the sequence of **SEQ ID NO: 13**.

[0370] In one embodiment, the *lpd* gene has at least about 80% identity with **SEQ ID NO: 14**. In another embodiment, the *lpd* gene has at least about 85% identity with **SEQ ID NO: 14**. In one embodiment, the *lpd* gene has at least about 90% identity with **SEQ ID NO: 14**. In one embodiment, the *lpd* gene has at least about 95% identity with **SEQ ID NO: 14**. In another embodiment, the *lpd* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 14**. Accordingly, in one embodiment, the *lpd* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 14**. In another embodiment, the *lpd* gene comprises the sequence of **SEQ ID NO: 14**. In yet another embodiment the *lpd* gene consists of the sequence of **SEQ ID NO: 14**.

[0371] In one embodiment, the *tesB* gene has at least about 80% identity with **SEQ ID NO: 15**. In another embodiment, the *tesB* gene has at least about 85% identity with **SEQ ID NO: 15**. In one embodiment, the *tesB* gene has at least about 90% identity with **SEQ ID NO: 15**. In one embodiment, the *tesB* gene has at least about 95% identity with **SEQ ID NO: 15**. In another embodiment, the *tesB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 15**. Accordingly, in one embodiment, the *tesB* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 15**. In another embodiment, the

tesB gene comprises the sequence of **SEQ ID NO: 15**. In yet another embodiment the *tesB* gene consists of the sequence of **SEQ ID NO: 15**.

[0372] In one embodiment, the *acuI* gene has at least about 80% identity with **SEQ ID NO: 16**. In another embodiment, the *acuI* gene has at least about 85% identity with **SEQ ID NO: 16**. In one embodiment, the *acuI* gene has at least about 90% identity with **SEQ ID NO: 16**. In one embodiment, the *acuI* gene has at least about 95% identity with **SEQ ID NO: 16**. In another embodiment, the *acuI* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 16**. Accordingly, in one embodiment, the *acuI* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 16**. In another embodiment, the *acuI* gene comprises the sequence of **SEQ ID NO: 16**. In yet another embodiment the *acuI* gene consists of the sequence of **SEQ ID NO: 16**.

[0373] In one embodiment, the *sbm* gene has at least about 80% identity with **SEQ ID NO: 17**. In another embodiment, the *sbm* gene has at least about 85% identity with **SEQ ID NO: 17**. In one embodiment, the *sbm* gene has at least about 90% identity with **SEQ ID NO: 17**. In one embodiment, the *sbm* gene has at least about 95% identity with **SEQ ID NO: 17**. In another embodiment, the *sbm* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 17**.0. Accordingly, in one embodiment, the *sbm* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 17**. In another embodiment, the *sbm* gene comprises the sequence of **SEQ ID NO: 17**. In yet another embodiment the *sbm* gene consists of the sequence of **SEQ ID NO: 17**.

[0374] In one embodiment, the *ygfD* gene has at least about 80% identity with **SEQ ID NO: 18**. In another embodiment, the *ygfD* gene has at least about 85% identity with **SEQ ID NO: 18**. In one embodiment, the *ygfD* gene has at least about 90% identity with **SEQ ID NO: 18**. In one embodiment, the *ygfD* gene has at least about 95% identity with **SEQ ID NO: 18**. In another embodiment, the *ygfD* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 18**.. Accordingly, in one embodiment, the *ygfD* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 18**. In another embodiment, the *ygfD* gene comprises the sequence of **SEQ ID NO: 18**. In yet another embodiment the *ygfD* gene consists of the sequence of **SEQ ID NO: 18**.

[0375] In one embodiment, the *ygfG* gene has at least about 80% identity with **SEQ ID NO: 19**. In another embodiment, the *ygfG* gene has at least about 85% identity with **SEQ**

ID NO: 19. In one embodiment, the *ygfG* gene has at least about 90% identity with **SEQ ID NO: 19**. In one embodiment, the *ygfG* gene has at least about 95% identity with **SEQ ID NO: 19**. In another embodiment, the *ygfG* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 19**.. Accordingly, in one embodiment, the *ygfG* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 19**. In another embodiment, the *ygfG* gene comprises the sequence of **SEQ ID NO: 19**. In yet another embodiment the *ygfG* gene consists of the sequence of **SEQ ID NO: 19**.

[0376] In one embodiment, the *ygfH* gene has at least about 80% identity with **SEQ ID NO: 20**. In another embodiment, the *ygfH* gene has at least about 85% identity with **SEQ ID NO: 20**. In one embodiment, the *ygfH* gene has at least about 90% identity with **SEQ ID NO: 20**. In one embodiment, the *ygfH* gene has at least about 95% identity with **SEQ ID NO: 20**. In another embodiment, the *ygfH* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 20**.. Accordingly, in one embodiment, the *ygfH* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 20**. In another embodiment, the ygfH gene comprises the sequence of **SEQ ID NO: 20**. In yet another embodiment the ygfH gene consists of the sequence of **SEQ ID NO: 20**.

[0377] In one embodiment, one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 27 through SEQ ID NO: 52**. In another embodiment, one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria have at least about 85% identity with one or more of **SEQ ID NO: 27 through SEQ ID NO: 52**. In one embodiment, one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria have at least about 90% identity with one or more of **SEQ ID NO: 27 through SEQ ID NO: 52**. In one embodiment, one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria have at least about 95% identity with one or more of **SEQ ID NO: 27 through SEQ ID NO: 52**. In another embodiment, one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria have at least about 96%, 97%, 98%, or 99% identity with one or more of **SEQ ID NO: 27 through SEQ ID NO: 52**. Accordingly, in one embodiment, one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria have at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%,

95%, 96%, 97%, 98%, or 99% identity with one or more of **SEQ ID NO: 27 through SEQ ID NO: 52**. In another embodiment, one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria comprise the sequence of one or more of **SEQ ID NO: 27 through SEQ ID NO: 52**. In yet another embodiment one or more polypeptides encoded by the propionate circuits and expressed by the genetically engineered bacteria consist of or or more of **SEQ ID NO: 27 through SEQ ID NO: 52**.

[0378] In some embodiments, one or more of the propionate biosynthesis genes is a synthetic propionate biosynthesis gene. In some embodiments, one or more of the propionate biosynthesis genes is an *E. coli* propionate biosynthesis gene. In some embodiments, one or more of the propionate biosynthesis genes is a *C. glutamicum* propionate biosynthesis gene. In some embodiments, one or more of the propionate biosynthesis genes is a *C. propionicum* propionate biosynthesis gene. In some embodiments, one or more of the propionate biosynthesis genes is a *R. sphaeroides* propionate biosynthesis gene. The propionate gene cassette may comprise genes for the aerobic biosynthesis of propionate and/or genes for the anaerobic or microaerobic biosynthesis of propionate.

[0379] In some embodiments, the genetically engineered bacteria comprise a combination of propionate biosynthesis genes from different species, strains, and/or substrains of bacteria, and are capable of producing propionate. In some embodiments, one or more of the propionate biosynthesis genes is functionally replaced, modified, and/or mutated in order to enhance stability and/or increase propionate production. In some embodiments, the local production of propionate reduces food intake and ameliorates metabolic disease (Lin *et al.,* 2012). In some embodiments, the genetically engineered bacteria are capable of expressing the propionate biosynthesis cassette and producing propionate in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0380] To improve acetate production, while maintaining high levels of propionate production, targeted one or more deletions can be introduced in competing metabolic arms of mixed acid fermentation to prevent the production of alternative metabolic fermentative byproducts (thereby increasing acetate production). Non-limiting examples of competing such competing metabolic arms are frdA (converts phosphoenolpyruvate to succinate), ldhA

(converts pyruvate to lactate) and adhE (converts Acetyl-CoA to Ethanol). Deletions which may be introduced therefore include deletion of adhE, ldh, and frd. Thus, in certain embodiments, the genetically engineered bacteria comprise one or more propionate cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0381] In some embodiments, the genetically engineered bacteria comprise one or more propionate cassette(s) described herein and one or more mutation(s) and/or deletion(s) in one or more genes selected from the ldhA gene, the frdA gene and the adhE gene.

[0382] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous frdA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous ldhA and rdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further

comprise a mutation and/or deletion in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE genes.

[0383] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH gene cassette(s) and further comprise a mutation and/or deletion in the endogenous adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous frdA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH gene cassette(s) and further comprise a mutation and/or deletion in the endogenous frdA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH gene cassette(s) and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some

embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes.

[0384] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, fourty-fold, or fifty-fold, more acetate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0385] In some embodiments, the genetically engineered bacteria produce 0% to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more propionate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more propionate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more propionate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0386] In certain situations, the need may arise to prevent and/or reduce acetate production by of an engineered or naturally occurring strain, e.g., E. coli Nissle, while maintaining high levels of propionate production. Without wishing to be bound by theory, one or more mutations and/or deletions in one or more gene(s) encoding in one or more

enzymes which function in the acetate producing metabolic arm of fermentation should reduce and/or prevent production of acetate. A non-limiting example of such an enzyme is phosphate acetyltransferase (Pta), which is the first enzyme in the metabolic arm converting acetyl-CoA to acetate. Deletion and/or mutation of the Pta gene or a gene encoding another enzyme in this metabolic arm may also allow for more acetyl-CoA to be used for propionate production. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentaion, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for propionate synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0387] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation in the endogenous pta and ldhA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of propionate and further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more

enzyme(s) for the production of propionate and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0388] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfH and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation in the endogenous pta and ldhA genes.

[0389] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation in the endogenous pta and ldhA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the

genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from sbm, ygfD, ygfG, and/or ygfHand further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more sbm-ygfD-ygfG-ygfH propionate cassette(s) and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes.

[0390] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% less acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold less acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, less acetate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0391] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to

50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more propionate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more propionate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more propionate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0392] In some embodiments, the genetically engineered bacteria comprise a combination of propionate biosynthesis genes from different species, strains, and/or substrains of bacteria, and are capable of producing propionate. In some embodiments, one or more of the propionate biosynthesis genes is functionally replaced, modified, and/or mutated in order to enhance stability and/or increase propionate production. In some embodiments, the local production of propionate reduces food intake and improves gut barrier function and reduces inflammation In some embodiments, the genetically engineered bacteria are capable of expressing the propionate biosynthesis cassette and producing propionate in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, such molecules or metabolites specific to certain conditions, e.g., conditions associated with hyperammonemia, such as HE-related molecules, e.g., bilirubin, ammonia, manganese, blood coagulation factors, certain antigens and antibodies, and others described herein or known in the art, or their metabolites.

[0393] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of propionate is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, liver damage, or inflammation. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule

or metabolite associated with liver damage, e.g., bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0394] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0395] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of propionate is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present as a consequence of liver disease, e.g., NASH, e.g., hyperammonemia liver damage, or inflammation.. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of propionate is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in Table IX or **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of propionate is modified and/or mutated, e.g., to enhance stability, or increase propionate production.

[0396] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of propionate may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of propionate are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0397] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences described herein for the consumption of ammonia.

[0398] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0399] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0400] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production or catabolism of tryptophan and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above

and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of propionate further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

Butyrate

[0401] In some embodiments, the genetically engineered bacteria of the invention comprise a butyrogenic gene cassette and are capable of producing butyrate under particular exogenous environmental conditions. The bacterial cells described herein may comprise a butyrate gene cassette and are capable of producing butyrate in order to treat liver disease, such as NASH. In one embodiment, the butyrate gene cassette increases the level of butyrate in the cell or in the subject as compared to the level of butyrate in the cell or in the subject prior to expression of the butyrate gene cassette.

[0402] The genetically engineered bacteria may include any suitable set of butyrogenic genes (*see, e.g.,* Table 3). Unmodified bacteria comprising butyrate biosynthesis genes are known and include, but are not limited to, *Peptoclostridium, Clostridium, Fusobacterium, Butyrivibrio, Eubacterium,* and *Treponema*. In some embodiments, the genetically engineered bacteria of the invention comprise butyrate biosynthesis genes from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise the eight genes of the butyrate biosynthesis pathway from *Peptoclostridium difficile, e.g., Peptoclostridium difficile* strain 630: *bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt,* and *buk* (Aboulnaga *et al.*, 2013) and are capable of producing butyrate. *Peptoclostridium difficile* strain 630 and strain 1296 are both capable of producing butyrate, but comprise different nucleic acid sequences for *etfA3, thiA1, hbd, crt2, pbt,* and *buk*. In some embodiments, the genetically engineered bacteria comprise a combination of butyrogenic genes from different species, strains, and/or substrains of bacteria and are capable of producing butyrate. For example, in some embodiments, the genetically engineered bacteria comprise *bcd2, etfB3, etfA3,* and *thiA1* from *Peptoclostridium difficile* strain 630, and *hbd, crt2, pbt,* and *buk* from *Peptoclostridium difficile* strain 1296. Alternatively, a single gene from Treponema denticola (ter, encoding trans-2-enoynl-CoA reductase) is capable of functionally replacing all three of the bcd2, etfB3, and etfA3 genes from Peptoclostridium difficile. Thus, a butyrogenic gene cassette may comprise thiA1, hbd,

crt2, pbt, and buk from Peptoclostridium difficile and ter from Treponema denticola. In another example of a butyrate gene cassette, the pbt and buk genes are replaced with tesB (*e.g.*, from E coli). Thus a butyrogenic gene cassette may comprise ter, thiA1, hbd, crt2, and tesB. In some embodiments, the genetically engineered bacteria are capable of expressing the butyrate biosynthesis cassette and producing butyrate in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. One or more of the butyrate biosynthesis genes may be functionally replaced or modified, *e.g.*, codon optimized.

[0403] In some embodiments, additional genes may be mutated or knocked out, to further increase the levels of butyrate production. Production under anaerobic conditions depends on endogenous NADH pools. Therefore, the flux through the butyrate pathway may be enhanced by eliminating competing routes for NADH utilization. Non-limiting examples of such competing routes are frdA (converts phosphoenolpyruvate to succinate), ldhA (converts pyruvate to lactate) and adhE (converts Acetyl-CoA to Ethanol). Thus, in certain embodiments, the genetically engineered bacteria further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0404] **Table 5** depicts the nucleic acid sequences of exemplary genes in exemplary butyrate biosynthesis gene cassettes.

Table 5. Exemplary Butyrate Cassette Sequences

Description	Sequence
bcd2 **SEQ ID NO: 53**	ATGGATTTAAATTCTAAAAAATATCAGATGCTTAAAGAGCTATA TGTAAGCTTCGCTGAAAATGAAGTTAAACCTTTAGCAACAGAAC TTGATGAAGAAGAAAGATTTCCTTATGAAACAGTGGAAAAAAT GGCAAAAGCAGGAATGATGGGTATACCATATCCAAAAGAATAT GGTGGAGAAGGTGGAGACACTGTAGGATATATAATGGCAGTTG AAGAATTGTCTAGAGTTTGTGGTACTACAGGAGTTATATTATCA GCTCATACATCTCTTGGCTCATGGCCTATATATCAATATGGTAAT GAAGAACAAAAACAAAAATTCTTAAGACCACTAGCAAGTGGAG AAAAATTAGGAGCATTTGGTCTTACTGAGCCTAATGCTGGTACA GATGCGTCTGGCCAACAAACAACTGCTGTTTTAGACGGGGATGA ATACATACTTAATGGCTCAAAAATATTTATAACAAACGCAATAG CTGGTGACATATATGTAGTAATGGCAATGACTGATAAATCTAAG GGGAACAAAGGAATATCAGCATTTATAGTTGAAAAAGGAACTC CTGGGTTTAGCTTTGGAGTTAAAGAAAAGAAAATGGGTATAAG AGGTTCAGCTACGAGTGAATTAATATTTGAGGATTGCAGAATAC CTAAAGAAAATTTACTTGGAAAAGAAGGTCAAGGATTTAAGAT

Description	Sequence
	AGCAATGTCTACTCTTGATGGTGGTAGAATTGGTATAGCTGCAC AAGCTTTAGGTTTAGCACAAGGTGCTCTTGATGAAACTGTTAAA TATGTAAAAGAAAGAGTACAATTTGGTAGACCATTATCAAAATT CCAAAATACACAATTCCAATTAGCTGATATGGAAGTTAAGGTAC AAGCGGCTAGACACCTTGTATATCAAGCAGCTATAAATAAAGA CTTAGGAAAACCTTATGGAGTAGAAGCAGCAATGGCAAAATTA TTTGCAGCTGAAACAGCTATGGAAGTTACTACAAAAGCTGTACA ACTTCATGGAGGATATGGATACACTCGTGACTATCCAGTAGAAA GAATGATGAGAGATGCTAAGATAACTGAAATATATGAAGGAAC TAGTGAAGTTCAAAGAATGGTTATTTCAGGAAAACTATTAAAAT AG
etfB3 **SEQ ID NO: 54**	ATGAATATAGTCGTTTGTATAAAACAAGTTCCAGATACAACAGA AGTTAAACTAGATCCTAATACAGGTACTTTAATTAGAGATGGAG TACCAAGTATAATAAACCCTGATGATAAAGCAGGTTTAGAAGA AGCTATAAAATTAAAAGAAGAAATGGGTGCTCATGTAACTGTTA TAACAATGGGACCTCCTCAAGCAGATATGGCTTTAAAAGAAGCT TTAGCAATGGGTGCAGATAGAGGTATATTATTAACAGATAGAGC ATTTGCGGGTGCTGATACTTGGGCAACTTCATCAGCATTAGCAG GAGCATTAAAAAATATAGATTTTGATATTATAATAGCTGGAAGA CAGGCGATAGATGGAGATACTGCACAAGTTGGACCTCAAATAG CTGAACATTTAAATCTTCCATCAATAACATATGCTGAAGAAATA AAAACTGAAGGTGAATATGTATTAGTAAAAAGACAATTTGAAG ATTGTTGCCATGACTTAAAAGTTAAAATGCCATGCCTTATAACA ACTCTTAAAGATATGAACACACCAAGATACATGAAAGTTGGAA GAATATATGATGCTTTCGAAAATGATGTAGTAGAAACATGGACT GTAAAAGATATAGAAGTTGACCCTTCTAATTTAGGTCTTAAAGG TTCTCCAACTAGTGTATTTAAATCATTTACAAAATCAGTTAAACC AGCTGGTACAATATACAATGAAGATGCGAAAACATCAGCTGGA ATTATCATAGATAAATTAAAAGAGAAGTATATCATATAA
etfA3 **SEQ ID NO: 55**	ATGGGTAACGTTTTAGTAGTAATAGAACAAAGAGAAAATGTAA TTCAAACTGTTTCTTTAGAATTACTAGGAAAGGCTACAGAAATA GCAAAAGATTATGATACAAAAGTTTCTGCATTACTTTTAGGTAG TAAGGTAGAAGGTTTAATAGATACATTAGCACACTATGGTGCAG ATGAGGTAATAGTAGTAGATGATGAAGCTTTAGCAGTGTATACA ACTGAACCATATACAAAAGCAGCTTATGAAGCAATAAAAGCAG CTGACCCTATAGTTGTATTATTTGGTGCAACTTCAATAGGTAGA GATTTAGCGCCTAGAGTTTCTGCTAGAATACATACAGGTCTTAC TGCTGACTGTACAGGTCTTGCAGTAGCTGAAGATACAAAATTAT TATTAATGACAAGACCTGCCTTTGGTGGAAATATAATGGCAACA ATAGTTTGTAAAGATTTCAGACCTCAAATGTCTACAGTTAGACC AGGGGTTATGAAGAAAAATGAACCTGATGAAACTAAAGAAGCT GTAATTAACCGTTTCAAGGTAGAATTTAATGATGCTGATAAATT AGTTCAAGTTGTACAAGTAATAAAAGAAGCTAAAAAACAAGTT AAAATAGAAGATGCTAAGATATTAGTTTCTGCTGGACGTGGAAT GGGTGGAAAAGAAAACTTAGACATACTTTATGAATTAGCTGAA ATTATAGGTGGAGAAGTTTCTGGTTCTCGTGCCACTATAGATGC AGGTTGGTTAGATAAAGCAAGACAAGTTGGTCAAACTGGTAAA ACTGTAAGACCAGACCTTTATATAGCATGTGGTATATCTGGAGC

Description	Sequence
	AATACAACATATAGCTGGTATGGAAGATGCTGAGTTTATAGTTG CTATAAATAAAAATCCAGAAGCTCCAATATTTAAATATGCTGAT GTTGGTATAGTTGGAGATGTTCATAAAGTGCTTCCAGAACTTAT CAGTCAGTTAAGTGTTGCAAAAGAAAAAGGTGAAGTTTTAGCTA ACTAA
thiA1 **SEQ ID NO: 56**	ATGAGAGAAGTAGTAATTGCCAGTGCAGCTAGAACAGCAGTAG GAAGTTTTGGAGGAGCATTTAAATCAGTTTCAGCGGTAGAGTTA GGGGTAACAGCAGCTAAAGAAGCTATAAAAAGAGCTAACATAA CTCCAGATATGATAGATGAATCTCTTTTAGGGGGAGTACTTACA GCAGGTCTTGGACAAAATATAGCAAGACAAATAGCATTAGGAG CAGGAATACCAGTAGAAAAACCAGCTATGACTATAAATATAGT TTGTGGTTCTGGATTAAGATCTGTTTCAATGGCATCTCAACTTAT AGCATTAGGTGATGCTGATATAATGTTAGTTGGTGGAGCTGAAA ACATGAGTATGTCTCCTTATTTAGTACCAAGTGCGAGATATGGT GCAAGAATGGGTGATGCTGCTTTTGTTGATTCAATGATAAAAGA TGGATTATCAGACATATTTAATAACTATCACATGGGTATTACTG CTGAAAACATAGCAGAGCAATGGAATATAACTAGAGAAGAACA AGATGAATTAGCTCTTGCAAGTCAAAATAAAGCTGAAAAAGCT CAAGCTGAAGGAAAATTTGATGAAGAAATAGTTCCTGTTGTTAT AAAAGGAAGAAAAGGTGACACTGTAGTAGATAAAGATGAATAT ATTAAGCCTGGCACTACAATGGAGAAACTTGCTAAGTTAAGACC TGCATTTAAAAAAGATGGAACAGTTACTGCTGGTAATGCATCAG GAATAAATGATGGTGCTGCTATGTTAGTAGTAATGGCTAAAGAA AAAGCTGAAGAACTAGGAATAGAGCCTCTTGCAACTATAGTTTC TTATGGAACAGCTGGTGTTGACCCTAAAATAATGGGATATGGAC CAGTTCCAGCAACTAAAAAAGCTTTAGAAGCTGCTAATATGACT ATTGAAGATATAGATTTAGTTGAAGCTAATGAGGCATTTGCTGC CCAATCTGTAGCTGTAATAAGAGACTTAAATATAGATATGAATA AAGTTAATGTTAATGGTGGAGCAATAGCTATAGGACATCCAATA GGATGCTCAGGAGCAAGAATACTTACTACACTTTTATATGAAAT GAAGAGAAGAGATGCTAAAACTGGTCTTGCTACACTTTGTATAG GCGGTGGAATGGGAACTACTTTAATAGTTAAGAGATAG
hbd **SEQ ID NO: 57**	ATGAAATTAGCTGTAATAGGTAGTGGAACTATGGGAAGTGGTAT TGTACAAACTTTTGCAAGTTGTGGACATGATGTATGTTTAAAGA GTAGAACTCAAGGTGCTATAGATAAATGTTTAGCTTTATTAGAT AAAAATTTAACTAAGTTAGTTACTAAGGGAAAAATGGATGAAG CTACAAAAGCAGAAATATTAAGTCATGTTAGTTCAACTACTAAT TATGAAGATTTAAAAGATATGGATTTAATAATAGAAGCATCTGT AGAAGACATGAATATAAAGAAAGATGTTTTCAAGTTACTAGAT GAATTATGTAAAGAAGATACTATCTTGGCAACAAATACTTCATC ATTATCTATAACAGAAATAGCTTCTTCTACTAAGCGCCCAGATA AAGTTATAGGAATGCATTTCTTTAATCCAGTTCCTATGATGAAA TTAGTTGAAGTTATAAGTGGTCAGTTAACATCAAAAGTTACTTT TGATACAGTATTTGAATTATCTAAGAGTATCAATAAAGTACCAG TAGATGTATCTGAATCTCCTGGATTTGTAGTAAATAGAATACTT ATACCTATGATAAATGAAGCTGTTGGTATATATGCAGATGGTGT TGCAAGTAAAGAAGAAATAGATGAAGCTATGAAATTAGGAGCA AACCATCCAATGGGACCACTAGCATTAGGTGATTTAATCGGATT

Description	Sequence
	AGATGTTGTTTTAGCTATAATGAACGTTTTATATACTGAATTTGG AGATACTAAATATAGACCTCATCCACTTTTAGCTAAAATGGTTA GAGCTAATCAATTAGGAAGAAAAACTAAGATAGGATTCTATGA TTATAATAAATAA
crt2 **SEQ ID NO: 58**	ATGAGTACAAGTGATGTTAAAGTTTATGAGAATGTAGCTGTTGA AGTAGATGGAAATATATGTACAGTGAAAATGAATAGACCTAAA GCCCTTAATGCAATAAATTCAAAGACTTTAGAAGAACTTTATGA AGTATTTGTAGATATTAATAATGATGAAACTATTGATGTTGTAA TATTGACAGGGGAAGGAAAGGCATTTGTAGCTGGAGCAGATAT TGCATACATGAAAGATTTAGATGCTGTAGCTGCTAAAGATTTTA GTATCTTAGGAGCAAAAGCTTTTGGAGAAATAGAAAATAGTAA AAAAGTAGTGATAGCTGCTGTAAACGGATTTGCTTTAGGTGGAG GATGTGAACTTGCAATGGCATGTGATATAAGAATTGCATCTGCT AAAGCTAAATTTGGTCAGCCAGAAGTAACTCTTGGAATAACTCC AGGATATGGAGGAACTCAAAGGCTTACAAGATTGGTTGGAATG GCAAAAGCAAAAGAATTAATCTTTACAGGTCAAGTTATAAAAG CTGATGAAGCTGAAAAAATAGGGCTAGTAAATAGAGTCGTTGA GCCAGACATTTTAATAGAAGAAGTTGAGAAATTAGCTAAGATA ATAGCTAAAAATGCTCAGCTTGCAGTTAGATACTCTAAAGAAGC AATACAACTTGGTGCTCAAACTGATATAAATACTGGAATAGATA TAGAATCTAATTTATTTGGTCTTTGTTTTTCAACTAAAGACCAAA AAGAAGGAATGTCAGCTTTCGTTGAAAAGAGAGAAGCTAACTT TATAAAAGGGTAA
pbt **SEQ ID NO: 59**	ATGAGAAGTTTTGAAGAAGTAATTAAGTTTGCAAAAGAAAGAG GACCTAAAACTATATCAGTAGCATGTTGCCAAGATAAAGAAGTT TTAATGGCAGTTGAAATGGCTAGAAAAGAAAAAATAGCAAATG CCATTTTAGTAGGAGATATAGAAAAGACTAAAGAAATTGCAAA AAGCATAGACATGGATATCGAAAATTATGAACTGATAGATATA AAAGATTTAGCAGAAGCATCTCTAAAATCTGTTGAATTAGTTTC ACAAGGAAAAGCCGACATGGTAATGAAAGGCTTAGTAGACACA TCAATAATACTAAAAGCAGTTTTAAATAAAGAAGTAGGTCTTAG AACTGGAAATGTATTAAGTCACGTAGCAGTATTTGATGTAGAGG GATATGATAGATTATTTTTCGTAACTGACGCAGCTATGAACTTA GCTCCTGATACAAATACTAAAAAGCAAATCATAGAAAATGCTTG CACAGTAGCACATTCATTAGATATAAGTGAACCAAAAGTTGCTG CAATATGCGCAAAAGAAAAAGTAAATCCAAAAATGAAAGATAC AGTTGAAGCTAAAGAACTAGAAGAAATGTATGAAAGAGGAGAA ATCAAAGGTTGTATGGTTGGTGGGCCTTTTGCAATTGATAATGC AGTATCTTTAGAAGCAGCTAAACATAAAGGTATAAATCATCCTG TAGCAGGACGAGCTGATATATTATTAGCCCCAGATATTGAAGGT GGTAACATATTATATAAAGCTTTGGTATTCTTCTCAAAATCAAA AAATGCAGGAGTTATAGTTGGGGCTAAAGCACCAATAATATTA ACTTCTAGAGCAGACAGTGAAGAAACTAAACTAAACTCAATAG CTTTAGGTGTTTTAATGGCAGCAAAGGCATAA

Description	Sequence
buk **SEQ ID NO: 60**	ATGAGCAAAATATTTAAAATCTTAACAATAAATCCTGGTTCGAC ATCAACTAAAATAGCTGTATTTGATAATGAGGATTTAGTATTTG AAAAAACTTTAAGACATTCTTCAGAAGAAATAGGAAAATATGA GAAGGTGTCTGACCAATTTGAATTTCGTAAACAAGTAATAGAAG AAGCTCTAAAAGAAGGTGGAGTAAAAACATCTGAATTAGATGC TGTAGTAGGTAGAGGAGGACTTCTTAAACCTATAAAAGGTGGTA CTTATTCAGTAAGTGCTGCTATGATTGAAGATTTAAAAGTGGGA GTTTTAGGAGAACACGCTTCAAACCTAGGTGGAATAATAGCAA AACAAATAGGTGAAGAAGTAAATGTTCCTTCATACATAGTAGAC CCTGTTGTTGTAGATGAATTAGAAGATGTTGCTAGAATTTCTGG TATGCCTGAAATAAGTAGAGCAAGTGTAGTACATGCTTTAAATC AAAAGGCAATAGCAAGAAGATATGCTAGAGAAATAAACAAGA AATATGAAGATATAAATCTTATAGTTGCACACATGGGTGGAGGA GTTTCTGTTGGAGCTCATAAAAATGGTAAAATAGTAGATGTTGC AAACGCATTAGATGGAGAAGGACCTTTCTCTCCAGAAAGAAGT GGTGGACTACCAGTAGGTGCATTAGTAAAAATGTGCTTTAGTGG AAAATATACTCAAGATGAAATTAAAAAGAAAATAAAAGGTAAT GGCGGACTAGTTGCATACTTAAACACTAATGATGCTAGAGAAGT TGAAGAAAGAATTGAAGCTGGTGATGAAAAAGCTAAATTAGTA TATGAAGCTATGGCATATCAAATCTCTAAAGAAATAGGAGCTAG TGCTGCAGTTCTTAAGGGAGATGTAAAAGCAATATTATTAACTG GTGGAATCGCATATTCAAAAATGTTTACAGAAATGATTGCAGAT AGAGTTAAATTTATAGCAGATGTAAAAGTTTATCCAGGTGAAGA TGAAATGATTGCATTAGCTCAAGGTGGACTTAGAGTTTTAACTG GTGAAGAAGAGGCTCAAGTTTATGATAACTAA
ter **SEQ ID NO: 61**	ATGATCGTAAAACCTATGGTACGCAACAATATCTGCCTGAACGC CCATCCTCAGGGCTGCAAGAAGGGAGTGGAAGATCAGATTGAA TATACCAAGAAACGCATTACCGCAGAAGTCAAAGCTGGCGCAA AAGCTCCAAAAAACGTTCTGGTGCTTGGCTGCTCAAATGGTTAC GGCCTGGCGAGCCGCATTACTGCTGCGTTCGGATACGGGGCTGC GACCATCGGCGTGTCCTTTGAAAAAGCGGGTTCAGAAACCAAAT ATGGTACACCGGGATGGTACAATAATTTGGCATTTGATGAAGCG GCAAAACGCGAGGGTCTTTATAGCGTGACGATCGACGGCGATG CGTTTTCAGACGAGATCAAGGCCCAGGTAATTGAGGAAGCCAA AAAAAAAGGTATCAAATTTGATCTGATCGTATACAGCTTGGCCA GCCCAGTACGTACTGATCCTGATACAGGTATCATGCACAAAAGC GTTTTGAAACCCTTTGGAAAAACGTTCACAGGCAAAACAGTAGA TCCGTTTACTGGCGAGCTGAAGGAAATCTCCGCGGAACCAGCAA ATGACGAGGAAGCAGCCGCCACTGTTAAAGTTATGGGGGGTGA AGATTGGGAACGTTGGATTAAGCAGCTGTCGAAGGAAGGCCTC TTAGAAGAAGGCTGTATTACCTTGGCCTATAGTTATATTGGCCC TGAAGCTACCCAAGCTTTGTACCGTAAAGGCACAATCGGCAAG GCCAAAGAACACCTGGAGGCCACAGCACACCGTCTCAACAAAG AGAACCCGTCAATCCGTGCCTTCGTGAGCGTGAATAAAGGCCTG GTAACCCGCGCAAGCGCCGTAATCCCGGTAATCCCTCTGTATCT CGCCAGCTTGTTCAAAGTAATGAAAGAGAAGGGCAATCATGAA GGTTGTATTGAACAGATCACGCGTCTGTACGCCGAGCGCCTGTA CCGTAAAGATGGTACAATTCCAGTTGATGAGGAAAATCGCATTC

Description	Sequence
	GCATTGATGATTGGGAGTTAGAAGAAGACGTCCAGAAAGCGGT ATCCGCGTTGATGGAGAAAGTCACGGGTGAAAACGCAGAATCT CTCACTGACTTAGCGGGGTACCGCCATGATTTCTTAGCTAGTAA CGGCTTTGATGTAGAAGGTATTAATTATGAAGCGGAAGTTGAAC GCTTCGACCGTATCTGA
tesB **SEQ ID NO: 15**	ATGAGTCAGGCGCTAAAAAATTTACTGACATTGTTAAATCTGGA AAAAATTGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTTA GGTTTACGCCAGGTGTTTGGCGGCCAGGTCGTGGGTCAGGCCTT GTATGCTGCAAAAGAGACCGTCCCTGAAGAGCGGCTGGTACATT CGTTTCACAGCTACTTTCTTCGCCCTGGCGATAGTAAGAAGCCG ATTATTTATGATGTCGAAACGCTGCGTGACGGTAACAGCTTCAG CGCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCGATTTTTT ATATGACTGCCTCTTTCCAGGCACCAGAAGCGGGTTTCGAACAT CAAAAAACAATGCCGTCCGCGCCAGCGCCTGATGGCCTCCCTTC GGAAACGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCA GTGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCG TCCGGTGGAGTTTCATAACCCACTGAAAGGTCACGTCGCAGAAC CACATCGTCAGGTGTGGATCCGCGCAAATGGTAGCGTGCCGGAT GACCTGCGCGTTCATCAGTATCTGCTCGGTTACGCTTCTGATCTT AACTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCT CGAACCGGGGATTCAGATTGCCACCATTGACCATTCCATGTGGT TCCATCGCCCGTTTAATTTGAATGAATGGCTGCTGTATAGCGTG GAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGA GTTTTATACCCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGG AAGGGGTGATGCGTAATCACAATTAA

[0405] Exemplary polypeptide sequences for the production of butyrate by the genetically engineered bacteria are provided in **Table 6**.

Table 6. Exemplary Polypeptide Sequences for Butyrate Production

Description	Sequence
Bcd2 **SEQ ID NO: 62**	MDLNSKKYQMLKELYVSFAENEVKPLATELDEEER FPYETVEKMAKAGMMGIPYPKEYGGEGGDTVGYIM AVEELSRVCGTTGVILSAHTSLGSWPIYQYGNEEQK QKFLRPLASGEKLGAFGLTEPNAGTDASGQQTTAVL DGDEYILNGSKIFITNAIAGDIYVVMAMTDKSKGNK GISAFIVEKGTPGFSFGVKEKKMGIRGSATSELIFEDC RIPKENLLGKEGQGFKIAMSTLDGGRIGIAAQALGLA QGALDETVKYVKERVQFGRPLSKFQNTQFQLADME VKVQAARHLVYQAAINKDLGKPYGVEAAMAKLFA AETAMEVTTKAVQLHGGYGYTRDYPVERMMRDAK ITEIYEGTSEVQRMVISGKLLK
etfB3 **SEQ ID NO: 63**	MNIVVCIKQVPDTTEVKLDPNTGTLIRDGVPSIINPDD KAGLEEAIKLKEEMGAHVTVITMGPPQADMALKEA LAMGADRGILLTDRAFAGADTWATSSALAGALKNI DFDIIIAGRQAIDGDTAQVGPQIAEHLNLPSITYAEEIK TEGEYVLVKRQFEDCCHDLKVKMPCLITTLKDMNT

	PRYMKVGRIYDAFENDVVETWTVKDIEVDPSNLGL KGSPTSVFKSFTKSVKPAGTIYNEDAKTSAGIIIDKLK EKYII
etfA3 **SEQ ID NO: 64**	MGNVLVVIEQRENVIQTVSLELLGKATEIAKDYDTK VSALLLGSKVEGLIDTLAHYGADEVIVVDDEALAVY TTEPYTKAAYEAIKAADPIVVLFGATSIGRDLAPRVS ARIHTGLTADCTGLAVAEDTKLLLMTRPAFGGNIMA TIVCKDFRPQMSTVRPGVMKKNEPDETKEAVINRFK VEFNDADKLVQVVQVIKEAKKQVKIEDAKILVSAGR GMGGKENLDILYELAEIIGGEVSGSRATIDAGWLDK ARQVGQTGKTVRPDLYIACGISGAIQHIAGMEDAEFI VAINKNPEAPIFKYADVGIVGDVHKVLPELISQLSVA KEKGEVLAN
Ter **SEQ ID NO: 65**	MIVKPMVRNNICLNAHPQGCKKGVEDQIEYTKKRIT AEVKAGAKAPKNVLVLGCSNGYGLASRITAAFGYG AATIGVSFEKAGSETKYGTPGWYNNLAFDEAAKRE GLYSVTIDGDAFSDEIKAQVIEEAKKKGIKFDLIVYSL ASPVRTDPDTGIMHKSVLKPFGKTFTGKTVDPFTGEL KEISAEPANDEEAAATVKVMGGEDWERWIKQLSKE GLLEEGCITLAYSYIGPEATQALYRKGTIGKAKEHLE ATAHRLNKENPSIRAFVSVNKGLVTRASAVIPVIPLY LASLFKVMKEKGNHEGCIEQITRLYAERLYRKDGTIP VDEENRIRIDDWELEEDVQKAVSALMEKVTGENAES LTDLAGYRHDFLASNGFDVEGINYEAEVERFDRI
ThiA **SEQ ID NO: 66**	MREVVIASAARTAVGSFGGAFKSVSAVELGVTAAK EAIKRANITPDMIDESLLGGVLTAGLGQNIARQIALG AGIPVEKPAMTINIVCGSGLRSVSMASQLIALGDADI MLVGGAENMSMSPYLVPSARYGARMGDAAFVDSM IKDGLSDIFNNYHMGITAENIAEQWNITREEQDELAL ASQNKAEKAQAEGKFDEEIVPVVIKGRKGDTVVDK DEYIKPGTTMEKLAKLRPAFKKDGTVTAGNASGIND GAAMLVVMAKEKAEELGIEPLATIVSYGTAGVDPKI MGYGPVPATKKALEAANMTIEDIDLVEANEAFAAQ SVAVIRDLNIDMNKVNVNGGAIAIGHPIGCSGARILT TLLYEMKRRDAKTGLATLCIGGGMGTTLIVKR
Hbd **SEQ ID NO: 67**	MKLAVIGSGTMGSGIVQTFASCGHDVCLKSRTQGAI DKCLALLDKNLTKLVTKGKMDEATKAEILSHVSSTT NYEDLKDMDLIIEASVEDMNIKKDVFKLLDELCKED TILATNTSSLSITEIASSTKRPDKVIGMHFFNPVPMMK LVEVISGQLTSKVTFDTVFELSKSINKVPVDVSESPGF VVNRILIPMINEAVGIYADGVASKEEIDEAMKLGAN HPMGPLALGDLIGLDVVLAIMNVLYTEFGDTKYRPH PLLAKMVRANQLGRKTKIGFYDYNK
Crt2 **SEQ ID NO: 68**	MSTSDVKVYENVAVEVDGNICTVKMNRPKALNAIN SKTLEELYEVFVDINNDETIDVVILTGEGKAFVAGAD IAYMKDLDAVAAKDFSILGAKAFGEIENSKKVVIAA VNGFALGGGCELAMACDIRIASAKAKFGQPEVTLGI TPGYGGTQRLTRLVGMAKAKELIFTGQVIKADEAEK IGLVNRVVEPDILIEEVEKLAKIIAKNAQLAVRYSKE AIQLGAQTDINTGIDIESNLFGLCFSTKDQKEGMSAF

	VEKREANFIKG
Pbt **SEQ ID NO: 69**	MRSFEEVIKFAKERGPKTISVACCQDKEVLMAVEMA RKEKIANAILVGDIEKTKEIAKSIDMDIENYELIDIKD LAEASLKSVELVSQGKADMVMKGLVDTSIILKAVLN KEVGLRTGNVLSHVAVFDVEGYDRLFFVTDAAMNL APDTNTKKQIIENACTVAHSLDISEPKVAAICAKEKV NPKMKDTVEAKELEEMYERGEIKGCMVGGPFAIDN AVSLEAAKHKGINHPVAGRADILLAPDIEGGNILYKA LVFFSKSKNAGVIVGAKAPIILTSRADSEETKLNSIAL GVLMAAKA
Buk **SEQ ID NO: 70**	MSKIFKILTINPGSTSTKIAVFDNEDLVFEKTLRHSSE EIGKYEKVSDQFEFRKQVIEEALKEGGVKTSELDAV VGRGGLLKPIKGGTYSVSAAMIEDLKVGVLGEHASN LGGIIAKQIGEEVNVPSYIVDPVVVDELEDVARISGM PEISRASVVHALNQKAIARRYAREINKKYEDINLIVA HMGGGVSVGAHKNGKIVDVANALDGEGPFSPERSG GLPVGALVKMCFSGKYTQDEIKKKIKGNGGLVAYL NTNDAREVEERIEAGDEKAKLVYEAMAYQISKEIGA SAAVLKGDVKAILLTGGIAYSKMFTEMIADRVKFIA DVKVYPGEDEMIALAQGGLRVLTGEEEAQVYDN
TesB **SEQ ID NO: 41**	MSQALKNLLTLLNLEKIEEGLFRGQSEDLGLRQVFG GQVVGQALYAAKETVPEERLVHSFHSYFLRPGDSKK PIIYDVETLRDGNSFSARRVAAIQNGKPIFYMTASFQ APEAGFEHQKTMPSAPAPDGLPSETQIAQSLAHLLPP VLKDKFICDRPLEVRPVEFHNPLKGHVAEPHRQVWI RANGSVPDDLRVHQYLLGYASDLNFLPVALQPHGIG FLEPGIQIATIDHSMWFHRPFNLNEWLLYSVESTSAS SARGFVRGEFYTQDGVLVASTVQEGVMRNHN*

[0406] The gene products of the *bcd2, etfA3,* and *etfB3* genes in *Clostridium difficile* form a complex that converts crotonyl-CoA to butyryl-CoA, which may function as an oxygen-dependent co-oxidant. In some embodiments, because the genetically engineered bacteria of the invention are designed to produce butyrate in a microaerobic or oxygen-limited environment, *e.g.*, the mammalian gut, oxygen dependence could have a negative effect on butyrate production in the gut. It has been shown that a single gene from *Treponema denticola* (*ter,* encoding *trans*-2-enoynl-CoA reductase) can functionally replace this three-gene complex in an oxygen-independent manner. In some embodiments, the genetically engineered bacteria comprise a *ter* gene, *e.g.*, from *Treponema denticola,* which can functionally replace all three of the *bcd2, etfB3,* and *etfA3* genes, *e.g.*, from *Peptoclostridium difficile*. In this embodiment, the genetically engineered bacteria comprise

thiA1, hbd, crt2, pbt, and *buk*, *e.g.*, from *Peptoclostridium difficile,* and *ter*, *e.g.*, from *Treponema denticola,* and produce butyrate in low-oxygen conditions, in the presence of certain molecules or metabolites , in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose..

[0407] In some embodiments, the genetically engineered bacteria of the invention comprise *thiA1, hbd, crt2, pbt,* and *buk*, *e.g.*, from *Peptoclostridium difficile; ter*, *e.g.*, from *Treponema denticola;* one or more of *bcd2, etfB3,* and *etfA3*, *e.g.*, from *Peptoclostridium difficile;* and produce butyrate in low-oxygen conditions, in the presence of certain molecules or metabolites , in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, one or more of the butyrate biosynthesis genes is functionally replaced, modified, and/or mutated in order to enhance stability and/or increase butyrate production in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0408] The gene products of *pbt* and *buk* convert butyrylCoA to Butyrate. In some embodiments, the *pbt* and *buk* genes can be replaced by a *tesB* gene. tesB can be used to cleave off the CoA from butyryl-coA. In one embodiment, the genetically engineered bacteria comprise *bcd2, etfB3, etfA3, thiA1, hbd, and crt2, e.g.*, from *Peptoclostridium difficile,* and *tesB* from *E. coli* and produce butyrate in low-oxygen conditions, in the presence of molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In one embodiment, the genetically engineered bacteria comprise *ter* gene (encoding *trans*-2-enoynl-CoA reductase) *e.g.*, from *Treponema denticola, thiA1, hbd, crt2, pbt,* and *buk*, *e.g.*, from *Peptoclostridium difficile,* and *tesB* from *E. coli* , and produce butyrate in low-oxygen conditions,in the presence of specific molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, one or more

of the butyrate biosynthesis genes is functionally replaced, modified, and/or mutated in order to enhance stability and/or increase butyrate production in low-oxygen conditions or in the presence of specific molecules or metabolites, or molecules or metabolites associated with liver damage, e.g., as seen in NASH, , or other condition(s) such as inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0409] In some embodiments, the local production of butyrate induces the differentiation of regulatory T cells in the gut and/or promotes the barrier function of colonic epithelial cells. In some embodiments, the genetically engineered bacteria comprise genes for aerobic butyrate biosynthesis and/or genes for anaerobic or microaerobic butyrate biosynthesis.

[0410] In one embodiment, the *bcd2* gene has at least about 80% identity with **SEQ ID NO: 53**. In another embodiment, the *bcd2* gene has at least about 85% identity with **SEQ ID NO: 53**. In one embodiment, the *bcd2* gene has at least about 90% identity with **SEQ ID NO: 53**. In one embodiment, the *bcd2* gene has at least about 95% identity with **SEQ ID NO: 53**. In another embodiment, the *bcd2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 53**. Accordingly, in one embodiment, the *bcd2* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 53**. In another embodiment, the *bcd2* gene comprises the sequence of **SEQ ID NO: 53**. In yet another embodiment the *bcd2* gene consists of the sequence of **SEQ ID NO: 53**.

[0411] In one embodiment, the *etfB3* gene has at least about 80% identity with **SEQ ID NO: 54**. In another embodiment, the *etfB3* gene has at least about 85% identity with **SEQ ID NO: 54**. In one embodiment, the *etfB3* gene has at least about 90% identity with **SEQ ID NO: 54**. In one embodiment, the *etfB3* gene has at least about 95% identity with **SEQ ID NO: 54**. In another embodiment, the *etfB3* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 54**. Accordingly, in one embodiment, the *etfB3* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 54**. In another embodiment, the *etfB3* gene comprises the sequence of **SEQ ID NO: 54**. In yet another embodiment the *etfB3* gene consists of the sequence of **SEQ ID NO: 54**.

[0412] In one embodiment, the *etfA3* gene has at least about 80% identity with **SEQ ID NO: 55**. In another embodiment, the *etfA3* gene has at least about 85% identity with **SEQ ID NO: 55**. In one embodiment, the *etfA3* gene has at least about 90% identity with **SEQ ID**

NO: 55. In one embodiment, the *etfA3* gene has at least about 95% identity with **SEQ ID NO: 55**. In another embodiment, the *etfA3* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 55**. Accordingly, in one embodiment, the *etfA3* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 55**. In another embodiment, the *etfA3* gene comprises the sequence of **SEQ ID NO: 55**. In yet another embodiment the *etfA3* gene consists of the sequence of **SEQ ID NO: 55**.

[0413] In one embodiment, the *thiA1* gene has at least about 80% identity with **SEQ ID NO: 56**. In another embodiment, the *thiA1* gene has at least about 85% identity with **SEQ ID NO: 56**. In one embodiment, the *thiA1* gene has at least about 90% identity with **SEQ ID NO: 56**. In one embodiment, the *thiA1* gene has at least about 95% identity with **SEQ ID NO: 56**. In another embodiment, the *thiA1* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 56**. Accordingly, in one embodiment, the *thiA1* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 56**. In another embodiment, the *thiA1* gene comprises the sequence of **SEQ ID NO: 56**. In yet another embodiment the *thiA1* gene consists of the sequence of **SEQ ID NO: 56**.

[0414] In one embodiment, the *hbd* gene has at least about 80% identity with **SEQ ID NO: 57**. In another embodiment, the *hbd* gene has at least about 85% identity with **SEQ ID NO: 57**. In one embodiment, the *hbd* gene has at least about 90% identity with **SEQ ID NO: 57**. In one embodiment, the *hbd* gene has at least about 95% identity with **SEQ ID NO: 57**. In another embodiment, the *hbd* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 57**. Accordingly, in one embodiment, the *hbd* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 57**. In another embodiment, the *hbd* gene comprises the sequence of **SEQ ID NO: 57**. In yet another embodiment the *hbd* gene consists of the sequence of **SEQ ID NO: 57**.

[0415] In one embodiment, the *crt2* gene has at least about 80% identity with **SEQ ID NO: 58**. In another embodiment, the *crt2* gene has at least about 85% identity with **SEQ ID NO: 58**. In one embodiment, the *crt2* gene has at least about 90% identity with **SEQ ID NO: 58**. In one embodiment, the *crt2* gene has at least about 95% identity with **SEQ ID NO: 58**. In another embodiment, the *crt2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 58**. Accordingly, in one embodiment, the *crt2* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,

96%, 97%, 98%, or 99% identity with **SEQ ID NO: 58**. In another embodiment, the *crt2* gene comprises the sequence of **SEQ ID NO: 58**. In yet another embodiment the *crt2* gene consists of the sequence of **SEQ ID NO: 58**.

[0416] In one embodiment, the *pbt* gene has at least about 80% identity with **SEQ ID NO: 59**. In another embodiment, the *pbt* gene has at least about 85% identity with **SEQ ID NO: 59**. In one embodiment, the *pbt* gene has at least about 90% identity with **SEQ ID NO: 59**. In one embodiment, the *pbt* gene has at least about 95% identity with **SEQ ID NO: 59**. In another embodiment, the *pbt* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 59**. Accordingly, in one embodiment, the *pbt* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 59**. In another embodiment, the *pbt* gene comprises the sequence of **SEQ ID NO: 59**. In yet another embodiment the *pbt* gene consists of the sequence of **SEQ ID NO: 59**.

[0417] In one embodiment, the *buk* gene has at least about 80% identity with **SEQ ID NO: 60**. In another embodiment, the *buk* gene has at least about 85% identity with **SEQ ID NO: 60**. In one embodiment, the *buk* gene has at least about 90% identity with **SEQ ID NO: 60**. In one embodiment, the *buk* gene has at least about 95% identity with **SEQ ID NO: 60**. In another embodiment, the *buk* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 60**. Accordingly, in one embodiment, the *buk* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 60**. In another embodiment, the *buk* gene comprises the sequence of **SEQ ID NO: 60**. In yet another embodiment the *buk* gene consists of the sequence of **SEQ ID NO: 60**.

[0418] In one embodiment, the *ter* gene has at least about 80% identity with **SEQ ID NO: 61**. In another embodiment, the *ter* gene has at least about 85% identity with **SEQ ID NO: 61**. In one embodiment, the *ter* gene has at least about 90% identity with **SEQ ID NO: 61**. In one embodiment, the *ter* gene has at least about 95% identity with **SEQ ID NO: 61**. In another embodiment, the *ter* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 61**. Accordingly, in one embodiment, the *ter* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 61**. In another embodiment, the *ter* gene comprises the sequence of **SEQ ID NO: 61**. In yet another embodiment the *ter* gene consists of the sequence of **SEQ ID NO: 61**.

[0419] In one embodiment, the *tesB* gene has at least about 80% identity with **SEQ ID NO: 15**. In another embodiment, the *tesB* gene has at least about 85% identity with **SEQ ID NO: 15**. In one embodiment, the *tesB* gene has at least about 90% identity with **SEQ ID NO: 15**. In one embodiment, the *tesB* gene has at least about 95% identity with **SEQ ID NO: 15**. In another embodiment, the *tesB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 15**. Accordingly, in one embodiment, the *tesB* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 15**. In another embodiment, the *tesB* gene comprises the sequence of **SEQ ID NO: 15**. In yet another embodiment the *tesB* gene consists of the sequence of **SEQ ID NO: 15**.

[0420] In one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**. In another embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 85% identity with with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**. In one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 90% identity with with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**. In one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 95% identity with with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**. In another embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**. Accordingly, in one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**. In another embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria comprise the sequence of with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**. In yet another embodiment one or more polypeptides encoded by the butyrate circuits and expressed by the

genetically engineered bacteria consist of the sequence of with one or more of **SEQ ID NO: 62** through **SEQ ID NO: 70**, and **SEQ ID NO: 41**.

[0421] In some embodiments, one or more of the butyrate biosynthesis genes is a synthetic butyrate biosynthesis gene. In some embodiments, one or more of the butyrate biosynthesis genes is a *Treponema denticola* butyrate biosynthesis gene. In some embodiments, one or more of the butyrate biosynthesis genes is a *C. glutamicum* butyrate biosynthesis gene. In some embodiments, one or more of the butyrate biosynthesis genes is a *Peptoclostridicum difficile* butyrate biosynthesis gene. The butyrate gene cassette may comprise genes for the aerobic biosynthesis of butyrate and/or genes for the anaerobic or microaerobic biosynthesis of butyrate.

[0422] In some embodiments, the genetically engineered bacteria comprise a combination of butyrate biosynthesis genes from different species, strains, and/or substrains of bacteria, and are capable of producing butyrate. In some embodiments, one or more of the butyrate biosynthesis genes is functionally replaced, modified, and/or mutated in order to enhance stability and/or increase butyrate production. In some embodiments, the local production of butyrate reduces food intake and ameliorates metabolic disease (Lin *et al.,* 2012). In some embodiments, the genetically engineered bacteria are capable of expressing the butyrate biosynthesis cassette and producing butyrate in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0423] In one embodiment, the butyrate gene cassette is directly operably linked to a first promoter. In another embodiment, the butyrate gene cassette is indirectly operably linked to a first promoter. In one embodiment, the promoter is not operably linked with the butyrate gene cassette in nature.

[0424] In some embodiments, the butyrate gene cassette is expressed under the control of a constitutive promoter. In another embodiment, the butyrate gene cassette is expressed under the control of an inducible promoter. In some embodiments, the butyrate gene cassette is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the butyrate gene cassette is expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the butyrate gene cassette is

activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. Inducible promoters are described in more detail *infra*.

[0425] The butyrate gene cassette may be present on a plasmid or chromosome in the bacterial cell. In one embodiment, the butyrate gene cassette is located on a plasmid in the bacterial cell. In another embodiment, the butyrate gene cassette is located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the butyrate gene cassette is located in the chromosome of the bacterial cell, and a butyrate gene cassette from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the butyrate gene cassette is located on a plasmid in the bacterial cell, and a butyrate gene cassette from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the butyrate gene cassette is located in the chromosome of the bacterial cell, and a butyrate gene cassette from a different species of bacteria is located in the chromosome of the bacterial cell.

[0426] In some embodiments, the butyrate gene cassette is expressed on a low-copy plasmid. In some embodiments, the butyrate gene cassette is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of butyrate.

[0427] In one embodiment, the bacterial cell comprises a heterologous butyrate gene cassette. In some embodiments, the disclosure provides a bacterial cell that comprises a heterologous butyrate gene cassette operably linked to a first promoter. In one embodiment, the first promoter is an inducible promoter. In one embodiment, the bacterial cell comprises a butyrate gene cassette from a different organism, *e.g.,* a different species of bacteria. In another embodiment, the bacterial cell comprises more than one copy of a native gene encoding a butyrate gene cassette. In yet another embodiment, the bacterial cell comprises at least one native gene encoding a butyrate gene cassette, as well as at least one copy of a butyrate gene cassette from a different organism, *e.g.,* a different species of bacteria. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of a gene encoding a butyrate gene cassette. In one embodiment, the bacterial cell comprises multiple copies of a gene or genes encoding a butyrate gene cassette.

[0428] Multiple distinct butyrate gene cassettes are known in the art. In some embodiments, a butyrate gene cassette is encoded by a gene cassette derived from a bacterial species. In some embodiments, a butyrate gene cassette is encoded by a gene cassette derived from a non-bacterial species. In some embodiments, a butyrate gene cassette is encoded by a gene derived from a eukaryotic species, *e.g.,* a fungi. In one embodiment, the

gene encoding the butyrate gene cassette is derived from an organism of the genus or species that includes, but is not limited to, *Peptoclostridium, Clostridium, Fusobacterium, Butyrivibrio, Eubacterium,* or *Treponema.*

[0429] In one embodiment, the butyrate gene cassette has been codon-optimized for use in the engineered bacterial cell. In one embodiment, the butyrate gene cassette has been codon-optimized for use in *Escherichia coli.* In another embodiment, the butyrate gene cassette has been codon-optimized for use in *Lactococcus.* When the butyrate gene cassette is expressed in the engineered bacterial cells, the bacterial cells produce more butyrate than unmodified bacteria of the same bacterial subtype under the same conditions (*e.g.,* culture or environmental conditions). Thus, the genetically engineered bacteria comprising a heterologous butyrate gene cassette may be used to generate butyrate to treat liver disease, such as nonalcoholic steatohepatitis (NASH). The present disclosure also encompasses butyrate biosynthesis enzymes comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein.

[0430] Assays for testing the activity of a butyrate biosynthesis enzyme, a butyrate biosynthesis enzyme functional variant, or a butyrate biosynthesis enzyme functional fragment are well known to one of ordinary skill in the art.

[0431] In some embodiments, a butyrate biosynthesis enzyme is mutagenized; mutants exhibiting increased activity are selected; and the mutagenized gene encoding the butyrate biosynthesis enzyme is isolated and inserted into the bacterial cell of the disclosure. The gene comprising the modifications described herein may be present on a plasmid or chromosome.

[0432] In one embodiment, the butyrate biosynthesis gene cassette is from *Clostridium* spp. In one embodiment, the *Clostridium* spp. is *Clostridium propionicum.* In another embodiment, the butyrate biosynthesis gene cassette is from a *Peptoclostridium* spp. In one embodiment, the *Peptoclostridium* spp. is *Peptoclostridium difficile.* In another embodiment, the butyrate biosynthesis gene cassette is from *Fusobacterium* spp. In another embodiment, the butyrate biosynthesis gene cassette is from *Butyrivibrio* spp. In another embodiment, the butyrate biosynthesis gene cassette is from *Eubacterium* spp. In another embodiment, the butyrate biosynthesis gene cassette is from *Treponema* spp. Other butyrate biosynthesis gene cassettes are well-known to one of ordinary skill in the art.

[0433] In some embodiments, the genetically engineered bacteria comprise the genes *pct, lcd,* and *acr* from *Clostridium propionicum.* In some embodiments, the genetically engineered bacteria comprise the eight genes of the butyrate biosynthesis pathway from

Peptoclostridium difficile, e.g., Peptoclostridium difficile strain 630: *bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt,* and *buk*. The genes may be codon-optimized, and translational and transcriptional elements may be added.

[0434] In some embodiments, butyrate production by the genetically engineered bacteria can be further enhanced by additional modifications. Butyrate production under anaerobic conditions depends on endogenous NADH pools. In some embodiments, the flux through the butyrate pathway may be enhanced by eliminating competing routes for NADH utilization. A non-limiting example is the mutation/deletion of frdA, which utilizes NADH to catalyze the conversion of phosphoenolpyruvate to succinate. In some embodiments, any of the genetically engineered bacteria described herein further comprise a mutation, which eliminates competing routes for NADH utilization, *e.g.*, a mutation/deletion of frdA.

[0435] To improve or maintain acetate production, while maintaining or improving levels of butyrate production, one or more targeted deletions can be introduced in competing metabolic arms of mixed acid fermentation to prevent the production of alternative metabolic fermentative byproducts (thereby simultaneously increasing butyrate and acetate production). Non-limiting examples of such competing metabolic arms are frdA (converts phosphoenolpyruvate to succinate), ldhA (converts pyruvate to lactate) and adhE (converts Acetyl-CoA to Ethanol). Deletions which may be introduced therefore include deletion of adhE, ldh, and frd. Thus, in certain embodiments, the genetically engineered bacteria comprise one or more butyrate-producing cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE genes.

[0436] In some embodiments, the genetically engineered bacteria comprise one or more butyrate producing cassette(s) described herein and one or more mutation(s) and/or deletion(s) in one or more genes selected from the ldhA gene, the frdA gene and the adhE gene.

[0437] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous adhE gene. In some

embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous frdA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous ldhA and rdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE genes.

[0438] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk gene cassette(s) and further comprise a mutation and/or deletion in the endogenous adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous frdA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk gene cassette(s) and further comprise a mutation and/or deletion in the endogenous frdA gene. In

some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk gene cassette(s) and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes.

[0439] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB gene cassette(s) and further comprise a mutation and/or deletion in the endogenous adhE gene. In some embodiments, the genetically engineered bacteria comprise

one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous frdA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB gene cassette(s) and further comprise a mutation and/or deletion in the endogenous frdA gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA genes and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB gene cassette(s) and further comprise a mutation and/or deletion in the endogenous frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB gene cassette(s) and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes.

[0440] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another

embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, fourty-fold, or fifty-fold, more acetate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0441] In some embodiments, the genetically engineered bacteria produce 0% to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more butyrate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more butyrate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more butyrate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0442] In certain situations, the need may arise to prevent and/or reduce acetate production of an engineered or naturally occurring strain, e.g., E. coli Nissle, while maintaining high levels of butyrate production. Without wishing to be bound by theory, one or more mutations and/or deletions in one or more gene(s) encoding in one or more enzymes which function in the acetate producing metabolic arm of fermentation should reduce and/or prevent production of acetate. A non-limiting example of such an enzyme is phosphate acetyltransferase (Pta), which is the first enzyme in the metabolic arm converting acetyl-CoA to acetate. Deletion and/or mutation of the Pta gene or a gene encoding another enzyme in this metabolic arm may also allow for more acetyl-CoA to be used for butyrate production. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentaion, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for butyrate synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0443] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s)

encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation in the endogenous pta and ldhA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of butyrate and further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzyme(s) for the production of butyrate and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0444] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta gene and in one or more

endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation in the endogenous pta and ldhA genes.

[0445] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation in the endogenous pta and ldhA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more

gene sequence(s) selected from ter, thiA1, hbd, crt2, pbt, and/or buk and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-pbt-buk butyrate cassette(s) and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes.

[0446] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta gene and in one or more endogenous genes selected from in the ldhA gene, the frdA gene and the adhE gene. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation in the endogenous pta and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation in the endogenous pta and ldhA genes.

[0447] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation in the endogenous pta and ldhA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation in the endogenous pta and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s)

selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation and/or deletion in the endogenous pta, ldhA and frdA genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation in the endogenous pta, ldhA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation in the endogenous pta, frdA and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from ter, thiA1, hbd, crt2, tesB and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising one or more ter-thiA1-hbd-crt2-tesB butyrate cassette(s) and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes.

[0448] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% less acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold less acetate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, less acetate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0449] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to

18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more butyrate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more butyrate than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more butyrate than unmodified bacteria of the same bacterial subtype under the same conditions.

[0450] In some embodiments, the genetically engineered bacteria comprise a combination of butyrate biosynthesis genes from different species, strains, and/or substrains of bacteria, and are capable of producing butyrate, alone or in combination with various mutations in genes of the mixed acid fermentation pathway, as described herein. In some embodiments, one or more of the butyrate biosynthesis genes is functionally replaced, modified, and/or mutated in order to enhance stability and/or increase butyrate production. In some embodiments, the local production of butyrate reduces food intake and ameliorates improves gut barrier function and reduces inflammation. In some embodiments, such molecules or metabolites specific to certain conditions, e.g., conditions associated with NASH, e.g., hyperammonemia, inflammation and liver damage related molecules, e.g., bilirubin, ammonia, manganese, blood coagulation factors, certain antigens and antibodies, and others described herein or known in the art, or their metabolites. In some embodiments, the genetically engineered bacteria are capable of expressing the butyrate biosynthesis cassette and producing butyrate in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0451] In one embodiment, the butyrate gene cassette is directly operably linked to a first promoter. In another embodiment, the butyrate gene cassette is indirectly operably linked to a first promoter. In one embodiment, the promoter is not operably linked with the butyrate gene cassette in nature.

[0452] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of butyrate is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or

indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, such as liver damage-related molecules. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with hyperammonemia, inflammation, or liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0453] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0454] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of butyrate is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of butyrate is operably

linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of butyrate is modified and/or mutated, e.g., to enhance stability, or increase butyrate production.

[0455] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of butyrate may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of butyrate are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0456] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences described herein for the consumption of ammonia.

[0457] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0458] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate.

[0459] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production or catabolism of tryptophan and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides

for the production of butyrate further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of butyrate further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

IL-22

[0460] In some embodiments, the genetically engineered bacteria are capable of producing IL-22. Interleukin 22 (IL-22) cytokine can be produced by dendritic cells, lymphoid tissue inducer-like cells, natural killer cells and expressed on adaptive lymphocytes. Through initiation of Jak-STAT signaling pathways, IL-22 expression can trigger expression of antimicrobial compounds as well as a range of cell growth related pathways, both of which enhance tissue repair mechanisms. IL-22 is critical in promoting intestinal barrier fidelity and healing, while modulating inflammatory states. Murine models have demonstrated improved intestinal inflammation states following administration of Il-22. Additionally, IL-22 activates STAT3 signaling to promote enhanced mucus production to preserve barrier function.

[0461] As described by Wang et al, (Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes, Nature 514, 237–241 (09 October 2014)) mice which are deficient in IL-22 receptor and are fed a high-fat diet have a propensity to the development of metabolic disorders. Moreover, Wang et al found that administration of exogenous IL-22 in genetically obese leptin-receptor-deficient (db/db) mice and mice fed with high-fat diet reverses many of the metabolic symptoms, including hyperglycaemia and insulin resistance. These results indicate that IL-22 shows metabolic benefits, from positively affecting insulin sensitivity to the preservation of gut barreier integrity. IL-22 further affects endocrine functions, decreases endotoxaemia and chronic inflammation, and regulates lipid metabolism in liver and adipose tissues.

[0462] In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence encoding **SEQ ID NO: 1050** or a functional fragment thereof. In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to a nucleic acid sequence encoding **SEQ ID NO: 1050** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria are capable of producing IL-22 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing IL-22 in low-oxygen conditions.

Table D. SEQ ID NO: 1050

SEQ ID NO: 1050
MAALQKSVSSFLMGTLATSCLLLLALLVQGGAAAPISSHCRLDKSNFQQPYITNRTF MLAKEASLADNNTDVRLIGEKLFHGVSMSERCYLMKQVLNFTLEEVLFPQSDRFQP YMQEVVPFLARLSNRLSTCHIEGDDLHIQRNVQKLKDTVKKLGESGEIKAIGELDLLF MSLRNACI

[0463] In some embodiments, the construct comprising IL-22 further comprises a secretion tag, such as any secretion tag described herein or known in the art. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-22, OmpF-IL-22, and TorA-IL-22.

[0464] In some embodiments, the genetically engineered bacteria for the secretion of of IL-22 comprise one or more mutations and or deletion in endogenous genes to generate a "leaky" membrane. Non-limiting examples of such endogenous genes which are mutated or deleted are lpp, nlpI, tolA, or PAL.

[0465] In some embodiments, the IL-22 construct comprises a membrane anchor for display of IL-22 on the cell surface.

[0466] Non-limiting examples of such membrane anchors are described herein. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated

with hyperammonemia, inflammation, and/or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0467] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0468] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia, inflammation, or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or

surface display of IL-22 is modified and/or mutated, e.g., to enhance stability, or increase IL-22 production, secretion or display.

[0469] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0470] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences described herein for the consumption of ammonia.

[0471] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0472] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0473] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0474] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production or catabolism of tryptophan and/or

one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0475] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise a GABA transport circuit and/or a GABA metabolic circuit.. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of IL-22 further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0476] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

GLP-1 circuits

[0477] As used herein, the term "glucagon-like peptide 1" refers to an insulinotropic gut hormone, or incretin, which stimulates insulin and suppresses plasma glucose levels. Glucagon-like peptide 1 (GLP-1) is used to treat those suffering from non-alcoholic steatohepatitis by reducing the degree of lipotoxic metabolites, pro-inflammatory substrate, and hepatic lipid deposition. Glucagon-like peptide 1 is well known to those of skill in the art. For example, glucagon-like peptide 1 has been used to stimulate insulin secretion in the

treatment of type-two diabetes and non-alcoholic steatohepatitis (NASH). See, for example, Armstrong, *et al.*, *J. of Hepatology*, 64:399-408 (2016); Bernsmeier, *et al.*, *PLOS One*, 9(1): e87488 (2014); Kjems, *et al.*, *Diabetes*, 52:380-386 (2003); Knudsen *et al.*, *J. Med. Chem.*, 43:1664-1669 (2000); MacDonald, *et al.*, *Diabetes*, 51(supp. 3):S434-S442 (2002); Werner, *et al.*, *Regulatory Peptides*, 164:58-34 (2010); Drucker and Nauck, *Lancet*, 368:1696-1705 (2006); Jiminez-Solem, *et al.*, *Cur. Opinion in Mol. Therap.*, 12(6):760-797 (2010); Schnabel, *et al.*, *Vasc. Health and Risk Mgmt.*, 2(1):69-77 (2006); and WO1995/017510, the entire contents of each of which are expressly incorporated herein by reference.

[0478] The engineered bacteria described herein comprise a heterologous gene encoding glucagon-like peptide 1, and are capable of reducing the degree of lipotoxic metabolites, pro-inflammatory substrate, and hepatic lipid deposition prevalent to those suffering from non-alcoholic steatohepatitis.

[0479] In some embodiments, the genetically engineered bacteria of the invention are capable of producing GLP-1 or proglucagon. GLP-1 and several other insulin and satiety regulating peptides result from cleaved of preproglucagon. Preproglucagon is proteolytically cleaved in a tissue-specific manner. Post-translational processing in the gut and brain by prohormone convertases results in the secretion of GLP-1 and GLP-2, while the glucagon sequence remains in a larger peptide, glicentin or glicentin-related pancreatic peptide (GRPP) and oxyntomodulin. Glucagon-like peptide 1 (GLP-1) is produced by intestinal cells, *e.g.*, ileal L cells, and is capable of stimulating insulin secretion and the differentiation of insulin-secreting cells and inhibiting glucagon secretion. GLP-1 is capable of restoring glucose sensitivity and increasing satiety.

[0480] Glucagon-like peptide 1 (GLP-1) is also used to treat those suffering from non-alcoholic steatohepatitis by reducing the degree of lipotoxic metabolites, pro-inflammatory substrate, and hepatic lipid deposition. Glucagon-like peptide 1 is well known to those of skill in the art. For example, glucagon-like peptide 1 has been used to stimulate insulin secretion in the treatment of type-two diabetes and non-alcoholic steatohepatitis (NASH). See, for example, Armstrong, et al., J. of Hepatology, 64:399-408 (2016); Bernsmeier, et al., PLOS One, 9(1): e87488 (2014); Kjems, et al., Diabetes, 52:380-386 (2003); Knudsen et al., J. Med. Chem., 43:1664-1669 (2000); MacDonald, et al., Diabetes, 51(supp. 3):S434-S442 (2002); Werner, et al., Regulatory Peptides, 164:58-34 (2010); Drucker and Nauck, Lancet, 368:1696-1705 (2006); Jiminez-Solem, et al., Cur. Opinion in Mol. Therap., 12(6):760-797 (2010); Schnabel, et al., Vasc. Health and Risk Mgmt., 2(1):69-

77 (2006); and WO1995/017510, the entire contents of each of which are expressly incorporated herein by reference.

[0481] Proteolytic cleavage of proglucagon produces GLP-1 and GLP-2. GLP-1 adminstration has therapeutic potential in treating type 2 diabetes (Gallwitz *et al.*, 2000). The genetically engineered bacteria may comprise any suitable gene encoding GLP-1 or proglucagon, *e.g.*, human GLP-1 or proglucagon. In some embodiments, a protease inhibitor, *e.g.*, an inhibitor of dipeptidyl peptidase, is also administered to decrease GLP-1 degradation. In some embodiments, the genetically engineered bacteria express a degradation resistant GLP-1 analog (*see, e.g.,* Gallwitz *et al.*, 2000). In some embodiments, the gene encoding GLP-1 or proglucagon is modified and/or mutated, *e.g.*, to enhance stability, increase GLP-1 production, and/or increase metabolic disease attenuation potency. In some embodiments, the local production of GLP-1 induces insulin secretion and/or differentiation of insulin-secreting cells. In some embodiments, the local production of GLP-1 produces satiety in a subject and ameliorates obesity. In some embodiments, the genetically engineered bacteria are capable of expressing GLP-1 or proglucagon in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

Table 7. GLP-1 Polynucleotide Sequences

Description	**Sequence**
GLP-1 (1-37), with initiation met codon; codon optimized for expression in *E. coli*. **SEQ ID NO: 71**	ATGGACGAGTTCGAACGCCACGC GGAGGGAACTTTCACTTCTGATGT TTCTAGCTATTTGGAGGGCCAGGC TGCGAAAGAGTTTATTGCTTGGCT GGTTAAAGGTCGTGGTTAA
GLP1 (1-37) codon optimized for expression in *E. coli*. **SEQ ID NO: 72**	GACGAGTTCGAACGCCACGCGGA GGGAACTTTCACTTCTGATGTTTC TAGCTATTTGGAGGGCCAGGCTGC GAAAGAGTTTATTGCTTGGCTGGT TAAAGGTCGTGGTTAA

Table 8. GLP-1 Polypeptide Sequences

Description	Sequence
GLP-1 (1-37) SEQ ID NO: 73	HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
GLP-1 (1-37) H->M ıtion SEQ ID NO: 74	MDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
GLP-1-(7-37) SEQ ID NO: 75	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
GLP-1-(7-36)NH2 SEQ ID NO: 76	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR
glucagon preproprotein (NP_002045.1) 1-20 is signal peptide SEQ ID NO: 77	MKSIYFVAGLFVMLVQGSWQRSLQDTEEKSRSFSASQ ADPLSDPDQMNEDKRHSQGTFTSDYSKYLDSRRAQDF VQWLMNTKRNRNNIAKRHDEFERHAEGTFTSDVSSYL EGQAAKEFIAWLVKGRGRRDFPEEVAIVEELGRRHAD GSFSDEMNTILDNLAARDFINWLIQTKITDRK
Proglucagon (Signal peptide 1 – 20; Glucagon-like peptide 1 (92-128); Glucagon-like peptide 2 146-178 SEQ ID NO: 78	RSLQDTEEKSRSFSASQADPLSDPDQMNEDKRHSQGTF TSDYSKYLDSRRAQDFVQWLMNTKRNRNNIAKRHDEF ERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRGRRDFP EEVAIVEELGRRHADGSFSDEMNTILDNLAARDFINWLI QTKITDRK
Glucagon SEQ ID NO: 79	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
Glicentin SEQ ID NO: 80	RSLQDTEEKSRSFSASQADPLSDPDQMNEDKRHSQGTF TSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA
Glicentin related peptide SEQ ID NO: 81	RSLQDTEEKSRSFSASQADPLSDPDQMNED
Oxyntomodulin SEQ ID NO: 82	HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA

[01] In some embodiments, the construct comprising GLP-1 further comprises a secretion tag, such as any secretion tag described herein or known in the art. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA- GLP-1, OmpF- GLP-1, and TorA- GLP-1. Other exemplary secretion tages are described herein.

[02] In some embodiments, the genetically engineered bacteria for the secretion of of GLP-1 comprise one or more mutations and or deletion in endogenous genes to generate a “leaky” membrane. Non-limiting examples of such endogenous genes which are mutated or deleted are lpp, nlpI, tolA, or PAL.

[03] The circulating active form of GLP-1 is GLP-1(7-37), which has a very short biological half-life of the order of just a few minutes in blood. The relatively low stability of GLP-1 (3–5 min) has significantly limited its clinical utility because of the rapid degradation catalyzed by the enzyme dipeptidyl peptidase IV (DPP-IV), but also other enzymes such as neutral endopeptidase (NEP), plasma kallikrein or plasmin. One strategy to prolong *in vivo* half-life is stabilization towards degradation by DPPIV, which preferably cleaves N-terminal Xaa–Pro or Xaa–Ala dipeptide sequences. Alteration of that N-terminal sequence, especially the second amino acid, has proven to reduce degradation by DPPIV (*e.g.*, reviewed in Lorenz et al., Recent progress and future options in the development of GLP-1 receptor agonists for the treatment of diabesity; Bioorganic & Medicinal Chemistry Letters, 23 (14);4011–4018). In some embodiments, the genetically engineered bacteria comprise a cassette encoding GLP-1 fragment or variant, in which the DPP-IV is mutated, such that it can no longer be cleaved by the enzyme.

[04] GLP-1 is released in a tissue specific manner, though post-translational processing of pre-pro-glucagon, from the neuroendocrine L-cells predominantly in two forms, GLP-1 (7–36) amide, which constitutes approximately 80% of circulating GLP-1, and GLP-1 (7–37) amide. GLP-1 (1–36 amide) is predominantly secreted in the pancreas, whereas GLP-1 (1–37) is secreted in the ileum and hypothalamus.

[05] In addition, full length GLP-1-(1–37) is produced in much smaller amounts. This full-length form of GLP-1(1-37), was previously thought to be inactive, but was found to stimulate rat intestinal epithelial cells to become glucose-responsive insulin-secreting cells, *i.e.*, full length GLP-1 could convert intestinal epithelial progenitors in the small intestine into insulin-producing cells (Suzuki et al., Glucagon-like peptide 1 (1–37) converts intestinal epithelial cells into insulin-producing cells; Proc Natl Acad Sci U S A. 2003 Apr 29; 100(9): 5034–5039). While the amounts of GLP-1 (1-37) produced endogenously likely are not sufficient for these effects, secretion of large amounts of GLP-1, *e.g.*, by the genetically engineered bacteria, are likely sufficient to alter a balance in the developmental environment of the intestinal epithelia, leading to the induction of insulin-producing cells from intestinal epithelial progenitors. As such, secretion of full-length GLP-1 by the genetically engineered bacteria of the disclosure is a novel therapeutic strategy for the treatment of a number of diseases related to dysregulation of insulin production and/or secretion, including diabetes.

[06] GLP-1 analogs, which exhibit extended stability in serum, have become important in the clinic. Exendin-4, a peptide produced in the salivary glands of the Gila monster (Heloderma suspectum), possesses similar glucose regulatory function to the human

GLP-1 peptide. In exendin-4, the second amino acid is a Gly rendering it resistant to DPPIV mediated degradation. Furthermore, the Leu21–Ser39 span of exendin-4 forms a compact tertiary fold (the Trp-cage) which shields the side chain of Trp25 from solvent exposure, leading to enhanced helicity and stability of the peptide (see Lorenz et al. for review). Exenatide BID is a synthetic version of exendin-4, represents the first GLP-1 RA approved in 2005 as antidiabetic therapy for the treatment of T2DM. Following the FDA approval of exendin-4, liraglutide and albiglutide, which are long-acting GLP-1 analogs using palmitic acid conjugation and albumin fusion, respectively, were approved. Many other strategies have also been employed to achieve long-acting activity of GLP-1, including dimerization, intra-molecular conjugation, and additional variant positive charged amino acids on the N terminus. **Table 9** lists non-limiting examples of GLP-1R agonists. In some embodiments, the genetically engineered bacteria comprise a gene encoding Exenatide. In some embodiments, the genetically engineered bacteria comprise a gene encoding Liraglutide. In some embodiments, the genetically engineered bacteria comprise a gene encoding Lixisenatide. In some embodiments, the genetically engineered bacteria comprise a gene encoding Albiglutide. In some embodiments, the genetically engineered bacteria comprise a gene encoding Dulaglutide. In some embodiments, the genetically engineered bacteria comprise a gene encoding Taspoglutide. In some embodiments, the genetically engineered bacteria comprise a gene encoding Semaglutide.

Table 9. Non-limiting examples of GLP-1R agonists

Name and SEQ ID NO	Sequence	Short description
Exenatide **SEQ ID NO: 83**	HGEGTFTSDLSKQMEEEA VRLFIEWLKNGGPSSGAP PPS	Second amino acid is a Gly rendering it resistant to DPPIV mediated degradation. Furthermore, the Leu21–Ser39 span of exendin-4 forms a compact tertiary fold (the Trp-cage) which shields the side chain of Trp25 from solvent exposure, leading to enhanced helicity and stability of the peptide
Liraglutide **SEQ ID NO: 84**	HAEGTFTSDVSSYLEGQA AKEEFIIAWLVKGRG	a close structural homolog to GLP-1(7–37) with 97% sequence identity to the native hormone. Lys in position 34 is substituted by Arg and a palmitic acid is

		conjugated to Lys in position 26 via a glutamate spacer
Lixisenatide **SEQ ID NO: 85**	HGEGTFTSDLSKQMEEEA VRLFIEWLKNGGPSSGAP PSKKKKKK	synthetic analog of exendin-4. Compared to exendin-4, six Lys residues have been added to the C-terminus (also amidated), while one Pro in the C-terminal region has been deleted.
Albiglutide **SEQ ID NO: 86**	HGEGTFTSDVSSYLEGQA AKEFIAWLVKGRHGEGTF TSDVSSYLEGQAAKEFIA WLVKGRDAHKSEVAHRF KDLGEENFKALVLIAFAQ YLQQCPFEDHVKLVNEVT EFAKTCVADESAENCDKS LHTLFGDKLCTVATLRET YGEMADCCAKQEPERNE CFLQHKDDNPNLPRLVRP EVDVMCTAFHDNEETFL KKYLYEIARRHPYFYAPE LLFFAKRYKAAFTECCQA ADKAACLLPKLDELRDEG KASSAKQRLKCASLQKFG ERAFKAWAVARLSQRFP KAEFAEVSKLVTDLTKVH TECCHGDLLECADDRAD LAKYICENQDSISSKLKEC CEKPLLEKSHCIAEVEND EMPADLPSLAADFVESKD VCKNYAEAKDVFLGMFL YEYARRHPDYSVVLLLRL AKTYETTLEKCCAAADPH ECYAKVFDEFKPLVEEPQ NLIKQNCELFEQLGEYKF QNALLVRYTKKVPQVSTP TLVEVSRNLGKVGSKCCK HPEAKRMPCAEDYLSVV LNQLCVLHEKTPVSDRVT KCCTESLVNRRPCFSALE VDETYVPKEFNAETFTFH ADICTLSEKERQIKKQTAL VELVKHKPKATKEQLKA VMDDFAAFVEKCCKADD KETCFAEEGKKLVAASQA ALGL	two copies of GLP-1 are fused as tandem repeat to the N-terminus of albumin. DPPIV-resistance is achieved by a single substitution, Ala for Gly, at the DPPIV cleavage site.
Dulaglutide **SEQ ID NO: 87**	HGEGTFTSDVSSYLEEQA AKEFIAWLVKGGGGGGG SGGGGSGGGGSAESKYGP PCPPCPAPEAAGGPSVFLF	A recombinant fusion protein, which consists of two GLP-1 peptides covalently linked by a small peptide [tetraglycyl-l-

	PPKPKDTLMISRTPEVTCV VVDVSQEDPEVQFNWYV DGVEVHNAKTKPREEQF NSTYRVVSVLTVLHQDW LNGKEYKCKVSNKGLPSS IEKTISKAKGQPREPQVYT LPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSF FLYSRLTVDKSRWQEGN VFSCSVMHEALHNHYTQ KSLSLSLG	seryltetraglycyl-l-seryltetraglycyl-l-seryl-l-alanyl] to a human IgG4-Fc heavy chain variant. Compared to natural GLP-1, the GLP-1 moieties contain amino acid substitutions (Ala8→Gly, Gly26→Glu, Arg36→Gly) to ensure protection from DPPIV cleavage as well as maintenance of the potency of the construct.
Taspoglutide **SEQ ID NO: 88**	His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu- Phe-Ile-Ala-Trp-Leu-Val-Lys-Aib-Arg-NH_2	a close analog of natural GLP-1(7–36) in which the unnatural amino acid aminoisobutyric acid (Aib) has been introduced in position 8 and 35 in order to avoid degradation by DPPIV, but also by other serine proteases such as plasma kallikrein and plasmin.
Semaglutide **SEQ ID NO: 89**	MAGAPGPLRLALLLLGM VGRAGPRPQGATVSLWE TVQKWREYRRQCQRSLT EDPPPATDLFCNRTFDEY ACWPDGEPGSFVNVSCP WYLPWASSVPQGHVYRF CTAEGLWLQKDNSSLPW RDLSECEESKRGERSSPEE QLLFLYIIYTVGYALSFSA LVIASAILLGFRHLHCTRN YIHLNLFASFILRALSVFIK DAALKWMYSTAAQQHQ WDGLLSYQDSLSCRLVFL LMQYCVAANYYWLLVE GVYLYTLLAFSVLSEQWI FRLYVSIGWGVPLLFVVP WGIVKYLYEDEGCWTRN SNMNYWLIIRLPILFAIGV NFLIFVRVICIVVSKLKAN LMCKTDIKCRLAKSTLTLI PLLGTHEVIFAFVMDEHA RGTLRFIKLFTELSFTSFQ GLMVAILYCFVNNEVQLE FRKSWERWRLEHLHIQRD SSMKPLKCPTSSLSSGATA GSSMYTATCQASCS	

[07] In one embodiment, GLP-1 and/or a GLP-1R agonist of **Table 9** stimulates the rate of insulin secretion in the body. In one embodiment, GLP-1 and/or a GLP-1R agonist of **Table 9** inhibits and lowers plasma glucose produced in the body. In one embodiment, GLP-1 and/or a GLP-1R agonist of Table 10 decreases the level of lipotoxic metabolites in the body. In one embodiment, GLP-1 and/or a GLP-1R agonist of Table 10 decreases the degree of pro-inflammatory substrate in the body. In one embodiment, GLP-1 decreases the level of insulin resistance (IR) in the body. In one embodiment, GLP-1 and/or a GLP-1R agonist of **Table 9** decreases the level of hepatic lipid deposition in the body. Methods for measuring the insulin secretion rates and glucose levels are well known to one of ordinary skill in the art. For example, blood samples taken periodically, and standard statistical analysis methods may be used to determine the insulin secretion rates and plasma glucose levels in a subject.

[08] GLP-1 and/or a GLP-1R agonist of **Table 9** may be expressed or modified in bacteria of this disclosure in order to enhance insulin stimulation and reduce plasma glucose levels in subjects having liver disease, such as NASH. Specifically, when GLP-1 and/or a GLP-1R agonist of **Table 9** is expressed in the engineered bacterial cells of the disclosure, the expressed GLP-1 and/or a GLP-1R agonist of **Table 9** will reduce the degree of lipotoxic metabolites, pro-inflammatory substrate, and hepatic lipid deposition in the subject. Thus, the genetically engineered bacteria comprising a heterologous gene encoding GLP-1 can be used to treat non-alcoholic steatohepatitis.

[09] GLP-1 and/or a GLP-1R agonist of **Table 9** may be expressed or modified in bacteria of this disclosure in order to enhance insulin stimulation and reduce plasma glucose levels in subjects having type two diabetes, obesity, and/or metabolic syndrome, or metabolic syndrome related disorders, including cardiovascular disorders, and obesity in a subject.

[010] In one embodiment, the bacterial cell comprises one or more genes encoding a GLP-1 and/or a GLP-1R agonist of **Table 9**. In some embodiments, the disclosure provides a bacterial cell that comprises a heterologous gene encoding a glucagon-like peptide 1 operably linked to a first promoter. In one embodiment, the first promoter is an inducible promoter. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of a gene encoding a glucagon-like peptide 1. In one embodiment, the bacterial cell comprises multiple copies of a gene or genes encoding a glucagon-like peptide 1.

[011] Multiple distinct embodiments of GLP-1 and/or a GLP-1R agonist of **Table 9** are known in the art. In some embodiments, the glucagon-like peptide 1 is encoded by a gene derived from a bacterial species. In some embodiments, a glucagon-like peptide 1 is encoded by a gene derived from a non-bacterial species. In some embodiments, a glucagon-like

peptide 1 is encoded by a gene derived from a eukaryotic species, *e.g.* homo sapiens. In one embodiment, the gene encoding the glucagon-like peptide 1 is expressed in an organism of the genus or species that includes, but is not limited to, Lactobacillus spp., such as Lactobacillus plantarum, Lactobacillus johnsonii, Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus brevis, or Lactobacillus gasseri; Bifidobacterium spp., such as Bifidobacterium longum; Bacillus spp., such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus; and Streptomyces spp., such as Streptomyces lividans.

[012] The present disclosure encompasses genes encoding a GLP-1 and/or a GLP-1R agonist of **Table 9** comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein.

[013] In one embodiment, the gene encoding the GLP-1 and/or a GLP-1R agonist of **Table 9** has been codon-optimized for use in the engineered bacterial cell. In one embodiment, the gene encoding the glucagon-like peptide 1 has been codon-optimized for use in *Escherichia coli*. In another embodiment, the gene encoding the glucagon-like peptide 1 has been codon-optimized for use in Lactococcus. When the gene encoding the GLP-1 and/or a GLP-1R agonist of **Table 9** is expressed in the engineered bacterial cells, the bacterial cells express more GLP-1 and/or a GLP-1R agonist of **Table 9** than unmodified bacteria of the same bacterial subtype under the same conditions (*e.g.*, culture or environmental conditions). Thus, the genetically engineered bacteria comprising a heterologous gene encoding a GLP-1 and/or a GLP-1R agonist of **Table 9** may be used to express more GLP-1 and/or a GLP-1R agonist of **Table 9** to treat liver disease, such as nonalcoholic steatohepatitis, in a subject.

[014] Assays for testing the activity of a GLP-1 and/or a GLP-1R agonist of **Table 9** or a glucagon-like peptide 1 receptor are well known to one of ordinary skill in the art. For example, glucose and insulin levels can be assessed by drawing plasma samples from subjects previously administered intravenous infusions of the glucagon-like peptide 1 as described in Kjems, et al., Diabetes, 52:380-386 (2003), the entire contents of which are expressly incorporated herein by reference. Briefly, plasma samples from a subject are treated with heparin and sodium fluoride, centrifuged, and plasma glucose levels measured by a glucose oxidase technique. Likewise, the plasma insulin concentrations are measured by a two-site insulin enzyme linked immunosorbent method. Alternatively, baby hamster kidney cells can be used to assay structure-activity relationships of glucagon-like peptide 1

derivatives (see, for example, Knudsen et al., J. Med. Chem., 43:1664-1669 (2000), the entire contents of which are expressly incorporated herein by reference). The present disclosure encompasses genes encoding a GLP-1 and/or a GLP-1R agonist of **Table 9** comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein.

[015] In some embodiments, the gene encoding a GLP-1 and/or a GLP-1R agonist of **Table 9** is mutagenized; mutants exhibiting increased activity are selected; and the mutagenized gene encoding the GLP-1 and/or a GLP-1R agonist of **Table 9** is isolated and inserted into the bacterial cell of the disclosure. The gene comprising the modifications described herein may be present on a plasmid or chromosome.

[016] In one embodiment, the gene encoding the glucagon-like peptide 1 is from *Homo sapiens*. Other genes encoding glucagon-like peptide 1 are well-known to one of ordinary skill in the art and described in, for example, MacDonald, *et al.*, *Diabetes*, 51(supp. 3):S434-S442 (2002) and WO1995/017510.

[017] In one embodiment, the gene encoding the glucagon-like peptide 1 has at least about 80% identity with a nucleic acid sequence encoding **SEQ ID NO: 71** or **SEQ ID NO: 72**. In another embodiment, the gene encoding the glucagon-like peptide 1 has at least about 85% identity with a nucleic acid sequence encoding **SEQ ID NO: 71** or **SEQ ID NO: 72**. In one embodiment, the gene encoding the glucagon-like peptide 1 has at least about 90% identity with a nucleic acid sequence encoding **SEQ ID NO: 71** or **SEQ ID NO: 72**. In one embodiment, the gene encoding the glucagon-like peptide 1 has at least about 95% identity with a nucleic acid sequence encoding **SEQ ID NO: 71** or **SEQ ID NO: 72**. In another embodiment, the gene encoding the glucagon-like peptide 1 has at least about 96%, 97%, 98%, or 99% identity with a nucleic acid sequence encoding **SEQ ID NO: 71** or **SEQ ID NO: 72**. Accordingly, in one embodiment, the gene encoding the glucagon-like peptide 1 has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with a nucleic acid sequence encoding SEQ ID NO:40. In another embodiment, the gene encoding the glucagon-like peptide 1 comprises a nucleic acid sequence encoding **SEQ ID NO: 71** or **SEQ ID NO: 72**. In yet another embodiment the gene encoding the glucagon-like peptide 1 consists of a nucleic acid sequence encoding **SEQ ID NO: 71** or **SEQ ID NO: 72**.

[018] In one embodiment, the gene encoding the glucagon-like peptide 1 is directly operably linked to a first promoter. In another embodiment, the gene encoding the glucagon-

like peptide 1 is indirectly operably linked to a first promoter. In one embodiment, the promoter is not operably linked with the gene encoding the glucagon-like peptide 1 in nature.

[019] In some embodiments, the gene encoding the glucagon-like peptide 1 is expressed under the control of a constitutive promoter. In another embodiment, the gene encoding the glucagon-like peptide 1 is expressed under the control of an inducible promoter. In some embodiments, the gene encoding the glucagon-like peptide 1 is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the gene encoding the glucagon-like peptide 1 is expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the gene encoding the glucagon-like peptide 1 is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. In one embodiment, the gene encoding the glucagon-like peptide 1 is expressed under the control of a promoter that is directly or indirectly induced in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, , inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. Inducible promoters are described in more detail infra.

[020] The gene encoding the glucagon-like peptide 1 may be present on a plasmid or chromosome in the bacterial cell. In one embodiment, the gene encoding the glucagon-like peptide 1 is located on a plasmid in the bacterial cell. In another embodiment, the gene encoding the glucagon-like peptide 1 is located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the gene encoding the glucagon-like peptide 1 is located in the chromosome of the bacterial cell, and a second gene encoding a second glucagon-like peptide 1 is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene encoding the glucagon-like peptide 1 is located on a plasmid in the bacterial cell, and a second gene encoding a second glucagon-like peptide 1 is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene encoding the glucagon-like peptide 1 is located in the chromosome of the bacterial cell, and a second gene encoding a second glucagon-like peptide 1 is located in the chromosome of the bacterial cell.

[021] In some embodiments, the gene encoding the glucagon-like peptide 1 is expressed on a low-copy plasmid. In some embodiments, the gene encoding the glucagon-like peptide 1 is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of the glucagon-like peptide 1, thereby

reducing the degree of lipotoxic metabolites, pro-inflammatory substrate, and hepatic lipid deposition prevalent to those suffering from non-alcoholic steatohepatitis.

[022] In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding GLP-1 (1-37), or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 73**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding GLP-1 (1-37) H->M substitution), or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 74**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding GLP-1-(7-37), or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 75**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding GLP-1-(7-36), or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 76**.

[023] In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding glucagon preproprotein (NP_002045.1), or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Proglucagon, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 78**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Glucagon, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 79**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Glicentin), or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 80** In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Glicentin related peptide), or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 81**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Oxyntomodulin. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 82**.

[024] In one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 73** through **SEQ ID NO: 82**. In another embodiment, one or

more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 85% identity with with one or more of **SEQ ID NO: 73** through **SEQ ID NO: 82**. In one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 90% identity with with one or more of **SEQ ID NO: 73** through **SEQ ID NO: 82**. In one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 95% identity with with one or more of **SEQ ID NO: 73** through **SEQ ID NO: 82**. In another embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 73** through **SEQ ID NO: 82**. Accordingly, in one embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria have at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 62 through SEQ ID NO: 70, and SEQ ID NO: 41**. In another embodiment, one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria comprise the sequence of with one or more of **SEQ ID NO: 73** through **SEQ ID NO: 82**. In yet another embodiment one or more polypeptides encoded by the butyrate circuits and expressed by the genetically engineered bacteria consist of the sequence of with one or more of **SEQ ID NO: 73** through **SEQ ID NO: 82**.

[025] In embodiments, the pro-glucagon derived polypeptides, GLP-1 polypeptides, GLP-1 analogs described herein, and functional variants or fragments thereof are secreted. In some embodiments, the genetically engineered bacteria comprise one or more cassettes encoding pro-glucagon derived polypeptides, GLP-1 polypeptides, GLP-1 analogs, and/or functional variants or fragments and a secretion gene cassette and/or mutations generating a leaky phenotype. In some embodiments, a flagellar type III secretion pathway is used to secrete pro-glucagon derived polypeptides, GLP-1 polypeptides, and/or GLP-1 analogs described herein.

In some embodiments, a Type V Autotransporter Secretion System is used to secrete pro-glucagon derived polypeptides, GLP-1 polypeptides, and/or GLP-1 analogs described herein.

In some embodiments, a Hemolysin-based Secretion System is used to secrete the pro-glucagon derived polypeptides, GLP-1 polypeptides, and/or GLP-1 analogs described herein..

In alternate embodiments, the genetically engineered bacteria expressing the pro-glucagon

derived polypeptides, GLP-1 polypeptides, and/or GLP-1 analogs described herein. further comprise a non-native single membrane-spanning secretion system. As descriebed herein. In some embodiments, the engineered bacteria expressing the pro-glucagon derived polypeptides, GLP-1 polypeptides, and/or GLP-1 analogs described herein. have one or more deleted or mutated membrane genes to generate a leaky phenotype as described herein.

[026] In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Exenatide, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 83**.

[027] In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Liraglutide, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 84**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Lixisenatide, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 85**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Albiglutide, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 86**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Dulaglutide, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 87**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Taspoglutide, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 88**. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding Semaglutide, or a functional fragment or variant thereof. In one embodiment, the genetically engineered bacteria comprise a gene cassette encoding **SEQ ID NO: 89**.

[028] In one embodiment, one or more polypeptides encoded by the and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**. In another embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 85% identity with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**. In one embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 90% identity with with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**. In one embodiment, one or more polypeptides

encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 95% identity with with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**. In another embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**. Accordingly, in one embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**. In another embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria comprise the sequence of with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**. In yet another embodiment one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria consist of the sequence of with one or more of **SEQ ID NO: 83** through **SEQ ID NO: 89**.

[029] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation or liver damage, e.g., as seen in NASH. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[030] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production

and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[031] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof is modified and/or mutated, e.g., to enhance stability, or increase GLP-1 production, secretion, or display.

[032] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[033] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides

for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences described herein for the consumption of ammonia.

[034] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[035] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[036] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[037] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production or catabolism of tryptophan and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the secretion or surface display of GLP-1 or an analog thereof further comprise one or more gene sequences for the secretion of IL-22.

Bile Salts

[038] Bile salts (also called conjugated bile acids) are cholesterol derivatives synthesized in the liver which comprise a steroid ring component conjugated with either taurine (taurocholic acid; TCA) or glycine (glycochenodeoxycholic acid; GCDCA). Bile salts act as signaling molecules to regulate systemic endocrine functions, including triglyceride, cholesterol, and glucose homeostasis (Houten *et al., EMBO J.,* 25:1419-1425 (2006) and Watanabe *et al., Nature,* 439:484-489 (2006)) and may trigger cellular farnesoid X receptor (FXR)- and G-protein coupled receptor (TGR4)-mediated host responses. Additionally, bile salts have been shown to facilitate lipid absorption and repress bacterial cell growth in the small intestine, thereby influencing both host metabolic pathways and the microflora present in the gut (Jones *et al., PNAS*, 105(36):13580-13585 (2008) and Ridlon *et al., J. Lipid Research*, 47(2):241-259 (2006)).

[039] Bile salts are stored in the gallbladder and then subsequently released into the duodenum via the common bile duct. In the small intestine, microbial bile salt hydrolase (BSH) enzymes remove the glycine or taurine molecules, a process referred to as deconjugation, to produce the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). In the gut, bile acids are reabsorbed within the terminal ileum, while non-reabsorbed bile acids enter the large intestine. Once in the large intestine, bile acids are amenable to further modification by microbial 7α-dehydroxylating enzymes to yield secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA) (Joyce *et al., Gut Microbes,* 5(5):669-674 (2014); Bhowmik *et al.,* Accepted Article, doi:10.1002/prot.24971 (2015); see also Figure 1).

[040] Bile acids (also called unconjugated bile salts) are also ligands for the FXR nuclear hormone receptor.T he bile acid-FXR interaction regulates bile acid synthesis, transport and cholesterol metabolism. Furthermore, bile acid-FXR regulation has been shown to affect hepatic and intestinal inflammation, atherosclerosis, and inflammation and autoimmune disease in the CNS. For example, CDCA and obeticholic acid, also known as 6α-ethyl-chenodeoxycholic acid (6-ECDCA), a synthetic bile acid analogue that is a 6α-ethyl derivative of CDCA, have each been shown to ameliorate experimental autoimmune encephalomyelitis (EAE) in mice (Ho and Steinman, *Proc. Natl. Acad. Sci. U.S.A.*, 113(6):1600-1605 (2016)). In various other studies using animal models for fatty liver disease, 6-ECDCA has also been shown to reduce liver fat and fibrosis. More specifically, 6-ECDCA has been found to improve glucose and insulin tolerance and decrease steatohepatitis (Vignozzi *et al., Journal of Sexual Medicine*, 8:57-77 (2011); Cipriani *et al., J. Lipid Res.*,

51:771-784 (2010)), decrease hepatic expression of genes involved in fatty acid synthesis and reduce TNF-α and elevated peroxisome-proliferator activated receptor alpha expression, thereby improving NASH phenotype (Carr *et al.*, *Pharm. Res.*, 17(16):1-16 (2015); Cipriani *et al.*, *J. Lipid Res.*, 51:771-784 (2010)), prevent hepatic stellate cell activation by inhibiting osteopontin production (Fiorucci *et al.*, *Gastroenterology*, 127:1497-1512 (2004)), and prevent fibrosis progression, reverse fibrosis and cirrhosis development and reduce portal hypertension (Carr *et al.*, *Pharm. Res.*, 17(16):1-16 (2015); *see also* Khalid *et al.*, *Liver Res. Open J.*, 1:32-40 (2015)). Furthermore, a small number of clinical trial studies have shown that the FXR agonist, 6-ECDCA, can improve insulin sensitivity and decrease the levels of markers for inflammation and fibrosis in patients with type II diabetes and NAFLD (Mudaliar *et al.*, *Gastroenterology*, 127:1497-1512 (2013)), and improve liver histology in patients with non-alcoholic steatohepatitis (Neuschwander-Tetri *et al.*, (*Lancet*, 385:956-1065 (2014)).

[041] It has been shown that bile salt metabolism is involved in host physiology (Ridlon *et al., Current Opinion Gastroenterol.,* 30(3):332 (2014) and Jones *et al.,* 2008). For example, it is known that the expression of bile salt hydrolase enzymes functionally regulate host lipid metabolism and play a role in cholesterol metabolism and transport, circadian rhythm, gut homeostasis/barrier function, weight gain, adiposity, and possibly gastrointestinal cancers in the host (Joyce *et al., PNAS,* 111(20):7421-7426 (2014); Zhou and Hylemon, *Steroids*, 86:62-68, (2014); Mitchell *et al., Expert Opinion Biolog. Therapy,* 13(5):631-642 (2013); and WO14/198857, the entire contents of each of which are expressly incorporated herein by reference). Specifically, potential effects of bile salt hydrolase-expressing bacteria on cholesterol metabolic pathways have been shown to upregulate the ATP binding cassette A1 (ABCA1), the ATP binding cassette G1 (ABCG1), the ATP binding cassette G5/G8 (ABCG5/G8), cholesterol 7 alpha-hydroxylase (CYP7A1), and liver X receptor (LXR), and to downregulate farnesoid X receptor (FXR), Niemann-Pick C1-like 1 (NPC1L1), and small heterodimer partner (SHP), which impacts cholesterol efflux, plasma HDL-C levels, biliary excretion, cholesterol catabolism, bile acid synthesis, cholesterol levels, and decreased intestinal cholesterol absorption, among other effects (Mitchel *et al.* (2014) and Zhou and Hylemon (2014)). Additionally, bile salt hydrolase activity has been shown to impact bile detoxification, gastrointestinal persistence, nutrition, membrane alterations, altered digestive functions (lipid malabsorption, weight loss), cholesterol lowering, cancer, and formation of gallstones (see Begley *et al., Applied and Environmental Microbiology,* 72(3):1729-1738 (2006)).

[042] Moreover, a *Clostridium scindens* bacterium expressing a 7α-dehydroxylating enzyme has been shown to produce resistance to *C. difficile* infection in hosts (Buffie *et al., Nature,* 517:205-208 (2015), and bile salt metabolism has been shown to play a role in both regulating the microbiome as well as in cirrhosis (Ridlon *et al., Gut Microbes,* 4(5):382-387 (2013) and Kakiyama *et al., J. Hepatol.,* 58(5):949-955 (2013)). Thus, a need exists for treatments which address the metabolism of bile salts in subjects in order to treat and prevent diseases and disorders in which bile salts play a role, such as cardiovascular disease, metabolic and/or liver disease, e.g., NASH and NAFLD , cirrhosis, gastrointestinal cancer, and *C. difficile* infection.

[043] The present disclosure provides recombinant bacterial cells, pharmaceutical compositions thereof, and methods of modulating and treating disorders associated with bile salts. Specifically, the recombinant bacteria disclosed herein have been engineered to comprise genetic circuits encoding, for example, a bile salt hydrolase enzyme and/or a 7α-dehydrolase enzyme to treat disease, disorders, and/or conditions associated with bile salts and bile salt metabolism. In some embodiments, the recombinant bacteria comprise genetic circuits encoding a bile salt hydrolase enzyme and/or a 7α-dehydrolase enzyme, eas well as other circuitry in order to guarantee the safety and non-colonization of a subject, such as auxotrophies, kill switches, *etc.* These recombinant bacteria are safe and well tolerated and augment the innate activities of the subject's microbiome to achieve a therapeutic effect.

[044] In some embodiments, the disclosure provides a bacterial cell genetically engineered to comprise gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s). In some embodiments, the disclosure provides a bacterial cell genetically engineered to comprise a heterologous gene encoding a 7α-dehydrolase enzyme. In some embodiments, the disclosure provides a bacterial cell genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme and a 7α-dehydrolase enzyme. In some embodiments, the disclosure provides a bacterial cell genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme and/or a 7α-dehydrolase enzyme and is capable of processing and reducing levels of bile salts, e.g., deconjugation of bile salts. In some embodiments, the disclosure provides a bacterial cell genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme and/or a 7α-dehydrolase enzyme and is capable of processing and reducing levels of bile salts, e.g., deconjugation of bile salts. In some embodiments, the disclosure provides a bacterial cell genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme and/or a 7α-dehydrolase enzyme and is capable

of processing and reducing levels of bile salts, e.g., deconjugation of bile salts in low-oxygen environments, *e.g.,* the gut. Thus, the genetically engineered bacterial cells and pharmaceutical compositions comprising the bacterial cells disclosed herein may be used to convert excess bile salts into non-toxic molecules (e.g., bile salt metabolites) in order to treat and/or prevent disorders associated with bile salts, such as cardiovascular disease, metabolic and/or liver disease, e.g., NASH and NAFLD , cirrhosis, cancer, liver disease, and *C. difficile* infection.

[045] In some embodiments, a recombinant bacterial cell disclosed herein has been genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme. In some embodiments, the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s). In some embodiments, a recombinant bacterial cell disclosed herein has been genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme that removes the glycine or taurine molecules from a bile salt to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA). In some embodiments, the recombinant bacterial cell engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme is capable of removing the glycine or taurine molecules from a bile salt to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA). In some embodiments, the recombinant bacterial cell is engineered to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA). In some embodiments, the recombinant bacterial cell is genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme that removes the glycine from glycochenodeoxycholic acid (GCDCA) to produce chenodeoxycholic acid (CDCA). In some embodiments, the recombinant bacterial cell is genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme that removes the taurine from taurocholic acid (TCA) to produce cholic acid (CA).

[046] In some embodiments, a recombinant bacterial cell is engineered to comprise a heterologous gene sequence encoding a bile salt hydrolase (BSH) enzyme that removes the glycine or taurine molecules from a bile salt to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA) in low-oxygen environments, *e.g.,* the gut. In some embodiments, a recombinant bacterial cell is engineered to comprise a heterologous gene sequence encoding a bile salt hydrolase (BSH) enzyme to produce the primary bile acids cholic acid (CA) and/or chenodeoxycholic acid (CDCA), wherein the primary bile acid stimulates FXR.

[047] In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s), the gene sequence encoding one or more bile salt hydrolase (BSH) enzymes is operably linked to an inducible promoter. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s), the gene sequence encoding one or more bile salt hydrolase (BSH) enzymes is operably linked to an inducible promoter that is induced directly or indirectly induced by exogenous environmental conditions. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s), the gene sequence encoding one or more bile salt hydrolase (BSH) enzymes is operably linked to an inducible promoter that is directly or indirectly induced by exogenous environmental conditions found in a mammalian gut. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s), the gene sequence encoding one or more bile salt hydrolase (BSH) enzymes is operably linked to an inducible promoter that is directly or indirectly induced by low oxygen or anaerobic conditions. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s), the gene sequence encoding one or more bile salt hydrolase (BSH) enzymes is operably linked to a constitutive promoter. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s), the gene sequence encoding one or more bile salt hydrolase (BSH) enzymes is present on a plasmid. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt hydrolase (BSH) enzyme(s), the gene sequence encoding one or more bile salt hydrolase (BSH) enzymes is present on a chromosome in the bacterial cell.

[048] In some embodiments, a recombinant bacterial cell disclosed herein has been genetically engineered to comprise a heterologous gene encoding one or more 7α-dehydroxylating enzyme(s). In some embodiments, the recombinant bacterial cell is genetically engineered to comprise gene sequence encoding one or more 7α-dehydroxylating enzyme(s) to produce secondary bile salts, e.g., deoxycholic acid (DCA) and/or lithocholic acid (LCA). In some embodiments, the recombinant bacterial cell is genetically engineered to comprise gene sequence encoding a 7α-dehydroxylating enzyme to produce deoxycholic acid (DCA). In some embodiments, the recombinant bacterial cell is genetically engineered

to comprise gene sequence encoding a 7α-dehydroxylating enzyme to produce lithocholic acid (LCA). In some embodiments, the 7α-dehydroxylating enzyme is a bacterial enzyme. In some embodiments, the 7α-dehydroxylating enzyme is a *bai* gene. In some embodiments, the 7α-dehydroxylating enzyme is produced by a bacterial *bai* operon. In some embodiments, the *bai* operon is from *C. scindens*. In some embodiments, the *bai* operon of *C. scindens* encodes *baiB*, *baiCD*, *baiE*, *baiA1*, *baiA2*, *baiA3*, *baiF*, *baiG*, *baiH*, and/or *baiI*. In some embodiments, the *bai* operon is from *C. hiranonis*.

[049] In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more 7α-dehydroxylating enzyme(s), the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) is operably linked to an inducible promoter. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more 7α-dehydroxylating enzyme(s), the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) is operably linked to an inducible promoter that is induced directly or indirectly induced by exogenous environmental conditions. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more 7α-dehydroxylating enzyme(s), the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) is operably linked to an inducible promoter that is directly or indirectly induced by exogenous environmental conditions found in a mammalian gut. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more 7α-dehydroxylating enzyme(s), the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) is operably linked to an inducible promoter that is directly or indirectly induced by low oxygen or anaerobic conditions. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more 7α-dehydroxylating enzyme(s), the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) is operably linked to a constitutive promoter. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more 7α-dehydroxylating enzyme(s), the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) is present on a plasmid. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more 7α-dehydroxylating enzyme(s), the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) is present on a chromosome in the bacterial cell.

[050] In some embodiments, the recombinant bacterial cell is genetically engineered to comprise gene sequence encoding one or more bile salt hydrolase enzyme(s) and to further

comprise gene sequence encoding one or more 7α-dehydroxylating enzyme(s). In some embodiments, the recombinant bacterial cell is capable of producing secondary bile acids, e.g., DCA and/or LCA. In some embodiments, the recombinant bacterial cell is engineered to comprise gene sequence encoding one or more bile salt hydrolase enzyme(s) to produce the primary bile acids cholic acid (CA) and/or chenodeoxycholic acid (CDCA) and to further comprise gene sequence encoding one or more 7α-dehydroxylating enzyme(s) to produce deoxycholic acid (DCA) and/or lithocholic acid (LCA). In some embodiments, the recombinant bacterial cell is genetically engineered to comprise gene sequence encoding one or more bile salt hydrolase enzyme(s) and to further comprise gene sequence encoding one or more 7α-dehydroxylating enzyme(s) to produce primary bile acids and/or secondary bile acids in low-oxygen environments, *e.g.,* the gut.

[051] In some embodiments, the recombinant bacterial cell further comprises gene sequence encoding one or more bile salt and/or bile acid transporters. In some embodiments, the recombinant bacterial cell comprises gene sequence encoding one or more bile salt hydrolase enzymes and gene sequence encoding one or more bile salt and/or bile acid transporters. In some embodiments, the recombinant bacterial cell comprises gene sequence encoding one or more 7α-dehydroxylating enzymes and gene sequence encoding one or more bile salt and/or bile acid transporters. In some embodiments, the recombinant bacterial cell comprises gene sequence encoding one or more bile salt hydrolase enzymes, gene sequence encoding one or more 7α-dehydroxylating enzymes and gene sequence encoding one or more bile salt and/or bile acid transporters.

[052] In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt and/or bile acid transporters, the gene sequence encoding one or more bile salt and/or bile acid transporters is operably linked to an inducible promoter. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt and/or bile acid transporters, the gene sequence encoding one or more bile salt and/or bile acid transporters is operably linked to an inducible promoter that is induced directly or indirectly induced by exogenous environmental conditions. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt and/or bile acid transporters, the gene sequence encoding one or more 7α-dehydroxylating enzyme(s) bile salt and/or bile acid transporters is operably linked to an inducible promoter that is directly or indirectly induced by exogenous environmental conditions found in a mammalian gut. In some embodiments in which the recombinant bacterial cell comprises a

heterologous gene sequence encoding one or more bile salt and/or bile acid transporters, the gene sequence encoding one or more bile salt and/or bile acid transporters is operably linked to an inducible promoter that is directly or indirectly induced by low oxygen or anaerobic conditions. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt and/or bile acid transporters, the gene sequence encoding one or more bile salt and/or bile acid transporters is operably linked to a constitutive promoter. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt and/or bile acid transporters, the gene sequence encoding one or more bile salt and/or bile acid transporters is present on a plasmid. In some embodiments in which the recombinant bacterial cell comprises a heterologous gene sequence encoding one or more bile salt and/or bile acid transporters, the gene sequence encoding one or more bile salt and/or bile acid transporters is present on a chromosome in the bacterial cell.

[053] In some embodiments, the recombinant bacterial cell further comprises gene sequence encoding one or more bile acid exporters. In some embodiments, the recombinant bacterial cell comprises gene sequence encoding one or more bile salt hydrolase enzymes and gene sequence encoding one or more bile acid exporters. In some embodiments, the recombinant bacterial cell comprises gene sequence encoding one or more 7α-dehydroxylating enzymes and gene sequence encoding one or more bile acid exporters. In some embodiments, the recombinant bacterial cell comprises gene sequence encoding one or more bile salt and/or bile acid transporters and gene sequence encoding one or more bile acid exporters. In some embodiments, the recombinant bacterial cell comprises gene sequence encoding one or more bile salt hydrolase enzymes, and/or gene sequence encoding one or more 7α-dehydroxylating enzymes and/or gene sequence encoding one or more bile salt and/or bile acid transporters and/or gene sequence encoding one or more bile acid exporters.

[054] As used herein, the term “bile salt” or “conjugated bile acid” refers to a cholesterol derivative that is synthesized in the liver and consists of a steroid ring component that is conjugated with either glycine (glycochenodeoxycholic acid; GCDCA) or taurine (taurocholic acid; TCA). Examples of bile salts include, but are not limited to, taurocholic acid (TCA) and glycochenodeoxycholic acid (GCDCA). Bile salts are stored in the gallbladder and then subsequently released into the duodenum. Bile salts act as signaling molecules to regulate systemic endocrine functions including triglyceride, cholesterol, and glucose homeostasis, and also facilitate lipid absorption. In the small intestine, microbial bile

salt hydrolase (BSH) enzymes remove the glycine or taurine molecules to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA).

[055] As used herein, the term "bile acid" or "unconjugated bile salt" refers to a cholesterol moiety that that consists of a steroid ring that is synthesized in the liver via a classic bile acid biosynthetic pathway wherein cholesterol is converted to 7α-hydroxycholesterol by the cholesterol 7α-hydroxylase enzyme (CYP7A1), or via an alternative pathway carried out by the microsomal enzyme sterol 12-hydroxylase (CYP8B1) (*see* Khalid *et al.*, *Liver Res. Open J.*, 1:32-40 (2015)). The immediate products of each of these pathways are the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). The term "primary bile acids" refers to, for example, CA and CDCA. CA and CDCA can subsequently be conjugated with taurine or glycine by the bile acid transferase and bile acid coenzyme A synthesas to form the bile acid salts taurocholic acid (TCA) and glycochenodeoxycholic acid (GCDCA). In the gut, bile acids are reabsorbed within the terminal ileum, while non-reabsorbed bile acids enter the large intestine. In the large intestine, bile acids are amenable to further modification by microbial 7α-dehydroxylating enzymes to yield secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA). The term "secondary bile acids" refers to, for example, deoxycholic acid (DCA) and lithocholic acid (LCA).

[056] As used herein, the term "farnesoid X receptor" or "FXR" refers to a nuclear bile acid receptor that is expressed in liver, intestine, kidneys, and adrenal glands mainly, and also at lower levels in adipose tissue and the heart (*see* Ho and Steinman, *Proc. Natl. Acad. Sci. U.S.A.*, 113(6):1600-1605 (2016)). FXR is the primary receptor for bile acid. The CDCA, DCA and LCA bile acids are the natural ligands that bind to and activate FXR. The bile acid-FXR interaction has been shown to regulate, for example, hepatic inflammation and regeneration, liver injury, bacterial outgrowth and inflammatory responses in the intestinal tract, preservation of intestinal barriers, and inflammation in the central nervous system (*see* Khalid *et al.*, *Liver Res. Open J.*, 1:32-40 (2015); Ho and Steinman, *Proc. Natl. Acad. Sci. U.S.A.*, 113(6):1600-1605 (2016); Neuschwander-Tetri *et al.*, (*Lancet*, 385:956-1065 (2014); and Joyce *et al.*, *PNAS*, 111(20):7421-7426 (2014)).

[057] As used herein, the term "FXR agonist" or "FXR activator" refers to a molecule that activates the farnesoid X receptor (FXR), thereby stimulating FXR activity. As used herein, the FXR agonist can be a naturally occurring molecule, such as a natural FXR ligand, or the FXR agonist can be a molecule that is not naturally produced *in vivo*. FXR can be stimulated to varying degrees by many bile acids. CDCA is the highest affinity natural

ligand for FXR, and stimulates FXR with the highest potency, with an EC50 of about 10 μM (*see* Khalid *et al.*, *Liver Res. Open J.*, 1:32-40 (2015); Ho and Steinman, *Proc. Natl. Acad. Sci. U.S.A.*, 113(6):1600-1605 (2016)). CDCA stimulates FXR to a greater degree than LCA and DCA, and LCA and DCA each stimulate FXR to a greater degree than CA. In some embodiments, the FXR agonist can be a naturally occurring ligand such as CDCA, LCA, DCA, or CA. In some embodiments, the FXR agonist can be a molecule that is not naturally produced in a mammal, such as a bile acid analogue, including, *e.g.*, obeticholic acid (OCA), a 6α-ethyl derivative of CDCA (*see, e.g.*, Pellicciari *et al.*, *J. Med. Chem.*, 45:3569-3572 (2002)), GW4064 (*see, e.g.*, Zhang *et al.*, *Proc. Natl. Acad. Sci., U.S.A.*, 103:1006-1011 (2006)), or WAY-362450 (Zhang *et al.*, *J. Hepatol.*, 51:380-388 (2009)). In some embodiments, the recombinant bacterial cell comprises one FXR agonist. In some embodiments, the recombinant bacterial cell comprises two or more FXR agonists. In some embodiments, the recombinant bacterial cell FXR agonist is CDCA.

[058] In some embodiments, a recombinant bacterial cell disclosed herein has been genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme. In some embodiments, a recombinant bacterial cell disclosed herein has been genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme that removes the glycine or taurine molecules from a bile salt to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA). In some embodiments, a recombinant bacterial cell disclosed herein has been genetically engineered to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA). In some embodiments, a recombinant bacterial cell disclosed herein has been genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme that removes the glycine or taurine molecules from a bile salt to produce the primary bile acids cholic acid (CA) or chenodeoxycholic acid (CDCA) in low-oxygen environments, *e.g.*, the gut, wherein the primary bile acid stimulates FXR. In some embodiments, the recombinant bacterial cell is genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme that removes the glycine from glycochenodeoxycholic acid (GCDCA) to produce chenodeoxycholic acid (CDCA). In some embodiments, the recombinant bacterial cell is genetically engineered to comprise a heterologous gene encoding a bile salt hydrolase (BSH) enzyme that removes the taurine from taurocholic acid (TCA) to produce cholic acid (CA). In some embodiments, the recombinant bacterial cell is genetically engineered to additionally comprise a 7α-dehydroxylating enzyme to produce deoxycholic acid (DCA) or lithocholic acid (LCA).

[059] As used herein, the term "7α-dehydroxylating enzyme" or "7α-dehydroxylase" refers to an enzyme that is involved in the biosynthesis of secondary bile acids. In some embodiments, the 7α-dehydroxylating enzyme is necessary for the biosynthesis of the secondary bile acid(s), deoxycholic acid (DCA) and/or lithocholic acid (LCA). DCA and LCA are produced through the action of microbial enzymes in the human large intestines (Ridlon *et al., J. Lipid Res.,* 47(2):241-259 (2006)). In some embodiments, the 7α-dehydroxylating enzyme is a bacterial enzyme. In some embodiments, the 7α-dehydroxylating enzyme is a *bai* gene. In some embodiments, the 7α-dehydroxylating enzyme is produced by a bacterial *bai* operon. In some embodiments, the *bai* operon is from *C. scindens*. In some embodiments, the *bai* operon of *C. scindens* encodes *baiB*, *baiCD*, *baiE*, *baiA1*, *baiA2*, *baiA3*, *baiF*, *baiG*, *baiH*, and/or *baiI*. In some embodiments, the *bai* operon is from *C. hiranonis*.

[060] As used herein, the term "catabolism" refers to the processing, breakdown and/or degradation of a complex molecule, such as a bile salt and/or bile acid, into compounds that are non-toxic or which can be utilized by the bacterial cell. In one embodiment, the term "bile salt catabolism" refers to the processing, breakdown, and/or degradation of bile salts into unconjugated bile acid(s).

[061] In one embodiment, "abnormal catabolism" refers to any condition(s), disorder(s), disease(s), predisposition(s), and/or genetic mutations(s) that result in increased levels of bile salts. In one embodiment, "abnormal catabolism" refers to an inability and/or decreased capacity of a cell, organ, and/or system to process, degrade, and/or secrete bile salts. In healthy adult humans, 600 mg of bile salts are secreted daily. In one embodiment, said inability or decreased capacity of a cell, organ, and/or system to process and/or degrade bile salts is caused by the decreased endogenous deconjugation of bile salts, *e.g.,* decreased endogenous deconjugation of bile salts into bile acids by the intestinal microbiota in the gut. In one embodiment, the inability or decreased capacity of a cell, organ, and/or system to process and/or degrade bile salts results from a decrease in the number of or activity of intestinal bile salt hydrolase (BSH)-producing microorganisms.

[062] In one embodiment, a "disease associated with bile salts" or a "disorder associated with bile salts" is a disease or disorder involving the abnormal, *e.g.,* increased, levels of bile salts in a subject. In one embodiment, a "disease associated with bile salts" may also refer to a disease or disorder involving the abnormal levels of bile salts and bile acids in a subject. Alternatively, a disease or disorder associated with bile salts is a disease or disorder wherein a subject exhibits normal levels of bile salts, but wherein the subject would

benefit from decreased levels of bile salts. Bile salts function to solubilize dietary fat and enable its absorption into host circulation, and healthy adult humans secrete about 600 mg of bile salts daily through the stool. Thus, decreasing increased levels of bile salts, or normal levels of bile salts, in a subject would result in less uptake of dietary fat, causing the subject's liver to pull cholesterol from systemic circulation as it attempts to synthesize more. Thus, in one embodiment, a subject having a disease or disorder associated with bile salts secretes about 600 mg of bile salts in their stool daily. In another embodiment, a subject having a disease or disorder associated with bile salts secretes more than 600 mg, 700 mg, 800 mg, 900 mg, or 1 g of bile salts in their stool daily.

[063] In one embodiment, a disease or disorder associated with bile salts is a cardiovascular disease. In another embodiment, a disease or disorder associated with bile salts is a metabolic disease. In another embodiment, the disease or disorder associated with bile salts is an inflammatory and/or autoimmune disease. In another embodiment, a disease or disorder associated with bile salts is a liver disease, such as cirrhosis, nonalcoholic steatohepatitis (NASH), or progressive familialintrahepatic cholestasis type 2 (PFIC2). In another embodiment, the disease or disorder associated with bile salts is a disease of the central nervous system (CNS), such as an autoimmune disease, a multiple sclerosis, and/or experimental autoimmune encephalomyelitis (EAE). In another embodiment, a disease or disorder associated with bile salts is a cancer, such as a gastrointestinal cancer, hepatocellular carcinoma, or colon cancer. In another embodiment, a disease or disorder associated with bile salts is a *C. difficile* infection. In another embodiment, a disease or disorder associated with bile salts is inflammatory bowel disease (IBD) or colitis.

[064] As used herein, the terms "cardiovascular disease" or "cardiovascular disorder" are terms used to classify numerous conditions affecting the heart, heart valves, and vasculature (*e.g.,* veins and arteries) of the body, and encompasses diseases and conditions including, but not limited to hypercholesterolemia, diabetic dyslipidemia, hypertension, arteriosclerosis, atherosclerosis, myocardial infarction, acute coronary syndrome, angina, congestive heart failure, aortic aneurysm, aortic dissection, iliac or femoral aneurysm, pulmonary embolism, primary hypertension, atrial fibrillation, stroke, transient ischemic attack, systolic dysfunction, diastolic dysfunction, myocarditis, atrial tachycardia, ventricular fibrillation, endocarditis, arteriopathy, vasculitis, atherosclerotic plaque, vulnerable plaque, acute coronary syndrome, acute ischemic attack, sudden cardiac death, peripheral vascular disease, coronary artery disease (CAD), peripheral artery disease (PAD), and cerebrovascular disease. As used herein, a subject having "hypercholesterolemia" may have a total

cholesterol of greater than 4 mmol/L, and a low-density lipoprotein cholesterol (LDL) of greater than 3mmol/L.

[065] As used herein, the terms "metabolic disease" or "metabolic disorder" refer to diseases caused by lipid and cholesterol metabolic pathways that are regulated by or affected by bile salts and bile acids. For example, cholesterol metabolic diseases and disorders include diabetes (including Type 1 diabetes, Type 2 diabetes, and maturity onset diabetes of the young (MODY)), obesity, weight gain, gallstones, hypertriglyceridemia, hyperfattyacidemia, and hyperinsulinemia.

[066] As used herein, the term "bile salt hydrolase" enzyme refers to an enzyme involved in the cleavage of the amino acid sidechain of glycol- or tauro-conjugated bile acids to generate unconjugated bile acids (Figure 2). Bile salt hydrolase (BSH) enzymes are well known to those of skill in the art. For example, bile salt hydrolase activity has been detected in *Lactobacillus spp., Bifidobacterium spp., Enterococcus spp., Clostridum spp., Bacteroides spp., Methanobrevibacter spp.,* and *Listeria spp.* See, for example, Begley *et al., Applied and Environmental Microbiology,* 72(3):1729-1738 (2006); Jones *et al., Proc. Natl. Acad. Sci.,* 105(36):13580-13585 (2008); Ridlon *et al., J. Lipid Res.,* 47(2):241-259 (2006); and WO2014/198857, the entire contents of each of which are expressly incorporated herein by reference.

Bile Salt Hydrolases

[067] The bacterial cells described herein comprise a heterologous gene encoding a bile salt hydrolase enzyme and are capable of deconjugating bile salts into unconjugated bile acids (see Figures 1 and 2).

[068] In one embodiment, the bile salt hydrolase enzyme increases the rate of bile salt catabolism in the cell. In one embodiment, the bile salt hydrolase enzyme decreases the level of bile salts in the cell or in the subject. In one embodiment, the bile salt hydrolase enzyme decreases the level of taurocholic acid (TCA) in the cell or in the subject. In one embodiment, the bile salt hydrolase enzyme decreases the level of glycochenodeoxycholic acid (GCDCA) in the cell or in the subject. Methods for measuring the rate of bile salt catabolism and the level of bile salts and bile acids are well known to one of ordinary skill in the art. For example, bile salts and acids may be extracted from a sample, and standard LC/MS methods may be used to determine the rate of bile salt catabolism and/or level of bile salts and bile acids.

[069] In another embodiment, the bile salt hydrolase enzyme increases the level of bile acids in the cell or in the subject as compared to the level of bile salts in the cell or in the

subject. In another embodiment, the bile salt hydrolase enzyme increases the level of cholic acid (CA) in the cell. In another embodiment, the bile salt hydrolase enzyme increases the level of chenodeoxycholic acid (CDCA) in the cell.

[070] Enzymes involved in the catabolism of bile salts may be expressed or modified in the bacteria of the disclosure in order to enhance catabolism of bile salts. Specifically, when a bile salt hydrolase enzyme is expressed in the recombinant bacterial cells of the disclosure, the bacterial cells convert more bile salts into unconjugated bile acids when the bile salt hydrolase enzyme is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In another embodiment, when a bile salt hydrolase enzyme is expressed in the recombinant bacterial cells of the disclosure, the bacterial cells convert more bile salts, such as TCA or GCDCA, into CA and CDCA when the bile salt hydrolase enzyme is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the genetically engineered bacteria comprising a heterologous gene encoding a bile salt hydrolase enzyme can catabolize bile salts to treat disorders associated with bile salts, including cardiovascular diseases, metabolic diseases, liver disease, such as cirrhosis or NASH, gastrointestinal cancers, and *C. difficile* infection.

[071] In one embodiment, the bacterial cell comprises a heterologous gene encoding a bile salt hydrolase enzyme. In some embodiments, the disclosure provides a bacterial cell that comprises a heterologous gene encoding a bile salt hydrolase enzyme operably linked to a first promoter. In one embodiment, the first promoter is an inducible promoter. In one embodiment, the bacterial cell comprises a gene encoding a bile salt hydrolase enzyme from a different organism, *e.g.,* a different species of bacteria. In another embodiment, the bacterial cell comprises more than one copy of a native gene encoding a bile salt hydrolase enzyme. In yet another embodiment, the bacterial cell comprises at least one native gene encoding a bile salt hydrolase enzyme, as well as at least one copy of a gene encoding a bile salt hydrolase enzyme from a different organism, *e.g.,* a different species of bacteria. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of a gene encoding a bile salt hydrolase enzyme. In one embodiment, the bacterial cell comprises multiple copies of a gene or genes encoding a bile salt hydrolase enzyme.

[072] Multiple distinct bile salt hydrolase enzymes are known in the art. In some embodiments, bile salt hydrolase enzyme is encoded by a gene encoding a bile salt hydrolase enzyme derived from a bacterial species. In some embodiments, a bile salt hydrolase enzyme is encoded by a gene encoding a bile salt hydrolase enzyme derived from a non-bacterial species. In some embodiments, a bile salt hydrolase enzyme is encoded by a gene derived

from a eukaryotic species, *e.g.,* a fungi. In one embodiment, the gene encoding the bile salt hydrolase enzyme is derived from an organism of the genus or species that includes, but is not limited to, *Lactobacillus spp.,* such as *Lactobacillus plantarum, Lactobacillus johnsonii, Lactobacillus acidophilus, Lactobacillus brevis, or Lactobacillus gasseri; Bifidobacterium spp.,* such as *Bifidobacterium longum, Bifidobacterium bifidum,* or *Bifidobacterium adolescentis*; *Bacteroides spp.,* such as *Bacteroides fragilis or Bacteroides vlugatus*; *Clostridium spp.,* such as *Clostridium perfringens*; *Listeria spp.,* such as *Listeria monocytogenes, Enterococcus* spp., such as *Enterococcus faecium* or *Enterococcus faecalis; Brucella spp.,* such as *Brucella abortus; Methanobrevibacter spp.,* such as *Methanobrevibacter smithii, Staphylococcus* spp., such as *Staphylococcus aureus*, *Mycobacterium* spp., such as *Mycobacterium tuberculosis; Salmonella* spp., such as *Salmonella enterica; Listeria* spp., such as *Listeria monocytogenes.*

[073] In one embodiment, the gene encoding the bile salt hydrolase enzyme has been codon-optimized for use in the recombinant bacterial cell. In one embodiment, the gene encoding the bile salt hydrolase enzyme has been codon-optimized for use in *Escherichia coli.* In another embodiment, the gene encoding the bile salt hydrolase enzyme has been codon-optimized for use in *Lactococcus.* When the gene encoding the bile salt hydrolase enzyme is expressed in the recombinant bacterial cells, the bacterial cells catabolize more bile salt than unmodified bacteria of the same bacterial subtype under the same conditions (*e.g.,* culture or environmental conditions). Thus, the genetically engineered bacteria comprising a heterologous gene encoding a bile salt hydrolase enzyme may be used to catabolize excess bile salts to treat a disorder associated with bile salts, such as cardiovascular disease, metabolic disease, liver disease, such as cirrhosis or NASH.

[074] The present disclosure further comprises genes encoding functional fragments of a bile salt hydrolase enzyme or functional variants of a bile salt hydrolase enzyme. As used herein, the term "functional fragment thereof" or "functional variant thereof" of a bile salt hydrolase enzyme relates to an element having qualitative biological activity in common with the wild-type bile salt hydrolase enzyme from which the fragment or variant was derived. For example, a functional fragment or a functional variant of a mutated bile salt hydrolase enzyme is one which retains essentially the same ability to catabolize bile salts as the bile salt hydrolase enzyme from which the functional fragment or functional variant was derived. For example, a polypeptide having bile salt hydrolase enzyme activity may be truncated at the N-terminus or C-terminus and the retention of bile salt hydrolase enzyme activity assessed using assays known to those of skill in the art, including the exemplary

assays provided herein. In one embodiment, the recombinant bacterial cell comprises a heterologous gene encoding a bile salt hydrolase enzyme functional variant. In another embodiment, the recombinant bacterial cell comprises a heterologous gene encoding a bile salt hydrolase enzyme functional fragment.

[075] Assays for testing the activity of a bile salt hydrolase enzyme, a bile salt hydrolase enzyme functional variant, or a bile salt hydrolase enzyme functional fragment are well known to one of ordinary skill in the art. For example, bile salt catabolism can be assessed by expressing the protein, functional variant, or fragment thereof, in a recombinant bacterial cell that lacks endogenous bile salt hydrolase enzyme activity. Bile salt hydrolase activity can be assessed using a plate assay as described in Dashkevicz and Feighner, *Applied Environ. Microbiol.,* 55:11-16 (1989) and Christiaens *et al., Appl. Environ. Microbiol.,* 58:3792-3798 (1992), the entire contents of each of which are expressly incorporated herein by reference. Briefly, bacterial cultures that are grown overnight can be spotted onto LB bile agar supplemented with either 0.5% (wt/vol) TDCA, 0.5% (wt/vol) GDCA, or 3% (vol/vol) human bile. BSH activity can be indicated by halos of precipitated deconjugated bile acids (see, also, Jones *et al., PNAS,* 105(36):13580-13585 (2008), the entire contents of which are expressly incorporated herein by reference). A ninhydrine assay for free taurine has also been described (see, for example, Clarke *et al., Gut Microbes,* 3(3):186-202 (2012), the entire contents of which are expressly incorporated herein by reference. Alternatively, a mouse model can be used to assay bile salt and bile acid signatures *in vivo* (see, for example, Joyce *et al., PNAS,* 111(20):7421-7426 (2014), the entire contents of which are expressly incorporated herein by reference). The present disclosure encompasses genes encoding a bile salt hydrolase enzyme comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein.

[076] In some embodiments, the gene encoding a bile salt hydrolase enzyme is mutagenized; mutants exhibiting increased activity are selected; and the mutagenized gene encoding the bile salt hydrolase enzyme is isolated and inserted into the bacterial cell of the disclosure. The gene comprising the modifications described herein may be present on a plasmid or chromosome.

[077] In one embodiment, the gene encoding the bile salt hydrolase enzyme is from *Lactobacillus* spp. In one embodiment, the *Lacotbacillus* spp. is *Lactobacillus plantarum* WCFS1, *Lactobacillus plantarum* 80, *Lactobacillus johnsonii* NCC533, *Lactobacillus johnsonii* 100-100, *Lactobacillus acidophilus* NCFM ATCC700396, *Lactobacillus brevis* ATCC 367, *Lactobacillus gasseri* ATCC 33323, or *Lactobacillus acidophilus.* In another

embodiment, the gene encoding the bile salt hydrolase enzyme is from a *Bifidobacterium* spp. In one embodiment, the *Bifidobacterium* spp. is *Bifidobacterium longum* NCC2705, *Bifidobacterium longum* DJO10A, *Bifidobacterium longum* BB536, *Bifidobacterium longum* SBT2928, *Bifidobacterium bifidum* ATCC 11863, or *Bifidobacterium adolescentis*. In another embodiment, the gene encoding the bile salt hydrolase enzyme is from *Bacteroides* spp. In one embodiment, the *Bacteroides* spp. is *Bacteroides fragilis* or *Bacteroides vlugatus*. In another embodiment, the gene encoding the bile salt hydrolase enzyme is from *Clostridium* spp. In one embodiment, the *Clostridum* spp. is *Clostridum perfringens* MCV 185 or *Clostridum perfringens* 13. In another embodiment, the gene encoding the bile salt hydrolase enzyme is from *Listeria* spp. In one embodiment, the *Listeria* spp. is *Listeria monocytogenes*. In one embodiment, the gene encoding the bile salt hydrolase enzyme is from *Methanobrevibacter spp.* In one embodiment, the *Methanobrevibacter* spp. is *Methanobrevibacter smithii*. Other genes encoding bile salt hydrolase enzymes are well-known to one of ordinary skill in the art and described in, for example, Jones *et al., PNAS*, 105(36):13580-13585 (2008) and WO2014/198857. **Table 10** lists non-limiting examples of bile salt hydrolases.

Table 10. Bile Salt Hydrolases

Gene or Operon	Sequence
Bile salt hydrolase from *Lactobacillus plantarum* **SEQ ID NO: 90**	ATGTGTACTGCCATAACTTATCAATCTTATAATAATTAC TTCGGTAGAAATTTCGATTATGAAATTTCATACAATGAA ATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTT CGTAAGGTGGAGAACTTAGATCACCATTATGCAATAAT TGGAATTACTGCTGATGTAGAAAGCTATCCACTTTACTA CGATGCGATGAATGAAAAAGGCTTGTGTATTGCGGGAT TAAATTTTGCAGGTTATGCTGATTATAAAAAATATGATG CTGATAAAGTTAATATCACACCATTTGAATTAATTCCTT GGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAA AAGAACATACAAAAACTAAACTTGGTTAATATTAATTT TAGTGAACAATTACCATTATCACCGCTACATTGGTTGGT TGCTGATAAACAGGAATCGATAGTTATTGAAAGTGTCA AAGAAGGACTAAAAATTTACGACAATCCAGTAGGTGTG TTAACAAACAATCCTAATTTTGACTACCAATTATTTAAT TTGAACAACTATCGTGCCTTATCAAATAGCACACCCCA AAATAGTTTTTCGGAAAAAGTGGATTTAGATAGTTATA GTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTG TCCTCAATGTCTAGATTTGTCAGAGCCGCTTTTACTAAA TTAAACTCGTTGTCGATGCAGACAGAGAGTGGCAGTGT TAGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACA AAAAGGGCTATGTGAAGTTACTGACGGAAAGTACGAAT ATACAATCTATTCTTCTTGTTGTGATATGGACAAAGGAG TTTATTACTATAGAACTTATGACAATAGTCAAATTAACA

	GTGTCAGTTTAAACCATGAGCACTTGGATACGACTGAA TTAATTTCTTATCCATTACGATCAGAAGCACAATACTAT GCAGTTAACTAA
Bile salt hydrolase protein from *Lactobacillus plantarum* **SEQ ID NO: 91**	MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFR KVENLDHHYAIIGITADVESYPLYYDAMNEKGLCIAGLNF AGYADYKKYDADKVNITPFELIPWLLGQFSSVREVKKNIQ KLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIY DNPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVD LDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLSMQTES GSVSQFFHILGSVEQQKGLCEVTDGKYEYTIYSSCCDMDK GVYYYRTYDNSQINSVSLNHEHLDTTELISYPLRSEAQYY AVN
Bile salt hydrolase from *Methanobrevibacter smithii 3142* **SEQ ID NO: 92**	ATGTGTACTGCTGCAAATTATTTAACAAAATGCCATTAT TTTGGCCGTAATTTTGACTATGAAATTTCATATAATGAA AGAGTAACGATAACTCCTAGAAACTATCCTTTAATATTC AGGGATACTGAGGACATTGAAAATCATTATGGGATTAT TGGCATAGCTGCAGGTATTGATGAATATCCTTTGTATTA TGATGCATGTAATGAGAAAGGATTAGCTATGGGGGGAT TAAACTTTCCGGATTACTGTGACTACAAACCACTAGATA AATCTAAAGTTAACATAGCTTCTTTTGAGATTATTCCAT ATATATTATCTCAAGCAAAAACCATCAGTGATGCCGAA AGGTTATTGGAAAACTTAAATATTTCAGATGAGAAATT TTCCGCCCAGTTGCCTCCATCTCCACTTCATTGGATTATT TCAGATAGGAATGCTTCAATTGTTGTAGAGGTTGTAGA GGAAGGACTGGATATTTATGATAATCCTGTAGGAGTTTT AACAAACAACCCTCCTTTTGATAAACAGCTATTTAATTT AAATAATTATATGGCATTATCAAACAGAACGCCTGAAA ATACCTTTGGAGGCAATTTGGATTTGGCAACTTATAGTC GGGGAATGGGTTCAATTGGTCTTCCGGGGGATGTTTCTT CACAGTCCCGTTTTGTAAAAGCAGCTTTTGTTAAAGAAA ATTCCGTTTCCGGAGATTCTGAAAAAGAAAGTGTGTCTC AGTTTTTCCATATTCTGGCATCTGTTGAACAGCAAAAAG GATGTACGTTAGTGGAAGAACCTGATAAATTTGAGTAT ACTATTTATTCAGACTGTTACAATACAGATAAGGGAAT ATTGTATTATAAAACATATGATGGTCCTCAAACATCTGT TAATATACATGATGAGGATTTGGAAACCAATCAGTTAA TTAATTTTGAGTTGGTTGATTAA
Bile salt hydrolase protein from *Methanobrevibacter smithii 3142* **SEQ ID NO: 93**	MCTAANYLTKCHYFGRNFDYEISYNERVTITPRNYPLIFRD TEDIENHYGIIGIAAGIDEYPLYYDACNEKGLAMGGLNFPD YCDYKPLDKSKVNIASFEIIPYILSQAKTISDAERLLENLNIS DEKFSAQLPPSPLHWIISDRNASIVVEVVEEGLDIYDNPVG VLTNNPPFDKQLFNLNNYMALSNRTPENTFGGNLDLATYS RGMGSIGLPGDVSSQSRFVKAAFVKENSVSGDSEKESVSQ FFHILASVEQQKGCTLVEEPDKFEYTIYSDCYNTDKGILYY KTYDGPQTSVNIHDEDLETNQLINFELVD
Bile salt hydrolase from *Bacteroides vulgatus* **SEQ ID NO: 94**	ATGGTTATGAAAAAGATTTTGATAGCTTTGGCCTTATTG CTGACAGGCATTGCAAGCGGATCGGCATGTACCGGTAT TTCATTCCTCGCTGAAGATGGCGGATATGTGCAGGCAC GTACTATAGAGTGGGGGAACAGTTATCTTCCGAGTGAA

	TATGTTATTGTTCCCAGAGGACAGGATTTGGTATCTTAT ACTCCAACGGGTGTAAATGGCTTGAGATTTCGGGCTAA ATATGGTCTGGTAGGACTGGCTATCATTCAGAAAGAGT TTGTGGCTGAAGGACTGAATGAAGTAGGGCTTTCGGCT GGATTGTTTTATTTTCCCCATTATGGGAAGTATGAAGAA TATGATGAGGCTCAAAATGCAATTACTTTGTCGGATTTG CAGGTGGTGAACTGGATGCTTTCCCAATTTGCTACTATA GACGAAGTGAGAGAAGCTATAGAAGGGGTGAAGGTGG TGTCTCTTGATAAACCTGGTAAAAGTTCTACGGTACATT GGCGCATTGGCGATGCTAAAGGAAATCAAATGGTGTTG GAATTTGTAGGTGGTGTTCCTTATTTTTATGAAAATAAA GTAGGAGTACTCACCAATTCTCCCGATTTTCCATGGCAG GTGATTAACTTGAATAATTATGTAAATCTATATCCGGGA GCTGTCACTCCACAGCAATGGGGTGGGGTGACTATTTTC CCTTTTGGCGCAGGTGCCGGATTTCATGGTATTCCGGGG GATGTAACTCCTCCATCCCGTTTTGTTCGTGTAGCGTTTT ATAAGGCAACAGCTCCGGTGTGTCCTACAGCGTATGAC GCTATATTACAAAGCTTTCATATCCTGAATAATTTTGAT ATTCCTATTGGTATAGAATATGCGTTAGGGAAAGCACC TGATATTCCTAGTGCCACACAATGGACTTCGGCTATTGA TTTGACAAACAGGAAAGTGTATTATAAAACAGCATACA ATAACAATATTCGTTGTATTAGTATGAAGAAGATTGATT TTGATAAAGTGAAGTATCAGTCGTATCCATTGGATAAG GAGTTGAAACAGCCTGTAGAAGAGATTATTGTGAAATA G
Bile salt hydrolase protein from *Bacteroides vulgatus* **SEQ ID NO: 95**	MVMKKILIALALLLTGIASGSACTGISFLAEDGGYVQARTI EWGNSYLPSEYVIVPRGQDLVSYTPTGVNGLRFRAKYGL VGLAIIQKEFVAEGLNEVGLSAGLFYFPHYGKYEEYDEAQ NAITLSDLQVVNWMLSQFATIDEVREAIEGVKVVSLDKPG KSSTVHWRIGDAKGNQMVLEFVGGVPYFYENKVGVLTNS PDFPWQVINLNNYVNLYPGAVTPQQWGGVTIFPFGAGAG FHGIPGDVTPPSRFVRVAFYKATAPVCPTAYDAILQSFHIL NNFDIPIGIEYALGKAPDIPSATQWTSAIDLTNRKVYYKTA YNNNIRCISMKKIDFDKVKYQSYPLDKELKQPVEEIIVK
Bile salt hydrolase from *Bifidobacterium longum* **SEQ ID NO: 96**	ATGTGCACTGGTGTCCGTTTCTCCGATGATGAGGGCAA CACCTATTTCGGCCGTAATCTCGACTGGAGTTTCTCAT ATGGGGAGACCATCCTGGTTACTCCGCGCGGCTACCA CTATGACACGGTGTTTGGTGCGGGCGGCAAGGCGAAG CCGAACGCGGTGATCGGCGTGGGTGTGGTCATGGCCG ATAGGCCGATGTATTTCGACTGCGCCAATGAACATGG TCTGGCCATCGCCGGCTTGAATTTCCCCGGCTACGCCT CGTTCGTCCACGAACCGGTCGAAGGCACGGAAAACGT CGCCACGTTCGAATTTCCGCTGTGGGTGGCGCGTAATT TCGACTCCGTCGACGAGGTCGAGGAGGCGCTCAGGAA CGTGACGCTCGTCTCCCAGATCGTGCCGGGACAGCAG GAGTCTCTGCTGCACTGGTTCATCGGCGACGGCAAGC GCAGCATCGTCGTCGAGCAGATGGCCGATGGCATGCA CGTGCATCATGATGACGTCGATGTGCTGACCAATCAG CCGACGTTCGACTTCCATATGGAAAACCTGCGCAACT ACATGTGCGTCAGCAACGAGATGGCCGAACCGACTTC

	ATGGGGCAAGGCCTCCTTGACCGCCTGGGGTGCGGGT GTGGGCATGCATGGCATCCCGGGCGACGTGAGTTCCC CGTCGCGCTTCGTTCGTGTGGCCTACACCAACGCGCAT TACCCGCAGCAGAACGATGAAGCCGCCAATGTGTCGC GCCTGTTCCACACCCTCGGCTCCGTGCAGATGGTGGAC GGCATGGCGAAGATGGGCGACGGCCAGTTCGAACGCA CGCTGTTCACCAGCGGATATTCGTCCAAGACCAACAC CTATTACATGAACACCTATGATGACCCCGCCATCCGTT CCTACGCCATGGCCGATTACGATATGGATTCCTCGGAG CTCATCAGCGTCGCCCGATGA
Bile salt hydrolase protein from *Bifidobacterium longum* **SEQ ID NO: 97**	MCTGVRFSDDEGNTYFGRNLDWSFSYGETILVTPRGYHY DTVFGAGGKAKPNAVIGVGVVMADRPMYFDCANEHGLA IAGLNFPGYASFVHEPVEGTENVATFEFPLWVARNFDSVD EVEEALRNVTLVSQIVPGQQESLLHWFIGDGKRSIVVEQM ADGMHVHHDDVDVLTNQPTFDFHMENLRNYMCVSNEM AEPTSWGKASLTAWGAGVGMHGIPGDVSSPSRFVRVAYT NAHYPQQNDEAANVSRLFHTLGSVQMVDGMAKMGDGQ FERTLFTSGYSSKTNTYYMNTYDDPAIRSYAMADYDMDS SELISVAR
Bile salt hydrolase from *Listeria monocytogenes* **SEQ ID NO: 98**	ATGTGTACGTCAATAACTTATACAACGAAGGATCACT ATTTTGGAAGGAATTTCGATTATGAACTTTCTTACAAA GAAGTTGTGGTTGTTACGCCGAAAAATTACCCGTTCCA TTTTCGCAAGGTAGAGGATATAGAGAAGCATTATGCA CTTATTGGTATTGCTGCTGTGATGGAAAACTACCCGTT GTATTACGATGCTACCAATGAAAAAGGCCTTAGTATG GCAGGACTCAATTTCTCAGGAAATGCGGATTACAAGG ATTTTGCAGAAGGTAAGGACAATGTGACCCCCTTTGA ATTTATTCCGTGGATTCTTGGTCAATGCGCTACTGTAA AAGAAGCAAGAAGATTACTTCAGAGAATCAATCTCGT GAATATTAGTTTTAGTGAAAATTTACCGCTGTCTCCAT TACATTGGTTGATGGCTGATCAAACAGAATCTATTGTA GTGGAATGTGTGAAAGATGGACTTCACATTTATGATA ATCCTGTTGGCGTGTTAACAAATAATCCAACATTTGAT TACCAACTATTTAATTTAAACAATTATCGCGTTCTTTC GAGTGAAACCCCAGAAAATAATTTTTCCAAAGAGATT GATTTGGATGCTTATAGTCGTGGGATGGGCGGAATTG GCTTACCTGGTGATTTATCTTCTATGTCTCGTTTTGTGA AAGCAACTTTTACCAAATTGAATTCTGTTTCAGGTGAT TCTGAATCAGAAAGTATTAGCCAATTTTTCCATATTTT AGGCTCGGTGGAACAACAAAAAGGTCTTTGTGATGTT GGTGGGGGAAAATACGAGCATACTATTTATTCCTCGT GTTGCAATATCGATAAAGGAATTTATTATTATAGAAC ATACGGAAACAGTCAAATTACTGGTGTGGATATGCAC CAAGAGGATTTAGAGAGCAAAGAACTAGCTATTTATC CACTCGTCAATGAGCAACGACTAAACATTGTTAACAA ATAA
Bile salt hydrolase protein from *Listeria monocytogenes* **SEQ ID NO: 99**	MCTSITYTTKDHYFGRNFDYELSYKEVVVVTPKNYPFHFR KVEDIEKHYALIGIAAVMENYPLYYDATNEKGLSMAGLN FSGNADYKDFAEGKDNVTPFEFIPWILGQCATVKEARRLL QRINLVNISFSENLPLSPLHWLMADQTESIVVECVKDGLHI

	YDNPVGVLTNNPTFDYQLFNLNNYRVLSSETPENNFSKEI DLDAYSRGMGGIGLPGDLSSMSRFVKATFTKLNSVSGDSE SESISQFFHILGSVEQQKGLCDVGGGKYEHTIYSSCCNIDK GIYYYRTYGNSQITGVDMHQEDLESKELAIYPLVNEQRLN IVNK
Bile salt hydrolase from *Clostridium perfringens* **SEQ ID NO: 100**	ATGTGTACAGGATTAGCCTTAGAAACAAAAGATGGATT ACATTTGTTTGGAAGAAATATGGATATTGAATATTCATT TAATCAATCTATTATATTTATTCCTAGGAATTTTAAATG TGTAAACAAATCAAACAAAAAAGAATTAACAACAAAA TATGCTGTTCTTGGAATGGGAACTATTTTTGATGATTAT CCTACCTTTGCAGATGGTATGAATGAAAAGGGATTAGG GTGTGCTGGCTTAAATTTCCCTGTTTATGTTAGCTATTCT AAAGAAGATATAGAAGGTAAAACTAATATTCCAGTATA TAATTTCTTATTATGGGTTTTAGCTAATTTTAGCTCAGTA GAAGAGGTAAAGGAAGCATTAAAAAATGCTAATATAGT GGATATACCTATTAGCGAAAATATTCCTAATACAACTCT TCATTGGATGATAAGCGATATAACAGGAAAGTCTATTG TGGTTGAACAAACAAAGGAAAAATTAAATGTATTTGAT AATAATATTGGAGTATTAACTAATTCACCTACTTTTGAT TGGCATGTAGCAAATTTAAATCAATATGTAGGTTTGAG ATATAATCAAGTTCCAGAATTTAAGTTAGGAGATCAAT CTTTAACTGCTTTAGGTCAAGGAACTGGTTTAGTAGGAT TACCAGGGGACTTTACACCTGCATCTAGATTTATAAGA GTAGCATTTTTAAGAGATGCAATGATAAAAAATGATAA AGATTCAATAGACTTAATTGAATTTTTCCATATATTAAA TAATGTTGCTATGGTAAGAGGATCAACTAGAACTGTAG AAGAAAAAAGTGATCTTACTCAATATACAAGTTGCATG TGTTTAGAAAAAGGAATTTATTATTATAATACCTATGAA AATAATCAAATTAATGCAATAGACATGAATAAAGAAAA CTTAGATGGAAATGAAATTAAAACATATAAATACAACA AAACTTTAAGTATTAATCATGTAAATTAG
Bile salt hydrolase protein from *Clostridium perfringens* **SEQ ID NO: 101**	MCTGLALETKDGLHLFGRNMDIEYSFNQSIIFIPRNFKCVN KSNKKELTTKYAVLGMGTIFDDYPTFADGMNEKGLGCAG LNFPVYVSYSKEDIEGKTNIPVYNFLLWVLANFSSVEEVKE ALKNANIVDIPISENIPNTTLHWMISDITGKSIVVEQTKEKL NVFDNNIGVLTNSPTFDWHVANLNQYVGLRYNQVPEFKL GDQSLTALGQGTGLVGLPGDFTPASRFIRVAFLRDAMIKN DKDSIDLIEFFHILNNVAMVRGSTRTVEEKSDLTQYTSCMC LEKGIYYYNTYENNQINAIDMNKENLDGNEIKTYKYNKTL SINHVN
Bile salt hydrolase from *Enterococcus faecium* **SEQ ID NO: 102**	ATGTGTACGTCTATTACTTATGTAACAAGTGATCATTAT TTTGGAAGGAATTTTGATTATGAAATATCTTACAATGAA GTAGTTACTGTTACTCCAAGAAATTATAAGTTGAATTTT CGAAAGGTAAATGATTTGGATACTCATTATGCAATGAT TGGTATTGCCGCTGGTATAGCTGACTACCCTCTTTATTA CGATGCGACAAATGAAAAAGGATTGAGTATGGCTGGGC TAAATTTTTCTGGGTATGCTGATTATAAAGAAATACAAG AAGGGAAAGACAATGTATCTCCTTTTGAATTTATTCCTT GGATTTTAGGACAATGCTCAACAGTAGGAGAAGCTAAA AAATTGTTAAAAAATATCAATTTAGCAAATATAAATTA

	TAGTGACGAACTTCCTTTATCCCCTTTACATTGGCTATT AGCTGATAAAGAAAAATCAATTGTCATTGAAAGTATGA AAGATGGACTTCATATATATGATAACCCTGTGGGCGTTC TTACCAATAATCCTTCATTTGACTATCAATTATTTAATTT AAACAATTATCGTGTCTTATCGAGTGAAACTCCTAAAA ATAATTTTTCAAATCAAATAAGTTTGAATGCCTATAGCC GCGGTATGGGAGGGATAGGCTTGCCTGGAGATTTATCC TCAGTATCTCGTTTTGTTAAAGCGACTTTTACGAAGCTG AATTCTGTATCTGGAGATTCAGAGTCAGAAAGTATTAG TCAATTTTTCCATATCTTAGGTTCAGTAGAACAACAAAA AGGTTTGTGTGATGTAGGTGATGGAAAATATGAATATA CAATTTATTCTTCTTGTTGCAATGTTGACAAAGGAATCT ATTATTATCGAACATATGAAGACAGTCAAATTACTGCA ATTGATATGAATAAAGAAGACTTAGATAGTCATAAGTT AATTAGTTATCCAATTATAGAAAAACAACAAATTAAAT ATATAAATTAG
Bile salt hydrolase protein from *Enterococcus faecium* **SEQ ID NO: 103**	MCTSITYVTSDHYFGRNFDYEISYNEVVTVTPRNYKLNFR KVNDLDTHYAMIGIAAGIADYPLYYDATNEKGLSMAGLN FSGYADYKEIQEGKDNVSPFEFIPWILGQCSTVGEAKKLLK NINLANINYSDELPLSPLHWLLADKEKSIVIESMKDGLHIY DNPVGVLTNNPSFDYQLFNLNNYRVLSSETPKNNFSNQISL NAYSRGMGGIGLPGDLSSVSRFVKATFTKLNSVSGDSESE SISQFFHILGSVEQQKGLCDVGDGKYEYTIYSSCCNVDKGI YYYRTYEDSQITAIDMNKEDLDSHKLISYPIIEKQQIKYIN
Bile salt hydrolase A from *Lacotbacillus acidophilus* **SEQ ID NO: 104**	AAGAGAAAAATATGTGTACATCAATTATATTCAGTCCC AAAGATCATTACTTTGGTCGTAACCTTGATTTAGAAATT ACTTTTGGTCAACAAGTTGTTATTACGCCACGCAATTAC ACTTTTAAATTCCGTAAGATGCCCAGTTTAAAAAAGCA CTATGCAATGATTGGTATCTCATTAGATATGGATGATTA TCCCCTATATTTCGACGCTACAAATGAAAAAGGTTTAG GTATGGCCGGACTCAACTATCCAGGAAATGCTACATAT TATGAAGAAAAAGAAAATAAAGATAATATTGCTTCCTT TGAATTCATCCCTTGGATTTTAGGACAGTGTAGCACTAT TAGCGAAGTAAAGGATTTACTTAGCAGAATCAACATCG CCGATTTAAATTTCAGCGAAAAAATGCAAGCCTCCTCTC TTCACTGGCTTATTGCAGATAAAACAGGTACATCATTAG TTGTTGAAACAGACAAAGATGGAATGCATATTTATGAT AATCCAGTTGGCTGCTTAACTAATAATCCACAATTTCCA AAGCAATTATTCAATTTAAATAACTATGCTGACGTATCT CCAAAAATGCCTAAAAATAACTTCTCAGATAAAGTAAA TATGGCTGGCTACAGCCGTGGATTAGGGTCTCACAACTT ACCAGGTGGAATGGATTCTGAATCACGTTTTGTCAGAG TAGCTTTCAATAAATTTAATGCTCCAATTGCTGAAACCG AAGAAGAAAATATTGATACTTACTTCCACATTTTACATT CGGTTGAACAACAAAAGGGACTGGATGAAGTTGGTCCA AACTCATTTGAATATACAATTTATTCTGATGGAACTAAC TTAGACAAAGGTATTTTCTACTACACCACTTATTCAAAC AAACAAATTAACGTTGTTGATATGAATAAAGAAGATCT AGATAGCAGCAATTTGATCACTTATGATATGCTTGATAA AACTAAATTTAACCATCAAAACTAA

Bile salt hydrolase A protein from *Lacotbacillus acidophilus* **SEQ ID NO: 105**	MCTSIIFSPKDHYFGRNLDLEITFGQQVVITPRNYTFKFRK MPSLKKHYAMIGISLDMDDYPLYFDATNEKGLGMAGLNY PGNATYYEEKENKDNIASFEFIPWILGQCSTISEVKDLLSRI NIADLNFSEKMQASSLHWLIADKTGTSLVVETDKDGMHIY DNPVGCLTNNPQFPKQLFNLNNYADVSPKMPKNNFSDKV NMAGYSRGLGSHNLPGGMDSESRFVRVAFNKFNAPIAET EEENIDTYFHILHSVEQQKGLDEVGPNSFEYTIYSDGTNLD KGIFYYTTYSNKQINVVDMNKEDLDSSNLITYDMLDKTKF NHQN
Bile salt hydrolase B from *Lacotbacillus acidophilus* **SEQ ID NO: 106**	AGAAAGCGTGCAGTAAATGTGTACATCAATTTGTTAT AATCCTAACGATCATTATTTTGGTAGAAATCTTGACTA TGAAATTGCTTATGGTCAAAAAGTAGTCATTGTACCA AGAAACTACGAATTTAAGTATAGAGAAATGCCCTCTC AAAAGATGCATTATGCTTTTATCGGAGTATCTGTAGTT AATGATGATTATCCATTATTATGTGATGCAATTAATGA AAAGGGGCTTGGTATTGCAGGATTAAATTTTCAAGGT CCTAATCATTACTTTCCTAAAATCGAAGGTAAGAAGA ATATTGCTTCTTTTGAATTAATGCCATACTTATTAAGT AATTGTGAAAATACTGACGATGTTAAAGAAATCTTAG ATAATGCAAATATTTTAAATATTAGCTTTTCAGCAAAT TATCCTGCAGCTGATTTACATTGGATTTTAAGTGATAA AGCTGGTAAGAGTATCGTAGTTGAATCAACCAATTCA GGTTTACATATTTATGATAATCCAGTGAATGTCTTAAC TAACAATCCTGAATTTCCGGATCAATTAATTAAATTAA GTGACTACGCCGACGTTACTCCACATAATCCTAAGAA TACATTGGTTCCTAATGTTGATCTTAATCTATATAGTA GAGGCTTAGGTACTCACCACTTACCTGGTGGAATGGA TTCTAGCTCTCGATTTGTTAAGGTAGCTTTTGTCTTGGC ACACACTCCACAAGGAAAAAATGAAGTGGAAAATGTT ACTAATTATTTCCATATTCTGCATTCAGTAGAACAACC TGATGGTTTAGATGAAGTAGAAGATAATCGCTATGAA TATACTATGTATACAGATTGTATGAACTTAGATAAAG GTATTTTGTACTTTACTACTTATGACAATAATCGGATT AATGCAGTAGATATGCATAAAGCAGATTTAGATTCAG AAGATTTAATCTGCTACGATTTGTTTAAGAAACAAGAT ATTGAATATATGAATTAA
Bile salt hydrolase B protein from *Lacotbacillus acidophilus* **SEQ ID NO: 107**	MCTSICYNPNDHYFGRNLDYEIAYGQKVVIVPRNYEFKYR EMPSQKMHYAFIGVSVVNDDYPLLCDAINEKGLGIAGLNF QGPNHYFPKIEGKKNIASFELMPYLLSNCENTDDVKEILDN ANILNISFSANYPAADLHWILSDKAGKSIVVESTNSGLHIY DNPVNVLTNNPEFPDQLIKLSDYADVTPHNPKNTLVPNVD LNLYSRGLGTHHLPGGMDSSSRFVKVAFVLAHTPQGKNE VENVTNYFHILHSVEQPDGLDEVEDNRYEYTMYTDCMNL DKGILYFTTYDNNRINAVDMHKADLDSEDLICYDLFKKQ DIEYMN
Bile salt hydrolase from *Brucella abortus* **SEQ ID NO: 108**	ATGGAAACGAAAAGCTCTCTCTGGAAATCATCGCGCC GCGTGCTTGCACATGGGGCTGCAACTGTTCTGGTCGCG GCGGGCCTTATCGTTCCCCAGGCGGCTATGGCTTGCAC GAGCTTCGTTCTGCCGACGAGCGACGGTGGTATGGTC TATGGTCGCACGATGGAATTCGGGTTCAATCTCAAATC

	CGACATGATTGCCATTCCGCGCAATTACACCATCACG GCAAGCGGGCCGGACGGTGCTGCGGGCAAGAAATGG AAGGGCAAATATGCCACGATCGGCATGAATGCTTTTG GTATCGTCGCTCTCACCGACGGTATGAACGAGAAGGG GCTTGCAGGCGGGCTTCTCTATTTCCCGGAATATGCCA AGTATCAGGACCCATCCACGGCGAAGCCGGAAGACAG CCTCGCTCCGTGGGATTTCCTGACCTGGGCGCTGGCCA ATTTTTCGACAGTGGCCGAAGTCAAGGATGCTTTGAG CACCATTTCCATCGTCGATGTGAAACAAAAGGACCTG GGATTTACCCCGCCCGCTCACTACACGCTGCATGATGC GACCGGCGCATCCATCGTGATCGAACCGATCGACGGC AAGCTCAAGGTTTACGACAACAAGCTCGGTGTCATGA CCAATTCGCCGTCTTTCGACTGGCACATGACCAATCTG CGCAACTATGTCTATCTCTCGCGTGAAAATCCGAAGCC GTTGCAGATCCTTGGCGAGACGATCCAGTCATTCGGG CAAGGCGCCGGTATGCATGGTATTCCGGGCGACACCA CGCCGCCATCGCGTTTCGTGCGTGCAAGCGCCTACGTC CTTTCCGCCAAGAAGGTGCCGAGCGGCCTTGAAAGCG TGCGGCTGGCCGAGCATATTGCCAATAACTTCGACATT CCAAAGGGATGGAGCGAAGAGCAGAATATGTTTGAAT ATACCCAGTGGACCGCCTTTGCGGACATGAAGAACGA TGTCTATTACATCAAGACCTATGACGATCAGGTTCTGC GCAGCTTCAGCTTCAAGGATTTTGATGTCGATAGCAA AGATATTCTAACGATCAAGTTCGAGCCAAAACTGGAC GCGCCGTCACTGAAAAAGTAA
Bile salt hydrolase protein from *Brucella abortus* **SEQ ID NO: 109**	METKSSLWKSSRRVLAHGAATVLVAAGLIVPQAAMACTS FVLPTSDGGMVYGRTMEFGFNLKSDMIAIPRNYTITASGP DGAAGKKWKGKYATIGMNAFGIVALTDGMNEKGLAGGL LYFPEYAKYQDPSTAKPEDSLAPWDFLTWALANFSTVAE VKDALSTISIVDVKQKDLGFTPPAHYTLHDATGASIVIEPID GKLKVYDNKLGVMTNSPSFDWHMTNLRNYVVLSRENPK PLQILGETIQSFGQGAGMHGIPGDTTPPSRFVRASAYVLSA KKVPSGLESVRLAEHIANNFDIPKGWSEEQNMFEYTQWT AFADMKNDVYYIKTYDDQVLRSFSFKDFDVDSKDILTIKF EPKLDAPSLKK

[078] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 90.** In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 90**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 90**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 90**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 90**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 90**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 90**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 90**.

[079] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 92**. In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 92**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 92**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 92**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 92**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 92**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 92**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 92**.

[080] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 94** In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 94**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 93**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 94**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 94**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 94**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 94**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 94**.

[081] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 96** In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 96**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the

entire sequence of **SEQ ID NO: 96**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 96**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 96**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 96**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 96**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 96**.

[082] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 98**. In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 98**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 98**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 98**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 98**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 98**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 98**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 98**.

[083] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 100**. In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 100**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 100**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 100**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 100**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 100**. In another embodiment, the bile salt hydrolase gene comprises the

sequence of **SEQ ID NO: 100**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 100**.

[084] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 102**. In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 102**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 102**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 102**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 102**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 102**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 102**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 102**.

[085] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 104**. In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 104**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 104**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 104**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 104**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 104**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 104**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 104**.

[086] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 106**. In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 106**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 106**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 106**. In another

embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 106**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 106**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 106**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 106**.

[087] In one embodiment, the bile salt hydrolase gene has at least about 80% identity with the entire sequence of **SEQ ID NO: 108**. In another embodiment, the bile salt hydrolase gene has at least about 85% identity with the entire sequence of **SEQ ID NO: 108**. In one embodiment, the bile salt hydrolase gene has at least about 90% identity with the entire sequence of **SEQ ID NO: 108**. In one embodiment, the bile salt hydrolase gene has at least about 95% identity with the entire sequence of **SEQ ID NO: 108**. In another embodiment, the bile salt hydrolase gene has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 108**. Accordingly, in one embodiment, the bile salt hydrolase gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 108**. In another embodiment, the bile salt hydrolase gene comprises the sequence of **SEQ ID NO: 108**. In yet another embodiment the bile salt hydrolase gene consists of the sequence of **SEQ ID NO: 108**.

[088] In one embodiment, one or more polypeptides encoded by the and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**. In another embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 85% identity with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**. In one embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 90% identity with with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**. In one embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 95% identity with with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**. In another embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**.

Accordingly, in one embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria have at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**. In another embodiment, one or more polypeptides encoded by gene sequencesand expressed by the genetically engineered bacteria one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria comprise the sequence of with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**. In yet another embodiment one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria consist of the sequence of with one or more of **SEQ ID NO: 91, 93, 95, 97, 99, 101, 103, 105, 107,** and **109**.

[089] In one embodiment, the gene encoding the bile salt hydrolase enzyme is directly operably linked to a first promoter. In another embodiment, the gene encoding the bile salt hydrolase enzyme is indirectly operably linked to a first promoter. In one embodiment, the promoter is not operably linked with the gene encoding the bile salt hydrolase enzyme in nature.

[090] In some embodiments, the gene encoding the bile salt hydrolase enzyme is expressed under the control of a constitutive promoter. In another embodiment, the gene encoding the bile salt hydrolase enzyme is expressed under the control of an inducible promoter. In some embodiments, the gene encoding the bile salt hydrolase enzyme is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the gene encoding the bile salt hydrolase enzyme is expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the gene encoding the bile salt hydrolase enzyme is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. Inducible promoters are described in more detail *infra*.

[091] In some embodiments, the genetically engineered bacteria are capable of expressing bile sale hydrolase under inducing conditions, *e.g.*, under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of expressing bile sale hydrolase in low-oxygen conditions, in the presence of certain molecules or metabolites , in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, metabolic disease, inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[092] The gene encoding the bile salt hydrolase enzyme may be present on a plasmid or chromosome in the bacterial cell. In one embodiment, the gene encoding the bile salt hydrolase enzyme is located on a plasmid in the bacterial cell. In another embodiment, the gene encoding the bile salt hydrolase is located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the gene encoding the bile salt hydrolase enzyme is located in the chromosome of the bacterial cell, and a gene encoding a bile salt hydrolase enzyme from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene encoding the bile salt hydrolase enzyme is located on a plasmid in the bacterial cell, and a gene encoding the bile salt hydrolase enzyme from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene encoding the bile salt hydrolase enzyme is located in the chromosome of the bacterial cell, and a gene encoding the bile salt hydrolase enzyme from a different species of bacteria is located in the chromosome of the bacterial cell. For example, *E. coli* comprises a native bile salt hydrolase gene.

[093] In some embodiments, the gene encoding the bile salt hydrolase enzyme is expressed on a low-copy plasmid. In some embodiments, the gene encoding the bile salt hydrolase enzyme is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of the bile salt hydrolase enzyme, thereby increasing the catabolism of bile salts.

Transporters of Bile Salts and Bile Acids

[094] The uptake of bile salts into the *Lactobacillus* and *Bifidobacterium* has been found to occur via the bile salt transporters CbsT1 and CbsT2 (see, *e.g.,* Elkins *et al., Microbiology*, 147(Pt. 12):3403-3412 (2001), the entire contents of which are expressly incorporated herein by reference). The uptake of bile acids into the *Neisseria meningitides* has been found to occur via the bile acid sodium symporter ASBT (*see, e.g.,* Hu *et al.*, Nature, 478(7369):408-411 (2011), the contents of which are expressly incorporated herein by reference. Other proteins that mediate the import of bile salts or acids into cells are well known to those of skill in the art. For the purposes of this invention, a bile salt transporter includes bile salt importers and bile acid symporters.

[095] Bile salt transporters, *e.g.,* bile salt importers or bile acid symporters, may be expressed or modified in the bacteria in order to enhance bile salt or acid transport into the cell. Specifically, when the transporter of bile salts is expressed in the recombinant bacterial cells, the bacterial cells import more bile salts into the cell when the transporter is expressed

than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the genetically engineered bacteria comprising a heterologous gene encoding a transporter of bile salts may be used to import bile salts into the bacteria so that any gene encoding a bile salt hydrolase (BSH) enzyme expressed in the organism can be used to treat disorders associated with bile salts, such as cardiac disease, metabolic disease, liver disease, cancer, and *C. difficile* infection. In one embodiment, the bacterial cell comprises a heterologous gene encoding a transporter of a bile salt. In one embodiment, the bacterial cell comprises a heterologous gene encoding a transporter of a bile salt and a heterologous gene encoding a bile salt hydrolase (BSH) enzyme.

[096] Thus, in some embodiments, the disclosure provides a bacterial cell that comprises a heterologous gene encoding a bile salt hydrolase enzyme operably linked to a first promoter and a heterologous gene encoding a transporter of a bile salt. In some embodiments, the disclosure provides a bacterial cell that comprises a heterologous gene encoding a transporter of a bile salt operably linked to the first promoter. In another embodiment, the disclosure provides a bacterial cell that comprises a heterologous gene encoding at least one bile salt hydrolase enzyme operably linked to a first promoter and a heterologous gene encoding transporter of a bile salt operably linked to a second promoter. In one embodiment, the first promoter and the second promoter are separate copies of the same promoter. In another embodiment, the first promoter and the second promoter are different promoters.

[097] In one embodiment, the bacterial cell comprises a gene encoding a transporter of a bile salt from a different organism, *e.g.,* a different species of bacteria. In one embodiment, the bacterial cell comprises at least one native gene encoding transporter of a bile salt. In some embodiments, the at least one native gene encoding atransporter of a bile salt is not modified. In another embodiment, the bacterial cell comprises more than one copy of at least one native gene encoding a transporter of a bile salt. In yet another embodiment, the bacterial cell comprises a copy of a gene encoding a native transporter of a bile salt, as well as at least one copy of a heterologous gene encoding a transporter of a bile salt from a different bacterial species. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of the heterologous gene encoding a tarnsporter of a bile salt. In one embodiment, the bacterial cell comprises multiple copies of the heterologous gene encoding a transporter of a bile salt.

[098] In some embodiments, the transporterof a bile salt is encoded by a transporter of a bile salt gene derived from a bacterial genus or species, including but not limited to,

Lactobacillus. In some embodiments, the transporterof a bile salt gene is derived from a bacteria of the species *Lactobacillus johnsonni* strain 100-100.

[099] The present disclosure further comprises genes encoding functional fragments of a transporter of a bile salt or functional variants of a transporter of a bile salt. As used herein, the term "functional fragment thereof" or "functional variant thereof" of a transporter of a bile salt relates to an element having qualitative biological activity in common with the wild-type transporter of a bile salt from which the fragment or variant was derived. For example, a functional fragment or a functional variant of a mutated transporter of bile salt protein is one which retains essentially the same ability to import the bile salt into the bacterial cell as does the transporter protein from which the functional fragment or functional variant was derived. In one embodiment, the recombinant bacterial cell comprises a heterologous gene encoding a functional fragment of a transporter of a bile salt. In another embodiment, the recombinant bacterial cell comprises a heterologous gene encoding a functional variant of a transporter of a bile salt.

[0100] Assays for testing the activity of a transporter of a bile salt, a functional variant of a transporter of a bile salt, or a functional fragment of a transporter of a bile salt are well known to one of ordinary skill in the art. For example, bile salt import can be assessed as described in Elkins *et al., Microbiology*, 147:3403-3412 (2001), the entire contents of which are expressly incorporated herein by reference.

[0101] In one embodiment the gene(s) encoding the transporter of a bile salt have been codon-optimized for use in the host organism. In one embodiment, the genes encoding the transporter of a bile salt have been codon-optimized for use in *Escherichia coli*.

[0102] The present disclosure also encompasses genes encoding a transporter of a bile salt comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein. Amino acid sequences that are substantially the same as the sequences described herein include sequences comprising conservative amino acid substitutions, as well as amino acid deletions and/or insertions.

[0103] In some embodiments, the gene encoding a transporter of a bile salt is mutagenized; mutants exhibiting increased bile salt transport are selected; and the mutagenized a gene encoding a transporter of a bile salt is isolated and inserted into the bacterial cell. In some embodiments, the gene encoding a transporter of a bile salt is mutagenized; mutants exhibiting decreased bile salt transport are selected; and the mutagenized a gene encoding a transporter of the bile salt is isolated and inserted into the bacterial cell. The transporter modifications described herein may be present on a plasmid or

chromosome. Non-limiting examples of bile salt transporters, which are encoded in the genetically engineered bacteria, are in **Table 11**.

Table 11. Bile Salt Transport and Export Sequences

Description	Sequence
cbsT1 from *Lactobacillus johnsonii* **SEQ ID NO: 110**	ATGTCGACCACACCGACACAGCCATCATCACGAAAACAGGC TGTTTACCCGTACTTGATCGTGCTGTCGGGCATCGTCTTCAC GGCCATCCCGGTATCGCTGGTCTGCAGTTGCGCAGGTATCTT CTTCACGCCTGTCAGCAGCTACTTCCATGTTCCCAAGGCCGC ATTCACCGGATATTTCAGCATATTCAGCATCACCATGGTCGC CTTCCTGCCGGTGGCCGGATGGCTGATGCACCGCTACGATC TGCGCATCGTACTGACCGCAAGCACCGTCCTGGCTGGACTG GGCTGCCTGGGTATGTCCCGATCATCCGCCATGTGGCAGTT CTATCTATGCGGAGTGGTTCTGGGAATCGGCATGCCGGCCG TCCTCTATCTGTCAGTGCCAACACTCATCAACGCCTGGTTCC GCAAGCGGGTCGGGTTCTTCATCGGCCTGTGCATGGCCTTC ACCGGCATAGGCGGCGTGATCTTCAACCAGATAGGCACCAT GATCATCAGATCCGCCCCTGATGGATGGAGGCGGGGATATC TGGTTTTCGCTATTCTCATCCTGGTGATCACCCTGCCCTTCA CCATTTTCGTCATTCGCAGCACACCCGAACAGATGGGTCTG CATCCCTACGGCGCCGACCAGGAGCCTGATGCAGCTGAGAC GGCCACCAATAGTGCAGGCACCGGGAGCAAAGACCAAAAG AGTCCTGAGCCTGCAGCGTCAACCGTAGGCATGACTGCCTC CCAGGCCTTGCGCTCCCCTGCCTTCTGGGCGCTGGCGCTCTT CTGCGGTCTGATCACCATGAATCAGACCATTTACCAGTTCCT GCCCTCCTACGCGGCATCCCTGCCATCCATGGCAGCCTACA CGGGACTGATCGCCTCCTCCTGCATGGCCGGCCAGGCCATC GGCAAGATCATCCTGGGCATGGTCAACGACGGCAGCATCGT AGGCGGTCTCTGTCTGGGCATCGGCGGCGGCATTCTCGGCG TCTGCCTCATGGTCGCCTTCCCCGGATTGCCCGTGCTCCTCC TGCTGGGAGCCTTTGCCTTCGGCCTTGTCTACGCCTGCACTA CTGTGCAGACACCAATCCTGGTTACAGCGGTCTTCGGCTCG CGCGACTACACCAACATCTATGCACGTATCCAGATGGTTGG GTCCCTAGCCTCGGCCTTCGCAGCTCTCTTCTGGGGCGCCAT CGCTGACCAGCCCCACGGCTACATCATCATGTTCGGTCTGA GCATCCTGATCATGGTTGTGGCCTTGTTCCTAGGCATTATCC CTCTGAAAGGTACGCGCAAGTTGACCGATCAGATCGCCTGA
CbsT1 protein from *Lactobacillus johnsonii* **SEQ ID NO: 111**	MSTTPTQPSSRKQAVYPYLIVLSGIVFTAIPVSLVCSCAGIFFTP VSSYFHVPKAAFTGYFSIFSITMVAFLPVAGWLMHRYDLRIVL TASTVLAGLGCLGMSRSSAMWQFYLCGVVLGIGMPAVLYLSV PTLINAWFRKRVGFFIGLCMAFTGIGGVIFNQIGTMIIRSAPDG WRRGYLVFAILILVITLPFTIFVIRSTPEQMGLHPYGADQEPDAA ETATNSAGTGSKDQKSPEPAASTVGMTASQALRSPAFWALAL FCGLITMNQTIYQFLPSYAASLPSMAAYTGLIASSCMAGQAIGK IILGMVNDGSIVGGLCLGIGGGILGVCLMVAFPGLPVLLLLGAF AFGLVYACTTVQTPILVTAVFGSRDYTNIYARIQMVGSLASAF AALFWGAIADQPHGYIIMFGLSILIMVVALFLGIIPLKGTRKLTD QIA
cbsT2 from	ATGTCTACTGATGCCGCTACTAAAGATAAAGTAGTAAGCAA

Lactobacillus johnsonii **SEQ ID NO: 112**	GGGCTATAAATACTTCATGGTTTTCCTTTGTATGTTAACCCA AGCTATTCCTTATGGAATTGCTCAAAACATTCAGCCTTTGTT TATCCACCCTTTAGTTAATACTTTCCACTTTACCTTAGCATC GTACACATTAATTTTTACGTTTGGTGCGGTTTTTGCTTCAGT TGCTTCTCCATTTATTGGTAAGGCATTAGAAAAAGTTAACTT CCGACTAATGTATTTAATTGGTATTGGTCTTTCTGCTATTGC CTACGTAATTTTTGGAATTAGTACAAAACTACCCGGTTTCTA TATTGCCGCTATCATTTGTATGGTTGGTTCAACCTTTTACTC CGGCCAAGGTGTTCCCTGGGTTATTAACCACTGGTTCCCAG CAAAGGGACGTGGGGCTGCCTTAGGAATTGCCTTCTGCGGT GGTTCTATTGGTAATATCTTTTTACAACCAGCAACCCAAGCT ATTTTAAAACACTACATGACAGGTAATACTAAGACCGGTCA TTTAACCTCTATGGCACCATTCTTTATCTTTGCCGTAGCTTTA TTAGTAATCGGTGTAATTATCGCCTGCTTCATTAGAACCCCT AAGAAAGACGAAATTGTTGTTTCTGATGCAGAACTAGCTGA AAGCAAGAAAGCTGAAGCCGCAGCCAAAGCTAAAGAGTTT AAAGGCTGGACTAGTAAACAAGTGTTACAAATGAAATGGTT CTGGATTTTCAGCCTTGGTTTCTTAATCATTGGTTTAGGCTT AGCTTCTTTAAATGAAGACTATGCCGCCTTCCTTGATACTAA GCTTTCTTTAACCGATGTTGGTTTAGTTGGGTCAATGTACGG TGTTGGTTGTTTAATCGGAAATATTTCTGGTGGTTTCTTATTT GATAAATTTGGTACAGCAAAATCAATGACCTATGCTGGTTG TATGTATATTTTATCTATTCTGATGATGATCTTTATTAGCTTC CAGCCATATGGTTCATCTATTAGTAAGGCTGCTGGCATTGG CTATGCTATCTTTTGCGGCTTAGCTGTATTTAGTTACATGTC AGGCCCAGCCTTCATGGCAAAAGACCTCTTTGGTTCAAGAG ATCAAGGTGTCATGCTTGGATACGTTGGTTTAGCTTATGCAA TTGGCTATGCCATTGGTGCTCCACTATTTGGGATTATTAAGG GAGCGGCAAGCTTTACAGTTGCTTGGTACTTTATGATTGCCT TTGTTGCAATTGGTTTTATCATTTTAGTATTTGCCGTTATCCA AATTAAGAGATACCAAAAGAAATACATTGCAGAGCAAGCA GCAAAAGCTAATGCTAAATAA
CbsT2 protein from *Lactobacillus johnsonii* **SEQ ID NO: 113**	MSTDAATKDKVVSKGYKYFMVFLCMLTQAIPYGIAQNIQPLFI HPLVNTFHFTLASYTLIFTFGAVFASVASPFIGKALEKVNFRLM YLIGIGLSAIAYVIFGISTKLPGFYIAAIICMVGSTFYSGQGVPW VINHWFPAKGRGAALGIAFCGGSIGNIFLQPATQAILKHYMTG NTKTGHLTSMAPFFIFAVALLVIGVIIACFIRTPKKDEIVVSDAE LAESKKAEAAAKAKEFKGWTSKQVLQMKWFWIFSLGFLIIGL GLASLNEDYAAFLDTKLSLTDVGLVGSMYGVGCLIGNISGGFL FDKFGTAKSMTYAGCMYILSILMMIFISFQPYGSSISKAAGIGY AIFCGLAVFSYMSGPAFMAKDLFGSRDQGVMLGYVGLAYAIG YAIGAPLFGIIKGAASFTVAWYFMIAFVAIGFIILVFAVIQIKRY QKKYIAEQAAKANAK
ABCB11 bile salt exporter *Homo sapiens* **SEQ ID NO: 114**	GAATGATGAAAACCGAGGTTGGAAAAGGTTGTGAAACCTTT TAACTCTCCACAGTGGAGTCCATTATTTCCTCTGGCTTCCTC AAATTCATATTCACAGGGTCGTTGGCTGTGGGTTGCAATTA CCATGTCTGACTCAGTAATTCTTCGAAGTATAAAGAAATTT GGAGAGGAGAATGATGGTTTTGAGTCAGATAAATCATATAA TAATGATAAGAAATCAAGGTTACAAGATGAGAAGAAAGGT GATGGCGTTAGAGTTGGCTTCTTTCAATTGTTTCGGTTTTCT

	TCATCAACTGACATTTGGCTGATGTTTGTGGGAAGTTTGTGT GCATTTCTCCATGGAATAGCCCAGCCAGGCGTGCTACTCAT TTTTGGCACAATGACAGATGTTTTTATTGACTACGACGTTGA GTTACAAGAACTCCAGATTCCAGGAAAAGCATGTGTGAATA ACACCATTGTATGGACTAACAGTTCCCTCAACCAGAACATG ACAAATGGAACACGTTGTGGGTTGCTGAACATCGAGAGCGA AATGATCAAATTTGCCAGTTACTATGCTGGAATTGCTGTCGC AGTACTTATCACAGGATATATTCAAATATGCTTTTGGGTCAT TGCCGCAGCTCGTCAGATACAGAAAATGAGAAAATTTTACT TTAGGAGAATAATGAGAATGGAAATAGGGTGGTTTGACTGC AATTCAGTGGGGGAGCTGAATACAAGATTCTCTGATGATAT TAATAAAATCAATGATGCCATAGCTGACCAAATGGCCCTTT TCATTCAGCGCATGACCTCGACCATCTGTGGTTTCCTGTTGG GATTTTTCAGGGGTTGGAAACTGACCTTGGTTATTATTTCTG TCAGCCCTCTCATTGGGATTGGAGCAGCCACCATTGGTCTG AGTGTGTCCAAGTTTACGGACTATGAGCTGAAGGCCTATGC CAAAGCAGGGGTGGTGGCTGATGAAGTCATTTCATCAATGA GAACAGTGGCTGCTTTTGGTGGTGAGAAAAGAGAGGTTGAA AGGTATGAGAAAAATCTTGTGTTCGCCCAGCGTTGGGGAAT TAGAAAAGGAATAGTGATGGGATTCTTTACTGGATTCGTGT GGTGTCTCATCTTTTTGTGTTATGCACTGGCCTTCTGGTACG GCTCCACACTTGTCCTGGATGAAGGAGAATATACACCAGGA ACCCTTGTCCAGATTTTCCTCAGTGTCATAGTAGGAGCTTTA AATCTTGGCAATGCCTCTCCTTGTTTGGAAGCCTTTGCAACT GGACGTGCAGCAGCCACCAGCATTTTTGAGACAATAGACAG GAAACCCATCATTGACTGCATGTCAGAAGATGGTTACAAGT TGGATCGAATCAAGGGTGAAATTGAATTCCATAATGTGACC TTCCATTATCCTTCCAGACCAGAGGTGAAGATTCTAAATGA CCTCAACATGGTCATTAAACCAGGGGAAATGACAGCTCTGG TAGGACCCAGTGGAGCTGGAAAAAGTACAGCACTGCAACT CATTCAGCGATTCTATGACCCCTGTGAAGGAATGGTGACCG TGGATGGCCATGACATTCGCTCTCTTAACATTCAGTGGCTTA GAGATCAGATTGGGATAGTGGAGCAAGAGCCAGTTCTGTTC TCTACCACCATTGCAGAAAATATTCGCTATGGCAGAGAAGA TGCAACAATGGAAGACATAGTCCAAGCTGCCAAGGAGGCC AATGCCTACAACTTCATCATGGACCTGCCACAGCAATTTGA CACCCTTGTTGGAGAAGGAGGAGGCCAGATGAGTGGTGGC CAGAAACAAAGGGTAGCTATCGCCAGAGCCCTCATCCGAA ATCCCAAGATTCTGCTTTTGGACATGGCCACCTCAGCTCTGG ACAATGAGAGTGAAGCCATGGTGCAAGAAGTGCTGAGTAA GATTCAGCATGGGCACACAATCATTTCAGTTGCTCATCGCTT GTCTACGGTCAGAGCTGCAGATACCATCATTGGTTTTGAAC ATGGCACTGCAGTGGAAAGAGGGACCCATGAAGAATTACT GGAAAGGAAAGGTGTTTACTTCACTCTAGTGACTTTGCAAA GCCAGGGAAATCAAGCTCTTAATGAAGAGGACATAAAGGA TGCAACTGAAGATGACATGCTTGCGAGGACCTTTAGCAGAG GGAGCTACCAGGATAGTTTAAGGGCTTCCATCCGGCAACGC TCCAAGTCTCAGCTTTCTTACCTGGTGCACGAACCTCCATTA GCTGTTGTAGATCATAAGTCTACCTATGAAGAAGATAGAAA GGACAAGGACATTCCTGTGCAGGAAGAAGTTGAACCTGCCC

	CAGTTAGGAGGATTCTGAAATTCAGTGCTCCAGAATGGCCC TACATGCTGGTAGGGTCTGTGGGTGCAGCTGTGAACGGGAC AGTCACACCCTTGTATGCCTTTTTATTCAGCCAGATTCTTGG GACTTTTTCAATTCCTGATAAAGAGGAACAAAGGTCACAGA TCAATGGTGTGTGCCTACTTTTTGTAGCAATGGGCTGTGTAT CTCTTTTCACCCAATTTCTACAGGGATATGCCTTTGCTAAAT CTGGGGAGCTCCTAACAAAAAGGCTACGTAAATTTGGTTTC AGGGCAATGCTGGGGCAAGATATTGCCTGGTTTGATGACCT CAGAAATAGCCCTGGAGCATTGACAACAAGACTTGCTACAG ATGCTTCCCAAGTTCAAGGGGCTGCCGGCTCTCAGATCGGG ATGATAGTCAATTCCTTCACTAACGTCACTGTGGCCATGATC ATTGCCTTCTCCTTTAGCTGGAAGCTGAGCCTGGTCATCTTG TGCTTCTTCCCCTTCTTGGCTTTATCAGGAGCCACACAGACC AGGATGTTGACAGGATTTGCCTCTCGAGATAAGCAGGCCCT GGAGATGGTGGGACAGATTACAAATGAAGCCCTCAGTAAC ATCCGCACTGTTGCTGGAATTGGAAAGGAGAGGCGGTTCAT TGAAGCACTTGAGACTGAGCTGGAGAAGCCCTTCAAGACAG CCATTCAGAAAGCCAATATTTACGGATTCTGCTTTGCCTTTG CCCAGTGCATCATGTTTATTGCGAATTCTGCTTCCTACAGAT ATGGAGGTTACTTAATCTCCAATGAGGGGCTCCATTTCAGC TATGTGTTCAGGGTGATCTCTGCAGTTGTACTGAGTGCAAC AGCTCTTGGAAGAGCCTTCTCTTACACCCCAAGTTATGCAA AAGCTAAAATATCAGCTGCACGCTTTTTTCAACTGCTGGAC CGACAACCCCCAATCAGTGTATACAATACTGCAGGTGAAAA ATGGGACAACTTCCAGGGGAAGATTGATTTTGTTGATTGTA AATTTACATATCCTTCTCGACCTGACTCGCAAGTTCTGAATG GTCTCTCAGTGTCGATTAGTCCAGGGCAGACACTGGCGTTT GTTGGGAGCAGTGGATGTGGCAAAAGCACTAGCATTCAGCT GTTGGAACGTTTCTATGATCCTGATCAAGGGAAGGTGATGA TAGATGGTCATGACAGCAAAAAAGTAAATGTCCAGTTCCTC CGCTCAAACATTGGAATTGTTTCCCAGGAACCAGTGTTGTTT GCCTGTAGCATAATGGACAATATCAAGTATGGAGACAACAC CAAAGAAATTCCCATGGAAAGAGTCATAGCAGCTGCAAAA CAGGCTCAGCTGCATGATTTTGTCATGTCACTCCCAGAGAA ATATGAAACTAACGTTGGGTCCCAGGGGTCTCAACTCTCTA GAGGGGAGAAACAACGCATTGCTATTGCTCGGGCCATTGTA CGAGATCCTAAAATCTTGCTACTAGATGAAGCCACTTCTGC CTTAGACACAGAAAGTGAAAAGACGGTGCAGGTTGCTCTAG ACAAAGCCAGAGAGGGTCGGACCTGCATTGTCATTGCCCAT CGCTTGTCCACCATCCAGAACGCGGATATCATTGCTGTCAT GGCACAGGGGGTGGTGATTGAAAAGGGGACCCATGAAGAA CTGATGGCCCAAAAAGGAGCCTACTACAAACTAGTCACCAC TGGATCCCCCATCAGTTGACCCAATGCAAGAATCTCAGACA CACATGACGCACCAGTTACAGGGGTTGTTTTTAAAGAAAAA AACAATCCCAGCAGGAGGGATTGCTGGGATTGTTTTTTCTTT AAAGAAGAATGTTAATATTTTACTTTTACAGTCATTTTCCTA CATCGGAATCCAAGCTAATTTCTAATGGCCTTCCATAATAAT TCTGCTTTAGATGTGTATACAGAAAATGAAAGAAACTAGGG TCCATATGAGGGAAAACCCAATGTCAAGTGGCAGCTCAGCC ACCACTCAGTGCTTCTCTGTGCAGGAGCCAGTCCTGATTAAT

	ATGTGGGAATTAGTGAGACATCAGGGAGTAAGTGACACTTT GAACTCCTCAAGGGCAGAGAACTGTCTTTCATTTTTGAACC CTCGGTGTACACAGAGGCGGGTCTATAACAGGCAATCAACA AACGTTTCTTGAGCTAGACCAAGGTCAGATTTGAAAAGAAC AGAAGGACTGAAGACCAGCTGTGTTTCTTAACTAAATTTGT CTTTCAAGTGAAACCAGCTTCCTTCATCTCTAAGGCTAAGG ATAGGGAAAGGGTGGATGCTCTCAGGCTGAGGGAGGCAGA AAGGGAAAGTATTAGCATGAGCTTTCCAGTTAGGGCTGTTG ATTTATGCTTTAACTTCAGAGTGAGTGTAGGGGTGGTGATG CT
ABCB11 bile salt exporter protein *Homo sapiens* **SEQ ID NO: 115**	MSDSVILRSIKKFGEENDGFESDKSYNNDKKSRLQDEKKGDGV RVGFFQLFRFSSSTDIWLMFVGSLCAFLHGIAQPGVLLIFGTMT DVFIDYDVELQELQIPGKACVNNTIVWTNSSLNQNMTNGTRC GLLNIESEMIKFASYYAGIAVAVLITGYIQICFWVIAAARQIQK MRKFYFRRIMRMEIGWFDCNSVGELNTRFSDDINKINDAIADQ MALFIQRMTSTICGFLLGFFRGWKLTLVIISVSPLIGIGAATIGLS VSKFTDYELKAYAKAGVVADEVISSMRTVAAFGGEKREVERY EKNLVFAQRWGIRKGIVMGFFTGFVWCLIFLCYALAFWYGST LVLDEGEYTPGTLVQIFLSVIVGALNLGNASPCLEAFATGRAA ATSIFETIDRKPIIDCMSEDGYKLDRIKGEIEFHNVTFHYPSRPEV KILNDLNMVIKPGEMTALVGPSGAGKSTALQLIQRFYDPCEGM VTVDGHDIRSLNIQWLRDQIGIVEQEPVLFSTTIAENIRYGRED ATMEDIVQAAKEANAYNFIMDLPQQFDTLVGEGGGQMSGGQ KQRVAIARALIRNPKILLLDMATSALDNESEAMVQEVLSKIQH GHTIISVAHRLSTVRAADTIIGFEHGTAVERGTHEELLERKGVY FTLVTLQSQGNQALNEEDIKDATEDDMLARTFSRGSYQDSLRA SIRQRSKSQLSYLVHEPPLAVVDHKSTYEEDRKDKDIPVQEEV EPAPVRRILKFSAPEWPYMLVGSVGAAVNGTVTPLYAFLFSQI LGTFSIPDKEEQRSQINGVCLLFVAMGCVSLFTQFLQGYAFAKS GELLTKRLRKFGFRAMLGQDIAWFDDLRNSPGALTTRLATDA SQVQGAAGSQIGMIVNSFTNVTVAMIIAFSFSWKLSLVILCFFPF LALSGATQTRMLTGFASRDKQALEMVGQITNEALSNIRTVAGI GKERRFIEALETELEKPFKTAIQKANIYGFCFAFAQCIMFIANSA SYRYGGYLISNEGLHFSYVFRVISAVVLSATALGRAFSYTPSYA KAKISAARFFQLLDRQPPISVYNTAGEKWDNFQGKIDFVDCKF TYPSRPDSQVLNGLSVSISPGQTLAFVGSSGCGKSTSIQLLERFY DPDQGKVMIDGHDSKKVNVQFLRSNIGIVSQEPVLFACSIMDN IKYGDNTKEIPMERVIAAAKQAQLHDFVMSLPEKYETNVGSQ GSQLSRGEKQRIAIARAIVRDPKILLLDEATSALDTESEKTVQV ALDKAREGRTCIVIAHRLSTIQNADIIAVMAQGVVIEKGTHEEL MAQKGAYYKLVTTGSPIS
Streptococcus thermophilus Msba subfamily ABC transporter ATP-binding protein *STH8232_1633* **SEQ ID NO:**	MEGRTVFVIAHRLSTIVNSDVILVMDHGRIIKRGDHDTLMEQG GTYYRLYTGSLEID

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Nostoc spp. Asl1293 ABC transporter gene **SEQ ID NO: 117**	ATGTGGGGGAAACAAAGACAAAGAATCGCCATTGCACGAG GGGGTTTTAAGAATTTGCAGGTTTTGATTTTAGATAAAGCA ACCTCGGCATTGGATAATAAAACAGAAGCAGCTATTGAGCG ATCGCTGGTGTTGACTGTTGACCAATGA
Nostoc spp. Asl1293 ABC transporter protein **SEQ ID NO: 118**	MWGKQRQRIAIARGGFKNLQVLILDKATSALDNKTEAAIERSL VLTVDQ
Neisseria meningitides (MC58) $ASBT_{NM}$ bile acid sodium symporter (NMB0705) **SEQ ID NO: 119**	ATGAATATCCTCAGTAAAATCAGCAGCTTTATCGGAAAAAC ATTTTCCCTCTGGGCCGCGCTCTTTGCCGCCGCCGCTTTTTTC GCGCCCGACACCTTCAAATGGGCGGGGCCTTATATTCCTTG GCTGTTGGGCATTATTATGTTCGGTATGGGTTTGACGCTCAA ACCTTCCGACTTCGATATTTTGTTCAAACATCCCAAAGTCGT CATCATCGGCGTAATCGCACAATTCGCCATTATGCCGGCAA CCGCCTGGCTGCTGTCCAAACTGTTGAACCTGCCTGCCGAA ATCGCGGTCGGCGTGATTTTGGTCGGCTGCTGCCCGGGCGG TACGGCTTCCAATGTGATGACCTATCTGGCGCGTGGCAATG TGGCTTTGTCGGTTGCCGTTACGTCTGTTTCCACCCTGATTT CCCCATTGCTGACTCCCGCCATCTTCCTGATGCTTGCCGGCG AAATGCTGGAAATCCAAGCGGCCGGTATGTTGATGTCCATC GTCAAAATGGTTTTGCTCCCCATTGTTTTGGGTTTGATTGTC CATAAGGTTTTGGGCAGTAAAACCGAAAAGCTGACCGATGC GCTGCCGCTGGTTTCCGTTGCCGCCATCGTGCTGATTATCGG CGCGGTTGTTGGGGCAAGCAAAGGCAAGATTATGGAAAGC GGCCTGCTGATTTTTGCGGTTGTCGTACTCCACAACGGCATC GGCTACCTGCTCGGCTTCTTTGCCGCCAAATGGACCGGCCT GCCTTATGATGCACAAAAAACGCTGACCATCGAAGTCGGTA TGCAAAACTCGGGCCTGGCCGCCGCGCTTGCCGCCGCACAC TTTGCCGCCGCGCCGGTCGTTGCCGTTCCGGGCGCATTGTTC AGCGTGTGGCACAATATCTCCGGCTCGCTGCTGGCAACTTA TTGGGCGGCCAAAGCCGGTAAACATAAAAAACCCTAA
Neisseria meningitides (MC58) $ASBT_{NM}$ bile acid sodium symporter protein **SEQ ID NO: 120**	MNILSKISSFIGKTFSLWAALFAAAAFFAPDTFKWAGPYIPWLL GIIMFGMGLTLKPSDFDILFKHPKVVIIGVIAQFAIMPATAWLLS KLLNLPAEIAVGVILVGCCPGGTASNVMTYLARGNVALSVAV TSVSTLISPLLTPAIFLMLAGEMLEIQAAGMLMSIVKMVLLPIV LGLIVHKVLGSKTEKLTDALPLVSVAAIVLIIGAVVGASKGKIM ESGLLIFAVVVLHNGIGYLLGFFAAKWTGLPYDAQKTLTIEVG MQNSGLAAALAAAHFAAAPVVAVPGALFSVWHNISGSLLATY WAAKAGKHKKPGSENLYFQ

[0104] In one embodiment, the bile salt transporter is the bile salt importer CbsT1. In one embodiment, the *cbsT1* gene has at least about 80% identity to **SEQ ID NO: 110**. Accordingly, in one embodiment, the *cbsT1* gene has at least about 90% identity to **SEQ ID**

NO: 110. Accordingly, in one embodiment, the *cbsT1* gene has at least about 95% identity to **SEQ ID NO: 110**. Accordingly, in one embodiment, the *cbsT1* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to **SEQ ID NO: 110**. In another embodiment, the *cbsT1* gene comprises the sequence of **SEQ ID NO: 110**. In yet another embodiment the *cbsT1* gene consists of the sequence of **SEQ ID NO: 110**.

[0105] In one embodiment, the bile salt transporter is the bile salt importer CbsT2. In one embodiment, the *cbsT2* gene has at least about 80% identity to **SEQ ID NO: 112**. Accordingly, in one embodiment, the *cbsT2* gene has at least about 90% identity to **SEQ ID NO: 112**. Accordingly, in one embodiment, the *cbsT2* gene has at least about 95% identity to **SEQ ID NO: 112**. Accordingly, in one embodiment, the *cbsT2* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to **SEQ ID NO: 112**. In another embodiment, the *cbsT2* gene comprises the sequence of **SEQ ID NO: 112**. In yet another embodiment the *cbsT2* gene consists of the sequence of **SEQ ID NO: 112**.

[0106] In one embodiment, the bile acid transporter is the bile acid sodium symporter $ASBT_{NM}$. In one embodiment, the NMB0705 gene of Neisseria meningitides has at least about 80% identity to **SEQ ID NO: 117.** Accordingly, in one embodiment, the NMB0705 gene has at least about 90% identity to **SEQ ID NO: 117**. Accordingly, in one embodiment, the NMB0705 gene has at least about 95% identity to **SEQ ID NO: 117**. Accordingly, in one embodiment, the NMB0705 gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to **SEQ ID NO: 117**. In another embodiment, the NMB0705 gene comprises the sequence of **SEQ ID NO: 117**. In yet another embodiment the NMB0705 gene consists of the sequence of **SEQ ID NO: 117**.

[0107] In one embodiment, one or more polypeptides encoded by the and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 111, 113, 115, 116, 118** and **120**. In another embodiment, one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria have at least about 85% identity with one or more of **SEQ ID NO: 111, 113, 115, 116, 118** and **120**. In one embodiment, one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria have at least about 90% identity with with one or more of **SEQ ID NO: 111, 113, 115, 116, 118** and **120**. In one embodiment, one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria have at least about 95% identity with with one or more of **SEQ ID NO:**

111, 113, 115, 116, 118 and **120**. In another embodiment, one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria have at least about 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 111, 113, 115, 116, 118** and **120**. Accordingly, in one embodiment, one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria have at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 111, 113, 115, 116, 118** and **120**. In another embodiment, one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria comprise the sequence of with one or more of **SEQ ID NO: 111, 113, 115, 116, 118** and **120**. In yet another embodiment one or more polypeptides encoded by gene sequences and expressed by the genetically engineered bacteria consist of the sequence of with one or more of **SEQ ID NO: 111, 113, 115, 116, 118** and **120**.

[0108] In some embodiments, the bacterial cell comprises a heterologous gene encoding a bile salt hydrolase enzyme operably linked to a first promoter and a heterologous gene encoding a transporter of a bile salt. In some embodiments, the heterologous gene encoding a transporter of the bile salt is operably linked to the first promoter. In other embodiments, the heterologous gene encoding a transporter of the bile salt is operably linked to a second promoter. In one embodiment, the gene encoding a transporter of the bile salt is directly operably linked to the second promoter. In another embodiment, the gene encoding a transporter of the bile salt is indirectly operably linked to the second promoter.

[0109] In some embodiments, expression of a gene encoding a transporter of a bile salt is controlled by a different promoter than the promoter that controls expression of the gene encoding the bile salt hydrolase enzyme. In some embodiments, expression of the gene encoding a transporter of a bile salt is controlled by the same promoter that controls expression of the bile salt hydrolase enzyme. In some embodiments, a gene encoding a transporter of a bile salt and the bile salt hydrolase enzyme are divergently transcribed from a promoter region. In some embodiments, expression of each of genes encoding the gene encoding a transporter of a bile salt and the gene encoding the bile salt hydrolase enzyme is controlled by different promoters.

[0110] In one embodiment, the the gene encoding a transporter of a bile salt is not operably linked with its natural promoter. In some embodiments, the gene encoding the transporter of the bile salt is controlled by its native promoter. In some embodiments, the

gene encoding the transporter of the bile salt is controlled by an inducible promoter. In some embodiments, the gene encoding the transporter of the bile salt is controlled by a promoter that is stronger than its native promoter. In some embodiments, the gene encoding the transporter of the bile salt is controlled by a constitutive promoter.

[0111] In another embodiment, the promoter is an inducible promoter. Inducible promoters are described in more detail *infra*.

[0112] In one embodiment, the gene encoding a transporter of a bile salt is located on a plasmid in the bacterial cell. In another embodiment, the gene encoding a transporter of a bile salt is located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the gene encoding a transporter of a bile salt is located in the chromosome of the bacterial cell, and a copy of a gene encoding a transporter of a bile salt from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene encoding a transporter of a bile salt is located on a plasmid in the bacterial cell, and a copy of a gene encoding a transporter of a bile salt from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene encoding a transporter of a bile salt is located in the chromosome of the bacterial cell, and a copy of the gene encoding a transporter of a bile salt from a different species of bacteria is located in the chromosome of the bacterial cell.

[0113] In some embodiments, the at least one native gene encoding the transporter of a bile salt in the bacterial cell is not modified, and one or more additional copies of the native transporter of a bile salt are inserted into the genome. In one embodiment, the one or more additional copies of the native transporter that is inserted into the genome are under the control of the same inducible promoter that controls expression of the gene encoding the bile salt hydrolase enzyme, *e.g.,* the FNR responsive promoter, or a different inducible promoter than the one that controls expression of the bile salt hydrolase enzyme, or a constitutive promoter. In alternate embodiments, the at least one native gene encoding the transporter is not modified, and one or more additional copies of the transporter from a different bacterial species is inserted into the genome of the bacterial cell. In one embodiment, the one or more additional copies of the transporter inserted into the genome of the bacterial cell are under the control of the same inducible promoter that controls expression of the gene encoding the bile salt hydrolase enzyme, *e.g.,* the FNR responsive promoter, or a different inducible promoter than the one that controls expression of the gene encoding the bile salt hydrolase enzyme, or a constitutive promoter.

[0114] In one embodiment, when the transporter of a bile salt is expressed in the recombinant bacterial cells, the bacterial cells import 10% more bile salt into the bacterial cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In another embodiment, when the transporter of a bile salt is expressed in the recombinant bacterial cells, the bacterial cells import 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% more bile salt into the bacterial cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the transporter of a bile salt is expressed in the recombinant bacterial cells, the bacterial cells import two-fold more bile salt into the cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the transporter of a bile salt is expressed in the recombinant bacterial cells, the bacterial cells import three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold more bile salt into the cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions.

Generation of Bacterial Strains with Enhanced Ability to Transport Bile Salts

[0115] Due to their ease of culture, short generation times, very high population densities and small genomes, microbes can be evolved to unique phenotypes in abbreviated timescales. Adaptive laboratory evolution (ALE) is the process of passaging microbes under selective pressure to evolve a strain with a preferred phenotype. Most commonly, this is applied to increase utilization of carbon/energy sources or adapting a strain to environmental stresses (*e.g.*, temperature, pH), whereby mutant strains more capable of growth on the carbon substrate or under stress will outcompete the less adapted strains in the population and will eventually come to dominate the population.

[0116] This same process can be extended to any essential metabolite by creating an auxotroph. An auxotroph is a strain incapable of synthesizing an essential metabolite and must therefore have the metabolite provided in the media to grow. In this scenario, by making an auxotroph and passaging it on decreasing amounts of the metabolite, the resulting dominant strains should be more capable of obtaining and incorporating this essential metabolite.

[0117] For example, if the biosynthetic pathway for producing an amino acid is disrupted a strain capable of high-affinity capture of said amino acid can be evolved via ALE. First, the strain is grown in varying concentrations of the auxotrophic amino acid, until a minimum concentration to support growth is established. The strain is then passaged at that

concentration, and diluted into lowering concentrations of the amino acid at regular intervals. Over time, cells that are most competitive for the amino acid – at growth-limiting concentrations – will come to dominate the population. These strains will likely have mutations in their amino acid-transporters resulting in increased ability to import the essential and limiting amino acid.

[0118] Similarly, by using an auxotroph that cannot use an upstream metabolite to form an amino acid, a strain can be evolved that not only can more efficiently import the upstream metabolite, but also convert the metabolite into the essential downstream metabolite. These strains will also evolve mutations to increase import of the upstream metabolite, but may also contain mutations which increase expression or reaction kinetics of downstream enzymes, or that reduce competitive substrate utilization pathways.

[0119] A metabolite innate to the microbe can be made essential via mutational auxotrophy and selection applied with growth-limiting supplementation of the endogenous metabolite. However, phenotypes capable of consuming non-native compounds can be evolved by tying their consumption to the production of an essential compound. For example, if a gene from a different organism is isolated which can produce an essential compound or a precursor to an essential compound this gene can be recombinantly introduced and expressed in the heterologous host. This new host strain will now have the ability to synthesize an essential nutrient from a previously non-metabolizable substrate. Hereby, a similar ALE process can be applied by creating an auxotroph incapable of converting an immediately downstream metabolite and selecting in growth-limiting amounts of the non-native compound with concurrent expression of the recombinant enzyme. This will result in mutations in the transport of the non-native substrate, expression and activity of the heterologous enzyme and expression and activity of downstream native enzymes. It should be emphasized that the key requirement in this process is the ability to tether the consumption of the non-native metabolite to the production of a metabolite essential to growth.

[0120] Once the basis of the selection mechanism is established and minimum levels of supplementation have been established, the actual ALE experimentation can proceed. Throughout this process several parameters must be vigilantly monitored. It is important that the cultures are maintained in an exponential growth phase and not allowed to reach saturation/stationary phase. This means that growth rates must be check during each passaging and subsequent dilutions adjusted accordingly. If growth rate improves to such a degree that dilutions become large, then the concentration of auxotrophic supplementation should be decreased such that growth rate is slowed, selection pressure is increased and

dilutions are not so severe as to heavily bias subpopulations during passaging. In addition, at regular intervals cells should be diluted, grown on solid media and individual clones tested to confirm growth rate phenotypes observed in the ALE cultures.

[0121] Predicting when to halt the stop the ALE experiment also requires vigilance. As the success of directing evolution is tied directly to the number of mutations "screened" throughout the experiment and mutations are generally a function of errors during DNA replication, the cumulative cell divisions (CCD) acts as a proxy for total mutants which have been screened. Previous studies have shown that beneficial phenotypes for growth on different carbon sources can be isolated in about $10^{11.2}$ CCD[1]. This rate can be accelerated by the addition of chemical mutagens to the cultures – such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) – which causes increased DNA replication errors. However, when continued passaging leads to marginal or no improvement in growth rate the population has converged to some fitness maximum and the ALE experiment can be halted.

[0122] At the conclusion of the ALE experiment, the cells should be diluted, isolated on solid media and assayed for growth phenotypes matching that of the culture flask. Best performers from those selected are then prepped for genomic DNA and sent for whole genome sequencing. Sequencing with reveal mutations occurring around the genome capable of providing improved phenotypes, but will also contain silent mutations (those which provide no benefit but do not detract from desired phenotype). In cultures evolved in the presence of NTG or other chemical mutagen, there will be significantly more silent, background mutations. If satisfied with the best performing strain in its current state, the user can proceed to application with that strain. Otherwise the contributing mutations can be deconvoluted from the evolved strain by reintroducing the mutations to the parent strain by genome engineering techniques. See Lee, D.-H., Feist, A. M., Barrett, C. L. & Palsson, B. Ø. Cumulative Number of Cell Divisions as a Meaningful Timescale for Adaptive Laboratory Evolution of *Escherichia coli*. *PLoS ONE* **6,** e26172 (2011).

[0123] Similar methods can be used to generate E.Coli Nissle mutants that consume bile salts and/or over-produce bile salt hydrolase.

Exporters of Bile Salts

[0124] The export of bile salts is mediated by proteins well known to those of skill in the art. For example, the ATP-binding cassette, sub-family B member 11 (ABCB11, also called BSEP or "bile salt export pump") is responsible for the export of taurochoate and other cholate conjugates from hepatocytes to the bile in mammals, and mutations in this gene have been associated with progressive familial intrahepatic cholestasis type 2 (PFIC2) and

hepatocellular carcinoma (see Strautnieks *et al., Nature Genetics,* 20(3):233-238, 1998; Knisely *et al., Hepatology,* 44(2):478-486, 2006; and Ho *et al., Pharmacogenet. Genomics,* 20(1):45-57, 2010; **SEQ ID NO: 113 and SEQ ID NO:114**). In bacteria, *Streptococcus thermophilus* comprises a bile salt export pump (Msba subfamily ABC transporter ATP-binding protein; accession F8LYG6; **SEQ ID NO: 116**), and *Nostoc spp.* are known to comprise a bile salt export pump (Asl1293; accession Q8YXC2; **SEQ ID NO: 117 and SEQ ID NO: 118**). Multiple other bile salt exporters are known in the art.

[0125] Thus, in one embodiment of the invention, when the recombinant bacterial cell comprises an endogenous bile salt exporter gene, the recombinant bacterial cells may comprise a genetic modification that reduces export of one or more bile salts from the bacterial cell. In another embodiment, the recombinant bacterial cell comprises a genetic modification that reduces export of one or more bile salts from the bacterial cell and a heterologous gene encoding a bile salt catabolism enzyme. When the recombinant bacterial cells comprise a genetic modification that reduces export of a bile salt, the bacterial cells retain more bile salts in the bacterial cell than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the recombinant bacteria comprising a genetic modification that reduces export of a bile salt may be used to retain more bile salts in the bacterial cell so that any bile salt catabolism enzyme expressed in the organism can catabolize the bile salt(s) to treat diseases associated with bile salts, including cardiovascular disease. In one embodiment, the recombinant bacteria further comprise a heterologous gene encoding a transporter of one or more bile salts.

