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(71) **Applicant**: **SYNLOGIC, INC.** [US/US]; 130 Brookline Street #201, Cambridge, MA 02139 (US).

(72) **Inventors**: **MILLER, Paul, F.**; 39 Emerald Glen Lane, Salem, CT 06420 (US). **ISABELLA, Vincent, M.**; 465 Putnam Avenue, Unit 1, Cambridge, MA 02139 (US). **KOTULA, Jonathan, W.**; 345 Washington Street, Somerville, MA 02143 (US). **FALB, Dean**; 180 Lake Street, Sherborn, MA 01770 (US). **FISHER, Adam, B.**; 240 Sidney Street, #306, Cambridge, MA 02139 (US). **MILLET, Yves**; 15 Frederick Street, Newton, MA 02460 (US). **LI, Ning**; 322 Cambridge Street, Winchester, MA 01890 (US).

(74) **Agents**: **HANLEY, Elizabeth, A.** et al.; McCarter & English, LLP, 265 Franklin Street, Boston, MA 02110 (US).

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(54) **Title**: BACTERIA ENGINEERED TO TREAT DISEASES THAT BENEFIT FROM REDUCED GUT INFLAMMATION AND/OR TIGHTENED GUT MUCOSAL BARRIER

(57) **Abstract**: Genetically engineered bacteria, pharmaceutical compositions thereof, and methods of treating or preventing autoimmune disorders, inhibiting inflammatory mechanisms in the gut, and/or tightening gut mucosal barrier function are disclosed.

Bacteria Engineered to Treat Diseases that Benefit from Reduced Gut Inflammation and/or Tightened Gut Mucosal Barrier

RELATED APPLICATIONS

[01] This application is a continuation-in-part of PCT Application No. PCT/US2016/020530, filed March 2, 2016; PCT Application No. PCT/US2016/050836, filed September 8, 2016, and U.S. Application No. 15/260,319, filed September 8, 2016; and claims the benefit of U.S. Provisional Application No. 62/291,461 filed February 4, 2016; U.S. Provisional Application No. 62/291,468 filed February 4, 2016; U.S. Provisional Application No. 62/291,470 filed February 4, 2016; U.S. Provisional Application No. 62/347,508, filed June 8, 2016; U.S. Provisional Application No. 62/354,682, filed June 24, 2016; U.S. Provisional Application No. 62/362,954, filed July 15, 2016; U.S. Provisional Application No. 62/385,235, filed September 8, 2016; U.S. Provisional Application No. 62/423,170, filed November 16, 2016; U.S. Provisional Application No. 62/439,871, filed December 28, 2016; PCT Application No. PCT/US2016/032565, filed May 13, 2016; U.S. Provisional Application No. 62/347,576, filed June 8, 2016; U.S. Provisional Application No. 62/348,620, filed June 10, 2016; PCT Application No. PCT/US2016/039444, filed June 24, 2016; and PCT Application No. PCT/US2016/069052, filed December 28, 2016. The entire contents of each of the foregoing applications are expressly incorporated herein by reference in their entireties to provide continuity of disclosure.

BACKGROUND OF THE INVENTION

[02] This disclosure relates to compositions and therapeutic methods for inhibiting inflammatory mechanisms in the gut, restoring and tightening gut mucosal barrier function, and/or treating and preventing autoimmune disorders. In certain aspects, the disclosure relates to genetically engineered bacteria that are capable of reducing inflammation in the gut and/or enhancing gut barrier function. In some embodiments, the genetically engineered bacteria are capable of reducing gut inflammation and/or enhancing gut barrier function, thereby ameliorating or preventing an autoimmune disorder. In some aspects, the compositions and methods disclosed herein may be used for treating or preventing autoimmune disorders as well as diseases and conditions associated with gut

inflammation and/or compromised gut barrier function, e.g., diarrheal diseases, inflammatory bowel diseases, and related diseases.

[03] Inflammatory bowel diseases (IBDs) are a group of diseases characterized by significant local inflammation in the gastrointestinal tract typically driven by T cells and activated macrophages and by compromised function of the epithelial barrier that separates the luminal contents of the gut from the host circulatory system (Ghishan et al., 2014). IBD pathogenesis is linked to both genetic and environmental factors and may be caused by altered interactions between gut microbes and the intestinal immune system. Current approaches to treat IBD are focused on therapeutics that modulate the immune system and suppress inflammation. These therapies include steroids, such as prednisone, and tumor necrosis factor (TNF) inhibitors, such as Humira® (Cohen et al., 2014). Drawbacks from this approach are associated with systemic immunosuppression, which includes greater susceptibility to infectious disease and cancer.

[04] Other approaches have focused on treating compromised barrier function by supplying the short-chain fatty acid butyrate via enemas. Recently, several groups have demonstrated the importance of short-chain fatty acid production by commensal bacteria in regulating the immune system in the gut (Smith et al., 2013), showing that butyrate plays a direct role in inducing the differentiation of regulatory T cells and suppressing immune responses associated with inflammation in IBD (Atarashi et al., 2011; Furusawa et al., 2013). Butyrate is normally produced by microbial fermentation of dietary fiber and plays a central role in maintaining colonic epithelial cell homeostasis and barrier function (Hamer et al., 2008). Studies with butyrate enemas have shown some benefit to patients, but this treatment is not practical for long term therapy. More recently, patients with IBD have been treated with fecal transfer from healthy patients with some success (Ianiro et al., 2014). This success illustrates the central role that gut microbes play in disease pathology and suggests that certain microbial functions are associated with ameliorating the IBD disease process. However, this approach raises safety concerns over the transmission of infectious disease from the donor to the recipient. Moreover, the nature of this treatment has a negative stigma and thus is unlikely to be widely accepted.

[05] Compromised gut barrier function also plays a central role in autoimmune diseases pathogenesis (Lerner et al., 2015a; Lerner et al., 2015b; Fasano et al., 2005; Fasano, 2012). A single layer of epithelial cells separates the gut lumen from the immune

cells in the body. The epithelium is regulated by intercellular tight junctions and controls the equilibrium between tolerance and immunity to nonself-antigens (Fasano et al., 2005). Disrupting the epithelial layer can lead to pathological exposure of the highly immunoreactive subepithelium to the vast number of foreign antigens in the lumen (Lerner et al., 2015a) resulting in increased susceptibility to and both intestinal and extraintestinal autoimmune disorders can occur" (Fasano et al., 2005). Some foreign antigens are postulated to resemble self-antigens and can induce epitope-specific cross-reactivity that accelerates the progression of a pre-existing autoimmune disease or initiates an autoimmune disease (Fasano, 2012). Rheumatoid arthritis and celiac disease, for example, are autoimmune disorders that are thought to involve increased intestinal permeability (Lerner et al., 2015b). In individuals who are genetically susceptible to autoimmune disorders, dysregulation of intercellular tight junctions can lead to disease onset (Fasano, 2012). In fact, the loss of protective function of mucosal barriers that interact with the environment is necessary for autoimmunity to develop (Lerner et al., 2015a).

[06] Changes in gut microbes can alter the host immune response (Paun et al., 2015; Sanz et al., 2014; Sanz et al., 2015; Wen et al., 2008). For example, in children with high genetic risk for type 1 diabetes, there are significant differences in the gut microbiome between children who develop autoimmunity for the disease and those who remain healthy (Richardson et al., 2015). Others have shown that gut bacteria are a potential therapeutic target in the prevention of asthma and exhibit strong immunomodulatory capacity… in lung inflammation (Arrieta et al., 2015). Thus, enhancing barrier function and reducing inflammation in the gastrointestinal tract are potential therapeutic mechanisms for the treatment or prevention of autoimmune disorders.

[07] Recently there has been an effort to engineer microbes that produce anti-inflammatory molecules, such as IL-10, and administer them orally to a patient in order to deliver the therapeutic directly to the site of inflammation in the gut. The advantage of this approach is that it avoids systemic administration of immunosuppressive drugs and delivers the therapeutic directly to the gastrointestinal tract. However, while these engineered microbes have shown efficacy in some pre-clinical models, efficacy in patients has not been observed. One reason for the lack of success in treating patients is that the viability and stability of the microbes are compromised due to the constitutive production of large amounts of non-native proteins, e.g., human interleukin. Thus, there remains a

great need for additional therapies to reduce gut inflammation, enhance gut barrier function, and/or treat autoimmune disorders, and that avoid undesirable side effects.

Summary

[08] The genetically engineered bacteria disclosed herein are capable of producing therapeutic anti-inflammation and/or gut barrier enhancer molecules. In some embodiments, the genetically engineered bacteria are functionally silent until they reach an inducing environment, e.g., a mammalian gut, wherein expression of the therapeutic molecule is induced. In certain embodiments, the genetically engineered bacteria are naturally non-pathogenic and may be introduced into the gut in order to reduce gut inflammation and/or enhance gut barrier function and may thereby further ameliorate or prevent an autoimmune disorder. In certain embodiments, the anti-inflammation and/or gut barrier enhancer molecule is stably produced by the genetically engineered bacteria, and/or the genetically engineered bacteria are stably maintained in vivo and/or in vitro. The invention also provides pharmaceutical compositions comprising the genetically engineered bacteria, and methods of treating diseases that benefit from reduced gut inflammation and/or tightened gut mucosal barrier function, e.g., an inflammatory bowel disease or an autoimmune disorder.

[09] In some embodiments, the genetically engineered bacteria of the invention produce one or more therapeutic molecule(s) under the control of one or more promoters induced by an environmental condition, e.g., an environmental condition found in the mammalian gut, such as an inflammatory condition or a low oxygen condition. In on-limiting exemplary embodiments, the genetically engineered bacteria produce one or more therapeutic molecule(s) 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. In some embodiments, the therapeutic molecule is butyrate; in an inducing environment, the butyrate biosynthetic gene cassette is activated, and butyrate is produced. 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 produce their therapeutic effect only in inducing environments such as the gut, thereby lowering the safety issues associated with systemic exposure.

[010] Disclosed herein is a butyrate-producing bacterium comprising at least one gene or gene cassette encoding one or more non-native biosynthetic pathways for producing butyrate, wherein the bacteria produces acetyl CoA and wherein the bacterium has at least one mutation in or deletion of an endogenous pta gene. Such bacterium is capable of producing butyrate, but does not produce acetate. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous ldhA gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous frd gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene and an endogenous ldhA gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene and an endogenous frd gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous ldhA gene and an endogenous frd gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene, an endogenous frd gene, and an endogenous ldhA gene. In certain specific embodiments, the butyrate-producing bacterium comprises at least one gene or gene cassette encoding one or more non-native biosynthetic pathways for producing butyrate, wherein the bacteria produces acetyl CoA and wherein the bacterium has at least one mutation in or deletion of an endogenous pta gene and at least one mutation in or deletion of an endogenous gene selected from adhE gene and/or ldhA gene and/or frd gene.

[011] In any of the above described embodiments of butyrate-producing bacteria, the at least one gene or gene cassette for producing butyrate is operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature. In any of the above described embodiments of butyrate-producing bacteria, the at least one gene or gene cassette for producing butyrate is operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature and is induced by exogenous environmental conditions found in a mammalian gut.

[012] In some embodiments, the butyrate-producing bacterium may produce an increased level of butyrate as compared to a bacterium which produces butyrate naturally or which comprises a gene or gene cassette for producing butyrate, but does not comprise

at least one mutation in or deletion of an endogenous ldhA gene. In some embodiments, the butyrate-producing bacterium may produce an increased level of butyrate as compared to a bacterium which produces butyrate naturally or which comprises a gene or gene cassette for producing butyrate, but does not comprise at least one mutation in or deletion of an endogenous adhE gene. In some embodiments, the butyrate-producing bacterium may produce an increased level of butyrate as compared to a bacterium which produces butyrate naturally or which comprises a gene or gene cassette for producing butyrate, but does not comprise at least one mutation in or deletion of an endogenous frd gene. In some embodiments, the butyrate-producing bacterium may produce an increased level of butyrate as compared to a bacterium which produces butyrate naturally or which comprises a gene or gene cassette for producing butyrate, but does not comprise at least one mutation in or deletion of an endogenous pta gene. In some embodiments, the butyrate-producing bacterium may produce an increased level of butyrate as compared to a bacterium which produces butyrate naturally or which comprises a gene or gene cassette for producing butyrate, but does not comprise at least one mutation in or deletion of an endogenous gene selected from frd and/or ldhA and/or adhE and/or pta. In some embodiments, the butyrate-producing bacterium may produce an increased level of butyrate as compared to a bacterium which produces butyrate naturally or which comprises a gene or gene cassette for producing butyrate, but does not comprise at least one mutation in or deletion of an endogenous ldhA gene, frd gene, adhE gene, and pta gene.

[013] In some embodiments, the bacterium described above comprises an endogenous pta gene and produces acetate. In these embodiments, the bacterium comprises at least one gene or gene cassette encoding one or more non-native biosynthetic pathways for producing butyrate, wherein the bacteria produces acetyl CoA and wherein the bacterium has an endogenous pta gene. Such bacterium is capable of producing butyrate and acetate. In some embodiments of this bacterium, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous ldhA gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous frd gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene and an endogenous ldhA gene. In

some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene and an endogenous frd gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous ldhA gene and an endogenous frd gene. In some embodiments, the bacterium further has at least one mutation in or deletion of an endogenous adhE gene, an endogenous frd gene, and an endogenous ldhA gene. In certain specific embodiments, the butyrate-producing bacterium comprises at least one gene or gene cassette encoding one or more non-native biosynthetic pathways for producing butyrate, wherein the bacteria produces acetyl CoA and wherein the bacterium has an endogenous pta gene and at least one mutation in or deletion of an endogenous gene selected from adhE gene and/or ldhA gene and/or frd gene.

[014] In any of the above-described embodiments of butyrate-producing bacterium, the at least one gene or gene cassette for producing butyrate may comprise ter, thiA1, hbd, crt2, pbt, and buk genes. In any of the above-described embodiments of butyrate-producing bacterium, the at least one gene or gene cassette for producing butyrate may comprise ter, thiA1, hbd, crt2, and tesB genes.

[015] In any of the above described embodiments of butyrate- and acetate-producing bacteria, the at least one gene or gene cassette for producing butyrate is operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature. In any of the above described embodiments of butyrate- and acetate-producing bacteria, the at least one gene or gene cassette for producing butyrate is operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature and is induced by exogenous environmental conditions found in a mammalian gut.

[016] In another aspect, disclosed herein is an acetate-producing bacterium that produces acetate but not butyrate. In any of these embodiments, the acetate-producing bacterium produces acetyl CoA and comprises a wild-type pta gene. In some embodiments, the acetate-producing bacterium comprises at least one mutation in or deletion of a ldhA gene. In some embodiments, the acetate-producing bacterium comprises at least one mutation in or deletion of an adhE gene. In some embodiments, the acetate-producing bacterium comprises at least one mutation in or deletion of a frd gene. In some embodiments, the acetate-producing bacterium comprises at least one mutation in

or deletion of an ldhA gene and at least one mutation in or deletion of an adhE gene. In some embodiments, the acetate-producing bacterium comprises at least one mutation in or deletion of a ldhA gene and at least one mutation in or deletion of an frd gene. In some embodiments, the acetate-producing bacterium comprises at least one mutation in or deletion of an adhA gene and at least one mutation in or deletion of an frd gene. In some embodiments, the acetate-producing bacterium comprises at least one mutation in or deletion of an adhA gene, at least one mutation in or deletion of an frd gene, and at least one mutation in or deletion of an ldhA gene.

[017] The bacterium may produce an increased level of acetate as compared to a bacterium which produces Acetyl CoA and comprises an endogenous pta gene, and has an endogenous frd gene and/or endogenous ldhA gene and/or endogenous adhA gene. The bacterium may produce an increased level of acetate as compared to a bacterium which produces Acetyl CoA and comprises an endogenous pta gene, and does not comprise at least one mutation in or deletion of an ldhA gene, an adhE gene, and/or a frd gene.

[018] In any of the above-described embodiments comprising a gene or gene cassette for producing butyrate in which the gene or gene cassette is operably linked to a directly or indirectly inducible promoter, the promoter may be induced under low-oxygen or anaerobic conditions. In some embodiments, the promoter is selected from an FNR-responsive promoter, an ANR-responsive promoter, and a DNR-responsive promoter. In some embodiments, the promoter is an FNR-responsive promoter. In some embodiments, the promoter may be induced by the presence of reactive nitrogen species. In some embodiments, the promoter is selected from an NsrR-responsive promoter, NorR-responsive promoter, and a DNR-responsive promoter. In some embodiments, the promoter may be induced by the presence of reactive oxygen species. In some embodiments, the promoter is selected from an OxyR-responsive promoter, PerR-responsive promoter, OhrR-responsive promoter, SoxR-responsive promoter, or a RosR-responsive promoter.

[019] In some embodiments, the gene and/or gene cassette is located on a chromosome in the bacterium. In some embodiments, the at least one gene and/or gene cassette is located on a plasmid in the bacterium.

[020] In some embodiments, the bacterium is a probiotic bacterium. In some embodiments, the bacterium is selected from the group consisting of *Bacteroides,*

Bifidobacterium, Clostridium, Escherichia, Lactobacillus, and *Lactococcus*. In some embodiments, thebacterium is *Escherichia coli* strain Nissle.

[021] In some embodiments, the bacterium is an an auxotroph in a gene that is complemented when the bacterium is present in a mammalian gut. The bacterium may be an auxotroph in diaminopimelic acid or an enzyme in the thymine biosynthetic pathway.

[022] Disclosed herein is a pharmaceutically acceptable composition comprising one or more of any of the bacterium disclosed herein; and a pharmaceutically acceptable carrier. In some embodiments, the composition is formulated for oral or rectal administration.

[023] Disclosed herein is a method of treating or preventing an autoimmune disorder, comprising the step of administering to a patient in need thereof, a composition disclosed herein.

[024] Disclosed herein is a method of treating a disease or condition associated with gut inflammation and/or compromised gut barrier function comprising the step of administering to a patient in need thereof, a composition.

[025] The autoimmune disorder may be selected from the group consisting of acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, Addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune oophoritis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroid disease, autoimmune urticarial, Axonal & neuronal neuropathies, Balo disease, Behcet's disease, Bullous pemphigoid, Cardiomyopathy, Castleman disease, Celiac disease, Chagas disease, Chronic inflammatory demyelinating polyneuropathy (CIDP), Chronic recurrent multifocal ostomyelitis (CRMO), Churg-Strauss syndrome, Cicatricial pemphigoid/benign mucosal pemphigoid, Crohn's disease, Cogan syndrome, Cold agglutinin disease, Congenital heart block, Coxsackie myocarditis, CREST disease, Essential mixed cryoglobulinemia, Demyelinating neuropathies, Dermatitis herpetiformis, Dermatomyositis, Devic's disease (neuromyelitis optica), Discoid lupus, Dressler's syndrome, Endometriosis, Eosinophilic

esophagitis, Eosinophilic fasciitis, Erythema nodosum, Experimental allergic encephalomyelitis, Evans syndrome, Fibrosing alveolitis, Giant cell arteritis (temporal arteritis), Giant cell myocarditis, Glomerulonephritis, Goodpasture's syndrome, Granulomatosis with Polyangiitis (GPA), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, Hemolytic anemia, Henoch-Schonlein purpura, Herpes gestationis, Hypogammaglobulinemia, Idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, Immunoregulatory lipoproteins, Inclusion body myositis, Interstitial cystitis, Juvenile arthritis, Juvenile idiopathic arthritis, Juvenile myositis, Kawasaki syndrome, Lambert-Eaton syndrome, Leukocytoclastic vasculitis, Lichen planus, Lichen sclerosus, Ligneous conjunctivitis, Linear IgA disease (LAD), Lupus (Systemic Lupus Erythematosus), chronic Lyme disease, Meniere's disease, Microscopic polyangiitis, Mixed connective tissue disease (MCTD), Mooren's ulcer, Mucha-Habermann disease, Multiple sclerosis, Myasthenia gravis, Myositis, Narcolepsy, Neuromyelitis optica (Devic's), Neutropenia, Ocular cicatricial pemphigoid, Optic neuritis, Palindromic rheumatism, PANDAS (Pediatric autoimmune Neuropsychiatric Disorders Associated with Streptococcus), Paraneoplastic cerebellar degeneration, Paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, Pars planitis (peripheral uveitis), Pemphigus, Peripheral neuropathy, Perivenous encephalomyelitis, Pernicious anemia, POEMS syndrome, Polyarteritis nodosa, Type I, II, & III autoimmune polyglandular syndromes, Polymyalgia rheumatic, Polymyositis, Postmyocardial infarction syndrome, Postpericardiotomy syndrome, Progesterone dermatitis, Primary biliary cirrhosis, Primary sclerosing cholangitis, Psoriasis, Psoriatic arthritis, Idiopathic pulmonary fibrosis, Pyoderma gangrenosum, Pure red cell aplasia, Raynauds phenomenon, reactive arthritis, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis, scleroderma, Sjogren's syndrome, sperm & testicular autoimmunity, stiff person syndrome, subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, temporal arteritis/giant cell arteritis, thrombocytopenic purpura (TTP), Tolosa-Hunt syndrome, transverse myelitis, type 1 diabetes, asthma, ulcerative colitis, undifferentiated connective tissue disease

(UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, and Wegener's granulomatosis.

[026] The autoimmune disorder may be selected from the group consisting of type 1 diabetes, lupus, rheumatoid arthritis, ulcerative colitis, juvenile arthritis, psoriasis, psoriatic arthritis, celiac disease, and ankylosing spondylitis.

[027] The disease or condition may be selected from an inflammatory bowel disease, including Crohn's disease and ulcerative colitis, and a diarrheal disease.

Brief Description of the Figures

[028] **FIG. 1A, FIG. 1B, FIG 1C, FIG. 1D, FIG. 1E, FIG. 1F, FIG. 1G, FIG. 1H, FIG. 1I, FIG. 1J, and FIG. 1K** depict schematics of *E. coli* that are genetically engineered to express a propionate biosynthesis cassette (**FIG. 1A**), a butyrate biosynthesis cassette (**FIG. 1B**), an acetate biosynthesis cassette (**FIG. 1C**), a cassette for the expression of GLP-2 (**FIG. 1D**), a cassette for the expression of human IL-10 (**FIG. 1E**) or v-IL-22 or hIL-22 (**FIG. 1F**) under the control of a FNR-responsive promoter. The genetically engineered E. coli depicted in FIG. 1D, FIG. 1E, and FIG. 1F may further comprise a secretion system for secretion of the expressed polypeptide out of the cell. **FIG. 1G**depicts bacteria overexpressing butyrate (and not expressing acetate) by expressing a butyrate biosynthesis cassette in combination with deletions in adhE and pta (**FIG. 1G**), **FIG. 1H** depicts bacteria overexpressing butyrate by expressing a butyrate biosynthesis cassette in combination with deletions in ldhA, **FIG. 1I** depicts bacteria overexpressing butyrate by expressing a butyrate biosynthesis cassette in combination with deletions in adhE and frdA (**FIG. 1I**). **FIG. 1J** depicts bacteria overexpressing acetate by deletion in ldhA. **FIG. 1K** depicts bacteria overexpressing GLP-2 in combination with a deletion in adhE and pta.

[029] **FIG. 2A, FIG. 2B, FIG. 2C, and FIG.2D** depict schematics of a butyrate production pathway and schematics of different butyrate producing circuits. **FIG. 2A** depicts a metabolic pathway for butyrate production. **FIG. 2B** and **FIG. 2C** depict schematics of two different exemplary butyrate producing circuits, both under the control of a tetracycline inducible promoter. **FIG. 2B** depicts 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. **FIG. 2C** depicts 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**. FIG. 2D** depicts a schematic of a third exemplary butyrate gene cassette under the control of a tetracycline inducible promoter, specifically, 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 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.

[030] **FIG. 3A, FIG. 3B, FIG. 3C, FIG. 3D, FIG. 3E, and FIG. 3F** depict schematics of the gene organization of exemplary bacteria of the disclosure. **FIG. 3A** and **FIG. 3B** depict the gene organization of an exemplary engineered bacterium of the invention and its induction of butyrate production under low-oxygen conditions. **FIG. 3A** depicts relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (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. 3B** depicts increased butyrate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate. **FIG. 3C** and **FIG. 3D** depict the gene organization of an exemplary recombinant bacterium of the invention and its derepression in the presence of nitric oxide (NO). In **FIG. 3C**, in the absence of NO, the NsrR transcription factor (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) is expressed. In **FIG. 3D**, 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 black arrows and black squiggles) and ultimately to the production of butyrate.

[031] **FIG. 3E and FIG. 3F** depict the gene organization of an exemplary recombinant bacterium of the invention and its induction in the presence of H2O2. In **FIG. 3E**, in the absence of H2O2, the OxyR transcription factor (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) is expressed. In **FIG. 3F**, 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 black arrows and black squiggles) and ultimately to the production of butyrate.

[032] **FIG. 4A, FIG. 4B, FIG. 4C, FIG. 4D, FIG. 4E, and FIG. 4F** depict schematics of the gene organization of exemplary bacteria of the disclosure. **FIG. 4A** and **FIG. 4B** 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. 3A, FIG 3B, FIG. 3C, FIG. 3D, FIG. 3E, and FIG. 3F**. **FIG. 4A** depicts relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (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. 4B** depicts increased butyrate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate. **FIG. 4C** and **FIG. 4D** depict the gene organization of another exemplary recombinant bacterium of the invention and its derepression in the presence of NO. In **FIG. 4C**, in the absence of NO, the NsrR transcription factor (circle, "NsrR") binds to and represses a corresponding regulatory region. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2, pbt, buk*) 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 black arrows and black squiggles) and ultimately to the production of butyrate. **FIG. 4E** and **FIG. 4F** depict the gene organization of another exemplary recombinant bacterium of the invention and its induction in the presence of H_2O_2. In **FIG. 4E**, in the absence of H_2O_2, the OxyR transcription factor (circle, "OxyR") binds to, but does not induce, the *oxyS* promoter. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2, pbt, buk*) is expressed. In **FIG. 4F**, 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 black arrows and black squiggles) and ultimately to the production of butyrate.

[033] **FIG. 5A, FIG. 5B, FIG. 5C, FIG. 5D, FIG. 5E, and FIG. 5F** depict schematics of the gene organization of exemplary bacteria of the disclosure. **FIG. 5A** and **FIG. 5B** depict the gene organization of an exemplary recombinant bacterium of the invention and its induction under low-oxygen conditions. **FIG. 5A** depicts relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (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*) is expressed. **FIG. 5B** depicts increased butyrate production under low-oxygen conditions due to FNR dimerizing (two boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate. **FIG. 5C** and **FIG. 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 ("NsrR") binds to and represses a corresponding regulatory region. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2, tesB*) 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 black arrows and black squiggles) and ultimately to the production of butyrate. **FIG. 5E** and **FIG. 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 (circle, "OxyR")

binds to, but does not induce, the *oxyS* promoter. Therefore, none of the butyrate biosynthesis enzymes (*ter, thiA1, hbd, crt2, tesB*) 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 black arrows and black squiggles) and ultimately to the production of butyrate.

[034] **FIG. 6A** and **FIG. 6B** depict schematics of the gene organization of exemplary bacteria of the disclosure for inducible propionate production. **FIG. 6A** depicts relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (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*) is expressed. **FIG. 6B** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two 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. 3C-3F, FIG. 4C-4F, FIG. 5C-5F**.

[035] **FIG. 7** depicts an exemplary propionate biosynthesis gene cassette.

[036] **FIG. 8A, FIG. 8B, and FIG. 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 (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*) is expressed. **FIG. 8B** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two 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. 3C-3F, FIG. 4C-4F, FIG. 5C-5F**.

[037] **FIG. 9A** and **FIG. 9B** depict schematics of the gene organization of exemplary bacteria of the disclosure for inducible propionate production. **FIG. 9A** depicts relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (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*) is expressed. **FIG. 9B** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two 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. 3C-3F, FIG. 4C-4F, FIG. 5C-5F**.

[038] **FIG. 10A, FIG. 10B,** and **FIG. 10C** depict schematics of the sleeping beauty pathway and the gene organization of an exemplary bacterium of the disclosure. **FIG. 10A** 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. **FIG. 10B** and **FIG. 10C** 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. 10B** depicts relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*sbm, ygfD, ygfG, ygfH*) is expressed. **FIG. 10C** depicts increased propionate production under low-oxygen or anaerobic conditions due to FNR dimerizing (two 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. 3C-3F, FIG. 4C-4F, FIG. 5C-5F**.

[039] **FIG. 11** depicts a bar graph showing butyrate production of butyrate producing strains of the disclosure. **FIG. 11** 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.

[040] **FIG. 12** depicts a bar graph showing butyrate production of butyrate producing strains of the disclosure. **FIG. 12** 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.

[041] **FIG. 13** 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.

[042] **FIG. 14A, FIG. 14B, FIG.14C,** and **FIG. 14D** depict schematics and graphs showing butyrate or biomarker production of a butyrate producing circuit under the control of an FNR promoter. **FIG. 14A** depicts a schematic showing a butyrate producing circuit under the control of an FNR promoter. **FIG. 14B** 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-501 led to significant butyrate production under anaerobic conditions. **FIG. 14C** depicts SYN-501 in the presence and absence of glucose and oxygen *in vitro*. SYN-501 comprises pSC101 PydfZ-ter butyrate plasmid; SYN-500 comprises pSC101 PydfZ-bcd butyrate plasmid; SYN-506 comprises pSC101 nirB-bcd butyrate plasmid. **FIG. 14D** depict levels of mouse lipocalin 2 (left) and calprotectin (right) quantified by ELISA using the fecal samples in an *in vivo* model. SYN-501 reduces inflammation and/or protects gut barrier function as compared to wild type Nissle control.

[043] **FIG. 15** depicts a graph measuring gut-barrier function in dextran sodium sulfate (DSS)-induced mouse models of IBD. The amount of FITC dextran found in the plasma of mice administered different concentrations of DSS was measured as an indicator of gut barrier function.

[044] **FIG. 16** depicts serum levels of FITC-dextran analyzed by spectrophotometry. FITC-dextran is a readout for gut barrier function in the DSS-induced mouse model of IBD.

[045] **FIG. 17** 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.

[046] **FIG. 18** 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.

[047] **FIG. 19A** 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.

[048] **FIG. 19B** and **FIG. 19C** 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. 19B)** and nonlinear fit **(FIG. 19C)** graphical formats.

[049] **FIG. 20A** and **FIG. 20B** depicts the construction and gene organization of an exemplary plasmids. **FIG. 20A** depicts the construction and gene organization of an exemplary plasmids comprising a gene encoding NsrR, a regulatory sequence from *norB,* and a butyrogenic gene cassette (pLogic031-nsrR-norB-butyrate construct). **FIG. 20B** depicts the construction and gene organization of another exemplary plasmid comprising a gene encoding NsrR, a regulatory sequence from *norB,* and a butyrogenic gene cassette (pLogic046- nsrR-norB-butyrogenic gene cassette).

[050] **FIG. 21** 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).

[051] **FIG. 22** depicts butyrate production by genetically engineered Nissle comprising the pLogic031-nsrR-norB-butyrate construct (SYN133) or the pLogic046-nsrR-norB-butyrate construct (SYN145), which produce more butyrate as compared to wild-type Nissle (SYN001).

[052] **FIG. 23** depicts the construction and gene organization of an exemplary plasmid comprising an *oxyS* promoter and butyrogenic gene cassette (pLogic031-oxyS-butyrogenic gene cassette).

[053] **FIG. 24** depicts the construction and gene organization of another exemplary plasmid comprising an *oxyS* promoter and butyrogenic gene cassette (pLogic046-oxyS- butyrogenic gene cassette).

[054] **FIG. 25** 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.

[055] **FIG. 26A and FIG. 26B** depict line graphs showing acetate production over a 6 hour time course post-induction in 0.5% glucose MOPS (pH6.8) (**FIG. 26A**) and in 0.5% glucuronic acid MOPS (pH6.3) (**FIG. 26B**). 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).

[056] **FIG. 26C** and **FIG. 26D** depict bar graphs showing acetate and butyrate production in 0.5% glucose MOPS (pH6.8) (**FIG. 26C**) and acetate and butyrate production in 0.5% glucuronic acid MOPS (pH6.3) (**FIG. 26D**). 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.

[057] **FIG. 26E** 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).

[058] **FIG. 26F** 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.

[059] **FIG. 26G and FIG. 26H** depict line graphs showing the effect of supernatants from the engineered acetate-producing strain, SYN2001, on LPS-induced IFNγ secretion in primary human PBMC cells from donor 1 (D1) (**Fig. 26G**) and donor 2 (D2) (**FIG. 26H**).

[060] **FIG. 27** depicts a schematic of an exemplary propionate biosynthesis gene cassette.

[061] **FIG. 28** depicts a schematic of a construct comprising the sleeping beauty mutase operon from E. coli under the control of a heterologous FnrS promoter.

[062] **FIG. 29** depicts a bar graph of proprionate 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. 28**.

[063] **FIG. 30A, FIG. 30B**, and **FIG. 30C** depict schematics of the gene organization of exemplary circuits of the disclosure for the expression of therapeutic polypeptides, which are secreted using components of the flagellar type III secretion system. A therapeutic polypeptide of interest, such as, GLP-2, IL-10, and IL-22, is assembled behind a fliC-5'UTR, and is driven by the native fliC and/or fliD promoter (**FIG. 30A** and **FIG. 30B**) or a tet-inducible promoter (**FIG. 30C**). 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 can be used. 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. 30B** and **FIG. 30D**.

[064] **FIG. 31A and FIG. 31B** depict schematics of the gene organization of exemplary circuits of the disclosure for the expression of therapeutic polypeptides, 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. 31A**) or an inducible promoter, such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter, **FIG. 31B**), 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.

[065] **FIG. 32A, FIG. 32B, FIG. 32C, FIG. 32D, and FIG. 32E** depict schematics of non-limiting examples of constructs for the expression of GLP2 for bacterial secretion. **FIG. 32A** depicts a schematic of a human GLP2 construct inserted into the FliC locus, under the control of the native FliC promoter. **FIG. 32B** depicts a schematic of a human GLP2 construct, including the N terminal 20 amino acids of FliC, inserted into the FliC locus under the control of the native FliC promoter. **FIG. 32C** depicts a schematic of a human GLP2 construct, including the N-terminal 20 amino acids of FliC, inserted into the FliC locus under the control of a tet inducible promoter. **FIG. 32D** depicts a schematic of a human GLP2 construct with a N terminal OmpF secretion tag (sec-dependent secretion system) under the control of a tet inducible promoter. **FIG. 32E** depicts a schematic of a human GLP2 construct with a N terminal TorA secretion tag (tat secretion system) under the control of a tet inducible promoter.

[066] **FIG. 33A** and **FIG. 33B** depict line graphs of ELISA results. **FIG. 33A** 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. 33B** 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.

[067] **FIG. 33C** depicts a line graph showing SYN3001 (PhoA-IL-22 in *pal* mutant chassi), but not SYN3000 (*pal* mutant chassi) supernatant induces STAT3 activation.

[068] **FIG. 33D** depicts a line graph showing that anti IL-22 neutralizing antibody inhibits SYN3001-induced STAT3 activation (n=3).

[069] **FIG. 33E** depicts a Western blot analysis of bacterial supernatants from strain SYN2980 and SYN2982, using IL-10 antibody (IL-10 (D13A11) XP® Rabbit mAb #12163, Cell Signaling Technology). The secreted polypepetide has the same molecular weight as the standards, indicating that the signal sequence is cleaved from the native peptide.

[070] **FIG. 34** depicts a schematic of tryptophan metabolism along the kynurenine and the serotonin arms 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.

[071] **FIG. 35** depicts a schematic of 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. 35**. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes which produce one or more of the metabolites depicted in **FIG.**

35, including but not limited to, kynurenine, indole-3-aldehyde, indole-3-acetic acid, and/or indole-3 acetaldehyde.

[072] **FIG. 36A and FIG. 36B** depict schematics of indole metabolite mode of action (**FIG.36A**) and indole biosynthesis (**FIG. 36B**). **FIG.36A** 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). **FIG. 36B** depicts a schematic of the trypophan catabolic pathway/indole biosynthesis pathways. 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 **FIGs. 36A** and **36B**. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes which produce one or more of the metabolites depicted in **FIGs. 36A** and **36B**, including but not limited to, kynurenine, indole-3-aldehyde, indole-3-acetic acid, and/or indole-3 acetaldehyde.

[073] **FIG. 37A** and **FIG. 37B** depict diagrams of bacterial tryptophan metabolism pathways. **FIG. 37A** 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. 37B** 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 **FIGs. 37A** and **37B**. In certain embodiments, the genetically engineered bacteria comprise one or more gene cassettes which produce one or more of the metabolites depicted in **FIGs. 37A** and **37B**. 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 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.

[074] **FIG. 38** depicts a schematic of the E. coli tryptophan synthesis pathway. In Escherichia coli, tryptophan is biosynthesized from chorismate, the principal common precursor of the aromatic amino acids tryptophan, tyrosine and phenylalanine, as well as the essential compounds tetrahydrofolate, ubiquinone-8, menaquinone-8 and enterobactin (enterochelin), as shown in the superpathway of chorismate metabolism. Five genes

encode five enzymes that catalyze tryptophan biosynthesis from chorismate. The five genes trpE trpD trpC trpB trpA form a single transcription unit, the trp operon. A weak internal promoter also exists within the trpD structural gene that provides low, constitutive levels of mRNA.

[075] **FIG. 39** depicts one embodiment of the disclosure in which the E. coli TRP synthesis enzymes are expressed from a construct under the control of a tetracycline inducible system.

[076] **FIG. 40A, FIG. 40B, FIG. 40C,** and **FIG. 40D** 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. 40A** 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. 40B**, and/or **FIG. 40C**, and/or **FIG. 40D. FIG. 40B** 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. 40A** and/or described in the description of **FIG. 40A**. The

bacteria may optionally also comprise one or more gene sequence(s) depicted or described in **FIG. 40C**, and/or **FIG. 40D.** Optionally, trpR and/or the tnaA gene (encoding a tryptophanase converting tryptophan into indole) are deleted to further increase levels of tryptophan produced. **FIG. 40C** 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. 40A** and/or described in the description of **FIG. 40A**. The bacteria may optionally also comprise one or more gene sequence(s) depicted or described in **FIG. 40B**, and/or **FIG. 40D.** 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. 40D** 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. 40A** and/or described in the description of **FIG. 40A**. The bacteria may optionally also comprise one or more gene sequence(s) depicted or described in **FIG. 40B**, and/or **FIG. 40C.** 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.

[077] **FIG. 41A, FIG. 41B, FIG. 41D, FIG. 41D, FIG. 41E, FIG. 41F, FIG. 41G, and FIG. 41H** 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. 41A** 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. 40A** and/or and/or **FIG. 40B,** and/or **FIG. 40C,** and/or **FIG. 40D** 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. 41B** 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. 40A** and/or **FIG. 40B,** and/or **FIG. 40C,** and/or **FIG. 40D** 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. 41C** 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. 40A** and/or and/or **FIG. 40B,** and/or **FIG. 40C,** and/or **FIG. 40D** 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. 41D** 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. 40A** and/or and/or **FIG. 40B,** and/or **FIG. 40C,** and/or **FIG. 40D** 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. 41E** 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. 40A** and/or and/or **FIG. 40B,** and/or **FIG. 40C,** and/or **FIG. 40D** 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. 41F** 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. 40A** and/or and/or **FIG. 40B,** and/or **FIG. 40C,** and/or **FIG. 40D** 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. 41G** 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. 40A** and/or and/or **FIG. 40B,** and/or **FIG. 40C,** and/or **FIG. 40D** 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. 41H** 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. 41A, FIG. 41B, FIG. 41D, FIG. 41D, FIG. 41E, FIG. 41F, FIG. 41G and FIG. 41H** 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] **FIG. 42A, FIG. 42B, FIG. 42C, FIG. 42D,** and **FIG. 42E** 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. 42A**, the optional circuits for tryptophan production are as depicted and described in **FIG. 40A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D.** 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. 42B** the optional circuits for tryptophan production are as depicted and described in **FIG. 40A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D.** 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. 42C** the optional circuits for tryptophan production are as depicted and described in **FIG. 40A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D.** 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. 42D** the optional circuits for tryptophan production are as depicted and described in **FIG. 40A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D.** 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. 42E** the optional circuits for tryptophan production are as depicted and described in **FIG. 40A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D.** 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. 42A, FIG. 42B, FIG. 42C, FIG. 42D, and FIG. 42E** 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.

[079] In **FIG. 42F** the optional circuits for tryptophan production are as depicted and described in **FIG. 40A**. The strain optionally comprises additional circuits as depicted and/or described in **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D.** 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.

[080] **FIG. 43A, FIG. 43B, and FIG. 43C** 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. 43A** 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. 40A** and/or **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D**. Additionally, the strain comprises tdc (tryptophan decarboxylase, e.g., from *Catharanthus roseus* and/or Clostridium sporogenes), which converts tryptophan into tryptamine. **FIG. 43B** 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. 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. **FIG. 43C** 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. 40A** and/or **FIG. 40B** and/or **FIG. 40C** and/or **FIG. 40D**. Additionally, the strain comprises a circuit as described in **FIG. 48**, 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).

[081] **FIG. 44A and FIG. 44B** depict schematics showing exemplary engineering strategies which can be employed for tryptophan production. **FIG. 44A** 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. 44B** 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. 44B** are engineered into a bacterial strain. Alternatively, other gene products in this pathway may be mutated or overexpressed.

[082] **FIG.45A and FIG. 45B and FIG. 45C** depict bar graphs showing tryptophan production by various engineered bacterial strains. **FIG.45A** 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. 45B** 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. 45C** 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.

[083] **FIG. 46** 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.

[084] **FIG. 47** 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 **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.

[085] **FIG. 48** 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.*

[086] 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), 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.

[087] **FIG. 49** 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.

[088] **FIG. 50** 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-P_{tet}-trpE^{fbr}DCBA (pSC101), tetR-P_{tet}-aroG^{fbr} (p15A). SYN2340 comprises ΔtrpRΔtnaA, tetR-P_{tet}-trpE^{fbr}DCBA (pSC101), tetR-P_{tet}-aroG^{fbr}-tdc_{Cr} (p15A). SYN2794 comprises ΔtrpRΔtnaA, tetR-P_{tet}-trpE^{fbr}DCBA (pSC101), tetR-P_{tet}-aroG^{fbr}-tdc_{Cs} (p15A). Results indicate that Tdc_{Cs} from *Clostridium sporogenes* is more efficient the Tdc_{Cr} from *Catharanthus roseus* in tryptamine production and converts all the tryptophan produced into tryptamine.

[089] **FIG. 51A, FIG. 51B, FIG. 51C, FIG. 51D, FIG. 51E** depict schematics of non-limiting examples of genetically engineered bacteria of the disclosure which comprises one or more gene sequence(s) and/or gene cassette(s) as described herein.

[090] **FIG. 52** 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.

[091] **FIG. 53** depicts an exemplary schematic of the E. coli 1917 Nissle chromosome comprising multiple mechanisms of action (MoAs).

[092] **FIG. 54A and FIG. 54B** depict schematics of bacterial chromosomes, for example the *E. coli* Nissle 1917 Chromosome. For example, **FIG. 54A** depicts a schematic of an engineered bacterium comprising, a circuit for butyrate production, a circuit for propionate production, and a circuit for production of one or more interleukins relevant to IBD. **Fig. 54B** depicts a schematic of an engineered bacterium comprising three circuits, a circuit for butyrate production, a circuit for GLP-2 expression and and a circuit for production of one or more interleukins relevant to IBD.

[093] **FIG. 55** 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.

[094] **FIG. 56** 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.

[095] **FIG. 57** 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.

[096] **FIG. 58** 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, nlpl, generates a leaky phenotype. Combinations of mutations may synergistically enhance the leaky phenotype.

[097] **FIG. 59** 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).

[098] **FIGs. 60A- 60C** depict other non-limiting embodiments 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 ParaBAD promoter (P_{araBAD}), which induces expression of the Tet repressor (TetR) 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. 60A** 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. **FIG. 60B** depicts 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. 60C** 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 the Tet repressor (TetR) 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 either under the control of a constitutive promoter or an inducible promoter (e.g., AraC promoter) in this circuit.

[099] **FIG. 61** depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition 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.

[0100] **FIG. 62** depicts another non-limiting embodiment of the disclosure, where an exogenous environmental condition 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.

[0101] **FIG. 63** depicts another non-limiting embodiment of the disclosure, where an exogenous environmental condition 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.

[0102] **FIG. 64** depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and a first recombinase from an inducible promoter or inducible promoters. The recombinase then flips a second recombinase from an inverted orientation to an active conformation. The activated second recombinase flips the 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.

[0103] **FIG. 65** 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.

[0104] **FIG. 66** 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 the tables (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.

[0105] **FIGs. 67A-67C** depict a schematic representation of the *lacZ* gene under the control of an exemplary FNR promoter (P_{fnrS}) and corresponding graphical data. **FIGs. 67A** 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. 67B** depicts 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. 67C** depicts the growth of bacterial cell cultures expressing *lacZ* over time, both in the presence and absence of oxygen.

[0106] **FIGs. 68A-68D** depict bar graphs, schematic, and dot blot, respectively, showing the structure or activity of reporter constructs. **FIG. 68A** and **FIG. 68B** depict bar graphs of reporter constructs activity. **FIG. 68A** depicts a graph of an ATC-inducible reporter construct expression and **FIG. 68B** depicts a graph of a nitric oxide-inducible reporter construct expression. 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. 68C** depicts a schematic of the constructs. **FIG. 68D** 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.

[0107] **FIG. 69** 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 6 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.

[0108] **FIG. 70** 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.

[0109] **FIG. 71A and FIG. 71B** depict a schematic diagrams of a wild-type clbA construct (**FIG. 71A**) and a schematic diagram of a clbA knockout construct (**FIG. 71B**).

[0110] **FIG. 72** depicts a schematic of a design-build-test cycle. Steps are as follows: 1: Define the disease pathway; 2. Identify target metabolites; 3. Design genetic circuits; 4. Build synthetic biotic; 5. Activate circuit in vivo; 6. Characterize circuit activation kinetics; 7. Optimize in vitro productivity to disease threshold; 8. Test optimize circuit in animla disease model; 9. Assimilate into the microbiome; 10. Develop understanding of in vivo PK and dosing regimen.

[0111] **FIG. 73** depicts a schematic of non-limiting manufacturing processes for upstream and downstream production of the genetically engineered bacteria of the present disclosure. Step 1 depicts the parameters for starter culture 1 (SC1): loop full – glycerol stock, duration overnight, temperature 37° C, shaking at 250 rpm. Step 2 depicts the parameters for starter culture 2 (SC2): 1/100 dilution from SC1, duration 1.5 hours, temperature 37° C, shaking at 250 rpm. Step 3 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. Step 4 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. Step 5 depicts the parameters for vial fill/storage: 1-2 mL aliquots, -80° C.

[0112] **Fig. 74** depicts three bacterial strains which constitutively express red fluorescent protein (RFP). In strains 1-3, the *rfp* gene has been inserted into different sites within the bacterial chromosome, and results in varying degrees of brightness under fluorescent light. Unmodified *E. coli* Nissle (strain 4) is non-fluorescent.

[0113] **Fig. 75A** 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.

[0114] **Fig. 75B** 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.

[0115] **Fig. 76** depicts a one non-limiting embodiment of the disclosure, which comprises a plasmid stability system with a plasmid that produces both a short-lived anti-toxin and a long-lived toxin. When the cell loses the plasmid, the anti-toxin is no longer produced, and the toxin kills the cell. In one embodiment, the genetically engineered bacteria produce an equal amount of a Hok toxin and a short-lived Sok antitoxin. 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.

[0116] **Figs. 77A-77D** depict schematics of non-limiting examples of the gene organization of plasmids, which function as a component of a biosafety system (**Fig. 77A and Fig. 77B**), which also contains a chromosomal component (shown in **Fig. 77C and Fig. 77D**). The bosafety plasmid system vector comprises Kid Toxin and R6K minimal ori, dapA (**Fig. 77A**) and thyA (**Fig. 77B**) 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. 77C** and **Fig. 77D** depict schematics of the gene organization of the chromosomal component of a biosafety system. **Fig. 77C** 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. 77D** 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. 77A**), then the chromosomal constructs shown in **Fig. 77C** and **Fig. 77D** are knocked into the DapA locus. If the plasmid containing the functional ThyA is used (as shown in **Fig. 77B**), then the chromosomal constructs shown in **Fig. 77C** and **Fig. 77D** 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.

[0117] **Fig. 78** 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.

[0118] **Fig. 79** depicts the gene organization of exemplary construct comprising FNRS24Y driven by the arabinose inducible promoter and araC in reverse direction.

[0119] **Fig. 80A** 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 anti-cancer molecules or immune modulatory effectors (POI1) and a second set of one or more proteins of interest (POI2), *e.g.*, one or more transporter(s)/importer(s) and/or exporter(s), 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 effector molecules prior to administration. This can be done by pre-inducing the expression of these effectors 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 effectors or 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 effectors or proteins of interest. This allows more efficient growth and, in some cases, reduces the build-up of toxic metabolites.

[0120] 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 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*. 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.

[0121] **Fig. 80B** 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 **Fnr^{S24Y}** 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.

[0122] **Fig. 80C** 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 P_{fnrS}-POI constructs are maintained to allow for strong *in vivo* induction.

[0123] **Fig. 81** depicts the gene organization of an exemplary construct, *e.g.*, comprised in SYN-PKU401, comprising a cloned POI gene under the control of a Tet promoter sequence and a Tet repressor gene.

[0124] **Fig. 82** depicts the gene organization of an exemplary construct comprising LacI in reverse orientation, and a IPTG inducible promoter driving the expression of one or more POIs. 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.

[0125] 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 construct expressing a second POI, *e.g.*, a transporter, which can either be provided on a plasmid or is integrated into the bacterial chromosome at one or more locations. POI2 expression may be constitutive or driven by an inducible promoter, *e.g.*, low-oxygen, arabinose, or IPTG. In some embodiments, the construct is located on a plasmid, *e.g.*, a low or high copy plasmid. In some embodiments, the construct is employed in a biosafety system, such as the system shown in **Fig. 77A**, **Fig. 77B**, **Fig. 77C**, and **Fig. 77D**. In some embodiments, the construct is integrated into the genome at one or more locations described herein.

[0126] **Fig. 83A, Fig. 83B, and Fig. 83C** depict schematics of non-limiting examples of constructs for the expression of proteins of interest POI(s). **Fig 83A** 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, 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 a POI1 and/or a POI2 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 a POI2 construct, which can either be provided on a plasmid or is integrated into the bacterial chromosome at one or more locations. POI2 expression may be constitutive or driven by an inducible promoter, *e.g.*, low-oxygen, arabinose, rhamnose,

or temperature sensitive. In some embodiments, the construct is used in combination with a POI3 expression construct.

[0127] 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).

[0128] **Fig. 84A** 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.

[0129] **Fig. 84B** depicts a bar 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).

[0130] **Fig. 85A** depicts a schematic diagram of a wild-type *clbA* construct.

[0131] **Fig. 85B** depicts a schematic diagram of a *clbA* knockout construct.

Description of Embodiments

[0132] The present disclosure includes genetically engineered bacteria, pharmaceutical compositions thereof, and methods of reducing gut inflammation, enhancing gut barrier function, and/or treating or preventing autoimmune disorders. In some embodiments, the genetically engineered bacteria comprise at least one non-native gene and/or gene cassette for producing a non-native anti-inflammation and/or gut barrier function enhancer molecule(s). In some embodiments, the at least one gene and/or gene cassette is further operably linked to a regulatory region that is controlled by a transcription factor that is capable of sensing an inducing condition, e.g., a low-oxygen environment, the presence of ROS, or the presence of RNS. The genetically engineered bacteria are capable of producing the anti-inflammation and/or gut barrier function enhancer molecule(s) in inducing environments, e.g., in the gut. Thus, the genetically engineered bacteria and pharmaceutical compositions comprising those bacteria may be used to treat or prevent autoimmune disorders and/or diseases or conditions associated with gut inflammation and/or compromised gut barrier function, including IBD.

[0133] 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.

[0134] As used herein, "diseases and conditions associated with gut inflammation and/or compromised gut barrier function" include, but are not limited to, inflammatory bowel diseases, diarrheal diseases, and related diseases. "Inflammatory bowel diseases" and "IBD" are used interchangeably herein to refer to a group of diseases associated with gut inflammation, which include, but are not limited to, Crohn's disease, ulcerative colitis,

collagenous colitis, lymphocytic colitis, diversion colitis, Behcet's disease, and indeterminate colitis. As used herein, "diarrheal diseases" include, but are not limited to, acute watery diarrhea, e.g., cholera; acute bloody diarrhea, e.g., dysentery; and persistent diarrhea. As used herein, related diseases include, but are not limited to, short bowel syndrome, ulcerative proctitis, proctosigmoiditis, left-sided colitis, pancolitis, and fulminant colitis.

[0135] Symptoms associated with the aforementioned diseases and conditions include, but are not limited to, one or more of diarrhea, bloody stool, mouth sores, perianal disease, abdominal pain, abdominal cramping, fever, fatigue, weight loss, iron deficiency, anemia, appetite loss, weight loss, anorexia, delayed growth, delayed pubertal development, inflammation of the skin, inflammation of the eyes, inflammation of the joints, inflammation of the liver, and inflammation of the bile ducts.

[0136] A disease or condition associated with gut inflammation and/or compromised gut barrier function may be an autoimmune disorder. A disease or condition associated with gut inflammation and/or compromised gut barrier function may be co-morbid with an autoimmune disorder. As used herein, "autoimmune disorders" include, but are not limited to, acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, Addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune oophoritis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroid disease, autoimmune urticarial, axonal & neuronal neuropathies, Balo disease, Behcet's disease, bullous pemphigoid, cardiomyopathy, Castleman disease, celiac disease, Chagas disease, chronic inflammatory demyelinating polyneuropathy (CIDP), chronic recurrent multifocal ostomyelitis (CRMO), Churg-Strauss syndrome, cicatricial pemphigoid/benign mucosal pemphigoid, Crohn's disease, Cogan's syndrome, cold agglutinin disease, congenital heart block, Coxsackie myocarditis, CREST disease, essential mixed cryoglobulinemia, demyelinating neuropathies, dermatitis herpetiformis, dermatomyositis, Devic's disease (neuromyelitis optica), discoid lupus, Dressler's syndrome, endometriosis, eosinophilic

esophagitis, eosinophilic fasciitis, erythema nodosum, experimental allergic encephalomyelitis, Evans syndrome, fibrosing alveolitis, giant cell arteritis (temporal arteritis), giant cell myocarditis, glomerulonephritis, Goodpasture's syndrome, granulomatosis with polyangiitis (GPA), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, herpes gestationis, hypogammaglobulinemia, idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, immunoregulatory lipoproteins, inclusion body myositis, interstitial cystitis, juvenile arthritis, juvenile idiopathic arthritis, juvenile myositis, Kawasaki syndrome, Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, ligneous conjunctivitis, linear IgA disease (LAD), lupus (systemic lupus erythematosus), chronic Lyme disease, Meniere's disease, microscopic polyangiitis, mixed connective tissue disease (MCTD), Mooren's ulcer, Mucha-Habermann disease, multiple sclerosis, myasthenia gravis, myositis, narcolepsy, neuromyelitis optica (Devic's), neutropenia, ocular cicatricial pemphigoid, optic neuritis, palindromic rheumatism, PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus), paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, pars planitis (peripheral uveitis), pemphigus, peripheral neuropathy, perivenous encephalomyelitis, pernicious anemia, POEMS syndrome, polyarteritis nodosa, type I, II, & III autoimmune polyglandular syndromes, polymyalgia rheumatic, polymyositis, postmyocardial infarction syndrome, postpericardiotomy syndrome, progesterone dermatitis, primary biliary cirrhosis, primary sclerosing cholangitis, psoriasis, psoriatic arthritis, idiopathic pulmonary fibrosis, pyoderma gangrenosum, pure red cell aplasia, Raynaud's phenomenon, reactive arthritis, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis, scleroderma, Sjogren's syndrome, sperm & testicular autoimmunity, stiff person syndrome, subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, temporal arteritis/giant cell arteritis, thrombocytopenic purpura (TTP), Tolosa-Hunt syndrome, transverse myelitis, type 1 diabetes, asthma, ulcerative colitis, undifferentiated connective tissue disease

(UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, and Wegener's granulomatosis.

[0137] As used herein, "anti-inflammation molecules" and/or "gut barrier function enhancer molecules" include, but are not limited to, short-chain fatty acids, butyrate, propionate, acetate, IL-2, IL-22, superoxide dismutase (SOD), GLP-2 and analogs, GLP-1, IL-10, IL-27, TGF-β1, TGF-β2, N-acylphosphatidylethanolamines (NAPEs), elafin (also called peptidase inhibitor 3 and SKALP), trefoil factor, melatonin, tryptophan, PGD_2, and kynurenic acid, indole metabolites, and other tryptophan metabolites, as well as other molecules disclosed herein. Such molecules may also include compounds that inhibit pro-inflammatory molecules, e.g., a single-chain variable fragment (scFv), antisense RNA, siRNA, or shRNA that neutralizes TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-17, and/or chemokines, e.g., CXCL-8 and CCL2. Such molecules also include AHR agonists (e.g., which result in IL-22 production, e.g., indole acetic acid, indole-3-aldehyde, and indole) and and PXR agonists (e.g., IPA), as described herein. Such molecules also include HDAC inhibitors (e.g., butyrate), activators of GPR41 and/or GPR43 (e.g., butyrate and/or propionate and/or acetate), activtators of GPR109A (e.g., butyrate), inhibitors of NF-kappaB signaling (e.g., butyrate), and modulators of PPARgamma (e.g., butyrate), activators of AMPK signaling (e.g., acetate), and modulators of GLP-1 secretion. Such molecules also include hydroxyl radical scavengers and antioxidants (e.g., IPA). A molecule may be primarily anti-inflammatory, e.g., IL-10, or primarily gut barrier function enhancing, e.g., GLP-2. A molecule may be both anti-inflammatory and gut barrier function enhancing. An anti-inflammation and/or gut barrier function enhancer molecule may be encoded by a single gene, e.g., elafin is encoded by the *PI3* gene. Alternatively, an anti-inflammation and/or gut barrier function enhancer molecule may be synthesized by a biosynthetic pathway requiring multiple genes, e.g., butyrate. These molecules may also be referred to as therapeutic molecules. In some instances, the "anti-inflammation molecules" and/or "gut barrier function enhancer molecules" are referred to herein as "effector molecules" or "therapeutic molecules" or "therapeutic polypeptides".

[0138] As used herein, the term "recombinant microorganism" refers to a microorganism, e.g., bacterial, yeast, or viral cell, or bacteria, yeast, or virus, that has been genetically modified from its native state. Thus, a "recombinant bacterial cell" or "recombinant bacteria" refers to a bacterial cell or bacteria that have been genetically

modified from their native state. For instance, a recombinant 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. Recombinant bacterial cells disclosed herein may comprise exogenous nucleotide sequences on plasmids. Alternatively, recombinant bacterial cells may comprise exogenous nucleotide sequences stably incorporated into their chromosome.

[0139] A "programmed or engineered microorganism" refers to a microorganism, e.g., bacterial or viral cell, or bacteria or virus, that has been genetically modified from its native state to perform a specific function. Thus, a "programmed or engineered bacterial cell" or "programmed or engineered bacteria" refers to a bacterial cell or bacteria 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.

[0140] 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.

[0141] As used herein, the term "gene sequence" is meant to refer to a genetic sequence, e.g., a nucleic acid sequence. The gene sequence or genetic sequence is meant to include a complete gene sequence or a partial gene sequence. The gene sequence or

genetic sequence is meant to include sequence that encodes a protein or polypeptide and is also menat to include genetic sequence that does not encode a protein or polypeptide, e.g., a regulatory sequence, leader sequence, signal sequence, or other non-protein coding sequence.

[0142] In some embodiments, the term "gene" or "gene sequence" is meant to refer to a nucleic acid sequence encoding any of the anti-inflammatory and gut barrier function enhancing molecules described herein, e.g., IL-2, IL-22, superoxide dismutase (SOD), kynurenine, GLP-2, GLP-1, IL-10, IL-27, TGF-β1, TGF-β2, N-acylphosphatidylethanolamines (NAPEs), elafin, and trefoil factor, as well as others. The nucleic acid sequence may comprise the entire gene sequence or a partial gene sequence encoding a functional molecule. The nucleic acid sequence may be a natural sequence or a synthetic sequence. The nucleic acid sequence may comprise a native or wild-type sequence or may comprise a modified sequence having one or more insertions, deletions, substitutions, or other modifications, for example, the nucleic acid sequence may be codon-optimized.

[0143] 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.

[0144] 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.

[0145] 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, signal sequences, and stem-loop structures. In one embodiment, the regulatory sequence comprises a promoter, *e.g.,* an FNR responsive promoter or other promoter disclosed herein.

[0146] As used herein, a "gene cassette" or "operon" encoding a biosynthetic pathway refers to the two or more genes that are required to produce an anti-inflammatory or gut barrier enhancer molecule. 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.

[0147] A "butyrogenic gene cassette," "butyrate biosynthesis 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.

[0148] Likewise, 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.

[0149] 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.

[0150] 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.

[0151] 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.

[0152] 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.

[0153] 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.

[0154] 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.

[0155] "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, Ptac promoter, BBa_J23100, a constitutive Escherichia coli σS 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 σ32 promoter (e.g., htpG heat shock promoter (BBa_J45504)), a constitutive Escherichia coli σ70 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 σA promoter (e.g., promoter veg (BBa_K143013), promoter 43 (BBa_K143013), PliaG (BBa_K823000), PlepA (BBa_K823002), Pveg (BBa_K823003)), a constitutive Bacillus subtilis σB 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)).

[0156] 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.

[0157] 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 encoding one or more anti-inflammation and/or gut barrier enhancer molecule(s), which 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 encoding a encoding a payload, e.g., one or more anti-inflammation and/or gut barrier enhancer molecule(s), in which the plasmid or chromosome carrying the gene is stably maintained in the bacterium, such that the payload 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.

[0158] 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.

[0159] 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.

[0160] 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.

[0161] 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, base substitution, 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 an anti-inflammatory or gut barrier enhancer molecule 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.

[0162] 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.

[0163] 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.

[0164] 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.

[0165] 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 1A**.

[0166] 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 1A. 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*

[0167] 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.

[0168] 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.

[0169] 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 a recombinant 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). "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.

[0170] 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. 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. 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 1B 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/>. 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 1B.

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)

[0171] "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, yeast, e.g., Saccharomyces, and protozoa. In some aspects, the microorganism is engineered ("engineered microorganism") to produce one or more therpauetic molecules, e.g., an antinflammatory or barrier enhancer molecule. In certain embodiments, the engineered microorganism is an engineered bacterium. In certain embodiments, the engineered microorganism is an engineered virus.

[0172] "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 Gram-negative bacteria. In some embodiments, non-pathogenic bacteria are Gram-positive bacteria. In some embodiments, non-pathogenic bacteria do not contain lipopolysaccharides (LPS). In some embodiments, non-pathogenic bacteria are commensal bacteria. Examples of non-pathogenic bacteria include, but are not limited to certain strains belonging to the genus *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, Escherichia coli, Escherichia coli Nissle, 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). Non-pathogenic bacteria also include commensal bacteria, which are present in the indigenous microbiota of the gut. In one embodiment, the disclosure further includes non-pathogenic *Saccharomyces*, such as

Saccharomyces boulardii. Naturally pathogenic bacteria may be genetically engineered to reduce or eliminate pathogenicity.

[0173] "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. In some embodiments, the probiotic bacteria are Gram-negative bacteria. In some embodiments, the probiotic bacteria are Gram-positive bacteria. 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, certain strains belonging to the genus *Bifidobacteria*, *Escherichia Coli*, *Lactobacillus,* and S*accharomyces e.g., Bifidobacterium bifidum, Enterococcus faecium, Escherichia coli* strain Nissle, *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus paracasei*, and *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.

[0174] 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).

[0175] 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*).

[0176] 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.

[0177] Those in need of treatment may include individuals already having a particular medical disorder, as well as those at risk of having, or who may ultimately acquire the disorder. The need for treatment is assessed, for example, by the presence of one or more risk factors associated with the development of a disorder, the presence or progression of a disorder, or likely receptiveness to treatment of a subject having the disorder. Treating autoimmune disorders and/or diseases and conditions associated with gut inflammation and/or compromised gut barrier function may encompass reducing or

eliminating excess inflammation and/or associated symptoms, and does not necessarily encompass the elimination of the underlying disease.

[0178] Treating the diseases described herein may encompass increasing levels of butyrate, increasing levels of acetate, increasing levels of butyrate and increasing GLP-2, IL-22, and/o rIL-10, and/or modulating levels of tryptophan and/or its metabolites (e.g., kynurenine), and/or providing any other anti-inflammation and/or gut barrier enhancer molecule and does not necessarily encompass the elimination of the underlying disease.

[0179] As used herein a "pharmaceutical composition" refers to a preparation of genetically engineered microorganism of the disclosure, e.g., genetically engineered bacteria or virus, with other components such as a physiologically suitable carrier and/or excipient.

[0180] 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 or viral compound. An adjuvant is included under these phrases.

[0181] 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.

[0182] 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 condition, e.g., inflammation, diarrhea.an autoimmune disorder. 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 an autoimmune a disorder and/or a disease or condition associated with gut inflammation and/or compromised gut barrier function. 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.

[0183] 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 recombinant bacterial cell of the disclosure.

[0184] As used herein, the term "bactericidal" refers to a molecule or protein which is capable of killing the recombinant bacterial cell of the disclosure.

[0185] 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 recombinant bacterial cell of the disclosure, or which is capable of killing the recombinant 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*.

[0186] 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.

[0187] 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.

[0188] 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.

[0189] 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.

[0190] 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.

[0191] An 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.

[0192] 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 framents), 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 anti cancer 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.

[0193] 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.

[0194] 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).

[0195] 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.

[0196] 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.

[0197] 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.

[0198] 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.

[0199] The articles "a" and "an," as used herein, should be understood to mean "at least one," unless clearly indicated to the contrary.

[0200] 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.

[0201] 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.

Bacteria

[0202] The genetically engineered microorganisms, or programmed microorganisms, such as genetically engineered bacteria of the disclosure are capable of producing one or more non-native anti-inflammation and/or gut barrier function enhancer molecules. In certain embodiments, the genetically engineered bacteria are obligate anaerobic bacteria. In certain embodiments, the genetically engineered bacteria are facultative anaerobic bacteria. In certain embodiments, the genetically engineered bacteria are aerobic bacteria. In some embodiments, the genetically engineered bacteria are Gram-positive bacteria. In some embodiments, the genetically engineered bacteria are Gram-positive bacteria and lack LPS. In some embodiments, the genetically engineered bacteria are Gram-negative bacteria. In some embodiments, the genetically engineered bacteria are Gram-positive and obligate anaerobic bacteria. In some embodiments, the genetically engineered bacteria are Gram-positive and facultative anaerobic bacteria. In some embodiments, the genetically engineered bacteria are non-pathogenic bacteria. In some embodiments, the genetically engineered bacteria are commensal bacteria. In some embodiments, the genetically engineered bacteria are probiotic bacteria. In some embodiments, the genetically engineered bacteria are naturally pathogenic bacteria that are modified or mutated to reduce or eliminate pathogenicity. Exemplary bacteria include, but are not limited to, Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Caulobacter, Clostridium, Enterococcus, Escherichia coli, Lactobacillus, Lactococcus, Listeria, Mycobacterium, Saccharomyces, Salmonella, Staphylococcus, Streptococcus, Vibrio,

Bacillus coagulans, Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium breve UCC2003, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium acetobutylicum, Clostridium butyricum, Clostridium butyricum M-55, Clostridium cochlearum, Clostridium felsineum, Clostridium histolyticum, Clostridium multifermentans, Clostridium novyi-NT, Clostridium paraputrificum, Clostridium pasteureanum, Clostridium pectinovorum, Clostridium perfringens, Clostridium roseum, Clostridium sporogenes, Clostridium tertium, Clostridium tetani, Clostridium tyrobutyricum, Corynebacterium parvum, Escherichia coli MG1655, Escherichia coli Nissle 1917, Listeria monocytogenes, Mycobacterium bovis, Salmonella choleraesuis, Salmonella typhimurium, and Vibrio cholera. In certain embodiments, the genetically engineered bacteria are selected from the group consisting of Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis, and Saccharomyces boulardii, Clostridium clusters IV and XIVa of Firmicutes (including species of Eubacterium), Roseburia, Faecalibacterium, Enterobacter, Faecalibacterium prausnitzii, Clostridium difficile, Subdoligranulum, Clostridium sporogenes, Campylobacter jejuni, Clostridium saccharolyticum, Klebsiella, Citrobacter, Pseudobutyrivibrio, and Ruminococcus. In certain embodiments, the the genetically engineered bacteria are selected from Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides subtilis, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Clostridium butyricum, Escherichia coli, Escherichia coli Nissle, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus reuteri, and Lactococcus lactis

[0203] In some embodiments, the genetically engineered bacterium is a Gram-positive bacterium, e.g., Clostridium, that is naturally capable of producing high levels of butyrate. In some embodiments, the genetically engineered bacterium is selected from the group consisting of C. butyricum ZJUCB, C. butyricum S21, C. thermobutyricum ATCC 49875, C. beijerinckii, C. populeti ATCC 35295, C. tyrobutyricum JM1, C. tyrobutyricum CIP 1-776, C. tyrobutyricum ATCC 25755, C. tyrobutyricum CNRZ 596, and C. tyrobutyricum ZJU 8235. In some embodiments, the genetically engineered bacterium is C. butyricum CBM588, a probiotic bacterium that is highly amenable to protein secretion

and has demonstrated efficacy in treating IBD (Kanai et al., 2015). In some embodiments, the genetically engineered bacterium is Bacillus, a probiotic bacterium that is highly genetically tractable and has been a popular chassis for industrial protein production; in some embodiments, the bacterium has highly active secretion and/or no toxic byproducts (Cutting, 2011).

[0204] 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 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 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.

[0205] In some embodiments, the genetically engineered bacteria are 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). In addition, it has been shown that E. coli Nissle does not carry pathogenic adhesion factors, does not produce any enterotoxins or cytotoxins, is not invasive, and not uropathogenic (Sonnenborn et al., 2009). As early as in 1917, E. coli Nissle was packaged into medicinal capsules, called Mutaflor, for therapeutic use. E. coli Nissle has since been used to treat ulcerative colitis in humans in vivo (Rembacken et al., 1999), to treat inflammatory bowel disease, Crohn's disease, and pouchitis in humans in vivo (Schultz, 2008), and to inhibit enteroinvasive Salmonella, Legionella, Yersinia, and Shigella in vitro (Altenhoefer et al., 2004). It is commonly accepted that E. coli Nissle's therapeutic efficacy and safety have

convincingly been proven (Ukena et al., 2007). In some embodiments, the genetically engineered bacteria are E. coli Nissle and are naturally capable of promoting tight junctions and gut barrier function. In some embodiments, the genetically engineered bacteria are E. coli and are highly amenable to recombinant protein technologies.

[0206] 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. It is known, for example, that the clostridial butyrogenic pathway genes are widespread in the genome-sequenced clostridia and related species (Aboulnaga et al., 2013). Furthermore, genes from one or more different species of bacteria can be introduced into one another, e.g., the butyrogenic genes fromPeptoclostridium difficile have been expressed in Escherichia coli (Aboulnaga et al., 2013).

[0207] . In one embodiment, the recombinant bacterial cell does not colonize the subject having the disorder. 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 (Sonnenborn et al., 2009) or by activation of a kill switch, several hours or days after administration. Thus, the genetically engineered bacteria may require continued administration. Residence time in vivo may be calculated for the genetically engineered bacteria. In some embodiments, the residence time is calculated for a human subject. In some embodiments, residence time in vivo is calculated for the genetically engineered bacteria of the invention, e.g. as described herein.

[0208] In some embodiments, the bacterial cell is a genetically engineered bacterial cell. In another embodiment, the bacterial cell is a recombinant bacterial cell. In some embodiments, the disclosure comprises a colony of bacterial cells disclosed herein.

[0209] In another aspect, the disclosure provides a recombinant bacterial culture which comprises bacterial cells disclosed herein.

[0210] In some embodiments, the genetically engineered bacteria comprising an anti-inflammatory or gut barrier enhancer molecule 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 a 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 ParaBAD. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin.

[0211] In some embodiments, the genetically engineered bacteria is an auxotroph comprising gene sequence encoding an anti-inflammatory or gut barrier enhancer molecule and further comprises a kill-switch circuit, such as any of the kill-switch circuits described herein.

[0212] In some embodiments of the above described genetically engineered bacteria, the gene encoding an anti-inflammatory or gut barrier enhancer molecule is present on a plasmid in the bacterium. In some embodiments, the gene sequence(s) encoding an anti-inflammatory or gut barrier enhancer molecule is present in the bacterial chromosome. In some embodiments, a gene sequence encoding a secretion protein or protein complex, such as any of the secretion systems disclosed herein, for secreting a biomolecule (e.g. an anti-inflammatory or gut barrier enhancer molecule), is present on a plasmid in the bacterium. In some embodiments, the gene sequence encoding a secretion protein or protein complex for secreting a biomolecule, such as any of the secretion systems disclosed herein, is present in the bacterial chromosome. In some embodiments, the gene sequence(s) encoding an antibiotic resistance gene is present on a plasmid in the bacterium. In some embodiments, the gene sequence(s) encoding an antibiotic resistance gene is present in the bacterial chromosome.

Anti-inflammation and/or gut barrier function enhancer molecules

[0213] The genetically engineered bacteria comprise one or more gene sequence(s) and/or gene cassette(s) for producing a non-native anti-inflammation and/or gut barrier function enhancer molecule. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) for producing a non-native anti-inflammation and/or gut barrier function enhancer molecule. For example, the genetically engineered

bacteria may comprise two or more gene sequence(s) for producing a non-native anti-inflammation and/or gut barrier function enhancer molecule. In some embodiments, the two or more gene sequences are multiple copies of the same gene. In some emodiments, the two or more gene sequences are sequences encoding different genes. In some emodiments, the two or more gene sequences are sequences encoding multiple copies of one or more different genes. In some embodiments, the genetically engineered bacteria comprise one or more gene cassette(s) for producing a non-native anti-inflammation and/or gut barrier function enhancer molecule. For example, the genetically engineered bacteria may comprise two or more gene cassette(s) for producing a non-native anti-inflammation and/or gut barrier function enhancer molecule. In some embodiments, the two or more gene cassettes are multiple copies of the same gene cassette. In some emodiments, the two or more gene cassettes are different gene cassettes for producing either the same or different anti-inflammation and/or gut barrier function enhancer molecule(s). In some emodiments, the two or more gene cassettes are gene cassettes for producing multiple copies of one or more different anti-inflammation and/or gut barrier function enhancer molecule(s). In some embodiments, the anti-inflammation and/or gut barrier function enhancer molecule is selected from the group consisting of a short-chain fatty acid, butyrate, propionate, acetate, IL-2, IL-22, superoxide dismutase (SOD), GLP-2, GLP-1, IL-10 (human or viral), IL-27, TGF-β1, TGF-β2, N-acylphosphatidylethanolamines (NAPEs), elafin (also known as peptidase inhibitor 3 or SKALP), trefoil factor, melatonin, PGD2, kynurenic acid, kynurenine, typtophan metabolite, indole, indole metabolite, a single-chain variable fragment (scFv), antisense RNA, siRNA, or shRNA that neutralizes TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-17, and/or chemokines, e.g., CXCL-8 and CCL2, AHR agonist (e.g., indole acetic acid, indole-3-aldehyde, and indole), PXR agonist (e.g., IPA), HDAC inhibitor (e.g., butyrate), GPR41 and/or GPR43 activator (e.g., butyrate and/or propionate and/or acetate), GPR109A activator (e.g., butyrate), inhibitor of NF-kappaB signaling (e.g., butyrate), modulator of PPARgamma (e.g., butyrate), activator of AMPK signaling (e.g., acetate), modulator of GLP-1 secretion, and hydroxyl radical scavengers and antioxidants (e.g., IPA). A molecule may be primarily anti-inflammatory, e.g., IL-10, or primarily gut barrier function enhancing, e.g., GLP-2. Alternatively, a molecule may be both anti-inflammatory and gut barrier function enhancing.

[0214] In some embodiments, the genetically engineered bacteria of the invention express one or more anti-inflammation and/or gut barrier function enhancer molecule(s) that is encoded by a single gene, e.g., the molecule is elafin and encoded by the PI3 gene, or the molecule is interleukin-10 and encoded by the IL10 gene. In alternate embodiments, the genetically engineered bacteria of the invention encode one or more an anti-inflammation and/or gut barrier function enhancer molecule(s), e.g., butyrate, that is synthesized by a biosynthetic pathway requiring multiple genes.

[0215] The one or more gene sequence(s) and/or gene cassette(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s). In some embodiments, expression from the chromosome may be useful for increasing stability of expression of the anti-inflammation and/or gut barrier function enhancer molecule(s). In some embodiments, the gene sequence(s)or gene cassette(s) for producing the anti-inflammation and/or gut barrier function enhancer molecule(s) 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 butyrate biosynthesis gene cassette may be integrated into the bacterial chromosome. In some embodiments, the gene sequence(s) or gene cassette(s) for producing the anti-inflammation and/or gut barrier function enhancer molecule(s) is expressed from a plasmid in the genetically engineered bacteria. In some embodiments, the gene sequence(s) or gene cassette(s) for producing the anti-inflammation and/or gut barrier function enhancer molecule(s) 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. 52 for exemplary insertion sites). 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.

Short chain Fatty Acids and Tryptophan Metabolites

[0216] One strategy in the treatment, prevention, and/or management of inflammatory bowel disorders 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.

[0217] 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.

[0218] 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.

[0219] 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.

[0220] 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.

[0221] 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.

[0222] 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

[0223] 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 eomprise 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.

[0224] 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).

[0225] 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.

[0226] In some embodiments, the genetically engineered bacteria producing acetate comprise a mutation and/or deletion in the endogenous ldhA gene.

[0227] 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).

[0228] In some embodiments, the genetically engineered bacteria producing acetate comprise a mutation and/or deletion in the endogenous adhE gene.

[0229] 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 subinut 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.

[0230] 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.

[0231] 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 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.

[0232] 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.

[0233] 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).

[0234] In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta gene. In some embodiments, the gentically engineered bacteria produce butyrate.In some embodiments, the genetically engineered bacteria comprise a mutation and/or deletion in the endogenous pta gene and also 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 mutationand/or deletion in the endogenous pta, ldhA, frdA, and adhE genes. In some embodiments, the gentically engineered bacteris produce butyrate.

[0235] 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 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.

Butyrate

[0236] 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 genetically engineered bacteria may include any suitable set of butyrogenic genes (*see, e.g.,* **Table 2 and 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.n 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. One or more of the butyrate biosynthesis genes may be functionally replaced or modified, e.g., codon optimized.

[0237] 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.

[0238] **Table 2** depicts the nucleic acid sequences of exemplary genes in exemplary butyrate biosynthesis gene cassettes.

Table 2. Exemplary Butyrate Cassette Sequences

Description	Sequence
bcd2 **SEQ ID NO: 1**	ATGGATTTAAATTCTAAAAAATATCAGATGCTTAAAGAGCTATATGTAAG CTTCGCTGAAAATGAAGTTAAACCTTTAGCAACAGAACTTGATGAAGAAG AAAGATTTCCTTATGAAACAGTGGAAAAAATGGCAAAAGCAGGAATGATG GGTATACCATATCCAAAAGAATATGGTGGAGAAGGTGGAGACACTGTAGG ATATATAATGGCAGTTGAAGAATTGTCTAGAGTTTGTGGTACTACAGGAG TTATATTATCAGCTCATACATCTCTTGGCTCATGGCCTATATATCAATAT GGTAATGAAGAACAAAAACAAAAATTCTTAAGACCACTAGCAAGTGGAGA AAAATTAGGAGCATTTGGTCTTACTGAGCCTAATGCTGGTACAGATGCGT CTGGCCAACAAACAACTGCTGTTTTAGACGGGGATGAATACATACTTAAT GGCTCAAAAATATTTATAACAAACGCAATAGCTGGTGACATATATGTAGT AATGGCAATGACTGATAAATCTAAGGGGAACAAAGGAATATCAGCATTTA TAGTTGAAAAAGGAACTCCTGGGTTTAGCTTTGGAGTTAAAGAAAAGAAA ATGGGTATAAGAGGTTCAGCTACGAGTGAATTAATATTTGAGGATTGCAG AATACCTAAAGAAAATTTACTTGGAAAAGAAGGTCAAGGATTTAAGATAG CAATGTCTACTCTTGATGGTGGTAGAATTGGTATAGCTGCACAAGCTTTA GGTTTAGCACAAGGTGCTCTTGATGAAACTGTTAAATATGTAAAAGAAAG AGTACAATTTGGTAGACCATTATCAAAATTCCAAAATACACAATTCCAAT TAGCTGATATGGAAGTTAAGGTACAAGCGGCTAGACACCTTGTATATCAA

Description	Sequence
	GCAGCTATAAATAAAGACTTAGGAAAACCTTATGGAGTAGAAGCAGCAAT GGCAAAATTATTTGCAGCTGAAACAGCTATGGAAGTTACTACAAAAGCTG TACAACTTCATGGAGGATATGGATACACTCGTGACTATCCAGTAGAAAGA ATGATGAGAGATGCTAAGATAACTGAAATATATGAAGGAACTAGTGAAGT TCAAAGAATGGTTATTTCAGGAAAACTATTAAAATAG
etfB3 **SEQ ID NO: 2**	ATGAATATAGTCGTTTGTATAAAACAAGTTCCAGATACAACAGAAGTTAA ACTAGATCCTAATACAGGTACTTTAATTAGAGATGGAGTACCAAGTATAA TAAACCCTGATGATAAAGCAGGTTTAGAAGAAGCTATAAAATTAAAAGAA GAAATGGGTGCTCATGTAACTGTTATAACAATGGGACCTCCTCAAGCAGA TATGGCTTTAAAAGAAGCTTTAGCAATGGGTGCAGATAGAGGTATATTAT TAACAGATAGAGCATTTGCGGGTGCTGATACTTGGGCAACTTCATCAGCA TTAGCAGGAGCATTAAAAAATATAGATTTTGATATTATAATAGCTGGAAG ACAGGCGATAGATGGAGATACTGCACAAGTTGGACCTCAAATAGCTGAAC ATTTAAATCTTCCATCAATAACATATGCTGAAGAAATAAAAACTGAAGGT GAATATGTATTAGTAAAAAGACAATTTGAAGATTGTTGCCATGACTTAAA AGTTAAAATGCCATGCCTTATAACAACTCTTAAAGATATGAACACACCAA GATACATGAAAGTTGGAAGAATATATGATGCTTTCGAAAATGATGTAGTA GAAACATGGACTGTAAAAGATATAGAAGTTGACCCTTCTAATTTAGGTCT TAAAGGTTCTCCAACTAGTGTATTTAAATCATTTACAAAATCAGTTAAAC CAGCTGGTACAATATACAATGAAGATGCGAAAACATCAGCTGGAATTATC ATAGATAAATTAAAAGAGAAGTATATCATATAA
etfA3 **SEQ ID NO: 3**	ATGGGTAACGTTTTAGTAGTAATAGAACAAAGAGAAAATGTAATTCAAAC TGTTTCTTTAGAATTACTAGGAAAGGCTACAGAAATAGCAAAAGATTATG ATACAAAAGTTTCTGCATTACTTTTAGGTAGTAAGGTAGAAGGTTTAATA GATACATTAGCACACTATGGTGCAGATGAGGTAATAGTAGTAGATGATGA AGCTTTAGCAGTGTATACAACTGAACCATATACAAAAGCAGCTTATGAAG CAATAAAAGCAGCTGACCCTATAGTTGTATTATTTGGTGCAACTTCAATA GGTAGAGATTTAGCGCCTAGAGTTTCTGCTAGAATACATACAGGTCTTAC TGCTGACTGTACAGGTCTTGCAGTAGCTGAAGATACAAAATTATTATTAA TGACAAGACCTGCCTTTGGTGGAAATATAATGGCAACAATAGTTTGTAAA GATTTCAGACCTCAAATGTCTACAGTTAGACCAGGGGTTATGAAGAAAAA TGAACCTGATGAAACTAAAGAAGCTGTAATTAACCGTTTCAAGGTAGAAT TTAATGATGCTGATAAATTAGTTCAAGTTGTACAAGTAATAAAAGAAGCT AAAAAACAAGTTAAAATAGAAGATGCTAAGATATTAGTTTCTGCTGGACG TGGAATGGGTGGAAAAGAAAACTTAGACATACTTTATGAATTAGCTGAAA TTATAGGTGGAGAAGTTTCTGGTTCTCGTGCCACTATAGATGCAGGTTGG TTAGATAAAGCAAGACAAGTTGGTCAAACTGGTAAAACTGTAAGACCAGA CCTTTATATAGCATGTGGTATATCTGGAGCAATACAACATATAGCTGGTA TGGAAGATGCTGAGTTTATAGTTGCTATAAATAAAAATCCAGAAGCTCCA ATATTTAAATATGCTGATGTTGGTATAGTTGGAGATGTTCATAAAGTGCT TCCAGAACTTATCAGTCAGTTAAGTGTTGCAAAAGAAAAAGGTGAAGTTT TAGCTAACTAA
thiA1 **SEQ ID NO: 4**	ATGAGAGAAGTAGTAATTGCCAGTGCAGCTAGAACAGCAGTAGGAAGTTT TGGAGGAGCATTTAAATCAGTTTCAGCGGTAGAGTTAGGGGTAACAGCAG CTAAAGAAGCTATAAAAAGAGCTAACATAACTCCAGATATGATAGATGAA TCTCTTTTAGGGGGAGTACTTACAGCAGGTCTTGGACAAAATATAGCAAG ACAAATAGCATTAGGAGCAGGAATACCAGTAGAAAAACCAGCTATGACTA

Description	Sequence
	TAAATATAGTTTGTGGTTCTGGATTAAGATCTGTTTCAATGGCATCTCAA CTTATAGCATTAGGTGATGCTGATATAATGTTAGTTGGTGGAGCTGAAAA CATGAGTATGTCTCCTTATTTAGTACCAAGTGCGAGATATGGTGCAAGAA TGGGTGATGCTGCTTTTGTTGATTCAATGATAAAAGATGGATTATCAGAC ATATTTAATAACTATCACATGGGTATTACTGCTGAAAACATAGCAGAGCA ATGGAATATAACTAGAGAAGAACAAGATGAATTAGCTCTTGCAAGTCAAA ATAAAGCTGAAAAAGCTCAAGCTGAAGGAAAATTTGATGAAGAAATAGTT CCTGTTGTTATAAAAGGAAGAAAAGGTGACACTGTAGTAGATAAAGATGA ATATATTAAGCCTGGCACTACAATGGAGAAACTTGCTAAGTTAAGACCTG CATTTAAAAAAGATGGAACAGTTACTGCTGGTAATGCATCAGGAATAAAT GATGGTGCTGCTATGTTAGTAGTAATGGCTAAAGAAAAAGCTGAAGAACT AGGAATAGAGCCTCTTGCAACTATAGTTTCTTATGGAACAGCTGGTGTTG ACCCTAAAATAATGGGATATGGACCAGTTCCAGCAACTAAAAAAGCTTTA GAAGCTGCTAATATGACTATTGAAGATATAGATTTAGTTGAAGCTAATGA GGCATTTGCTGCCCAATCTGTAGCTGTAATAAGAGACTTAAATATAGATA TGAATAAAGTTAATGTTAATGGTGGAGCAATAGCTATAGGACATCCAATA GGATGCTCAGGAGCAAGAATACTTACTACACTTTTATATGAAATGAAGAG AAGAGATGCTAAAACTGGTCTTGCTACACTTTGTATAGGCGGTGGAATGG GAACTACTTTAATAGTTAAGAGATAG
hbd **SEQ ID NO: 5**	ATGAAATTAGCTGTAATAGGTAGTGGAACTATGGGAAGTGGTATTGTACA AACTTTTGCAAGTTGTGGACATGATGTATGTTTAAAGAGTAGAACTCAAG GTGCTATAGATAAATGTTTAGCTTTATTAGATAAAAATTTAACTAAGTTA GTTACTAAGGGAAAAATGGATGAAGCTACAAAAGCAGAAATATTAAGTCA TGTTAGTTCAACTACTAATTATGAAGATTTAAAAGATATGGATTTAATAA TAGAAGCATCTGTAGAAGACATGAATATAAAGAAAGATGTTTTCAAGTTA CTAGATGAATTATGTAAAGAAGATACTATCTTGGCAACAAATACTTCATC ATTATCTATAACAGAAATAGCTTCTTCTACTAAGCGCCCAGATAAAGTTA TAGGAATGCATTTCTTTAATCCAGTTCCTATGATGAAATTAGTTGAAGTT ATAAGTGGTCAGTTAACATCAAAAGTTACTTTTGATACAGTATTTGAATT ATCTAAGAGTATCAATAAAGTACCAGTAGATGTATCTGAATCTCCTGGAT TTGTAGTAAATAGAATACTTATACCTATGATAAATGAAGCTGTTGGTATA TATGCAGATGGTGTTGCAAGTAAAGAAGAAATAGATGAAGCTATGAAATT AGGAGCAAACCATCCAATGGGACCACTAGCATTAGGTGATTTAATCGGAT TAGATGTTGTTTTAGCTATAATGAACGTTTTATATACTGAATTTGGAGAT ACTAAATATAGACCTCATCCACTTTTAGCTAAAATGGTTAGAGCTAATCA ATTAGGAAGAAAAACTAAGATAGGATTCTATGATTATAATAAATAA
crt2 **SEQ ID NO: 6**	ATGAGTACAAGTGATGTTAAAGTTTATGAGAATGTAGCTGTTGAAGTAGA TGGAAATATATGTACAGTGAAAATGAATAGACCTAAAGCCCTTAATGCAA TAAATTCAAAGACTTTAGAAGAACTTTATGAAGTATTTGTAGATATTAAT AATGATGAAACTATTGATGTTGTAATATTGACAGGGGAAGGAAAGGCATT TGTAGCTGGAGCAGATATTGCATACATGAAAGATTTAGATGCTGTAGCTG CTAAAGATTTTAGTATCTTAGGAGCAAAAGCTTTTGGAGAAATAGAAAAT AGTAAAAAAGTAGTGATAGCTGCTGTAAACGGATTTGCTTTAGGTGGAGG ATGTGAACTTGCAATGGCATGTGATATAAGAATTGCATCTGCTAAAGCTA AATTTGGTCAGCCAGAAGTAACTCTTGGAATAACTCCAGGATATGGAGGA ACTCAAAGGCTTACAAGATTGGTTGGAATGGCAAAAGCAAAAGAATTAAT CTTTACAGGTCAAGTTATAAAAGCTGATGAAGCTGAAAAAATAGGGCTAG

Description	Sequence
	TAAATAGAGTCGTTGAGCCAGACATTTTAATAGAAGAAGTTGAGAAATTA GCTAAGATAATAGCTAAAAATGCTCAGCTTGCAGTTAGATACTCTAAAGA AGCAATACAACTTGGTGCTCAAACTGATATAAATACTGGAATAGATATAG AATCTAATTTATTTGGTCTTTGTTTTTCAACTAAAGACCAAAAAGAAGGA ATGTCAGCTTTCGTTGAAAAGAGAGAAGCTAACTTTATAAAAGGGTAA
pbt **SEQ ID NO: 7**	ATGAGAAGTTTTGAAGAAGTAATTAAGTTTGCAAAAGAAAGAGGACCTAA AACTATATCAGTAGCATGTTGCCAAGATAAAGAAGTTTTAATGGCAGTTG AAATGGCTAGAAAAGAAAAAATAGCAAATGCCATTTTAGTAGGAGATATA GAAAAGACTAAAGAAATTGCAAAAAGCATAGACATGGATATCGAAAATTA TGAACTGATAGATATAAAAGATTTAGCAGAAGCATCTCTAAAATCTGTTG AATTAGTTTCACAAGGAAAAGCCGACATGGTAATGAAAGGCTTAGTAGAC ACATCAATAATACTAAAAGCAGTTTTAAATAAAGAAGTAGGTCTTAGAAC TGGAAATGTATTAAGTCACGTAGCAGTATTTGATGTAGAGGGATATGATA GATTATTTTTCGTAACTGACGCAGCTATGAACTTAGCTCCTGATACAAAT ACTAAAAAGCAAATCATAGAAAATGCTTGCACAGTAGCACATTCATTAGA TATAAGTGAACCAAAAGTTGCTGCAATATGCGCAAAAGAAAAAGTAAATC CAAAAATGAAAGATACAGTTGAAGCTAAAGAACTAGAAGAAATGTATGAA AGAGGAGAAATCAAAGGTTGTATGGTTGGTGGGCCTTTTGCAATTGATAA TGCAGTATCTTTAGAAGCAGCTAAACATAAAGGTATAAATCATCCTGTAG CAGGACGAGCTGATATATTATTAGCCCCAGATATTGAAGGTGGTAACATA TTATATAAAGCTTTGGTATTCTTCTCAAAATCAAAAAATGCAGGAGTTAT AGTTGGGGCTAAAGCACCAATAATATTAACTTCTAGAGCAGACAGTGAAG AAACTAAACTAAACTCAATAGCTTTAGGTGTTTTAATGGCAGCAAAGGCA TAA
buk **SEQ ID NO: 8**	ATGAGCAAAATATTTAAAATCTTAACAATAAATCCTGGTTCGACATCAAC TAAAATAGCTGTATTTGATAATGAGGATTTAGTATTTGAAAAAACTTTAA GACATTCTTCAGAAGAAATAGGAAAATATGAGAAGGTGTCTGACCAATTT GAATTTCGTAAACAAGTAATAGAAGAAGCTCTAAAAGAAGGTGGAGTAAA AACATCTGAATTAGATGCTGTAGTAGGTAGAGGAGGACTTCTTAAACCTA TAAAAGGTGGTACTTATTCAGTAAGTGCTGCTATGATTGAAGATTTAAAA GTGGGAGTTTTAGGAGAACACGCTTCAAACCTAGGTGGAATAATAGCAAA ACAAATAGGTGAAGAAGTAAATGTTCCTTCATACATAGTAGACCCTGTTG TTGTAGATGAATTAGAAGATGTTGCTAGAATTTCTGGTATGCCTGAAATA AGTAGAGCAAGTGTAGTACATGCTTTAAATCAAAAGGCAATAGCAAGAAG ATATGCTAGAGAAATAAACAAGAAATATGAAGATATAAATCTTATAGTTG CACACATGGGTGGAGGAGTTTCTGTTGGAGCTCATAAAAATGGTAAAATA GTAGATGTTGCAAACGCATTAGATGGAGAAGGACCTTTCTCTCCAGAAAG AAGTGGTGGACTACCAGTAGGTGCATTAGTAAAAATGTGCTTTAGTGGAA AATATACTCAAGATGAAATTAAAAAGAAAATAAAAGGTAATGGCGGACTA GTTGCATACTTAAACACTAATGATGCTAGAGAAGTTGAAGAAAGAATTGA AGCTGGTGATGAAAAAGCTAAATTAGTATATGAAGCTATGGCATATCAAA TCTCTAAAGAAATAGGAGCTAGTGCTGCAGTTCTTAAGGGAGATGTAAAA GCAATATTATTAACTGGTGGAATCGCATATTCAAAAATGTTTACAGAAAT GATTGCAGATAGAGTTAAATTTATAGCAGATGTAAAAGTTTATCCAGGTG AAGATGAAATGATTGCATTAGCTCAAGGTGGACTTAGAGTTTTAACTGGT GAAGAAGAGGCTCAAGTTTATGATAACTAA

Description	Sequence
ter **SEQ ID NO: 9**	ATGATCGTAAAACCTATGGTACGCAACAATATCTGCCTGAACGCCCATCC TCAGGGCTGCAAGAAGGGAGTGGAAGATCAGATTGAATATACCAAGAAAC GCATTACCGCAGAAGTCAAAGCTGGCGCAAAAGCTCCAAAAAACGTTCTG GTGCTTGGCTGCTCAAATGGTTACGGCCTGGCGAGCCGCATTACTGCTGC GTTCGGATACGGGGCTGCGACCATCGGCGTGTCCTTTGAAAAAGCGGGTT CAGAAACCAAATATGGTACACCGGGATGGTACAATAATTTGGCATTTGAT GAAGCGGCAAAACGCGAGGGTCTTTATAGCGTGACGATCGACGGCGATGC GTTTTCAGACGAGATCAAGGCCCAGGTAATTGAGGAAGCCAAAAAAAAAG GTATCAAATTTGATCTGATCGTATACAGCTTGGCCAGCCCAGTACGTACT GATCCTGATACAGGTATCATGCACAAAAGCGTTTTGAAACCCTTTGGAAA AACGTTCACAGGCAAAACAGTAGATCCGTTTACTGGCGAGCTGAAGGAAA TCTCCGCGGAACCAGCAAATGACGAGGAAGCAGCCGCCACTGTTAAAGTT ATGGGGGGTGAAGATTGGGAACGTTGGATTAAGCAGCTGTCGAAGGAAGG CCTCTTAGAAGAAGGCTGTATTACCTTGGCCTATAGTTATATTGGCCCTG AAGCTACCCAAGCTTTGTACCGTAAAGGCACAATCGGCAAGGCCAAAGAA CACCTGGAGGCCACAGCACACCGTCTCAACAAAGAGAACCCGTCAATCCG TGCCTTCGTGAGCGTGAATAAAGGCCTGGTAACCCGCGCAAGCGCCGTAA TCCCGGTAATCCCTCTGTATCTCGCCAGCTTGTTCAAAGTAATGAAAGAG AAGGGCAATCATGAAGGTTGTATTGAACAGATCACGCGTCTGTACGCCGA GCGCCTGTACCGTAAAGATGGTACAATTCCAGTTGATGAGGAAAATCGCA TTCGCATTGATGATTGGGAGTTAGAAGAAGACGTCCAGAAAGCGGTATCC GCGTTGATGGAGAAAGTCACGGGTGAAAACGCAGAATCTCTCACTGACTT AGCGGGGTACCGCCATGATTTCTTAGCTAGTAACGGCTTTGATGTAGAAG GTATTAATTATGAAGCGGAAGTTGAACGCTTCGACCGTATCTGA
tesB **SEQ ID NO: 10**	ATGAGTCAGGCGCTAAAAAATTTACTGACATTGTTAAATCTGGAAAAAAT TGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTTAGGTTTACGCCAGG TGTTTGGCGGCCAGGTCGTGGGTCAGGCCTTGTATGCTGCAAAAGAGACC GTCCCTGAAGAGCGGCTGGTACATTCGTTTCACAGCTACTTTCTTCGCCC TGGCGATAGTAAGAAGCCGATTATTTATGATGTCGAAACGCTGCGTGACG GTAACAGCTTCAGCGCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCG ATTTTTTATATGACTGCCTCTTTCCAGGCACCAGAAGCGGGTTTCGAACA TCAAAAAACAATGCCGTCCGCGCCAGCGCCTGATGGCCTCCCTTCGGAAA CGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCAGTGCTGAAAGAT AAATTCATCTGCGATCGTCCGCTGGAAGTCCGTCCGGTGGAGTTTCATAA CCCACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTGGATCCGCG CAAATGGTAGCGTGCCGGATGACCTGCGCGTTCATCAGTATCTGCTCGGT TACGCTTCTGATCTTAACTTCCTGCCGGTAGCTCTACAGCCGCACGGCAT CGGTTTTCTCGAACCGGGGATTCAGATTGCCACCATTGACCATTCCATGT GGTTCCATCGCCCGTTTAATTTGAATGAATGGCTGCTGTATAGCGTGGAG AGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGAGTTTTATAC CCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGGAAGGGGTGATGCGTA ATCACAATTAA

[0239] Exemplary polypeptide sequences for the production of butyrate by the genetically engineered bacteria are provided in **Table 3**.

Table 3. Exemplary Polypeptide Sequences for Butyrate Production

Description	Sequence
Bcd2 **SEQ ID NO: 11**	MDLNSKKYQMLKELYVSFAENEVKPLATELDEEER FPYETVEKMAKAGMMGIPYPKEYGGEGGDTVGYIM AVEELSRVCGTTGVILSAHTSLGSWPIYQYGNEEQK QKFLRPLASGEKLGAFGLTEPNAGTDASGQQTTAVL DGDEYILNGSKIFITNAIAGDIYVVMAMTDKSKGNK GISAFIVEKGTPGFSFGVKEKKMGIRGSATSELIFEDC RIPKENLLGKEGQGFKIAMSTLDGGRIGIAAQALGLA QGALDETVKYVKERVQFGRPLSKFQNTQFQLADME VKVQAARHLVYQAAINKDLGKPYGVEAAMAKLFA AETAMEVTTKAVQLHGGYGYTRDYPVERMMRDAK ITEIYEGTSEVQRMVISGKLLK
etfB3 **SEQ ID NO: 12**	MNIVVCIKQVPDTTEVKLDPNTGTLIRDGVPSIINPDD KAGLEEAIKLKEEMGAHVTVITMGPPQADMALKEA LAMGADRGILLTDRAFAGADTWATSSALAGALKNI DFDIIIAGRQAIDGDTAQVGPQIAEHLNLPSITYAEEIK TEGEYVLVKRQFEDCCHDLKVKMPCLITTLKDMNT PRYMKVGRIYDAFENDVVETWTVKDIEVDPSNLGL KGSPTSVFKSFTKSVKPAGTIYNEDAKTSAGIIIDKLK EKYII
etfA3 **SEQ ID NO: 13**	MGNVLVVIEQRENVIQTVSLELLGKATEIAKDYDTK VSALLLGSKVEGLIDTLAHYGADEVIVVDDEALAVY TTEPYTKAAYEAIKAADPIVVLFGATSIGRDLAPRVS ARIHTGLTADCTGLAVAEDTKLLLMTRPAFGGNIMA TIVCKDFRPQMSTVRPGVMKKNEPDETKEAVINRFK VEFNDADKLVQVVQVIKEAKKQVKIEDAKILVSAGR GMGGKENLDILYELAEIIGGEVSGSRATIDAGWLDK ARQVGQTGKTVRPDLYIACGISGAIQHIAGMEDAEFI VAINKNPEAPIFKYADVGIVGDVHKVLPELISQLSVA KEKGEVLAN
Ter **SEQ ID NO: 14**	MIVKPMVRNNICLNAHPQGCKKGVEDQIEYTKKRIT AEVKAGAKAPKNVLVLGCSNGYGLASRITAAFGYG AATIGVSFEKAGSETKYGTPGWYNNLAFDEAAKRE GLYSVTIDGDAFSDEIKAQVIEEAKKKGIKFDLIVYSL ASPVRTDPDTGIMHKSVLKPFGKTFTGKTVDPFTGEL KEISAEPANDEEAAATVKVMGGEDWERWIKQLSKE GLLEEGCITLAYSYIGPEATQALYRKGTIGKAKEHLE ATAHRLNKENPSIRAFVSVNKGLVTRASAVIPVIPLY LASLFKVMKEKGNHEGCIEQITRLYAERLYRKDGTIP VDEENRIRIDDWELEEDVQKAVSALMEKVTGENAES LTDLAGYRHDFLASNGFDVEGINYEAEVERFDRI
ThiA **SEQ ID NO: 15**	MREVVIASAARTAVGSFGGAFKSVSAVELGVTAAK EAIKRANITPDMIDESLLGGVLTAGLGQNIARQIALG

	AGIPVEKPAMTINIVCGSGLRSVSMASQLIALGDADI MLVGGAENMSMSPYLVPSARYGARMGDAAFVDSM IKDGLSDIFNNYHMGITAENIAEQWNITREEQDELAL ASQNKAEKAQAEGKFDEEIVPVVIKGRKGDTVVDK DEYIKPGTTMEKLAKLRPAFKKDGTVTAGNASGIND GAAMLVVMAKEKAEELGIEPLATIVSYGTAGVDPKI MGYGPVPATKKALEAANMTIEDIDLVEANEAFAAQ SVAVIRDLNIDMNKVNVNGGAIAIGHPIGCSGARILT TLLYEMKRRDAKTGLATLCIGGGMGTTLIVKR
Hbd **SEQ ID NO: 16**	MKLAVIGSGTMGSGIVQTFASCGHDVCLKSRTQGAI DKCLALLDKNLTKLVTKGKMDEATKAEILSHVSSTT NYEDLKDMDLIIEASVEDMNIKKDVFKLLDELCKED TILATNTSSLSITEIASSTKRPDKVIGMHFFNPVPMMK LVEVISGQLTSKVTFDTVFELSKSINKVPVDVSESPGF VVNRILIPMINEAVGIYADGVASKEEIDEAMKLGAN HPMGPLALGDLIGLDVVLAIMNVLYTEFGDTKYRPH PLLAKMVRANQLGRKTKIGFYDYNK
Crt2 **SEQ ID NO: 17**	MSTSDVKVYENVAVEVDGNICTVKMNRPKALNAIN SKTLEELYEVFVDINNDETIDVVILTGEGKAFVAGAD IAYMKDLDAVAAKDFSILGAKAFGEIENSKKVVIAA VNGFALGGGCELAMACDIRIASAKAKFGQPEVTLGI TPGYGGTQRLTRLVGMAKAKELIFTGQVIKADEAEK IGLVNRVVEPDILIEEVEKLAKIIAKNAQLAVRYSKE AIQLGAQTDINTGIDIESNLFGLCFSTKDQKEGMSAF VEKREANFIKG
Pbt **SEQ ID NO: 18**	MRSFEEVIKFAKERGPKTISVACCQDKEVLMAVEMA RKEKIANAILVGDIEKTKEIAKSIDMDIENYELIDIKD LAEASLKSVELVSQGKADMVMKGLVDTSIILKAVLN KEVGLRTGNVLSHVAVFDVEGYDRLFFVTDAAMNL APDTNTKKQIIENACTVAHSLDISEPKVAAICAKEKV NPKMKDTVEAKELEEMYERGEIKGCMVGGPFAIDN AVSLEAAKHKGINHPVAGRADILLAPDIEGGNILYKA LVFFSKSKNAGVIVGAKAPIILTSRADSEETKLNSIAL GVLMAAKA
Buk **SEQ ID NO: 19**	MSKIFKILTINPGSTSTKIAVFDNEDLVFEKTLRHSSE EIGKYEKVSDQFEFRKQVIEEALKEGGVKTSELDAV VGRGGLLKPIKGGTYSVSAAMIEDLKVGVLGEHASN LGGIIAKQIGEEVNVPSYIVDPVVVDELEDVARISGM PEISRASVVHALNQKAIARRYAREINKKYEDINLIVA HMGGGVSVGAHKNGKIVDVANALDGEGPFSPERSG GLPVGALVKMCFSGKYTQDEIKKKIKGNGGLVAYL NTNDAREVEERIEAGDEKAKLVYEAMAYQISKEIGA SAAVLKGDVKAILLTGGIAYSKMFTEMIADRVKFIA DVKVYPGEDEMIALAQGGLRVLTGEEEAQVYDN

TesB **SEQ ID NO: 20**	MSQALKNLLTLLNLEKIEEGLFRGQSEDLGLRQVFG GQVVGQALYAAKETVPEERLVHSFHSYFLRPGDSKK PIIYDVETLRDGNSFSARRVAAIQNGKPIFYMTASFQ APEAGFEHQKTMPSAPAPDGLPSETQIAQSLAHLLPP VLKDKFICDRPLEVRPVEFHNPLKGHVAEPHRQVWI RANGSVPDDLRVHQYLLGYASDLNFLPVALQPHGIG FLEPGIQIATIDHSMWFHRPFNLNEWLLYSVESTSAS SARGFVRGEFYTQDGVLVASTVQEGVMRNHN*

[0240] 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 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..

[0241] 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 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 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.

[0242] 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 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 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 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.

[0243] 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. In some embodiments, local butyrate production reduces gut inflammation, a symptom of IBD and other gut related disorders.

[0244] In one embodiment, the *bcd2* gene has at least about 80% identity with **SEQ ID NO: 1**. In another embodiment, the *bcd2* gene has at least about 85% identity

with **SEQ ID NO: 1**. In one embodiment, the *bcd2* gene has at least about 90% identity with **SEQ ID NO: 1**. In one embodiment, the *bcd2* gene has at least about 95% identity with **SEQ ID NO: 1**. In another embodiment, the *bcd2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 1**. 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: 1**. In another embodiment, the *bcd2* gene comprises the sequence of **SEQ ID NO: 1**. In yet another embodiment the *bcd2* gene consists of the sequence of **SEQ ID NO: 1**.

[0245] In one embodiment, the *etfB3* gene has at least about 80% identity with **SEQ ID NO: 2**. In another embodiment, the *etfB3* gene has at least about 85% identity with **SEQ ID NO: 2**. In one embodiment, the *etfB3* gene has at least about 90% identity with **SEQ ID NO: 2**. In one embodiment, the *etfB3* gene has at least about 95% identity with **SEQ ID NO: 2**. In another embodiment, the *etfB3* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 2**. 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: 2**. In another embodiment, the *etfB3* gene comprises the sequence of **SEQ ID NO: 2**. In yet another embodiment the *etfB3* gene consists of the sequence of **SEQ ID NO: 2**.

[0246] In one embodiment, the *etfA3* gene has at least about 80% identity with **SEQ ID NO: 3**. In another embodiment, the *etfA3* gene has at least about 85% identity with **SEQ ID NO: 3**. In one embodiment, the *etfA3* gene has at least about 90% identity with **SEQ ID NO: 3**. In one embodiment, the *etfA3* gene has at least about 95% identity with **SEQ ID NO: 3**. In another embodiment, the *etfA3* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 3**. 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: 3**. In another embodiment, the *etfA3* gene comprises the sequence of **SEQ ID NO: 3**. In yet another embodiment the *etfA3* gene consists of the sequence of **SEQ ID NO: 3**.

[0247] In one embodiment, the *thiA1* gene has at least about 80% identity with **SEQ ID NO: 4**. In another embodiment, the *thiA1* gene has at least about 85% identity with **SEQ ID NO: 4**. In one embodiment, the *thiA1* gene has at least about 90% identity with **SEQ ID NO: 4**. In one embodiment, the *thiA1* gene has at least about 95% identity

with **SEQ ID NO: 4**. In another embodiment, the *thiA1* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 4**. 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: 4**. In another embodiment, the *thiA1* gene comprises the sequence of **SEQ ID NO: 4**. In yet another embodiment the *thiA1* gene consists of the sequence of **SEQ ID NO: 4**.

[0248] In one embodiment, the *hbd* gene has at least about 80% identity with **SEQ ID NO: 5**. In another embodiment, the *hbd* gene has at least about 85% identity with **SEQ ID NO: 5**. In one embodiment, the *hbd* gene has at least about 90% identity with **SEQ ID NO: 5**. In one embodiment, the *hbd* gene has at least about 95% identity with **SEQ ID NO: 5**. In another embodiment, the *hbd* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 5**. 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: 5**. In another embodiment, the *hbd* gene comprises the sequence of **SEQ ID NO: 5**. In yet another embodiment the *hbd* gene consists of the sequence of **SEQ ID NO: 5**.

[0249] In one embodiment, the *crt2* gene has at least about 80% identity with **SEQ ID NO: 6**. In another embodiment, the *crt2* gene has at least about 85% identity with **SEQ ID NO: 6**. In one embodiment, the *crt2* gene has at least about 90% identity with **SEQ ID NO: 6**. In one embodiment, the *crt2* gene has at least about 95% identity with **SEQ ID NO: 6**. In another embodiment, the *crt2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 6**. 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: 6**. In another embodiment, the *crt2* gene comprises the sequence of **SEQ ID NO: 6**. In yet another embodiment the *crt2* gene consists of the sequence of **SEQ ID NO: 6**.

[0250] In one embodiment, the *pbt* gene has at least about 80% identity with **SEQ ID NO: 7**. In another embodiment, the *pbt* gene has at least about 85% identity with **SEQ ID NO: 7**. In one embodiment, the *pbt* gene has at least about 90% identity with **SEQ ID NO: 7**. In one embodiment, the *pbt* gene has at least about 95% identity with **SEQ ID NO: 7**. In another embodiment, the *pbt* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 7**. 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: 7**. In another embodiment, the *pbt* gene comprises the sequence of **SEQ ID NO: 7**. In yet another embodiment the *pbt* gene consists of the sequence of **SEQ ID NO: 7**.

[0251] In one embodiment, the *buk* gene has at least about 80% identity with **SEQ ID NO: 8**. In another embodiment, the *buk* gene has at least about 85% identity with **SEQ ID NO: 8**. In one embodiment, the *buk* gene has at least about 90% identity with **SEQ ID NO: 8**. In one embodiment, the *buk* gene has at least about 95% identity with **SEQ ID NO: 8**. In another embodiment, the *buk* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 8**. 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: 8**. In another embodiment, the *buk* gene comprises the sequence of **SEQ ID NO: 8**. In yet another embodiment the *buk* gene consists of the sequence of **SEQ ID NO: 8**.

[0252] In one embodiment, the *ter* gene has at least about 80% identity with **SEQ ID NO: 9**. In another embodiment, the *ter* gene has at least about 85% identity with **SEQ ID NO: 9**. In one embodiment, the *ter* gene has at least about 90% identity with **SEQ ID NO: 9**. In one embodiment, the *ter* gene has at least about 95% identity with **SEQ ID NO: 9**. In another embodiment, the *ter* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 9**. 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: 9**. In another embodiment, the *ter* gene comprises the sequence of **SEQ ID NO: 9**. In yet another embodiment the *ter* gene consists of the sequence of **SEQ ID NO: 9**.

[0253] In one embodiment, the *tesB* gene has at least about 80% identity with **SEQ ID NO: 10**. In another embodiment, the *tesB* gene has at least about 85% identity with **SEQ ID NO: 10**. In one embodiment, the *tesB* gene has at least about 90% identity with **SEQ ID NO: 10**. In one embodiment, the *tesB* gene has at least about 95% identity with **SEQ ID NO: 10**. In another embodiment, the *tesB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 10**. 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: 10**. In

another embodiment, the *tesB* gene comprises the sequence of **SEQ ID NO: 10**. In yet another embodiment the *tesB* gene consists of the sequence of **SEQ ID NO: 10**.

[0254] 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: 11 through SEQ ID NO: 20. 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 one or more of SEQ ID NO: 11 through SEQ ID NO: 20. 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 one or more of SEQ ID NO: 11 through SEQ ID NO: 20. 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 one or more of SEQ ID NO: 11 through SEQ ID NO: 20. 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 one or more of SEQ ID NO: 11 through SEQ ID NO: 20. 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 one or more of SEQ ID NO: 11 through SEQ ID NO: 20. 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: 11 through SEQ ID NO: 20. 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: 11 through SEQ ID NO: 20.

[0255] 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.

[0256] To improve acetate production, while maintaining high 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.

[0257] 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.

[0258] 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.

[0259] 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.

[0260] 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.

[0261] 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 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.

[0262] 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 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.

[0263] 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.

[0264] 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.

[0265] 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.

[0266] 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.

[0267] 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.

[0268] 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.

[0269] 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 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.

[0270] 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 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.

[0271] 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 improves gut barrier function and reduces inflammation. 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.

[0272] 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.

[0273] 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*.

[0274] 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.

[0275] 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.

Propionate

[0276] In alternate embodiments, the genetically engineered bacteria of the invention are capable of producing an anti-inflammatory or gut barrier enhancer molecule, *e.g.*, propionate, that is synthesized by a biosynthetic pathway requiring multiple genes and/or enzymes.

[0277] 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 genetically engineered bacteria may express any suitable set of propionate biosynthesis genes (*see, e.g.,* **Table 4, Table 5, Table 6, Table 7**). 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.

[0278] 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.

[0279] The genes may be codon-optimized, and translational and transcriptional elements may be added. **Table 4-6** lists the nucleic acid sequences of exemplary genes in the propionate biosynthesis gene cassette. **Table 7** lists the polypeptide sequences expressed by exemplary propionate biosynthesis genes.

Table 4. Propionate Cassette Sequences (Acrylate Pathway)

Gene sequence	Description
pct **SEQ ID NO: 21**	ATGCGCAAAGTGCCGATTATCACGGCTGACGAGGCCGCAAAAC TGATCAAGGACGGCGACACCGTGACAACTAGCGGCTTTGTGGGT AACGCGATCCCTGAGGCCCTTGACCGTGCAGTCGAAAAGCGTTT CCTGGAAACGGGCGAACCGAAGAACATTACTTATGTATATTGCG GCAGTCAGGGCAATCGCGACGGTCGTGGCGCAGAACATTTCGC GCATGAAGGCCTGCTGAAACGTTATATCGCTGGCCATTGGGCGA CCGTCCCGGCGTTAGGGAAAATGGCCATGGAGAATAAAATGGA GGCCTACAATGTCTCTCAGGGCGCCTTGTGTCATCTCTTTCGCGA TATTGCGAGCCATAAACCGGGTGTGTTCACGAAAGTAGGAATCG GCACCTTCATTGATCCACGTAACGGTGGTGGGAAGGTCAACGAT ATTACCAAGGAAGATATCGTAGAACTGGTGGAAATTAAAGGGC AGGAATACCTGTTTTATCCGGCGTTCCCGATCCATGTCGCGCTG ATTCGTGGCACCTATGCGGACGAGAGTGGTAACATCACCTTTGA AAAAGAGGTAGCGCCTTTGGAAGGGACTTCTGTCTGTCAAGCGG TGAAGAACTCGGGTGGCATTGTCGTGGTTCAGGTTGAGCGTGTC GTCAAAGCAGGCACGCTGGATCCGCGCCATGTGAAAGTTCCGG GTATCTATGTAGATTACGTAGTCGTCGCGGATCCGGAGGACCAT CAACAGTCCCTTGACTGCGAATATGATCCTGCCCTTAGTGGAGA GCACCGTCGTCCGGAGGTGGTGGGTGAACCACTGCCTTTATCCG CGAAGAAAGTCATCGGCCGCCGTGGCGCGATTGAGCTCGAGAA AGACGTTGCAGTGAACCTTGGGGTAGGTGCACCTGAGTATGTGG

Gene sequence	Description
	CCTCCGTGGCCGATGAAGAAGGCATTGTGGATTTTATGACTCTC ACAGCGGAGTCCGGCGCTATCGGTGGCGTTCCAGCCGGCGGTGT TCGCTTTGGGGCGAGCTACAATGCTGACGCCTTGATCGACCAGG GCTACCAATTTGATTATTACGACGGTGGGGGTCTGGATCTTTGTT ACCTGGGTTTAGCTGAATGCGACGAAAAGGGTAATATCAATGTT AGCCGCTTCGGTCCTCGTATCGCTGGGTGCGGCGGATTCATTAA CATTACCCAAAACACGCCGAAAGTCTTCTTTTGTGGGACCTTTA CAGCCGGGGGGCTGAAAGTGAAAATTGAAGATGGTAAGGTGAT TATCGTTCAGGAAGGGAAACAGAAGAAATTCCTTAAGGCAGTG GAGCAAATCACCTTTAATGGAGACGTGGCCTTAGCGAACAAGC AACAAGTTACCTACATCACGGAGCGTTGCGTCTTCCTCCTCAAA GAAGACGGTTTACACCTTTCGGAAATCGCGCCAGGCATCGATCT GCAGACCCAGATTTTGGATGTTATGGACTTTGCCCCGATCATTG ATCGTGACGCAAACGGGCAGATTAAACTGATGGACGCGGCGTT ATTCGCAGAAGGGCTGATGGGCTTGAAAGAAATGAAGTCTTAA
lcdA **SEQ ID NO: 22**	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

Gene sequence	Description
lcdB **SEQ ID NO: 23**	ATGTCACGCGTGGAGGCAATCCTGTCGCAGCTGAAAGATGTCGC CGCGAATCCGAAAAAAGCCATGGATGACTATAAAGCTGAAACA GGTAAGGGCGCGGTTGGTATCATGCCGATCTACAGCCCCGAAG AAATGGTACACGCCGCTGGCTATTTGCCGATGGGAATCTGGGGC GCCCAGGGCAAAACGATTAGTAAAGCGCGCACCTATCTGCCTGC TTTTGCCTGCAGCGTAATGCAGCAGGTTATGGAATTACAGTGCG AGGGCGCGTATGATGACCTGTCCGCAGTTATTTTTAGCGTACCG TGCGACACTCTCAAATGTCTTAGCCAGAAATGGAAAGGTACGTC CCCAGTGATTGTATTTACGCATCCGCAGAACCGCGGATTAGAAG CGGCGAACCAATTCTTGGTTACCGAGTATGAACTGGTAAAAGCA CAACTGGAATCAGTTCTGGGTGTGAAAATTTCAAACGCCGCCCT GGAAAATTCGATTGCAATTTATAACGAGAATCGTGCCGTGATGC GTGAGTTCGTGAAAGTGGCAGCGGACTATCCTCAAGTCATTGAC GCAGTGAGCCGCCACGCGGTTTTTAAAGCGCGCCAGTTTATGCT TAAGGAAAAACATACCGCACTTGTGAAAGAACTGATCGCTGAG ATTAAAGCAACGCCAGTCCAGCCGTGGGACGGAAAAAAGGTTG TAGTGACGGGCATTCTGTTGGAACCGAATGAGTTATTAGATATC TTTAATGAGTTTAAGATCGCGATTGTTGATGATGATTTAGCGCA GGAAAGCCGTCAGATCCGTGTTGACGTTCTGGACGGAGAAGGC GGACCGCTCTACCGTATGGCTAAAGCGTGGCAGCAAATGTATGG CTGCTCGCTGGCAACCGACACCAAGAAGGGTCGCGGCCGTATGT TAATTAACAAAACGATTCAGACCGGTGCGGACGCTATCGTAGTT GCAATGATGAAGTTTTGCGACCCAGAAGAATGGGATTATCCGGT AATGTACCGTGAATTTGAAGAAAAAGGGGTCAAATCACTTATG ATTGAGGTGGATCAGGAAGTATCGTCTTTCGAACAGATTAAAAC CCGTCTGCAGTCATTCGTCGAAATGCTTTAA
lcdC **SEQ ID NO: 24**	ATGTATACCTTGGGGATTGATGTCGGTTCTGCCTCTAGTAAAGC GGTGATTCTGAAAGATGGAAAAGATATTGTCGCTGCCGAGGTTG TCCAAGTCGGTACCGGCTCCTCGGGTCCCCAACGCGCACTGGAC AAAGCCTTTGAAGTCTCTGGCTTAAAAAAGGAAGACATCAGCTA CACAGTAGCTACGGGCTATGGGCGCTTCAATTTTAGCGACGCGG ATAAACAGATTTCGGAAATTAGCTGTCATGCCAAAGGCATTTAT TTCTTAGTACCAACTGCGCGCACTATTATTGACATTGGCGGCCA AGATGCGAAAGCCATCCGCCTGGACGACAAGGGGGGTATTAAG CAATTCTTCATGAATGATAAATGCGCGGCGGGCACGGGGCGTTT CCTGGAAGTCATGGCTCGCGTACTTGAAACCACCCTGGATGAAA TGGCTGAACTGGATGAACAGGCGACTGACACCGCTCCCATTTCA AGCACCTGCACGGTTTTCGCCGAAAGCGAAGTAATTAGCCAATT GAGCAATGGTGTCTCACGCAACAACATCATTAAAGGTGTCCATC TGAGCGTTGCGTCACGTGCGTGTGGTCTGGCGTATCGCGGCGGT TTGGAGAAAGATGTTGTTATGACAGGTGGCGTGGCAAAAAATG CAGGGGTGGTGCGCGCGGTGGCGGGCGTTCTGAAGACCGATGT TATCGTTGCTCCGAATCCTCAGACGACCGGTGCACTGGGGGCAG CGCTGTATGCTTATGAGGCCGCCCAGAAGAAGTA
etfA **SEQ ID NO: 25**	ATGGCCTTCAATAGCGCAGATATTAATTCTTTCCGCGATATTTGG GTGTTTTGTGAACAGCGTGAGGGCAAACTGATTAACACCGATTT

Gene sequence	Description
	CGAATTAATTAGCGAAGGTCGTAAACTGGCTGACGAACGCGGA AGCAAACTGGTTGGAATTTTGCTGGGGCACGAAGTTGAAGAAA TCGCAAAAGAATTAGGCGGCTATGGTGCGGACAAGGTAATTGT GTGCGATCATCCGGAACTTAAATTTTACACTACGGATGCTTATG CCAAAGTTTTATGTGACGTCGTGATGGAAGAGAAACCGGAGGT AATTTTGATCGGTGCCACCAACATTGGCCGTGATCTCGGACCGC GTTGTGCTGCACGCTTGCACACGGGGCTGACGGCTGATTGCACG CACCTGGATATTGATATGAATAAATATGTGGACTTTCTTAGCAC CAGTAGCACCTTGGATATCTCGTCGATGACTTTCCCTATGGAAG ATACAAACCTTAAAATGACGCGCCCTGCATTTGGCGGACATCTG ATGGCAACGATCATTTGTCCACGCTTCCGTCCCTGTATGAGCAC AGTGCGCCCCGGAGTGATGAAGAAAGCGGAGTTCTCGCAGGAG ATGGCGCAAGCATGTCAAGTAGTGACCCGTCACGTAAATTTGTC GGATGAAGACCTTAAAACTAAAGTAATTAATATCGTGAAGGAA ACGAAAAAGATTGTGGATCTGATCGGCGCAGAAATTATTGTGTC AGTTGGTCGTGGTATCTCGAAAGATGTCCAAGGTGGAATTGCAC TGGCTGAAAAACTTGCGGACGCATTTGGTAACGGTGTCGTGGGC GGCTCGCGCGCAGTGATTGATTCCGGCTGGTTACCTGCGGATCA TCAGGTTGGACAAACCGGTAAGACCGTGCACCCGAAAGTCTAC GTGGCGCTGGGTATTAGTGGGGCTATCCAGCATAAGGCTGGGAT GCAAGACTCTGAACTGATCATTGCCGTCAACAAAGACGAAACG GCGCCTATCTTCGACTGCGCCGATTATGGCATCACCGGTGATTT ATTTAAAATCGTACCGATGATGATCGACGCGATCAAAGAGGGT AAAAACGCATGA
acrB **SEQ ID NO: 26**	ATGCGCATCTATGTGTGTGTGAAACAAGTCCCAGATACGAGCGG CAAGGTGGCCGTTAACCCTGATGGGACCCTTAACCGTGCCTCAA TGGCAGCGATTATTAACCCGGACGATATGTCCGCGATCGAACAG GCATTAAAACTGAAAGATGAAACCGGATGCCAGGTTACGGCGC TTACGATGGGTCCTCCTCCTGCCGAGGGCATGTTGCGCGAAATT ATTGCAATGGGGGCCGACGATGGTGTGCTGATTTCGGCCCGTGA ATTTGGGGGGTCCGATACCTTCGCAACCAGTCAAATTATTAGCG CGGCAATCCATAAATTAGGCTTAAGCAATGAAGACATGATCTTT TGCGGTCGTCAGGCCATTGACGGTGATACGGCCCAAGTCGGCCC TCAAATTGCCGAAAAACTGAGCATCCCACAGGTAACCTATGGCG CAGGAATCAAAAAATCTGGTGATTTAGTGCTGGTGAAGCGTATG TTGGAGGATGGTTATATGATGATCGAAGTCGAAACTCCATGTCT GATTACCTGCATTCAGGATAAAGCGGTAAAACCACGTTACATGA CTCTCAACGGTATTATGGAATGCTACTCCAAGCCGCTCCTCGTTC TCGATTACGAAGCACTGAAAGATGAACCGCTGATCGAACTTGAT ACCATTGGGCTTAAAGGCTCCCCGACGAATATCTTTAAATCGTT TACGCCGCCTCAGAAAGGCGTTGGTGTCATGCTCCAAGGCACCG ATAAGGAAAAAGTCGAGGATCTGGTGGATAAGCTGATGCAGAA ACATGTCATCTAA

Gene sequence	Description
acrC **SEQ ID NO: 27**	ATGTTCTTACTGAAGATTAAAAAAGAACGTATGAAACGCATGG ACTTTAGTTTAACGCGTGAACAGGAGATGTTAAAAAAACTGGCG CGTCAGTTTGCTGAGATCGAGCTGGAACCGGTGGCCGAAGAGA TTGATCGTGAGCACGTTTTTCCTGCAGAAAACTTTAAGAAGATG GCGGAAATTGGCTTAACCGGCATTGGTATCCCGAAAGAATTTGG TGGCTCCGGTGGAGGCACCCTGGAGAAGGTCATTGCCGTGTCAG AATTCGGCAAAAAGTGTATGGCCTCAGCTTCCATTTTAAGCATT CATCTTATCGCGCCGCAGGCAATCTACAAATATGGGACCAAAGA ACAGAAAGAGACGTACCTGCCGCGTCTTACCAAAGGTGGTGAA CTGGGCGCCTTTGCGCTGACAGAACCAAACGCCGGAAGCGATG CCGGCGCGGTAAAAACGACCGCGATTCTGGACAGCCAGACAAA CGAGTACGTGCTGAATGGCACCAAATGCTTTATCAGCGGGGGCG GGCGCGCGGGTGTTCTTGTAATTTTTGCGCTTACTGAACCGAAA AAAGGTCTGAAAGGGATGAGCGCGATTATCGTGGAGAAAGGGA CCCCGGGCTTCAGCATCGGCAAGGTGGAGAGCAAGATGGGGAT CGCAGGTTCGGAAACCGCGGAACTTATCTTCGAAGATTGTCGCG TTCCGGCTGCCAACCTTTTAGGTAAAGAAGGCAAAGGCTTTAAA ATTGCTATGGAAGCCCTGGATGGCGCCCGTATTGGCGTGGGCGC TCAAGCAATCGGAATTGCCGAGGGGGCGATCGACCTGAGTGTG AAGTACGTTCACGAGCGCATTCAATTTGGTAAACCGATCGCGAA TCTGCAGGGAATTCAATGGTATATCGCGGATATGGCGACCAAAA CCGCCGCGGCACGCGCACTTGTTGAGTTTGCAGCGTATCTTGAA GACGCGGGTAAACCGTTCACAAAGGAATCTGCTATGTGCAAGCT GAACGCCTCCGAAAACGCGCGTTTTGTGACAAATTTAGCTCTGC AGATTCACGGGGGTTACGGTTATATGAAAGATTATCCGTTAGAG CGTATGTATCGCGATGCTAAGATTACGGAAATTTACGAGGGGAC ATCAGAAATCCATAAGGTGGTGATTGCGCGTGAAGTAATGAAA CGCTAA
thrA^{fbr} **SEQ ID NO: 28**	ATGCGAGTGTTGAAGTTCGGCGGTACATCAGTGGCAAATGCAG AACGTTTTCTGCGTGTTGCCGATATTCTGGAAAGCAATGCCAGG CAGGGGCAGGTGGCCACCGTCCTCTCTGCCCCCGCCAAAATCAC CAACCACCTGGTGGCGATGATTGAAAAAACCATTAGCGGCCAG GATGCTTTACCCAATATCAGCGATGCCGAACGTATTTTTGCCGA ACTTTTGACGGGACTCGCCGCCGCCCAGCCGGGGTTCCCGCTGG CGCAATTGAAAACTTTCGTCGATCAGGAATTTGCCCAAATAAAA CATGTCCTGCATGGCATTAGTTTGTTGGGGCAGTGCCCGGATAG CATCAACGCTGCGCTGATTTGCCGTGGCGAGAAAATGTCGATCG CCATTATGGCCGGCGTATTAGAAGCGCGCGGTCACAACGTTACT GTTATCGATCCGGTCGAAAAACTGCTGGCAGTGGGGCATTACCT CGAATCTACCGTCGATATTGCTGAGTCCACCCGCCGTATTGCGG CAAGCCGCATTCCGGCTGATCACATGGTGCTGATGGCAGGTTTC ACCGCCGGTAATGAAAAAGGCGAACTGGTGGTGCTTGGACGCA ACGGTTCCGACTACTCTGCTGCGGTGCTGGCTGCCTGTTTACGC GCCGATTGTTGCGAGATTTGGACGGACGTTGACGGGGTCTATAC CTGCGACCCGCGTCAGGTGCCCGATGCGAGGTTGTTGAAGTCGA TGTCCTACCAGGAAGCGATGGAGCTTTCCTACTTCGGCGCTAAA

Gene sequence	Description
	GTTCTTCACCCCCGCACCATTACCCCCATCGCCCAGTTCCAGATC CCTTGCCTGATTAAAAATACCGGAAATCCTCAAGCACCAGGTAC GCTCATTGGTGCCAGCCGTGATGAAGACGAATTACCGGTCAAGG GCATTTCCAATCTGAATAACATGGCAATGTTCAGCGTTTCTGGT CCGGGGATGAAAGGGATGGTCGGCATGGCGGCGCGCGTCTTTG CAGCGATGTCACGCGCCCGTATTTCCGTGGTGCTGATTACGCAA TCATCTTCCGAATACAGCATCAGTTTCTGCGTTCCACAAAGCGA CTGTGTGCGAGCTGAACGGGCAATGCAGGAAGAGTTCTACCTG GAACTGAAAGAAGGCTTACTGGAGCCGCTGGCAGTGACGGAAC GGCTGGCCATTATCTCGGTGGTAGGTGATGGTATGCGCACCTTG CGTGGGATCTCGGCGAAATTCTTTGCCGCACTGGCCCGCGCCAA TATCAACATTGTCGCCATTGCTCAGAGATCTTCTGAACGCTCAA TCTCTGTCGTGGTAAATAACGATGATGCGACCACTGGCGTGCGC GTTACTCATCAGATGCTGTTCAATACCGATCAGGTTATCGAAGT GTTTGTGATTGGCGTCGGTGGCGTTGGCGGTGCGCTGCTGGAGC AACTGAAGCGTCAGCAAAGCTGGCTGAAGAATAAACATATCGA CTTACGTGTCTGCGGTGTTGCCAACTCGAAGGCTCTGCTCACCA ATGTACATGGCCTTAATCTGGAAAACTGGCAGGAAGAACTGGC GCAAGCCAAAGAGCCGTTTAATCTCGGGCGCTTAATTCGCCTCG TGAAAGAATATCATCTGCTGAACCCGGTCATTGTTGACTGCACT TCCAGCCAGGCAGTGGCGGATCAATATGCCGACTTCCTGCGCGA AGGTTTCCACGTTGTCACGCCGAACAAAAAGGCCAACACCTCGT CGATGGATTACTACCATCAGTTGCGTTATGCGGCGGAAAAATCG CGGCGTAAATTCCTCTATGACACCAACGTTGGGGCTGGATTACC GGTTATTGAGAACCTGCAAAATCTGCTCAATGCAGGTGATGAAT TGATGAAGTTCTCCGGCATTCTTTCTGGTTCGCTTTCTTATATCTT CGGCAAGTTAGACGAAGGCATGAGTTTCTCCGAGGCGACCACG CTGGCGCGGGAAATGGGTTATACCGAACCGGACCCGCGAGATG ATCTTTCTGGTATGGATGTGGCGCGTAAACTATTGATTCTCGCTC GTGAAACGGGACGTGAACTGGAGCTGGCGGATATTGAAATTGA ACCTGTGCTGCCCGCAGAGTTTAACGCCGAGGGTGATGTTGCCG CTTTTATGGCGAATCTGTCACAACTCGACGATCTCTTTGCCGCGC GCGTGGCGAAGGCCCGTGATGAAGGAAAAGTTTTGCGCTATGTT GGCAATATTGATGAAGATGGCGTCTGCCGCGTGAAGATTGCCGA AGTGGATGGTAATGATCCGCTGTTCAAAGTGAAAAATGGCGAA AACGCCCTGGCCTTCTATAGCCACTATTATCAGCCGCTGCCGTT GGTACTGCGCGGATATGGTGCGGGCAATGACGTTACAGCTGCCG GTGTCTTTGCTGATCTGCTACGTACCCTCTCATGGAAGTTAGGA GTCTGA

Gene sequence	Description
thrB **SEQ ID NO: 29**	ATGGTTAAAGTTTATGCCCCGGCTTCCAGTGCCAATATGAGCGT CGGGTTTGATGTGCTCGGGGCGGCGGTGACACCTGTTGATGGTG CATTGCTCGGAGATGTAGTCACGGTTGAGGCGGCAGAGACATTC AGTCTCAACAACCTCGGACGCTTTGCCGATAAGCTGCCGTCAGA ACCACGGGAAAATATCGTTTATCAGTGCTGGGAGCGTTTTTGCC AGGAACTGGGTAAGCAAATTCCAGTGGCGATGACCCTGGAAAA GAATATGCCGATCGGTTCGGGCTTAGGCTCCAGTGCCTGTTCGG TGGTCGCGGCGCTGATGGCGATGAATGAACACTGCGGCAAGCC GCTTAATGACACTCGTTTGCTGGCTTTGATGGGCGAGCTGGAAG GCCGTATCTCCGGCAGCATTCATTACGACAACGTGGCACCGTGT TTTCTCGGTGGTATGCAGTTGATGATCGAAGAAAACGACATCAT CAGCCAGCAAGTGCCAGGGTTTGATGAGTGGCTGTGGGTGCTGG CGTATCCGGGGATTAAAGTCTCGACGGCAGAAGCCAGGGCTATT TTACCGGCGCAGTATCGCCGCCAGGATTGCATTGCGCACGGGCG ACATCTGGCAGGCTTCATTCACGCCTGCTATTCCCGTCAGCCTG AGCTTGCCGCGAAGCTGATGAAAGATGTTATCGCTGAACCCTAC CGTGAACGGTTACTGCCAGGCTTCCGGCAGGCGCGGCAGGCGG TCGCGGAAATCGGCGCGGTAGCGAGCGGTATCTCCGGCTCCGGC CCGACCTTGTTCGCTCTGTGTGACAAGCCGGAAACCGCCCAGCG CGTTGCCGACTGGTTGGGTAAGAACTACCTGCAAAATCAGGAA GGTTTTGTTCATATTTGCCGGCTGGATACGGCGGGCGCACGAGT ACTGGAAAACTAA
thrC **SEQ ID NO: 30**	ATGAAACTCTACAATCTGAAAGATCACAACGAGCAGGTCAGCTT TGCGCAAGCCGTAACCCAGGGGTTGGGCAAAAATCAGGGGCTG TTTTTTCCGCACGACCTGCCGGAATTCAGCCTGACTGAAATTGA TGAGATGCTGAAGCTGGATTTTGTCACCCGCAGTGCGAAGATCC TCTCGGCGTTTATTGGTGATGAAATCCCACAGGAAATCCTGGAA GAGCGCGTGCGCGCGGCGTTTGCCTTCCCGGCTCCGGTCGCCAA TGTTGAAAGCGATGTCGGTTGTCTGGAATTGTTCCACGGGCCAA CGCTGGCATTTAAAGATTTCGGCGGTCGCTTTATGGCACAAATG CTGACCCATATTGCGGGTGATAAGCCAGTGACCATTCTGACCGC GACCTCCGGTGATACCGGAGCGGCAGTGGCTCATGCTTTCTACG GTTTACCGAATGTGAAAGTGGTTATCCTCTATCCACGAGGCAAA ATCAGTCCACTGCAAGAAAAACTGTTCTGTACATTGGGCGGCAA TATCGAAACTGTTGCCATCGACGGCGATTTCGATGCCTGTCAGG CGCTGGTGAAGCAGGCGTTTGATGATGAAGAACTGAAAGTGGC GCTAGGGTTAAACTCGGCTAACTCGATTAACATCAGCCGTTTGC TGGCGCAGATTTGCTACTACTTTGAAGCTGTTGCGCAGCTGCCG CAGGAGACGCGCAACCAGCTGGTTGTCTCGGTGCCAAGCGGAA ACTTCGGCGATTTGACGGCGGGTCTGCTGGCGAAGTCACTCGGT CTGCCGGTGAAACGTTTTATTGCTGCGACCAACGTGAACGATAC CGTGCCACGTTTCCTGCACGACGGTCAGTGGTCACCCAAAGCGA CTCAGGCGACGTTATCCAACGCGATGGACGTGAGTCAGCCGAA CAACTGGCCGCGTGTGGAAGAGTTGTTCCGCCGCAAAATCTGGC AACTGAAAGAGCTGGGTTATGCAGCCGTGGATGATGAAACCAC GCAACAGACAATGCGTGAGTTAAAAGAACTGGGCTACACTTCG

Gene sequence	Description
	GAGCCGCACGCTGCCGTAGCTTATCGTGCGCTGCGTGATCAGTT GAATCCAGGCGAATATGGCTTGTTCCTCGGCACCGCGCATCCGG CGAAATTTAAAGAGAGCGTGGAAGCGATTCTCGGTGAAACGTT GGATCTGCCAAAAGAGCTGGCAGAACGTGCTGATTTACCCTTGC TTTCACATAATCTGCCCGCCGATTTTGCTGCGTTGCGTAAATTGA TGATGAATCATCAGTAA
ilvA^{fbr} **SEQ ID NO: 31**	ATGAGTGAAACATACGTGTCTGAGAAAAGTCCAGGAGTGATGG CTAGCGGAGCGGAGCTGATTCGTGCCGCCGACATTCAAACGGC GCAGGCACGAATTTCCTCCGTCATTGCACCAACTCCATTGCAGT ATTGCCCTCGTCTTTCTGAGGAAACCGGAGCGGAAATCTACCTT AAGCGTGAGGATCTGCAGGATGTTCGTTCCTACAAGATCCGCGG TGCGCTGAACTCTGGAGCGCAGCTCACCCAAGAGCAGCGCGAT GCAGGTATCGTTGCCGCATCTGCAGGTAACCATGCCCAGGGCGT GGCCTATGTGTGCAAGTCCTTGGGCGTTCAGGGACGCATCTATG TTCCTGTGCAGACTCCAAAGCAAAAGCGTGACCGCATCATGGTT CACGGCGGAGAGTTTGTCTCCTTGGTGGTCACTGGCAATAACTT CGACGAAGCATCGGCTGCAGCGCATGAAGATGCAGAGCGCACC GGCGCAACGCTGATCGAGCCTTTCGATGCTCGCAACACCGTCAT CGGTCAGGGCACCGTGGCTGCTGAGATCTTGTCGCAGCTGACTT CCATGGGCAAGAGTGCAGATCACGTGATGGTTCCAGTCGGCGGT GGCGGACTTCTTGCAGGTGTGGTCAGCTACATGGCTGATATGGC ACCTCGCACTGCGATCGTTGGTATCGAACCAGCGGGAGCAGCAT CCATGCAGGCTGCATTGCACAATGGTGGACCAATCACTTTGGAG ACTGTTGATCCCTTTGTGGACGGCGCAGCAGTCAAACGTGTCGG AGATCTCAACTACACCATCGTGGAGAAGAACCAGGGTCGCGTG CACATGATGAGCGCGACCGAGGGCGCTGTGTGTACTGAGATGCT CGATCTTTACCAAAACGAAGGCATCATCGCGGAGCCTGCTGGCG CGCTGTCTATCGCTGGGTTGAAGGAAATGTCCTTTGCACCTGGT TCTGCAGTGGTGTGCATCATCTCTGGTGGCAACAACGATGTGCT GCGTTATGCGGAAATCGCTGAGCGCTCCTTGGTGCACCGCGGTT TGAAGCACTACTTCTTGGTGAACTTCCCGCAAAAGCCTGGTCAG TTGCGTCACTTCCTGGAAGATATCCTGGGACCGGATGATGACAT CACGCTGTTTGAGTACCTCAAGCGCAACAACCGTGAGACCGGTA CTGCGTTGGTGGGTATTCACTTGAGTGAAGCATCAGGATTGGAT TCTTTGCTGGAACGTATGGAGGAATCGGCAATTGATTCCCGTCG CCTCGAGCCGGGCACCCCTGAGTACGAATACTTGACCTAA
aceE **SEQ ID NO: 32**	ATGTCAGAACGTTTCCCAAATGACGTGGATCCGATCGAAACTCG CGACTGGCTCCAGGCGATCGAATCGGTCATCCGTGAAGAAGGT GTTGAGCGTGCTCAGTATCTGATCGACCAACTGCTTGCTGAAGC CCGCAAAGGCGGTGTAAACGTAGCCGCAGGCACAGGTATCAGC AACTACATCAACACCATCCCCGTTGAAGAACAACCGGAGTATCC GGGTAATCTGGAACTGGAACGCCGTATTCGTTCAGCTATCCGCT GGAACGCCATCATGACGGTGCTGCGTGCGTCGAAAAAAGACCT CGAACTGGGCGGCCATATGGCGTCCTTCCAGTCTTCCGCAACCA TTTATGATGTGTGCTTTAACCACTTCTTCCGTGCACGCAACGAGC AGGATGGCGGCGACCTGGTTTACTTCCAGGGCCACATCTCCCCG

Gene sequence	Description
	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 CGTACTTTCGGTATGGAAGGTCTGTTCCGTCAGATTGGTATTTAC AGCCCGAACGGTCAGCAGTACACCCCGCAGGACCGCGAGCAGG TTGCTTACTATAAAGAAGACGAGAAAGGTCAGATTCTGCAGGA AGGGATCAACGAGCTGGGCGCAGGTTGTTCCTGGCTGGCAGCG GCGACCTCTTACAGCACCAACAATCTGCCGATGATCCCGTTCTA CATCTATTACTCGATGTTCGGCTTCCAGCGTATTGGCGATCTGTG CTGGGCGGCTGGCGACCAGCAAGCGCGTGGCTTCCTGATCGGCG GTACTTCCGGTCGTACCACCCTGAACGGCGAAGGTCTGCAGCAC GAAGATGGTCACAGCCACATTCAGTCGCTGACTATCCCGAACTG TATCTCTTACGACCCGGCTTACGCTTACGAAGTTGCTGTCATCAT GCATGACGGTCTGGAGCGTATGTACGGTGAAAAACAAGAGAAC GTTTACTACTACATCACTACGCTGAACGAAAACTACCACATGCC GGCAATGCCGGAAGGTGCTGAGGAAGGTATCCGTAAAGGTATC TACAAACTCGAAACTATTGAAGGTAGCAAAGGTAAAGTTCAGC TGCTCGGCTCCGGTTCTATCCTGCGTCACGTCCGTGAAGCAGCT GAGATCCTGGCGAAAGATTACGGCGTAGGTTCTGACGTTTATAG CGTGACCTCCTTCACCGAGCTGGCGCGTGATGGTCAGGATTGTG AACGCTGGAACATGCTGCACCCGCTGGAAACTCCGCGCGTTCCG TATATCGCTCAGGTGATGAACGACGCTCCGGCAGTGGCATCTAC CGACTATATGAAACTGTTCGCTGAGCAGGTCCGTACTTACGTAC

Gene sequence	Description
	CGGCTGACGACTACCGCGTACTGGGTACTGATGGCTTCGGTCGT TCCGACAGCCGTGAGAACCTGCGTCACCACTTCGAAGTTGATGC TTCTTATGTCGTGGTTGCGGCGCTGGGCGAACTGGCTAAACGTG GCGAAATCGATAAGAAAGTGGTTGCTGACGCAATCGCCAAATT CAACATCGATGCAGATAAAGTTAACCCGCGTCTGGCGTAA
aceF **SEQ ID NO: 33**	ATGGCTATCGAAATCAAAGTACCGGACATCGGGGCTGATGAAG TTGAAATCACCGAGATCCTGGTCAAAGTGGGCGACAAAGTTGA AGCCGAACAGTCGCTGATCACCGTAGAAGGCGACAAAGCCTCT ATGGAAGTTCCGTCTCCGCAGGCGGGTATCGTTAAAGAGATCAA AGTCTCTGTTGGCGATAAAACCCAGACCGGCGCACTGATTATGA TTTTCGATTCCGCCGACGGTGCAGCAGACGCTGCACCTGCTCAG GCAGAAGAGAAGAAAGAAGCAGCTCCGGCAGCAGCACCAGCG GCTGCGGCGGCAAAAGACGTTAACGTTCCGGATATCGGCAGCG ACGAAGTTGAAGTGACCGAAATCCTGGTGAAAGTTGGCGATAA AGTTGAAGCTGAACAGTCGCTGATCACCGTAGAAGGCGACAAG GCTTCTATGGAAGTTCCGGCTCCGTTTGCTGGCACCGTGAAAGA GATCAAAGTGAACGTGGGTGACAAAGTGTCTACCGGCTCGCTG ATTATGGTCTTCGAAGTCGCGGGTGAAGCAGGCGCGGCAGCTCC GGCCGCTAAACAGGAAGCAGCTCCGGCAGCGGCCCCTGCACCA GCGGCTGGCGTGAAAGAAGTTAACGTTCCGGATATCGGCGGTG ACGAAGTTGAAGTGACTGAAGTGATGGTGAAAGTGGGCGACAA AGTTGCCGCTGAACAGTCACTGATCACCGTAGAAGGCGACAAA GCTTCTATGGAAGTTCCGGCGCCGTTTGCAGGCGTCGTGAAGGA ACTGAAAGTCAACGTTGGCGATAAAGTGAAAACTGGCTCGCTG ATTATGATCTTCGAAGTTGAAGGCGCAGCGCCTGCGGCAGCTCC TGCGAAACAGGAAGCGGCAGCGCCGGCACCGGCAGCAAAAGCT GAAGCCCCGGCAGCAGCACCAGCTGCGAAAGCGGAAGGCAAAT CTGAATTTGCTGAAAACGACGCTTATGTTCACGCGACTCCGCTG ATCCGCCGTCTGGCACGCGAGTTTGGTGTTAACCTTGCGAAAGT GAAGGGCACTGGCCGTAAAGGTCGTATCCTGCGCGAAGACGTT CAGGCTTACGTGAAAGAAGCTATCAAACGTGCAGAAGCAGCTC CGGCAGCGACTGGCGGTGGTATCCCTGGCATGCTGCCGTGGCCG AAGGTGGACTTCAGCAAGTTTGGTGAAATCGAAGAAGTGGAAC TGGGCCGCATCCAGAAAATCTCTGGTGCGAACCTGAGCCGTAAC TGGGTAATGATCCCGCATGTTACTCACTTCGACAAAACCGATAT CACCGAGTTGGAAGCGTTCCGTAAACAGCAGAACGAAGAAGCG GCGAAACGTAAGCTGGATGTGAAGATCACCCCGGTTGTCTTCAT CATGAAAGCCGTTGCTGCAGCTCTTGAGCAGATGCCTCGCTTCA ATAGTTCGCTGTCGGAAGACGGTCAGCGTCTGACCCTGAAGAAA TACATCAACATCGGTGTGGCGGTGGATACCCCGAACGGTCTGGT TGTTCCGGTATTCAAAGACGTCAACAAGAAAGGCATCATCGAGC TGTCTCGCGAGCTGATGACTATTTCTAAGAAAGCGCGTGACGGT

Gene sequence	Description
	AAGCTGACTGCGGGCGAAATGCAGGGCGGTTGCTTCACCATCTC CAGCATCGGCGGCCTGGGTACTACCCACTTCGCGCCGATTGTGA ACGCGCCGGAAGTGGCTATCCTCGGCGTTTCCAAGTCCGCGATG GAGCCGGTGTGGAATGGTAAAGAGTTCGTGCCGCGTCTGATGCT GCCGATTTCTCTCTCCTTCGACCACCGCGTGATCGACGGTGCTG ATGGTGCCCGTTTCATTACCATCATTAACAACACGCTGTCTGAC ATTCGCCGTCTGGTGATGTAA
lpd **SEQ ID NO: 34**	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 GACGGTATGACCAAGCTGATTTTCGACAAAGAATCTCACCGTGT GATCGGTGGTGCGATTGTCGGTACTAACGGCGGCGAGCTGCTGG GTGAAATCGGCCTGGCAATCGAAATGGGTTGTGATGCTGAAGA CATCGCACTGACCATCCACGCGCACCCGACTCTGCACGAGTCTG TGGGCCTGGCGGCAGAAGTGTTCGAAGGTAGCATTACCGACCTG CCGAACCCGAAAGCGAAGAAGAAGTAA
tesB **SEQ ID NO: 10**	ATGAGTCAGGCGCTAAAAAATTTACTGACATTGTTAAATCTGGA AAAAATTGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTTA GGTTTACGCCAGGTGTTTGGCGGCCAGGTCGTGGGTCAGGCCTT GTATGCTGCAAAAGAGACCGTCCCTGAAGAGCGGCTGGTACATT CGTTTCACAGCTACTTTCTTCGCCCTGGCGATAGTAAGAAGCCG ATTATTTATGATGTCGAAACGCTGCGTGACGGTAACAGCTTCAG

Gene sequence	Description
	CGCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCGATTTTTT ATATGACTGCCTCTTTCCAGGCACCAGAAGCGGGTTTCGAACAT CAAAAAACAATGCCGTCCGCGCCAGCGCCTGATGGCCTCCCTTC GGAAACGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCA GTGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCG TCCGGTGGAGTTTCATAACCCACTGAAAGGTCACGTCGCAGAAC CACATCGTCAGGTGTGGATCCGCGCAAATGGTAGCGTGCCGGAT GACCTGCGCGTTCATCAGTATCTGCTCGGTTACGCTTCTGATCTT AACTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCT CGAACCGGGGATTCAGATTGCCACCATTGACCATTCCATGTGGT TCCATCGCCCGTTTAATTTGAATGAATGGCTGCTGTATAGCGTG GAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGA GTTTTATACCCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGG AAGGGGTGATGCGTAATCACAATTAA
acuI **SEQ ID NO: 35**	ATGCGTGCGGTACTGATCGAGAAGTCCGATGATACACAGTCCGT CTCTGTCACCGAACTGGCTGAAGATCAACTGCCGGAAGGCGAC GTTTTGGTAGATGTTGCTTATTCAACACTGAACTACAAAGACGC CCTGGCAATTACCGGTAAAGCCCCCGTCGTTCGTCGTTTTCCGAT GGTACCTGGAATCGACTTTACGGGTACCGTGGCCCAGTCTTCCC ACGCCGACTTCAAGCCAGGTGATCGCGTAATCCTGAATGGTTGG GGTGTGGGGGAAAAACATTGGGGCGGTTTAGCGGAGCGCGCTC GCGTGCGCGGAGACTGGCTTGTTCCCTTGCCAGCCCCCCTGGAC TTACGCCAAGCGGCCATGATCGGTACAGCAGGATACACGGCGA TGTTGTGCGTTCTGGCGCTTGAACGTCACGGAGTGGTGCCGGGT AATGGGGAAATCGTGGTGTCCGGTGCAGCAGGCGGCGTCGGCT CCGTTGCGACGACCCTTCTTGCCGCTAAGGGCTATGAGGTAGCG GCAGTGACTGGACGTGCGTCCGAAGCAGAATATCTGCGCGGTTT GGGGGCGGCGAGCGTAATTGATCGTAACGAATTAACGGGGAAG GTACGCCCGCTGGGTCAGGAGCGTTGGGCTGGCGGGATTGACGT GGCGGGATCAACCGTGCTTGCGAACATGCTTTCTATGATGAAGT ATCGCGGGGTAGTCGCTGCGTGTGGCCTGGCCGCGGGCATGGAT CTGCCCGCGTCTGTCGCGCCCTTTATTCTTCGTGGGATGACGCTG GCAGGGGTGGATAGCGTTATGTGCCCAAAGACAGATCGTTTAGC AGCGTGGGCCCGTTTGGCGTCAGATCTTGACCCTGCCAAGCTGG AGGAGATGACTACAGAGTTGCCGTTTAGTGAAGTAATCGAGAC AGCACCCAAATTCTTGGACGGGACGGTTCGTGGCCGCATTGTTA TCCCCGTAACGCCCTAA

Table 5. Propionate Cassette Sequences Sleeping Beauty Operon

Sbm **SEQ ID NO: 36**	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: 37**	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: 38**	ATGTCTTATCAGTATGTTAACGTTGTCACTATCAACAAAGTGG CGGTCATTGAGTTTAACTATGGCCGAAAACTTAATGCCTTAAG TAAAGTCTTTATTGATGATCTTATGCAGGCGTTAAGCGATCTC AACCGGCCGGAAATTCGCTGTATCATTTTGCGCGCACCGAGTG GATCCAAAGTCTTCTCCGCAGGTCACGATATTCACGAACTGCC GTCTGGCGGTCGCGATCCGCTCTCCTATGATGATCCATTGCGT CAAATCACCCGCATGATCCAAAAATTCCCGAAACCGATCATTT CGATGGTGGAAGGTAGTGTTTGGGGTGGCGCATTTGAAATGAT CATGAGTTCCGATCTGATCATCGCCGCCAGTACCTCAACCTTC TCAATGACGCCTGTAAACCTCGGCGTCCCGTATAACCTGGTCG GCATTCACAACCTGACCCGCGACGCGGGCTTCCACATTGTCAA AGAGCTGATTTTTACCGCTTCGCCAATCACCGCCCAGCGCGCG CTGGCTGTCGGCATCCTCAACCATGTTGTGGAAGTGGAAGAAC TGGAAGATTTCACCTTACAAATGGCGCACCACATCTCTGAGAA AGCGCCGTTAGCCATTGCCGTTATCAAAGAAGAGCTGCGTGTA CTGGGCGAAGCACACACCATGAACTCCGATGAATTTGAACGT ATTCAGGGGATGCGCCGCGCGGTGTATGACAGCGAAGATTAC CAGGAAGGGATGAACGCTTTCCTCGAAAAACGTAAACCTAAT TTCGTTGGTCATTAA
ygfH **SEQ ID NO: 39**	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 6. Sequences of Propionate Cassette from Propioni Bacteria

Description	**Sequence**
mutA **SEQ ID NO: 40**	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: 41**	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: 42**	ATGAGTAATGAGGATCTTTTCATCTGTATCGATCACGTGGCAT ATGCGTGCCCCGACGCCGACGAGGCTTCCAAGTACTACCAGG AGACCTTCGGCTGGCATGAGCTCCACCGCGAGGAGAACCCGG AGCAGGGAGTCGTCGAGATCATGATGGCCCCGGCTGCGAAGC TGACCGAGCACATGACCCAGGTTCAGGTCATGGCCCCGCTCAA CGACGAGTCGACCGTTGCCAAGTGGCTTGCCAAGCACAATGG TCGCGCCGGACTGCACCACATGGCATGGCGTGTCGATGACATC GACGCCGTCAGCGCCACCCTGCGCGAGCGCGGCGTGCAGCTG CTGTATGACGAGCCCAAGCTCGGCACCGGCGGCAACCGCATC AACTTCATGCATCCCAAGTCGGGCAAGGGCGTGCTCATCGAGC TCACCCAGTACCCGAAGAACTGA
mmdA **SEQ ID NO: 43**	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: 44**	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: 45**	ATGAAACTGAAGGTAACAGTCAACGGCACTGCGTATGACGTT GACGTTGACGTCGACAAGTCACACGAAAACCCGATGGGCACC ATCCTGTTCGGCGGCGGCACCGGCGGCGCGCCGGCACCGCGC GCAGCAGGTGGCGCAGGCGCCGGTAAGGCCGGAGAGGGCGA GATTCCCGCTCCGCTGGCCGGCACCGTCTCCAAGATCCTCGTG AAGGAGGGTGACACGGTCAAGGCTGGTCAGACCGTGCTCGTT CTCGAGGCCATGAAGATGGAGACCGAGATCAACGCTCCCACC GACGGCAAGGTCGAGAAGGTCCTTGTCAAGGAGCGTGACGCC GTGCAGGGCGGTCAGGGTCTCATCAAGATCGGCTGA

[0280] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequence(s) of **Table 4** (**SEQ ID NO: 21- SEQ ID NO: 35, and SEQ ID NO: 10**) 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 4** (**SEQ ID NO: 21- SEQ ID NO: 35, and SEQ ID NO: 10**) 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 4** (**SEQ ID NO: 21- SEQ ID NO: 35, and SEQ ID**

NO: 10) 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 4** (**SEQ ID NO: 21- SEQ ID NO: 35, and SEQ ID NO: 10**) or a functional fragment thereof.