[0126] In one embodiment, the recombinant bacterial cell comprises a genetic modification in a gene encoding a bile salt exporter wherein said bile salt exporter comprises an amino acid sequence that has at least 80%, 81%, 82%, 83% 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence of a polypeptide encoded by a bile salt exporter gene disclosed herein. In one embodiment, the bile salt exporter has at least 80%, 81%, 82%, 83% 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence of **SEQ ID NO: 115**. In another embodiment, the bile salt exporter has at least 80%, 81%, 82%, 83% 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the nucleotide sequence of **SEQ ID NO: 117**.

[0127] In one embodiment, the genetic modification reduces export of a bile salt from the bacterial cell. In one embodiment, the bacterial cell is from a bacterial genus or species that includes but is not limited to, *Streptococcus thermophilus* or *Nostoc* spp.

[0128] In one embodiment, the genetic modification is a mutation in an endogenous gene encoding an exporter of one or more bile salts. In one embodiment, the genetic mutation results in an exporter having reduced activity as compared to a wild-type exporter protein. In one embodiment, the activity of the exporter is reduced at least 50%, at least 75%, or at least 100%. In another embodiment, the activity of the exporter is reduced at least two-fold, three-fold, four-fold, or five-fold. In another embodiment, the genetic mutation results in an exporter having no activity, *i.e.,* results in an exporter which cannot export one or more bile salts from the bacterial cell.

[0129] It is routine for one of ordinary skill in the art to make mutations in a gene of interest. Mutations include substitutions, insertions, deletions, and/or truncations of one or more specific amino acid residues or of one or more specific nucleotides or codons in the polypeptide or polynucleotide of the exporter of an amino acid. Mutagenesis and directed evolution methods are well known in the art for creating variants. See, *e.g.,* U.S. Pat. No. 7,783,428; U.S. Pat. No. 6,586,182; U.S. Pat. No. 6,117,679; and Ling, *et al.,* 1999, "Approaches to DNA mutagenesis: an overview," *Anal. Biochem.,* 254(2):157-78; Smith, 1985, "*In vitro* mutagenesis," *Ann. Rev. Genet.,* 19:423-462; Carter, 1986, "Site-directed mutagenesis," *Biochem. J.,* 237:1-7; and Minshull, *et al.*, 1999, "Protein evolution by molecular breeding," *Current Opinion in Chemical Biology,* 3:284-290. For example, the lambda red system can be used to knock-out genes in *E. coli* (see, for example, Datta *et al., Gene*, 379:109-115 (2006)).

[0130] The term "inactivated" as applied to a gene refers to any genetic modification that decreases or eliminates the expression of the gene and/or the functional activity of the corresponding gene product (mRNA and/or protein). The term “inactivated” encompasses complete or partial inactivation, suppression, deletion, interruption, blockage, promoter alterations, antisense RNA, dsRNA, or down-regulation of a gene. This can be accomplished, for example, by gene "knockout," inactivation, mutation (*e.g.,* insertion, deletion, point, or frameshift mutations that disrupt the expression or activity of the gene product), or by use of inhibitory RNAs (*e.g.,* sense, antisense, or RNAi technology). A deletion may encompass all or part of a gene's coding sequence. The term "knockout" refers to the deletion of most (at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%) or all (100%) of the coding sequence of a gene. In some

embodiments, any number of nucleotides can be deleted, from a single base to an entire piece of a chromosome.

[0131] Assays for testing the activity of an exporter of one or more bile salts are well known to one of ordinary skill in the art. For example, export of one or more bile salts may be determined using the methods described by Telbisz and Homolya, *Expert Opinion Ther. Targets,* 1-14, 2015, the entire contents of which are expressly incorporated herein by reference.

[0132] In another embodiment, the genetic modification is a mutation in a promoter of an endogenous gene encoding an exporter of one or more bile salts. In one embodiment, the genetic mutation results in decreased expression of the exporter gene. In one embodiment, exporter gene expression is reduced by about 50%, 75%, or 100%. In another embodiment, exporter gene expression is reduced about two-fold, three-fold, four-fold, or five-fold. In another embodiment, the genetic mutation completely inhibits expression of the exporter gene.

[0133] Assays for testing the level of expression of a gene, such as an exporter of one or more bile salts are well known to one of ordinary skill in the art. For example, reverse-transcriptase polymerase chain reaction may be used to detect the level of mRNA expression of a gene. Alternatively, Western blots using antibodies directed against a protein may be used to determine the level of expression of the protein.

[0134] In another embodiment, the genetic modification is an overexpression of a repressor of an exporter of one or more bile salts. In one embodiment, the overexpression of the repressor of the exporter is caused by a mutation which renders the promoter of the repressor constitutively active. In another embodiment, the overexpression of the repressor of the exporter is caused by the insertion of an inducible promoter in front of the repressor so that the expression of the repressor can be induced. Inducible promoters are described in more detail herein.

[0135] In one embodiment, the recombinant bacterial cells described herein comprise at least one genetic modification that reduces export of one or more bile salts from the bacterial cell. In another embodiment, the recombinant bacterial cells described herein comprise two genetic modifications that reduce export of one or more bile salts from the bacterial cell. In another embodiment, the recombinant bacterial cells described herein comprise three genetic modifications that reduce export of one or more bile salts from the bacterial cell. In another embodiment, the recombinant bacterial cells described herein comprise four genetic modifications that reduce export of one or more bile salts from the

bacterial cell. In another embodiment, the recombinant bacterial cells described herein comprise five genetic modifications that reduce export of one or more bile salts from the bacterial cell.

Tryptophan, Tryptophan Metabolism, and Tryptophan Metabolites

Tryptophan and the Kynurenine Pathway

[0136] Tryptophan (TRP) is an essential amino acid that, after consumption, is either incorporated into proteins via new protein synthesis, or converted a number of biologically active metabolites with a number of differing roles in health and disease (Perez-De La Cruz *et al.*, 2007 Kynurenine Pathway and Disease: An Overview; CNS&Neurological Disorders - Drug Targets 2007, 6,398-410). Along one arm of tryptophan catabolism, trytophan is converted to the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) by tryptophan hydroxylase. Serotonin can further be converted into the hormone melatonin. The a large share of tryptophan, , however, is metabolized to a number of bioactive metabolites, collectively called kynurenines, along a second arm called the kynurenine pathway (KP). In the first step of catabolism, TRP is converted to Kynurenine, (KYN), which has well-documented immune suppressive functions in several types of immune cells, and has recently been shown to be an activating ligand for the arylcarbon receptor (AhR; also known as dioxin receptor). KYN was initially shown in the cancer setting as an endogenous AHR ligand in immune and tumor cells, acting both in an autocrine and paracrine manner, and promoting tumor cell survival. The tryptophan kynurenine pathway is also expressed in a large number of microbiota, most prominently in Enterobacteriaceae, and kynurenine and metabolites may be synthesized in the gut (Sci Transl Med. 2013 July 10; 5(193): 193ra91).

[0137] More recently, additional tryptophan metabolites, collectively termed "indoles", herein, including for example, indole-3 aldehyde, indole-3-acetic acid, indole-3-acetaldehyde, some of which are generated by the microbiota, some by the human host, which are also able to function as AhR agonists, see *e.g.*, **Table 12 and Figure 13** and elsewhere herein, and Lama et al., Nat Med. 2016 Jun;22(6):598-605; CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands.

[0138] Ahr best known as a receptor for xenobiotics such as polycyclic aromatic hydrocarbons AhR is a ligand-dependent cytosolic transcription factor that is able to translocate to the cell nucleus after ligand binding. The in additiona to kynurenine, tryptophan metabolites L-kynurenine, 6-formylindolcarbazole (FICZ, a photoproduct of TRP), and KYNA are have recently been identified as endogenous AhR ligands mediating

immunosuppressive functions. To induce transcription of AhR target genes in the nucleus, AhR partners with proteins such as AhR nuclear translocator (ARNT) or NF-κB subunit RelB. Studies on human cancer cells have shown that KYN activates the AhR-ARNT associated transcription of IL-6, which induced autocrine activation of IDO1 via STAT3. This AhR-IL-6-STAT3 loop is associated with a poor prognosis in lung cancer, supporting the idea that IDO/kynurenine-mediated immunosuppression enables the immune escape of tumor cells.

[0139] In the gut, tryptophan may also be transported across the epithelium by transport machinery comprising angiotensin I converting enzyme 2 (ACE2), and converted to kynurenine, where it functions in the suppression of T cell respononse and promotion of Treg cells.

[0140] Ahr in the gut. The rate-limiting conversion of TRP to KYN may be mediated by either of two forms of indoleamine 2, 3-dioxygenase (IDO) or by tryptophan 2,3-dioxygenase (TDO). One characteristic of TRP metabolism is that the rate-limiting step of the catalysis from TRP to KYN is generated by both the hepatic enzyme tryptophan 2,3-dioxygenase (TDO) and the ubiquitous expressed enzyme IDO1. TDO is essential for homeostasis of TRP concentrations in organisms and has a lower affinity to TRP than IDO1. Its expression is activated mainly by increased plasma TRP concentrations but can also be activated by glucocorticoids and glucagon.

[0141] The tryptophan kynurenine pathway is also expressed in a large number of microbiota, most prominently in Enterobacteriaceae, and kynurenine and metabolites may be synthesized in the gut (**FIG. 15** and Sci Transl Med. 2013 July 10; 5(193): 193ra91). In some embodiments, the genetically engineered bacteria comprise one or more heterologous bacterially derived genes from Enterobacteriaceae, *e.g.* whose gene products catalyze the conversion of TRP:KYN.

[0142] Along one side arm, KYN may be further metabolized to another bioactive metabolite, kynurenic acid, (KYNA) which can antagonize glutamate receptors and is generally considered neuroprotective or along a second arm to 3-hydroxykynurenine (3-HK). In one embodiment, KYNA can bind to an aryl hydrocarbon receptor and a GPCR. KYNA has agonistic activity on the G protein-coupled receptor GPR35 [11] and antagonistic effects on glutamate receptors, in particular, the glycine co-agonist site of the N-methyl D-aspartate (NMDA)-receptor, and the cholinergic a7 nicotinic receptor, but it is also a ligand for the aryl hydrocarbon receptor (AHR) [12,13]. Along a third side arm of the KP, KYN can be

converted to anthranilic acid (AA) and further downstream quinolinic acid (QUIN), which is a glutamate receptor agonist and has a neurotoxic role. Consequently, the KP has two responsibilities in regard to TRP in the body; it depletes serum levels of TRP and converts TRP into other biologically active metabolites. These metabolites, along with the enzymes responsible for their production, have implications in a broad range of diseases, including, but not limited to, Non-alcoholic steatohepatitis (NASH) advanced lipotoxic metabolites, pro-inflammatory substrate, fibrosis, and increased hepatic lipid deposition..

[0143] Therefore, finding a means to upregulate and/or downregulate the levels of flux through the KP and to reset relative amounts and/or ratios of tryptophan and its various bioactive metabolites may be useful in the prevention, treatment and/or management of a number of diseases as described herein. The present disclosure describes compositions for modulating, regulating and fine tuning trypophan and tryptophan metabolite levels, *e.g.*, in the serum or in the gastrointestinal system, through genetically engineered bacteria which comprise circuitry enabling the synthesis, bacterial uptake and catabolism of tryptophan and/or tryptophan metabolites. and provides methods for using these compositions in the treatment, management and/or prevention of a number of different diseases.

Other Indole Tryptophan Metabolites

[0144] In addition to kynurenine and KYNA, numerous compounds have been proposed as endogenous AHR ligands, many of which are generated through pathways involved in the metabolism of tryptophan and indole (Bittinger et al., 2003; Chung and Gadupudi, 2011) A large number of metabolites generated through the tryptophan indole pathway are generated by microbiota in the gut. For example, bacteria take up tryptophan, which .can be converted to mono-substituted indole compounds, such as indole acetic acid (IAA) and tryptamine, and other compounds, which have been found to activate the AHR (Hubbard et al., 2015, Adaptation of the human aryl hydrocarbon receptor to sense microbiota-derived indoles; Nature Scientific Reoports 5:12689).

[0145] In the gastronintestinal tract, diet derived AhR ligands promote Il-22 production by innate lymphoid cells, referred to as group 3 ILCs (Spits *et al.*, 2013, Zelante *et al.*, *Tryptophan Catabolites from Microbiota Engage Aryl Hydrocarbon Receptor and Balance Mucosal Reactivity via Interleukin-22*; Immunity 39, 372–385, August 22, 2013). Ahr is essential for IL-22-production in the intestinal lamina propria (Lee *et al.*, Nature Immunology 13, 144–151 (2012); Ahr drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch.

[0146] **Table 12** lists exemplary tryptophan metabolites which have been shown to bind to AHR and which can be produced by the genetically engineered bacteria of the disclosure.Thus, in some embodiments, the engineered bacteria comprises gene sequence(s) encoding one or more enzymes for the production of one or more metabolites listed in Table 13.

Table 12. Indole Tryptophan Metabolites

Origin	Compound
Exogenous	2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
Dietary	Indole-3-carbinol (I3C)
Dietary	Indole-3-acetonitrile (I3ACN)
Dietary	3.3'-Diindolylmethane (DIM)
Dietary	2-(indol-3-ylmethyl)-3.3'-diindolylmethane (Ltr-1)
Dietary	Indolo(3,2-b)carbazole (ICZ)
Dietary	2-(1'H-indole-3'-carbony)-thiazole-4-carboxylic acid methyl ester (ITE)
Microbial	Indole
Microbial	Indole-3-acetic acid (IAA)
Microbial	Indole-3-aldehyde (IAId)
Microbial	Tryptamine
Microbial	3-methyl-indole (Skatole)
Yeast	Tryptanthrin
Microbial/Host Metabolism	Indigo
Microbial/Host Metabolism	Indirubin
Microbial/Host Metabolism	Indoxyl-3-sulfate (I3S)
Host Metabolism	Kynurenine (Kyn)
Host Metabolism	Kynurenic acid (KA)
Host Metabolism	Xanthurenic acid
Host Metabolism	Cinnabarinic acid (CA)
UV-Light Oxidation	6-formylindolo(3,2-b)carbazole (FICZ)

[0147] Through initiation of Jak-STAT signaling pathways, IL-22 expression can trigger expression of antimicrobial compounds as well as a range of cell growth related pathways, both of which enhance tissue repair mechanisms. IL-22 is critical in promoting

intestinal barrier fidelity and healing, while modulating inflammatory states. Murine models have demonstrated improved intestinal inflammation states following administration of IL-22. Additionally, IL-22 activates STAT3 signaling to promote enhanced mucus production to preserve barrier function.

[0148] Additionally, indole metabolites have been suggested to be beneficial in the treatment of metabolic disease, such as type2 diabetes or liver disease, e.g., NASH or NAFLD. For example, in addition to its enhancement of the gut barrier function, indole has been found to promote GLP-1 secretion by intestinal enteroendocrine cells, i.e, indole inhibits voltage-gated K+ channels, and changes the action potential properties of L cells, ultimately triggering GLP-1 secretion (Chimerel C, et a., (2014) Bacterial metabolite indole modulates incretin secretion from intestinal enteroendocrine L cells. Cell Rep 9:1202–1208).

[0149] In addition, some indole metabolites may exert their effect through Pregnane X receptor (PXR), which is thought to play a key role as an essential regulator of intestinal barrier function. PXR-deficient (Nr1i2-/-) mice showed a distinctly ‘‘leaky’’gut physiology coupled with upregulation of the Toll-like receptor 4 (TLR4), a receptor well known for recognizing LPS and activating the innate immune system (Venkatesh et al., 2014 Symbiotic Bacterial Metabolites Regulate Gastrointestinal Barrier Function via the Xenobiotic Sensor PXR and Toll-like Receptor 4; Immunity 41, 296–310, August 21, 2014). In particular, indole 3-propionic acid (IPA), produced by microbiota in the gut, has been shown to be a ligand for PXR *in vivo*.

[0150] As a result, indole levels *e.g.*, produced by commensal bacteria, or by genetically engineered bacteria, may through the activation of PXR regulate and balance the levels of TLR4 expression to promote homeostasis and gut barrier health. Ie., low levels of IPA and/or PXR and an excess of TLR4 may lead to intestinaly barrier dysfunction, while increasing levels of IPA may promote PXR activation and TLR4 downregulation, and improved gut barrier health.

Methoxyindole pathway, Serotonin and Melatonin

[0151] The methoxyindole pathway leads to formation of serotonin (5-HT) and melatonin. Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic amine synthesized in a two-step enzymatic reaction: First, enzymes encoded by one of two tryptophan hydroxylase genes (Tph1 or Tph2) catalyze the rate-limiting conversion of tryptophan to 5-hydroxytryptophan (5-HTP), thus allocating the bioactivity of serotonin into either the brain (Tph2) or the periphery (Tph1). Then, 5-HTP undergoes decarboxylation to serotonin. Intestinal serotonin (5-hydroxytryptamine, 5-HT) is released by enterochromaffin cells and neurons and is

regulated via the serotonin re-uptake transporter (SERT). The SERT is located on epithelial cells and neurons in the intestine. In certain embodiments, the genetically engineered bacteria described herein may modulate serotonin levels in the intestine, *e.g.*, decrease serotonin levels.

[0152] 5-HT also functions a substrate for melatonin biosynthesis. The rate-limiting step of melatonin biosynthesis is 5-HT-N-acetylation resulting in the formation of N-acetyl-serotonin (NAS) with subsequent Omethylation into 5-methoxy-N-acetyltryptamine (melatonin). The deficient production of 5-HT, NAS, and melatonin contribute to depressed mood, disturbances of sleep and circadian rhythms. Melatonin acts as a neurohormone and is associated with the development of circadian rhythm and the sleep-wake cycle.

[0153] In certain embodiments, the genetically engineered bacteria influence 5-HT synthesis, release, and/or degradation. Gut microbiota are interconnected with serotonin signaling and care capable of increasing serotonin levels through host serotonin production (Jano *et al.*, Cell. 2015 Apr 9;161(2):264-76. doi: 10.1016/j.cell.2015.02.047. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis). In some embodiments, the genetically engineered bacteria may modulate the serotonin levels in the gut to ameliorate symptoms of a metabolic disease, e.g., liver inflammation and damage as seen in e.g., NASH and NAFLD. In some embodimetns, the genetically engineered bacteria take up serotonin from the environment, *e.g.*, the gut. In a non limiting example, serotonin can be converted to melatonin by, *e.g.*, , tryptophan hydroxylase (TPH), hydroxyl-O-methyltransferase (HIOMT), N-acetyltransferase (NAT), aromatic –amino acid decarboxylase (AAAD), or bacterial equivalents thereof. In some embodiments, the genetically engineered influence serotonin levels produced by the host.

[0154] In bacteria, melatonin is synthesized indirectly with tryptophan as an intermediate product of the shikimic acid pathway. In these cells, synthesis starts with d-erythrose-4-phosphate and phosphoenolpyruvate. In some embodiments the genetically engineered bacteria comprise an endogenous or exogenous cassette for the production of melatonin. As anon-limiting example, one pathway or cassette is described in Bochkov, Denis V.; Sysolyatin, Sergey V.; Kalashnikov, Alexander I.; Surmacheva, Irina A. (2011). "Shikimic acid: review of its analytical, isolation, and purification techniques from plant and microbial sources". Journal of Chemical Biology 5 (1): 5–17. doi:10.1007/s12154-011-0064-8.

Exemplary Tryptophan and Tryptophan Metabolite Circuits

Decreasing Exogenous Tryptophan

[0155] In some embodiments, the genetically engineered bacteria are capable of decreasing the level of tryptophan and/or the level of a tryptophan metabolite. In some embodiments, the engineered bacteria comprise gene sequence(s) for encoding one or more aromatic amino acid transporter(s). In one embodiment, the amino acid transporter is a tryptophan transporter. Tryptophan transporters may be expressed or modified in the recombinant bacteria described herein in order to enhance tryptophan transport into the cell. Specifically, when the tryptophan transporter is expressed in the recombinant bacterial cells described herein, the bacterial cells import more tryptophan into the cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the genetically engineered bacteria comprising a heterologous gene encoding a tryptophan transporter which may be used to import tryptophan into the bacteria.

[0156] The uptake of tryptophan into bacterial cells is mediated by proteins well known to those of skill in the art. For example, three different tryptophan transporters, distinguishable on the basis of their affinity for tryptophan have been identified in E. coli (see, e.g., Yanofsky et al. (1991) J. Bacteriol. 173: 6009-17). The bacterial genes mtr, aroP, and tnaB encode tryptophan permeases responsible for tryptophan uptake in bacteria. High affinity permease, Mtr, is negatively regulated by the trp repressor and positively regulated by the TyR product (see, e.g., Yanofsky et al. (1991) J. Bacteriol. 173: 6009-17 and Heatwole, et al. (1991) J. Bacteriol. 173: 3601-04), while AroP is negatively regulated by the tyR product (Chye et al. (1987) J. Bacteriol. 169:386-93).

[0157] In some embodiments, the engineered bacteria comprise gene sequence(s) for encoding one or more aromatic amino acid transporter(s). In one embodiment, the amino acid transporter is a tryptophan transporter. In one embodiment, the at least one gene encoding a tryptophan transporter is a gene selected from the group consisting of mtr, aroP and tnaB. In one embodiment, the bacterial cell described herein has been genetically engineered to comprise at least one heterologous gene selected from the group consisting of mtr, aroP and tnaB. In one embodiment, the at least one gene encoding a tryptophan transporter is the Escherichia coli mtr gene. In one embodiment, the at least one gene encoding a tryptophan transporter is the Escherichia coli aroP gene. In one embodiment, the at least one gene encoding a tryptophan transporter is the Escherichia coli tnaB gene.

[0158] In some embodiments, the tryptophan transporter is encoded by a tryptophan transporter gene derived from a bacterial genus or species, including but not limited to,

Escherichia, Corynebacterium, Escherichia coli, Saccharomyces cerevisiae or Corynebacterium glutamicum. In some embodiments, the bacterial species is Escherichia coli. In some embodiments, the bacterial species is Escherichia coli strain Nissle.

[0159] Assays for testing the activity of a tryptophan transporter, a functional variant of a tryptophan transporter, or a functional fragment of transporter of tryptophan are well known to one of ordinary skill in the art. For example, import of tryptophan may be determined using the methods as described in Shang et al. (2013) J. Bacteriol. 195:5334-42, the entire contents of each of which are expressly incorporated by reference herein.

[0160] In one embodiment, when the tryptophan transporter is expressed in the recombinant bacterial cells described herein, the bacterial cells import 10% more tryptophan into the bacterial cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In another embodiment, when the tryptophan transporter is expressed in the recombinant bacterial cells described herein, the bacterial cells import 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% more tryptophan into the bacterial cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the tryptophan transporter is expressed in the recombinant bacterial cells described herein, the bacterial cells import two-fold more tryptophan into the cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the tryptophan transporter is expressed in the recombinant bacterial cells described herein, the bacterial cells import three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more tryptophan into the cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions.

[0161] In addition to the tryptophan uptake transporters, in some embodiments, the genetically engineered bacteria further comprise a circuit for the production of tryptophan metabolites, as described herein, e.g., for the production of kynurenine, kynurenine metabolites, or indole tryptophan metabolites as shown in **Table 12, FIG. 16 and 17.**

[0162] In some embodiments, the genetically engineered bacteria are capable of decreasing the level of tryptophan. In some embodiments, the engineered bacteria comprises one or more gene sequences for converting tryptophan to kynurenine. In some embodiments, the engineered bacteria comprise gene sequence(s) for encoding the enzyme indoleamine 2,3-dioxygenase (IDO-1). In some embodiments, the engineered bacteria comprises gene sequence(s) for encoding the enzyme tryptophan dioxygenase (TDO). In some embodiments,

the engineered bacteria comprise gene sequence(s) for encoding the enzyme indoleamine 2,3-dioxygenase (IDO-1) and the enzyme tryptophan dioxygenase (TDO). In some embodiments, the genetically engineered bacteria comprise a gene cassette encoding Indoleamine 2, 3 dioxygenase (EC 1.13.11.52; producing N-formyl kynurenine from tryptophan) and Kynurenine formamidase (EC3.5.1.9) producing kynurenine from n-formylkynurenine). In some embodiments, the enzymes are bacterially derived, e.g., as described in Vujkovi-Cvijin et al. 2013.

[0163] In some embodiments, the genetically engineered bacteria are capable of decreasing the level of tryptophan, e.g., in combination with the production of indole metabolites, through expression of gene(s) and gene cassette(s) described herein. In some embodiments, expression of the gene sequences(s) is driven by an inducible promoter, described in more detail herein. In some embodiments, the expression of the gene sequences(s) is driven by a constitutive promoter.

Increasing Kynurenine

[0164] In some embodiments, the genetically engineered bacteria are capable of producing kynurenine.

[0165] In some embodiments, the genetically engineered bacteria are capable of decreasing the level of tryptophan. In some embodiments, the engineered bacteria comprise one or more gene sequences for converting tryptophan to kynurenine. In some embodiments, the engineered bacteria comprise gene sequence(s) for encoding the enzyme indoleamine 2,3-dioxygenase (IDO-1). In some embodiments, the engineered bacteria comprise gene sequence(s) for encoding the enzyme tryptophan dioxygenase (TDO). In some embodiments, the engineered bacteria comprise on or more gene sequence(s) for encoding the enzyme indoleamine 2,3-dioxygenase (IDO-1) and the enzyme tryptophan dioxygenase (TDO). In some embodiments, the genetically engineered bacteria comprise a gene cassette encoding Indoleamine 2, 3 dioxygenase (EC 1.13.11.52; producing N-formyl kynurenine from tryptophan) and Kynurenine formamidase (EC3.5.1.9) producing kynurenine from n-formylkynurenine). In some embodiments, the enzymes are bacterially derived, e.g., as described in Vujkovi-Cvijin et al. 2013.

[0166] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce kynurenine from tryptophan. Non-limiting example of such gene sequence(s) are shown the figures and described elsewhere herein. In one embodiment, the genetically engineered

bacteria comprise one or more gene sequence(s) which encode IDO1(indoleamine 2,3-dioxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode IDO1 from homo sapiens. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode TDO2 (tryptophan 2,3-dioxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode TDO2 from homo sapiens. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 (indoleamine 2,3-dioxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 from S. cerevisiae). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid: Kynurenine formamidase. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid: Kynurenine formamidase from mouse. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid in combination with one or more of ido1 and/or tdo2 and/or bna2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid in combination with ido1. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with tdo2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid in combination with bna2.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA3 (kynurenine--oxoglutarate transaminase. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA3 from S. cerevisae. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with one or more of ido1 and/or tdo2 and/or bna2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with ido1. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with tdo2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with bna2.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of ido1 and/or tdo2 and/or bna2.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of afmid and/or bna3.

[0167] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of ido1 and/or tdo2 and/or bna2, in combination with one or more of afmid and/or bna3.

[0168] In any of these embodiments, the genetically engineered bacteria which produce kynurenine from tryptophan also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in the figures and described elsewhere herein. In some embodiments, the genetically engineered bacteria which produce kynurenine from tryptophan also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria which produce kynurenine from tryptophan also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0169] The genetically engineered bacteria may comprise any suitable gene for producing kynurenine. In some embodiments, the gene for producing kynurenine is modified and/or mutated, e.g., to enhance stability, increase kynurenine production, and/or increase anti-inflammatory potency under inducing conditions. In some embodiments, the engineered bacteria also have enhanced uptake or import of tryptophan, e.g., comprise a transporter or other mechanism for increasing the uptake of tryptophan into the bacterial cell, as discussed in detail above. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose and others described herein. In some embodiments, the gene sequences(s) are controlled by an inducible promoter. In some embodiments, the gene sequences(s) are controlled by a constitutive promoter. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constitutive promoter, and are expressed during bacterial culture in vitro, e.g., for bacterial expansion, production and/or manufacture, as described herein.

[0170] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) for the consumption of tryptophan and production of kynurenine, which are bacterially derived. In some embodiments, the enzymes for TRP to

KYN conversion are derived from one or more of Pseudomonas, Xanthomonas, Burkholderia, Stenotrophomonas, Shewanella, and Bacillus, and/or members of the families Rhodobacteraceae, Micrococcaceae, and Halomonadaceae, In some embodiments the enzymes are derived from the species listed in table S7 of Vujkovic-Cvijin et al. (Dysbiosis of the gut microbiota is associated with HIV diseaseprogression and tryptophan catabolism Sci Transl Med. 2013 July 10; 5(193): 193ra91), the contents of which is herein incorporated by reference in its entirety.

[0171] In some embodiments, the one or more genes for producing kynurenine are modified and/or mutated, e.g., to enhance stability, increase kynurenine production, and/or increase anti-inflammatory potency under inducing conditions. In some embodiments, the engineered bacteria have enhanced uptake or import of tryptophan, e.g., comprise a transporter or other mechanism for increasing the uptake of tryptophan into the bacterial cell. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose and others described herein.

[0172] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria are capable of expressing any one or more of the described circuits in low-oxygen conditions, in the presence of disease or tissue specific molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response or immune suppression, liver damage, or metabolic disease, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose and others described herein. In some embodiments, any one or more of the described circuits are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the bacterial chromosome. Also, in some embodiments, the genetically engineered bacteria are further capable of expressing any one or more of the described circuits and further comprise one or more of the following: (1) one or more auxotrophies, such as any auxotrophies known in the art and provided herein, e.g., thyA auxotrophy, (2) one or more kill switch circuits, such as any of the kill-switches described herein or otherwise known in the art, (3) one or more antibiotic resistance circuits, (4) one or more transporters for importing biological molecules or substrates, such any of the

transporters described herein or otherwise known in the art, (5) one or more secretion circuits, such as any of the secretion circuits described herein and otherwise known in the art, and (6) combinations of one or more of such additional circuits.

Increasing Tryptophan

[0173] In some embodiments, the genetically engineered microorganisms of the present disclosure are capable of producing tryptophan. Exemplary circuits for the production of tryptophan are shown in the figures.

[0174] In some embodiments, the genetically engineered bacteria that produce tryptophan comprise one or more gene sequences encoding one or more enzymes of the tryptophan biosynthetic pathway. In some embodiments, the genetically engineered bacteria comprise a tryptophan operon. In some embodiments, the genetically engineered bacteria comprise the tryptophan operon of E. coli. (Yanofsky, RNA (2007), 13:1141-1154). In some embodiments, the genetically engineered bacteria comprise the tryptophan operon of B. subtilis. (Yanofsky, RNA (2007), 13:1141-1154). In some embodiments, the genetically engineered bacteria comprise sequence(s) encoding trypE, trypG-D, trypC-F, trypB, and trpA genes. In some embodiments, the genetically engineered bacteria comprise sequence(s) encoding trypE, trypG-D, trypC-F, trypB, and trpA genes from E. Coli. In some embodiments, the genetically engineered bacteria comprise sequence(s) encoding trypE, trypD, trypC, trypF, trypB, and trpA genes from B. subtilis.

[0175] Also, in any of these embodiments, the genetically engineered bacteria optionally comprise gene sequence(s) to produce the tryptophan precursor, chorismate. Thus, in some embodiments, the genetically engineered bacteria optionally comprise sequence(s) encoding aroG, aroF, aroH, aroB, aroD, aroE, aroK, and AroC. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding one or more enzymes of the tryptophan biosynthetic pathway and one or more gene sequences encoding one or more enzymes of the chorismate biosynthetic pathway. In some embodiments, the genetically engineered bacteria comprise sequence(s) encoding trypE, trypG-D, trypC-F, trypB, and trpA genes from E. Coli and sequence(s) encoding aroG, aroF, aroH, aroB, aroD, aroE, aroK, and AroC genes. In some embodiments, the genetically engineered bacteria comprise sequence(s) encoding trypE, trypD, trypC, trypF, trypB, and trpA genes from B. subtilis and sequence(s) encoding aroG, aroF, aroH, aroB, aroD, aroE, aroK, and AroC genes.

[0176] In some embodiments, the genetically engineered bacteria comprise sequence(s) encoding either a wild type or a feedback resistant SerA gene (**Table 10**).

Escherichia coli serA-encoded 3-phosphoglycerate (3PG) dehydrogenase catalyzes the first step of the major phosphorylated pathway of L-serine (Ser) biosynthesis. This step is an oxidation of 3PG to 3-phosphohydroxypyruvate (3PHP) with the concomitant reduction of NAD+ to NADH. As part of Tryptophan biosynthesis, E. coli uses one serine for each tryptophan produced. As a result, by expressing serA, tryptophan production is improved.

[0177] In any of these embodiments, AroG and TrpE are optionally replaced with feedback resistant versions to improve tryptophan production (**Table 10**

[0178] In any of these embodiments, the tryptophan repressor (trpR) optionally may be deleted, mutated, or modified so as to diminish or obliterate its repressor function.

[0179] In any of these embodiments the tnaA gene (encoding a tryptophanase converting Trp into indole) optionally may be deleted to prevent tryptophan catabolism along this pathway and to further increase levels of tryptophan produced (**Table 10**.

[0180] The inner membrane protein YddG of Escherichia coli, encoded by the yddG gene, is a homologue of the known amino acid exporters RhtA and YdeD. Studies have shown that YddG is capable of exporting aromatic amino acids, including tryptophan. Thus, YddG can function as a tryptophan exporter or a tryptophan secretion system (or tryptophan secretion protein). Other aromatic amino acid exporters are described in Doroshenko *et al.*, FEMS Microbial Lett., 275:312-318 (2007). Thus, in some embodiments, the engineered bacteria optionally further comprise gene sequence(s) encoding YddG. In some embodiments, the engineered bacteria can over-express YddG. In some embodiments, the engineered bacteria optionally comprise one or more copies of yddG gene.

[0181] In some embodiments, the genetically engineered bacterium or genetically engineered microorganism comprises one or more genes for producing tryptophan, under the control of a promoter that is activated by low-oxygen conditions, by inflammatory conditions, liver damage, and.or metabolic disease, such as any of the promoters activated by said conditions and described herein. In some embodiments, the genetically engineered bacteria expresses one or more genes for producing tryptophan. In some embodiments, the gene sequences(s) are controlled by an inducible promoter. In some embodiments, the gene sequences(s) are controlled by a constitutive promoter. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constitutive promoter, and are expressed during bacterial culture in vitro, e.g., for bacterial expansion, production and/or manufacture, as described herein.

Table 13. Tyrptophan Synthesis Cassette Sequences

Description	Sequence
Tet-regulated Tryptophan operon **SEQ ID NO: 121**	taagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataagaaggctggctct gcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttct tctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatata atgcattctctagtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtagg ccgtgtacctaaatgtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaa aaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcg tcgagcaaagcccgcttatttttttacatgccaatacaatgtaggctgctctacacctagcttctgggcgagtttacg ggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttacttttatctaatctagaca tcattaattcctaatttttgttgacactctatcattgatagagttatttaccactccctatcagtgatagagaaaagtg aactctagaaataattttgtttaactttaagaaggagatatacatatgcaaacacaaaaaccgactctcgaactgct aacctgcgaaggcgcttatcgcgacaacccgactgcgcttttcaccagttgtgtggggatcgtccggcaacg ctgctgctggaatccgcagatatcgacagcaaagatgatttaaaaagcctgctgctggtagacagtgcgctgc gcattacagcattaagtgacactgtcacaatccaggcgctttccggcaatggagaagccctgttgacactactg gataacgccttgcctgcgggtgtggaaaatgaacaatcaccaaactgccgcgtactgcgcttcccgcctgtca gtccactgctggatgaagacgcccgcttatgctccctttcggtttttgacgctttccgcttattacagaatctgttga atgtaccgaaggaagaacgagaagcaatgttcttcggcggcctgttctcttatgaccttgtggcgggatttgaaa atttaccgcaactgtcagcggaaaatagctgccctgatttctgtttttatctcgctgaaacgctgatggtgattgac catcagaaaaaaagcactcgtattcaggccagcctgtttgctccgaatgaagaagaaaaacaacgtctcactgc tcgcctgaacgaactacgtcagcaactgaccgaagccgcgccgccgctgccggtggtttccgtgccgcatat gcgttgtgaatgtaaccagagcgatgaagagttcggtggtgtagtgcgtttgttgcaaaaagcgattcgcgccg gagaaattttccaggtggtgccatctcgccgtttctctctgccctgcccgtcaccgctggcagcctattacgtgct gaaaaagagtaatcccagcccgtacatgttttttatgcaggataatgatttcaccctgtttggcgcgtcgccggaa agttcgctcaagtatgacgccaccagccgccagattgagatttacccgattgccggaacacgtccacgcggtc gtcgtgccgatggttcgctggacagagacctcgacagccgcatcgaactggagatgcgtaccgatcataaag agctttctgaacatctgatgctggtggatctcgcccgtaatgacctggcacgcatttgcacacccggcagccgc tacgtcgccgatctcaccaaagttgaccgttactcttacgtgatgcacctagtctcccgcgttgttggtgagctgc gccacgatctcgacgccctgcacgcttaccgcgcctgtatgaatatggggacgttaagcggtgcaccgaaagt acgcgctatgcagttaattgccgaagcagaaggtcgtcgacgcggcagctacggcggcgcggtaggttatttt accgcgcatggcgatctcgacacctgcattgtgatccgctcggcgctggtggaaaacggtatcgccaccgtgc aagccggtgctggcgtagtccttgattctgttccgcagtcggaagccgacgaaactcgtaataaagcccgcgc tgtactgcgcgctattgccaccgcgcatcatgcacaggagacgttctaatggctgacattctgctgctcgataat atcgactcttttacgtacaacctggcagatcagttgcgcagcaatggtcataacgtggtgatttaccgcaaccata ttccggcgcagaccttaattgaacgcctggcgacgatgagcaatccggtgctgatgctttctcctggccccggt gtgccgagcgaagccggttgtatgccggaactcctcacccgcttgcgtggcaagctgccaattattggcatttg cctcggacatcaggcgattgtcgaagcttacgggggctatgtcggtcaggcgggcgaaattcttcacggtaaa gcgtcgagcattgaacatgacggtcaggcgatgtttgccggattaacaaacccgctgccagtggcgcgttatc actcgctggttggcagtaacattccggccggtttaaccatcaacgcccattttaatggcatggtgatggcggtgc gtcacgatgcagatcgcgtttgtggattccagttccatccggaatccattcttactacccagggcgctcgcctgct ggaacaaacgctggcctgggcgcagcagaaactagagccaaccaacacgctgcaaccgattctggaaaaa ctgtatcaggcacagacgcttagccaacaagaaagccaccagctgttttcagcggtggtacgtggcgagctga agccggaacaactggcggcggcgctggtgagcatgaaaattcgcggtgaacacccgaacgagatcgccgg ggcagcaaccgcgctactggaaaacgccgcgccattcccgcgcccggattatctgtttgccgatatcgtcggt actggcggtgacggcagcaacagcatcaatatttctaccgccagtgcgtttgtcgccgcggcctgcgggctga aagtggcgaaacacggcaaccgtagcgtctccagtaaatccggctcgtcggatctgctggcggcgttcggtat taatcttgatatgaacgccgataaatcgcgccaggcgctggatgagttaggcgtctgtttcctctttgcgccgaa gtatcacaccggattccgccatgcgatgccggttcgccagcaactgaaaaccccgcactctgttcaacgtgctg ggaccattgattaacccggcgcatccgccgctggcgctaattggtgtttatagtccggaactggtgctgccgatt

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Tet repressor **SEQ ID NO:122**	taagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataagaaggctggctct gcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttct tctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatata atgcattctctagtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtagg ccgtgtacctaaatgtacttttgctccatcgcgatgacttagtaaagcacatctaaaactttagcgttattacgtaa aaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcg tcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctctacacctagcttctgggcgagtttacg ggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttacttttatctaatctagaca t
tetR/tetA promoters and RBS and leader region **SEQ ID NO 123:**	cattaattcctaatttttgttgacactctatcattgatagagttattttaccactccctatcagtgatagagaaaagtga actctagaaataattttgtttaactttaagaaggagatatacat
trpE **SEQ ID NO: 124**	atgcaaacacaaaaaccgactctcgaactgctaacctgcgaaggcgcttatcgcgacaacccgactgcgctttt tcaccagttgtgtggggatcgtccggcaacgctgctgctggaatccgcagatatcgacagcaaagatgatttaa aaagcctgctgctggtagacagtgcgctgcgcattacagcattaagtgacactgtcacaatccaggcgctttcc ggcaatggagaagccctgttgacactactggataacgccttgcctgcgggtgtggaaaatgaacaatcaccaa actgccgcgtactgcgcttcccgcctgtcagtccactgctggatgaagacgcccgcttatgctccctttcggtttt tgacgctttccgcttattacagaatctgttgaatgtaccgaaggaagaacgagaagcaatgttcttcggcggcct gttctcttatgaccttgtggcgggatttgaaaatttaccgcaactgtcagcggaaaatagctgccctgatttctgttt ttatctcgctgaaacgctgatggtgattgaccatcagaaaaaaagcactcgtattcaggccagcctgtttgctcc gaatgaagaagaaaaacaacgtctcactgctcgcctgaacgaactacgtcagcaactgaccgaagccgcgc cgccgctgccggtggtttccgtgccgcatatgcgttgtgaatgtaaccagagcgatgaagagttcggtggtgta gtgcgtttgttgcaaaaagcgattcgcgccggagaaattttccaggtggtgccatctcgccgtttctctctgccct gcccgtcaccgctggcagcctattacgtgctgaaaaagagtaatcccagcccgtacatgttttttatgcaggata atgatttcaccctgtttggcgcgtcgccggaaagttcgctcaagtatgacgccaccagccgccagattgagattt acccgattgccggaacacgtccacgcggtcgtcgtgccgatggttcgctggacagagacctcgacagccgc atcgaactggagatgcgtaccgatcataaagagctttctgaacatctgatgctggtggatctcgcccgtaatgac ctggcacgcatttgcacacccggcagccgctacgtcgccgatctcaccaaagttgaccgttactcttacgtgat gcacctagtctcccgcgttgttggtgagctgcgccacgatctcgacgccctgcacgcttaccgcgcctgtatga atatggggacgttaagcggtgcaccgaaagtacgcgctatgcagttaattgccgaagcagaaggtcgtcgac gcggcagctacggcggcgcggtaggttattttaccgcgcatggcgatctcgacacctgcattgtgatccgctc ggcgctggtggaaaacggtatcgccaccgtgcaagccggtgctggcgtagtccttgattctgttccgcagtcg gaagccgacgaaactcgtaataaagcccgcgctgtactgcgcgctattgccaccgcgcatcatgcacaggag acgttcta
TrpE **SEQ ID NO: 125**	MQTQKPTLELLTCEGAYRDNPTALFHQLCGDRPATLLLESADIDSKD DLKSLLLVDSALRITALSDTVTIQALSGNGEALLTLLDNALPAGVENE QSPNCRVLRFPPVSPLLDEDARLCSLSVFDAFRLLQNLLNVPKEEREA MFFGGLFSYDLVAGFENLPQLSAENSCPDFCFYLAETLMVIDHQKKST RIQASLFAPNEEEKQRLTARLNELRQQLTEAAPPLPVVSVPHMRCECN

	QSDEEFGGVVRLLQKAIRAGEIFQVVPSRRFSLPCPSPLAAYYVLKKS NPSPYMFFMQDNDFTLFGASPESSLKYDATSRQIEIYPIAGTRPRGRRA DGSLDRDLDSRIELEMRTDHKELSEHLMLVDLARNDLARICTPGSRY VADLTKVDRYSYVMHLVSRVVGELRHDLDALHAYRACMNMGTLSG APKVRAMQLIAEAEGRRRGSYGGAVGYFTAHGDLDTCIVIRSALVEN GIATVQAGAGVVLDSVPQSEADETRNKARAVLRAIATAHHAQETF
trpD **SEQ ID NO: 126**	atggctgacattctgctgctcgataatatcgactcttttacgtacaacctggcagatcagttgcgcagcaatggtc ataacgtggtgatttaccgcaaccatattccggcgcagaccttaattgaacgcctggcgacgatgagcaatccg gtgctgatgctttctcctggccccggtgtgccgagcgaagccggttgtatgccggaactcctcacccgcttgcg tggcaagctgccaattattggcatttgcctcggacatcaggcgattgtcgaagcttacgggggctatgtcggtca ggcgggcgaaattcttcacggtaaagcgtcgagcattgaacatgacggtcaggcgatgtttgccggattaaca aacccgctgccagtggcgcgttatcactcgctggttggcagtaacattccggccggtttaaccatcaacgccca ttttaatggcatggtgatggcggtgcgtcacgatgcagatcgcgtttgtggattccagttccatccggaatccatt cttactacccagggcgctcgcctgctggaacaaacgctggcctgggcgcagcagaaactagagccaaccaa cacgctgcaaccgattctggaaaaactgtatcaggcacagacgcttagccaacaagaaagccaccagctgttt tcagcggtggtacgtggcgagctgaagccggaacaactggcggcggcgctggtgagcatgaaaattcgcgg tgaacacccgaacgagatcgccggggcagcaaccgcgctactggaaaacgccgcgccattcccgcgcccg gattatctgtttgccgatatcgtcggtactggcggtgacggcagcaacagcatcaatatttctaccgccagtgcg tttgtcgccgcggcctgcgggctgaaagtggcgaaacacggcaaccgtagcgtctccagtaaatccggctcg tcggatctgctggcggcgttcggtattaatcttgatatgaacgccgataaatcgcgccaggcgctggatgagtta ggcgtctgtttcctctttgcgccgaagtatcacaccggattccgccatgcgatgccggttcgccagcaactgaa aacccgcactctgttcaacgtgctgggaccattgattaacccggcgcatccgccgctggcgctaattggtgttta tagtccggaactggtgctgccgattgccgaaaccttgcgcgtgctggggtatcaacgcgcggcagtggtgca cagcggcgggatggatgaagtttcattacacgcgccgacaatcgttgccgaactacatgacggcgaaattaag agctatcaattgaccgctgaagattttggcctgacaccctaccaccaggagcaattggcaggcggaacaccgg aagaaaaccgtgacatttttaacacgcttgttacaaggtaaaggcgacgccgcccatgaagcagccgtcgcgg cgaatgtcgccatgttaatgcgcctgcatggccatgaagatctgcaagccaatgcgcaaaccgttcttgaggta ctgcgcagtggttccgcttacgacagagtcaccgcactggcggcacgagggtaa
TrpD **SEQ ID NO: 127**	MADILLLDNIDSFTYNLADQLRSNGHNVVIYRNHIPAQTLIERLATMS NPVLMLSPGPGVPSEAGCMPELLTRLRGKLPIIGICLGHQAIVEAYGG YVGQAGEILHGKASSIEHDGQAMFAGLTNPLPVARYHSLVGSNIPAG LTINAHFNGMVMAVRHDADRVCGFQFHPESILTTQGARLLEQTLAW AQQKLEPTNTLQPILEKLYQAQTLSQQESHQLFSAVVRGELKPEQLAA ALVSMKIRGEHPNEIAGAATALLENAAPFPRPDYLFADIVGTGGDGSN SINISTASAFVAAACGLKVAKHGNRSVSSKSGSSDLLAAFGINLDMNA DKSRQALDELGVCFLFAPKYHTGFRHAMPVRQQLKTRTLFNVLGPLI NPAHPPLALIGVYSPELVLPIAETLRVLGYQRAAVVHSGGMDEVSLH APTIVAELHDGEIKSYQLTAEDFGLTPYHQEQLAGGTPEENRDILTRLL QGKGDAAHEAAVAANVAMLMRLHGHEDLQANAQTVLEVLRSGSA YDRVTALAARG
trpC **SEQ ID NO: 128**	atgcaaaccgttttagcgaaaatcgtcgcagacaaggcgatttgggtagaaacccgcaaagagcagcaaccg ctggccagttttcagaatgaggttcagccgagcacgcgacatttttatgatgcacttcagggcgcacgcacggc gtttattctggagtgtaaaaaagcgtcgccgtcaaaaggcgtgatccgtgatgatttcgatccggcacgcattgc cgccatttataaacattacgcttcggcaatttcagtgctgactgatgagaaatattttcaggggagctttgatttcct ccccatcgtcagccaaatcgccccgcagccgattttatgtaaagacttcattatcgatccttaccagatctatctg gcgcgctattaccaggccgatgcctgcttattaatgctttcagtactggatgacgaacaatatcgccagcttgca gccgtcgcccacagtctggagatgggtgtgctgaccgaagtcagtaatgaagaggaactggagcgcgccatt gcattgggggcaaaggtcgttggcatcaacaaccgcgatctgcgcgatttgtcgattgatctcaaccgtacccg cgagcttgcgccgaaactggggcacaacgtgacggtaatcagcgaatccggcatcaatacttacgctcaggt

	gcgcgagttaagccacttcgctaacggctttctgattggttcggcgttgatggcccatgacgatttgaacgccgc cgtgcgtcgggtgttgctgggtgagaataaagtatgtggcctgacacgtgggcaagatgctaaagcagcttat gacgcgggcgcgatttacggtgggttgatttttgttgcgacatcaccgcgttgcgtcaacgttgaacaggcgca ggaagtgatggctgcagcaccgttgcagtatgttggcgtgttccgcaatcacgatattgccgatgtggcggaca aagctaaggtgttatcgctggcggcagtgcaactgcatggtaatgaagatcagctgtatatcgacaatctgcgt gaggctctgccagcacacgtcgccatctggaaggctttaagtgtcggtgaaactcttcccgcgcgcgattttca gcacatcgataaatatgtattcgacaacggtcagggcgggagcggacaacgtttcgactggtcactattaaatg gtcaatcgcttggcaacgttctgctggcgggggcttaggcgcagataactgcgtggaagcggcacaaaccg gctgcgccgggcttgattttaattctgctgtagagtcgcaaccgggtatcaaagacgcacgtcttttggcctcggt tttccagacgctgcgcgcatattaa
TrpC **SEQ ID NO: 129**	MQTVLAKIVADKAIWVETRKEQQPLASFQNEVQPSTRHFYDALQGA RTAFILECKKASPSKGVIRDDFDPARIAAIYKHYASAISVLTDEKYFQG SFDFLPIVSQIAPQPILCKDFIIDPYQIYLARYYQADACLLMLSVLDDEQ YRQLAAVAHSLEMGVLTEVSNEEELERAIALGAKVVGINNRDLRDLS IDLNRTRELAPKLGHNVTVISESGINTYAQVRELSHFANGFLIGSALM AHDDLNAAVRRVLLGENKVCGLTRGQDAKAAYDAGAIYGGLIFVAT SPRCVNVEQAQEVMAAAPLQYVGVFRNHDIADVADKAKVLSLAAV QLHGNEDQLYIDNLREALPAHVAIWKALSVGETLPARDFQHIDKYVF DNGQGGSGQRFDWSLLNGQSLGNVLLAGGLGADNCVEAAQTGCAG LDFNSAVESQPGIKDARLLASVFQTLRAY
trpB **SEQ ID NO: 130**	atgacaacattacttaacccctattttggtgagtttggcggcatgtacgtgccacaaatcctgatgcctgctctgcg ccagctggaagaagcttttgtcagcgcgcaaaaagatcctgaatttcaggctcagttcaacgacctgctgaaaa actatgccgggcgtccaaccgcgctgaccaaatgccagaacattacagccgggacgaacaccacgctgtatc tgaagcgcgaagatttgctgcacggcggcgcgcataaaactaaccaggtgctcggtcaggctttactggcga agcggatgggtaaaactgaaattattgccgaaaccggtgccggtcagcatggcgtggcgtcggcccttgcca gcgccctgctcggcctgaaatgccgaatttatatgggtgccaaagacgttgaacgccagtcgcccaacgttttc cggatgcgcttaatgggtgcggaagtgatcccggtacatagcggttccgcgaccctgaaagatgcctgtaatg aggcgctacgcgactggtccggcagttatgaaaccgcgcactatatgctgggtaccgcagctggcccgcatc cttacccgaccattgtgcgtgagtttcagcggatgattggcgaagaaacgaaagcgcagattctggaaagaga aggtcgcctgccggatgccgttatcgcctgtgttggcggtggttcgaatgccatcggtatgtttgcagatttcatc aacgaaaccgacgtcggcctgattggtgtggagcctggcggccacggtatcgaaactggcgagcacggcgc accgttaaaacatggtcgcgtgggcatctatttcggtatgaaagcgccgatgatgcaaaccgaagacgggcaa attgaagagtcttactccatttctgccgggctggatttcccgtccgtcggcccgcaacatgcgtatctcaacagc actggacgcgctgattacgtgtctattaccgacgatgaagccctggaagcctttaaaacgctttgcctgcatgaa gggatcatcccggcgctggaatcctcccacgccctggcccatgcgctgaaaatgatgcgcgaaaatccggaa aaagagcagctactggtggttaacctttccggtcgcggcgataaagacatcttcaccgttcacgatattttgaaa gcacgaggggaaatctga
TrpB **SEQ ID NO: 131**	MTTLLNPYFGEFGGMYVPQILMPALRQLEEAFVSAQKDPEFQAQFND LLKNYAGRPTALTKCQNITAGTNTTLYLKREDLLHGGAHKTNQVLG QALLAKRMGKTEIIAETGAGQHGVASALASALLGLKCRIYMGAKDV ERQSPNVFRMRLMGAEVIPVHSGSATLKDACNEALRDWSGSYETAH YMLGTAAGPHPYPTIVREFQRMIGEETKAQILEREGRLPDAVIACVGG GSNAIGMFADFINETDVGLIGVEPGGHGIETGEHGAPLKHGRVGIYFG MKAPMMQTEDGQIEESYSISAGLDFPSVGPQHAYLNSTGRADYVSIT DDEALEAFKTLCLHEGIIPALESSHALAHALKMMRENPEKEQLLVVN LSGRGDKDIFTVHDILKARGEI
trpA **SEQ ID NO: 132**	atggaacgctacgaatctctgtttgcccagttgaaggagcgcaaagaaggcgcattcgttcctttcgtcaccctc ggtgatccgggcattgagcagtcgttgaaaattatcgatacgctaattgaagccggtgctgacgcgctggagtt aggcatccccttctccgacccactggcggatggcccgacgattcaaaacgccacactgcgtgcttttgcggcg ggagtaaccccggcgcagtgctttgagatgctggcactcattcgccagaagcacccgaccattcccatcggcc

	ttttgatgtatgccaacctggtgtttaacaaaggcattgatgagttttatgccgagtgcgagaaagtcggcgtcga ttcggtgctggttgccgatgtgcccgtggaagagtccgcgcccttccgccaggccgcgttgcgtcataatgtcg cacctatctttatttgcccgccgaatgccgacgatgatttgctgcgccagatagcctcttacggtcgtggttacac ctatttgctgtcgcgagcgggcgtgaccggcgcagaaaaccgcgccgcgttacccctcaatcatctggttgcg aagctgaaagagtacaacgctgcgcctccattgcagggatttggtatttccgccccggatcaggtaaaagccg cgattgatgcaggagctgcgggcgcgatttctggttcggccatcgttaaaatcatcgagcaacatattaatgagc cagagaaaatgctggcggcactgaaagcttttgtacaaccgatgaaagcggcgacgcgcagttaa
TrpA **SEQ ID NO: 133**	MERYESLFAQLKERKEGAFVPFVTLGDPGIEQSLKIIDTLIEAGADALE LGIPFSDPLADGPTIQNATLRAFAAGVTPAQCFEMLALIRQKHPTIPIGL LMYANLVFNKGIDEFYAECEKVGVDSVLVADVPVEESAPFRQAALR HNVAPIFICPPNADDDLLRQIASYGRGYTYLLSRAGVTGAENRAALPL NHLVAKLKEYNAAPPLQGFGISAPDQVKAAIDAGAAGAISGSAIVKII EQHINEPEKMLAALKAFVQPMKAATRS

[0182] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequence of **Table 13** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as one or more nucleic acid sequence of **Table 13** or a functional fragment thereof. In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of one or more nucleic acid sequence of **Table 13** or a functional fragment thereof, or a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as one or more nucleic acid sequence of **Table 13** or a functional fragment thereof.

[0183] In one embodiment, one or more polypeptides and/polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 121** through **SEQ ID NO: 133**. In one embodiment, one or more polypeptides and/polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 85% identity with with one or more of **SEQ ID NO: 121** through **SEQ ID NO: 133**. In one embodiment, one or more polypeptides and/polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 90% identity with with one or more of **SEQ ID NO: 121** through **SEQ ID NO: 133**. In one embodiment, one or more polypeptides and/polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 95% identity with with one or more of **SEQ ID NO: 121** through **SEQ ID NO: 133**. In one embodiment, one or more polypeptides and/polynucleotides encoded and expressed by the genetically engineered bacteria have have at least about 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 121**

through **SEQ ID NO: 133**. Accordingly, in one embodiment, one or more polypeptides and/or polynucleotides expressed by the genetically engineered bacteria have at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with with one or more of **SEQ ID NO: 121** through **SEQ ID NO: 133**. In another embodiment, one or more polynucleotides and/or polypeptides encoded and expressed by the genetically engineered bacteria comprise the sequence of one or more of **SEQ ID NO: 121** through **SEQ ID NO: 133**. In another embodiment, one or more polynucleotides and/or polypeptides encoded and expressed by the genetically engineered bacteria comprise the sequence of one or more of **SEQ ID NO: 121** through **SEQ ID NO: 133**.

[0324] **Table 14 and 15** depicts exemplary polypeptide sequences feedback resistant AroG and TrpE. **Table 14 and 15** also depicts an exemplary TnaA (tryptophanase from E. coli) sequence. IN some embodiments, the sequence is encoded in circuits for tryptophan catabolism to indole; in other embodimetns, the sequence is deleted from the E coli chromosome to increase levels of tryptophan.

Table 14 Feedback resistant AroG and TrpE and tryptophanase sequences

Description	Sequence
AroGfbr: feedback resistant 2-dehydro-3-deoxyphosphoheptonate aldolase from E. coli **SEQ ID NO: 221**	MNYQNDDLRIKEIKELLPPVALLEKFPATENAANTVAHARKAI HKILKGNDDRLLVVIGPCSIHDPVAAKEYATRLLTLREELQDE LEIVMRVYFEKPRTTVGWKGLINDPHMDNSFQINDGLRIARK LLLDINDSGLPAAGEFLDMITLQYLADLMSWGAIGARTTESQ VHRELASGLSCPVGFKNGTDGTIKVAIDAINAAGAPHCFLSVT KWGHSAIVNTSGNGDCHIILRGGKEPNYSAKHVAEVKEGLNK AGLPAQVMIDFSHANSSKQFKKQMDVCTDVCQQIAGGEKAII GVMVESHLVEGNQSLESGEPLAYGKSITDACIGWDDTDALLR QLASAVKARRG
TrpEfbr: feedback resistant anthranilate synthase component I from E. coli **SEQ ID NO: 222**	MQTQKPTLELLTCEGAYRDNPTALFHQLCGDRPATLLLEFADI DSKDDLKSLLLVDSALRITALSDTVTIQALSGNGEALLTLLDN ALPAGVENEQSPNCRVLRFPPVSPLLDEDARLCSLSVFDAFRL LQNLLNVPKEEREAMFFGGLFSYDLVAGFENLPQLSAENSCP DFCFYLAETLMVIDHQKKSTRIQASLFAPNEEEKQRLTARLNE LRQQLTEAAPPLPVVSVPHMRCECNQSDEEFGGVVRLLQKAI RAGEIFQVVPSRRFSLPCPSPLAAYYVLKKSNPSPYMFFMQDN DFTLFGASPESSLKYDATSRQIEIYPIAGTRPRGRRADGSLDRD LDSRIELEMRTDHKELSEHLMLVDLARNDLARICTPGSRYVA DLTKVDRYSYVMHLVSRVVGELRHDLDALHAYRACMNMGT LSGAPKVRAMQLIAEAEGRRRGSYGGAVGYFTAHGDLDTCIV IRSALVENGIATVQAGAGVVLDSVPQSEADETRNKARAVLRA IATAHHAQETF

SerA: 2-oxoglutarate reductase from E. coli Nissle **SEQ ID NO: 223**	MAKVSLEKDKIKFLLVEGVHQKALESLRAAGYTNIEFHKGAL DDEQLKESIRDAHFIGLRSRTHLTEDVINAAEKLVAIGCFCIGT NQVDLDAAAKRGIPVFNAPFSNTRSVAELVIGELLLLLRGVPE ANAKAHRGVWNKLAAGSFEARGKKLGIIGYGHIGTQLGILAE SLGMYVYFYDIENKLPLGNATQVQHLSDLLNMSDVVSLHVPE NPSTKNMMGAKEISLMKPGSLLINASRGTVVDIPALCDALASK HLAGAAIDVFPTEPATNSDPFTSPLCEFDNVLLTPHIGGSTQEA QENIGLEVAGKLIKYSDNGSTLSAVNFPEVSLPLHGGRRLMHI HENRPGVLTALNKIFAEQGVNIAAQYLQTSAQMGYVVIDIEA DEDVAEKALQAMKAIPGTIRARLLY
SerAfbr: feedback resistant 2-oxoglutarate reductase from E. coli Nissle **SEQ ID NO: 224**	MAKVSLEKDKIKFLLVEGVHQKALESLRAAGYTNIEFHKGAL DDEQLKESIRDAHFIGLRSRTHLTEDVINAAEKLVAIGCFCIGT NQVDLDAAAKRGIPVFNAPFSNTRSVAELVIGELLLLLRGVPE ANAKAHRGVWNKLAAGSFEARGKKLGIIGYGHIGTQLGILAE SLGMYVYFYDIENKLPLGNATQVQHLSDLLNMSDVVSLHVPE NPSTKNMMGAKEISLMKPGSLLINASRGTVVDIPALCDALASK HLAGAAIDVFPTEPATNSDPFTSPLCEFDNVLLTPHIGGSTQEA QENIGLEVAGKLIKYSDNGSTLSAVNFPEVSLPLHGGRRLMHI AEARPGVLTALNKIFAEQGVNIAAQYLQTSAQMGYVVIDIEA DEDVAEKALQAMKAIPGTIRARLLY
TnaA: tryptophanase from E. coli **SEQ ID NO: 225**	MENFKHLPEPFRIRVIEPVKRTTRAYREEAIIKSGMNPFLLDSE DVFIDLLTDSGTGAVTQSMQAAMMRGDEAYSGSRSYYALAE SVKNIFGYQYTIPTHQGRGAEQIYIPVLIKKREQEKGLDRSKM VAFSNYFFDTTQGHSQINGCTVRNVYIKEAFDTGVRYDFKGN FDLEGLERGIEEVGPNNVPYIVATITSNSAGGQPVSLANLKVM YSIAKKYDIPVVMDSARFAENAYFIKQREAEYKDWTIEQITRE TYKYADMLAMSAKKDAMVPMGGLLCMKDDSFFDVYTECRT LCVVQEGFPTYGGLEGGAMERLAVGLYDGMNLDWLAYRIA QVQYLVDGLEEIGVVCQQAGGHAAFVDAGKLLPHIPADQFPA QALACELYKVAGIRAVEIGSFLLGRDPKTGKQLPCPAELLRLTI PRATYTQTHMDFIIEAFKHVKENAANIKGLTFTYEPKVLRHFT AKLKEV

[0657] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide of Table 14. In some embodiments, polypeptide of Table 14 has at least about 80% identity with one or more sequences **in Table 14**. In some embodiments, polypeptide of Table 14 has at least about 85% identity with one or more of one or more sequences **in Table 14**. In some embodiments, polypeptide of Table 14 has at least about 90% identity with one or more sequences **in Table 14**. In some embodiments, polypeptide of Table 14 has at least about 95% identity with one or more sequences **in Table 14**. In some embodiments, polypeptide of Table 14 has at least about 96%, 97%, 98%, or 99% identity with SEQ ID NO:84. Accordingly, In some embodiments, polypeptide of Table 14 r has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%,

88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with one or more sequences **in Table 14**. In some embodiments, AroGfbr comprises the sequence of one or more sequences **in Table 14**. In some embodiments, polypeptide of Table 14 consists of one of the sequences **in Table 14**.

[0658] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding AroGfbr. In some embodiments, AroGfbr has at least about 80% identity with **SEQ ID NO: 221**. In some embodiments, AroGfbr has at least about 85% identity with one or more of **SEQ ID NO: 221**. In some embodiments, AroGfbr has at least about 90% identity with **SEQ ID NO: 221**. In some embodiments, AroGfbr has at least about 95% identity with **SEQ ID NO: 221**. In some embodiments, AroGfbr has at least about 96%, 97%, 98%, or 99% identity with SEQ ID NO:84. Accordingly, In some embodiments, AroGfbr has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 221**. In some embodiments, AroGfbr comprises the sequence of **SEQ ID NO: 221**. In some embodiments, AroGfbr consists of the sequence of one or more of **SEQ ID NO: 221**.

[0659] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding TrpEfbr. In some embodiments, TrpEfbr has at least about 80% identity with **SEQ ID NO: 222**. In some embodiments, TrpEfbr has at least about 85% identity with one or more of **SEQ ID NO: 222**. In some embodiments, TrpEfbr has at least about 90% identity with **SEQ ID NO: 222**. In some embodiments, TrpEfbr has at least about 95% identity with **SEQ ID NO: 222**. In some embodiments, TrpEfbr has at least about 96%, 97%, 98%, or 99% identity with SEQ ID NO:85. Accordingly, In some embodiments, TrpEfbr has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 222**. In some embodiments,TrpEfbr comprises the sequence of **SEQ ID NO: 222**. In some embodiments, TrpEfbr consists of the sequence of one or more of **SEQ ID NO: 222**.

[0660] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding SerA. In some embodiments, SerA has at least about 80% identity with **SEQ ID NO: 223**. In some embodiments, SerA has at least about 85% identity with one or more of **SEQ ID NO: 223**. In some embodiments, SerA has at least about 90% identity with **SEQ ID NO: 223**. In some embodiments, SerA has at least about 95% identity with **SEQ ID NO: 223**. In some embodiments, SerA has at least about 96%, 97%, 98%, or 99% identity with SEQ ID NO:86. Accordingly, In some embodiments, SerA has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%,

95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 223**. In some embodiments, SerA comprises the sequence of **SEQ ID NO: 223**. In some embodiments, SerA consists of the sequence of one or more of **SEQ ID NO: 223**.

[0661] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding SerAfbr. In some embodiments, SerAfbr has at least about 80% identity with **SEQ ID NO: 224**. In some embodiments, SerAfbr has at least about 85% identity with one or more of **SEQ ID NO: 224**. In some embodiments, SerAfbr has at least about 90% identity with **SEQ ID NO: 224**. In some embodiments, SerAfbr has at least about 95% identity with **SEQ ID NO: 224**. In some embodiments, SerAfbr has at least about 96%, 97%, 98%, or 99% identity with SEQ ID NO:87. Accordingly, In some embodiments, SerAfbr has at least about 80%, 81%, 82%, 83%, 87%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 224**. In some embodiments,SerAfbr comprises the sequence of **SEQ ID NO: 224**. In some embodiments, SerAfbr consists of the sequence of one or more of **SEQ ID NO: 224**.

[0662] In one embodiment, TnaA is mutated or deleted.

[0663] **Table 15** lists exemplary polynucleotide sequences useful for tryptophan production.

Table 15. Sequences Useful for Tryptophan Production

Description	Sequence
fbrTrpE SEQ ID NO: 225	atgcaaacacaaaaaccgactctcgaactgctaacctgcgaaggcgcttatcgcgacaacccgactg cgctttttcaccagttgtgtggggatcgtccggcaacgctgctgctggaattcgcagatatcgacagca aagatgatttaaaaagcctgctgctggtagacagtgcgctgcgcattacagcattaagtgacactgtca caatccaggcgctttccggcaatggagaagccctgttgacactactggataacgccttgcctgcgggt gtggaaaatgaacaatcaccaaactgccgcgtactgcgcttcccgcctgtcagtccactgctggatga agacgcccgcttatgctccctttcggtttttgacgctttccgcttattacagaatctgttgaatgtaccgaag gaagaacgagaagcaatgttcttcggcggcctgttctcttatgaccttgtggcgggatttgaaaatttacc gcaactgtcagcggaaaatagctgccctgatttctgtttttatctcgctgaaacgctgatggtgattgacc atcagaaaaaaagcactcgtattcaggccagcctgtttgctccgaatgaagaagaaaaacaacgtctc actgctcgcctgaacgaactacgtcagcaactgaccgaagccgcgccgccgctgccggtggtttccg tgccgcatatgcgttgtgaatgtaaccagagcgatgaagagttcggtggtgtagtgcgtttgttgcaaaa agcgattcgcgccggagaaattttccaggtggtgccatctcgccgtttctctctgccctgcccgtcaccg ctggcagcctattacgtgctgaaaaagagtaatcccagcccgtacatgttttttatgcaggataatgatttc accctgtttggcgcgtcgccggaaagttcgctcaagtatgacgccaccagccgccagattgagatttac ccgattgccggaacacgtccacgcggtcgtcgtgccgatggttcgctggacagagacctcgacagcc gcatcgaactggagatgcgtaccgatcataaagagctttctgaacatctgatgctggtggatctcgccc gtaatgacctggcacgcatttgcacacccggcagccgctacgtcgccgatctcaccaaagttgaccgt tactcttacgtgatgcacctagtctcccgcgttgttggtgagctgcgccacgatctcgacgccctgcacg cttaccgcgcctgtatgaatatggggacgttaagcggtgcaccgaaagtacgcgctatgcagttaattg ccgaagcagaaggtcgtcgacgcggcagctacggcggcgcggtaggttattttaccgcgcatggcg atctcgacacctgcattgtgatccgctcggcgctggtggaaaacggtatcgccaccgtgcaagccggt gctggcgtagtccttgattctgttccgcagtcggaagccgacgaaactcgtaataaagcccgcgctgta

	ctgcgcgctattgccaccgcgcatcatgcacaggagacgttcta
fbrAroG **SEQ ID NO:** 226	atgaattatcagaacgacgatttacgcatcaaagaaatcaaagagttacttcctcctgtcgcattgctgg aaaaattccccgctactgaaaatgccgcgaatacggtcgcccatgcccgaaaagcgatccataagat cctgaaaggtaatgatgatcgcctgttggtggtgattggcccatgctcaattcatgatcctgtcgcggct aaagagtatgccactcgcttgctgacgctgcgtgaagagctgcaagatgagctggaaatcgtgatgc gcgtctattttgaaaagccgcgtactacggtgggctggaaagggctgattaacgatccgcatatggat aacagcttccagatcaacgacggtctgcgtattgcccgcaaattgctgctcgatattaacgacagcgg tctgccagcggcgggtgaattcctggatatgatcaccctacaatatctcgctgacctgatgagctggg gcgcaattggcgcacgtaccaccgaatcgcaggtgcaccgcgaactggcgtctggtctttcttgtccg gtaggtttcaaaaatggcactgatggtacgattaaagtggctatcgatgccattaatgccgccggtgcg ccgcactgcttcctgtccgtaacgaaatgggggcattcggcgattgtgaataccagcggtaacggcg attgccatatcattctgcgcggcggtaaagagcctaactacagcgcgaagcacgttgctgaagtgaa agaagggctgaacaaagcaggcctgccagcgcaggtgatgatcgatttcagccatgctaactcgtca aaacaattcaaaaagcagatggatgtttgtactgacgtttgccagcagattgccggtggcgaaaaggc cattattggcgtgatggtggaaagccatctggtggaaggcaatcagagcctcgagagcggggaacc gctggcctacggtaagagcatcaccgatgcctgcattggctgggatgataccgatgctctgttacgtc aactggcgagtgcagtaaaagcgcgtcgcgggtaa
SerA SEQ ID NO: 227	atggcaaaggtatcgctggagaaagacaagattaagtttctgctggtagaaggcgtgcaccaaaagg cgctggaaagccttcgtgcagctggttacaccaacatcgaatttcacaaaggcgcgctggatgatgaa caattaaaagaatccatccgcgatgcccacttcatcggcctgcgatcccgtacccatctgactgaagac gtgatcaacgccgcagaaaaactggtcgctattggctgtttctgtatcggaacaaatcaggttgatctgg atgcggcggcaaagcgcgggatcccggtatttaacgcaccgttctcaaatacgcgctctgttgcggag ctggtgattggcgaactgctgctgctattgcgcggcgtgccagaagccaatgctaaagcgcatcgtgg cgtgtggaacaaactggcggcgggttcttttgaagcgcgcggcaaaaagctgggtatcatcggctac ggtcatattggtacgcaattgggcattctggctgaatcgctgggaatgtatgtttacttttatgatattgaaa acaaactgccgctgggcaacgccactcaggtacagcatctttctgacctgctgaatatgagcgatgtgg tgagtctgcatgtaccagagaatccgtccaccaaaaatatgatgggcgcgaaagagatttcgctaatga agcccggctcgctgctgattaatgcttcgcgcggtactgtggtggatattccagcgctgtgtgacgcgc tggcgagcaaacatctggcgggggcggcaatcgacgtattcccgacggaaccggcgaccaatagc gatccatttacctctccgctgtgtgaattcgacaatgtccttctgacgccacacattggcggttcgactca ggaagcgcaggagaatatcggcttggaagttgcgggtaaattgatcaagtattctgacaatggctcaac gctctctgcggtgaacttcccggaagtctcgctgccactgcacggtgggcgtcgtctgatgcacatcca cgaaaaccgtccgggcgtgctaactgcgctcaacaaaatttttgccgagcagggcgtcaacatcgcc gcgcaatatctacaaacttccgcccagatgggttatgtagttattgatattgaagccgacgaagacgttg ccgaaaaagcgctgcaggcaatgaaagctattccgggtaccattcgcgcccgtctgctgtactaa
SerAfbr SEQ ID NO: 228	atggcaaaggtatcgctggagaaagacaagattaagtttctgctggtagaaggcgtgcaccaaaaggcgctggaaag ccttcgtgcagctggttacaccaacatcgaatttcacaaaggcgcgctggatgatgaacaattaaaagaatccatccgc gatgcccacttcatcggcctgcgatcccgtacccatctgactgaagacgtgatcaacgccgcagaaaaactggtcgct attggctgtttctgtatcggaacaaatcaggttgatctggatgcggcggcaaagcgcgggatcccggtatttaacgcac cgttctcaaatacgcgctctgttgcggagctggtgattggcgaactgctgctgctattgcgcggcgtgccagaagccaa tgctaaagcgcatcgtggcgtgtggaacaaactggcggcgggttcttttgaagcgcgcggcaaaaagctgggtatcat cggctacggtcatattggtacgcaattgggcattctggctgaatcgctgggaatgtatgtttacttttatgatattgaaaac aaactgccgctgggcaacgccactcaggtacagcatctttctgacctgctgaatatgagcgatgtggtgagtctgcatg taccagagaatccgtccaccaaaaatatgatgggcgcgaaagagatttcgctaatgaagcccggctcgctgctgatta atgcttcgcgcggtactgtggtggatattccagcgctgtgtgacgcgctggcgagcaaacatctggcgggggcggca atcgacgtattcccgacggaaccggcgaccaatagcgatccatttacctctccgctgtgtgaattcgacaatgtccttct gacgccacacattggcggttcgactcaggaagcgcaggagaatatcggcttggaagttgcgggtaaattgatcaagt attctgacaatggctcaacgctctctgcggtgaacttcccggaagtctcgctgccactgcacggtgggcgtcgtctgat gcacatc**GCT**gaa**GCT**cgtccgggcgtgctaactgcgctcaacaaaatttttgccgagcagggcgtcaacatc gccgcgcaatatctacaaacttccgcccagatgggttatgtagttattgatattgaagccgacgaagacgttgccgaaa aagcgctgcaggcaatgaaagctattccgggtaccattcgcgcccgtctgctgtactaa

[0184] In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with one or more sequences of **Table 15**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with one or more sequences of **Table 15**. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with one or more sequences of **Table 15**. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with one or more sequences of Table 19B. In another embodiment, the gene has at least about 96%, 97%, 98%, or 99% identity with one or more sequences of **Table 15**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with one or more sequences of **Table 15**. In another embodiment, the genetically engineered bacteria comprise the sequence of **Table 15.** embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of with one or more sequences of **Table 15**.

[0185] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding TrpEfbr. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 225**. In another embodiment, the genetically engineered bacteria comprise a TrpEfbr gene sequence which has at least about 85% identity with **SEQ ID NO: 225**. In some embodiments, the genetically engineered bacteria comprise a TrpEfbr gene sequence which has at least about 90% identity with **SEQ ID NO: 225**. In some embodiments, the genetically engineered bacteria comprise a TrpEfbr gene sequence which has at least about 95% identity with **SEQ ID NO: 225**. In another embodiment, the TrpEfbr gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 225**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a TrpEfbr gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 225**. In another embodiment, the genetically engineered bacteria comprise the TrpEfbr gene sequence of **SEQ ID NO: 225**. In yet another embodiment the genetically engineered bacteria comprise a TrpEfbr gene sequence which consists of the sequence of **SEQ ID NO: 225**.

[0186] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding feedback resistant AroG. In some embodiments, the genetically engineered bacteria comprise a AroGfbr gene sequence which has at least about 80% identity

with **SEQ ID NO: 226**. In another embodiment, the genetically engineered bacteria comprise a AroGfbr sequence which has at least about 85% identity with **SEQ ID NO: 226**. In some embodiments, the genetically engineered bacteria comprise a AroGfbr gene sequence which has at least about 90% identity with **SEQ ID NO: 226**. In some embodiments, the genetically engineered bacteria comprise a AroGfbr gene sequence which has at least about 95% identity with **SEQ ID NO: 226**. In another embodiment, the a AroGfbr gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 226**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a AroGfbr gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 226**. In another embodiment, the genetically engineered bacteria comprise the a AroGfbr gene sequence of **SEQ ID NO: 226**. In yet another embodiment the genetically engineered bacteria comprise a AroGfbr gene sequence which consists of the sequence of **SEQ ID NO: 226**.

[0187] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding SerA. In some embodiments, the genetically engineered bacteria comprise a SerA gene sequence which has at least about 80% identity with **SEQ ID NO: 227**. In another embodiment, the genetically engineered bacteria comprise a SerA gene sequence which has at least about 85% identity with **SEQ ID NO: 227**. In some embodiments, the genetically engineered bacteria comprise a SerA gene sequence which has at least about 90% identity with **SEQ ID NO: 227**. In some embodiments, the genetically engineered bacteria comprise a SerA gene sequence which has at least about 95% identity with **SEQ ID NO: 227**. In another embodiment, the SerA gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 227**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a SerA gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 227**. In another embodiment, the genetically engineered bacteria comprise the SerA gene sequence of **SEQ ID NO: 227**. In yet another embodiment the genetically engineered bacteria comprise a SerA gene sequence which consists of the sequence of **SEQ ID NO: 227**.

[0188] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding feedback resistant SerA. In some embodiments, the genetically engineered bacteria comprise a SerAfbr gene sequence which has at least about 80% identity with **SEQ ID NO: 228**. In another embodiment, the genetically engineered bacteria comprise a SerAfbr gene sequence which has at least about 85% identity with **SEQ ID NO:**

228. In some embodiments, the genetically engineered bacteria comprise a SerAfbr gene sequence which has at least about 90% identity with **SEQ ID NO: 228**. In some embodiments, the genetically engineered bacteria comprise a SerAfbr gene sequence which has at least about 95% identity with **SEQ ID NO: 228**. In another embodiment, the SerAfbr gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 228**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a SerAfbr gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 228**. In another embodiment, the genetically engineered bacteria comprise the SerAfbr gene sequence of **SEQ ID NO: 228**. In yet another embodiment the genetically engineered bacteria comprise a SerAfbr gene sequence which consists of the sequence of **SEQ ID NO: 228**.

[0189] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA. In some embodiments, the genetically engineered bacteria comprise and of the trypophan production combinations described herein.

[0190] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production or catabolism of tryptophan and/or one of its metabolites is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0191] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other

chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0192] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptophan is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table IX or Table X**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptophan is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table IX** or **Table X** or is listed in **Table XI**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptophan is modified and/or mutated, e.g., to enhance stability, or increase tryptophan production.

[0193] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptophan may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptophan are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0194] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides

for the production of tryptophan further comprise one or more gene sequences described herein for the consumption of ammonia.

[0195] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0196] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0197] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0198] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production or catabolism of tryptophan and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptophan further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one

or more polypeptides for the production of tryptophan further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

Producing Kynurenic Acid

[0664] In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid. Kynurenic acid is produced from the irreversible transamination of kynurenine in a reaction catalyzed by the enzyme kynurenine-oxoglutarate transaminase. Kynurenic acid acts as an antagonist of ionotropic glutamate receptors (Turski et al., 2013). While glutamate is known to be a major excitatory neurotransmitter in the central nervous system, there is now evidence to suggest an additional role for glutamate in the peripheral nervous system. For example, the activation of NMDA glutamate receptors in the major nerve supply to the GI tract (i.e., the myenteric plexus) leads to an increase in gut motility (Forrest et al., 2003), but rats treated with kynurenic acid exhibit decreased gut motility and inflammation in the early phase of acute colitis (Varga et al., 2010). Thus, the elevated levels of kynurenic acid reported in IBD patients may represent a compensatory response to the increased activation of enteric neurons (Forrest et al., 2003). The genetically engineered bacteria may comprise any suitable gene or genes for producing kynurenic acid. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more kynurenine-oxoglutarate transaminases (also referred to as kynurenine aminotransferases (e.g., KAT I, II, III)).

[0665] In some embodiments, the gene or genes for producing kynurenic acid is modified and/or mutated, e.g., to enhance stability, increase kynurenic acid production under inducing conditions. In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, the gene sequences(s) are controlled by a constitutive promoter. In some embodiments, the gene sequences(s) are controlled by an inducible promoter. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constitutive promoter, and are expressed during bacterial culture in vitro, e.g., for bacterial expansion, production and/or manufacture, as described herein.

[0666] In some embodiments, the genetically engineered bacteria comprising one or more gene(s) or gene cassette(s) can alter the TRP:KYNA ratio, e.g. in the circulation. In some embodiments the TRP:KYNA ratio is increased. In some embodiments, TRP:KYNA ratio is decreased.

[0667] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) for the consumption of tryptophan and production of kynurenic acid, which are bacterially derived. In some embodiments, the enzymes for producing kynureic acid are derived from one or more of Pseudomonas, Xanthomonas, Burkholderia, Stenotrophomonas, Shewanella, and Bacillus, and/or members of the families Rhodobacteraceae, Micrococcaceae, and Halomonadaceae, In some embodiments the enzymes are derived from the species listed in table S7 of Vujkovic-Cvijin et al. (Dysbiosis of the gut microbiota is associated with HIV diseaseprogression and tryptophan catabolism Sci Transl Med. 2013 July 10; 5(193): 193ra91), the contents of which is herein incorporated by reference in its entirety.

[0668] In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding one or more tryptophan transporters and gene sequence(s) encoding kynureninase. In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding one or more tryptophan transporters and gene sequence(s) encoding one or more kynurenine-oxoglutarate transaminases (kynurenine aminotransferases). In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding one or more tryptophan transporters, gene sequence(s) encoding kynureninase, and gene sequence(s) encoding one or more kynurenine-oxoglutarate transaminases (kynurenine aminotransferases). In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding kynureninase and gene sequence(s) encoding one or more kynurenine aminotransferases.

[0669] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce kynurenic acid from tryptophan. Non-limiting example of such gene sequence(s) are shown in the figures and described elsewhere herein. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode IDO1(indoleamine 2,3-dioxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode IDO1 from homo sapiens. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode TDO2 (tryptophan 2,3-dioxygenase). In one embodiment, the genetically engineered bacteria

comprise one or more gene sequence(s) which encode TDO2 from homo sapiens. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 (indoleamine 2,3-dioxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 from S. cerevisiae). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid: Kynurenine formamidase. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid: Kynurenine formamidase from mouse. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid in combination with one or more of ido1 and/or tdo2 and/or bna2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid in combination with IDO1. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with TDO2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode Afmid in combination with bna2. In one embodiment, the genetically engineered bacteria further comprise one or more gene sequence(s) which encode cclb1 and/or cclb2 and/or aadat and/or got2.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA3 (kynurenine--oxoglutarate transaminase. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA3 from S. cerevisae. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with one or more of ido1 and/or tdo2 and/or bna2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with ido1. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with tdo2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode BNA2 in combination with bna2. In one embodiment, the genetically engineered bacteria further comprise one or more gene sequence(s) which encode cclb1 and/or cclb2 and/or aadat and/or got2.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of ido1 and/or tdo2 and/or bna2.

[0670] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of afmid and/or bna3.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of ido1 and/or tdo2 and/or bna2, in combination with one or more of afmid and/or

bna3.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode GOT2 (Aspartate aminotransferase, mitochondrial). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode GOT2 from homo sapiens.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode AADAT (Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode AADAT from homo sapiens. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode CCLB1 (Kynurenine--oxoglutarate transaminase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode CCLB1 from homo sapiens). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode CCLB2 (kynurenine--oxoglutarate transaminase 3) In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode CCLB2 from homo sapiens.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cclb1 and/or cclb2 and/or aadat and/or got2.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of ido1 and/or tdo2 and/or bna2, in combination with one or more of afmid and/or bna3, and in combination with one or more of . cclb1 and/or cclb2 and/or aadat and/or got2.

[0671] In any of these embodiments, the genetically engineered bacteria which produce kynurenic acid from tryptophan also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in the figures and the examples and described elsewhere herein. In some embodiments, the genetically engineered bacteria which produce kynurenic acid from tryptophan also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria which produce kynurenic acid from tryptophan also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0672] In some embodiments, the one or more genes for producing kynurenic acid are modified and/or mutated, e.g., to enhance stability, increase kynurenic acid production under inducing conditions. In some embodiments, the engineered bacteria have enhanced uptake or

import of tryptophan, e.g., comprise a transporter or other mechanism for increasing the uptake of tryptophan into the bacterial cell.

[0673] In some embodiments, the genetically engineered bacteria produce 0% to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more kynurenic acid than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more kynurenic acid than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more kynurenic acid than unmodified bacteria of the same bacterial subtype under the same conditions.

[0674] In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damate, e.g., bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0675] . In some embodiments, the gene sequences(s) are controlled by an inducible promoter. In some embodiments, the gene sequences(s) are controlled by a constitutive promoter. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constritutive promoter, and are expressed during bacterial culture in vitro, e.g., for bacterial expansion, production and/or manufacture, as described herein.

[0676] In some embodiments, the genetically engineered bacteria are capable of expressing any one or more of the described circuits in low-oxygen conditions, in the

presence of disease or tissue specific molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response or immune suppression or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose and others described herein. In some embodiments, any one or more of the described circuits are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the bacterial chromosome. Also, in some embodiments, the genetically engineered bacteria are further capable of expressing any one or more of the described circuits and further comprise one or more of the following: (1) one or more auxotrophies, such as any auxotrophies known in the art and provided herein, e.g., thyA auxotrophy, (2) one or more kill switch circuits, such as any of the kill-switches described herein or otherwise known in the art, (3) one or more antibiotic resistance circuits, (4) one or more transporters for importing biological molecules or substrates, such any of the transporters described herein or otherwise known in the art, (5) one or more secretion circuits, such as any of the secretion circuits described herein and otherwise known in the art, and (6) combinations of one or more of such additional circuits.

Producing Indole Tryptophan Metabolites and Tryptamine

[0677] In some embodiments, the genetically engineered bacteria comprise genetic circuits for the production of indole metabolites and/or tryptamine. Exemplary circuits for the production of indole metabolites/derivatives are shown in the figures.

[0678] In some embodiments, the genetically engineered bacteria comprise genetic circuitry for converting tryptophan to tryptamine. In some embodiments, the engineered bacteria comprise gene sequence encoding Tryptophan decarboxylase, e.g., from *Catharanthus roseus*. In some embodiments, the engineered bacteria comprise genetic circuitry for producing indole-3-acetaldehyde and FICZ from tryptophan. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: aro9 (L-tryptophan aminotransferase, e.g., from S. cerevisae), aspC (aspartate aminotransferase, e.g., from E. coli, taa1 (L-tryptophan-pyruvate aminotransferase, e.g., from Arabidopsis thaliana), staO (L-tryptophan oxidase, e.g., from streptomyces sp. TP-A0274), trpDH (Tryptophan dehydrogenase, e.g., from Nostoc punctiforme NIES-2108) and ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae). In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: tdc (Tryptophan decarboxylase, e.g., from Catharanthus roseus and/or Clostridium sporogenes), and tynA (Monoamine oxidase,

e.g., from E. coli). In some embodiments, the engineered bacteria comprise genetic circuitry for producing indole-3-acetonitrile from tryptophan. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: cyp79B2, (tryptophan N-monooxygenase, e.g., from Arabidopsis thaliana), cyp79B3 (tryptophan N-monooxygenase, e.g., from Arabidopsis thaliana). In some embodiments, the engineered bacteria comprise genetic circuitry for producing kynurenine from tryptophan. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: IDO1(indoleamine 2,3-dioxygenase, e.g., from homo sapiens or TDO2 (tryptophan 2,3-dioxygenase, e.g., from homo sapiens), BNA2 (indoleamine 2,3-dioxygenase, e.g., from S. cerevisiae) and Afmid: Kynurenine formamidase, e.g., from mouse), BNA3 (kynurenine--oxoglutarate transaminase, e.g., from S. cerevisae). In some embodiments, the engineered bacteria comprise genetic circuitry for producing kynureninic acid from tryptophan. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: IDO1(indoleamine 2,3-dioxygenase, e.g., from homo sapiens or TDO2 (tryptophan 2,3-dioxygenase, e.g., from homo sapiens), BNA2 (indoleamine 2,3-dioxygenase, e.g., from S. cerevisiae) and Afmid: Kynurenine formamidase, e.g., from mouse), BNA3 (kynurenine--oxoglutarate transaminase, e.g., from S. cerevisae) and GOT2 (Aspartate aminotransferase, mitochondrial, e.g.,from homo sapiens or AADAT (Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial, e.g., from homo sapiens), or CCLB1 (Kynurenine--oxoglutarate transaminase 1, e.g., from homo sapiens) or CCLB2 (kynurenine--oxoglutarate transaminase 3, e.g., from homo sapiens. In some embodiments, the engineered bacteria comprise genetic circuitry for producing indole from tryptophan. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: tnaA (tryptophanase, e.g., from E. coli). In some embodiments, the engineered bacteria comprise genetic circuitry for producing indole-3-carbinol, indole-3-aldehyde, 3,3' diindolylmethane (DIM), indolo(3,2-b) carbazole (ICZ) from indole glucosinolate (taken up through the diet). The genetically engineered bacteria comprise a gene sequence encoding pne2 (myrosinase, e.g., from Arabidopsis thaliana). In some embodiments, the engineered bacteria comprise genetic circuitry for producing indole-3-acetic acid from tryptophan. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: aro9 (L-tryptophan aminotransferase, e.g., from S. cerevisae), aspC (aspartate aminotransferase, e.g., from E. coli, taa1 (L-tryptophan-pyruvate aminotransferase, e.g., from Arabidopsis thaliana), staO (L-tryptophan oxidase, e.g., from streptomyces sp. TP-A0274), trpDH (Tryptophan

dehydrogenase, e.g., from Nostoc punctiforme NIES-2108), ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae), iad1 (Indole-3-acetaldehyde dehydrogenase, e.g., from Ustilago maydis), AAO1 (Indole-3-acetaldehyde oxidase, e.g., from Arabidopsis thaliana). In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: tdc (Tryptophan decarboxylase, e.g.,from Catharanthus roseus and/or Clostridium sporogenes), tynA (Monoamine oxidase, e.g., from E. coli), iad1 (Indole-3-acetaldehyde dehydrogenase, e.g., from Ustilago maydis), AAO1 (Indole-3-acetaldehyde oxidase, e.g., from Arabidopsis thaliana). In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: aro9 (L-tryptophan aminotransferase, e.g., from S. cerevisae), aspC (aspartate aminotransferase, e.g., from E. coli, taa1 (L-tryptophan-pyruvate aminotransferase, e.g., from Arabidopsis thaliana), staO (L-tryptophan oxidase, e.g., from streptomyces sp. TP-A0274), trpDH (Tryptophan dehydrogenase, e.g., from Nostoc punctiforme NIES-2108) and yuc2 (indole-3-pyruvate monoxygenase, e.g., from Arabidopsis thaliana). In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: IaaM (Tryptophan 2-monooxygenase e.g., from Pseudomonas savastanoi), iaaH (Indoleacetamide hydrolase, e.g., from Pseudomonas savastanoi). In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more of the following: cyp79B2 (tryptophan N-monooxygenase, e.g., from Arabidopsis thaliana), cyp79B3 (tryptophan N-monooxygenase, e.g., from Arabidopsis thaliana, cyp71a13 (indoleacetaldoxime dehydratase, e.g., from Arabidopis thaliana), nit1 (Nitrilase, e.g., from Arabidopsis thaliana), iaaH (Indoleacetamide hydrolase, e.g., from Pseudomonas savastanoi). In some embodiments, the genetically engineered bacteria comprises trpDH (Tryptophan dehydrogenase, e.g., from Nostoc punctiforme NIES-2108), ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae) which together produce indole-3-acetaldehyde and FICZ though an (indol-3yl)pyruvate intermediate, and iad1 (Indole-3-acetaldehyde dehydrogenase, e.g., from Ustilago maydis), which converts indole-3-acetaldehyde into indole-3-acetate.

[0679] In some embodiments, the genetically engineered bacteria comprise genetic circuits for the production of tryptophan, tryptamine, indole acetic acid, and indole propionic acid. In some embodiments, the engineered bacteria produces tryptamine. Tryptophan is optionally produced from chorismate precursor, and the bacteria optionally comprises circuits as depicted and/or described in **FIG. 40A** and/or **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D**. Additionally, the bacteria comprises tdc (tryptophan decarboxylase, e.g., from

Catharanthus roseus and/or Clostridium sporogenes), which converts tryptophan into tryptamine.

[0199] In some embodiments, the engineered bacteria comprise genetic circuits for the production of indole-3-acetate. Tryptophan is optionally produced from chorismate precursor, and the strain optionally comprises circuits as depicted and/or described in **FIG. 40A** and/or **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D**. Additionally, the strain comprises trpDH (Tryptophan dehydrogenase, e.g., from *Nostoc punctiforme* NIES-2108) and ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae) which together produce indole-3-acetaldehyde and FICZ though an (indol-3yl)pyruvate intermediate, and iad1 (Indole-3-acetaldehyde dehydrogenase, e.g., from Ustilago maydis), which converts indole-3-acetaldehyde into indole-3-acetate.

[0200] In some embodiments, the engineered bacteria comprise genetic circuits for the production of indole-3-propionate. Tryptophan is optionally produced from chorismate precursor, and the strain optionally comprises circuits as depicted and/or described in **FIG. 40A** and/or **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D**. Additionally, the strain comprises a circuit as described in **FIG. 98**, comprising trpDH (Tryptophan dehydrogenase, e.g., from Nostoc punctiforme NIES-2108, which produces (indol-3yl)pyruvate from tryptophan), fldA (indole-3-propionyl-CoA:indole-3-lactate CoA transferase, e.g., from Clostridium sporogenes, which converts indole-3-lactate and indol-3-propionyl-CoA to indole-3-propionic acid and indole-3-lactate-CoA), fldB and fldC (indole-3-lactate dehydratase e.g., from Clostridium sporogenes, which converts indole-3-lactate-CoA to indole-3-acrylyl-CoA) fldD and/or AcuI: (indole-3-acrylyl-CoA reductase, e.g., from Clostridium sporogenes and/or acrylyl-CoA reductase, e.g., from Rhodobacter sphaeroides, which convert indole-3-acrylyl-CoA to indole-3-propionyl-CoA). The circuits further comprise *fldH1 and/or fldH2 (*indole-3-lactate dehydrogenase 1 and/or 2, e.g., from Clostridium sporogenes), which converts (indol-3-yl)pyruvate into indole-3-lactate).

[0201] In some embodiments, the engineered bacteria comprises genetic circuitry for the production of indole-3-propionic acid (IPA). In some embodiments, the engineered bacteria comprises gene sequence encoding tryptophan ammonia lyase and an indole-3-acrylate reductase (e.g., Tryptophan ammonia lyase (WAL) (e.g., from *Rubrivivax benzoatilyticus*) and indole-3-acrylate reductase (e.g., from *Clostridum botulinum*). Tryptophan ammonia lyase converts tryptophan to indole-3-acrylic acid, and indole-3-acrylate reductase converts indole-3-acrylic acid into IPA. Without wishing to be bound by theory, no oxygen is needed for this reaction, allowing it to proceed under low or no oxygen

conditions, e.g., as those found in the mammalian gut. In some embodiments, the genetically engineered bacteria further comprise one or more circuits for the production of tryptophan, e.g., as shown in **FIG. 40 (A-D)** and **FIG. 44** and as described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced.

[0202] In some embodiments, the engineered bacteria comprise genetic circuitry for producing indole-3-propionic acid (IPA), indole acetic acid (IAA), and/or tryptamine synthesis(TrA) circuits. In some embodiments, the engineered bacteria comprise gene sequence encoding one or more of the following: TrpDH: tryptophan dehydrogenase, e.g., from Nostoc punctiforme NIES-2108; FldH1/FldH2: indole-3-lactate dehydrogenase, e.g., from Clostridium sporogenes; FldA: indole-3-propionyl-CoA:indole-3-lactate CoA transferase, e.g., from Clostridium sporogenes; FldBC: indole-3-lactate dehydratase, e.g., from Clostridium sporogenes; FldD: indole-3-acrylyl-CoA reductase, e.g., from Clostridium sporogenes; AcuI: acrylyl-CoA reductase, e.g., from *Rhodobacter sphaeroides*. lpdC: Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae; lad1: Indole-3-acetaldehyde dehydrogenase, e.g., from Ustilago maydis; Tdc: Tryptophan decarboxylase, e.g., from *Catharanthus roseus* or from *Clostridium sporogenes.*

[0203] In some embodiments, the engineered bacteria comprise genetic circuitry for producing (indol-3-yl)pyruvate (IPyA). In some embodiments, the engineered bacteria comprise gene sequence encoing one or more of the following: tryptophan dehydrogenase (EC 1.4.1.19) (enzyme that catalyzes the reversible chemical reaction converting L-tryptophan, NAD(P) and water to (indol-3-yl)pyruvate (IPyA), NH_3, NAD(P)H and H^+)); Indole-3-lactate dehydrogenase ((EC 1.1.1.110, e.g., Clostridium sporogenes or Lactobacillus casei) (converts (indol-3yl)pyruvate (IpyA) and NADH and H+ to indole-3-lactate (ILA) and NAD+); Indole-3-propionyl-CoA:indole-3-lactate CoA transferase (FldA) (converts indole-3-lactate (ILA) and indol-3-propionyl-CoA to indole-3-propionic acid (IPA) and indole-3-lactate-CoA); Indole-3-acrylyl-CoA reductase (FldD) and acrylyl-CoA reductase (AcuI) (convert indole-3-acrylyl-CoA to indole-3-propionyl-CoA); Indole-3-lactate dehydratase (FldBC) (converts indole-3-lactate-CoA to indole-3-acrylyl-CoA); Indole-3-pyruvate decarboxylase (lpdC:) (converts Indole-3-pyruvic acid (IPyA) into Indole-3-acetaldehyde (IAAld)); lad1: Indole-3-acetaldehyde dehydrogenase (coverts Indole-3-acetaldehyde (IAAld) into Indole-3-acetic acid (IAA)); Tdc: Tryptophan decarboxylase (converts

tryptophan (Trp) into tryptamine (TrA)). In some embodiments, the genetically engineered bacteria further comprise one or more circuits for the production of tryptophan, e.g., as shown in **FIG. 40 (A-D)** and **FIG. 44** and as described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced.

[0204] In any of the described embodiments, any of the gene(s), gene sequence(s) and/or gene circuit(s) or cassette(s) are optionally expressed from an inducible promoter. In certain embodiments, the one or more cassettes are under the control of constitutive promoters. Exemplary inducible promoters which may control the expression of the gene(s), gene sequence(s) and/or gene circuit(s) or cassette(s) include oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose and tetracycline. The bacteria may also include an auxotrophy, *e.g.*, deletion of thyA (Δ thyA; thymidine dependence).

[0205] In some embodiments, the genetically engineered bacteria further comprise one or more circuits for the production of tryptophan, e.g., as shown in **FIG. 40 (A-D)** and **FIG. 44** and as described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced.

[0206] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more tryptophan metabolites bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more tryptophan metabolites than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold,

twenty-fold, thirty-fold, forty-fold, or fifty-fold, more tryptophan metabolites than unmodified bacteria of the same bacterial subtype under the same conditions.

[0207] In in any of these embodiments the expression of the gene sequences for the production of the indole and other tryptophan metabolites, including, but not limited to, tryptamine and/or indole-3 acetaladehyde, indole-3acetonitrile, indole, indole acetic acid FICZ, indole-3-propionic acid, is under the control of an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0208] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0209] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia, inflammation, or as a consequence of

liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0210] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0211] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences described herein for the consumption of ammonia.

[0212] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0213] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more

gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0214] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0215] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of indoles and other tryptophan metabolites and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0216] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise a GABA transport circuit and/or a GABA metabolic circuit.. In some embodiments, the genetically

engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of indoles and other tryptophan metabolites further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0217] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Tryptamine

[0218] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce tryptamine from tryptophan. The monoamine alkaloid, tryptamine, is derived from the direct decarboxylation of tryptophan. Tryptophan is converted to indole-3-acetic acid (IAA) via the enzymes tryptophan monooxygenase (*IaaM*) and indole-3- acetamide hydrolase (*IaaH*), which constitute the indole-3-acetamide (IAM) pathway, as described in the figures and examples.

[0219] A non-limiting example of such as strain is shown in **FIG. 91A.** Another non-limiting example of such as strain is shown in **FIG. 43A.** In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more Tryptophan decarboxylase(s), e.g., from *Catharanthus roseus*. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more Tryptophan decarboxylase(s), e.g., from *Clostridium sporgenenes*. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more Tryptophan decarboxylase(s) e.g., from Ruminococcus Gnavus.

[0220] **Table 18-19 and Table A-C** lists exemplary sequences for tryptamine production in genetically engineered bacteria.

[0221] In some embodiments, the genetically engineered bacteria which produce tryptamine from tryptophan also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in **FIG. 40, FIG. 44A and/or FIG. 44B** and described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant

versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced. In some embodiments, the genetically engineered bacteria which produce tryptamine from tryptophan also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, In some embodiments, the genetically engineered bacteria which produce tryptamine from tryptophan also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0222] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more tryptamine than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more tryptamine than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more tryptamine than unmodified bacteria of the same bacterial subtype under the same conditions.

[0223] In some embodiments, the genetically engineered bacteria are capable of producing Tryptamine under inducing conditions, *e.g.*, under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose and others described herein.

[0224] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptamine is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the

presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0225] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0226] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptamine is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptamine is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more

polypeptides for the production of tryptamine is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0227] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptamine may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of tryptamine are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0228] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences described herein for the consumption of ammonia.

[0229] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0230] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0231] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0232] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of tryptamine and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the

genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0233] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise a GABA transport circuit and/or a GABA metabolic circuit.. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of tryptamine further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0234] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Indole-3-acetaldehyde and FICZ

[0235] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce indole-3-acetaldehyde and FICZ from tryptophan. Exemplary gene cassettes for the production of produce indole-3-acetaldehyde and FICZ from tryptophan are shown in **FIG. 91B**.

[0236] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aro9 (L-tryptophan aminotransferase). In one embodiment, the (L-tryptophan aminotransferase is from S. cerevisiae. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aro9 and ipdC. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC (aspartate aminotransferase. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC from E. coli. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC and ipdC. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 (L-tryptophan-pyruvate aminotransferase, In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 and ipdC. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO (L-tryptophan oxidase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO from streptomyces sp. TP-A0274. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO and ipdC. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH (Tryptophan dehydrogenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH from Nostoc punctiforme NIES-2108. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH and ipdC. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of aro9 or aspC or taa1 or staO or trpDH. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of aro9 or aspC or taa1 or staO or trpDH and ipdC.

[0237] Further exemplary gene cassettes for the production of produce indole-3-acetaldehyde and FICZ from tryptophan are shown in **FIG. 91C.** In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc (Tryptophan decarboxylase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc from Catharanthus roseus. In one

embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tynA (Monoamine oxidase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tynA from E. coli. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc and tynA.

[0238] In any of these embodiments, the genetically engineered bacteria which produce produce indole-3-acetaldehyde and FICZ also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in **FIG. 40, FIG. 44A and/or FIG. 44B** and described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced. In some embodiments, the genetically engineered bacteria which produce indole-3-acetaldehyde and FICZ also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria which produce indole-3-acetaldehyde and FICZ also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0239] In some embodiments, the genetically engineered bacteria produce 0% to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more Indole-3-acetaldehyde and/or FICZ than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more Indole-3-acetaldehyde and/or FICZ than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more Indole-3-acetaldehyde and/or FICZ than unmodified bacteria of the same bacterial subtype under the same conditions.

[0240] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0241] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0242] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by

methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0243] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0244] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences described herein for the consumption of ammonia.

[0245] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0246] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0247] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene

sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0248] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of Indole-3-acetaldehyde and/or FICZ and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0249] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise a GABA transport circuit and/or a GABA metabolic circuit.. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetaldehyde and/or FICZ further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a “manganese transport circuit”).

[0250] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Indole-3-acetic acid

[0251] In some embodiments, the genetically engineered bacteria comprise one or more gene cassettes which convert tryptophan to Indole-3-aldehyde and Indole Acetic Acid, e.g., via a tryptophan aminotransferase cassette. A non-limiting example of such a tryptophan aminotransferase expressed by the genetically engineered bacteria is in the tables. In some embodiments, the genetically engineered bacteria take up tryptophan through an endogenous or exogenous transporter, and further produce Indole-3-aldehyde and Indole Acetic Acid from tryptophan. In some embodiments, the genetically engineered bacteria optionally comprise a tryptophan and/or indole metabolite exporter.

[0252] The genetically engineered bacteria may comprise any suitable gene for producing Indole-3-aldehyde and/or Indole Acetic Acid and/or Tryptamine. In some embodiments, the gene for producing kynurenine is modified and/or mutated, *e.g.*, to enhance stability, increase Indole-3-aldehyde and/or Indole Acetic Acid and/or Tryptamine production, and/or increase anti-inflammatory potency under inducing conditions. In some embodiments, the engineered bacteria also have enhanced uptake or import of tryptophan, *e.g.*, comprise a transporter or other mechanism for increasing the uptake of tryptophan into the bacterial cell, as discussed in detail above. In some embodiments, the engineered bacteria also have enhanced export of a indole tryptophan metabolite , *e.g.*, comprise an exporter or other mechanism for increasing the uptake of tryptophan into the bacterial cell, as discussed in detail above. In some embodiments, the genetically engineered bacteria are capable of producing Indole-3-aldehyde and/or Indole Acetic Acid and/or Tryptamine under inducing conditions, *e.g.*, under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0253] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce indole-3-acetic acid.

[0254] Non-limiting example of such genes encoding tryptophan catabolism enzymes are shown in **FIG. 92A, FIG. 92B, FIG. 92C, FIG. 92D, and FIG. 92E, and FIG. 43B and FIG. 43E.**

[0255] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aro9 (L-tryptophan aminotransferase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aro9 from S. cerevisae). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC (aspartate aminotransferase), In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC from E. coli. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 (L-tryptophan-pyruvate aminotransferase. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 from Arabidopsis thaliana). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO (L-tryptophan oxidase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO from streptomyces sp. TP-A0274). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH (Tryptophan dehydrogenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH from Nostoc punctiforme NIES-2108). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iad1 (Indole-3-acetaldehyde dehydrogenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iad1 from Ustilago maydis. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode AAO1 (Indole-3-acetaldehyde oxidase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode AAO1 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae) in combination with one or more sequences encoding enzymes selected from aro9 and/or aspC

and/or taa1 and/or staO and/or trpDH. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae) in combination with one or more sequences encoding enzymes selected from iad1 and/or aao1.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae) in combination with one or more sequences encoding enzymes selected from aro9 and/or aspC and/or taa1 and/or staO and in combination with one or more sequences encoding enzymes selected from iad1 and/or aao1 (see, e.g., **FIG. 92A**).

[0256] Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 92B.** In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc (Tryptophan decarboxylase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc from Catharanthus roseus). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tynA (Monoamine oxidase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tynA from E. coli). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iad1 (Indole-3-acetaldehyde dehydrogenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iad1 from Ustilago maydis). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode AAO1 (Indole-3-acetaldehyde oxidase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode AAO1 from Arabidopsis thaliana). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc and tynA. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc and one or more sequence(s) selected from iad1 and/or aao1. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tynA and one or more sequence(s) selected from iad1 and/or aao1. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tdc and tynA and one or more sequence(s) selected from iad1 and/or aao1.

[0257] Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 92C.** In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode yuc2 (indole-3-pyruvate

monooxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode yuc2 from Enterobacter cloacae. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aro9 (L-tryptophan aminotransferase). In one embodiment, the (L-tryptophan aminotransferase is from S. cerevisiae. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aro9 and yuc2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC (aspartate aminotransferase. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC from E. coli. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode aspC and yuc2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 (L-tryptophan-pyruvate aminotransferase, In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode taa1 and yuc2.In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO (L-tryptophan oxidase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO from streptomyces sp. TP-A0274. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode staO and yuc2. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH (Tryptophan dehydrogenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH from Nostoc punctiforme NIES-2108. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH and yuc2.. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of aro9 or aspC or taa1 or staO or trpDH. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of aro9 or aspC or taa1 or staO or trpDH and yuc2.

[0258] Another non-limiting example of gene sequence(s) for the production of acetic acid are shown in **FIG. 92D.** In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode IaaM (Tryptophan 2-monooxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode IaaM from Pseudomonas savastanoi). In one embodiment, the

genetically engineered bacteria comprise one or more gene sequence(s) which encode iaaH (Indoleacetamide hydrolase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iaaH from Pseudomonas savastanoi). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode IaaM and iaaH.

[0259] Another non-limiting example of gene sequence(s) for the production of acetic acid are shown in **FIG. 92E.** In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp71a13 (indoleacetaldoxime dehydratase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp71a13 from Arabidopis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode nit1 (Nitrilase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode nit1 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iaaH (Indoleacetamide hydrolase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iaaH from Pseudomonas savastanoi).In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 (tryptophan N-monooxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 and cyp71a13. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 and nit1 and/or iaaH. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 (tryptophan N-monooxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 and cyp71a13. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 and cyp71a13 and nit1 and/or iaaH. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3, cyp79B2 and cyp71a13. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3, cyp79B2 and cyp71a13, and nit1 and/or iaaH. In

one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 and cyp71a13 and nit1 and iaaH. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3, cyp79B2 and cyp71a13 and nit1 and iaaH.

[0260] Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 92F.** Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 43E**. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH (Tryptophan dehydrogenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode trpDH from Nostoc punctiforme NIES-2108. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode ipdC (Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iad1 (Indole-3-acetaldehyde dehydrogenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode iad1 from Ustilago maydis. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of trpDH and/or ipdC and/or iad1. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more of trpDH and ipdC and iad1.

[0261] In any of these embodiments, the genetically engineered bacteria which produce indole acetic acid also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in **FIG. 40, FIG. 44A and/or FIG. 44B** and described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced. In some embodiments, the genetically engineered bacteria which produce indole acetic acid also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria which produce indole acetic acid also optionally comprise one or more

gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0262] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more indole-3-acetic acid than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more indole-3-acetic acid than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more indole-3-acetic acid than unmodified bacteria of the same bacterial subtype under the same conditions.

[0263] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA.

[0264] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0265] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other

chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0266] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0267] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0268] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences described herein for the consumption of ammonia.

[0269] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0270] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0271] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0272] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of Indole-3-acetic acid and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-

acetic acid further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0273] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise a GABA transport circuit and/or a GABA metabolic circuit.. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetic acid further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0274] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Indole-3-acetonitrile

[0275] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce indole-3-acetonitrile from tryptophan. A non-limiting example of such gene sequence(s) which allow in which the genetically engineered bacteria to produce indole-3-acetonitrile from tryptophan is depicted in the figures and examples.

[0276] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 (tryptophan N-monooxygenase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp71a13 (indoleacetaldoxime dehydratase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp71a13 from Arabidopis thaliana. In

one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B2 and cyp71a13.

[0277] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 (tryptophan N-monooxygenase) In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 from Arabidopsis thaliana. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3 and cyp71a13. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode cyp79B3, cyp79B2 and cyp71a13.

[0278] In any of these embodiments, the genetically engineered bacteria which produce indole-3-acetonitrile from tryptophan also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in **FIG. 40, FIG. 44A and/or FIG. 44B** and described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced.

[0279] In some embodiments, the genetically engineered bacteria which produce indole-3-acetonitrile from tryptophan also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria which produce indole-3-acetonitrile from tryptophan also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0280] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more Indole-3-acetonitrile than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more Indole-3-acetonitrile than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-

fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more Indole-3-acetonitrile than unmodified bacteria of the same bacterial subtype under the same conditions.

[0281] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0282] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0283] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain

culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0284] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0285] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences described herein for the consumption of ammonia.

[0286] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0287] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0288] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides

for the production of Indole-3-acetonitrile further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0289] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of Indole-3-acetonitrile and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0290] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise a GABA transport circuit and/or a GABA metabolic circuit.. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-acetonitrile further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0291] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused

primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Indole-3-propionic acid (IPA)

[0680] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce indole-3-propionic acid from tryptophan. **FIG. 97 and FIG 98, and FIG. 43C** depict schematics of exemplary circuits for the production of indole-3-propionic acid.

[0681] In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding tryptophan ammonia lyase. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding tryptophan ammonia lyase from *Rubrivivax benzoatilyticus.* In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding indole-3-acrylate reductase. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding indole-3-acrylate reductase from *Clostridum botulinum.* In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding a tryptophan ammonia lyase and an indole-3-acrylate reductase. In some embodiments, the indole-3-propionate-producing strain optionally produces tryptophan from a chorismate precursor, and the strain optionally comprises additional circuits for tryptophan production and/or tryptophan uptake/transport s described herein.

[0292] The genetically engineered bacteria comprise a circuit, comprising trpDH (Tryptophan dehydrogenase, e.g., from Nostoc punctiforme NIES-2108, which produces (indol-3yl)pyruvate from tryptophan), fldA (indole-3-propionyl-CoA:indole-3-lactate CoA transferase, e.g., from Clostridium sporogenes, which converts converts indole-3-lactate and indol-3-propionyl-CoA to indole-3-propionic acid and indole-3-lactate-CoA), fldB and fldC (indole-3-lactate dehydratase e.g., from Clostridium sporogenes, which converts indole-3-lactate-CoA to indole-3-acrylyl-CoA) fldD and/or AcuI: (indole-3-acrylyl-CoA reductase, e.g., from Clostridium sporogenes and/or acrylyl-CoA reductase, e.g., from Rhodobacter sphaeroides, which convert indole-3-acrylyl-CoA to indole-3-propionyl-CoA). The circuits further comprise *fldH1 and/or fldH2* (indole-3-lactate dehydrogenase 1 and/or 2, e.g., from Clostridium sporogenes), which converts (indol-3-yl)pyruvate into indole-3-lactate) (see, e.g., **FIG. 98**).

[0293] Another embodiment of the IPA producing strain is shown in **FIG. 97**.

[0294] In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH (Tryptophan dehydrogenase). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH from Nostoc punctiforme NIES-2108. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding fldA (indole-3-propionyl-CoA:indole-3-lactate CoA transferase). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding fldA from Clostridium sporogenes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding fldB and fldC (indole-3-lactate dehydratase). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding fldB and fldC Clostridium sporogenes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding fldD (indole-3-acrylyl-CoA reductase). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding fldD from Clostridium sporogenes. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding AcuI (acrylyl-CoA reductase). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding AcuI from Rhodobacter sphaeroides. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding *fldH1* (3-lactate dehydrogenase 1). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding *fldH1* from *Clostridium sporogenes*. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding *fldH2 (*indole-3-lactate dehydrogenase 2). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding *fldH2* from Clostridium sporogenes). In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH and/or fldA and/or fldB and/or flD and/or fldH1. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH and/or fldA and/or fldB and/or flD and/or fldH2. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH and/or fldA and/or fldB and/or acul and/or fldH1. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH and/or fldA and/or fldB and/or acul and/or fldH2. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH and fldA and fldB and flD and fldH1. In some embodiments, the genetically engineered bacteria comprise one or more gene

sequences encoding trpDH and fldA and fldB and flD and fldH2. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH and fldA and fldB and acul and fldH1. In some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding trpDH and fldA and fldB and acul and fldH2.

[0295] In any of these embodiments, the genetically engineered bacteria which produce indole-3-propionic acid also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in **FIG. 40, FIG. 44A and/or FIG. 44B** and described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced. In some embodiments, the genetically engineered bacteria which produce indole-3-propionic acid also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria which produce indole-3-propionic acid also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0296] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more Indole-3-propionic acidthan unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more Indole-3-propionic acidthan unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more Indole-3-propionic acidthan unmodified bacteria of the same bacterial subtype under the same conditions.

[0297] In certain embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of tryptophan metabolites. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 different tryptophan metabolites. In

certain embodiments the bacteria comprise one or more gene sequence(s) encoding one or more enzymes for the production of tryptophan metabolites selected from tryptamine and/or indole-3 acetaldehyde, indole-3acetonitrile, kynurenine, kynurenic acid, indole, indole acetic acid FICZ, indole-3-propionic acid.

[0298] In some embodiments, the genetically engineered bacteria are capable of producing such tryptophan metabolites under inducing conditions, *e.g.*, under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing such tryptophan metabolites in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0299] In some embodiments, the gene sequences(s) are controlled by an inducible promoter. In some embodiments, the gene sequences(s) are controlled by a constitutive promoter. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constritutive promoter, and are expressed during bacterial culture in vitro, e.g., for bacterial expansion, production and/or manufacture, as described herein.

[0300] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0301] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or

indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0302] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or inflammation, or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0303] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0304] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides

for the production of Indole-3-propionic acid further comprise one or more gene sequences described herein for the consumption of ammonia.

[0305] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0306] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0307] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0308] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of Indole-3-propionic acid and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically

engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0309] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise a GABA transport circuit and/or a GABA metabolic circuit. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole-3-propionic acid further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0310] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Indole

[0682] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce indole from tryptophan. Non-limiting example of such gene sequence(s) are shown **FIG. 91G** and described elsewhere herein. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tnaA (tryptophanase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode tnaA from E. coli.

[0683] In any of these embodiments, the genetically engineered bacteria which produce indole from tryptophan also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in **FIG. 40, FIG. 44A and/or FIG. 44B** and described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced. In some

embodiments, the genetically engineered bacteria which produce indole from tryptophan also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria which produce indole from tryptophan also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0684] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more Indole than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more Indole than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more Indole than unmodified bacteria of the same bacterial subtype under the same conditions.

[0685] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, e.g., bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0686] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0687] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia, or inflammation, or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0688] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Indole are present on one or more plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0689] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences described herein for the consumption of ammonia.

[0690] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0691] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0692] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0693] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of Indole and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences for the secretion of IL-22. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences for the secretion of GLP2. In any of the

embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0694] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise a GABA transport circuit and/or a GABA metabolic circuit. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Indole further comprise one or more circuits for producing a manganese membrane transport protein, e.g., MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0695] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Other indole metabolites

[0311] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more tryptophan catabolism enzymes, which produce indole-3-carbinol, indole-3-aldehyde, 3,3' diindolylmethane (DIM), indolo(3,2-b) carbazole (ICZ) from indole glucosinolate taken up through the diet. Non-limiting example of such gene sequence(s) are shown **FIG. 91H** and described elsewhere herein. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode pne2 (myrosinase). In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) which encode pne2from Arabidopsis thaliana.

[0312] In any of these embodiments, the genetically engineered bacteria also optionally comprise one or more gene sequence(s) comprising one or more enzymes for tryptophan production, and gene deletions/or mutations as depicted and described in **FIG. 40, FIG. 44A and/or FIG. 44B** and described elsewhere herein. In some embodiments, AroG and/or TrpE are replaced with feedback resistant versions to improve tryptophan production in the genetically engineered bacteria. In some embodiments, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase

levels of tryptophan produced. In some embodiments, the genetically engineered bacteria also optionally comprise one or more gene sequence(s) which encode one or more transporter(s) as described herein, through which tryptophan can be imported. Optionally, in some embodiments, the genetically engineered bacteria also optionally comprise one or more gene sequence(s) which encode an exporter as described herein, which can export tryptophan or any of its metabolites.

[0313] In some embodiments, the genetically engineered bacteria produce 0% to to 2% to 4%, 4% to 6%,6% to 8%, 8% to 10%, 10% to 12%, 12% to 14%, 14% to 16%, 16% to 18%, 18% to 20%, 20% to 25%,25% to 30%, 30% to 35%, 35% to 40%,40% to 45% 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70% to 80%, 80% to 90%, or 90% to 100% more Other indoles than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce 1.0-1.2-fold, 1.2-1.4-fold, 1.4-1.6-fold, 1.6-1.8-fold, 1.8-2-fold, or two-fold more Other indoles than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the genetically engineered bacteria produce three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, fifteen-fold, twenty-fold, thirty-fold, forty-fold, or fifty-fold, more Other indoles than unmodified bacteria of the same bacterial subtype under the same conditions.

[0314] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Other indole metabolites is operably linked to an inducible promoter. In some embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In some embodiments, the inducible promoter is directly or indirectly induced under condition(s) found in the gut in vivo, e.g., low oxygen conditions. In some embodiments, the promoter is induced in the presence of certain molecules or metabolites, e.g., metabolites found in the gut. In some embodiments, such molecules or metabolites are specific to certain conditions, e.g., conditions associated with hyperammonemia, inflammation, or liver damage. In some embodiments, the genetically engineered bacteria comprise a promoter induced by a molecule or metabolite associated with liver damage, *e.g.*, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, or manganese.

[0315] In some embodiments, the promoter is induced in the presence of some other metabolite that may or may not be present in vivo, e.g., the gut, such as arabinose and other chemical inducers described herein. In some embodiments, the promoter is directly or indirectly induced under in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the promoter is induced in vitro by one or more chemical and/or nutritional inducers, such as arabinose and others described herein. In some embodiments, the promoter is directly or indirectly induced in vitro under low oxygen conditions or other conditions described herein. In some embodiments, the promoter is directly or indirectly induced in vitro and/or in vivo, under certain conditions described herein.

[0316] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Other indole metabolites is operably linked to a constitutive promoter. In some embodiments, the constitutive promoter is active under exogenous in vivo conditions, e.g., found in the gut, or under conditions present during hyperammonemia or as a consequence of liver damage or disease. In some embodiments, the constitutive promoter is active in in vitro conditions, e.g., during strain culture, expansion, production and/or manufacture. In some embodiments, the constitutive promoter is selected from a promoter provided in **Table 27-37**. In any of the embodiments described above and elsewhere herein, gene expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Other indole metabolites is operably linked to a RBS, enhancer or other regulatory sequence. In some embodiments, the RBS is selected from a promoter provided in **Table 27-37** or is listed in **Table 38-39**. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Other indole metabolites is modified and/or mutated, e.g., to enhance stability, or increase tryptophan/tryptophan metabolite production or catalysis.

[0317] In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Other indole metabolites may be codon optimized, e.g., to improve expression in the host microorganism. In any of the embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides for the production of Other indole metabolites are present on one or more

plasmids (e.g., high copy or low copy) or are integrated into one or more sites in the microorganism chromosome.

[0318] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences described herein for the consumption of ammonia.

[0319] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences for the production of one or more gut barrier enhancer molecules and/or anti-inflammatory molecules known in the art or described herein.

[0320] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of butyrate.

[0321] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of propionate.

[0322] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of acetate. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences and/or deletions of endogenous genes described herein, for the production of Other indole metabolites and/or one or more of its metabolites described herein. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences for the secretion of an anti-inflammatory cytokine. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences for the secretion of IL-22. In

any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences for the secretion of GLP2. In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more gene sequences for the secretion of a satiety effector, e.g., GLP1.

[0323] In any of the embodiments described above and elsewhere herein, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise a GABA transport circuit and/or a GABA metabolic circuit.. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding one or more polypeptides for the production of Other indole metabolites further comprise one or more circuits for producing a manganese membrane transport protein, *e.g.*, MntH, and are capable of transporting manganese ions into the cell (a "manganese transport circuit").

[0324] In any of the embodiments described herein, the genetically engineered bacteria may further comprise a resistance to rifaximin. Resistance to rifaximin is caused primarily by mutations in the rpoB gene. In some embodiments, the genetically engineered bacteria comprise a known rifaximin resistance mutation, e.g., in the rpoB gene. In other embodiments, a screen can be employed, exposing the genetically engineered bacteria to increasing amounts of rifaximin, to identify a useful mutation which confers rifaximin resistance.

Tryptophan Catabolic Pathway Enzymes

[0696] **Table D and E** comprises sequences of such enzymes which are encoded by the genetically engineered bacteria of the disclosure.

Table D. Tryptophan Pathway Enzymes

Description	**Sequence**
Trp aminotransferase (EC 2.6.1.27); tryptophan aminotransferase [Cryptococcus deuterogattii R265], codon optimized for expression in E. coli	ATGACGGCAACTACAATTTCTATTGAGACCGTACCTCAGGC CCCGGCGGCGGGGACCAAAACTAATGGGACTTCAGGAAAA TACAACCCCCGCACTTACCTGTCCGACCGCGCCAAAGTCAC TGAGATTGATGGATCTGACGCCGGTCGCCCCAATCCCGATA CTTTCCCATTTAACTCGATTACCTTAAATTTGAAACCACCTT TAGGCTTGCCCGAGAGTTCAAATAACATGCCGGTCTCTATC ACGATTGAAGACCCCGATTTAGCGACGGCCTTACAATATG CACCTAGCGCCGGTATTCCTAAGCTGCGCGAATGGCTGGCT GACTTACAAGCTCACGTTCATGAGCGCCCCCGTGGCGATTA

SEQ ID NO: 229	TGCCATCTCGGTCGGGTCGGGGTCACAGGATTTGATGTTTA AGGGCTTCCAAGCTGTCTTGAATCCAGGTGATCCAGTCCTT CTGGAAACCCCAATGTATTCAGGTGTTCTGCCAGCGCTGCG CATTCTGAAGGCGGATTATGCAGAAGTTGATGTAGACGAC CAGGGGTTATCTGCTAAAAACCTTGAAAAAGTTTTATCAGA GTGGCCCGCAGATAAGAAGCGTCCTCGTGTCCTGTATACGT CGCCAATCGGCTCCAATCCTTCCGGATGTTCAGCATCCAAG GAACGCAAGTTAGAGGTACTGAAAGTCTGTAAGAAGTACG ATGTGCTGATCTTCGAAGACGATCCGTATTATTACCTTGCT CAAGAGCTTATTCCATCCTATTTTGCGTTGGAAAAACAAGT TTATCCGGAGGGTGGGCACGTTGTACGCTTTGACTCATTTA GTAAATTGCTTTCTGCTGGGATGCGCTTGGGATTTGCTACA GGGCCGAAGGAAATTCTTCATGCGATTGACGTCAGTACAG CAGGCGCAAATTTACATACTTCAGCGGTCTCTCAAGGTGTC GCTCTTCGCCTGATGCAGTATTGGGGGATCGAGGGATTCCT TGCACATGGCCGCGCGGTGGCCAAACTTTACACGGAGCGC CGCGCTCAGTTCGAGGCAACCGCACATAAGTACCTGGACG GGCTGGCCACTTGGGTATCTCCCGTAGCGGGAATGTTTTTA TGGATCGATCTTCGTCCAGCAGGAATCGAAGATTCTTACGA ATTAATTCGCCATGAAGCATTAGCCAAAGGCGTTTTAGGCG TTCCAGGGATGGCGTTTTATCCGACAGGCCGTAAGTCTTCC CATGTTCGTGTCAGTTTCAGTATCGTCGACCTGGAAGACGA ATCTGACCTTGGTTTTCAACGCCTGGCTGAAGCTATTAAGG ATAAACGCAAGGCTTTAGGGCTGGCT
Tryptophan Decarboxylase (EC 4.1.1.28) Chain A, Ruminococcus Gnavus **Tryptophan Decarboxylase** Rum gna_01526 (alpha-fmt); codon optimized for the expression in E. coli **SEQ ID NO: 230**	ATGAGTCAAGTGATTAAGAAGAAACGTAACACCTTTATGA TCGGAACGGAGTACATTCTTAACAGTACACAATTGGAGGA AGCGATTAAATCATTCGTACATGATTTCTGCGCAGAGAAGC ATGAGATCCATGATCAACCTGTGGTAGTAGAAGCTAAAGA ACATCAGGAGGACAAAATCAAACAAATCAAAATCCCGGAA AAGGGACGTCCTGTAAATGAAGTCGTTTCTGAGATGATGA ATGAAGTGTATCGCTACCGCGGAGACGCCAACCATCCTCG CTTTTTTTCTTTTGTGCCCGGACCTGCAAGCAGTGTGTCGTG GTTGGGGGATATTATGACGTCCGCCTACAATATTCATGCTG GAGGCTCAAAGCTGGCACCGATGGTTAACTGCATTGAGCA GGAAGTTCTGAAGTGGTTAGCAAAGCAAGTGGGGTTCACA GAAAATCCAGGTGGCGTATTTGTGTCGGGCGGTTCAATGG CGAATATTACGGCACTTACTGCGGCTCGTGACAATAAACTG ACCGACATTAACCTTCATTTGGGAACTGCTTATATTAGTGA CCAGACTCATAGTTCAGTTGCGAAAGGATTACGCATTATTG GAATCACTGACAGTCGCATCCGTCGCATTCCCACTAACTCC CACTTCCAGATGGATACCACCAAGCTGGAGGAAGCCATCG AGACCGACAAGAAGTCTGGCTACATTCCGTTCGTCGTTATC GGAACAGCAGGTACCACCAACACTGGTTCGATTGACCCCC TGACAGAAATCTCTGCGTTATGTAAGAAGCATGACATGTG GTTTCATATCGACGGAGCGTATGGAGCTAGTGTTCTGCTGT CACCTAAGTACAAGAGCCTTCTTACCGGAACCGGCTTGGCT GACAGTATTTCGTGGGATGCTCATAAATGGTTGTTCCAAAC

	GTACGGCTGTGCAATGGTACTTGTCAAAGATATCCGTAATT TATTCCACTCTTTTCATGTGAATCCCGAGTATCTTAAGGAT CTGGAAAACGACATCGATAACGTTAATACATGGGACATCG GCATGGAGCTGACGCGCCCTGCACGCGGTCTTAAATTGTG GCTTACTTTACAGGTCCTTGGATCTGACTTGATTGGGAGTG CCATTGAACACGGTTTCCAGCTGGCAGTTTGGGCTGAGGA AGCATTGAATCCAAAGAAAGACTGGGAGATCGTTTCTCCA GCTCAGATGGCTATGATTAATTTCCGTTATGCCCCTAAGGA TTTAACCAAAGAGGAACAGGATATTCTGAATGAAAAGATC TCCCACCGCATTTTAGAGAGCGGATACGCTGCAATTTTCAC TACTGTATTAAACGGCAAGACCGTTTTACGCATCTGTGCAA TTCACCCGGAGGCAACTCAAGAGGATATGCAACACACAAT CGACTTATTAGACCAATACGGTCGTGAAATCTATACCGAG ATGAAGAAAGCG
Tdc (tdc from C. roseus) **SEQ ID NO: 231**	ATGGGTTCTATTGACTCGACGAATGTGGCCATGTCTAATTC TCCTGTTGGCGAGTTTAAGCCCCTTGAAGCAGAAGAGTTCC GTAAACAGGCACACCGCATGGTGGATTTTATTGCGGATTAT TACAAGAACGTAGAAACATACCCGGTCCTTTCCGAGGTTG AACCCGGCTATCTGCGCAAACGTATTCCCGAAACCGCACC ATACCTGCCGGAGCCACTTGATGATATTATGAAGGATATTC AAAAGGACATTATCCCCGGAATGACGAACTGGATGTCCCC GAACTTTTACGCCTTCTTCCCGGCCACAGTTAGCTCAGCAG CTTTCTTGGGGGAAATGCTTTCAACGGCCCTTAACAGCGTA GGATTTACCTGGGTCAGTTCCCCGGCAGCGACTGAATTAGA GATGATCGTTATGGATTGGCTTGCGCAAATTTTGAAACTTC CAAAAAGCTTTATGTTCTCCGGAACCGGGGGTGGTGTCATC CAAAACACTACGTCAGAGTCGATCTTGTGCACTATTATCGC GGCCCGTGAACGCGCCTTGGAAAAATTGGGCCCTGATTCA ATTGGTAAGCTTGTCTGCTATGGGTCCGATCAAACGCACAC AATGTTTCCGAAAACCTGTAAGTTAGCAGGAATTTATCCGA ATAATATCCGCCTTATCCCTACCACGGTAGAAACCGACTTT GGCATCTCACCGCAGGTACTTCGCAAGATGGTCGAAGACG ACGTCGCTGCGGGGTACGTTCCCTTATTTTTGTGTGCCACC TTGGGAACGACATCAACTACGGCAACAGATCCTGTAGATT CGCTGTCCGAAATCGCAAACGAGTTTGGTATCTGGATTCAT GTCGACGCCGCATATGCTGGATCGGCTTGCATCTGCCCAGA ATTTCGTCACTACCTTGATGGCATCGAACGTGTGGATTCCT TATCGCTGTCTCCCCACAAATGGCTTTTAGCATATCTGGAT TGCACGTGCTTGTGGGTAAAACAACCTCACCTGCTGCTTCG CGCTTTAACGACTAATCCCGAATACTTGAAGAATAAACAG AGTGATTTAGATAAGGTCGTGGATTTTAAGAACTGGCAGA TCGCAACAGGACGTAAGTTCCGCTCTTTAAAACTTTGGTTA ATTCTGCGTTCCTACGGGGTAGTTAACCTGCAAAGTCATAT CCGTAGTGATGTAGCGATGGGGAAGATGTTTGAGGAATGG GTCCGTTCCGATAGCCGCTTTGAAATCGTCGTGCCACGTAA TTTTTCGCTTGTATGCTTTCGCTTGAAACCGGATGTATCTAG TTTACATGTCGAGGAGGTCAACAAGAAGTTGTTGGATATG CTTAACTCCACCGGTCGCGTATATATGACGCATACAATTGT

	TGGCGGAATCTATATGTTACGTTTGGCTGTAGGTAGCAGCT TGACAGAGGAACATCACGTGCGCCGCGTTTGGGACTTGAT CCAGAAGCTTACGGACGACCTGCTTAAAGAGGCGTGA
Tdc (tdc from *Clostridium sporogenes*) **SEQ ID NO: 232**	ATGAAATTTTGGCGCAAGTATACGCAACAGGAGATGGATG AGAAAATCACAGAATCGCTTGAGAAGACATTAAATTACGA TAACACGAAAACCATCGGCATCCCAGGTACTAAGCTGGAT GATACTGTATTTTATGACGATCACTCCTTCGTTAAGCACTC TCCCTATTTACGTACGTTCATCCAAAACCCTAATCACATTG GTTGTCACACGTACGATAAAGCAGACATCTTGTTTGGCGGC ACGTTTGACATCGAACGCGAACTGATTCAGCTTTTGGCCAT CGATGTCTTAAACGGAAATGATGAGGAATTCGATGGATAT GTGACACAGGGGGGAACCGAGGCGAATATTCAGGCAATGT GGGTTTATCGTAACTATTTCAAAAAAGAACGTAAAGCAAA ACATGAGGAAATCGCAATCATCACGAGCGCGGATACCCAT TACAGTGCATATAAGGGGAGCGACTTGCTGAACATTGATA TTATCAAGGTCCCAGTAGACTTCTATTCGCGTAAGATCCAG GAGAACACGTTAGACTCGATTGTCAAGGAGGCGAAGGAAA TTGGAAAGAAGTACTTCATTGTCATCTCAAACATGGGTACG ACTATGTTTGGCAGTGTAGACGACCCTGATCTTTATGCTAA CATTTTTGATAAGTATAACTTAGAATACAAAATCCACGTCG ATGGAGCTTTTGGGGGTTTCATTTATCCTATCGATAATAAG GAGTGCAAAACAGATTTCTCGAACAAGAACGTCTCATCCA TCACGCTTGACGGTCACAAAATGCTTCAAGCCCCCTATGGG ACTGGTATCTTCGTGTCACGTAAGAACTTGATCCATAACAC CCTGACAAAGGAAGCAACGTATATTGAAAACCTGGACGTT ACCCTGAGTGGGTCCCGCTCCGGATCCAACGCCGTTGCGAT CTGGATGGTTTTAGCCTCTTATGGCCCCTACGGGTGGATGG AGAAGATTAACAAGTTGCGCAATCGCACTAAGTGGCTTTG CAAGCAGCTTAACGACATGCGCATCAAATACTATAAGGAG GATAGCATGAATATCGTCACGATTGAAGAGCAATACGTAA ATAAAGAGATTGCAGAGAAATACTTCCTTGTGCCTGAAGT ACACAATCCTACCAACAATTGGTACAAGATTGTAGTCATG GAACATGTTGAACTTGACATCTTGAACTCCCTTGTTTATGA TTTACGTAAATTCAACAAGGAGCACCTGAAGGCAATGTGA
trpDH **SEQ ID NO: 233**	ATGCTGTTATTCGAGACTGTGCGTGAAATGGGTCATGAGCA AGTCCTTTTCTGTCATAGCAAGAATCCCGAGATCAAGGCAA TTATCGCAATCCACGATACCACCTTAGGACCGGCTATGGGC GCAACTCGTATCTTACCTTATATTAATGAGGAGGCTGCCCT GAAAGATGCATTACGTCTGTCCCGCGGAATGACTTACAAA GCAGCCTGCGCCAATATTCCCGCCGGGGGCGGCAAAGCCG TCATCATCGCTAACCCCGAAAACAAGACCGATGACCTGTT ACGCGCATACGGCCGTTTCGTGGACAGCTTGAACGGCCGTT TCATCACCGGGCAGGACGTTAACATTACGCCCGACGACGT TCGCACTATTTCGCAGGAGACTAAGTACGTGGTAGGCGTCT CAGAAAAGTCGGGAGGGCCGGCACCTATCACCTCTCTGGG AGTATTTTTAGGCATCAAAGCCGCTGTAGAGTCGCGTTGGC AGTCTAAACGCCTGGATGGCATGAAAGTGGCGGTGCAAGG ACTTGGGAACGTAGGAAAAAATCTTTGTCGCCATCTGCATG AACACGATGTACAACTTTTTGTGTCTGATGTCGATCCAATC AAGGCCGAGGAAGTAAAACGCTTATTCGGGGCGACTGTTG

	TCGAACCGACTGAAATCTATTCTTTAGATGTTGATATTTTT GCACCGTGTGCACTTGGGGGTATTTTGAATAGCCATACCAT CCCGTTCTTACAAGCCTCAATCATCGCAGGAGCAGCGAAT AACCAGCTGGAGAACGAGCAACTTCATTCGCAGATGCTTG CGAAAAAGGGTATTCTTTACTCACCAGACTACGTTATCAAT GCAGGAGGACTTATCAATGTTTATAACGAAATGATCGGAT ATGACGAGGAAAAAGCATTCAAACAAGTTCATAACATCTA CGATACGTTATTAGCGATTTTCGAAATTGCAAAAGAACAA GGTGTAACCACCAACGACGCGGCCCGTCGTTTAGCAGAGG ATCGTATCAACAACTCCAAACGCTCAAAGAGTAAAGCGAT TGCGGCGTGA
ipdC **SEQ ID NO: 234**	ATGCGTACACCCTACTGTGTCGCCGATTATCTTTTAGATCG TCTGACGGACTGCGGGGCCGATCACCTGTTTGGCGTACCGG GCGATTACAACTTGCAGTTTCTGGACCACGTCATTGACTCA CCAGATATCTGCTGGGTAGGGTGTGCGAACGAGCTTAACG CGAGCTACGCTGCTGACGGATATGCGCGTTGTAAAGGCTTT GCTGCACTTCTTACTACCTTCGGGGTCGGTGAGTTATCGGC GATGAACGGTATCGCAGGCTCGTACGCTGAGCACGTCCCG GTATTACACATTGTGGGAGCTCCGGGTACCGCAGCTCAAC AGCGCGGAGAACTGTTACACCACACGCTGGGCGACGGAGA ATTCCGCCACTTTTACCATATGTCCGAGCCAATTACTGTAG CCCAGGCTGTACTTACAGAGCAAAATGCCTGTTACGAGAT CGACCGTGTTTTGACCACGATGCTTCGCGAGCGCCGTCCCG GGTATTTGATGCTGCCAGCCGATGTTGCCAAAAAAGCTGC GACGCCCCCAGTGAATGCCCTGACGCATAAACAAGCTCAT GCCGATTCCGCCTGTTTAAAGGCTTTTCGCGATGCAGCTGA AAATAAATTAGCCATGTCGAAACGCACCGCCTTGTTGGCG GACTTTCTGGTCCTGCGCCATGGCCTTAAACACGCCCTTCA GAAATGGGTCAAAGAAGTCCCGATGGCCCACGCTACGATG CTTATGGGTAAGGGGATTTTTGATGAACGTCAAGCGGGATT TTATGGAACTTATTCCGGTTCGGCGAGTACGGGGGCGGTA AAGGAAGCGATTGAGGGAGCCGACACAGTTCTTTGCGTGG GGACACGTTTCACCGATACACTGACCGCTGGATTCACACAC CAACTTACTCCGGCACAAACGATTGAGGTGCAACCCCATG CGGCTCGCGTGGGGGATGTATGGTTTACGGGCATTCCAATG AATCAAGCCATTGAGACTCTTGTCGAGCTGTGCAAACAGC ACGTCCACGCAGGACTGATGAGTTCGAGCTCTGGGGCGAT TCCTTTTCCACAACCAGATGGTAGTTTAACTCAAGAAAACT TCTGGCGCACATTGCAAACCTTTATCCGCCCAGGTGATATC ATCTTAGCAGACCAGGGTACTTCAGCCTTTGGAGCAATTGA CCTGCGCTTACCAGCAGACGTGAACTTTATTGTGCAGCCGC TGTGGGGGTCTATTGGTTATACTTTAGCTGCGGCCTTCGGA GCGCAGACAGCGTGTCCAAACCGTCGTGTGATCGTATTGA CAGGAGATGGAGCAGCGCAGTTGACCATTCAGGAGTTAGG CTCGATGTTACGCGATAAGCAGCACCCCATTATCCTGGTCC TGAACAATGAGGGGTATACAGTTGAACGCGCCATTCATGG TGCGGAACAACGCTACAATGACATCGCTTTATGGAATTGG ACGCACATCCCCCAAGCCTTATCGTTAGATCCCCAATCGGA ATGTTGGCGTGTGTCTGAAGCAGAGCAACTGGCTGATGTTC TGGAAAAAGTTGCTCATCATGAACGCCTGTCGTTGATCGAG

	GTAATGTTGCCCAAGGCCGATATCCCTCCGTTACTGGGAGC CTTGACCAAGGCTTTAGAAGCCTGCAACAACGCTTAA
Iad1 **SEQ ID NO: 235**	ATGCCCACCTTGAACTTGGACTTACCCAACGGTATTAAGAG CACGATTCAGGCAGACCTTTTCATCAATAATAAGTTTGTGC CGGCGCTTGATGGGAAAACGTTCGCAACTATTAATCCGTCT ACGGGGAAAGAGATCGGACAGGTGGCAGAGGCTTCGGCG AAGGATGTGGATCTTGCAGTTAAGGCCGCGCGTGAGGCGT TTGAAACTACTTGGGGGGAAAACACGCCAGGTGATGCTCG TGGCCGTTTACTGATTAAGCTTGCTGAGTTGGTGGAAGCGA ATATTGATGAGTTAGCGGCAATTGAATCACTGGACAATGG GAAAGCGTTCTCTATTGCTAAGTCATTCGACGTAGCTGCTG TGGCCGCAAACTTACGTTACTACGGCGGTTGGGCTGATAA AAACCACGGTAAAGTCATGGAGGTAGACACAAAGCGCCTG AACTATACCCGCCACGAGCCGATCGGGGTTTGCGGACAAA TCATTCCGTGGAATTTCCCGCTTTTGATGTTTGCATGGAAG CTGGGTCCCGCTTTAGCCACAGGGAACACAATTGTGTTAAA GACTGCCGAGCAGACTCCCTTAAGTGCTATCAAGATGTGTG AATTAATCGTAGAAGCCGGCTTTCCGCCCGGAGTAGTTAAT GTGATCTCGGGATTCGGACCGGTGGCGGGGGCCGCGATCT CGCAACACATGGACATCGATAAGATTGCCTTTACAGGATC GACATTGGTTGGCCGCAACATTATGAAGGCAGCTGCGTCG ACTAACTTAAAAAAGGTTACACTTGAGTTAGGAGGAAAAT CCCCGAATATCATTTTCAAAGATGCCGACCTTGACCAAGCT GTTCGCTGGAGCGCCTTCGGTATCATGTTTAACCACGGACA ATGCTGCTGCGCTGGATCGCGCGTATATGTGGAAGAATCC ATCTATGACGCCTTCATGGAAAAAATGACTGCGCATTGTAA GGCGCTTCAAGTTGGAGATCCTTTCAGCGCGAACACCTTCC AAGGACCACAAGTCTCGCAGTTACAATACGACCGTATCAT GGAATACATCGAATCAGGGAAAAAAGATGCAAATCTTGCT TTAGGCGGCGTTCGCAAAGGGAATGAGGGGTATTTCATTG AGCCAACTATTTTTACAGACGTGCCGCACGACGCGAAGAT TGCCAAAGAGGAGATCTTCGGTCCAGTGGTTGTTGTGTCGA AATTTAAGGACGAAAAAGATCTGATCCGTATCGCAAATGA TTCTATTTATGGTTTAGCTGCGGCAGTCTTTTCCCGCGACAT CAGCCGCGCGATCGAGACAGCACACAAACTGAAAGCAGGC ACGGTCTGGGTCAACTGCTATAATCAGCTTATTCCGCAGGT GCCATTCGGAGGGTATAAGGCTTCCGGTATCGGCCGTGAG TTGGGGGAATATGCCTTGTCTAATTACACAAATATCAAGGC CGTCCACGTTAACCTTTCTCAACCGGCGCCCATTTGA
fldA **SEQ ID NO: 236**	ATGGAAAACAACACCAATATGTTCTCTGGAGTGAAGGTGA TCGAACTGGCCAACTTTATCGCTGCTCCGGCGGCAGGTCGC TTCTTTGCTGATGGGGGAGCAGAAGTAATTAAGATCGAAT CTCCAGCAGGCGACCCGCTGCGCTACACGGCCCCATCAGA AGGACGCCCGCTTTCTCAAGAGGAAAACACAACGTATGAT TTGGAAAACGCGAATAAGAAAGCAATTGTTCTGAACTTAA AATCGGAAAAAGGAAAGAAAATTCTTCACGAGATGCTTGC TGAGGCAGACATCTTGTTAACAAATTGGCGCACGAAAGCG TTAGTCAAACAGGGGTTAGATTACGAAACACTGAAAGAGA AGTATCCAAAATTGGTATTTGCACAGATTACAGGATACGG GGAGAAAGGACCCGACAAAGACCTGCCTGGTTTCGACTAC

	ACGGCGTTTTTCGCCCGCGGAGGAGTCTCCGGTACATTATA TGAAAAAGGAACTGTCCCTCCTAATGTGGTACCGGGTCTG GGTGACCACCAGGCAGGAATGTTCTTAGCTGCCGGTATGG CTGGTGCGTTGTATAAGGCCAAAACCACCGGACAAGGCGA CAAAGTCACCGTTAGTCTGATGCATAGCGCAATGTACGGC CTGGGAATCATGATTCAGGCAGCCCAGTACAAGGACCATG GGCTGGTGTACCCGATCAACCGTAATGAAACGCCTAATCCT TTCATCGTTTCATACAAGTCCAAAGATGATTACTTTGTCCA AGTTTGCATGCCTCCCTATGATGTGTTTTATGATCGCTTTAT GACGGCCTTAGGACGTGAAGACTTGGTAGGTGACGAACGC TACAATAAGATCGAGAACTTGAAGGATGGTCGCGCAAAAG AAGTCTATTCCATCATCGAACAACAAATGGTAACGAAGAC GAAGGACGAATGGGACAAGATTTTTCGTGATGCAGACATT CCATTCGCTATTGCCCAAACGTGGGAAGATCTTTTAGAAGA CGAGCAGGCATGGGCCAACGACTACCTGTATAAAATGAAG TATCCCACAGGCAACGAACGTGCCCTGGTACGTTTACCTGT GTTCTTCAAAGAAGCTGGACTTCCTGAATACAACCAGTCGC CACAGATTGCTGAGAATACCGTGGAAGTGTTAAAGGAGAT GGGATATACCGAGCAAGAAATTGAGGAGCTTGAGAAAGAC AAAGACATCATGGTACGTAAAGAGAAATGA
fldB **SEQ ID NO: 237**	ATGTCAGACCGCAACAAAGAAGTGAAAGAAAAGAAGGCT AAACACTATCTGCGCGAGATCACAGCTAAACACTACAAGG AAGCGTTAGAGGCTAAAGAGCGTGGGGAGAAAGTGGGTTG GTGTGCCTCTAACTTCCCCCAAGAGATTGCAACCACGTTGG GTGTAAAGGTTGTTTATCCCGAAAACCACGCCGCCGCCGTA GCGGCACGTGGCAATGGGCAAAATATGTGCGAACACGCGG AGGCTATGGGATTCAGTAATGATGTGTGTGGATATGCACGT GTAAATTTAGCCGTAATGGACATCGGCCATAGTGAAGATC AACCTATTCCAATGCCTGATTTCGTTCTGTGCTGTAATAAT ATCTGCAATCAGATGATTAAATGGTATGAACACATTGCAA AAACGTTGGATATTCCTATGATCCTTATCGATATTCCATAT AATACTGAGAACACGGTGTCTCAGGACCGCATTAAGTACA TCCGCGCCCAGTTCGATGACGCTATCAAGCAACTGGAAGA AATCACTGGCAAAAAGTGGGACGAGAATAAATTCGAAGAA GTGATGAAGATTTCGCAAGAATCGGCCAAGCAATGGTTAC GCGCCGCGAGCTACGCGAAATACAAACCATCACCGTTTTC GGGCTTTGACCTTTTTAATCACATGGCTGTAGCCGTTTGTG CTCGCGGCACCCAGGAAGCCGCCGATGCATTCAAAATGTT AGCAGATGAATATGAAGAGAACGTTAAGACAGGAAAGTCT ACTTATCGCGGCGAGGAGAAGCAGCGTATCTTGTTCGAGG GCATCGCTTGTTGGCCTTATCTGCGCCACAAGTTGACGAAA CTGAGTGAATATGGAATGAACGTCACAGCTACGGTGTACG CCGAAGCTTTTGGGGTTATTTACGAAAACATGGATGAACTG ATGGCCGCTTACAATAAAGTGCCTAACTCAATCTCCTTCGA GAACGCGCTGAAGATGCGTCTTAATGCCGTTACAAGCACC AATACAGAAGGGGCTGTTATCCACATTAATCGCAGTTGTA AGCTGTGGTCAGGATTCTTATACGAACTGGCCCGTCGTTTG GAAAAGGAGACGGGGATCCCTGTTGTTTCGTTCGACGGAG ATCAAGCGGATCCCCGTAACTTCTCCGAGGCTCAATATGAC ACTCGCATCCAAGGTTTAAATGAGGTGATGGTCGCGAAAA

	AAGAAGCAGAGTGA
fldC **SEQ ID NO: 238**	ATGTCGAATAGTGACAAGTTTTTTAACGACTTCAAGGACAT TGTGGAAAACCCAAAGAAGTATATCATGAAGCATATGGAA CAAACGGGACAAAAAGCCATCGGTTGCATGCCTTTATACA CCCCAGAAGAGCTTGTCTTAGCGGCGGGTATGTTTCCTGTT GGAGTATGGGGCTCGAATACTGAGTTGTCAAAAGCCAAGA CCTACTTTCCGGCTTTTATCTGTTCTATCTTGCAAACTACTT TAGAAAACGCATTGAATGGGGAGTATGACATGCTGTCTGG TATGATGATCACAAACTATTGCGATTCGCTGAAATGTATGG GACAAAACTTCAAACTTACAGTGGAAAATATCGAATTCAT CCCGGTTACGGTTCCACAAAACCGCAAGATGGAGGCGGGT AAAGAATTTCTGAAATCCCAGTATAAAATGAATATCGAAC AACTGGAAAAAATCTCAGGGAATAAGATCACTGACGAGAG CTTGGAGAAGGCTATTGAAATTTACGATGAGCACCGTAAA GTCATGAACGATTTCTCTATGCTTGCGTCCAAGTACCCTGG TATCATTACGCCAACGAAACGTAACTACGTGATGAAGTCA GCGTATTATATGGACAAGAAAGAACATACAGAGAAGGTAC GTCAGTTGATGGATGAAATCAAGGCCATTGAGCCTAAACC ATTCGAAGGAAAACGCGTGATTACCACTGGGATCATTGCA GATTCGGAGGACCTTTTGAAAATCTTGGAGGAGAATAACA TTGCTATCGTGGGAGATGATATTGCACACGAGTCTCGCCAA TACCGCACTTTGACCCCGGAGGCCAACACACCTATGGACC GTCTTGCTGAACAATTTGCGAACCGCGAGTGTTCGACGTTG TATGACCCTGAAAAAAAACGTGGACAGTATATTGTCGAGA TGGCAAAAGAGCGTAAGGCCGACGGAATCATCTTCTTCAT GACAAAATTCTGCGATCCCGAAGAATACGATTACCCTCAG ATGAAAAAAGACTTCGAAGAAGCCGGTATTCCCCACGTTC TGATTGAGACAGACATGCAAATGAAGAACTACGAACAAGC TCGCACCGCTATTCAAGCATTTTCAGAAACCCTTTG
AcuI **SEQ ID NO: 239**	ATGCGTGCTGTCTTAATCGAGAAGTCAGATGACACCCAGA GTGTTTCAGTTACGGAGTTGGCTGAAGACCAATTACCCGAA GGTGACGTCCTTGTGGATGTCGCGTACAGCACATTGAATTA CAAGGATGCTCTTGCGATTACTGGAAAAGCACCCGTTGTAC GCCGTTTTCCTATGGTCCCCGGAATTGACTTTACTGGGACT GTCGCACAGAGTTCCCATGCTGATTTCAAGCCAGGCGACC GCGTAATTCTGAACGGATGGGGAGTTGGTGAGAAACACTG GGGCGGTCTTGCAGAACGCGCACGCGTACGTGGGGACTGG CTTGTCCCGTTGCCAGCCCCCTTAGACTTGCGCCAGGCTGC AATGATTGGCACTGCGGGGTACACAGCTATGCTGTGCGTG CTTGCCCTTGAGCGCCATGGAGTCGTACCTGGGAACGGCG AGATTGTCGTCTCAGGCGCAGCAGGAGGGGTAGGTTCTGT AGCAACCACACTGTTAGCAGCCAAAGGCTACGAAGTGGCC GCCGTGACCGGGCGCGCAAGCGAGGCCGAATATTTACGCG GATTAGGCGCCGCGTCGGTCATTGATCGCAATGAATTAAC GGGGAAGGTGCGTCCATTAGGGCAGGAACGCTGGGCAGGA GGAATCGATGTAGCAGGATCAACCGTACTTGCTAATATGTT GAGCATGATGAAATACCGTGGCGTGGTGGCGGCCTGTGGC CTGGCGGCTGGAATGGACTTGCCCGCGTCTGTCGCCCCTTT TATTCTGCGTGGTATGACTTTGGCAGGGGTAGATTCAGTCA TGTGCCCCAAAACTGATCGTCTGGCTGCTTGGGCACGCCTG

	GCATCCGACCTGGACCCTGCAAAGCTGGAAGAGATGACAA CTGAATTACCGTTCTCTGAGGTGATTGAAACGGCTCCGAAG TTCTTGGATGGAACAGTGCGTGGGCGTATTGTCATTCCGGT AACACCTTGA
fldH1 **SEQ ID NO: 240**	ATGAAAATCTTGGCATACTGCGTCCGCCCAGACGAGGTAG ACTCCTTTAAGAAATTTAGTGAAAAGTACGGGCATACAGTT GATCTTATTCCAGACTCTTTTGGACCTAATGTCGCTCATTTG GCGAAGGGTTACGATGGGATTTCTATTCTGGGCAACGACA CGTGTAACCGTGAGGCACTGGAGAAGATCAAGGATTGCGG GATCAAATATCTGGCAACCCGTACAGCCGGAGTGAACAAC ATTGACTTCGATGCAGCAAAGGAGTTCGGTATTAACGTGG CTAATGTTCCCGCATATTCCCCCAACTCGGTCAGCGAATTT ACCATTGGATTGGCATTAAGTCTGACGCGTAAGATTCCATT TGCCCTGAAACGCGTGGAACTGAACAATTTTGCGCTTGGCG GCCTTATTGGTGTGGAATTGCGTAACTTAACTTTAGGAGTC ATCGGTACTGGTCGCATCGGATTGAAAGTGATTGAGGGCTT CTCTGGGTTTGGAATGAAAAAAATGATCGGTTATGACATTT TTGAAAATGAAGAAGCAAAGAAGTACATCGAATACAAATC ATTAGACGAAGTTTTTAAAGAGGCTGATATTATCACTCTGC ATGCGCCTCTGACAGACGACAACTATCATATGATTGGTAA AGAATCCATTGCTAAAATGAAGGATGGGGTATTTATTATCA ACGCAGCGCGTGGAGCCTTAATCGATAGTGAGGCCCTGAT TGAAGGGTTAAAATCGGGGAAGATTGCGGGCGCGGCTCTG GATAGCTATGAGTATGAGCAAGGTGTCTTTCACAACAATA AGATGAATGAAATTATGCAGGATGATACCTTGGAACGTCT GAAATCTTTTCCCAACGTCGTGATCACGCCGCATTTGGGTT TTTATACTGATGAGGCGGTTTCCAATATGGTAGAGATCACA CTGATGAACCTTCAGGAATTCGAGTTGAAAGGAACCTGTA AGAACCAGCGTGTTTGTAAATGA
FldD **SEQ ID NO: 241**	ATGTTCTTTACGGAGCAACACGAACTTATTCGCAAACTGGC GCGTGACTTTGCCGAACAGGAAATCGAGCCTATCGCAGAC GAAGTAGATAAAACCGCAGAGTTCCCAAAAGAAATCGTGA AGAAGATGGCTCAAAATGGATTTTTCGGCATTAAAATGCCT AAAGAATACGGAGGGGCGGGTGCGGATAACCGCGCTTATG TCACTATTATGGAGGAAATTTCACGTGCTTCCGGGGTAGCG GGTATCTACCTGAGCTCGCCGAACAGTTTGTTAGGAACTCC CTTCTTATTGGTCGGAACCGATGAGCAAAAAGAAAAGTAC CTTAAGCCTATGATCCGCGGCGAGAAGACTCTGGCGTTCGC CCTGACAGAGCCTGGTGCTGGCTCTGATGCGGGTGCGTTGG CTACTACTGCCCGTGAAGAGGGCGACTATTATATCTTAAAT GGCCGCAAGACGTTTATTACAGGGGCTCCTATTAGCGACA ATATTATTGTGTTCGCAAAAACCGATATGAGCAAAGGGAC CAAAGGTATCACCACTTTCATTGTGGACTCAAAGCAGGAA GGGGTAAGTTTTGGTAAGCCAGAGGACAAAATGGGAATGA TTGGTTGTCCGACAAGCGACATCATCTTGGAAAACGTTAAA GTTCATAAGTCCGACATCTTGGGAGAAGTCAATAAGGGGT TTATTACCGCGATGAAAACACTTTCCGTTGGTCGTATCGGA GTGGCGTCACAGGCGCTTGGAATTGCACAGGCCGCCGTAG ATGAGGCGGTAAAGTACGCCAAGCAACGTAAACAATTCAA TCGCCCAATCGCGAAATTTCAGGCCATTCAATTTAAACTTG

	CCAATATGGAGACTAAATTAAATGCCGCTAAACTTCTTGTT TATAACGCAGCGTACAAAATGGATTGTGGAGAAAAAGCCG ACAAGGAAGCCTCTATGGCTAAATACTTTGCTGCTGAATCA GCGATCCAAATCGTTAACGACGCGCTGCAAATCCATGGCG GGTATGGCTATATCAAAGACTACAAGATTGAACGTTTGTAC CGCGATGTGCGTGTGATCGCTATTTATGAGGGCACTTCCGA GGTCCAACAGATGGTTATCGCGTCCAATCTGCTGAAGTAA

[0231] In some embodiments, the disclosure provides novel nucleic acids for producing one or more tryptophan metabolites. In some embodiments, the nucleic acid comprises gene sequence encoding one or more **Trp aminotransferase.** Accordingly, In some embodiments, the nucleic acid sequence comprising the **Trp aminotransferase** gene has at least about 80% identity with SEQ ID NO: 229. In some embodiments, the gene sequence comprising the **Trp aminotransferase** gene has at least about 90% identity with SEQ ID NO: 229. In another embodiment, the gene sequence comprising the **Trp aminotransferase** gene has at least about 95% identity with SEQ ID NO: 229. Accordingly, In some embodiments, the gene sequence comprising the **Trp aminotransferase** gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 229. In another embodiment, the gene sequence comprising the **Trp aminotransferase** gene comprises SEQ ID NO: 229. In yet another embodiment the gene sequence comprising the **Trp aminotransferase** gene consists of SEQ ID NO: 229.

[0697] In some embodiments, the disclosure provides novel nucleic acids for producing one or more tryptophan metabolites. In some embodiments, the nucleic acid comprises gene sequence encoding one or more Tryptophan Decarboxylase Accordingly, In some embodiments, the nucleic acid sequence comprising the Tryptophan Decarboxylase gene has at least about 80% identity with **SEQ ID NO: 230**. In some embodiments, the gene sequence comprising the Tryptophan Decarboxylase gene has at least about 90% identity with **SEQ ID NO: 230**. In another embodiment, the gene sequence comprising the Tryptophan Decarboxylase gene has at least about 95% identity with **SEQ ID NO: 230**. Accordingly, In some embodiments, the gene sequence comprising the Tryptophan Decarboxylase gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 230**. In another embodiment, the gene sequence comprising the Tryptophan Decarboxylase gene comprises **SEQ ID NO: 230**. In yet another embodiment the gene sequence comprising the Tryptophan Decarboxylase gene consists of **SEQ ID NO: 230**.

[0698] In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with one or more sequences of **Table D**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with one or more sequences of **Table D**. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with one or more sequences of **Table D**. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with one or more sequences of **Table D**. In another embodiment, the gene has at least about 96%, 97%, 98%, or 99% identity with one or more sequences of **Table D**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with one or more sequences of **Table D**. In another embodiment, the genetically engineered bacteria comprise the sequence of **Table D.** In some embodiments, the genetically engineered bacteria comprise a sequence which consists of the sequence of with one or more sequences of **Table D**.

[0699] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding tryptophan amino transferase. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding tdc from C. roseus. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 231**. In another embodiment, the genetically engineered bacteria comprise a tryptophan amino transferase gene sequence which has at least about 85% identity with **SEQ ID NO: 231**. In some embodiments, the genetically engineered bacteria comprise a tryptophan amino transferase gene sequence which has at least about 90% identity with **SEQ ID NO: 231**. In some embodiments, the genetically engineered bacteria comprise a tryptophan amino transferase gene sequence which has at least about 95% identity with **SEQ ID NO: 231**. In another embodiment, the tryptophan amino transferase gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 231**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a tryptophan amino transferase gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 231**. In another embodiment, the genetically engineered bacteria comprise the tryptophan amino transferase gene sequence of **SEQ ID NO: 231**. In yet another embodiment the genetically engineered bacteria comprise a

tryptophan amino transferase gene sequence which consists of the sequence of **SEQ ID NO: 231**.

[0700] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding tryptophan decarboxylase. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding tdc from C. roseus. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 96**. In another embodiment, the genetically engineered bacteria comprise a tryptophan decarboxylase gene sequence which has at least about 85% identity with **SEQ ID NO: 96**. In some embodiments, the genetically engineered bacteria comprise a tryptophan decarboxylase gene sequence which has at least about 90% identity with **SEQ ID NO: 96**. In some embodiments, the genetically engineered bacteria comprise a tryptophan decarboxylase gene sequence which has at least about 95% identity with **SEQ ID NO: 96**. In another embodiment, the tryptophan decarboxylase gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 96**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a tryptophan decarboxylase gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 96**. In another embodiment, the genetically engineered bacteria comprise the tryptophan decarboxylase gene sequence of **SEQ ID NO: 96**. In yet another embodiment the genetically engineered bacteria comprise a tryptophan decarboxylase gene sequence which consists of the sequence of **SEQ ID NO: 96**.

[0701] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding Tdc. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding tdc from C. sporogenes. In some embodiments, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 232**. In another embodiment, the genetically engineered bacteria comprise a Tdc gene sequence which has at least about 85% identity with **SEQ ID NO: 232**. In some embodiments, the genetically engineered bacteria comprise a Tdc gene sequence which has at least about 90% identity with **SEQ ID NO: 232**. In some embodiments, the genetically engineered bacteria comprise a Tdc gene sequence which has at least about 95% identity with **SEQ ID NO: 232**. In another embodiment, the Tdc gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 232**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a Tdc gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,

97%, 98%, or 99% identity with **SEQ ID NO: 232**. In another embodiment, the genetically engineered bacteria comprise the Tdc gene sequence of **SEQ ID NO: 232**. In yet another embodiment the genetically engineered bacteria comprise a Tdc gene sequence which consists of the sequence of **SEQ ID NO: 232**.

[0702] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding tryptophan dehydrogenase (trpDH). In some embodiments, the genetically engineered bacteria comprise a trpDH gene sequence which has at least about 80% identity with **SEQ ID NO: 233**. In another embodiment, the genetically engineered bacteria comprise a trpDH sequence which has at least about 85% identity with **SEQ ID NO: 233**. In some embodiments, the genetically engineered bacteria comprise a trpDH gene sequence which has at least about 90% identity with **SEQ ID NO: 233**. In some embodiments, the genetically engineered bacteria comprise a trpDH gene sequence which has at least about 95% identity with **SEQ ID NO: 233**. In another embodiment, the a trpDH gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 233**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a trpDH gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 233**. In another embodiment, the genetically engineered bacteria comprise the a trpDH gene sequence of **SEQ ID NO: 233**. In yet another embodiment the genetically engineered bacteria comprise a trpDH gene sequence which consists of the sequence of **SEQ ID NO: 233**.

[0703] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding ipdC. In some embodiments, the genetically engineered bacteria comprise a ipdC gene sequence which has at least about 80% identity with **SEQ ID NO: 234**. In another embodiment, the genetically engineered bacteria comprise a ipdC gene sequence which has at least about 85% identity with **SEQ ID NO: 234**. In some embodiments, the genetically engineered bacteria comprise a ipdC gene sequence which has at least about 90% identity with **SEQ ID NO: 234**. In some embodiments, the genetically engineered bacteria comprise a ipdC gene sequence which has at least about 95% identity with **SEQ ID NO: 234**. In another embodiment, the ipdC gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 234**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a ipdC gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 234**. In another embodiment, the genetically engineered bacteria comprise the ipdC gene sequence of **SEQ ID NO: 234**. In yet another embodiment

the genetically engineered bacteria comprise a ipdC gene sequence which consists of the sequence of **SEQ ID NO: 234**.

[0704] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding Iad1. In some embodiments, the genetically engineered bacteria comprise a Iad1 gene sequence which has at least about 80% identity with **SEQ ID NO: 235**. In another embodiment, the genetically engineered bacteria comprise a Iad1 gene sequence which has at least about 85% identity with **SEQ ID NO: 235**. In some embodiments, the genetically engineered bacteria comprise a Iad1 gene sequence which has at least about 90% identity with **SEQ ID NO: 235**. In some embodiments, the genetically engineered bacteria comprise a Iad1 gene sequence which has at least about 95% identity with **SEQ ID NO: 235**. In another embodiment, the Iad1 gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 235**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a Iad1 gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 235**. In another embodiment, the genetically engineered bacteria comprise the Iad1 gene sequence of **SEQ ID NO: 235**. In yet another embodiment the genetically engineered bacteria comprise a Iad1 gene sequence which consists of the sequence of **SEQ ID NO: 235**.

[0705] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding fldA. In some embodiments, the genetically engineered bacteria comprise a fldA gene sequence which has at least about 80% identity with **SEQ ID NO: 236**. In another embodiment, the genetically engineered bacteria comprise a fldA gene sequence which has at least about 85% identity with **SEQ ID NO: 236**. In some embodiments, the genetically engineered bacteria comprise a fldA gene sequence which has at least about 90% identity with **SEQ ID NO: 236**. In some embodiments, the genetically engineered bacteria comprise a fldA gene sequence which has at least about 95% identity with **SEQ ID NO: 236**. In another embodiment, the fldA gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 236**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a fldA gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 236**. In another embodiment, the genetically engineered bacteria comprise the fldA gene sequence of **SEQ ID NO: 236**. In yet another embodiment the genetically engineered bacteria comprise a fldA gene sequence which consists of the sequence of **SEQ ID NO: 236**.

[0706] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding fldB. In some embodiments, the genetically engineered bacteria comprise a fldB gene sequence which has at least about 80% identity with **SEQ ID NO: 237**. In another embodiment, the genetically engineered bacteria comprise a fldB gene sequence which has at least about 85% identity with **SEQ ID NO: 237**. In some embodiments, the genetically engineered bacteria comprise a fldB gene sequence which has at least about 90% identity with **SEQ ID NO: 237**. In some embodiments, the genetically engineered bacteria comprise a fldB gene sequence which has at least about 95% identity with **SEQ ID NO: 237**. In another embodiment, the fldB gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 237**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a fldB gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 237**. In another embodiment, the genetically engineered bacteria comprise the fldB gene sequence of **SEQ ID NO: 237**. In yet another embodiment the genetically engineered bacteria comprise a fldB gene sequence which consists of the sequence of **SEQ ID NO: 237**.

[0707] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding fldC. In some embodiments, the genetically engineered bacteria comprise a fldC gene sequence which has at least about 80% identity with **SEQ ID NO: 238**. In another embodiment, the genetically engineered bacteria comprise a fldC gene sequence which has at least about 85% identity with **SEQ ID NO: 238**. In some embodiments, the genetically engineered bacteria comprise a fldC gene sequence which has at least about 90% identity with **SEQ ID NO: 238**. In some embodiments, the genetically engineered bacteria comprise a fldC gene sequence which has at least about 95% identity with **SEQ ID NO: 238**. In another embodiment, the fldC gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 238**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a fldC gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 238**. In another embodiment, the genetically engineered bacteria comprise the fldC gene sequence of **SEQ ID NO: 238**. In yet another embodiment the genetically engineered bacteria comprise a fldC gene sequence which consists of the sequence of **SEQ ID NO: 238**.

[0708] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding Acul. In some embodiments, the genetically engineered bacteria comprise

a AcuI gene sequence which has at least about 80% identity with **SEQ ID NO: 239**. In another embodiment, the genetically engineered bacteria comprise a AcuI gene sequence which has at least about 85% identity with **SEQ ID NO: 239**. In some embodiments, the genetically engineered bacteria comprise a AcuI gene sequence which has at least about 90% identity with **SEQ ID NO: 239**. In some embodiments, the genetically engineered bacteria comprise a AcuI gene sequence which has at least about 95% identity with **SEQ ID NO: 239**. In another embodiment, the AcuI gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 239**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a AcuI gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 239**. In another embodiment, the genetically engineered bacteria comprise the AcuI gene sequence of **SEQ ID NO: 239**. In yet another embodiment the genetically engineered bacteria comprise a AcuI gene sequence which consists of the sequence of **SEQ ID NO: 239**.

[0709] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding fldH1. In some embodiments, the genetically engineered bacteria comprise a fldH1 gene sequence which has at least about 80% identity with **SEQ ID NO: 240**. In another embodiment, the genetically engineered bacteria comprise a fldH1 gene sequence which has at least about 85% identity with **SEQ ID NO: 240**. In some embodiments, the genetically engineered bacteria comprise a fldH1 gene sequence which has at least about 90% identity with **SEQ ID NO: 240**. In some embodiments, the genetically engineered bacteria comprise a fldH1 gene sequence which has at least about 95% identity with **SEQ ID NO: 240**. In another embodiment, the fldH1 gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 240**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a fldH1 gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 240**. In another embodiment, the genetically engineered bacteria comprise the fldH1 gene sequence of **SEQ ID NO: 240**. In yet another embodiment the genetically engineered bacteria comprise a fldH1 gene sequence which consists of the sequence of **SEQ ID NO: 240**.

[0710] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding fldD. In some embodiments, the genetically engineered bacteria comprise a fldD gene sequence which has at least about 80% identity with **SEQ ID NO: 241**. In another embodiment, the genetically engineered bacteria comprise a fldD gene sequence

which has at least about 85% identity with **SEQ ID NO: 241**. In some embodiments, the genetically engineered bacteria comprise a fldD gene sequence which has at least about 90% identity with **SEQ ID NO: 241**. In some embodiments, the genetically engineered bacteria comprise a fldD gene sequence which has at least about 95% identity with **SEQ ID NO: 241**. In another embodiment, the fldD gene sequence has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 241**. Accordingly, In some embodiments, the genetically engineered bacteria comprise a fldD gene sequence which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 241**. In another embodiment, the genetically engineered bacteria comprise the fldD gene sequence of **SEQ ID NO: 241**. In yet another embodiment the genetically engineered bacteria comprise a fldD gene sequence which consists of the sequence of **SEQ ID NO: 241**.

Table E. Tryptophan Pathway Catabolic Enzymes

Description	**Sequence**
TDC: Tryptophan decarboxylase from Catharanthus roseus **SEQ ID NO: 242**	MGSIDSTNVAMSNSPVGEFKPLEAEEFRKQAHRMVDFIADYY KNVETYPVLSEVEPGYLRKRIPETAPYLPEPLDDIMKDIQKDII PGMTNWMSPNFYAFFPATVSSAAFLGEMLSTALNSVGFTWV SSPAATELEMIVMDWLAQILKLPKSFMFSGTGGGVIQNTTSES ILCTIIAARERALEKLGPDSIGKLVCYGSDQTHTMFPKTCKLA GIYPNNIRLIPTTVETDFGISPQVLRKMVEDDVAAGYVPLFLC ATLGTTSTTATDPVDSLSEIANEFGIWIHVDAAYAGSACICPEF RHYLDGIERVDSLSLSPHKWLLAYLDCTCLWVKQPHLLLRAL TTNPEYLKNKQSDLDKVVDFKNWQIATGRKFRSLKLWLILRS YGVVNLQSHIRSDVAMGKMFEEWVRSDSRFEIVVPRNFSLVC FRLKPDVSSLHVEEVNKKLLDMLNSTGRVYMTHTIVGGIYML RLAVGSSLTEEHHVRRVWDLIQKLTDDLLKEA
TDC: Tryptophan decarboxylase from Clostridium sporogenes **SEQ ID NO: 243**	MKFWRKYTQQEMDEKITESLEKTLNYDNTKTIGIPGTKLDDT VFYDDHSFVKHSPYLRTFIQNPNHIGCHTYDKADILFGGTFDIE RELIQLLAIDVLNGNDEEFDGYVTQGGTEANIQAMWVYRNY FKKERKAKHEEIAIITSADTHYSAYKGSDLLNIDIIKVPVDFYS RKIQENTLDSIVKEAKEIGKKYFIVISNMGTTMFGSVDDPDLY ANIFDKYNLEYKIHVDGAFGGFIYPIDNKECKTDFSNKNVSSIT LDGHKMLQAPYGTGIFVSRKNLIHNTLTKEATYIENLDVTLSG SRSGSNAVAIWMVLASYGPYGWMEKINKLRNRTKWLCKQL NDMRIKYYKEDSMNIVTIEEQYVNKEIAEKYFLVPEVHNPTN NWYKIVVMEHVELDILNSLVYDLRKFNKEHLKAM
Tryptophan Decarboxylase (EC 4.1.1.28) Chain A, Ruminococcus Gnavus **Tryptophan**	MSQVIKKKRNTFMIGTEYILNSTQLEEAIKSFVHDFCAEKHEIH DQPVVVEAKEHQEDKIKQIKIPEKGRPVNEVVSEMMNEVYRY RGDANHPRFFSFVPGPASSVSWLGDIMTSAYNIHAGGSKLAP MVNCIEQEVLKWLAKQVGFTENPGGVFVSGGSMANITALTA ARDNKLTDINLHLGTAYISDQTHSSVAKGLRIIGITDSRIRRIPT NSHFQMDTTKLEEAIETDKKSGYIPFVVIGTAGTTNTGSIDPLT

SEQ ID NO: 244	EISALCKKHDMWFHIDGAYGASVLLSPKYKSLLTGTGLADSIS WDAHKWLFQTYGCAMVLVKDIRNLFHSFHVNPEYLKDLEN DIDNVNTWDIGMELTRPARGLKLWLTLQVLGSDLIGSAIEHG FQLAVWAEEALNPKKDWEIVSPAQMAMINFRYAPKDLTKEE QDILNEKISHRILESGYAAIFTTVLNGKTVLRICAIHPEATQED MQHTIDLLDQYGREIYTEMKKa
Trp aminotransferase (EC 2.6.1.27); tryptophan aminotransferase [Cryptococcus deuterogattii R265] **SEQ ID NO: 245**	MTATTISIETVPQAPAAGTKTNGTSGKYNPRTYLSDRAKVTEI DGSDAGRPNPDTFPFNSITLNLKPPLGLPESSNNMPVSITIEDPD LATALQYAPSAGIPKLREWLADLQAHVHERPRGDYAISVGSG SQDLMFKGFQAVLNPGDPVLLETPMYSGVLPALRILKADYAE VDVDDQGLSAKNLEKVLSEWPADKKRPRVLYTSPIGSNPSGC SASKERKLEVLKVCKKYDVLIFEDDPYYYLAQELIPSYFALEK QVYPEGGHVVRFDSFSKLLSAGMRLGFATGPKEILHAIDVSTA GANLHTSAVSQGVALRLMQYWGIEGFLAHGRAVAKLYTERR AQFEATAHKYLDGLATWVSPVAGMFLWIDLRPAGIEDSYELI RHEALAKGVLGVPGMAFYPTGRKSSHVRVSFSIVDLEDESDL GFQRLAEAIKDKRKALGLA
ARO9: L-tryptophan aminotransferase from S. cerevisae **SEQ ID NO: 246**	MTAGSAPPVDYTSLKKNFQPFLSRRVENRSLKSFWDASDISD DVIELAGGMPNERFFPIESMDLKISKVPFNDNPKWHNSFTTAH LDLGSPSELPIARSFQYAETKGLPPLLHFVKDFVSRINRPAFSD ETESNWDVILSGGSNDSMFKVFETICDESTTVMIEEFTFTPAM SNVEATGAKVIPIKMNLTFDRESQGIDVEYLTQLLDNWSTGP YKDLNKPRVLYTIATGQNPTGMSVPQWKREKIYQLAQRHDF LIVEDDPYGYLYFPSYNPQEPLENPYHSSDLTTERYLNDFLMK SFLTLDTDARVIRLETFSKIFAPGLRLSFIVANKFLLQKILDLAD ITTRAPSGTSQAIVYSTIKAMAESNLSSSLSMKEAMFEGWIRW IMQIASKYNHRKNLTLKALYETESYQAGQFTVMEPSAGMFIII KINWGNFDRPDDLPQQMDILDKFLLKNGVKVVLGYKMAVCP NYSKQNSDFLRLTIAYARDDDQLIEASKRIGSGIKEFFDNYKS
TYNA: Monoamine oxidase from E. coli **SEQ ID NO: 247**	MGSPSLYSARKTTLALAVALSFAWQAPVFAHGGEAHMVPM DKTLKEFGADVQWDDYAQLFTLIKDGAYVKVKPGAQTAIVN GQPLALQVPVVMKDNKAWVSDTFINDVFQSGLDQTFQVEKR PHPLNALTADEIKQAVEIVKASADFKPNTRFTEISLLPPDKEAV WAFALENKPVDQPRKADVIMLDGKHIIEAVVDLQNNKLLSW QPIKDAHGMVLLDDFASVQNIINNSEEFAAAVKKRGITDAKK VITTPLTVGYFDGKDGLKQDARLLKVISYLDVGDGNYWAHPI ENLVAVVDLEQKKIVKIEEGPVVPVPMTARPFDGRDRVAPAV KPMQIIEPEGKNYTITGDMIHWRNWDFHLSMNSRVGPMISTV TYNDNGTKRKVMYEGSLGGMIVPYGDPDIGWYFKAYLDSGD YGMGTLTSPIARGKDAPSNAVLLNETIADYTGVPMEIPRAIAV FERYAGPEYKHQEMGQPNVSTERRELVVRWISTVGNYDYIFD WIFHENGTIGIDAGATGIEAVKGVKAKTMHDETAKDDTRYGT LIDHNIVGTTHQHIYNFRLDLDVDGENNSLVAMDPVVKPNTA GGPRTSTMQVNQYNIGNEQDAAQKFDPGTIRLLSNPNKENRM GNPVSYQIIPYAGGTHPVAKGAQFAPDEWIYHRLSFMDKQLW VTRYHPGERFPEGKYPNRSTHDTGLGQYSKDNESLDNTDAV VWMTTGTTHVARAEEWPIMPTEWVHTLLKPWNFFDETPTLG ALKKDK

AAO1: Indole-3-acetaldehyde oxidase from Arabidopsis thaliana **SEQ ID NO: 248**	MGEKAIDEDKVEAMKSSKTSLVFAINGQRFELELSSIDPSTTL VDFLRNKTPFKSVKLGCGEGGCGACVVLLSKYDPLLEKVDEF TISSCLTLLCSIDGCSITTSDGLGNSRVGFHAVHERIAGFHATQ CGFCTPGMSVSMFSALLNADKSHPPPRSGFSNLTAVEAEKAV SGNLCRCTGYRPLVDACKSFAADVDIEDLGFNAFCKKGENRD EVLRRLPCYDHTSSHVCTFPEFLKKEIKNDMSLHSRKYRWSSP VSVSELQGLLEVENGLSVKLVAGNTSTGYYKEEKERKYERFI DIRKIPEFTMVRSDEKGVELGACVTISKAIEVLREEKNVSVLA KIATHMEKIANRFVRNTGTIGGNIMMAQRKQFPSDLATILVA AQATVKIMTSSSSQEQFTLEEFLQQPPLDAKSLLLSLEIPSWHS AKKNGSSEDSILLFETYRAAPRPLGNALAFLNAAFSAEVTEAL DGIVVNDCQLVFGAYGTKHAHRAKKVEEFLTGKVISDEVLM EAISLLKDEIVPDKGTSNPGYRSSLAVTFLFEFFGSLTKKNAKT TNGWLNGGCKEIGFDQNVESLKPEAMLSSAQQIVENQEHSPV GKGITKAGACLQASGEAVYVDDIPAPENCLYGAFIYSTMPLA RIKGIRFKQNRVPEGVLGIITYKDIPKGGQNIGTNGFFTSDLLF AEEVTHCAGQIIAFLVADSQKHADIAANLVVIDYDTKDLKPPI LSLEEAVENFSLFEVPPPLRGYPVGDITKGMDEAEHKILGSKIS FGSQYFFYMETQTALAVPDEDNCMVVYSSTQTPEFVHQTIAG CLGVPENNVRVITRRVGGGFGGKAVKSMPVAAACALAASK MQRPVRTYVNRKTDMITTGGRHPMKVTYSVGFKSNGKITAL DVEVLLDAGLTEDISPLMPKGIQGALMKYDWGALSFNVKVC KTNTVSRTALRAPGDVQGSYIGEAIIEKVASYLSVDVDEIRKV NLHTYESLRLFHSAKAGEFSEYTLPLLWDRIDEFSGFNKRRKV VEEFNASNKWRKRGISRVPAVYAVNMRSTPGRVSVLGDGSIV VEVQGIEIGQGLWTKVKQMAAYSLGLIQCGTTSDELLKKIRVI QSDTLSMVQGSMTAGSTTSEASSEAVRICCDGLVERLLPVKT ALVEQTGGPVTWDSLISQAYQQSINMSVSSKYMPDSTGEYLN YGIAASEVEVNVLTGETTILRTDIIYDCGKSLNPAVDLGQIEGA FVQGLGFFMLEEFLMNSDGLVVTDSTWTYKIPTVDTIPRQFN VEILNSGQHKNRVLSSKASGEPPLLLAASVHCAVRAAVKEAR KQILSWNSNKQGTDMYFELPVPATMPIVKEFCGLDVVEKYLE WKIQQRKNV
aspC: aspartate aminotransferase from E. coli **SEQ ID NO: 249**	MFENITAAPADPILGLADLFRADERPGKINLGIGVYKDETGKT PVLTSVKKAEQYLLENETTKNYLGIDGIPEFGRCTQELLFGKG SALINDKRARTAQTPGGTGALRVAADFLAKNTSVKRVWVSN PSWPNHKSVFNSAGLEVREYAYYDAENHTLDFDALINSLNEA QAGDVVLFHGCCHNPTGIDPTLEQWQTLAQLSVEKGWLPLF DFAYQGFARGLEEDAEGLRAFAAMHKELIVASSYSKNFGLYN ERVGACTLVAADSETVDRAFSQMKAAIRANYSNPPAHGASV VATILSNDALRAIWEQELTDMRQRIQRMRQLFVNTLQEKGAN RDFSFIIKQNGMFSFSGLTKEQVLRLREEFGVYAVASGRVNVA GMTPDNMAPLCEAIVAVL
TAA1: L-tryptophan-pyruvate aminotransferase from Arabidopsis thaliana **SEQ ID NO: 250**	MVKLENSRKPEKISNKNIPMSDFVVNLDHGDPTAYEEYWRK MGDRCTVTIRGCDLMSYFSDMTNLCWFLEPELEDAIKDLHGV VGNAATEDRYIVVGTGSTQLCQAAVHALSSLARSQPVSVVA AAPFYSTYVEETTYVRSGMYKWEGDAWGFDKKGPYIELVTS PNNPDGTIRETVVNRPDDDEAKVIHDFAYYWPHYTPITRRQD HDIMLFTFSKITGHAGSRIGWALVKDKEVAKKMVEYIIVNSIG VSKESQVRTAKILNVLKETCKSESESENFFKYGREMMKNRWE

	KLREVVKESDAFTLPKYPEAFCNYFGKSLESYPAFAWLGTKE ETDLVSELRRHKVMSRAGERCGSDKKHVRVSMLSREDVFNV FLERLANMKLIKSIDL
STAO: L-tryptophan oxidase from streptomyces sp. TP-A0274 **SEQ ID NO: 251**	MTAPLQDSDGPDDAIGGPKQVTVIGAGIAGLVTAYELERLGH HVQIIEGSDDIGGRIHTHRFSGAGGPGPFAEMGAMRIPAGHRL TMHYIAELGLQNQVREFRTLFSDDAAYLPSSAGYLRVREAHD TLVDEFATGLPSAHYRQDTLLFGAWLDASIRAIAPRQFYDGL HNDIGVELLNLVDDIDLTPYRCGTARNRIDLHALFADHPRVR ASCPPRLERFLDDVLDETSSSIVRLKDGMDELPRRLASRIRGKI SLGQEVTGIDVHDDTVTLTVRQGLRTVTRTCDYVVCTIPFTVL RTLRLTGFDQDKLDIVHETKYWPATKIAFHCREPFWEKDGIS GGASFTGGHVRQTYYPPAEGDPALGAVLLASYTIGPDAEALA RMDEAERDALVAKELSVMHPELRRPGMVLAVAGRDWGARR WSRGAATVRWGQEAALREAERRECARPQKGLFFAGEHCSSK PAWIEGAIESAIDAAHEIEWYEPRASRVFAASRLSRSDRSA
ipdC: Indole-3-pyruvate decarboxylase from Enterobacter cloacae **SEQ ID NO: 252**	MRTPYCVADYLLDRLTDCGADHLFGVPGDYNLQFLDHVIDS PDICWVGCANELNASYAADGYARCKGFAALLTTFGVGELSA MNGIAGSYAEHVPVLHIVGAPGTAAQQRGELLHHTLGDGEFR HFYHMSEPITVAQAVLTEQNACYEIDRVLTTMLRERRPGYLM LPADVAKKAATPPVNALTHKQAHADSACLKAFRDAAENKLA MSKRTALLADFLVLRHGLKHALQKWVKEVPMAHATMLMG KGIFDERQAGFYGTYSGSASTGAVKEAIEGADTVLCVGTRFT DTLTAGFTHQLTPAQTIEVQPHAARVGDVWFTGIPMNQAIET LVELCKQHVHAGLMSSSSGAIPFPQPDGSLTQENFWRTLQTFI RPGDIILADQGTSAFGAIDLRLPADVNFIVQPLWGSIGYTLAA AFGAQTACPNRRVIVLTGDGAAQLTIQELGSMLRDKQHPIILV LNNEGYTVERAIHGAEQRYNDIALWNWTHIPQALSLDPQSEC WRVSEAEQLADVLEKVAHHERLSLIEVMLPKADIPPLLGALT KALEACNNA
IAD1: Indole-3-acetaldehyde dehydrogenase from Ustilago maydis **SEQ ID NO: 253**	MPTLNLDLPNGIKSTIQADLFINNKFVPALDGKTFATINPSTGK EIGQVAEASAKDVDLAVKAAREAFETTWGENTPGDARGRLLI KLAELVEANIDELAAIESLDNGKAFSIAKSFDVAAVAANLRY YGGWADKNHGKVMEVDTKRLNYTRHEPIGVCGQIIPWNFPL LMFAWKLGPALATGNTIVLKTAEQTPLSAIKMCELIVEAGFPP GVVNVISGFGPVAGAAISQHMDIDKIAFTGSTLVGRNIMKAA ASTNLKKVTLELGGKSPNIIFKDADLDQAVRWSAFGIMFNHG QCCCAGSRVYVEESIYDAFMEKMTAHCKALQVGDPFSANTF QGPQVSQLQYDRIMEYIESGKKDANLALGGVRKGNEGYFIEP TIFTDVPHDAKIAKEEIFGPVVVVSKFKDEKDLIRIANDSIYGL AAAVFSRDISRAIETAHKLKAGTVWVNCYNQLIPQVPFGGYK ASGIGRELGEYALSNYTNIKAVHVNLSQPAPI
YUC2: indole-3-pyruvate monoxygenase from Arabidopsis thaliana **SEQ ID NO: 254**	MEFVTETLGKRIHDPYVEETRCLMIPGPIIVGSGPSGLATAACL KSRDIPSLILERSTCIASLWQHKTYDRLRLHLPKDFCELPLMPF PSSYPTYPTKQQFVQYLESYAEHFDLKPVFNQTVEEAKFDRR CGLWRVRTTGGKKDETMEYVSRWLVVATGENAEEVMPEID GIPDFGGPILHTSSYKSGEIFSEKKILVVGCGNSGMEVCLDLCN FNALPSLVVRDSVHVLPQEMLGISTFGISTSLLKWFPVHVVDR FLLRMSRLVLGDTDRLGLVRPKLGPLERKIKCGKTPVLDVGT LAKIRSGHIKVYPELKRVMHYSAEFVDGRVDNFDAIILATGY KSNVPMWLKGVNMFSEKDGFPHKPFPNGWKGESGLYAVGF

	TKLGLLGAAIDAKKIAEDIEVQRHFLPLARPQHC
IaaM: Tryptophan 2-monooxygenase from Pseudomonas savastanoi **SEQ ID NO: 255**	MYDHFNSPSIDILYDYGPFLKKCEMTGGIGSYSAGTPTPRVAI VGAGISGLVAATELLRAGVKDVVLYESRDRIGGRVWSQVFD QTRPRYIAEMGAMRFPPSATGLFHYLKKFGISTSTTFPDPGVV DTELHYRGKRYHWPAGKKPPELFRRVYEGWQSLLSEGYLLE GGSLVAPLDITAMLKSGRLEEAAIAWQGWLNVFRDCSFYNAI VCIFTGRHPPGGDRWARPEDFELFGSLGIGSGGFLPVFQAGFT EILRMVINGYQSDQRLIPDGISSLAARLADQSFDGKALRDRVC FSRVGRISREAEKIIIQTEAGEQRVFDRVIVTSSNRAMQMIHCL TDSESFLSRDVARAVRETHLTGSSKLFILTRTKFWIKNKLPTTI QSDGLVRGVYCLDYQPDEPEGHGVVLLSYTWEDDAQKMLA MPDKKTRCQVLVDDLAAIHPTFASYLLPVDGDYERYVLHHD WLTDPHSAGAFKLNYPGEDVYSQRLFFQPMTANSPNKDTGL YLAGCSCSFAGGWIEGAVQTALNSACAVLRSTGGQLSKGNPL DCINASYRY
iaaH: Indoleacetamide hydrolase from Pseudomonas savastanoi **SEQ ID NO: 256**	MHEIITLESLCQALADGEIAAAELRERALDTEARLARLNCFIRE GDAVSQFGEADHAMKGTPLWGMPVSFKDNICVRGLPLTAGT RGMSGFVSDQDAAIVSQLRALGAVVAGKNNMHELSFGVTSI NPHWGTVGNPVAPGYCAGGSSGGSAAAVASGIVPLSVGTDT GGSIRIPAAFCGITGFRPTTGRWSTAGIIPVSHTKDCVGLLTRT AGDAGFLYGLLSGKQQSFPLSRTAPCRIGLPVSMWSDLDGEV ERACVNALSLLRKTGFEFIEIDDADIVELNQTLTFTVPLYEFFA DLAQSLLSLGWKHGIHHIFAQVDDANVKGIINHHLGEGAIKP AHYLSSLQNGELLKRKMDELFARHNIELLGYPTVPCRVPHLD HADRPEFFSQAIRNTDLASNAMLPSITIPVGPEGRLPVGLSFDA LRGRDALLLSRVSAIEQVLGFVRKVLPHTT
TrpDH: Tryptophan dehydrogenase from Nostoc punctiforme NIES-2108 **SEQ ID NO: 257**	MLLFETVREMGHEQVLFCHSKNPEIKAIIAIHDTTLGPAMGAT RILPYINEEAALKDALRLSRGMTYKAACANIPAGGGKAVIIAN PENKTDDLLRAYGRFVDSLNGRFITGQDVNITPDDVRTISQET KYVVGVSEKSGGPAPITSLGVFLGIKAAVESRWQSKRLDGMK VAVQGLGNVGKNLCRHLHEHDVQLFVSDVDPIKAEEVKRLF GATVVEPTEIYSLDVDIFAPCALGGILNSHTIPFLQASIIAGAAN NQLENEQLHSQMLAKKGILYSPDYVINAGGLINVYNEMIGYD EEKAFKQVHNIYDTLLAIFEIAKEQGVTTNDAARRLAEDRINN SKRSKSKAIAA
CYP79B2: tryptophan N-monooxygenase from Arabidopsis thaliana **SEQ ID NO: 258**	MNTFTSNSSDLTTTATETSSFSTLYLLSTLQAFVAITLVMLLKK LMTDPNKKKPYLPPGPTGWPIIGMIPTMLKSRPVFRWLHSIMK QLNTEIACVKLGNTHVITVTCPKIAREILKQQDALFASRPLTY AQKILSNGYKTCVITPFGDQFKKMRKVVMTELVCPARHRWL HQKRSEENDHLTAWVYNMVKNSGSVDFRFMTRHYCGNAIK KLMFGTRTFSKNTAPDGGPTVEDVEHMEAMFEALGFTFAFCI SDYLPMLTGLDLNGHEKIMRESSAIMDKYHDPIIDERIKMWR EGKRTQIEDFLDIFISIKDEQGNPLLTADEIKPTIKELVMAAPDN PSNAVEWAMAEMVNKPEILRKAMEEIDRVVGKERLVQESDIP KLNYVKAILREAFRLHPVAAFNLPHVALSDTTVAGYHIPKGS QVLLSRYGLGRNPKVWADPLCFKPERHLNECSEVTLTENDLR FISFSTGKRGCAAPALGTALTTMMLARLLQGFTWKLPENETR VELMESSHDMFLAKPLVMVGDLRLPEHLYPTVK

CYP79B3: tryptophan N-monooxygenase from Arabidopsis thaliana **SEQ ID NO: 259**	MDTLASNSSDLTTKSSLGMSSFTNMYLLTTLQALAALCFLMI LNKIKSSSRNKKLHPLPPGPTGFPIVGMIPAMLKNRPVFRWLH SLMKELNTEIACVRLGNTHVIPVTCPKIAREIFKQQDALFASRP LTYAQKILSNGYKTCVITPFGEQFKKMRKVIMTEIVCPARHR WLHDNRAEETDHLTAWLYNMVKNSEPVDLRFVTRHYCGNA IKRLMFGTRTFSEKTEADGGPTLEDIEHMDAMFEGLGFTFAFC ISDYLPMLTGLDLNGHEKIMRESSAIMDKYHDPIIDERIKMWR EGKRTQIEDFLDIFISIKDEAGQPLLTADEIKPTIKELVMAAPDN PSNAVEWAIAEMINKPEILHKAMEEIDRVVGKERFVQESDIPK LNYVKAIIREAFRLHPVAAFNLPHVALSDTTVAGYHIPKGSQV LLSRYGLGRNPKVWSDPLSFKPERHLNECSEVTLTENDLRFIS FSTGKRGCAAPALGTAITTMMLARLLQGFKWKLAGSETRVE LMESSHDMFLSKPLVLVGELRLSEDLYPMVK
CYP71A13: indoleacetaldoxime dehydratase from Arabidopis thaliana **SEQ ID NO: 260**	MSNIQEMEMILSISLCLTTLITLLLLRRFLKRTATKVNLPPSPW RLPVIGNLHQLSLHPHRSLRSLSLRYGPLMLLHFGRVPILVVSS GEAAQEVLKTHDHKFANRPRSKAVHGLMNGGRDVVFAPYG EYWRQMKSVCILNLLTNKMVESFEKVREDEVNAMIEKLEKA SSSSSSENLSELFITLPSDVTSRVALGRKHSEDETARDLKKRVR QIMELLGEFPIGEYVPILAWIDGIRGFNNKIKEVSRGFSDLMDK VVQEHLEASNDKADFVDILLSIEKDKNSGFQVQRNDIKFMILD MFIGGTSTTSTLLEWTMTELIRSPKSMKKLQDEIRSTIRPHGSY IKEKEVENMKYLKAVIKEVLRLHPSLPMILPRLLSEDVKVKGY NIAAGTEVIINAWAIQRDTAIWGPDAEEFKPERHLDSGLDYHG KNLNYIPFGSGRRICPGINLALGLAEVTVANLVGRFDWRVEA GPNGDQPDLTEAIGIDVCRKFPLIAFPSSVV
PEN2: myrosinase from Arabidopsis thaliana **SEQ ID NO: 261**	MAHLQRTFPTEMSKGRASFPKGFLFGTASSSYQYEGAVNEGA RGQSVWDHFSNRFPHRISDSSDGNVAVDFYHRYKEDIKRMK DINMDSFRLSIAWPRVLPYGKRDRGVSEEGIKFYNDVIDELLA NEITPLVTIFHWDIPQDLEDEYGGFLSEQIIDDFRDYASLCFERF GDRVSLWCTMNEPWVYSVAGYDTGRKAPGRCSKYVNGASV AGMSGYEAYIVSHNMLLAHAEAVEVFRKCDHIKNGQIGIAHN PLWYEPYDPSDPDDVEGCNRAMDFMLGWHQHPTACGDYPE TMKKSVGDRLPSFTPEQSKKLIGSCDYVGINYYSSLFVKSIKH VDPTQPTWRTDQGVDWMKTNIDGKQIAKQGGSEWSFTYPTG LRNILKYVKKTYGNPPILITENGYGEVAEQSQSLYMYNPSIDT ERLEYIEGHIHAIHQAIHEDGVRVEGYYVWSLLDNFEWNSGY GVRYGLYYIDYKDGLRRYPKMSALWLKEFLRFDQEDDSSTS KKEEKKESYGKQLLHSVQDSQFVHSIKDSGALPAVLGSLFVV SATVGTSLFFKGANN
Nit1: Nitrilase from Arabidopsis thaliana **SEQ ID NO: 262**	MSSTKDMSTVQNATPFNGVAPSTTVRVTIVQSSTVYNDTPATI DKAEKYIVEAASKGAELVLFPEGFIGGYPRGFRFGLAVGVHN EEGRDEFRKYHASAIHVPGPEVARLADVARKNHVYLVMGAI EKEGYTLYCTVLFFSPQGQFLGKHRKLMPTSLERCIWGQGDG STIPVYDTPIGKLGAAICWENRMPLYRTALYAKGIELYCAPTA DGSKEWQSSMLHIAIEGGCFVLSACQFCQRKHFPDHPDYLFT DWYDDKEHDSIVSQGGSVIISPLGQVLAGPNFESEGLVTADID LGDIARAKLYFDSVGHYSRPDVLHLTVNEHPRKSVTFVTKVE KAEDDSNK
IDO1: indoleamine 2,3-dioxygenase from	MAHAMENSWTISKEYHIDEEVGFALPNPQENLPDFYNDWMFI AKHLPDLIESGQLRERVEKLNMLSIDHLTDHKSQRLARLVLG

homo sapiens **SEQ ID NO: 263**	CITMAYVWGKGHGDVRKVLPRNIAVPYCQLSKKLELPPILVY ADCVLANWKKKDPNKPLTYENMDVLFSFRDGDCSKGFFLVS LLVEIAAASAIKVIPTVFKAMQMQERDTLLKALLEIASCLEKA LQVFHQIHDHVNPKAFFSVLRIYLSGWKGNPQLSDGLVYEGF WEDPKEFAGGSAGQSSVFQCFDVLLGIQQTAGGGHAAQFLQ DMRRYMPPAHRNFLCSLESNPSVREFVLSKGDAGLREAYDA CVKALVSLRSYHLQIVTKYILIPASQQPKENKTSEDPSKLEAK GTGGTDLMNFLKTVRSTTEKSLLKEG
TDO2: tryptophan 2,3-dioxygenase from homo sapiens **SEQ ID NO: 264**	MSGCPFLGNNFGYTFKKLPVEGSEEDKSQTGVNRASKGGLIY GNYLHLEKVLNAQELQSETKGNKIHDEHLFIITHQAYELWFK QILWELDSVREIFQNGHVRDERNMLKVVSRMHRVSVILKLLV QQFSILETMTALDFNDFREYLSPASGFQSLQFRLLENKIGVLQ NMRVPYNRRHYRDNFKGEENELLLKSEQEKTLLELVEAWLE RTPGLEPHGFNFWGKLEKNITRGLEEEFIRIQAKEESEEKEEQV AEFQKQKEVLLSLFDEKRHEHLLSKGERRLSYRALQGALMIY FYREEPRFQVPFQLLTSLMDIDSLMTKWRYNHVCMVHRMLG SKAGTGGSSGYHYLRSTVSDRYKVFVDLFNLSTYLIPRHWIPK MNPTIHKFLYTAEYCDSSYFSSDESD
BNA2: indoleamine 2,3-dioxygenase from S. cerevisiae **SEQ ID NO: 265**	MNNTSITGPQVLHRTKMRPLPVLEKYCISPHHGFLDDRLPLTR LSSKKYMKWEEIVADLPSLLQEDNKVRSVIDGLDVLDLDETIL GDVRELRRAYSILGFMAHAYIWASGTPRDVLPECIARPLLETA HILGVPPLATYSSLVLWNFKVTDECKKTETGCLDLENITTINTF TGTVDESWFYLVSVRFEKIGSACLNHGLQILRAIRSGDKGDA NVIDGLEGLAATIERLSKALMEMELKCEPNVFYFKIRPFLAGW TNMSHMGLPQGVRYGAEGQYRIFSGGSNAQSSLIQTLDILLG VKHTANAAHSSQGDSKINYLDEMKKYMPREHREFLYHLESV CNIREYVSRNASNRALQEAYGRCISMLKIFRDNHIQIVTKYIIL PSNSKQHGSNKPNVLSPIEPNTKASGCLGHKVASSKTIGTGGT RLMPFLKQCRDETVATADIKNEDKN
Afmid: Kynurenine formamidase from mouse **SEQ ID NO: 266**	MAFPSLSAGQNPWRNLSSEELEKQYSPSRWVIHTKPEEVVGN FVQIGSQATQKARATRRNQLDVPYGDGEGEKLDIYFPDEDSK AFPLFLFLHGGYWQSGSKDDSAFMVNPLTAQGIVVVIVAYDI APKGTLDQMVDQVTRSVVFLQRRYPSNEGIYLCGHSAGAHL AAMVLLARWTKHGVTPNLQGFLLVSGIYDLEPLIATSQNDPL RMTLEDAQRNSPQRHLDVVPAQPVAPACPVLVLVGQHDSPE FHRQSKEFYETLLRVGWKASFQQLRGVDHFDIIENLTREDDV LTQIILKTVFQKL
BNA3: kynurenine--oxoglutarate transaminase from S. cerevisae **SEQ ID NO: 267**	MKQRFIRQFTNLMSTSRPKVVANKYFTSNTAKDVWSLTNEA AAKAANNSKNQGRELINLGQGFFSYSPPQFAIKEAQKALDIPM VNQYSPTRGRPSLINSLIKLYSPIYNTELKAENVTVTTGANEGI LSCLMGLLNAGDEVIVFEPFFDQYIPNIELCGGKVVYVPINPPK ELDQRNTRGEEWTIDFEQFEKAITSKTKAVIINTPHNPIGKVFT REELTTLGNICVKHNVVIISDEVYEHLYFTDSFTRIATLSPEIGQ LTLTVGSAGKSFAATGWRIGWVLSLNAELLSYAAKAHTRICF ASPSPLQEACANSINDALKIGYFEKMRQEYINKFKIFTSIFDEL GLPYTAPEGTYFVLVDFSKVKIPEDYPYPEEILNKGKDFRISH WLINELGVVAIPPTEFYIKEHEKAAENLLRFAVCKDDAYLEN AVERLKLLKDYL
GOT2: Aspartate aminotransferase,	MALLHSGRVLPGIAAAFHPGLAAAASARASSWWTHVEMGPP DPILGVTEAFKRDTNSKKMNLGVGAYRDDNGKPYVLPSVRK

mitochondrial from homo sapiens **SEQ ID NO: 268**	AEAQIAAKNLDKEYLPIGGLAEFCKASAELALGENSEVLKSG RFVTVQTISGTGALRIGASFLQRFFKFSRDVFLPKPTWGNHTPI FRDAGMQLQGYRYYDPKTCGFDFTGAVEDISKIPEQSVLLLH ACAHNPTGVDPRPEQWKEIATVVKKRNLFAFFDMAYQGFAS GDGDKDAWAVRHFIEQGINVCLCQSYAKNMGLYGERVGAFT MVCKDADEAKRVESQLKILIRPMYSNPPLNGARIAAAILNTPD LRKQWLQEVKVMADRIIGMRTQLVSNLKKEGSTHNWQHITD QIGMFCFTGLKPEQVERLIKEFSIYMTKDGRISVAGVTSSNVG YLAHAIHQVTK
AADAT: Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial **SEQ ID NO: 269**	MNYARFITAASAARNPSPIRTMTDILSRGPKSMISLAGGLPNP NMFPFKTAVITVENGKTIQFGEEMMKRALQYSPSAGIPELLSW LKQLQIKLHNPPTIHYPPSQGQMDLCVTSGSQQGLCKVFEMII NPGDNVLLDEPAYSGTLQSLHPLGCNIINVASDESGIVPDSLR DILSRWKPEDAKNPQKNTPKFLYTVPNGNNPTGNSLTSERKK EIYELARKYDFLIIEDDPYYFLQFNKFRVPTFLSMDVDGRVIRA DSFSKIISSGLRIGFLTGPKPLIERVILHIQVSTLHPSTFNQLMIS QLLHEWGEEGFMAHVDRVIDFYSNQKDAILAAADKWLTGLA EWHVPAAGMFLWIKVKGINDVKELIEEKAVKMGVLMLPGN AFYVDSSAPSPYLRASFSSASPEQMDVAFQVLAQLIKESL
CCLB1: Kynurenine--oxoglutarate transaminase 1 from homo sapiens **SEQ ID NO: 270**	MAKQLQARRLDGIDYNPWVEFVKLASEHDVVNLGQGFPDFP PPDFAVEAFQHAVSGDFMLNQYTKTFGYPPLTKILASFFGELL GQEIDPLRNVLVTVGGYGALFTAFQALVDEGDEVIIIEPFFDC YEPMTMMAGGRPVFVSLKPGPIQNGELGSSSNWQLDPMELA GKFTSRTKALVLNTPNNPLGKVFSREELELVASLCQQHDVVCI TDEVYQWMVYDGHQHISIASLPGMWERTLTIGSAGKTFSATG WKVGWVLGPDHIMKHLRTVHQNSVFHCPTQSQAAVAESFER EQLLFRQPSSYFVQFPQAMQRCRDHMIRSLQSVGLKPIIPQGS YFLITDISDFKRKMPDLPGAVDEPYDRRFVKWMIKNKGLVAI PVSIFYSVPHQKHFDHYIRFCFVKDEATLQAMDEKLRKWKVE L
CCLB2: kynurenine--oxoglutarate transaminase 3 from homo sapiens **SEQ ID NO: 271**	MFLAQRSLCSLSGRAKFLKTISSSKILGFSTSAKMSLKFTNAKR IEGLDSNVWIEFTKLAADPSVVNLGQGFPDISPPTYVKEELSKI AAIDSLNQYTRGFGHPSLVKALSYLYEKLYQKQIDSNKEILVT VGAYGSLFNTIQALIDEGDEVILIVPFYDCYEPMVRMAGATPV FIPLRSKPVYGKRWSSSDWTLDPQELESKFNSKTKAIILNTPHN PLGKVYNREELQVIADLCIKYDTLCISDEVYEWLVYSGNKHL KIATFPGMWERTITIGSAGKTFSVTGWKLGWSIGPNHLIKHLQ TVQQNTIYTCATPLQEALAQAFWIDIKRMDDPECYFNSLPKEL EVKRDRMVRLLESVGLKPIVPDGGYFIIADVSLLDPDLSDMK NNEPYDYKFVKWMTKHKKLSAIPVSAFCNSETKSQFEKFVRF CFIKKDSTLDAAEEIIKAWSVQKS
TnaA: tryptophanase from E. coli **SEQ ID NO: 272**	MENFKHLPEPFRIRVIEPVKRTTRAYREEAIIKSGMNPFLLDSE DVFIDLLTDSGTGAVTQSMQAAMMRGDEAYSGSRSYYALAE SVKNIFGYQYTIPTHQGRGAEQIYIPVLIKKREQEKGLDRSKM VAFSNYFFDTTQGHSQINGCTVRNVYIKEAFDTGVRYDFKGN FDLEGLERGIEEVGPNNVPYIVATITSNSAGGQPVSLANLKAM YSIAKKYDIPVVMDSARFAENAYFIKQREAEYKDWTIEQITRE TYKYADMLAMSAKKDAMVPMGGLLCMKDDSFFDVYTECRT LCVVQEGFPTYGGLEGGAMERLAVGLYDGMNLDWLAYRIA QVQYLVDGLEEIGVVCQQAGGHAAFVDAGKLLPHIPADQFP

	AQALACELYKVAGIRAVEIGSFLLGRDPKTGKQLPCPAELLRL TIPRATYTQTHMDFIIEAFKHVKENAANIKGLTFTYEPKVLRH FTAKLKEV

[0325] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence or nucleic acid sequence encoding a polypeptide of **Table E** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as one or more nucleic acid sequence of **Table E** or a functional fragment thereof. In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of one or more nucleic acid sequence of **Table E** or a functional fragment thereof, or a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as one or more nucleic acid sequence of **Table E** or a functional fragment thereof.

[0326] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding tryptophan decarboxylase. In some embodiments, the Tryptophan Decarboxylase encoded by bacterium has at least about 80% identity with the entire sequence selected from **SEQ ID NO: 242, 243, 244**. In some embodiments, the Tryptophan Decarboxylase gene has at least about 85% identity with the entire sequence selected from **SEQ ID NO: 242, 243, 244**. In some embodiments, the Tryptophan Decarboxylase polypeptide encoded by the bacteria has at least about 90% identity with the entire sequence selected from **SEQ ID NO: 242, 243, 244**. In some embodiments, the Tryptophan Decarboxylase polypeptide encoded by the bacteria has at least about 95% identity with the entire sequence selected from **SEQ ID NO: 242, 243, 244**. In another embodiment, the Tryptophan Decarboxylase polypeptide encoded by the bacteria has at least about 96%, 97%, 98%, or 99% identity with the entire sequence selected from **SEQ ID NO: 242, 243, 244**. Accordingly, In some embodiments, the Tryptophan Decarboxylase polypeptide encoded by the bacteria has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence selected from **SEQ ID NO: 242, 243, 244**. In some embodiments, the Tryptophan Decarboxylase polypeptide encoded by the bacteria comprises the sequence selected from **SEQ ID NO: 242, 243, 244**. In some embodiments, the Tryptophan

Decarboxylase polypeptide encoded by the bacteria consists of the sequence of selected from **SEQ ID NO: 242, 243, 244**..

[0327] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding tryptophan aminotransferase. In some embodiments, the Trp aminotransferase polypeptide encoded by the gene sequence has at least about 80% identity with the entire sequence of selected form **SEQ ID NO: 245 and 246**. In another embodiment, the Trp aminotransferase polypeptide encoded by the gene sequence has at least about 85% identity with the entire sequence of **SEQ ID NO: 245 and 246**. In some embodiments, the Trp aminotransferase polypeptide encoded by the gene sequence has at least about 90% identity with the entire sequence of **SEQ ID NO: 245 and 246**. In some embodiments, the Trp aminotransferase polypeptide encoded by the gene sequence has at least about 95% identity with the entire sequence of **SEQ ID NO: 245 and 246**. In another embodiment, the Trp aminotransferase polypeptide encoded by the gene sequence has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 245 and 246**. Accordingly, In some embodiments, the Trp aminotransferase polypeptide encoded by the gene sequence has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 245 and 246**. In another embodiment, the Trp aminotransferase polypeptide encoded by the gene sequence comprises the sequence of **SEQ ID NO: 245 and 246**. In yet another embodiment the Trp aminotransferase polypeptide encoded by the gene sequence consists of the sequence of **SEQ ID NO: 245 and 246.**

[0328] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding Monoamine oxidase (TYNA), e.g., from E. coli. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 247**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 247**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 247**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 247**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 247**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 247**.

[0329] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding AAO1: Indole-3-acetaldehyde oxidase, e.g., from Arabidopsis thaliana. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 248**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 248**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 248**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 248**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 248**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 248**.

[0330] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding aspC: aspartate aminotransferase, e.g., from E. coli. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 249**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 249**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 249**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 249**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 249**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 249**.

[0331] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding TAA1: L-tryptophan-pyruvate aminotransferase, e.g., from Arabidopsis thaliana. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 250**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 250**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 250**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 250**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a

sequence which encodes **SEQ ID NO: 250**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 250**.

[0332] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding STAO: L-tryptophan oxidase, e.g., from streptomyces sp. TP-A0274. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 251**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 251**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 251**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 251**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 251**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 251**.

[0333] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding ipdC: Indole-3-pyruvate decarboxylase, e.g., from Enterobacter cloacae. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 252**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 252**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 252**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 252**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 252**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 252**.

[0334] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding IAD1: Indole-3-acetaldehyde dehydrogenase, e.g., from Ustilago maydis. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 253**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 253**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 253**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%,

91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 253**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 253**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 253**.

[0335] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding YUC2: indole-3-pyruvate monooxygenase, e.g., from Arabidopsis thaliana. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 254**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 254**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 254**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 254**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 254**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 254**.

[0336] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding IaaM: Tryptophan 2-monooxygenase, e.g., from Pseudomonas savastanoi. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 255**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 255**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 255**. Accordingly, in some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 255**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 255**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 255**.

[0337] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding iaaH: Indoleacetamide hydrolase, e.g., from Pseudomonas savastanoi. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 256**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 256**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95%

identity with **SEQ ID NO: 256**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 256**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 256**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 256**.

[0338] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding TrpDH: Tryptophan dehydrogenase, e.g., from Nostoc punctiforme NIES-2108. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 257**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 257**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 257**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 257**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 257**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 257**.

[0339] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding CYP79B2: tryptophan N-monooxygenase, e.g., from Arabidopsis thaliana. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 258**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 258**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 258**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 258**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 258**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 258**.

[0340] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding CYP79B3: tryptophan N-monooxygenase, e.g., from Arabidopsis thaliana. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 259**. In some embodiments, the

gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 259**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 259**. Accordingly, in some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 259**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 259**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 259**.

[0341] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding CYP71A13: indoleacetaldoxime dehydratase, eg., from Arabidopis thaliana. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 260**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 260**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 260**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 260**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 260**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 260**.

[0342] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding PEN2: myrosinase, e.g, from Arabidopsis thaliana. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 261**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 261**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 261**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 261**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 261**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 261**.

[0343] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding Nit1: Nitrilase, e.g., from Arabidopsis thaliana. In some

embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 262**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 262**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 262**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 262**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 262**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 262**.

[0344] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding IDO1: indoleamine 2,3-dioxygenase, e.g.,, from homo sapiens In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 263**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 263**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 263**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 263**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 263**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 263**.

[0345] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding TDO2: tryptophan 2,3-dioxygenase, e.g., from homo sapiens. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 264**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 264**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 264**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 264**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 264**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 264**.

[0346] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding BNA2: indoleamine 2,3-dioxygenase, e.g., from S. cerevisiae. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 265**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 265**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 265**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 265**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 265**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 265**.

[0347] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding Afmid: Kynurenine formamidase, e.g., from mouse. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 266**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 266**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 266**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 266**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 266**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 266**.

[0348] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding BNA3: kynurenine--oxoglutarate transaminase, e.g., from S. cerevisae. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 267**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 267**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 267**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 267**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which

encodes **SEQ ID NO: 267**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 267**.

[0349] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding GOT2: Aspartate aminotransferase, mitochondrial, e.g., from homo sapiens. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 268**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 268**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 268**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 268**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 268**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 268**.

[0350] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding AADAT: Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial, e.g., from homo sapiens. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 269**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 269**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 269**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 269**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 269**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 269**.

[0351] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding CCLB1: Kynurenine--oxoglutarate transaminase 1, e.g., from homo sapiens. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 270**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 270**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 270**. Accordingly, In some embodiments, the gene

sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 270**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 270**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 270**.

[0352] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding CCLB2: kynurenine--oxoglutarate transaminase 3, e.g., from homo sapiens. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 271**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 271**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 271**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 271**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 271**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 271**.

[0353] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding TnaA: tryptophanase, e.g., from E. coli. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 80% identity with **SEQ ID NO: 272**. In some embodiments, the gene sequence encodes a polypeptide, which has at least about 90% identity with **SEQ ID NO: 272**. In another embodiment, the gene sequence encodes a polypeptide, which has at least about 95% identity with **SEQ ID NO: 272**. Accordingly, In some embodiments, the gene sequence encodes a polypeptide, which has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 272**. In another embodiment, the gene sequence encodes a polypeptide, which comprises a sequence which encodes **SEQ ID NO: 272**. In yet another embodiment, the gene sequence encodes a polypeptide, which consists of a sequence which encodes **SEQ ID NO: 272**.

[0354] In some embodiments, TNA (e.g., **SEQ ID NO: 272**) is mutated or deleted.

[0355] In some embodiments, the genetically engineered bacteria comprise a gene cassette for the production of tryptamine from tryptophan. In some embodiments, the genetically engineered bacteria take up tryptophan through an endogenous or exogenous transporter as described above herein. In som embodiments, the bacteria further produce

tryptamine from tryptophan. In some embodiments, the genetically engineered bacteria optionally comprise a tryptamine exporter. In some embodiments, the genetically engineered bacteria comprise an exporter of one or more indole metabolites, in order to increase the export of indole metabolites produced.

[0356] **Table F** depicts non-limiting examples of contemplated polypeptide sequences, which are encoded by indole-3-propionate producing bacteria.

Table F. Non-limiting Examples of Sequences for indole-3-propionate Production

Description	Sequence
FldA: indole-3-propionyl-CoA:indole-3-lactate CoA transferase from Clostridium sporogenes **SEQ ID NO: 273**	MENNTNMFSGVKVIELANFIAAPAAGRFFADGGAEVIKIESPA GDPLRYTAPSEGRPLSQEENTTYDLENANKKAIVLNLKSEKGK KILHEMLAEADILLTNWRTKALVKQGLDYETLKEKYPKLVFA QITGYGEKGPDKDLPGFDYTAFFARGGVSGTLYEKGTVPPNV VPGLGDHQAGMFLAAGMAGALYKAKTTGQGDKVTVSLMHS AMYGLGIMIQAAQYKDHGLVYPINRNETPNPFIVSYKSKDDYF VQVCMPPYDVFYDRFMTALGREDLVGDERYNKIENLKDGRA KEVYSIIEQQMVTKTKDEWDKIFRDADIPFAIAQTWEDLLEDE QAWANDYLYKMKYPTGNERALVRLPVFFKEAGLPEYNQSPQI AENTVEVLKEMGYTEQEIEELEKDKDIMVRKEK
FldB: subunit of indole-3-lactate dehydratase from Clostridium sporogenes **SEQ ID NO: 274**	MSDRNKEVKEKKAKHYLREITAKHYKEALEAKERGEKVGWC ASNFPQEIATTLGVKVVYPENHAAAVAARGNGQNMCEHAEA MGFSNDVCGYARVNLAVMDIGHSEDQPIPMPDFVLCCNNICN QMIKWYEHIAKTLDIPMILIDIPYNTENTVSQDRIKYIRAQFDD AIKQLEEITGKKWDENKFEEVMKISQESAKQWLRAASYAKYK PSPFSGFDLFNHMAVAVCARGTQEAADAFKMLADEYEENVKT GKSTYRGEEKQRILFEGIACWPYLRHKLTKLSEYGMNVTATV YAEAFGVIYENMDELMAAYNKVPNSISFENALKMRLNAVTST NTEGAVIHINRSCKLWSGFLYELARRLEKETGIPVVSFDGDQA DPRNFSEAQYDTRIQGLNEVMVAKKEAE
FldC: subunit of indole-3-lactate dehydratase from Clostridium sporogenes **SEQ ID NO: 275**	MSNSDKFFNDFKDIVENPKKYIMKHMEQTGQKAIGCMPLYTP EELVLAAGMFPVGVWGSNTELSKAKTYFPAFICSILQTTLENA LNGEYDMLSGMMITNYCDSLKCMGQNFKLTVENIEFIPVTVPQ NRKMEAGKEFLKSQYKMNIEQLEKISGNKITDESLEKAIEIYDE HRKVMNDFSMLASKYPGIITPTKRNYVMKSAYYMDKKEHTE KVRQLMDEIKAIEPKPFEGKRVITTGIIADSEDLLKILEENNIAIV GDDIAHESRQYRTLTPEANTPMDRLAEQFANRECSTLYDPEKK RGQYIVEMAKERKADGIIFFMTKFCDPEEYDYPQMKKDFEEA GIPHVLIETDMQMKNYEQARTAIQAFSETL
FldD: indole-3-acrylyl-CoA reductase from Clostridium sporogenes **SEQ ID NO: 276**	MFFTEQHELIRKLARDFAEQEIEPIADEVDKTAEFPKEIVKKMA QNGFFGIKMPKEYGGAGADNRAYVTIMEEISRASGVAGIYLSS PNSLLGTPFLLVGTDEQKEKYLKPMIRGEKTLAFALTEPGAGS DAGALATTAREEGDYYILNGRKTFITGAPISDNIIVFAKTDMSK GTKGITTFIVDSKQEGVSFGKPEDKMGMIGCPTSDIILENVKVH KSDILGEVNKGFITAMKTLSVGRIGVASQALGIAQAAVDEAVK YAKQRKQFNRPIAKFQAIQFKLANMETKLNAAKLLVYNAAYK MDCGEKADKEASMAKYFAAESAIQIVNDALQIHGGYGYIKDY

	KIERLYRDVRVIAIYEGTSEVQQMVIASNLLK
FldH1: indole-3-lactate dehydrogenase from Clostridium sporogenes **SEQ ID NO: 277**	MKILAYCVRPDEVDSFKKFSEKYGHTVDLIPDSFGPNVAHLAK GYDGISILGNDTCNREALEKIKDCGIKYLATRTAGVNNIDFDA AKEFGINVANVPAYSPNSVSEFTIGLALSLTRKIPFALKRVELN NFALGGLIGVELRNLTLGVIGTGRIGLKVIEGFSGFGMKKMIGY DIFENEEAKKYIEYKSLDEVFKEADIITLHAPLTDDNYHMIGKE SIAKMKDGVFIINAARGALIDSEALIEGLKSGKIAGAALDSYEY EQGVFHNNKMNEIMQDDTLERLKSFPNVVITPHLGFYTDEAVS NMVEITLMNLQEFELKGTCKNQRVCK
FldH2: indole-3-lactate dehydrogenase from Clostridium sporogenes **SEQ ID NO: 278**	MKILMYSVREHEKPAIKKWLEANPGVQIDLCNNALSEDTVCK AKEYDGIAIQQTNSIGGKAVYSTLKEYGIKQIASRTAGVDMIDL KMASDSNILVTNVPAYSPNAIAELAVTHTMNLLRNIKTLNKRI AYGDYRWSADLIAREVRSVTVGVVGTGKIGRTSAKLFKGLGA NVIGYDAYPDKKLEENNLLTYKESLEDLLREADVVTLHTPLLE STKYMINKNNLKYMKPDAFIVNTGRGGIINTEDLIEALEQNKIA GAALDTFENEGLFLNKVVDPTKLPDSQLDKLLKMDQVLITHH VGFFTTTAVQNIVDTSLDSVVEVLKTNNSVNKVN
AcuI: acrylyl-CoA reductase from Rhodobacter sphaeroides **SEQ ID NO: 279**	MRAVLIEKSDDTQSVSVTELAEDQLPEGDVLVDVAYSTLNYK DALAITGKAPVVRRFPMVPGIDFTGTVAQSSHADFKPGDRVIL NGWGVGEKHWGGLAERARVRGDWLVPLPAPLDLRQAAMIG TAGYTAMLCVLALERHGVVPGNGEIVVSGAAGGVGSVATTLL AAKGYEVAAVTGRASEAEYLRGLGAASVIDRNELTGKVRPLG QERWAGGIDVAGSTVLANMLSMMKYRGVVAACGLAAGMDL PASVAPFILRGMTLAGVDSVMCPKTDRLAAWARLASDLDPAK LEEMTTELPFSEVIETAPKFLDGTVRGRIVIPVTP

[0357] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding FldA: indole-3-propionyl-CoA:indole-3-lactate CoA transferase, e.g., from Clostridium sporogenes. In some embodiments, FldA has at least about 80% identity with **SEQ ID NO: 273**. In some embodiments, FldA has at least about 85% identity with one or more of **SEQ ID NO: 273**. In some embodiments, FldA has at least about 90% identity with **SEQ ID NO: 273**. In some embodiments, FldA has at least about 95% identity with **SEQ ID NO: 273**. In some embodiments, FldA has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 273**. Accordingly, In some embodiments, FldA has at least about 80%, 81%, 82%, 83%, 127%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 273**. In some embodiments,FldA comprises the sequence of **SEQ ID NO: 273**. In some embodiments, FldA consists of the sequence of one or more of SEQ ID NO: 84.

[0358] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding FldB: subunit of indole-3-lactate dehydratase, e.g., from Clostridium sporogenes. In some embodiments, FldB has at least about 80% identity with **SEQ ID NO: 274**. In some embodiments, FldB has at least about 85% identity with one or

more of **SEQ ID NO: 274**. In some embodiments, FldB has at least about 90% identity with **SEQ ID NO: 274**. In some embodiments, FldB has at least about 95% identity with **SEQ ID NO: 274**. In some embodiments, FldB has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 274**. Accordingly, In some embodiments, FldB has at least about 80%, 81%, 82%, 83%, 128%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 274**. In some embodiments,FldB comprises the sequence of **SEQ ID NO: 274**. In some embodiments, FldB consists of the sequence of one or more of **SEQ ID NO: 274**.

[0359] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding FldC: subunit of indole-3-lactate dehydratase from Clostridium sporogenes. In some embodiments, FldC has at least about 80% identity with **SEQ ID NO: 275**. In some embodiments, FldC has at least about 85% identity with one or more of **SEQ ID NO: 275**. In some embodiments, FldC has at least about 90% identity with **SEQ ID NO: 275**. In some embodiments, FldC has at least about 95% identity with **SEQ ID NO: 275**. In some embodiments, FldC has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 275**. Accordingly, In some embodiments, FldC has at least about 80%, 81%, 82%, 83%, 129%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 275**. In some embodiments,FldC comprises the sequence of **SEQ ID NO: 275**. In some embodiments, FldC consists of the sequence of one or more of **SEQ ID NO: 275**.

[0360] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding FldD: indole-3-acrylyl-CoA reductase, e.g., from Clostridium sporogenes. In some embodiments, FldD has at least about 80% identity with **SEQ ID NO: 276**. In some embodiments, FldD has at least about 85% identity with one or more of **SEQ ID NO: 276**. In some embodiments, FldD has at least about 90% identity with **SEQ ID NO: 276**. In some embodiments, FldD has at least about 95% identity with **SEQ ID NO: 276**. In some embodiments, FldD has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 276**. Accordingly, In some embodiments, FldD has at least about 80%, 81%, 82%, 83%, 130%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 276**. In some embodiments,FldD comprises the sequence of **SEQ ID NO: 276**. In some embodiments, FldD consists of the sequence of one or more of **SEQ ID NO: 276**.

[0361] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding FldH1: indole-3-lactate dehydrogenase, e.g., from

Clostridium sporogenes. In some embodiments, FldH1 has at least about 80% identity with **SEQ ID NO: 277**. In some embodiments, FldH1 has at least about 85% identity with one or more of **SEQ ID NO: 277**. In some embodiments, FldH1 has at least about 90% identity with **SEQ ID NO: 277**. In some embodiments, FldH1 has at least about 95% identity with **SEQ ID NO: 277**. In some embodiments, FldH1 has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 277**. Accordingly, In some embodiments, FldH1 has at least about 80%, 81%, 82%, 83%, 131%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 277**. In some embodiments,FldH1 comprises the sequence of **SEQ ID NO: 277**. In some embodiments, FldH1 consists of the sequence of one or more of **SEQ ID NO: 277**.

[0362] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding FldH2: indole-3-lactate dehydrogenase from Clostridium sporogenes. In some embodiments, FldH2 has at least about 80% identity with **SEQ ID NO: 278**. In some embodiments, FldH2 has at least about 85% identity with one or more of **SEQ ID NO: 278**. In some embodiments, FldH2 has at least about 90% identity with **SEQ ID NO: 278**. In some embodiments, FldH2 has at least about 95% identity with **SEQ ID NO: 278**. In some embodiments, FldH2 has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 278**. Accordingly, In some embodiments, FldH2 has at least about 80%, 81%, 82%, 83%, 132%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 278**. In some embodiments,FldH2 comprises the sequence of **SEQ ID NO: 278**. In some embodiments, FldH2 consists of the sequence of one or more of **SEQ ID NO: 278**.

[0363] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding AcuI: acrylyl-CoA reductase from Rhodobacter sphaeroides. In some embodiments, AcuI has at least about 80% identity with **SEQ ID NO: 279**. In some embodiments, AcuI has at least about 85% identity with one or more of **SEQ ID NO: 279**. In some embodiments, AcuI has at least about 90% identity with **SEQ ID NO: 279**. In some embodiments, AcuI has at least about 95% identity with **SEQ ID NO: 279**. In some embodiments, AcuI has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 279**. Accordingly, In some embodiments, AcuI has at least about 80%, 81%, 82%, 83%, 133%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 279**. In some embodiments,AcuI comprises the sequence of **SEQ ID NO: 279**. In some embodiments, AcuI consists of the sequence of one or more of **SEQ ID NO: 279**.

[0364] In some embodiments, the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding the tryptophan pathway catabolic enzyme which has at least about 80% identity with the entire sequence of one or more of **SEQ ID NO: 273 through SEQ ID NO: 279**. In another embodiment, the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding the tryptophan pathway catabolic enzyme which has at least about 85% identity with the entire sequence of one or more **SEQ ID NO: 273 through SEQ ID NO: 279**. In some embodiments, the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding the tryptophan pathway catabolic enzyme which has at least about 90% identity with the entire sequence of one or more **SEQ ID NO: 273 through SEQ ID NO: 279**. In some embodiments, the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding the tryptophan pathway catabolic enzyme which has at least about 95% identity with the entire sequence of one or more **SEQ ID NO: 273 through SEQ ID NO: 279**. In another embodiment, the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding the tryptophan pathway catabolic enzyme which has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of one or more **SEQ ID NO: 273 through SEQ ID NO: 279**. Accordingly, In some embodiments, the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding the tryptophan pathway catabolic enzyme which which has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with the entire sequence of one or more **SEQ ID NO: 273 through SEQ ID NO: 279**. In another embodiment, the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding tryptophan pathway catabolic enzyme which comprises the sequence of one or more **SEQ ID NO: 273 through SEQ ID NO: 279**. In yet another embodiment the genetically engineered bactierum comprises a gene sequence or nucleic acid sequence encoding the tryptophan pathway catabolic enzyme which consists of the sequence of one or more **SEQ ID NO: 273 through SEQ ID NO: 279.**

[0365] In some embodiments, the genetically engineered bacteria comprise a gene cassette for the production of one or more indole pathway metabolites described herein from tryptophan or a tryptophan metabolite. In some embodiments, the genetically engineered bacteria take up tryptophan through an endogenous or exogenous transporter as described above herein. In some embodiments, the genetically engineered bacteria additionally produce tryptophan and/or chorismate through any of the pathways described herein, e.g. **FIG. 40, FIG. 44A and FIG. 44B.** In some embodiments, the genetically engineered bacteria

comprise an exporter of one or more indole metabolites, in order to increase the export of indole metabolites produced.

[0366] In some embodiments, the genetically engineered bacteria are capable of expressing any one or more of the described circuits in low-oxygen conditions, in the presence of disease or tissue specific molecules or metabolites, in the presence of molecules or metabolites associated with inflammation or an inflammatory response or immune suppression or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose or tetracycline. In some embodiments, any one or more of the described circuits are present on one or more plasmids (*e.g.*, high copy or low copy) or are integrated into one or more sites in the bacterial chromosome. In some embodiments, the tryptophan synthesis and/or tryptophan catabolism cassette(s) is under control of an inducible promoter. Exemplary inducible promoters which may control the expression of the al teast one sequence(s) include oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose and tetracycline.

[0367] Also, in some embodiments, the genetically engineered bacteria are further capable of expressing any one or more of the described circuits and further comprise one or more of the following: (1) one or more auxotrophies, such as any auxotrophies known in the art and provided herein, *e.g.*, thyA auxotrophy, (2) one or more kill switch circuits, such as any of the kill-switches described herein or otherwise known in the art, (3) one or more antibiotic resistance circuits, (4) one or more transporters for importing biological molecules or substrates, such any of the transporters described herein or otherwise known in the art, (5) one or more exporters for exporting biological molecules or substrates, such any of the exporters described herein or otherwise known in the art, (6) one or more secretion circuits, such as any of the secretion circuits described herein and otherwise known in the art, and (7) combinations of one or more of such additional circuits.

Tryptophan Repressor (TrpR)

[0368] In any of these embodiments, the tryptophan repressor (trpR) optionally may be deleted, mutated, or modified so as to diminish or obliterate its repressor function. Also, in any of these embodiments, the genetically engineered bacteria optionally comprise gene sequence(s) to produce the tryptophan precursor, Chorismate, *e.g.*, sequence(s) encoding aroG, aroF, aroH, aroB, aroD, aroE, aroK, and AroC.

Tryptophan and Tryptophan MetaboliteTransport

[0369] Metabolite transporters may further be expressed or modified in the genetically engineered bacteria of the invention in order to enhance tryptophan or KP metabolite transport into the cell.

[0370] The inner membrane protein YddG of *E. coli*, encoded by the *yddG* gene, is a homologue of the known amino acid exporters RhtA and YdeD. Studies have shown that YddG is capable of exporting aromatic amino acids, including tryptophan. Thus, YddG can function as a tryptophan exporter or a tryptophan secretion system (or tryptophan secretion protein). Other aromatic amino acid exporters are described in Doroshenko *et al.*, FEMS Microbiol. Lett., 275:312-318 (2007). Thus, in some embodiments, the engineered bacteria optionally further comprise gene sequence(s) encoding YddG. In some embodiments, the engineered bacteria can over-express YddG. In some embodiments, the engineered bacteria optionally comprise one or more copies of yddG gene.

[0371] In some embodiments, the engineered microbe has a mechanism for importing (transporting) Kynurenine from the local environment into the cell. Thus, in some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding a kynureninase secreter. In some embodiments, the genetically engineered bacteria comprise one or more copies of *aroP, tnaB* or *mtr* gene.

[0372] In some embodiments the genetically engineered bacteria comprise a transporter to facilitate uptake of tryptophan into the cell. Three permeases, Mtr, TnaB, and AroP, are involved in the uptake of L-tryptophan in *Escherichia coli*. In some embodiments, the genetically engineered bacteria comprise one or more copies of one or more of Mtr, TnaB, and AroP.

[0373] In some embodiments, the genetically engineered bacteria of the invention also comprise multiple copies of the the transporter gene. In some embodiments, the genetically engineered bacteria of the invention also comprise a transporte gene from a different bacterial species. In some embodiments, the genetically engineered bacteria of the invention comprise multiple copies of a transporter gene from a different bacterial species. In some embodiments, the native transporter gene in the genetically engineered bacteria of the invention is not modified. In some embodiments, the genetically engineered bacteria of the invention comprise a transporter gene that is controlled by its native promoter, an inducible promoter, or a promoter that is stronger than the native promoter, *e.g.*, a GlnRS promoter, a P(Bla) promoter, or a constitutive promoter.

[0374] In some embodiments, the native transporter gene in the genetically engineered bacteria is not modified, and one or more additional copies of the native transporter gene are inserted into the genome under the control of the same inducible promoter that controls expression of the payload, *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload or a constitutive promoter. In alternate embodiments, the native transporter gene is not modified, and a copy of a non-native transporter gene from a different bacterial species is inserted into the genome under the control of the same inducible promoter that controls expression of the payload, *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload or a constitutive promoter.

[0375] In some embodiments, the native transporter gene in the genetically engineered bacteria is not modified, and one or more additional copies of the native transporter gene are present in the bacteria on a plasmid and under the control of the same inducible promoter that controls expression of the payload *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload or a constitutive promoter. In alternate embodiments, the native transporter gene is not modified, and a copy of a non-native transporter gene from a different bacterial species is present in the bacteria on a plasmid and under the control of the same inducible promoter that controls expression of the payload , *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload or a constitutive promoter.

[0376] In some embodiments, the native transporter gene is mutagenized, the mutants exhibiting increased ammonia transport are selected, and the mutagenized transporter gene is isolated and inserted into the genetically engineered bacteria. In some embodiments, the native transporter gene is mutagenized, mutants exhibiting increased ammonia transport are selected, and those mutants are used to produce the bacteria of the invention. The transporter modifications described herein may be present on a plasmid or chromosome.

[0377] In some embodiments, the genetically engineered bacterium is *E. coli* Nissle, and the native transporter gene in *E. coli* Nissle is not modified; one or more additional copies the native *E. coli* Nissle transporter genes are inserted into the *E. coli* Nissle genome under the control of the same inducible promoter that controls expression of the payload *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload or a constitutive promoter. In an alternate embodiment, the native *transporter* gene in *E. coli* Nissle is not modified, and a copy of a non-native *transporter* gene from a different bacterium, *e.g.*, *Lactobacillus plantarum,* is inserted into the *E. coli* Nissle genome under the

control of the same inducible promoter that controls expression of the payload, *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload or a constitutive promoter.

[0378] In some embodiments, the genetically engineered bacterium is *E. coli* Nissle, and the native *transporter* gene in *E. coli* Nissle is not modified; one or more additional copies the native *E. coli* Nissle *transporter* genes are present in the bacterium on a plasmid and under the control of the same inducible promoter that controls expression of the payload , *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload, or a constitutive promoter. In an alternate embodiment, the native *transporter* gene in *E. coli* Nissle is not modified, and a copy of a non-native *transporter* gene from a different bacterium, *e.g.*, *Lactobacillus plantarum,* are present in the bacterium on a plasmid and under the control of the same inducible promoter that controls expression of the payload, *e.g.*, a FNR promoter, or a different inducible promoter than the one that controls expression of the payload, or a constitutive promoter.

ALE

[0379] *E. coli* Nissle can be engineered to efficiently import KYN and convert it to TRP. While Nissle does not typically utilize KYN, by introducing the Kynureninase (KYNase) from *Pseudomonas fluorescens* (*kynU*) on a medium-copy plasmid under the control of the tetracycline promoter (Ptet) a new strain with this plasmid (Ptet-KYNase) is able to convert L-kynurenine into anthranilate. *E. coli* naturally utilizes anthranilate in its TRP biosynthetic pathway. Briefly, the TrpE (in complex with TrpD) enzyme converts chorismate into anthranilate. TrpD, TrpC, TrpA and TrpB then catalyzes a five-step reaction ending with the condensation of an indole with serine to form tryptophan. By replacing the TrpE enzyme via lambda-RED recombineering, the subsequent strain of Nissle (Δ*trpE*::Cm) is an auxotroph unable to grow in minimal media without supplementation of TRP or anthranilate. By expressing kynureninase in Δ*trpE*::Cm (KYNase-trpE), this auxotrophy can be alternatively rescued by providing KYN.

[0380] Leveraging the growth-limiting nature of KYN in KYNase-trpE, adaptive laboratory evolution was employed to further evolve a strain capable of increasingly efficient utilization of KYN. First a lower limit of KYN concentration was established and mutants were evolved by passaging in lowering concentrations of KYN. While this can select for mutants capable of increasing KYN import, the bacterial cells still prefer to utilize free, exogenous TRP. In the tumor environment, dual-therapeutic functions can be provided by depletion of KYN and increasing local concentrations of TRP. Therefore, to evolve a strain

which prefers KYN over TRP, a toxic analogue of TRP – 5-fluoro-L-tryptophan (ToxTRP) – can be incorporated into the ALE experiment. The resulting best performing strain is then whole genome sequenced in order to deconvolute the contributing mutations. Lambda-RED can be performed in order to reintroduce TrpE, to inactivate Trp regulation (*trpR, tyrR*, transcriptional attenuators) to up-regulate TrpABCDE expression and increase chorismate production. The resulting strain is now insensitive to external TRP, efficiently converts KYN into TRP, and also now overproduces TRP.

[0381] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequences , or a functional fragment thereof, as described herein. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as one or more nucleic acid sequence, or a functional fragment thereof, as described herein. In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of one or more nucleic acid sequence, or a functional fragment thereof, or a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as one or more nucleic acid sequence, or a functional fragment thereof, as described herein.

[0382] In some embodiments, the genetically engineered bacteria encode a gene or gene cassette, which promotes anti-inflammatory activity. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine.

[0383] In some embodiments, this step involves the conversion of tryptophan to kynurenine, and may be catalyzed by the ubiquitously-expressed enzyme indoleamine 2,3-dioxygenase (IDO-1), or by tryptophan dioxygenase (TDO), an enzyme which is primarily localized to the liver (Alvarado *et al.*, 2015). The genetically engineered bacteria may comprise any suitable gene for producing kynurenine. In some embodiments, the genetically engineered bacteria may comprise one or more gene(s) or gene cassette(s) for producing a tryptophan transporter, a gene or gene cassette for producing IDO-1, and a gene or gene cassette for producing TDO. In some embodiments, the genetically engineered bacteria comprise a gene encoding kynurenine formamidase.

[0384] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) for the consumption of tryptophan and production of kynurenine, which are bacterially derived. In some embodiments, the enzymes for TRP to KYN conversion are derived from one or more of Pseudomonas, Xanthomonas,

Burkholderia, Stenotrophomonas, Shewanella, and Bacillus, and/or members of the families Rhodobacteraceae, Micrococcaceae, and Halomonadaceae, In some embodiments the enzymes are derived from the species listed in table S7 of Vujkovic-Cvijin *et al.* (Dysbiosis of the gut microbiota is associated with HIV diseaseprogression and tryptophan catabolism Sci Transl Med. 2013 July 10; 5(193): 193ra91), the contents of which is herein incorporated by reference in its entirety.

[0385] In some embodiments, the one or more genes for producing kynurenine are modified and/or mutated, *e.g.*, to enhance stability, increase kynurenine production, and/or increase anti-inflammatory potency under inducing conditions. In some embodiments, the engineered bacteria have enhanced uptake or import of tryptophan, *e.g.*, comprise a transporter or other mechanism for increasing the uptake of tryptophan into the bacterial cell. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine under inducing conditions, *e.g.*, under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenine in low-oxygen conditions. In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid. Kynurenic acid is produced from the irreversible transamination of kynurenine in a reaction catalyzed by the enzyme kynurenine-oxoglutarate transaminase. In some embodiments,

[0386] The genetically engineered bacteria may comprise any suitable gene for producing kynurenic acid. In some embodiments, the gene for producing kynurenic acid is modified and/or mutated, *e.g.*, to enhance stability, increase kynurenic acid production, and/or increase anti-inflammatory potency under inducing conditions. In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid under inducing conditions, *e.g.*, under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing kynurenic acid in low-oxygen conditions.

[0387] In some embodiments, the genetically engineered bacteria comprising one or more gene(s) or gene cassette(s) can alter the TRP:KYN ratio, *e.g.* in the circulation. In some embodimetns the TRP:KYN ratio is increased. In some embodiments, TRP:KYN ratio is decreased. some embodiments, the genetically engineered bacteria the genetically engineered bacteria comprising one or more gene(s) or gene cassette(s) can alter the KYNA:QUIN ratio.

[0388] In some embodiments, the genetically engineered bacteria are capable of expressing any one or more of the described circuits in low-oxygen conditions, in the presence of disease or tissue specific molecules or metabolites, in the presence of molecules

or metabolites associated with inflammation or an inflammatory response or liver damage, e.g., as seen in NASH,, metabolic disease, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, any one or more of the described circuits are present on one or more plasmids (*e.g.*, high copy or low copy) or are integrated into one or more sites in the bacterial chromosome. Also, in some embodiments, the genetically engineered bacteria are further capable of expressing any one or more of the described circuits and further comprise one or more of the following: (1) one or more auxotrophies, such as any auxotrophies known in the art and provided herein, *e.g.*, thyA auxotrophy, (2) one or more kill switch circuits, such as any of the kill-switches described herein or otherwise known in the art, (3) one or more antibiotic resistance circuits, (4) one or more transporters for importing biological molecules or substrates, such any of the transporters described herein or otherwise known in the art, (5) one or more secretion circuits, such as any of the secretion circuits described herein and otherwise known in the art, and (6) combinations of one or more of such additional circuits.

Inhibitory and targeting molecules

[0389] In some embodiments, the genetically engineered bacteria of the invention are capable of producing a molecule that is capable of inhibiting a NASH-promoting molecule. The genetically engineered bacteria may express any suitable inhibitory molecule, *e.g.*, a single-chain variable fragment (scFv), antisense RNA, siRNA, or shRNA, that is capable of neutralizing one or more NASH-promoting molecules, *e.g.*, dipeptidyl peptidase-4 (DPP-4) or ghrelin receptor. The genetically engineered bacteria may inhibit one or more NASH-promoting molecules.

[0390] RNA interference (RNAi) is a post-transcriptional gene silencing mechanism in plants and animals. RNAi is activated when microRNA (miRNA), double-stranded RNA (dsRNA), or short hairpin RNA (shRNA) is processed into short interfering RNA (siRNA) duplexes (Keates et al., 2008). RNAi can be "activated *in vitro* and *in vivo* by non-pathogenic bacteria engineered to manufacture and deliver shRNA to target cells" such as mammalian cells (Keates et al., 2008). In some embodiments, the genetically engineered bacteria of the invention induce RNAi-mediated gene silencing of one or more NASH-promoting molecules in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, NASH, inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.In some embodiments, the genetically engineered bacteria produce siRNA targeting DPP-4 in low-

oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose.

[0391] Single-chain variable fragments (scFv) are "widely used antibody fragments… produced in prokaryotes" (Frenzel et al., 2013). scFv lacks the constant domain of a traditional antibody and expresses the antigen-binding domain as a single peptide. Bacteria such as *Escherichia coli* are capable of producing scFv that target a variety of molecules, *e.g.*, TNF (Hristodorov et al., 2014). In some embodiments, the genetically engineered bacteria of the invention express a binding protein for neutralizing one or more metabolic disease-promoting molecules in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH,, metabolic disease, inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, the genetically engineered bacteria produce scFv targeting DPP-4 in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, metabolic disease, inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose. In some embodiments, the genetically engineered bacteria produce both scFv and siRNA targeting one or more metabolic disease-promoting molecules in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, e.g., as seen in NASH, metabolic disease, inflammation or an inflammatory response, or in the presence of some other metabolite that may or may not be present in the gut, such as arabinose (*see, e.g.,* Xiao *et al.*, 2014).

Generation of Bacterial Strains with Enhance Ability to Transport Amino Acids

[0392] Due to their ease of culture, short generation times, very high population densities and small genomes, microbes can be evolved to unique phenotypes in abbreviated timescales. Adaptive laboratory evolution (ALE) is the process of passaging microbes under selective pressure to evolve a strain with a preferred phenotype. Most commonly, this is applied to increase utilization of carbon/energy sources or adapting a strain to environmental stresses (e.g., temperature, pH), whereby mutant strains more capable of growth on the

carbon substrate or under stress will outcompete the less adapted strains in the population and will eventually come to dominate the population.

[0393] This same process can be extended to any essential metabolite by creating an auxotroph. An auxotroph is a strain incapable of synthesizing an essential metabolite and must therefore have the metabolite provided in the media to grow. In this scenario, by making an auxotroph and passaging it on decreasing amounts of the metabolite, the resulting dominant strains should be more capable of obtaining and incorporating this essential metabolite.

[0394] For example, if the biosynthetic pathway for producing an amino acid is disrupted a strain capable of high-affinity capture of said amino acid can be evolved via ALE. First, the strain is grown in varying concentrations of the auxotrophic amino acid, until a minimum concentration to support growth is established. The strain is then passaged at that concentration, and diluted into lowering concentrations of the amino acid at regular intervals. Over time, cells that are most competitive for the amino acid – at growth-limiting concentrations – will come to dominate the population. These strains will likely have mutations in their amino acid-transporters resulting in increased ability to import the essential and limiting amino acid.

[0395] Similarly, by using an auxotroph that cannot use an upstream metabolite to form an amino acid, a strain can be evolved that not only can more efficiently import the upstream metabolite, but also convert the metabolite into the essential downstream metabolite. These strains will also evolve mutations to increase import of the upstream metabolite, but may also contain mutations which increase expression or reaction kinetics of downstream enzymes, or that reduce competitive substrate utilization pathways.

[0396] A metabolite innate to the microbe can be made essential via mutational auxotrophy and selection applied with growth-limiting supplementation of the endogenous metabolite. However, phenotypes capable of consuming non-native compounds can be evolved by tying their consumption to the production of an essential compound. For example, if a gene from a different organism is isolated which can produce an essential compound or a precursor to an essential compound this gene can be recombinantly introduced and expressed in the heterologous host. This new host strain will now have the ability to synthesize an essential nutrient from a previously non-metabolizable substrate.

[0397] Hereby, a similar ALE process can be applied by creating an auxotroph incapable of converting an immediately downstream metabolite and selecting in growth-limiting amounts of the non-native compound with concurrent expression of the recombinant

enzyme. This will result in mutations in the transport of the non-native substrate, expression and activity of the heterologous enzyme and expression and activity of downstream native enzymes. It should be emphasized that the key requirement in this process is the ability to tether the consumption of the non-native metabolite to the production of a metabolite essential to growth.

[0398] Once the basis of the selection mechanism is established and minimum levels of supplementation have been established, the actual ALE experimentation can proceed. Throughout this process several parameters must be vigilantly monitored. It is important that the cultures are maintained in an exponential growth phase and not allowed to reach saturation/stationary phase. This means that growth rates must be check during each passaging and subsequent dilutions adjusted accordingly. If growth rate improves to such a degree that dilutions become large, then the concentration of auxotrophic supplementation should be decreased such that growth rate is slowed, selection pressure is increased and dilutions are not so severe as to heavily bias subpopulations during passaging. In addition, at regular intervals cells should be diluted, grown on solid media and individual clones tested to confirm growth rate phenotypes observed in the ALE cultures.

[0399] Predicting when to halt the stop the ALE experiment also requires vigilance. As the success of directing evolution is tied directly to the number of mutations "screened" throughout the experiment and mutations are generally a function of errors during DNA replication, the cumulative cell divisions (CCD) acts as a proxy for total mutants which have been screened. Previous studies have shown that beneficial phenotypes for growth on different carbon sources can be isolated in about 1011.2 CCD1. This rate can be accelerated by the addition of chemical mutagens to the cultures – such as N-methyl-N-nitro-N-nitrosoguanidine (NTG) – which causes increased DNA replication errors. However, when continued passaging leads to marginal or no improvement in growth rate the population has converged to some fitness maximum and the ALE experiment can be halted.

[0400] At the conclusion of the ALE experiment, the cells should be diluted, isolated on solid media and assayed for growth phenotypes matching that of the culture flask. Best performers from those selected are then prepped for genomic DNA and sent for whole genome sequencing. Sequencing with reveal mutations occurring around the genome capable of providing improved phenotypes, but will also contain silent mutations (those which provide no benefit but do not detract from desired phenotype). In cultures evolved in the presence of NTG or other chemical mutagen, there will be significantly more silent, background mutations. If satisfied with the best performing strain in its current state, the user

can proceed to application with that strain. Otherwise the contributing mutations can be deconvoluted from the evolved strain by reintroducing the mutations to the parent strain by genome engineering techniques. See Lee, D.-H., Feist, A. M., Barrett, C. L. & Palsson, B. Ø. Cumulative Number of Cell Divisions as a Meaningful Timescale for Adaptive Laboratory Evolution of *Escherichia coli*. PLoS ONE 6, e26172 (2011).

[0401] Similar methods can be used to generate E.Coli Nissle mutants that consume or import tryptophan.

Inducible Regulatory Regions

[0611] In some embodiments, the bacterial cell comprises a stably maintained plasmid or chromosome carrying the gene(s) encoding metabolic and/or satiety effector molecule(s), such that the molecule(s) can be expressed in the host cell, and the host cell is capable of survival and/or growth *in vitro*, *e.g.,* in medium, and/or *in vivo*, *e.g.,* in the gut or in the liver. In some embodiments, bacterial cell comprises two or more distinct metabolic and/or satiety effector molecule(s) or operons, *e.g.,* two or more metabolic and/or satiety effector molecule genes. In some embodiments, bacterial cell comprises three or more distinct transporters or operons, *e.g.,* three or more metabolic and/or satiety effector molecules genes. In some embodiments, bacterial cell comprises 4, 5, 6, 7, 8, 9, 10, or more distinct metabolic and/or satiety effector molecules or operons, *e.g.,* 4, 5, 6, 7, 8, 9, 10, or more metabolic and/or satiety effector molecules genes.

[0612] Herein the terms "payload" "polypeptide of interest" or "polypeptides of interest", "protein of interest", "proteins of interest", "payloads" "effector molecule", "effector" refers to one or more effector molecules described herein and/or one or more enzyme(s) or polypeptide(s0 function as enyzmes for the production of such effector molecules. Non-limiting examples of payloads include propionate, butyrate, GLP-1, a tryptophan transporter, aromatic amino acid transporter, IDO, TDO, polypeptide(s) for producing kynurenine or kynurenic acid, polypeptides for metabolizing tryptophan. .As used herein, the term "polypeptide of interest" or "polypeptides of interest", "protein of interest", "proteins of interest", "payload", "payloads" further includes any or a plurality of any of the gene, gene(s), or gene cassettes for producing the payload(s) that are present on a plasmid and operably linked to a directly or indirectly inducible promoter described herein. As used herein, the term "gene of interest" or "gene sequence of interest" includes any or a plurality of any of the gene(s) an/or gene sequence(s) and or gene cassette(s) encoding one or more stably maintained plasmid or chromosome carrying the gene or genes for producing one or

more payload molecules or the gene or genes for encoding one or more payload polypeptides described herein.

[0613] In some embodiments, the genetically engineered bacteria comprise multiple copies of the same metabolic and/or satiety effector molecules gene(s). In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on a plasmid and operably linked to a directly or indirectly inducible promoter. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on a plasmid and operably linked to a constitutive promoter. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on a plasmid and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on plasmid and operably linked to a promoter that is induced by exposure to tetracycline or arabinose, or another chemical or nutritional inducer described herein.

[0614] In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on a chromosome and operably linked to a directly or indirectly inducible promoter. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on a chromosome and operably linked to a constitutive promoter. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present in the chromosome and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on chromosome and operably linked to a promoter that is induced by exposure to tetracycline or arabinose, or another chemical or nutritional inducer described herein.

[0615] In some embodiments, the genetically engineered bacteria comprise two or more metabolic and/or satiety effector molecules, all of which are present on the chromosome. In some embodiments, the genetically engineered bacteria comprise two or more metabolic and/or satiety effector molecules, all of which are present on one or more same or different plasmids. In some embodiments, the genetically engineered bacteria comprise two or more metabolic and/or satiety effector molecules, some of which are present on the chromosome and some of which are present on one or more same or different plasmids.

[0616] In any of the nucleic acid embodiments described above, the one or more metabolic and/or satiety effector molecules for producing the at least one gene, gene(s), or gene cassettes for producing the metabolic and/or satiety effector molecule combination(s)

are operably linked to one or more directly or indirectly inducible promoter(s). In some embodiments, the one or more metabolic and/or satiety effector molecules are operably linked to a directly or indirectly inducible promoter that is induced under exogeneous environmental conditions, *e.g.*, conditions found in the gut, or liver. In some embodiments, the one or more metabolic and/or satiety effector molecules are operably linked to a directly or indirectly inducible promoter that is induced by metabolites found in the gut, or conditions associated with liver damage or inflammation. In some embodiments, the one or more metabolic and/or satiety effector molecules are operably linked to a directly or indirectly inducible promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the one or more metabolic and/or satiety effector molecules are operably linked to a directly or indirectly inducible promoter that is induced under inflammatory conditions (*e.g.*, RNS, ROS), as described herein. In some embodiments, the one or more metabolic and/or satiety effector molecules are operably linked to a directly or indirectly inducible promoter that is induced under immunosuppressive conditions, *e.g.*, as found in the tumor, as described herein. In some embodiments, the two or more gene sequence(s) are linked to a directly or indirectly inducible promoter that is induced by exposure a chemical or nutritional inducer, which may or may not be present under *in vivo* conditions and which may be present during *in vitro* conditions (such as strain culture, expansion, manufacture), such as tetracycline or arabinose, or others described herein. In some embodiments, the two or more payloads are all linked to a constitutive promoter. Such constitutive promoters are described in **Table 30 - Table 40** herein.

[0617] In some embodiments, the promoter is induced under *in vivo* conditions, *e.g.*, the gut, as described herein. In some embodiments, the promoters is induced under *in vitro* conditions, *e.g.*, various cell culture and/or cell manufacturing conditions, as described herein. In some embodiments, the promoter is induced under *in vivo* conditions, *e.g.*, the gut, as described herein, and under *in vitro* conditions, *e.g.*, various cell culture and/or cell production and/or manufacturing conditions, as described herein.

[0618] In some embodiments, the promoter that is operably linked to the gene encoding the payload is directly induced by exogenous environmental conditions (*e.g.*, *in vivo* and/or *in vitro* and/or production/manufacturing conditions). In some embodiments, the promoter that is operably linked to the gene encoding the payload is indirectly induced by exogenous environmental conditions (*e.g.*, *in vivo* and/or *in vitro* and/or production/manufacturing conditions).

[0619] In some embodiments, the promoter is directly or indirectly induced by exogenous environmental conditions specific to the gut and liver of a mammal. In some embodiments, the promoter is directly or indirectly induced by exogenous environmental conditions specific to the hypoxic environment of a tumor and/or the small intestine of a mammal. In some embodiments, the promoter is directly or indirectly induced by low-oxygen or anaerobic conditions such as the environment of the mammalian gut. In some embodiments, the promoter is directly or indirectly induced by molecules or metabolites that are specific to the tumor, a particular tissue or the gut of a mammal. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the bacterial cell.

FNR dependent Regulation

[0620] The genetically engineered bacteria of the invention comprise a gene or gene cassette for producing metabolic and/or satiety effector molecules that are expressed under the control of the fumarate and nitrate reductase regulator (FNR) promoter, wherein the gene or gene cassette is operably linked to a directly or indirectly inducible promoter that is controlled by exogenous environmental condition(s). In some embodiments, the inducible promoter is an oxygen level-dependent promoter and the bacterial cell comprises at least one gene, gene(s), or gene cassettes for producing the metabolic and/or satiety effector molecules is expressed in low-oxygen, microaerobic, or anaerobic conditions. For example, in low oxygen conditions, the oxygen level-dependent promoter is activated by a corresponding oxygen level-sensing transcription factor, thereby driving production of the bacterial cell that comprises at least one gene, gene(s), or gene cassettes for producing the metabolic and/or satiety effector molecules.

[0621] Bacteria have evolved transcription factors that are capable of sensing oxygen levels. Different signaling pathways may be triggered by different oxygen levels and occur with different kinetics. An oxygen level-dependent promoter is a nucleic acid sequence to which one or more oxygen level-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression. In one embodiment, the genetically engineered bacteria comprise a gene or gene cassette for producing a payload under the control of an oxygen level-dependent promoter. In a more specific aspect, the genetically engineered bacteria that comprise a gene or gene cassette for producing a payload under the control of an oxygen level-dependent

promoter that is activated under low-oxygen or anaerobic environments, such as the hypoxic environment of a tumor and/or the environment of the mammalian gut.

[0622] In certain embodiments, the bacterial cell comprises a gene encoding a metabolic and/or satiety effector molecule expressed under the control of a fumarate and nitrate reductase regulator (FNR) responsive promoter. In *E. coli,* FNR is a major transcriptional activator that controls the switch from aerobic to anaerobic metabolism (Unden *et al.*, 1997). In the anaerobic state, FNR dimerizes into an active DNA binding protein that activates hundreds of genes responsible for adapting to anaerobic growth. In the aerobic state, FNR is prevented from dimerizing by oxygen and is inactive. FNR responsive promoters include, but are not limited to, the FNR responsive promoters listed in **Table 23** and **Table 24** below. Underlined sequences are predicted ribosome binding sites, and bolded sequences are restriction sites used for cloning.

Table 20. FNR Promoter Sequences

FNR Responsive Promoter	Sequence
SEQ ID NO: 563	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTA CATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATACCCCTTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGT AATAGAAAAGAAATCGAGGCAAAA
SEQ ID NO: 564	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTT ATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAA ACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCCGT TACGTGGGCTTCGACTGTAAATCAGAAAGGAGAAAACACCT
SEQ ID NO: 565	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTA CATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATACCCCTTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAA**GGATCC**CTCT AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
SEQ ID NO: 566	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACT TATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAA ACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCC**GG ATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CAT

SEQ ID NO: 567	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAA ATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAA ACGCCGTAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTC AGGGCAATATCTCTCTT**GGATCC**CTCTAGAAATAATTTTGTTTA ACTTTAAGAAGGAGATATACAT

Table 21. FNR Promoter sequences

FNR-responsive regulatory region	**12345678901234567890123456789012345678901234567890**
SEQ ID NO: 568	ATCCCCATCACTCTTGATGGAGATCAATTCCCCAAGCTGCTA GAGCGTTACCTTGCCCTTAAACATTAGCAATGTCGATTTATC AGAGGGCCGACAGGCTCCCACAGGAGAAAACCG
SEQ ID NO: 569	CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACC TTGCCCTTAAACATTAGCAATGTCGATTTATCAGAGGGCCGA CAGGCTCCCACAGGAGAAAACCG
nirB1 **SEQ ID NO: 570**	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGG GCGGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTA CGTACATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTT GCACAAACATGAAATATCAGACAATTCCGTGACTTAAGAAA ATTTATACAAATCAGCAATATACCCCTTAAGGAGTATATAAA GGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGT TAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA
nirB2 **SEQ ID NO: 571**	CGGCCCGATCGTTGAACATAGCGGTCCGCAGGCGGCACTGC TTACAGCAAACGGTCTGTACGCTGTCGTCTTTGTGATGTGCT TCCTGTTAGGTTTCGTCAGCCGTCACCGTCAGCATAACACCC TGACCTCTCATTAATTGCTCATGCCGGACGGCACTATCGTCG TCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTATTTCT ATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGAAA TATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAG CAATATACCCATTAAGGAGTATATAAAGGTGAATTTGATTTA CATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGTA ATAGAAAAGAAATCGAGGCAAAAatgtttgtttaactttaagaaggagatatac at
nirB3 **SEQ ID NO: 572**	GTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGG ACGGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTA CGTGCATCTATTTCTATAAACCCGCTCATTTTGTCTATTTTTT GCACAAACATGAAATATCAGACAATTCCGTGACTTAAGAAA ATTTATACAAATCAGCAATATACCCATTAAGGAGTATATAAA GGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGT TAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA

ydfZ **SEQ ID NO: 573**	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGAC TTATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCA AAAACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCG CCCGTTACGTGGGCTTCGACTGTAAATCAGAAAGGAGAAAA CACCT
nirB+RBS **SEQ ID NO: 574**	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGG GCGGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTA CGTACATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTT GCACAAACATGAAATATCAGACAATTCCGTGACTTAAGAAA ATTTATACAAATCAGCAATATACCCCTTAAGGAGTATATAAA GGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGT TAA**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGG AGATATACAT
ydfZ+RBS **SEQ ID NO: 575**	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGA CTTATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATC AAAAACAAAAAATATTTCACTCGACAGGAGTATTTATATTGC GCCC**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAG GAGATATACAT
fnrS1 **SEQ ID NO: 576**	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGT AAATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATAC AAAAACGCCGTAAAGTTTGAGCGAAGTCAATAAACTCTCTA CCCATTCAGGGCAATATCTCTCTT**GGATCC**CTCTAGAAATAA TTTTGTTTAACTTTAAGAAGGAGATATACAT
fnrS2 **SEQ ID NO: 577**	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGT AAATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATAC AAAAACGCCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTA CCCATTCAGGGCAATATCTCTCTTGGATCCAAAGTGAACTCT AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
nirB+crp **SEQ ID NO: 578**	TCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTCAGCCGTCA CCGTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCC GGACGGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGC TACGTGCATCTATTTCTATAAACCCGCTCATTTTGTCTATTTT TTGCACAAACATGAAATATCAGACAATTCCGTGACTTAAGA AAATTTATACAAATCAGCAATATACCCATTAAGGAGTATATA AAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATC GTTAAGGTAGaaatgtgatctagttcacatttGCGGTAATAGAAAAGAAAT CGAGGCAAAA*atgtttgtttaactttaagaaggagatatacat*
fnrS+crp **SEQ ID NO: 579**	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGT AAATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATAC AAAAACGCCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTA CCCATTCAGGGCAATATCTCTCaaatgtgatctagttcacattt*tttgtttaactttaa gaaggagatatacat*

[0623] FNR promoter sequences are known in the art, and any suitable FNR promoter sequence(s) may be used in the genetically engineered bacteria of the invention. Any suitable FNR promoter(s) may be combined with any suitable metabolic and/or satiety effector molecule.

[0624] Non-limiting FNR promoter sequences are provided in **Table 20** and **Table 21**. **Table 20 and Table 21** depicts the nucleic acid sequences of exemplary regulatory region sequences comprising a FNR-responsive promoter sequence. Underlined sequences are predicted ribosome binding sites, and bolded sequences are restriction sites used for cloning. In some embodiments, the genetically engineered bacteria of the invention comprise one or more of: **SEQ ID NO: 563, SEQ ID NO: 564, SEQ ID NO: 565, SEQ ID NO: 566, SEQ ID NO: 567, SEQ ID NO: 568, SEQ ID NO: 569**, nirB1 promoter (**SEQ ID NO: 570**), nirB2 promoter (**SEQ ID NO: 571**), nirB3 promoter (**SEQ ID NO: 572**), ydfZ promoter (**SEQ ID NO: 573**), nirB promoter fused to a strong ribosome binding site (**SEQ ID NO: 574**), ydfZ promoter fused to a strong ribosome binding site (**SEQ ID NO: 575**), fnrS, an anaerobically induced small RNA gene (fnrS1 promoter **SEQ ID NO: 576** or fnrS2 promoter **SEQ ID NO: 577**), *nirB* promoter fused to a crp binding site (**SEQ ID NO: 578**), and fnrS fused to a crp binding site (**SEQ ID NO: 579**). In some embodiments, the FNR-responsive promoter is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the sequence of any one of **SEQ ID NOs: 563-579**.

[0625] In some embodiments, multiple distinct FNR nucleic acid sequences are inserted in the genetically engineered bacteria. In alternate embodiments, the genetically engineered bacteria comprise a gene encoding a metabolic and/or satiety effector molecule expressed under the control of an alternate oxygen level-dependent promoter, *e.g.*, DNR (Trunk *et al.*, 2010) or ANR (Ray *et al.*, 1997). In these embodiments, expression of the metabolic and/or satiety effector molecule gene is particularly activated in a low-oxygen or anaerobic environment, such as in the gut. In some embodiments, gene expression is further optimized by methods known in the art, *e.g.*, by optimizing ribosomal binding sites and/or increasing mRNA stability. In one embodiment, the mammalian gut is a human mammalian gut.

[0626] In another embodiment, the genetically engineered bacteria comprise the gene or gene cassette for producing the metabolic and/or satiety effector molecules expressed under the control of anaerobic regulation of arginine deiminiase and nitrate reduction transcriptional regulator (ANR). In *P. aeruginosa,* ANR is "required for the expression of physiological functions which are inducible under oxygen-limiting or anaerobic conditions"

(Winteler *et al.*, 1996; Sawers 1991). *P. aeruginosa* ANR is homologous with *E. coli* FNR, and "the consensus FNR site (TTGAT----ATCAA) was recognized efficiently by ANR and FNR" (Winteler *et al.*, 1996). Like FNR, in the anaerobic state, ANR activates numerous genes responsible for adapting to anaerobic growth. In the aerobic state, ANR is inactive. *Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas syringae*, and *Pseudomonas mendocina* all have functional analogs of ANR (Zimmermann *et al.*, 1991). Promoters that are regulated by ANR are known in the art, *e.g.*, the promoter of the arcDABC operon (*see, e.g.,* Hasegawa *et al.*, 1998).

[0627] ln other embodiments, the one or more gene sequence(s) for producing a metabolic and/or satiety effector molecule are expressed under the control of an oxygen level-dependent promoter fused to a binding site for a transcriptional activator, *e.g.*, CRP. CRP (cyclic AMP receptor protein or catabolite activator protein or CAP) plays a major regulatory role in bacteria by repressing genes responsible for the uptake, metabolism, and assimilation of less favorable carbon sources when rapidly metabolizable carbohydrates, such as glucose, are present (Wu et al., 2015). This preference for glucose has been termed glucose repression, as well as carbon catabolite repression (Deutscher, 2008; Görke and Stülke, 2008). In some embodiments, the gene or gene cassette for producing a payload is controlled by an oxygen level-dependent promoter fused to a CRP binding site. In some embodiments, the one or more gene sequence(s) for a payload are controlled by a FNR promoter fused to a CRP binding site. In these embodiments, cyclic AMP binds to CRP when no glucose is present in the environment. This binding causes a conformational change in CRP, and allows CRP to bind tightly to its binding site. CRP binding then activates transcription of the gene or gene cassette by recruiting RNA polymerase to the FNR promoter via direct protein-protein interactions. In the presence of glucose, cyclic AMP does not bind to CRP and transcription of the gene or gene cassette for producing an payload is repressed. In some embodiments, an oxygen level-dependent promoter (*e.g.*, an FNR promoter) fused to a binding site for a transcriptional activator is used to ensure that the gene or gene cassette for producing a payload is not expressed under anaerobic conditions when sufficient amounts of glucose are present, *e.g.*, by adding glucose to growth media *in vitro*.

[0628] In some embodiments, the genetically engineered bacteria comprise an oxygen level-dependent promoter from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise an oxygen level-sensing transcription factor, *e.g.*, FNR, ANR or DNR, from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise an oxygen

level-sensing transcription factor and corresponding promoter from a different species, strain, or substrain of bacteria. The heterologous oxygen-level dependent transcriptional regulator and/or promoter increases the transcription of genes operably linked to said promoter, *e.g.*, one or more gene sequence(s) for producing the payload(s) in a low-oxygen or anaerobic environment, as compared to the native gene(s) and promoter in the bacteria under the same conditions. In certain embodiments, the non-native oxygen-level dependent transcriptional regulator is an FNR protein from N. gonorrhoeae (see, *e.g.*, Isabella et al., 2011). In some embodiments, the corresponding wild-type transcriptional regulator is left intact and retains wild-type activity. In alternate embodiments, the corresponding wild-type transcriptional regulator is deleted or mutated to reduce or eliminate wild-type activity.

[0629] In some embodiments, the genetically engineered bacteria comprise a wild-type oxygen-level dependent transcriptional regulator, *e.g.*, FNR, ANR, or DNR, and corresponding promoter that is mutated relative to the wild-type promoter from bacteria of the same subtype. The mutated promoter enhances binding to the wild-type transcriptional regulator and increases the transcription of genes operably linked to said promoter, *e.g.,* the gene encoding the metabolic and/or satiety effector molecules, in a low-oxygen or anaerobic environment, as compared to the wild-type promoter under the same conditions. In some embodiments, the genetically engineered bacteria comprise a wild-type oxygen-level dependent promoter, *e.g.,* FNR, ANR, or DNR promoter, and corresponding transcriptional regulator that is mutated relative to the wild-type transcriptional regulator from bacteria of the same subtype. The mutated transcriptional regulator enhances binding to the wild-type promoter and increases the transcription of genes operably linked to said promoter, *e.g.,* the gene encoding the metabolic and/or satiety effector molecules, in a low-oxygen or anaerobic environment, as compared to the wild-type transcriptional regulator under the same conditions. In certain embodiments, the mutant oxygen-level dependent transcriptional regulator is an FNR protein comprising amino acid substitutions that enhance dimerization and FNR activity (*see, e.g.,* Moore *et al.,* (2006). In some embodiments, both the oxygen level-sensing transcriptional regulator and corresponding promoter are mutated relative to the wild-type sequences from bacteria of the same subtype in order to increase expression of the payload in low-oxygen conditions.

[0630] In some embodiments, the bacterial cells comprise multiple copies of the endogenous gene encoding the oxygen level-sensing transcriptional regulator, *e.g.,* the *FNR* gene. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator is present on a plasmid. In some embodiments, the gene encoding the oxygen level-

sensing transcriptional regulator and the gene encoding the payload are present on different plasmids. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the payload are present on the same plasmid.

[0631] In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator is present on a chromosome. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the metabolic and/or satiety effector molecules are present on different chromosomes. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the metabolic and/or satiety effector molecules are present on the same chromosome. In some instances, it may be advantageous to express the oxygen level-sensing transcriptional regulator under the control of an inducible promoter in order to enhance expression stability. In some embodiments, expression of the transcriptional regulator is controlled by a different promoter than the promoter that controls expression of the gene encoding the metabolic and/or satiety effector molecules. In some embodiments, expression of the transcriptional regulator is controlled by the same promoter that controls expression of the metabolic and/or satiety effector molecules. In some embodiments, the transcriptional regulator and the payload are divergently transcribed from a promoter region.

RNS-dependent regulation

[0632] In some embodiments, the genetically engineered bacteria or genetically engineered virus comprise a gene encoding a metabolic and/or satiety effector molecule that is expressed under the control of an inducible promoter. In some embodiments, the genetically engineered bacterium or genetically engineered virus that expresses a metabolic and/or satiety effector molecule under the control of a promoter that is activated by inflammatory conditions. In one embodiment, the gene for producing the metabolic and/or satiety effector molecules is expressed under the control of an inflammatory-dependent promoter that is activated in inflammatory environments, *e.g.*, a reactive nitrogen species or RNS promoter.

[0633] As used herein, "reactive nitrogen species" and "RNS" are used interchangeably to refer to highly active molecules, ions, and/or radicals derived from molecular nitrogen. RNS can cause deleterious cellular effects such as nitrosative stress. RNS includes, but is not limited to, nitric oxide (NO•), peroxynitrite or peroxynitrite anion (ONOO-), nitrogen dioxide (•NO2), dinitrogen trioxide (N2O3), peroxynitrous acid (ONOOH), and nitroperoxycarbonate (ONOOCO2-) (unpaired electrons denoted by •).

Bacteria have evolved transcription factors that are capable of sensing RNS levels. Different RNS signaling pathways are triggered by different RNS levels and occur with different kinetics.

[0634] As used herein, "RNS-inducible regulatory region" refers to a nucleic acid sequence to which one or more RNS-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression; in the presence of RNS, the transcription factor binds to and/or activates the regulatory region. In some embodiments, the RNS-inducible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor senses RNS and subsequently binds to the RNS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the RNS-inducible regulatory region in the absence of RNS; in the presence of RNS, the transcription factor undergoes a conformational change, thereby activating downstream gene expression. The RNS-inducible regulatory region may be operatively linked to a gene or genes, *e.g.*, a payload gene sequence(s), *e.g.*, any of the payloads described herein. For example, in the presence of RNS, a transcription factor senses RNS and activates a corresponding RNS-inducible regulatory region, thereby driving expression of an operatively linked gene sequence. Thus, RNS induces expression of the gene or gene sequences.

[0635] As used herein, "RNS-derepressible regulatory region" refers to a nucleic acid sequence to which one or more RNS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene expression; in the presence of RNS, the transcription factor does not bind to and does not repress the regulatory region. In some embodiments, the RNS-derepressible regulatory region comprises a promoter sequence. The RNS-derepressible regulatory region may be operatively linked to a gene or genes, *e.g.*, a payload gene sequence(s). For example, in the presence of RNS, a transcription factor senses RNS and no longer binds to and/or represses the regulatory region, thereby derepressing an operatively linked gene sequence or gene cassette. Thus, RNS derepresses expression of the gene or genes.

[0636] As used herein, "RNS-repressible regulatory region" refers to a nucleic acid sequence to which one or more RNS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene expression; in the presence of RNS, the transcription factor binds to and represses the regulatory region. In some embodiments, the RNS-repressible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor that senses RNS is

capable of binding to a regulatory region that overlaps with part of the promoter sequence. In alternate embodiments, the transcription factor that senses RNS is capable of binding to a regulatory region that is upstream or downstream of the promoter sequence. The RNS-repressible regulatory region may be operatively linked to a gene sequence or gene cassette. For example, in the presence of RNS, a transcription factor senses RNS and binds to a corresponding RNS-repressible regulatory region, thereby blocking expression of an operatively linked gene sequence or gene sequences. Thus, RNS represses expression of the gene or gene sequences.

[0637] As used herein, a "RNS-responsive regulatory region" refers to a RNS-inducible regulatory region, a RNS-repressible regulatory region, and/or a RNS-derepressible regulatory region. In some embodiments, the RNS-responsive regulatory region comprises a promoter sequence. Each regulatory region is capable of binding at least one corresponding RNS-sensing transcription factor. Examples of transcription factors that sense RNS and their corresponding RNS-responsive genes, promoters, and/or regulatory regions include, but are not limited to, those shown in **Table 22.**

Table 22. Examples of RNS-sensing transcription factors and RNS-responsive genes

RNS-sensing transcription factor:	**Primarily capable of sensing:**	**Examples of responsive genes, promoters, and/or regulatory regions:**
NsrR	NO	*norB, aniA, nsrR, hmpA, ytfE, ygbA, hcp, hcr, nrfA, aox*
NorR	NO	*norVW, norR*
DNR	NO	*norCB, nir, nor, nos*

[0638] In some embodiments, the genetically engineered bacteria of the invention comprise a tunable regulatory region that is directly or indirectly controlled by a transcription factor that is capable of sensing at least one reactive nitrogen species. The tunable regulatory region is operatively linked to a gene or genes capable of directly or indirectly driving the expression of a payload, thus controlling expression of the payload relative to RNS levels. For example, the tunable regulatory region is a RNS-inducible regulatory region, and the payload is a metabolic and/or satiety effector molecule, such as any of the payloads provided herein; when RNS is present, *e.g.*, in an inflamed tissue, a RNS-sensing transcription factor binds to and/or activates the regulatory region and drives expression of the payload gene or genes. Subsequently, when inflammation is ameliorated, RNS levels are reduced, and production of the metabolic and/or satiety effector molecules is decreased or eliminated.

[0639] In some embodiments, the tunable regulatory region is a RNS-inducible regulatory region; in the presence of RNS, a transcription factor senses RNS and activates the RNS-inducible regulatory region, thereby driving expression of an operatively linked gene or genes. In some embodiments, the transcription factor senses RNS and subsequently binds to the RNS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the RNS-inducible regulatory region in the absence of RNS; when the transcription factor senses RNS, it undergoes a conformational change, thereby inducing downstream gene expression.

[0640] In some embodiments, the tunable regulatory region is a RNS-inducible regulatory region, and the transcription factor that senses RNS is NorR. NorR "is an NO-responsive transcriptional activator that regulates expression of the norVW genes encoding flavorubredoxin and an associated flavoprotein, which reduce NO to nitrous oxide" (Spiro 2006). The genetically engineered bacteria of the invention may comprise any suitable RNS-responsive regulatory region from a gene that is activated by NorR. Genes that are capable of being activated by NorR are known in the art (see, *e.g.*, Spiro 2006; Vine *et al.*, 2011; Karlinsey *et al.*, 2012). In certain embodiments, the genetically engineered bacteria of the invention comprise a RNS-inducible regulatory region from norVW that is operatively linked to a gene or genes, *e.g.*, one or more payload gene sequence(s). In the presence of RNS, a NorR transcription factor senses RNS and activates to the norVW regulatory region, thereby driving expression of the operatively linked gene(s) and producing the payload(s).

[0641] In some embodiments, the tunable regulatory region is a RNS-inducible regulatory region, and the transcription factor that senses RNS is DNR. DNR (dissimilatory nitrate respiration regulator) "promotes the expression of the nir, the nor and the nos genes" in the presence of nitric oxide (Castiglione *et al.*, 2009). The genetically engineered bacteria of the invention may comprise any suitable RNS-responsive regulatory region from a gene that is activated by DNR. Genes that are capable of being activated by DNR are known in the art (see, *e.g.*, Castiglione *et al.*, 2009; Giardina *et al.*, 2008). In certain embodiments, the genetically engineered bacteria of the invention comprise a RNS-inducible regulatory region from norCB that is operatively linked to a gene or gene cassette, *e.g.*, a butyrogenic gene cassette. In the presence of RNS, a DNR transcription factor senses RNS and activates to the norCB regulatory region, thereby driving expression of the operatively linked gene or genes and producing one or more metabolic and/or satiety effector molecules. In some embodiments, the DNR is Pseudomonas aeruginosa DNR.

[0642] In another embodiment, the genetically engineered bacteria comprise the gene or gene cassette for producing metabolic and/or satiety effector molecules expressed under the control of the dissimilatory nitrate respiration regulator (DNR). DNR is a member of the FNR family (Arai *et al.*, 1995) and is a transcriptional regulator that is required in conjunction with ANR for "anaerobic nitrate respiration of *Pseudomonas aeruginosa*" (Hasegawa *et al.*, 1998). For certain genes, the FNR-binding motifs "are probably recognized only by DNR" (Hasegawa *et al.*, 1998). Any suitable transcriptional regulator that is controlled by exogenous environmental conditions and corresponding regulatory region may be used. Non-limiting examples include ArcA/B, ResD/E, NreA/B/C, and AirSR, and others are known in the art.

[0643] In some embodiments, the tunable regulatory region is a RNS-derepressible regulatory region, and binding of a corresponding transcription factor represses downstream gene expression; in the presence of RNS, the transcription factor no longer binds to the regulatory region, thereby derepressing the operatively linked gene or gene cassette.

[0644] In some embodiments, the tunable regulatory region is a RNS-derepressible regulatory region, and the transcription factor that senses RNS is NsrR. NsrR is "an Rrf2-type transcriptional repressor [that] can sense NO and control the expression of genes responsible for NO metabolism" (Isabella *et al.*, 2009). The genetically engineered bacteria of the invention may comprise any suitable RNS-responsive regulatory region from a gene that is repressed by NsrR. In some embodiments, the NsrR is Neisseria gonorrhoeae NsrR. Genes that are capable of being repressed by NsrR are known in the art (see, *e.g.*, Isabella *et al.*, 2009; Dunn *et al.*, 2010). In certain embodiments, the genetically engineered bacteria of the invention comprise a RNS-derepressible regulatory region from norB that is operatively linked to a gene or genes, *e.g.*, a payload gene or genes. In the presence of RNS, an NsrR transcription factor senses RNS and no longer binds to the norB regulatory region, thereby derepressing the operatively linked a payload gene or genes and producing the encoding a metabolic and/or satiety effector molecules.

[0645] In some embodiments, it is advantageous for the genetically engineered bacteria to express a RNS-sensing transcription factor that does not regulate the expression of a significant number of native genes in the bacteria. In some embodiments, the genetically engineered bacterium of the invention expresses a RNS-sensing transcription factor from a different species, strain, or substrain of bacteria, wherein the transcription factor does not bind to regulatory sequences in the genetically engineered bacterium of the invention. In some embodiments, the genetically engineered bacterium of the invention is *Escherichia coli*,

and the RNS-sensing transcription factor is NsrR, *e.g.*, from is Neisseria gonorrhoeae, wherein the *Escherichia coli* does not comprise binding sites for said NsrR. In some embodiments, the heterologous transcription factor minimizes or eliminates off-target effects on endogenous regulatory regions and genes in the genetically engineered bacteria.

[0646] In some embodiments, the tunable regulatory region is a RNS-repressible regulatory region, and binding of a corresponding transcription factor represses downstream gene expression; in the presence of RNS, the transcription factor senses RNS and binds to the RNS-repressible regulatory region, thereby repressing expression of the operatively linked gene or gene cassette. In some embodiments, the RNS-sensing transcription factor is capable of binding to a regulatory region that overlaps with part of the promoter sequence. In alternate embodiments, the RNS-sensing transcription factor is capable of binding to a regulatory region that is upstream or downstream of the promoter sequence.

[0647] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express a metabolic and/or satiety effector molecule. The two repressor activation regulatory circuit comprises a first RNS-sensing repressor and a second repressor, which is operatively linked to a gene or gene cassette, *e.g.*, encoding a payload. In one aspect of these embodiments, the RNS-sensing repressor inhibits transcription of the second repressor, which inhibits the transcription of the gene or gene cassette. Examples of second repressors useful in these embodiments, include, but are not limited to, TetR, C1, and LexA. In the absence of binding by the first repressor (which occurs in the absence of RNS), the second repressor is transcribed, which represses expression of the gene or genes. In the presence of binding by the first repressor (which occurs in the presence of RNS), expression of the second repressor is repressed, and the gene or genes, *e.g.*, a metabolic and/or satiety effector molecule gene or genes is expressed.

[0648] A RNS-responsive transcription factor may induce, derepress, or repress gene expression depending upon the regulatory region sequence used in the genetically engineered bacteria. One or more types of RNS-sensing transcription factors and corresponding regulatory region sequences may be present in genetically engineered bacteria. In some embodiments, the genetically engineered bacteria comprise one type of RNS-sensing transcription factor, *e.g.*, NsrR, and one corresponding regulatory region sequence, *e.g.*, from norB. In some embodiments, the genetically engineered bacteria comprise one type of RNS-sensing transcription factor, *e.g.*, NsrR, and two or more different corresponding regulatory region sequences, *e.g.*, from norB and aniA. In some embodiments, the genetically engineered bacteria comprise two or more types of RNS-sensing transcription factors, *e.g.*,

NsrR and NorR, and two or more corresponding regulatory region sequences, *e.g.*, from norB and norR, respectively. One RNS-responsive regulatory region may be capable of binding more than one transcription factor. In some embodiments, the genetically engineered bacteria comprise two or more types of RNS-sensing transcription factors and one corresponding regulatory region sequence. Nucleic acid sequences of several RNS-regulated regulatory regions are known in the art (see, *e.g.*, Spiro 2006; Isabella *et al.*, 2009; Dunn *et al.*, 2010; Vine *et al.*, 2011; Karlinsey *et al.*, 2012).

[0649] In some embodiments, the genetically engineered bacteria of the invention comprise a gene encoding a RNS-sensing transcription factor, *e.g.*, the nsrR gene, that is controlled by its native promoter, an inducible promoter, a promoter that is stronger than the native promoter, *e.g.*, the GlnRS promoter or the P(Bla) promoter, or a constitutive promoter. In some instances, it may be advantageous to express the RNS-sensing transcription factor under the control of an inducible promoter in order to enhance expression stability. In some embodiments, expression of the RNS-sensing transcription factor is controlled by a different promoter than the promoter that controls expression of the therapeutic molecule. In some embodiments, expression of the RNS-sensing transcription factor is controlled by the same promoter that controls expression of the therapeutic molecule. In some embodiments, the RNS-sensing transcription factor and therapeutic molecule are divergently transcribed from a promoter region.

[0650] In some embodiments, the genetically engineered bacteria of the invention comprise a gene for a RNS-sensing transcription factor from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise a RNS-responsive regulatory region from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise a RNS-sensing transcription factor and corresponding RNS-responsive regulatory region from a different species, strain, or substrain of bacteria. The heterologous RNS-sensing transcription factor and regulatory region may increase the transcription of genes operatively linked to said regulatory region in the presence of RNS, as compared to the native transcription factor and regulatory region from bacteria of the same subtype under the same conditions.

[0651] In some embodiments, the genetically engineered bacteria comprise a RNS-sensing transcription factor, NsrR, and corresponding regulatory region, nsrR, from Neisseria gonorrhoeae. In some embodiments, the native RNS-sensing transcription factor, *e.g.*, NsrR, is left intact and retains wild-type activity. In alternate embodiments, the native RNS-sensing

transcription factor, *e.g.*, NsrR, is deleted or mutated to reduce or eliminate wild-type activity.

[0652] In some embodiments, the genetically engineered bacteria of the invention comprise multiple copies of the endogenous gene encoding the RNS-sensing transcription factor, *e.g.*, the nsrR gene. In some embodiments, the gene encoding the RNS-sensing transcription factor is present on a plasmid. In some embodiments, the gene encoding the RNS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on different plasmids. In some embodiments, the gene encoding the RNS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on the same plasmid. In some embodiments, the gene encoding the RNS-sensing transcription factor is present on a chromosome. In some embodiments, the gene encoding the RNS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on different chromosomes. In some embodiments, the gene encoding the RNS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on the same chromosome.

[0653] In some embodiments, the genetically engineered bacteria comprise a wild-type gene encoding a RNS-sensing transcription factor, *e.g.*, the NsrR gene, and a corresponding regulatory region, *e.g.*, a norB regulatory region, that is mutated relative to the wild-type regulatory region from bacteria of the same subtype. The mutated regulatory region increases the expression of the payload in the presence of RNS, as compared to the wild-type regulatory region under the same conditions. In some embodiments, the genetically engineered bacteria comprise a wild-type RNS-responsive regulatory region, *e.g.*, the norB regulatory region, and a corresponding transcription factor, *e.g.*, NsrR, that is mutated relative to the wild-type transcription factor from bacteria of the same subtype. The mutant transcription factor increases the expression of the payload in the presence of RNS, as compared to the wild-type transcription factor under the same conditions. In some embodiments, both the RNS-sensing transcription factor and corresponding regulatory region are mutated relative to the wild-type sequences from bacteria of the same subtype in order to increase expression of the payload in the presence of RNS.

[0654] In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecules is present on a plasmid and operably linked to a promoter that is induced by RNS. In some embodiments, expression is further optimized by methods known in the art, *e.g.*, by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability.

[0655] In some embodiments, any of the gene(s) of the present disclosure may be integrated into the bacterial chromosome at one or more integration sites. For example, one or more copies of one or more encoding a payload gene(s) may be integrated into the bacterial chromosome. Having multiple copies of the gene or gen(s) integrated into the chromosome allows for greater production of the payload(s) and also permits fine-tuning of the level of expression. Alternatively, different circuits described herein, such as any of the secretion or exporter circuits, in addition to the therapeutic gene(s) or gene cassette(s) could be integrated into the bacterial chromosome at one or more different integration sites to perform multiple different functions.

[0656] In some embodiments, the genetically engineered bacteria of the invention produce at least one payload in the presence of RNS to reduce local gut inflammation by at least about 1.5-fold, at least about 2-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, at least about 600-fold, at least about 700-fold, at least about 800-fold, at least about 900-fold, at least about 1,000-fold, or at least about 1,500-fold as compared to unmodified bacteria of the same subtype under the same conditions. Inflammation may be measured by methods known in the art, *e.g.*, counting disease lesions using endoscopy; detecting T regulatory cell differentiation in peripheral blood, *e.g.*, by fluorescence activated sorting; measuring T regulatory cell levels; measuring cytokine levels; measuring areas of mucosal damage; assaying inflammatory biomarkers, *e.g.*, by qPCR; PCR arrays; transcription factor phosphorylation assays; immunoassays; and/or cytokine assay kits (Mesoscale, Cayman Chemical, Qiagen).

[0657] In some embodiments, the genetically engineered bacteria produce at least about 1.5-fold, at least about 2-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, at least about 600-fold, at least about 700-fold, at least about 800-fold, at least about 900-fold, at least about 1,000-fold, or at least about 1,500-fold more of payload in the presence of RNS than unmodified bacteria of the same subtype under the same conditions. Certain unmodified bacteria will not have detectable levels of the payload. In embodiments using genetically modified forms of these bacteria, payload will be detectable in the presence of RNS.

ROS-dependent regulation

[0658] In some embodiments, the genetically engineered bacteria or genetically engineered virus comprise a gene for producing a metabolic and/or satiety effector molecule that is expressed under the control of an inducible promoter. In some embodiments, the genetically engineered bacterium or genetically engineered virus that expresses a metabolic and/or satiety effector molecule under the control of a promoter that is activated by conditions of cellular damage. In one embodiment, the gene for producing the metabolic and/or satiety effector molecule is expressed under the control of an cellular damaged-dependent promoter that is activated in environments in which there is cellular or tissue damage, *e.g.*, a reactive oxygen species or ROS promoter.

[0659] As used herein, "reactive oxygen species" and "ROS" are used interchangeably to refer to highly active molecules, ions, and/or radicals derived from molecular oxygen. ROS can be produced as byproducts of aerobic respiration or metal-catalyzed oxidation and may cause deleterious cellular effects such as oxidative damage. ROS includes, but is not limited to, hydrogen peroxide (H2O2), organic peroxide (ROOH), hydroxyl ion (OH-), hydroxyl radical (•OH), superoxide or superoxide anion (•O2-), singlet oxygen (1O2), ozone (O3), carbonate radical, peroxide or peroxyl radical (•O2-2), hypochlorous acid (HOCl), hypochlorite ion (OCl-), sodium hypochlorite (NaOCl), nitric oxide (NO•), and peroxynitrite or peroxynitrite anion (ONOO-) (unpaired electrons denoted by •). Bacteria have evolved transcription factors that are capable of sensing ROS levels. Different ROS signaling pathways are triggered by different ROS levels and occur with different kinetics (Marinho *et al.*, 2014).

[0660] As used herein, "ROS-inducible regulatory region" refers to a nucleic acid sequence to which one or more ROS-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression; in the presence of ROS, the transcription factor binds to and/or activates the regulatory region. In some embodiments, the ROS-inducible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor senses ROS and subsequently binds to the ROS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the ROS-inducible regulatory region in the absence of ROS; in the presence of ROS, the transcription factor undergoes a conformational change, thereby activating downstream gene expression. The ROS-inducible regulatory region may be operatively linked to a gene sequence or gene sequence, *e.g.*, a sequence or sequences encoding one or more payload(s).

For example, in the presence of ROS, a transcription factor, *e.g.*, OxyR, senses ROS and activates a corresponding ROS-inducible regulatory region, thereby driving expression of an operatively linked gene sequence or gene sequences. Thus, ROS induces expression of the gene or genes.

[0661] As used herein, "ROS-derepressible regulatory region" refers to a nucleic acid sequence to which one or more ROS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene expression; in the presence of ROS, the transcription factor does not bind to and does not repress the regulatory region. In some embodiments, the ROS-derepressible regulatory region comprises a promoter sequence. The ROS-derepressible regulatory region may be operatively linked to a gene or genes, *e.g.*, one or more genes encoding one or more payload(s). For example, in the presence of ROS, a transcription factor, *e.g.*, OhrR, senses ROS and no longer binds to and/or represses the regulatory region, thereby derepressing an operatively linked gene sequence or gene cassette. Thus, ROS derepresses expression of the gene or gene cassette.

[0662] As used herein, "ROS-repressible regulatory region" refers to a nucleic acid sequence to which one or more ROS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene expression; in the presence of ROS, the transcription factor binds to and represses the regulatory region. In some embodiments, the ROS-repressible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor that senses ROS is capable of binding to a regulatory region that overlaps with part of the promoter sequence. In alternate embodiments, the transcription factor that senses ROS is capable of binding to a regulatory region that is upstream or downstream of the promoter sequence. The ROS-repressible regulatory region may be operatively linked to a gene sequence or gene sequences. For example, in the presence of ROS, a transcription factor, *e.g.*, PerR, senses ROS and binds to a corresponding ROS-repressible regulatory region, thereby blocking expression of an operatively linked gene sequence or gene sequences. Thus, ROS represses expression of the gene or genes.

[0663] As used herein, a "ROS-responsive regulatory region" refers to a ROS-inducible regulatory region, a ROS-repressible regulatory region, and/or a ROS-derepressible regulatory region. In some embodiments, the ROS-responsive regulatory region comprises a promoter sequence. Each regulatory region is capable of binding at least one corresponding ROS-sensing transcription factor. Examples of transcription factors that sense ROS and their

corresponding ROS-responsive genes, promoters, and/or regulatory regions include, but are not limited to, those shown in **Table 23**.

Table 23. Examples of ROS-sensing transcription factors and ROS-responsive genes

ROS-sensing transcription factor:	Primarily capable of sensing:	Examples of responsive genes, promoters, and/or regulatory regions:
OxyR	H_2O_2	*ahpC; ahpF; dps; dsbG; fhuF; flu; fur; gor; grxA; hemH; katG; oxyS; sufA; sufB; sufC; sufD; sufE; sufS; trxC; uxuA; yaaA; yaeH; yaiA; ybjM; ydcH; ydeN; ygaQ; yljA; ytfK*
PerR	H_2O_2	*katA; ahpCF; mrgA; zoaA; fur; hemAXCDBL; srfA*
OhrR	Organic peroxides NaOCl	*ohrA*
SoxR	$\bullet O_2^-$ NO• (also capable of sensing H_2O_2)	*soxS*
RosR	H_2O_2	*rbtT; tnp16a; rluC1; tnp5a; mscL; tnp2d; phoD; tnp15b; pstA; tnp5b; xylC; gabD1; rluC2; cgtS9; azlC; narKGHJI; rosR*

[0664] In some embodiments, the genetically engineered bacteria comprise a tunable regulatory region that is directly or indirectly controlled by a transcription factor that is capable of sensing at least one reactive oxygen species. The tunable regulatory region is operatively linked to a gene or gene cassette capable of directly or indirectly driving the expression of a payload, thus controlling expression of the payload relative to ROS levels. For example, the tunable regulatory region is a ROS-inducible regulatory region, and the molecule is a metabolic and/or satiety effector molecule; when ROS is present, *e.g.*, in an inflamed tissue, a ROS-sensing transcription factor binds to and/or activates the regulatory region and drives expression of the gene sequence for the payload, thereby producing the payload. Subsequently, when inflammation is ameliorated, ROS levels are reduced, and production of the metabolic and/or satiety effector molecule is decreased or eliminated.

[0665] In some embodiments, the tunable regulatory region is a ROS-inducible regulatory region; in the presence of ROS, a transcription factor senses ROS and activates the ROS-inducible regulatory region, thereby driving expression of an operatively linked gene or

gene cassette. In some embodiments, the transcription factor senses ROS and subsequently binds to the ROS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the ROS-inducible regulatory region in the absence of ROS; when the transcription factor senses ROS, it undergoes a conformational change, thereby inducing downstream gene expression.

[0666] In some embodiments, the tunable regulatory region is a ROS-inducible regulatory region, and the transcription factor that senses ROS is OxyR. OxyR "functions primarily as a global regulator of the peroxide stress response" and is capable of regulating dozens of genes, *e.g.*, "genes involved in H2O2 detoxification (katE, ahpCF), heme biosynthesis (hemH), reductant supply (grxA, gor, trxC), thiol-disulfide isomerization (dsbG), Fe-S center repair (sufA-E, sufS), iron binding (yaaA), repression of iron import systems (fur)" and "OxyS, a small regulatory RNA" (Dubbs *et al.*, 2012). The genetically engineered bacteria may comprise any suitable ROS-responsive regulatory region from a gene that is activated by OxyR. Genes that are capable of being activated by OxyR are known in the art (see, *e.g.*, Zheng *et al.*, 2001; Dubbs *et al.*, 2012). In certain embodiments, the genetically engineered bacteria of the invention comprise a ROS-inducible regulatory region from oxyS that is operatively linked to a gene, *e.g.*, a payload gene. In the presence of ROS, *e.g.*, H2O2, an OxyR transcription factor senses ROS and activates to the oxyS regulatory region, thereby driving expression of the operatively linked payload gene and producing the payload. In some embodiments, OxyR is encoded by an *E. coli* oxyR gene. In some embodiments, the oxyS regulatory region is an *E. coli* oxyS regulatory region. In some embodiments, the ROS-inducible regulatory region is selected from the regulatory region of katG, dps, and ahpC.

[0667] In alternate embodiments, the tunable regulatory region is a ROS-inducible regulatory region, and the corresponding transcription factor that senses ROS is SoxR. When SoxR is "activated by oxidation of its [2Fe–2S] cluster, it increases the synthesis of SoxS, which then activates its target gene expression" (Koo *et al.*, 2003). "SoxR is known to respond primarily to superoxide and nitric oxide" (Koo *et al.*, 2003), and is also capable of responding to H2O2. The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is activated by SoxR. Genes that are capable of being activated by SoxR are known in the art (see, *e.g.*, Koo *et al.*, 2003). In certain embodiments, the genetically engineered bacteria of the invention comprise a ROS-inducible regulatory region from soxS that is operatively linked to a gene, *e.g.*, ametabolic

and/or satiety effector molecule. In the presence of ROS, the SoxR transcription factor senses ROS and activates the soxS regulatory region, thereby driving expression of the operatively linked a payload gene and producing the metabolic and/or satiety effector molecule.

[0668] In some embodiments, the tunable regulatory region is a ROS-derepressible regulatory region, and binding of a corresponding transcription factor represses downstream gene expression; in the presence of ROS, the transcription factor no longer binds to the regulatory region, thereby derepressing the operatively linked gene or gene cassette.

[0669] In some embodiments, the tunable regulatory region is a ROS-derepressible regulatory region, and the transcription factor that senses ROS is OhrR. OhrR "binds to a pair of inverted repeat DNA sequences overlapping the ohrA promoter site and thereby represses the transcription event," but oxidized OhrR is "unable to bind its DNA target" (Duarte *et al.*, 2010). OhrR is a "transcriptional repressor [that]… senses both organic peroxides and NaOCl" (Dubbs *et al.*, 2012) and is "weakly activated by H2O2 but it shows much higher reactivity for organic hydroperoxides" (Duarte *et al.*, 2010). The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is repressed by OhrR. Genes that are capable of being repressed by OhrR are known in the art (see, *e.g.*, Dubbs *et al.*, 2012). In certain embodiments, the genetically engineered bacteria of the invention comprise a ROS-derepressible regulatory region from ohrA that is operatively linked to a gene or gene cassette, *e.g.*, a metabolic and/or satiety effector molecule gene. In the presence of ROS, *e.g.*, NaOCl, an OhrR transcription factor senses ROS and no longer binds to the ohrA regulatory region, thereby derepressing the operatively linked payload gene and producing themetabolic and/or satiety effector molecule.

[0670] OhrR is a member of the MarR family of ROS-responsive regulators. "Most members of the MarR family are transcriptional repressors and often bind to the -10 or -35 region in the promoter causing a steric inhibition of RNA polymerase binding" (Bussmann *et al.*, 2010). Other members of this family are known in the art and include, but are not limited to, OspR, MgrA, RosR, and SarZ. In some embodiments, the transcription factor that senses ROS is OspR, MgRA, RosR, and/or SarZ, and the genetically engineered bacteria of the invention comprises one or more corresponding regulatory region sequences from a gene that is repressed by OspR, MgRA, RosR, and/or SarZ. Genes that are capable of being repressed by OspR, MgRA, RosR, and/or SarZ are known in the art (see, *e.g.*, Dubbs *et al.*, 2012).

[0671] In some embodiments, the tunable regulatory region is a ROS-derepressible regulatory region, and the corresponding transcription factor that senses ROS is RosR. RosR is "a MarR-type transcriptional regulator" that binds to an "18-bp inverted repeat with the consensus sequence TTGTTGAYRYRTCAACWA" and is "reversibly inhibited by the oxidant H2O2" (Bussmann *et al.*, 2010). RosR is capable of repressing numerous genes and putative genes, including but not limited to "a putative polyisoprenoid-binding protein (cg1322, gene upstream of and divergent from rosR), a sensory histidine kinase (cgtS9), a putative transcriptional regulator of the Crp/FNR family (cg3291), a protein of the glutathione S-transferase family (cg1426), two putative FMN reductases (cg1150 and cg1850), and four putative monooxygenases (cg0823, cg1848, cg2329, and cg3084)" (Bussmann *et al.*, 2010). The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is repressed by RosR. Genes that are capable of being repressed by RosR are known in the art (see, *e.g.*, Bussmann *et al.*, 2010). In certain embodiments, the genetically engineered bacteria of the invention comprise a ROS-derepressible regulatory region from cgtS9 that is operatively linked to a gene or gene cassette, *e.g.*, a metabolic and/or satiety effector molecule. In the presence of ROS, *e.g.*, H2O2, a RosR transcription factor senses ROS and no longer binds to the cgtS9 regulatory region, thereby derepressing the operatively linked payload gene and producing the metabolic and/or satiety effector molecule.

[0672] In some embodiments, it is advantageous for the genetically engineered bacteria to express a ROS-sensing transcription factor that does not regulate the expression of a significant number of native genes in the bacteria. In some embodiments, the genetically engineered bacterium of the invention expresses a ROS-sensing transcription factor from a different species, strain, or substrain of bacteria, wherein the transcription factor does not bind to regulatory sequences in the genetically engineered bacterium of the invention. In some embodiments, the genetically engineered bacterium of the invention is *Escherichia coli*, and the ROS-sensing transcription factor is RosR, *e.g.*, from Corynebacterium glutamicum, wherein the *Escherichia coli* does not comprise binding sites for said RosR. In some embodiments, the heterologous transcription factor minimizes or eliminates off-target effects on endogenous regulatory regions and genes in the genetically engineered bacteria.

[0673] In some embodiments, the tunable regulatory region is a ROS-repressible regulatory region, and binding of a corresponding transcription factor represses downstream gene expression; in the presence of ROS, the transcription factor senses ROS and binds to the

ROS-repressible regulatory region, thereby repressing expression of the operatively linked gene or gene cassette. In some embodiments, the ROS-sensing transcription factor is capable of binding to a regulatory region that overlaps with part of the promoter sequence. In alternate embodiments, the ROS-sensing transcription factor is capable of binding to a regulatory region that is upstream or downstream of the promoter sequence.

[0674] In some embodiments, the tunable regulatory region is a ROS-repressible regulatory region, and the transcription factor that senses ROS is PerR. In Bacillus subtilis, PerR "when bound to DNA, represses the genes coding for proteins involved in the oxidative stress response (katA, ahpC, and mrgA), metal homeostasis (hemAXCDBL, fur, and zoaA) and its own synthesis (perR)" (Marinho *et al.*, 2014). PerR is a "global regulator that responds primarily to H2O2" (Dubbs *et al.*, 2012) and "interacts with DNA at the per box, a specific palindromic consensus sequence (TTATAATNATTATAA) residing within and near the promoter sequences of PerR-controlled genes" (Marinho *et al.*, 2014). PerR is capable of binding a regulatory region that "overlaps part of the promoter or is immediately downstream from it" (Dubbs *et al.*, 2012). The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is repressed by PerR. Genes that are capable of being repressed by PerR are known in the art (see, *e.g.*, Dubbs *et al.*, 2012).

[0675] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express a metabolic and/or satiety effector molecule. The two repressor activation regulatory circuit comprises a first ROS-sensing repressor, *e.g.*, PerR, and a second repressor, *e.g.*, TetR, which is operatively linked to a gene or gene cassette, *e.g.*, a payload. In one aspect of these embodiments, the ROS-sensing repressor inhibits transcription of the second repressor, which inhibits the transcription of the gene or gene cassette. Examples of second repressors useful in these embodiments include, but are not limited to, TetR, C1, and LexA. In some embodiments, the ROS-sensing repressor is PerR. In some embodiments, the second repressor is TetR. In this embodiment, a PerR-repressible regulatory region drives expression of TetR, and a TetR-repressible regulatory region drives expression of the gene or gene cassette, *e.g.*, a payload. In the absence of PerR binding (which occurs in the absence of ROS), tetR is transcribed, and TetR represses expression of the gene or gene cassette, *e.g.*, a metabolic and/or satiety effector molecule. In the presence of PerR binding (which occurs in the presence of ROS),

tetR expression is repressed, and the gene or gene cassette, *e.g.*, a metabolic and/or satiety effector molecule, is expressed.

[0676] A ROS-responsive transcription factor may induce, derepress, or repress gene expression depending upon the regulatory region sequence used in the genetically engineered bacteria. For example, although "OxyR is primarily thought of as a transcriptional activator under oxidizing conditions . . . OxyR can function as either a repressor or activator under both oxidizing and reducing conditions" (Dubbs *et al.*, 2012), and OxyR "has been shown to be a repressor of its own expression as well as that of fhuF (encoding a ferric ion reductase) and flu (encoding the antigen 43 outer membrane protein)" (Zheng *et al.*, 2001). The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is repressed by OxyR. In some embodiments, OxyR is used in a two repressor activation regulatory circuit, as described above. Genes that are capable of being repressed by OxyR are known in the art (see, *e.g.*, Zheng *et al.*, 2001). Or, for example, although RosR is capable of repressing a number of genes, it is also capable of activating certain genes, *e.g.*, the narKGHJI operon. In some embodiments, the genetically engineered bacteria comprise any suitable ROS-responsive regulatory region from a gene that is activated by RosR. In addition, "PerR-mediated positive regulation has also been observed…and appears to involve PerR binding to distant upstream sites" (Dubbs *et al.*, 2012). In some embodiments, the genetically engineered bacteria comprise any suitable ROS-responsive regulatory region from a gene that is activated by PerR.

[0677] One or more types of ROS-sensing transcription factors and corresponding regulatory region sequences may be present in genetically engineered bacteria. For example, "OhrR is found in both Gram-positive and Gram-negative bacteria and can coreside with either OxyR or PerR or both" (Dubbs *et al.*, 2012). In some embodiments, the genetically engineered bacteria comprise one type of ROS-sensing transcription factor, *e.g.*, OxyR, and one corresponding regulatory region sequence, *e.g.*, from oxyS. In some embodiments, the genetically engineered bacteria comprise one type of ROS-sensing transcription factor, *e.g.*, OxyR, and two or more different corresponding regulatory region sequences, *e.g.*, from oxyS and katG. In some embodiments, the genetically engineered bacteria comprise two or more types of ROS-sensing transcription factors, *e.g.*, OxyR and PerR, and two or more corresponding regulatory region sequences, *e.g.*, from oxyS and katA, respectively. One ROS-responsive regulatory region may be capable of binding more than one transcription factor. In some embodiments, the genetically engineered bacteria comprise two or more

types of ROS-sensing transcription factors and one corresponding regulatory region sequence.

[0678] Nucleic acid sequences of several exemplary OxyR-regulated regulatory regions are shown in **Table 24**. OxyR binding sites are underlined and bolded. In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of **SEQ ID NO: 580, SEQ ID NO: 581, SEQ ID NO: 582**, or **SEQ ID NO: 583**, or a functional fragment thereof.

Table 24. Nucleotide sequences of exemplary OxyR-regulated regulatory regions

Regulatory sequence	Sequence
katG **(SEQ ID NO: 580)**	TGTGGCTTTTATGAAAATCACACAGTGATCACAAATTTTAAACA GAGCACAAAATGCTGCCTCGAAATGAGGGCGGGAAAATAAGGT TATCAGCCTTGTTTTCTCCCTCATTACTTGAAGGATATGAAGCTA AAACCCTTTTTTATAAAGCATTTGTCCGAATTCGGACATAATCA AAAAAGCTTAATTAAGATCAATTTGATCTACATCTCTTTAACCA ACAATATGTAAGATCTCAACTATCGCATCCGTGGATTAATTCAA TTATAACTTCTCTCTAACGCTGTGTATCGTAACGGTAACACTGTA GAGGGGAGCACATTGATGCGAATTCATTAAAGAGGAGAAAGGT ACC
dps **(SEQ ID NO: 581)**	TTCCGAAAATTCCTGGCGAGCAGATAAATAAGAATTGTTCTTAT CAATATATCTAACTCATTGAATCTTTATTAGTTTTGTTTTTCACG CTTGTTACCACTATTAGTGTGATAGGAACAGCCAGAATAGCGGA ACACATAGCCGGTGCTATACTTAATCTCGTTAATTACTGGGACA TAACATCAAGAGGATATGAAATTCGAATTCATTAAAGAGGAGA AAGGTACC
ahpC **(SEQ ID NO: 582)**	GCTTAGATCAGGTGATTGCCCTTTGTTTATGAGGGTGTTGTAATC CATGTCGTTGTTGCATTTGTAAGGGCAACACCTCAGCCTGCAGG CAGGCACTGAAGATACCAAAGGGTAGTTCAGATTACACGGTCA CCTGGAAAGGGGGCCATTTTACTTTTTATCGCCGCTGGCGGTGC AAAGTTCACAAAGTTGTCTTACGAAGGTTGTAAGGTAAAACTTA TCGATTTGATAATGGAAACGCATTAGCCGAATCGGCAAAAATTG GTTACCTTACATCTCATCGAAAACACGGAGGAAGTATAGATGCG AATTCATTAAAGAGGAGAAAGGTACC
oxyS **(SEQ ID NO: 583)**	CTCGAGTTCATTATCCATCCTCCATCGCCACGATAGTTCATGGCG ATAGGTAGAATAGCAATGAACGATTATCCCTATCAAGCATTCTG ACTGATAATTGCTCACACGAATTCATTAAAGAGGAGAAAGGTA CC

[0679] In some embodiments, the regulatory region sequence is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to

the sequence of **SEQ ID NO: 580, SEQ ID NO: 581, SEQ ID NO: 582**, and/or **SEQ ID NO: 583**.

[0680] In some embodiments, the genetically engineered bacteria of the invention comprise a gene encoding a ROS-sensing transcription factor, *e.g.*, the oxyR gene, that is controlled by its native promoter, an inducible promoter, a promoter that is stronger than the native promoter, *e.g.*, the GlnRS promoter or the P(Bla) promoter, or a constitutive promoter. In some instances, it may be advantageous to express the ROS-sensing transcription factor under the control of an inducible promoter in order to enhance expression stability. In some embodiments, expression of the ROS-sensing transcription factor is controlled by a different promoter than the promoter that controls expression of the therapeutic molecule. In some embodiments, expression of the ROS-sensing transcription factor is controlled by the same promoter that controls expression of the therapeutic molecule. In some embodiments, the ROS-sensing transcription factor and therapeutic molecule are divergently transcribed from a promoter region.

[0681] In some embodiments, the genetically engineered bacteria of the invention comprise a gene for a ROS-sensing transcription factor from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise a ROS-responsive regulatory region from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise a ROS-sensing transcription factor and corresponding ROS-responsive regulatory region from a different species, strain, or substrain of bacteria. The heterologous ROS-sensing transcription factor and regulatory region may increase the transcription of genes operatively linked to said regulatory region in the presence of ROS, as compared to the native transcription factor and regulatory region from bacteria of the same subtype under the same conditions.

[0682] In some embodiments, the genetically engineered bacteria comprise a ROS-sensing transcription factor, OxyR, and corresponding regulatory region, oxyS, from *Escherichia coli*. In some embodiments, the native ROS-sensing transcription factor, *e.g.*, OxyR, is left intact and retains wild-type activity. In alternate embodiments, the native ROS-sensing transcription factor, *e.g.*, OxyR, is deleted or mutated to reduce or eliminate wild-type activity.

[0683] In some embodiments, the genetically engineered bacteria of the invention comprise multiple copies of the endogenous gene encoding the ROS-sensing transcription factor, *e.g.*, the oxyR gene. In some embodiments, the gene encoding the ROS-sensing transcription factor is present on a plasmid. In some embodiments, the gene encoding the

ROS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on different plasmids. In some embodiments, the gene encoding the ROS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on the same. In some embodiments, the gene encoding the ROS-sensing transcription factor is present on a chromosome. In some embodiments, the gene encoding the ROS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on different chromosomes. In some embodiments, the gene encoding the ROS-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on the same chromosome.

[0684] In some embodiments, the genetically engineered bacteria comprise a wild-type gene encoding a ROS-sensing transcription factor, *e.g.*, the soxR gene, and a corresponding regulatory region, *e.g.*, a soxS regulatory region, that is mutated relative to the wild-type regulatory region from bacteria of the same subtype. The mutated regulatory region increases the expression of the metabolic and/or satiety effector molecules in the presence of ROS, as compared to the wild-type regulatory region under the same conditions. In some embodiments, the genetically engineered bacteria comprise a wild-type ROS-responsive regulatory region, *e.g.*, the oxyS regulatory region, and a corresponding transcription factor, *e.g.*, OxyR, that is mutated relative to the wild-type transcription factor from bacteria of the same subtype. The mutant transcription factor increases the expression of the metabolic and/or satiety effector molecules in the presence of ROS, as compared to the wild-type transcription factor under the same conditions. In some embodiments, both the ROS-sensing transcription factor and corresponding regulatory region are mutated relative to the wild-type sequences from bacteria of the same subtype in order to increase expression of the metabolic and/or satiety effector molecules in the presence of ROS.

[0685] In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a plasmid and operably linked to a promoter that is induced by ROS. In some embodiments, the gene or gene cassette for producing the payload is present in the chromosome and operably linked to a promoter that is induced by ROS. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a chromosome and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments,

expression is further optimized by methods known in the art, *e.g.*, by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability.

[0686] In some embodiments, the genetically engineered bacteria may comprise multiple copies of the gene(s) capable of producing metabolic and/or satiety effector molecules. In some embodiments, the gene(s) capable of producing metabolic and/or satiety effector molecules is present on a plasmid and operatively linked to a ROS-responsive regulatory region. In some embodiments, the gene(s) capable of producing a metabolic and/or satiety effector molecule is present in a chromosome and operatively linked to a ROS-responsive regulatory region.

[0687] Thus, in some embodiments, the genetically engineered bacteria or genetically engineered virus produce one or more metabolic and/or satiety effector molecules under the control of an oxygen level-dependent promoter, a reactive oxygen species (ROS)-dependent promoter, or a reactive nitrogen species (RNS)-dependent promoter, and a corresponding transcription factor.

[0688] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying a gene for producing a metabolic and/or satiety effector molecule, such that the metabolic and/or satiety effector molecule can be expressed in the host cell, and the host cell is capable of survival and/or growth *in vitro*, *e.g.*, in medium, and/or *in vivo*. In some embodiments, a bacterium may comprise multiple copies of the gene encoding the metabolic and/or satiety effector molecules. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is expressed on a low-copy plasmid. In some embodiments, the low-copy plasmid may be useful for increasing stability of expression. In some embodiments, the low-copy plasmid may be useful for decreasing leaky expression under non-inducing conditions. In some embodiments, the gene encoding the payload is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of the metabolic and/or satiety effector molecules. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is expressed on a chromosome.

Propionate and other promoters

[0689] In some embodiments, the genetically engineered bacteria comprise the gene or gene cassette for producing the metabolic and/or satiety effector molecules expressed under the control of an inducible promoter that is responsive to specific molecules or metabolites in the environment, the mammalian gut. For example, the short-chain fatty acid propionate is a major microbial fermentation metabolite localized to the gut (Hosseini et al.,

2011). In one embodiment, the gene or gene cassette for producing the payload is under the control of a propionate-inducible promoter. In a more specific embodiment, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is under the control of a propionate-inducible promoter that is activated by the presence of propionate in the mammalian gut. Any molecule or metabolite found in the mammalian gut, in a healthy and/or disease state, may be used to induce payload expression. Non-limiting examples of inducers include propionate, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, and manganese. In alternate embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule and/or nodulator of inflammation is under the control of a pBAD promoter, which is activated in the presence of the sugar arabinose.

[0690] In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a plasmid and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule of inflammation is present in the chromosome and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a plasmid and operably linked to a promoter that is induced by molecules or metabolites that are specific to the to the tumor and/or the mammalian gut. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a chromosome and operably linked to a promoter that is induced by molecules or metabolites that are specific to the tumor and/or the mammalian gut. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a chromosome and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the gene or gene cassette for producing the metabolic and/or satiety effector molecule is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, expression is further optimized by methods known in the art, *e.g.*, by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability.

[0691] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying the gene or gene cassette for producing the

metabolic and/or satiety effector molecules, such that the gene or gene cassette can be expressed in the host cell, and the host cell is capable of survival and/or growth *in vitro*, *e.g.*, in medium, and/or *in vivo*, *e.g.*, in the gut. In some embodiments, a bacterium may comprise multiple copies of the gene or gene cassette for producing the metabolic and/or satiety effector molecules. In some embodiments, gene or gene cassette for producing the payload is expressed on a low-copy plasmid. In some embodiments, the low-copy plasmid may be useful for increasing stability of expression. In some embodiments, the low-copy plasmid may be useful for decreasing leaky expression under non-inducing conditions. In some embodiments, gene or gene cassette for producing the metabolic and/or satiety effector molecules is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing gene or gene cassette expression. In some embodiments, gene or gene cassette for producing the metabolic and/or satiety effector molecules is expressed on a chromosome.

[0692] **Table 25** lists a propionate promoter sequence. In some embodiments, the propionate promoter is induced in the mammalian gut. In some embodiments, the propionate promoter sequence is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the sequence of SEQ ID NO: 584.

Table 25. Propionate promoter sequence

Description	Sequence
Prp (Propionate) promoter Bold: prpR Lower case: ribosome binding site ATG underlined: start of gene of interest **SEQ ID NO: 584**	**TTACCCGTCTGGATTTTCAGTACGCGCTTTTAAAC** **GACGCCACAGCGTGGTACGGCTGATCCCCAAATA** **ACGTGCGGCGGCGCGCTTATCGCCATTAAAGCGT** **GCGAGCACCTCCTGCAATGGAAGCGCTTCTGCTG** **ACGAGGGCGTGATTTCTGCTGTGGTCCCCACCAGT** **TCAGGTAATAATTGCCGCATAAATTGTCTGTCCAG** **TGTTGGTGCGGGATCGACGCTTAAAAAAAGCGCC** **AGGCGTTCCATCATATTCCGCAGTTCGCGAATATT** **ACCGGGCCAATGATAGTTCAGTAGAAGCGGCTGA** **CACTGCGTCAGCCCATGACGCACCGATTCGGTAAA** **AGGGATCTCCATCGCGGCCAGCGATTGTTTTAAAA** **AGTTTTCCGCCAGAGGCAGAATATCAGGCTGTCGC** **TCGCGCAAGGGGGGAAGCGGCAGACGCAGAATGC** **TCAAACGGTAAAACAGATCGGTACGAAAACGTCCT** **TGCGTTATCTCCCGATCCAGATCGCAATGCGTGGC** **GCTGATCACCCGGACATCTACCGGGATCGGCTGA** **TGCCCGCCAACGCGGGTGACGGCTTTTTCCTCCAG** **TACGCGTAGAAGGCGGGTTTGTAACGGCAGCGGC** **ATTTCGCCAATTTCGTCAAGAAACAGCGTGCCGCC** **GTGGGCGACCTCAAACAGCCCCGCACGTCCACCT** **CGTCTTGAGCCGGTAAACGCTCCCTCCTCATAGCC** **AAACAGTTCAGCCTCCAGCAACGACTCGGTAATCG**

	CGCCGCAATTAACGGCGACAAAGGGCGGAGAAGG **CTTGTTCTGACGGTGGGGCTGACGGTTAAACAAC** **GCCTGATGAATCGCTTGCGCCGCCAGCTCTTTCCC** **GGTCCCTGTTTCCCCCTGAATCAGCACTGCCGCGC** **GGGAACGGGCATAGAGTGTAATCGTATGGCGAAC** **CTGCTCCATTTGTGGTGAATCGCCGAGGATATCGC** **TCAGCGCATAACGGGTCTGTAATCCCTTGCTGGAG** **GTATGCTGGCTATACTGACGCCGTGTCAGGCGGG** **TCATATCCAGCGCATCATGGAAAGCCTGACGTACG** **GTGGCCGCTGAATAAATAAAGATGGCGGTCATTCC** **TGCCTCTTCCGCCAGGTCGGTAATTAGTCCTGCCC** **CAATTACAGCCTCAATGCCGTTAGCTTTGAGCTCG** **TTAATTTGCCCGCGAGCATCCTCTTCAGTGATATA** **GCTTCGCTGTTCAAGACGGAGGTGAAACGTTTTCT** **GAAAGGCGACCAGAGCCGGAATGGTCTCCTGATA** **GGTCACGATTCCCATTGAGGAAGTCAGCTTTCCCG** **CTTTTGCCAGAGCCTGTAATACATCGAATCCGCTG** **GGTTTGATGAGGATGACAGGTACCGACAGTCGGC** **TTTTTAAATAAGCGCCGTTGGAACCTGCCGCGATA** **ATCGCGTCGCAGCGTTCGGTTGCCAGTTTTTTGCG** **AATGTAGGCTACTGCCTTTTCAAAACCGAGCTGAA** **TAGGCGTGATCGTCGCCAGATGATCAAACTCCAG** **GCTGATATCCCGAAATAGTTCGAACAGGCGCGTTA** **CCGAGACCGTCCAGATCACCGGTTTATCGCTATTA** **TCGCGCGAAGCGCTATGCACAGTAACCAT**CGTCGT AGATTCATGTTTAAGGAACGAATTCTTGTTTTATAGA TGTTTCGTTAATGTTGCAATGAAACACAGGCCTCCGT TTCATGAAACGTTAGCTGACTCGTTTTTCTTGTGACTC GTCTGTCAGTATTAAAAAAGATTTTTCATTTAACTGA TTGTTTTTAAATTGAATTTTATTTAATGGTTTCTCGGT TTTTGGGTCTGGCATATCCCTTGCTTTAATGAGTGCAT CTTAATTAACAATTCAATAACAAGAGGGCTGAATagtaa tttcaacaaaataacgagcattcgaatg

Other Inducible Promoters

[0703] In some embodiments, the gene encoding the payload is present on a plasmid and operably linked to a promoter that is induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present in the chromosome and operably linked to a promoter that is induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s).

[0693] In some embodiments, the bacterial cell comprises a stably maintained plasmid or chromosome carrying the one or more gene sequences(s), inducible by one or more nutritional and/or chemical inducer(s) and/or metabolite(s), encoding the payload, such

that the metabolic and/or satiety effector molecules can be expressed in the host cell, and the host cell is capable of survival and/or growth *in vitro*, *e.g.*, in medium, and/or *in vivo*, *e.g.*, in the tumor or in the gut. In some embodiments, bacterial cell comprises two or more distinct copies of the one or more gene sequences(s) encoding the metabolic and/or satiety effector molecules, which is controlled by a promoter inducible one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, the genetically engineered bacteria comprise multiple copies of the same one or more gene sequences(s) encoding the metabolic and/or satiety effector molecules, which is controlled by a promoter inducible one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, the one or more gene sequences(s) encoding the metabolic and/or satiety effector molecules, is present on a plasmid and operably linked to a directly or indirectly inducible promoter inducible by one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, the one or more gene sequences(s) encoding the metabolic and/or satiety effector molecules, is present on a chromosome and operably linked to a directly or indirectly inducible by one or more nutritional and/or chemical inducer(s) and/or metabolite(s).

[0694] In some embodiments, one or more gene sequence(s) encoding polypeptides of interest described herein is present on a plasmid and operably linked to promoter a directly or indirectly inducible by one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, the bacterial cell comprises a stably maintained plasmid or chromosome carrying the gene encoding the metabolic and/or satiety effector molecules, which is induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s), such that the payload can be expressed in the host cell, and the host cell is capable of survival and/or growth *in vitro*, *e.g.*, under culture conditions, and/or *in vivo*, *e.g.*, in the gut, or healthy or under conditions associated with inflammation or liver damage. In some embodiments, bacterial cell comprises two or more gene sequence(s) for the production of a polypeptide of interest, one or more of which are induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, the genetically engineered bacteria comprise multiple copies of the same gene sequence(s) for the production of a polypeptide of interest which are induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, the genetically engineered bacteria comprise multiple copies of different gene sequence(s) for the production of a polypeptide of interest, one or more of which are induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s).

[0695] In some embodiments, the gene sequence(s) for the production of a polypeptide of interest is present on a plasmid and operably linked to a promoter that is induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s). In some embodiments, gene sequence(s) for the production of a polypeptide of interest is present in the chromosome and operably linked to a promoter that is induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s).

[0696] In some embodiments, the promoter that is operably linked to the gene encoding the polypeptide of interest is directly or indirectly induced by one or more nutritional and/or chemical inducer(s) and/or metabolite(s).

[0697] In some embodiments, one or more inducible promoter(s) are useful for or induced during *in vivo* expression of the one or more protein(s) of interest. In some embodiments, the promoters are induced during *in vivo* expression of one or more payload(s) and/or other polypeptide(s) of interest. In some embodiments, expression of one or more payload(s) and/or other polypeptide(s) of interest is driven directly or indirectly by one or more arabinose inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a chemical and/or nutritional inducer and/or metabolite which is co-administered with the genetically engineered bacteria of the invention.

[0698] In some embodiments, expression of one or more payload and/or other polypeptide(s) of interest, is driven directly or indirectly by one or more promoter(s) induced by a chemical and/or nutritional inducer and/or metabolite during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the promoter(s) induced by a chemical and/or nutritional inducer and/or metabolite are induced in culture, *e.g.*, grown in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with payload(s) and/or other polypeptide(s) of interest prior to administration. In some embodiments, the cultures, which are induced by a chemical and/or nutritional inducer and/or metabolite, are grown aerobically. In some embodiments, the cultures, which are induced by a chemical and/or nutritional inducer and/or metabolite, are grown anaerobically.

[0402] The genes of arabinose metabolism are organized in one operon, AraBAD, which is controlled by the PAraBAD promoter. The PAraBAD (or Para) promoter suitably fulfills the criteria of inducible expression systems. PAraBAD displays tighter control of

payload gene expression than many other systems, likely due to the dual regulatory role of AraC, which functions both as an inducer and as a repressor. Additionally, the level of ParaBAD-based expression can be modulated over a wide range of L-arabinose concentrations to fine-tune levels of expression of the payload. However, the cell population exposed to sub-saturating L-arabinose concentrations is divided into two subpopulations of induced and uninduced cells, which is determined by the differences between individual cells in the availability of L-arabinose transporter (Zhang et al., Development and Application of an Arabinose-Inducible Expression System by Facilitating Inducer Uptake in Corynebacterium glutamicum; Appl. Environ. Microbiol. August 2012 vol. 78 no. 16 5831-5838). Alternatively, inducible expression from the ParaBad can be controlled or fine-tuned through the optimization of the ribosome binding site (RBS), as described herein. An exemplary construct is depicted in **FIG. 57C.**

[0699] In one embodiment, expression of one or more payload(s) of interest, *e.g.*, one or more therapeutic polypeptide(s), is driven directly or indirectly by one or more arabinose inducible promoter(s).

[0700] In some embodiments, the arabinose inducible promoter is useful for or induced during *in vivo* expression of the one or more protein(s) of interest. In some embodiments, expression of one or more payload(s) of interest is driven directly or indirectly by one or more arabinose inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, *e.g.*, arabinose.

[0701] In some embodiments, expression of one or more protein(s) of interest, is driven directly or indirectly by one or more arabinose inducible promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the arabinose inducible promoter(s) are induced in culture, *e.g.*, grown in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with the payload prior to administration, *e.g.*, arabinose. In some embodiments, the cultures, which are induced by arabinose, are grown aerobically. In some embodiments, the cultures, which are induced by arabinose, are grown anaerobically.

[0702] In one embodiment, the arabinose inducible promoter drives the expression of a construct comprising one or more protein(s) of interest, jointly with a second promoter, *e.g.*,

a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the arabinose inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (*i.e.*, during preparation of the culture in a flask, fermenter or other appropriate culture vessel, *e.g.*, arabinose and IPTG). In another non-limiting example, the first inducing conditions may be culture conditions, *e.g.*, including arabinose presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of liver metabolites, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more arabinose promoters drive expression of one or more protein(s) of interest, in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0703] In some embodiments, the arabinose inducible promoter drives the expression of one or more protein(s) of interest from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the arabinose inducible promoter drives the expression of one or more protein(s) of interest from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0704] In some embodiments, one or more protein(s) of interest are knocked into the arabinose operon and are driven by the native arabinose inducible promoter

[0705] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 585**. In some embodiments, the arabinose inducible construct further comprises a gene encoding AraC, which is divergently transcribed from the same promoter as the one or more one or more protein(s) of interest. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 586**. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of **SEQ ID NO: 587**.

[0706] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are inducible through a rhamnose inducible system. The genes rhaBAD are organized in one operon which is controlled by the rhaP BAD promoter. The rhaP BAD promoter is regulated by two activators, RhaS and RhaR, and the corresponding genes belong to one transcription unit which divergently transcribed in the opposite direction of rhaBAD. In the presence of L-rhamnose, RhaR binds to the rhaP RS promoter and activates the production of RhaR and RhaS. RhaS together with L-rhamnose then bind to the rhaP BAD and the rhaP T promoter and activate the transcription of the structural genes. In contrast to the arabinose system, in which AraC is provided and divergently transcribed in the gene sequence(s), it is not necessary to express the regulatory proteins in larger quantities in the rhamnose expression system because the amounts expressed from the chromosome are sufficient to activate transcription even on multi-copy plasmids. Therefore, only the rhaP BAD promoter is cloned upstream of the gene that is to be expressed. Full induction of rhaBAD transcription also requires binding of the CRP-cAMP complex, which is a key regulator of catabolite repression. Alternatively, inducible expression from the rhaBAD can be controlled or fine-tuned through the optimization of the ribosome binding site (RBS), as described herein.

[0707] In one embodiment, expression of one or more protein(s) of interest is driven directly or indirectly by one or more rhamnose inducible promoter(s). In one embodiment, expression of the payload is driven directly or indirectly by a rhamnose inducible promoter.

[0708] In some embodiments, the rhamnose inducible promoter is useful for or induced during *in vivo* expression of the one or more protein(s) of interest. In some embodiments, expression of one or more protein(s) of interest is driven directly or indirectly by one or more rhamnose inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, *e.g.*, rhamnose

[0709] In some embodiments, expression of one or more protein(s) of interest, is driven directly or indirectly by one or more rhamnose inducible promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the rhamnose inducible promoter(s) are induced in culture, *e.g.*, grown in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with the payload prior to

administration, *e.g.*, rhamnose. In some embodiments, the cultures, which are induced by rhamnose, are grown arerobically. In some embodiments, the cultures, which are induced by rhamnose, are grown anaerobically.

[0710] In one embodiment, the rhamnose inducible promoter drives the expression of a construct comprising one or more protein(s) of interest jointly with a second promoter, *e.g.*, a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the rhamnose inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (*i.e.*, during preparation of the culture in a flask, fermenter or other appropriate culture vessel, *e.g.*, rhamnose and arabinose). In another non-limiting example, the first inducing conditions may be culture conditions, *e.g.*, including rhamnose presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of liver metabolites, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more rhamnose promoters drive expression of one or more protein(s) of interest and/or transcriptional regulator(s), *e.g.*, FNRS24Y, in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0711] In some embodiments, the rhamnose inducible promoter drives the expression of one or more protein(s) of interest , from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the rhamnose inducible promoter drives the expression of one or more protein(s) of interest , from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0712] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 588**.

[0713] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are inducible through an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible system or other compound which induced transcription from the Lac Promoter. IPTG is a molecular mimic of allolactose, a lactose metabolite that activates transcription of the lac operon. In contrast to allolactose, the sulfur atom in IPTG creates a non-hydrolyzable chemical blond, which prevents the degradation of

IPTG, allowing the concentration to remain constant. IPTG binds to the lac repressor and releases the tetrameric repressor (lacI) from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon. Since IPTG is not metabolized by *E. coli*, its concentration stays constant and the rate of expression of Lac promoter-controlled is tightly controlled, both *in vivo* and *in vitro*. IPTG intake is independent on the action of lactose permease, since other transport pathways are also involved. Inducible expression from the PLac can be controlled or fine-tuned through the optimization of the ribosome binding site (RBS), as described herein. Other compounds which inactivate LacI, can be used instead of IPTG in a similar manner.

[0714] In one embodiment, expression of one or more protein(s) of interest is driven directly or indirectly by one or more IPTG inducible promoter(s).

[0715] In some embodiments, the IPTG inducible promoter is useful for or induced during *in vivo* expression of the one or more protein(s) of interest. In some embodiments, expression of one or more protein(s) of interest is driven directly or indirectly by one or more IPTG inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, *e.g.*, IPTG.

[0716] In some embodiments, expression of one or more protein(s) of interest is driven directly or indirectly by one or more IPTG inducible promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the IPTG inducible promoter(s) are induced in culture, *e.g.*, grown in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with the payload prior to administration, *e.g.*, IPTG. In some embodiments, the cultures, which are induced by IPTG, are grown arerobically. In some embodiments, the cultures, which are induced by IPTG, are grown anaerobically.

[0717] In one embodiment, the IPTG inducible promoter drives the expression of a construct comprising one or more protein(s) of interest jointly with a second promoter, *e.g.*, a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the IPTG inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting

example, the first and second conditions may be two sequential culture conditions (*i.e.*, during preparation of the culture in a flask, fermenter or other appropriate culture vessel, *e.g.*, arabinose and IPTG). In another non-limiting example, the first inducing conditions may be culture conditions, *e.g.*, including IPTG presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of liver metabolites, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more IPTG inducible promoters drive expression of one or more protein(s) of interest in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0718] In some embodiments, the IPTG inducible promoter drives the expression of one or more protein(s) of interest from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the IPTG inducible promoter drives the expression of one or more protein(s) of interest from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0719] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 589**. In some embodiments, the IPTG inducible construct further comprises a gene encoding lacI, which is divergently transcribed from the same promoter as the one or more one or more protein(s) of interest. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 590**. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of **SEQ ID NO: 591**.

[0720] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are inducible through a tetracycline inducible system. The initial system Gossen and Bujard (Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Gossen M & Bujard H.*PNAS,* 1992 Jun 15;89(12):5547-51) developed is known as tetracycline off: in the presence of tetracycline, expression from a tet-inducible promoter is reduced. Tetracycline-controlled transactivator (tTA) was created by fusing tetR with the C-terminal domain of VP16 (virion protein 16) from herpes simplex

virus.In the absence of tetracycline, the tetR portion of tTA will bind tetO sequences in the tet promoter, and the activation domain promotes expression. In the presence of tetracycline, tetracycline binds to tetR, precluding tTA from binding to the tetO sequences. Next, a reverse Tet repressor (rTetR), was developed which created a reliance on the presence of tetracycline for induction, rather than repression. The new transactivator rtTA (reverse tetracycline-controlled transactivator) was created by fusing rTetR with VP16. The tetracycline on system is also known as the rtTA-dependent system.

[0721] In one embodiment, expression of one or more protein(s) of interest is driven directly or indirectly by one or more tetracycline inducible promoter(s).

[0722] In some embodiments, the tetracycline inducible promoter is useful for or induced during *in vivo* expression of the one or more protein(s) of interest. In some embodiments, expression of one or more protein(s) of interest and/or transcriptional regulator(s), *e.g.*, FNRS24Y, is driven directly or indirectly by one or more tetracycline inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, *e.g.*, tetracycline

[0723] In some embodiments, expression of one or more protein(s) of interest is driven directly or indirectly by one or more tetracycline inducible promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the tetracycline inducible promoter(s) are induced in culture, *e.g.*, grown in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with the payload prior to administration, *e.g.*, tetracycline. In some embodiments, the cultures, which are induced by tetracycline, are grown arerobically. In some embodiments, the cultures, which are induced by tetracycline, are grown anaerobically.

[0724] In one embodiment, the tetracycline inducible promoter drives the expression of a construct comprising one or more protein(s) of interest jointly with a second promoter, *e.g.*, a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the tetracycline inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions

(*i.e.*, during preparation of the culture in a flask, fermenter or other appropriate culture vessel, *e.g.*, tetracycline and IPTG). In another non-limiting example, the first inducing conditions may be culture conditions, *e.g.*, including tetracycline presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of liver metabolites, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more tetracycline promoters drive expression of one or more protein(s) of interest in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0725] In some embodiments, the tetracycline inducible promoter drives the expression of one or more protein(s) of interest from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the tetracycline inducible promoter drives the expression of one or more protein(s) of interest from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0726] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the bolded sequences of **SEQ ID NO: 596** (tet promoter is in bold). In some embodiments, the tetracycline inducible construct further comprises a gene encoding AraC, which is divergently transcribed from the same promoter as the one or more one or more protein(s) of interest In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 596** in italics (Tet repressor is in italics). In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of **SEQ ID NO: 596** in italics (Tet repressor is in italics).

[0727] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) whose expression is controlled by a temperature sensitive mechanism. Thermoregulators are advantageous because of strong transcriptional control without the use of external chemicals or specialized media (see, *e.g.*, Nemani et al., Magnetic nanoparticle hyperthermia induced cytosine deaminase expression in microencapsulated *E. coli* for

enzyme-prodrug therapy; J Biotechnol. 2015 Jun 10; 203: 32–40, and references therein). Thermoregulated protein expression using the mutant cI857 repressor and the pL and/or pR phage λ promoters have been used to engineer recombinant bacterial strains. The gene of interest cloned downstream of the λ promoters can then be efficiently regulated by the mutant thermolabile cI857 repressor of bacteriophage λ. At temperatures below 37 °C, cI857 binds to the oL or oR regions of the pR promoter and blocks transcription by RNA polymerase. At higher temperatures, the functional cI857 dimer is destabilized, binding to the oL or oR DNA sequences is abrogated, and mRNA transcription is initiated. An exemplary construct is depicted in **FIG. 88A**. Inducible expression from the ParaBad can be controlled or further fine-tuned through the optimization of the ribosome binding site (RBS), as described herein.

[0728] In one embodiment, expression of one or more protein(s) of interest is driven directly or indirectly by one or more thermoregulated promoter(s).

[0729] In some embodiments, the thermoregulated promoter is useful for or induced during *in vivo* expression of the one or more protein(s) of interest. In some embodiments, expression of one or more protein(s) of interest is driven directly or indirectly by one or more thermoregulated promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, *e.g.*, temperature.

[0730] In some embodiments, expression of one or more protein(s) of interest is driven directly or indirectly by one or more thermoregulated promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, it may be advantageous to shup off production of the one or more protein(s) of interest. This can be done in a thermoregulated system by growing the strain at lower temperatures, *e.g.*, 30 C. Expression can then be induced by elevating the temperature to 37 C and/or 42 C. In some embodiments, the thermoregulated promoter(s) are induced in culture, *e.g.*, grown in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In some embodiments, the cultures, which are induced by temperatures between 37 C and 42 C, are grown arerobically. In some embodiments, the cultures, which are induced by induced by temperatures between 37 C and 42 C, are grown anaerobically.

[0731] In one embodiment, the thermoregulated promoter drives the expression of a construct comprising one or more protein(s) of interest jointly with a second promoter, *e.g.*, a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the thermoregulated

promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (*i.e.*, during preparation of the culture in a flask, fermenter or other appropriate culture vessel, *e.g.*, thermoregulation and arabinose). In another non-limiting example, the first inducing conditions may be culture conditions, *e.g.*, permissive temperature, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of liver metabolites, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more thermoregulated promoters drive expression of one or more protein(s) of interest in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0732] In some embodiments, the thermoregulated promoter drives the expression of one or more protein(s) of interest from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the thermoregulated promoter drives the expression of one or more protein(s) of interest from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0733] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 592**. In some embodiments, the thermoregulated construct further comprises a gene encoding mutant cI857 repressor, which is divergently transcribed from the same promoter as the one or more one or more protein(s) of interest . In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 593**. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of **SEQ ID NO: 595**.

[0734] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are indirectly inducible through a system driven by the PssB promoter. The Pssb promoter is active under aerobic conditions, and shuts off under anaerobic conditions.

[0735] This promoter can be used to express a gene of interest under aerobic conditions. This promoter can also be used to tightly control the expression of a gene product such that it is only expressed under anaerobic conditions. In this case, the oxygen induced PssB promoter induces the expression of a repressor, which represses the expression of a gene of interest. As a result, the gene of interest is only expressed in the absence of the repressor, *i.e.*, under anaerobic conditions. This strategy has the advantage of an additional level of control for improved fine-tuning and tighter control. **FIG. 89A** depicts a schematic of the gene organization of a PssB promoter.

[0736] In one embodiment, expression of one or more protein(s) of interest is indirectly regulated by a repressor expressed under the control of one or more PssB promoter(s).

[0737] In some embodiments, induction of the RssB promoter(s) indirectly drives the *in vivo* expression of one or more protein(s) of interest. In some embodiments, induction of the RssB promoter(s) indirectly drives the expression of one or more protein(s) of interest during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, conditions for induction of the RssB promoter(s) are provided in culture, *e.g.*, in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture.

[0738] In some embodiments, the PssB promoter indirectly drives the expression of one or more protein(s) of interest from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the PssB promoter indirectly drives the expression of one or more protein(s) of interest from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0739] In another non-limiting example, this strategy can be used to control expression of thyA and/or dapA, *e.g.*, to make a conditional auxotroph. The chromosomal copy of dapA or ThyA is knocked out. Under anaerobic conditions, dapA or thyA -as the case may be- are expressed, and the strain can grow in the absence of dap or thymidine. Under aerobic conditions, dapA or thyA expression is shut off, and the strain cannot grow in the absence of dap or thymidine. Such a strategy can, for example be employed to allow survival of bacteria under anaerobic conditions, *e.g.*, the gut or liver, but prevent survival under aerobic conditions (biosafety switch). In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%,

85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of **SEQ ID NO: 597**.

[0740] Sequences useful for expression from inducible promoters are listed in **Table 26.**

Table 26. Inducible promoter construct sequences

Description	Sequence
Arabinose Promoter region **SEQ ID NO: 585**	CAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTCGC TAACCCAACCGGTAACCCCGCTTATTAAAAGCATTCTGTA ACAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAA AAGTGTCTATAATCACGGCAGAAAAGTCCACATTGATTAT TTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATC CATAAGATTAGCGGATCCAGCCTGACGCTTTTTTTCGCAA CTCTCTACTGTTTCTCCATACCTCTAGAAATAATTTTGTTT AACTTTAAGAAGGAGATATACAT
AraC (reverse orientation) **SEQ ID NO: 586**	TTATTCACAACCTGCCCTAAACTCGCTCGGACTCGCCCCG GTGCATTTTTTAAATACTCGCGAGAAATAGAGTTGATCGT CAAAACCGACATTGCGACCGACGGTGGCGATAGGCATCC GGGTGGTGCTCAAAAGCAGCTTCGCCTGACTGATGCGCTG GTCCTCGCGCCAGCTTAATACGCTAATCCCTAACTGCTGG CGGAACAAATGCGACAGACGCGACGGCGACAGGCAGACA TGCTGTGCGACGCTGGCGATATCAAAATTACTGTCTGCCA GGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCGATT ATCCATCGGTGGATGGAGCGACTCGTTAATCGCTTCCATG CGCCGCAGTAACAATTGCTCAAGCAGATTTATCGCCAGCA ATTCCGAATAGCGCCCTTCCCCTTGTCCGGCATTAATGATT TGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCAT CCGGGCGAAAGAAACCGGTATTGGCAAATATCGACGGCC AGTTAAGCCATTCATGCCAGTAGGCGCGCGGACGAAAGT AAACCCACTGGTGATACCATTCGTGAGCCTCCGGATGACG ACCGTAGTGATGAATCTCTCCAGGCGGGAACAGCAAAAT ATCACCCGGTCGGCAGACAAATTCTCGTCCCTGATTTTTCA CCACCCCCTGACCGCGAATGGTGAGATTGAGAATATAACC TTTCATTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAA CCGTTGGCCTCAATCGGCGTTAAACCCGCCACCAGATGGG CGTTAAACGAGTATCCCGGCAGCAGGGGATCATTTTGCGC TTCAGCCATACTTTTCATACTCCCGCCATTCAGAGAAGAA ACCAATTGTCCATATTGCAT
AraC polypeptide **SEQ ID NO: 587**	MQYGQLVSSLNGGSMKSMAEAQNDPLLPGYSFNAHLVAGL TPIEANGYLDFFIDRPLGMKGYILNLTIRGQGVVKNQGREFV CRPGDILLFPPGEIHHYGRHPEAHEWYHQWVYFRPRAYWHE WLNWPSIFANTGFFRPDEAHQPHFSDLFGQIINAGQGEGRYS ELLAINLLEQLLLRRMEAINESLHPPMDNRVREACQYISDHL ADSNFDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRIS QAKLLLSTTRMPIATVGRNVGFDDQLYFSRVFKKCTGASPSE FRAGCE*
Region comprising	CGGTGAGCATCACATCACCACAATTCAGCAAATTGTGAAC ATCATCACGTTCATCTTTCCCTGGTTGCCAATGGCCCATTT

rhamnose inducible promoter **SEQ ID NO: 588**	TCCTGTCAGTAACGAGAAGGTCGCGAATCAGGCGCTTTTT AGACTGGTCGTAATGAAATTCAGCTGTCACCGGATGTGCT TTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCCTCTAC AAATAATTTTGTTTAAAACAACACCCACTAAGATAACTCT AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
Lac Promoter region **SEQ ID NO: 589**	ATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATG CCATACCGCGAAAGGTTTTGCGCCATTCGATGGCGCGCCG CTTCGTCAGGCCACATAGCTTTCTTGTTCTGATCGGAACGA TCGTTGGCTGTGTTGACAATTAATCATCGGCTCGTATAATG TGTGGAATTGTGAGCGCTCACAATTAGCTGTCACCGGATG TGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCCT CTACAAATAATTTTGTTTAAAACAACACCCACTAAGATAA CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CAT
LacO	GGAATTGTGAGCGCTCACAATT
LacI (in reverse orientation) **SEQ ID NO: 590**	TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTT GCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGA GACTGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGA GAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCA GGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATA ACATGAGCTATCTTCGGTATCGTCGTATCCCACTACCGAG ATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGC GCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCAT CGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTT TGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTT CCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATG CCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAA TGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCG ACCAGATGCTCCACGCCCAGTCGCGTACCGTCCTCATGGG AGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATC AAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCAC AGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATC AGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCG CTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACC ACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCG CCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGG AGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAG TTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCC ATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTG GCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAG ACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTT TCAT
LacI polypeptide sequence **SEQ ID NO: 591**	MKPVTLYDVAEYAGVSYQTVSRVVNQASHVSAKTREKVEA AMAELNYIPNRVAQQLAGKQSLLIGVATSSLALHAPSQIVAA IKSRADQLGASVVVSMVERSGVEACKAAVHNLLAQRVSGLI INYPLDDQDAIAVEAACTNVPALFLDVSDQTPINSIIFSHEDGT RLGVEHLVALGHQQIALLAGPLSSVSARLRLAGWHKYLTRN QIQPIAEREGDWSAMSGFQQTMQMLNEGIVPTAMLVANDQ

	MALGAMRAITESGLRVGADISVVGYDDTEDSSCYIPPLTTIK QDFRLLGQTSVDRLLQLSQGQAVKGNQLLPVSLVKRKTTLA PNTQTASPRALADSLMQLARQVSRLESGQ
Region comprising Temperature sensitive promoter **SEQ ID NO: 592**	ACGTTAAATCTATCACCGCAAGGGATAAATATCTAACACC GTGCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTG CATAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCC GTGAGGACGAAACAGCCTCTACAAATAATTTTGTTTAAAA CAACACCCACTAAGATAACTCTAGAAATAATTTTGTTTAA CTTTAAGAAGGAGATATACAT
mutant cI857 repressor **SEQ ID NO: 593**	TCAGCCAAACGTCTCTTCAGGCCACTGACTAGCGATAACT TTCCCCACAACGGAACAACTCTCATTGCATGGGATCATTG GGTACTGTGGGTTTAGTGGTTGTAAAAACACCTGACCGCT ATCCCTGATCAGTTTCTTGAAGGTAAACTCATCACCCCCA AGTCTGGCTATGCAGAAATCACCTGGCTCAACAGCCTGCT CAGGGTCAACGAGAATTAACATTCCGTCAGGAAAGCTTGG CTTGGAGCCTGTTGGTGCGGTCATGGAATTACCTTCAACC TCAAGCCAGAATGCAGAATCACTGGCTTTTTTGGTTGTGC TTACCCATCTCTCCGCATCACCTTTGGTAAAGGTTCTAAGC TTAGGTGAGAACATCCCTGCCTGAACATGAGAAAAAACA GGGTACTCATACTCACTTCTAAGTGACGGCTGCATACTAA CCGCTTCATACATCTCGTAGATTTCTCTGGCGATTGAAGG GCTAAATTCTTCAACGCTAACTTTGAGAATTTTTGTAAGCA ATGCGGCGTTATAAGCATTTAATGCATTGATGCCATTAAA TAAAGCACCAACGCCTGACTGCCCCATCCCCATCTTGTCT GCGACAGATTCCTGGGATAAGCCAAGTTCATTTTTCTTTTT TTCATAAATTGCTTTAAGGCGACGTGCGTCCTCAAGCTGC TCTTGTGTTAATGGTTTCTTTTTTGTGCTCAT
RBS and leader region **SEQ ID NO: 594**	CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CAT
mutant cI857 repressor polypeptide sequence **SEQ ID NO: 595**	MSTKKKPLTQEQLEDARRLKAIYEKKKNELGLSQESVADKM GMGQSGVGALFNGINALNAYNAALLTKILKVSVEEFSPSIAR EIYEMYEAVSMQPSLRSEYEYPVFSHVQAGMFSPKLRTFTKG DAERWVSTTKKASDSAFWLEVEGNSMTAPTGSKPSFPDGML ILVDPEQAVEPGDFCIARLGGDEFTFKKLIRDSGQVFLQPLNP QYPMIPCNESCSVVGKVIASQWPEETFG
TetR-Tet promoter construct **SEQ ID NO: 596**	*Ttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataa gaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcata ctatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaacct aaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgttgg cataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgta cttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaat cttgccagctttcccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggct aaggcgtcgagcaaagcccgcttatttttttacatgccaatacaatgtaggctgctctacaccta gcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcg*

	*ctgttaatcactttacttttatctaatctagacat*cattaattcctaatttttt**gttgacactctatcattg atagagttattttaccactccctatcagtgatagagaa**aagtgaa*ctctagaaataattttgttt aactttaagaaggagatatacat*
PssB promoter **SEQ ID NO: 597**	tcacctttcccggattaaacgctttttgcccggtggcatggtgctaccggcgatcacaaacggtta attatgacacaaattgacctgaatgaatatacagtattggaatgcattacccggagtgttgtgtaac aatgtctggccaggtttgtttcccggaaccgaggtcacaacatagtaaaagcgctattggtaatgg tacaatcgcgcgtttacacttattc

Constitutive promoters

[0741] In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on a plasmid and operably linked to a constitutive promoter. In some embodiments, the gene encoding the metabolic and/or satiety effector molecules is present on a chromosome and operably linked to a constitutive promoter.

[0742] In some embodiments, the constitutive promoter is active under *in vivo* conditions, *e.g.*, the gut and/or conditions of the liver, as described herein. In some embodiments, the promoters is active under *in vitro* conditions, *e.g.*, various cell culture and/or cell manufacturing conditions, as described herein. In some embodiments, the constitutive promoter is active under *in vivo* conditions, *e.g.*, the gut and/or conditions of the liver, as described herein, and under *in vitro* conditions, *e.g.*, various cell culture and/or cell production and/or manufacturing conditions, as described herein.

[0743] In some embodiments, the constitutive promoter that is operably linked to the gene encoding the metabolic and/or satiety effector molecules is active in various exogenous environmental conditions (*e.g.*, *in vivo* and/or *in vitro* and/or production/manufacturing conditions).

[0744] In some embodiments, the constitutive promoter is active in exogenous environmental conditions specific to the gut of a mammal and/or conditions of the liver. In some embodiments, the constitutive promoter is active in exogenous environmental conditions specific to the small intestine of a mammal. In some embodiments, the constitutive promoter is active in low-oxygen or anaerobic conditions such as the environment of the mammalian gut and/or conditions of the liver. In some embodiments, the constitutive promoter is active in the presence of molecules or metabolites that are specific to the gut of a mammal and/or conditions of the liver. In some embodiments, the constitutive promoter is directly or indirectly induced by a molecule that is co-administered with the bacterial cell. In some embodiments, the constitutive promoter is active in the presence of

molecules or metabolites or other conditions, that are present during *in vitro* culture, cell production and/or manufacturing conditions.

[0745] Bacterial constitutive promoters are known in the art. Examplary constitutive promoters are listed in the following Tables.

Table 27. Constitutive *E. coli* σ70 promoters

Name	Description	Promoter Sequence	Length
BBa_I14018 SEQ ID NO: 598	P(Bla)	. . . gtttatacataggcgagtactctgttatgg	35
BBa_I14033 SEQ ID NO: 599	P(Cat)	. . . agaggttccaactttcaccataatgaaaca	38
BBa_I14034 SEQ ID NO: 600	P(Kat)	. . . taaacaactaacggacaattctacctaaca	45
BBa_I732021 SEQ ID NO: 601	Template for Building Primer Family Member	. . . acatcaagccaaattaaacaggattaacac	159
BBa_I742126 SEQ ID NO: 602	Reverse lambda cI-regulated promoter	. . . gaggtaaaatagtcaacacgcacggtgtta	49
BBa_J01006 SEQ ID NO: 603	Key Promoter absorbs 3	. . . caggccggaataactccctataatgcgcca	59
BBa_J23100 SEQ ID NO: 604	constitutive promoter family member	. . . ggctagctcagtcctaggtacagtgctagc	35
BBa_J23101 SEQ ID NO: 605	constitutive promoter family member	. . . agctagctcagtcctaggtattatgctagc	35
BBa_J23102 SEQ ID NO: 606	constitutive promoter family member	. . . agctagctcagtcctaggtactgtgctagc	35
BBa_J23103 SEQ ID NO: 607	constitutive promoter family member	. . . agctagctcagtcctagggattatgctagc	35
BBa_J23104 SEQ ID NO: 608	constitutive promoter family member	. . . agctagctcagtcctaggtattgtgctagc	35
BBa_J23105	constitutive promoter	. . .	35

SEQ ID NO: 609	family member	ggctagctcagtcctaggtactatgctagc	
BBa_J23106 SEQ ID NO: 610	constitutive promoter family member	. . . ggctagctcagtcctaggtatagtgctagc	35
BBa_J23107 SEQ ID NO: 611	constitutive promoter family member	. . . ggctagctcagccctaggtattatgctagc	35
BBa_J23108 SEQ ID NO: 612	constitutive promoter family member	. . . agctagctcagtcctaggtataatgctagc	35
BBa_J23109 SEQ ID NO: 613	constitutive promoter family member	. . . agctagctcagtcctagggactgtgctagc	35
BBa_J23110 SEQ ID NO: 614	constitutive promoter family member	. . . ggctagctcagtcctaggtacaatgctagc	35
BBa_J23111 SEQ ID NO: 615	constitutive promoter family member	. . . ggctagctcagtcctaggtatagtgctagc	35
BBa_J23112 SEQ ID NO: 616	constitutive promoter family member	. . . agctagctcagtcctagggattatgctagc	35
BBa_J23113 SEQ ID NO: 617	constitutive promoter family member	. . . ggctagctcagtcctagggattatgctagc	35
BBa_J23114 SEQ ID NO: 618	constitutive promoter family member	. . . ggctagctcagtcctaggtacaatgctagc	35
BBa_J23115 SEQ ID NO: 619	constitutive promoter family member	. . . agctagctcagcccttggtacaatgctagc	35
BBa_J23116 SEQ ID NO: 620	constitutive promoter family member	. . . agctagctcagtcctagggactatgctagc	35
BBa_J23117 SEQ ID NO: 621	constitutive promoter family member	. . . agctagctcagtcctagggattgtgctagc	35
BBa_J23118 SEQ ID NO: 622	constitutive promoter family member	. . . ggctagctcagtcctaggtattgtgctagc	35
BBa_J23119	constitutive promoter family member	. . . agctagctcagtcctaggtataatgctagc	35

SEQ ID NO: 623			
BBa_J23150 SEQ ID NO: 624	1bp mutant from J23107	. . . ggctagctcagtcctaggtattatgctagc	35
BBa_J23151 SEQ ID NO: 625	1bp mutant from J23114	. . . ggctagctcagtcctaggtacaatgctagc	35
BBa_J44002 SEQ ID NO: 626	pBAD reverse	. . . aaagtgtgacgccgtgcaaataatcaatgt	130
BBa_J48104 SEQ ID NO: 627	NikR promoter, a protein of the ribbon helix-helix family of trancription factors that repress expre	. . . gacgaatacttaaaatcgtcatacttattt	40
BBa_J54200 SEQ ID NO: 628	lacq_Promoter	. . . aaacctttcgcggtatggcatgatagcgcc	50
BBa_J56015 SEQ ID NO: 629	lacIQ - promoter sequence	. . . tgatagcgcccggaagagagtcaattcagg	57
BBa_J64951 SEQ ID NO: 630	*E. coli* CreABCD phosphate sensing operon promoter	. . . ttatttaccgtgacgaactaattgctcgtg	81
BBa_K088007 SEQ ID NO: 631	GlnRS promoter	. . . catacgccgttatacgttgtttacgctttg	38
BBa_K119000 SEQ ID NO: 632	Constitutive weak promoter of lacZ	. . . ttatgcttccggctcgtatgttgtgtggac	38
BBa_K119001 SEQ ID NO: 633	Mutated LacZ promoter	. . . ttatgcttccggctcgtatggtgtgtggac	38
BBa_K1330002 SEQ ID NO: 634	Constitutive promoter (J23105)	. . . ggctagctcagtcctaggtactatgctagc	35
BBa_K137029 SEQ ID NO: 635	constitutive promoter with (TA)10 between -10 and -35 elements	. . . atatatatatatataatggaagcgtttt	39
BBa_K137030 SEQ ID NO: 636	constitutive promoter with (TA)9 between -10 and -35 elements	. . . atatatatatatataatggaagcgtttt	37
BBa_K137031	constitutive promoter with	. . .	62

SEQ ID NO: 637	(C)10 between -10 and -35 elements	ccccgaaagcttaagaatataattgtaagc	
BBa_K137032 SEQ ID NO: 638	constitutive promoter with (C)12 between -10 and -35 elements	. . . ccccgaaagcttaagaatataattgtaagc	64
BBa_K137085 SEQ ID NO: 639	optimized (TA) repeat constitutive promoter with 13 bp between -10 and -35 elements	. . . tgacaatatatatatatataatgctagc	31
BBa_K137086 SEQ ID NO: 640	optimized (TA) repeat constitutive promoter with 15 bp between -10 and -35 elements	. . . acaatatatatatatatataatgctagc	33
BBa_K137087 SEQ ID NO: 641	optimized (TA) repeat constitutive promoter with 17 bp between -10 and -35 elements	. . . aatatatatatatatatataatgctagc	35
BBa_K137088 SEQ ID NO: 642	optimized (TA) repeat constitutive promoter with 19 bp between -10 and -35 elements	. . . tatatatatatatatatataatgctagc	37
BBa_K137089 SEQ ID NO: 643	optimized (TA) repeat constitutive promoter with 21 bp between -10 and -35 elements	. . . tatatatatatatatatataatgctagc	39
BBa_K137090 SEQ ID NO: 644	optimized (A) repeat constitutive promoter with 17 bp between -10 and -35 elements	. . . aaaaaaaaaaaaaaaaaatataatgctagc	35
BBa_K137091 SEQ ID NO: 645	optimized (A) repeat constitutive promoter with 18 bp between -10 and -35 elements	. . . aaaaaaaaaaaaaaaaaatataatgctagc	36
BBa_K1585100 SEQ ID NO: 646	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585101 SEQ ID NO: 647	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585102 SEQ ID NO: 648	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585103 SEQ ID NO: 649	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78

BBa_K1585104 SEQ ID NO: 650	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585105 SEQ ID NO: 651	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585106 SEQ ID NO: 652	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585110 SEQ ID NO: 653	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585113 SEQ ID NO: 654	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585115 SEQ ID NO: 655	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585116 SEQ ID NO: 656	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585117 SEQ ID NO: 657	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585118 SEQ ID NO: 658	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1585119 SEQ ID NO: 659	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	78
BBa_K1824896 SEQ ID NO: 660	J23100 + RBS	. . . gattaaagaggagaaatactagagtactag	88
BBa_K256002 SEQ ID NO: 661	J23101:GFP	. . . caccttcgggtgggcctttctgcgtttata	918
BBa_K256018 SEQ ID NO: 662	J23119:IFP	. . . caccttcgggtgggcctttctgcgtttata	1167
BBa_K256020 SEQ ID NO: 663	J23119:HO1	. . . caccttcgggtgggcctttctgcgtttata	949
BBa_K256033	Infrared signal reporter	. . .	2124

SEQ ID NO: 664	(J23119:IFP:J23119:HO1)	caccttcgggtgggcctttctgcgtttata	
BBa_K292000 SEQ ID NO: 665	Double terminator + constitutive promoter	... ggctagctcagtcctaggtacagtgctagc	138
BBa_K292001 SEQ ID NO: 666	Double terminator + Constitutive promoter + Strong RBS	... tgctagctactagagattaaagaggagaaa	161
BBa_K418000 SEQ ID NO: 667	IPTG inducible Lac promoter cassette	... ttgtgagcggataacaagatactgagcaca	1416
BBa_K418002 SEQ ID NO: 668	IPTG inducible Lac promoter cassette	... ttgtgagcggataacaagatactgagcaca	1414
BBa_K418003 SEQ ID NO: 669	IPTG inducible Lac promoter cassette	... ttgtgagcggataacaagatactgagcaca	1416
BBa_K823004 SEQ ID NO: 670	Anderson promoter J23100	... ggctagctcagtcctaggtacagtgctagc	35
BBa_K823005 SEQ ID NO: 671	Anderson promoter J23101	... agctagctcagtcctaggtattatgctagc	35
BBa_K823006 SEQ ID NO: 672	Anderson promoter J23102	... agctagctcagtcctaggtactgtgctagc	35
BBa_K823007 SEQ ID NO: 673	Anderson promoter J23103	... agctagctcagtcctagggattatgctagc	35
BBa_K823008 SEQ ID NO: 674	Anderson promoter J23106	... ggctagctcagtcctaggtatagtgctagc	35
BBa_K823010 SEQ ID NO: 675	Anderson promoter J23113	... ggctagctcagtcctagggattatgctagc	35
BBa_K823011 SEQ ID NO: 676	Anderson promoter J23114	... ggctagctcagtcctaggtacaatgctagc	35
BBa_K823013 SEQ ID NO: 677	Anderson promoter J23117	... agctagctcagtcctagggattgtgctagc	35
BBa_K823014	Anderson promoter J23118	... ggctagctcagtcctaggtattgtgctagc	35

SEQ ID NO: 678			
BBa_M13101 SEQ ID NO: 679	M13K07 gene I promoter	. . . cctgtttttatgttattctctctgtaaagg	47
BBa_M13102 SEQ ID NO: 680	M13K07 gene II promoter	. . . aaatatttgcttatacaatcttcctgtttt	48
BBa_M13103 SEQ ID NO: 681	M13K07 gene III promoter	. . . gctgataaaccgatacaattaaaggctcct	48
BBa_M13104 SEQ ID NO: 682	M13K07 gene IV promoter	. . . ctcttctcagcgtcttaatctaagctatcg	49
BBa_M13105 SEQ ID NO: 683	M13K07 gene V promoter	. . . atgagccagttcttaaaatcgcataaggta	50
BBa_M13106 SEQ ID NO: 684	M13K07 gene VI promoter	. . . ctattgattgtgacaaaataaacttattcc	49
BBa_M13108 SEQ ID NO: 685	M13K07 gene VIII promoter	. . . gtttcgcgcttggtataatcgctgggggtc	47
BBa_M13110 SEQ ID NO: 686	M13110	. . . ctttgcttctgactataatagtcagggtaa	48
BBa_M31519 SEQ ID NO: 687	Modified promoter sequence of g3.	. . . aaaccgatacaattaaaggctcctgctagc	60
BBa_R1074 SEQ ID NO: 688	Constitutive Promoter I	. . . caccacactgatagtgctagtgtagatcac	74
BBa_R1075 SEQ ID NO: 689	Constitutive Promoter II	. . . gccggaataactccctataatgcgccacca	49
BBa_S03331 SEQ ID NO: 690	--Specify Parts List--	ttgacaagcttttcctcagctccgtaaact	

Table 28. Constitutive *E. coli* σ^S promoters

Name	Description	Promoter Sequence	Length

BBa_J45992 SEQ ID NO: 691	Full-length stationary phase osmY promoter	. . . ggtttcaaaattgtgatctatatttaacaa	199
BBa_J45993 SEQ ID NO: 692	Minimal stationary phase osmY promoter	. . . ggtttcaaaattgtgatctatatttaacaa	57

Table 29. Constitutive *E. coli* σ^{32} promoters

Name	Description	Promoter Sequence	Length
BBa_J45504 SEQ ID NO: 693	htpG Heat Shock Promoter	. . . tctattccaataaagaaatcttcctgcgtg	405
BBa_K1895002 SEQ ID NO: 694	dnaK Promoter	. . . gaccgaatatatagtggaaacgtttagatg	182
BBa_K1895003 SEQ ID NO: 695	htpG Promoter	. . . ccacatcctgtttttaaccttaaaatggca	287

Table 30. Constitutive *B. subtilis* σ^{A} promoters

Name	Description	Promoter Sequence	Length
BBa_K143012 SEQ ID NO: 696	Promoter veg a constitutive promoter for *B. subtilis*	. . . aaaaatgggctcgtgttgtacaataaatgt	97
BBa_K143013 SEQ ID NO: 697	Promoter 43 a constitutive promoter for *B. subtilis*	. . . aaaaaaagcgcgcgattatgtaaaatataa	56
BBa_K780003 SEQ ID NO: 698	Strong constitutive promoter for Bacillus subtilis	. . . aattgcagtaggcatgacaaaatggactca	36
BBa_K823000 SEQ ID NO: 699	P_{liaG}	. . . caagcttttccttttataatagaatgaatga	121
BBa_K823002 SEQ ID NO: 700	P_{lepA}	. . . tctaagctagtgtattttgcgtttaatagt	157
BBa_K823003 SEQ ID NO: 701	P_{veg}	. . . aatgggctcgtgttgtacaataaatgtagt	237

Table 31. Constitutive *B. subtilis* σ^{B} promoters

Name	Description	Promoter Sequence	Length
BBa_K143010 SEQ ID NO: 702	Promoter ctc for *B. subtilis*	. . . atccttatcgttatgggtattgtttgtaat	56
BBa_K143011 SEQ ID NO: 703	Promoter gsiB for *B. subtilis*	. . . taaaagaattgtgagcgggaatacaacaac	38

BBa_K143013 SEQ ID NO: 704	Promoter 43 a constitutive promoter for *B. subtilis*	. . . aaaaaaagcgcgcgattatgtaaaatataa	56

Table 32. Constitutive promoters from miscellaneous prokaryotes

Name	Description	Promoter Sequence	Length
BBa_K112706 SEQ ID NO: 705	Pspv2 from *Salmonella*	. . . tacaaaataattcccctgcaaacattatca	474
BBa_K112707 SEQ ID NO: 706	Pspv from *Salmonella*	. . . tacaaaataattcccctgcaaacattatcg	1956

Table 33. Constitutive promoters from bacteriophage T7

Name	Description	Promoter Sequence	Length
BBa_I712074 SEQ ID NO: 707	T7 promoter (strong promoter from T7 bacteriophage)	. . . agggaatacaagctacttgttctttttgca	46
BBa_I719005 SEQ ID NO: 708	T7 Promoter	taatacgactcactatagggaga	23
BBa_J34814 SEQ ID NO: 709	T7 Promoter	gaatttaatacgactcactatagggaga	28
BBa_J64997 SEQ ID NO: 710	T7 consensus -10 and rest	taatacgactcactatagg	19
BBa_K113010 SEQ ID NO: 711	overlapping T7 promoter	. . . gagtcgtattaatacgactcactatagggg	40
BBa_K113011 SEQ ID NO: 712	more overlapping T7 promoter	. . . agtgagtcgtactacgactcactatagggg	37
BBa_K113012 SEQ ID NO: 713	weaken overlapping T7 promoter	. . . gagtcgtattaatacgactctctatagggg	40
BBa_K1614000 SEQ ID NO: 714	T7 promoter for expression of functional RNA	taatacgactcactatag	18
BBa_R0085 SEQ ID NO: 715	T7 Consensus Promoter Sequence	taatacgactcactatagggaga	23
BBa_R0180 SEQ ID NO: 716	T7 RNAP promoter	ttatacgactcactatagggaga	23
BBa_R0181 SEQ ID NO: 717	T7 RNAP promoter	gaatacgactcactatagggaga	23
BBa_R0182 SEQ ID NO: 718	T7 RNAP promoter	taatacgtctcactatagggaga	23
BBa_R0183	T7 RNAP promoter	tcatacgactcactatagggaga	23

SEQ ID NO: 719			
BBa_Z0251 SEQ ID NO: 720	T7 strong promoter	. . . taatacgactcactatagggagaccacaac	35
BBa_Z0252 SEQ ID NO: 721	T7 weak binding and processivity	. . . taattgaactcactaaagggagaccacagc	35
BBa_Z0253 SEQ ID NO: 722	T7 weak binding promoter	. . . cgaagtaatacgactcactattagggaaga	35

Table 34. Constitutive promoters from bacteriophage SP6

Name	Description	Promoter Sequence	Length
BBa_J64998 SEQ ID NO: 723	consensus -10 and rest from SP6	atttaggtgacactataga	19

Table 35. Constitutive promoters from yeast

Name	Description	Promoter Sequence	Length
BBa_I766555 SEQ ID NO: 724	pCyc (Medium) Promoter	. . . acaaacacaaatacacacactaaattaata	244
BBa_I766556 SEQ ID NO: 725	pAdh (Strong) Promoter	. . . ccaagcatacaatcaactatctcatataca	1501
BBa_I766557 SEQ ID NO: 726	pSte5 (Weak) Promoter	. . . gatacaggatacagcggaaacaacttttaa	601
BBa_J63005 SEQ ID NO: 727	yeast ADH1 promoter	. . . tttcaagctataccaagcatacaatcaact	1445
BBa_K105027 SEQ ID NO: 728	cyc100 minimal promoter	. . . cctttgcagcataaattactatacttctat	103
BBa_K105028 SEQ ID NO: 729	cyc70 minimal promoter	. . . cctttgcagcataaattactatacttctat	103
BBa_K105029 SEQ ID NO: 730	cyc43 minimal promoter	. . . cctttgcagcataaattactatacttctat	103
BBa_K105030 SEQ ID NO: 731	cyc28 minimal promoter	. . . cctttgcagcataaattactatacttctat	103
BBa_K105031 SEQ ID NO: 732	cyc16 minimal promoter	. . . cctttgcagcataaattactatacttctat	103
BBa_K122000 SEQ ID NO: 733	pPGK1	. . . ttatctactttttacaacaaatataaaaca	1497
BBa_K124000 SEQ ID NO: 734	pCYC Yeast Promoter	. . . acaaacacaaatacacacactaaattaata	288

BBa_K124002 SEQ ID NO: 735	Yeast GPD (TDH3) Promoter	. . . gtttcgaataaacacacataaacaaacaaa	681
BBa_K319005 SEQ ID NO: 736	yeast mid-length ADH1 promoter	. . . ccaagcatacaatcaactatctcatataca	720
BBa_M31201 SEQ ID NO: 737	Yeast CLB1 promoter region, G2/M cell cycle specific	. . . accatcaaaggaagctttaatcttctcata	500

Table 36. Constitutive promoters from miscellaneous eukaryotes

Name	Description	Promoter Sequence	Length
BBa_I712004 SEQ ID NO: 738	CMV promoter	. . . agaacccactgcttactggcttatcgaaat	654
BBa_K076017 SEQ ID NO: 739	Ubc Promoter	. . . ggccgtttttggcttttttgttagacgaag	1219

Table 37. Promoters

Name	Sequence	Description
Plpp SEQ ID NO: 740	ataagtgccttcccatcaaaaaaatattctc aacataaaaaactttgtgtaatacttgtaac gcta	The Plpp promoter is a natural promoter taken from the Nissle genome. In situ it is used to drive production of lpp, which is known to be the most abundant protein in the cell. Also, in some previous RNAseq experiments I was able to confirm that the lpp mRNA is one of the most abundant mRNA in Nissle during exponential growth.
PapFAB46 SEQ ID NO: 741	AAAAAGAGTATTGACTTC GCATCTTTTTGTACCTATA ATAGATTCATTGCTA	See, *e.g.*, Kosuri, S., Goodman, D. B. & Cambray, G. Composability of regulatory sequences controlling transcription and translation in *Escherichia coli*. in 1–20 (2013). doi:10.1073/pnas.
PJ23101+ UP element SEQ ID NO: 742	ggaaaatttttttaaaaaaaaaactttacag ctagctcagtcctaggtattatgctagc	UP element helps recruit RNA polymerase (ggaaaatttttttaaaaaaaaaac)
PJ23107+ UP element SEQ ID	ggaaaatttttttaaaaaaaaaactttacgg ctagctcagccctaggtattatgctagc	UP element helps recruit RNA polymerase (ggaaaatttttttaaaaaaaaaac)

NO: 743		
PSYN23119 SEQ ID NO: 744	ggaaaatttttttaaaaaaaaaacTTGACAGCTAGCTCAGTCCTTGGTATAATGCTAGCACGAA	UP element at 5' end; consensus -10 region is TATAAT; the consensus -35 is TTGACA; the extended -10 region is generally TGNTATAAT (TGGTATAAT in this sequence)

[0746] In some embodiments, the constitutive promoter is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the sequence of any one of SEQ ID NOs: 598-744.

Ribosome Binding Sites

[0747] In some embodiments, ribosome binding sites are added, switched out or replaced. By testing a few ribosome binding sites, expression levels can be fine-tuned to the desired level. **Table 38** and **Table 39** lists a number RBS which are suitable for prokaryotic expression and can be used to achieve the desired expression levels (See, *e.g.*, Registry of standard biological parts).

Table 38. Selected Ribosome Binding Sites

Identifier	Sequence[a]	SEQ ID NO
Master Sequence	TCTAGAGAAAGANNNGANNNACTAGATG	1018
BBa_J61100	TCTAGAGAAAGAGGGGACAAACTAGATG	1019
BBa_J61101	TCTAGAGAAAGACAGGACCCACTAGATG	1020
BBa_J61102	TCTAGAGAAAGATCCGATGTACTAGATG	1021
BBa_J61103	TCTAGAGAAAGATTAGACAAACTAGATG	1022
BBa_J61104	TCTAGAGAAAGAAGGGACAGACTAGATG	1023
BBa_J61105	TCTAGAGAAAGACATGACGTACTAGATG	1024
BBa_J61106	TCTAGAGAAAGATAGGAGACACTAGATG	1025
BBa_J61107	TCTAGAGAAAGAAGAGACTCACTAGATG	1026
BBa_J61108	TCTAGAGAAAGACGAGATATACTAGATG	1027
BBa_J61109	TCTAGAGAAAGACTGGAGACACTAGATG	1028
BBa_J61110	TCTAGAGAAAGAGGCGAATTACTAGATG	1029
BBa_J61111	TCTAGAGAAAGAGGCGATACACTAGATG	1030
BBa_J61112	TCTAGAGAAAGAGGTGACATACTAGATG	1031

BBa_J61113	TCTAGAGAAAGAGTGGAAAAACTAGATG	1032
BBa_J61114	TCTAGAGAAAGATGAGAAGAACTAGATG	1033
BBa_J61115	TCTAGAGAAAGAAGGGATACACTAGATG	1034
BBa_J61116	TCTAGAGAAAGACATGAGGCACTAGATG	1035
BBa_J61117	TCTAGAGAAAGACATGAGTTACTAGATG	1036
BBa_J61118	TCTAGAGAAAGAGACGAATCACTAGATG	1037
BBa_J61119	TCTAGAGAAAGATTTGATATACTAGATG	1038
BBa_J61120	TCTAGAGAAAGACGCGAGAAACTAGATG	1039
BBa_J61121	TCTAGAGAAAGAGACGAGTCACTAGATG	1040
BBa_J61122	TCTAGAGAAAGAGAGGAGCCACTAGATG	1041
BBa_J61123	TCTAGAGAAAGAGATGACTAACTAGATG	1042
BBa_J61124	TCTAGAGAAAGAGCCGACATACTAGATG	1043
BBa_J61125	TCTAGAGAAAGAGCCGAGTTACTAGATG	1044
BBa_J61126	TCTAGAGAAAGAGGTGACTCACTAGATG	1045
BBa_J61127	TCTAGAGAAAGAGTGGAACTACTAGATG	1046
BBa_J61128	TCTAGAGAAAGATAGGACTCACTAGATG	1047
BBa_J61129	TCTAGAGAAAGATTGGACGTACTAGATG	1048
BBa_J61130	TCTAGAGAAAGAAACGACATACTAGATG	1049
BBa_J61131	TCTAGAGAAAGAACCGAATTACTAGATG	1050
BBa_J61132	TCTAGAGAAAGACAGGATTAACTAGATG	873
BBa_J61133	TCTAGAGAAAGACCCGAGACACTAGATG	869
BBa_J61134	TCTAGAGAAAGACCGGAAATACTAGATG	870
BBa_J61135	TCTAGAGAAAGACCGGAGACACTAGATG	871
BBa_J61136	TCTAGAGAAAGAGCTGAGCAACTAGATG	874
BBa_J61137	TCTAGAGAAAGAGTAGATCAACTAGATG	875
BBa_J61138	TCTAGAGAAAGATATGAATAACTAGATG	876
BBa_J61139	TCTAGAGAAAGATTAGAGTCACTAGATG	877

Table 39. Selected Ribosome Binding Sites

Identifier	Sequence[a]	SEQ ID NO
BBa_B0029	TCTAGAGTTCACACAGGAAACCTACTAGATG	880
BBa_B0030	TCTAGAGATTAAAGAGGAGAAATACTAGATG	881
BBa_B0031	TCTAGAGTCACACAGGAAACCTACTAGATG	882
BBa_B0032	TCTAGAGTCACACAGGAAAGTACTAGATG	883
BBa_B0033	TCTAGAGTCACACAGGACTACTAGATG	884
BBa_B0034	TCTAGAGAAAGAGGAGAAATACTAGATG	885

BBa_B0035	TCTAGAGATTAAAGAGGAGAATACTAGATG	886
BBa_B0064	TCTAGAGAAAGAGGGGAAATACTAGATG	887

Essential Genes and Auxotrophs

[0748] As used herein, the term "essential gene" refers to a gene which is necessary to for cell growth and/or survival. Bacterial essential genes are well known to one of ordinary skill in the art, and can be identified by directed deletion of genes and/or random mutagenesis and screening (see, for example, Zhang and Lin, 2009, DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes, Nucl. Acids Res., 37:D455-D458 and Gerdes *et al.,* Essential genes on metabolic maps, Curr. Opin. Biotechnol., 17(5):448-456, the entire contents of each of which are expressly incorporated herein by reference).

[0749] An "essential gene" may be dependent on the circumstances and environment in which an organism lives. For example, a mutation of, modification of, or excision of an essential gene may result in the engineered bacteria of the disclosure becoming an auxotroph, *e.g.,* the bacteria may be an auxotroph depending on the environmental conditions (a conditional auxotroph). An auxotrophic modification is intended to cause bacteria to die in the absence of an exogenously added nutrient essential for survival or growth because they lack the gene(s) necessary to produce that essential nutrient.

[0750] An auxotrophic modification is intended to cause bacteria to die in the absence of an exogenously added nutrient essential for survival or growth because they lack the gene(s) necessary to produce that essential nutrient (see Figures 7 and 8). In some embodiments, any of the genetically engineered bacteria described herein also comprise a deletion or mutation in a gene required for cell survival and/or growth. In one embodiment, the essential gene is an oligonucleotide synthesis gene, for example, *thyA*. In another embodiment, the essential gene is a cell wall synthesis gene, for example, *dapA*. In yet another embodiment, the essential gene is an amino acid gene, for example, *serA* or *MetA*. Any gene required for cell survival and/or growth may be targeted, including but not limited to, *cysE, glnA, ilvD, leuB, lysA, serA, metA, glyA, hisB, ilvA, pheA, proA, thrC, trpC, tyrA, thyA, uraA, dapA, dapB, dapD, dapE, dapF, flhD, metB, metC, proAB,* and *thi1*, as long as the corresponding wild-type gene product is not produced in the bacteria.

[0751] **Table 40** lists depicts exemplary bacterial genes which may be disrupted or deleted to produce an auxotrophic strain. These include, but are not limited to, genes required for oligonucleotide synthesis, amino acid synthesis, and cell wall synthesis.

Table 40. Non-limiting Examples of Bacterial Genes Useful for Generation of an Auxotroph

Amino Acid	Oligonucleotide	Cell Wall
cysE	thyA	dapA
glnA	uraA	dapB
ilvD		dapD
leuB		dapE
lysA		dapF
serA		
metA		
glyA		
hisB		
ilvA		
pheA		
proA		
thrC		
trpC		
tyrA		

[0752] **Table 41** shows the survival of various amino acid auxotrophs in the mouse gut, as detected 24 hrs and 48 hrs post-gavage. These auxotrophs were generated using BW25113, a non-Nissle strain of *E. coli*.

Table 41. Survival of amino acid auxotrophs in the mouse gut

Gene	AA Auxotroph	Pre-Gavage	24 hours	48 hours
argA	**Arginine**	Present	Present	Absent
cysE	**Cysteine**	Present	Present	Absent
glnA	**Glutamine**	Present	Present	Absent
glyA	**Glycine**	Present	Present	Absent
hisB	**Histidine**	Present	Present	Present
ilvA	**Isoleucine**	Present	Present	Absent
leuB	**Leucine**	Present	Present	Absent
lysA	**Lysine**	Present	Present	Absent
metA	**Methionine**	Present	Present	Present

pheA	**Phenylalanine**	Present	Present	Present
proA	**Proline**	Present	Present	Absent
serA	**Serine**	Present	Present	Present
thrC	**Threonine**	Present	Present	Present
trpC	**Tryptophan**	Present	Present	Present
tyrA	**Tyrosine**	Present	Present	Present
ilvD	**Valine/Isoleucine/ Leucine**	Present	Present	Absent
thyA	**Thiamine**	Present	Absent	Absent
uraA	**Uracil**	Present	Absent	Absent
flhD	**FlhD**	Present	Present	Present

[0753] For example, thymine is a nucleic acid that is required for bacterial cell growth; in its absence, bacteria undergo cell death. The *thyA* gene encodes thimidylate synthetase, an enzyme that catalyzes the first step in thymine synthesis by converting dUMP to dTMP (Sat *et al.,* 2003). In some embodiments, the bacterial cell of the disclosure is a *thyA* auxotroph in which the *thyA* gene is deleted and/or replaced with an unrelated gene. A *thyA* auxotroph can grow only when sufficient amounts of thymine are present, *e.g.,* by adding thymine to growth media *in vitro*, or in the presence of high thymine levels found naturally in the human gut *in vivo*. In some embodiments, the bacterial cell of the disclosure is auxotrophic in a gene that is complemented when the bacterium is present in the mammalian gut. Without sufficient amounts of thymine, the *thyA* auxotroph dies. In some embodiments, the auxotrophic modification is used to ensure that the bacterial cell does not survive in the absence of the auxotrophic gene product (*e.g.,* outside of the gut).

[0754] Diaminopimelic acid (DAP) is an amino acid synthetized within the lysine biosynthetic pathway and is required for bacterial cell wall growth (Meadow *et al.,* 1959; Clarkson *et al.,* 1971). In some embodiments, any of the genetically engineered bacteria described herein is a *dapD* auxotroph in which *dapD* is deleted and/or replaced with an unrelated gene. A *dapD* auxotroph can grow only when sufficient amounts of DAP are present, *e.g.*, by adding DAP to growth media *in vitro*. Without sufficient amounts of DAP, the *dapD* auxotroph dies. In some embodiments, the auxotrophic modification is used to ensure that the bacterial cell does not survive in the absence of the auxotrophic gene product (*e.g.,* outside of the gut).

[0755] In other embodiments, the genetically engineered bacterium of the present disclosure is a *uraA* auxotroph in which *uraA* is deleted and/or replaced with an unrelated gene. The *uraA* gene codes for UraA, a membrane-bound transporter that facilitates the

uptake and subsequent metabolism of the pyrimidine uracil (Andersen *et al.,* 1995). A *uraA* auxotroph can grow only when sufficient amounts of uracil are present, *e.g.,* by adding uracil to growth media *in vitro*. Without sufficient amounts of uracil, the *uraA* auxotroph dies. In some embodiments, auxotrophic modifications are used to ensure that the bacteria do not survive in the absence of the auxotrophic gene product (*e.g.,* outside of the gut).

[0756] In complex communities, it is possible for bacteria to share DNA. In very rare circumstances, an auxotrophic bacterial strain may receive DNA from a non-auxotrophic strain, which repairs the genomic deletion and permanently rescues the auxotroph. Therefore, engineering a bacterial strain with more than one auxotroph may greatly decrease the probability that DNA transfer will occur enough times to rescue the auxotrophy. In some embodiments, the genetically engineered bacteria comprise a deletion or mutation in two or more genes required for cell survival and/or growth.

[0757] Other examples of essential genes include, but are not limited to yhbV, yagG, hemB, secD, secF, ribD, ribE, thiL, dxs, ispA, dnaX, adk, hemH, lpxH, cysS, fold, rplT, infC, thrS, nadE, gapA, yeaZ, aspS, argS, pgsA, yefM, metG, folE, yejM, gyrA, nrdA, nrdB, folC, accD, fabB, gltX, ligA, zipA, dapE, dapA, der, hisS, ispG, suhB, tadA, acpS, era, rnc, ftsB, eno, pyrG, chpR, lgt, fbaA, pgk, yqgD, metK, yqgF, plsC, ygiT, pare, ribB, cca, ygjD, tdcF, yraL, yihA, ftsN, murI, murB, birA, secE, nusG, rplJ, rplL, rpoB, rpoC, ubiA, plsB, lexA, dnaB, ssb, alsK, groS, psd, orn, yjeE, rpsR, chpS, ppa, valS, yjgP, yjgQ, dnaC, ribF, lspA, ispH, dapB, folA, imp, yabQ, ftsL, ftsI, murE, murF, mraY, murD, ftsW, murG, murC, ftsQ, ftsA, ftsZ, lpxC, secM, secA, can, folK, hemL, yadR, dapD, map, rpsB, infB ,nusA, ftsH, obgE, rpmA, rplU, ispB, murA, yrbB, yrbK, yhbN, rpsI, rplM, degS, mreD, mreC, mreB, accB, accC, yrdC, def, fmt, rplQ, rpoA, rpsD, rpsK, rpsM, entD, mrdB, mrdA, nadD, hlepB, rpoE, pssA, yfiO, rplS, trmD, rpsP, ffh, grpE, yfjB, csrA, ispF, ispD, rplW, rplD, rplC, rpsJ, fusA, rpsG, rpsL, trpS, yrfF, asd, rpoH, ftsX, ftsE, ftsY, frr, dxr, ispU, rfaK, kdtA, coaD, rpmB, dfp, dut, gmk, spot, gyrB, dnaN, dnaA, rpmH, rnpA, yidC, tnaB, glmS, glmU, wzyE, hemD, hemC, yigP, ubiB, ubiD, hemG, secY, rplO, rpmD, rpsE, rplR, rplF, rpsH, rpsN, rplE, rplX, rplN, rpsQ, rpmC, rplP, rpsC, rplV, rpsS, rplB, cdsA, yaeL, yaeT, lpxD, fabZ, lpxA, lpxB, dnaE, accA, tilS, proS, yafF, tsf, pyrH, olA, rlpB, leuS, lnt, glnS, fldA, cydA, infA, cydC, ftsK, lolA, serS, rpsA, msbA, lpxK, kdsB, mukF, mukE, mukB, asnS, fabA, mviN, rne, yceQ, fabD, fabG, acpP, tmk, holB, lolC, lolD, lolE, purB, ymfK, minE, mind, pth, rsA, ispE, lolB, hemA, prfA, prmC, kdsA, topA, ribA, fabI, racR, dicA, ydfB, tyrS, ribC, ydiL, pheT, pheS, yhhQ, bcsB, glyQ, yibJ, and gpsA. Other essential genes are known to those of ordinary skill in the art.

[0758] In some embodiments, the genetically engineered bacterium of the present disclosure is a synthetic ligand-dependent essential gene (SLiDE) bacterial cell. SLiDE bacterial cells are synthetic auxotrophs with a mutation in one or more essential genes that only grow in the presence of a particular ligand (see Lopez and Anderson "Synthetic Auxotrophs with Ligand-Dependent Essential Genes for a BL21 (DE3 Biosafety Strain, "ACS Synthetic Biology (2015) DOI: 10.1021/acssynbio.5b00085, the entire contents of which are expressly incorporated herein by reference).

[0759] In some embodiments, the SLiDE bacterial cell comprises a mutation in an essential gene. In some embodiments, the essential gene is selected from the group consisting of *pheS, dnaN, tyrS, metG* and *adk*. In some embodiments, the essential gene is *dnaN* comprising one or more of the following mutations: H191N, R240C, I317S, F319V, L340T, V347I, and S345C. In some embodiments, the essential gene is *dnaN* comprising the mutations H191N, R240C, I317S, F319V, L340T, V347I, and S345C. In some embodiments, the essential gene is *pheS* comprising one or more of the following mutations: F125G, P183T, P184A, R186A, and I188L. In some embodiments, the essential gene is *pheS* comprising the mutations F125G, P183T, P184A, R186A, and I188L. In some embodiments, the essential gene is *tyrS* comprising one or more of the following mutations: L36V, C38A and F40G. In some embodiments, the essential gene is *tyrS* comprising the mutations L36V, C38A and F40G. In some embodiments, the essential gene is *metG* comprising one or more of the following mutations: E45Q, N47R, I49G, and A51C. In some embodiments, the essential gene is *metG* comprising the mutations E45Q, N47R, I49G, and A51C. In some embodiments, the essential gene is *adk* comprising one or more of the following mutations: I4L, L5I and L6G. In some embodiments, the essential gene is *adk* comprising the mutations I4L, L5I and L6G.

[0760] In some embodiments, the genetically engineered bacterium is complemented by a ligand. In some embodiments, the ligand is selected from the group consisting of benzothiazole, indole, 2-aminobenzothiazole, indole-3-butyric acid, indole-3-acetic acid, and L-histidine methyl ester. For example, bacterial cells comprising mutations in metG (E45Q, N47R, I49G, and A51C) are complemented by benzothiazole, indole, 2-aminobenzothiazole, indole-3-butyric acid, indole-3-acetic acid or L-histidine methyl ester. Bacterial cells comprising mutations in dnaN (H191N, R240C, I317S, F319V, L340T, V347I, and S345C) are complemented by benzothiazole, indole or 2-aminobenzothiazole. Bacterial cells comprising mutations in pheS (F125G, P183T, P184A, R186A, and I188L) are complemented by benzothiazole or 2-aminobenzothiazole. Bacterial cells comprising

mutations in tyrS (L36V, C38A, and F40G) are complemented by benzothiazole or 2-aminobenzothiazole. Bacterial cells comprising mutations in adk (I4L, L5I and L6G) are complemented by benzothiazole or indole.

[0761] In some embodiments, the genetically engineered bacterium comprises more than one mutant essential gene that renders it auxotrophic to a ligand. In some embodiments, the bacterial cell comprises mutations in two essential genes. For example, in some embodiments, the bacterial cell comprises mutations in *tyrS* (L36V, C38A, and F40G) and *metG* (E45Q, N47R, I49G, and A51C). In other embodiments, the bacterial cell comprises mutations in three essential genes. For example, in some embodiments, the bacterial cell comprises mutations in *tyrS* (L36V, C38A, and F40G), *metG* (E45Q, N47R, I49G, and A51C), and *pheS* (F125G, P183T, P184A, R186A, and I188L).

[0762] In some embodiments, the genetically engineered bacterium is a conditional auxotroph whose essential gene(s) is replaced using the arabinose system described herein.

[0763] In some embodiments, the genetically engineered bacterium of the disclosure is an auxotroph and also comprises kill-switch circuitry, such as any of the kill-switch components and systems described herein. For example, the engineered bacteria may comprise a deletion or mutation in an essential gene required for cell survival and/or growth, for example, in a DNA synthesis gene, for example, *thyA*, cell wall synthesis gene, for example, *dapA* and/or an amino acid gene, for example, *serA* or *MetA* and may also comprise a toxin gene that is regulated by one or more transcriptional activators that are expressed in response to an environmental condition(s) and/or signal(s) (such as the described arabinose system) or regulated by one or more recombinases that are expressed upon sensing an exogenous environmental condition(s) and/or signal(s) (such as the recombinase systems described herein). Other embodiments are described in Wright *et al.,* "GeneGuard: A Modular Plasmid System Designed for Biosafety," ACS Synthetic Biology (2015) 4: 307-16, the entire contents of which are expressly incorporated herein by reference). In some embodiments, the genetically engineered bacterium of the disclosure is an auxotroph and also comprises kill-switch circuitry, such as any of the kill-switch components and systems described herein, as well as another biosecurity system, such a conditional origin of replication (see Wright *et al., supra*).

Genetic Regulatory Circuits

[0764] In some embodiments, the genetically engineered bacteria comprise multi-layered genetic regulatory circuits for expressing the constructs described herein (*see, e.g.,*

U.S. Provisional Application No. 62/184,811, incorporated herein by reference in its entirety).

[0765] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a metabolic or satiety effector molecule and a T7 polymerase-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a first gene encoding a T7 polymerase, wherein the first gene is operably linked to a fumarate and nitrate reductase regulator (FNR)-responsive promoter; a second gene or gene cassette for producing a metabolic or satiety effector molecule, wherein the second gene or gene cassette is operably linked to a T7 promoter that is induced by the T7 polymerase; and a third gene encoding an inhibitory factor, lysY, that is capable of inhibiting the T7 polymerase. In the presence of oxygen, FNR does not bind the FNR-responsive promoter, and the metabolic or satiety effector molecule is not expressed. LysY is expressed constitutively (P-lac constitutive) and further inhibits T7 polymerase. In the absence of oxygen, FNR dimerizes and binds to the FNR-responsive promoter, T7 polymerase is expressed at a level sufficient to overcome lysY inhibition, and the effector molecule is expressed. In some embodiments, the lysY gene is operably linked to an additional FNR binding site. In the absence of oxygen, FNR dimerizes to activate T7 polymerase expression as described above, and also inhibits lysY expression.

[0766] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing an effector molecule and a protease-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a first gene encoding an mf-lon protease, wherein the first gene is operably linked to a FNR-responsive promoter; a second gene or gene cassette for producing an effector molecule operably linked to a tet regulatory region (tetO); and a third gene encoding an mf-lon degradation signal linked to a tet repressor (tetR), wherein the tetR is capable of binding to the tet regulatory region and repressing expression of the second gene or gene cassette. The mf-lon protease is capable of recognizing the mf-lon degradation signal and degrading the tetR. In the presence of oxygen, FNR does not bind the FNR-responsive promoter, the repressor is not degraded, and the metabolic or satiety effector molecule is not expressed. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, thereby inducing expression of mf-lon protease. The mf-lon protease recognizes the mf-lon degradation signal and degrades the tetR, and the metabolic or satiety effector molecule is expressed.

[0767] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing an effector molecule and a repressor-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a first gene encoding a first repressor, wherein the first gene is operably linked to a FNR-responsive promoter; a second gene or gene cassette for producing an effector molecule operably linked to a first regulatory region comprising a constitutive promoter; and a third gene encoding a second repressor, wherein the second repressor is capable of binding to the first regulatory region and repressing expression of the second gene or gene cassette. The third gene is operably linked to a second regulatory region comprising a constitutive promoter, wherein the first repressor is capable of binding to the second regulatory region and inhibiting expression of the second repressor. In the presence of oxygen, FNR does not bind the FNR-responsive promoter, the first repressor is not expressed, the second repressor is expressed, and the metabolic or satiety effector molecule is not expressed. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, the first repressor is expressed, the second repressor is not expressed, and the metabolic or satiety effector molecule is expressed.

[0768] Examples of repressors useful in these embodiments include, but are not limited to, ArgR, TetR, ArsR, AscG, LacI, CscR, DeoR, DgoR, FruR, GalR, GatR, CI, LexA, RafR, QacR, and PtxS (US20030166191).

[0769] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing an effector molecule and a regulatory RNA-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a first gene encoding a regulatory RNA, wherein the first gene is operably linked to a FNR-responsive promoter, and a second gene or gene cassette for producing an effector molecule. The second gene or gene cassette is operably linked to a constitutive promoter and further linked to a nucleotide sequence capable of producing an mRNA hairpin that inhibits translation of the metabolic or satiety effector molecule. The regulatory RNA is capable of eliminating the mRNA hairpin and inducing translation via the ribosomal binding site. In the presence of oxygen, FNR does not bind the FNR-responsive promoter, the regulatory RNA is not expressed, and the mRNA hairpin prevents the metabolic or satiety effector molecule from being translated. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, the regulatory RNA is expressed, the mRNA hairpin is eliminated, and the metabolic or satiety effector molecule is expressed.

[0770] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing an effector molecule and a CRISPR-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a Cas9 protein; a first gene encoding a CRISPR guide RNA, wherein the first gene is operably linked to a FNR-responsive promoter; a second gene or gene cassette for producing an effector molecule, wherein the second gene or gene cassette is operably linked to a regulatory region comprising a constitutive promoter; and a third gene encoding a repressor operably linked to a constitutive promoter, wherein the repressor is capable of binding to the regulatory region and repressing expression of the second gene or gene cassette. The third gene is further linked to a CRISPR target sequence that is capable of binding to the CRISPR guide RNA, wherein said binding to the CRISPR guide RNA induces cleavage by the Cas9 protein and inhibits expression of the repressor. In the presence of oxygen, FNR does not bind the FNR-responsive promoter, the guide RNA is not expressed, the repressor is expressed, and the metabolic or satiety effector molecule is not expressed. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, the guide RNA is expressed, the repressor is not expressed, and the metabolic or satiety effector molecule is expressed.

[0771] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing an effector molecule and a recombinase-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a first gene encoding a recombinase, wherein the first gene is operably linked to a FNR-responsive promoter, and a second gene or gene cassette for producing an effector molecule operably linked to a constitutive promoter. The second gene or gene cassette is inverted in orientation (3' to 5') and flanked by recombinase binding sites, and the recombinase is capable of binding to the recombinase binding sites to induce expression of the second gene or gene cassette by reverting its orientation (5' to 3'). In the presence of oxygen, FNR does not bind the FNR-responsive promoter, the recombinase is not expressed, the gene or gene cassette remains in the 3' to 5' orientation, and no functional metabolic or satiety effector molecule is produced. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, the recombinase is expressed, the gene or gene cassette is reverted to the 5' to 3' orientation, and functional metabolic or satiety effector molecule is produced.

[0772] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing an effector molecule and a polymerase- and

recombinase-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a first gene encoding a recombinase, wherein the first gene is operably linked to a FNR-responsive promoter; a second gene or gene cassette for producing an effector molecule operably linked to a T7 promoter; a third gene encoding a T7 polymerase, wherein the T7 polymerase is capable of binding to the T7 promoter and inducing expression of the metabolic or satiety effector molecule. The third gene encoding the T7 polymerase is inverted in orientation (3' to 5') and flanked by recombinase binding sites, and the recombinase is capable of binding to the recombinase binding sites to induce expression of the T7 polymerase gene by reverting its orientation (5' to 3'). In the presence of oxygen, FNR does not bind the FNR-responsive promoter, the recombinase is not expressed, the T7 polymerase gene remains in the 3' to 5' orientation, and the metabolic or satiety effector molecule is not expressed. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, the recombinase is expressed, the T7 polymerase gene is reverted to the 5' to 3' orientation, and the metabolic or satiety effector molecule is expressed.

Kill Switches

[0773] In some embodiments, the genetically engineered bacteria also comprise a kill switch (*see, e.g.,* U.S. Provisional Application Nos. 62/183,935 and 62/263,329, each of which are expressly incorporated herein by reference in their entireties). The kill switch is intended to actively kill engineered microbes in response to external stimuli. As opposed to an auxotrophic mutation where bacteria die because they lack an essential nutrient for survival, the kill switch is triggered by a particular factor in the environment that induces the production of toxic molecules within the microbe that cause cell death.

[0774] Bacteria engineered with kill switches have been engineered for *in vitro* research purposes, *e.g.,* to limit the spread of a biofuel-producing microorganism outside of a laboratory environment. Bacteria engineered for *in vivo* administration to treat a disease or disorder may also be programmed to die at a specific time after the expression and delivery of a heterologous gene or genes, for example, a therapeutic gene(s) or after the subject has experienced the therapeutic effect. For example, in some embodiments, the kill switch is activated to kill the bacteria after a period of time following expression of the biosynthetic cassette(s) and/or gene(s) present in the engineered bacteria, for example, following expression of a butyrate cassette, propionate cassette, GLP-1, butyrate cassette and propionate cassette, butyrate cassette and GLP-1, propionate cassette and GLP-1, or butyrate and propionate cassettes and GLP-1. In some embodiments, the kill switch is activated in a

delayed fashion following expression of the heterologous gene(s) or gene cassette(s), for example, after the production of the corresponding protein(s) or molecule(s). Alternatively, the bacteria may be engineered to die after the bacteria has spread outside of a disease site. Specifically, it may be useful to prevent long-term colonization of subjects by the microorganism, spread of the microorganism outside the area of interest (for example, outside the gut) within the subject, or spread of the microorganism outside of the subject into the environment (for example, spread to the environment through the stool of the subject).

[0775] Examples of such toxins that can be used in kill-switches include, but are not limited to, bacteriocins, lysins, and other molecules that cause cell death by lysing cell membranes, degrading cellular DNA, or other mechanisms. Such toxins can be used individually or in combination. The switches that control their production can be based on, for example, transcriptional activation (toggle switches; *see, e.g.,* Gardner *et al.,* 2000), translation (riboregulators), or DNA recombination (recombinase-based switches), and can sense environmental stimuli such as anaerobiosis or reactive oxygen species. These switches can be activated by a single environmental factor or may require several activators in AND, OR, NAND and NOR logic configurations to induce cell death. For example, an AND riboregulator switch is activated by tetracycline, isopropyl β-D-1-thiogalactopyranoside (IPTG), and arabinose to induce the expression of lysins, which permeabilize the cell membrane and kill the cell. IPTG induces the expression of the endolysin and holin mRNAs, which are then derepressed by the addition of arabinose and tetracycline. All three inducers must be present to cause cell death. Examples of kill switches are known in the art (Callura *et al.,* 2010). In some embodiments, the kill switch is activated to kill the bacteria after a period of time following oxygen level-dependent expression of a heterologous gene(s) or gene cassette(s). In some embodiments, the kill switch is activated in a delayed fashion following oxygen level-dependent expression of a heterologous gene(s) or gene cassette(s).

[0776] Kill-switches can be designed such that a toxin is produced in response to an environmental condition or external signal (*e.g.,* the bacteria is killed in response to an external cue; *i.e.,* an activation-based kill switch, see Figures 18-22) or, alternatively designed such that a toxin is produced once an environmental condition no longer exists or an external signal is ceased (*i.e.,* a repression-based kill switch, see Figures 14-17).

[0777] Thus, in some embodiments, the genetically engineered bacteria of the disclosure are further programmed to die after sensing an exogenous environmental signal, for example, in a low oxygen environment. In some embodiments, the genetically engineered bacteria of the present disclosure comprise one or more genes encoding one or more

recombinase(s), whose expression is induced in response to an environmental condition or signal and causes one or more recombination events that ultimately leads to the expression of a toxin which kills the cell. In some embodiments, the at least one recombination event is the flipping of an inverted heterologous gene encoding a bacterial toxin which is then constitutively expressed after it is flipped by the first recombinase. In one embodiment, constitutive expression of the bacterial toxin kills the genetically engineered bacterium. In these types of kill-switch systems once the engineered bacterial cell senses the exogenous environmental condition and expresses the heterologous gene of interest, the engineered bacterial cell is no longer viable.

[0778] In another embodiment in which the genetically engineered bacteria of the present disclosure express one or more recombinase(s) in response to an environmental condition or signal causing at least one recombination event, the genetically engineered bacterium further expresses a heterologous gene encoding an anti-toxin in response to an exogenous environmental condition or signal. In one embodiment, the at least one recombination event is flipping of an inverted heterologous gene encoding a bacterial toxin by a first recombinase. In one embodiment, the inverted heterologous gene encoding the bacterial toxin is located between a first forward recombinase recognition sequence and a first reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the bacterial toxin is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the anti-toxin inhibits the activity of the toxin, thereby delaying death of the genetically engineered bacterium. In one embodiment, the genetically engineered bacterium is killed by the bacterial toxin when the heterologous gene encoding the anti-toxin is no longer expressed when the exogenous environmental condition is no longer present.

[0779] In another embodiment, the at least one recombination event is flipping of an inverted heterologous gene encoding a second recombinase by a first recombinase, followed by the flipping of an inverted heterologous gene encoding a bacterial toxin by the second recombinase. In one embodiment, the inverted heterologous gene encoding the second recombinase is located between a first forward recombinase recognition sequence and a first reverse recombinase recognition sequence. In one embodiment, the inverted heterologous gene encoding the bacterial toxin is located between a second forward recombinase recognition sequence and a second reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the second recombinase is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the heterologous

gene encoding the bacterial toxin is constitutively expressed after it is flipped by the second recombinase. In one embodiment, the genetically engineered bacterium is killed by the bacterial toxin. In one embodiment, the genetically engineered bacterium further expresses a heterologous gene encoding an anti-toxin in response to the exogenous environmental condition. In one embodiment, the anti-toxin inhibits the activity of the toxin when the exogenous environmental condition is present, thereby delaying death of the genetically engineered bacterium. In one embodiment, the genetically engineered bacterium is killed by the bacterial toxin when the heterologous gene encoding the anti-toxin is no longer expressed when the exogenous environmental condition is no longer present.

[0780] In one embodiment, the at least one recombination event is flipping of an inverted heterologous gene encoding a second recombinase by a first recombinase, followed by flipping of an inverted heterologous gene encoding a third recombinase by the second recombinase, followed by flipping of an inverted heterologous gene encoding a bacterial toxin by the third recombinase. Accordingly, in one embodiment, the disclosure provides at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 recombinases that can be used serially.

[0781] In one embodiment, the at least one recombination event is flipping of an inverted heterologous gene encoding a first excision enzyme by a first recombinase. In one embodiment, the inverted heterologous gene encoding the first excision enzyme is located between a first forward recombinase recognition sequence and a first reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the first excision enzyme is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the first excision enzyme excises a first essential gene. In one embodiment, the programmed engineered bacterial cell is not viable after the first essential gene is excised.

[0782] In one embodiment, the first recombinase further flips an inverted heterologous gene encoding a second excision enzyme. In one embodiment, the wherein the inverted heterologous gene encoding the second excision enzyme is located between a second forward recombinase recognition sequence and a second reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the second excision enzyme is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the genetically engineered bacterium dies or is no longer viable when the first essential gene and the second essential gene are both excised. In one embodiment, the genetically engineered bacterium dies or is no longer viable when either the first essential gene is excised or the second essential gene is excised by the first recombinase.

[0783] In one embodiment, the first excision enzyme is Xis1. In one embodiment, the first excision enzyme is Xis2. In one embodiment, the first excision enzyme is Xis1, and the second excision enzyme is Xis2.

[0784] In one embodiment, the genetically engineered bacterium dies after the at least one recombination event occurs. In another embodiment, the genetically engineered bacterium is no longer viable after the at least one recombination event occurs.

[0785] In any of these embodiment, the recombinase can be a recombinase selected from the group consisting of: BxbI, PhiC31, TP901, BxbI, PhiC31, TP901, HK022, HP1, R4, Int1, Int2, Int3, Int4, Int5, Int6, Int7, Int8, Int9, Int10, Int11, Int12, Int13, Int14, Int15, Int16, Int17, Int18, Int19, Int20, Int21, Int22, Int23, Int24, Int25, Int26, Int27, Int28, Int29, Int30, Int31, Int32, Int33, and Int34, or a biologically active fragment thereof.

[0786] In the above-described kill-switch circuits, a toxin is produced in the presence of an environmental factor or signal. In another aspect of kill-switch circuitry, a toxin may be repressed in the presence of an environmental factor (not produced) and then produced once the environmental condition or external signal is no longer present. Such kill switches are called repression-based kill switches and represent systems in which the bacterial cells are viable only in the presence of an external factor or signal, such as arabinose or other sugar. Exemplary kill switch designs in which the toxin is repressed in the presence of an external factor or signal (and activated once the external signal is removed) is shown in **FIG. 56-57**. The disclosure provides engineered bacterial cells which express one or more heterologous gene(s) upon sensing arabinose or other sugar in the exogenous environment. In this aspect, the engineered bacterial cells contain the *araC* gene, which encodes the AraC transcription factor, as well as one or more genes under the control of the *araBAD* promoter. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription of genes under the control of the *araBAD* promoter. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the *araBAD* promoter, which induces expression of the desired gene, for example tetR, which represses expression of a toxin gene. In this embodiment, the toxing gene is repressed in the presence of arabinose or other sugar. In an environment where arabinose is not present, the tetR gene is not activated and the toxin is expressed, thereby killing the bacteria. The arbinoase system can also be used to express an essential gene, in which the essential gene is only expressed in the presence of arabinose or other sugar and is not expressed when arabinose or other sugar is absent from the environment.

[0787] Thus, in some embodiments in which one or more heterologous gene(s) are expressed upon sensing arabinose in the exogenous environment, the one or more heterologous genes are directly or indirectly under the control of the *araBAD* promoter. In some embodiments, the expressed heterologous gene is selected from one or more of the following: a heterologous therapeutic gene, a heterologous gene encoding an antitoxin, a heterologous gene encoding a repressor protein or polypeptide, for example, a TetR repressor, a heterologous gene encoding an essential protein not found in the bacterial cell, and/or a heterologous encoding a regulatory protein or polypeptide.

[0788] Arabinose inducible promoters are known in the art, including P_{ara}, P_{araB}, P_{araC}, and P_{araBAD}. In one embodiment, the arabinose inducible promoter is from *E. coli*. In some embodiments, the P_{araC} promoter and the P_{araBAD} promoter operate as a bidirectional promoter, with the P_{araBAD} promoter controlling expression of a heterologous gene(s) in one direction, and the P_{araC} (in close proximity to, and on the opposite strand from the P_{araBAD} promoter), controlling expression of a heterologous gene(s) in the other direction. In the presence of arabinose, transcription of both heterologous genes from both promoters is induced. However, in the absence of arabinose, transcription of both heterologous genes from both promoters is not induced.

[0789] In one exemplary embodiment of the disclosure, the engineered bacteria of the present disclosure contains a kill-switch having at least the following sequences: a P_{araBAD} promoter operably linked to a heterologous gene encoding a Tetracycline Repressor Protein (TetR), a P_{araC} promoter operably linked to a heterologous gene encoding AraC transcription factor, and a heterologous gene encoding a bacterial toxin operably linked to a promoter which is repressed by the Tetracycline Repressor Protein (P_{TetR}). In the presence of arabinose, the AraC transcription factor activates the P_{araBAD} promoter, which activates transcription of the TetR protein which, in turn, represses transcription of the toxin. In the absence of arabinose, however, AraC suppresses transcription from the P_{araBAD} promoter and no TetR protein is expressed. In this case, expression of the heterologous toxin gene is activated, and the toxin is expressed. The toxin builds up in the engineered bacterial cell, and the engineered bacterial cell is killed. In one embodiment, the *araC* gene encoding the AraC transcription factor is under the control of a constitutive promoter and is therefore constitutively expressed.

[0790] In one embodiment of the disclosure, the engineered bacterial cell further comprises an antitoxin under the control of a constitutive promoter. In this situation, in the presence of arabinose, the toxin is not expressed due to repression by TetR protein, and the

antitoxin protein builds-up in the cell. However, in the absence of arabinose, TetR protein is not expressed, and expression of the toxin is induced. The toxin begins to build-up within the engineered bacterial cell. The engineered bacterial cell is no longer viable once the toxin protein is present at either equal or greater amounts than that of the anti-toxin protein in the cell, and the engineered bacterial cell will be killed by the toxin.

[0791] In another embodiment of the disclosure, the engineered bacterial cell further comprises an antitoxin under the control of the P_{araBAD} promoter. In this situation, in the presence of arabinose, TetR and the anti-toxin are expressed, the anti-toxin builds up in the cell, and the toxin is not expressed due to repression by TetR protein. However, in the absence of arabinose, both the TetR protein and the anti-toxin are not expressed, and expression of the toxin is induced. The toxin begins to build-up within the engineered bacterial cell. The engineered bacterial cell is no longer viable once the toxin protein is expressed, and the engineered bacterial cell will be killed by the toxin.

[0792] In another exemplary embodiment of the disclosure, the engineered bacteria of the present disclosure contains a kill-switch having at least the following sequences: a P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the engineered bacterial cell (and required for survival), and a P_{araC} promoter operably linked to a heterologous gene encoding AraC transcription factor. In the presence of arabinose, the AraC transcription factor activates the P_{araBAD} promoter, which activates transcription of the heterologous gene encoding the essential polypeptide, allowing the engineered bacterial cell to survive. In the absence of arabinose, however, AraC suppresses transcription from the P_{araBAD} promoter and the essential protein required for survival is not expressed. In this case, the engineered bacterial cell dies in the absence of arabinose. In some embodiments, the sequence of P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the engineered bacterial cell can be present in the bacterial cell in conjunction with the TetR/toxin kill-switch system described directly above. In some embodiments, the sequence of P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the engineered bacterial cell can be present in the bacterial cell in conjunction with the TetR/toxin/anto-toxin kill-switch system described directly above.

[0793] In yet other embodiments, the bacteria may comprise a plasmid stability system with a plasmid that produces both a short-lived anti-toxin and a long-lived toxin. In this system, the bacterial cell produces equal amounts of toxin and anti-toxin to neutralize the toxin. However, if/when the cell loses the plasmid, the short-lived anti-toxin begins to decay.

When the anti-toxin decays completely the cell dies as a result of the longer-lived toxin killing it.

[0794] In some embodiments, the engineered bacteria of the present disclosure, for example, bacteria described herein may further comprise the gene(s) encoding the components of any of the above-described kill-switch circuits.

[0795] In any of the above-described embodiments, the bacterial toxin is selected from the group consisting of a lysin, Hok, Fst, TisB, LdrD, Kid, SymE, MazF, FlmA, Ibs, XCV2162, dinJ, CcdB, MazF, ParE, YafO, Zeta, hicB, relB, yhaV, yoeB, chpBK, hipA, microcin B, microcin B17, microcin C, microcin C7-C51, microcin J25, microcin ColV, microcin 24, microcin L, microcin D93, microcin L, microcin E492, microcin H47, microcin I47, microcin M, colicin A, colicin E1, colicin K, colicin N, colicin U, colicin B, colicin Ia, colicin Ib, colicin 5, colicin10, colicin S4, colicin Y, colicin E2, colicin E7, colicin E8, colicin E9, colicin E3, colicin E4, colicin E6; colicin E5, colicin D, colicin M, and cloacin DF13, or a biologically active fragment thereof.

[0796] In any of the above-described embodiments, the anti-toxin is selected from the group consisting of an anti-lysin, Sok, RNAII, IstR, RdlD, Kis, SymR, MazE, FlmB, Sib, ptaRNA1, yafQ, CcdA, MazE, ParD, yafN, Epsilon, HicA, relE, prlF, yefM, chpBI, hipB, MccE, $MccE^{CTD}$, MccF, Cai, ImmE1, Cki, Cni, Cui, Cbi, Iia, Imm, Cfi, Im10, Csi, Cyi, Im2, Im7, Im8, Im9, Im3, Im4, ImmE6, cloacin immunity protein (Cim), ImmE5, ImmD, and Cmi, or a biologically active fragment thereof.

[0797] In one embodiment, the bacterial toxin is bactericidal to the genetically engineered bacterium. In one embodiment, the bacterial toxin is bacteriostatic to the genetically engineered bacterium.

[0798] In one embodiment, the method further comprises administering a second engineered bacterial cell to the subject, wherein the second engineered bacterial cell comprises a heterologous reporter gene operably linked to an inducible promoter that is directly or indirectly induced by an exogenous environmental condition. In one embodiment, the heterologous reporter gene is a fluorescence gene. In one embodiment, the fluorescence gene encodes a green fluorescence protein (GFP). In another embodiment, the method further comprises administering a second engineered bacterial cell to the subject, wherein the second engineered bacterial cell expresses a *lacZ* reporter construct that cleaves a substrate to produce a small molecule that can be detected in urine (see, for example, Danio *et al.*, *Science Translational Medicine,* 7(289):1-12, 2015, the entire contents of which are expressly incorporated herein by reference).

Multiple Mechanisms of Action

[0799] In some embodiments, the bacteria are genetically engineered to include multiple mechanisms of action (MOAs), *e.g.*, circuits producing multiple copies of the same product (*e.g.*, to enhance copy number) or circuits performing multiple different functions. Examples of insertion sites include, but are not limited to, *malE/K, insB/I, araC/BAD, lacZ, dapA, cea,* and other shown in **FIG. 42**. For example, the genetically engineered bacteria may include four copies of a butyrate biosynthetic cassette, four copies of a propionate biosynthetic cassette, or four copies of GLP-1 inserted at four different insertion sites, *e.g.*, *malE/K, insB/I, araC/BAD,* and *lacZ.* Alternatively, the genetically engineered bacteria may include one or more copies of GLP-1 inserted at one or more different insertion sites, *e.g.*, *malE/K, insB/I,* and *lacZ*, one or more copies of a butyrogenic gene cassette inserted at one or more different insertion sites, *e.g., dapA, cea,* and *araC/BAD* and/or one or more copies of a propionaic gene cassette inserted at one or more different insertion sites. Examples of genetically engineered bacteria having different combinations of gene(s) and/or gene cassette(s) are depicted in **Figs. 1-10**.

[0800] In some embodiments, the genetically engineered bacteria further comprise a mutation or deletion in ldhA. In some embodiments, the genetically engineered bacteria further comprise a mutation or deletion in frdA. In some embodiments, the genetically engineered bacteria further comprise a mutation or deletion in Adhe.

[0801] In some embodiments, the bacteria are genetically engineered to include multiple mechanisms of action (MOAs), *e.g.*, circuits producing multiple copies of the same product (*e.g.*, to enhance copy number) or circuits performing multiple different functions. For example, the genetically engineered bacteria may include four copies of the gene, gene(s), or gene cassettes for producing the payload(s) inserted at four different insertion sites. Alternatively, the genetically engineered bacteria may include three copies of the gene, gene(s), or gene cassettes for producing the payload(s) inserted at three different insertion sites and three copies of the gene, gene(s), or gene cassettes for producing the payload(s) inserted at three different insertion sites.

[0802] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) and/or gene cassette(s) selected from gene(s) and/or gene cassette(s):

(1) for the production of propionate, as described herein;

(2) for the production of butyrate, as described herein;

(3) for the production of acetate, as described herein;

(4) for the exxpression of one or more of GLP-1 and/or GLP-1 analogs and/or other GLP-1R agonists, as described herein;

(5) for the expression of one or more bile salt hydrolases including but not limited to, tryptophan and/or tryptophan metabolites, as described herein;

(6) for the expression of one or more transporters, *e.g.* for the import of bile salts and/or bile acids, as described herein;

(7) for the expression of one or more exporters, *e.g.*, for the export of bile salts and/or bile acids, as described herein;

(8) for the production of tryptophan and/or one or more of its metabolites, as described herein, including but not limited to kynurenine pathway metabolites ann/or indole metabolites.

(**9**) for the catabolism of tryptophan and/or one or more of its metabolites, as described herein, including but not limited to kynurenine pathway metabolites.

(10) for the expression of one or more transporters, *e.g.* for the import of metabolites, *e.g.* tryptophan and/or tryptophan metabolites, as described herein;

(**11**) for the expression of one or more exporters, *e.g.* for the export of metabolites, *e.g.* tryptophan and/or tryptophan metabolites, as described herein;

(**12**) for the expression of one or more polypeptides for secretion, including but not limited to, GLP-1 and/or GLP-1 analogs and/or other GLP-1R agonists, bile salt hydrolases, including but not limited to, tryptophan and/or tryptophan metabolites; and tryptophan synthesis and/or tryptophan catabolic enzymes, any of which may be in wild type or in mutated form (*e.g.*, for increased stability or metabolic activity) , as described herein;

(13) for the expression of one or more components of secretion machinery, as described herein

(**12**) for the expression of one more more antibiotic resistance genes, including but not limited to, kanamycin or chloramphenicol resistance

(**13**) one or more kill switches, as described herein.

[0803] (**14**) for the expression and production of one or more effector molecules selected from satiety molecules, anti-inflammatory molecules, gut-barrier healing molecules, and or modulators of inflammation described herein. In addition to any of the one or more gene cassettes described in **(1)-(11)**, the genetically engineered bacteria may also comprise one or more mutations and/or deletions selected from

(**1**) one or more auxotrophies, *e.g.*, deltaThyA and/or dapA;

(**2**) one or more mutations/deletions to increase the flux through a metabolic pathway encoded by one or more genes or gene cassette(s), e.g mutations/deletions in genes in NADH consuming pathways, genes involved in feedback inhibition of a metabolic pathway encoded by the gene(s) or gene cassette(s) genes, as described herein

(**3**) one or more mutations/deletions in one or more genes of the endogenous metabolic pathways, *e.g.*, tryptophan synthesis pathway and/or one or more tryptophan catabolism pathway(s).

(**4**) one or more mutations in membrane proteins to generate a leaky phenotype, as described herein.

[0804] In some embodiments, under conditions where the gene, gene(s), or gene cassettes for producing the payload(s) is expressed, the genetically engineered bacteria of the disclosure produce at least about 1.5-fold, at least about 2-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, at least about 600-fold, at least about 700-fold, at least about 800-fold, at least about 900-fold, at least about 1,000-fold, or at least about 1,500-fold more of the payload(s) as compared to unmodified bacteria of the same subtype under the same conditions.

[0805] In any of the embodiments described in this section or elsewhere in the specification, any of the one or more gene(s) and or gene cassette(s) can be operably linked to a directly or indirectly inducible promoter, such as any of the promoters described herein, *e.g.*, induced by low oxygen or anaerobic conditions, such as those found in the mammalian gut, or induced by a disease or tissue-specific molecule or metabolite, *e.g.*, a molecule or metabolite indicative of liver damage, e.g., as seen in NASH, or metabolic disease, *e.g.* a gut specific metabolite, or induced by inflammation or an inflammatory response, *e.g.*, a gut-specfic metabolite indicative or inflammation or an inflammatory response, or induced by some other metabolite that may or may not be present in the gut, such as arabinose.

[0806] In one embodiment, each component within the gene, or gene cassette, *e.g.* butyrate cassette, can be controlled by the same or different promoter. Ine one embodiment, the order of the gene(s) within the gene cassette can be mixed up and the ribosome binding sites can be replaced to increase or decrease the expression of the payload; *e.g.* rate limiting enzymes.

Induction of Payloads During Strain Culture

[0807] In some embodiments, it is desirable to pre-induce payload or protein of interest expression and/or payload activity prior to administration. Such payload or protein of interest may be an effector intended for secretion or may be an enzyme which catalyzes a metabolic reaction to produce an effector. In other embodiments, the protein of interest is an enzyme which catabolizes a harmful metabolite. In such situations, the strains are pre-loaded with active payload or protein of interest. In such instances, the genetically engineered bacteria of the invention express one or more protein(s) of interest, under conditions provided in bacterial culture during cell growth, expansion, purification, fermentation, and/or manufacture prior to administration *in vivo*. Such culture conditions can be provided in a flask, fermenter or other appropriate culture vessel, *e.g.*, used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. As used herein, the term "bacterial culture" or bacterial cell culture" or "culture" refers to bacterial cells or microorganisms, which are maintained or grown *in vitro* during several production processes, including cell growth, cell expansion, recovery, purification, fermentation, and/or manufacture. As used herein, the term "fermentation" refers to the growth, expansion, and maintenance of bacteria under defined conditions. Fermentation may occur under a number of cell culture conditions, including anaerobic or low oxygen or oxygenated conditions, in the presence of inducers, nutrients, at defined temperatures, and the like.

[0808] Culture conditions are selected to achieve optimal activity and viability of the cells, while maintaining a high cell density (high biomass) yield. A number of cell culture conditions and operating parameters are monitored and adjusted to achieve optimal activity, high yield and high viability, including oxygen levels (*e.g.*, low oxygen, microaerobic, aerobic), temperature of the medium, and nutrients and/or different growth media, chemical and/or nutritional inducers and other components provided in the medium.

[0809] In some embodiments, the one or more protein(s) of interest and are directly or indirectly induced, while the strains is grown up for *in vivo* administration. Without wishing to be bound by theory, pre-induction may boost *in vivo* activity. This is particularly important in proximal regions of the gut which are reached first by the bacteria, *e.g.*, the small intestine. If the bacterial residence time in this compartment is relatively short, the bacteria may pass through the small intestine without reaching full *in vivo* induction capacity. In contrast, if a strain is pre-induced and preloaded, the strains are already fully active, allowing for greater activity more quickly as the bacteria reach the intestine. Ergo, no transit time is "wasted", in which the strain is not optimally active. As the bacteria continue to move through the

intestine, *in vivo* induction occurs under environmental conditions of the gut (*e.g.*, low oxygen, or in the presence of gut metabolites).

[0810] In one embodiment, expression of one or more payload(s), is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of several different proteins of interest is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more payload(s), is driven from the same promoter as a multicistronic message. In one embodiment, expression of one or more payload(s) is driven from the same promoter as two or more separate messages. In one embodiment, expression of one or more payload(s) is driven from the one or more different promoters.

[0811] In some embodiments, the strains are administered without any pre-induction protocols during strain growth prior to *in vivo* administration.

Anaerobic induction

[0812] In some embodiments, cells are induced under anaerobic or low oxygen conditions in culture. In such instances, cells are grown (*e.g.*, for 1.5 to 3 hours) until they have reached a certain OD, *e.g.*, ODs within the range of 0.1 to 10, indicating a certain density *e.g.*, ranging from 1X10^8 to 1X10^11, and exponential growth and are then switched to anaerobic or low oxygen conditions for approximately 3 to 5 hours. In some embodiments, strains are induced under anaerobic or low oxygen conditions, *e.g.* to induce FNR promoter activity and drive expression of one or more payload(s) under the control of one or more FNR promoters.

[0813] In one embodiment, expression of one or more payload(s), is under the control of one or more anaerobic or low oxygen inducible promoter(s), *e.g.*, FNR promoter(s), and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of several different proteins of interest is under the control of one or more anaerobic or low oxygen inducible promoter(s), *e.g.*, FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions.

[0814] In one embodiment, expression of two or more payload(s), is under the control of one or more anaerobic or low oxygen inducible promoter(s), *e.g.*, FNR promoter(s), and is driven from the same promoter in the form of a multicistronic message under anaerobic or low oxygen conditions. In one embodiment, expression of one or more payload(s), is under

the control of one or more anaerobic or low oxygen inducible promoter(s), *e.g.*, FNR promoter(s), and is driven from the same promoter as two or more separate messages under anaerobic or low oxygen conditions. In one embodiment, expression of one or more payload(s) under the control of one or more anaerobic or low oxygen inducible promoter(s), *e.g.*, FNR promoter(s), and is driven from the one or more different promoters under anaerobic or low oxygen conditions.

[0815] Without wishing to be bound by theory, strains that comprise one or more payload(s) under the control of an FNR promoter, may allow expression of payload(s) from these promoters *in vitro*, under anaerobic or low oxygen culture conditions, and *in vivo*, under the low oxygen conditions found in the gut and/or liver.

[0816] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers can be induced under anaerobic or low oxygen conditions in the presence of the chemical and/or nutritional inducer. In some embodiments, strains may comprise a combination of gene sequence(s), some of which are under control of FNR promoters and others which are under control of promoters induced by chemical and/or nutritional inducers. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of one or more FNR promoter(s) and one or more payload gene sequence(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. In some embodiments, strains may comprise one or more payload gene sequence(s) and/or under the control of one or more FNR promoter(s), and one or more payload gene sequence(s) under the control of a one or more constitutive promoter(s) described herein. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of an FNR promoter and one or more payload gene sequence(s) under the control of a one or more thermoregulated promoter(s) described herein.

[0817] In one embodiment, expression of one or more payload gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, the chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one embodiment, the

chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0818] In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or more separate messages under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promoters under anaerobic and/or low oxygen conditions.

[0819] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers, under anaerobic or low oxygen conditions. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers. In some embodiments, the strains comprise gene sequence(s) under the control of a a third inducible promoter, *e.g.*, an anaerobic/low oxygen promoter, *e.g.*, FNR promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a chemically induced promoter or a low oxygen promoter and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a FNR promoter and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a chemically induced and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of an FNR promoter and one or more payload gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or

nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria strains further comprise payload sequence(s) under the control of one or more constitutive promoter(s) active under low oxygen conditions.