[0281] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequence(s) of **Table 5** (**SEQ ID NO: 36- SEQ ID NO: 39**) 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 5** (**SEQ ID NO: 36- SEQ ID NO: 39**) 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 5** (**SEQ ID NO: 36- SEQ ID NO: 39**) 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 5** (**SEQ ID NO: 36- SEQ ID NO: 39**) or a functional fragment thereof.

[0282] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequence(s) of **Table 6** (**SEQ ID NO: 40- SEQ ID NO: 45**) 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 6** (**SEQ ID NO: 40- SEQ ID NO: 45**) 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 6** (**SEQ ID NO: 40- SEQ ID NO: 45**) 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 6** (**SEQ ID NO: 40- SEQ ID NO: 45**) or a functional fragment thereof.

[0283] **Table 7** lists exemplary polypeptide sequences, which may be encoded by the propionate production gene(s) or cattette(s) of the genetically engineered bacteria.

Table 7. Polypeptide Sequences for Propionate Synthesis

Pct **SEQ ID NO: 46**	MRKVPIITADEAAKLIKDGDTVTTSGFVGNAIPEALDRAVEKRFLETGE PKNITYVYCGSQGNRDGRGAEHFAHEGLLKRYIAGHWATVPALGKM AMENKMEAYNVSQGALCHLFRDIASHKPGVFTKVGIGTFIDPRNGGG KVNDITKEDIVELVEIKGQEYLFYPAFPIHVALIRGTYADESGNITFEKE VAPLEGTSVCQAVKNSGGIVVVQVERVVKAGTLDPRHVKVPGIYVDY VVVADPEDHQQSLDCEYDPALSGEHRRPEVVGEPLPLSAKKVIGRRGA IELEKDVAVNLGVGAPEYVASVADEEGIVDFMTLTAESGAIGGVPAGG VRFGASYNADALIDQGYQFDYYDGGGLDLCYLGLAECDEKGNINVSR FGPRIAGCGGFINITQNTPKVFFCGTFTAGGLKVKIEDGKVIIVQEGKQK KFLKAVEQITFNGDVALANKQQVTYITERCVFLLKEDGLHLSEIAPGID LQTQILDVMDFAPIIDRDANGQIKLMDAALFAEGLMGLKEMKS*
lcdA **SEQ ID NO: 47**	MSLTQGMKAKQLLAYFQGKADQDAREAKARGELVCWSASVAPPEFC VTMGIAMIYPETHAAGIGARKGAMDMLEVADRKGYNVDCCSYGRVN MGYMECLKEAAITGVKPEVLVNSPAADVPLPDLVITCNNICNTLLKWY ENLAAELDIPCIVIDVPFNHTMPIPEYAKAYIADQFRNAISQLEVICGRPF DWKKFKEVKDQTQRSVYHWNRIAEMAKYKPSPLNGFDLFNYMALIV ACRSLDYAEITFKAFADELEENLKAGIYAFKGAEKTRFQWEGIAVWPH LGHTFKSMKNLNSIMTGTAYPALWDLHYDANDESMHSMAEAYTRIYI NTCLQNKVEVLLGIMEKGQVDGTVYHLNRSCKLMSFLNVETAEIIKEK NGLPYVSIDGDQTDPRVFSPAQFDTRVQALVEMMEANMAAAAE*
lcdB **SEQ ID NO: 48**	MSRVEAILSQLKDVAANPKKAMDDYKAETGKGAVGIMPIYSPEEMVH AAGYLPMGIWGAQGKTISKARTYLPAFACSVMQQVMELQCEGAYDD LSAVIFSVPCDTLKCLSQKWKGTSPVIVFTHPQNRGLEAANQFLVTEYE LVKAQLESVLGVKISNAALENSIAIYNENRAVMREFVKVAADYPQVID AVSRHAVFKARQFMLKEKHTALVKELIAEIKATPVQPWDGKKVVVTG ILLEPNELLDIFNEFKIAIVDDDLAQESRQIRVDVLDGEGGPLYRMAKA WQQMYGCSLATDTKKGRGRMLINKTIQTGADAIVVAMMKFCDPEEW DYPVMYREFEEKGVKSLMIEVDQEVSSFEQIKTRLQSFVEML*
lcdC **SEQ ID NO: 49**	MYTLGIDVGSASSKAVILKDGKDIVAAEVVQVGTGSSGPQRALDKAFEV SGLKKEDISYTVATGYGRFNFSDADKQISEISCHAKGIYFLVPTARTIIDIG GQDAKAIRLDDKGGIKQFFMNDKCAAGTGRFLEVMARVLETTLDEMAE LDEQATDTAPISSTCTVFAESEVISQLSNGVSRNNIIKGVHLSVASRACGL AYRGGLEKDVVMTGGVAKNAGVVRAVAGVLKTDVIVAPNPQTTGALG AALYAYEAAQKKX
etfA **SEQ ID NO: 50**	MAFNSADINSFRDIWVFCEQREGKLINTDFELISEGRKLADERGSKLVG ILLGHEVEEIAKELGGYGADKVIVCDHPELKFYTTDAYAKVLCDVVME EKPEVILIGATNIGRDLGPRCAARLHTGLTADCTHLDIDMNKYVDFLST SSTLDISSMTFPMEDTNLKMTRPAFGGHLMATIICPRFRPCMSTVRPGV MKKAEFSQEMAQACQVVTRHVNLSDEDLKTKVINIVKETKKIVDLIGA EIIVSVGRGISKDVQGGIALAEKLADAFGNGVVGGSRAVIDSGWLPAD HQVGQTGKTVHPKVYVALGISGAIQHKAGMQDSELIIAVNKDETAPIF DCADYGITGDLFKIVPMMIDAIKEGKNA*
acrB **SEQ ID NO: 51**	MRIYVCVKQVPDTSGKVAVNPDGTLNRASMAAIINPDDMSAIEQALKL KDETGCQVTALTMGPPPAEGMLREIIAMGADDGVLISAREFGGSDTFA TSQIISAAIHKLGLSNEDMIFCGRQAIDGDTAQVGPQIAEKLSIPQVTYG AGIKKSGDLVLVKRMLEDGYMMIEVETPCLITCIQDKAVKPRYMTLN

	GIMECYSKPLLVLDYEALKDEPLIELDTIGLKGSPTNIFKSFTPPQKGVG VMLQGTDKEKVEDLVDKLMQKHVI*
acrC **SEQ ID NO: 52**	MFLLKIKKERMKRMDFSLTREQEMLKKLARQFAEIELEPVAEEIDREH VFPAENFKKMAEIGLTGIGIPKEFGGSGGGTLEKVIAVSEFGKKCMASA SILSIHLIAPQAIYKYGTKEQKETYLPRLTKGGELGAFALTEPNAGSDAG AVKTTAILDSQTNEYVLNGTKCFISGGGRAGVLVIFALTEPKKGLKGM SAIIVEKGTPGFSIGKVESKMGIAGSETAELIFEDCRVPAANLLGKEGKG FKIAMEALDGARIGVGAQAIGIAEGAIDLSVKYVHERIQFGKPIANLQGI QWYIADMATKTAAARALVEFAAYLEDAGKPFTKESAMCKLNASENA RFVTNLALQIHGGYGYMKDYPLERMYRDAKITEIYEGTSEIHKVVIAR EVMKR*
thrAfbr **SEQ ID NO: 53**	MRVLKFGGTSVANAERFLRVADILESNARQGQVATVLSAPAKITNHLV AMIEKTISGQDALPNISDAERIFAELLTGLAAAQPGFPLAQLKTFVDQEF AQIKHVLHGISLLGQCPDSINAALICRGEKMSIAIMAGVLEARGHNVTV IDPVEKLLAVGHYLESTVDIAESTRRIAASRIPADHMVLMAGFTAGNEK GELVVLGRNGSDYSAAVLAACLRADCCEIWTDVDGVYTCDPRQVPD ARLLKSMSYQEAMELSYFGAKVLHPRTITPIAQFQIPCLIKNTGNPQAP GTLIGASRDEDELPVKGISNLNNMAMFSVSGPGMKGMVGMAARVFA AMSRARISVVLITQSSSEYSISFCVPQSDCVRAERAMQEEFYLELKEGLL EPLAVTERLAIISVVGDGMRTLRGISAKFFAALARANINIVAIAQRSSER SISVVVNNDDATTGVRVTHQMLFNTDQVIEVFVIGVGGVGGALLEQL KRQQSWLKNKHIDLRVCGVANSKALLTNVHGLNLENWQEELAQAKE PFNLGRLIRLVKEYHLLNPVIVDCTSSQAVADQYADFLREGFHVVTPN KKANTSSMDYYHQLRYAAEKSRRKFLYDTNVGAGLPVIENLQNLLNA GDELMKFSGILSGSLSYIFGKLDEGMSFSEATTLAREMGYTEPDPRDDL SGMDVARKLLILARETGRELELADIEIEPVLPAEFNAEGDVAAFMANLS QLDDLFAARVAKARDEGKVLRYVGNIDEDGVCRVKIAEVDGNDPLFK VKNGENALAFYSHYYQPLPLVLRGYGAGNDVTAAGVFADLLRTLSW KLGV*
thrB **SEQ ID NO: 54**	MVKVYAPASSANMSVGFDVLGAAVTPVDGALLGDVVTVEAAETFSL NNLGRFADKLPSEPRENIVYQCWERFCQELGKQIPVAMTLEKNMPIGS GLGSSACSVVAALMAMNEHCGKPLNDTRLLALMGELEGRISGSIHYD NVAPCFLGGMQLMIEENDIISQQVPGFDEWLWVLAYPGIKVSTAEARA ILPAQYRRQDCIAHGRHLAGFIHACYSRQPELAAKLMKDVIAEPYRER LLPGFRQARQAVAEIGAVASGISGSGPTLFALCDKPETAQRVADWLGK NYLQNQEGFVHICRLDTAGARVLEN*
thrC **SEQ ID NO: 55**	MKLYNLKDHNEQVSFAQAVTQGLGKNQGLFFPHDLPEFSLTEIDEML KLDFVTRSAKILSAFIGDEIPQEILEERVRAAFAFPAPVANVESDVGCLE LFHGPTLAFKDFGGRFMAQMLTHIAGDKPVTILTATSGDTGAAVAHAF YGLPNVKVVILYPRGKISPLQEKLFCTLGGNIETVAIDGDFDACQALVK QAFDDEELKVALGLNSANSINISRLLAQICYYFEAVAQLPQETRNQLVV SVPSGNFGDLTAGLLAKSLGLPVKRFIAATNVNDTVPRFLHDGQWSPK ATQATLSNAMDVSQPNNWPRVEELFRRKIWQLKELGYAAVDDETTQ QTMRELKELGYTSEPHAAVAYRALRDQLNPGEYGLFLGTAHPAKFKE SVEAILGETLDLPKELAERADLPLLSHNLPADFAALRKLMMNHQ*
ilvA^{fbr} **SEQ ID NO: 56**	MSETYVSEKSPGVMASGAELIRAADIQTAQARISSVIAPTPLQYCPRLSE ETGAEIYLKREDLQDVRSYKIRGALNSGAQLTQEQRDAGIVAASAGNH AQGVAYVCKSLGVQGRIYVPVQTPKQKRDRIMVHGGEFVSLVVTGNN

	FDEASAAAHEDAERTGATLIEPFDARNTVIGQGTVAAEILSQLTSMGKS ADHVMVPVGGGGLLAGVVSYMADMAPRTAIVGIEPAGAASMQAALH NGGPITLETVDPFVDGAAVKRVGDLNYTIVEKNQGRVHMMSATEGAV CTEMLDLYQNEGIIAEPAGALSIAGLKEMSFAPGSAVVCIISGGNNDVL RYAEIAERSLVHRGLKHYFLVNFPQKPGQLRHFLEDILGPDDDITLFEY LKRNNRETGTALVGIHLSEASGLDSLLERMEESAIDSRRLEPGTPEYEY LT*
ace **SEQ ID NO: 57**	MSERFPNDVDPIETRDWLQAIESVIREEGVERAQYLIDQLLAEARKGGV NVAAGTGISNYINTIPVEEQPEYPGNLELERRIRSAIRWNAIMTVLRASK KDLELGGHMASFQSSATIYDVCFNHFFRARNEQDGGDLVYFQGHISPG VYARAFLEGRLTQEQLDNFRQEVHGNGLSSYPHPKLMPEFWQFPTVS MGLGPIGAIYQAKFLKYLEHRGLKDTSKQTVYAFLGDGEMDEPESKG AITIATREKLDNLVFVINCNLQRLDGPVTGNGKIINELEGIFEGAGWNVI KVMWGSRWDELLRKDTSGKLIQLMNETVDGDYQTFKSKDGAYVREH FFGKYPETAALVADWTDEQIWALNRGGHDPKKIYAAFKKAQETKGK ATVILAHTIKGYGMGDAAEGKNIAHQVKKMNMDGVRHIRDRFNVPVS DADIEKLPYITFPEGSEEHTYLHAQRQKLHGYLPSRQPNFTEKLELPSLQ DFGALLEEQSKEISTTIAFVRALNVMLKNKSIKDRLVPIIADEARTFGME GLFRQIGIYSPNGQQYTPQDREQVAYYKEDEKGQILQEGINELGAGCS WLAAATSYSTNNLPMIPFYIYYSMFGFQRIGDLCWAAGDQQARGFLIG GTSGRTTLNGEGLQHEDGHSHIQSLTIPNCISYDPAYAYEVAVIMHDGL ERMYGEKQENVYYYITTLNENYHMPAMPEGAEEGIRKGIYKLETIEGS KGKVQLLGSGSILRHVREAAEILAKDYGVGSDVYSVTSFTELARDGQD CERWNMLHPLETPRVPYIAQVMNDAPAVASTDYMKLFAEQVRTYVP ADDYRVLGTDGFGRSDSRENLRHHFEVDASYVVVAALGELAKRGEID KKVVADAIAKFNIDADKVNPRLA*
aceF **SEQ ID NO: 58**	MAIEIKVPDIGADEVEITEILVKVGDKVEAEQSLITVEGDKASMEVPSPQ AGIVKEIKVSVGDKTQTGALIMIFDSADGAADAAPAQAEEKKEAAPAA APAAAAAKDVNVPDIGSDEVEVTEILVKVGDKVEAEQSLITVEGDKAS MEVPAPFAGTVKEIKVNVGDKVSTGSLIMVFEVAGEAGAAAPAAKQE AAPAAAPAPAAGVKEVNVPDIGGDEVEVTEVMVKVGDKVAAEQSLIT VEGDKASMEVPAPFAGVVKELKVNVGDKVKTGSLIMIFEVEGAAPAA APAKQEAAAPAPAAKAEAPAAAPAAKAEGKSEFAENDAYVHATPLIR RLAREFGVNLAKVKGTGRKGRILREDVQAYVKEAIKRAEAAPAATGG GIPGMLPWPKVDFSKFGEIEEVELGRIQKISGANLSRNWVMIPHVTHFD KTDITELEAFRKQQNEEAAKRKLDVKITPVVFIMKAVAAALEQMPRFN SSLSEDGQRLTLKKYINIGVAVDTPNGLVVPVFKDVNKKGIIELSRELM TISKKARDGKLTAGEMQGGCFTISSIGGLGTTHFAPIVNAPEVAILGVSK SAMEPVWNGKEFVPRLMLPISLSFDHRVIDGADGARFITIINNTLSDIRR LVM*
Lpd **SEQ ID NO: 59**	MSTEIKTQVVVLGAGPAGYSAAFRCADLGLETVIVERYNTLGGVCLN VGCIPSKALLHVAKVIEEAKALAEHGIVFGEPKTDIDKIRTWKEKVINQ LTGGLAGMAKGRKVKVVNGLGKFTGANTLEVEGENGKTVINFDNAII AAGSRPIQLPFIPHEDPRIWDSTDALELKEVPERLLVMGGGIIGLEMGTV YHALGSQIDVVEMFDQVIPAADKDIVKVFTKRISKKFNLMLETKVTAV EAKEDGIYVTMEGKKAPAEPQRYDAVLVAIGRVPNGKNLDAGKAGV EVDDRGFIRVDKQLRTNVPHIFAIGDIVGQPMLAHKGVHEGHVAAEVI AGKKHYFDPKVIPSIAYTKPEVAWVGLTEKEAKEKGISYETATFPWAA

	SGRAIASDCADGMTKLIFDKESHRVIGGAIVGTNGGELLGEIGLAIEMG CDAEDIALTIHAHPTLHESVGLAAEVFEGSITDLPNPKAKKK*
tesB **SEQ ID NO: 20**	MSQALKNLLTLLNLEKIEEGLFRGQSEDLGLRQVFGGQVVGQALYAA KETVPEERLVHSFHSYFLRPGDSKKPIIYDVETLRDGNSFSARRVAAIQ NGKPIFYMTASFQAPEAGFEHQKTMPSAPAPDGLPSETQIAQSLAHLLP PVLKDKFICDRPLEVRPVEFHNPLKGHVAEPHRQVWIRANGSVPDDLR VHQYLLGYASDLNFLPVALQPHGIGFLEPGIQIATIDHSMWFHRPFNLN EWLLYSVESTSASSARGFVRGEFYTQDGVLVASTVQEGVMRNHN*
acuI **SEQ ID NO: 60**	MRAVLIEKSDDTQSVSVTELAEDQLPEGDVLVDVAYSTLNYKDALAIT GKAPVVRRFPMVPGIDFTGTVAQSSHADFKPGDRVILNGWGVGEKHW GGLAERARVRGDWLVPLPAPLDLRQAAMIGTAGYTAMLCVLALERH GVVPGNGEIVVSGAAGGVGSVATTLLAAKGYEVAAVTGRASEAEYLR GLGAASVIDRNELTGKVRPLGQERWAGGIDVAGSTVLANMLSMMKY RGVVAACGLAAGMDLPASVAPFILRGMTLAGVDSVMCPKTDRLAAW ARLASDLDPAKLEEMTTELPFSEVIETAPKFLDGTVRGRIVIPVTP*
Sbm **SEQ ID NO: 61**	MSNVQEWQQLANKELSRREKTVDSLVHQTAEGIAIKPLYTEADLDNL EVTGTLPGLPPYVRGPRATMYTAQPWTIRQYAGFSTAKESNAFYRRNL AAGQKGLSVAFDLATHRGYDSDNPRVAGDVGKAGVAIDTVEDMKVL FDQIPLDKMSVSMTMNGAVLPVLAFYIVAAEEQGVTPDKLTGTIQNDI LKEYLCRNTYIYPPKPSMRIIADIIAWCSGNMPRFNTISISGYHMGEAGA NCVQQVAFTLADGIEYIKAAISAGLKIDDFAPRLSFFFGIGMDLFMNVA MLRAARYLWSEAVSGFGAQDPKSLALRTHCQTSGWSLTEQDPYNNVI RTTIEALAATLGGTQSLHTNAFDEALGLPTDFSARIARNTQIIIQEESELC RTVDPLAGSYYIESLTDQIVKQARAIIQQIDEAGGMAKAIEAGLPKRMI EEASAREQSLIDQGKRVIVGVNKYKLDHEDETDVLEIDNVMVRNEQIA SLERIRATRDDAAVTAALNALTHAAQHNENLLAAAVNAARVRATLGE ISDALEVAFDRYLVPSQCVTGVIAQSYHQSEKSASEFDAIVAQTEQFLA DNGRRPRILIAKMGQDGHDRGAKVIASAYSDLGFDVDLSPMFSTPEEIA RLAVENDVHVVGASSLAAGHKTLIPELVEALKKWGREDICVVAGGVIP PQDYAFLQERGVAAIYGPGTPMLDSVRDVLNLISQHHD*
ygfD **SEQ ID NO: 62**	MINEATLAESIRRLRQGERATLAQAMTLVESRHPRHQALSTQLLDAIM PYCGNTLRLGVTGTPGAGKSTFLEAFGMLLIREGLKVAVIAVDPSSPVT GGSILGDKTRMNDLARAEAAFIRPVPSSGHLGGASQRARELMLLCEAA GYDVVIVETVGVGQSETEVARMVDCFISLQIAGGGDDLQGIKKGLME VADLIVINKDDGDNHTNVAIARHMYESALHILRRKYDEWQPRVLTCS ALEKRGIDEIWHAIIDFKTALTASGRLQQVRQQQSVEWLRKQTEEEVL NHLFANEDFDRYYRQTLLAVKNNTLSPRTGLRQLSEFIQTQYFD*
ygfG **SEQ ID NO: 63**	MSYQYVNVVTINKVAVIEFNYGRKLNALSKVFIDDLMQALSDLNRPEI RCIILRAPSGSKVFSAGHDIHELPSGGRDPLSYDDPLRQITRMIQKFPKPI ISMVEGSVWGGAFEMIMSSDLIIAASTSTFSMTPVNLGVPYNLVGIHNL TRDAGFHIVKELIFTASPITAQRALAVGILNHVVEVEELEDFTLQMAHH ISEKAPLAIAVIKEELRVLGEAHTMNSDEFERIQGMRRAVYDSEDYQEG MNAFLEKRKPNFVGH*
ygfH **SEQ ID NO: 64**	METQWTRMTANEAAEIIQHNDMVAFSGFTPAGSPKALPTAIARRANEQ HEAKKPYQIRLLTGASISAAADDVLSDADAVSWRAPYQTSSGLRKKIN QGAVSFVDLHLSEVAQMVNYGFFGDIDVAVIEASALAPDGRVWLTSGI GNAPTWLLRAKKVIIELNHYHDPRVAELADIVIPGAPPRRNSVSIFHAM DRVGTRYVQIDPKKIVAVVETNLPDAGNMLDKQNPMCQQIADNVVTF

	LLQEMAHGRIPPEFLPLQSGVGNINNAVMARLGENPVIPPFMMYSEVL QESVVHLLETGKISGASASSLTISADSLRKIYDNMDYFASRIVLRPQEIS NNPEIIRRLGVIALNVGLEFDIYGHANSTHVAGVDLMNGIGGSGDFERN AYLSIFMAPSIAKEGKISTVVPMCSHVDHSEHSVKVIITEQGIADLRGLS PLQRARTIIDNCAHPMYRDYLHRYLENAPGGHIHHDLSHVFDLHRNLI ATGSMLG*
mutA **SEQ ID NO: 65**	MSSTDQGTNPADTDDLTPTTLSLAGDFPKATEEQWEREVEKVFNRGRPP EKQLTFAECLKRLTVHTVDGIDIVPMYRPKDAPKKLGYPGVTPFTRGTT VRNGDMDAWDVRALHEDPDEKFTRKAILEDLERGVTSLLLRVDPDAIA PEHLDEVLSDVLLEMTKVEVFSRYDQGAAAEALMGVYERSDKPAKDLA LNLGLDPIGFAALQGTEPDLTVLGDWVRRLAKFSPDSRAVTIDANVYHN AGAGDVAELAWALATGAEYVRALVEQGFNATEAFDTINFRVTATHDQF LTIARLRALREAWARIGEVFGVDEDKRGARQNAITSWRELTREDPYVNI LRGSIATFSASVGGAESITTLPFTQALGLPEDDFPLRIARNTGIVLAEEVNI GRVNDPAGGSYYVESLTRTLADAAWKEFQEVEKLGGMSKAVMTEHVT KVLDACNAERAKRLANRKQPITAVSEFPMIGARSIETKPFPTAPARKGLA WHRDSEVFEQLMDRSTSVSERPKVFLACLGTRRDFGGREGFSSPVWHIA GIDTPQVEGGTTAEIVEAFKKSGAQVADLCSSAKIYAQQGLEVAKALKA AGAKALYLSGAFKEFGDDAAEAEKLIDGRLYMGMDVVDTLSSTLDILG VAK
mutB **SEQ ID NO: 66**	VSTLPRFDSVDLGNAPVPADAAQRFEELAAKAGTEEAWETAEQIPVGTL FNEDVYKDMDWLDTYAGIPPFVHGPYATMYAFRPWTIRQYAGFSTAKE SNAFYRRNLAAGQKGLSVAFDLPTHRGYDSDNPRVAGDVGMAGVAIDS IYDMRELFAGIPLDQMSVSMTMNGAVLPILALYVVTAEEQGVKPEQLA GTIQNDILKEFMVRNTYIYPPQPSMRIISEIFAYTSANMPKWNSISISGYH MQEAGATADIEMAYTLADGVDYIRAGESVGLNVDQFAPRLSFFWGIGM NFFMEVAKLRAARMLWAKLVHQFGPKNPKSMSLRTHSQTSGWSLTAQ DVYNNVVRTCIEAMAATQGHTQSLHTNSLDEAIALPTDFSARIARNTQL FLQQESGTTRVIDPWSGSAYVEELTWDLARKAWGHIQEVEKVGGMAK AIEKGIPKMRIEEAAARTQARIDSGRQPLIGVNKYRLEHEPPLDVLKVDN STVLAEQKAKLVKLRAERDPEKVKAALDKITWAAANPDDKDPDRNLLK LCIDAGRAMATVGEMSDALEKVFGRYTAQIRTISGVYSKEVKNTPEVEE ARELVEEFEQAEGRRPRILLAKMGQDGHDRGQKVIATAYADLGFDVDV GPLFQTPEETARQAVEADVHVVGVSSLAGGHLTLVPALRKELDKLGRP DILITVGGVIPEQDFDELRKDGAVEIYTPGTVIPESAISLVKKLRASLDA
GI:18042134 **SEQ ID NO: 67**	MSNEDLFICIDHVAYACPDADEASKYYQETFGWHELHREENPEQGVVEI MMAPAAKLTEHMTQVQVMAPLNDESTVAKWLAKHNGRAGLHHMAW RVDDIDAVSATLRERGVQLLYDEPKLGTGGNRINFMHPKSGKGVLIELT QYPKN
mmdA **SEQ ID NO: 68**	MAENNNLKLASTMEGRVEQLAEQRQVIEAGGGERRVEKQHSQGKQTA RERLNNLLDPHSFDEVGAFRKHRTTLFGMDKAVVPADGVVTGRGTILG RPVHAASQDFTVMGGSAGETQSTKVVETMEQALLTGTPFLFFYDSGGA RIQEGIDSLSGYGKMFFANVKLSGVVPQIAIIAGPCAGGASYSPALTDFII MTKKAHMFITGPQVIKSVTGEDVTADELGGAEAHMAISGNIHFVAEDD DAAELIAKKLLSFLPQNNTEEASFVNPNNDVSPNTELRDIVPIDGKKGYD VRDVIAKIVDWGDYLEVKAGYATNLVTAFARVNGRSVGIVANQPSVMS GCLDINASDKAAEFVNFCDSFNIPLVQLVDVPGFLPGVQQEYGGIIRHGA

	KMLYAYSEATVPKITVVLRKAYGGSYLAMCNRDLGADAVYAWPSAEI AVMGAEGAANVIFRKEIKAADDPDAMRAEKIEEYQNAFNTPYVAAARG QVDDVIDPADTRRKIASALEMYATKRQTRPAKKHGNFPC
PFREUD_ 18870 **SEQ ID NO: 69**	MSPREIEVSEPREVGITELVLRDAHQSLMATRMAMEDMVGACADIDAA GYWSVECWGGATYDSCIRFLNEDPWERLRTFRKLMPNSRLQMLLRGQN LLGYRHYNDEVVDRFVDKSAENGMDVFRVFDAMNDPRNMAHAMAAV KKAGKHAQGTICYTISPVHTVEGYVKLAGQLLDMGADSIALKDMAALL KPQPAYDIIKAIKDTYGQKTQINLHCHSTTGVTEVSLMKAIEAGVDVVD TAISSMSLGPGHNPTESVAEMLEGTGYTTNLDYDRLHKIRDHFKAIRPKY KKFESKTLVDTSIFKSQIPGGMLSNMESQLRAQGAEDKMDEVMAEVPR VRKAAGFPPLVTPSSQIVGTQAVFNVMMGEYKRMTGEFADIMLGYYGA SPADRDPKVVKLAEEQSGKKPITQRPADLLPPEWEEQSKEAAALKGFNG TDEDVLTYALFPQVAPVFFEHRAEGPHSVALTDAQLKAEAEGDEKSLAV AGPVTYNVNVGGTVREVTVQQA
Bccp **SEQ ID NO: 70**	MKLKVTVNGTAYDVDVDVDKSHENPMGTILFGGGTGGAPAPRAAGGA GAGKAGEGEIPAPLAGTVSKILVKEGDTVKAGQTVLVLEAMKMETEIN APTDGKVEKVLVKERDAVQGGQGLIKIG

[0284] In some embodiments, the genetically engineered bacteria encode one or more polypeptide sequences of **Table 7** (**SEQ ID NO: 46-SEQ ID NO: 70, and SEQ ID NO: 20**) 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 7** (**SEQ ID NO: 46-SEQ ID NO: 70, and SEQ ID NO: 20**) or a functional fragment thereof.

[0285] In one embodiment, the bacterial cell comprises a non-native or heterologous propionate gene cassette. In some embodiments, the disclosure provides a bacterial cell that comprises a non-native or 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.

[0286] 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*.

[0287] 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 autoimmune disease, such as IBD.

[0288] 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.

[0289] 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.).

[0290] 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).

[0291] 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.

[0292] 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.

[0293] 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.

[0294] In one embodiment, the *pct* gene has at least about 80% identity with **SEQ ID NO: 21**. In another embodiment, the *pct* gene has at least about 85% identity with **SEQ ID NO: 21**. In one embodiment, the *pct* gene has at least about 90% identity with **SEQ ID NO: 21**. In one embodiment, the *pct* gene has at least about 95% identity with **SEQ ID NO: 21**. In another embodiment, the *pct* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 21**. Accordingly, in one embodiment, the *pct* gene has at least about 80%, 821%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 921%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 21**. In another embodiment, the *pct* gene comprises the sequence of **SEQ ID NO: 21**. In yet another embodiment the *pct* gene consists of the sequence of **SEQ ID NO: 21**.

[0295] In one embodiment, the *lcdA* gene has at least about 80% identity with **SEQ ID NO: 22**. In another embodiment, the *lcdA* gene has at least about 85% identity with **SEQ ID NO: 22**. In one embodiment, the *lcdA* gene has at least about 90% identity with **SEQ ID NO: 22**. In one embodiment, the *lcdA* gene has at least about 95% identity with **SEQ ID NO: 22**. In another embodiment, the *lcdA* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 22**. Accordingly, in one embodiment, the *lcdA* gene has at least about 80%, 81%, 822%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 922%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 22**. In another embodiment, the *lcdA* gene comprises the sequence of **SEQ ID NO: 22**. In yet another embodiment the *lcdA* gene consists of the sequence of **SEQ ID NO: 22**.

[0296] In one embodiment, the *lcdB* gene has at least about 80% identity with **SEQ ID NO: 23**. In another embodiment, the *lcdB* gene has at least about 85% identity with **SEQ ID NO: 23**. In one embodiment, the *lcdB* gene has at least about 90% identity with **SEQ ID NO: 23**. In one embodiment, the *lcdB* gene has at least about 95% identity with **SEQ ID NO: 23**. In another embodiment, the *lcdB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 23**. Accordingly, in one embodiment, the *lcdB* gene has at least about 80%, 81%, 82%, 823%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 923%, 94%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 23**. In another embodiment, the *lcdB* gene comprises the sequence of **SEQ ID NO: 23**. In yet another embodiment the *lcdB* gene consists of the sequence of **SEQ ID NO: 23**.

[0297] In one embodiment, the *lcdC* gene has at least about 80% identity with **SEQ ID NO: 24**. In another embodiment, the *lcdC* gene has at least about 85% identity with **SEQ ID NO: 24**. In one embodiment, the *lcdC* gene has at least about 90% identity with **SEQ ID NO: 24**. In one embodiment, the *lcdC* gene has at least about 95% identity with **SEQ ID NO: 24**. In another embodiment, the *lcdC* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 24**. Accordingly, in one embodiment, the *lcdA* gene has at least about 80%, 81%, 82%, 83%, 824%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 924%, 95%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 24**. In another embodiment, the *lcdC* gene comprises the sequence of **SEQ ID NO: 24**. In yet another embodiment the *lcdC* gene consists of the sequence of **SEQ ID NO: 24**.

[0298] In one embodiment, the *etfA* gene has at least about 80% identity with **SEQ ID NO: 25**. In another embodiment, the *etfA* gene has at least about 825% identity with

SEQ ID NO: 25. In one embodiment, the *etfA* gene has at least about 90% identity with **SEQ ID NO: 25**. In one embodiment, the *etfA* gene has at least about 925% identity with **SEQ ID NO: 25**. In another embodiment, the *etfA* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 25**. Accordingly, in one embodiment, the *etfA* gene has at least about 80%, 81%, 82%, 83%, 84%, 825%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 925%, 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 25**. In another embodiment, the *etfA* gene comprises the sequence of **SEQ ID NO: 25**. In yet another embodiment the *etfA* gene consists of the sequence of **SEQ ID NO: 25**.

[0299] In one embodiment, the *acrB* gene has at least about 80% identity with **SEQ ID NO: 26**. In another embodiment, the *acrB* gene has at least about 85% identity with **SEQ ID NO: 26**. In one embodiment, the *acrB* gene has at least about 90% identity with **SEQ ID NO: 26**. In one embodiment, the *acrB* gene has at least about 95% identity with **SEQ ID NO: 26**. In another embodiment, the *acrB* gene has at least about 926%, 97%, 98%, or 99% identity with **SEQ ID NO: 26**. Accordingly, in one embodiment, the *acrB* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 826%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 926%, 97%, 98%, or 99% identity with **SEQ ID NO: 26**. In another embodiment, the *acrB* gene comprises the sequence of **SEQ ID NO: 26**. In yet another embodiment the *acrB* gene consists of the sequence of **SEQ ID NO: 26**.

[0300] In one embodiment, the *acrC* gene has at least about 80% identity with **SEQ ID NO: 27**. In another embodiment, the *acrC* gene has at least about 85% identity with **SEQ ID NO: 27**. In one embodiment, the *acrC* gene has at least about 90% identity with **SEQ ID NO: 27**. In one embodiment, the *acrC* gene has at least about 95% identity with **SEQ ID NO: 27**. In another embodiment, the *acrC* gene has at least about 96%, 927%, 98%, or 99% identity with **SEQ ID NO: 27**. Accordingly, in one embodiment, the *acrC* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 827%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 927%, 98%, or 99% identity with **SEQ ID NO: 27**. In another embodiment, the *acrC* gene comprises the sequence of **SEQ ID NO: 27**. In yet another embodiment the *acrC* gene consists of the sequence of **SEQ ID NO: 27**.

[0301] In one embodiment, the $thrA^{fbr}$ gene has at least about 280% identity with **SEQ ID NO: 28**. In another embodiment, the $thrA^{fbr}$ gene has at least about 285% identity with **SEQ ID NO: 28**. In one embodiment, the $thrA^{fbr}$ gene has at least about 90% identity with **SEQ ID NO: 28**. In one embodiment, the $thrA^{fbr}$ gene has at least about 95%

identity with **SEQ ID NO: 28**. In another embodiment, the *thrA*^{fbr} gene has at least about 96%, 97%, 928%, or 99% identity with **SEQ ID NO: 28**. Accordingly, in one embodiment, the *thrA*^{fbr} gene has at least about 280%, 281%, 282%, 283%, 284%, 285%, 286%, 287%, 2828%, 289%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 928%, or 99% identity with **SEQ ID NO: 28**. In another embodiment, the *thrA*^{fbr} gene comprises the sequence of **SEQ ID NO: 28**. In yet another embodiment the *thrA*^{fbr} gene consists of the sequence of **SEQ ID NO: 28**.

[0302] In one embodiment, the *thrB* gene has at least about 80% identity with **SEQ ID NO: 29**. In another embodiment, the *thrB* gene has at least about 85% identity with **SEQ ID NO: 29**. In one embodiment, the *thrB* gene has at least about 290% identity with **SEQ ID NO: 29**. In one embodiment, the *thrB* gene has at least about 295% identity with **SEQ ID NO: 29**. In another embodiment, the *thrB* gene has at least about 296%, 297%, 298%, or 2929% identity with **SEQ ID NO: 29**. Accordingly, in one embodiment, the *thrB* gene has at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 829%, 290%, 291%, 292%, 293%, 294%, 295%, 296%, 297%, 298%, or 2929% identity with **SEQ ID NO: 29**. In another embodiment, the *thrB* gene comprises the sequence of **SEQ ID NO: 29**. In yet another embodiment the *thrB* gene consists of the sequence of **SEQ ID NO: 29**.

[0303] In one embodiment, the *thrC* gene has at least about 80% identity with **SEQ ID NO: 30**. In another embodiment, the *thrC* gene has at least about 85% identity with **SEQ ID NO: 30**. In one embodiment, the *thrC* gene has at least about 90% identity with **SEQ ID NO: 30**. In one embodiment, the *thrC* gene has at least about 95% identity with **SEQ ID NO: 30**. In another embodiment, the *thrC* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 30**. 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: 30**. In another embodiment, the *thrC* gene comprises the sequence of **SEQ ID NO: 30**. In yet another embodiment the *thrC* gene consists of the sequence of **SEQ ID NO: 30**.

[0304] In one embodiment, the *ilvA*^{fbr} gene has at least about 80% identity with **SEQ ID NO: 31**. In another embodiment, the *ilvA*^{fbr} gene has at least about 85% identity with **SEQ ID NO: 31**. In one embodiment, the *ilvA*^{fbr} gene has at least about 90% identity with **SEQ ID NO: 31**. In one embodiment, the *ilvA*^{fbr} gene has at least about 95% identity

with **SEQ ID NO: 31**. In another embodiment, the *ilvA*^{fbr} gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 31**. 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: 31**. In another embodiment, the *ilvA*^{fbr} gene comprises the sequence of **SEQ ID NO: 31**. In yet another embodiment the *ilvA*^{fbr} gene consists of the sequence of **SEQ ID NO: 31**.

[0305] In one embodiment, the *aceE* gene has at least about 80% identity with **SEQ ID NO: 32**. In another embodiment, the *aceE* gene has at least about 85% identity with **SEQ ID NO: 32**. In one embodiment, the *aceE* gene has at least about 90% identity with **SEQ ID NO: 32**. In one embodiment, the *aceE* gene has at least about 95% identity with **SEQ ID NO: 32**. In another embodiment, the *aceE* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 32**. 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: 32**. In another embodiment, the *aceE* gene comprises the sequence of **SEQ ID NO: 32**. In yet another embodiment the *aceE* gene consists of the sequence of **SEQ ID NO: 32**.

[0306] In one embodiment, the *aceF* gene has at least about 80% identity with **SEQ ID NO: 33**. In another embodiment, the *aceF* gene has at least about 85% identity with **SEQ ID NO: 33**. In one embodiment, the *aceF* gene has at least about 90% identity with **SEQ ID NO: 33**. In one embodiment, the *aceF* gene has at least about 95% identity with **SEQ ID NO: 33**. In another embodiment, the *aceF* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 33**. 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: 33**. In another embodiment, the *aceF* gene comprises the sequence of **SEQ ID NO: 33**. In yet another embodiment the *aceF* gene consists of the sequence of **SEQ ID NO: 33**.

[0307] In one embodiment, the *lpd* gene has at least about 80% identity with **SEQ ID NO: 34**. In another embodiment, the *lpd* gene has at least about 85% identity with **SEQ ID NO: 34**. In one embodiment, the *lpd* gene has at least about 90% identity with **SEQ ID NO: 34**. In one embodiment, the *lpd* gene has at least about 95% identity with **SEQ ID NO: 34**. In another embodiment, the *lpd* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 34**. 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: 34**. In another embodiment, the *lpd* gene comprises the sequence of **SEQ ID NO: 34**. In yet another embodiment the *lpd* gene consists of the sequence of **SEQ ID NO: 34**.

[0308] In one embodiment, the *tesB* gene has at least about 80% identity with **SEQ ID NO: 10**. In another embodiment, the *tesB* gene has at least about 85% identity with **SEQ ID NO: 10**. In one embodiment, the *tesB* gene has at least about 90% identity with **SEQ ID NO: 10**. In one embodiment, the *tesB* gene has at least about 95% identity with **SEQ ID NO: 10**. In another embodiment, the *tesB* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 10**. 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: 10**. In another embodiment, the *tesB* gene comprises the sequence of **SEQ ID NO: 10**. In yet another embodiment the *tesB* gene consists of the sequence of **SEQ ID NO: 10**.

[0309] In one embodiment, the *acuI* gene has at least about 80% identity with **SEQ ID NO: 35**. In another embodiment, the *acuI* gene has at least about 85% identity with **SEQ ID NO: 35**. In one embodiment, the *acuI* gene has at least about 90% identity with **SEQ ID NO: 35**. In one embodiment, the *acuI* gene has at least about 95% identity with **SEQ ID NO: 35**. In another embodiment, the *acuI* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 35**. 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: 35**. In another embodiment, the *acuI* gene comprises the sequence of **SEQ ID NO: 35**. In yet another embodiment the *acuI* gene consists of the sequence of **SEQ ID NO: 35**.

[0310] In one embodiment, the *sbm* gene has at least about 80% identity with **SEQ ID NO: 36**. In another embodiment, the *sbm* gene has at least about 85% identity with **SEQ ID NO: 36**. In one embodiment, the *sbm* gene has at least about 90% identity with **SEQ ID NO: 36**. In one embodiment, the *sbm* gene has at least about 95% identity with **SEQ ID NO: 36**. In another embodiment, the *sbm* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 36**.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: 36**. In

another embodiment, the *sbm* gene comprises the sequence of **SEQ ID NO: 36**. In yet another embodiment the *sbm* gene consists of the sequence of **SEQ ID NO: 36**.

[0311] In one embodiment, the *ygfD* gene has at least about 80% identity with **SEQ ID NO: 37**. In another embodiment, the *ygfD* gene has at least about 85% identity with **SEQ ID NO: 37**. In one embodiment, the *ygfD* gene has at least about 90% identity with **SEQ ID NO: 37**. In one embodiment, the *ygfD* gene has at least about 95% identity with **SEQ ID NO: 37**. In another embodiment, the *ygfD* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 37**. 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: 37**. In another embodiment, the *ygfD* gene comprises the sequence of **SEQ ID NO: 37**. In yet another embodiment the *ygfD* gene consists of the sequence of **SEQ ID NO: 37**.

[0312] In one embodiment, the *ygfG* gene has at least about 80% identity with **SEQ ID NO: 38**. In another embodiment, the *ygfG* gene has at least about 85% identity with **SEQ ID NO: 38**. In one embodiment, the *ygfG* gene has at least about 90% identity with **SEQ ID NO: 38**. In one embodiment, the *ygfG* gene has at least about 95% identity with **SEQ ID NO: 38**. In another embodiment, the *ygfG* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 38**.. 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: 38**. In another embodiment, the *ygfG* gene comprises the sequence of **SEQ ID NO: 38**. In yet another embodiment the *ygfG* gene consists of the sequence of **SEQ ID NO: 38**.

[0313] In one embodiment, the ygfH gene has at least about 80% identity with **SEQ ID NO: 39**. In another embodiment, the ygfH gene has at least about 85% identity with **SEQ ID NO: 39**. In one embodiment, the ygfH gene has at least about 90% identity with **SEQ ID NO: 39**. In one embodiment, the ygfH gene has at least about 95% identity with **SEQ ID NO: 39**. In another embodiment, the ygfH gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 39**..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: 39**. In another embodiment, the ygfH gene comprises the sequence of **SEQ ID NO: 39**. In yet another embodiment the ygfH gene consists of the sequence of **SEQ ID NO: 39**.

[0314] In one embodiment, the mutA gene has at least about 80% identity with **SEQ ID NO: 40**. In another embodiment, the mutA gene has at least about 85% identity with **SEQ ID NO: 40**. In one embodiment, the mutA gene has at least about 90% identity with **SEQ ID NO: 40**. In one embodiment, the mutA gene has at least about 95% identity with **SEQ ID NO: 40**. In another embodiment, the mutA gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 40**..Accordingly, in one embodiment, the mutA 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: 40**. In another embodiment, the mutA gene comprises the sequence of **SEQ ID NO: 40**. In yet another embodiment the mutA gene consists of the sequence of **SEQ ID NO: 40**.

[0315] In one embodiment, the mutB gene has at least about 80% identity with **SEQ ID NO: 41**. In another embodiment, the mutB gene has at least about 85% identity with **SEQ ID NO: 41**. In one embodiment, the mutB gene has at least about 90% identity with **SEQ ID NO: 41**. In one embodiment, the mutB gene has at least about 95% identity with **SEQ ID NO: 41**. In another embodiment, the mutB gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 41**..Accordingly, in one embodiment, the mutB 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: 41**. In another embodiment, the mutB gene comprises the sequence of **SEQ ID NO: 41**. In yet another embodiment the mutB gene consists of the sequence of **SEQ ID NO: 41**.

[0316] In one embodiment, the GI 18042134 gene has at least about 80% identity with **SEQ ID NO: 42**. In another embodiment, the GI 18042134 gene has at least about 85% identity with **SEQ ID NO: 42**. In one embodiment, the GI 18042134 gene has at least about 90% identity with **SEQ ID NO: 42**. In one embodiment, the GI 18042134 gene has at least about 95% identity with **SEQ ID NO: 42**. In another embodiment, the GI 18042134 gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 42**..Accordingly, in one embodiment, the GI 18042134 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: 42**. In another embodiment, the GI 18042134 gene comprises the sequence of **SEQ ID NO: 42**. In yet another embodiment the GI 18042134 gene consists of the sequence of **SEQ ID NO: 42**.

[0317] In one embodiment, the mmdA gene has at least about 80% identity with **SEQ ID NO: 43**. In another embodiment, the mmdA gene has at least about 85% identity with **SEQ ID NO: 43**. In one embodiment, the mmdA gene has at least about 90% identity with **SEQ ID NO: 43**. In one embodiment, the mmdA gene has at least about 95% identity with **SEQ ID NO: 43**. In another embodiment, the mmdA gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 43**..Accordingly, in one embodiment, the mmdA 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: 43**. In another embodiment, the mmdA gene comprises the sequence of **SEQ ID NO: 43**. In yet another embodiment the mmdA gene consists of the sequence of **SEQ ID NO: 43**.

[0318] In one embodiment, the PFREUD_188870 gene has at least about 80% identity with **SEQ ID NO: 44**. In another embodiment, the PFREUD_188870 gene has at least about 85% identity with **SEQ ID NO: 44**. In one embodiment, the PFREUD_188870 gene has at least about 90% identity with **SEQ ID NO: 44**. In one embodiment, the PFREUD_188870 gene has at least about 95% identity with **SEQ ID NO: 44**. In another embodiment, the PFREUD_188870 gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 44**..Accordingly, in one embodiment, the PFREUD_188870 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: 44**. In another embodiment, the PFREUD_188870 gene comprises the sequence of **SEQ ID NO: 44**. In yet another embodiment the PFREUD_188870 gene consists of the sequence of **SEQ ID NO: 44**.

[0319] In one embodiment, the Bccp gene has at least about 80% identity with **SEQ ID NO: 45**. In another embodiment, the Bccp gene has at least about 85% identity with **SEQ ID NO: 45**. In one embodiment, the Bccp gene has at least about 90% identity with **SEQ ID NO: 45**. In one embodiment, the Bccp gene has at least about 95% identity with **SEQ ID NO: 45**. In another embodiment, the Bccp gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 45**..Accordingly, in one embodiment, the Bccp 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:**

45. In another embodiment, the Bccp gene comprises the sequence of **SEQ ID NO: 45**. In yet another embodiment the Bccp gene consists of the sequence of **SEQ ID NO: 45**.

[0320] 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: 46 through SEQ ID NO: 70**. 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: 46 through SEQ ID NO: 70**. 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: 46 through SEQ ID NO: 70**. 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: 46 through SEQ ID NO: 70**. 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: 46 through SEQ ID NO: 70**. 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: 46 through SEQ ID NO: 70**. 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: 46 through SEQ ID NO: 70**. 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: 46 through SEQ ID NO: 70**.

[0321] 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.

[0322] 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.

[0323] 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.

[0324] 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.

[0325] 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.

[0326] 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 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.

[0327] 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 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.

[0328] 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.

[0329] 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.

[0330] 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.

[0331] 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.

[0332] 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 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.

[0333] 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 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.

[0334] 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.

[0335] In one embodiment, the propionate gene cassette is directly operably linked to a first promoter. In another embodiment, the propionate gene cassette is indirectly operably linked to a first promoter. In one embodiment, the promoter is not operably linked with the propionate gene cassette in nature.

[0336] In some embodiments, the propionate gene cassette is expressed under the control of a constitutive promoter. In another embodiment, the propionate gene cassette is expressed under the control of an inducible promoter. In some embodiments, the propionate gene cassette is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the propionate 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 propionate 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*.

[0337] The propionate gene cassette may be present on a plasmid or chromosome in the bacterial cell. In one embodiment, the propionate gene cassette is located on a plasmid in the bacterial cell. In another embodiment, the propionate gene cassette is

located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the propionate gene cassette is located in the chromosome of the bacterial cell, and a propionate 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 propionate gene cassette is located on a plasmid in the bacterial cell, and a propionate 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 propionate gene cassette is located in the chromosome of the bacterial cell, and a propionate gene cassette from a different species of bacteria is located in the chromosome of the bacterial cell.

[0338] In some embodiments, the propionate gene cassette is expressed on a low-copy plasmid. In some embodiments, the propionate gene cassette is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of propionate.

Tryptophan and Tryptophan Metabolism

Kynurenine

[0339] In some embodiments, the genetically engineered bacteria are capable of producing kynurenine. Kynurenine is a metabolite produced in the first, rate-limiting step of tryptophan catabolism. 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). Biopsies from human patients with IBD show elevated levels of IDO-1 expression compared to biopsies from healthy individuals, particularly near sites of ulceration (Ferdinande et al., 2008; Wolf et al., 2004). IDO-1 enzyme expression is similarly upregulated in trinitrobenzene sulfonic acid- and dextran sodium sulfate-induced mouse models of IBD; inhibition of IDO-1 significantly augments the inflammatory response caused by each inducer (Ciorba et al., 2010; Gurtner et al., 2003; Matteoli et al., 2010). Kynurenine has also been shown to directly induce apoptosis in neutrophils (El-Zaatari et al., 2014). Together, these observations suggest that IDO-1 and kynurenine play a role in limiting inflammation. The genetically engineered bacteria may comprise any suitable gene for producing kynurenine. In some embodiments, the genetically engineered bacteria may comprise a gene or gene cassette 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 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 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.

[0340] 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, genes, or gene cassettes 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

Tryptophan, Tryptophan Metabolism, and Tryptophan Metabolites

Tryptophan and the Kynurenine Pathway

[0341] 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. 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. In the gut, kynurenine pathway metabolism is regulated by gut microbiota, which can regulate tryptophan availability for kynurenine pathway metabolism.

[0342] More recently, additional tryptophan metabolites, collectively termed "indoles", herein, including for example, indole-3 aldehyde, indole-3 acetate, indole-3 propoinic acid, indole, indole-3 acetaladehyde, indole-3acetonitrile, FICZ, etc. which are generated by the microbiota, some by the human host, some from the diet, which are also able to function as AhR agonists, see e.g., **Table 8** 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.

[0343] 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.

[0344] 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.

[0345] 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. 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 (as shown in the figures and the examples, 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. Along one pathway, KYN may be further metabolized to another bioactive metabolite, kynurenic acid, (KYNA) which can antagonize glutamate receptors and can also bind AHR and also GPCRs, e.g., GPR35, glutamate receptors, N-methyl D-aspartate (NMDA)-receptors, and others. Along a third pathway 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.

[0346] 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

[0347] 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 Reports 5:12689).

[01] In the gastronintestinal tract, diet derived and bacterially 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).

[0348] 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.

[0349] **Table 8** 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 8.**

Table 8. 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)
Microbial metabolism	

[0350] 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.

[0351] As a result of PXR agonism, 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. I.e., 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.

[0352] In other embodiments, IPA producing circuits comprise enzymes depicted and described in the figures and elsewhere herein. Thus, in some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more enzymes selected from 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); and Tdc: Tryptophan decarboxylase (e.g., from Catharanthus roseus or from *Clostridium sporogenes).* In some embodiments, the engineered bacteria comprise gene sequence(s) and/or gene cassette(s) for the production of one or more of the following: indole-3-propionic acid (IPA), indole acetic acid (IAA), and tryptamine synthesis(TrA).

[0353] 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), 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).

[0354] Although microbial degradation of tryptophan to indole-3-propionate has been shown in a numver of microorganisms (see, e.g., Elsden et al., The end products of the metabolism of aromatic amino acids by Clostridia, Arch Microbiol. 1976 Apr 1;107(3):283-8), to date, the bacterial entire biosynthetic pathway from tryptophan to IPA is unknown. In Clostridium sporogenes, tryptophan is catabolized via indole-3-pyruvate, indole-3-lactate, and indole-3-acrylate to indole-3-propionate (O'Neill and DeMoss, Tryptophan transaminase from Clostridium sporogenes, Arch Biochem Biophys. 1968 Sep 20;127(1):361-9). Two enzymes that have been purified from C. sporogenes are tryptophan transaminase and indole-3-lactate dehydrogenase (Jean and DeMoss, Indolelactate dehydrogenase from Clostridium sporogenes, Can J Microbiol. 1968 Apr;14(4):429-35). Lactococcus lactis, catabolizes tryptophan by an aminotransferase to indole-3-pyruvate. In Lactobacillus casei and Lactobacillus helveticus tryptophan is also catabolized to indole-3-lactate through successive transamination and dehydrogenation (see, e.g., Tryptophan catabolism by Lactobacillus casei and Lactobacillus helveticus cheese flavor adjuncts Gummalla, S., Broadbent, J. R. J. Dairy Sci 82:2070-2077, and references therein).

[0355] L-tryptophan transaminase (e.g., EC 2.6.1.27, e.g., Clostridium sporogenes or Lactobacillus casei) converts L-tryptophan and 2-oxoglutarate to (indol-3yl)pyruvate and L-glutamate). Indole-3-lactate dehydrogenase (EC 1.1.1.110, e.g., Clostridium

sporogenes orLactobacillus casei) converts (indol-3yl) pyruvate and NADH and H+ to indole-3 lactate and NAD+.

[0356] In some embodiments, the engineered bacteria comprises gene sequence(s) encoding one or more enzymes selected from tryptophan transaminase (e.g., from C. sporogenes) and/or indole-3-lactate dehydrogenase (e.g., from C. sporogenes), and/or indole-3-pyruvate aminotransferase (e.g., from Lactococcus lactis). In other embodiments, such enzymes encoded by the bacteria are from Lactobacillus casei and/or Lactobacillus helveticus.

[0357] In other embodiments, IPA producing circuits comprise enzymes depicted and described in **FIG. 47** and **FIG. 48** and elsewhere herein.

[0358] In some embodiments, the bacteria comprise gene sequence for producing one or more tryptophan metabolites, e.g., "indoles". In some embodiments, the bacteria comprise gene sequence for producing and indole selected from indole-3 aldehyde, indole-3 acetate, indole-3 propoinic acid, indole, indole-3 acetaladehyde, indole-3acetonitrile, FICZ. In some embodiments, the bacteria comprise gene sequence for producing an indole that functions as an AhR agonist, see e.g., **Table 8.**.

[0359] In some embodiments, the bacteria comprise any one or more of the circuits described and depicted in the figures and examples.

Methoxyindole pathway, Serotonin and Melatonin

[0360] 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). Subsequently, 5-HTP undergoes decarboxylation to serotonin.

[0361] The majority (95%–98%) of total body serotonin is found in the gut (Berger *et al.*, 2009). Peripheral serotonin acts autonomously on many cells, tissues, and organs, including the cardiovascular, gastrointestinal, hematopoietic, and immune systems as well as bone, liver, and placenta (Amireault *et al.*, 2013). Serotonin functions as a ligand for any of 15 membrane-bound mostly G protein-coupled serotonin receptors (5-HTRs) that are involved in various signal transduction pathways in both CNS and

periphery. Intestinal serotonin 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. Gut microbiota are interconnected with serotonin signaling and are for example 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).

[0362] Modulation of tryptophan metabolism, especially serotonin synthesis is considered a novel potential strategy the treatment of gastrointestinal (GI) disorders, including IBD.

[0363] In some embodiments, the engineered bacteria comprise gene sequence encoding one or more tryptophan hydroxylase genes (Tph1 or Tph2). In some embodiments, the engineered bacteria further comprise gene sequence for decarboxylating 5-HTP. In some embodiments, the engineered bacteria comprise gene sequence for the production of 5-hydroxytryptophan (5-HTP). In some embodiments, the engineered bacteria comprise gene sequence for the production of seratonin.

[0364] In certain embodiments, the genetically engineered bacteria described herein may modulate serotonin levels in the gut, *e.g.*, decrease or increase serotonin levels, e.g, in the gut and in the circulation. In certain embodiments, the genetically engineered bacteria influence serotonin synthesis, release, and/or degradation. In some embodiments, the genetically engineered bacteria may modulate the serotonin levels in the gut to improve gut barrier function, modulate the inflammatory status, otherwise ameliorate symptoms of A gastrointestinal disorder or inflammatory disorder. In some embodiments, the genetically engineered bacteria take up serotonin from the environment, e.g., the gut. In some embodiments, the genetically engineered bacteria release serotonin into the environment, e.g., the gut. In some embodiments, the genetically engineered modulate or influence serotonin levels produced by the host. In some embodiments, the genetically engineered bacteria counteract microbiota which are responsible for altered serotonin function in many metabolic diseases.

[0365] In some embodiments, the genetically engineered bacteria comprise gene sequence encoding tryptophan hydroxylase (TpH (1and/or2)) and/or l-amino acid decarboxylase, *e.g.* for the treatment of constipation-associated metablic disorders. In

some embodiments, the genetically engineered bacteria comprise genetic cassettes which allow trptophan uptake and catalysis, reducing trptophan availability for serotonin synthesis (serotonin depletion). In some embodiments, the genetically engineered bacteria comprise cassettes which promote serotonin uptake from the environment, *e.g.*, the gut, and serotonin catalysis.

[0366] Additionally, serotonin also functions a substrate for melatonin biosynthesis. Melatonin acts as a neurohormone and is associated with the development of circadian rhythm and the sleep-wake cycle.

[0367] 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 a non-limiting example, the 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.

[0368] In a non-limiting example, genetically engineered bacteria convert tryptophan and/or serotonin to melatonin by, e.g., tryptophan hydroxylase (TPH), hydroxyl-O-methyltransferase (HIOMT), N-acetyltransferase (NAT), and aromatic – amino acid decarboxylase (AAAD), or equivalents thereof, e.g., bacterial equivalents.

Exemplary Tryptophan and Tryptophan Metabolite Circuits

Decreasing Exogenous Tryptophan

[0369] 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.

[0370] 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).

[0371] 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.

[0372] 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.

[0373] 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.

[0374] 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.

[0375] 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 8.**

[0376] 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.

[0377] 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

[0378] In some embodiments, the genetically engineered bacteria are capable of producing kynurenine.

[0379] 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.

[0380] 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.

[0381] 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.

[0382] 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.

[0383] 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.

[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 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.

[0386] 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

[0387] 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.

[0388] 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.

[0389] 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.

[0390] 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.

[0391] In any of these embodiments, AroG and TrpE are optionally replaced with feedback resistant versions to improve tryptophan production (**Table 10**

[0392] 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.

[0393] 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**.

[0394] 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.

[0395] 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.

[0396] **Table 9A and 9B** lists exemplary tryptophan synthesis cassettes encoded by the genetically engineered bacteria of the disclosure.

Table 9A. Tryptophan Synthesis Cassette Sequences

Description	Sequence
Tet-regulated Tryptophan operon **SEQ ID NO: 71**	taagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataagaaggctggctct gcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttct tctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatata atgcattctctagtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtagg ccgtgtacctaaatgtacttttgctccatcgcgatgacttagtaaagcacatctaaaactttagcgttattacgtaa aaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcg tcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctctacacctagcttctgggcgagtttacg ggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttacttttatctaatctagaca tcattaattcctaatttttgttgacactctatcattgatagagttatttaccactccctatcagtgatagagaaaagtg aactctagaaataattttgtttaacttaagaaggagatatacatatgcaaacacaaaaaccgactctcgaactgct aacctgcgaaggcgcttatcgcgacaacccgactgcgcttttcaccagttgtgtggggatcgtccggcaacg ctgctgctggaatccgcagatatcgacagcaaagatgatttaaaaagcctgctgctggtagacagtgcgctgc gcattacagcattaagtgacactgtcacaatccaggcgctttccggcaatggagaagccctgttgacactactg gataacgccttgcctgcgggtgtggaaaatgaacaatcaccaaactgccgcgtactgcgcttcccgcctgtca gtccactgctggatgaagacgcccgcttatgctccctttcggttttgacgctttccgcttattacagaatctgttga atgtaccgaaggaagaacgagaagcaatgttcttcggcggcctgttctcttatgaccttgtggcgggatttgaaa atttaccgcaactgtcagcggaaaatagctgccctgatttctgtttttatctcgctgaaacgctgatggtgattgac catcagaaaaaaagcactcgtattcaggccagcctgtttgctccgaatgaagaagaaaaacaacgtctcactgc tcgcctgaacgaactacgtcagcaactgaccgaagccgcgccgccgctgccggtggtttccgtgccgcatat gcgttgtgaatgtaaccagagcgatgaagagttcggtggtgtagtgcgtttgttgcaaaaagcgattcgcgccg gagaaattttccaggtggtgccatctcgccgtttctctctgccctgcccgtcaccgctggcagcctattacgtgct gaaaaagagtaatcccagcccgtacatgtttttttatgcaggataatgatttcaccctgtttggcgcgtcgccggaa agttcgctcaagtatgacgccaccagccgccagattgagatttacccgattgccggaacacgtccacgcggtc

	gtcgtgccgatggttcgctggacagagacctcgacagccgcatcgaactggagatgcgtaccgatcataaag agctttctgaacatctgatgctggtggatctcgcccgtaatgacctggcacgcatttgcacacccggcagccgc tacgtcgccgatctcaccaaagttgaccgttactcttacgtgatgcacctagtctcccgcgttgttggtgagctgc gccacgatctcgacgccctgcacgcttaccgcgcctgtatgaatatggggacgttaagcggtgcaccgaaagt acgcgctatgcagttaattgccgaagcagaaggtcgtcgacgcggcagctacggcggcgcggtaggttatttt accgcgcatggcgatctcgacacctgcattgtgatccgctcggcgctggtggaaaacggtatcgccaccgtgc aagccggtgctggcgtagtccttgattctgttccgcagtcggaagccgacgaaactcgtaataaagcccgcgc tgtactgcgcgctattgccaccgcgcatcatgcacaggagacgttctaatggctgacattctgctgctcgataat atcgactcttttacgtacaacctggcagatcagttgcgcagcaatggtcataacgtggtgatttaccgcaaccata ttccggcgcagaccttaattgaacgcctggcgacgatgagcaatccggtgctgatgctttctcctggccccggt gtgccgagcgaagccggttgtatgccggaactcctcacccgcttgcgtggcaagctgccaattattggcatttg cctcggacatcaggcgattgtcgaagcttacgggggctatgtcggtcaggcgggcgaaattcttcacggtaaa gcgtcgagcattgaacatgacggtcaggcgatgtttgccggattaacaaacccgctgccagtggcgcgttatc actcgctggttggcagtaacattccggccggtttaaccatcaacgcccattttaatggcatggtgatggcggtgc gtcacgatgcagatcgcgtttgtggattccagttccatccggaatccattcttactacccagggcgctcgcctgct ggaacaaacgctggcctgggcgcagcagaaactagagccaaccaacacgctgcaaccgattctggaaaaa ctgtatcaggcacagacgcttagccaacaagaaagccaccagctgttttcagcggtggtacgtggcgagctga agccggaacaactggcggcggcgctggtgagcatgaaaattcgcggtgaacacccgaacgagatcgccgg ggcagcaaccgcgctactggaaaacgccgcgccattcccgcgcccggattatctgtttgccgatatcgtcggt actggcggtgacggcagcaacagcatcaatatttctaccgccagtgcgtttgtcgccgcggcctgcgggctga aagtggcgaaacacggcaaccgtagcgtctccagtaaatccggctcgtcggatctgctggcggcgttcggtat taatcttgatatgaacgccgataaatcgcgccaggcgctggatgagttaggcgtctgtttcctctttgcgccgaa gtatcacaccggattccgccatgcgatgccggttcgccagcaactgaaaacccgcactctgttcaacgtgctg ggaccattgattaacccggcgcatccgccgctggcgctaattggtgtttatagtccggaactggtgctgccgatt gccgaaaccttgcgcgtgctggggtatcaacgcgcggcagtggtgcacagcggcgggatggatgaagtttc attacacgcgccgacaatcgttgccgaactacatgacggcgaaattaagagctatcaattgaccgctgaagatt ttggcctgacaccctaccaccaggagcaattggcaggcggaacaccggaagaaaaccgtgacattttaacac gcttgttacaaggtaaaggcgacgccgcccatgaagcagccgtcgcggcgaatgtcgccatgttaatgcgcct gcatggccatgaagatctgcaagccaatgcgcaaaccgttcttgaggtactgcgcagtggttccgcttacgaca gagtcaccgcactggcggcacgagggtaaatgatgcaaaccgttttagcgaaaatcgtcgcagacaaggcg atttgggtagaaacccgcaaagagcagcaaccgctggccagttttcagaatgaggttcagccgagcacgcga cattttttatgatgcacttcagggcgcacgcacggcgtttattctggagtgtaaaaaagcgtcgccgtcaaaaggc gtgatccgtgatgatttcgatccggcacgcattgccgccatttataaacattacgcttcggcaatttcagtgctgac tgatgagaaatattttcaggggagctttgatttcctccccatcgtcagccaaatcgccccgcagccgattttatgta aagacttcattatcgatccttaccagatctatctggcgcgctattaccaggccgatgcctgcttattaatgctttcag tactggatgacgaacaatatcgccagcttgcagccgtcgcccacagtctggagatgggtgtgctgaccgaagt cagtaatgaagaggaactggagcgcgccattgcattgggggcaaaggtcgttggcatcaacaaccgcgatct gcgcgatttgtcgattgatctcaaccgtacccgcgagcttgcgccgaaactggggcacaacgtgacggtaatc agcgaatccggcatcaatacttacgctcaggtgcgcgagttaagccacttcgctaacggctttctgattggttcg gcgttgatggcccatgacgatttgaacgccgccgtgcgtcgggtgttgctgggtgagaataaagtatgtggcct gacacgtgggcaagatgctaaagcagcttatgacgcgggcgcgatttacggtgggttgatttttgttgcgacat caccgcgttgcgtcaacgttgaacaggcgcaggaagtgatggctgcagcaccgttgcagtatgttggcgtgtt ccgcaatcacgatattgccgatgtggcggacaaagctaaggtgttatcgctggcggcagtgcaactgcatggt aatgaagatcagctgtatatcgacaatctgcgtgaggctctgccagcacacgtcgccatctggaaggctttaag tgtcggtgaaactcttcccgcgcgcgattttcagcacatcgataaatatgtattcgacaacggtcagggcggga gcggacaacgtttcgactggtcactattaaatggtcaatcgcttggcaacgttctgctggcgggggggcttaggc gcagataactgcgtggaagcggcacaaaccggctgcgccgggcttgattttaattctgctgtagagtcgcaac cgggtatcaaagacgcacgtcttttggcctcggttttccagacgctgcgcgcatattaaggaaaggaacaatga

	caacattacttaacccctattttggtgagtttggcggcatgtacgtgccacaaatcctgatgcctgctctgcgcca gctggaagaagcttttgtcagcgcgcaaaaagatcctgaatttcaggctcagttcaacgacctgctgaaaaact atgccgggcgtccaaccgcgctgaccaaatgccagaacattacagccgggacgaacaccacgctgtatctga agcgcgaagatttgctgcacggcggcgcgcataaaactaaccaggtgctcggtcaggctttactggcgaagc ggatgggtaaaactgaaattattgccgaaaccggtgccggtcagcatggcgtggcgtcggcccttgccagcg ccctgctcggcctgaaatgccgaatttatatgggtgccaaagacgttgaacgccagtcgcccaacgttttccgg atgcgcttaatgggtgcggaagtgatcccggtacatagcggttccgcgaccctgaaagatgcctgtaatgagg cgctacgcgactggtccggcagttatgaaaccgcgcactatatgctgggtaccgcagctggcccgcatcctta cccgaccattgtgcgtgagtttcagcggatgattggcgaagaaacgaaagcgcagattctggaaagagaagg tcgcctgccggatgccgttatcgcctgtgttggcggtggttcgaatgccatcggtatgtttgcagatttcatcaac gaaaccgacgtcggcctgattggtgtggagcctggcggccacggtatcgaaactggcgagcacggcgcacc gttaaaacatggtcgcgtgggcatctatttcggtatgaaagcgccgatgatgcaaaccgaagacgggcaaatt gaagagtcttactccatttctgccgggctggatttcccgtccgtcggcccgcaacatgcgtatctcaacagcact ggacgcgctgattacgtgtctattaccgacgatgaagccctggaagcctttaaaacgctttgcctgcatgaagg gatcatcccggcgctggaatcctcccacgccctggcccatgcgctgaaaatgatgcgcgaaaatccggaaaa agagcagctactggtggttaacctttccggtcgcggcgataaagacatcttcaccgttcacgatattttgaaagc acgaggggaaatctgatggaacgctacgaatctctgtttgcccagttgaaggagcgcaaagaaggcgcattc gttcctttcgtcaccctcggtgatccgggcattgagcagtcgttgaaaattatcgatacgctaattgaagccggtg ctgacgcgctggagttaggcatccccttctccgacccactggcggatggcccgacgattcaaaacgccacact gcgtgcttttgcggcgggagtaaccccggcgcagtgctttgagatgctggcactcattcgccagaagcacccg accattcccatcggccttttgatgtatgccaacctggtgtttaacaaaggcattgatgagttttatgccgagtgcga gaaagtcggcgtcgattcggtgctggttgccgatgtgcccgtggaagagtccgcgcccttccgccaggccgc gttgcgtcataatgtcgcacctatctttatttgcccgccgaatgccgacgatgatttgctgcgccagatagcctctt acggtcgtggttacacctatttgctgtcgcgagcgggcgtgaccggcgcagaaaaccgcgccgcgttacccc tcaatcatctggttgcgaagctgaaagagtacaacgctgcgcctccattgcagggatttggtatttccgccccgg atcaggtaaaagccgcgattgatgcaggagctgcgggcgcgatttctggttcggccatcgttaaaatcatcgag caacatattaatgagccagagaaaatgctggcggcactgaaagcttttgtacaaccgatgaaagcggcgacgc gcagttaatacgcatggcatggatgaCCGATGGTAGTGTGGGGTCTCCCCATGCG AGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGT CGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGC TCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGC GAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAA CTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCC TTTTTGCGTGGCCAGTGCCAAGCTTGCATGCGTGC
trpE **SEQ ID NO: 74**	atgcaaacacaaaaaccgactctcgaactgctaacctgcgaaggcgcttatcgcgacaacccgactgcgctttt tcaccagttgtgtggggatcgtccggcaacgctgctgctggaatccgcagatatcgacagcaaagatgatttaa aaagcctgctgctggtagacagtgcgctgcgcattacagcattaagtgacactgtcacaatccaggcgctttcc ggcaatggagaagccctgttgacactactggataacgccttgcctgcgggtgtggaaaatgaacaatcaccaa actgccgcgtactgcgcttcccgcctgtcagtccactgctggatgaagacgcccgcttatgctccctttcggtttt tgacgctttccgcttattacagaatctgttgaatgtaccgaaggaagaacgagaagcaatgttcttcggcggcct gttctcttatgaccttgtggcgggatttgaaaatttaccgcaactgtcagcggaaaatagctgccctgatttctgttt ttatctcgctgaaacgctgatggtgattgaccatcagaaaaaaagcactcgtattcaggccagcctgtttgctcc gaatgaagaagaaaaacaacgtctcactgctcgcctgaacgaactacgtcagcaactgaccgaagccgcgc cgccgctgccggtggtttccgtgccgcatatgcgttgtgaatgtaaccagagcgatgaagagttcggtggtgta gtgcgtttgttgcaaaaagcgattcgcgccggagaaatttttccaggtggtgccatctcgccgtttctctctgccct gcccgtcaccgctggcagcctattacgtgctgaaaaagagtaatcccagcccgtacatgttttttatgcaggata atgatttcaccctgtttggcgcgtcgccggaaagttcgctcaagtatgacgccaccagccgccagattgagattt acccgattgccggaacacgtccacgcggtcgtcgtgccgatggttcgctggacagagacctcgacagccgc

	atcgaactggagatgcgtaccgatcataaagagctttctgaacatctgatgctggtggatctcgcccgtaatgac ctggcacgcatttgcacacccggcagccgctacgtcgccgatctcaccaaagttgaccgttactcttacgtgat gcacctagtctcccgcgttgttggtgagctgcgccacgatctcgacgccctgcacgcttaccgcgcctgtatga atatggggacgttaagcggtgcaccgaaagtacgcgctatgcagttaattgccgaagcagaaggtcgtcgac gcggcagctacggcggcgcggtaggttattttaccgcgcatggcgatctcgacacctgcattgtgatccgctc ggcgctggtggaaaacggtatcgccaccgtgcaagccggtgctggcgtagtccttgattctgttccgcagtcg gaagccgacgaaactcgtaataaagcccgcgctgtactgcgcgctattgccaccgcgcatcatgcacaggag acgttcta
trpD **SEQ ID NO: 76**	atggctgacattctgctgctcgataatatcgactcttttacgtacaacctggcagatcagttgcgcagcaatggtc ataacgtggtgatttaccgcaaccatattccggcgcagaccttaattgaacgcctggcgacgatgagcaatccg gtgctgatgctttctcctggccccggtgtgccgagcgaagccggttgtatgccggaactcctcacccgcttgcg tggcaagctgccaattattggcatttgcctcggacatcaggcgattgtcgaagcttacgggggctatgtcggtca ggcgggcgaaattcttcacggtaaagcgtcgagcattgaacatgacggtcaggcgatgtttgccggattaaca aacccgctgccagtggcgcgttatcactcgctggttggcagtaacattccggccggtttaaccatcaacgccca ttttaatggcatggtgatggcggtgcgtcacgatgcagatcgcgtttgtggattccagttccatccggaatccatt cttactacccagggcgctcgcctgctggaacaaacgctggcctgggcgcagcagaaactagagccaaccaa cacgctgcaaccgattctggaaaaactgtatcaggcacagacgcttagccaacaagaaagccaccagctgttt tcagcggtggtacgtggcgagctgaagccggaacaactggcggcggcgctggtgagcatgaaaattcgcgg tgaacacccgaacgagatcgccggggcagcaaccgcgctactggaaaacgccgcgccattcccgcgcccg gattatctgtttgccgatatcgtcggtactggcggtgacggcagcaacagcatcaatatttctaccgccagtgcg tttgtcgccgcggcctgcgggctgaaagtggcgaaacacggcaaccgtagcgtctccagtaaatccggctcg tcggatctgctggcggcgttcggtattaatcttgatatgaacgccgataaatcgcgccaggcgctggatgagtta ggcgtctgtttcctctttgcgccgaagtatcacaccggattccgccatgcgatgccggttcgccagcaactgaa aacccgcactctgttcaacgtgctgggaccattgattaacccggcgcatccgccgctggcgctaattggtgttta tagtccggaactggtgctgccgattgccgaaaccttgcgcgtgctggggtatcaacgcgcggcagtggtgca cagcggcgggatggatgaagtttcattacacgcgccgacaatcgttgccgaactacatgacggcgaaattaag agctatcaattgaccgctgaagattttggcctgacaccctaccaccaggagcaattggcaggcggaacaccgg aagaaaaccgtgacattttaacacgcttgttacaaggtaaaggcgacgccgcccatgaagcagccgtcgcgg cgaatgtcgccatgttaatgcgcctgcatggccatgaagatctgcaagccaatgcgcaaaccgttcttgaggta ctgcgcagtggttccgcttacgacagagtcaccgcactggcggcacgagggtaa
trpC **SEQ ID NO: 78**	atgcaaaccgttttagcgaaaatcgtcgcagacaaggcgatttgggtagaaacccgcaaagagcagcaaccg ctggccagttttcagaatgaggttcagccgagcacgcgacatttttatgatgcacttcagggcgcacgcacggc gtttattctggagtgtaaaaaagcgtcgccgtcaaaaggcgtgatccgtgatgatttcgatccggcacgcattgc cgccatttataaacattacgcttcggcaatttcagtgctgactgatgagaaatattttcaggggagctttgatttcct ccccatcgtcagccaaatcgccccgcagccgattttatgtaaagacttcattatcgatccttaccagatctatctg gcgcgctattaccaggccgatgcctgcttattaatgctttcagtactggatgacgaacaatatcgccagcttgca gccgtcgcccacagtctggagatgggtgtgctgaccgaagtcagtaatgaagaggaactggagcgcgccatt gcattgggggcaaaggtcgttggcatcaacaaccgcgatctgcgcgatttgtcgattgatctcaaccgtacccg cgagcttgcgccgaaactggggcacaacgtgacggtaatcagcgaatccggcatcaatacttacgctcaggt gcgcgagttaagccacttcgctaacggctttctgattggttcggcgttgatggcccatgacgatttgaacgccgc cgtgcgtcgggtgttgctgggtgagaataaagtatgtggcctgacacgtgggcaagatgctaaagcagcttat gacgcgggcgcgatttacggtgggttgatttttgttgcgacatcaccgcgttgcgtcaacgttgaacaggcgca ggaagtgatggctgcagcaccgttgcagtatgttggcgtgttccgcaatcacgatattgccgatgtggcggaca aagctaaggtgttatcgctggcggcagtgcaactgcatggtaatgaagatcagctgtatatcgacaatctgcgt gaggctctgccagcacacgtcgccatctggaaggctttaagtgtcggtgaaactcttcccgcgcgcgattttca gcacatcgataaatatgtattcgacaacggtcagggcgggagcggacaacgtttcgactggtcactattaaatg gtcaatcgcttggcaacgttctgctggcgggggggcttaggcgcagataactgcgtggaagcggcacaaaccg gctgcgccgggcttgattttaattctgctgtagagtcgcaaccgggtatcaaagacgcacgtcttttggcctcggt

	tttccagacgctgcgcgcatattaa
trpB **SEQ ID NO: 80**	atgacaacattacttaacccctattttggtgagtttggcggcatgtacgtgccacaaatcctgatgcctgctctgcg ccagctggaagaagcttttgtcagcgcgcaaaaagatcctgaatttcaggctcagttcaacgacctgctgaaaa actatgccgggcgtccaaccgcgctgaccaaatgccagaacattacagccgggacgaacaccacgctgtatc tgaagcgcgaagatttgctgcacggcggcgcgcataaaactaaccaggtgctcggtcaggctttactggcga agcggatgggtaaaactgaaattattgccgaaaccggtgccggtcagcatggcgtggcgtcggcccttgcca gcgccctgctcggcctgaaatgccgaatttatatgggtgccaaagacgttgaacgccagtcgcccaacgttttc cggatgcgcttaatgggtgcggaagtgatcccggtacatagcggttccgcgaccctgaaagatgcctgtaatg aggcgctacgcgactggtccggcagttatgaaaccgcgcactatatgctgggtaccgcagctggcccgcatc cttacccgaccattgtgcgtgagtttcagcggatgattggcgaagaaacgaaagcgcagattctggaaagaga aggtcgcctgccggatgccgttatcgcctgtgttggcggtggttcgaatgccatcggtatgtttgcagatttcatc aacgaaaccgacgtcggcctgattggtgtggagcctggcggccacggtatcgaaactggcgagcacggcgc accgttaaaacatggtcgcgtgggcatctatttcggtatgaaagcgccgatgatgcaaaccgaagacgggcaa attgaagagtcttactccatttctgccgggctggatttcccgtccgtcggcccgcaacatgcgtatctcaacagc actggacgcgctgattacgtgtctattaccgacgatgaagccctggaagcctttaaaacgctttgcctgcatgaa gggatcatcccggcgctggaatcctcccacgccctggcccatgcgctgaaaatgatgcgcgaaaatccggaa aaagagcagctactggtggttaacctttccggtcgcggcgataaagacatcttcaccgttcacgatattttgaaa gcacgaggggaaatctga
trpA **SEQ ID NO: 82**	atggaacgctacgaatctctgtttgcccagttgaaggagcgcaaagaaggcgcattcgttcctttcgtcaccctc ggtgatccgggcattgagcagtcgttgaaaattatcgatacgctaattgaagccggtgctgacgcgctggagtt aggcatccccttctccgacccactggcggatggcccgacgattcaaaacgccacactgcgtgcttttgcggcg ggagtaaccccggcgcagtgctttgagatgctggcactcattcgccagaagcacccgaccattcccatcggcc ttttgatgtatgccaacctggtgtttaacaaaggcattgatgagttttatgccgagtgcgagaaagtcggcgtcga ttcggtgctggttgccgatgtgcccgtggaagagtccgcgcccttccgccaggccgcgttgcgtcataatgtcg cacctatctttatttgcccgccgaatgccgacgatgatttgctgcgccagatagcctcttacggtcgtggttacac ctatttgctgtcgcgagcgggcgtgaccggcgcagaaaaccgcgccgcgttacccctcaatcatctggttgcg aagctgaaagagtacaacgctgcgcctccattgcagggatttggtatttccgccccggatcaggtaaaagccg cgattgatgcaggagctgcgggcgcgatttctggttcggccatcgttaaaatcatcgagcaacatattaatgagc cagagaaaatgctggcggcactgaaagcttttgtacaaccgatgaaagcggcgacgcgcagttaa

Table 9B. Tryptophan Synthesis Polypeptide Sequences

Description	Sequence
TrpE **SEQ ID NO: 75**	MQTQKPTLELLTCEGAYRDNPTALFHQLCGDRPATLL LESADIDSKDDLKSLLLVDSALRITALSDTVTIQALSGN GEALLTLLDNALPAGVENEQSPNCRVLRFPPVSPLLDE DARLCSLSVFDAFRLLQNLLNVPKEEREAMFFGGLFS YDLVAGFENLPQLSAENSCPDFCFYLAETLMVIDHQK KSTRIQASLFAPNEEEKQRLTARLNELRQQLTEAAPPL PVVSVPHMRCECNQSDEEFGGVVRLLQKAIRAGEIFQ VVPSRRFSLPCPSPLAAYYVLKKSNPSPYMFFMQDND FTLFGASPESSLKYDATSRQIEIYPIAGTRPRGRRADGS LDRDLDSRIELEMRTDHKELSEHLMLVDLARNDLARI CTPGSRYVADLTKVDRYSYVMHLVSRVVGELRHDLD ALHAYRACMNMGTLSGAPKVRAMQLIAEAEGRRRGS YGGAVGYFTAHGDLDTCIVIRSALVENGIATVQAGAG VVLDSVPQSEADETRNKARAVLRAIATAHHAQETF

TrpD **SEQ ID NO: 77**	MADILLLDNIDSFTYNLADQLRSNGHNVVIYRNHIPAQ TLIERLATMSNPVLMLSPGPGVPSEAGCMPELLTRLRG KLPIIGICLGHQAIVEAYGGYVGQAGEILHGKASSIEHD GQAMFAGLTNPLPVARYHSLVGSNIPAGLTINAHFNG MVMAVRHDADRVCGFQFHPESILTTQGARLLEQTLA WAQQKLEPTNTLQPILEKLYQAQTLSQQESHQLFSAV VRGELKPEQLAAALVSMKIRGEHPNEIAGAATALLEN AAPFPRPDYLFADIVGTGGDGSNSINISTASAFVAAAC GLKVAKHGNRSVSSKSGSSDLLAAFGINLDMNADKSR QALDELGVCFLFAPKYHTGFRHAMPVRQQLKTRTLF NVLGPLINPAHPPLALIGVYSPELVLPIAETLRVLGYQR AAVVHSGGMDEVSLHAPTIVAELHDGEIKSYQLTAED FGLTPYHQEQLAGGTPEENRDILTRLLQGKGDAAHEA AVAANVAMLMRLHGHEDLQANAQTVLEVLRSGSAY DRVTALAARG
TrpC **SEQ ID NO: 79**	MQTVLAKIVADKAIWVETRKEQQPLASFQNEVQPSTR HFYDALQGARTAFILECKKASPSKGVIRDDFDPARIAA IYKHYASAISVLTDEKYFQGSFDFLPIVSQIAPQPILCK DFIIDPYQIYLARYYQADACLLMLSVLDDEQYRQLAA VAHSLEMGVLTEVSNEEELERAIALGAKVVGINNRDL RDLSIDLNRTRELAPKLGHNVTVISESGINTYAQVREL SHFANGFLIGSALMAHDDLNAAVRRVLLGENKVCGL TRGQDAKAAYDAGAIYGGLIFVATSPRCVNVEQAQE VMAAAPLQYVGVFRNHDIADVADKAKVLSLAAVQL HGNEDQLYIDNLREALPAHVAIWKALSVGETLPARDF QHIDKYVFDNGQGGSGQRFDWSLLNGQSLGNVLLAG GLGADNCVEAAQTGCAGLDFNSAVESQPGIKDARLL ASVFQTLRAY
TrpB **SEQ ID NO: 81**	MTTLLNPYFGEFGGMYVPQILMPALRQLEEAFVSAQK DPEFQAQFNDLLKNYAGRPTALTKCQNITAGTNTTLY LKREDLLHGGAHKTNQVLGQALLAKRMGKTEIIAET GAGQHGVASALASALLGLKCRIYMGAKDVERQSPNV FRMRLMGAEVIPVHSGSATLKDACNEALRDWSGSYE TAHYMLGTAAGPHPYPTIVREFQRMIGEETKAQILERE GRLPDAVIACVGGGSNAIGMFADFINETDVGLIGVEPG GHGIETGEHGAPLKHGRVGIYFGMKAPMMQTEDGQI EESYSISAGLDFPSVGPQHAYLNSTGRADYVSITDDEA LEAFKTLCLHEGIIPALESSHALAHALKMMRENPEKEQ LLVVNLSGRGDKDIFTVHDILKARGEI
TrpA **SEQ ID NO: 83**	MERYESLFAQLKERKEGAFVPFVTLGDPGIEQSLKIID TLIEAGADALELGIPFSDPLADGPTIQNATLRAFAAGV TPAQCFEMLALIRQKHPTIPIGLLMYANLVFNKGIDEF YAECEKVGVDSVLVADVPVEESAPFRQAALRHNVAPI FICPPNADDDLLRQIASYGRGYTYLLSRAGVTGAENR AALPLNHLVAKLKEYNAAPPLQGFGISAPDQVKAAID AGAAGAISGSAIVKIIEQHINEPEKMLAALKAFVQPMK AATRS

[0397] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequence of **Table 9A** 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 9B** 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 9A** 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 9B** or a functional fragment thereof.

[0398] In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 71 through SEQ ID NO: 83**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 85% identity with one or more of **SEQ ID NO: 71 through SEQ ID NO: 83**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 90% identity with one or more of **SEQ ID NO: 71 through SEQ ID NO: 83**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 95% identity with one or more of **SEQ ID NO: 71 through SEQ ID NO: 83**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have have at least about 96%, 97%, 98%, or 99% identity with one or more of **SEQ ID NO: 71 through SEQ ID NO: 83**. 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 one or more of **SEQ ID NO: 71 through SEQ ID NO: 83**. 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:**

71 through SEQ ID NO: 83. In another embodiment, one or more polynucleotides and/or polypeptides encoded and expressed by the genetically engineered bacteria consist of the sequence of one or more of **SEQ ID NO: 71 through SEQ ID NO: 83**.

[0399] **Table 10A** depicts exemplary polypeptide sequences feedback resistant AroG and TrpE. **Table 10A** 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 10A. Feedback resistant AroG and TrpE and tryptophanase sequences

Description	Sequence
AroGfbr: feedback resistant 2-dehydro-3-deoxyphosphoheptonate aldolase from E. coli **SEQ ID NO: 84**	MNYQNDDLRIKEIKELLPPVALLEKFPATENAANTVAHARKAI HKILKGNDDRLLVVIGPCSIHDPVAAKEYATRLLTLREELQDE LEIVMRVYFEKPRTTVGWKGLINDPHMDNSFQINDGLRIARK LLLDINDSGLPAAGEFLDMITLQYLADLMSWGAIGARTTESQ VHRELASGLSCPVGFKNGTDGTIKVAIDAINAAGAPHCFLSVT KWGHSAIVNTSGNGDCHIILRGGKEPNYSAKHVAEVKEGLNK AGLPAQVMIDFSHANSSKQFKKQMDVCTDVCQQIAGGEKAII GVMVESHLVEGNQSLESGEPLAYGKSITDACIGWDDTDALLR QLASAVKARRG
TrpEfbr: feedback resistant anthranilate synthase component I from E. coli **SEQ ID NO: 85**	MQTQKPTLELLTCEGAYRDNPTALFHQLCGDRPATLLLEFADI DSKDDLKSLLLVDSALRITALSDTVTIQALSGNGEALLTLLDN ALPAGVENEQSPNCRVLRFPPVSPLLDEDARLCSLSVFDAFRL LQNLLNVPKEEREAMFFGGLFSYDLVAGFENLPQLSAENSCP DFCFYLAETLMVIDHQKKSTRIQASLFAPNEEEKQRLTARLNE LRQQLTEAAPPLPVVSVPHMRCECNQSDEEFGGVVRLLQKAI RAGEIFQVVPSRRFSLPCPSPLAAYYVLKKSNPSPYMFFMQDN DFTLFGASPESSLKYDATSRQIEIYPIAGTRPRGRRADGSLDRD LDSRIELEMRTDHKELSEHLMLVDLARNDLARICTPGSRYVA DLTKVDRYSYVMHLVSRVVGELRHDLDALHAYRACMNMGT LSGAPKVRAMQLIAEAEGRRRGSYGGAVGYFTAHGDLDTCIV IRSALVENGIATVQAGAGVVLDSVPQSEADETRNKARAVLRA IATAHHAQETF
SerA: 2-oxoglutarate reductase from E. coli Nissle **SEQ ID NO: 86**	MAKVSLEKDKIKFLLVEGVHQKALESLRAAGYTNIEFHKGAL DDEQLKESIRDAHFIGLRSRTHLTEDVINAAEKLVAIGCFCIGT NQVDLDAAAKRGIPVFNAPFSNTRSVAELVIGELLLLLRGVPE ANAKAHRGVWNKLAAGSFEARGKKLGIIGYGHIGTQLGILAE SLGMYVYFYDIENKLPLGNATQVQHLSDLLNMSDVVSLHVPE NPSTKNMMGAKEISLMKPGSLLINASRGTVVDIPALCDALASK HLAGAAIDVFPTEPATNSDPFTSPLCEFDNVLLTPHIGGSTQEA QENIGLEVAGKLIKYSDNGSTLSAVNFPEVSLPLHGGRRLMHI HENRPGVLTALNKIFAEQGVNIAAQYLQTSAQMGYVVIDIEA DEDVAEKALQAMKAIPGTIRARLLY

SerAfbr: feedback resistant 2-oxoglutarate reductase from E. coli Nissle **SEQ ID NO: 87**	MAKVSLEKDKIKFLLVEGVHQKALESLRAAGYTNIEFHKGAL DDEQLKESIRDAHFIGLRSRTHLTEDVINAAEKLVAIGCFCIGT NQVDLDAAAKRGIPVFNAPFSNTRSVAELVIGELLLLLRGVPE ANAKAHRGVWNKLAAGSFEARGKKLGIIGYGHIGTQLGILAE SLGMYVYFYDIENKLPLGNATQVQHLSDLLNMSDVVSLHVPE NPSTKNMMGAKEISLMKPGSLLINASRGTVVDIPALCDALASK HLAGAAIDVFPTEPATNSDPFTSPLCEFDNVLLTPHIGGSTQEA QENIGLEVAGKLIKYSDNGSTLSAVNFPEVSLPLHGGRRLMHI AEARPGVLTALNKIFAEQGVNIAAQYLQTSAQMGYVVIDIEA DEDVAEKALQAMKAIPGTIRARLLY
TnaA: tryptophanase from E. coli **SEQ ID NO: 88**	MENFKHLPEPFRIRVIEPVKRTTRAYREEAIIKSGMNPFLLDSE DVFIDLLTDSGTGAVTQSMQAAMMRGDEAYSGSRSYYALAE SVKNIFGYQYTIPTHQGRGAEQIYIPVLIKKREQEKGLDRSKM VAFSNYFFDTTQGHSQINGCTVRNVYIKEAFDTGVRYDFKGN FDLEGLERGIEEVGPNNVPYIVATITSNSAGGQPVSLANLKVM YSIAKKYDIPVVMDSARFAENAYFIKQREAEYKDWTIEQITRE TYKYADMLAMSAKKDAMVPMGGLLCMKDDSFFDVYTECRT LCVVQEGFPTYGGLEGGAMERLAVGLYDGMNLDWLAYRIA QVQYLVDGLEEIGVVCQQAGGHAAFVDAGKLLPHIPADQFPA QALACELYKVAGIRAVEIGSFLLGRDPKTGKQLPCPAELLRLTI PRATYTQTHMDFIIEAFKHVKENAANIKGLTFTYEPKVLRHFT AKLKEV

Table 10B.

fbrAroG **SEQ ID NO: 256**	atgaattatcagaacgacgatttacgcatcaaagaaatcaaagagttacttcctcctgtcg cattgctggaaaaattccccgctactgaaaatgccgcgaatacggtcgcccatgcccga aaagcgatccataagatcctgaaaggtaatgatgatcgcctgttggtggtgattggccca tgctcaattcatgatcctgtcgcggctaaagagtatgccactcgcttgctgacgctgcgtg aagagctgcaagatgagctggaaatcgtgatgcgcgtctattttgaaaagccgcgtacta cggtgggctggaaagggctgattaacgatccgcatatggataacagcttccagatcaac gacggtctgcgtattgcccgcaaattgctgctcgatattaacgacagcggtctgccagcg gcgggtgaattcctggatatgatcaccctacaatatctcgctgacctgatgagctggggc gcaattggcgcacgtaccaccgaatcgcaggtgcaccgcgaactggcgtctggtctttc ttgtccggtaggtttcaaaaatggcactgatggtacgattaaagtggctatcgatgccatta atgccgccggtgcgccgcactgcttcctgtccgtaacgaaatgggggcattcggcgatt gtgaataccagcggtaacggcgattgccatatcattctgcgcggcggtaaagagcctaa ctacagcgcgaagcacgttgctgaagtgaaagaagggctgaacaaagcaggcctgcc agcgcaggtgatgatcgatttcagccatgctaactcgtcaaaacaattcaaaaagcagat ggatgtttgtactgacgtttgccagcagattgccggtggcgaaaaggccattattggcgt gatggtggaaagccatctggtggaaggcaatcagagcctcgagagcggggaaccgct ggcctacggtaagagcatcaccgatgcctgcattggctgggatgataccgatgctctgtt acgtcaactggcgagtgcagtaaaagcgcgtcgcgggtaa
fbrTrpE **SEQ ID NO: 274**	atgcaaacacaaaaaccgactctcgaactgctaacctgcgaaggcgcttatcgcgacaa cccgactgcgctttttcaccagttgtgtggggatcgtccggcaacgctgctgctggaattc gcagatatcgacagcaaagatgatttaaaaagcctgctgctggtagacagtgcgctgcg cattacagcattaagtgacactgtcacaatccaggcgctttccggcaatggagaagccct

	gttgacactactggataacgccttgcctgcgggtgtggaaaatgaacaatcaccaaactg ccgcgtactgcgcttcccgcctgtcagtccactgctggatgaagacgcccgcttatgctc cctttcggtttttgacgctttccgcttattacagaatctgttgaatgtaccgaaggaagaacg agaagcaatgttcttcggcggcctgttctcttatgaccttgtggcgggatttgaaaatttac cgcaactgtcagcggaaaatagctgccctgatttctgtttttatctcgctgaaacgctgatg gtgattgaccatcagaaaaaaagcactcgtattcaggccagcctgtttgctccgaatgaa gaagaaaaacaacgtctcactgctcgcctgaacgaactacgtcagcaactgaccgaag ccgcgccgccgctgccggtggtttccgtgccgcatatgcgttgtgaatgtaaccagagc gatgaagagttcggtggtgtagtgcgtttgttgcaaaaagcgattcgcgccggagaaatt ttccaggtggtgccatctcgccgtttctctctgccctgcccgtcaccgctggcagcctatta cgtgctgaaaaagagtaatcccagcccgtacatgtttttatgcaggataatgatttcaccc tgtttggcgcgtcgccggaaagttcgctcaagtatgacgccaccagccgccagattgag atttacccgattgccggaacacgtccacgcggtcgtcgtgccgatggttcgctggacag agacctcgacagccgcatcgaactggagatgcgtaccgatcataaagagctttctgaac atctgatgctggtggatctcgcccgtaatgacctggcacgcatttgcacacccggcagc cgctacgtcgccgatctcaccaaagttgaccgttactcttacgtgatgcacctagtctccc gcgttgttggtgagctgcgccacgatctcgacgccctgcacgcttaccgcgcctgtatga atatggggacgttaagcggtgcaccgaaagtacgcgctatgcagttaattgccgaagca gaaggtcgtcgacgcggcagctacggcggcgcggtaggttattttaccgcgcatggcg atctcgacacctgcattgtgatccgctcggcgctggtggaaaacggtatcgccaccgtg caagccggtgctggcgtagtccttgattctgttccgcagtcggaagccgacgaaactcgt aataaagcccgcgctgtactgcgcgctattgccaccgcgcatcatgcacaggagacgtt cta
SerA **SEQ ID NO: 258**	atggcaaaggtatcgctggagaaagacaagattaagtttctgctggtagaaggcgtgca ccaaaaggcgctggaaagccttcgtgcagctggttacaccaacatcgaatttcacaaag gcgcgctggatgatgaacaattaaaagaatccatccgcgatgcccacttcatcggcctg cgatcccgtacccatctgactgaagacgtgatcaacgccgcagaaaaactggtcgctat tggctgtttctgtatcggaacaaatcaggttgatctggatgcggcggcaaagcgcgggat cccggtatttaacgcaccgttctcaaatacgcgctctgttgcggagctggtgattggcga actgctgctgctattgcgcggcgtgccagaagccaatgctaaagcgcatcgtggcgtgt ggaacaaactggcggcgggttcttttgaagcgcgcggcaaaaagctgggtatcatcgg ctacggtcatattggtacgcaattgggcattctggctgaatcgctgggaatgtatgtttactt ttatgatattgaaaacaaactgccgctgggcaacgccactcaggtacagcatctttctgac ctgctgaatatgagcgatgtggtgagtctgcatgtaccagagaatccgtccaccaaaaat atgatgggcgcgaaagagatttcgctaatgaagcccggctcgctgctgattaatgcttcg cgcggtactgtggtggatattccagcgctgtgtgacgcgctggcgagcaaacatctggc gggggcggcaatcgacgtattcccgacggaaccggcgaccaatagcgatccatttacc tctccgctgtgtgaattcgacaatgtccttctgacgccacacattggcggttcgactcagg aagcgcaggagaatatcggcttggaagttgcgggtaaattgatcaagtattctgacaatg gctcaacgctctctgcggtgaacttcccggaagtctcgctgccactgcacggtgggcgt cgtctgatgcacatccacgaaaaccgtccgggcgtgctaactgcgctcaacaaaatttttt gccgagcagggcgtcaacatcgccgcgcaatatctacaaacttccgcccagatgggtt atgtagttattgatattgaagccgacgaagacgttgccgaaaaagcgctgcaggcaatg aaagctattccgggtaccattcgcgcccgtctgctgtactaa
SerAfbr **SEQ ID NO: 1510**	atggcaaaggtatcgctggagaaagacaagattaagtttctgctggtagaaggcgtgca ccaaaaggcgctggaaagccttcgtgcagctggttacaccaacatcgaatttcacaaag gcgcgctggatgatgaacaattaaaagaatccatccgcgatgcccacttcatcggcctg cgatcccgtacccatctgactgaagacgtgatcaacgccgcagaaaaactggtcgctat tggctgtttctgtatcggaacaaatcaggttgatctggatgcggcggcaaagcgcgggat

	cccggtatttaacgcaccgttctcaaatacgcgctctgttgcggagctggtgattggcgaa ctgctgctgctattgcgcggcgtgccagaagccaatgctaaagcgcatcgtggcgtgtgg aacaaactggcggcgggttcttttgaagcgcgcggcaaaaagctgggtatcatcggcta cggtcatattggtacgcaattgggcattctggctgaatcgctgggaatgtatgtttacttttatg atattgaaaacaaactgccgctgggcaacgccactcaggtacagcatctttctgacctgc tgaatatgagcgatgtggtgagtctgcatgtaccagagaatccgtccaccaaaaatatga tgggcgcgaaagagatttcgctaatgaagcccggctcgctgctgattaatgcttcgcgcg gtactgtggtggatattccagcgctgtgtgacgcgctggcgagcaaacatctggcgggg gcggcaatcgacgtattcccgacggaaccggcgaccaatagcgatccatttacctctcc gctgtgtgaattcgacaatgtccttctgacgccacacattggcggttcgactcaggaagcg caggagaatatcggcttggaagttgcgggtaaattgatcaagtattctgacaatggctcaa cgctctctgcggtgaacttcccggaagtctcgctgccactgcacggtgggcgtcgtctgat gcacatc**GCT**gaa**GCT**cgtccgggcgtgctaactgcgctcaacaaaatttttgccga gcagggcgtcaacatcgccgcgcaatatctacaaacttccgcccagatgggttatgtagt tattgatattgaagccgacgaagacgttgccgaaaaagcgctgcaggcaatgaaagct attccgggtaccattcgcgcccgtctgctgtactaa

[0400] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with one or more sequences of **Table 10B**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with one or more sequences of **Table 10B**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with one or more sequences of **Table 10B**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with one or more sequences of **Table 10B**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 96%, 97%, 98%, or 99% identity with one or more sequences of **Table 10B**. Accordingly, in one embodiment, 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 10B**. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of with one or more sequences of **Table 10B**.

[0401] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 256**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with **SEQ ID NO: 256**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with **SEQ ID NO: 256**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with **SEQ ID NO: 256**. In another embodiment, the

bcd2 gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 256**. Accordingly, in one embodiment, 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 **SEQ ID NO: 256**. In another embodiment, the genetically engineered bacteria comprise the sequence of **SEQ ID NO: 256**. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of **SEQ ID NO: 256**.

[0402] In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 80% identity with one or more of **SEQ ID NO: 84 through SEQ ID NO: 87**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 85% identity with one or more of **SEQ ID NO: 84 through SEQ ID NO: 87**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 90% identity with one or more of **SEQ ID NO: 84 through SEQ ID NO: 87**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have at least about 95% identity with one or more of **SEQ ID NO: 84 through SEQ ID NO: 87**. In one embodiment, one or more polypeptides and/or polynucleotides encoded and expressed by the genetically engineered bacteria have have at least about 96%, 97%, 98%, or 99% identity with one or more of **SEQ ID NO: 84 through SEQ ID NO: 87**. 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 one or more of **SEQ ID NO: 84 through SEQ ID NO: 87**. 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: 84 through SEQ ID NO: 87**. In another embodiment, one or more polynucleotides and/or polypeptides encoded and expressed by the genetically engineered bacteria consist of the sequence of one or more of **SEQ ID NO: 84 through SEQ ID NO: 87**.

[0403] In some embodiments, the endogenous TnaA polypeptide comprising **SEQ ID NO: 88** is mutated or deleted.

[0404] To improve acetate production, while maintaining high levels of tryptophan 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 tryptophan production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0405] In some embodiments, the genetically engineered bacteria comprise one or more tryptophan production 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.

[0406] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of

tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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.

[0407] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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

trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes.

[0408] 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 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.

[0409] 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 tryptophan 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 tryptophan than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the 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 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0410] 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 tryptophan 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 described herein 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 tryptophan 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 tryptophan synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0411] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 described herein for the production of tryptophan 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 tryptophan and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0412] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, and further comprise a mutation in the endogenous pta and ldhA genes.

[0413] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr, trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, 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 trpEfbr,

trpDCBA, aroGfbr, SerAfbr and ΔtrpR, ΔtnaA, and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes.

[0414] 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.

[0415] 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 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 tryptophan 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 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0416] 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,

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.

[0417] n 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 Kynurenic Acid

[0418] 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)).

[0419] 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.

[0420] 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.

[0421] 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.

[0422] 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.

[0423] 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.

[0424] 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.

[0425] 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.

[0426] 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.

[0427] To improve acetate production, while maintaining high levels of kynurenic acid 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 kynurenic acid production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0428] In some embodiments, the genetically engineered bacteria comprise one or more kynurenic acid production 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.

[0429] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described

herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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.

[0430] 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 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.

[0431] 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 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.

[0432] 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 kynurenic acid 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 described herein 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 kynurenic acid 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 kynurenic acid synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0433] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 described herein for the production of kynurenic acid 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 kynurenic acid and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0434] 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.

[0435] 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 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.

[0436] 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.

[0437] . 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.

[0438] 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

[0439] 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.

[0440] 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-acetat

[0441] 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.

[0442] 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.

[0443] 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. 48**, 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).

[0444] 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.

[0445] 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 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.*

[0446] 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.

[0447] 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).

[0448] 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.

[0449] 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. Exemplary inducible promoters which may control the expression of the biosynthetic cassettes 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 characteristic of a disorder described herein, or that

may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose and tetracycline. 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.

[0450] 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. 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.

Tryptamine

[0451] 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.

[0452] A non-limiting example of such as strain is shown in **FIG. 41A.** 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.

[0453] **Table 15, Table 11A,** and **Table 12** lists exemplary sequences for tryptamine production in genetically engineered bacteria.

[0454] 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.

[0455] To improve acetate production, while maintaining high levels of tryptamine 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 tryptamine production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0456] In some embodiments, the genetically engineered bacteria comprise one or more tryptamine production 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.

[0457] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described

herein for the production of tryptamine 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 described herein for the production of tryptamine 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.

[0458] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes.

[0459] 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 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.

[0460] 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 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 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.

[0461] 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 tryptamine 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 described herein 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 tryptamine 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 tryptamine synthesis. Such mutations and/or deletions,

include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0462] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 described herein for the production of tryptamine 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 tryptamine and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0463] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, and further comprise a mutation in the endogenous pta and ldhA genes.

[0464] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, 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 trpEfbrDCBA, aroGfbr, tdc, SerAfbr, and ΔtrpR and ΔtnaA, and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes.

[0465] 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.

[0466] 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.

[0467] 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.

Indole-3-acetaldehyde and FICZ

[0468] 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. 41B**.

[0469] 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.

[0470] Further exemplary gene cassettes for the production of produce indole-3-acetaldehyde and FICZ from tryptophan are shown in **FIG. 41C.** 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.

[0471] 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.

[0472] To improve acetate production, while maintaining high levels of Indole-3-acetaldehyde and/or FICZ 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 Indole-3-acetaldehyde and/or FICZ production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0473] In some embodiments, the genetically engineered bacteria comprise one or more Indole-3-acetaldehyde and/or FICZ production 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.

[0474] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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.

[0475] 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 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.

[0476] 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 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.

[0477] 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 Indole-3-acetaldehyde and/or FICZ 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 described herein 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 Indole-3-acetaldehyde and/or FICZ 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 Indole-3-acetaldehyde and/or FICZ synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0478] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 described herein for the production of Indole-3-acetaldehyde and/or FICZ 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 Indole-3-acetaldehyde and/or FICZ and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0479] 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.

[0480] 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-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.

[0481] In some embodiments, the genetically engineered bacteria are capable of producing Indole-3-aldehyde 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.

[0482] 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.

Indole-3-acetic acid

[0483] 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.

[0484] 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.

[0485] 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.

[0486] Non-limiting example of such gene sequence(s) are shown in **FIG. 42A, FIG. 42B, FIG. 42C, FIG. 42D, and FIG. 42E, and FIG. 43B and FIG. 43E.**

[0487] 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. 42A**).

[0488] Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 42B.** 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.

[0489] Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 42C.** 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.

[0490] Another non-limiting example of gene sequence(s) for the production of acetic acid are shown in **FIG. 42D.** 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.

[0491] Another non-limiting example of gene sequence(s) for the production of acetic acid are shown in **FIG. 42E.** 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.

[0492] Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 42F.** Another non-limiting example of gene sequence(s) for the production of indole-3-acetic acid are shown in **FIG. 343E**. 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.

[0493] 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.

[0494] To improve acetate production, while maintaining high levels of indole-3-acetic acid 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 indole-3-acetic acid production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0495] In some embodiments, the genetically engineered bacteria comprise one or more indole-3-acetic acid production 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.

[0496] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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.

[0497] 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, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes.

[0498] 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 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.

[0499] 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-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 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.

[0500] 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 indole-3-acetic acid 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 described herein 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 indole-3-acetic acid 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 indole-3-acetic acid synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0501] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described herein for the production of indole-3-acetic acid 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 described

herein for the production of indole-3-acetic acid 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 indole-3-acetic acid and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0502] 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, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, and further comprise a mutation in the endogenous pta and ldhA genes.

[0503] 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, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and ΔtrpR, ΔtnaA, 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 trpEfbrDCBA, aroGfbr, SerAfbr, trpDH, ipdC, iad, and

ΔtrpR, ΔtnaA, and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes.

[0504] 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.

[0505] 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.

[0506] In some embodiments, the genetically engineered bacteria are capable of producing Indole Acetic Acid and 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.

[0507] 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.

Indole-3-acetonitrile

[0508] 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.

[0509] 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.

[0510] 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.

[0511] 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.

[0512] 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.

[0513] To improve acetate production, while maintaining high levels of Indole-3-acetonitrile 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 Indole-3-acetonitrile production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0514] In some embodiments, the genetically engineered bacteria comprise one or more Indole-3-acetonitrile production 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.

[0515] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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.

[0516] 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 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.

[0517] 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-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 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.

[0518] 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 Indole-3-acetonitrile 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 described herein 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 Indole-3-acetonitrile 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 Indole-3-acetonitrile synthesis. Such

mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0519] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 described herein for the production of Indole-3-acetonitrile 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 Indole-3-acetonitrile and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0520] 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.

[0521] 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.

[0522] 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.

Indole-3-propionic acid (IPA)

[0523] 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. 47 and FIG 48, and FIG. 43C** depict schematics of exemplary circuits for the production of indole-3-propionic acid.

[0524] 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.

[0525] 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. 48**).

[0526] Another embodiment of the IPA producing strain is shown in **FIG. 47**.

[0527] 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.

[0528] 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.

[0529] To improve acetate production, while maintaining high levels of Indole-3-propionic acid 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 Indole-3-propionic acid production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0530] In some embodiments, the genetically engineered bacteria comprise one or more Indole-3-propionic acid production 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.

[0531] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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.

[0532] 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 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.

[0533] 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-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 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.

[0534] 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 Indole-3-propionic acid 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 described herein 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 Indole-3-propionic acid 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 Indole-3-propionic acidsynthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0535] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 described herein for the production of Indole-3-propionic acidand 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 Indole-3-propionic acidand further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0536] 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.

[0537] 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.

[0538] 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 acetaladehyde, indole-3acetonitrile, kynurenine, kynurenic acid, indole, indole acetic acid FICZ, indole-3-propionic acid.

[0539] 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.

[0540] 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.

Indole

[0541] 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. 41G** 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.

[0542] 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.

[0543] To improve acetate production, while maintaining high levels of Indole 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 Indole production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0544] In some embodiments, the genetically engineered bacteria comprise one or more Indole production 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.

[0545] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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.

[0546] 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 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.

[0547] 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 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 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.

[0548] 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 Indole 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 described herein 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 Indole 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 Indole synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0549] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production

of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 described herein for the production of Indole 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 Indole and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0550] 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.

[0551] 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.

[0552] In some embodiments, the genetically engineered bacteria are capable of producing Indole-3-acetonitrile 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.

Other indole metabolites

[0553] 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. 41H** 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.

[0554] 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.

[0555] To improve acetate production, while maintaining high levels of Other indoles 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 Other indoles production cassette(s) and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0556] In some embodiments, the genetically engineered bacteria comprise one or more Other indoles production 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.

[0557] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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.

[0558] 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 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.

[0559] 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 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 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.

[0560] 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 Other indoles 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 described herein 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 Other indoles 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 Other indoles synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0561] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more enzymes described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 described herein for the production of Other indoles 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 Other indoles and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0562] 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.

[0563] 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.

[0564] In some embodiments, the genetically engineered bacteria are capable of producing these metabolites 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.

Tryptophan Catabolic Pathway Enzymes

[0565] **Table 11A and Table 11B** comprise polypeptide and polynucleotide sequences of such enzymes which are encoded by the genetically engineered bacteria of the disclosure.

Table 11A. Tryptophan Pathway Catabolic Enzymes

Description	**Sequence**
TDC: Tryptophan decarboxylase from Catharanthus roseus **SEQ ID NO: 141**	MGSIDSTNVAMSNSPVGEFKPLEAEEFRKQAHRMVDFIADYY KNVETYPVLSEVEPGYLRKRIPETAPYLPEPLDDIMKDIQKDII PGMTNWMSPNFYAFFPATVSSAAFLGEMLSTALNSVGFTWV SSPAATELEMIVMDWLAQILKLPKSFMFSGTGGGVIQNTTSES ILCTIIAARERALEKLGPDSIGKLVCYGSDQTHTMFPKTCKLA GIYPNNIRLIPTTVETDFGISPQVLRKMVEDDVAAGYVPLFLC ATLGTTSTTATDPVDSLSEIANEFGIWIHVDAAYAGSACICPEF RHYLDGIERVDSLSLSPHKWLLAYLDCTCLWVKQPHLLLRAL TTNPEYLKNKQSDLDKVVDFKNWQIATGRKFRSLKLWLILRS YGVVNLQSHIRSDVAMGKMFEEWVRSDSRFEIVVPRNFSLVC FRLKPDVSSLHVEEVNKKLLDMLNSTGRVYMTHTIVGGIYML RLAVGSSLTEEHHVRRVWDLIQKLTDDLLKEA
TDC: Tryptophan decarboxylase from Clostridium sporogenes **SEQ ID NO: 142**	MKFWRKYTQQEMDEKITESLEKTLNYDNTKTIGIPGTKLDDT VFYDDHSFVKHSPYLRTFIQNPNHIGCHTYDKADILFGGTFDIE RELIQLLAIDVLNGNDEEFDGYVTQGGTEANIQAMWVYRNY FKKERKAKHEEIAIITSADTHYSAYKGSDLLNIDIIKVPVDFYS RKIQENTLDSIVKEAKEIGKKYFIVISNMGTTMFGSVDDPDLY ANIFDKYNLEYKIHVDGAFGGFIYPIDNKECKTDFSNKNVSSIT LDGHKMLQAPYGTGIFVSRKNLIHNTLTKEATYIENLDVTLSG SRSGSNAVAIWMVLASYGPYGWMEKINKLRNRTKWLCKQL NDMRIKYYKEDSMNIVTIEEQYVNKEIAEKYFLVPEVHNPTN NWYKIVVMEHVELDILNSLVYDLRKFNKEHLKAM
TYNA: Monoamine oxidase from E. coli **SEQ ID NO: 143**	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: 144**	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
ARO9: L-tryptophan aminotransferase from S. cerevisae **SEQ ID NO: 145**	MTAGSAPPVDYTSLKKNFQPFLSRRVENRSLKSFWDASDISD DVIELAGGMPNERFFPIESMDLKISKVPFNDNPKWHNSFTTAH LDLGSPSELPIARSFQYAETKGLPPLLHFVKDFVSRINRPAFSD ETESNWDVILSGGSNDSMFKVFETICDESTTVMIEEFTFTPAM SNVEATGAKVIPIKMNLTFDRESQGIDVEYLTQLLDNWSTGP YKDLNKPRVLYTIATGQNPTGMSVPQWKREKIYQLAQRHDF LIVEDDPYGYLYFPSYNPQEPLENPYHSSDLTTERYLNDFLMK SFLTLDTDARVIRLETFSKIFAPGLRLSFIVANKFLLQKILDLAD

	ITTRAPSGTSQAIVYSTIKAMAESNLSSSLSMKEAMFEGWIRW IMQIASKYNHRKNLTLKALYETESYQAGQFTVMEPSAGMFIII KINWGNFDRPDDLPQQMDILDKFLLKNGVKVVLGYKMAVCP NYSKQNSDFLRLTIAYARDDDQLIEASKRIGSGIKEFFDNYKS
aspC: aspartate aminotransferase from E. coli **SEQ ID NO: 146**	MFENITAAPADPILGLADLFRADERPGKINLGIGVYKDETGKT PVLTSVKKAEQYLLENETTKNYLGIDGIPEFGRCTQELLFGKG SALINDKRARTAQTPGGTGALRVAADFLAKNTSVKRVWVSN PSWPNHKSVFNSAGLEVREYAYYDAENHTLDFDALINSLNEA QAGDVVLFHGCCHNPTGIDPTLEQWQTLAQLSVEKGWLPLF DFAYQGFARGLEEDAEGLRAFAAMHKELIVASSYSKNFGLYN ERVGACTLVAADSETVDRAFSQMKAAIRANYSNPPAHGASV VATILSNDALRAIWEQELTDMRQRIQRMRQLFVNTLQEKGAN RDFSFIIKQNGMFSFSGLTKEQVLRLREEFGVYAVASGRVNVA GMTPDNMAPLCEAIVAVL
TAA1: L-tryptophan-pyruvate aminotransferase from Arabidopsis thaliana **SEQ ID NO: 147**	MVKLENSRKPEKISNKNIPMSDFVVNLDHGDPTAYEEYWRK MGDRCTVTIRGCDLMSYFSDMTNLCWFLEPELEDAIKDLHGV VGNAATEDRYIVVGTGSTQLCQAAVHALSSLARSQPVSVVA AAPFYSTYVEETTYVRSGMYKWEGDAWGFDKKGPYIELVTS PNNPDGTIRETVVNRPDDDEAKVIHDFAYYWPHYTPITRRQD HDIMLFTFSKITGHAGSRIGWALVKDKEVAKKMVEYIIVNSIG VSKESQVRTAKILNVLKETCKSESESENFFKYGREMMKNRWE KLREVVKESDAFTLPKYPEAFCNYFGKSLESYPAFAWLGTKE ETDLVSELRRHKVMSRAGERCGSDKKHVRVSMLSREDVFNV FLERLANMKLIKSIDL
STAO: L-tryptophan oxidase from streptomyces sp. TP-A0274 **SEQ ID NO: 148**	MTAPLQDSDGPDDAIGGPKQVTVIGAGIAGLVTAYELERLGH HVQIIEGSDDIGGRIHTHRFSGAGGPGPFAEMGAMRIPAGHRL TMHYIAELGLQNQVREFRTLFSDDAAYLPSSAGYLRVREAHD TLVDEFATGLPSAHYRQDTLLFGAWLDASIRAIAPRQFYDGL HNDIGVELLNLVDDIDLTPYRCGTARNRIDLHALFADHPRVR ASCPPRLERFLDDVLDETSSSIVRLKDGMDELPRRLASRIRGKI SLGQEVTGIDVHDDTVTLTVRQGLRTVTRTCDYVVCTIPFTVL RTLRLTGFDQDKLDIVHETKYWPATKIAFHCREPFWEKDGIS GGASFTGGHVRQTYYPPAEGDPALGAVLLASYTIGPDAEALA RMDEAERDALVAKELSVMHPELRRPGMVLAVAGRDWGARR WSRGAATVRWGQEAALREAERRECARPQKGLFFAGEHCSSK PAWIEGAIESAIDAAHEIEWYEPRASRVFAASRLSRSDRSA
ipdC: Indole-3-pyruvate decarboxylase from Enterobacter cloacae **SEQ ID NO: 149**	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: 150**	MPTLNLDLPNGIKSTIQADLFINNKFVPALDGKTFATINPSTGK EIGQVAEASAKDVDLAVKAAREAFETTWGENTPGDARGRLLI KLAELVEANIDELAAIESLDNGKAFSIAKSFDVAAVAANLRY YGGWADKNHGKVMEVDTKRLNYTRHEPIGVCGQIIPWNFPL LMFAWKLGPALATGNTIVLKTAEQTPLSAIKMCELIVEAGFPP GVVNVISGFGPVAGAAISQHMDIDKIAFTGSTLVGRNIMKAA ASTNLKKVTLELGGKSPNIIFKDADLDQAVRWSAFGIMFNHG QCCCAGSRVYVEESIYDAFMEKMTAHCKALQVGDPFSANTF QGPQVSQLQYDRIMEYIESGKKDANLALGGVRKGNEGYFIEP TIFTDVPHDAKIAKEEIFGPVVVVSKFKDEKDLIRIANDSIYGL AAAVFSRDISRAIETAHKLKAGTVWVNCYNQLIPQVPFGGYK ASGIGRELGEYALSNYTNIKAVHVNLSQPAPI
YUC2: indole-3-pyruvate monoxygenase from Arabidopsis thaliana **SEQ ID NO: 151**	MEFVTETLGKRIHDPYVEETRCLMIPGPIIVGSGPSGLATAACL KSRDIPSLILERSTCIASLWQHKTYDRLRLHLPKDFCELPLMPF PSSYPTYPTKQQFVQYLESYAEHFDLKPVFNQTVEEAKFDRR CGLWRVRTTGGKKDETMEYVSRWLVVATGENAEEVMPEID GIPDFGGPILHTSSYKSGEIFSEKKILVVGCGNSGMEVCLDLCN FNALPSLVVRDSVHVLPQEMLGISTFGISTSLLKWFPVHVVDR FLLRMSRLVLGDTDRLGLVRPKLGPLERKIKCGKTPVLDVGT LAKIRSGHIKVYPELKRVMHYSAEFVDGRVDNFDAIILATGY KSNVPMWLKGVNMFSEKDGFPHKPFPNGWKGESGLYAVGF TKLGLLGAAIDAKKIAEDIEVQRHFLPLARPQHC
IaaM: Tryptophan 2-monooxygenase from Pseudomonas savastanoi **SEQ ID NO: 152**	MYDHFNSPSIDILYDYGPFLKKCEMTGGIGSYSAGTPTPRVAI VGAGISGLVAATELLRAGVKDVVLYESRDRIGGRVWSQVFD QTRPRYIAEMGAMRFPPSATGLFHYLKKFGISTSTTFPDPGVV DTELHYRGKRYHWPAGKKPPELFRRVYEGWQSLLSEGYLLE GGSLVAPLDITAMLKSGRLEEAAIAWQGWLNVFRDCSFYNAI VCIFTGRHPPGGDRWARPEDFELFGSLGIGSGGFLPVFQAGFT EILRMVINGYQSDQRLIPDGISSLAARLADQSFDGKALRDRVC FSRVGRISREAEKIIIQTEAGEQRVFDRVIVTSSNRAMQMIHCL TDSESFLSRDVARAVRETHLTGSSKLFILTRTKFWIKNKLPTTI QSDGLVRGVYCLDYQPDEPEGHGVVLLSYTWEDDAQKMLA MPDKKTRCQVLVDDLAAIHPTFASYLLPVDGDYERYVLHHD WLTDPHSAGAFKLNYPGEDVYSQRLFFQPMTANSPNKDTGL YLAGCSCSFAGGWIEGAVQTALNSACAVLRSTGGQLSKGNPL DCINASYRY
iaaH: Indoleacetamide hydrolase from Pseudomonas savastanoi **SEQ ID NO: 153**	MHEIITLESLCQALADGEIAAAELRERALDTEARLARLNCFIRE GDAVSQFGEADHAMKGTPLWGMPVSFKDNICVRGLPLTAGT RGMSGFVSDQDAAIVSQLRALGAVVAGKNNMHELSFGVTSI NPHWGTVGNPVAPGYCAGGSSGGSAAAVASGIVPLSVGTDT GGSIRIPAAFCGITGFRPTTGRWSTAGIIPVSHTKDCVGLLTRT AGDAGFLYGLLSGKQQSFPLSRTAPCRIGLPVSMWSDLDGEV ERACVNALSLLRKTGFEFIEIDDADIVELNQTLTFTVPLYEFFA DLAQSLLSLGWKHGIHHIFAQVDDANVKGIINHHLGEGAIKP AHYLSSLQNGELLKRKMDELFARHNIELLGYPTVPCRVPHLD HADRPEFFSQAIRNTDLASNAMLPSITIPVGPEGRLPVGLSFDA

	LRGRDALLLSRVSAIEQVLGFVRKVLPHTT
TrpDH: Tryptophan dehydrogenase from Nostoc punctiforme NIES-2108 **SEQ ID NO: 154**	MLLFETVREMGHEQVLFCHSKNPEIKAIIAIHDTTLGPAMGAT RILPYINEEAALKDALRLSRGMTYKAACANIPAGGGKAVIIAN PENKTDDLLRAYGRFVDSLNGRFITGQDVNITPDDVRTISQET KYVVGVSEKSGGPAPITSLGVFLGIKAAVESRWQSKRLDGMK VAVQGLGNVGKNLCRHLHEHDVQLFVSDVDPIKAEEVKRLF GATVVEPTEIYSLDVDIFAPCALGGILNSHTIPFLQASIIAGAAN NQLENEQLHSQMLAKKGILYSPDYVINAGGLINVYNEMIGYD EEKAFKQVHNIYDTLLAIFEIAKEQGVTTNDAARRLAEDRINN SKRSKSKAIAA
CYP79B2: tryptophan N-monooxygenase from Arabidopsis thaliana **SEQ ID NO: 155**	MNTFTSNSSDLTTTATETSSFSTLYLLSTLQAFVAITLVMLLKK LMTDPNKKKPYLPPGPTGWPIIGMIPTMLKSRPVFRWLHSIMK QLNTEIACVKLGNTHVITVTCPKIAREILKQQDALFASRPLTY AQKILSNGYKTCVITPFGDQFKKMRKVVMTELVCPARHRWL HQKRSEENDHLTAWVYNMVKNSGSVDFRFMTRHYCGNAIK KLMFGTRTFSKNTAPDGGPTVEDVEHMEAMFEALGFTFAFCI SDYLPMLTGLDLNGHEKIMRESSAIMDKYHDPIIDERIKMWR EGKRTQIEDFLDIFISIKDEQGNPLLTADEIKPTIKELVMAAPDN PSNAVEWAMAEMVNKPEILRKAMEEIDRVVGKERLVQESDIP KLNYVKAILREAFRLHPVAAFNLPHVALSDTTVAGYHIPKGS QVLLSRYGLGRNPKVWADPLCFKPERHLNECSEVTLTENDLR FISFSTGKRGCAAPALGTALTTMMLARLLQGFTWKLPENETR VELMESSHDMFLAKPLVMVGDLRLPEHLYPTVK
CYP79B3: tryptophan N-monooxygenase from Arabidopsis thaliana **SEQ ID NO: 156**	MDTLASNSSDLTTKSSLGMSSFTNMYLLTTLQALAALCFLMI LNKIKSSSRNKKLHPLPPGPTGFPIVGMIPAMLKNRPVFRWLH SLMKELNTEIACVRLGNTHVIPVTCPKIAREIFKQQDALFASRP LTYAQKILSNGYKTCVITPFGEQFKKMRKVIMTEIVCPARHR WLHDNRAEETDHLTAWLYNMVKNSEPVDLRFVTRHYCGNA IKRLMFGTRTFSEKTEADGGPTLEDIEHMDAMFEGLGFTFAFC ISDYLPMLTGLDLNGHEKIMRESSAIMDKYHDPIIDERIKMWR EGKRTQIEDFLDIFISIKDEAGQPLLTADEIKPTIKELVMAAPDN PSNAVEWAIAEMINKPEILHKAMEEIDRVVGKERFVQESDIPK LNYVKAIIREAFRLHPVAAFNLPHVALSDTTVAGYHIPKGSQV LLSRYGLGRNPKVWSDPLSFKPERHLNECSEVTLTENDLRFIS FSTGKRGCAAPALGTAITTMMLARLLQGFKWKLAGSETRVE LMESSHDMFLSKPLVLVGELRLSEDLYPMVK
CYP71A13: indoleacetaldoxime dehydratase from Arabidopis thaliana **SEQ ID NO: 157**	MSNIQEMEMILSISLCLTTLITLLLLRRFLKRTATKVNLPPSPW RLPVIGNLHQLSLHPHRSLRSLSLRYGPLMLLHFGRVPILVVSS GEAAQEVLKTHDHKFANRPRSKAVHGLMNGGRDVVFAPYG EYWRQMKSVCILNLLTNKMVESFEKVREDEVNAMIEKLEKA SSSSSSENLSELFITLPSDVTSRVALGRKHSEDETARDLKKRVR QIMELLGEFPIGEYVPILAWIDGIRGFNNKIKEVSRGFSDLMDK VVQEHLEASNDKADFVDILLSIEKDKNSGFQVQRNDIKFMILD MFIGGTSTTSTLLEWTMTELIRSPKSMKKLQDEIRSTIRPHGSY IKEKEVENMKYLKAVIKEVLRLHPSLPMILPRLLSEDVKVKGY NIAAGTEVIINAWAIQRDTAIWGPDAEEFKPERHLDSGLDYHG KNLNYIPFGSGRRICPGINLALGLAEVTVANLVGRFDWRVEA

	GPNGDQPDLTEAIGIDVCRKFPLIAFPSSVV
PEN2: myrosinase from Arabidopsis thaliana **SEQ ID NO: 158**	MAHLQRTFPTEMSKGRASFPKGFLFGTASSSYQYEGAVNEGA RGQSVWDHFSNRFPHRISDSSDGNVAVDFYHRYKEDIKRMK DINMDSFRLSIAWPRVLPYGKRDRGVSEEGIKFYNDVIDELLA NEITPLVTIFHWDIPQDLEDEYGGFLSEQIIDDFRDYASLCFERF GDRVSLWCTMNEPWVYSVAGYDTGRKAPGRCSKYVNGASV AGMSGYEAYIVSHNMLLAHAEAVEVFRKCDHIKNGQIGIAHN PLWYEPYDPSDPDDVEGCNRAMDFMLGWHQHPTACGDYPE TMKKSVGDRLPSFTPEQSKKLIGSCDYVGINYYSSLFVKSIKH VDPTQPTWRTDQGVDWMKTNIDGKQIAKQGGSEWSFTYPTG LRNILKYVKKTYGNPPILITENGYGEVAEQSQSLYMYNPSIDT ERLEYIEGHIHAIHQAIHEDGVRVEGYYVWSLLDNFEWNSGY GVRYGLYYIDYKDGLRRYPKMSALWLKEFLRFDQEDDSSTS KKEEKKESYGKQLLHSVQDSQFVHSIKDSGALPAVLGSLFVV SATVGTSLFFKGANN
Nit1: Nitrilase from Arabidopsis thaliana **SEQ ID NO: 159**	MSSTKDMSTVQNATPFNGVAPSTTVRVTIVQSSTVYNDTPATI DKAEKYIVEAASKGAELVLFPEGFIGGYPRGFRFGLAVGVHN EEGRDEFRKYHASAIHVPGPEVARLADVARKNHVYLVMGAI EKEGYTLYCTVLFFSPQGQFLGKHRKLMPTSLERCIWGQGDG STIPVYDTPIGKLGAAICWENRMPLYRTALYAKGIELYCAPTA DGSKEWQSSMLHIAIEGGCFVLSACQFCQRKHFPDHPDYLFT DWYDDKEHDSIVSQGGSVIISPLGQVLAGPNFESEGLVTADID LGDIARAKLYFDSVGHYSRPDVLHLTVNEHPRKSVTFVTKVE KAEDDSNK
IDO1: indoleamine 2,3-dioxygenase from homo sapiens **SEQ ID NO: 160**	MAHAMENSWTISKEYHIDEEVGFALPNPQENLPDFYNDWMFI AKHLPDLIESGQLRERVEKLNMLSIDHLTDHKSQRLARLVLG CITMAYVWGKGHGDVRKVLPRNIAVPYCQLSKKLELPPILVY ADCVLANWKKKDPNKPLTYENMDVLFSFRDGDCSKGFFLVS LLVEIAAASAIKVIPTVFKAMQMQERDTLLKALLEIASCLEKA LQVFHQIHDHVNPKAFFSVLRIYLSGWKGNPQLSDGLVYEGF WEDPKEFAGGSAGQSSVFQCFDVLLGIQQTAGGGHAAQFLQ DMRRYMPPAHRNFLCSLESNPSVREFVLSKGDAGLREAYDA CVKALVSLRSYHLQIVTKYILIPASQQPKENKTSEDPSKLEAK GTGGTDLMNFLKTVRSTTEKSLLKEG
TDO2: tryptophan 2,3-dioxygenase from homo sapiens **SEQ ID NO: 161**	MSGCPFLGNNFGYTFKKLPVEGSEEDKSQTGVNRASKGGLIY GNYLHLEKVLNAQELQSETKGNKIHDEHLFIITHQAYELWFK QILWELDSVREIFQNGHVRDERNMLKVVSRMHRVSVILKLLV QQFSILETMTALDFNDFREYLSPASGFQSLQFRLLENKIGVLQ NMRVPYNRRHYRDNFKGEENELLLKSEQEKTLLELVEAWLE RTPGLEPHGFNFWGKLEKNITRGLEEEFIRIQAKEESEEKEEQV AEFQKQKEVLLSLFDEKRHEHLLSKGERRLSYRALQGALMIY FYREEPRFQVPFQLLTSLMDIDSLMTKWRYNHVCMVHRMLG SKAGTGGSSGYHYLRSTVSDRYKVFVDLFNLSTYLIPRHWIPK MNPTIHKFLYTAEYCDSSYFSSDESD
BNA2: indoleamine 2,3-dioxygenase from S. cerevisiae	MNNTSITGPQVLHRTKMRPLPVLEKYCISPHHGFLDDRLPLTR LSSKKYMKWEEIVADLPSLLQEDNKVRSVIDGLDVLDLDETIL GDVRELRRAYSILGFMAHAYIWASGTPRDVLPECIARPLLETA

SEQ ID NO: 162	HILGVPPLATYSSLVLWNFKVTDECKKTETGCLDLENITTINTF TGTVDESWFYLVSVRFEKIGSACLNHGLQILRAIRSGDKGDA NVIDGLEGLAATIERLSKALMEMELKCEPNVFYFKIRPFLAGW TNMSHMGLPQGVRYGAEGQYRIFSGGSNAQSSLIQTLDILLG VKHTANAAHSSQGDSKINYLDEMKKYMPREHREFLYHLESV CNIREYVSRNASNRALQEAYGRCISMLKIFRDNHIQIVTKYIIL PSNSKQHGSNKPNVLSPIEPNTKASGCLGHKVASSKTIGTGGT RLMPFLKQCRDETVATADIKNEDKN
Afmid: Kynurenine formamidase from mouse **SEQ ID NO: 163**	MAFPSLSAGQNPWRNLSSEELEKQYSPSRWVIHTKPEEVVGN FVQIGSQATQKARATRRNQLDVPYGDGEGEKLDIYFPDEDSK AFPLFLFLHGGYWQSGSKDDSAFMVNPLTAQGIVVVIVAYDI APKGTLDQMVDQVTRSVVFLQRRYPSNEGIYLCGHSAGAHL AAMVLLARWTKHGVTPNLQGFLLVSGIYDLEPLIATSQNDPL RMTLEDAQRNSPQRHLDVVPAQPVAPACPVLVLVGQHDSPE FHRQSKEFYETLLRVGWKASFQQLRGVDHFDIIENLTREDDV LTQIILKTVFQKL
BNA3: kynurenine--oxoglutarate transaminase from S. cerevisae **SEQ ID NO: 164**	MKQRFIRQFTNLMSTSRPKVVANKYFTSNTAKDVWSLTNEA AAKAANNSKNQGRELINLGQGFFSYSPPQFAIKEAQKALDIPM VNQYSPTRGRPSLINSLIKLYSPIYNTELKAENVTVTTGANEGI LSCLMGLLNAGDEVIVFEPFFDQYIPNIELCGGKVVYVPINPPK ELDQRNTRGEEWTIDFEQFEKAITSKTKAVIINTPHNPIGKVFT REELTTLGNICVKHNVVIISDEVYEHLYFTDSFTRIATLSPEIGQ LTLTVGSAGKSFAATGWRIGWVLSLNAELLSYAAKAHTRICF ASPSPLQEACANSINDALKIGYFEKMRQEYINKFKIFTSIFDEL GLPYTAPEGTYFVLVDFSKVKIPEDYPYPEEILNKGKDFRISH WLINELGVVAIPPTEFYIKEHEKAAENLLRFAVCKDDAYLEN AVERLKLLKDYL
GOT2: Aspartate aminotransferase, mitochondrial from homo sapiens **SEQ ID NO: 165**	MALLHSGRVLPGIAAAFHPGLAAAASARASSWWTHVEMGPP DPILGVTEAFKRDTNSKKMNLGVGAYRDDNGKPYVLPSVRK AEAQIAAKNLDKEYLPIGGLAEFCKASAELALGENSEVLKSG RFVTVQTISGTGALRIGASFLQRFFKFSRDVFLPKPTWGNHTPI FRDAGMQLQGYRYYDPKTCGFDFTGAVEDISKIPEQSVLLLH ACAHNPTGVDPRPEQWKEIATVVKKRNLFAFFDMAYQGFAS GDGDKDAWAVRHFIEQGINVCLCQSYAKNMGLYGERVGAFT MVCKDADEAKRVESQLKILIRPMYSNPPLNGARIAAAILNTPD LRKQWLQEVKVMADRIIGMRTQLVSNLKKEGSTHNWQHITD QIGMFCFTGLKPEQVERLIKEFSIYMTKDGRISVAGVTSSNVG YLAHAIHQVTK
AADAT: Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial **SEQ ID NO: 166**	MNYARFITAASAARNPSPIRTMTDILSRGPKSMISLAGGLPNP NMFPFKTAVITVENGKTIQFGEEMMKRALQYSPSAGIPELLSW LKQLQIKLHNPPTIHYPPSQGQMDLCVTSGSQQGLCKVFEMII NPGDNVLLDEPAYSGTLQSLHPLGCNIINVASDESGIVPDSLR DILSRWKPEDAKNPQKNTPKFLYTVPNGNNPTGNSLTSERKK EIYELARKYDFLIIEDDPYYFLQFNKFRVPTFLSMDVDGRVIRA DSFSKIISSGLRIGFLTGPKPLIERVILHIQVSTLHPSTFNQLMIS QLLHEWGEEGFMAHVDRVIDFYSNQKDAILAAADKWLTGLA EWHVPAAGMFLWIKVKGINDVKELIEEKAVKMGVLMLPGN AFYVDSSAPSPYLRASFSSASPEQMDVAFQVLAQLIKESL

CCLB1: Kynurenine--oxoglutarate transaminase 1 from homo sapiens **SEQ ID NO: 167**	MAKQLQARRLDGIDYNPWVEFVKLASEHDVVNLGQGFPDFP PPDFAVEAFQHAVSGDFMLNQYTKTFGYPPLTKILASFFGELL GQEIDPLRNVLVTVGGYGALFTAFQALVDEGDEVIIIEPFFDC YEPMTMMAGGRPVFVSLKPGPIQNGELGSSSNWQLDPMELA GKFTSRTKALVLNTPNNPLGKVFSREELELVASLCQQHDVVCI TDEVYQWMVYDGHQHISIASLPGMWERTLTIGSAGKTFSATG WKVGWVLGPDHIMKHLRTVHQNSVFHCPTQSQAAVAESFER EQLLFRQPSSYFVQFPQAMQRCRDHMIRSLQSVGLKPIIPQGS YFLITDISDFKRKMPDLPGAVDEPYDRRFVKWMIKNKGLVAI PVSIFYSVPHQKHFDHYIRFCFVKDEATLQAMDEKLRKWKVE L
CCLB2: kynurenine--oxoglutarate transaminase 3 from homo sapiens **SEQ ID NO: 168**	MFLAQRSLCSLSGRAKFLKTISSSKILGFSTSAKMSLKFTNAKR IEGLDSNVWIEFTKLAADPSVVNLGQGFPDISPPTYVKEELSKI AAIDSLNQYTRGFGHPSLVKALSYLYEKLYQKQIDSNKEILVT VGAYGSLFNTIQALIDEGDEVILIVPFYDCYEPMVRMAGATPV FIPLRSKPVYGKRWSSSDWTLDPQELESKFNSKTKAIILNTPHN PLGKVYNREELQVIADLCIKYDTLCISDEVYEWLVYSGNKHL KIATFPGMWERTITIGSAGKTFSVTGWKLGWSIGPNHLIKHLQ TVQQNTIYTCATPLQEALAQAFWIDIKRMDDPECYFNSLPKEL EVKRDRMVRLLESVGLKPIVPDGGYFIIADVSLLDPDLSDMK NNEPYDYKFVKWMTKHKKLSAIPVSAFCNSETKSQFEKFVRF CFIKKDSTLDAAEEIIKAWSVQKS
TnaA: tryptophanase from E. coli **SEQ ID NO: 140**	MENFKHLPEPFRIRVIEPVKRTTRAYREEAIIKSGMNPFLLDSE DVFIDLLTDSGTGAVTQSMQAAMMRGDEAYSGSRSYYALAE SVKNIFGYQYTIPTHQGRGAEQIYIPVLIKKREQEKGLDRSKM VAFSNYFFDTTQGHSQINGCTVRNVYIKEAFDTGVRYDFKGN FDLEGLERGIEEVGPNNVPYIVATITSNSAGGQPVSLANLKAM YSIAKKYDIPVVMDSARFAENAYFIKQREAEYKDWTIEQITRE TYKYADMLAMSAKKDAMVPMGGLLCMKDDSFFDVYTECRT LCVVQEGFPTYGGLEGGAMERLAVGLYDGMNLDWLAYRIA QVQYLVDGLEEIGVVCQQAGGHAAFVDAGKLLPHIPADQFP AQALACELYKVAGIRAVEIGSFLLGRDPKTGKQLPCPAELLRL TIPRATYTQTHMDFIIEAFKHVKENAANIKGLTFTYEPKVLRH FTAKLKEV
Trp aminotransferase (EC 2.6.1.27); tryptophan aminotransferase [Cryptococcus deuterogattii R265] **SEQ ID NO: 169**	MTATTISIETVPQAPAAGTKTNGTSGKYNPRTYLSDRAKVTEI DGSDAGRPNPDTFPFNSITLNLKPPLGLPESSNNMPVSITIEDPD LATALQYAPSAGIPKLREWLADLQAHVHERPRGDYAISVGSG SQDLMFKGFQAVLNPGDPVLLETPMYSGVLPALRILKADYAE VDVDDQGLSAKNLEKVLSEWPADKKRPRVLYTSPIGSNPSGC SASKERKLEVLKVCKKYDVLIFEDDPYYYLAQELIPSYFALEK QVYPEGGHVVRFDSFSKLLSAGMRLGFATGPKEILHAIDVSTA GANLHTSAVSQGVALRLMQYWGIEGFLAHGRAVAKLYTERR AQFEATAHKYLDGLATWVSPVAGMFLWIDLRPAGIEDSYELI RHEALAKGVLGVPGMAFYPTGRKSSHVRVSFSIVDLEDESDL GFQRLAEAIKDKRKALGLA
Tryptophan Decarboxylase (EC 4.1.1.28) Chain A,	MSQVIKKKRNTFMIGTEYILNSTQLEEAIKSFVHDFCAEKHEIH DQPVVVEAKEHQEDKIKQIKIPEKGRPVNEVVSEMMNEVYRY RGDANHPRFFSFVPGPASSVSWLGDIMTSAYNIHAGGSKLAP

Ruminococcus Gnavus **SEQ ID NO: 170**	MVNCIEQEVLKWLAKQVGFTENPGGVFVSGGSMANITALTA ARDNKLTDINLHLGTAYISDQTHSSVAKGLRIIGITDSRIRRIPT NSHFQMDTTKLEEAIETDKKSGYIPFVVIGTAGTTNTGSIDPLT EISALCKKHDMWFHIDGAYGASVLLSPKYKSLLTGTGLADSIS WDAHKWLFQTYGCAMVLVKDIRNLFHSFHVNPEYLKDLEN DIDNVNTWDIGMELTRPARGLKLWLTLQVLGSDLIGSAIEHG FQLAVWAEEALNPKKDWEIVSPAQMAMINFRYAPKDLTKEE QDILNEKISHRILESGYAAIFTTVLNGKTVLRICAIHPEATQED MQHTIDLLDQYGREIYTEMKKa

Table 11B. Tryptophan Pathway Catabolic Enzymes

Description	Sequence
Trp aminotransferase (EC 2.6.1.27); tryptophan aminotransferase [Cryptococcus deuterogattii R265], codon optimized for expression in E. coli **SEQ ID NO: 171**	ATGACGGCAACTACAATTTCTATTGAGACCGTACCTC AGGCCCCGGCGGCGGGGACCAAAACTAATGGGACTT CAGGAAAATACAACCCCCGCACTTACCTGTCCGACC GCGCCAAAGTCACTGAGATTGATGGATCTGACGCCG GTCGCCCCAATCCCGATACTTTCCCATTTAACTCGAT TACCTTAAATTTGAAACCACCTTTAGGCTTGCCCGAG AGTTCAAATAACATGCCGGTCTCTATCACGATTGAA GACCCCGATTTAGCGACGGCCTTACAATATGCACCT AGCGCCGGTATTCCTAAGCTGCGCGAATGGCTGGCT GACTTACAAGCTCACGTTCATGAGCGCCCCCGTGGC GATTATGCCATCTCGGTCGGGTCGGGGTCACAGGAT TTGATGTTTAAGGGCTTCCAAGCTGTCTTGAATCCAG GTGATCCAGTCCTTCTGGAAACCCCAATGTATTCAGG TGTTCTGCCAGCGCTGCGCATTCTGAAGGCGGATTAT GCAGAAGTTGATGTAGACGACCAGGGGTTATCTGCT AAAAACCTTGAAAAAGTTTTATCAGAGTGGCCCGCA GATAAGAAGCGTCCTCGTGTCCTGTATACGTCGCCA ATCGGCTCCAATCCTTCCGGATGTTCAGCATCCAAGG AACGCAAGTTAGAGGTACTGAAAGTCTGTAAGAAGT ACGATGTGCTGATCTTCGAAGACGATCCGTATTATTA CCTTGCTCAAGAGCTTATTCCATCCTATTTTGCGTTG GAAAAACAAGTTTATCCGGAGGGTGGGCACGTTGTA CGCTTTGACTCATTTAGTAAATTGCTTTCTGCTGGGA TGCGCTTGGGATTTGCTACAGGGCCGAAGGAAATTC TTCATGCGATTGACGTCAGTACAGCAGGCGCAAATT TACATACTTCAGCGGTCTCTCAAGGTGTCGCTCTTCG CCTGATGCAGTATTGGGGGATCGAGGGATTCCTTGC ACATGGCCGCGCGGTGGCCAAACTTTACACGGAGCG CCGCGCTCAGTTCGAGGCAACCGCACATAAGTACCT GGACGGGCTGGCCACTTGGGTATCTCCCGTAGCGGG AATGTTTTTATGGATCGATCTTCGTCCAGCAGGAATC GAAGATTCTTACGAATTAATTCGCCATGAAGCATTA GCCAAAGGCGTTTTAGGCGTTCCAGGGATGGCGTTTT ATCCGACAGGCCGTAAGTCTTCCCATGTTCGTGTCAG TTTCAGTATCGTCGACCTGGAAGACGAATCTGACCTT

	GGTTTTCAACGCCTGGCTGAAGCTATTAAGGATAAA CGCAAGGCTTTAGGGCTGGCT
Tryptophan Decarboxylase (EC 4.1.1.28) Chain A, Ruminococcus Gnavus **Tryptophan Decarboxylase** Rumgna_01526 (alpha-fmt); codon optimized for the expression in E. coli **SEQ ID NO: 172**	ATGAGTCAAGTGATTAAGAAGAAACGTAACACCTTT ATGATCGGAACGGAGTACATTCTTAACAGTACACAA TTGGAGGAAGCGATTAAATCATTCGTACATGATTTCT GCGCAGAGAAGCATGAGATCCATGATCAACCTGTGG TAGTAGAAGCTAAAGAACATCAGGAGGACAAAATC AAACAAATCAAAATCCCGGAAAAGGGACGTCCTGTA AATGAAGTCGTTTCTGAGATGATGAATGAAGTGTAT CGCTACCGCGGAGACGCCAACCATCCTCGCTTTTTTT CTTTTGTGCCCGGACCTGCAAGCAGTGTGTCGTGGTT GGGGGATATTATGACGTCCGCCTACAATATTCATGCT GGAGGCTCAAAGCTGGCACCGATGGTTAACTGCATT GAGCAGGAAGTTCTGAAGTGGTTAGCAAAGCAAGTG GGGTTCACAGAAAATCCAGGTGGCGTATTTGTGTCG GGCGGTTCAATGGCGAATATTACGGCACTTACTGCG GCTCGTGACAATAAACTGACCGACATTAACCTTCATT TGGGAACTGCTTATATTAGTGACCAGACTCATAGTTC AGTTGCGAAAGGATTACGCATTATTGGAATCACTGA CAGTCGCATCCGTCGCATTCCCACTAACTCCCACTTC CAGATGGATACCACCAAGCTGGAGGAAGCCATCGAG ACCGACAAGAAGTCTGGCTACATTCCGTTCGTCGTTA TCGGAACAGCAGGTACCACCAACACTGGTTCGATTG ACCCCCTGACAGAAATCTCTGCGTTATGTAAGAAGC ATGACATGTGGTTTCATATCGACGGAGCGTATGGAG CTAGTGTTCTGCTGTCACCTAAGTACAAGAGCCTTCT TACCGGAACCGGCTTGGCTGACAGTATTTCGTGGGA TGCTCATAAATGGTTGTTCCAAACGTACGGCTGTGCA ATGGTACTTGTCAAAGATATCCGTAATTTATTCCACT CTTTTCATGTGAATCCCGAGTATCTTAAGGATCTGGA AAACGACATCGATAACGTTAATACATGGGACATCGG CATGGAGCTGACGCGCCCTGCACGCGGTCTTAAATT GTGGCTTACTTTACAGGTCCTTGGATCTGACTTGATT GGGAGTGCCATTGAACACGGTTTCCAGCTGGCAGTT TGGGCTGAGGAAGCATTGAATCCAAAGAAAGACTGG GAGATCGTTTCTCCAGCTCAGATGGCTATGATTAATT TCCGTTATGCCCCTAAGGATTTAACCAAAGAGGAAC AGGATATTCTGAATGAAAAGATCTCCCACCGCATTTT AGAGAGCGGATACGCTGCAATTTTCACTACTGTATTA AACGGCAAGACCGTTTTACGCATCTGTGCAATTCACC CGGAGGCAACTCAAGAGGATATGCAACACACAATCG ACTTATTAGACCAATACGGTCGTGAAATCTATACCG AGATGAAGAAAGCG
Tdc (tdc from C.	ATGGGTTCTATTGACTCGACGAATGTGGCCATGTCT

roseus) **SEQ ID NO: 260**	AATTCTCCTGTTGGCGAGTTTAAGCCCCTTGAAGCA GAAGAGTTCCGTAAACAGGCACACCGCATGGTGGA TTTTATTGCGGATTATTACAAGAACGTAGAAACATA CCCGGTCCTTTCCGAGGTTGAACCCGGCTATCTGCG CAAACGTATTCCCGAAACCGCACCATACCTGCCGG AGCCACTTGATGATATTATGAAGGATATTCAAAAG GACATTATCCCCGGAATGACGAACTGGATGTCCCCG AACTTTTACGCCTTCTTCCCGGCCACAGTTAGCTCA GCAGCTTTCTTGGGGGAAATGCTTTCAACGGCCCTT AACAGCGTAGGATTTACCTGGGTCAGTTCCCCGGCA GCGACTGAATTAGAGATGATCGTTATGGATTGGCTT GCGCAAATTTTGAAACTTCCAAAAAGCTTTATGTTC TCCGGAACCGGGGGTGGTGTCATCCAAAACACTAC GTCAGAGTCGATCTTGTGCACTATTATCGCGGCCCG TGAACGCGCCTTGGAAAAATTGGGCCCTGATTCAAT TGGTAAGCTTGTCTGCTATGGGTCCGATCAAACGCA CACAATGTTTCCGAAAACCTGTAAGTTAGCAGGAAT TTATCCGAATAATATCCGCCTTATCCCTACCACGGT AGAAACCGACTTTGGCATCTCACCGCAGGTACTTCG CAAGATGGTCGAAGACGACGTCGCTGCGGGGTACG TTCCCTTATTTTTGTGTGCCACCTTGGGAACGACATC AACTACGGCAACAGATCCTGTAGATTCGCTGTCCGA AATCGCAAACGAGTTTGGTATCTGGATTCATGTCGA CGCCGCATATGCTGGATCGGCTTGCATCTGCCCAGA ATTTCGTCACTACCTTGATGGCATCGAACGTGTGGA TTCCTTATCGCTGTCTCCCCACAAATGGCTTTTAGCA TATCTGGATTGCACGTGCTTGTGGGTAAAACAACCT CACCTGCTGCTTCGCGCTTTAACGACTAATCCCGAA TACTTGAAGAATAAACAGAGTGATTTAGATAAGGT CGTGGATTTTAAGAACTGGCAGATCGCAACAGGAC GTAAGTTCCGCTCTTTAAAACTTTGGTTAATTCTGC GTTCCTACGGGGTAGTTAACCTGCAAAGTCATATCC GTAGTGATGTAGCGATGGGGAAGATGTTTGAGGAA TGGGTCCGTTCCGATAGCCGCTTTGAAATCGTCGTG CCACGTAATTTTTCGCTTGTATGCTTTCGCTTGAAAC CGGATGTATCTAGTTTACATGTCGAGGAGGTCAACA AGAAGTTGTTGGATATGCTTAACTCCACCGGTCGCG TATATATGACGCATACAATTGTTGGCGGAATCTATA TGTTACGTTTGGCTGTAGGTAGCAGCTTGACAGAGG AACATCACGTGCGCCGCGTTTGGGACTTGATCCAGA AGCTTACGGACGACCTGCTTAAAGAGGCGTGA
Tdc (tdc from *Clostridium sporogenes*) **SEQ ID NO: 262**	ATGAAATTTTGGCGCAAGTATACGCAACAGGAGAT GGATGAGAAAATCACAGAATCGCTTGAGAAGACAT TAAATTACGATAACACGAAAACCATCGGCATCCCA GGTACTAAGCTGGATGATACTGTATTTTATGACGAT CACTCCTTCGTTAAGCACTCTCCCTATTTACGTACGT TCATCCAAAACCCTAATCACATTGGTTGTCACACGT ACGATAAAGCAGACATCTTGTTTGGCGGCACGTTTG