Aerobic induction

[0820] In some embodiments, it is desirable to prepare, pre-load and pre-induce the strains under aerobic conditions. This allows more efficient growth and viability, and, in some cases, reduces the build-up of toxic metabolites. In such instances, cells are grown (*e.g.*, for 1.5 to 3 hours) until they have reached a certain OD, *e.g.*, ODs within the range of 0.1 to 10, indicating a certain density *e.g.*, ranging from 1X10^8 to 1X10^11, and exponential growth and are then induced through the addition of the inducer or through other means, such as shift to a permissive temperature, for approximately 3 to 5 hours.

[0821] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art can be induced under aerobic conditions in the presence of the chemical and/or nutritional inducer during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more payload(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions.

[0822] In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message under aerobic conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or more separate messages under aerobic conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promoters under aerobic conditions.

[0823] In one embodiment, the chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one

embodiment, the chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0824] In some embodiments, promoters regulated by temperature are induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more payload(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions.

[0825] In one embodiment, expression of one or more payload(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is driven from the same promoter in the form of a multicistronic message under aerobic conditions. In one embodiment, expression of one or more payload(s) is driven directly or indirectly by one or more thermoregulated promoter(s)and is driven from the same promoter as two or more separate messages under aerobic conditions. In one embodiment, expression of one or more payload(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is driven from the one or more different promoters under aerobic conditions.

[0826] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced under aerobic conditions. In some embodiments, a strain comprises three or more different promoters which are induced under aerobic culture conditions.

[0827] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.* a chemically inducible promoter, and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter under aerobic culture conditions. In some embodiments two or more chemically induced promoter gene sequence(s) are combined with a thermoregulated construct described herein. In one embodiment, the chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one

embodiment, the chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0828] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a FNR promoter and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a chemically induced and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of an FNR promoter and one or more payload gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria strains further comprise payload sequences under the control of one or more constitutive promoter(s) active under aerobic conditions.

[0829] In some embodiments, genetically engineered strains comprise gene sequence(s) which are induced under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut and/or liver. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut liver.

[0830] In some embodiments, genetically engineered strains comprise gene sequence(s), which are arabinose inducible under aerobic culture conditions. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut and/or liver.

[0831] In some embodiments, genetically engineered strains comprise gene sequence(s), which are IPTG inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut, or under certain conditions, e.g., associated with liver damage or inflammation . In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut liver.

[0832] In some embodiments, genetically engineered strains comprise gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises sequence(s) which are IPTG inducible under

aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene payload sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut and/or liver.

[0833] As evident from the above non-limiting examples, genetically engineered strains comprise inducible gene sequence(s) which can be induced numerous combinations. For example, rhamnose or tetracycline can be used as an inducer with the appropriate promoters in addition or in lieu of arabinose and/or IPTG or with thermoregulation. Additionally, such bacterial strains can also be induced with the chemical and/or nutritional inducers under anaerobic conditions.

Microaerobic Induction

[0834] In some embodiments, viability, growth, and activity are optimized by pre-inducing the bacterial strain under microaerobic conditions. In some embodiments, microaerobic conditions are best suited to "strike a balance" between optimal growth, activity and viability conditions and optimal conditions for induction; in particular, if the expression of the one or more payload(s) are driven by an anaerobic and/or low oxygen promoter, *e.g.*, a FNR promoter. In such instances, cells are grown (*e.g.*, for 1.5 to 3 hours) until they have reached a certain OD, *e.g.*, ODs within the range of 0.1 to 10, indicating a certain density *e.g.*, ranging from 1X10^8 to 1X10^11, and exponential growth and are then induced through the addition of the inducer or through other means, such as shift to at a permissive temperature, for approximately 3 to 5 hours.

[0835] In one embodiment, expression of one or more payload(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions.

[0836] In one embodiment, expression of one or more payload(s), is under the control of one or more FNR promoter(s) and is driven from the same promoter in the form of a multicistronic message under microaerobic conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more FNR promoter(s) and is driven from the same promoter as two or more separate messages under microaerobic conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more FNR promoter(s) and is driven from the one or more different promoters under microaerobic conditions.

[0837] Without wishing to be bound by theory, strains that comprise one or more payload(s) under the control of an FNR promoter, may allow expression of payload(s) from these promoters *in vitro*, under microaerobic culture conditions, and *in vivo*, under the low oxygen conditions found in the gut and/or liver.

[0838] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers can be induced under microaerobic conditions in the presence of the chemical and/or nutritional inducer. In particular, strains may comprise a combination of gene sequence(s), some of which are under control of FNR promoters and others which are under control of promoters induced by chemical and/or nutritional inducers. In some embodiments, strains may comprise one or more payload gene sequence(s) sequence(s) under the control of one or more FNR promoter(s) and one or more payload gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of one or more FNR promoter(s), and one or more payload gene sequence(s) under the control of a one or more constitutive promoter(s) described herein. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of an FNR promoter and one or more payload gene sequence(s) under the control of a one or more thermoregulated promoter(s) described herein.

[0839] In one embodiment, expression of one or more payload(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions.

[0840] In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message under microaerobic conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or more separate messages under microaerobic conditions. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promoters under microaerobic conditions.

[0841] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers, under microaerobic conditions. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers. In some embodiments, the strains comprise gene sequence(s) under the control of a third inducible promoter, *e.g.*, an anaerobic/low oxygen promoter or microaerobic promoter, *e.g.*, FNR promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a chemically induced promoter or a low oxygen or microaerobic promoter and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a FNR promoter and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a chemically induced and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of an FNR promoter and one or more payload gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria strains further comprise payload under the control of one or more constitutive promoter(s) active under low oxygen conditions.

Induction of Strains using Phasing, Pulsing and/or Cycling

[0842] In some embodiments, cycling, phasing, or pulsing techniques are emplyed during cell growth, expansion, recovery, purification, fermentation, and/or manufacture to efficienty induce and grow the strains prior to *in vivo* administration. This method is used to "strike a balance" between optimal growth, activity, cell health, and viability conditions and optimal conditions for induction; in particular, if growth, cell health or viability are negatively affected under inducing conditions. In such instances, cells are grown (*e.g.*, for 1.5 to 3 hours) in a first phase or cycle until they have reached a certain OD, *e.g.*, ODs within the

range of 0.1 to 10, indicating a certain density *e.g.*, ranging from 1X10^8 to 1X10^11, and are then induced through the addition of the inducer or through other means, such as shift to a permissive temperature (if a promoter is thermoregulated), or change in oxygen levels (*e.g.*, reduction of oxygen level in the case of induction of an FNR promoter driven construct) for approximately 3 to 5 hours. In a second phase or cycle, conditions are brought back to the original conditions which support optimal growth, cell health and viability. Alternatively, if a chemical and/or nutritional inducer is used, then the culture can be spiked with a second dose of the inducer in the second phase or cycle.

[0843] In some embodiments, two cycles of optimal conditions and inducing conditions are employed (i.e, growth, induction, recovery and growth, induction). In some embodiments, three cycles of optimal conditions and inducing conditions are employed. In some embodiments, four or more cycles of optimal conditions and inducing conditions are employed. In a non-liming example, such cycling and/or phasing is used for induction under anaerobic and/or low oxygen conditions (*e.g.*, induction of FNR promoters). In one embodiment, cells are grown to the optimal density and then induced under anaerobic and/or low oxygen conditions. Before growth and/or viability are negatively impacted due to stressful induction conditions, cells are returned to oxygenated conditions to recover, after which they are then returned to inducing anaerobic and/or low oxygen conditions for a second time. In some embodiments, these cycles are repeated as needed.

[0844] In some embodiments, growing cultures are spiked once with the chemical and/or nutritional inducer. In some embodiments, growing cultures are spiked twice with the chemical and/or nutritional inducer. In some embodiments, growing cultures are spiked three or more times with the chemical and/or nutritional inducer. In a non-limiting example, cells are first grown under optimal growth conditions up to a certain density, *e.g.*, for 1.5 to 3 hour) to reached an of 0.1 to 10, until the cells are at a density ranging from 1X10^8 to 1X10^11. Then the chemical inducer, *e.g.*, arabinose or IPTG, is added to the culture. After 3 to 5 hours, an additional dose of the inducer is added to re-initiate the induction. Spiking can be repeated as needed.

[0845] In some embodiments, phasing or cycling changes in temperature in the culture. In another embodiment, adjustment of temperature may be used to improve the activity of a payload. For example, lowering the temperature during culture may improve the proper folding of the payload. In such instances, cells are first grown at a temperature optimal for growth (*e.g.*, 37 C). In some embodiments, the cells are then induced, *e.g.*, by a chemical inducer, to express the payload. Concurrently or after a set amount of induction time, the

temperature in the media is lowered, *e.g.*, between 25 and 35 C, to allow improved folding of the expressed payload .

[0846] In some embodiments, payload(s) are under the control of different inducible promoters, for example two different chemical inducers. In other embodiments, the payload is induced under low oxygen conditions or microaerobic conditions and a second payload is induced by a chemical inducer.

[0847] In one embodiment, expression of one or more payload(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture by using phasing or cycling or pulsing or spiking techniques.

[0848] In one embodiment, expression of one or more payload(s), is under the control of one or more FNR promoter(s) and is driven from the same promoter in the form of a multicistronic message through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more payload(s), is under the control of one or more FNR promoter(s) and is driven from the same promoter as two or more separate messages through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more payload(s), is under the control of one or more FNR promoter(s) and is driven from the one or more different promoters through the employment of phasing or cycling or pulsing or spiking techniques.

[0849] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers can be induced through the employment of phasing or cycling or pulsing or spiking techniques in the presence of the chemical and/or nutritional inducer. In particular, strains may comprise a combination of gene sequence(s), some of which are under control of FNR promoters and others which are under control of promoters induced by chemical and/or nutritional inducers. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of one or more FNR promoter(s) and one or more payload gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of one or more FNR promoter(s), and one or more payload gene sequence(s) under the control of a one or more constitutive promoter(s) described herein and are induced through the employment of phasing or cycling or pulsing or spiking techniques. In some

embodiments, strains may comprise one or more payload gene sequence(s) under the control of an FNR promoter and one or more payload gene sequence(s) under the control of a one or more thermoregulated promoter(s) described herein, and are induced through the employment of phasing or cycling or pulsing or spiking techniques.

[0850] Any of the strains described herein can be grown through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more payload(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions.

[0851] In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message and which are induced through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or more separate messages and is grown through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more payload(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promoters, all of which are induced through the employment of phasing or cycling or pulsing or spiking techniques.

[0852] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers, through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers through the employment of phasing or cycling or pulsing or spiking techniques. In some embodiments, the strains comprise gene sequence(s) under the control of a a third inducible promoter, *e.g.*, an anaerobic/low oxygen promoter, *e.g.*, FNR promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a chemically induced promoter or a low oxygen promoter and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s),

some of which are under control of a first inducible promoter, *e.g.*, a FNR promoter and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, *e.g.*, a chemically induced and others which are under control of a second inducible promoter, *e.g.* a temperature sensitive promoter. In some embodiments, strains may comprise one or more payload gene sequence(s) under the control of an FNR promoter and one or more payload gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria strains further comprise payload sequence(s) under the control of one or more constitutive promoter(s) active under low oxygen conditions. Any of the strains described in these embodiments may be induced through the employment of phasing or cycling or pulsing or spiking techniques.

Aerobic induction of the FNR promoter

[0853] FNRS24Y is a mutated form of FNR which is more resistant to inactivation by oxygen, and therefore can activate FNR promoters under aerobic conditions (see *e.g.*, Jervis AJ The O2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe-4S] to [2Fe-2S] conversion, Proc Natl Acad Sci U S A. 2009 Mar 24;106(12):4659-64, the contents of which is herein incorporated by reference in its entirety). In some embodiments, an oxygen bypass system shown and described in **FIG. 85A** is used. In this oxygen bypass system, FNRS24Y is induced by addition of arabinose and then drives the expression of the protein of interest (*e.g.*, one or more effector(s) described herein) by binding and activating the FNR promoter under aerobic conditions. Thus, strains can be grown, produced or manufactured efficiently under aerobic conditions, while being effectively pre-induced and pre-loaded, as the system takes advantage of the strong FNR promoter resulting in of high levels of expression of the protein of interest. This system does not interfere with or compromise *in vivo* activation, since the mutated FNRS24Y is no longer expressed in the absence of arabinose, and wild type FNR then binds to the FNR promoter and drives expression of the protein of interest, *e.g.*, one or more effector(s) described herein.

[0854] In some embodiments, FNRS24Y is expressed during aerobic culture growth and induces a gene of interest. In other embodiments described herein, a second payload expression can also be induced aerobically, *e.g.*, by arabinose. In a non-limiting example, a

protein of interest and FNRS24Y can in some embodiments be induced simultaneously, *e.g.*, from an arabinose inducible promoter. In some embodiments, FNRS24Y and the protein of interest (*e.g.*, one or more effector(s) described herein) are transcribed as a bicistronic message whose expression is driven by an arabinose promoter. In some embodiments, FNRS24Y is knocked into the arabinose operon, allowing expression to be driven from the endogenous Para promoter.

[0855] In some embodiments, a LacI promoter and IPTG induction are used in this system (in lieu of Para and arabinose induction). In some embodiments, a rhamnose inducible promoter is used in this system. In some embodiments, a temperature sensitive promoter is used to drive expression of FNRS24Y.

Secretion

[0856] In any of the embodiments described herein, in which the genetically engineered organism, *e.g.*, engineered bacteria or engineered virus, produces a protein, polypeptide, peptide, or other payload, DNA, RNA, small molecule or other molecule intended to be secreted from the microorganism, the engineered microorganism may comprise a secretion mechanism and corresponding gene sequence(s) encoding the secretion system.

[0857] In some embodiments, the genetically engineered bacteria further comprise a native secretion mechanism or non-native secretion mechanism that is capable of secreting the payload from the bacterial cytoplasm in the extracellular environment. Many bacteria have evolved sophisticated secretion systems to transport substrates across the bacterial cell envelope. Substrates, such as small molecules, proteins, and DNA, may be released into the extracellular space or periplasm (such as the gut lumen or other space), injected into a target cell, or associated with the bacterial membrane.

[0858] In Gram-negative bacteria, secretion machineries may span one or both of the inner and outer membranes. In some embodiments, the genetically engineered bacteria further comprise a non-native double membrane-spanning secretion system. Double membrane-spanning secretion systems include, but are not limited to, the type I secretion system (T1SS), the type II secretion system (T2SS), the type III secretion system (T3SS), the type IV secretion system (T4SS), the type VI secretion system (T6SS), and the resistance-nodulation-division (RND) family of multi-drug efflux pumps (Pugsley 1993; Gerlach et al., 2007; Collinson et al., 2015; Costa et al., 2015; Reeves et al., 2015; WO2014138324A1, incorporated herein by reference). Examples of such secretion systems are shown in **Fig. 63, Fig. 64, Fig. 65, Fig. 66,** and **Fig. 67, Fig. 78A, Fig. 78B, Fig. 77A, Fig. 77B, Fig. 77C**.

Mycobacteria, which have a Gram-negative-like cell envelope, may also encode a type VII secretion system (T7SS) (Stanley et al., 2003). With the exception of the T2SS, double membrane-spanning secretions generally transport substrates from the bacterial cytoplasm directly into the extracellular space or into the target cell. In contrast, the T2SS and secretion systems that span only the outer membrane may use a two-step mechanism, wherein substrates are first translocated to the periplasm by inner membrane-spanning transporters, and then transferred to the outer membrane or secreted into the extracellular space. Outer membrane-spanning secretion systems include, but are not limited to, the type V secretion or autotransporter system or autosecreter system (T5SS), the curli secretion system, and the chaperone-usher pathway for pili assembly (Saier, 2006; Costa et al., 2015).

[0859] In some embodiments in which the one or more proteins of interest or therapeutic proteins are secreted or exported from the microorganism, the engineered microorganism comprises gene sequence(s) that includes a secretion tag. In some embodiments, the one or more proteins of interest or therapeutic proteins include a "secretion tag" of either RNA or peptide origin to direct the one or more proteins of interest or therapeutic proteins to specific secretion systems. For example, a secretion tag for the Type I Hemolysin secretion system is encoded in the C-terminal 53 amino acids of the alpha hemolysin protein (HlyA).

[0860] In some embodiments, a Hemolysin-based Secretion System is used to secrete the molecule of interest, *e.g.*, therapeutic peptide. Type I Secretion systems offer the advantage of translocating their passenger peptide directly from the cytoplasm to the extracellular space, obviating the two-step process of other secretion types. **Fig. 65** shows the alpha-hemolysin (HlyA) of uropathogenic *Escherichia coli*. This pathway uses HlyB, an ATP-binding cassette transporter; HlyD, a membrane fusion protein; and TolC, an outer membrane protein. The assembly of these three proteins forms a channel through both the inner and outer membranes. HlyB inserts into inner membrane to form a pore, HlyD aligns HlyB with TolC (outer membrane pore) thereby forming a channel through inner and outer membrane. Natively, this channel is used to secrete HlyA, however, to secrete the therapeutic peptide of the present disclosure, the secretion signal-containing C-terminal portion of HlyA is fused to the C-terminal portion of a therapeutic peptide (star) to mediate secretion of this peptide. The C-terminal secretion tag can be removed by either an autocatalytic or protease-catalyzed *e.g.*, OmpT cleavage thereby releasing the one or more proteins of interest or therapeutic proteins into the extracellular milieu. In some embodiments the one or more proteins of interest or therapeutic proteins contain expressed as fusion protein with the 53

amino acids of the C termini of alpha-hemolysin (hlyA) of *E. coli* CFT073 (C terminal secretion tag).

[0861] In some embodiments, a Type V Autotransporter Secretion System is used to secrete the molecule of interest, *e.g.*, therapeutic peptide. The Type V Auto-secretion System utilizes an N-terminal Sec-dependent peptide tag (inner membrane) and C-terminal tag (outer-membrane). This system uses the Sec-system to get from the cytoplasm to the periplasm. The C-terminal tag then inserts into the outer membrane forming a pore through which the "passenger protein" threads through. Due to the simplicity of the machinery and capacity to handle relatively large protein fluxes, the Type V secretion system is attractive for the extracellular production of recombinant proteins. As shown in **Fig. 64**, a therapeutic peptide (star) can be fused to an N-terminal secretion signal, a linker, and the beta-domain of an autotransporter. The N-terminal, Sec-dependent signal sequence directs the protein to the SecA-YEG machinery which moves the protein across the inner membrane into the periplasm, followed by subsequent cleavage of the signal sequence. The Beta-domain is recruited to the Bam complex ('Beta-barrel assembly machinery') where the beta-domain is folded and inserted into the outer membrane as a beta-barrel structure. The therapeutic peptide is threaded through the hollow pore of the beta-barrel structure ahead of the linker sequence. Once across the outer membrane, the passenger is released from the membrane-embedded C-terminal tag by either an autocatalytic, intein-like mechanism (left side of Bam complex) or via a membrane-bound protease (black scissors; right side of Bam complex) (*i.e.*, OmpT). For example, a membrane-associated peptidase to a complimentary protease cut site in the linker. Thus, in some embodiments, the secreted molecule, such as a heterologous protein or peptide comprises an N-terminal secretion signal, a linker, and beta-domain of an autotransporter so as to allow the molecule to be secreted from the bacteria.

[0862] The N-terminal tag is removed by the Sec system. Thus, in some embodiments, the secretion system is able to remove this tag before secreting the one or more proteins of interest or therapeutic proteins, from the engineered bacteria. In the Type V auto-secretion-mediated secretion the N-terminal peptide secretion tag is removed upon translocation of the "passenger" peptide from the cytoplasm into the periplasmic compartment by the native Sec system. Further, once the auto-secretor is translocated across the outer membrane the C-terminal secretion tag can be removed by either an autocatalytic or protease-catalyzed *e.g.*, OmpT cleavage thereby releasing the payload(s) into the extracellular milieu.

[0863] In some embodiments, the genetically engineered bacteria of the invention comprise a type III or a type III-like secretion system (T3SS) from Shigella, Salmonella, *E. coli*, Bivrio, Burkholderia, Yersinia, Chlamydia, or Pseudomonas. The traditional T3SS is capable of transporting a protein from the bacterial cytoplasm to the host cytoplasm through a needle complex. In the Type III traditional secretion system, the basal body closely resembles the flagella, however, instead of a "tail"/whip, the traditional T3SS has a syringe to inject the passenger proteins into host cells. The secretion tag is encoded by an N-terminal peptide (lengths vary and there are several different tags, see PCT/US14/020972). The N-terminal tag is not removed from the polypeptides in this secretion system.

[0864] The T3SS may be modified to secrete the molecule from the bacterial cytoplasm, but not inject the molecule into the host cytoplasm. Thus, the molecule is secreted into the gut lumen or other extracellular space. In some embodiments, the genetically engineered bacteria comprise said modified T3SS and are capable of secreting the molecule of interest from the bacterial cytoplasm. In some embodiments, the secreted molecule, such as a heterologous protein or peptide comprises a type III secretion sequence that allows the molecule of interest to be secreted from the bacteria.

[0865] As used herein, a "heterologous" gene or "heterologous sequence" refers to a nucleotide sequence that is not normally found in a given cell in nature. As used herein, a heterologous sequence encompasses a nucleic acid sequence that is exogenously introduced into a given cell and can be a native sequence (naturally found or expressed in the cell) or non-native sequence (not naturally found or expressed in the cell) and can be a natural or wild-type sequence or a variant, non-natural, or synthetic sequence. "Heterologous gene" includes a native gene, or fragment thereof, that has been introduced into the host cell in a form that is different from the corresponding native gene. For example, a heterologous gene may include a native coding sequence that is a portion of a chimeric gene to include non-native regulatory regions that is reintroduced into the host cell. A heterologous gene may also include a native gene, or fragment thereof, introduced into a non-native host cell. Thus, a heterologous gene may be foreign or native to the recipient cell; a nucleic acid sequence that is naturally found in a given cell but expresses an unnatural amount of the nucleic acid and/or the polypeptide which it encodes; and/or two or more nucleic acid sequences that are not found in the same relationship to each other in nature. As used herein, the term "endogenous gene" refers to a native gene in its natural location in the genome of an organism. As used herein, the term "transgene" refers to a gene that has been introduced into the host organism, *e.g.*, host bacterial cell, genome.

[0866] In the Flagellar modified Type III Secretion, the tag is encoded in 5'untranslated region of the mRNA and thus there is no peptide tag to cleave/remove. This modified system does not contain the "syringe" portion and instead uses the basal body of the flagella structure as the pore to translocate across both membranes and out through the forming flagella. If the fliC/fliD genes (encoding the flagella "tail"/whip) are disrupted the flagella cannot fully form and this promotes overall secretion. In some embodiments, the tail portion can be removed entirely.

[0867] In some embodiments, a flagellar type III secretion pathway is used to secrete the molecule of interest. In some embodiments, an incomplete flagellum is used to secrete a therapeutic peptide of interest by recombinantly fusing the peptide to an N-terminal flagellar secretion signal of a native flagellar component. In this manner, the intracellularly expressed chimeric peptide can be mobilized across the inner and outer membranes into the surrounding host environment.

[0868] For example, a modified flagellar type III secretion apparatus in which untranslated DNA fragment upstream of the gene fliC (encoding flagellin), *e.g.*, a 173-bp region, is fused to the gene encoding the heterologous protein or peptide can be used to secrete polypeptides of interest (See, *e.g.*, Majander et al., Extracellular secretion of polypeptides using a modified *Escherichia coli* flagellar secretion apparatus. Nat Biotechnol. 2005 Apr;23(4):475-81). In some cases, the untranslated region from the fliC loci may not be sufficient to mediate translocation of the passenger peptide through the flagella. Here it may be necessary to extend the N-terminal signal into the amino acid coding sequence of FliC, for example, by using the 173 bp of untranslated region along with the first 20 amino acids of FliC (see, *e.g.*, Duan et al., Secretion of Insulinotropic Proteins by Commensal Bacteria: Rewiring the Gut To Treat Diabetes, Appl. Environ. Microbiol. December 2008 vol. 74 no. 23 7437-7438).

[0869] In alternate embodiments, the genetically engineered bacteria further comprise a non-native single membrane-spanning secretion system. Single membrane-spanning transporters may act as a component of a secretion system, or may export substrates independently. Such transporters include, but are not limited to, ATP-binding cassette translocases, flagellum/virulence-related translocases, conjugation-related translocases, the general secretory system (*e.g.*, the SecYEG complex in *E. coli*), the accessory secretory system in mycobacteria and several types of Gram-positive bacteria (*e.g.*, Bacillus anthracis, Lactobacillus johnsonii, Corynebacterium glutamicum, Streptococcus gordonii, Staphylococcus aureus), and the twin-arginine translocation (TAT) system (Saier, 2006;

Rigel and Braunstein, 2008; Albiniak et al., 2013). It is known that the general secretory and TAT systems can both export substrates with cleavable N-terminal signal peptides into the periplasm, and have been explored in the context of biopharmaceutical production. The TAT system may offer particular advantages, however, in that it is able to transport folded substrates, thus eliminating the potential for premature or incorrect folding. In certain embodiments, the genetically engineered bacteria comprise a TAT or a TAT-like system and are capable of secreting the payload of interest from the bacterial cytoplasm. One of ordinary skill in the art would appreciate that the secretion systems disclosed herein may be modified to act in different species, strains, and subtypes of bacteria, and/or adapted to deliver different payloads.

[0870] In order to translocate a protein, *e.g.*, therapeutic polypeptide, to the extracellular space, the polypeptide must first be translated intracellularly, mobilized across the inner membrane and finally mobilized across the outer membrane. Many effector proteins (*e.g.*, therapeutic polypeptides) – particularly those of eukaryotic origin – contain disulphide bonds to stabilize the tertiary and quaternary structures. While these bonds are capable of correctly forming in the oxidizing periplasmic compartment with the help of periplasmic chaperones, in order to translocate the polypeptide across the outer membrane the disulphide bonds must be reduced and the protein unfolded again.

[0871] One way to secrete properly folded proteins in gram-negative bacteria– particularly those requiring disulphide bonds – is to target the reducing-environment periplasm in conjunction with a destabilizing outer membrane. In this manner the protein is mobilized into the oxidizing environment and allowed to fold properly. In contrast to orchestrated extracellular secretion systems, the protein is then able to escape the periplasmic space in a correctly folded form by membrane leakage. These "leaky" gram-negative mutants are therefore capable of secreting bioactive, properly disulphide-bonded polypeptides. In some embodiments, the genetically engineered bacteria have a "leaky" or de-stabilized outer membrane. Destabilizing the bacterial outer membrane to induce leakiness can be accomplished by deleting or mutagenizing genes responsible for tethering the outer membrane to the rigid peptidoglycan skeleton, including for example, lpp, ompC, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl. Lpp is the most abundant polypeptide in the bacterial cell existing at ~500,000 copies per cell and functions as the primary 'staple' of the bacterial cell wall to the peptidoglycan. 1. Silhavy, T. J., Kahne, D. & Walker, S. The bacterial cell envelope. Cold Spring Harb Perspect Biol 2, a000414 (2010). TolA-PAL and OmpA complexes function similarly to Lpp and are other deletion targets to generate a leaky

phenotype. Additionally, leaky phenotypes have been observed when periplasmic proteases are inactivated. The periplasm is very densely packed with protein and therefore encode several periplasmic proteins to facilitate protein turnover. Removal of periplasmic proteases such as degS, degP or nlpI can induce leaky phenotypes by promoting an excessive build-up of periplasmic protein. Mutation of the proteases can also preserve the effector polypeptide by preventing targeted degradation by these proteases. Moreover, a combination of these mutations may synergistically enhance the leaky phenotype of the cell without major sacrifices in cell viability. Thus, in some embodiments, the engineered bacteria have one or more deleted or mutated membrane genes. In some embodiments, the engineered bacteria have a deleted or mutated lpp gene. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from ompA, ompA, and ompF genes. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from tolA, tolB, and pal genes. in some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes. In some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes selected from degS, degP, and nlpl. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from lpp, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl genes.

[0872] To minimize disturbances to cell viability, the leaky phenotype can be made inducible by placing one or more membrane or periplasmic protease genes, *e.g.*, selected from lpp, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl, under the control of an inducible promoter. For example, expression of lpp or other cell wall stability protein or periplasmic protease can be repressed in conditions where the therapeutic polypeptide needs to be delivered (secreted). For instance, under inducing conditions a transcriptional repressor protein or a designed antisense RNA can be expressed which reduces transcription or translation of a target membrane or periplasmic protease gene. Conversely, overexpression of certain peptides can result in a destabilized phenotype, *e.g.*, overexpression of colicins or the third topological domain of TolA, wherein peptide overexpression can be induced in conditions in which the therapeutic polypeptide needs to be delivered (secreted). These sorts of strategies would decouple the fragile, leaky phenotypes from biomass production. Thus, in some embodiments, the engineered bacteria have one or more membrane and/or periplasmic protease genes under the control of an inducible promoter.

[0873] **Table 42 and Table 43** below lists secretion systems for Gram positive bacteria and Gram negative bacteria.

Table 42 Secretion systems for gram positive bacteria

Bacterial Strain	Relevant Secretion System
C. novyi-NT (Gram+)	Sec pathway Twin- arginine (TAT) pathway
C. butryicum (Gram+)	Sec pathway Twin- arginine (TAT) pathway
Listeria monocytogenes (Gram +)	Sec pathway Twin- arginine (TAT) pathway

Table 43. Secretion Systems for Gram negative bacteria

Protein secretary pathways (SP) in gram-negative bacteria and their descendants							
Type (Abbreviation)	Name	TC#[2]	Bacteria	Archaea	Eukarya	# Proteins/System	Energy Source
IMPS – Gram-negative bacterial inner membrane channel-forming translocases							
ABC (SIP)	ATP binding cassette translocase	3.A.1	+	+	+	3-4	ATP
SEC (IISP)	General secretory translocase	3.A.5	+	+	+	~12	GTP OR ATP + PMF
Fla/Path (IIISP)	Flagellum/virulence-related translocase	3.A.6	+	-	-	>10	ATP
Conj (IVSP)	Conjugation-related translocase	3.A.7	+	-	-	>10	ATP
Tat (IISP)	Twin-arginine targeting translocase	2.A.64	+	+	+ (chloroplasts)	2-4	PMF
Oxa1 (YidC)	Cytochrome oxidase biogenesis family	2.A.9	+	+	+ (mitochondria chloroplasts)	1	None or PMF
MscL	Large conductance mechanosensitive channel family	1.A.22	+	+	+	1	None

Holins	Holin functional superfamily	1.E.1 •21	+	-	-	1	None
Eukaryotic Organelles							
MPT	Mitochondrial protein translocase	3.A.B	-	-	+ (mitochondrial)	>20	ATP
CEPT	Chloroplast envelope protein translocase	3.A.9	(+)	-	+ (chloroplasts)	≥3	GTP
Bcl-2	Eukaryotic Bcl-2 family (programmed cell death)	1.A.21	-	-	+	1?	None
Gram-negative bacterial outer membrane channel-forming translocases							
MTB (IISP)	Main terminal branch of the general secretory translocase	3.A.15	+[b]	-	-	~14	ATP; PMF
FUP AT-1	Fimbrial usher protein Autotransporter-1	1.B.11 1.B.12	+[b] +[b]	-	- -	1 1	None None
AT-2 OMF (ISP)	Autotransporter-2	1.B.40 1.B.17	+[b] +[b]	-	- +(?)	1 1	None None
TPS Secretin (IISP and IISP)		1.B.20 1.B.22	+ +[b]	-	+ -	1 1	None None
OmpIP	Outer membrane insertion porin	1.B.33	+	-	+ (mitochondria; chloroplasts)	≥4	None ?

[0874] The above tables for gram positive and gram negative bacteria list secretion systems that can be used to secrete polypeptides and other molecules from the engineered bacteria, which are reviewed in Milton H. Saier, Jr. Microbe / Volume 1, Number 9, 2006 "Protein Secretion Systems in Gram-Negative Bacteria Gram-negative bacteria possess many protein secretion-membrane insertion systems that apparently evolved independently", the contents of which is herein incorporated by reference in its entirety.

[0875] In some embodiments, the genetically engineered bacterial comprise a native or non-native secretion system described herein for the secretion of a payload, *e.g.*, a cytokine, antibody (*e.g.*, scFv), metabolic enzyme, *e.g.*, kynureninase, an others described herein.

Table 44. Polypeptide Sequences of exemplary secretion tags

Description	**Sequence**
PhoA **SEQ ID NO: 1000**	MKQSTIALALLPLLFTPVTKA
PhoA **SEQ ID NO: 1001**	KQSTIALALLPLLFTPVTKA
OmpF **SEQ ID NO: 1002**	MMKRNILAVIVPALLVAGTANA
cvaC **SEQ ID NO: 1003**	MRTLTLNELDSVSGG
TorA **SEQ ID NO: 1004**	MNNNDLFQASRRRFLAQLGGLTVAGMLGTSLLTPRRATAAQA A
fdnG **SEQ ID NO: 1005**	MDVSRRQFFKICAGGMAGTTVAALGFAPKQALA
dmsA **SEQ ID NO: 1006**	MKTKIPDAVLAAEVSRRGLVKTTAIGGLAMASSALTLPFSRIAH A
PelB **SEQ ID NO: 1007**	KYLLPTAAAGLLLLAAQPAMA
HlyA secretion signal **SEQ ID NO: 1008**	LNPLINEISKIISAAGNFDVKEERAAASLLQLSGNASDFSYGRNSI TLTASA
HlyA secretion signal **SEQ ID NO: 1009**	CTTAATCCATTAATTAATGAAATCAGCAAAATCATTTCAGCT GCAGGTAATTTTGATGTTAAAGAGGAAAGAGCTGCAGCTTC TTTATTGCAGTTGTCCGGTAATGCCAGTGATTTTTCATATGG ACGGAACTCAATAACTTTGACAGCATCAGCATAA.

Table B. Additionals secretion tag sequences (native to E coli.)

Description	Sequences
ECOLIN_05715 Secretion signal **SEQ ID NO: 1010**	MKRHLNTSYRLVWNHITGAFVVASELARARGKRAGVA VALSLAAATSLPALA

ECOLIN_16495 Secretion signal **SEQ ID NO: 1011**	MFWRDMTLSVWRKKTTGLKTKKRLLALVLAAALCSSPV WA
ECOLIN_19410 Secretion signal **SEQ ID NO:1012**	MGYKMNISSLRKAFIFMGAVAALSLVNAQSALA
ECOLIN_19880 Secretion signal **SEQ ID NO:1013**	MNKIFKVIWNPATGSYTVASETAKSRGKKSGRSKLLISAL VAGGLLSSFGASA

[0876] In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that encodes a polypeptide which is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of **SEQ ID NO: 1000, SEQ ID NO: 1001, SEQ ID NO: 1002, SEQ ID NO: 1003, SEQ ID NO: 1004, SEQ ID NO: 1005, SEQ ID NO: 1006, SEQ ID NO: 1007, SEQ ID NO: 1008, and/or SEQ ID NO: 1009, SEQ ID NO: 1010, SEQ ID NO: 1011, SEQ ID NO: 1012, SEQ ID NO: 1013.**

[0877] Any secretion tag or secretion system can be combined with any cytokine described herein, and can be used to generate a construct (plasmid based or integrated) which is driven by an directly or indirectly inducible or constitutive promoter described herein. In some embodiments, the secretion system is used in combination with one or more genomic mutations, which leads to the leaky or diffusible outer membrane phenotype (DOM), including but not limited to, lpp, nlP, tolA, PAL.

[0878] In some embodiments, the secretion system is selected from the type III flagellar, modified type III flagellar, type I (*e.g.*, hemolysin system), type II, type IV, type V, type VI, and type VII secretion systems, resistance-nodulation-division (RND) multi-drug efflux pumps, a single membrane secretion system, Sec and, TAT secretion systems.

[0879] Any of the secretion systems described herein may according to the disclosure be employed to secrete the polypeptides of interest. In some embodiments, the therapeutic proteins secreted by the genetically engineered bacteria are modified to increase resistance to proteases, *e.g.* intestinal proteases.

[0880] In some embodiments, the genetically engineered microorganisms are capable of expressing any one or more of the described circuits in low-oxygen conditions, tissue specific molecules or metabolites, and/or in the presence of molecules or metabolites associated with inflammation or liver damage, and/or in the presence of metabolites that may

be present in the gut, and/or in the presence of metabolites that may or may not be present *in vivo*, and may be present *in vitro* during strain culture, expansion, production and/or manufacture, such as arabinose and others described herein. In some embodiments, the gene sequences(s) are controlled by a promoter inducible by such conditions and/or inducers. In some embodiments, the gene sequences(s) are controlled by a constitutive promoter, as described herein. In some embodiments, the gene sequences(s) are controlled by a constitutive promoter, and are expressed in *in vivo* conditions and/or *in vitro* conditions, *e.g.*, during expansion, production and/or manufacture, as described herein.

[0881] In some embodiments, any one or more of the described circuits are present on one or more plasmids (*e.g.*, high copy or low copy) or are integrated into one or more sites in the microorganisms chromosome. Also, in some embodiments, the genetically engineered microorganisms are further capable of expressing any one or more of the described circuits and further comprise one or more of the following: (1) one or more auxotrophies, such as any auxotrophies known in the art and provided herein, *e.g.*, thyA auxotrophy, (2) one or more kill switch circuits, such as any of the kill-switches described herein or otherwise known in the art, (3) one or more antibiotic resistance circuits, (4) one or more transporters for importing biological molecules or substrates, such any of the transporters described herein or otherwise known in the art, (5) one or more secretion circuits, such as any of the secretion circuits described herein and otherwise known in the art, (6) one or more surface display circuits, such as any of the surface display circuits described herein and otherwise known in the art and (7) one or more circuits for the production or degradation of one or more metabolites (*e.g.*, kynurenine, tryptophan, adenosine, arginine) described herein (8) combinations of one or more of such additional circuits.

[0882] Non-limiting examples of payloads include propionate, butyrate, GLP-1, a tryptophan transporter, aromatic amino acid transporter, IDO, TDO, polypeptide(s) for producing kynurenine or kynurenic acid, polypeptides for metabolizing tryptophan. These polypeptides may be mutated to increase stability, resistance to protease digestion, and/or activity.

Table 45. Comparison of Secretion systems for secretion of polypeptide from engineered bacteria

Secretion System	Tag	Cleavage	Advantages	Other features
Modified Type III (flagellar)	mRNA (or N-terminal)	No cleavage necessary	- No peptide tag - Endogenous	May not be as suited for larger proteins Deletion of flagellar

				genes
Type V autotransport	N- and C-terminal	Yes	Large proteins Endogenous Cleavable	2-step secretion
Type I	C-terminal	No		Tag; Exogenous Machinery
Diffusible Outer Membrane (DOM)	N-terminal	Yes	Disulfide bond formation	May affect cell fragility/survivability/ growth/yield

[0883] In some embodiments, the therapeutic polypeptides of interest are secreted using components of the flagellar type III secretion system. In a non-limiting examples, *e.g.*, propionate, butyrate, GLP-1, a tryptophan transporter, aromatic amino acid transporter, IDO, TDO, polypeptide(s) for producing kynurenine or kynurenic acid, polypeptides for metabolizing tryptophan are assembled behind a fliC-5'UTR (*e.g.*, 173-bp untranslated region from the fliC loci), and is driven by the native promoter. In other embodiments, the expression of the therapeutic peptide of interested secreted using components of the flagellar type III secretion system is driven by a tet-inducible promoter. In alternate embodiments, an inducible promoter such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), promoters induced by IBD specific molecules or promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (*e.g.*, can be exogenously added) in the gut, *e.g.*, arabinose is used. In some embodiments, the therapeutic polypeptide of interest is expressed from a plasmid (*e.g.*, a medium copy plasmid). In some embodiments, the therapeutic polypeptide of interest is expressed from a construct which is integrated into fliC locus (thereby deleting fliC), where it is driven by the native FliC promoter. In some embodiments, an N terminal part of FliC (*e.g.*, the first 20 amino acids of FliC) is included in the construct, to further increase secretion efficiency.

[0884] In some embodiments, the therapeutic polypeptides of interest, include propionate, butyrate, GLP-1, a tryptophan transporter, aromatic amino acid transporter, IDO, TDO, polypeptide(s) for producing kynurenine or kynurenic acid, polypeptides for metabolizing tryptophan. , are secreted using via a diffusible outer membrane (DOM) system. In some embodiments, the therapeutic polypeptide of interest is fused to a N-terminal Sec-dependent secretion signal. Non-limiting examples of such N-terminal Sec-dependent

secretion signals include PhoA, OmpF, OmpA, and cvaC. In alternate embodiments, the therapeutic polypeptide of interest is fused to a Tat-dependent secretion signal. Exemplary Tat-dependent tags include TorA, FdnG, and DmsA.

[0885] In certain embodiments, the genetically engineered bacteria comprise deletions or mutations in one or more of the outer membrane and/or periplasmic proteins. Non-limiting examples of such proteins, one or more of which may be deleted or mutated, include lpp, pal, tolA, and/or nlpI. In some embodiments, lpp is deleted or mutated. In some embodiments, pal is deleted or mutated. In some embodiments, tolA is deleted or mutated. In other embodiments, nlpI is deleted or mutated. In yet other embodiments, certain periplasmic proteases are deleted or mutated, *e.g.*, to increase stability of the polypeptide in the periplasm. Non-limiting examples of such proteases include degP and ompT. In some embodiments, degP is deleted or mutated. In some embodiments, ompT is deleted or mutated. In some embodiments, degP and ompT are deleted or mutated.

[0886] In some embodiments, the therapeutic polypeptides of interest, *e.g.*, propionate, butyrate, GLP-1, a tryptophan transporter, aromatic amino acid transporter, IDO, TDO, polypeptide(s) for producing kynurenine or kynurenic acid, polypeptides for metabolizing tryptophan are secreted via a Type V Auto-secreter (*pic* Protein) Secretion. In some embodiments, the therapeutic protein of interest is expressed as a fusion protein with the native Nissle auto-secreter *E. coli*_01635 (where the original passenger protein is replaced with the therapeutic polypeptides of interest.

[0887] In some embodiments, the therapeutic polypeptides of interest, propionate, butyrate, GLP-1, a tryptophan transporter, aromatic amino acid transporter, IDO, TDO, polypeptide(s) for producing kynurenine or kynurenic acid, polypeptides for metabolizing tryptophan , are secreted via Type I Hemolysin Secretion. In one embodiment, therapeutic polypeptide of interest is expressed as fusion protein with the 53 amino acids of the C terminus of alpha-hemolysin (hlyA) of *E. coli* CFT073.

Host-Plasmid Mutual Dependency

[0888] In some embodiments, the genetically engineered bacteria also comprise a plasmid that has been modified to create a host-plasmid mutual dependency. In certain embodiments, the mutually dependent host-plasmid platform is GeneGuard (Wright *et al.*, 2015). In some embodiments, the GeneGuard plasmid comprises (i) a conditional origin of replication, in which the requisite replication initiator protein is provided *in trans*; (ii) an auxotrophic modification that is rescued by the host via genomic translocation and is also compatible for use in rich media; and/or (iii) a nucleic acid sequence which encodes a broad-

spectrum toxin. The toxin gene may be used to select against plasmid spread by making the plasmid DNA itself disadvantageous for strains not expressing the anti-toxin (*e.g.,* a wild-type bacterium). In some embodiments, the GeneGuard plasmid is stable for at least 100 generations without antibiotic selection. In some embodiments, the GeneGuard plasmid does not disrupt growth of the host. The GeneGuard plasmid is used to greatly reduce unintentional plasmid propagation in the genetically engineered bacteria described herein.

[0889] The mutually dependent host-plasmid platform may be used alone or in combination with other biosafety mechanisms, such as those described herein (*e.g.,* kill switches, auxotrophies). In some embodiments, the genetically engineered bacteria comprise a GeneGuard plasmid. In other embodiments, the genetically engineered bacteria comprise a GeneGuard plasmid and/or one or more kill switches. In other embodiments, the genetically engineered bacteria comprise a GeneGuard plasmid and/or one or more auxotrophies. In still other embodiments, the genetically engineered bacteria comprise a GeneGuard plasmid, one or more kill switches, and/or one or more auxotrophies.

[0890] In some embodiments, the vector comprises a conditional origin of replication. In some embodiments, the conditional origin of replication is a R6K or ColE2-P9. In embodiments where the plasmid comprises the conditional origin of replication R6K, the host cell expresses the replication initiator protein π. In embodiments where the plasmid comprises the conditional origin or replication ColE2, the host cell expresses the replication initiator protein RepA. It is understood by those of skill in the art that the expression of the replication initiator protein may be regulated so that a desired expression level of the protein is achieved in the host cell to thereby control the replication of the plasmid. For example, in some embodiments, the expression of the gene encoding the replication initiator protein may be placed under the control of a strong, moderate, or weak promoter to regulate the expression of the protein.

[0891] In some embodiments, the vector comprises a gene encoding a protein required for complementation of a host cell auxotrophy, preferably a rich-media compatible auxotrophy. In some embodiments, the host cell is auxotrophic for thymidine (Δ*thyA*), and the vector comprises the thymidylate synthase (*thyA*) gene. In some embodiments, the host cell is auxotrophic for diaminopimelic acid (Δ*dapA*) and the vector comprises the 4-hydroxy-tetrahydrodipicolinate synthase (*dapA*) gene. It is understood by those of skill in the art that the expression of the gene encoding a protein required for complementation of the host cell auxotrophy may be regulated so that a desired expression level of the protein is achieved in the host cell.

[0892] In some embodiments, the vector comprises a toxin gene. In some embodiments, the host cell comprises an anti-toxin gene encoding and/or required for the expression of an anti-toxin. In some embodiments, the toxin is Zeta and the anti-toxin is Epsilon. In some embodiments, the toxin is Kid, and the anti-toxin is Kis. In preferred embodiments, the toxin is bacteriostatic. Any of the toxin/antitoxin pairs described herein may be used in the vector systems of the present disclosure. It is understood by those of skill in the art that the expression of the gene encoding the toxin may be regulated using art known methods to prevent the expression levels of the toxin from being deleterious to a host cell that expresses the anti-toxin. For example, in some embodiments, the gene encoding the toxin may be regulated by a moderate promoter. In other embodiments, the gene encoding the toxin may be cloned adjacent to ribosomal binding site of interest to regulate the expression of the gene at desired levels (see, *e.g.*, Wright *et al.* (2015)).

Integration

[0893] In some embodiments, any of the gene(s) or gene cassette(s) of the present disclosure may be integrated into the bacterial chromosome at one or more integration sites. One or more copies of the heterologous gene or heterologous gene cassette may be integrated into the bacterial chromosome. Having multiple copies of the gene or gene cassette integrated into the chromosome allows for greater production of the corresponding protein(s) and also permits fine-tuning of the level of expression. Alternatively, different circuits described herein, such as any of the kill-switch circuits, in addition to the therapeutic gene(s) or gene cassette(s) could be integrated into the bacterial chromosome at one or more different integration sites to perform multiple different functions.

[0894] For example, **FIG. 42** depicts a map of integration sites within the *E. coli* Nissle chromosome. **FIG. 43** depicts three bacterial strains wherein the RFP gene has been successfully integrated into the bacterial chromosome at an integration site.

In vivo Models

[0895] The engineered bacteria may be evaluated *in vivo*, *e.g.*, in an animal model. Any suitable animal model of a disease associated with Non-Alcoholic Fatty Liver Disease/Non-Alcoholic Steatohepatitis (NAFLD/NASH) may be used. For example, the effects of liver steatosis and hepatic inflammation in an *in vivo* mouse model have been described (Jun Jin, et al., Brit. J. Nutrition, 114:145-1755 (2015)).

[0896] Several studies have employed genetic leptin-deficient (ob/ob) or leptin-resistant (db/db) mouse and dietary methionine-/choline-deficient (MCD) models (*e.g.*,

reviewed in Anstee and Goldin, Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int. J. Exp. Pathol. 2006;87:1–16).

[0897] The MCD model of feeding a diet deficient in both methionine and choline is one of the most widely adopted in NASH research. Because methionine- and choline are essential for hepatic beta-oxidation and the production of very low-density lipoprotein, deficiency causes excess hepatic lipid accumulation and subsequent steatohepatitis, which resembles the pathology of human NASH. The phenotype resulting from feeding with the MCD diet (MCDD) is characterized by macrovesicular steatosis, hepatocellular death, inflammation, oxidative stress and fibrosis (Caballero et al. 2010). However, the mice show dramatic systemic weight loss during the MCDD induction of NASH, with the greatest weight loss observed in the A/J mouse strain, followed by strain C57BL/6 (Rangnekar et al. Quantitative trait loci analysis of mice administered the methionine-choline deficient dietary model of experimental steatohepatitis. Liver Int. 2006;26:1000–1005).

[0898] The choline-deficient, l-amino acid-defined (CDAA) dietary model overcomes the weight loss problem, and has been demonstrated to mimic human NASH in both mice and rats by sequentially producing steatohepatitis, liver fibrosis and liver cancer without any loss of body weight (Denda et al. Development of hepatocellular adenomas and carcinomas associated with fibrosis in C57BL/6J male mice given a choline-deficient, l-amino acid-defined diet. Jpn. J. Cancer Res. 2002;93:125–132.). An *in vivo* rat model of choline deficient/L-amino acid defined (CDAA) diet has also been described (Endo, *et al.*, PLoS One, 8(5):e63388 (2013)). In this model, rats are fed the CDAA diet for eight weeks. The diet induces NAFLD/NASH symptoms such as liver steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocarcinogenesis. With rats, optimized feeding with the CDAA diet results in rapid progression of fibrosis followed by a rise in alanine aminotransferase (**ALT**), which is a parameter indicating liver injury. In mice, feeding with the CDAA diet does not result in significant increases in ALT, and long-term feeding of 20 weeks or more is required before liver fibrosis is observed (Denda et al. 2007).

[0899] Matsumoto et al. developed an improved mouse model of rapidly progressive liver fibrosis, buy optimizing the methionine content in the diet (Matsumoto et al. Int J Exp Pathol. 2013 Apr; 94(2): 93–103). An improved mouse model that rapidly develops fibrosis in non-alcoholic steatohepatitis). In this study, C57BL/6J mice and A/J mice mice were fed a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) consisting of 60 kcal% fat and 0.1% methionine by weight.. In both strains of mice, plasma levels of alanine aminotransferase increased from week 1, when hepatic steatosis was also observed. In both

C57BL/6J mice and A/J mice, histopathological fatty liver started developing as steatosis 1 week after starting the diet, accompanied with a marked rise of ALT. By week 6, C57BL/6J mice had developed enlarged fatty liver with fibrosis as assessed by Masson's trichrome staining and by hydroxyproline assay. Moreover, when C57BL/6J mice continued to be fed in the same way for 14 weeks, transient loss of body weight recovered and continued to increase and fibrosis further developed. In A/J mice, cell infiltration was weaker than in C57BL/6J mice, and fibrosis was identified not in the sixth week but in the ninth week. It was clear that in this model, feeding with CDAHFD rapidly induced fibrosis to develop within 6–9 weeks of beginning the diet, regardless of the mouse strain.

[0900] Yet another dietary model combines a HFD with administration of high fructose corn syrup. NAFLD and NASH were induced in a "American life style obesity syndrome" model through long-term feeding (12 months) of a HFD in combination with fructose corn syrup. Features of early nonalcoholic steatohepatitis at 6 months (mean NAFLD activity score = 2.4) and features of more advanced nonalcoholic steatohepatitis at 12 months, including liver inflammation and bridging fibrosis (mean NAFLD activity score = 5.0) (Dowman et al., Development of hepatocellular carcinoma in a murine model of nonalcoholic steatohepatitis induced by use of a high-fat/fructose diet and sedentary lifestyle; Am J Pathol. 184(5):1550-61).

[0901] Alternatively, Asgharpour et al., developed an isogenic strain derived from a cross of two common mouse strains, (129S1/SvImJ and C57BL/6J), for which a simple HFD accompanied by ad libitum consumption of water with a high fructose and glucose content (Western diet sugar water (WD SW)) sequentially induces steatosis, steatohepatitis, progressive fibrosis and hepatocellular carcinoma (HCC) (Asgharpour et al., A diet-induced animal model of non-alcoholic fatty liver disease and hepatocellular cancer. J Hepatol. 2016 May 31. pii: S0168-8278(16)30190-8). At a transcriptomic level, there was a concordance in terms of the pathways activated in humans with NAFLD and mice with corresponding histologic phenotypes. Several key protein pathways relevant to human NASH were also activated in these mice.

[0902] A number of NASH/HCC models utilize chemotoxic agents in combination with dietary models (*e.g.*, reviewed in Nakagawa, Recent advances in mouse models of obesity- and nonalcoholic steatohepatitis-associated hepatocarcinogenesis; World J Hepatol. 2015 Aug 18; 7(17): 2110–2118). As a non-limiting example, Fujii and co-workers developed a novel NASH model for diabetes-based NASH, which sequentially progresses from fatty liver, to NASH, fibrosis, and finally to HCC on a diabetic background. Neonatal

male mice were immediately exposed to low-dose streptozotocin (STZ; injection of 200 lg STZ at 2 days post birth). When fed a high fat diet (HFD) at 4 weeks, these mice developed liver steatosis with diabetes after 1 week. Continuous HFD decreased hepatic fat deposit while lobular inflammation with foam cell-like macrophages increased, consistent with NASH pathology (Fujii et al., A murine model for non-alcoholic steatohepatitis showing evidence of association between diabetes and hepatocellular carcinoma; Med Mol Morphol. 2013 Sep;46(3):141-52). Macrophage phagocytosis was decreased and fibroblasts accumulated to form ''chicken-wired'' fibrosis. All mice later developed multiple HCC. In contrast, in this model, female mice treated with STZ–HFD (and male mice treated with STZ alone) showed diabetes did not exhibit NASH-related fibrosis and also never developed HCC, and as such may be useful as controls.

[0903] Other models are known in the art, including a *Lepob/Lepob* and C57BL6 (B6) mouse model used to study the effects of high fat diet and GLP-1 administration within the NASH setting. See, for example, Trevaskis *et al.,* Am. J. Physiology-Gastrointestinal and Liver Physiology, 302(8):G762-G772, 2012, and Takahashi *et al.,* World J. Gastroenterol., 18(19):2300-2308, 2012, the entire contents of each of which are expressly incorporated herein by reference. Animal models known in the art are reviewed extensively (*e.g.*, see Takahashi et al., Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, World J Gastroenterol 2012 May 21; 18(19): 2300-2308; Kanuri and Bergheim, *In vitro* and *in vivo* Models of Non-Alcoholic Fatty Liver Disease (NAFLD), Int. J. Mol. Sci. 2013, 14, 11963-11980; and Sanches et al., Nonalcoholic Steatohepatitis: A Search for Factual Animal Models, BioMed Research International Volume 2015 (2015)).

Nucleic Acids

[0904] In some embodiments, the disclosure provides novel nucleic acids for producing butyrate. In some embodiments, the nucleic acids comprises gene sequence encoding one or more butyrogenic genes. In some embodiments, the nucleic acids comprises gene sequence encoding one or more butyrate gene cassettes. In some embodiments, the nucleic acids comprise one or more butyrate genes from Table 5. In some embodiments, the nucleic acids comprises gene sequence encoding one or more butyrogenic genes selected from *bcd2, etfB3, etfA3*, *thiA1*, *hbd, crt2*, *pbt*, *buk*, *ter*, and *TesB*.

[0905] In some embodiments, the nucleic acid comprises gene sequence encoding a Bcd2 polypeptide. In some embodiments, the nucleic acid comprises a *bcd2* gene sequence. In certain embodiments, the nucleic acid comprising the *bcd2* gene sequence has at least

about 80% identity with SEQ ID NO: 53. In certain embodiments, the nucleic acid comprising the *bcd2* gene sequence has at least about 90% identity with SEQ ID NO: 53. In certain embodiments, the nucleic acid comprising the *bcd2* gene sequence has at least about 95% identity with SEQ ID NO: 53. In some embodiments, the nucleic acid comprising the *bcd2* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 53. In some specific embodiments, the nucleic acid comprising the *bcd2* gene sequence comprises SEQ ID NO: 1. In other specific embodiments the nucleic acid comprising the *bcd2* gene sequence consists of SEQ ID NO: 53.

[0906] In some embodiments, the nucleic acid comprises gene sequence encoding a EtfB3 polypeptide. In some embodiments, the nucleic acid comprises a *etfB3* gene sequence. In certain embodiments, the nucleic acid comprising the *etfB3* gene sequence has at least about 80% identity with SEQ ID NO: 54. In certain embodiments, the nucleic acid comprising the *etfB3* gene sequence has at least about 90% identity with SEQ ID NO: 54. In certain embodiments, the nucleic acid comprising the *etfB3* gene sequence has at least about 95% identity with SEQ ID NO: 54. In some embodiments, the nucleic acid comprising the *etfB3* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 54. In some specific embodiments, the nucleic acid comprising the *etfB3* gene sequence comprises SEQ ID NO: 54. In other specific embodiments the nucleic acid comprising the *etfB3* gene sequence consists of SEQ ID NO: 54.

[0907] In some embodiments, the nucleic acid comprises gene sequence encoding a EtfA3 polypeptide. In some embodiments, the nucleic acid comprises a *etfA3* gene sequence. In certain embodiments, the nucleic acid comprising the *etfA3* gene sequence has at least about 80% identity with SEQ ID NO: 55. In certain embodiments, the nucleic acid comprising the *etfA3* gene sequence has at least about 90% identity with SEQ ID NO: 55. In certain embodiments, the nucleic acid comprising the *etfA3* gene sequence has at least about 95% identity with SEQ ID NO: 55. In some embodiments, the nucleic acid comprising the *etfA3* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 55. In some specific embodiments, the nucleic acid comprising the *etfA3* gene sequence comprises SEQ ID NO: 55. In other specific embodiments the nucleic acid comprising the *etfA3* gene sequence consists of SEQ ID NO: 55.

[0908] In some embodiments, the nucleic acid comprises gene sequence encoding a ThiA1 polypeptide. In some embodiments, the nucleic acid comprises a *thiA1* gene sequence. In certain embodiments, the nucleic acid comprising the *thiA1* gene sequence has at least about 80% identity with SEQ ID NO: 56. In certain embodiments, the nucleic acid comprising the *thiA1* gene sequence has at least about 90% identity with SEQ ID NO: 56. In certain embodiments, the nucleic acid comprising the *thiA1* gene sequence has at least about 95% identity with SEQ ID NO: 4. In some embodiments, the nucleic acid comprising the *thiA1* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 56. In some specific embodiments, the nucleic acid comprising the *thiA1* gene sequence comprises SEQ ID NO: 56. In other specific embodiments the nucleic acid comprising the *thiA1* gene sequence consists of SEQ ID NO: 56.

[0909] In some embodiments, the nucleic acid comprises gene sequence encoding a Hbd polypeptide. In some embodiments, the nucleic acid comprises a *hbd* gene sequence. In certain embodiments, the nucleic acid comprising the *hbd* gene sequence has at least about 80% identity with SEQ ID NO: 57. In certain embodiments, the nucleic acid comprising the *hbd* gene sequence has at least about 90% identity with SEQ ID NO: 57. In certain embodiments, the nucleic acid comprising the *hbd* gene sequence has at least about 95% identity with SEQ ID NO: 57. In some embodiments, the nucleic acid comprising the *hbd* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 57. In some specific embodiments, the nucleic acid comprising the *hbd* gene sequence comprises SEQ ID NO: 57. In other specific embodiments the nucleic acid comprising the *hbd* gene sequence consists of SEQ ID NO: 57.

[0910] In some embodiments, the nucleic acid comprises gene sequence encoding a Crt2 polypeptide. In some embodiments, the nucleic acid comprises a *crt2* gene sequence. In certain embodiments, the nucleic acid comprising the *crt2* gene sequence has at least about 80% identity with SEQ ID NO: 58. In certain embodiments, the nucleic acid comprising the *crt2* gene sequence has at least about 90% identity with SEQ ID NO: 58. In certain embodiments, the nucleic acid comprising the *crt2* gene sequence has at least about 95% identity with SEQ ID NO: 58. In some embodiments, the nucleic acid comprising the *crt2* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 58. In some specific embodiments, the nucleic acid comprising the *crt2* gene sequence comprises SEQ ID NO: 58. In other

specific embodiments the nucleic acid comprising the *crt2* gene sequence consists of SEQ ID NO: 58.

[0911] In some embodiments, the nucleic acid comprises gene sequence encoding a Pbt polypeptide. In some embodiments, the nucleic acid comprises a *pbt* gene sequence. In certain embodiments, the nucleic acid comprising the *pbt* gene sequence has at least about 80% identity with SEQ ID NO: 59 In certain embodiments, the nucleic acid comprising the *pbt* gene sequence has at least about 90% identity with SEQ ID NO: 59. In certain embodiments, the nucleic acid comprising the *pbt* gene sequence has at least about 95% identity with SEQ ID NO: 59. In some embodiments, the nucleic acid comprising the *pbt* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 59. In some specific embodiments, the nucleic acid comprising the *pbt* gene sequence comprises SEQ ID NO: 59. In other specific embodiments the nucleic acid comprising the *pbt* gene sequence consists of SEQ ID NO: 59.

[0912] In some embodiments, the nucleic acid comprises gene sequence encoding a Buk polypeptide. In some embodiments, the nucleic acid comprises a *buk* gene sequence. In certain embodiments, the nucleic acid comprising the *buk* gene sequence has at least about 80% identity with SEQ ID NO: 60. In certain embodiments, the nucleic acid comprising the *buk* gene sequence has at least about 90% identity with SEQ ID NO: 60. In certain embodiments, the nucleic acid comprising the *buk* gene sequence has at least about 95% identity with SEQ ID NO: 60. In some embodiments, the nucleic acid comprising the *buk* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 60. In some specific embodiments, the nucleic acid comprising the *buk* gene sequence comprises SEQ ID NO: 60. In other specific embodiments the nucleic acid comprising the *buk* gene sequence consists of SEQ ID NO: 60.

[0913] In some embodiments, the nucleic acid comprises gene sequence encoding a Ter polypeptide. In some embodiments, the nucleic acid comprises a *ter* gene sequence. In certain embodiments, the nucleic acid comprising the *ter* gene sequence has at least about 80% identity with SEQ ID NO: 61. In certain embodiments, the nucleic acid comprising the *ter* gene sequence has at least about 90% identity with SEQ ID NO: 61. In certain embodiments, the nucleic acid comprising the *ter* gene sequence has at least about 95% identity with SEQ ID NO: 61. In some embodiments, the nucleic acid comprising the *ter* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%,

95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 61. In some specific embodiments, the nucleic acid comprising the *ter* gene sequence comprises SEQ ID NO: 61. In other specific embodiments the nucleic acid comprising the *ter* gene sequence consists of SEQ ID NO: 61.

[0914] In some embodiments, the nucleic acid comprises gene sequence encoding a TesB polypeptide. In some embodiments, the nucleic acid comprises a *tesB* gene sequence. In certain embodiments, the nucleic acid comprising the *tesB* gene sequence has at least about 80% identity with SEQ ID NO: 15. In certain embodiments, the nucleic acid comprising the *tesB* gene sequence has at least about 90% identity with SEQ ID NO: 15. In certain embodiments, the nucleic acid comprising the *tesB* gene sequence has at least about 95% identity with SEQ ID NO: 15. In some embodiments, the nucleic acid comprising the *tesB* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 15. In some specific embodiments, the nucleic acid comprising the *tesB* gene sequence comprises SEQ ID NO: 15. In other specific embodiments the nucleic acid comprising the *tesB* gene sequence consists of SEQ ID NO: 15.

[0915] In other embodiments, the disclosure provides novel nucleic acids for producing butyrate in which the nucleic acid comprises gene sequence encoding one or more butyrogenic gene cassette(s). In some embodiments, the nucleic acid comprises gene sequence encoding a butyrogenic gene cassette comprising Bcd2, EtfB3, EtfA3, ThiA1, Hbd, Crt2, Pbt, and Buk. In some embodiments, the nucleic acid comprises a butyrogenic gene cassette(s) cassette comprising *bcd2, etfB3, etfA3*, *thiA1*, *hbd, crt2*, *pbt*, and *buk* gene sequence. In some embodiments, the nucleic acid comprises gene sequence encoding a butyrogenic gene cassette comprising ThiA1, Hbd, Crt2, Pbt, Buk, and Ter. In some embodiments, the nucleic acid comprises a butyrogenic gene cassette(s) cassette comprising *thiA1*, *hbd, crt2*, *pbt*, *buk*, and *ter* gene sequence. In some embodiments, the nucleic acid comprises gene sequence encoding a butyrogenic gene cassette comprising Ter, ThiA1, Hbd, Crt2, and TesB. In some embodiments, the nucleic acid comprises a butyrogenic gene cassette(s) cassette comprising *ter*, *thiA1*, *hbd, crt2*, and *tesB* gene sequence.

[0916] In some embodiments, the disclosure provides novel nucleic acids for producing propionate In some embodiments, the nucleic acids comprises gene sequence encoding one or more propionate genes. In some embodiments, the nucleic acids comprises gene sequence encoding one or more propionate gene cassettes. In some embodiments, the nucleic acids comprise one or more propionate genes from Table 1. In some embodiments,

the nucleic acids comprises gene sequence encoding one or more propionate genes selected from *pct, lcdA, lcdB, lcdC, etfA, acrB*, and *acrC*.

[0917] In some embodiments, the nucleic acid comprises gene sequence encoding a Pct polypeptide. In some embodiments, the nucleic acid comprises a *pct* gene sequence. In certain embodiments, the nucleic acid comprising the *pct* gene sequence has at least about 80% identity with SEQ ID NO: 1. In certain embodiments, the nucleic acid comprising the *pct* gene sequence has at least about 90% identity with SEQ ID NO: 1. In certain embodiments, the nucleic acid comprising the *pct* gene sequence has at least about 95% identity with SEQ ID NO: 1. In some embodiments, the nucleic acid comprising the *pct* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 1. In some specific embodiments, the nucleic acid comprising the *pct* gene sequence comprises SEQ ID NO: 1. In other specific embodiments the nucleic acid comprising the *pct* gene sequence consists of SEQ ID NO: 1.

[0918] In some embodiments, the nucleic acid comprises gene sequence encoding a lcdA polypeptide. In some embodiments, the nucleic acid comprises a *lcdA* gene sequence. In certain embodiments, the nucleic acid comprising the *lcdA* gene sequence has at least about 80% identity with SEQ ID NO: 2. In certain embodiments, the nucleic acid comprising the *lcdA* gene sequence has at least about 90% identity with SEQ ID NO: 2. In certain embodiments, the nucleic acid comprising the *lcdA* gene sequence has at least about 95% identity with SEQ ID NO: 2. In some embodiments, the nucleic acid comprising the *lcdA* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 2. In some specific embodiments, the nucleic acid comprising the *lcdA* gene sequence comprises SEQ ID NO: 2. In other specific embodiments the nucleic acid comprising the *lcdA* gene sequence consists of SEQ ID NO: 2.

[0919] In some embodiments, the nucleic acid comprises gene sequence encoding a lcdB polypeptide. In some embodiments, the nucleic acid comprises a *lcdB* gene sequence. In certain embodiments, the nucleic acid comprising the *lcdB* gene sequence has at least about 80% identity with SEQ ID NO: 3. In certain embodiments, the nucleic acid comprising the *lcdB* gene sequence has at least about 90% identity with SEQ ID NO: 3. In certain embodiments, the nucleic acid comprising the *lcdB* gene sequence has at least about 95% identity with SEQ ID NO: 3. In some embodiments, the nucleic acid comprising the *lcdB* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 3. In some specific embodiments,

the nucleic acid comprising the *lcdB* gene sequence comprises SEQ ID NO: 3. In other specific embodiments the nucleic acid comprising the *lcdB* gene sequence consists of SEQ ID NO: 3.

[0920] In some embodiments, the nucleic acid comprises gene sequence encoding a lcdC polypeptide. In some embodiments, the nucleic acid comprises a *lcdC* gene sequence. In certain embodiments, the nucleic acid comprising the *lcdC* gene sequence has at least about 80% identity with SEQ ID NO: 4. In certain embodiments, the nucleic acid comprising the *lcdC* gene sequence has at least about 90% identity with SEQ ID NO: 4. In certain embodiments, the nucleic acid comprising the *lcdC* gene sequence has at least about 95% identity with SEQ ID NO: 4. In some embodiments, the nucleic acid comprising the *lcdC* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 4. In some specific embodiments, the nucleic acid comprising the *lcdC* gene sequence comprises SEQ ID NO: 4. In other specific embodiments the nucleic acid comprising the *lcdC* gene sequence consists of SEQ ID NO: 4.

[0921] In some embodiments, the nucleic acid comprises gene sequence encoding a etfA polypeptide. In some embodiments, the nucleic acid comprises a *etfA* gene sequence. In certain embodiments, the nucleic acid comprising the *etfA* gene sequence has at least about 80% identity with SEQ ID NO: 5. In certain embodiments, the nucleic acid comprising the *etfA* gene sequence has at least about 90% identity with SEQ ID NO: 5. In certain embodiments, the nucleic acid comprising the *etfA* gene sequence has at least about 95% identity with SEQ ID NO: 5. In some embodiments, the nucleic acid comprising the *etfA* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 5. In some specific embodiments, the nucleic acid comprising the *etfA* gene sequence comprises SEQ ID NO: 5. In other specific embodiments the nucleic acid comprising the *etfA* gene sequence consists of SEQ ID NO: 5.

[0922] In some embodiments, the nucleic acid comprises gene sequence encoding an acrB polypeptide. In some embodiments, the nucleic acid comprises a *acrB* gene sequence. In certain embodiments, the nucleic acid comprising the *acrB* gene sequence has at least about 80% identity with SEQ ID NO: 6. In certain embodiments, the nucleic acid comprising the *acrB* gene sequence has at least about 90% identity with SEQ ID NO: 6. In certain embodiments, the nucleic acid comprising the *acrB* gene sequence has at least about 95% identity with SEQ ID NO: 6. In some embodiments, the nucleic acid comprising the *acrB*

gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 6. In some specific embodiments, the nucleic acid comprising the *acrB* gene sequence comprises SEQ ID NO: 6. In other specific embodiments the nucleic acid comprising the *acrB* gene sequence consists of SEQ ID NO: 6.

[0923] In some embodiments, the nucleic acid comprises gene sequence encoding an acrC polypeptide. In some embodiments, the nucleic acid comprises a *acrC* gene sequence. In certain embodiments, the nucleic acid comprising the *acrC* gene sequence has at least about 80% identity with SEQ ID NO: 7. In certain embodiments, the nucleic acid comprising the *acrC* gene sequence has at least about 90% identity with SEQ ID NO: 7. In certain embodiments, the nucleic acid comprising the *acrC* gene sequence has at least about 95% identity with SEQ ID NO: 7. In some embodiments, the nucleic acid comprising the *acrC* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 7. In some specific embodiments, the nucleic acid comprising the *acrC* gene sequence comprises SEQ ID NO: 7. In other specific embodiments the nucleic acid comprising the *acrC* gene sequence consists of SEQ ID NO: 7.

[0924] In other embodiments, the disclosure provides novel nucleic acids for producing propionate in which the nucleic acid comprises gene sequence encoding one or more propionate gene cassette(s). In some embodiments, the nucleic acid comprises gene sequence encoding a propionate gene cassette comprising Pct, lcdA, lcdB, lcdC, etfA, acrB, and acrC. In some embodiments, the nucleic acid comprises a propionate gene cassette(s) cassette comprising *pct, lcdA, lcdB, lcdC, etfA, acrB, and acrC* gene sequence. In some embodiments, the nucleic acid comprises gene sequence encoding a propionate gene cassette comprising Pct, lcdA, lcdB, lcdC, and etfA. In some embodiments, the nucleic acid comprises a propionate gene cassette(s) cassette comprising *pct, lcdA, lcdB, lcdC, and etfA* gene sequence. In some embodiments, the nucleic acid comprises gene sequence encoding a propionate gene cassette comprising lcdB, lcdC, etfA, acrB, and acrC. In some embodiments, the nucleic acid comprises a propionate gene cassette(s) cassette comprising *lcdB, lcdC, etfA, acrB, and acrC* gene sequence.

[0925] In any of the nucleic acid embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides that produce butyrate is operably linked to an inducible promoter. In said embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In any of the nucleic acid

embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides that produce butyrate is operably linked to an constitutive promoter. In some embodiments, the nucleic acid is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the nucleic acid is expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the nucleic acid is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. Inducible promoters and constitutive promoters are described in more detail *infra*.

[0926] One or more of the nucleic acids encoding butyrate biosynthesis genes may be functionally replaced or modified, *e.g.*, codon optimized.

[0927] In some embodiments, the disclosure provides novel nucleic acids for producing propionate In some embodiments, the nucleic acids comprises gene sequence encoding one or more propionate genes. In some embodiments, the nucleic acids comprises gene sequence encoding one or more propionate gene cassettes. In some embodiments, the nucleic acids comprise one or more propionate genes from Table 1. In some embodiments, the nucleic acids comprises gene sequence encoding one or more propionate genes selected from *thrA*^{fbr}, *thrB, thrC, ilvA*^{fbr}, *aceE, aceF, and lpd.*

[0928] In some embodiments, the nucleic acid comprises gene sequence encoding a thrA^{fbr} polypeptide. In some embodiments, the nucleic acid comprises a *thrA*^{fbr} gene sequence. In certain embodiments, the nucleic acid comprising the *thrA*^{fbr} gene sequence has at least about 80% identity with SEQ ID NO: 8. In certain embodiments, the nucleic acid comprising the *thrA*^{fbr} gene sequence has at least about 90% identity with SEQ ID NO: 8. In certain embodiments, the nucleic acid comprising the *thrA*^{fbr} gene sequence has at least about 95% identity with SEQ ID NO: 8. In some embodiments, the nucleic acid comprising the *pct* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 8. In some specific embodiments, the nucleic acid comprising the *thrA*^{fbr} gene sequence comprises SEQ ID NO: 8. In other specific embodiments the nucleic acid comprising the *thrA*^{fbr}gene sequence consists of SEQ ID NO: 8.

[0929] In some embodiments, the nucleic acid comprises gene sequence encoding a thrB polypeptide. In some embodiments, the nucleic acid comprises a *thrB* gene sequence. In certain embodiments, the nucleic acid comprising the *thrB* gene sequence has at least about 80% identity with SEQ ID NO: 9. In certain embodiments, the nucleic acid comprising the *thrB* gene sequence has at least about 90% identity with SEQ ID NO: 9. In certain

embodiments, the nucleic acid comprising the *thrB* gene sequence has at least about 95% identity with SEQ ID NO: 9. In some embodiments, the nucleic acid comprising the *thrB* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 9. In some specific embodiments, the nucleic acid comprising the *thrB* gene sequence comprises SEQ ID NO: 2. In other specific embodiments the nucleic acid comprising the *thrB* gene sequence consists of SEQ ID NO: 9.

[0930] In some embodiments, the nucleic acid comprises gene sequence encoding a thrC polypeptide. In some embodiments, the nucleic acid comprises a *thrC* gene sequence. In certain embodiments, the nucleic acid comprising the *thrC* gene sequence has at least about 80% identity with SEQ ID NO: 10. In certain embodiments, the nucleic acid comprising the *thrC* gene sequence has at least about 90% identity with SEQ ID NO: 10. In certain embodiments, the nucleic acid comprising the *thrC* gene sequence has at least about 95% identity with SEQ ID NO: 10. In some embodiments, the nucleic acid comprising the *thrC* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 10. In some specific embodiments, the nucleic acid comprising the *thrC* gene sequence comprises SEQ ID NO: 10. In other specific embodiments the nucleic acid comprising the *thrC* gene sequence consists of SEQ ID NO: 10.

[0931] In some embodiments, the nucleic acid comprises gene sequence encoding a ilvA^{fbr} polypeptide. In some embodiments, the nucleic acid comprises a *ilvA*^{fbr} gene sequence. In certain embodiments, the nucleic acid comprising the *ilvA*^{fbr} gene sequence has at least about 80% identity with SEQ ID NO: 11. In certain embodiments, the nucleic acid comprising the *ilvA*^{fbr} gene sequence has at least about 90% identity with SEQ ID NO: 11. In certain embodiments, the nucleic acid comprising the *ilvA*^{fbr} gene sequence has at least about 95% identity with SEQ ID NO: 11. In some embodiments, the nucleic acid comprising the *ilvA*^{fbr} gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 11. In some specific embodiments, the nucleic acid comprising the *ilvA*^{fbr} gene sequence comprises SEQ ID NO: 11. In other specific embodiments the nucleic acid comprising the *ilvA*^{fbr} gene sequence consists of SEQ ID NO: 11.

[0932] In some embodiments, the nucleic acid comprises gene sequence encoding a aceE polypeptide. In some embodiments, the nucleic acid comprises a *aceE* gene sequence. In certain embodiments, the nucleic acid comprising the *aceE* gene sequence has at least

about 80% identity with SEQ ID NO: 12. In certain embodiments, the nucleic acid comprising the *aceE* gene sequence has at least about 90% identity with SEQ ID NO: 12. In certain embodiments, the nucleic acid comprising the *aceE* gene sequence has at least about 95% identity with SEQ ID NO: 12. In some embodiments, the nucleic acid comprising the *aceE* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 12. In some specific embodiments, the nucleic acid comprising the *aceE* gene sequence comprises SEQ ID NO: 12. In other specific embodiments the nucleic acid comprising the *aceE* gene sequence consists of SEQ ID NO: 12.

[0933] In some embodiments, the nucleic acid comprises gene sequence encoding an aceF polypeptide. In some embodiments, the nucleic acid comprises a *aceF* gene sequence. In certain embodiments, the nucleic acid comprising the *aceF* gene sequence has at least about 80% identity with SEQ ID NO: 13. In certain embodiments, the nucleic acid comprising the *aceF* gene sequence has at least about 90% identity with SEQ ID NO: 13. In certain embodiments, the nucleic acid comprising the *aceF* gene sequence has at least about 95% identity with SEQ ID NO: 13. In some embodiments, the nucleic acid comprising the *aceF* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 13. In some specific embodiments, the nucleic acid comprising the *aceF* gene sequence comprises SEQ ID NO: 13. In other specific embodiments the nucleic acid comprising the *aceF* gene sequence consists of SEQ ID NO: 13.

[0934] In some embodiments, the nucleic acid comprises gene sequence encoding an lpd polypeptide. In some embodiments, the nucleic acid comprises a *lpd* gene sequence. In certain embodiments, the nucleic acid comprising the *lpd* gene sequence has at least about 80% identity with SEQ ID NO: 14. In certain embodiments, the nucleic acid comprising the *lpd* gene sequence has at least about 90% identity with SEQ ID NO: 14. In certain embodiments, the nucleic acid comprising the *lpd* gene sequence has at least about 95% identity with SEQ ID NO: 14. In some embodiments, the nucleic acid comprising the *lpd* gene sequence has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 14. In some specific embodiments, the nucleic acid comprising the *lpd* gene sequence comprises SEQ ID NO: 14. In other specific embodiments the nucleic acid comprising the *lpd* gene sequence consists of SEQ ID NO: 14.

[0935] In other embodiments, the disclosure provides novel nucleic acids for producing propionate in which the nucleic acid comprises gene sequence encoding one or more propionate gene cassette(s). In some embodiments, the nucleic acid comprises gene sequence encoding a propionate gene cassette comprising $thrA^{fbr}$, thrB, thrC, $ilvA^{fbr}$, aceE, aceF, and lpd. In some embodiments, the nucleic acid comprises a propionate gene cassette(s) cassette comprising *$thrA^{fbr}$, thrB, thrC, $ilvA^{fbr}$, aceE, aceF, and lpd* gene sequence. In some embodiments, the nucleic acid comprises gene sequence encoding a propionate gene cassette comprising $thrA^{fbr}$, thrB, thrC, $ilvA^{fbr}$, and aceE. In some embodiments, the nucleic acid comprises a propionate gene cassette(s) cassette comprising *$thrA^{fbr}$, thrB, thrC, $ilvA^{fbr}$, and aceE* gene sequence. In some embodiments, the nucleic acid comprises gene sequence encoding a propionate gene cassette comprising thrC, $ilvA^{fbr}$, aceE, aceF, and lpd. In some embodiments, the nucleic acid comprises a propionate gene cassette(s) cassette comprising *thrC, $ilvA^{fbr}$, aceE, aceF, and lpd* gene sequence.

[0936] In any of the nucleic acid embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides that produce propionate is operably linked to an inducible promoter. In said embodiments, the inducible promoter is directly or indirectly induced by exogenous environmental conditions. In any of the nucleic acid embodiments described above and elsewhere herein, the gene sequence encoding one or more polypeptides that produce propionate is operably linked to an constitutive promoter. In some embodiments, the nucleic acid is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the nucleic acid is expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the nucleic acid is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. Inducible promoters and constitutive promoters are described in more detail *infra*.

[0937] One or more of the nucleic acids encoding propionate biosynthesis genes may be functionally replaced or modified, *e.g.*, codon optimized.

Surface Display

[0938] In some embodiments, the genetically engineered bacteria and/or microorganisms encode one or more gene(s) and/or gene cassette(s) encoding an anti-inflammatory effector molecule which is anchored or displayed on the surface of the bacteria and/or microorganisms. Examples of the anti-inflammatory effector molecules which are displayed or anchored to the bacteria and/or microorganism, are any of the anti-inflammatory

effector molecules described herein, and include but are not limited to antibodies, *e.g.*, scFv fragments, and tumor-specific antigens or neoantigens. In a non-limiting example, the antibodies or scFv fragments which are anchored or displayed on the bacterial cell surface are directed against checkpoint inhibitors described herein, including, but not limited to, CLTLA4, PD-1, PD-L1, and others described herein.

[0939] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding therapeutic polypeptide or effector molecule, *e.g.*, a ScFv, which is anchored or displayed on the surface of the bacteria, and which remains anchored while exerting its effector function. In other embodiments, the genetically engineered bacteria encoding the surface-displayed therapeutic polypeptide, *e.g.*, the antibodies or scFv fragments, lyse before, during or after exerting their effector function. In some embodiments, the genetically engineered bacteria encode a therapeutic peptide that is temporarily attached to the cell surface and which dissociates from the bacterium before, during, or after exerting its function.

[0940] In some embodiments, shorter peptides or polypeptides, *e.g.* peptides or polypeptides of less than 60 amino acids of length, are displayed on the cell surface of the genetically engineered bacteria. In some embodiments, such shorter peptides or polypeptides comprise a immune modulatory effector molecule. Non-limiting examples of such therapeutic polypeptides are described herein.

[0941] Several strategies for the display of shorter peptides or polypeptides on the surface of gram negative bacteria are known in the art, and are for example described in Georgiou et al., Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines: Nat Biotechnol. 1997 Jan;15(1):29-34, the contents of which is herein incorporated by reference in its entirety. These systems all share a common theme, targeting recombinant proteins to the cell surface by the construction of gene fusions using sequences from membrane-anchoring domains of surface proteins.

[0942] Non-limiting examples of such strategies are described in **Table 46 and Table 47**.

Table 46. Exemplary Cell Surface Display Strategies

Carrier protein	Exemplary carrier organism	Type of fusion	Localization of heterologous polypeptide

LamB	*E. coli*	Sandwich fusion	Cell surface
PhoE	*E. coli*	Sandwich fusion	Cell surface
OprF	*Pseudomonas*	Sandwich fusion	Cell surface
Gram negative lipoproteins	*E. coli*	C-terminal or sandwich fusion	Periplasmic side or outer membrane/Cell surface
Lpp-OmpA	*E. coli*	C-terminal fusion	Cell surface
VirG	*Shigella*	N-terminal fusion	Cell surface
IgA	*Neisseria*	N-terminal fusion	Cell surface
Flagellin (FliC)	*E. coli*	Sandwich fusion	Cell surface
Flagellin (FliC)	*E. coli*	Sandwich fusion	Cell surface
FimH (type I pili)	*E. coli*	Sandwich fusion	Cell surface
PapA (Pap pili)	*E. coli*	Sandwich fusion	Cell surface
PulA	*Klebsiella*	C-terminal fusion	Cell surface/extracellular fluid

Table 47. Exemplary Cell Surface Display Strategies

Carrier	Passenger size
Outer membrane Proteins	
OmpA	15-514 aa
OmprF	17-43 aa
LamB	11-232 aa
OmpS	38-115 aa
OmpC	162 aa
PhoE	8-32 aa

Invasin	18 aa
LppOmpA	< or = 40 kDa
Lipoproteins	
TraT	11-98 aa
PAL	Approx.. 250 aa
OprI	16 aa
Inp	Less than or equal 47 kDa
Autotransporters	
Igabeta	12 kDa
VirGbeta	Approx.. 50 kDa
AIDA-1	12-40 kDa
Secreted	
Pullulanase	
Subunits of Surface Appendages	
Flagellae	11-115 aa
Fimbriae	7-52 aa
S-layer proteins	
RsaA	12 aa

Table 48. Exemplary Cell Surface Strategies

Outer membrane protein	**Type of fusion**	**Passenger size (kDa)**
Outer membrane protein		
eCPX derived from OmpX	Biterminal	0.8-1.6
FhuA	Insertional	1.1-3.3
LamB	Insertional	1.2-25.5
Omp1	C-terminal	56
OmpA	Insertional	1-50
OmpC	Insertional, C-terminal	18-52
OmpT		35
OprF	C-terminal	50
PgsA	C-terminal	34-77
Wza-omp orf1/OmpU/Omp26La	C-terminal	27-50
Surface Appendages		

F Pillin	Insertional	1.6
Fimbria (FimH and FimA)	Insertional	1-4
Flagellin (FliC and FliD)	Insertional	1.2-33
Lipoproteins		
INP	C-terminal	7-119
Lpp=OmpA	C-terminal	27-74
PAL	N-terminal	29
Tat-dependent lipoprotein	C-terminal	27
TraT	Insertional, C-terminal	1.2-11
Virulence Factors		
AIDA-1	N-terminal	12-65
EaeA	C-terminal	3.9-31.6
EspP	N-terminal	20
EstA	N-terminal	38-60
Invasin	C-terminal	1.1
MSP1a	N-terminal	4.6

[0943] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more short therapeutic peptides or polypeptides fused into surface exposed loops of outer membrane proteins (OMPs), *e.g.*, from enteric bacteria. In a non-limiting example, the short therapeutic peptides or polypeptides expressed by the genetically engineered bacteria are inserted into the outer membrane protein LamB, *e.g.*, from *E. coli*, and displayed on the bacterial cell surface. Extracellular display of peptides through Insertion of peptides into surface exposed loops of LamB is for example described in Hofnung et al., Expression of foreign polypeptides at the *Escherichia coli* cell surface; Methods Cell Biol. 34:77-105, and Charbit, A. et al., 1987. Presentation of two epitopes of the preS2 region of hepatitis B virus on live recombinant bacteria, J. Immunol. 139:1658–1664.

[0944] In another non-limiting example, the short therapeutic peptides or polypeptides encoded by one or more gene sequence(s) comprised in the genetically engineered bacteria are inserted into the outer membrane protein PhoE, *e.g.*, from *E. coli*, and displayed on the bacterial cell surface. The PhoE protein is another abundant outer membrane protein of *E. coli* K-12, which has a trimeric structure and functions as a pore for small molecules. Analysis of the primary structure of PhoE revealed 16 beta sheets which traverse through the membranes, and eight hypervariable regions exposed at the surface of the cell. One or more of these cell surface exposed regions of PhoE protein can be used to insert

heterologous peptides. For example, antigenic determinants of pathogenic organisms have been presented in one or more cell surface exposed regions of PhoE protein (*e.g.*, as described in Aterberg et al., 1990; Outer membrane PhoE protein of *Escherichia coli* as a carrier for foreign antigenic determinants: immunogenicity of epitopes of foot-and-mouth disease virus; Vaccine. 1990 Feb;8(1):85-91).

[0945] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more short therapeutic peptides or polypeptides fused to protein components of extracellular appendages. Several systems have been described, in which extracellular appendages, such as pili and flagella are used to display peptides of interest at the bacterial cell surface. Examples of flagellar and pilar proteins used include FliC, a major structural component of the *E. coli* flagellum, and PapA, the major subunit of the Pap pilus. In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more components of a FLITRX system. The FLITRX system is an *E. coli* display system based on the use of fusion protein of FliC and thioredoxin, a small redox protein which represents a highly versatile scaffold that allows peptide inserts to assume a confirmation compatible with binding to other proteins. In the FLITRX system, thioredoxin is fused into a dispensable region of FliC. Then, heterologous peptides can be inserted within the thioredoxin domain in the FliC fusion, and are surface exposed. Other scaffolding proteins are known in the art, some of which may replace thioredoxin as a scaffolding protein in this system.

[0946] In some embodiments, the genetically engineered bacteria comprise a FimH fusion protein, in which the therapeutic peptide of interest is fused to FimH, an adhesin of type 1 fimbriae, *e.g.*, from *E. coli*. FimH adhesin chimeras containing as many as 56 foreign amino acids in certain positions are transported to the bacterial surface as components of the fimbrial organelles (Pallesen et al., Chimeric FimH adhesion of type I fimbriae: a bacterial surface display system for heterologous sequences. Microbiology 141: 2839-2848).

[0947] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a fusion protein in which the therapeutic peptide of interest is fused to the major subunit of F11 fimbriae, *e.g.*, from *E. coli*. Hypervariable regions of the major subunit of F11 fimbriae can be used for insertion of heterologous peptides, *e.g.*, antigenic epitopes (Van Die et al., Expression of foreign epitopes in P-fimbriae of *Escherichia coli*. Mol. Gen. Genet. 222: 297-303).

[0948] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a papA fusion protein, in which the therapeutic peptide of interest

is fused to papA. In some embodiments, peptides of interest are inserted following either codon 7 or 68 of the coding sequence for the mature portion of PapA, as peptides in the area of amino acids 7 and 68 of PapA are localized at the external side of the pilus (Steidler et al., Pap pili as a vector system for surface exposition of an immunoglobulin G-binding domain of protein A of Staphylococcus aureus in *Escherichia coli*; J Bacteriol. 1993 Dec;175(23):7639-43).

[0949] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s), which encode polypeptides larger than 60 amino acids, *e.g.*, immune modulatory effector, and which are displayed on the bacterial cell surface. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s), which encode a fusion protein, in which a therapeutic peptide of interest, *e.g.*, a polypeptide greater than 60 amino acids in length, is fused to a lipoprotein from a gram negative bacterium, or one or more fragments thereof.

[0950] In one embodiment, the genetically engineered bacteria comprise one or more gene sequence(s), which encode a fusion protein, in which a therapeutic protein of interest is fused to peptidoglycan associated lipoprotein (PAL) or a fragment thereof. The fusion protein in located in the periplasm and can be displayed externaly upon permeablization of the outer membrane. For example, a PAL-scFv fusion protein was shown to bind its antigen and to be tightly bound to the murein layer of the cell envelope (Fuchs et al., Targeting recombinant antibodies to the surface of *Escherichia coli* fusion to a peptidoglycan-associated lipoprotein; Biotechnology (N Y). 1991 Dec;9(12):1369-72). The PAL-scFv fusion was located in the periplasm and bound to the murein layer, and after permeabilization of the outer membrane, the scFv became accessible to externally added antigen. In some embodiments, the genetically engineered bacteria comprising a fusion protein for surface display further have a permeable outer membrane. Mutations and/or deletions resulting in a leaky outer membrane are described elsewhere herein.

[0951] In one embodiment, the genetically engineered bacteria encode a fusion protein, in which a therapeutic protein of interest, *e.g.*, a immune modulatory effector, is fused to residues of the major lipoprotein of a gram negative bacterium, *e.g.*, *E. coli*. In one embodiment, the genetically engineered bacteria encode a fusion protein, in which a therapeutic protein of interest, is fused to the signal peptide and the nine N-terminal amino acid residues of the major lipoprotein of a gram negative bacterium, *e.g.*, *E. coli*. These residues of the *E. coli* major lipoprotein function as a hydrophobic membrane anchor. For example, a fusion construct of these residues with a therapeutic polypeptide, in this case a

scFv fragment, resulted in specific accumulation of an immunoreactive and cell-bound polypeptide in *E. coli* (Laukkanen et al., Lipid-tagged antibodies: bacterial expression and characterization of a lipoprotein-single-chain antibody fusion protein. Mol. Microbiol. 4:1259-1268).

[0952] In one embodiment, the genetically engineered bacteria encode a fusion protein, in which a therapeutic protein of interest, is inserted into the TraT protein of a gram negative bacterium, *e.g.*, *E. coli*, *e.g.* at position 180. The TraT protein is a surface-exposed lipoprotein, specified by plasmids of the IncF group, that mediates serum resistance and surface exclusion. Taylor et al. showed that insertion of the C3 epitope of polio virus, *e.g.*, at position 180, allowed exposure of the antigen to the cell surface, while the oligomeric conformation of the wild-type protein was maintained (Taylor et al., The TraT lipoprotein as a vehicle for the transport of foreign antigenic determinants to the cell surface of *Escherichia coli* K12: structure-function relationship in the TraT protein. Mol Microbiol. 1990 Aug;4(8):1259-68).

[0953] In one embodiment, the genetically engineered bacteria comprise one or more genes and/or gene cassettes encoding a fusion protein comprising a Lpp-OmpA display vehicle comprising the N terminal outer membrane signal from the major lipoprotein (Lpp) fused to a domain from the outer membrane protein OmpA, fused to the therapeutic polypeptide of interest. In this system, the Lpp signal peptide mediates localization, and OmpA provides the framework for the display of the therapeutic protein of interest. Lpp-OmpA fusions have been used to display several proteins between 20 and 54 kDa in size on the surface of *E. coli* (see, *e.g.*, Staphopoulos et al., Characterization of *Escherichia coli* expressing and Lpp-OpmA (46-159)-PhoA fusion protein localized in the outer membrane). For example, Fransco et al fused beta -lactamase to the N-terminal targeting sequence of Lpp and an OmpA fragment containing 5 of the 8 membrane spanning loops of the native protein. This fusion protein was assembled on the cell surface and the beta-lactamase domain was stably anchored in the cell wall (Fransisco et al., Transport ansd anchoring of beta-lactamase to the external surface of *Escherichia coli*; Proc. Natl. Acad. Sci. USA Vol 89, pp. 2713-2717, 1992).

[0954] In one embodiment, the Type II secretion pathway or a variation thereof is used to for transient or longer duration display of therapeutic proteins of interest on the bacterial cell surface, *e.g.*, the IgA protease secretion pathway of Neisseria or the VirG protein pathway of Shigella. In one embodiment, the IgA protease secretion pathway is used to export and display therapeutic peptides of interest on the cell surface of gram negative

bacteria. The IgA proteases of Neisseria gonorrhoeae and Hemophilus influenza use a variation of the most common, Type II secretion pathway, to achieve extracellular export independent of any other gene products. The IgA genes of Neisseria species encode extracellular proteins that cleave human IgA1 antibody. The iga gene alone is sufficient to direct selected extracellular secretion of IgA protease in Neisseria, Salmonella, and *E. coli* species (Klauser et al., 1993, Extracellular transport of cholera toxin B subunit using Neisseria IgA protease beta-domain: conformation-dependent outer membrane translocation. EMBO J 9:1991-1999, and references therein). The mature IgA protease is processed in several steps from a large precursor by signal peptidase and autoproteolytic cleavage. The precursor consists of four domains: (1) an aminoterminal signal peptide which mediates inner membrane transport; (2) the protease domain (3) the alpha domain, a basic alpha helical region which is secreted with the protease and (4) the autotransporter beta domain which harbors the essential function for outer membrane transport. Essentially, the C-terminal beta autotransporter domain of the IgA protease forms a channel in the outer membrane that mediates the export of the N terminal domain across the membrane, which in turn becomes transiently displayed on the external surface of the bacteria. The alpha domain and protease domain are then released through proteolytic cleavage. Klauser et al. (1993), showed that replacement of the native N-terminal domains of IgA protease of N. gonorrhoeae with the cholera toxin B resulted in the surface presentation of the passenger polypeptide in S. typhymurium. In another study, the signal sequence and the C-terminal beta autotransporter domain of the IgA protease of Neisseria gonorrhoeae was used to translocate and display a scFv directed against a porcine epidemic diarrhea virus epitope on the bacterial cell surface of *E. coli* (Pyo et al., *Escherichia coli* expressing single chain Fv on the cell surface as a potential prophylactic of porcine epidemic diarrhea virus; Vaccine (27) (2009) 2030-2036.).

[0955] Thus, in one embodiment, the genetically engineered bacteria encode a IgA protease fragment in which the alpha domain is substituted with a therapeutic protein of interest, and fused to a functional IgA protease beta-domain, which mediates export through the outer membrane. Without wishing to be bound by theory, IgA protease activity is eliminated in such a fusion protein, and therefore the autoproteoulytic release of the fusion protein into the medium does not occur, resulting in the display of the therapeutic protein of interest on the cell surface of the gram-negative host bacterium.

[0956] The secretion of VirG protein from Shigella is similar to the export system utilized by the IgA protease of Neisseria (see., *e.g.*, Suzuki et al., 1995; Extracellular transport of VirG protein in Shigella J Biol. Chem 270:30874-30880, and references therein).

Thus, in some embodiments, the genetically engineered bacteria encode a fusion protein comprising a therapeutic protein of interest fused to the membrane spanning region of VirG, resulting in surface display of the therapeutic protein of interest. The VirG gene on the large plasmid of Shigella has been shown to be responsible for the localized deposition of filamentous actin (F-actin) trailing from one pole of invading bacterial cells and extending in a filament through the host epithelial cytoplasm. VirG is a surface-exposed outer membrane protein consisting of three distinctive domains, the N-terminal signal sequence (amino acids 1-52), the id α-domain (amino acids 53-758), and the dC-terminal β-core (amino acids 759-1102) (see, *e.g.*, Suzuki et al., 1996; Functional Analysis of Shigella VirG Domains Essential for Interaction with Vinculin and Actin-based Motility; J. Biol. Chem., 271, 21878-21885, and references therein). Suzuki et al. (1995); showed that the fusion of a foreign protein such as MalE or PhoA protein to the N terminus 37-kDa VirG portion resulted in the transport of the passenger polypeptides from the periplasm to the external side of the outer membrane, indicating that the C-terminal 37-kDa VirG portion embedded in the outer membrane is involved in the translocation of the preceding VirG portion or the heterologous or passenger polypeptide from the periplasmic space to the external side of the outer membrane, in a manner homologous to the IgA protease beta-domain. In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a fusion protein, in which a C-terminal 37-kDa VirG protein fragment is fused to a therapeutic protein of interest.

[0957] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a fusion protein, in which a therapeutic protein of interest is fused to pullulanase for temporary surface display. Pullulanase is specifically released into the medium by Klebsiella pneumonieae, and exists as a fully exposed, cell surface-bound intermediate before it is released into the medium from early stationary growth phase onwards. Cell-surface anchoring is accomplished by an N-terminal fatty acyl modification whose chemical composition is identical to that of other bacterial protein.

[0958] Unlike the IgA protease, the lipoprotein pullulanase (PulA) of Klebsiella pneumoniae, which is also exported via a type II secretion mechanism, requires 14 genes for its translocation across the outer membrane. For example, Pugsley and coworkers have shown that the lipoprotein pullulanase (PulA) can facilitate translocation of the periplasmic enzyme beta-lactamase across the outer membrane. In particular, in *E. coli* strains expressing all pullulanase secretion genes, pullulanase-beta-lactamase hybrid protein molecules containing an N-terminal 834-amino-acid pullulanase segment were efficiently transported to

the cell surface. Of note, pullulanase hybrids remain only temporarily attached to the bacterial surface and are subsequently released into the medium (Kornacker and Pugsley: The normally periplasmic enzyme beta-lactamase is specifically and efficiently translocated through the *Escherichia coli* outer membrane when it is fused to the cell surface enzyme pullulanase. Mol. Microbiol. 4:1101-1109, and references therein). Accordingly, in some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising a complete set of pullulanase genes required for secretion and fusion protein comprising a therapeutic protein of interest fused to a N-terminal pullulanase polypeptide fragment, *e.g.*, as described by Kornacker and Pugsley. In some embodiments, the fusion proteins comprising N-terminal pullulanase polypeptide fused to the therapeutic protein of interest, are transiently displayed on the surface of the bacterial cell, and subsequently released into the media or extracellular space.

[0959] In one embodiment, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a fusion protein in which the ice nucleation protein (INP) from Pseudomonas syringae anchors a therapeutic protein of interest in the cell wall. INP is a secretory protein that catalyzes extracellular ice formation as the ice nuclei. INP has been found in a number of Gram-negative species, including *P. syringae*, *Erwinia herbicola*, *Xanthomonas campestris*, and *Pseudomonas fluorescens*. Four genes in *P. syringae* strains , *inaK*, *inaV* , and *inaZ* , and inaQ exhibit high similarities in sequences and in primary organization (Li et al., Molecular Characterization of an Ice Nucleation Protein Variant (InaQ) from Pseudomonas syringae and the Analysis of Its Transmembrane Transport Activity in *Escherichia coli* Int J Biol Sci. 2012; 8(8): 1097–1108). All INPs (1200 aa to 1500 aa) comprise of three distinct structural domains: (1) the N-terminal domain (approximately 15% of the total sequence), which is relatively hydrophobic and which is are potentially capable of being coupled to the mannan-phosphatidylinositol group in the outer membrane through *N*-glycan (Asp) or *O*-glycan (Ser, Thr) linkages; (2) the C-terminal domain (approximately 4%), which is a relatively hydrophilic terminus; and (3) the central repeating domain (CRD) (approximately 81%), which constitutes contiguous repeats given by 16-residue (or 48-residue) periodicities with a consensus octapeptide (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr). INPs have been employed in various bacterial cell-surface display systems including *E. coli*, *Zymomonas mobilis*, *Salmonella*s sp., *Vibrio anguillarum*, *Pseudomonas putida*, and cyanobacteria, in all od which INPs were able to target a heterologous protein onto the surface of the host cell. Moreover, the N-terminal region alone was shown to direct translocation of foreign proteins to the cell surface and can be employed as a potential

cell surface display motif (Li et al., 2004 Functional display of foreign protein on surface of *Escherichia coli* using N-terminal domain of ice nucleation protein; Biotechnol Bioeng. 2004 Jan 20;85(2):214-21). Accordingly, in some embodiments, the genetically engineered bacteria comprise IMP fusions for surface display of a therapeutic peptide of interest. In some embodiment,s the N-terminal region of the INP protein is fused to the polypeptide of interest for surface display.

[0960] IMP proteins further have modifiable internal repeating units, ie., CRD length is adjustable, which is allows flexibility in protein fusion length (Jung et al., 1998), and also can accommodate larger polypeptides. For example, the INP-based display systems were used to successfully express a 90 kDA protein on the cell surface of *E. coli* (Wu et al., 2006; Cell surface display of Chi92 on *Escherichia coli* using ice nucleation protein for improved catalytic and antifungal activity; FEMS Bicrobiology Letters, Volume 256, Issue 1; Pages 119–125).

[0961] It is understood by those skilled in the art that translocation of such fusion or hybrid proteins described herein requires a "translocation-competent" conformation, *e.g.*, the formation of disulfide bonds, *e.g.*, in the periplasmic space, may be undesirable and inhibit translocation through the outer membrane (see, *e.g.*, Klauser et al., 1990), or alternatively may be required for, (or at least not impede) translocation through the outer membrane (see, *e.g.*, Puggsley, 1992; Translocation of a folded protein across the outer membrane in *Escherichia coli*; Proc Natl Acad Sci U S A. 1992 Dec 15; 89(24): 12058–12062). In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding for a fusion protein in which disulfide bonds are prevented from forming prior to the translocation to the cell surface. In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding for a fusion protein in which disulfide bonds are formed prior to translocation to the cell surface.

[0962] Expression systems for the display of proteins in Gram-positive bacteria have also been developed. Consequently, in some embodiments, gram positive bacteria are engineered to display therapeutic proteins of interest on their cell surface. Uhlen et al. used fusions to the cell-wall bound, X-domain of protein A, for the display of foreign peptides up to 88 amino acids long to the surface of Staphylococcus strains. For example one study describes an expression system to allow targeting of heterologous proteins to the cell surface of Staphylococcus xylosus, a coagulase-negative gram-positive bacterium (Hansson et al., Expression of recombinant proteins on the surface of the coagulase-negative bacterium Staphylococcus xylosus; J Bacteriol. 1992 Jul;174(13):4239-45).

[0963] The expression of recombinant gene fragments, fused between gene fragments encoding the signal peptide and the cell surface-binding regions of staphylococcal protein A, targets the resulting fusion proteins to the outer bacterial cell surface via the membrane-anchoring region and the highly charged cell wall-spanning region of staphylococcal protein A. Accordingly, in some embodiments, the genetically engineered bacteria comprise one or more gene sequences encoding a therapeutic polypeptide fused between gene fragments encoding the signal peptide and the cell surface-binding regions of staphylococcal protein A

[0964] *E. coli*-staphylococcus shuttle vectors have been constructed by taking advantage of the promoter, signal sequence, and propeptide region from the lipase gene construct derived from S. hyicus and the cell surface attachment part of staphylococcal protein A. This system has been investigated for the surface display of heterologous polypeptides on S. carnosus (Samuelson et al., Cell surface display of recombinant proteins on Staphylococcus carnosus; J Bacteriol. 1995 Mar;177(6):1470-6). In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a therapeutic polypeptide fusion protein comprising promoter, signal sequence, and propeptide region from the lipase gene construct derived from S. hyicus and the cell surface attachment part of staphylococcal protein A.

[0965] In other studies, the fibrillary M6 proteins of Streptococcus pyrogenes was employed as a carrier for antigen delivery in Streptococcus cells. (Pozzi et al., 1992; Delivery and expression of a heterologous antigen on the surface of streptococci. Infect. Immunm. 60: 1902-1907). In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) comprising therapeutic polypeptide fusion proteins comprising the fibrillary M6 proteins of Streptococcus pyrogenes for cell surface display of the therapeutic polypeptide.

[0966] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a polypeptide of interest which is displayed on the cell surface through a fusion with an intimin or invasin. Intimins and invasins belong to a family of bacterial adhesins which specifically interact with various eukaryotic cell surface receptors, thereby mediating bacterial adherence and invasion. Both intimins and invasins provide a structural scaffold ideally suited to the cell surface display.

[0967] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a polypeptide of interest which is displayed on the cell surface through a fusion with an intimin, *e.g.*, with the Enterohemorragic *E. coli* Intimin EaeA protein or a carboxy-terminal truncation thereof (*e.g.*, as described inWentzel et al,

Display of Passenger Proteins on the Surface of *Escherichia coli* K-12 by the Enterohemorrhagic *E. coli* Intimin EaeA J Bacteriol. 2001 Dec; 183(24): 7273–7284). For example, N-terminal 489 amino acids of invasin are sufficient to promote the localization of a fusion protein to the cell surface. [030] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a polypeptide of interest which is displayed on the cell surface through a fusion with an invasin, *e.g.* Enterohemorrhagic *E. coli* invasion, or a carboxyterminal truncation thereof. For example, N-terminal 539 amino acids of intimin were sufficient to promote outer membrane localization of a fusion protein (Liu et al., The Tir-binding region of enterohaemorrhagic *Escherichia coli* intimin is sufficient to trigger actin condensation after bacterial-induced host cell signaling; Mol Microbiol. 1999 Oct;34(1):67-81).

[0968] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a polypeptide of interest which is displayed on the cell surface through a fusion with Bacillus anthracis exosporal protein (BclA) as an anchoring motif. The BclA is an exosporium protein, a hair-like protein surrounding the B. anthracis spore. In a nonlimiting example, a polypeptide of interest is linked to the C-terminus of N-terminal domain (21 amino acids) of BclA, *e.g.*, as described in Park et al. (Surface display of recombinant proteins on *Escherichia coli* by BclA exosporium of Bacillus anthracis).

[0969] Various other anchoring motifs have been developed including OprF, OmpC, and OmpX. In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding a polypeptide of interest which is displayed on the cell surface through a fusion with OprF, OmpC, and OmpX.

[0970] In some embodiments, the therapeutic polypeptides of interest are permanently displayed on the cell surface of the genetically engineered bacterium. In some embodiments, the therapeutic polypeptides of interest are transiently displayed on the cell surface of the genetically engineered bacterium.

[0971] In some embodiments, the therapeutic polypeptides are displayed in strains, *e.g.*, described herein which display a leaky phenotype. Such strains have deactivating mutations in one or more of genes encoding a protein that tethers the outer membrane to the peptidoglycan skeleton, *e.g.*, lpp, ompC, ompA, ompF, tolA, tolB, pal, and/or one or more genes encoding a periplasmic protease, *e.g.*, degS, degP, nlpI.

[0972] In some embodiments, one or more ScFvs are displayed on the bacterial cell surface, alone or in combination with other therapeutic polypeptides of interest.

[0973] In some embodiments, a cell surface display strategy or circuit is combined with a secretion strategy or circuit in one bacterium. In some embodiments, the same polypeptide is both displayed and secreted. In some embodiments, a first polypeptide is displayed and a second is secreted. In some embodiments, a display strategy or circuit strategy is combined with a circuit for the intracellular production of an enzyme and consequentially intracellular catabolism of its substrate. In some embodiments, a display strategy or display circuit is combined with a circuit for the intracellular production of a gut barrier enhancer molecule and/or an anti-inflammatory effector molecule.

[0974] In some embodiments, the expression of the surface displayed polypeptide or fusion protein is driven by an inducible promoter. In some embodiments, the inducible promoter is an oxygen level-dependent promoter (*e.g.*, FNR-inducible promoter). In some embodiments, the inducible promoter is induced by gut-specific and/or tumor-specific or promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), or promoters induced by a metabolite that may or may not be naturally present (*e.g.*, can be exogenously added) in the gut, *e.g.*, arabinose. In alternate embodiments, expression of the surface displayed polypeptides or polypeptide fusion proteins is driven by a constitutive promoter.

[0975] In some embodiments, the expression of the surface displayed polypeptide or fusion protein is plasmid based. In some embodiments, the gene sequence(s) encoding the antibodies or scFv fragments for surface display is chromosomally inserted.

Table 49. Selected display anchors

Invasin **display tag SEQ ID NO: 990**	MVFQPISEFLLIRNAGMSMYFNKIISFNIISRIVICIFLICGMFMAGASEKYDANAPQQV QPYSVSSSAFENLHPNNEMESSINPFSASDTERNAAIIDRANKEQETEAVNKMISTGARL AASGRASDVAHSMVGDAVNQEIKQWLNRFGTAQVNLNFDKNFSLKESSLDWLAPWYDSAS FLFFSQLGIRNKDSRNTLNLGVGIRTLENGWLYGLNTFYDNDLTGHNHRIGLGAEAWTDY LQLAANGYFRLNGWHSSRDFSDYKERPATGGDLRANAYLPALPQLGGKLMYEQYTGERVA LFGKDNLQRNPYAVTAGINYTPVPLLTVGVDQRMGKSSKHETQWNLQMNYRLGESFQSQL SPSAVAGTRLLAESRYNLVDRNNNIVLEYQKQQVVKLTLSPATISGLPGQVYQVNAQVQG ASAVREIVWSDAELIAAGGTLTPLSTTQFNLVLPPYKRTAQVSRVTDDLTANFYSLSALA VDHQGNRSNSFTLSVTVQQPQLTLTAAVIGDGAPANGKTAITVEFTVADFEGKPLAGQEV VITTNNGALPNKITEKTDANGVARIALTNTTDGVTVVTAEVEGQRQSVDTHFVKGTIAAD KSTLAAV
LppOmpA **display tag SEQ ID NO: 991**	KATKLVLGAVILGSTLLAGCSSNAKIDQGINPYVGFEMGYDWLGRMPYKGSVENGAYKAQ GVQLTAKLGYPITDDLDIYTRLGGMVWRADTKSNVYGKNHDTGVSPVFAGGVEYAITPEI ATRLEYQWTNNIGDAHTIGTRPDNGIPG
IntiminN **display tag SEQ ID NO: 992**	ITHGCYTRTRHKHKLKKTLIMLSAGLGLFFYVNQNSFANGENYFKLGSDSKLLTHDSYQN RLFYTLKTGETVADLSKSQDINLSTIWSLNKHLYSSESEMMKAAPGQQIILPLKKLPFEY SALPLLGSAPLVAAGGVAGHTNKLTKMSPDVTKSNMTDDKALNYAAQQAASLGSQLQSRS LNGDYAKDTALGIAGNQASSQLQAWLQHYGTAEVNLQSGNNFDGSSLDFLLPFYDSEKML

	AFGQVGARYIDSRFTANLGAGQRFFLPANMLGYNVFIDQDFSGDNTRLGIGGEYWRDYFK SSVNGYFRMSGWHESYNKKDYDERPANGFDIRFNGYLPSYPALGAKLIYEQYYGDNVALF NSDKLQSNPGAATVGVNYTPIPLVTMGIDYRHGTGNENDLLYSMQFRYQFDKSWSQQIEP QYVNELRTLSGSRYDLVQRNNNIILEYKKQDILSLNIPHDINGTEHSTQKIQLIVKSKYG LDRIVWDDSALRSQGGQIQHSGSQSAQDYQAILPAYVQGGSNIYKVTARAYYRNGNSSNN VQLTITVLSNGQVVDQVGVTDFTADKTSAKADNADTITYTATVKKNGVAQANVPVSFNIV SGTATLGANSAKTDANGKATVTLKSSTPGQVVVSAKTAEMTSALNASAVIFFDQTKAS

[0976] In some embodiments, the scFv Display Construct Sequence is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the sequence of **SEQ ID NO: 987, SEQ ID NO: 988**, and/or **SEQ ID NO: 989**.

[0977] In some embodiments, the display anchor is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the sequence of **SEQ ID NO: 990, SEQ ID NO: 991**, and/or **SEQ ID NO: 992**.

Pharmaceutical Compositions and Formulations

[0978] Pharmaceutical compositions comprising the genetically engineered bacteria described herein may be used to treat, manage, ameliorate, and/or prevent a liver disorder, such as NASH, or symptom(s) associated with liver disorders, such as NASH. Pharmaceutical compositions comprising one or more genetically engineered bacteria, alone or in combination with prophylactic agents, therapeutic agents, and/or pharmaceutically acceptable carriers are provided.

[0979] In certain embodiments, the pharmaceutical composition comprises one species, strain, or subtype of bacteria that are engineered to comprise the genetic modifications described herein, *e.g.*, to express a propionate gene cassette, a butyrate gene cassette, GLP-1, or combinations thereof. In alternate embodiments, the pharmaceutical composition comprises two or more species, strains, and/or subtypes of bacteria that are each engineered to comprise the genetic modifications described herein, *e.g.*, to express a propionate gene cassette, a butyrate gene cassette, GLP-1, or combinations thereof.

[0980] The pharmaceutical compositions described herein may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into compositions for pharmaceutical use. Methods of formulating pharmaceutical compositions are known in the art (*see, e.g.,* "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA). In some embodiments, the pharmaceutical compositions are subjected to

tabletting, lyophilizing, direct compression, conventional mixing, dissolving, granulating, levigating, emulsifying, encapsulating, entrapping, or spray drying to form tablets, granulates, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. Appropriate formulation depends on the route of administration.

[0981] The genetically engineered bacteria described herein may be formulated into pharmaceutical compositions in any suitable dosage form (*e.g.,* liquids, capsules, sachet, hard capsules, soft capsules, tablets, enteric coated tablets, suspension powders, granules, or matrix sustained release formations for oral administration) and for any suitable type of administration (*e.g.,* oral, topical, injectable, immediate-release, pulsatile-release, delayed-release, or sustained release). Suitable dosage amounts for the genetically engineered bacteria may range from about 10^5 to 10^{12} bacteria, *e.g.*, approximately 10^5 bacteria, approximately 10^6 bacteria, approximately 10^7 bacteria, approximately 10^8 bacteria, approximately 10^9 bacteria, approximately 10^{10} bacteria, approximately 10^{11} bacteria, or approximately 10^{11} bacteria. The composition may be administered once or more daily, weekly, or monthly. The composition may be administered before, during, or following a meal. In one embodiment, the pharmaceutical composition is administered before the subject eats a meal. In one embodiment, the pharmaceutical composition is administered currently with a meal. In one embodiment, the pharmaceutical composition is administered after the subject eats a meal.

[0982] The genetically engineered bacteria may be formulated into pharmaceutical compositions comprising one or more pharmaceutically acceptable carriers, thickeners, diluents, buffers, buffering agents, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers or agents. For example, the pharmaceutical composition may include, but is not limited to, the addition of calcium bicarbonate, sodium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20. In some embodiments, the genetically engineered bacteria may be formulated in a solution of sodium bicarbonate, *e.g.,* 1 molar solution of sodium bicarbonate (to buffer an acidic cellular environment, such as the stomach, for example). The genetically engineered bacteria may be administered and formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium,

ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. The genetically engineered bacteria disclosed herein may be administered topically and formulated in the form of an ointment, cream, transdermal patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form well-known to one of skill in the art. *See, e.g.,* "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA. In an embodiment, for non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity greater than water are employed. Suitable formulations include, but are not limited to, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, etc., which may be sterilized or mixed with auxiliary agents (*e.g.,* preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, *e.g.,* osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (*e.g.,* a gaseous propellant, such as freon) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms. Examples of such additional ingredients are well known in the art. In one embodiment, the pharmaceutical composition comprising the engineered bacteria may be formulated as a hygiene product. For example, the hygiene product may be an antibacterial formulation, or a fermentation product such as a fermentation broth. Hygiene products may be, for example, shampoos, conditioners, creams, pastes, lotions, and lip balms.

[0983] The genetically engineered bacteria disclosed herein may be administered orally and formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, etc. Pharmacological compositions for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients include, but are not limited to, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose compositions such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP) or polyethylene glycol (PEG). Disintegrating agents may also be added, such as cross-linked polyvinylpyrrolidone, agar, alginic acid or a salt thereof such as sodium alginate.

[0984] Tablets or capsules can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.,* pregelatinised maize starch, polyvinylpyrrolidone, hydroxypropyl methylcellulose, carboxymethylcellulose, polyethylene glycol, sucrose, glucose, sorbitol, starch, gum, kaolin, and tragacanth); fillers (*e.g., l*actose, microcrystalline cellulose, or calcium hydrogen phosphate); lubricants (*e.g.,* calcium, aluminum, zinc, stearic acid, polyethylene glycol, sodium lauryl sulfate, starch, sodium benzoate, L-leucine, magnesium stearate, talc, or silica); disintegrants (*e.g.,* starch, potato starch, sodium starch glycolate, sugars, cellulose derivatives, silica powders); or wetting agents (*e.g.,* sodium lauryl sulphate). The tablets may be coated by methods well known in the art. A coating shell may be present, and common membranes include, but are not limited to, polylactide, polyglycolic acid, polyanhydride, other biodegradable polymers, alginate-polylysine-alginate (APA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroymethylacrylate-methyl methacrylate (HEMA-MMA), multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitrile/sodium methallylsulfonate (AN-69), polyethylene glycol/poly pentamethylcyclopentasiloxane/polydimethylsiloxane (PEG/PD5/PDMS), poly N,N-dimethyl acrylamide (PDMAAm), siliceous encapsulates, cellulose sulphate/sodium alginate/polymethylene-co-guanidine (CS/A/PMCG), cellulose acetate phthalate, calcium alginate, k-carrageenan-locust bean gum gel beads, gellan-xanthan beads, poly(lactide-co-glycolides), carrageenan, starch poly-anhydrides, starch polymethacrylates, polyamino acids, and enteric coating polymers.

[0985] In some embodiments, the genetically engineered bacteria are enterically coated for release into the gut or a particular region of the gut, for example, the large intestine. The typical pH profile from the stomach to the colon is about 1-4 (stomach), 5.5-6 (duodenum), 7.3-8.0 (ileum), and 5.5-6.5 (colon). In some diseases, the pH profile may be modified. In some embodiments, the coating is degraded in specific pH environments in order to specify the site of release. In some embodiments, at least two coatings are used. In some embodiments, the outside coating and the inside coating are degraded at different pH levels.

[0986] In some embodiments, enteric coating materials may be used, in one or more coating layers (*e.g.*, outer, inner and/o intermediate coating layers). Enteric coated polymers remain unionised at low pH, and therefore remain insoluble. But as the pH increases in the gastrointestinal tract, the acidic functional groups are capable of ionisation, and the polymer swells or becomes soluble in the intestinal fluid.

[0987] Materials used for enteric coatings include Cellulose acetate phthalate (CAP), Poly(methacrylic acid-co-methyl methacrylate), Cellulose acetate trimellitate (CAT), Poly(vinyl acetate phthalate) (PVAP) and Hydroxypropyl methylcellulose phthalate (HPMCP), fatty acids, waxes, Shellac (esters of aleurtic acid), plastics and plant fibers. Additionally, Zein, Aqua-Zein (an aqueous zein formulation containing no alcohol), amylose starch and starch derivatives, and dextrins (*e.g.*, maltodextrin) are also used. Other known enteric coatings include ethylcellulose, methylcellulose, hydroxypropyl methylcellulose, amylose acetate phthalate, cellulose acetate phthalate, hydroxyl propyl methyl cellulose phthalate, an ethylacrylate, and a methylmethacrylate.

[0988] Coating polymers also may comprise one or more of, phthalate derivatives, CAT, HPMCAS, polyacrylic acid derivatives, copolymers comprising acrylic acid and at least one acrylic acid ester, Eudragit™ S (poly(methacrylic acid, methyl methacrylate)1:2); Eudragit L100™ S (poly(methacrylic acid, methyl methacrylate)1:1); Eudragit L30D™, (poly(methacrylic acid, ethyl acrylate)1:1); and (Eudragit L100-55) (poly(methacrylic acid, ethyl acrylate)1:1) (Eudragit™ L is an anionic polymer synthesized from methacrylic acid and methacrylic acid methyl ester), polymethyl methacrylate blended with acrylic acid and acrylic ester copolymers, alginic acid, ammonia alginate, sodium, potassium, magnesium or calcium alginate, vinyl acetate copolymers, polyvinyl acetate 30D (30% dispersion in water), a neutral methacrylic ester comprising poly(dimethylaminoethylacrylate) ("Eudragit E™), a copolymer of methylmethacrylate and ethylacrylate with trimethylammonioethyl methacrylate chloride, a copolymer of methylmethacrylate and ethylacrylate, Zein, shellac, gums, or polysaccharides, or a combination thereof.

[0989] Coating layers may also include polymers which contain Hydroxypropylmethylcellulose (HPMC), Hydroxypropylethylcellulose (HPEC), Hydroxypropylcellulose (HPC), hydroxypropylethylcellulose (HPEC), hydroxymethylpropylcellulose (HMPC), ethylhydroxyethylcellulose (EHEC) (Ethulose), hydroxyethylmethylcellulose (HEMC), hydroxymethylethylcellulose (HMEC), propylhydroxyethylcellulose (PHEC), methylhydroxyethylcellulose (M H EC), hydrophobically modified hydroxyethylcellulose (NEXTON), carboxymethyl hydroxyethylcellulose (CMHEC), Methylcellulose, Ethylcellulose, water soluble vinyl acetate copolymers, gums, polysaccharides such as alginic acid and alginates such as ammonia alginate, sodium alginate, potassium alginate, acid phthalate of carbohydrates, amylose acetate phthalate, cellulose acetate phthalate (CAP), cellulose ester phthalates, cellulose ether phthalates, hydroxypropylcellulose phthalate (HPCP),

hydroxypropylethylcellulose phthalate (HPECP), hydroxyproplymethylcellulose phthalate (HPMCP), hydroxyproplymethylcellulose acetate succinate (HPMCAS).

[0990] Liquid preparations for oral administration may take the form of solutions, syrups, suspensions, or a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable agents such as suspending agents (*e.g.,* sorbitol syrup, cellulose derivatives, or hydrogenated edible fats); emulsifying agents (*e.g.,* lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils); and preservatives (*e.g.,* methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring, and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated for slow release, controlled release, or sustained release of the genetically engineered bacteria described herein.

[0991] In one embodiment, the genetically engineered bacteria of the disclosure may be formulated in a composition suitable for administration to pediatric subjects. As is well known in the art, children differ from adults in many aspects, including different rates of gastric emptying, pH, gastrointestinal permeability, etc. (Ivanovska *et al., Pediatrics*, 134(2):361-372, 2014). Moreover, pediatric formulation acceptability and preferences, such as route of administration and taste attributes, are critical for achieving acceptable pediatric compliance. Thus, in one embodiment, the composition suitable for administration to pediatric subjects may include easy-to-swallow or dissolvable dosage forms, or more palatable compositions, such as compositions with added flavors, sweeteners, or taste blockers. In one embodiment, a composition suitable for administration to pediatric subjects may also be suitable for administration to adults.

[0992] In one embodiment, the composition suitable for administration to pediatric subjects may include a solution, syrup, suspension, elixir, powder for reconstitution as suspension or solution, dispersible/effervescent tablet, chewable tablet, gummy candy, lollipop, freezer pop, troche, chewing gum, oral thin strip, orally disintegrating tablet, sachet, soft gelatin capsule, sprinkle oral powder, or granules. In one embodiment, the composition is a gummy candy, which is made from a gelatin base, giving the candy elasticity, desired chewy consistency, and longer shelf-life. In some embodiments, the gummy candy may also comprise sweeteners or flavors.

[0993] In one embodiment, the composition suitable for administration to pediatric subjects may include a flavor. As used herein, "flavor" is a substance (liquid or solid) that

provides a distinct taste and aroma to the formulation. Flavors also help to improve the palatability of the formulation. Flavors include, but are not limited to, strawberry, vanilla, lemon, grape, bubble gum, and cherry.

[0994] In certain embodiments, the genetically engineered bacteria may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

[0995] In another embodiment, the pharmaceutical composition comprising the engineered bacteria may be a comestible product, for example, a food product. In one embodiment, the food product is milk, concentrated milk, fermented milk (yogurt, sour milk, frozen yogurt, lactic acid bacteria-fermented beverages), milk powder, ice cream, cream cheeses, dry cheeses, soybean milk, fermented soybean milk, vegetable-fruit juices, fruit juices, sports drinks, confectionery, candies, infant foods (such as infant cakes), nutritional food products, animal feeds, or dietary supplements. In one embodiment, the food product is a fermented food, such as a fermented dairy product. In one embodiment, the fermented dairy product is yogurt. In another embodiment, the fermented dairy product is cheese, milk, cream, ice cream, milk shake, or kefir. In another embodiment, the engineered bacteria are combined in a preparation containing other live bacterial cells intended to serve as probiotics. In another embodiment, the food product is a beverage. In one embodiment, the beverage is a fruit juice-based beverage or a beverage containing plant or herbal extracts. In another embodiment, the food product is a jelly or a pudding. Other food products suitable for administration of the engineered bacteria are well known in the art. For example, see U.S. 2015/0359894 and US 2015/0238545, the entire contents of each of which are expressly incorporated herein by reference. In yet another embodiment, the pharmaceutical composition is injected into, sprayed onto, or sprinkled onto a food product, such as bread, yogurt, or cheese.

[0996] In some embodiments, the composition is formulated for intraintestinal administration, intrajejunal administration, intraduodenal administration, intraileal administration, gastric shunt administration, or intracolic administration, via nanoparticles,

nanocapsules, microcapsules, or microtablets, which are enterically coated or uncoated. The pharmaceutical compositions may also be formulated in rectal compositions such as suppositories or retention enemas, using, *e.g.,* conventional suppository bases such as cocoa butter or other glycerides. The compositions may be suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain suspending, stabilizing and/or dispersing agents.

[0997] The genetically engineered bacteria described herein may be administered intranasally, formulated in an aerosol form, spray, mist, or in the form of drops, and conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant (*e.g.,* dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas). Pressurized aerosol dosage units may be determined by providing a valve to deliver a metered amount. Capsules and cartridges (*e.g.,* of gelatin) for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0998] The genetically engineered bacteria may be administered and formulated as depot preparations. Such long acting formulations may be administered by implantation or by injection, including intravenous injection, subcutaneous injection, local injection, direct injection, or infusion. For example, the compositions may be formulated with suitable polymeric or hydrophobic materials (*e.g.,* as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives (*e.g.,* as a sparingly soluble salt).

[0999] In some embodiments, disclosed herein are pharmaceutically acceptable compositions in single dosage forms. Single dosage forms may be in a liquid or a solid form. Single dosage forms may be administered directly to a patient without modification or may be diluted or reconstituted prior to administration. In certain embodiments, a single dosage form may be administered in bolus form, *e.g.,* single injection, single oral dose, including an oral dose that comprises multiple tablets, capsule, pills, etc. In alternate embodiments, a single dosage form may be administered over a period of time, *e.g.,* by infusion.

[01000] Single dosage forms of the pharmaceutical composition may be prepared by portioning the pharmaceutical composition into smaller aliquots, single dose containers, single dose liquid forms, or single dose solid forms, such as tablets, granulates, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. A single dose in a solid form may be reconstituted by adding liquid, typically sterile water or saline solution, prior to administration to a patient.

[01001] In other embodiments, the composition can be delivered in a controlled release or sustained release system. In one embodiment, a pump may be used to achieve controlled or sustained release. In another embodiment, polymeric materials can be used to achieve controlled or sustained release of the therapies of the present disclosure (see *e.g.*, U.S. Patent No. 5,989,463). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N- vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. The polymer used in a sustained release formulation may be inert, free of leachable impurities, stable on storage, sterile, and biodegradable. In some embodiments, a controlled or sustained release system can be placed in proximity of the prophylactic or therapeutic target, thus requiring only a fraction of the systemic dose. Any suitable technique known to one of skill in the art may be used.

[01002] Dosage regimens may be adjusted to provide a therapeutic response. Dosing can depend on several factors, including severity and responsiveness of the disease, route of administration, time course of treatment (days to months to years), and time to amelioration of the disease. For example, a single bolus may be administered at one time, several divided doses may be administered over a predetermined period of time, or the dose may be reduced or increased as indicated by the therapeutic situation. The specification for the dosage is dictated by the unique characteristics of the active compound and the particular therapeutic effect to be achieved. Dosage values may vary with the type and severity of the condition to be alleviated. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgment of the treating clinician. Toxicity and therapeutic efficacy of compounds provided herein can be determined by standard pharmaceutical procedures in cell culture or animal models. For example, LD_{50}, ED_{50}, EC_{50}, and IC_{50} may be determined, and the dose ratio between toxic and therapeutic effects (LD_{50}/ED_{50}) may be calculated as the therapeutic index. Compositions that exhibit toxic side effects may be used, with careful modifications to minimize potential damage to reduce side effects. Dosing may be estimated initially from cell culture assays and animal models. The data obtained from *in vitro* and *in vivo* assays and animal studies can be used in formulating a range of dosage for use in humans. The ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such

as an ampoule or sachet indicating the quantity of active agent. If the mode of administration is by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[01003] The pharmaceutical compositions may be packaged in a hermetically sealed container such as an ampoule or sachet indicating the quantity of the agent. In one embodiment, one or more of the pharmaceutical compositions is supplied as a dry sterilized lyophilized powder or water-free concentrate in a hermetically sealed container and can be reconstituted (*e.g.,* with water or saline) to the appropriate concentration for administration to a subject. In an embodiment, one or more of the prophylactic or therapeutic agents or pharmaceutical compositions is supplied as a dry sterile lyophilized powder in a hermetically sealed container stored between 2° C and 8° C and administered within 1 hour, within 3 hours, within 5 hours, within 6 hours, within 12 hours, within 24 hours, within 48 hours, within 72 hours, or within one week after being reconstituted. Cryoprotectants can be included for a lyophilized dosage form, principally 0-10% sucrose (optimally 0.5-1.0%). Other suitable cryoprotectants include trehalose and lactose. Other suitable bulking agents include glycine and arginine, either of which can be included at a concentration of 0-0.05%, and polysorbate-80 (optimally included at a concentration of 0.005-0.01%). Additional surfactants include but are not limited to polysorbate 20 and BRIJ surfactants. The pharmaceutical composition may be prepared as an injectable solution and can further comprise an agent useful as an adjuvant, such as those used to increase absorption or dispersion, *e.g.,* hyaluronidase.

[01004] In one embodiment, the composition comprises the disclosure provides a composition (e.g., two or more) of different genetically engineered bacteria, each bacteria encoding and producing a different effector molecule.

[01005] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and two or more distinct genetically engineered bacteria comprising gene sequence(s) encoding one or more GLP-1 polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more GLP-1 polypeptide(s).

[01006] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) comprising gene sequence(s) encoding one or more GLP-1 polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and sequence(s) encoding one or more GLP-1 polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more GLP-1 polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more GLP-1 polypeptide(s).

[01007] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition

comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s).

[01008] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), for producing GLP-1, and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s).

[01009] In one embodiment, the composition comprises the bacteria comprising gene sequence(s) encoding one or more bile salt transporter(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria

comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt transporter(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more bile salt transporter(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more bile salt transporter(s).

[01010] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more

biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), and gene sequence(s) encoding GLP-1 and a transporter.

[01011] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). For example, In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least

one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s).

[01012] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), gene sequence(s) encoding GLP-1, gene sequences encoding a transporter and gene sequence(s) encoding one or more bile salt hydrolase polypeptide(s). .

[01013] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene sequence(s) encoding GLP-1 and one or more bile salt hydrolase polypeptide(s).

[01014] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene sequence(s) encoding GLP-1 and a transporter.

[01015] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene

cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and at least one gene cassette for the production or catabolism of tryptophan and/or one of its metabolites. In any of these embodiments, two or more distinct genetically engineered bacteriamay further comprise one or more cassettes for the consumption of ammonia. Suitable gene sequences and circuits for the consumption of ammonia are described in pending International Patent Application PCT/US2015/64140 (published as WO/2016/090343) and International Patent Application PCT/US2016/34200, the contents of which is herein incorporated by reference in its entirety.

[01016] In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In one

embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s) and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, *e.g.*, acetate biosynthesis gene cassette(s), and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid. In one embodiment, the composition comprises two or more distinct genetically engineered bacteria comprising at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing butyrate, *e.g.*, butyrate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing propionate, *e.g.*, propionate biosynthesis gene cassette(s), at least one gene cassette(s) encoding one or more biosynthetic pathway(s) for producing acetate, and at least one gene cassette for the production of a tryptophan metabolite selected from tryptamine and/or indole-3-acetic acid and indole-3-propionic acid.

Methods of Treatment

[01017] Further disclosed herein are methods of treating liver disease, such as nonalcoholic steatohepatitis (NASH). In some embodiments, disclosed herein are methods for reducing, ameliorating, or eliminating one or more symptom(s) associated with these diseases or disorders. In some embodiments, the disclosure provides methods for reducing, ameliorating, or eliminating one or more symptom(s) associated with these diseases, including but not limited to chest pain, heart failure, or weight gain. In some embodiments, the disease is secondary to other conditions, *e.g.,* liver disease.

[01018] In certain embodiments, the bacterial cells are capable of producing propionate, butyrate, and/or GLP-1 in a subject in order to treat nonalcoholic steatohepatitis (NASH). In these embodiments, a patient suffering from nonalcoholic steatohepatitis (NASH) may be able to resume a substantially normal diet, or a diet that is less restrictive.

[01019] The method may comprise preparing a pharmaceutical composition with at least one genetically engineered species, strain, or subtype of bacteria described herein, and administering the pharmaceutical composition to a subject in a therapeutically effective amount. In some embodiments, the genetically engineered bacteria disclosed herein are administered orally, *e.g.,* in a liquid suspension. In some embodiments, the genetically engineered bacteria are lyophilized in a gel cap and administered orally. In some embodiments, the genetically engineered bacteria are administered via a feeding tube or gastric shunt. In some embodiments, the genetically engineered bacteria are administered rectally, *e.g.,* by enema. In some embodiments, the genetically engineered bacteria are administered topically, intraintestinally, intrajejunally, intraduodenally, intraileally, and/or intracolically.

[01020] Before, during, and after the administration of the pharmaceutical composition, ammonia concentrations in the subject may be measured in a biological sample, such as blood, serum, plasma, urine, fecal matter, peritoneal fluid, intestinal mucosal scrapings, a sample collected from a tissue, and/or a sample collected from the contents of one or more of the following: the stomach, duodenum, jejunum, ileum, cecum, colon, rectum, and anal canal.

[01021] In certain embodiments, the genetically engineered bacteria is *E. coli* Nissle. The genetically engineered bacteria may be destroyed, *e.g.,* by defense factors in the gut or blood serum (Sonnenborn *et al.,* 2009), or by activation of a kill switch, several hours or days after administration. Thus, the pharmaceutical composition comprising the engineered bacteria may be re-administered at a therapeutically effective dose and frequency. Length of Nissle residence *in vivo* in mice can be determined. In alternate embodiments, the genetically engineered bacteria are not destroyed within hours or days after administration and may propagate and colonize the gut.

[01022] The methods disclosed herein may comprise administration of a composition alone or in combination with one or more additional therapies, *e.g.,* pioglitazone, which has been shown to improve steatosis, inflammation, and fibrosis; vitamin E, which has been shown to improve steatohepatitis; or orlistat, which has been shown to improve alanine transaminase and steatosis (see, for example, Dyson *et al.,* Frontline Gastroenterology,

5(4):277-286, 2014). The pharmaceutical composition may be administered alone or in combination with one or more additional therapeutic agents. In another embodiment, the methods disclosed herein may comprise a caloric restricted diet (600 calories less than the daily requirement), exercise, or bariatric surgery.

[01023] An important consideration in the selection of the one or more additional therapeutic agents is that the agent(s) should be compatible with the bacteria, *e.g.,* the agent(s) must not interfere with or kill the bacteria. In some embodiments, the pharmaceutical composition is administered with food. In alternate embodiments, the pharmaceutical composition is administered before or after eating food. The pharmaceutical composition may be administered in combination with one or more dietary modifications, *e.g.,* low-protein diet and amino acid supplementation. The dosage of the pharmaceutical composition and the frequency of administration may be selected based on the severity of the symptoms and the progression of the disorder. The appropriate therapeutically effective dose and/or frequency of administration can be selected by a treating clinician.

Examples

[01024] The present disclosure is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references, including literature references, issued patents, and published patent applications, as cited throughout this application are hereby expressly incorporated herein by reference. It should further be understood that the contents of all the figures and tables attached hereto are also expressly incorporated herein by reference.

Development of engineered bacterial cells

Example 1. Construction of Plasmids Encoding Circuits

[01025] The propionate gene circuit genes, butyrate gene circuit genes, or GLP-1 gene are synthesized (Genewiz), fused to the Tet promoter, cloned into the high-copy plasmid pUC57-Kan by Gibson assembly, and transformed into *E. coli* DH5α as described herein to generate the plasmid pTet-BSH.

Example 2. Generation of Engineered Bacteria

[01026] The pTet-BSH plasmid described above is transformed into *E. coli* Nissle, DH5α, or PIR1. All tubes, solutions, and cuvettes are pre-chilled to 4° C. An overnight culture of *E. coli* (Nissle, DH5α or PIR1) is diluted 1:100 in 4 mL of LB and grown

until it reaches an OD_{600} of 0.4-0.6. 1mL of the culture is then centrifuged at 13,000 rpm for 1 min in a 1.5mL microcentrifuge tube and the supernatant is removed. The cells are then washed three times in pre-chilled 10% glycerol and resuspended in 40uL pre-chilled 10% glycerol. The electroporator is set to 1.8kV. 1uL of a pTet-BSH miniprep is added to the cells, mixed by pipetting, and pipetted into a sterile, chilled 1mm cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. 500uL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 37° C for 1 hr. The cells are spread out on an LB plate containing 50ug/mL Kanamycin for pTet-BSH.

Functional assays using engineered bacterial cells

Example 3. Functional Assay Demonstrating that the Engineered Bacterial Cells Produce Propionate, Butyrate, and/or GLP-1

[01027] For *in vitro* studies, all incubations will be performed at 37° C. Cultures of *E. coli* Nissle containing pTet-BSH are grown overnight in LB and then diluted 1:100 in LB. The cells are grown with shaking (250 rpm) to early log phase with the appropriate antibiotics. Anhydrous tetracycline (ATC) is added to cultures at a concentration of 100 ng/mL to induce expression of genes and gene circuits, and bacteria are grown for another 3 hours. Culture broths are then inoculated at 20% in flasks containing fresh LB culture media and grown for 16 hours with shaking (250 rpm). A "medium blank" for each culture condition broth is also prepared whereby the "medium blank" is not inoculated with bacteria but treated under the same conditions as the inoculated broths. Following the 16 hour incubation period, broth cultures are pasteurized at 90°C for 15 minutes, centrifuged at 5,000 rpm for 10 minutes, and supernatants filtered with a 0.45 micron filter. Butyrate, propionate, and GLP-1 levels and activity in the supernatants is determined.

Example 4. In vivo NASH Studies

[01028] For *in vivo* studies, a mouse model is used to study the effects of liver steatosis and hepatic inflammation (Jun Jin, *et al.*, Brit. J. Nutrition, 114:145-1755 (2015)). To briefly summarize, female C57BL/6J mice are fasted and fed either a standard liquid diet of carbohydrates, fat, and protein; or a liquid Western style diet (WSD) fortified with fructose, fat, cholesterol, and a sodium butyrate supplement for six weeks. Butyrate is a short chain fatty acid naturally produced by intestinal bacteria effective in maintaining intestinal

homoeostasis. Body weight and plasma samples can be taken throughout the duration of the study. Upon conclusion of the study, the mice can be killed, and the liver and intestine can be removed and assayed. A decrease in liver damage, e.g., as seen in NASH, after treatment with the engineered bacterial cells indicates that the engineered bacterial cells described herein are effective for treating nonalcoholic steatohepatitis (NASH).

[01029] Additionally, throughout the study, phenotypes of the mice can also be analyzed. A decrease in the number of symptoms associated with nonalcoholic steatohepatitis (NASH), for example, weight loss, further indicates the efficacy of the engineered bacterial cells described herein for treating nonalcoholic steatohepatitis (NASH).

Example 5. Construction of vectors for overproducing butyrate

[01030] To facilitate inducible production of butyrate in *Escherichia coli* Nissle, the eight genes of the butyrate production pathway from *Peptoclostridium difficile 630* (*bcd2, etfB3, etfA3, thiA1, hbd, crt2, bpt,* and *buk*; NCBI), as well as transcriptional and translational elements, were synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. The butyrate gene cassette is placed under the control of a FNR regulatory region selected from SEQ ID NOs: 31-38. In certain constructs, an FNR-responsive promoter is further fused to a strong ribosome binding site sequence. For efficient translation of butyrate genes, each synthetic gene in the operon was separated by a 15 base pair ribosome binding site derived from the T7 promoter/translational start site.

[01031] The gene products of the *bcd2-etfA3-etfB3* genes form a complex that converts crotonyl-CoA to butyryl-CoA and may exhibit dependence on oxygen as a co-oxidant. Because the recombinant bacteria of the invention are designed to produce butyrate in an oxygen-limited environment (*e.g.* the mammalian gut), that dependence on oxygen could have a negative effect of butyrate production in the gut. It has been shown that a single gene from *Treponema denticola*, trans-2-enoynl-CoA reductase (*ter*), can functionally replace this three gene complex in an oxygen-independent manner. Therefore, a second butyrate gene cassette in which the *ter* gene replaces the *bcd2-etfA3-etfB3* genes of the first butyrate cassette is synthesized (Genewiz, Cambridge, MA). The *ter* gene is codon-optimized for *E. coli* codon usage using Integrated DNA Technologies online codon optimization tool (https://www.idtdna.com/CodonOpt). The second butyrate gene cassette, as well as transcriptional and translational elements, is synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. The second butyrate gene cassette is placed under control of a FNR regulatory region as described above.

Example 6. Construction of vectors for overproducing butyrate using an inducible tet promoter- butyrate circuit

[01032] To facilitate inducible production of butyrate in *Escherichia coli* Nissle, the eight genes of the butyrate production pathway from *Peptoclostridium difficile 630* (*bcd2, etfB3, etfA3, thiA1, hbd, crt2, bpt,* and *buk*; NCBI), as well as transcriptional and translational elements, were synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322 to create pLogic031. As synthesized, the genes were placed under control of a tetracycline-inducible promoter, with the *tet* repressor (*tetR*) expressed constitutively, divergent from the tet-inducible synthetic butyrate operon. For efficient translation of butyrate genes, each synthetic gene in the operon was separated by a 15 base pair ribosome binding site derived from the T7 promoter.

[01033] The gene products of *bcd2-etfA3-etfB3* form a complex that convert crotonyl-CoA to butyryl-CoA, and may show some dependence on oxygen as a co-oxidant. Because an effective probiotic should be able to function in an oxygen-limited environment (*e.g.* the mammalian gut), and because it has been shown that a single gene from *Treponema denticola* can functionally replace this three gene complex in an oxygen-independent manner (*trans*-2-enoynl-CoA reductase; *ter*), we created a second plasmid capable of butyrate production in *E. coli*. Inverse PCR was used to amplify the entire sequence of pLogic031 outside of the *bcd-etfA3-etfB3* region. The *ter* gene was codon optimized for *E. coli* codon usage using Integrated DNA technologies online codon optimization tool, synthesized (Genewiz, Cambridge, MA), and cloned into this inverse PCR fragment using Gibson assembly to create pLogic046.

Example 7. Transforming E. coli

[01034] Each plasmid is transformed into *E. coli* Nissle or *E. coli* DH5a. All tubes, solutions, and cuvettes are pre-chilled to 4° C. An overnight culture of *E. coli* Nissle or *E. coli* DH5a is diluted 1:100 in 5 mL of lysogeny broth (LB) and grown until it reached an OD_{600} of 0.4-0.6. The cell culture medium contains a selection marker, *e.g.*, ampicillin, that is suitable for the plasmid. The *E. coli* cells are then centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 1 mL of 4° C water. The *E. coli* are again centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.5 mL of 4° C water. The *E. coli* are again centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are finally resuspended in

0.1 mL of 4° C water. The electroporator is set to 2.5 kV. 0.5 μg of one of the above plasmids is added to the cells, mixed by pipetting, and pipetted into a sterile, chilled cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. One mL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 37° C for 1 hr. The cells are spread out on an LB plate containing ampicillin and incubated overnight.

[01035] In alternate embodiments, the butyrate cassette can be inserted into the Nissle genome through homologous recombination (Genewiz, Cambridge, MA). Organization of the constructs and nucleotide sequences are shown in **FIG. 3**. To create a vector capable of integrating the synthesized butyrate cassette construct into the chromosome, Gibson assembly was first used to add 1000bp sequences of DNA homologous to the Nissle lacZ locus into the R6K origin plasmid pKD3. This targets DNA cloned between these homology arms to be integrated into the lacZ locus in the Nissle genome. Gibson assembly was used to clone the fragment between these arms. PCR was used to amplify the region from this plasmid containing the entire sequence of the homology arms, as well as the butyrate cassette between them. This PCR fragment was used to transform electrocompetent Nissle-pKD46, a strain that contains a temperature-sensitive plasmid encoding the lambda red recombinase genes. After transformation, cells were grown out for 2 hours before plating on chloramphenicol at 20ug/mL at 37°C. Growth at 37°C also cures the pKD46 plasmid. Transformants containing cassette were chloramphenicol resistant and lac-minus (lac-).

Example 8. Production of Butyrate in Recombinant E. coli

[01036] Production of butyrate wase assessed in *E. coli* Nissle strains containing butyrate cassettes described above in order to determine the effect of oxygen on butyrate production. All incubations are performed at 37° C. Cultures of *E. coli* strains DH5a and Nissle transformed with the butyrate cassettes are grown overnight in LB and then diluted 1:200 into 4 mL of M9 minimal medium containing 0.5% glucose. The cells were grown with shaking (250 rpm) for 4-6 h and incubated aerobically or anaerobically in a Coy anaerobic chamber (supplying 90% N_2, 5% CO_2, 5%H_2). One mL culture aliquots were prepared in 1.5 mL capped tubes and incubated in a stationary incubator to limit culture aeration. One tube is removed at each time point (0, 1, 2, 4, and 20 hours) and analyzed for butyrate concentration by LC-MS to confirm that butyrate production in these recombinant strains can be achieved in a low-oxygen environment. See **FIG. 31** which depicts a graph of butyrate production using the different butyrate-producing circuits shown in **FIG. 3**.

Example 9. Production of butyrate in Recombinant E. coli using an inducible tet promoter- butyrate circuit

[01037] All incubations were performed at 37°C. Lysogeny broth (LB)-grown overnight cultures of *E. coli* strains DH5a and Nissle containing pLogic031 or pLogic046 were subcultured 1:100 into 10mL of M9 minimal medium containing 0.2% glucose and grown shaking (200 rpm) for 2h, at which time anhydrous tetracycline (ATC) was added to cultures at a concentration of 100ng/mL to induce expression the butyrate operon from pLogic031 or pLogic046. Cultures were incubated either shaking in flasks ($+O_2$) or in the anaerobic chamber ($-O_2$) and samples were removed, and butyrate was quantitated at 2, 4, and 24hr via LC-MS. See **FIG. 32**, which depicts a graph of butyrate production using different butyrate-producing circuits comprising a *nuoB* gene deletion.

Example 10. Intestinal Residence and Survival of Bacterial Strains in vivo

[01038] Localization and intestinal residence time of streptomycin resistant Nissle, **FIG. 71,** was determined. Mice were gavaged, sacrificed at various time points, and effluents were collected from various areas of the small intestine cecum and colon.

[01039] Bacterial cultures were grown overnight and pelleted. The pellets were resuspended in PBS at a final concentration of approximately 10^{10} CFU/mL. Mice (C57BL6/J, 10-12 weeks old) were gavaged with 100 µL of bacteria (approximately 10^9 CFU). Drinking water for the mice was changed to contain 0.1 mg/mL anhydrotetracycline (ATC) and 5% sucrose for palatability. At each timepoint (1, 4, 8, 12, 24, and 30 hours post-gavage), animals (n=4) were euthanized, and intestine, cecum, and colon were removed. The small intestine was cut into three sections, and the large intestine and colon each into two sections. Each section was flushed with 0.5 ml cold PBS and collected in separate 1.5 ml tubes. The cecum was harvested, contents were squeezed out, and flushed with 0.5 ml cold PBS and collected in a 1.5 ml tube. Intestinal effluents were placed on ice for serial dilution plating.

[01040] In order to determine the CFU of bacteria in each effluent, the effluent was serially diluted, and plated onto LB plates containing kanamycin. The plates were incubated at 37°C overnight, and colonies were counted. The amount of bacteria and residence time in each compartment is shown in **FIG. 71**.

Example 11. Further Construction of vectors for overproducing butyrate

[01041] In addition to the ammonia conversion circuit, GABA transport circuit, GABA metabolic circuit, and/or manganese transport circuit described above, the *E. coli* Nissle bacteria further comprise one or more circuits for producing a gut barrier enhancer molecule.

[01042] To facilitate inducible production of butyrate in *E. coli* Nissle, the eight genes of the butyrate production pathway from *Peptoclostridium difficile 630* (*bcd2, etfB3, etfA3, thiA1, hbd, crt2, bpt,* and *buk*; NCBI), as well as transcriptional and translational elements, were synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. The butyrate gene cassette is placed under the control of a FNR regulatory region selected from (**SEQ ID NOs: 568-579**) (**Table 23**) In certain constructs, the FNR-responsive promoter is further fused to a strong ribosome binding site sequence. For efficient translation of butyrate genes, each synthetic gene in the operon was separated by a 15 base pair ribosome binding site derived from the T7 promoter/translational start site.

[01043] In certain constructs, the butyrate gene cassette is placed under the control of an RNS-responsive regulatory region, *e.g.*, *norB*, and the bacteria further comprises a gene encoding a corresponding RNS-responsive transcription factor, *e.g.*, *nsrR* (see, *e.g.*, **Tables 50 and 51**). In certain constructs, the butyrate gene cassette is placed under the control of an ROS-responsive regulatory region, *e.g.*, *oxyS*, and the bacteria further comprises a gene encoding a corresponding ROS-responsive transcription factor, *e.g.*, *oxyR* (see, *e.g.*, Tables 14-17). In certain constructs, the butyrate gene cassette is placed under the control of a tetracycline-inducible or constitutive promoter.

Table 50. pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 200)

Description	Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 200)
Nucleic acid sequence of an exemplary RNS-regulated construct comprising a gene encoding *nsrR*, a regulatory region of *norB*, and a butyrogenic gene cassette (pLogic031-nsrR-norB-butyrate construct; SEQ ID	ttatta**tcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgttg agcaggtcttgcagcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgc cgagtatgcccgtcagccggcaggacggcgtaatcaggcattcgttgttcgggcccat acactcgaccagctgcatcggttcgaggtggcggacgaccgcgccgatattgatgcgt tcgggcggcgcggccagcctcagcccgccgcctttcccgcgtacgctgtgcaagaacc cgcctttgaccagcgcggtaaccactttcatcaaatggcttttggaaatgccgtaggtcg aggcgatggtggcgatattgaccagcgcgtcgtcgttgacggcggtgtagatgaggac gcgcagcccgtagtcggtatgttgggtcagatacat**acaacctccttagtacatgcaaaat tatttctagagcaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtt gagttgaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggttgttgac aattaatcatcggctcgtataatgtataacattcatattttgtgaattttaaactctagaaataatttt gtttaactttaagaaggagatatacatatggatttaaattctaaaaaatatcagatgcttaaagag

Description	Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 200)
NO: 79). The sequence encoding NsrR is **underlined and bolded**, and the NsrR binding site, *i.e.*, a regulatory region of *norB* is boxed.	ctatatgtaagcttcgctgaaaatgaagttaaacctttagcaacagaacttgatgaagaagaaa gatttccttatgaaacagtggaaaaaatggcaaaagcaggaatgatgggtataccatatccaa aagaatatggtggagaaggtggagacactgtaggatatataatggcagttgaagaattgtcta gagtttgtggtactacaggagttatattatcagctcatacatctcttggctcatggcctatatatc aatatggtaatgaagaacaaaaacaaaaattcttaagaccactagcaagtggagaaaaatta ggagcatttggtcttactgagcctaatgctggtacagatgcgtctggccaacaaacaactgct gttttagacggggatgaatacatacttaatggctcaaaaatatttataacaaacgcaatagctg gtgacatatatgtagtaatggcaatgactgataaatctaaggggaacaaaggaatatcagcat ttatagttgaaaaaggaactcctgggtttagctttggagttaaagaaaagaaaatgggtataag aggttcagctacgagtgaattaatatttgaggattgcagaatacctaaagaaaatttacttgga aaagaaggtcaaggatttaagatagcaatgtctactcttgatggtggtagaattggtatagctg cacaagctttaggtttagcacaaggtgctcttgatgaaactgttaaatatgtaaaagaaagagt acaatttggtagaccattatcaaaattccaaaatacacaattccaattagctgatatggaagtta aggtacaagcggctagacaccttgtatatcaagcagctataaataaagacttaggaaaaccctt atggagtagaagcagcaatggcaaaattatttgcagctgaaacagctatggaagttactaca aaagctgtacaacttcatggaggatatggatacactcgtgactatccagtagaaagaatgatg agagatgctaagataactgaaatatatgaaggaactagtgaagttcaaagaatggttatttcag gaaaactattaaaatagtaagaaggagatatacatatggaggaaggatttatgaatatagtcgt ttgtataaaacaagttccagatacaacagaagttaaactagatcctaatacaggtactttaatta gagatggagtaccaagtataataaaccctgatgataaagcaggtttagaagaagctataaaat taaaagaagaaatgggtgctcatgtaactgttataacaatgggacctcctcaagcagatatgg ctttaaaagaagctttagcaatgggtgcagatagaggtatattattaacagatagagcatttgc gggtgctgatacttgggcaacttcatcagcattagcaggagcattaaaaaatatagattttgat attataatagctggaagacaggcgatagatggagatactgcacaagttggacctcaaatagc tgaacatttaaatcttccatcaataacatatgctgaagaaataaaaactgaaggtgaatatgtat tagtaaaaagacaatttgaagattgttgccatgacttaaaagttaaaatgccatgccttataaca actcttaaagatatgaacacaccaagatacatgaaagttggaagaatatatgatgctttcgaaa atgatgtagtagaaacatggactgtaaaagatatagaagttgacccttctaatttaggtcttaaa ggttctccaactagtgtatttaaatcatttacaaaatcagttaaaccagctggtacaatatacaat gaagatgcgaaaacatcagctggaattatcatagataaattaaaagagaagtatatcatataat aagaaggagatatacatatgggtaacgttttagtagtaatagaacaaagagaaaatgtaattc aaactgtttctttagaattactaggaaaggctacagaaatagcaaaagattatgatacaaaagt ttctgcattacttttaggtagtaaggtagaaggtttaatagatacattagcacactatggtgcag atgaggtaatagtagtagatgatgaagctttagcagtgtatacaactgaaccatatacaaaag cagcttatgaagcaataaaagcagctgaccctatagttgtattatttggtgcaacttcaataggt agagatttagcgcctagagtttctgctagaatacatacaggtcttactgctgactgtacaggtct tgcagtagctgaagatacaaaattattattaatgacaagacctgcctttggtggaaatataatg gcaacaatagtttgtaaagatttcagacctcaaatgtctacagttagaccaggggttatgaaga aaaatgaacctgatgaaactaaagaagctgtaattaaccgtttcaaggtagaatttaatgatgc tgataaattagttcaagttgtacaagtaataaaagaagctaaaaaacaagttaaaatagaagat gctaagatattagtttctgctggacgtggaatgggtggaaaagaaaacttagacatactttatg aattagctgaaattataggtggagaagtttctggttctcgtgccactatagatgcaggttggtta gataaagcaagacaagttggtcaaactggtaaaactgtaagaccagacctttatatagcatgt ggtatatctggagcaatacaacatatagctggtatggaagatgctgagtttatagttgctataaa taaaaatccagaagctccaatatttaaatatgctgatgttggtatagttggagatgttcataaagt gcttccagaacttatcagtcagttaagtgttgcaaaagaaaaaggtgaagttttagctaactaat aagaaggagatatacatatgagagaagtagtaattgccagtgcagctagaacagcagtagg

Description	Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 200)
	aagttttggaggagcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaaga agctataaaaagagctaacataactccagatatgatagatgaatctcttttaggggagtactt acagcaggtcttggacaaaatatagcaagacaaatagcattaggagcaggaataccagtag aaaaaccagctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatctc aacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatgagtatgtct ccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaat gataaaagatggattatcagacatatttaataactatcacatgggtattactgctgaaaacatag cagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaataaa gctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgttataaaagga agaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactacaatggagaa acttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcagga ataaatgatggtgctgctatgttagtagtaatggctaaagaaaaagctgaagaactaggaata gagcctcttgcaactatagtttcttatggaacagctggtgttgaccctaaaataatgggatatgg accagttccagcaactaaaaaagctttagaagctgctaatatgactattgaagatatagattta gttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaatatagatat gaataaagttaatgttaatggtggagcaatagctataggacatccaataggatgctcaggagc aagaatacttactacacttttatatgaaatgaagagaagagatgctaaaactggtcttgctaca ctttgtataggcggtggaatgggaactactttaatagttaagagatagtaagaaggagatatac atatgaaattagctgtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaagt tgtggacatgatgtatgtttaaagagtagaactcaaggtgctatagataaatgtttagctttatta gataaaaatttaactaagttagttactaagggaaaaatggatgaagctacaaaagcagaaata ttaagtcatgttagttcaactactaattatgaagatttaaaagatatggatttaataatagaagcat ctgtagaagacatgaatataaagaaagatgttttcaagttactagatgaattatgtaaagaagat actatcttggcaacaaatacttcatcattatctataacagaaatagcttcttctactaagcgccca gataaagttataggaatgcatttctttaatccagttcctatgatgaaattagttgaagttataagtg gtcagttaacatcaaaagttacttttgatacagtatttgaattatctaagagtatcaataaagtacc agtagatgtatctgaatctcctggatttgtagtaaatagaatacttatacctatgataaatgaagc tgttggtatatatgcagatggtgttgcaagtaaagaagaaatagatgaagctatgaaattagga gcaaaccatccaatgggaccactagcattaggtgatttaatcggattagatgttgttttagctat aatgaacgttttatatactgaatttggagatactaaatatagacctcatccacttttagctaaaatg gttagagctaatcaattaggaagaaaaactaagataggattctatgattataataaataataag aaggagatatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtaga tggaaatatatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagact ttagaagaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgtaatattgac aggggaaggaaaggcatttgtagctggagcagatattgcatacatgaaagatttagatgctgt agctgctaaagattttagtatcttaggagcaaaagcttttggagaaatagaaaatagtaaaaaa gtagtgatagctgctgtaaacggatttgctttaggtggaggatgtgaacttgcaatggcatgtg atataagaattgcatctgctaaagctaaatttggtcagccagaagtaactcttggaataactcc aggatatggaggaactcaaaggcttacaagattggttggaatggcaaaagcaaaagaatta atctttacaggtcaagttataaaagctgatgaagctgaaaaaatagggctagtaaatagagtc gttgagccagacattttaatagaagaagttgagaaattagctaagataatagctaaaaatgctc agcttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactgg aatagatatagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtc agctttcgttgaaaagagagaagctaactttataaaagggtaataagaaggagatatacatat gagaagttttgaagaagtaattaagtttgcaaaagaaagaggacctaaaactatatcagtagc atgttgccaagataaagaagttttaatggcagttgaaatggctagaaaagaaaaaatagcaaa tgccattttagtaggagatatagaaaagactaaagaaattgcaaaaagcatagacatggatat

Description	Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 200)
	cgaaaattatgaactgatagatataaaagatttagcagaagcatctctaaaatctgttgaattag tttcacaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaaa gcagttttaaataaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttga tgtagagggatatgatagattatttttcgtaactgacgcagctatgaacttagctcctgatacaa atactaaaaagcaaatcatagaaaatgcttgcacagtagcacattcattagatataagtgaacc aaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagttgaag ctaaagaactagaagaaatgtatgaaagaggagaaatcaaaggttgtatggttggtgggcct tttgcaattgataatgcagtatctttagaagcagctaaacataaaggtataaatcatcctgtagc aggacgagctgatatattattagccccagatattgaaggtggtaacatattatataaagctttgg tattcttctcaaaatcaaaaaatgcaggagttatagttggggctaaagcaccaataatattaact tctagagcagacagtgaagaaactaaactaaactcaatagctttaggtgttttaatggcagca aaggcataataagaaggagatatacatatgagcaaaatatttaaaatcttaacaataaatcctg gttcgacatcaactaaaatagctgtatttgataatgaggatttagtatttgaaaaaactttaagac attcttcagaagaaataggaaaatatgagaaggtgtctgaccaatttgaatttcgtaaacaagt aatagaagaagctctaaaagaaggtggagtaaaaacatctgaattagatgctgtagtaggta gaggaggacttcttaaacctataaaaggtggtacttattcagtaagtgctgctatgattgaagat ttaaaagtgggagttttaggagaacacgcttcaaacctaggtggaataatagcaaaacaaata ggtgaagaagtaaatgttccttcatacatagtagaccctgttgttgtagatgaattagaagatgt tgctagaatttctggtatgcctgaaataagtagagcaagtgtagtacatgctttaaatcaaaag gcaatagcaagaagatatgctagagaaataaacaagaaatatgaagatataaatcttatagtt gcacacatgggtggaggagtttctgttggagctcataaaaatggtaaaatagtagatgttgca aacgcattagatggagaaggacctttctctccagaaagaagtggtggactaccagtaggtgc attagtaaaaatgtgctttagtggaaaatatactcaagatgaaattaaaaagaaaataaaaggt aatggcggactagttgcatacttaaacactaatgatgctagagaagttgaagaaagaattgaa gctggtgatgaaaaagctaaattagtatatgaagctatggcatatcaaatctctaaagaaatag gagctagtgctgcagttcttaagggagatgtaaaagcaatattattaactggtggaatcgcata ttcaaaaatgtttacagaaatgattgcagatagagttaaatttatagcagatgtaaaagtttatcc aggtgaagatgaaatgattgcattagctcaaggtggacttagagttttaactggtgaagaaga ggctcaagtttatgataactaataa

Table 51. Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct

Description	Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 201)
Nucleic acid sequence of an exemplary RNS-regulated construct comprising a gene encoding *nsrR*, a regulatory region of *norB*, and a butyrogenic gene	ttatta**tcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgttgagcagg tcttgcagcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgccgagtatgcccgt cagccggcaggacggcgtaatcaggcattcgttgttcgggcccatacactcgaccagctgcatc ggttcgaggtggcggacgaccgcgccgatattgatgcgttcgggcggcgcggccagcctcagc ccgccgcctttcccgcgtacgctgtgcaagaacccgcctttgaccagcgcggtaaccactttcat caaatggcttttggaaatgccgtaggtcgaggcgatggtggcgatattgaccagcgcgtcgtcgt tgacggcggtgtagatgaggacgcgcagcccgtagtcggtatgttgggtcagatacat**acaacc tccttagtacatgcaaaattatttctagagcaacatacgagccggaagcataaagtgtaaagcctgggg tgcctaatgagttgagttgaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggtt gttgacaattaatcatcggctcgtataatgtataacattcatattttgtgaattttaaactctagaaataatttt gtttaactttaagaaggagatatacatatgatcgtaaaacctatggtacgcaacaatatctgcctgaacgc

Description	Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 201)
cassette (pLogic046-nsrR-norB-butyrate construct; SEQ ID NO: 80).	ccatcctcagggctgcaagaagggagtggaagatcagattgaatataccaagaaacgcattaccgca gaagtcaaagctggcgcaaaagctccaaaaaacgttctggtgcttggctgctcaaatggttacggcct ggcgagccgcattactgctgcgttcggatacggggctgcgaccatcggcgtgtcctttgaaaaagcg ggttcagaaaccaaatatggtacaccgggatggtacaataatttggcatttgatgaagcggcaaaacg cgagggtctttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaaggcccaggtaattg aggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatacagcttggccagcccagtacgtactg atcctgatacaggtatcatgcacaaaagcgttttgaaaccctttggaaaaacgttcacaggcaaaacag tagatccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagccg ccactgttaaagttatgggggtgaagattgggaacgttggattaagcagctgtcgaaggaaggcctc ttagaagaaggctgtattaccttggcctatagttatattggcccctgaagctacccaagctttgtaccgtaa aggcacaatcggcaaggccaaagaacacctggaggccacagcacaccgtctcaacaaagagaaccc cgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggta atccctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtattgaac agatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggtacaattccagttgatgaggaaaat cgcattcgcattgatgattgggagttagaagaagacgtccagaaagcggtatccgcgttgatggagaa agtcacgggtgaaaacgcagaatctctcactgacttagcggggtaccgccatgatttcttagctagtaa cggctttgatgtagaaggtattaattatgaagcggaagttgaacgcttcgaccgtatctgataagaagga gatatacatatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagc atttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagctaacat aactccagatatgatagatgaatctcttttaggggagtacttacagcaggtcttggacaaaatatagca agacaaatagcattaggagcaggaataccagtagaaaaaccagctatgactataaatatagtttgtggtt ctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatgctgatataatgttagttggtgg agctgaaaacatgagtatgtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgct gcttttgttgattcaatgataaaagatggattatcagacatatttaataactatcacatgggtattactgctga aaacatagcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaata aagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgttataaaaggaagaa aaggtgacactgtagtagataaagatgaatatattaagcctggcactacaatggagaaacttgctaagtt aagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaataaatgatggtgctgct atgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatagtttctta tggaacagctggtgttgaccctaaaataatgggatatggaccagttccagcaactaaaaaagctttaga agctgctaatatgactattgaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgtagc tgtaataagagacttaaatatagatatgaataaagttaatgttaatggtggagcaatagctataggacatc caataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaagagatgctaaaa ctggtcttgctacactttgtataggcggtggaatgggaactactttaatagttaagagatagtaagaagg agatatacatatgaaattagctgtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaa gttgtggacatgatgtatgtttaaagagtagaactcaaggtgctatagataaatgtttagctttattagataa aaatttaactaagttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgtta gttcaactactaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaagacatgaata taaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatcttggcaacaaatacttcat cattatctataacagaaatagcttcttctactaagcgcccagataaagttataggaatgcatttctttaatcc agttcctatgatgaaattagttgaagttataagtggtcagttaacatcaaaagttacttttgatacagtatttg aattatctaagagtatcaataaagtaccagtagatgtatctgaatctcctggatttgtagtaaatagaatact tatacctatgataaatgaagctgttggtatatatgcagatggtgttgcaagtaaagaagaaatagatgaa gctatgaaattaggagcaaaccatccaatgggaccactagcattaggtgatttaatcggattagatgttg ttttagctataatgaacgttttatatactgaatttggagatactaaatatagacctcatccacttttagctaaa atggttagagctaatcaattaggaagaaaaactaagataggattctatgattataataaataataagaag gagatatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatggaaatata

Description	Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 201)
	tgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagactttagaagaactttatga agtatttgtagatattaataatgatgaaactattgatgttgtaatattgacaggggaaggaaaggcatttgt agctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatcttaggag caaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgctttagg tggaggatgtgaacttgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggtcagcca gaagtaactcttggaataactccaggatatggaggaactcaaaggcttacaagattggttggaatggca aaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagctgaaaaaatagggctagta aatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaagataatagctaaaaatg ctcagcttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaata gatatagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttga aaagagagaagctaactttataaaagggtaataagaaggagatatacatatgagaagttttgaagaagt aattaagtttgcaaaagaaagaggacctaaaactatatcagtagcatgttgccaagataaagaagtttta atggcagttgaaatggctagaaaagaaaaaatagcaaatgccattttagtaggagatatagaaaagact aaagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgatagatataaaagatttagca gaagcatctctaaaatctgttgaattagtttcacaaggaaaagccgacatggtaatgaaaggcttagtag acacatcaataatactaaaagcagttttaaataaagaagtaggtcttagaactggaaatgtattaagtcac gtagcagtatttgatgtagagggatatgatagattatttttcgtaactgacgcagctatgaacttagctcct gatacaaatactaaaaagcaaatcatagaaaatgcttgcacagtagcacattcattagatataagtgaac caaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagttgaagctaaa gaactagaagaaatgtatgaaagaggagaaatcaaaggttgtatggttggtgggccttttgcaattgata atgcagtatctttagaagcagctaaacataaaggtataaatcatcctgtagcaggacgagctgatatatt attagccccagatattgaaggtggtaacatattatataaagctttggtattcttctcaaaatcaaaaaatgc aggagttatagttggggctaaagcaccaataatattaacttctagagcagacagtgaagaaactaaact aaactcaatagctttaggtgttttaatggcagcaaaggcataataagaaggagatatacatatgagcaaa atatttaaaatcttaacaataaatcctggttcgacatcaactaaaatagctgtatttgataatgaggatttagt atttgaaaaaactttaagacattcttcagaagaaataggaaaatatgagaaggtgtctgaccaatttgaat ttcgtaaacaagtaatagaagaagctctaaaagaaggtggagtaaaaacatctgaattagatgctgtag taggtagaggaggacttcttaaacctataaaaggtggtacttattcagtaagtgctgctatgattgaagat ttaaaagtgggagttttaggagaacacgcttcaaacctaggtggaataatagcaaaacaaataggtgaa gaagtaaatgttccttcatacatagtagaccctgttgttgtagatgaattagaagatgttgctagaatttctg gtatgcctgaaataagtagagcaagtgtagtacatgctttaaatcaaaaggcaatagcaagaagatatg ctagagaaataaacaagaaatatgaagatataaatcttatagttgcacacatgggtggaggagtttctgtt ggagctcataaaaatggtaaaatagtagatgttgcaaacgcattagatggagaaggacctttctctcca gaaagaagtggtggactaccagtaggtgcattagtaaaaatgtgctttagtggaaaatatactcaagat gaaattaaaaagaaaataaaaggtaatggcggactagttgcatacttaaacactaatgatgctagagaa gttgaagaaagaattgaagctggtgatgaaaaagctaaattagtatatgaagctatggcatatcaaatct ctaaagaaataggagctagtgctgcagttcttaagggagatgtaaaagcaatattattaactggtggaat cgcatattcaaaaatgtttacagaaatgattgcagatagagttaaatttatagcagatgtaaaagtttatcc aggtgaagatgaaatgattgcattagctcaaggtggacttagagttttaactggtgaagaagaggctca agtttatgataactaataa

[01044] The gene products of the bcd2-etfA3-etfB3 genes form a complex that converts crotonyl-CoA to butyryl-CoA and may exhibit dependence on oxygen as a co-oxidant. Because the recombinant bacteria of the invention are designed to produce butyrate

in an oxygen-limited environment (*e.g.* the mammalian gut), that dependence on oxygen could have a negative effect of butyrate production in the gut. It has been shown that a single gene from Treponema denticola, trans-2-enoynl-CoA reductase (ter), can functionally replace this three gene complex in an oxygen-independent manner. Therefore, a second butyrate gene cassette in which the ter gene replaces the bcd2-etfA3-etfB3 genes of the first butyrate cassette is synthesized (Genewiz, Cambridge, MA). The ter gene is codon-optimized for *E. coli* codon usage using Integrated DNA Technologies online codon optimization tool (https://www.idtdna.com/CodonOpt). The second butyrate gene cassette, as well as transcriptional and translational elements, is synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. The second butyrate gene cassette is placed under control of a FNR regulatory region as described above. In certain constructs, the butyrate gene cassette is placed under the control of an RNS-responsive regulatory region, *e.g.*, norB, and the bacteria further comprises a gene encoding a corresponding RNS-responsive transcription factor, *e.g.*, nsrR (see, *e.g.*, **Table 25**). In certain constructs, the butyrate gene cassette is placed under the control of an ROS-responsive regulatory region, *e.g.*, oxyS, and the bacteria further comprises a gene encoding a corresponding ROS-responsive transcription factor, *e.g.*, oxyR (see, *e.g.*, **Table 26** and **Table 55**).

Table 52. ROS regulated constructs, OxyR construct, Tet-regulated constructs

Description	Sequence
Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 202)	ctcgagttcattatccatcctccatcgccacgatagttcatggcgataggtagaatagcaatgaacgattatccctatcaagcattc tgactgataattgctcacacgaattcattaaagaggagaaaggtaccatggatttaaattctaaaaaatatcagatgcttaaagagc tatatgtaagcttcgctgaaaatgaagttaaacctttagcaacagaacttgatgaagaagaaagatttccttatgaaacagtggaa aaaatggcaaaagcaggaatgatgggtataccatatccaaaagaatatggtggagaaggtggagacactgtaggatatataat ggcagttgaagaattgtctagagtttgtggtactacaggagttatattatcagctcatacatctcttggctcatggcctatatatcaat atggtaatgaagaacaaaaacaaaaattcttaagaccactagcaagtggagaaaaattaggagcatttggtcttactgagcctaa tgctggtacagatgcgtctggccaacaaacaactgctgttttagacggggatgaatacatacttaatggctcaaaaatatttataac aaacgcaatagctggtgacatatatgtagtaatggcaatgactgataaatctaaggggaacaaaggaatatcagcatttatagttg aaaaaggaactcctgggtttagctttggagttaaagaaaagaaaatgggtataagaggttcagctacgagtgaattaatatttgag gattgcagaatacctaaagaaaatttacttggaaaagaaggtcaaggatttaagatagcaatgtctactcttgatggtggtagaatt ggtatagctgcacaagctttaggtttagcacaaggtgctcttgatgaaactgttaaatatgtaaaagaaagagtacaatttggtaga ccattatcaaaattccaaaatacacaattccaattagctgatatggaagttaaggtacaagcggctagacaccttgtatatcaagca gctataaataaagacttaggaaaaccttatggagtagaagcagcaatggcaaaattatttgcagctgaaacagctatggaagtta ctacaaaagctgtacaacttcatggaggatatggatacactcgtgactatccagtagaaagaatgatgagagatgctaagataac tgaaatatatgaaggaactagtgaagttcaaagaatggttatttcaggaaaactattaaaatagtaagaaggagatatacatatgg aggaaggatttatgaatatagtcgtttgtataaaacaagttccagatacaacagaagttaaactagatcctaatacaggtactttaat tagagatggagtaccaagtataataaaccctgatgataaagcaggtttagaagaagctataaaattaaaagaagaaatgggtgct catgtaactgttataacaatgggacctcctcaagcagatatggctttaaaagaagctttagcaatgggtgcagatagaggtatatt attaacagatagagcatttgcgggtgctgatacttgggcaacttcatcagcattagcaggagcattaaaaaatatagattttgatatt ataatagctggaagacaggcgatagatggagatactgcacaagttggacctcaaatagctgaacatttaaatcttccatcaataa catatgctgaagaaataaaaactgaaggtgaatatgtattagtaaaaagacaatttgaagattgttgccatgacttaaaagttaaaa

Description	Sequence
	tgccatgccttataacaactcttaaagatatgaacacaccaagatacatgaaagttggaagaatatatgatgctttcgaaaatgatg tagtagaaacatggactgtaaaagatatagaagttgacccttctaatttaggtcttaaaggttctccaactagtgtatttaaatcattta caaaatcagttaaaccagctggtacaatatacaatgaagatgcgaaaacatcagctggaattatcatagataaattaaaagagaa gtatatcatataataagaaggagatatacatatgggtaacgttttagtagtaatagaacaaagagaaaatgtaattcaaactgtttct ttagaattactaggaaaggctacagaaatagcaaaagattatgatacaaaagtttctgcattacttttaggtagtaaggtagaaggt ttaatagatacattagcacactatggtgcagatgaggtaatagtagtagatgatgaagctttagcagtgtatacaactgaaccatat acaaaagcagcttatgaagcaataaaagcagctgaccctatagttgtattatttggtgcaacttcaataggtagagatttagcgcct agagtttctgctagaatacatacaggtcttactgctgactgtacaggtcttgcagtagctgaagatacaaaattattattaatgacaa gacctgcctttggtggaaatataatggcaacaatagtttgtaaagatttcagacctcaaatgtctacagttagaccaggggttatga agaaaaatgaacctgatgaaactaaagaagctgtaattaaccgtttcaaggtagaatttaatgatgctgataaattagttcaagttgt acaagtaataaaagaagctaaaaaacaagttaaaatagaagatgctaagatattagtttctgctggacgtggaatgggtggaaaa gaaaacttagacatactttatgaattagctgaaattataggtggagaagtttctggttctcgtgccactatagatgcaggttggttag ataaagcaagacaagttggtcaaactggtaaaactgtaagaccagacctttatatagcatgtggtatatctggagcaatacaacat atagctggtatggaagatgctgagtttatagttgctataaataaaaatccagaagctccaatatttaaatatgctgatgttggtatagt tggagatgttcataaagtgcttccagaacttatcagtcagttaagtgttgcaaaagaaaaaggtgaagttttagctaactaataaga aggagatatacatatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagtt tcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagctaacataactccagatatgatagatgaatctcttt tagggggagtacttacagcaggtcttggacaaaatatagcaagacaaatagcattaggagcaggaataccagtagaaaaacca gctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatgctgatataatgt tagttggtggagctgaaaacatgagtatgtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgt tgattcaatgataaaagatggattatcagacatatttaataactatcacatgggtattactgctgaaaacatagcagagcaatggaa tataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaaagctcaagctgaaggaaaatttgatgaa gaaatagttcctgttgttataaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactacaatgga gaaacttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaataaatgatggtgctgcta tgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctggtgttg accctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgactattgaagatatagatttag ttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaatatagatatgaataaagttaatgttaatggtgg agcaatagctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaagagatgct aaaactggtcttgctacactttgtataggcggtggaatgggaactactttaatagttaagagatagtaagaaggagatatacatatg aaattagctgtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtatgtttaaagagta gaactcaaggtgctatagataaatgtttagctttattagataaaaatttaactaagttagttactaagggaaaaatggatgaagctac aaaagcagaaatattaagtcatgttagttcaactactaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaa gacatgaatataaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatcttggcaacaaatacttcatcattatc tataacagaaatagcttcttctactaagcgcccagataaagttataggaatgcatttctttaatccagttcctatgatgaaattagttg aagttataagtggtcagttaacatcaaaagttacttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtat ctgaatctcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatatatgcagatggtgttgcaagtaaa gaagaaatagatgaagctatgaaattaggagcaaaccatccaatgggaccactagcattaggtgatttaatcggattagatgttgt tttagctataatgaacgttttatatactgaatttggagatactaaatatagacctcatccacttttagctaaaatggttagagctaatca attaggaagaaaaactaagataggattctatgattataataaataataagaaggagatatacatatgagtacaagtgatgttaaagt ttatgagaatgtagctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaa gactttagaagaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgtaatattgacaggggaaggaaaggca tttgtagctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatcttaggagcaaaagcttttg gagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgctttaggtggaggatgtgaacttgcaatggcatg tgatataagaattgcatctgctaaagctaaatttggtcagccagaagtaactcttggaataactccaggatatggaggaactcaaa ggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagctgaaaaaat agggctagtaaatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaagataatagctaaaaatgctcag cttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaatagatatagaatctaatttatttgg tctttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactttataaaagggtaataaga aggagatatacatatgagaagttttgaagaagtaattaagtttgcaaaagaaagaggacctaaaactatatcagtagcatgttgcc aagataaagaagttttaatggcagttgaaatggctagaaaagaaaaaatagcaaatgccattttagtaggagatatagaaaagac taaagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgatagatataaaagatttagcagaagcatctctaaaat ctgttgaattagtttcacaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaaagcagttttaaata aagaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttgatgtagagggatatgatagattatttttcgtaactga cgcagctatgaacttagctcctgatacaaatactaaaaagcaaatcatagaaaatgcttgcacagtagcacattcattagatataa gtgaaccaaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagttgaagctaaagaactagaa gaaatgtatgaaagaggagaaatcaaaggttgtatggttggtgggccttttgcaattgataatgcagtatctttagaagcagctaa acataaaggtataaatcatcctgtagcaggacgagctgatatattattagccccagatattgaaggtggtaacatattatataaagc

Description	Sequence
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Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 205) The sequence encoding TetR is underlined, and the overlapping tetR/tetA promoters are boxed.	gtaaaacgacggccagtgaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaat aagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttcc ctttcttctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcatt ctctagtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtac ttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatcttgccagctttccccttctaaagg gcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttttacatgccaatacaatgt aggctgctctacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgtta atcactttacttttatctaatctagacatcattaattcctaatttttgttgacactctatcattgatagagttatttttaccactccctatcagt gatagagaaaagtgaactctagaaataatttttgtttaactttaagaaggagatatacatatggatttaaattctaaaaaatatcagat gcttaaagagctatatgtaagcttcgctgaaaatgaagttaaaccctttagcaacagaacttgatgaagaagaaagatttccttatga aacagtggaaaaaatggcaaaagcaggaatgatgggtataccatatccaaaagaatatggtggagaaggtggagacactgta ggatatataatggcagttgaagaattgtctagagtttgtggtactacaggagttatattatcagctcatacatctcttggctcatggcc tatatatcaatatggtaatgaagaacaaaaacaaaaattcttaagaccactagcaagtggagaaaaattaggagcatttggtctta ctgagcctaatgctggtacagatgcgtctggccaacaaacaactgctgttttagacggggatgaatacatacttaatggctcaaa aatatttataacaaacgcaatagctggtgacatatatgtagtaatggcaatgactgataaatctaaggggaacaaaggaatatcag catttatagttgaaaaaggaactcctgggtttagctttggagttaaagaaaagaaaatgggtataagaggttcagctacgagtgaa ttaatatttgaggattgcagaatacctaaagaaaatttacttggaaaagaaggtcaaggatttaagatagcaatgtctactcttgatg gtggtagaattggtatagctgcacaagctttaggtttagcacaaggtgctcttgatgaaactgttaaatatgtaaaagaaagagtac aatttggtagaccattatcaaaattccaaaatacacaattccaattagctgatatggaagttaaggtacaagcggctagacaccttg tatatcaagcagctataaataaagacttaggaaaaccttatggagtagaagcagcaatggcaaaattatttgcagctgaaacagc tatggaagttactacaaaagctgtacaacttcatggaggatatggatacactcgtgactatccagtagaaagaatgatgagagat gctaagataactgaaatatatgaaggaactagtgaagttcaaagaatggttatttcaggaaaactattaaaatagtaagaaggag atatacatatggaggaaggatttatgaatatagtcgtttgtataaaacaagttccagatacaacagaagttaaactagatcctaata caggtactttaattagagatggagtaccaagtataataaaccctgatgataaagcaggtttagaagaagctataaaattaaaagaa gaaatgggtgctcatgtaactgttataacaatgggacctcctcaagcagatatggctttaaaagaagctttagcaatgggtgcag atagaggtatattattaacagatagagcatttgcgggtgctgatacttgggcaacttcatcagcattagcaggagcattaaaaaat atagattttgatattataatagctggaagacaggcgatagatggagatactgcacaagttggacctcaaatagctgaacatttaaat cttccatcaataacatatgctgaagaaataaaaactgaaggtgaatatgtattagtaaaaagacaatttgaagattgttgccatgac ttaaaagttaaaatgccatgccttataacaactcttaaagatatgaacacaccaagatacatgaaagttggaagaatatatgatgct ttcgaaaatgatgtagtagaaacatggactgtaaaagatatagaagttgacccttctaatttaggtcttaaaggttctccaactagtg tatttaaatcatttacaaaatcagttaaaccagctggtacaatatacaatgaagatgcgaaaacatcagctggaattatcatagata aattaaaagagaagtatatcatataataagaaggagatatacatatgggtaacgttttagtagtaatagaacaaagagaaaatgta attcaaactgtttctttagaattactaggaaaggctacagaaatagcaaaagattatgatacaaaagtttctgcattacttttaggtag taaggtagaaggtttaatagatacattagcacactatggtgcagatgaggtaatagtagtagatgatgaagctttagcagtgtata caactgaaccatatacaaaagcagcttatgaagcaataaaagcagctgaccctatagttgtattatttggtgcaacttcaataggta gagatttagcgcctagagtttctgctagaatacatacaggtcttactgctgactgtacaggtcttgcagtagctgaagatacaaaat tattattaatgacaagacctgcctttggtggaaatataatggcaacaatagtttgtaaagatttcagacctcaaatgtctacagttag accaggggttatgaagaaaaatgaacctgatgaaactaaagaagctgtaattaaccgtttcaaggtagaatttaatgatgctgata aattagttcaagttgtacaagtaataaaagaagctaaaaaacaagttaaaatagaagatgctaagatattagtttctgctggacgtg gaatgggtggaaaagaaaacttagacatactttatgaattagctgaaattataggtggagaagtttctggttctcgtgccactatag atgcaggttggttagataaagcaagacaagttggtcaaactggtaaaactgtaagaccagacctttatatagcatgtggtatatct ggagcaatacaacatatagctggtatggaagatgctgagtttatagttgctataaataaaaatccagaagctccaatatttaaatat gctgatgttggtatagttggagatgttcataaagtgcttccagaacttatcagtcagttaagtgttgcaaaagaaaaaggtgaagttt tagctaactaataagaaggagatatacatatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggag gagcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagctaacataactccagatat

Description	Sequence
	gatagatgaatctcttttaggggagtacttacagcaggtcttggacaaaatatagcaagacaaatagcattaggagcaggaata ccagtagaaaaaccagctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcattagg tgatgctgatataatgttagttggtggagctgaaaacatgagtatgtctccttatttagtaccaagtgcgagatatggtgcaagaat gggtgatgctgcttttgttgattcaatgataaaagatggattatcagacatatttaataactatcacatgggtattactgctgaaaaca tagcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaaagctcaagctg aaggaaaatttgatgaagaaatagttcctgttgttataaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaag cctggcactacaatggagaaacttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaa taaatgatggtgctgctatgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatagtttctt atggaacagctggtgttgaccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgac tattgaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaatatagatatgaata aagttaatgttaatggtggagcaatagctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatgaaa tgaagagaagagatgctaaaactggtcttgctacactttgtataggcggtggaatgggaactactttaatagttaagagatagtaa gaaggagatatacatatgaaattagctgtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacat gatgtatgtttaaagagtagaactcaaggtgctatagataaatgtttagctttattagataaaaatttaactaagttagttactaaggg aaaaatggatgaagctacaaaagcagaaatattaagtcatgttagttcaactactaattatgaagatttaaaagatatggatttaata atagaagcatctgtagaagacatgaatataaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatcttggca acaaatacttcatcattatctataacagaaatagcttcttctactaagcgcccagataaagttataggaatgcatttcttttaatccagtt cctatgatgaaattagttgaagttataagtggtcagttaacatcaaaagttacttttgatacagtatttgaattatctaagagtatcaat aaagtaccagtagatgtatctgaatctcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatatatgc agatggtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaatgggaccactagcattaggtga tttaatcggattagatgttgttttagctataatgaacgttttatatactgaatttggagatactaaatatagacctcatccacttttagcta aaatggttagagctaatcaattaggaagaaaaactaagataggattctatgattataataaataataagaaggagatatacatatga gtacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaagcc cttaatgcaataaattcaaagactttagaagaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgtaatattga caggggaaggaaaggcatttgtagctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatc ttaggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgctttaggtggaggat gtgaacttgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggtcagccagaagtaactcttggaataactccag gatatggaggaactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaagc tgatgaagctgaaaaaatagggctagtaaatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaagata atagctaaaaatgctcagcttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaatagat atagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactt tataaaagggtaataagaaggagatatacatatgagaagttttgaagaagtaattaagtttgcaaaagaaagaggacctaaaact atatcagtagcatgttgccaagataaagaagttttaatggcagttgaaatggctagaaaagaaaaaatagcaaatgccatttttagt aggagatatagaaaagactaaagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgatagatataaaagatttta gcagaagcatctctaaaatctgttgaattagtttcacaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataat actaaaagcagttttaaataaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttgatgtagagggatatga tagattatttttcgtaactgacgcagctatgaacttagctcctgatacaaatactaaaaagcaaatcatagaaaatgcttgcacagta gcacattcattagatataagtgaaccaaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagttg aagctaaagaactagaagaaatgtatgaaagaggagaaatcaaaggttgtatggttggtgggccttttgcaattgataatgcagt atctttagaagcagctaaacataaaggtataaatcatcctgtagcaggacgagctgatatattattagccccagatattgaaggtg gtaacatattatataaagctttggtattcttctcaaaatcaaaaaatgcaggagttatagttggggctaaagcaccaataatattaact tctagagcagacagtgaagaaactaaactaaactcaatagctttaggtgttttaatggcagcaaaggcataataagaaggagata tacatatgagcaaaatatttaaaatcttaacaataaatcctggttcgacatcaactaaaatagctgtatttgataatgaggatttagtat ttgaaaaaacttaagacattcttcagaagaaatagaaaatatgagaaggtgtctgaccaatttgaatttcgtaaacaagtaatag aagaagctctaaaagaaggtggagtaaaaacatctgaattagatgctgtagtaggtagaggaggacttcttaaacctataaaag gtggtacttattcagtaagtgctgctatgattgaagatttaaaagtgggagttttaggagaacacgcttcaaacctaggtggaataa tagcaaaacaaataggtgaagaagtaaatgttccttcatacatagtagaccctgttgttgtagatgaattagaagatgttgctagaa tttctggtatgcctgaaataagtagagcaagtgtagtacatgctttaaatcaaaaggcaatagcaagaagatatgctagagaaata aacaagaaatatgaagatataaatcttatagttgcacacatgggtggaggagtttctgttggagctcataaaaatggtaaaatagt agatgttgcaaacgcattagatggagaaggacctttctctccagaaagaagtggtggactaccagtaggtgcattagtaaaaatg tgctttagtggaaaatatactcaagatgaaattaaaaagaaaataaaaggtaatggcggactagttgcatacttaaacactaatgat gctagagaagttgaagaaagaattgaagctggtgatgaaaaagctaaattagtatatgaagctatggcatatcaaatctctaaag aaataggagctagtgctgcagttcttaagggagatgtaaaagcaatattattaactggtggaatcgcatattcaaaaatgtttacag aaatgattgcagatagagttaaatttatagcagatgtaaaagtttatccaggtgaagatgaaatgattgcattagctcaaggtggac ttagagttttaactggtgaagaagaggctcaagtttatgataactaataa
Nucleotide sequences of	gtaaaacgacggccagtgaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaat aagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttcc

Description	Sequence
pLogic046-tet-butyrate construct (SEQ ID NO: 206) The sequence encoding TetR is underlined, and the overlapping tetR/tetA promoters are boxed.	ctttcttctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcatt ctctagtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtac ttttgctccatcgcgatgacttagtaaagcacatctaaaactttagcgttattacgtaaaaaatcttgccagctttccccttctaaagg gcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttttacatgccaatacaatgt aggctgctctacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgtta atcactttacttttatctaatctagacatcattaattcctaatttttgttgacactctatcattgatagagttatttttaccactccctatcagt gatagagaaaagtgaactctagaaataattttgtttaactttaagaaggagatatacatatgatcgtaaaacctatggtacgcaaca atatctgcctgaacgcccatcctcagggctgcaagaagggagtggaagatcagattgaatataccaagaaacgcattaccgca gaagtcaaagctggcgcaaaagctccaaaaaacgttctggtgcttggctgctcaaatggttacggcctggcgagccgcattact gctgcgttcggatacggggctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaatatggtacaccgggatg gtacaataatttggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgatcgacggcgatgcgttttcagacgag atcaaggcccaggtaattgaggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatacagcttggccagcccagtacgt actgatcctgatacaggtatcatgcacaaaagcgttttgaaaccctttggaaaaacgttcacaggcaaaacagtagatccgtttac tggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagccgccactgttaaagttatgggggtgaaga ttgggaacgttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggcctatagttatattggccctg aagctacccaagctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagcacaccgtctcaacaa agagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggtaatccctc tgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtattgaacagatcacgcgtctgtacgccga gcgcctgtaccgtaaagatggtacaattccagttgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtc cagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttagcggggtaccgccatg atttcttagctagtaacggctttgatgtagaaggtattaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggag atatacatatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagtttcagc ggtagagttaggggtaacagcagctaaagaagctataaaaagagctaacataactccagatatgatagatgaatctcttttaggg ggagtacttacagcaggtcttggacaaaatatagcaagacaaatagcattaggagcaggaataccagtagaaaaaccagctat gactataaatatagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatgctgatataatgttagtt ggtggagctgaaaacatgagtatgtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgatt caatgataaaagatggattatcagacatatttaataactatcacatgggtattactgctgaaaacatagcagagcaatggaatataa ctagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaa tagttcctgttgttataaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactacaatggagaaa cttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaataaatgatggtgctgctatgtta gtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctggtgttgaccc taaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgactattgaagatatagatttagttga agctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaatatagatatgaataaagttaatgttaatggtggagc aatagctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaagagatgctaaa actggtcttgctacactttgtataggcggtggaatgggaactactttaatagttaagagatagtaagaaggagatatacatatgaaa ttagctgtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtatgtttaaagagtagaa ctcaaggtgctatagataaatgtttagctttattagataaaaatttaactaagttagttactaagggaaaaatggatgaagctacaaa agcagaaatattaagtcatgttagttcaactactaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaagac atgaatataaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatcttggcaacaaatacttcatcattatctata acagaaatagcttcttctactaagcgcccagataaagttataggaatgcatttctttaatccagttcctatgatgaaattagttgaagt tataagtggtcagttaacatcaaaagttacttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtatctga atctcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatatatgcagatggtgttgcaagtaaagaa gaaatagatgaagctatgaaattaggagcaaaccatccaatgggaccactagcattaggtgatttaatcggattagatgttgtttta gctataatgaacgttttatatactgaatttggagatactaaatatagacctcatccacttttagctaaaatggttagagctaatcaatta ggaagaaaaactaagataggattctatgattataataaataataagaaggagatatacatatgagtacaagtgatgttaaagtttat gagaatgtagctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagac tttagaagaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgtaatattgacaggggaaggaaaggcatttg tagctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatcttaggagcaaaagcttttggag aaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgctttaggtggaggatgtgaacttgcaatggcatgtgat ataagaattgcatctgctaaagctaaatttggtcagccagaagtaactcttggaataactccaggatatggaggaactcaaaggc ttacaagattggttggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagctgaaaaaatagg gctagtaaatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaagataatagctaaaaatgctcagcttg cagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaatagatatagaatctaatttatttggtctt tgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactttataaaagggtaataagaag gagatatacatatgagaagttttgaagaagtaattaagtttgcaaaagaaagaggacctaaaactatatcagtagcatgttgccaa gataaagaagttttaatggcagttgaaatggctagaaaagaaaaaatagcaaatgccatttttagtaggagatatagaaaagacta aagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgatagatataaaagatttagcagaagcatctctaaaatct

Description	Sequence
	gttgaattagtttcacaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaaagcagttttaaataa agaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttgatgtagagggatatgatagattatttttcgtaactgac gcagctatgaacttagctcctgatacaaatactaaaaagcaaatcatagaaaatgcttgcacagtagcacattcattagatataagt gaaccaaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagttgaagctaaagaactagaaga aatgtatgaaagaggagaaatcaaaggttgtatggttggtgggccttttgcaattgataatgcagtatctttagaagcagctaaac ataaaggtataaatcatcctgtagcaggacgagctgatatattattagccccagatattgaaggtggtaacatattatataaagcttt ggtattcttctcaaaatcaaaaaatgcaggagttatagttggggctaaagcaccaataatattaacttctagagcagacagtgaag aaactaaactaaactcaatagctttaggtgttttaatggcagcaaaggcataataagaaggagatatacatatgagcaaaatattta aaatcttaacaataaatcctggttcgacatcaactaaaatagctgtatttgataatgaggatttagtatttgaaaaaactttaagacatt cttcagaagaaataggaaaatatgagaaggtgtctgaccaatttgaatttcgtaaacaagtaatagaagaagctctaaaagaagg tggagtaaaaacatctgaattagatgctgtagtaggtagaggaggacttcttaaacctataaaaggtggtacttattcagtaagtgc tgctatgattgaagatttaaaagtgggagttttaggagaacacgcttcaaacctaggtggaataatagcaaaacaaataggtgaa gaagtaaatgttccttcatacatagtagaccctgttgttgtagatgaattagaagatgttgctagaatttctggtatgcctgaaataag tagagcaagtgtagtacatgctttaaatcaaaaggcaatagcaagaagatatgctagagaaataaacaagaaatatgaagatata aatcttatagttgcacacatgggtggaggagtttctgttggagctcataaaaatggtaaaatagtagatgttgcaaacgcattagat ggagaaggacctttctctccagaaagaagtggtggactaccagtaggtgcattagtaaaaatgtgctttagtggaaaatatactc aagatgaaattaaaaagaaaataaaaggtaatggcggactagttgcatacttaaacactaatgatgctagagaagttgaagaaa gaattgaagctggtgatgaaaaagctaaattagtatatgaagctatggcatatcaaatctctaaagaaataggagctagtgctgca gttcttaagggagatgtaaaagcaatattattaactggtggaatcgcatattcaaaaatgtttacagaaatgattgcagatagagtta aatttatagcagatgtaaaagtttatccaggtgaagatgaaatgattgcattagctcaaggtggacttagagttttaactggtgaag aagaggctcaagtttatgataactaataa

[01045] In certain constructs, the butyrate gene cassette is placed under the control of a tetracycline-inducible or constitutive promoter.

[01046] In a third butyrate gene cassette, the *pbt* and *buk* genes are replaced with *tesB*. TesB is a thioesterase found in *E. coli* that cleaves off the butyrate from butyryl-coA, thus obviating the need for pbt-buk.

[01047] In one embodiment, the *tesB* cassette is placed under the control of a FNR regulatory region selected from any of the sequences in **Table 20**. In an alternate embodiment, the *tesB* cassette is placed under the control of an RNS-responsive regulatory region, *e.g.*, *norB*, and the bacteria further comprises a gene encoding a corresponding RNS-responsive transcription factor, *e.g.*, *nsrR*. In yet another embodiment, the *tesB* cassette is placed under the control of an ROS-responsive regulatory region, *e.g.*, *oxyS*, and the bacteria further comprises a gene encoding a corresponding ROS-responsive transcription factor, *e.g.*, *oxyR*. In certain constructs, the different described butyrate gene cassettes are each placed under the control of a tetracycline-inducible or constitutive promoter. For example, genetically engineered Nissle are generated comprising a butyrate gene cassette in which the *pbt* and *buk* genes are replaced with *tesB* expressed under the control of a nitric oxide-responsive regulatory element. **SEQ ID NO: 207** comprises a reverse complement of the *nsrR* repressor gene from *Neisseria gonorrhoeae* (underlined), intergenic region containing

divergent promoters controlling *nsrR* and the butyrogenic gene cassette and their respective RBS (bold), and the butyrate genes (*ter-thiA-hbd-crt-tesB)* separated by RBS.

[01048] **Table 53: SEQ ID NO: 207** respective RBS (bold), and the butyrate genes (*ter-thiA-hbd-crt-tesB)* separated by RBS.

Table 53. SEQ ID NO: 207

SEQ ID NO: 207
ttattatcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgttgagcaggtcttgcagcgtgaaaccgtccag atacgtgaaaaacgacttcattgcaccgccgagtatgcccgtcagccggcaggacggcgtaatcaggcattcgttgttcgggcccata cactcgaccagctgcatcggttcgaggtggcggacgaccgcgccgatattgatgcgttcgggcggcgcggccagcctcagcccgc cgcctttcccgcgtacgctgtgcaagaacccgcctttgaccagcgcggtaaccactttcatcaaatggcttttggaaatgccgtaggtcg aggcgatggtggcgatattgaccagcgcgtcgtcgttgacggcggtgtagatgaggacgcgcagcccgtagtcggtatgttgggtca gatacat**acaacctccttagtacatgcaaaattatttctagagcaacatacgagccggaagcataaagtgtaaagcctggggtg cctaatgagttgagttgaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggttgttgacaattaatca tcggctcgtataatgtataacattcatattttgtgaattttaaactctagaaataattttgtttaactttaagaaggagatatacat**a tgatcgtaaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggctgcaagaagggagtggaagatcagattgaatat accaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaaacgttctggtgcttggctgctcaaatggttacggcc tggcgagccgcattactgctgcgttcggatacggggctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaatatgg tacaccgggatggtacaataatttggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgatcgacggcgatgcgtttt cagacgagatcaaggcccaggtaattgaggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatacagcttggccagcccagt acgtactgatcctgatacaggtatcatgcacaaaagcgttttgaaacccttggaaaaacgttcacaggcaaaacagtagatccgtttact ggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagccgccactgttaaagttatgggggtgaagattggg aacgttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggcctatagttatattggccctgaagctaccc aagctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagcacaccgtctcaacaaagagaacccgtca atccgtgccttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggtaatccctctgtatctcgccagcttgttc aaagtaatgaaagagaagggcaatcatgaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggta caattccagttgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtccagaaagcggtatccgcgttgatggag aaagtcacgggtgaaaacgcagaatctctcactgacttagcggggtaccgccatgatttcttagctagtaacggctttgatgtagaaggt attaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatatacatatgagagaagtagtaattgccagtgcagct agaacagcagtaggaagttttggaggagcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaaaa gagctaacataactccagatatgatagatgaatctcttttaggggagtacttacagcaggtcttggacaaaatatagcaagacaaatag cattaggagcaggaataccagtagaaaaaccagctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatctcaa cttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatgagtatgtctccttatttagtaccaagtgcgagatatggt gcaagaatgggtgatgctgcttttgttgattcaatgataaaagatggattatcagacatatttaataactatcacatgggtattactgctgaa aacatagcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaaagctcaagctg aaggaaaatttgatgaagaaatagttcctgttgttataaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctg gcactacaatggagaaacttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaataaatgatg gtgctgctatgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctgg tgttgaccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgactattgaagatatagatttag ttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaatatagatatgaataaagttaatgttaatggtggagca atagctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaagagatgctaaaactggt cttgctacactttgtataggcggtggaatgggaactactttaatagttaagagatagtaagaaggagatatacatatgaaattagctgtaat aggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtatgtttaaagagtagaactcaaggtgctata gataaatgtttagctttattagataaaaatttaactaagttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcat gttagttcaactactaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaagacatgaatataaagaaagatgttttc aagttactagatgaattatgtaaagaagatactatcttggcaacaaatacttcatcattatctataacagaaatagcttcttctactaagcgcc cagataaagttataggaatgcatttctttaatccagttcctatgatgaaattagttgaagttataagtggtcagttaacatcaaaagttacttttt gatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtatctgaatctcctggatttgtagtaaatagaatacttatacctat

gataaatgaagctgttggtatatatgcagatggtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaat
gggaccactagcattaggtgatttaatcggattagatgttgttttagctataatgaacgttttatatactgaatttggagatactaaatataga
cctcatccacttttagctaaaatggttagagctaatcaattaggaagaaaaactaagataggattctatgattataataaataataagaagg
agatatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatggaaatatatgtacagtgaaaatgaatagac
ctaaagcccttaatgcaataaattcaaagactttagaagaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgtaata
ttgacaggggaaggaaaggcatttgtagctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatctt
aggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgctttaggtggaggatgtgaact
tgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggtcagccagaagtaactcttggaataactccaggatatggagg
aactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagctgaaaa
aatagggctagtaaatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaagataatagctaaaaatgctcagctt
gcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaatagatatagaatctaatttatttggtctttgttt
ttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactttataaaagggtaataagaaggagatatac
atatgAGTCAGGCGCTAAAAAATTTACTGACATTGTTAAATCTGGAAAAAATTGAGG
AAGGACTCTTTCGCGGCCAGAGTGAAGATTTAGGTTTACGCCAGGTGTTTGGCGG
CCAGGTCGTGGGTCAGGCCTTGTATGCTGCAAAAGAGACCGTCCCTGAAGAGCG
GCTGGTACATTCGTTTCACAGCTACTTTCTTCGCCCTGGCGATAGTAAGAAGCCG
ATTATTTATGATGTCGAAACGCTGCGTGACGGTAACAGCTTCAGCGCCCGCCGGG
TTGCTGCTATTCAAAACGGCAAACCGATTTTTTATATGACTGCCTCTTTCCAGGCA
CCAGAAGCGGGTTTCGAACATCAAAAAACAATGCCGTCCGCGCCAGCGCCTGAT
GGCCTCCCTTCGGAAACGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCAG
TGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCGTCCGGTGGAGTT
TCATAACCCACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTGGATCCG
CGCAAATGGTAGCGTGCCGGATGACCTGCGCGTTCATCAGTATCTGCTCGGTTAC
GCTTCTGATCTTAACTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCT
CGAACCGGGGATTCAGATTGCCACCATTGACCATTCCATGTGGTTCCATCGCCCG
TTTAATTTGAATGAATGGCTGCTGTATAGCGTGGAGAGCACCTCGGCGTCCAGCG
CACGTGGCTTTGTGCGCGGTGAGTTTTATACCCAAGACGGCGTACTGGTTGCCTC
GACCGTTCAGGAAGGGGTGATGCGTAATCACAATtaa

Example 12. Further Construction of vectors for overproducing butyrate using a tet-inducible promoter

[01049] To facilitate inducible production of butyrate in *Escherichia coli* Nissle, the eight genes of the butyrate production pathway from *Peptoclostridium difficile* (*bcd, etfB, etfA, thiA, hbd, crt, bpt,* and *buk*; NCBI), as well as transcriptional and translational elements, were synthesized (Gen, Cambridge, MA) and cloned into vector pBR to create pLogic. As synthesized, the genes were placed under control of a tetracycline-inducible promoter, with the *tet* repressor (*tetR*) expressed constitutively, divergent from the tet-inducible synthetic butyrate operon. For efficient translation of butyrate genes, each synthetic gene in the operon was separated by a base pair ribosome binding site derived from the T promoter.

[01050] The gene products of *bcd-etfA-etfB* form a complex that convert crotonyl-CoA to butyryl-CoA, and may show some dependence on oxygen as a co-oxidant.

Because an effective probiotic should be able to function in an oxygen-limited environment (*e.g.* the mammalian gut), and because it has been shown that a single gene from *Treponema denticola* can functionally replace this three gene complex in an oxygen-independent manner (*trans*--enoynl-CoA reductase; *ter*), we created a second plasmid capable of butyrate production in *E. coli*. Inverse PCR was used to amplify the entire sequence of pLogic outside of the *bcd-etfA-etfB* region. The *ter* gene was codon optimized for *E. coli* codon usage using Integrated DNA technologies online codon optimization tool (https://www.idtdna.com/CodonOpt), synthesized (Genewiz, Cambridge, MA), and cloned into this inverse PCR fragment using Gibson assembly to create pLogic.

Example 13. Transforming E. coli

[01051] Each plasmid is transformed into *E. coli* Nissle or *E. coli* DH5a. All tubes, solutions, and cuvettes are pre-chilled to 4° C. An overnight culture of *E. coli* Nissle or *E. coli* DH5a is diluted 1:100 in 5 mL of lysogeny broth (LB) and grown until it reached an OD_{600} of 0.4-0.6. The cell culture medium contains a selection marker, *e.g.*, ampicillin, that is suitable for the plasmid. The *E. coli* cells are then centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 1 mL of 4° C water. The *E. coli* are again centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.5 mL of 4° C water. The *E. coli* are again centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are finally resuspended in 0.1 mL of 4° C water. The electroporator is set to 2.5 kV. 0.5 µg of one of the above plasmids is added to the cells, mixed by pipetting, and pipetted into a sterile, chilled cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. One mL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 37° C for 1 hr. The cells are spread out on an LB plate containing ampicillin and incubated overnight.

[01052] In alternate embodiments, the butyrate cassette can be inserted into the Nissle genome through homologous recombination (Genewiz, Cambridge, MA). Organization of the constructs and nucleotide sequences are provided herein. To create a vector capable of integrating the synthesized butyrate cassette construct into the chromosome, Gibson assembly was first used to add 1000bp sequences of DNA homologous to the Nissle lacZ locus into the R6K origin plasmid pKD3. This targets DNA cloned between these homology arms to be integrated into the lacZ locus in the Nissle genome. Gibson assembly was used to clone the fragment between these arms. PCR was used to amplify the region from this plasmid containing the entire sequence of the homology arms, as well as the

butyrate cassette between them. This PCR fragment was used to transform electrocompetent Nissle-pKD46, a strain that contains a temperature-sensitive plasmid encoding the lambda red recombinase genes. After transformation, cells were grown out for 2 hours before plating on chloramphenicol at 20ug/mL at 37 degrees C. Growth at 37 degrees C also cures the pKD46 plasmid. Transformants containing cassette were chloramphenicol resistant and lac-minus (lac-).

Example 14. Production of butyrate in recombinant E. coli

[01053] Production of butyrate is assessed in *E. coli* Nissle strains containing the butyrate cassettes described above in order to determine the effect of oxygen on butyrate production. All incubations are performed at 37° C. Cultures of *E. coli* strains DH5a and Nissle transformed with the butyrate cassettes are grown overnight in LB and then diluted 1:200 into 4 mL of M9 minimal medium containing 0.5% glucose. The cells are grown with shaking (250 rpm) for 4-6 h and incubated aerobically or anaerobically in a Coy anaerobic chamber (supplying 90% N2, 5% CO2, 5%H2). One mL culture aliquots are prepared in 1.5 mL capped tubes and incubated in a stationary incubator to limit culture aeration. One tube is removed at each time point (0, 1, 2, 4, and 20 hrs) and analyzed for butyrate concentration by LC-MS to confirm that butyrate production in these recombinant strains can be achieved in a low-oxygen environment.

Example 15. Production of butyrate in recombinant E. coli

[01054] Production of butyrate is assessed in *E. coli* Nissle strains containing the butyrate cassettes described above in order to determine the effect of oxygen on butyrate production. All incubations are performed at 37° C. Cultures of *E. coli* strains DH5a and Nissle transformed with the butyrate cassettes are grown overnight in LB and then diluted 1:200 into 4 mL of M9 minimal medium containing 0.5% glucose. The cells are grown with shaking (250 rpm) for 4-6 h and incubated aerobically or anaerobically in a Coy anaerobic chamber (supplying 90% N2, 5% CO2, 5%H2). One mL culture aliquots are prepared in 1.5 mL capped tubes and incubated in a stationary incubator to limit culture aeration. One tube is removed at each time point (0, 1, 2, 4, and 20 hrs) and analyzed for butyrate concentration by LC-MS to confirm that butyrate production in these recombinant strains can be achieved in a low-oxygen environment.

Example 16. Production of Butyrate in Recombinant E. coli using tet-inducible promoter

[01055] **FIG. 3** shows butyrate cassettes described above under the control of a tet-inducible promoter. Production of butyrate is assessed using the methods described below in **Example 22**. The tet-inducible cassettes tested include (1) tet-butyrate cassette comprising all eight genes (pLOGIC031); (2) tet-butyrate cassette in which the ter is substituted (pLOGIC046) and (3) tet-butyarte cassette in which tesB is substituted in place of pbt and buk genes.

[01056] **FIG. 31A** shows butyrate production in strains pLOGIC031 and pLOGIC046 in the presence and absence of oxygen, in which there is no significant difference in butyrate production. Enhanced butyrate production was shown in Nissle in low copy plasmid expressing pLOGIC046 which contain a deletion of the final two genes (ptb-buk) and their replacement with the endogenous *E. coli* tesB gene (a thioesterase that cleaves off the butyrate portion from butyryl CoA).

[01057] Overnight cultures of cells were diluted 1:100 in Lb and grown for 1.5 hours until early log phase was reached at which point anhydrous tet was added at a final concentration of 100ng/ml to induce plasmid expression. After 2 hours induction, cells were washed and resuspended in M9 minimal media containing 0.5% glucose at OD600=0.5. Samples were removed at indicated times and cells spun down. The supernatant was tested for butyrate production using LC-MS. **FIG. 31B** shows butyrate production in strains comprising a tet–butyrate cassette having ter substitution (pLOGIC046) or the tesB substitution (ptb-buk deletion), demonstrating that the tesB substituted strain has greater butyrate production.

[01058] **FIG. 32** shows the BW25113 strain of *E. coli*, which is a common cloning strain and the background of the KEIO collection of *E. coli* mutants. NuoB mutants having NuoB deletion were obtained. NuoB is a protein complex involved in the oxidation of NADH during respiratory growth (form of growth requiring electron transport). Preventing the coupling of NADH oxidation to electron transport allows an increase in the amount of NADH being used to support butyrate production. **FIG. 32** shows that compared with wild-type Nissle, deletion of NuoB results in grater production of butyrate.

Table 54. pLOGIC046-tesB-butyrate

pLOGIC046-tesB-butyrate: **SEQ ID NO:208**
gtaaaacgacggccagtgaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccg aataagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccct ttcttctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagt gaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtacttttgctccatc gcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatcttgccagctttccccttctaaagggcaaaagtgagtat ggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttttacatgccaatacaatgtaggctgctctacacctag cttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttacttttatctaatctag acatcattaattcctaatttttgttgacactctatcattgatagagttatttttaccactccctatcagtgatagagaaaagtgaactctagaaat aattttgtttaactttaagaaggagatatacatatgatcgtaaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggctg caagaagggagtggaagatcagattgaatataccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaaacg ttctggtgcttggctgctcaaatggttacggcctggcgagccgcattactgctgcgttcggatacggggctgcgaccatcggcgtgtcc tttgaaaaagcgggttcagaaaccaaatatggtacaccgggatggtacaataatttggcatttgatgaagcggcaaaacgcgagggtct ttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaaggcccaggtaattgaggaagccaaaaaaaaaggtatcaaattt gatctgatcgtatacagcttggccagcccagtacgtactgatcctgatacaggtatcatgcacaaaagcgttttgaaaccctttggaaaa acgttcacaggcaaaacagtagatccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagccgc cactgttaaagttatgggggtgaagattgggaacgttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttt ggcctatagttatattggccctgaagctacccaagctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccaca gcacaccgtctcaacaaagagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatc ccggtaatccctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtattgaacagatcacgcgtctg tacgccgagcgcctgtaccgtaaagatggtacaattccagttgatgaggaaaatcgcattcgcattgatgattgggagttagaagaaga cgtccagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttagcggggtaccgccatga tttcttagctagtaacggctttgatgtagaaggtattaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatatac atatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagtttcagcggtagagtta ggggtaacagcagctaaagaagctataaaaagagctaacataactccagatatgatagatgaatctcttttaggggagtacttacagc aggtcttggacaaaatatagcaagacaaatagcattaggagcaggaataccagtagaaaaaccagctatgactataaatatagtttgtg gttctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatgagtat gtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaatgataaaagatggattatcagacat atttaataactatcacatgggtattactgctgaaaacatagcagagcaatggaatataactagagaagaacaagatgaattagctcttgca agtcaaaataaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgttataaaaggaagaaaaggtgacac tgtagtagataaagatgaatatattaagcctggcactacaatggagaaacttgctaagttaagacctgcatttaaaaaagatggaacagtt actgctggtaatgcatcaggaataaatgatggtgctgctatgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcc

tcttgcaactatagtttcttatggaacagctggtgttgaccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaag
ctgctaatatgactattgaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaatatag
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ataagtggtcagttaacatcaaaagttacttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtatctgaatctc
ctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatatatgcagatggtgttgcaagtaaagaagaaatagat
gaagctatgaaattaggagcaaaccatccaatgggaccactagcattaggtgatttaatcggattagatgttgttttagctataatgaacgt
tttatatactgaatttggagatactaaatatagacctcatccacttttagctaaaatggttagagctaatcaattaggaagaaaaactaagat
aggattctatgattataataaataataagaaggagatatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtag
atggaaatatatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagactttagaagaactttatgaagtatttgta
gatattaataatgatgaaactattgatgttgtaatattgacaggggaaggaaaggcatttgtagctggagcagatattgcatacatgaaag
atttagatgctgtagctgctaaagattttagtatcttaggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgct
gtaaacggatttgctttaggtggaggatgtgaacttgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggtcagccag
aagtaactcttggaataactccaggatatggaggaactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaatcttta
caggtcaagttataaaagctgatgaagctgaaaaaatagggctagtaaatagagtcgttgagccagacattttaatagaagaagttgag
aaattagctaagataatagctaaaaatgctcagcttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaata
ctggaatagatatagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaag
ctaactttataaaagggtaataagaaggagatatacatatgAGTCAGGCGCTAAAAAATTTACTGACATTG
TTAAATCTGGAAAAAATTGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTTA
GGTTTACGCCAGGTGTTTGGCGGCCAGGTCGTGGGTCAGGCCTTGTATGCTGCAA
AAGAGACCGTCCCTGAAGAGCGGCTGGTACATTCGTTTCACAGCTACTTTCTTCG
CCCTGGCGATAGTAAGAAGCCGATTATTTATGATGTCGAAACGCTGCGTGACGGT
AACAGCTTCAGCGCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCGATTTTTT
ATATGACTGCCTCTTTCCAGGCACCAGAAGCGGGTTTCGAACATCAAAAAACAAT
GCCGTCCGCGCCAGCGCCTGATGGCCTCCCTTCGGAAACGCAAATCGCCCAATCG
CTGGCGCACCTGCTGCCGCCAGTGCTGAAAGATAAATTCATCTGCGATCGTCCGC
TGGAAGTCCGTCCGGTGGAGTTTCATAACCCACTGAAAGGTCACGTCGCAGAAC
CACATCGTCAGGTGTGGATCCGCGCAAATGGTAGCGTGCCGGATGACCTGCGCG

TTCATCAGTATCTGCTCGGTTACGCTTCTGATCTTAACTTCCTGCCGGTAGCTCTA
CAGCCGCACGGCATCGGTTTTCTCGAACCGGGGATTCAGATTGCCACCATTGACC
ATTCCATGTGGTTCCATCGCCCGTTTAATTTGAATGAATGGCTGCTGTATAGCGTG
GAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGAGTTTTATACCC
AAGACGGCGTACTGGTTGCCTCGACCGTTCAGGAAGGGGTGATGCGTAATCACA
ATtaa

Example 17. Production of Butyrate in Recombinant E. coli

[01059] Production of butyrate is assessed in *E. coli* Nissle strains containing the butyrate cassettes described above in order to determine the effect of oxygen on butyrate production. All incubations are performed at 37° C. Cultures of *E. coli* strains DH5a and Nissle transformed with the butyrate cassettes are grown overnight in LB and then diluted 1:200 into 4 mL of M9 minimal medium containing 0.5% glucose. The cells are grown with shaking (250 rpm) for 4-6 h and incubated aerobically or anaerobically in a Coy anaerobic chamber (supplying 90% N_2, 5% CO_2, 5%H_2). One mL culture aliquots are prepared in 1.5 mL capped tubes and incubated in a stationary incubator to limit culture aeration. One tube is removed at each time point (0, 1, 2, 4, and 20 hours) and analyzed for butyrate concentration by LC-MS to confirm that butyrate production in these recombinant strains can be achieved in a low-oxygen environment.

[01060] In an alternate embodiment, overnight bacterial cultures were diluted 1:100 into fresh LB and grown for 1.5 hrs to allow entry into early log phase. At this point, long half-life nitric oxide donor (DETA-NO; diethylenetriamine-nitric oxide adduct) was added to cultures at a final concentration of 0.3mM to induce expression from plasmid. After 2 hours of induction, cells were spun down, supernatant was discarded, and the cells were resuspended in M9 minimal media containing 0.5% glucose. Culture supernatant was then analyzed at indicated time points to assess levels of butyrate production. Genetically engineered Nissle comprising pLogic031-nsrR-norB-butyrate operon construct; SYN507) or (pLogic046-nsrR-norB-butyrate operon construct; SYN--508) produce significantly more butyrate as compared to wild-type Nissle.

[01061] Genetically engineered Nissle were generated comprising a butyrate gene cassette in which the *pbt* and *buk* genes are replaced with *tesB* (**SEQ ID NO: 15**) expressed under the control of a tetracycline promoter (pLOGIC046-tesB-butyrate; **SEQ ID NO: 208**). **SEQ ID NO: 208** comprises a reverse complement of the tetR repressor

(underlined), an intergenic region containing divergent promoters controlling tetR and the butyrate operon and their respective RBS (bold), and the butyrate genes (*ter-thiA1-hbd-crt2-tesB)* separated by RBS.

[01062] Overnight bacterial cultures were diluted 1:100 into fresh LB and grown for 1.5 hrs to allow entry into early log phase. At this point, anhydrous tetracycline (ATC) was added to cultures at a final concentration of 100 ng/mL to induce expression of butyrate genes from plasmid. After 2 hours of induction, cells were spun down, supernatant was discarded, and the cells were resuspended in M9 minimal media containing 0.5% glucose. Culture supernatant was then analyzed at indicated time points to assess levels of butyrate production. Replacement of pbt and buk with tesB leads to greater levels of butyrate production.

[01063] **FIG. 33C** shows butyrate production in strains comprising an FNR-butyrate cassette SYN501 (having the ter substitution) in the presence/absence of glucose and oxygen. **FIG. 33C** shows that bacteria need both glucose and anaerobic conditions for butyrate production from the FNR promoter. Cells were grown aerobically or anaerobically in media containg no glucose (LB) or in media containing glucose at 0.5% (RMC). Culture samples were taken at indicaed time pints and supernatant fractions were assessed for butyrate concentration using LC-MS. These data show that SYN501 requires glucose for butyrate production and that in the presence of glucose butyrate production can be enhanced under anaerobic conditions when under the control of the anaerobic FNR-regulated ydfZ promoter.

Table 55. Butyrate cassette sequences

Description	**Sequence**	**SEQ ID NO**
ydfZ +RBS (RBS is bolded)	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCC CCCGACTTATGGCTCATGCATGCATCAAAAAAGATGT GAGCTTGATCAAAAACAAAAAATATTTCACTCGACAG GAGTATTTATATTGCGCCCGGATCCCTCTAGAAATAAT **TTTGTTTAACTTTAAGAAGGAGATATACAT**	**SEQ ID NO: 209**
First RBS (in ydfZ=RBS)	TTTGTTTAACTTTAAGAAGGAGA	**SEQ ID NO: 210**
Internal RBS between genes	taagaaggagatatacat	**SEQ ID NO: 211**
Butryate cassette under the control	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCC CCCGACTTATGGCTCATGCATGCATCAAAAAAGATGT	**SEQ ID NO:**

of the ydfZ promoter (uppercase: ydfZ promoter, with RBS in bold; lower case: coding regions in the following order: ter, thiA, hbd, crt2, pbt, buk, separated by internal RBS (uppercase and underlined)	GAGCTTGATCAAAAACAAAAAATATTTCACTCGACAG GAGTATTTATATTGCGCCCGGATCCCTCTAGAAATAAT **TTTGTTTAACTTTAAGAAGGAGATATACAT**atgatcgtaa aacctatggtacgcaacaatatctgcctgaacgcccatcctcagggctgcaagaaggga gtggaagatcagattgaatataccaagaaacgcattaccgcagaagtcaaagctggcgc aaaagctccaaaaaacgttctggtgcttggctgctcaaatggttacggcctggcgagccg cattactgctgcgttcggatacggggctgcgaccatcggcgtgtcctttgaaaaagcgggt tcagaaaccaaatatggtacaccgggatggtacaataatttggcatttgatgaagcggcaa aacgcgagggtctttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaagg cccaggtaattgaggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatacagctt ggccagcccagtacgtactgatcctgatacaggtatcatgcacaaaagcgttttgaaaccc tttggaaaaacgttcacaggcaaaacagtagatccgtttactggcgagctgaaggaaatct ccgcggaaccagcaaatgacgaggaagcagccgccactgttaaagttatgggggggtga agattgggaacgttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtatt accttggcctatagttatattggccctgaagctacccaagctttgtaccgtaaaggcacaat cggcaaggccaaagaacacctggaggccacagcacaccgtctcaacaaagagaaccc gtcaatccgtgccttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaat cccggtaatccctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcat gaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggt acaattccagttgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacg tccagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctc actgacttagcggggtaccgccatgatttcttagctagtaacggctttgatgtagaaggtatt aattatgaagcggaagttgaacgcttcgaccgtatctga<u>TAAGAAGGAGATA</u> <u>TACAT</u>atgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttg gaggagcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagcta taaaaagagctaacataactccagatatgatagatgaatctcttttaggggagtacttaca gcaggtcttggacaaaatatagcaagacaaatagcattaggagcaggaataccagtaga aaaaccagctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatctc aacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatgagtatgt ctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattc aatgataaaagatggattatcagacatatttaataactatcacatgggtattactgctgaaaa catagcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtca aaataaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgtta taaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactac aatggagaaacttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggta atgcatcaggaataaatgatggtgctgctatgttagtagtaatggctaaagaaaaagctga agaactaggaatagagcctcttgcaactatagtttcttatggaacagctggtgttgacccta aaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgact attgaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgtagctgtaataa gagacttaaatatagatatgaataaagttaatgttaatggtggagcaatagctataggacat ccaataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaagag atgctaaaactggtcttgctacactttgtataggcggtggaatgggaactactttaatagtta agagatag<u>TAAGAAGGAGATATACAT</u>atgaaattagctgtaataggtagt ggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtatgtttaaag agtagaactcaaggtgctatagataaatgtttagctttattagataaaaatttaactaagttagt tactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgttagttcaacta ctaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaagacatgaatat aaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatcttggcaacaaa tacttcatcattatctataacagaaatagcttcttctactaagcgcccagataaagttatagga atgcatttctttaatccagttcctatgatgaaattagttgaagttataagtggtcagttaacatc	**212**

	aaaagttacttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtat ctgaatctcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtata tatgcagatggtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaac catccaatgggaccactagcattaggtgatttaatcggattagatgttgttttagctataatga acgttttatatactgaatttggagatactaaatatagacctcatccacttttagctaaaatggtt agagctaatcaattaggaagaaaaactaagataggattctatgattataataaataaTAA GAAGGAGATATACATatgagtacaagtgatgttaaagtttatgagaatgtag ctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaagcccttaatgca ataaattcaaagactttagaagaactttatgaagtatttgtagatattaataatgatgaaactat tgatgttgtaatattgacaggggaaggaaaggcatttgtagctggagcagatattgcatac atgaaagatttagatgctgtagctgctaaagattttagtatcttaggagcaaaagcttttgga gaaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgctttaggtggag gatgtgaacttgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggtcagc cagaagtaactcttggaataactccaggatatggaggaactcaaaggcttacaagattggt tggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagct gaaaaaatagggctagtaaatagagtcgttgagccagacattttaatagaagaagttgaga aattagctaagataatagctaaaaatgctcagcttgcagttagatactctaaagaagcaata caacttggtgctcaaactgatataaatactggaatagatatagaatctaatttatttggtctttg tttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaact ttataaaagggtaaTAAGAAGGAGATATACATatgagaagttttgaagaa gtaattaagtttgcaaaagaaagaggacctaaaactatatcagtagcatgttgccaagataa agaagttttaatggcagttgaaatggctagaaaagaaaaaatagcaaatgccattttagtag gagatatagaaaagactaaagaaattgcaaaaagcatagacatggatatcgaaaattatg aactgatagatataaaagatttagcagaagcatctctaaaatctgttgaattagtttcacaag gaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaaagcagtttt aaataaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttgatgtag agggatatgatagattatttttcgtaactgacgcagctatgaacttagctcctgatacaaata ctaaaaagcaaatcatagaaaatgcttgcacagtagcacattcattagatataagtgaacca aaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagttgaa gctaaagaactagaagaaatgtatgaaagaggagaaatcaaaggttgtatggttggtggg ccttttgcaattgataatgcagtatctttagaagcagctaaacataaaggtataaatcatcctg tagcaggacgagctgatatattattagccccagatattgaaggtggtaacatattatataaa gctttggtattcttctcaaaatcaaaaaatgcaggagttatagttggggctaaagcaccaata atattaacttctagagcagacagtgaagaaactaaactaaactcaatagctttaggtgtttta atggcagcaaaggcataaTAAGAAGGAGATATACATatgagcaaaata tttaaaatcttaacaataaatcctggttcgacatcaactaaaatagctgtatttgataatgagg atttagtatttgaaaaaactttaagacattcttcagaagaaataggaaaatatgagaaggtgt ctgaccaatttgaatttcgtaaacaagtaatagaagaagctctaaaagaaggtggagtaaa aacatctgaattagatgctgtagtaggtagaggaggacttcttaaacctataaaaggtggta cttattcagtaagtgctgctatgattgaagatttaaaagtgggagttttaggagaacacgctt caaacctaggtggaataatagcaaaacaaataggtgaagaagtaaatgttccttcatacat agtagaccctgttgttgtagatgaattagaagatgttgctagaatttctggtatgcctgaaata agtagagcaagtgtagtacatgctttaaatcaaaaggcaatagcaagaagatatgctaga gaaataaacaagaaatatgaagatataaatcttatagttgcacacatgggtggaggagtttc tgttggagctcataaaaatggtaaaatagtagatgttgcaaacgcattagatggagaagga cctttctctccagaaagaagtggtggactaccagtaggtgcattagtaaaaatgtgctttagt ggaaaatatactcaagatgaaattaaaaagaaaataaaaggtaatggcggactagttgcat acttaaacactaatgatgctagagaagttgaagaaagaattgaagctggtgatgaaaaag ctaaattagtatatgaagctatggcatatcaaatctctaaagaaataggagctagtgctgca gttcttaagggagatgtaaaagcaatattattaactggtggaatcgcatattcaaaaatgttta	

	cagaaatgattgcagatagagttaaatttatagcagatgtaaaagtttatccaggtgaagat gaaatgattgcattagctcaaggtggacttagagttttaactggtgaagaagaggctcaagt ttatgataactaataa	

[01064] In some embodiments, the genetically engineered bacteria comprise the nucleic acid sequence of **SEQ ID NO: 212** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as **SEQ ID NO: 212** or a functional fragment thereof. In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of **SEQ ID NO: 212** or a functional fragment thereof, or a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide as **SEQ ID NO: 212** or a functional fragment thereof.

[01065] In alternate embodiments, pbt and buk are replaced with TesB (**SEQ ID NO: 15**)

[01066] In some embodiments, the butyrate cassette is driven by an inducible promoter. For example, other FNR promotors can be used in lieu of ydfZ, *e.g.*, in **SEQ ID NO: 177-188**.

[01067] Non-limiting FNR promoter sequences are provided herein. In some embodiments, the genetically engineered bacteria of the invention comprise a butyrate cassette under the control of one or more of promoter sequences found in Table 6, *e.g.*, *nirB* promoter, *ydfZ* promoter, *nirB* promoter fused to a strong ribosome binding site, *ydfZ* promoter fused to a strong ribosome binding site, *fnrS*, an anaerobically induced small RNA gene (*fnrS* promoter), *nirB* promoter fused to a *crp* binding site, and *fnrS* fused to a *crp* binding site.

[01068] In some embodiments, the butyrate cassette is under the control of a promoter which is inducible by metabolites present in the gut. In some embodiments the butyrate cassette is induced by metabolites indicative of liver damage, *e.g.*, bilirubin. In some embodiments, the butyrate cassette is placed under the control of promoter, which is inducible by inflammation or an inflammatory response (*e.g.*, RNS or ROS promoter).

[01069] In some embodiments, the genetically engineered bacteria comprise a butyrate cassette driven by a promoter induced by a molecule or metabolite. Promoters that

respond to one of these molecules or their metabolites may be used in the genetically engineered bacteria provided herein.

[01070] In some embodiments, the butyrate cassette is inducible by arabinose and is driven by the AraBAD promoter.

Example 18. Comparison of in vitro butyrate production efficacy of chromosomal insertion and plasmid-bearing engineered bacterial strains

[01071] The *in vitro* butyrate production efficacy of engineered bacterial strains harboring a chromosomal insertion of a butyrate cassette was compared to a strain strain bearing a butyrate cassette on a plasmid. SYN1001 and SYN1002 harbor a chromosomal insertion between the *agaI/rsmI* locus of a butyrate cassette (either ter→tesB or ter→pbt-buk, respectively) driven by an fnr inducible promoter. These strains were compared side by side with the low copy plasmid strain SYN501 (Logic156 (pSC101 PydfZ-ter ->pbt-buk butyrate plasmid) also driven by an fnr inducible promoter. Butyrate levels in the media were measured at 4 and 24 hours post anaerobic induction.

[01072] Briefly, 3ml LB was inoculated with bacteria from frozen glycerol stocks. Bacteria were grown overnight at 37 C with shaking. Overnight cultures were diluted 1:100 dilution into 10ml LB (containing antibiotics) in a 125ml baffled flask. Cultures were grown aerobically at 37 C with shaking for about 1.5h, and then transferred to the anaerobic chamber at 37 C for 4h. Bacteria ($2X10^8$ CFU) were added to 1ml M9 media containing 50mM MOPS with 0.5% glucose in microcentrifuge tubes. Cells were plated to determine cell counts. The assay tubes were placed in the anaerobic chamber at 37 C. At indicated times (4 and 24h), 120 ul cells were removed and pelleted at 14,000rpm for 1min, and 100ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for butyrate concentrations (as described in **Example 22**). Results are depicted in **FIG. 38,** and show that SYN1001 and SYN1002 give comparable butyrate production to the plasmid strain SYN501.

Table 56. FRNRs Butyrate Cassette Sequences

Description	**Sequence**
Pfnrs-ter-thiA1-hbd-ctr2-tesB **SEQ ID NO:213** , *e.g.* integrated into the chromosome in SYN1001 Pfnrs:uppercase; butyrate cassette: lower case	GGTACCAGTTGTTCTTATTGGTGGTGTTGCTTTATGGTT GCATCGTAGTAAATGGTTGTAACAAAAGCAATTTTTCC GGCTGTCTGTATACAAAAACGCCGCAAAGTTTGAGCGA AGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTC TTGGATCCAAAGTGAACTCTAGAAATAATTTTGTTTAAC TTTAAGAAGGAGATATACATatgatcgtaaaacctatggtacgcaacaat atctgcctgaacgcccatcctcagggctgcaagaagggagtggaagatcagattgaatata

	ccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaaacgttctggt gcttggctgctcaaatggttacggcctggcgagccgcattactgctgcgttcggatacgggg ctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaatatggtacaccggg atggtacaataatttggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgat cgacggcgatgcgttttcagacgagatcaaggcccaggtaattgaggaagccaaaaaaaa aggtatcaaatttgatctgatcgtatacagcttggccagcccagtacgtactgatcctgataca ggtatcatgcacaaaagcgttttgaaaccctttggaaaaacgttcacaggcaaaacagtagat ccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagcc gccactgttaaagttatgggggggtgaagattgggaacgttggattaagcagctgtcgaagga aggcctcttagaagaaggctgtattaccttggcctatagttatattggccctgaagctacccaa gctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagcacac cgtctcaacaaagagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaac ccgcgcaagcgccgtaatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaa agagaagggcaatcatgaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgt accgtaaagatggtacaattccagttgatgaggaaaatcgcattcgcattgatgattgggagtt agaagaagacgtccagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgc agaatctctcactgacttagcggggtaccgccatgatttcttagctagtaacggctttgatgtag aaggtattaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatatac atatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagc atttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagag ctaacataactccagatatgatagatgaatctcttttaggggggagtacttacagcaggtcttgg acaaaatatagcaagacaaatagcattaggagcaggaataccagtagaaaaaccagctatg actataaatatagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcattag gtgatgctgatataatgttagttggtggagctgaaaacatgagtatgtctccttatttagtaccaa gtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaatgataaaagatggatt atcagacatatttaataactatcacatgggtattactgctgaaaacatagcagagcaatggaat ataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaaagctca agctgaaggaaaatttgatgaagaaatagttcctgttgttataaaaggaagaaaaggtgacac tgtagtagataaagatgaatatattaagcctggcactacaatggagaaacttgctaagttaaga cctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaataaatgatggtgct gctatgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaact atagtttcttatggaacagctggtgttgaccctaaaataatgggatatggaccagttccagcaa ctaaaaaagctttagaagctgctaatatgactattgaagatatagatttagttgaagctaatgag gcatttgctgcccaatctgtagctgtaataagagacttaaatatagatatgaataaagttaatgtt aatggtggagcaatagctataggacatccaataggatgctcaggagcaagaatacttactac acttttatatgaaatgaagagaagagatgctaaaactggtcttgctacactttgtataggcggtg gaatgggaactactttaatagttaagagatagtaagaaggagatatacatatgaaattagctgt aataggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtat gtttaaagagtagaactcaaggtgctatagataaatgtttagctttattagataaaaatttaacta agttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgttagttc aactactaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaagacatga atataaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatcttggcaaca aatacttcatcattatctataacagaaatagcttcttctactaagcgcccagataaagttatagga atgcatttctttaatccagttcctatgatgaaattagttgaagttataagtggtcagttaacatcaa aagttacttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtatctga atctcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatatatgca gatggtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaat gggaccactagcattaggtgatttaatcggattagatgttgttttagctataatgaacgttttatat actgaatttggagatactaaatatagacctcatccacttttagctaaaatggttagagctaatca attaggaagaaaaactaagataggattctatgattataataaataataagaaggagatatacat

	atgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatggaaatatatgtac agtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagactttagaagaactttat gaagtatttgtagatattaataatgatgaaactattgatgttgtaatattgacaggggaaggaaa ggcatttgtagctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagat tttagtatcttaggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctg ctgtaaacggatttgctttaggtggaggatgtgaacttgcaatggcatgtgatataagaattgc atctgctaaagctaaatttggtcagccagaagtaactcttggaataactccaggatatggagg aactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacaggtca agttataaaagctgatgaagctgaaaaaatagggctagtaaatagagtcgttgagccagaca ttttaatagaagaagttgagaaattagctaagataatagctaaaaatgctcagcttgcagttaga tactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaatagatatagaat ctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaa gagagaagctaactttataaaagggtaataagaaggagatatacatatgagtcaggcgctaa aaaatttactgacattgttaaatctggaaaaaattgaggaaggactctttcgcggccagagtga agatttaggtttacgccaggtgtttggcggccaggtcgtgggtcaggccttgtatgctgcaaa agagaccgtccctgaagagcggctggtacattcgtttcacagctactttcttcgccctggcga tagtaagaagccgattatttatgatgtcgaaacgctgcgtgacggtaacagcttcagcgcccg ccgggttgctgctattcaaaacggcaaaccgatttttttatatgactgcctctttccaggcaccag aagcgggtttcgaacatcaaaaaacaatgccgtccgcgccagcgcctgatggcctcccttc ggaaacgcaaatcgcccaatcgctggcgcacctgctgccgccagtgctgaaagataaattc atctgcgatcgtccgctggaagtccgtccggtggagtttcataacccactgaaaggtcacgtc gcagaaccacatcgtcaggtgtggatccgcgcaaatggtagcgtgccggatgacctgcgc gttcatcagtatctgctcggttacgcttctgatcttaacttcctgccggtagctctacagccgca cggcatcggttttctcgaaccggggattcagattgccaccattgaccattccatgtggttccat cgcccgtttaatttgaatgaatggctgctgtatagcgtggagagcacctcggcgtccagcgc acgtggctttgtgcgcggtgagttttatacccaagacggcgtactggttgcctcgaccgttca ggaaggggtgatgcgtaatcacaattaa
Pfnrs-ter-thiA1-hbd-crt2-pbt-buk (**SEQ ID NO: 214**), *e.g.* integrated into the chromosome in SYN1002 Pfnrs:uppercase; butyrate cassette: lower case	GGTACCAGTTGTTCTTATTGGTGGTGTTGCTTTATGGTT GCATCGTAGTAAATGGTTGTAACAAAAGCAATTTTTCC GGCTGTCTGTATACAAAAACGCCGCAAAGTTTGAGCGA AGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTC TTGGATCCAAAGTGAACTCTAGAAATAATTTTGTTTAAC TTTAAGAAGGAGATATACATatgatcgtaaaacctatggtacgcaacaat atctgcctgaacgcccatcctcagggctgcaagaagggagtggaagatcagattgaatata ccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaaacgttctggt gcttggctgctcaaatggttacggcctggcgagccgcattactgctgcgttcggatacgggg ctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaatatggtacaccggg atggtacaataatttggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgat cgacggcgatgcgttttcagacgagatcaaggcccaggtaattgaggaagccaaaaaaaa aggtatcaaatttgatctgatcgtatacagcttggccagcccagtacgtactgatcctgataca ggtatcatgcacaaaagcgttttgaaacccttggaaaaacgttcacaggcaaaacagtagat ccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagcc gccactgttaaagttatgggggtgaagattgggaacgttggattaagcagctgtcgaagga aggcctcttagaagaaggctgtattaccttggcctatagttatattggccctgaagctacccaa gctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagcacac cgtctcaacaaagagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaac ccgcgcaagcgccgtaatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaa agagaagggcaatcatgaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgt accgtaaagatggtacaattccagttgatgaggaaaatcgcattcgcattgatgattgggagtt agaagaagacgtccagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgc

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PfNRS (ribosome binding site is underlined) (**SEQ ID NO: 215**)	GGTACCAGTTGTTCTTATTGGTGGTGTTGCTTTATGGTT GCATCGTAGTAAATGGTTGTAACAAAAGCAATTTTTCC GGCTGTCTGTATACAAAAACGCCGCAAAGTTTGAGCGA AGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTC TTGGATCCAAAGTGAACTCTAGAAATAATTTTGTTTAAC TTTAAGAAGGAGATATACAT
Ribosome binding site and leader region (**SEQ ID NO:216**)	CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATAT ACAT

Example 19. Assessment of intestinal butyrate levels in response to SYN501 administration in mice

[01073] To determine efficacy of butyrate production by the genetically engineered bacteria *in vivo*, the levels of butyrate upon administration of SYN501 (Logic156 (pSC101 PydfZ-ter ->pbt-buk butyrate plasmid)) to C57BL6 mice was first assessed in the feces. Water containing 100 mM butyrate was used as a control.

[01074] On day 1, C57BL6 mice (24 total animals) were weighed and randomized into 4 groups; Group 1: H20 control (n=6); Group 2-100 mM butyrate (n=6); Group 3-streptomycin resistant Nissle (n=6); Group 4-SYN501 (n=6). Mice were either gavaged with 100 ul streptomycin resistant Nissle or SYN501, and group 2 was changed to H20(+)100 mM butyrate at a dose of 10e10 cells/100ul. On days 2-4, mice were weighted and Groups 3 and 4 were gavaged in the AM and the PM with streptomycin resistant Nissle or SYN501. On day 5, mice were weighed and Groups 3 and 4 were gavaged in the am with streptomycin resistant Nissle or SYN501, and feces was collected and butyrate concentrations determined as described in **Example 23**. Results are depicted in **FIG. 37** Significantly greater levels of butyrate were detected in the feces of the mice gavaged with SYN501 as compared mice gavaged with the Nissle control or those given water only. Levels are close to 2 mM and higher than the levels seen in the mice fed with H20 (+) 200 mM butyrate.

[01075] Next the effects of SYN501 on levels of butyrate in the cecum, cecal effluent, large intestine, and large intestine effluent are assessed. Because baseline concentrations of butyrate are high in these compartments, an antibiotic treatment is administered in advance to clear out the bacteria responsible for butyrate production in the intestine. As a result, smaller differences in butyrate levels can be more accurately observed and measured. Water containing 100 mM butyrate is used as a control.

[01076] During week 1 of the study, animals are treated with an antibiotic cocktail in the drinking water to reduce the baseline levels of resident microflora. The antibiotic cocktail is composed of ABX-ampicillin, vancomycin, neomycin, and metronidazole. During week 2 animals are orally administered 100 ul of streptomycin resistant Nissle or engineered strain SYN501 twice a day for five days (at a dose of 10e10 cells/100ul).

[01077] On day 1, C57BL6 (Female, 8 weeks) are separated into four groups as follows: Group 1: H20 control (n=10); Group 2: 100 mM butyrate (n=10); Group 3: streptomycin resistant Nissle (n=10); Group 4: SYN501 (n=10). Animals are weighed and feces is collected from the animals (T=0-time point). Animals are changed to H2O (+) antibiotic cocktail. On day 5, animals are weighed and feces is collected (time point T=5d). The H2O (+) antibiotic cocktail bottles are changed. On day 8, the mice are weighed and feces is collected. Mice of Group 3 and Group 4 are gavaged in the AM and PM with streptomycin resistant Nissle or SYN501. The water in all cages is changed to water without antibiotic. Group 2 is provided with 100 mM butyrate in H2O. On days 9-11, mice are weighed, and mice of Group 3 and Group 4 are gavaged in the AM and PM with

streptomycin resistant Nissle or SYN501. On day 12, mice are gavaged with streptomycin resistant Nissle or SYN501 in the AM, and 4 hours post dose, blood is harvested, and cecal and large intestinal contents, and tissue, and feces are collected and processed for analysis.

Example 20. Measurement of Satiety Markers upon administration of SYN501 in vivo

[01078] To determine whether administration of a butyrate producing strain might result in increased levels of satiety markers, SYN501 is administered to 10-week old C57BL6 (10 weeks) and blood levels of GLP1 and insulin are measured. Butyrate in H20 at 100 mM is used as a control (*e.g.*, as described in Lin et al., Butyrate and Propionate Protect against Diet-Induced Obesity and Regulate Gut Hormones via Free Fatty Acid Receptor 3-Independent Mechanisms, PLOS One, April 2012 | Volume 7 | Issue 4 | e35240).

[01079] On day 1, animals are randomized and distributed into 5 groups as follows: Group 1: Time 0 control (n=6); Group 2-H20 (+) 100 mM butyrate, 10 min (n=6); Group 3-SYN501, 30 min (n=6); Group 4-SYN501, 4h (n=6); Group 5-H20 (+) 100 mM butyrate, 4h (n=6). Mice are fasted overnight. On day 2, mice are gavaged with either H20(+)100 mM butyrate or SYN501. Then, blood is harvested via cardiac bleed at the following time points post dose: Group 1 is Time 0; Group 2 (H20 (+) 100 mM butyrate) at 10 min; Group 3 (SYN501) at 30 min; Group 4 (SYN501) at 4h; Group 5 (H20 (+) 100 mM butyrate) at 4h. Serum is analyzed by ELISA for GLP-1 and insulin. Fecal samples are analyzed for butyrate by MS as described herein.

Example 21. Comparison of Butyrate production levels between the genetically engineered bacteria encoding a butyrate cassette and selected Clostridia strains

[01080] The efficacy of pbutyrate production in SYN501 (pSC101 PydfZ-ter ->pbt-buk butyrate plasmid) was compared to CBM588 (Clostridia butyricum MIYARISAN, a Japanese probiotic strain), Clostridium tyrobutyricum VPI 5392 (Type Strain), and Clostridium butyricum NCTC 7423 (Type Strain).

[01081] Briefly, overnight cultures of SYN501 were diluted 1:100 dilution and was grown in RCM (Reinforced Clostridial Media, which is similar to LB but contains 05% glucose) at 37 C with shaking for 2 hours, then either moved into the anaerobic chamber or left aerobically shaking. Clostridial strains were only grown anaerobically. At indicated times (2, 8, 24, and 48h), 120 ul cells were removed and pelleted at 14,000rpm for 1min, and 100ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for butyrate concentrations (as described in **Example**

22). Results are depicted in **FIG. 39**, and show that SYN501 produces butyrate levels comparable to *Clostridium spp.* in RCM media

Example 22. Quantification of Butyrate by LC-MS/MS

[01082] To obtain the butyrate measurements in Example 37 a LC-MS/MS protocol for butyrate quantification was used.

Sample preparation

[01083] First, fresh 1000, 500, 250, 100, 20, 4 and 0.8µg/mL sodium butyrate standards were prepared in water. Then, 10µL of sample (bacterial supernatants and standards) were pipetted into a V-bottom polypropylene 96-well plate, and 90µL of 67% ACN (60uL ACN+30uL water per reaction) with 4ug/mL of butyrate-d7 (CDN isotope) internal standard in final solution were added to each sample. The plate was heat-sealed, mixed well, and centrifuged at 4000rpm for 5 minutes. In a round-bottom 96-well polypropylene plate, 20µL of diluted samples were added to 180µL of a buffer containing 10mM MES pH4.5, 20mM EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide), and 20mM TFEA (2,2,2-trifluroethylamine). The plate was again heat-sealed and mixed well, and samples were incubated at room temperature for 1 hour.

LC-MS/MS method

[01084] Butyrate was measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. HPLC Details are listed in **Table 57A** and **Table 57B**. Tandem Mass Spectrometry details are found in **Table 42**.

Table 57A. HPLC Details

Column	Thermo Aquasil C18 column, 5 µm (50 x 2.1 mm)
Mobile Phase A	100% H2O, 0.1% Formic Acid
Mobile Phase B	100% ACN, 0.1% Formic Acid
Injection volume	10uL

Table 57B. HPLC Method

Total Time (min)	Flow Rate (µL/min)	A%	B%
0	0.5	100	0
1	0.5	100	0
2	0.5	10	90
4	0.5	10	90
4.01	0.5	100	0
4.25	0.5	100	0

Table 57C. Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM transitions	Butyrate 170.0/71.1, Butyrate d7 177.1/78.3

Example 23. Quantification of Butyrate in feces by LC-MS/MS

Sample preparation

[01085] Fresh 1000, 500, 250, 100, 20, 4 and 0.8µg/mL sodium butyrate standards were prepared in water. Single fecal pellets were ground in 100uL water and centrifuged at 15,000 rpm for 5min at 4°C. 10µL of the sample (fecal supernatant and standards) were pipetted into a V-bottom polypropylene 96-well plate, and 90µL of the derivatizing solution containing 50mM of 2-Hydrazinoquinoline (2-HQ), dipyridyl disulfide, and triphenylphospine in acetonitrile with 5ug/mL of butyrate-d_7 were added to each sample. The plate was heat-sealed and incubated at 60°C for 1hr. The plate was then centrifuged at 4,000rpm for 5min and 20µL of the derivatized samples mixed to 180µL of 22% acetonitrile with 0.1% formic acid.

LC-MS/MS method

[01086] Butyrate was measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. HPLC Details are listed in **Table 58A** and **Table 58B**. Tandem Mass Spectrometry details are found in **Table 61C**.

Table 58A. HPLC Details

Column	Luna phenomenex C18 column, 5 µm (100 x 2.1 mm)
Mobile Phase A	100% H2O, 0.1% Formic Acid
Mobile Phase B	100% ACN, 0.1% Formic Acid
Injection volume	10uL

Table 58B. HPLC Method

Total Time (min)	**Flow Rate (µL/min)**	**A%**	**B%**
0	0.5	95	5
0.5	0.5	95	5
1.5	0.5	10	90
3.5	0.5	10	90
3.51	0.5	95	5
3.75	0.5	95	5

Table 58C. Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM transitions	Butyrate 230.1/143.1, Butyrate d7 237.1/143.1

Example 24. Production of Propionate through the Sleeping Beauty Mutase Pathway in genetically engineered E. coli BW25113 and Nissle

[01087] In *E. coli*, a four gene operon, *sbm-ygfD-ygfG-ygfH* (sleeping beauty mutase pathway) has been shown to encode a putative cobalamin-dependent pathway with the ability to produce propionate from succinate *in vitro.* While the sleeping beauty mutase pathway is present in *E. coli*, it is not under the control of a strong promoter and has shown low activity *in vivo.*

[01088] The utility of this operon for the production of propionate was assessed. Because *E. coli* Nissle does not have the complete operon, initial experiments were conducted in *E. coli* K12 (*BW25113).*

[01089] First, the native promoter for the sleeping beauty mutase operon on the chromosome in the BW25113 strain was replaced with a fnr promoter (BW25113 ldhA::frt; PfnrS-SBM-cam). The sequence for this construct is provided in **Table 59.** Mutation of the lactate dehydrogenase gene (ldhA) reportedly increases propionate production, and this mutation is therefore also added in certain embodiments.

Table 59 SBM Construct Sequences

Description	Sequence
BW25113 fnrS SBM construct (BW25113 frt-cam-frt-PfnrS- sbm, ygfD, ygfG, ygfH), comprising rrnB terminator 1, rrnB terminator 2 (both italic, uppercase), cat promoter and cam resistance gene (encoded on the lagging strand underlined uppercase), frt sites (italic underlined), FNRS promoter bold lowercase, with RBS and leader region bold and underlined and FNR binding site in bold and italics); sleeping beauty operon (sbm, ygfD, ygfG, ygfH) bold and uppercase (**SEQ ID NO: 217**)	***CAAATAAAACGAAAGGCTCAGTCGAAAGACTGG GCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACG CTCTCCTGAGTAGGACAAAT*****CCGCCGGGAGCG GATTTGAACGTTGCGAAGCAACGGCCCGGA GGGTGGCGGGCAGGACGCCCGCCATAAACT GCCAGGCATCAAATTAAGC*****AGAAGGCCATCCT GACGGATGGCCTTTT*****TGCGTGGCCAGTGCCAA GCTTGCATGCAGATTGCAGCATTACACGTCT TGAGCGATTGTGTAGGCTGGAGCTGCTTC*****GA AGTTCCTATACTTTCTAGAGAATAGGAACTTCGG AATAGGAACTTC*****ATTTAAATGGCGCGCCTTAC GCCCCGCCCTGCCACTCATCGCAGTACTGTT GTATTCATTAAGCATCTGCCGACATGGAAGC CATCACAAACGGCATGATGAACCTGAATCGC CAGCGGCATCAGCACCTTGTCGCCTTGCGTA TAATATTTGCCCATGGTGAAAACGGGGGCGA AGAAGTTGTCCATATTGGCCACGTTTAAATC AAAACTGGTGAAACTCACCCAGGGATTGGCT GAGACGAAAAACATATTCTCAATAAACCCTT TAGGGAAATAGGCCAGGTTTTCACCGTAACA CGCCACATCTTGCGAATATATGTGTAGAAAC TGCCGGAAATCGTCGTGGTATTCACTCCAGA GCGATGAAAACGTTTCAGTTTGCTCATGGAA AACGGTGTAACAAGGGTGAACACTATCCCAT ATCACCAGCTCACCGTCTTTCATTGCCATAC GTAATTCCGGATGAGCATTCATCAGGCGGGC AAGAATGTGAATAAAGGCCGGATAAAACTTG TGCTTATTTTTCTTTACGGTCTTTAAAAAGGC CGTAATATCCAGCTGAACGGTCTGGTTATAG GTACATTGAGCAACTGACTGAAATGCCTCAA AATGTTCTTTACGATGCCATTGGGATATATC AACGGTGGTATATCCAGTGATTTTTTTCTCC ATTTTAGCTTCCTTAGCTCCTGAAAATCTCGA CAACTCAAAAAATACGCCCGGTAGTGATCTT ATTTCATTATGGTGAAAGTTGGAACCTCTTA CGTGCCGATCAACGTCTCATTTTCGCCAAAA GTTGGCCCAGGGCTTCCCGGTATCAACAGGG ACACCAGGATTTATTTATTCTGCGAAGTGAT**

	CTTCCGTCACAGGTAGGCGCGCC*GAAGTTCC* *TATACTTTCTAGAGAATAGGAACTTC*GGAATAG GAACTAAGGAGGATATTCATATGGACCATGG CTAATTCCCAGGTACCagttgttcttattggtggtgttgcttt atggttgcatcgtagtaaatggttgtaacaaaagcaatttttccggctgtct gtatacaaaaacgccgcaaagt*ttgagcgaagtcaa*taaactctctaccc attcagggcaatatctctcttggatccaaagtgaactctagaaataatttttg tttaactttaagaaggagatatacatATGTCTAACGTGCAG GAGTGGCAACAGCTTGCCAACAAGGAATTGA GCCGTCGGGAGAAAACTGTCGACTCGCTGGT TCATCAAACCGCGGAAGGGATCGCCATCAAG CCGCTGTATACCGAAGCCGATCTCGATAATC TGGAGGTGACAGGTACCCTTCCTGGTTTGCC GCCCTACGTTCGTGGCCCGCGTGCCACTATG TATACCGCCCAACCGTGGACCATCCGTCAGT ATGCTGGTTTTTCAACAGCAAAAGAGTCCAA CGCTTTTTATCGCCGTAACCTGGCCGCCGGG CAAAAAGGTCTTTCCGTTGCGTTTGACCTTG CCACCCACCGTGGCTACGACTCCGATAACCC GCGCGTGGCGGGCGACGTCGGCAAAGCGGG CGTCGCTATCGACACCGTGGAAGATATGAAA GTCCTGTTCGACCAGATCCCGCTGGATAAAA TGTCGGTTTCGATGACCATGAATGGCGCAGT GCTACCAGTACTGGCGTTTTATATCGTCGCC GCAGAAGAGCAAGGTGTTACACCTGATAAAC TGACCGGCACCATTCAAAACGATATTCTCAA AGAGTACCTCTGCCGCAACACCTATATTTAC CCACCAAAACCGTCAATGCGCATTATCGCCG ACATCATCGCCTGGTGTTCCGGCAACATGCC GCGATTTAATACCATCAGTATCAGCGGTTAC CACATGGGTGAAGCGGGTGCCAACTGCGTG CAGCAGGTAGCATTTACGCTCGCTGATGGGA TTGAGTACATCAAAGCAGCAATCTCTGCCGG ACTGAAAATTGATGACTTCGCTCCTCGCCTG TCGTTCTTCTTCGGCATCGGCATGGATCTGT TTATGAACGTCGCCATGTTGCGTGCGGCACG TTATTTATGGAGCGAAGCGGTCAGTGGATTT GGCGCACAGGACCCGAAATCACTGGCGCTG CGTACCCACTGCCAGACCTCAGGCTGGAGCC TGACTGAACAGGATCCGTATAACAACGTTAT CCGCACCACCATTGAAGCGCTGGCTGCGACG CTGGGCGGTACTCAGTCACTGCATACCAACG CCTTTGACGAAGCGCTTGGTTTGCCTACCGA TTTCTCAGCACGCATTGCCCGCAACACCCAG ATCATCATCCAGGAAGAATCAGAACTCTGCC GCACCGTCGATCCACTGGCCGGATCCTATTA CATTGAGTCGCTGACCGATCAAATCGTCAAA CAAGCCAGAGCTATTATCCAACAGATCGACG AAGCCGGTGGCATGGCGAAAGCGATCGAAG CAGGTCTGCCAAAACGAATGATCGAAGAGGC

	CTCAGCGCGCGAACAGTCGCTGATCGACCAG GGCAAGCGTGTCATCGTTGGTGTCAACAAGT ACAAACTGGATCACGAAGACGAAACCGATGT ACTTGAGATCGACAACGTGATGGTGCGTAAC GAGCAAATTGCTTCGCTGGAACGCATTCGCG CCACCCGTGATGATGCCGCCGTAACCGCCGC GTTGAACGCCCTGACTCACGCCGCACAGCAT AACGAAAACCTGCTGGCTGCCGCTGTTAATG CCGCTCGCGTTCGCGCCACCCTGGGTGAAAT TTCCGATGCGCTGGAAGTCGCTTTCGACCGT TATCTGGTGCCAAGCCAGTGTGTTACCGGCG TGATTGCGCAAAGCTATCATCAGTCTGAGAA ATCGGCCTCCGAGTTCGATGCCATTGTTGCG CAAACGGAGCAGTTCCTTGCCGACAATGGTC GTCGCCCGCGCATTCTGATCGCTAAGATGGG CCAGGATGGACACGATCGCGGCGCGAAAGT GATCGCCAGCGCCTATTCCGATCTCGGTTTC GACGTAGATTTAAGCCCGATGTTCTCTACAC CTGAAGAGATCGCCCGCCTGGCCGTAGAAAA CGACGTTCACGTAGTGGGCGCATCCTCACTG GCTGCCGGTCATAAAACGCTGATCCCGGAAC TGGTCGAAGCGCTGAAAAAATGGGGACGCG AAGATATCTGCGTGGTCGCGGGTGGCGTCAT TCCGCCGCAGGATTACGCCTTCCTGCAAGAG CGCGGCGTGGCGGCGATTTATGGTCCAGGT ACACCTATGCTCGACAGTGTGCGCGACGTAC TGAATCTGATAAGCCAGCATCATGATTAATG AAGCCACGCTGGCAGAAAGTATTCGCCGCTT ACGTCAGGGTGAGCGTGCCACACTCGCCCA GGCCATGACGCTGGTGGAAAGCCGTCACCC GCGTCATCAGGCACTAAGTACGCAGCTGCTT GATGCCATTATGCCGTACTGCGGTAACACCC TGCGACTGGGCGTTACCGGCACCCCCGGCG CGGGGAAAAGTACCTTTCTTGAGGCCTTTGG CATGTTGTTGATTCGAGAGGGATTAAAGGTC GCGGTTATTGCGGTCGATCCCAGCAGCCCGG TCACTGGCGGTAGCATTCTCGGGGATAAAAC CCGCATGAATGACCTGGCGCGTGCCGAAGC GGCGTTTATTCGCCCGGTACCATCCTCCGGT CATCTGGGCGGTGCCAGTCAGCGAGCGCGG GAATTAATGCTGTTATGCGAAGCAGCGGGTT ATGACGTAGTGATTGTCGAAACGGTTGGCGT CGGGCAGTCGGAAACAGAAGTCGCCCGCAT GGTGGACTGTTTTATCTCGTTGCAAATTGCC GGTGGCGGCGATGATCTGCAGGGCATTAAA AAAGGGCTGATGGAAGTGGCTGATCTGATCG TTATCAACAAAGACGATGGCGATAACCATAC CAATGTCGCCATTGCCCGGCATATGTACGAG AGTGCCCTGCATATTCTGCGACGTAAATACG ACGAATGGCAGCCACGGGTTCTGACTTGTAG

	CGCACTGGAAAAACGTGGAATCGATGAGATC TGGCACGCCATCATCGACTTCAAAACCGCGC TAACTGCCAGTGGTCGTTTACAACAAGTGCG GCAACAACAATCGGTGGAATGGCTGCGTAAG CAGACCGAAGAAGAAGTACTGAATCACCTGT TCGCGAATGAAGATTTCGATCGCTATTACCG CCAGACGCTTTTAGCGGTCAAAAACAATACG CTCTCACCGCGCACCGGCCTGCGGCAGCTCA GTGAATTTATCCAGACGCAATATTTTGATTA AAGGAATTTTTATGTCTTATCAGTATGTTAAC GTTGTCACTATCAACAAAGTGGCGGTCATTG AGTTTAACTATGGCCGAAAACTTAATGCCTT AAGTAAAGTCTTTATTGATGATCTTATGCAG GCGTTAAGCGATCTCAACCGGCCGGAAATTC GCTGTATCATTTTGCGCGCACCGAGTGGATC CAAAGTCTTCTCCGCAGGTCACGATATTCAC GAACTGCCGTCTGGCGGTCGCGATCCGCTCT CCTATGATGATCCATTGCGTCAAATCACCCG CATGATCCAAAAATTCCCGAAACCGATCATT TCGATGGTGGAAGGTAGTGTTTGGGGTGGC GCATTTGAAATGATCATGAGTTCCGATCTGA TCATCGCCGCCAGTACCTCAACCTTCTCAAT GACGCCTGTAAACCTCGGCGTCCCGTATAAC CTGGTCGGCATTCACAACCTGACCCGCGACG CGGGCTTCCACATTGTCAAAGAGCTGATTTT TACCGCTTCGCCAATCACCGCCCAGCGCGCG CTGGCTGTCGGCATCCTCAACCATGTTGTGG AAGTGGAAGAACTGGAAGATTTCACCTTACA AATGGCGCACCACATCTCTGAGAAAGCGCCG TTAGCCATTGCCGTTATCAAAGAAGAGCTGC GTGTACTGGGCGAAGCACACACCATGAACTC CGATGAATTTGAACGTATTCAGGGGATGCGC CGCGCGGTGTATGACAGCGAAGATTACCAG GAAGGGATGAACGCTTTCCTCGAAAAACGTA AACCTAATTTCGTTGGTCATTAATCCCTGCGA ACGAAGGAGTAAAAATGGAAACTCAGTGGAC AAGGATGACCGCCAATGAAGCGGCAGAAATT ATCCAGCATAACGACATGGTGGCATTTAGCG GCTTTACCCCGGCGGGTTCGCCGAAAGCCCT ACCCACCGCGATTGCCCGCAGAGCTAACGAA CAGCATGAGGCCAAAAAGCCGTATCAAATTC GCCTTCTGACGGGTGCGTCAATCAGCGCCGC CGCTGACGATGTACTTTCTGACGCCGATGCT GTTTCCTGGCGTGCGCCATATCAAACATCGT CCGGTTTACGTAAAAAGATCAATCAGGGCGC GGTGAGTTTCGTTGACCTGCATTTGAGCGAA GTGGCGCAAATGGTCAATTACGGTTTCTTCG GCGACATTGATGTTGCCGTCATTGAAGCATC GGCACTGGCACCGGATGGTCGAGTCTGGTTA ACCAGCGGGATCGGTAATGCGCCGACCTGG

	CTGCTGCGGGCGAAGAAAGTGATCATTGAAC TCAATCACTATCACGATCCGCGCGTTGCAGA ACTGGCGGATATTGTGATTCCTGGCGCGCCA CCGCGGCGCAATAGCGTGTCGATCTTCCATG CAATGGATCGCGTCGGTACCCGCTATGTGCA AATCGATCCGAAAAAGATTGTCGCCGTCGTG GAAACCAACTTGCCCGACGCCGGTAATATGC TGGATAAGCAAAATCCCATGTGCCAGCAGAT TGCCGATAACGTGGTCACGTTCTTATTGCAG GAAATGGCGCATGGGCGTATTCCGCCGGAAT TTCTGCCGCTGCAAAGTGGCGTGGGCAATAT CAATAATGCGGTAATGGCGCGTCTGGGGGA AAACCCGGTAATTCCTCCGTTTATGATGTAT TCGGAAGTGCTACAGGAATCGGTGGTGCATT TACTGGAAACCGGCAAAATCAGCGGGGCCA GCGCCTCCAGCCTGACAATCTCGGCCGATTC CCTGCGCAAGATTTACGACAATATGGATTAC TTTGCCAGCCGCATTGTGTTGCGTCCGCAGG AGATTTCCAATAACCCGGAAATCATCCGTCG TCTGGGCGTCATCGCTCTGAACGTCGGCCTG GAGTTTGATATTTACGGGCATGCCAACTCAA CACACGTAGCCGGGGTCGATCTGATGAACG GCATCGGCGGCAGCGGTGATTTTGAACGCAA CGCGTATCTGTCGATCTTTATGGCCCCGTCG ATTGCTAAAGAAGGCAAGATCTCAACCGTCG TGCCAATGTGCAGCCATGTTGATCACAGCGA ACACAGCGTCAAAGTGATCATCACCGAACAA GGGATCGCCGATCTGCGCGGTCTTTCCCCGC TTCAACGCGCCCGCACTATCATTGATAATTG TGCACATCCTATGTATCGGGATTATCTGCAT CGCTATCTGGAAAATGCGCCTGGCGGACATA TTCACCACGATCTTAGCCACGTCTTCGACTT ACACCGTAATTTAATTGCAACCGGCTCGATG CTGGGTTAA
FNRS promoter bold lowercase, with RBS and leader region bold and underlined, and FNR binding site bold and italics); sleeping beauty operon (sbm, ygfD, ygfG, ygfH) bold and uppercase (**SEQ ID NO: 218**)	**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgta acaaaagcaatttttccggctgtctgtatacaaaaacgccgcaaagt*ttga gcgaagtcaat*taaactctctacccattcagggcaatatctctcttggatcc aaagtgaactctagaaataattttgtttaactttaagaaggagatatacat ATGTCTAACGTGCAGGAGTGGCAACAGCTTG CCAACAAGGAATTGAGCCGTCGGGAGAAAA CTGTCGACTCGCTGGTTCATCAAACCGCGGA AGGGATCGCCATCAAGCCGCTGTATACCGAA GCCGATCTCGATAATCTGGAGGTGACAGGTA CCCTTCCTGGTTTGCCGCCCTACGTTCGTGG CCCGCGTGCCACTATGTATACCGCCCAACCG TGGACCATCCGTCAGTATGCTGGTTTTTCAA CAGCAAAAGAGTCCAACGCTTTTTATCGCCG TAACCTGGCCGCCGGGCAAAAAGGTCTTTCC GTTGCGTTTGACCTTGCCACCCACCGTGGCT ACGACTCCGATAACCCGCGCGTGGCGGGCG**

	ACGTCGGCAAAGCGGGCGTCGCTATCGACA CCGTGGAAGATATGAAAGTCCTGTTCGACCA GATCCCGCTGGATAAAATGTCGGTTTCGATG ACCATGAATGGCGCAGTGCTACCAGTACTGG CGTTTTATATCGTCGCCGCAGAAGAGCAAGG TGTTACACCTGATAAACTGACCGGCACCATT CAAAACGATATTCTCAAAGAGTACCTCTGCC GCAACACCTATATTTACCCACCAAAACCGTC AATGCGCATTATCGCCGACATCATCGCCTGG TGTTCCGGCAACATGCCGCGATTTAATACCA TCAGTATCAGCGGTTACCACATGGGTGAAGC GGGTGCCAACTGCGTGCAGCAGGTAGCATTT ACGCTCGCTGATGGGATTGAGTACATCAAAG CAGCAATCTCTGCCGGACTGAAAATTGATGA CTTCGCTCCTCGCCTGTCGTTCTTCTTCGGC ATCGGCATGGATCTGTTTATGAACGTCGCCA TGTTGCGTGCGGCACGTTATTTATGGAGCGA AGCGGTCAGTGGATTTGGCGCACAGGACCC GAAATCACTGGCGCTGCGTACCCACTGCCAG ACCTCAGGCTGGAGCCTGACTGAACAGGATC CGTATAACAACGTTATCCGCACCACCATTGA AGCGCTGGCTGCGACGCTGGGCGGTACTCA GTCACTGCATACCAACGCCTTTGACGAAGCG CTTGGTTTGCCTACCGATTTCTCAGCACGCA TTGCCCGCAACACCCAGATCATCATCCAGGA AGAATCAGAACTCTGCCGCACCGTCGATCCA CTGGCCGGATCCTATTACATTGAGTCGCTGA CCGATCAAATCGTCAAACAAGCCAGAGCTAT TATCCAACAGATCGACGAAGCCGGTGGCATG GCGAAAGCGATCGAAGCAGGTCTGCCAAAA CGAATGATCGAAGAGGCCTCAGCGCGCGAA CAGTCGCTGATCGACCAGGGCAAGCGTGTCA TCGTTGGTGTCAACAAGTACAAACTGGATCA CGAAGACGAAACCGATGTACTTGAGATCGAC AACGTGATGGTGCGTAACGAGCAAATTGCTT CGCTGGAACGCATTCGCGCCACCCGTGATGA TGCCGCCGTAACCGCCGCGTTGAACGCCCTG ACTCACGCCGCACAGCATAACGAAAACCTGC TGGCTGCCGCTGTTAATGCCGCTCGCGTTCG CGCCACCCTGGGTGAAATTTCCGATGCGCTG GAAGTCGCTTTCGACCGTTATCTGGTGCCAA GCCAGTGTGTTACCGGCGTGATTGCGCAAAG CTATCATCAGTCTGAGAAATCGGCCTCCGAG TTCGATGCCATTGTTGCGCAAACGGAGCAGT TCCTTGCCGACAATGGTCGTCGCCCGCGCAT TCTGATCGCTAAGATGGGCCAGGATGGACAC GATCGCGGCGCGAAAGTGATCGCCAGCGCC TATTCCGATCTCGGTTTCGACGTAGATTTAA GCCCGATGTTCTCTACACCTGAAGAGATCGC CCGCCTGGCCGTAGAAAACGACGTTCACGTA

	GTGGGCGCATCCTCACTGGCTGCCGGTCATA AAACGCTGATCCCGGAACTGGTCGAAGCGCT GAAAAAATGGGGACGCGAAGATATCTGCGT GGTCGCGGGTGGCGTCATTCCGCCGCAGGA TTACGCCTTCCTGCAAGAGCGCGGCGTGGCG GCGATTTATGGTCCAGGTACACCTATGCTCG ACAGTGTGCGCGACGTACTGAATCTGATAAG CCAGCATCATGATTAATGAAGCCACGCTGGC AGAAAGTATTCGCCGCTTACGTCAGGGTGAG CGTGCCACACTCGCCCAGGCCATGACGCTGG TGGAAAGCCGTCACCCGCGTCATCAGGCACT AAGTACGCAGCTGCTTGATGCCATTATGCCG TACTGCGGTAACACCCTGCGACTGGGCGTTA CCGGCACCCCCGGCGCGGGGAAAAGTACCT TTCTTGAGGCCTTTGGCATGTTGTTGATTCG AGAGGGATTAAAGGTCGCGGTTATTGCGGTC GATCCCAGCAGCCCGGTCACTGGCGGTAGC ATTCTCGGGGATAAAACCCGCATGAATGACC TGGCGCGTGCCGAAGCGGCGTTTATTCGCCC GGTACCATCCTCCGGTCATCTGGGCGGTGCC AGTCAGCGAGCGCGGGAATTAATGCTGTTAT GCGAAGCAGCGGGTTATGACGTAGTGATTGT CGAAACGGTTGGCGTCGGGCAGTCGGAAAC AGAAGTCGCCCGCATGGTGGACTGTTTTATC TCGTTGCAAATTGCCGGTGGCGGCGATGATC TGCAGGGCATTAAAAAAGGGCTGATGGAAGT GGCTGATCTGATCGTTATCAACAAAGACGAT GGCGATAACCATACCAATGTCGCCATTGCCC GGCATATGTACGAGAGTGCCCTGCATATTCT GCGACGTAAATACGACGAATGGCAGCCACG GGTTCTGACTTGTAGCGCACTGGAAAAACGT GGAATCGATGAGATCTGGCACGCCATCATCG ACTTCAAAACCGCGCTAACTGCCAGTGGTCG TTTACAACAAGTGCGGCAACAACAATCGGTG GAATGGCTGCGTAAGCAGACCGAAGAAGAA GTACTGAATCACCTGTTCGCGAATGAAGATT TCGATCGCTATTACCGCCAGACGCTTTTAGC GGTCAAAAACAATACGCTCTCACCGCGCACC GGCCTGCGGCAGCTCAGTGAATTTATCCAGA CGCAATATTTTGATTAAAGGAATTTTTATGTC TTATCAGTATGTTAACGTTGTCACTATCAACA AAGTGGCGGTCATTGAGTTTAACTATGGCCG AAAACTTAATGCCTTAAGTAAAGTCTTTATTG ATGATCTTATGCAGGCGTTAAGCGATCTCAA CCGGCCGGAAATTCGCTGTATCATTTTGCGC GCACCGAGTGGATCCAAAGTCTTCTCCGCAG GTCACGATATTCACGAACTGCCGTCTGGCGG TCGCGATCCGCTCTCCTATGATGATCCATTG CGTCAAATCACCCGCATGATCCAAAAATTCC CGAAACCGATCATTTCGATGGTGGAAGGTAG

	TGTTTGGGGTGGCGCATTTGAAATGATCATG AGTTCCGATCTGATCATCGCCGCCAGTACCT CAACCTTCTCAATGACGCCTGTAAACCTCGG CGTCCCGTATAACCTGGTCGGCATTCACAAC CTGACCCGCGACGCGGGCTTCCACATTGTCA AAGAGCTGATTTTTACCGCTTCGCCAATCAC CGCCCAGCGCGCGCTGGCTGTCGGCATCCTC AACCATGTTGTGGAAGTGGAAGAACTGGAAG ATTTCACCTTACAAATGGCGCACCACATCTC TGAGAAAGCGCCGTTAGCCATTGCCGTTATC AAAGAAGAGCTGCGTGTACTGGGCGAAGCA CACACCATGAACTCCGATGAATTTGAACGTA TTCAGGGGATGCGCCGCGCGGTGTATGACA GCGAAGATTACCAGGAAGGGATGAACGCTTT CCTCGAAAAACGTAAACCTAATTTCGTTGGT CATTAATCCCTGCGAACGAAGGAGTAAAAATG GAAACTCAGTGGACAAGGATGACCGCCAATG AAGCGGCAGAAATTATCCAGCATAACGACAT GGTGGCATTTAGCGGCTTTACCCCGGCGGGT TCGCCGAAAGCCCTACCCACCGCGATTGCCC GCAGAGCTAACGAACAGCATGAGGCCAAAA AGCCGTATCAAATTCGCCTTCTGACGGGTGC GTCAATCAGCGCCGCCGCTGACGATGTACTT TCTGACGCCGATGCTGTTTCCTGGCGTGCGC CATATCAAACATCGTCCGGTTTACGTAAAAA GATCAATCAGGGCGCGGTGAGTTTCGTTGAC CTGCATTTGAGCGAAGTGGCGCAAATGGTCA ATTACGGTTTCTTCGGCGACATTGATGTTGC CGTCATTGAAGCATCGGCACTGGCACCGGAT GGTCGAGTCTGGTTAACCAGCGGGATCGGTA ATGCGCCGACCTGGCTGCTGCGGGCGAAGA AAGTGATCATTGAACTCAATCACTATCACGA TCCGCGCGTTGCAGAACTGGCGGATATTGTG ATTCCTGGCGCGCCACCGCGGCGCAATAGC GTGTCGATCTTCCATGCAATGGATCGCGTCG GTACCCGCTATGTGCAAATCGATCCGAAAAA GATTGTCGCCGTCGTGGAAACCAACTTGCCC GACGCCGGTAATATGCTGGATAAGCAAAATC CCATGTGCCAGCAGATTGCCGATAACGTGGT CACGTTCTTATTGCAGGAAATGGCGCATGGG CGTATTCCGCCGGAATTTCTGCCGCTGCAAA GTGGCGTGGGCAATATCAATAATGCGGTAAT GGCGCGTCTGGGGGAAAACCCGGTAATTCCT CCGTTTATGATGTATTCGGAAGTGCTACAGG AATCGGTGGTGCATTTACTGGAAACCGGCAA AATCAGCGGGGCCAGCGCCTCCAGCCTGAC AATCTCGGCCGATTCCCTGCGCAAGATTTAC GACAATATGGATTACTTTGCCAGCCGCATTG TGTTGCGTCCGCAGGAGATTTCCAATAACCC GGAAATCATCCGTCGTCTGGGCGTCATCGCT

	CTGAACGTCGGCCTGGAGTTTGATATTTACG GGCATGCCAACTCAACACACGTAGCCGGGGT CGATCTGATGAACGGCATCGGCGGCAGCGG TGATTTTGAACGCAACGCGTATCTGTCGATC TTTATGGCCCCGTCGATTGCTAAAGAAGGCA AGATCTCAACCGTCGTGCCAATGTGCAGCCA TGTTGATCACAGCGAACACAGCGTCAAAGTG ATCATCACCGAACAAGGGATCGCCGATCTGC GCGGTCTTTCCCCGCTTCAACGCGCCCGCAC TATCATTGATAATTGTGCACATCCTATGTATC GGGATTATCTGCATCGCTATCTGGAAAATGC GCCTGGCGGACATATTCACCACGATCTTAGC CACGTCTTCGACTTACACCGTAATTTAATTG CAACCGGCTCGATGCTGGGTTAA

[01090] Next, this strain was tested for propionate production.

[01091] Briefly, 3ml LB (containing selective antibiotics (cam) where necessary was inoculated from frozen glycerol stocks with either wild type *E. coli* K12 or the genetically engineered bacteria comprising the chromosomal sleeping beauty mutase operon under the control of a FNR promoter. Bacteria were grown overnight at 37 C with shaking. Overnight cultures were diluted 1:100 into 10ml LB in a 125ml baffled flask. Cultures were grown aerobically at 37 C with shaking for about 1.5 h, and then transferred to the anaerobic chamber at 37 C for 4h. Bacteria ($2X10^8$ CFU) were added to 1ml M9 media containing 50mM MOPS with 0.5% glucose in microcentrifuge tubes. Cells were plated to determine cell counts. The assay tubes were placed in the anaerobic chamber at 37 C. At 1, 2, and 24 hours, 120 ul of cells were removed and pelleted at 14,000rpm for 1 min, and 100 ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for propionate concentrations, as described in

[01092] Results are depicted in **FIG. 40B** and show that the genetically engineered strain produces ~2.5mM after 24h, while very little or no propionate production was detected from the *E. coli* K12 wild type strain. Propionate was measured as described in [[**Example 27**]].

Example 25. Evaluation of the Sleeping Beauty Mutase Pathway for the Production of Propionate in E coli Nissle

[01093] Next, the SBM pathway is evaluated for propionate production in *E. coli Nissle*. Nissle does not have the full 4-gene sleeping beauty mutase operon; it only has the first gene and a partial gene of the second, and genes 3 and 4 are missing. Therefore,

recombineering is used to introduce this pathway into Nissle. The frt-cam-frt-PfnrS- sbm, ygfD, ygfG, ygfH construct is inserted at the location of the endogenous, truncated Nissle SBM. Next, the construct is transformed into E coli Nissle and tested for propionate production essentially as described above.

Example 26. Evaluation of the Acrylate Pathway from Clostridium propionicum for Propionate Production

[01094] The acrylate pathway from *Clostridium propionicum* is evaluated for adaptation to propionate production in *E. coli*. A construct (Ptet-pct-lcdABC-acrABC), codon optimized for *E. coli*, was synthesized by Genewiz and placed in a high copy plasmid (Logic051). Additionally, another construct is generated for side by side testing, in which the acrABC genes (which may be the rate limiting step of the pathway) are replaced with the acuI gene from *Rhodobacter sphaeroides* (*Ptet- acuI-pct-lcdABC*). Subsequently these constructs are transformed into BW25113 and are assessed for their ability to produce propionate, as compared to the type BW5113 strain as described above in **Example 24**. Propionate was measured as described in **Example 27**.

Table 60 of Exemplary Propionate Cassette Sequences

Description and SEQ ID NO	**Sequence**
Ptet-pct-lcdABC-acrABC; Ptet: lower case; tertR/tetA promoter within Ptet: lower case bold, with tet operator: lower case bold underlined; ribosome binding site and leader: lowe case italic; ribosome binding sites: lower case underlined; coding regions: upper case; (**SEQ ID NO: 219**)	ttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataa gaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcat actatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaa cctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgt tggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaa atgtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaa aaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctca atggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctct acacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagct ctaatgcgctgttaatcactttacttttatctaatctagacatcattaattcctaatttttt**gttgaca ctctatcattgatagagttattttaccactccctatcagtgatagagaa**aagtgaa*ctct agaaataattttgtttaactttaagaaggagatatacat*ATGCGCAAAGTGCC GATTATCACGGCTGACGAGGCCGCAAAACTGATCAAG GACGGCGACACCGTGACAACTAGCGGCTTTGTGGGTA ACGCGATCCCTGAGGCCCTTGACCGTGCAGTCGAAAA GCGTTTCCTGGAAACGGGCGAACCGAAGAACATTACTT ATGTATATTGCGGCAGTCAGGGCAATCGCGACGGTCGT GGCGCAGAACATTTCGCGCATGAAGGCCTGCTGAAAC GTTATATCGCTGGCCATTGGGCGACCGTCCCGGCGTTA GGGAAAATGGCCATGGAGAATAAAATGGAGGCCTACA ATGTCTCTCAGGGCGCCTTGTGTCATCTCTTTCGCGATA

	TTGCGAGCCATAAACCGGGTGTGTTCACGAAAGTAGG AATCGGCACCTTCATTGATCCACGTAACGGTGGTGGGA AGGTCAACGATATTACCAAGGAAGATATCGTAGAACT GGTGGAAATTAAAGGGCAGGAATACCTGTTTTATCCGG CGTTCCCGATCCATGTCGCGCTGATTCGTGGCACCTAT GCGGACGAGAGTGGTAACATCACCTTTGAAAAAGAGG TAGCGCCTTTGGAAGGGACTTCTGTCTGTCAAGCGGTG AAGAACTCGGGTGGCATTGTCGTGGTTCAGGTTGAGCG TGTCGTCAAAGCAGGCACGCTGGATCCGCGCCATGTGA AAGTTCCGGGTATCTATGTAGATTACGTAGTCGTCGCG GATCCGGAGGACCATCAACAGTCCCTTGACTGCGAATA TGATCCTGCCCTTAGTGGAGAGCACCGTCGTCCGGAGG TGGTGGGTGAACCACTGCCTTTATCCGCGAAGAAAGTC ATCGGCCGCCGTGGCGCGATTGAGCTCGAGAAAGACG TTGCAGTGAACCTTGGGGTAGGTGCACCTGAGTATGTG GCCTCCGTGGCCGATGAAGAAGGCATTGTGGATTTTAT GACTCTCACAGCGGAGTCCGGCGCTATCGGTGGCGTTC CAGCCGGCGGTGTTCGCTTTGGGGCGAGCTACAATGCT GACGCCTTGATCGACCAGGGCTACCAATTTGATTATTA CGACGGTGGGGGTCTGGATCTTTGTTACCTGGGTTTAG CTGAATGCGACGAAAAGGGTAATATCAATGTTAGCCG CTTCGGTCCTCGTATCGCTGGGTGCGGCGGATTCATTA ACATTACCCAAAACACGCCGAAAGTCTTCTTTTGTGGG ACCTTTACAGCCGGGGGGCTGAAAGTGAAAATTGAAG ATGGTAAGGTGATTATCGTTCAGGAAGGGAAACAGAA GAAATTCCTTAAGGCAGTGGAGCAAATCACCTTTAATG GAGACGTGGCCTTAGCGAACAAGCAACAAGTTACCTA CATCACGGAGCGTTGCGTCTTCCTCCTCAAAGAAGACG GTTTACACCTTTCGGAAATCGCGCCAGGCATCGATCTG CAGACCCAGATTTTGGATGTTATGGACTTTGCCCCGAT CATTGATCGTGACGCAAACGGGCAGATTAAACTGATG GACGCGGCGTTATTCGCAGAAGGGCTGATGGGCTTGA AAGAAATGAAGTCTTGAtaagaaggagatatacatATGAGCTTA ACCCAAGGCATGAAAGCTAAACAACTGTTAGCATACTT TCAGGGTAAAGCCGATCAGGATGCACGTGAAGCGAAA GCCCGCGGTGAGCTGGTCTGCTGGTCGGCGTCAGTCGC GCCGCCGGAATTTTGCGTAACAATGGGCATTGCCATGA TCTACCCGGAGACTCATGCAGCGGGCATCGGTGCCCGC AAAGGTGCGATGGACATGCTGGAAGTTGCGGACCGCA AAGGCTACAACGTGGATTGTTGTTCCTACGGCCGTGTA AATATGGGTTACATGGAATGTTTAAAAGAAGCCGCCAT CACGGGCGTCAAGCCGGAAGTTTTGGTTAATTCCCCTG CTGCTGACGTTCCGCTTCCCGATTTGGTGATTACGTGTA ATAATATCTGTAACACGCTGCTGAAATGGTACGAAAAC TTAGCAGCAGAACTCGATATTCCTTGCATCGTGATCGA CGTACCGTTTAATCATACCATGCCGATTCCGGAATATG CCAAGGCCTACATCGCGGACCAGTTCCGCAATGCAATT TCTCAGCTGGAAGTTATTTGTGGCCGTCCGTTCGATTG GAAGAAATTTAAGGAGGTCAAAGATCAGACCCAGCGT AGCGTATACCACTGGAACCGCATTGCCGAGATGGCGA

	AATACAAGCCTAGCCCGCTGAACGGCTTCGATCTGTTC AATTACATGGCGTTAATCGTGGCGTGCCGCAGCCTGGA TTATGCAGAAATTACCTTTAAAGCGTTCGCGGACGAAT TAGAAGAGAATTTGAAGGCGGGTATCTACGCCTTTAAA GGTGCGGAAAAAACGCGCTTTCAATGGGAAGGTATCG CGGTGTGGCCACATTTAGGTCACACGTTTAAATCTATG AAGAATCTGAATTCGATTATGACCGGTACGGCATACCC CGCCCTTTGGGACCTGCACTATGACGCTAACGACGAAT CTATGCACTCTATGGCTGAAGCGTACACCCGTATTTAT ATTAATACTTGTCTGCAGAACAAAGTAGAGGTCCTGCT TGGGATCATGGAAAAAGGCCAGGTGGATGGTACCGTA TATCATCTGAATCGCAGCTGCAAACTGATGAGTTTCCT GAACGTGGAAACGGCTGAAATTATTAAAGAGAAGAAC GGTCTTCCTTACGTCTCCATTGATGGCGATCAGACCGA TCCTCGCGTTTTTTCTCCGGCCCAGTTTGATACCCGTGT TCAGGCCCTGGTTGAGATGATGGAGGCCAATATGGCG GCAGCGGAATAAtaagaaggagatatacatATGTCACGCGTGGA GGCAATCCTGTCGCAGCTGAAAGATGTCGCCGCGAATC CGAAAAAAGCCATGGATGACTATAAAGCTGAAACAGG TAAGGGCGCGGTTGGTATCATGCCGATCTACAGCCCCG AAGAAATGGTACACGCCGCTGGCTATTTGCCGATGGG AATCTGGGGCGCCCAGGGCAAAACGATTAGTAAAGCG CGCACCTATCTGCCTGCTTTTGCCTGCAGCGTAATGCA GCAGGTTATGGAATTACAGTGCGAGGGCGCGTATGAT GACCTGTCCGCAGTTATTTTTAGCGTACCGTGCGACAC TCTCAAATGTCTTAGCCAGAAATGGAAAGGTACGTCCC CAGTGATTGTATTTACGCATCCGCAGAACCGCGGATTA GAAGCGGCGAACCAATTCTTGGTTACCGAGTATGAACT GGTAAAAGCACAACTGGAATCAGTTCTGGGTGTGAAA ATTTCAAACGCCGCCCTGGAAAATTCGATTGCAATTTA TAACGAGAATCGTGCCGTGATGCGTGAGTTCGTGAAA GTGGCAGCGGACTATCCTCAAGTCATTGACGCAGTGAG CCGCCACGCGGTTTTTAAAGCGCGCCAGTTTATGCTTA AGGAAAAACATACCGCACTTGTGAAAGAACTGATCGC TGAGATTAAAGCAACGCCAGTCCAGCCGTGGGACGGA AAAAAGGTTGTAGTGACGGGCATTCTGTTGGAACCGA ATGAGTTATTAGATATCTTTAATGAGTTTAAGATCGCG ATTGTTGATGATGATTTAGCGCAGGAAAGCCGTCAGAT CCGTGTTGACGTTCTGGACGGAGAAGGCGGACCGCTCT ACCGTATGGCTAAAGCGTGGCAGCAAATGTATGGCTG CTCGCTGGCAACCGACACCAAGAAGGGTCGCGGCCGT ATGTTAATTAACAAAACGATTCAGACCGGTGCGGACG CTATCGTAGTTGCAATGATGAAGTTTTGCGACCCAGAA GAATGGGATTATCCGGTAATGTACCGTGAATTTGAAGA AAAAGGGGTCAAATCACTTATGATTGAGGTGGATCAG GAAGTATCGTCTTTCGAACAGATTAAAACCCGTCTGCA GTCATTCGTCGAAATGCTTTAAtaagaaggagatatacatATGTA TACCTTGGGGATTGATGTCGGTTCTGCCTCTAGTAAAG CGGTGATTCTGAAAGATGGAAAAGATATTGTCGCTGCC GAGGTTGTCCAAGTCGGTACCGGCTCCTCGGGTCCCCA

	ACGCGCACTGGACAAAGCCTTTGAAGTCTCTGGCTTAA AAAAGGAAGACATCAGCTACACAGTAGCTACGGGCTA TGGGCGCTTCAATTTTAGCGACGCGGATAAACAGATTT CGGAAATTAGCTGTCATGCCAAAGGCATTTATTTCTTA GTACCAACTGCGCGCACTATTATTGACATTGGCGGCCA AGATGCGAAAGCCATCCGCCTGGACGACAAGGGGGGT ATTAAGCAATTCTTCATGAATGATAAATGCGCGGCGGG CACGGGGCGTTTCCTGGAAGTCATGGCTCGCGTACTTG AAACCACCCTGGATGAAATGGCTGAACTGGATGAACA GGCGACTGACACCGCTCCCATTTCAAGCACCTGCACGG TTTTCGCCGAAAGCGAAGTAATTAGCCAATTGAGCAAT GGTGTCTCACGCAACAACATCATTAAAGGTGTCCATCT GAGCGTTGCGTCACGTGCGTGTGGTCTGGCGTATCGCG GCGGTTTGGAGAAAGATGTTGTTATGACAGGTGGCGTG GCAAAAAATGCAGGGGTGGTGCGCGCGGTGGCGGGCG TTCTGAAGACCGATGTTATCGTTGCTCCGAATCCTCAG ACGACCGGTGCACTGGGGGCAGCGCTGTATGCTTATGA GGCCGCCCAGAAGAAGTAAtaagaaggagatatacatATGGCCT TCAATAGCGCAGATATTAATTCTTTCCGCGATATTTGG GTGTTTTGTGAACAGCGTGAGGGCAAACTGATTAACAC CGATTTCGAATTAATTAGCGAAGGTCGTAAACTGGCTG ACGAACGCGGAAGCAAACTGGTTGGAATTTTGCTGGG GCACGAAGTTGAAGAAATCGCAAAAGAATTAGGCGGC TATGGTGCGGACAAGGTAATTGTGTGCGATCATCCGGA ACTTAAATTTTACACTACGGATGCTTATGCCAAAGTTT TATGTGACGTCGTGATGGAAGAGAAACCGGAGGTAAT TTTGATCGGTGCCACCAACATTGGCCGTGATCTCGGAC CGCGTTGTGCTGCACGCTTGCACACGGGGCTGACGGCT GATTGCACGCACCTGGATATTGATATGAATAAATATGT GGACTTTCTTAGCACCAGTAGCACCTTGGATATCTCGT CGATGACTTTCCCTATGGAAGATACAAACCTTAAAATG ACGCGCCCTGCATTTGGCGGACATCTGATGGCAACGAT CATTTGTCCACGCTTCCGTCCCTGTATGAGCACAGTGC GCCCCGGAGTGATGAAGAAAGCGGAGTTCTCGCAGGA GATGGCGCAAGCATGTCAAGTAGTGACCCGTCACGTA AATTTGTCGGATGAAGACCTTAAAACTAAAGTAATTAA TATCGTGAAGGAAACGAAAAAGATTGTGGATCTGATC GGCGCAGAAATTATTGTGTCAGTTGGTCGTGGTATCTC GAAAGATGTCCAAGGTGGAATTGCACTGGCTGAAAAA CTTGCGGACGCATTTGGTAACGGTGTCGTGGGCGGCTC GCGCGCAGTGATTGATTCCGGCTGGTTACCTGCGGATC ATCAGGTTGGACAAACCGGTAAGACCGTGCACCCGAA AGTCTACGTGGCGCTGGGTATTAGTGGGGCTATCCAGC ATAAGGCTGGGATGCAAGACTCTGAACTGATCATTGCC GTCAACAAAGACGAAACGGCGCCTATCTTCGACTGCG CCGATTATGGCATCACCGGTGATTTATTTAAAATCGTA CCGATGATGATCGACGCGATCAAAGAGGGTAAAAACG CATGAtaagaaggagatatacatATGCGCATCTATGTGTGTGTGA AACAAGTCCCAGATACGAGCGGCAAGGTGGCCGTTAA CCCTGATGGGACCCTTAACCGTGCCTCAATGGCAGCGA

	TTATTAACCCGGACGATATGTCCGCGATCGAACAGGCA TTAAAACTGAAAGATGAAACCGGATGCCAGGTTACGG CGCTTACGATGGGTCCTCCTCCTGCCGAGGGCATGTTG CGCGAAATTATTGCAATGGGGGCCGACGATGGTGTGCT GATTTCGGCCCGTGAATTTGGGGGGTCCGATACCTTCG CAACCAGTCAAATTATTAGCGCGGCAATCCATAAATTA GGCTTAAGCAATGAAGACATGATCTTTTGCGGTCGTCA GGCCATTGACGGTGATACGGCCCAAGTCGGCCCTCAA ATTGCCGAAAAACTGAGCATCCCACAGGTAACCTATG GCGCAGGAATCAAAAAATCTGGTGATTTAGTGCTGGTG AAGCGTATGTTGGAGGATGGTTATATGATGATCGAAGT CGAAACTCCATGTCTGATTACCTGCATTCAGGATAAAG CGGTAAAACCACGTTACATGACTCTCAACGGTATTATG GAATGCTACTCCAAGCCGCTCCTCGTTCTCGATTACGA AGCACTGAAAGATGAACCGCTGATCGAACTTGATACC ATTGGGCTTAAAGGCTCCCCGACGAATATCTTTAAATC GTTTACGCCGCCTCAGAAAGGCGTTGGTGTCATGCTCC AAGGCACCGATAAGGAAAAAGTCGAGGATCTGGTGGA TAAGCTGATGCAGAAACATGTCATCTAAtaagaaggagatatac atATGTTCTTACTGAAGATTAAAAAAGAACGTATGAAA CGCATGGACTTTAGTTTAACGCGTGAACAGGAGATGTT AAAAAAACTGGCGCGTCAGTTTGCTGAGATCGAGCTG GAACCGGTGGCCGAAGAGATTGATCGTGAGCACGTTTT TCCTGCAGAAAACTTTAAGAAGATGGCGGAAATTGGC TTAACCGGCATTGGTATCCCGAAAGAATTTGGTGGCTC CGGTGGAGGCACCCTGGAGAAGGTCATTGCCGTGTCA GAATTCGGCAAAAAGTGTATGGCCTCAGCTTCCATTTT AAGCATTCATCTTATCGCGCCGCAGGCAATCTACAAAT ATGGGACCAAAGAACAGAAAGAGACGTACCTGCCGCG TCTTACCAAAGGTGGTGAACTGGGCGCCTTTGCGCTGA CAGAACCAAACGCCGGAAGCGATGCCGGCGCGGTAAA AACGACCGCGATTCTGGACAGCCAGACAAACGAGTAC GTGCTGAATGGCACCAAATGCTTTATCAGCGGGGGCG GGCGCGCGGGTGTTCTTGTAATTTTTGCGCTTACTGAA CCGAAAAAAGGTCTGAAAGGGATGAGCGCGATTATCG TGGAGAAAGGGACCCCGGGCTTCAGCATCGGCAAGGT GGAGAGCAAGATGGGGATCGCAGGTTCGGAAACCGCG GAACTTATCTTCGAAGATTGTCGCGTTCCGGCTGCCAA CCTTTTAGGTAAAGAAGGCAAAGGCTTTAAAATTGCTA TGGAAGCCCTGGATGGCGCCCGTATTGGCGTGGGCGCT CAAGCAATCGGAATTGCCGAGGGGGCGATCGACCTGA GTGTGAAGTACGTTCACGAGCGCATTCAATTTGGTAAA CCGATCGCGAATCTGCAGGGAATTCAATGGTATATCGC GGATATGGCGACCAAAACCGCCGCGGCACGCGCACTT GTTGAGTTTGCAGCGTATCTTGAAGACGCGGGTAAACC GTTCACAAAGGAATCTGCTATGTGCAAGCTGAACGCCT CCGAAAACGCGCGTTTTGTGACAAATTTAGCTCTGCAG ATTCACGGGGGTTACGGTTATATGAAAGATTATCCGTT AGAGCGTATGTATCGCGATGCTAAGATTACGGAAATTT ACGAGGGGACATCAGAAATCCATAAGGTGGTGATTGC

	GCGTGAAGTAATGAAACGCTAA
pct-lcdABC-acrABC (ribosome binding sites: lower case underlined; coding regions: upper case) (**SEQ ID NO: 220**)	ATGCGCAAAGTGCCGATTATCACGGCTGACGAGGCCG CAAAACTGATCAAGGACGGCGACACCGTGACAACTAG CGGCTTTGTGGGTAACGCGATCCCTGAGGCCCTTGACC GTGCAGTCGAAAAGCGTTTCCTGGAAACGGGCGAACC GAAGAACATTACTTATGTATATTGCGGCAGTCAGGGCA ATCGCGACGGTCGTGGCGCAGAACATTTCGCGCATGA AGGCCTGCTGAAACGTTATATCGCTGGCCATTGGGCGA CCGTCCCGGCGTTAGGGAAAATGGCCATGGAGAATAA AATGGAGGCCTACAATGTCTCTCAGGGCGCCTTGTGTC ATCTCTTTCGCGATATTGCGAGCCATAAACCGGGTGTG TTCACGAAAGTAGGAATCGGCACCTTCATTGATCCACG TAACGGTGGTGGGAAGGTCAACGATATTACCAAGGAA GATATCGTAGAACTGGTGGAAATTAAAGGGCAGGAAT ACCTGTTTTATCCGGCGTTCCCGATCCATGTCGCGCTG ATTCGTGGCACCTATGCGGACGAGAGTGGTAACATCAC CTTTGAAAAAGAGGTAGCGCCTTTGGAAGGGACTTCTG TCTGTCAAGCGGTGAAGAACTCGGGTGGCATTGTCGTG GTTCAGGTTGAGCGTGTCGTCAAAGCAGGCACGCTGG ATCCGCGCCATGTGAAAGTTCCGGGTATCTATGTAGAT TACGTAGTCGTCGCGGATCCGGAGGACCATCAACAGTC CCTTGACTGCGAATATGATCCTGCCCTTAGTGGAGAGC ACCGTCGTCCGGAGGTGGTGGGTGAACCACTGCCTTTA TCCGCGAAGAAAGTCATCGGCCGCCGTGGCGCGATTG AGCTCGAGAAAGACGTTGCAGTGAACCTTGGGGTAGG TGCACCTGAGTATGTGGCCTCCGTGGCCGATGAAGAAG GCATTGTGGATTTTATGACTCTCACAGCGGAGTCCGGC GCTATCGGTGGCGTTCCAGCCGGCGGTGTTCGCTTTGG GGCGAGCTACAATGCTGACGCCTTGATCGACCAGGGCT ACCAATTTGATTATTACGACGGTGGGGGTCTGGATCTT TGTTACCTGGGTTTAGCTGAATGCGACGAAAAGGGTAA TATCAATGTTAGCCGCTTCGGTCCTCGTATCGCTGGGT GCGGCGGATTCATTAACATTACCCAAAACACGCCGAA AGTCTTCTTTTGTGGGACCTTTACAGCCGGGGGGCTGA AAGTGAAAATTGAAGATGGTAAGGTGATTATCGTTCA GGAAGGGAAACAGAAGAAATTCCTTAAGGCAGTGGAG CAAATCACCTTTAATGGAGACGTGGCCTTAGCGAACAA GCAACAAGTTACCTACATCACGGAGCGTTGCGTCTTCC TCCTCAAAGAAGACGGTTTACACCTTTCGGAAATCGCG CCAGGCATCGATCTGCAGACCCAGATTTTGGATGTTAT GGACTTTGCCCCGATCATTGATCGTGACGCAAACGGGC AGATTAAACTGATGGACGCGGCGTTATTCGCAGAAGG GCTGATGGGCTTGAAAGAAATGAAGTCTTGAtaagaaggag atatacatATGAGCTTAACCCAAGGCATGAAAGCTAAACAA CTGTTAGCATACTTTCAGGGTAAAGCCGATCAGGATGC ACGTGAAGCGAAAGCCCGCGGTGAGCTGGTCTGCTGG TCGGCGTCAGTCGCGCCGCCGGAATTTTGCGTAACAAT GGGCATTGCCATGATCTACCCGGAGACTCATGCAGCGG GCATCGGTGCCCGCAAAGGTGCGATGGACATGCTGGA AGTTGCGGACCGCAAAGGCTACAACGTGGATTGTTGTT

	CCTACGGCCGTGTAAATATGGGTTACATGGAATGTTTA AAAGAAGCCGCCATCACGGGCGTCAAGCCGGAAGTTT TGGTTAATTCCCCTGCTGCTGACGTTCCGCTTCCCGATT TGGTGATTACGTGTAATAATATCTGTAACACGCTGCTG AAATGGTACGAAAACTTAGCAGCAGAACTCGATATTC CTTGCATCGTGATCGACGTACCGTTTAATCATACCATG CCGATTCCGGAATATGCCAAGGCCTACATCGCGGACCA GTTCCGCAATGCAATTTCTCAGCTGGAAGTTATTTGTG GCCGTCCGTTCGATTGGAAGAAATTTAAGGAGGTCAA AGATCAGACCCAGCGTAGCGTATACCACTGGAACCGC ATTGCCGAGATGGCGAAATACAAGCCTAGCCCGCTGA ACGGCTTCGATCTGTTCAATTACATGGCGTTAATCGTG GCGTGCCGCAGCCTGGATTATGCAGAAATTACCTTTAA AGCGTTCGCGGACGAATTAGAAGAGAATTTGAAGGCG GGTATCTACGCCTTTAAAGGTGCGGAAAAAACGCGCTT TCAATGGGAAGGTATCGCGGTGTGGCCACATTTAGGTC ACACGTTTAAATCTATGAAGAATCTGAATTCGATTATG ACCGGTACGGCATACCCCGCCCTTTGGGACCTGCACTA TGACGCTAACGACGAATCTATGCACTCTATGGCTGAAG CGTACACCCGTATTTATATTAATACTTGTCTGCAGAAC AAAGTAGAGGTCCTGCTTGGGATCATGGAAAAAGGCC AGGTGGATGGTACCGTATATCATCTGAATCGCAGCTGC AAACTGATGAGTTTCCTGAACGTGGAAACGGCTGAAA TTATTAAAGAGAAGAACGGTCTTCCTTACGTCTCCATT GATGGCGATCAGACCGATCCTCGCGTTTTTTCTCCGGC CCAGTTTGATACCCGTGTTCAGGCCCTGGTTGAGATGA TGGAGGCCAATATGGCGGCAGCGGAATAAtaagaaggagata tacatATGTCACGCGTGGAGGCAATCCTGTCGCAGCTGAA AGATGTCGCCGCGAATCCGAAAAAAGCCATGGATGAC TATAAAGCTGAAACAGGTAAGGGCGCGGTTGGTATCA TGCCGATCTACAGCCCCGAAGAAATGGTACACGCCGCT GGCTATTTGCCGATGGGAATCTGGGGCGCCCAGGGCA AAACGATTAGTAAAGCGCGCACCTATCTGCCTGCTTTT GCCTGCAGCGTAATGCAGCAGGTTATGGAATTACAGTG CGAGGGCGCGTATGATGACCTGTCCGCAGTTATTTTTA GCGTACCGTGCGACACTCTCAAATGTCTTAGCCAGAAA TGGAAAGGTACGTCCCCAGTGATTGTATTTACGCATCC GCAGAACCGCGGATTAGAAGCGGCGAACCAATTCTTG GTTACCGAGTATGAACTGGTAAAAGCACAACTGGAAT CAGTTCTGGGTGTGAAAATTTCAAACGCCGCCCTGGAA AATTCGATTGCAATTTATAACGAGAATCGTGCCGTGAT GCGTGAGTTCGTGAAAGTGGCAGCGGACTATCCTCAA GTCATTGACGCAGTGAGCCGCCACGCGGTTTTTAAAGC GCGCCAGTTTATGCTTAAGGAAAAACATACCGCACTTG TGAAAGAACTGATCGCTGAGATTAAAGCAACGCCAGT CCAGCCGTGGGACGGAAAAAAGGTTGTAGTGACGGGC ATTCTGTTGGAACCGAATGAGTTATTAGATATCTTTAA TGAGTTTAAGATCGCGATTGTTGATGATGATTTAGCGC AGGAAAGCCGTCAGATCCGTGTTGACGTTCTGGACGG AGAAGGCGGACCGCTCTACCGTATGGCTAAAGCGTGG

	CAGCAAATGTATGGCTGCTCGCTGGCAACCGACACCA AGAAGGGTCGCGGCCGTATGTTAATTAACAAAACGAT TCAGACCGGTGCGGACGCTATCGTAGTTGCAATGATGA AGTTTTGCGACCCAGAAGAATGGGATTATCCGGTAATG TACCGTGAATTTGAAGAAAAAGGGGTCAAATCACTTAT GATTGAGGTGGATCAGGAAGTATCGTCTTTCGAACAGA TTAAAACCCGTCTGCAGTCATTCGTCGAAATGCTTTAAt aagaaggagatatacatATGTATACCTTGGGGATTGATGTCGGT TCTGCCTCTAGTAAAGCGGTGATTCTGAAAGATGGAAA AGATATTGTCGCTGCCGAGGTTGTCCAAGTCGGTACCG GCTCCTCGGGTCCCCAACGCGCACTGGACAAAGCCTTT GAAGTCTCTGGCTTAAAAAAGGAAGACATCAGCTACA CAGTAGCTACGGGCTATGGGCGCTTCAATTTTAGCGAC GCGGATAAACAGATTTCGGAAATTAGCTGTCATGCCAA AGGCATTTATTTCTTAGTACCAACTGCGCGCACTATTA TTGACATTGGCGGCCAAGATGCGAAAGCCATCCGCCTG GACGACAAGGGGGGTATTAAGCAATTCTTCATGAATG ATAAATGCGCGGCGGGCACGGGGCGTTTCCTGGAAGT CATGGCTCGCGTACTTGAAACCACCCTGGATGAAATGG CTGAACTGGATGAACAGGCGACTGACACCGCTCCCATT TCAAGCACCTGCACGGTTTTCGCCGAAAGCGAAGTAAT TAGCCAATTGAGCAATGGTGTCTCACGCAACAACATCA TTAAAGGTGTCCATCTGAGCGTTGCGTCACGTGCGTGT GGTCTGGCGTATCGCGGCGGTTTGGAGAAAGATGTTGT TATGACAGGTGGCGTGGCAAAAAATGCAGGGGTGGTG CGCGCGGTGGCGGGCGTTCTGAAGACCGATGTTATCGT TGCTCCGAATCCTCAGACGACCGGTGCACTGGGGGCA GCGCTGTATGCTTATGAGGCCGCCCAGAAGAAGTAAtaa gaaggagatatacatATGGCCTTCAATAGCGCAGATATTAATTC TTTCCGCGATATTTGGGTGTTTTGTGAACAGCGTGAGG GCAAACTGATTAACACCGATTTCGAATTAATTAGCGAA GGTCGTAAACTGGCTGACGAACGCGGAAGCAAACTGG TTGGAATTTTGCTGGGGCACGAAGTTGAAGAAATCGCA AAAGAATTAGGCGGCTATGGTGCGGACAAGGTAATTG TGTGCGATCATCCGGAACTTAAATTTTACACTACGGAT GCTTATGCCAAAGTTTTATGTGACGTCGTGATGGAAGA GAAACCGGAGGTAATTTTGATCGGTGCCACCAACATTG GCCGTGATCTCGGACCGCGTTGTGCTGCACGCTTGCAC ACGGGGCTGACGGCTGATTGCACGCACCTGGATATTGA TATGAATAAATATGTGGACTTTCTTAGCACCAGTAGCA CCTTGGATATCTCGTCGATGACTTTCCCTATGGAAGAT ACAAACCTTAAAATGACGCGCCCTGCATTTGGCGGACA TCTGATGGCAACGATCATTTGTCCACGCTTCCGTCCCT GTATGAGCACAGTGCGCCCCGGAGTGATGAAGAAAGC GGAGTTCTCGCAGGAGATGGCGCAAGCATGTCAAGTA GTGACCCGTCACGTAAATTTGTCGGATGAAGACCTTAA AACTAAAGTAATTAATATCGTGAAGGAAACGAAAAAG ATTGTGGATCTGATCGGCGCAGAAATTATTGTGTCAGT TGGTCGTGGTATCTCGAAAGATGTCCAAGGTGGAATTG CACTGGCTGAAAAACTTGCGGACGCATTTGGTAACGGT

	GTCGTGGGCGGCTCGCGCGCAGTGATTGATTCCGGCTG GTTACCTGCGGATCATCAGGTTGGACAAACCGGTAAG ACCGTGCACCCGAAAGTCTACGTGGCGCTGGGTATTAG TGGGGCTATCCAGCATAAGGCTGGGATGCAAGACTCT GAACTGATCATTGCCGTCAACAAAGACGAAACGGCGC CTATCTTCGACTGCGCCGATTATGGCATCACCGGTGAT TTATTTAAAATCGTACCGATGATGATCGACGCGATCAA AGAGGGTAAAAACGCATGAtaagaaggagatatacatATGCGCA TCTATGTGTGTGTGAAACAAGTCCCAGATACGAGCGGC AAGGTGGCCGTTAACCCTGATGGGACCCTTAACCGTGC CTCAATGGCAGCGATTATTAACCCGGACGATATGTCCG CGATCGAACAGGCATTAAAACTGAAAGATGAAACCGG ATGCCAGGTTACGGCGCTTACGATGGGTCCTCCTCCTG CCGAGGGCATGTTGCGCGAAATTATTGCAATGGGGGC CGACGATGGTGTGCTGATTTCGGCCCGTGAATTTGGGG GGTCCGATACCTTCGCAACCAGTCAAATTATTAGCGCG GCAATCCATAAATTAGGCTTAAGCAATGAAGACATGA TCTTTTGCGGTCGTCAGGCCATTGACGGTGATACGGCC CAAGTCGGCCCTCAAATTGCCGAAAAACTGAGCATCCC ACAGGTAACCTATGGCGCAGGAATCAAAAAATCTGGT GATTTAGTGCTGGTGAAGCGTATGTTGGAGGATGGTTA TATGATGATCGAAGTCGAAACTCCATGTCTGATTACCT GCATTCAGGATAAAGCGGTAAAACCACGTTACATGAC TCTCAACGGTATTATGGAATGCTACTCCAAGCCGCTCC TCGTTCTCGATTACGAAGCACTGAAAGATGAACCGCTG ATCGAACTTGATACCATTGGGCTTAAAGGCTCCCCGAC GAATATCTTTAAATCGTTTACGCCGCCTCAGAAAGGCG TTGGTGTCATGCTCCAAGGCACCGATAAGGAAAAAGT CGAGGATCTGGTGGATAAGCTGATGCAGAAACATGTC ATCTAAtaagaaggagatatacatATGTTCTTACTGAAGATTAAA AAAGAACGTATGAAACGCATGGACTTTAGTTTAACGC GTGAACAGGAGATGTTAAAAAAACTGGCGCGTCAGTT TGCTGAGATCGAGCTGGAACCGGTGGCCGAAGAGATT GATCGTGAGCACGTTTTTCCTGCAGAAAACTTTAAGAA GATGGCGGAAATTGGCTTAACCGGCATTGGTATCCCGA AAGAATTTGGTGGCTCCGGTGGAGGCACCCTGGAGAA GGTCATTGCCGTGTCAGAATTCGGCAAAAAGTGTATGG CCTCAGCTTCCATTTTAAGCATTCATCTTATCGCGCCGC AGGCAATCTACAAATATGGGACCAAAGAACAGAAAGA GACGTACCTGCCGCGTCTTACCAAAGGTGGTGAACTGG GCGCCTTTGCGCTGACAGAACCAAACGCCGGAAGCGA TGCCGGCGCGGTAAAAACGACCGCGATTCTGGACAGC CAGACAAACGAGTACGTGCTGAATGGCACCAAATGCT TTATCAGCGGGGGCGGGCGCGCGGGTGTTCTTGTAATT TTTGCGCTTACTGAACCGAAAAAAGGTCTGAAAGGGA TGAGCGCGATTATCGTGGAGAAAGGGACCCCGGGCTT CAGCATCGGCAAGGTGGAGAGCAAGATGGGGATCGCA GGTTCGGAAACCGCGGAACTTATCTTCGAAGATTGTCG CGTTCCGGCTGCCAACCTTTTAGGTAAAGAAGGCAAAG GCTTTAAAATTGCTATGGAAGCCCTGGATGGCGCCCGT

	ATTGGCGTGGGCGCTCAAGCAATCGGAATTGCCGAGG GGGCGATCGACCTGAGTGTGAAGTACGTTCACGAGCG CATTCAATTTGGTAAACCGATCGCGAATCTGCAGGGAA TTCAATGGTATATCGCGGATATGGCGACCAAAACCGCC GCGGCACGCGCACTTGTTGAGTTTGCAGCGTATCTTGA AGACGCGGGTAAACCGTTCACAAAGGAATCTGCTATG TGCAAGCTGAACGCCTCCGAAAACGCGCGTTTTGTGAC AAATTTAGCTCTGCAGATTCACGGGGGTTACGGTTATA TGAAAGATTATCCGTTAGAGCGTATGTATCGCGATGCT AAGATTACGGAAATTTACGAGGGGACATCAGAAATCC ATAAGGTGGTGATTGCGCGTGAAGTAATGAAACGCTA A
Ptet-acuI-pct-lcdABC (Ptet: lower case; tetA/R promoter within Ptet: lower case bold, with tet operator underlined; RBS and leader region lower case italic; ribosome binding site: lower case underlined italic; coding region: upper case, rrnB T1 and T2 terminors : lower case bold underline italics) (**SEQ ID NO: 221**)	caactgttgggaagggcgatcggtgcgggcctcttcgctattacgccagctggcgaaagg gggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgacgttgta aaacgacggccagtgaattgacgcgtattgggatgtaaaacgacggccagtgaattcgtta agacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataaga aggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatac tatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaacc taaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgttg gcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaat gtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaa aaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaa tggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctcta cacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctc taatgcgctgttaatcactttacttttatctaatctagacatcattaattcctaatttttt**gttgacac** **tctatcattgatagagttattttaccactccctatcagtgatagaga**aaagtgaa*actcta* *gaaataatttgtttaactttaagaaggagatatacat*ATGCGTGCGGTACTG ATCGAGAAGTCCGATGATACACAGTCCGTCTCTGTCAC CGAACTGGCTGAAGATCAACTGCCGGAAGGCGACGTT TTGGTAGATGTTGCTTATTCAACACTGAACTACAAAGA CGCCCTGGCAATTACCGGTAAAGCCCCCGTCGTTCGTC GTTTTCCGATGGTACCTGGAATCGACTTTACGGGTACC GTGGCCCAGTCTTCCCACGCCGACTTCAAGCCAGGTGA TCGCGTAATCCTGAATGGTTGGGGTGTGGGGGAAAAA CATTGGGGCGGTTTAGCGGAGCGCGCTCGCGTGCGCG GAGACTGGCTTGTTCCCTTGCCAGCCCCCCTGGACTTA CGCCAAGCGGCCATGATCGGTACAGCAGGATACACGG CGATGTTGTGCGTTCTGGCGCTTGAACGTCACGGAGTG GTGCCGGGTAATGGGGAAATCGTGGTGTCCGGTGCAG CAGGCGGCGTCGGCTCCGTTGCGACGACCCTTCTTGCC GCTAAGGGCTATGAGGTAGCGGCAGTGACTGGACGTG CGTCCGAAGCAGAATATCTGCGCGGTTTGGGGGCGGC GAGCGTAATTGATCGTAACGAATTAACGGGGAAGGTA CGCCCGCTGGGTCAGGAGCGTTGGGCTGGCGGGATTG ACGTGGCGGGATCAACCGTGCTTGCGAACATGCTTTCT ATGATGAAGTATCGCGGGGTAGTCGCTGCGTGTGGCCT GGCCGCGGGCATGGATCTGCCCGCGTCTGTCGCGCCCT TTATTCTTCGTGGGATGACGCTGGCAGGGGTGGATAGC GTTATGTGCCCAAAGACAGATCGTTTAGCAGCGTGGGC CCGTTTGGCGTCAGATCTTGACCCTGCCAAGCTGGAGG

	AGATGACTACAGAGTTGCCGTTTAGTGAAGTAATCGAG ACAGCACCCAAATTCTTGGACGGGACGGTTCGTGGCCG CATTGTTATCCCCGTAACGCCCTAAgaa*ctctagaaataattttgttt* *aactttaagaaggagatatacat*ATGCGCAAAGTGCCGATTATCAC GGCTGACGAGGCCGCAAAACTGATCAAGGACGGCGAC ACCGTGACAACTAGCGGCTTTGTGGGTAACGCGATCCC TGAGGCCCTTGACCGTGCAGTCGAAAAGCGTTTCCTGG AAACGGGCGAACCGAAGAACATTACTTATGTATATTGC GGCAGTCAGGGCAATCGCGACGGTCGTGGCGCAGAAC ATTTCGCGCATGAAGGCCTGCTGAAACGTTATATCGCT GGCCATTGGGCGACCGTCCCGGCGTTAGGGAAAATGG CCATGGAGAATAAAATGGAGGCCTACAATGTCTCTCA GGGCGCCTTGTGTCATCTCTTTCGCGATATTGCGAGCC ATAAACCGGGTGTGTTCACGAAAGTAGGAATCGGCAC CTTCATTGATCCACGTAACGGTGGTGGGAAGGTCAACG ATATTACCAAGGAAGATATCGTAGAACTGGTGGAAAT TAAAGGGCAGGAATACCTGTTTTATCCGGCGTTCCCGA TCCATGTCGCGCTGATTCGTGGCACCTATGCGGACGAG AGTGGTAACATCACCTTTGAAAAAGAGGTAGCGCCTTT GGAAGGGACTTCTGTCTGTCAAGCGGTGAAGAACTCG GGTGGCATTGTCGTGGTTCAGGTTGAGCGTGTCGTCAA AGCAGGCACGCTGGATCCGCGCCATGTGAAAGTTCCG GGTATCTATGTAGATTACGTAGTCGTCGCGGATCCGGA GGACCATCAACAGTCCCTTGACTGCGAATATGATCCTG CCCTTAGTGGAGAGCACCGTCGTCCGGAGGTGGTGGGT GAACCACTGCCTTTATCCGCGAAGAAAGTCATCGGCCG CCGTGGCGCGATTGAGCTCGAGAAAGACGTTGCAGTG AACCTTGGGGTAGGTGCACCTGAGTATGTGGCCTCCGT GGCCGATGAAGAAGGCATTGTGGATTTTATGACTCTCA CAGCGGAGTCCGGCGCTATCGGTGGCGTTCCAGCCGGC GGTGTTCGCTTTGGGGCGAGCTACAATGCTGACGCCTT GATCGACCAGGGCTACCAATTTGATTATTACGACGGTG GGGGTCTGGATCTTTGTTACCTGGGTTTAGCTGAATGC GACGAAAAGGGTAATATCAATGTTAGCCGCTTCGGTCC TCGTATCGCTGGGTGCGGCGGATTCATTAACATTACCC AAAACACGCCGAAAGTCTTCTTTTGTGGGACCTTTACA GCCGGGGGGCTGAAAGTGAAAATTGAAGATGGTAAGG TGATTATCGTTCAGGAAGGGAAACAGAAGAAATTCCTT AAGGCAGTGGAGCAAATCACCTTTAATGGAGACGTGG CCTTAGCGAACAAGCAACAAGTTACCTACATCACGGA GCGTTGCGTCTTCCTCCTCAAAGAAGACGGTTTACACC TTTCGGAAATCGCGCCAGGCATCGATCTGCAGACCCAG ATTTTGGATGTTATGGACTTTGCCCCGATCATTGATCGT GACGCAAACGGGCAGATTAAACTGATGGACGCGGCGT TATTCGCAGAAGGGCTGATGGGCTTGAAAGAAATGAA GTCTTGAtaa*gaaggagatatacat*ATGAGCTTAACCCAAGGCA TGAAAGCTAAACAACTGTTAGCATACTTTCAGGGTAAA GCCGATCAGGATGCACGTGAAGCGAAAGCCCGCGGTG AGCTGGTCTGCTGGTCGGCGTCAGTCGCGCCGCCGGAA TTTTGCGTAACAATGGGCATTGCCATGATCTACCCGGA

	GACTCATGCAGCGGGCATCGGTGCCCGCAAAGGTGCG ATGGACATGCTGGAAGTTGCGGACCGCAAAGGCTACA ACGTGGATTGTTGTTCCTACGGCCGTGTAAATATGGGT TACATGGAATGTTTAAAAGAAGCCGCCATCACGGGCG TCAAGCCGGAAGTTTTGGTTAATTCCCCTGCTGCTGAC GTTCCGCTTCCCGATTTGGTGATTACGTGTAATAATATC TGTAACACGCTGCTGAAATGGTACGAAAACTTAGCAG CAGAACTCGATATTCCTTGCATCGTGATCGACGTACCG TTTAATCATACCATGCCGATTCCGGAATATGCCAAGGC CTACATCGCGGACCAGTTCCGCAATGCAATTTCTCAGC TGGAAGTTATTTGTGGCCGTCCGTTCGATTGGAAGAAA TTTAAGGAGGTCAAAGATCAGACCCAGCGTAGCGTAT ACCACTGGAACCGCATTGCCGAGATGGCGAAATACAA GCCTAGCCCGCTGAACGGCTTCGATCTGTTCAATTACA TGGCGTTAATCGTGGCGTGCCGCAGCCTGGATTATGCA GAAATTACCTTTAAAGCGTTCGCGGACGAATTAGAAG AGAATTTGAAGGCGGGTATCTACGCCTTTAAAGGTGCG GAAAAAACGCGCTTTCAATGGGAAGGTATCGCGGTGT GGCCACATTTAGGTCACACGTTTAAATCTATGAAGAAT CTGAATTCGATTATGACCGGTACGGCATACCCCGCCCT TTGGGACCTGCACTATGACGCTAACGACGAATCTATGC ACTCTATGGCTGAAGCGTACACCCGTATTTATATTAAT ACTTGTCTGCAGAACAAAGTAGAGGTCCTGCTTGGGAT CATGGAAAAAGGCCAGGTGGATGGTACCGTATATCAT CTGAATCGCAGCTGCAAACTGATGAGTTTCCTGAACGT GGAAACGGCTGAAATTATTAAAGAGAAGAACGGTCTT CCTTACGTCTCCATTGATGGCGATCAGACCGATCCTCG CGTTTTTTCTCCGGCCCAGTTTGATACCCGTGTTCAGGC CCTGGTTGAGATGATGGAGGCCAATATGGCGGCAGCG GAATAAtaa*gaaggagatatacat*ATGTCACGCGTGGAGGCAAT CCTGTCGCAGCTGAAAGATGTCGCCGCGAATCCGAAA AAAGCCATGGATGACTATAAAGCTGAAACAGGTAAGG GCGCGGTTGGTATCATGCCGATCTACAGCCCCGAAGAA ATGGTACACGCCGCTGGCTATTTGCCGATGGGAATCTG GGGCGCCCAGGGCAAAACGATTAGTAAAGCGCGCACC TATCTGCCTGCTTTTGCCTGCAGCGTAATGCAGCAGGT TATGGAATTACAGTGCGAGGGCGCGTATGATGACCTGT CCGCAGTTATTTTTAGCGTACCGTGCGACACTCTCAAA TGTCTTAGCCAGAAATGGAAAGGTACGTCCCCAGTGAT TGTATTTACGCATCCGCAGAACCGCGGATTAGAAGCGG CGAACCAATTCTTGGTTACCGAGTATGAACTGGTAAAA GCACAACTGGAATCAGTTCTGGGTGTGAAAATTTCAAA CGCCGCCCTGGAAAATTCGATTGCAATTTATAACGAGA ATCGTGCCGTGATGCGTGAGTTCGTGAAAGTGGCAGCG GACTATCCTCAAGTCATTGACGCAGTGAGCCGCCACGC GGTTTTTAAAGCGCGCCAGTTTATGCTTAAGGAAAAAC ATACCGCACTTGTGAAAGAACTGATCGCTGAGATTAAA GCAACGCCAGTCCAGCCGTGGGACGGAAAAAAGGTTG TAGTGACGGGCATTCTGTTGGAACCGAATGAGTTATTA GATATCTTTAATGAGTTTAAGATCGCGATTGTTGATGA

	TGATTTAGCGCAGGAAAGCCGTCGGATCCGTGTTGACG TTCTGGACGGAGAAGGCGGACCGCTCTACCGTATGGCT AAAGCGTGGCAGCAAATGTATGGCTGCTCGCTGGCAA CCGACACCAAGAAGGGTCGCGGCCGTATGTTAATTAA CAAAACGATTCAGACCGGTGCGGACGCTATCGTAGTTG CAATGATGAAGTTTTGCGACCCAGAAGAATGGGATTAT CCGGTAATGTACCGTGAATTTGAAGAAAAAGGGGTCA AATCACTTATGATTGAGGTGGATCAGGAAGTATCGTCT TTCGAACAGATTAAAACCCGTCTGCAGTCATTCGTCGA AATGCTTTAAtaa*gaaggagatatacat*ATGTATACCTTGGGGA TTGATGTCGGTTCTGCCTCTAGTAAAGCGGTGATTCTG AAAGATGGAAAAGATATTGTCGCTGCCGAGGTTGTCC AAGTCGGTACCGGCTCCTCGGGTCCCCAACGCGCACTG GACAAAGCCTTTGAAGTCTCTGGCTTAAAAAAGGAAG ACATCAGCTACACAGTAGCTACGGGCTATGGGCGCTTC AATTTTAGCGACGCGGATAAACAGATTTCGGAAATTAG CTGTCATGCCAAAGGCATTTATTTCTTAGTACCAACTG CGCGCACTATTATTGACATTGGCGGCCAAGATGCGAAA GCCATCCGCCTGGACGACAAGGGGGGTATTAAGCAAT TCTTCATGAATGATAAATGCGCGGCGGGCACGGGGCG TTTCCTGGAAGTCATGGCTCGCGTACTTGAAACCACCC TGGATGAAATGGCTGAACTGGATGAACAGGCGACTGA CACCGCTCCCATTTCAAGCACCTGCACGGTTTTCGCCG AAAGCGAAGTAATTAGCCAATTGAGCAATGGTGTCTC ACGCAACAACATCATTAAAGGTGTCCATCTGAGCGTTG CGTCACGTGCGTGTGGTCTGGCGTATCGCGGCGGTTTG GAGAAAGATGTTGTTATGACAGGTGGCGTGGCAAAAA ATGCAGGGGTGGTGCGCGCGGTGGCGGGCGTTCTGAA GACCGATGTTATCGTTGCTCCGAATCCTCAGACGACCG GTGCACTGGGGGGCAGCGCTGTATGCTTATGAGGCCGCC CAGAAGAAGTAgatggtagtgtggggtctccccatgcgagagtagggaactgc caggcat***caaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgt ttgtcggtgaacgctctcctgagtaggacaaat***ccgccgggagcggatttgaacgttgcg aagcaacggcccggagggtggcgggcaggacgcccgccataaactgccaggcatcaa attaagc***agaaggccatcctgacggatggcctttt***
acuI-pct-lcdABC (**SEQ ID NO: 222**)	ATGCGTGCGGTACTGATCGAGAAGTCCGATGATACAC AGTCCGTCTCTGTCACCGAACTGGCTGAAGATCAACTG CCGGAAGGCGACGTTTTGGTAGATGTTGCTTATTCAAC ACTGAACTACAAAGACGCCCTGGCAATTACCGGTAAA GCCCCCGTCGTTCGTCGTTTTCCGATGGTACCTGGAAT CGACTTTACGGGTACCGTGGCCCAGTCTTCCCACGCCG ACTTCAAGCCAGGTGATCGCGTAATCCTGAATGGTTGG GGTGTGGGGGAAAAACATTGGGGCGGTTTAGCGGAGC GCGCTCGCGTGCGCGGAGACTGGCTTGTTCCCTTGCCA GCCCCCCTGGACTTACGCCAAGCGGCCATGATCGGTAC AGCAGGATACACGGCGATGTTGTGCGTTCTGGCGCTTG AACGTCACGGAGTGGTGCCGGGTAATGGGGAAATCGT GGTGTCCGGTGCAGCAGGCGGCGTCGGCTCCGTTGCGA CGACCCTTCTTGCCGCTAAGGGCTATGAGGTAGCGGCA GTGACTGGACGTGCGTCCGAAGCAGAATATCTGCGCG

	GTTTGGGGGCGGCGAGCGTAATTGATCGTAACGAATTA ACGGGGAAGGTACGCCCGCTGGGTCAGGAGCGTTGGG CTGGCGGGATTGACGTGGCGGGATCAACCGTGCTTGCG AACATGCTTTCTATGATGAAGTATCGCGGGGTAGTCGC TGCGTGTGGCCTGGCCGCGGGCATGGATCTGCCCGCGT CTGTCGCGCCCTTTATTCTTCGTGGGATGACGCTGGCA GGGGTGGATAGCGTTATGTGCCCAAAGACAGATCGTTT AGCAGCGTGGGCCCGTTTGGCGTCAGATCTTGACCCTG CCAAGCTGGAGGAGATGACTACAGAGTTGCCGTTTAGT GAAGTAATCGAGACAGCACCCAAATTCTTGGACGGGA CGGTTCGTGGCCGCATTGTTATCCCCGTAACGCCCTAA gaa*ctctagaaataatttgtttaactttaagaaggagatatacat*ATGCGCAAA GTGCCGATTATCACGGCTGACGAGGCCGCAAAACTGA TCAAGGACGGCGACACCGTGACAACTAGCGGCTTTGT GGGTAACGCGATCCCTGAGGCCCTTGACCGTGCAGTCG AAAAGCGTTTCCTGGAAACGGGCGAACCGAAGAACAT TACTTATGTATATTGCGGCAGTCAGGGCAATCGCGACG GTCGTGGCGCAGAACATTTCGCGCATGAAGGCCTGCTG AAACGTTATATCGCTGGCCATTGGGCGACCGTCCCGGC GTTAGGGAAAATGGCCATGGAGAATAAAATGGAGGCC TACAATGTCTCTCAGGGCGCCTTGTGTCATCTCTTTCGC GATATTGCGAGCCATAAACCGGGTGTGTTCACGAAAGT AGGAATCGGCACCTTCATTGATCCACGTAACGGTGGTG GGAAGGTCAACGATATTACCAAGGAAGATATCGTAGA ACTGGTGGAAATTAAAGGGCAGGAATACCTGTTTTATC CGGCGTTCCCGATCCATGTCGCGCTGATTCGTGGCACC TATGCGGACGAGAGTGGTAACATCACCTTTGAAAAAG AGGTAGCGCCTTTGGAAGGGACTTCTGTCTGTCAAGCG GTGAAGAACTCGGGTGGCATTGTCGTGGTTCAGGTTGA GCGTGTCGTCAAAGCAGGCACGCTGGATCCGCGCCAT GTGAAAGTTCCGGGTATCTATGTAGATTACGTAGTCGT CGCGGATCCGGAGGACCATCAACAGTCCCTTGACTGCG AATATGATCCTGCCCTTAGTGGAGAGCACCGTCGTCCG GAGGTGGTGGGTGAACCACTGCCTTTATCCGCGAAGA AAGTCATCGGCCGCCGTGGCGCGATTGAGCTCGAGAA AGACGTTGCAGTGAACCTTGGGGTAGGTGCACCTGAGT ATGTGGCCTCCGTGGCCGATGAAGAAGGCATTGTGGAT TTTATGACTCTCACAGCGGAGTCCGGCGCTATCGGTGG CGTTCCAGCCGGCGGTGTTCGCTTTGGGGCGAGCTACA ATGCTGACGCCTTGATCGACCAGGGCTACCAATTTGAT TATTACGACGGTGGGGGTCTGGATCTTTGTTACCTGGG TTTAGCTGAATGCGACGAAAAGGGTAATATCAATGTTA GCCGCTTCGGTCCTCGTATCGCTGGGTGCGGCGGATTC ATTAACATTACCCAAAACACGCCGAAAGTCTTCTTTTG TGGGACCTTTACAGCCGGGGGGCTGAAAGTGAAAATT GAAGATGGTAAGGTGATTATCGTTCAGGAAGGGAAAC AGAAGAAATTCCTTAAGGCAGTGGAGCAAATCACCTTT AATGGAGACGTGGCCTTAGCGAACAAGCAACAAGTTA CCTACATCACGGAGCGTTGCGTCTTCCTCCTCAAAGAA GACGGTTTACACCTTTCGGAAATCGCGCCAGGCATCGA

	TCTGCAGACCCAGATTTTGGATGTTATGGACTTTGCCC CGATCATTGATCGTGACGCAAACGGGCAGATTAAACT GATGGACGCGGCGTTATTCGCAGAAGGGCTGATGGGC TTGAAAGAAATGAAGTCTTGAtaa*gaaggagatatacat*ATGAG CTTAACCCAAGGCATGAAAGCTAAACAACTGTTAGCAT ACTTTCAGGGTAAAGCCGATCAGGATGCACGTGAAGC GAAAGCCCGCGGTGAGCTGGTCTGCTGGTCGGCGTCA GTCGCGCCGCCGGAATTTTGCGTAACAATGGGCATTGC CATGATCTACCCGGAGACTCATGCAGCGGGCATCGGTG CCCGCAAAGGTGCGATGGACATGCTGGAAGTTGCGGA CCGCAAAGGCTACAACGTGGATTGTTGTTCCTACGGCC GTGTAAATATGGGTTACATGGAATGTTTAAAAGAAGCC GCCATCACGGGCGTCAAGCCGGAAGTTTTGGTTAATTC CCCTGCTGCTGACGTTCCGCTTCCCGATTTGGTGATTAC GTGTAATAATATCTGTAACACGCTGCTGAAATGGTACG AAAACTTAGCAGCAGAACTCGATATTCCTTGCATCGTG ATCGACGTACCGTTTAATCATACCATGCCGATTCCGGA ATATGCCAAGGCCTACATCGCGGACCAGTTCCGCAATG CAATTTCTCAGCTGGAAGTTATTTGTGGCCGTCCGTTC GATTGGAAGAAATTTAAGGAGGTCAAAGATCAGACCC AGCGTAGCGTATACCACTGGAACCGCATTGCCGAGAT GGCGAAATACAAGCCTAGCCCGCTGAACGGCTTCGAT CTGTTCAATTACATGGCGTTAATCGTGGCGTGCCGCAG CCTGGATTATGCAGAAATTACCTTTAAAGCGTTCGCGG ACGAATTAGAAGAGAATTTGAAGGCGGGTATCTACGC CTTTAAAGGTGCGGAAAAAACGCGCTTTCAATGGGAA GGTATCGCGGTGTGGCCACATTTAGGTCACACGTTTAA ATCTATGAAGAATCTGAATTCGATTATGACCGGTACGG CATACCCCGCCCTTTGGGACCTGCACTATGACGCTAAC GACGAATCTATGCACTCTATGGCTGAAGCGTACACCCG TATTTATATTAATACTTGTCTGCAGAACAAAGTAGAGG TCCTGCTTGGGATCATGGAAAAAGGCCAGGTGGATGG TACCGTATATCATCTGAATCGCAGCTGCAAACTGATGA GTTTCCTGAACGTGGAAACGGCTGAAATTATTAAAGAG AAGAACGGTCTTCCTTACGTCTCCATTGATGGCGATCA GACCGATCCTCGCGTTTTTTCTCCGGCCCAGTTTGATAC CCGTGTTCAGGCCCTGGTTGAGATGATGGAGGCCAATA TGGCGGCAGCGGAATAAtaa*gaaggagatatacat*ATGTCACGC GTGGAGGCAATCCTGTCGCAGCTGAAAGATGTCGCCG CGAATCCGAAAAAAGCCATGGATGACTATAAAGCTGA AACAGGTAAGGGCGCGGTTGGTATCATGCCGATCTAC AGCCCCGAAGAAATGGTACACGCCGCTGGCTATTTGCC GATGGGAATCTGGGGCGCCCAGGGCAAAACGATTAGT AAAGCGCGCACCTATCTGCCTGCTTTTGCCTGCAGCGT AATGCAGCAGGTTATGGAATTACAGTGCGAGGGCGCG TATGATGACCTGTCCGCAGTTATTTTTAGCGTACCGTG CGACACTCTCAAATGTCTTAGCCAGAAATGGAAAGGT ACGTCCCCAGTGATTGTATTTACGCATCCGCAGAACCG CGGATTAGAAGCGGCGAACCAATTCTTGGTTACCGAGT ATGAACTGGTAAAAGCACAACTGGAATCAGTTCTGGG

	TGTGAAAATTTCAAACGCCGCCCTGGAAAATTCGATTG CAATTTATAACGAGAATCGTGCCGTGATGCGTGAGTTC GTGAAAGTGGCAGCGGACTATCCTCAAGTCATTGACGC AGTGAGCCGCCACGCGGTTTTTAAAGCGCGCCAGTTTA TGCTTAAGGAAAAACATACCGCACTTGTGAAAGAACT GATCGCTGAGATTAAAGCAACGCCAGTCCAGCCGTGG GACGGAAAAAAGGTTGTAGTGACGGGCATTCTGTTGG AACCGAATGAGTTATTAGATATCTTTAATGAGTTTAAG ATCGCGATTGTTGATGATGATTTAGCGCAGGAAAGCCG TCGGATCCGTGTTGACGTTCTGGACGGAGAAGGCGGA CCGCTCTACCGTATGGCTAAAGCGTGGCAGCAAATGTA TGGCTGCTCGCTGGCAACCGACACCAAGAAGGGTCGC GGCCGTATGTTAATTAACAAAACGATTCAGACCGGTGC GGACGCTATCGTAGTTGCAATGATGAAGTTTTGCGACC CAGAAGAATGGGATTATCCGGTAATGTACCGTGAATTT GAAGAAAAAGGGGTCAAATCACTTATGATTGAGGTGG ATCAGGAAGTATCGTCTTTCGAACAGATTAAAACCCGT CTGCAGTCATTCGTCGAAATGCTTTAAtaa*gaaggagatataca* *t*ATGTATACCTTGGGGATTGATGTCGGTTCTGCCTCTAG TAAAGCGGTGATTCTGAAAGATGGAAAAGATATTGTC GCTGCCGAGGTTGTCCAAGTCGGTACCGGCTCCTCGGG TCCCCAACGCGCACTGGACAAAGCCTTTGAAGTCTCTG GCTTAAAAAAGGAAGACATCAGCTACACAGTAGCTAC GGGCTATGGGCGCTTCAATTTTAGCGACGCGGATAAAC AGATTTCGGAAATTAGCTGTCATGCCAAAGGCATTTAT TTCTTAGTACCAACTGCGCGCACTATTATTGACATTGG CGGCCAAGATGCGAAAGCCATCCGCCTGGACGACAAG GGGGGTATTAAGCAATTCTTCATGAATGATAAATGCGC GGCGGGCACGGGGCGTTTCCTGGAAGTCATGGCTCGC GTACTTGAAACCACCCTGGATGAAATGGCTGAACTGG ATGAACAGGCGACTGACACCGCTCCCATTTCAAGCACC TGCACGGTTTTCGCCGAAAGCGAAGTAATTAGCCAATT GAGCAATGGTGTCTCACGCAACAACATCATTAAAGGT GTCCATCTGAGCGTTGCGTCACGTGCGTGTGGTCTGGC GTATCGCGGCGGTTTGGAGAAAGATGTTGTTATGACAG GTGGCGTGGCAAAAAATGCAGGGGTGGTGCGCGCGGT GGCGGGCGTTCTGAAGACCGATGTTATCGTTGCTCCGA ATCCTCAGACGACCGGTGCACTGGGGGCAGCGCTGTAT GCTTATGAGGCCGCCCAGAAGAAGTA

Example 27. Quantification of Propionate by LC-MS/MS

Sample preparation

[01095] First, fresh 1000, 500, 250, 100, 20, 4 and 0.8μg/mL sodium propionate standards were prepared in water. Then, 25μL of sample (bacterial supernatants and standards) were pipetted into a V-bottom polypropylene 96-well plate, and 75μL of 60% ACN (45uL ACN+30uL water per reaction) with 10ug/mL of butyrate-d5 (CDN isotope) internal standard in final solution were added to each sample. The plate was heat-sealed,

mixed well, and centrifuged at 4000rpm for 5 minutes. In a round-bottom 96-well polypropylene plate, 5µL of diluted samples were added to 95µL of a buffer containing 10mM MES pH4.5, 20mM EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide), and 20mM TFEA (2,2,2-trifluroethylamine). The plate was again heat-sealed and mixed well, and samples were incubated at room temperature for 1 hour

LC-MS/MS method

[01096] Propionate was measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. HPLC Details are listed in **Table 61A** and **Table 61B**. Tandem Mass Spectrometry details are found in **Table 61C.**

Table 61A. HPLC Details

Column	Thermo Aquasil C18 column, 5 µm (50 x 2.1 mm)
Mobile Phase A	100% H2O, 0.1% Formic Acid
Mobile Phase B	100% ACN, 0.1% Formic Acid
Injection volume	10uL

Table 61B. HPLC Method

Total Time (min)	**Flow Rate (µL/min)**	**A%**	**B%**
0	0.5	100	0
1	0.5	100	0
2	0.5	10	90
4	0.5	10	90
4.01	0.5	100	0
4.25	0.5	100	0

Table 61C. Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM transitions	Propionate 156.2/57.1, Propionate-d5 161/62.1

Example 28. GLP-1 production from genetically engineered bacteria and Activity Measurements

[01097] To determine whether GLP-1 can be expressed by the genetically engineered bacteria, a construct expressing GLP-1 in conjunction with a modified flagellar type III secretion system shown in **FIG. 80** was generated and integrated into the E coli Nissle chromosome. The construct comprises GLP-1 under control of the native FliC promoter and 5'UTR (untranslated region containing the N-terminal flagellar secretion signal) with an optimized ribosome binding site **FIG. 80** and **Table 62**).

Table 62. GLP-1 construct sequences

Description and SEQ ID NO	Sequence
GLP-1 under control of the native FliC promoter and 5'UTR with an optimized ribosome binding site (in reverse orientation) **(SEQ ID NO: 223)**	ttaaccacgacctttaaccagccaagcaataaactctttcgcagcctggccctccaaat agctagaaacatcagaagtgaaagttccctccgcgtggcgttcgaactcgtccatatt acctcctgactgtgtctacttcgttgattacgttttgggtttccacccgtcggctcaatcg ccgtca
GLP-1 (in reverse orientation) **(SEQ ID NO: 224)**	ttaaccacgacctttaaccagccaagcaataaactctttcgcagcctggccctccaaat agctagaaacatcagaagtgaaagttccctccgcgtggcgttcgaactcgtccat
FliC 5' UTR (in reverse orientation) **(SEQ ID NO: 225)**	attacctcctgactgtgtctacttcgttgattacgttttgggtttccacccgtcggctcaat cgccgtca
Optimized RBS (in reverse orientation) **(SEQ ID NO: 226)**	attacctcctgactgtgtctacttc
Putative terminator **(SEQ ID NO: 227)**	gggcagaaaaaaccccgccgttggcggggaagcacgttgc
GLP-1 construct comprising terminator (lower case italic) GLP-1 (lower case bold) under control of the native FliC promoter and 5'UTR (upper case bold, with optimized RBS underlined) and a chloramphenicol resistance gene under the control of the cat promoter (upper case italic bold), frt homology (upper case underlined) **(SEQ ID NO: 228)**	*Gggcagaaaaaaccccgccgttggcggggaagcacgttgc*tggcaaattaccat tcatgttgccggatgcggcgtaaacgccttatccggcctacaaaaatgtgcaaattca ataaattgcaattccccttgtaggcctgataagcgcagcgcatcaggcaatttggcgtt gccgtcagtctcagttaatcaggttacggcga**ttaaccacgacctttaaccagccaa gcaataaactctttcgcagcctggccctccaaatagctagaaacatcagaagtg aaagttccctccgcgtggcgttcgaactcgtccat**ATTACCTCCTGAC TGTGTCTACTTCGTTGATTACGTTTTGGGTTTCCACC CGTCGGCTCAATCGCCGTCAACCCTGTTATCGTCTG TCGTAAAACAACCTTTAGAATTTTTTTCACAAACAG CCATTTTTTGTTAGTCGACGAAATACTCTTTTCTCTG CCCCTTATTCCCGCTATTAAAAAAAACAATTAAACG TAAACTTTGCGCAATTCAGGCCGATAACCCCGGTAT TCGTTTTACGTGTCGAAAGATAAACGAAGTTCCTAT ACTTTCTAGAGAATAGGAACTTCGGAATAGGAACT TCATTTCTCGTTCGCTGCCACCTAAGAATACTCTAC GGTCACATACAAATGGCGCGCCTTACGCCCCGCCCT GCCA***CTCATCGCAGTACTGTTGTATTCATTAAGCATC TGCCGACATGGAAGCCATCACAAACGGCATGATGAA CCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTT GCGTATAATATTTGCCCATGGTGAAAACGGGGGCGA AGAAGTTGTCCATATTGGCCACGTTTAAATCAAAACT GGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAA CATATTCTCAATAAACCCTTTAGGGAAATAGGCCAG GTTTTCACCGTAACACGCCACATCTTGCGAATATATG***

	TGTAGAAACTGCCGGAAATCGTCGTGGTATTCACTC *CAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAA* *ACGGTGTAACAAGGGTGAACACTATCCCATATCACC* *AGCTCACCGTCTTTCATTGCCATACGTAATTCCGGAT* *GAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGG* *CCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTT* *TAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTT* *ATAGGTACATTGAGCAACTGACTGAAATGCCTCAAA* *ATGTTCTTTACGATGCCATTGGGATATATCAACGGTG* *GTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTT* *AGCTCCTGAAAATCTCGACAACTCAAAAAATACGCC* *CGGTAGTGATCTTATTTCATTATGGTGAAAGTTGGAA* *CCTCTTACGTGCCGATCA*ACGTCTCATTTTCGCCAA AAGTTGGCCCAGGGCTTCCCGGTATCAACAGGGAC ACCAGGATTTATTTATTCTGCGAAGTGATCTTCCGT CACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAG AGAATAGGAACTTCGGAATAGGAACT

[01098] Cultures (the genetically engineered bacteria comprising the GLP-1 construct or streptomycin resistant control Nissle) are grown overnight in F-12K medium (Mediatech, Manassas, VA) without glucose (containing selective antibiotics (chloramphenicol or streptomycin) and then diluted 1:200. The cells are grown with shaking at 250 rpm, and at indicated times (0, 3, 6, and 12h), the supernatant aliquots are collected for GLP-1 quantification.

[01099] Additionally, bacteria are pelleted, washed, and harvested, resuspended in 25 mL sonication buffer (50 mM Tris-HCl, 30 mM NaCl, pH 8.0) with protease inhibitors, and lysed by sonication on ice. Unsoluble debris is spun down twice for 20 min at 12,000 rpm at 4◦C to detect any intracellular recombinant protein.

[01100] To generate cell free medium, the supernatant is centrifuged, and filtered through a 0.2 microm filter to remove any remaining bacteria. The cell-free culture medium (CFM) is diluted to OD600=1 with F-12K, and 10 ng/ml leupeptin, 200μM PMSF and 5 ng/mL aproitinin was added to the CFM to inhibit proteases prior storage at 4°C.

Western blotting

[01101] The cell-free culture medium (CFM) was diluted to the same OD600 with F-12K, and 10 ng/ml leupeptin, PMSF and 5 ng/mL aprotinin was added to inhibit proteases. Clarified supernant (14 ml) is precipitated with 10% trichloroacetic acid (TCA, VWR) for 30 min on ice, and the pellet was washed twice in ice-cold ethanol/ether (1:1). The supernatant pellet is dried under vacuum, dissolved in 50 μl sample buffer (2% SDS, 50mM Tris, pH 6.8, 20%glycerol, 10% mercaptoethanol, bromophenol blue) and boiled for 5 min at 95 °C. The cell pellet is resuspended (From 14 ml culture) in room temperature BugBuster Master Mix by gentle vortexing, using 500 μl BugBuster Master Mix with protease inhibitors

(10 ng/ml Leupeptin, 200μMPMSF and 5 ng/mL aprotinin). The cell suspension is incubated on a shaking platform (VWR, Bristol, CT) at a slow setting for 10–20 min at room temperature. 125 μl 5X sample buffer is added to each sample before and boiling for 10 min at 95 °C.

[01102] Protein concentration is determined by BCA protein assay, and isolated proteins are analyzed by Western blot. Proteins are transferred onto PVDF membranes are detected with an HRP-conjugated Glucagon Antibody (24HCLC), ABfinity™ Rabbit Oligoclonal, Thermo Fisher.

Example 29. Co-culture with Caco-2 cells and ELISA for Insulin

[01103] To determine whether the GLP-1 expressed by the genetically engineered bacteria is functional, a co-culture experiment is conducted in which the bacterial supernatant containing GLP-1 is added to the growth medium of a mammalian intestinal cell line, Caco-2. Caco-2 cells are an intestinal cell line derived from a human colorectal carcinoma that spontaneously differentiates under standard culture conditions, and which lends itself to the *in vitro* study of human gut. The ability of the Caco-2 cells to produce insulin upon exposure to the bacterial cell free supernatant is measured.

[01104] Caco-2 epithelial cells (ATCC# CRL-2102, Manassas, VA) are maintained in Dulbecco's Modified Eagle Media (DMEM, Cellgro, Herndon,VA) plus 10% FBS (Cellgro) at 37°C in a humidified incubator supplemented with 5% CO2. For co-culture experiments, Caco-2 cells are grown in F-12K supplemented with 10% FBS at 37°C in a humidified incubator supplemented with 5% CO2. All co-culture experiments are performed in F-12K plus 10% FBS with Caco-2 cells in passages between 15 and 22.

[01105] Approximately 80% confluent monolayers of Caco-2 cells in 12-well plates are washed with fresh F-12K plus 10% FBS once and covered with 1 mL 50% CFM in F-12K with 10% FBS and incubated at 37°C with 5% CO_2. 200 nM. As a control, the same volume of recombinant GLP-1 (200 nM) in F-12K with 10% FBS is added as a positive control in separate wells. Following a 16 h incubation, an additional 1 mL of 50% CFM in F-12K with 10% FBS or GLP-1 is added to the cells, supplemented with 0.4% Glucose or 0.4% Glycerol before incubation for an additional 2 h. The media is removed from the cells, supplemented with Leupeptin (10ng/mL), 0.2 mM PMSF and aprotinin (10ng/mL), centrifuged (12,000 x rpm), and kept briefly at 4°C prior to ELISA analysis for insulin expression (see "Immuno-blot and ELISA" section).

[01106] In order to estimate the amount of insulin secreted from Caco-2 cells activated by Glp-1, cell free supernatants are assayed using standard ELISA procedures using the Insulin ELISA Kit, Human (KAQ125, Thermo Fisher), according to manufacturer's instructions.

Example 30. In vivo NASH Studies

[01107] For *in vivo* studies, a mouse model is used to study the effects of liver steatosis and hepatic inflammation (Jun Jin, *et al.*, Brit. J. Nutrition, 114:145-1755 (2015)). To briefly summarize, female C57BL/6J mice are fasted and fed either a standard liquid diet of carbohydrates, fat, and protein; or a liquid Western style diet (WSD) fortified with fructose, fat, cholesterol, and a sodium butyrate supplement for six weeks. Butyrate is a short chain fatty acid naturally produced by intestinal bacteria effective in maintaining intestinal homoeostasis. Body weight and plasma samples can be taken throughout the duration of the study. Upon conclusion of the study, the mice can be killed, and the liver and intestine can be removed and assayed. A decrease in liver damage after treatment with the engineered bacterial cells indicates that the engineered bacterial cells described herein are effective for treating nonalcoholic steatohepatitis (NASH).

[01108] Additionally, throughout the study, phenotypes of the mice can also be analyzed. A decrease in the number of symptoms associated with nonalcoholic steatohepatitis (NASH), for example, weight loss, further indicates the efficacy of the engineered bacterial cells described herein for treating nonalcoholic steatohepatitis (NASH).

Example 31. Construction of Plasmids Encoding Bile Salt Hydrolase Enzymes

[01109] The bile salt hydrolase genes from *Lactobacillus plantarum* (SEQ ID NO:1) is synthesized (Genewiz), fused to the Tet promoter, cloned into the high-copy plasmid pUC57-Kan by Gibson assembly, and transformed into *E. coli* DH5α as described herein to generate the plasmid pTet-BSH.

Example 32. Generation of Recombinant Bacteria Comprising a Bile Salt Hydrolase Enzyme

[01110] The pTet-BSH plasmid described above is transformed into *E. coli* Nissle, DH5α, or PIR1. All tubes, solutions, and cuvettes are pre-chilled to 4° C. An overnight culture of *E. coli* (Nissle, DH5α or PIR1) is diluted 1:100 in 4 mL of LB and grown until it reaches an OD_{600} of 0.4-0.6. 1mL of the culture is then centrifuged at 13,000 rpm for 1 min in a 1.5mL microcentrifuge tube and the supernatant is removed. The cells are then

washed three times in pre-chilled 10% glycerol and resuspended in 40uL pre-chilled 10% glycerol. The electroporator is set to 1.8kV. 1uL of a pTet-BSH miniprep is added to the cells, mixed by pipetting, and pipetted into a sterile, chilled 1mm cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. 500uL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 37° C for 1 hr. The cells are spread out on an LB plate containing 50ug/mL Kanamycin for pTet-BSH.

Example 33. Functional Assay Demonstrating that the Recombinant Bacterial Cells Decrease Bile Salt Concentration

[01111] For *in vitro* studies, all incubations will be performed at 37° C. Cultures of *E. coli* Nissle containing pTet-BSH are grown overnight in LB and then diluted 1:100 in LB. The cells are grown with shaking (250 rpm) to early log phase with the appropriate antibiotics. Anhydrous tetracycline (ATC) is added to cultures at a concentration of 100 ng/mL to induce expression of bile salt hydrolase, and bacteria are grown for another 3 hours. Culture broths are then inoculated at 20% in flasks containing fresh LB culture media containing excess bile salts (either 0.5% (wt/vol) TDCA, 0.5% (wt/vol) GDCA, or 3% (vol/vol) human bile) and grown for 16 hours with shaking (250 rpm). A "medium blank" for each culture condition broth is also prepared whereby the "medium blank" is not inoculated with bacteria but treated under the same conditions as the inoculated broths. Following the 16 hour incubation period, broth cultures are pasteurized at 90°C for 15 minutes, centrifuged at 5,000 rpm for 10 minutes, and supernatants filtered with a 0.45 micron filter.

[01112] Bile salt levels and activity in the supernatants is determined. Briefly, bile salt hydrolase activity can be assessed using a plate assay as described in Dashkevicz and Feighner, *Applied Environ. Microbiol.*, 55:11-16 (1989) and Christiaens *et al., Appl. Environ. Microbiol.*, 58:3792-3798 (1992). BSH activity can also be indicated by halos of precipitated deconjugated bile acids (see, also, Jones *et al., PNAS*, 105(36):13580-13585 (2008)). A ninhydrine assay for free taurine has also been described (see, for example, Clarke *et al., Gut Microbes,* 3(3):186-202 (2012)).

Example 34. In vivo Studies Demonstrating that the Recombinant Bacterial Cells Decrease Bile Salt Concentration

[01113] For *in vivo* studies, a mouse model of weight gain and lipid metabolism (as described by Joyce *et al., PNAS*, 111(20):7421-7426 (2014)) is used. To briefly summarize, C57BL/6J mice and germ-free Swiss Webster mice can be fasted and fed either a normal low-fat diet or a high-fat diet for ten weeks. After ten weeks, the mice can be inoculated with recombinant bacteria comprising a bile salt hydrolase enzyme (as described herein) or control bacteria. Body weight, plasma samples, and fecal samples can be taken throughout the duration of the study. Upon conclusion of the study, the mice can be killed, and internal organs (liver, spleen, intestines) and fat pads can be removed and assayed. Treatment efficacy is determined, for example, by measuring levels of bile salts and bile acids. A decrease in levels of bile salts after treatment with the recombinant bacterial cells indicates that the recombinant bacterial cells described herein are effective for treating disorders associated with bile salts.

[01114] Additionally, throughout the study, phenotypes of the mice can also be analyzed. A decrease in the number of symptoms associated with disorders associated with bile salts, for example, weight loss, further indicates the efficacy of the recombinant bacterial cells described herein for treating disorders associated with bile salts.

Example 35. Generation of E.Coli Mutants with ability to consume L-Kynurenine and produce tryptophan from kyrurenine

[0851] *E. coli* Nissle can be engineered to efficiently import KYN and convert it to TRP. A strain was constructed (tryptophan auxotroph) that also expresses exogenous Pseudomonas fluorescens kynureninase mutiation, with the goal of generating a strain that is capable of converting L-kynurenine to anthranilate. Anthranilate can then be converted tryptophan through the enzymes of the tryptophan biosynthetic pathway.

[01115] *E. coli* naturally utilizes anthranilate in its TRP biosynthetic pathway. Briefly, the TrpE (in complex with TrpD) enzyme converts chorismate into anthranilate. TrpD, TrpC, TrpA and TrpB then catalyze a five-step reaction ending with the condensation of an indole with serine to form tryptophan. Next, the kynureninase si introduced into a strain which harbors ΔtrpE (trypophan auxotrophy) deletion. By deleting the TrpE enzyme via lambda-RED recombineering, the subsequent strain of Nissle (Δ*trpE*::Cm) is an auxotroph unable to grow in minimal media without supplementation of TRP or anthranilate.

By expressing kynureninase in Δ*trpE*::Cm (KYNase-trpE), this auxotrophy should alternatively rescued by providing KYN.

[01116] Indeed, as a proof of concept, we were able to show that -while Nissle does not typically utilize KYN - by introducing the Kynureninase (KYNase) from *Pseudomonas fluorescens* (*kynU*) on a medium-copy plasmid under the control of the tetracycline promoter (Ptet) a new strain with this plasmid (Ptet-KYNase) was able to convert L-kynurenine into anthranilate in the presence of a Tet inducer.

Table 63.

STRAIN	**Rich Media**	**Min Media**	**Min + Anthranilate**	**Min + KYNU+ aTc**
Wild type Nissle	+	+	+	+
trpE	+	-	+	-
***trpE* pseudoKYNase**	+	-	+	+
***trpE* hKYNase**	+	-	+	-

[01117] In a preliminary assay (**Table 63**), wildtype Nissle (SYN094), Nissle with a deletion of *trpE*, and *trpE* mutants expressing either the human kynureninase (hKYNase) or the *Pseudomonas fluorescens* kynureninase (pseudoKYNase) from a Ptet promoter on a medium-copy plasmid were grown in either rich media, minimal media (min media), minimal media with 5 mM anthranilate (Min + anthranilate) or minimal media with 10 mM kynurenine and 100 ng/uL aTc (Min + KYNU + aTc). These were grown in 1 mL of media in a deep well plate with shaking at 37°C. A positive for growth (+) in **Table 63** indicates a change in optical density of >5-fold from inoculation.

[01118] The results show that in a mutant *trpE* (which is typically used in the tryptophan biosynthetic pathway to convert chorismate into anthranilate) background, Nissle is unable to grow in minimal media without supplementation with anthranilate (or tryptophan). When minimal media was supplemented with KYNU, the *trpE* mutant was also unable to grow. However, when the pseudoKYNase was expressed in the *trpE* tryptophan-auxotroph the cells were able to grow in Min+KYNU. This indicates that Nissle is able to import L-kynurenine from the media and convert it into anthranilate using the pseudoKYNase. The hKYNase homolog was unable to support growth on M9+KYNU, most likely due to differences in substrate specificity as it has been documented that the human

kynureninase prefers 3-hydroxykynurenine as a substrate (Phillips, Structure and mechanism of kynureninase.. Arch Biochem Biophys. 2014 Feb 15;544:69-74).

Example 36. Generation of E.Coli Mutants with enhanced ability to consume L-Kynurenine and produce tryptophan from kyrurenine

[01119] Adaptive Laboratory Evolution was used to produce mutant bacterial strains with improved Kynurenine consumption and reduced tryptophan uptake. First a lower limit of KYN concentration was established and mutants were evolved by passaging in lowering concentrations of KYN.

[01120] While this can select for mutants capable of increasing KYN import, the bacterial cells still prefer to utilize free, exogenous TRP. In the tumor environment, dual-therapeutic functions can be provided by depletion of KYN and increasing local concentrations of TRP. Therefore, to evolve a strain which prefers KYN over TRP, a toxic analogue of TRP – 5-fluoro-L-tryptophan (ToxTRP) – can be incorporated into the ALE experiment. The resulting best performing strain is then whole genome sequenced in order to deconvolute the contributing mutations. Lambda-RED can be performed in order to reintroduce TrpE, to inactivate Trp regulation (*trpR, tyrR*, transcriptional attenuators) to up-regulate TrpABCDE expression and increase chorismate production. The resulting strain prefers external KYN over to external TRP, efficiently converts KYN into TRP, and also now overproduces TRP.

[01121] Moving forward with the knowledge that Nissle is able to grow on KYNU supplemented minimal media in a *trpE* auxotroph by importing and converting kynurenine, the next step was to establish the minimal concentrations of kynurenine capable of supporting growth. Additionally, in our selection experiment if 5-fluoro-L-tryptophan (ToxTrp) was employed the concentrations of both KYNU and ToxTrp capable of still sustaining growth.

[01122] A growth assay was performed in 96-well plates using streptomycin resistant Nissle, *trpE* and *trpE* pseudoKYNase with and without induction of pseudoKYNase expression using 100 ng/uL aTc. These strains were inoculated at very dilute concentrations into M9 minimal media with varying concentrations of KYNU across columns (2-fold dilutions starting at 2000 ug/mL) and varying concentrations of ToxTrp across rows (2-fold dilutions starting at 200 ug/mL). On a separate plate, the strains were grown in M9+KYNU (at the same concentrations) in the absence of ToxTrp, as described in Example 15.

[01123] The results of the initial checkerboard assay showed the the control sand *trpE* strains are shown in M9+KYNU without any ToxTrp, as there was no growth

detected from either strain at any concentration of ToxTrp. The results of the assay found that expression of the pseudoKYNase provides protection against toxicity of ToxTrp. More importantly, growth is permitted between 250-62.5 ug/mL of KYNU and 6.3-1.55 ug/mL of ToxTrp.

[01124] Together these experiments establish that expression of the *Pseudomonas fluorescens* kynureninase is sufficient to rescue a *trpE* auxotrophy in the presence of kynurenine, as the strain ia able to consume KYN into anthranilate, and upstream metabolite in the TRP biosynthetic pathway. In addition, the KYNase is also capable of providing increased resistance to the toxic tryptophan, 5-fluoro-L-tryptophan. Using the information attained here it is possible to proceed to an adapative laboratory evolution experiment to select for mutants with highly efficient and selective conversion of kynurenine to tryptophan.

Example 37. Checkerboard Assay and ALE Parameters

[01125] To establish the minimum concentration of L-kynurenine and maximum concentration of 5-fluoro-L-tryptophan (ToxTrp) capable of sustaining growth of the KYNase strain, using a checkerboard assay, the following protocol was used. Using a 96-well plate with M9 minimal media with glucose, KYN is supplemented decreasing across columns in 2-fold dilutions from 2000 ug/mL down to ~1 ug/mL. In the rows, ToxTrp concentration decreases by 2-fold from 200 ug/mL down to ~1.5 ug/mL. In one plate, Anhydrous Tetracycline (aTc) was added to a final concentration of 100 ng/uL to induce production of the KYNase. From an overnight culture cells were diluted to an OD600 = 0.5 in 12 mL of TB (plus appropriate antibiotics and inducers, where applicable) and grown for 4 hours. 100 uL of cells were spun down and resuspended to an OD600 = 1.0. These were diluted 2000-fold and 25 uL was added to each well to bring the final volumes in each well to 100 uL. Cells were grown for roughly 20 hours with static incubation at 37C then growth was assessed by OD600, making sure readings fell within linear range (.05-1.0).

[01126] Once identified, the highest concentrations of ToxTrp and lowest concentration of kynurenine capable of supporting growth becomes the starting point for ALE. The ALE parental strain was chosen by culturing the KYNase strain on M9 minimal media supplemented with glucose and L-kynurenine (referred to as M9+KYNU from here on). A single colony was selected, resuspended in 20 uL of sterile phosphate-buffered saline solution. This colony was then used to inoculate three cultures of M9+KYNU, grown into late-logarithmic phase and optical density determined at 600 nm. These cultures were then diluted to 10^3 in 4 rows of a 96-well deep-well plate with 1 mL of M9+KYNU. Each one of

the four rows has a different ToxTrp (increasing 2-fold), while each column has decreasing concentrations of KYNU (by 2-fold). Each morning and evening this plate is diluted back to 10^3 using the well in which the culture has grown to just below saturation so that the culture is always in logarithmic growth. This process is repeated until a change in growth rate is no longer detected. Once no growth rate increases are detected (usually around 10^{11} Cumulative Cell Divisions) the culture is plated onto M9+KYNU (Lee, *et al.*, Cumulative Number of Cell Divisions as a Meaningful Timescale for Adaptive Laboratory Evolution of *Escherichia coli*. PLoS ONE 6, e26172; 2011). Individual colonies are selected and screened in M9+KYNU+ToxTrp media to confirm increased growth rate phenotype. Once mutants with significantly increased growth rate on M9+KYNU are isolated, genomic DNA can be isolated and sent for whole genome sequencing to reveal the mutations responsible for phenotype. All culturing is done shaking at 350 RPM at 37°C.

Example 38. Nitric oxide-inducible reporter constructs

[01127] ATC and nitric oxide-inducible reporter constructs were synthesized (Genewiz, Cambridge, MA). When induced by their cognate inducers, these constructs express GFP, which is detected by monitoring fluorescence in a plate reader at an excitation/emission of 395/509 nm, respectively. Nissle cells harboring plasmids with either the control, ATC-inducible Ptet-GFP reporter construct, or the nitric oxide inducible PnsrR-GFP reporter construct were first grown to early log phase (OD600 of about 0.4-0.6), at which point they were transferred to 96-well microtiter plates containing LB and two-fold decreased inducer (ATC or the long half-life NO donor, DETA-NO (Sigma)). Both ATC and NO were able to induce the expression of GFP in their respective constructs across a range of concentrations (**FIG. 55**); promoter activity is expressed as relative florescence units. An exemplary sequence of a nitric oxide-inducible reporter construct is shown. The bsrR sequence is **bolded**. The gfp sequence is underlined. The PnsrR (NO regulated promoter and RBS) is *italicized*. The constitutive promoter and RBS are boxed.

Table 64. SEQ ID NO: 249

SEQ ID NO: 249
ttatta**tcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgttgagcaggtcttgcagcgtgaaaccgtccagatacgtgaaaaa cgacttcattgcaccgccgagtatgcccgtcagccggcaggacggcgtaatcaggcattcgttgttcgggcccatacactcgaccagctgcatcggttcga ggtggcggacgaccgcgccgatattgatgcgttcgggcggcgcggccagcctcagcccgccgcctttcccgcgtacgctgtgcaagaacccgcctttgac cagcgcggtaaccactttcatcaaatggcttttggaaatgccgtaggtcgaggcgatggtggcgatattgaccagcgcgtcgtcgttgacggcggtgtag atgaggacgcgcagcccgtagtcggtatgttgggtcagatacat**acaacctccttagtacatgcaaaattatttctagagcaacatacgagccggaagcataaa gtgtaaagcctggggtgcctaatgagttgagttgaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggttg*ttgacaattaatcatcggctc*

*gtataatgtataacattcatattttgtgaattttaaactctagaaataattttgtttaactttaagaaggagatatacata***tggctagcaaaggcgaagaattgttca cgggcgttgttcctattttggttgaattggatggcgatgttaatggccataaattcagcgttagcggcgaaggcgaaggcgatgctacgtatggcaaattg acgttgaaattcatttgtacgacgggcaaattgcctgttccttggcctacgttggttacgacgttcagctatggcgttcaatgtttcagccgttatcctgatcat atgaaacgtcatgatttcttcaaaagcgctatgcctgaaggctatgttcaagaacgtacgattagcttcaaagatgatggcaattataaaacgcgtgctga agttaaattcgaaggcgatacgttggttaatcgtattgaattgaaaggcattgatttcaaagaagatggcaatattttgggccataaattggaatataatta taatagccataatgtttatattacggctgataaacaaaaaaatggcattaaagctaatttcaaaattcgtcataatattgaagatggcagcgttcaattggc tgatcattatcaacaaaatacgcctattggcgatggccctgttttgttgcctgataatcattatttgagcacgcaaagcgctttgagcaaagatcctaatgaa aaacgtgatcatatggttttgttggaattcgttacggctgctggcattacgcatggcatggatgaattgtataaa**taataa

[01128] These constructs, when induced by their cognate inducer, lead to high level expression of GFP, which is detected by monitoring fluorescence in a plate reader at an excitation/emission of 395/509 nm, respectively. Nissle cells harboring plasmids with either the ATC-inducible Ptet-GFP reporter construct or the nitric oxide inducible PnsrR-GFP reporter construct were first grown to early log phase (OD600= ~0.4-0.6), at which point they were transferred to 96-well microtiter plates containing LB and 2-fold decreases in inducer (ATC or the long half-life NO donor, DETA-NO (Sigma)). It was observed that both the ATC and NO were able to induce the expression of GFP in their respective construct across a wide range of concentrations. Promoter activity is expressed as relative florescence units.

[01129] **FIG. 55** shows NO-GFP constructs (the dot blot) *E. coli* Nissle harboring the nitric oxide inducible NsrR-GFP reporter fusion were grown overnight in LB supplemented with kanamycin. Bacteria were then diluted 1:100 into LB containing kanamycin and grown to an optical density of 0.4-0.5 and then pelleted by centrifugation. Bacteria were resuspended in phosphate buffered saline and 100 microliters were administered by oral gavage to mice. IBD is induced in mice by supplementing drinking water with 2-3% dextran sodium sulfate for 7 days prior to bacterial gavage. At 4 hours post-gavage, mice were sacrificed and bacteria were recovered from colonic samples. Colonic contents were boiled in SDS, and the soluble fractions were used to perform a dot blot for GFP detection (induction of NsrR-regulated promoters). Detection of GFP was performed by binding of anti-GFP antibody conjugated to HRP (horse radish peroxidase). Detection was visualized using Pierce chemiluminescent detection kit. It is shown in the figure that NsrR-regulated promoters are induced in DSS-treated mice, but are not shown to be induced in untreated mice. This is consistent with the role of NsrR in response to NO, and thus inflammation.

[01130] Bacteria harboring a plasmid expressing NsrR under control of a constitutive promoter and the reporter gene *gfp* (green fluorescent protein) under control of an NsrR-inducible promoter were grown overnight in LB supplemented with kanamycin. Bacteria are then diluted 1:100 into LB containing kanamycin and grown to an

optical density of about 0.4-0.5 and then pelleted by centrifugation. Bacteria are resuspended in phosphate buffered saline and 100 microliters were administered by oral gavage to mice. IBD is induced in mice by supplementing drinking water with 2-3% dextran sodium sulfate for 7 days prior to bacterial gavage. At 4 hours post-gavage, mice were sacrificed and bacteria were recovered from colonic samples. Colonic contents were boiled in SDS, and the soluble fractions were used to perform a dot blot for GFP detection (induction of NsrR-regulated promoters) Detection of GFP was performed by binding of anti-GFP antibody conjugated to to HRP (horse radish peroxidase). Detection was visualized using Pierce chemiluminescent detection kit. **Fig. 55D** shows NsrR-regulated promoters are induced in DSS-treated mice, but not in untreated mice.

Example 39. FNR promoter activity

[01131] In order to measure the promoter activity of different FNR promoters, the *lacZ* gene, as well as transcriptional and translational elements, were synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. The *lacZ* gene was placed under the control of any of the exemplary FNR promoter sequences disclosed in **Table 20**. The nucleotide sequences of these constructs are shown in **Tables 67-71** (**(SEQ ID NO: 228-229**). However, as noted above, the *lacZ* gene may be driven by other inducible promoters in order to analyze activities of those promoters, and other genes may be used in place of the *lacZ* gene as a readout for promoter activity, exemplary results are shown in **FIG. 63**

[01132] **Table 65** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr1} **(SEQ ID NO: 228).** The construct comprises a translational fusion of the Nissle *nirB1* gene and the *lacZ* gene, in which the translational fusions are fused in frame to the 8th codon of the *lacZ* coding region. The P_{fnr1} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[01133] **Table 66** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr2} (**(SEQ ID NO: 229**). The construct comprises a translational fusion of the Nissle *ydfZ* gene and the *lacZ* gene, in which the translational fusions are fused in frame to the 8th codon of the *lacZ* coding region. The P_{fnr2} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is

bolded upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[01134] **Table 67** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr3} (**(SEQ ID NO: 230**). The construct comprises a transcriptional fusion of the Nissle *nirB* gene and the *lacZ* gene, in which the transcriptional fusions use only the promoter region fused to a strong ribosomal binding site. The P_{fnr3} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[01135] **Table 68** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr4} (**(SEQ ID NO: 2318**). The construct comprises a transcriptional fusion of the Nissle *ydfZ* gene and the *lacZ* gene. The P_{fnr4} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[01136] **Table 69** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr5} (**(SEQ ID NO: 232**). The construct comprises a transcriptional fusion of the anaerobically induced small RNA gene, *fnrS1*, fused to *lacZ*. The P_{fnrs} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

Table 65. Pfnr1-lacZ construct Sequences

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 228)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatcgtcgtccggcct tttcctctcttactctgctacgtacatctatttctataaatccgttcaatttgtctgtttttttgcacaaacatgaaatat cagacaattccgtgacttaagaaaatttatacaaatcagcaatatacccccttaaggagtatataaaggtgaatt tgatttacatcaataagcggggttgctgaatcgttaaggtaggcggtaatagaaaagaaatcgaggcaaaa ATGagcaaagtcagactcgcaattat**GGATCCTCTGGCCGTCGTATTACAACGTCGTGA CTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTT CGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGT TGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCG GTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGACGCCGATACTGTCGTCGT

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 228)
CCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCTATCTACACCAACGTGA
CCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCGCGGAGAATCCGACAGGTT
GTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACAGGAAGGCCAGACGC
GAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCT
GGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGCGCA
TTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGA
CGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTG
ACGTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCA
CTCTCTTTAATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGT
ACGGCGAGCTGCGCGATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGA
AACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGATGAGC
GTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTTGAAAATCCGGAA
CTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGCACAC
CGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCGAG
GTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCG
CGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGC
AGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTG
CGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGCTA
CGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCAA
TGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGAGCGAACGCGTA
ACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCT
GGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCA
AATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCCGACACC
ACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCC
CTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCTGCCTGGAG
AAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTAACAGTCTT
GGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGG
CGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACG
GCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGC
CAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCT
GACGGAAGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAAC
CATCGAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCA
CTGGATGGTGGCACTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTG
GATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACTGCCTGAACTGCCGCAGCC
GGAGAGCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCAACCAAACGCG
ACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTCTGG
CGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAACTG
ACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTT
AACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTG
CTGACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGG
CGTAAGTGAAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGG
CGGCGGGCCATTACCAGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATAC
ACTTGCCGACGCGGTGCTGATTACAACCGCCCACGCGTGGCAGCATCAGGGG
AAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGGCACGGTGAGATG
GTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCCGGCGCGGAT
TGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTCGGC
CTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCG
CTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAA

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 228)
CGGTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGC GGCGACTTCCAGTTCAACATCAGCCGCTACAGCCAACAACAACTGATGGAAAC CAGCCATCGCCATCTGCTGCACGCGGAAGAAGGCACATGGCTGAATATCGACG GTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCG GAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAAAAA TAA

Table 66. Pfnr2-lacZ construct sequences

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 229)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctcatgcatgcatcaa aaaagatgtgagcttgatcaaaaacaaaaaatatttcactcgacaggagtatttatattgcgcccgttacgtgg gcttcgactgtaaatcagaaaggagaaaacacctATGacgacctacgatcg**GGATCCTCTGGCCG TCGTATTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCC TTGCGGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACC GATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTG GTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTG ACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCG CCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCC GCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGG CTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCAT CTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGT CTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTG ATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAGGATATGTGGCG GATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACCACGCAAAT CAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCAGCCGCGCGGTACT GGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGGTGACG GTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGG CGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGCC TGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTG CAGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGC GACGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGG CAAGCCGTTGCTGATTCGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATG GTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAG AACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTAC ACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAAC CCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCG CGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAG TGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGACG CGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAG GCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCG CGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAAT GGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCAC GCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCA GTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGA TTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGC

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 229)
GATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCG CACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAGCAGTATTTCCAGT TCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTTCCGTCATA GCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAGCCGCTGGCA AGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACT GCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGTACGC GTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTG GCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCC ACGCCATCCCTCAACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGT AATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATT GGCGATGAAAAACAACTGCTGACCCCGCTGCGCGATCAGTTCACCCGTGCGCC GCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCATTGACCCTAACGCCT GGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCGGCGTTGTT GCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACCGCCCACG CGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATT GATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATAC ACCGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAG CGGGTAAACTGGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTAC TGCAGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTA CGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCGAATTGAATTATG GCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCGCTACAGCCAA CAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGAAGGCAC ATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGA GCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAG TTGGTCTGGTGTCAAAAATAA

Table 67. Pfnr3-lacZ construct Sequences

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 230)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatcgtcgtccggcct** **tttcctctcttactctgctacgtacatctatttctataaatccgttcaatttgtctgtttttttgcacaaacatgaaatat** **cagacaattccgtgacttaagaaaatttatacaaatcagcaatatacccccttaaggagtatataaaggtgaatt** **tgatttacatcaataagcggggttgctgaatcgttaa**GGATCC**ctctagaaataattttgtttaactttaagaa** **ggagatatacat****ATG**ACTATGATTACGGATTCTCTGGCCGTCGTATTACAACGTCGT GACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCC TTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC AGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAA GCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGACGCCGATACTGTCGT CGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCTATCTACACCAACG TGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCGCGGAGAATCCGACAG GTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACAGGAAGGCCAGA CGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGC GCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGC GCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAG TGACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCC GTGACGTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTA

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 230)

CCACTCTCTTTAATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGA
TGTACGGCGAGCTGCGCGATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGT
GAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGATGA
GCGTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTTGAAAATCCGG
AACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGCAC
ACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCG
AGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATT
CGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGA
GCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCG
TGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGC
TACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCC
AATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGAGCGAACGCG
TAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCG
CTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGAT
CAAATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCCGACA
CCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAG
CCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCTGCCTGG
AGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTAACAGTC
TTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAG
GGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAA
CGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATC
GCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCG
CTGACGGAAGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGA
ACCATCGAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTG
CACTGGATGGTGGCACTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTC
TGGATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACTGCCTGAACTGCCGCAG
CCGGAGAGCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCAACCAAACG
CGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTCT
GGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAAC
TGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAA
TTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAA
CTGCTGACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACAT
TGGCGTAAGTGAAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGA
AGGCGGCGGGCCATTACCAGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGA
TACACTTGCCGACGCGGTGCTGATTACAACCGCCCACGCGTGGCAGCATCAGG
GGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGGCACGGTGAG
ATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCCGGCGCG
GATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTC
GGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGA
CCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCG
AAAACGGTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGG
CGCGGCGACTTCCAGTTCAACATCAGCCGCTACAGCCAACAACAACTGATGGA
AACCAGCCATCGCCATCTGCTGCACGCGGAAGAAGGCACATGGCTGAATATCG
ACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGCCCGTCAGTATCG
GCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCA
AAAATAA

Table 68. Pfnr4-lacZ construct Sequences

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 231)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctcatgcatgcatcaa aaaagatgtgagcttgatcaaaaacaaaaaatatttcactcgacaggagtatttatattgcgccc**GGATCC **ctctagaaataattttgtttaactttaagaaggagatatacatATG**ACTATGATTACGGATTCTCTG GCCGTCGTATTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAAT CGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCG CACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTG CCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTT CCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGA TGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGT TCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAG CTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTT TCATCTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTG CCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGC GGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAGGATATGT GGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACCACG CAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCAGCCGCGCG GTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGG TGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCC TTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACAC TACGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTC TATCGTGCAGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGA AGCCTGCGACGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGC TGAACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCGTCACGAGCATCATCCT CTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGAT GAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCT GTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATA TTGAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGG CTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATCA CCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATC ACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAG TATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTA CGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATC AAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATAT GCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTT TCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGT CGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGAT TTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCC GACCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAGCAGTATTT CCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTTCC GTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAGCCG CTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGTTGAT TGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACG GTACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAG CGCCTGGCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTCC GCGTCCCACGCCATCCCTCAACTGACCACCAGCGGAACGGATTTTTGCATCGA

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 231)
GCTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTTCACAGAT GTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGCGCGATCAGTTCACCC GTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCATTGACCCT AACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCGG CGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACC GCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTA CCGGATTGATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAA GCGATACACCGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGT CTCAGAGCGGGTAAACTGGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGAC CGCCTTACTGCAGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGACATGTAT ACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCGAATT GAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCGCT ACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAA GAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGA CTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACC ATTACCAGTTGGTCTGGTGTCAAAAATAA

Table 69 Pfnrs-lacZ construct Sequences

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 232)
GGTACC**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgtaacaaaagcaatttttc** **cggctgtctgtatacaaaaacgccgtaaagtttgagcgaagtcaataaactctctacccattcagggcaatat** **ctctctt**GGATCC**ctctagaaataattttgtttaactttaagaaggagatatacat**ATGCTATGATTACG GATTCTCTGGCCGTCGTATTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC CAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA GAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATG GCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAG TGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCA CGGTTACGATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCC GCCGTTTGTTCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATAT TGATGAAAGCTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAA CTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGAC AGCCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAAAC CGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCA GGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAAC CGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCAG CCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGAA CTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCA CCGCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGC GTCACACTACGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCC GAATCTCTATCGTGCAGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTG AAGCAGAAGCCTGCGACGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTG CTGCTGCTGAACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCGTCACGAGCA TCATCCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCC TGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACC ATCCGCTGTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAA

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 232)
GCCAATATTGAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCC GCGCTGGCTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGAT CGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGG CGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCC GGTACAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCC CGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATG GTCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTG CGAATATGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGC AGGCGTTTCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTG GATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGG CGGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGG TCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAG CAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATAC CTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGG CAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAG CAGTTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTG GCTAACGGTACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGA CACATCAGCGCCTGGCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACT CCCCTCCGCGTCCCACGCCATCCCTCAACTGACCACCAGCGGAACGGATTTTT GCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTT CACAGATGTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGCGCGATCAG TTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCAT TGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCC GAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGAT TACAACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGA AAACCTACCGGATTGATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCG GTGGCAAGCGATACACCGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGG CGCAGGTCTCAGAGCGGGTAAACTGGCTCGGCCTGGGGCCGCAAGAAAACTA TCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGA CATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGC GCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATC AGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCA CGCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTG GCGACGACTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGG TCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAA

Example 40. In vivo NASH Model Development

[01137] For *in vivo* studies, a mouse model to study the effects of liver steatosis and hepatic inflammation was developed according to a choline deficient, L-amino acid defined, high-fat diet (CDAHFD) (as described in Matsumoto et al., 2013, An improved mouse model that rapidly develops fibrosis in non-alcoholic steatohepatitis; Int J Exp Pathol. 2013 Apr; 94(2): 93–103, the contents of which is herein incorporated by reference in its entirety).

[01138] Briefly, C57BL6 mice (20 total animals) were weighed on day 1 and randomized into treatment two groups (Group 1- normal chow, n=10; Group 2 – CDAHFD, n=10) based on weight. Group 2 was changed to a CDAHFD diet (60 kcal% fat, 0.1% methionine, and no choline). Animals were weighed on days 2 through 5. On day 9, animals were weighed, and blood was collected for serum alanine aminotransferase (ALT) / aspartate transaminase (AST) and MCP-1 evaluation, and liver tissue was collected for hematoxylin and eosin (H&E) staining, and for analysis of RNA and protein markers of fibrosis.

[01139] Low levels of body weight change (approximately three percent change) over the 9 day time course were observed. Serum MCP-1 levels were determined by ELISA (ThermoFisher KMC1011, according to manufacturer's instructions) and a 50% change in serum MCP-1 levels were observed. Changes in expression of Col1A1 (Collagen Type I Alpha 1), Col3A1 (Collagen Type III Alpha 1), Col4A1 (Collagen Type IV Alpha 1), and ACTA2 (Actin, Alpha 2, Smooth Muscle, Aorta) were evaluated by qRT-PCR. Briefly, total RNA was isolated from liver using RNeasy kit (QIAGEN), and concentration and purity were measured by measuring the OD_{260} and OD_{280} of the sample on a Eppendorf BioSpectrometer Basic. 1 μg of total RNA was transcribed to cDNA using the SuperScript III System (Invitrogen). Real-time PCR was carried out on each sample in triplicate using SYBR Green PCR Master Mix (MM), (Thermo Fisher Scientific: 4368577). Cycling conditions were as follows: 95°C for 10 seconds, 55–60°C (target dependent) for 50 seconds, and 72°C for 1 second. Serum levels of ALT and AST are measured using a colorimetric assay kit.

Example 41. In vitro Activity of Bacterial Strain Comprising Ammonia-Metabolizing and Butyrate Producing Circuits

[01140] To determine whether ammonia uptake and conversion to arginine and production of butyrate could be accomplished in one strain, a plasmid (Logic156) comprising a butyrate production cassette construct, was used for initial proof-of-concept experiments. The following strains were generated using the plasmid: SYN-UCD501 (comprising Logic156 (pSC101 PydfZ-ter butyrate plasmid; amp resistance); and SYN-UCD601, which is SYN-UCD-305, additionally comprising Logic156 (*i.e.*, SYN-UCD601 comprises ΔArgR, PfnrS- ArgAfbr integrated into the chromosome at the malEK locus, ΔThyA, and Logic156 (pSC101 PydfZ-ter butyrate plasmid; amp resistance)). Arginine and butyrate production was compared between the butyrate only producer SYN-UCD501, the arginine only producer SYN-UCD305, and the combined butyrate/arginine producer SYN-UCD-601. Sequences for the butyrate cassette used are shown in **Tables 30 and 31**.

[01141] Briefly, 3ml LB (containing selective antibiotics (Amp for SYN-UCD501 and SYN-UCD601) and 3mM thymidine for SYN-UCD-305 and SYN-UCD601) with bacteria from frozen glycerol stocks. Bacteria were grown overnight at 37 C with shaking. Overnight cultures were diluted 1:100 dilution into 10ml LB (containing antibiotics and thymidine where necessary as above) in a 125ml baffled flask. Cultures were grown aerobically at 37 C with shaking for about 1.5h, and then transferred to the anaerobic chamber at 37 C for 4h. Bacteria (2X108 CFU) were added to 1ml M9 media containing 50mM MOPS with 0.5% glucose in microcentrifuge tubes. Cells were plated to determine cell counts. The assay tubes were placed in the anaerobic chamber at 37 C. At indicated times (1, 2, 24h), 120 ul cells were removed and pelleted at 14,000rpm for 1min, and 100ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for arginine and butyrate concentrations (as described in Example 13 and Example 40).

[01142] Results are depicted in **Fig. 34A** and **Fig. 34B**, and show that SYN-UCD601 is able to produce similar levels of arginine as SYN-UCD305 and similar levels of butyrate as SYN-UCD501 *in vitro*.

Table. 70 Other Sequences of interest

Wild-type *clbA* **(SEQ ID NO: 233)**	caaatatcacataatcttaacatatcaataaacacagtaaagtttcatgtgaaaaacatcaaacataaa atacaagctcggaatacgaatcacgctatacacattgctaacaggaatgagattatctaaatgagga ttgatatattaattggacatactagtttttttcatcaaaccagtagagataacttccttcactatctcaatg aggaagaaataaaacgctatgatcagtttcattttgtgagtgataaagaactctatattttaagccgtat cctgctcaaaacagcactaaaaagatatcaacctgatgtctcattacaatcatggcaatttagtacgt gcaaatatggcaaaccatttatagtttttcctcagttggcaaaaaagatttttttttaacctttcccatacta tagatacagtagccgttgctattagttctcactgcgagcttggtgtcgatattgaacaaataagagatt tagacaactcttatctgaatatcagtcagcatttttttactccacaggaagctactaacatagtttcactt cctcgttatgaaggtcaattacttttttggaaaatgtggacgctcaaagaagcttacatcaaatatcga ggtaaaggcctatctttaggactggattgtattgaatttcatttaacaaataaaaaactaacttcaaaat atagaggttcacctgtttatttctctcaatggaaaatatgtaactcatttctcgcattagcctctccactc atcacccctaaaataactattgagctatttcctatgcagtcccaactttatcaccacgactatcagcta attcattcgtcaaatgggcagaattgaatcgccacggataatctagacacttctgagccgtcgataat attgattttcatattccgtcggtggtgtaagtatcccgcataatcgtgccattcacatttag
clbA knock-out **(SEQ ID NO: 234)**	ggatgggggggaaacatggataagttcaaagaaaaaaacccgttatctctgcgtgaaagacaagtatt gcgcatgctggcacaaggtgatgagtactctcaaatatcacataatcttaacatatcaataaacacagt aaagtttcatgtgaaaaacatcaaacataaaatacaagctcggaatacgaatcacgctatacacattgc taacaggaatgagattatctaaatgaggattgaTGTGTAGGCTGGAGCTGCTTCG AAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAA CTTCGGAATAGGAACTAAGGAGGATATTCATATGtcgtcaaatgggca gaattgaatcgccacggataatctagacacttctgagccgtcgataatattgattttcatattccgtcggt gg

Example 42. Biochemical Analysis of Butyrate Production in SYN1001

[01143] SYN1001 was assessed for its ability to produce butyrate *in vitro*. An overnight culture of LB-grown SYN1001 was diluted 1:100 into fresh LB (10mL in a 125mL baffled flask). The culture was grown aerobically with shaking at 250 rpm, 37°C for 1.5h. The culture was then moved into an anaerobic chamber (Coy Lab Products, MI) supplying an atmosphere of 85% N_2, 10% CO_2, and 5% H_2. Anaerobic incubation commenced at 37°C for 4 hours in order to induce the expression of the butyrate operon from the P_{fnrS} promoter.

[01144] After the 4 hour anaerobic induction of the butyrate operon, the culture was removed from the anaerobic chamber and approximately $2x10^8$ activated cells were used to inoculate 1 mL of M9 minimal medium containing 0.5% glucose. Assay cultures were incubated statically at 37°C for 18 hours in the presence of O_2. For sample collection, 200uL aliquots were removed from assay cultures and spun down at maximum speed for 1 min in a microcentrifuge. The culture supernatant was retained, and LC-MS-MS was used to determine the concentration of butyrate in the supernatant fraction (**Table 73**-data are average of assay performed in triplicate for three different manufacturing runs).

Table 71: Butyrate production in SYN1001 from three different experiments

Strain	Butyrate (mM) Run 1	SD	Butyrate (mM) Run 2	SD	Butyrate (mM) Run 3	SD
SYN94	NA	NA	NA	NA	0.474	0.002
SYN2001	NA	NA	NA	NA	0.389	0.003
SYN1001	6.371	0.530	6.131	0.100	6.982	0.577

[01145] Equivalent concentrations of butyrate were obtained from 3 independent production runs of SYN1001. In production run 3, SYN94 and SYN2001 control strains were included and supernatants from these strains contained negligible amounts of butyrate (0.47 and 0.38mM respectively) compared to SYN1001, which contained significantly higher levels (6.98mM; n=3). SYN94 is a streptomycin-resistant version of the parental *E.coli* Nissle strain. SYN2001 is an engineered *E.coli* strain that has been modified to over-produce acetate and does not contain a synthetic butyrate operon, described elsewhere herein. Run 3 culture supernatants were used to generate bioactivity in cell based assays described below.

Example 43. Cell-based Assay Development and In-vitro Butyrate Strain Assessment

Methods

[01146] **Mammalian Cell Culture:** HT-29 colon adenocarcinoma cells were obtained from ATCC (Cat#: HTB38). Cells were cultured *at 37^{0}C*, 5% CO_2 in RPMI media supplemented with 10% FBS, 1% pen-strep (complete media). Cells were allowed to grow to ~80% confluency before passaging for activity assays.

[01147] **Alkaline Phosphatase (AP) Activity Assay:** HT-29 colon adenocarcinoma cells were plated in complete media at either 1x10^5 cells/well (24 well plates) *or 1x10^4 cells/well (96 well plates) and allowed to recover overnight at 37^{0}C,* 5% CO_2. The following day media was replaced with fresh complete media containing either PBS, synthetic acetate (SIGMA-Cat#S8750) or butyrate (SIGMA-Cat#B5887), or bacterial supernatants of interest. Cells were incubated for 4 days under these conditions and then media was removed and cellular lysates were prepared (10 min on ice with vendor-supplied lysis buffer (BioVision-see below) followed by clarification for 10 min @14K rpm, 4^{0}C). Lysates from each condition were then assessed for AP activity using an alkaline phosphatase activity kit (BioVision, Cat#K412-500) according to manufacturer's recommendations.

[01148] **Cell Viability Assay:**HT-29 colon adenocarcinoma cells were plated in 2 separate plates in complete media at either 1x10^5 cells/well (24-well plates) *or 1x10^4 cells/well (96-well plates) and allowed to recover overnight at 37^{0}C,* 5% CO_2. The following day, one plate of cells, which served as the day 1 time point read out (input), was treated with trypsin (5 min at *37^{0}C,* 5% CO_2) and cells were counted using a Cellometer K2 instrument (Nexcelom). Live and dead cells were distinguished by trypan blue exclusion. For the remaining plate, media was replaced with fresh complete media containing either PBS, synthetic acetate or butyrate, or bacterial supernatants of interest. Cells were incubated for 4 days under these conditions and then media was removed. Cells were detached from plates with trypsin and counted using the Cellometer K2 as described above.

In vitro Assessment of Engineered Butyrate-producing Strain SYN1001

[01149] To assess the activity of the butyrate-producing strain SYN1001 *in vitro*, we employed the AP cell-based assay. HT-29 cells were plated in triplicate at 1x10^4 cells/well 96-well plates in complete media and allowed to recover overnight. The following day, media was removed and fresh media containing a dilution series of exogenous synthetic butyrate (5mM-0.3mM), or culture supernatants from the SYN94 control (0.26mM-0.016mM), SYN1001 butyrate-producing strain (3.5mM-0.11mM) or SYN2001 acetate-producing strain (0.22mM-0.01mM) were added, and the cells were incubated for 4 days. After the incubation period, media was removed and the plates were processed for assessment

of AP activity. **FIG. 38B** shows that incubation of HT-29 cells with the supernatants from the butyrate-producing SYN1001 strain demonstrated a similar AP activity profile to cells incubated with synthetic butyrate. In contrast, the unengineered strain SYN94 or the acetate-producing strain SYN2001 had little to no effect on AP activity at any concentration tested. To better visualize the similarity in AP activity induction between synthetic butyrate and SYN1001-produced butyrate, the values from the AP activity assay were fit to a non-linear equation algorithm and graphed. As shown in **FIG. 38C**, the activity profile for butyrate produced by SYN1001 is comparable to synthetic butyrate. Incubation with synthetic butyrate, SYN94, SYN1001 or SYN2001 did not have any appreciable effect on cell viability (Data not shown)

Summary

[01150] The results describe the design and evaluation of an engineered, butyrate-producing strain, SYN1001, which contains a modified butyrate module comprised of the *trans*-2-enoyl-CoA reductase *(ter)* gene from *Treponema denticola,* the thiolase *(thiA1)*, 3-hydroxyybutyryl-CoA dehydrogenase *(hbd),* and crotonase *(crt2)* genes from *Clostridium difficile,* and the thioesterase B gene (*tesB*), which is endogenous to *E. coli.* SYN1001 is capable of producing ~7mM butyrate *in vitro* under the conditions described here. This *in vitro* butyrate production translates to activity in a cell-based assay that is comparable on an equimolar basis to that observed with pure, synthetic butyrate. **Table 74**. summarizes the final pharmacological characteristics of the SYN1001.

Table 72. Final characterization of the pharmacological characteristics of SYN1001

	Run 3 [Butyrate] secreted (in mM)	SD	AP Activity at max dose (in U/mg prot)	SD
SYN94	0.474	0.002	0.96	0.052
SYN2001	0.389	0.003	0.95	0.047
SYN1001	6.982	0.577	3.97	0.36

Example 44. In vitro Assessment of the Engineered Acetate-producing Strain SYN2001

[01151] To evaluate the activity of acetate-producing strains, we employed a cell-based assay based on work by Cox et al. (Cox et al., WGJ, 15(44), 2009) where the authors demonstrated that the addition of acetate inhibited LPS-induced secretion of IFN□ in human PBMC cells.

[01152] To assess the activity of the acetate-producing strain SYN2001 *in vitro*, we employed the LPS-induction of IFN□ cell-based assay. Frozen normal human

PBMCs from two independent donors (Lot#'s A4956 and A4924) were plated in triplicate at $1x10^6$ cells/mL in 96-well plates in complete media. The cells were then incubated for 15 minutes with media containing either a dilution series of synthetic acetate (40mM-0.08Mm), SYN2001 supernatant (30mM-0.03mM acetate concentrations based on LC-MS determination) or untreated (negative control). After the 15-minute incubation period, complete media containing LPS was added to the cells to a final concentration of 100ng/mL and the cells were further incubated overnight under these conditions. The following day supernatants were harvested from each of the different conditions and the IFN□ levels assessed by ELISA. **FIG. 84G** and **FIG. 84H** show the results from 3 independent experiments (each performed in triplicate) with the two different donors (donor 1=D1; donor 2=D2) in which incubation of primary human PBMC cells with exogenous acetate that was either synthetic or derived from SYN2001 supernatants led to a dose-dependent decrease in the LPS-induced secretion of IFN□ by the cells. We noted that the absolute levels of IFN□ □production in the SYN2001 experiments was higher than in the purified acetate experiments, likely due to residual additional LPS in the supernatants from the bacterially-derived acetate. Nonetheless, the IC50s observed for the two acetate sources were very similar. **Table 73** summarizes the data from the 3 experiments using the 2 separate donors.

Table 73. Summary of EC50's for SYN2001 on LPS-induced IFN□ secretion from 3 experiments performed in triplicate with human PBMC cells from 2 separate donors.

	DONOR 1	DONOR 2
Acetate (mM)	3.12	2.18
SEM	0.29	0.38
SYN1592 (mM)	5.44	3.96
SEM	0.92	0.19

[01153] In conclusion, results presented describe the design and evaluation of an engineered, acetate-producing strain, SYN2001, which contains an enhanced acetate biosynthetic program resulting from deletion of the L-lactate dehydrogenase A (*ldhA*) gene to block the carbon flux from pyruvate to lactate, greatly improving acetate biosynthesis in *E. coli* Nissle. This strain is capable of producing >30mM acetate *in vitro* under the conditions described here. This *in vitro* acetate production translates to activity in a cell-based assay that is comparable on an equimolar basis to that observed with pure, synthetic acetate. The final pharmacological characterization of SYN2001 is summarized in **Table 74**.

Table 74. Final pharmacological characterization of SYN2001

	Run 3 [Acetate] secreted (mM)	EC50-donor 1 (mM)	SEM	EC50-donor 2 (mM)	SEM
SYN2001	31.58	5.44	0.92	3.96	0.19
Acetate (synthetic)	NA	3.12	0.39	2.18	0.28

Example 45. Generation and Analysis of an engineered IL-22-producing E. coli Nissle strain
Engineering and Production of IL-22

[01154] A synthetic construct was generated in which expression of IL-22 is controlled by the tetracycline-inducible promoter (P_{tet}), which is derepressed via the addition of the tetracycline analog anhydrotetracycline (aTc), and translation is driven by a strong ribosome binding site (RBS) located immediately upstream from the IL-22 coding sequence. To promote translocation to the periplasm, a 21-amino acid PhoA-secretion tag was added to the N-terminus of IL-22.

[01155] The corresponding engineered element was constructed using a synthetic DNA cassette encoding the IL-22 protein coding sequence (IDT Technologies, Coralville, Iowa) which was cloned into an initial plasmid vector, creating the plasmid Logic435. The IL-22 sequence was later amplified and cloned using Gibson assembly technology and the NEBuilder Hifi Mastermix (NEB). The final pBR322-based plasmid was sequence-verified by Sanger sequencing (Genewiz) and designated Logic522.

[01156] To create a Gram-negative bacterium capable of secreting bioactive proteins, a diffusible outer membrane (DOM) phenotype was engineneered in the *E. coli* Nissle background. A series of DOM mutants were created by deleting different periplasmic proteins leading to a 'leaky' phenotype. Deletions of several different genes were tested including *lpp*, *pal*, *tolA* and *nlpI*. For example, the *pal* mutant (SYN3000) showed a good secretion phenotype with little-to-no deleterious effect on growth rate while supporting strong production of effectors in the extracellular medium. Logic522 was inserted into SYN3000 to create the IL-22 secretion strain, SYN3001.

[01157] To assay for production of IL-22, cultures were grown and induced, then supernatants were harvested and quantified using ELISA. Overnight cultures were harvested by centrifugation at 12.5K x g for 5 minutes. The supernatants of the cultures were removed from the cell pellet and filtered through a 0.22 μm filter to separate any remaining

bacteria from the supernatant. This supernatant was run immediately in the ELISA, stored short-term at 4°C, or aliquoted and stored at -20°C.

[01158] To evaluate the production of IL-22 in the filtered supernatants, samples of SYN3000 and SYN3001 were diluted in triplicate and run on an R&D Systems IL-22 Quantikine® ELISA Kit (Minneapolis, MN). The results from 3 independent production runs are shown in **Table 75**. The results demonstrated that the SYN3001 supernatants contained an average of 312 ng/ml (+/- 11.38) of material that reacted positively in the IL-22 ELISA assay. In contrast, the SYN3000 supernatants had undetectable levels (not shown). Culture supernatant from run 3 was then used to generate the bioactivity results from the cell based assays described below.

Table 75. SYN3001 supernatant results from three different production runs.

	Run1		Run 2		Run 3	
Strain	**[IL-22] in ng/ml**	**SD**	**[IL-22] in ng/ml**	**SD**	**[IL-22] in ng/ml**	**SD**
SYN3001	325.01	8.40	303.56	2.94	307.67	6.21

In vitro Assessment of IL-22 Produced by the Engineered Strain SYN3001

[01159] To assess the biological activity of IL-22 produced by SYN3001 (IL-22 secreting strain), titrations of SYN3001 and SYN3000 (DOM mutant, non IL-22 secreting negative control strain) supernatants (starting at 150ng/mL and titrated in 1:3 dilutions) were added to Colo205 cells and the activation of STAT3 was assessed. **FIG. 83C** shows the results from 5 independent experiments (each performed in triplicate). Supernatants from SYN3001 induced activation of STAT3 with an average EC50 of 4.8 ng/mL (+/- 1.74 ng/mL). In contrast, SYN3000 had no effect on STAT3 activity.

[01160] To verify that the STAT3 activation elicited by supernatants from SYN3001 was indeed due to IL-22 signaling, Colo205 cells were stimulated with IL-22 supernatants derived from SYN3001 at 3 ng/mL in the presence of increasing concentrations of an anti-IL-22 neutralizing antibody. rLI-22 in the absence of the neutralizing antibody served as a positive control. **FIG. 83D** shows the results from 3 independent experiments (performed in triplicate), demonstrating that the anti-IL-22 antibody inhibited SYN3001-induced activation of STAT3 in a dose-dependent manner. The average IC50 for the anti-IL-22 antibody mediated inhibition of SYN3001-derived IL-22 was 3.45 ng/mL for SYN3001, in line with the value observed using rIL-22, 3.70 ng/mL.

Summary

[01161] The results describe the design and evaluation of an engineered IL- 22 producing strain, SYN3001, which contains a tetracycline-inducible promoter driving the expression of IL-22 fused to a cleavable PhoA-secretion tag to mediate Sec-dependent secretion into the periplasm and a *pal* mutation to create a diffusible outer membrane phenotype (DOM) that facilitates extracellular secretion. This strain is capable of producing >300 ng/mL IL-22 *in vitro* under the conditions described here. This *in vitro* IL-22 production translates to biological activity in a cell-based assay that is comparable to that observed with recombinant IL-22. In addition, the specific activity of the bacterially-produced IL-22 was verified by demonstrating that this signal could be inhibited by a neutralizing antibody against IL-22. **Table 76** summarizes the final pharmacological characteristics of SYN3001.

Table 76. Final characterization of the pharmacological characteristics of SYN300

	Run 3 [IL-22] secreted (ng/mL)	**SD**	**EC50 (ng/mL)**	**SD**	**IC50 (ng/mL)**	**SD**
SYN3001	307.7	6.21	4.80	1.74	3.45	0.37
rIL-22	NA	NA	1.56	0.82	3.70	0.52

Example 46. Bacterial Secretion of GLP-1

[01162] The concentration of secreted GLP-1in the bacterial supernatant from four engineered strains comprising GLP-1 constructs/strains with different ribosome binding site (RBS) strength and two different secretion tags (PhoA or OmpF) were measured and compared.

[01163] All of the constructs were tested in a deltaLpp background. Strains are described in **Table 77** (and shown in **FIG. 81** and **FIG. 82**). 20K, 100K and 67K are numbers indicating the strengths of the RBS as determined by bioinformatics on an arbitrary scale, *e.g.*, strength of 20K<67K<100K.

[01164] Strains were grown overnight in LB medium. Cultures were diluted 1:200 in LB and grown shaking (200 rpm). Cultures were diluted to an optical density of 0.5 at which time strains were induced with ATC (100ng/mL). After 12 hours of induction, cells were spun down, and supernatant was collected. To generate cell free medium, the clarified supernatant was further filtered through a 0.22 micron filter to remove any remaining bacteria and placed on ice. Additionally, to detect intracellular recombinant protein production, pelleted were bacteria washed and resuspended in BugBuster™ (Millipore) with protease

inhibitors and Ready-Lyse Lysozyme Solution (Epicentre), resulting in lysate concentrated 10-fold compared to original culture conditions. After incubation at room temperature for 10 minutes insoluble debris was spun down at 20 min at 12,000 rcf at 4◦C then placed on ice until further processing.

[01165] The concentration of GLP-1in the cell-free medium and in the bacterial cell extract was measured by Abcam kit (ab184857) according to the manufacturers protocol. Standard curves were generated using recombinant GLP-1. Wild type Nissle was included in the ELISA as a negative control, and no signal was observed. Results are shown in **Table 77** and **FIG. 82C.**

Table 77. GLP-1 Secretion

Strain	**Genotype**	**ng/mL GLP1**
SYN2627	□lpp TetR-pTet-20K RBS -PhoA-Glp1	3.6
SYN2643	□lpp TetR-pTet-100K RBS -PhoA-Glp1	26.3
SYN2672	□lpp TetR-pTet-20K RBS -OmpF-Glp1	2
SYN2673	□lpp TetR-pTet-67K RBS -OmpF-Glp1	57.6

Table 78. Glp1 Secretion Sequences

Description	Sequence
pTet-20K RBS -PhoA-Glp1 **SEQ ID NO: 337**	TAATTCCTAATTTTTGTTGACACTCTATCATTGATAG AGTTATTTTACCACTCCCTATCAGTGATAGAGAAAA GTGAACCAAAACAGAGTCATATTTAAAGGAAGGTA CAAAATGAAGCAGAGCACCATCGCGCTTGCCCTGCT GCCGTTGCTTTTCACGCCTGTCACCAAGGCTCACGA TGAATTTGAGAGACATGCAGAAGGAACGTTCACAT CTGATGTGTCATCATATTTGGAAGGCCAAGCTGCCA AAGAATTCATCGCATGGTTGGTGAAAGGCCGAGGA TGA
pTet-100K RBS -PhoA-Glp1 **SEQ ID NO: 338**	TAATTCCTAATTTTTGTTGACACTCTATCATTGATAG AGTTATTTTACCACTCCCTATCAGTGATAGAGAAAA GTGAAATAAGTTTATCAAAATAAAAGGAGGTAATA TATGAAGCAGAGCACCATCGCGCTTGCCCTGCTGCC GTTGCTTTTCACGCCTGTCACCAAGGCTCACGATGA ATTTGAGAGACATGCAGAAGGAACGTTCACATCTG

	ATGTGTCATCATATTTGGAAGGCCAAGCTGCCAAAG AATTCATCGCATGGTTGGTGAAAGGCCGAGGATGA
pTet-20K RBS -OmpF-Glp1 **SEQ ID NO: 339**	TAATTCCTAATTTTTGTTGACACTCTATCATTGATAG AGTTATTTTACCACTCCCTATCAGTGATAGAGAAAA GTGAAGTCTTCCCGATCCTTTCCCGAGCGTACAAAA TGATGAAGCGTAACATCTTAGCCGTTATTGTCCCCG CATTGCTTGTGGCCGGGACGGCTAACGCACACGATG AATTTGAGAGACATGCAGAAGGAACGTTCACATCT GATGTGTCATCATATTTGGAAGGCCAAGCTGCCAAA GAATTCATCGCATGGTTGGTGAAAGGCCGAGGATG A
pTet-67K RBS -OmpF-Glp1 **SEQ ID NO: 340**	TAATTCCTAATTTTTGTTGACACTCTATCATTGATAG AGTTATTTTACCACTCCCTATCAGTGATAGAGAAAA GTGAAAAAACCGCCATCAAGAGTTAAGGAGGAGAA TATGATGAAGCGTAACATCTTAGCCGTTATTGTCCC CGCATTGCTTGTGGCCGGGACGGCTAACGCACACGA TGAATTTGAGAGACATGCAGAAGGAACGTTCACAT CTGATGTGTCATCATATTTGGAAGGCCAAGCTGCCA AAGAATTCATCGCATGGTTGGTGAAAGGCCGAGGA TGA
20K RBS **SEQ ID NO: 341**	CCAAAACAGAGTCATATTTAAAGGAAGGTACAAA
67K RBS **SEQ ID NO: 342**	AAAACCGCCATCAAGAGTTAAGGAGGAGAAT
100K RBS **SEQ ID NO: 343**	ATAAGTTTATCAAAATAAAAGGAGGTAATAT
PhoA **SEQ ID NO: 344**	ATGAAGCAGAGCACCATCGCGCTTGCCCTGCTGCCG TTGCTTTTCACGCCTGTCACCAAGGCT
OmpF **SEQ ID NO: 345**	ATGATGAAGCGTAACATCTTAGCCGTTATTGTCCCC GCATTGCTTGTGGCCGGGACGGCTAACGCA
Glp1 **SEQ ID NO: 346**	CACGATGAATTTGAGAGACATGCAGAAGGAACGTT CACATCTGATGTGTCATCATATTTGGAAGGCCAAGC TGCCAAAGAATTCATCGCATGGTTGGTGAAAGGCCG AGGATGA
PhoA-Glp1 **SEQ ID NO: 347**	ATGAAGCAGAGCACCATCGCGCTTGCCCTGCTGCCG TTGCTTTTCACGCCTGTCACCAAGGCTCACGATGAA TTTGAGAGACATGCAGAAGGAACGTTCACATCTGAT GTGTCATCATATTTGGAAGGCCAAGCTGCCAAAGAA TTCATCGCATGGTTGGTGAAAGGCCGAGGATGA
OmpF-Glp1 **SEQ ID NO: 348**	ATGATGAAGCGTAACATCTTAGCCGTTATTGTCCCC GCATTGCTTGTGGCCGGGACGGCTAACGCACACGAT GAATTTGAGAGACATGCAGAAGGAACGTTCACATC TGATGTGTCATCATATTTGGAAGGCCAAGCTGCCAA AGAATTCATCGCATGGTTGGTGAAAGGCCGAGGAT

	GA
Terminator **SEQ ID NO: 349**	AACGATTGGTAAACCCGGTGaacgcatgagAAAGCCCCC GGAAGATCACCTTCCGGGGGCTTTtttattgcgcGGACCA AAACGAAAAAAGACGCTCGAAAGCGTCTCTTTTCTG GAATTTGGTACCGAGG

[01166] In some embodiments, genetically engineered bacteria comprise a nucleic acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the DNA sequence of **SEQ ID NO: 337, SEQ ID NO: 338, SEQ ID NO: 339, SEQ ID NO: 340, SEQ ID NO: 341, SEQ ID NO: 342, SEQ ID NO: 343, SEQ ID NO: 344, SEQ ID NO: 345, SEQ ID NO: 346, SEQ ID NO: 347, SEQ ID NO: 348**, and **SEQ ID NO: 349.**

Example 47. GLP-1 production from genetically engineered bacteria and Activity Measurements

[01167] To determine whether GLP-1 can be expressed by the genetically engineered bacteria, a construct expressing GLP-1 in conjunction with a modified flagellar type III secretion system shown in **FIG. 63** was generated and integrated into the E coli Nissle chromosome. The construct comprises GLP-1 under control of the native FliC promoter and 5'UTR (untranslated region containing the N-terminal flagellar secretion signal) with an optimized ribosome binding site **FIG. 80** and **Table 79**).

Table 79. GLP-1 construct sequences

Description and SEQ ID NO	Sequence
GLP-1 under control of the native FliC promoter and 5'UTR with an optimized ribosome binding site (in reverse orientation) (**SEQ ID NO: 243**)	ttaaccacgacctttaaccagccaagcaataaactctttcgcagc ctggccctccaaatagctagaaacatcagaagtgaaagttccct ccgcgtggcgttcgaactcgtccatattacctcctgactgtgtcta cttcgttgattacgttttgggtttccacccgtcggctcaatcgccgt ca
GLP-1 (in reverse orientation) (**SEQ ID NO: 244**)	ttaaccacgacctttaaccagccaagcaataaactctttcgcagc ctggccctccaaatagctagaaacatcagaagtgaaagttccct ccgcgtggcgttcgaactcgtccat
FliC 5' UTR (in reverse orientation) (**SEQ ID NO: 245**)	attacctcctgactgtgtctacttcgttgattacgttttgggtttcca cccgtcggctcaatcgccgtca
Optimized RBS (in reverse orientation) (**SEQ ID NO: 246**)	attacctcctgactgtgtctacttc
Putative terminator	gggcagaaaaaaccccgccgttggcggggaagcacgttgc

(**SEQ ID NO: 247**)	
GLP-1 construct comprising terminator (lower case italic) GLP-1 (lower case bold) under control of the native FliC promoter and 5'UTR (upper case bold, with optimized RBS underlined) and a chloramphenicol resistance gene under the control of the cat promoter (upper case italic bold), frt homology (upper case underlined) (**SEQ ID NO: 248**)	*Gggcagaaaaaaccccgccgttggcggggaagcacgttgc* tggcaaattaccattcatgttgccggatgcggcgtaaacgcctta tccggcctacaaaaatgtgcaaattcaataaattgcaattcccctt gtaggcctgataagcgcagcgcatcaggcaatttggcgttgcc gtcagtctcagttaatcaggttacggcga**ttaaccacgacctttaaccagccaagcaataaactctttcgcagcctggccctccaaatagctagaaacatcagaagtgaaagttccctccgcgtggcgttcgaactcgtccat**ATTACCTCCTGACTGTG TCTACTTCGTTGATTACGTTTTGGGTTT CCACCCGTCGGCTCAATCGCCGTCAAC CCTGTTATCGTCTGTCGTAAAACAACC TTTAGAATTTTTTTCACAAACAGCCATT TTTTGTTAGTCGACGAAATACTCTTTTC TCTGCCCCTTATTCCCGCTATTAAAAAA AACAATTAAACGTAAACTTTGCGCAAT TCAGGCCGATAACCCCGGTATTCGTTT TACGTGTCGAAAGATAAACGAAGTTCC TATACTTTCTAGAGAATAGGAACTTCG GAATAGGAACTTCATTTCTCGTTCGCT GCCACCTAAGAATACTCTACGGTCACA TACAAATGGCGCGCCTTACGCCCCGCC CTGCCA***CTCATCGCAGTACTGTTGTATT CATTAAGCATCTGCCGACATGGAAGCCA TCACAAACGGCATGATGAACCTGAATCG CCAGCGGCATCAGCACCTTGTCGCCTTG CGTATAATATTTGCCCATGGTGAAAACG GGGGCGAAGAAGTTGTCCATATTGGCC ACGTTTAAATCAAAACTGGTGAAACTCA CCCAGGGATTGGCTGAGACGAAAAACA TATTCTCAATAAACCCTTTAGGGAAATA GGCCAGGTTTTCACCGTAACACGCCACA TCTTGCGAATATATGTGTAGAAACTGCC GGAAATCGTCGTGGTATTCACTCCAGAG CGATGAAAACGTTTCAGTTTGCTCATGG AAAACGGTGTAACAAGGGTGAACACTA TCCCATATCACCAGCTCACCGTCTTTCA TTGCCATACGTAATTCCGGATGAGCATT CATCAGGCGGGCAAGAATGTGAATAAA GGCCGGATAAAACTTGTGCTTATTTTTC TTTACGGTCTTTAAAAAGGCCGTAATAT CCAGCTGAACGGTCTGGTTATAGGTACA TTGAGCAACTGACTGAAATGCCTCAAAA TGTTCTTTACGATGCCATTGGGATATAT CAACGGTGGTATATCCAGTGATTTTTTT CTCCATTTTAGCTTCCTTAGCTCCTGAA AATCTCGACAACTCAAAAAATACGCCCG GTAGTGATCTTATTTCATTATGGTGAAA GTTGGAACCTCTTACGTGCCGATCA***ACG TCTCATTTTCGCCAAAAGTTGGCCCAG GGCTTCCCGGTATCAACAGGGACACCA GGATTTATTTATTCTGCGAAGTGATCTT CCGTCACAGGTAGGCGCGCCGAAGTTC CTATACTTTCTAGAGAATAGGAACTTC GGAATAGGAACT

[01168] Cultures (the genetically engineered bacteria comprising the GLP-1 construct or streptomycin resistant control Nissle) are grown overnight in F-12K medium (Mediatech, Manassas, VA) without glucose (containing selective antibiotics (chloramphenicol or streptomycin) and then diluted 1:200. The cells are grown with shaking at 250 rpm, and at indicated times (0, 3, 6, and 12h), the supernatant aliquots are collected for GLP-1 quantification.

[01169] Additionally, bacteria are pelleted, washed, and harvested, resuspended in 25 mL sonication buffer (50 mM Tris-HCl, 30 mM NaCl, pH 8.0) with protease inhibitors, and lysed by sonication on ice. Insoluble debris is spun down twice for 20 min at 12,000 rpm at 4◦C to detect any intracellular recombinant protein.

[01170] To generate cell free medium, the supernatant is centrifuged, and filtered through a 0.2-micron filter to remove any remaining bacteria. The cell-free culture medium (CFM) is diluted to OD600=1 with F-12K, and 10 ng/ml leupeptin, 200μM PMSF and 5 ng/mL aprotinin was added to the CFM to inhibit proteases prior storage at 4°C.

Western blotting

[01171] The cell-free culture medium (CFM) was diluted to the same OD600 with F-12K, and 10 ng/ml leupeptin, PMSF and 5 ng/mL aprotinin was added to inhibit proteases. Clarified supernatant (14 ml) is precipitated with 10% trichloroacetic acid (TCA, VWR) for 30 min on ice, and the pellet was washed twice in ice-cold ethanol/ether (1:1). The supernatant pellet is dried under vacuum, dissolved in 50 μl sample buffer (2% SDS, 50mM Tris, pH 6.8, 20%glycerol, 10% mercaptoethanol, bromophenol blue) and boiled for 5 min at 95 °C. The cell pellet is resuspended (From 14 ml culture) in room temperature BugBuster Master Mix by gentle vortexing, using 500 μl BugBuster Master Mix with protease inhibitors (10 ng/ml Leupeptin, 200μMPMSF and 5 ng/mL aprotinin). The cell suspension is incubated on a shaking platform (VWR, Bristol, CT) at a slow setting for 10–20 min at room temperature. 125 μl 5X sample buffer is added to each sample before and boiling for 10 min at 95 °C.

[01172] Protein concentration is determined by BCA protein assay, and isolated proteins are analyzed by Western blot. Proteins are transferred onto PVDF membranes are detected with an HRP-conjugated Glucagon Antibody (24HCLC), ABfinity™ Rabbit Oligoclonal, Thermo Fisher.

Co-culture with Caco-2 cells and ELISA for Insulin

[01173] To determine whether the GLP-1 expressed by the genetically engineered bacteria is functional, a co-culture experiment is conducted in which the bacterial supernatant containing GLP-1 is added to the growth medium of a mammalian intestinal cell line, Caco-2. Caco-2 cells are an intestinal cell line derived from a human colorectal carcinoma that spontaneously differentiates under standard culture conditions, and which lends itself to the *in vitro* study of human gut. The ability of the Caco-2 cells to produce insulin upon exposure to the bacterial cell free supernatant is measured.

[01174] Caco-2 epithelial cells (ATCC# CRL-2102, Manassas, VA) are maintained in Dulbecco's Modified Eagle Media (DMEM, Cellgro, Herndon,VA) plus 10% FBS (Cellgro) at 37°C in a humidified incubator supplemented with 5% CO2. For co-culture experiments, Caco-2 cells are grown in F-12K supplemented with 10% FBS at 37°C in a humidified incubator supplemented with 5% CO2. All co-culture experiments are performed in F-12K plus 10% FBS with Caco-2 cells in passages between 15 and 22.

[01175] Approximately 80% confluent monolayers of Caco-2 cells in 12-well plates are washed with fresh F-12K plus 10% FBS once and covered with 1 mL 50% CFM in F-12K with 10% FBS and incubated at 37°C with 5% CO_2. 200 nM. As a control, the same volume of recombinant GLP-1 (200 nM) in F-12K with 10% FBS is added as a positive control in separate wells. Following a 16 h incubation, an additional 1 mL of 50% CFM in F-12K with 10% FBS or GLP-1 is added to the cells, supplemented with 0.4% Glucose or 0.4% Glycerol before incubation for an additional 2 h. The media is removed from the cells, supplemented with Leupeptin (10ng/mL), 0.2 mM PMSF and aprotinin (10ng/mL), centrifuged (12,000 x rpm), and kept briefly at 4°C prior to ELISA analysis for insulin expression (see "Immuno-blot and ELISA" section).

[01176] In order to estimate the amount of insulin secreted from Caco-2 cells activated by Glp-1, cell free supernatants are assayed using standard ELISA procedures using the Insulin ELISA Kit, Human (KAQ125, Thermo Fisher), according to manufacturer's instructions.

Example 48. Construction of Vectors for Producing Propionate

[01177] To facilitate inducible production of propionate in *Escherichia coli* Nissle, a propionate gene cassette comprising the genes encoding the enzymes of the acrylate pathway, *i.e.*, *pct, lcdA, lcdB, lcdC, etfA, acrB,* and *acrC*, as well as transcriptional and translational elements, are synthesized (Gen9, Cambridge, MA) and cloned into vector

pBR322. The genes are codon-optimized for *E. coli* codon usage using Integrated DNA Technologies online codon optimization tool (https://www.idtdna.com/CodonOpt). A second clone is generated as described above using a propionate gene cassette comprising the genes encoding the enzymes of the pyruvate pathway, *i.e.*, $thrA^{fbr}$, *thrB, thrC,* $ilvA^{fbr}$, *aceE, aceF,* and *lpd*; NCBI; Tseng *et al.*, 2012). A third clone is generated as described above that comprises $thrA^{fbr}$, *thrB, thrC,* $ilvA^{fbr}$, *aceE, aceF, lpd,* and *E. coli tesB* . Each propionate gene cassette is expressed under the control of each of the following regulatory regions: a FNR-inducible regulatory region selected from the sequences listed in **Table 20** a tetracycline-inducible promoter, and an arabinose-inducible promoter. In certain constructs, the FNR-responsive promoter is further fused to a strong ribosome binding site sequence. For efficient translation of propionate genes, each synthetic gene in the operon was separated by a 15 base pair ribosome binding site derived from the T7 promoter/translational start site. Each gene cassette and regulatory region construct is expressed on a high-copy plasmid, a low-copy plasmid, or a chromosome.

[01178] The propionate construct is inserted into the bacterial genome at one or more of the following insertion sites in *E. coli* Nissle: *malE/K, araC/BAD, lacZ, thyA, malP/T.* Any suitable insertion site may be used (*see, e.g.,* **FIG. 42**). The insertion site may be anywhere in the genome, *e.g.*, in a gene required for survival and/or growth, such as *thyA* (to create an auxotroph); in an active area of the genome, such as near the site of genome replication; and/or in between divergent promoters in order to reduce the risk of unintended transcription, such as between AraB and AraC of the arabinose operon. At the site of insertion, DNA primers that are homologous to the site of insertion and to the propionate construct are designed. A linear DNA fragment containing the construct with homology to the target site is generated by PCR, and lambda red recombination is performed as described below. The resulting *E. coli* Nissle bacteria are genetically engineered to express a propionate biosynthesis cassette and produce propionate.

Example 49. Lambda red recombination

[01179] Lambda red recombination is used to make chromosomal modifications, *e.g.*, to express a propionate biosynthesis cassette in *E. coli* Nissle. Lambda red is a procedure using recombination enzymes from a bacteriophage lambda to insert a piece of custom DNA into the chromosome of *E. coli.* A pKD46 plasmid is transformed into the *E. coli* Nissle host strain. *E. coli* Nissle cells are grown overnight in LB media. The overnight culture is diluted 1:100 in 5 mL of LB media and grown until it reaches an OD_{600}

of 0.4-0.6. All tubes, solutions, and cuvettes are pre-chilled to 4° C. The *E. coli* cells are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 1 mL of 4° C water. The *E. coli* are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.5 mL of 4° C water. The *E. coli* are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.1 mL of 4° C water. The electroporator is set to 2.5 kV. 1 ng of pKD46 plasmid DNA is added to the *E. coli* cells, mixed by pipetting, and pipetted into a sterile, chilled cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. 1 mL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 30° C for 1 hr. The cells are spread out on a selective media plate and incubated overnight at 30° C.

[01180] DNA sequences comprising the desired propionate biosynthesis genes shown above were ordered from a gene synthesis company. The lambda enzymes are used to insert this construct into the genome of *E. coli* Nissle through homologous recombination. The construct is inserted into a specific site in the genome of *E. coli* Nissle based on its DNA sequence. In some embodiments, the construct is in the *E. coli* Nissle genome at the *malP/T* site (**FIG. 57**). To insert the construct into a specific site, the homologous DNA sequence flanking the construct is identified, and includes approximately 50 bases on either side of the sequence. The homologous sequences are ordered as part of the synthesized gene. Alternatively, the homologous sequences may be added by PCR. The construct includes an antibiotic resistance marker that may be removed by recombination. The resulting construct comprises approximately 50 bases of homology upstream, a kanamycin resistance marker that can be removed by recombination, the propionate biosynthesis genes, and approximately 50 bases of homology downstream.

Example 50. Further Transforming E. coli

[01181] Each of the constructs above is transformed into *E. coli* Nissle comprising pKD46. All tubes, solutions, and cuvettes are pre-chilled to 4° C. An overnight culture is diluted 1:100 in 5 mL of LB media containing ampicillin and grown until it reaches an OD_{600} of 0.1. 0.05 mL of 100X L-arabinose stock solution is added to induce pKD46 lambda red expression. The culture is grown until it reaches an OD_{600} of 0.4-0.6. The *E. coli* cells are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 1 mL of 4° C water. The *E. coli* are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.5 mL of 4° C water. The

E. coli are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.1 mL of 4° C water. The electroporator is set to 2.5 kV. 0.5 µg of the construct is added to the cells, mixed by pipetting, and pipetted into a sterile, chilled cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. 1 mL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 37° C for 1 hr. The cells are spread out on an LB plate containing kanamycin and incubated overnight.

[01182] In alternate embodiments, the propionate cassette may be inserted into the Nissle genome through homologous recombination (Genewiz, Cambridge, MA). Organization of the constructs and nucleotide sequences are shown in Figs. 1-5. To create a vector capable of integrating the synthesized propionate cassette construct into the chromosome, Gibson assembly was first used to add 1000bp sequences of DNA homologous to the Nissle lacZ locus into the R6K origin plasmid pKD3. This targets DNA cloned between these homology arms to be integrated into the lacZ locus in the Nissle genome. Gibson assembly was used to clone the fragment between these arms. PCR was used to amplify the region from this plasmid containing the entire sequence of the homology arms, as well as the propionate cassette between them. This PCR fragment was used to transform electrocompetent Nissle-pKD46, a strain that contains a temperature-sensitive plasmid encoding the lambda red recombinase genes. After transformation, cells were grown out for 2 hours before plating on chloramphenicol at 20ug/mL at 37 degrees C. Growth at 37 degrees C also cures the pKD46 plasmid. Transformants containing cassette were chloramphenicol resistant and lac-minus
(lac-).

Example 51. Verifying mutants

[01183] The presence of the propionate gene cassette is verified by colony PCR. Colonies are picked with a pipette tip and resuspended in 20 µl of cold ddH_2O by pipetting up and down. 3 µl of the suspension is pipetted onto an index plate with appropriate antibiotic for use later. The index plate is grown at 37° C overnight. A PCR master mix is made using 5 µl of 10X PCR buffer, 0.6 µl of 10 mM dNTPs, 0.4 µl of 50 mM Mg_2SO_4, 6.0 µl of 10X enhancer, and 3.0 µl of ddH_2O (15 µl of master mix per PCR reaction). A 10 µM primer mix is made by mixing 2 µL of primers unique to the propionate construct (100 µM stock) into 16 µL of ddH_2O. For each 20 µl reaction, 15µL of the PCR master mix, 2.0 µL of the colony suspension (template), 2.0 µL of the primer mix, and 1.0 µL of Pfx Platinum DNA

Pol are mixed in a PCR tube. The PCR thermocycler is programmed as follows, with steps 2-4 repeating 34 times: 1) 94° C at 5:00 min., 2) 94° C at 0:15 min., 3) 55° C at 0:30 min., 4) 68° C at 2:00 min., 5) 68° C at 7:00 min., and then cooled to 4° C. The PCR products are analyzed by gel electrophoresis using 10 µL of each amplicon and 2.5 µL 5X dye. The PCR product only forms if the heterologous sequence has been inserted.

Example 52. Generation of ΔThyA

[01184] An auxotrophic mutation causes bacteria to die in the absence of an exogenously added nutrient essential for survival or growth because they lack the gene(s) necessary to produce that essential nutrient. In order to generate genetically engineered bacteria with an auxotrophic modification, the thyA, a gene essential for oligonucleotide synthesis was deleted. Deletion of the thyA gene in E. coli Nissle yields a strain that cannot form a colony on LB plates unless they are supplemented with thymidine.