	ACATCGAACGCGAACTGATTCAGCTTTTGGCCATCG ATGTCTTAAACGGAAATGATGAGGAATTCGATGGA TATGTGACACAGGGGGGAACCGAGGCGAATATTCA GGCAATGTGGGTTTATCGTAACTATTTCAAAAAAGA ACGTAAAGCAAAACATGAGGAAATCGCAATCATCA CGAGCGCGGATACCCATTACAGTGCATATAAGGGG AGCGACTTGCTGAACATTGATATTATCAAGGTCCCA GTAGACTTCTATTCGCGTAAGATCCAGGAGAACAC GTTAGACTCGATTGTCAAGGAGGCGAAGGAAATTG GAAAGAAGTACTTCATTGTCATCTCAAACATGGGTA CGACTATGTTTGGCAGTGTAGACGACCCTGATCTTT ATGCTAACATTTTTGATAAGTATAACTTAGAATACA AAATCCACGTCGATGGAGCTTTTGGGGGTTTCATTT ATCCTATCGATAATAAGGAGTGCAAAACAGATTTCT CGAACAAGAACGTCTCATCCATCACGCTTGACGGTC ACAAAATGCTTCAAGCCCCCTATGGGACTGGTATCT TCGTGTCACGTAAGAACTTGATCCATAACACCCTGA CAAAGGAAGCAACGTATATTGAAAACCTGGACGTT ACCCTGAGTGGGTCCCGCTCCGGATCCAACGCCGTT GCGATCTGGATGGTTTTAGCCTCTTATGGCCCCTAC GGGTGGATGGAGAAGATTAACAAGTTGCGCAATCG CACTAAGTGGCTTTGCAAGCAGCTTAACGACATGCG CATCAAATACTATAAGGAGGATAGCATGAATATCG TCACGATTGAAGAGCAATACGTAAATAAAGAGATT GCAGAGAAATACTTCCTTGTGCCTGAAGTACACAAT CCTACCAACAATTGGTACAAGATTGTAGTCATGGAA CATGTTGAACTTGACATCTTGAACTCCCTTGTTTATG ATTTACGTAAATTCAACAAGGAGCACCTGAAGGCA ATGTGA
ipdC **SEQ ID NO: 265**	ATGCGTACACCCTACTGTGTCGCCGATTATCTTTTA GATCGTCTGACGGACTGCGGGGCCGATCACCTGTTT GGCGTACCGGGCGATTACAACTTGCAGTTTCTGGAC CACGTCATTGACTCACCAGATATCTGCTGGGTAGGG TGTGCGAACGAGCTTAACGCGAGCTACGCTGCTGA CGGATATGCGCGTTGTAAAGGCTTTGCTGCACTTCT TACTACCTTCGGGGTCGGTGAGTTATCGGCGATGAA CGGTATCGCAGGCTCGTACGCTGAGCACGTCCCGGT ATTACACATTGTGGGAGCTCCGGGTACCGCAGCTCA ACAGCGCGGAGAACTGTTACACCACACGCTGGGCG ACGGAGAATTCCGCCACTTTTACCATATGTCCGAGC CAATTACTGTAGCCCAGGCTGTACTTACAGAGCAAA ATGCCTGTTACGAGATCGACCGTGTTTTGACCACGA TGCTTCGCGAGCGCCGTCCCGGGTATTTGATGCTGC CAGCCGATGTTGCCAAAAAAGCTGCGACGCCCCCA GTGAATGCCCTGACGCATAAACAAGCTCATGCCGA TTCCGCCTGTTTAAAGGCTTTTCGCGATGCAGCTGA AAATAAATTAGCCATGTCGAAACGCACCGCCTTGTT GGCGGACTTTCTGGTCCTGCGCCATGGCCTTAAACA

	CGCCCTTCAGAAATGGGTCAAAGAAGTCCCGATGG CCCACGCTACGATGCTTATGGGTAAGGGGATTTTTG ATGAACGTCAAGCGGGATTTTATGGAACTTATTCCG GTTCGGCGAGTACGGGGGCGGTAAAGGAAGCGATT GAGGGAGCCGACACAGTTCTTTGCGTGGGGACACG TTTCACCGATACACTGACCGCTGGATTCACACACCA ACTTACTCCGGCACAAACGATTGAGGTGCAACCCC ATGCGGCTCGCGTGGGGGATGTATGGTTTACGGGC ATTCCAATGAATCAAGCCATTGAGACTCTTGTCGAG CTGTGCAAACAGCACGTCCACGCAGGACTGATGAG TTCGAGCTCTGGGGCGATTCCTTTTCCACAACCAGA TGGTAGTTTAACTCAAGAAAACTTCTGGCGCACATT GCAAACCTTTATCCGCCCAGGTGATATCATCTTAGC AGACCAGGGTACTTCAGCCTTTGGAGCAATTGACCT GCGCTTACCAGCAGACGTGAACTTTATTGTGCAGCC GCTGTGGGGGTCTATTGGTTATACTTTAGCTGCGGC CTTCGGAGCGCAGACAGCGTGTCCAAACCGTCGTGT GATCGTATTGACAGGAGATGGAGCAGCGCAGTTGA CCATTCAGGAGTTAGGCTCGATGTTACGCGATAAGC AGCACCCCATTATCCTGGTCCTGAACAATGAGGGGT ATACAGTTGAACGCGCCATTCATGGTGCGGAACAA CGCTACAATGACATCGCTTTATGGAATTGGACGCAC ATCCCCCAAGCCTTATCGTTAGATCCCCAATCGGAA TGTTGGCGTGTGTCTGAAGCAGAGCAACTGGCTGAT GTTCTGGAAAAAGTTGCTCATCATGAACGCCTGTCG TTGATCGAGGTAATGTTGCCCAAGGCCGATATCCCT CCGTTACTGGGAGCCTTGACCAAGGCTTTAGAAGCC TGCAACAACGCTTAA
Iad1 **SEQ ID NO: 266**	ATGCCCACCTTGAACTTGGACTTACCCAACGGTATT AAGAGCACGATTCAGGCAGACCTTTTCATCAATAAT AAGTTTGTGCCGGCGCTTGATGGGAAAACGTTCGCA ACTATTAATCCGTCTACGGGGAAAGAGATCGGACA GGTGGCAGAGGCTTCGGCGAAGGATGTGGATCTTG CAGTTAAGGCCGCGCGTGAGGCGTTTGAAACTACTT GGGGGGAAAACACGCCAGGTGATGCTCGTGGCCGT TTACTGATTAAGCTTGCTGAGTTGGTGGAAGCGAAT ATTGATGAGTTAGCGGCAATTGAATCACTGGACAAT GGGAAAGCGTTCTCTATTGCTAAGTCATTCGACGTA GCTGCTGTGGCCGCAAACTTACGTTACTACGGCGGT TGGGCTGATAAAAACCACGGTAAAGTCATGGAGGT AGACACAAAGCGCCTGAACTATACCCGCCACGAGC CGATCGGGGTTTGCGGACAAATCATTCCGTGGAATT TCCCGCTTTTGATGTTTGCATGGAAGCTGGGTCCCG CTTTAGCCACAGGGAACACAATTGTGTTAAAGACTG CCGAGCAGACTCCCTTAAGTGCTATCAAGATGTGTG AATTAATCGTAGAAGCCGGCTTTCCGCCCGGAGTAG TTAATGTGATCTCGGGATTCGGACCGGTGGCGGGG GCCGCGATCTCGCAACACATGGACATCGATAAGAT

	TGCCTTTACAGGATCGACATTGGTTGGCCGCAACAT TATGAAGGCAGCTGCGTCGACTAACTTAAAAAAGG TTACACTTGAGTTAGGAGGAAAATCCCCGAATATCA TTTTCAAAGATGCCGACCTTGACCAAGCTGTTCGCT GGAGCGCCTTCGGTATCATGTTTAACCACGGACAAT GCTGCTGCGCTGGATCGCGCGTATATGTGGAAGAAT CCATCTATGACGCCTTCATGGAAAAAATGACTGCGC ATTGTAAGGCGCTTCAAGTTGGAGATCCTTTCAGCG CGAACACCTTCCAAGGACCACAAGTCTCGCAGTTAC AATACGACCGTATCATGGAATACATCGAATCAGGG AAAAAAGATGCAAATCTTGCTTTAGGCGGCGTTCGC AAAGGGAATGAGGGGTATTTCATTGAGCCAACTAT TTTTACAGACGTGCCGCACGACGCGAAGATTGCCA AAGAGGAGATCTTCGGTCCAGTGGTTGTTGTGTCGA AATTTAAGGACGAAAAAGATCTGATCCGTATCGCA AATGATTCTATTTATGGTTTAGCTGCGGCAGTCTTTT CCCGCGACATCAGCCGCGCGATCGAGACAGCACAC AAACTGAAAGCAGGCACGGTCTGGGTCAACTGCTA TAATCAGCTTATTCCGCAGGTGCCATTCGGAGGGTA TAAGGCTTCCGGTATCGGCCGTGAGTTGGGGGAAT ATGCCTTGTCTAATTACACAAATATCAAGGCCGTCC ACGTTAACCTTTCTCAACCGGCGCCCATTTGA
fldA **SEQ ID NO: 276**	ATGGAAAACAACACCAATATGTTCTCTGGAGTGAA GGTGATCGAACTGGCCAACTTTATCGCTGCTCCGGC GGCAGGTCGCTTCTTTGCTGATGGGGGAGCAGAAG TAATTAAGATCGAATCTCCAGCAGGCGACCCGCTGC GCTACACGGCCCCATCAGAAGGACGCCCGCTTTCTC AAGAGGAAAACACAACGTATGATTTGGAAAACGCG AATAAGAAAGCAATTGTTCTGAACTTAAAATCGGA AAAAGGAAAGAAAATTCTTCACGAGATGCTTGCTG AGGCAGACATCTTGTTAACAAATTGGCGCACGAAA GCGTTAGTCAAACAGGGGTTAGATTACGAAACACT GAAAGAGAAGTATCCAAAATTGGTATTTGCACAGA TTACAGGATACGGGGAGAAAGGACCCGACAAAGAC CTGCCTGGTTTCGACTACACGGCGTTTTTCGCCCGC GGAGGAGTCTCCGGTACATTATATGAAAAAGGAAC TGTCCCTCCTAATGTGGTACCGGGTCTGGGTGACCA CCAGGCAGGAATGTTCTTAGCTGCCGGTATGGCTGG TGCGTTGTATAAGGCCAAAACCACCGGACAAGGCG ACAAAGTCACCGTTAGTCTGATGCATAGCGCAATGT ACGGCCTGGGAATCATGATTCAGGCAGCCCAGTAC AAGGACCATGGGCTGGTGTACCCGATCAACCGTAA TGAAACGCCTAATCCTTTCATCGTTTCATACAAGTC CAAAGATGATTACTTTGTCCAAGTTTGCATGCCTCC CTATGATGTGTTTTATGATCGCTTTATGACGGCCTTA GGACGTGAAGACTTGGTAGGTGACGAACGCTACAA TAAGATCGAGAACTTGAAGGATGGTCGCGCAAAAG AAGTCTATTCCATCATCGAACAACAAATGGTAACG

	AAGACGAAGGACGAATGGGACAAGATTTTTCGTGA TGCAGACATTCCATTCGCTATTGCCCAAACGTGGGA AGATCTTTTAGAAGACGAGCAGGCATGGGCCAACG ACTACCTGTATAAAATGAAGTATCCCACAGGCAAC GAACGTGCCCTGGTACGTTTACCTGTGTTCTTCAAA GAAGCTGGACTTCCTGAATACAACCAGTCGCCACA GATTGCTGAGAATACCGTGGAAGTGTTAAAGGAGA TGGGATATACCGAGCAAGAAATTGAGGAGCTTGAG AAAGACAAAGACATCATGGTACGTAAAGAGAAATG A
fldB **SEQ ID NO: 277**	ATGTCAGACCGCAACAAAGAAGTGAAAGAAAAGA AGGCTAAACACTATCTGCGCGAGATCACAGCTAAA CACTACAAGGAAGCGTTAGAGGCTAAAGAGCGTGG GGAGAAAGTGGGTTGGTGTGCCTCTAACTTCCCCCA AGAGATTGCAACCACGTTGGGTGTAAAGGTTGTTTA TCCCGAAAACCACGCCGCCGCCGTAGCGGCACGTG GCAATGGGCAAAATATGTGCGAACACGCGGAGGCT ATGGGATTCAGTAATGATGTGTGTGGATATGCACGT GTAAATTTAGCCGTAATGGACATCGGCCATAGTGA AGATCAACCTATTCCAATGCCTGATTTCGTTCTGTG CTGTAATAATATCTGCAATCAGATGATTAAATGGTA TGAACACATTGCAAAAACGTTGGATATTCCTATGAT CCTTATCGATATTCCATATAATACTGAGAACACGGT GTCTCAGGACCGCATTAAGTACATCCGCGCCCAGTT CGATGACGCTATCAAGCAACTGGAAGAAATCACTG GCAAAAAGTGGGACGAGAATAAATTCGAAGAAGTG ATGAAGATTTCGCAAGAATCGGCCAAGCAATGGTT ACGCGCCGCGAGCTACGCGAAATACAAACCATCAC CGTTTTCGGGCTTTGACCTTTTTAATCACATGGCTGT AGCCGTTTGTGCTCGCGGCACCCAGGAAGCCGCCG ATGCATTCAAAATGTTAGCAGATGAATATGAAGAG AACGTTAAGACAGGAAAGTCTACTTATCGCGGCGA GGAGAAGCAGCGTATCTTGTTCGAGGGCATCGCTTG TTGGCCTTATCTGCGCCACAAGTTGACGAAACTGAG TGAATATGGAATGAACGTCACAGCTACGGTGTACG CCGAAGCTTTTGGGGTTATTTACGAAAACATGGATG AACTGATGGCCGCTTACAATAAAGTGCCTAACTCAA TCTCCTTCGAGAACGCGCTGAAGATGCGTCTTAATG CCGTTACAAGCACCAATACAGAAGGGGCTGTTATC CACATTAATCGCAGTTGTAAGCTGTGGTCAGGATTC TTATACGAACTGGCCCGTCGTTTGGAAAAGGAGAC GGGGATCCCTGTTGTTTCGTTCGACGGAGATCAAGC GGATCCCCGTAACTTCTCCGAGGCTCAATATGACAC TCGCATCCAAGGTTTAAATGAGGTGATGGTCGCGA AAAAAGAAGCAGAGTGA
fldC **SEQ ID NO: 278**	ATGTCGAATAGTGACAAGTTTTTTAACGACTTCAAG GACATTGTGGAAAACCCAAAGAAGTATATCATGAA GCATATGGAACAAACGGGACAAAAAGCCATCGGTT

	GCATGCCTTTATACACCCCAGAAGAGCTTGTCTTAG CGGCGGGTATGTTTCCTGTTGGAGTATGGGGCTCGA ATACTGAGTTGTCAAAAGCCAAGACCTACTTTCCGG CTTTTATCTGTTCTATCTTGCAAACTACTTTAGAAAA CGCATTGAATGGGGAGTATGACATGCTGTCTGGTAT GATGATCACAAACTATTGCGATTCGCTGAAATGTAT GGGACAAAACTTCAAACTTACAGTGGAAAATATCG AATTCATCCCGGTTACGGTTCCACAAAACCGCAAGA TGGAGGCGGGTAAAGAATTTCTGAAATCCCAGTAT AAAATGAATATCGAACAACTGGAAAAAATCTCAGG GAATAAGATCACTGACGAGAGCTTGGAGAAGGCTA TTGAAATTTACGATGAGCACCGTAAAGTCATGAAC GATTTCTCTATGCTTGCGTCCAAGTACCCTGGTATC ATTACGCCAACGAAACGTAACTACGTGATGAAGTC AGCGTATTATATGGACAAGAAAGAACATACAGAGA AGGTACGTCAGTTGATGGATGAAATCAAGGCCATT GAGCCTAAACCATTCGAAGGAAAACGCGTGATTAC CACTGGGATCATTGCAGATTCGGAGGACCTTTTGAA AATCTTGGAGGAGAATAACATTGCTATCGTGGGAG ATGATATTGCACACGAGTCTCGCCAATACCGCACTT TGACCCCGGAGGCCAACACACCTATGGACCGTCTTG CTGAACAATTTGCGAACCGCGAGTGTTCGACGTTGT ATGACCCTGAAAAAAAACGTGGACAGTATATTGTC GAGATGGCAAAAGAGCGTAAGGCCGACGGAATCAT CTTCTTCATGACAAAATTCTGCGATCCCGAAGAATA CGATTACCCTCAGATGAAAAAAGACTTCGAAGAAG CCGGTATTCCCCACGTTCTGATTGAGACAGACATGC AAATGAAGAACTACGAACAAGCTCGCACCGCTATT CAAGCATTTTCAGAAACCCTTTG
AcuI **SEQ ID NO: 279**	ATGCGTGCTGTCTTAATCGAGAAGTCAGATGACACC CAGAGTGTTTCAGTTACGGAGTTGGCTGAAGACCA ATTACCCGAAGGTGACGTCCTTGTGGATGTCGCGTA CAGCACATTGAATTACAAGGATGCTCTTGCGATTAC TGGAAAAGCACCCGTTGTACGCCGTTTTCCTATGGT CCCCGGAATTGACTTTACTGGGACTGTCGCACAGAG TTCCCATGCTGATTTCAAGCCAGGCGACCGCGTAAT TCTGAACGGATGGGGAGTTGGTGAGAAACACTGGG GCGGTCTTGCAGAACGCGCACGCGTACGTGGGGAC TGGCTTGTCCCGTTGCCAGCCCCCTTAGACTTGCGC CAGGCTGCAATGATTGGCACTGCGGGGTACACAGC TATGCTGTGCGTGCTTGCCCTTGAGCGCCATGGAGT CGTACCTGGGAACGGCGAGATTGTCGTCTCAGGCG CAGCAGGAGGGGTAGGTTCTGTAGCAACCACACTG TTAGCAGCCAAAGGCTACGAAGTGGCCGCCGTGAC CGGGCGCGCAAGCGAGGCCGAATATTTACGCGGAT TAGGCGCCGCGTCGGTCATTGATCGCAATGAATTAA CGGGGAAGGTGCGTCCATTAGGGCAGGAACGCTGG GCAGGAGGAATCGATGTAGCAGGATCAACCGTACT

	TGCTAATATGTTGAGCATGATGAAATACCGTGGCGT GGTGGCGGCCTGTGGCCTGGCGGCTGGAATGGACT TGCCCGCGTCTGTCGCCCCTTTTATTCTGCGTGGTAT GACTTTGGCAGGGGTAGATTCAGTCATGTGCCCCAA AACTGATCGTCTGGCTGCTTGGGCACGCCTGGCATC CGACCTGGACCCTGCAAAGCTGGAAGAGATGACAA CTGAATTACCGTTCTCTGAGGTGATTGAAACGGCTC CGAAGTTCTTGGATGGAACAGTGCGTGGGCGTATTG TCATTCCGGTAACACCTTGA
fldH1 **SEQ ID NO: 280**	ATGAAAATCTTGGCATACTGCGTCCGCCCAGACGA GGTAGACTCCTTTAAGAAATTTAGTGAAAAGTACG GGCATACAGTTGATCTTATTCCAGACTCTTTTGGAC CTAATGTCGCTCATTTGGCGAAGGGTTACGATGGGA TTTCTATTCTGGGCAACGACACGTGTAACCGTGAGG CACTGGAGAAGATCAAGGATTGCGGGATCAAATAT CTGGCAACCCGTACAGCCGGAGTGAACAACATTGA CTTCGATGCAGCAAAGGAGTTCGGTATTAACGTGGC TAATGTTCCCGCATATTCCCCCAACTCGGTCAGCGA ATTTACCATTGGATTGGCATTAAGTCTGACGCGTAA GATTCCATTTGCCCTGAAACGCGTGGAACTGAACAA TTTTGCGCTTGGCGGCCTTATTGGTGTGGAATTGCG TAACTTAACTTTAGGAGTCATCGGTACTGGTCGCAT CGGATTGAAAGTGATTGAGGGCTTCTCTGGGTTTGG AATGAAAAAAATGATCGGTTATGACATTTTTGAAA ATGAAGAAGCAAAGAAGTACATCGAATACAAATCA TTAGACGAAGTTTTTAAAGAGGCTGATATTATCACT CTGCATGCGCCTCTGACAGACGACAACTATCATATG ATTGGTAAAGAATCCATTGCTAAAATGAAGGATGG GGTATTTATTATCAACGCAGCGCGTGGAGCCTTAAT CGATAGTGAGGCCCTGATTGAAGGGTTAAAATCGG GGAAGATTGCGGGCGCGGCTCTGGATAGCTATGAG TATGAGCAAGGTGTCTTTCACAACAATAAGATGAAT GAAATTATGCAGGATGATACCTTGGAACGTCTGAA ATCTTTTCCCAACGTCGTGATCACGCCGCATTTGGG TTTTTATACTGATGAGGCGGTTTCCAATATGGTAGA GATCACACTGATGAACCTTCAGGAATTCGAGTTGAA AGGAACCTGTAAGAACCAGCGTGTTTGTAAATGA
FldD **SEQ ID NO: 282**	ATGTTCTTTACGGAGCAACACGAACTTATTCGCAAA CTGGCGCGTGACTTTGCCGAACAGGAAATCGAGCC TATCGCAGACGAAGTAGATAAAACCGCAGAGTTCC CAAAAGAAATCGTGAAGAAGATGGCTCAAAATGGA TTTTTCGGCATTAAAATGCCTAAAGAATACGGAGGG GCGGGTGCGGATAACCGCGCTTATGTCACTATTATG GAGGAAATTTCACGTGCTTCCGGGGTAGCGGGTATC TACCTGAGCTCGCCGAACAGTTTGTTAGGAACTCCC TTCTTATTGGTCGGAACCGATGAGCAAAAAGAAAA GTACCTTAAGCCTATGATCCGCGGCGAGAAGACTCT GGCGTTCGCCCTGACAGAGCCTGGTGCTGGCTCTGA

	TGCGGGTGCGTTGGCTACTACTGCCCGTGAAGAGG GCGACTATTATATCTTAAATGGCCGCAAGACGTTTA TTACAGGGGCTCCTATTAGCGACAATATTATTGTGT TCGCAAAAACCGATATGAGCAAAGGGACCAAAGGT ATCACCACTTTCATTGTGGACTCAAAGCAGGAAGG GGTAAGTTTTGGTAAGCCAGAGGACAAAATGGGAA TGATTGGTTGTCCGACAAGCGACATCATCTTGGAAA ACGTTAAAGTTCATAAGTCCGACATCTTGGGAGAA GTCAATAAGGGGTTTATTACCGCGATGAAAACACTT TCCGTTGGTCGTATCGGAGTGGCGTCACAGGCGCTT GGAATTGCACAGGCCGCCGTAGATGAGGCGGTAAA GTACGCCAAGCAACGTAAACAATTCAATCGCCCAA TCGCGAAATTTCAGGCCATTCAATTTAAACTTGCCA ATATGGAGACTAAATTAAATGCCGCTAAACTTCTTG TTTATAACGCAGCGTACAAAATGGATTGTGGAGAA AAAGCCGACAAGGAAGCCTCTATGGCTAAATACTT TGCTGCTGAATCAGCGATCCAAATCGTTAACGACGC GCTGCAAATCCATGGCGGGTATGGCTATATCAAAG ACTACAAGATTGAACGTTTGTACCGCGATGTGCGTG TGATCGCTATTTATGAGGGCACTTCCGAGGTCCAAC AGATGGTTATCGCGTCCAATCTGCTGAAGTAA

[0566] In some embodiments, the genetically engineered bacteria comprise one or more nucleic acid sequence of **Table 11B** 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 listed in **Table 11A** 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 11B** or a functional fragment thereof, or a nucleic acid sequence that, but for the redundancy of the genetic code, encodes the same polypeptide the polypeptide sequences listed in **Table 11A** or a functional fragment thereof.

[0567] In one embodiment, the Tryptophan Decarboxylase gene encodes a polypeptide which has at least about 80% identity with the entire sequence of **SEQ ID NO: 141.** In another embodiment, the Tryptophan Decarboxylase gene encodes a polypeptide which has at least about 85% identity with the entire sequence of **SEQ ID NO: 141**. In one embodiment, the Tryptophan Decarboxylase gene encodes a polypeptide which has at least about 90% identity with the entire sequence of **SEQ ID NO: 141**. In

one embodiment, the Tryptophan Decarboxylase gene encodes a polypeptide which has at least about 95% identity with the entire sequence of **SEQ ID NO: 141**. In another embodiment, the Tryptophan Decarboxylase gene encodes a polypeptide which has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 141**. Accordingly, in one embodiment, the Tryptophan Decarboxylase gene encodes a polypeptide 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 **SEQ ID NO: 141**. In another embodiment, the Tryptophan Decarboxylase gene encodes a polypeptide which comprises the sequence of **SEQ ID NO: 141**. In yet another embodiment the Tryptophan Decarboxylase gene encodes a polypeptide which consists of the sequence of **SEQ ID NO: 141**.

[0568] In one embodiment, the Indole-3-pyruvate decarboxylase gene encodes a polypeptide which has at least about 80% identity with the entire sequence of **SEQ ID NO: 149.** In another embodiment, the Indole-3-pyruvate decarboxylase gene encodes a polypeptide which has at least about 85% identity with the entire sequence of **SEQ ID NO: 149**. In one embodiment, the Indole-3-pyruvate decarboxylase gene encodes a polypeptide which has at least about 90% identity with the entire sequence of **SEQ ID NO: 149**. In one embodiment, the Indole-3-pyruvate decarboxylase gene encodes a polypeptide which has at least about 95% identity with the entire sequence of **SEQ ID NO: 149**. In another embodiment, the Indole-3-pyruvate decarboxylase gene encodes a polypeptide which has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 149**. Accordingly, in one embodiment, the Indole-3-pyruvate decarboxylase gene encodes a polypeptide 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 **SEQ ID NO: 149**. In another embodiment, the Indole-3-pyruvate decarboxylase gene encodes a polypeptide which comprises the sequence of **SEQ ID NO: 149**. In yet another embodiment the Indole-3-pyruvate decarboxylase gene encodes a polypeptide which consists of the sequence of **SEQ ID NO: 149**.

[0569] In one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 80% identity with the entire sequence of **SEQ ID NO: 150.** In another embodiment, the Indole-3-acetaldehyde dehydrogenase

gene encodes a polypeptide which has at least about 85% identity with the entire sequence of **SEQ ID NO: 150**. In one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 90% identity with the entire sequence of **SEQ ID NO: 150**. In one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 95% identity with the entire sequence of **SEQ ID NO: 150**. In another embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 150**. Accordingly, in one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide 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 **SEQ ID NO: 150**. In another embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which comprises the sequence of **SEQ ID NO: 150**. In yet another embodiment the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which consists of the sequence of **SEQ ID NO: 150**.

[0570] In one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 80% identity with the entire sequence of **SEQ ID NO: 154.** In another embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 85% identity with the entire sequence of **SEQ ID NO: 154**. In one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 90% identity with the entire sequence of **SEQ ID NO: 154**. In one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 95% identity with the entire sequence of **SEQ ID NO: 154**. In another embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of **SEQ ID NO: 154**. Accordingly, in one embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide 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 **SEQ ID NO: 154**. In another embodiment, the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which comprises the sequence of **SEQ ID NO: 154**. In yet another

embodiment the Indole-3-acetaldehyde dehydrogenase gene encodes a polypeptide which consists of the sequence of **SEQ ID NO: 154**.

[0571] In one embodiment, genetically engineered bacteria comprise one or more gene sequence(s) which encode one or more polypeptide(s) which has at least about 80% identity with the entire sequence of one or more sequence(s) of **Table 11A.** In another embodiment, the one or more gene sequence(s) which encode one or more polypeptide(s) which has at least about 85% identity with the entire sequence of one or more sequence(s) of **Table 11A.** In one embodiment, the one or more gene sequence(s) which encode one or more polypeptide(s) which has at least about 90% identity with the entire sequence of one or more sequence(s) of **Table 11A.** In one embodiment, the one or more gene sequence(s) which encode one or more polypeptide(s) which has at least about 95% identity with the entire sequence of one or more sequence(s) of **Table 11A.** In another embodiment, the one or more gene sequence(s) which encode one or more polypeptide(s) which has at least about 96%, 97%, 98%, or 99%identity with the entire sequence of one or more sequence(s) of **Table 11A.** Accordingly, in one embodiment, the one or more gene sequence(s) which encode one or more polypeptide(s) 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 sequence(s) of **Table 11A**. In another embodiment, the one or more gene sequence(s) which encode one or more polypeptide(s) which comprises the entire sequence of one or more sequence(s) of **Table 11A**.

[0572] 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..

[0573] **Table 12** depicts non-limiting examples of contemplated polypeptide sequences, which are encoded by indole-3-propionate producing bacteria.

Table 12. 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:173**	MENNTNMFSGVKVIELANFIAAPAAGRFFADGGAEVIKIESPA GDPLRYTAPSEGRPLSQEENTTYDLENANKKAIVLNLKSEKGK KILHEMLAEADILLTNWRTKALVKQGLDYETLKEKYPKLVFA QITGYGEKGPDKDLPGFDYTAFFARGGVSGTLYEKGTVPPNV VPGLGDHQAGMFLAAGMAGALYKAKTTGQGDKVTVSLMHS AMYGLGIMIQAAQYKDHGLVYPINRNETPNPFIVSYKSKDDYF VQVCMPPYDVFYDRFMTALGREDLVGDERYNKIENLKDGRA KEVYSIIEQQMVTKTKDEWDKIFRDADIPFAIAQTWEDLLEDE QAWANDYLYKMKYPTGNERALVRLPVFFKEAGLPEYNQSPQI AENTVEVLKEMGYTEQEIEELEKDKDIMVRKEK
FldB: subunit of indole-3-lactate dehydratase from Clostridium sporogenes **SEQ ID NO:174**	MSDRNKEVKEKKAKHYLREITAKHYKEALEAKERGEKVGWC ASNFPQEIATTLGVKVVYPENHAAAVAARGNGQNMCEHAEA MGFSNDVCGYARVNLAVMDIGHSEDQPIPMPDFVLCCNNICN QMIKWYEHIAKTLDIPMILIDIPYNTENTVSQDRIKYIRAQFDD AIKQLEEITGKKWDENKFEEVMKISQESAKQWLRAASYAKYK PSPFSGFDLFNHMAVAVCARGTQEAADAFKMLADEYEENVKT GKSTYRGEEKQRILFEGIACWPYLRHKLTKLSEYGMNVTATV YAEAFGVIYENMDELMAAYNKVPNSISFENALKMRLNAVTST NTEGAVIHINRSCKLWSGFLYELARRLEKETGIPVVSFDGDQA DPRNFSEAQYDTRIQGLNEVMVAKKEAE
FldC: subunit of indole-3-lactate dehydratase from Clostridium sporogenes **SEQ ID NO:175**	MSNSDKFFNDFKDIVENPKKYIMKHMEQTGQKAIGCMPLYTP EELVLAAGMFPVGVWGSNTELSKAKTYFPAFICSILQTTLENA LNGEYDMLSGMMITNYCDSLKCMGQNFKLTVENIEFIPVTVPQ NRKMEAGKEFLKSQYKMNIEQLEKISGNKITDESLEKAIEIYDE HRKVMNDFSMLASKYPGIITPTKRNYVMKSAYYMDKKEHTE KVRQLMDEIKAIEPKPFEGKRVITTGIIADSEDLLKILEENNIAIV GDDIAHESRQYRTLTPEANTPMDRLAEQFANRECSTLYDPEKK RGQYIVEMAKERKADGIIFFMTKFCDPEEYDYPQMKKDFEEA GIPHVLIETDMQMKNYEQARTAIQAFSETL
FldD: indole-3-acrylyl-CoA reductase from Clostridium sporogenes **SEQ ID NO:176**	MFFTEQHELIRKLARDFAEQEIEPIADEVDKTAEFPKEIVKKMA QNGFFGIKMPKEYGGAGADNRAYVTIMEEISRASGVAGIYLSS PNSLLGTPFLLVGTDEQKEKYLKPMIRGEKTLAFALTEPGAGS DAGALATTAREEGDYYILNGRKTFITGAPISDNIIVFAKTDMSK GTKGITTFIVDSKQEGVSFGKPEDKMGMIGCPTSDIILENVKVH KSDILGEVNKGFITAMKTLSVGRIGVASQALGIAQAAVDEAVK YAKQRKQFNRPIAKFQAIQFKLANMETKLNAAKLLVYNAAYK MDCGEKADKEASMAKYFAAESAIQIVNDALQIHGGYGYIKDY KIERLYRDVRVIAIYEGTSEVQQMVIASNLLK
FldH1: indole-3-lactate dehydrogenase from Clostridium sporogenes **SEQ ID NO: 177**	MKILAYCVRPDEVDSFKKFSEKYGHTVDLIPDSFGPNVAHLAK GYDGISILGNDTCNREALEKIKDCGIKYLATRTAGVNNIDFDA AKEFGINVANVPAYSPNSVSEFTIGLALSLTRKIPFALKRVELN NFALGGLIGVELRNLTLGVIGTGRIGLKVIEGFSGFGMKKMIGY DIFENEEAKKYIEYKSLDEVFKEADIITLHAPLTDDNYHMIGKE SIAKMKDGVFIINAARGALIDSEALIEGLKSGKIAGAALDSYEY EQGVFHNNKMNEIMQDDTLERLKSFPNVVITPHLGFYTDEAVS NMVEITLMNLQEFELKGTCKNQRVCK
FldH2: indole-3-	MKILMYSVREHEKPAIKKWLEANPGVQIDLCNNALSEDTVCK

lactate dehydrogenase from Clostridium sporogenes **SEQ ID NO: 178**	AKEYDGIAIQQTNSIGGKAVYSTLKEYGIKQIASRTAGVDMIDL KMASDSNILVTNVPAYSPNAIAELAVTHTMNLLRNIKTLNKRI AYGDYRWSADLIAREVRSVTVGVVGTGKIGRTSAKLFKGLGA NVIGYDAYPDKKLEENNLLTYKESLEDLLREADVVTLHTPLLE STKYMINKNNLKYMKPDAFIVNTGRGGIINTEDLIEALEQNKIA GAALDTFENEGLFLNKVVDPTKLPDSQLDKLLKMDQVLITHH VGFFTTTAVQNIVDTSLDSVVEVLKTNNSVNKVN
AcuI: acrylyl-CoA reductase from Rhodobacter sphaeroides **SEQ ID NO: 179**	MRAVLIEKSDDTQSVSVTELAEDQLPEGDVLVDVAYSTLNYK DALAITGKAPVVRRFPMVPGIDFTGTVAQSSHADFKPGDRVIL NGWGVGEKHWGGLAERARVRGDWLVPLPAPLDLRQAAMIG TAGYTAMLCVLALERHGVVPGNGEIVVSGAAGGVGSVATTLL AAKGYEVAAVTGRASEAEYLRGLGAASVIDRNELTGKVRPLG QERWAGGIDVAGSTVLANMLSMMKYRGVVAACGLAAGMDL PASVAPFILRGMTLAGVDSVMCPKTDRLAAWARLASDLDPAK LEEMTTELPFSEVIETAPKFLDGTVRGRIVIPVTP

[0574] In one embodiment, the tryptophan pathway catabolic enzyme encoded by the genetically engineered bacteria has at least about 80% identity with the entire sequence of one or more of **SEQ ID NO: 173 through SEQ ID NO: 179**. In another embodiment, the tryptophan pathway catabolic enzyme has at least about 85% identity with the entire sequence of one or more **SEQ ID NO: 173 through SEQ ID NO: 179**. In one embodiment, the tryptophan pathway catabolic enzyme has at least about 90% identity with the entire sequence of one or more **SEQ ID NO: 173 through SEQ ID NO: 179**. In one embodiment, the tryptophan pathway catabolic enzyme has at least about 95% identity with the entire sequence of one or more **SEQ ID NO: 173 through SEQ ID NO: 179**. In another embodiment, the tryptophan pathway catabolic enzyme has at least about 96%, 97%, 98%, or 99% identity with the entire sequence of one or more **SEQ ID NO: 173 through SEQ ID NO: 179**. Accordingly, in one embodiment, the tryptophan pathway catabolic enzyme 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: 173 through SEQ ID NO: 179**. In another embodiment, the tryptophan pathway catabolic enzyme comprises the sequence of one or more **SEQ ID NO: 173 through SEQ ID NO: 179**. In yet another embodiment the tryptophan pathway catabolic enzyme consists of the sequence of one or more **SEQ ID NO: 173 through SEQ ID NO: 179.**

[0575] 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. 43, FIG 49A and FIG. 49B.** 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.

[0576] 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.

[0577] 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)

[0578] 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.

[0579] In some embodiments, the expression of the gene sequences(s) is controlled by an inducible promoter. In some embodiments, the expression of the gene sequences(s) is 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. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constitutive promoter, and are expressed in vivo, e.g., in the gut.

Tryptophan and Tryptophan MetaboliteTransport

[0580] 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.

[0581] 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.

[0582] 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.

[0583] 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.

[0584] 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.

[0585] 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.

[0586] In some embodiments, the expression of the gene sequences(s) is controlled by an inducible promoter. In some embodiments, the expression of the gene sequences(s) is 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. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constitutive promoter, and are expressed in vivo, e.g., in the gut.

[0587] In some embodiments, the expression of the gene sequences(s) is controlled by an inducible promoter. In some embodiments, the expression of the gene sequences(s) is 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. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constitutive promoter, and are expressed in vivo, e.g., in the gut.

[0588] 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.

[0589] In some embodiments, the expression of the gene sequences(s) is controlled by an inducible promoter. In some embodiments, the expression of the gene sequences(s) is 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. In some embodiments, the gene sequences(s) are controlled by an inducible and/or constitutive promoter, and are expressed in vivo, e.g., in the gut.

[0590] 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.

[0591] 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.

[0592] In some embodiments, the expression of the gene sequences(s) encoding the transporter is controlled by an inducible promoter. In some embodiments, the expression of the gene sequences(s) encoding the transporter is controlled by a constitutive promoter. In some embodiments, the expression of the gene sequences(s) encoding the transporter is 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. In some embodiments, the expression of the gene sequences(s) encoding the transporter is controlled by an inducible and/or constitutive promoter, and are expressed in vivo, e.g., in the gut.

[0593] 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.

[0594] In some embodiments, the expression of the gene sequences(s) encoding the transporter is controlled by an inducible promoter. In some embodiments, the expression of the gene sequences(s) encoding the transporter is controlled by a constitutive promoter. In some embodiments, the expression of the gene sequences(s) encoding the transporter is 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. In some embodiments, the expression of the gene sequences(s) encoding the transporter is controlled by an inducible and/or constitutive promoter, and are expressed in vivo, e.g., in the gut.

Secreted Polypeptides

IL-10

[0595] In some embodiments, the genetically engineered bacteria of the invention are capable of producing IL-10. Interleukin-10 (IL-10) is a class 2 cytokine, a category which includes cytokines, interferons, and interferon-like molecules, such as IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, IL-29, IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, IFN-ω, and limitin. IL-10 is an anti-inflammatory cytokine that signals through two receptors, IL-10R1 and IL-10R2. Anti-inflammatory properties of human IL-10 include down-regulation of pro-inflammatory cytokines, inhibition of antigen presentation on dendritic cells or suppression of major histocompatibility complex expression. Deficiencies in IL-10 and/or its receptors are associated with IBD and intestinal sensitivity (Nielsen, 2014). Bacteria expressing IL-10 or protease inhibitors may ameliorate conditions such as Crohn's disease and ulcerative colitis (Simpson et al., 2014). The genetically engineered bacteria may comprise any suitable gene encoding IL-10, e.g., human IL-10. In some embodiments, the gene encoding IL-10 is modified and/or mutated, e.g., to enhance stability, increase IL-10 production, and/or increase anti-inflammatory potency under inducing conditions. In some embodiments, the genetically engineered bacteria are capable of producing IL-10 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing IL-10 in low-oxygen conditions. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that

encodes IL-10. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence comprising **SEQ ID NO: 134** 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 comprising **SEQ ID NO: 49** or a functional fragment thereof.

Table 13. IL-10 (SEQ ID NO: 134):

ATGAGCCCCGGACAGGGAACTCAAAGCGAGAACAGCTGCACACATTTTCCAGGTAATCTTCCAAAT
ATGCTTCGTGACTTGCGTGACGCTTTCTCTCGCGTGAAAACCTTTTTTCAGATGAAGGATCAGTTA
GATAATCTGCTGCTGAAAGAATCGCTTCTTGAGGACTTCAAGGGATATCTGGGATGTCAGGCGTTA
TCTGAGATGATTCAGTTTTATTTGGAAGAAGTTATGCCCCAGGCTGAGAATCAAGACCCTGACATC
AAAGCGCATGTGAATAGCCTGGGCGAGAATCTGAAGACACTGCGCCTGCGTCTTCGCCGCTGTCAC
CGTTTTCTGCCTTGCGAAAATAAGAGTAAGGCCGTTGAGCAAGTGAAAAATGCTTTCAACAAGTTA
CAAGAAAAAGGGATTTACAAAGCTATGTCTGAGTTTGACATTTTCATTAATTACATTGAGGCCTAC
ATGACTATGAAGATTCGCAAT

[0596] Wild type IL-10 (wtIL-10) is a domain swapped dimer whose structural integrity depends on the dimerization of two peptide chains. wtIL-10 was converted to a monomeric isomer by inserting 6 amino acids into the loop connecting the swapped secondary structural elements (see, e.g., Josephson, K. et al. Design and analysis of an engineered human interleukin-10 monomer. J. Biol. Chem. 275, 13552–13557 (2000), and Yoon, S. I. et al. Epstein-Barr Virus IL-10 Engages IL-10R1 by a Two-step Mechanism Leading to Altered Signaling Properties. J. Biol. Chem. 287, 26586–26595 (2012). Monomoerized IL-10 therefore comnprises a small linker which deviates from the wild-type human IL-10 sequence. This linker causes the IL10 to become active as a monomer rather than a dimer (see, e.g., Josephson, K. et al. Design and analysis of an engineered human interleukin-10 monomer. J. Biol. Chem. 275, 13552–13557 (2000), and Yoon, S. I. et al. Epstein-Barr Virus IL-10 Engages IL-10R1 by a Two-step Mechanism Leading to Altered Signaling Properties. J. Biol. Chem. 287, 26586–26595 (2012)).

[0597] Secretion of a monomeric protein may have advantages, avoiding the extra step of dimerization in the periplasmic space. Moreover, there is more flexibility in the selection of appropriate secretion systems. For example, the tat-dependent secretion system secretes polypeptides in a folded fashion. Dimers cannot fold correctly without the

formation of disulfide bonds. Disulfide bonds, however, cannot form in the reducing intracellular environment and require the oxidizing environment of the periplasm to form. Therefore, the tat-dependent system may no be appropriate for the secretion of proteins which require dimerization to function properly.

[0598] In some embodiments, the genetically engineered bacteria of the invention are capable of producing monomerized human IL-10. In some embodiments, the genetically engineered bacteria are capable of producing monomerized IL-10 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing monomerized IL-10 in low-oxygen conditions. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that encodes monomerized IL-10. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence comprising **SEQ ID NO: 198** 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 comprising **SEQ ID NO: 198** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a sequence which encodes the polypeptide encoded by **SEQ ID NO: 198** or a fragment or functiona variant thereof. In some embodiments, the monomerized IL-10 expressed by the bacteria stimulates IL-10R1 and IL-10R2 and initiates signal transduction. Signaling includes Stat signaling, e.g. through the phosphorylation of Tyr705 and/or Ser727.

[0599] In some embodiments, the genetically engineered bacteria of the invention are capable of producing viral IL-10. Exemplary viral IL-10 homologues encoded by the bacteria include human cytomegalo- (HCMV) and Epstein-Barr virus (EBV) IL-10. Apart from its anti-inflammatory effects, human IL-10 also possesses pro-inflammatory activity, e.g., stimulation of B-cell maturation and proliferation of natural killer cells (Foerster et al., Secretory expression of biologically active human Herpes virus interleukin-10 analogues in Escherichia coli via a modified Sec-dependent transporter construct, BMC Biotechnol. 2013; 13: 82, and references therein). In contrast, viral IL-10 homologues share many biological activities of hIL-10 but, due to selective pressure during virus evolution and the need to escape the host immune system, also display unique traits, including increased stability and lack of immunostimulatory functions (Foerster et al, and

references therein). As such, viral counterparts may be useful and possibly more effective than hIL-10 with respect to anti-inflammatory and/or immune suppressing effects.

[0600] In some embodiments, the genetically engineered bacteria are capable of producing viral IL-10 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing viral IL-10 in low-oxygen conditions. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that encodes viral IL-10. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence comprising **SEQ ID NO: 193 and/or SEQ ID NO: 194** 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 comprising **SEQ ID NO: 193 and/or SEQ ID NO: 194** or a functional fragment thereof. In some embodiments, the viral d IL-10 expressed by the bacteria stimulates IL-10R1 and IL-10R2 and initiates signal transduction. Signaling includes Stat signaling, e.g. through the phosphorylation of Tyr705 and/or Ser727.

[0601] To improve acetate production, while maintaining high levels of IL-10 secretion, 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 gene sequence(s) encoding one or more IL-10 polypeptides for secretion and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0602] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-10- polypeptides for secretion 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.

[0603] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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.

[0604] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10,

OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. 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.

[0605] 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 IL-10 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 IL-10 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 IL-10 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0606] 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 IL-10 secretion. 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 IL-10 secretion. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentation, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for IL-10 synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0607] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion 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 IL-10 polypeptides for secretion and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0608] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 and further comprise a mutation in the endogenous pta and ldhA genes.

[0609] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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

PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 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 PhoA-IL-10, OmpF-IL-10, and TorA-IL-10 and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes

[0610] 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 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.

[0611] 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 IL-10 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 IL-10 than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the 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 IL-10 than unmodified bacteria of the same bacterial subtype under the same conditions.

IL-2

[0612] In some embodiments, the genetically engineered bacteria are capable of producing IL-2. Interleukin 2 (IL-2) mediates autoimmunity by preserving health of regulatory T cells (Treg). Treg cells, including those expressing Foxp3, typically suppress effector T cells that are active against self-antigens, and in doing so, can dampen autoimmune activity. IL-2 functions as a cytokine to enhance Treg cell differentiation and activity while diminished IL-2 activity can promote autoimmunity events. IL-2 is generated by activated CD4+ T cells, and by other immune mediators including activated CD8+ T cells, activated dendritic cells, natural killer cells, and NK T cells. IL-2 binds to IL-2R, which is composed of three chains including CD25, CD122, and CD132. IL-2 promotes growth of Treg cells in the thymus, while preserving their function and activity in systemic circulation. Treg cell activity plays an intricate role in the IBD setting, with murine studies suggesting a protective role in disease pathogenesis. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence encoding **SEQ ID NO: 135** 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: 135** or a functional fragment thereof.

[0613] In some embodiments, the genetically engineered bacteria are capable of producing IL-2 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing IL-2 in low-oxygen conditions.

Table 14. SEQ ID NO: 135

SEQ ID NO: 135
MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN RWITFCQSII STLT

[0614] To improve acetate production, while maintaining high levels of IL-2 secretion, 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 gene sequence(s) encoding one or more IL-2 polypeptides for secretion and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0615] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-2- polypeptides for secretion 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.

[0616] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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.

[0617] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. 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.

[0618] 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 IL-2 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 IL-2 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 IL-2 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0619] 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 IL-2 secretion. 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 IL-2 secretion. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentation, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for IL-2 synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0620] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion 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 IL-2 polypeptides for secretion and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0621] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 and further comprise a mutation in the endogenous pta and ldhA genes.

[0622] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 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 PhoA-IL-2, OmpF-IL-2, and TorA-IL-2 and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes

[0623] 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 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.

[0624] 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 IL-2 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 IL-2 than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the 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 IL-2 than unmodified bacteria of the same bacterial subtype under the same conditions.

IL-22

[0625] 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. IL-22's association with IBD susceptibility genes may modulate phenotypic expression of disease as well. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence encoding **SEQ ID NO: 136** 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: 136** 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 15. SEQ ID NO: 136

SEQ ID NO: 136
MAALQKSVSS FLMGTLATSC LLLLALLVQG GAAAPISSHC RLDKSNFQQP YITNRTFMLA KEASLADNNT DVRLIGEKLF HGVSMSERCY LMKQVLNFTL EEVLFPQSDR FQPYMQEVVP FLARLSNRLS TCHIEGDDLH IQRNVQKLKD TVKKLGESGE IKAIGELDLL FMSLRNACI

[0626] To improve acetate production, while maintaining high levels of IL-22 secretion, 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 gene sequence(s) encoding one or more IL-22 polypeptides for secretion and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0627] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-22- polypeptides for secretion 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.

[0628] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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.

[0629] 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 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. 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.

[0630] 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 IL-22 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 IL-22 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 IL-22 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0631] 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 IL-22 secretion. 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 IL-22 secretion. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentation, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for IL-22 synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0632] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion 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 IL-22 polypeptides for secretion and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0633] 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 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 and further comprise a mutation in the endogenous pta and ldhA genes.

[0634] 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 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 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 PhoA-IL-22, OmpF-IL-22, and TorA-IL-22 and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes

[0635] 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 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.

[0636] 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 IL-22 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 IL-22 than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the 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 IL-22 than unmodified bacteria of the same bacterial subtype under the same conditions.

IL-27

[0637] In some embodiments, the genetically engineered bacteria are capable of producing IL-27. Interleukin 27 (IL-27) cytokine is predominately expressed by activated antigen presenting cells, while IL-27 receptor is found on a range of cells including T cells, NK cells, among others. In particular, IL-27 suppresses development of pro-inflammatory T helper 17 (Th17) cells, which play a critical role in IBD pathogenesis. Further, IL-27 can promote differentiation of IL-10 producing Tr1 cells and enhance IL-10

output, both of which have anti-inflammatory effects. IL-27 has protective effects on epithelial barrier function via activation of MAPK and STAT signaling within intestinal epithelial cells. Additionally, IL-27 enhances production of antibacterial proteins that curb bacterial growth. Improvement in barrier function and reduction in bacterial growth suggest a favorable role for IL-27 in IBD pathogenesis. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence encoding **SEQ ID NO: 137** 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: 137** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria are capable of producing IL-27 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing IL-27 in low-oxygen conditions.

Table 16. SEQ ID NO: 137

SEQ ID NO: 137
MGQTAGDLGW RLSLLLLPLL LVQAGVWGFP RPPGRPQLSL QELRREFTVS
LHLARKLLSE VRGQAHRFAE SHLPGVNLYL LPLGEQLPDV SLTFQAWRRL
SDPERLCFIS TTLQPFHALL GGLGTQGRWT NMERMQLWAM RLDLRDLQRH
LRFQVLAAGF NLPEEEEEEE EEEEEERKGL LPGALGSALQ GPAQVSWPQL
LSTYRLLHSL ELVLSRAVRE LLLLSKAGHS VWPLGFPTLS PQP

[0638] To improve acetate production, while maintaining high levels of IL-27 secretion, 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 gene sequence(s) encoding one or more IL-27 polypeptides for secretion and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0639] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-27- polypeptides for secretion 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.

[0640] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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.

[0641] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. 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.

[0642] 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 IL-27 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 IL-27 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 IL-27 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0643] 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 IL-27 secretion. 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 IL-27 secretion. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentation, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for IL-27 synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0644] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion 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 IL-27 polypeptides for secretion and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0645] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 and further comprise a mutation in the endogenous pta and ldhA genes.

[0646] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 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 PhoA-IL-27, OmpF-IL-27, and TorA-IL-27 and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes

[0647] 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 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.

[0648] 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 IL-27 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 IL-27 than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the 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 IL-27 than unmodified bacteria of the same bacterial subtype under the same conditions.

SOD

[0649] In some embodiments, the genetically engineered bacteria of the invention are capable of producing SOD. Increased ROS levels contribute to pathophysiology of inflammatory bowel disease. Increased ROS levels may lead to enhanced expression of vascular cell adhesion molecule 1 (VCAM-1), which can facilitate translocation of inflammatory mediators to disease affected tissue, and result in a greater degree of inflammatory burden. Antioxidant systems including superoxide dismutase (SOD) can function to mitigate overall ROS burden. However, studies indicate that the expression of SOD in the setting of IBD may be compromised, e.g., produced at lower levels in IBD, thus allowing disease pathology to proceed. Further studies have shown that supplementation with SOD to rats within a colitis model is associated with reduced colonic lipid peroxidation and endothelial VCAM-1 expression as well as overall improvement in inflammatory environment. Thus, in some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence encoding **SEQ ID NO: 138** 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: 138** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria are capable of producing SOD under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing SOD in low-oxygen conditions.

Table 17. SEQ ID NO: 138

SEQ ID NO: 138
MATKAVCVLK GDGPVQGIIN FEQKESNGPV KVWGSIKGLT EGLHGFHVHE

FGDNTAGCTS AGPHFNPLSR KHGGPKDEER HVGDLGNVTA DKDGVADVSI EDSVISLSGD HCIIGRTLVV HEKADDLGKG GNEESTKTGN AGSRLACGVI GIAQ

GLP2

[0650] In some embodiments, the genetically engineered bacteria are capable of producing GLP-2 or proglucagon. Glucagon-like peptide 2 (GLP-2) is produced by intestinal endocrine cells and stimulates intestinal growth and enhances gut barrier function. GLP-2 administration has therapeutic potential in treating IBD, short bowel syndrome, and small bowel enteritis (Yazbeck et al., 2009). The genetically engineered bacteria may comprise any suitable gene encoding GLP-2 or proglucagon, e.g., human GLP-2 or proglucagon. In some embodiments, a protease inhibitor, e.g., an inhibitor of dipeptidyl peptidase, is also administered to decrease GLP-2 degradation. In some embodiments, the genetically engineered bacteria express a degradation resistant GLP-2 analog, e.g., Teduglutide (Yazbeck et al., 2009). In some embodiments, the gene encoding GLP-2 or proglucagon is modified and/or mutated, e.g., to enhance stability, increase GLP-2 production, and/or increase gut barrier enhancing potency under inducing conditions. In some embodiments, the genetically engineered bacteria of the invention are capable of producing GLP-2 or proglucagon under inducing conditions. GLP-2 administration in a murine model of IBD is associated with reduced mucosal damage and inflammation, as well as a reduction in inflammatory mediators, such as TNF-α and IFN-y. Further, GLP-2 supplementation may also lead to reduced mucosal myeloperoxidase in colitis/ileitis models. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence encoding **SEQ ID NO: 139** 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: 139** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria are capable of producing GLP-2 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing GLP-2 in low-oxygen conditions.

Table 18. SEQ ID NO: 139 GLP-2

SEQ ID NO: 139
HADGSFSDEMNTILDNLAARDFINWLIQTKITD

[0651] In some embodiments, the genetically engineered bacteria are capable of producing GLP-2 analogs, including but not limited to, Gattex and teduglutide. Teduglutide is a protease resistan analog of GLP-2. It is made up of 33 amino acids and differs from GLP-2 by one amino acid (alanine is substituted by glycine). The significance of this substitution is that teduglutide is longer acting than endogenous GLP-2 as it is more resistant to proteolysis from dipeptidyl peptidase-4.

Table 19. SEQ ID NO: 140 Teduglutide

SEQ ID NO: 140
HGDGSFSDEMNTILDNLAARDFINWLIQTKITD

[0652] In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence encoding **SEQ ID NO: 140** 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: 140** or a functional fragment thereof. In some embodiments, the genetically engineered bacteria are capable of producing Teduglutide under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing Teduglutide in low-oxygen conditions.

[0653] To improve acetate production, while maintaining high levels of GLP-2 secretion, 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 gene sequence(s) encoding one or more GLP-2 polypeptides for secretion and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0654] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more GLP-2- polypeptides for secretion 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.

[0655] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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.

[0656] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. 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.

[0657] 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 GLP-2 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 GLP-2 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 GLP-2 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0658] 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 GLP-2 secretion. 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 GLP-2 secretion. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentation, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for GLP-2 synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0659] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion 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 GLP-2 polypeptides for secretion and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0660] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-

2, and TorA-GLP-2 and further comprise a mutation in the endogenous pta and ldhA genes.

[0661] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 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 PhoA-GLP-2, OmpF-GLP-2, and TorA-GLP-2 and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes

[0662] 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 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.

[0663] 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 GLP-2 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 GLP-2 than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the 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 GLP-2 than unmodified bacteria of the same bacterial subtype under the same conditions.

IL-19, IL-20, and/or IL-24

[0664] In some embodiments, the genetically engineered bacteria are capable of producing IL-19, IL-20, and/or IL-24. In some embodiments, the genetically engineered bacteria are capable of producing IL-19, IL-20, and/or IL-24 under inducing conditions, e.g., under a condition(s) associated with inflammation. In some embodiments, the genetically engineered bacteria are capable of producing IL-19, IL-20 and/or IL-24 in low-oxygen conditions.

[0665] To improve acetate production, while maintaining high levels of IL-19, IL-20, AND/OR IL-24 secretion, 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 gene sequence(s) encoding one or more IL-19, IL-20, AND/OR IL-24 polypeptides for secretion and further comprise mutations and/or deletions in one or more of frdA, ldhA, and adhE.

[0666] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-19, IL-20, AND/OR IL-24- polypeptides for secretion 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.

[0667] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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.

[0668] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 and further comprise a mutation and/or deletion in the endogenous ldhA, the frdA, and adhE genes. 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.

[0669] 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 IL-19, IL-20, AND/OR IL-24 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 IL-19, IL-20, AND/OR IL-24 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 IL-19, IL-20, AND/OR IL-24 than unmodified bacteria of the same bacterial subtype under the same conditions.

[0670] 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 IL-19, IL-20, AND/OR IL-24 secretion. 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 IL-19, IL-20, AND/OR IL-24 secretion. Additionally, one or more mutations preventing or reducing the flow through other metabolic arms of mixed acid fermentation, such as those which produce succinate, lactate, and/or ethanol can increase the production of acetyl-CoA, which is available for IL-19, IL-20, AND/OR IL-24 synthesis. Such mutations and/or deletions, include but are not limited to mutations and/or deletions in the frdA, ldhA, and/or adhE genes.

[0671] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion 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 IL-19, IL-20, AND/OR IL-24 polypeptides for secretion and further comprise a mutation and/or deletion in the endogenous pta, ldhA, frdA, and adhE genes.

[0672] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 and further comprise a mutation in the endogenous pta and ldhA genes.

[0673] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) selected from PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 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 PhoA-IL-19, IL-20, AND/OR IL-24, OmpF-IL-19, IL-20, AND/OR IL-24, and TorA-IL-19, IL-20, AND/OR IL-24 and further comprise a mutation in the endogenous pta, ldhA, frdA, and adhE genes

[0674] 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 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.

[0675] 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 IL-19, IL-20, AND/OR IL-24 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 IL-19, IL-20, AND/OR IL-24 than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, the 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 IL-19, IL-20, AND/OR IL-24 than unmodified bacteria of the same bacterial subtype under the same conditions.

Inhibition of pro-inflammatory molecules

[0676] In some embodiments, the genetically engineered bacteria of the invention are capable of producing a molecule that is capable of inhibiting a pro-inflammatory 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 pro-inflammatory molecules, e.g., TNF, IFN-γ, IL-1β, IL-6, IL-8, IL-17, IL-18, IL-21, IL-23, IL-26, IL-32, Arachidonic acid, prostaglandins (e.g., PGE_2), PGI_2, serotonin, thromboxanes (e.g., TXA_2), leukotrienes (e.g., LTB_4), hepoxillin A_3, or chemokines (Keates et al., 2008; Ahmad et al., 2012). The

genetically engineered bacteria may inhibit one or more pro-inflammatory molecules, e.g., TNF, IL-17. In some embodiments, the genetically engineered bacteria are capable of modulating one or more molecule(s) shown in **Table 20**. In some embodiments, the genetically engineered bacteria are capable of inhibiting, removing, degrading, and/or metabolizing one or more inflammatory molecules.

Table 20

Metabolites	Related bacteria	Potential biological functions
Bile acids: cholate, hyocholate, deoxycholate, chenodeoxycholate, a-muricholate, b-muricholate, w-muricholate, taurocholate, glycocholate, taurochenoxycholate, glycochenodeoxycholate, taurocholate, tauro-a-muricholate, tauro-b-muricholate, lithocholate, ursodeoxycholate, hyodeoxycholate, glycodeoxylcholate	Lactobacillus, Bifidobacteria, Enterobacter, Bacteroides, Clostridium	Absorb dietary fats and lipid-soluble vitamins, facilitate lipid absorption, maintain intestinal barrier function, signal systemic endocrine functions to regulate triglycerides, cholesterol, glucose and energy homeostasis.
Choline metabolites: methylamine, dimethylamine, trimethylamine, trimethylamine-N-oxide, dimethylglycine, betaine	Faecalibacterium prausnitzii, Bifidobacterium	Modulate lipid metabolism and glucose homeostasis. Involved in nonalcoholic fatty liver disease, dietary induced obesity, diabetes, and cardiovascular disease.
Phenolic, benzoyl, and phenyl derivatives: benzoic acid, hippuric acid, 2-hydroxyhippuric acid, 2-hydroxybenzoic acid, 3-hydroxyhippuric acid, 3-hydroxybenzoic acid, 4 hydroxybenzoic acid, 3hydroxyphenylpropionate, 4-hydroxyphenylpropionate, 3-hydroxycinnamate, 4-methylphenol, tyrosine, phenylalanine, 4-cresol, 4-cresyl sulfate, 4-cresyl glucuronide, 4-hydroxyphenylacetate	Clostridium difficile, F. prausnitzii, Bifidobacterium, Subdoligranulum, Lactobacillus	Detoxification of xenobiotics; indicate gut microbial composition and activity; utilize polyphenols. Urinary hippuric acid may be a biomarker of hypertension and obesity in humans. Urinary 4-hydroxyphenylacetate, 4-cresol, and phenylacetate are elevated in colorectal cancer. Urinary 4-cresyl sulfate is elevated in children with severe autism.
Indole derivatives: N-acetyltryptophan, indoleacetate, indoleacetylglycine (IAG), indole, indoxyl sulfate, indole-3-propionate, melatonin, melatonin 6-sulfate, serotonin, 5-hydroxyindole	Clostridium sporogenes, E. coli	Protect against stress-induced lesions in the GI tract; modulate expression of proinflammatory genes, increase expression of anti-inflammatory genes, strengthen epithelial cell barrier properties. Implicated in GI pathologies, brain-gut axis, and a few neurological conditions.

Vitamins: vitamin K, vitamin B12, biotin, folate, thiamine, riboflavin, pyridoxine	Bifidobacterium	Provide complementary endogenous sources of vitamins, strengthen immune function, exert epigenetic effects to regulate cell proliferation.
Polyamines: putrescine, cadaverine, spermidine, spermine	Campylobacter jejuni, Clostridium saccharolyticum	Exert genotoxic effects on the host, anti-inflammatory and antitumoral effects. Potential tumor markers.
Lipids: conjugated fatty acids, LPS, peptidoglycan, acylglycerols, sphingomyelin, cholesterol, phosphatidylcholines, phosphoethanolamines, triglycerides	Bifidobacterium, Roseburia, Lactobacillus, Klebsiella, Enterobacter, Citrobacter, Clostridium	Impact intestinal permeability, activate intestinebrain- liver neural axis to regulate glucose homeostasis; LPS induces chronic systemic inflammation; conjugated fatty acids improve hyperinsulinemia, enhance the immune system and alter lipoprotein profiles.
Others: D-lactate, formate, methanol, ethanol, succinate, lysine, glucose, urea, a-ketoisovalerate, creatine, creatinine, endocannabinoids, 2-arachidonoylglycerol (2-AG), N-arachidonoylethanolamide, LPS	Bacteroides, Pseudobutyrivibrio, Ruminococcus, Faecalibacterium	Direct or indirect synthesis or utilization of compounds or modulation of linked pathways including endocannabinoid system.

[0677] In some embodiments, the genetically engineered bacteria are capable of producing an anti-inflammation and/or gut barrier enhancer molecule and further producing a molecule that is capable of inhibiting an inflammatory molecule. In some embodiments, the genetically engineered bacteria of the invention are capable of producing an anti-inflammation and/or gut barrier enhancer molecule and further producing an enzyme that is capable of degrading an inflammatory molecule. For example, the genetically engineered bacteria of the invention are capable of expressing a gene cassette for producing butyrate, as well as a molecule or biosynthetic pathway for inhibiting, removing, degrading, and/or metabolizing an inflammatory molecule, e.g., PGE_2.

RNAi, scFV, other mechanisms

[0678] 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 pro-inflammatory molecules in low-oxygen conditions. In some embodiments, the genetically engineered bacteria produce siRNA targeting TNF in low-oxygen conditions.

[0679] 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 pro-inflammatory cytokines, 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 pro-inflammatory molecules in low-oxygen conditions. In some embodiments, the genetically engineered bacteria produce scFv targeting TNF in low-oxygen conditions. In some embodiments, the genetically engineered bacteria produce both scFv and siRNA targeting one or more pro-inflammatory molecules in low-oxygen conditions (*see, e.g.,* Xiao et al., 2014).

[0680] One of skill in the art would appreciate that additional genes and gene cassettes capable of producing anti-inflammation and/or gut barrier function enhancer molecules 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.

[0681] In some embodiments, the genetically engineered bacteria produce two or more anti-inflammation and/or gut barrier function enhancer molecules. In certain embodiments, the two or more molecules behave synergistically to reduce gut inflammation and/or enhance gut barrier function. In some embodiments, the genetically engineered bacteria express at least one anti-inflammation molecule and at least one gut barrier function enhancer molecule. In certain embodiments, the genetically engineered bacteria express IL-10 and GLP-2. In alternate embodiments, the genetically engineered bacteria express IL-10 and butyrate.

[0682] In some embodiments, the genetically engineered bacteria are capable of producing IL-2, IL-10, IL-22, IL-27, propionate, and butyrate. In some embodiments, the genetically engineered bacteria are capable of producing IL-10, IL-27, GLP-2, and

butyrate. In some embodiments, the genetically engineered bacteria are capable of producing GLP-2, IL-10, IL-22, SOD, butyrate, and propionate. In some embodiments, the genetically engineered bacteria are capable of GLP-2, IL-2, IL-10, IL-22, IL-27, SOD, butyrate, and propionate. Any suitable combination of therapeutic molecules may be produced by the genetically engineered bacteria.

Generation of Bacterial Strains with Enhance Ability to Transport Amino Acids

[0683] 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.

[0684] 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.

[0685] 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.

[0686] 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.

[0687] 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.

[0688] 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.

[0689] 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.

[0690] 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.

[0691] 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).

[0692] Similar methods can be used to generate E.Coli Nissle mutants that consume or import tryptophan.

Inducible regulatory regions

[0693] In some embodiments, the bacterial cell comprises a stably maintained plasmid or chromosome carrying the gene(s) encoding payload (s), such that the payload(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. In some embodiments, bacterial cell comprises two or more distinct payloads or operons, *e.g.,* two or more payload genes. In some embodiments, bacterial cell comprises three or more distinct transporters or operons, *e.g.,* three or more payload genes. In some embodiments, bacterial cell comprises 4, 5, 6, 7, 8, 9, 10, or more distinct payloads or operons, *e.g.,* 4, 5, 6, 7, 8, 9, 10, or more payload genes.

[0694] 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(s) function as enyzmes for the production of such effector molecules. Non-limiting examples of payloads include anti-inflammation and/or gut barrier function enhancer molecule(s), including but not limited to, butyrate, propionate, acetate, IL10, IL-2, IL-22, IL-27, IL-20, IL-24, IL-19, SOD, GLP2, and/or tryptophan and/or its metabolites. 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 anti-inflammation and/or gut barrier function enhancer molecule(s). 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 anti-inflammation and/or gut barrier function enhancer molecule(s) described herein.

[0695] In some embodiments, the genetically engineered bacteria comprise multiple copies of the same payload gene(s). In some embodiments, the gene encoding the payload is present on a plasmid and operably linked to a directly or indirectly inducible promoter. In some embodiments, the gene encoding the payload is present on a plasmid and operably linked to a constitutive promoter. In some embodiments, the gene encoding the payload 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 payload 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.

[0696] In some embodiments, the gene encoding the payload is present on a chromosome and operably linked to a directly or indirectly inducible promoter. In some

embodiments, the gene encoding the payload is present on a chromosome and operably linked to a constitutive promoter. In some embodiments, the gene encoding the payload 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 payload 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.

[0697] In some embodiments, the genetically engineered bacteria comprise two or more payloads, all of which are present on the chromosome. In some embodiments, the genetically engineered bacteria comprise two or more payloads, all of which are present on one or more same or different plasmids. In some embodiments, the genetically engineered bacteria comprise two or more payloads, some of which are present on the chromosome and some of which are present on one or more same or different plasmids.

[0698] In any of the nucleic acid embodiments described above, the one or more payload(s) for producing the anti-inflammation and/or gut barrier function enhancer molecule combinations are operably linked to one or more directly or indirectly inducible promoter(s). In some embodiments, the one or more payload(s) are operably linked to a directly or indirectly inducible promoter that is induced under exogeneous environmental conditions, e.g., conditions found in the gut. In some embodiments, the one or more payload(s) are operably linked to a directly or indirectly inducible promoter that is induced by metabolites found in the gut, or other specific conditions. In some embodiments, the one or more payload(s) 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 payload(s) 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 payload(s) 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 the tables herein.

[0699] 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.

[0700] 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).

[0701] In some embodiments, the promoter is directly or indirectly induced by exogenous environmental conditions specific to the gut 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

[0702] The genetically engineered bacteria of the invention comprise a gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule(s), 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 anti-inflammation and/or gut barrier function enhancer molecule(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s).

[0703] 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 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 environment of the mammalian gut.

[0704] In certain embodiments, the bacterial cell comprises a gene encoding a payload 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 21** and **Table 22** below. Underlined sequences are predicted ribosome binding sites, and bolded sequences are restriction sites used for cloning.

Table 21. FNR Promoter Sequences

FNR Responsive Promoter	Sequence
SEQ ID NO: 563	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCA CTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATCTATTT CTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGAAATATCA GACAATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCC TTAAGGAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTT GCTGAATCGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA

SEQ ID NO: 564	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATGG CTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACAAAAAA TATTTCACTCGACAGGAGTATTTATATTGCGCCCGTTACGTGGGCTTCG ACTGTAAATCAGAAAGGAGAAAACACCT
SEQ ID NO: 565	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCA CTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATCTATTT CTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGAAATATCA GACAATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCC TTAAGGAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTT GCTGAATCGTTAA**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAG AAGGAGATATACAT
SEQ ID NO: 566	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATG GCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACAAAAA ATATTTCACTCGACAGGAGTATTTATATTGCGCCC**GGATCC**CTCTAGA AATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
SEQ ID NO: 567	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGG TTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGTA AAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCT CTCTT**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGAT ATACAT

Table 22. FNR Promoter sequences

FNR-responsive regulatory region	**12345678901234567890123456789012345678901234567890**
SEQ ID NO: 568	ATCCCCATCACTCTTGATGGAGATCAATTCCCCAAGCTGCTAGAGC GTTACCTTGCCCTTAAACATTAGCAATGTCGATTTATCAGAGGGCC GACAGGCTCCCACAGGAGAAAACCG
SEQ ID NO: 569	CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGC CCTTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCC CACAGGAGAAAACCG
nirB1 **SEQ ID NO: 570**	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGG CACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATC TATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGA AATATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGC AATATACCCCTTAAGGAGTATATAAAGGTGAATTTGATTTACATCA ATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGTAATAGAAAAG AAATCGAGGCAAAA

nirB2 **SEQ ID NO: 571**	CGGCCCGATCGTTGAACATAGCGGTCCGCAGGCGGCACTGCTTAC AGCAAACGGTCTGTACGCTGTCGTCTTTGTGATGTGCTTCCTGTTA GGTTTCGTCAGCCGTCACCGTCAGCATAACACCCTGACCTCTCATT AATTGCTCATGCCGGACGGCACTATCGTCGTCCGGCCTTTTCCTCT CTTCCCCCGCTACGTGCATCTATTTCTATAAACCCGCTCATTTTGTC TATTTTTTGCACAAACATGAAATATCAGACAATTCCGTGACTTAAG AAAATTTATACAAATCAGCAATATACCCATTAAGGAGTATATAAA GGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTAAG GTAGGCGGTAATAGAAAAGAAATCGAGGCAAAAatgtttgtttaactttaagaa ggagatatacat
nirB3 **SEQ ID NO: 572**	GTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGG CACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATC TATTTCTATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGA AATATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGC AATATACCCATTAAGGAGTATATAAAGGTGAATTTGATTTACATCA ATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGTAATAGAAAAG AAATCGAGGCAAAA
ydfZ **SEQ ID NO: 573**	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTAT GGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACAA AAAATATTTCACTCGACAGGAGTATTTATATTGCGCCCGTTACGTG GGCTTCGACTGTAAATCAGAAAGGAGAAAACACCT
nirB+RBS **SEQ ID NO: 574**	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGG CACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATC TATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGA AATATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGC AATATACCCCTTAAGGAGTATATAAAGGTGAATTTGATTTACATCA ATAAGCGGGGTTGCTGAATCGTTAA**GGATCC**CTCTAGAAATAATT TTGTTTAACTTTAAGAAGGAGATATACAT
ydfZ+RBS **SEQ ID NO: 575**	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTA TGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACA AAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCC**GGATCC** CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
fnrS1 **SEQ ID NO: 576**	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAAT GGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGC CGTAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGC AATATCTCTCTT**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAA GAAGGAGATATACAT

fnrS2 **SEQ ID NO: 577**	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAAT GGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGC CGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGC AATATCTCTCTTGGATCCAAAGTGAACTCTAGAAATAATTTTGTTT AACTTTAAGAAGGAGATATACAT
nirB+crp **SEQ ID NO: 578**	TCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTCAGCCGTCACCGT CAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGGCA CTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTA TTTCTATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGAAA TATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGCAAT ATACCCATTAAGGAGTATATAAAGGTGAATTTGATTTACATCAATA AGCGGGGTTGCTGAATCGTTAAGGTAGaaatgtgatctagttcacatttGCGGTA ATAGAAAAGAAATCGAGGCAAAA*atgtttgtttaactttaagaaggagatatacat*
fnrS+crp **SEQ ID NO: 579**	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAAT GGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGC CGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGC AATATCTCTCaaatgtgatctagttcacattt*tttgtttaactttaagaaggagatatacat*

[0705] 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 payload.

[0706] Non-limiting FNR promoter sequences are provided in **Table 21** and **Table 22**. **Table 21 and Table 22** 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.

[0707] 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 payload 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 payload 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.

[0708] In another embodiment, the genetically engineered bacteria comprise the gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule(s) 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-N4-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).

[0709] ln other embodiments, the one or more gene sequence(s) for producing a payload 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 an anti-inflammation and/or gut barrier function enhancer molecule(s) 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*.

[0710] 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.

[0711] 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 payload, 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 payload, 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.

[0712] 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.

[0713] 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 payload are present on different chromosomes. In some embodiments, the gene encoding

the oxygen level-sensing transcriptional regulator and the gene encoding the payload 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 payload. In some embodiments, expression of the transcriptional regulator is controlled by the same promoter that controls expression of the payload. In some embodiments, the transcriptional regulator and the payload are divergently transcribed from a promoter region

RNS-dependent regulation

[0714] In some embodiments, the genetically engineered bacteria or genetically engineered virus comprise a gene encoding a payload that is expressed under the control of an inducible promoter. In some embodiments, the genetically engineered bacterium or genetically engineered virus that expresses a payload under the control of a promoter that is activated by inflammatory conditions. In one embodiment, the gene for producing the payload 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.

[0715] 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.

[0716] 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.

[0717] 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.

[0718] 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.

[0719] 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 23.**

Table 23. 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*

[0720] 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 payload, 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 payload is decreased or eliminated.

[0721] 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.

[0722] 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).

[0723] 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 payloads. In some embodiments, the DNR is Pseudomonas aeruginosa DNR.

[0724] In another embodiment, the genetically engineered bacteria comprise the gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule(s) 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.

[0725] 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.

[0726] 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 payload(s).

[0727] 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.

[0728] 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.

[0729] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express a payload. 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 payload gene or genes is expressed.

[0730] 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).

[0731] 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.

[0732] 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.

[0733] 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.

[0734] 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.

[0735] 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.

[0736] In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule(s) 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.

[0737] 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.

[0738] 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).

[0739] 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

[0740] In some embodiments, the genetically engineered bacteria or genetically engineered virus comprise a gene for producing a payload that is expressed under the control of an inducible promoter. In some embodiments, the genetically engineered bacterium or genetically engineered virus that expresses a payload under the control of a promoter that is activated by conditions of cellular damage. In one embodiment, the gene for producing the payload 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.

[0741] 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).

[0742] 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.

[0743] 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.

[0744] 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.

[0745] 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 24**.

Table 24. 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	$\bullet O_2^-$	*soxS*

ROS-sensing transcription factor:	Primarily capable of sensing:	Examples of responsive genes, promoters, and/or regulatory regions:
	NO• (also capable of sensing H_2O_2)	
RosR	H_2O_2	*rbtT; tnp16a; rluC1; tnp5a; mscL; tnp2d; phoD; tnp15b; pstA; tnp5b; xylC; gabD1; rluC2; cgtS9; azlC; narKGHJI; rosR*

[0746] 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 payload; 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 payload is decreased or eliminated.

[0747] 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.

[0748] 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.

[0749] 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.*, a payload. 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 a payload.

[0750] 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.

[0751] 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 payload 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 the a payload.

[0752] 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).

[0753] 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 payload. 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 payload.

[0754] 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.

[0755] 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.

[0756] 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).

[0757] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express a payload. 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 payload. 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 payload, is expressed.

[0758] 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.

[0759] 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.

[0760] Nucleic acid sequences of several exemplary OxyR-regulated regulatory regions are shown in **Table 25**. 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 25. 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

[0761] 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.

[0762] 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.

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.

[0763] 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.

[0764] 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.

[0765] 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 payload 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 payload 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 payload in the presence of ROS.

[0766] In some embodiments, the gene or gene cassette for producing the payload 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 payload 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 payload 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.

[0767] In some embodiments, the genetically engineered bacteria may comprise multiple copies of the gene(s) capable of producing a payload(s). In some embodiments, the gene(s) capable of producing a payload(s) is present on a plasmid and operatively linked to a ROS-responsive regulatory region. In some embodiments, the gene(s) capable of producing a payload is present in a chromosome and operatively linked to a ROS-responsive regulatory region.

[0768] Thus, in some embodiments, the genetically engineered bacteria or genetically engineered virus produce one or more payloads 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.

[0769] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying a gene for producing a payload, 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.*, in medium, and/or *in vivo*. In some embodiments, a bacterium may comprise multiple copies of the gene encoding the payload. In some embodiments, the gene encoding 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, 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 payload. In some embodiments, the gene encoding the payload is expressed on a chromosome.

Propionate and other promoters

[0770] In some embodiments, the genetically engineered bacteria comprise the gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule(s) expressed under the control of an inducible promoter that is responsive to specific molecules or metabolites in the environment, *e.g.*, the tumor microenvironment, a specific tissue, or 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 an anti-inflammation and/or gut barrier function enhancer molecule(s) is under the control of a propionate-inducible promoter. In a more specific embodiment, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule(s) 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 an anti-inflammation and/or gut barrier function enhancer molecule(s) is under the control of a pBAD promoter, which is activated in the presence of the sugar arabinose.

[0771] In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s) is present on a plasmid and operably linked to a promoter that is induced by molecules or metabolites that are specific to the mammalian gut. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s) 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.

[0772] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule(s), 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 anti-inflammation and/or gut barrier function enhancer molecule(s). 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 anti-inflammation and/or gut barrier function enhancer molecule(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s) is expressed on a chromosome.

[0773] **Table 26** 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 26. Propionate promoter sequence

Description	Sequence
Prp (Propionate) promoter Bold: prpR Lower case:	**TTACCCGTCTGGATTTTCAGTACGCGCTTTTAAACGACGCCA** **CAGCGTGGTACGGCTGATCCCCAAATAACGTGCGGCGGCGCG** **CTTATCGCCATTAAAGCGTGCGAGCACCTCCTGCAATGGAAG** **CGCTTCTGCTGACGAGGGCGTGATTTCTGCTGTGGTCCCCAC**

ribosome binding site ATG underlined: start of gene of interest **SEQ ID NO: 584**	**CAGTTCAGGTAATAATTGCCGCATAAATTGTCTGTCCAGTGT** **TGGTGCGGGATCGACGCTTAAAAAAAGCGCCAGGCGTTCCAT** **CATATTCCGCAGTTCGCGAATATTACCGGGCCAATGATAGTT** **CAGTAGAAGCGGCTGACACTGCGTCAGCCCATGACGCACCGA** **TTCGGTAAAAGGGATCTCCATCGCGGCCAGCGATTGTTTTAA** **AAAGTTTTCCGCCAGAGGCAGAATATCAGGCTGTCGCTCGCG** **CAAGGGGGGAAGCGGCAGACGCAGAATGCTCAAACGGTAAAA** **CAGATCGGTACGAAAACGTCCTTGCGTTATCTCCCGATCCAG** **ATCGCAATGCGTGGCGCTGATCACCCGGACATCTACCGGGAT** **CGGCTGATGCCCGCCAACGCGGGTGACGGCTTTTTCCTCCAG** **TACGCGTAGAAGGCGGGTTTGTAACGGCAGCGGCATTTCGCC** **AATTTCGTCAAGAAACAGCGTGCCGCCGTGGGCGACCTCAAA** **CAGCCCCGCACGTCCACCTCGTCTTGAGCCGGTAAACGCTCC** **CTCCTCATAGCCAAACAGTTCAGCCTCCAGCAACGACTCGGT** **AATCGCGCCGCAATTAACGGCGACAAAGGGCGGAGAAGGCTT** **GTTCTGACGGTGGGGCTGACGGTTAAACAACGCCTGATGAAT** **CGCTTGCGCCGCCAGCTCTTTCCCGGTCCCTGTTTCCCCCTG** **AATCAGCACTGCCGCGCGGGAACGGGCATAGAGTGTAATCGT** **ATGGCGAACCTGCTCCATTTGTGGTGAATCGCCGAGGATATC** **GCTCAGCGCATAACGGGTCTGTAATCCCTTGCTGGAGGTATG** **CTGGCTATACTGACGCCGTGTCAGGCGGGTCATATCCAGCGC** **ATCATGGAAAGCCTGACGTACGGTGGCCGCTGAATAAATAAA** **GATGGCGGTCATTCCTGCCTCTTCCGCCAGGTCGGTAATTAG** **TCCTGCCCCAATTACAGCCTCAATGCCGTTAGCTTTGAGCTC** **GTTAATTTGCCCGCGAGCATCCTCTTCAGTGATATAGCTTCG** **CTGTTCAAGACGGAGGTGAAACGTTTTCTGAAAGGCGACCAG** **AGCCGGAATGGTCTCCTGATAGGTCACGATTCCCATTGAGGA** **AGTCAGCTTTCCCGCTTTTGCCAGAGCCTGTAATACATCGAA** **TCCGCTGGGTTTGATGAGGATGACAGGTACCGACAGTCGGCT** **TTTTAAATAAGCGCCGTTGGAACCTGCCGCGATAATCGCGTC** **GCAGCGTTCGGTTGCCAGTTTTTTGCGAATGTAGGCTACTGC** **CTTTTCAAAACCGAGCTGAATAGGCGTGATCGTCGCCAGATG** **ATCAAACTCCAGGCTGATATCCCGAAATAGTTCGAACAGGCG** **CGTTACCGAGACCGTCCAGATCACCGGTTTATCGCTATTATC** **GCGCGAAGCGCTATGCACAGTAACCAT**CGTCGTAGATTCATG TTTAAGGAACGAATTCTTGTTTTATAGATGTTTCGTTAATGT TGCAATGAAACACAGGCCTCCGTTTCATGAAACGTTAGCTGA CTCGTTTTTCTTGTGACTCGTCTGTCAGTATTAAAAAAGATT TTTCATTTAACTGATTGTTTTTAAATTGAATTTTATTTAATG GTTTCTCGGTTTTTGGGTCTGGCATATCCCTTGCTTTAATGA GTGCATCTTAATTAACAATTCAATAACAAGAGGGCTGAATag taatttcaacaaaataacgagcattcgaatg

Other Inducible Promoters

[0774] In some embodiments, the gene encoding the anti-inflammation and/or gut barrier function enhancer molecule(s) 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 anti-inflammation and/or gut barrier function enhancer molecule(s) 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).

[0775] 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 anti-inflammation and/or gut barrier function enhancer molecule(s), such that the anti-inflammation and/or gut barrier function enhancer 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. In some embodiments, bacterial cell comprises two or more distinct copies of the one or more gene sequences(s) encoding the anti-inflammation and/or gut barrier function enhancer molecule(s), 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 anti-inflammation and/or gut barrier function enhancer molecule(s), 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 anti-inflammation and/or gut barrier function enhancer molecule(s), 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 anti-inflammation and/or gut barrier function enhancer molecule(s), 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).

[0776] 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 anti-inflammation and/or gut barrier function enhancer molecule(s), which is induced by one

or more nutritional and/or chemical inducer(s) and/or metabolite(s), such that the anti-inflammation and/or gut barrier function enhancer molecule(s) 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 and/or the tumor microenvironment. 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).

[0777] 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).

[0778] 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).

[0779] 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 anti-inflammation and/or gut barrier function enhancer molecule(s) and/or other polypeptide(s) of interest. In some embodiments, expression of one or more anti-inflammation and/or gut barrier function enhancer molecule(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.

[0780] In some embodiments, expression of one or more anti-inflammation and/or gut barrier function enhancer molecule(s) 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 the anti-inflammation and/or gut barrier function enhancer molecule(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.

[0781] 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 the figures and examples.

[0782] In one embodiment, expression of one or more anti-inflammation and/or gut barrier function enhancer molecule(s) of interest, *e.g.*, one or more therapeutic polypeptide(s), is driven directly or indirectly by one or more arabinose inducible promoter(s).

[0783] 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 anti-inflammation and/or gut barrier function enhancer molecule(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.

[0784] 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.

[0785] 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 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).

[0786] 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.

[0787] 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

[0788] 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**.

[0789] 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.

[0790] 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.

[0791] 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

[0792] 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.

[0793] 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 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).

[0794] 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.

[0795] 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**.

[0796] 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.

[0797] 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).

[0798] 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.

[0799] 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.

[0800] 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 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).

[0801] 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.

[0802] 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**.

[0803] 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.

[0804] 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).

[0805] 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

[0806] 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.

[0807] 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 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).

[0808] 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.

[0809] 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).

[0810] 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 in the figures and examples. Inducible expression from the ParaBad can be controlled or further fine-tuned through the optimization of the ribosome binding site (RBS), as described herein.

[0811] In one embodiment, expression of one or more protein(s) of interest is driven directly or indirectly by one or more thermoregulated promoter(s).

[0812] 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.

[0813] 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.

[0814] 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 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).

[0815] 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.

[0816] 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**.

[0817] 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.

[0818] 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. 84A** depicts a schematic of the gene organization of a PssB promoter.

[0819] 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).

[0820] 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.

[0821] 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.

[0822] 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, 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**.

[0823] Sequences useful for expression from inducible promoters are listed in **Table 27.**

Table 27. Inducible promoter construct sequences

Description	Sequence
Arabinose	CAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTCGC

Promoter region **SEQ ID NO: 585**	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 rhamnose inducible promoter **SEQ ID NO: 588**	CGGTGAGCATCACATCACCACAATTCAGCAAATTGTGAAC ATCATCACGTTCATCTTTCCCTGGTTGCCAATGGCCCATTT TCCTGTCAGTAACGAGAAGGTCGCGAATCAGGCGCTTTTT AGACTGGTCGTAATGAAATTCAGCTGTCACCGGATGTGCT TTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCCTCTAC AAATAATTTTGTTTAAAACAACACCCACTAAGATAACTCT AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
Lac Promoter region	ATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATG CCATACCGCGAAAGGTTTTGCGCCATTCGATGGCGCGCCG

SEQ ID NO: 589	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	ACGTTAAATCTATCACCGCAAGGGATAAATATCTAACACC GTGCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTG CATAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCC

sensitive promoter **SEQ ID NO: 592**	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**	*Ttaagacccactttcacatttaagttgttttctaatccgcatatgatcaattcaaggccgaataa gaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcata ctatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaacct aaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgttgg cataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgta cttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaat cttgccagctttcccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggct aaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctctacaccta gcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcg ctgttaatcactttacttttatctaatctagacat*cattaattcctaatttttt**gttgacactctatcattg atagagttattttaccactccctatcagtgatagagaa**aagtgaa*ctctagaaataattttgttt aactttaagaaggagatatacat*</u>

PssB promoter **SEQ ID NO: 597**	tcacctttcccggattaaacgctttttttgcccggtggcatggtgctaccggcgatcacaaacggtta attatgacacaaattgacctgaatgaatatacagtattggaatgcattacccggagtgttgtgtaac aatgtctggccaggtttgtttcccggaaccgaggtcacaacatagtaaaagcgctattggtaatgg tacaatcgcgcgtttacacttattc

[0824] In some embodiments, the anti-inflammation and/or gut barrier enhancer molecule is butyrate. Methods of measuring butyrate levels, e.g., by mass spectrometry, gas chromatography, high-performance liquid chromatography (HPLC), are known in the art (see, e.g., Aboulnaga et al., 2013). In some embodiments, butyrate is measured as butyrate level/bacteria optical density (OD). In some embodiments, measuring the activity and/or expression of one or more gene products in the butyrogenic gene cassette serves as a proxy measurement for butyrate production. In some embodiments, the bacterial cells of the invention are harvested and lysed to measure butyrate production. In alternate embodiments, butyrate production is measured in the bacterial cell medium. In some embodiments, the genetically engineered bacteria produce at least about 1 nM/OD, at least about 10 nM/OD, at least about 100 nM/OD, at least about 500 nM/OD, at least about 1 μM/OD, at least about 10 μM/OD, at least about 100 μM/OD, at least about 500 μM/OD, at least about 1 mM/OD, at least about 2 mM/OD, at least about 3 mM/OD, at least about 5 mM/OD, at least about 10 mM/OD, at least about 20 mM/OD, at least about 30 mM/OD, or at least about 50 mM/OD of butyrate in the presence of ROS.

Constitutive promoters

[0825] In some embodiments, the gene encoding the payload is present on a plasmid and operably linked to a constitutive promoter. In some embodiments, the gene encoding the payload is present on a chromosome and operably linked to a constitutive promoter.

[0826] In some embodiments, the constitutive promoter is active under *in vivo* conditions, *e.g.*, the gut, 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, as described herein, and under *in vitro* conditions, *e.g.*, various cell culture and/or cell production and/or manufacturing conditions, as described herein.

[0827] In some embodiments, the constitutive promoter that is operably linked to the gene encoding the payload is active in various exogenous environmental conditions (*e.g.*, *in vivo* and/or *in vitro* and/or production/manufacturing conditions).

[0828] In some embodiments, the constitutive promoter is active in exogenous environmental conditions specific to the gut of a mammal. 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. In some embodiments, the constitutive promoter is active in the presence of molecules or metabolites that are specific to the gut of a mammal. 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.

[0829] Bacterial constitutive promoters are known in the art. Examplary constitutive promoters are listed in the following Tables.

Table 28A. 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:	Key Promoter absorbs 3	. . . caggccggaataactccctataatgcgcca	59

603			
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 SEQ ID NO: 609	constitutive promoter family member	. . . ggctagctcagtcctaggtactatgctagc	35
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	constitutive promoter	. . .	35

SEQ ID NO: 617	family member	ggctagctcagtcctagggattatgctagc	
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 SEQ ID NO: 623	constitutive promoter family member	. . . agctagctcagtcctaggtataatgctagc	35
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:	*E. coli* CreABCD phosphate sensing operon promoter	. . . ttatttaccgtgacgaactaattgctcgtg	81

630			
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 SEQ ID NO: 637	constitutive promoter with (C)10 between -10 and -35 elements	. . . ccccgaaagcttaagaatataattgtaagc	62
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	optimized (TA) repeat	. . . tatatatatatatatatataatgctagc	39

SEQ ID NO: 643	constitutive promoter with 21 bp between -10 and -35 elements		
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	Anderson Promoter with	. . .	78

SEQ ID NO: 656	lacI binding site	ggaattgtgagcggataacaatttcacaca	
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 SEQ ID NO: 664	Infrared signal reporter (J23119:IFP:J23119:HO1)	. . . caccttcgggtgggcctttctgcgtttata	2124
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 SEQ ID NO: 678	Anderson promoter J23118	. . . ggctagctcagtcctaggtattgtgctagc	35
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:	M13K07 gene V promoter	. . . atgagccagttcttaaaatcgcataaggta	50

683			
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 28B. 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 28C. 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 28D. 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 28E. 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 28F. 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 28G. 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 SEQ ID NO: 719	T7 RNAP promoter	tcatacgactcactatagggaga	23
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 28H. Constitutive promoters from bacteriophage SP6

Name	Description	Promoter Sequence	Length
BBa_J64998	consensus -10 and rest from SP6	atttaggtgacactataga	19

SEQ ID NO: 723			

Table 28I. 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 28J. Constitutive promoters from miscellaneous eukaryotes

Name	Description	Promoter Sequence	Length
BBa_I712004	CMV promoter	. . . agaacccactgcttactggcttatcgaaat	654

SEQ ID NO: 738			
BBa_K076017 SEQ ID NO: 739	Ubc Promoter	. . . ggccgtttttggctttttgttagacgaag	1219

Table 28K. 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 NO: 743	ggaaaatttttttaaaaaaaaaactttacgg ctagctcagccctaggtattatgctagc	UP element helps recruit RNA polymerase (ggaaaatttttttaaaaaaaaaac)

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)

[0830] Bacterial constitutive promoters are known in the art. Examplary constitutive promoters are listed in the following Tables.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

[0831] 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 A and Table B 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 29A. 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 29B. 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

Multiple mechanisms of action

[0832] 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. 52**. For example, the genetically engineered bacteria may include four copies of GLP-2 inserted at four different insertion sites, *e.g.*, *malE/K, insB/I, araC/BAD,* and *lacZ.* Alternatively, the genetically engineered bacteria may include three copies of GLP-2 inserted at three different insertion sites, *e.g.*, *malE/K, insB/I,* and *lacZ,* and three copies of a butyrogenic gene cassette inserted at three different insertion sites, *e.g.*, *dapA, cea,* and *araC/BA*

[0833] 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.

[0834] In some embodiments, the genetically engineered bacteria comprise one or more of **(1)** one or more gene(s) or gene cassette(s) for the production of propionate, as described herein **(2)** one or more gene(s) or gene cassette(s) for the production of butyrate, as described herein **(3)** one or more gene(s) or gene cassette(s) for the production of acetate, as described herein **(4)** one or more gene(s) or gene cassette(s) for the production of tryptophan and/or its metabolites (including but not limited to kynurenine, indole, indole-3-acetic acid, indole-3 aldehyde, and IPA) , as described herein **(5)** one or more

gene(s) or gene cassette(s) for the production of one or more of GLP-2 and GLP-2 analogs, as described herein (6) one or more gene(s) or gene cassette(s) for the production of human or viral or monommerized IL-10, as described herein (7) one or more gene(s) or gene cassette(s) for the production of human IL-22, as described herein (8) one or more gene(s) or gene cassette(s) for the production of IL-2, and./or SOD, and/or IL-27 and other interleukins, as described herein (9) one or more gene(s) or gene cassette(s) for the production of one or more transporters, e.g. for the import of tryptophan and/or metabolites as described herein (10) one or more polypepides for secretion, including but not limited to GLP-2 and its analogs, IL-10, and/or IL-22, SCFA and/or tryptophan synthesis and/or catabolic enzymes in wild type or in mutated form (for increased stability or metabolic activity) (11) one or more components of secretion machinery, as described herein (12) one or more auxotrophies, e.g., deltaThyA (13) one more more antibiotic resistances, including but not limited to, kanamycin or chloramphenicol resistance (14) 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 (15) one or more mutations/deletions in one or more genes of the endogenous metabolic pathways, e.g., tryptophan synthesis pathway.

[0835] In some embodiments, the genetically engineered bacteria promote one or more of the following effector functions: (1) neutralizes TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-17, and/or chemokines, e.g., CXCL-8 and CCL2 (2) activates include AHR (e.g., which result in IL-22 production) and (3) activates PXR, (4) inhibits HDACs, (5) activates GPR41 and/or GPR43 and/or GPR109A, (6) inhibits NF-kappaB signaling, (7) modulators of PPARgamma, (8) activates of AMPK signaling, (9) modulates GLP-1 secretion and/or (10). scavenges hydroxyl radicals and functions as antioxidants.

[0836] 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.

[0837] 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 a payload under inducing conditions 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, the payload will be detectable under inducing conditions.

[0838] In certain embodiments, the immune modulator molecule is butyrate. Methods of measuring butyrate levels, *e.g.*, by mass spectrometry, gas chromatography, high-performance liquid chromatography (HPLC), are known in the art (*see, e.g.,* Aboulnaga *et al.*, 2013). In some embodiments, butyrate is measured as butyrate level/bacteria optical density (OD). In some embodiments, measuring the activity and/or expression of one or more gene products in the butyrogenic gene cassette serves as a proxy measurement for butyrate production. In some embodiments, the bacterial cells of the invention are harvested and lysed to measure butyrate production. In alternate embodiments, butyrate production is measured in the bacterial cell medium. In some embodiments, the genetically engineered bacteria produce at least about 1 nM/OD, at least about 10 nM/OD, at least about 100 nM/OD, at least about 500 nM/OD, at least about 1 μM/OD, at least about 10 μM/OD, at least about 100 μM/OD, at least about 500 μM/OD, at least about 1 mM/OD, at least about 2 mM/OD, at least about 3 mM/OD, at least about 5 mM/OD, at least about 10 mM/OD, at least about 20 mM/OD, at least about 30 mM/OD, or at least about 50 mM/OD of butyrate in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, 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.

[0839] In certain embodiments, the immune modulator molecule is propionate. Methods of measuring propionate levels, *e.g.*, by mass spectrometry, gas chromatography, high-performance liquid chromatography (HPLC), are known in the art (*see, e.g.,* Hillman 1978; Lukovac *et al.*, 2014). In some embodiments, measuring the activity and/or expression of one or more gene products in the propionate gene cassette serves as a proxy measurement for propionate production. In some embodiments, the bacterial cells of the invention are harvested and lysed to measure propionate production. In alternate embodiments, propionate production is measured in the bacterial cell medium. In some embodiments, the genetically engineered bacteria produce at least about 1 μM, at least about 10 μM, at least about 100 μM, at least about 500 μM, at least about 1 mM, at least about 2 mM, at least about 3 mM, at least about 5 mM, at least about 10 mM, at least about 15 mM, at least about 20 mM, at least about 30 mM, at least about 40 mM, or at least about 50 mM of propionate in low-oxygen conditions, in the presence of certain molecules or metabolites, in the presence of molecules or metabolites associated with liver damage, 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.

[0840] In some embodiments, quantitative PCR (qPCR) is used to amplify, detect, and/or quantify mRNA expression levels of the gene, gene(s), or gene cassettes for producing the payload(s). Primers may be designed and used to detect mRNA in a sample according to methods known in the art. In some embodiments, a fluorophore is added to a sample reaction mixture that may contain payload RNA, and a thermal cycler is used to illuminate the sample reaction mixture with a specific wavelength of light and detect the subsequent emission by the fluorophore. The reaction mixture is heated and cooled to predetermined temperatures for predetermined time periods. In certain embodiments, the heating and cooling is repeated for a predetermined number of cycles. In some embodiments, the reaction mixture is heated and cooled to 90-100°C, 60-70°C, and 30-50°C for a predetermined number of cycles. In a certain embodiment, the reaction mixture is heated and cooled to 93-97°C, 55-65°C, and 35-45°C for a predetermined number of cycles. In some embodiments, the accumulating amplicon is quantified after each cycle of the qPCR. The number of cycles at which fluorescence exceeds the threshold is the threshold cycle (CT). At least one CT result for each sample is generated, and the CT result(s) may be used to determine mRNA expression levels of the payload(s).

[0841] In some embodiments, quantitative PCR (qPCR) is used to amplify, detect, and/or quantify mRNA expression levels of the payload(s). Primers may be designed and used to detect mRNA in a sample according to methods known in the art. In some embodiments, a fluorophore is added to a sample reaction mixture that may contain payload mRNA, and a thermal cycler is used to illuminate the sample reaction mixture with a specific wavelength of light and detect the subsequent emission by the fluorophore. The reaction mixture is heated and cooled to predetermined temperatures for predetermined time periods. In certain embodiments, the heating and cooling is repeated for a predetermined number of cycles. In some embodiments, the reaction mixture is heated and cooled to 90-100°C, 60-70°C, and 30-50°C for a predetermined number of cycles. In a certain embodiment, the reaction mixture is heated and cooled to 93-97°C, 55-65°C, and 35-45°C for a predetermined number of cycles. In some embodiments, the accumulating amplicon is quantified after each cycle of the qPCR. The number of cycles at which fluorescence exceeds the threshold is the threshold cycle (CT). At least one CT result for each sample is generated, and the CT result(s) may be used to determine mRNA expression levels of the payload(s).

[0842] In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding short chain fatty acid production enzymes described herein and/or one or more gene sequence(s) encoding tryptophan catabolism enzyme(s) described herein and one or more gene sequence(s) encoding metabolite transporters described herein, and/or one or more gene sequence(s) encoding one or more therapeutic peptides for secretion, as described herein.

[0843] In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate. In some embodiments, the genetically engineered bacteria comprise a propionate gene cassette and are capable of producing propionate. In some embodiments, the genetically engineered bacteria comprise a acetate gene cassette and are capable of producing acetate. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding IL-10. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding IL-2. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding IL-22. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding IL-27. In some embodiments, the genetically

engineered bacteria comprise a gene sequence encoding SOD. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding GLP-2. In some embodiments, the genetically engineered bacteria are capable of producing kyurenine.

[0844] In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-10. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-2. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-22. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-27. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding SOD. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding GLP-2. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and are capable of producing kyurenine.

[0845] In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-10 and one or more gene sequences encoding IL-2, IL-22, IL-27, GLP-2, and SOD. In any of these embodiments the bacteria comprise a propionate gene cassette and can produce propionate. In any of these embodiments, the bacteria can produce kyuernine.

[0846] In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-2 and one or more gene sequences encoding IL-10, IL-22, IL-27, GLP-2, and SOD. In any of these embodiments the bacteria comprise a propionate gene cassette and can produce propionate. In any of these embodiments, the bacteria can produce kyuernine. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-22 and one or more gene sequences encoding IL-2, IL-10, IL-27,

GLP-2, and SOD. In any of these embodiments the bacteria comprise a propionate gene cassette and can produce propionate. In any of these embodiments, the bacteria can produce kyuernine. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding IL-27 and one or more gene sequences encoding IL-2, IL-22, IL-10, GLP-2, and SOD. In any of these embodiments the bacteria comprise a propionate gene cassette and can produce propionate. In any of these embodiments, the bacteria can produce kyuernine. In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding GLP-2 and one or more gene sequences encoding IL-2, IL-22, IL-27, IL-10, and SOD. In any of these embodiments the bacteria comprise a propionate gene cassette and can produce propionate. In any of these embodiments, the bacteria can produce kyuernine.

[0847] In some embodiments, the genetically engineered bacteria comprise a butyrate gene cassette and are capable of producing butyrate and comprise a gene sequence encoding SOD and one or more gene sequences encoding IL-2, IL-22, IL-27, GLP-2, and IL-10. In any of these embodiments the bacteria comprise a propionate gene cassette and can produce propionate. In any of these embodiments, the bacteria can produce kyuernine.

[0848] In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding IL-10 and a gene sequence(s) encoding one or more molecules selected from IL-2, IL-22, IL-27, GLP-2, and SOD. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding IL-2 and a gene sequence(s) encoding one or more molecules selected from IL-10, IL-22, IL-27, GLP-2, and SOD. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding IL-22 and a gene sequence(s) encoding one or more molecules selected from IL-2, IL-27, IL-10, GLP-2, and SOD. In some embodiments, the genetically engineered bacteria comprise a gene sequence(s) encoding IL-27 and a gene sequence encoding one or more molecules selected from IL-2, IL-22, IL-10, GLP-2, and SOD. In some embodiments, the genetically engineered bacteria comprise a gene sequence encoding SOD and a gene sequence(s) encoding one or more molecules selected from IL-2, IL-22, IL-27, GLP-2, and IL-10. In some embodiments, the genetically engineered bacteria

comprise a gene sequence encoding GLP-2 and a gene sequence(s) encoding one or more molecules selected from IL-2, IL-22, IL-27, IL-10, and SOD. In any of these embodiments, the genetically engineered bacteria are capable of producing kyurenine. In any of these embodiments, the genetically engineered bacteria are capable of producing butyrate. In any of these embodiments, the genetically engineered bacteria are capable of producing propionate. In any of these embodiments, the genetically engineered bacteria are capable of producing acetate.

[0849] In some embodiments, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed under the control of a constitutive promoter. In another embodiment, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed under the control of an inducible promoter. In some embodiments, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. In some embodiments, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed under the control of a promoter that is

directly or indirectly induced by inflammatory conditions. 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 P_{araC} promoter, a P_{araBAD} promoter, and a P_{TetR} promoter, each of which are described in more detail herein. Inducible promoters are described in more detail *infra*.

[0850] The at least one gene encoding the at least one short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion may be present on a plasmid or chromosome in the bacterial cell. In one embodiment, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are located on a plasmid in the bacterial cell. In another embodiment, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are located in the chromosome of the bacterial cell, and at least one gene encoding at least one short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion from a different species of bacteria are located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are located on a plasmid in the bacterial cell, and at least

one gene encoding the at least one one short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion from a different species of bacteria are located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are located in the chromosome of the bacterial cell, and at least one gene encoding the at least one one short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion from a different species of bacteria are located in the chromosome of the bacterial cell.

[0851] In some embodiments, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed on a low-copy plasmid. In some embodiments, the gene sequence(s) encoding the one or more short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of the at least one short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion .

[0852] In some embodiments, a recombinant bacterial cell of the invention comprising at least one gene encoding at least one short chain fatty acid production enzyme(s) and/or tryptophan catabolism enzyme(s) and/or tryptophan biosynthesis enzyme(s) and/or metabolite transporters and/or therapeutic peptides for secretion are expressed on a high-copy plasmid do not increase tryptophan catabolism as compared to a recombinant bacterial cell comprising the same gene expressed on a low-copy plasmid in the absence of a heterologous importer of tryptophan and/or its metabolites and additional copies of a native importer of tryptophanand/or its metabolites. In alternate embodiments,

the importer of tryptophan and/or its metabolites is used in conjunction with a high-copy plasmid.

[0853] In some embodiments, the genetically engineered bacteria described above further comprise one or more of the modifications, mutations, and/or deletions in endogenous genes described herein.

[0854] In some embodiments, the the genetically engineered microorganism further comprises a mutation and/or deletion in ldhA. In some embodiments, the genetically engineered microorganism further comprises a mutation and/or deletion in frdA. In some embodiments, the genetically engineered microorganism further comprises a mutation and/or deletion in adhE. In some embodiments, the the genetically engineered microorganism further comprises a mutation and/or deletion in one or more of ldhA, frdA, and adhE.

[0855] In some embodiments, surface display could be used to display a protein of interest on the surface of the genetically modified bacterium. In some embodiments, the genetically engineered bacteria and/or microorganisms encode one or more gene(s) and/or gene cassette(s) encoding a protein of interest, e.g., an anti-inflammation and/or gut barrier function enhancer molecule, which is anchored or displayed on the surface of the bacteria and/or microorganisms.

Induction of Payloads During Strain Culture

[0856] 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.

[0857] 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.

[0858] 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).

[0859] 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.

[0860] In some embodiments, the strains are administered without any pre-induction protocols during strain growth prior to *in vivo* administration.

Anaerobic induction

[0861] 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.

[0862] 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.

[0863] 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.

[0864] 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 .

[0865] 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.

[0866] 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.

[0867] 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.

[0868] 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

[0869] 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.

[0870] 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.

[0871] 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.

[0872] 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.

[0873] 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.

[0874] 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.

[0875] 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.

[0876] 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.

[0877] 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.

[0878] 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. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0879] 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.

[0880] 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. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0881] 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.

[0882] 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

[0883] 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.

[0884] 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.

[0885] 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.

[0886] 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.

[0887] 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.

[0888] 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.

[0889] 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.

[0890] 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

[0891] In some embodiments, cycling, phasing, or pulsing techniques are employed 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.

[0892] 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.

[0893] 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.

[0894] 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 .

[0895] 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.

[0896] 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.

[0897] 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.

[0898] 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.

[0899] 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.

[0900] 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.

[0901] 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

[0902] 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 figures and examples 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 anti-cancer 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 anti-cancer effector(s) described herein.

[0903] 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 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.

[0904] 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

[0905] In any of the embodiments described herein, in which the genetically engineered organism, e.g., engineered bacteria or engineered virus, produces a protein, polypeptide, or peptide, 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.

[0906] In some embodiments, the genetically engineered bacteria further comprise a native secretion mechanism or non-native secretion mechanism that is capable of secreting the molecule 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.

[0907] 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 figures and examples. 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).

[0908] 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).

[0909] 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. 57** 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).

[0910] 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. 56**, 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.

[0911] 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 molecule(s) into the extracellular milieu.

[0912] 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.

[0913] 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, tumor microenvironment, 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.

[0914] 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.

[0915] 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.

[0916] 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).

[0917] 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 molecule 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.

[0918] 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.

[0919] 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.

[0920] 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.

[0921] **Table 30 and Table 31A** below lists secretion systems for Gram positive bacteria and Gram negative bacteria.

Table 30 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 31A. 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	1.E.1 •21	+	-	-	1	None

	superfamily						
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 ?

[0922] 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.

[0923] In some embodiments, the genetically engineered bacterial comprise a native or non-native secretion system described herein for the secretion of a molecule, e.g., a cytokine, antibody (e.g., scFv), metabolic enzyme, e.g., kynureninase, an others described herein.

Table 31B. Polypeptide Sequences of exemplary secretion tags

Description	**Sequence**
PhoA **SEQ ID NO: 1500**	MKQSTIALALLPLLFTPVTKA
PhoA **SEQ ID NO: 1501**	KQSTIALALLPLLFTPVTKA
OmpF **SEQ ID NO: 1502**	MMKRNILAVIVPALLVAGTANA
cvaC **SEQ ID NO: 1503**	MRTLTLNELDSVSGG
TorA **SEQ ID NO: 1504**	MNNNDLFQASRRRFLAQLGGLTVAGMLGTSLLTPRRATAAQA A
fdnG **SEQ ID NO: 1505**	MDVSRRQFFKICAGGMAGTTVAALGFAPKQALA
dmsA **SEQ ID NO: 1506**	MKTKIPDAVLAAEVSRRGLVKTTAIGGLAMASSALTLPFSRIAH A
PelB **SEQ ID NO: 1507**	KYLLPTAAAGLLLLAAQPAMA
HlyA secretion signal **SEQ ID NO: 1508**	LNPLINEISKIISAAGNFDVKEERAAASLLQLSGNASDFSYGRNSI TLTASA
HlyA secretion signal **SEQ ID NO: 1509**	CTTAATCCATTAATTAATGAAATCAGCAAAATCATTTCAGCT GCAGGTAATTTTGATGTTAAAGAGGAAAGAGCTGCAGCTTC TTTATTGCAGTTGTCCGGTAATGCCAGTGATTTTTCATATGG ACGGAACTCAATAACTTTGACAGCATCAGCATAA.

Table 31C. Additionals secretion tag sequences (native to E coli.)

Description	Sequences

ECOLIN_05715 Secretion signal **SEQ ID NO: 1511**	MKRHLNTSYRLVWNHITGAFVVASELARARGKRAGVA VALSLAAATSLPALA
ECOLIN_16495 Secretion signal **SEQ ID NO: 1512**	MFWRDMTLSVWRKKTTGLKTKKRLLALVLAAALCSSPV WA
ECOLIN_19410 Secretion signal **SEQ ID NO:1513**	MGYKMNISSLRKAFIFMGAVAALSLVNAQSALA
ECOLIN_19880 Secretion signal **SEQ ID NO:1514**	MNKIFKVIWNPATGSYTVASETAKSRGKKSGRSKLLISAL VAGGLLSSFGASA

[0924] 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: 1500, SEQ ID NO: 1501, SEQ ID NO: 1502, SEQ ID NO: 1503, SEQ ID NO: 1504, SEQ ID NO: 1505, SEQ ID NO: 1506, SEQ ID NO: 1507, SEQ ID NO: 1508, SEQ ID NO: 1509, SEQ ID NO: 1511, SEQ ID NO: 1512, SEQ ID NO: 1513, and/or SEQ ID NO: 1514.**

[0925] 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.

[0926] 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.

[0927] 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.

[0928] In some embodiments, the genetically engineered microorganisms are capable of expressing any one or more of the described circuits in low-oxygen conditions,

and/or in the presence of cancer and/or the tumor microenvironment, or tissue specific molecules or metabolites, and/or in the presence of molecules or metabolites associated with inflammation or immune suppression, 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.

[0929] 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 described herein (8) combinations of one or more of such additional circuits.

[0930] Non-limiting examples of proteins of interest include GLP-2 peptides, GLP-2 analogs, IL-22, vIL-10, hIL-10, monomerized IL-10, IL-27, IL-19, IL-20, IL-24, tryptophan synthesies enzymes, SCFA biosynthesis enzymes, tryptophan catabolic enzymes, including but not limited to IDO, TDO, kynureninase, other tryptophan pathway catabolic enzymes, e.g. in the indole pathway and/or the kynurenine pathway as described

herein. These polypeptides may be mutated to increase stability, resistance to protease digestion, and/or activity.

Table 32. 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

[0931] In some embodiments, the therapeutic polypeptides of interest are secreted using components of the flagellar type III secretion system. In a non-limiting example, such a therapeutic polypeptide of interest, such as, GLP-2 peptides, GLP-2 analogs, IL-22, vIL-10, hIL-10, monomerized IL-10, IL-27, IL-19, IL-20, IL-24, are secreted via Type I Hemolysin Secretion, is 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.

[0932] In some embodiments, the therapeutic polypeptides of interest, e.g., GLP-2 peptides, GLP-2 analogs, IL-22, vIL-10, hIL-10, monomerized IL-10, IL-27, IL-19, IL-20, IL-24, are secreted via Type I Hemolysin Secretion, 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.

[0933] 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.

[0934] In some embodiments, the therapeutic polypeptides of interest, e.g., GLP-2 peptides, GLP-2 analogs, IL-22, vIL-10, hIL-10, monomerized IL-10, IL-27, IL-19, IL-20, IL-24, are secreted via Type I Hemolysin Secretion, 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.

[0935] In some embodiments, the therapeutic polypeptides of interest, e.g., GLP-2 peptides, GLP-2 analogs, IL-22, vIL-10, hIL-10, monomerized IL-10, IL-27, IL-19, IL-20, IL-24, are secreted via Type I Hemolysin Secretion, 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.

Essential genes and auxotrophs

[0936] 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, e.g., 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).

[0937] 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 genetically engineered bacteria of the disclosure becoming an 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.

[0938] 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. 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 a DNA 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.

[0939] . **Table 33** 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 33. 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		

[0940] **Table 34** 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 34. 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

[0941] 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).

[0942] 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).

[0943] 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).

[0944] 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 of the invention comprise a deletion or mutation in two or more genes required for cell survival and/or growth.

[0945] 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.

[0946] 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).

[0947] 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.

[0948] 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.

[0949] 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).

[0950] In some embodiments, the genetically engineered bacterium is a conditional auxotroph whose essential gene(s) is replaced using the arabinose system shown in Fig. 60.

[0951] 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 genetically 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 (Wright et al., 2015). In other embodiments, auxotrophic modifications may also be used to screen for mutant bacteria that produce the anti-inflammatory or gut barrier enhancer molecule.

Genetic regulatory circuits

[0952] 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 and PCT/US2016/39434, both of which are incorporated herein by reference in their entireties). The genetic regulatory circuits are useful to screen for mutant bacteria that produce an anti-inflammation and/or gut barrier enhancer molecule or rescue an auxotroph. In certain embodiments, the invention provides methods for selecting genetically engineered bacteria that produce one or more genes of interest.

[0953] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a therapeutic molecule (e.g., butyrate) 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 FNR-responsive promoter; a second gene or gene cassette for producing a therapeutic molecule (e.g., butyrate), 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 therapeutic molecule (e.g., butyrate) 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 therapeutic molecule (e.g., butyrate) 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.

[0954] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a therapeutic molecule (e.g., butyrate) 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 a therapeutic 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 therapeutic molecule is not expressed. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, thereby inducing expression of the mf-lon protease. The mf-lon protease recognizes the mf-lon degradation signal and degrades the TetR, and the therapeutic molecule is expressed.

[0955] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a therapeutic 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 a therapeutic 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 therapeutic 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 therapeutic molecule is expressed.

[0956] 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).

[0957] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a therapeutic 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 a therapeutic 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 therapeutic 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 therapeutic 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 therapeutic molecule is expressed.

[0958] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a therapeutic 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 a therapeutic 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 therapeutic 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 therapeutic molecule is expressed.

[0959] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a therapeutic 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 a therapeutic 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 therapeutic 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 a functional therapeutic molecule is produced.

[0960] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a therapeutic 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 a therapeutic 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 therapeutic 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 therapeutic 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 therapeutic molecule is expressed.

[0961] Synthetic gene circuits expressed on plasmids may function well in the short term but lose ability and/or function in the long term (Danino et al., 2015). In some embodiments, the genetically engineered bacteria comprise stable circuits for expressing genes of interest over prolonged periods. In some embodiments, the genetically engineered bacteria are capable of producing a therapeutic molecule and further comprise a toxin-anti-toxin system that simultaneously produces a toxin (hok) and a short-lived anti-toxin (sok), wherein loss of the plasmid causes the cell to be killed by the long-lived toxin (Danino et al., 2015). In some embodiments, the genetically engineered bacteria further comprise alp7 from B. subtilis plasmid pL20 and produces filaments that are capable of pushing plasmids to the poles of the cells in order to ensure equal segregation during cell division (Danino et al., 2015).

Host-plasmid mutual dependency

[0962] In some embodiments, the genetically engineered bacteria of the invention 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 of the invention.

[0963] 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.

[0964] Synthetic gene circuits express on plasmids may function well in the short term but lose ability and/or function in the long term (Danino et al., 2015). In some embodiments, the genetically engineered bacteria comprise stable circuits for expressing genes of interest over prolonged periods. In some embodiments, the genetically engineered bacteria are capable of producing an anti-inflammation and/or gut enhancer molecule and further comprise a toxin-anti-toxin system that simultaneously produces a toxin (hok) and a short-lived anti-toxin (sok), wherein loss of the plasmid causes the cell to be killed by the long-lived toxin (Danino et al., 2015; as shown in the figures and examples). In some embodiments, the genetically engineered bacteria further comprise alp7 from B. subtilis plasmid pL20 and produces filaments that are capable of pushing plasmids to the poles of the cells in order to ensure equal segregation during cell division (Danino et al., 2015).

Kill switch

[0965] In some embodiments, the genetically engineered bacteria of the invention also comprise a kill switch (see, e.g., U.S. Provisional Application Nos. 62/183,935, 62/263,329, and 62/277,654, each of which is incorporated herein by reference in their entireties). The kill switch is intended to actively kill genetically engineered bacteria 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.

[0966] Bacteria comprising 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 may also be programmed to die at a specific time after the expression and delivery

of a heterologous gene or genes, for example, an anti-inflammation and/or gut barrier enhancer molecule, 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 anti-inflammation and/or gut barrier enhancer molecule, e.g., GLP-2. In some embodiments, the kill switch is activated in a delayed fashion following expression of the anti-inflammation and/or gut barrier enhancer molecule. Alternatively, the bacteria may be engineered to die after the bacterium 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). 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).

[0967] 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) or, alternatively designed such that a toxin is produced once an environmental condition no longer exists or an external signal is ceased.

[0968] 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 low-oxygen conditions, in the presence of ROS, or in the presence of RNS. 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 recombinant bacterial cell is no longer viable.

[0969] 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.

[0970] 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.

[0971] 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.

[0972] 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 recombinant bacterial cell is not viable after the first essential gene is excised.

[0973] In one embodiment, the first recombinase further flips an inverted heterologous gene encoding a second excision enzyme. In one embodiment, 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.

[0974] 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.

[0975] 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.

[0976] 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 **Figs. 57, 60, 65**. The disclosure provides recombinant bacterial cells which express one or more heterologous gene(s) upon sensing arabinose or other sugar in the exogenous environment. In this aspect, the recombinant 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 toxin 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 arabinose 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.

[0977] 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 (P_{araBAD}). 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 anti-toxin, 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.

[0978] 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.

[0979] In one exemplary embodiment of the disclosure, the genetically 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 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 recombinant bacterial cell, and the recombinant 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.

[0980] In one embodiment of the disclosure, the genetically engineered bacterium further comprises an anti-toxin 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 anti-toxin 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 recombinant bacterial cell. The recombinant 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 recombinant bacterial cell will be killed by the toxin.

[0981] In another embodiment of the disclosure, the genetically engineered bacterium further comprises an anti-toxin 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 recombinant bacterial cell. The recombinant bacterial cell is no longer viable once the toxin protein is expressed, and the recombinant bacterial cell will be killed by the toxin.

[0982] In another exemplary embodiment of the disclosure, the genetically 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 recombinant 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 recombinant bacterial cell to survive. In the absence of arabinose, however, AraC suppresses transcription from the the P_{araBAD} promoter and the essential protein required for survival is not expressed. In this case, the recombinant 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 recombinant 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 recombinant bacterial cell can be present in the bacterial cell in conjunction with the TetR/toxin/anti-toxin kill-switch system described directly above.

[0983] 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.

[0984] In some embodiments, the engineered bacteria of the present disclosure further comprise the gene(s) encoding the components of any of the above-described kill-switch circuits.

[0985] In any of the above-described embodiments, the bacterial toxin may be 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.

[0986] In any of the above-described embodiments, the anti-toxin may be 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.

[0987] 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.

[0988] In some embodiments, the genetically engineered bacterium provided herein is an auxotroph. In one embodiment, the genetically engineered bacterium 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.

[0989] In some embodiments, the genetically engineered bacterium provided herein further comprises 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 anti-toxin. 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 anti-toxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding a toxin under the control of a 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 anti-toxin.

[0990] In some embodiments, the genetically engineered bacterium is an auxotroph comprising a therapeutic payload and further comprises a kill-switch circuit, such as any of the kill-switch circuits described herein.

[0991] In some embodiments of the above described genetically engineered bacteria, the gene or gene cassette for producing the anti-inflammation and/or gut barrier enhancer molecule is present on a plasmid in the bacterium and operatively linked on the plasmid to the inducible promoter. In other embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier enhancermolecule is present in the bacterial chromosome and is operatively linked in the chromosome to the inducible promoter.

Methods of Screening

Mutagenesis

[0992] In some embodiments, the inducible promoter is operably linked to a detectable product, e.g., GFP, and can be used to screen for mutants. In some embodiments, the inducible promoter is mutagenized, and mutants are selected based upon the level of detectable product, e.g., by flow cytometry, fluorescence-activated cell sorting (FACS) when the detectable product fluoresces. In some embodiments, one or more transcription factor binding sites is mutagenized to increase or decrease binding. In alternate embodiments, the wild-type binding sites are left intact and the remainder of the regulatory region is subjected to mutagenesis. In some embodiments, the mutant promoter is inserted into the genetically engineered bacteria of the invention to increase expression of the anti-inflammation and/or gut barrier enhancer molecule under inducing conditions, as compared to unmutated bacteria of the same subtype under the same conditions. In some embodiments, the inducible promoter and/or corresponding transcription factor is a synthetic, non-naturally occurring sequence.

[01] In some embodiments, the gene encoding an anti-inflammation and/or gut barrier enhancer molecule is mutated to increase expression and/or stability of said molecule under inducing conditions, as compared to unmutated bacteria of the same subtype under the same conditions. In some embodiments, one or more of the genes in a gene cassette for producing an anti-inflammation and/or gut barrier enhancer molecule is mutated to increase expression of said molecule under inducing conditions, as compared to unmutated bacteria of the same subtype under the same conditions. In some embodiments,

the efficacy or activity of any of the importers and exporters for metabolites of interest can be improved through mutations in any of these genes. Mutations increase uptake or export of such metabolites, including but not limited to, tryptophan, e.g., under inducing conditions, as compared to unmutated bacteria of the same subtype under the same conditions. Methods for directed mutation and screening are known in the art.

Generation of Bacterial Strains with Enhance Ability to Transport Metabolites of Interest

[0993] 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.

[0994] 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.

[0995] For example, if the biosynthetic pathway for producing a metabolite of interest is disrupted a strain capable of high-affinity capture of the metabolite of interest can be evolved via ALE. First, the strain is grown in varying concentrations of the auxotrophic metabolite of interest, until a minimum concentration to support growth is established. The strain is then passaged at that concentration, and diluted into lowering concentrations of the metabolite of interest at regular intervals. Over time, cells that are most competitive for the metabolite of interest – at growth-limiting concentrations – will come to dominate the population. These strains will likely have mutations in their metabolite of interest-transporters resulting in increased ability to import the essential and limiting metabolite of interest.

[0996] Similarly, by using an auxotroph that cannot use an upstream metabolite to form the metabolite of interest, 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 of interest. 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.

[0997] 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.

[0998] 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.

[0999] 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.

[01000] 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.

[01001] 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).

[01002] Similar methods can be used to generate E. coli Nissle mutants that consume or import metabolites, including, but not limited to, tryptophan.

Nucleic Acids

[01003] 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 butyrogenic gene cassettes. In some embodiments, the nucleic acids comprise one or more butyrogenic genes from Table 2. 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*.

[01004] 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: 1. In certain embodiments, the nucleic acid comprising the *bcd2* gene sequence has at least about 90% identity with SEQ ID NO: 1. In certain embodiments, the nucleic acid comprising the *bcd2* gene sequence has at least about 95% identity with SEQ ID NO: 1. 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: 1. 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: 1.

[01005] 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: 2. In certain embodiments, the nucleic acid comprising the *etfB3* gene sequence has at least about 90% identity with SEQ ID NO: 2. In certain embodiments, the nucleic acid comprising the *etfB3* gene sequence has at least about 95% identity with SEQ ID NO: 2. 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: 2. In some specific embodiments, the nucleic acid comprising the *etfB3* gene sequence

comprises SEQ ID NO: 2. In other specific embodiments the nucleic acid comprising the *etfB3* gene sequence consists of SEQ ID NO: 2.

[01006] 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: 3. In certain embodiments, the nucleic acid comprising the *etfA3* gene sequence has at least about 90% identity with SEQ ID NO: 3. In certain embodiments, the nucleic acid comprising the *etfA3* gene sequence has at least about 95% identity with SEQ ID NO: 3. 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: 3. In some specific embodiments, the nucleic acid comprising the *etfA3* gene sequence comprises SEQ ID NO: 3. In other specific embodiments the nucleic acid comprising the *etfA3* gene sequence consists of SEQ ID NO: 3.

[01007] 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: 4. In certain embodiments, the nucleic acid comprising the *thiA1* gene sequence has at least about 90% identity with SEQ ID NO: 4. 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: 4. In some specific embodiments, the nucleic acid comprising the *thiA1* gene sequence comprises SEQ ID NO: 4. In other specific embodiments the nucleic acid comprising the *thiA1* gene sequence consists of SEQ ID NO: 4.

[01008] 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: 5. In certain embodiments, the nucleic acid comprising the *hbd*gene sequence has at least about 90% identity with SEQ ID NO: 5. In certain embodiments, the nucleic acid comprising the *hbd*gene sequence has at least about

95% identity with SEQ ID NO: 5. 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: 5. In some specific embodiments, the nucleic acid comprising the *hbd* gene sequence comprises SEQ ID NO: 5. In other specific embodiments the nucleic acid comprising the *hbd* gene sequence consists of SEQ ID NO: 5.

[01009] 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: 6. In certain embodiments, the nucleic acid comprising the *crt2* gene sequence has at least about 90% identity with SEQ ID NO: 6. In certain embodiments, the nucleic acid comprising the *crt2* gene sequence has at least about 95% identity with SEQ ID NO: 6. 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: 6. In some specific embodiments, the nucleic acid comprising the *crt2* gene sequence comprises SEQ ID NO: 6. In other specific embodiments the nucleic acid comprising the *crt2* gene sequence consists of SEQ ID NO: 6.

[01010] 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: 7. In certain embodiments, the nucleic acid comprising the *pbt* gene sequence has at least about 90% identity with SEQ ID NO: 7. In certain embodiments, the nucleic acid comprising the *pbt* gene sequence has at least about 95% identity with SEQ ID NO: 7. 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: 7. In some specific embodiments, the nucleic acid comprising the *pbt* gene sequence comprises SEQ ID NO: 7. In other specific embodiments the nucleic acid comprising the *pbt* gene sequence consists of SEQ ID NO: 7.

[01011] 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: 8. In certain embodiments, the nucleic acid comprising the *buk* gene sequence has at least about 90% identity with SEQ ID NO: 8. In certain embodiments, the nucleic acid comprising the *buk* gene sequence has at least about 95% identity with SEQ ID NO: 8. 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: 8. In some specific embodiments, the nucleic acid comprising the *buk* gene sequence comprises SEQ ID NO: 8. In other specific embodiments the nucleic acid comprising the *buk* gene sequence consists of SEQ ID NO: 8.

[01012] 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: 9. In certain embodiments, the nucleic acid comprising the *ter* gene sequence has at least about 90% identity with SEQ ID NO: 9. In certain embodiments, the nucleic acid comprising the *ter* gene sequence has at least about 95% identity with SEQ ID NO: 9. 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: 9. In some specific embodiments, the nucleic acid comprising the *ter* gene sequence comprises SEQ ID NO: 9. In other specific embodiments the nucleic acid comprising the *ter* gene sequence consists of SEQ ID NO: 9.

[01013] 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: 10. In certain embodiments, the nucleic acid comprising the *tesB* gene sequence has at least about 90% identity with SEQ ID NO: 10. In certain embodiments, the nucleic acid comprising the *tesB* gene sequence has at least about 95% identity with SEQ ID NO: 10. 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: 10. In some specific embodiments, the nucleic acid comprising the *tesB* gene

sequence comprises SEQ ID NO: 10. In other specific embodiments the nucleic acid comprising the *tesB* gene sequence consists of SEQ ID NO: 10.

[01014] 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.

[01015] 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*.

[01016] One or more of the nucleic acids encoding butyrate biosynthesis genes may be functionally replaced or modified, e.g., codon optimized.

Pharmaceutical Compositions and Formulations

[01017] Pharmaceutical compositions comprising the genetically engineered microorganisms of the invention may be used to inhibit inflammatory mechanisms in the gut, restore and tighten gut mucosal barrier function, and/or treat or prevent autoimmunedisorders. Pharmaceutical compositions comprising one or more genetically engineered bacteria, and/or one or more genetically engineered virus, alone or in combination with prophylactic agents, therapeutic agents, and/or pharmaceutically acceptable carriers are provided.

[01018] 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 produce an anti-inflammation and/or gut barrier enhancer molecule. 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 produce an anti-inflammation and/or gut barrier enhancer molecule.

[01019] I:n certain embodiments, a combination of two or more different genetically engineered microorganisms can be administered at the same time. In some embodiments, the method comprises administering the a subject a combination of two or more genetically engineered microorganisms, a first microorganism which expresses a first payload, and at least a second microorganism which expresses a second payload. In some embodiments, the method comprises compositions comprising a combination of two or more genetically engineered microorganisms, a first microorgainsms which expresses a first payload, and at least a second microorganism which expresses a second payload.

[01020] 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.

[01021] The genetically engineered microorganisms 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, intravenous, sub-cutaneous, immediate-release, pulsatile-release, delayed-release, or sustained release). Suitable dosage amounts for the genetically engineered bacteria may range from about 105 to 1012 bacteria, e.g., approximately 105 bacteria, approximately 106 bacteria, approximately 107 bacteria, approximately 108 bacteria, approximately 109 bacteria, approximately 1010 bacteria, approximately 1011 bacteria, or approximately 1011 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 on embodiment, the pharmaceutical composition is administered after the subject eats a meal

[01022] The genetically engineered bacteria or genetically engineered virus 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 of the invention 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.

[01023] The genetically engineered microorganisms may be administered intravenously, e.g., by infusion or injection.

[01024] The genetically engineered microroganisms of the disclosure may be administered intrathecally. In some embodiments, the genetically engineered microorganisms of the invention may be administered orally. The genetically engineered microorganisms 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 recombinant bacteria of the invention 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.

[01025] The genetically engineered microorganisms 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.

[01026] 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., lactose, 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.

[01027] In some embodiments, the genetically engineered microorganisms 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.

[01028] 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 microorganisms described herein.

[01029] In one embodiment, the genetically engineered microorganisms 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.

[01030] 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.

[01031] 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.

[01032] In certain embodiments, the genetically engineered microorganisms 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.

[01033] In another embodiment, the pharmaceutical composition comprising the recombinant bacteria of the invention 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 recombinant bacteria of the invention 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 recombinant bacteria of the invention 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 of the invention is injected into, sprayed onto, or sprinkled onto a food product, such as bread, yogurt, or cheese.

[01034] 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.

[01035] The genetically engineered microorganisms 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.

[01036] The genetically engineered microorganisms 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).

[01037] 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.

[01038] 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.

[01039] 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.

[01040] 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, LD50, ED50, EC50, and IC50 may be determined, and the dose ratio between toxic and therapeutic effects (LD50/ED50) 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.

[01041] 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.

[01042] 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.

Methods of treatment

[01043] Another aspect of the invention provides methods of treating autoimmune disorders, diarrheal diseases, IBD, related diseases, and other diseases that benefit from reduced gut inflammation and/or enhanced gut barrier function. In some embodiments, the invention provides for the use of at least one genetically engineered species, strain, or subtype of bacteria described herein for the manufacture of a medicament. In some embodiments, the invention provides for the use of at least one genetically engineered species, strain, or subtype of bacteria described herein for the manufacture of a medicament for treating autoimmune disorders, diarrheal diseases, IBD, related diseases, and other diseases that benefit from reduced gut inflammation and/or enhanced gut barrier function. In some embodiments, the invention provides at least one genetically engineered species, strain, or subtype of bacteria described herein for use in treating autoimmune disorders, diarrheal diseases, IBD, related diseases, and other diseases that benefit from reduced gut inflammation and/or enhanced gut barrier function.

[01044] In some embodiments, the diarrheal disease is selected from the group consisting of acute watery diarrhea, e.g., cholera, acute bloody diarrhea, e.g., dysentery, and persistent diarrhea. In some embodiments, the IBD or related disease is selected from the group consisting of Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, diversion colitis, Behcet's disease, intermediate colitis, short bowel syndrome, ulcerative proctitis, proctosigmoiditis, left-sided colitis, pancolitis, and fulminant colitis. In some embodiments, the disease or condition is an autoimmune disorder selected from the group consisting of acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, Addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune oophoritis,

autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroid disease, autoimmune urticarial, axonal & neuronal neuropathies, Balo disease, Behcet's disease, bullous pemphigoid, cardiomyopathy, Castleman disease, celiac disease, Chagas disease, chronic inflammatory demyelinating polyneuropathy (CIDP), chronic recurrent multifocal ostomyelitis (CRMO), Churg-Strauss syndrome, cicatricial pemphigoid/benign mucosal pemphigoid, Crohn's disease, Cogan's syndrome, cold agglutinin disease, congenital heart block, Coxsackie myocarditis, CREST disease, essential mixed cryoglobulinemia, demyelinating neuropathies, dermatitis herpetiformis, dermatomyositis, Devic's disease (neuromyelitis optica), discoid lupus, Dressler's syndrome, endometriosis, eosinophilic esophagitis, eosinophilic fasciitis, erythema nodosum, experimental allergic encephalomyelitis, Evans syndrome, fibrosing alveolitis, giant cell arteritis (temporal arteritis), giant cell myocarditis, glomerulonephritis, Goodpasture's syndrome, granulomatosis with polyangiitis (GPA), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, herpes gestationis, hypogammaglobulinemia, idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, immunoregulatory lipoproteins, inclusion body myositis, interstitial cystitis, juvenile arthritis, juvenile idiopathic arthritis, juvenile myositis, Kawasaki syndrome, Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, ligneous conjunctivitis, linear IgA disease (LAD), lupus (systemic lupus erythematosus), chronic Lyme disease, Meniere's disease, microscopic polyangiitis, mixed connective tissue disease (MCTD), Mooren's ulcer, Mucha-Habermann disease, multiple sclerosis, myasthenia gravis, myositis, narcolepsy, neuromyelitis optica (Devic's), neutropenia, ocular cicatricial pemphigoid, optic neuritis, palindromic rheumatism, PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus), paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, pars planitis (peripheral uveitis), pemphigus, peripheral neuropathy, perivenous encephalomyelitis, pernicious anemia, POEMS syndrome, polyarteritis nodosa, type I, II, & III autoimmune polyglandular syndromes, polymyalgia rheumatic, polymyositis, postmyocardial infarction syndrome, postpericardiotomy syndrome, progesterone dermatitis, primary biliary cirrhosis, primary sclerosing cholangitis, psoriasis, psoriatic

arthritis, idiopathic pulmonary fibrosis, pyoderma gangrenosum, pure red cell aplasia, Raynaud's phenomenon, reactive arthritis, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis, scleroderma, Sjogren's syndrome, sperm & testicular autoimmunity, stiff person syndrome, subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, temporal arteritis/giant cell arteritis, thrombocytopenic purpura (TTP), Tolosa-Hunt syndrome, transverse myelitis, type 1 diabetes, asthma, ulcerative colitis, undifferentiated connective tissue disease (UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, and Wegener's granulomatosis. In some embodiments, the invention provides methods for reducing, ameliorating, or eliminating one or more symptom(s) associated with these diseases, including but not limited to diarrhea, bloody stool, mouth sores, perianal disease, abdominal pain, abdominal cramping, fever, fatigue, weight loss, iron deficiency, anemia, appetite loss, weight loss, anorexia, delayed growth, delayed pubertal development, and inflammation of the skin, eyes, joints, liver, and bile ducts. In some embodiments, the invention provides methods for reducing gut inflammation and/or enhancing gut barrier function, thereby ameliorating or preventing a systemic autoimmune disorder, e.g., asthma (Arrieta et al., 2015).

[01045] 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 of the invention are administered orally in a liquid suspension. In some embodiments, the genetically engineered bacteria of the invention are lyophilized in a gel cap and administered orally. In some embodiments, the genetically engineered bacteria of the invention are administered via a feeding tube. In some embodiments, the genetically engineered bacteria of the invention are administered rectally, e.g., by enema. In some embodiments, the genetically engineered bacteria of the invention are administered topically, intraintestinally, intrajejunally, intraduodenally, intraileally, and/or intracolically.

[01046] In some embodiments, the genetically engineered viruses are prepared for delivery, taking into consideration the need for efficient delivery and for

overcoming the host antiviral immune response. Approaches to evade antiviral response include the administration of different viral serotypes as par of the treatment regimen (serotype switching), formulation, such as polymer coating to mask the virus from antibody recognition and the use of cells as delivery vehicles.

[01047] In another embodiment, 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.

[01048] The genetically engineered bacteria of the invention 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.

[01049] In certain embodiments, the pharmaceutical composition described herein is administered to reduce gut inflammation, enhance gut barrier function, and/or treat or prevent an autoimmune disorder in a subject. In some embodiments, the methods of the present disclosure may reduce gut inflammation in a subject by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or more as compared to levels in an untreated or control subject. In some embodiments, the methods of the present disclosure may enhance gut barrier function in a subject by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or more as compared

to levels in an untreated or control subject. In some embodiments, changes in inflammation and/or gut barrier function are measured by comparing a subject before and after administration of the pharmaceutical composition. In some embodiments, the method of treating or ameliorating the autoimmune disorder and/or the disease or condition associated with gut inflammation and/or compromised gut barrier function allows one or more symptoms of the disease or condition to improve by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more.

[01050] In some embodiments, reduction is measured by comparing the levels of inflammation in a subject before and after administration of the pharmaceutical composition. In one embodiment, the levels of inflammation is reduced in the gut of the subject. In one embodiment, gut barrier function is enhanced in the gut of the subject. In another embodiment, levels of inflammation is reduced in the blood of the subject. In another embodiment, the levels of inflammation is reduced in the plasma of the subject. In another embodiment, levels of inflammation is reduced in the brain of the subject.

[01051] In one embodiment, the pharmaceutical composition described herein is administered to reduce levels of inflammation in a subject to normal levels. In another embodiment, the pharmaceutical composition described herein is administered to reduce levels of inflammation in a subject below normal.

[01052] In some embodiments, the method of treating the autoimmune disorder allows one or more symptoms of the condition or disorder to improve by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more. In some embodiments, the method of treating the disorder, allows one or more symptoms of the condition or disorder to improve by at least about two-fold, three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold.

[01053] Before, during, and after the administration of the pharmaceutical composition, gut inflammation and/or barrier function 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. In some embodiments, the methods may include administration of the compositions of the invention to enhance gut barrier function and/or to reduce gut inflammation to baseline levels, e.g., levels comparable to those of a

healthy control, in a subject. In some embodiments, the methods may include administration of the compositions of the invention to reduce gut inflammation to undetectable levels in a subject, or to less than about 1%, 2%, 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, or 80% of the subject's levels prior to treatment. In some embodiments, the methods may include administration of the compositions of the invention to enhance gut barrier function in a subject by about 1%, 2%, 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 100% or more of the subject's levels prior to treatment.

[01054] In certain embodiments, the recombinant bacteria are E. coli Nissle. The recombinant 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 recombinant bacteria may be re-administered at a therapeutically effective dose and frequency. In alternate embodiments, the recombinant bacteria are not destroyed within hours or days after administration and may propagate and colonize the gut.

[01055] The pharmaceutical composition may be administered alone or in combination with one or more additional therapeutic agents, e.g., corticosteroids, aminosalicylates, anti-inflammatory agents. In some embodiments, the pharmaceutical composition is administered in conjunction with an anti-inflammatory drug (e.g., mesalazine, prednisolone, methylprednisolone, butesonide), an immunosuppressive drug (e.g., azathioprine, 6-mercaptopurine, methotrexate, cyclosporine, tacrolimus), an antibiotic (e.g., metronidazole, ornidazole, clarithromycin, rifaximin, ciprofloxacin, anti-TB), other probiotics, and/or biological agents (e.g., infliximab, adalimumab, certolizumab pegol) (Triantafillidis et al., 2011). An important consideration in the selection of the one or more additional therapeutic agents is that the agent(s) should be compatible with the genetically engineered bacteria of the invention, e.g., the agent(s) must not kill the bacteriaIn one embodiments, the bacterial cells disclosed herein are administered to a subject once daily. In another embodiment, the bacterial cells disclosed herein are administered to a subject twice daily. In another embodiment, the bacterial cells disclosed herein are administered to a subject in combination with a meal. In another embodiment, the bacterial cells disclosed herein are administered to a subject prior to a meal. In another embodiment, the bacterial cells disclosed herein are administered to a subject after a meal.

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 disease. The appropriate therapeutically effective dose and/or frequency of administration can be selected by a treating clinician.

Treatment *in vivo*

[01056] The genetically engineered bacteria of the invention may be evaluated *in vivo,* e.g., in an animal model. Any suitable animal model of a disease or condition associated with gut inflammation, compromised gut barrier function, and/or an autoimmune disorder may be used (*see, e.g.,* Mizoguchi, 2012). The animal model may be a mouse model of IBD, e.g., a $CD45RB^{Hi}$ T cell transfer model or a dextran sodium sulfate (DSS) model. The animal model may be a mouse model of type 1 diabetes (T1D), and T1D may be induced by treatment with streptozotocin.

[01057] Colitis is characterized by inflammation of the inner lining of the colon, and is one form of IBD. In mice, modeling colitis often involves the aberrant expression of T cells and/or cytokines. One exemplary mouse model of IBD can be generated by sorting CD4+ T cells according to their levels of CD45RB expression, and adoptively transferring CD4+ T cells with high CD45RB expression from normal donor mice into immunodeficient mice. Non-limiting examples of immunodeficient mice that may be used for transfer include severe combined immunodeficient (SCID) mice (Morrissey et al., 1993; Powrie et al., 1993), and recombination activating gene 2 (RAG2)-deficient mice (Corazza et al., 1999). The transfer of $CD45RB^{Hi}$ T cells into immunodeficient mice, e.g., via intravenous or intraperitoneal injection, results in epithelial cell hyperplasia, tissue damage, and severe mononuclear cell infiltration within the colon (Byrne et al., 2005; Dohi et al., 2004; Wei et al., 2005). In some embodiments, the genetically engineered bacteria of the invention may be evaluated in a $CD45RB^{Hi}$ T cell transfer mouse model of IBD.

[01058] Another exemplary animal model of IBD can be generated by supplementing the drinking water of mice with dextran sodium sulfate (DSS) (Martinez et al., 2006; Okayasu et al., 1990; Whittem et al., 2010). Treatment with DSS results in epithelial damage and robust inflammation in the colon lasting several days. Single treatments may be used to model acute injury, or acute injury followed by repair. Mice treated acutely show signs of acute colitis, including bloody stool, rectal bleeding,

diarrhea, and weight loss (Okayasu et al., 1990). In contrast, repeat administration cycles of DSS may be used to model chronic inflammatory disease. Mice that develop chronic colitis exhibit signs of colonic mucosal regeneration, such as dysplasia, lymphoid follicle formation, and shortening of the large intestine (Okayasu et al., 1990). In some embodiments, the genetically engineered bacteria of the invention may be evaluated in a DSS mouse model of IBD.

[01059] In some embodiments, the genetically engineered bacteria of the invention is administered to the animal, e.g., by oral gavage, and treatment efficacy is determined, e.g., by endoscopy, colon translucency, fibrin attachment, mucosal and vascular pathology, and/or stool characteristics. In some embodiments, the animal is sacrificed, and tissue samples are collected and analyzed, e.g., colonic sections are fixed and scored for inflammation and ulceration, and/or homogenized and analyzed for myeloperoxidase activity and cytokine levels (e.g., IL-1β, TNF-α, IL-6, IFN-γ and IL-10).

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Examples

[01060] The following examples provide illustrative embodiments of the disclosure. One of ordinary skill in the art will recognize the numerous modifications and variations that may be performed without altering the spirit or scope of the disclosure. Such modifications and variations are encompassed within the scope of the disclosure. The Examples do not in any way limit the disclosure.

Example 1. Construction of Vectors for Producing Therapeutic Molecules

Butyrate

[01061] 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, pbt,* and *buk*; NCBI; **Table 2** and **Table 36**), as well as transcriptional and translational elements, are synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322 to create pLogic031 (bcd2-etfB3-etfA3-thiA1-hbd- crt2-pbt buk butyrate cassette, also referred to as bcd2-etfB3-etfA3 butyrate cassette, **SEQ ID NO: 162**).

[01062] 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,* **Table 2** and **Table 36**), 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 to create pLogic046 (ter-thiA1-hbd- crt2-pbt buk butyrate cassette, also referred to herein as ter butyrate cassette or pbt buk butyrate cassette, **SEQ ID NO: 163**).

[01063] In a third butyrate gene cassette, the *pbt* and *buk* genes are replaced with *tesB* (**SEQ ID NO: 10**). TesB is a thioesterase found in E. Coli that cleaves off the

butyrate from butyryl-coA, thus obviating the need for pbt-buk (see,, e.g., **FIG. 2** and **Table 2** and **Table 36**). The third butyrate gene cassette, as well as transcriptional and translational elements, is synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322 to create **pLOGIC046-delta pbt.buk/tesB+** (ter-thiA1-hbd- crt2-tesb butyrate cassette, also referred to herein as tesB butyrate cassette, **SEQ ID NO: 164**). **Table 36** lists non-limiting examples for sequences of the three cassettes.

Table 36. Butyrate Cassette Sequences

Description	Sequence	SEQ ID NO
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ter-thiA1-hbd-crt2-tesb butyrate cassette	atgatcgtaaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggctg caagaagggagtggaagatcagattgaatataccaagaaacgcattaccgcagaagt caaagctggcgcaaaagctccaaaaaacgttctggtgcttggctgctcaaatggttacg gcctggcgagccgcattactgctgcgttcggatacggggctgcgaccatcggcgtgtc ctttgaaaaagcgggttcagaaaccaaatatggtacaccgggatggtacaataatttgg catttgatgaagcggcaaaacgcgagggtctttatagcgtgacgatcgacggcgatgc gttttcagacgagatcaaggcccaggtaattgaggaagccaaaaaaaaaggtatcaaa tttgatctgatcgtatacagcttggccagcccagtacgtactgatcctgatacaggtatca tgcacaaaagcgttttgaaacccttggaaaaacgttcacaggcaaaacagtagatccg tttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagcc gccactgttaaagttatgggggtgaagattgggaacgttggattaagcagctgtcgaa ggaaggcctcttagaagaaggctgtattaccttggcctatagttatattggccctgaagc tacccaagctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggc cacagcacaccgtctcaacaaagagaacccgtcaatccgtgccttcgtgagcgtgaat aaaggcctggtaacccgcgcaagcgccgtaatcccggtaatccctctgtatctcgcca gcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtattgaacagatcacg cgtctgtacgccgagcgcctgtaccgtaaagatggtacaattccagttgatgaggaaaa tcgcattcgcattgatgattgggagttagaagaagacgtccagaaagcggtatccgcgt tgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttagcggggtaccg ccatgatttcttagctagtaacggctttgatgtagaaggtattaattatgaagcggaagttg aacgcttcgaccgtatctgataagaaggagatatacatatgagagaagtagtaattgcc agtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagtttcagcggt agagttaggggtaacagcagctaaagaagctataaaaagagctaacataactccagat atgatagatgaatctcttttaggggagtacttacagcaggtcttggacaaaatatagca agacaaatagcattaggagcaggaataccagtagaaaaaccagctatgactataaata tagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatg ctgatataatgttagttggtggagctgaaaacatgagtatgtctccttatttagtaccaagt gcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaatgataaaagatgga ttatcagacatatttaataactatcacatgggtattactgctgaaaacatagcagagcaat ggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaa aaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgttataaaaggaaga aaaggtgacactgtagtagataaagatgaatatattaagcctggcactacaatggagaa acttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatca ggaataaatgatggtgctgctatgttagtagtaatggctaaagaaaaagctgaagaact aggaatagagcctcttgcaactatagtttcttatggaacagctggtgttgaccctaaaata atgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgactatt gaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgtagctgtaataa gagacttaaatatagatatgaataaagttaatgttaatggtggagcaatagctataggac atccaataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaa gagatgctaaaactggtcttgctacactttgtataggcggtggaatgggaactactttaat agttaagagatagtaagaaggagatatacatatgaaattagctgtaataggtagtggaa ctatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtatgtttaaagagt agaactcaaggtgctatagataaatgtttagctttattagataaaaatttaactaagttagtt	**SEQ ID NO: 164**

	actaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgttagttcaac tactaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaagacatg aatataaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatcttggc aacaaatacttcatcattatctataacagaaatagcttcttctactaagcgcccagataaa gttataggaatgcatttctttaatccagttcctatgatgaaattagttgaagttataagtggt cagttaacatcaaaagttacttttgatacagtatttgaattatctaagagtatcaataaagta ccagtagatgtatctgaatctcctggatttgtagtaaatagaatacttatacctatgataaat gaagctgttggtatatatgcagatggtgttgcaagtaaagaagaaatagatgaagctat gaaattaggagcaaaccatccaatgggaccactagcattaggtgatttaatcggattag atgttgttttagctataatgaacgttttatatactgaatttggagatactaaatatagacctca tccacttttagctaaaatggttagagctaatcaattaggaagaaaaactaagataggattc tatgattataataaataataagaaggagatatacatatgagtacaagtgatgttaaagttta tgagaatgtagctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaa agcccttaatgcaataaattcaaagactttagaagaactttatgaagtatttgtagatatta ataatgatgaaactattgatgttgtaatattgacaggggaaggaaaggcatttgtagctg gagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatctta ggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaa cggatttgctttaggtggaggatgtgaacttgcaatggcatgtgatataagaattgcatct gctaaagctaaatttggtcagccagaagtaactcttggaataactccaggatatggagg aactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacag gtcaagttataaaagctgatgaagctgaaaaaatagggctagtaaatagagtcgttgag ccagacattttaatagaagaagttgagaaattagctaagataatagctaaaaatgctcag cttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactg gaatagatatagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaagga atgtcagctttcgttgaaaagagagaagctaactttataaaagggtaataagaaggaga tatacatatgAGTCAGGCGCTAAAAAATTTACTGACATTGT TAAATCTGGAAAAAATTGAGGAAGGACTCTTTCGCG GCCAGAGTGAAGATTTAGGTTTACGCCAGGTGTTTG GCGGCCAGGTCGTGGGTCAGGCCTTGTATGCTGCAA AAGAGACCGTCCCTGAAGAGCGGCTGGTACATTCGT TTCACAGCTACTTTCTTCGCCCTGGCGATAGTAAGAA GCCGATTATTTATGATGTCGAAACGCTGCGTGACGG TAACAGCTTCAGCGCCCGCCGGGTTGCTGCTATTCA AAACGGCAAACCGATTTTTTATATGACTGCCTCTTTC CAGGCACCAGAAGCGGGTTTCGAACATCAAAAAAC AATGCCGTCCGCGCCAGCGCCTGATGGCCTCCCTTC GGAAACGCAAATCGCCCAATCGCTGGCGCACCTGCT GCCGCCAGTGCTGAAAGATAAATTCATCTGCGATCG TCCGCTGGAAGTCCGTCCGGTGGAGTTTCATAACCC ACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGT GTGGATCCGCGCAAATGGTAGCGTGCCGGATGACCT GCGCGTTCATCAGTATCTGCTCGGTTACGCTTCTGAT CTTAACTTCCTGCCGGTAGCTCTACAGCCGCACGGC ATCGGTTTTCTCGAACCGGGGATTCAGATTGCCACC ATTGACCATTCCATGTGGTTCCATCGCCCGTTTAATT TGAATGAATGGCTGCTGTATAGCGTGGAGAGCACCT CGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGAGT TTTATACCCAAGACGGCGTACTGGTTGCCTCGACCG	

	TTCAGGAAGGGGTGATGCGTAATCACAATtaa	

[01064] In certain constructs, the butyrate gene cassette (e.g., bcd2-etfB3-etfA3-thiA1-hbd- crt2-pbt buk butyrate cassette (pLogic031), and/or ter-thiA1-hbd- crt2-pbt buk butyrate cassette (pLogic046) and/or ter-thiA1-hbd- crt2-tesb butyrate cassette (pLOGIC046-delta pbt.buk/tesB+)) is placed under the control of an RNS-responsive regulatory region, e.g., *norB*. In some embodiments, 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 37 and Table 38 and SEQ ID NO: 167**).