[01185] A *thyA::cam* PCR fragment was amplified using 3 rounds of PCR as follows. Sequences of the primers used at a 100um concentration are found in **Table 80.**

Table 80. Primer Sequences

Name	Sequence	Description	SEQ ID NO
SR36	tagaactgatgcaaaaagtgctcgacgaaggcacacagaTGTGTAGGCTGGAG CTGCTTC	Round 1: binds on pKD3	**SEQ ID NO: 215**
SR38	gtttcgtaattagatagccaccggcgctttaatgcccggaCATATGAATATCCTCC TTAG	Round 1: binds on pKD3	**SEQ ID NO: 216**
SR33	caacacgtttcctgaggaaccatgaaacagtatttagaactgatgcaaaaag	Round 2: binds to round 1 PCR product	**SEQ ID NO: 217**
SR34	cgcacactggcgtcggctctggcaggatgtttcgtaattagatagc	Round 2: binds to round 1 PCR product	**SEQ ID NO: 218**
SR43	atatcgtcgcagcccacagcaacacgtttcctgagg	Round 3: binds to round 2 PCR product	**SEQ ID NO: 219**
SR44	aagaatttaacggagggcaaaaaaaaccgacgcacactggcgtcggc	Round 3: binds to round 2 PCR product	**SEQ ID NO: 220**

[01186] For the first PCR round, 4x50ul PCR reactions containing 1ng pKD3 as template, 25ul 2xphusion, 0.2ul primer SR36 and SR38, and either 0, 0.2, 0.4 or 0.6ul DMSO were brought up to 50 ul volume with nuclease free water and amplified under the following cycle conditions:

step1: 98c for 30s

step2: 98c for 10s

step3: 55c for 15s

step4: 72c for 20s

repeat step 2-4 for 30 cycles

step5: 72c for 5min

[01187] Subsequently, 5ul of each PCR reaction was run on an agarose gel to confirm PCR product of the appropriate size. The PCR product was purified from the remaining PCR reaction using a Zymoclean gel DNA recovery kit according to the manufacturer's instructions and eluted in 30ul nuclease free water.

[01188] For the second round of PCR, 1ul purified PCR product from round 1 was used as template, in 4x50ul PCR reactions as described above except with 0.2ul of primers SR33 and SR34. Cycle conditions were the same as noted above for the first PCR reaction. The PCR product run on an agarose gel to verify amplification, purified, and eluted in 30ul as described above.

[01189] For the third round of PCR, 1ul of purified PCR product from round 2 was used as template in 4x50ul PCR reactions as described except with primer SR43 and SR44. Cycle conditions were the same as described for rounds 1 and 2. Amplification was verified, the PCR product purified, and eluted as described above. The concentration and purity was measured using a spectrophotometer. The resulting linear DNA fragment, which contains 92 bp homologous to upstream of *thyA*, the chloramphenicol cassette flanked by frt sites, and 98 bp homologous to downstream of the *thyA* gene, was transformed into a *E. coli* Nissle 1917 strain containing pKD46 grown for recombineering. Following electroporation, 1ml SOC medium containing 3mM thymidine was added, and cells were allowed to recover at 37 C for 2h with shaking. Cells were then pelleted at 10,000xg for 1 minute, the supernatant was discarded, and the cell pellet was resuspended in 100ul LB containing 3mM thymidine and spread on LB agar plates containing 3mM thy and 20ug/ml chloramphenicol. Cells were incubated at 37 C overnight. Colonies that appeared on LB plates were restreaked. + cam 20ug/ml + or - thy 3mM. (*thyA* auxotrophs will only grow in media supplemented with thy 3mM).

[01190] Next, the antibiotic resistance was removed with pCP20 transformation. pCP20 has the yeast Flp recombinase gene, FLP, chloramphenicol and ampicillin resistant genes, and temperature sensitive replication. Bacteria were grown in LB media containing the selecting antibiotic at 37°C until OD600 = 0.4 - 0.6. 1mL of cells were washed as follows: cells were pelleted at 16,000xg for 1 minute. The supernatant was discarded and the pellet was resuspended in 1mL ice-cold 10% glycerol. This wash step was repeated 3x times. The final pellet was resuspended in 70ul ice-cold 10% glycerol. Next, cells

were electroporated with 1ng pCP20 plasmid DNA, and 1mL SOC supplemented with 3mM thymidine was immediately added to the cuvette. Cells were resuspended and transferred to a culture tube and grown at 30°C for 1hours. Cells were then pelleted at 10,000xg for 1 minute, the supernatant was discarded, and the cell pellet was resuspended in 100ul LB containing 3mM thymidine and spread on LB agar plates containing 3mM thy and 100ug/ml carbenicillin and grown at 30°C for 16-24 hours. Next, transformants were colony purified non-selectively (no antibiotics) at 42°C.

[01191] To test the colony-purified transformants, a colony was picked from the 42°C plate with a pipette tip and resuspended in 10μL LB. 3μL of the cell suspension was pipetted onto a set of 3 plates: Cam, (37°C; tests for the presence/absence of CamR gene in the genome of the host strain), Amp, (30°C, tests for the presence/absence of AmpR from the pCP20 plasmid) and LB only (desired cells that have lost the chloramphenicol cassette and the pCP20 plasmid), 37°C. Colonies were considered cured if there is no growth in neither the Cam or Amp plate, picked, and re-streaked on an LB plate to get single colonies, and grown overnight at 37°C.

Example 53. Production of Propionate in genetically engineered E. coli

[01192] Production of propionate is assessed in E. coli Nissle strains containing the propionate cassettes described above. All incubations are performed at 37° C. Cultures of E. coli strains DH5a and Nissle transformed with the propionate cassettes are grown overnight in LB and then diluted 1:200 into 4 mL of M9 minimal medium containing 0.5% glucose. The cells are grown with shaking (250 rpm) for 4-6 h, and the inducible constructs are induced as follows: (1) bacteria comprising a propionate gene cassette driven by a FNR-inducible promoter are induced in LB at 37C for up to 4 hours in anaerobic conditions in a Coy anaerobic chamber (supplying 90% N2, 5% CO2, 5%H2, and 20mM nitrate) at 37° C; (2) bacteria comprising a propionate gene cassette driven by a tetracycline-inducible promoter are induced with anhydrotetracycline (100ng/mL); (3) bacteria comprising a propionate gene cassette driven by a arabinose-inducible promoter are induced with 1% arabinose in media lacking glucose. One mL culture aliquots are prepared in 1.5 mL capped tubes and FNR-inducible constructs are incubated in a stationary incubator to limit culture aeration. One tube is removed at each time point (0, 1, 2, 4, and 20 hours) and analyzed for propionate concentration by LC-MS to confirm that propionate production in these recombinant strains can be achieved in a low-oxygen environment.

Example 54. Nissle residence

[01193] Unmodified *E. coli* Nissle and the genetically engineered bacteria of the invention may be destroyed, e.g., by defense factors in the gut or blood serum. The residence time of bacteria *in vivo* may be calculated. A non-limiting example using a streptomycin-resistant strain of *E. coli* Nissle is described below. In alternate embodiments, residence time is calculated for the genetically engineered bacteria of the invention.

[01194] C57BL/6 mice were acclimated in the animal facility for 1 week. After one week of acclimation (i.e., day 0), streptomycin-resistant Nissle (SYN-103) was administered to the mice via oral gavage on days 1-3. Mice were not pre-treated with antibiotic. The amount of bacteria administered, i.e., the inoculant, is shown in **Table 81**. In order to determine the CFU of the inoculant, the inoculant was serially diluted, and plated onto LB plates containing streptomycin (300 μg/mL). The plates were incubated at 37°C overnight, and colonies were counted.

Table 81. CFU administered via oral gavage

CFU administered via oral gavage			
Strain	Day 1	Day 2	Day 3
SYN-103	1.30E+08	8.50E+08	1.90E+09

[01195] On days 2-10, fecal pellets were collected from up to 6 mice (ID NOs. 1-6; **Table 11**). The pellets were weighed in tubes containing PBS and homogenized. In order to determine the CFU of Nissle in the fecal pellet, the homogenized fecal pellet was serially diluted, and plated onto LB plates containing streptomycin (300 μg/mL). The plates were incubated at 37°C overnight, and colonies were counted.

[01196] Fecal pellets from day 1 were also collected and plated on LB plates containing streptomycin (300 μg/mL) to determine if there were any strains native to the mouse gastrointestinal tract that were streptomycin resistant. The time course and amount of administered Nissle still residing within the mouse gastrointestinal tract is shown in **Table 82**.

[01197] **FIG. 70** depicts a graph of Nissle residence *in vivo*. Streptomycin-resistant Nissle was administered to mice via oral gavage without antibiotic pre-treatment. Fecal pellets from six total mice were monitored post-administration to determine the amount of administered Nissle still residing within the mouse gastrointestinal tract. The bars

represent the number of bacteria administered to the mice. The line represents the number of Nissle recovered from the fecal samples each day for 10 consecutive days.

Table 82. Nissle residence *in vivo*

ID	Day 2	Day 3	Day 4	Day 5
1	2.40E+05	6.50E+03	6.00E+04	2.00E+03
2	1.00E+05	1.00E+04	3.30E+04	3.00E+03
3	6.00E+04	1.70E+04	6.30E+04	2.00E+02
4	3.00E+04	1.50E+04	1.10E+05	3.00E+02
5		1.00E+04	3.00E+05	1.50E+04
6		1.00E+06	4.00E+05	2.30E+04
Avg	1.08E+05	1.76E+05	1.61E+05	7.25E+03

ID	Day 6	Day 7	Day 8	Day 9	Day 10
1	9.10E+03	1.70E+03	4.30E+03	6.40E+03	2.77E+03
2	6.00E+03	7.00E+02	6.00E+02	0.00E+00	0.00E+00
3	1.00E+02	2.00E+02	0.00E+00	0.00E+00	0.00E+00
4	1.50E+03	1.00E+02		0.00E+00	0.00E+00
5	3.10E+04	3.60E+03		0.00E+00	0.00E+00
6	1.50E+03	1.40E+03	4.20E+03	1.00E+02	0.00E+00
Avg	8.20E+03	1.28E+03	2.28E+03	1.08E+03	4.62E+02

Example 55. Assessment of in vitro and in vivo activity of Biosafety System Containing Strain

[01198] The activity of the following strains is tested:

[01199] SYN-1001 comprises a construct shown in **FIG. 69C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69A**, except that the bla gene is replaced with the construct of **FIG. 81C** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01200] SYN-1002 comprises a construct shown in **FIG. 69C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69A**, except that the bla gene is replaced with the construct of **FIG. 81D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01201] SYN-1003 comprises a construct shown in **FIG. 69D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69A**, except that the bla gene is replaced with the construct of **FIG. 81C** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01202] SYN-1004 comprises a construct shown in **FIG. 69D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown **in FIG. 69A**, except that the bla gene is replaced with the construct of **FIG. 81D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01203] SYN-1005 comprises a construct shown in **FIG. 69C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69B**, except that the bla gene is replaced with the construct of **FIG. 81C** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01204] SYN-1006 comprises a construct shown in **FIG. 69C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69B**, except that the bla gene is replaced with the construct of **FIG. 81D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01205] SYN-1007 comprises a construct shown in **FIG. 69D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69B**, except that the bla gene is replaced with the

construct of **FIG. 81D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01206] SYN-1008 a construct shown in **FIG. 69D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69B**, except that the bla gene is replaced with the construct of **FIG. 81D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01207] SYN-1009 a construct shown in **FIG. 69C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69A**, except that the bla gene is replaced with the construct of **FIG. 5A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

[01208] SYN-1011 comprises a construct shown in **FIG. 69D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69A**, except that the bla gene is replaced with the construct of **FIG. 5A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

[01209] SYN-1013 comprises a construct shown in **FIG. 69C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69B**, except that the bla gene is replaced with the construct of **FIG. 5A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

[01210] SYN-1014 comprises a construct shown in **FIG. 69D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **FIG. 69B**, except that the bla gene is replaced with the construct of **FIG. 5A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

Table 83. Biosafety System Constructs and Sequence Components

Description	Sequence	SEQ ID NO
Biosafety Plasmid System Component – dap A Biosafety Plasmid System Vector sequences, comprising dapA, Kid Toxin and R6K minimal ori, and promoter elements driving expression of these components, as shown in **FIG. 74A**	ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATT GTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAA ACAAATAGGGGAATTAAAAAAAAGCCCGCTCATTAGGCGGGC TACTACCTAGGCCGCGGCCGCGCGAATTCGAGCTCGGTACCCG GGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGGCC GCGTCGTGACTGGGAAAACCCTGGCGACTAGTCTTGGACTCCT GTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATTTG TTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATTGGTGAGA ATCCAGGGGTCCCCAATAATTACGATTTAAATCACAGCAAACA CCACGTCGGCCCTATCAGCTGCGTGCTTTCTATGAGTCGTTGCT GCATAACTTGACAATTAACATCCGGCTCGTAGGGTTTGTGGAG GGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAAT TTTCGTACTGAAACATCTTAATCATGCTGGGGAGGGTTTCTAA TGTTCACGGGAAGTATTGTCGCGATTGTTACTCCGATGGATGA AAAAGGTAATGTCTGTCGGGCTAGCTTGAAAAAACTGATTGAT TATCATGTCGCCAGCGGTACTTCGGCGATCGTTTCTGTTGGCA CCACTGGCGAGTCCGCTACCTTAAATCATGACGAACATGCTGA TGTGGTGATGATGACGCTGGATCTGGCTGATGGGCGCATTCCG GTAATTGCCGGGACCGGCGCTAACGCTACTGCGGAAGCCATTA GCCTGACGCAGCGCTTCAATGACAGTGGTATCGTCGGCTGCCT GACGGTAACCCCTTACTACAATCGTCCGTCGCAAGAAGGTTTG TATCAGCATTTCAAAGCCATCGCTGAGCATACTGACCTGCCGC AAATTCTGTATAATGTGCCGTCCCGTACTGGCTGCGATCTGCT CCCGGAAACGGTGGGCCGTCTGGCGAAAGTAAAAAATATTAT CGGAATCAAAGAGGCAACAGGGAACTTAACGCGTGTAAACCA GATCAAAGAGCTGGTTTCAGATGATTTTGTTCTGCTGAGCGGC GATGATGCGAGCGCGCTGGACTTCATGCAATTGGGCGGTCATG GGGTTATTTCCGTTACGGCTAACGTCGCAGCGCGTGATATGGC CCAGATGTGCAAACTGGCAGCAGAAGGGCATTTTGCCGAGGC ACGCGTTATTAATCAGCGTCTGATGCCATTACACAACAAACTA TTTGTCGAACCCAATCCAATCCCGGTGAAATGGGCATGTAAGG AACTGGGTCTTGTGGCGACCGATACGCTGCGCCTGCCAATGAC ACCAATCACCGACAGTGGCCGTGAGACGGTCAGAGCGGCGCT TAAACATGCCGGTTTGCTGTAAGACTTTTGTCAGGTTCCTACTG TGACGACTACCACCGATAGACTGGAGTGTTGCTGCGAAAAAA CCCCGCCGAAGCGGGGTTTTTTGCGAGAAGTCACCACGATTGT GCTTTACACGGAGTAGTCGGCAGTTCCTTAAGTCAGAATAGTG GACAGGCGGCCAAGAACTTCGTTCATGATAGTCTCCGGAACCC GTTCGAGTCGTTTTCCGCCCCGTGCTTTCATATCAATTGTCCGG GGTTGATCGCAACGTACAACACCTGTGGTACGTATGCCAACAC CATCCAACGACACCGCAAAGCCGGCAGTGCGGGCAAAATTGC CTCCGCTGGTTACGGGCACAACAACAGGCAGGCGGGTCACGC GATTAAAGGCCGCCGGTGTGACAATCAGCACCGGCCGCGTTCC CTGCTGCTCATGACCTGCGGTAGGATCAAGCGAGACAAGCCA GATTTCCCCTCTTTCCATCTAGTATAACTATTGTTTCTCTAGTA ACATTTATTGTACAACACGAGCCCATTTTTGTCAAATAAATTTT AAATTATATCAACGTTAATAAGACGTTGTCAATAAAATTATTT TGACAAAATTGGCCGGCCGGCGCGCCGATCTGAAGATCAGCA GTTCAACCTGTTGATAGTACGTACTAAGCTCTCATGTTTCACGT ACTAAGCTCTCATGTTTAACGTACTAAGCTCTCATGTTTAACG AACTAAACCCTCATGGCTAACGTACTAAGCTCTCATGGCTAAC GTACTAAGCTCTCATGTTTCACGTACTAAGCTCTCATGTTTGAA CAATAAAATTAATATAAATCAGCAACTTAAATAGCCTCTAAGG TTTTAAGTTTTATAAGAAAAAAAAGAATATATAAGGCTTTTAA AGCCTTTAAGGTTTAACGGTTGTGGACAACAAGCCAGGGATGT AACGCACTGAGAAGCCCTTAGAGCCTCTCAAAGCAATTTTGAG TGACACAGGAACACTTAACGGCTGACATGGGGCGCGCCCAGC TGTCTAGGGCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACC	352

	GACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGA GCCTTTCGTTTTATTTGATGCCT	
Biosafety Plasmid System Component – ThyA Biosafety Plasmid System Vector sequences, comprising ThyA, Kid Toxin and R6K minimal ori, and promoter elements driving expression of these components, as shown in **FIG. 74B**	ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATT GTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAA ACAAATAGGGGAATTAAAAAAAAGCCCGCTCATTAGGCGGGC TACTACCTAGGCCGCGGCCGCGCGAATTCGAGCTCGGTACCCG GGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGGCC GCGTCGTGACTGGGAAAACCCTGGCGACTAGTCTTGGACTCCT GTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATTTG TTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATTGGTGAGA ATCCAGGGGTCCCCAATAATTACGATTTAAATCACAGCAAACA CCACGTCGGCCCTATCAGCTGCGTGCTTTCTATGAGTCGTTGCT GCATAACTTGACAATTAATCATCCGGCTCGTAGGGTTTGTGGA GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAA TTTTCGTACTGAAACATCTTAATCATGCTGGGGAGGGTTTCTA ATGAAACAGTATTTAGAACTGATGCAAAAAGTGCTCGACGAA GGCACACAGAAAAACGACCGTACCGGAACCGGAACGCTTTCC ATTTTTGGTCATCAGATGCGTTTTAACCTGCAAGATGGATTCCC GCTGGTGACAACTAAACGTTGCCACCTGCGTTCCATCATCCAT GAACTGCTGTGGTTTCTTCAGGGCGACACTAACATTGCTTATC TACACGAAAACAATGTCACCATCTGGGACGAATGGGCCGATG AAAACGGCGACCTCGGGCCAGTGTATGGTAAACAGTGGCGTG CCTGGCCAACGCCAGATGGTCGTCATATTGACCAGATCACTAC GGTACTGAACCAGCTGAAAAACGACCCGGATTCGCGCCGCAT TATTGTTTCAGCGTGGAACGTAGGCGAACTGGATAAAATGGCG CTGGCACCGTGCCATGCATTCTTCCAGTTCTATGTGGCAGACG GCAAACTCTCTTGCCAGCTTTATCAGCGCTCCTGTGACGTCTTC CTCGGCCTGCCGTTCAACATTGCCAGCTACGCGTTATTGGTGC ATATGATGGCGCAGCAGTGCGATCTGGAAGTGGGTGATTTTGT CTGGACCGGTGGCGACACGCATCTGTACAGCAACCATATGGAT CAAACTCATCTGCAATTAAGCCGCGAACCGCGTCCGCTGCCGA AGTTGATTATCAAACGTAAACCCGAATCCATCTTCGACTACCG TTTCGAAGACTTTGAGATTGAAGGCTACGATCCGCATCCGGGC ATTAAAGCGCCGGTGGCTATCTAAGACTTTTGTCAGGTTCCTA CTGTGACGACTACCACCGATAGACTGGAGTGTTGCTGCGAAAA AACCCCGCCGAAGCGGGGTTTTTTGCGAGAAGTCACCACGATT GTGCTTTACACGGAGTAGTCGGCAGTTCCTTAAGTCAGAATAG TGGACAGGCGGCCAAGAACTTCGTTCATGATAGTCTCCGGAAC CCGTTCGAGTCGTTTTCCGCCCCGTGCTTTCATATCAATTGTCC GGGGTTGATCGCAACGTACAACACCTGTGGTACGTATGCCAAC ACCATCCAACGACACCGCAAAGCCGGCAGTGCGGGCAAAATT GCCTCCGCTGGTTACGGGCACAACAACAGGCAGGCGGGTCAC GCGATTAAAGGCCGCCGGTGTGACAATCAGCACCGGCCGCGT TCCCTGCTGCTCATGACCTGCGGTAGGATCAAGCGAGACAAGC CAGATTTCCCCTCTTTCCATCTAGTATAACTATTGTTTCTCTAG TAACATTTATTGTACAACACGAGCCCATTTTTGTCAAATAAAT TTTAAATTATATCAACGTTAATAAGACGTTGTCAATAAAATTA TTTTGACAAAATTGGCCGGCCGGCGCGCCGATCTGAAGATCAG CAGTTCAACCTGTTGATAGTACGTACTAAGCTCTCATGTTTCAC GTACTAAGCTCTCATGTTTAACGTACTAAGCTCTCATGTTTAAC GAACTAAACCCTCATGGCTAACGTACTAAGCTCTCATGGCTAA CGTACTAAGCTCTCATGTTTCACGTACTAAGCTCTCATGTTTGA ACAATAAAATTAATATAAATCAGCAACTTAAATAGCCTCTAAG GTTTTAAGTTTTATAAGAAAAAAAAGAATATATAAGGCTTTTA AAGCCTTTAAGGTTTAACGGTTGTGGACAACAAGCCAGGGAT GTAACGCACTGAGAAGCCCTTAGAGCCTCTCAAAGCAATTTTG AGTGACACAGGAACACTTAACGGCTGACATGGGGCGCGCCCA GCTGTCTAGGGCGGCGGATTTGTCCTACTCAGGAGAGCGTTCA CCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACT GAGCCTTTCGTTTTATTTGATGCCT	353

Kid toxin (reverse orientation)	TTAAGTCAGAATAGTGGACAGGCGGCCAAGAACTTCGTTCATG ATAGTCTCCGGAACCCGTTCGAGTCGTTTTCCGCCCCGTGCTTT CATATCAATTGTCCGGGGTTGATCGCAACGTACAACACCTGTG GTACGTATGCCAACACCATCCAACGACACCGCAAAGCCGGCA GTGCGGGCAAAATTGCCTCCGCTGGTTACGGGCACAACAACA GGCAGGCGGGTCACGCGATTAAAGGCCGCCGGTGTGACAATC AGCACCGGCCGCGTTCCCTGCTGCTCATGACCTGCGGTAGGAT CAAGCGAGACAAGCCAGATTTCCCCTCTTTCCAT	354
dapA	ATGTTCACGGGAAGTATTGTCGCGATTGTTACTCCGATGGATG AAAAAGGTAATGTCTGTCGGGCTAGCTTGAAAAAACTGATTG ATTATCATGTCGCCAGCGGTACTTCGGCGATCGTTTCTGTTGGC ACCACTGGCGAGTCCGCTACCTTAAATCATGACGAACATGCTG ATGTGGTGATGATGACGCTGGATCTGGCTGATGGGCGCATTCC GGTAATTGCCGGGACCGGCGCTAACGCTACTGCGGAAGCCATT AGCCTGACGCAGCGCTTCAATGACAGTGGTATCGTCGGCTGCC TGACGGTAACCCCTTACTACAATCGTCCGTCGCAAGAAGGTTT GTATCAGCATTTCAAAGCCATCGCTGAGCATACTGACCTGCCG CAAATTCTGTATAATGTGCCGTCCCGTACTGGCTGCGATCTGC TCCCGGAAACGGTGGGCCGTCTGGCGAAAGTAAAAAATATTA TCGGAATCAAAGAGGCAACAGGGAACTTAACGCGTGTAAACC AGATCAAAGAGCTGGTTTCAGATGATTTTGTTCTGCTGAGCGG CGATGATGCGAGCGCGCTGGACTTCATGCAATTGGGCGGTCAT GGGGTTATTTCCGTTACGGCTAACGTCGCAGCGCGTGATATGG CCCAGATGTGCAAACTGGCAGCAGAAGGGCATTTTGCCGAGG CACGCGTTATTAATCAGCGTCTGATGCCATTACACAACAAACT ATTTGTCGAACCCAATCCAATCCCGGTGAAATGGGCATGTAAG GAACTGGGTCTTGTGGCGACCGATACGCTGCGCCTGCCAATGA CACCAATCACCGACAGTGGCCGTGAGACGGTCAGAGCGGCGC TTAAACATGCCGGTTTGCTGTAA	355
thyA	ATGAAACAGTATTTAGAACTGATGCAAAAAGTGCTCGACGAA GGCACACAGAAAAACGACCGTACCGGAACCGGAACGCTTTCC ATTTTTGGTCATCAGATGCGTTTTAACCTGCAAGATGGATTCCC GCTGGTGACAACTAAACGTTGCCACCTGCGTTCCATCATCCAT GAACTGCTGTGGTTTCTTCAGGGCGACACTAACATTGCTTATC TACACGAAAACAATGTCACCATCTGGGACGAATGGGCCGATG AAAACGGCGACCTCGGGCCAGTGTATGGTAAACAGTGGCGTG CCTGGCCAACGCCAGATGGTCGTCATATTGACCAGATCACTAC GGTACTGAACCAGCTGAAAAACGACCCGGATTCGCGCCGCAT TATTGTTTCAGCGTGGAACGTAGGCGAACTGGATAAAATGGCG CTGGCACCGTGCCATGCATTCTTCCAGTTCTATGTGGCAGACG GCAAACTCTCTTGCCAGCTTTATCAGCGCTCCTGTGACGTCTTC CTCGGCCTGCCGTTCAACATTGCCAGCTACGCGTTATTGGTGC ATATGATGGCGCAGCAGTGCGATCTGGAAGTGGGTGATTTTGT CTGGACCGGTGGCGACACGCATCTGTACAGCAACCATATGGAT CAAACTCATCTGCAATTAAGCCGCGAACCGCGTCCGCTGCCGA AGTTGATTATCAAACGTAAACCCGAATCCATCTTCGACTACCG TTTCGAAGACTTTGAGATTGAAGGCTACGATCCGCATCCGGGC ATTAAAGCGCCGGTGGCTATCTAA	356
Kid toxin polypeptide	MERGEIWLVSLDPTAGHEQQGTRPVLIVTPAAFNRVTRLPVVVPV TSGGNFARTAGFAVSLDGVGIRTTGVVRCDQPRTIDMKARGGKR LERVPETIMNEVLGRLSTILT*	357
dapA polypeptide	MFTGSIVAIVTPMDEKGNVCRASLKKLIDYHVASGTSAIVSVGTT GESATLNHDEHADVVMMTLDLADGRIPVIAGTGANATAEAISLT QRFNDSGIVGCLTVTPYYNRPSQEGLYQHFKAIAEHTDLPQILYN VPSRTGCDLLPETVGRLAKVKNIIGIKEATGNLTRVNQIKELVSDD FVLLSGDDASALDFMQLGGHGVISVTANVAARDMAQMCKLAAE GHFAEARVINQRLMPLHNKLFVEPNPIPVKWACKELGLVATDTL RLPMTPITDSGRETVRAALKHAGLL	358
ThyA polypeptide	MKQYLELMQKVLDEGTQKNDRTGTGTLSIFGHQMRFNLQDGFP LVTTKRCHLRSIIHELLWFLQGDTNIAYLHENNVTIWDEWADEN	359

	GDLGPVYGKQWRAWPTPDGRHIDQITTVLNQLKNDPDSRRIIVSA WNVGELDKMALAPCHAFFQFYVADGKLSCQLYQRSCDVFLGLP FNIASYALLVHMMAQQCDLEVGDFVWTGGDTHLYSNHMDQTH LQLSREPRPLPKLIIKRKPESIFDYRFEDFEIEGYDPHPGIKAPVAI*	

Table 84. Chromosomally Inserted Biosafety System Constructs

Description	Sequence	SEQ ID NO
Biosafety Chromosomal Construct - low copy Rep (Pi) and Kis antitoxin (as shown in **FIG. 74C**)	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGGATCTGCTGGA ACAGGTGGTGAGACTCAAGGTCATGATGGACGTGAACAAAAAAACG AAAATTCGCCACCGAAACGAGCTAAATCACACCCTGGCTCAACTTCC TTTGCCCGCAAAGCGAGTGATGTATATGGCGCTTGCTCCCATTGATA GCAAAGAACCTCTTGAACGAGGGCGAGTTTTCAAAATTAGGGCTGA AGACCTTGCAGCGCTCGCCAAAATCACCCCATCGCTTGCTTATCGAC AATTAAAAGAGGGTGGTAAATTACTTGGTGCCAGCAAAATTTCGCTA AGAGGGGATGATATCATTGCTTTAGCTAAAGAGCTTAACCTGCTCTT TACTGCTAAAAACTCCCCTGAAGAGTTAGACCTTAACATTATTGAGT GGATAGCTTATTCAAATGATGAAGGATACTTGTCTTTAAAATTCACC AGAACCATAGAACCATATATCTCTAGCCTTATTGGGAAAAAAAATA AATTCACAACGCAATTGTTAACGGCAAGCTTACGCTTAAGTAGCCAG TATTCATCTTCTCTTTATCAACTTATCAGGAAGCATTACTCTAATTTT AAGAAGAAAAATTATTTTATTATTTCCGTTGATGAGTTAAAGGAAGA GTTAATAGCTTATACTTTTGATAAAGATGGAAATATTGAGTACAAAT ACCCTGACTTTCCTATTTTTAAAAGGGATGTGTTAAATAAAGCCATT GCTGAAATTAAAAAGAAAACAGAAATATCGTTTGTTGGCTTCACTGT TCATGAAAAAGAAGGAAGAAAAATTAGTAAGCTGAAGTTCGAATTT GTCGTTGATGAAGATGAATTTTCTGGCGATAAAGATGATGAAGCTTT TTTTATGAATTTATCTGAAGCTGATGCAGCTTTTCTCAAGGTATTTGA TGAAACCGTACCTCCCAAAAAAGCTAAGGGGTGAGGATCTCCAGGC ATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTT ATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTC ACCTTCGGGTGGGCCTTTCTGCGTTTATACCCGGGAAAAAGAGTATT GACTtaaagtctaacctataggTATAATGTGTGGAGACCAGAGGTAAGGAGGT AACAACCATGCGAGTGTTGAAGAAACATCTTAATCATGCTAAGGAG GTTTTCTAATGCATACCACCCGACTGAAGAGGGTTGGCGGCTCAGTT ATGCTGACCGTCCCACCGGCACTGCTGAATGCGCTGTCTCTGGGCAC AGATAATGAAGTTGGCATGGTCATTGATAATGGCCGGCTGATTGTTG AGCCGTACAGACGCCCGCAATATTCACTGGCTGAGCTACTGGCACA GTGTGATCCGAATGCTGAAATATCAGCTGAAGAACGAGAATGGCTG GATGCACCGGCGACTGGTCAGGAGGAAATCTGA	360
Biosafety Chromosomal Construct - medium copy Rep (Pi) and Kis antitoxin (as shown in **FIG. 74D**)	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGGATCTTCCGGA AGACTAGGTGAGACTCAAGGTCATGATGGACGTGAACAAAAAAACG AAAATTCGCCACCGAAACGAGCTAAATCACACCCTGGCTCAACTTCC TTTGCCCGCAAAGCGAGTGATGTATATGGCGCTTGCTCCCATTGATA GCAAAGAACCTCTTGAACGAGGGCGAGTTTTCAAAATTAGGGCTGA AGACCTTGCAGCGCTCGCCAAAATCACCCCATCGCTTGCTTATCGAC AATTAAAAGAGGGTGGTAAATTACTTGGTGCCAGCAAAATTTCGCTA AGAGGGGATGATATCATTGCTTTAGCTAAAGAGCTTAACCTGCTCTT TACTGCTAAAAACTCCCCTGAAGAGTTAGACCTTAACATTATTGAGT GGATAGCTTATTCAAATGATGAAGGATACTTGTCTTTAAAATTCACC AGAACCATAGAACCATATATCTCTAGCCTTATTGGGAAAAAAAATA AATTCACAACGCAATTGTTAACGGCAAGCTTACGCTTAAGTAGCCAG TATTCATCTTCTCTTTATCAACTTATCAGGAAGCATTACTCTAATTTT AAGAAGAAAAATTATTTTATTATTTCCGTTGATGAGTTAAAGGAAGA GTTAATAGCTTATACTTTTGATAAAGATGGAAATATTGAGTACAAAT	361

	ACCCTGACTTTCCTATTTTTAAAAGGGATGTGTTAAATAAAGCCATT GCTGAAATTAAAAAGAAAACAGAAATATCGTTTGTTGGCTTCACTGT TCATGAAAAAGAAGGAAGAAAAATTAGTAAGCTGAAGTTCGAATTT GTCGTTGATGAAGATGAATTTTCTGGCGATAAAGATGATGAAGCTTT TTTTATGAATTTATCTGAAGCTGATGCAGCTTTTCTCAAGGTATTTGA TGAAACCGTACCTCCCAAAAAAGCTAAGGGGTGAGGATCTCCAGGC ATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTT ATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTC ACCTTCGGGTGGGCCTTTCTGCGTTTATACCCGGGAAAAAGAGTATT GACTtaaagtctaacctataggTATAATGTGTGGAGACCAGAGGTAAGGAGGT AACAACCATGCGAGTGTTGAAGAAACATCTTAATCATGCTAAGGAG GTTTTCTAATGCATACCACCCGACTGAAGAGGGTTGGCGGCTCAGTT ATGCTGACCGTCCCACCGGCACTGCTGAATGCGCTGTCTCTGGGCAC AGATAATGAAGTTGGCATGGTCATTGATAATGGCCGGCTGATTGTTG AGCCGTACAGACGCCCGCAATATTCACTGGCTGAGCTACTGGCACA GTGTGATCCGAATGCTGAAATATCAGCTGAAGAACGAGAATGGCTG GATGCACCGGCGACTGGTCAGGAGGAAATCTGA	
Rep (Pi)	TGAGACTCAAGGTCATGATGGACGTGAACAAAAAAACGAAAATTCG CCACCGAAACGAGCTAAATCACACCCTGGCTCAACTTCCTTTGCCCG CAAAGCGAGTGATGTATATGGCGCTTGCTCCCATTGATAGCAAAGA ACCTCTTGAACGAGGGCGAGTTTTCAAAATTAGGGCTGAAGACCTTG CAGCGCTCGCCAAAATCACCCCATCGCTTGCTTATCGACAATTAAAA GAGGGTGGTAAATTACTTGGTGCCAGCAAAATTTCGCTAAGAGGGG ATGATATCATTGCTTTAGCTAAAGAGCTTAACCTGCTCTTTACTGCTA AAAACTCCCCTGAAGAGTTAGACCTTAACATTATTGAGTGGATAGCT TATTCAAATGATGAAGGATACTTGTCTTTAAAATTCACCAGAACCAT AGAACCATATATCTCTAGCCTTATTGGGAAAAAAAATAAATTCACAA CGCAATTGTTAACGGCAAGCTTACGCTTAAGTAGCCAGTATTCATCT TCTCTTTATCAACTTATCAGGAAGCATTACTCTAATTTTAAGAAGAA AAATTATTTTATTATTTCCGTTGATGAGTTAAAGGAAGAGTTAATAG CTTATACTTTTGATAAAGATGGAAATATTGAGTACAAATACCCTGAC TTTCCTATTTTTAAAAGGGATGTGTTAAATAAAGCCATTGCTGAAAT TAAAAAGAAAACAGAAATATCGTTTGTTGGCTTCACTGTTCATGAAA AAGAAGGAAGAAAAATTAGTAAGCTGAAGTTCGAATTTGTCGTTGA TGAAGATGAATTTTCTGGCGATAAAGATGATGAAGCTTTTTTTATGA ATTTATCTGAAGCTGATGCAGCTTTTCTCAAGGTATTTGATGAAACC GTACCTCCCAAAAAAGCTAAGGGGTGA	362
Kis antitoxin	CATACCACCCGACTGAAGAGGGTTGGCGGCTCAGTTATGCTGACCGT CCCACCGGCACTGCTGAATGCGCTGTCTCTGGGCACAGATAATGAAG TTGGCATGGTCATTGATAATGGCCGGCTGATTGTTGAGCCGTACAGA CGCCCGCAATATTCACTGGCTGAGCTACTGGCACAGTGTGATCCGAA TGCTGAAATATCAGCTGAAGAACGAGAATGGCTGGATGCACCGGCG ACTGGTCAGGAGGAAATCTGA	363
RBS (low copy)	GCTGGAACAGGTGG	364
RBS (medium copy)	TCCGGAAGACTAGG	365

Example 56. Generation of Butyrate and Acetate Producing Strains

A. Generation of an Acetate Overproducing Strain

[0704] E. coli generates high levels of acetate as an end product of fermentation. In order generate enhanced acetate production, strain SYN2001 was generated, which harbors a deletion in the endogenous ldh (lactate dehydrogenase) gene, with the intention to prevent or reduce flux through the metabolic arm generating lactate, and thereby enhancing the flux through the metabolic arm generating acetate (see, e.g., **FIG. 85**).

[0705] Briefly, We deleted the gene encoding L-lactate dehydrogenase A (*ldhA*) to block carbon flux from pyruvate to lactate and improve acetate biosynthetic yield in *E. coli* Nissle. Knockout primers were synthesized (IDT) and a chloramphenicol-resistance antibiotic marker was inserted in place of the *ldhA* coding region to ensure the removal of the targeted gene. The *ldhA* gene on the *E. coli* Nissle genome was knocked out and replaced with the chloramphenicol resistance gene through allelic exchange, which was facilitated by the lambda red recombinase system. Proper knockout of the target gene in the Nissle genome was validated by the ability of the resulting Nissle strain to grow on chloramphenicol-containing LB plates or medium and further confirmed by PCR. This strain was designated SYN2001.

[0706] For this study, media M9 media containing 50mM MOPS with 0.5% glucose was compared to media containing 0.5/% glucuronic acid, as glucuronic acid better mimics available carbon sources in the gut.

[0707] SYN2001 and streptomycin resistant E coli Nissle (SYN094) were grown overnight at 37 C with shaking. Overnight cultures were diluted 1:100 into 10 ml LB (containing antibiotics) in a 125 ml baffled flask. Cultures were grown aerobically at 37 C with shaking for about 1.5h, and then transferred to the anaerobic chamber at 37 C for 4h. Bacteria ($2X10^8$ CFU) were added to 1ml M9 media containing 50mM MOPS with 0.5% glucose or 0.5% glucuronic acid in microcentrifuge tubes. Cells were plated to determine cell counts. The assay tubes were placed in the anaerobic chamber at 37 C. At 1, 2, 3, 4, 5, and 6 hours, cells were removed and pelleted at 14,000rpm for 1 min, and 100 ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for acetate concentrations as described herein, e.g., in **Example 56**.

Table 85. Acetate production by SYN2001 from three different manufacturing experiments

	Run1		Run 2		Run 3	
Strain	**[Acetate] in mM**	**SD**	**[Acetate] in mM**	**SD**	**[Acetate] in mM**	**SD**
SYN2001	24.43737	2.970942327	21.26342667	1.719791084	31.58750134	6.6846157 5

[0708] Culture supernatants of SYN2001 produced between 21.2 and 31.5 mM acetate and an undetectable amount of butyrate (data not shown) under the above conditions

in 3 independent production runs. Culture supernatant from run 3 was then used to generate the bioactivity results from cell based assays presented below in **Example 63**.

[0709] As seen in **FIG. 84A** and **FIG. 84B**, the ldhA knockout E. coli Nissle strain SYN2001 has improved acetate productivity during over a 6 hour time course using either glucose or glucuronic acid as the main carbon source.

B. Generation of strains which produces butyrate and acetate

a. Knock out of the endogenous adhE and ldhA genes

[0710] In order to improve acetate production while also producing high levels butyrate production, deletions in endogenous adhE (Aldehyde-alcohol dehydrogenase) and ldh (lactate dehydrogenase) were generated to prevent or reduce metabolic flux through pathways which do not result in acetate or butyrate production (see, e.g., **FIG. 85**). Aldehyde-alcohol dehydrogenase converts acetylCoA into acetaldehyde, which is then converted to ethanol. As a result, a mutation or deletion of adhE is expected to prevent the metabolic flux towards ethanol production and consequently allow for additional acetylCoA to be used for butyrate production. For this study, Nissle strains with either integrated FNRS ter-tesB or FNRS-ter-pbt-buk butyrate cassettes were used. Additionally, media M9 media containing 50mM MOPS with 0.5% glucose was compared to media containing 0.5/% glucuronic acid, as glucuronic acid better mimics available carbon sources in the gut.

[0711] Briefly, bacteria were grown overnight at 37 C with shaking. Overnight cultures were diluted 1:100 into 10ml LB (containing antibiotics) in a 125ml baffled flask. Cultures were grown aerobically at 37 C with shaking for about 1.5 h, and then transferred to the anaerobic chamber at 37 C for 4h. Bacteria ($2X10^8$ CFU) were added to 1ml M9 media containing 50mM MOPS with 0.5% glucose or 0.5% glucuronic acid in microcentrifuge tubes. Cells were plated to determine cell counts. The assay tubes were placed in the anaerobic chamber at 37 C. At 18 hours, cells were removed and pelleted at 14,000 rpm for 1 min, and 100 ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for butyrate and acetate concentrations as described herein, e.g., in **Example 57**.

[0712] As seen in **FIG. 84C and FIG. 84D**, both integrated strains made similar amounts of acetate, and FNRS-ter-pbt-buk butyrate cassettes produced slightly more butyrate. Deletions in adhE and ldhA have similar effects on butyrate and acetate production. Acetate production was much greater in media containing 0.5% glucuronic acid.

b. Knock out of the endogenous frdA gene

[0713] FrdA is one of two catalytic subunits in the four subunit fumarate reductase complex. Fumarate reductase converts fumarate (derived from phosphoenolpyruvate) to succinate along one arm of anaerobic metabolism. In a second study, the effect of a deletion in the endogenous frdA gene, which prevents metabolic flux through the phosphoenolpyruvate -> succinate pathway, on acetate and butyrate production was assessed. For this study, SYN2005 (comprising FNRS-ter-tesB butyrate cassette integrated at the HA1/2 site and a deletion in the endogenous frd gene) was compared to SYN1004 (comprising the FNRS-ter-tesB butyrate cassette integrated at the HA1/2 site).

[0714] Bacteria were grown overnight at 37 C with shaking. Overnight cultures were diluted 1:100 into 10ml LB (containing antibiotics) in a 125ml baffled flask. Cultures were grown aerobically at 37 C with shaking for about 1.5h, and then transferred to the anaerobic chamber at 37 C for 4h. Bacteria ($2X10^8$ CFU) were added to 1ml M9 media containing 50mM MOPS with 0.5% glucose in microcentrifuge tubes. Cells were plated to determine cell counts. The assay tubes were placed in the anaerobic chamber at 37 C. At 18 hours, cells were removed and pelleted at 14,000rpm for 1 min, and 100 ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for butyrate and acetate concentrations as described herein, e.g., in **Example 21**.

[0715] Results are depicted in **FIG. 84E** and indicate that the frdA mutation in SYN2005 allowed increased acetate production relative to SYN1173. SYN1173 produces greater levels of butyrate than acetate, while SYN2005 produces similar levels of both acetate and butyrate.

[0716] In other studies, strains are generated with combinations of deletions in two or more of the aldE, ldhA, and frd genes and the effect of the deletions on acetate and butyrate production are assessed.

C. Butyrate only producing strains

[0717] In order to generate a strain which can produce butyrate, but has a reduced ability to produce acetate, a deletion in the pta gene was introduced into a strain that contains an integrated butyrate cassette (Ter/TesB cassette) under the control of an FNR promoter (SYN2002). Phosphate acetyltransferase (Pta) catalyzes the conversion between acetyl-CoA and acetylphosphate, the first step in the metabolic arm leading to the generation of acetate (see., e.g., **FIG. 85**). As such inhibition of this step was assumed to help prevent accumulation of acetate. Additionally, a mutation in the adhE (aldehyde-alcohol dehydrogenase) gene was introduced.

[0718] Acetate and butyrate production in both strains was compared to a third strain which contains both the FNR-driven ter-pbt-buk butyrate cassette and the deletion in the endogenous ldhA gene (e.g., as described above).

[0719] For this study, bacteria from all three strains were grown overnight at 37 C with shaking. Overnight cultures were diluted 1:100 into 10ml LB (containing antibiotics) in a 125ml baffled flask. Cultures were grown aerobically at 37 C with shaking for about 1.5h, and then transferred to the anaerobic chamber at 37 C for 4h. Bacteria ($2X10^8$ CFU) were added to 1ml M9 media containing 50mM MOPS with 0.5% glucose in microcentrifuge tubes. Cells were plated to determine cell counts. The assay tubes were placed in the anaerobic chamber at 37 C. At 18 hours, cells were removed and pelleted at 14,000rpm for 1 min, and 100 ul of the supernatant was transferred to a 96-well assay plate and sealed with aluminum foil, and stored at -80 C until analysis by LC-MS for butyrate and acetate concentrations as described herein, e.g., in **Example 21**.

[0720] Results are depicted in **FIG. 84F**, and show that the strain comprising the deletion in the endogenous ldhA gene produced acetate but no butyrate, the strain comprising the FNR-ter-tesB butyrate cassette and the aldhE deletion produced butyrate, but very low levels of acetate. The third strain, comprising the FNRter-tesB butyrate cassette and the deletions in the adhE and pta genes, made equal amounts of acetate and butyrate.

Example 57. Acetate and Butyrate quantification in bacterial supernatant by LC-MS/MS

Sample Preparation

[0721] Ammonium acetate and Sodium butyrate stock (10 mg/mL) was prepared in water and aliquoted in 1.5 mL microcentrifuge tubes (100 µL) and stored at -20°C. Standards (1000, 500, 250, 100, 20, 4, 0.8 µg/mL) were prepared in water. Sample and standards (10µL) were pipetted in a V-bottom polypropylene 96-well plate on ice. Derivatizing solution (90µL) containing 50mM of 2-Hydrazinoquinoline (2-HQ), dipyridyl disulfide, and triphenylphosphine in acetonitrile with 2 ug/mL of Sodium butyrate-d7 was added into the final solution. The plate was then heat-sealed with a ThermASeal foil and mixed well, and the samples were incubated at 60°C for 1hr for derivatization and centrifuged at 4000rpm for 5min. The derivatized samples (20µL) were added to 180µL of 0.1% formic acid in water/ACN (140:40) in a round-bottom 96-well plate. The plate was then heat-sealed with a ClearASeal sheet and mixed well.

LC-MS/MS method

[0722] Derivatized metabolites were measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. **Table 55** and **Table 56** provides the summary of the LC-MS/MS method.

Table 55.

Column:	C18 column, 3 µm (100 x 2 mm)
Mobile Phase A:	100% H2O, 0.1% Formic Acid
Mobile Phase B:	100% ACN, 0.1% Formic Acid
Injection volume:	10uL

Table 56. HPLC Method:

Time (min)	Flow Rate (µL/min)	A%	B%
0	500	95	5
0.5	500	95	5
2.0	500	10	90
3.0	500	10	90
3.01	500	95	5
3.25	500	95	5

Table 57. Tandem Mass Spectrometry:

Ion Source:	HESI-II
Polarity:	Positive
SRM transitions:	
Acetate:	202.1/143.1
Butyrate:	230.1/160.2
Butyrate-d7:	237.1/160.2

CLAIMS

1. A bacterium comprising a gene or gene cassette for producing a gut barrier enhancer molecule and a gene or gene cassette for producing a satiety effector molecule, wherein the gene or gene cassettes are operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature.

2. The bacterium of claim 1, wherein the gene or gene cassette for producing a gut barrier enhancer encodes a non-native gut barrier enhancer molecule.

3. The bacterium of claim 1, wherein the gene or gene cassette for producing a satiety effector molecule encodes a non-native satiety effector molecule.

4. The bacterium of any of claims 1-3, wherein the gene or gene cassette encoding a non-native satiety effector molecule produces GLP-1.

5. The bacterium of claim 4, wherein gene or gene cassette encoding GLP-1 comprises a secretion tag.

6. The bacterium of claim 5, wherein gene or gene cassette encoding GLP-1 comprises a PhoA secretion tag.

7. The bacterium of claim 5, wherein gene or gene cassette encoding GLP-1 comprises a OmpF secretion tag.

8. The bacterium of claim 5, wherein gene or gene cassette encoding GLP-1 comprises a TorA-secretion tag.

9. The bacterium of any of claims 1-8, wherein the bacterium comprises an endogenous lpp gene which is knocked down via mutation or deletion.

10. The bacterium of any of claims 1-9, wherein the bacterium comprises an endogenous pal gene which is knocked down via mutation or deletion.

11. The bacterium of any of claims 1-10, wherein the bacterium comprises an endogenous tolA gene which is knocked down via mutation or deletion.

12. The bacterium of any of claims 1-11, wherein the bacterium comprises an endogenous nlPI gene which is knocked down via mutation or deletion.

13. The bacterium of any of claims 1-12, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces a short chain fatty acid selected from butyrate, acetate and propionate.

14. The bacterium of any of claims 1-12, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces butyrate.

15. The bacterium of claim 14, wherein the at least one gene cassette for producing butyrate comprises ter, thiA1, hbd, crt2, pbt, and buk genes.

16. The bacterium of claim 14, wherein the at least one gene cassette for producing butyrate comprises ter, thiA1, hbd, crt2, and tesb genes.

17. The bacterium of any of claims 1-12, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces propionate.

18. The bacterium of any of claims 1-12, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces acetate.

19. The bacterium of any of claims 14-18, wherein the bacterium comprises an endogenous adhE gene which is knocked down via mutation or deletion.

20. The bacterium of any of claims 14-19, wherein the bacterium comprises an endogenous frd gene which is knocked down via mutation or deletion.

21. The bacterium of any of claims 14-20, wherein the bacterium comprises an endogenous ldhA gene which is knocked down via mutation or deletion.

22. The bacterium of any of claims 14-21, wherein the bacterium comprises an endogenous pta gene which is knocked down via mutation or deletion.

23. The bacterium of any of claims 1-12, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces a tryptophan metabolite.

24. The bacterium of claim 23, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces a tryptophan metabolite selected from tryptamine, indole-3-acetic acid and indole-3-propionic acid.

25. The bacterium of claim 24, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces tryptamine.

26. The bacterium of claim 24, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces indole-3 acetic acid.

27. The bacterium of claim 24, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces indole-3-propionic acid.

28. The bacterium of any of claims 1-27, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by exogenous environmental conditions.

29. The bacterium of claim 28, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by low-oxygen or anaerobic conditions.

30. The bacterium of claim 28, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is selected from a FNR-inducible promoter, an ANR-inducible promoter, and a DNR-inducible promoter.

31. The bacterium of claim 28, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by one or more molecules or metabolites indicative of liver damage.

32. The bacterium of claim 28, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by the presence of reactive nitrogen species.

33. The bacterium of claim 28, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by the presence of reactive oxygen species.

34. The bacterium of claim 28, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by an environmental factor that is not naturally present in a mammalian gut.

35. The bacterium of any of claims 1-34, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is induced by exogenous environmental conditions.

36. The bacterium of claim 35, wherein the promoter operably linked to the at least one gene or gene cassette for producing satiety effector molecule is induced by low-oxygen or anaerobic conditions.

37. The bacterium of claim 36, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is selected from a FNR-inducible promoter, an ANR-inducible promoter, and a DNR-inducible promoter.

38. The bacterium of claim 35, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is induced by one or more molecules or metabolites indicative of liver damage.

39. The bacterium of claim 35, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is induced by the presence of reactive nitrogen species.

40. The bacterium of claim 35, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is induced by the presence of reactive oxygen species.

41. The bacterium of claim 35, wherein the promoter operably linked to the at least one gene or gene cassette for producing satiety effector molecule is induced by an environmental factor that is not naturally present in a mammalian gut.

42. A bacterium comprising two or more gene or gene cassettes for producing two or more gut barrier enhancer effector molecule(s) and optionally at least one gene or gene cassette for producing a satiety effector molecule, wherein the gene or gene cassettes are operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature.

43. The bacterium of claim 42, wherein the two or more gene or gene cassettes encode two or more non-native gut barrier enhancer molecules.

44. The bacterium of any of claims 42-43, wherein a gene or gene cassette encoding a first non-native gut barrier enhancer produces butyrate.

45. The bacterium of any of claims 42-43, wherein a gene or gene cassette encoding a first non-native gut barrier enhancer produces propionate.

46. The bacterium of any of claims 42-43, wherein a gene or gene cassette encoding a first non-native gut barrier enhancer produces acetate.

47. The bacterium of any of claims 42-46, wherein a gene or gene cassette encoding a second non-native gut barrier enhancer produces a tryptophan metabolite.

48. The bacterium of claim 47, wherein the gene or gene cassette encoding a second non-native gut barrier enhancer produces a tryptophan metabolite selected from tryptamine, indole-3-acetic acid and indole-3-propionic acid.

49. The bacterium of claim 47, wherein the gene or gene cassette encoding a second non-native gut barrier enhancer produces tryptamine.

50. The bacterium of claim 47, wherein the gene or gene cassette encoding a second non-native gut barrier enhancer produces indole-3 acetic acid.

51. The bacterium of claim 47, wherein the gene or gene cassette encoding a second non-native gut barrier enhancer produces indole-3-propionic acid.

52. The bacterium of any of claims 42-51, wherein the gene or gene cassette encoding the optional a non-native satiety effector molecule produces GLP-1.

53. The bacterium of any of claims 42-52, wherein the promoter operably linked to the first and second gene or gene cassettes for producing gut barrier enhancer effector molecules are induced by exogenous environmental conditions.

54. The bacterium of claim 53, wherein the promoter operably linked to the first and second gene cassettes for producing gut barrier enhancer effector molecules are induced by low-oxygen or anaerobic conditions.

55. The bacterium of claim 54, wherein the promoter operably linked to the first and second gene gene cassettes for producing gut barrier enhancer effector molecules is selected from a FNR-inducible promoter, an ANR-inducible promoter, and a DNR-inducible promoter.

56. The bacterium of claim 53, wherein the promoter operably linked to the first and second gene or gene cassettes for producing gut barrier enhancer effector molecules are induced by one or more molecules or metabolites indicative of liver damage.

57. The bacterium of claim 53, wherein the promoter operably linked to the first and second gene or gene cassettes for producing gut barrier enhancer effector molecules are induced by the presence of reactive nitrogen species.

58. The bacterium of claim 53, wherein the promoter operably linked to the first and second gene or gene cassettes for producing gut barrier enhancer effector molecules are induced by the presence of reactive oxygen species.

59. The bacterium of claim 53, wherein the promoter operably linked to to the first and second gene or gene cassettes for producing gut barrier enhancer effector molecules are induced by an environmental factor that is not naturally present in a mammalian gut.

60. The bacterium of any of claims 42-59, wherein the promoter operably linked to the at least one optional gene or gene cassette for producing a satiety effector molecule is induced by exogenous environmental conditions.

61. The bacterium of claim 60, wherein the promoter operably linked to the at least one gene or gene cassettes for producing satiety effector molecule are induced by low-oxygen or anaerobic conditions.

62. The bacterium of claim 61, wherein the promoter operably linked to the at least one gene or gene cassettes for producing a satiety effector molecule is selected from a FNR-inducible promoter, an ANR-inducible promoter, and a DNR-inducible promoter.

63. The bacterium of claim 60, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is induced by one or more molecules or metabolites indicative of liver damage.

64. The bacterium of claim 60, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is induced by the presence of reactive nitrogen species.

65. The bacterium of claim 60, wherein the promoter operably linked to the at least one gene or gene cassette for producing a satiety effector molecule is induced by the presence of reactive oxygen species.

66. The bacterium of claim 60, wherein the promoter operably linked to the at least one gene or gene cassette for producing satiety effector molecule is induced by an environmental factor that is not naturally present in a mammalian gut.

67. A bacterium comprising a gene or gene cassette for producing a gut barrier enhancer effector molecule and a gene or gene cassette for producing bile salt hydrolase, wherein the gene or gene cassettes are operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature.

68. The bacterium of claim 67, wherein the gene or gene cassette encodes a non-native gut barrier enhancer.

69. The bacterium of any of claims 67-68, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces a short chain fatty acid selected from butyrate, acetate and propionate.

70. The bacterium of any of claims 67-69, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces butyrate.

71. The bacterium of any of claims 67-69, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces propionate.

72. The bacterium of any of claims 67-69, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces acetate.

73. The bacterium of any of claims 67-69, wherein the gene or gene cassette encoding a non-native gut barrier enhancer produces acetate.

74. The bacterium of any of claims 67-73, wherein the gene or gene cassette encoding bile salt hydrolase encodes a 7α-dehydroxylating enzyme.

75. The bacterium of any of claims 67-74, wherein the bacterium comprises at least one gene or gene cassette encoding bile salt and/or bile acid transporter(s).

76. The bacterium of any of claims 67-75, wherein the bacterium comprises at least one gene or gene cassette encoding at least one satiety effector molecule.

77. The bacterium of claim 76, wherein the bacterium comprises at least one gene or gene cassette encoding GLP-1.

78. The bacterium of any of claims 67-77, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by exogenous environmental conditions.

79. The bacterium of claim 78, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by low-oxygen or anaerobic conditions.

80. The bacterium of claim 79, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is selected from a FNR-inducible promoter, an ANR-inducible promoter, and a DNR-inducible promoter.

81. The bacterium of claim 78, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by one or more molecules or metabolites indicative of liver damage.

82. The bacterium of claim 78, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by the presence of reactive nitrogen species.

83. The bacterium of claim 78, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by the presence of reactive oxygen species.

84. The bacterium of claim 78, wherein the promoter operably linked to the at least one gene or gene cassette for producing a gut barrier enhancer effector molecule is induced by an environmental factor that is not naturally present in a mammalian gut.

85. The bacterium of any of claims 67-84, wherein the promoter operably linked to the at least one gene or gene cassette for producing the bile salt hydrolase is induced by exogenous environmental conditions.

86. The bacterium of claim 85, wherein the promoter operably linked to the at least one gene or gene cassette for producing the bile salt hydrolase is induced by low-oxygen or anaerobic conditions.

87. The bacterium of claim 86, wherein the promoter operably linked to the at least one gene or gene cassette for producing the bile salt hydrolase is selected from a FNR-inducible promoter, an ANR-inducible promoter, and a DNR-inducible promoter.

88. The bacterium of claim 85, wherein the promoter operably linked to the at least one gene or gene cassette for producing the bile salt hydrolase is induced by one or more molecules or metabolites indicative of liver damage.

89. The bacterium of claim 85, wherein the promoter operably linked to the at least one gene or gene cassette for producing the bile salt hydrolase is induced by the presence of reactive nitrogen species.

90. The bacterium of claim 85, wherein the promoter operably linked to the at least one gene or gene cassette for producing the bile salt hydrolase is induced by the presence of reactive oxygen species.

91. The bacterium of claim 85, wherein the promoter operably linked to the at least one gene or gene cassette for producing the bile salt hydrolase is induced by an environmental factor that is not naturally present in a mammalian gut.

92. A pharmaceutically acceptable composition comprising the bacterium of any one of claims 1-91.

93. The pharmaceutically acceptable composition of claim 92, wherein the composition is formulated for oral or rectal administration.

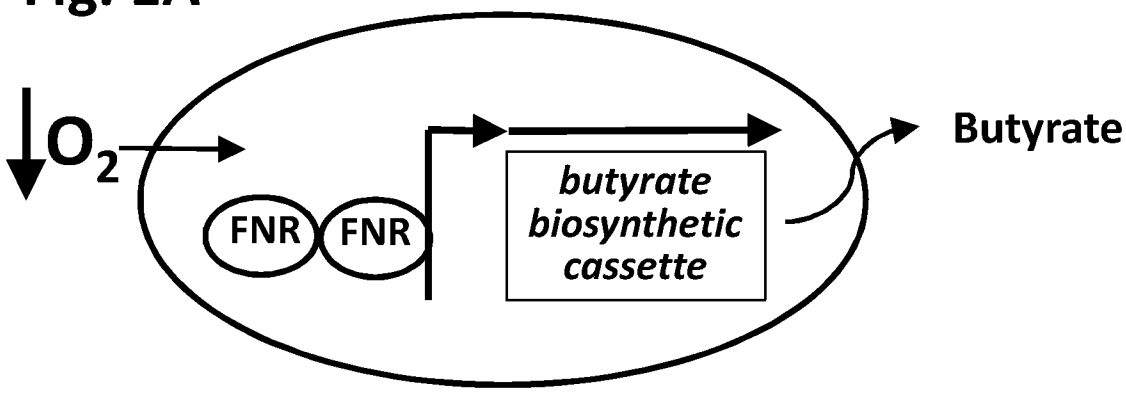
94. A method of treating a liver disease, disorder or condition, or symptom(s) thereof in a subject in need thereof comprising the step of administering to the subject the composition of claim 80 for a period of time sufficient to lessen the severity of the disease or symptom(s).

95. The method of claim 94, wherein the disease, disorder or condition is selected from the group consisting of: Nonalcoholic Steatohepatitis (NASH); Non-alcoholic fatty liver disease (NAFLD); type 1 diabetes; type 2 diabetes; metabolic syndrome; Bardet-Biedel syndrome; Prader-Willi syndrome; tuberous sclerosis; Albright hereditary osteodystrophy; brain-derived neurotrophic factor (BDNF) deficiency; Single-minded 1 (SIM1) deficiency; leptin deficiency; leptin receptor deficiency; pro-opiomelanocortin (POMC) defects; proprotein convertase subtilisin/kexin type 1 (PCSK1) deficiency; Src homology 2B1 (SH2B1) deficiency; pro-hormone convertase 1/3 deficiency; melanocortin-4-receptor (MC4R) deficiency; Wilms tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) syndrome; pseudohypoparathyroidism type 1A; Fragile X syndrome; Borjeson-Forsmann-Lehmann syndrome; Alstrom syndrome; Cohen syndrome; and ulnar-mammary syndrome.

96. The method of claim 95, wherein the disease is non-alcoholic fatty liver disease.

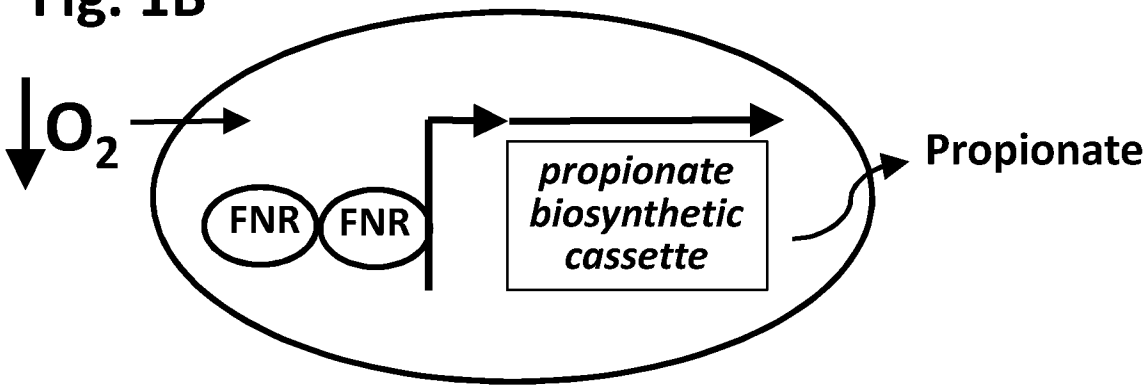
97. The method of claim 95 or 96, wherein the disease is nonalcoholic Steatohepatitis.

Fig. 1A



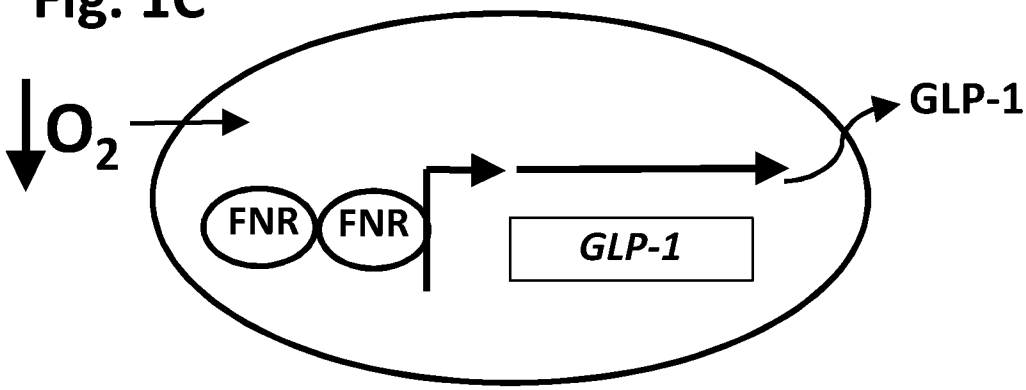
E. Coli Nissle 1917

Fig. 1B



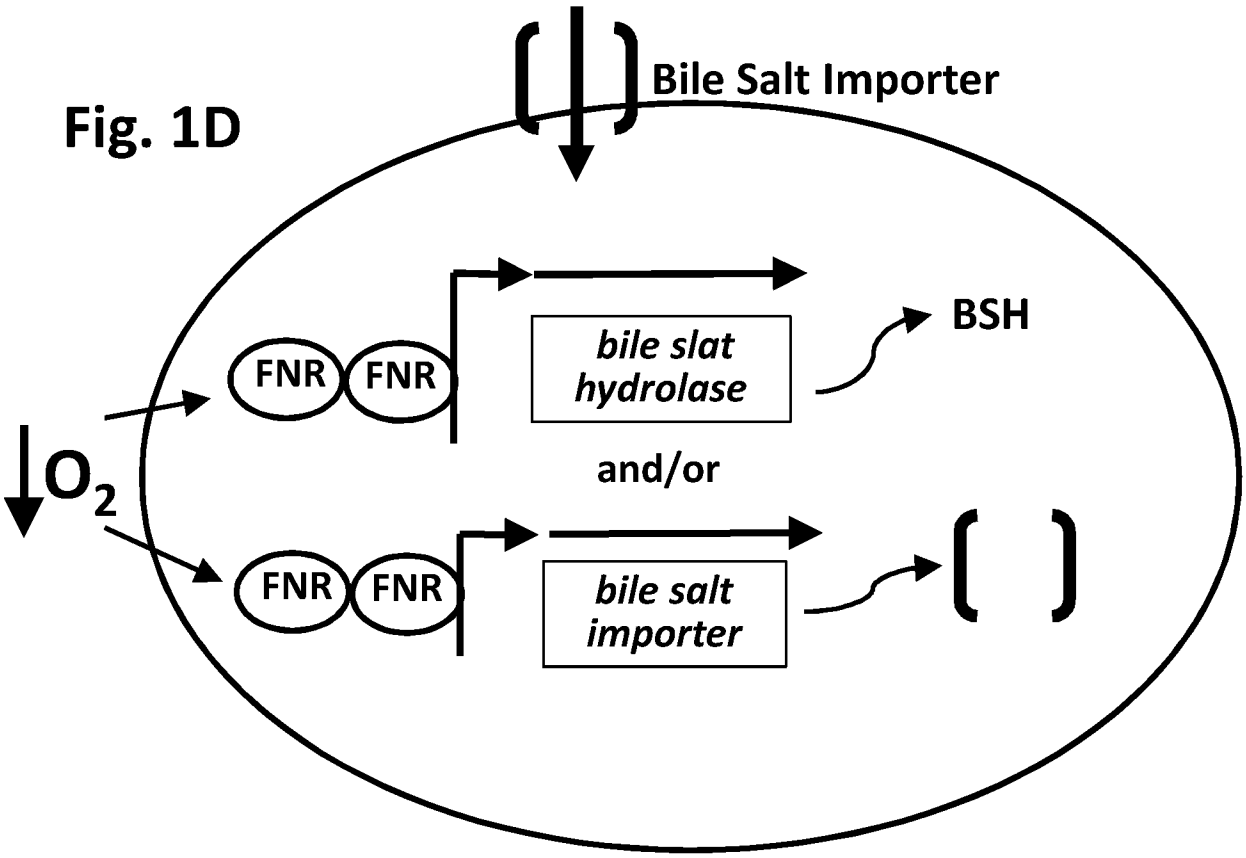
E. Coli Nissle 1917

Fig. 1C



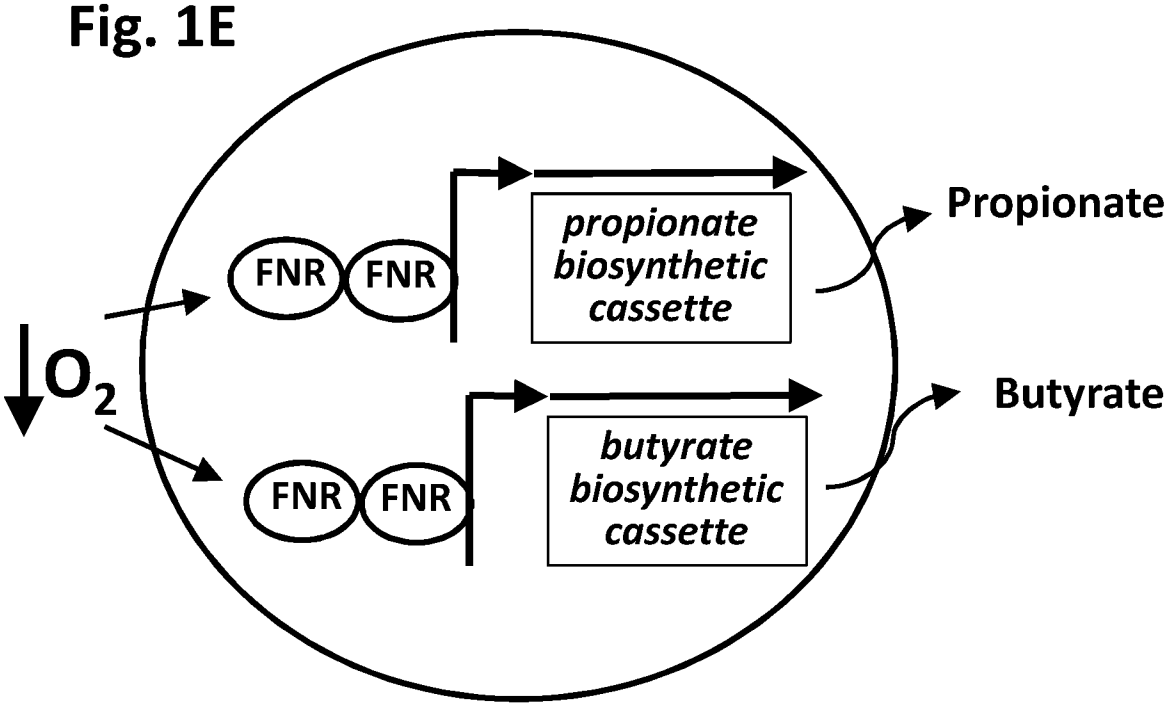
E. Coli Nissle 1917

Fig. 1D



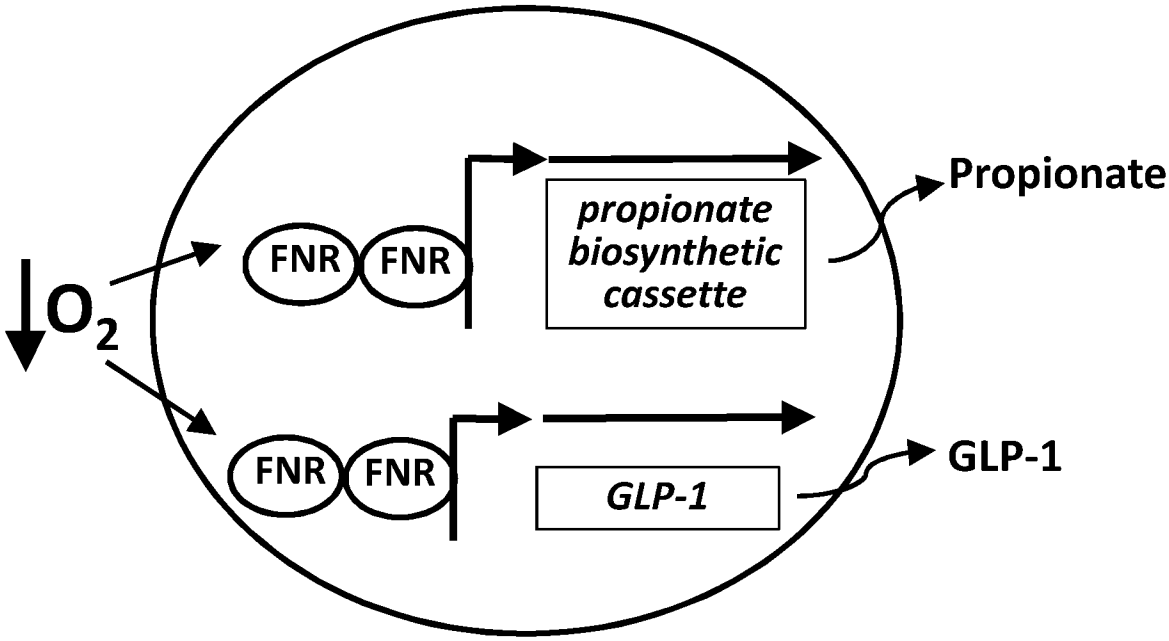
E. Coli Nissle 1917

Fig. 1E



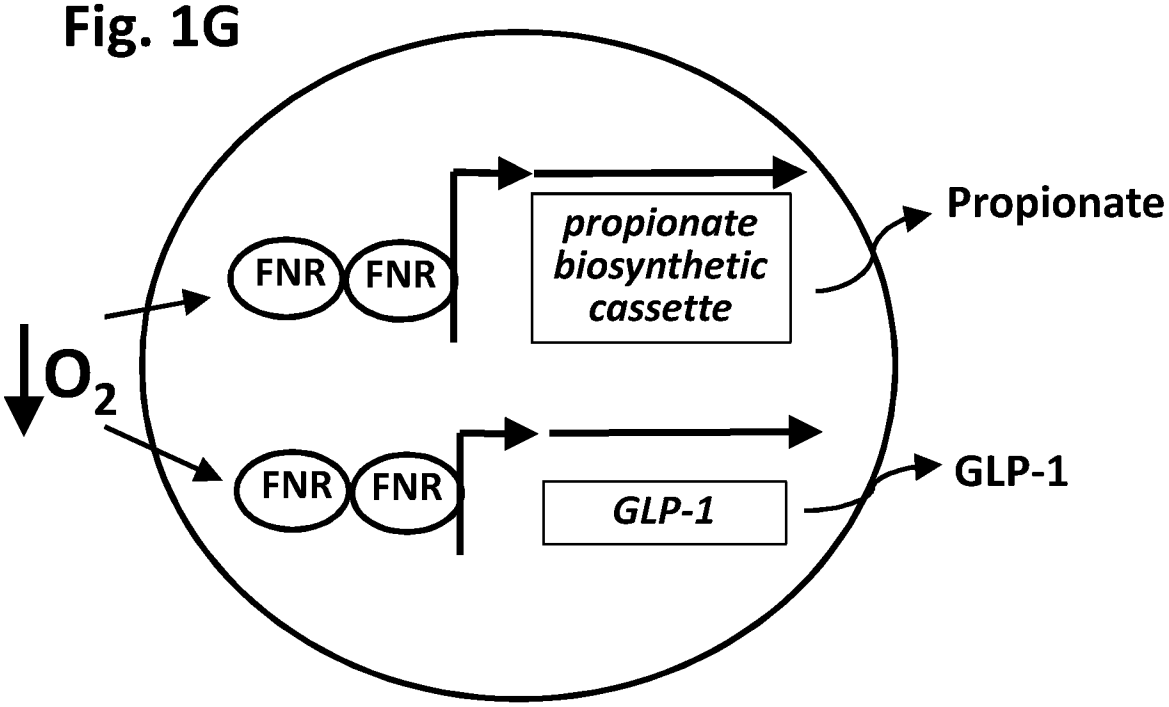
E. Coli Nissle 1917

Fig. 1F



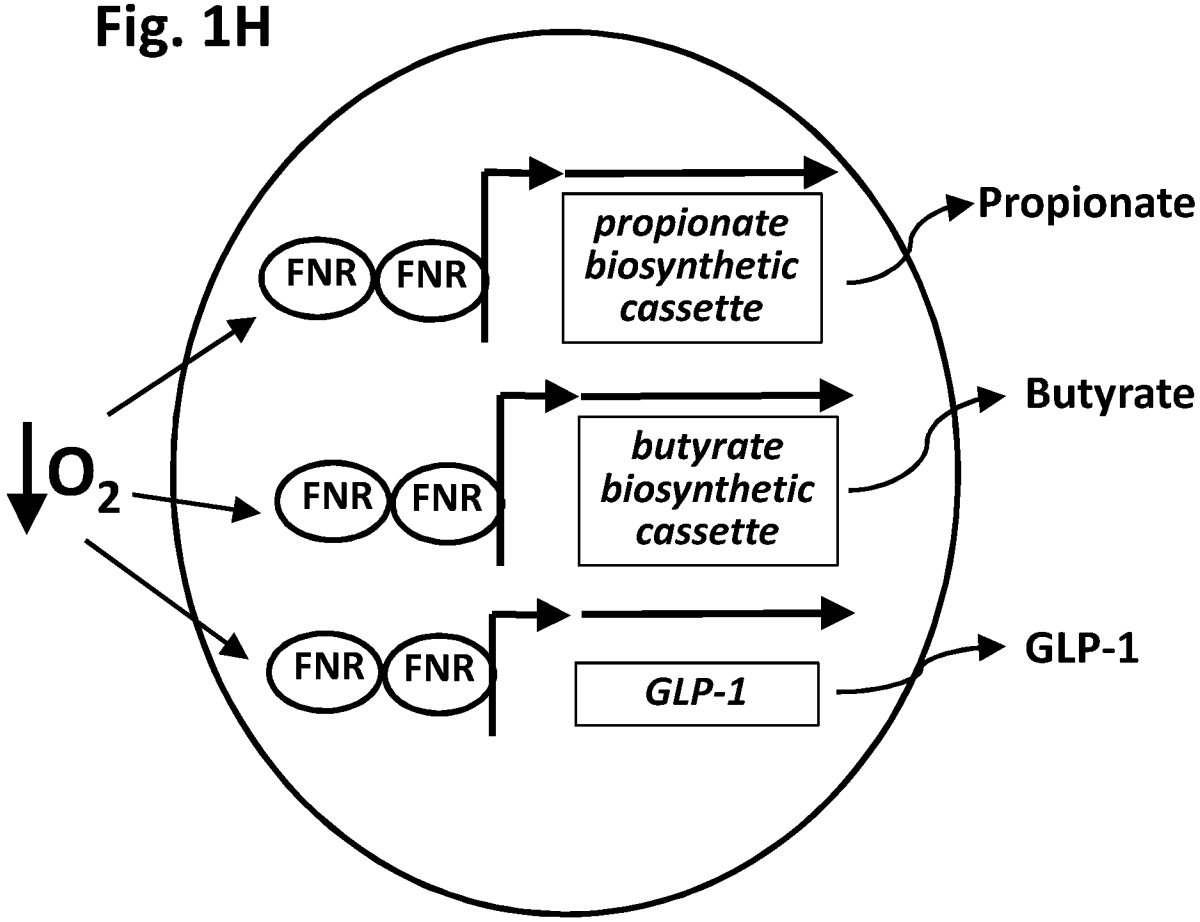
E. Coli Nissle 1917

Fig. 1G



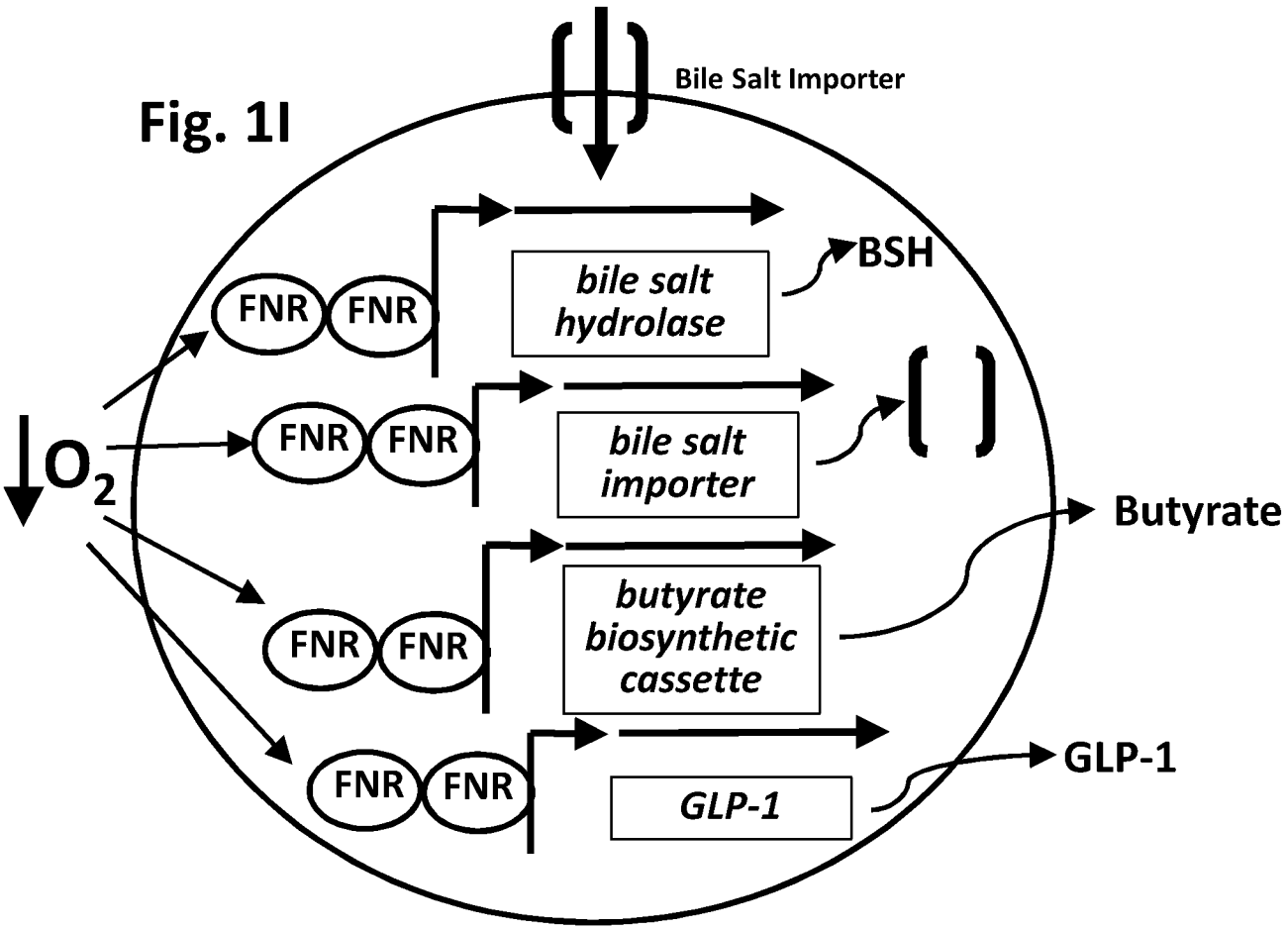
E. Coli Nissle 1917

Fig. 1H



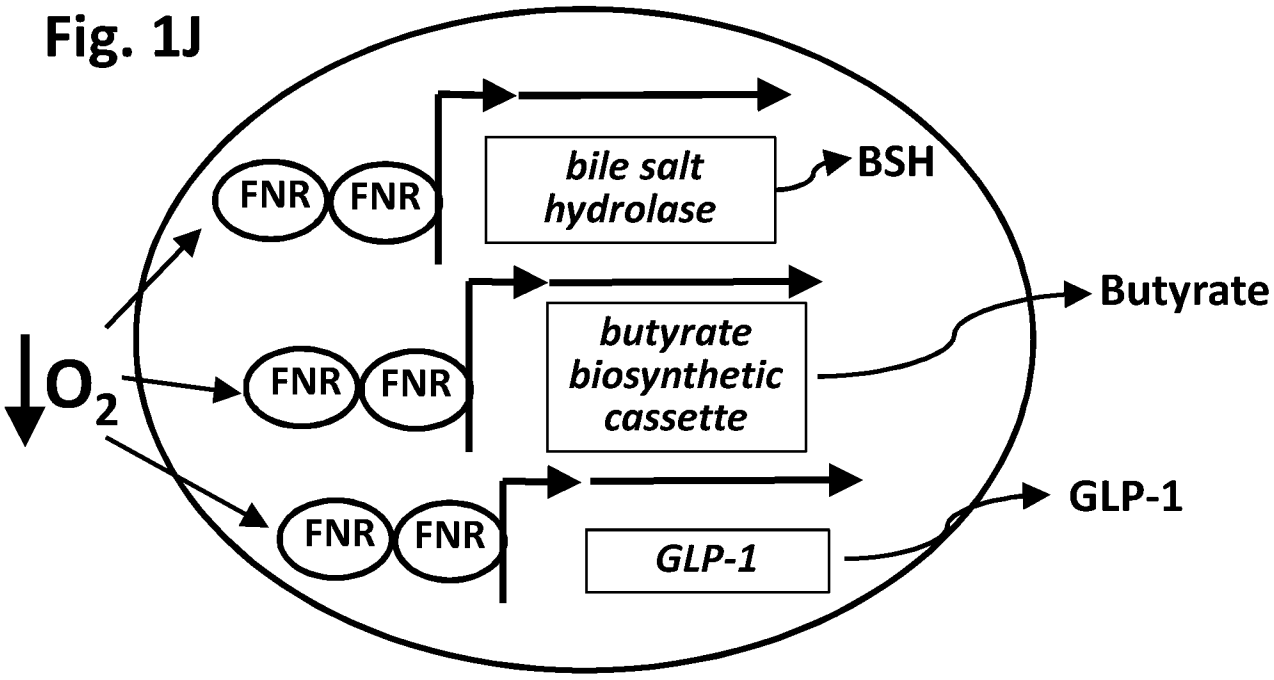
E. Coli Nissle 1917

Fig. 1I



E. Coli Nissle 1917

Fig. 1J



E. Coli Nissle 1917

Fig. 2A

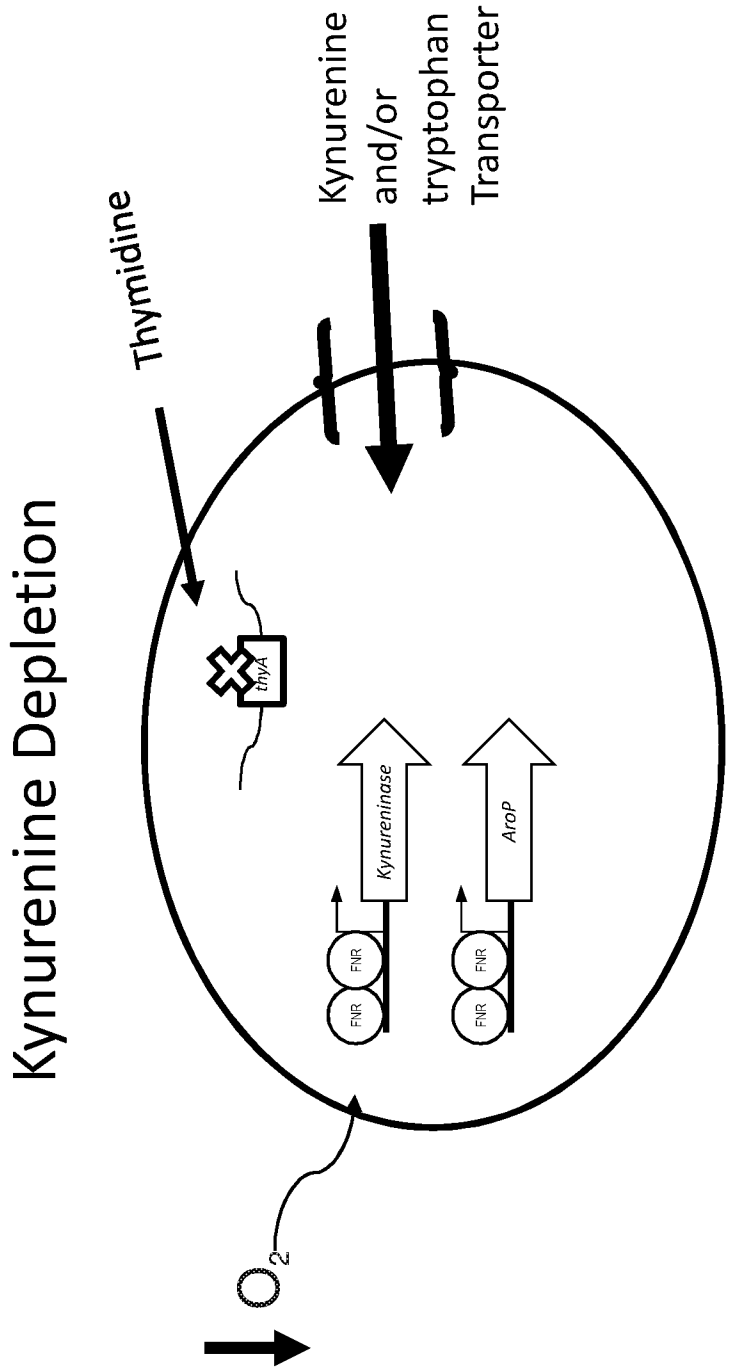

Kynurenine Depletion
Thymidine
Kynurenine and/or tryptophan Transporter
thyA
Kynureninase
AroP
FNR
FNR
FNR
FNR
O2

FIG. 2B

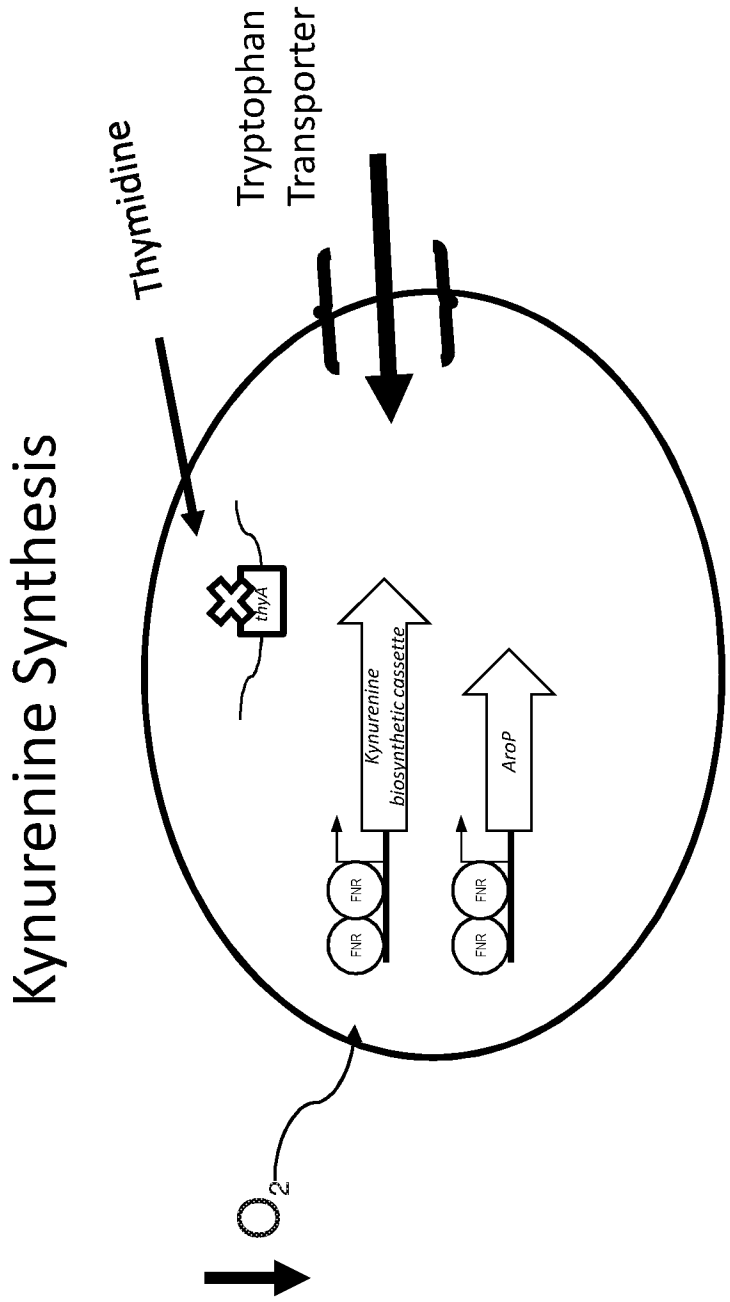

Kynurenine Synthesis
Thymidine
Tryptophan Transporter
thyA
Kynurenine biosynthetic cassette
AroP
FNR
FNR
FNR
FNR
O2

FIG. 2C

Kynurenine Synthesis

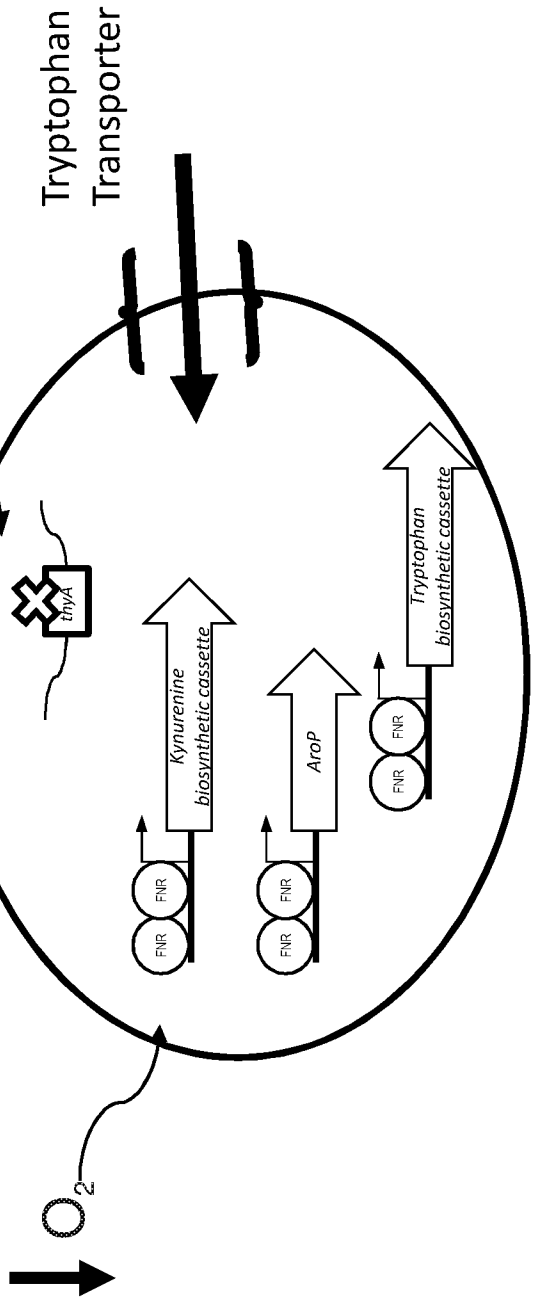


FIG. 2D

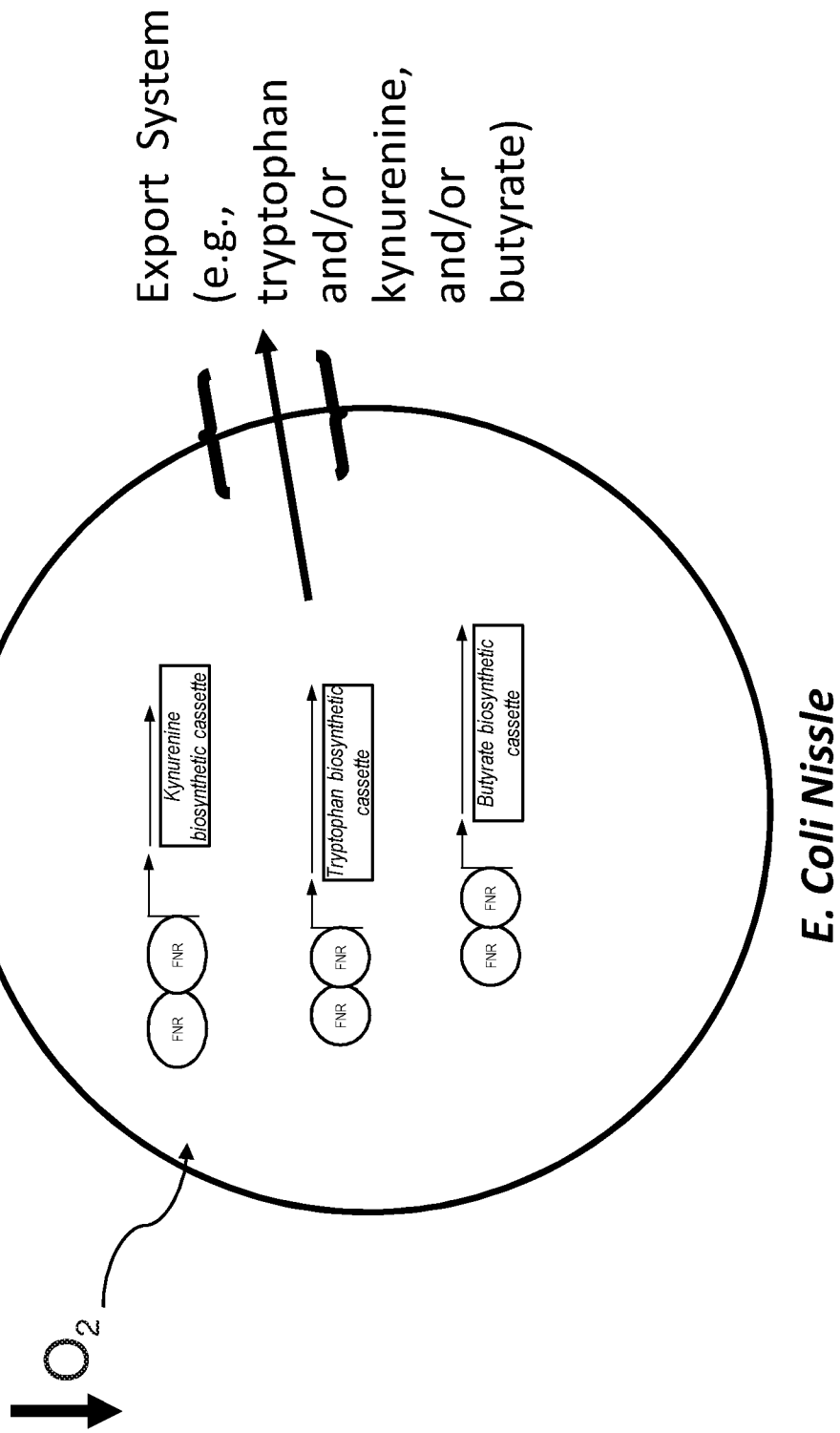

Export System (e.g., tryptophan and/or kynurenine, and/or butyrate)
Kynurenine biosynthetic cassette
Tryptophan biosynthetic cassette
Butyrate biosynthetic cassette
FNR
FNR
FNR
FNR
FNR
FNR
O_2
E. Coli Nissle

FIG. 2E

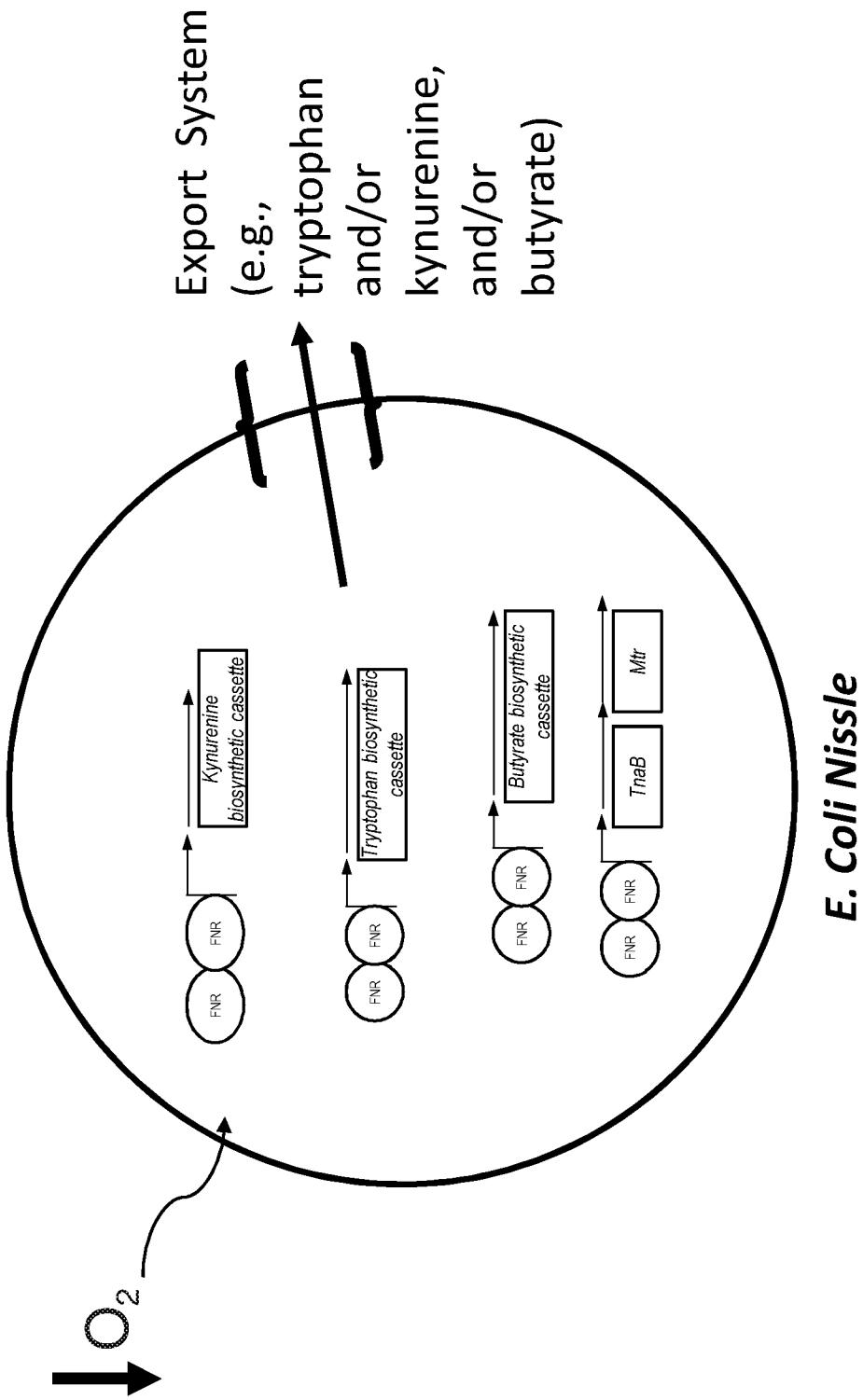

Export System (e.g., tryptophan and/or kynurenine, and/or butyrate)
Kynurenine biosynthetic cassette
Tryptophan biosynthetic cassette
Butyrate biosynthetic cassette
TnaB
Mtr
FNR
O_2
E. Coli Nissle

FIG. 2F

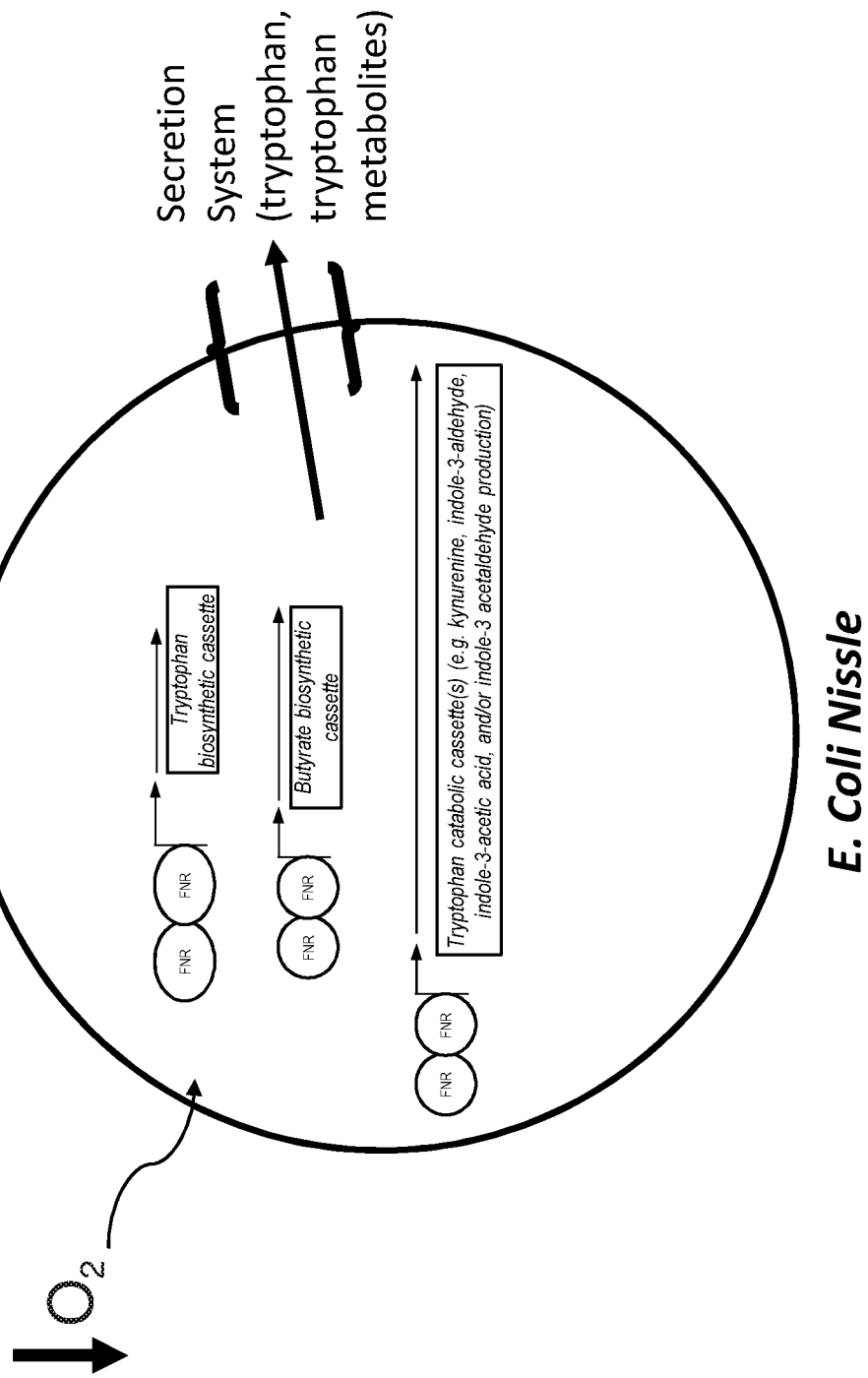

Secretion System (tryptophan, tryptophan metabolites)
Tryptophan biosynthetic cassette
Butyrate biosynthetic cassette
Tryptophan catabolic cassette(s) (e.g. kynurenine, indole-3-aldehyde, indole-3-acetic acid, and/or indole-3 acetaldehyde production)
FNR
FNR
FNR
FNR
FNR
FNR
O2
E. Coli Nissle

FIG. 2G

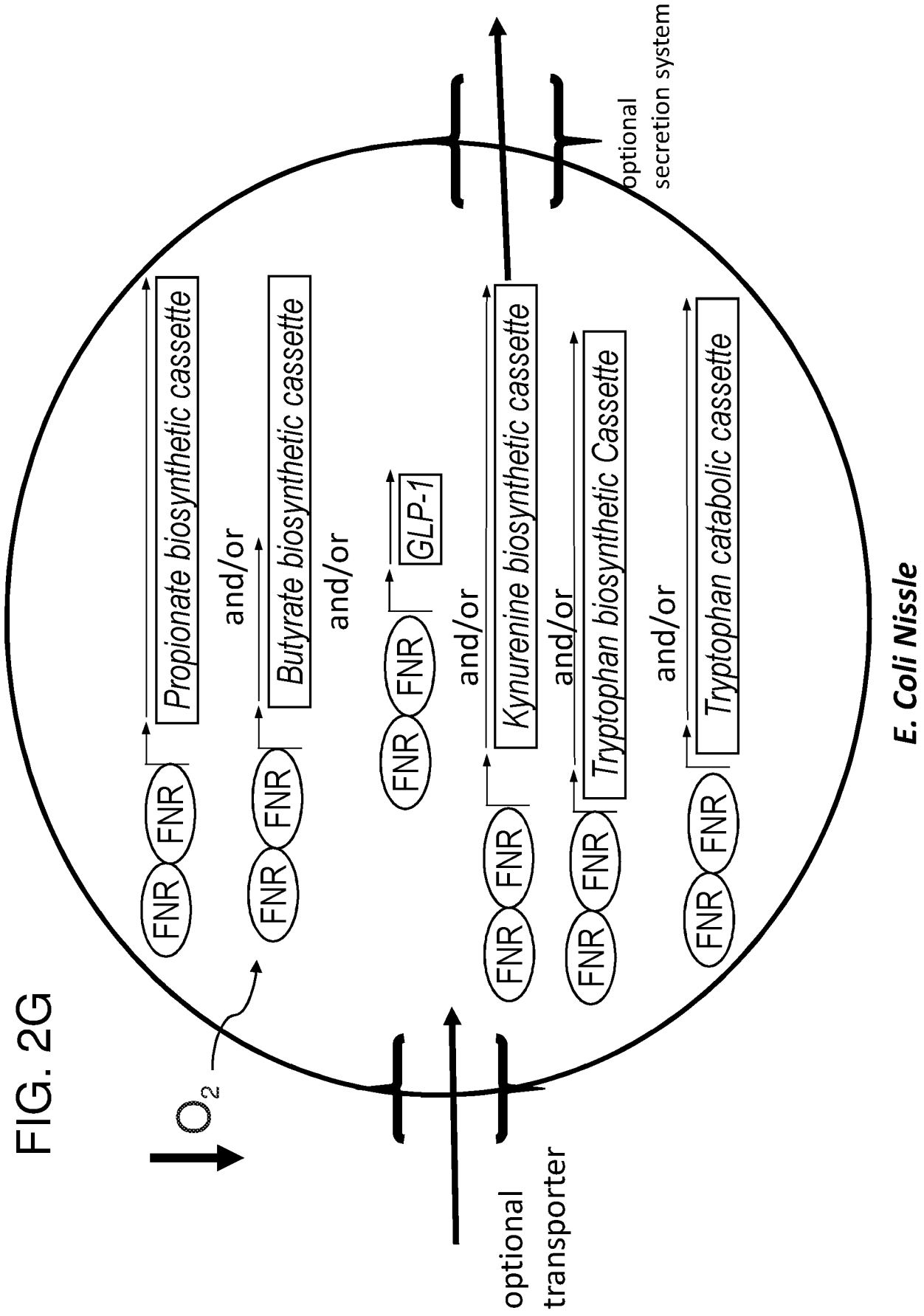

FNR
FNR
Propionate biosynthetic cassette
and/or
FNR
FNR
Butyrate biosynthetic cassette
and/or
FNR
FNR
GLP-1
and/or
FNR
FNR
Kynurenine biosynthetic cassette
and/or
FNR
FNR
Tryptophan biosynthetic Cassette
and/or
FNR
FNR
Tryptophan catabolic cassette
optional secretion system
optional transporter
O_2
E. Coli Nissle

Fig. 3A

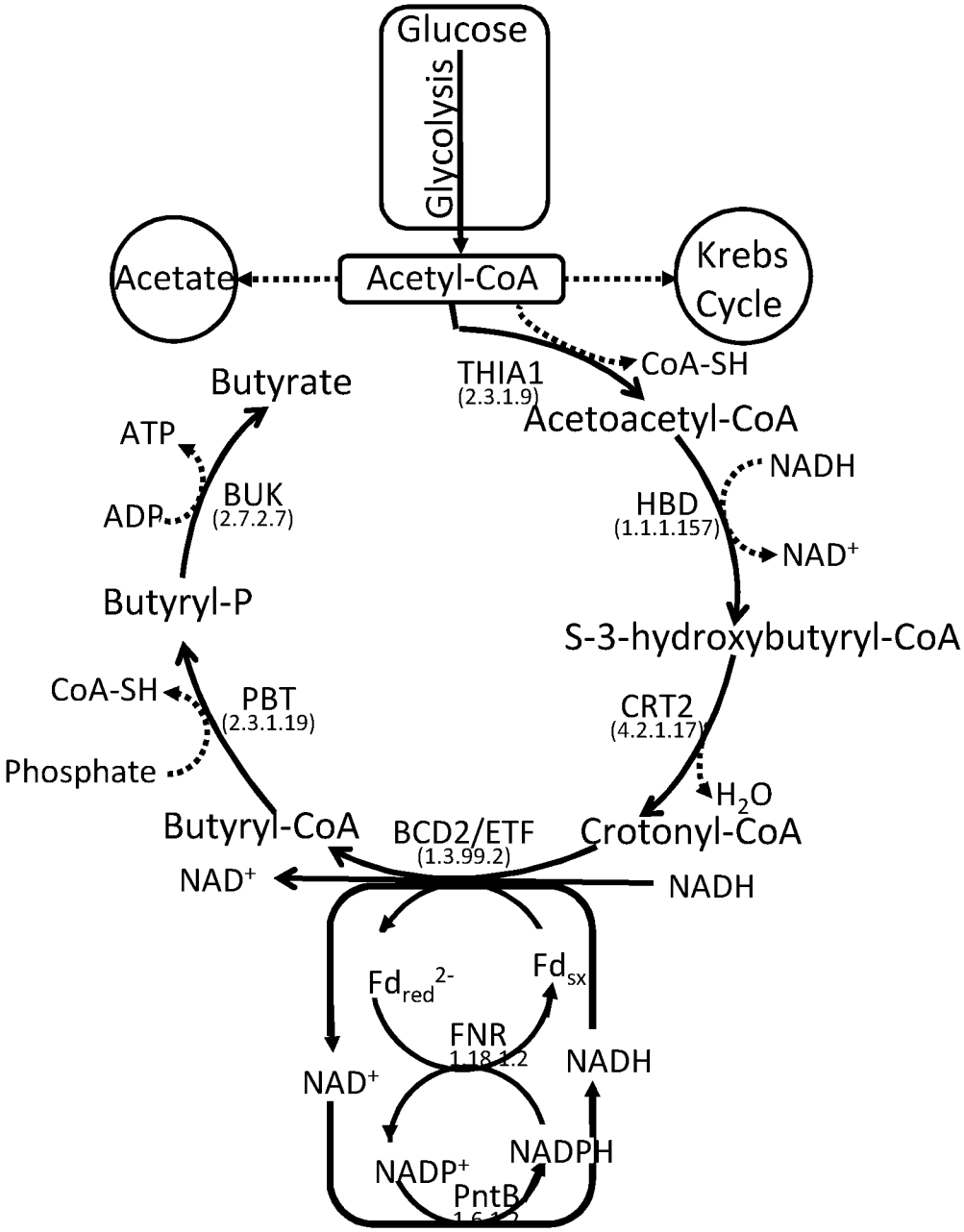

Glucose
Glycolysis
Acetate
Acetyl-CoA
Krebs Cycle
THIA1
(2.3.1.9)
CoA-SH
Acetoacetyl-CoA
NADH
HBD
(1.1.1.157)
NAD+
S-3-hydroxybutyryl-CoA
CRT2
(4.2.1.17)
H2O
Crotonyl-CoA
BCD2/ETF
(1.3.99.2)
NADH
NAD+
Butyryl-CoA
PBT
(2.3.1.19)
CoA-SH
Phosphate
Butyryl-P
BUK
(2.7.2.7)
ATP
ADP
Butyrate
Fdsx
Fdred2-
FNR
1.18.1.2
NADH
NAD+
NADPH
NADP+
PntB

Fig. 3B

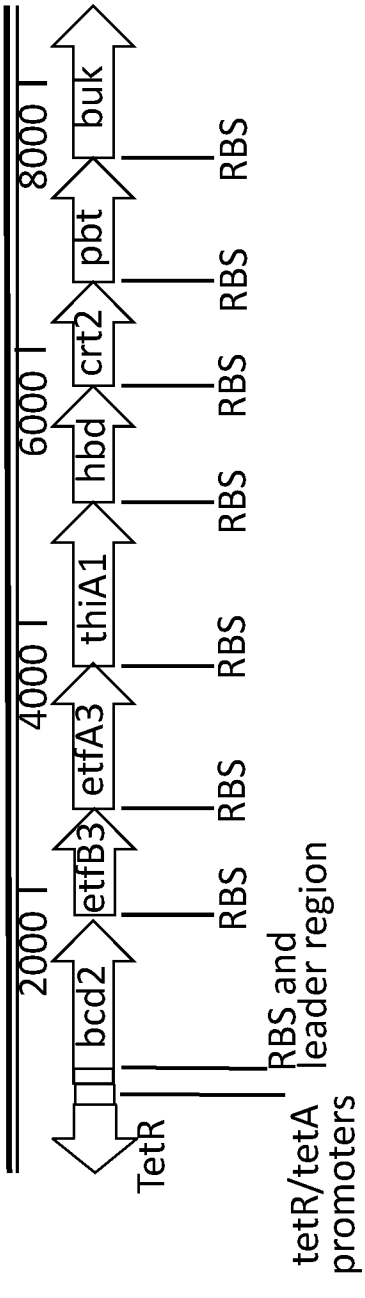

pLogic031
2000
4000
6000
8000
TetR
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
RBS
RBS and leader region
tetR/tetA promoters

Fig. 3C

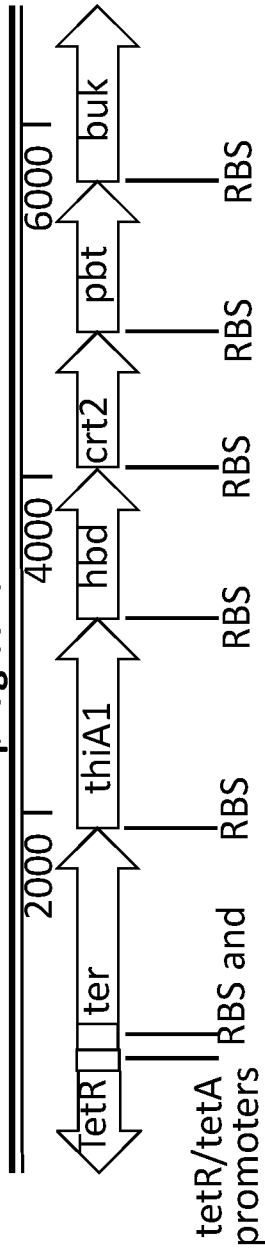

pLogic046
2000
4000
6000
TetR
ter
thiA1
hbd
crt2
pbt
buk
RBS
RBS and leader region
tetR/tetA promoters

Fig. 3D

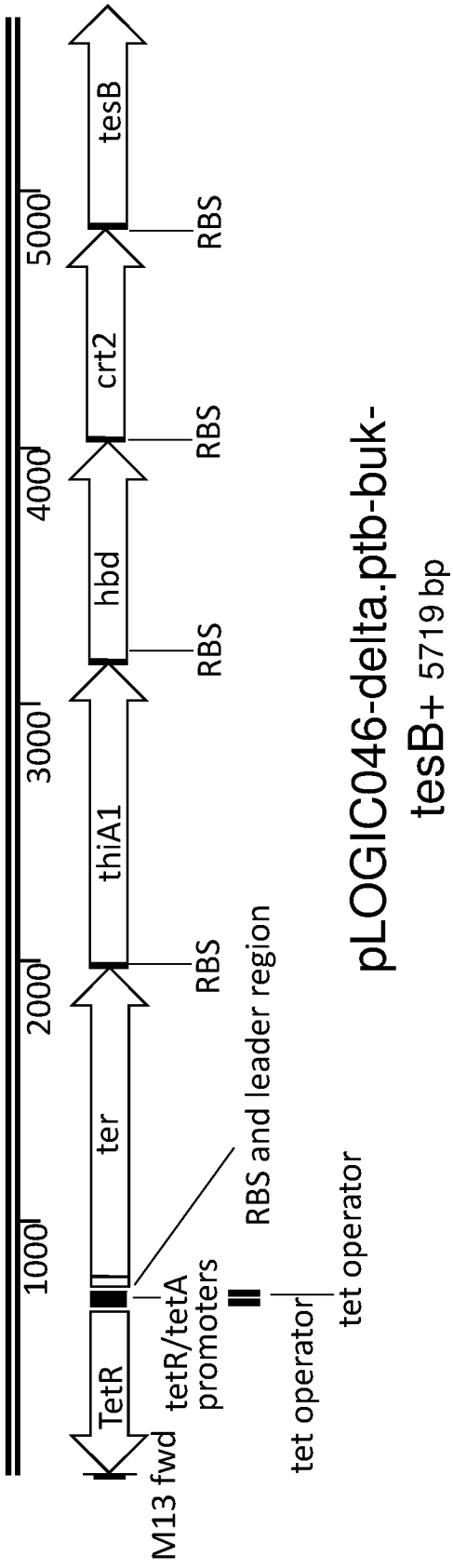

1000
2000
3000
4000
5000
TetR
ter
thiA1
hbd
crt2
tesB
RBS
RBS
RBS
RBS
M13 fwd
tetR/tetA promoters
RBS and leader region
tet operator
tet operator
pLOGIC046-delta.ptb-buk-tesB+ 5719 bp

Fig. 4A

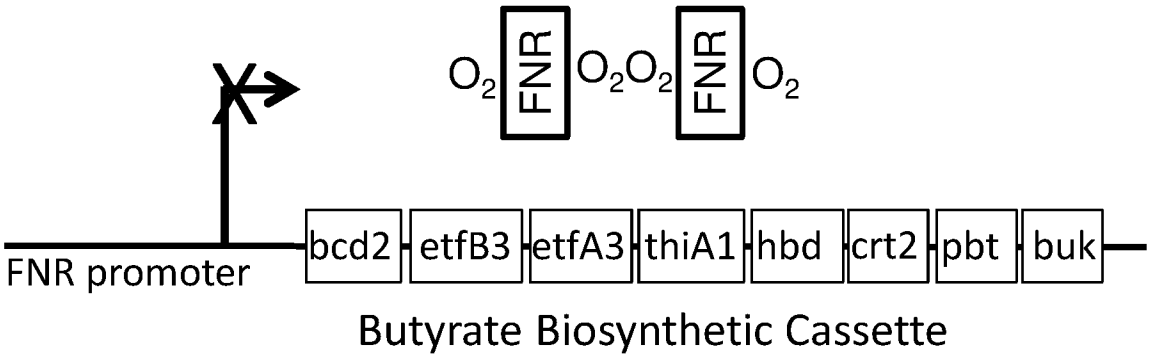
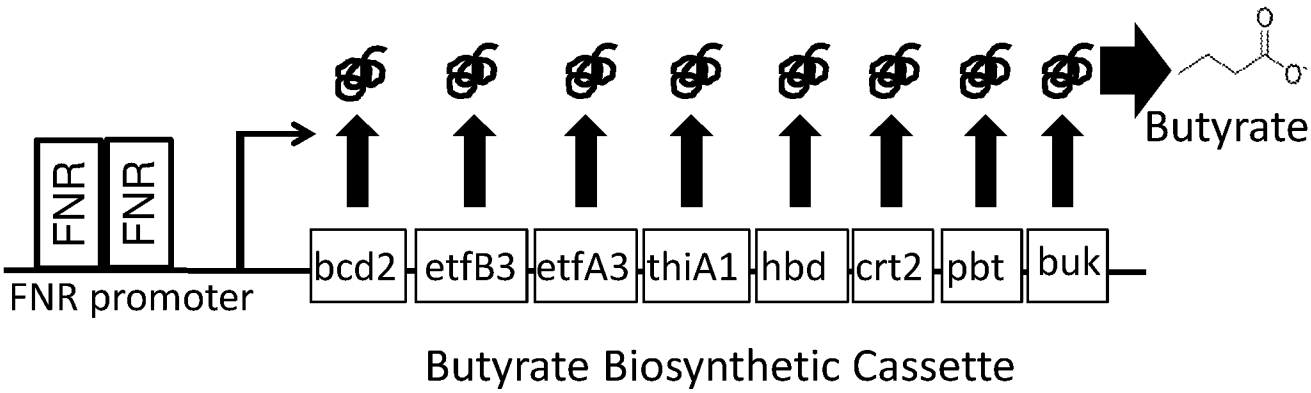

O2
FNR
O2O2
FNR
O2
FNR promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 4B


FNR
FNR
FNR promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate
Butyrate Biosynthetic Cassette


bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette
NsrR
NsrR

Fig. 4C

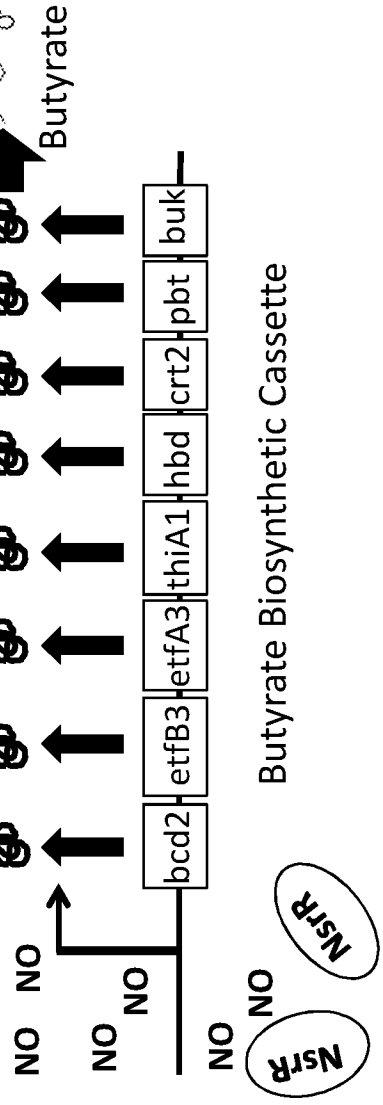

Butyrate
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette
NO
NO
NO
NO
NO
NO
NsrR
NsrR

Fig. 4D

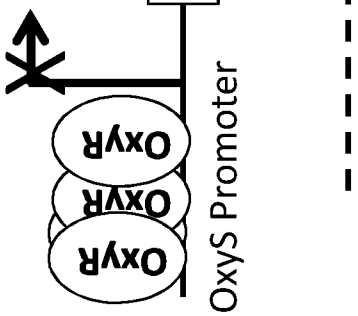

OxyR
OxyR
OxyR
OxyS Promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 4E

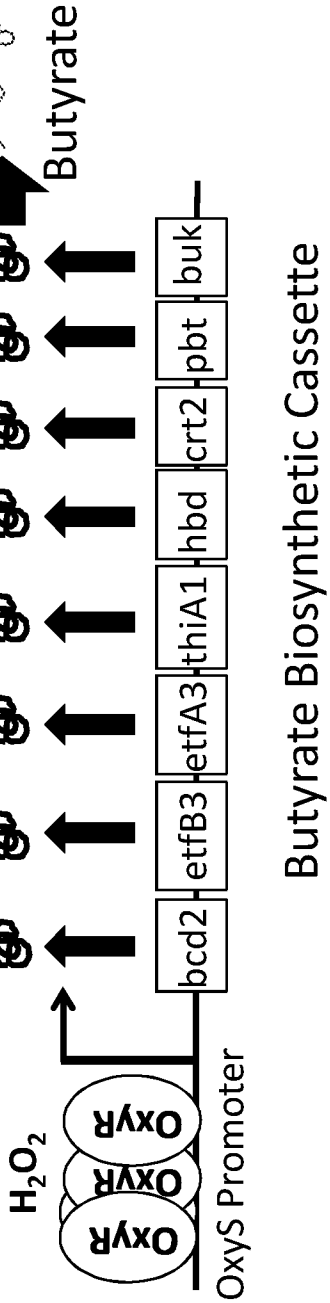

Butyrate
H_2O_2
OxyR
OxyR
OxyR
OxyS Promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 4F

Fig. 5A

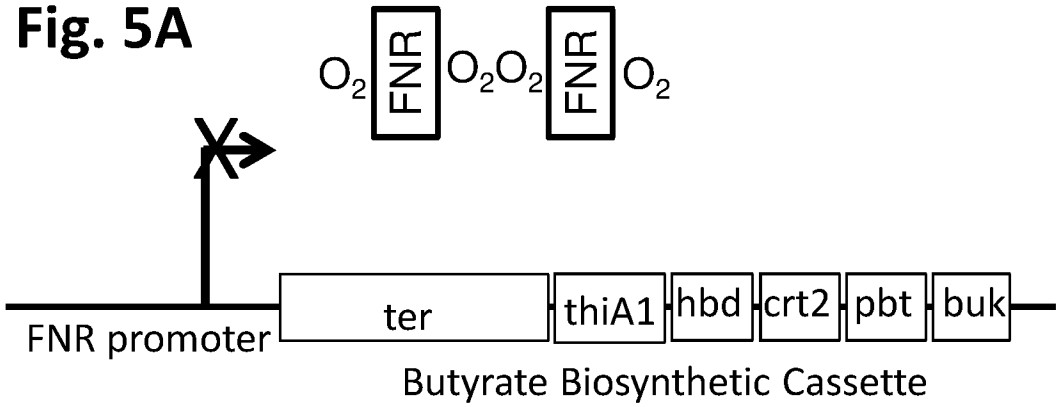
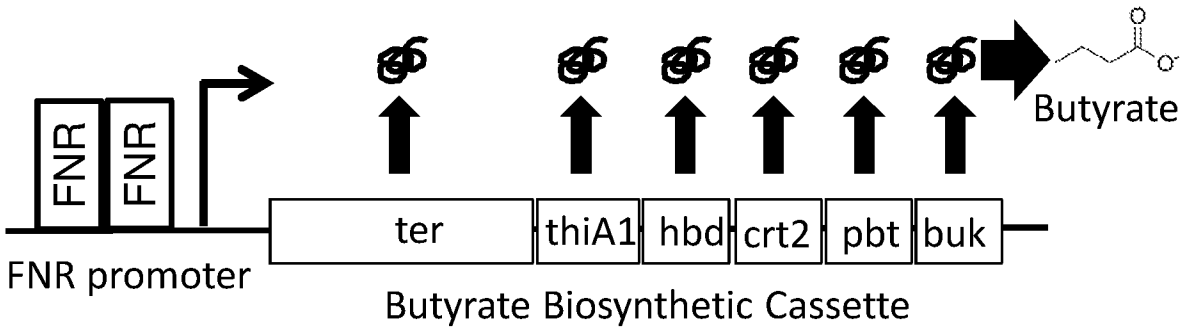

O2
FNR
O2O2
FNR
O2
FNR promoter
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 5B


FNR
FNR
Butyrate
ter
thiA1
hbd
crt2
pbt
buk
FNR promoter
Butyrate Biosynthetic Cassette

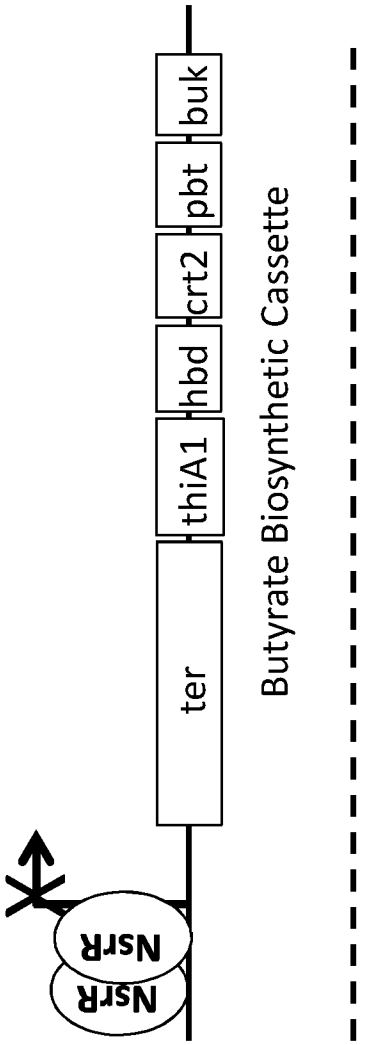

NsrR
NsrR
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 5C


Butyrate
NO
NO
NO
NO
NO
NO
NsrR
NsrR
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 5D

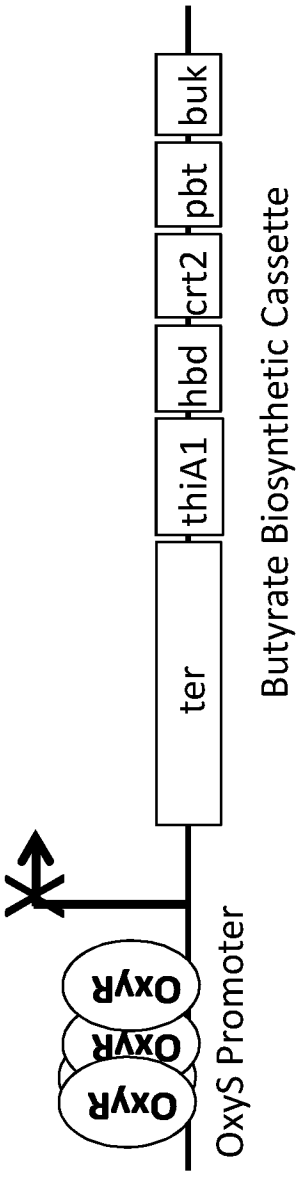

OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 5E

Butyrate
H_2O_2
OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

Fig. 5F

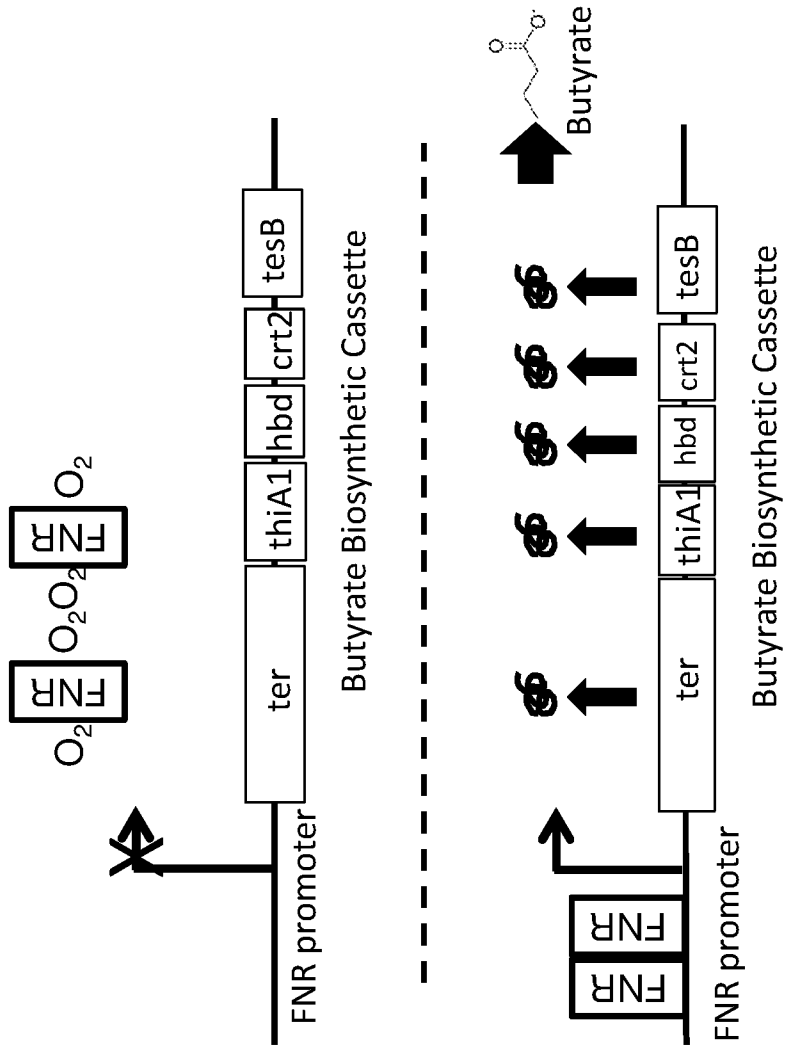

FNR
FNR
O_2
O_2
O_2
O_2
FNR promoter
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette
Butyrate

Fig. 6A

Fig. 6B

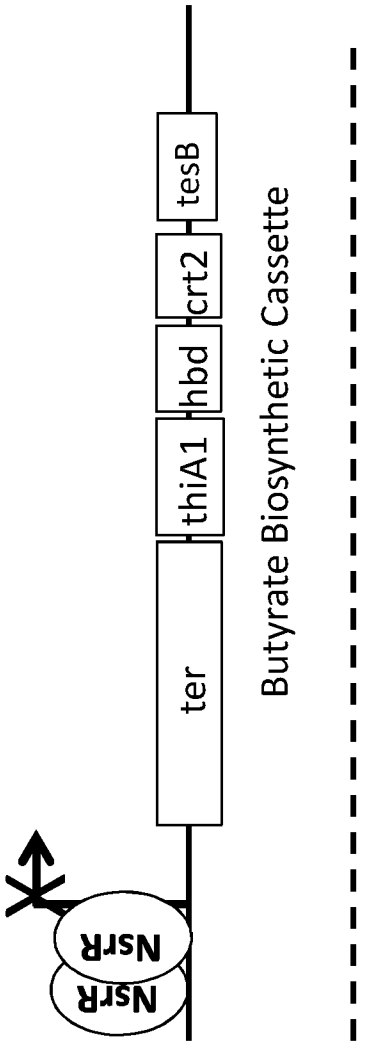

NsrR
NsrR
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

Fig. 6C


Butyrate
NO
NO
NO
NO
NO
NO
NsrR
NsrR
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

Fig. 6D

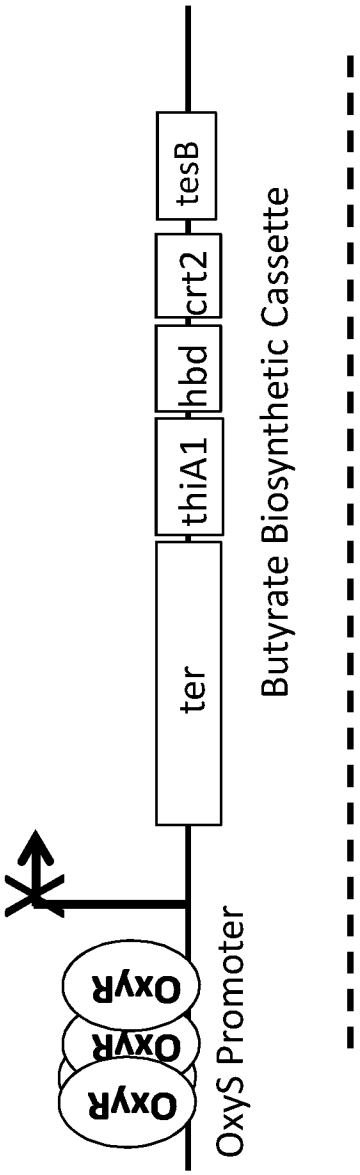

OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

Fig. 6E

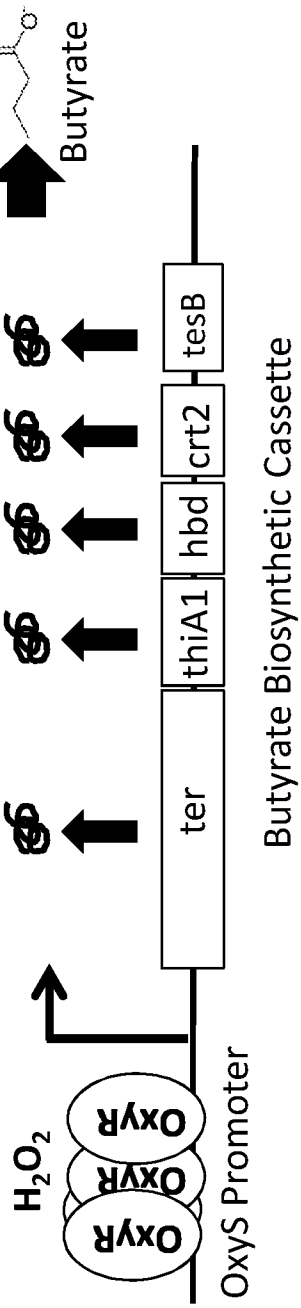

Butyrate
H_2O_2
OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

Fig. 6F

Fig. 7A

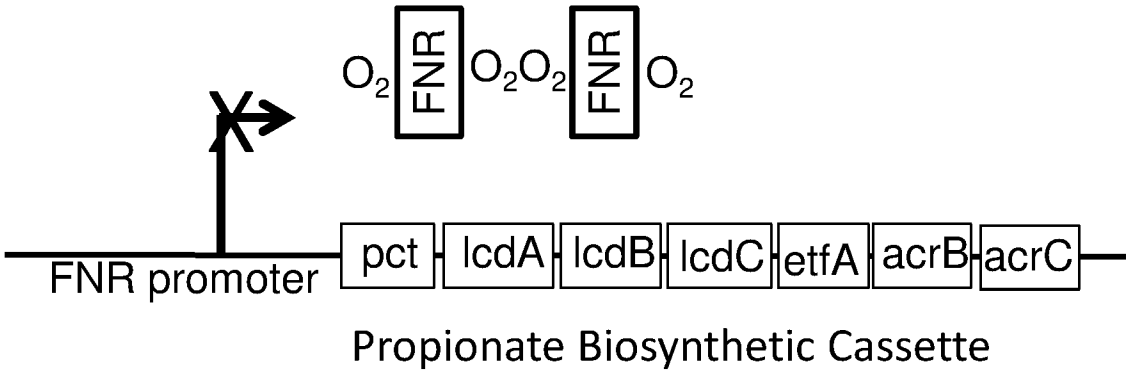

O2
FNR
O2O2
FNR
O2
FNR promoter
pct
lcdA
lcdB
lcdC
etfA
acrB
acrC
Propionate Biosynthetic Cassette

Fig. 7B

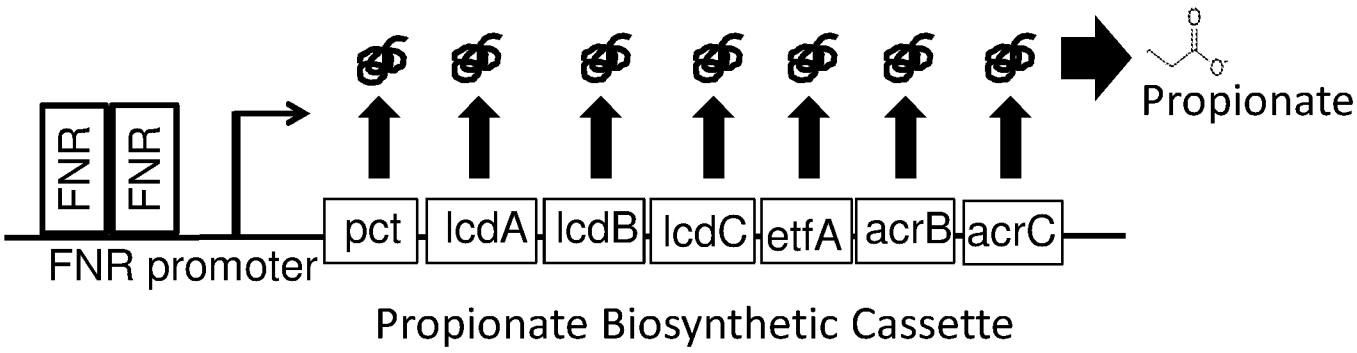

FNR
FNR
FNR promoter
pct
lcdA
lcdB
lcdC
etfA
acrB
acrC
Propionate
Propionate Biosynthetic Cassette

Fig. 7C

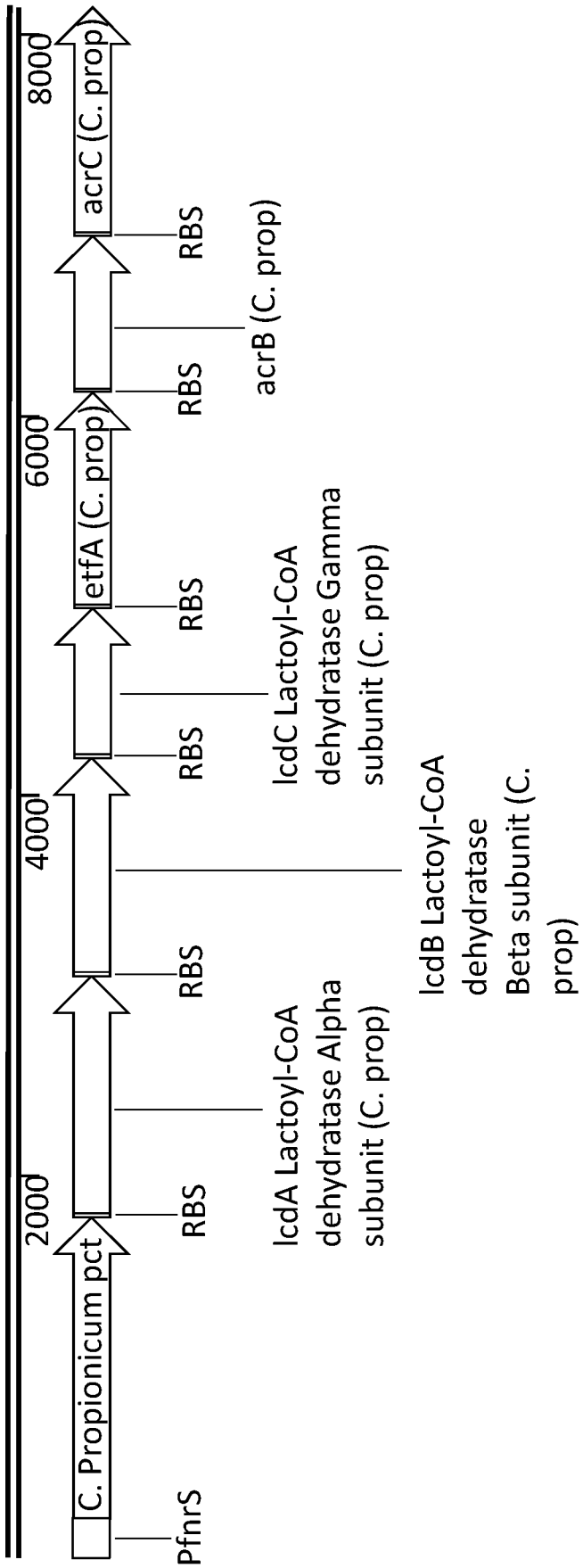

2000
4000
6000
8000
PfnrS
C. Propionicum pct
RBS
lcdA Lactoyl-CoA dehydratase Alpha subunit (C. prop)
RBS
lcdB Lactoyl-CoA dehydratase Beta subunit (C. prop)
RBS
lcdC Lactoyl-CoA dehydratase Gamma subunit (C. prop)
etfA (C. prop)
RBS
acrB (C. prop)
RBS
acrC (C. prop)

Fig. 8A

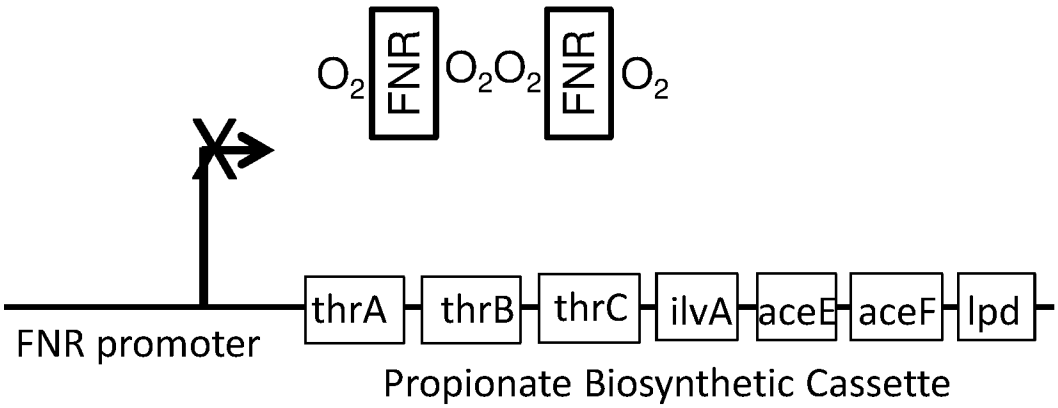
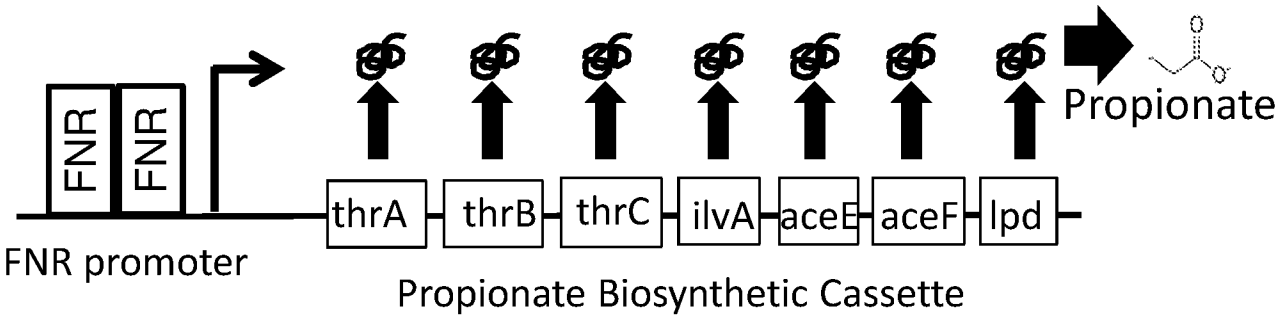

O2
FNR
O2O2
FNR
O2
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
FNR promoter
Propionate Biosynthetic Cassette

Fig. 8B


FNR
FNR
Propionate
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
FNR promoter
Propionate Biosynthetic Cassette

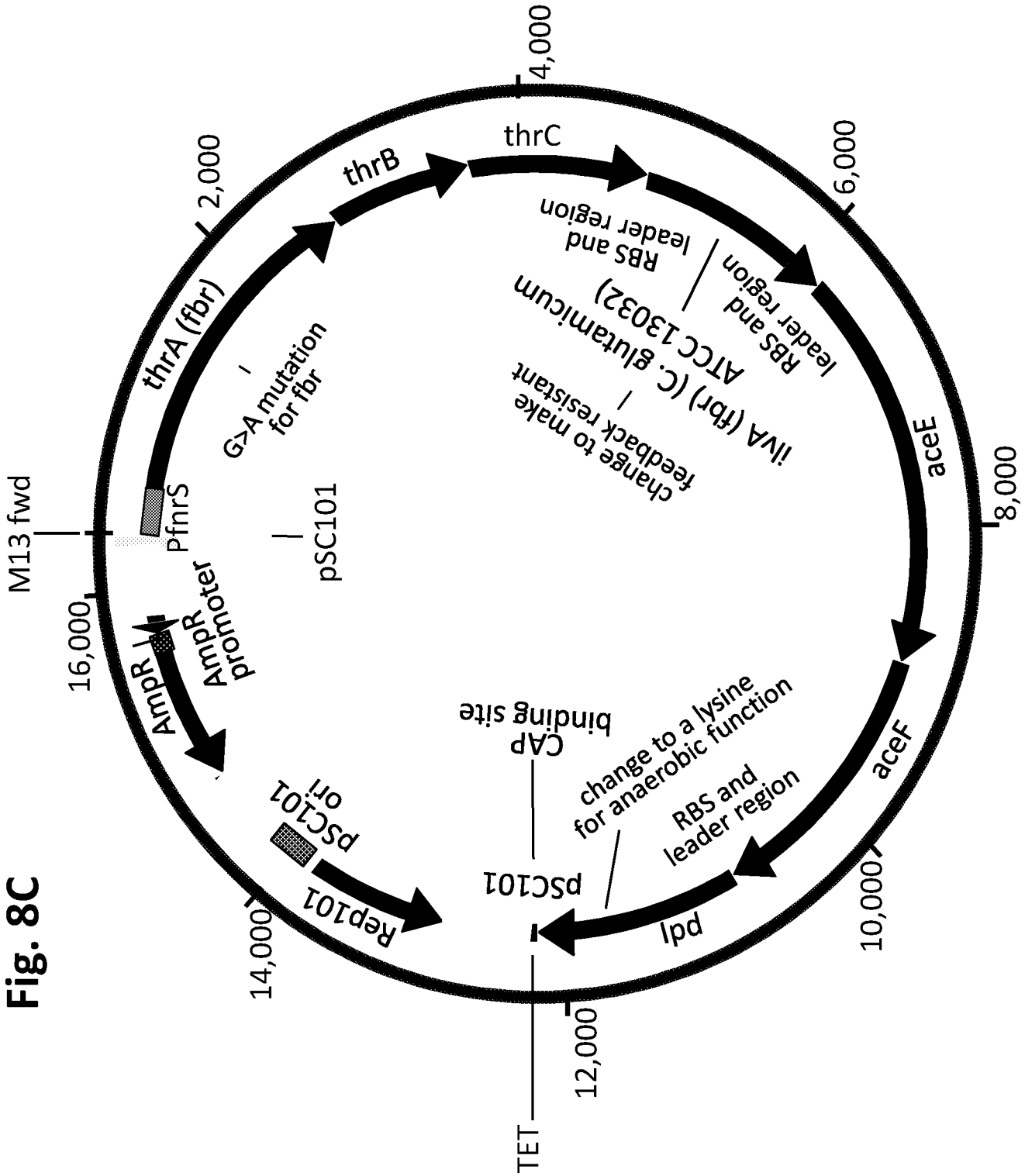

2,000
4,000
6,000
8,000
10,000
12,000
14,000
16,000
M13 fwd
TET
thrB
thrC
thrA (fbr)
aceE
aceF
lpd
PfnrS
pSC101
pSC101 ori
Rep101
AmpR
AmpR promoter
CAP binding site
RBS and leader region
RBS and leader region
ilvA (fbr) (C. glutamicum ATCC 13032)
change to make feedback resistant
G>A mutation for fbr
change to a lysine for anaerobic function

Fig. 8C

Fig. 8D

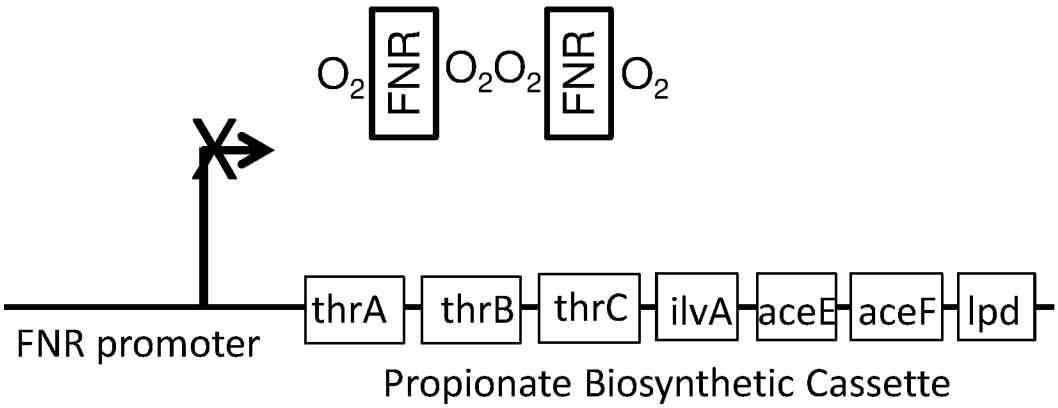
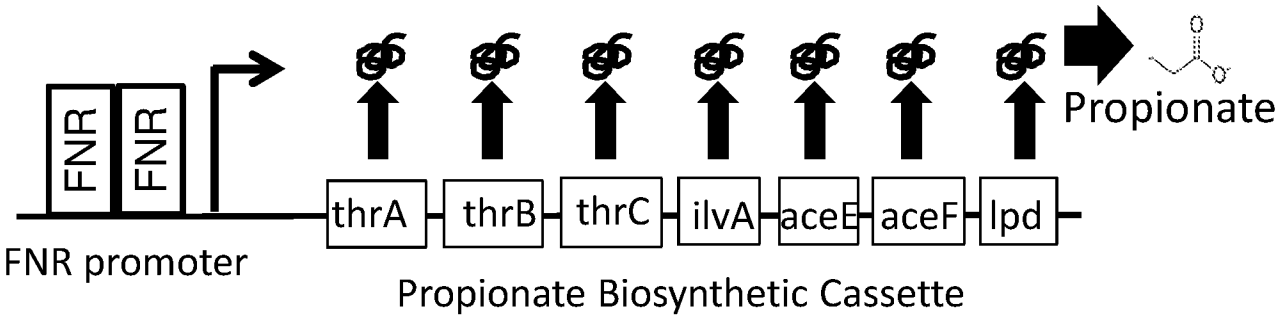

O2
FNR
O2O2
FNR
O2
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
FNR promoter
Propionate Biosynthetic Cassette

Fig. 8E


FNR
FNR
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
Propionate
FNR promoter
Propionate Biosynthetic Cassette

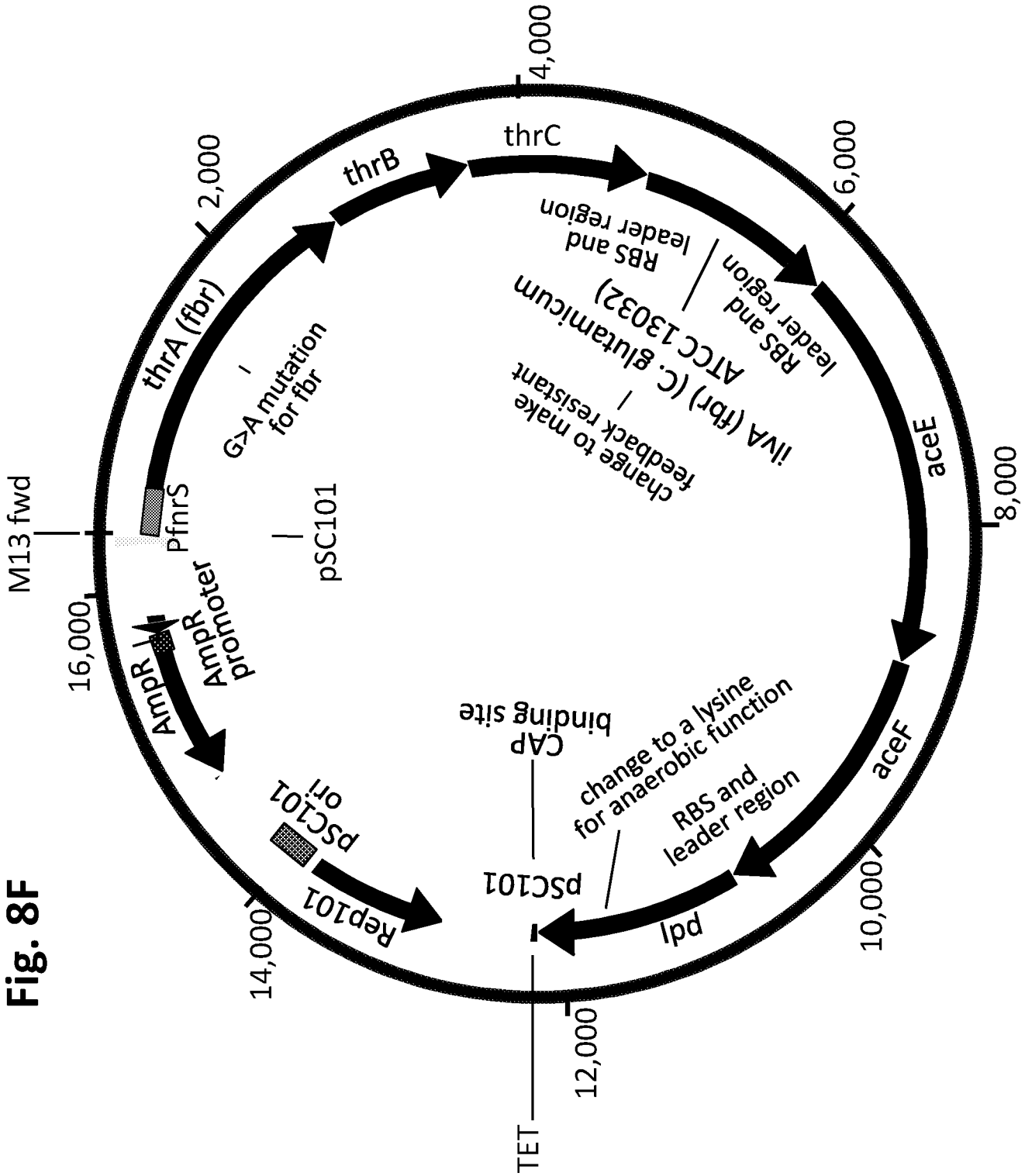

M13 fwd
2,000
4,000
6,000
8,000
10,000
12,000
14,000
16,000
TET
thrB
thrC
thrA (fbr)
PfnrS
G>A mutation for fbr
pSC101
change to make feedback resistant
ilvA (fbr) (C. glutamicum ATCC 13032)
RBS and leader region
RBS and leader region
aceE
aceF
pdh
change to a lysine for anaerobic function
RBS and leader region
CAP binding site
pSC101
pSC101 ori
Rep101
AmpR
AmpR promoter

Fig. 8F

FIG. 9A

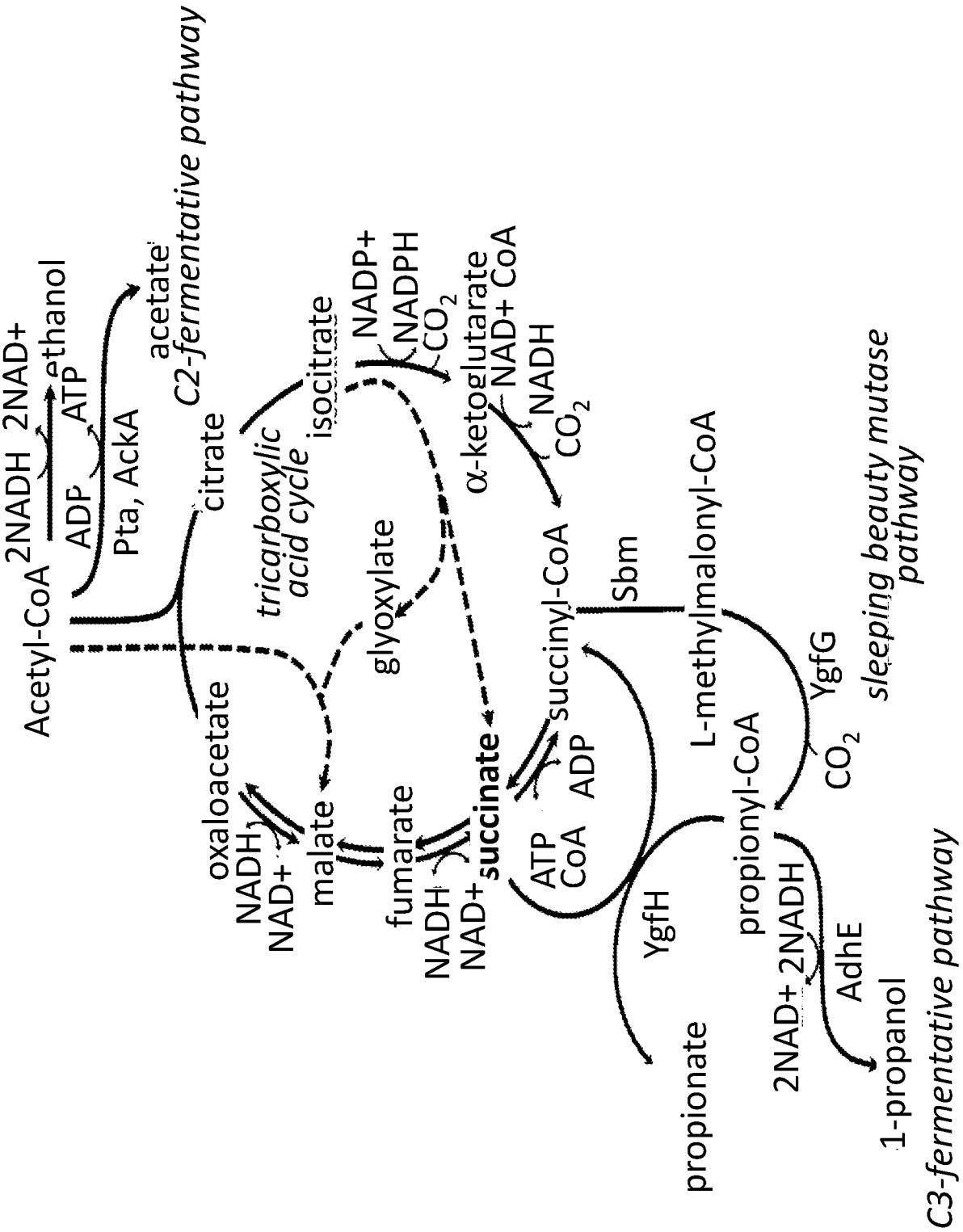

AdhE
Acetyl-CoA
2NADH
2NAD+
ethanol
ATP
ADP
Pta, AckA
acetate
C2-fermentative pathway
citrate
tricarboxylic acid cycle
isocitrate
NADP+
NADPH
CO2
α-ketoglutarate
NAD+ CoA
NADH
CO2
oxaloacetate
NADH
NAD+
malate
glyoxylate
fumarate
NADH
NAD+
succinate
succinyl-CoA
ATP
CoA
ADP
Sbm
L-methylmalonyl-CoA
YgfH
propionyl-CoA
YgfG
CO2
sleeping beauty mutase pathway
propionate
2NAD+ 2NADH
AdhE
1-propanol
C3-fermentative pathway

Fig. 9B

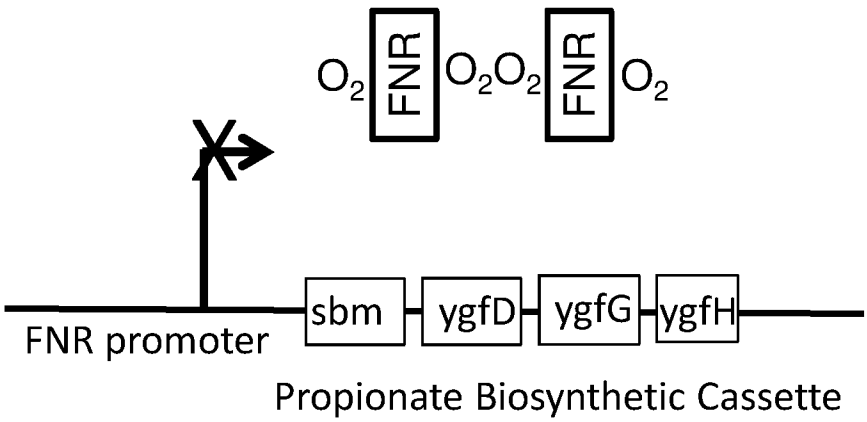

O2
FNR
O2O2
FNR
O2
sbm
ygfD
ygfG
ygfH
FNR promoter
Propionate Biosynthetic Cassette

Fig. 9C

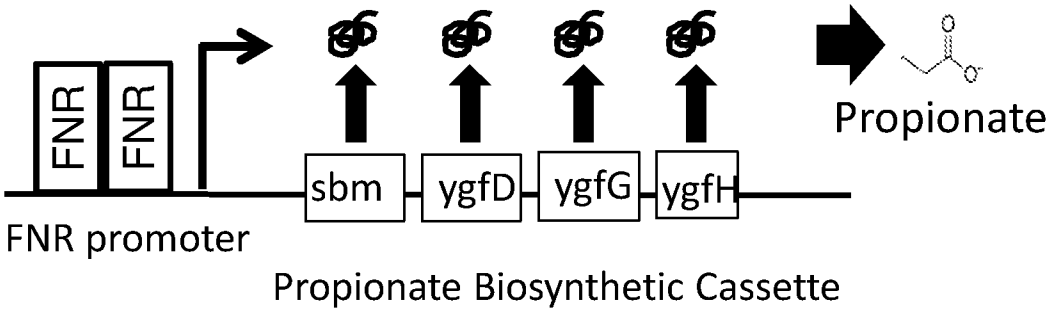

FNR
FNR
sbm
ygfD
ygfG
ygfH
Propionate
FNR promoter
Propionate Biosynthetic Cassette

FIG. 10

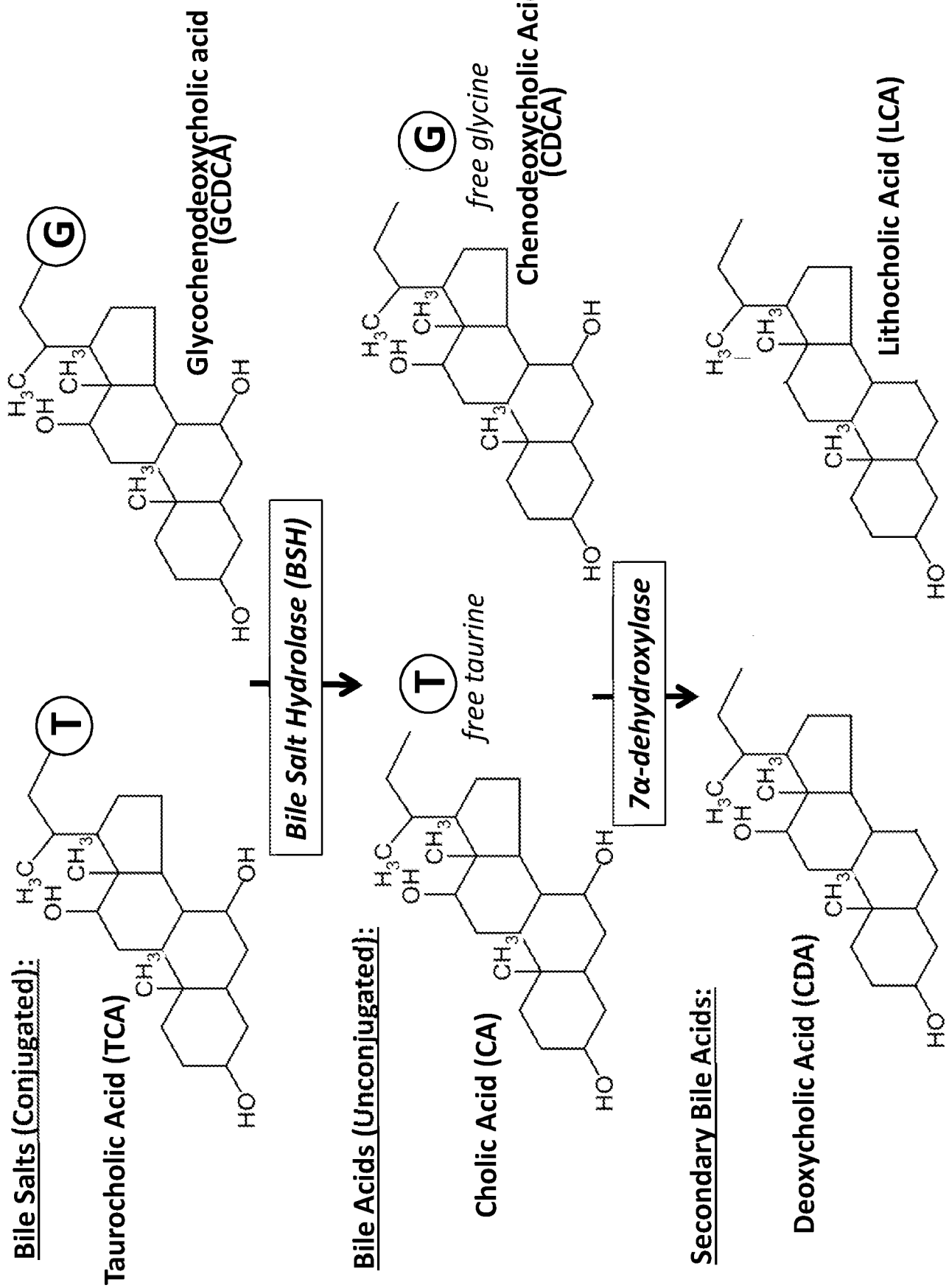

Bile Salts (Conjugated):
Taurocholic Acid (TCA)
T
Glycochenodeoxycholic acid (GCDCA)
G
Bile Salt Hydrolase (BSH)
Bile Acids (Unconjugated):
Cholic Acid (CA)
free taurine
Chenodeoxycholic Acid (CDCA)
free glycine
7α-dehydroxylase
Secondary Bile Acids:
Deoxycholic Acid (CDA)
Lithocholic Acid (LCA)

FIG. 11

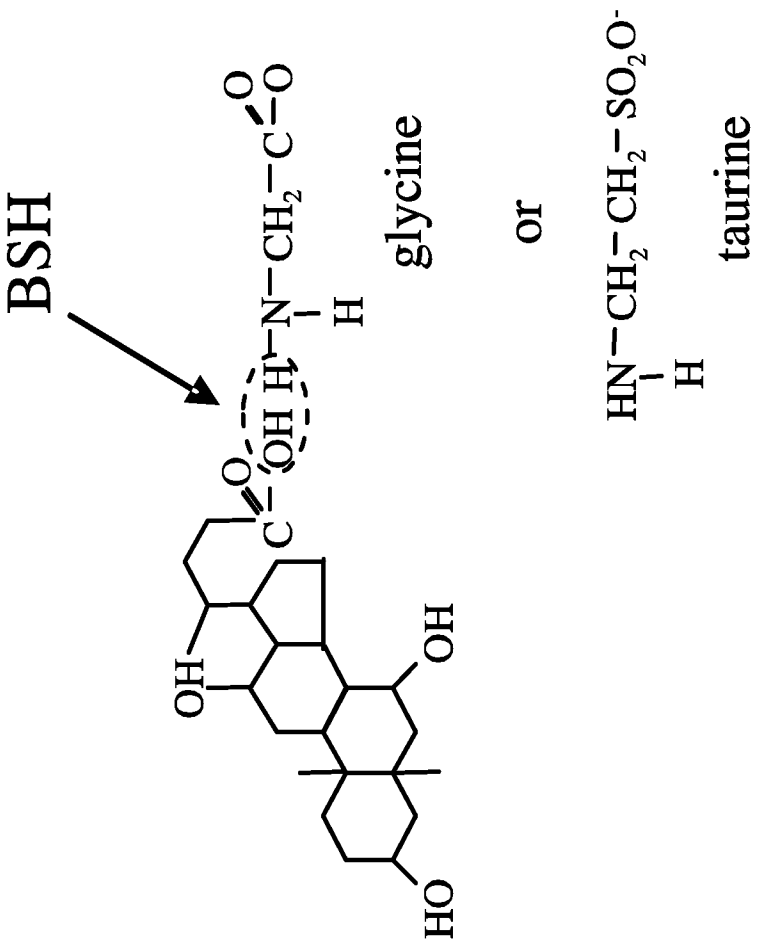


FIG. 12

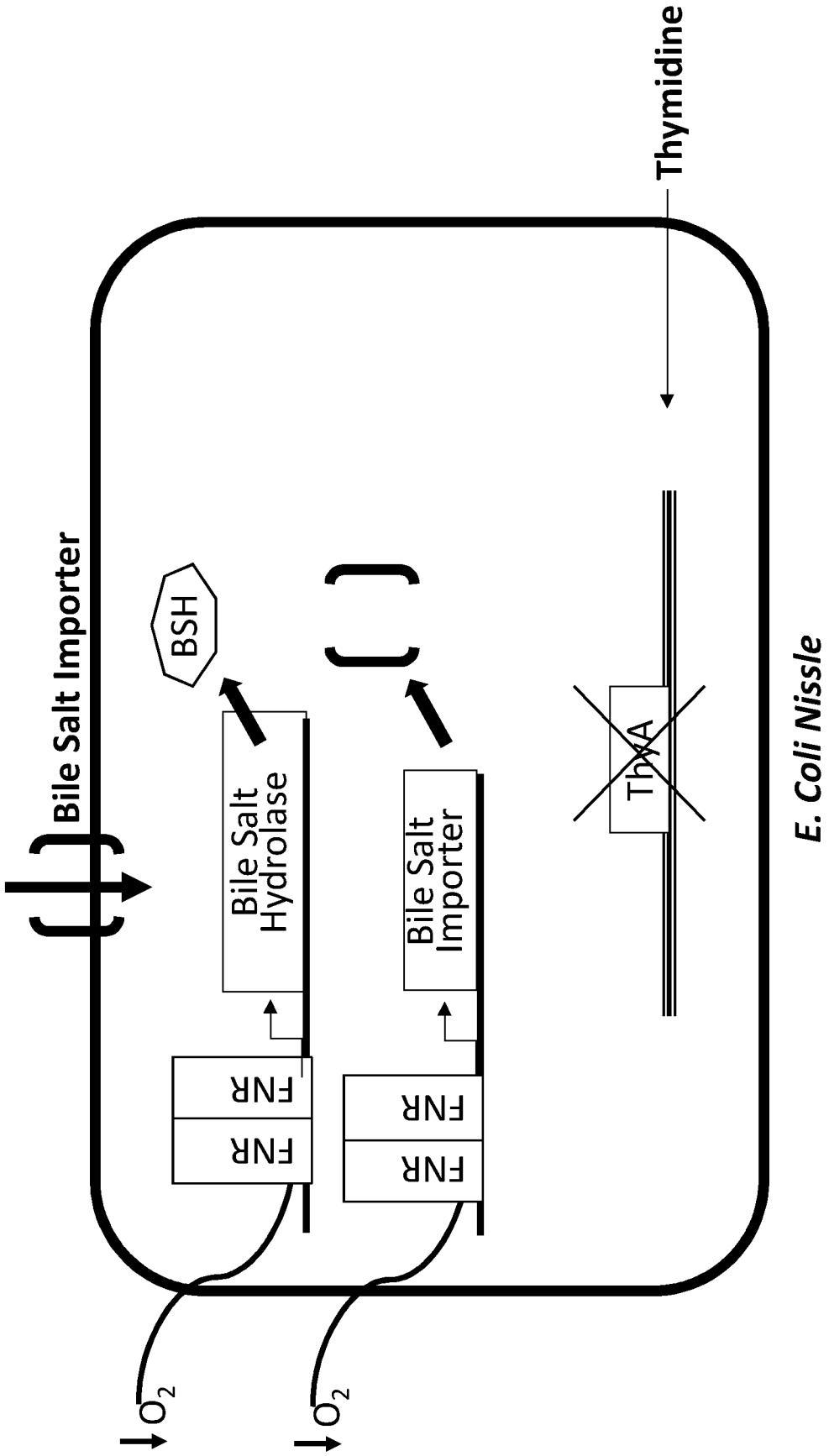

Thymidine
Bile Salt Importer
BSH
Bile Salt Hydrolase
Bile Salt Importer
FNR
FNR
FNR
FNR
ThyA
E. Coli Nissle
O2
O2

FIG. 13

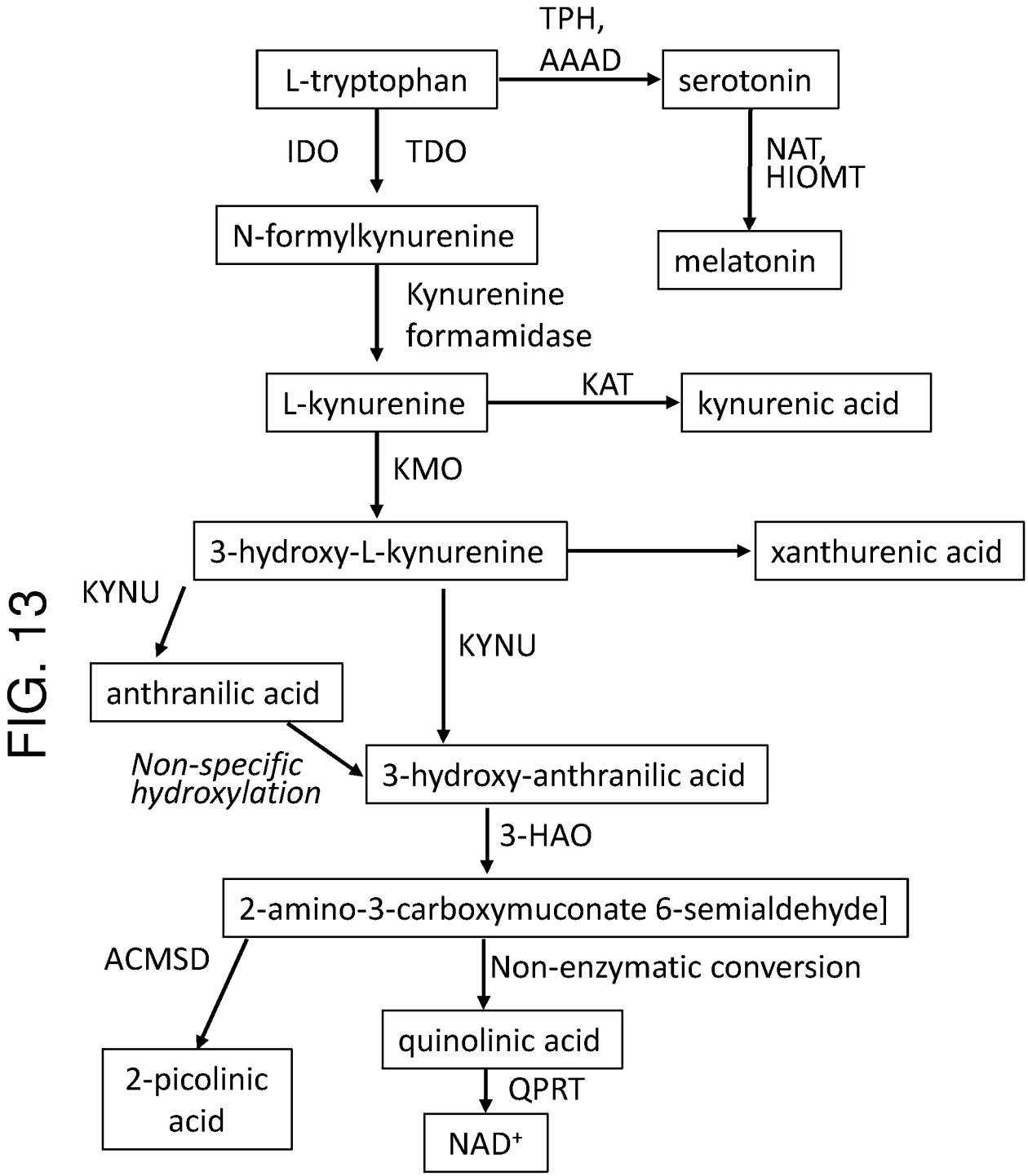
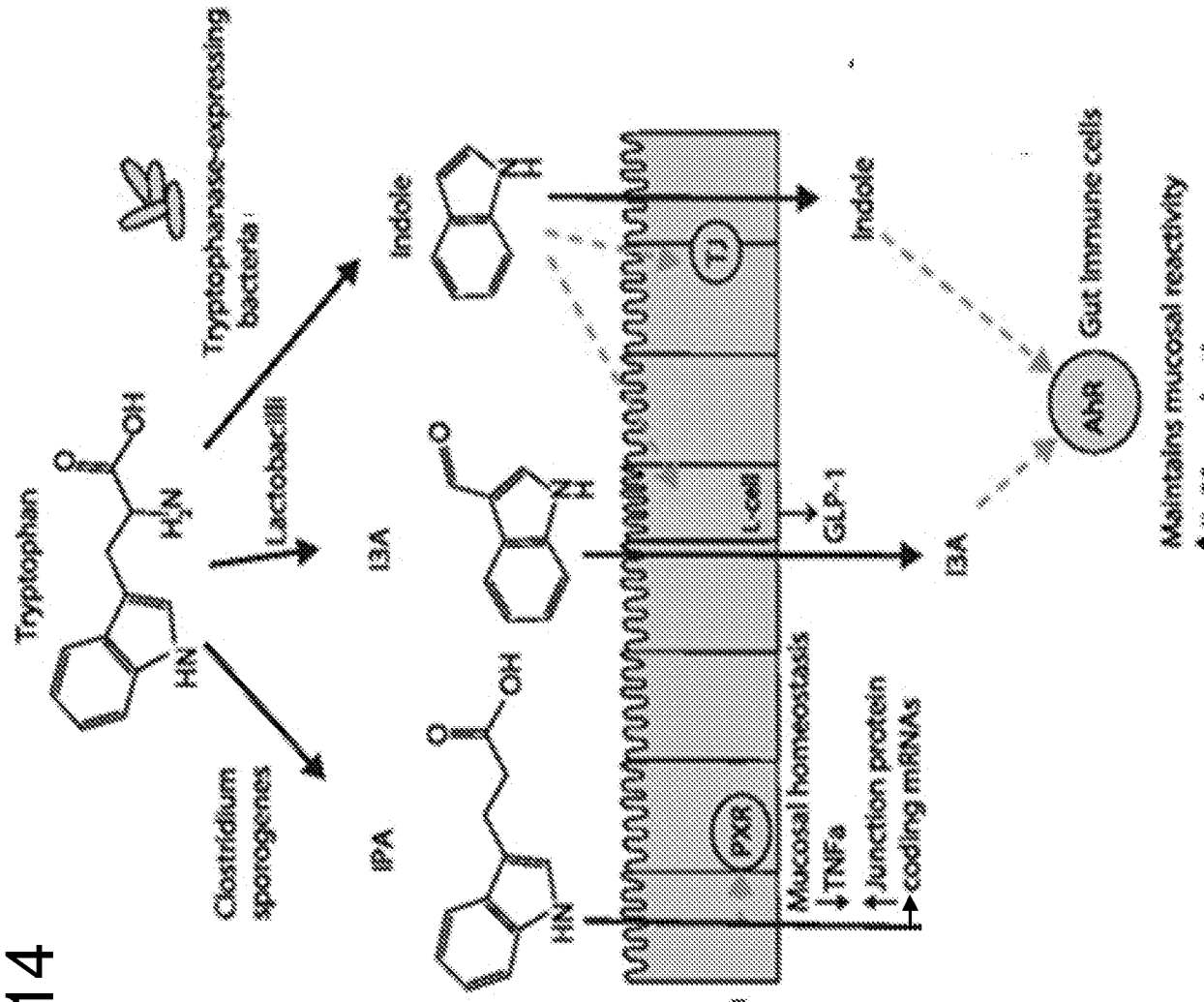

L-tryptophan
TPH,
AAAD
serotonin
IDO
TDO
NAT,
HIOMT
N-formylkynurenine
melatonin
Kynurenine
formamidase
L-kynurenine
KAT
kynurenic acid
KMO
3-hydroxy-L-kynurenine
xanthurenic acid
KYNU
KYNU
anthranilic acid
Non-specific
hydroxylation
3-hydroxy-anthranilic acid
3-HAO
2-amino-3-carboxymuconate 6-semialdehyde]
ACMSD
Non-enzymatic conversion
quinolinic acid
2-picolinic
acid
QPRT
NAD+

FIG. 14



Zhang et al. Genome Med. 2016; 8: 46.

FIG. 15

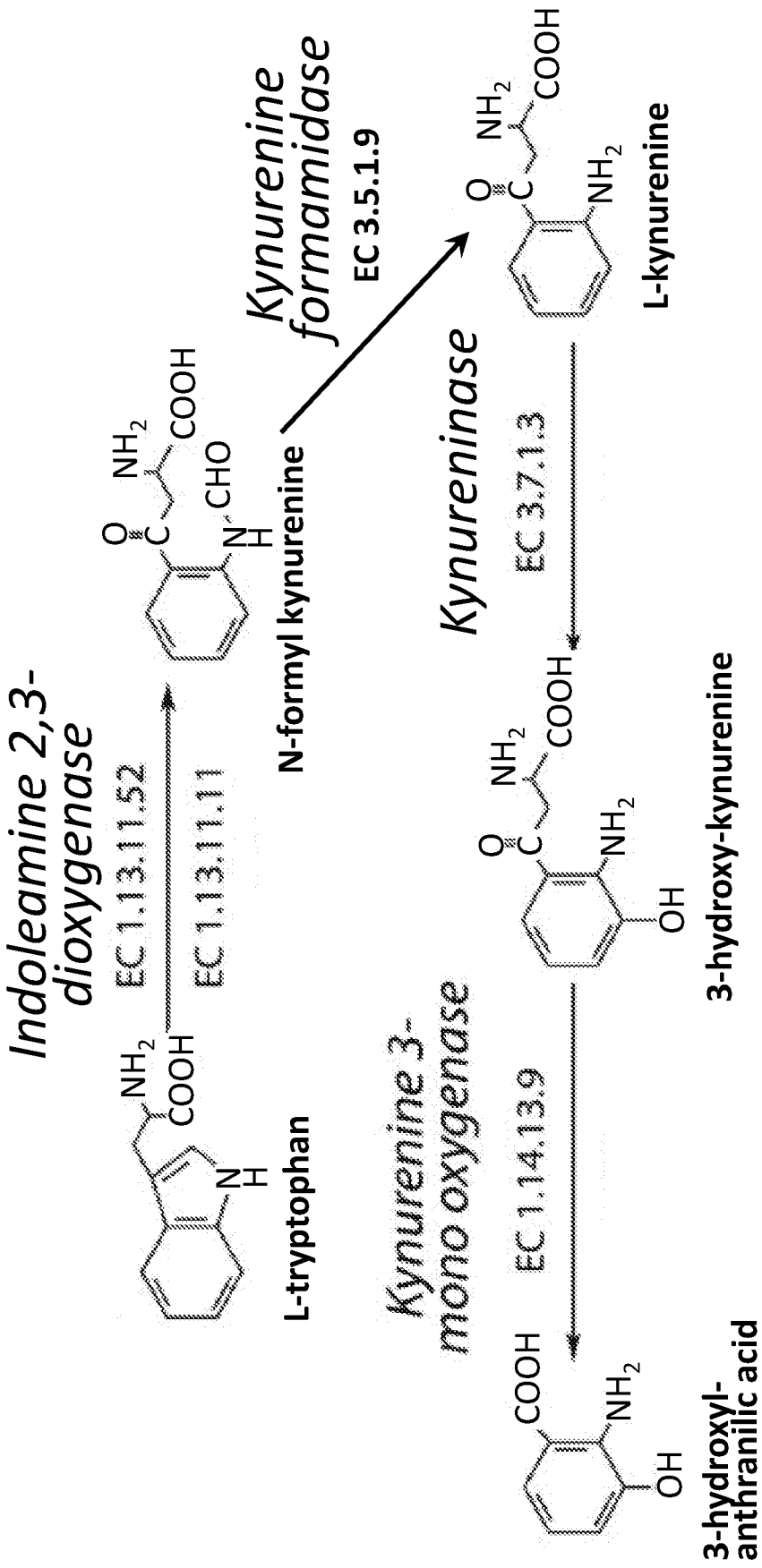

Indoleamine 2,3-dioxygenase
EC 1.13.11.52
EC 1.13.11.11
L-tryptophan
N-formyl kynurenine
Kynurenine formamidase
EC 3.5.1.9
L-kynurenine
Kynureninase
EC 3.7.1.3
3-hydroxy-kynurenine
Kynurenine 3-mono oxygenase
EC 1.14.13.9
3-hydroxyl-anthranilic acid

FIG. 16

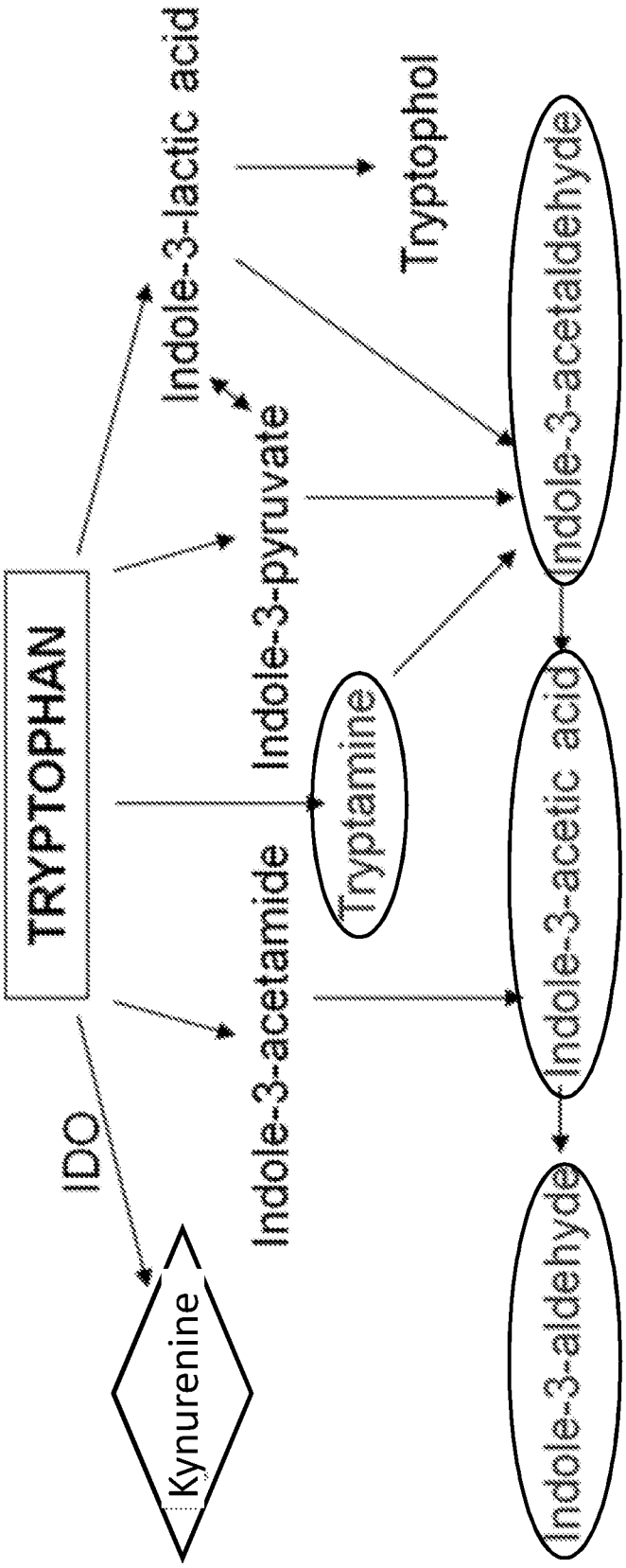

TRYPTOPHAN
IDO
Kynurenine
Indole-3-lactic acid
Indole-3-pyruvate
Indole-3-acetamide
Tryptophol
Tryptamine
Indole-3-acetaldehyde
Indole-3-acetic acid
Indole-3-aldehyde

FIG. 17A

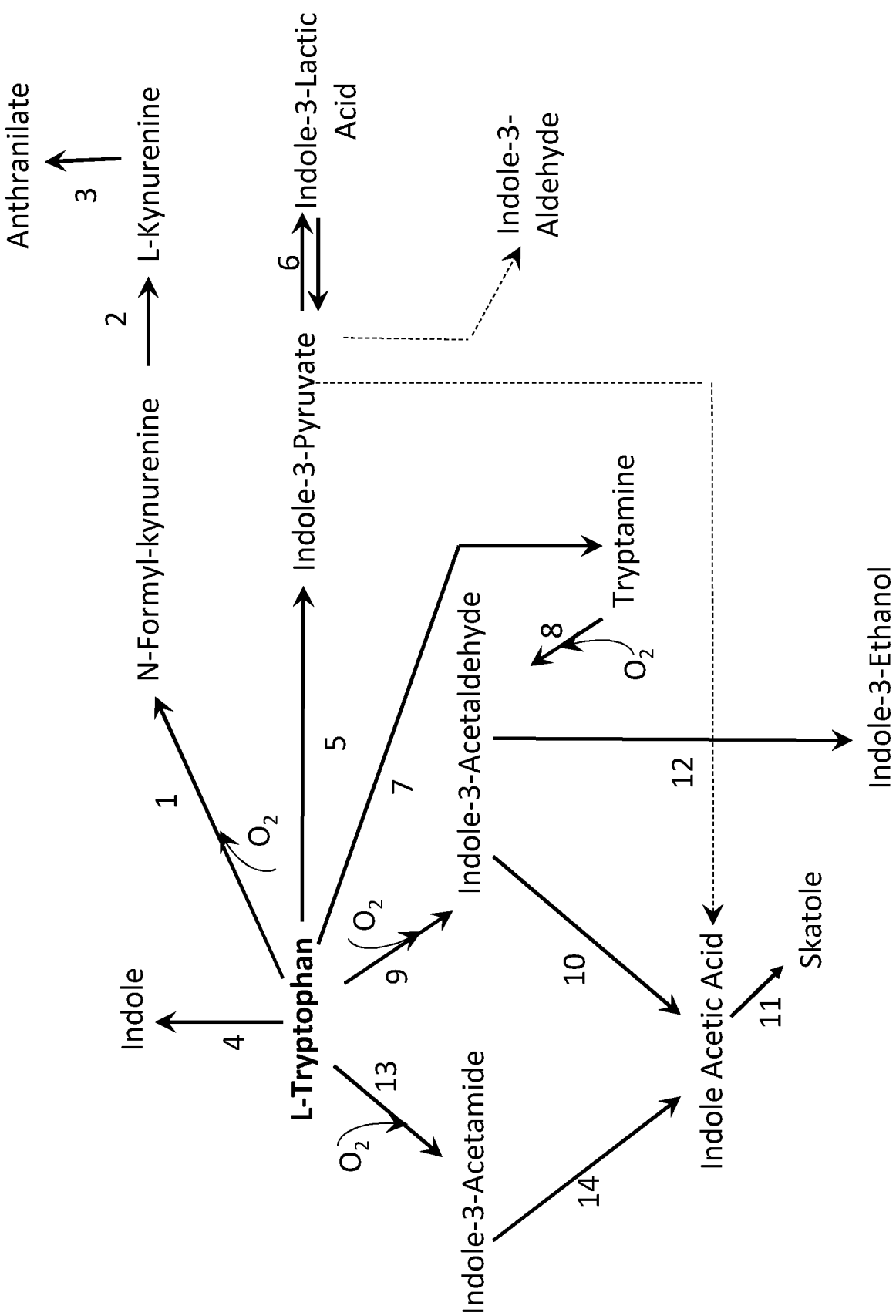

Anthranilate
3
L-Kynurenine
2
N-Formyl-kynurenine
1
O_2
Indole
4
L-Tryptophan
5
Indole-3-Pyruvate
6
Indole-3-Lactic Acid
Indole-3-Aldehyde
7
Indole-3-Acetaldehyde
8
Tryptamine
O_2
9
O_2
10
Indole Acetic Acid
11
Skatole
12
Indole-3-Ethanol
13
O_2
Indole-3-Acetamide
14

FIG. 17B

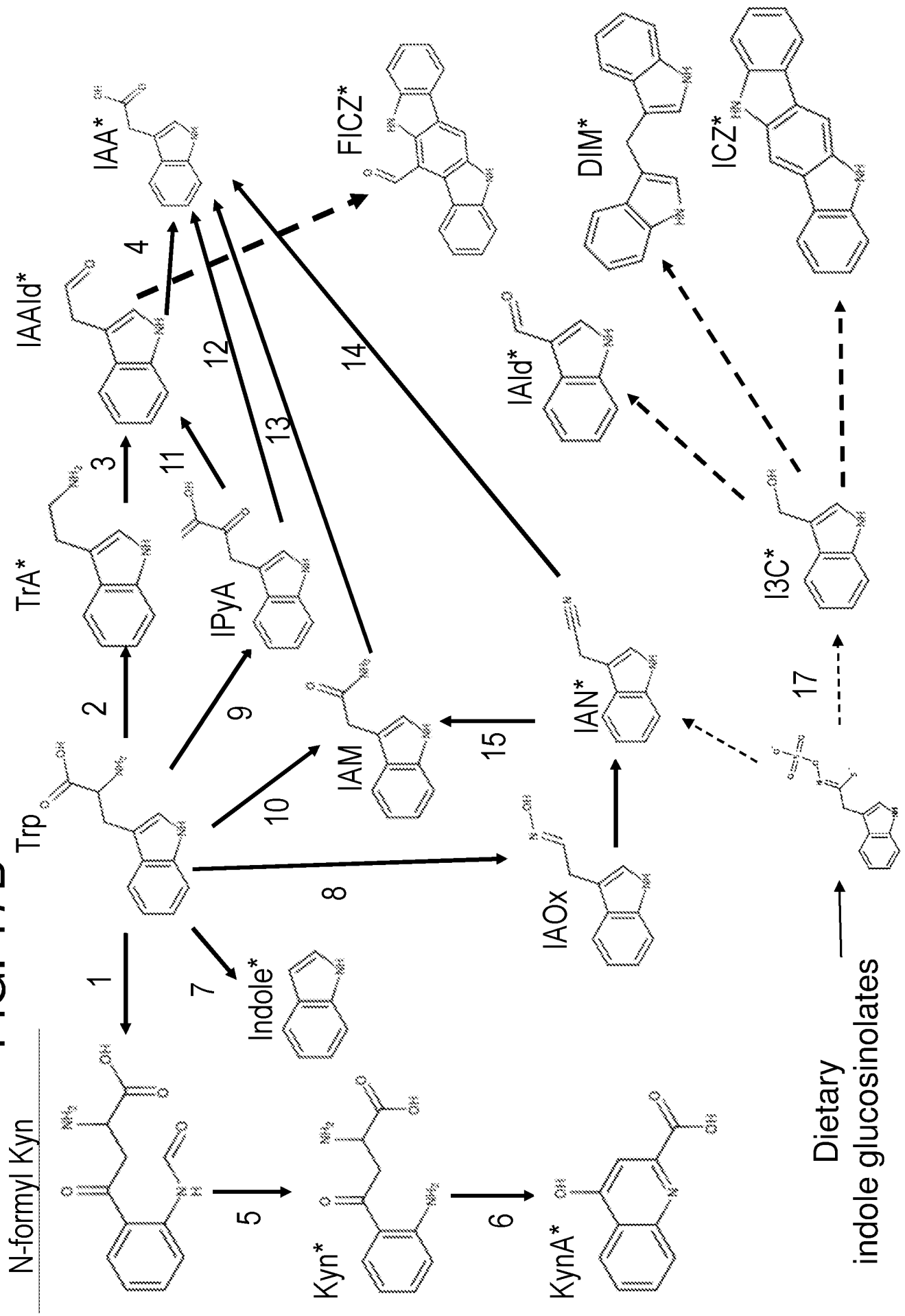

N-formyl Kyn
Trp
TrA*
IAAld*
IAA*
IPyA
IAM
IAN*
IAOx
Indole*
Kyn*
KynA*
FICZ*
IAld*
DIM*
I3C*
ICZ*
Dietary
indole glucosinolates
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
17

FIG. 18

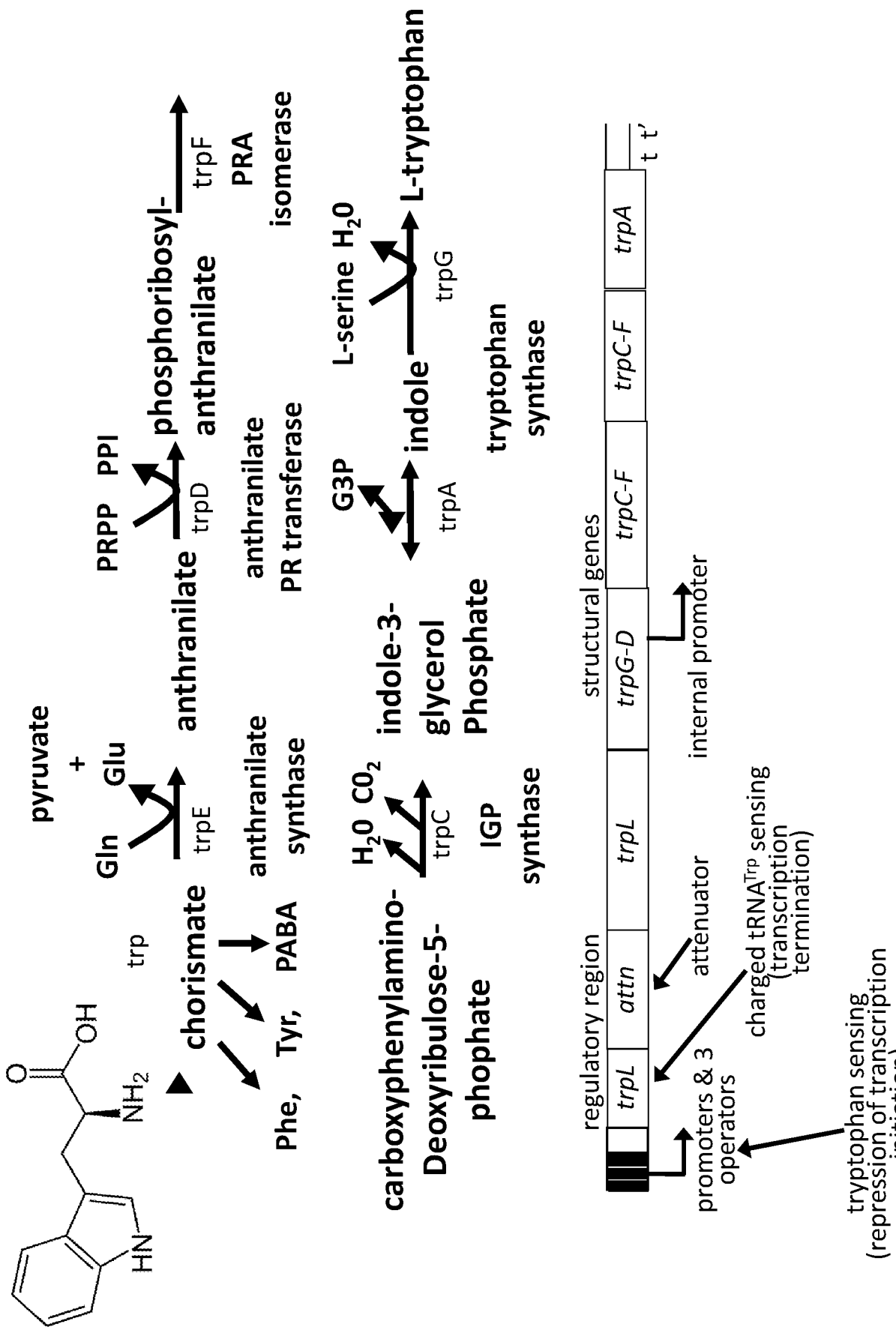

pyruvate
+
Gln Glu
trp
trpE
anthranilate
synthase
chorismate
Phe, Tyr, PABA
anthranilate
PRPP PPI
trpD
phosphoribosyl-
anthranilate
trpF
PRA
isomerase
anthranilate
PR transferase
H2O CO2
trpC
carboxyphenylamino-
Deoxyribulose-5-
phophate
IGP
synthase
G3P
trpA
indole
L-serine H2O
trpG
L-tryptophan
tryptophan
synthase
regulatory region
structural genes
trpL
attn
trpL
trpG-D
trpC-F
trpC-F
trpA
t t'
attenuator
internal promoter
promoters & 3
operators
charged tRNATrp sensing
(transcription
termination)
tryptophan sensing
(repression of transcription
initiation)

FIG. 19

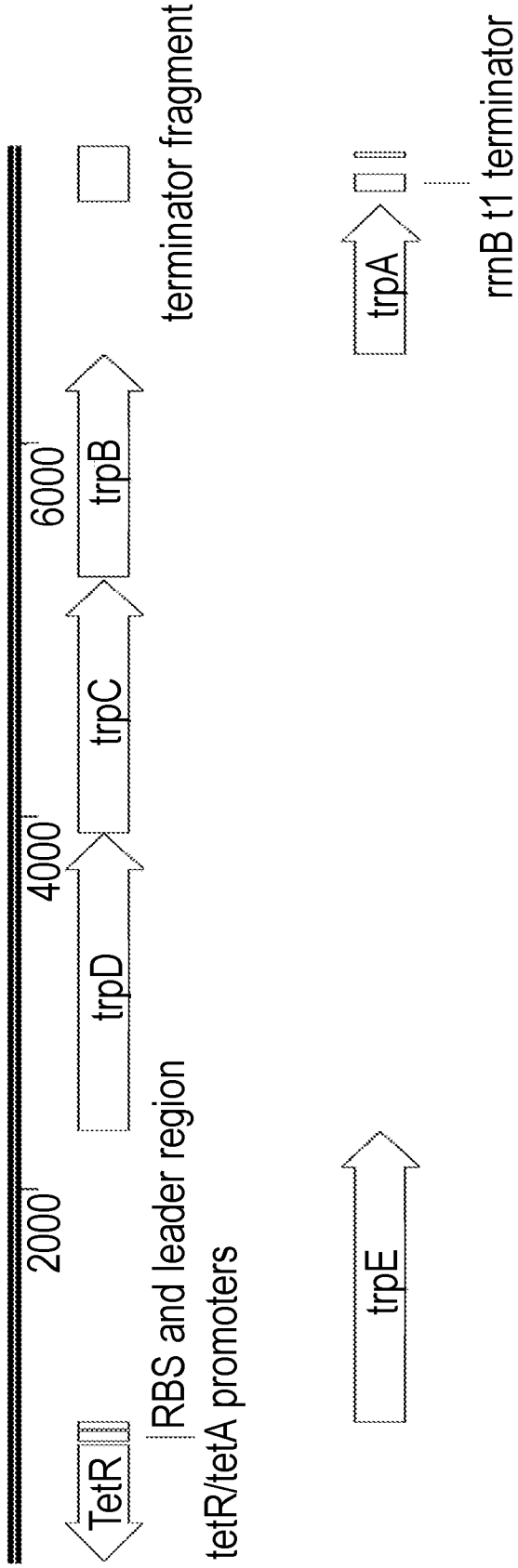

2000
4000
6000
TetR
RBS and leader region
tetR/tetA promoters
trpD
trpC
trpB
terminator fragment
trpE
trpA
rrnB t1 terminator
Tet-TrpEDCBA_Nissle_ter
7592 bp

FIG. 20A

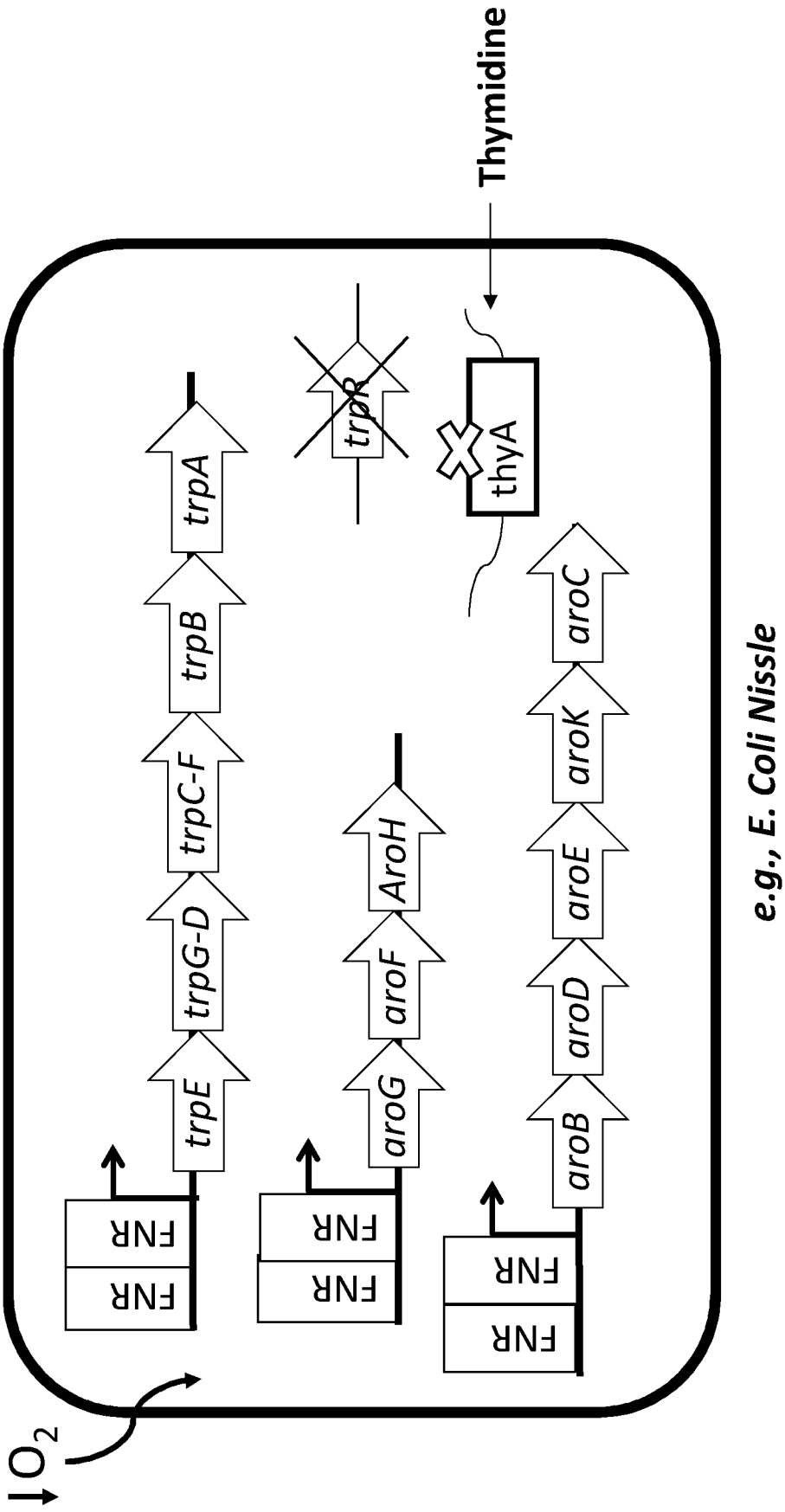

Thymidine
Tryptophan Production
trpA
trpB
trpC-F
trpG-D
trpE
trpR
thyA
AroH
aroF
aroG
aroC
aroK
aroE
aroD
aroB
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 20B

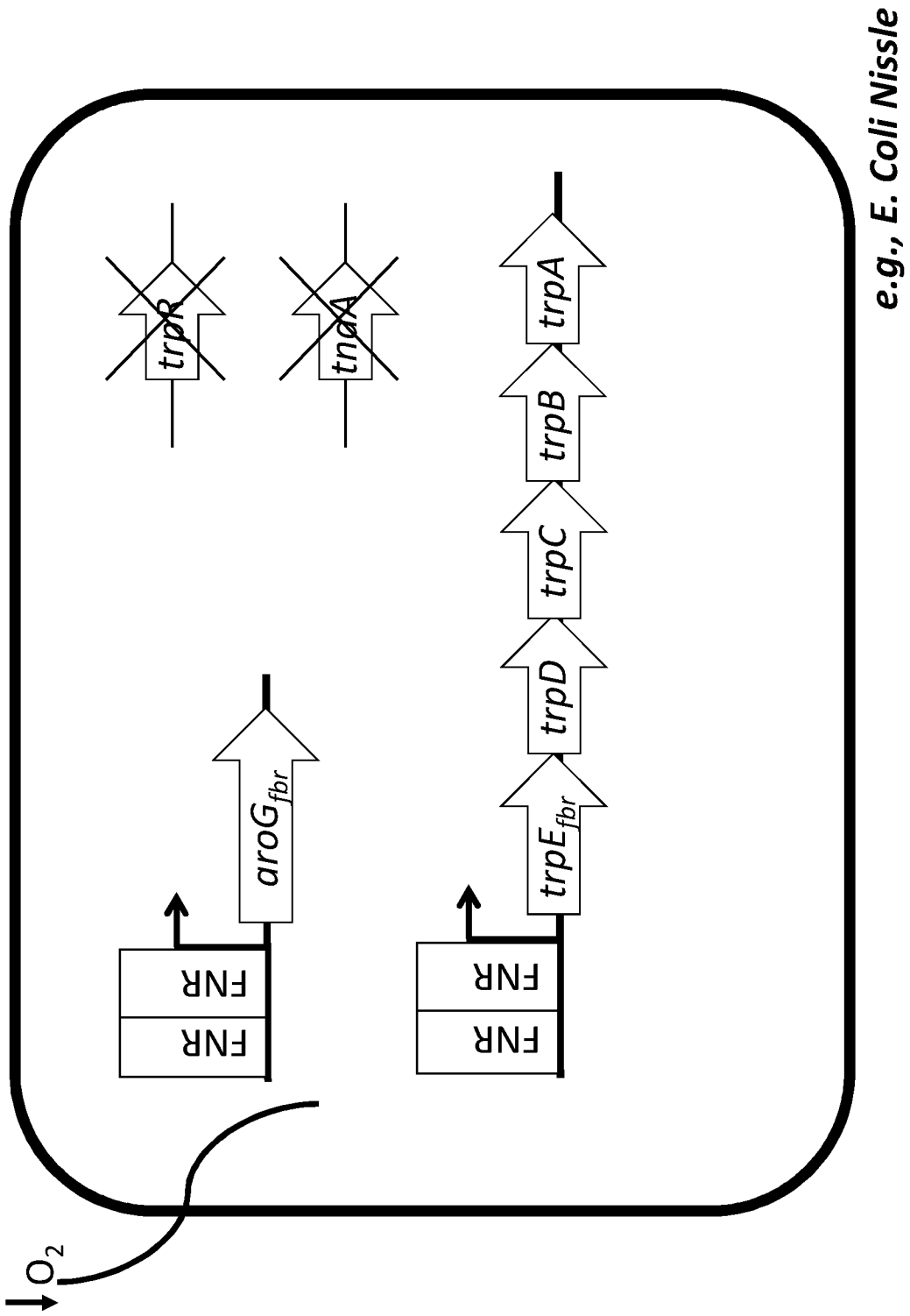
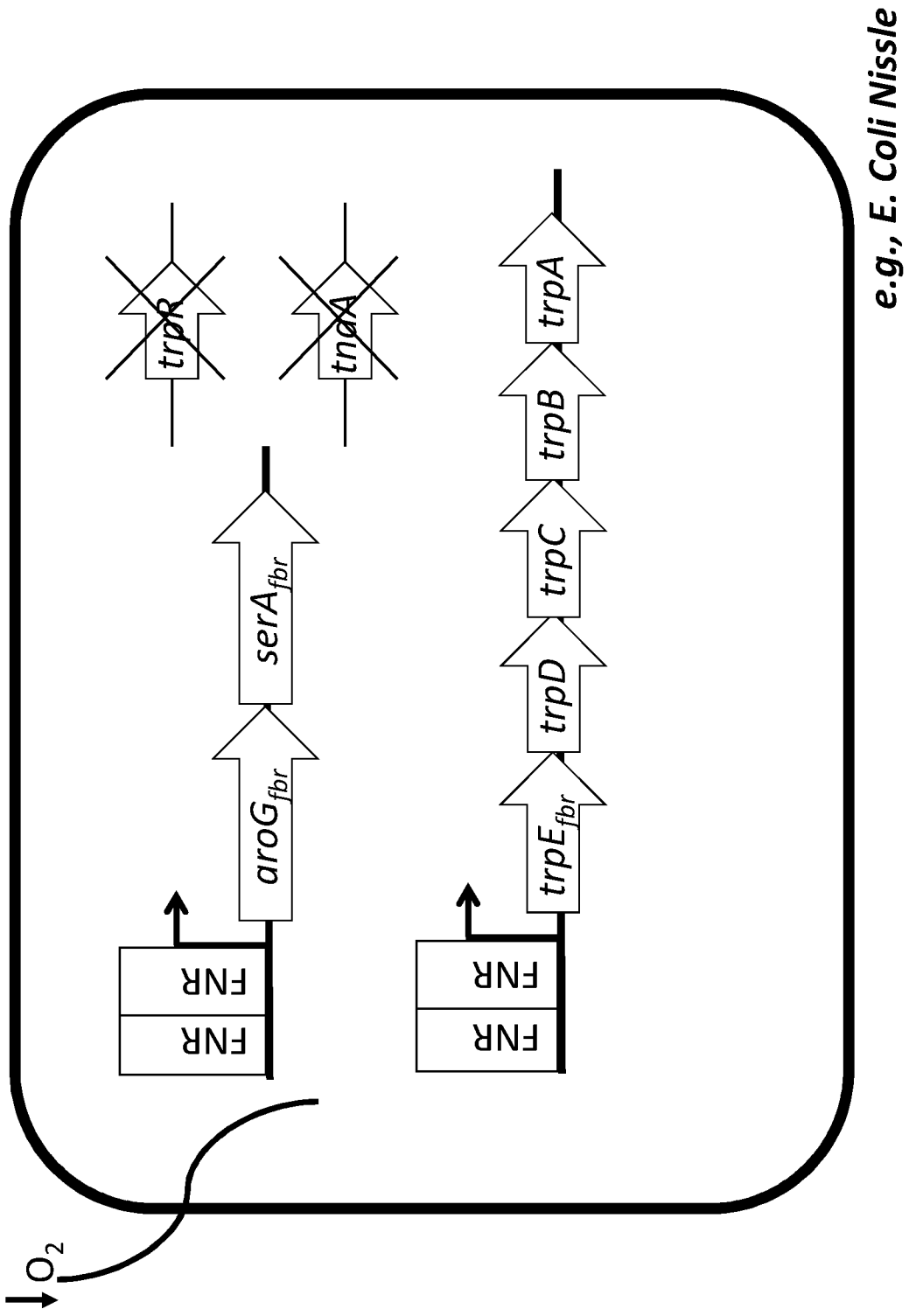

e.g., E. Coli Nissle
trpA
trpB
trpC
trpD
trpE$_{fbr}$
FNR
FNR
aroG$_{fbr}$
FNR
FNR
tnaA
trpR
O$_2$

FIG. 20C


e.g., E. Coli Nissle
O_2
FNR
FNR
$aroG_{fbr}$
$serA_{fbr}$
trpR
tnaA
FNR
FNR
$trpE_{fbr}$
trpD
trpC
trpB
trpA

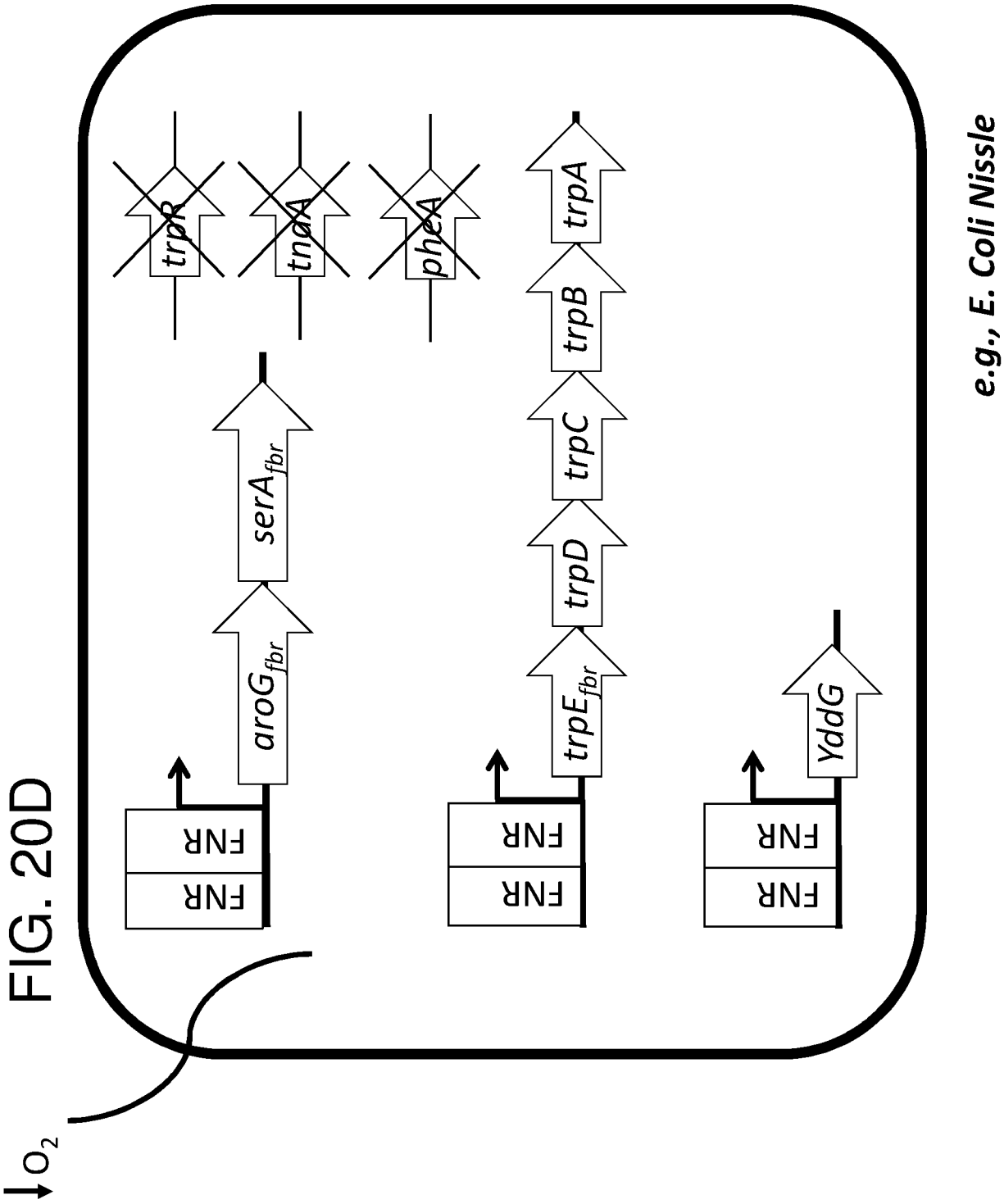


FIG. 20D
O_2
FNR
FNR
$aroG_{fbr}$
$serA_{fbr}$
trpR
tnaA
pheA
FNR
FNR
$trpE_{fbr}$
trpD
trpC
trpB
trpA
FNR
FNR
YddG
e.g., E. Coli Nissle

FIG. 21A

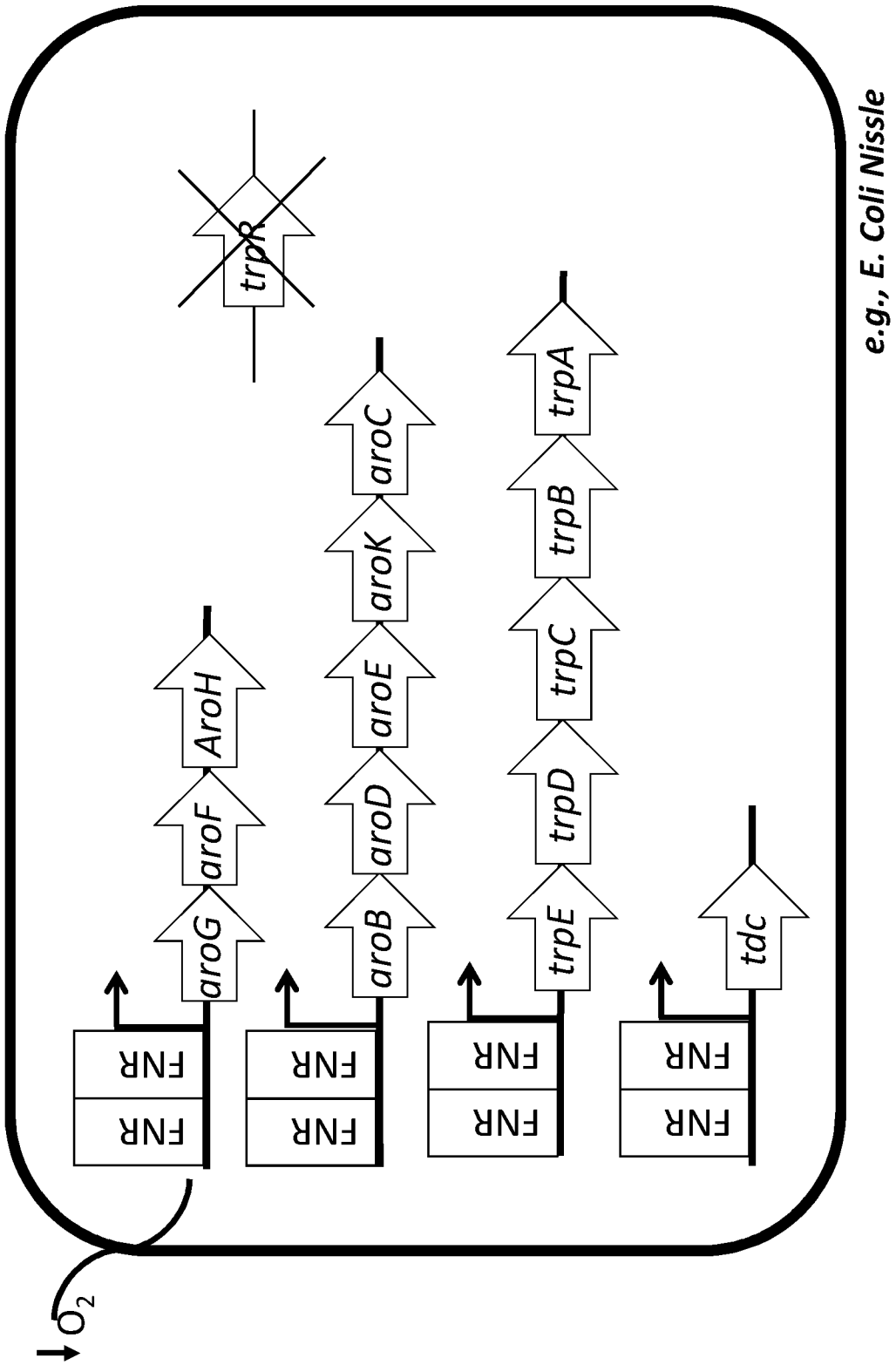

O_2
FNR
FNR
aroG
aroF
AroH
FNR
FNR
aroB
aroD
aroE
aroK
aroC
trpR
FNR
FNR
trpE
trpD
trpC
trpB
trpA
FNR
FNR
tdc
e.g., E. Coli Nissle

FIG. 21B

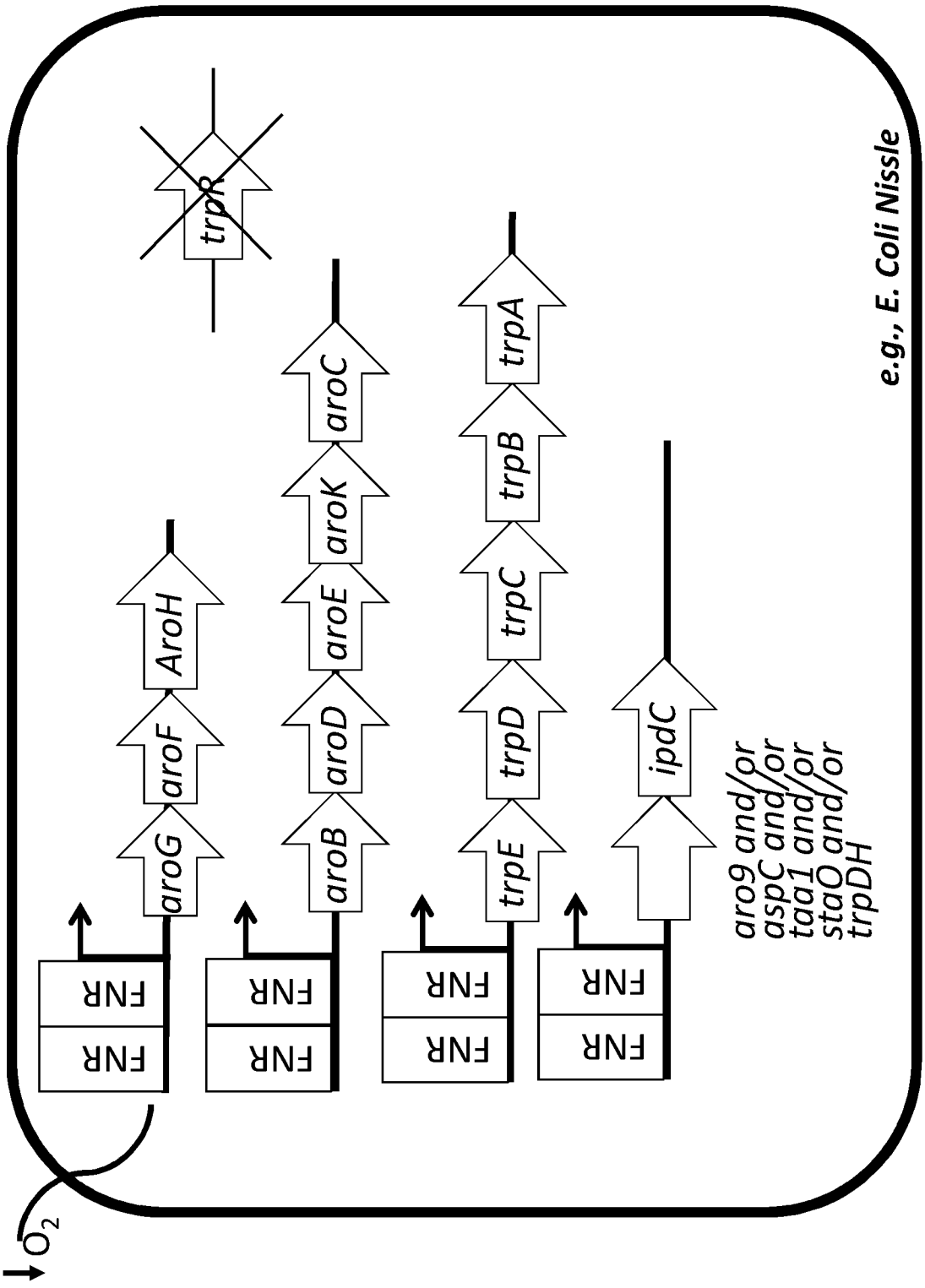

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
ipdC
FNR
aro9 and/or
aspC and/or
taa1 and/or
staO and/or
trpDH
e.g., E. Coli Nissle
O2

FIG. 21C

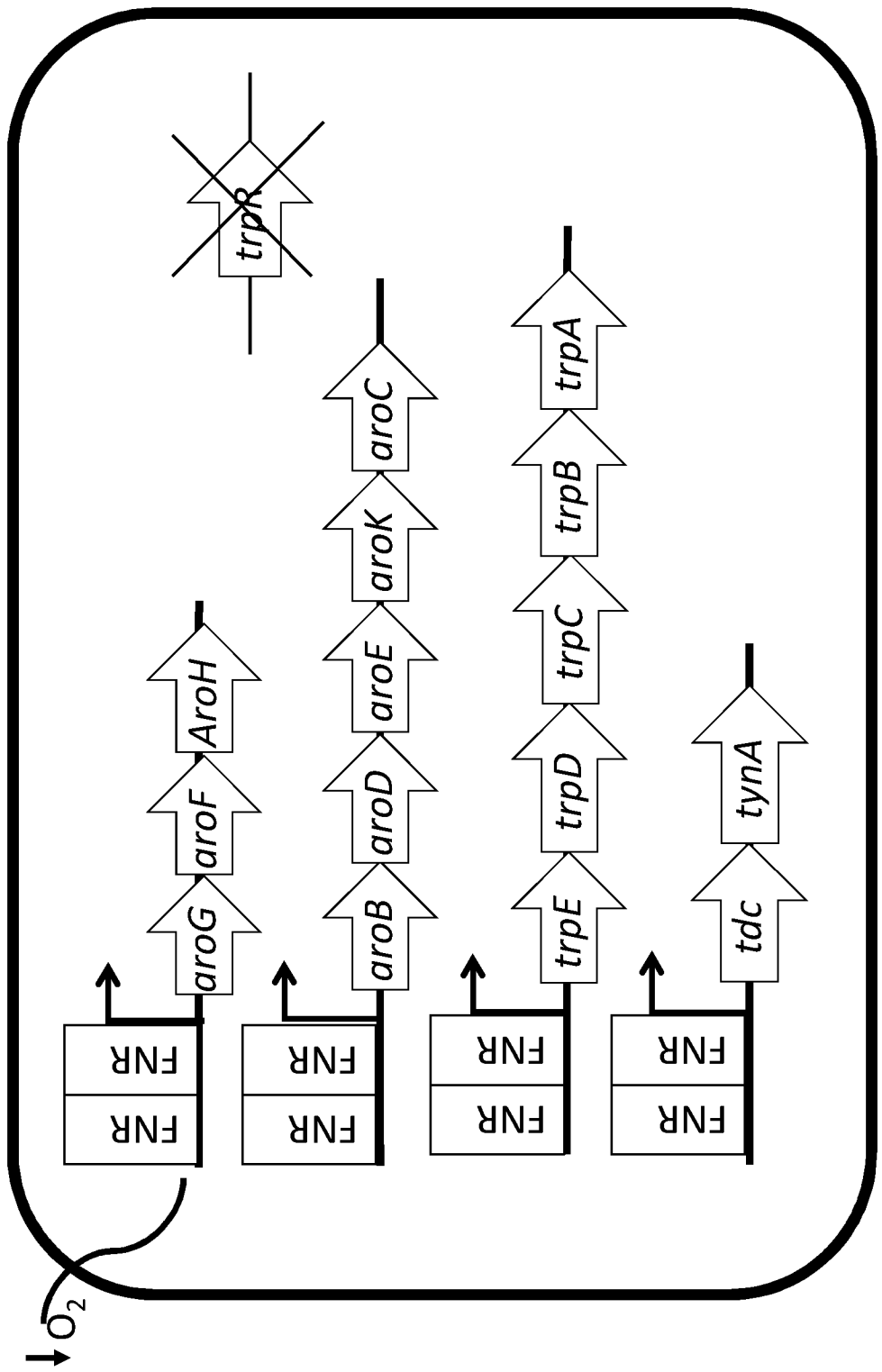

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
tdc
tynA
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 21D

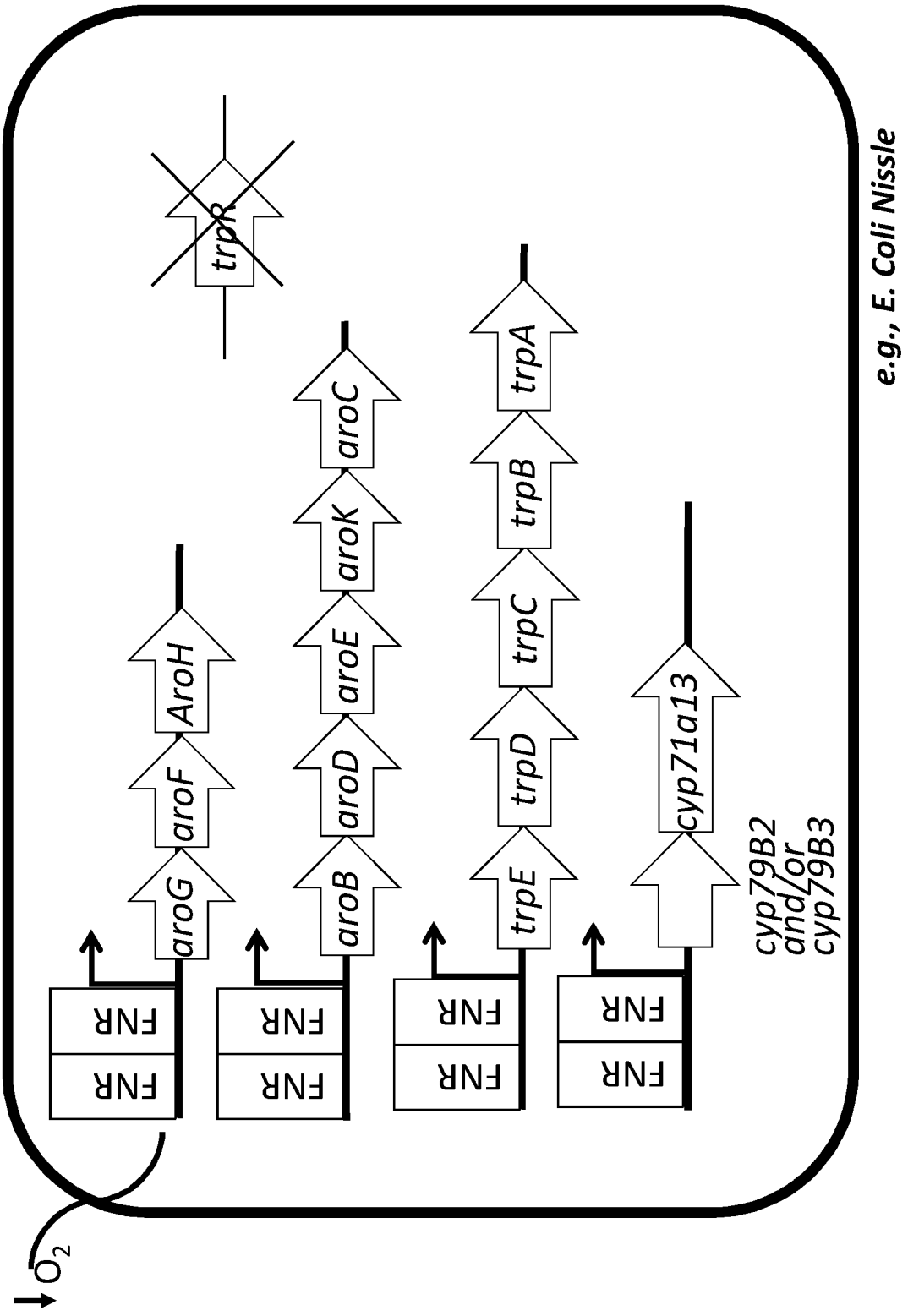

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
cyp71a13
cyp79B2
and/or
cyp79B3
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O2
e.g., E. Coli Nissle

FIG. 21E

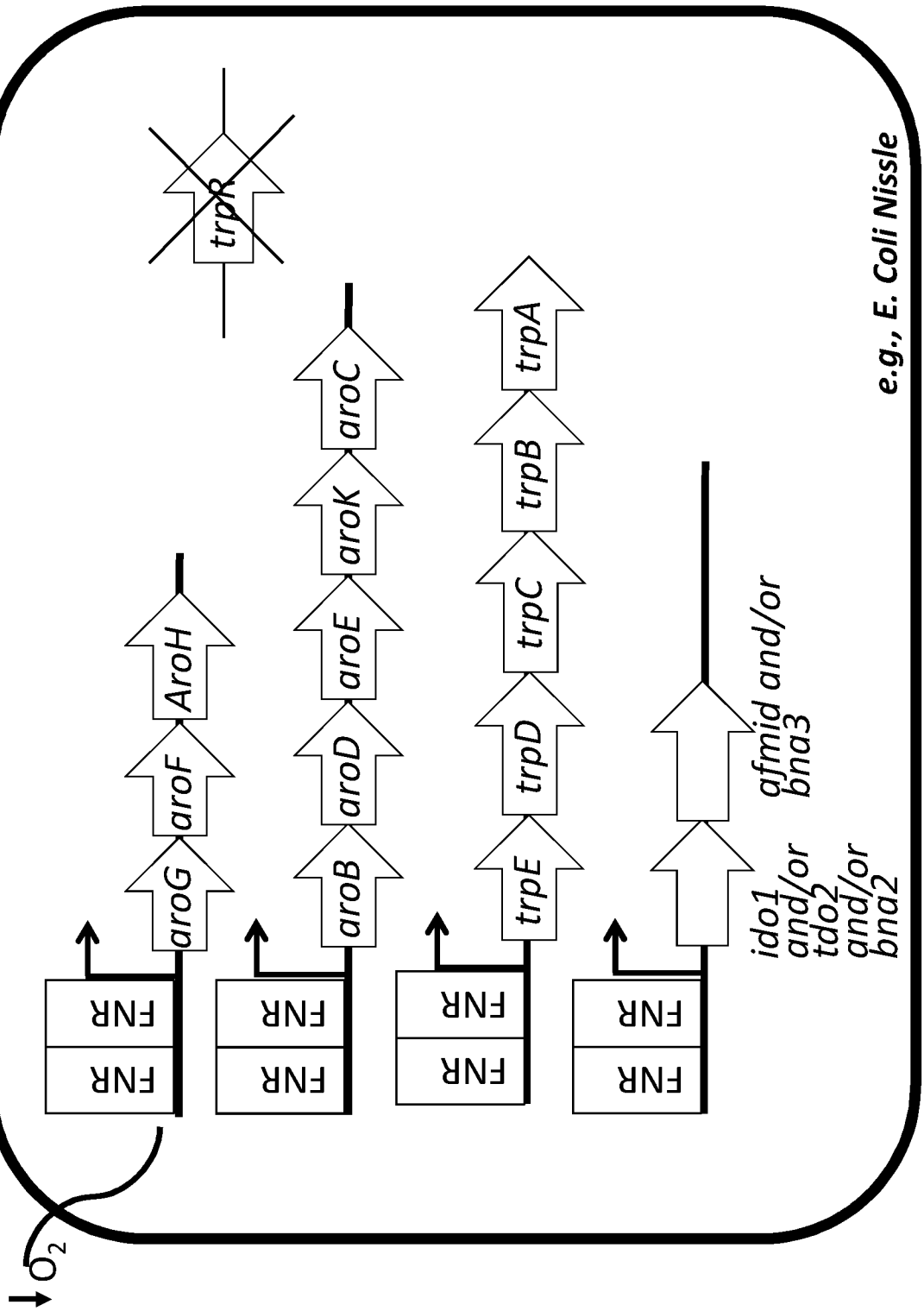

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
ido1 and/or tdo2 and/or bna2
qfmid and/or bna3
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2

FIG. 21F

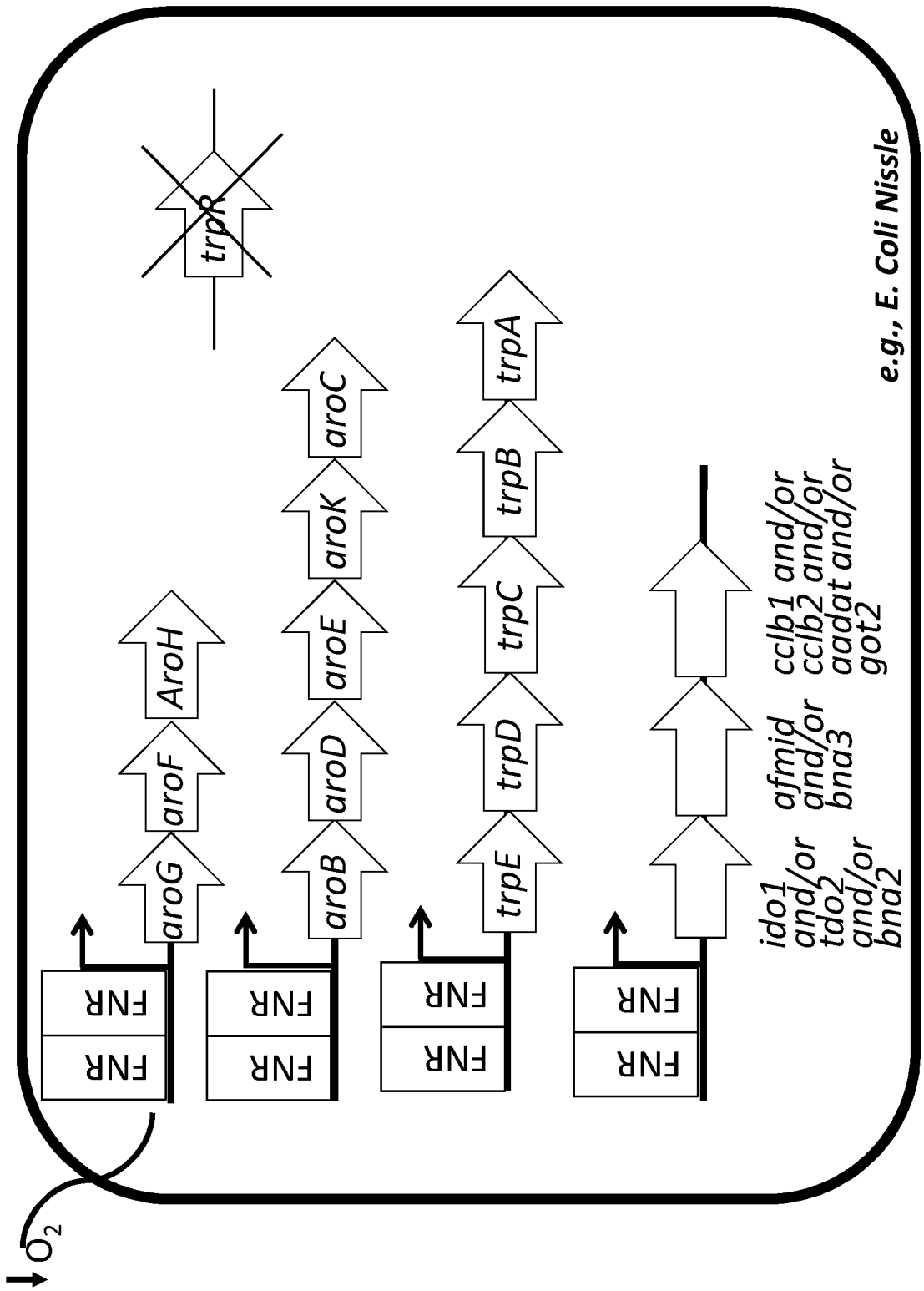

O_2
FNR
FNR
aroG
aroF
AroH
FNR
FNR
aroB
aroD
aroE
aroK
aroC
FNR
FNR
trpE
trpD
trpC
trpB
trpA
FNR
FNR
ido1 and/or tdo2 and/or bna2
afmid and/or bna3
cclb1 and/or cclb2 and/or aadat and/or got2
trpR
e.g., E. Coli Nissle

FIG. 21G

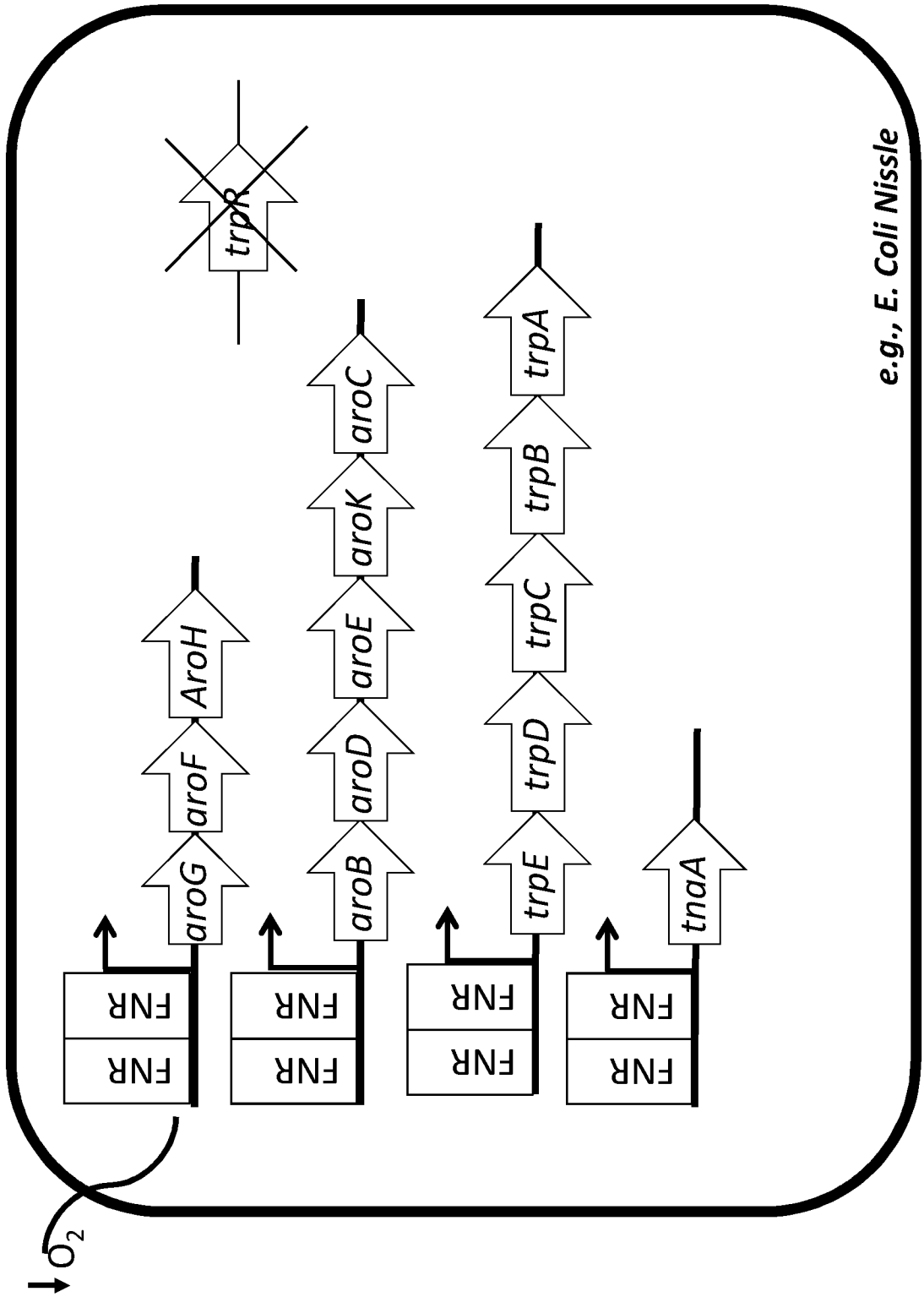

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
tnaA
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2

FIG. 21H

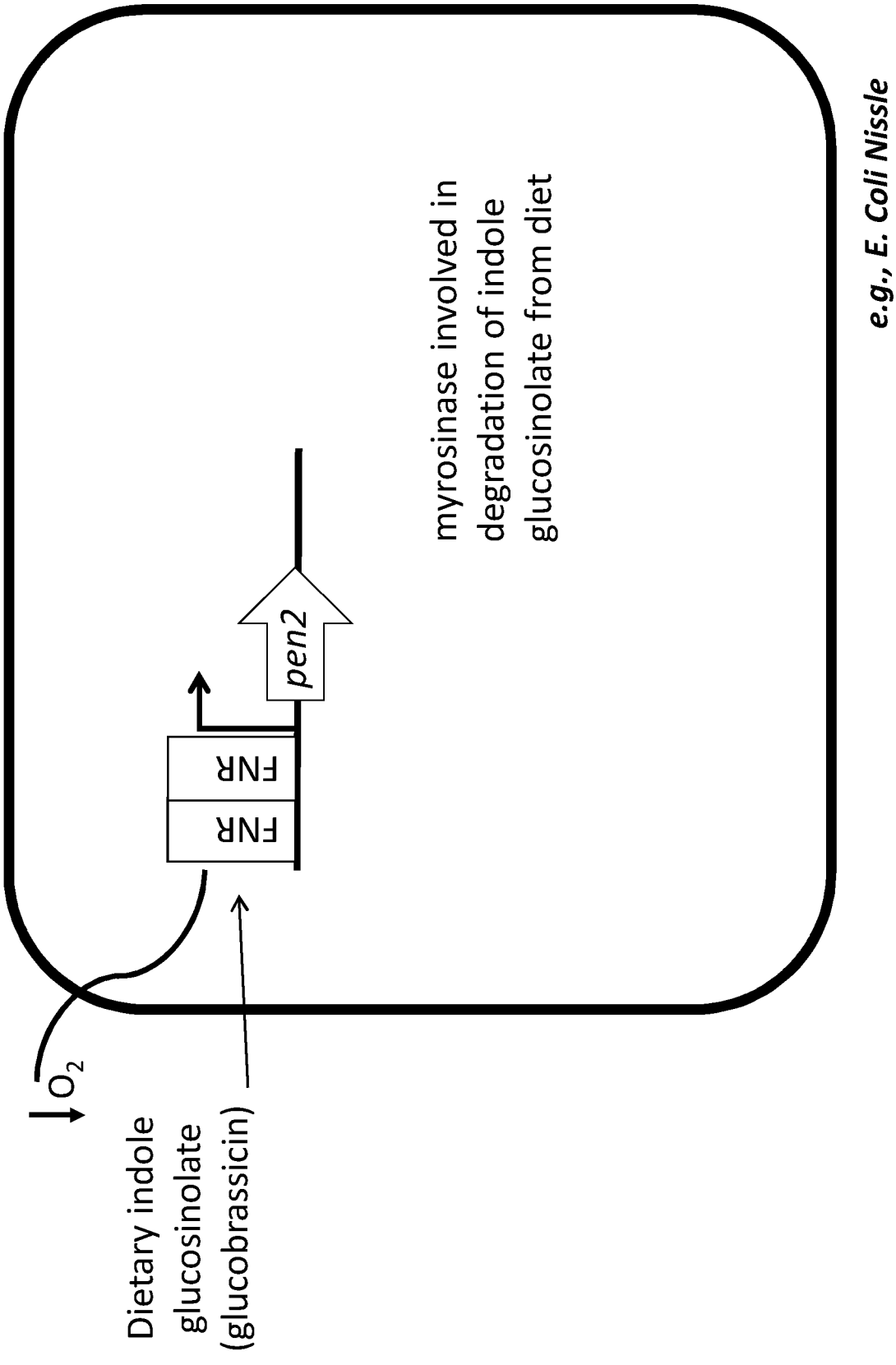

O_2
Dietary indole glucosinolate (glucobrassicin)
FNR
FNR
pen2
myrosinase involved in degradation of indole glucosinolate from diet
e.g., E. Coli Nissle

FIG. 22A

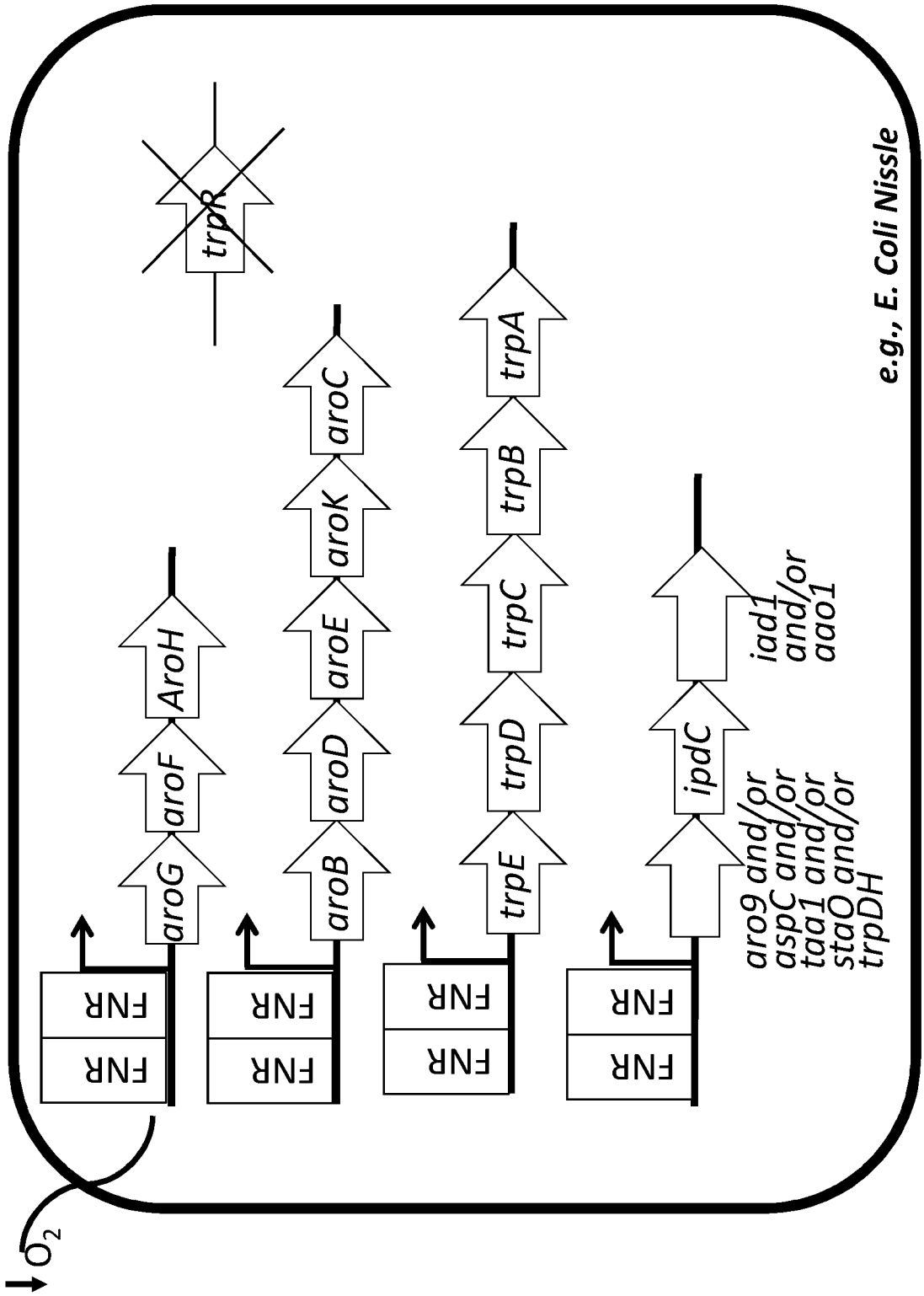

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
ipdC
iad1
and/or
aao1
aro9 and/or
aspC and/or
taa1 and/or
staO and/or
trpDH
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2

FIG. 22B

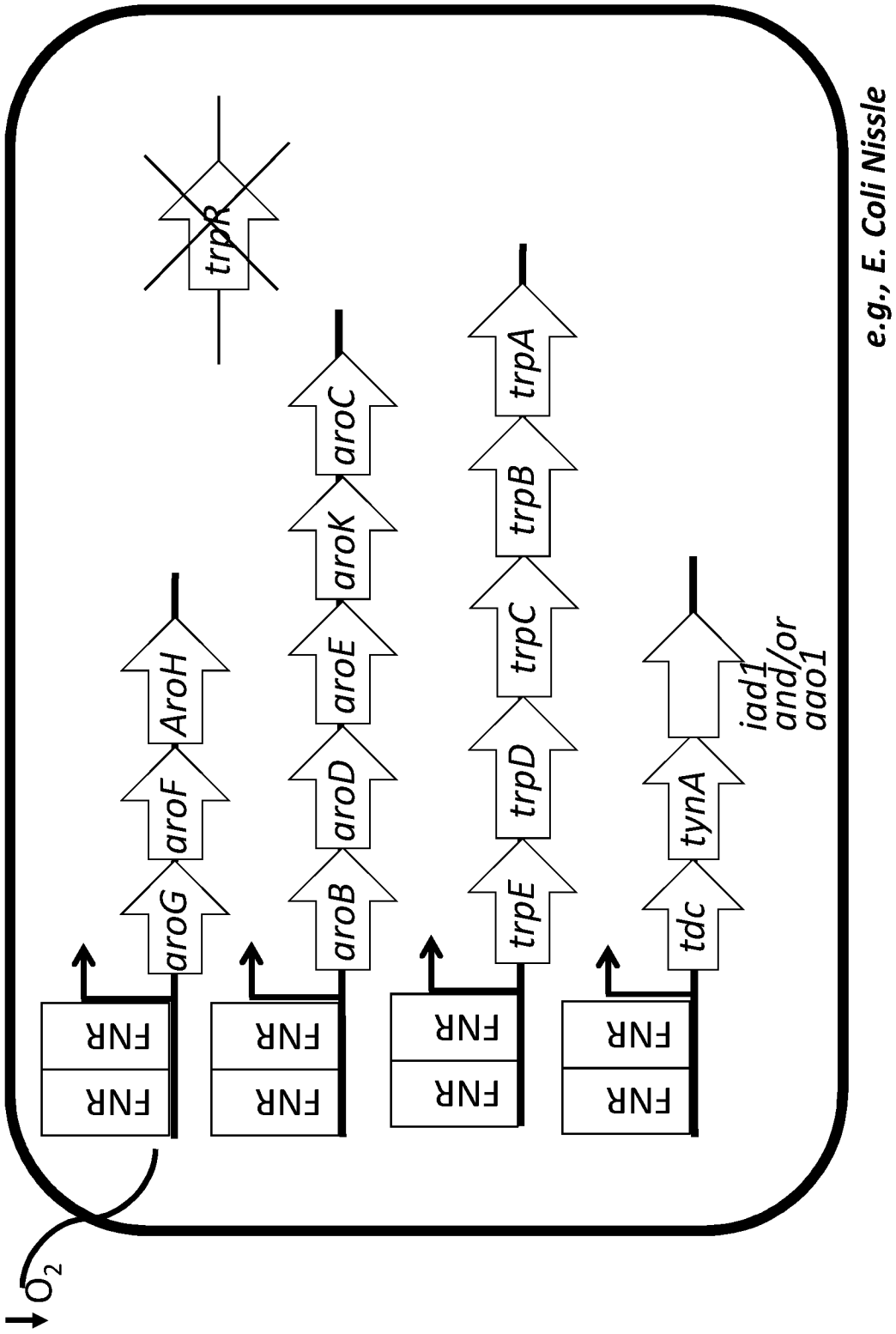

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
tdc
tynA
iad1
and/or
aao1
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O2
e.g., E. Coli Nissle

FIG. 22C

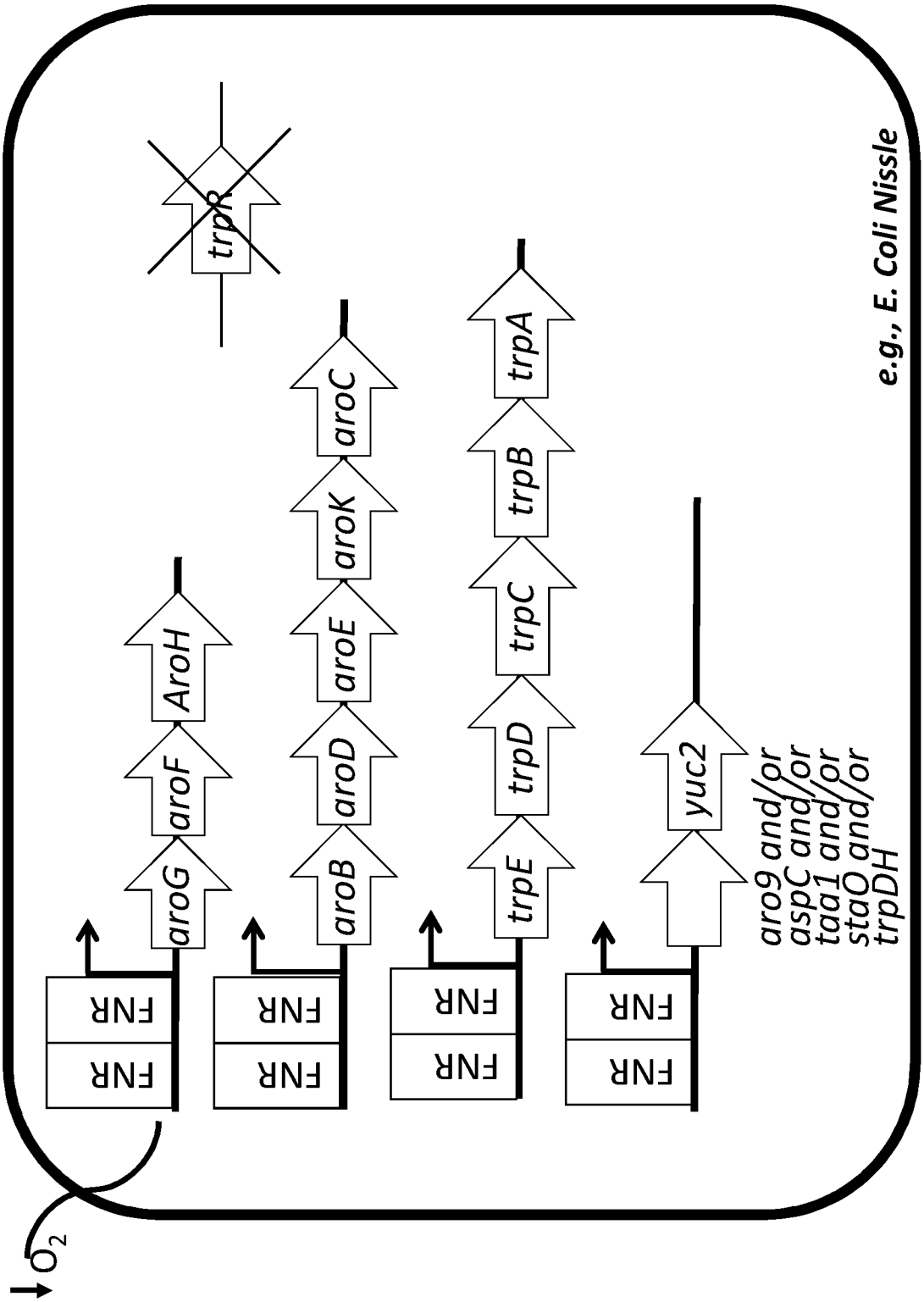

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
yuc2
aro9 and/or
aspC and/or
taa1 and/or
staO and/or
trpDH
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O2

FIG. 22D

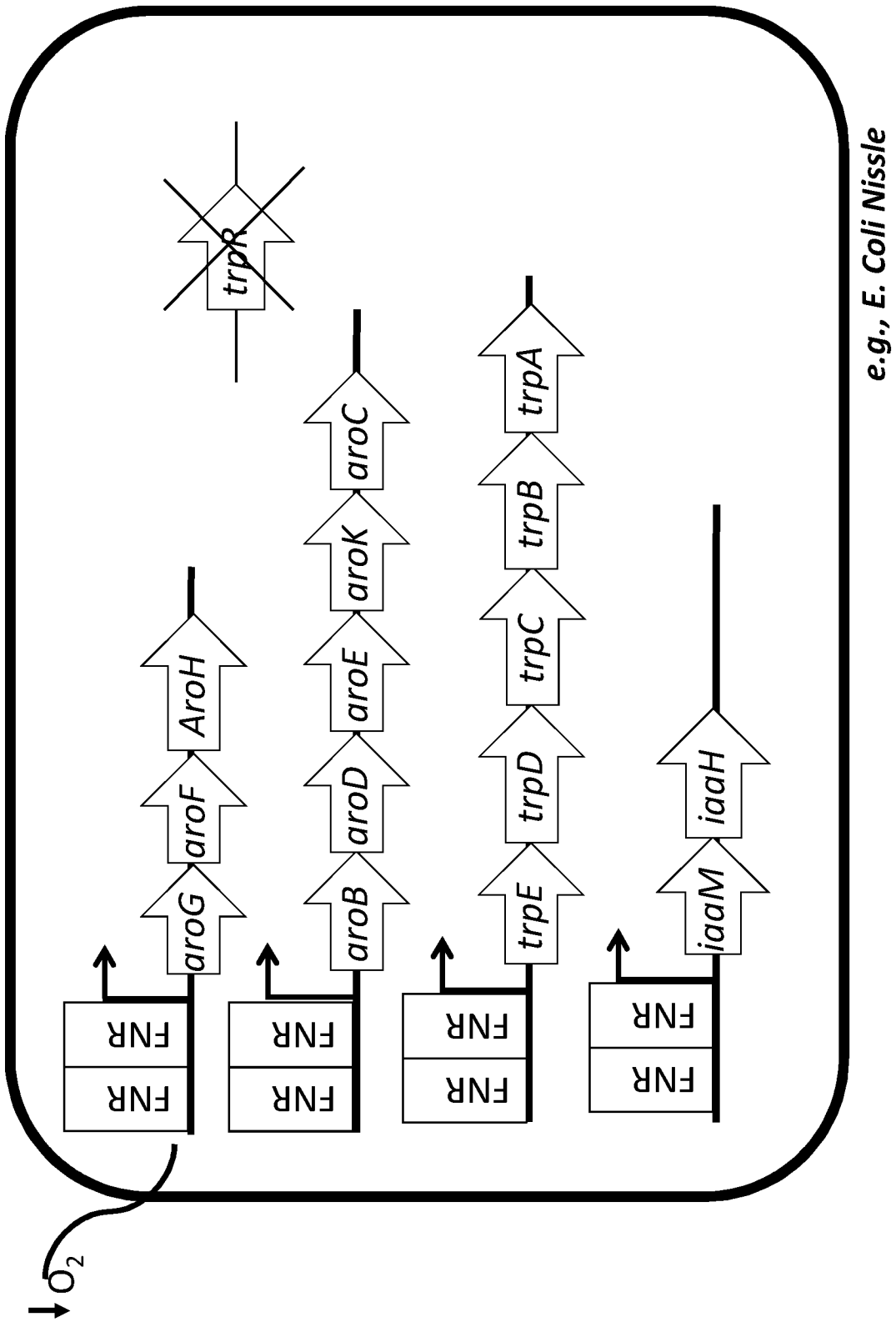

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
iaaM
iaaH
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 22E

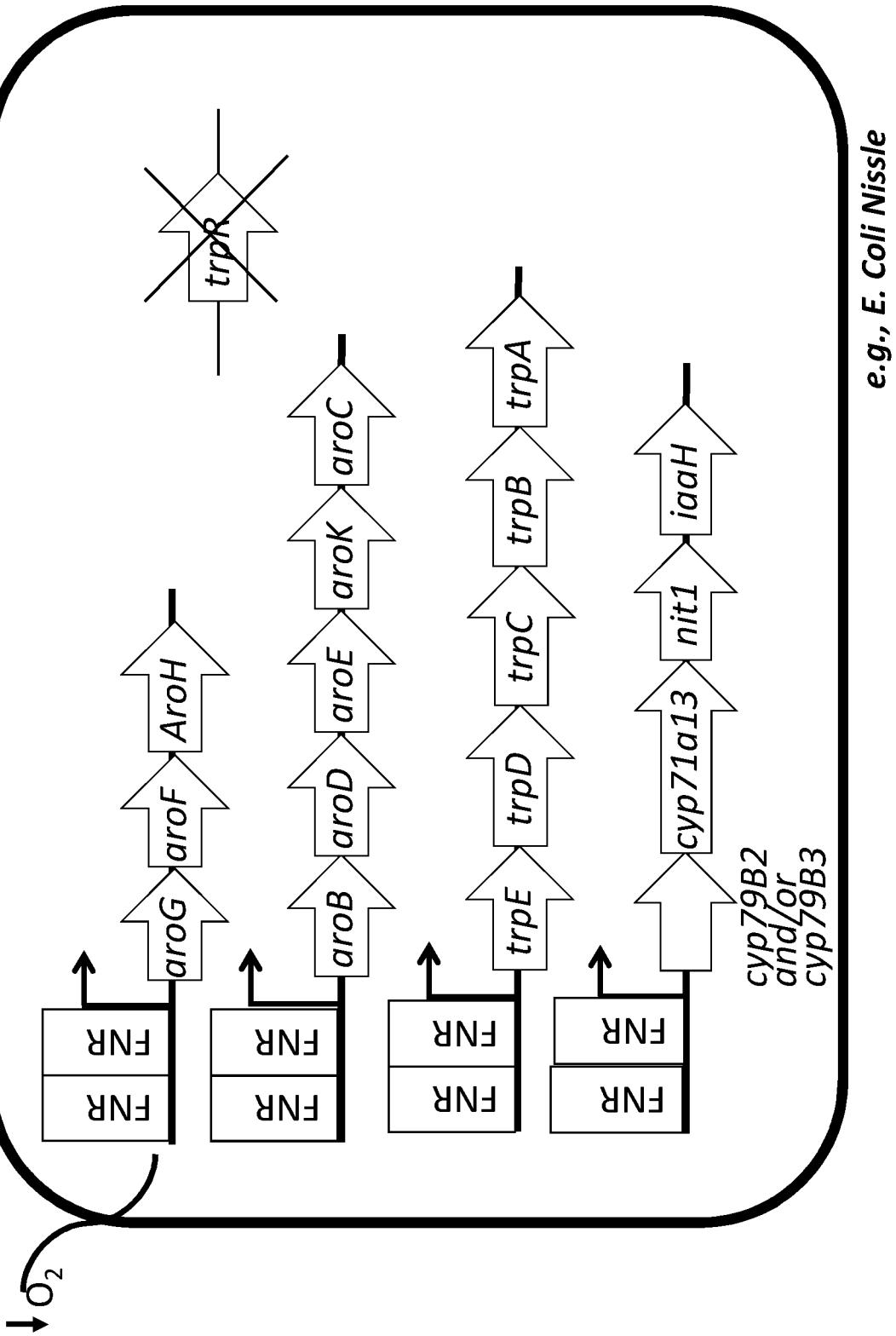

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
cyp71a13
nit1
iaaH
cyp79B2
and/or
cyp79B3
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O2
e.g., E. Coli Nissle

FIG. 22F

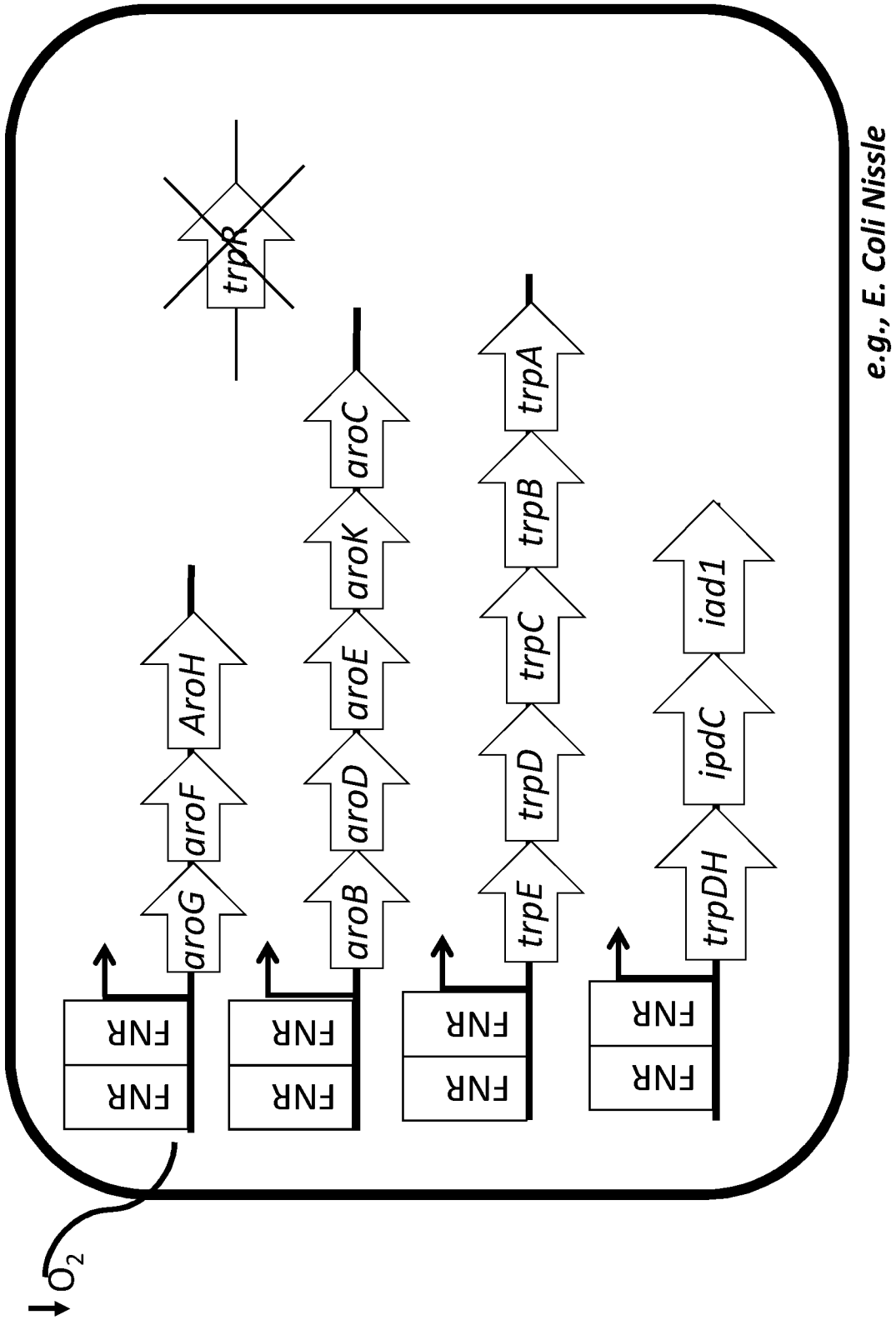

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
trpDH
ipdC
iad1
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 23A

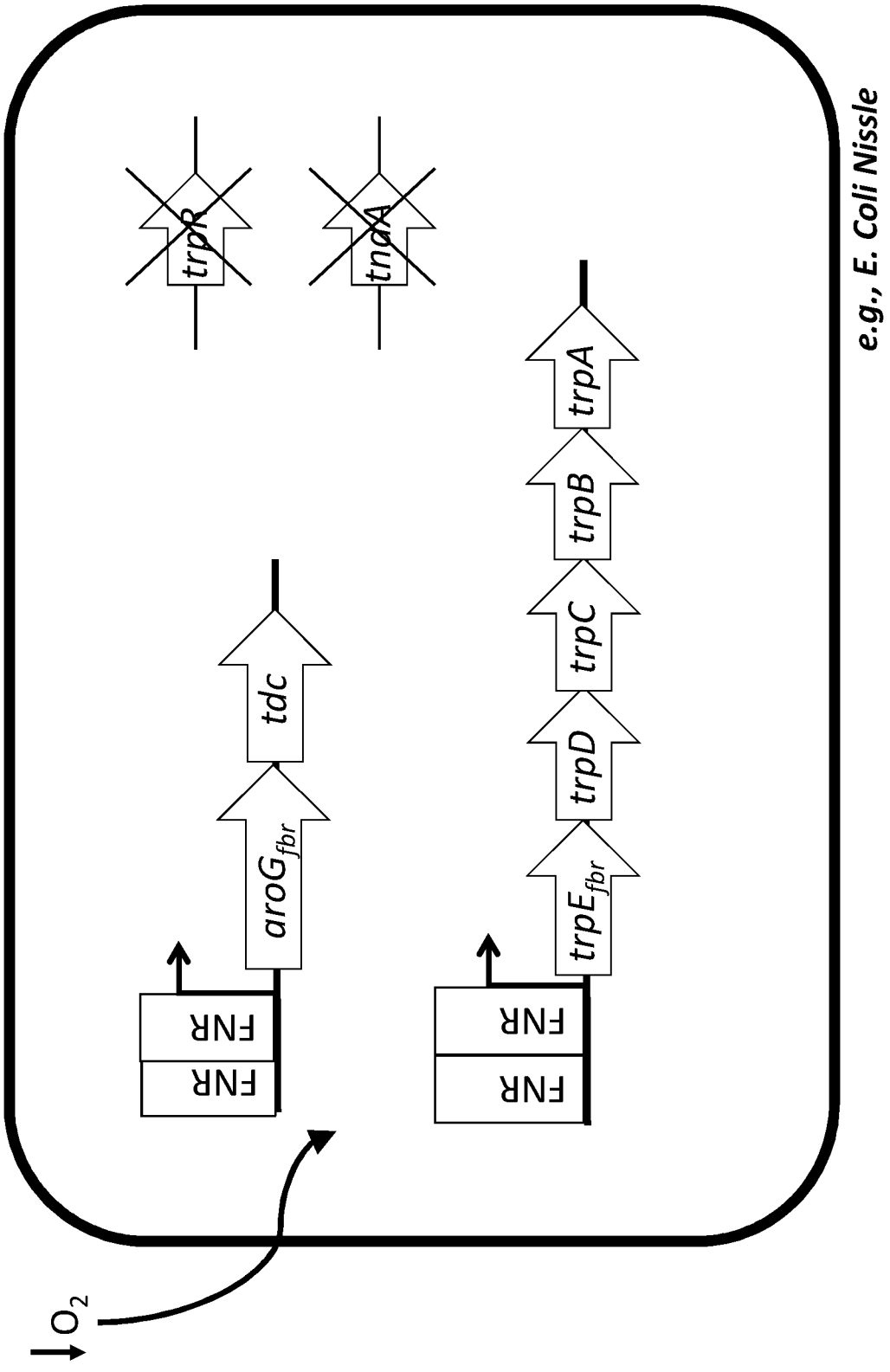

e.g., E. Coli Nissle
trpR
tnaA
tdc
aroG$_{fbr}$
FNR
FNR
trpA
trpB
trpC
trpD
trpE$_{fbr}$
FNR
FNR
O$_2$

FIG. 23B

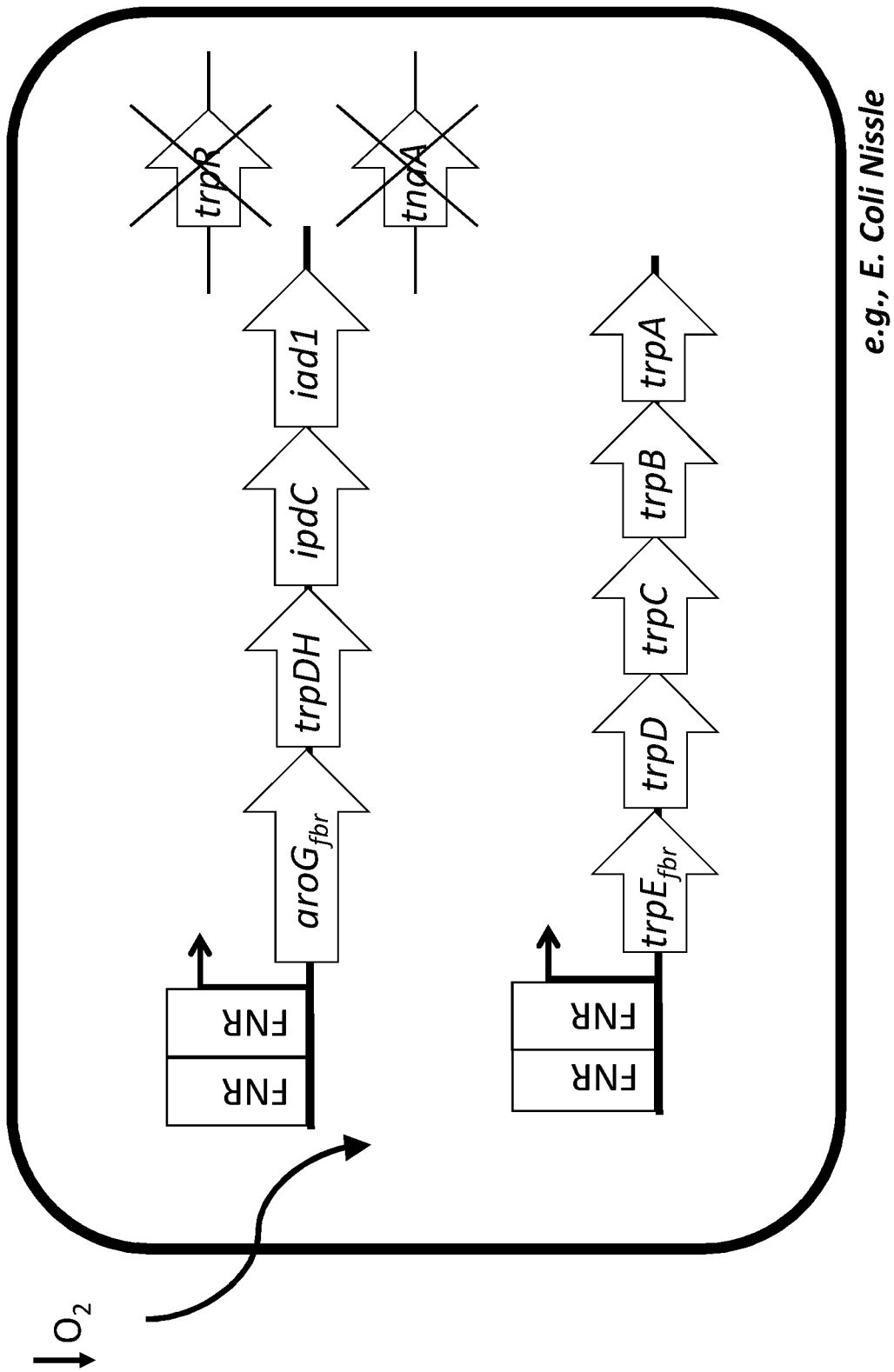

O_2
FNR
FNR
$aroG_{fbr}$
trpDH
ipdC
iad1
trpR
tnaA
FNR
FNR
$trpE_{fbr}$
trpD
trpC
trpB
trpA
e.g., E. Coli Nissle

FIG. 23C

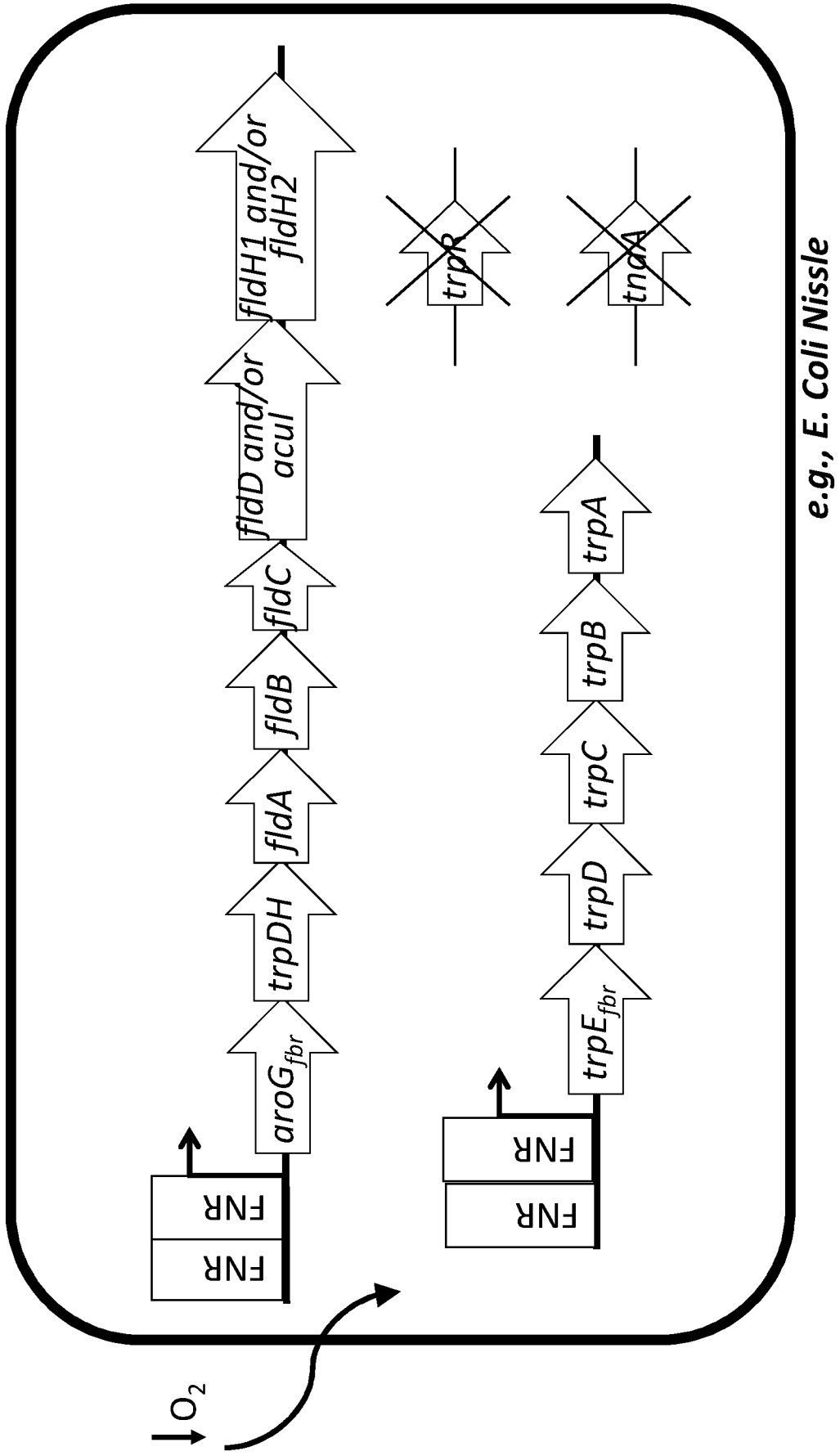

FNR
FNR
$aroG_{fbr}$
trpDH
fldA
fldB
fldC
fldD and/or acuI
fldH1 and/or fldH2
FNR
FNR
$trpE_{fbr}$
trpD
trpC
trpB
trpA
trpR
tnaA
O_2
e.g., E. Coli Nissle

FIG. 24A

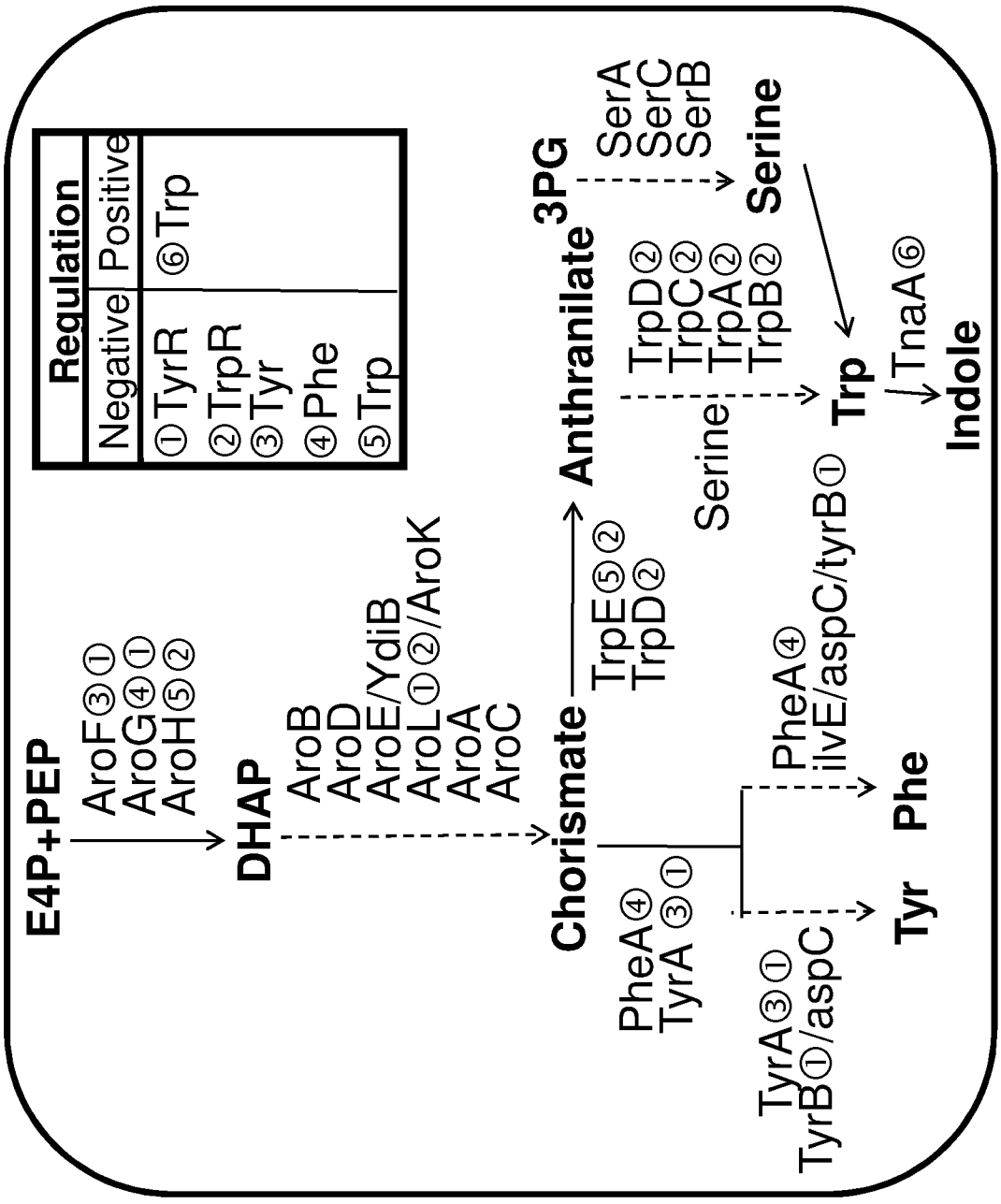

Regulation
Negative
Positive
①TyrR
②TrpR
③Tyr
④Phe
⑤Trp
⑥Trp
E4P+PEP
AroF③①
AroG④①
AroH⑤②
DHAP
AroB
AroD
AroE/YdiB
AroL①②/AroK
AroA
AroC
Chorismate
TrpE⑤②
TrpD②
Anthranilate
3PG
SerA
SerC
SerB
Serine
TrpD②
TrpC②
TrpA②
TrpB②
Serine
Trp
TnaA⑥
Indole
PheA④
TyrA③①
PheA④
ilvE/aspC/tyrB①
TyrA③①
TyrB①/aspC
Tyr
Phe

FIG. 24B

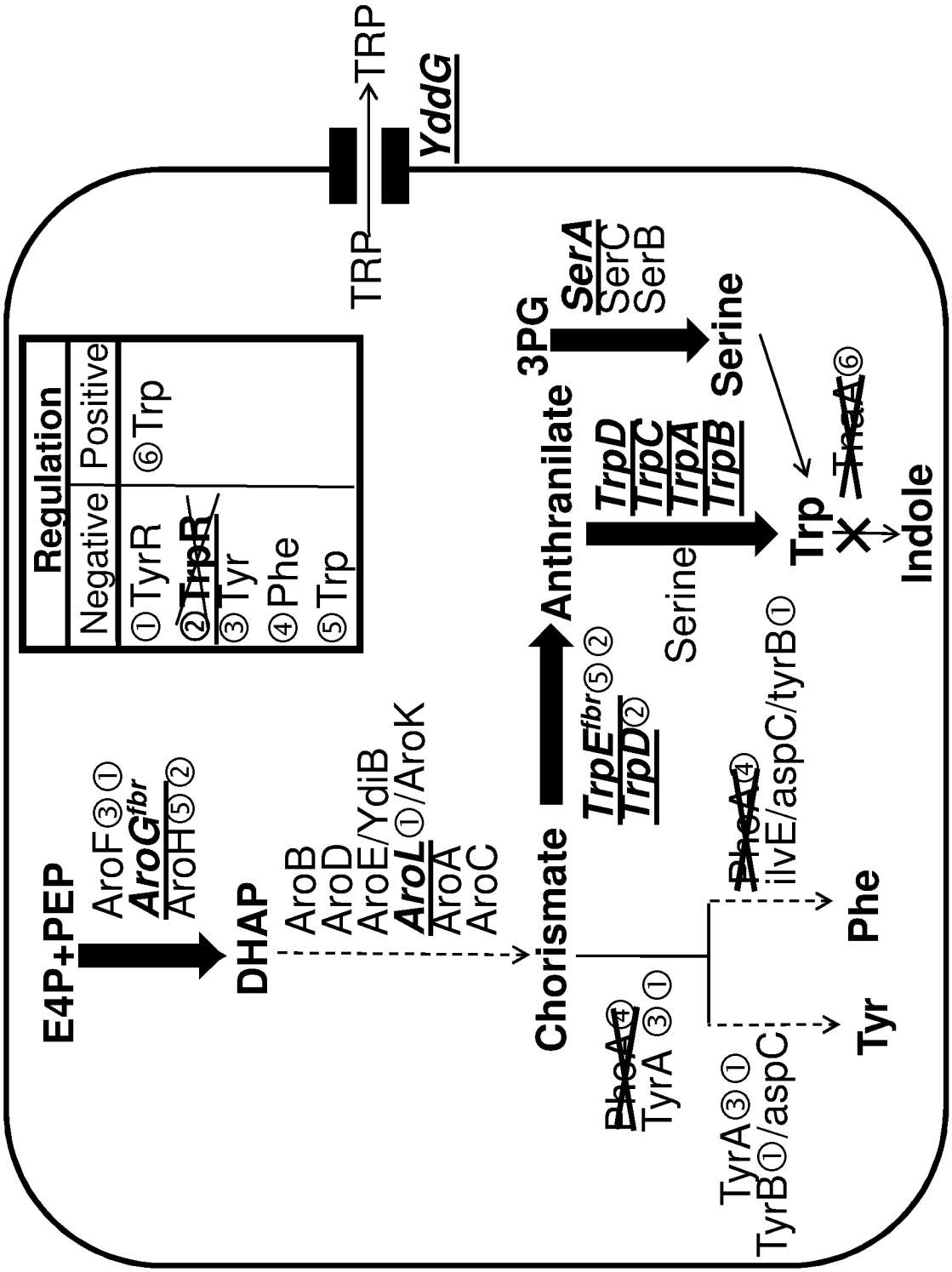

Regulation
Negative
Positive
①TyrR
②TrpR
③Tyr
④Phe
⑤Trp
⑥Trp
TRP
TRP
YddG
E4P+PEP
AroF③①
AroG^fbr
AroH⑤②
DHAP
AroB
AroD
AroE/YdiB
AroL①/AroK
AroA
AroC
Chorismate
TrpE^fbr⑤②
TrpD②
Anthranilate
3PG
SerA
SerC
SerB
Serine
Serine
TrpD
TrpC
TrpA
TrpB
Trp
TrpA⑥
Indole
PheA④
TyrA③①
TyrB①/aspC
ilvE/aspC/tyrB①
Phe
Tyr

FIG. 25A

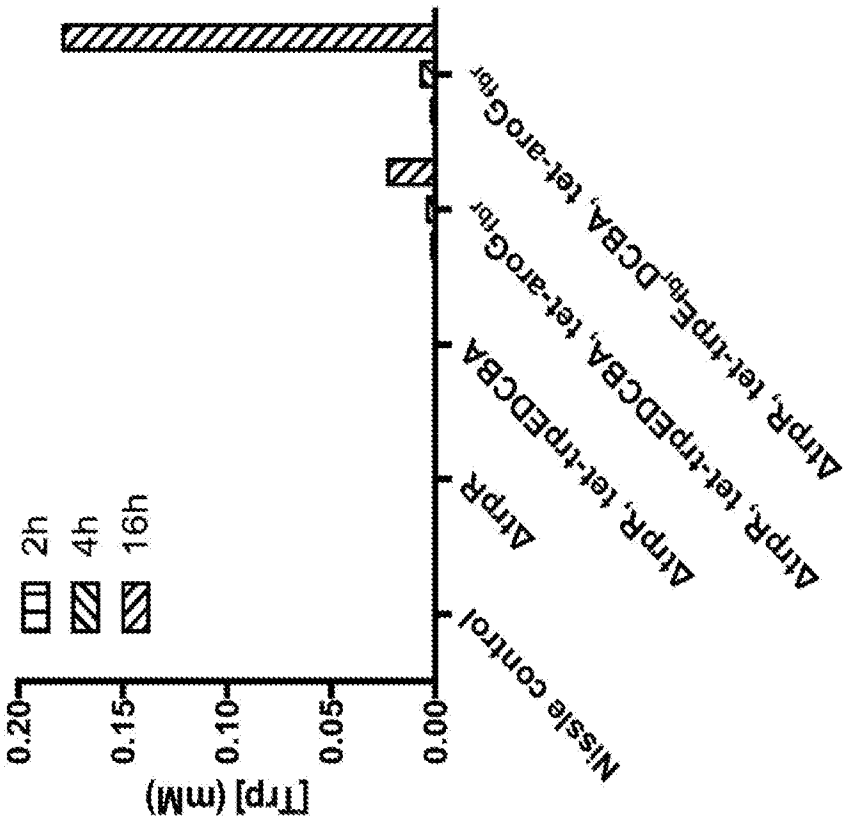

2h
4h
16h
[Trp] (mM)
0.20
0.15
0.10
0.05
0.0
Nissle control
AtrpR
AtrpR, tet-trpEDCBA
AtrpR, tet-trpEDCBA, tet-aroG$_{fbr}$
AtrpR, tet-trpE$_{fbr}$DCBA, tet-aroG$_{fbr}$

FIG. 25B

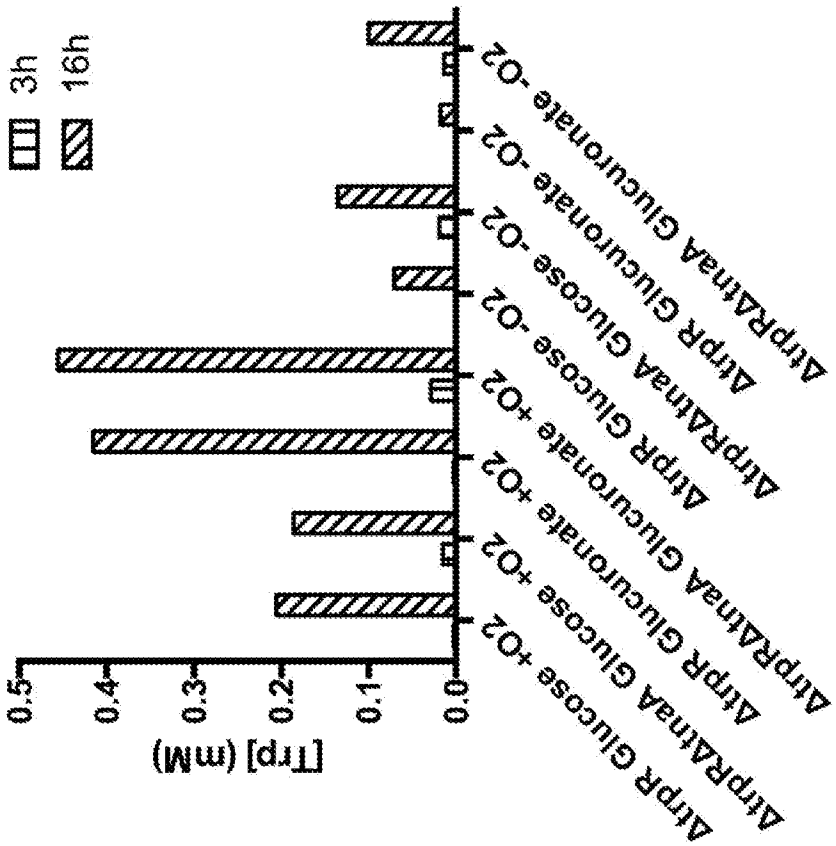

3h
16h
[Trp] (mM)
0.5
0.4
0.3
0.2
0.1
0.0
ΔtrpR Glucose +O2
ΔtrpRΔtnaA Glucose +O2
ΔtrpR Glucuronate +O2
ΔtrpRΔtnaA Glucuronate +O2
ΔtrpR Glucose -O2
ΔtrpRΔtnaA Glucose -O2
ΔtrpR Glucuronate -O2
ΔtrpRΔtnaA Glucuronate -O2

FIG. 25C

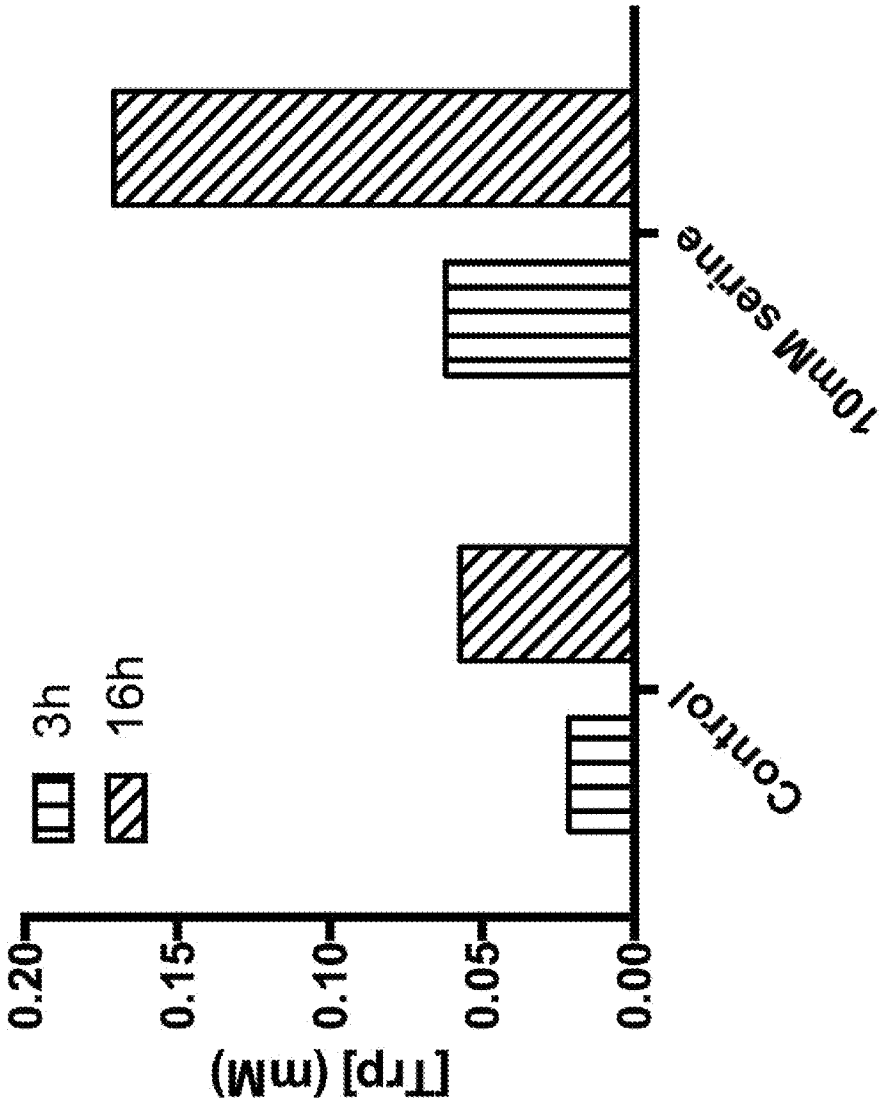


FIG. 26

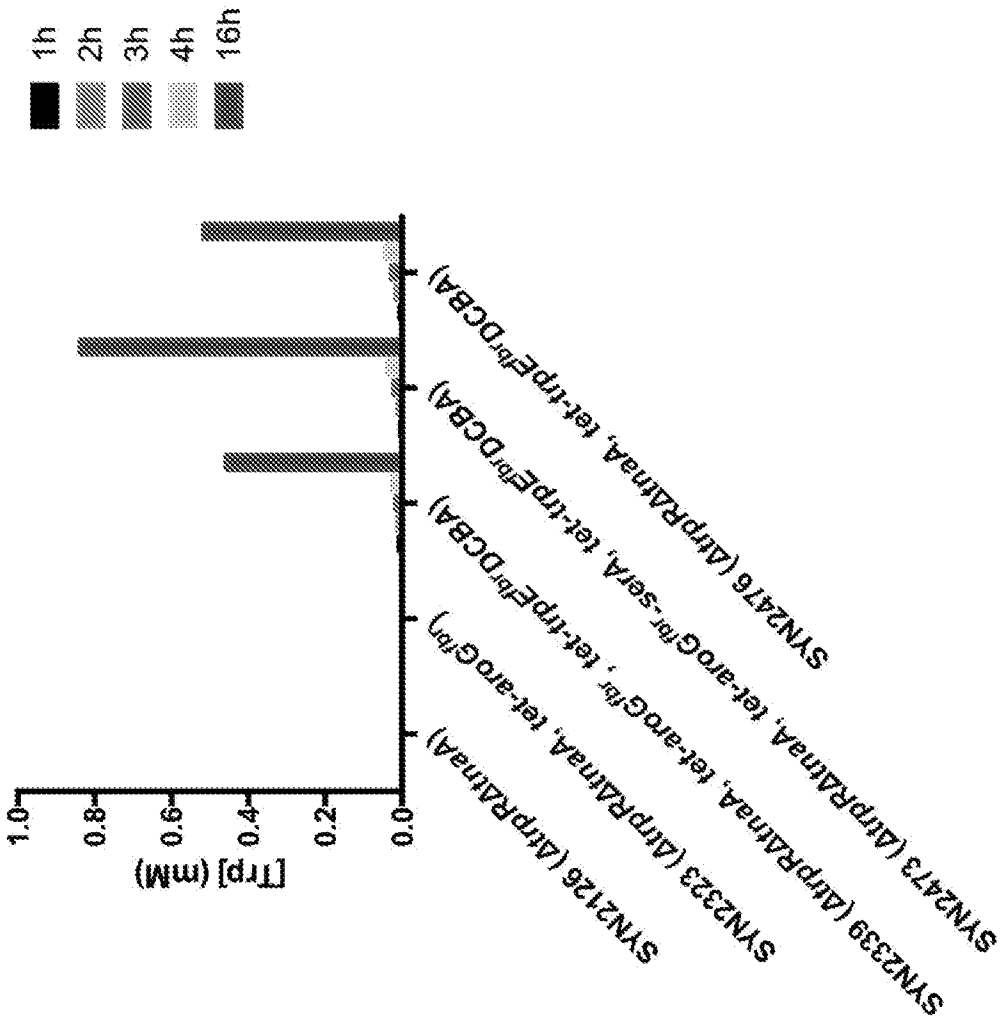

1h
2h
3h
4h
16h
[Trp] (mM)
1.0
0.8
0.6
0.4
0.2
0.0
SYN2126 (ΔtrpRΔtnaA, tet-aroGfbr)
SYN2323 (ΔtrpRΔtnaA, tet-aroGfbr, tet-trpEfbrDCBA)
SYN2339 (ΔtrpRΔtnaA, tet-aroGfbr-serA, tet-trpEfbrDCBA)
SYN2473 (ΔtrpRΔtnaA, tet-aroGfbr-serA, tet-trpEfbrDCBA)
SYN2476 (ΔtrpRΔtnaA, tet-trpEfbrDCBA)

FIG. 27

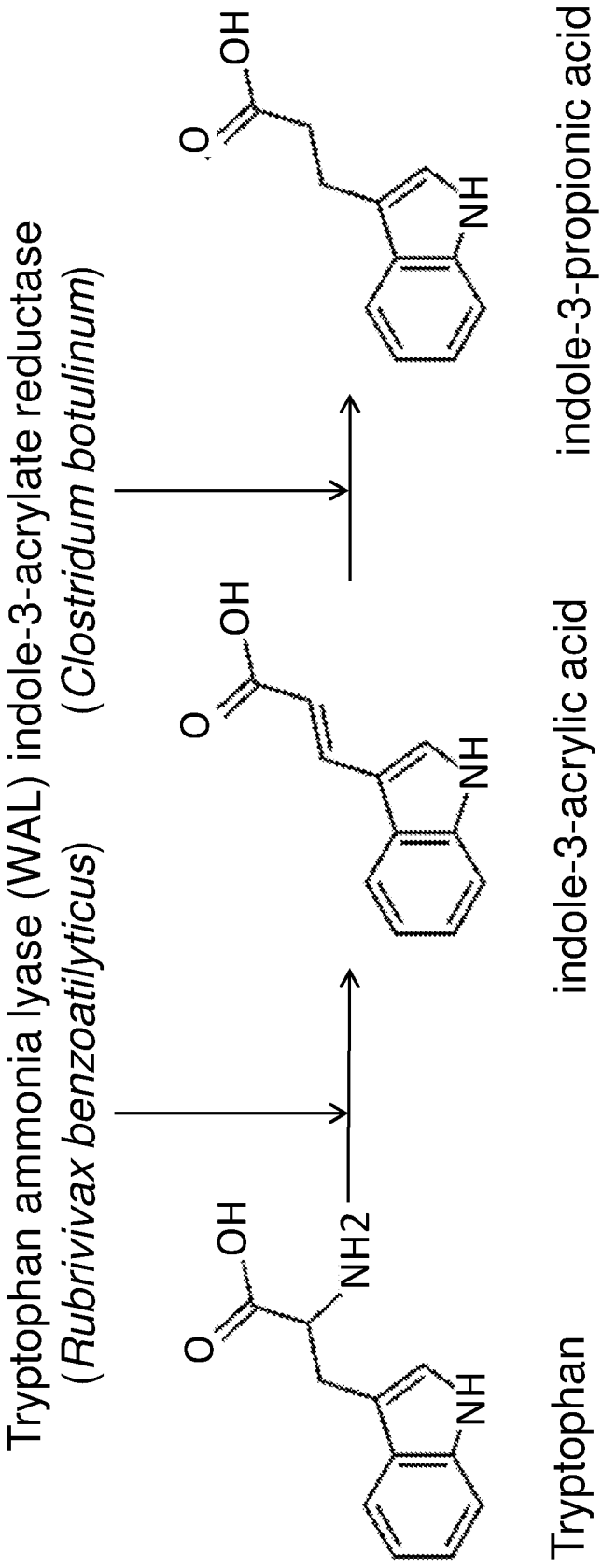
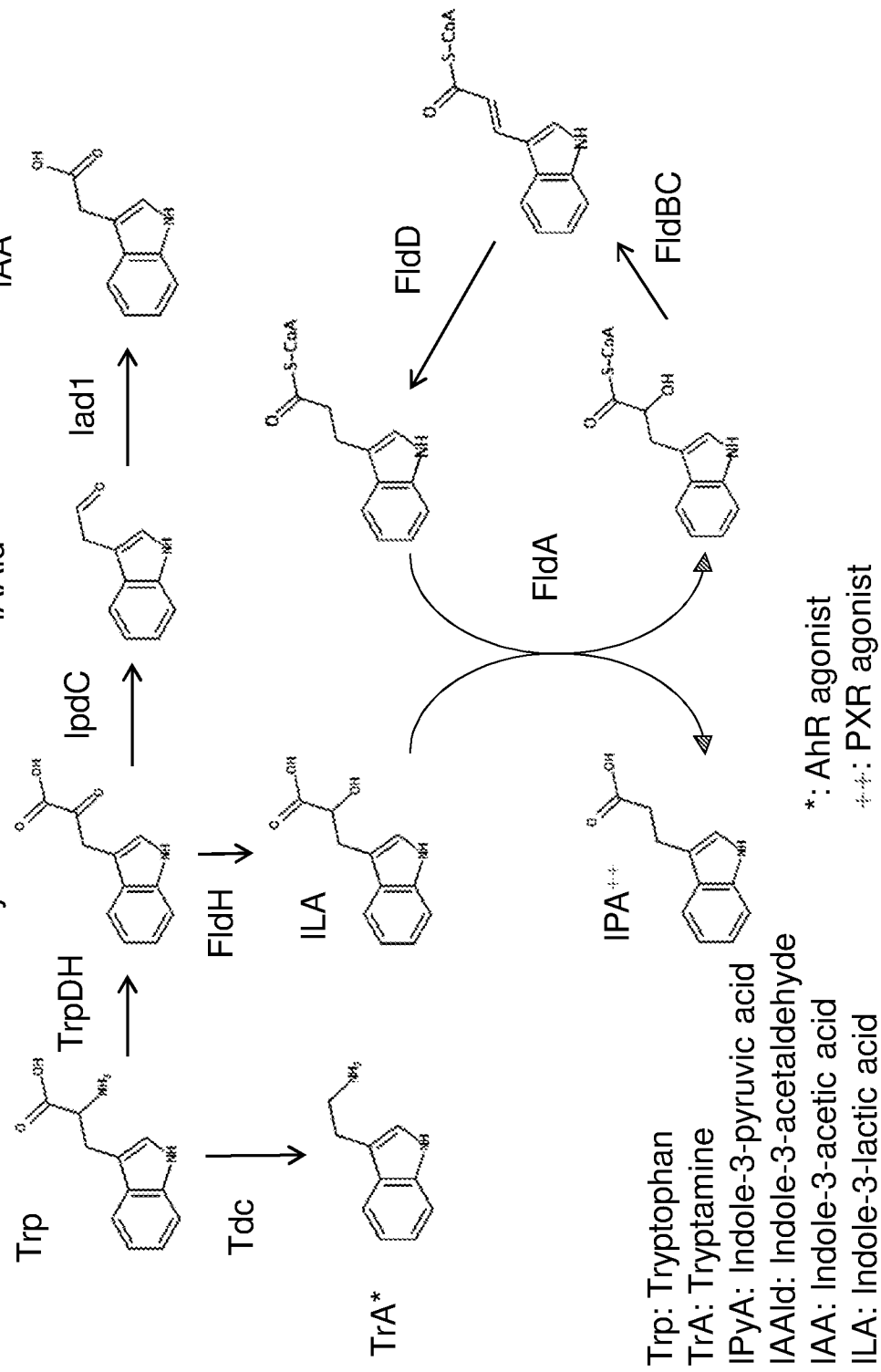

Tryptophan ammonia lyase (WAL) indole-3-acrylate reductase
(Rubrivivax benzoatilyticus) (Clostridium botulinum)
OH
O
NH2
NH
OH
O
NH
OH
O
NH
Tryptophan
indole-3-acrylic acid
indole-3-propionic acid

FIG. 28



Trp: Tryptophan
TrA: Tryptamine
IPyA: Indole-3-pyruvic acid
IAAld: Indole-3-acetaldehyde
IAA: Indole-3-acetic acid
ILA: Indole-3-lactic acid
IPA: Indole-3-propionic acid

FIG. 29

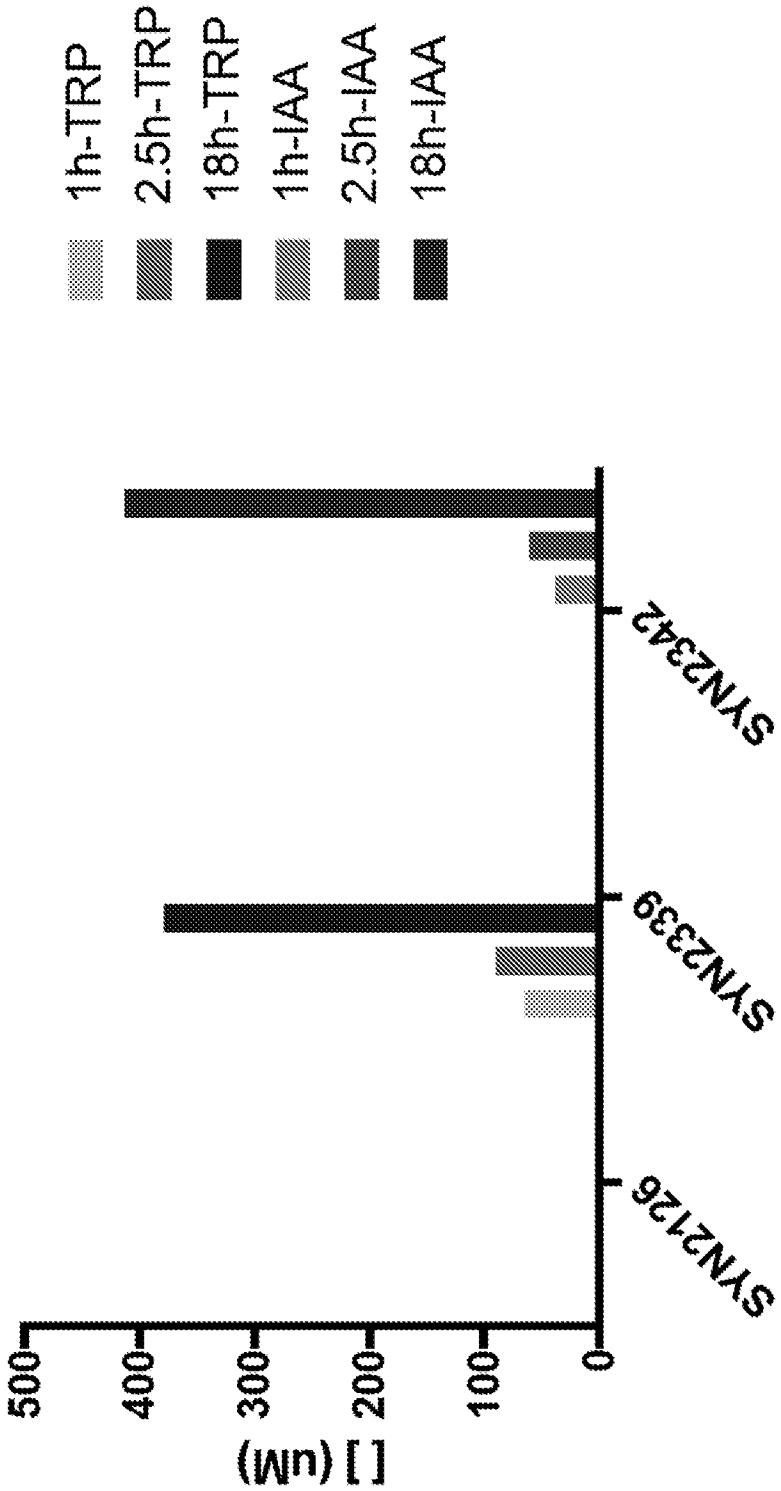

1h-TRP
2.5h-TRP
18h-TRP
1h-IAA
2.5h-IAA
18h-IAA
[ ] (uM)
500
400
300
200
100
0
SYN2126
SYN2339
SYN2342

FIG. 30

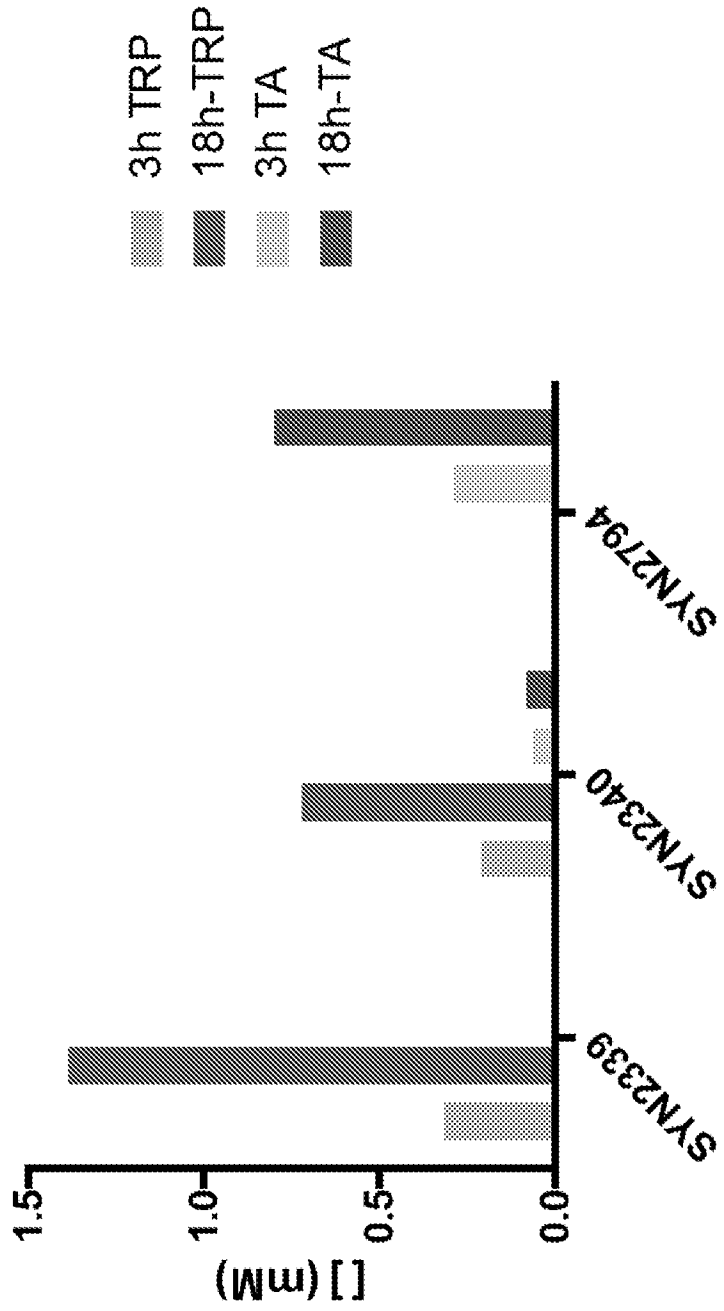
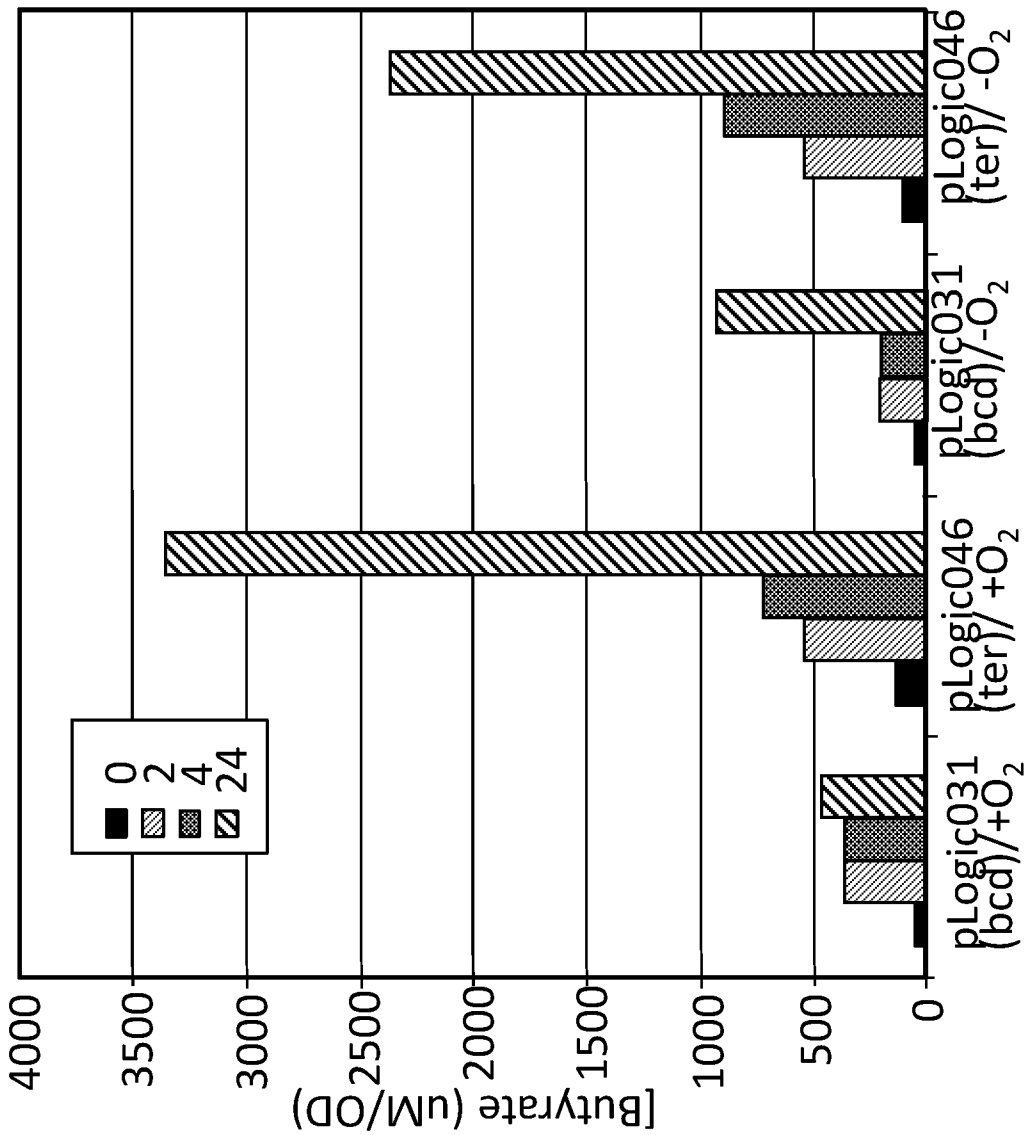

3h TRP
18h-TRP
3h TA
18h-TA
SYN2794
SYN2340
SYN2339
0.0
0.5
1.0
1.5
[ ] (mM)

Fig. 31A


[Butyrate (uM/OD)
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
24
pLogic031 (bcd)/+O2
pLogic046 (ter)/ +O2
pLogic031 (bcd)/-O2
pLogic046 (ter)/ -O2

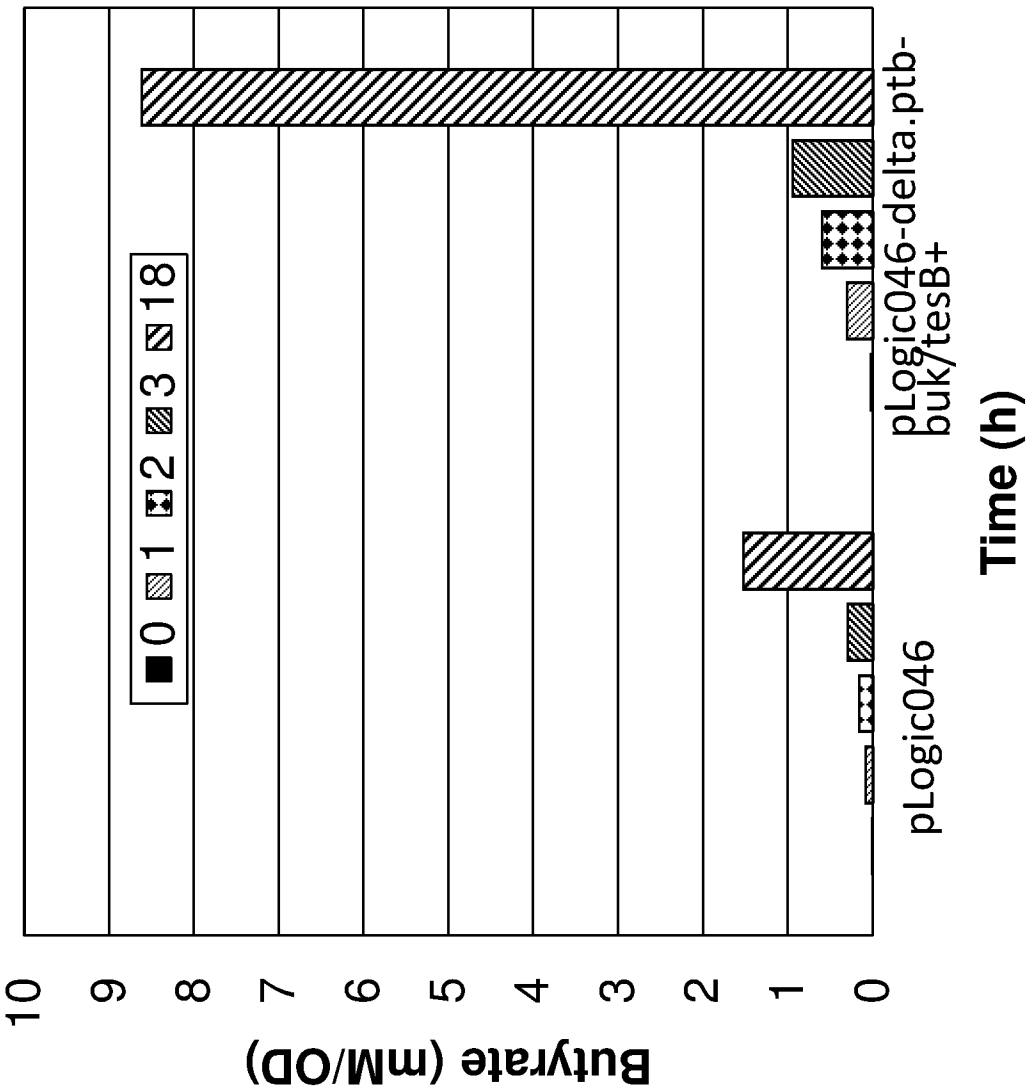

Butyrate (mM/OD)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
18
pLogic046
pLogic046-delta.ptb-buk/tesB+
Time (h)

Fig. 31B

Fig. 32

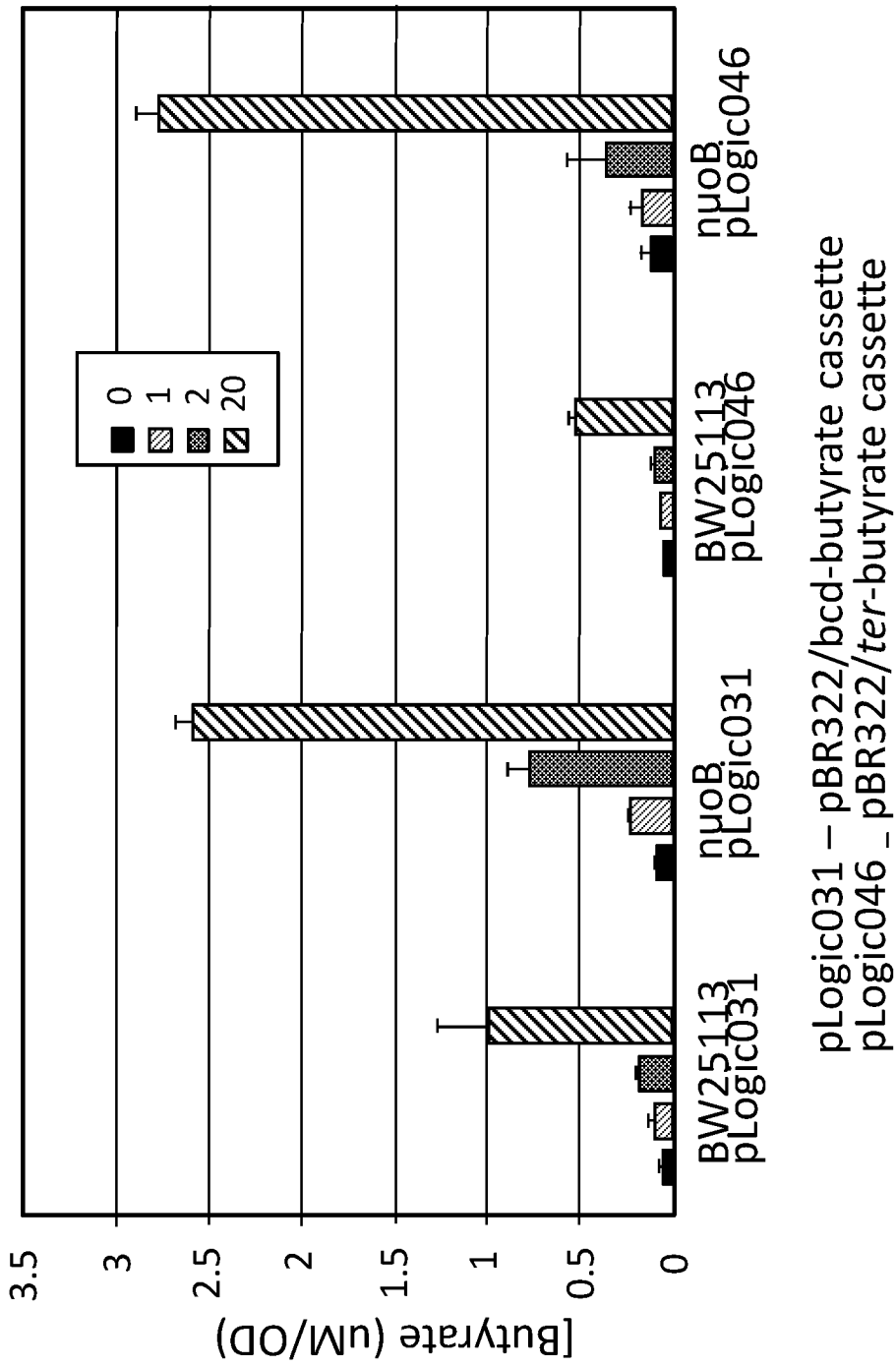

[Butyrate (uM/OD)
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
20
BW25113 pLogic031
nuoB pLogic031
BW25113 pLogic046
nuoB pLogic046
pLogic031 – pBR322/bcd-butyrate cassette
pLogic046 _ pBR322/ter-butyrate cassette

FIG. 33A

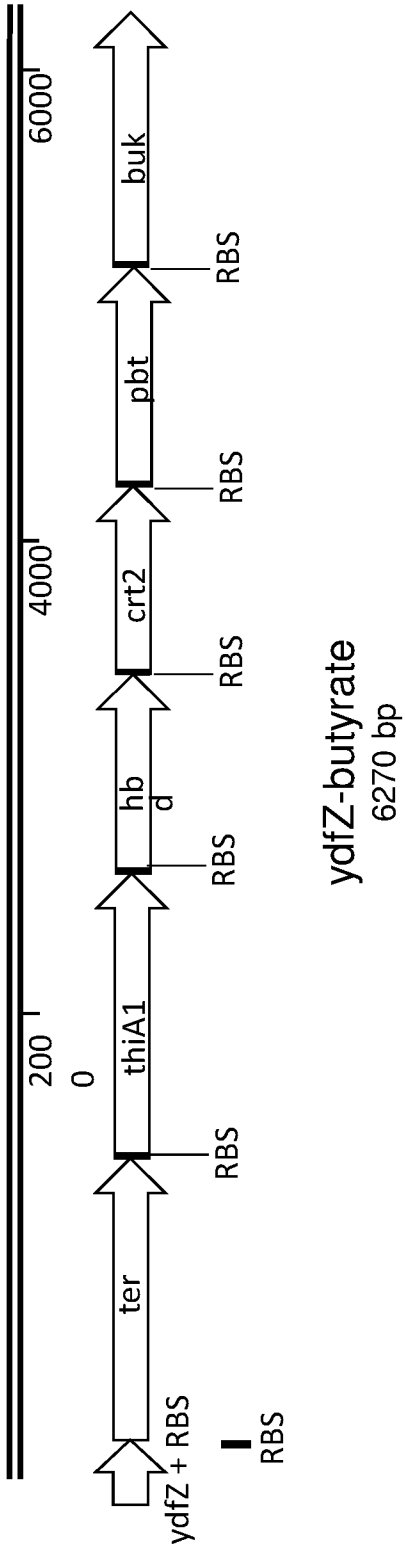

6000
4000
200
0
ydfZ + RBS
ter
RBS
thiA1
RBS
hbd
RBS
crt2
RBS
pbt
RBS
buk
RBS
RBS
ydfZ-butyrate
6270 bp

FIG. 33B

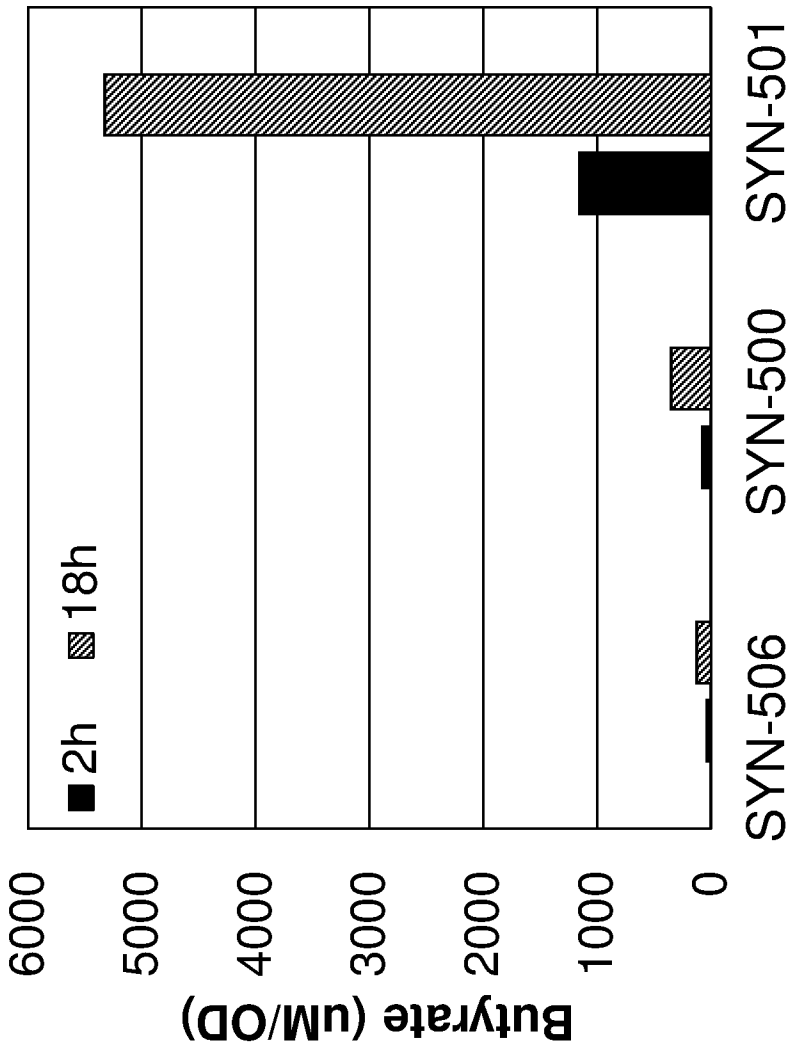

Butyrate (uM/OD)
6000
5000
4000
3000
2000
1000
0
SYN-506
SYN-500
SYN-501
2h
18h

FIG. 33C

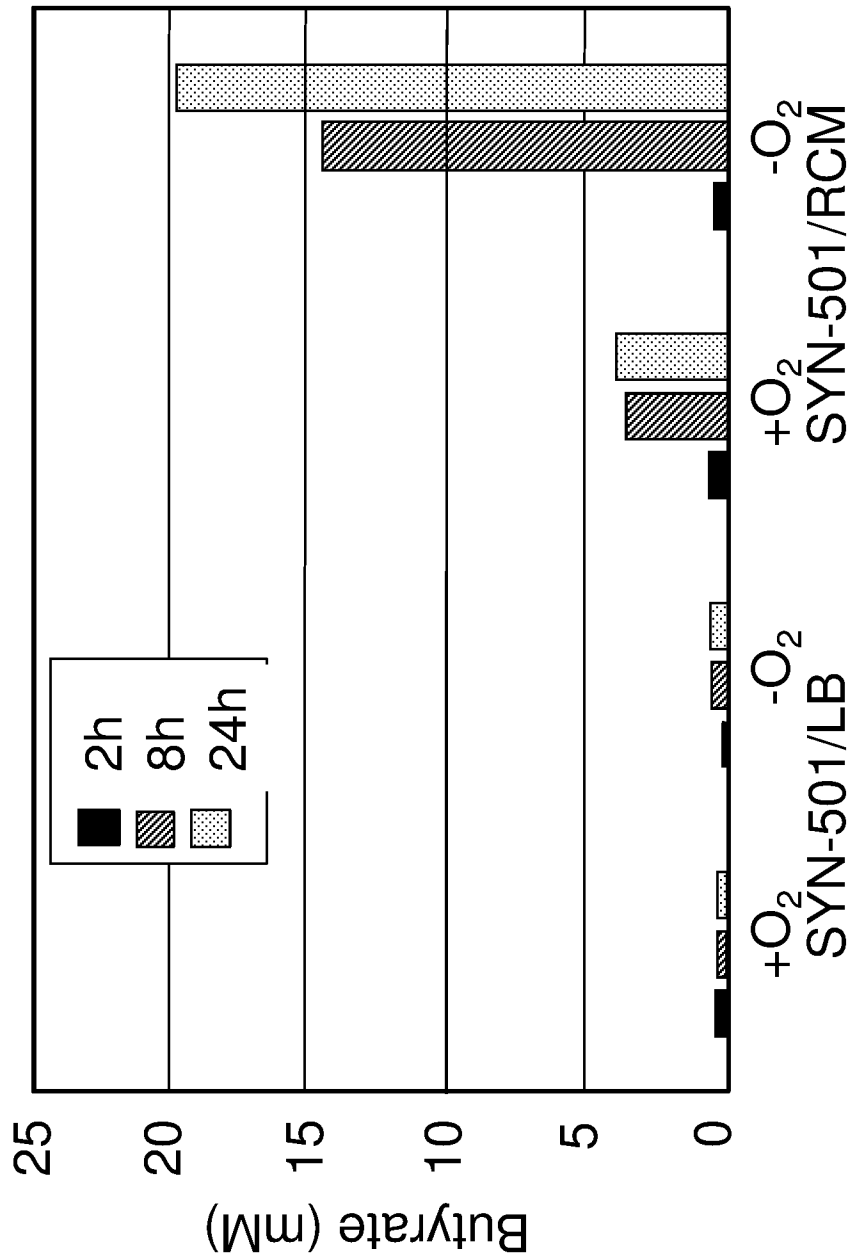

Butyrate (mM)
25
20
15
10
5
0
2h
8h
24h
+O2 SYN-501/LB
-O2
+O2 SYN-501/RCM
-O2

FIG. 33D

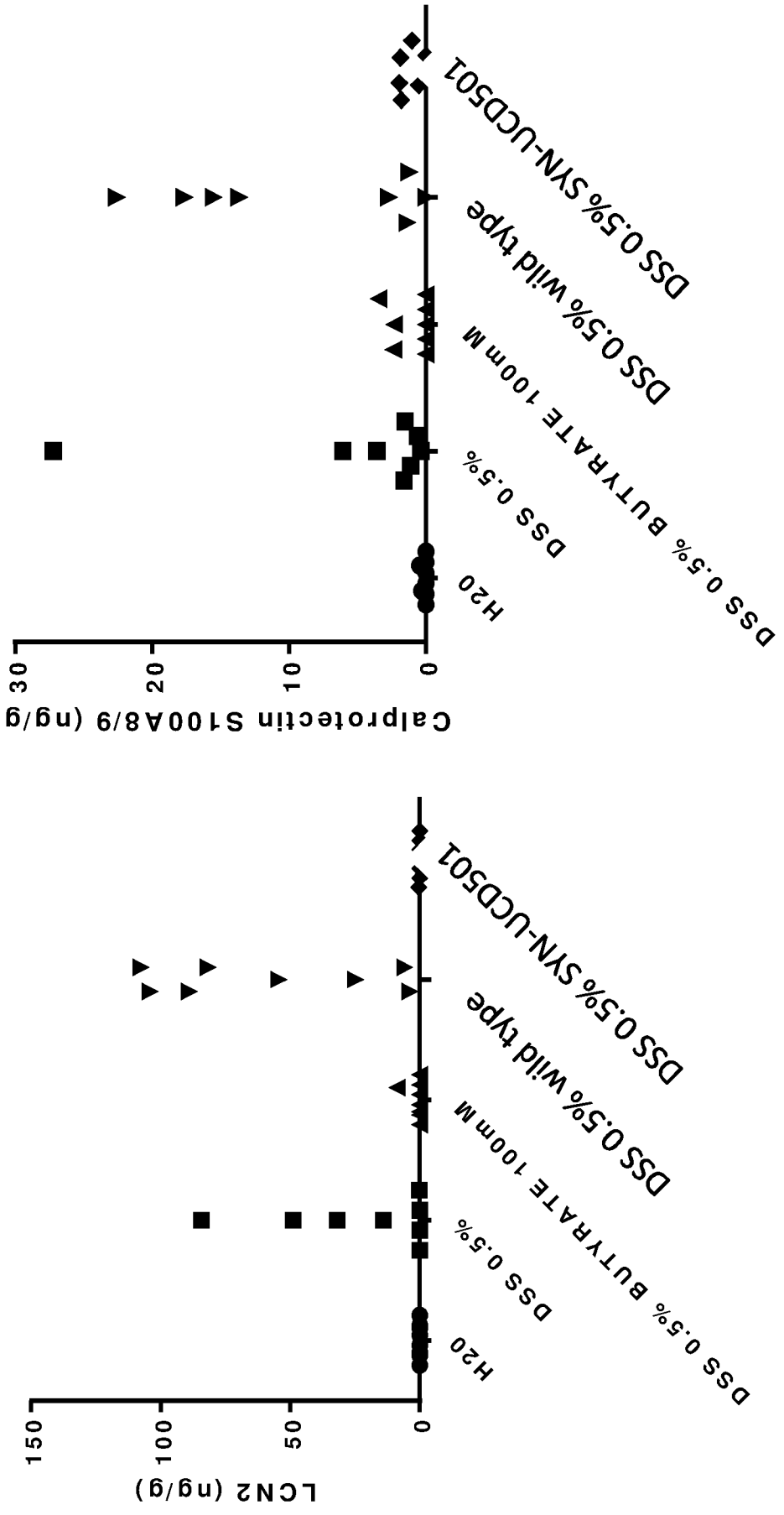

Calprotectin S100A8/9 (ng/g
0
10
20
30
H20
DSS 0.5%
DSS 0.5% BUTYRATE 100m M
DSS 0.5% wild type
DSS 0.5% SYN-UCD501
LCN2 (ng/g)
0
50
100
150
H20
DSS 0.5%
DSS 0.5% BUTYRATE 100m M
DSS 0.5% wild type
DSS 0.5% SYN-UCD501

FIG. 34A

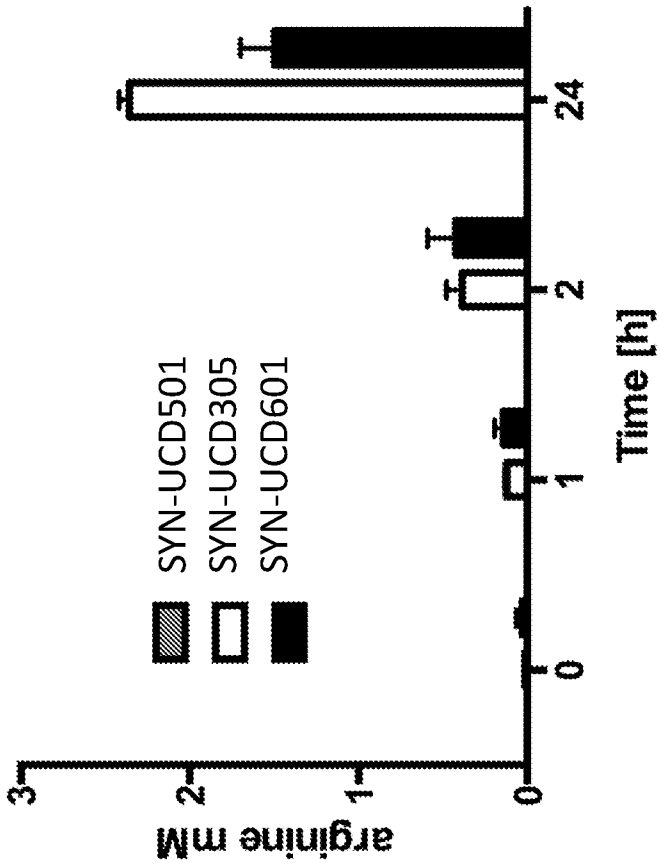

Arginine production
SYN-UCD501
SYN-UCD305
SYN-UCD601
arginine mM
0
1
2
3
0
1
2
24
Time [h]

FIG. 34B

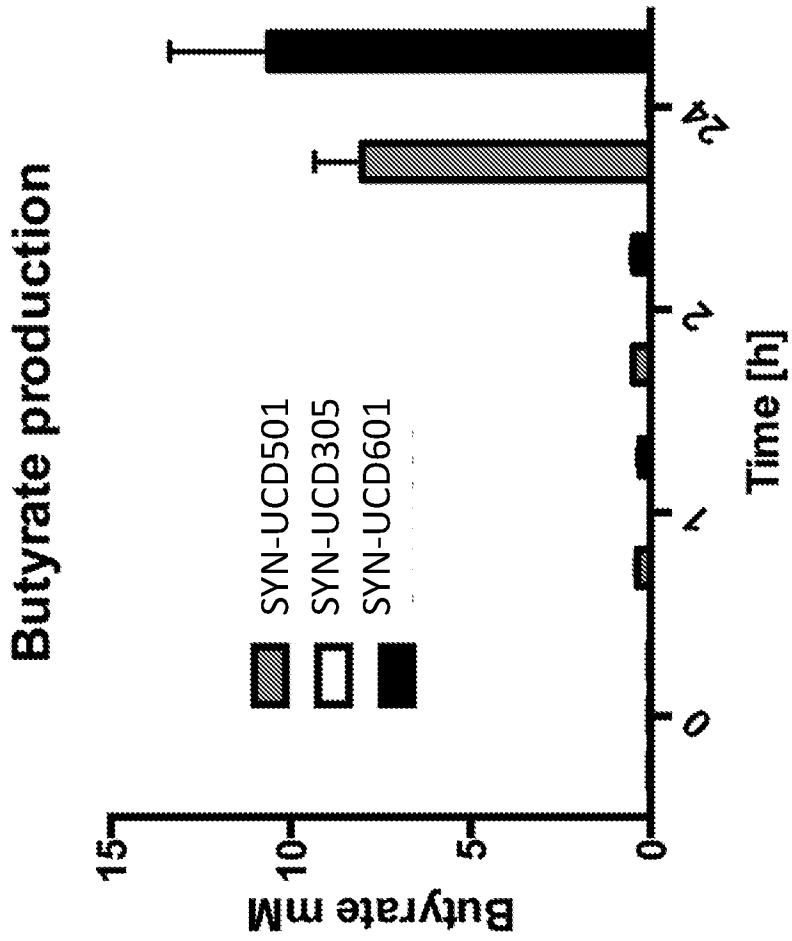

Butyrate production
SYN-UCD501
SYN-UCD305
SYN-UCD601
Butyrate mM
15
10
5
0
0
1
2
24
Time [h]

FIG. 35

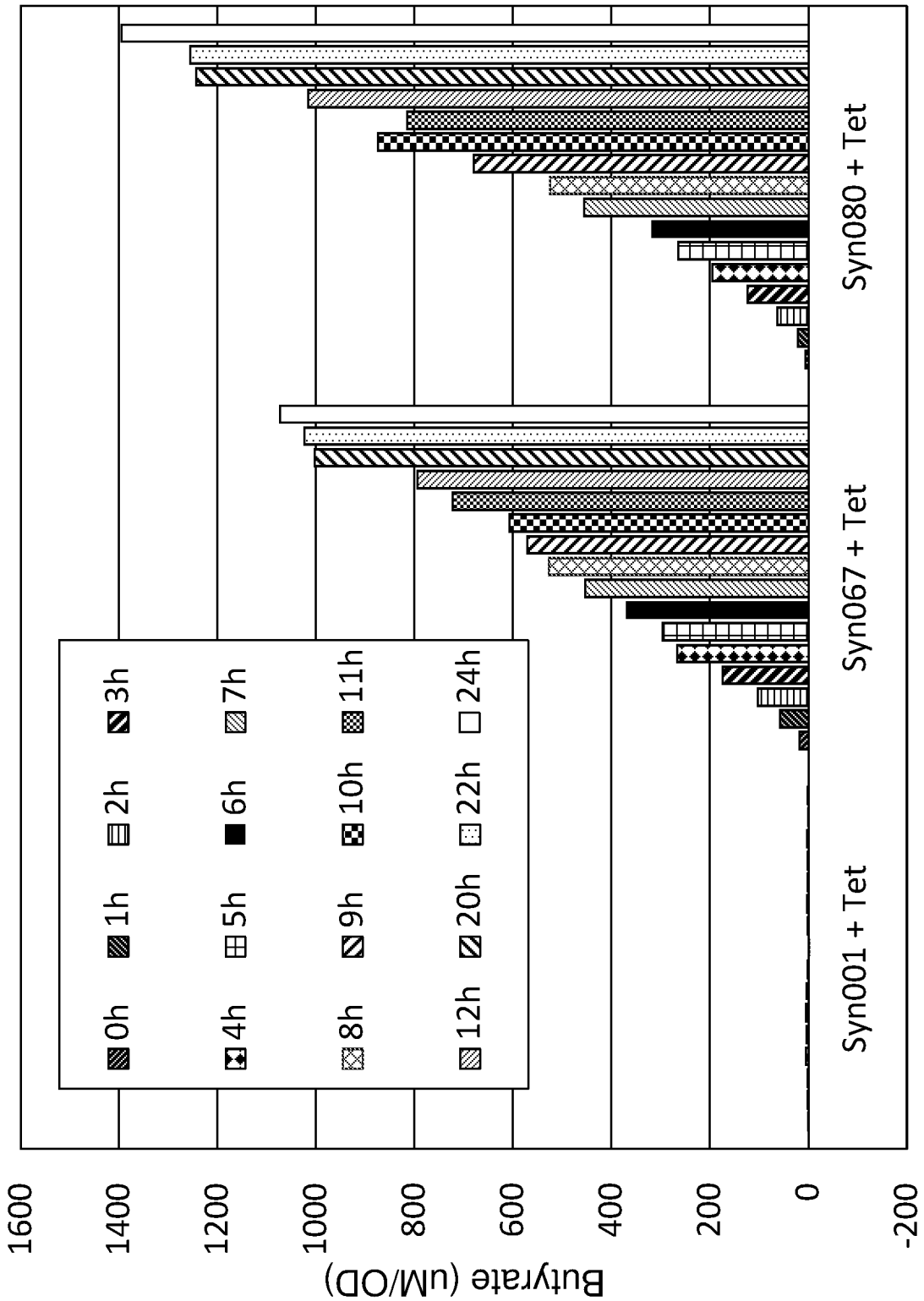

Butyrate (uM/OD)
1600
1400
1200
1000
800
600
400
200
0
-200
Syn001 + Tet
Syn067 + Tet
Syn080 + Tet
0h
1h
2h
3h
4h
5h
6h
7h
8h
9h
10h
11h
12h
20h
22h
24h

FIG. 36

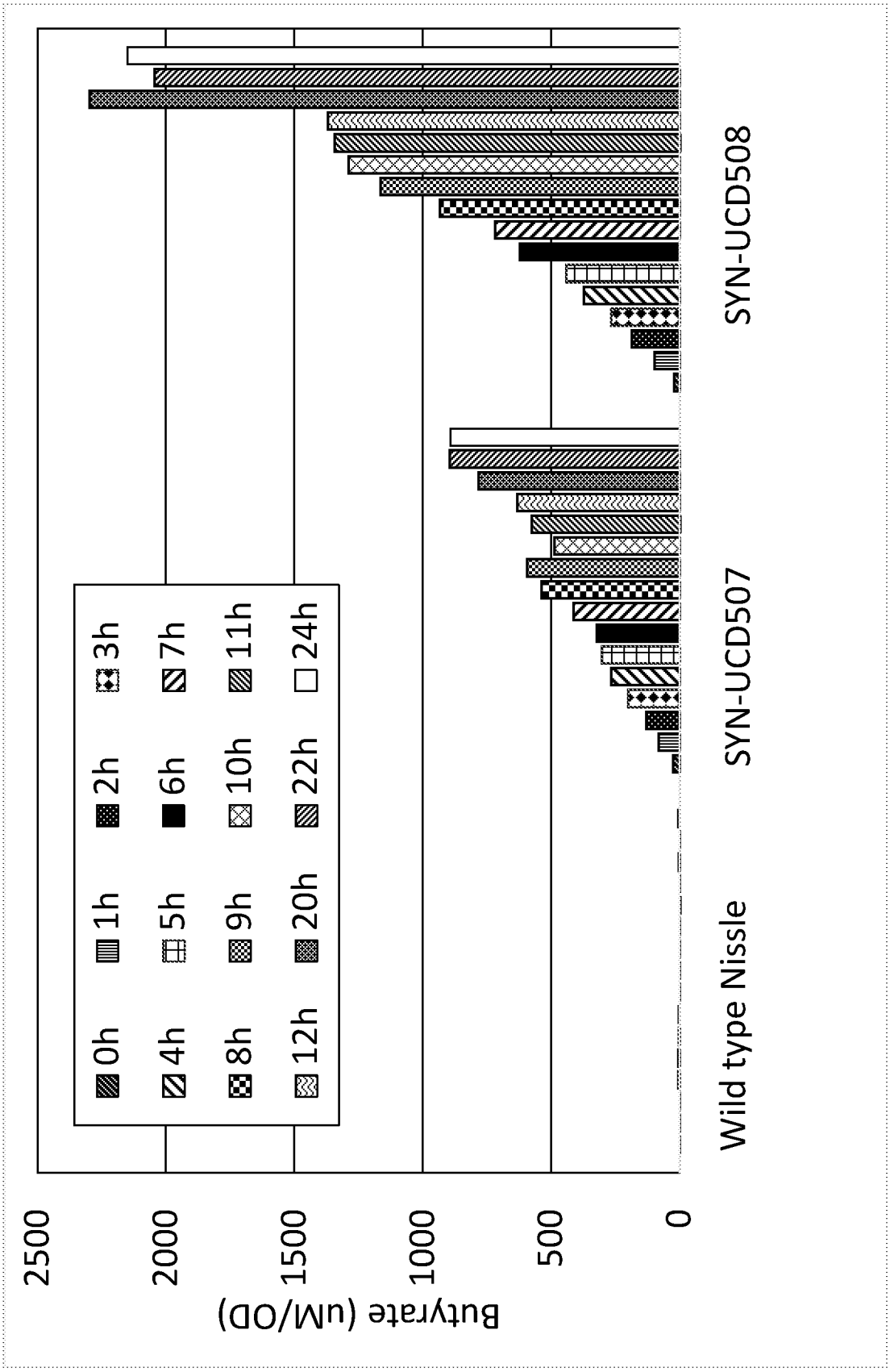

Butyrate (uM/OD)
2500
2000
1500
1000
500
0
0h
1h
2h
3h
4h
5h
6h
7h
8h
9h
10h
11h
12h
20h
22h
24h
Wild type Nissle
SYN-UCD507
SYN-UCD508

FIG. 37

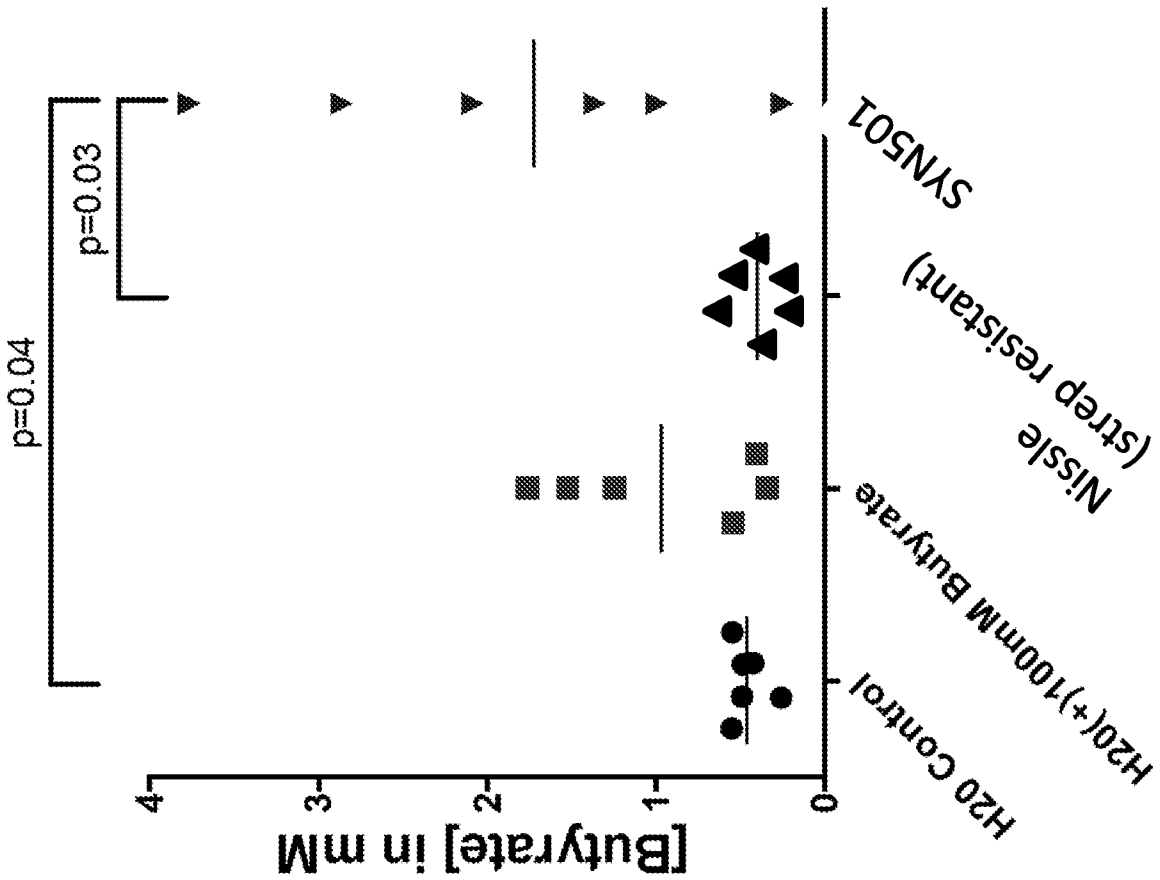

p=0.04
p=0.03
[Butyrate] in mM
0
1
2
3
4
H20 Control
H20(+)100mM Butyrate
Nissle
(strep resistant)
SYN501

FIG. 38A

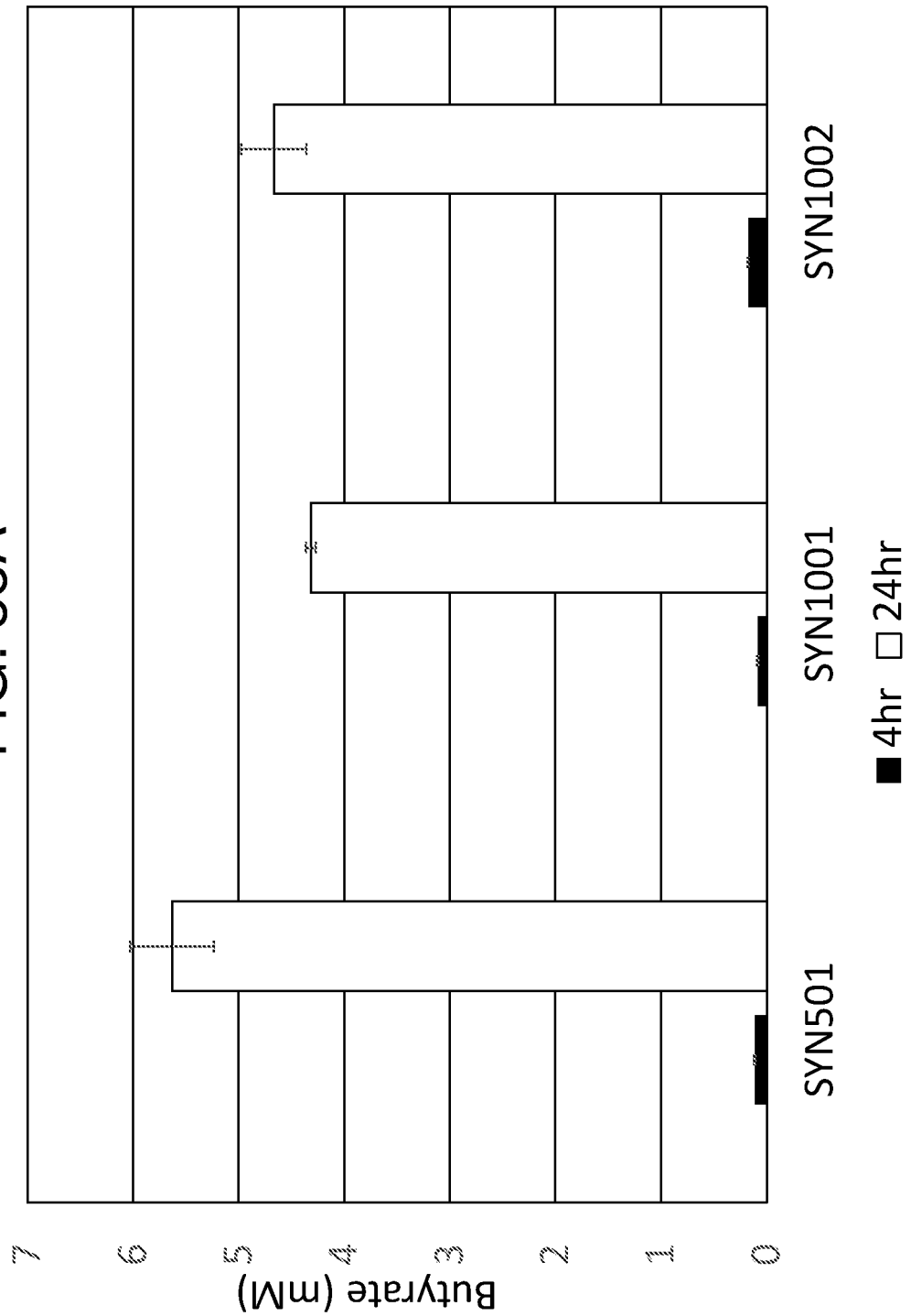


FIG. 38B

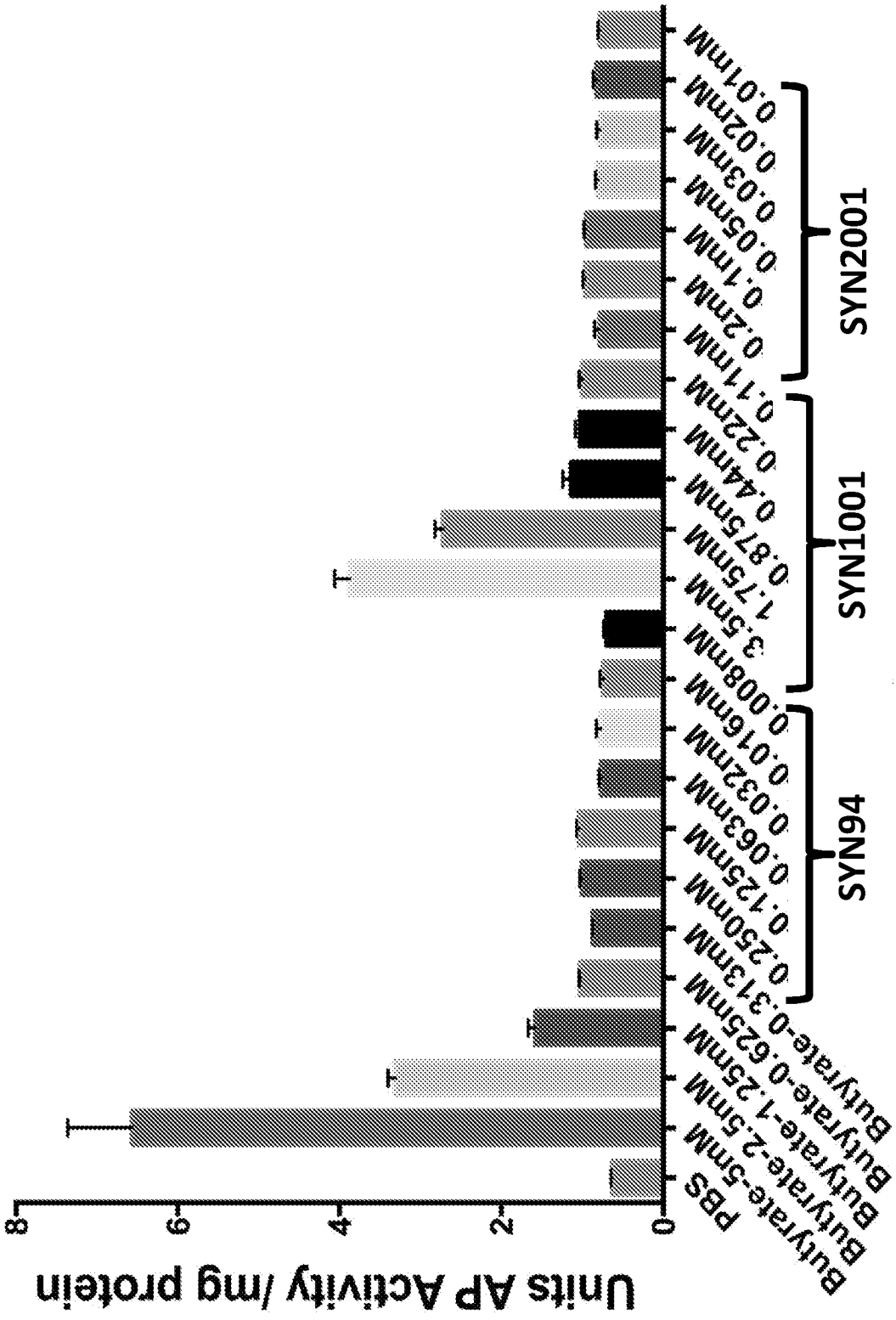

Units AP Activity /mg protein
0
2
4
6
8
PBS
Butyrate-5mM
Butyrate-2.5mM
Butyrate-1.25mM
Butyrate-0.625mM
Butyrate-0.313mM
0.250mM
0.125mM
0.063mM
0.032mM
0.016mM
0.008mM
3.5mM
1.75mM
0.875mM
0.44mM
0.22mM
0.11mM
0.2mM
0.1mM
0.05mM
0.03mM
0.02mM
0.01mM
SYN94
SYN1001
SYN2001

FIG. 38C

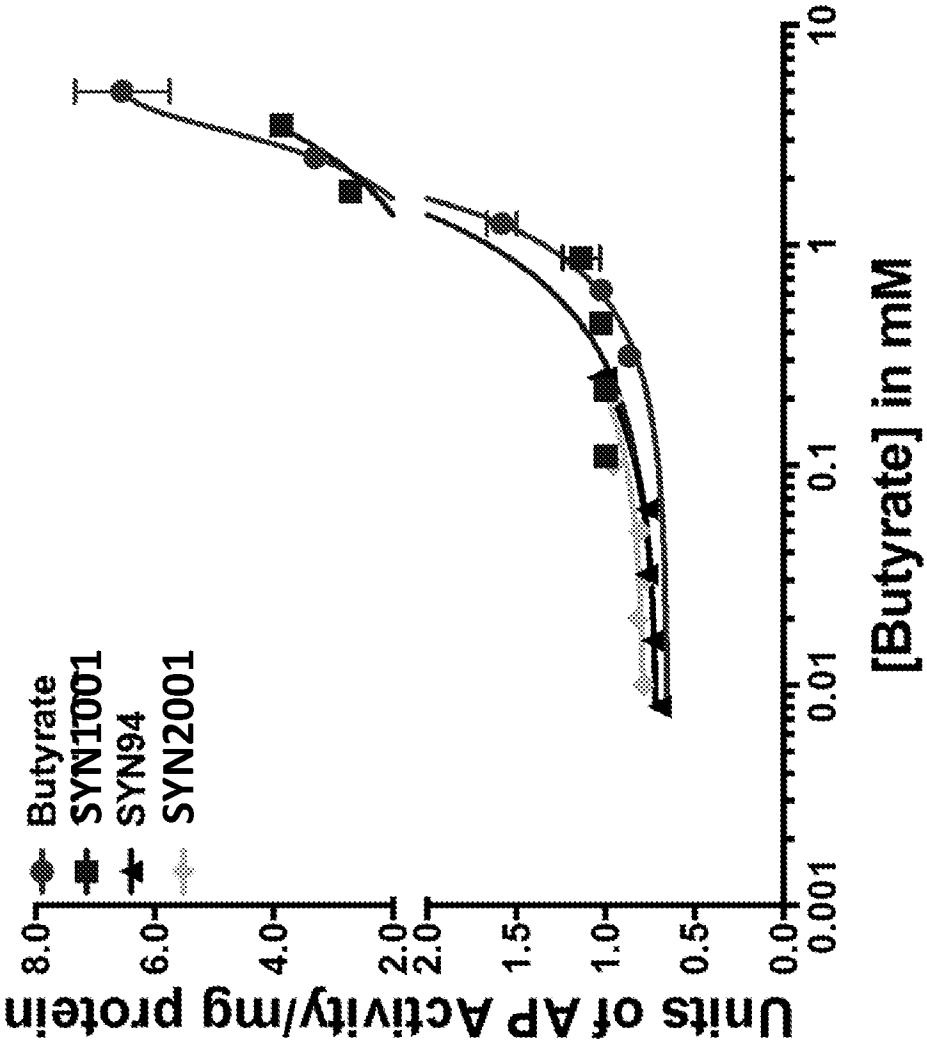

Units of AP Activity/mg protein
8.0
6.0
4.0
2.0
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
[Butyrate] in mM
Butyrate
SYN1001
SYN94
SYN2001

FIG. 39

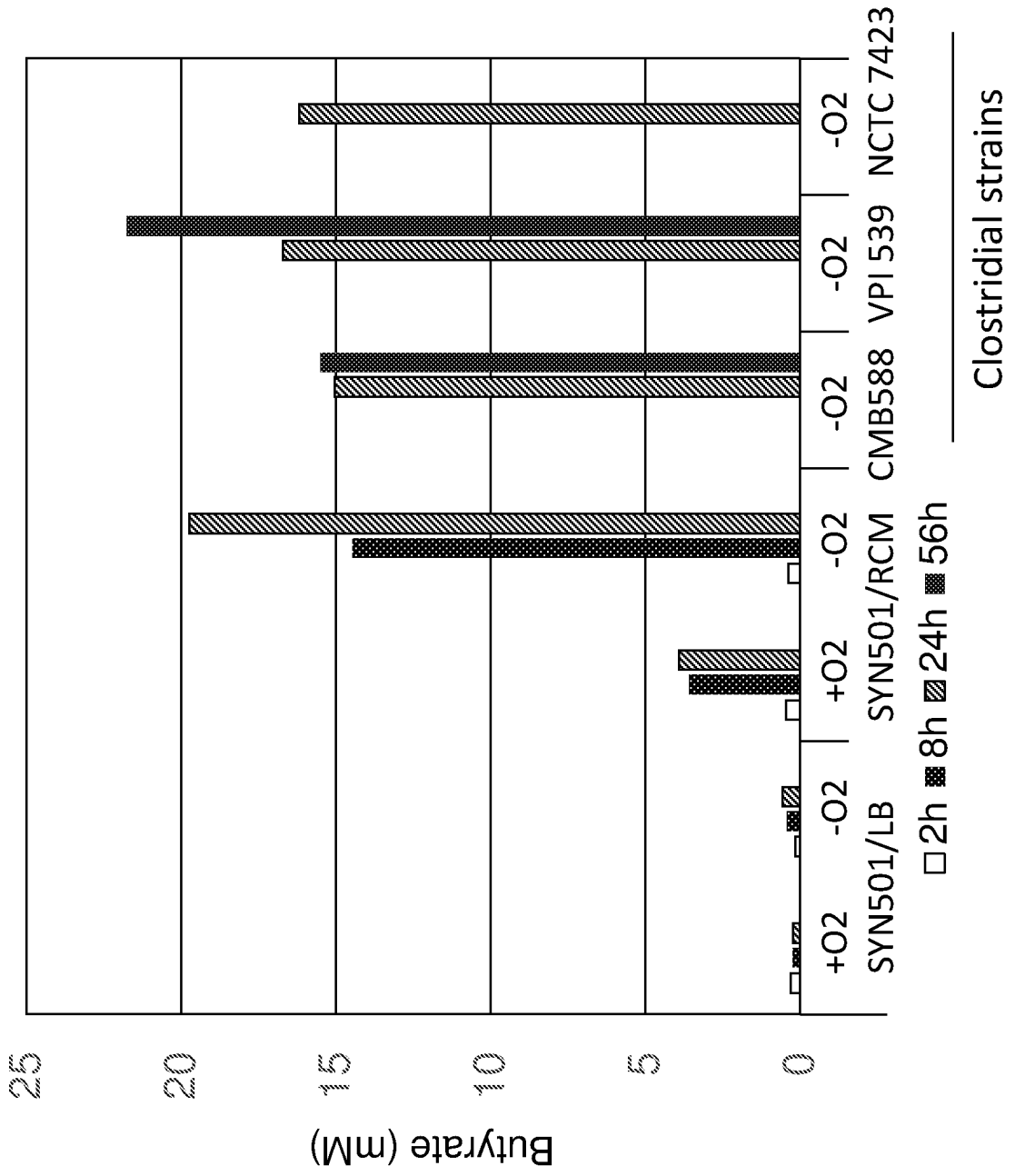

Butyrate (mM)
0
5
10
15
20
25
+O2
-O2
+O2
-O2
+O2
-O2
+O2
-O2
SYN501/LB
SYN501/RCM
CMB588
VPI 539
NCTC 7423
Clostridial strains
2h
8h
24h
56h

FIG. 40A

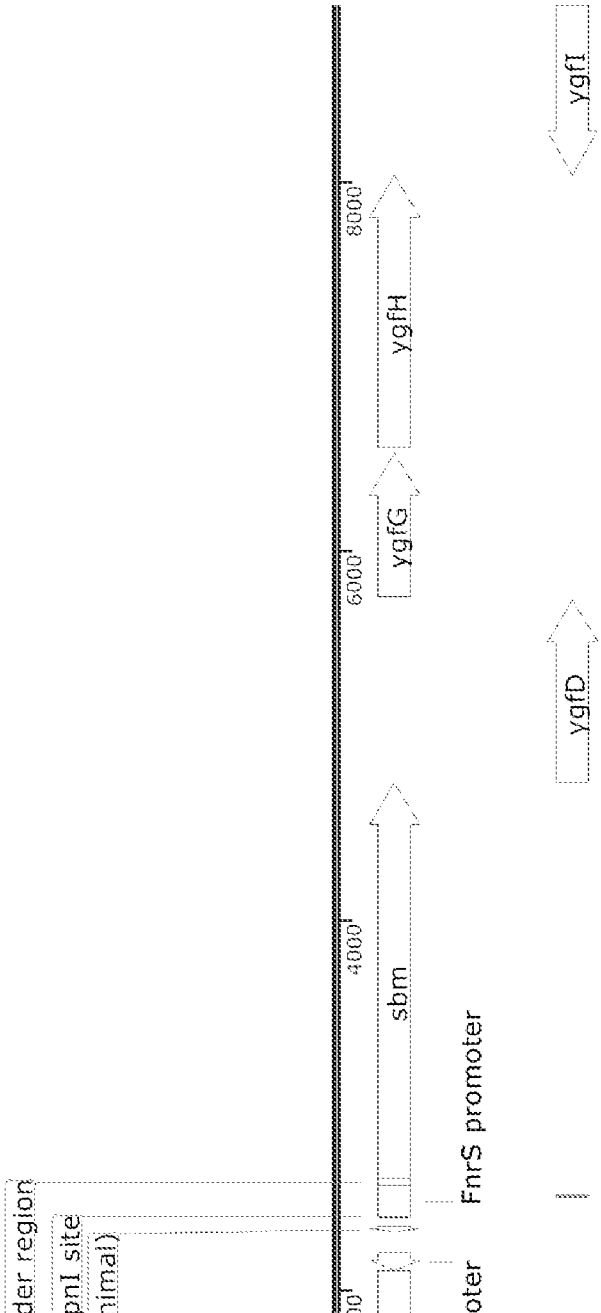
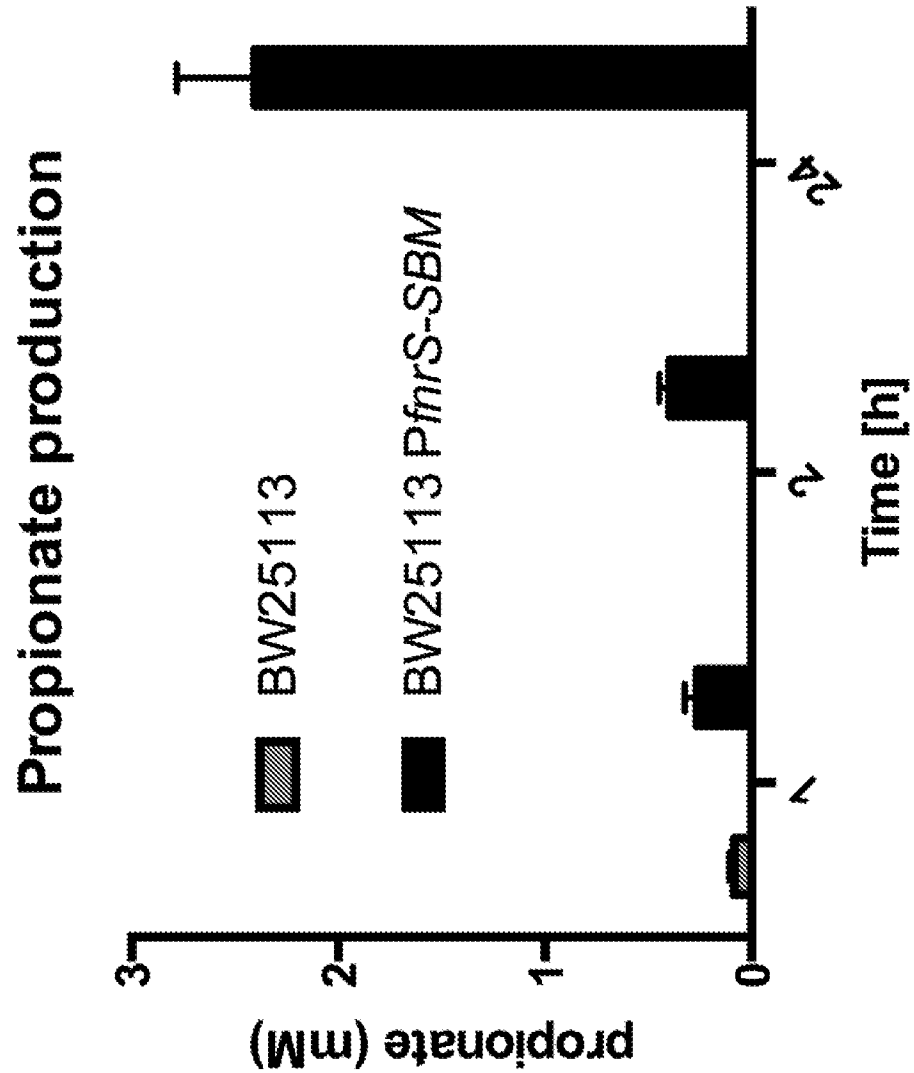

RBS and leader region
kpnI site
FRT (minimal)
Chloramphenicol Resistance
FRT
rrnB T2 terminator
rrnB T1 terminator
argP
2000
cat promoter
4000
sbm
FnrS promoter
6000
ygfG
ygfH
8000
ygfD
ygfI
FNR binding site

FIG. 40B


Propionate production
BW25113
BW25113 PfnrS-SBM
propionate (mM)
0
1
2
3
7
24
Time [h]

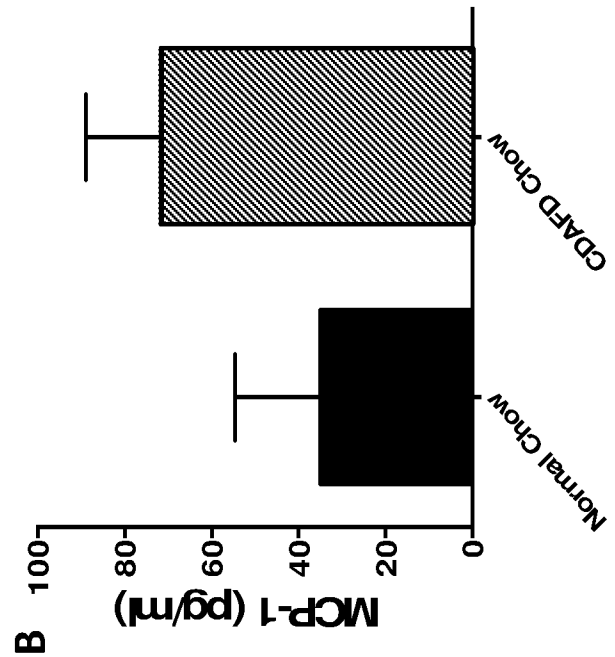


FIG. 41B

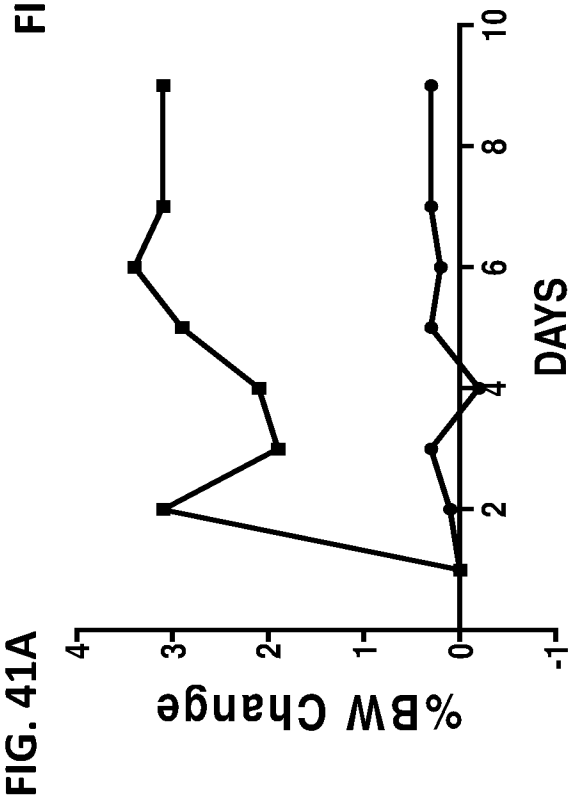


FIG. 41A

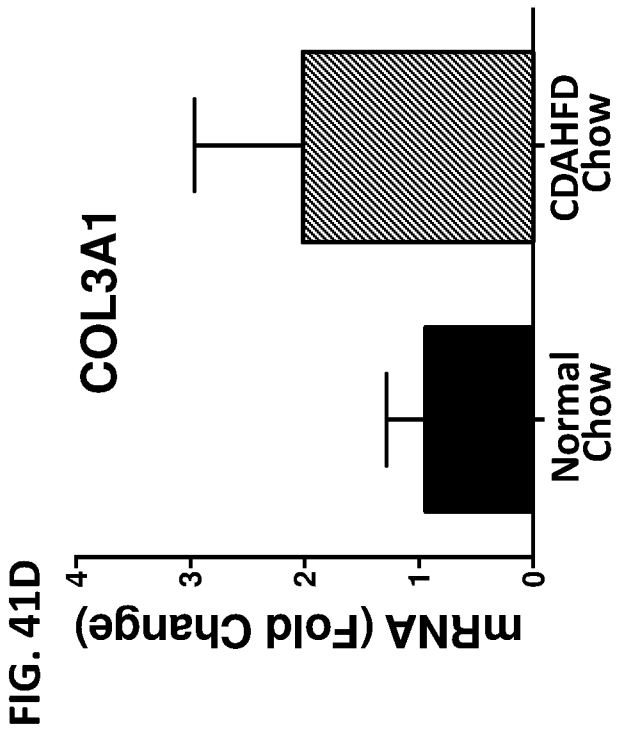


FIG. 41D

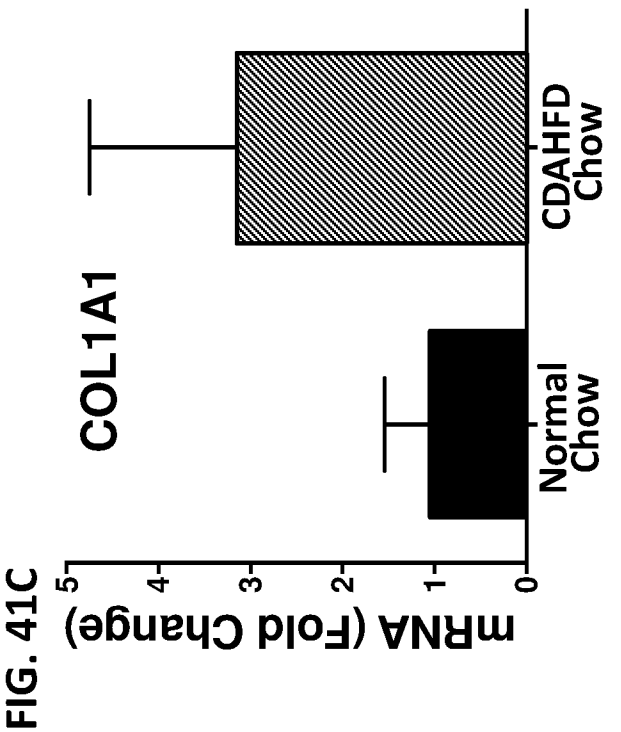


FIG. 41C

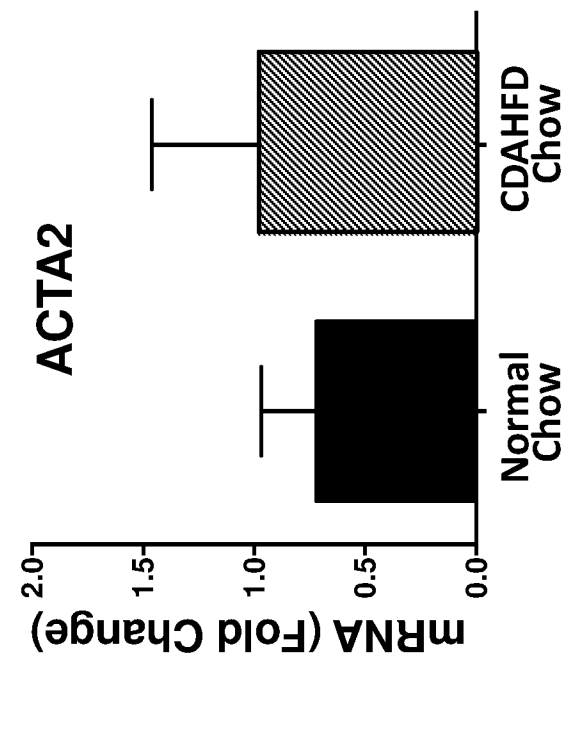


FIG. 41F

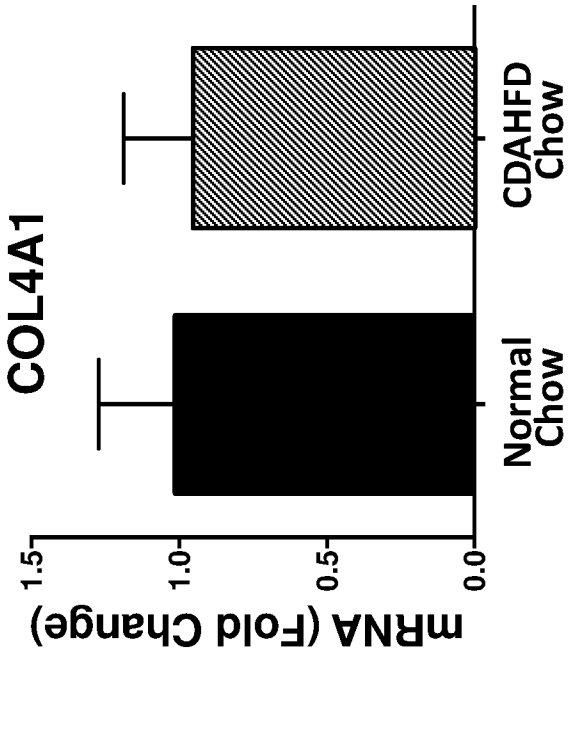


FIG 41E

Fig. 42

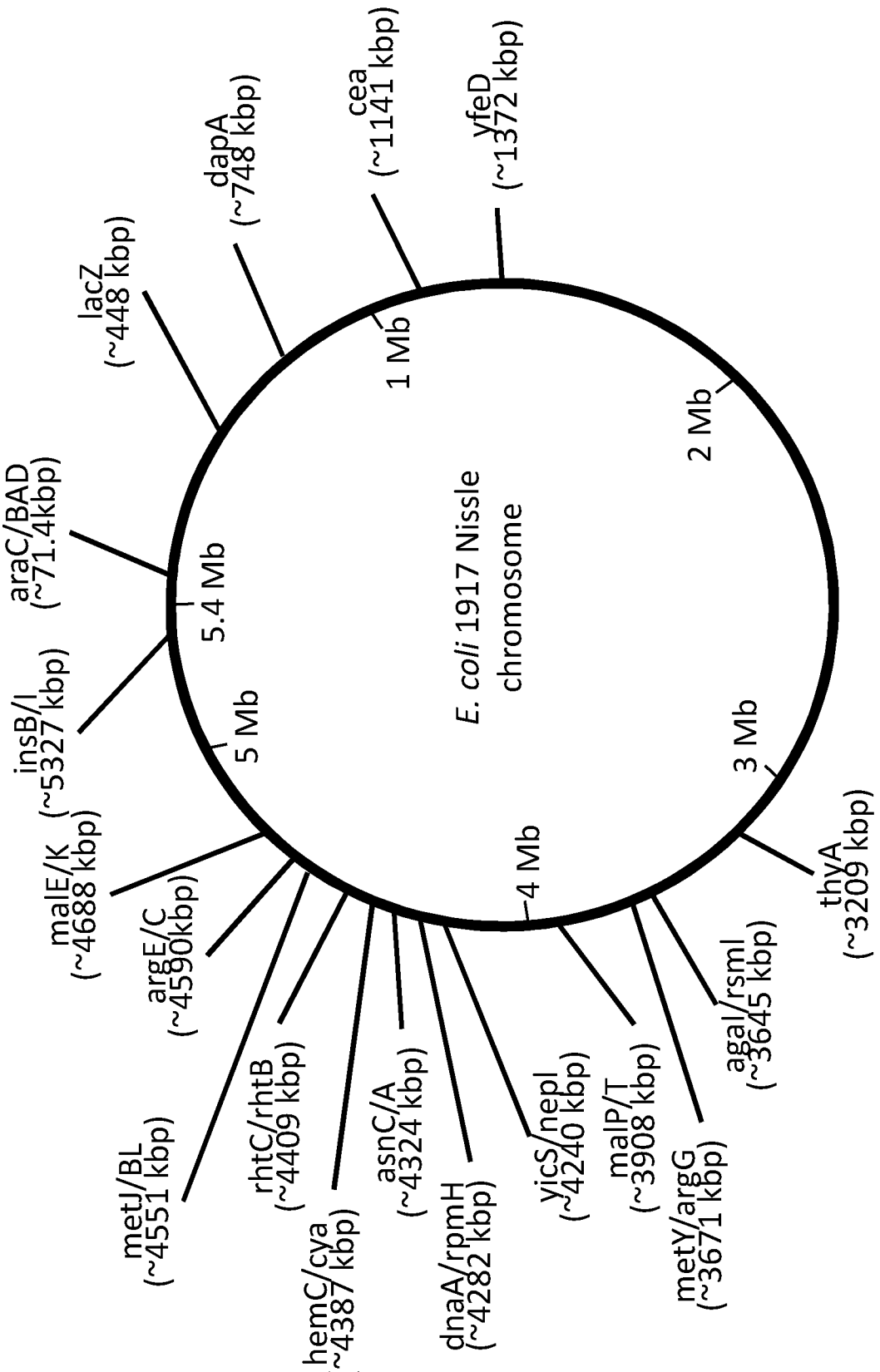

E. coli 1917 Nissle chromosome
1 Mb
2 Mb
3 Mb
4 Mb
5 Mb
5.4 Mb
cea (~1141 kbp)
yfeD (~1372 kbp)
dapA (~748 kbp)
lacZ (~448 kbp)
araC/BAD (~71.4kbp)
insB/I (~5327 kbp)
malE/K (~4688 kbp)
argE/C (~4590kbp)
rhtC/rhtB (~4409 kbp)
hemC/cya (~4387 kbp)
asnC/A (~4324 kbp)
dnaA/rpmH (~4282 kbp)
yicS/nepI (~4240 kbp)
malP/T (~3908 kbp)
metY/argG (~3671 kbp)
agaI/rsmI (~3645 kbp)
thyA (~3209 kbp)
metJ/BL (~4551 kbp)

Fig. 43

Brightness of constitutive RFP integrated in three locations:

1. AraB/C
2. MalE/K
3. MetY/ArgG
4. Nissle (non-fluorescent)

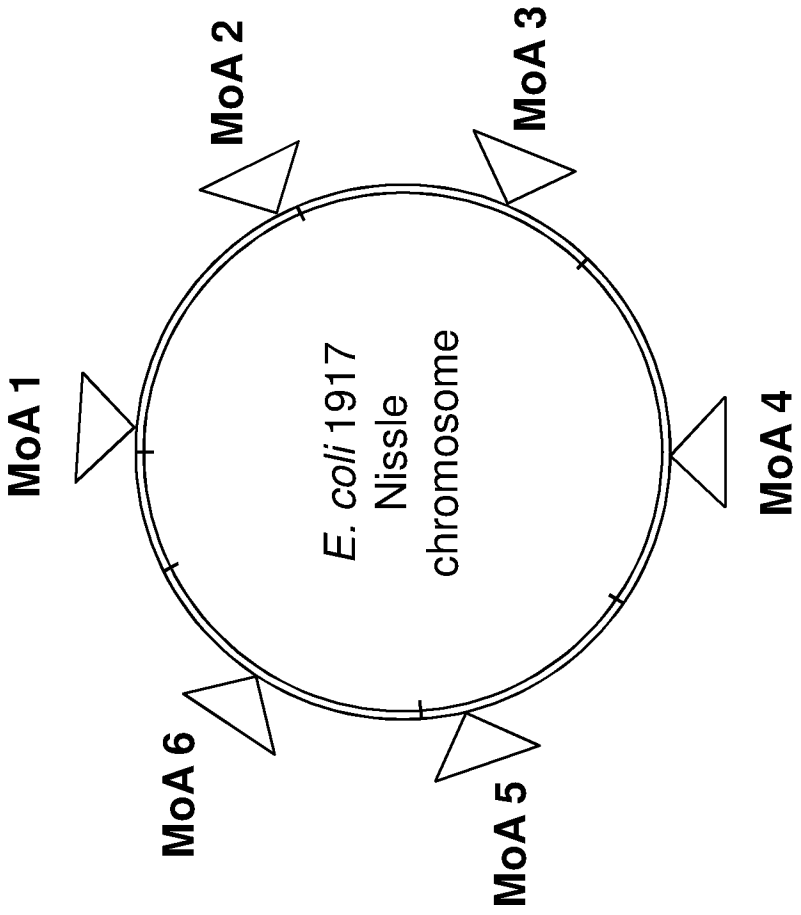


Fig. 44

Fig. 45A

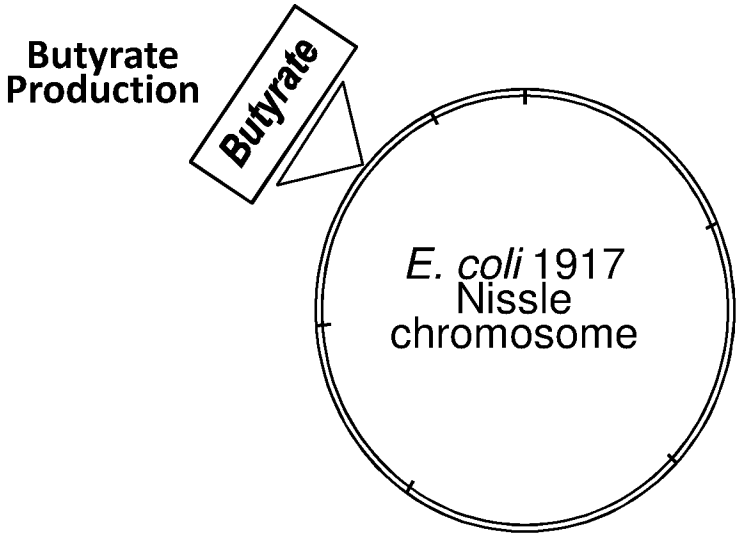


Fig. 45B

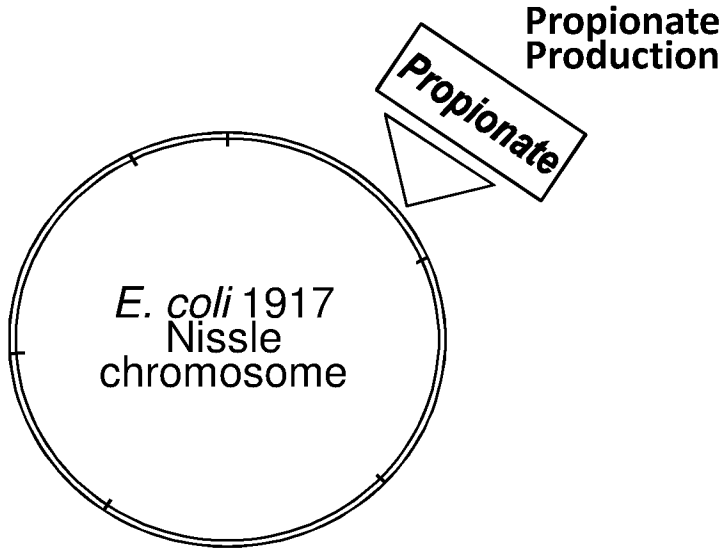


Fig. 45C

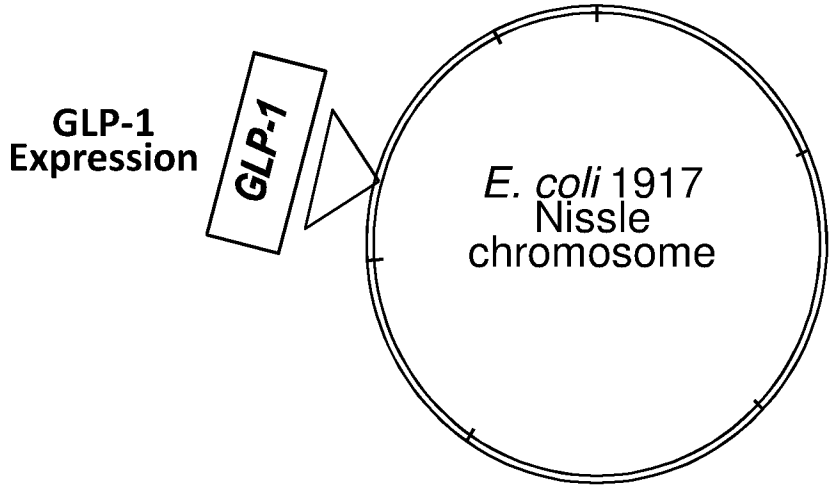


Fig. 45D

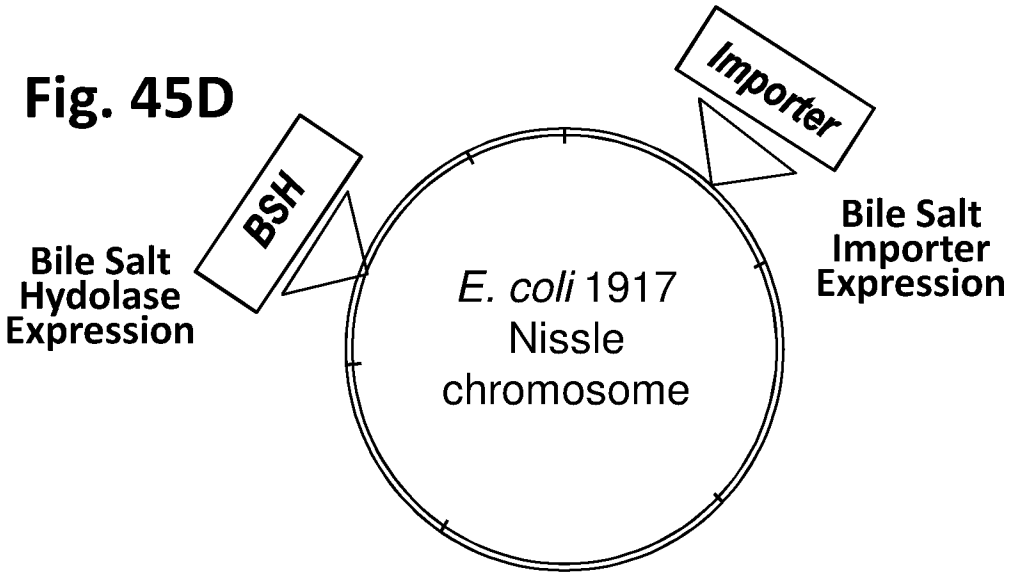


Fig. 45E

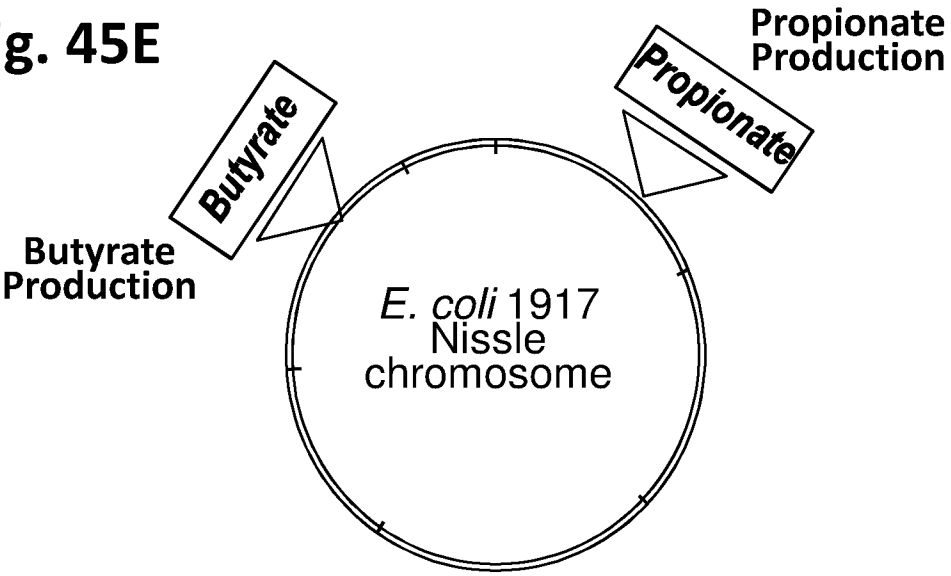


Fig. 45F

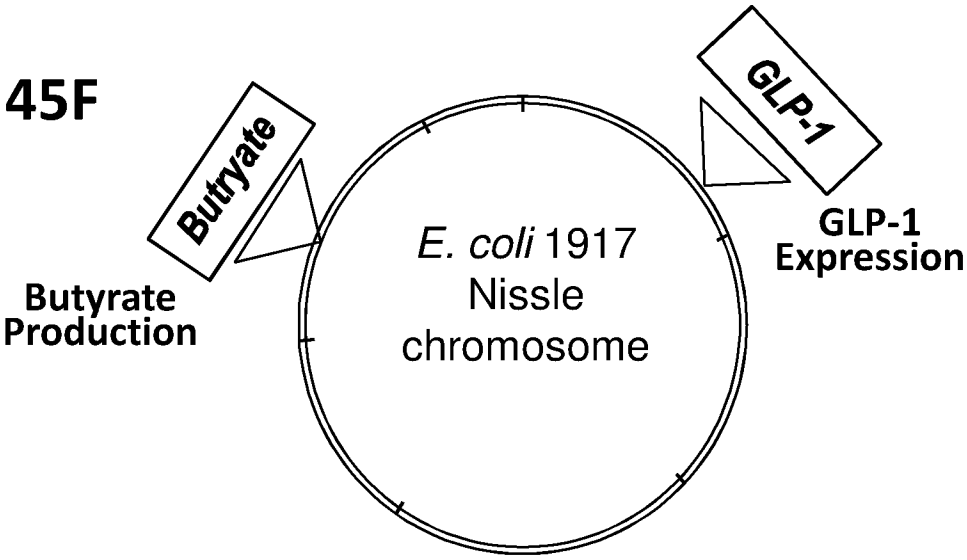


Fig. 45G

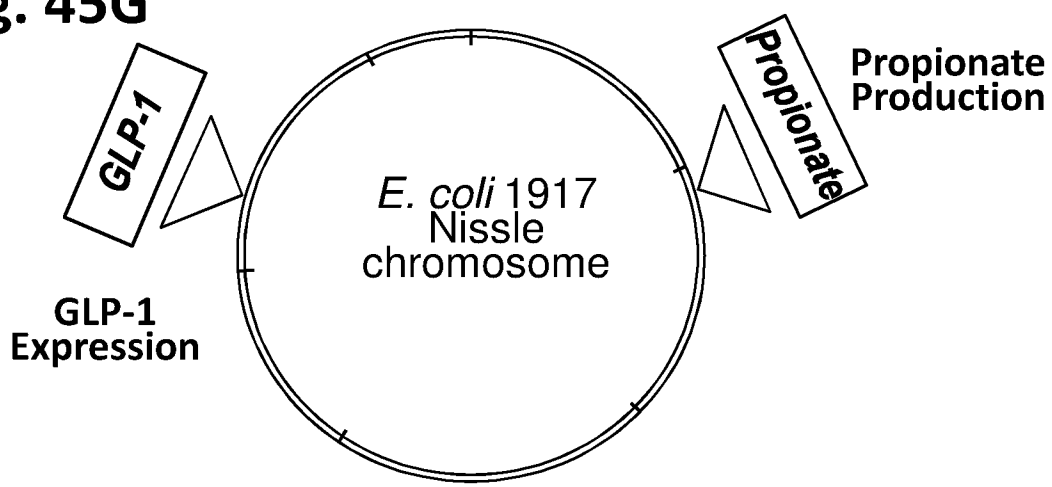


Fig. 45H

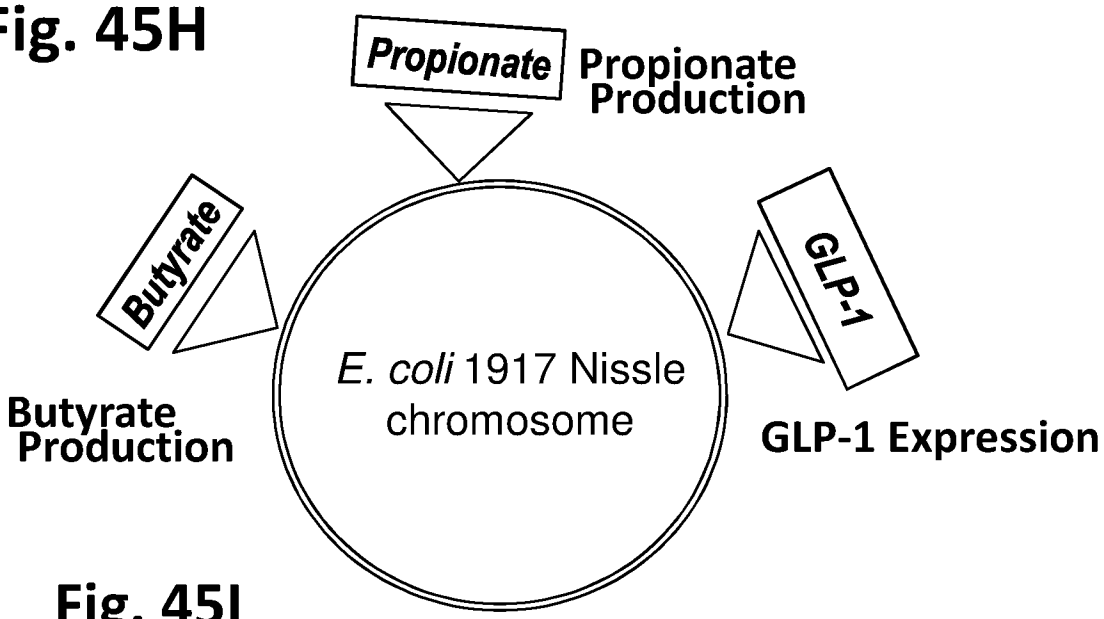


Fig. 45I

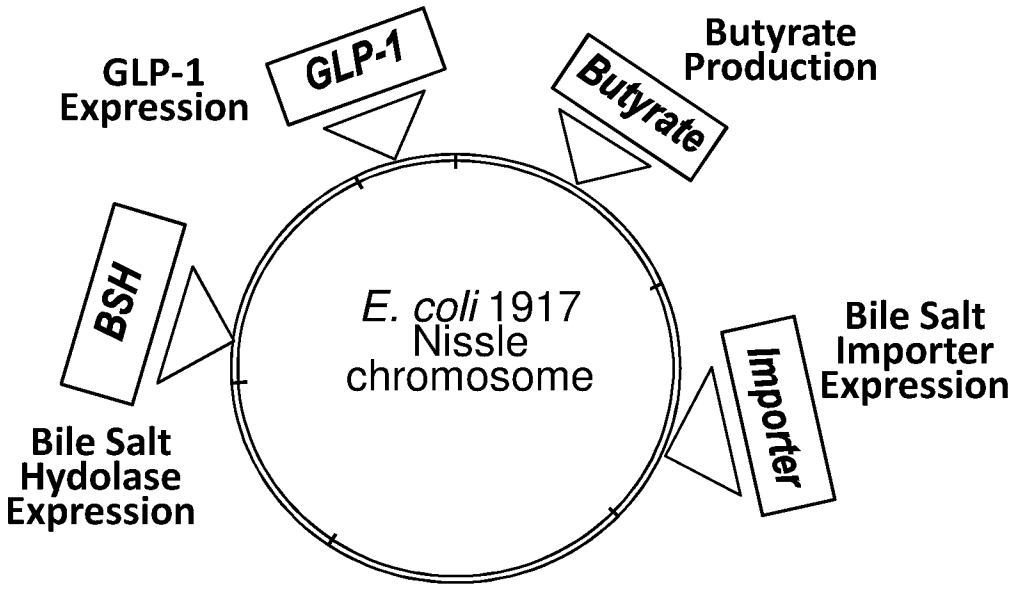


FIG. 46

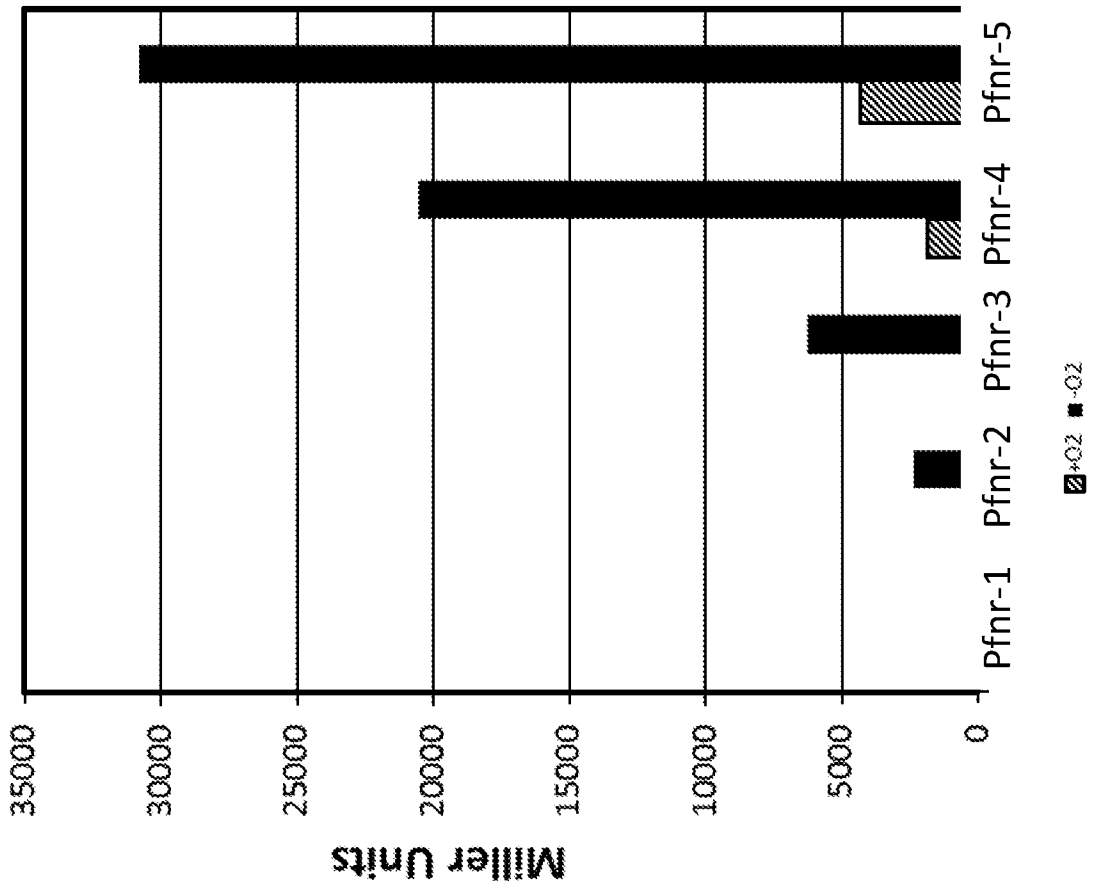

Miller Units
35000
30000
25000
20000
15000
10000
5000
0
Pfnr-1
Pfnr-2
Pfnr-3
Pfnr-4
Pfnr-5

FIG. 47A and 47B

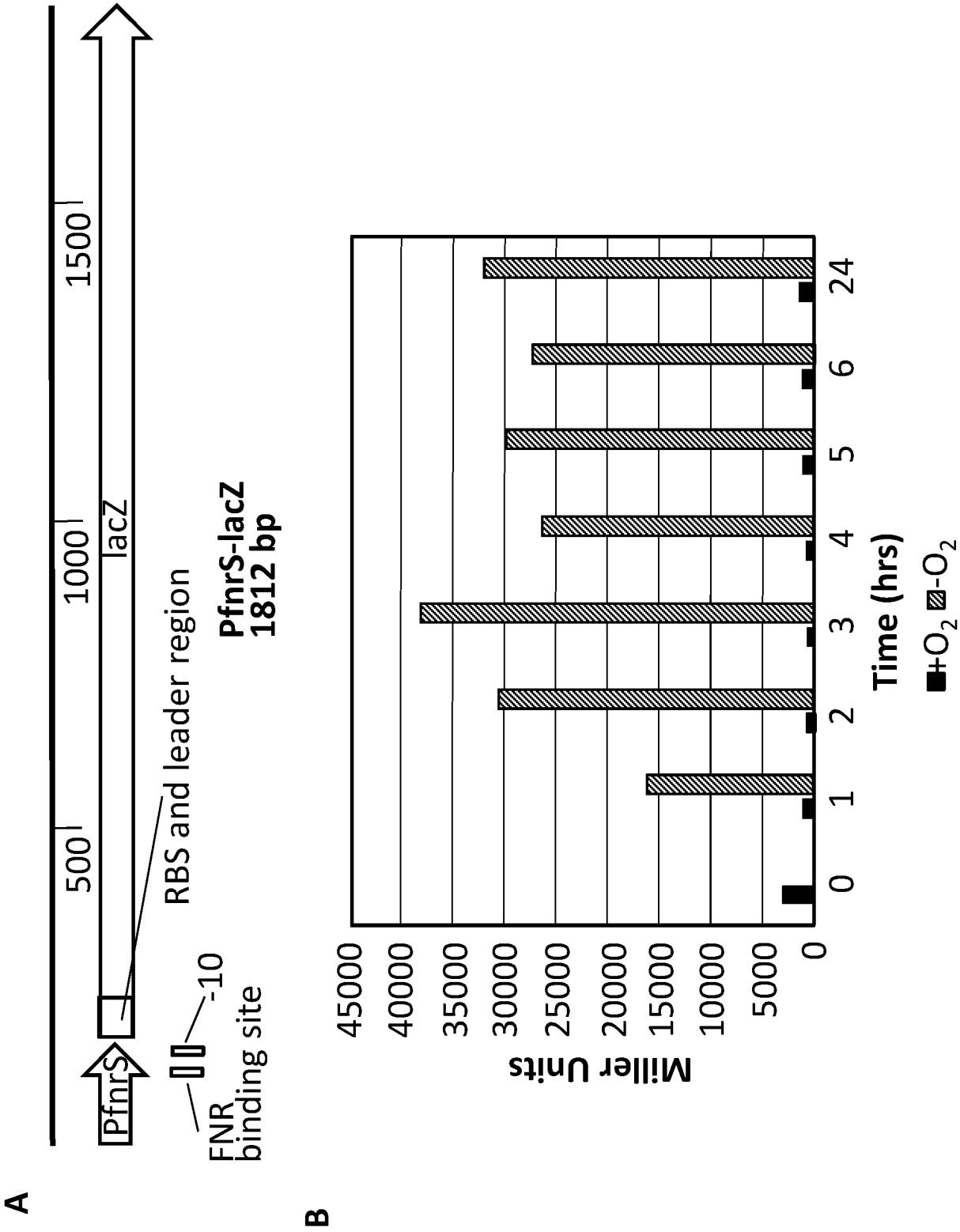


FIG. 47C

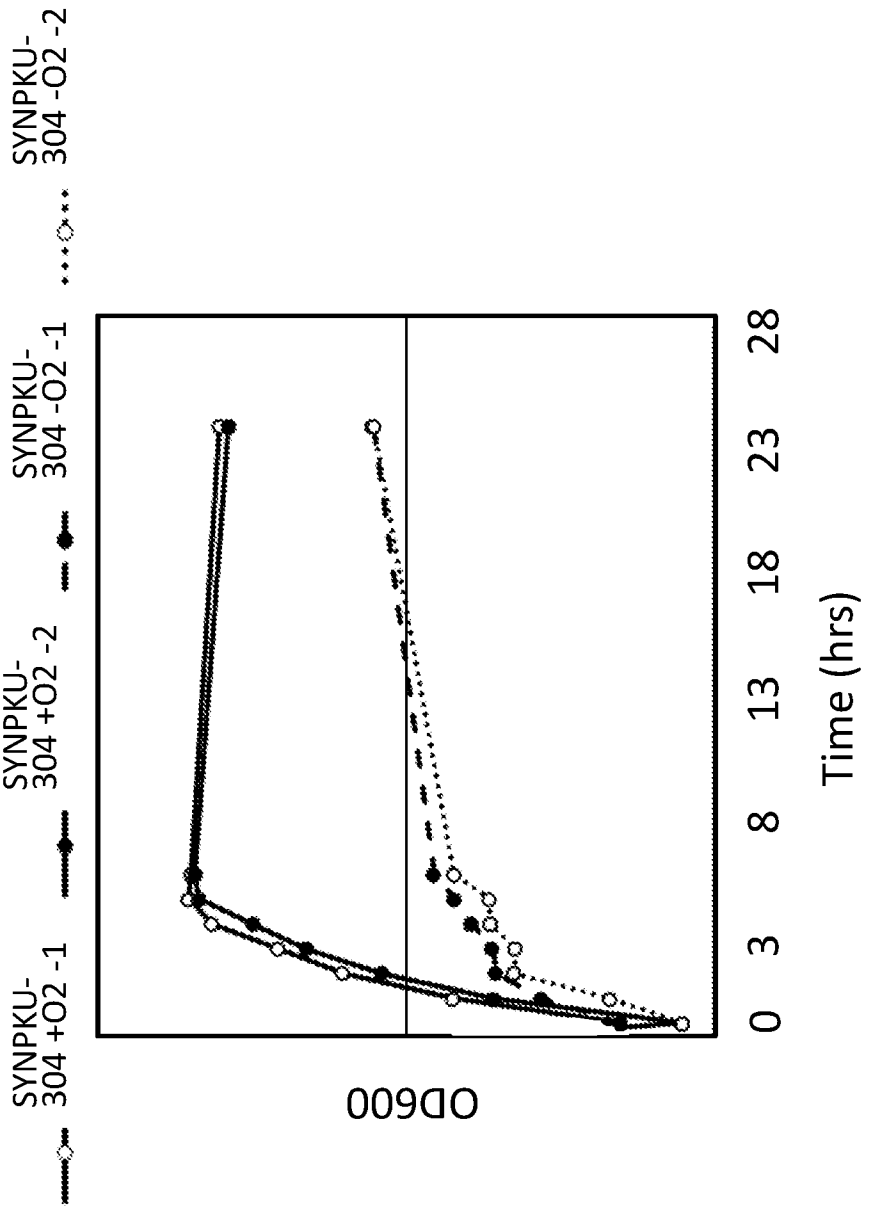

SYNPKU-304 +O2 -1
SYNPKU-304 +O2 -2
SYNPKU-304 -O2 -1
SYNPKU-304 -O2 -2
OD600
0
3
8
13
18
23
28
Time (hrs)

FIG. 48A

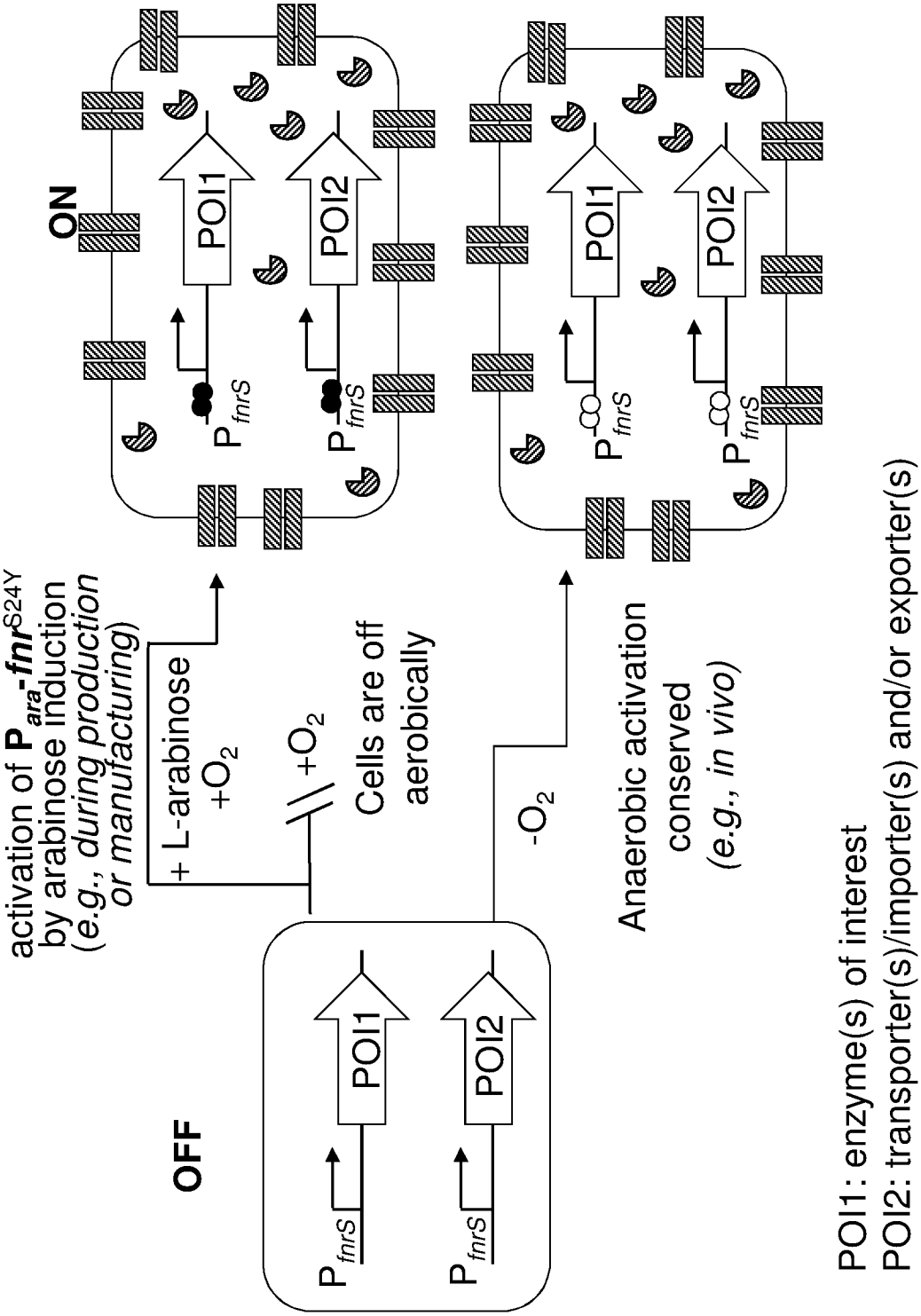

activation of P_{ara}-fnr^{S24Y} by arabinose induction (e.g., during production or manufacturing)
+ L-arabinose
+O2
+O2
Cells are off aerobically
-O2
Anaerobic activation conserved (e.g., in vivo)
ON
OFF
POI1
POI2
PfnrS
POI1: enzyme(s) of interest
POI2: transporter(s)/importer(s) and/or exporter(s)

FIG. 48B

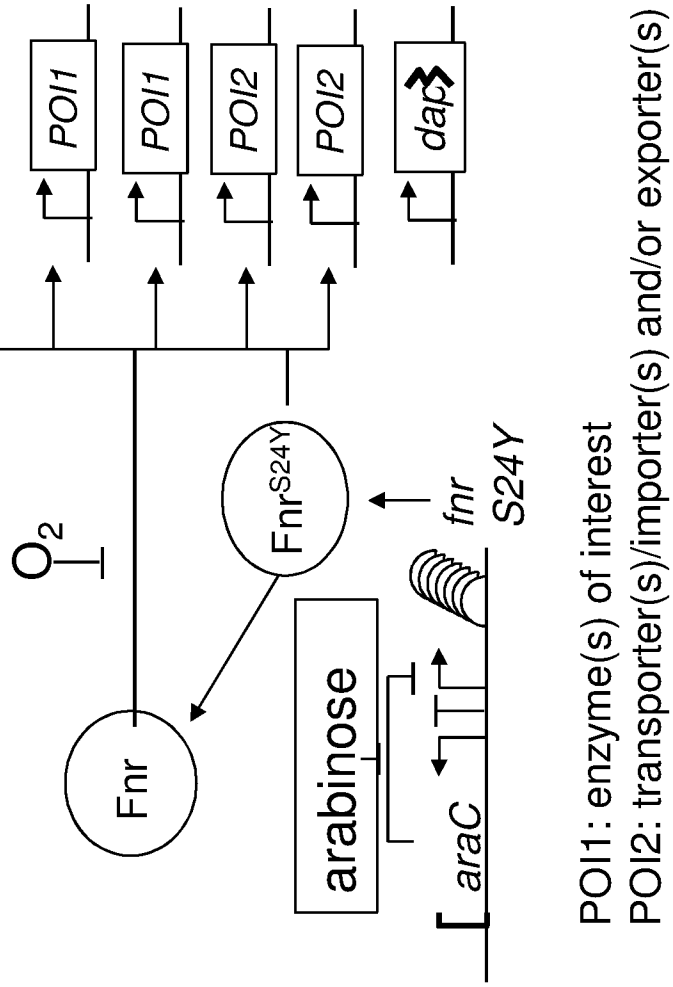

PfnrS
POI1
POI1
POI1
POI2
POI2
dapA
O2
Fnr
FnrS24Y
fnr
S24Y
arabinose
araC
POI1: enzyme(s) of interest
POI2: transporter(s)/importer(s) and/or exporter(s)