[01065] **Table 37** depicts the 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 NO: 165**). The sequence encoding NsrR is underlined and bolded, and the NsrR binding site, i.e., a regulatory region of *norB* is boxed. **Table 38** depicts the nucleic acid sequence of an exemplary RNS-regulated construct comprising a gene encoding *nsrR*, a regulatory region of *norB*, and a butyrogenic gene cassette (pLogic046-nsrR-norB-butyrate construct; **SEQ ID NO: 166**). The sequence encoding NsrR is underlined and bolded, and the NsrR binding site, i.e., a regulatory region of *norB* is boxed.

[01066] **. Table 39 (SEQ ID NO: 167)** depicts the nucleic acid sequence of an exemplary RNS-regulated construct comprising a gene encoding *nsrR*, a regulatory region of *norB*, and a butyrogenic gene cassette (**pLOGIC046-delta pbt.buk/tesB+**-nsrR-norB-butyrate construct (**SEQ ID NO: 167).**

[01067] 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: 165, 166, 167**, or a functional fragment thereof.

Table 37. Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct

Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 165)
ttatta**tcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgtt gagcaggtcttgcagcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgcc gagtatgcccgtcagccggcaggacggcgtaatcaggcattcgttgttcgggcccataca**

Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 165)

ctcgaccagctgcatcggttcgaggtggcggacgaccgcgccgatattgatgcgttcggg
cggcgcggccagcctcagcccgccgcctttcccgcgtacgctgtgcaagaacccgccttt
gaccagcgcggtaaccactttcatcaaatggcttttggaaatgccgtaggtcgaggcgat
ggtggcgatattgaccagcgcgtcgtcgttgacggcggtgtagatgaggacgcgcagccc
gtagtcggtatgttgggtcagatacatacaacctccttagtacatgcaaaattatttcta
gagcaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagttgagtt
gaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggttgttga
caattaatcatcggctcgtataatgtataacattcatattttgtgaattttaaactctag
aaataattttgtttaactttaagaaggagatatacatatggatttaaattctaaaaaata
tcagatgcttaaagagctatatgtaagcttcgctgaaaatgaagttaaacctttagcaac
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tacattagcacactatggtgcagatgaggtaatagtagtagatgatgaagctttagcagt
gtatacaactgaaccatatacaaaagcagcttatgaagcaataaaagcagctgaccctat
agttgtattatttggtgcaacttcaataggtagagatttagcgcctagagtttctgctag

Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 165)
aatacatacaggtcttactgctgactgtacaggtcttgcagtagctgaagatacaaaatt
attattaatgacaagacctgcctttggtggaaatataatggcaacaatagtttgtaaaga
tttcagacctcaaatgtctacagttagaccaggggttatgaagaaaaatgaacctgatga
aactaaagaagctgtaattaaccgtttcaaggtagaatttaatgatgctgataaattagt
tcaagttgtacaagtaataaaagaagctaaaaaacaagttaaaatagaagatgctaagat
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tggttctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatgctga
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gaatataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaa
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agttactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgttagttc
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cttggcaacaaatacttcatcattatctataacagaaatagcttcttctactaagcgccc
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tataagtggtcagttaacatcaaaagttacttttgatacagtatttgaattatctaagag
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aatagatgaagctatgaaattaggagcaaaccatccaatgggaccactagcattaggtga
tttaatcggattagatgttgttttagctataatgaacgttttatatactgaatttggaga
tactaaatatagacctcatccacttttagctaaaatggttagagctaatcaattaggaag
aaaaactaagataggattctatgattataataaataataagaaggagatatacatatgag
tacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatggaaatatatgtac

Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 165)

agtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagactttagaagaact
ttatgaagtatttgtagatattaataatgatgaaactattgatgttgtaatattgacagg
ggaaggaaaggcatttgtagctggagcagatattgcatacatgaaagatttagatgctgt
agctgctaaagattttagtatcttaggagcaaaagcttttggagaaatagaaaatagtaa
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tggaataactccaggatatggaggaactcaaaggcttacaagattggttggaatggcaaa
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gctagtaaatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaa
gataatagctaaaaatgctcagcttgcagttagatactctaaagaagcaatacaacttgg
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ttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactt
tataaaagggtaataagaaggagatatacatatgagaagttttgaagaagtaattaagtt
tgcaaaagaaagaggacctaaaactatatcagtagcatgttgccaagataaagaagtttt
aatggcagttgaaatggctagaaaagaaaaaatagcaaatgccattttagtaggagatat
agaaaagactaaagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgat
agatataaaagatttagcagaagcatctctaaaatctgttgaattagtttcacaaggaaa
agccgacatggtaatgaaaggcttagtagacacatcaataatactaaaagcagttttaaa
taaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttgatgtaga
gggatatgatagattatttttcgtaactgacgcagctatgaacttagctcctgatacaaa
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accaaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagt
tgaagctaaagaactagaagaaatgtatgaaagaggagaaatcaaaggttgtatggttgg
tgggccttttgcaattgataatgcagtatctttagaagcagctaaacataaaggtataaa
tcatcctgtagcaggacgagctgatatattattagccccagatattgaaggtggtaacat
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agctttaggtgttttaatggcagcaaaggcataataagaaggagatatacatatgagcaa
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tgagaaggtgtctgaccaatttgaatttcgtaaacaagtaatagaagaagctctaaaaga
aggtggagtaaaaacatctgaattagatgctgtagtaggtagaggaggacttcttaaacc
tataaaaggtggtacttattcagtaagtgctgctatgattgaagatttaaaagtgggagt
tttaggagaacacgcttcaaacctaggtggaataatagcaaaacaaataggtgaagaagt
aaatgttccttcatacatagtagaccctgttgttgtagatgaattagaagatgttgctag
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tgcaaacgcattagatggagaaggacctttctctccagaaagaagtggtggactaccagt
aggtgcattagtaaaaatgtgctttagtggaaaatatactcaagatgaaattaaaaagaa
aataaaaggtaatggcggactagttgcatacttaaacactaatgatgctagagaagttga
agaaagaattgaagctggtgatgaaaaagctaaattagtatatgaagctatggcatatca
aatctctaaagaaataggagctagtgctgcagttcttaagggagatgtaaaagcaatatt
attaactggtggaatcgcatattcaaaaatgtttacagaaatgattgcagatagagttaa
atttatagcagatgtaaaagtttatccaggtgaagatgaaatgattgcattagctcaagg
tggacttagagttttaactggtgaagaagaggctcaagtttatgataactaataa

Table 38. pLogic046-nsrR-norB-butyrate construct

Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 166)
ttatta**tcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgtt gagcaggtcttgcagcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgcc gagtatgcccgtcagccggcaggacggcgtaatcaggcattcgttgttcgggcccataca ctcgaccagctgcatcggttcgaggtggcggacgaccgcgccgatattgatgcgttcggg cggcgcggccagcctcagcccgccgcctttcccgcgtacgctgtgcaagaacccgccttt gaccagcgcggtaaccactttcatcaaatggcttttggaaatgccgtaggtcgaggcgat ggtggcgatattgaccagcgcgtcgtcgttgacggcggtgtagatgaggacgcgcagccc gtagtcggtatgttgggtcagatacat**acaacctccttagtacatgcaaaattatttcta gagcaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagttgagtt gaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggttgttga caattaatcatcggctcgtataatgtataacattcatattttgtgaattttaaactctag aaataatttttgtttaactttaagaaggagatatacatatgatcgtaaaacctatggtacg caacaatatctgcctgaacgcccatcctcagggctgcaagaagggagtggaagatcagat tgaatataccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaaa cgttctggtgcttggctgctcaaatggttacggcctggcgagccgcattactgctgcgtt cggatacggggctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaata tggtacaccgggatggtacaataatttggcatttgatgaagcggcaaaacgcgagggtct ttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaaggcccaggtaattga ggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatacagcttggccagcccagt acgtactgatcctgatacaggtatcatgcacaaaagcgttttgaaacccttggaaaaac gttcacaggcaaaacagtagatccgtttactggcgagctgaaggaaatctccgcggaacc agcaaatgacgaggaagcagccgccactgttaaagttatgggggtgaagattgggaacg ttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggccta tagttatattggccctgaagctacccaagctttgtaccgtaaaggcacaatcggcaaggc caaagaacacctggaggccacagcacaccgtctcaacaaagagaacccgtcaatccgtgc cttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggtaatccc tctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtat tgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggtacaattccagt tgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtccagaaagc ggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttagc ggggtaccgccatgatttcttagctagtaacggctttgatgtagaaggtattaattatga agcggaagttgaacgcttcgaccgtatctgataagaaggagatatacatatgagagaagt agtaattgccagtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagt ttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagctaacataac tccagatatgatagatgaatctcttttaggggagtacttacagcaggtcttggacaaaa tatagcaagacaaatagcattaggagcaggaataccagtagaaaaaccagctatgactat aaatatagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcatt aggtgatgctgatataatgttagttggtggagctgaaaacatgagtatgtctccttattt agtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaatgat aaaagatggattatcagacatatttaataactatcacatgggtattactgctgaaaacat agcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaa taaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgttat aaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactac aatggagaaacttgctaagttaagacctgcatttaaaaaagatggaacagttactgctgg

Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 166)
taatgcatcaggaataaatgatggtgctgctatgttagtagtaatggctaaagaaaaagc
tgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctggtgttga
ccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaa
tatgactattgaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgt
agctgtaataagagacttaaatatagatatgaataaagttaatgttaatggtggagcaat
agctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatga
aatgaagagaagagatgctaaaactggtcttgctacactttgtataggcggtggaatggg
aactactttaatagttaagagatagtaagaaggagatatacatatgaaattagctgtaat
aggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgt
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tttaactaagttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaag
tcatgttagttcaactactaattatgaagatttaaaagatatggatttaataatagaagc
atctgtagaagacatgaatataaagaaagatgttttcaagttactagatgaattatgtaa
agaagatactatcttggcaacaaatacttcatcattatctataacagaaatagcttcttc
tactaagcgcccagataaagttataggaatgcatttctttaatccagttcctatgatgaa
attagttgaagttataagtggtcagttaacatcaaaagttacttttgatacagtatttga
attatctaagagtatcaataaagtaccagtagatgtatctgaatctcctggatttgtagt
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aagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaatgggaccact
agcattaggtgatttaatcggattagatgttgttttagctataatgaacgttttatatac
tgaatttggagatactaaatatagacctcatccacttttagctaaaatggttagagctaa
tcaattaggaagaaaaactaagataggattctatgattataataaataataagaaggaga
tatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatgg
aaatatatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagac
tttagaagaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgt
aatattgacaggggaaggaaaggcatttgtagctggagcagatattgcatacatgaaaga
tttagatgctgtagctgctaaagattttagtatcttaggagcaaaagcttttggagaaat
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tgaacttgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggtcagcc
agaagtaactcttggaataactccaggatatggaggaactcaaaggcttacaagattggt
tggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagc
tgaaaaaatagggctagtaaatagagtcgttgagccagacattttaatagaagaagttga
gaaattagctaagataatagctaaaaatgctcagcttgcagttagatactctaaagaagc
aatacaacttggtgctcaaactgatataaatactggaatagatatagaatctaatttatt
tggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagag
agaagctaactttataaaagggtaataagaaggagatatacatatgagaagttttgaaga
agtaattaagtttgcaaaagaaagaggacctaaaactatatcagtagcatgttgccaaga
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ttatgaactgatagatataaaagatttagcagaagcatctctaaaatctgttgaattagt
ttcacaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaa
agcagttttaaataaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagt
atttgatgtagagggatatgatagattatttttcgtaactgacgcagctatgaacttagc
tcctgatacaaatactaaaaagcaaatcatagaaaatgcttgcacagtagcacattcatt
agatataagtgaaccaaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaat
gaaagatacagttgaagctaaagaactagaagaaatgtatgaaagaggagaaatcaaagg
ttgtatggttggtgggccttttgcaattgataatgcagtatctttagaagcagctaaaca

Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 166)
taaaggtataaatcatcctgtagcaggacgagctgatatattattagccccagatattga aggtggtaacatattatataaagctttggtattcttctcaaaatcaaaaaatgcaggagt tatagttggggctaaagcaccaataatattaacttctagagcagacagtgaagaaactaa actaaactcaatagctttaggtgttttaatggcagcaaaggcataataagaaggagatat acatatgagcaaaatatttaaaatcttaacaataaatcctggttcgacatcaactaaaat agctgtatttgataatgaggatttagtatttgaaaaaactttaagacattcttcagaaga aataggaaaatatgagaaggtgtctgaccaatttgaatttcgtaaacaagtaatagaaga agctctaaaagaaggtggagtaaaaacatctgaattagatgctgtagtaggtagaggagg acttcttaaacctataaaaggtggtacttattcagtaagtgctgctatgattgaagattt aaaagtgggagttttaggagaacacgcttcaaacctaggtggaataatagcaaaacaaat aggtgaagaagtaaatgttccttcatacatagtagaccctgttgttgtagatgaattaga agatgttgctagaatttctggtatgcctgaaataagtagagcaagtgtagtacatgcttt aaatcaaaaggcaatagcaagaagatatgctagagaaataaacaagaaatatgaagatat aaatcttatagttgcacacatgggtggaggagtttctgttggagctcataaaaatggtaa aatagtagatgttgcaaacgcattagatggagaaggacctttctctccagaaagaagtgg tggactaccagtaggtgcattagtaaaaatgtgctttagtggaaaatatactcaagatga aattaaaaagaaaataaaaggtaatggcggactagttgcatacttaaacactaatgatgc tagagaagttgaagaaagaattgaagctggtgatgaaaaagctaaattagtatatgaagc tatggcatatcaaatctctaaagaaataggagctagtgctgcagttcttaagggagatgt aaaagcaatattattaactggtggaatcgcatattcaaaaatgtttacagaaatgattgc agatagagttaaatttatagcagatgtaaaagtttatccaggtgaagatgaaatgattgc attagctcaaggtggacttagagttttaactggtgaagaagaggctcaagtttatgataa ctaataa

Table 39. pLOGIC046-delta pbt.buk/tesB+ -nsrR-norB-butyrate construct

pLOGIC046-delta pbt.buk/tesB+-nsrR-norB-butyrate construct SEQ ID NO: 167
ttattatcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgtt gagcaggtcttgcagcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgcc gagtatgcccgtcagccggcaggacggcgtaatcaggcattcgttgttcgggcccataca ctcgaccagctgcatcggttcgaggtggcggacgaccgcgccgatattgatgcgttcggg cggcgcggccagcctcagcccgccgcctttcccgcgtacgctgtgcaagaacccgccttt gaccagcgcggtaaccactttcatcaaatggcttttggaaatgccgtaggtcgaggcgat ggtggcgatattgaccagcgcgtcgtcgttgacggcggtgtagatgaggacgcgcagccc gtagtcggtatgttgggtcagatacat**acaacctccttagtacatgcaaaattatttcta** **gagcaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagttgagtt** **gaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggttgttga** **caattaatcatcggctcgtataatgtataacattcatattttgtgaattttaaactctag** **aaataattttgtttaactttaagaaggagatatacat**atgatcgtaaaacctatggtacg caacaatatctgcctgaacgcccatcctcagggctgcaagaagggagtggaagatcagat tgaatataccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaaa cgttctggtgcttggctgctcaaatggttacggcctggcgagccgcattactgctgcgtt cggatacggggctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaata tggtacaccgggatggtacaataatttggcatttgatgaagcggcaaaacgcgagggtct ttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaaggcccaggtaattga ggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatacagcttggccagcccagt

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agcaaatgacgaggaagcagccgccactgttaaagttatgggggtgaagattgggaacg
ttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggccta
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caaagaacacctggaggccacagcacaccgtctcaacaaagagaacccgtcaatccgtgc
cttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggtaatccc
tctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtat
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tgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtccagaaagc
ggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttagc
ggggtaccgccatgatttcttagctagtaacggctttgatgtagaaggtattaattatga
agcggaagttgaacgcttcgaccgtatctgataagaaggagatatacatatgagagaagt
agtaattgccagtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagt
ttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagctaacataac
tccagatatgatagatgaatctcttttaggggagtacttacagcaggtcttggacaaaa
tatagcaagacaaatagcattaggagcaggaataccagtagaaaaaccagctatgactat
aaatatagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcatt
aggtgatgctgatataatgttagttggtggagctgaaaacatgagtatgtctccttattt
agtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaatgat
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agcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaa
taaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgttat
aaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactac
aatggagaaacttgctaagttaagacctgcatttaaaaaagatggaacagttactgctgg
taatgcatcaggaataaatgatggtgctgctatgttagtagtaatggctaaagaaaaagc
tgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctggtgttga
ccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaa
tatgactattgaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgt
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agctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatga
aatgaagagaagagatgctaaaactggtcttgctacactttgtataggcggtggaatggg
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aggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgt
atgtttaaagagtagaactcaaggtgctatagataaatgtttagctttattagataaaaa
tttaactaagttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaag
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agaagatactatcttggcaacaaatacttcatcattatctataacagaaatagcttcttc
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attatctaagagtatcaataaagtaccagtagatgtatctgaatctcctggatttgtagt
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tcaattaggaagaaaaactaagataggattctatgattataataaataataagaaggaga
tatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatgg
aaatatatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagac

```
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aatattgacaggggaaggaaaggcatttgtagctggagcagatattgcatacatgaaaga
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agaagtaactcttggaataactccaggatatggaggaactcaaaggcttacaagattggt
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aatacaacttggtgctcaaactgatataaatactggaatagatatagaatctaatttatt
tggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagag
agaagctaactttataaaagggtaataagaaggagatatacatatgAGTCAGGCGCTAAA
AAATTTACTGACATTGTTAAATCTGGAAAAAATTGAGGAAGGACTCTTTCGCGGCCAGAG
TGAAGATTTAGGTTTACGCCAGGTGTTTGGCGGCCAGGTCGTGGGTCAGGCCTTGTATGC
TGCAAAAGAGACCGTCCCTGAAGAGCGGCTGGTACATTCGTTTCACAGCTACTTTCTTCG
CCCTGGCGATAGTAAGAAGCCGATTATTTATGATGTCGAAACGCTGCGTGACGGTAACAG
CTTCAGCGCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCGATTTTTTATATGACTGC
CTCTTTCCAGGCACCAGAAGCGGGTTTCGAACATCAAAAAACAATGCCGTCCGCGCCAGC
GCCTGATGGCCTCCCTTCGGAAACGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCC
AGTGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCGTCCGGTGGAGTTTCA
TAACCCACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTGGATCCGCGCAAATGG
TAGCGTGCCGGATGACCTGCGCGTTCATCAGTATCTGCTCGGTTACGCTTCTGATCTTAA
CTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCTCGAACCGGGGATTCAGAT
TGCCACCATTGACCATTCCATGTGGTTCCATCGCCCGTTTAATTTGAATGAATGGCTGCT
GTATAGCGTGGAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGAGTTTTA
TACCCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGGAAGGGGTGATGCGTAATCACAA
Ttaa
```

[01068] In certain constructs, the butyrate gene cassette(e.g., bcd2-etfB3-etfA3-thiA1-hbd- crt2-pbt buk butyrate cassette (pLogic031), and/or ter-thiA1-hbd- crt2-pbt buk butyrate cassette (pLogic046) and/or ter-thiA1-hbd- crt2-tesb butyrate cassette (pLOGIC046-delta pbt.buk/tesB+)) is placed under the control of an ROS-responsive regulatory region, e.g., *oxyS*. In certain constructs, the butyrate gene cassette (e.g., bcd2-etfB3-etfA3-thiA1-hbd- crt2-pbt buk butyrate cassette (pLogic031), and/or ter-thiA1-hbd-crt2-pbt buk butyrate cassette (pLogic046) and/or ter-thiA1-hbd- crt2-tesb butyrate cassette (pLOGIC046-delta pbt.buk/tesB+)) 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., the tables and elsewhere herein).

[01069] Nucleic acid sequences of exemplary ROS-regulated constructs comprising an *oxyS* promoter are shown in **Table 40** and **Table 41 and Table 43.**. The

nucleic acid sequence of an exemplary construct encoding OxyR is shown in **Table 42**. **Table 40** depicts the nucleic acid sequence of an exemplary ROS-regulated construct comprising an *oxyS* promoter and a butyrogenic gene cassette (pLogic031-oxyS-butyrate construct; **SEQ ID NO: 168**). **Table 41** depicts the nucleic acid sequence of an exemplary ROS-regulated construct comprising an *oxyS* promoter and a butyrogenic gene cassette (pLogic046-oxyS-butyrate construct; **SEQ ID NO: 169**). **Table 42** depicts the nucleic acid sequence of an exemplary construct encoding OxyR (pZA22-oxyR construct; **SEQ ID NO: 170**). **Table 43** depicts the nucleic acid sequence of an exemplary ROS-regulated construct comprising an *oxyS* promoter and a butyrogenic gene cassette (pLOGIC046-delta pbt.buk/tesB+ -oxyS-butyrate construct; **SEQ ID NO: 171**).

[01070] 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: 168, 169, 170, or 171**, or a functional fragment thereof.

Table 40. pLogic031-oxyS-butyrate construct (SEQ ID NO: 168)

Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 168)
ctcgagttcattatccatcctccatcgccacgatagttcatggcgataggtagaatagcaatgaacgatt
atccctatcaagcattctgactgataattgctcacacgaattcattaaagaggagaaaggtaccatggat
ttaaattctaaaaaatatcagatgcttaaagagctatatgtaagcttcgctgaaaatgaagttaaaccttt
tagcaacagaacttgatgaagaagaaagatttccttatgaaacagtggaaaaaatggcaaaagcaggaat
gatgggtataccatatccaaaagaatatggtggagaaggtggagacactgtaggatatataatggcagtt
gaagaattgtctagagtttgtggtactacaggagttatattatcagctcatacatctcttggctcatggc
ctatatatcaatatggtaatgaagaacaaaaacaaaaattcttaagaccactagcaagtggagaaaaatt
aggagcatttggtcttactgagcctaatgctggtacagatgcgtctggccaacaaacaactgctgtttta
gacggggatgaatacatacttaatggctcaaaaatatttataacaaacgcaatagctggtgacatatatg
tagtaatggcaatgactgataaatctaaggggaacaaaggaatatcagcatttatagttgaaaaaggaac
tcctgggtttagctttggagttaaagaaaagaaaatgggtataagaggttcagctacgagtgaattaata
tttgaggattgcagaatacctaaagaaaatttacttggaaaagaaggtcaaggatttaagatagcaatgt
ctactcttgatggtggtagaattggtatagctgcacaagctttaggtttagcacaaggtgctcttgatga
aactgttaaatatgtaaaagaaagagtacaatttggtagaccattatcaaaattccaaaatacacaattc
caattagctgatatggaagttaaggtacaagcggctagacaccttgtatatcaagcagctataaataaag
acttaggaaaaccttatggagtagaagcagcaatggcaaaattatttgcagctgaaacagctatggaagt
tactacaaaagctgtacaacttcatggaggatatggatacactcgtgactatccagtagaaagaatgatg
agagatgctaagataactgaaatatatgaaggaactagtgaagttcaaagaatggttatttcaggaaaac
tattaaaatagtaagaaggagatatacatatggaggaaggatttatgaatatagtcgtttgtataaaaca
agttccagatacaacagaagttaaactagatcctaatacaggtactttaattagagatggagtaccaagt
ataataaaccctgatgataaagcaggtttagaagaagctataaaattaaaagaagaaatgggtgctcatg
taactgttataacaatgggacctcctcaagcagatatggctttaaaagaagctttagcaatgggtgcaga
tagaggtatattattaacagatagagcatttgcgggtgctgatacttgggcaacttcatcagcattagca
ggagcattaaaaaatatagattttgatattataatagctggaagacaggcgatagatggagatactgcac
aagttggacctcaaatagctgaacatttaaatcttccatcaataacatatgctgaagaaataaaaactga
aggtgaatatgtattagtaaaaagacaatttgaagattgttgccatgacttaaaagttaaaatgccatgc
cttataacaactcttaaagatatgaacacaccaagatacatgaaagttggaagaatatatgatgctttcg
aaaatgatgtagtagaaacatggactgtaaaagatatagaagttgacccttctaatttaggtcttaaagg

Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 168)

ttctccaactagtgtatttaaatcatttacaaaatcagttaaaccagctggtacaatatacaatgaagat
gcgaaaacatcagctggaattatcatagataaattaaaagagaagtatatcatataataagaaggagata
tacatatgggtaacgttttagtagtaatagaacaaagagaaaatgtaattcaaactgtttctttagaatt
actaggaaaggctacagaaatagcaaaagattatgatacaaaagtttctgcattacttttaggtagtaag
gtagaaggtttaatagatacattagcacactatggtgcagatgaggtaatagtagtagatgatgaagctt
tagcagtgtatacaactgaaccatatacaaaagcagcttatgaagcaataaaagcagctgaccctatagt
tgtattatttggtgcaacttcaataggtagagatttagcgcctagagtttctgctagaatacatacaggt
cttactgctgactgtacaggtcttgcagtagctgaagatacaaaattattattaatgacaagacctgcct
ttggtggaaatataatggcaacaatagtttgtaaagatttcagacctcaaatgtctacagttagaccagg
ggttatgaagaaaaatgaacctgatgaaactaaagaagctgtaattaaccgtttcaaggtagaatttaat
gatgctgataaattagttcaagttgtacaagtaataaaagaagctaaaaaacaagttaaaatagaagatg
ctaagatattagtttctgctggacgtggaatgggtggaaaagaaaacttagacatactttatgaattagc
tgaaattataggtggagaagtttctggttctcgtgccactatagatgcaggttggttagataaagcaaga
caagttggtcaaactggtaaaactgtaagaccagacctttatatagcatgtggtatatctggagcaatac
aacatatagctggtatggaagatgctgagtttatagttgctataaataaaaatccagaagctccaatatt
taaatatgctgatgttggtatagttggagatgttcataaagtgcttccagaacttatcagtcagttaagt
gttgcaaaagaaaaaggtgaagttttagctaactaataagaaggagatatacatatgagagaagtagtaa
ttgccagtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagtttcagcggtagagtt
aggggtaacagcagctaaagaagctataaaaagagctaacataactccagatatgatagatgaatctctt
ttaggggggagtacttacagcaggtcttggacaaaatatagcaagacaaatagcattaggagcaggaatac
cagtagaaaaaccagctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatc
tcaacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatgagtatgtctcct
tatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaatgataaaag
atggattatcagacatatttaataactatcacatgggtattactgctgaaaacatagcagagcaatggaa
tataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaaagctcaagctgaa
ggaaaatttgatgaagaaatagttcctgttgttataaaaggaagaaaaggtgacactgtagtagataaag
atgaatatattaagcctggcactacaatggagaaacttgctaagttaagacctgcatttaaaaaagatgg
aacagttactgctggtaatgcatcaggaataaatgatggtgctgctatgttagtagtaatggctaaagaa
aaagctgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctggtgttgacccta
aaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgactattgaaga
tatagatttagttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaatata
gatatgaataaagttaatgttaatggtggagcaatagctataggacatccaataggatgctcaggagcaa
gaatacttactacacttttatatgaaatgaagagaagagatgctaaaactggtcttgctacactttgtat
aggcggtggaatgggaactactttaatagttaagagatagtaagaaggagatatacatatgaaattagct
gtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtatgtt
taaagagtagaactcaaggtgctatagataaatgtttagctttattagataaaaatttaactaagttagt
tactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgttagttcaactactaattat
gaagatttaaaagatatggatttaataatagaagcatctgtagaagacatgaatataaagaaagatgttt
tcaagttactagatgaattatgtaaagaagatactatcttggcaacaaatacttcatcattatctataac
agaaatagcttcttctactaagcgcccagataaagttataggaatgcatttctttaatccagttcctatg
atgaaattagttgaagttataagtggtcagttaacatcaaaagttacttttgatacagtatttgaattat
ctaagagtatcaataaagtaccagtagatgtatctgaatctcctggatttgtagtaaatagaatacttat
acctatgataaatgaagctgttggtatatatgcagatggtgttgcaagtaaagaagaaatagatgaagct
atgaaattaggagcaaaccatccaatgggaccactagcattaggtgatttaatcggattagatgttgttt
tagctataatgaacgttttatatactgaatttggagatactaaatatagacctcatccacttttagctaa
aatggttagagctaatcaattaggaagaaaaactaagataggattctatgattataataaataataagaa
ggagatatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtagatggaaata
tatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagactttagaagaacttta
tgaagtatttgtagatattaataatgatgaaactattgatgttgtaatattgacaggggaaggaaaggca
tttgtagctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatct
taggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgc
tttaggtggaggatgtgaacttgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggt
cagccagaagtaactcttggaataactccaggatatggaggaactcaaaggcttacaagattggttggaa
tggcaaaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagctgaaaaaatagggct
agtaaatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaagataatagctaaa
aatgctcagcttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactg

Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 168)
gaatagatatagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagc tttcgttgaaaagagagaagctaactttataaaagggtaataagaaggagatatacatatgagaagtttt gaagaagtaattaagtttgcaaaagaaagaggacctaaaactatatcagtagcatgttgccaagataaag aagttttaatggcagttgaaatggctagaaaagaaaaaatagcaaatgccattttagtaggagatataga aaagactaaagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgatagatataaaagat ttagcagaagcatctctaaaatctgttgaattagtttcacaaggaaaagccgacatggtaatgaaaggct tagtagacacatcaataatactaaaagcagttttaaataaagaagtaggtcttagaactggaaatgtatt aagtcacgtagcagtatttgatgtagagggatatgatagattatttttcgtaactgacgcagctatgaac ttagctcctgatacaaatactaaaaagcaaatcatagaaaatgcttgcacagtagcacattcattagata taagtgaaccaaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaatgaaagatacagttga agctaaagaactagaagaaatgtatgaaagaggagaaatcaaaggttgtatggttggtgggccttttgca attgataatgcagtatctttagaagcagctaaacataaaggtataaatcatcctgtagcaggacgagctg atatattattagccccagatattgaaggtggtaacatattatataaagctttggtattcttctcaaaatc aaaaaatgcaggagttatagttggggctaaagcaccaataatattaacttctagagcagacagtgaagaa actaaactaaactcaatagctttaggtgttttaatggcagcaaaggcataataagaaggagatatacata tgagcaaaatatttaaaatcttaacaataaatcctggttcgacatcaactaaaatagctgtatttgataa tgaggatttagtatttgaaaaaactttaagacattcttcagaagaaataggaaaatatgagaaggtgtct gaccaatttgaatttcgtaaacaagtaatagaagaagctctaaaagaaggtggagtaaaaacatctgaat tagatgctgtagtaggtagaggaggacttcttaaacctataaaaggtggtacttattcagtaagtgctgc tatgattgaagatttaaaagtgggagttttaggagaacacgcttcaaacctaggtggaataatagcaaaa caaataggtgaagaagtaaatgttccttcatacatagtagaccctgttgttgtagatgaattagaagatg ttgctagaatttctggtatgcctgaaataagtagagcaagtgtagtacatgctttaaatcaaaaggcaat agcaagaagatatgctagagaaataaacaagaaatatgaagatataaatcttatagttgcacacatgggt ggaggagtttctgttggagctcataaaaatggtaaaatagtagatgttgcaaacgcattagatggagaag gacctttctctccagaaagaagtggtggactaccagtaggtgcattagtaaaaatgtgctttagtggaaa atatactcaagatgaaattaaaaagaaaataaaaggtaatggcggactagttgcatacttaaacactaat gatgctagagaagttgaagaaagaattgaagctggtgatgaaaaagctaaattagtatatgaagctatgg catatcaaatctctaaagaaataggagctagtgctgcagttcttaagggagatgtaaaagcaatattatt aactggtggaatcgcatattcaaaaatgtttacagaaatgattgcagatagagttaaatttatagcagat gtaaaagtttatccaggtgaagatgaaatgattgcattagctcaaggtggacttagagttttaactggtg aagaagaggctcaagtttatgataactaataa

Table 41. pLogic046-oxyS-butyrate construct (SEQ ID NO: 169)

Nucleotide sequences of pLogic046-oxyS-butyrate construct (SEQ ID NO: 169)
ctcgagttcattatccatcctccatcgccacgatagttcatggcgataggtagaatagcaatgaacgatt atccctatcaagcattctgactgataattgctcacacgaattcattaaagaggagaaaggtaccatgatc gtaaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggctgcaagaagggagtggaag atcagattgaatataccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaaacgt tctggtgcttggctgctcaaatggttacggcctggcgagccgcattactgctgcgttcggatacggggct gcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaatatggtacaccgggatggtacaata atttggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgatcgacggcgatgcgttttc agacgagatcaaggcccaggtaattgaggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatac agcttggccagcccagtacgtactgatcctgatacaggtatcatgcacaaaagcgttttgaaaccctttg gaaaaacgttcacaggcaaaacagtagatccgtttactggcgagctgaaggaaatctccgcggaaccagc aaatgacgaggaagcagccgccactgttaaagttatgggggtgaagattgggaacgttggattaagcag ctgtcgaaggaaggcctcttagaagaaggctgtattaccttggcctatagttatattggccctgaagcta cccaagctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagcacaccgtct caacaaagagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgcc gtaatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaag gttgtattgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggtacaattccagttga tgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtccagaaagcggtatccgcgttg atggagaaagtcacgggtgaaaacgcagaatctctcactgacttagcggggtaccgccatgatttcttag ctagtaacggctttgatgtagaaggtattaattatgaagcggaagttgaacgcttcgaccgtatctgata agaaggagatatacatatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttgga

Nucleotide sequences of pLogic046-oxyS-butyrate construct (SEQ ID NO: 169)

ggagcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagcta
acataactccagatatgatagatgaatctcttttaggggagtacttacagcaggtcttggacaaaatat
agcaagacaaatagcattaggagcaggaataccagtagaaaaaccagctatgactataaatatagtttgt
ggttctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatgctgatataatgttag
ttggtggagctgaaaacatgagtatgtctccttatttagtaccaagtgcgagatatggtgcaagaatggg
tgatgctgcttttgttgattcaatgataaaagatggattatcagacatatttaataactatcacatgggt
attactgctgaaaacatagcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaa
gtcaaaataaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttgttataaa
aggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactacaatggagaaactt
gctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaataaatgatg
gtgctgctatgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactat
agtttcttatggaacagctggtgttgaccctaaaataatgggatatggaccagttccagcaactaaaaaa
gctttagaagctgctaatatgactattgaagatatagatttagttgaagctaatgaggcatttgctgccc
aatctgtagctgtaataagagacttaaatatagatatgaataaagttaatgttaatggtggagcaatagc
tataggacatccaataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaaga
gatgctaaaactggtcttgctacactttgtataggcggtggaatgggaactactttaatagttaagagat
agtaagaaggagatatacatatgaaattagctgtaataggtagtggaactatgggaagtggtattgtaca
aacttttgcaagttgtggacatgatgtatgtttaaagagtagaactcaaggtgctatagataaatgtttta
gctttattagataaaaatttaactaagttagttactaagggaaaaatggatgaagctacaaaagcagaaa
tattaagtcatgttagttcaactactaattatgaagatttaaaagatatggatttaataatagaagcatc
tgtagaagacatgaatataaagaaagatgttttcaagttactagatgaattatgtaaagaagatactatc
ttggcaacaaatacttcatcattatctataacagaaatagcttcttctactaagcgcccagataaagtta
taggaatgcatttctttaatccagttcctatgatgaaattagttgaagttataagtggtcagttaacatc
aaaagttacttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtatctgaa
tctcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatatatgcagatg
gtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaatgggaccactagc
attaggtgatttaatcggattagatgttgttttagctataatgaacgttttatatactgaatttggagat
actaaatatagacctcatccacttttagctaaaatggttagagctaatcaattaggaagaaaaactaaga
taggattctatgattataataaataataagaaggagatatacatatgagtacaagtgatgttaaagttta
tgagaatgtagctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaagcccttaat
gcaataaattcaaagactttagaagaactttatgaagtatttgtagatattaataatgatgaaactattg
atgttgtaatattgacaggggaaggaaaggcatttgtagctggagcagatattgcatacatgaaagattt
agatgctgtagctgctaaagattttagtatcttaggagcaaaagcttttggagaaatagaaaatagtaaa
aaagtagtgatagctgctgtaaacggatttgctttaggtggaggatgtgaacttgcaatggcatgtgata
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aggaactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacaggtcaagtt
ataaaagctgatgaagctgaaaaaatagggctagtaaatagagtcgttgagccagacattttaatagaag
aagttgagaaattagctaagataatagctaaaaatgctcagcttgcagttagatactctaaagaagcaat
acaacttggtgctcaaactgatataaatactggaatagatatagaatctaatttatttggtctttgtttt
tcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactttataaaagggt
aataagaaggagatatacatatgagaagttttgaagaagtaattaagtttgcaaaagaaagaggacctaa
aactatatcagtagcatgttgccaagataaagaagttttaatggcagttgaaatggctagaaaagaaaaa
atagcaaatgccattttagtaggagatatagaaaagactaaagaaattgcaaaaagcatagacatggata
tcgaaaattatgaactgatagatataaaagatttagcagaagcatctctaaaatctgttgaattagtttc
acaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaaagcagttttaaat
aaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttgatgtagagggatatgata
gattatttttcgtaactgacgcagctatgaacttagctcctgatacaaatactaaaaagcaaatcataga
aaatgcttgcacagtagcacattcattagatataagtgaaccaaaagttgctgcaatatgcgcaaaagaa
aaagtaaatccaaaaatgaaagatacagttgaagctaaagaactagaagaaatgtatgaaagaggagaaa
tcaaaggttgtatggttggtgggccttttgcaattgataatgcagtatctttagaagcagctaaacataa
aggtataaatcatcctgtagcaggacgagctgatatattattagccccagatattgaaggtggtaacata
ttatataaagctttggtattcttctcaaaatcaaaaaatgcaggagttatagttggggctaaagcaccaa
taatattaacttctagagcagacagtgaagaaactaaactaaactcaatagctttaggtgttttaatggc
agcaaaggcataataagaaggagatatacatatgagcaaaatatttaaaatcttaacaataaatcctggt
tcgacatcaactaaaatagctgtatttgataatgaggatttagtatttgaaaaaactttaagacattctt
cagaagaaataggaaaatatgagaaggtgtctgaccaatttgaatttcgtaaacaagtaatagaagaagc
tctaaaagaaggtggagtaaaaacatctgaattagatgctgtagtaggtagaggaggacttcttaaacct

Nucleotide sequences of pLogic046-oxyS-butyrate construct (SEQ ID NO: 169)
ataaaaggtggtacttattcagtaagtgctgctatgattgaagatttaaaagtgggagttttaggagaac acgcttcaaacctaggtggaataatagcaaaacaaataggtgaagaagtaaatgttccttcatacatagt agaccctgttgttgtagatgaattagaagatgttgctagaatttctggtatgcctgaaataagtagagca agtgtagtacatgctttaaatcaaaaggcaatagcaagaagatatgctagagaaataaacaagaaatatg aagatataaatcttatagttgcacacatgggtggaggagtttctgttggagctcataaaaatggtaaaat agtagatgttgcaaacgcattagatggagaaggacctttctctccagaaagaagtggtggactaccagta ggtgcattagtaaaaatgtgctttagtggaaaatatactcaagatgaaattaaaaagaaaataaaaggta atggcggactagttgcatacttaaacactaatgatgctagagaagttgaagaaagaattgaagctggtga tgaaaaagctaaattagtatatgaagctatggcatatcaaatctctaaagaaataggagctagtgctgca gttcttaagggagatgtaaaagcaatattattaactggtggaatcgcatattcaaaaatgtttacagaaa tgattgcagatagagttaaatttatagcagatgtaaaagtttatccaggtgaagatgaaatgattgcatt agctcaaggtggacttagagttttaactggtgaagaagaggctcaagtttatgataactaataa

Table 42. pZA22-oxyR construct (SEQ ID NO: 170)

Nucleotide sequences of pZA22-oxyR construct (SEQ ID NO: 170)
ctcgagatgctagcaattgtgagcggataacaattgacattgtgagcggataacaagatactgagcacat cagcaggacgcactgaccttaattaaaagaattcattaaagaggagaaaggtaccatgaatattcgtgat cttgagtacctggtggcattggctgaacaccgccattttcggcgtgcggcagattcctgccacgttagcc agccgacgcttagcgggcaaattcgtaagctggaagatgagctgggcgtgatgttgctggagcggaccag ccgtaaagtgttgttcacccaggcgggaatgctgctggtggatcaggcgcgtaccgtgctgcgtgaggtg aaagtccttaaagagatggcaagccagcagggcgagacgatgtccggaccgctgcacattggtttgattc ccacagttggaccgtacctgctaccgcatattatccctatgctgcaccagacctttccaaagctggaaat gtatctgcatgaagcacagacccaccagttactggcgcaactggacagcggcaaactcgattgcgtgatc ctcgcgctggtgaaagagagcgaagcattcattgaagtgccgttgtttgatgagccaatgttgctggcta tctatgaagatcacccgtgggcgaaccgcgaatgcgtaccgatggccgatctggcaggggaaaaactgct gatgctggaagatggtcactgtttgcgcgatcaggcaatgggtttctgttttgaagccggggcggatgaa gatacacacttccgcgcgaccagcctggaaactctgcgcaacatggtggcggcaggtagcgggatcactt tactgccagcgctggctgtgccgccggagcgcaaacgcgatggggttgtttatctgccgtgcattaagcc ggaaccacgccgcactattggcctggtttatcgtcctggctcaccgctgcgcagccgctatgagcagctg gcagaggccatccgcgcaagaatggatggccatttcgataaagttttaaaacaggcggtttaaggatccc atggtacgcgtgctagaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatc tgttgtttgtcggtgaacgctctcctgagtaggacaaatccgccgccctagacctaggggatatattccg cttcctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaaatggcttacgaacgggg cggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggccgcggcaaagccgtttt tccataggctccgccccctgacaagcatcacgaaatctgacgctcaaatcagtggtggcgaaacccgac aggactataaagataccaggcgtttccccctggcggctccctcgtgcgctctcctgttcctgcctttcgg tttaccggtgtcattccgctgttatggccgcgtttgtctcattccacgcctgacactcagttccgggtag gcagttcgctccaagctggactgtatgcacgaacccccccgttcagtccgaccgctgcgccttatccggta actatcgtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatt tagaggagttagtcttgaagtcatgcgccggttaaggctaaactgaaaggacaagttttggtgactgcgc tcctccaagccagttacctcggttcaaagagttggtagctcagagaaccttcgaaaaaccgccctgcaag gcggtttttcgttttcagagcaagagattacgcgcagaccaaaacgatctcaagaagatcatcttatta atcagataaaatatttctagatttcagtgcaatttatctcttcaaatgtagcacctgaagtcagccccat acgatataagttgttactagtgcttggattctcaccaataaaaaacgcccggcggcaaccgagcgttctg aacaaatccagatggagttctgaggtcattactggatctatcaacaggagtccaagcgagctctcgaacc ccagagtcccgctcagaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcggcg ataccgtaaagcacgaggaagcggtcagcccattcgccgccaagctcttcagcaatatcacgggtagcca acgctatgtcctgatagcggtccgccacacccagccggccacagtcgatgaatccagaaaagcggccatt ttccaccatgatattcggcaagcaggcatcgccatgggtcacgacgagatcctcgccgtcgggcatgcgc gccttgagcctggcgaacagttcggctggcgcgagcccctgatgctcttcgtccagatcatcctgatcga caagaccggcttccatccgagtacgtgctcgctcgatgcgatgtttcgcttggtggtcgaatgggcaggt agccggatcaagcgtatgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaagg tgagatgacaggagatcctgccccggcacttcgcccaatagcagccagtcccttcccgcttcagtgacaa cgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgcgctgcctcgtcctgcag ttcattcagggcaccggacaggtcggtcttgacaaaaagaaccgggcgcccctgcgctgacagccggaac

Nucleotide sequences of pZA22-oxyR construct (SEQ ID NO: 170)
acggcggcatcagagcagccgattgtctgttgtgcccagtcatagccgaatagcctctccacccaagcgg ccggagaacctgcgtgcaatccatcttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcaga tcttgatcccctgcgccatcagatccttggcggcaagaaagccatccagtttactttgcagggcttccca accttaccagagggcgccccagctggcaattccgacgtctaagaaaccattattatcatgacattaacct ataaaaataggcgtatcacgaggccctttcgtcttcac

Table 43. pLOGIC046-delta pbt.buk/tesB+ -oxyS-butyrate construct

Nucleotide sequences of pLOGIC046-delta pbt.buk/tesB+ -oxyS-butyrate construct (SEQ ID NO: 171)
Ctcgagttcattatccatcctccatcgccacgatagttcatggcgataggtagaatagcaatgaacgatt atccctatcaagcattctgactgataattgctcacacgaattcattaaagaggagaaaggtacc atgatcgtaaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggct gcaagaagggagtggaagatcagattgaatataccaagaaacgcattaccgcagaagt caaagctggcgcaaaagctccaaaaaacgttctggtgcttggctgctcaaatggttac ggcctggcgagccgcattactgctgcgttcggatacggggctgcgaccatcggcgtgt cctttgaaaaagcgggttcagaaaccaaatatggtacaccgggatggtacaataattt ggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgatcgacggcgat gcgttttcagacgagatcaaggcccaggtaattgaggaagccaaaaaaaaaggtatca aatttgatctgatcgtatacagcttggccagcccagtacgtactgatcctgatacagg tatcatgcacaaaagcgttttgaaacccttggaaaaacgttcacaggcaaaacagta gatccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaag cagccgccactgttaaagttatgggggtgaagattgggaacgttggattaagcagct gtcgaaggaaggcctcttagaagaaggctgtattaccttggcctatagttatattggc cctgaagctacccaagctttgtaccgtaaaggcacaatcggcaaggccaaagaacacc tggaggccacagcacaccgtctcaacaaagagaacccgtcaatccgtgccttcgtgag cgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggtaatccctctgtat ctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtattgaac agatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggtacaattccagttga tgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtccagaaagcg gtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttag cggggtaccgccatgatttcttagctagtaacggctttgatgtagaaggtattaatta tgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatatacatatgaga gaagtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagcattta aatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagc taacataactccagatatgatagatgaatctcttttaggggggagtacttacagcaggt cttggacaaaatatagcaagacaaatagcattaggagcaggaataccagtagaaaaac cagctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatc tcaacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatg agtatgtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctg cttttgttgattcaatgataaaagatggattatcagacatatttaataactatcacat gggtattactgctgaaaacatagcagagcaatggaatataactagagaagaacaagat gaattagctcttgcaagtcaaaataaagctgaaaaagctcaagctgaaggaaaatttg atgaagaaatagttcctgttgttataaaaggaagaaaaggtgacactgtagtagataa agatgaatatattaagcctggcactacaatggagaaacttgctaagttaagacctgca tttaaaaaagatggaacagttactgctggtaatgcatcaggaataaatgatggtgctg

Nucleotide sequences of pLOGIC046-delta pbt.buk/tesB+ -oxyS-butyrate construct

(SEQ ID NO: 171)

ctatgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgc
aactatagtttcttatggaacagctggtgttgaccctaaaataatgggatatggacca
gttccagcaactaaaaaagctttagaagctgctaatatgactattgaagatatagatt
tagttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagacttaaa
tatagatatgaataaagttaatgttaatggtggagcaatagctataggacatccaata
ggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaagagatg
ctaaaactggtcttgctacactttgtataggcggtggaatgggaactactttaatagt
taagagatagtaagaaggagatatacatatgaaattagctgtaataggtagtggaact
atgggaagtggtattgtacaaacttttgcaagttgtggacatgatgtatgtttaaaga
gtagaactcaaggtgctatagataaatgtttagctttattagataaaaatttaactaa
gttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgtt
agttcaactactaattatgaagatttaaaagatatggatttaataatagaagcatctg
tagaagacatgaatataaagaaagatgttttcaagttactagatgaattatgtaaaga
agatactatcttggcaacaaatacttcatcattatctataacagaaatagcttcttct
actaagcgcccagataaagttataggaatgcatttctttaatccagttcctatgatga
aattagttgaagttataagtggtcagttaacatcaaaagttacttttgatacagtatt
tgaattatctaagagtatcaataaagtaccagtagatgtatctgaatctcctggattt
gtagtaaatagaatacttatacctatgataaatgaagctgttggtatatatgcagatg
gtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaat
gggaccactagcattaggtgatttaatcggattagatgttgttttagctataatgaac
gttttatatactgaatttggagatactaaatatagacctcatccacttttagctaaaa
tggttagagctaatcaattaggaagaaaaactaagataggattctatgattataataa
ataataagaaggagatatacatatgagtacaagtgatgttaaagtttatgagaatgta
gctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaagccctta
atgcaataaattcaaagactttagaagaactttatgaagtatttgtagatattaataa
tgatgaaactattgatgttgtaatattgacaggggaaggaaaggcatttgtagctgga
gcagatattgcatacatgaaagatttagatgctgtagctgctaaagattttagtatct
taggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgt
aaacggatttgctttaggtggaggatgtgaacttgcaatggcatgtgatataagaatt
gcatctgctaaagctaaatttggtcagccagaagtaactcttggaataactccaggat
atggaggaactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaat
ctttacaggtcaagttataaaagctgatgaagctgaaaaaatagggctagtaaataga
gtcgttgagccagacattttaatagaagaagttgagaaattagctaagataatagcta
aaaatgctcagcttgcagttagatactctaaagaagcaatacaacttggtgctcaaac
tgatataaatactggaatagatatagaatctaatttatttggtctttgtttttcaact
aaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactttataa
aagggtaataagaaggagatatacatatgAGTCAGGCGCTAAAAAATTTACTGACATT
GTTAAATCTGGAAAAAATTGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTTAGGT
TTACGCCAGGTGTTTGGCGGCCAGGTCGTGGGTCAGGCCTTGTATGCTGCAAAAGAGA
CCGTCCCTGAAGAGCGGCTGGTACATTCGTTTCACAGCTACTTTCTTCGCCCTGGCGA
TAGTAAGAAGCCGATTATTTATGATGTCGAAACGCTGCGTGACGGTAACAGCTTCAGC
GCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCGATTTTTTATATGACTGCCTCTT
TCCAGGCACCAGAAGCGGGTTTCGAACATCAAAAAACAATGCCGTCCGCGCCAGCGCC
TGATGGCCTCCCTTCGGAAACGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCA
GTGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCGTCCGGTGGAGTTTC
ATAACCCACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTGGATCCGCGCAAA

Nucleotide sequences of pLOGIC046-delta pbt.buk/tesB+ -oxyS-butyrate construct (SEQ ID NO: 171)
TGGTAGCGTGCCGGATGACCTGCGCGTTCATCAGTATCTGCTCGGTTACGCTTCTGAT CTTAACTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCTCGAACCGGGGA TTCAGATTGCCACCATTGACCATTCCATGTGGTTCCATCGCCCGTTTAATTTGAATGA ATGGCTGCTGTATAGCGTGGAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGC GGTGAGTTTTATACCCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGGAAGGGGTGA TGCGTAATCACAATtaa

[01071] In some embodiments, the butyrate gene cassette (e.g., bcd2-etfB3-etfA3-thiA1-hbd- crt2-pbt buk butyrate cassette (pLogic031), and/or ter-thiA1-hbd- crt2-pbt buk butyrate cassette (pLogic046) and/or ter-thiA1-hbd- crt2-tesb butyrate cassette (pLOGIC046-delta pbt.buk/tesB+)) is placed under the control of a FNR regulatory region selected from **Table 21 or 22 and SEQ ID NOs: 141-157.** 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.

Example 2. Construction of vectors for overproducing butyrate using an inducible tet promoter- butyrate circuit

[01072] 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. 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.

[01073] 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. For reasone described in Example 1, a second plasmid was generated, in which *bcd2-etfA3-etfB3* was replaced with (trans-2-enoynl-CoA reductase; *ter* from *Treponema denticola* 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.

[01074] A third butyrate gene cassette was further genereated, in which the *pbt* and *buk* genes were replaced with *tesB* (SEQ ID NO: 10). TesB is a thioesterase found in E. Coli that cleaves off the butyrate from butyryl-coA, thus obviating the need for pbt-buk (see Fig. 2). The third butyrate gene cassette, as well as transcriptional and translational elements, is synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322 to create **pLOGIC046-delta pbt.buk/tesB+** (ter-thiA1-hbd- crt2-tesb butyrate cassette, also referred to herein as tesB butyrate cassette).

[01075] As synthesized, the all three butyrate gene cassettes were placed under control of a tetracycline-inducible promoter, with the *tet* repressor (*tetR*) expressed constitutively, divergent from the tet-inducible synthetic butyrate operon.

[01076] Nucleic acid sequences of tetracycline-regulated constructs comprising a *tet* promoter are shown in **Table 44** and **Table 45 and Table 46**. **Table 44** depicts the nucleic acid sequence of an exemplary tetracycline-regulated construct comprising a *tet* promoter and a butyrogenic gene cassette (pLogic031-tet-butyrate construct; SEQ ID NO: 78). The sequence encoding TetR is underlined, and the overlapping tetR/tetA promoters are boxed. **Table 45** depicts the nucleic acid sequence of an exemplary tetracycline-regulated construct comprising a *tet* promoter and a butyrogenic gene cassette (pLogic046-tet-butyrate construct; SEQ ID NO: 79). The sequence encoding TetR is underlined, and the overlapping tetR/tetA promoters are boxed.

[01077] **Table 46** depicts the nucleic acid sequence of an exemplary tetracycline-regulated construct (pLOGIC046-delta pbt.buk/tesB+- tet-butyrate construct) comprising 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 separated by RBS.

[01078] 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: 172, 173, or 174**, or a functional fragment thereof.

Table 44. pLogic031-tet-butyrate construct (SEQ ID NO: 172)

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 172)

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 172)

gtaaaacgacggccagtgaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatg
atcaattcaaggccgaataagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgta
ataatggcggcatactatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttc
caatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaacct
tgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaat
gtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatc
ttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcg
tcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctctacacctagcttctgggcga
gtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttact
tttatctaatctagacatcattaattcctaatttttgttgacactctatcattgatagagttattttacc
actccctatcagtgatagagaaaagtgaactctagaaataattttgtttaactttaagaaggagatatac
atatggatttaaattctaaaaaatatcagatgcttaaagagctatatgtaagcttcgctgaaaatgaagt
taaacctttagcaacagaacttgatgaagaagaaagatttccttatgaaacagtggaaaaaatggcaaaa
gcaggaatgatgggtataccatatccaaaagaatatggtggagaaggtggagacactgtaggatatataa
tggcagttgaagaattgtctagagtttgtggtactacaggagttatattatcagctcatacatctcttgg
ctcatggcctatatatcaatatggtaatgaagaacaaaaacaaaaattcttaagaccactagcaagtgga
gaaaaattaggagcatttggtcttactgagcctaatgctggtacagatgcgtctggccaacaaacaactg
ctgttttagacggggatgaatacatacttaatggctcaaaaatatttataacaaacgcaatagctggtga
catatatgtagtaatggcaatgactgataaatctaaggggaacaaaggaatatcagcatttatagttgaa
aaaggaactcctgggtttagctttggagttaaagaaaagaaaatgggtataagaggttcagctacgagtg
aattaatatttgaggattgcagaatacctaaagaaaatttacttggaaaagaaggtcaaggatttaagat
agcaatgtctactcttgatggtggtagaattggtatagctgcacaagctttaggtttagcacaaggtgct
cttgatgaaactgttaaatatgtaaaagaaagagtacaatttggtagaccattatcaaaattccaaaata
cacaattccaattagctgatatggaagttaaggtacaagcggctagacaccttgtatatcaagcagctat
aaataaagacttaggaaaaccttatggagtagaagcagcaatggcaaaattatttgcagctgaaacagct
atggaagttactacaaaagctgtacaacttcatggaggatatggatacactcgtgactatccagtagaaa
gaatgatgagagatgctaagataactgaaatatatgaaggaactagtgaagttcaaagaatggttatttc
aggaaaactattaaaatagtaagaaggagatatacatatggaggaaggatttatgaatatagtcgtttgt
ataaaacaagttccagatacaacagaagttaaactagatcctaatacaggtactttaattagagatggag
taccaagtataataaaccctgatgataaagcaggtttagaagaagctataaaattaaaagaagaaatggg
tgctcatgtaactgttataacaatgggacctcctcaagcagatatggctttaaaagaagctttagcaatg
ggtgcagatagaggtatattattaacagatagagcatttgcgggtgctgatacttgggcaacttcatcag
cattagcaggagcattaaaaaatatagattttgatattataatagctggaagacaggcgatagatggaga
tactgcacaagttggacctcaaatagctgaacatttaaatcttccatcaataacatatgctgaagaaata
aaaactgaaggtgaatatgtattagtaaaaagacaatttgaagattgttgccatgacttaaaagttaaaa
tgccatgccttataacaactcttaaagatatgaacacaccaagatacatgaaagttggaagaatatatga
tgctttcgaaaatgatgtagtagaaacatggactgtaaaagatatagaagttgacccttctaatttaggt
cttaaaggttctccaactagtgtatttaaatcatttacaaaatcagttaaaccagctggtacaatataca
atgaagatgcgaaaacatcagctggaattatcatagataaattaaaagagaagtatatcatataataaga
aggagatatacatatgggtaacgttttagtagtaatagaacaaagagaaaatgtaattcaaactgtttct
ttagaattactaggaaaggctacagaaatagcaaaagattatgatacaaaagtttctgcattacttttag
gtagtaaggtagaaggtttaatagatacattagcacactatggtgcagatgaggtaatagtagtagatga
tgaagctttagcagtgtatacaactgaaccatatacaaaagcagcttatgaagcaataaaagcagctgac
cctatagttgtattatttggtgcaacttcaataggtagagatttagcgcctagagtttctgctagaatac
atacaggtcttactgctgactgtacaggtcttgcagtagctgaagatacaaaattattattaatgacaag
acctgcctttggtggaaatataatggcaacaatagtttgtaaagatttcagacctcaaatgtctacagtt
agaccaggggttatgaagaaaaatgaacctgatgaaactaaagaagctgtaattaaccgtttcaaggtag
aatttaatgatgctgataaattagttcaagttgtacaagtaataaaagaagctaaaaaacaagttaaaat
agaagatgctaagatattagtttctgctggacgtggaatgggtggaaaagaaaacttagacatactttat
gaattagctgaaattataggtggagaagtttctggttctcgtgccactatagatgcaggttggttagata
aagcaagacaagttggtcaaactggtaaaactgtaagaccagacctttatatagcatgtggtatatctgg
agcaatacaacatatagctggtatggaagatgctgagtttatagttgctataaataaaaatccagaagct
ccaatatttaaatatgctgatgttggtatagttggagatgttcataaagtgcttccagaacttatcagtc
agttaagtgttgcaaaagaaaaaggtgaagttttagctaactaataagaaggagatatacatatgagaga
agtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagcatttaaatcagtttcagcg
gtagagttaggggtaacagcagctaaagaagctataaaaagagctaacataactccagatatgatagatg
aatctcttttaggggggagtacttacagcaggtcttggacaaaatatagcaagacaaatagcattaggagc

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 172)

aggaataccagtagaaaaaccagctatgactataaatatagtttgtggttctggattaagatctgtttca
atggcatctcaacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatgagta
tgtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaat
gataaaagatggattatcagacatatttaataactatcacatgggtattactgctgaaaacatagcagag
caatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaaagctc
aagctgaaggaaaatttgatgaagaaatagttcctgttgttataaaaggaagaaaaggtgacactgtagt
agataaagatgaatatattaagcctggcactacaatggagaaacttgctaagttaagacctgcatttaaa
aaagatggaacagttactgctggtaatgcatcaggaataaatgatggtgctgctatgttagtagtaatgg
ctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctggtgt
tgaccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgact
attgaagatatagatttagttgaagctaatgaggcatttgctgcccaatctgtagctgtaataagagact
taaatatagatatgaataaagttaatgttaatggtggagcaatagctataggacatccaataggatgctc
aggagcaagaatacttactacacttttatatgaaatgaagagaagagatgctaaaactggtcttgctaca
ctttgtataggcggtggaatgggaactactttaatagttaagagatagtaagaaggagatatacatatga
aattagctgtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatga
tgtatgtttaaagagtagaactcaaggtgctatagataaatgtttagctttattagataaaaatttaact
aagttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaagtcatgttagttcaacta
ctaattatgaagatttaaaagatatggatttaataatagaagcatctgtagaagacatgaatataaagaa
agatgttttcaagttactagatgaattatgtaaagaagatactatcttggcaacaaatacttcatcatta
tctataacagaaatagcttcttctactaagcgcccagataaagttataggaatgcatttctttaatccag
ttcctatgatgaaattagttgaagttataagtggtcagttaacatcaaaagttacttttgatacagtatt
tgaattatctaagagtatcaataaagtaccagtagatgtatctgaatctcctggatttgtagtaaataga
atacttatacctatgataaatgaagctgttggtatatatgcagatggtgttgcaagtaaagaagaaatag
atgaagctatgaaattaggagcaaaccatccaatgggaccactagcattaggtgatttaatcggattaga
tgttgttttagctataatgaacgttttatatactgaatttggagatactaaatatagacctcatccactt
ttagctaaaatggttagagctaatcaattaggaagaaaaactaagataggattctatgattataataaat
aataagaaggagatatacatatgagtacaagtgatgttaaagtttatgagaatgtagctgttgaagtaga
tggaaatatatgtacagtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagactttagaa
gaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgtaatattgacaggggaag
gaaaggcatttgtagctggagcagatattgcatacatgaaagatttagatgctgtagctgctaaagattt
tagtatcttaggagcaaaagcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaac
ggatttgctttaggtggaggatgtgaacttgcaatggcatgtgatataagaattgcatctgctaaagcta
aatttggtcagccagaagtaactcttggaataactccaggatatggaggaactcaaaggcttacaagatt
ggttggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaagctgatgaagctgaaaaa
atagggctagtaaatagagtcgttgagccagacattttaatagaagaagttgagaaattagctaagataa
tagctaaaaatgctcagcttgcagttagatactctaaagaagcaatacaacttggtgctcaaactgatat
aaatactggaatagatatagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaagga
atgtcagctttcgttgaaaagagagaagctaactttataaaagggtaataagaaggagatatacatatga
gaagttttgaagaagtaattaagtttgcaaaagaaagaggacctaaaactatatcagtagcatgttgcca
agataaagaagttttaatggcagttgaaatggctagaaaagaaaaaatagcaaatgccattttagtagga
gatatagaaaagactaaagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgatagata
taaaagatttagcagaagcatctctaaaatctgttgaattagtttcacaaggaaaagccgacatggtaat
gaaaggcttagtagacacatcaataatactaaaagcagttttaaataaagaagtaggtcttagaactgga
aatgtattaagtcacgtagcagtatttgatgtagagggatatgatagattatttttcgtaactgacgcag
ctatgaacttagctcctgatacaaatactaaaaagcaaatcatagaaaatgcttgcacagtagcacattc
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acgagctgatatattattagccccagatattgaaggtggtaacatattatataaagctttggtattcttc
tcaaaatcaaaaaatgcaggagttatagttggggctaaagcaccaataatattaacttctagagcagaca
gtgaagaaactaaactaaactcaatagctttaggtgttttaatggcagcaaaggcataataagaaggaga
tatacatatgagcaaaatatttaaaatcttaacaataaatcctggttcgacatcaactaaaatagctgta
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aggtgtctgaccaatttgaatttcgtaaacaagtaatagaagaagctctaaaagaaggtggagtaaaaac
atctgaattagatgctgtagtaggtagaggaggacttcttaaacctataaaaggtggtacttattcagta
agtgctgctatgattgaagatttaaaagtgggagttttaggagaacacgcttcaaacctaggtggaataa
tagcaaaacaaataggtgaagaagtaaatgttccttcatacatagtagaccctgttgttgtagatgaatt

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 172)
agaagatgttgctagaatttctggtatgcctgaaataagtagagcaagtgtagtacatgctttaaatcaa aaggcaatagcaagaagatatgctagagaaataaacaagaaatatgaagatataaatcttatagttgcac acatgggtggaggagtttctgttggagctcataaaaatggtaaaatagtagatgttgcaaacgcattaga tggagaaggacctttctctccagaaagaagtggtggactaccagtaggtgcattagtaaaaatgtgcttt agtggaaaatatactcaagatgaaattaaaaagaaaataaaaggtaatggcggactagttgcatacttaa acactaatgatgctagagaagttgaagaaagaattgaagctggtgatgaaaaagctaaattagtatatga agctatggcatatcaaatctctaaagaaataggagctagtgctgcagttcttaagggagatgtaaaagca atattattaactggtggaatcgcatattcaaaaatgtttacagaaatgattgcagatagagttaaattta tagcagatgtaaaagtttatccaggtgaagatgaaatgattgcattagctcaaggtggacttagagtttt aactggtgaagaagaggctcaagtttatgataactaataa

Table 45. pLogic046-tet-butyrate construct (SEQ ID NO: 173)

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 173)
gtaaaacgacggccagtgaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatg atcaattcaaggccgaataagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgta ataatggcggcatactatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttc caatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaacct tgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaat gtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatc ttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcg tcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctctacacctagcttctgggcga gtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttact tttatctaatctagacatcattaattcctaatttttgttgacactctatcattgatagagttattttacc actccctatcagtgatagagaaaagtgaactctagaaataattttgtttaactttaagaaggagatatac atatgatcgtaaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggctgcaagaaggg agtggaagatcagattgaatataccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctcca aaaaacgttctggtgcttggctgctcaaatggttacggcctggcgagccgcattactgctgcgttcggat acggggctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaatatggtacaccgggatg gtacaataatttggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgatcgacggcgat gcgttttcagacgagatcaaggcccaggtaattgaggaagccaaaaaaaaaggtatcaaatttgatctga tcgtatacagcttggccagcccagtacgtactgatcctgatacaggtatcatgcacaaaagcgttttgaa acccttttggaaaaacgttcacaggcaaaacagtagatccgtttactggcgagctgaaggaaatctccgcg gaaccagcaaatgacgaggaagcagccgccactgttaaagttatgggggggtgaagattgggaacgttgga ttaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggcctatagttatattggccc tgaagctacccaagctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagca caccgtctcaacaaagagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaacccgcg caagcgccgtaatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaa tcatgaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggtacaatt ccagttgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtccagaaagcggtat ccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttagcggggtaccgccatga tttcttagctagtaacggctttgatgtagaaggtattaattatgaagcggaagttgaacgcttcgaccgt atctgataagaaggagatatacatatgagagaagtagtaattgccagtgcagctagaacagcagtaggaa gttttggaggagcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaa aagagctaacataactccagatatgatagatgaatctcttttaggggagtacttacagcaggtcttgga caaaatatagcaagacaaatagcattaggagcaggaataccagtagaaaaaccagctatgactataaata tagtttgtggttctggattaagatctgtttcaatggcatctcaacttatagcattaggtgatgctgatat aatgttagttggtggagctgaaaacatgagtatgtctccttatttagtaccaagtgcgagatatggtgca agaatgggtgatgctgcttttgttgattcaatgataaaagatggattatcagacatatttaataactatc acatgggtattactgctgaaaacatagcagagcaatggaatataactagagaagaacaagatgaattagc tcttgcaagtcaaaataaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgtt gttataaaaggaagaaaaggtgacactgtagtagataaagatgaatatattaagcctggcactacaatgg agaaacttgctaagttaagacctgcatttaaaaaagatggaacagttactgctggtaatgcatcaggaat aaatgatggtgctgctatgttagtagtaatggctaaagaaaaagctgaagaactaggaatagagcctctt gcaactatagtttcttatggaacagctggtgttgaccctaaaataatgggatatggaccagttccagcaa

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 173)

ctaaaaaagctttagaagctgctaatatgactattgaagatatagatttagttgaagctaatgaggcatt
tgctgcccaatctgtagctgtaataagagacttaaatatagatatgaataaagttaatgttaatggtgga
gcaatagctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatgaaatga
agagaagagatgctaaaactggtcttgctacactttgtataggcggtggaatgggaactactttaatagt
taagagatagtaagaaggagatatacatatgaaattagctgtaataggtagtggaactatgggaagtggt
attgtacaaacttttgcaagttgtggacatgatgtatgtttaaagagtagaactcaaggtgctatagata
aatgtttagctttattagataaaaatttaactaagttagttactaagggaaaaatggatgaagctacaaa
agcagaaatattaagtcatgttagttcaactactaattatgaagatttaaaagatatggatttaataata
gaagcatctgtagaagacatgaatataaagaaagatgttttcaagttactagatgaattatgtaaagaag
atactatcttggcaacaaatacttcatcattatctataacagaaatagcttcttctactaagcgcccaga
taaagttataggaatgcatttctttaatccagttcctatgatgaaattagttgaagttataagtggtcag
ttaacatcaaaagttacttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatg
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tgcagatggtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaatggga
ccactagcattaggtgatttaatcggattagatgttgttttagctataatgaacgttttatatactgaat
ttggagatactaaatatagacctcatccacttttagctaaaatggttagagctaatcaattaggaagaaa
aactaagataggattctatgattataataaataataagaaggagatatacatatgagtacaagtgatgtt
aaagtttatgagaatgtagctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaag
cccttaatgcaataaattcaaagactttagaagaactttatgaagtatttgtagatattaataatgatga
aactattgatgttgtaatattgacaggggaaggaaaggcatttgtagctggagcagatattgcatacatg
aaagatttagatgctgtagctgctaaagattttagtatcttaggagcaaaagcttttggagaaatagaaa
atagtaaaaaagtagtgatagctgctgtaaacggatttgctttaggtggaggatgtgaacttgcaatggc
atgtgatataagaattgcatctgctaaagctaaatttggtcagccagaagtaactcttggaataactcca
ggatatggaggaactcaaaggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacag
gtcaagttataaaagctgatgaagctgaaaaaatagggctagtaaatagagtcgttgagccagacatttt
aatagaagaagttgagaaattagctaagataatagctaaaaatgctcagcttgcagttagatactctaaa
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tttgtttttcaactaaagaccaaaaagaaggaatgtcagctttcgttgaaaagagagaagctaactttat
aaaagggtaataagaaggagatatacatatgagaagttttgaagaagtaattaagtttgcaaaagaaaga
ggacctaaaactatatcagtagcatgttgccaagataaagaagttttaatggcagttgaaatggctagaa
aagaaaaaatagcaaatgccattttagtaggagatatagaaaagactaaagaaattgcaaaaagcataga
catggatatcgaaaattatgaactgatagatataaaagatttagcagaagcatctctaaaatctgttgaa
ttagtttcacaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaaagcag
ttttaaataaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagtatttgatgtagaggg
atatgatagattatttttcgtaactgacgcagctatgaacttagctcctgatacaaatactaaaaagcaa
atcatagaaaatgcttgcacagtagcacattcattagatataagtgaaccaaaagttgctgcaatatgcg
caaaagaaaaagtaaatccaaaaatgaaagatacagttgaagctaaagaactagaagaaatgtatgaaag
aggagaaatcaaaggttgtatggttggtgggccttttgcaattgataatgcagtatctttagaagcagct
aaacataaaggtataaatcatcctgtagcaggacgagctgatatattattagccccagatattgaaggtg
gtaacatattatataaagctttggtattcttctcaaaatcaaaaaatgcaggagttatagttggggctaa
agcaccaataatattaacttctagagcagacagtgaagaaactaaactaaactcaatagctttaggtgtt
ttaatggcagcaaaggcataataagaaggagatatacatatgagcaaaatatttaaaatcttaacaataa
atcctggttcgacatcaactaaaatagctgtatttgataatgaggatttagtatttgaaaaaactttaag
acattcttcagaagaaataggaaaatatgagaaggtgtctgaccaatttgaatttcgtaaacaagtaata
gaagaagctctaaaagaaggtggagtaaaaacatctgaattagatgctgtagtaggtagaggaggacttc
ttaaacctataaaaggtggtacttattcagtaagtgctgctatgattgaagatttaaaagtgggagtttt
aggagaacacgcttcaaacctaggtggaataatagcaaaacaaataggtgaagaagtaaatgttccttca
tacatagtagaccctgttgttgtagatgaattagaagatgttgctagaatttctggtatgcctgaaataa
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gaaatatgaagatataaatcttatagttgcacacatgggtggaggagtttctgttggagctcataaaaat
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taccagtaggtgcattagtaaaaatgtgctttagtggaaaatatactcaagatgaaattaaaaagaaaat
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gctggtgatgaaaaagctaaattagtatatgaagctatggcatatcaaatctctaaagaaataggagcta
gtgctgcagttcttaagggagatgtaaaagcaatattattaactggtggaatcgcatattcaaaaatgtt
tacagaaatgattgcagatagagttaaatttatagcagatgtaaaagtttatccaggtgaagatgaaatg
attgcattagctcaaggtggacttagagttttaactggtgaagaagaggctcaagtttatgataactaat

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 173)
aa

Table 46. pLOGIC046-delta pbt.buk/tesB+- tet-butyrate construct (**SEQ ID NO: 174**)

SEQ ID NO: 174
gtaaaacgacggccagtgaattcgttaagacccactttcacatttaagttgtttttctaa tccgcatatgatcaattcaaggccgaataagaaggctggctctgcaccttggtgatcaaa taattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttc ttctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccac agcgctgagtgcatataatgcattctctagtgaaaaaccttgttggcataaaaaggctaa ttgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtacttttgc tccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatc ttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaat ggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctc tacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaag cagctctaatgcgctgttaatcactttacttttatctaatctagacat**cattaattccta** **atttttgttgacactctatcattgatagagttattttaccactccctatcagtgatagag** **aaaagtgaactctagaaataattttgtttaactttaagaaggagatatacat**atgatcgt aaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggctgcaagaaggg agtggaagatcagattgaatataccaagaaacgcattaccgcagaagtcaaagctggcgc aaaagctccaaaaaacgttctggtgcttggctgctcaaatggttacggcctggcgagccg cattactgctgcgttcggatacggggctgcgaccatcggcgtgtcctttgaaaaagcggg ttcagaaaccaaatatggtacaccgggatggtacaataatttggcatttgatgaagcggc aaaacgcgagggtctttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaa ggcccaggtaattgaggaagccaaaaaaaaaggtatcaaatttgatctgatcgtatacag cttggccagcccagtacgtactgatcctgatacaggtatcatgcacaaaagcgttttgaa acccttggaaaaacgttcacaggcaaaacagtagatccgtttactggcgagctgaagga aatctccgcggaaccagcaaatgacgaggaagcagccgccactgttaaagttatgggggg tgaagattgggaacgttggattaagcagctgtcgaaggaaggcctcttagaagaaggctg tattaccttggcctatagttatattggccctgaagctacccaagctttgtaccgtaaagg cacaatcggcaaggccaaagaacacctggaggccacagcacaccgtctcaacaaagagaa cccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgt aatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaa tcatgaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaaga tggtacaattccagttgatgaggaaaatcgcattcgcattgatgattgggagttagaaga agacgtccagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatc tctcactgacttagcggggtaccgccatgatttcttagctagtaacggctttgatgtaga aggtattaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatat acatatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggagg agcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaa aagagctaacataactccagatatgatagatgaatctcttttaggggagtacttacagc aggtcttggacaaaatatagcaagacaaatagcattaggagcaggaataccagtagaaaa accagctatgactataaatatagtttgtggttctggattaagatctgtttcaatggcatc tcaacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacatgag tatgtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttt tgttgattcaatgataaaagatggattatcagacatatttaataactatcacatgggtat tactgctgaaaacatagcagagcaatggaatataactagagaagaacaagatgaattagc

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ggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatagtttcttatgg
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tgctgcccaatctgtagctgtaataagagacttaaatatagatatgaataaagttaatgt
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tacacttttatatgaaatgaagagaagagatgctaaaactggtcttgctacactttgtat
aggcggtggaatgggaactactttaatagttaagagatagtaagaaggagatatacatat
gaaattagctgtaataggtagtggaactatgggaagtggtattgtacaaacttttgcaag
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agcagaaatattaagtcatgttagttcaactactaattatgaagatttaaaagatatgga
tttaataatagaagcatctgtagaagacatgaatataaagaaagatgttttcaagttact
agatgaattatgtaaagaagatactatcttggcaacaaatacttcatcattatctataac
agaaatagcttcttctactaagcgcccagataaagttataggaatgcatttctttaatcc
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tgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtatctgaatc
tcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatata
tgcagatggtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaaacca
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aatggttagagctaatcaattaggaagaaaaactaagataggattctatgattataataa
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aactattgatgttgtaatattgacaggggaaggaaaggcatttgtagctggagcagatat
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taaatttggtcagccagaagtaactcttggaataactccaggatatggaggaactcaaag
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aatagaagaagttgagaaattagctaagataatagctaaaaatgctcagcttgcagttag
atactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaatagatat
agaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtcagc
tttcgttgaaaagagagaagctaactttataaaagggtaataagaaggagatatacatat
gAGTCAGGCGCTAAAAAATTTACTGACATTGTTAAATCTGGAAAAAATTGAGGAAGGACT
CTTTCGCGGCCAGAGTGAAGATTTAGGTTTACGCCAGGTGTTTGGCGGCCAGGTCGTGGG
TCAGGCCTTGTATGCTGCAAAAGAGACCGTCCCTGAAGAGCGGCTGGTACATTCGTTTCA
CAGCTACTTTCTTCGCCCTGGCGATAGTAAGAAGCCGATTATTTATGATGTCGAAACGCT
GCGTGACGGTAACAGCTTCAGCGCCCGCCGGGTTGCTGCTATTCAAAACGGCAAACCGAT
TTTTTATATGACTGCCTCTTTCCAGGCACCAGAAGCGGGTTTCGAACATCAAAAAACAAT
GCCGTCCGCGCCAGCGCCTGATGGCCTCCCTTCGGAAACGCAAATCGCCCAATCGCTGGC
GCACCTGCTGCCGCCAGTGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCG
TCCGGTGGAGTTTCATAACCCACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTG
GATCCGCGCAAATGGTAGCGTGCCGGATGACCTGCGCGTTCATCAGTATCTGCTCGGTTA