FIG. 48C

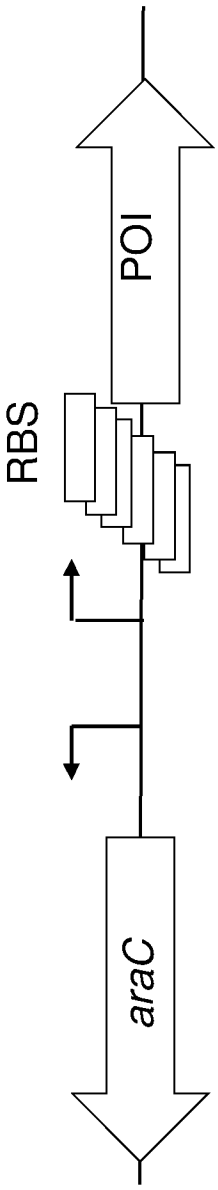


FIG. 49

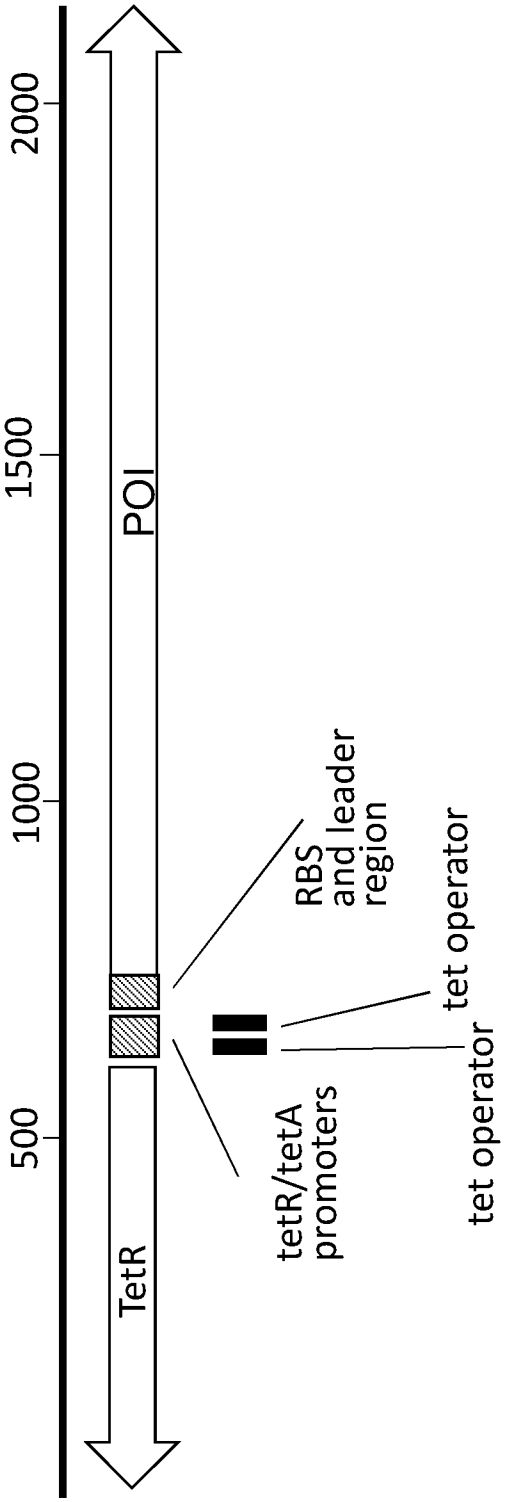

500
1000
1500
2000
TetR
POI
tetR/tetA promoters
RBS and leader region
tet operator
tet operator

FIG. 50

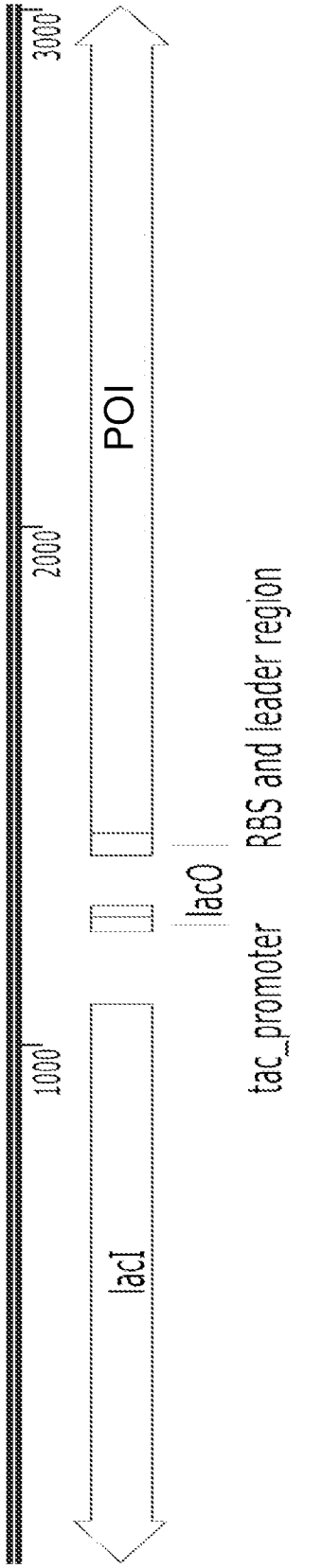
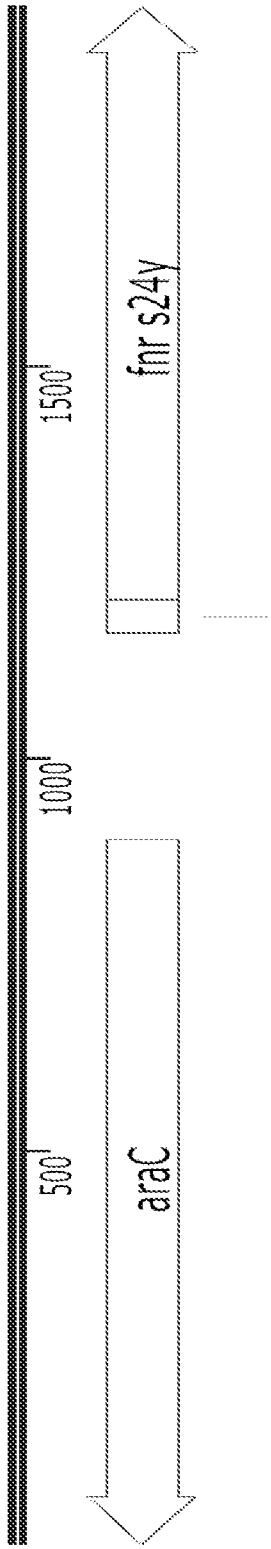
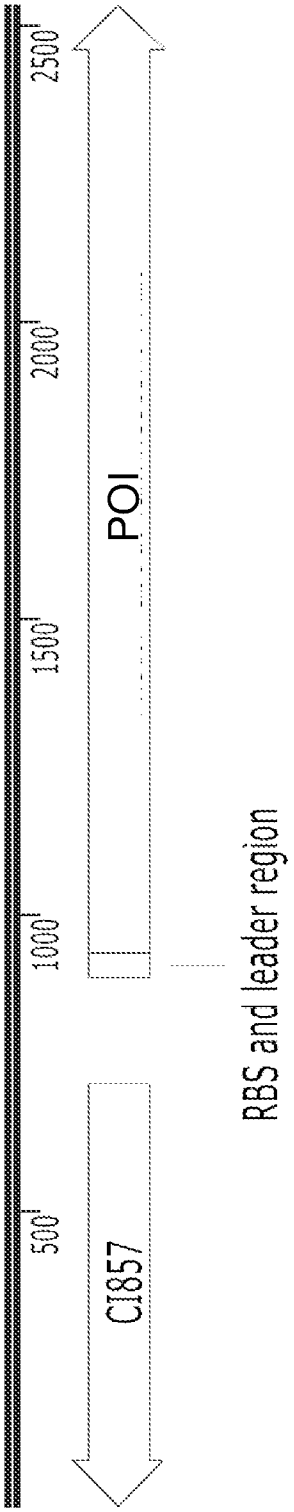

1000
2000
3000
lacI
tac_promoter
lacO
RBS and leader region
POI
Lac-PAL construct
3008 bp

FIG. 51



Para-FNRS24Y construct
1955 bp

FIG. 52A



Temperature-sensitive CI857 promoter – POI construct

FIG. 52B

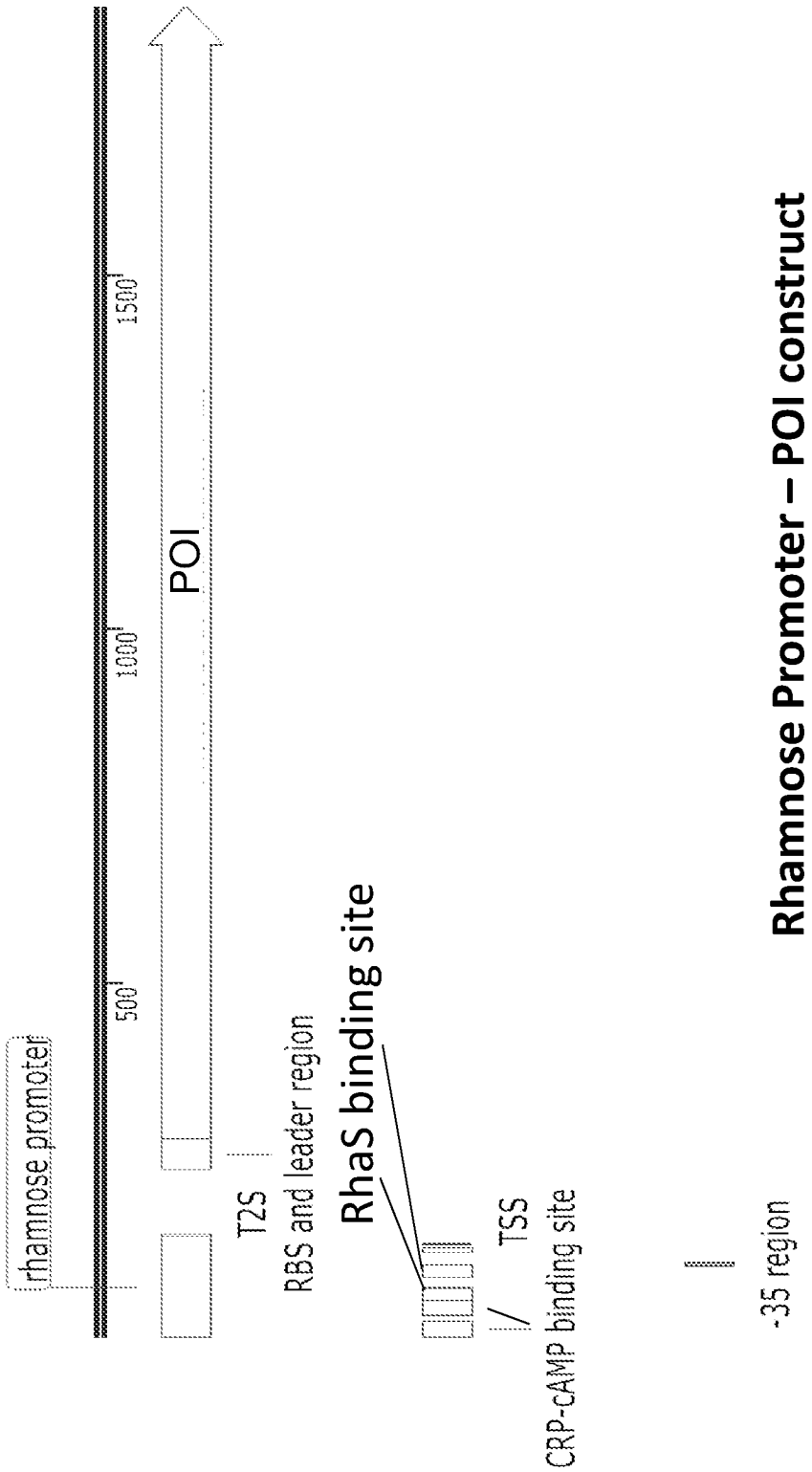
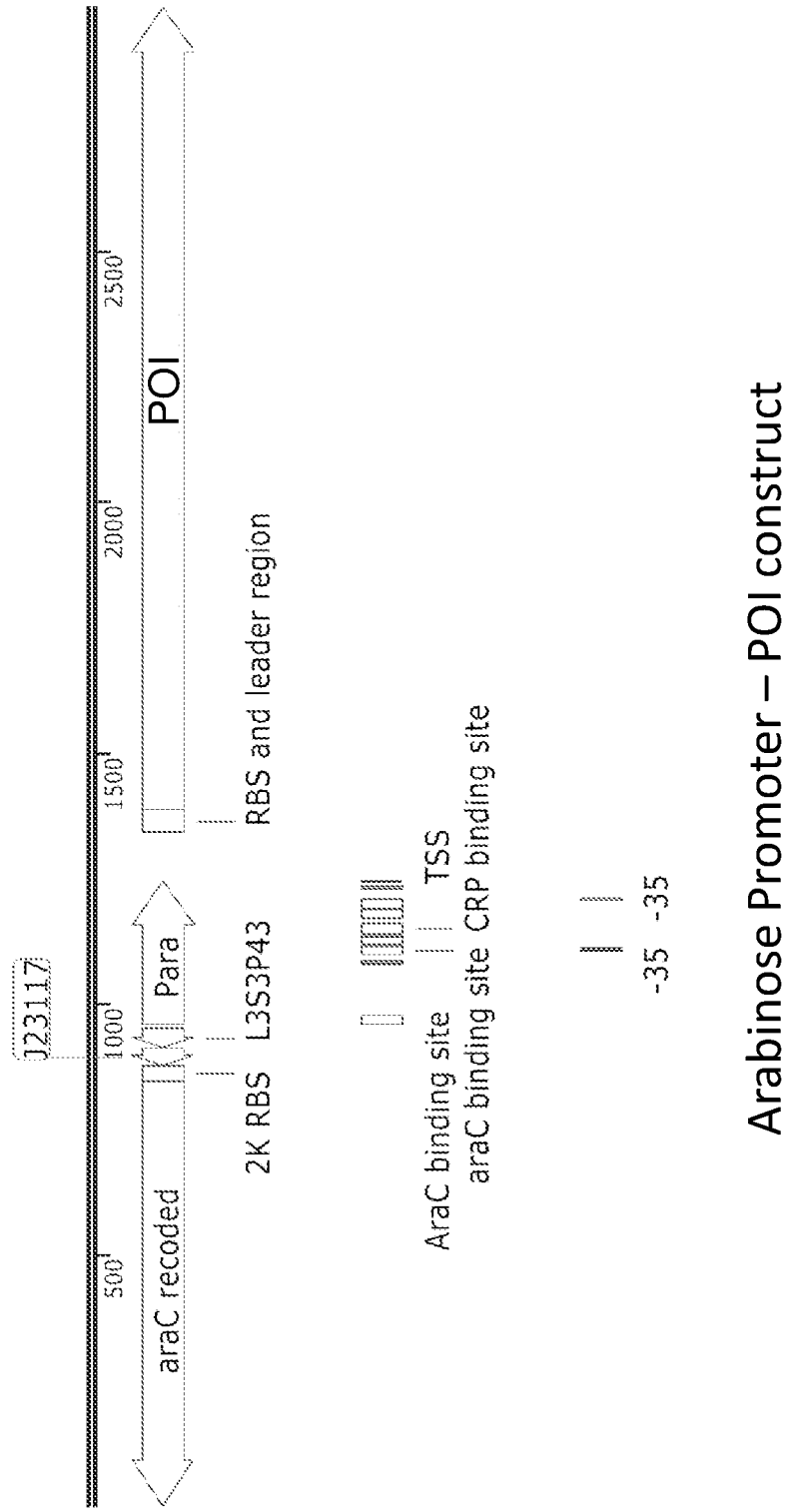

rhamnose promoter
500
1000
1500
POI
T2S
RBS and leader region
RhaS binding site
TSS
CRP-cAMP binding site
-35 region
Rhamnose Promoter – POI construct

FIG. 52C



Arabinose Promoter – POI construct
2986 bp

FIG. 53A

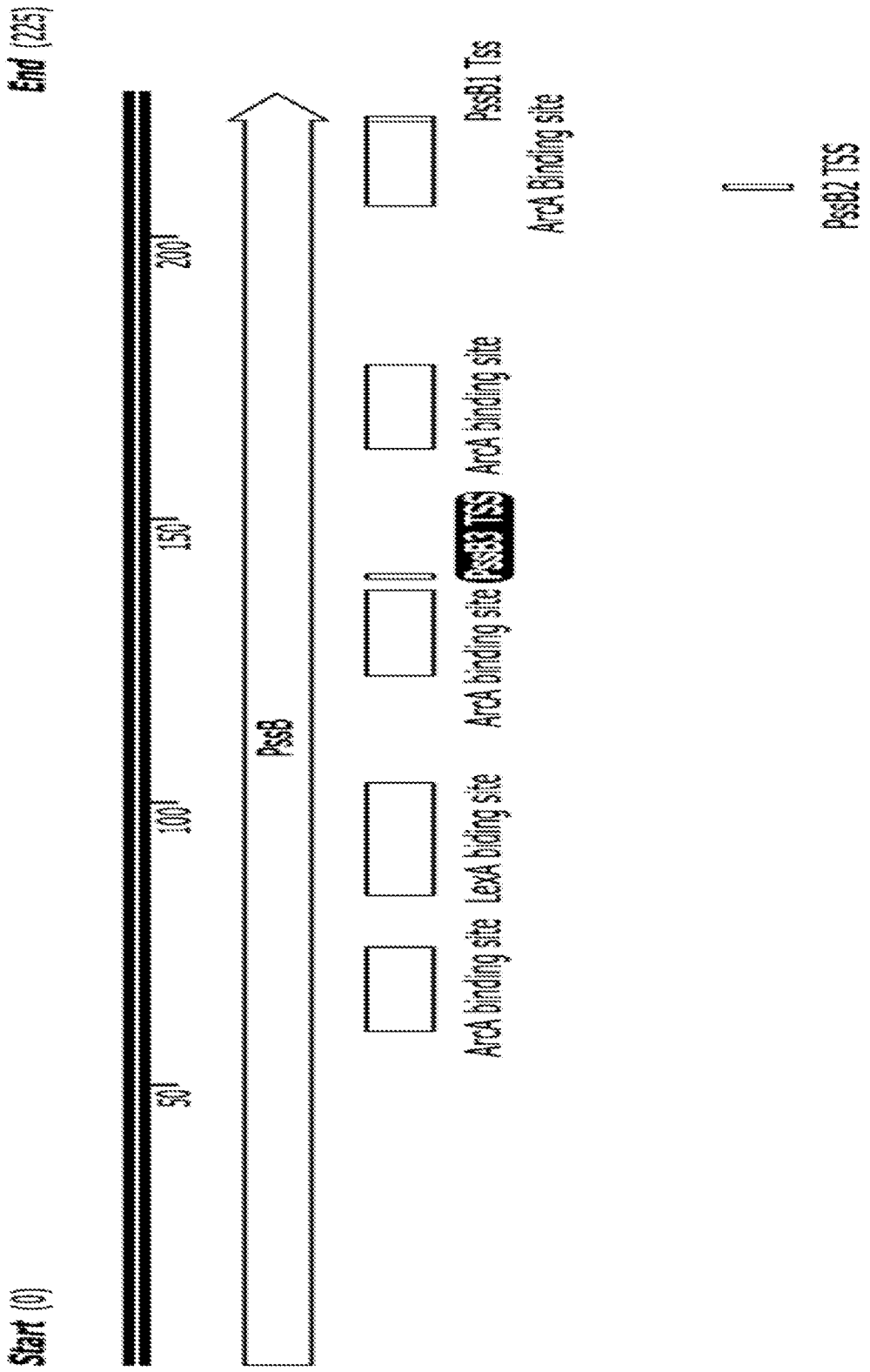

Start (0)
End (225)
50
100
150
200
PssB
ArcA binding site
LexA binding site
ArcA binding site
PssB1 TSS
ArcA Binding site
PssB2 TSS

FIG. 53B

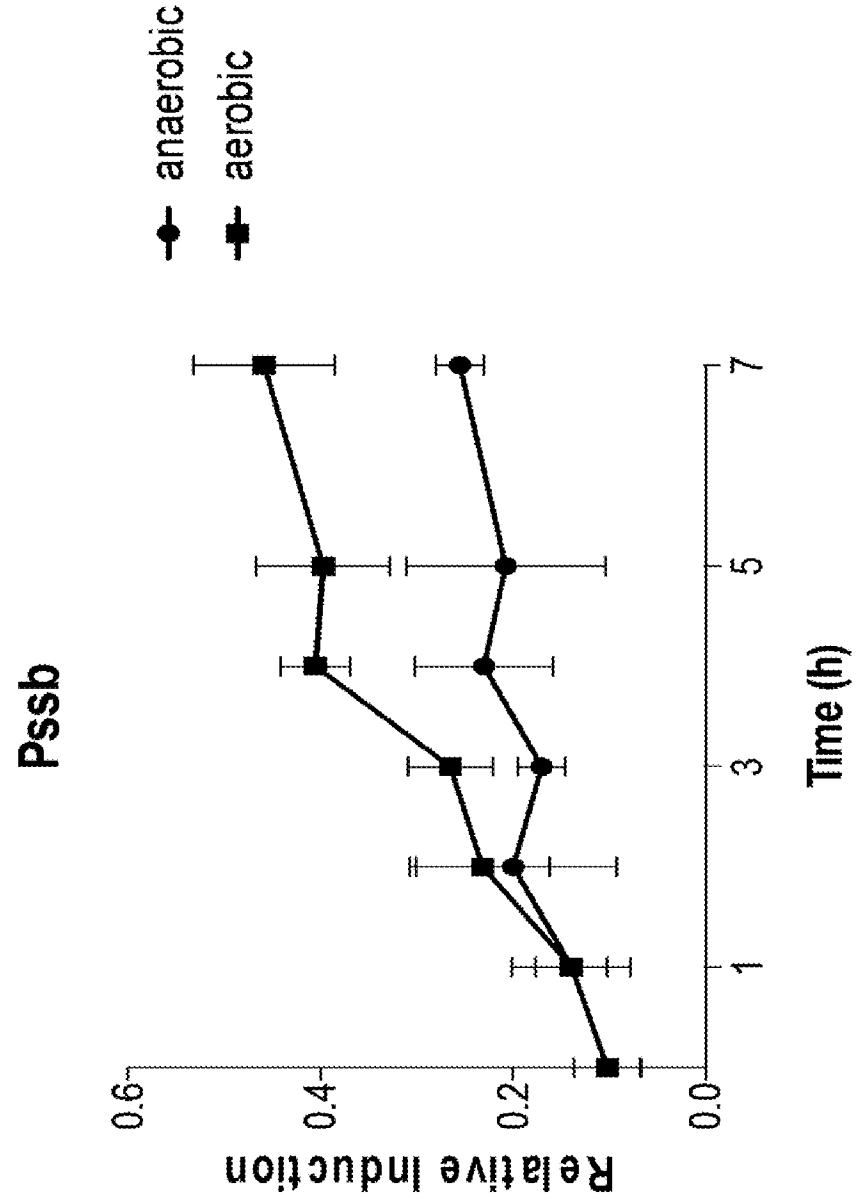

Pssb
anaerobic
aerobic
Relative Induction
0.0
0.2
0.4
0.6
1
3
5
7
Time (h)

FIG. 54A

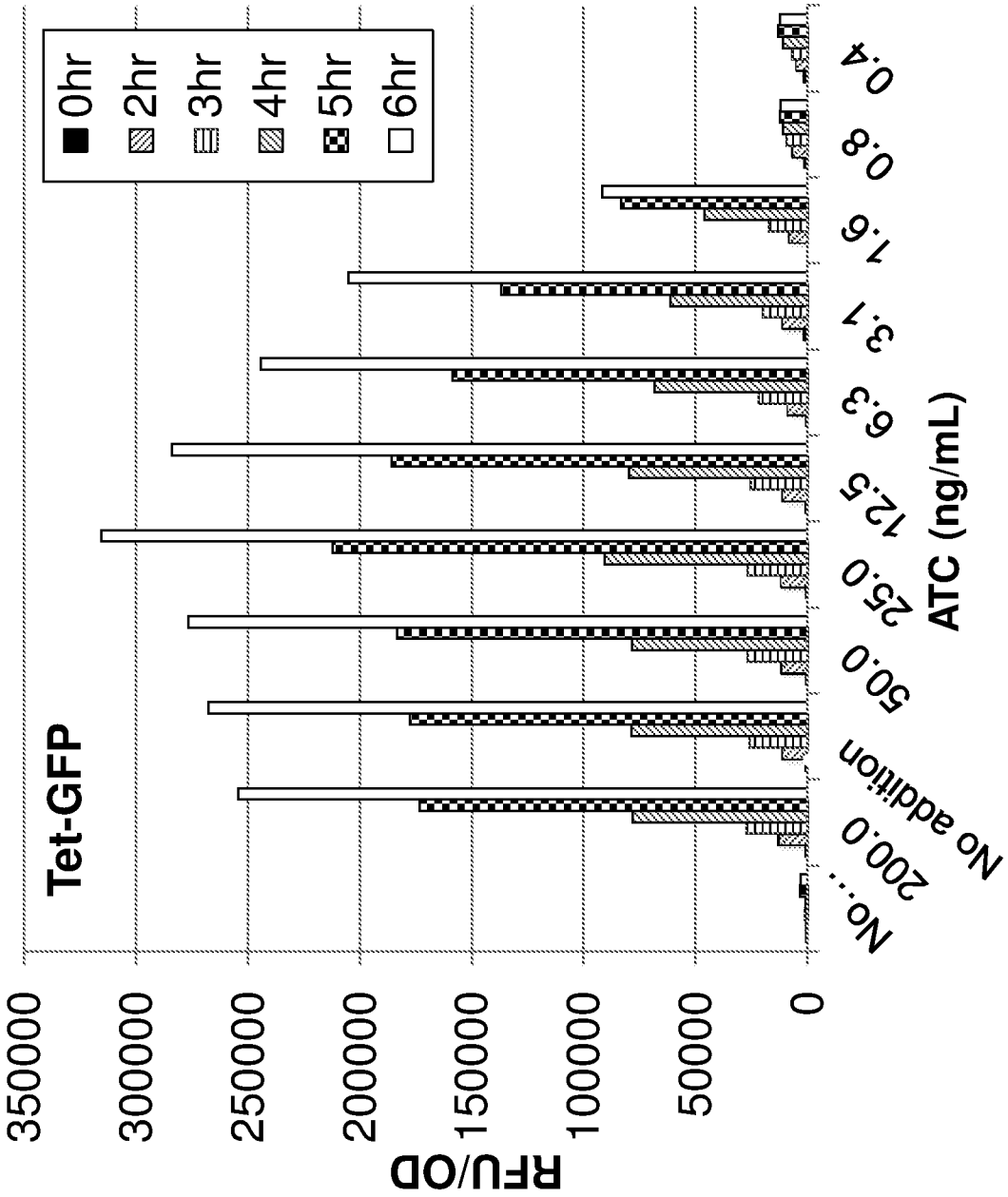

Tet-GFP
0hr
2hr
3hr
4hr
5hr
6hr
RFU/OD
350000
300000
250000
200000
150000
100000
50000
0
No...
200.0
No addition
50.0
25.0
12.5
6.3
3.1
1.6
0.8
0.4
ATC (ng/mL)

FIG. 54B

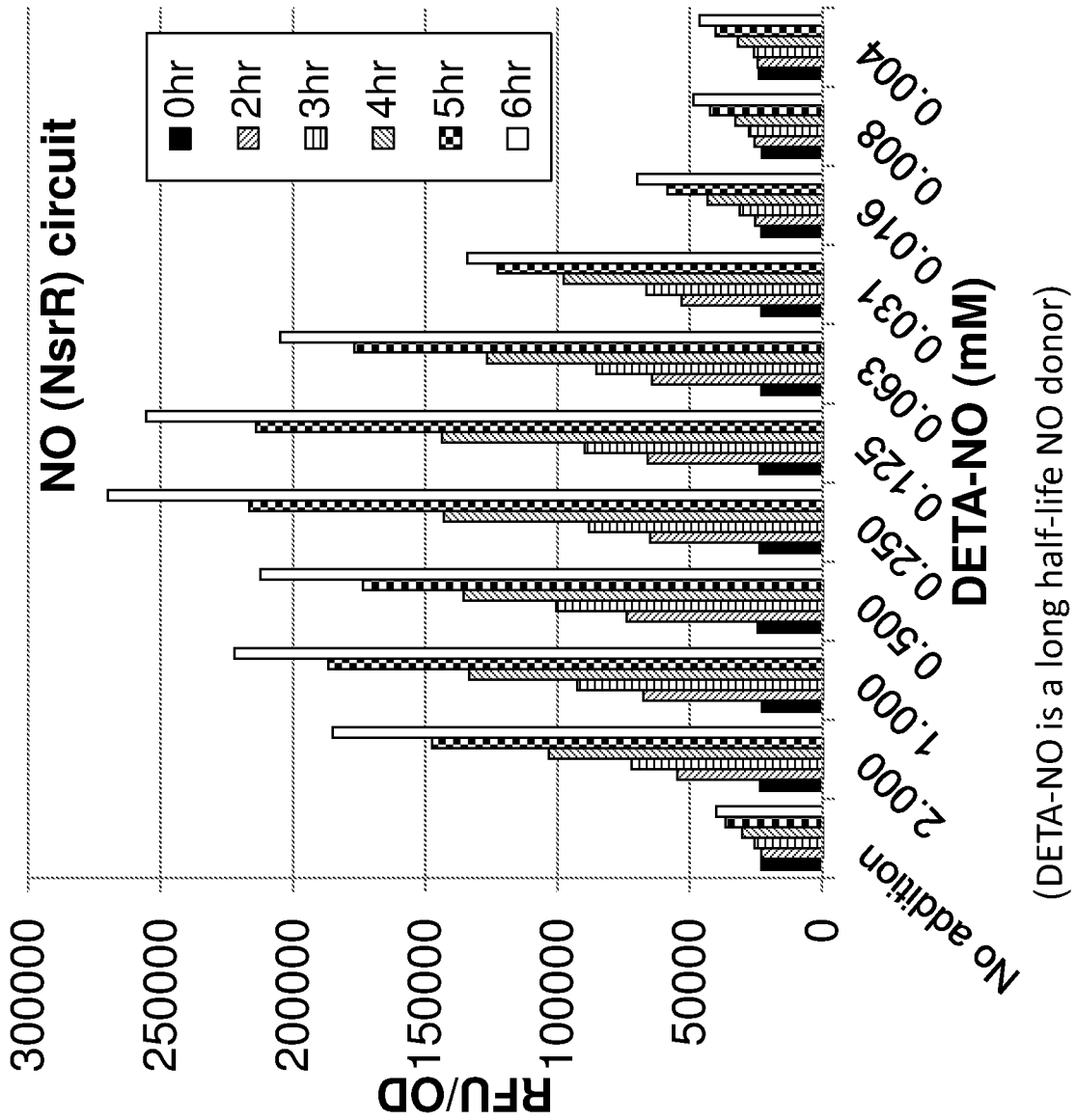

NO (NsrR) circuit
0hr
2hr
3hr
4hr
5hr
6hr
RFU/OD
300000
250000
200000
150000
100000
50000
0
No addition
2.000
1.000
0.500
0.250
0.125
0.063
0.031
0.016
0.008
0.004
DETA-NO (mM)
(DETA-NO is a long half-life NO donor)

FIG. 54C

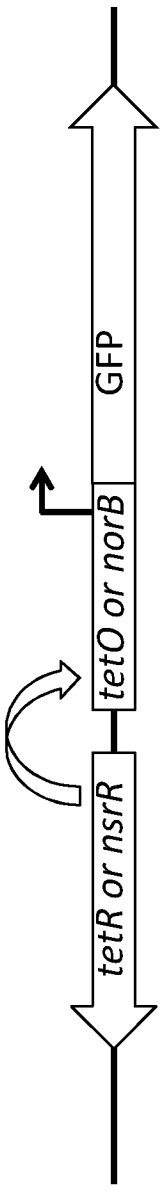


FIG. 55

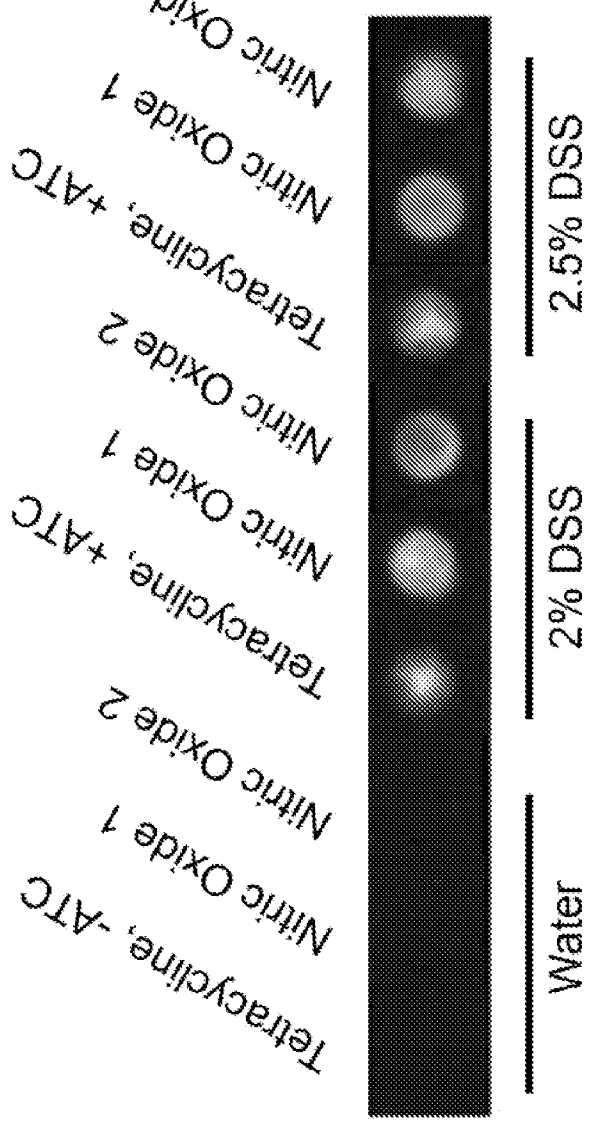

Tetracycline, -ATC
Nitric Oxide 1
Nitric Oxide 2
Tetracycline, +ATC
Nitric Oxide 1
Nitric Oxide 2
Tetracycline, +ATC
Nitric Oxide 1
Nitric Oxide 2
Water
2% DSS
2.5% DSS

Fig. 56

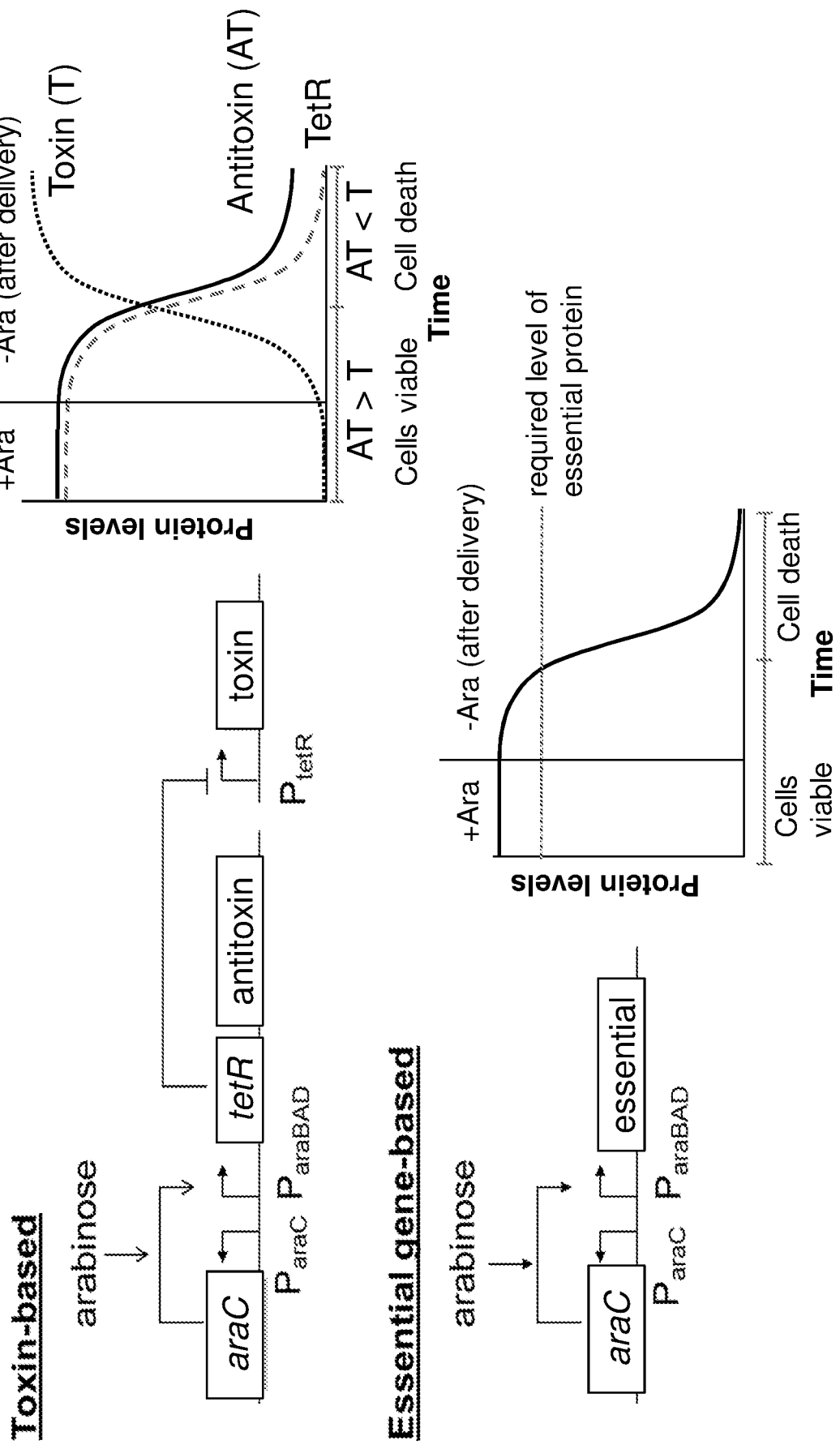

Toxin-based
arabinose
araC
tetR
antitoxin
toxin
P_{araC} P_{araBAD}
P_{tetR}
Protein levels
+Ara
-Ara (after delivery)
Toxin (T)
Antitoxin (AT)
TetR
AT > T
AT < T
Cells viable
Cell death
Time
Essential gene-based
arabinose
araC
essential
P_{araC} P_{araBAD}
Protein levels
+Ara
-Ara (after delivery)
required level of essential protein
Cells viable
Cell death
Time

Fig. 57A

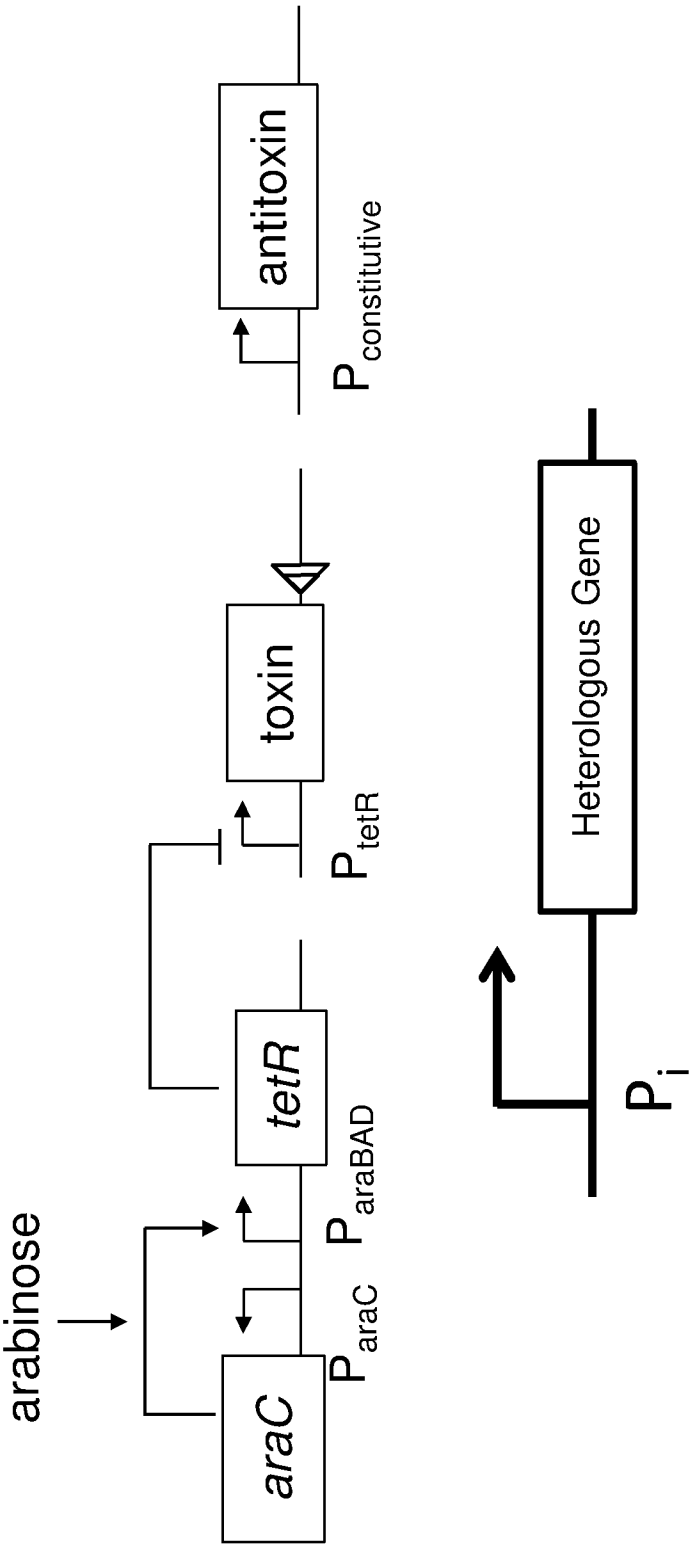

arabinose
araC
P araC
tetR
P araBAD
toxin
P tetR
antitoxin
P constitutive
Heterologous Gene
P i

Fig. 57B

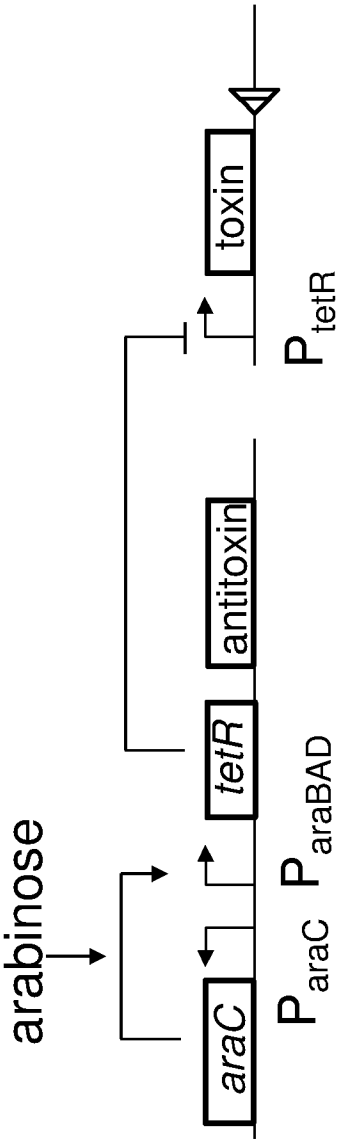


Fig. 57C

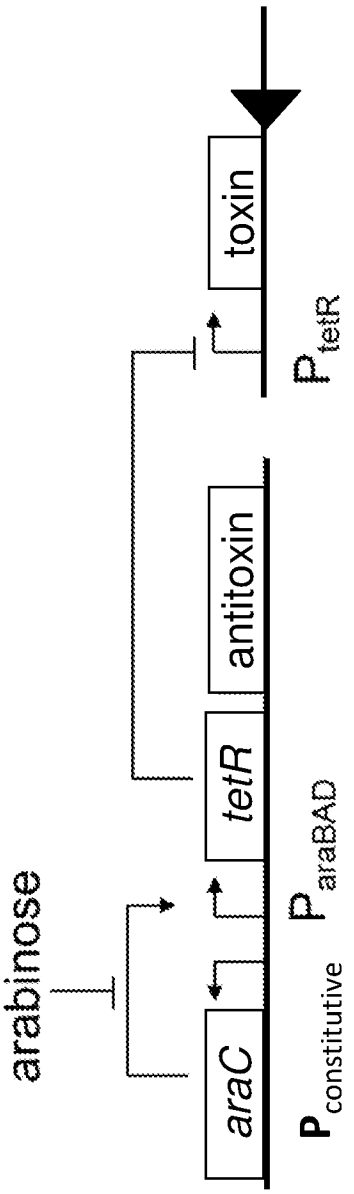


Fig. 58

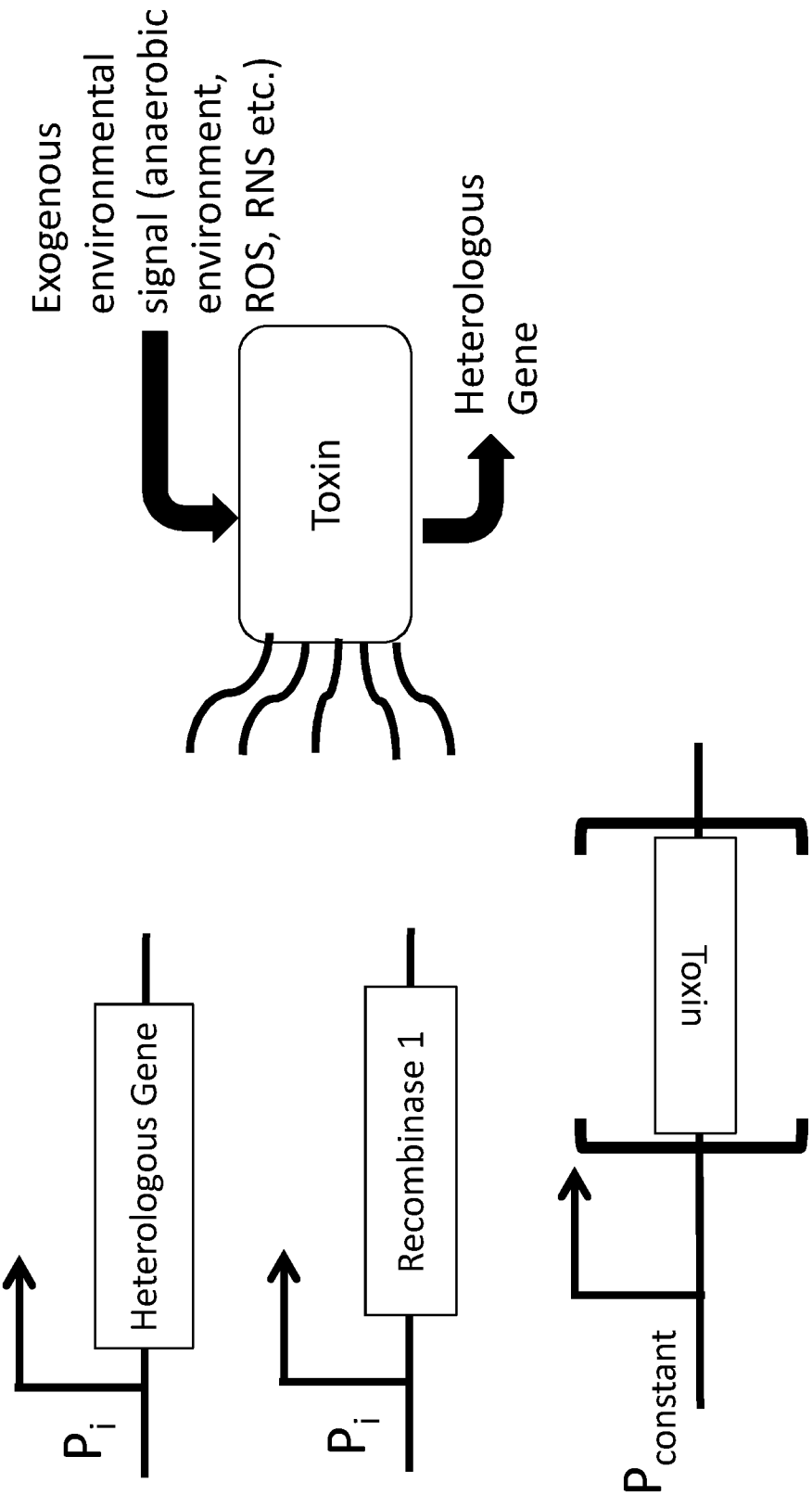

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
Heterologous Gene
Recombinase 1
Toxin
P_i
P_i
P_constant

Fig. 59

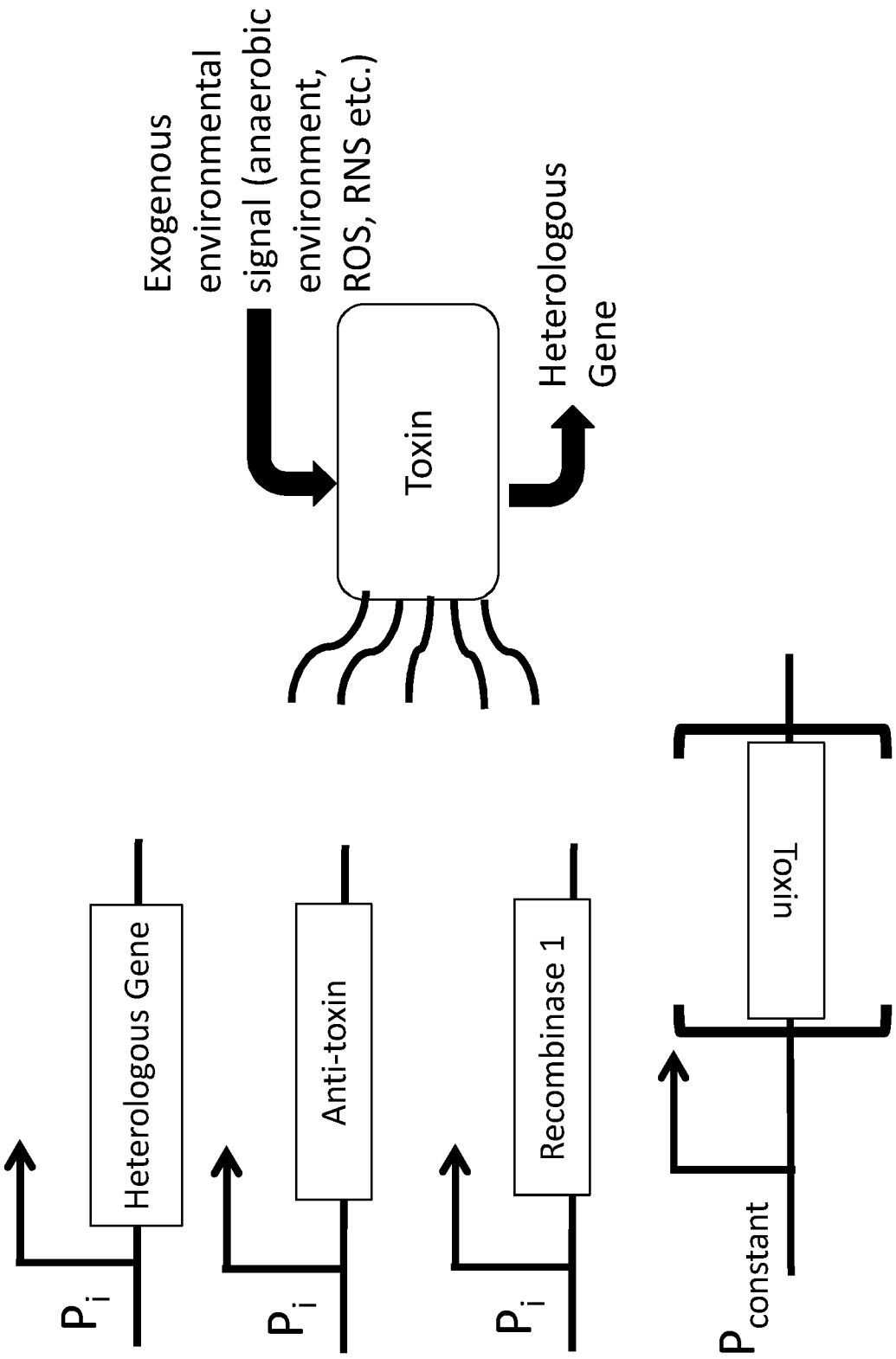

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
P_i
Heterologous Gene
Anti-toxin
P_i
Anti-toxin
Recombinase 1
P_i
Recombinase 1
Toxin
$P_{constant}$
Toxin

Fig. 60

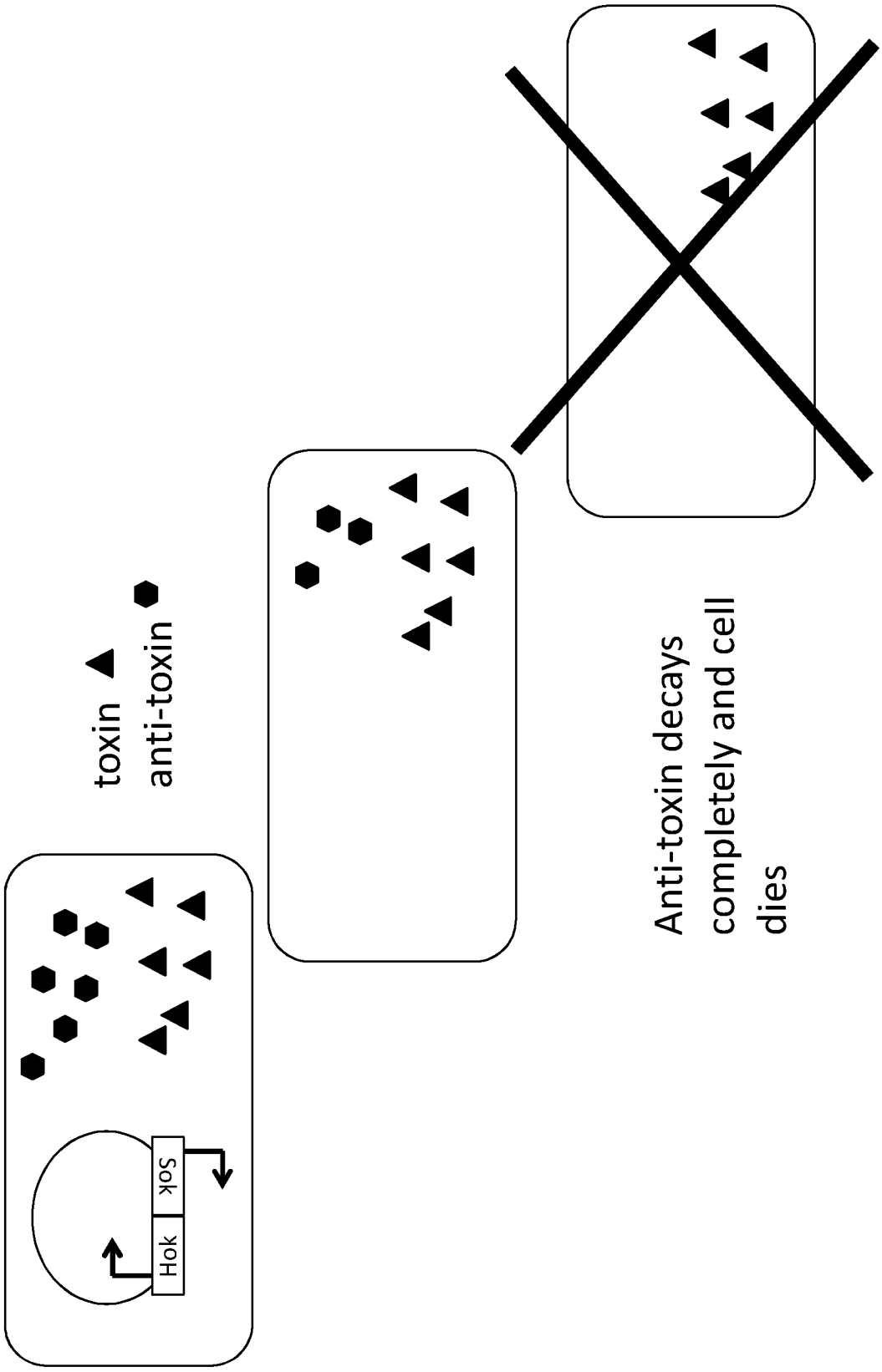

toxin
anti-toxin
Hok
SoK
Anti-toxin decays completely and cell dies

Fig. 61

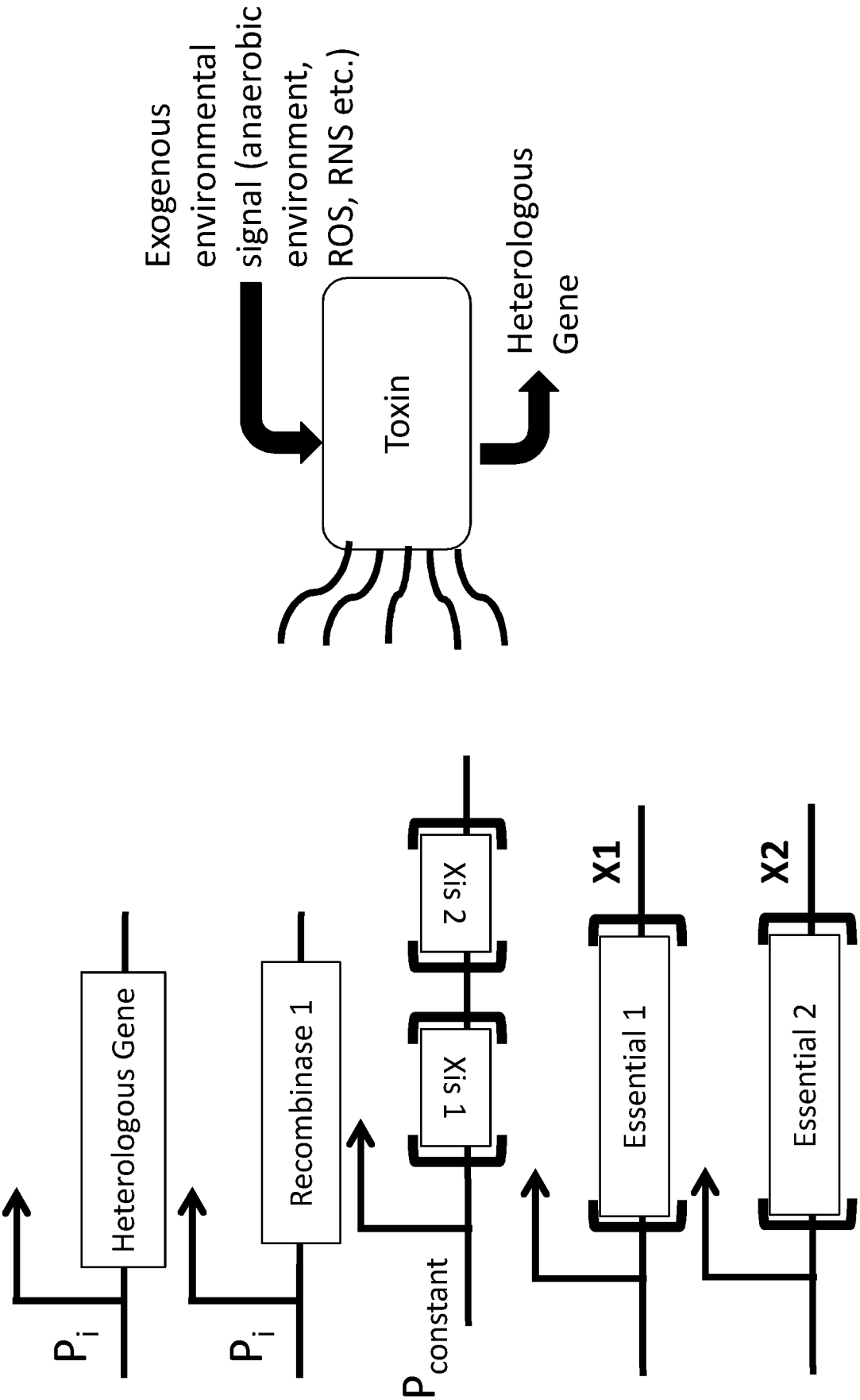

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
P_i
Heterologous Gene
P_i
Recombinase 1
$P_{constant}$
Xis 1
Xis 2
X1
Essential 1
X2
Essential 2

Fig. 62

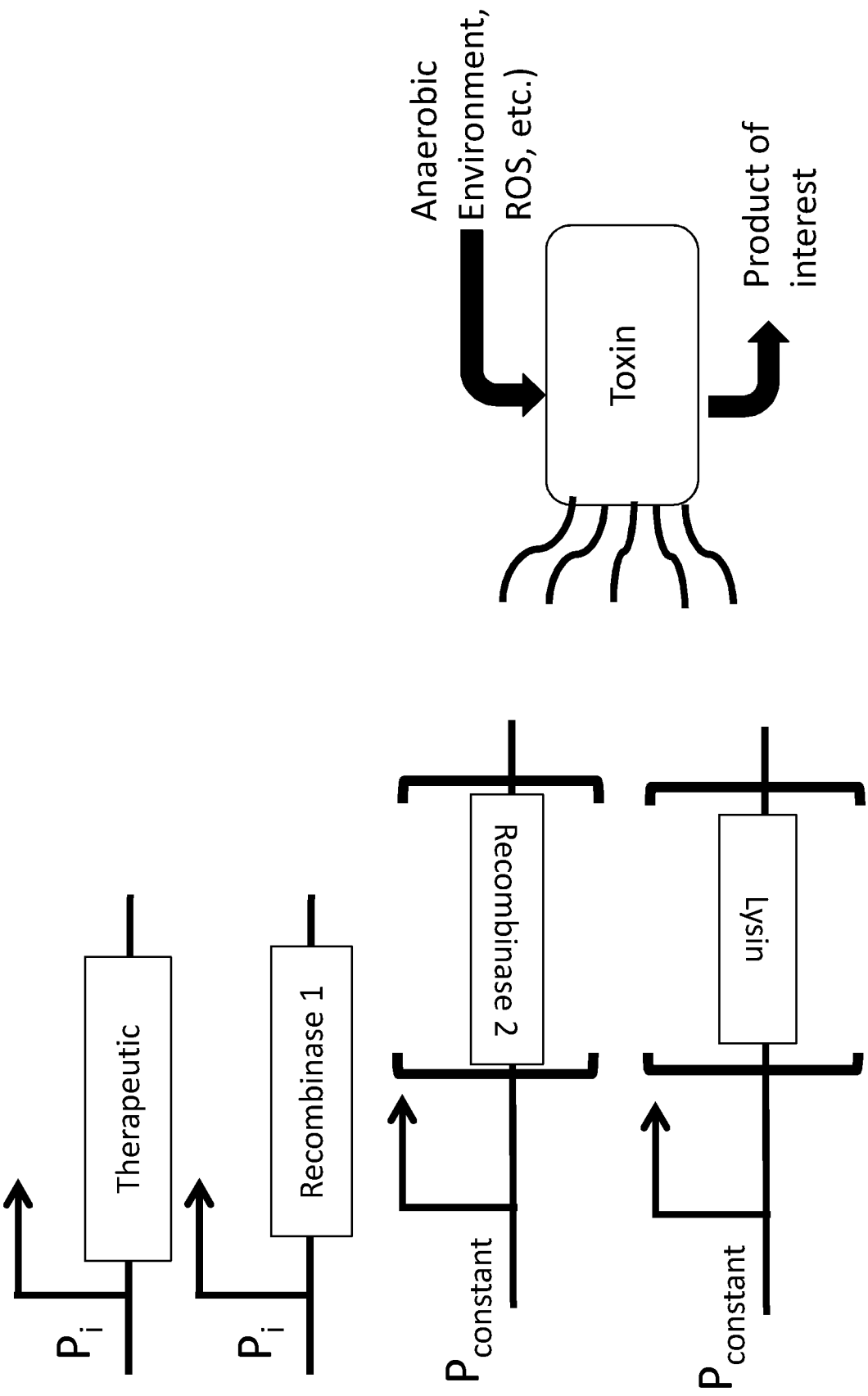

Anaerobic Environment, ROS, etc.)
Toxin
Product of interest
Therapeutic
Recombinase 1
Recombinase 2
Lysin
P_i
P_i
$P_{constant}$
$P_{constant}$

FIG. 63

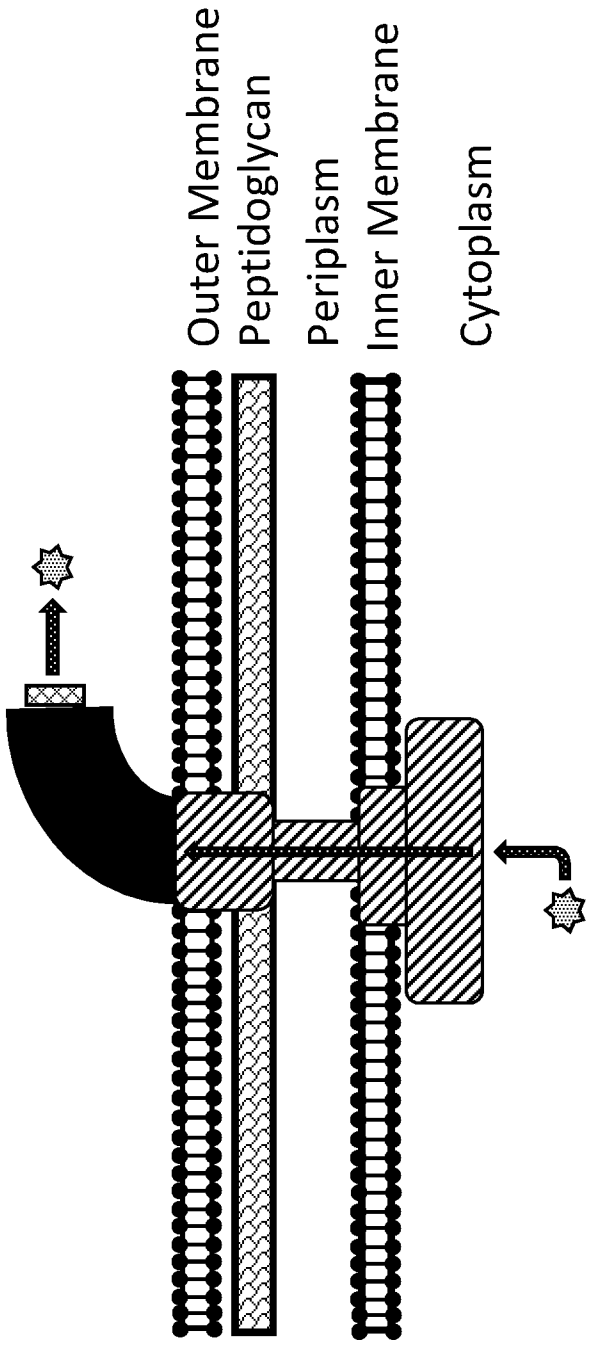

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm

FIG. 64

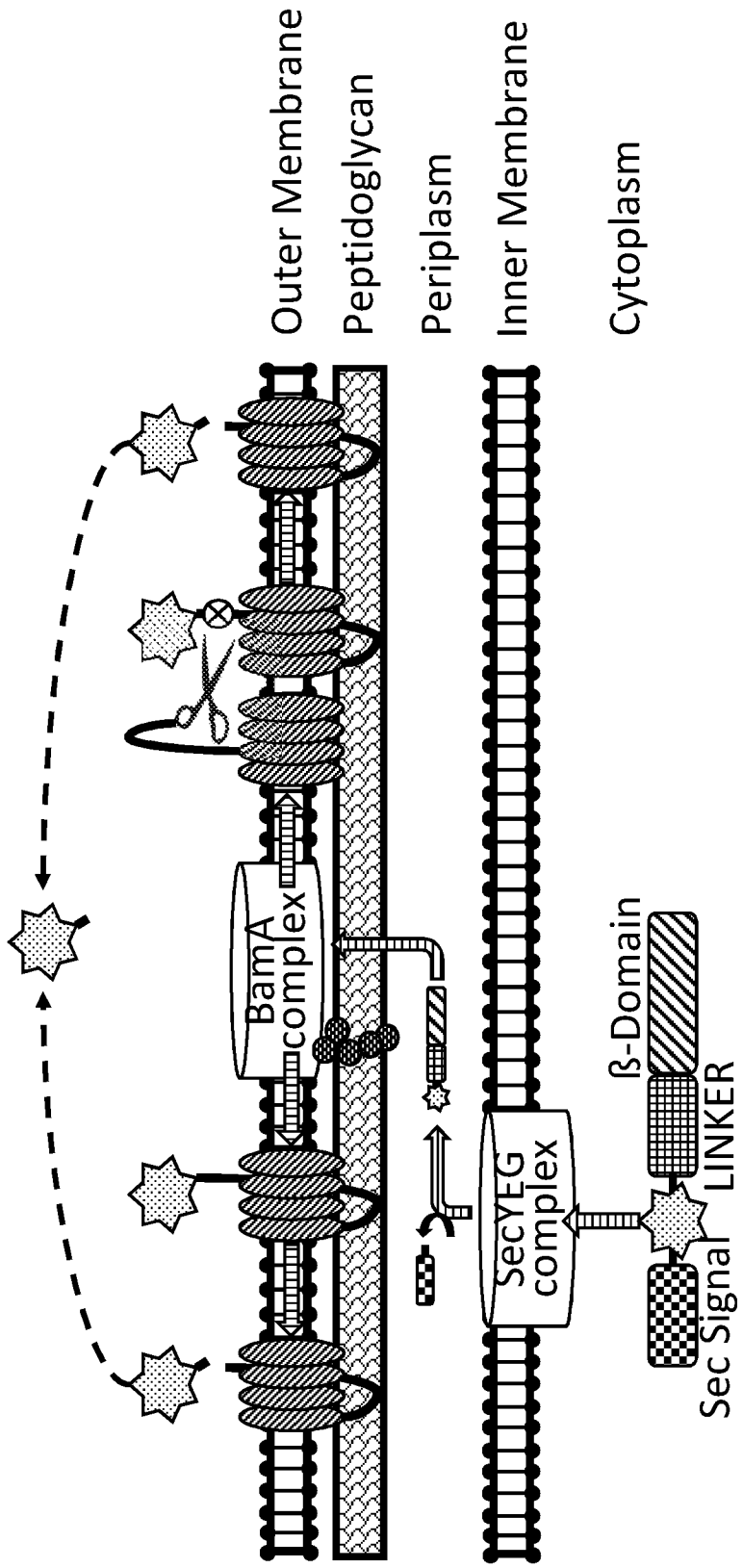

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
Bam complex
SecYEG complex
ß-Domain
LINKER
Sec Signal

FIG. 65

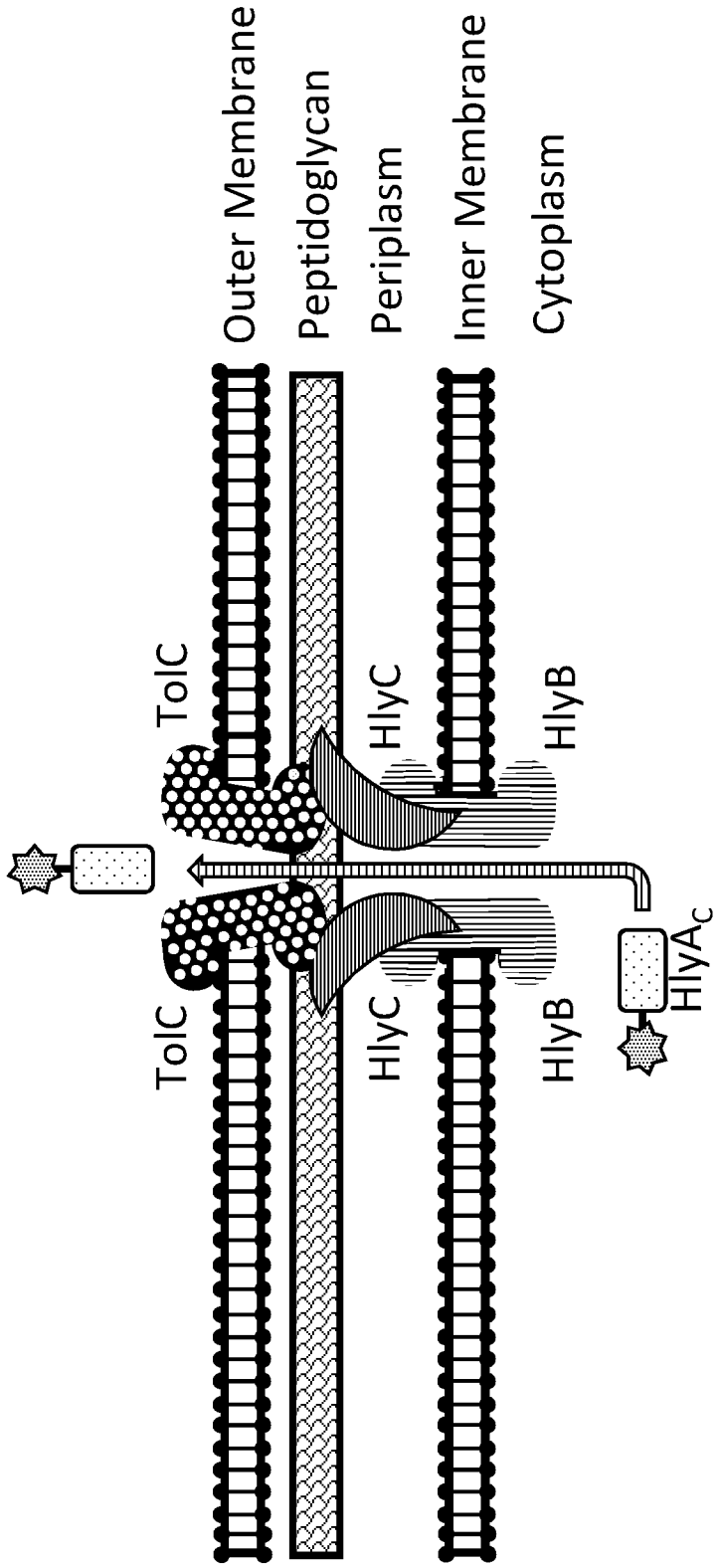
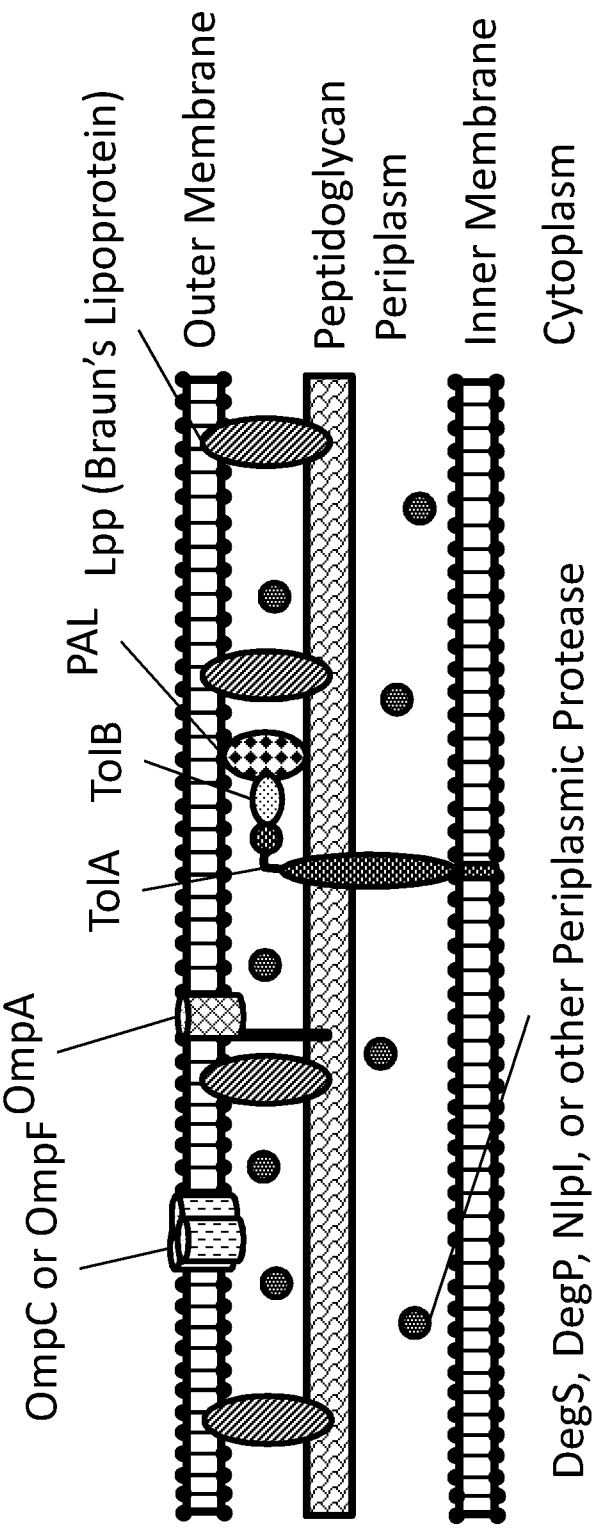

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
TolC
HlyC
HlyB
TolC
HlyC
HlyB
HlyA$_C$

FIG. 66


Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
Lpp (Braun's Lipoprotein)
PAL
TolB
TolA
OmpA
OmpF
OmpC or
DegS, DegP, NlpI, or other Periplasmic Protease

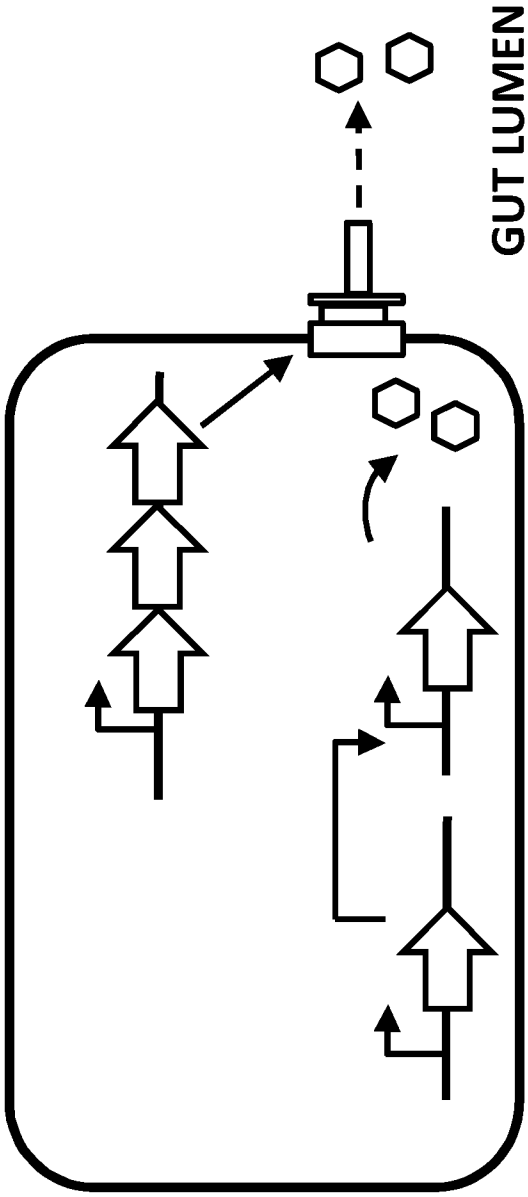
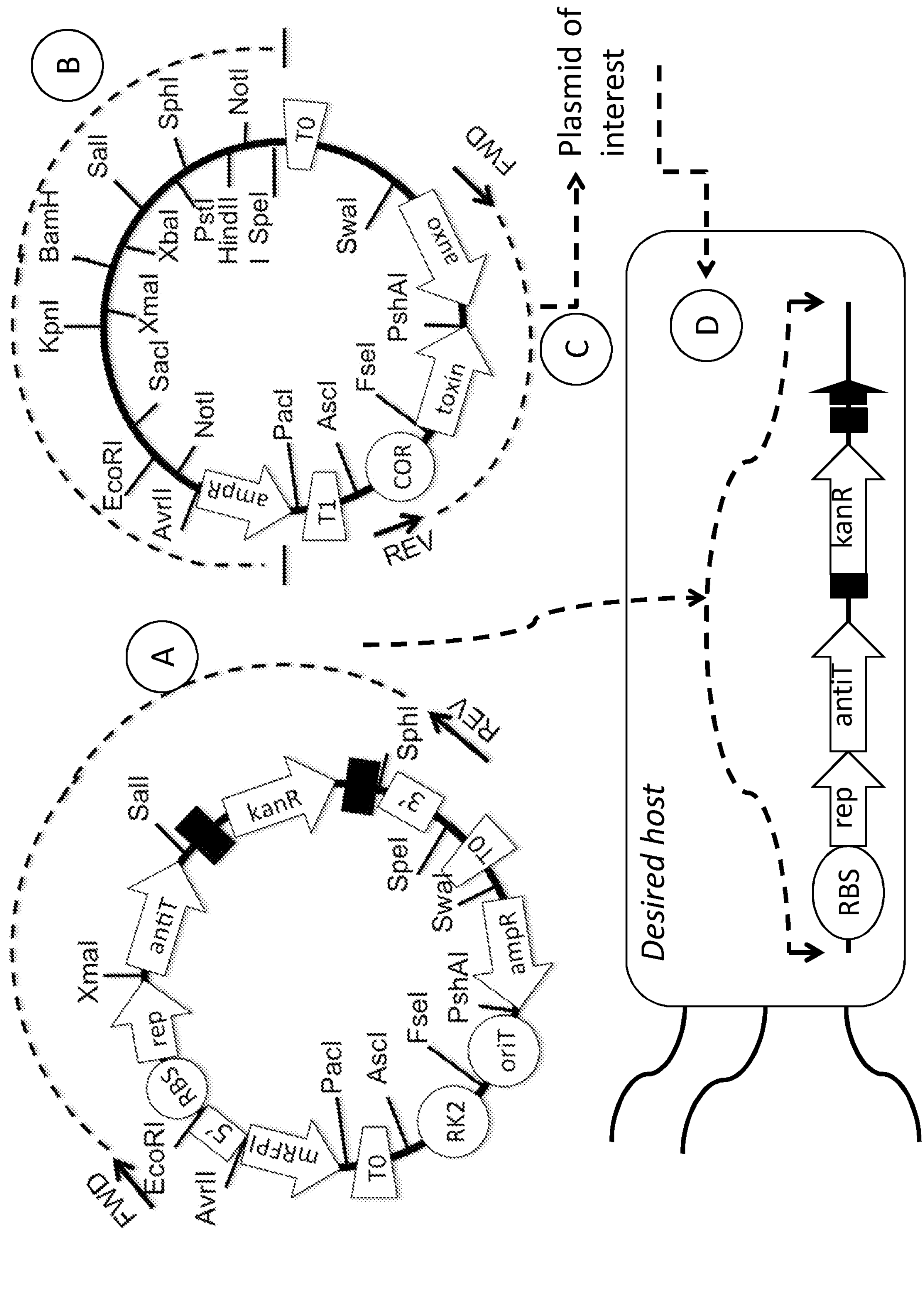


FIG. 67

Fig. 68


A
FWD
EcoRI
AvrII
RBS
rep
XmaI
antiT
SalI
kanR
SphI
REV
SpeI
SwaI
T0
ampR
PshAI
FseI
oriT
RK2
AscI
PacI
mRFPI
B
KpnI
BamH
SalI
SphI
NotI
XbaI
PstI
HindII
I SpeI
XmaI
SacI
EcoRI
NotI
AvrII
ampR
PacI
T1
AscI
COR
FseI
toxin
PshAI
auxo
SwaI
REV
FWD
C
Plasmid of
interest
D
Desired host
RBS
rep
antiT
kanR

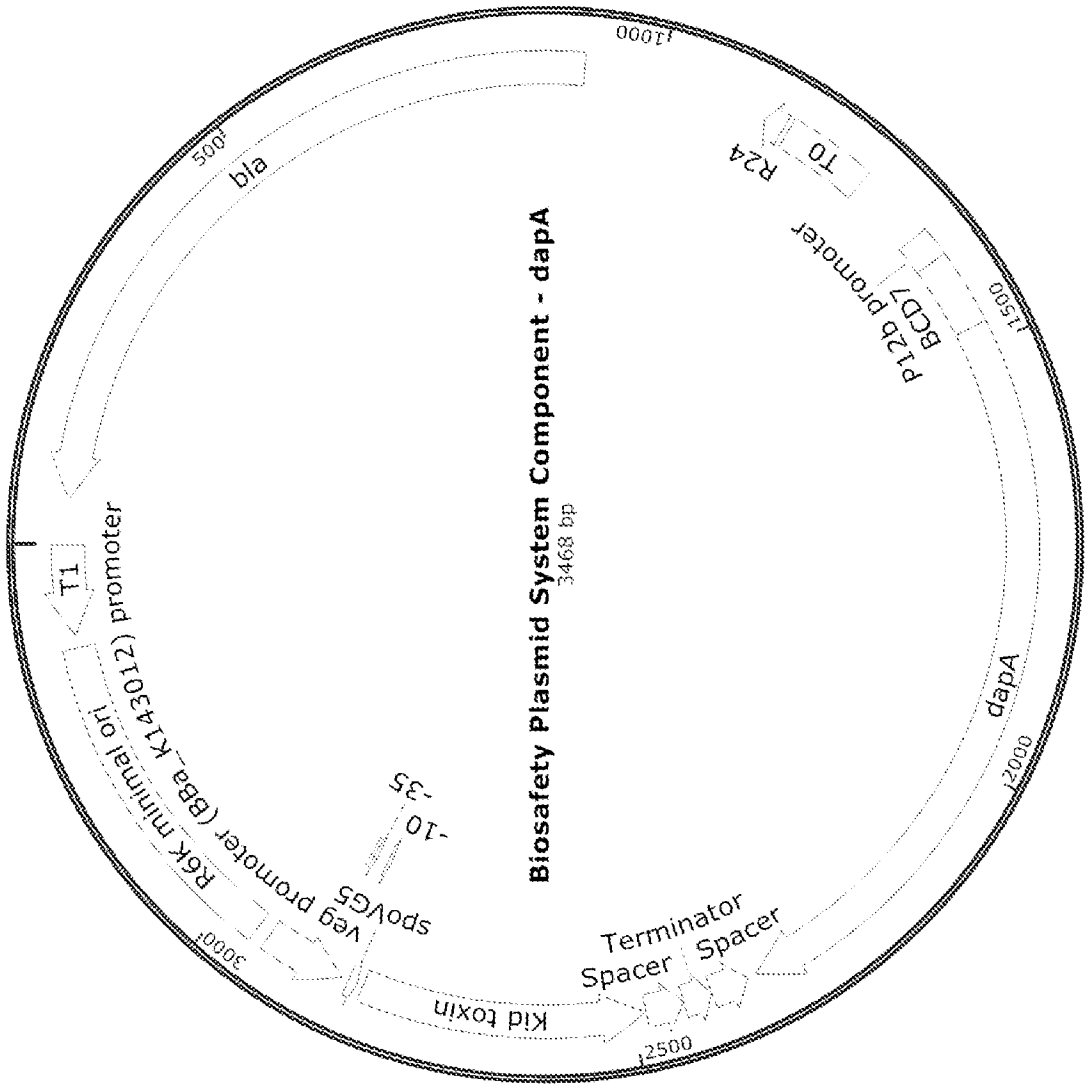

Biosafety Plasmid System Component - dapA
3468 bp
bla
T1
R6K minimal ori
Veg promoter (BBa_K143012)
spoVG5
-10
-35
Kid toxin
Terminator
Spacer
Spacer
dapA
P12b promoter
BCD7
R24
T0
500
1000
1500
2000
2500
3000

FIG. 69A

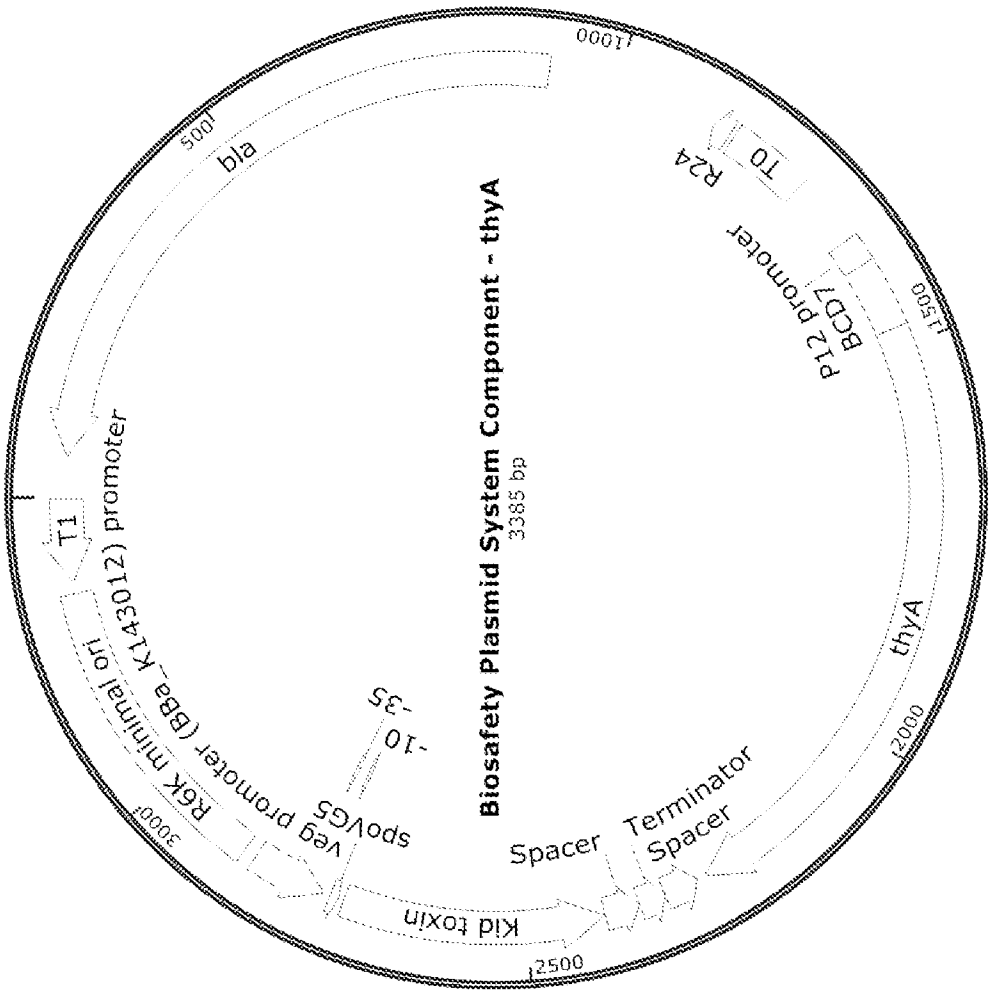
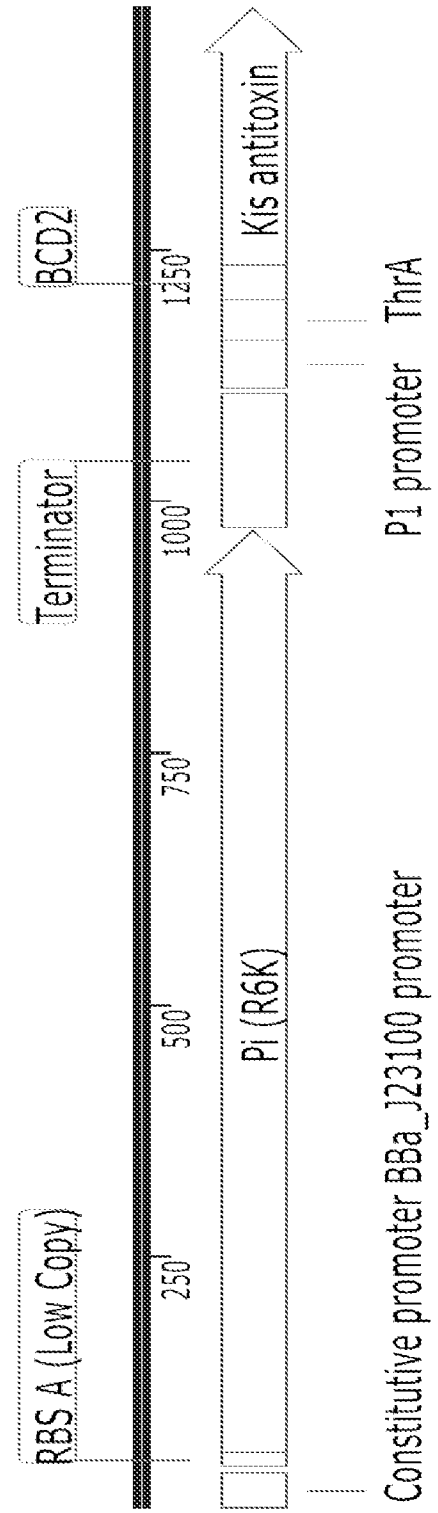

Biosafety Plasmid System Component - thyA
3385 bp
1000
500
bla
R2A
T0
P12 promoter
BCD7
1500
thyA
2000
Terminator
Spacer
Spacer
Kid toxin
2500
spoVG5
-10
-35
Veg promoter (Bba_K143012)
R6K minimal ori
3000
T1

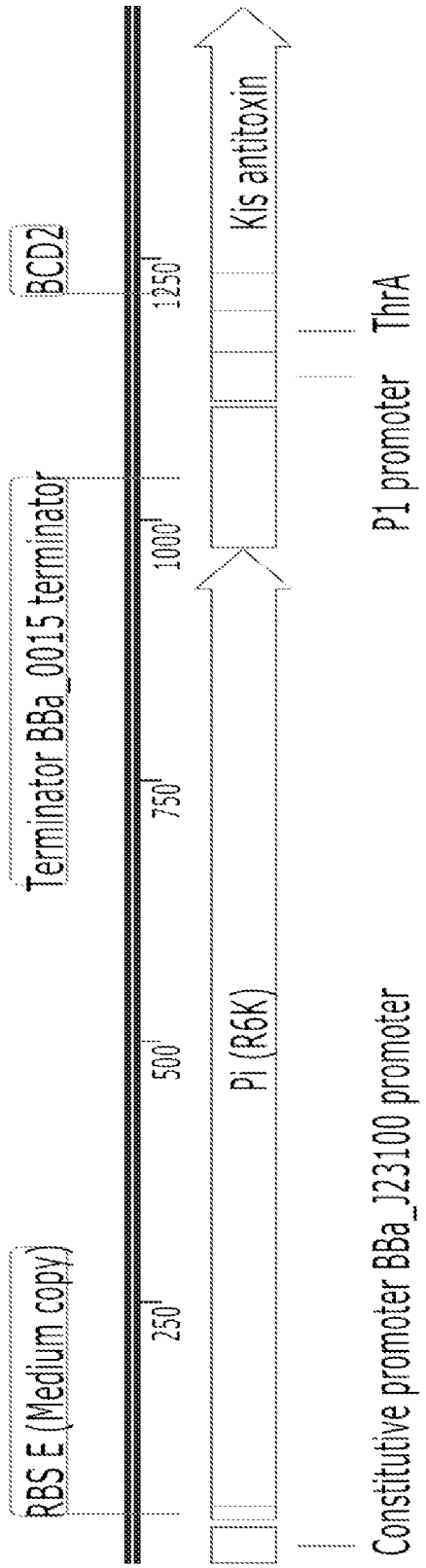
FIG. 69B

FIG. 69C



Biosafety Chromosomally Integrated Construct – low copy Rep (Pi) and Kis antitoxin
1490 bp

FIG. 69D



Biosafety Chromosomally Integrated Construct – medium copy Rep (Pi) and Kis antitoxin 1490 bp

FIG. 70

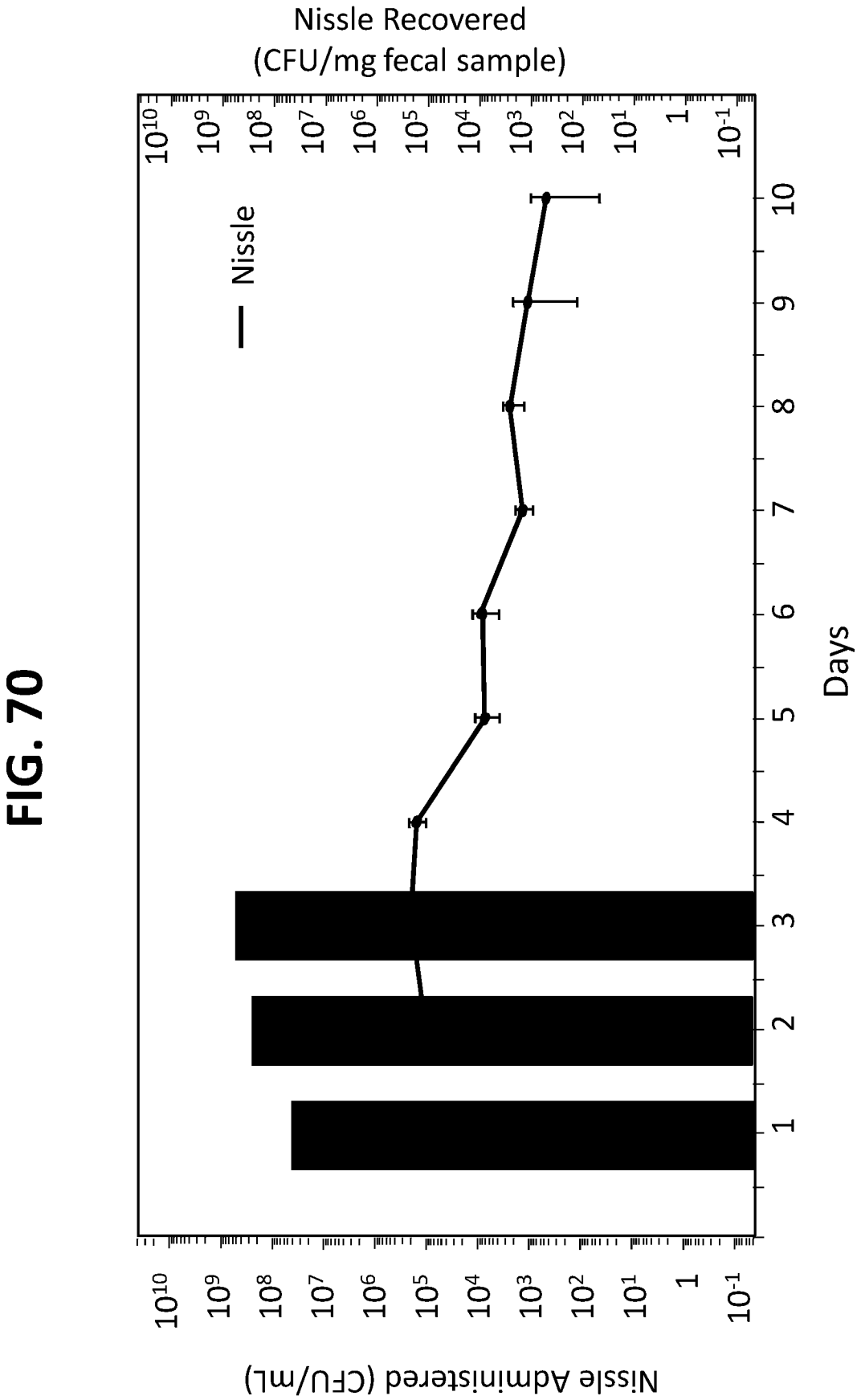

Nissle Recovered
(CFU/mg fecal sample)
Nissle
Days
Nissle Administered (CFU/mL)

FIG. 71

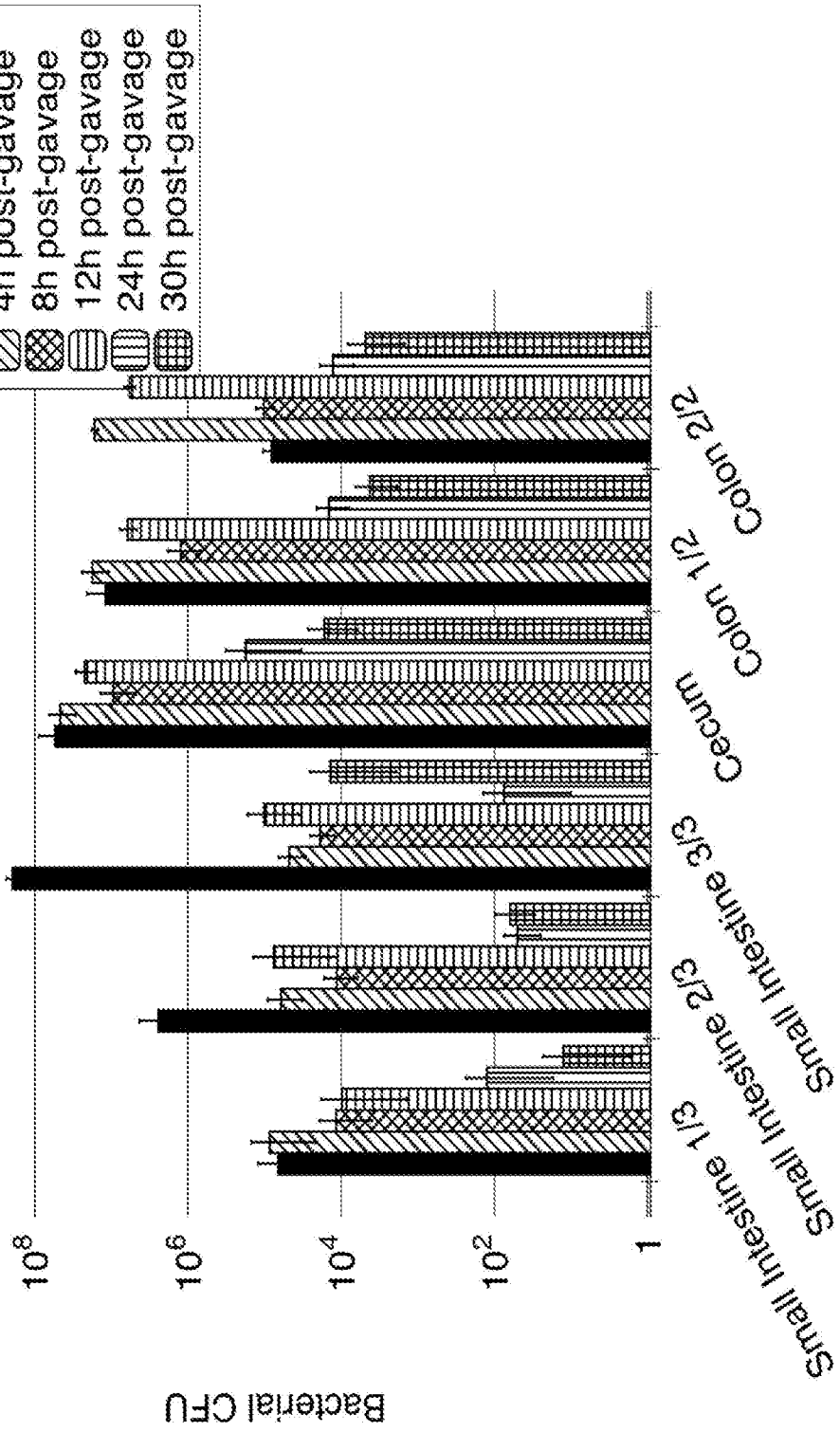

1h post-gavage
4h post-gavage
8h post-gavage
12h post-gavage
24h post-gavage
30h post-gavage
Bacterial CFU
10^8
10^6
10^4
10^2
1
Small Intestine 1/3
Small Intestine 2/3
Small Intestine 3/3
Cecum
Colon 1/2
Colon 2/2

FIG. 72A

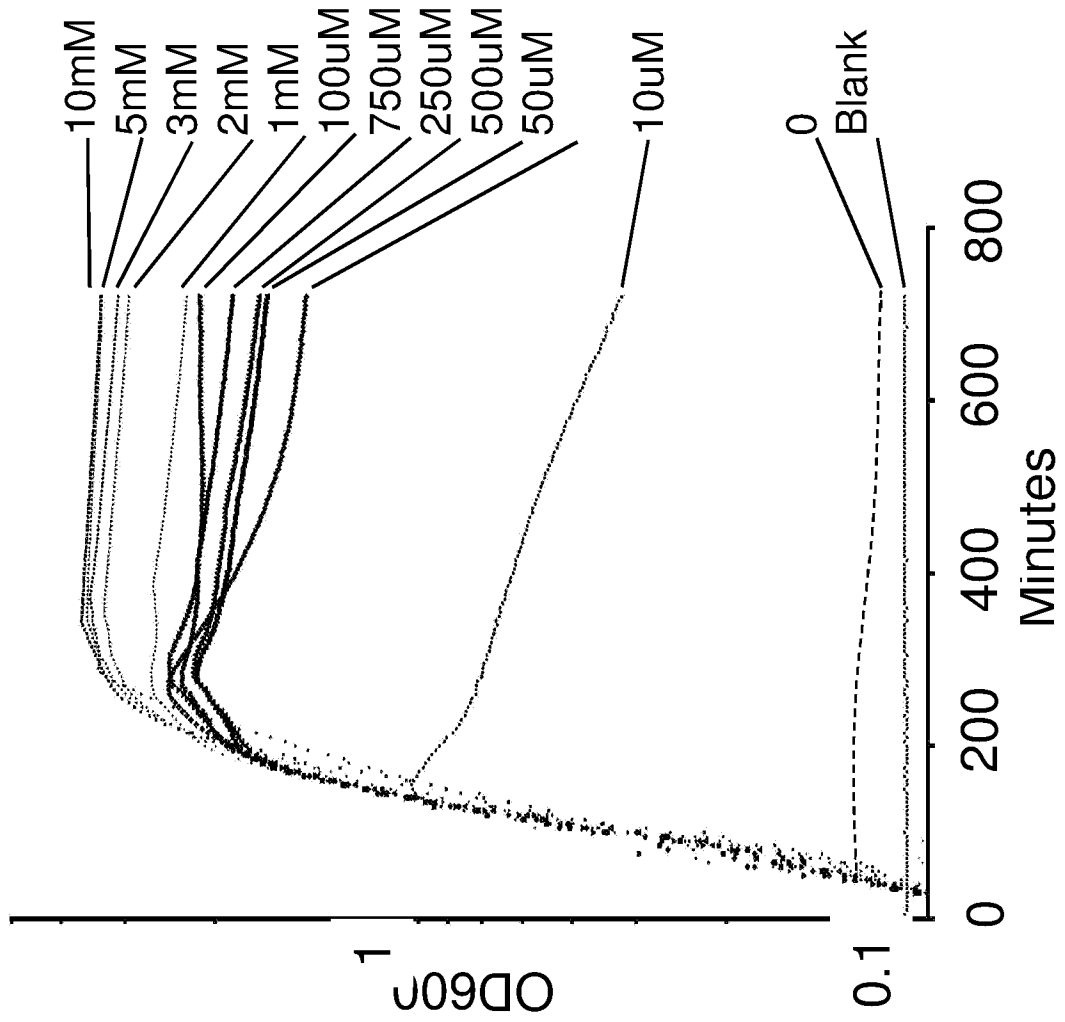
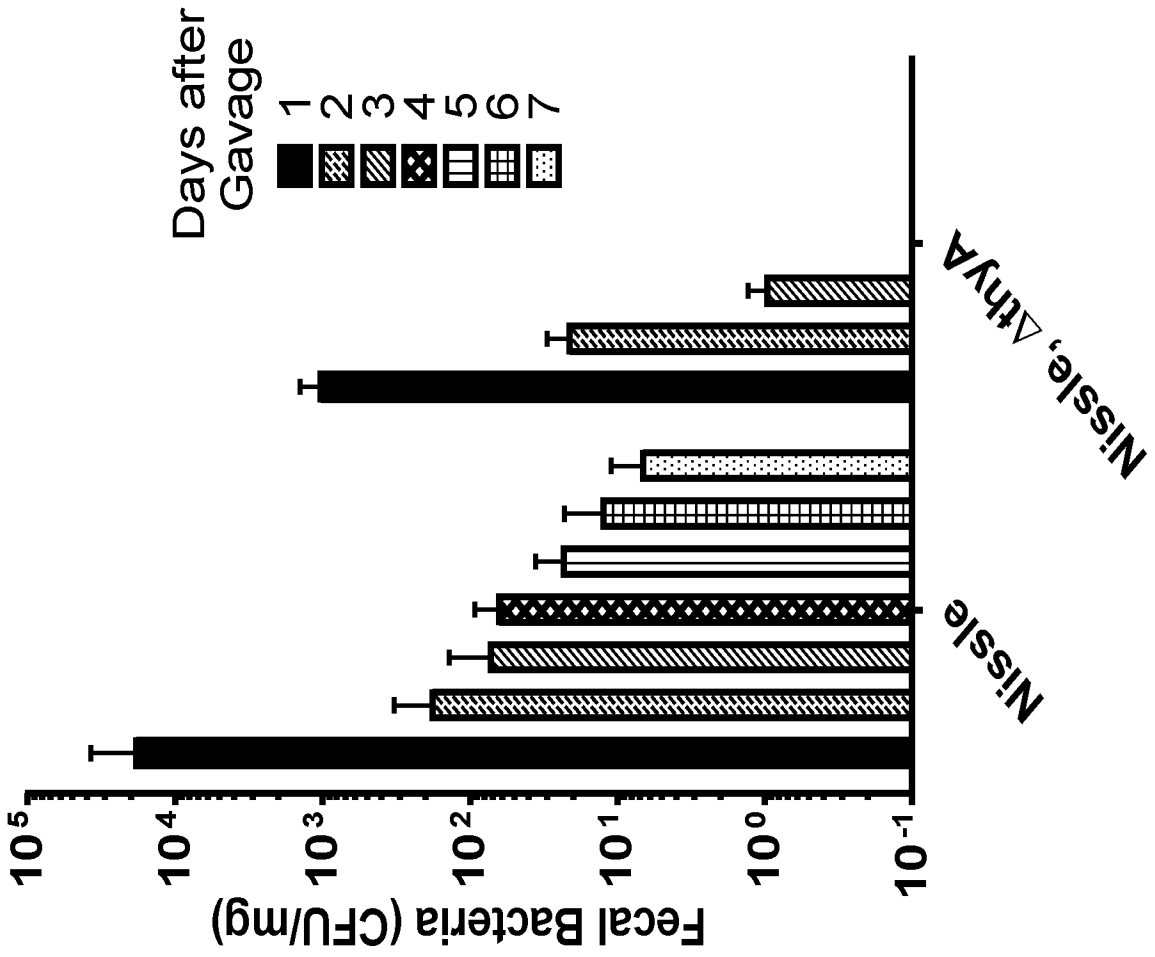

10mM
5mM
3mM
2mM
1mM
100uM
750uM
250uM
500uM
50uM
10uM
0
Blank
OD60nm
1
0.1
0
200
400
600
800
Minutes

FIG. 72B


Days after Gavage
1
2
3
4
5
6
7
Fecal Bacteria (CFU/mg)
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
Nissle
Nissle, ΔthyA

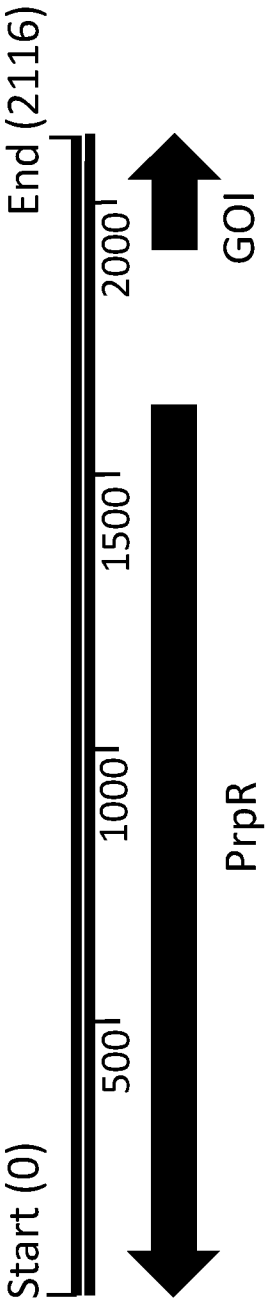
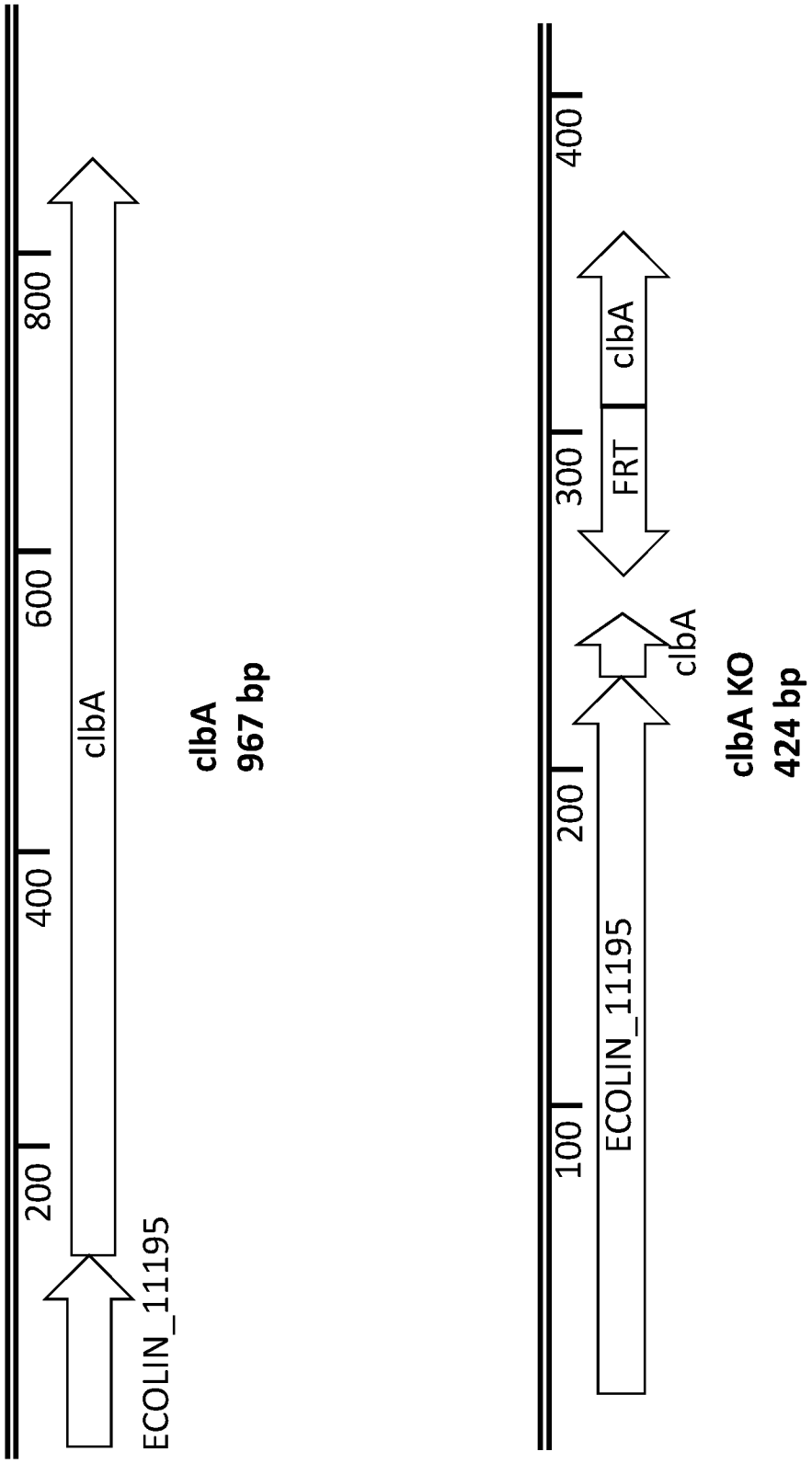


Fig. 73

Fig. 74


200
400
600
800
clbA
ECOLIN_11195
clbA
967 bp
100
200
300
400
ECOLIN_11195
FRT
clbA
clbA
clbA KO
424 bp

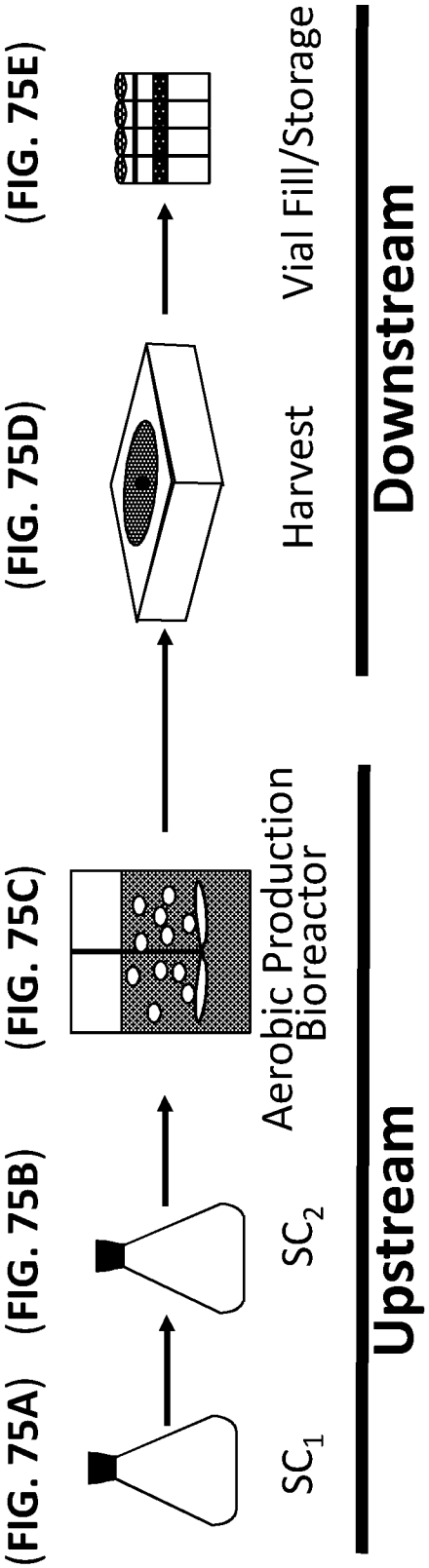

(FIG. 75A)
(FIG. 75B)
(FIG. 75C)
(FIG. 75D)
(FIG. 75E)
SC1
SC2
Aerobic Production Bioreactor
Harvest
Vial Fill/Storage
Upstream
Downstream

FIG. 76

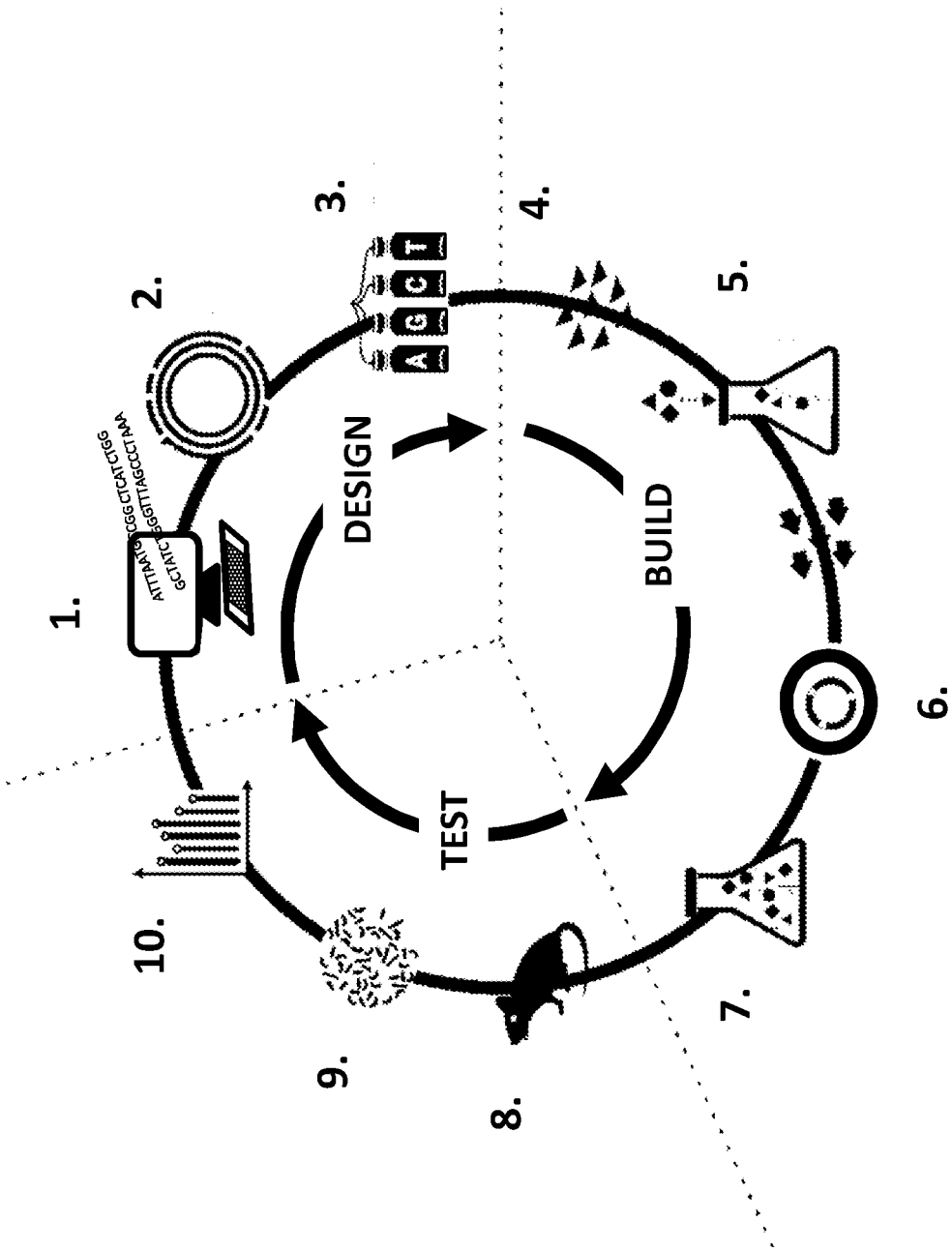


FIG. 77A

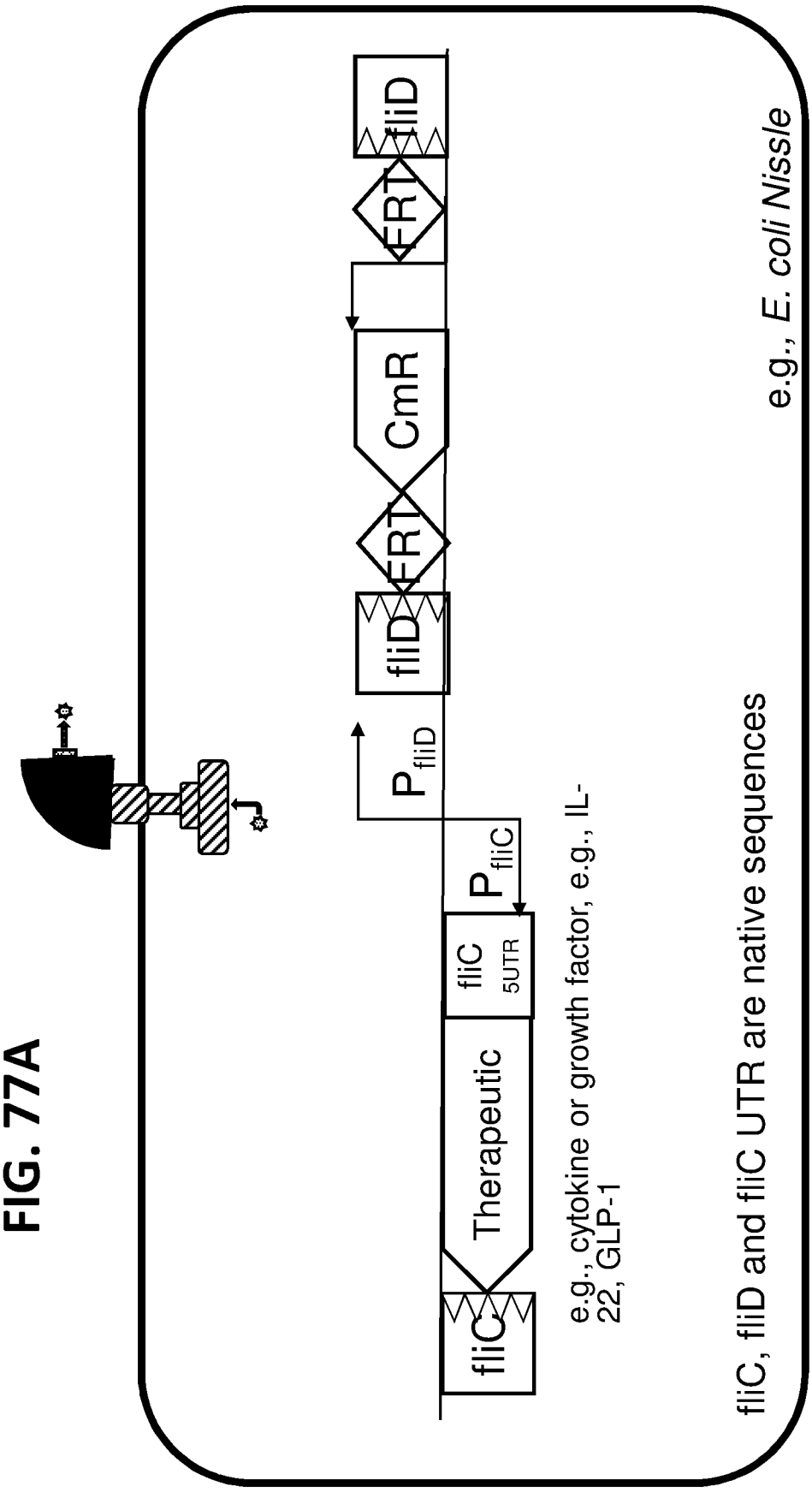

fliC, fliD and fliC UTR are native sequences
e.g., E. coli Nissle
fliC
Therapeutic
e.g., cytokine or growth factor, e.g., IL-22, GLP-1
fliC 5UTR
P fliC
P fliD
fliD
FRT
CmR
FRT
fliD

FIG. 77B

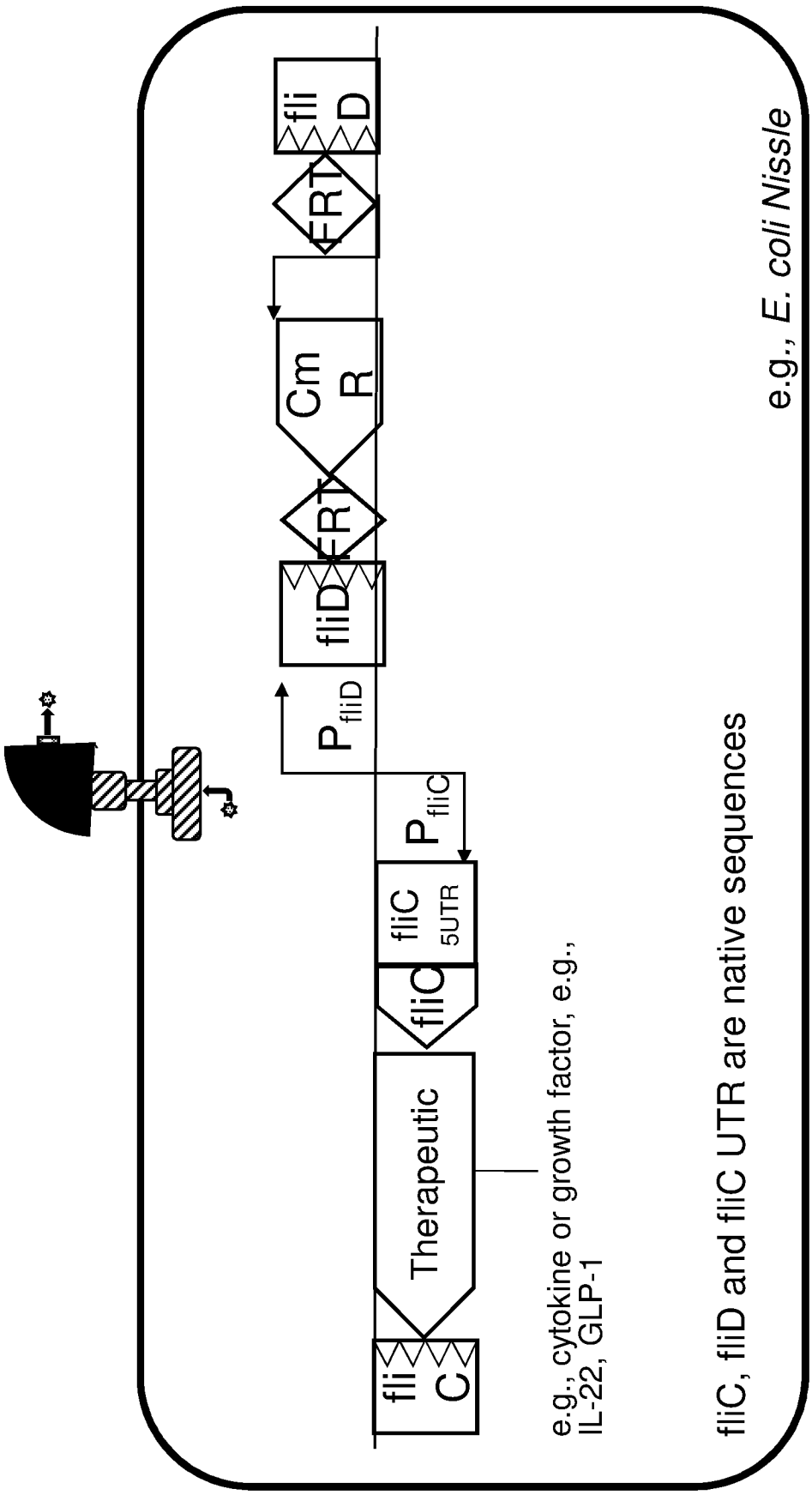

fliC, fliD and fliC UTR are native sequences
e.g., E. coli Nissle
fliC
Therapeutic
e.g., cytokine or growth factor, e.g., IL-22, GLP-1
fliC
fliC 5UTR
P fliC
P fliD
fliD
FRT
Cm R
FRT
fliD

FIG. 77C

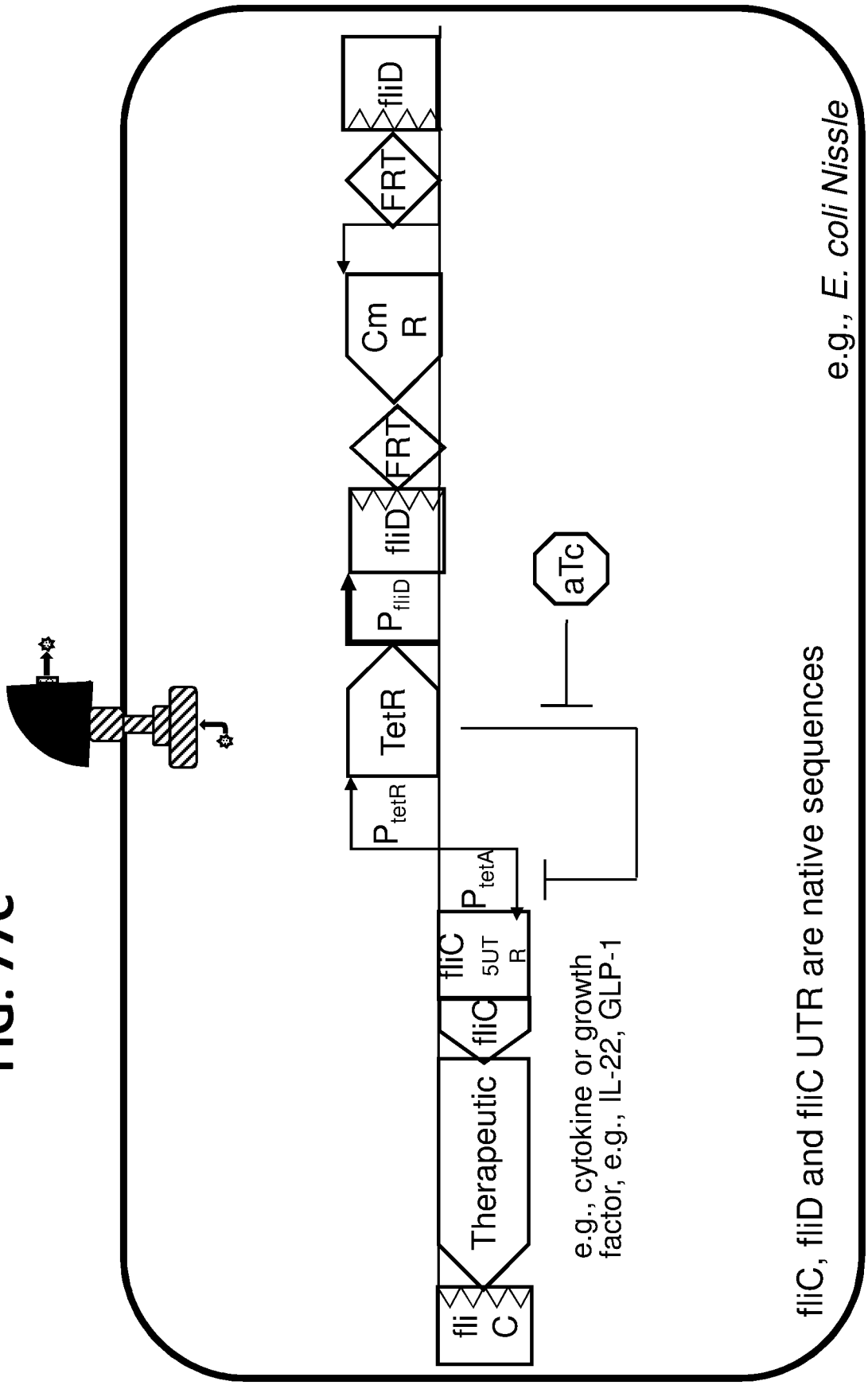

fliC, fliD and fliC UTR are native sequences
e.g., E. coli Nissle
fliC
Therapeutic
fliC
fliC 5UT R
P tetA
e.g., cytokine or growth factor, e.g., IL-22, GLP-1
aTc
P tetR
TetR
P fliD
fliD
FRT
Cm R
FRT
fliD

FIG. 78A

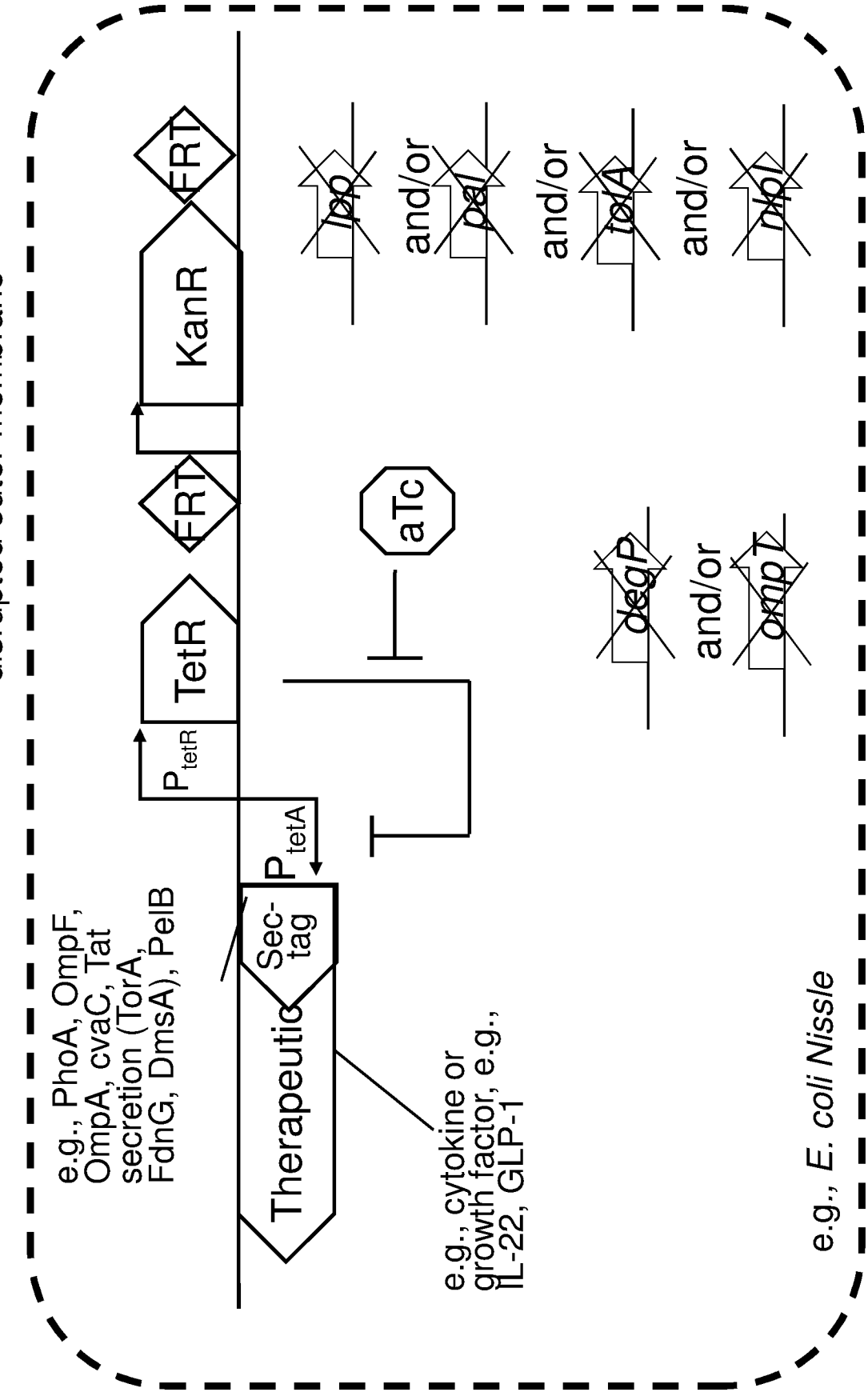

disrupted outer membrane
e.g., PhoA, OmpF, OmpA, cvaC, Tat secretion (TorA, FdnG, DmsA), PelB
Sec-tag
Therapeutic
e.g., cytokine or growth factor, e.g., IL-22, GLP-1
P tetA
P tetR
TetR
FRT
FRT
KanR
aTc
lpp
and/or
pal
and/or
tolA
and/or
nlpI
degP
and/or
ompT
e.g., E. coli Nissle

FIG. 78B

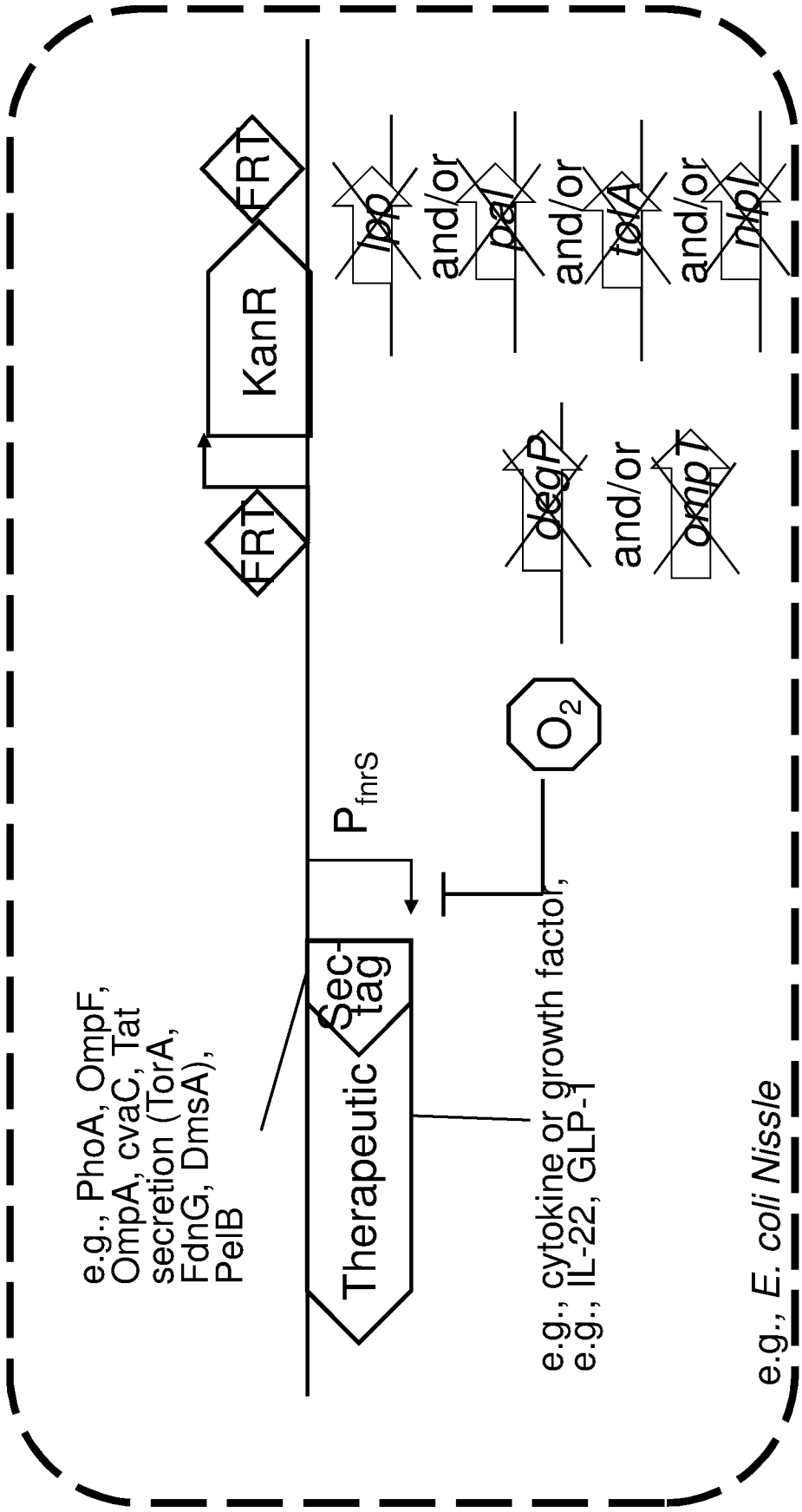

e.g., PhoA, OmpF, OmpA, cvaC, Tat secretion (TorA, FdnG, DmsA), PelB
Sec tag
Therapeutic
e.g., cytokine or growth factor, e.g., IL-22, GLP-1
P_{fnrS}
O_2
FRT
KanR
FRT
lpp
and/or
ral
and/or
tolA
and/or
rhtA
degP
and/or
ompT
e.g., E. coli Nissle

FIG. 79

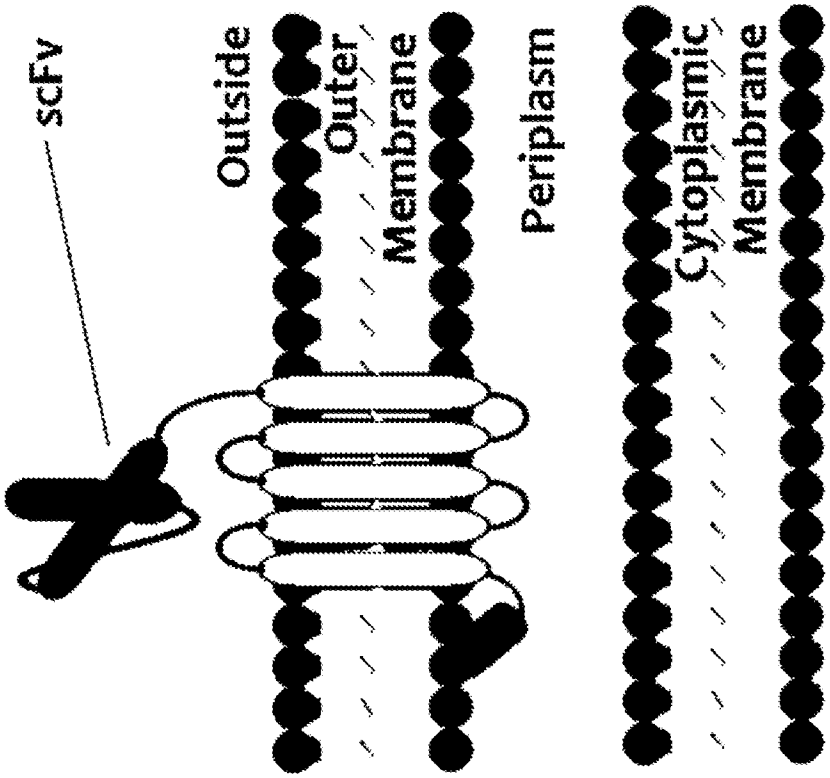
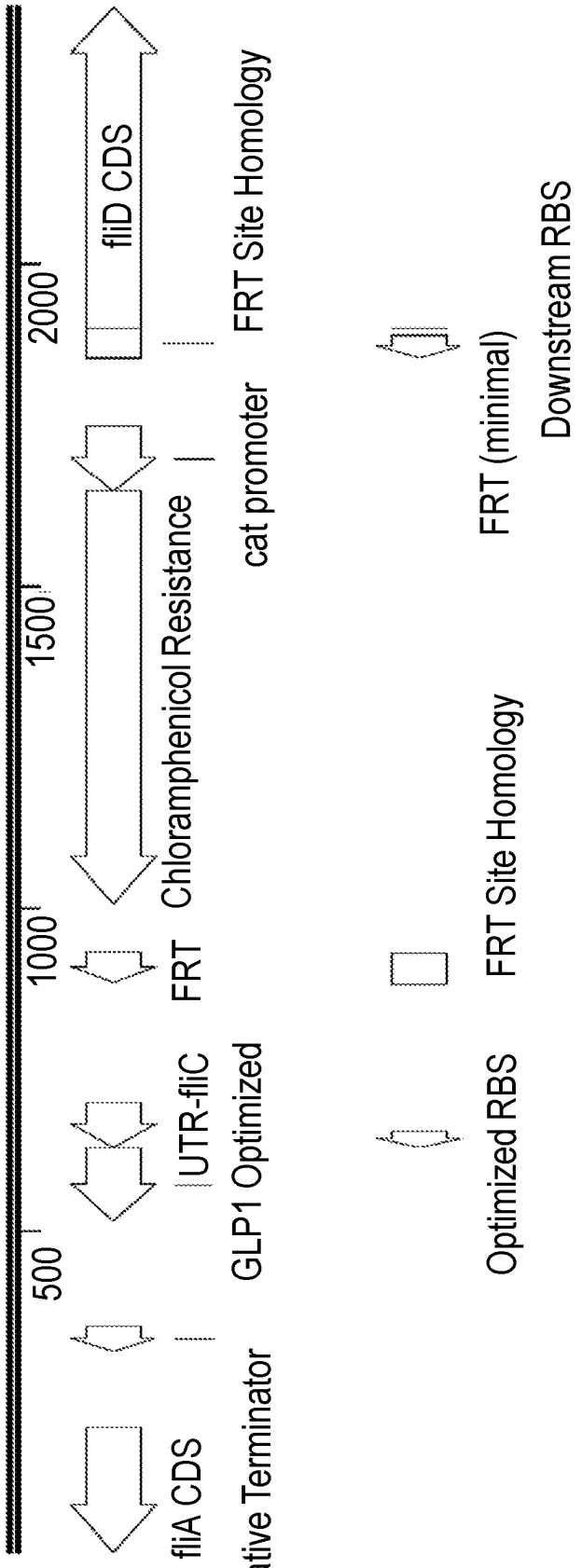
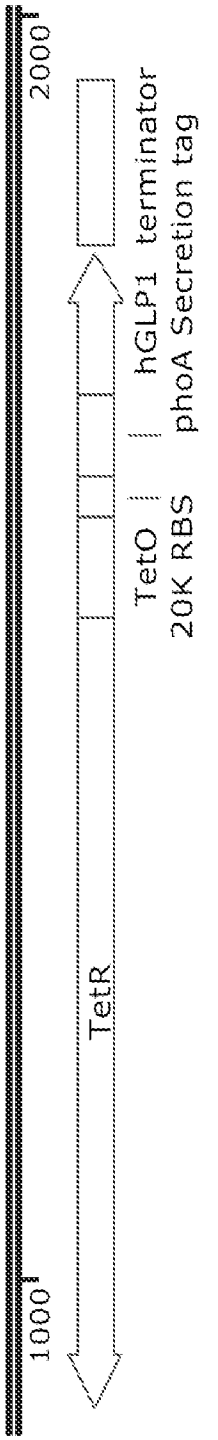

scFv
Outside
Outer
Membrane
Periplasm
Cytoplasmic
Membrane

FIG. 80



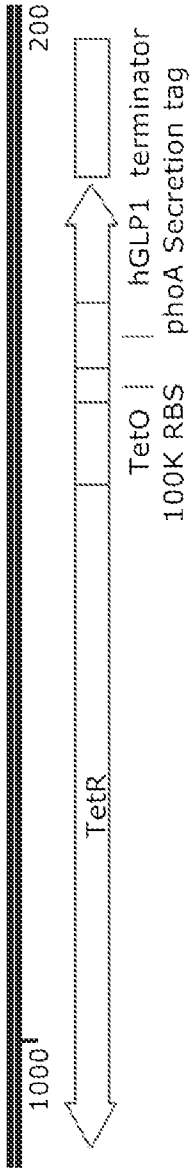
GLP-1 construct
2397 bp

FIG. 81A



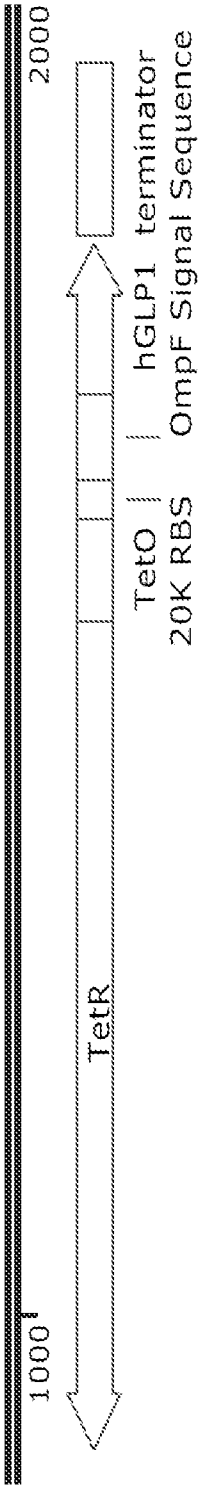
pTet-20K RBS -PhoA-Glp1 construct

FIG. 81B



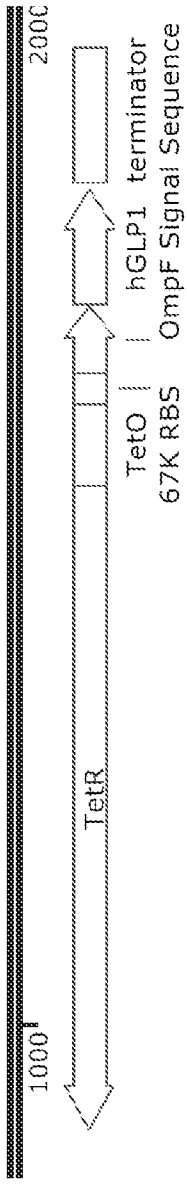
pTet-100K RBS -PhoA-Glp1 construct

FIG. 81C



pTet-20K RBS -OmpF-Glp1 construct

FIG. 81D



pTet-67K RBS -OmpF construct

FIG. 82A

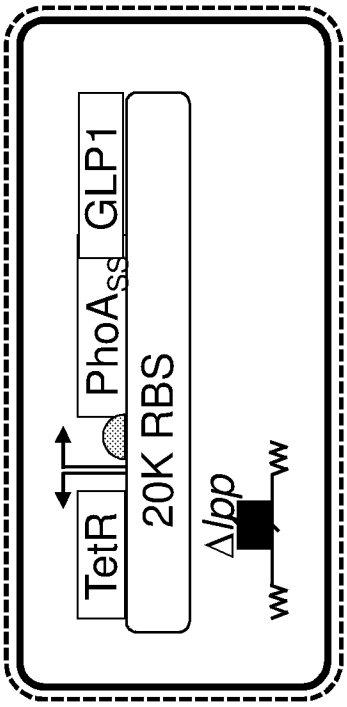


FIG. 82B

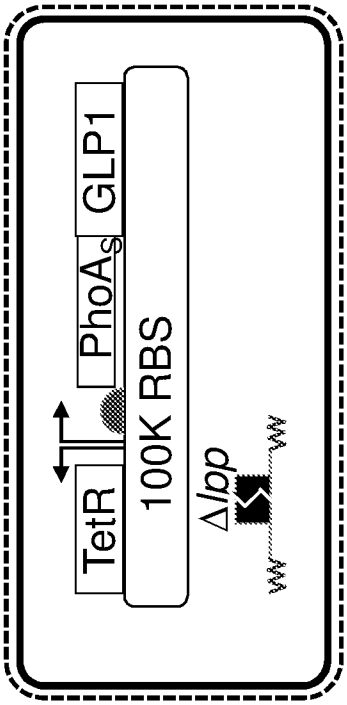


FIG. 82C

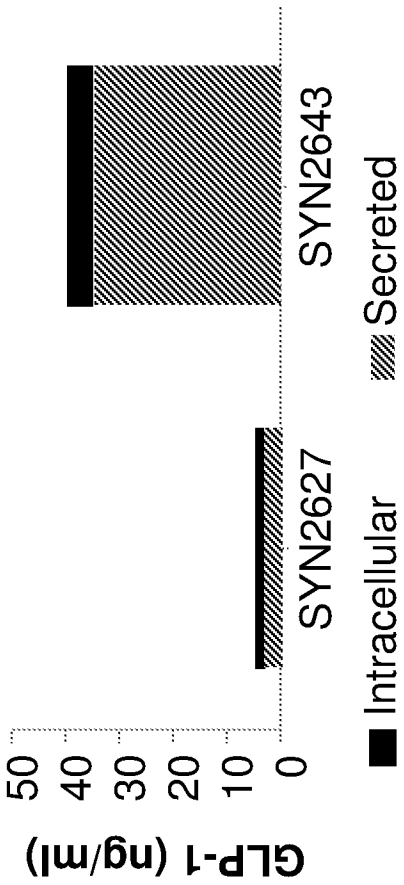


FIG. 83A

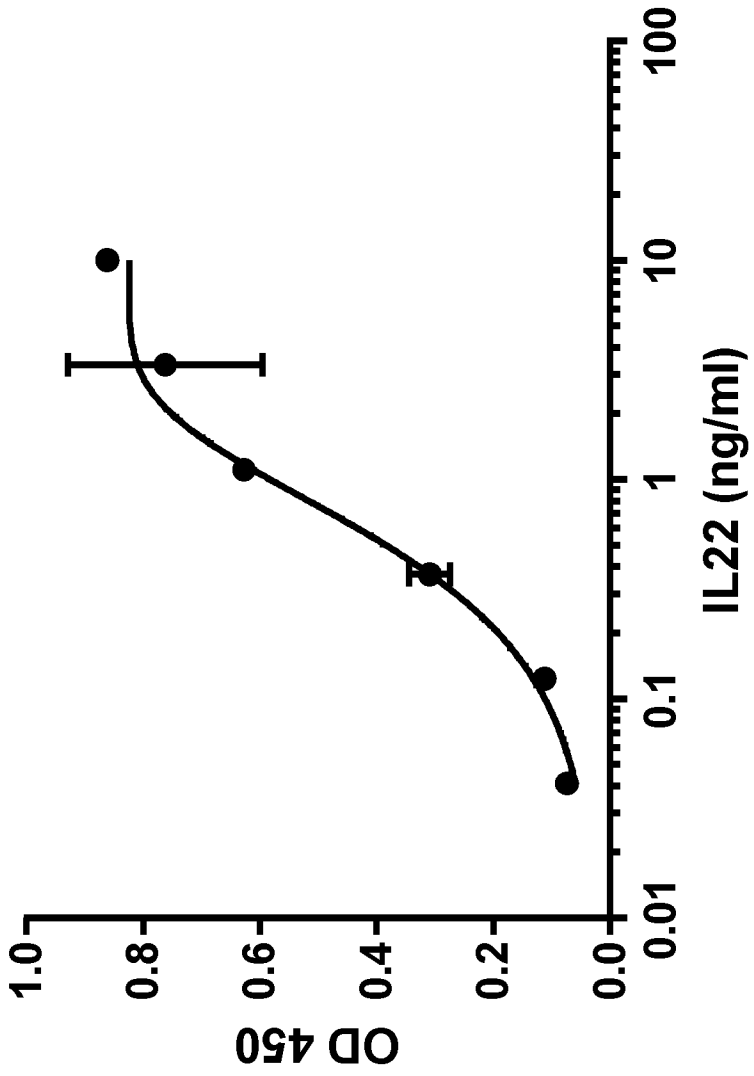

pSTAT3 Tyr705 ELISA
OD 450
1.0
0.8
0.6
0.4
0.2
0.0
0.01
0.1
1
10
100
IL22 (ng/ml)

FIG. 83B

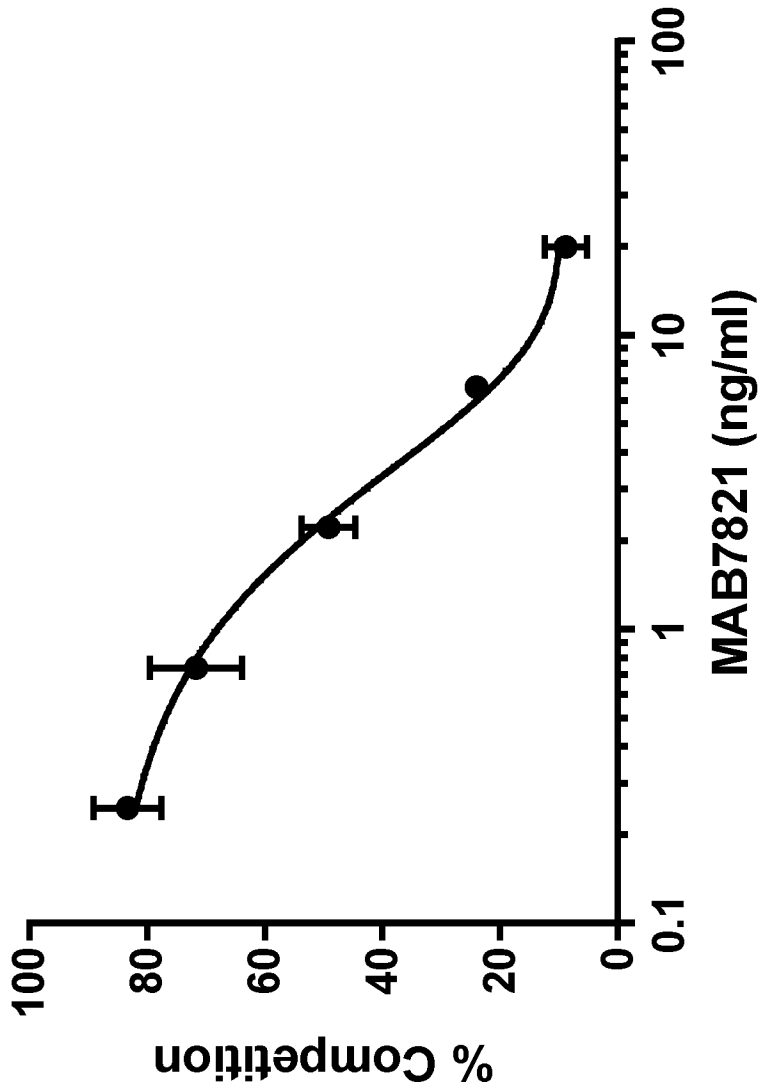

pSTAT3 Tyr705 ELISA
% Competition
100
80
60
40
20
0
0.1
1
10
100
MAB7821 (ng/ml)

FIG. 83C

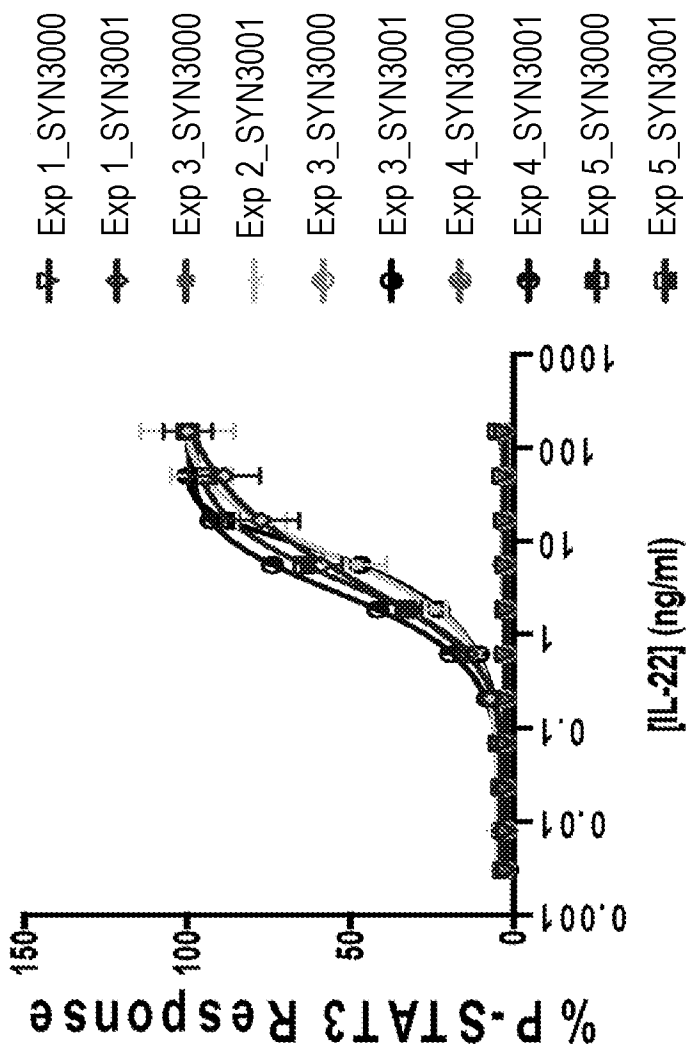

Exp 1_SYN3000
Exp 1_SYN3001
Exp 3_SYN3000
Exp 2_SYN3001
Exp 3_SYN3000
Exp 3_SYN3001
Exp 4_SYN3000
Exp 4_SYN3001
Exp 5_SYN3000
Exp 5_SYN3001
% P-STAT3 Response
150
100
50
0
0.001
0.01
0.1
1
10
100
1000
[IL-22] (ng/ml)

FIG. 83D

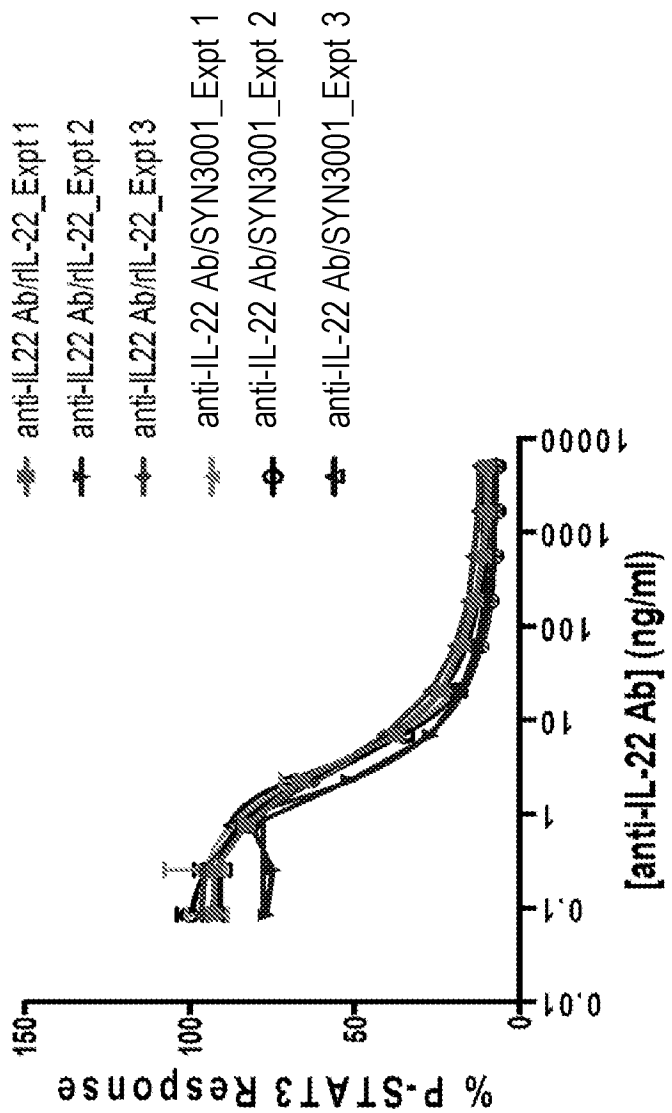

anti-IL22 Ab/rIL-22_Expt 1
anti-IL22 Ab/rIL-22_Expt 2
anti-IL22 Ab/rIL-22_Expt 3
anti-IL-22 Ab/SYN3001_Expt 1
anti-IL-22 Ab/SYN3001_Expt 2
anti-IL-22 Ab/SYN3001_Expt 3
% P-STAT3 Response
150
100
50
0
0.01
0.1
1
10
100
1000
10000
[anti-IL-22 Ab] (ng/ml)

FIG. 84A

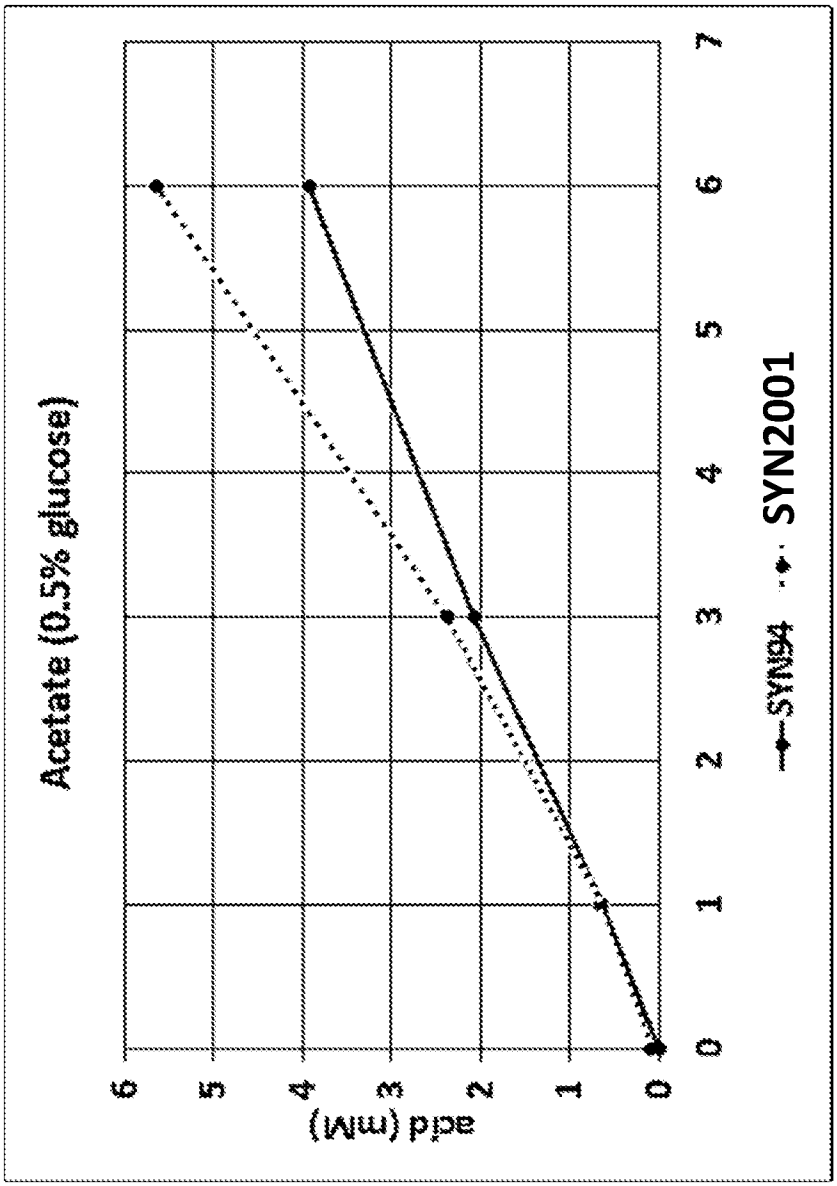
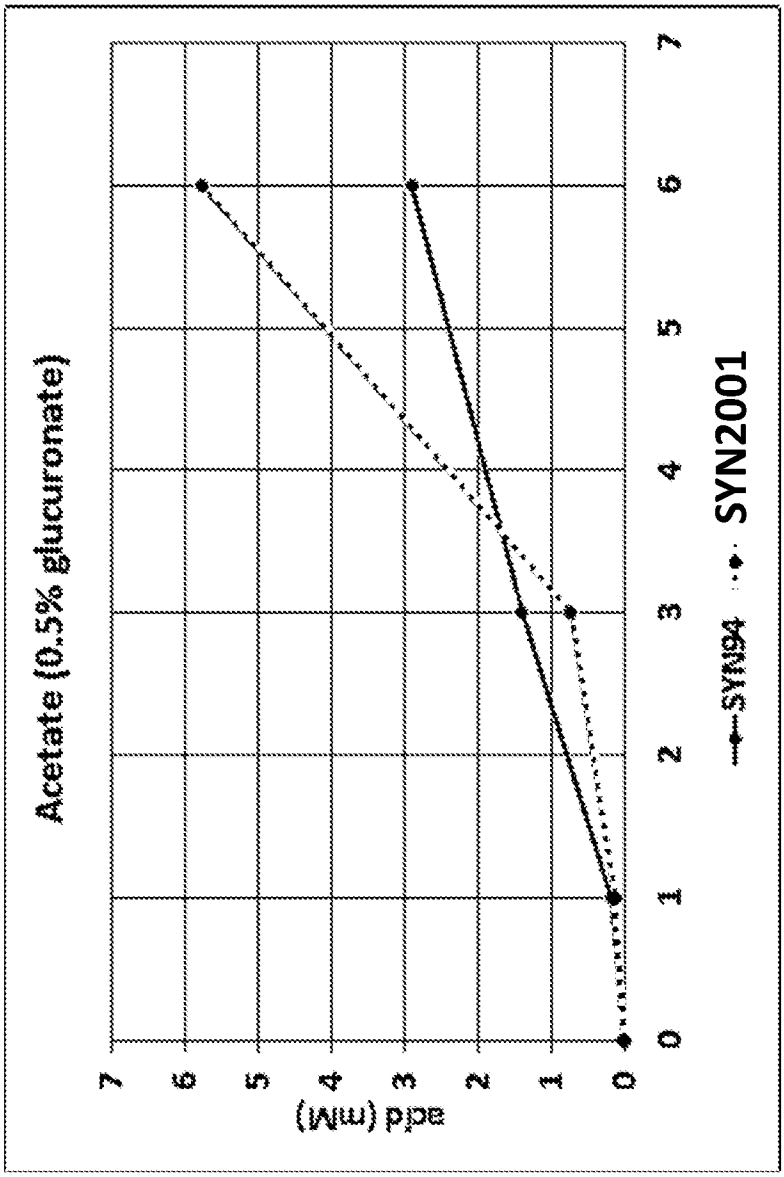


FIG. 84B



SYN94 Nissle
SYN2001 Nissle delta IdhA::CmR

FIG. 84C

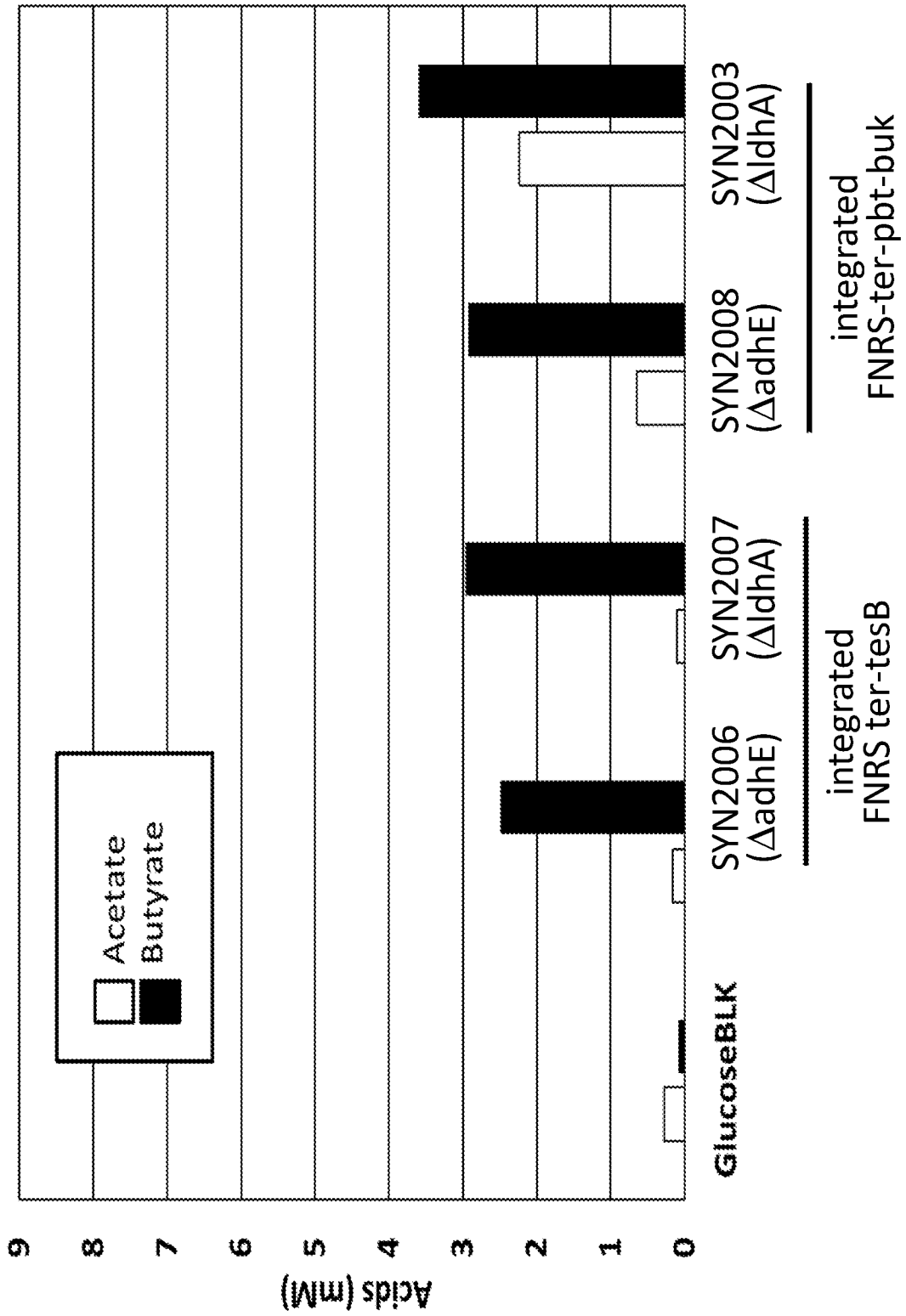

Acetate
Butyrate
Acids (mM)
0
1
2
3
4
5
6
7
8
9
GlucoseBLK
SYN2006 (ΔadhE)
SYN2007 (ΔldhA)
SYN2008 (ΔadhE)
SYN2003 (ΔldhA)
integrated FNRS ter-tesB
integrated FNRS-ter-pbt-buk

FIG. 84D

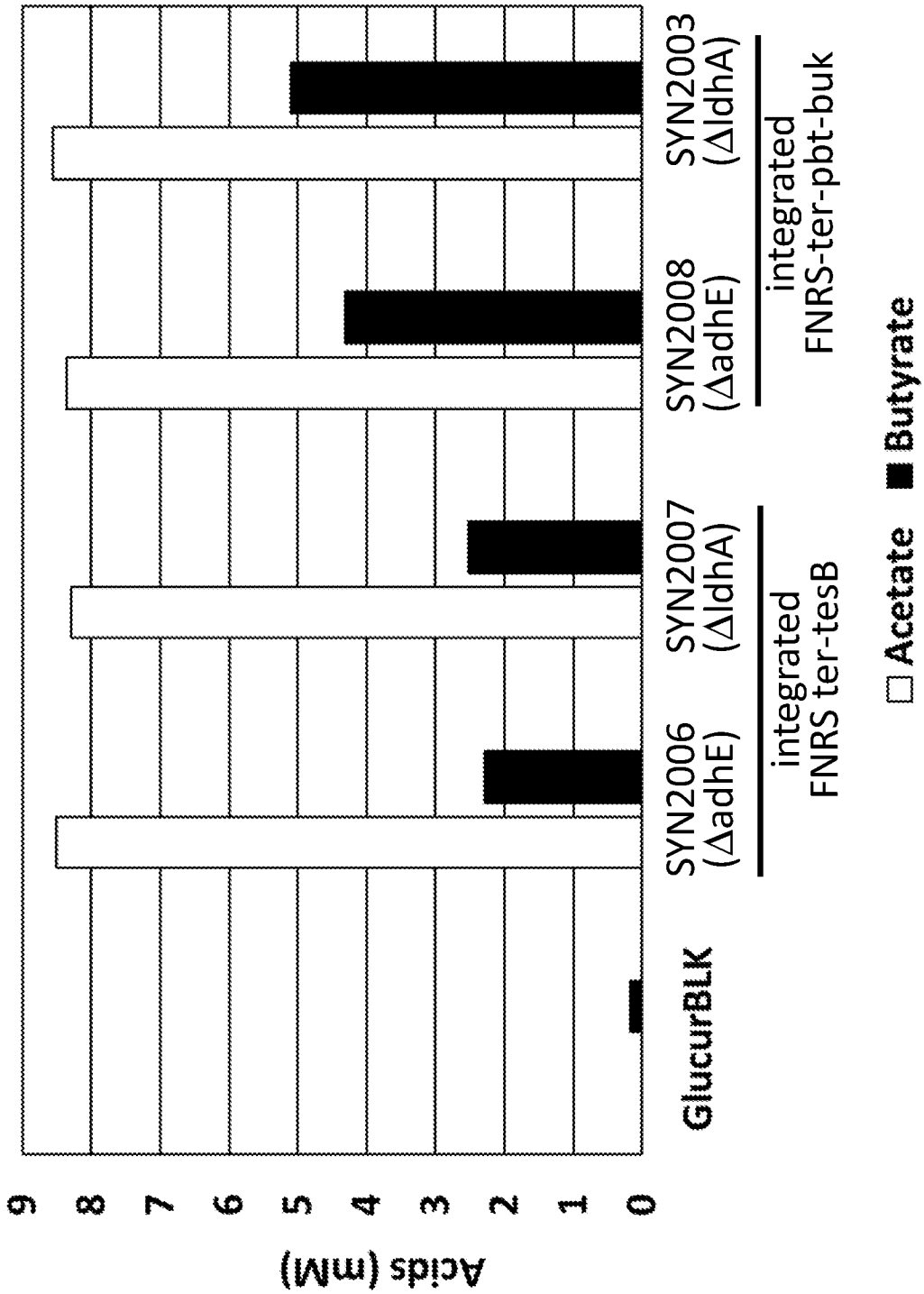

Acids (mM)
0
1
2
3
4
5
6
7
8
9
GlucurBLK
SYN2006 (ΔadhE)
SYN2007 (ΔldhA)
integrated FNRS ter-tesB
SYN2008 (ΔadhE)
SYN2003 (ΔldhA)
integrated FNRS-ter-pbt-buk
Acetate
Butyrate

FIG. 84E

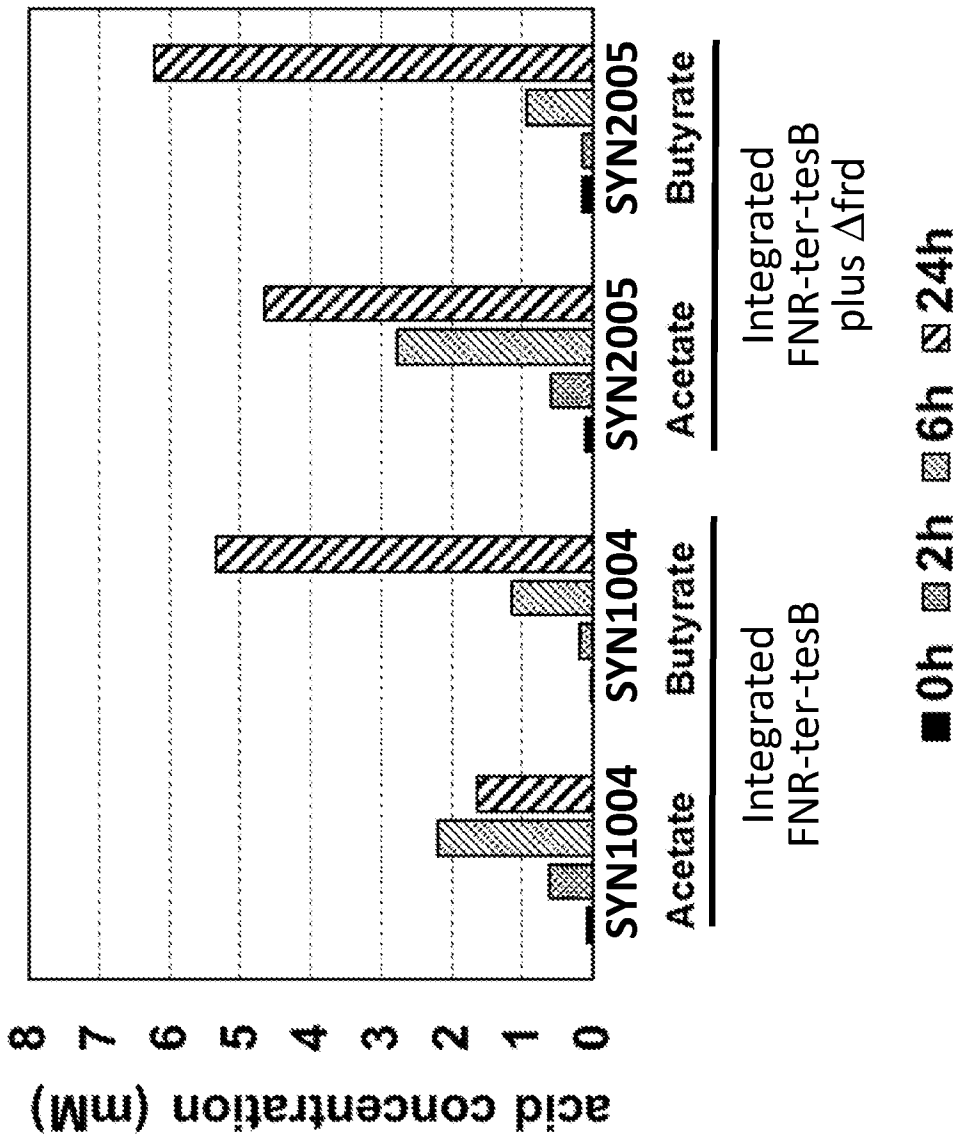
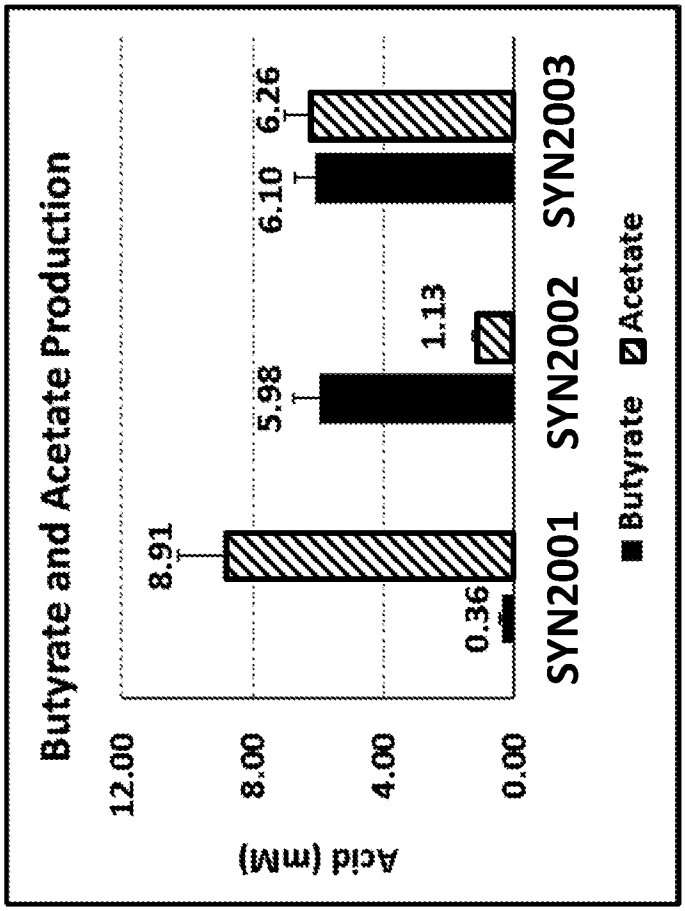

acid concentration (mM)
0
1
2
3
4
5
6
7
8
SYN1004
SYN1004
SYN2005
SYN2005
Acetate
Butyrate
Acetate
Butyrate
Integrated FNR-ter-tesB
Integrated FNR-ter-tesB plus Δfrd
0h
2h
6h
24h

FIG. 84F



SYN2001 Nissle ΔldhA::CmR
SYN2002 Nissle butyrate cassette ΔadhE/Δpta::CmR
SYN2003 Nissle butyrate cassette ΔldhA::CmR

FIG. 84G

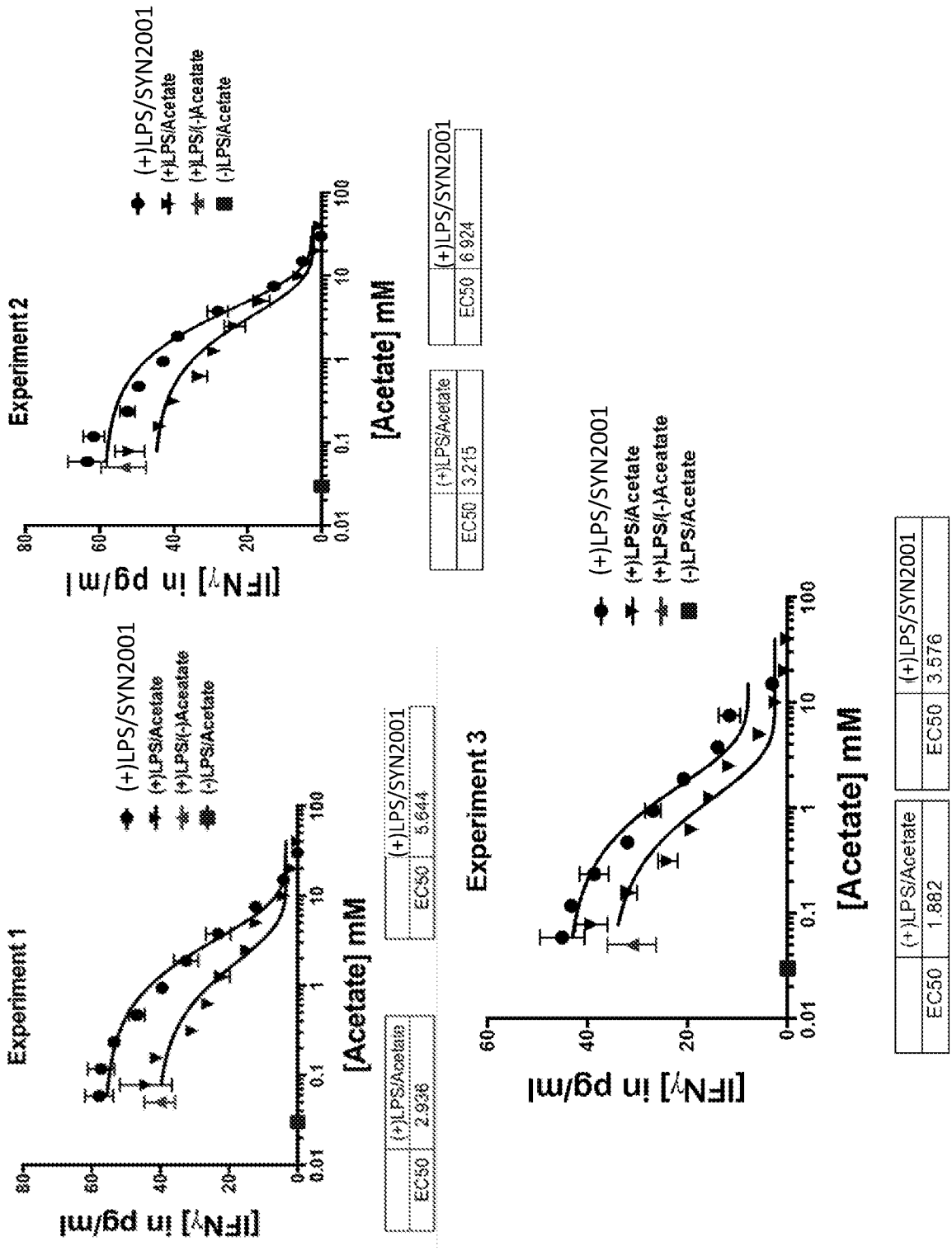

Experiment 1
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
(+)LPS/SYN2001
EC50
2.938
5.644
Experiment 2
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
(+)LPS/SYN2001
EC50
3.215
6.924
Experiment 3
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
(+)LPS/SYN2001
EC50
1.882
3.576

FIG. 84H

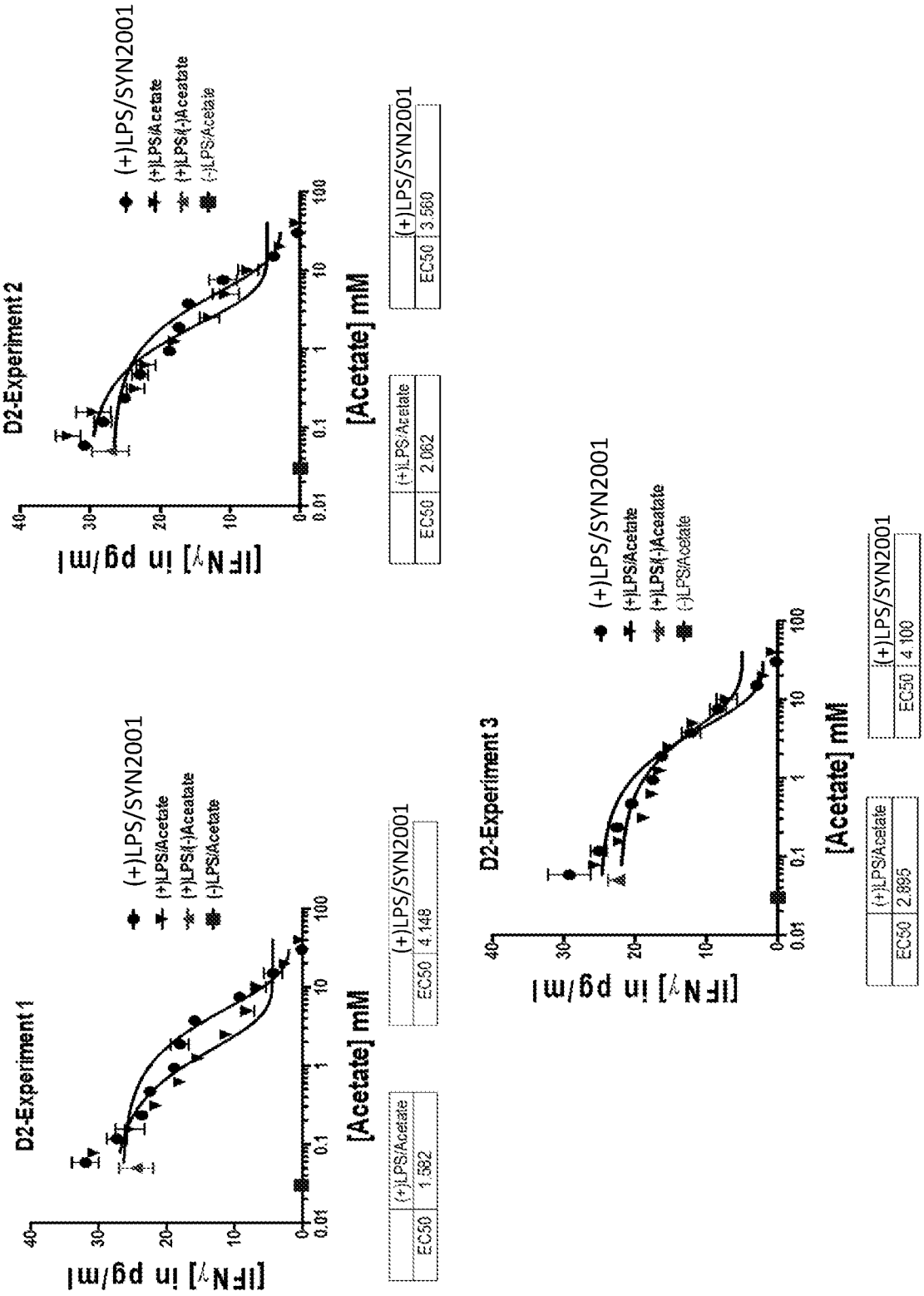

D2-Experiment 1
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/-)Acetate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 1.382
(+)LPS/SYN2001
EC50 4.148
D2-Experiment 2
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/-)Acetate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 2.062
(+)LPS/SYN2001
EC50 3.560
D2-Experiment 3
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/-)Acetate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 2.895
(+)LPS/SYN2001
EC50 4.100

FIG. 85

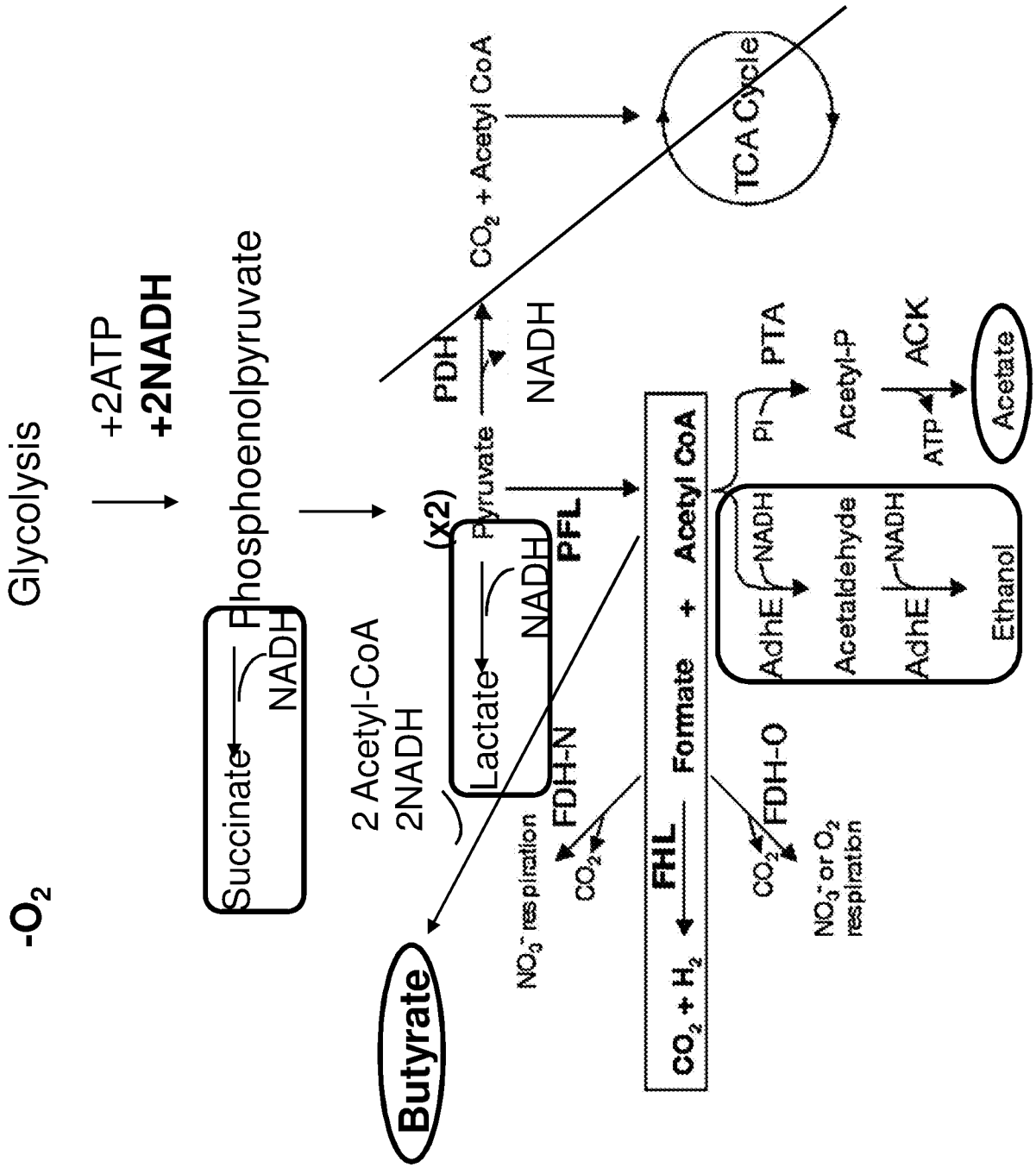

-O2
Glycolysis
+2ATP
+2NADH
Phosphoenolpyruvate
Succinate
NADH
2 Acetyl-CoA
2NADH
(x2)
Pyruvate
Lactate
NADH
PDH
NADH
CO2 + Acetyl CoA
TCA Cycle
PFL
FDH-N
NO3- respiration
CO2
Butyrate
FHL
CO2 + H2
Formate + Acetyl CoA
CO2
FDH-O
NO3- or O2 respiration
AdhE
NADH
Acetaldehyde
AdhE
NADH
Ethanol
Pi
PTA
Acetyl-P
ATP
ACK
Acetate

FIG. 86A

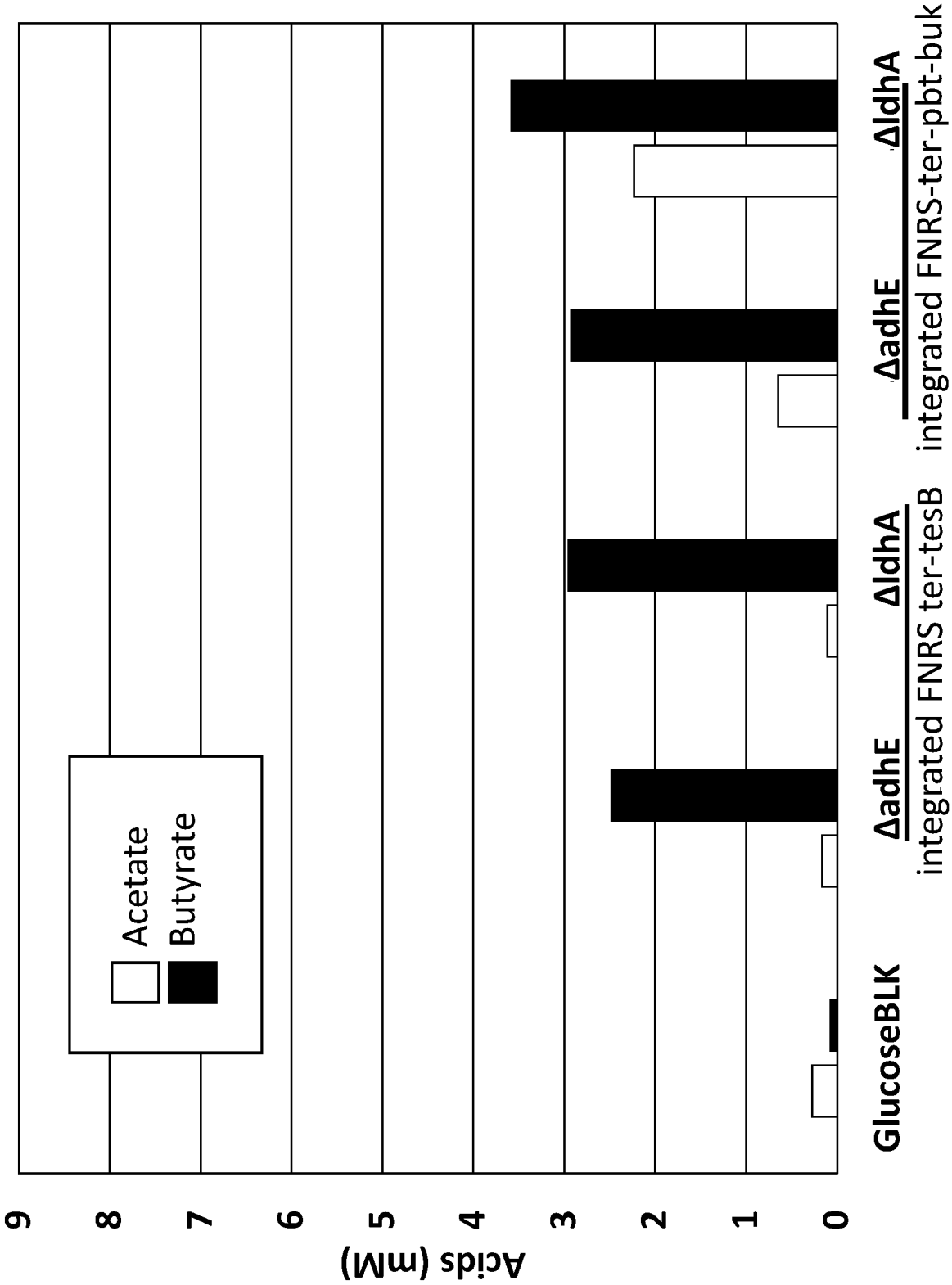

Acetate
Butyrate
Acids (mM)
0
1
2
3
4
5
6
7
8
9
GlucoseBLK
ΔadhE ΔldhA
integrated FNRS ter-tesB
ΔadhE ΔldhA
integrated FNRS-ter-pbt-buk

FIG. 86B

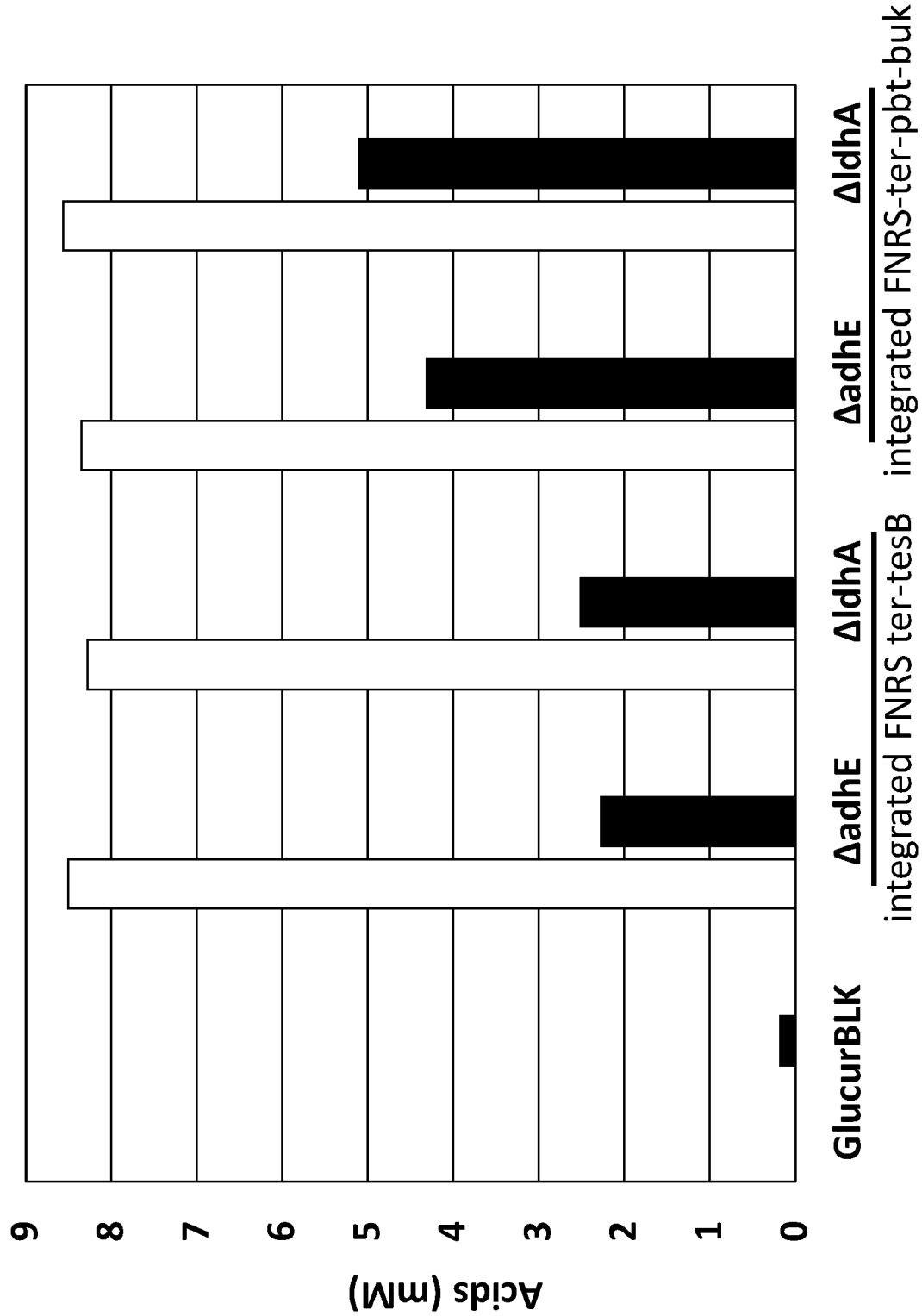

Acids (mM)
0
1
2
3
4
5
6
7
8
9
GlucurBLK
ΔadhE
ΔldhA
integrated FNRS ter-tesB
ΔadhE
ΔldhA
integrated FNRS-ter-pbt-buk
Acetate
Butyrate

FIG. 87

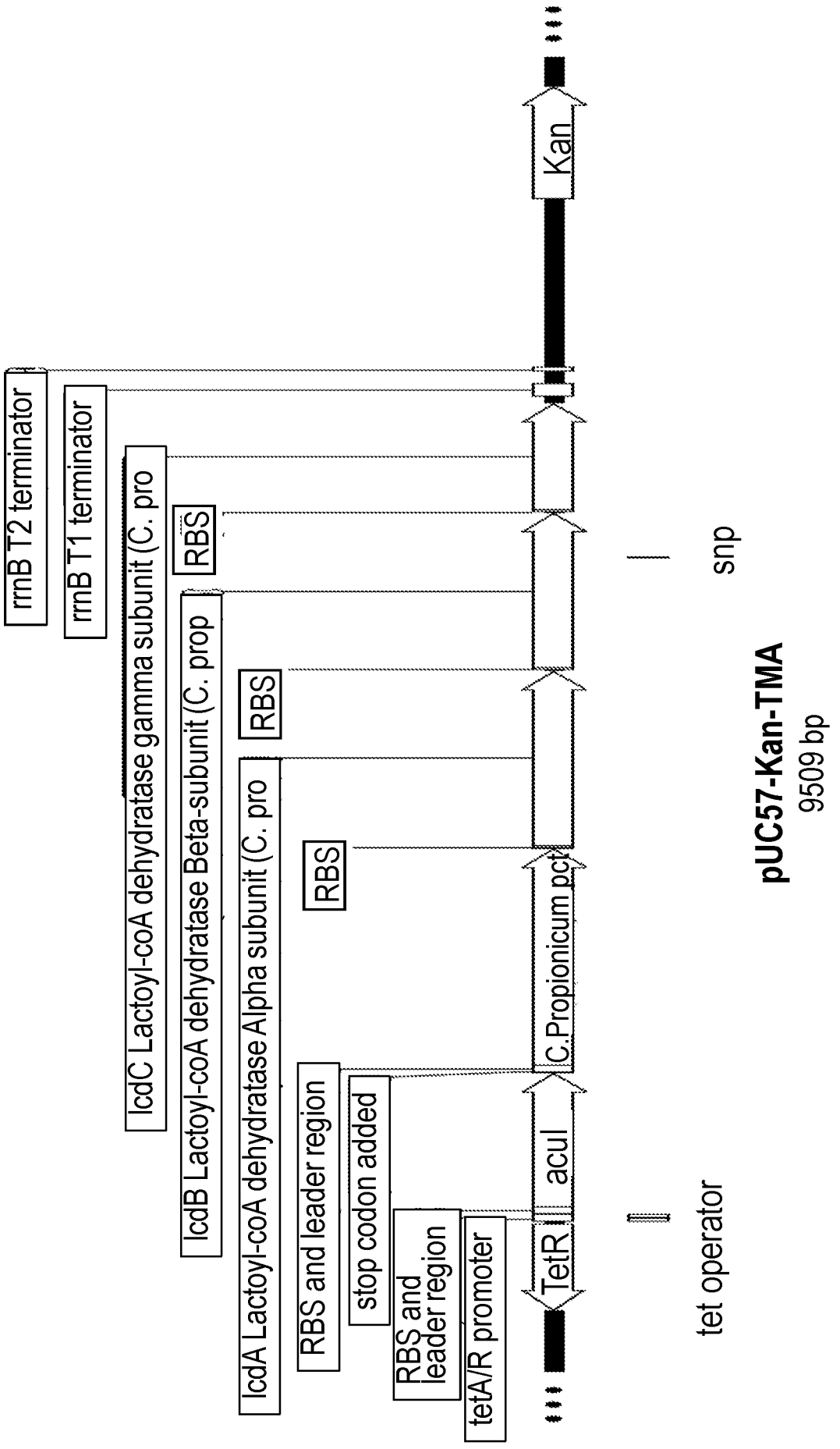
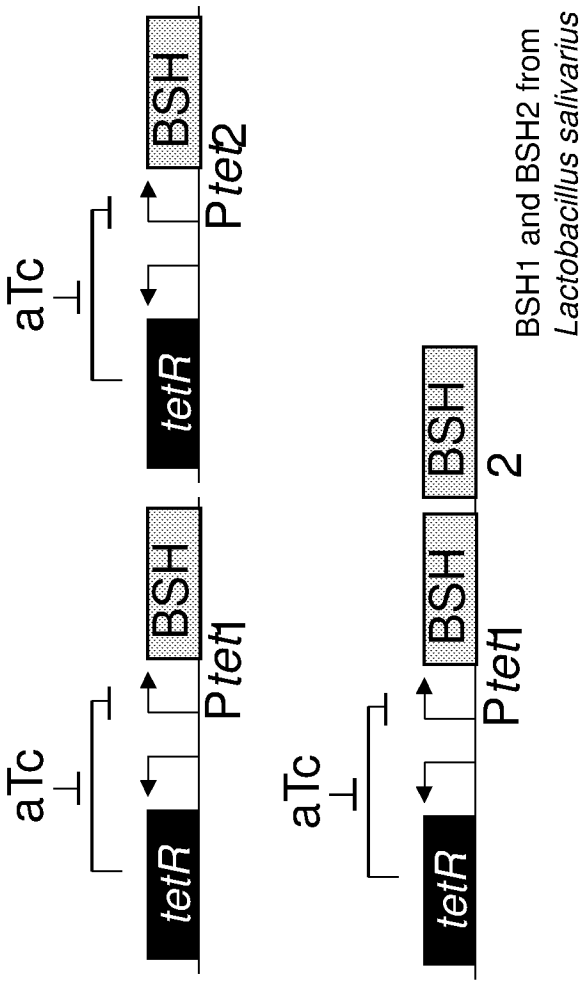

rrnB T2 terminator
rrnB T1 terminator
lcdC Lactoyl-coA dehydratase gamma subunit (C. pro
RBS
lcdB Lactoyl-coA dehydratase Beta-subunit (C. prop
RBS
lcdA Lactoyl-coA dehydratase Alpha subunit (C. pro
RBS
RBS and leader region
stop codon added
RBS and leader region
tetA/R promoter
TetR
acuI
C.Propionicum pct
Kan
snp
tet operator
pUC57-Kan-TMA
9509 bp

FIG. 88


aTc
tetR
BSH
Ptet1
aTc
tetR
BSH
Ptet2
aTc
tetR
BSH
BSH
Ptet1
2
BSH1 and BSH2 from
Lactobacillus salivarius

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/017563

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/00 A61K35/741
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	E.-H. ABOULNAGA ET AL: "Effect of an Oxygen-Tolerant Bifurcating Butyryl Coenzyme A Dehydrogenase/Electron-Transferring Flavoprotein Complex from Clostridium difficile on Butyrate Production in Escherichia coli", JOURNAL OF BACTERIOLOGY, vol. 195, no. 16, 14 June 2013 (2013-06-14), pages 3704-3713, XP055373221, US ISSN: 0021-9193, DOI: 10.1128/JB.00321-13 the whole document ----- -/--	1-41

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 2017	26/07/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brenz Verca, Stefano

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/017563

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-41(completely); 92-97(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BAEK J-M ET AL: "Butyrate production in engineered Escherichia coli with synthetic scaffolds", BIOTECHNOLOGY AND BIOENGINEERING OCTOBER 2013 JOHN WILEY AND SONS INC. USA,, vol. 110, no. 10, 1 October 2013 (2013-10-01), pages 2790-2794, XP002761845, DOI: 10.1002/BIT.24925 the whole document	1-41
A	FRANKLIN F. DUAN ET AL: "Engineered Commensal Bacteria Reprogram Intestinal Cells Into Glucose-Responsive Insulin-Secreting Cells for the Treatment of Diabetes", DIABETES, vol. 64, no. 5, 27 January 2015 (2015-01-27), pages 1794-1803, XP055356619, US ISSN: 0012-1797, DOI: 10.2337/db14-0635 the whole document	1,3-8
A,P	YIN LIN ET AL: "Oral Delivery of Pentameric Glucagon-Like Peptide-1 by Recombinant Lactobacillus in Diabetic Rats", PLOS ONE, vol. 11, no. 9, 9 September 2016 (2016-09-09), page e0162733, XP055356560, DOI: 10.1371/journal.pone.0162733 the whole document	1,3-8
A	CHEN ZHONGYI ET AL: "Incorporation of therapeutically modified bacteria into gut microbiota inhibits obesity", JOURNAL OF CLINICAL INVESTIGATION,, vol. 124, no. 8, 1 August 2014 (2014-08-01), pages 3391-3406, XP002761847, the whole document	1,3
A	HITOSHI ENDO ET AL: "Butyrate-Producing Probiotics Reduce Nonalcoholic Fatty Liver Disease Progression in Rats: New Insight into the Probiotics for the Gut-Liver Axis", PLOS ONE, vol. 8, no. 5, 16 May 2013 (2013-05-16), page e63388, XP055375486, DOI: 10.1371/journal.pone.0063388 cited in the application the whole document	1,13-16, 92-97

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JIN CHENG JUN ET AL: "Supplementation of sodium butyrate protects mice from the development of non-alcoholic steatohepatitis (NASH)", BRITISH JOURNAL OF NUTRITION, CAMBRIDGE UNIV. PRESS, UK, vol. 114, no. 11, 14 December 2015 (2015-12-14), pages 1745-1755, XP008180863, ISSN: 0007-1145, DOI: 10.1017/S0007114515003621 [retrieved on 2015-10-09] cited in the application the whole document	1,13-16, 92-97
A	YVONNE RITZE ET AL: "Effect of tryptophan supplementation on diet-induced non-alcoholic fatty liver disease in mice", BRITISH JOURNAL OF NUTRITION, vol. 112, no. 01, 8 April 2014 (2014-04-08), pages 1-7, XP055375951, UK ISSN: 0007-1145, DOI: 10.1017/S0007114514000440 the whole document	23
A	ALESSANDRA STACCHIOTTI ET AL: "Hepatic Macrosteatosis Is Partially Converted to Microsteatosis by Melatonin Supplementation in ob/ob Mice Non-Alcoholic Fatty Liver Disease", PLOS ONE, vol. 11, no. 1, 29 January 2016 (2016-01-29), page e0148115, XP055375962, DOI: 10.1371/journal.pone.0148115 the whole document	23
A	NI YE ET AL: "lpp deletion as a permeabilization method", BIOTECHNOLOGY AND BIOENGINEE, WILEY ETC, vol. 97, no. 6, 15 August 2007 (2007-08-15), pages 1347-1356, XP009190793, ISSN: 0006-3592, DOI: 10.1002/BIT.21375 the whole document	9-12

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Sabine Becker ET AL: "for Microbiology O 2 as the Regulatory Signal for FNR-Dependent Gene Regulation in Escherichia coli", JOURNAL OF BACTERIOLOGY, 1 August 1996 (1996-08-01), pages 4515-4521, XP055363883, Retrieved from the Internet: URL:http://jb.asm.org/content/178/15/4515.full.pdf#page=1&view=FitH [retrieved on 2017-04-11] the whole document -----	28-41

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-41(completely); 92-97(partially)

A bacterium comprising a gene or gene cassette for producing a gut barrier enhancer molecule and a gene or gene cassette for producing a satiety effector molecule, wherein the gene or gene cassettes are operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature; Pharmaceutically acceptable composition comprising said bacterium; Method of treating a liver disease, disorder or condition, or symptom(s) thereof in a subject in need thereof comprising the step of administering to a subject said composition.

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2. claims: 42-66(completely); 92-97(partially)

A bacterium comprising two or more gene or gene cassettes for producing two or more gut barrier enhancer effector molecule(s) and optionally at least one gene or gene cassette for producing a satiety effector molecule, wherein the gene or gene cassettes are operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature; Pharmaceutically acceptable composition comprising said bacterium; Method of treating a liver disease, disorder or condition, or symptom(s) thereof in a subject in need thereof comprising the step of administering to a subject said composition.

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3. claims: 67-91(completely); 92-97(partially)

A bacterium comprising a gene or gene cassette for producing a gut barrier enhancer effector molecule and a gene or gene cassette for producing bile salt hydrolase, wherein the gene or gene cassettes are operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature; Pharmaceutically acceptable composition comprising said bacterium; Method of treating a liver disease, disorder or condition, or symptom(s) thereof in a subject in need thereof comprising the step of administering to a subject said composition.