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CGCTTCTGATCTTAACTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCTCGA
ACCGGGGATTCAGATTGCCACCATTGACCATTCCATGTGGTTCCATCGCCCGTTTAATTT
GAATGAATGGCTGCTGTATAGCGTGGAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGT
GCGCGGTGAGTTTTATACCCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGGAAGGGGT
GATGCGTAATCACAATtaa
```

Butyrate, IL-10, IL-22, GLP-2

[01079] In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce one or more molecules selected from IL-10, IL-2, IL-22, IL-27, SOD, kyurenine, kyurenic acid, and GLP-2 using the methods described above. In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding IL-10 (see, e.g., **SEQ ID NO: 134**, **SEQ ID NO: 193, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 194**). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding IL-2 (see, e.g., **SEQ ID NO: 135**). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding IL-22 (see, e.g., **SEQ ID NO: 136, SEQ ID NO: 195, SEQ ID NO: 196**). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding IL-27 (see, e.g., **SEQ ID NO: 137**). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding SOD (see, e.g., **SEQ ID NO: 138**). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding GLP-2 (see, e.g., **SEQ ID NO: 139, SEQ ID NO: 140, SEQ ID NO: 136189, SEQ ID NO: 190, SEQ ID NO: 192**). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene or gene cassette for producing kyurenine or kyurenic acid. In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding IL-10, IL-22, and GLP-2. In one embodiment, each of the genes or gene cassettes is placed under the control of a FNR regulatory region selected from **SEQ ID NO: 141 therough SEQ ID NO: 157** (**Table 21** and **Table 22**). In an alternate embodiment, each of the genes or gene cassettes 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 27** and elsewhere herein). In yet another embodiment, each of the genes or gene cassettes

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 28** and **Table 29** and elsewhere herein). In certain constructs, one or more of the genes is placed under the control of a tetracycline-inducible or constitutive promoter.

Butyrate, Propionate, IL-10, IL-22, IL-2, IL-27

[01080] In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce propionate, and one or more molecules selected from IL-10, IL-2, IL-22, IL-27, SOD, kyurenine, kyurenic acid, and GLP-2 using the methods described above. In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce propionate, and one or more molecules selected from IL-10, IL-2, and IL-22. In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce propionate, and one or more molecules selected from IL-10, IL-2, and IL-27. In some embodiments, the genetically engineered bacteria further comprise acrylate pathway genes for propionate biosynthesis, *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*. In an alternate embodiment, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, and *lpd*. In another alternate embodiment, the genetically engineered bacteria comprise $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, *lpd*, and *tesB*.

[01081] The bacteria comprise a gene cassette for producing butyrate as described above, a gene cassette for producing propionate as described above, a gene encoding IL-10 (see, e.g., 49), a gene encoding IL-27 (see, e.g., **SEQ ID NO: 137**), a gene encoding IL-22 (see, e.g., **SEQ ID NO: 136, SEQ ID NO: 195, SEQ ID NO: 196**), and a gene encoding IL-2 (see, e.g., **SEQ ID NO: 135**). In one embodiment, each of the genes or gene cassettes is placed under the control of a FNR regulatory region selected from **SEQ ID NOs: 141-157** (**Table 21 and 22**). In an alternate embodiment, each of the genes or gene cassettes 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 23**). In yet another embodiment, each of the genes or gene cassettes 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 24** and elsewhere herein). In certain constructs, one or more of the genes is placed under the control of a tetracycline-inducible or constitutive promoter.

Butyrate, Propionate, IL-10, L-22, SOD, GLP-2, kynurenine

[01082] In certain constructs, in addition to the butyrate production pathways described above, the Escherichia *coli* Nissle are further engineered to produce one or more molecules selected from IL-10, IL-22, SOD, GLP-2, and kynurenine using the methods described above. In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce propionate, and one or more molecules selected from IL-10, IL-22, SOD, GLP-2, and kynurenine using the methods described above. In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce IL-10, IL-27, IL-22, SOD, GLP-2, and kynurenine using the methods described above. In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce propionate, IL-10, IL-27, IL-22, SOD, GLP-2, and kynurenine using the methods described above. In some embodiments, the genetically engineered bacteria further comprise acrylate pathway genes for propionate biosynthesis, *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*. In an alternate embodiment, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, and *lpd*. In another alternate embodiment, the genetically engineered bacteria comprise $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, *lpd*, and *tesB*.

[01083] The bacteria comprise a gene cassette for producing butyrate as described above, a gene cassette for producing propionate as described above, a gene encoding IL-10 (see, e.g., **SEQ ID NO: 134**), a gene encoding IL-22 (see, e.g., **SEQ ID NO: 136, SEQ ID NO: 195, SEQ ID NO: 196**), a gene encoding SOD (see, e.g., **SEQ ID NO: 138**), a gene encoding GLP-2 or a GLP-2 analog or GLP-2 polypppetide (see, e.g., **SEQ ID NO: 139**, **SEQ ID NO:140, SEQ ID NO:189, SEQ ID NO:190, SEQ ID NO: 192**), and a gene or gene cassette for producing kynurenine . In one embodiment, each of the genes or gene cassettes is placed under the control of a FNR regulatory region selected from **SEQ ID NO: 141 though SEQ ID NO: 157** (**Table 21 and Table 22**). In an

alternate embodiment, each of the genes or gene cassettes 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 23** and elsewhere herein). In yet another embodiment, each of the genes or gene cassettes 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 2**4 and **Table 25** and elsewhere herein). In certain constructs, one or more of the genes is placed under the control of a tetracycline-inducible or constitutive promoter.

Butyrate, Propionate, IL-10, IL-27, IL-22, IL-2, SOD, GLP-2, kynurenine

[01084] In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce one or more molecules selected from IL-10, IL-27, IL-22, IL-2, SOD, GLP-2, and kynurenine using the methods described above. In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce propionate and one or more molecules selected from IL-10, IL-27, IL-22, IL-2, SOD, GLP-2, and kynurenine using the methods described above. In certain constructs, in addition to the butyrate production pathways described above, the *Escherichia coli* Nissle are further engineered to produce IL-10, IL-27, IL-22, SOD, GLP-2, and kynurenine using the methods described above. In some embodiments, the genetically engineered bacteria further comprise acrylate pathway genes for propionate biosynthesis, *pct*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*. In an alternate embodiment, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, and *lpd*. In another alternate embodiment, the genetically engineered bacteria comprise $thrA^{fbr}$, *thrB*, *thrC*, $ilvA^{fbr}$, *aceE*, *aceF*, *lpd*, and *tesB*.

[01085] The bacteria comprise a gene cassette for producing butyrate as described above, a gene cassette for producing propionate as described above, a gene encoding IL-10 (see, e.g., **SEQ ID NO: 134, SEQ ID NO: 193, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 194**), a gene encoding IL-27 (see, e.g., **SEQ ID NO: 137**), a gene encoding IL-22 (see, e.g., SEQ ID NO: 51), a gene encoding IL-2 (see, e.g., SEQ ID NO: 50), a gene encoding SOD (see, e.g., SEQ ID NO: 53), a gene encoding GLP-2 (see,

e.g., SEQ ID NO: 54), and a gene or gene cassette for producing kynurenine . In one embodiment, each of the genes or gene cassettes is placed under the control of a FNR regulatory region selected from **SEQ ID NO: 141 through SEQ ID NO: 157** (**Table 21** and **Table 22**). In an alternate embodiment, each of the genes or gene cassettes 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 23** and **Table 24** and elsewhere herein). In yet another embodiment, each of the genes or gene cassettes 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 24** and **Table 25** and elsewhere herein). In certain constructs, one or more of the genes is placed under the control of a tetracycline-inducible or constitutive promoter.

[01086] In some embodiments, bacterial genes 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, as shown in **Table 33**.

Example 3. Transforming *E. coli*

[01087] 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.

[01088] 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. Organization of the constructs and nucleotide sequences are shown in **FIG. 2.** 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 4. Production of Butyrate in Recombinant E. coli using tet-inducible promoter

[01089] Production of butyrate was assessed in *E. coli* Nissle strains containing butyrate cassettes described above in order to determine the effect of oxygen on butyrate production. 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.

[01090] 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.

[01091] **FIG. 11** depicts bar graphs of butyrate production using the different butyrate-producing circuits shown in **FIG. 2**.

[01092] **FIG. 11A** 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).

Example 5. Tet-driven and RNS driven in vitro Butyrate Production in Recombinant E. coli

[01093] All incubations were performed at 37°C. Lysogeny broth (LB)-grown overnight cultures of *E. coli* Nissle transformed with pLogic031 or pLogic046 were subcultured 1:100 into 10mL of M9 minimal medium containing 0.5% 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. 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 ((0 up to 24 hours, as shown in **FIG. 21**) to assess levels of butyrate production by LC-MS. As seen in **FIG. 21** butyrate production is greater in the strain comprising the pLogic046 construct than the strain comprising the pLogic031 construct.

[01094] Production of butyrate was also assessed in *E. coli* Nissle strains containing the butyrate cassettes driven by an RNS promoter described above (pLogic031-nsrR-norB-butyrate operon construct and pLogic046-nsrR-norB-butyrate operon construct) in order to determine the effect of nitrogen on butyrate production. 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 (0 up to 24 hours, as shown in **FIG. 22**) to assess levels of butyrate production. As seen in **FIG. 22**, genetically engineered Nissle comprising pLogic031-nsrR-norB-butyrate operon construct) or (pLogic046-nsrR-norB-butyrate operon construct) produced significantly more butyrate as compared to wild-type Nissle.

Example 6. In vitro Production of butyrate in Recombinant *E. coli* using an Inducible tet Promoter Butyrate Circuit

[01095] 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. To test whether Preventing the coupling of NADH oxidation to electron transport would allow increased butyrate production, NuoB mutants having NuoB deletion were obtained.

[01096] 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. 13**, which depicts a graph of butyrate production using different butyrate-producing circuits comprising a *nuoB* gene deletion. **FIG. 13** shows the BW25113 strain of E. Coli, which is a common cloning strain and the background of the KEIO collection of E. Coli mutants. **FIG. 13** shows that compared with wild-type Nissle, deletion of NuoB results in greater production of butyrate.

Example 7. Production of Butyrate in Recombinant E. coli

[01097] In vitro production of butyrate under the control of a tetracycline promoter was compared between (1) Butyrate gene cassette (pLOGIC046- ter-thiA1-hbd-

crt2-pbt buk butyrate) and (2) butyrate cassette in which the *pbt* and *buk* genes were placed with *tesB* (pLOGIC046-deltapbt-buk/tesB+-butyrate; SEQ ID NO: 56).

[01098] 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. As shown in **FIG. 11B**, replacement of pbt and buk with tesB leads to greater levels of butyrate production.

Example 8. Construction of vectors for overproducing butyrate (FNR driven)

[01099] The three butyrate cassettes decribed in Example 1 (see, e.g., **Table 36**, **SEQ ID NO: 163**, **SEQ ID NO: 164, SEQ ID NO: 165**) are placed under the control of a FNR regulatory region selected from (**SEQ ID NO: 141 through SEQ ID NO: 157**) (**Table 21 and Table 22**) 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. In certain embodiments, a ydfZ promoter was used. In other embodiments, a FNRS promoter is used.

Example 9. FNR and RNS driven in vitro Production of Butyrate in Recombinant E. coli

[01100] Production of butyrate is assessed in *E. coli* Nissle strains containing the butyrate cassettes described above driven by an FNR promoter 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.

[01101] In an alternate embodiment, production of butyrate is assessed in *E. coli* Nissle strains containing the butyrate cassettes described above driven by an RNS promoter in order to determine the effect of nitrogen on butyrate production. Overnight bacterial cultures are 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) is added to cultures at a final concentration of 0.3mM to induce expression from plasmid. After 2 hours of induction, cells are spun down, supernatant is discarded, and the cells are resuspended in M9 minimal media containing 0.5% glucose. Culture supernatant is then analyzed at indicated time points to assess levels of butyrate production.

Example 10. Production of Butyrate in Recombinant E. coli

[01102] The effect of oxygen and glucose on FNR promoter driven butyrate production was compared between *E. coli* Nissle strains SYN501(comprises pSC101 PydfZ-ter butyrate plasmid, i.e., (ter-thiA1-hbd-crt2-pbt-buk genes under the control of a ydfZ promoter) SYN-UCD500 (comprises pSC101 PydfZ-bcd butyrate plasmid, i.e, bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt, and buk under control of the ydfZ promoter) and SYN-UCD506 (comprises pSC101 nirB-bcd butyrate plasmid, i.e., i.e, bcd2, etfB3, etfA3, thiA1, hbd, crt2, pbt, and buk under control of the nirB promoter.

[01103] All incubations were performed at 37° C. Cultures of *E. coli* Nissle strains transformed with the butyrate cassettes were 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 anaerobically in a Coy anaerobic chamber (supplying 90% N_2, 5% CO_2, 5%H_2) for 4 hours. Cells were washed and resuspended in minimal media w/ 0.5% glucose and incubated microaerobically to monitor butyrate production over time. One aliquot was removed at each time point (2, 8, and 24 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. As seen in **FIG. 14B**, SYN-501 led to significant butyrate production under anaerobic conditions..

[01104] 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: 175, 176, 177, or 178**, or a functional fragment thereof.

Table 47. ydfZ-butyrate cassettes

Description	Sequence	SEQ ID NO
YdfZ promoter	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTT CCCCCGACTTATGGCTCATGCATGCATCAAAAAAG ATGTGAGCTTGATCAAAAACAAAAAATATTTCACTC GACAGGAGTATTTATATTGCGCCCGGATCCCTCTAG AAATAATTTTGTTTAACTTTAAGAAGGAGATATACA T	**SEQ ID NO: 175**
YdfZ- bcd2-etfB3-etfA3-thiA1-hb-crt2-pbt- buk butyrate cassette	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTT CCCCCGACTTATGGCTCATGCATGCATCAAAAAAG ATGTGAGCTTGATCAAAAACAAAAAATATTTCACTC GACAGGAGTATTTATATTGCGCCCGGATCCCTCTAG AAATAATTTTGTTTAACTTTAAGAAGGAGATATACA T atggatttaaattctaaaaaatatcagatgcttaaagagctatatgtaagcttcgctgaaa atgaagttaaacctttagcaacagaacttgatgaagaagaaagatttccttatgaaaca gtggaaaaaatggcaaaagcaggaatgatgggtataccatatccaaaagaatatggt ggagaaggtggagacactgtaggatatataatggcagttgaagaattgtctagagttt gtggtactacaggagttatattatcagctcatacatctcttggctcatggcctatatatca atatggtaatgaagaacaaaaacaaaaattcttaagaccactagcaagtggagaaaa attaggagcatttggtcttactgagcctaatgctggtacagatgcgtctggccaacaaa caactgctgttttagacggggatgaatacatacttaatggctcaaaaatatttataacaa acgcaatagctggtgacatatatgtagtaatggcaatgactgataaatctaaggggaa caaaggaatatcagcatttatagttgaaaaaggaactcctgggtttagctttggagttaa agaaaagaaaatgggtataagaggttcagctacgagtgaattaatatttgaggattgca gaatacctaaagaaaatttacttggaaaagaaggtcaaggatttaagatagcaatgtct actcttgatggtggtagaattggtatagctgcacaagctttaggtttagcacaaggtgct cttgatgaaactgttaaatatgtaaaagaaagagtacaatttggtagaccattatcaaaa ttccaaaatacacaattccaattagctgatatggaagttaaggtacaagcggctagaca ccttgtatatcaagcagctataaataaagacttaggaaaaccttatggagtagaagcag caatggcaaaattatttgcagctgaaacagctatggaagttactacaaaagctgtacaa cttcatggaggatatggatacactcgtgactatccagtagaaagaatgatgagagatg ctaagataactgaaatatatgaaggaactagtgaagttcaaagaatggttatttcagga aaactattaaaatagtaagaaggagatatacatatggaggaaggatttatgaatatagt cgtttgtataaaacaagttccagatacaacagaagttaaactagatcctaatacaggta ctttaattagagatggagtaccaagtataataaaccctgatgataaagcaggtttagaa gaagctataaaattaaaagaagaaatgggtgctcatgtaactgttataacaatgggacc tcctcaagcagatatggctttaaaagaagctttagcaatgggtgcagatagaggtatat tattaacagatagagcatttgcgggtgctgatacttgggcaacttcatcagcattagca	**SEQ ID NO: 176**

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Ydfz- ter-thiA1-hbd-crt2-tesb butyrate cassette	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTT CCCCCGACTTATGGCTCATGCATGCATCAAAAAAG ATGTGAGCTTGATCAAAAACAAAAAATATTTCACTC GACAGGAGTATTTATATTGCGCCCGGATCCCTCTAG AAATAATTTTGTTTAACTTTAAGAAGGAGATATACA T atgatcgtaaaacctatggtacgcaacaatatctgcctgaacgcccatcctcagggct gcaagaagggagtggaagatcagattgaatataccaagaaacgcattaccgcagaa gtcaaagctggcgcaaaagctccaaaaaacgttctggtgcttggctgctcaaatggtt acggcctggcgagccgcattactgctgcgttcggatacggggctgcgaccatcggc gtgtcctttgaaaaagcgggttcagaaaccaaatatggtacaccgggatggtacaata atttggcatttgatgaagcggcaaaacgcgagggtctttatagcgtgacgatcgacgg cgatgcgttttcagacgagatcaaggcccaggtaattgaggaagccaaaaaaaaag gtatcaaatttgatctgatcgtatacagcttggccagcccagtacgtactgatcctgata caggtatcatgcacaaaagcgttttgaaacccttggaaaaacgttcacaggcaaaac agtagatccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacga ggaagcagccgccactgttaaagttatgggggtgaagattgggaacgttggattaa gcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggcctatagttata ttggccctgaagctacccaagctttgtaccgtaaaggcacaatcggcaaggccaaag aacacctggaggccacagcacaccgtctcaacaaagagaacccgtcaatccgtgcc ttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggtaatc cctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttg tattgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggtacaatt ccagttgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtcc	**SEQ ID NO: 178**

	agaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctca ctgacttagcggggtaccgccatgatttcttagctagtaacggctttgatgtagaaggta ttaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatatacat atgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggagga gcatttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataa aaagagctaacataactccagatatgatagatgaatctcttttaggggagtacttaca gcaggtcttggacaaaatatagcaagacaaatagcattaggagcaggaataccagta gaaaaaccagctatgactataaatatagtttgtggttctggattaagatctgtttcaatgg catctcaacttatagcattaggtgatgctgatataatgttagttggtggagctgaaaacat gagtatgtctccttatttagtaccaagtgcgagatatggtgcaagaatgggtgatgctg cttttgttgattcaatgataaaagatggattatcagacatatttaataactatcacatgggt attactgctgaaaacatagcagagcaatggaatataactagagaagaacaagatgaat tagctcttgcaagtcaaaataaagctgaaaaagctcaagctgaaggaaaatttgatga agaaatagttcctgttgttataaaaggaagaaaaggtgacactgtagtagataaagatg aatatattaagcctggcactacaatggagaaacttgctaagttaagacctgcatttaaaa aagatggaacagttactgctggtaatgcatcaggaataaatgatggtgctgctatgtta gtagtaatggctaaagaaaaagctgaagaactaggaatagagcctcttgcaactatag tttcttatggaacagctggtgttgaccctaaaataatgggatatggaccagttccagcaa ctaaaaaagctttagaagctgctaatatgactattgaagatatagatttagttgaagctaa tgaggcatttgctgcccaatctgtagctgtaataagagacttaaatatagatatgaataa agttaatgttaatggtggagcaatagctataggacatccaataggatgctcaggagca agaatacttactacacttttatatgaaatgaagagaagagatgctaaaactggtcttgct acactttgtataggcggtggaatgggaactactttaatagttaagagatagtaagaagg agatatacatatgaaattagctgtaataggtagtggaactatgggaagtggtattgtaca aacttttgcaagttgtggacatgatgtatgtttaaagagtagaactcaaggtgctatagat aaatgtttagctttattagataaaaatttaactaagttagttactaagggaaaaatggatg aagctacaaaagcagaaatattaagtcatgttagttcaactactaattatgaagatttaaa agatatggatttaataatagaagcatctgtagaagacatgaatataaagaaagatgtttt caagttactagatgaattatgtaaagaagatactatcttggcaacaaatacttcatcatta tctataacagaaatagcttcttctactaagcgcccagataaagttataggaatgcatttct ttaatccagttcctatgatgaaattagttgaagttataagtggtcagttaacatcaaaagtt acttttgatacagtatttgaattatctaagagtatcaataaagtaccagtagatgtatctga atctcctggatttgtagtaaatagaatacttatacctatgataaatgaagctgttggtatat atgcagatggtgttgcaagtaaagaagaaatagatgaagctatgaaattaggagcaa accatccaatgggaccactagcattaggtgatttaatcggattagatgttgttttagctat aatgaacgttttatatactgaatttggagatactaaatatagacctcatccacttttagcta aaatggttagagctaatcaattaggaagaaaaactaagataggattctatgattataata aataataagaaggagatatacatatgagtacaagtgatgttaaagtttatgagaatgtag ctgttgaagtagatggaaatatatgtacagtgaaaatgaatagacctaaagcccttaat gcaataaattcaaagactttagaagaactttatgaagtatttgtagatattaataatgatga aactattgatgttgtaatattgacaggggaaggaaaggcatttgtagctggagcagata ttgcatacatgaaagatttagatgctgtagctgctaaagattttagtatcttaggagcaaa agcttttggagaaatagaaaatagtaaaaaagtagtgatagctgctgtaaacggatttg ctttaggtggaggatgtgaacttgcaatggcatgtgatataagaattgcatctgctaaag ctaaatttggtcagccagaagtaactcttggaataactccaggatatggaggaactcaa aggcttacaagattggttggaatggcaaaagcaaaagaattaatctttacaggtcaagt tataaaagctgatgaagctgaaaaaatagggctagtaaatagagtcgttgagccagac attttaatagaagaagttgagaaattagctaagataatagctaaaaatgctcagcttgca	

	gttagatactctaaagaagcaatacaacttggtgctcaaactgatataaatactggaata gatatagaatctaatttatttggtctttgtttttcaactaaagaccaaaaagaaggaatgtc agctttcgttgaaaagagagaagctaactttataaaagggtaataagaaggagatata catatgAGTCAGGCGCTAAAAAATTTACTGACATTGTT AAATCTGGAAAAAATTGAGGAAGGACTCTTTCGCG GCCAGAGTGAAGATTTAGGTTTACGCCAGGTGTTTG GCGGCCAGGTCGTGGGTCAGGCCTTGTATGCTGCA AAAGAGACCGTCCCTGAAGAGCGGCTGGTACATTC GTTTCACAGCTACTTTCTTCGCCCTGGCGATAGTAA GAAGCCGATTATTTATGATGTCGAAACGCTGCGTGA CGGTAACAGCTTCAGCGCCCGCCGGGTTGCTGCTAT TCAAAACGGCAAACCGATTTTTTATATGACTGCCTC TTTCCAGGCACCAGAAGCGGGTTTCGAACATCAAA AAACAATGCCGTCCGCGCCAGCGCCTGATGGCCTC CCTTCGGAAACGCAAATCGCCCAATCGCTGGCGCA CCTGCTGCCGCCAGTGCTGAAAGATAAATTCATCTG CGATCGTCCGCTGGAAGTCCGTCCGGTGGAGTTTCA TAACCCACTGAAAGGTCACGTCGCAGAACCACATC GTCAGGTGTGGATCCGCGCAAATGGTAGCGTGCCG GATGACCTGCGCGTTCATCAGTATCTGCTCGGTTAC GCTTCTGATCTTAACTTCCTGCCGGTAGCTCTACAG CCGCACGGCATCGGTTTTCTCGAACCGGGGATTCAG ATTGCCACCATTGACCATTCCATGTGGTTCCATCGC CCGTTTAATTTGAATGAATGGCTGCTGTATAGCGTG GAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGT GCGCGGTGAGTTTTATACCCAAGACGGCGTACTGGT TGCCTCGACCGTTCAGGAAGGGGTGATGCGTAATC ACAATtaa	

Example 11. Production of Butyrate in Recombinant E. coli

[01105] The effect of oxygen and glucose on butyrate production was assessed in *E. coli* Nissle strains using a butyrate cassette driven by a FNR promoter (ter-thiA1-hbd-crt2-pbt-buk genes under the control of a ydfZ promoter).

[01106] All incubations were performed at 37° C. Cultures of *E. coli* strains DH5a and Nissle transformed with the butyrate cassettes were grown overnight in LB and then diluted 1:200 into 4 mL of LB containing no glucose or RCM 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 was 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.

[01107] **FIG. 14C** depicts butyrate production in strains comprising an FNR-butyrate cassette (having the ter substitution) in the presence/absence of glucose and oxygen and 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.

Example 12. Optimization of a Low-dose DSS-induced Colitis Model for the Detection of Compromised Barrier Function

[01108] To Determine the optimal DDS concentration to administer to mice to be able to investigate compromised barrier function, as study was conducted in mice using various concentrations of DSS.

[01109] Briefly, C57BL6 mice (12 weeks, N=25) were treated with 0.25%, 0.5%, 1% and 1.5% DSS and FITC-dextran (4kD).

[01110] On day 0 of the study, animlas were weighed, and randomized mice into 5 treatment groups (n=5/group) according to weight as follows: Group 1-H2O Control, n=5; Group 2-0.25% DSS n=5; Group 3-0.5% DSS, n=5; Group 4-1% DSS, n=5; Group 5-1.5% DSS, n=5. Fecal pellets were collected and water was changed to DSS-containing water. Animals were again weighed on day one and three. On day two, blood samples were collected for spectrophotometric analysis of FITC. On day four, mice were fasted for 4h and gavaged all mice with 0.6mg/g FITC-dextran (4kD). At 3h post FITC-dex administration, animals were weighed and bled. Fecal pellets were collected and colon samples were harvested. Blood samples were processed for spectrophotometric analysis of FITC, and serum was prepared from whole blood.

[01111] Fecal pellets are analyzed for levels of mouse lipocalin2 and calprotectin by ELISA (RnD systems), as seen in **FIG. 14D**. CRP levels are also analyzed

by ELISA (R&D Systems). Colon tissue is analyzed for increased levels of IL-1a/b, -6, -13, -18, CCL1, CXCL1, TNFa, IFNg EpCAM, MPO and G-CSF by qPCR. Serum was analyzed for FITC-dextran levels by spectrophotometry, and results are shown in **FIG. 15**. As seen in **FIG. 15**, 0.5% DSS is the lowest dose at which an increase in FITC dextran was observed.

Example 13. Comparison of Low-dose DSS Concentrations and Different FITC MW for the Detection of Compromised Barrier Function

[01112] A study was conducted to determine the optimal DSS concentration (0.75 or 1.5%) and molecular weight FITC-Dextran (4 or 40kDA) to administer to mice to be able to investigate compromised barrier function.

[01113] C57BL6 (9 weeks, n=18), were treated with DSS as follows DSS-0.75 and 1.5%; FITC-dextran (4 and 40kD) and effects on molecular markers of colitis (as assessed by Spectrophotometry and ELISA) assessed, and body weight and overall animal health were monitored.

[01114] On day 0, mice were weighed and randomized mice into 3 treatment groups (n=6/group) according to weight as follows: Group 1-H2O Control, n=6; Group 2-0.75% DSS, n=6; Group 3-1.5% DSS, n=6. Water was changed to DSS- containing water.

[01115] Mice wer again weighed on days 1-3. ON day 4, mice were fasted for 4 hours, and 3 mice from each group were gavaged with 0.6mg/g of either 4kDa or 40kDa FITC-dextran. Mice 1-3 and 4-6 (as designated by tail marks) from each group were used for 4kDa and 40kDa FITC-dex administration respectively. At 3h post FITC-dex administration, mice were weighed and bled, and fecal pellets were collected. Blood samples were processed for spectrophotometric analysis of FITC, and serum from whole blood was prepared.

[01116] Analysis of serum for FITC-dextran levels by spectrophotometry is shown in **FIG. 15**.

Example 14. Butyrate-Producing Bacterial Strain Reduces Gut Inflammation in a Low-Dose DSS-Induced Mouse Model of IBD

[01117] At Day 0, 40 C57BL6 mice (8 weeks of age) were weighed and randomized into the following five treatment groups (n=8 per group): H_2O control (group 1); 0.5% DSS control (group 2); 0.5% DSS + 100 mM butyrate (group 3); 0.5% DSS +

SYN94 (group 4); and 0.5% DSS + SYN363 (group 5). After randomization, the cage water for group 3 was changed to water supplemented with butyrate (100 mM), and groups 4 and 5 were administered 100 µL of SYN94 and SYN363 by oral gavage, respectively. At Day 1, groups 4 and 5 were gavaged with bacteria in the morning, weighed, and gavaged again in the evening. Groups 4 and 5 were also gavaged once per day for Day 2 and Day 3.

[01118] At Day 4, groups 4 and 5 were gavaged with bacteria, and then all mice were weighed. Cage water was changed to either H_2O + 0.5% DSS (groups 2, 4, and 5), or H_2O + 0.5% DSS supplemented with 100 mM butyrate (group 3). Mice from groups 4 and 5 were gavaged again in the evening. On Days 5-7, groups 4 and 5 were gavaged with bacteria in the morning, weighed, and gavaged again in the evening.

[01119] At Day 8, all mice were fasted for 4 hours, and groups 4 and 5 were gavaged with bacteria immediately following the removal of food. All mice were then weighed, and gavaged with a single dose of FITC-dextran tracer (4 kDa, 0.6 mg/g body weight). Fecal pellets were collected; however, if colitis was severe enough to prevent feces collection, feces were harvested after euthanization. All mice were euthanized at exactly 3 hours following FITC-dextran administration. Animals were then cardiac bled and blood samples were processed to obtain serum. Levels of mouse lipocalin 2, calprotectin, and CRP-1 were quantified by ELISA, and serum levels of FITC-dextran were analyzed by spectrophotometry (see also Example 8).

[01120] **FIG. 14D** shows lipocalin 2 (LCN2) levels in all treatment groups, as demonstrated by ELISA, on Day 8 of the study. Since LCN2 is a biomarker of inflammatory disease activity, these data suggest that SYN-501 produces enough butyrate to significantly reduce LCN2 concentrations, as well as gut inflammation, in a low-dose DSS-induced mouse model of IBD.

Example 15. Comparison of in vitro butyrate production efficacy of chromosomal insertion and plasmid-bearing engineered bacterial strains

[01121] The in vitro butyrate production efficacy of engineered bacterial strains harboring a chromosomal insertion of a butyrate cassette was compared to a 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.

[01122] 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. 19A,** and show that SYN1001 and SYN1002 give comparable butyrate production to the plasmid strain SYN501.

[01123] 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: 179, 180, 181, or 182**, or a functional fragment thereof.

Table 48. FRNRs Butyrate Cassette Sequences

Description	Sequence
Pfnrs-ter-thiA1-hbd-ctr2-tesB **SEQ ID NO:179** , 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

	ggtatcatgcacaaaagcgttttgaaacccttttggaaaaacgttcacaggcaaaacagtagat ccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagcc gccactgttaaagttatgggggggtgaagattgggaacgttggattaagcagctgtcgaagga aggcctcttagaagaaggctgtattaccttggcctatagttatattggccctgaagctacccaa gctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagcacac cgtctcaacaaagagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaac ccgcgcaagcgccgtaatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaa agagaagggcaatcatgaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgt accgtaaagatggtacaattccagttgatgaggaaaatcgcattcgcattgatgattgggagtt agaagaagacgtccagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgc agaatctctcactgacttagcggggtaccgccatgatttcttagctagtaacggctttgatgtag aaggtattaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatatac atatgagagaagtagtaattgccagtgcagctagaacagcagtaggaagttttggaggagc atttaaatcagtttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagag ctaacataactccagatatgatagatgaatctcttttaggggagtacttacagcaggtcttgg 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

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Pfnrs-ter-thiA1-hbd-crt2-pbt-buk (**SEQ ID NO: 180**), 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 ggtatcatgcacaaaagcgttttgaaaccctttggaaaaacgttcacaggcaaaacagtagat ccgtttactggcgagctgaaggaaatctccgcggaaccagcaaatgacgaggaagcagcc gccactgttaaagttatgggggtgaagattgggaacgttggattaagcagctgtcgaagga aggcctcttagaagaaggctgtattaccttggcctatagttatattggccctgaagctacccaa gctttgtaccgtaaaggcacaatcggcaaggccaaagaacacctggaggccacagcacac cgtctcaacaaagagaacccgtcaatccgtgccttcgtgagcgtgaataaaggcctggtaac ccgcgcaagcgccgtaatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaa agagaagggcaatcatgaaggttgtattgaacagatcacgcgtctgtacgccgagcgcctgt accgtaaagatggtacaattccagttgatgaggaaaatcgcattcgcattgatgattgggagtt agaagaagacgtccagaaagcggtatccgcgttgatggagaaagtcacgggtgaaaacgc agaatctctcactgacttagcggggtaccgccatgatttcttagctagtaacggctttgatgtag aaggtattaattatgaagcggaagttgaacgcttcgaccgtatctgataagaaggagatatac

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PfNRS (ribosome binding site is underlined) (**SEQ ID NO: 181**)	GGTACCAGTTGTTCTTATTGGTGGTGTTGCTTTATGGTT GCATCGTAGTAAATGGTTGTAACAAAAGCAATTTTTCC GGCTGTCTGTATACAAAAACGCCGCAAAGTTTGAGCGA AGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTC TTGGATCCAAAGTGAACTCTAGAAATAATTTTGTTTAAC TTTAAGAAGGAGATATACAT
Ribosome binding site and leader region (**SEQ ID NO:182**)	CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATAT ACAT

Example 16. Assessment of intestinal butyrate levels in response to SYN501 administration in mice

[01124] 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.

[01125] 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. 28**. 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.

[01126] 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.

[01127] 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).

[01128] 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 17. Comparison of Butyrate production levels between the genetically engineered bacteria encoding a butyrate cassette and selected Clostridia strains

[01129] 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).

[01130] Briefly, overnight cultures of SYN501 were diluted 1:100 were grown in RCM (Reinforced Clostridial Media, which is similar to LB but contains 0.5% 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 18**). Results are depicted in **FIG. 18**, and show that SYN501 produces butyrate levels comparable to *Clostridium spp.* in RCM media

Example 18. Quantification of Butyrate by LC-MS/MS

[01131] To obtain the butyrate measurements in Example 37 a LC-MS/MS protocol for butyrate quantification was used.

Sample preparation

[01132] 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

[01133] 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 49** and **Table 50**. Tandem Mass Spectrometry details are found in **Table 51**.

Table 49. 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 50. 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 51. Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM	Butyrate 170.0/71.1,

transitions	Butyrate d7 177.1/78.3

Example 19. Quantification of Butyrate in feces by LC-MS/MS

Sample preparation

[01134] 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

[01135] 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 52** and **Table 53**. Tandem Mass Spectrometry details are found in **Table 54**.

Table 52. 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

[01]

Table 53. 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 54A. Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM transitions	Butyrate 230.1/143.1, Butyrate d7 237.1/143.1

Example 20. Generation of Butyrate and Acetate Producing Strains

A. Generation of an Acetate Overproducing Strain

[01136] 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. 25**).

[01137] 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.

[01138] 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.

[01139] 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 21**.

Table 54B. 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	6.68461575

[01140] 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**.

[01141] As seen in **FIG. 26A** and **FIG. 26B**, 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

[01142] 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. 25**). 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.

[01143] 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 21**.

[01144] As seen in **FIG. 26C and FIG. 26D**, 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

[01145] 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).

[01146] 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**.

[01147] Results are depicted in **FIG. 26E** 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.

[01148] 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

[01149] 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. 25**). 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.

[01150] 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).

[01151] 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**.

[01152] Results are depicted in **FIG. 26F**, 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 21. Acetate and Butyrate quantification in bacterial supernatant by LC-MS/MS

Sample Preparation

[01153] 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

[01154] 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 summarizes Tandem Mass Spectrometry.

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

Example 22. Production of Propionate through the Sleeping Beauty Mutase Pathway in genetically engineered *E. coli* BW25113 and Nissle

[01155] 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.

[01156] 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).*

[01157] 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 58.** Mutation of the lactate dehydrogenase gene (ldhA) reportedly increases propionate production, and this mutation is therefore also added in certain embodiments.

[01158] 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: 184, or 184**, or a functional fragment thereof.

[01159]

Table 58. 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: 183**)	***CAAATAAAACGAAAGGCTCAGTCGAAAGACTGG GCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACG CTCTCCTGAGTAGGACAAAT*****CCGCCGGGAGCG GATTTGAACGTTGCGAAGCAACGGCCCGGA GGGTGGCGGGCAGGACGCCCGCCATAAACT GCCAGGCATCAAATTAAGC*****CAGAAGGCCATCCT 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 AACGGTGGTATATCCAGTGATTTTTTCTCC ATTTTAGCTTCCTTAGCTCCTGAAAATCTCGA CAACTCAAAAAATACGCCCGGTAGTGATCTT ATTTCATTATGGTGAAAGTTGGAACCTCTTA CGTGCCGATCAACGTCTCATTTTCGCCAAAA GTTGGCCCAGGGCTTCCCGGTATCAACAGGG ACACCAGGATTTATTTATTCTGCGAAGTGAT CTTCCGTCACAGGTAGGCGCGCC*GAAGTTCC* *TATACTTTCTAGAGAATAGGAACTTC*GGAATAG GAACTAAGGAGGATATTCATATGGACCATGG CTAATTCCCAGGTACCagttgttcttattggtggtgttgcttt atggttgcatcgtagtaaatggttgtaacaaaagcaatttttccggctgtct gtatacaaaaacgccgcaaagt*ttgagcgaagtcaa*taaactctctaccc attcagggcaatatctctcttggatccaaagtgaactctagaaataattttg 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: 184)**	**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgta acaaaagcaatttttccggctgtctgtatacaaaaacgccgcaaagt*ttga gcgaagtcaa*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

[01160] Next, this strain was tested for propionate production.

[01161] 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

[01162] Results are depicted in **FIG. 29** 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 25**.

Example 23. Evaluation of the Sleeping Beauty Mutase Pathway for the Production of Propionate in *E coli Nissle*

[01163] 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 24. Evaluation of the Acrylate Pathway from Clostridium propionicum for Propionate Production

[01164] 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*, is 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 59. 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: 185**)	ttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataa gaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcat actatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaa cctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgt tggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaa atgtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaa aaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctca atggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctct acacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagct ctaatgcgctgttaatcactttacttttatctaatctagacatcattaattcctaatttttt**gttgaca ctctatcattgatagagttattttaccactccctatcagtgatagagaa**aagtgaa*actct agaaataatttttgtttaactttaagaaggagatatacat*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: 186**)	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

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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: 187**)	caactgttgggaagggcgatcggtgcgggcctcttcgctattacgccagctggcgaaagg gggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgacgttgta aaacgacggccagtgaattgacgcgtattgggatgtaaaacgacggccagtgaattcgtta agacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataaga aggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatac tatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaacc taaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgttg gcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaat gtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaa aaatcttgccagctttcccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaa tggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtaggctgctcta cacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctc taatgcgctgttaatcactttacttttatctaatctagacatcattaattcctaatttttt**gttgacac** **tctatcattgatagagttattttaccactccctatcagtgatagaga**aaagtgaa*ctcta* *gaaataattttgtttaactttaagaaggagatatacat*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

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	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 GTGCACTGGGGGCAGCGCTGTATGCTTATGAGGCCGCC CAGAAGAAGTAgatggtagtgtggggtctccccatgcgagagtagggaactgc caggcat***caaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgt ttgtcggtgaacgctctcctgagtaggacaaat***ccgccgggagcggatttgaacgttgcg aagcaacggcccggagggtggcgggcaggacgcccgccataaactgccaggcatcaa attaagc***agaaggccatcctgacggatggcctttt***
acuI-pct-lcdABC (**SEQ ID NO: 188**)	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*ctctagaaataattttgtttaactttaagaaggagatatacat*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*gaaggagatatacat*ATGTATACCTTGGGGATTGATGTCGGTTCTGCCTCTAG TAAAGCGGTGATTCTGAAAGATGGAAAAGATATTGTC GCTGCCGAGGTTGTCCAAGTCGGTACCGGCTCCTCGGG TCCCCAACGCGCACTGGACAAAGCCTTTGAAGTCTCTG GCTTAAAAAAGGAAGACATCAGCTACACAGTAGCTAC GGGCTATGGGCGCTTCAATTTTAGCGACGCGGATAAAC AGATTTCGGAAATTAGCTGTCATGCCAAAGGCATTTAT TTCTTAGTACCAACTGCGCGCACTATTATTGACATTGG CGGCCAAGATGCGAAAGCCATCCGCCTGGACGACAAG GGGGGTATTAAGCAATTCTTCATGAATGATAAATGCGC GGCGGGCACGGGGCGTTTCCTGGAAGTCATGGCTCGC GTACTTGAAACCACCCTGGATGAAATGGCTGAACTGG ATGAACAGGCGACTGACACCGCTCCCATTTCAAGCACC TGCACGGTTTTCGCCGAAAGCGAAGTAATTAGCCAATT GAGCAATGGTGTCTCACGCAACAACATCATTAAAGGT GTCCATCTGAGCGTTGCGTCACGTGCGTGTGGTCTGGC GTATCGCGGCGGTTTGGAGAAAGATGTTGTTATGACAG GTGGCGTGGCAAAAAATGCAGGGGTGGTGCGCGCGGT GGCGGGCGTTCTGAAGACCGATGTTATCGTTGCTCCGA ATCCTCAGACGACCGGTGCACTGGGGGCAGCGCTGTAT GCTTATGAGGCCGCCCAGAAGAAGTA

[01165] 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: 185, 186, 187, or 188**, or a functional fragment thereof.

Example 25. Quantification of Propionate by LC-MS/MS

Sample preparation

[01166] 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

[01167] 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 60** and **Table 61**. Tandem Mass Spectrometry details are found in **Table 62.**

Table 60. 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 61. 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 62. Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM transitions	Propionate 156.2/57.1, Propionate-d5 161/62.1

Example 26. Generation of constructs for overproducing therapeutic molecules for secretion

[01168] To produce strain capable of secreting anti-inflammatory or gut barrier enhancer polypeptides, e.g., GLP2, IL-22, IL-10 (viral or human), several constructs are designed employing different secretion strategies. The organization of exemplary constructs is shown in **FIG. 30A, FIG.30B, FIG. 30C, and FIG 31A and FIG. 31B, FIG. 32A, FIG. 32B, FIG.32C, FIG. 32D, FIG.32E**. Various GLP2, IL-22, IL-10 (viral or human) constructs are synthesized, and cloned into vector pBR322 for transformation of E. coli. In some embodiments, the constructs encoding the effector molecules are integrated into the genome. In some embodiments, the constructs encoding the effector molecules are on a plasmid, e.g., a medium copy plasmid. **Table 63.** lists exemplary polypeptide coding sequences used in the constructs.

Table 63. Polypeptide coding sequences

Description	Sequence	SEQ ID NO
GLP2	CATGCTGATGGTTCTTTCTCTGATGAGAT GAACACCATTCTTGATAATCTTGCCGCCA	**SEQ ID NO: 189**

	GGGACTTTATAAACTGGTTGATTCAGACC AAAATCACTGAC	
GLP2 codon optimized	CATGCTGACGGCTCTTTTTCTGACGAAAT GAATACCATCCTGGATAATCTGGCGGCG CGTGATTTTATTAATTGGCTGATCCAAAC TAAAATTACTGATTAA	**SEQ ID NO: 190**
FliC20-GLP2 (FliC20, start of FliC gene preceding GLP2 sequence underlined)	ATGGCACAAGTCATTAATACCAACAGCC TCTCGCTGATCACTCAAAATAATATCAAC AAGCATGCTGACGGCTCTTTTTCTGACGA AATGAATACCATCCTGGATAATCTGGCG GCGCGTGATTTTATTAATTGGCTGATCCA AACTAAAATTACTGATTAA	**SEQ ID NO: 191**
GLP2 codon optimized (e.g., used in fliC construct)	ATGCATGCTGACGGCTCTTTTTCTGACGA AATGAATACCATCCTGGATAATCTGGCG GCGCGTGATTTTATTAATTGGCTGATCCA AACTAAAATTACTGATTAA	**SEQ ID NO: 192**
vIL10 codon optimized (e.g., used in fliC construct)	ATGGGTACTGACCAATGTGATAATTTCCC ACAAATGCTGCGTGATTTGCGCGACGCTT TCTCGCGTGTGAAAACTTTTTTTCAGACT AAAGATGAGGTGGATAATCTGCTGCTGA AAGAGAGCCTGTTGGAAGATTTTAAAGG CTACTTGGGCTGTCAAGCGCTGTCGGAG ATGATTCAATTTTATCTGGAAGAGGTGAT GCCGCAAGCTGAGAACCAAGATCCGGAA GCGAAAGATCACGTGAATTCGCTGGGCG AGAATCTGAAAACTCTGCGTCTGCGTCTG CGTCGTTGTCACCGTTTTTTGCCGTGCGA AAACAAAAGTAAAGCTGTTGAGCAAATT AAAAACGCTTTTAACAAACTGCAGGAAA AAGGTATCTATAAAGCGATGAGCGAATT TGATATTTTTATTAATTATATTGAAGCTT ATATGACTATTAAAGCTCGCTAA	**SEQ ID NO: 193**
vIL10	GGTACAGACCAATGTGACAATTTTCCCCA AATGTTGAGGGACCTAAGAGATGCCTTC AGTCGTGTTAAAACCTTTTTCCAGACAAA GGACGAGGTAGATAACCTTTTGCTCAAG GAGTCTCTGCTAGAGGACTTTAAGGGCT ACCTTGGATGCCAGGCCCTGTCAGAAAT GATCCAATTCTACCTGGAGGAAGTCATG CCACAGGCTGAAAACCAGGACCCTGAAG CCAAAGACCATGTCAATTCTTTGGGTGAA AATCTAAAGACCCTACGGCTCCGCCTGC GCCGTTGCCACAGGTTCCTGCCGTGTGAG AACAAGAGTAAAGCTGTGGAACAGATAA AAAATGCCTTTAACAAGCTGCAGGAAAA AGGAATTTACAAAGCCATGAGTGAATTT GACATTTTTATTAACTACATAGAAGCATA CATGACAATTAAAGCCAGG	**SEQ ID NO: 194**

IL-22 codon optimized (e.g., use with diffusible outer membrane construct)	GCACCGATCTCTTCCCACTGTCGCTTAGA TAAATCGAATTTTCAACAACCTTATATTA CGAATCGTACGTTTATGCTGGCTAAAGA AGCGTCATTAGCTGATAACAACACTGAT GTTCGCCTGATTGGTGAGAAATTGTTTCA CGGTGTGTCTATGTCAGAACGTTGCTACC TGATGAAACAAGTTCTGAATTTCACCCTG GAAGAAGTGTTGTTTCCGCAATCTGACCG CTTTCAACCGTATATGCAAGAGGTTGTGC CGTTTCTGGCGCGCCTGAGTAATCGCCTG AGCACTTGTCATATTGAGGGCGACGACC TGCATATTCAACGAAATGTTCAAAAATTG AAAGATACGGTGAAGAAACTGGGTGAAA GTGGTGAAATCAAAGCGATTGGTGAGCT GGATCTGCTGTTTATGTCATTGCGCAATG CGTGCATTTAA	**SEQ ID NO: 195**
IL-22 codon optimized (e.g., used in fliC construct)	ATGGCACCGATCTCTTCCCACTGTCGCTT AGATAAATCGAATTTTCAACAACCTTATA TTACGAATCGTACGTTTATGCTGGCTAAA GAAGCGTCATTAGCTGATAACAACACTG ATGTTCGCCTGATTGGTGAGAAATTGTTT CACGGTGTGTCTATGTCAGAACGTTGCTA CCTGATGAAACAAGTTCTGAATTTCACCC TGGAAGAAGTGTTGTTTCCGCAATCTGAC CGCTTTCAACCGTATATGCAAGAGGTTGT GCCGTTTCTGGCGCGCCTGAGTAATCGCC TGAGCACTTGTCATATTGAGGGCGACGA CCTGCATATTCAACGAAATGTTCAAAAAT TGAAAGATACGGTGAAGAAACTGGGTGA AAGTGGTGAAATCAAAGCGATTGGTGAG CTGGATCTGCTGTTTATGTCATTGCGCAA TGCGTGCATTTAA	**SEQ ID NO: 196**
hIL-10 codon optimized	TCGCCAGGTCAAGGAACGCAGTCAGAGA ATTCATGCACTCACTTTCCGGGCAATCTG CCGAATATGCTGCGCGATCTGCGAGATG CATTCTCTCGCGTGAAAACGTTCTTTCAA ATGAAAGATCAACTGGATAATCTGCTGC TGAAGGAGTCGTTGTTGGAGGATTTTAA GGGGTATCTGGGTTGTCAAGCACTGTCTG AAATGATTCAATTTTACTTGGAGGAAGTT ATGCCGCAAGCGGAAAACCAAGATCCGG ATATTAAGGCGCACGTGAACTCACTGGG CGAAAACCTGAAAACTTTGCGCCTGCGT CTGAGACGATGTCACCGATTCCTGCCGTG TGAAAACAAGTCAAAGGCGGTTGAGCAA GTTAAGAATGCTTTCAATAAGCTGCAAG AAAAGGGCATCTATAAAGCGATGTCTGA ATTTGATATCTTTATAAACTACATAGAAG	**SEQ ID NO: 197**

	CTTATATGACTATGAAGATTCGAAATTAA	
Monomerized hIL-10 (codon opt)	TCGCCAGGTCAAGGAACGCAGTCAGAGA ATTCATGCACTCACTTTCCGGGCAATCTG CCGAATATGCTGCGCGATCTGCGAGATG CATTCTCTCGCGTGAAAACGTTCTTTCAA ATGAAAGATCAACTGGATAATCTGCTGC TGAAGGAGTCGTTGTTGGAGGATTTTAA GGGGTATCTGGGTTGTCAAGCACTGTCTG AAATGATTCAATTTTACTTGGAGGAAGTT ATGCCGCAAGCGGAAAACCAAGATCCGG ATATTAAGGCGCACGTGAACTCACTGGG CGAAAACCTGAAAACTTTGCGCCTGCGT CTGAGACGATGTCACCGATTCCTGCCGTG TGAAAACGGAGGAGGAAGTGGTGGTAAG TCAAAGGCGGTTGAGCAAGTTAAGAATG CTTTCAATAAGCTGCAAGAAAAGGGCAT CTATAAAGCGATGTCTGAATTTGATATCT TTATAAACTACATAGAAGCTTATATGACT ATGAAGATTCGAAATTAA	**SEQ ID NO: 198**

[01169] 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: 189, SEQ ID NO: 190, SEQ ID NO: 191, SEQ ID NO: 192, SEQ ID NO: 193, SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, SEQ ID NO: 197,or SEQ ID NO: 198** or a functional fragment thereof.

[01170] Table 64 lists exemplary secretion tags, which can be added at the N-terminus when the diffusible outer membrane (DOM) method or the fliC method is used.

Table 64. Secretion Tags and FliC components

Sequence Name	Sequence	SEQ ID NO
fliC-FliC20 (e.g., used in GLP2 construct) FliC20: start of the fliC gene which (in some constructs) precedes the effector polypeptide sequence, see e.g., **FIG 30B** and **FIG. 30C** shown in italics fliC: native fliC UTR in bold, optimized RBS underlined	**tgacggcgattgagccgacgggtggaaaccc aaaacgtaatcaacGTGGGTACTC CTTAAATTGGGTTCGAATGG ACC***atggcacaagtcattaataccaacagc ctctcgctgatcactcaaaataatatcaacaag*	**SEQ ID NO: 199**

fliC-RBS (e.g., used in IL22 construct) fliC: native fliC UTR in bold, optimized RBS underlined	**tgacggcgattgagccgacgggtggaaaccc aaaacgtaatcaac<u>tacgaacacttacagga ggtaccca</u>**	**SEQ ID NO: 200**
fliC-RBS (e.g., used in GLP2 construct) fliC: native fliC UTR in bold, optimized RBS underlined	**tgacggcgattgagccgacgggtggaaaccc aaaacgtaatcaac<u>aagtataaactctggga ggttccta</u>**	
fliC-RBS (e.g., used in vIL10 construct) fliC: native fliC UTR in bold, optimized RBS underlined	**tgacggcgattgagccgacgggtggaaaccc aaaacgtaatcaac<u>tcaaatcccttaataagg aggtaaa</u>**	**SEQ ID NO: 201**
RBS-phoA RBS: underlined	<u>Ctctagaaataattttgtttaactttaagaaggaga tatacat</u>atgaaacaaagcactattgcactggca ctcttaccgttactgtttacccctgtgacaaaagc g	**SEQ ID NO: 202**
phoA	atgaaacaaagcactattgcactggcactcttac cgttactgtttaccctgtgacaaaagcg	**SEQ ID NO: 203**
RBS-ompF RBS: underlined	<u>Ctctagaaataattttgtttaactttaagaaggaga tatacat</u>atgatgaagcgcaatattctggcagtga tcgtccctgctctgttagtagcaggtactgcaaac gct	**SEQ ID NO: 204**
ompF	atgatgaagcgcaatattctggcagtgatcgtcc ctgctctgttagtagcaggtactgcaaacgct	**SEQ ID NO: 205**
RBS-cvaC RBS: underlined	<u>Ctctagaaataattttgtttaactttaagaaggaga tatacat</u>ATGAGAACTCTGACTCT AAATGAATTAGATTCTGTTTC TGGTGGT	**SEQ ID NO: 206**
cvaC	ATGAGAACTCTGACTCTAAAT GAATTAGATTCTGTTTCTGGT GGT	**SEQ ID NO: 207**
RBS-phoA (Opimized, e.g., used in IL10 construct) RBS: underlined	<u>GACGCCAGAGAGTTAAGGGG GTTAA</u>ATGAAACAATCGACC ATCGCATTGGCGCTGCTTCCT CTATTGTTCACACCGGTGACA AAGGCA	**SEQ ID NO: 208**
Optimized phoA	ATGAAACAATCGACCATCGC ATTGGCGCTGCTTCCTCTATT GTTCACACCGGTGACAAAGG CA	**SEQ ID NO: 209**
RBS-TorA RBS: underlined	<u>ctctagaaataattttgtttaactttaagaaggagat atacat</u>ATGAACAATAACGATCT CTTTCAGGCATCACGTCGGCG TTTTCTGGCACAACTCGGCGG CTTAACCGTCGCCGGGATGCT	**SEQ ID NO: 210**

	GGGGCCGTCATTGTTAACGCC GCGACGTGCGACTGCG	
TorA	ATGAACAATAACGATCTCTTT CAGGCATCACGTCGGCGTTTT CTGGCACAACTCGGCGGCTTA ACCGTCGCCGGGATGCTGGG GCCGTCATTGTTAACGCCGCG ACGTGCGACTGCG	**SEQ ID NO: 211**
RBS-TorA alternate	CCCACATTCGAGGTACTAAatg aacaataacgatctctttcaggcatcacgtcggc gttttctggcacaactcggcggcttaaccgtcgc cgggatgctggggacgtcattgttaacgccgcg ccgtgcgactgcggcgcaagcggcg	**SEQ ID NO: 212**
TorA (alternate)	atgaacaataacgatctctttcaggcatcacgtcg gcgttttctggcacaactcggcggcttaaccgtc gccgggatgctggggacgtcattgttaacgccg cgccgtgcgactgcggcgcaagcggcg	**SEQ ID NO: 213**
RBS-fdnG	ACCCTATTACACACCTAAGGA GGCCAAATACatggacgtcagtcgcag acaatttttttaaaatctgcgcgggcggtatggcg ggaacaacagtagcagcattgggctttgccccg aagcaagcactggct	**SEQ ID NO: 214**
fdnG	atggacgtcagtcgcagacaatttttttaaaatctg cgcgggcggtatggcgggaacaacagtagca gcattgggctttgccccgaagcaagcactggct	**SEQ ID NO: 215**
RBS-dmsA	TACGCAAAAAACATAATTTAA GAGAGGATAAACatgaaaacgaaaa tccctgatgcggtattggctgctgaggtgagtcg ccgtggtttggtaaaaacgacagcgatcggcgg cctggcaatggccagcagcgcattaacattacct ttttagtcggattgcgcacgct	**SEQ ID NO: 216**
dmsA	atgaaaacgaaaatccctgatgcggtattggctg ctgaggtgagtcgccgtggtttggtaaaaacgac agcgatcggcggcctggcaatggccagcagcg cattaacattacctttttagtcggattgcgcacgct	**SEQ ID NO: 217**

[01171] 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: 199, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 203, SEQ ID NO: 204, SEQ ID NO: 205, SEQ ID NO: 206, SEQ ID NO: 207, SEQ ID NO: 208, SEQ ID NO: 209, SEQ ID NO: 210, SEQ ID NO: 211, SEQ ID NO: 212, SEQ ID NO: 213, SEQ ID NO: 214, SEQ ID NO: 215, SEQ ID NO: 216, and SEQ ID NO: 217.** **Table 65** lists exemplary promoter sequences and miscellaneous construct sequences.

Table 65. Promoter Sequences and Miscellaneous Construct Sequences

Description	Sequence	SEQ ID NO
TetR/TetA Promoter	gaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaag gccgaataagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaata atggcggcatactatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatc ttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctct agtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttct gtaggccgtgtacctaaatgtacttttgctccatcgcgatgacttagtaaagcacatctaaa acttttagcgttattacgtaaaaaatcttgccagctttccccttctaaagggcaaaagtgag tatggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttacat gccaatacaatgtaggctgctctacacctagcttctgggcgagtttacgggttgttaaacctt cgattccgacctcattaagcagctctaatgcgctgttaatcactttacttttatctaatctaga catcattaattcctaatttttgttgacactctatcattgatagagttattttaccactccctatc agtgatagagaaaagtgaa	SEQ ID NO: 218
fliC Promoter	agcgggaataaggggcagagaaaagagtatttcgtcgactaacaaaaaatggctgtttgt gaaaaaaattctaaaggttgttttacgacagacgataacagggt	SEQ ID NO: 219
FnrS Promoter	ggtaccAGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGT AAATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAA CGCCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGG CAATATCTCTCTTggatcc	SEQ ID NO: 220
DOM Construct Terminator	cacatttccccgaaaagtgccgatggccccccgatggtagtgtggcccatgcgagagtagg gaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttat ctgttgtttgtcggtgaacgctctcctgagtaggacaaatccgccgggagcggatttgaacg ttgcgaagcaacggcccggagggtggcgggcaggacgcccgccataaactgccaggcat caaattaagcagaaggccatcctgacggatggccttttgcgtggccagtgccaagcttgc atgcagattgcagcattacacgtcttgagcgattgtgtaggctggagctgcttc	SEQ ID NO: 221
FRT Site	gaagttcctatactttctagagaataggaacttcggaataggaacttc	SEQ ID NO: 222
Kanamycin Resistance Cassette (for integration in between FRT sites)	aagatcccctcacgctgccgcaagcactcagggcgcaagggctgctaaaggaagcggaa cacgtagaaagccagtccgcagaaacggtgctgaccccggatgaatgtcagctactgggc tatctggacaagggaaaacgcaagcgcaaagagaaagcaggtagcttgcagtgggctta catggcgatagctagactgggcggttttatggacagcaagcgaaccggaattgccagctg gggcgccctctggtaaggttgggaagccctgcaaagtaaactggatggctttcttgccgcc aaggatctgatggcgcaggggatcaagatctgatcaagagacaggatgaggatcgtttcg catgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattc ggctatgactgggcacaacagacaatcggctgctctgatgccgccgtgttccggctgtcag cgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaactgcag	SEQ ID NO: 223

	gacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcg acgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatc tcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcgg ctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagc gagcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatc aggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcatgcccgacggcgag gatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgctt ttctggattcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttgg ctacccgtgatattgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttac ggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacgagttcttctga gcgggactctggggttcgaaatgaccgaccaagcgacgcccaacctgccatcacgagatt tcgattccaccgccgccttctatgaaaggttgggcttcggaatcgttttccgggacgccggc tggatgatcctccagcgcggggatctcatgctggagttcttcgcccaccccagcttcaaaag cgctct	

[01172] 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: 218, SEQ ID NO: 219, SEQ ID NO: 220, SEQ ID NO: 221, SEQ ID NO: 222, and SEQ ID NO: 223. Table 66** Lists exemplary secretion constructs.

Table 66. Non-limiting Examples of Secretion Constructs

Description	**Sequence**	**SEQ ID NO:**
FliC20-glp2; a human GLP2 construct inserted into the FliC locus, under the control of the native FliC promoter (as shown in FIG. 32A)	cgttccttgtagggcgtcatagcgttcgacggcattaagtaacccaatgcc gcccgcctgtagcagatcgtcaagttccacgctcgcgggcagtcgaacct gcaggcgcaatgcttcgtgacgcaccagcgggacataacgctgccacag cgagtgtttatccattacaccttcagcggtatagagtgaattcacgataaaca gccctgcgttatatgagttatcggcatgattatccgtttctgcagggtttttaat cggacgattagtgggtgaaatgaggggttatttgggggttaccggtaaatt gcgggcagaaaaaaccccgccgttggcggggaagcacgttgctggcaa attaccattcatgttgccggatgcggcgtaaacgccttatccggcctacaaa aatgtgcaaattcaataaattgcaattccccttgtaggcctgataagcgcag cgcatcaggcaatttggcgttgccgtcagtctcagttaatcaggttacggcg attaatcagtaattttagtttggatcagccaattaataaaatcacgcgccgcc agattatccaggatggtattcatttcgtcagaaaaagagccgtcagcATG cattaggaacctcccagagtttatacttgttgattacgttttgggtttccaccc gtcggctcaatcgccgtcaaccctgttatcgtctgtcgtaaaacaacctttag aatttttttcacaaacagccatttttttgttagtcgacgaaatactcttttctctgc ccttattcccgctattaaaaaaaacaattaaacgtaaactttgcgcaattca ggccgataaccccggtattcgttttacgtgtcgaaagataaaCGAAGT TCCTATACTTTCTAGAGAATAGGAACTTCGG AATAGGAACTTCATTTctcgttcgctgccacctaagaatact ctacggtcacatacAAATGGCGCGCCTTACGCCCCGC CCTGCCACTCATCGCAGTACTGTTGTATTCAT	**SEQ ID NO: 224**

	TAAGCATCTGCCGACATGGAAGCCATCACAA ACGGCATGATGAACCTGAATCGCCAGCGGCA TCAGCACCTTGTCGCCTTGCGTATAATATTTG CCCATGGTGAAAACGGGGGCGAAGAAGTTGT CCATATTGGCCACGTTTAAATCAAAACTGGT GAAACTCACCCAGGGATTGGCTGAGACGAAA AACATATTCTCAATAAACCCTTTAGGGAAAT AGGCCAGGTTTTCACCGTAACACGCCACATC TTGCGAATATATGTGTAGAAACTGCCGGAAA TCGTCGTGGTATTCACTCCAGAGCGATGAAA ACGTTTCAGTTTGCTCATGGAAAACGGTGTA ACAAGGGTGAACACTATCCCATATCACCAGC TCACCGTCTTTCATTGCCATACGTAATTCCGG ATGAGCATTCATCAGGCGGGCAAGAATGTGA ATAAAGGCCGGATAAAACTTGTGCTTATTTTT CTTTACGGTCTTTAAAAAGGCCGTAATATCC AGCTGAACGGTCTGGTTATAGGTACATTGAG CAACTGACTGAAATGCCTCAAAATGTTCTTT ACGATGCCATTGGGATATATCAACGGTGGTA TATCCAGTGATTTTTTTCTCCATTTTAGCTTCC TTAGCTCCTGAAAATCTCGACAACTCAAAAA ATACGCCCGGTAGTGATCTTATTTCATTATGG TGAAAGTTGGAACCTCTTACGTGCCGATCAA CGTCTCATTTTCGCCAAAAGTTGGCCCAGGG CTTCCCGGTATCAACAGGGACACCAGGATTT ATTTATTCTGCGAAGTGATCTTCCGTCACAGG TAGGCGCGCCGAAGTTCCTATACTTTCTAGA GAATAGGAACTTCGGAATAGGAACTctcaccgcc gcgcaaaaagcgacgctaacccctatttcaaatcagcaatcgtcgtttacc gctaaacttagcgcctacggtacgctgaaaagcgcgctgacgactttcca gaccgccaatactgcattgtctaaagccgatctttttccgctaccagcacc accagcagcaccaccgcgttcagtgccaccaccgcgggtaatgccatcg ccgggaaatacaccatcagcgtcacccatctggcgcaggcgcaaaccct gacaacgcgcaccaccagagacgatacgaaaacggcgatcgccacca gcgacagcaaactcaccattcaacaaggcggcgacaaagatccgatttcc attgatatcagcgcggctaactcgtctttaagcgggatccgtgatgccatca acaacgcaaaagcaggcgtaagcgcaagcatcattaacgtgggtaacgg tgaatatcgtctgtcagtcacatcaaatgacaccggcct	
FliC20 with optimized RBS-GLP2 and UTR-FliC (as shown in FIG. 32A, in reverse orientation)	attaatcagtaattttagtttggatcagccaattaataaaatcacgcgccgcc agattatccaggatggtattcatttcgtcagaaaaagagccgtcagcATG cattaggaacctcccagagtttatacttgttgattacgttttgggtttccaccc gtcggctcaatcgccgtca	**SEQ ID NO: 225**
human GLP2 construct, , including the N terminal 20 amino acids of FliC	cgttccttgtagggcgtcatagcgttcgacggcattaagtaacccaatgcc gcccgcctgtagcagatcgtcaagttccacgctcgcgggcagtcgaacct gcaggcgcaatgcttcgtgacgcaccagcgggacataacgctgccacag cgagtgtttatccattacaccttcagcggtatagagtgaattcacgataaaca	**SEQ ID NO: 226**

(reverse orientation), inserted into the FliC locus under the control of a tet inducible promoter, with TetR and chloramphenicol resistance. (as shown in FIG. 32C)	gccctgcgttatatgagttatcggcatgattatccgtttctgcagggtttttaat cggacgattagtgggtgaaatgaggggttatttgggggttaccggtaaatt gcgggcagaaaaaaaccccgccgttggcggggaagcacgttgctggcaa attaccattcatgttgccggatgcggcgtaaacgccttatccggcctacaaa aatgtgcaaattcaataaattgcaattccccttgtaggcctgataagcgcag cgcatcaggcaatttggcgttgccgtcagtctcagttaatcaggttacggcg attaatcagtaattttagtttggatcagccaattaataaaatcacgcgccgcc agattatccaggatggtattcatttcgtcagaaaaagagccgtcagcATG cttgttgatattattttgagtgatcagcgagaggctgttggtattaatgacttgt gccatGGTCCATTCGAACCCAATTTAAGGAGTA CCCACgttgattacgttttgggtttccacccgtcggctcaatcgccgtca ttctctatcactgatagggagtggtaaaataactctatcaatgatagagtgtc aacaaaaattaggaattaatgatgtctagattagataaaagtaaagtgattaa cagcgcattagagctgcttaatgaggtcggaatcgaaggtttaacaacccg taaactcgcccagaagctaggtgtagagcagcctacattgtattggcatgt aaaaaataagcgggctttgctcgacgccttagccattgagatgttagatag gcaccatactcacttttgccctttagaaggggaaagctggcaagatttttac gtaataacgctaaaagttttagatgtgctttactaagtcatcgcgatggagca aaagtacatttaggtacacggcctacagaaaaacagtatgaaactctcgaa aatcaattagccttttatgccaacaaggtttttcactagagaatgcattatatg cactcagcgctgtggggcattttactttaggttgcgtattggaagatcaaga gcatcaagtcgctaaagaagaaagggaaacacctactactgatagtatgc cgccattattacgacaagctatcgaattatttgatcaccaaggtgcagagcc agccttcttattcggccttgaattgatcatatgcggattagaaaaacaactta aatgtgaaagtgggtcttaagattttttcacaaacagccatttttgttagtc gacgaaatactcttttctctgccccttattcccgctattaaaaaaaacaattaa acgtaaactttgcgcaattcaggccgataaccccggtattcgttttacgtgtc gaaagataaaCGAAGTTCCTATACTTTCTAGAGAA TAGGAACTTCGGAATAGGAACTTCATTTctcgtt cgctgccacctaagaatactctacggtcacatacAAATGGCGCG CCTTACGCCCCGCCCTGCCACTCATCGCAGTA CTGTTGTATTCATTAAGCATCTGCCGACATGG AAGCCATCACAAACGGCATGATGAACCTGAA TCGCCAGCGGCATCAGCACCTTGTCGCCTTG CGTATAATATTTGCCCATGGTGAAAACGGGG GCGAAGAAGTTGTCCATATTGGCCACGTTTA AATCAAAACTGGTGAAACTCACCCAGGGATT GGCTGAGACGAAAAACATATTCTCAATAAAC CCTTTAGGGAAATAGGCCAGGTTTTCACCGT AACACGCCACATCTTGCGAATATATGTGTAG AAACTGCCGGAAATCGTCGTGGTATTCACTC CAGAGCGATGAAAACGTTTCAGTTTGCTCAT GGAAAACGGTGTAACAAGGGTGAACACTATC CCATATCACCAGCTCACCGTCTTTCATTGCCA TACGTAATTCCGGATGAGCATTCATCAGGCG GGCAAGAATGTGAATAAAGGCCGGATAAAA CTTGTGCTTATTTTTCTTTACGGTCTTTAAAA AGGCCGTAATATCCAGCTGAACGGTCTGGTT	

	ATAGGTACATTGAGCAACTGACTGAAATGCC TCAAAATGTTCTTTACGATGCCATTGGGATAT ATCAACGGTGGTATATCCAGTGATTTTTTTCT CCATTTTAGCTTCCTTAGCTCCTGAAAATCTC GACAACTCAAAAAATACGCCCGGTAGTGATC TTATTTCATTATGGTGAAAGTTGGAACCTCTT ACGTGCCGATCAACGTCTCATTTTCGCCAAA AGTTGGCCCAGGGCTTCCCGGTATCAACAGG GACACCAGGATTTATTTATTCTGCGAAGTGA TCTTCCGTCACAGGTAGGCGCGCCGAAGTTC CTATACTTTCTAGAGAATAGGAACTTCGGAA TAGGAACTctcaccgccgcgcaaaaagcgacgctaacccctatttcaaatcagcaatcgtcgtttaccgctaaacttagcgcctacggtacgctgaaaagcgcgctgacgactttccagaccgccaatactgcattgtctaaagccgatctttttttccgctaccagcaccaccagcagcaccaccgcgttcagtgccaccaccgcgggtaatgccatcgccgggaaatacaccatcagcgtcaccctctggcgcaggcgcaaaccctgacaacgcgcaccaccagagacgatacgaaaacggcgatcgccaccagcgacagcaaactcaccattcaacaaggcggcgacaaagatccgatttccattgatatcagcgcggctaactcgtcttaagcgggatccgtgatgccatcaacaacgcaaaagcaggcgtaagcgcaagcatcattaacgtgggtaacggtgaatatcgtctgtcagtcacatcaaatgacaccggcct	
human GLP2 construct, , including the N terminal 20 amino acids of FliC (reverse orientation)	ttaatcagtaattttagtttggatcagccaattaataaaatcacgcgccgccagattatccaggatggtattcatttcgtcagaaaaagagccgtcagcATGcttgttgatattattttgagtgatcagcgagaggctgttggtattaatgacttgtgccat	**SEQ ID NO: 227**
human GLP2 construct with a N terminal OmpF secretion tag (sec-dependent secretion system) under the control of a tet inducible promoter, includes TetR in reverse direction (as shown in FIG. 32C)	ttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtaccaaatgtacttttgctccatcgcgatgacttagtaaagcacatctaaaactttagcgttattacgtaaaaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttttacatgccaatacaatgtaggctgctctacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttacttttatctaatctagacatcattaattcctaatttttgttgacactctatcattgatagagttatttttaccactccctatcagtgatagagaaaagtgaactctagaaataatttttgtttaactttaagaaggagatatacatatgatgaagcgcaatattctggcagtgatcgtccctgctctgttagtagcaggtactgcaaacgctcatgctgatggttctttctctgatgagatgaacaccattcttgataatcttgccgccagggactttataaactggttgattcagaccaaaatcactgacaggtgacacatttccccgaaaagtgccgatggcccccccgatggtagtgtggccccatgcgagagtagggaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctcctgagtaggacaaatccgccgggagcggatttgaacgttgcgaagca	**SEQ ID NO: 228**

	acggcccggagggtggcgggcaggacgcccgccataaactgccaggc atcaaattaagcagaaggccatcctgacggatggccttttgcgtggccag tgccaagcttgcatgcagattgcagcattacacgtcttgagcgattgtgtag gctggagctgcttcgaagttcctatactttctagagaataggaacttcggaat aggaacttc	
human GLP2 construct with a N terminal OmpF secretion tag (sec-dependent secretion system) (as shown in FIG. 32C)	atgatgaagcgcaatattctggcagtgatcgtccctgctctgttagtagcag gtactgcaaacgctcatgctgatggttctttctctgatgagatgaacaccatt cttgataatcttgccgccagggactttataaactggttgattcagaccaaaat cactgacaggtga	**SEQ ID NO: 229**
human GLP2 construct with a N terminal TorA secretion tag (tat secretion system) under the control of a tet inducible promoter (as shown in FIG. 32E)	taagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaa ggccgaataagaaggctggctctgcaccttggtgatcaaataattcgatag cttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttcttctttt agcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgcccc acagcgctgagtgcatataatgcattctctagtgaaaaaccttgttggcata aaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacc taaatgtacttttgctccatcgcgatgacttagtaaagcacatctaaaactttt agcgttattacgtaaaaaatcttgccagctttccccttctaaagggcaaaagt gagtatggtgcctatctaacatctcaatggctaaggcgtcgagcaaagccc gcttatttttacatgccaatacaatgtaggctgctctacacctagcttctggg cgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaat gcgctgttaatcactttacttttatctaatctagacatcattaattcctaatttttgt tgacactctatcattgatagagttattttaccactccctatcagtgatagagaa aagtgaactctagaaataattttgtttaactttaagaaggagatatacatAT GAACAATAACGATCTCTTTCAGGCATCACGT CGGCGTTTTCTGGCACAACTCGGCGGCTTAA CCGTCGCCGGGATGCTGGGGCCGTCATTGTT AACGCCGCGACGTGCGACTGCGcatgctgatggttctt tctctgatgagatgaacaccattcttgataatcttgccgccagggactttata aactggttgattcagaccaaaatcactgactaataacacatttccccgaaaa gtgccgatggccccccgatggtagtgtggcccatgcgagagtagggaac tgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttc gttttatctgttgtttgtcggtgaacgctctcctgagtaggacaaatccgccg ggagcggatttgaacgttgcgaagcaacggcccggagggtggcgggca ggacgcccgccataaactgccaggcatcaaattaagcagaaggccatcc tgacggatggccttttgcgtggccagtgccaagcttgcatgcagattgca gcattacacgtcttgagcgattgtgtaggctggagctgcttcgaagttcctat actttctagagaataggaacttcggaataggaacttc	**SEQ ID NO: 230**
GLP-2 with TORA tag	ATGAACAATAACGATCTCTTTCAGGCATCAC GTCGGCGTTTTCTGGCACAACTCGGCGGCTT AACCGTCGCCGGGATGCTGGGGCCGTCATTG TTAACGCCGCGACGTGCGACTGCGcatgctgatggt tctttctctgatgagatgaacaccattcttgataatcttgccgccagggacttt ataaactggttgattcagaccaaaatcactgac	**SEQ ID NO: 231**

[01173] 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: 224, SEQ ID NO: 225, SEQ ID NO: 226, SEQ ID NO: 227, SEQ ID NO: 228, SEQ ID NO: 229, SEQ ID NO: 230, and SEQ ID NO: 231. Table 67** lists exemplary secretion constructs.

Table 67. Non-limiting Examples of Secretion Constructs

Description	Sequences	SEQ ID NO
Ptet-phoA-hIL10	gaattcgttaagacccactttcacatttaagttgtttttctaatccgcatat gatcaattcaaggccgaataagaaggctggctctgcaccttggtgatca aataattcgatagcttgtcgtaataatggcggcatactatcagtagtagg tgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaac ctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagt gaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcata ctgtttttctgtaggccgtgtacctaaatgtacttttgctccatcgcgatga cttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatcttgc cagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatc tcaatggctaaggcgtcgagcaaagcccgcttatttttacatgccaatac aatgtaggctgctctacacctagcttctgggcgagtttacgggttgttaaa ccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttac ttttatctaatctagacatcattaattcctaatttttgttgacactctatcatt gatagagttattttaccactccctatcagtgatagagaaaagtgaa GACGCCAGAGAGTTAAGGGGGTTAAATGAA ACAATCGACCATCGCATTGGCGCTGCTTCCTC TATTGTTCACACCGGTGACAAAGGCA TCGCCAGGTCAAGGAACGCAGTCAGAGAATT CATGCACTCACTTTCCGGGCAATCTGCCGAA TATGCTGCGCGATCTGCGAGATGCATTCTCTC GCGTGAAAACGTTCTTTCAAATGAAAGATCA ACTGGATAATCTGCTGCTGAAGGAGTCGTTG TTGGAGGATTTTAAGGGGTATCTGGGTTGTC AAGCACTGTCTGAAATGATTCAATTTTACTTG GAGGAAGTTATGCCGCAAGCGGAAAACCAA GATCCGGATATTAAGGCGCACGTGAACTCAC TGGGCGAAAACCTGAAAACTTTGCGCCTGCG TCTGAGACGATGTCACCGATTCCTGCCGTGT GAAAACAAGTCAAAGGCGGTTGAGCAAGTT AAGAATGCTTTCAATAAGCTGCAAGAAAAGG GCATCTATAAAGCGATGTCTGAATTTGATAT CTTTATAAACTACATAGAAGCTTATATGACT ATGAAGATTCGAAATTAA	**SEQ ID NO: 232**
phoA-hIL10	GACGCCAGAGAGTTAAGGGGGTTAAATGAA ACAATCGACCATCGCATTGGCGCTGCTTCCTC TATTGTTCACACCGGTGACAAAGGCA	**SEQ ID NO: 233**

	TCGCCAGGTCAAGGAACGCAGTCAGAGAATT CATGCACTCACTTTCCGGGCAATCTGCCGAA TATGCTGCGCGATCTGCGAGATGCATTCTCTC GCGTGAAAACGTTCTTTCAAATGAAAGATCA ACTGGATAATCTGCTGCTGAAGGAGTCGTTG TTGGAGGATTTTAAGGGGTATCTGGGTTGTC AAGCACTGTCTGAAATGATTCAATTTTACTTG GAGGAAGTTATGCCGCAAGCGGAAAACCAA GATCCGGATATTAAGGCGCACGTGAACTCAC TGGGCGAAAACCTGAAAACTTTGCGCCTGCG TCTGAGACGATGTCACCGATTCCTGCCGTGT GAAAACAAGTCAAAGGCGGTTGAGCAAGTT AAGAATGCTTTCAATAAGCTGCAAGAAAAGG GCATCTATAAAGCGATGTCTGAATTTGATAT CTTTATAAACTACATAGAAGCTTATATGACT ATGAAGATTCGAAATTAA	
fliC UTR-RBS - pvIL10	**tgacggcgattgagccgacgggtggaaacccaaaacgtaatcaact caaatcccttaataaggaggtaaa**ATGGGTACTGACCAA TGTGATAATTTCCCACAAATGCTGCGTGATTT GCGCGACGCTTTCTCGCGTGTGAAAACTTTTT TTCAGACTAAAGATGAGGTGGATAATCTGCT GCTGAAAGAGAGCCTGTTGGAAGATTTTAAA GGCTACTTGGGCTGTCAAGCGCTGTCGGAGA TGATTCAATTTTATCTGGAAGAGGTGATGCC GCAAGCTGAGAACCAAGATCCGGAAGCGAA AGATCACGTGAATTCGCTGGGCGAGAATCTG AAAACTCTGCGTCTGCGTCTGCGTCGTTGTCA CCGTTTTTTGCCGTGCGAAAACAAAAGTAAA GCTGTTGAGCAAATTAAAAACGCTTTTAACA AACTGCAGGAAAAAGGTATCTATAAAGCGAT GAGCGAATTTGATATTTTTATTAATTATATTG AAGCTTATATGACTATTAAAGCTCGCTAA	**SEQ ID NO: 2334**
Ptet-phoA-vIL10	Gaattcgttaagacccactttcacatttaagttgtttttctaatccgcatat gatcaattcaaggccgaataagaaggctggctctgcaccttggtgatca aataattcgatagcttgtcgtaataatggcggcatactatcagtagtagg tgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaac ctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagt gaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcata ctgtttttctgtaggccgtgtacctaaatgtacttttgctccatcgcgatga cttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatcttgc agctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatc tcaatggctaaggcgtcgagcaaagcccgcttatttttacatgccaatac aatgtaggctgctctacacctagcttctgggcgagtttacgggttgttaaa ccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttac ttttatctaatctagacatcattaattcctaatttttgttgacactctatcatt gatagagttatttaccactccctatcagtgatagagaaaagtgaa GACGCCAGAGAGTTAAGGGGGTTAAATGAA	**SEQ ID NO: 235**

	ACAATCGACCATCGCATTGGCGCTGCTTCCTC TATTGTTCACACCGGTGACAAAGGCA GGTACAGACCAATGTGACAATTTTCCCCAAA TGTTGAGGGACCTAAGAGATGCCTTCAGTCG TGTTAAAACCTTTTTCCAGACAAAGGACGAG GTAGATAACCTTTTGCTCAAGGAGTCTCTGCT AGAGGACTTTAAGGGCTACCTTGGATGCCAG GCCCTGTCAGAAATGATCCAATTCTACCTGG AGGAAGTCATGCCACAGGCTGAAAACCAGG ACCCTGAAGCCAAAGACCATGTCAATTCTTT GGGTGAAAATCTAAAGACCCTACGGCTCCGC CTGCGCCGTTGCCACAGGTTCCTGCCGTGTG AGAACAAGAGTAAAGCTGTGGAACAGATAA AAAATGCCTTTAACAAGCTGCAGGAAAAAGG AATTTACAAAGCCATGAGTGAATTTGACATT TTTATTAACTACATAGAAGCATACATGACAA TTAAAGCCAGG	
phoA-vIL10	<u>GACGCCAGAGAGTTAAGGGGGTTAA</u>ATGAA ACAATCGACCATCGCATTGGCGCTGCTTCCTC TATTGTTCACACCGGTGACAAAGGCA GGTACAGACCAATGTGACAATTTTCCCCAAA TGTTGAGGGACCTAAGAGATGCCTTCAGTCG TGTTAAAACCTTTTTCCAGACAAAGGACGAG GTAGATAACCTTTTGCTCAAGGAGTCTCTGCT AGAGGACTTTAAGGGCTACCTTGGATGCCAG GCCCTGTCAGAAATGATCCAATTCTACCTGG AGGAAGTCATGCCACAGGCTGAAAACCAGG ACCCTGAAGCCAAAGACCATGTCAATTCTTT GGGTGAAAATCTAAAGACCCTACGGCTCCGC CTGCGCCGTTGCCACAGGTTCCTGCCGTGTG AGAACAAGAGTAAAGCTGTGGAACAGATAA AAAATGCCTTTAACAAGCTGCAGGAAAAAGG AATTTACAAAGCCATGAGTGAATTTGACATT TTTATTAACTACATAGAAGCATACATGACAA TTAAAGCCAGG	**SEQ ID NO: 236**
Ptet- PhoA-IL22	Gaattcgttaagacccactttcacatttaagttgtttttctaatccgcatat gatcaattcaaggccgaataagaaggctggctctgcaccttggtgatca aataattcgatagcttgtcgtaataatggcggcatactatcagtagtagg tgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaac ctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagt gaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcata ctgtttttctgtaggccgtgtacctaaatgtacttttgctccatcgcgatga cttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatcttgc cagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatc tcaatggctaaggcgtcgagcaaagcccgcttatttttacatgccaatac aatgtaggctgctctacacctagcttctgggcgagtttacgggttgttaaa ccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttac	**SEQ ID NO: 237**

	ttttatctaatctagacatcattaattcctaatttttgttgacactctatcatt gatagagttattttaccactccctatcagtgatagagaaaagtgaa GACGCCAGAGAGTTAAGGGGGTTAAATGAA ACAATCGACCATCGCATTGGCGCTGCTTCCTC TATTGTTCACACCGGTGACAAAGGCA GCACCGATCTCTTCCCACTGTCGCTTAGATAA ATCGAATTTTCAACAACCTTATATTACGAATC GTACGTTTATGCTGGCTAAAGAAGCGTCATT AGCTGATAACAACACTGATGTTCGCCTGATT GGTGAGAAATTGTTTCACGGTGTGTCTATGTC AGAACGTTGCTACCTGATGAAACAAGTTCTG AATTTCACCCTGGAAGAAGTGTTGTTTCCGC AATCTGACCGCTTTCAACCGTATATGCAAGA GGTTGTGCCGTTTCTGGCGCGCCTGAGTAATC GCCTGAGCACTTGTCATATTGAGGGCGACGA CCTGCATATTCAACGAAATGTTCAAAAATTG AAAGATACGGTGAAGAAACTGGGTGAAAGT GGTGAAATCAAAGCGATTGGTGAGCTGGATC TGCTGTTTATGTCATTGCGCAATGCGTGCATT TAA	
PhoA-IL22	GACGCCAGAGAGTTAAGGGGGTTAAATGAA ACAATCGACCATCGCATTGGCGCTGCTTCCTC TATTGTTCACACCGGTGACAAAGGCA GCACCGATCTCTTCCCACTGTCGCTTAGATAA ATCGAATTTTCAACAACCTTATATTACGAATC GTACGTTTATGCTGGCTAAAGAAGCGTCATT AGCTGATAACAACACTGATGTTCGCCTGATT GGTGAGAAATTGTTTCACGGTGTGTCTATGTC AGAACGTTGCTACCTGATGAAACAAGTTCTG AATTTCACCCTGGAAGAAGTGTTGTTTCCGC AATCTGACCGCTTTCAACCGTATATGCAAGA GGTTGTGCCGTTTCTGGCGCGCCTGAGTAATC GCCTGAGCACTTGTCATATTGAGGGCGACGA CCTGCATATTCAACGAAATGTTCAAAAATTG AAAGATACGGTGAAGAAACTGGGTGAAAGT GGTGAAATCAAAGCGATTGGTGAGCTGGATC TGCTGTTTATGTCATTGCGCAATGCGTGCATT TAA	**SEQ ID NO: 238**
	GACGCCAGAGAGTTAAGGGGGTTAAATGAA ACAATCGACCATCGCATTGGCGCTGCTTCCTC TATTGTTCACACCGGTGACAAAGGCA	**SEQ ID NO: 239**

[01174] 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:

232, SEQ ID NO: 233, SEQ ID NO: 2334, SEQ ID NO: 235, SEQ ID NO: 236, SEQ ID NO: 237, SEQ ID NO: 238, and SEQ ID NO: 239.

Example 27. Bacterial Secretion of hIL-10 and vIL-10

[01175] To determine whether the human IL-10 and vIL-10 expressed by engineered bacteria is secreted, the concentration of IL-10 in the bacterial supernatant from a selection of engineered strains comprising various hIL-10 and vIL-10 constructs/strains was measured (see **Table 63, Table 64, Table 65, Table 66, Table 67** for components and sequences for hIL-10 and vIL-10 constructs/strains).

[01176] E. coli Nissle comprising various tet-inducible constructs or constructs under the native fliC promoter were grown overnight in LB medium. Cultures were diluted 1:200 in LB and grown shaking (200 rpm) for 2 hours. Cultures were diluted to an optical density of 0.5 at which time anhydrous tetracycline (ATC) was added to cultures at a concentration of 100ng/mL to induce expression of hIL-10. No tetracycline was added to cultures harboring the fliC constructs. 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 unsoluble debris is spun down at 20 min at 12,000 rcf at 4◦C then placed on ice until further processing.

[01177] The concentration of hIL-10 in the cell-free medium and in the bacterial cell extract was measured by hIL-10 ELISA (R&D Systems DY217B), according to manufacturer's instructions. Similarly, to determine the concentrations of vIL-10 an Ultrasensitive ELISA kit (Alpco, 45-I10HUU-E01) was employed using commercially available recombinant vIL-10 (R&D Systems, 915-VL-010). All samples were run in triplicate, and a standard curve was used to calculate secreted levels of IL-10. Standard curves were generated using both human and viral recombinant proteins. Wild type Nissle was included in the ELISA as a negative control, and no signal was observed. **Table 68** and **Table 69** summarize levels of hIL10 and vIL-10 measured in the supernatant and

intracellularly **Table 68** and extracellularly **Table 69**. The data show that both vIL-10 and hIL-10 are secreted at various levels from the different bacterial strains.

Table 68. hIL-10 Secretion

Sample	**hu IL-10 (ng/ml) (extracellular)**
WT	0
IL-10 Plasmid (Nissle pUC57.Ptet-phoA-hIL10)	8.4
IL-10 plasmid/lpp (lpp::Cm pUC57.Ptet-phoA-hIL10)	19.3
2083 IL-10 plasmid/nlpI (nlpI::Cm pUC57.Ptet-phoA-hIL10)	20.5
2084 IL-10 plasmid/tolA (tolA::Cm pUC57.Ptet-phoA-hIL10)	21.4
2085 IL-10 plasmid/pal (PAL::Cm pUC57.Ptet-phoA-hIL10)	28.4

Table 69. vIL-10 Secretion

Sample	**vIL-10 (ng/ml) (extracellular)**
WT	0
fliC-pvIL10 (Nissle pUN fli-vIL10 Kan Cm)	29
fliC ::vIL10 (Nissle fliC::vIL10 delta fliD CmR)	9
vIL-10 *lpp* (Nissle lpp mutant with vIL10 pBR3222 tet plasmid)	527
vIL-10 *nlpI* (Nissle delta nlpI::CmR pBR322.Ptet-phoA-vIL10)	982
vIL-10 *tolA* (Nissle delta tolA::CmR pBR322.Ptet-	428

phoA-vIL10)	
vIL-10 *pal* (Nissle delta PAL::CmR pBR322.Ptet-phoA-vIL10	1090

Co-culture studies

[01178] To determine whether the hIL-10 and viral IL-10 expressed by the genetically engineered bacteria shown in **Table 68** and **Table 69** is biologically functional, *in vitro* experimentation is conducted, in which the bacterial supernatant containing secreted human or viral IL-10 is added to the growth medium of a Raji cells (a hematopoietic cell line) and J774a1 cells (a macrophage cell line). IL-10 is known to induce the phosphorylation of STAT3 in these cells Functional activity of bacterially secreted IL-10 is therefore assessed by its ability to phosphorylate STAT3 in Raji and J774a1 cells.

[01179] Raji cells are grown in RPMI 1640 supplemented with 10% FBS supplemented with 10% fetal bovine serum at 37°C in a humidified incubator supplemented with 5% CO2. Prior to treatment with the bacterial supernatant, RPMI 1640 supplemented with 10% FBS (1e6/24 well) are serum starved overnight. Titrations of either recombinant human IL-10 diluted in LB or clarified supernatant from wild type Nissle or the engineered bacteria are added to cells for 30 minutes. Cells are harvested and resuspended in lysis buffer, and phospho-STAT3 ELISA (ELISA pSTAT3 (Tyr705) (Cell Signaling Technology)) is run in triplicate for all samples, according to manufacturer's instructions. PBS-treated cells and PBS are added as negative controls. Dilutions of samples are included to demonstrate linearity.

Competition studies

[01180] As an additional control for specificity, a competition assay is performed. Titrations of anti-IL10 antibody are pre-incubated with constant concentrations of either rhIL10 (data not shown) or supernatants from the engineered bacteria expressing human or viral IL-10 for 15 min. Next, the supernatants/ rhIL10 solutions are added to serum-starved Raji cells (1e6/well) and cells are incubated for 30 min followed by pSTAT3 ELISA as described above.

[01181] In other embodiments, similar studies are conducted with J774a1 cells.

Example 27. Bacterial Secretion of GLP-2

[01182] To determine whether the human GLP-2 expressed by engineered bacteria is secreted, the concentration of GLP-2 in the bacterial supernatant from two engineered strains comprising GLP-2 constructs/strains was measured. The first strain comprising a deletion in PAL and a plasmid expressing GLP-2 with an OmpF secretion tag from a tetracycline-inducible promoter and the second strain comprises the same PAL deletion and the same plasmid expressing GLP-2, further comprising a deletion in degP (see **Table 74**).

[01183] E. coli Nissle comprising various tet-inducible constructs or constructs under the native fliC promoter were grown overnight in LB medium. Cultures were diluted 1:200 in LB and grown shaking (200 rpm) for 2 hours. Cultures were diluted to an optical density of 0.5 at which time anhydrous tetracycline (ATC) was added to cultures at a concentration of 100ng/mL to induce expression of hIL-10. No tetracycline was added to cultures harboring the fliC constructs. 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 is spun down at 20 min at 12,000 rcf at 4◦C then placed on ice until further processing.

[01184] The concentration of GLP-2 in the cell-free medium and in the bacterial cell extract was measured by Human GLP2 ELISA Kit (Competitive EIA) (LSBio), according to manufacturer's instructions. All samples were run in triplicate, and a standard curve was used to calculate secreted levels of GLP-2. Standard curves were generated using recombinant GLP-2. Wild type Nissle was included in the ELISA as a negative control, and no signal was observed. As seen in **Table 70**, deletion of degP, a periplasmic protease, improved secretion levels over 3-fold.

Table 70. GLP-2 Secretion

DOM mut	ng/ml

WT	1.14
PAL::CmR Ptet-ompF-GLP2	1793.2
PAL::CmR ompT::Kan Ptet-ompF-GLP2	1142.1
PAL::CmR ompT::Kan phoA-GLP2 fusion	5360.4

Co-culture studies

[01185] To determine whether the hGLP-2 expressed by the genetically engineered bacteria is biologically functional, *in vitro* experimentation is conducted, in which the bacterial supernatant (from both strains shown above) containing secreted human GLP-2 is added to the growth medium of Caco-2 cells and CCD-18Co cells. The Caco-2 cell line is a continuous cell of heterogeneous human epithelial colorectal adenocarcinoma cells. As described e.g., in Jasleen et al. (Dig Dis Sci. 2002 May;47(5):1135-40) GLP-2 stimulates proliferation and [3H]thymidine incorporation in Caco-2 and T84 cells. Additionally, GLP-2 stimulates VEGFA secretion in these cells (see., e.g., Bulut et al, Eur J Pharmacol. 2008 Jan 14;578(2-3):279-85.

[01186] Functional activity of bacterially secreted GLP-2 is therefore assessed by its ability to induce proliferation and VEGF secretion.

[01187] Caco-2 are grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator supplemented with 5% CO2. Prior to treatment with the bacterial supernatant, Caco-2 cells (1e6/24 well) are serum starved overnight. Titrations of either recombinant human GLP-2 (50 and 250 nM) diluted in LB or clarified supernatant from wild type Nissle or the engineered bacteria are added to cells for a defined time.

[01188] For cell proliferation assays, cells are harvested and resuspended in lysis buffer. The cells are assayed after 12, 24, 48, and 72 hours of incubation. Cell proliferation is measured using a Cell proliferation assay kit according to manufacturers instruction (e.g., a Cell viability was assessed by a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT)-assay).

[01189] For the measurements of VEFA secretion, cells are harvested and resuspended in lysis buffer, and concentrations of GLP-2 in the medium are determined ELISA

[01190] PBS-treated cells and PBS are added as negative controls. Dilutions of samples are included to demonstrate linearity.

Competition studies

[01191] As an additional control for specificity, a competition assay is performed. Titrations of anti-GLP-2 antibody are pre-incubated with constant concentrations of either recombinant GLP-2 or supernatants from the engineered bacteria for 15min. Next, the supernatants/ rhIL2 solutions are added to serum-starved Cac-2 cells (1e6/well) and cells are incubated for 30 min followed by VEGFA ELISA as described above.

Example 28. Bacterial Secretion of IL-22

[01192] To determine whether the human IL-22 expressed by engineered bacteria is secreted, the concentration of IL-22 in the bacterial supernatant from a two engineered strains comprising IL-22 constructs/strains was measured. The first strain comprising a deletion in PAL and a plasmid expressing IL-22 with an OmpF secretion tag from a tetracycline-inducible promoter and the second strain comprises the same PAL deletion and the same plasmid expressing IL-22, further comprising a deletion in degP (**Table 71**).

[01193] E. coli Nissle comprising various tet-inducible constructs or constructs under the native fliC promoter were grown overnight in LB medium. Cultures were diluted 1:200 in LB and grown shaking (200 rpm) for 2 hours. Cultures were diluted to an optical density of 0.5 at which time anhydrous tetracycline (ATC) was added to cultures at a concentration of 100ng/mL to induce expression of hIL-10. No tetracycline was added to cultures harboring the fliC constructs. 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 BugBusterTM (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 unsoluble debris is spun down at 20 min at 12,000 rcf at 4◦C then placed on ice until further processing.

[01194] The concentration of IL-22 in the cell-free medium and in the bacterial cell extract was measured by hIL-22 ELISA (R&D Systems (DY782) ELISA for hIL-22), according to manufacturer's instructions. All samples were run in triplicate, and a standard curve was used to calculate secreted levels of IL-22. Standard curves were generated using recombinant IL-22. Wild type Nissle was included in the ELISA as a negative control, and no signal was observed. **Table 71** summarizes levels of IL-22 measured in the supernatant. The data show that both hIL-22 are secreted at various levels from the different bacterial strains.

Table 71. IL-22 Secretion

Genotype	**IL-22 Production/Secretion Dilution Corrected (ng/ml)**
WT	20.7
Lpp (delta lpp::CmR expressing PhoA-IL22 from Ptet)	87.6
nlpI (delta nlpI::CmR expressing PhoA-IL22 from Ptet)	105.4
tolA (delta tolA::CmR expressing PhoA-IL22 from Ptet)	623.2
PAL (delta pal::CmR expressing PhoA-IL22 from Ptet)	328.8

Example 29. Bacterial Secretion of IL-22 and Functional Assays

Generation of Bacterial Supernatant and Measurement of IL-22 concentration

[01195] To determine whether the human IL-22 expressed by engineered bacteria is secreted, the concentration of IL-22 in the bacterial supernatant was measured.

[01196] *E. coli* Nissle comprising a tet-inducible integrated construct (delta pal::CmR expressing PhoA-IL22 from Ptet) was grown overnight in LB medium. Cultures were diluted 1:200 in LB and grown shaking (200 rpm) for 2 hours. Cultures were diluted

to an optical density of 0.5 at which time anhydrous tetracycline (ATC) was added to cultures at a concentration of 100ng/mL to induce expression of hIL-22. After 12 hours of induction, cells were spun down, and supernatant was collected. To generate cell free medium, the supernatant was centrifuged, and filtered through a 0.22 micron filter to remove any remaining bacteria.

[01197] The concentration of hIL-22 in the cell-free medium was measured by hIL-22 ELISA (R&D Systems (DY782) ELISA for hIL-22), according to manufacturer's instructions. All samples were run in triplicate, and a standard curve was used to calculate secreted levels of IL-22. Additionally, samples were diluted to ensure absence of matrix effects and to demonstrate linearity. Wild type Nissle was included in the ELISA as a negative control, and no signal was observed. The engineered bacteria comprising a PAL deletion and the integrated construct encoding hIL-22 with a phoA secretion tag were determined to be secreting at 199 ng/ml supernatant.

Co-culture studies

[01198] To determine whether the hIL-22 expressed by the genetically engineered bacteria is biologically functional, *in vitro* experimentation was conducted, in which the bacterial supernatant containing secreted human IL-22 was added to the growth medium of a mammalian colonic epithelial cell line. IL-22 is known to induce the phosphorylation of STAT1 and STAT3 in Colo205 cells (see, e.g., Nagalakshmi et al., Interleukin-22 activates STAT3 and induces IL-10 by colon epithelial cells. Int Immunopharmacol. 2004 May;4(5):679-91). Functional activity of bacterially secreted IL-22 was therefore assessed by its ability to phosphorylate STAT3 in Colo205 cells.

[01199] Colo205 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator supplemented with 5% CO2. Prior to treatment with the bacterial supernatant, Colo205 (1e6/24 well) were serum starved overnight. Titrations of either recombinant human IL-22 diluted in LB or clarified supernatant from wild type Nissle or the engineered bacteria were added to cells for 30 minutes. Cells were harvested and resuspended in lysis buffer, and phospho-STAT3 ELISA (ELISA pSTAT3 (Tyr705) (Cell Signaling Technology)) was run in triplicate for all samples, according to manufacturer's instructions. PBS-treated cells and PBS were added as negative controls. Dilutions of samples were included to demonstrate linearity. No signal was observed for wild type Nissle. Results for the

engineered strain comprising a PAL deletion and the integrated construct encoding hIL-22 with a phoA secretion tag are shown in **FIG. 33A**, and demonstrate that hIL-22 secreted from the engineered bacteria is functionally active.

Competition studies

[01200] As an additional control for specificity, a competition assay was performed. Titrations of anti-IL22 antibody (MAB7821, R&D Systems) were pre-incubated with constant concentrations of either rhIL22 (data not shown) or supernatants from the engineered bacteria for 15min. Next, the supernatants/ rhIL2 solutions were added to serum-starved Colo205 cells (1e6/well) and cells were incubated for 30 min followed by pSTAT3 ELISA as described above. As shown in **FIG. 33B**, the phospho-Stat3 signal induced by the secreted hIL-22 is competed by the hIL-22 antibody MAB7821.

Example 30. Generation of Indole Propionic Acid Strain and in vitro indole production

[01201] To facilitate inducible production of indole propionic acid (IPA) in *Escherichia coli* Nissle, 6 genes allowing the production of indole propionic acid from tryptophan, as well as transcriptional and translational elements, are synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322 under a tet inducible promoter. In other embodiments, the IPA synthesis cassette is put under the control of an FNR, RNS or ROS promoter, e.g., described herein, or other promoter induced by conditions in the healthy or diseased gut, e.g., inflammatory conditions. For efficient translation of IPA synthesis genes, each synthetic gene in the cassette is separated by a 15 base pair ribosome binding site derived from the T7 promoter/translational start site.

[01202] The IPA synthesis cassette comprises TrpDH (tryptophan dehydrogenase from Nostoc punctiforme NIES-2108), FldH1/FldH2 (indole-3-lactate dehydrogenase from Clostridium sporogenes), FldA (indole-3-propionyl-CoA:indole-3-lactate CoA transferase from Clostridium sporogenes), FldBC (indole-3-lactate dehydratase from Clostridium sporogenes), FldD (indole-3-acrylyl-CoA reductase from Clostridium sporogenes), and AcuI (acrylyl-CoA reductase from Rhodobacter sphaeroides).

[01203] The tet inducible IPA construct described above is transformed into E.coli Nissle as described herein and production of IPA is assessed. In certain embodiments, *E. coli* Nissle strains containing the IPA synthesis cassette described further comprise a tryptophan synthesis cassette. In certain embodiments, the strains comprise a feedback resistant version of AroG and TrpE to achieve greater Trp production. In certain embodiments, additionally, the tnaA gene (tryptophanase converting Trp into indole) is deleted.

[01204] All incubations are performed at 37°C. LB-grown overnight cultures of *E. coli* Nissle transformed with the IPA biosynthesis construct alone or in combination with a tryptophan biosynthis construct and feedback resistant AroG and TrpE are subcultured 1:100 into 10mL of M9 minimal medium containing 0.5% glucose and grown shaking (200 rpm) for 2h, at which time anhydrous tetracycline (ATC) is added to cultures at a concentration of 100ng/mL to induce expression of the the IPA biosynthesis and tryptophan biosynthesis constructs. After 2 hours of induction, cells are spun down, supernatant is discarded, and the cells are resuspended in M9 minimal media containing 0.5% glucose. Culture supernatant is then analyzed at predetermined time points (e.g., 0 up to 24 hours) by LC-MS to assess levels of IPA.

[01205] Production of IPA is also assessed in E. coli Nissle strains containing the IPA and tryptophan cassettes both driven by an RNS promoter e.g., a nsrR-norB-IPA biosynthesis construct) in order to assess nitrogen dependent induction of IPA production. Overnight bacterial cultures are 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) wis added to cultures at a final concentration of 0.3mM to induce expression from plasmid. After 2 hours of induction, cells are spun down, supernatant is discarded, and the cells are resuspended in M9 minimal media containing 0.5% glucose. Culture supernatant is then analyzed at predetermined time points (0 up to 24 hours, as shown in Fig. 33) to assess IPA levels.

[01206] In alternate embodiments, production of IPA is also assessed in *E. coli* Nissle strains containing the IPA and tryptophan cassettes both driven by the low oxygen inducible FNR promoter, e.g., FNRS, or the the reactive oxygene regulated OxyS promoter.

Example 31. FNR promoter activity

[01207] 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 21**. The nucleotide sequences of these constructs are shown in **Table 72 through Table 76** ((**SEQ ID NO: 240-244**). 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 the figures**.**

[01208] **Table 72** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr1} **(SEQ ID NO: 240).** 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.

[01209] **Table 73** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr2} ((**SEQ ID NO: 241**). 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.

[01210] **Table 74** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr3} ((**SEQ ID NO: 242**). 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.

[01211] **Table 75** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr4} (**(SEQ ID NO: 243**). 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.

[01212] **Table 76** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, PfnrS (**(SEQ ID NO: 244**). 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 72. Pfnr1-lacZ construct Sequences

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 240)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatcg** **tcgtccggccttttcctctcttactctgctacgtacatctatttctataaatccgttcaa** **tttgtctgtttttgcacaaacatgaaatatcagacaattccgtgacttaagaaaattta** **tacaaatcagcaatatacccccttaaggagtatataaaggtgaatttgatttacatcaata** **agcggggttgctgaatcgttaaggtaggcggtaatagaaaagaaatcgaggcaaaaATGa** **gcaaagtcagactcgcaattat**GGATCCTCTGGCCGTCGTATTACAACGTCGTGACTGGG AAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGC GTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCG AATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCG ATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATG CGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCGCGG AGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACAGGAAG GCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGC GCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTT TACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATC TGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATA AACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCAGCC GCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGGTGA CGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTG AAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTTGAAA ATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGCACA

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 240)
CCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCGAGGTGCGGA TTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCGTC ACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCC TGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGC TGTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAA CCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGA GCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGT CGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAAT CTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCG ATATTATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGA AATGGTCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCG AATATGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTC GTCAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTA AATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGA ACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGC TGACGGAAGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCG AAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGG CACTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTA AGCAGTTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAA CGGTACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCT GGCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCA TCCCTCAACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGC AATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGC TGACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTG AAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACC AGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTA CAACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACC GGATTGATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACAC CGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACT GGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACC GCTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTC TGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGT TCAACATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGC ACGCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACG ACTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACC AGTTGGTCTGGTGTCAAAAATAA

Table 73. Pfnr2-lacZ construct sequences

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 241)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctca** **tgcatgcatcaaaaaagatgtgagcttgatcaaaaacaaaaaatatttcactcgacagga** **gtatttatattgcgcccgttacgtgggcttcgactgtaaatc**agaaaggagaaaacacct **ATGacgacctacgatcg**GGATCCTCTGGCCGTCGTATTACAACGTCGTGACTGGGAAAAC

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 241)
CCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGCGTAAT
AGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGG
CGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTT
CCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCT
ATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCGCGGAGAAT
CCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACAGGAAGGCCAG
ACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGG
GTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGC
GCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAA
GATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCG
ACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCAGCCGCGCG
GTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGGTGACGGTT
TCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATT
ATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTTGAAAATCCG
GAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGCACACCGCC
GACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCGAGGTGCGGATTGAA
AATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCGTCACGAG
CATCATCCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTG
ATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGG
TACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCAC
GGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGAGCGAA
CGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTG
GGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTC
GATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATT
ATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGG
TCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATAT
GCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAG
TACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATAT
GATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGAT
CGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCTGACG
GAAGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTG
ACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTG
GATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAG
TTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGTA
CGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAG
CAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCT
CAACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTT
AACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTGACC
CCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCG
ACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCC
GAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACC
GCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATT
GATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCAT
CCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTC
GGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCTGG
GATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGC
TGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAAC

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 241)
ATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCG GAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCC TGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTG GTCTGGTGTCAAAAATAA

Table 74. Pfnr3-lacZ construct Sequences

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 242)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatcg** **tcgtccggccttttcctctcttactctgctacgtacatctatttctataaatccgttcaa** **tttgtctgtttttgcacaaacatgaaatatcagacaattccgtgacttaagaaaattta** **tacaaatcagcaatatacccccttaaggagtatataaaggtgaatttgatttacatcaata** **agcggggttgctgaatcgttaa**GGATCC**ctctagaaataattttgtttaactttaagaag** **gagatatacat****ATG**ACTATGATTACGGATTCTCTGGCCGTCGTATTACAACGTCGTGACT GGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCT GGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATG GCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGT GCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACG ATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCG CGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACAGG AAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACG GGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGCGCAT TTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTT ATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGC ATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCA GCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGG TGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCG GTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTTG AAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGC ACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCGAGGTGC GGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGCGGCGTTAACC GTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATA TCCTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATC CGCTGTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTG AAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGA TGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCT GGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCA AATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCCGACACCACGGCCA CCGATATTATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGC CGAAATGGTCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTT GCGAATATGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGT TTCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGA TTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGC CGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGG

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 242)
CGCTGACGGAAGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCA TCGAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGG TGGCACTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAG GTAAGCAGTTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGC TAACGGTACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCG CCTGGCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACG CCATCCCTCAACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTT GGCAATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAAC TGCTGACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAA GTGAAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATT ACCAGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGA TTACAACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCT ACCGGATTGATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATA CACCGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAA ACTGGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTG ACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACG GTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCC AGTTCAACATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGC TGCACGCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCG ACGACTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATT ACCAGTTGGTCTGGTGTCAAAAATAA

Table 75. Pfnr4-lacZ construct Sequences

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 243)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctca** **tgcatgcatcaaaaaagatgtgagcttgatcaaaaacaaaaaatatttcactcgacagga** **gtatttatattgcgccc**GGATCC**ctctagaaataattttgtttaactttaagaaggagat** **atacat****ATG**ACTATGATTACGGATTCTCTGGCCGTCGTATTACAACGTCGTGACTGGGAA AACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGCGT AATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAA TGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGAT CTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCG CCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCGCGGAG AATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACAGGAAGGC CAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGC TGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTA CGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTG GAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAA CCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCAGCCGC GCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGGTGACG GTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAA ATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTTGAAAAT CCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGCACACC

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 243)
GCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCGAGGTGCGGATT GAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCGTCAC GAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTG CTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTG TGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACC CACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGAGC GAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCG CTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCT GTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGAT ATTATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAA TGGTCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAA TATGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGT CAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAA TATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAAC GATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCTG ACGGAAGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAA GTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCA CTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAG CAGTTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACG GTACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGG CAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATC CCTCAACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAA TTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTG ACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAA GCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAG GCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACA ACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGG ATTGATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCG CATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGG CTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGC TGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTG CGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTC AACATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCAC GCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGAC TCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAG TTGGTCTGGTGTCAAAAATAA

Table 76. Pfnrs-lacZ construct Sequences

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 244)
GGTACC**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgta** **acaaaagcaatttttccggctgtctgtatacaaaaacgccgtaaagtttgagcgaagtca** **ataaactctctacccattcagggcaatatctctcttGGATCC<u>ctctagaaataattttgt</u>** **<u>ttaactttaagaaggagatatacatATG</u>**CTATGATTACGGATTCTCTGGCCGTCGTATTA

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 244)
CAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCC
CCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTG
CGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAA
AGCTGGCTGGAGTGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAG
ATGCACGGTTACGATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCG
CCGTTTGTTCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAA
AGCTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCAT
CTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTT
GACCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGG
AGTGACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGAC
GTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTT
AATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGC
GATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACC
GCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTA
CGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCA
GTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGT
TTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATT
CGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCAGACG
ATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCAT
TATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGAT
GAAGCCAATATTGAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGC
TGGCTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCG
AGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTG
TATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCC
GACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCC
TTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGC
CCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAA
TACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTG
GATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGAT
TTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGC
ACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTA
TCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTC
CTGCACTGGATGGTGGCACTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGAT
GTTGGCCCGCAAGGTAAGCAGTTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCC
GGACAACTCTGGCTAACGGTACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCC
GGACACATCAGCGCCTGGCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCC
TCCGCGTCCCACGCCATCCCTCAACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTG
GGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGC
GATGAAAAACAACTGCTGACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAAC
GACATTGGCGTAAGTGAAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAG
GCGGCGGGCCATTACCAGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCC
GACGCGGTGCTGATTACAACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATC
AGCCGGAAAACCTACCGGATTGATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCG
GTGGCAAGCGATACACCGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTC
TCAGAGCGGGTAAACTGGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACT
GCAGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTC
CCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGG

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 244)
CGCGGCGACTTCCAGTTCAACATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGC CATCGCCATCTGCTGCACGCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATG GGGATTGGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCC GGTCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAA

Example 32. Nitric oxide-inducible reporter constructs

[01213] 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, as shown in the figures; 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 77. SEQ ID NO: 245

SEQ ID NO: 245
ttatta**tcgcaccgcaatcgggattttcgattcataaagcaggtcgtaggtcggcttgtt** **gagcaggtcttgcagcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgcc** **gagtatgcccgtcagccggcaggacggcgtaatcaggcattcgttgttcgggcccataca** **ctcgaccagctgcatcggttcgaggtggcggacgaccgcgccgatattgatgcgttcggg** **cggcgcggccagcctcagcccgccgcctttcccgcgtacgctgtgcaagaacccgccttt** **gaccagcgcggtaaccactttcatcaaatggcttttggaaatgccgtaggtcgaggcgat** **ggtggcgatattgaccagcgcgtcgtcgttgacggcggtgtagatgaggacgcgcagccc** **gtagtcggtatgttgggtcagatacat**acaacctccttagtacatgcaaaattatttcta gagcaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagttgagtt gaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatggttg*ttga* *caattaatcatcggctcgtataatgtataacattcatattttgtgaattttaaactctag* *aaataatttttgtttaactttaagaaggagatatacata***tggctagcaaaggcgaagaatt** **gttcacgggcgttgttcctattttggttgaattggatggcgatgttaatggccataaatt**

cagcgttagcggcgaaggcgaaggcgatgctacgtatggcaaattgacgttgaaattcat
ttgtacgacgggcaaattgcctgttccttggcctacgttggttacgacgttcagctatgg
cgttcaatgtttcagccgttatcctgatcatatgaaacgtcatgatttcttcaaaagcgc
tatgcctgaaggctatgttcaagaacgtacgattagcttcaaagatgatggcaattataa
aacgcgtgctgaagttaaattcgaaggcgatacgttggttaatcgtattgaattgaaagg
cattgatttcaaagaagatggcaatattttgggccataaattggaatataattataatag
ccataatgtttatattacggctgataaacaaaaaaatggcattaaagctaatttcaaaat
tcgtcataatattgaagatggcagcgttcaattggctgatcattatcaacaaaatacgcc
tattggcgatggccctgttttgttgcctgataatcattatttgagcacgcaaagcgcttt
gagcaaagatcctaatgaaaaacgtgatcatatggttttgttggaattcgttacggctgc
tggcattacgcatggcatggatgaattgtataaataataa

[01214] 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.

[01215] **FIG. 63D** 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.

[01216] 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. The figures shows NsrR-regulated promoters are induced in DSS-treated mice, but not in untreated mice.

Example 33. Generation of ΔThyA

[01217] 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.

[01218] 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 78.**

Table 78. Primer Sequences

Name	**Sequence**	**Description**	**SEQ ID NO**
SR36	tagaactgatgcaaaaagtgctcgacgaaggcacacagaTGTGTAGGCTGGAGCTGCTTC	Round 1: binds on pKD3	**SEQ ID NO: 246**
SR38	gtttcgtaattagatagccaccggcgctttaatgcccggaCATATGAATATCCTCCTTAG	Round 1: binds on pKD3	**SEQ ID NO: 247**
SR33	caacacgtttcctgaggaaccatgaaacagtatttagaactgatgcaaaaag	Round 2: binds to round 1 PCR product	**SEQ ID NO: 248**

SR34	cgcacactggcgtcggctctggcaggatgtttcgtaattagatagc	Round 2: binds to round 1 PCR product	**SEQ ID NO: 249**
SR43	atatcgtcgcagcccacagcaacacgtttcctgagg	Round 3: binds to round 2 PCR product	**SEQ ID NO: 250**
SR44	aagaatttaacggagggcaaaaaaaaccgacgcacactggcgtcggc	Round 3: binds to round 2 PCR product	**SEQ ID NO: 251**

[01219] 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:

[01220] step1: 98c for 30s

[01221] step2: 98c for 10s

[01222] step3: 55c for 15s

[01223] step4: 72c for 20s

[01224] repeat step 2-4 for 30 cycles

[01225] step5: 72c for 5min

[01226] 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.

[01227] 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.

[01228] 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).

[01229] 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.

[01230] 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 34. Nissle residence

[01231] 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.

[01232] 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-UCD103) 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 79**. 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 79: CFU administered via oral gavage

CFU administered via oral gavage			
Strain	Day 1	Day 2	Day 3
SYN-UCD103	1.30E+08	8.50E+08	1.90E+09

[01233] On days 2-10, fecal pellets were collected from up to 6 mice (ID NOs. 1-6; **Table 80**). 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.

[01234] 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 80**.

[01235] **FIG. 69** 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 80. 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 35. Intestinal Residence and Survival of Bacterial Strains in vivo

[01236] Localization and intestinal residence time of streptomycin resistant Nissle, **FIG. 70,** was determined. Mice were gavaged, sacrificed at various time points, and effluents were collected from various areas of the small intestine cecum and colon.

[01237] 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.

[01238] 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.70**.

Example 36. Efficacy of Butyrate-Expressing Bacteria in a Mouse Model of IBD

[01239] Bacteria harboring the butyrate cassettes described above are grown overnight in LB. Bacteria are then diluted 1:100 into LB containing a suitable selection marker, e.g., ampicillin, and grown to an optical density of 0.4-0.5 and then pelleted by centrifugation. Bacteria are resuspended in phosphate buffered saline and 100 microliters is administered by oral gavage to mice. IBD is induced in mice by supplementing drinking water with 3% dextran sodium sulfate for 7 days prior to bacterial gavage. Mice are treated daily for 1 week and bacteria in stool samples are detected by plating stool homogenate on agar plates supplemented with a suitable selection marker, e.g., ampicillin. After 5 days of bacterial treatment, colitis is scored in live mice using endoscopy. Endoscopic damage score is determined by assessing colon translucency, fibrin attachment, mucosal and vascular pathology, and/or stool characteristics. Mice are sacrificed and colonic tissues are isolated. Distal colonic sections are fixed and scored for inflammation and ulceration. Colonic tissue is homogenized and measurements are made for myeloperoxidase activity using an enzymatic assay kit and for cytokine levels (IL-1β, TNF-α, IL-6, IFN-γ and IL-10).

Example 37. Generating a DSS-Induced Mouse Model of IBD

[01240] The genetically engineered bacteria described in Example 1 can be tested in the dextran sodium sulfate (DSS)-induced mouse model of colitis. The administration of DSS to animals results in chemical injury to the intestinal epithelium, allowing proinflammatory intestinal contents (e.g., luminal antigens, enteric bacteria, bacterial products) to disseminate and trigger inflammation (Low et al., 2013). To prepare mice for DSS treatment, mice are labeled using ear punch, or any other suitable labeling method. Labeling individual mice allows the investigator to track disease progression in each mouse, since mice show differential susceptibilities and responsiveness to DSS

induction. Mice are then weighed, and if required, the average group weight is equilibrated to eliminate any significant weight differences between groups. Stool is also collected prior to DSS administration, as a control for subsequent assays. Exemplary assays for fecal markers of inflammation (e.g., cytokine levels or myeloperoxidase activity) are described below.

[01241] For DSS administration, a 3% solution of DSS (MP Biomedicals, Santa Ana, CA; Cat. No. 160110) in autoclaved water is prepared. Cage water bottles are then filled with 100 mL of DSS water, and control mice are given the same amount of water without DSS supplementation. This amount is generally sufficient for 5 mice for 2-3 days. Although DSS is stable at room temperature, both types of water are changed every 2 days, or when turbidity in the bottles is observed.

[01242] Acute, chronic, and resolving models of intestinal inflammation are achieved by modifying the dosage of DSS (usually 1-5%) and the duration of DSS administration (Chassaing et al., 2014). For example, acute and resolving colitis may be achieved after a single continuous exposure to DSS over one week or less, whereas chronic colitis is typically induced by cyclical administration of DSS punctuated with recovery periods (e.g., four cycles of DSS treatment for 7 days, followed by 7-10 days of water).

[01243] **FIG. 14D** shows that butyrate produced in vivo in DSS mouse models under the control of an FNR promoter can be gut protective. LCN2 and calprotectin are both a measure of gut barrier disruption (measure by ELISA in this assay). **FIG. 14D** shows that SYN-501 (ter substitution) reduces inflammation and/or protects gut barrier as conmpared to wildtype Nissle.

Example 38. Monitoring Disease Progression In Vivo

[01244] Following initial administration of DSS, stool is collected from each animal daily, by placing a single mouse in an empty cage (without bedding material) for 15-30 min. However, as DSS administration progresses and inflammation becomes more robust, the time period required for collection increases. Stool samples are collected using sterile forceps, and placed in a microfuge tube. A single pellet is used to monitor occult blood according to the following scoring system: 0, normal stool consistency with negative hemoccult; 1, soft stools with positive hemoccult; 2, very soft stools with traces

of blood; and 3, watery stools with visible rectal bleeding. This scale is used for comparative analysis of intestinal bleeding. All remaining stool is reserved for the measurement of inflammatory markers, and frozen at -20 ◦C.

[01245] The body weight of each animal is also measured daily. Body weights may increase slightly during the first three days following initial DSS administration, and then begin to decrease gradually upon initiation of bleeding. For mouse models of acute colitis, DSS is typically administered for 7 days. However, this length of time may be modified at the discretion of the investigator.

Example 39. In Vivo Efficacy of Genetically Engineered Bacteria Following DSS Induction

[01246] The genetically engineered bacteria described in Example 1 can be tested in DSS-induced animal models of IBD. Bacteria are grown overnight in LB supplemented with the appropriate antibiotic. Bacteria are then diluted 1:100 in fresh LB containing selective antibiotic, grown to an optical density of 0.4-0.5, and pelleted by centrifugation. Bacteria are then resuspended in phosphate buffered saline (PBS). IBD is induced in mice by supplementing drinking water with 3% DSS for 7 days prior to bacterial gavage. On day 7 of DSS treatment, 100 µL of bacteria (or vehicle) is administered to mice by oral gavage. Bacterial treatment is repeated once daily for 1 week, and bacteria in stool samples are detected by plating stool homogenate on selective agar plates.

[01247] After 5 days of bacterial treatment, colitis is scored in live mice using the Coloview system (Karl Storz Veterinary Endoscopy, Goleta, CA). In mice under 1.5-2.0% isoflurane anesthesia, colons are inflated with air and approximately 3 cm of the proximal colon can be visualized (Chassaing et al., 2014). Endoscopic damage is scored by assessing colon translucency (score 0-3), fibrin attachment to the bowel wall (score 0-3), mucosal granularity (score 0-3), vascular pathology (score 0-3), stool characteristics (normal to diarrhea; score 0-3), and the presence of blood in the lumen (score 0-3), to generate a maximum score of 18. Mice are sacrificed and colonic tissues are isolated using protocols described in Examples 8 and 9. Distal colonic sections are fixed and scored for inflammation and ulceration. Remaining colonic tissue is homogenized and cytokine levels (e.g., IL-1β, TNF-α, IL-6, IFN-γ, and IL-10), as well as myeloperoxidase activity, are measured using methods described below.

Example 40. Euthanasia Procedures for Rodent Models of IBD

[01248] Four and 24 hours prior to sacrifice, 5-bromo-2'-deooxyuridine (BrdU) (Invitrogen, Waltham, MA; Cat. No. B23151) may be intraperitoneally administered to mice, as recommended by the supplier. BrdU is used to monitor intestinal epithelial cell proliferation and/or migration via immunohistochemistry with standard anti-BrdU antibodies (Abcam, Cambridge, MA).

[01249] On the day of sacrifice, mice are deprived of food for 4 hours, and then gavaged with FITC-dextran tracer (4 kDa, 0.6 mg/g body weight). Fecal pellets are collected, and mice are euthanized 3 hours following FITC-dextran administration. Animals are then cardiac bled to collect hemolysis-free serum. Intestinal permeability correlates with fluorescence intensity of appropriately diluted serum (excitation, 488 nm; emission, 520 nm), and is measured using spectrophotometry. Serial dilutions of a known amount of FITC-dextran in mouse serum are used to prepare a standard curve.

[01250] Alternatively, intestinal inflammation is quantified according to levels of serum keratinocyte-derived chemokine (KC), lipocalin 2, calprotectin, and/or CRP-1. These proteins are reliable biomarkers of inflammatory disease activity, and are measured using DuoSet ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. For these assays, control serum samples are diluted 1:2 or 1:4 for KC, and 1:200 for lipocalin 2. Samples from DSS-treated mice require a significantly higher dilution.

Example 41. Isolation and Preservation of Colonic Tissues

[01251] To isolate intestinal tissues from mice, each mouse is opened by ventral midline incision. The spleen is then removed and weighed. Increased spleen weights generally correlate with the degree of inflammation and/or anemia in the animal. Spleen lysates (100 mg/mL in PBS) plated on non-selective agar plates are also indicative of disseminated intestinal bacteria. The extent of bacterial dissemination should be consistent with any FITC-dextran permeability data.

[01252] Mesenteric lymph nodes are then isolated. These may be used to characterize immune cell populations and/or assay the translocation of gut bacteria. Lymph node enlargement is also a reliable indicator of DSS-induced pathology. Finally, the colon is removed by lifting the organ with forceps and carefully pulling until the cecum is visible. Colon dissection from severely inflamed DSS-treated mice is

particularly difficult, since the inflammatory process causes colonic tissue to thin, shorten, and attach to extraintestinal tissues.

[01253] The colon and cecum are separated from the small intestine at the ileocecal junction, and from the anus at the distal end of the rectum. At this point, the mouse intestine (from cecum to rectum) may be imaged for gross analysis, and colonic length may be measured by straightening (but not stretching) the colon. The colon is then separated from the cecum at the ileocecal junction, and briefly flushed with cold PBS using a 5- or 10-mL syringe (with a feeding needle). Flushing removes any feces and/or blood. However, if histological staining for mucin layers or bacterial adhesion/translocation is ultimately anticipated, flushing the colon with PBS should be avoided. Instead, the colon is immersed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid; Johansson et al., 2008) to preserve mucosal architecture. The cecum can be discarded, as DSS-induced inflammation is generally not observed in this region.

[01254] After flushing, colon weights are measured. Inflamed colons exhibit reduced weights relative to normal colons due to tissue wasting, and reductions in colon weight correlate with the severity of acute inflammation. In contrast, in chronic models of colitis, inflammation is often associated with increased colon weight. Increased weight may be attributed to focal collections of macrophages, epithelioid cells, and multinucleated giant cells, and/or the accumulation of other cells, such as lymphocytes, fibroblasts, and plasma cells (Williams and Williams, 1983).

[01255] To obtain colon samples for later assays, colons are cut into the appropriate number of pieces. It is important to compare the same region of the colon from different groups of mice when performing any assay. For example, the proximal colon is frozen at -80 ◦C and saved for MPO analysis, the middle colon is stored in RNA later and saved for RNA isolation, and the rectal region is fixed in 10% formalin for histology. Alternatively, washed colons may be cultured ex vivo. Exemplary protocols for each of these assays are described below.

Example 42. Myeloperoxidase Activity Assay

[01256] Granulocyte infiltration in the rodent intestine correlates with inflammation, and is measured by the activity levels of myeloperoxidase, an enzyme abundantly expressed in neutrophil granulocytes. Myeloperoxidase (MPO) activity may

be quantified using either o-dianisidine dihydrochloride (Sigma, St. Louis, MO; Cat. No. D3252) or 3,3',5,5'-tetramethylbenzidine (Sigma; Cat. No. T2885) as a substrate.

[01257] Briefly, clean, flushed samples of colonic tissue (50-100 mg) are removed from storage at -80 ◦C and immediately placed on ice. Samples are then homogenized in 0.5% hexadecyltrimethylammonium bromide (Sigma; Cat. No. H6269) in 50 mM phosphate buffer, pH 6.0. Homogenates are then disrupted for 30 sec by sonication, snap-frozen in dry ice, and thawed for a total of three freeze-thaw cycles before a final sonication for 30 sec.

[01258] For assays with o-dianisidine dihydrochloride, samples are centrifuged for 6 min at high speed (13,400 g) at 4 °C. MPO in the supernatant is then assayed in a 96-well plate by adding 1 mg/mL of o-dianisidine dihydrochloride and 0.5x10-4 % H2O2, and measuring optical density at 450 nm. A brownish yellow color develops slowly over a period of 10-20 min; however, if color development is too rapid, the assay is repeated after further diluting the samples. Human neutrophil MPO (Sigma; Cat. No. M6908) is used as a standard, with a range of 0.5-0.015 units/mL. One enzyme unit is defined as the amount of enzyme needed to degrade 1.0 μmol of peroxide per minute at 25 ◦C. This assay is used to analyze MPO activity in rodent colonic samples, particularly in DSS-induced tissues.

[01259] For assays with 3,3',5,5'-tetramethylbenzidine (TMB), samples are incubated at 60 ◦C for 2 hours and then spun down at 4,000 g for 12 min. Enzymatic activity in the supernatant is quantified photometrically at 630 nm. The assay mixture consists of 20 mL supernatant, 10 mL TMB (final concentration, 1.6 mM) dissolved in dimethylsulfoxide, and 70 mL H2O2 (final concentration, 3.0 mM) diluted in 80 mM phosphate buffer, pH 5.4. One enzyme unit is defined as the amount of enzyme that produces an increase of one absorbance unit per minute. This assay is used to analyze MPO activity in rodent colonic samples, particularly in tissues induced by trinitrobenzene (TNBS) as described herein.

Example 43. RNA Isolation and Gene Expression Analysis

[01260] To gain further mechanistic insights into how the genetically engineered bacteria may reduce gut inflammation in vivo, gene expression is evaluated by semi-quantitative and/or real-time reverse transcription PCR.

[01261] For semi-quantitative analysis, total RNA is extracted from intestinal mucosal samples using the RNeasy isolation kit (Qiagen, Germantown, MD; Cat. No. 74106). RNA concentration and purity are determined based on absorbency measurements at 260 and 280 nm. Subsequently, 1 μg of total RNA is reverse-transcribed, and cDNA is amplified for the following genes: tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-2 (IL-2), or any other gene associated with inflammation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as the internal standard. Polymerase chain reaction (PCR) reactions are performed with a 2-min melting step at 95 ◦C, then 25 cycles of 30 sec at 94 ◦C, 30 sec at 63 ◦C, and 1 min at 75 ◦C, followed by a final extension step of 5 min at 65 ◦C. Reverse transcription (RT)-PCR products are separated by size on a 4% agarose gel and stained with ethidium bromide. Relative band intensities are analyzed using standard image analysis software.

[01262] For real-time, quantitative analysis, intestinal samples (50 mg) are stored in RNAlater solution (Sigma; Cat. No. R0901) until RNA extraction. Samples should be kept frozen at -20 ◦C for long-term storage. On the day of RNA extraction, samples are thawed, or removed from RNAlater, and total RNA is extracted using Trizol (Fisher Scientific, Waltham, MA; Cat. No. 15596026). Any suitable RNA extraction method may be used. When working with DSS-induced samples, it is necessary to remove all polysaccharides (including DSS) using the lithium chloride method (Chassaing et al., 2012). Traces of DSS in colonic tissues are known to interfere with PCR amplification in subsequent steps.

[01263] Primers are designed for various genes and cytokines associated with the immune response using Primer Express® software (Applied Biosystems, Foster City, CA). Following isolation of total RNA, reverse transcription is performed using random primers, dNTPs, and Superscript® II enzyme (Invitrogen; 18064014). cDNA is then used for real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems; 4309155) and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems), although any suitable detection method may be used. PCR products are validated by melt analysis.

Example 44. Histology

[01264] Standard histological stains are used to evaluate intestinal inflammation at the microscopic level. Hematoxylin-eosin (H&E) stain allows

visualization of the quality and dimension of cell infiltrates, epithelial changes, and mucosal architecture (Erben et al., 2014). Periodic Acid-Schiff (PAS) stain is used to stain for carbohydrate macromolecules (e.g., glycogen, glycoproteins, mucins). Goblet cells, for example, are PAS-positive due to the presence of mucin.

[01265] Swiss rolls are recommended for most histological stains, so that the entire length of the rodent intestine may be examined. This is a simple technique in which the intestine is divided into portions, opened longitudinally, and then rolled with the mucosa outwards (Moolenbeek and Ruitenberg, 1981). Briefly, individual pieces of colon are cut longitudinally, wrapped around a toothpick wetted with PBS, and placed in a cassette. Following fixation in 10% formalin for 24 hours, cassettes are stored in 70% ethanol until the day of staining. Formalin-fixed colonic tissue may be stained for BrdU using anti-BrdU antibodies (Abcam). Alternatively, Ki67 may be used to visualize epithelial cell proliferation. For stains using antibodies to more specific targets (e.g., immunohistochemistry, immunofluorescence), frozen sections are fixed in a cryoprotective embedding medium, such as Tissue-Tek® OCT (VWR, Radnor, PA; Cat. No. 25608-930).

[01266] For H&E staining, stained colonic tissues are analyzed by assigning each section four scores of 0-3 based on the extent of epithelial damage, as well as inflammatory infiltration into the mucosa, submucosa, and muscularis/serosa. Each of these scores is multiplied by: 1, if the change is focal; 2, if the change is patchy; and 3, if the change is diffuse. The four individual scores are then summed for each colon, resulting in a total scoring range of 0-36 per animal. Average scores for the control and affected groups are tabulated. Alternative scoring systems are detailed herein.

Example 45. Ex Vivo Culturing of Rodent Colons

[01267] Culturing colons ex vivo may provide information regarding the severity of intestinal inflammation. Longitudinally-cut colons (approximately 1.0 cm) are serially washed three times in Hanks' Balanced Salt Solution with 1.0% penicillin/streptomycin (Fisher; Cat. No. BP295950). Washed colons are then placed in the wells of a 24-well plate, each containing 1.0 mL of serum-free RPMI1640 medium (Fisher; Cat. No. 11875093) with 1.0% penicillin/streptomycin, and incubated at 37 ◦C with 5.0% CO2 for 24 hours. Following incubation, supernatants are collected and

centrifuged for 10 min at 4 ◦C. Supernatants are stored at -80 ◦C prior to analysis for proinflammatory cytokines.

Example 46. In Vivo Efficacy of Genetically Engineered Bacteria Following TNBS Induction

[01268] Apart from DSS, the genetically engineered bacteria described in 1 can also be tested in other chemically induced animal models of IBD. Non-limiting examples include those induced by oxazolone (Boirivant et al., 1998), acetic acid (MacPherson and Pfeiffer, 1978), indomethacin (Sabiu et al., 2016), sulfhydryl inhibitors (Satoh et al., 1997), and trinitrobenzene sulfonic acid (TNBS) (Gurtner et al., 2003; Seguí et al., 2004). To determine the efficacy of the genetically engineered bacteria in a TNBS-induced mouse model of colitis, bacteria are grown overnight in LB supplemented with the appropriate antibiotic. Bacteria are then diluted 1:100 in fresh LB containing selective antibiotic, grown to an optical density of 0.4-0.5, and pelleted by centrifugation. Bacteria are resuspended in PBS. IBD is induced in mice by intracolonic administration of 30 mg TNBS in 0.25 mL 50% (vol/vol) ethanol (Seguí et al., 2004). Control mice are administered 0.25 mL saline. Four hours post-induction, 100 µL of bacteria (or vehicle) is administered to mice by oral gavage. Bacterial treatment is repeated once daily for 1 week. Animals are weighed daily.

[01269] After 7 days of bacterial treatment, mice are sacrificed via intraperitoneal administration of thiobutabarbital (100 mg/kg). Colonic tissues are isolated by blunt dissection, rinsed with saline, and weighed. Blood samples are collected by open cardiac puncture under aseptic conditions using a 1-mL syringe, placed in Eppendorf vials, and spun at 1,500 g for 10 min at 4 ◦C. The supernatant serum is then pipetted into autoclaved Eppendorf vials and frozen at -80 ◦C for later assay of IL-6 levels using a quantitative, colorimetric commercial kit (R&D Systems).

[01270] Macroscopic damage is examined under a dissecting microscope by a blinded observer. An established scoring system is used to account for the presence/severity of intestinal adhesions (score 0-2), strictures (score 0-3), ulcers (score 0-3), and wall thickness (score 0-2) (Mourelle et al., 1996). Two colon samples (50 mg) are then excised, snap-frozen in liquid nitrogen, and stored at -80 ◦C for subsequent myeloperoxidase activity assay. If desired, additional samples are preserved in 10% formalin for histologic grading. Formalin-fixed colonic samples are then embedded in

paraffin, and 5 μm sections are stained with H&E. Microscopic inflammation of the colon is assessed on a scale of 0 to 11, according to previously defined criteria (Appleyard and Wallace, 1995).

Example 47. Generating a Cell Transfer Mouse Model of IBD

[01271] The genetically engineered bacteria described in Example 1 can be tested in cell transfer animal models of IBD. One exemplary cell transfer model is the CD45RBHi T cell transfer model of colitis (Bramhall et al., 2015; Ostanin et al., 2009; Sugimoto et al., 2008). This model is generated by sorting CD4+ T cells according to their levels of CD45RB expression, and adoptively transferring CD4+ T cells with high CD45RB expression (referred to as CD45RBHi T cells) from normal donor mice into immunodeficient mice (e.g., SCID or RAG-/- mice). Specific protocols are described below.

Enrichment for CD4 T Cells

[01272] Following euthanization of C57BL/6 wild-type mice of either sex (Jackson Laboratories, Bar Harbor, ME), mouse spleens are removed and placed on ice in a 100 mm Petri dish containing 10-15 mL of FACS buffer (1X PBS without Ca2+/Mg2+, supplemented with 4% fetal calf serum). Spleens are teased apart using two glass slides coated in FACS buffer, until no large pieces of tissue remain. The cell suspension is then withdrawn from the dish using a 10-mL syringe (no needle), and expelled out of the syringe (using a 26-gauge needle) into a 50-mL conical tube placed on ice. The Petri dish is washed with an additional 10 mL of FACS buffer, using the same needle technique, until the 50-mL conical tube is full. Cells are pelleted by centrifugation at 400 g for 10 min at 4 ◦C. After the cell pellet is gently disrupted with a stream of FACS buffer, cells are counted. Cells used for counting are kept on ice and saved for single-color staining described in the next section. All other cells (i.e., those remaining in the 50-mL conical tube) are transferred to new 50-mL conical tubes. Each tube should contain a maximum of $25x10^7$ cells.

[01273] To enrich for CD4+ T cells, the Dynal® Mouse CD4 Negative Isolation kit (Invitrogen; Cat. No. 114-15D) is used as per manufacturer's instructions. Any comparable CD4+ T cell enrichment method may be used. Following negative selection, CD4+ cells remain in the supernatant. Supernatant is carefully pipetted into a new 50-mL conical tube on ice, and cells are pelleted by centrifugation at 400 g for 10 min

at 4 ◦C. Cell pellets from all 50-mL tubes are then resuspended, pooled into a single 15-mL tube, and pelleted once more by centrifugation. Finally, cells are resuspended in 1 mL of fresh FACS buffer, and stained with anti-CD4-APC and anti-CD45RB-FITC antibodies.

Fluorescent Labeling of CD4+ T Cells

[01274] To label CD4+ T cells, an antibody cocktail containing appropriate dilutions of pre-titrated anti-CD4-APC and anti-CD45RB-FITC antibodies in FACS buffer (approximately 1 mL cocktail/5x107 cells) is added to a 1.5-mL Eppendorf tube, and the volume is adjusted to 1 mL with FACS buffer. Antibody cocktail is then combined with cells in a 15-mL tube. The tube is capped, gently inverted to ensure proper mixing, and incubated on a rocking platform for 15 min at 4 °C.

[01275] During the incubation period, a 96-well round-bottom staining plate is prepared by transferring equal aliquots of counted cells (saved from the previous section) into each well of the plate that corresponds to single-color control staining. These wells are then filled to 200 µL with FACs buffer, and the cells are pelleted at 300 g for 3 min at 4 °C using a pre-cooled plate centrifuge. Following centrifugation, the supernatant is discarded using a 21-gauge needle attached to a vacuum line, and 100 µL of anti-CD16/32 antibody (Fc receptor-blocking) solution is added to each well to prevent non-specific binding. The plate is incubated on a rocking platform at 4 °C for 15 min. Cells are then washed with 200 µL FACS buffer and pelleted by centrifugation. Supernatant is aspirated, discarded, and 100 µL of the appropriate antibody (i.e., pre-titrated anti-CD4-APC or anti-CD45RB-FITC) is added to wells corresponding to each single-color control. Cells in unstained control wells are resuspended in 100 µL FACS buffer. The plate is incubated on a rocking platform at 4 °C for 15 min. After two washes, cells are resuspended in 200 µL of FACS buffer, transferred into twelve 75-mm flow tubes containing 150-200 µL of FACS buffer, and the tubes are placed on ice.

[01276] Following incubation, cells in the 15-mL tube containing antibody cocktail are pelleted by centrifugation at 400 g for 10 min at 4 °C, and resuspended in FACS buffer to obtain a concentration of 25-50x10^6 cells/mL.

Purification of CD4+ CD45RBHi T Cells

[01277] Cell sorting of CD45RBHi and CD45RBLow populations is performed using flow cytometry. Briefly, a sample of unstained cells is used to establish

baseline autofluorescence, and for forward scatter vs. side scatter gating of lymphoid cells. Single-color controls are used to set the appropriate levels of compensation to apply to each fluorochrome. However, with FITC and APC fluorochromes, compensation is generally not required. A single-parameter histogram (gated on singlet lymphoid cells) is then used to gate CD4+ (APC+) singlet cells, and a second singlet-parameter (gated on CD4+ singlet cells) is collected to establish sort gates. The CD45RBHi population is defined as the 40% of cells which exhibit the brightest CD45RB staining, whereas the CD45RBLow population is defined as the 15% of cells with the dimmest CD45RB expression. Each of these populations is sorted individually, and the CD45RBHi cells are used for adoptive transfer.

Adoptive Transfer

[01278] Purified populations of CD4+ CD45RBHi cells are adoptively transferred into 6- to 8-week-old RAG-/- male mice. The collection tubes containing sorted cells are filled with FACS buffer, and the cells are pelleted by centrifugation. The supernatant is then discarded, and cells are resuspended in 500 µL PBS. Resuspended cells are transferred into an injection tube, with a maximum of 5x106 cells per tube, and diluted with cold PBS to a final concentration of 1x106 cells/mL. Injection tubes are kept on ice.

[01279] Prior to injection, recipient mice are weighed and injection tubes are gently inverted several times to mix the cells. Mixed cells (0.5 mL, ~0.5x106 cells) are carefully drawn into a 1-mL syringe with a 26G3/8 needle attached. Cells are then intraperitoneally injected into recipient mice.

Example 48. Efficacy of Genetically Engineered Bacteria in a CD45RBHi T Cell Transfer Model

[01280] To determine whether the genetically engineered bacteria of the disclosure are efficacious in CD45RBHi T cell transfer mice, disease progression following adoptive transfer is monitored by weighing each mouse on a weekly basis. Typically, modest weight increases are observed over the first 3 weeks post-transfer, followed by slow but progressive weight loss over the next 4-5 weeks. Weight loss is generally accompanied by the appearance of loose stools and diarrhea.

[01281] At weeks 4 or 5 post-transfer, as recipient mice begin to develop signs of disease, the genetically engineered bacteria described in Example 1 are grown

overnight in LB supplemented with the appropriate antibiotic. Bacteria are then diluted 1:100 in fresh LB containing selective antibiotic, grown to an optical density of 0.4-0.5, and pelleted by centrifugation. Bacteria are resuspended in PBS and 100 μL of bacteria (or vehicle) is administered by oral gavage to CD45RBHi T cell transfer mice. Bacterial treatment is repeated once daily for 1-2 weeks before mice are euthanized. Murine colonic tissues are isolated and analyzed using the procedures described above.

Example 49. Efficacy of Genetically Engineered Bacteria in a Genetic Mouse Model of IBD

[01282] The genetically engineered bacteria described in Example 1 can be tested in genetic (including congenic and genetically modified) animal models of IBD. For example, IL-10 is an anti-inflammatory cytokine and the gene encoding IL-10 is a susceptibility gene for both Crohn's disease and ulcerative colitis (Khor et al., 2011). Functional impairment of IL-10, or its receptor, has been used to create several mouse models for the study of inflammation (Bramhall et al., 2015). IL-10 knockout (IL-10-/-) mice housed under normal conditions develop chronic inflammation in the gut (Iyer and Cheng, 2012).

[01283] To determine whether the genetically engineered bacteria of the disclosure are efficacious in IL-10-/- mice, bacteria are grown overnight in LB supplemented with the appropriate antibiotic. Bacteria are then diluted 1:100 in fresh LB containing selective antibiotic, grown to an optical density of 0.4-0.5, and pelleted by centrifugation. Bacteria are resuspended in PBS and 100 μL of bacteria (or vehicle) is administered by oral gavage to IL-10-/- mice. Bacterial treatment is repeated once daily for 1-2 weeks before mice are euthanized. Murine colonic tissues are isolated and analyzed using the procedures described above.

[01284] Protocols for testing the genetically engineered bacteria are similar for other genetic animal models of IBD. Such models include, but are not limited to, transgenic mouse models, e.g., SAMP1/YitFc (Pizarro et al., 2011), dominant negative N-cadherin mutant (NCAD delta; Hermiston and Gordon, 1995), TNFΔARE (Wagner et al., 2013), IL-7 (Watanabe et al., 1998), C3H/HeJBir (Elson et al., 2000), and dominant negative TGF-β receptor II mutant (Zhang et al., 2010); and knockout mouse models, e.g., TCRα-/- (Mombaerts et al., 1993; Sugimoto et al., 2008), WASP-/- (Nguyen et al., 2007),

Mdr1a-/- (Wilk et al., 2005), IL-2 Rα-/- (Hsu et al., 2009), Gαi2-/- (Ohman et al., 2002), and TRUC (Tbet-/-Rag2-/-; Garrett et al., 2007).

Example 50. Efficacy of Genetically Engineered Bacteria in a Transgenic Rat Model of IBD

[01285] The genetically engineered bacteria described in Example 1 can be tested in non-murine animal models of IBD. The introduction of human leukocyte antigen B27 (HLA-B27) and the human β2-microglobulin gene into Fisher (F344) rats induces spontaneous, chronic inflammation in the GI tract (Alavi et al., 2000; Hammer et al., 1990). To investigate whether the genetically engineered bacteria of the invention are capable of ameliorating gut inflammation in this model, bacteria are grown overnight in LB supplemented with the appropriate antibiotic. Bacteria are then diluted 1:100 in fresh LB containing selective antibiotic, grown to an optical density of 0.4-0.5, and pelleted by centrifugation. Bacteria are resuspended in PBS and 100 μL of bacteria (or vehicle) is administered by oral gavage to transgenic F344-HLA-B27 rats. Bacterial treatment is repeated once daily for 2 weeks.

[01286] To determine whether bacterial treatment reduces the gross and histological intestinal lesions normally present in F344-HLA-B27 rats at 25 weeks of age, all animals are sacrificed at day 14 following the initial treatment. The GI tract is then resected from the ligament of Treitz to the rectum, opened along the antimesenteric border, and imaged using a flatbed scanner. Total mucosal damage, reported as a percent of the total surface area damaged, is quantified using standard image analysis software.

[01287] For microscopic analysis, samples (0.5-1.0 cm) are excised from both normal and diseased areas of the small and large intestine. Samples are fixed in formalin and embedded in paraffin before sections (5 μm) are processed for H&E staining. The stained sections are analyzed and scored as follows: 0, no inflammation; 1, mild inflammation extending into the submucosa; 2, moderate inflammation extending into the muscularis propria; and 3, severe inflammation. The scores are combined and reported as mean ± standard error.

Example 51. Synthesis of Constructs for Synthesis of Tryptophan, Tryptamine, and Other Indole Metabolites

[01288] Various constructs were synthesized, and cloned into vector pBR322 for transformation of E. coli. In some embodiments, the constructs encoding the effector molecules are integrated into the genome according to methods described herein, e.g., **Example 2**.

Table 81. Sequences

Description	Sequence
fbrAroG (RBS and leader region underlined) **SEQ ID NO: 255**	Ctctagaaataattttgtttaactttaagaaggagatatacat atgaattatcagaacgacgatttacgcatcaaagaaatcaaagagttacttcctcctgtcg cattgctggaaaaattccccgctactgaaaatgccgcgaatacggtcgcccatgcccga aaagcgatccataagatcctgaaaggtaatgatgatcgcctgttggtggtgattggccca tgctcaattcatgatcctgtcgcggctaaagagtatgccactcgcttgctgacgctgcgtg aagagctgcaagatgagctggaaatcgtgatgcgcgtctattttgaaaagccgcgtacta cggtgggctggaaagggctgattaacgatccgcatatggataacagcttccagatcaac gacggtctgcgtattgcccgcaaattgctgctcgatattaacgacagcggtctgccagcg gcgggtgaattcctggatatgatcaccctacaatatctcgctgacctgatgagctggggc gcaattggcgcacgtaccaccgaatcgcaggtgcaccgcgaactggcgtctggtctttc ttgtccggtaggtttcaaaaatggcactgatggtacgattaaagtggctatcgatgccatta atgccgccggtgcgccgcactgcttcctgtccgtaacgaaatgggggcattcggcgatt gtgaataccagcggtaacggcgattgccatatcattctgcgcggcggtaaagagcctaa ctacagcgcgaagcacgttgctgaagtgaaagaagggctgaacaaagcaggcctgcc agcgcaggtgatgatcgatttcagccatgctaactcgtcaaaacaattcaaaaagcagat ggatgtttgtactgacgtttgccagcagattgccggtggcgaaaaggccattattggcgt gatggtggaaagccatctggtggaaggcaatcagagcctcgagagcggggaaccgct ggcctacggtaagagcatcaccgatgcctgcattggctgggatgataccgatgctctgtt acgtcaactggcgagtgcagtaaaagcgcgtcgcgggtaa
fbrAroG **SEQ ID NO: 256**	atgaattatcagaacgacgatttacgcatcaaagaaatcaaagagttacttcctcctgtcg cattgctggaaaaattccccgctactgaaaatgccgcgaatacggtcgcccatgcccga aaagcgatccataagatcctgaaaggtaatgatgatcgcctgttggtggtgattggccca tgctcaattcatgatcctgtcgcggctaaagagtatgccactcgcttgctgacgctgcgtg aagagctgcaagatgagctggaaatcgtgatgcgcgtctattttgaaaagccgcgtacta cggtgggctggaaagggctgattaacgatccgcatatggataacagcttccagatcaac gacggtctgcgtattgcccgcaaattgctgctcgatattaacgacagcggtctgccagcg gcgggtgaattcctggatatgatcaccctacaatatctcgctgacctgatgagctggggc gcaattggcgcacgtaccaccgaatcgcaggtgcaccgcgaactggcgtctggtctttc ttgtccggtaggtttcaaaaatggcactgatggtacgattaaagtggctatcgatgccatta atgccgccggtgcgccgcactgcttcctgtccgtaacgaaatgggggcattcggcgatt gtgaataccagcggtaacggcgattgccatatcattctgcgcggcggtaaagagcctaa ctacagcgcgaagcacgttgctgaagtgaaagaagggctgaacaaagcaggcctgcc agcgcaggtgatgatcgatttcagccatgctaactcgtcaaaacaattcaaaaagcagat ggatgtttgtactgacgtttgccagcagattgccggtggcgaaaaggccattattggcgt gatggtggaaagccatctggtggaaggcaatcagagcctcgagagcggggaaccgct ggcctacggtaagagcatcaccgatgcctgcattggctgggatgataccgatgctctgtt acgtcaactggcgagtgcagtaaaagcgcgtcgcgggtaa

fbrAroG-serA (RBS and leader region underlined; SerA starts after second RBS) **SEQ ID NO: 257**	Ctctagaaataattttgtttaactttaagaaggagatatacatatgaattatcagaacgacg atttacgcatcaaagaaatcaaagagttacttcctcctgtcgcattgctggaaaaattcccc gctactgaaaatgccgcgaatacggtcgcccatgcccgaaaagcgatccataagatcct gaaaggtaatgatgatcgcctgttggtggtgattggcccatgctcaattcatgatcctgtc gcggctaaagagtatgccactcgcttgctgacgctgcgtgaagagctgcaagatgagct ggaaatcgtgatgcgcgtctattttgaaaagccgcgtactacggtgggctggaaagggc tgattaacgatccgcatatggataacagcttccagatcaacgacggtctgcgtattgccc gcaaattgctgctcgatattaacgacagcggtctgccagcggcgggtgaattcctggata tgatcaccctacaatatctcgctgacctgatgagctggggcgcaattggcgcacgtacca ccgaatcgcaggtgcaccgcgaactggcgtctggtctttcttgtccggtaggtttcaaaa atggcactgatggtacgattaaagtggctatcgatgccattaatgccgccggtgcgccgc actgcttcctgtccgtaacgaaatgggggcattcggcgattgtgaataccagcggtaacg gcgattgccatatcattctgcgcggcggtaaagagcctaactacagcgcgaagcacgtt gctgaagtgaaagaagggctgaacaaagcaggcctgccagcgcaggtgatgatcgat ttcagccatgctaactcgtcaaaacaattcaaaaagcagatggatgtttgtactgacgtttg ccagcagattgccggtggcgaaaaggccattattggcgtgatggtggaaagccatctg gtggaaggcaatcagagcctcgagagcggggaaccgctggcctacggtaagagcatc accgatgcctgcattggctgggatgataccgatgctctgttacgtcaactggcgagtgca gtaaaagcgcgtcgcgggtaaTACT taagaaggagatatacatatggcaaaggtatcgctggagaaagacaagattaagtttctg ctggtagaaggcgtgcaccaaaaggcgctggaaagccttcgtgcagctggttacacca acatcgaatttcacaaaggcgcgctggatgatgaacaattaaaagaatccatccgcgat gcccacttcatcggcctgcgatcccgtacccatctgactgaagacgtgatcaacgccgc agaaaaactggtcgctattggctgtttctgtatcggaacaaatcaggttgatctggatgcg gcggcaaagcgcgggatcccggtatttaacgcaccgttctcaaatacgcgctctgttgc ggagctggtgattggcgaactgctgctgctattgcgcggcgtgccagaagccaatgcta aagcgcatcgtggcgtgtggaacaaactggcggcgggttcttttgaagcgcgcggcaa aaagctgggtatcatcggctacggtcatattggtacgcaattgggcattctggctgaatcg ctgggaatgtatgtttacttttatgatattgaaaacaaactgccgctgggcaacgccactca ggtacagcatctttctgacctgctgaatatgagcgatgtggtgagtctgcatgtaccagag aatccgtccaccaaaaatatgatgggcgcgaaagagatttcgctaatgaagcccggctc gctgctgattaatgcttcgcgcggtactgtggtggatattccagcgctgtgtgacgcgctg gcgagcaaacatctggcgggggcggcaatcgacgtattcccgacggaaccggcgac caatagcgatccatttacctctccgctgtgtgaattcgacaatgtccttctgacgccacaca ttggcggttcgactcaggaagcgcaggagaatatcggcttggaagttgcgggtaaattg atcaagtattctgacaatggctcaacgctctctgcggtgaacttcccggaagtctcgctgc cactgcacggtgggcgtcgtctgatgcacatccacgaaaaccgtccgggcgtgctaact gcgctcaacaaaatttttgccgagcagggcgtcaacatcgccgcgcaatatctacaaact tccgcccagatgggttatgtagttattgatattgaagccgacgaagacgttgccgaaaaa gcgctgcaggcaatgaaagctattccgggtaccattcgcgcccgtctgctgtactaa
SerA **SEQ ID NO: 258**	atggcaaaggtatcgctggagaaagacaagattaagtttctgctggtagaaggcgtgca ccaaaaggcgctggaaagccttcgtgcagctggttacaccaacatcgaatttcacaaag gcgcgctggatgatgaacaattaaaagaatccatccgcgatgcccacttcatcggcctg cgatcccgtacccatctgactgaagacgtgatcaacgccgcagaaaaactggtcgctat tggctgtttctgtatcggaacaaatcaggttgatctggatgcggcggcaaagcgcgggat cccggtatttaacgcaccgttctcaaatacgcgctctgttgcggagctggtgattggcga actgctgctgctattgcgcggcgtgccagaagccaatgctaaagcgcatcgtggcgtgt ggaacaaactggcggcgggttcttttgaagcgcgcggcaaaaagctgggtatcatcgg

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fbrAroG-Tdc (tdc from C. roseus); RBS and leader region underlined **SEQ ID NO: 259**	ctctagaaataattttgtttaactttaagaaggagatatacatatgaattatcagaacgacga tttacgcatcaaagaaatcaaagagttacttcctcctgtcgcattgctggaaaaattccccg ctactgaaaatgccgcgaatacggtcgcccatgcccgaaaagcgatccataagatcctg aaaggtaatgatgatcgcctgttggtggtgattggcccatgctcaattcatgatcctgtcgc ggctaaagagtatgccactcgcttgctgacgctgcgtgaagagctgcaagatgagctgg aaatcgtgatgcgcgtctattttgaaaagccgcgtactacggtgggctggaaagggctg attaacgatccgcatatggataacagcttccagatcaacgacggtctgcgtattgcccgc aaattgctgctcgatattaacgacagcggtctgccagcggcgggtgaattcctggatatg atcaccctacaatatctcgctgacctgatgagctggggcgcaattggcgcacgtaccac cgaatcgcaggtgcaccgcgaactggcgtctggtctttcttgtccggtaggtttcaaaaat ggcactgatggtacgattaaagtggctatcgatgccattaatgccgccggtgcgccgca ctgcttcctgtccgtaacgaaatgggggcattcggcgattgtgaataccagcggtaacg gcgattgccatatcattctgcgcggcggtaaagagcctaactacagcgcgaagcacgtt gctgaagtgaaagaagggctgaacaaagcaggcctgccagcgcaggtgatgatcgat ttcagccatgctaactcgtcaaaacaattcaaaaagcagatggatgtttgtactgacgtttg ccagcagattgccggtggcgaaaaggccattattggcgtgatggtggaaagccatctg gtggaaggcaatcagagcctcgagagcggggaaccgctggcctacggtaagagcatc accgatgcctgcattggctgggatgataccgatgctctgttacgtcaactggcgagtgca gtaaaagcgcgtcgcgggtaaTACTtaagaaggagatatacatATGGGTTC TATTGACTCGACGAATGTGGCCATGTCTAATTCTCCT GTTGGCGAGTTTAAGCCCCTTGAAGCAGAAGAGTTCC GTAAACAGGCACACCGCATGGTGGATTTTATTGCGGA TTATTACAAGAACGTAGAAACATACCCGGTCCTTTCC GAGGTTGAACCCGGCTATCTGCGCAAACGTATTCCCG AAACCGCACCATACCTGCCGGAGCCACTTGATGATAT TATGAAGGATATTCAAAAGGACATTATCCCCGGAAT GACGAACTGGATGTCCCCGAACTTTTACGCCTTCTTC CCGGCCACAGTTAGCTCAGCAGCTTTCTTGGGGGAAA TGCTTTCAACGGCCCTTAACAGCGTAGGATTTACCTG GGTCAGTTCCCCGGCAGCGACTGAATTAGAGATGATC GTTATGGATTGGCTTGCGCAAATTTTGAAACTTCCAA AAAGCTTTATGTTCTCCGGAACCGGGGGTGGTGTCAT CCAAAACACTACGTCAGAGTCGATCTTGTGCACTATT ATCGCGGCCCGTGAACGCGCCTTGGAAAAATTGGGC CCTGATTCAATTGGTAAGCTTGTCTGCTATGGGTCCG

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Tdc (tdc from C. roseus) **SEQ ID NO: 260**	ATGGGTTCTATTGACTCGACGAATGTGGCCATGTCTA ATTCTCCTGTTGGCGAGTTTAAGCCCCTTGAAGCAGA AGAGTTCCGTAAACAGGCACACCGCATGGTGGATTTT ATTGCGGATTATTACAAGAACGTAGAAACATACCCG GTCCTTTCCGAGGTTGAACCCGGCTATCTGCGCAAAC GTATTCCCGAAACCGCACCATACCTGCCGGAGCCACT TGATGATATTATGAAGGATATTCAAAAGGACATTATC CCCGGAATGACGAACTGGATGTCCCCGAACTTTTACG CCTTCTTCCCGGCCACAGTTAGCTCAGCAGCTTTCTTG GGGGAAATGCTTTCAACGGCCCTTAACAGCGTAGGA TTTACCTGGGTCAGTTCCCCGGCAGCGACTGAATTAG AGATGATCGTTATGGATTGGCTTGCGCAAATTTTGAA ACTTCCAAAAAGCTTTATGTTCTCCGGAACCGGGGGT GGTGTCATCCAAAACACTACGTCAGAGTCGATCTTGT GCACTATTATCGCGGCCCGTGAACGCGCCTTGGAAAA ATTGGGCCCTGATTCAATTGGTAAGCTTGTCTGCTAT GGGTCCGATCAAACGCACACAATGTTTCCGAAAACCT GTAAGTTAGCAGGAATTTATCCGAATAATATCCGCCT TATCCCTACCACGGTAGAAACCGACTTTGGCATCTCA CCGCAGGTACTTCGCAAGATGGTCGAAGACGACGTC GCTGCGGGGTACGTTCCCTTATTTTTGTGTGCCACCTT GGGAACGACATCAACTACGGCAACAGATCCTGTAGA TTCGCTGTCCGAAATCGCAAACGAGTTTGGTATCTGG

	ATTCATGTCGACGCCGCATATGCTGGATCGGCTTGCA TCTGCCCAGAATTTCGTCACTACCTTGATGGCATCGA ACGTGTGGATTCCTTATCGCTGTCTCCCCACAAATGG CTTTTAGCATATCTGGATTGCACGTGCTTGTGGGTAA AACAACCTCACCTGCTGCTTCGCGCTTTAACGACTAA TCCCGAATACTTGAAGAATAAACAGAGTGATTTAGAT AAGGTCGTGGATTTTAAGAACTGGCAGATCGCAACA GGACGTAAGTTCCGCTCTTTAAAACTTTGGTTAATTC TGCGTTCCTACGGGGTAGTTAACCTGCAAAGTCATAT CCGTAGTGATGTAGCGATGGGGAAGATGTTTGAGGA ATGGGTCCGTTCCGATAGCCGCTTTGAAATCGTCGTG CCACGTAATTTTTCGCTTGTATGCTTTCGCTTGAAACC GGATGTATCTAGTTTACATGTCGAGGAGGTCAACAAG AAGTTGTTGGATATGCTTAACTCCACCGGTCGCGTAT ATATGACGCATACAATTGTTGGCGGAATCTATATGTT ACGTTTGGCTGTAGGTAGCAGCTTGACAGAGGAACA TCACGTGCGCCGCGTTTGGGACTTGATCCAGAAGCTT ACGGACGACCTGCTTAAAGAGGCGTGA
fbrAroG-Tdc (tdc from *Clostridium sporogenes*); RBS and leader region underlined **SEQ ID NO: 261**	ctctagaaataattttgtttaactttaagaaggagatatacatatgaattatcagaacgacga tttacgcatcaaagaaatcaaagagttacttcctcctgtcgcattgctggaaaaattccccg ctactgaaaatgccgcgaatacggtcgcccatgcccgaaaagcgatccataagatcctg aaaggtaatgatgatcgcctgttggtggtgattggcccatgctcaattcatgatcctgtcgc ggctaaagagtatgccactcgcttgctgacgctgcgtgaagagctgcaagatgagctgg aaatcgtgatgcgcgtctattttgaaaagccgcgtactacggtgggctggaaagggctg attaacgatccgcatatggataacagcttccagatcaacgacggtctgcgtattgcccgc aaattgctgctcgatattaacgacagcggtctgccagcggcgggtgaattcctggatatg atcaccctacaatatctcgctgacctgatgagctggggcgcaattggcgcacgtaccac cgaatcgcaggtgcaccgcgaactggcgtctggtctttcttgtccggtaggtttcaaaaat ggcactgatggtacgattaaagtggctatcgatgccattaatgccgccggtgcgccgca ctgcttcctgtccgtaacgaaatgggggcattcggcgattgtgaataccagcggtaacg gcgattgccatatcattctgcgcggcggtaaagagcctaactacagcgcgaagcacgtt gctgaagtgaaagaagggctgaacaaagcaggcctgccagcgcaggtgatgatcgat ttcagccatgctaactcgtcaaaacaattcaaaaagcagatggatgtttgtactgacgtttg ccagcagattgccggtggcgaaaaggccattattggcgtgatggtggaaagccatctg gtggaaggcaatcagagcctcgagagcggggaaccgctggcctacggtaagagcatc accgatgcctgcattggctgggatgataccgatgctctgttacgtcaactggcgagtgca gtaaaagcgcgtcgcgggtaaTACTtaagaaggagatatacatATGAAATT TTGGCGCAAGTATACGCAACAGGAGATGGATGAGAA AATCACAGAATCGCTTGAGAAGACATTAAATTACGA TAACACGAAAACCATCGGCATCCCAGGTACTAAGCT GGATGATACTGTATTTTATGACGATCACTCCTTCGTT AAGCACTCTCCCTATTTACGTACGTTCATCCAAAACC CTAATCACATTGGTTGTCACACGTACGATAAAGCAGA CATCTTGTTTGGCGGCACGTTTGACATCGAACGCGAA CTGATTCAGCTTTTGGCCATCGATGTCTTAAACGGAA ATGATGAGGAATTCGATGGATATGTGACACAGGGGG GAACCGAGGCGAATATTCAGGCAATGTGGGTTTATC GTAACTATTTCAAAAAAGAACGTAAAGCAAAACATG

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Tdc (tdc from *Clostridium sporogenes*) **SEQ ID NO: 262**	ATGAAATTTTGGCGCAAGTATACGCAACAGGAGATG GATGAGAAAATCACAGAATCGCTTGAGAAGACATTA AATTACGATAACACGAAAACCATCGGCATCCCAGGT ACTAAGCTGGATGATACTGTATTTTATGACGATCACT CCTTCGTTAAGCACTCTCCCTATTTACGTACGTTCATC CAAAACCCTAATCACATTGGTTGTCACACGTACGATA AAGCAGACATCTTGTTTGGCGGCACGTTTGACATCGA ACGCGAACTGATTCAGCTTTTGGCCATCGATGTCTTA AACGGAAATGATGAGGAATTCGATGGATATGTGACA CAGGGGGGAACCGAGGCGAATATTCAGGCAATGTGG GTTTATCGTAACTATTTCAAAAAAGAACGTAAAGCAA AACATGAGGAAATCGCAATCATCACGAGCGCGGATA CCCATTACAGTGCATATAAGGGGAGCGACTTGCTGA ACATTGATATTATCAAGGTCCCAGTAGACTTCTATTC GCGTAAGATCCAGGAGAACACGTTAGACTCGATTGT CAAGGAGGCGAAGGAAATTGGAAAGAAGTACTTCAT TGTCATCTCAAACATGGGTACGACTATGTTTGGCAGT GTAGACGACCCTGATCTTTATGCTAACATTTTTGATA AGTATAACTTAGAATACAAAATCCACGTCGATGGAG CTTTTGGGGGTTTCATTTATCCTATCGATAATAAGGA GTGCAAAACAGATTTCTCGAACAAGAACGTCTCATCC ATCACGCTTGACGGTCACAAAATGCTTCAAGCCCCCT ATGGGACTGGTATCTTCGTGTCACGTAAGAACTTGAT CCATAACACCCTGACAAAGGAAGCAACGTATATTGA

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fbrArG-trpDH-ipdC-iad1 (RBS and leader region underlined) **SEQ ID NO: 263**	Ctctagaaataattttgtttaactttaagaaggagatatacat atgaattatcagaacgacgatttacgcatcaaagaaatcaaagagttacttcctcctgtcg cattgctggaaaaattccccgctactgaaaatgccgcgaatacggtcgcccatgcccga aaagcgatccataagatcctgaaaggtaatgatgatcgcctgttggtggtgattggccca tgctcaattcatgatcctgtcgcggctaaagagtatgccactcgcttgctgacgctgcgtg aagagctgcaagatgagctggaaatcgtgatgcgcgtctattttgaaaagccgcgtacta cggtgggctggaaagggctgattaacgatccgcatatggataacagcttccagatcaac gacggtctgcgtattgcccgcaaattgctgctcgatattaacgacagcggtctgccagcg gcgggtgaattcctggatatgatcaccctacaatatctcgctgacctgatgagctggggc gcaattggcgcacgtaccaccgaatcgcaggtgcaccgcgaactggcgtctggtctttc ttgtccggtaggtttcaaaaatggcactgatggtacgattaaagtggctatcgatgccatta atgccgccggtgcgccgcactgcttcctgtccgtaacgaaatgggggcattcggcgatt gtgaataccagcggtaacggcgattgccatatcattctgcgcggcggtaaagagcctaa ctacagcgcgaagcacgttgctgaagtgaaagaagggctgaacaaagcaggcctgcc agcgcaggtgatgatcgatttcagccatgctaactcgtcaaaacaattcaaaaagcagat ggatgtttgtactgacgtttgccagcagattgccggtggcgaaaaggccattattggcgt gatggtggaaagccatctggtggaaggcaatcagagcctcgagagcggggaaccgct ggcctacggtaagagcatcaccgatgcctgcattggctgggatgataccgatgctctgtt acgtcaactggcgagtgcagtaaaagcgcgtcgcgggtaaTACTtaagaaggaga tatacatATGCTGTTATTCGAGACTGTGCGTGAAATGGGT CATGAGCAAGTCCTTTTCTGTCATAGCAAGAATCCCG AGATCAAGGCAATTATCGCAATCCACGATACCACCTT AGGACCGGCTATGGGCGCAACTCGTATCTTACCTTAT ATTAATGAGGAGGCTGCCCTGAAAGATGCATTACGTC TGTCCCGCGGAATGACTTACAAAGCAGCCTGCGCCA ATATTCCCGCCGGGGGCGGCAAAGCCGTCATCATCGC TAACCCCGAAAACAAGACCGATGACCTGTTACGCGC ATACGGCCGTTTCGTGGACAGCTTGAACGGCCGTTTC ATCACCGGGCAGGACGTTAACATTACGCCCGACGAC GTTCGCACTATTTCGCAGGAGACTAAGTACGTGGTAG GCGTCTCAGAAAAGTCGGGAGGGCCGGCACCTATCA CCTCTCTGGGAGTATTTTTAGGCATCAAAGCCGCTGT AGAGTCGCGTTGGCAGTCTAAACGCCTGGATGGCAT GAAAGTGGCGGTGCAAGGACTTGGGAACGTAGGAAA AAATCTTTGTCGCCATCTGCATGAACACGATGTACAA CTTTTTGTGTCTGATGTCGATCCAATCAAGGCCGAGG AAGTAAAACGCTTATTCGGGGCGACTGTTGTCGAACC

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trpDH **SEQ ID NO: 264**	ATGCTGTTATTCGAGACTGTGCGTGAAATGGGTCATG AGCAAGTCCTTTTCTGTCATAGCAAGAATCCCGAGAT CAAGGCAATTATCGCAATCCACGATACCACCTTAGGA CCGGCTATGGGCGCAACTCGTATCTTACCTTATATTA ATGAGGAGGCTGCCCTGAAAGATGCATTACGTCTGTC CCGCGGAATGACTTACAAAGCAGCCTGCGCCAATATT CCCGCCGGGGGCGGCAAAGCCGTCATCATCGCTAAC CCCGAAAACAAGACCGATGACCTGTTACGCGCATAC GGCCGTTTCGTGGACAGCTTGAACGGCCGTTTCATCA CCGGGCAGGACGTTAACATTACGCCCGACGACGTTC GCACTATTTCGCAGGAGACTAAGTACGTGGTAGGCGT CTCAGAAAAGTCGGGAGGGCCGGCACCTATCACCTC TCTGGGAGTATTTTTAGGCATCAAAGCCGCTGTAGAG TCGCGTTGGCAGTCTAAACGCCTGGATGGCATGAAA GTGGCGGTGCAAGGACTTGGGAACGTAGGAAAAAAT CTTTGTCGCCATCTGCATGAACACGATGTACAACTTT TTGTGTCTGATGTCGATCCAATCAAGGCCGAGGAAGT AAAACGCTTATTCGGGGCGACTGTTGTCGAACCGACT GAAATCTATTCTTTAGATGTTGATATTTTTGCACCGTG TGCACTTGGGGGTATTTTGAATAGCCATACCATCCCG TTCTTACAAGCCTCAATCATCGCAGGAGCAGCGAATA ACCAGCTGGAGAACGAGCAACTTCATTCGCAGATGC TTGCGAAAAAGGGTATTCTTTACTCACCAGACTACGT TATCAATGCAGGAGGACTTATCAATGTTTATAACGAA ATGATCGGATATGACGAGGAAAAAGCATTCAAACAA GTTCATAACATCTACGATACGTTATTAGCGATTTTCG AAATTGCAAAAGAACAAGGTGTAACCACCAACGACG CGGCCCGTCGTTTAGCAGAGGATCGTATCAACAACTC CAAACGCTCAAAGAGTAAAGCGATTGCGGCGTGA
ipdC **SEQ ID NO: 265**	ATGCGTACACCCTACTGTGTCGCCGATTATCTTTTAG ATCGTCTGACGGACTGCGGGGCCGATCACCTGTTTGG CGTACCGGGCGATTACAACTTGCAGTTTCTGGACCAC GTCATTGACTCACCAGATATCTGCTGGGTAGGGTGTG CGAACGAGCTTAACGCGAGCTACGCTGCTGACGGAT ATGCGCGTTGTAAAGGCTTTGCTGCACTTCTTACTAC CTTCGGGGTCGGTGAGTTATCGGCGATGAACGGTATC GCAGGCTCGTACGCTGAGCACGTCCCGGTATTACACA TTGTGGGAGCTCCGGGTACCGCAGCTCAACAGCGCG GAGAACTGTTACACCACACGCTGGGCGACGGAGAAT TCCGCCACTTTTACCATATGTCCGAGCCAATTACTGT AGCCCAGGCTGTACTTACAGAGCAAAATGCCTGTTAC GAGATCGACCGTGTTTTGACCACGATGCTTCGCGAGC GCCGTCCCGGGTATTTGATGCTGCCAGCCGATGTTGC CAAAAAAGCTGCGACGCCCCCAGTGAATGCCCTGAC GCATAAACAAGCTCATGCCGATTCCGCCTGTTTAAAG GCTTTTCGCGATGCAGCTGAAAATAAATTAGCCATGT

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TrpEDCBA (RBS and leader region underlined) **SEQ ID NO: 267**	Ctctagaaataattttgtttaactttaagaaggagatatacat atgcaaacacaaaaaccgactctcgaactgctaacctgcgaaggcgcttatcgcgacaa cccgactgcgctttttcaccagttgtgtggggatcgtccggcaacgctgctgctggaatc cgcagatatcgacagcaaagatgatttaaaaagcctgctgctggtagacagtgcgctgc gcattacagcattaagtgacactgtcacaatccaggcgctttccggcaatggagaagcc ctgttgacactactggataacgccttgcctgcgggtgtggaaaatgaacaatcaccaaac tgccgcgtactgcgcttcccgcctgtcagtccactgctggatgaagacgcccgcttatgc tccctttcggtttttgacgctttccgcttattacagaatctgttgaatgtaccgaaggaagaa cgagaagcaatgttcttcggcggcctgttctcttatgaccttgtggcgggatttgaaaattt accgcaactgtcagcggaaaatagctgccctgatttctgtttttatctcgctgaaacgctga tggtgattgaccatcagaaaaaaagcactcgtattcaggccagcctgtttgctccgaatg aagaagaaaaacaacgtctcactgctcgcctgaacgaactacgtcagcaactgaccga agccgcgccgccgctgccggtggtttccgtgccgcatatgcgttgtgaatgtaaccaga gcgatgaagagttcggtggtgtagtgcgtttgttgcaaaaagcgattcgcgccggagaa attttccaggtggtgccatctcgccgtttctctctgccctgcccgtcaccgctggcagccta ttacgtgctgaaaaagagtaatcccagcccgtacatgtttttatgcaggataatgatttcac cctgtttggcgcgtcgccggaaagttcgctcaagtatgacgccaccagccgccagattg agatttacccgattgccggaacacgtccacgcggtcgtcgtgccgatggttcgctggac agagacctcgacagccgcatcgaactggagatgcgtaccgatcataaagagctttctga acatctgatgctggtggatctcgcccgtaatgacctggcacgcatttgcacacccggca gccgctacgtcgccgatctcaccaaagttgaccgttactcttacgtgatgcacctagtctc ccgcgttgttggtgagctgcgccacgatctcgacgccctgcacgcttaccgcgcctgtat gaatatggggacgttaagcggtgcaccgaaagtacgcgctatgcagttaattgccgaag cagaaggtcgtcgacgcggcagctacggcggcgcggtaggttattttaccgcgcatgg cgatctcgacacctgcattgtgatccgctcggcgctggtggaaaacggtatcgccaccg tgcaagccggtgctggcgtagtccttgattctgttccgcagtcggaagccgacgaaactc

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	accgatgcctgcattggctgggatgataccgatgctctgttacgtcaactggcgagtgca gtaaaagcgcgtcgcgggtaaTACTtaagaaggagatatacatATGCTGTT ATTCGAGACTGTGCGTGAAATGGGTCATGAGCAAGT CCTTTTCTGTCATAGCAAGAATCCCGAGATCAAGGCA ATTATCGCAATCCACGATACCACCTTAGGACCGGCTA TGGGCGCAACTCGTATCTTACCTTATATTAATGAGGA GGCTGCCCTGAAAGATGCATTACGTCTGTCCCGCGGA ATGACTTACAAAGCAGCCTGCGCCAATATTCCCGCCG GGGGCGGCAAAGCCGTCATCATCGCTAACCCCGAAA ACAAGACCGATGACCTGTTACGCGCATACGGCCGTTT CGTGGACAGCTTGAACGGCCGTTTCATCACCGGGCAG GACGTTAACATTACGCCCGACGACGTTCGCACTATTT CGCAGGAGACTAAGTACGTGGTAGGCGTCTCAGAAA AGTCGGGAGGGCCGGCACCTATCACCTCTCTGGGAGT ATTTTTAGGCATCAAAGCCGCTGTAGAGTCGCGTTGG CAGTCTAAACGCCTGGATGGCATGAAAGTGGCGGTG CAAGGACTTGGGAACGTAGGAAAAAATCTTTGTCGC CATCTGCATGAACACGATGTACAACTTTTTGTGTCTG ATGTCGATCCAATCAAGGCCGAGGAAGTAAAACGCT TATTCGGGGCGACTGTTGTCGAACCGACTGAAATCTA TTCTTTAGATGTTGATATTTTTGCACCGTGTGCACTTG GGGGTATTTTGAATAGCCATACCATCCCGTTCTTACA AGCCTCAATCATCGCAGGAGCAGCGAATAACCAGCT GGAGAACGAGCAACTTCATTCGCAGATGCTTGCGAA AAAGGGTATTCTTTACTCACCAGACTACGTTATCAAT GCAGGAGGACTTATCAATGTTTATAACGAAATGATCG GATATGACGAGGAAAAAGCATTCAAACAAGTTCATA ACATCTACGATACGTTATTAGCGATTTTCGAAATTGC AAAAGAACAAGGTGTAACCACCAACGACGCGGCCCG TCGTTTAGCAGAGGATCGTATCAACAACTCCAAACGC TCAAAGAGTAAAGCGATTGCGGCGTGAAATGtaagaagg agatatacatATGGAAAACAACACCAATATGTTCTCTGGAG TGAAGGTGATCGAACTGGCCAACTTTATCGCTGCTCC GGCGGCAGGTCGCTTCTTTGCTGATGGGGGAGCAGA AGTAATTAAGATCGAATCTCCAGCAGGCGACCCGCT GCGCTACACGGCCCCATCAGAAGGACGCCCGCTTTCT CAAGAGGAAAACACAACGTATGATTTGGAAAACGCG AATAAGAAAGCAATTGTTCTGAACTTAAAATCGGAA AAAGGAAAGAAAATTCTTCACGAGATGCTTGCTGAG GCAGACATCTTGTTAACAAATTGGCGCACGAAAGCG TTAGTCAAACAGGGGTTAGATTACGAAACACTGAAA GAGAAGTATCCAAAATTGGTATTTGCACAGATTACAG GATACGGGGAGAAAGGACCCGACAAAGACCTGCCTG GTTTCGACTACACGGCGTTTTTCGCCCGCGGAGGAGT CTCCGGTACATTATATGAAAAAGGAACTGTCCCTCCT AATGTGGTACCGGGTCTGGGTGACCACCAGGCAGGA ATGTTCTTAGCTGCCGGTATGGCTGGTGCGTTGTATA AGGCCAAAACCACCGGACAAGGCGACAAAGTCACCG

	TTAGTCTGATGCATAGCGCAATGTACGGCCTGGGAAT CATGATTCAGGCAGCCCAGTACAAGGACCATGGGCT GGTGTACCCGATCAACCGTAATGAAACGCCTAATCCT TTCATCGTTTCATACAAGTCCAAAGATGATTACTTTG TCCAAGTTTGCATGCCTCCCTATGATGTGTTTTATGAT CGCTTTATGACGGCCTTAGGACGTGAAGACTTGGTAG GTGACGAACGCTACAATAAGATCGAGAACTTGAAGG ATGGTCGCGCAAAAGAAGTCTATTCCATCATCGAACA ACAAATGGTAACGAAGACGAAGGACGAATGGGACA AGATTTTTCGTGATGCAGACATTCCATTCGCTATTGC CCAAACGTGGGAAGATCTTTTAGAAGACGAGCAGGC ATGGGCCAACGACTACCTGTATAAAATGAAGTATCCC ACAGGCAACGAACGTGCCCTGGTACGTTTACCTGTGT TCTTCAAAGAAGCTGGACTTCCTGAATACAACCAGTC GCCACAGATTGCTGAGAATACCGTGGAAGTGTTAAA GGAGATGGGATATACCGAGCAAGAAATTGAGGAGCT TGAGAAAGACAAAGACATCATGGTACGTAAAGAGAA ATGAAGGTtaagaaggagatatacatATGTCAGACCGCAACAA AGAAGTGAAAGAAAAGAAGGCTAAACACTATCTGCG CGAGATCACAGCTAAACACTACAAGGAAGCGTTAGA GGCTAAAGAGCGTGGGGAGAAAGTGGGTTGGTGTGC CTCTAACTTCCCCCAAGAGATTGCAACCACGTTGGGT GTAAAGGTTGTTTATCCCGAAAACCACGCCGCCGCCG TAGCGGCACGTGGCAATGGGCAAAATATGTGCGAAC ACGCGGAGGCTATGGGATTCAGTAATGATGTGTGTG GATATGCACGTGTAAATTTAGCCGTAATGGACATCGG CCATAGTGAAGATCAACCTATTCCAATGCCTGATTTC GTTCTGTGCTGTAATAATATCTGCAATCAGATGATTA AATGGTATGAACACATTGCAAAAACGTTGGATATTCC TATGATCCTTATCGATATTCCATATAATACTGAGAAC ACGGTGTCTCAGGACCGCATTAAGTACATCCGCGCCC AGTTCGATGACGCTATCAAGCAACTGGAAGAAATCA CTGGCAAAAAGTGGGACGAGAATAAATTCGAAGAAG TGATGAAGATTTCGCAAGAATCGGCCAAGCAATGGT TACGCGCCGCGAGCTACGCGAAATACAAACCATCAC CGTTTTCGGGCTTTGACCTTTTTAATCACATGGCTGTA GCCGTTTGTGCTCGCGGCACCCAGGAAGCCGCCGATG CATTCAAAATGTTAGCAGATGAATATGAAGAGAACG TTAAGACAGGAAAGTCTACTTATCGCGGCGAGGAGA AGCAGCGTATCTTGTTCGAGGGCATCGCTTGTTGGCC TTATCTGCGCCACAAGTTGACGAAACTGAGTGAATAT GGAATGAACGTCACAGCTACGGTGTACGCCGAAGCT TTTGGGGTTATTTACGAAAACATGGATGAACTGATGG CCGCTTACAATAAAGTGCCTAACTCAATCTCCTTCGA GAACGCGCTGAAGATGCGTCTTAATGCCGTTACAAGC ACCAATACAGAAGGGGCTGTTATCCACATTAATCGCA GTTGTAAGCTGTGGTCAGGATTCTTATACGAACTGGC CCGTCGTTTGGAAAAGGAGACGGGGATCCCTGTTGTT

	TCGTTCGACGGAGATCAAGCGGATCCCCGTAACTTCT CCGAGGCTCAATATGACACTCGCATCCAAGGTTTAAA TGAGGTGATGGTCGCGAAAAAAGAAGCAGAGTGAGC TTtaagaaggagatatacatATGTCGAATAGTGACAAGTTTTTT AACGACTTCAAGGACATTGTGGAAAACCCAAAGAAG TATATCATGAAGCATATGGAACAAACGGGACAAAAA GCCATCGGTTGCATGCCTTTATACACCCCAGAAGAGC TTGTCTTAGCGGCGGGTATGTTTCCTGTTGGAGTATG GGGCTCGAATACTGAGTTGTCAAAAGCCAAGACCTA CTTTCCGGCTTTTATCTGTTCTATCTTGCAAACTACTT TAGAAAACGCATTGAATGGGGAGTATGACATGCTGT CTGGTATGATGATCACAAACTATTGCGATTCGCTGAA ATGTATGGGACAAAACTTCAAACTTACAGTGGAAAA TATCGAATTCATCCCGGTTACGGTTCCACAAAACCGC AAGATGGAGGCGGGTAAAGAATTTCTGAAATCCCAG TATAAAATGAATATCGAACAACTGGAAAAAATCTCA GGGAATAAGATCACTGACGAGAGCTTGGAGAAGGCT ATTGAAATTTACGATGAGCACCGTAAAGTCATGAAC GATTTCTCTATGCTTGCGTCCAAGTACCCTGGTATCAT TACGCCAACGAAACGTAACTACGTGATGAAGTCAGC GTATTATATGGACAAGAAAGAACATACAGAGAAGGT ACGTCAGTTGATGGATGAAATCAAGGCCATTGAGCCT AAACCATTCGAAGGAAAACGCGTGATTACCACTGGG ATCATTGCAGATTCGGAGGACCTTTTGAAAATCTTGG AGGAGAATAACATTGCTATCGTGGGAGATGATATTG CACACGAGTCTCGCCAATACCGCACTTTGACCCCGGA GGCCAACACACCTATGGACCGTCTTGCTGAACAATTT GCGAACCGCGAGTGTTCGACGTTGTATGACCCTGAAA AAAAACGTGGACAGTATATTGTCGAGATGGCAAAAG AGCGTAAGGCCGACGGAATCATCTTCTTCATGACAAA ATTCTGCGATCCCGAAGAATACGATTACCCTCAGATG AAAAAAGACTTCGAAGAAGCCGGTATTCCCCACGTT CTGATTGAGACAGACATGCAAATGAAGAACTACGAA CAAGCTCGCACCGCTATTCAAGCATTTTCAGAAACCC TTTGACGCTtaagaaggagatatacatATGCGTGCTGTCTTAAT CGAGAAGTCAGATGACACCCAGAGTGTTTCAGTTAC GGAGTTGGCTGAAGACCAATTACCCGAAGGTGACGT CCTTGTGGATGTCGCGTACAGCACATTGAATTACAAG GATGCTCTTGCGATTACTGGAAAAGCACCCGTTGTAC GCCGTTTTCCTATGGTCCCCGGAATTGACTTTACTGG GACTGTCGCACAGAGTTCCCATGCTGATTTCAAGCCA GGCGACCGCGTAATTCTGAACGGATGGGGAGTTGGT GAGAAACACTGGGGCGGTCTTGCAGAACGCGCACGC GTACGTGGGGACTGGCTTGTCCCGTTGCCAGCCCCCT TAGACTTGCGCCAGGCTGCAATGATTGGCACTGCGGG GTACACAGCTATGCTGTGCGTGCTTGCCCTTGAGCGC CATGGAGTCGTACCTGGGAACGGCGAGATTGTCGTCT CAGGCGCAGCAGGAGGGGTAGGTTCTGTAGCAACCA

	CACTGTTAGCAGCCAAAGGCTACGAAGTGGCCGCCG TGACCGGGCGCGCAAGCGAGGCCGAATATTTACGCG GATTAGGCGCCGCGTCGGTCATTGATCGCAATGAATT AACGGGGAAGGTGCGTCCATTAGGGCAGGAACGCTG GGCAGGAGGAATCGATGTAGCAGGATCAACCGTACT TGCTAATATGTTGAGCATGATGAAATACCGTGGCGTG GTGGCGGCCTGTGGCCTGGCGGCTGGAATGGACTTGC CCGCGTCTGTCGCCCCTTTTATTCTGCGTGGTATGACT TTGGCAGGGGTAGATTCAGTCATGTGCCCCAAAACTG ATCGTCTGGCTGCTTGGGCACGCCTGGCATCCGACCT GGACCCTGCAAAGCTGGAAGAGATGACAACTGAATT ACCGTTCTCTGAGGTGATTGAAACGGCTCCGAAGTTC TTGGATGGAACAGTGCGTGGGCGTATTGTCATTCCGG TAACACCTTGATACTtaagaaggagatatacatATGAAAATCTT GGCATACTGCGTCCGCCCAGACGAGGTAGACTCCTTT AAGAAATTTAGTGAAAAGTACGGGCATACAGTTGAT CTTATTCCAGACTCTTTTGGACCTAATGTCGCTCATTT GGCGAAGGGTTACGATGGGATTTCTATTCTGGGCAAC GACACGTGTAACCGTGAGGCACTGGAGAAGATCAAG GATTGCGGGATCAAATATCTGGCAACCCGTACAGCC GGAGTGAACAACATTGACTTCGATGCAGCAAAGGAG TTCGGTATTAACGTGGCTAATGTTCCCGCATATTCCC CCAACTCGGTCAGCGAATTTACCATTGGATTGGCATT AAGTCTGACGCGTAAGATTCCATTTGCCCTGAAACGC GTGGAACTGAACAATTTTGCGCTTGGCGGCCTTATTG GTGTGGAATTGCGTAACTTAACTTTAGGAGTCATCGG TACTGGTCGCATCGGATTGAAAGTGATTGAGGGCTTC TCTGGGTTTGGAATGAAAAAAATGATCGGTTATGACA TTTTTGAAAATGAAGAAGCAAAGAAGTACATCGAAT ACAAATCATTAGACGAAGTTTTTAAAGAGGCTGATAT TATCACTCTGCATGCGCCTCTGACAGACGACAACTAT CATATGATTGGTAAAGAATCCATTGCTAAAATGAAG GATGGGGTATTTATTATCAACGCAGCGCGTGGAGCCT TAATCGATAGTGAGGCCCTGATTGAAGGGTTAAAATC GGGGAAGATT
fldA **SEQ ID NO: 276**	ATGGAAAACAACACCAATATGTTCTCTGGAGTGAAG GTGATCGAACTGGCCAACTTTATCGCTGCTCCGGCGG CAGGTCGCTTCTTTGCTGATGGGGGAGCAGAAGTAAT TAAGATCGAATCTCCAGCAGGCGACCCGCTGCGCTAC ACGGCCCCATCAGAAGGACGCCCGCTTTCTCAAGAG GAAAACACAACGTATGATTTGGAAAACGCGAATAAG AAAGCAATTGTTCTGAACTTAAAATCGGAAAAAGGA AAGAAAATTCTTCACGAGATGCTTGCTGAGGCAGAC ATCTTGTTAACAAATTGGCGCACGAAAGCGTTAGTCA AACAGGGGTTAGATTACGAAACACTGAAAGAGAAGT ATCCAAAATTGGTATTTGCACAGATTACAGGATACGG GGAGAAAGGACCCGACAAAGACCTGCCTGGTTTCGA CTACACGGCGTTTTTCGCCCGCGGAGGAGTCTCCGGT

	ACATTATATGAAAAAGGAACTGTCCCTCCTAATGTGG TACCGGGTCTGGGTGACCACCAGGCAGGAATGTTCTT AGCTGCCGGTATGGCTGGTGCGTTGTATAAGGCCAAA ACCACCGGACAAGGCGACAAAGTCACCGTTAGTCTG ATGCATAGCGCAATGTACGGCCTGGGAATCATGATTC AGGCAGCCCAGTACAAGGACCATGGGCTGGTGTACC CGATCAACCGTAATGAAACGCCTAATCCTTTCATCGT TTCATACAAGTCCAAAGATGATTACTTTGTCCAAGTT TGCATGCCTCCCTATGATGTGTTTTATGATCGCTTTAT GACGGCCTTAGGACGTGAAGACTTGGTAGGTGACGA ACGCTACAATAAGATCGAGAACTTGAAGGATGGTCG CGCAAAAGAAGTCTATTCCATCATCGAACAACAAAT GGTAACGAAGACGAAGGACGAATGGGACAAGATTTT TCGTGATGCAGACATTCCATTCGCTATTGCCCAAACG TGGGAAGATCTTTTAGAAGACGAGCAGGCATGGGCC AACGACTACCTGTATAAAATGAAGTATCCCACAGGC AACGAACGTGCCCTGGTACGTTTACCTGTGTTCTTCA AAGAAGCTGGACTTCCTGAATACAACCAGTCGCCAC AGATTGCTGAGAATACCGTGGAAGTGTTAAAGGAGA TGGGATATACCGAGCAAGAAATTGAGGAGCTTGAGA AAGACAAAGACATCATGGTACGTAAAGAGAAATGA
fldB **SEQ ID NO: 277**	ATGTCAGACCGCAACAAAGAAGTGAAAGAAAAGAA GGCTAAACACTATCTGCGCGAGATCACAGCTAAACA CTACAAGGAAGCGTTAGAGGCTAAAGAGCGTGGGGA GAAAGTGGGTTGGTGTGCCTCTAACTTCCCCCAAGAG ATTGCAACCACGTTGGGTGTAAAGGTTGTTTATCCCG AAAACCACGCCGCCGCCGTAGCGGCACGTGGCAATG GGCAAAATATGTGCGAACACGCGGAGGCTATGGGAT TCAGTAATGATGTGTGTGGATATGCACGTGTAAATTT AGCCGTAATGGACATCGGCCATAGTGAAGATCAACC TATTCCAATGCCTGATTTCGTTCTGTGCTGTAATAATA TCTGCAATCAGATGATTAAATGGTATGAACACATTGC AAAAACGTTGGATATTCCTATGATCCTTATCGATATT CCATATAATACTGAGAACACGGTGTCTCAGGACCGC ATTAAGTACATCCGCGCCCAGTTCGATGACGCTATCA AGCAACTGGAAGAAATCACTGGCAAAAAGTGGGACG AGAATAAATTCGAAGAAGTGATGAAGATTTCGCAAG AATCGGCCAAGCAATGGTTACGCGCCGCGAGCTACG CGAAATACAAACCATCACCGTTTTCGGGCTTTGACCT TTTTAATCACATGGCTGTAGCCGTTTGTGCTCGCGGC ACCCAGGAAGCCGCCGATGCATTCAAAATGTTAGCA GATGAATATGAAGAGAACGTTAAGACAGGAAAGTCT ACTTATCGCGGCGAGGAGAAGCAGCGTATCTTGTTCG AGGGCATCGCTTGTTGGCCTTATCTGCGCCACAAGTT GACGAAACTGAGTGAATATGGAATGAACGTCACAGC TACGGTGTACGCCGAAGCTTTTGGGGTTATTTACGAA AACATGGATGAACTGATGGCCGCTTACAATAAAGTG CCTAACTCAATCTCCTTCGAGAACGCGCTGAAGATGC

	GTCTTAATGCCGTTACAAGCACCAATACAGAAGGGG CTGTTATCCACATTAATCGCAGTTGTAAGCTGTGGTC AGGATTCTTATACGAACTGGCCCGTCGTTTGGAAAAG GAGACGGGGATCCCTGTTGTTTCGTTCGACGGAGATC AAGCGGATCCCCGTAACTTCTCCGAGGCTCAATATGA CACTCGCATCCAAGGTTTAAATGAGGTGATGGTCGCG AAAAAAGAAGCAGAGTGA
fldC **SEQ ID NO: 278**	ATGTCGAATAGTGACAAGTTTTTTAACGACTTCAAGG ACATTGTGGAAAACCCAAAGAAGTATATCATGAAGC ATATGGAACAAACGGGACAAAAAGCCATCGGTTGCA TGCCTTTATACACCCCAGAAGAGCTTGTCTTAGCGGC GGGTATGTTTCCTGTTGGAGTATGGGGCTCGAATACT GAGTTGTCAAAAGCCAAGACCTACTTTCCGGCTTTTA TCTGTTCTATCTTGCAAACTACTTTAGAAAACGCATT GAATGGGGAGTATGACATGCTGTCTGGTATGATGATC ACAAACTATTGCGATTCGCTGAAATGTATGGGACAA AACTTCAAACTTACAGTGGAAAATATCGAATTCATCC CGGTTACGGTTCCACAAAACCGCAAGATGGAGGCGG GTAAAGAATTTCTGAAATCCCAGTATAAAATGAATAT CGAACAACTGGAAAAAATCTCAGGGAATAAGATCAC TGACGAGAGCTTGGAGAAGGCTATTGAAATTTACGA TGAGCACCGTAAAGTCATGAACGATTTCTCTATGCTT GCGTCCAAGTACCCTGGTATCATTACGCCAACGAAAC GTAACTACGTGATGAAGTCAGCGTATTATATGGACAA GAAAGAACATACAGAGAAGGTACGTCAGTTGATGGA TGAAATCAAGGCCATTGAGCCTAAACCATTCGAAGG AAAACGCGTGATTACCACTGGGATCATTGCAGATTCG GAGGACCTTTTGAAAATCTTGGAGGAGAATAACATT GCTATCGTGGGAGATGATATTGCACACGAGTCTCGCC AATACCGCACTTTGACCCCGGAGGCCAACACACCTAT GGACCGTCTTGCTGAACAATTTGCGAACCGCGAGTGT TCGACGTTGTATGACCCTGAAAAAAAACGTGGACAG TATATTGTCGAGATGGCAAAAGAGCGTAAGGCCGAC GGAATCATCTTCTTCATGACAAAATTCTGCGATCCCG AAGAATACGATTACCCTCAGATGAAAAAAGACTTCG AAGAAGCCGGTATTCCCCACGTTCTGATTGAGACAGA CATGCAAATGAAGAACTACGAACAAGCTCGCACCGC TATTCAAGCATTTTCAGAAACCCTTTG
AcuI **SEQ ID NO: 279**	ATGCGTGCTGTCTTAATCGAGAAGTCAGATGACACCC AGAGTGTTTCAGTTACGGAGTTGGCTGAAGACCAATT ACCCGAAGGTGACGTCCTTGTGGATGTCGCGTACAGC ACATTGAATTACAAGGATGCTCTTGCGATTACTGGAA AAGCACCCGTTGTACGCCGTTTTCCTATGGTCCCCGG AATTGACTTTACTGGGACTGTCGCACAGAGTTCCCAT GCTGATTTCAAGCCAGGCGACCGCGTAATTCTGAACG GATGGGGAGTTGGTGAGAAACACTGGGGCGGTCTTG CAGAACGCGCACGCGTACGTGGGGACTGGCTTGTCC CGTTGCCAGCCCCCTTAGACTTGCGCCAGGCTGCAAT

	GATTGGCACTGCGGGGTACACAGCTATGCTGTGCGTG CTTGCCCTTGAGCGCCATGGAGTCGTACCTGGGAACG GCGAGATTGTCGTCTCAGGCGCAGCAGGAGGGGTAG GTTCTGTAGCAACCACACTGTTAGCAGCCAAAGGCTA CGAAGTGGCCGCCGTGACCGGGCGCGCAAGCGAGGC CGAATATTTACGCGGATTAGGCGCCGCGTCGGTCATT GATCGCAATGAATTAACGGGGAAGGTGCGTCCATTA GGGCAGGAACGCTGGGCAGGAGGAATCGATGTAGCA GGATCAACCGTACTTGCTAATATGTTGAGCATGATGA AATACCGTGGCGTGGTGGCGGCCTGTGGCCTGGCGG CTGGAATGGACTTGCCCGCGTCTGTCGCCCCTTTTATT CTGCGTGGTATGACTTTGGCAGGGGTAGATTCAGTCA TGTGCCCCAAAACTGATCGTCTGGCTGCTTGGGCACG CCTGGCATCCGACCTGGACCCTGCAAAGCTGGAAGA GATGACAACTGAATTACCGTTCTCTGAGGTGATTGAA ACGGCTCCGAAGTTCTTGGATGGAACAGTGCGTGGG CGTATTGTCATTCCGGTAACACCTTGA
fldH1 **SEQ ID NO: 280**	ATGAAAATCTTGGCATACTGCGTCCGCCCAGACGAG GTAGACTCCTTTAAGAAATTTAGTGAAAAGTACGGGC ATACAGTTGATCTTATTCCAGACTCTTTTGGACCTAAT GTCGCTCATTTGGCGAAGGGTTACGATGGGATTTCTA TTCTGGGCAACGACACGTGTAACCGTGAGGCACTGG AGAAGATCAAGGATTGCGGGATCAAATATCTGGCAA CCCGTACAGCCGGAGTGAACAACATTGACTTCGATGC AGCAAAGGAGTTCGGTATTAACGTGGCTAATGTTCCC GCATATTCCCCCAACTCGGTCAGCGAATTTACCATTG GATTGGCATTAAGTCTGACGCGTAAGATTCCATTTGC CCTGAAACGCGTGGAACTGAACAATTTTGCGCTTGGC GGCCTTATTGGTGTGGAATTGCGTAACTTAACTTTAG GAGTCATCGGTACTGGTCGCATCGGATTGAAAGTGAT TGAGGGCTTCTCTGGGTTTGGAATGAAAAAAATGATC GGTTATGACATTTTTGAAAATGAAGAAGCAAAGAAG TACATCGAATACAAATCATTAGACGAAGTTTTTAAAG AGGCTGATATTATCACTCTGCATGCGCCTCTGACAGA CGACAACTATCATATGATTGGTAAAGAATCCATTGCT AAAATGAAGGATGGGGTATTTATTATCAACGCAGCG CGTGGAGCCTTAATCGATAGTGAGGCCCTGATTGAAG GGTTAAAATCGGGGAAGATTGCGGGCGCGGCTCTGG ATAGCTATGAGTATGAGCAAGGTGTCTTTCACAACAA TAAGATGAATGAAATTATGCAGGATGATACCTTGGA ACGTCTGAAATCTTTTCCCAACGTCGTGATCACGCCG CATTTGGGTTTTTATACTGATGAGGCGGTTTCCAATA TGGTAGAGATCACACTGATGAACCTTCAGGAATTCGA GTTGAAAGGAACCTGTAAGAACCAGCGTGTTTGTAA ATGA
fbrAroG-TrpDH-fldABCDH (RBS and leader region	Ctctagaaataattttgtttaactttaagaaggagatatacat atgaattatcagaacgacgatttacgcatcaaagaaatcaaagagttacttcctcctgtcg cattgctggaaaaattccccgctactgaaaatgccgcgaatacggtcgcccatgcccga

underlined) **SEQ ID NO: 281**	aaagcgatccataagatcctgaaaggtaatgatgatcgcctgttggtggtgattggccca tgctcaattcatgatcctgtcgcggctaaagagtatgccactcgcttgctgacgctgcgtg aagagctgcaagatgagctggaaatcgtgatgcgcgtctattttgaaaagccgcgtacta cggtgggctggaaagggctgattaacgatccgcatatggataacagcttccagatcaac gacggtctgcgtattgcccgcaaattgctgctcgatattaacgacagcggtctgccagcg gcgggtgaattcctggatatgatcaccctacaatatctcgctgacctgatgagctggggc gcaattggcgcacgtaccaccgaatcgcaggtgcaccgcgaactggcgtctggtctttc ttgtccggtaggtttcaaaaatggcactgatggtacgattaaagtggctatcgatgccatta atgccgccggtgcgccgcactgcttcctgtccgtaacgaaatgggggcattcggcgatt gtgaataccagcggtaacggcgattgccatatcattctgcgcggcggtaaagagcctaa ctacagcgcgaagcacgttgctgaagtgaaagaagggctgaacaaagcaggcctgcc agcgcaggtgatgatcgatttcagccatgctaactcgtcaaaacaattcaaaaagcagat ggatgtttgtactgacgtttgccagcagattgccggtggcgaaaaggccattattggcgt gatggtggaaagccatctggtggaaggcaatcagagcctcgagagcggggaaccgct ggcctacggtaagagcatcaccgatgcctgcattggctgggatgataccgatgctctgtt acgtcaactggcgagtgcagtaaaagcgcgtcgcgggtaaTACTtaagaaggaga tatacatATGCTGTTATTCGAGACTGTGCGTGAAATGGGT CATGAGCAAGTCCTTTTCTGTCATAGCAAGAATCCCG AGATCAAGGCAATTATCGCAATCCACGATACCACCTT AGGACCGGCTATGGGCGCAACTCGTATCTTACCTTAT ATTAATGAGGAGGCTGCCCTGAAAGATGCATTACGTC TGTCCCGCGGAATGACTTACAAAGCAGCCTGCGCCA ATATTCCCGCCGGGGGCGGCAAAGCCGTCATCATCGC TAACCCCGAAAACAAGACCGATGACCTGTTACGCGC ATACGGCCGTTTCGTGGACAGCTTGAACGGCCGTTTC ATCACCGGGCAGGACGTTAACATTACGCCCGACGAC GTTCGCACTATTTCGCAGGAGACTAAGTACGTGGTAG GCGTCTCAGAAAAGTCGGGAGGGCCGGCACCTATCA CCTCTCTGGGAGTATTTTTAGGCATCAAAGCCGCTGT AGAGTCGCGTTGGCAGTCTAAACGCCTGGATGGCAT GAAAGTGGCGGTGCAAGGACTTGGGAACGTAGGAAA AAATCTTTGTCGCCATCTGCATGAACACGATGTACAA CTTTTTGTGTCTGATGTCGATCCAATCAAGGCCGAGG AAGTAAAACGCTTATTCGGGGCGACTGTTGTCGAACC GACTGAAATCTATTCTTTAGATGTTGATATTTTTGCAC CGTGTGCACTTGGGGGTATTTTGAATAGCCATACCAT CCCGTTCTTACAAGCCTCAATCATCGCAGGAGCAGCG AATAACCAGCTGGAGAACGAGCAACTTCATTCGCAG ATGCTTGCGAAAAAGGGTATTCTTTACTCACCAGACT ACGTTATCAATGCAGGAGGACTTATCAATGTTTATAA CGAAATGATCGGATATGACGAGGAAAAAGCATTCAA ACAAGTTCATAACATCTACGATACGTTATTAGCGATT TTCGAAATTGCAAAAGAACAAGGTGTAACCACCAAC GACGCGGCCCGTCGTTTAGCAGAGGATCGTATCAAC AACTCCAAACGCTCAAAGAGTAAAGCGATTGCGGCG TGAAATGtaagaaggagatatacatATGGAAAACAACACCAAT ATGTTCTCTGGAGTGAAGGTGATCGAACTGGCCAACT TTATCGCTGCTCCGGCGGCAGGTCGCTTCTTTGCTGA

	TGGGGGAGCAGAAGTAATTAAGATCGAATCTCCAGC AGGCGACCCGCTGCGCTACACGGCCCCATCAGAAGG ACGCCCGCTTTCTCAAGAGGAAAACACAACGTATGA TTTGGAAAACGCGAATAAGAAAGCAATTGTTCTGAA CTTAAAATCGGAAAAAGGAAAGAAAATTCTTCACGA GATGCTTGCTGAGGCAGACATCTTGTTAACAAATTGG CGCACGAAAGCGTTAGTCAAACAGGGGTTAGATTAC GAAACACTGAAAGAGAAGTATCCAAAATTGGTATTT GCACAGATTACAGGATACGGGGAGAAAGGACCCGAC AAAGACCTGCCTGGTTTCGACTACACGGCGTTTTTCG CCCGCGGAGGAGTCTCCGGTACATTATATGAAAAAG GAACTGTCCCTCCTAATGTGGTACCGGGTCTGGGTGA CCACCAGGCAGGAATGTTCTTAGCTGCCGGTATGGCT GGTGCGTTGTATAAGGCCAAAACCACCGGACAAGGC GACAAAGTCACCGTTAGTCTGATGCATAGCGCAATGT ACGGCCTGGGAATCATGATTCAGGCAGCCCAGTACA AGGACCATGGGCTGGTGTACCCGATCAACCGTAATG AAACGCCTAATCCTTTCATCGTTTCATACAAGTCCAA AGATGATTACTTTGTCCAAGTTTGCATGCCTCCCTAT GATGTGTTTTATGATCGCTTTATGACGGCCTTAGGAC GTGAAGACTTGGTAGGTGACGAACGCTACAATAAGA TCGAGAACTTGAAGGATGGTCGCGCAAAAGAAGTCT ATTCCATCATCGAACAACAAATGGTAACGAAGACGA AGGACGAATGGGACAAGATTTTTCGTGATGCAGACA TTCCATTCGCTATTGCCCAAACGTGGGAAGATCTTTT AGAAGACGAGCAGGCATGGGCCAACGACTACCTGTA TAAAATGAAGTATCCCACAGGCAACGAACGTGCCCT GGTACGTTTACCTGTGTTCTTCAAAGAAGCTGGACTT CCTGAATACAACCAGTCGCCACAGATTGCTGAGAAT ACCGTGGAAGTGTTAAAGGAGATGGGATATACCGAG CAAGAAATTGAGGAGCTTGAGAAAGACAAAGACATC ATGGTACGTAAAGAGAAATGAAGGTtaagaaggagatatacat ATGTCAGACCGCAACAAAGAAGTGAAAGAAAAGAA GGCTAAACACTATCTGCGCGAGATCACAGCTAAACA CTACAAGGAAGCGTTAGAGGCTAAAGAGCGTGGGGA GAAAGTGGGTTGGTGTGCCTCTAACTTCCCCCAAGAG ATTGCAACCACGTTGGGTGTAAAGGTTGTTTATCCCG AAAACCACGCCGCCGCCGTAGCGGCACGTGGCAATG GGCAAAATATGTGCGAACACGCGGAGGCTATGGGAT TCAGTAATGATGTGTGTGGATATGCACGTGTAAATTT AGCCGTAATGGACATCGGCCATAGTGAAGATCAACC TATTCCAATGCCTGATTTCGTTCTGTGCTGTAATAATA TCTGCAATCAGATGATTAAATGGTATGAACACATTGC AAAAACGTTGGATATTCCTATGATCCTTATCGATATT CCATATAATACTGAGAACACGGTGTCTCAGGACCGC ATTAAGTACATCCGCGCCCAGTTCGATGACGCTATCA AGCAACTGGAAGAAATCACTGGCAAAAAGTGGGACG AGAATAAATTCGAAGAAGTGATGAAGATTTCGCAAG

	AATCGGCCAAGCAATGGTTACGCGCCGCGAGCTACG CGAAATACAAACCATCACCGTTTTCGGGCTTTGACCT TTTTAATCACATGGCTGTAGCCGTTTGTGCTCGCGGC ACCCAGGAAGCCGCCGATGCATTCAAAATGTTAGCA GATGAATATGAAGAGAACGTTAAGACAGGAAAGTCT ACTTATCGCGGCGAGGAGAAGCAGCGTATCTTGTTCG AGGGCATCGCTTGTTGGCCTTATCTGCGCCACAAGTT GACGAAACTGAGTGAATATGGAATGAACGTCACAGC TACGGTGTACGCCGAAGCTTTTGGGGTTATTTACGAA AACATGGATGAACTGATGGCCGCTTACAATAAAGTG CCTAACTCAATCTCCTTCGAGAACGCGCTGAAGATGC GTCTTAATGCCGTTACAAGCACCAATACAGAAGGGG CTGTTATCCACATTAATCGCAGTTGTAAGCTGTGGTC AGGATTCTTATACGAACTGGCCCGTCGTTTGGAAAAG GAGACGGGGATCCCTGTTGTTTCGTTCGACGGAGATC AAGCGGATCCCCGTAACTTCTCCGAGGCTCAATATGA CACTCGCATCCAAGGTTTAAATGAGGTGATGGTCGCG AAAAAAGAAGCAGAGTGAGCTTtaagaaggagatatacatATG TCGAATAGTGACAAGTTTTTTAACGACTTCAAGGACA TTGTGGAAAACCCAAAGAAGTATATCATGAAGCATA TGGAACAAACGGGACAAAAAGCCATCGGTTGCATGC CTTTATACACCCCAGAAGAGCTTGTCTTAGCGGCGGG TATGTTTCCTGTTGGAGTATGGGGCTCGAATACTGAG TTGTCAAAAGCCAAGACCTACTTTCCGGCTTTTATCT GTTCTATCTTGCAAACTACTTTAGAAAACGCATTGAA TGGGGAGTATGACATGCTGTCTGGTATGATGATCACA AACTATTGCGATTCGCTGAAATGTATGGGACAAAACT TCAAACTTACAGTGGAAAATATCGAATTCATCCCGGT TACGGTTCCACAAAACCGCAAGATGGAGGCGGGTAA AGAATTTCTGAAATCCCAGTATAAAATGAATATCGAA CAACTGGAAAAAATCTCAGGGAATAAGATCACTGAC GAGAGCTTGGAGAAGGCTATTGAAATTTACGATGAG CACCGTAAAGTCATGAACGATTTCTCTATGCTTGCGT CCAAGTACCCTGGTATCATTACGCCAACGAAACGTAA CTACGTGATGAAGTCAGCGTATTATATGGACAAGAA AGAACATACAGAGAAGGTACGTCAGTTGATGGATGA AATCAAGGCCATTGAGCCTAAACCATTCGAAGGAAA ACGCGTGATTACCACTGGGATCATTGCAGATTCGGAG GACCTTTTGAAAATCTTGGAGGAGAATAACATTGCTA TCGTGGGAGATGATATTGCACACGAGTCTCGCCAATA CCGCACTTTGACCCCGGAGGCCAACACACCTATGGAC CGTCTTGCTGAACAATTTGCGAACCGCGAGTGTTCGA CGTTGTATGACCCTGAAAAAAAACGTGGACAGTATA TTGTCGAGATGGCAAAAGAGCGTAAGGCCGACGGAA TCATCTTCTTCATGACAAAATTCTGCGATCCCGAAGA ATACGATTACCCTCAGATGAAAAAAGACTTCGAAGA AGCCGGTATTCCCCACGTTCTGATTGAGACAGACATG CAAATGAAGAACTACGAACAAGCTCGCACCGCTATT

	CAAGCATTTTCAGAAACCCTTTGACGCTtaagaaggagatata catATGTTCTTTACGGAGCAACACGAACTTATTCGCAA ACTGGCGCGTGACTTTGCCGAACAGGAAATCGAGCC TATCGCAGACGAAGTAGATAAAACCGCAGAGTTCCC AAAAGAAATCGTGAAGAAGATGGCTCAAAATGGATT TTTCGGCATTAAAATGCCTAAAGAATACGGAGGGGC GGGTGCGGATAACCGCGCTTATGTCACTATTATGGAG GAAATTTCACGTGCTTCCGGGGTAGCGGGTATCTACC TGAGCTCGCCGAACAGTTTGTTAGGAACTCCCTTCTT ATTGGTCGGAACCGATGAGCAAAAAGAAAAGTACCT TAAGCCTATGATCCGCGGCGAGAAGACTCTGGCGTTC GCCCTGACAGAGCCTGGTGCTGGCTCTGATGCGGGTG CGTTGGCTACTACTGCCCGTGAAGAGGGCGACTATTA TATCTTAAATGGCCGCAAGACGTTTATTACAGGGGCT CCTATTAGCGACAATATTATTGTGTTCGCAAAAACCG ATATGAGCAAAGGGACCAAAGGTATCACCACTTTCA TTGTGGACTCAAAGCAGGAAGGGGTAAGTTTTGGTA AGCCAGAGGACAAAATGGGAATGATTGGTTGTCCGA CAAGCGACATCATCTTGGAAAACGTTAAAGTTCATAA GTCCGACATCTTGGGAGAAGTCAATAAGGGGTTTATT ACCGCGATGAAAACACTTTCCGTTGGTCGTATCGGAG TGGCGTCACAGGCGCTTGGAATTGCACAGGCCGCCGT AGATGAGGCGGTAAAGTACGCCAAGCAACGTAAACA ATTCAATCGCCCAATCGCGAAATTTCAGGCCATTCAA TTTAAACTTGCCAATATGGAGACTAAATTAAATGCCG CTAAACTTCTTGTTTATAACGCAGCGTACAAAATGGA TTGTGGAGAAAAAGCCGACAAGGAAGCCTCTATGGC TAAATACTTTGCTGCTGAATCAGCGATCCAAATCGTT AACGACGCGCTGCAAATCCATGGCGGGTATGGCTAT ATCAAAGACTACAAGATTGAACGTTTGTACCGCGATG TGCGTGTGATCGCTATTTATGAGGGCACTTCCGAGGT CCAACAGATGGTTATCGCGTCCAATCTGCTGAAGTAA TACTtaagaaggagatatacatATGAAAATCTTGGCATACTGCG TCCGCCCAGACGAGGTAGACTCCTTTAAGAAATTTAG TGAAAAGTACGGGCATACAGTTGATCTTATTCCAGAC TCTTTTGGACCTAATGTCGCTCATTTGGCGAAGGGTT ACGATGGGATTTCTATTCTGGGCAACGACACGTGTAA CCGTGAGGCACTGGAGAAGATCAAGGATTGCGGGAT CAAATATCTGGCAACCCGTACAGCCGGAGTGAACAA CATTGACTTCGATGCAGCAAAGGAGTTCGGTATTAAC GTGGCTAATGTTCCCGCATATTCCCCCAACTCGGTCA GCGAATTTACCATTGGATTGGCATTAAGTCTGACGCG TAAGATTCCATTTGCCCTGAAACGCGTGGAACTGAAC AATTTTGCGCTTGGCGGCCTTATTGGTGTGGAATTGC GTAACTTAACTTTAGGAGTCATCGGTACTGGTCGCAT CGGATTGAAAGTGATTGAGGGCTTCTCTGGGTTTGGA ATGAAAAAAATGATCGGTTATGACATTTTTGAAAATG AAGAAGCAAAGAAGTACATCGAATACAAATCATTAG

	ACGAAGTTTTTAAAGAGGCTGATATTATCACTCTGCA TGCGCCTCTGACAGACGACAACTATCATATGATTGGT AAAGAATCCATTGCTAAAATGAAGGATGGGGTATTT ATTATCAACGCAGCGCGTGGAGCCTTAATCGATAGTG AGGCCCTGATTGAAGGGTTAAAATCGGGGAAGATTG CGGGCGCGGCTCTGGATAGCTATGAGTATGAGCAAG GTGTCTTTCACAACAATAAGATGAATGAAATTATGCA GGATGATACCTTGGAACGTCTGAAATCTTTTCCCAAC GTCGTGATCACGCCGCATTTGGGTTTTTATACTGATG AGGCGGTTTCCAATATGGTAGAGATCACACTGATGA ACCTTCAGGAATTCGAGTTGAAAGGAACCTGTAAGA ACCAGCGTGTTTGTAAATGA
FldD **SEQ ID NO: 282**	ATGTTCTTTACGGAGCAACACGAACTTATTCGCAAAC TGGCGCGTGACTTTGCCGAACAGGAAATCGAGCCTAT CGCAGACGAAGTAGATAAAACCGCAGAGTTCCCAAA AGAAATCGTGAAGAAGATGGCTCAAAATGGATTTTT CGGCATTAAAATGCCTAAAGAATACGGAGGGGCGGG TGCGGATAACCGCGCTTATGTCACTATTATGGAGGAA ATTTCACGTGCTTCCGGGGTAGCGGGTATCTACCTGA GCTCGCCGAACAGTTTGTTAGGAACTCCCTTCTTATT GGTCGGAACCGATGAGCAAAAAGAAAAGTACCTTAA GCCTATGATCCGCGGCGAGAAGACTCTGGCGTTCGCC CTGACAGAGCCTGGTGCTGGCTCTGATGCGGGTGCGT TGGCTACTACTGCCCGTGAAGAGGGCGACTATTATAT CTTAAATGGCCGCAAGACGTTTATTACAGGGGCTCCT ATTAGCGACAATATTATTGTGTTCGCAAAAACCGATA TGAGCAAAGGGACCAAAGGTATCACCACTTTCATTGT GGACTCAAAGCAGGAAGGGGTAAGTTTTGGTAAGCC AGAGGACAAAATGGGAATGATTGGTTGTCCGACAAG CGACATCATCTTGGAAAACGTTAAAGTTCATAAGTCC GACATCTTGGGAGAAGTCAATAAGGGGTTTATTACCG CGATGAAAACACTTTCCGTTGGTCGTATCGGAGTGGC GTCACAGGCGCTTGGAATTGCACAGGCCGCCGTAGA TGAGGCGGTAAAGTACGCCAAGCAACGTAAACAATT CAATCGCCCAATCGCGAAATTTCAGGCCATTCAATTT AAACTTGCCAATATGGAGACTAAATTAAATGCCGCTA AACTTCTTGTTTATAACGCAGCGTACAAAATGGATTG TGGAGAAAAAGCCGACAAGGAAGCCTCTATGGCTAA ATACTTTGCTGCTGAATCAGCGATCCAAATCGTTAAC GACGCGCTGCAAATCCATGGCGGGTATGGCTATATCA AAGACTACAAGATTGAACGTTTGTACCGCGATGTGCG TGTGATCGCTATTTATGAGGGCACTTCCGAGGTCCAA CAGATGGTTATCGCGTCCAATCTGCTGAAGTAA
RBS **SEQ ID NO: 283**	taagaaggagatatacat

RBS **SEQ ID NO: 284**	ctctagaaataattttgtttaactttaagaaggagatatacat

[01289] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with one or more sequences of **Table 81**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with one or more sequences of **Table 81**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with one or more sequences of **Table 81**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with one or more sequences of **Table 81**. In another embodiment, the bcd2 gene has at least about 96%, 97%, 98%, or 99% identity with one or more sequences of **Table 81**. Accordingly, in one embodiment, 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 81**. In another embodiment, the genetically engineered bacteria comprise the sequence of SEQ ID NO: 263. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of with one or more sequences of **Table 81**.

[01290] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 263**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with **SEQ ID NO: 263**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with **SEQ ID NO: 263**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with **SEQ ID NO: 263**. In another embodiment, the *bcd2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 263**. Accordingly, in one embodiment, 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 **SEQ ID NO: 263**. In another embodiment, the genetically engineered bacteria comprise the sequence of **SEQ**

ID NO: 263. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of **SEQ ID NO: 263**.

[01291] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 261**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with **SEQ ID NO: 261**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with **SEQ ID NO: 261**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with **SEQ ID NO: 261**. In another embodiment, the *bcd2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 261**. Accordingly, in one embodiment, 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 **SEQ ID NO: 261**. In another embodiment, the genetically engineered bacteria comprise the sequence of **SEQ ID NO: 261**. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of **SEQ ID NO: 261**.

[01292] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 273**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with **SEQ ID NO: 273**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with **SEQ ID NO: 273**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with **SEQ ID NO: 273**. In another embodiment, the *bcd2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 273**. Accordingly, in one embodiment, 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 **SEQ ID NO: 273**. In another embodiment, the genetically engineered bacteria comprise the sequence of **SEQ ID NO: 273**. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of **SEQ ID NO: 273**.

[01293] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 256**. In another

embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with **SEQ ID NO: 256**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with **SEQ ID NO: 256**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with **SEQ ID NO: 256**. In another embodiment, the *bcd2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 256**. Accordingly, in one embodiment, 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 **SEQ ID NO: 256**. In another embodiment, the genetically engineered bacteria comprise the sequence of **SEQ ID NO: 256**. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of **SEQ ID NO: 256**.

[01294] In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 80% identity with **SEQ ID NO: 257**. In another embodiment, the genetically engineered bacteria comprise a sequence which has at least about 85% identity with **SEQ ID NO: 257**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 90% identity with **SEQ ID NO: 257**. In one embodiment, the genetically engineered bacteria comprise a sequence which has at least about 95% identity with **SEQ ID NO: 257**. In another embodiment, the *bcd2* gene has at least about 96%, 97%, 98%, or 99% identity with **SEQ ID NO: 257**. Accordingly, in one embodiment, 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 **SEQ ID NO: 257**. In another embodiment, the genetically engineered bacteria comprise the sequence of **SEQ ID NO: 257**. In yet another embodiment the genetically engineered bacteria comprise a sequence which consists of the sequence of **SEQ ID NO: 257**.

Example 52. Tryptophan Production in an Engineered Strain of E. coli Nissle

[01295] A number of tryptophan metabolites, either host-derived (such as tryptamine or kynurenine) or intestinal bacteria-derived (such as indole acetate or indole), have been shown to downregulate inflammation and promote gut barrier health, via the activation of the AhR receptor. Other tryptophan metabolites, such as the bacteria-derived

indole propionate, have been shown to help restore intestinal barrier integrity, in experimental models of colitis. In this example, the *E. coli* strain Nissle was engineered to produce tryptophan, the precursor to all those beneficial metabolites.

[01296] First, in order to remove the negative regulation of tryptophan biosynthetic genes mediated by the transcription factor TrpR, the *trpR* gene was deleted form the *E. coli* Nissle genome. The tryptophan operon *trpEDCBA* was amplified by PCR from the *E. coli* Nissle genomic DNA and cloned in the low-copy plasmid pSC101 under the control of the tet promoter, downstream of the *tetR* repressor gene. This *tet-trpEDCBA* plasmid was then transformed into the *ΔtrpR* mutant to obtain the *ΔtrpR, tet-trpEDCBA* strain. Subsequently, a feedback resistant version of the *aroG* gene (*aroG*^{fbr}) from *E. coli* Nissle, coding for the enzyme catalyzing the first committing step towards aromatic amino acid production, was synthetized and cloned into the medium copy plasmid p15A, under the control of the tet promoter, downstream of the *tetR* repressor. This plasmid was transformed into the *ΔtrpR, tet-trpEDCBA* strain to obtain the *ΔtrpR, tet-trpEDCBA, tet-aroG*^{fbr} strain. Finally, a feedback resistant version of the *tet-trpEBCDA* construct *(tet-trpE*^{fbr}*BCDA)* was generated from the *tet-trpEBCDA*. Both the *tet-aroG*^{fbr} *and the tet-trpE*^{fbr}*BCDA* constructs were transformed into the *ΔtrpR* mutant to obtain the *ΔtrpR, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr} strain.

[01297] All generated strains were grown in LB overnight with the appropriate antibiotics and subcultured 1/100 in 3mL LB with antibiotics in culture tubes. After two hours of growth at 37C at 250rpm, 100ng/mL anhydrotetracycline (ATC) was added to the culture to induce expression of the constructs. Two hours after induction, the bacterial cells were pelleted by centrifugation at 4,000rpm for 5min and resuspended in 3mL M9 minimal media. Cells were spun down again at 4,000rpm for 5min, resuspended in 3mL M9 minimal media with 0.5% glucose and placed at 37C at 250rpm. 200uL were collected at 2h, 4h and 16h and tryptophan was quantified by LC-MS/MS in the bacterial supernatant. **FIG. 45A** shows that tryptophan is being produced and secreted by the *ΔtrpR, tet-trpEDCBA, tet-aroG*^{fbr} strain. The production of tryptophan is significantly enhanced by expressing the feedback resistant version of *trpE*.

Example 53. Improved Tryptophan by Using a non-PTS Carbon Source and by Deleting the *tnaA* Gene Encoding Tryptophanase

[01298] One of the precursor molecule to tryptophan in *E. coli* is phosphoenolpyruvate (PEP). Only 3% of available PEP is normally used to produce aromatic acids (that include tryptophan, phenylalanine and tyrosine). When *E. coli* is grown using glucose as a sole carbon source, 50% of PEP is used to import glucose into the cell using the phosphotransferase system (PTS). In order to increase tryptophan production, a non-PTS oxidized sugar, glucuronate, was used to test tryptophan secretion by the engineered *E. coli* Nissle strain *ΔtrpR, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr}. In addition, the *tnaA* gene, encoding the tryptophanase enzyme, was deleted in the *ΔtrpR, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr} strain in order to block the conversion of tryptophan into indole to obtain the *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr} strain.

[01299] The *ΔtrpR, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr} and *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr} strains were grown in LB overnight with the appropriate antibiotics and subcultured 1/100 in 3mL LB with antibiotics in culture tubes. After two hours of growth at 37C at 250rpm, 100ng/mL anhydrotetracycline (ATC) was added to the culture to induce expression of the constructs. Two hours after induction, the bacterial cells were pelleted by centrifugation at 4,000rpm for 5min and resuspended in 3mL M9 minimal media. Cells were spun down again at 4,000rpm for 5min, resuspended in 3mL M9 minimal media with 1% glucose or 1% glucuronate and placed at 37C at 250rpm or at 37C in an anaerobic chamber. 200uL were collected at 3h and 16h and tryptophan was quantified by LC-MS/MS in the bacterial supernatant. **FIG. 45B** shows that tryptophan production is doubled in aerobic condition when the non-PTS oxidized sugar glucoronate was used. In addition, the deletion of *tnaA* had a positive effect on tryptophan production at the 3h time point in both aerobic and anaerobic conditions and at the 16h time point, only in anaerobic condition.

Example 54. Improved Tryptophan Production by Increasing the Rate of Serine Biosynthesis in *E. coli* Nissle

[01300] The last step in the tryptophan biosynthesis in *E. coli* consumes one molecule of serine. In this example, we demonstrate that serine availability is a limiting factor for tryptophan production and describe the construction of the tryptophan producing

E. coli Nissle strains *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr}*serA* and *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr}*serA*^{fbr} strains.

[01301] The *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr} strain was grown in LB overnight with the appropriate antibiotics and subcultured 1/100 in 3mL LB with antibiotics in culture tubes. After two hours of growth at 37C at 250rpm, 100ng/mL anhydrotetracycline (ATC) was added to the culture to induce expression of the constructs. Two hours after induction, the bacterial cells were pelleted by centrifugation at 4,000rpm for 5min and resuspended in 3mL M9 minimal media. Cells were spun down again at 4,000rpm for 5min, resuspended in 3mL M9 minimal media with 1% glucuronate or 1% glucuronate and 10mM serine and placed at 37C an anaerobic chamber. 200uL were collected at 3h and 16h and tryptophan was quantified by LC-MS/MS in the bacterial supernatant. **FIG. 45C** shows that tryptophan production is improved three-fold by serine addition.

[01302] In order to increase the rate of serine biosynthesis in the *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr} strain, the *serA* gene from *E. coli* Nissle encoding the enzyme catalyzing the first step in the serine biosynthetic pathway was amplified by PCR and cloned into the *tet-aroG*^{fbr} plasmid by Gibson assembly. The newly generated *tet-aroG*^{fbr}*-serA* construct was then transformed into a *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA* strain to generate the *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr}*-serA* strain. The *tet-aroG*^{fbr}*-serA* construct was further modified to encode a feedback resistant version of *serA* (*serA*^{fbr}). The newly generated *tet-aroG*^{fbr}*-serA*^{fbr} construct was used to produce the *ΔtrpRΔtnaA, tet-trpE*^{fbr}*DCBA, tet-aroG*^{fbr}*-serA*^{fbr} strain, optimized to improve the rate of serine biosynthesis and maximize tryptophan production.

Example 55. Comparison of Various Tryptophan Producing Strains

[01303] Compare the rates of tryptophan production in the different strains generated, the following constructs and strains were generated according to methods and sequences described herein (e.g. **Example 43**), and assayed for tryptophan production in the presence of glucuronate as a carbon source under aerobic conditions. SYN2126 comprises ΔtrpRΔtnaA (ΔtrpRΔtnaA). SYN2323 comprises ΔtrpRΔtnaA and a tetracycline inducible construct for the expression of feedback resistant aroG on a plasmid (ΔtrpRΔtnaA, tet-aroGfbr). SYN2339 comprises ΔtrpRΔtnaA and a first tetracycline

inducible construct for the expression of feedback resistant aroG on a first plasmid and a second tetracycline inducible construct with the genes of the trp operon with a feedback resistant form of trpE on a second plasmid (ΔtrpRΔtnaA, tet-aroGfbr, tet-trpEfbrDCBA). SYN2473 comprises ΔtrpRΔtnaA and a first tetracycline inducible construct for the expression of feedback resistant aroG and SerA on a first plasmid and a second tetracycline inducible construct with the genes of the trp operon with a feedback resistant form of trpE on a second plasmid (ΔtrpRΔtnaA, tet-aroGfbr-serA, tet-trpEfbrDCBA). SYN2476 comprises ΔtrpRΔtnaA and a tetracycline inducible construct with the genes of the trp operon with a feedback resistant form of trpE on a plasmid (ΔtrpRΔtnaA, tet-trpEfbrDCBA).

[01304] Overnight cultures were diluted 1/100 in 3mL LB plus antibiotics and grown for 2 hours (37C, 250rpm). Next, cells were induced with 100ng/mL ATC for 2 hours (37C, 250rpm), spun down, washed with cmL M9, spun down again and resuspended in 3mL M9+1% glucuronate. Cells were plated for CFU counting. For the assay, the cells were placed af 37C with shaking at 250rpm. Supernatants were collected at 1h, 2h, 3h, 4h 16h for HPLC analysis for tryptophan. As seen in **FIG. 46**, 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.

Example 56. Bacterial Production of Indole Acetic Acid (IAA)

[01305] The ability of a strain comprising tryptophan production circuits and additionally Indole-3-pyruvate decarboxylase from Enterobacter cloacae (IpdC) and Indole-3-acetaldehyde dehydrogenase from Ustilago maydis (Iad1) to produce indole acetic acid (IAA) was tested. The following strains were generated according to methods described herein and tested.

[01306] SYN2126: comprises ΔtrpR and ΔtnaA (ΔtrpRΔtnaA). SYN2339 comprises circuitry for the production of tryptophan; ΔtrpR and ΔtnaA, a first tetracline inducible trpEfbrDCBA construct on a first plasmid(pSC101), and a second tetracycline inducible aroGfbr construct on a second plasmid (ΔtrpRΔtnaA, tetR-Ptet-trpEfbrDCBA (pSC101), tetR-Ptet-aroGfbr (p15A)) (**FIG. 40B**). SYN2342 comprises the same tryptophan production circuitry as the parental strain SYN2339, and additionally comprises trpDH-ipdC-iad1 incorporated at the end of the second construct (ΔtrpRΔtnaA,

tetR-Ptet-trpEfbrDCBA (pSC101), tetR-Ptet-aroGfbr-trpDH-ipdC-iad1 (p15A))(**FIG. 43B**).

[01307] Overnight cultures of the strains were diluted 1/100 in 3mL LB plus antibiotics and grown for 2 hours (37C, 250rpm). Strains were then induced with 100ng/mL ATC for 2 hours (37C, 250rpm). Cells were spun down, and resuspended in 1mL M9+1% glucuronic acid and CFUs were quantified CFUs using the cellometer. Supernatants were collected at 1h, 2.5h and 18h for LCMS analysis of tryptophan and indole acetic acid as described herein.

[01308] As seen in **FIG. 49**, SYN2126 produced no tryptophan, SYN2339 produces increasing tryptophan over the time points measured, and SYN2342 containing the additional IAA producing circuitry produces amounts of IAA that are comparable to the amounts of tryptophan produced in its parent SYN2339. No tryptophan is measured, indicating that all tryptophan produced in SYN2342 is efficiently converted into IAA.

Example 57. Tryptamine Production Comparing Two Tryptophan Decarboxylases

[01309] The efficacy of two tryptophan decarboxylases (tdc), one from *Catharanthus roseus* (tdc_{Cr})and a second from *Clostridium sporogenes* (tdc_{Cs}) in producing tryptamine from tryptophan was tested. The following strains were generated according to methods described herein and tested.

[01310] SYN2339 comprises ΔtrpR and ΔtnaA and a tetracycline inducible $trpE^{fbr}$DCBA construct on a plasmid and another tetracycline inducible construct expressing $aroG^{fbr}$ on a second plasmid (ΔtrpRΔtnaA, tetR-P_{tet}-$trpE^{fbr}$DCBA (pSC101), tetR-P_{tet}-$aroG^{fbr}$ (p15A)). SYN2339 is used as a control which can produce tryptophan but cannot convert it to tryptamine. SYN2340 comprises ΔtrpR and ΔtnaA and a tetracycline inducible $trpE^{fbr}$DCBA construct on a plasmid and another tetracycline inducible construct expressing $aroG^{fbr}$ tdc_{Cr} on a second plasmid (ΔtrpRΔtnaA, tetR-P_{tet}-$trpE^{fbr}$DCBA (pSC101), tetR-P_{tet}-$aroG^{fbr}$-tdc_{Cr} (p15A)). SYN2794 comprises ΔtrpR and ΔtnaA and a tetracycline inducible $trpE^{fbr}$DCBA construct on a plasmid and another tetracycline inducible construct expressing $aroG^{fbr}$ tdc_{Cs} on a second plasmid (ΔtrpRΔtnaA, tetR-P_{tet}-$trpE^{fbr}$DCBA (pSC101), tetR-P_{tet}-$aroG^{fbr}$-tdc_{Cs} (p15A)).

[01311] Overnight cultures of the strains were diluted 1/100 in 3mL LB plus antibiotics and grown for 2 hours (37C, 250rpm). Strains were then induced with

100ng/mL ATC for 2 hours (37C, 250rpm). Cells were spun down, and resuspended in 1mL M9+1% glucuronic acid and CFUs were quantified CFUs using the cellometer. Supernatants were collected at 3h and 18h for LCMS analysis of tryptophan and tryptamine, as described herein.

[01312] As seen in **FIG. 51**, Tdc_{Cs} from *Clostridium sporogenes* is more efficient than Tdc_{Cr} from *Catharanthus roseus* in tryptamine production and converts all the *tryptophan* produced into tryptamine

Example 58. Tryptophan and Anthranilic Acid Quantification in Bacterial Supernatant by LC-MS/MS

[01313] Tryptophan and Anthranilic acid stock (10 mg/mL) were prepared in 0.5N HCl, aliquoted in 1.5 mL microcentrifuge tubes (100 µL), and stored at -20°C. Standards (250, 100, 20, 4, 0.8, 0.16, 0.032 µg/mL) were prepared in water. Samples (10 µL) and standards were mixed with 90 µL of ACN/H2O (60:30, v/v) containing 1µg/mL of Tryptophan-d5 in the final solution in a V-bottom 96-well plate. The plate was heat-sealed with a AlumASeal foil, mixed well, and centrifuged at 4000rpm for 5min. The solution (10µL) was transferred into a round-bottom 96-well plate 90 uL 0.1% formic acid in water was added to the sample. The plate was again heat-sealed with a ClearASeal sheet and mixed well.

LC-MS/MS method

[01314] Tryptophan and Anthranilic acid were measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. **Table 82.**, **Table 83**, and **Table 84** provide the summary of the LC-MS/MS method.

Table 82. HPLC Method

Column	Accucore aQ column, 2.6 µm (100 x 2.1 mm)
Mobile Phase A	99.9% H2O, 0.1% Formic Acid
Mobile Phase B	99.9% ACN, 0.1% Formic Acid
Injection volume	10uL

Table 83. HPLC Method:

Time (min)	Flow Rate (µL/min)	A%	B%
-0.5	350	100	0
0.5	350	100	0
1.0	350	10	90
2.5	350	10	90
2.51	350	100	10

Table 84. Tandem Mass Spectrometry

Ion Source	HESI-II
Polarity	Positive
SRM transitions	
Tryptophan	205.1/118.2
Anthranilic acid	138.1/92.2
Tryptophan-d5	210.1/151.1

Example 59. Quantification of Tryptamine in Bacterial Supernatant by Liquid Chromatography-Mass Spectrometry (LC-MS)

[01315] Tryptamine acid stock (10 mg/mL) were prepared in 0.5N HCl, aliquoted in 1.5 mL microcentrifuge tubes (100 µL), and stored at -20°C. Standards (250, 100, 20, 4, 0.8, 0.16, 0.032 µg/mL) were prepared. Samples (10 µL) and standards were mixed with 90 µL of ACN/H2O (60:30, v/v) containing 1µg/mL of tryptamine-d5 in the final solution in a V-bottom 96-well plate. The plate was heat-sealed with a AlumASeal foil, mixed well, and centrifuged at 4000rpm for 5min. The solution (10µL) was transferred into a round-bottom 96-well plate 90 uL 0.1% formic acid in water was added to the sample. The plate was again heat-sealed with a ClearASeal sheet and mixed well.

LC-MS/MS method

[01316] Tryptamine was measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. **Table 85**., **Table 86**, and **Table 87** provide the summary of the LC-MS/MS method.

Table 85. HPLC Method

Column	Accucore aQ column, 2.6 µm (100 x 2.1 mm)
Mobile Phase A	99.9% H2O, 0.1% Formic Acid

Mobile Phase B	99.9% ACN, 0.1% Formic Acid
Injection volume	10uL

Table 86. HPLC Method:

Time (min)	Flow Rate (µL/min)	A%	B%
-0.5	350	100	0
0.5	350	100	0
1.0	350	10	90
2.5	350	10	90
2.51	350	100	10

Table 87. Tandem Mass Spectrometry

Ion Source	HESI-II
Polarity	Positive
SRM transitions	
Tryptamine	161.1/144.1

Example 60. Quantification of Tryptophan, Indole-3-acetate, Indole-3-lactate, Indole-3-propionate in Bacterial Supernatant by High-pressure Liquid Chromatography (HPLC)

[01317] Samples were thawed on ice and centrifuged at 3,220 x g for 5min at 4°C. 80µL of the supernatant was pipetted, mixed with 20µL 0.5% formic acid in water, and analyzed by HPLC using a Shimadzu Prominence-I. HPLC conditions used for the quantification of tryptophan, indole-3-acetate, indole-3-lactate and indole-3-propionate are described in Table 88.

Table 88. HPLC Analysis

Chromatography	
Calibration standards	250, 100, 20, 4, 0.8 µg/mL
Column	Luna 3 µm C18(2) 100Å, 100 x 2 mm (catalog# 00D-4251-B0)
Column Temperature	40 °C
Injection Volume	10 µL
Autosampler Temperature	10°C
Flow Rate	0.5 mL/min
Mobile Phases	A: water, 0.1% FA

	B: acetonitrile, 0.1% FA		
Gradient	Time (min)	% A	% B
	0	90	10
	0.5	90	10
	3	10	90
	5	10	90
	5.01	90	10
	7	(end)	
Detection: Photodiode Array Detector (PDA)			
Polarity	Positive		
Start Wavelength	190 nm		
End Wavelength	800 nm		
Spectrum resolution	512		
Slit Width	8 nm		
Compound	Wavelength (nm)	Retention time (min)	
Tryptophan	274	1.3	
Indole-3-acetate	274	3.5	
Indole-lactate	274	3.3	
Indole-3-propionate	274	3.7	

Example 61. Biochemical Analysis of Butyrate Production in SYN1001

[01318] 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.

[01319] 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 89**-data are average of assay performed in triplicate for three different manufacturing runs).

Table 89: 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

[01320] 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 62. Cell-based Assay Development and *In-vitro* Butyrate Strain Assessment

Methods

[01321] **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.

[01322] **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.

[01323] **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 37^0C,* 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 *37^0C,* 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

[01324] 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. 19B** 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. 19C**, 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

[01325] 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 90**. summarizes the final pharmacological characteristics of the SYN1001.

Table 90. 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 63. *In Vitro* Assessment of the Engineered Acetate-producing Strain SYN2001

[01326] 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 IFNγ in human PBMC cells.

[01327] To assess the activity of the acetate-producing strain SYN2001 *in vitro*, we employed the LPS-induction of IFNγ 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 IFNγ levels assessed by ELISA. **FIG. 26G** and **FIG. 26H** 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 IFNγ by the cells. We noted that the absolute levels of IFNγ 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 91** summarizes the data from the 3 experiments using the 2 separate donors.

Table 91. Summary of EC50's for SYN2001 on LPS-induced IFNγ 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

[01328] 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 92**.

Table 92. 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 64. Generation and Analysis of an engineered IL-22-producing *E. coli* Nissle strain

Engineering and Production of IL-22

[01329] 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.

[01330] 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.

[01331] 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.

[01332] 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.

[01333] 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 93**. 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 93 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

[01334] 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. 33C** 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.

[01335] 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. 33D** 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

[01336] 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 94** summarizes the final pharmacological characteristics of SYN3001.

Table 94. 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 65. Generation and Testing of an IL-10 producing strain

Strain construction

[01337] In order to generate strains which secrete human IL-10, a base strain was used which has a "leaky membrane" phenotype (SYN1557), comprising delta PAL DOM background). For plasmid-based secretion construct, a codon optimized human IL10 sequence was combined with a number of secretion signals to determine the optimal configuration. Recently the Nissle genome was mined bioinformatically for signal sequences larger than the 21 AA PhoA tag. This yielded several candidates including the signal sequences from: ECOLIN_05715 (52 AA), ECOLIN_16495 (40 AA), ECOLIN_19410 (33 AA) and ECOLIN_19880 (53 AA). These signal sequences were codon optimized and synthesized along with optimized RBS sequences, then inserted upstream of an optimized hIL10 sequence in a high copy pUC57 backbone. All of the candidate hIL10 constructs were then transformed into the delta PAL DOM background to test for secretion.

Production of hIL10 for in vitro quantification

[01338] To assay for production of bioactive hIL10 from *E. coli* Nissle, candidate strains were grown, induced and supernatants harvested and filter sterilized. These supernatants were then quantified via ELISA for hIL10 concentration corresponding to secreted hIL10.

[01339] Briefly, overnight cultures were used to inoculate 50mL starter cultures of 2YT broth at a 1:50 dilution, and bacteria were grown for 2 hours and harvested by centrifugation at 12.8Kxg for 5 minutes. The pellet was resuspended in 50mL of fresh 2YT media with aTc (100ug/mL) and appropriate antibiotic, and cells were induced at 30C for an additional 4 hours to allow expression and secretion of hIL10. Supernatants were harvested via centrifugation and filtration through a .22 micron PVDF filter then used for Western blot analysis and in BD OptEIA Human IL10 ELISA Kit II (Cat. No. 550613) both according to manufacturers instruction. **FIG. 33E** depicts a Western blot analysis of bacterial supernatants from strain SYN2980 and SYN2982, using IL-10 antibody (IL-10 (D13A11) XP® Rabbit mAb #12163, Cell Signaling Technology). The secreted polypepetide has the same molecular weight as the standards, indicating that the signal sequence is cleaved from the native peptide. Results from the ELISA are shown in **Table 95A**. Selected secretion sequences are shown in **Table 95B**.

Table 95A. ELISA results

STRAIN	**Genotype**	**Circuit**	**[hIL10] (ng/mL)**
SYN1557	*pal*::Cm	None	0
SYN2898	*pal*::Cm	Ptet-OmpFss-hIL10 p15a	37
SYN2890	*pal*::Cm	Ptet-ECOLIN_05715ss-hIL10 pUC57	84
SYN2892	*pal*::Cm	Ptet-ECOLIN_19410ss-hIL10 pUC57	306
SYN2893	*pal*::Cm	Ptet-ECOLIN_19880ss-hIL10 pUC57	224

Table 95B. Selected Sequences

Description	Sequences
Construct comprising RBS-ECOLIN_05715 Secretion signal-codon optimized IL-10 SEQ ID NO:	CAATATCCTCGTAATCCTAAGGACGCCACTATGAAACGCCATCTGAAC ACCTCCTATCGCTTAGTCTGGAACCATATTACCGGTGCTTTCGTTGTTG CATCAGAGCTGGCCCGCGCTCGTGGTAAGCGTGCAGGCGTGGCGGTA GCCTTAAGCCTGGCAGCAGCAACATCTTTGCCTGCGTTAGCATCGCCA GGTCAAGGAACGCAGTCAGAGAATTCATGCACTCACTTTCCGGGCAAT CTGCCGAATATGCTGCGCGATCTGCGAGATGCATTCTCTCGCGTGAAA ACGTTCTTTCAAATGAAAGATCAACTGGATAATCTGCTGCTGAAGGAG TCGTTGTTGGAGGATTTTAAGGGGTATCTGGGTTGTCAAGCACTGTCT GAAATGATTCAATTTTACTTGGAGGAAGTTATGCCGCAAGCGGAAAAC CAAGATCCGGATATTAAGGCGCACGTGAACTCACTGGGCGAAAACCT GAAAACTTTGCGCCTGCGTCTGAGACGATGTCACCGATTCCTGCCGTG TGAAAACAAGTCAAAGGCGGTTGAGCAAGTTAAGAATGCTTTCAATA AGCTGCAAGAAAAGGGCATCTATAAAGCGATGTCTGAATTTGATATCT TTATAAACTACATAGAAGCTTATATGACTATGAAGATTCGAAATTAA
RBS	CAATATCCTCGTAATCCTAAGGACGCCACT

SEQ ID NO:	
ECOLIN_05715 Secretion signal SEQ ID NO:	ATGAAACGCCATCTGAACACCTCCTATCGCTTAGTCTGGAACCATATT ACCGGTGCTTTCGTTGTTGCATCAGAGCTGGCCCGCGCTCGTGGTAAG CGTGCAGGCGTGGCGGTAGCCTTAAGCCTGGCAGCAGCAACATCTTTG CCTGCGTTAGCA
codon optimized IL-10 SEQ ID NO:	TCGCCAGGTCAAGGAACGCAGTCAGAGAATTCATGCACTCACTTTCCG GGCAATCTGCCGAATATGCTGCGCGATCTGCGAGATGCATTCTCTCGC GTGAAAACGTTCTTTCAAATGAAAGATCAACTGGATAATCTGCTGCTG AAGGAGTCGTTGTTGGAGGATTTTAAGGGGTATCTGGGTTGTCAAGCA CTGTCTGAAATGATTCAATTTTACTTGGAGGAAGTTATGCCGCAAGCG GAAAACCAAGATCCGGATATTAAGGCGCACGTGAACTCACTGGGCGA AAACCTGAAAACTTTGCGCCTGCGTCTGAGACGATGTCACCGATTCCT GCCGTGTGAAAACAAGTCAAAGGCGGTTGAGCAAGTTAAGAATGCTT TCAATAAGCTGCAAGAAAAGGGCATCTATAAAGCGATGTCTGAATTTG ATATCTTTATAAACTACATAGAAGCTTATATGACTATGAAGATTCGAA ATTAA
Construct comprising RBS-ECOLIN_16495 Secretion signal-codon optimized IL-10 SEQ ID NO:	TTAACAAAGATAGTTATCGCAGTAGGAGGCCCCCATGTTTTGGCGCGA CATGACACTTTCGGTGTGGCGCAAAAAAACGACTGGCCTTAAAACTAA GAAGCGTTTACTGGCTTTGGTATTGGCTGCTGCATTGTGCTCAAGCCCT GTCTGGGCGTCGCCAGGTCAAGGAACGCAGTCAGAGAATTCATGCAC TCACTTTCCGGGCAATCTGCCGAATATGCTGCGCGATCTGCGAGATGC ATTCTCTCGCGTGAAAACGTTCTTTCAAATGAAAGATCAACTGGATAA TCTGCTGCTGAAGGAGTCGTTGTTGGAGGATTTTAAGGGGTATCTGGG TTGTCAAGCACTGTCTGAAATGATTCAATTTTACTTGGAGGAAGTTAT GCCGCAAGCGGAAAACCAAGATCCGGATATTAAGGCGCACGTGAACT CACTGGGCGAAAACCTGAAAACTTTGCGCCTGCGTCTGAGACGATGTC ACCGATTCCTGCCGTGTGAAAACAAGTCAAAGGCGGTTGAGCAAGTT AAGAATGCTTTCAATAAGCTGCAAGAAAAGGGCATCTATAAAGCGAT GTCTGAATTTGATATCTTTATAAACTACATAGAAGCTTATATGACTATG AAGATTCGAAATTAA
RBS SEQ ID NO:	TTAACAAAGATAGTTATCGCAGTAGGAGGCCCCC
ECOLIN_16495 Secretion signal SEQ ID NO:	ATGTTTTGGCGCGACATGACACTTTCGGTGTGGCGCAAAAAAACGACT GGCCTTAAAACTAAGAAGCGTTTACTGGCTTTGGTATTGGCTGCTGCA TTGTGCTCAAGCCCTGTCTGGGCG
codon optimized IL-10 SEQ ID NO:	TCGCCAGGTCAAGGAACGCAGTCAGAGAATTCATGCACTCACTTTCCG GGCAATCTGCCGAATATGCTGCGCGATCTGCGAGATGCATTCTCTCGC GTGAAAACGTTCTTTCAAATGAAAGATCAACTGGATAATCTGCTGCTG AAGGAGTCGTTGTTGGAGGATTTTAAGGGGTATCTGGGTTGTCAAGCA CTGTCTGAAATGATTCAATTTTACTTGGAGGAAGTTATGCCGCAAGCG GAAAACCAAGATCCGGATATTAAGGCGCACGTGAACTCACTGGGCGA AAACCTGAAAACTTTGCGCCTGCGTCTGAGACGATGTCACCGATTCCT GCCGTGTGAAAACAAGTCAAAGGCGGTTGAGCAAGTTAAGAATGCTT TCAATAAGCTGCAAGAAAAGGGCATCTATAAAGCGATGTCTGAATTTG ATATCTTTATAAACTACATAGAAGCTTATATGACTATGAAGATTCGAA ATTAA
Construct comprising RBS-ECOLIN_19410 Secretion signal-codon optimized	TCAACTCAGTCTACAACATCGGAGGTTAAGAATGGGGTACAAAATGA ACATTAGCTCGCTTCGCAAAGCATTCATTTTTATGGGGGCTGTTGCAG CTTTAAGCCTTGTCAATGCCCAGTCAGCGCTTGCCTCGCCAGGTCAAG GAACGCAGTCAGAGAATTCATGCACTCACTTTCCGGGCAATCTGCCGA ATATGCTGCGCGATCTGCGAGATGCATTCTCTCGCGTGAAAACGTTCT

IL-10 SEQ ID NO:	TTCAAATGAAAGATCAACTGGATAATCTGCTGCTGAAGGAGTCGTTGT TGGAGGATTTTAAGGGGTATCTGGGTTGTCAAGCACTGTCTGAAATGA TTCAATTTTACTTGGAGGAAGTTATGCCGCAAGCGGAAAACCAAGATC CGGATATTAAGGCGCACGTGAACTCACTGGGCGAAAACCTGAAAACT TTGCGCCTGCGTCTGAGACGATGTCACCGATTCCTGCCGTGTGAAAAC AAGTCAAAGGCGGTTGAGCAAGTTAAGAATGCTTTCAATAAGCTGCA AGAAAAGGGCATCTATAAAGCGATGTCTGAATTTGATATCTTTATAAA CTACATAGAAGCTTATATGACTATGAAGATTCGAAATTAA
RBS SEQ ID NO:	TCAACTCAGTCTACAACATCGGAGGTTAAGA
ECOLIN_19410 Secretion signal SEQ ID NO:	ATGGGGTACAAAATGAACATTAGCTCGCTTCGCAAAGCATTCATTTTT ATGGGGGCTGTTGCAGCTTTAAGCCTTGTCAATGCCCAGTCAGCGCTT GCC
codon optimized IL-10 SEQ ID NO:	TCGCCAGGTCAAGGAACGCAGTCAGAGAATTCATGCACTCACTTTCCG GGCAATCTGCCGAATATGCTGCGCGATCTGCGAGATGCATTCTCTCGC GTGAAAACGTTCTTTCAAATGAAAGATCAACTGGATAATCTGCTGCTG AAGGAGTCGTTGTTGGAGGATTTTAAGGGGTATCTGGGTTGTCAAGCA CTGTCTGAAATGATTCAATTTTACTTGGAGGAAGTTATGCCGCAAGCG GAAAACCAAGATCCGGATATTAAGGCGCACGTGAACTCACTGGGCGA AAACCTGAAAACTTTGCGCCTGCGTCTGAGACGATGTCACCGATTCCT GCCGTGTGAAAACAAGTCAAAGGCGGTTGAGCAAGTTAAGAATGCTT TCAATAAGCTGCAAGAAAAGGGCATCTATAAAGCGATGTCTGAATTTG ATATCTTTATAAACTACATAGAAGCTTATATGACTATGAAGATTCGAA ATTAA
Construct comprising RBS- ECOLIN_19880 Secretion signal- codon optimized IL-10 SEQ ID NO:	CCCGTATAACACTAAGAGAGGCGAATTAGAGTATGAATAAGATTTTCA AGGTCATTTGGAATCCTGCGACCGGAAGCTACACGGTTGCAAGTGAA ACCGCTAAATCCCGTGGAAAAAAGAGTGGCCGCAGTAAATTACTTATC TCTGCTTTGGTCGCAGGAGGGTTATTATCCTCATTCGGCGCTTCAGCGT CGCCAGGTCAAGGAACGCAGTCAGAGAATTCATGCACTCACTTTCCGG GCAATCTGCCGAATATGCTGCGCGATCTGCGAGATGCATTCTCTCGCG TGAAAACGTTCTTTCAAATGAAAGATCAACTGGATAATCTGCTGCTGA AGGAGTCGTTGTTGGAGGATTTTAAGGGGTATCTGGGTTGTCAAGCAC TGTCTGAAATGATTCAATTTTACTTGGAGGAAGTTATGCCGCAAGCGG AAAACCAAGATCCGGATATTAAGGCGCACGTGAACTCACTGGGCGAA AACCTGAAAACTTTGCGCCTGCGTCTGAGACGATGTCACCGATTCCTG CCGTGTGAAAACAAGTCAAAGGCGGTTGAGCAAGTTAAGAATGCTTT CAATAAGCTGCAAGAAAAGGGCATCTATAAAGCGATGTCTGAATTTG ATATCTTTATAAACTACATAGAAGCTTATATGACTATGAAGATTCGAA ATTAA
RBS SEQ ID NO:	CCCGTATAACACTAAGAGAGGCGAATTAGAGT
ECOLIN_19880 Secretion signal SEQ ID NO:	ATGAATAAGATTTTCAAGGTCATTTGGAATCCTGCGACCGGAAGCTAC ACGGTTGCAAGTGAAACCGCTAAATCCCGTGGAAAAAAGAGTGGCCG CAGTAAATTACTTATCTCTGCTTTGGTCGCAGGAGGGTTATTATCCTCA TTCGGCGCTTCAGCG
codon optimized IL-10 SEQ ID NO:	TCGCCAGGTCAAGGAACGCAGTCAGAGAATTCATGCACTCACTTTCCG GGCAATCTGCCGAATATGCTGCGCGATCTGCGAGATGCATTCTCTCGC GTGAAAACGTTCTTTCAAATGAAAGATCAACTGGATAATCTGCTGCTG AAGGAGTCGTTGTTGGAGGATTTTAAGGGGTATCTGGGTTGTCAAGCA

	CTGTCTGAAATGATTCAATTTTACTTGGAGGAAGTTATGCCGCAAGCG GAAAACCAAGATCCGGATATTAAGGCGCACGTGAACTCACTGGGCGA AAACCTGAAAACTTTGCGCCTGCGTCTGAGACGATGTCACCGATTCCT GCCGTGTGAAAACAAGTCAAAGGCGGTTGAGCAAGTTAAGAATGCTT TCAATAAGCTGCAAGAAAAGGGCATCTATAAAGCGATGTCTGAATTTG ATATCTTTATAAACTACATAGAAGCTTATATGACTATGAAGATTCGAA ATTAA

Functional Assays

Co-culture studies

[01340] To determine whether the hIL-10 expressed by the genetically engineered bacteria is biologically functional, *in vitro* experimentation is conducted, in which the bacterial supernatant containing secreted human IL-10 is added to the growth medium of THP-1 cells. IL-10 is known to induce the phosphorylation of STAT1 and STAT3 in these cells. Functional activity of bacterially secreted IL-10 is therefore assessed by its ability to phosphorylate STAT3 in THP-1 cells.

[01341] THP-1 cells are grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator supplemented with 5% CO2. Prior to treatment with the bacterial supernatant, THP-1 (1e6/24 well) are serum starved overnight. Titrations of either recombinant human IL-10 diluted in LB or clarified supernatant from wild type Nissle or the engineered bacteria are added to cells for 30 minutes. Cells are harvested and resuspended in lysis buffer, and phospho-STAT3 ELISA (ELISA pSTAT3 (Tyr705) (Cell Signaling Technology)) is run in triplicate for all samples, according to manufacturer's instructions. PBS-treated cells and PBS are added as negative controls. Dilutions of samples are included to demonstrate linearity. No signal is observed for wild type Nissle. Activitiy for the engineered strain comprising a PAL deletion and the integrated construct encoding hIL-10 with a various secretion tags as listed in **Table 95** above are measured.

Competition studies

[01342] As an additional control for specificity, a competition assay is performed. Titrations of anti-IL10 antibody are pre-incubated with constant concentrations of either rhIL10 (data not shown) or supernatants from the engineered bacteria for 15min. Next, the supernatants/ rhIL-10solutions are added to serum-starved THP-1 cells (1e6/well) and cells are incubated for 30 min followed by pSTAT3 ELISA as described above.

Example 66. Assessment of *in vitro* and in vivo activity of Biosafety System Containing Strain

[01343] The activity of the following strains is tested:

[01344] SYN-1001 comprises a construct shown in **FIG. 74C** 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. 74A**, except that the bla gene is replaced with the construct of **FIG. 24C** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01345] SYN-1002 comprises a construct shown in **FIG. 74C** 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. 74A**, except that the bla gene is replaced with the construct of **FIG. 24D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01346] SYN-1003 comprises a construct shown in **FIG. 74D** 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. 74A**, except that the bla gene is replaced with the construct of **FIG. 24C** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01347] SYN-1004 comprises a construct shown in **FIG. 74D** 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. 74A**, except that the bla gene is replaced with the construct of **FIG. 24D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01348] SYN-1005 comprises a construct shown in **FIG. 74C** 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. 74B**, except that the bla gene is replaced with the

construct of **FIG. 24C** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01349] SYN-1006 comprises a construct shown in **FIG. 74C** 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. 74B**, except that the bla gene is replaced with the construct of **FIG. 24D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01350] SYN-1007 comprises a construct shown in **FIG. 74D** 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. 74B**, except that the bla gene is replaced with the construct of **FIG. 24D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01351] SYN-1008 a construct shown in **FIG. 74D** 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. 74B**, except that the bla gene is replaced with the construct of **FIG. 24D** (OmpF-hGLP-1). On other embodiments, other inducible or constitutive promoters are used.

[01352] SYN-1009 a construct shown in **FIG. 74C** 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. 74A**, except that the bla gene is replaced with the construct of **FIG. 8A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

[01353] SYN-1011 comprises a construct shown in **FIG. 74D** 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. 74A**, except that the bla gene is replaced with the construct of **FIG. 8A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

[01354] SYN-1013 comprises a construct shown in **FIG. 74C** 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. 74B**, except that the bla gene is replaced with the construct of **FIG. 8A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

[01355] SYN-1014 comprises a construct shown in **FIG. 74D** 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. 74B**, except that the bla gene is replaced with the construct of **FIG. 8A** (FNR-ter/pbt-buk butyrate cassette). On other embodiments, other inducible or constitutive promoters are used.

Table 96. 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**	ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAG GGTTATTGTCTCATGAGCGGATACATATTTGAATGT ATTTAGAAAAATAAACAAATAGGGGAATTAAAAAA AAGCCCGCTCATTAGGCGGGCTACTACCTAGGCCG CGGCCGCGCGAATTCGAGCTCGGTACCCGGGGATC CTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGG CCGCGTCGTGACTGGGAAAACCCTGGCGACTAGTC TTGGACTCCTGTTGATAGATCCAGTAATGACCTCAG AACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCC GCCGGGCGTTTTTTATTGGTGAGAATCCAGGGGTCC CCAATAATTACGATTTAAATCACAGCAAACACCAC GTCGGCCCTATCAGCTGCGTGCTTTCTATGAGTCGT TGCTGCATAACTTGACAATTAACATCCGGCTCGTAG GGTTTGTGGAGGGCCCAAGTTCACTTAAAAAGGAG ATCAACAATGAAAGCAATTTTCGTACTGAAACATCT TAATCATGCTGGGGAGGGTTTCTAATGTTCACGGGA AGTATTGTCGCGATTGTTACTCCGATGGATGAAAAA GGTAATGTCTGTCGGGCTAGCTTGAAAAAACTGATT GATTATCATGTCGCCAGCGGTACTTCGGCGATCGTT TCTGTTGGCACCACTGGCGAGTCCGCTACCTTAAAT CATGACGAACATGCTGATGTGGTGATGATGACGCT GGATCTGGCTGATGGGCGCATTCCGGTAATTGCCGG GACCGGCGCTAACGCTACTGCGGAAGCCATTAGCC TGACGCAGCGCTTCAATGACAGTGGTATCGTCGGCT GCCTGACGGTAACCCCTTACTACAATCGTCCGTCGC AAGAAGGTTTGTATCAGCATTTCAAAGCCATCGCTG AGCATACTGACCTGCCGCAAATTCTGTATAATGTGC	352

	CGTCCCGTACTGGCTGCGATCTGCTCCCGGAAACGG TGGGCCGTCTGGCGAAAGTAAAAAATATTATCGGA ATCAAAGAGGCAACAGGGAACTTAACGCGTGTAAA CCAGATCAAAGAGCTGGTTTCAGATGATTTTGTTCT GCTGAGCGGCGATGATGCGAGCGCGCTGGACTTCA TGCAATTGGGCGGTCATGGGGTTATTTCCGTTACGG CTAACGTCGCAGCGCGTGATATGGCCCAGATGTGC AAACTGGCAGCAGAAGGGCATTTTGCCGAGGCACG CGTTATTAATCAGCGTCTGATGCCATTACACAACAA ACTATTTGTCGAACCCAATCCAATCCCGGTGAAATG GGCATGTAAGGAACTGGGTCTTGTGGCGACCGATA CGCTGCGCCTGCCAATGACACCAATCACCGACAGT GGCCGTGAGACGGTCAGAGCGGCGCTTAAACATGC CGGTTTGCTGTAAGACTTTTGTCAGGTTCCTACTGT GACGACTACCACCGATAGACTGGAGTGTTGCTGCG AAAAAACCCCGCCGAAGCGGGGTTTTTTGCGAGAA GTCACCACGATTGTGCTTTACACGGAGTAGTCGGCA GTTCCTTAAGTCAGAATAGTGGACAGGCGGCCAAG AACTTCGTTCATGATAGTCTCCGGAACCCGTTCGAG TCGTTTTCCGCCCCGTGCTTTCATATCAATTGTCCGG GGTTGATCGCAACGTACAACACCTGTGGTACGTATG CCAACACCATCCAACGACACCGCAAAGCCGGCAGT GCGGGCAAAATTGCCTCCGCTGGTTACGGGCACAA CAACAGGCAGGCGGGTCACGCGATTAAAGGCCGCC GGTGTGACAATCAGCACCGGCCGCGTTCCCTGCTGC TCATGACCTGCGGTAGGATCAAGCGAGACAAGCCA GATTTCCCCTCTTTCCATCTAGTATAACTATTGTTTC TCTAGTAACATTTATTGTACAACACGAGCCCATTTT TGTCAAATAAATTTTAAATTATATCAACGTTAATAA GACGTTGTCAATAAAATTATTTTGACAAAATTGGCC GGCCGGCGCGCCGATCTGAAGATCAGCAGTTCAAC CTGTTGATAGTACGTACTAAGCTCTCATGTTTCACG TACTAAGCTCTCATGTTTAACGTACTAAGCTCTCAT GTTTAACGAACTAAACCCTCATGGCTAACGTACTAA GCTCTCATGGCTAACGTACTAAGCTCTCATGTTTCA CGTACTAAGCTCTCATGTTTGAACAATAAAATTAAT ATAAATCAGCAACTTAAATAGCCTCTAAGGTTTTAA GTTTTATAAGAAAAAAAAGAATATATAAGGCTTTT AAAGCCTTTAAGGTTTAACGGTTGTGGACAACAAG CCAGGGATGTAACGCACTGAGAAGCCCTTAGAGCC TCTCAAAGCAATTTTGAGTGACACAGGAACACTTA ACGGCTGACATGGGGCGCGCCCAGCTGTCTAGGGC GGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGAC AAACAACAGATAAAACGAAAGGCCCAGTCTTTCGA CTGAGCCTTTCGTTTTATTTGATGCCT	
Biosafety Plasmid System Component –	ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAG GGTTATTGTCTCATGAGCGGATACATATTTGAATGT ATTTAGAAAAATAAACAAATAGGGGAATTAAAAAA	353

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**	AAGCCCGCTCATTAGGCGGGCTACTACCTAGGCCG CGGCCGCGCGAATTCGAGCTCGGTACCCGGGGATC CTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGG CCGCGTCGTGACTGGGAAAACCCTGGCGACTAGTC TTGGACTCCTGTTGATAGATCCAGTAATGACCTCAG AACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCC GCCGGGCGTTTTTTATTGGTGAGAATCCAGGGGTCC CCAATAATTACGATTTAAATCACAGCAAACACCAC GTCGGCCCTATCAGCTGCGTGCTTTCTATGAGTCGT TGCTGCATAACTTGACAATTAATCATCCGGCTCGTA GGGTTTGTGGAGGGCCCAAGTTCACTTAAAAAGGA GATCAACAATGAAAGCAATTTTCGTACTGAAACAT CTTAATCATGCTGGGGAGGGTTTCTAATGAAACAGT ATTTAGAACTGATGCAAAAAGTGCTCGACGAAGGC ACACAGAAAAACGACCGTACCGGAACCGGAACGCT TTCCATTTTTGGTCATCAGATGCGTTTTAACCTGCA AGATGGATTCCCGCTGGTGACAACTAAACGTTGCC ACCTGCGTTCCATCATCCATGAACTGCTGTGGTTTC TTCAGGGCGACACTAACATTGCTTATCTACACGAAA ACAATGTCACCATCTGGGACGAATGGGCCGATGAA AACGGCGACCTCGGGCCAGTGTATGGTAAACAGTG GCGTGCCTGGCCAACGCCAGATGGTCGTCATATTGA CCAGATCACTACGGTACTGAACCAGCTGAAAAACG ACCCGGATTCGCGCCGCATTATTGTTTCAGCGTGGA ACGTAGGCGAACTGGATAAAATGGCGCTGGCACCG TGCCATGCATTCTTCCAGTTCTATGTGGCAGACGGC AAACTCTCTTGCCAGCTTTATCAGCGCTCCTGTGAC GTCTTCCTCGGCCTGCCGTTCAACATTGCCAGCTAC GCGTTATTGGTGCATATGATGGCGCAGCAGTGCGAT CTGGAAGTGGGTGATTTTGTCTGGACCGGTGGCGAC ACGCATCTGTACAGCAACCATATGGATCAAACTCAT CTGCAATTAAGCCGCGAACCGCGTCCGCTGCCGAA GTTGATTATCAAACGTAAACCCGAATCCATCTTCGA CTACCGTTTCGAAGACTTTGAGATTGAAGGCTACGA TCCGCATCCGGGCATTAAAGCGCCGGTGGCTATCTA AGACTTTTGTCAGGTTCCTACTGTGACGACTACCAC CGATAGACTGGAGTGTTGCTGCGAAAAAACCCCGC CGAAGCGGGGTTTTTTGCGAGAAGTCACCACGATT GTGCTTTACACGGAGTAGTCGGCAGTTCCTTAAGTC AGAATAGTGGACAGGCGGCCAAGAACTTCGTTCAT GATAGTCTCCGGAACCCGTTCGAGTCGTTTTCCGCC CCGTGCTTTCATATCAATTGTCCGGGGTTGATCGCA ACGTACAACACCTGTGGTACGTATGCCAACACCATC CAACGACACCGCAAAGCCGGCAGTGCGGGCAAAAT TGCCTCCGCTGGTTACGGGCACAACAACAGGCAGG CGGGTCACGCGATTAAAGGCCGCCGGTGTGACAAT CAGCACCGGCCGCGTTCCCTGCTGCTCATGACCTGC GGTAGGATCAAGCGAGACAAGCCAGATTTCCCCTC	

	TTTCCATCTAGTATAACTATTGTTTCTCTAGTAACAT TTATTGTACAACACGAGCCCATTTTTGTCAAATAAA TTTTAAATTATATCAACGTTAATAAGACGTTGTCAA TAAAATTATTTTGACAAAATTGGCCGGCCGGCGCGC CGATCTGAAGATCAGCAGTTCAACCTGTTGATAGTA CGTACTAAGCTCTCATGTTTCACGTACTAAGCTCTC ATGTTTAACGTACTAAGCTCTCATGTTTAACGAACT AAACCCTCATGGCTAACGTACTAAGCTCTCATGGCT AACGTACTAAGCTCTCATGTTTCACGTACTAAGCTC TCATGTTTGAACAATAAAATTAATATAAATCAGCAA CTTAAATAGCCTCTAAGGTTTTAAGTTTTATAAGAA AAAAAAGAATATATAAGGCTTTTAAAGCCTTTAAG GTTTAACGGTTGTGGACAACAAGCCAGGGATGTAA CGCACTGAGAAGCCCTTAGAGCCTCTCAAAGCAAT TTTGAGTGACACAGGAACACTTAACGGCTGACATG GGGCGCGCCCAGCTGTCTAGGGCGGCGGATTTGTC CTACTCAGGAGAGCGTTCACCGACAAACAACAGAT AAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCG TTTTATTTGATGCCT	
Kid toxin (reverse orientation)	TTAAGTCAGAATAGTGGACAGGCGGCCAAGAACTT CGTTCATGATAGTCTCCGGAACCCGTTCGAGTCGTT TTCCGCCCCGTGCTTTCATATCAATTGTCCGGGGTT GATCGCAACGTACAACACCTGTGGTACGTATGCCA ACACCATCCAACGACACCGCAAAGCCGGCAGTGCG GGCAAAATTGCCTCCGCTGGTTACGGGCACAACAA CAGGCAGGCGGGTCACGCGATTAAAGGCCGCCGGT GTGACAATCAGCACCGGCCGCGTTCCCTGCTGCTCA TGACCTGCGGTAGGATCAAGCGAGACAAGCCAGAT TTCCCCTCTTTCCAT	354
dapA	ATGTTCACGGGAAGTATTGTCGCGATTGTTACTCCG ATGGATGAAAAAGGTAATGTCTGTCGGGCTAGCTT GAAAAAACTGATTGATTATCATGTCGCCAGCGGTA CTTCGGCGATCGTTTCTGTTGGCACCACTGGCGAGT CCGCTACCTTAAATCATGACGAACATGCTGATGTGG TGATGATGACGCTGGATCTGGCTGATGGGCGCATTC CGGTAATTGCCGGGACCGGCGCTAACGCTACTGCG GAAGCCATTAGCCTGACGCAGCGCTTCAATGACAG TGGTATCGTCGGCTGCCTGACGGTAACCCCTTACTA CAATCGTCCGTCGCAAGAAGGTTTGTATCAGCATTT CAAAGCCATCGCTGAGCATACTGACCTGCCGCAAA TTCTGTATAATGTGCCGTCCCGTACTGGCTGCGATC TGCTCCCGGAAACGGTGGGCCGTCTGGCGAAAGTA AAAAATATTATCGGAATCAAAGAGGCAACAGGGAA CTTAACGCGTGTAAACCAGATCAAAGAGCTGGTTTC AGATGATTTTGTTCTGCTGAGCGGCGATGATGCGAG CGCGCTGGACTTCATGCAATTGGGCGGTCATGGGGT TATTTCCGTTACGGCTAACGTCGCAGCGCGTGATAT GGCCCAGATGTGCAAACTGGCAGCAGAAGGGCATT	355

	TTGCCGAGGCACGCGTTATTAATCAGCGTCTGATGC CATTACACAACAAACTATTTGTCGAACCCAATCCAA TCCCGGTGAAATGGGCATGTAAGGAACTGGGTCTT GTGGCGACCGATACGCTGCGCCTGCCAATGACACC AATCACCGACAGTGGCCGTGAGACGGTCAGAGCGG CGCTTAAACATGCCGGTTTGCTGTAA	
thyA	ATGAAACAGTATTTAGAACTGATGCAAAAAGTGCT CGACGAAGGCACACAGAAAAACGACCGTACCGGA ACCGGAACGCTTTCCATTTTTGGTCATCAGATGCGT TTTAACCTGCAAGATGGATTCCCGCTGGTGACAACT AAACGTTGCCACCTGCGTTCCATCATCCATGAACTG CTGTGGTTTCTTCAGGGCGACACTAACATTGCTTAT CTACACGAAAACAATGTCACCATCTGGGACGAATG GGCCGATGAAAACGGCGACCTCGGGCCAGTGTATG GTAAACAGTGGCGTGCCTGGCCAACGCCAGATGGT CGTCATATTGACCAGATCACTACGGTACTGAACCAG CTGAAAAACGACCCGGATTCGCGCCGCATTATTGTT TCAGCGTGGAACGTAGGCGAACTGGATAAAATGGC GCTGGCACCGTGCCATGCATTCTTCCAGTTCTATGT GGCAGACGGCAAACTCTCTTGCCAGCTTTATCAGCG CTCCTGTGACGTCTTCCTCGGCCTGCCGTTCAACAT TGCCAGCTACGCGTTATTGGTGCATATGATGGCGCA GCAGTGCGATCTGGAAGTGGGTGATTTTGTCTGGAC CGGTGGCGACACGCATCTGTACAGCAACCATATGG ATCAAACTCATCTGCAATTAAGCCGCGAACCGCGTC CGCTGCCGAAGTTGATTATCAAACGTAAACCCGAA TCCATCTTCGACTACCGTTTCGAAGACTTTGAGATT GAAGGCTACGATCCGCATCCGGGCATTAAAGCGCC GGTGGCTATCTAA	356
Kid toxin polypeptide	MERGEIWLVSLDPTAGHEQQGTRPVLIVTPAAFNRVT RLPVVVPVTSGGNFARTAGFAVSLDGVGIRTTGVVRC DQPRTIDMKARGGKRLERVPETIMNEVLGRLSTILT*	357
dapA polypeptide	MFTGSIVAIVTPMDEKGNVCRASLKKLIDYHVASGTS AIVSVGTTGESATLNHDEHADVVMMTLDLADGRIPVI AGTGANATAEAISLTQRFNDSGIVGCLTVTPYYNRPS QEGLYQHFKAIAEHTDLPQILYNVPSRTGCDLLPETVG RLAKVKNIIGIKEATGNLTRVNQIKELVSDDFVLLSGD DASALDFMQLGGHGVISVTANVAARDMAQMCKLAA EGHFAEARVINQRLMPLHNKLFVEPNPIPVKWACKEL GLVATDTLRLPMTPITDSGRETVRAALKHAGLL	358
ThyA polypeptide	MKQYLELMQKVLDEGTQKNDRTGTGTLSIFGHQMRF NLQDGFPLVTTKRCHLRSIIHELLWFLQGDTNIAYLHE NNVTIWDEWADENGDLGPVYGKQWRAWPTPDGRHI DQITTVLNQLKNDPDSRRIIVSAWNVGELDKMALAPC HAFFQFYVADGKLSCQLYQRSCDVFLGLPFNIASYAL LVHMMAQQCDLEVGDFVWTGGDTHLYSNHMDQTH LQLSREPRPLPKLIIKRKPESIFDYRFEDFEIEGYDPHPG IKAPVAI*	359

Table 97. 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**)	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGGAT CTGCTGGAACAGGTGGTGAGACTCAAGGTCATGATGGA CGTGAACAAAAAAACGAAAATTCGCCACCGAAACGAGC TAAATCACACCCTGGCTCAACTTCCTTTGCCCGCAAAGC GAGTGATGTATATGGCGCTTGCTCCCATTGATAGCAAAG AACCTCTTGAACGAGGGCGAGTTTTCAAAATTAGGGCTG AAGACCTTGCAGCGCTCGCCAAAATCACCCCATCGCTTG CTTATCGACAATTAAAAGAGGGTGGTAAATTACTTGGTG CCAGCAAAATTTCGCTAAGAGGGGATGATATCATTGCTT TAGCTAAAGAGCTTAACCTGCTCTTTACTGCTAAAAACT CCCCTGAAGAGTTAGACCTTAACATTATTGAGTGGATAG CTTATTCAAATGATGAAGGATACTTGTCTTTAAAATTCA CCAGAACCATAGAACCATATATCTCTAGCCTTATTGGGA AAAAAAATAAATTCACAACGCAATTGTTAACGGCAAGC TTACGCTTAAGTAGCCAGTATTCATCTTCTCTTTATCAAC TTATCAGGAAGCATTACTCTAATTTTAAGAAGAAAAATT ATTTTATTATTTCCGTTGATGAGTTAAAGGAAGAGTTAA TAGCTTATACTTTTGATAAAGATGGAAATATTGAGTACA AATACCCTGACTTTCCTATTTTTAAAAGGGATGTGTTAA ATAAAGCCATTGCTGAAATTAAAAAGAAAACAGAAATA TCGTTTGTTGGCTTCACTGTTCATGAAAAAGAAGGAAGA AAAATTAGTAAGCTGAAGTTCGAATTTGTCGTTGATGAA GATGAATTTTCTGGCGATAAAGATGATGAAGCTTTTTTT ATGAATTTATCTGAAGCTGATGCAGCTTTTCTCAAGGTA TTTGATGAAACCGTACCTCCCAAAAAAGCTAAGGGGTGA GGATCTCCAGGCATCAAATAAAACGAAAGGCTCAGTCG AAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGA ACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTG GGCCTTTCTGCGTTTATACCCGGGAAAAAGAGTATTGAC TtaaagtctaacctataggTATAATGTGTGGAGACCAGAGGTAAGG AGGTAACAACCATGCGAGTGTTGAAGAAACATCTTAATC ATGCTAAGGAGGTTTTCTAATGCATACCACCCGACTGAA GAGGGTTGGCGGCTCAGTTATGCTGACCGTCCCACCGGC ACTGCTGAATGCGCTGTCTCTGGGCACAGATAATGAAGT TGGCATGGTCATTGATAATGGCCGGCTGATTGTTGAGCC GTACAGACGCCCGCAATATTCACTGGCTGAGCTACTGGC ACAGTGTGATCCGAATGCTGAAATATCAGCTGAAGAAC GAGAATGGCTGGATGCACCGGCGACTGGTCAGGAGGAA ATCTGA	360
Biosafety Chromosomal Construct -	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGGAT CTTCCGGAAGACTAGGTGAGACTCAAGGTCATGATGGAC GTGAACAAAAAAACGAAAATTCGCCACCGAAACGAGCT	361

medium copy Rep (Pi) and Kis antitoxin (as shown in **FIG. 74D**)	AAATCACACCCTGGCTCAACTTCCTTTGCCCGCAAAGCG AGTGATGTATATGGCGCTTGCTCCCATTGATAGCAAAGA ACCTCTTGAACGAGGGCGAGTTTTCAAAATTAGGGCTGA AGACCTTGCAGCGCTCGCCAAAATCACCCCATCGCTTGC TTATCGACAATTAAAAGAGGGTGGTAAATTACTTGGTGC CAGCAAAATTTCGCTAAGAGGGGATGATATCATTGCTTT AGCTAAAGAGCTTAACCTGCTCTTTACTGCTAAAAACTC CCCTGAAGAGTTAGACCTTAACATTATTGAGTGGATAGC TTATTCAAATGATGAAGGATACTTGTCTTTAAAATTCAC CAGAACCATAGAACCATATATCTCTAGCCTTATTGGGAA AAAAAATAAATTCACAACGCAATTGTTAACGGCAAGCTT ACGCTTAAGTAGCCAGTATTCATCTTCTCTTTATCAACTT ATCAGGAAGCATTACTCTAATTTTAAGAAGAAAAATTAT TTTATTATTTCCGTTGATGAGTTAAAGGAAGAGTTAATA GCTTATACTTTTGATAAAGATGGAAATATTGAGTACAAA TACCCTGACTTTCCTATTTTTAAAAGGGATGTGTTAAATA AAGCCATTGCTGAAATTAAAAAGAAAACAGAAATATCG TTTGTTGGCTTCACTGTTCATGAAAAAGAAGGAAGAAAA ATTAGTAAGCTGAAGTTCGAATTTGTCGTTGATGAAGAT GAATTTTCTGGCGATAAAGATGATGAAGCTTTTTTTATG AATTTATCTGAAGCTGATGCAGCTTTTCTCAAGGTATTTG ATGAAACCGTACCTCCCAAAAAAGCTAAGGGGTGAGGA TCTCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAA GACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACG CTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGC CTTTCTGCGTTTATACCCGGGAAAAAGAGTATTGACTtaaa gtctaacctataggTATAATGTGTGGAGACCAGAGGTAAGGAGG TAACAACCATGCGAGTGTTGAAGAAACATCTTAATCATG CTAAGGAGGTTTTCTAATGCATACCACCCGACTGAAGAG GGTTGGCGGCTCAGTTATGCTGACCGTCCCACCGGCACT GCTGAATGCGCTGTCTCTGGGCACAGATAATGAAGTTGG CATGGTCATTGATAATGGCCGGCTGATTGTTGAGCCGTA CAGACGCCCGCAATATTCACTGGCTGAGCTACTGGCACA GTGTGATCCGAATGCTGAAATATCAGCTGAAGAACGAG AATGGCTGGATGCACCGGCGACTGGTCAGGAGGAAATC TGA	
Rep (Pi)	TGAGACTCAAGGTCATGATGGACGTGAACAAAAAAACG AAAATTCGCCACCGAAACGAGCTAAATCACACCCTGGCT CAACTTCCTTTGCCCGCAAAGCGAGTGATGTATATGGCG CTTGCTCCCATTGATAGCAAAGAACCTCTTGAACGAGGG CGAGTTTTCAAAATTAGGGCTGAAGACCTTGCAGCGCTC GCCAAAATCACCCCATCGCTTGCTTATCGACAATTAAAA GAGGGTGGTAAATTACTTGGTGCCAGCAAAATTTCGCTA AGAGGGGATGATATCATTGCTTTAGCTAAAGAGCTTAAC CTGCTCTTTACTGCTAAAAACTCCCCTGAAGAGTTAGAC CTTAACATTATTGAGTGGATAGCTTATTCAAATGATGAA GGATACTTGTCTTTAAAATTCACCAGAACCATAGAACCA TATATCTCTAGCCTTATTGGGAAAAAAAATAAATTCACA	362

	ACGCAATTGTTAACGGCAAGCTTACGCTTAAGTAGCCAG TATTCATCTTCTCTTTATCAACTTATCAGGAAGCATTACT CTAATTTTAAGAAGAAAAATTATTTTATTATTTCCGTTGA TGAGTTAAAGGAAGAGTTAATAGCTTATACTTTTGATAA AGATGGAAATATTGAGTACAAATACCCTGACTTTCCTAT TTTTAAAAGGGATGTGTTAAATAAAGCCATTGCTGAAAT TAAAAAGAAAACAGAAATATCGTTTGTTGGCTTCACTGT TCATGAAAAAGAAGGAAGAAAAATTAGTAAGCTGAAGT TCGAATTTGTCGTTGATGAAGATGAATTTTCTGGCGATA AAGATGATGAAGCTTTTTTTATGAATTTATCTGAAGCTG ATGCAGCTTTTCTCAAGGTATTTGATGAAACCGTACCTC CCAAAAAAGCTAAGGGGTGA	
Kis antitoxin	CATACCACCCGACTGAAGAGGGTTGGCGGCTCAGTTATG CTGACCGTCCCACCGGCACTGCTGAATGCGCTGTCTCTG GGCACAGATAATGAAGTTGGCATGGTCATTGATAATGGC CGGCTGATTGTTGAGCCGTACAGACGCCCGCAATATTCA CTGGCTGAGCTACTGGCACAGTGTGATCCGAATGCTGAA ATATCAGCTGAAGAACGAGAATGGCTGGATGCACCGGC GACTGGTCAGGAGGAAATCTGA	363
RBS (low copy)	GCTGGAACAGGTGG	364
RBS (medium copy)	TCCGGAAGACTAGG	365

Example 67.

Table 98. Other Sequences of interest

Wild-type *clbA* **(SEQ ID NO: 350)**	caaatatcacataatcttaacatatcaataaacacagtaaagtttcatgtgaaaaacat caaacataaaatacaagctcggaatacgaatcacgctatacacattgctaacagga atgagattatctaaatgaggattgatatattaattggacatactagttttttcatcaaac cagtagagataacttccttcactatctcaatgaggaagaaataaaacgctatgatca gtttcattttgtgagtgataaagaactctatattttaagccgtatcctgctcaaaacagc actaaaaagatatcaacctgatgtctcattacaatcatggcaatttagtacgtgcaaat atggcaaaccatttatagtttttcctcagttggcaaaaaagatttttttttaacctttcccat actatagatacagtagccgttgctattagttctcactgcgagcttggtgtcgatattga acaaataagagatttagacaactcttatctgaatatcagtcagcatttttttactccaca ggaagctactaacatagtttcacttcctcgttatgaaggtcaattacttttttggaaaat gtggacgctcaaagaagcttacatcaaatatcgaggtaaaggcctatctttaggact ggattgtattgaatttcatttaacaaataaaaaactaacttcaaaatatagaggttcacc tgtttatttctctcaatggaaaatatgtaactcatttctcgcattagcctctccactcatca cccctaaaataactattgagctatttcctatgcagtcccaactttatcaccacgactatc agctaattcattcgtcaaatgggcagaattgaatcgccacggataatctagacacttc tgagccgtcgataatattgattttcatattccgtcggtggtgtaagtatcccgcataatc gtgccattcacatttag
clbA knock-out **(SEQ ID NO: 351)**	ggatgggggaaacatggataagttcaaagaaaaaaacccgttatctctgcgtgaaa gacaagtattgcgcatgctggcacaaggtgatgagtactctcaaatatcacataatctt aacatatcaataaacacagtaaagtttcatgtgaaaaacatcaaacataaaatacaagc

	tcggaatacgaatcacgctatacacattgctaacaggaatgagattatctaaatgagga ttgaTGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATAC TTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCG GAATAGGAACTAAGGAGGATATTCATATGtcgtcaaatggg cagaattgaatcgccacggataatctagacacttctgagccgtcgataatattgattttc atattccgtcggtgg

CLAIMS

1. A bacterium comprising at least one gene or gene cassette encoding one or more non-native biosynthetic pathways for producing butyrate, wherein the bacterium comprises an endogenous pta gene which is knocked down via mutation or deletion, and wherein the at least one gene or gene cassette for producing butyrate is 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 bacterium comprises an endogenous adhE gene which is knocked down via mutation or deletion.

3. The bacterium of claim 1 or claim 2, wherein the bacterium comprises an endogenous frd gene which is knocked down via mutation or deletion.

4. The bacterium of any of claims 1-3, wherein the bacterium comprises an endogenous ldhA gene which is knocked down via mutation or deletion.

5. The bacterium of any of claims 1-4, wherein the at least one gene cassette comprises ter, thiA1, hbd, crt2, pbt, and buk genes.

6. The bacterium of any of claims 1-4, wherein the at least one gene cassette comprises ter, thiA1, hbd, crt2, and tesb genes.

7. A bacterium comprising a biosynthetic pathway for producing acetate, wherein the bacterium comprises an endogenous adhE gene which is knocked down via mutation or deletion.

8. A bacterium comprising a biosynthetic pathway for producing acetate, wherein the bacterium comprises an endogenous frd gene which is knocked down via mutation or deletion.

9. A bacterium comprising a biosynthetic pathway for producing acetate, wherein the bacterium comprises an endogenous ldhA gene which is knocked down via mutation or deletion.

10. The bacterium of claim 7 or claim 9, wherein the bacterium comprises an endogenous frd gene which is knocked down via mutation or deletion.

11. The bacterium of claim 7 or claim 8, wherein the bacterium comprises an endogenous ldhA gene which is knocked down via mutation or deletion.

12. The bacterium of claim 7, wherein the bacterium comprises an endogenous ldhA gene and an endogenous frd gene, both of which genes are knocked down via mutation and/or deletion.

13. The bacterium of any one of claims 7-12, wherein the biosynthetic pathway for producing acetate is a native biosynthetic pathway endogenous to the bacterium.

14. The bacterium of any one of claims 7-12, wherein the biosynthetic pathway for producing acetate is a non-native biosynthetic pathway.

15. The bacterium of claim 14, wherein the bacterium comprises at least one gene or gene cassette encoding the non-native biosynthetic pathway for producing acetate, wherein the at least one gene or gene cassette for producing acetate is operably linked to a directly or indirectly inducible promoter that is not associated with the gene or gene cassette in nature

16. The bacterium of any one of claims 1-6 and claim 15, wherein the promoter is induced by exogenous environmental conditions found in a mammalian gut.

17. The bacterium of claim 16, wherein the promoter is induced under low-oxygen or anaerobic conditions.

18. The bacterium of claim 17, wherein the promoter is a FNR-responsive promoter, an ANR-responsive promoter, or a DNR-responsive promoter.

19. The bacterium of claim 18, wherein the promoter is a FNR-responsive promoter.

20. The bacterium of any one of claims 1-6 and claim 15, wherein the promoter is induced by the presence of reactive nitrogen species.

21. The bacterium of claim 20, wherein the promoter is a NsrR-responsive promoter, NorR-responsive promoter, or a DNR-responsive promoter.

22. The bacterium of any one of claims 1-6 and claim 15, wherein the promoter is induced by the presence of reactive oxygen species.

23. The bacterium of claim 22, wherein the promoter is a OxyR-responsive promoter, PerR-responsive promoter, OhrR-responsive promoter, SoxR-responsive promoter, or a RosR-responsive promoter.

24. The bacterium of any one of claims 1-6 or claims 14-15, wherein the gene and/or gene cassette is located on a chromosome in the bacterium.

25. The bacterium of any one of claims 1-6 or claims 14-15, wherein the at least one gene and/or gene cassette is located on a plasmid in the bacterium.

26. The bacterium of any one of claims 1-25, wherein the bacterium is a probiotic bacterium.

27. The bacterium of claim 26, wherein the bacterium is selected from the group consisting of *Bacteroides, Bifidobacterium, Clostridium, Escherichia, Lactobacillus*, and *Lactococcus*.

28. The bacterium of claim 27, wherein the bacterium is *Escherichia coli* strain Nissle.

29. The bacterium of any one of claims 1-28, wherein the bacterium is an auxotroph in a gene that is complemented when the bacterium is present in a mammalian gut.

30. The bacterium of claim 29, wherein the bacterium is an auxotroph in diaminopimelic acid or an enzyme in the thymine biosynthetic pathway.

31. A pharmaceutically acceptable composition comprising the bacterium of any one of claims 1-30; and a pharmaceutically acceptable carrier.

32. The composition of claim 31 formulated for oral or rectal administration.

33. A method of treating or preventing an autoimmune disorder, comprising the step of administering to a patient in need thereof, the composition of any one of claims 31 or 32.

34. A method of treating a disease or condition associated with gut inflammation and/or compromised gut barrier function comprising the step of administering to a patient in need thereof, the composition of any one of claims 31 or 32.

35. The method of claim 33, wherein the autoimmune disorder is selected from the group consisting of acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, Addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune oophoritis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroid disease, autoimmune urticarial, Axonal & neuronal neuropathies, Balo disease, Behcet's disease, Bullous pemphigoid, Cardiomyopathy, Castleman disease, Celiac disease, Chagas disease, Chronic inflammatory demyelinating polyneuropathy (CIDP), Chronic recurrent

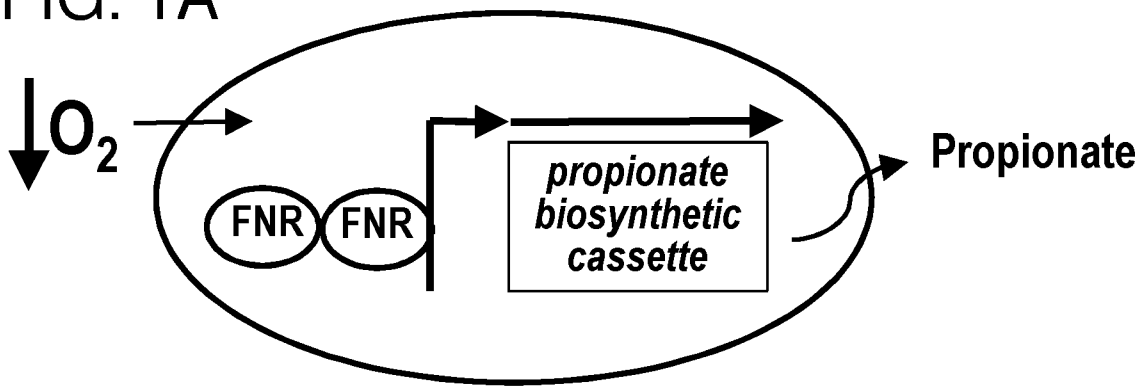
multifocal ostomyelitis (CRMO), Churg-Strauss syndrome, Cicatricial pemphigoid/benign mucosal pemphigoid, Crohn's disease, Cogan syndrome, Cold agglutinin disease, Congenital heart block, Coxsackie myocarditis, CREST disease, Essential mixed cryoglobulinemia, Demyelinating neuropathies, Dermatitis herpetiformis, Dermatomyositis, Devic's disease (neuromyelitis optica), Discoid lupus, Dressler's syndrome, Endometriosis, Eosinophilic esophagitis, Eosinophilic fasciitis, Erythema nodosum, Experimental allergic encephalomyelitis, Evans syndrome, Fibrosing alveolitis, Giant cell arteritis (temporal arteritis), Giant cell myocarditis, Glomerulonephritis, Goodpasture's syndrome, Granulomatosis with Polyangiitis (GPA), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, Hemolytic anemia, Henoch-Schonlein purpura, Herpes gestationis, Hypogammaglobulinemia, Idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, Immunoregulatory lipoproteins, Inclusion body myositis, Interstitial cystitis, Juvenile arthritis, Juvenile idiopathic arthritis, Juvenile myositis, Kawasaki syndrome, Lambert-Eaton syndrome, Leukocytoclastic vasculitis, Lichen planus, Lichen sclerosus, Ligneous conjunctivitis, Linear IgA disease (LAD), Lupus (Systemic Lupus Erythematosus), chronic Lyme disease, Meniere's disease, Microscopic polyangiitis, Mixed connective tissue disease (MCTD), Mooren's ulcer, Mucha-Habermann disease, Multiple sclerosis, Myasthenia gravis, Myositis, Narcolepsy, Neuromyelitis optica (Devic's), Neutropenia, Ocular cicatricial pemphigoid, Optic neuritis, Palindromic rheumatism, PANDAS (Pediatric autoimmune Neuropsychiatric Disorders Associated with Streptococcus), Paraneoplastic cerebellar degeneration, Paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, Pars planitis (peripheral uveitis), Pemphigus, Peripheral neuropathy, Perivenous encephalomyelitis, Pernicious anemia, POEMS syndrome, Polyarteritis nodosa, Type I, II, & III autoimmune polyglandular syndromes, Polymyalgia rheumatic, Polymyositis, Postmyocardial infarction syndrome, Postpericardiotomy syndrome, Progesterone dermatitis, Primary biliary cirrhosis, Primary sclerosing cholangitis, Psoriasis, Psoriatic arthritis, Idiopathic pulmonary fibrosis, Pyoderma gangrenosum, Pure red cell aplasia, Raynauds phenomenon, reactive arthritis, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis,

scleroderma, Sjogren's syndrome, sperm & testicular autoimmunity, stiff person syndrome, subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, temporal arteritis/giant cell arteritis, thrombocytopenic purpura (TTP), Tolosa-Hunt syndrome, transverse myelitis, type 1 diabetes, asthma, ulcerative colitis, undifferentiated connective tissue disease (UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, and Wegener's granulomatosis.

36. The method of claim 35, wherein the autoimmune disorder is selected from the group consisting of type 1 diabetes, lupus, rheumatoid arthritis, ulcerative colitis, juvenile arthritis, psoriasis, psoriatic arthritis, celiac disease, and ankylosing spondylitis.

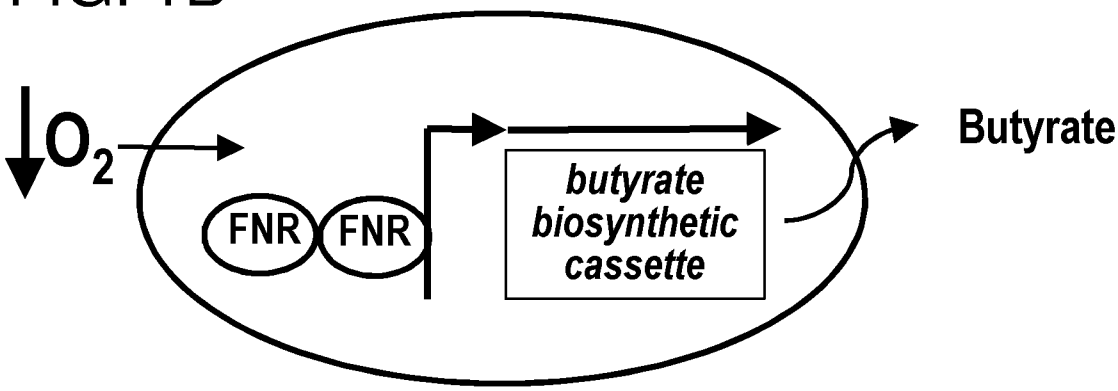
37. The method of claim 34, wherein the disease or condition is selected from an inflammatory bowel disease, including Crohn's disease and ulcerative colitis, and a diarrheal disease.

FIG. 1A



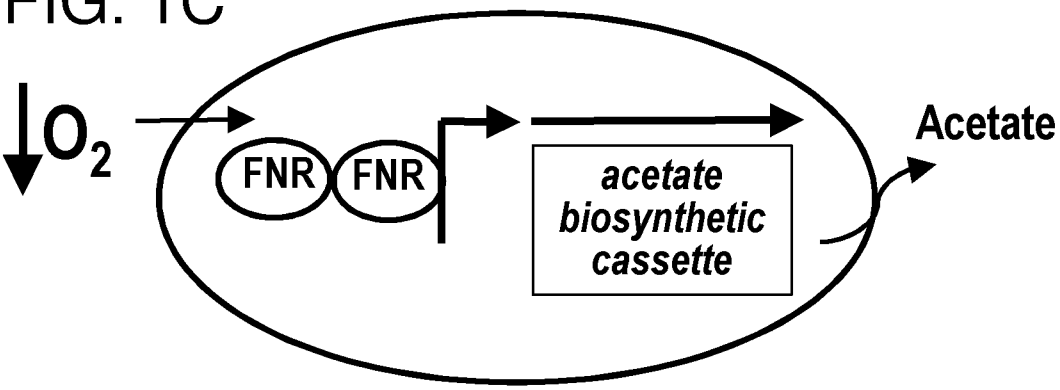
E. Coli Nissle 1917

FIG. 1B



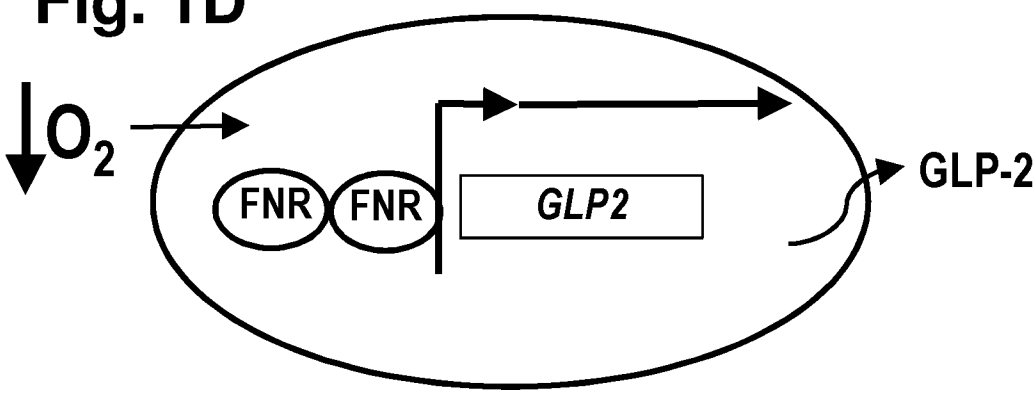
E. Coli Nissle 1917

FIG. 1C



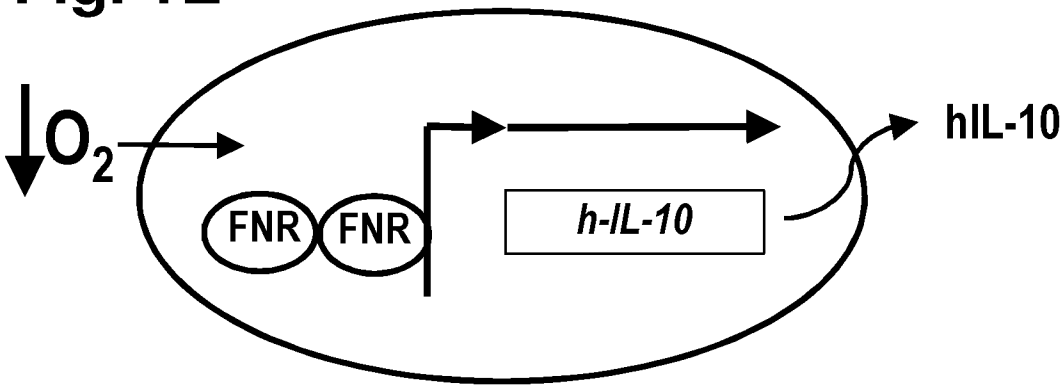
E. Coli Nissle 1917

Fig. 1D



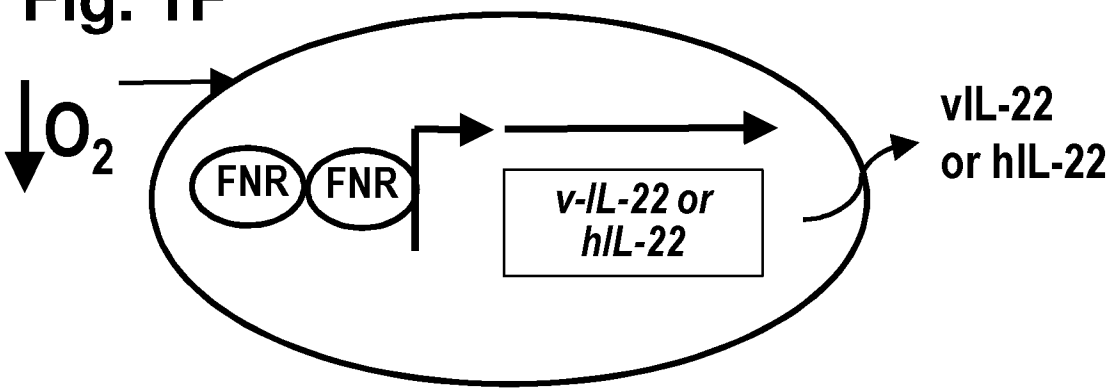
E. Coli Nissle 1917

Fig. 1E



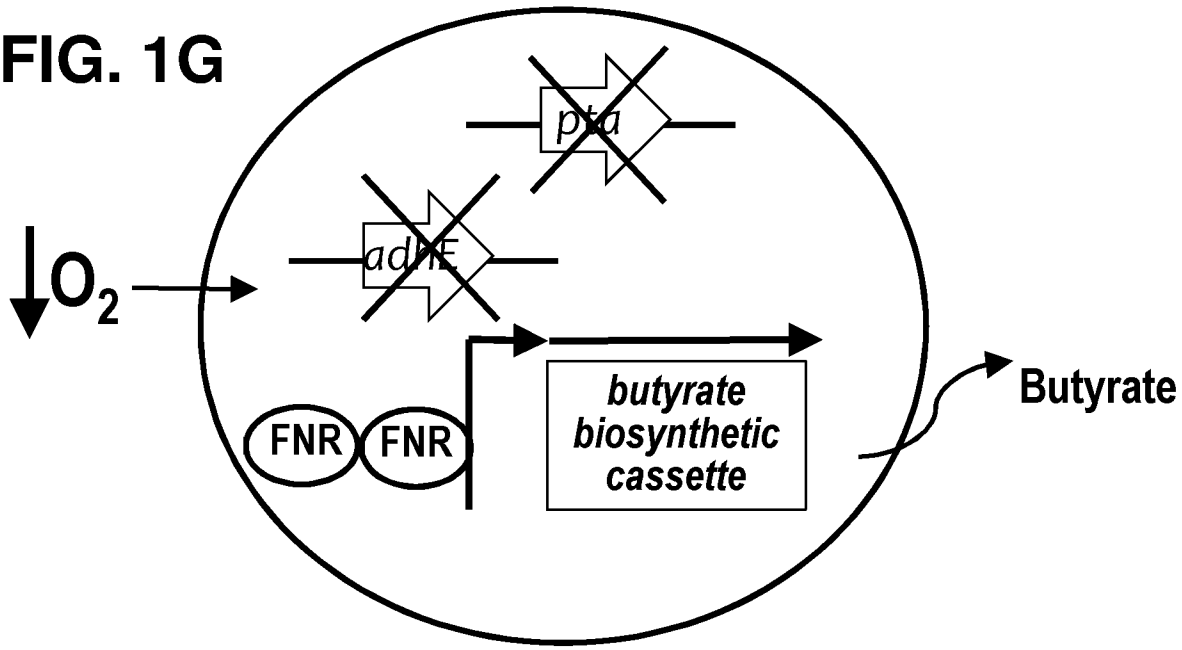
E. Coli Nissle 1917

Fig. 1F



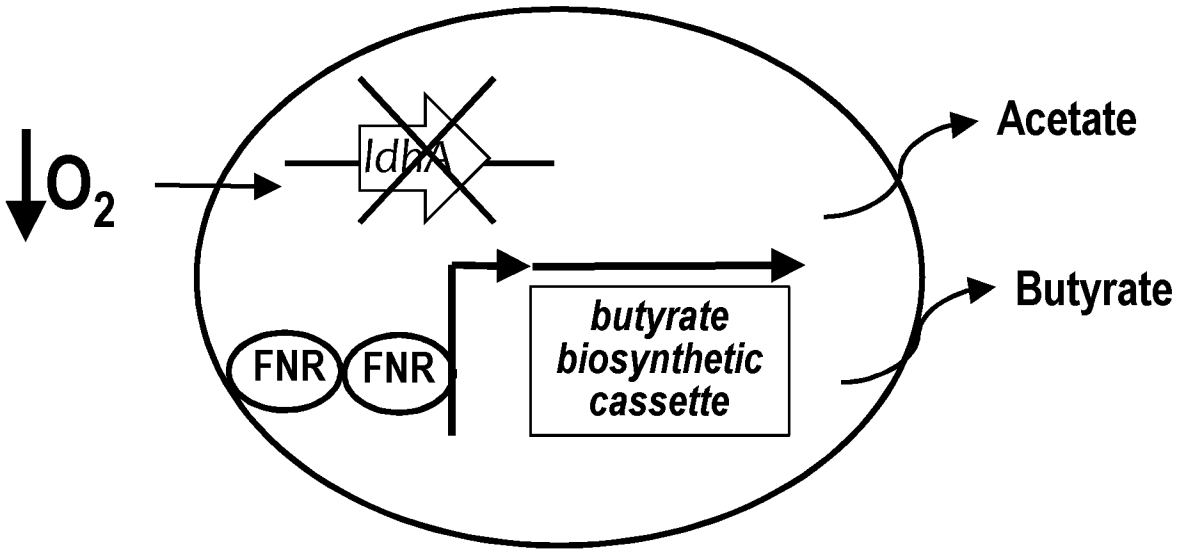
E. Coli Nissle 1917

FIG. 1G



E. Coli Nissle 1917

FIG. 1H



E. Coli Nissle 1917

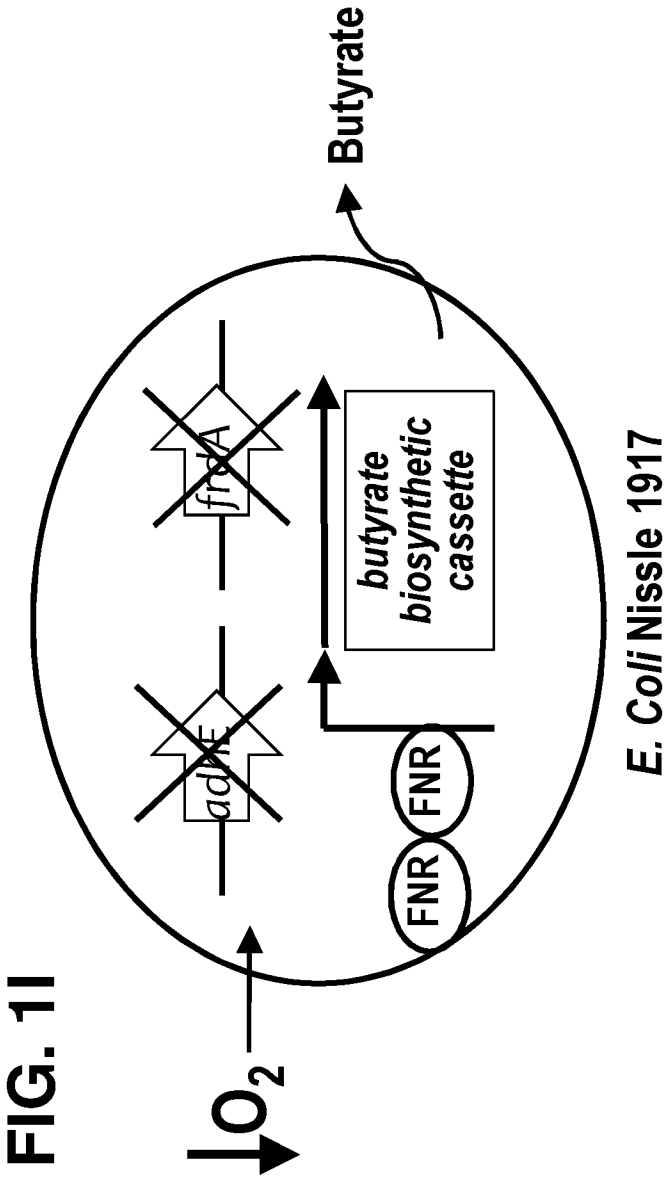
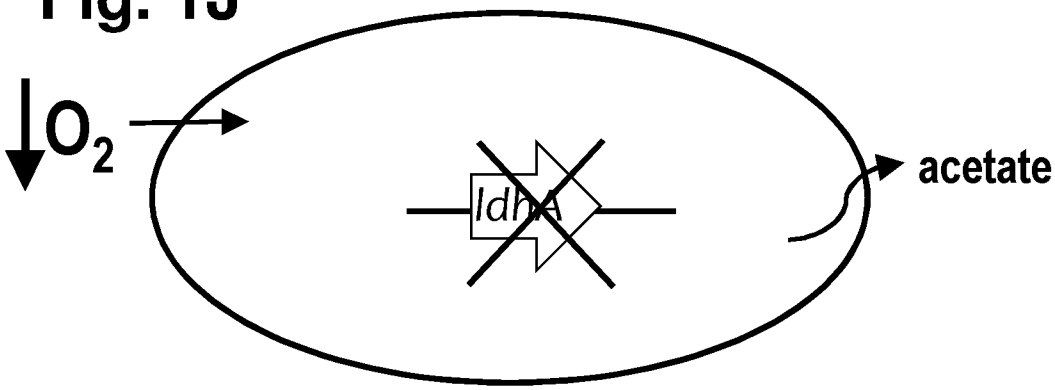

Butyrate
butyrate biosynthetic cassette
frdA
adhE
FNR
FNR
E. Coli Nissle 1917
O2

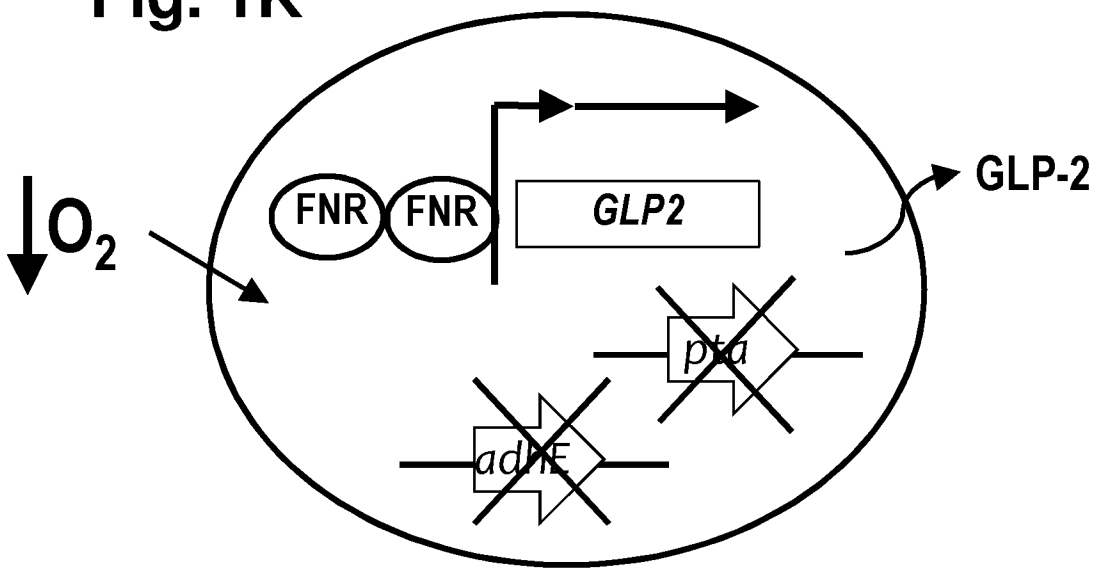
FIG. 1I

Fig. 1J



E. Coli Nissle 1917

Fig. 1K



E. Coli Nissle 1917

FIG. 2A

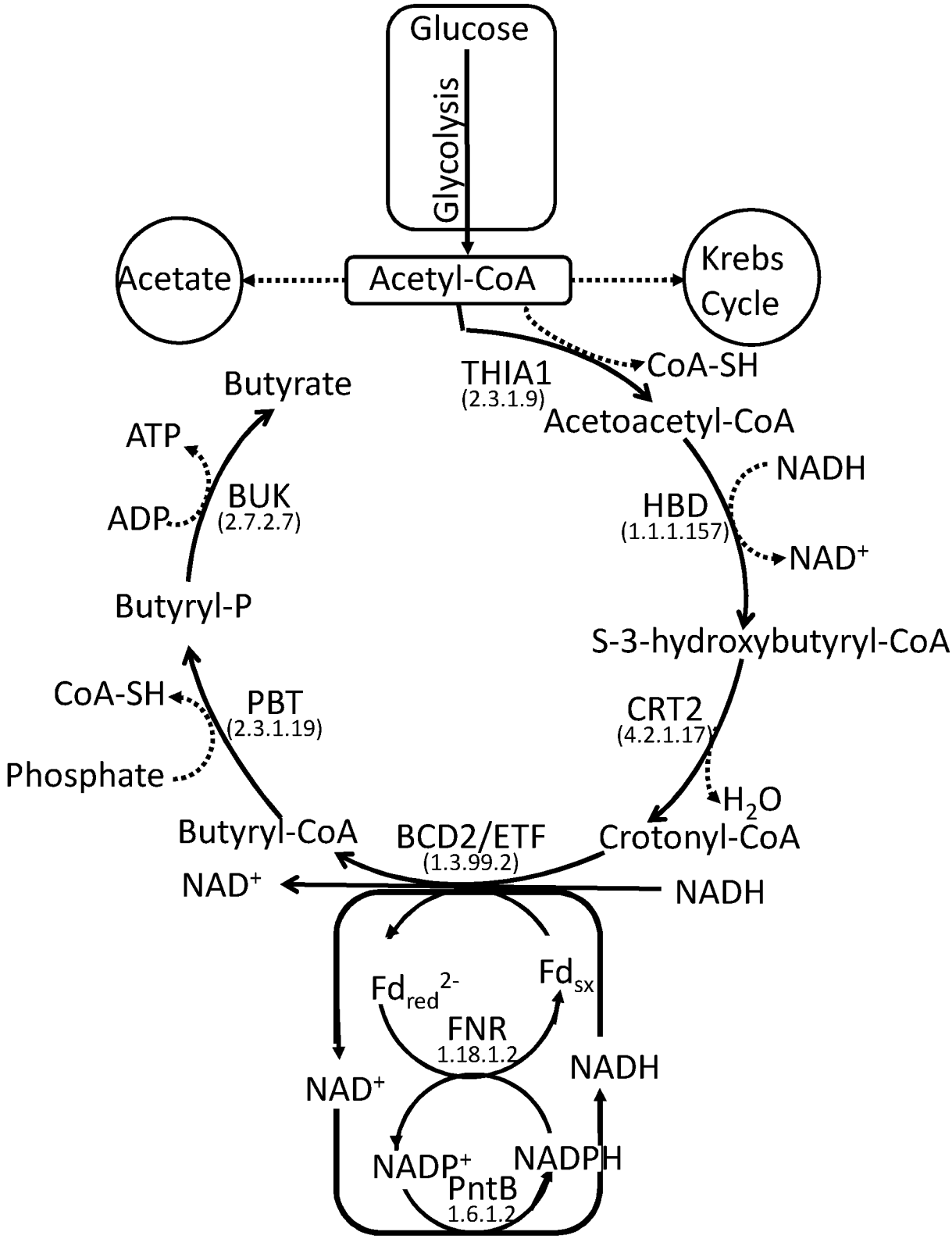

Glucose
Glycolysis
Acetate
Acetyl-CoA
Krebs Cycle
CoA-SH
THIA1
(2.3.1.9)
Acetoacetyl-CoA
NADH
HBD
(1.1.1.157)
NAD+
S-3-hydroxybutyryl-CoA
CRT2
(4.2.1.17)
H2O
Crotonyl-CoA
NADH
BCD2/ETF
(1.3.99.2)
NAD+
Butyryl-CoA
PBT
(2.3.1.19)
CoA-SH
Phosphate
Butyryl-P
BUK
(2.7.2.7)
ATP
ADP
Butyrate
Fdsx
Fdred2-
FNR
1.18.1.2
NADH
NAD+
NADP+
NADPH
PntB
1.6.1.2

FIG. 2B

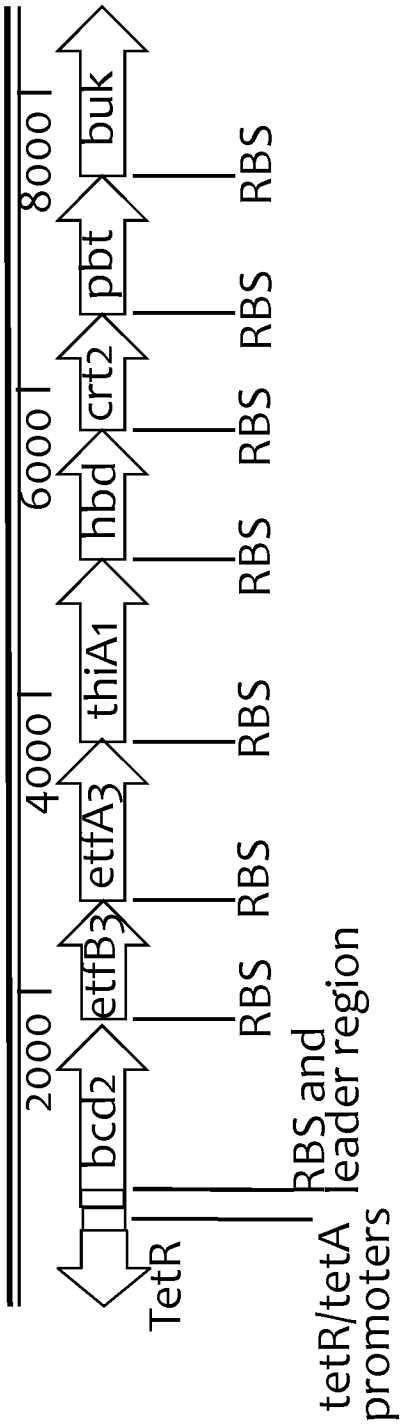

pLogic031
2000
4000
6000
8000
TetR
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
RBS
RBS and leader region
tetR/tetA promoters

FIG. 2C

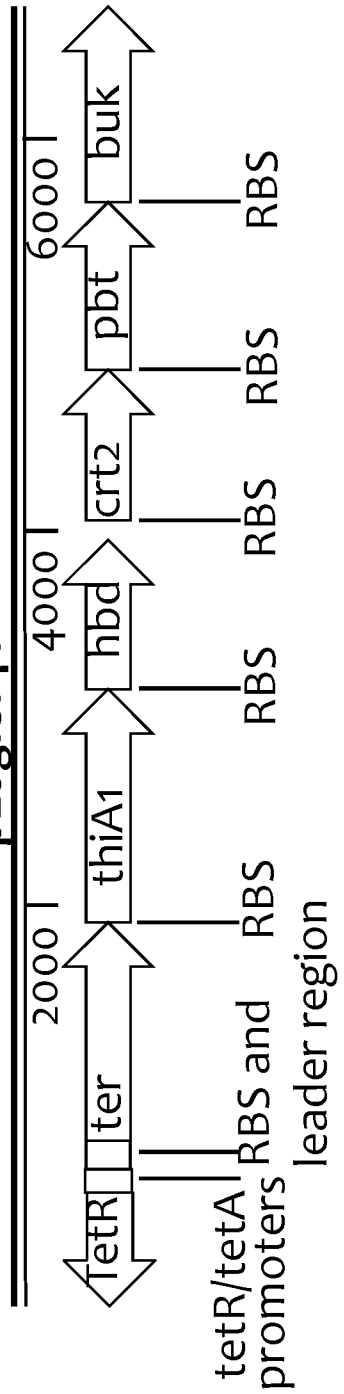

pLogic046
2000
4000
6000
TetR
ter
thiA1
hbd
crt2
pbt
buk
RBS
RBS and leader region
tetR/tetA promoters

FIG. 2D

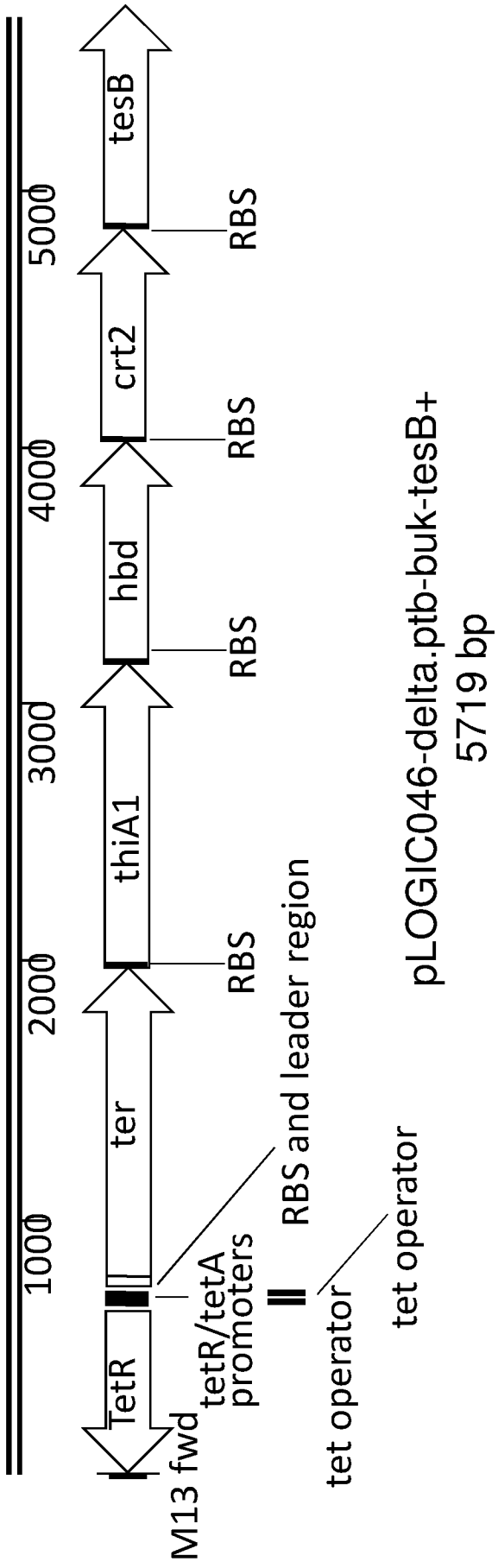

1000
2000
3000
4000
5000
TetR
ter
thiA1
hbd
crt2
tesB
RBS
RBS
RBS
RBS
RBS
M13 fwd
tetR/tetA promoters
RBS and leader region
tet operator
tet operator
pLOGIC046-delta.ptb-buk-tesB+
5719 bp

FIG. 3A

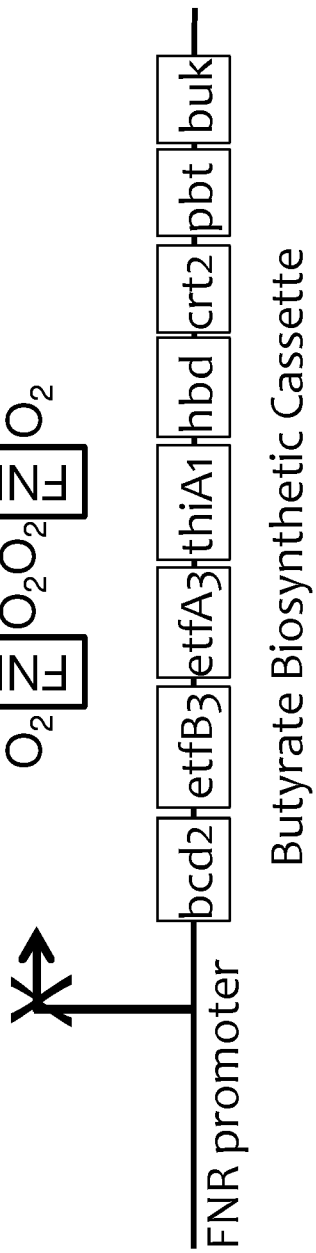

FNR
FNR
O_2
O_2
O_2
O_2
FNR promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 3B

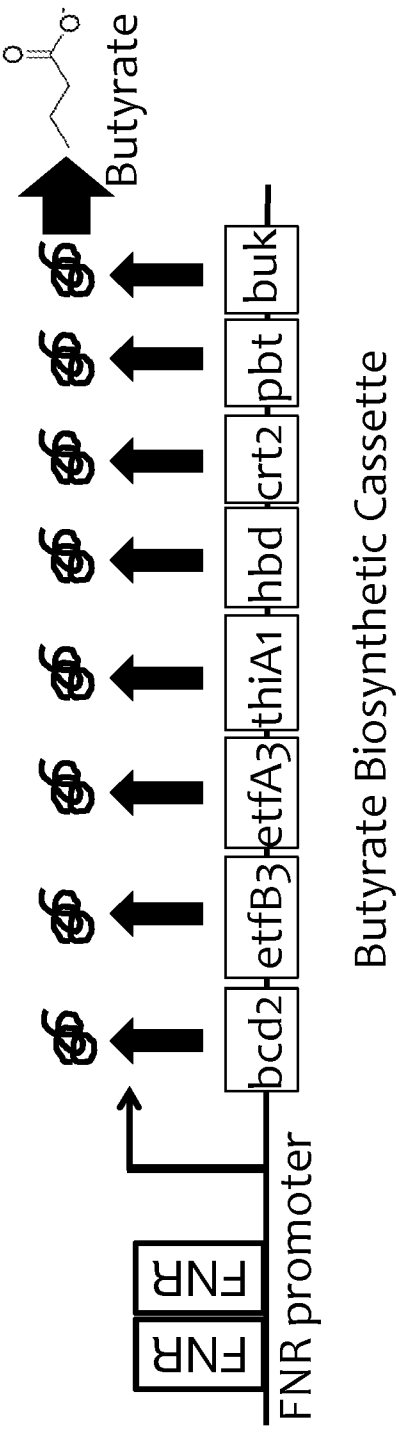

Butyrate
FNR
FNR
FNR promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 3C

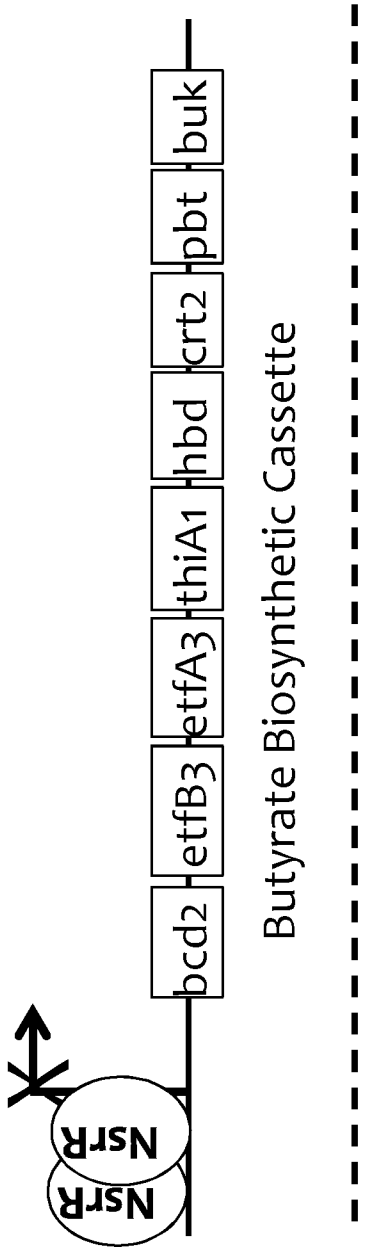

NsrR
NsrR
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 3D


Butyrate
NO
NO
NO
NO
NO
NO
NsrR
NsrR
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 3E

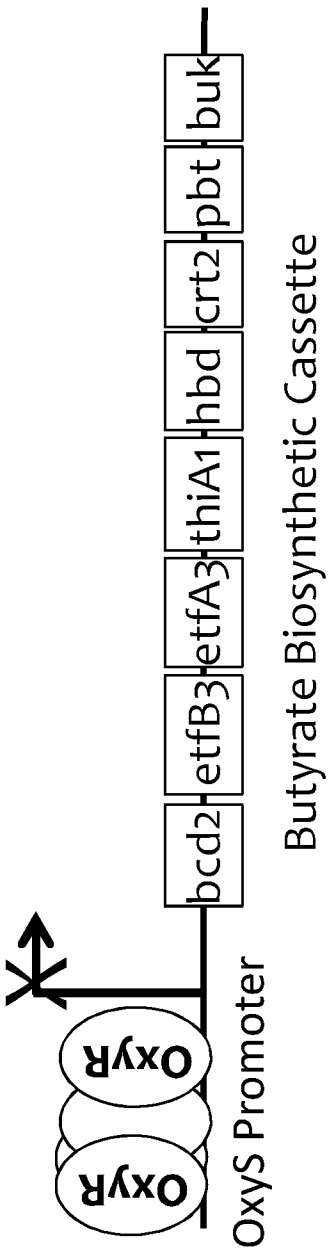

OxyR
OxyR
OxyS Promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 3F

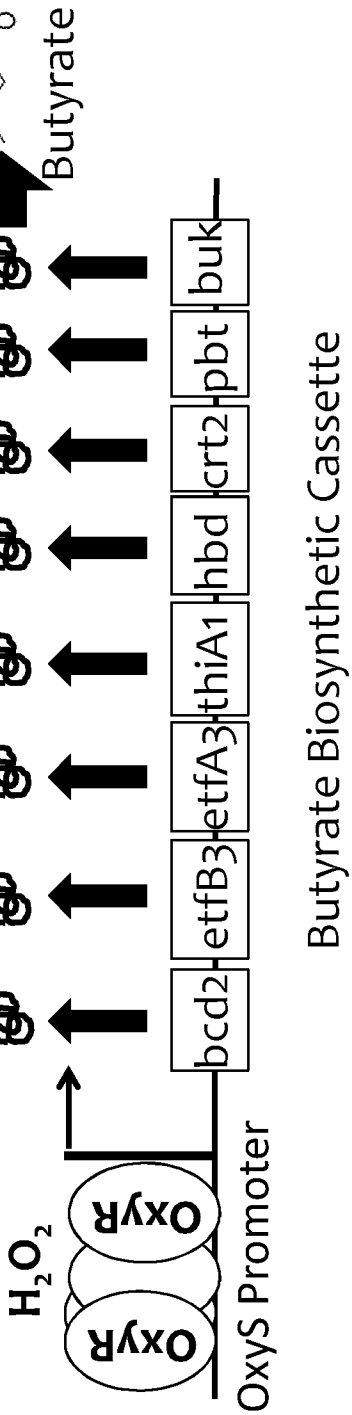

Butyrate
H_2O_2
OxyR
OxyR
OxyS Promoter
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 4A

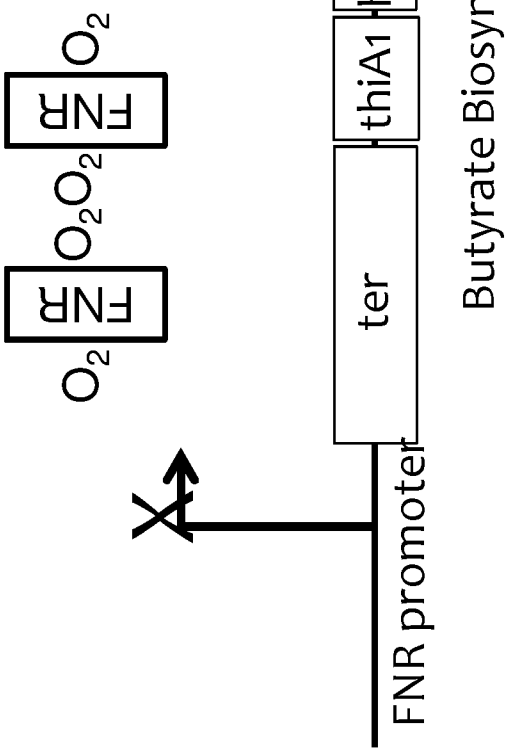

O2
FNR
O2
O2
O2
FNR
FNR promoter
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 4B

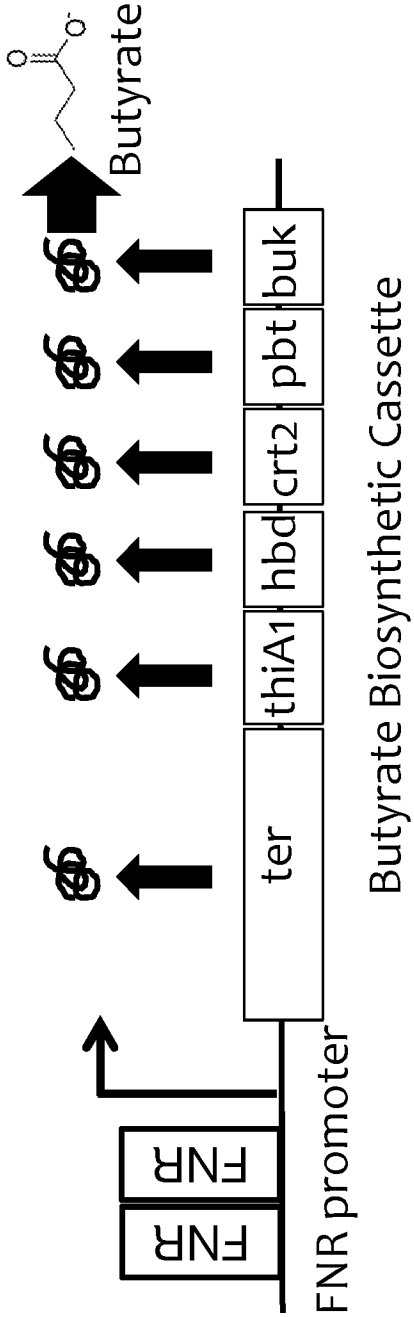

Butyrate
FNR
FNR
FNR promoter
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 4C

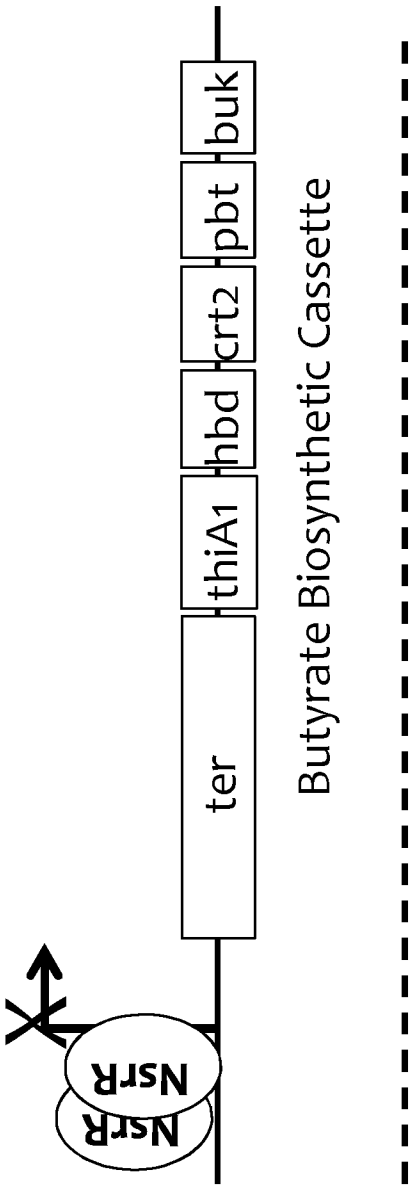

ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette
NsrR
NsrR

FIG. 4D

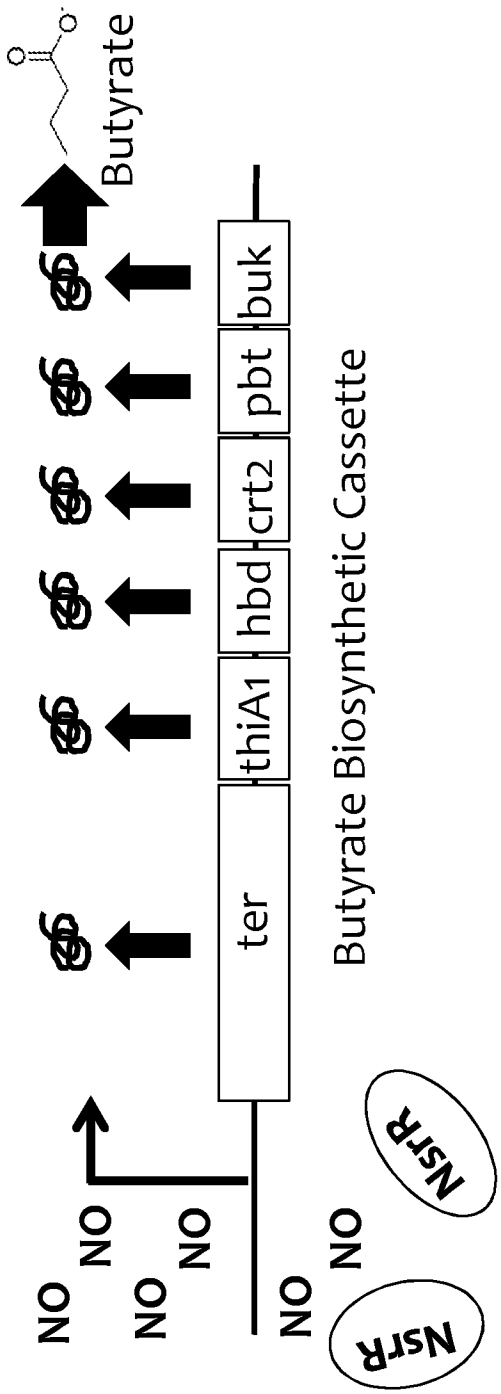

Butyrate
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette
NO
NO
NO
NO
NO
NO
NsrR
NsrR

FIG. 4E

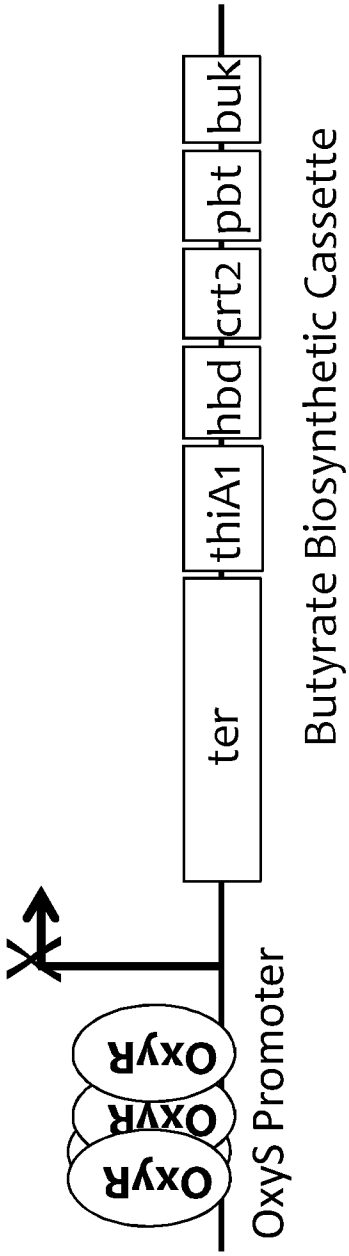

OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 4F

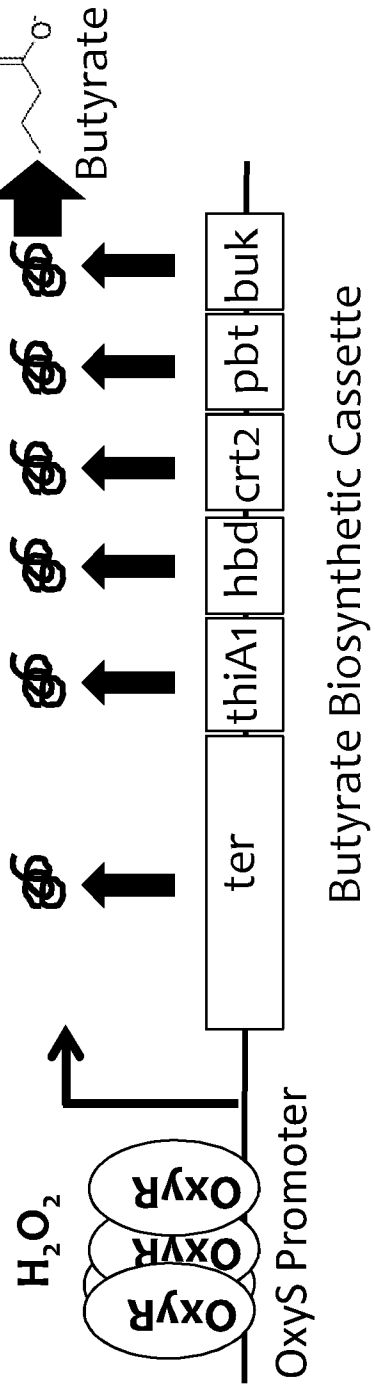

Butyrate
H_2O_2
OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
pbt
buk
Butyrate Biosynthetic Cassette

FIG. 5A

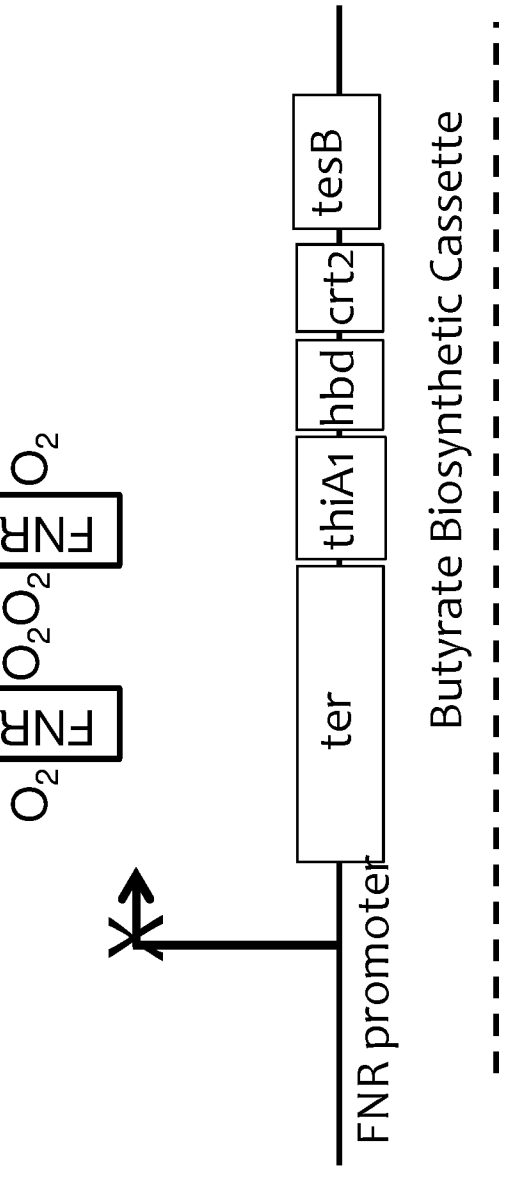

FNR
FNR
O_2
O_2
O_2
O_2
ter
thiA1
hbd
crt2
tesB
FNR promoter
Butyrate Biosynthetic Cassette

FIG. 5B

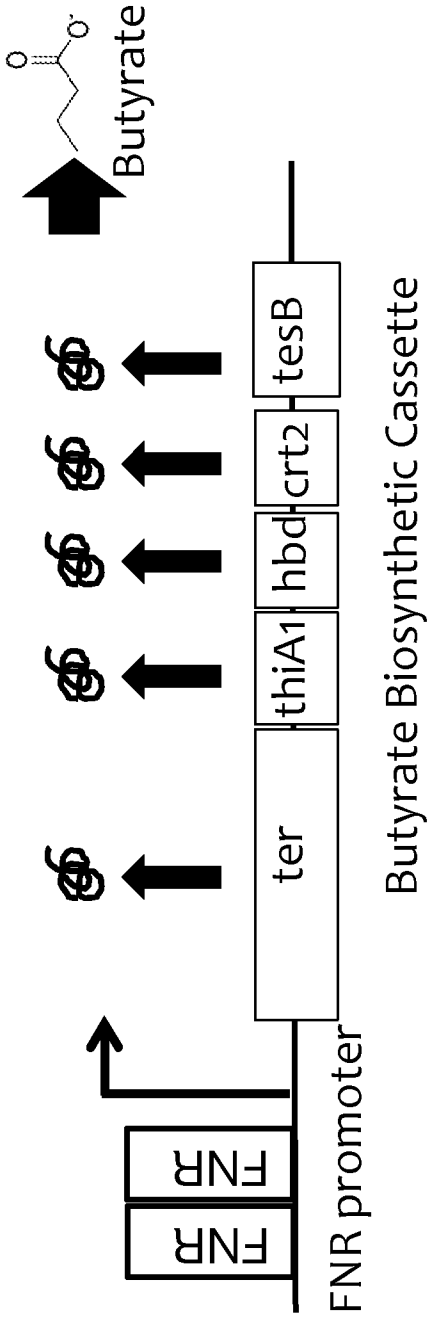

Butyrate
FNR
FNR
ter
thiA1
hbd
crt2
tesB
FNR promoter
Butyrate Biosynthetic Cassette

FIG. 5C

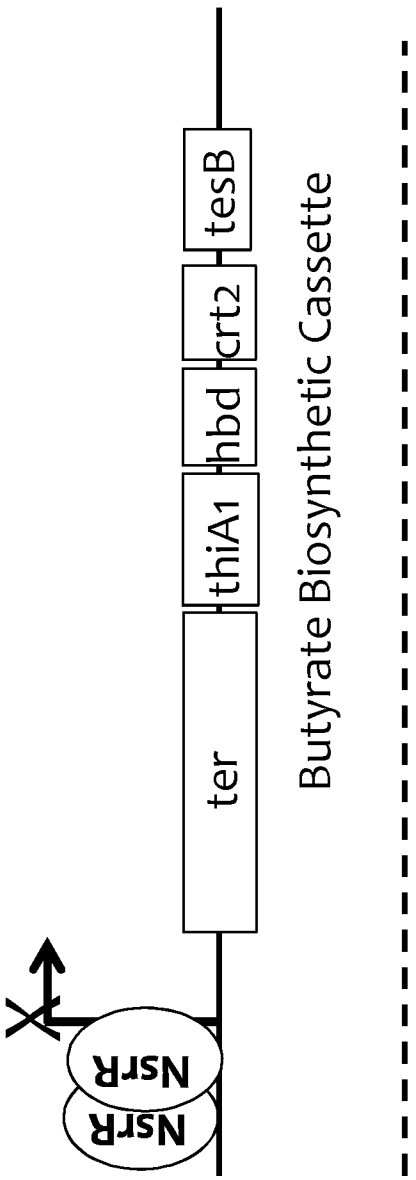

NsrR
NsrR
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

FIG. 5D

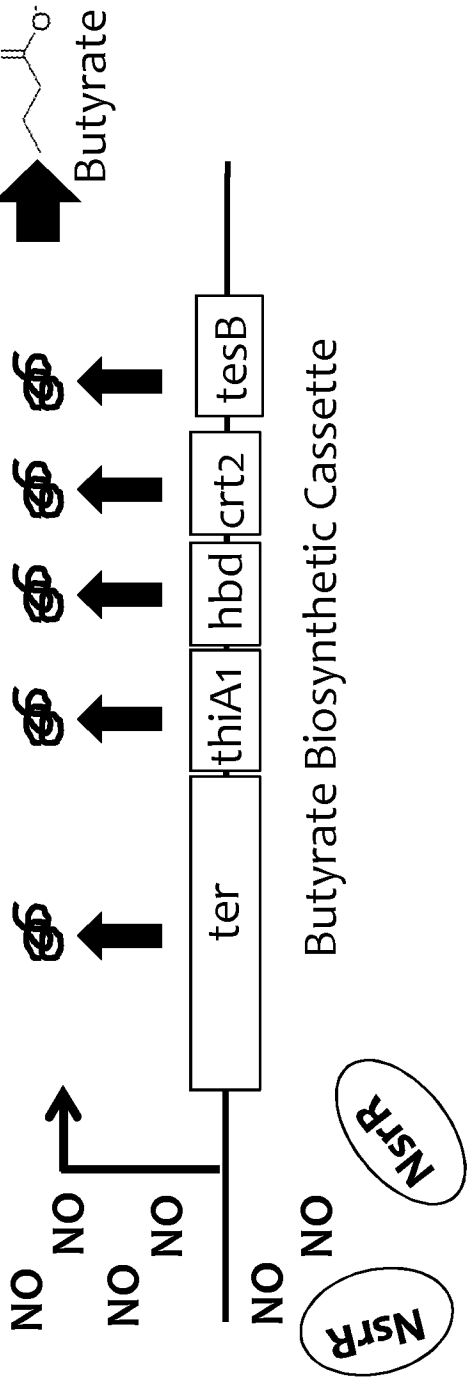

Butyrate
NO
NO
NO
NO
NO
NO
NsrR
NsrR
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

FIG. 5E

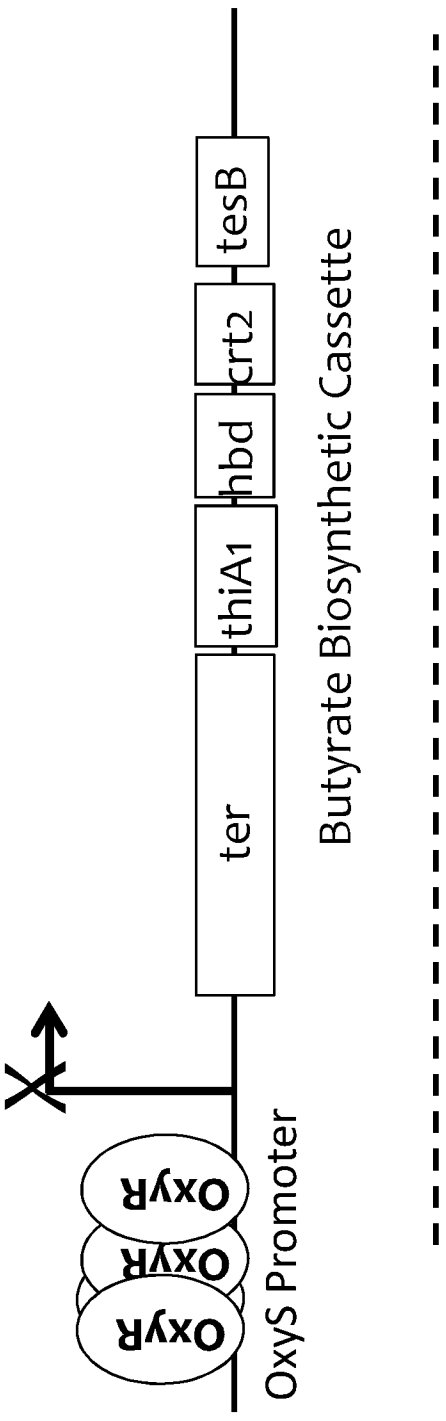

OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

FIG. 5F

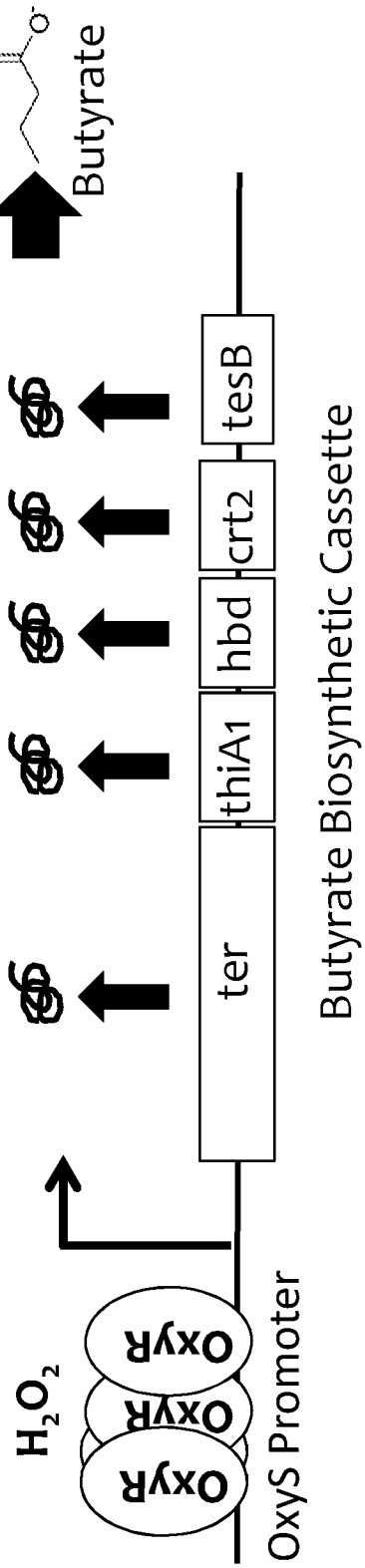

Butyrate
H_2O_2
OxyR
OxyR
OxyR
OxyS Promoter
ter
thiA1
hbd
crt2
tesB
Butyrate Biosynthetic Cassette

FIG. 6A

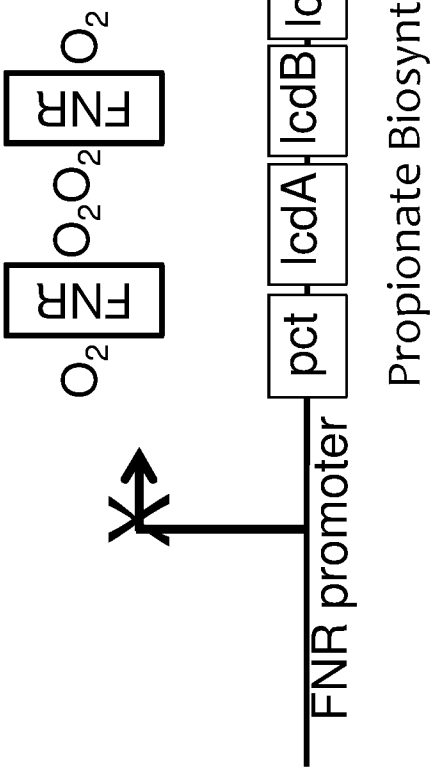

FNR
FNR
O_2
O_2
O_2
O_2
FNR promoter
pct
lcdA
lcdB
lcdC
etfA
acrB
acrC
Propionate Biosynthetic Cassette

FIG. 6B

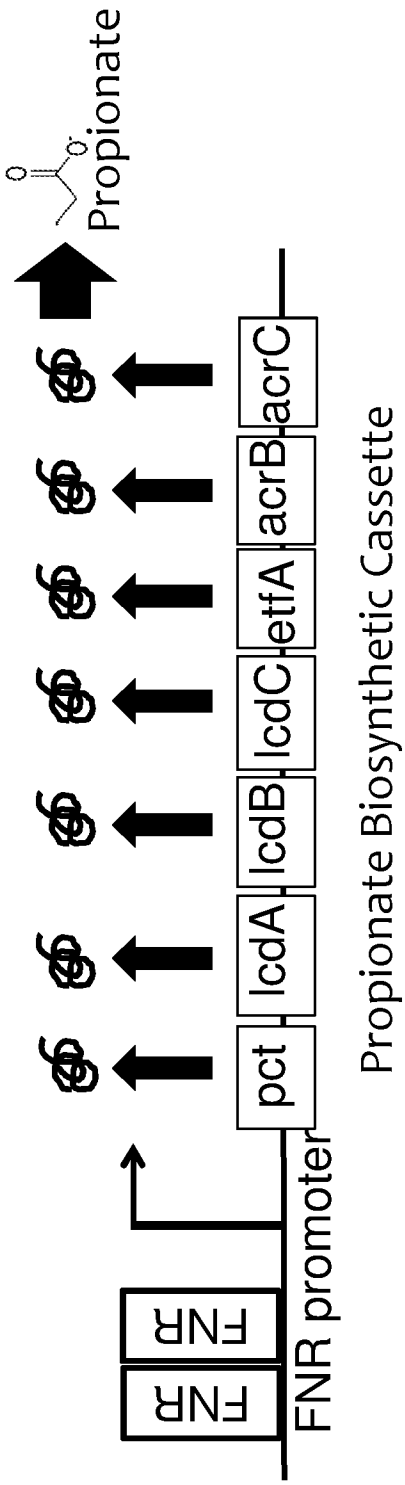

Propionate
FNR
FNR
FNR promoter
pct
lcdA
lcdB
lcdC
etfA
acrB
acrC
Propionate Biosynthetic Cassette

FIG. 7

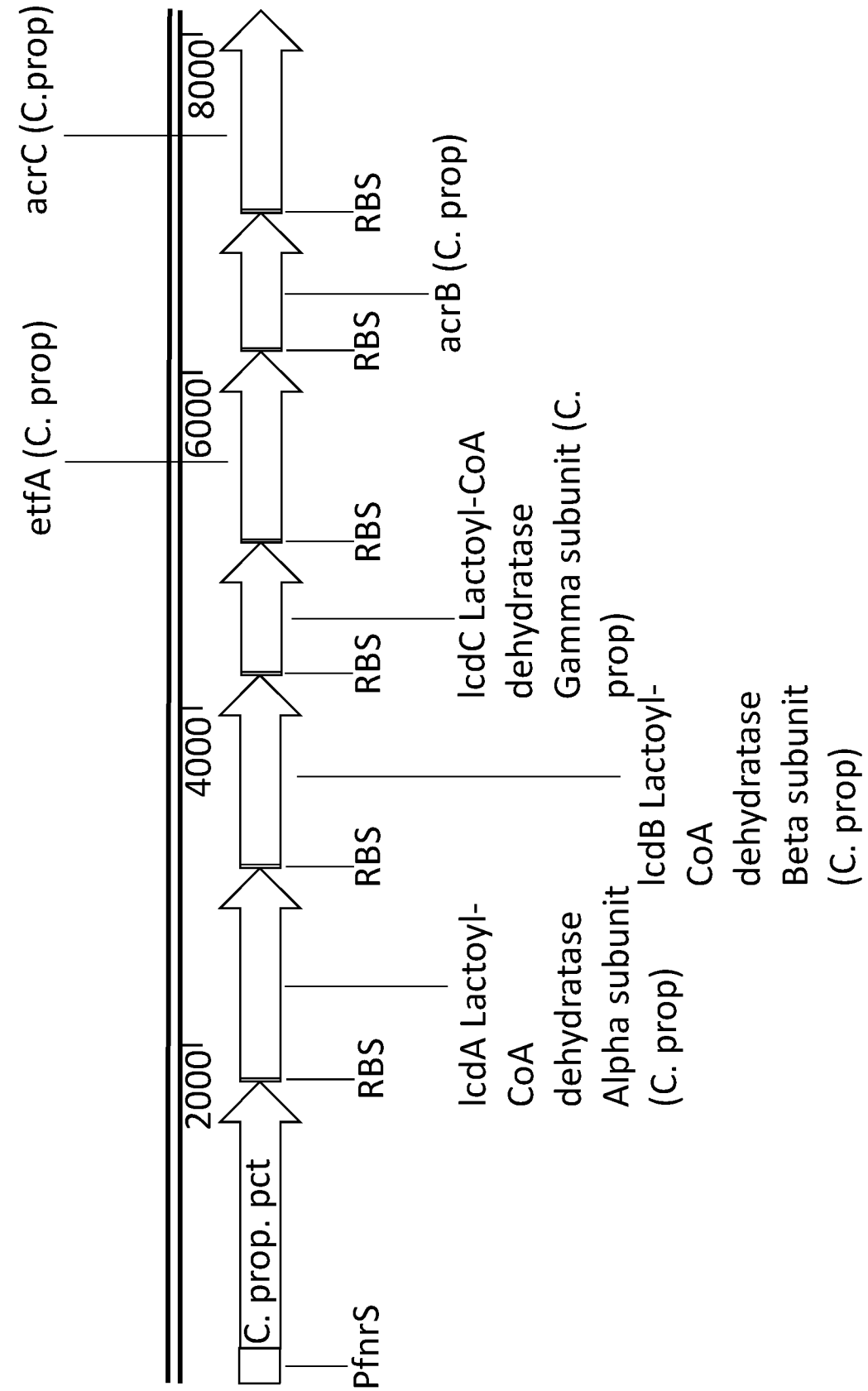

2000
4000
6000
8000
PfnrS
C. prop. pct
RBS
lcdA Lactoyl-CoA dehydratase Alpha subunit (C. prop)
RBS
lcdB Lactoyl-CoA dehydratase Beta subunit (C. prop)
RBS
lcdC Lactoyl-CoA dehydratase Gamma subunit (C. prop)
etfA (C. prop)
RBS
acrB (C. prop)
RBS
acrC (C.prop)

FIG. 8A

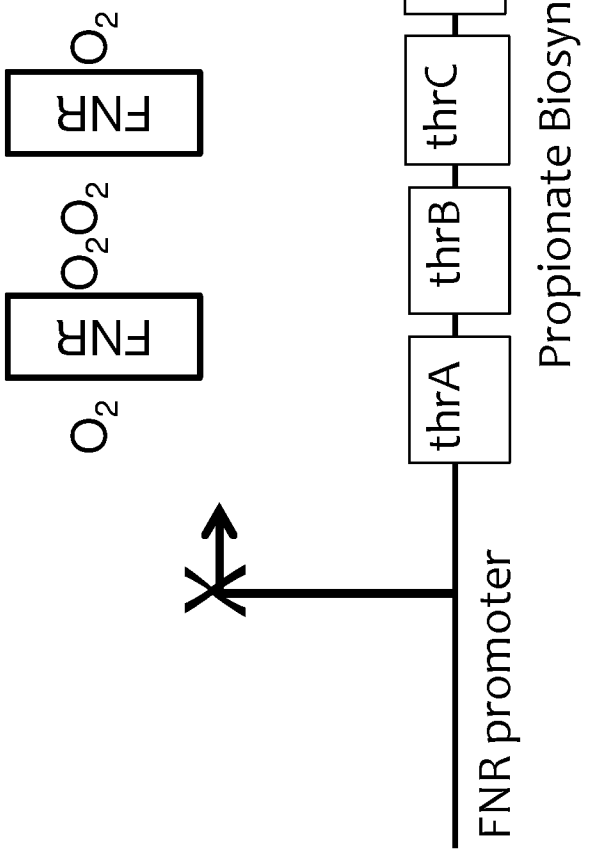
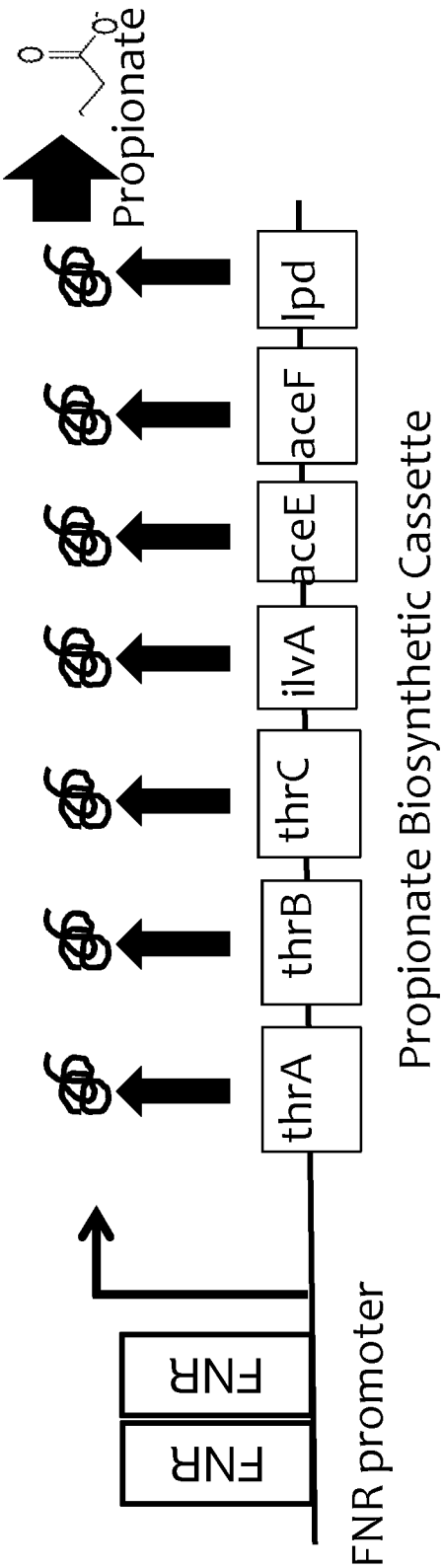

FNR
FNR
O_2
O_2O_2
O_2
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
FNR promoter
Propionate Biosynthetic Cassette

FIG. 8B


Propionate
FNR
FNR
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
FNR promoter
Propionate Biosynthetic Cassette

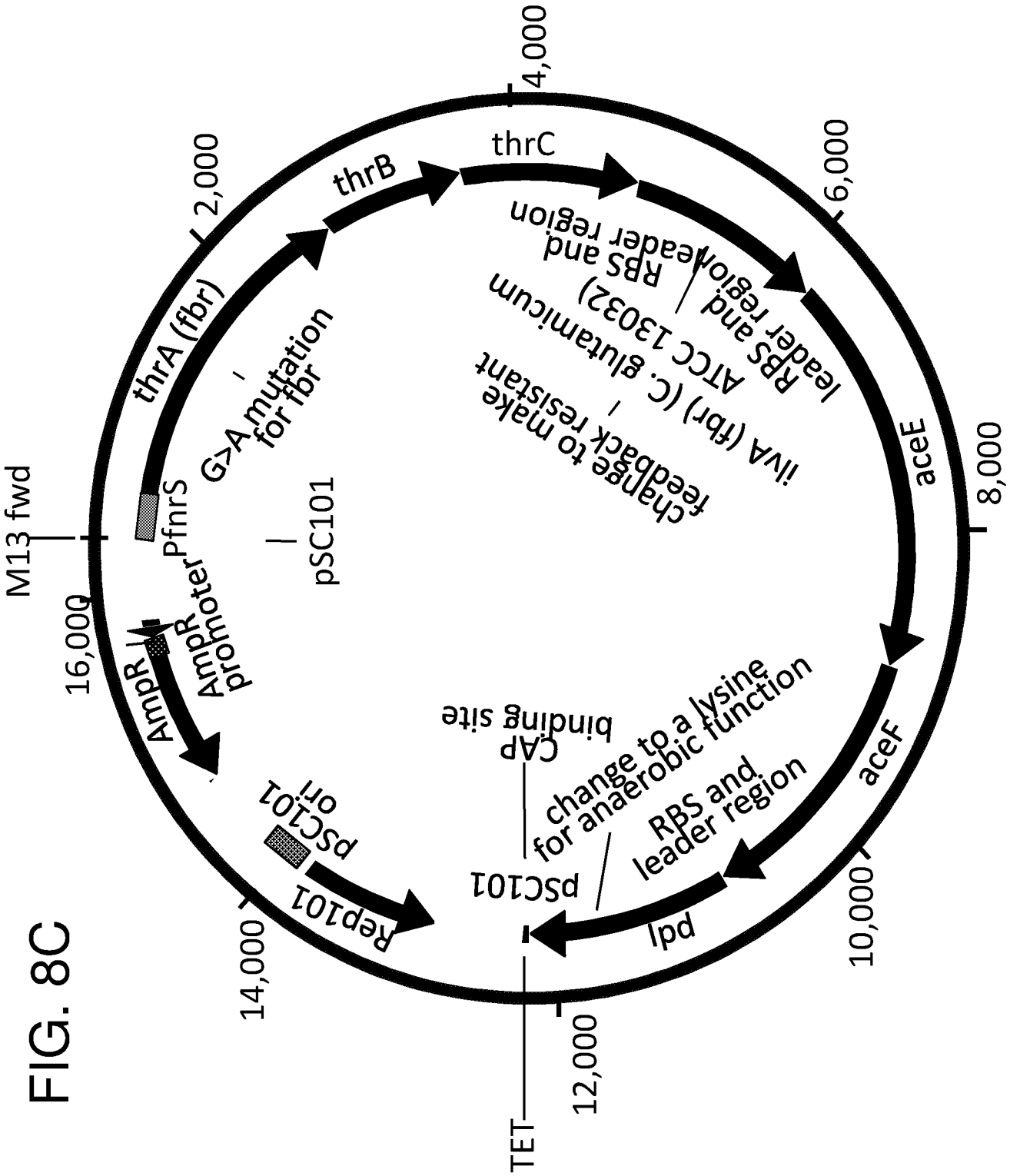

M13 fwd
2,000
4,000
6,000
8,000
10,000
12,000
14,000
16,000
TET
thrA (fbr)
thrB
thrC
aceE
aceF
lpd
PfnrS
G>A mutation for fbr
pSC101
change to a lysine for anaerobic function
RBS and leader region
CAP binding site
AmpR promoter
AmpR
pSC101 ori
Rep101

FIG. 8C

FIG. 9A

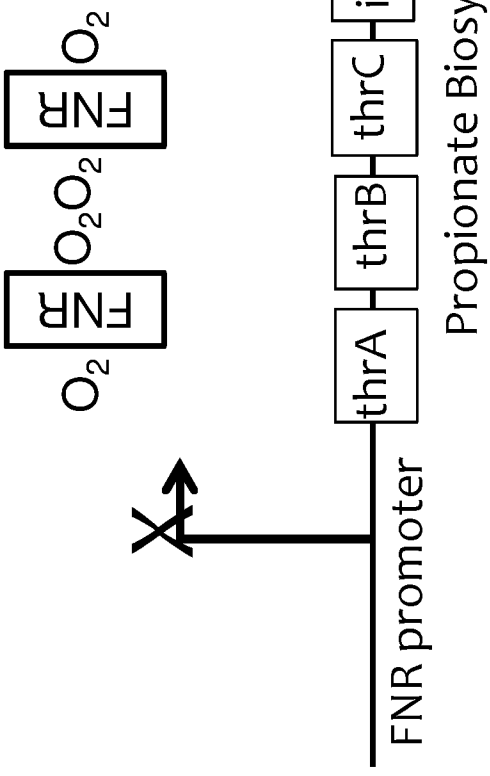

FNR
FNR
O_2
O_2
O_2
O_2
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
tesB
FNR promoter
Propionate Biosynthetic Cassette

FIG. 9B

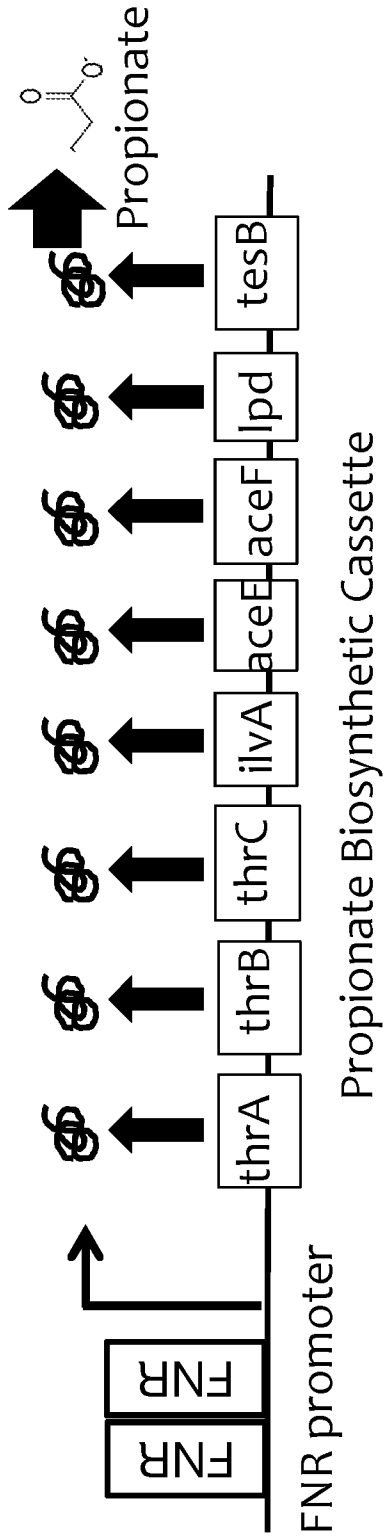

Propionate
FNR
FNR
thrA
thrB
thrC
ilvA
aceE
aceF
lpd
tesB
FNR promoter
Propionate Biosynthetic Cassette

FIG. 10A

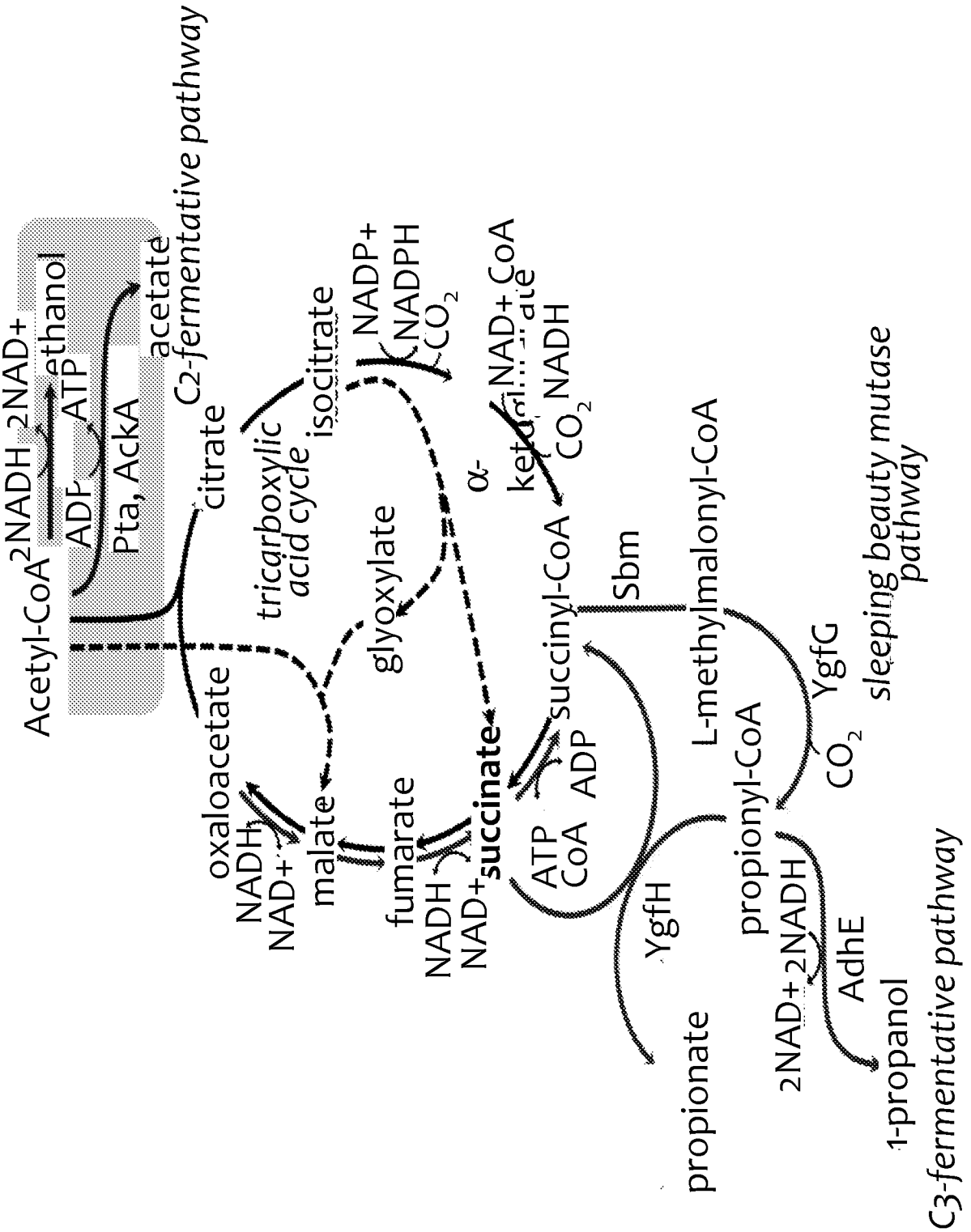

AdhE
2NADH
2NAD+
ethanol
ATP
ADP
Pta, AckA
acetate
C2-fermentative pathway
Acetyl-CoA
citrate
tricarboxylic acid cycle
isocitrate
NADP+
NADPH
CO2
glyoxylate
α-ketoglutarate
NAD+ CoA
CO2 NADH
oxaloacetate
NADH
NAD+
malate
fumarate
NADH
succinate
succinyl-CoA
Sbm
ATP
CoA
ADP
L-methylmalonyl-CoA
YgfH
propionyl-CoA
YgfG
CO2
sleeping beauty mutase pathway
propionate
2NAD+ 2NADH
AdhE
1-propanol
C3-fermentative pathway

FIG. 10B

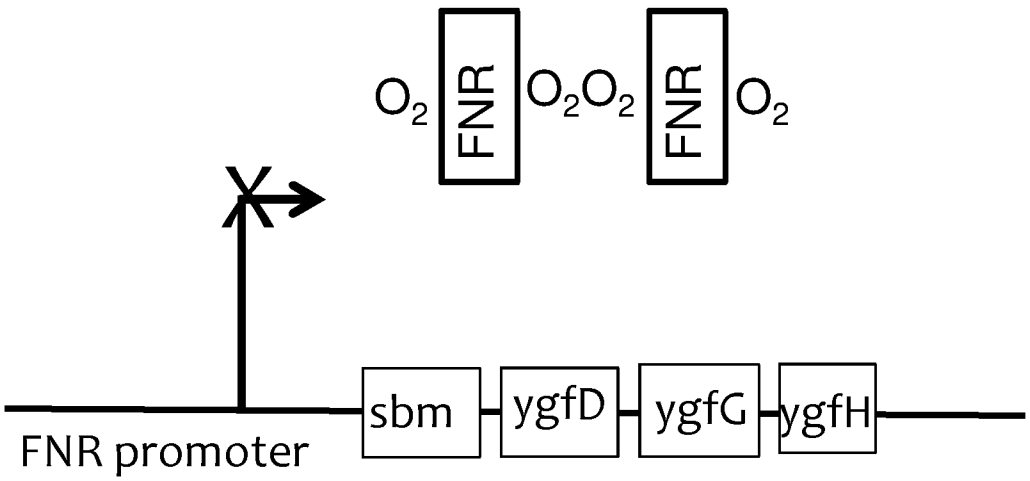


FIG. 10C

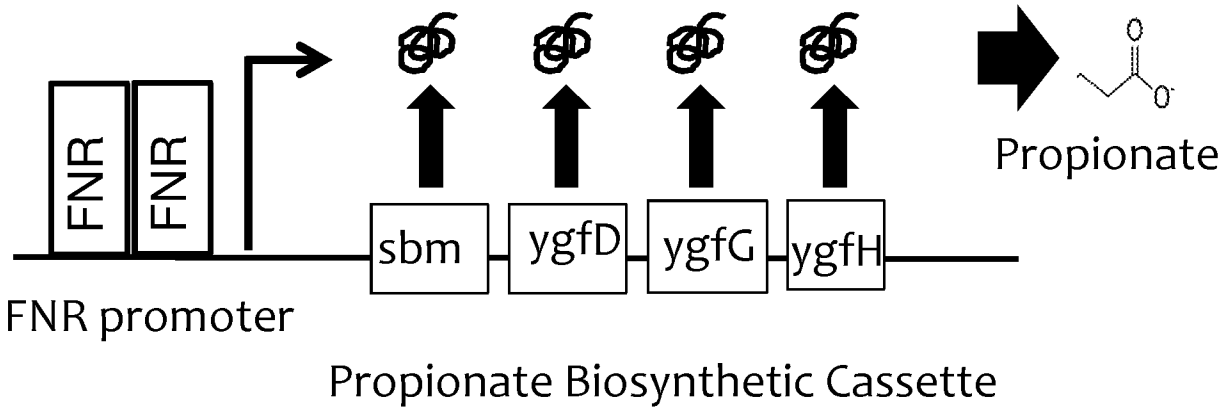


FIG. 11

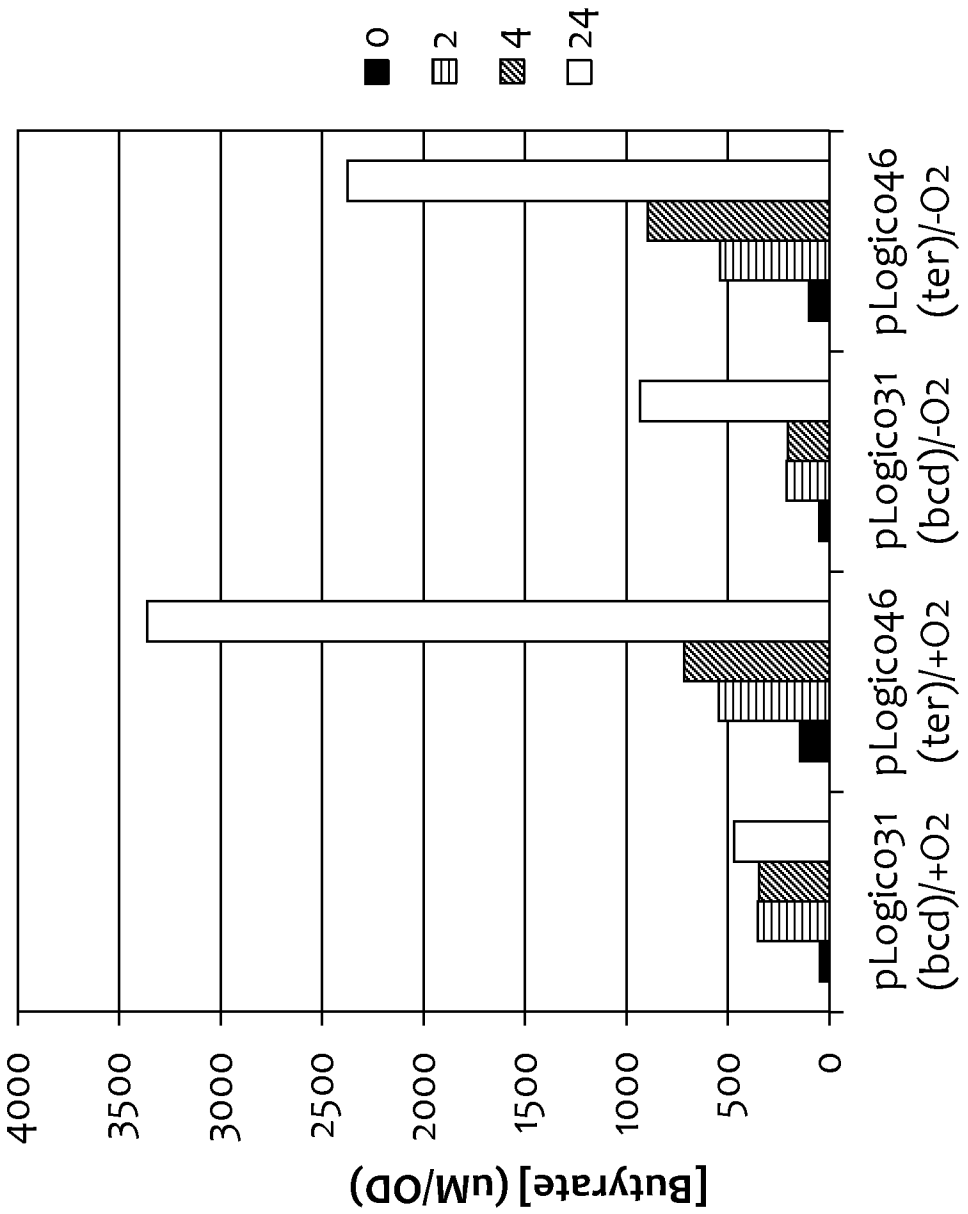

0
2
4
24
[Butyrate] (uM/OD)
4000
3500
3000
2500
2000
1500
1000
500
0
pLogic031 (bcd)/+O2
pLogic046 (ter)/+O2
pLogic031 (bcd)/-O2
pLogic046 (ter)/-O2

FIG. 12

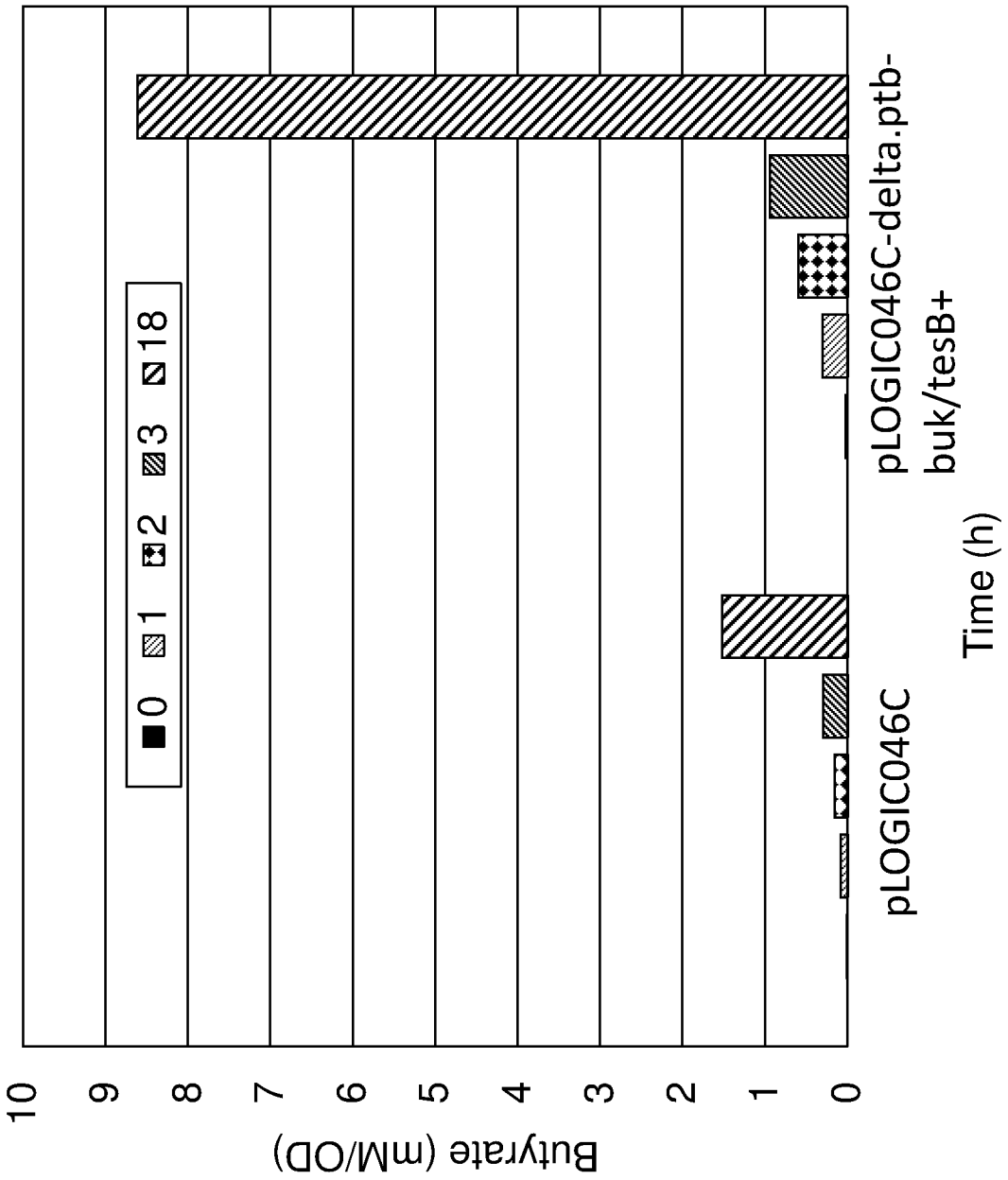

Butyrate (mM/OD)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
18
pLOGIC046C
pLOGIC046C-delta.ptb-buk/tesB+
Time (h)

FIG. 13

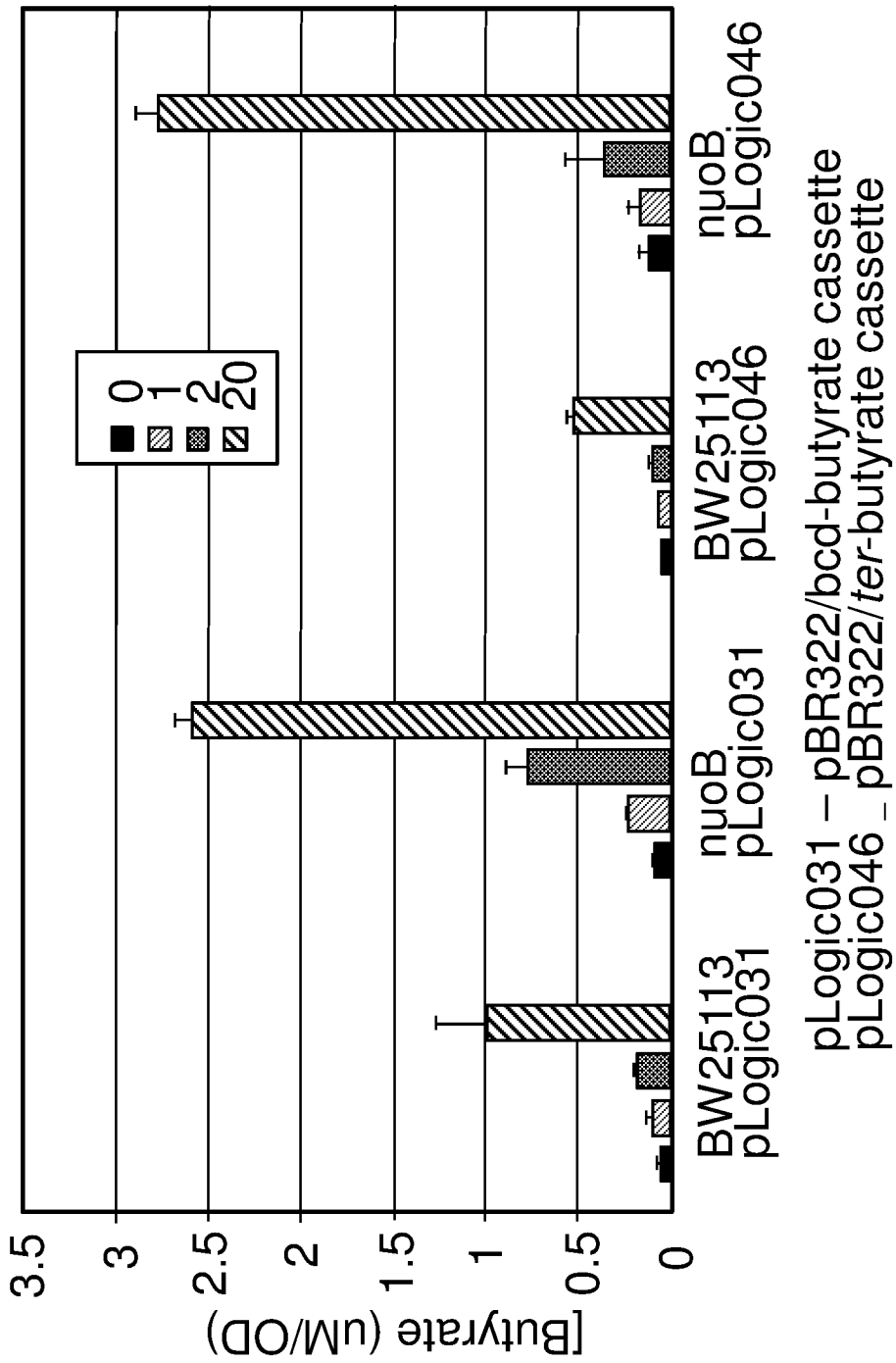

[Butyrate (uM/OD)
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
20
BW25113 pLogic031
nuoB pLogic031
BW25113 pLogic046
nuoB pLogic046
pLogic031 – pBR322/bcd-butyrate cassette
pLogic046 _ pBR322/ter-butyrate cassette

FIG. 14A

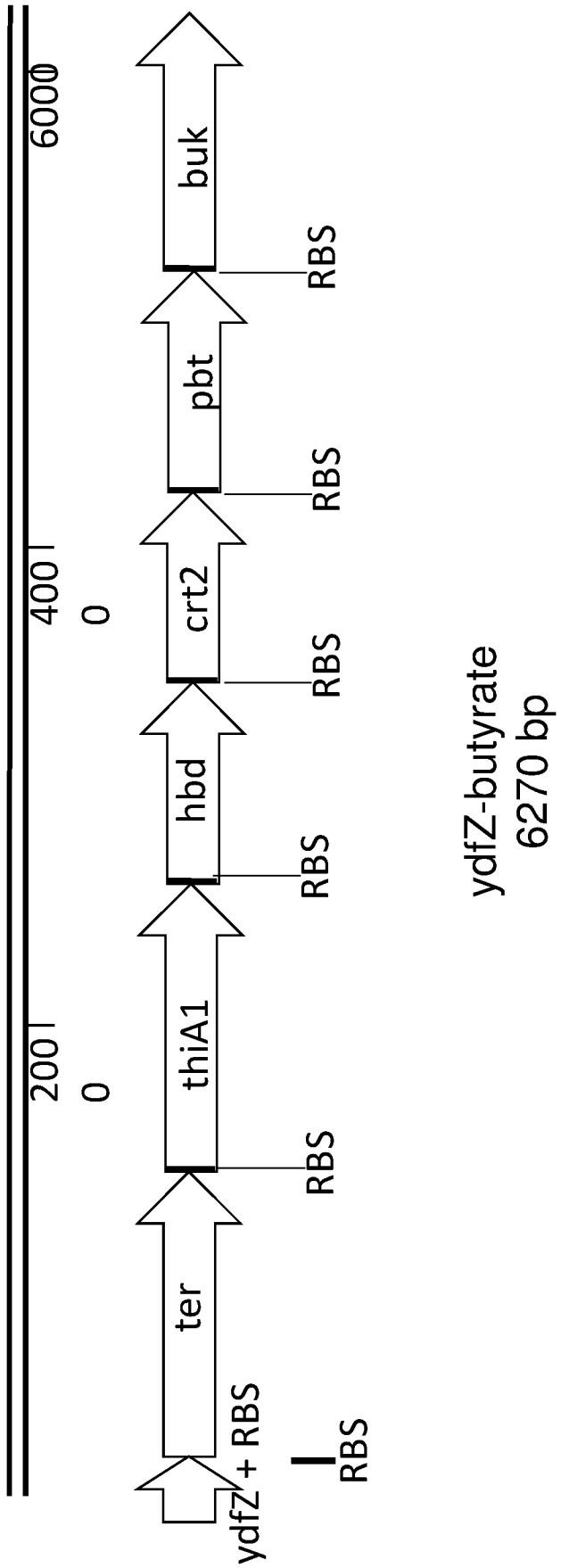

2000
4000
6000
ydfZ + RBS
ter
RBS
thiA1
RBS
hbd
RBS
crt2
RBS
pbt
RBS
buk
RBS
RBS
ydfZ-butyrate
6270 bp

FIG. 14B

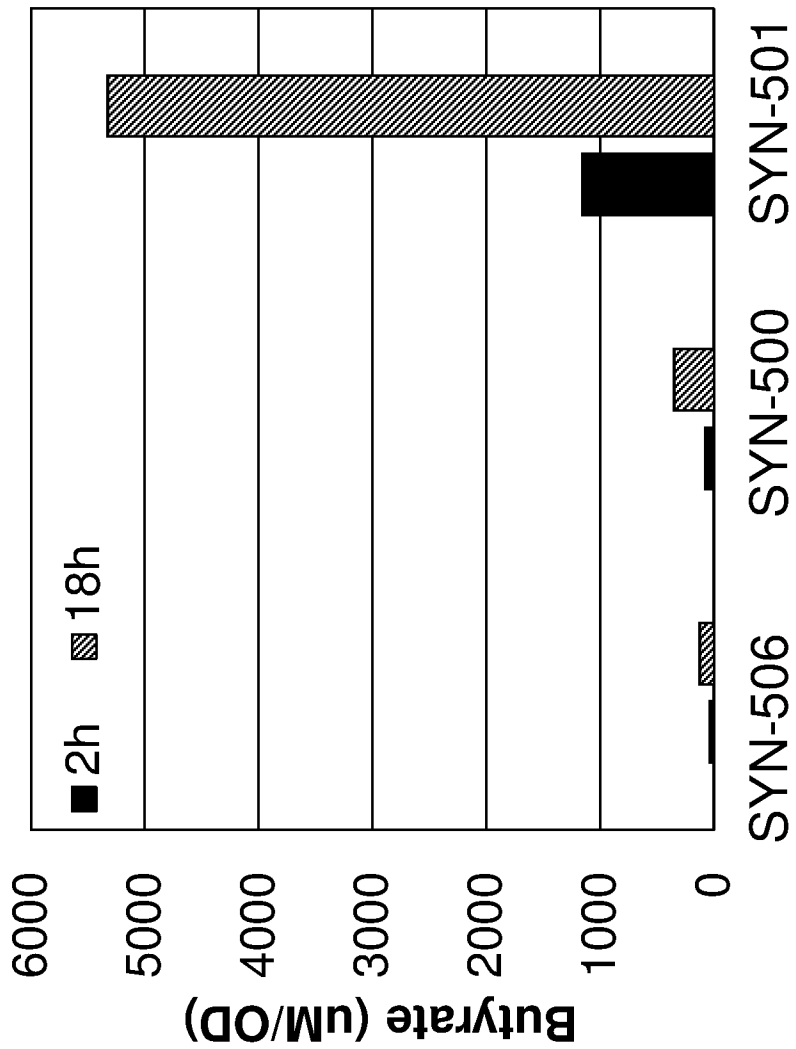

Butyrate (uM/OD)
6000
5000
4000
3000
2000
1000
0
2h
18h
SYN-506
SYN-500
SYN-501

FIG. 14C

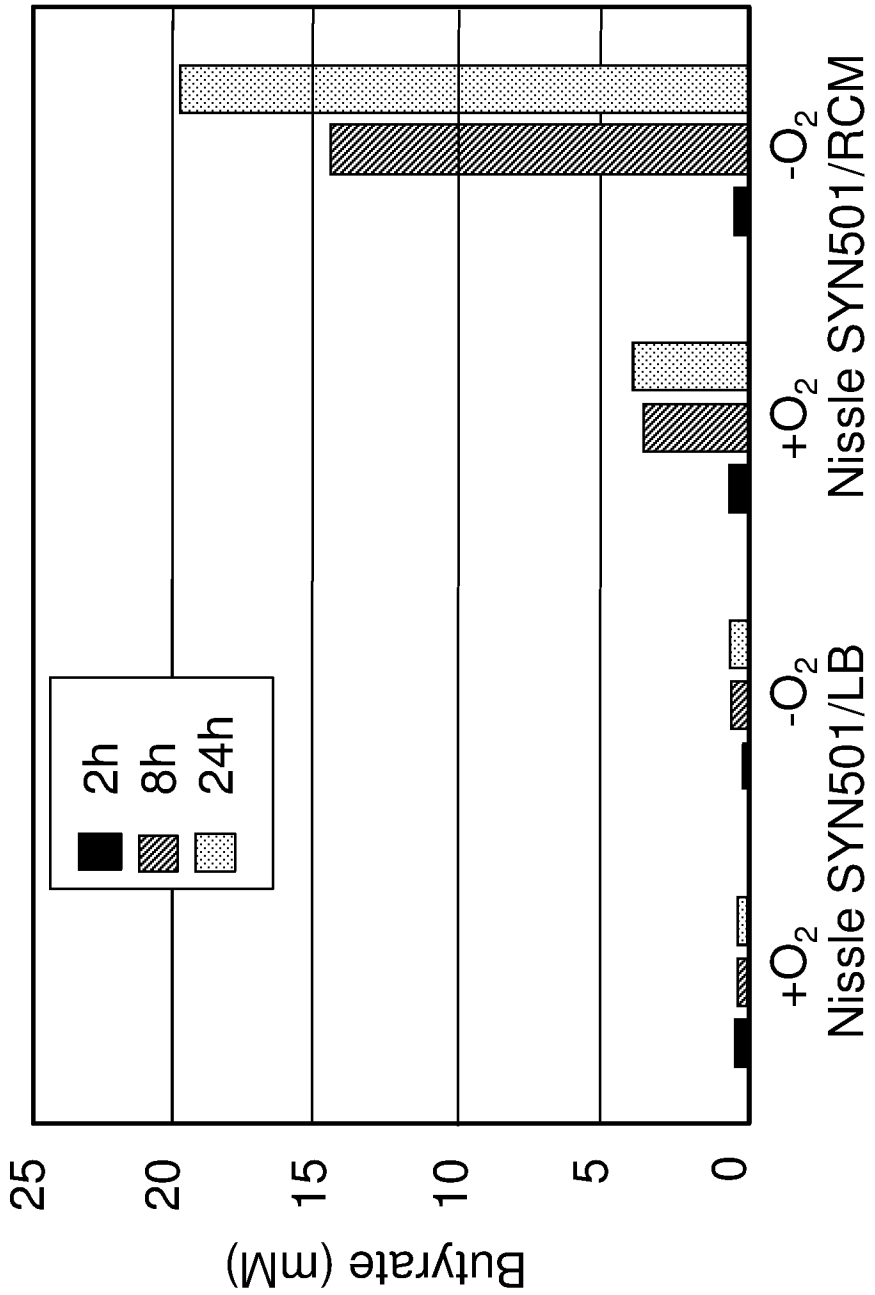
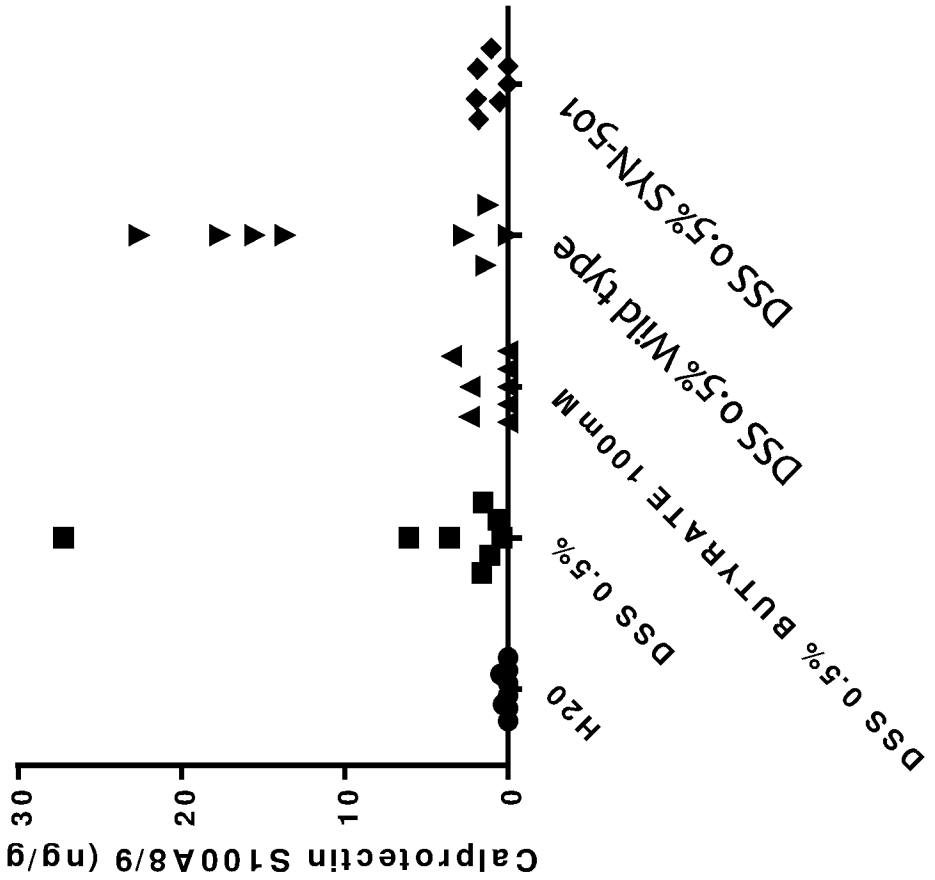

Butyrate (mM)
25
20
15
10
5
0
2h
8h
24h
+O2
-O2
+O2
-O2
Nissle SYN501/LB
Nissle SYN501/RCM

FIG. 14D


Calprotectin S100A8/9 (ng/g
0
10
20
30
H20
DSS 0.5%
DSS 0.5% BUTYRATE 100mM
DSS 0.5% Wild type
DSS 0.5% SYN-501

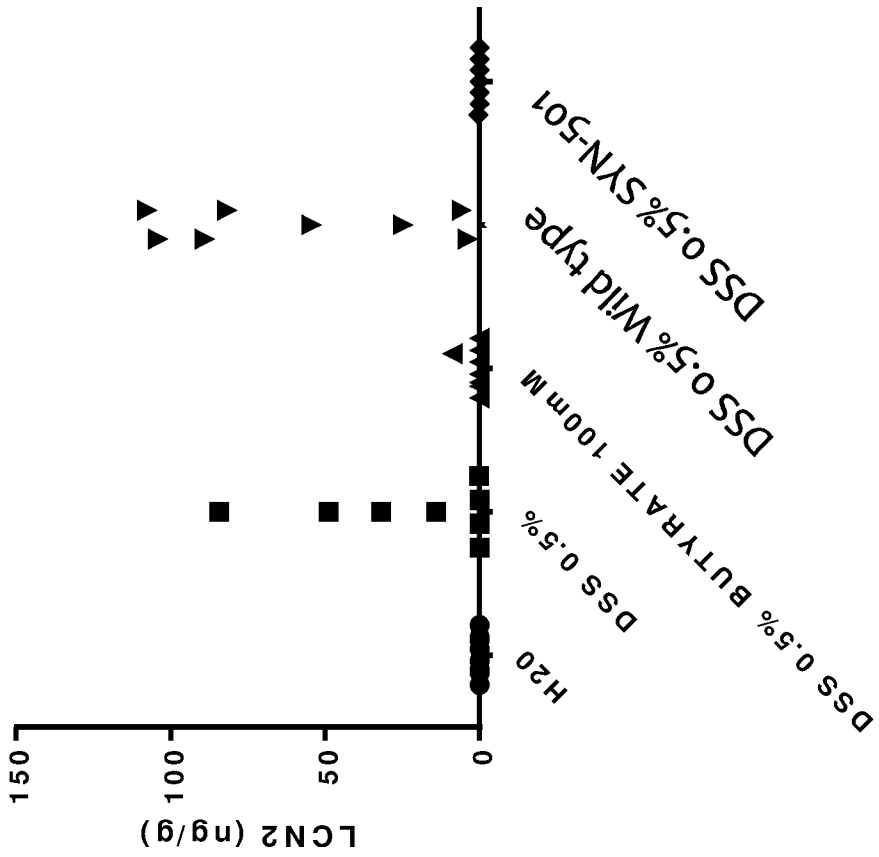

LCN2 (ng/g)
0
50
100
150
H20
DSS 0.5%
DSS 0.5% BUTYRATE 100mM
DSS 0.5% Wild type
DSS 0.5% SYN-501

FIG. 15

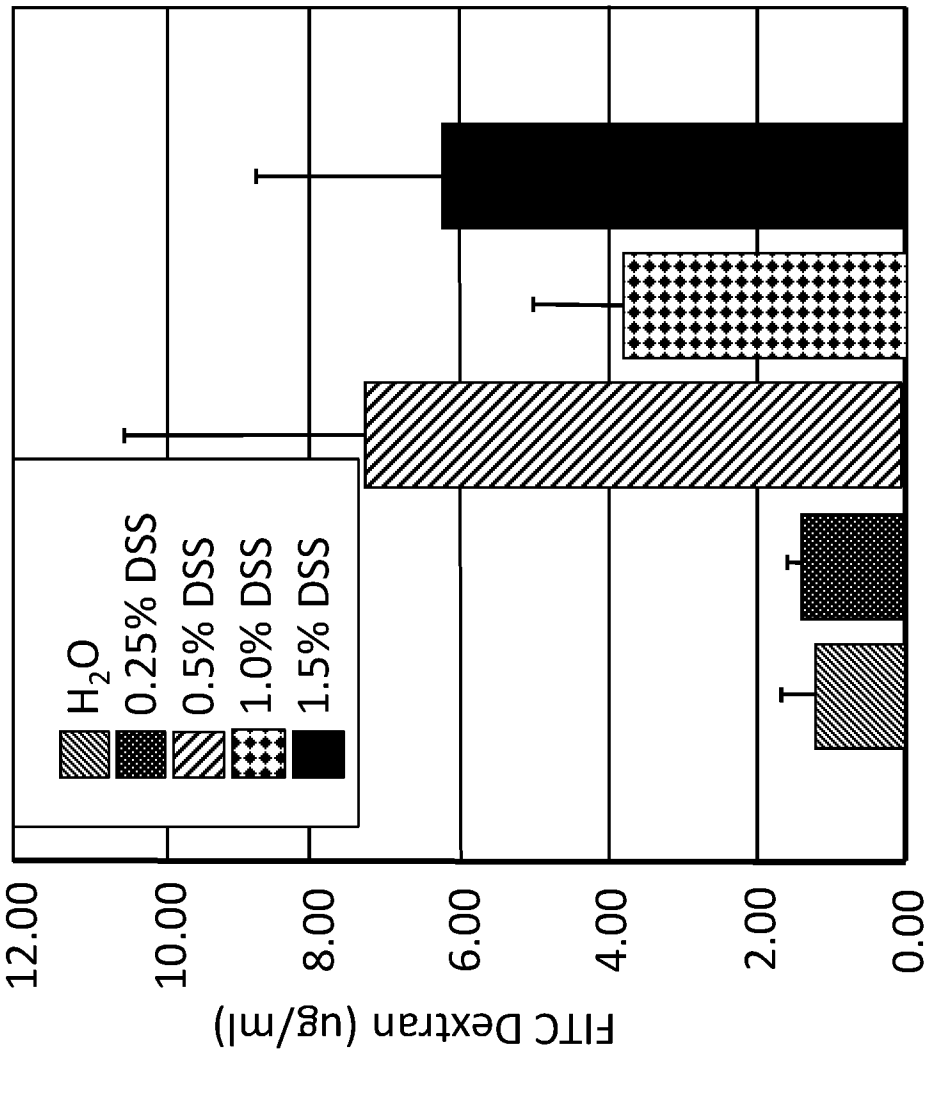

12.00
10.00
8.00
6.00
4.00
2.00
0.00
FITC Dextran (ug/ml)
H2O
0.25% DSS
0.5% DSS
1.0% DSS
1.5% DSS

FIG. 16

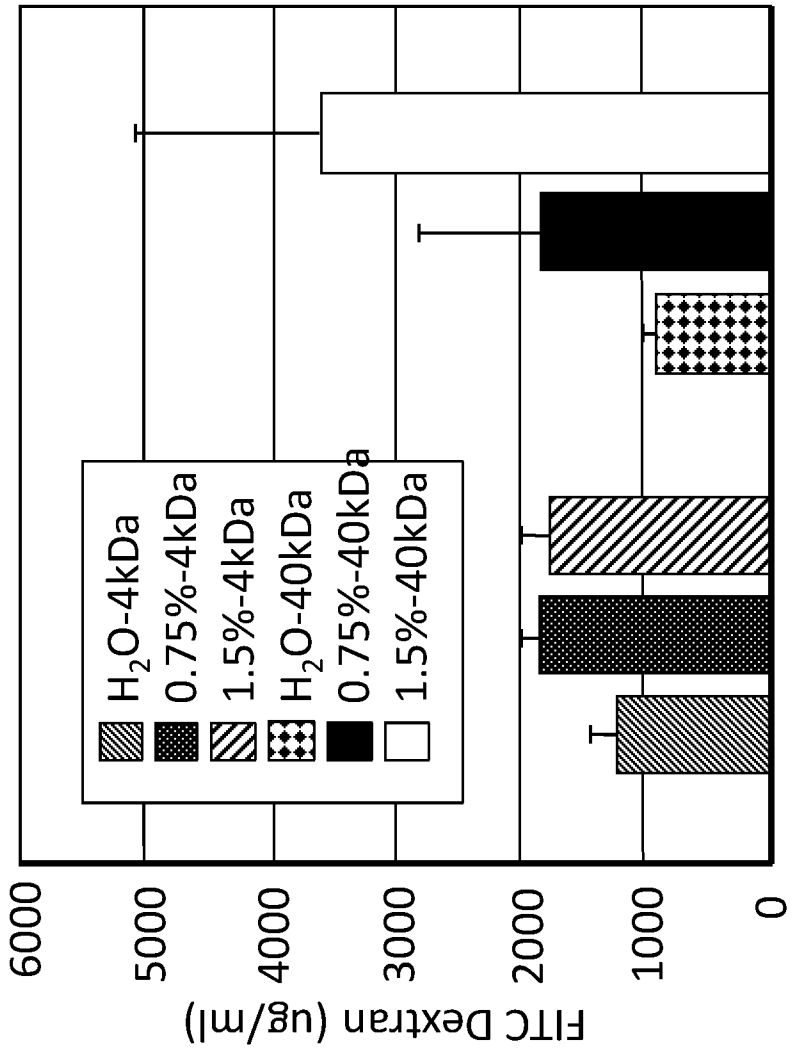

FITC Dextran (ug/ml)
6000
5000
4000
3000
2000
1000
0
H2O-4kDa
0.75%-4kDa
1.5%-4kDa
H2O-40kDa
0.75%-40kDa
1.5%-40kDa

FIG. 17

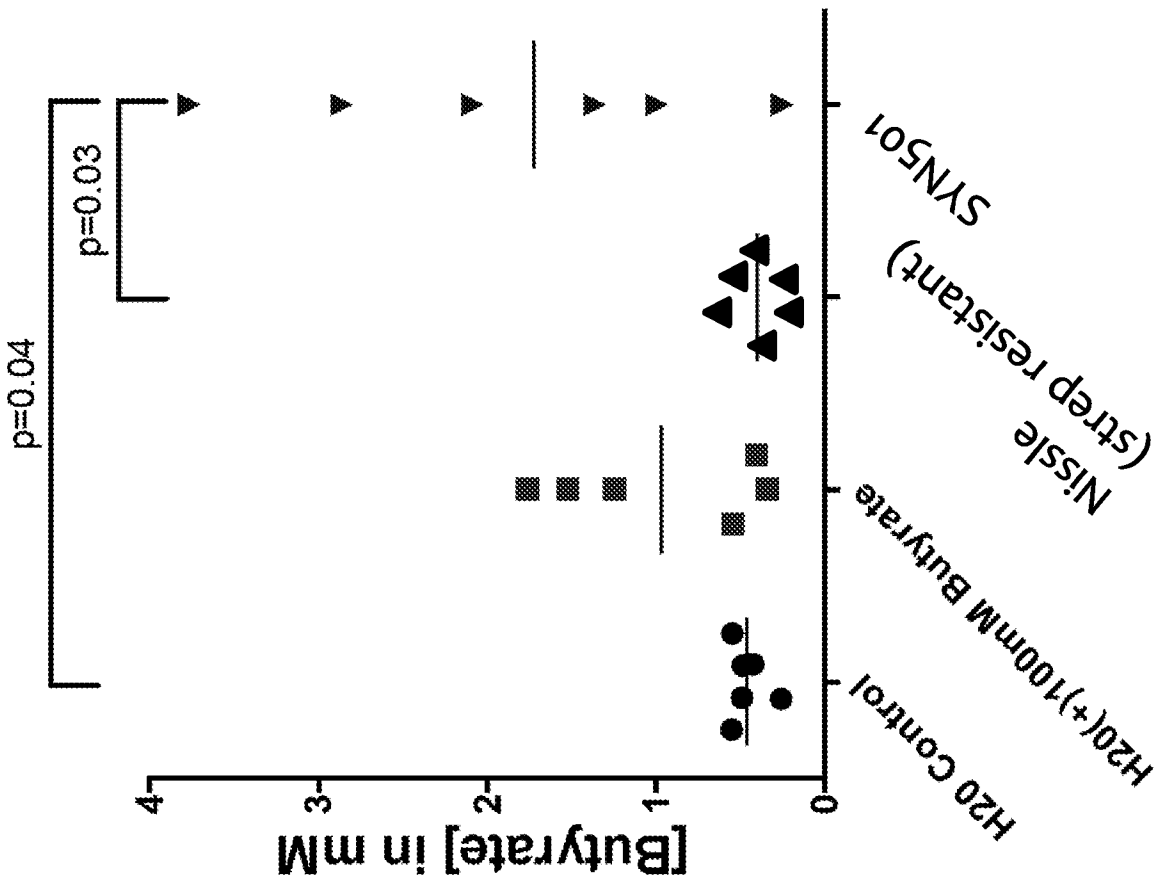
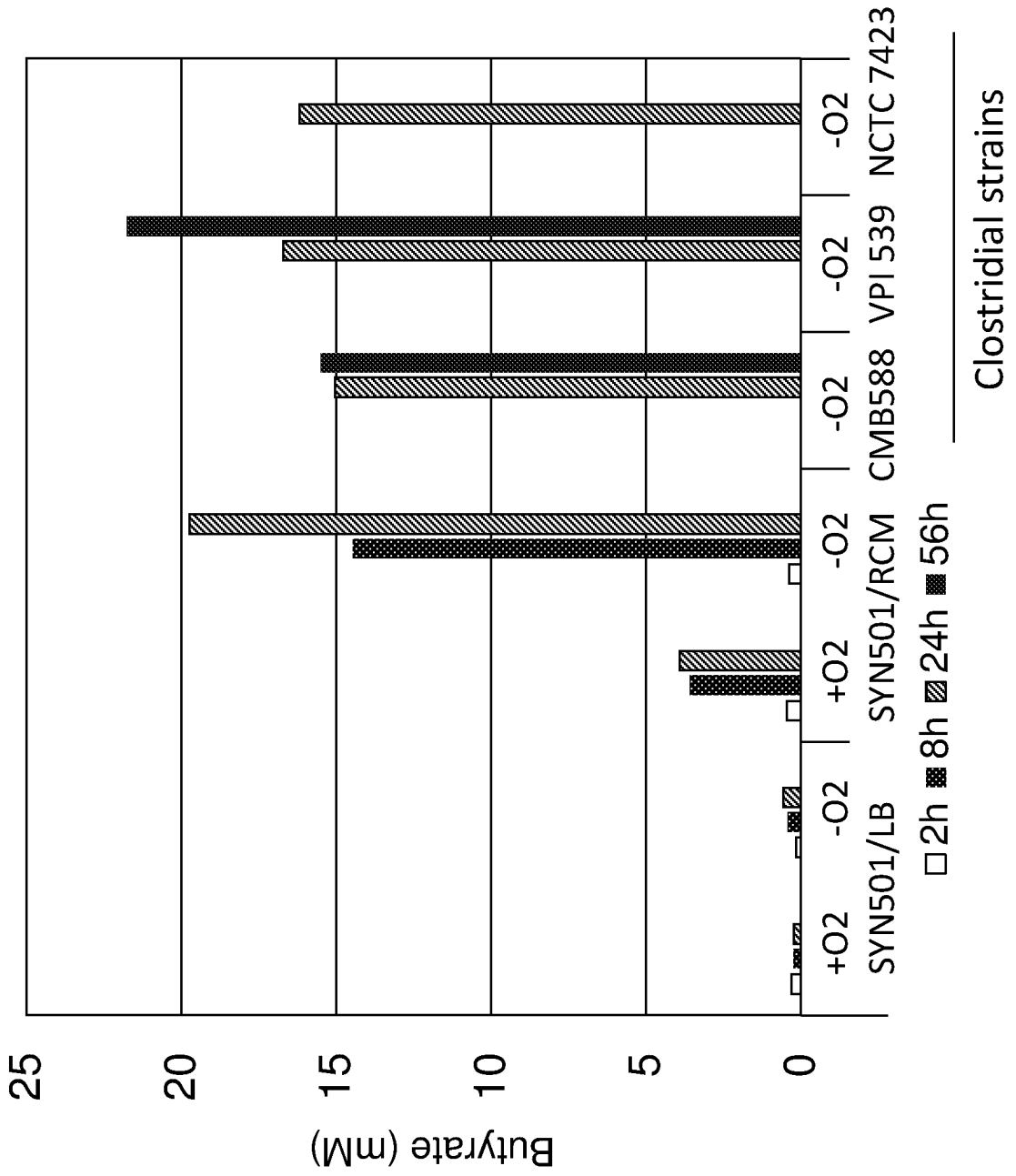

p=0.04
p=0.03
[Butyrate] in mM
0
1
2
3
4
H20 Control
H20(+)100mM Butyrate
Nissle (strep resistant)
SYN501

FIG. 18


Butyrate (mM)
0
5
10
15
20
25
SYN501/LB
+O2
-O2
SYN501/RCM
+O2
-O2
CMB588
-O2
VPI 539
-O2
NCTC 7423
-O2
2h
8h
24h
56h
Clostridial strains

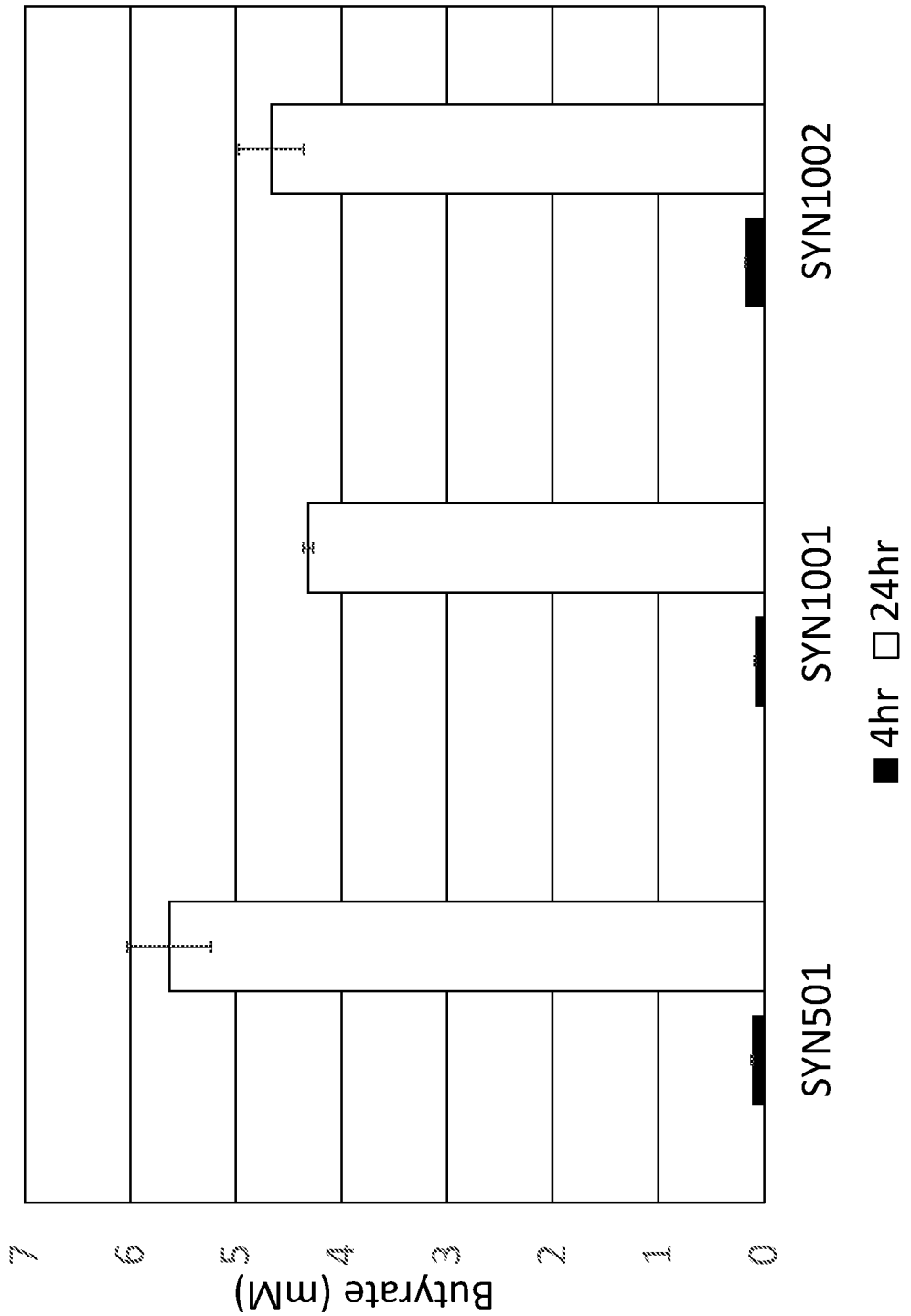


FIG. 19A

FIG. 19B

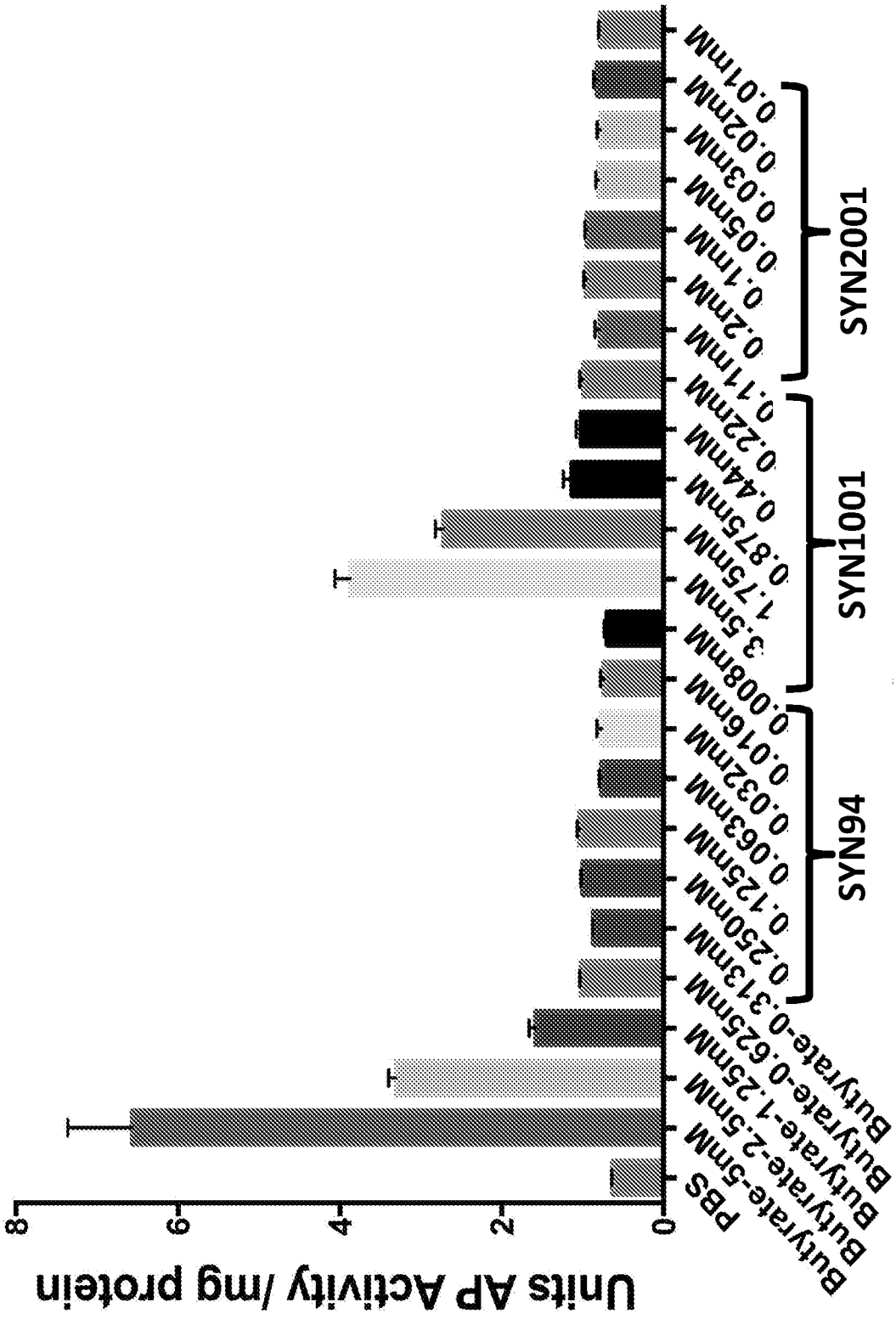

Units AP Activity /mg protein
0
2
4
6
8
PBS
Butyrate-5mM
Butyrate-2.5mM
Butyrate-1.25mM
Butyrate-0.625mM
Butyrate-0.313mM
0.250mM
0.125mM
0.063mM
0.032mM
0.016mM
0.008mM
3.5mM
1.75mM
0.875mM
0.44mM
0.22mM
0.11mM
0.2mM
0.1mM
0.05mM
0.03mM
0.02mM
0.01mM
SYN94
SYN1001
SYN2001

FIG. 19C

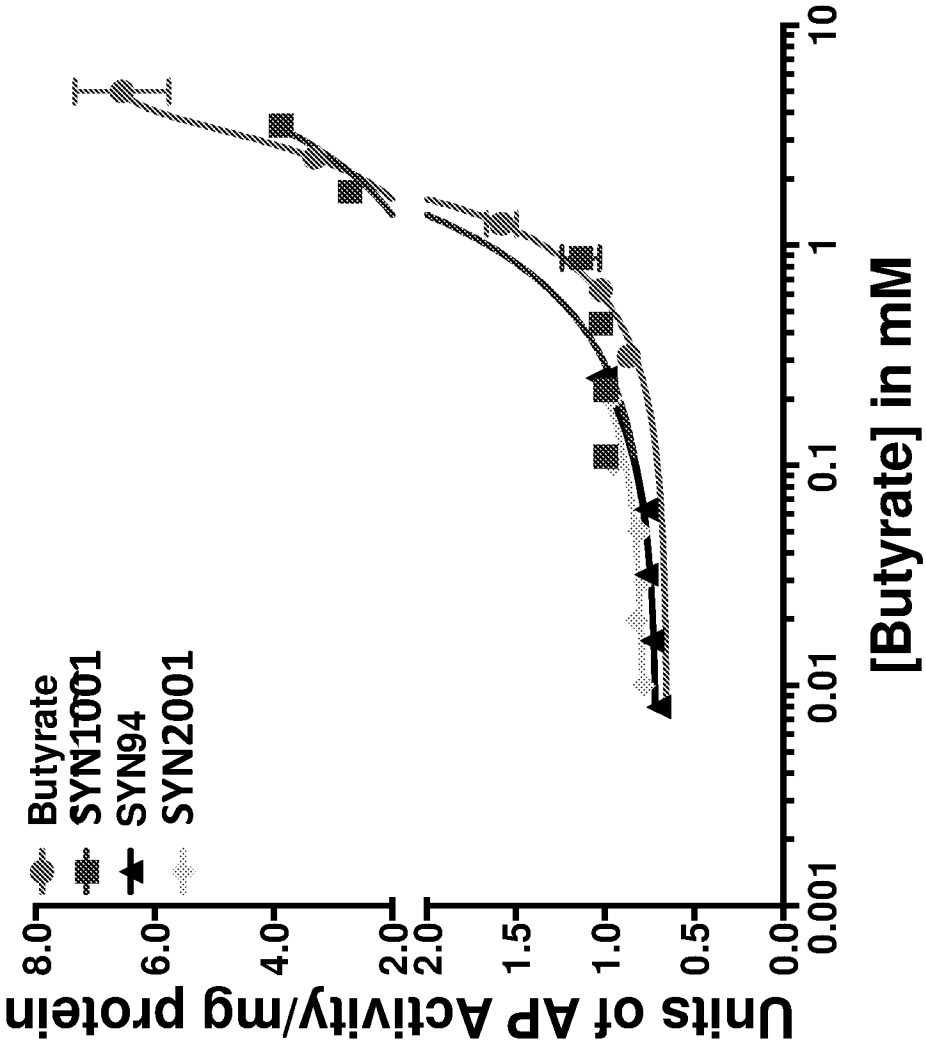

Butyrate
SYN1001
SYN94
SYN2001
Units of AP Activity/mg protein
8.0
6.0
4.0
2.0
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
[Butyrate] in mM

FIG. 20A

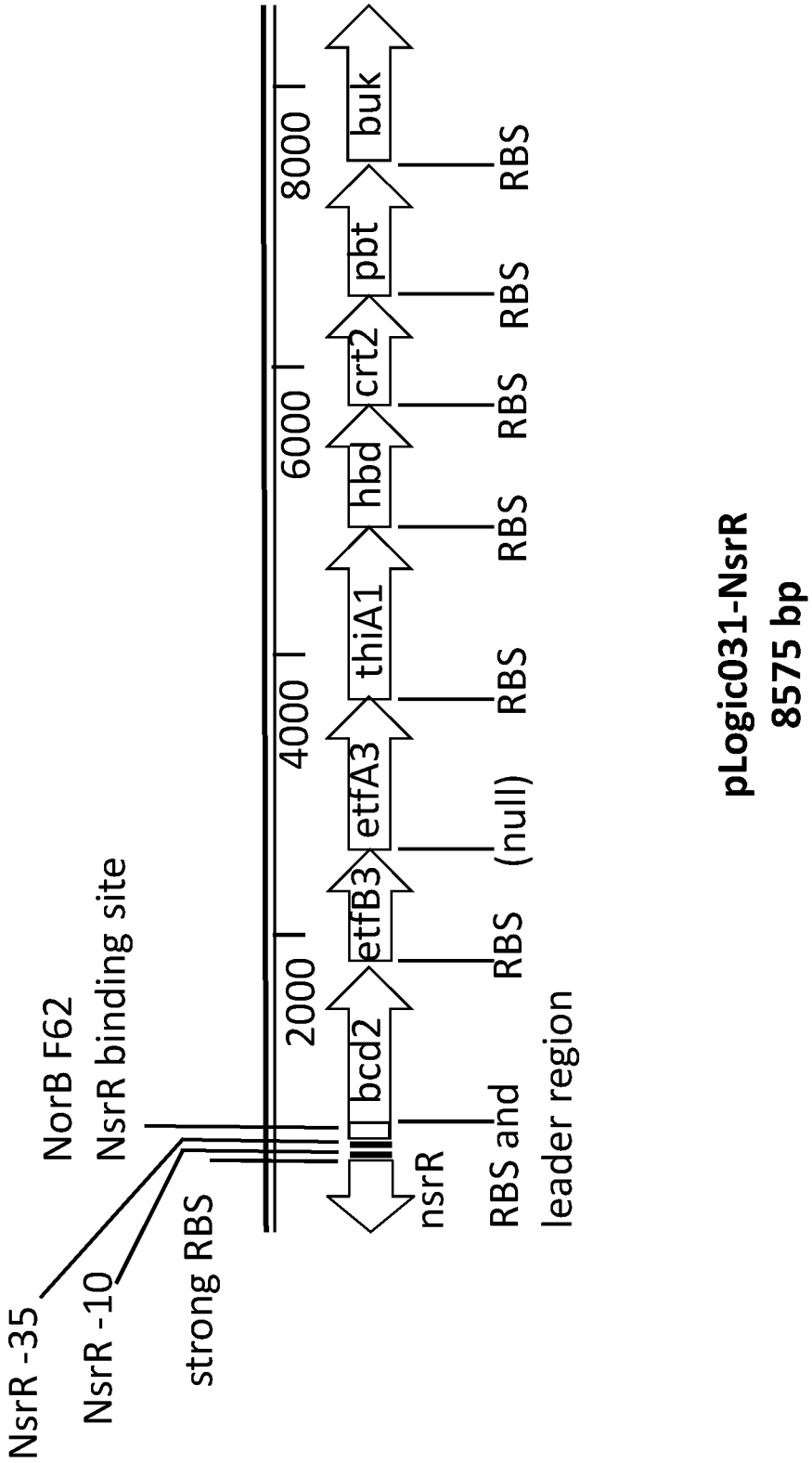

NsrR -35
NsrR -10
strong RBS
NorB F62
NsrR binding site
2000
4000
6000
8000
bcd2
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk
nsrR
RBS and
leader region
RBS
(null)
RBS
RBS
RBS
RBS
RBS
RBS
pLogic031-NsrR
8575 bp

FIG. 20B

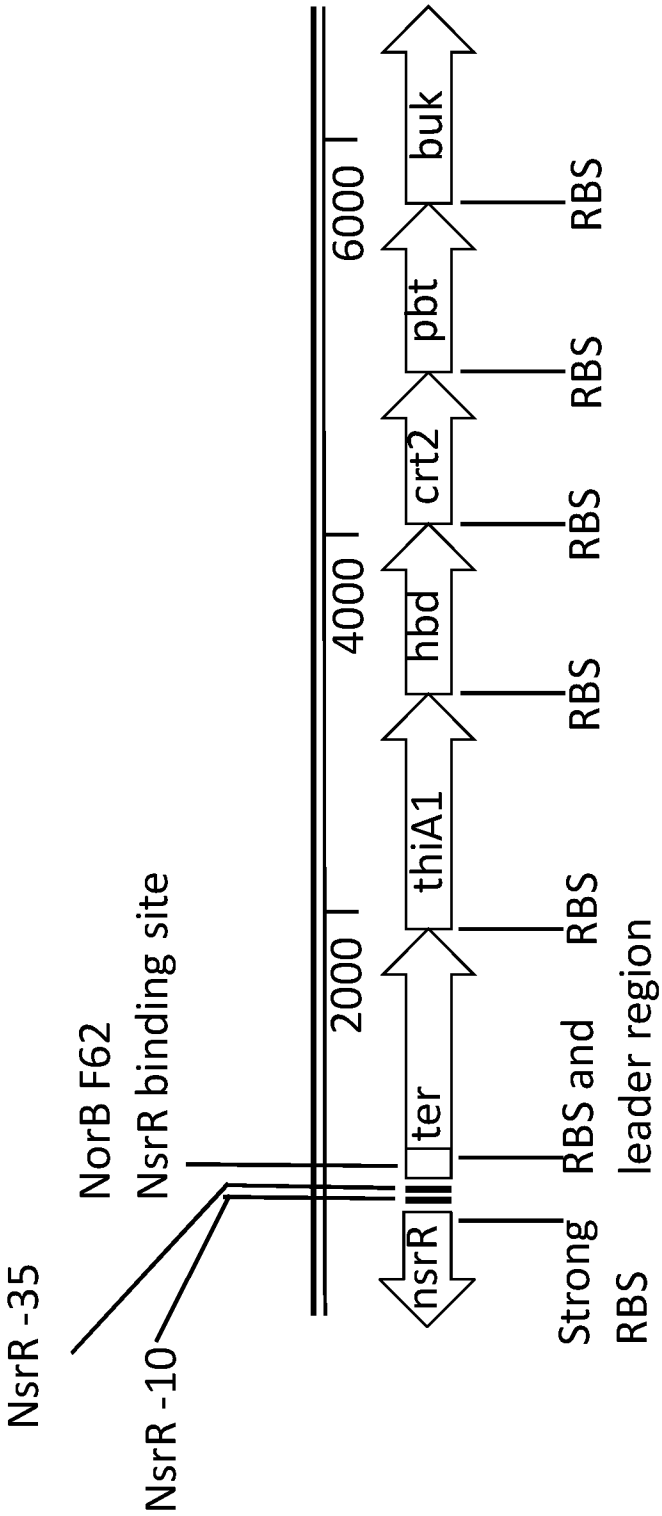

NsrR -35
NsrR -10
NorB F62
NsrR binding site
2000
4000
6000
nsrR
ter
thiA1
hbd
crt2
pbt
buk
Strong RBS
RBS and leader region
RBS
RBS
RBS
RBS
RBS
pLogic046-NsrR (2)
6787 bp

FIG. 21

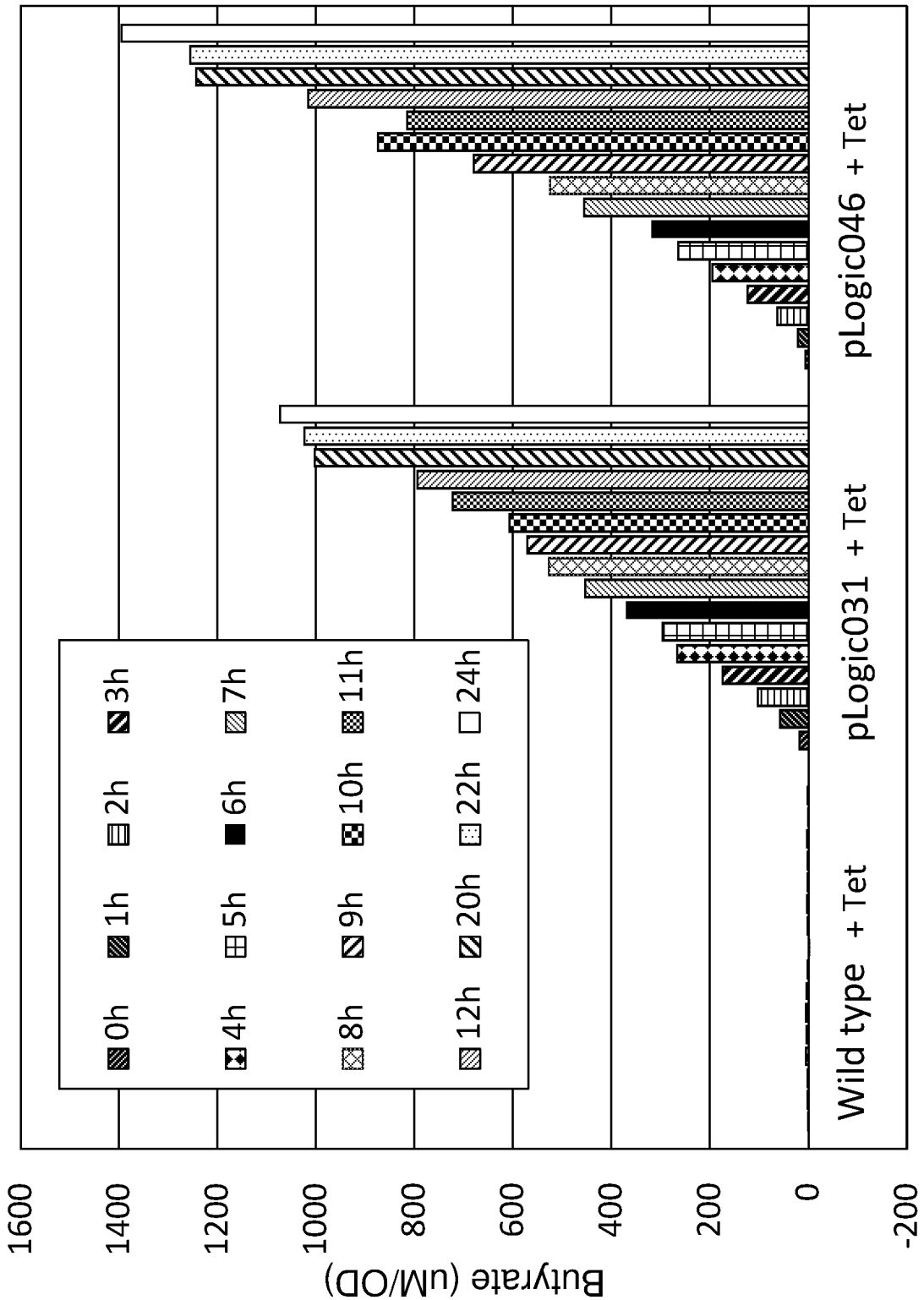
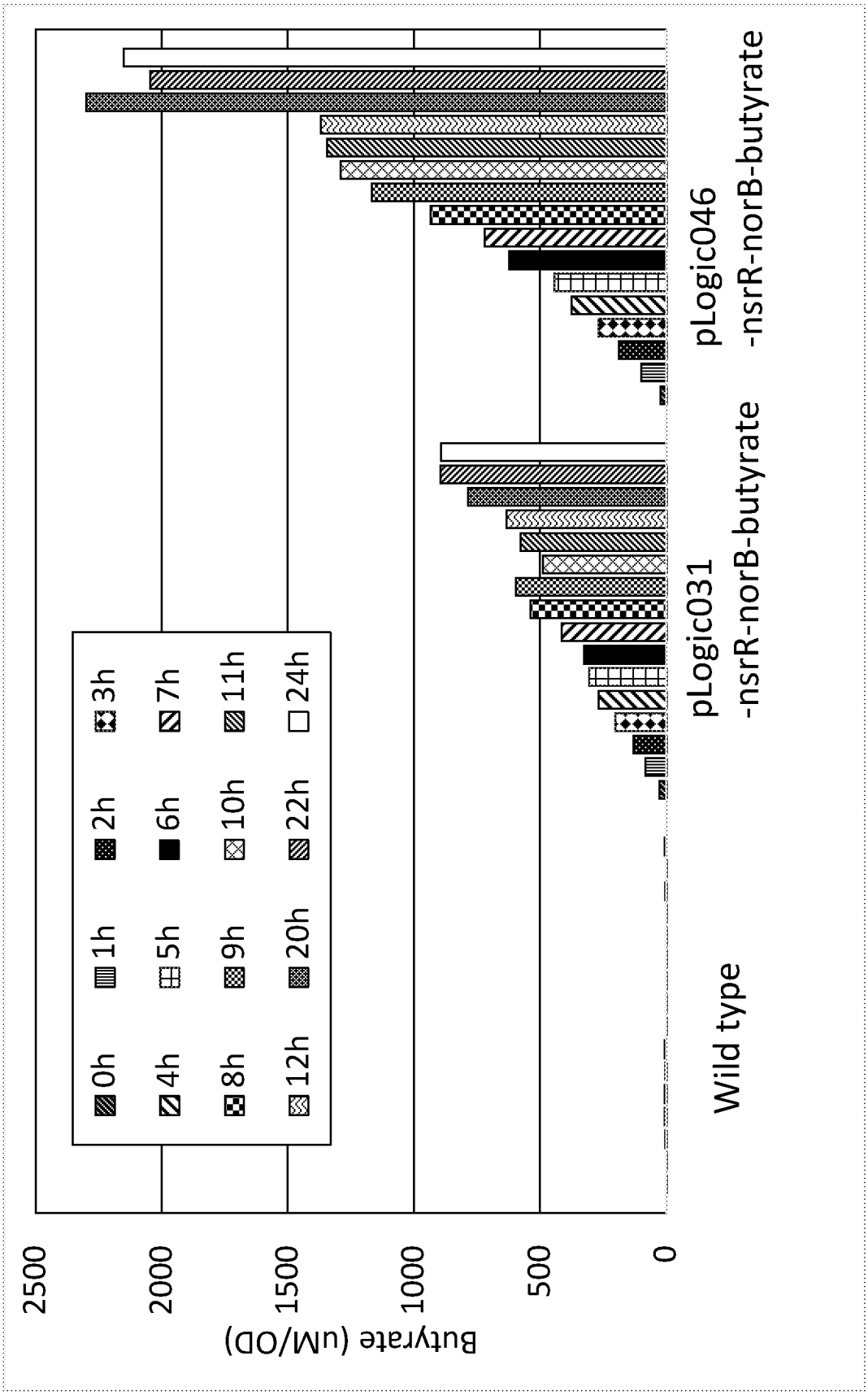

Butyrate (uM/OD)
1600
1400
1200
1000
800
600
400
200
0
-200
Wild type + Tet
pLogic031 + Tet
pLogic046 + Tet
0h
1h
2h
3h
4h
5h
6h
7h
8h
9h
10h
11h
12h
20h
22h
24h

FIG. 22


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
pLogic031
-nsrR-norB-butyrate
pLogic046
-nsrR-norB-butyrate

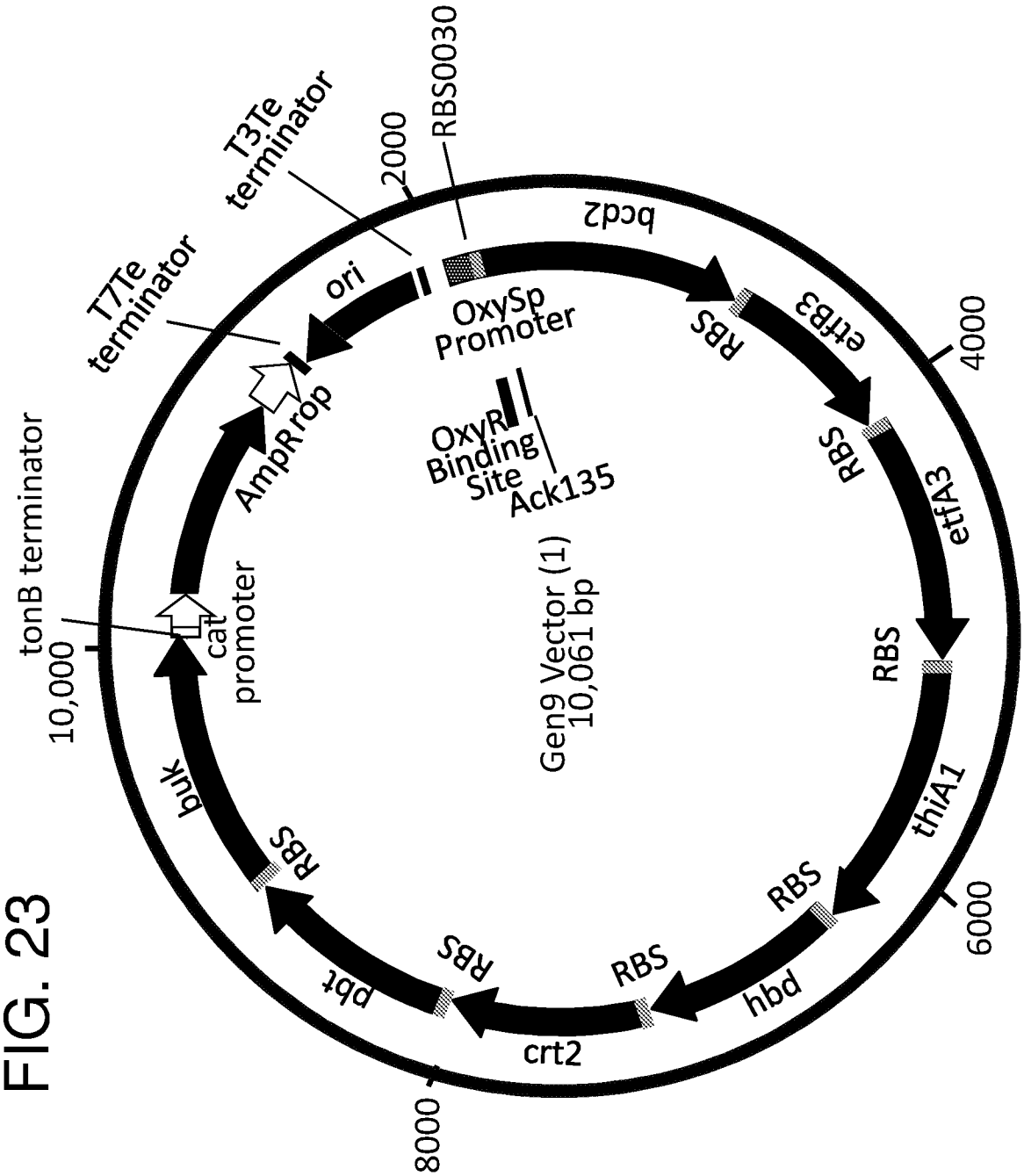

Gen9 Vector (1)
10,061 bp
2000
4000
6000
8000
10,000
RBS0030
T3Te terminator
T7Te terminator
tonB terminator
ori
OxySp Promoter
OxyR Binding Site
Ack135
AmpR rop
cat promoter
bcd2
RBS
etfB3
etfA3
thiA1
hbd
crt2
pbt
buk

FIG. 23

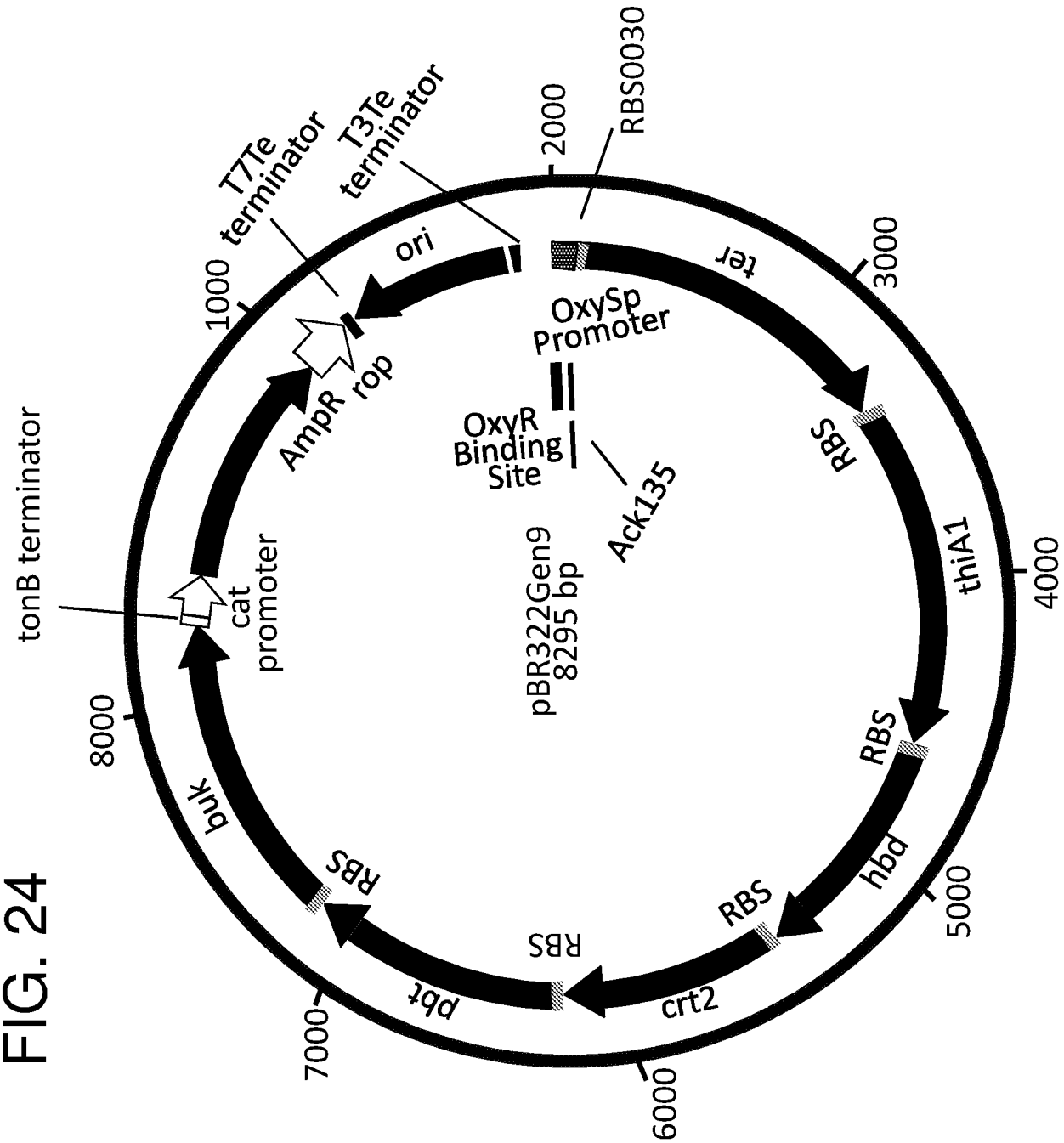

RBS0030
T3Te terminator
T7Te terminator
ori
ter
OxySp Promoter
OxyR Binding Site
Ack135
pBR322Gen9 8295 bp
AmpR
rop
tonB terminator
cat promoter
RBS
thiA1
RBS
hbd
RBS
crt2
RBS
pbt
RBS
bukl
2000
3000
4000
5000
6000
7000
8000
1000

FIG. 24

FIG. 25

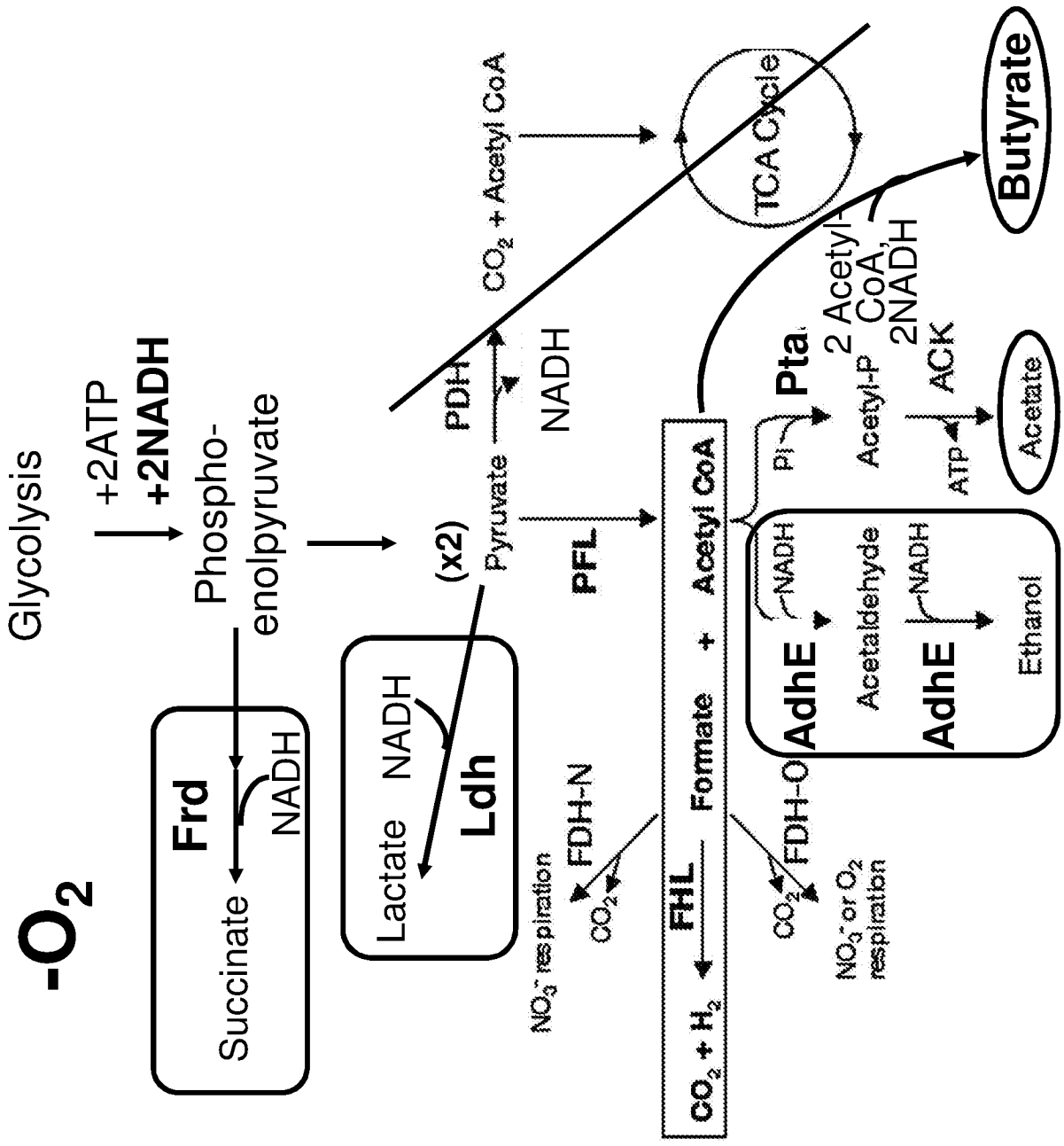

-O2
Glycolysis
+2ATP
+2NADH
Phospho-enolpyruvate
Frd
Succinate
NADH
Lactate
NADH
Ldh
(x2)
Pyruvate
PDH
CO2 + Acetyl CoA
NADH
TCA Cycle
PFL
Acetyl CoA
Formate
+
FHL
CO2 + H2
FDH-N
NO3- respiration
CO2
FDH-O
CO2
NO3- or O2 respiration
Pi
Pta
Acetyl-P
2 Acetyl-CoA, 2NADH
ACK
ATP
Acetate
Butyrate
NADH
AdhE
Acetaldehyde
NADH
AdhE
Ethanol

FIG. 26A

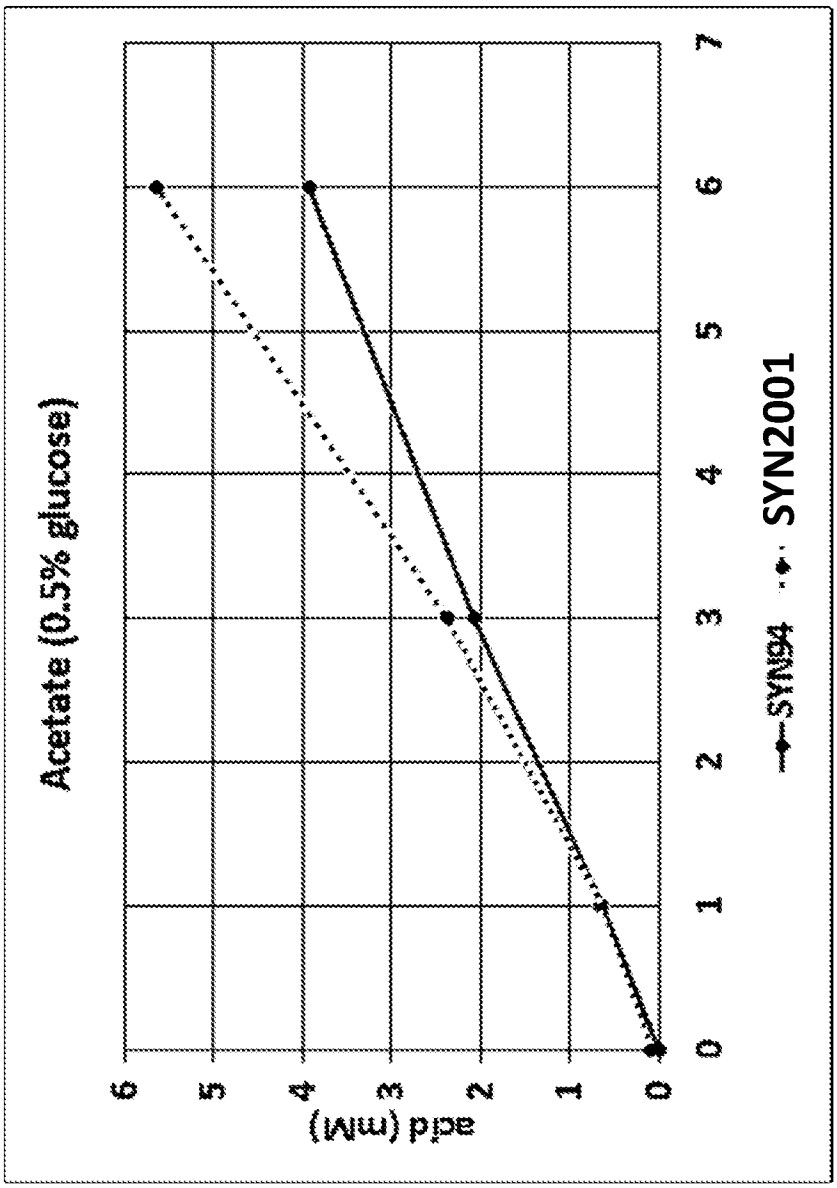
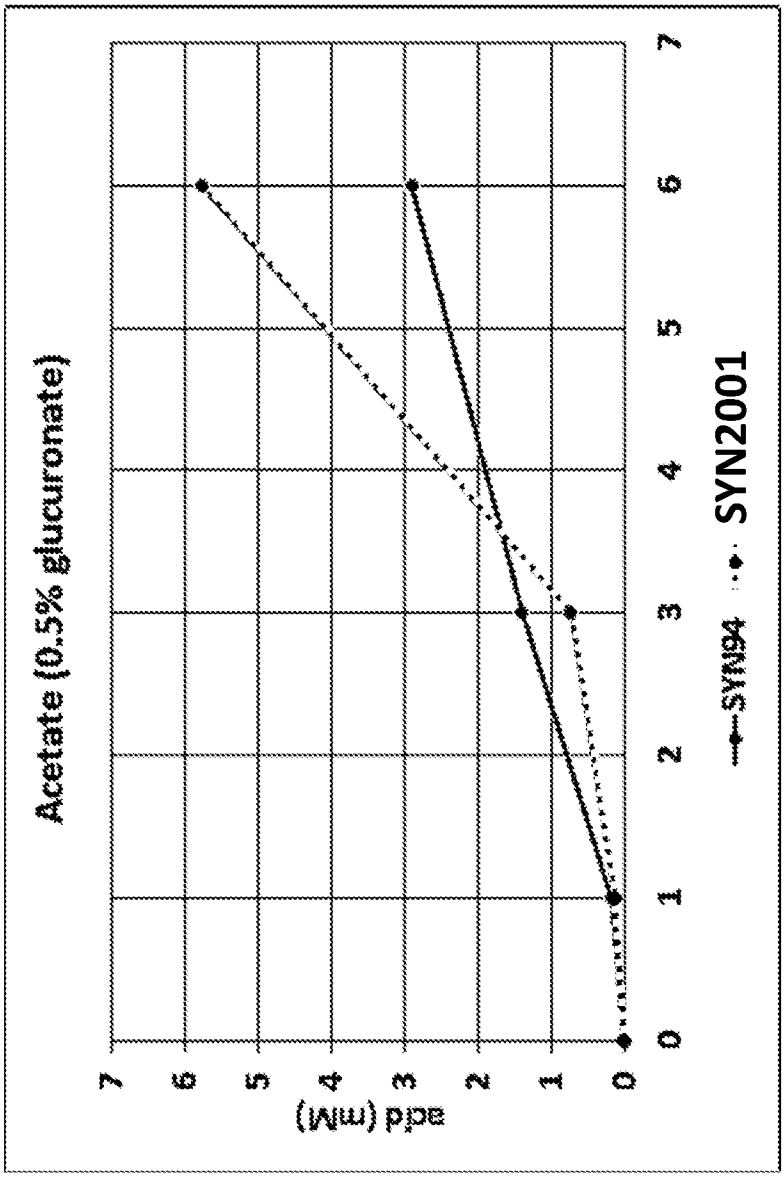


FIG. 26B



SYN94 Nissle
SYN2001 Nissle delta IdhA::CmR

FIG. 26C

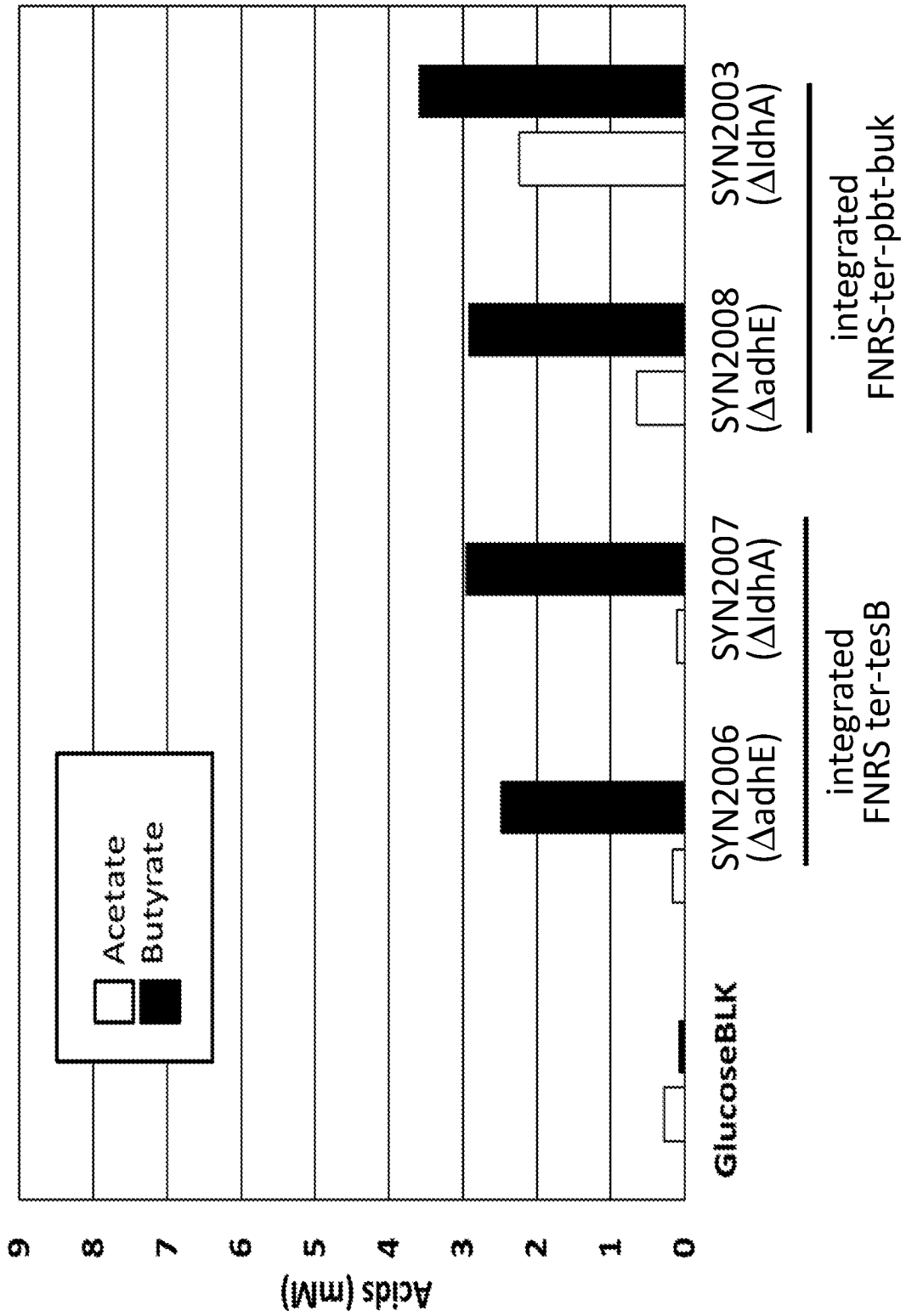

Acetate
Butyrate
Acids (mM)
0
1
2
3
4
5
6
7
8
9
GlucoseBLK
SYN2006 (ΔadhE)
SYN2007 (ΔldhA)
SYN2008 (ΔadhE)
SYN2003 (ΔldhA)
integrated FNRS ter-tesB
integrated FNRS-ter-pbt-buk

FIG. 26D

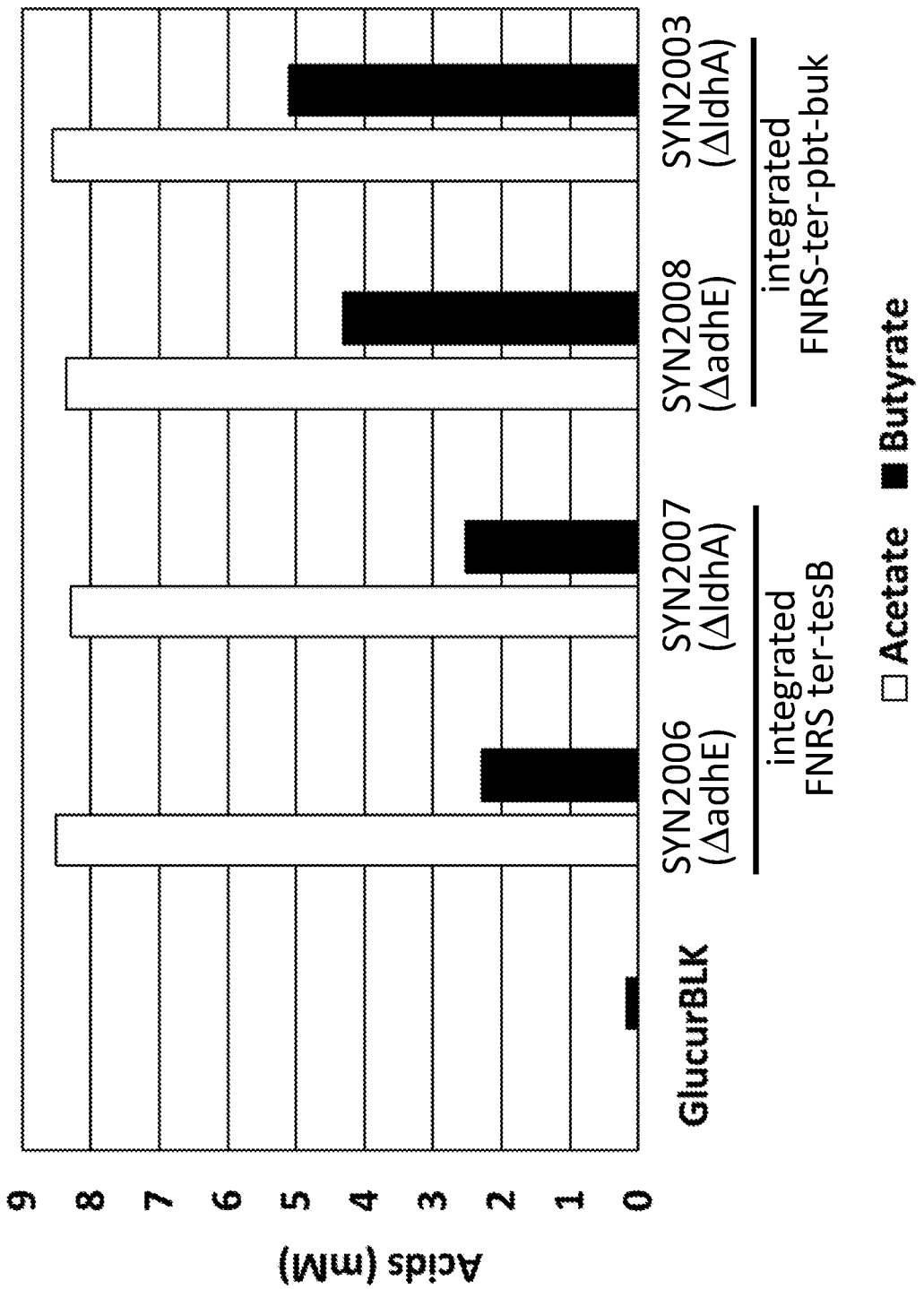

Acids (mM)
9
8
7
6
5
4
3
2
1
0
GlucurBLK
SYN2006 (ΔadhE)
SYN2007 (ΔldhA)
integrated FNRS ter-tesB
SYN2008 (ΔadhE)
SYN2003 (ΔldhA)
integrated FNRS-ter-pbt-buk
Acetate
Butyrate

FIG. 26E

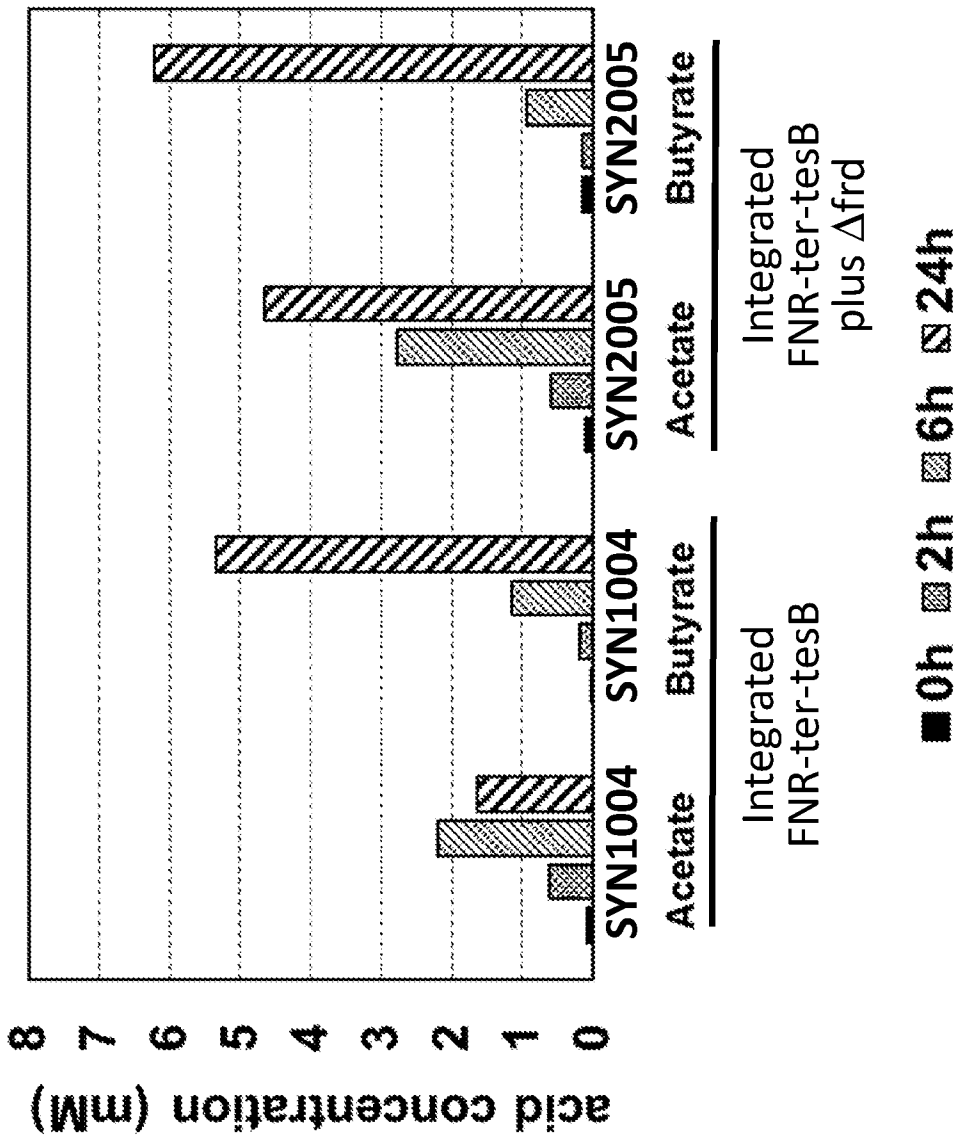
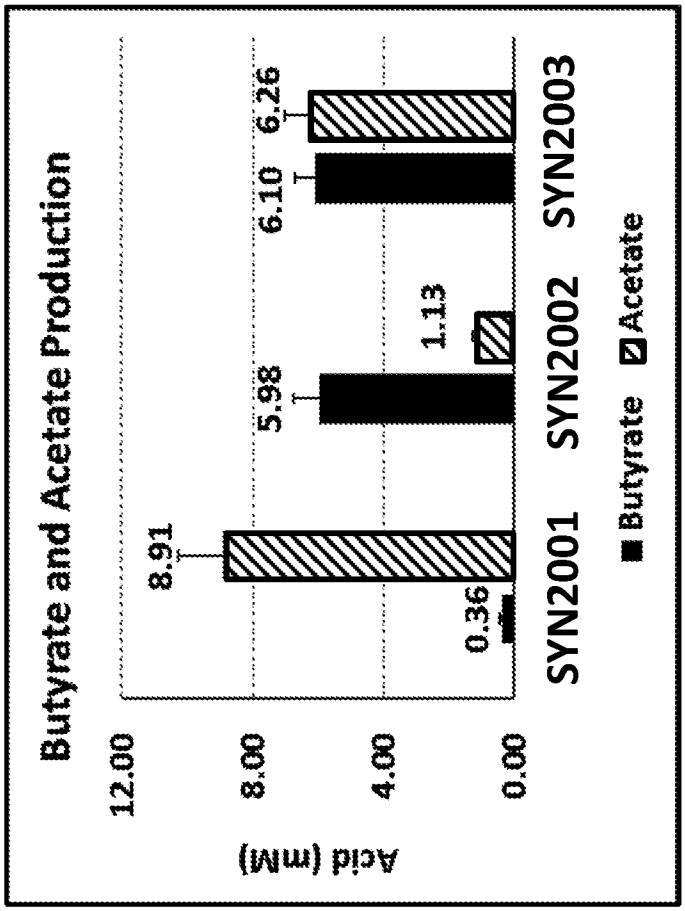

acid concentration (mM)
0
1
2
3
4
5
6
7
8
SYN1004
SYN1004
SYN2005
SYN2005
Acetate
Butyrate
Acetate
Butyrate
Integrated FNR-ter-tesB
Integrated FNR-ter-tesB plus Δfrd
0h
2h
6h
24h

FIG. 26F



SYN2001 Nissle ΔldhA::CmR
SYN2002 Nissle butyrate cassette ΔadhE/Δpta::CmR
SYN2003 Nissle butyrate cassette ΔldhA::CmR

FIG. 26G

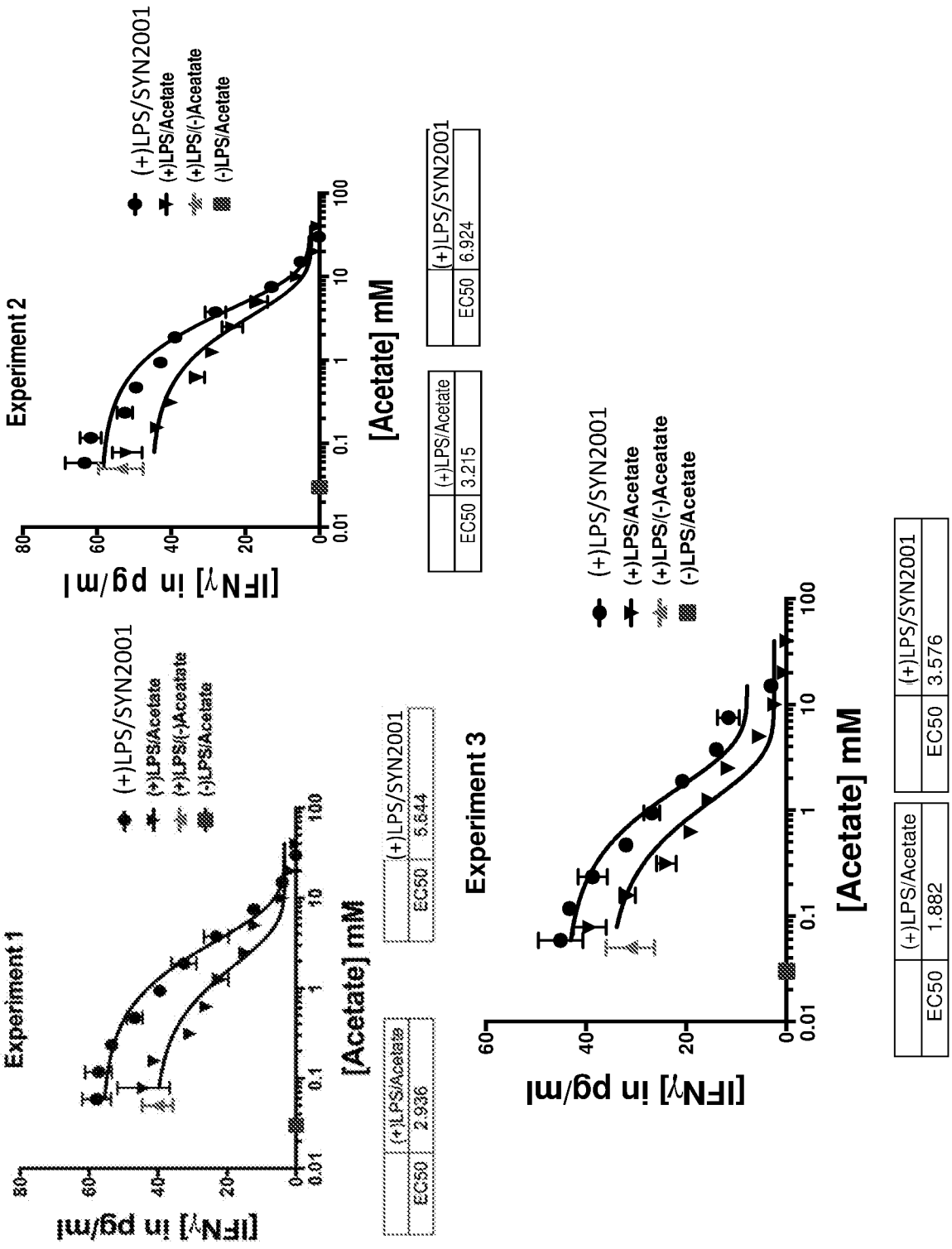

Experiment 1
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 2.936
(+)LPS/SYN2001
EC50 5.644
Experiment 2
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 3.215
(+)LPS/SYN2001
EC50 6.924
Experiment 3
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 1.882
(+)LPS/SYN2001
EC50 3.576

FIG. 26H

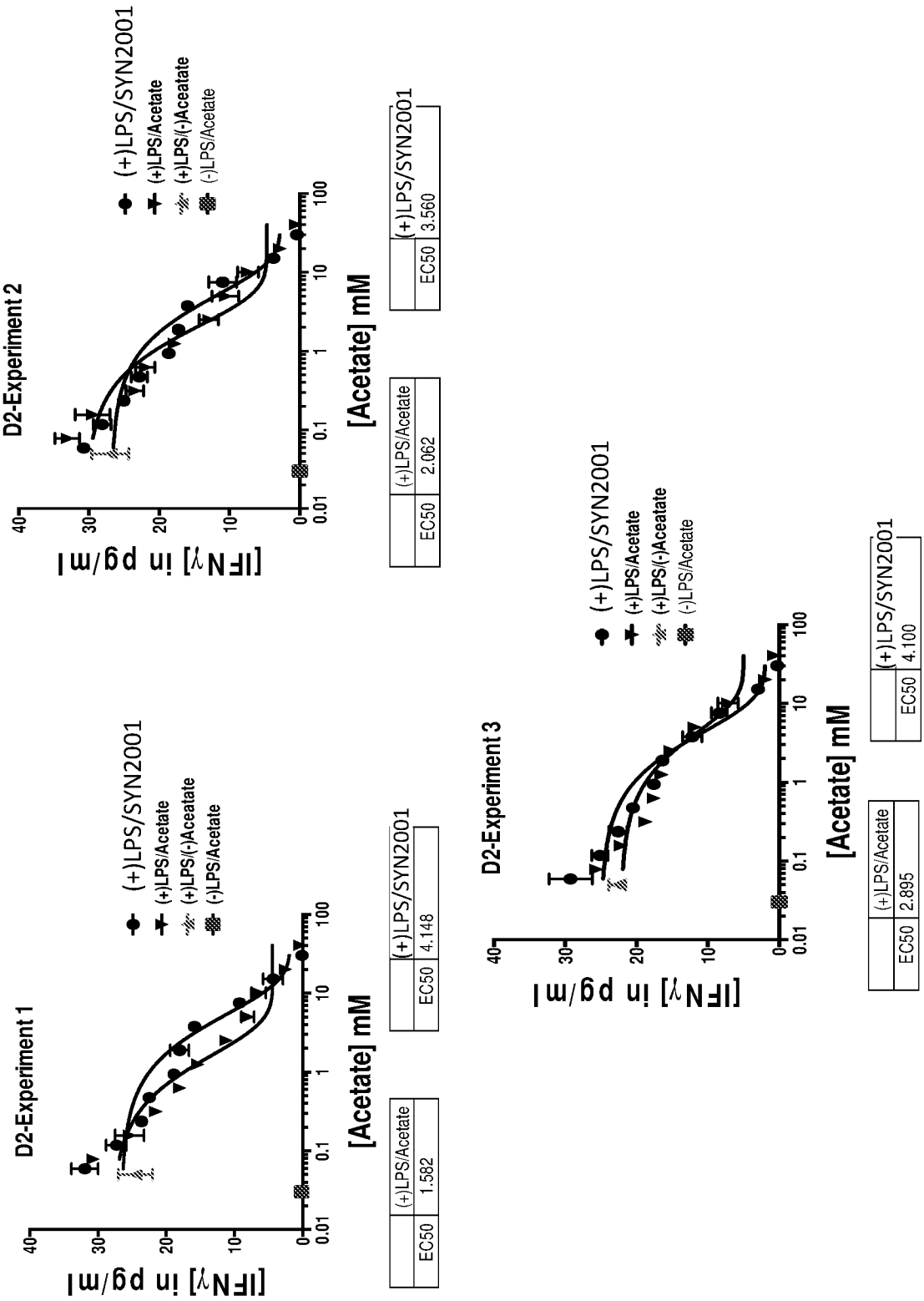

D2-Experiment 1
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 1.582
(+)LPS/SYN2001
EC50 4.148
D2-Experiment 2
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 2.062
(+)LPS/SYN2001
EC50 3.560
D2-Experiment 3
[IFNγ] in pg/ml
[Acetate] mM
(+)LPS/SYN2001
(+)LPS/Acetate
(+)LPS/(-)Aceatate
(-)LPS/Acetate
(+)LPS/Acetate
EC50 2.895
(+)LPS/SYN2001
EC50 4.100

FIG. 27

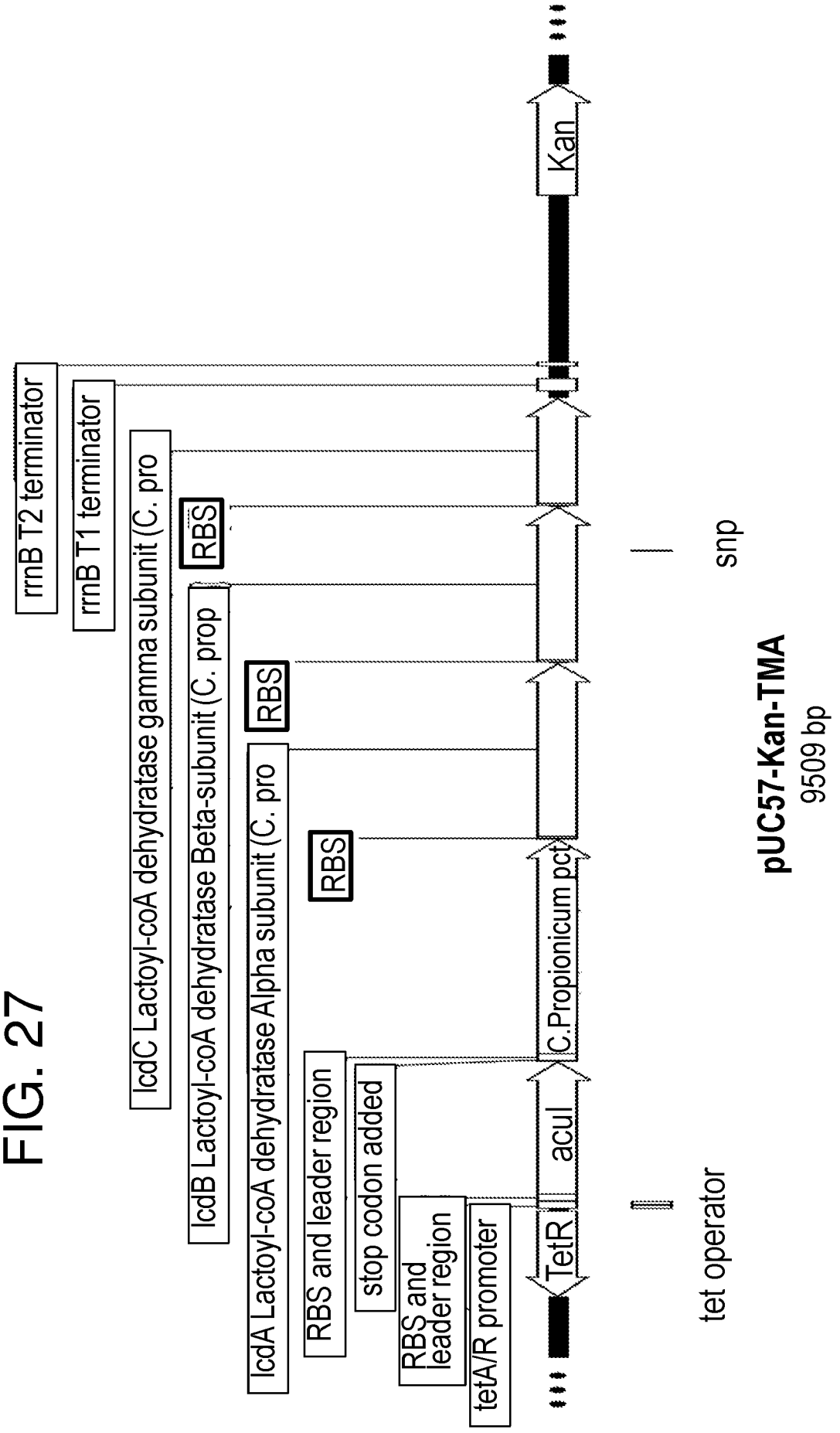

rrnB T2 terminator
rrnB T1 terminator
lcdC Lactoyl-coA dehydratase gamma subunit (C. pro
RBS
lcdB Lactoyl-coA dehydratase Beta-subunit (C. prop
RBS
lcdA Lactoyl-coA dehydratase Alpha subunit (C. pro
RBS
RBS and leader region
stop codon added
RBS and leader region
tetA/R promoter
TetR
acuI
C.Propionicum pct
Kan
snp
tet operator
pUC57-Kan-TMA
9509 bp

FIG. 28

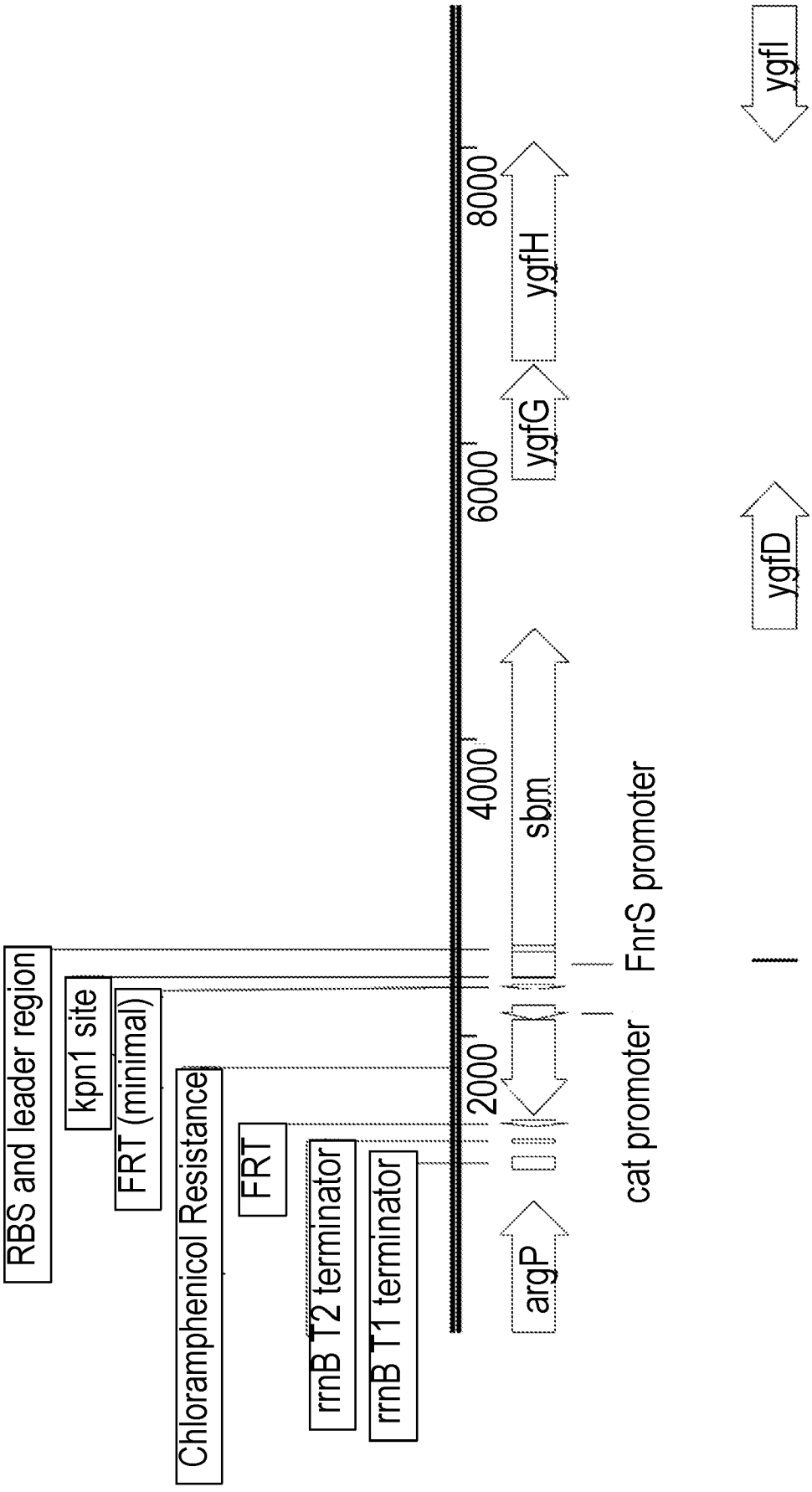

RBS and leader region
kpn1 site
FRT (minimal)
Chloramphenicol Resistance
FRT
rrnB T2 terminator
rrnB T1 terminator
argP
2000
cat promoter
FnrS promoter
4000
sbm
6000
ygfG
8000
ygfH
ygfD
ygfI
FNR binding site

FIG. 29

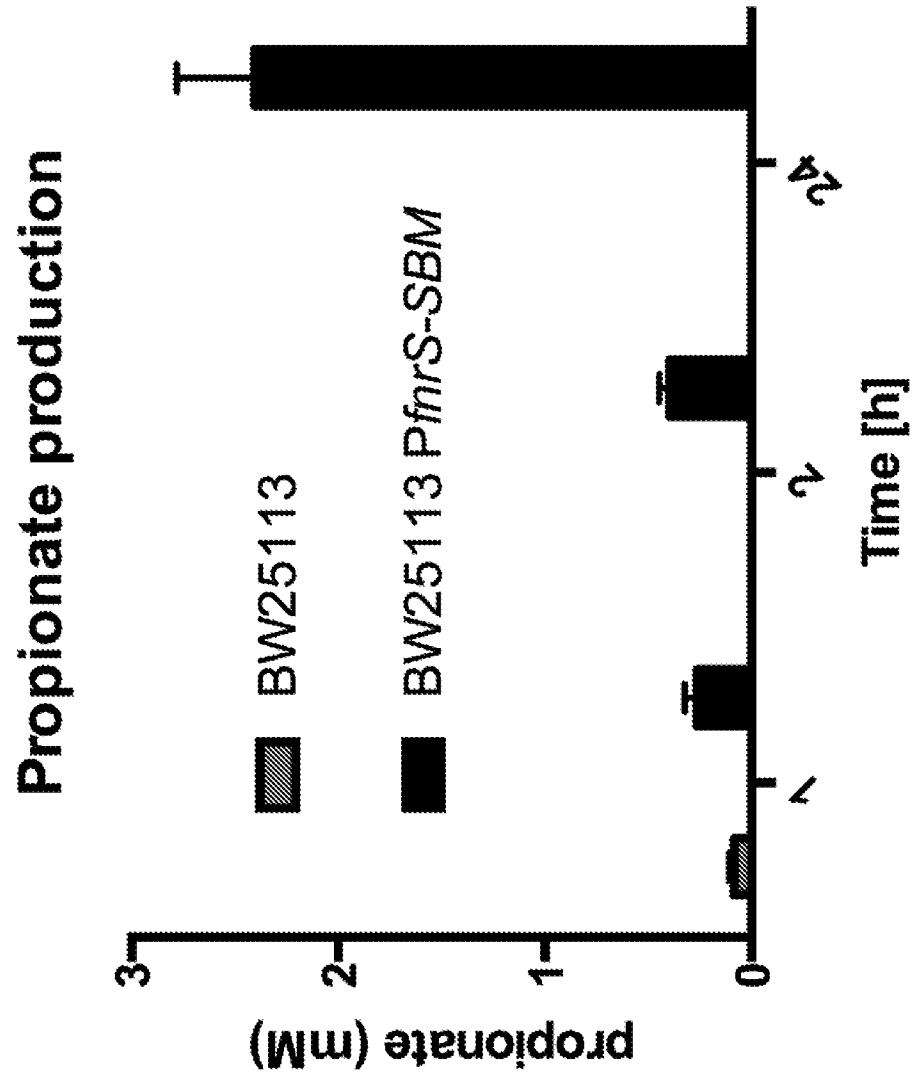

Propionate production
BW25113
BW25113 PfnrS-SBM
propionate (mM)
0
1
2
3
7
24
Time [h]

FIG. 30A

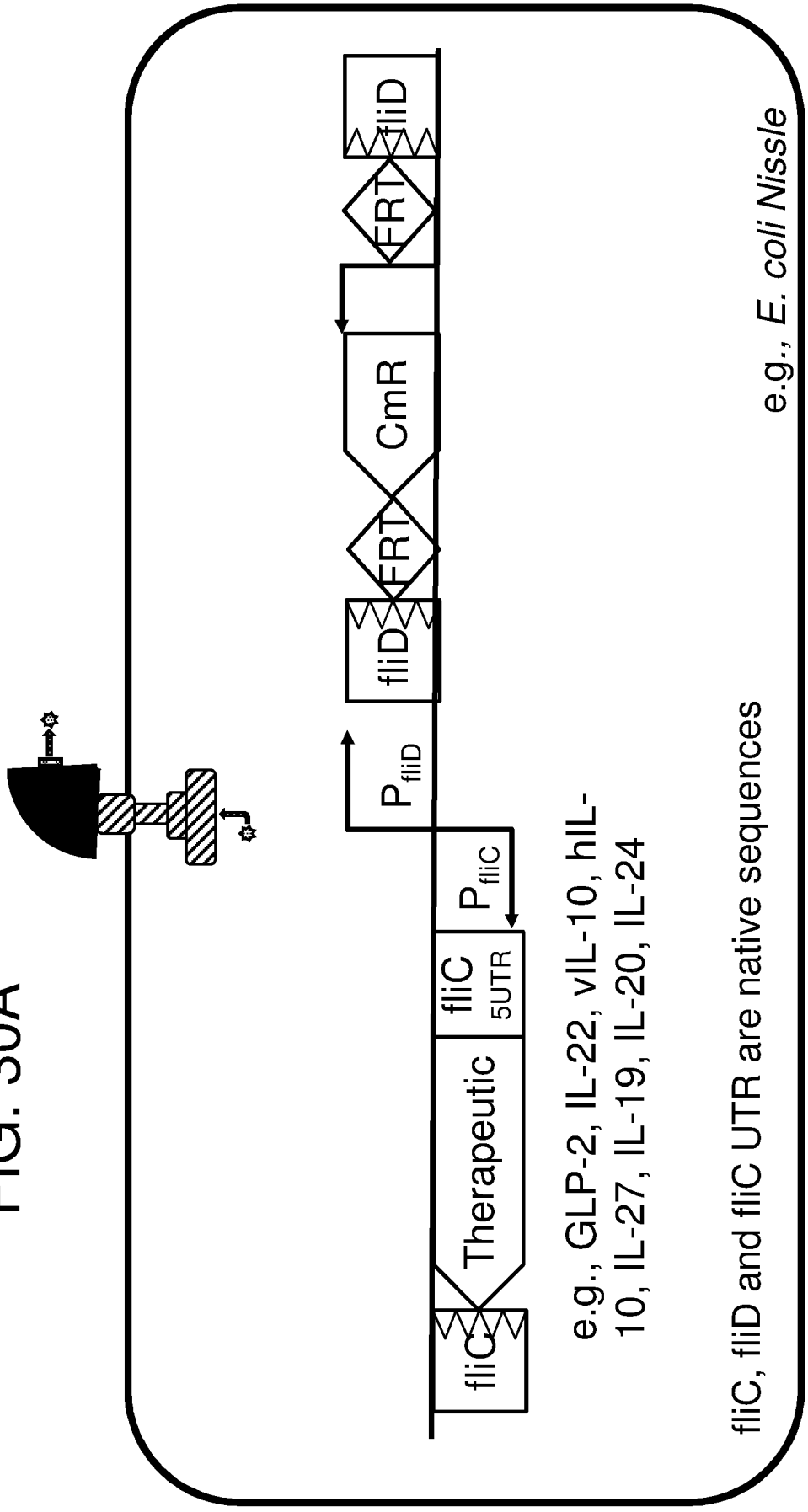

fliC, fliD and fliC UTR are native sequences
e.g., E. coli Nissle
fliC
Therapeutic
e.g., GLP-2, IL-22, vIL-10, hIL-10, IL-27, IL-19, IL-20, IL-24
fliC 5UTR
P fliC
P fliD
fliD
FRT
CmR
FRT
fliD

FIG. 30B

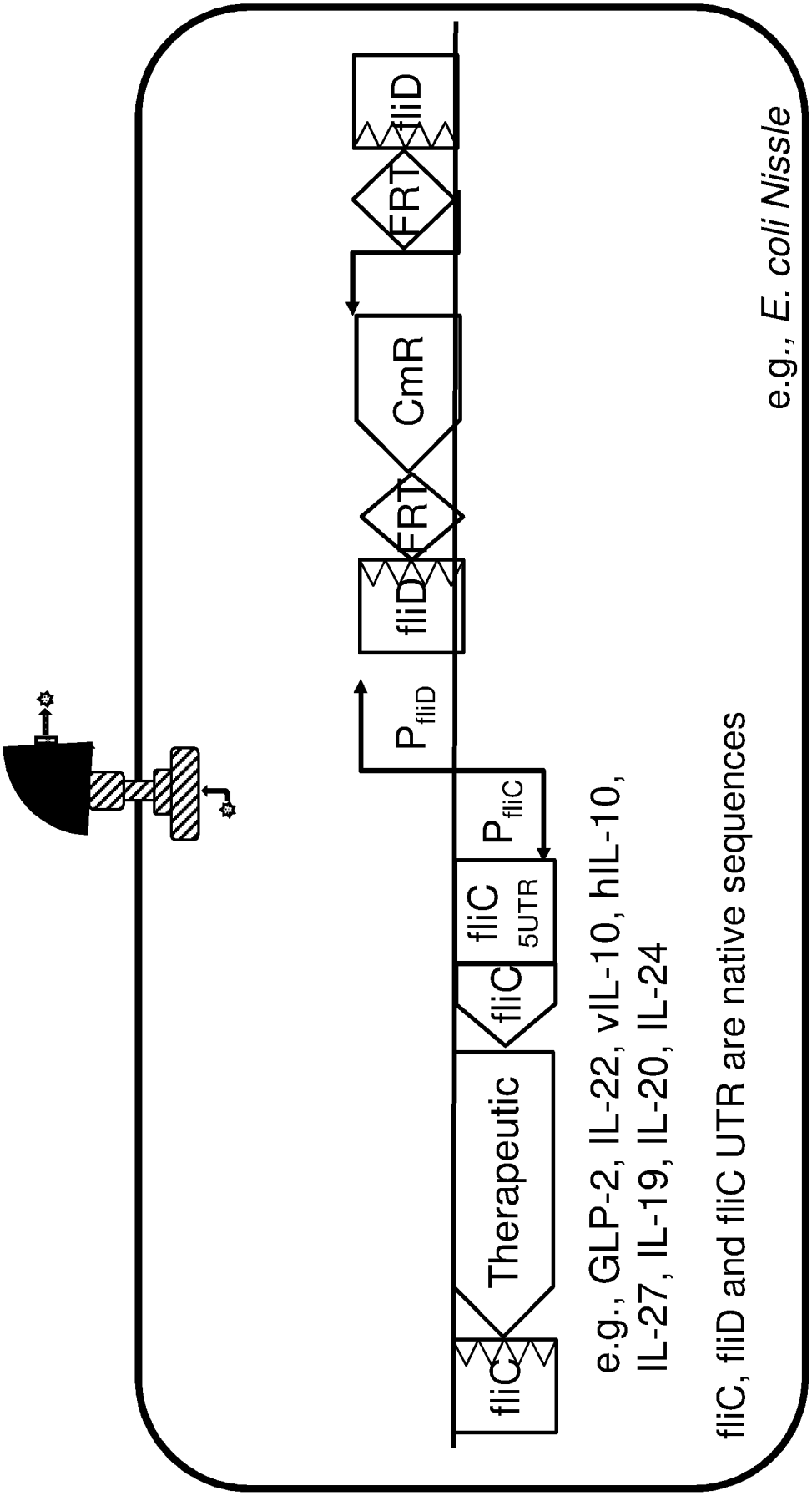
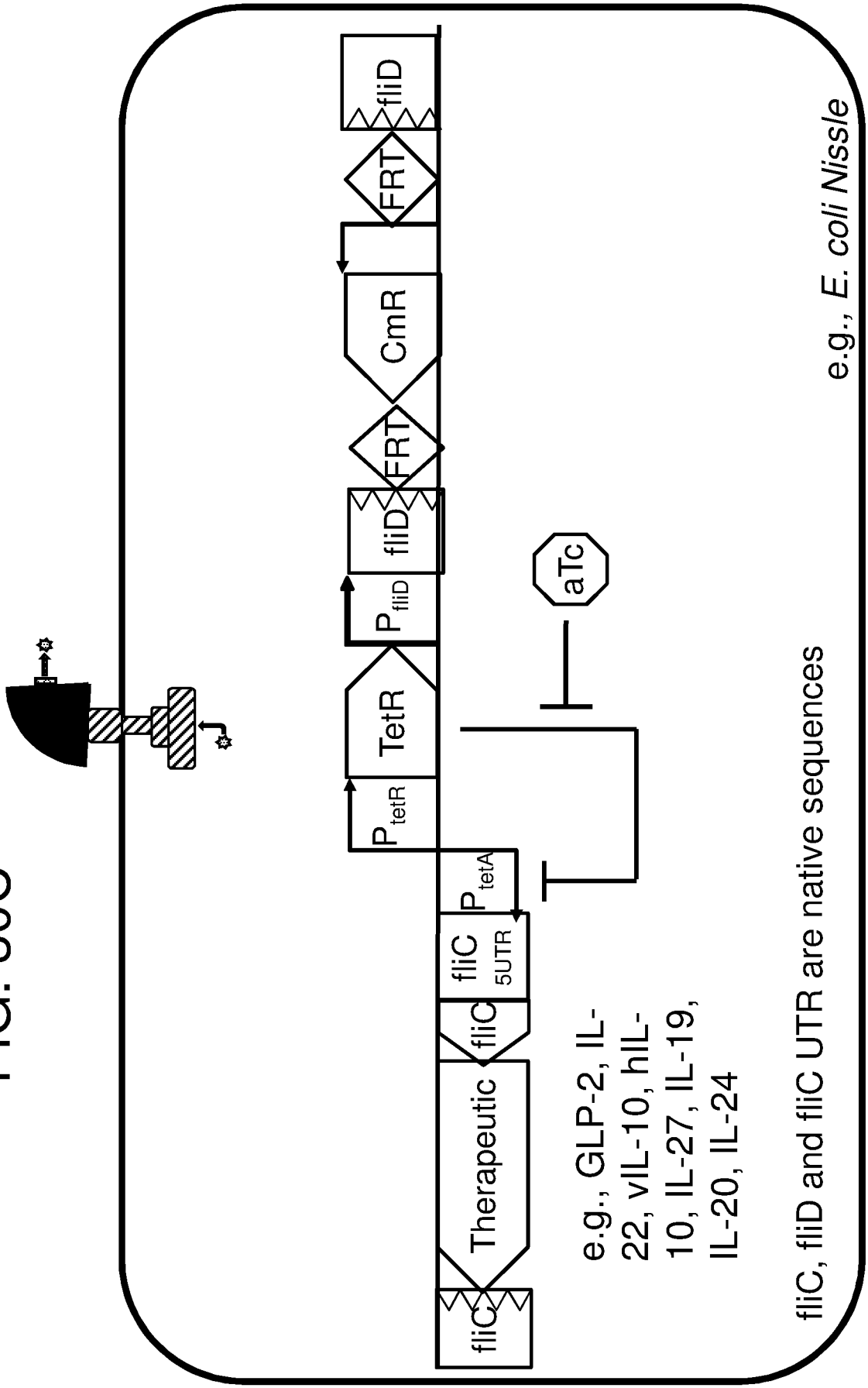

fliC
Therapeutic
fliC
fliC 5UTR
P_{fliC}
P_{fliD}
fliD
FRT
CmR
FRT
fliD
FRT
fliD
e.g., GLP-2, IL-22, vIL-10, hIL-10, IL-27, IL-19, IL-20, IL-24
fliC, fliD and fliC UTR are native sequences
e.g., *E. coli Nissle*

FIG. 30C


fliD
FRT
CmR
FRT
fliD
P_{fliD}
TetR
P_{tetR}
aTc
P_{tetA}
fliC 5UTR
fliC
Therapeutic
fliC
e.g., GLP-2, IL-22, vIL-10, hIL-10, IL-27, IL-19, IL-20, IL-24
fliC, fliD and fliC UTR are native sequences
e.g., E. coli Nissle

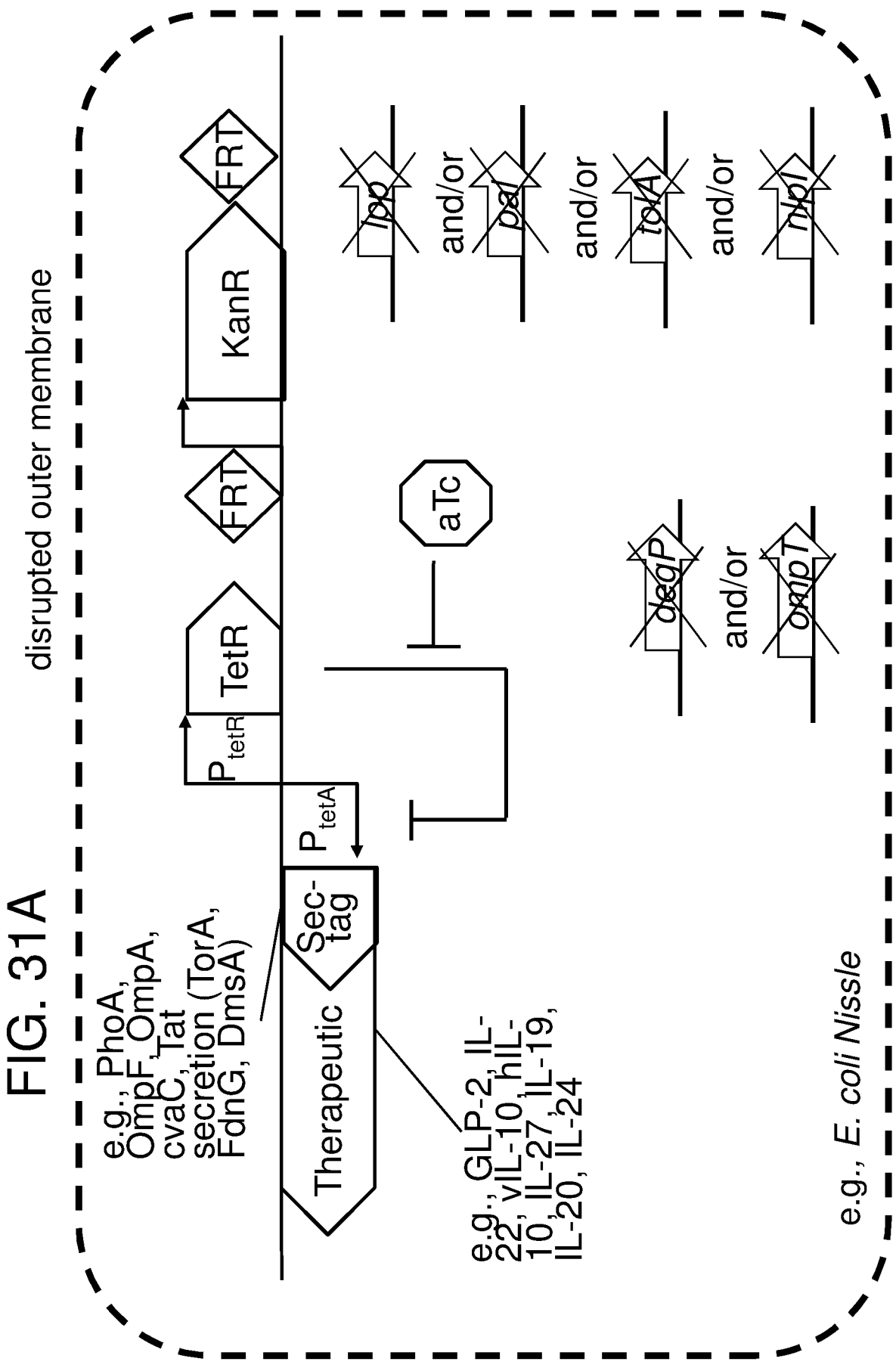

disrupted outer membrane
FRT
KanR
FRT
TetR
P_{tetR}
P_{tetA}
aTc
Sec-tag
Therapeutic
e.g., PhoA, OmpF, OmpA, cvaC, Tat secretion (TorA, FdnG, DmsA)
e.g., GLP-2, IL-22, vIL-10, hIL-10, IL-27, IL-19, IL-20, IL-24
lpp
and/or
pal
and/or
tolA
and/or
rhtI
degP
and/or
ompT
e.g., E. coli Nissle

FIG. 31A

FIG. 31B

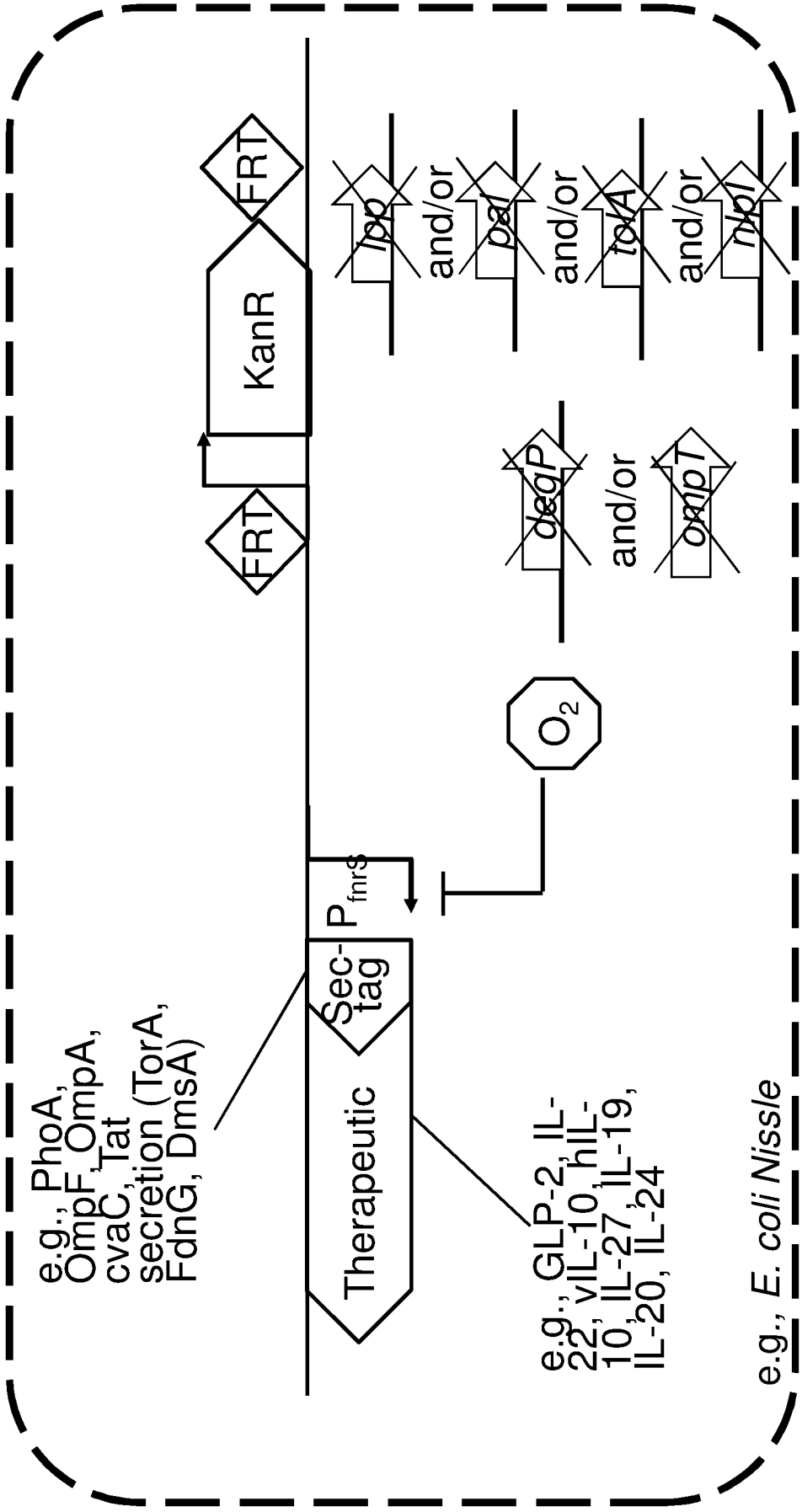

FRT
KanR
FRT
lpp
and/or
pal
and/or
tolA
and/or
nlpI
dsdP
and/or
ompT
O2
Pfnr
Sec-tag
Therapeutic
e.g., PhoA, OmpF, OmpA, cvaC, Tat secretion (TorA, FdnG, DmsA)
e.g., GLP-2, IL-22, vIL-10, hIL-10, IL-27, IL-19, IL-20, IL-24
e.g., E. coli Nissle

FIG. 32A

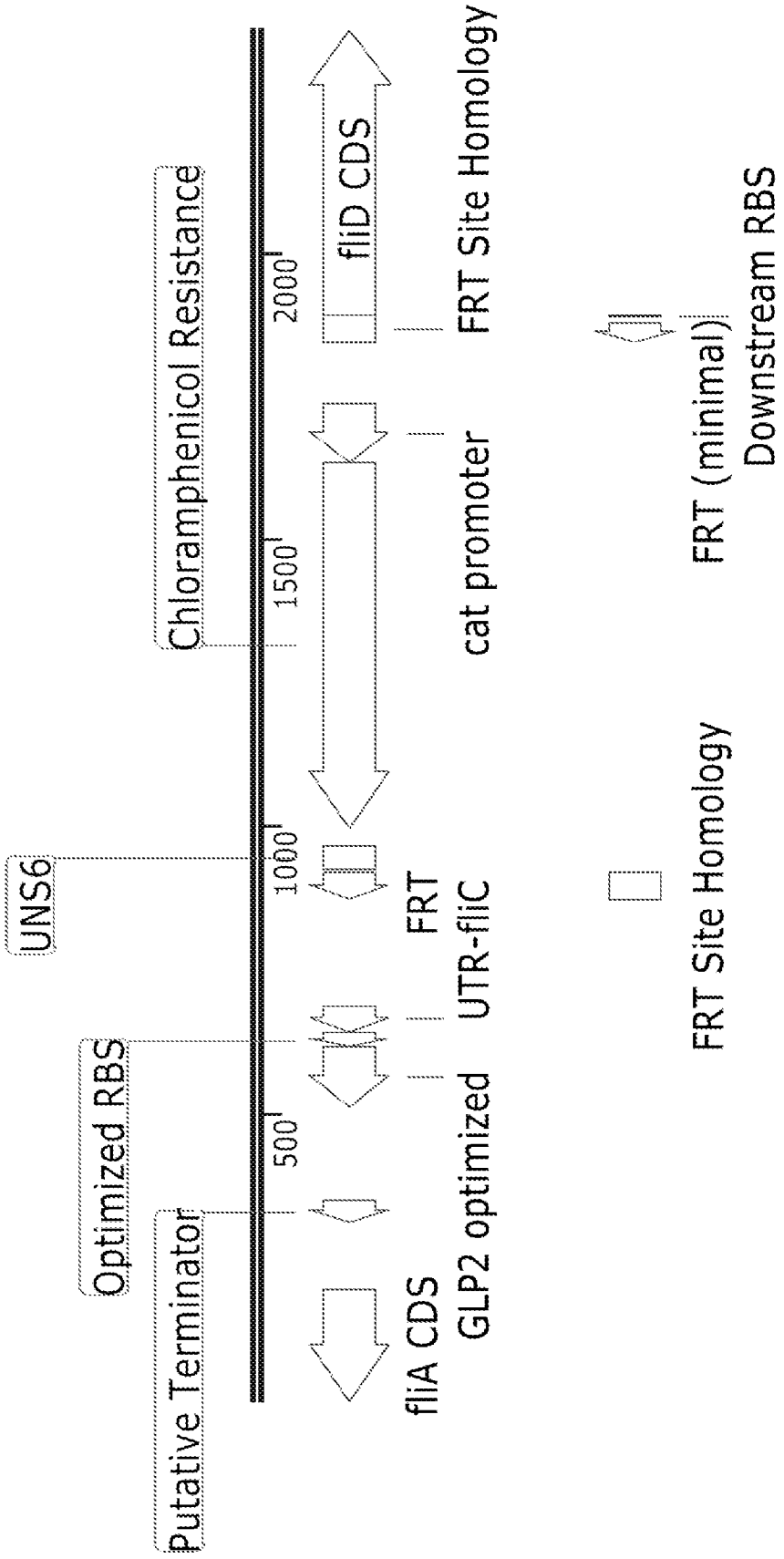

Chloramphenicol Resistance
UNS6
Optimized RBS
Putative Terminator
500
1000
1500
2000
fliA CDS
GLP2 optimized
FRT
UTR-fliC
cat promoter
fliD CDS
FRT Site Homology
FRT Site Homology
FRT (minimal)
Downstream RBS

FIG. 32B

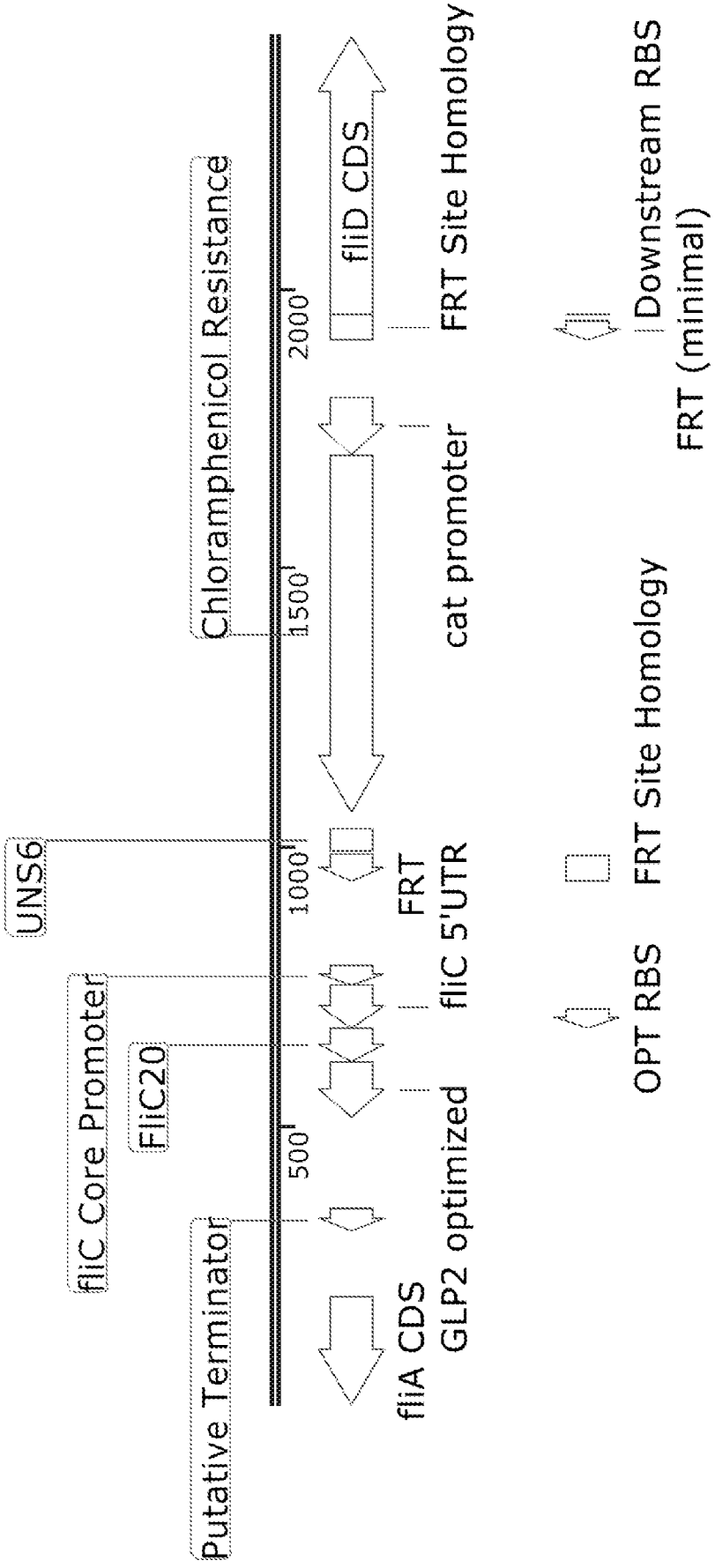

UNS6
fliC Core Promoter
FliC20
Putative Terminator
500
1000
1500
2000
Chloramphenicol Resistance
fliA CDS
GLP2 optimized
FRT
fliC 5'UTR
cat promoter
fliD CDS
FRT Site Homology
OPT RBS
FRT Site Homology
FRT (minimal)
Downstream RBS

FIG. 32C

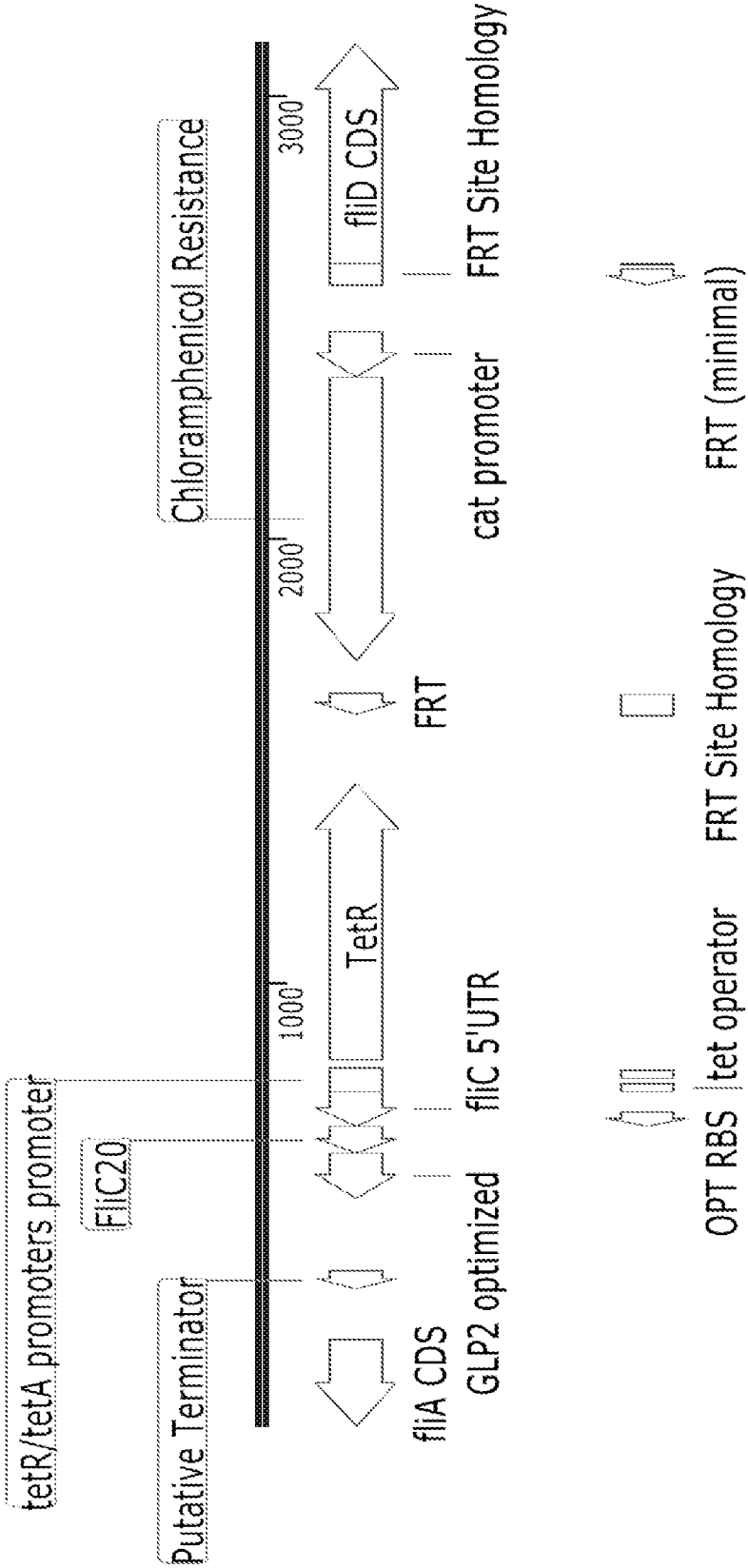

tetR/tetA promoters promoter
FliC20
Putative Terminator
Chloramphenicol Resistance
1000
2000
3000
TetR
fliA CDS
GLP2 optimized
fliC 5'UTR
FRT
cat promoter
FRT Site Homology
fliD CDS
OPT RBS
tet operator
tet operator
FRT Site Homology
FRT (minimal)

FIG. 32D

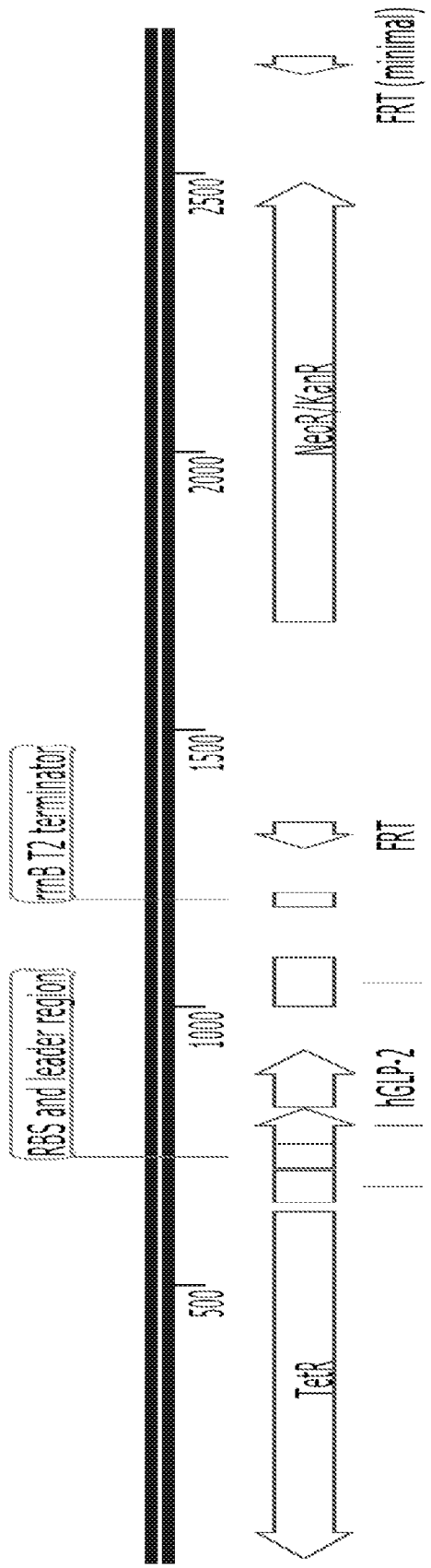

500
1000
1500
2000
2500
TetR
tetR/tetA promoters
promoter
hGLP-2
OmpF Tag
FRT
rrnB T1 terminator
RBS and leader region
rrnB T2 terminator
Neo3/KanR
FRT (minimal)

FIG. 32E

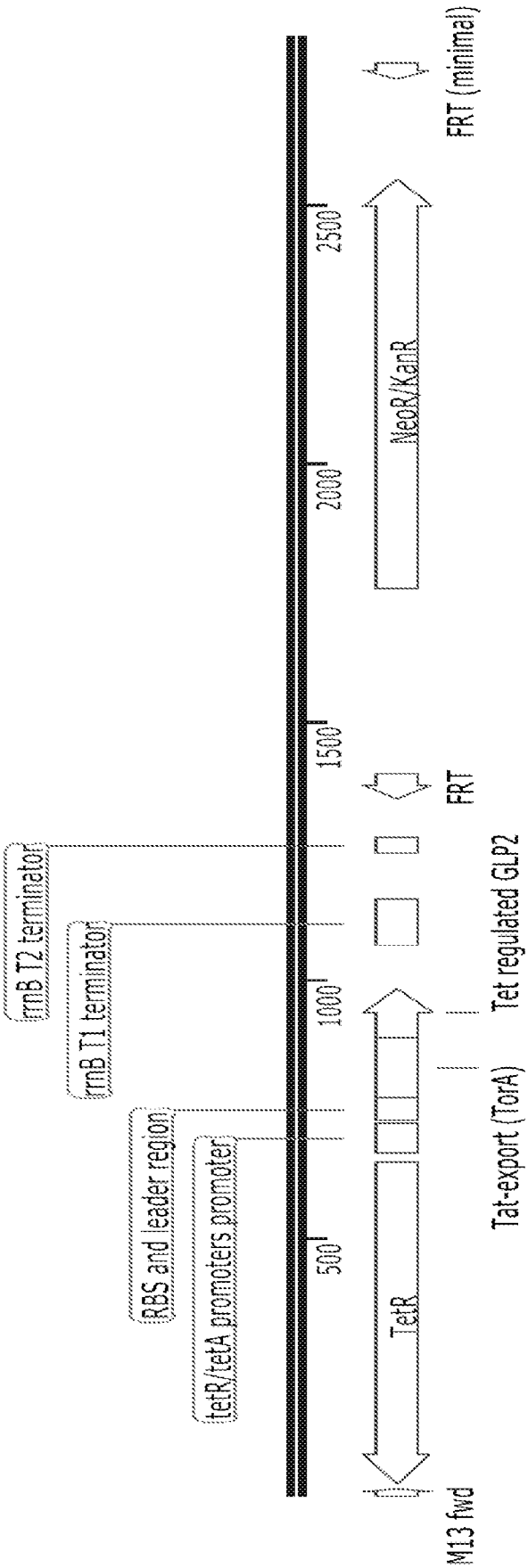

rrnB T2 terminator
rrnB T1 terminator
RBS and leader region
tetR/tetA promoters promoter
500
1000
1500
2000
2500
TetR
Tat-export (TorA)
Tet regulated GLP2
FRT
NeoR/KanR
FRT (minimal)
M13 fwd

FIG. 33A

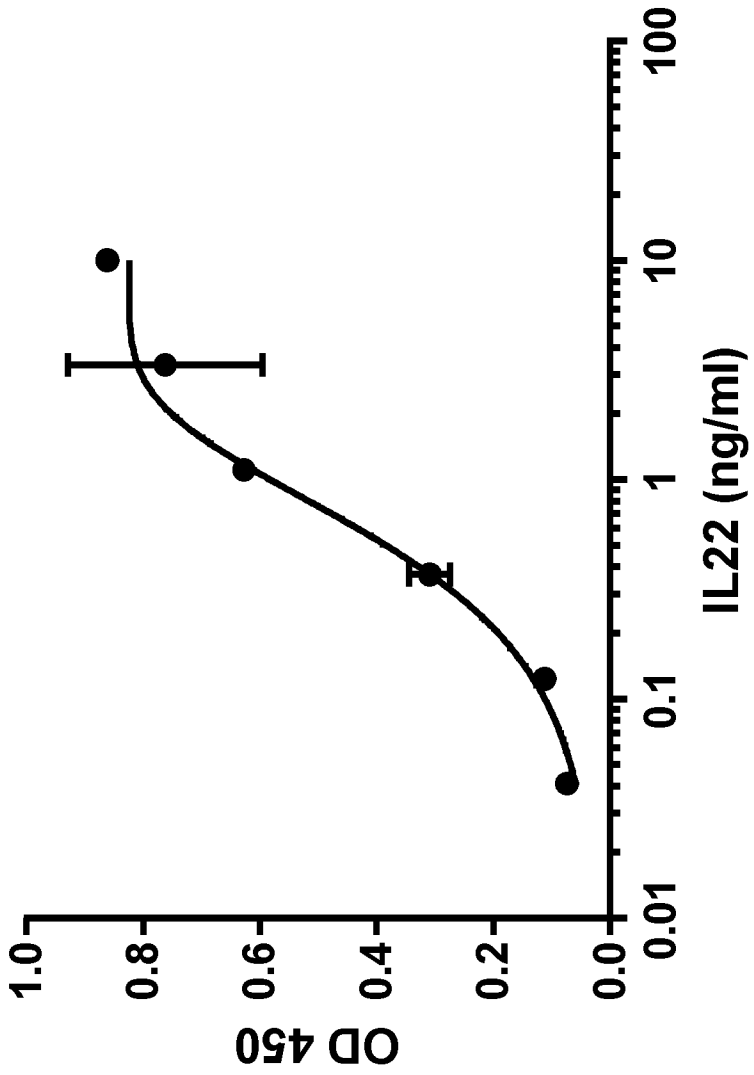

pSTAT3 Tyr705 ELISA
OD 450
1.0
0.8
0.6
0.4
0.2
0.0
0.01
0.1
1
10
100
IL22 (ng/ml)

FIG. 33B

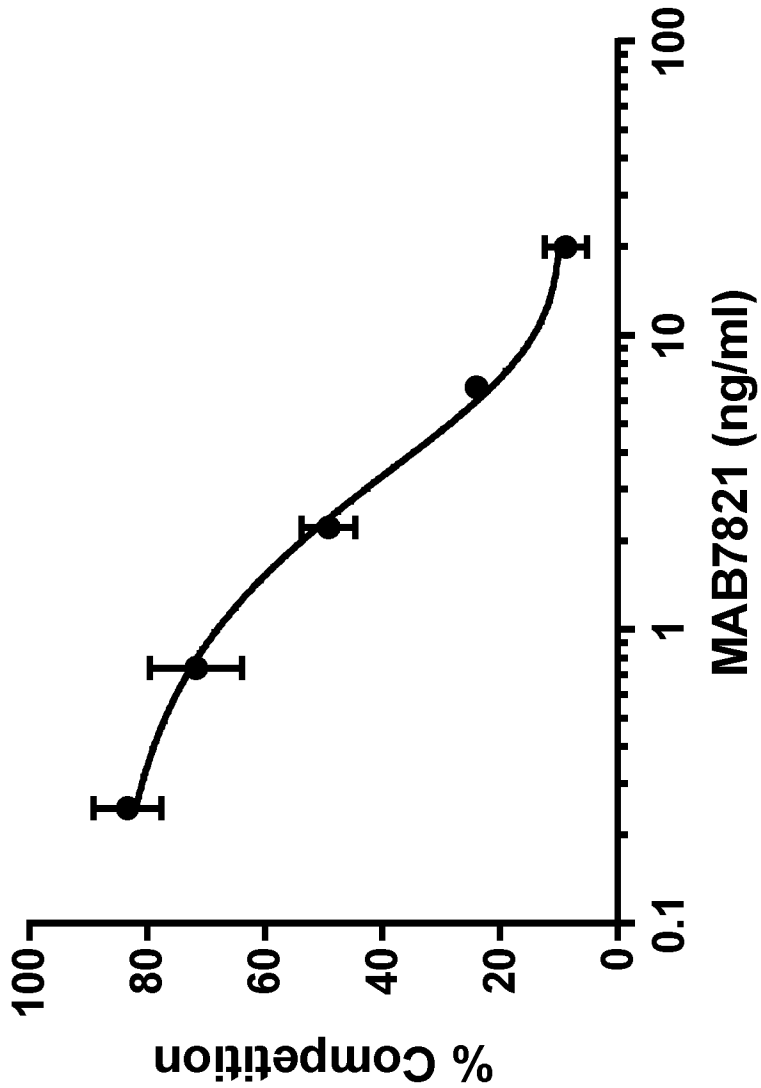

pSTAT3 Tyr705 ELISA
% Competition
100
80
60
40
20
0
0.1
1
10
100
MAB7821 (ng/ml)

FIG. 33C

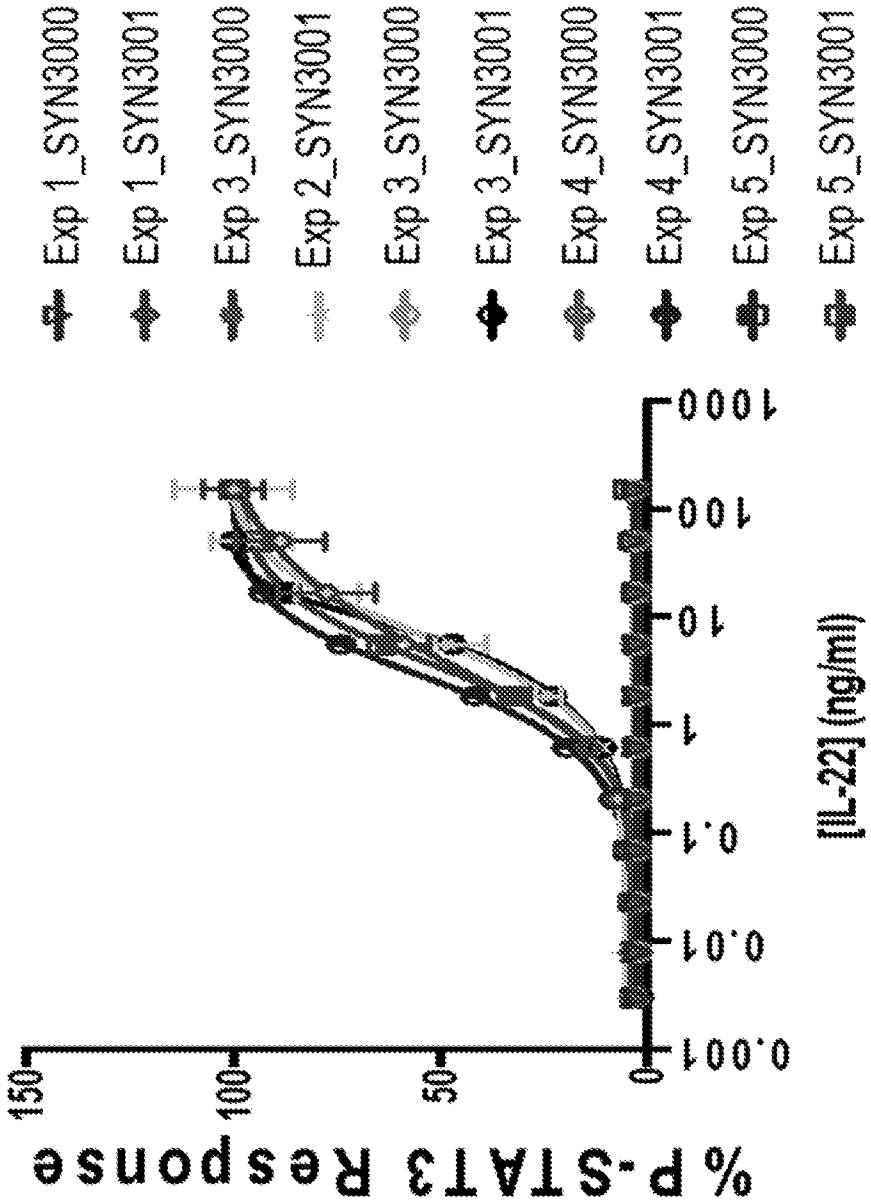

Exp 1_SYN3000
Exp 1_SYN3001
Exp 3_SYN3000
Exp 2_SYN3001
Exp 3_SYN3000
Exp 3_SYN3001
Exp 4_SYN3000
Exp 4_SYN3001
Exp 5_SYN3000
Exp 5_SYN3001
% P-STAT3 Response
150
100
50
0
0.001
0.01
0.1
1
10
100
1000
[IL-22] (ng/ml)

FIG. 33D

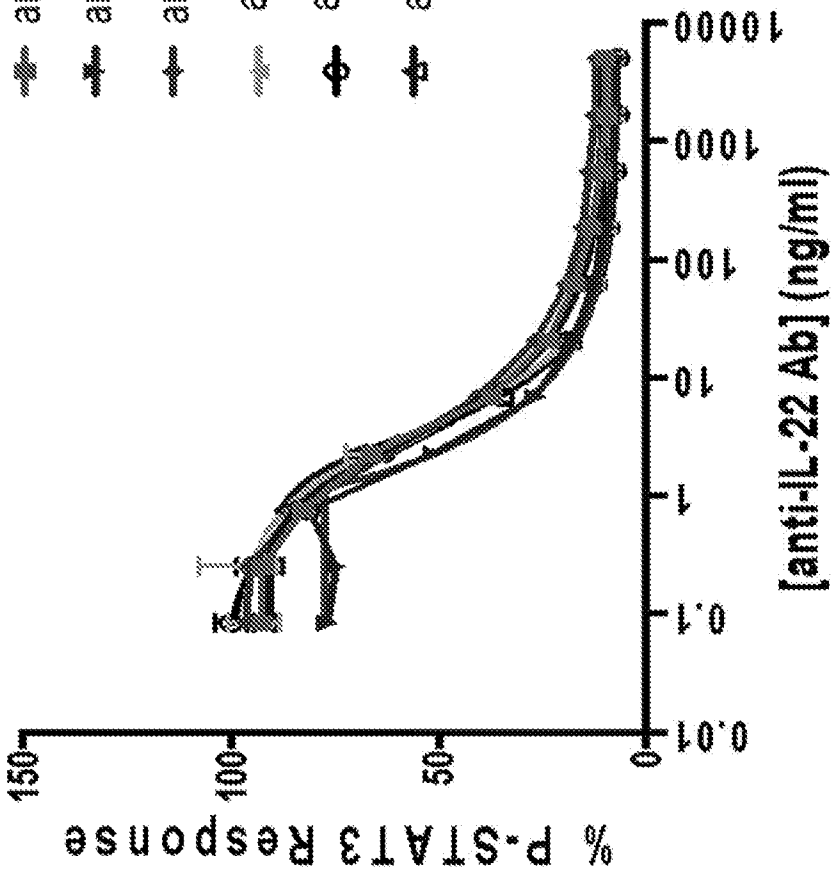

anti-IL22 Ab/rIL-22_Expt 1
anti-IL22 Ab/rIL-22_Expt 2
anti-IL22 Ab/rIL-22_Expt 3
anti-IL-22 Ab/SYN3001_Expt 1
anti-IL-22 Ab/SYN3001_Expt 2
anti-IL-22 Ab/SYN3001_Expt 3
% P-STAT3 Response
150
100
50
0
0.01
0.1
1
10
100
1000
10000
[anti-IL-22 Ab] (ng/ml)

FIG. 33E

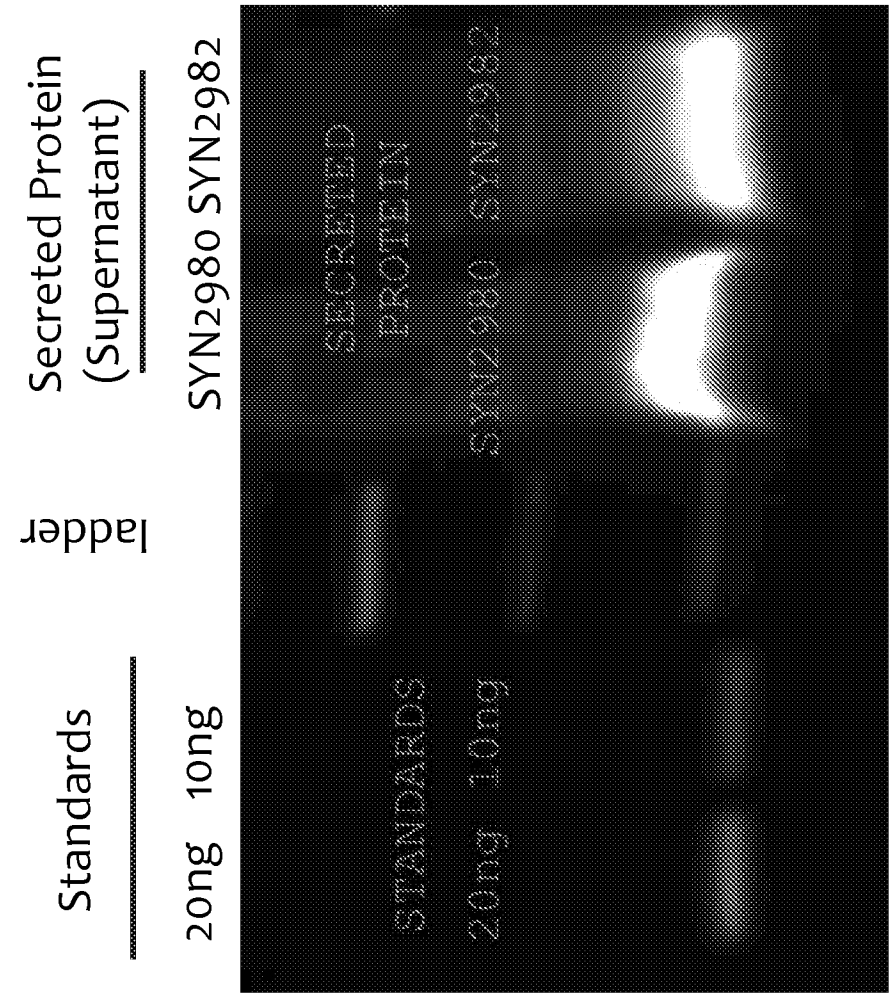

Standards
20ng 10ng
ladder
Secreted Protein
(Supernatant)
SYN2980 SYN2982
STANDARDS
20ng 10ng
SECRETED
PROTEIN
SYN2980 SYN2982

FIG. 34

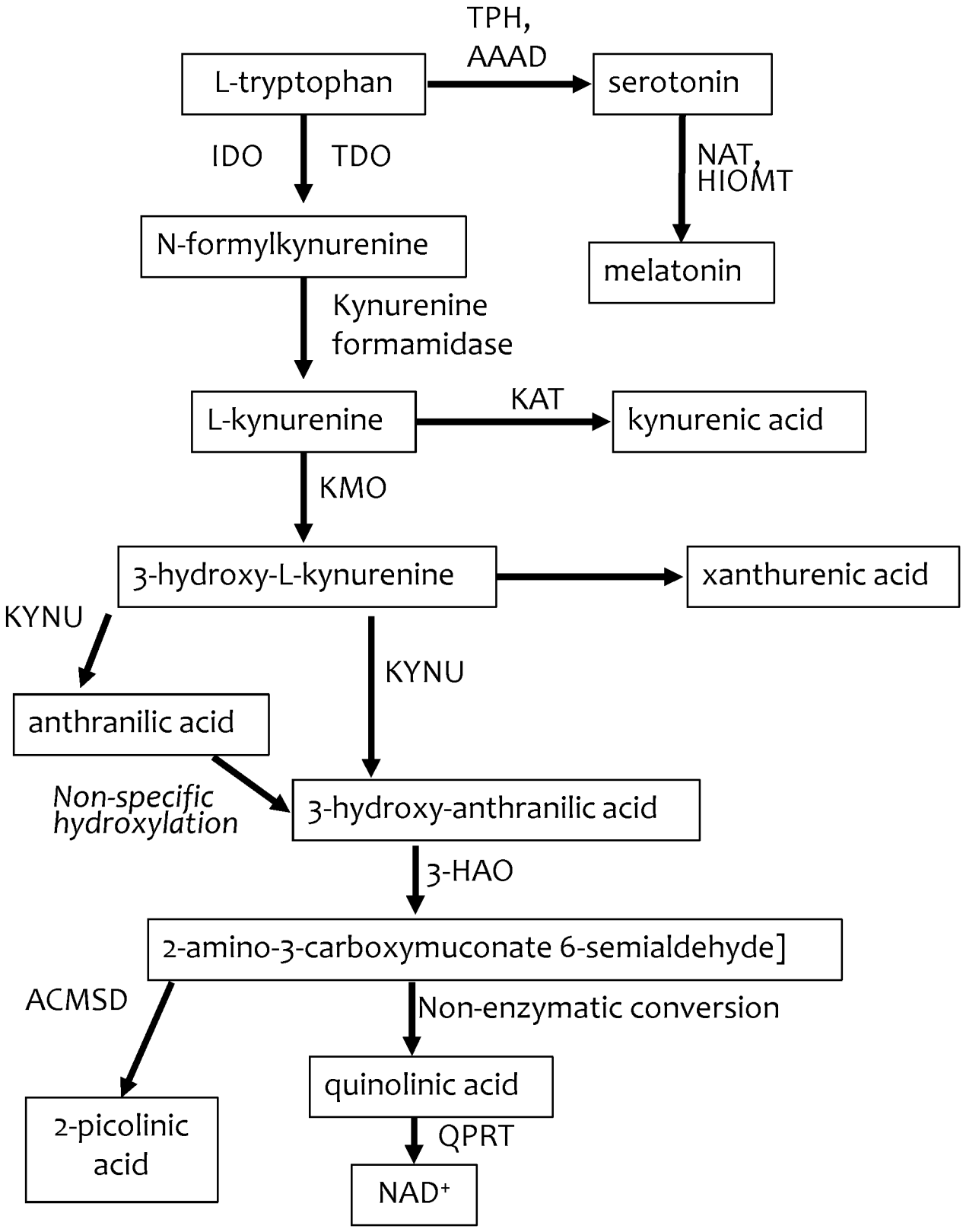

TPH,
AAAD
L-tryptophan
serotonin
IDO
TDO
NAT,
HIOMT
N-formylkynurenine
melatonin
Kynurenine
formamidase
L-kynurenine
KAT
kynurenic acid
KMO
3-hydroxy-L-kynurenine
xanthurenic acid
KYNU
KYNU
anthranilic acid
Non-specific
hydroxylation
3-hydroxy-anthranilic acid
3-HAO
2-amino-3-carboxymuconate 6-semialdehyde]
ACMSD
Non-enzymatic conversion
quinolinic acid
2-picolinic
acid
QPRT
NAD+

FIG. 35

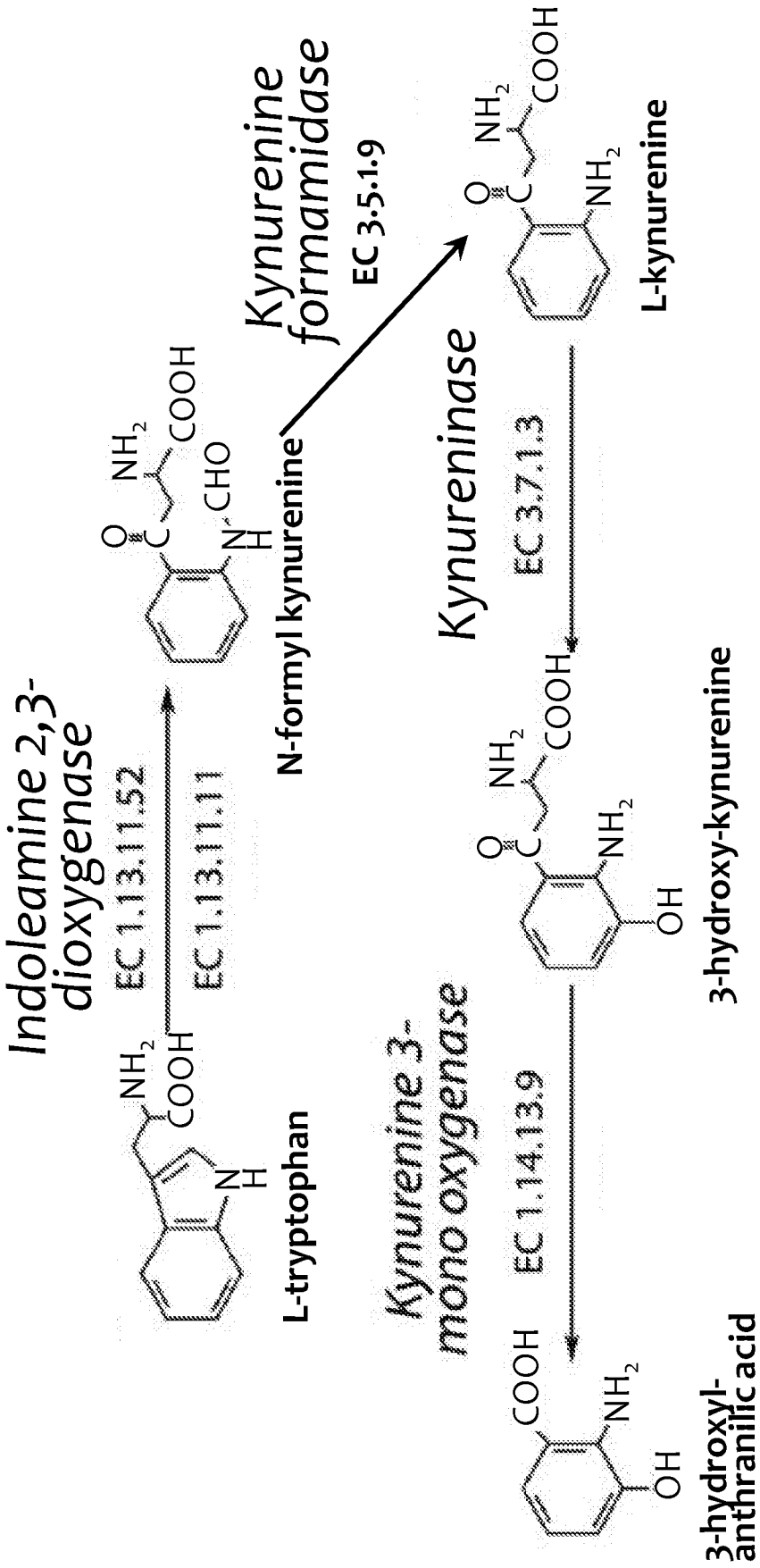
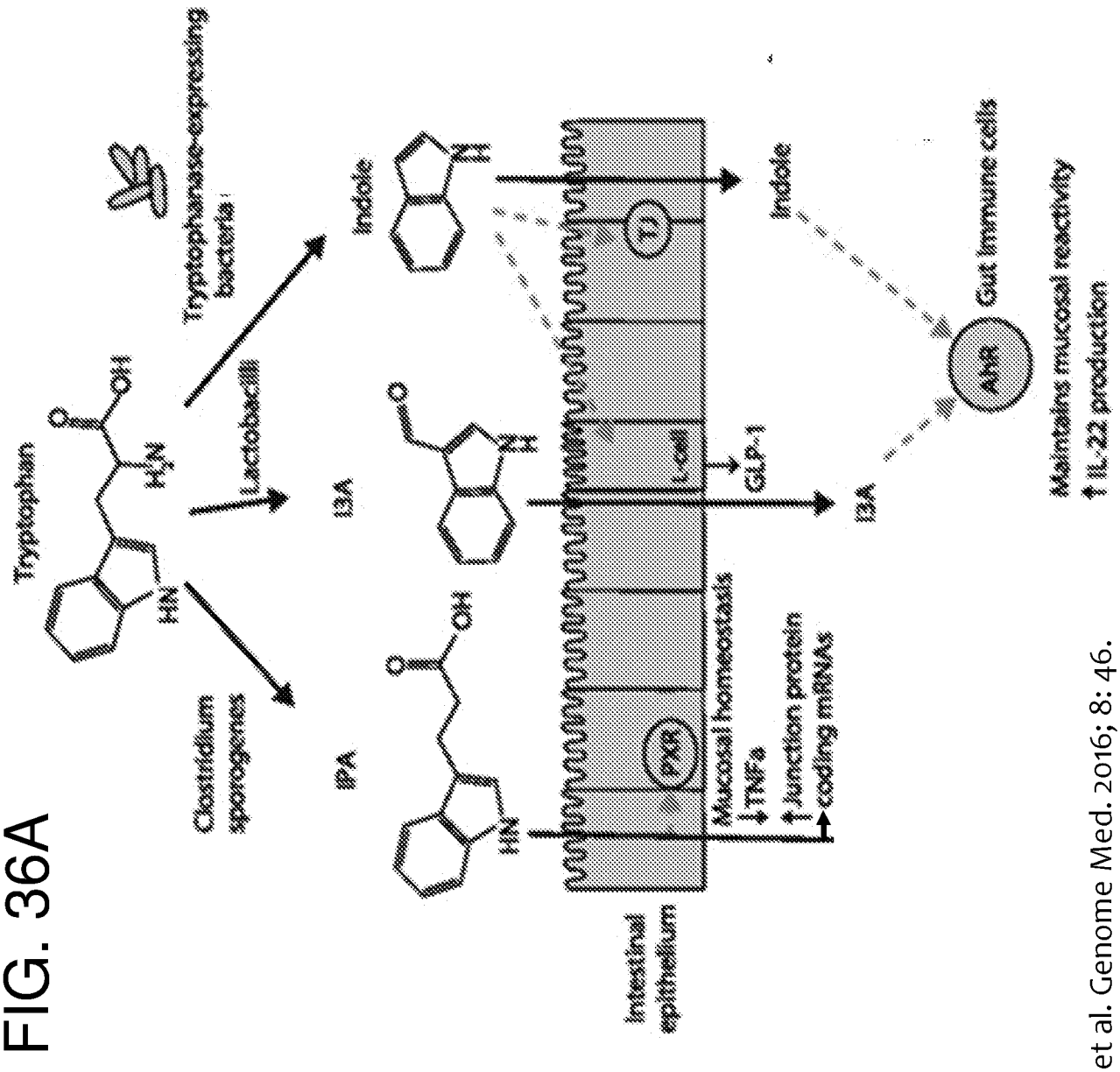

Indoleamine 2,3-dioxygenase
EC 1.13.11.52
EC 1.13.11.11
L-tryptophan
N-formyl kynurenine
Kynurenine formamidase
EC 3.5.1.9
Kynureninase
EC 3.7.1.3
L-kynurenine
Kynurenine 3-mono oxygenase
EC 1.14.13.9
3-hydroxy-kynurenine
3-hydroxyl-anthranilic acid

FIG. 36A



Zhang et al. Genome Med. 2016; 8: 46.

FIG. 36B

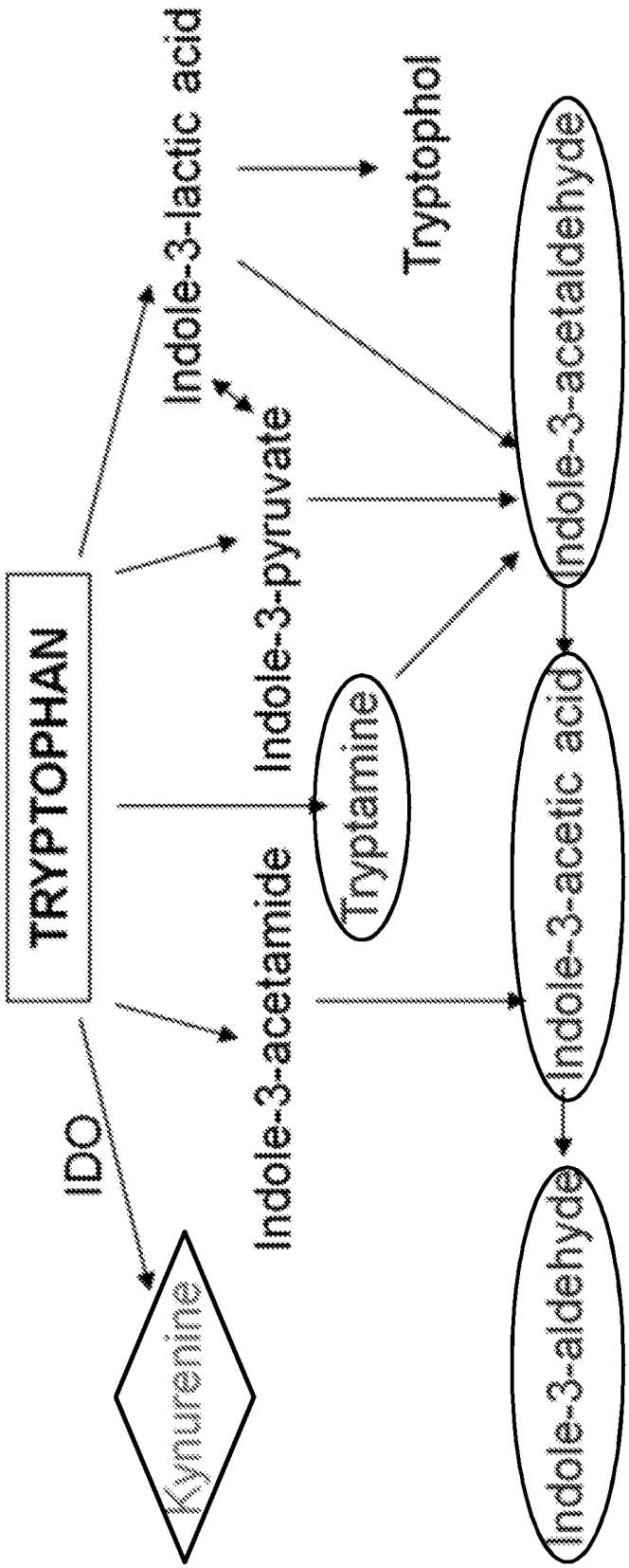

TRYPTOPHAN
IDO
Kynurenine
Indole-3-lactic acid
Indole-3-pyruvate
Indole-3-acetamide
Tryptophol
Tryptamine
Indole-3-acetaldehyde
Indole-3-acetic acid
Indole-3-aldehyde

FIG. 37A

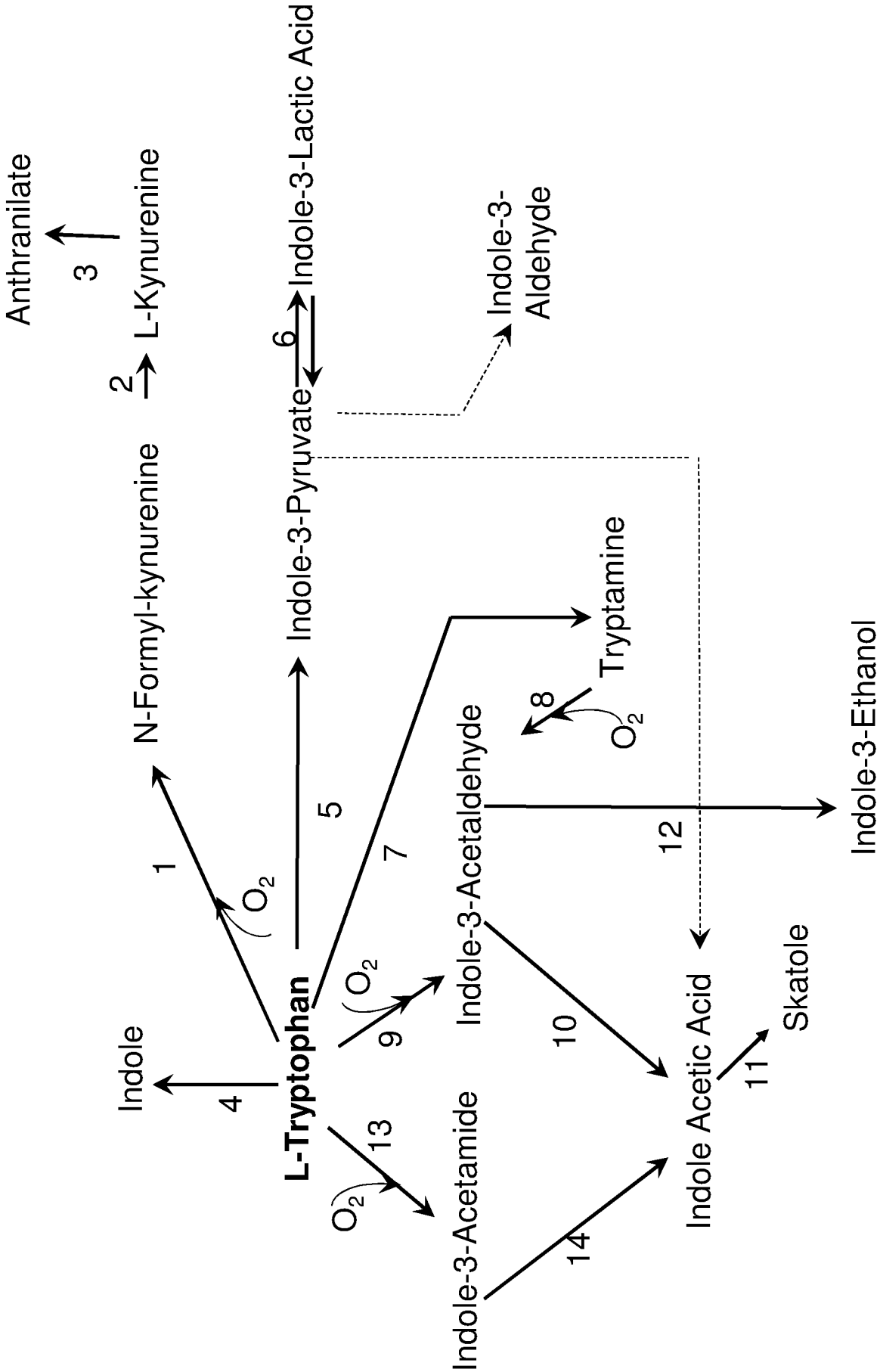

Anthranilate
3
L-Kynurenine
2
Indole-3-Lactic Acid
6
Indole-3-Aldehyde
N-Formyl-kynurenine
Indole-3-Pyruvate
1
O_2
5
7
Tryptamine
8
O_2
Indole
4
L-Tryptophan
O_2
9
Indole-3-Acetaldehyde
12
Indole-3-Ethanol
10
13
O_2
Indole-3-Acetamide
Indole Acetic Acid
11
Skatole
14

FIG. 37B

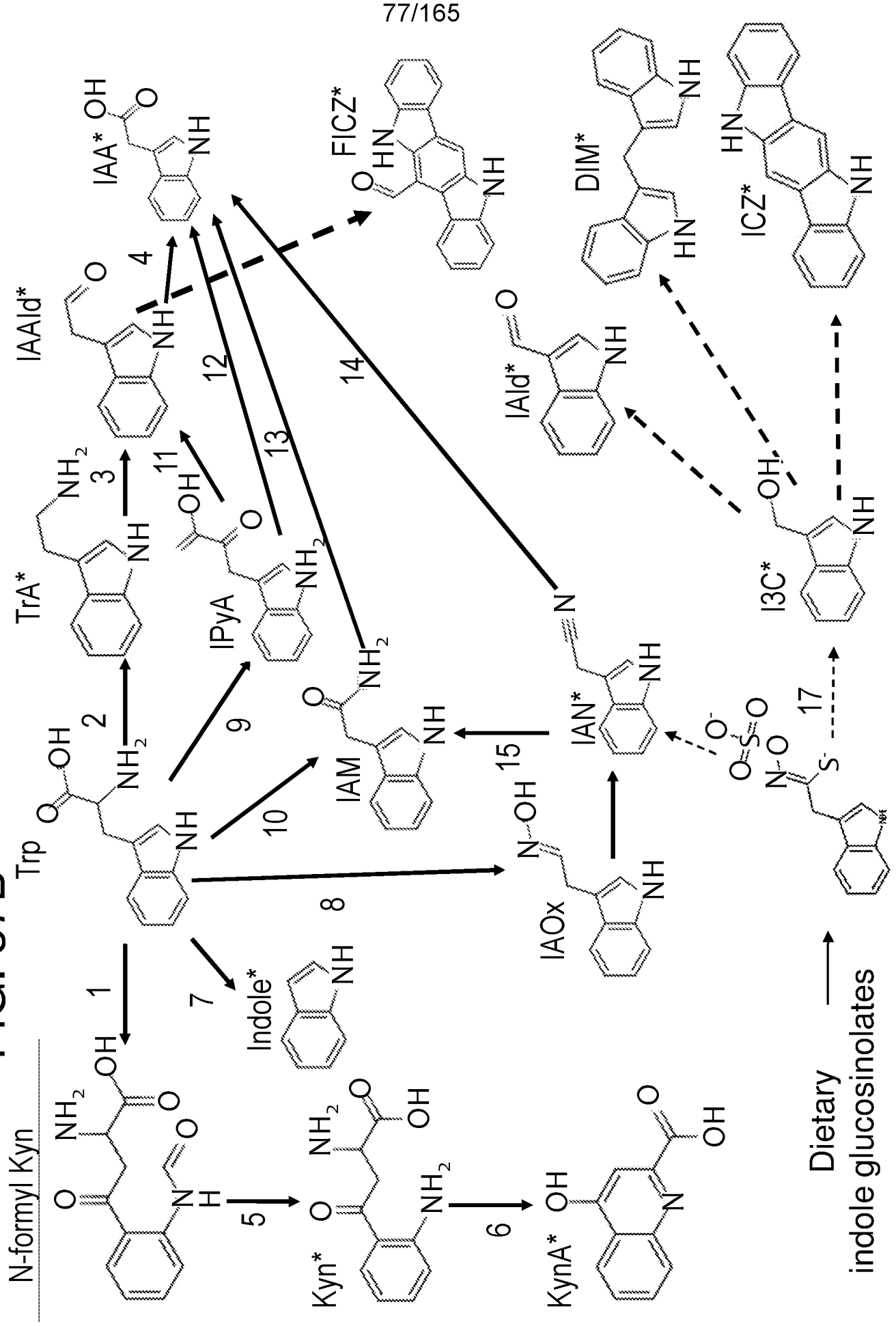

N-formyl Kyn
Trp
TrA*
IPyA
IAAld*
IAA*
FICZ*
DIM*
ICZ*
IAld*
I3C*
IAM
IAN*
IAOx
Indole*
Kyn*
KynA*
Dietary
indole glucosinolates
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
17

FIG. 38

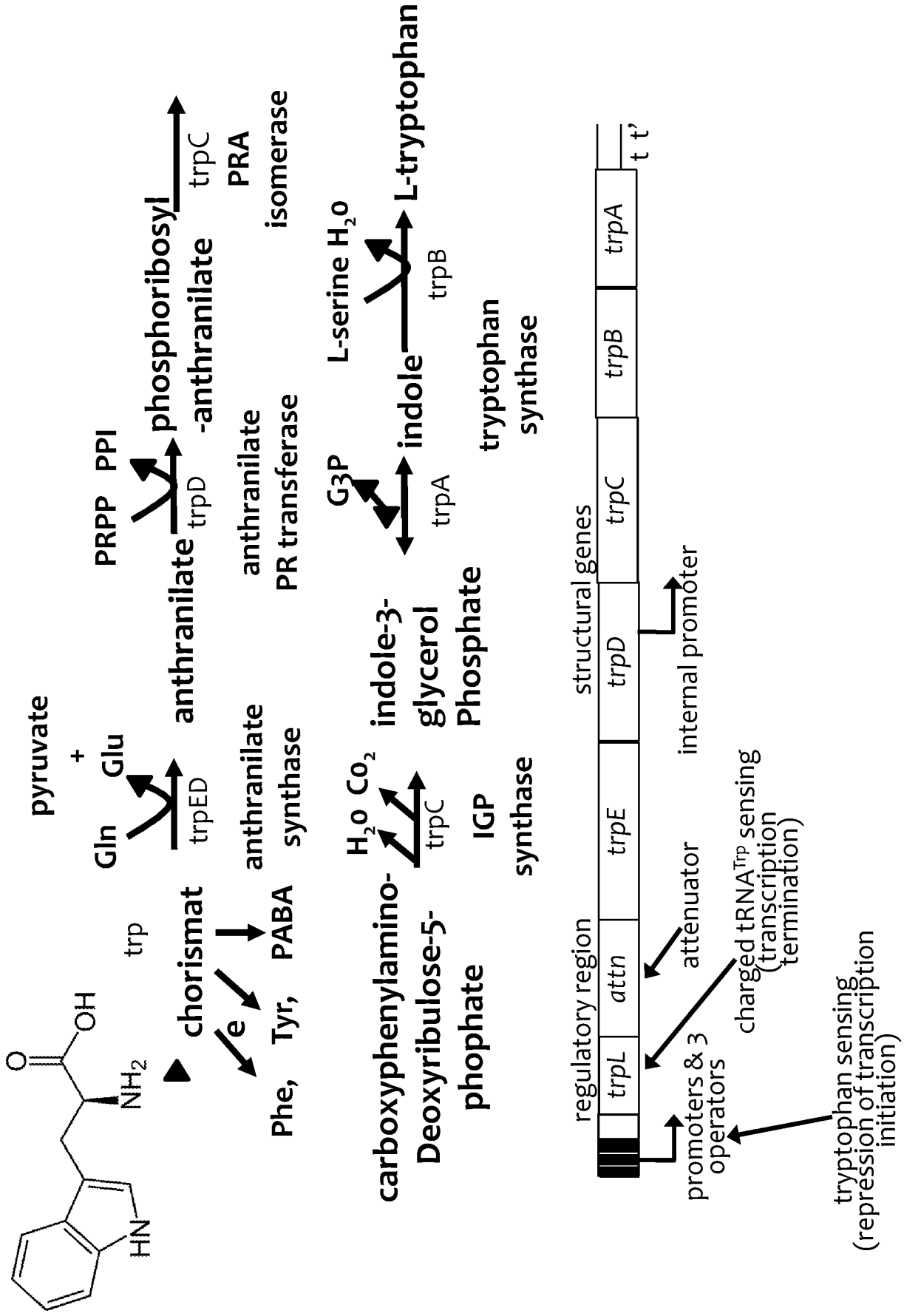

pyruvate
+
Glu
Gln
trpED
anthranilate
synthase
trp
chorismat
e
Phe, Tyr, PABA
PRPP PPI
trpD
anthranilate
PR transferase
phosphoribosyl
-anthranilate
trpC
PRA
isomerase
G3P
L-serine H2o
indole
trpA
trpB
L-tryptophan
tryptophan
synthase
H2o Co2
trpC
indole-3-
glycerol
Phosphate
IGP
synthase
carboxyphenylamino-
Deoxyribulose-5-
phophate
regulatory region
structural genes
trpL
attn
trpE
trpD
trpC
trpB
trpA
t t'
attenuator
internal promoter
charged tRNATrp sensing
(transcription
termination)
promoters & 3
operators
tryptophan sensing
(repression of transcription
initiation)

FIG. 39

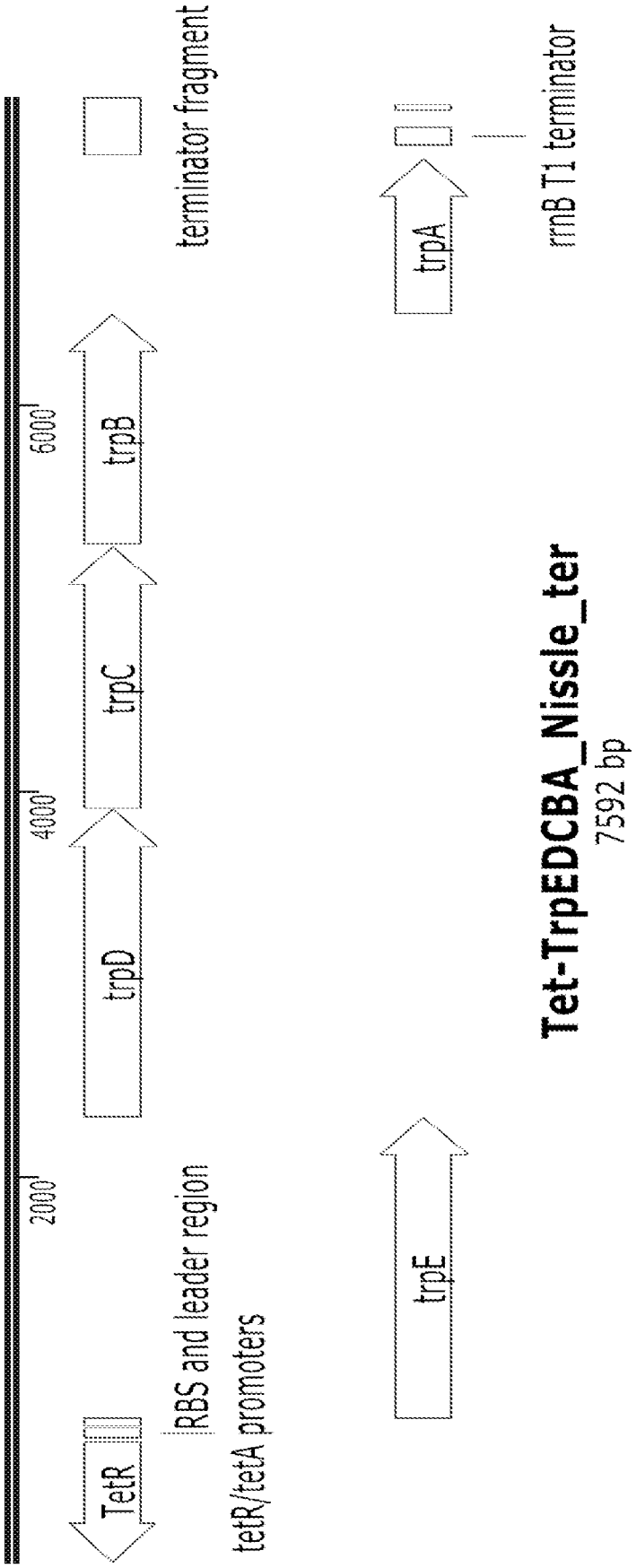

Tet-TrpEDCBA_Nissle_ter
7592 bp
2000
4000
6000
TetR
RBS and leader region
tetR/tetA promoters
trpD
trpC
trpB
terminator fragment
trpE
trpA
rrnB T1 terminator

FIG. 40A

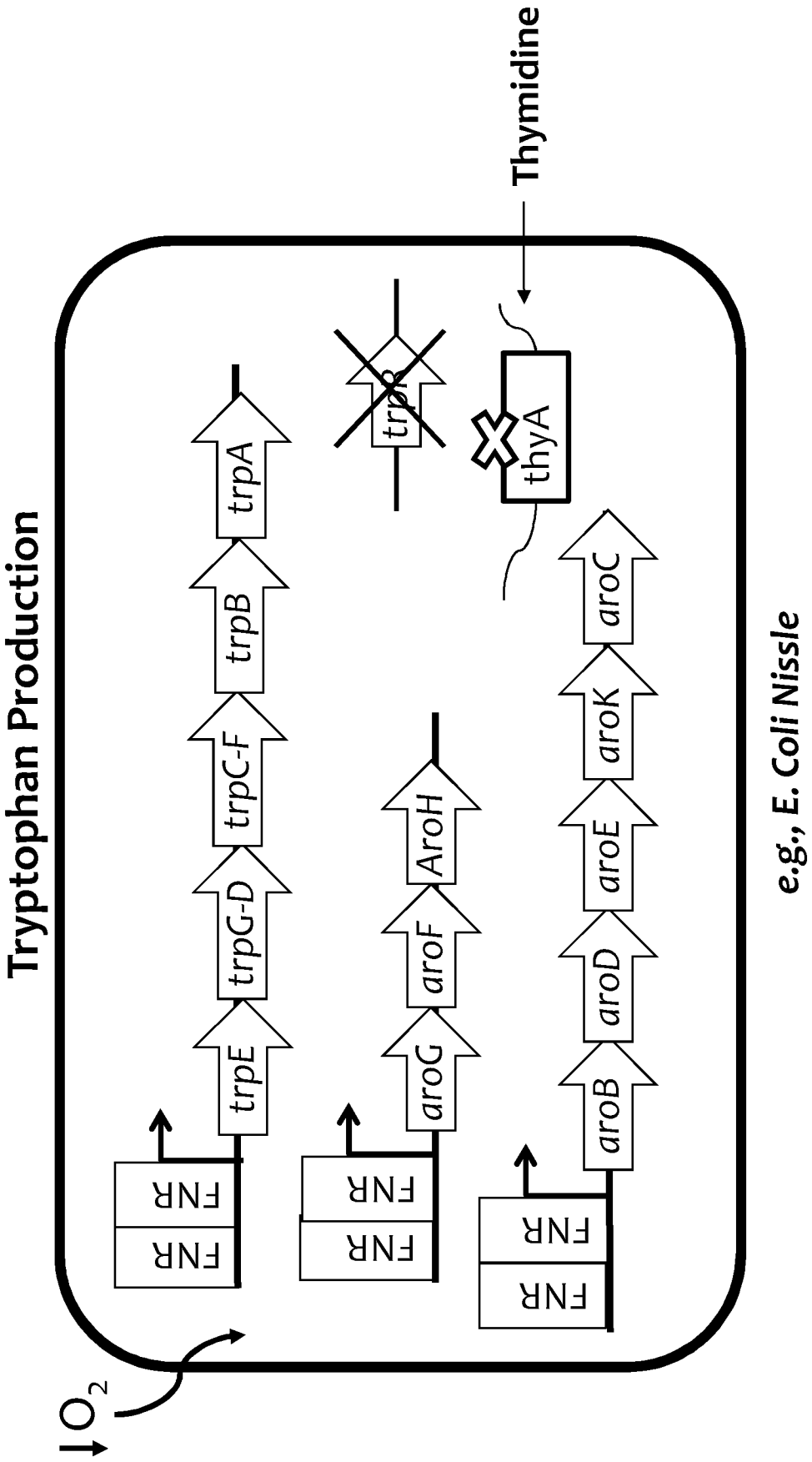

Thymidine
Tryptophan Production
trpA
trpB
trpC-F
trpG-D
trpE
trpR
thyA
AroH
aroF
aroG
aroC
aroK
aroE
aroD
aroB
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 40B

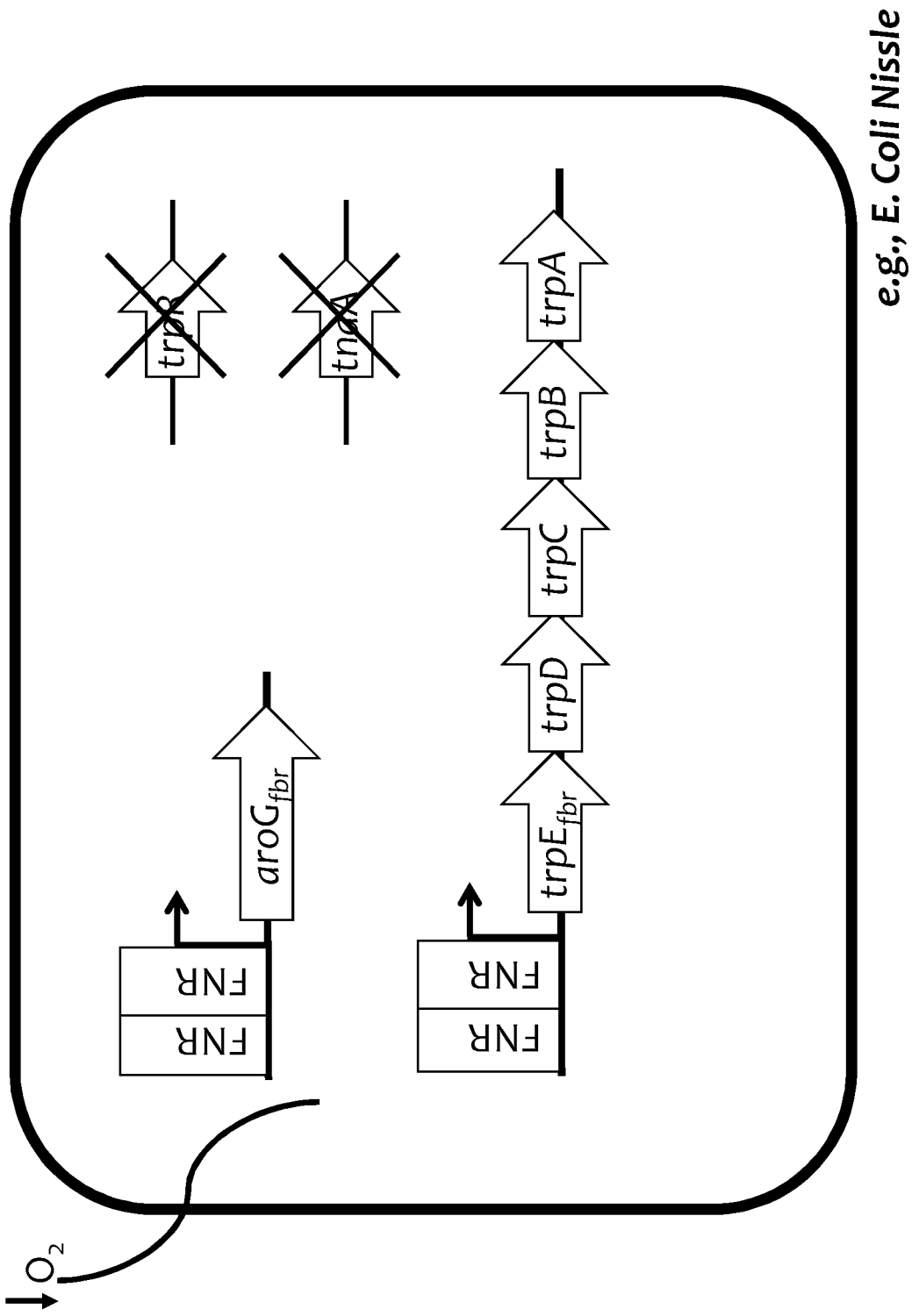
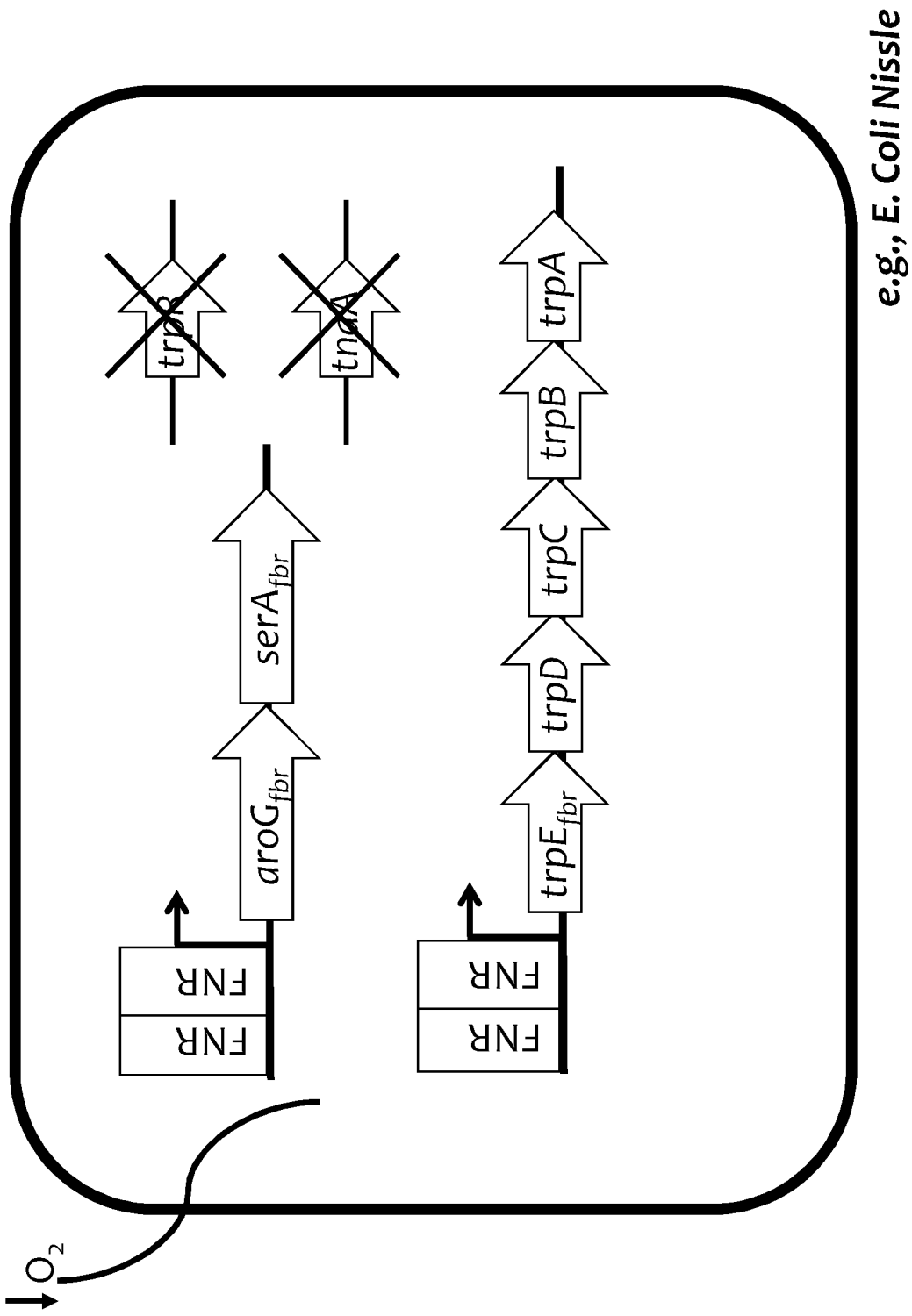
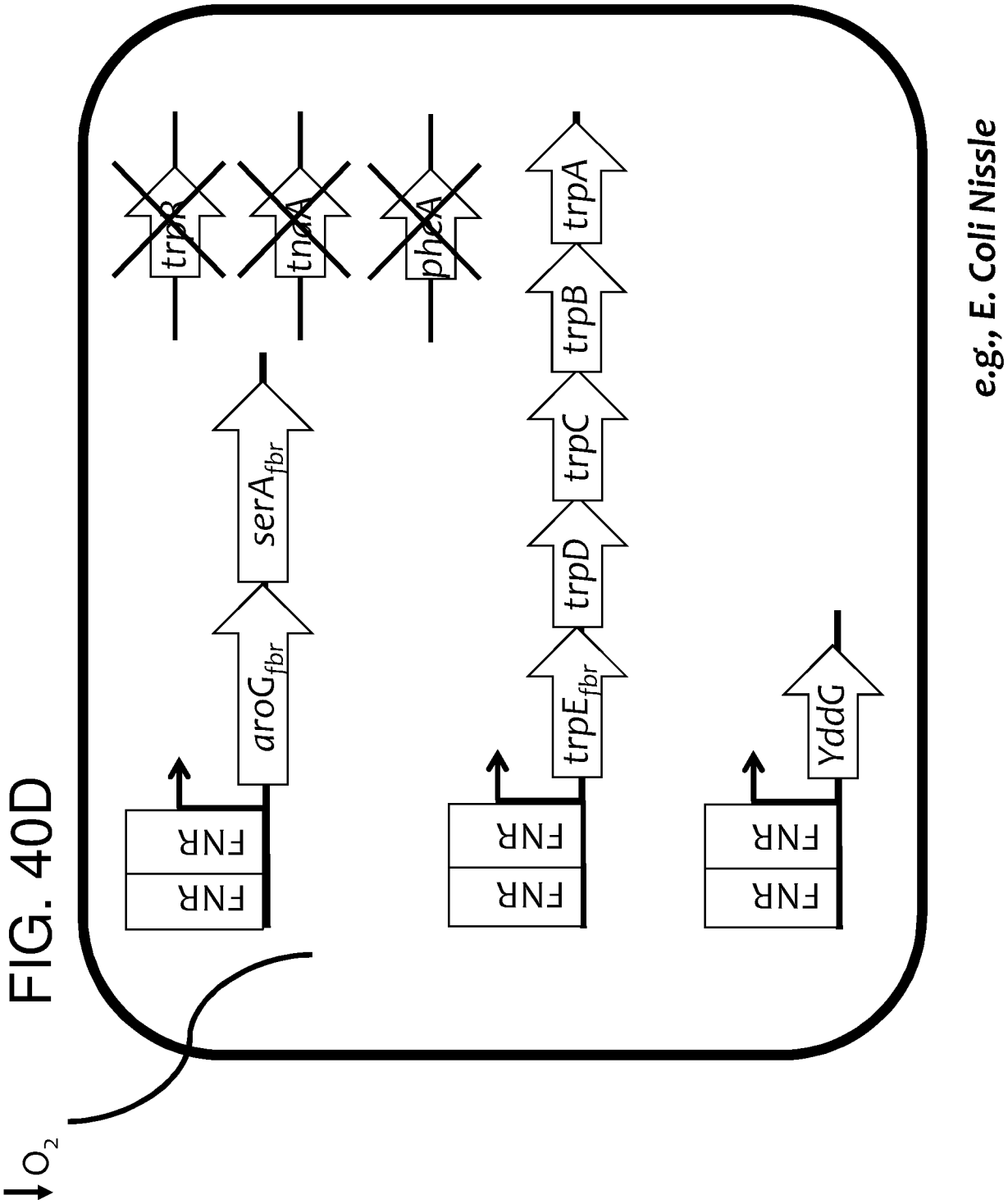

e.g., E. Coli Nissle
trpR
tnaA
trpA
trpB
trpC
trpD
trpE$_{fbr}$
aroG$_{fbr}$
FNR
FNR
FNR
FNR
O$_2$

FIG. 40C


e.g., E. Coli Nissle
trpE
tnaA
trpA
trpB
trpC
trpD
trpE$_{fbr}$
FNR
FNR
aroG$_{fbr}$
serA$_{fbr}$
FNR
FNR
O$_2$


FIG. 40D
O_2
FNR
FNR
$aroG_{fbr}$
$serA_{fbr}$
FNR
FNR
$trpE_{fbr}$
trpD
trpC
trpB
trpA
FNR
FNR
YddG
trpR
tnaA
pheA
e.g., E. Coli Nissle

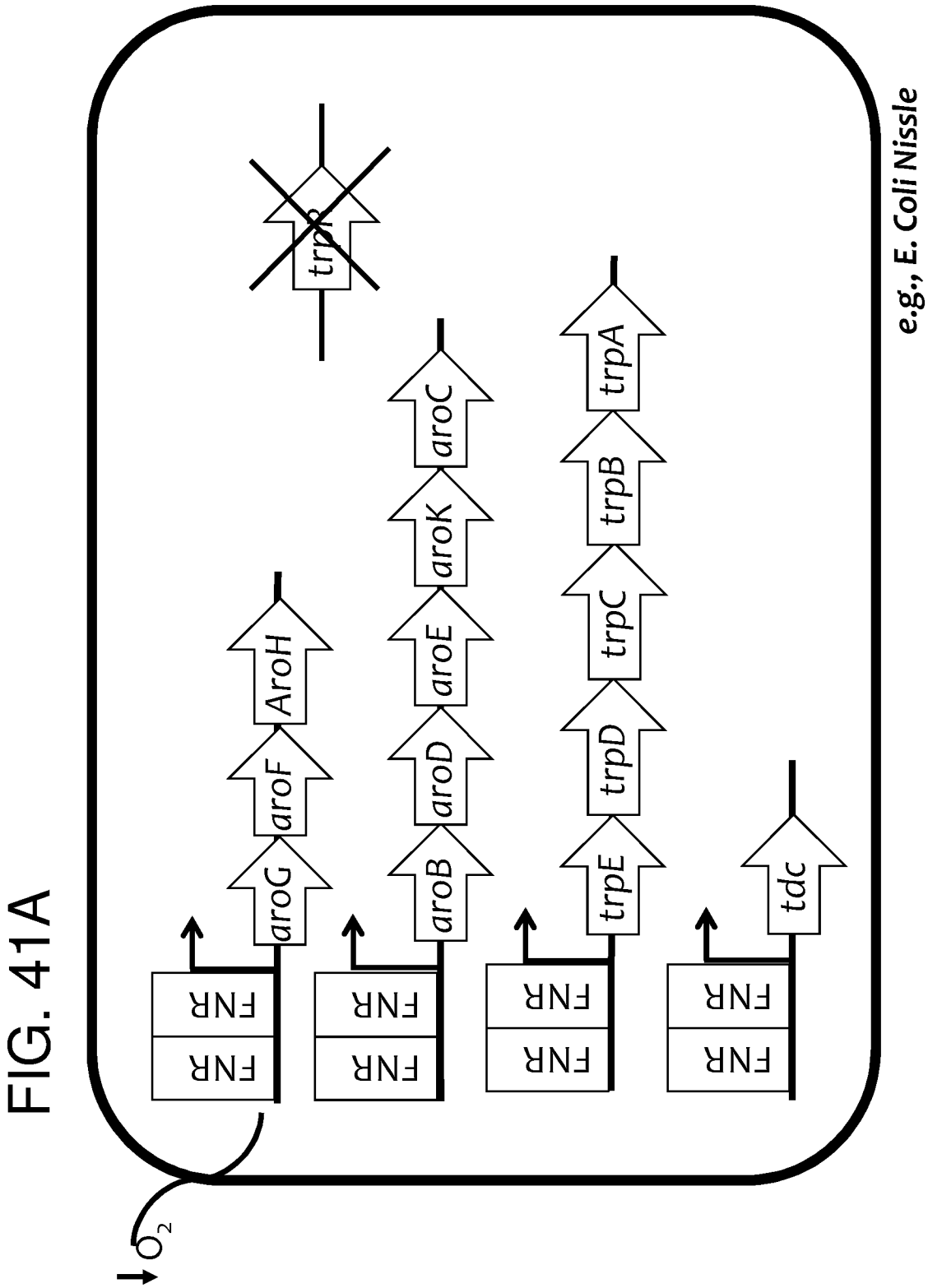

FIG. 41A
e.g., E. Coli Nissle
O_2
FNR
FNR
aroG
aroF
AroH
FNR
FNR
aroB
aroD
aroE
aroK
aroC
trpR
FNR
FNR
trpE
trpD
trpC
trpB
trpA
FNR
FNR
tdc

FIG. 41B

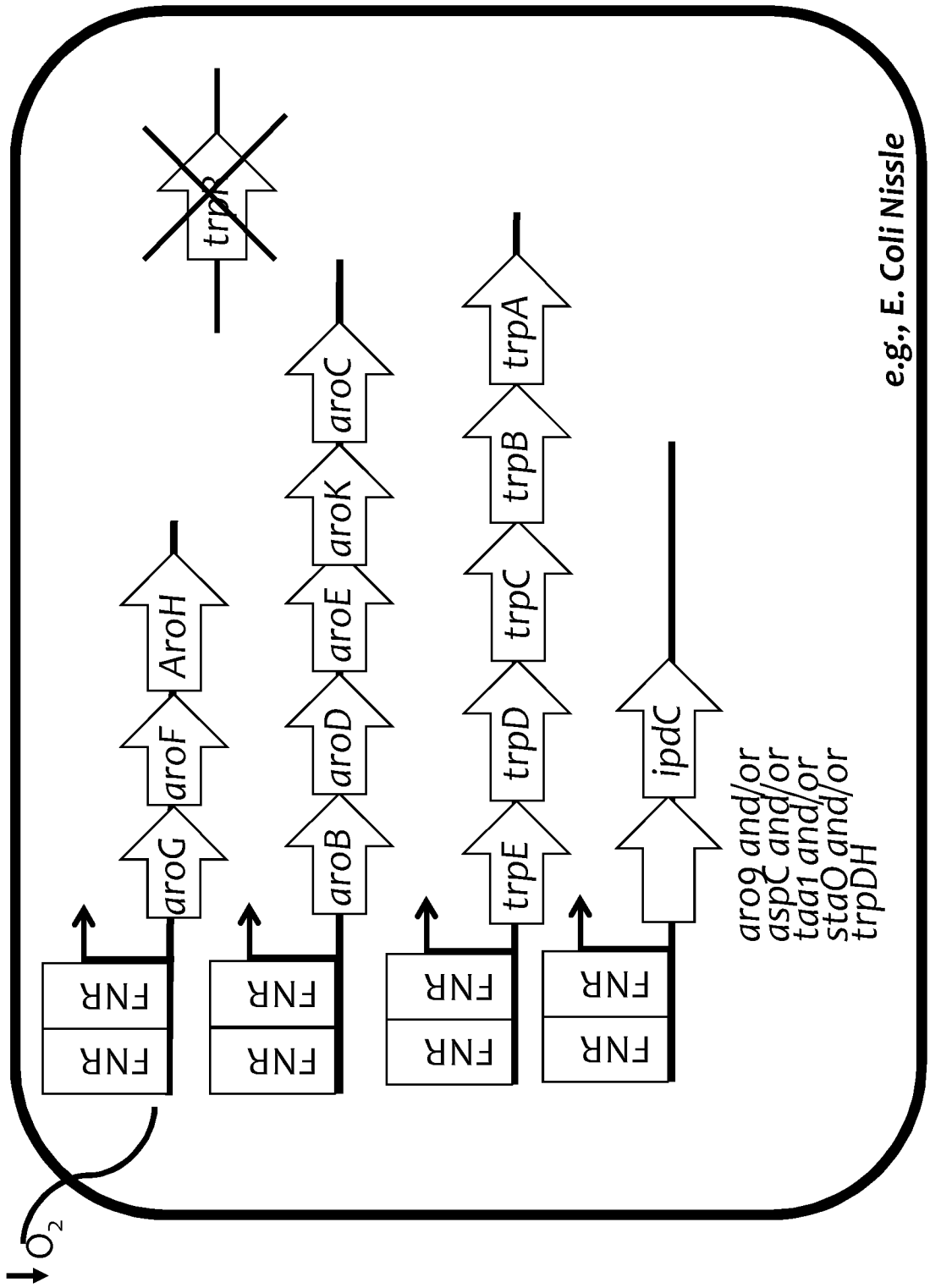

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
ipdC
aro9 and/or
aspC and/or
taa1 and/or
staO and/or
trpDH
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2

FIG. 41C

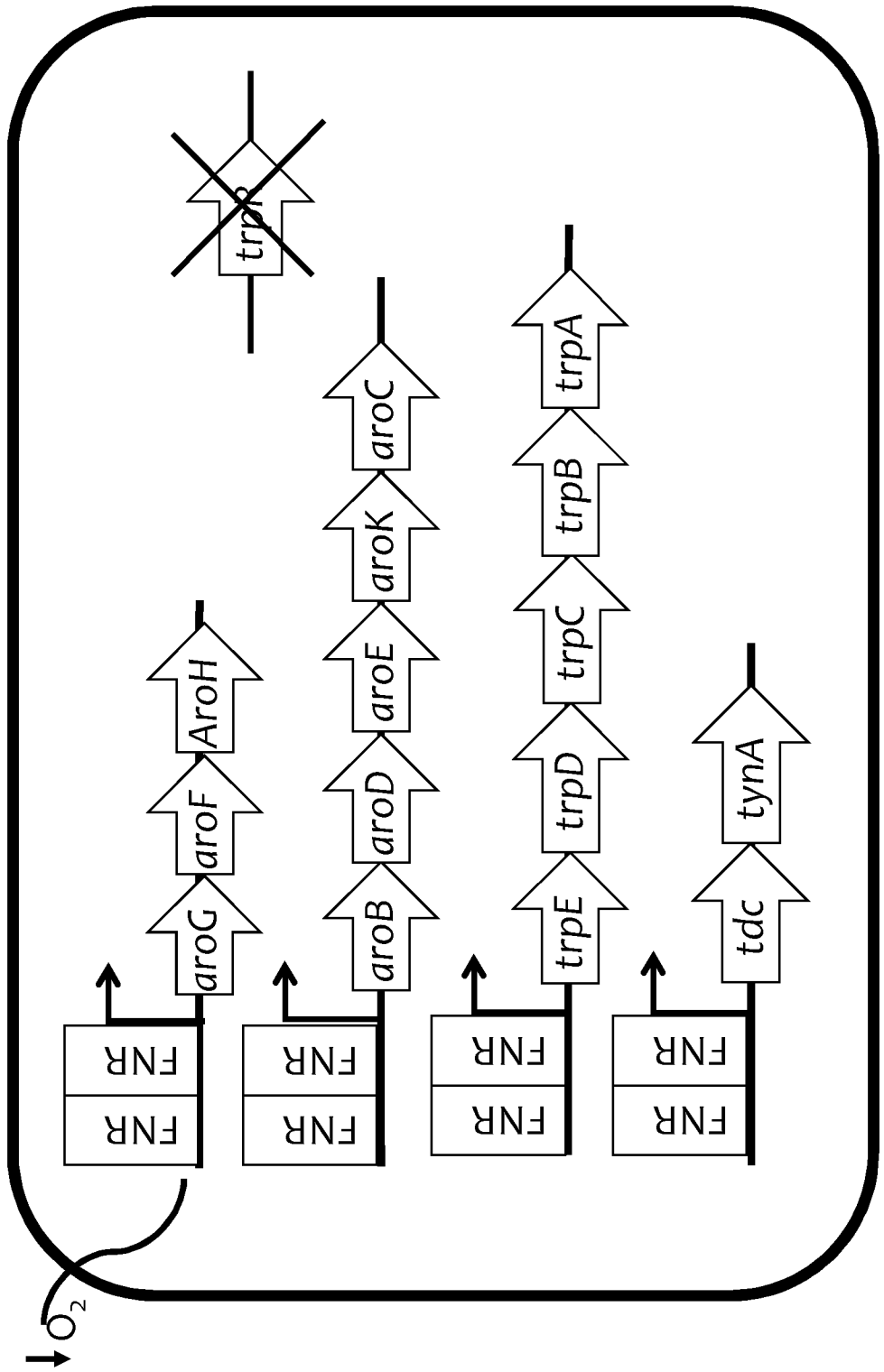

trpR
aroC
aroK
aroE
aroD
aroB
trpA
trpB
trpC
trpD
trpE
tynA
tdc
AroH
aroF
aroG
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 41D

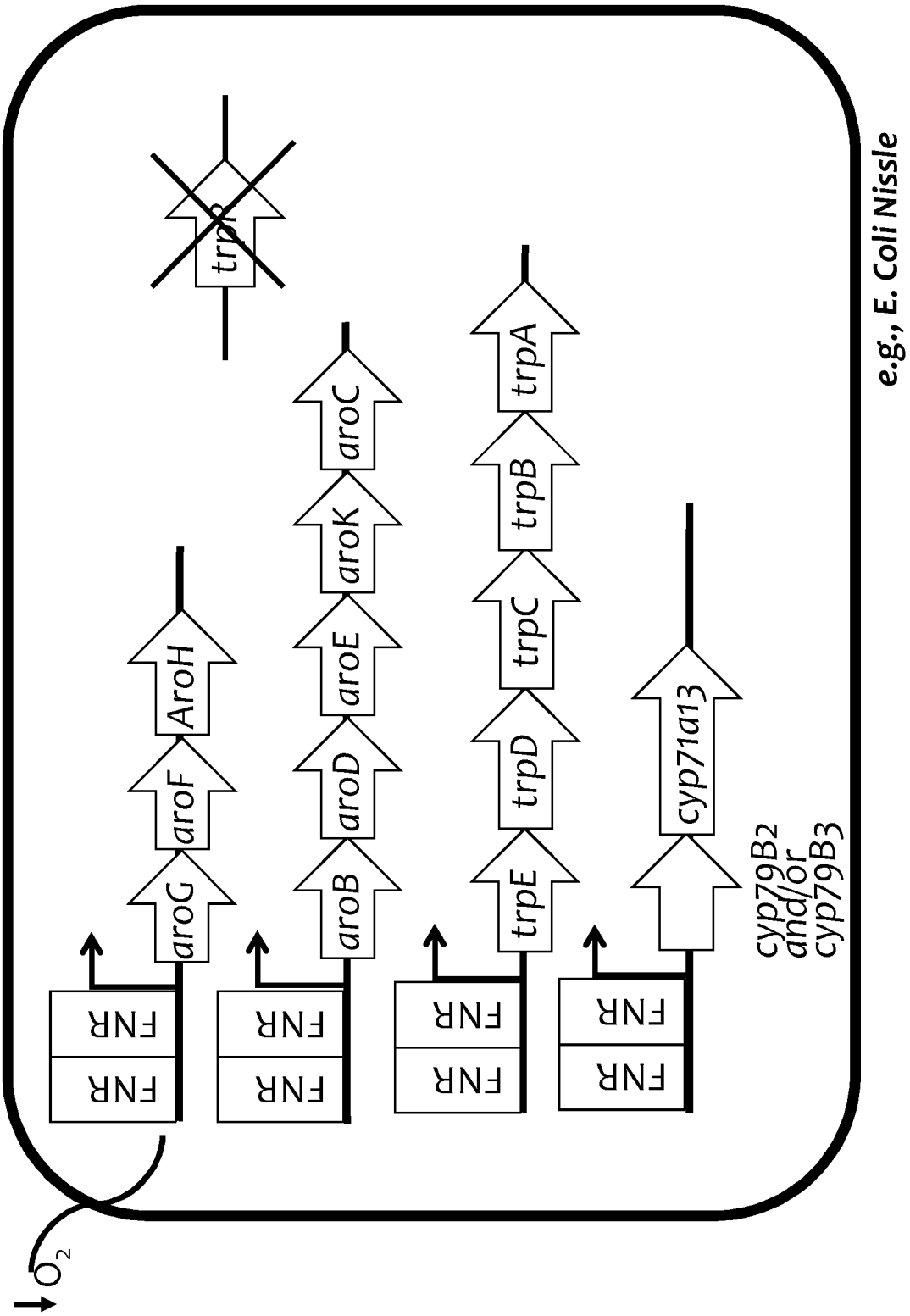

trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
cyp71a13
cyp79B2
and/or
cyp79B3
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 41E

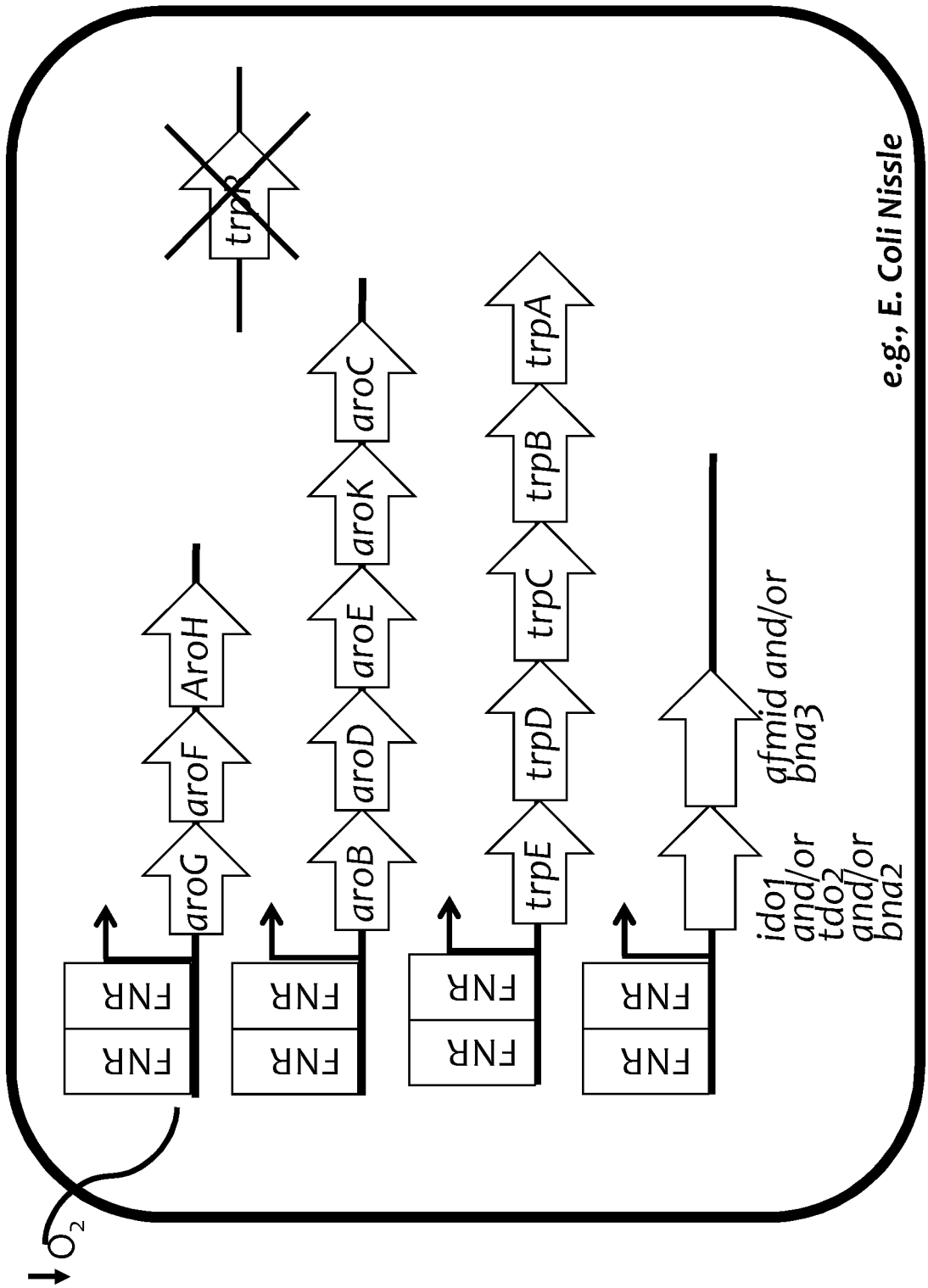
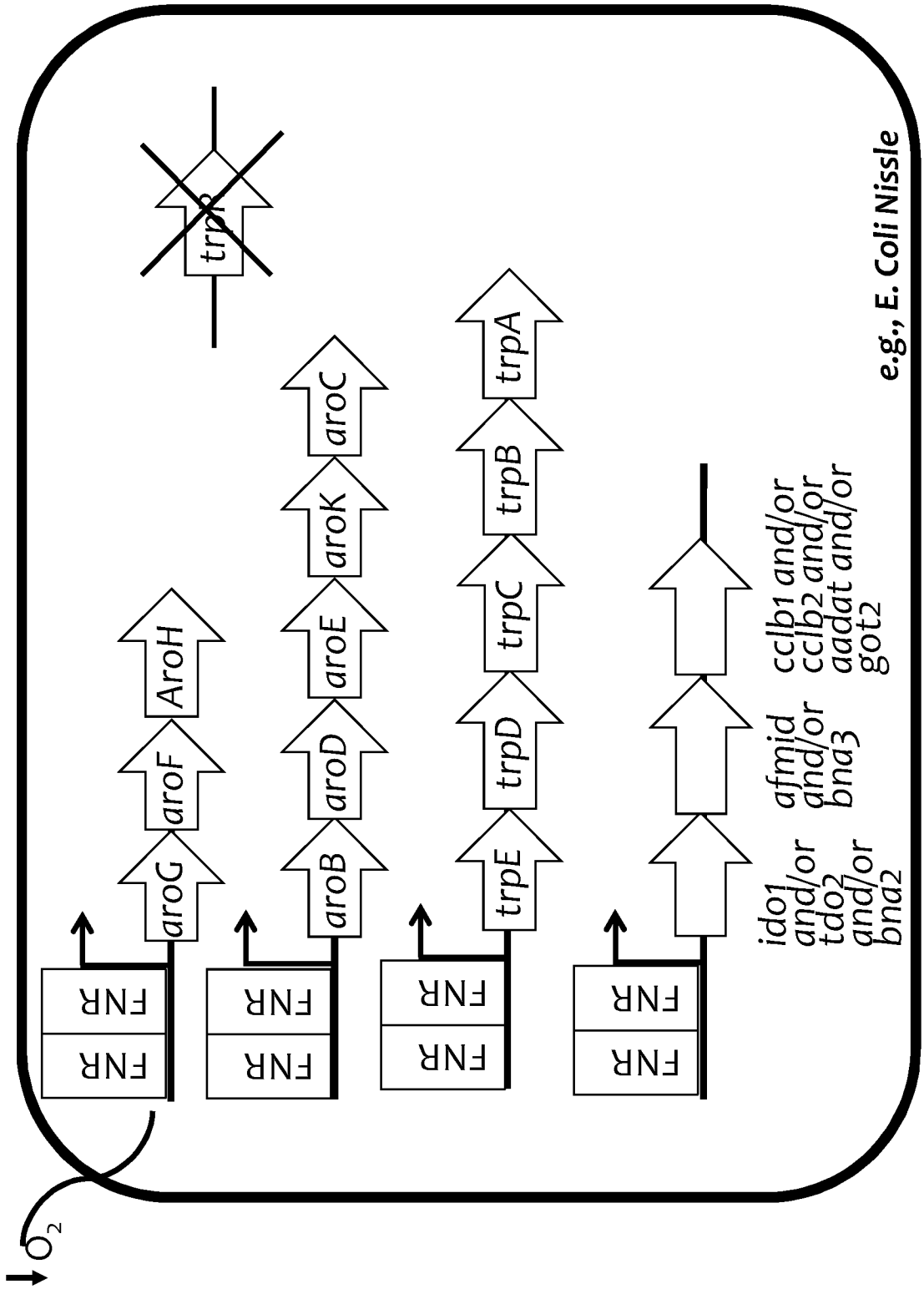

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
afmid and/or bna3
ido1 and/or tdo2 and/or bna2
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O2

FIG. 41F


e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
ido1 and/or tdo2 and/or bna2
afmid and/or bna3
cclb1 and/or cclb2 and/or aadat and/or got2
O2

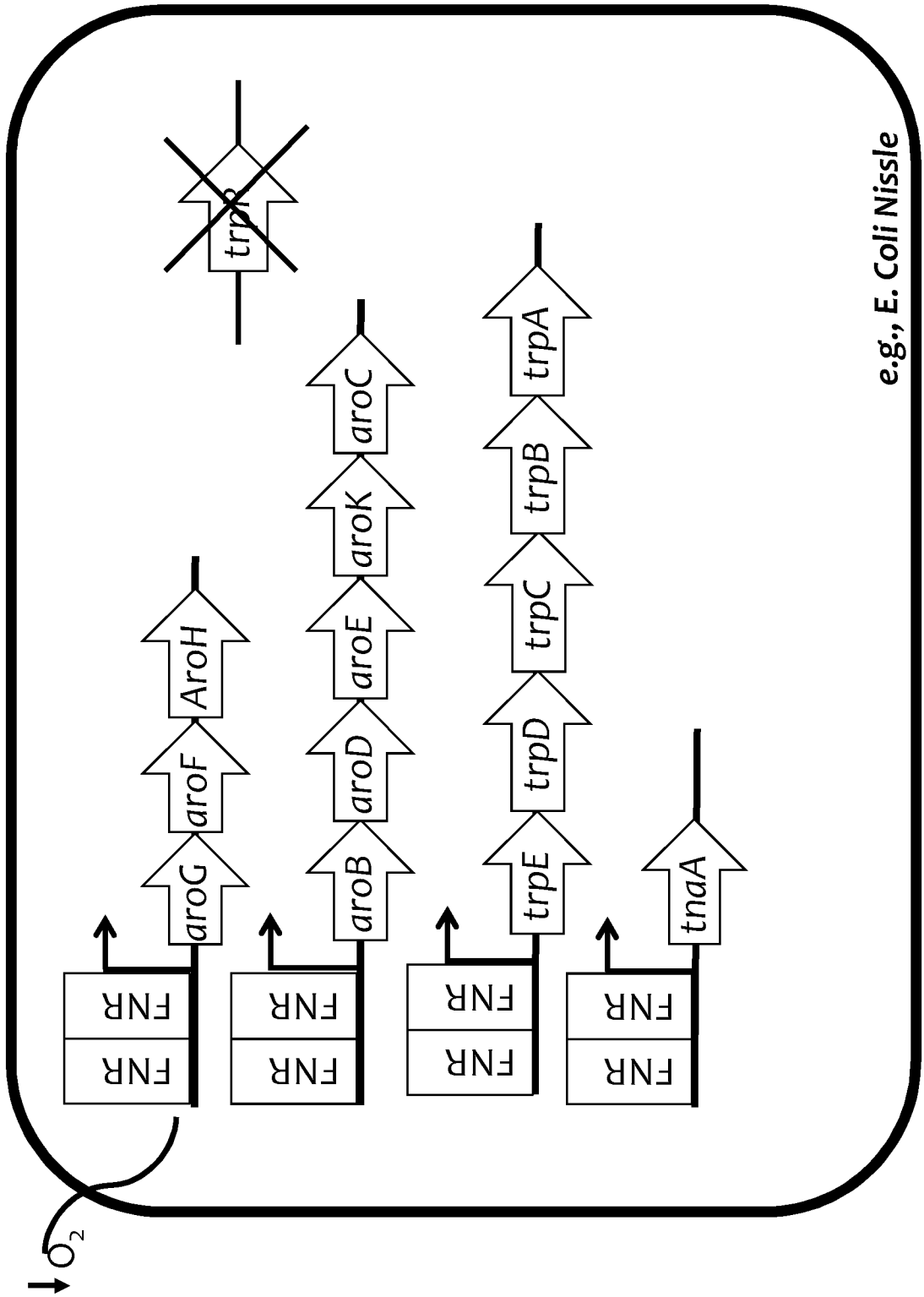

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
tnaA
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2

FIG. 41G

FIG. 41H

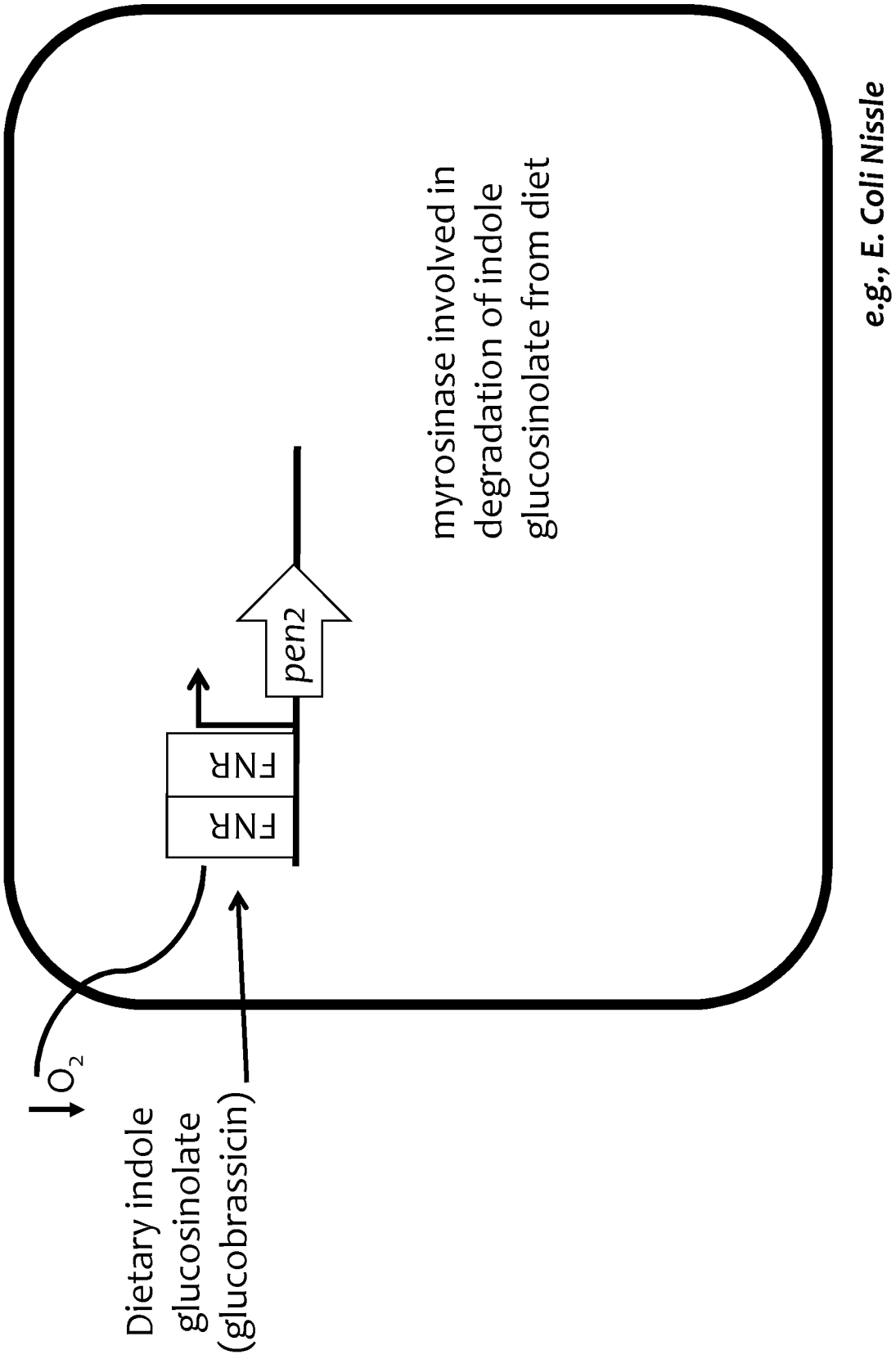

O_2
Dietary indole glucosinolate (glucobrassicin)
FNR
FNR
pen2
myrosinase involved in degradation of indole glucosinolate from diet
e.g., E. Coli Nissle

FIG. 42A

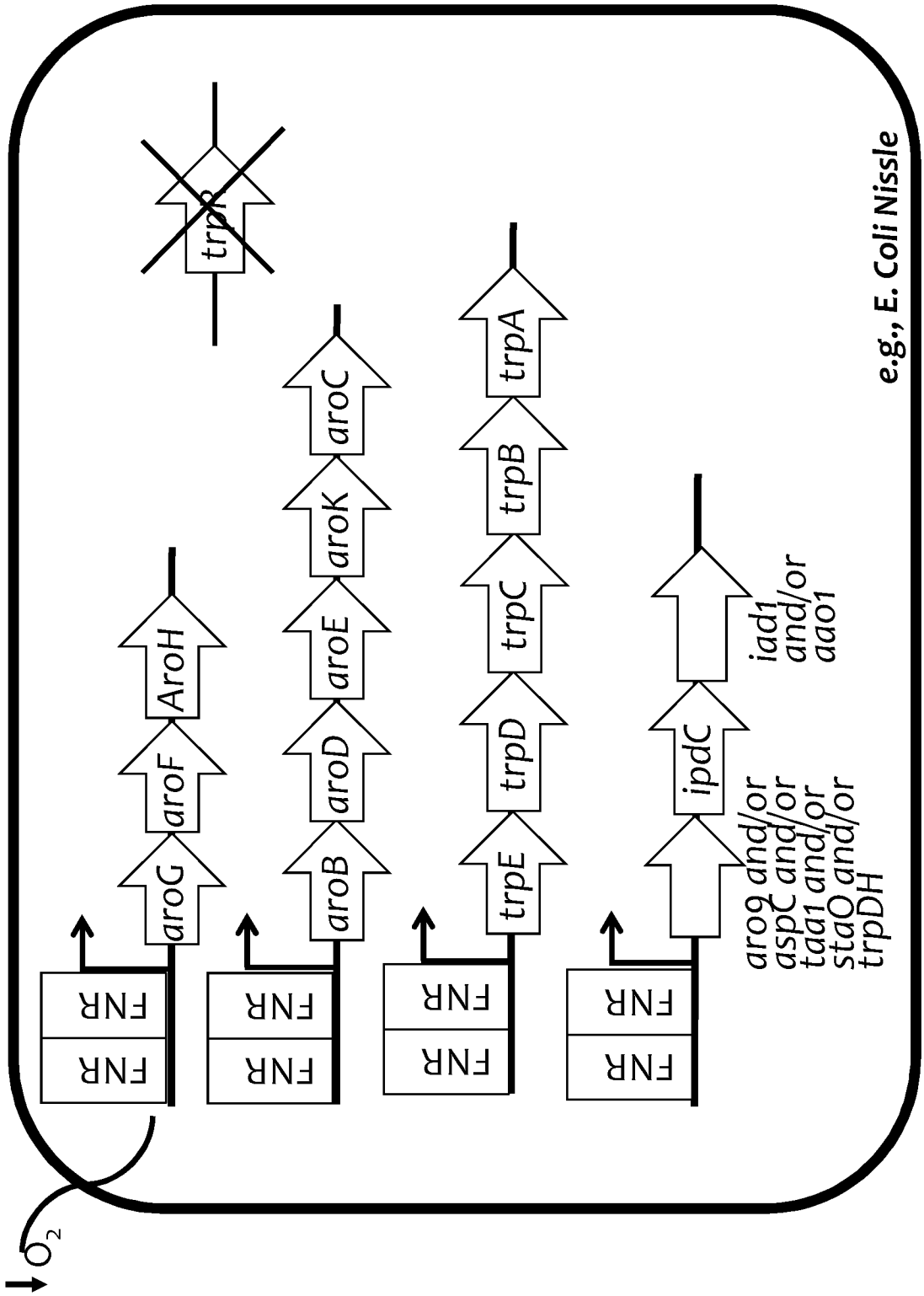

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
ipdC
iad1 and/or aao1
aro9 and/or aspC and/or taa1 and/or staO and/or trpDH
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O2

FIG. 42B

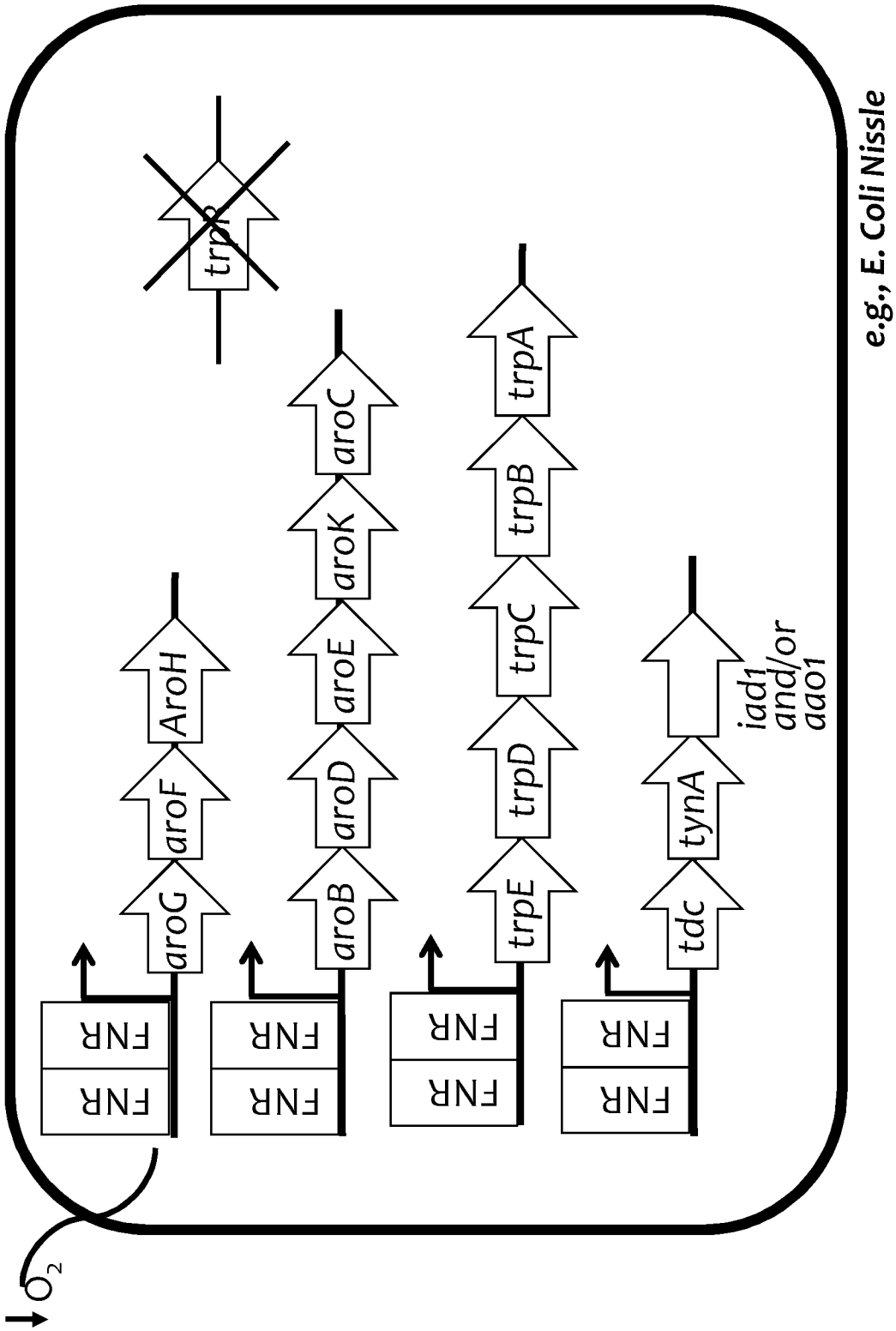
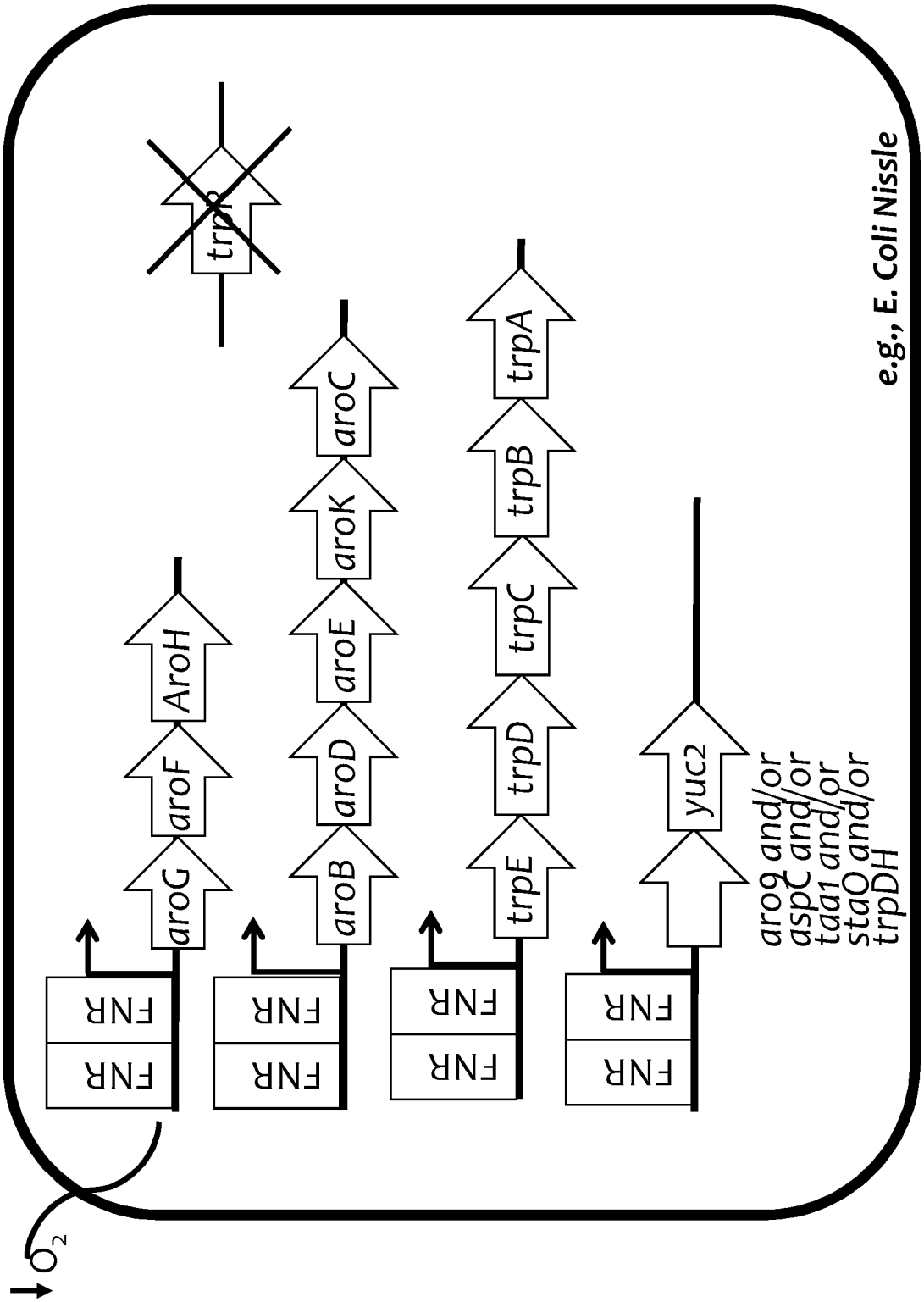

e.g., E. Coli Nissle
trpR
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
tdc
tynA
iad1
and/or
aao1
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O2

FIG. 42C


e.g., E. Coli Nissle
trpE
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
yuc2
FNR
aro9 and/or
aspC and/or
taa1 and/or
staO and/or
trpDH
O2

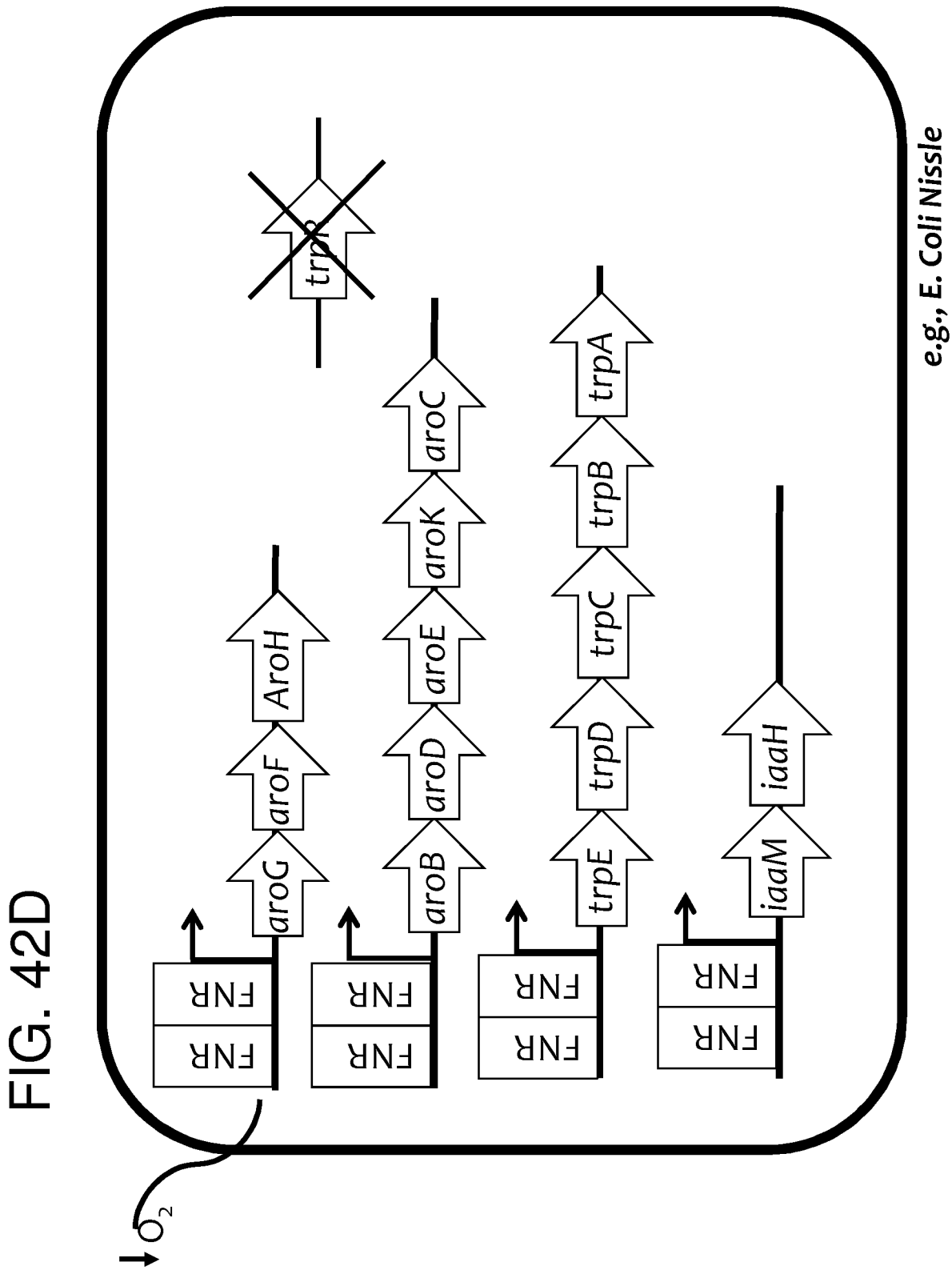
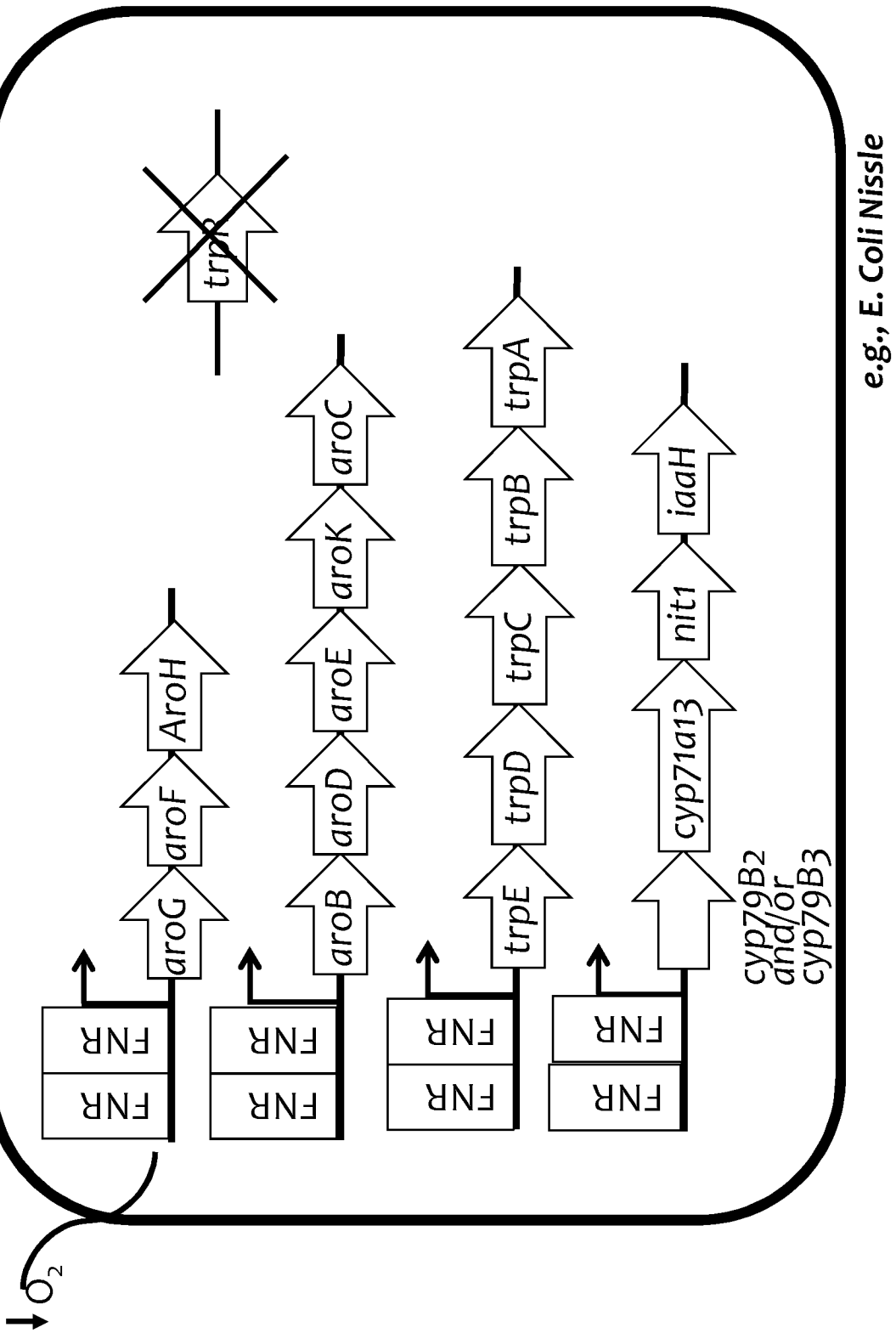

trpE
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
iaaM
iaaH
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 42D

FIG. 42E


e.g., E. Coli Nissle
trpE
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

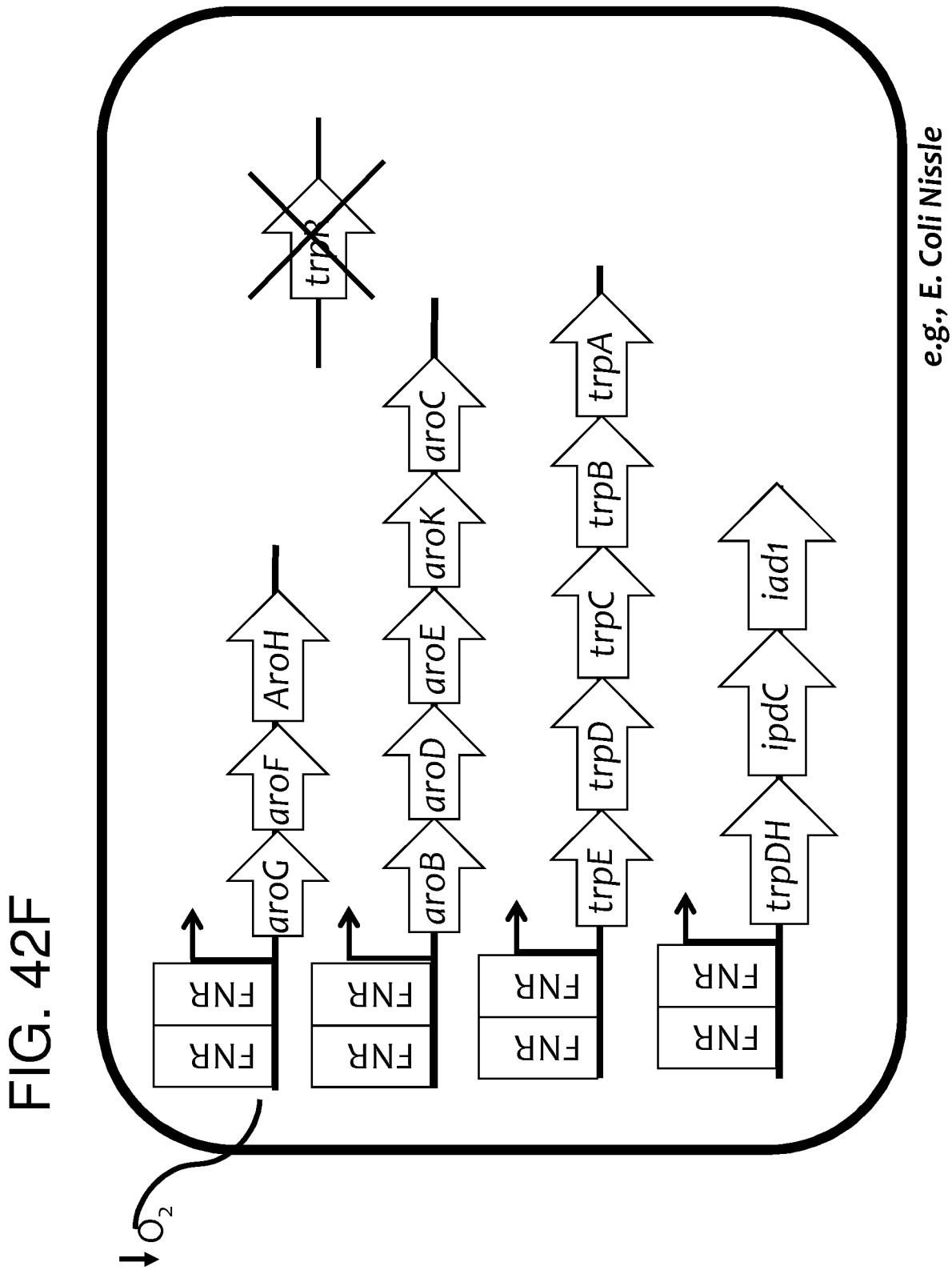
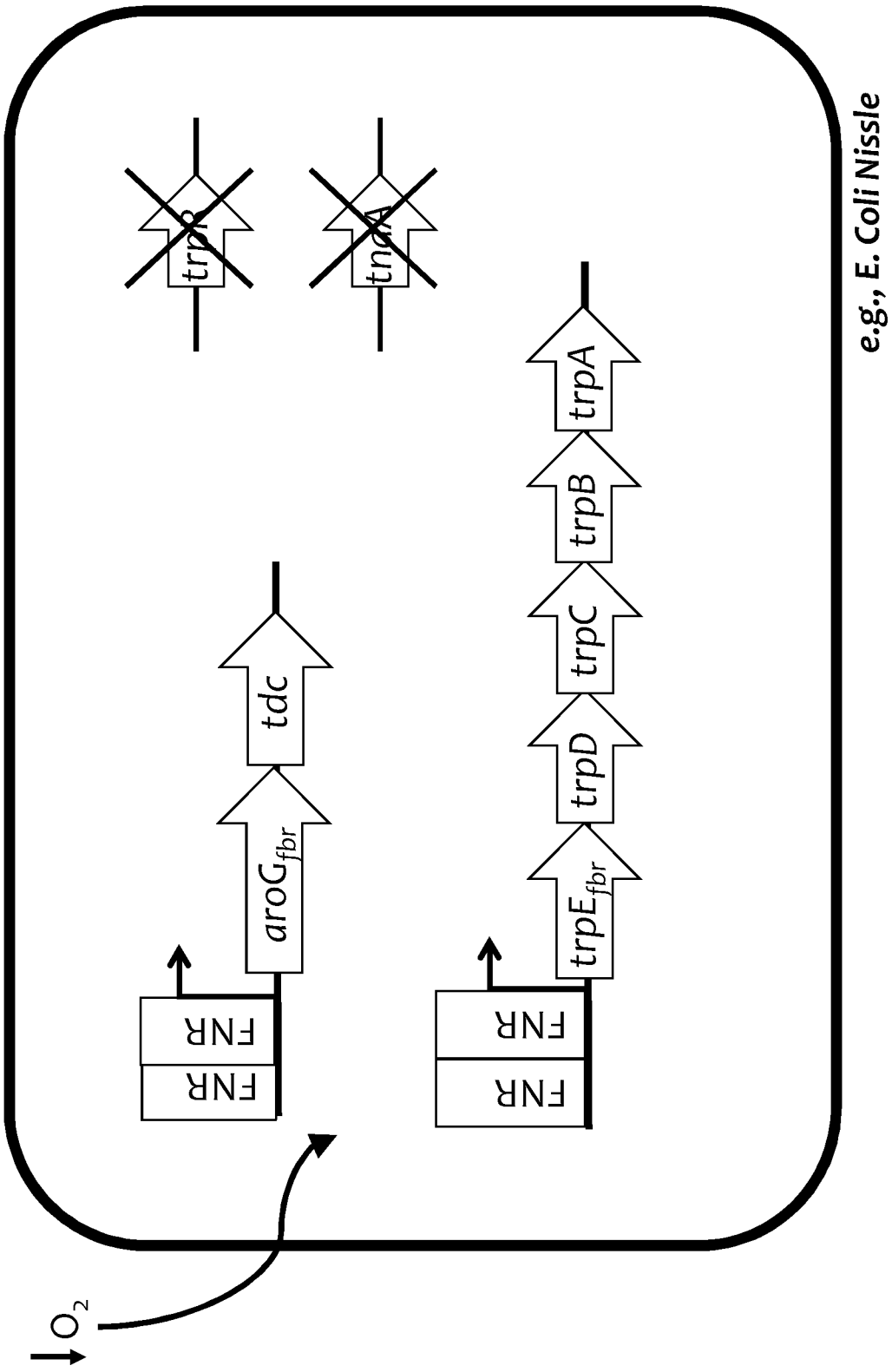

trpE
aroG
aroF
AroH
aroB
aroD
aroE
aroK
aroC
trpE
trpD
trpC
trpB
trpA
trpDH
ipdC
iad1
FNR
FNR
FNR
FNR
FNR
FNR
FNR
FNR
O_2
e.g., E. Coli Nissle

FIG. 42F

FIG. 43A


e.g., E. Coli Nissle
trpR
tnaA
tdc
aroG$_{fbr}$
FNR
FNR
trpA
trpB
trpC
trpD
trpE$_{fbr}$
FNR
FNR
O$_2$

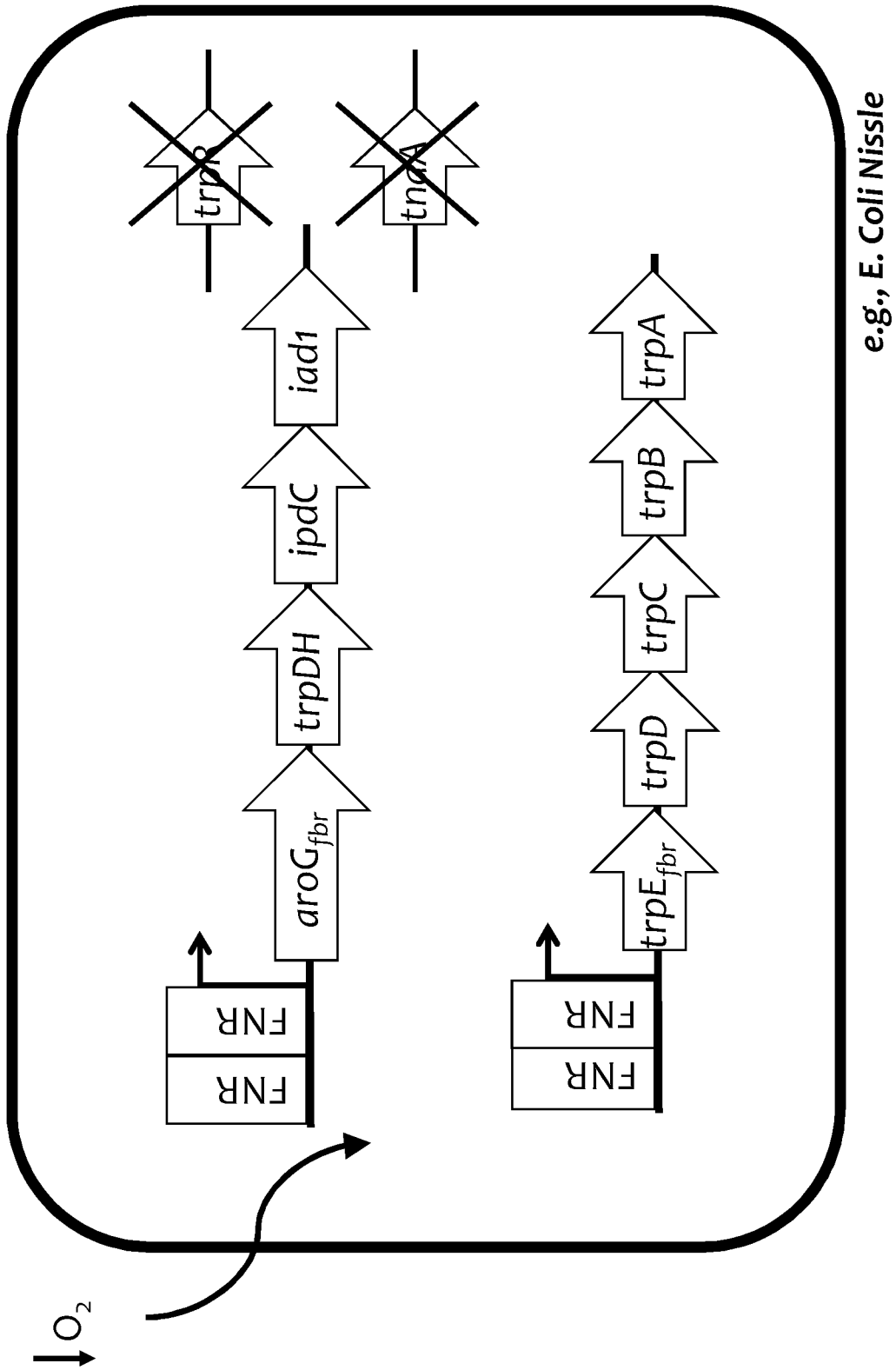

e.g., E. Coli Nissle
trpR
tnaA
iad1
ipdC
trpDH
aroG$_{fbr}$
FNR
FNR
trpA
trpB
trpC
trpD
trpE$_{fbr}$
FNR
FNR
O$_2$

FIG. 43B

FIG. 43C

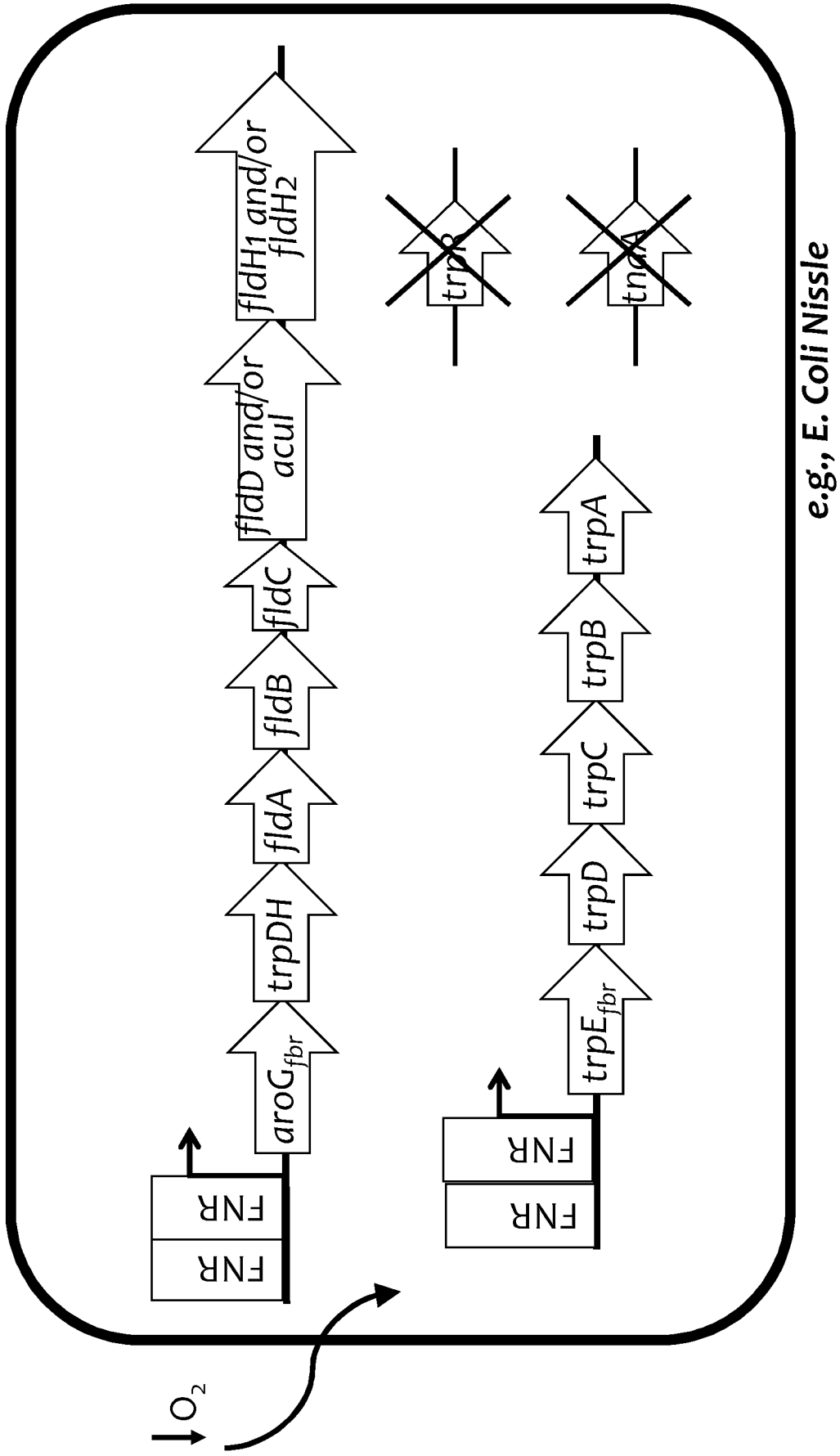

FNR
FNR
$aroG_{fbr}$
trpDH
fldA
fldB
fldC
fldD and/or acuI
fldH1 and/or fldH2
FNR
FNR
$trpE_{fbr}$
trpD
trpC
trpB
trpA
trpR
tnaA
O_2
e.g., E. Coli Nissle

FIG. 44A

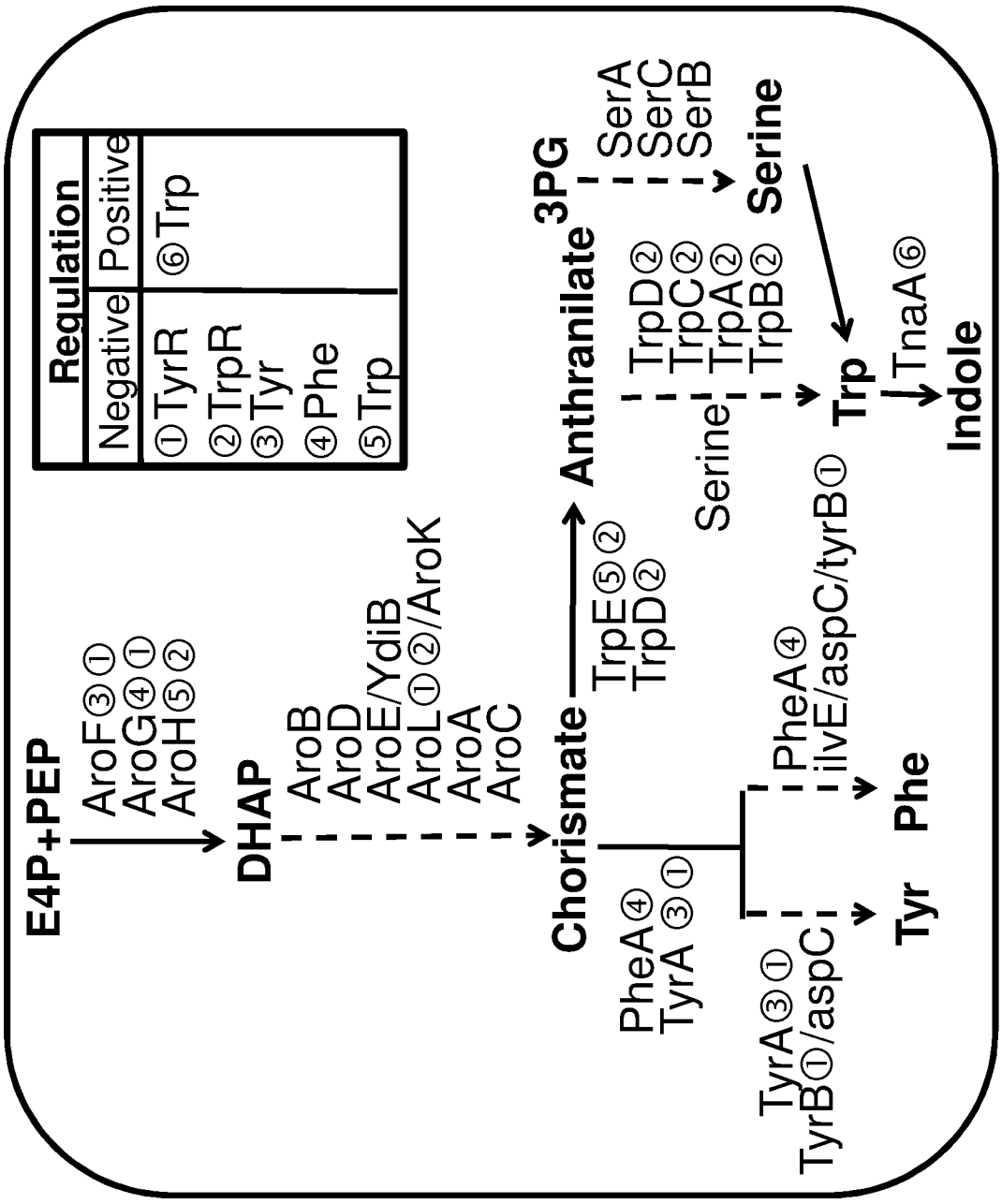

Regulation
Negative
Positive
①TyrR
②TrpR
③Tyr
④Phe
⑤Trp
⑥Trp
E4P+PEP
AroF③①
AroG④①
AroH⑤②
DHAP
AroB
AroD
AroE/YdiB
AroL①②/AroK
AroA
AroC
Chorismate
TrpE⑤②
TrpD②
Anthranilate
3PG
SerA
SerC
SerB
Serine
TrpD②
TrpC②
TrpA②
TrpB②
Serine
Trp
TnaA⑥
Indole
PheA④
TyrA③①
PheA④
ilvE/aspC/tyrB①
TyrA③①
TyrB①/aspC
Tyr
Phe

FIG. 44B

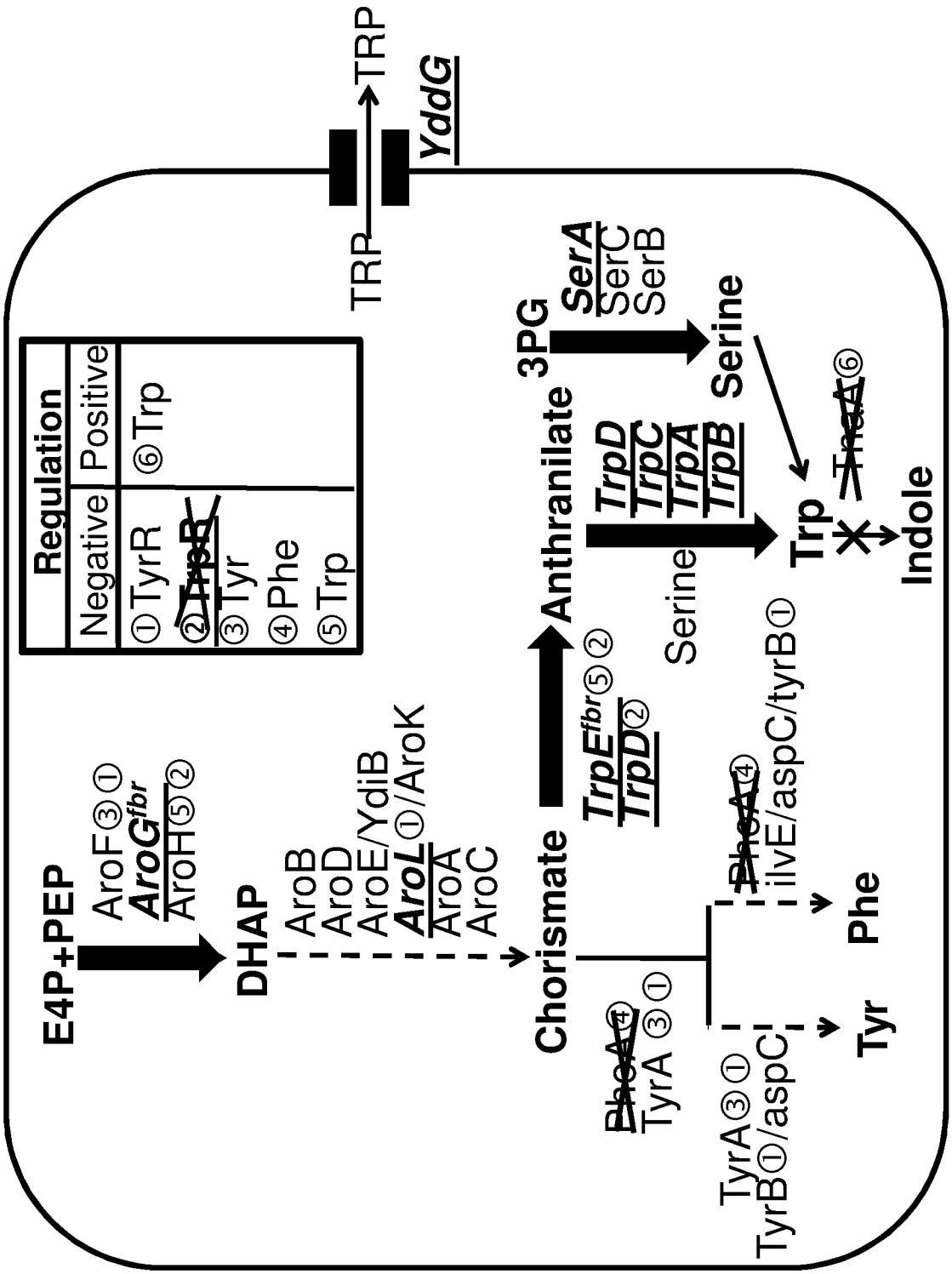

TRP
TRP
YddG
Regulation
Negative
Positive
①TyrR
②TrpR
③Tyr
④Phe
⑤Trp
⑥Trp
E4P+PEP
AroF③①
AroG^fbr
AroH⑤②
DHAP
AroB
AroD
AroE/YdiB
AroL①/AroK
AroA
AroC
Chorismate
TrpE^fbr⑤②
TrpD②
Anthranilate
3PG
SerA
SerC
SerB
Serine
TrpD
TrpC
TrpA
TrpB
Serine
Trp
TrpA⑥
Indole
PheA④
TyrA③①
TyrB①/aspC
ilvE/aspC/tyrB①
Phe
Tyr

FIG. 45A

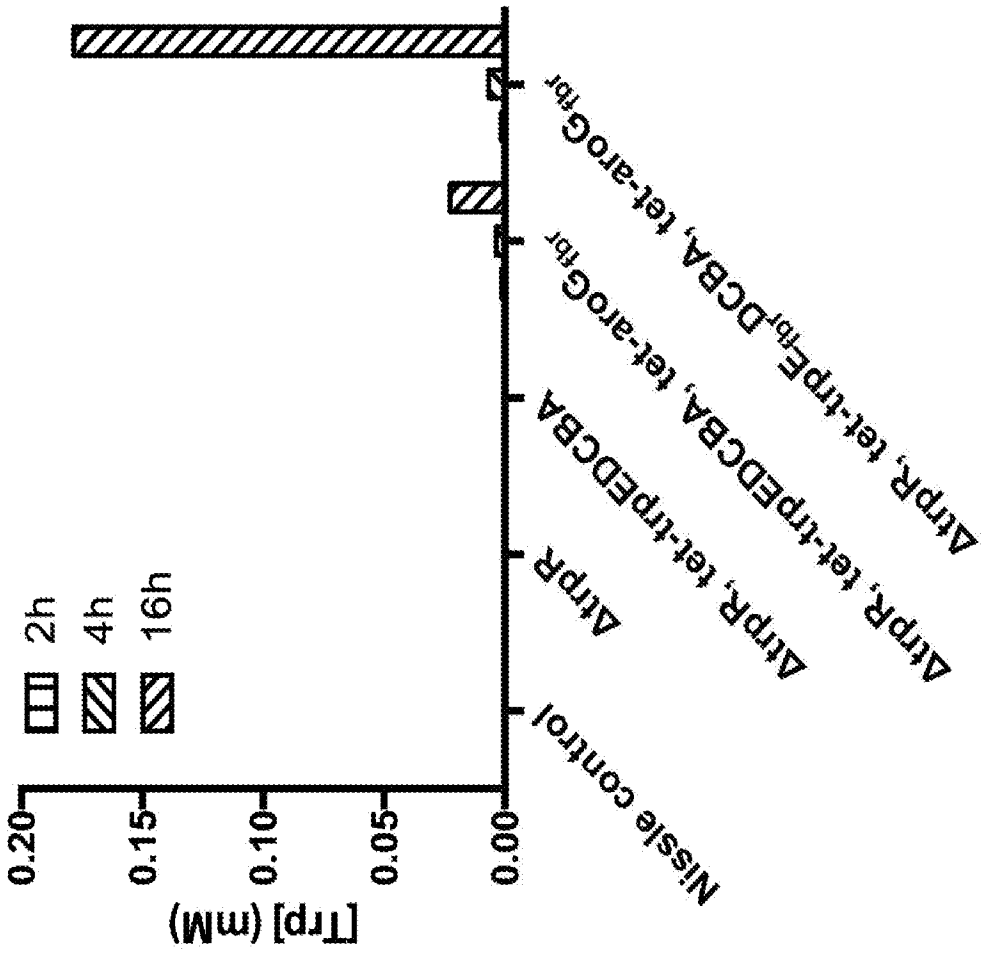

2h
4h
16h
[Trp] (mM)
0.20
0.15
0.10
0.05
0.00
Nissle control
AtrpR
AtrpR, tet-trpEDCBA
AtrpR, tet-trpEDCBA, tet-aroG$_{fbr}$
AtrpR, tet-trpE$_{fbr}$DCBA, tet-aroG$_{fbr}$

FIG. 45B

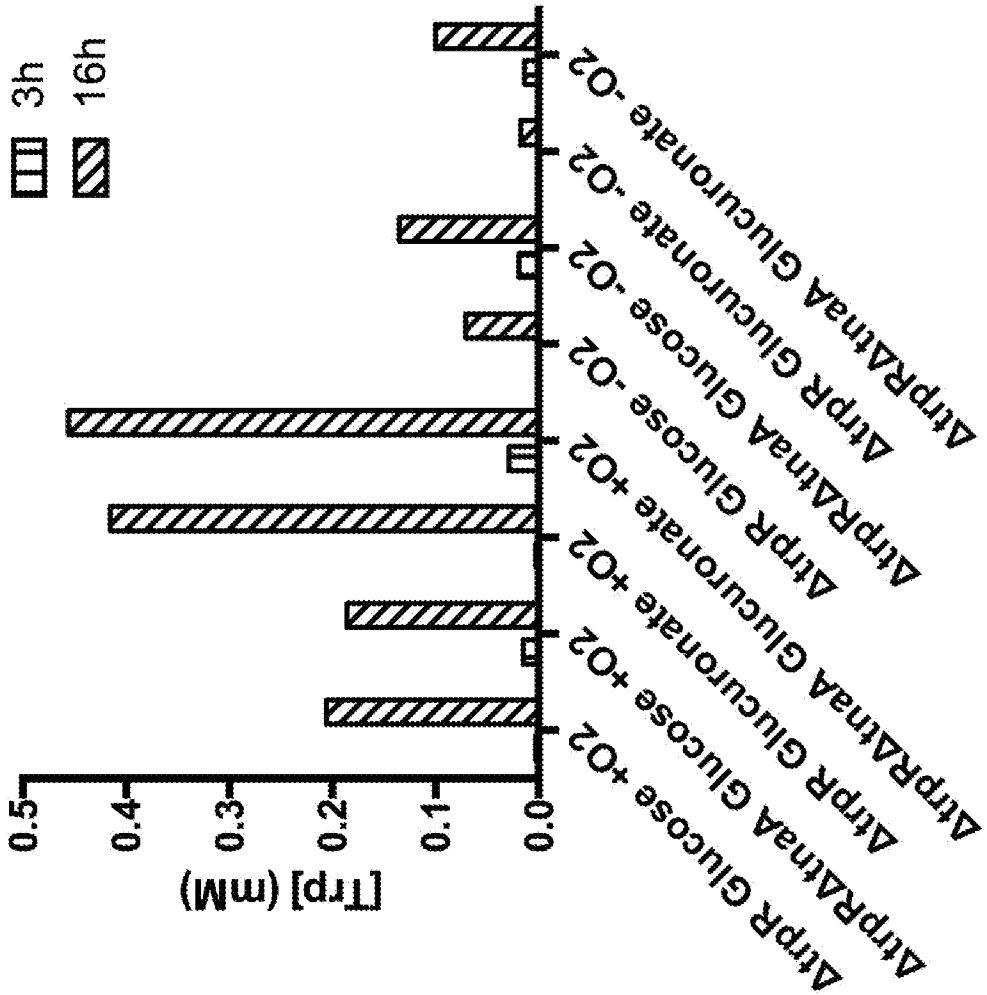

3h
16h
[Trp] (mM)
0.5
0.4
0.3
0.2
0.1
0.0
ΔtrpR Glucose +O2
ΔtrpRAtna Glucose +O2
ΔtrpR Glucuronate +O2
ΔtrpRAtna Glucuronate +O2
ΔtrpR Glucose -O2
ΔtrpRAtna Glucose -O2
ΔtrpR Glucuronate -O2
ΔtrpRAtna Glucuronate -O2

FIG. 45C

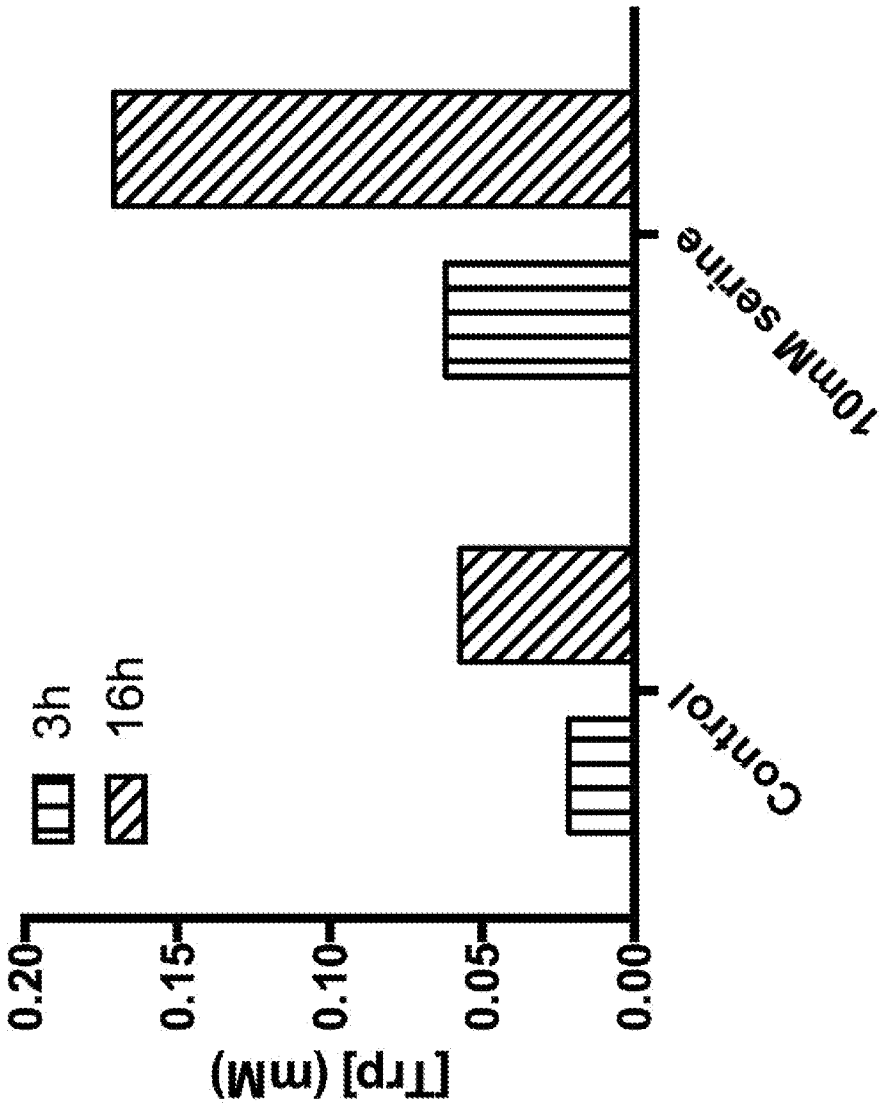


FIG. 46

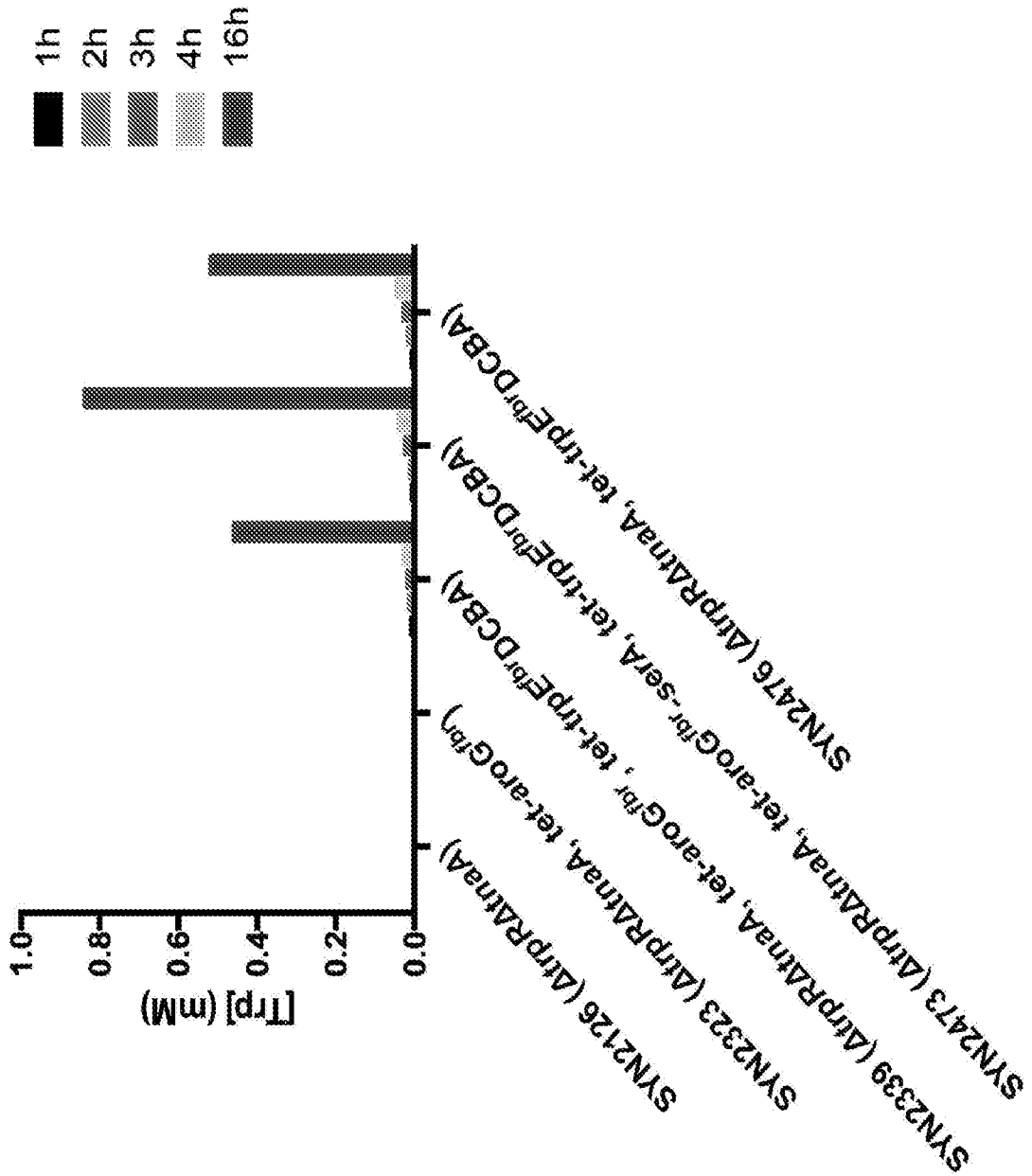

1h
2h
3h
4h
16h
[Trp] (mM)
1.0
0.8
0.6
0.4
0.2
0.0
SYN2126 (AtrpRAtna)
SYN2323 (AtrpRAtna, tet-aroG)
SYN2339 (AtrpRAtna, tet-aroG, tet-trpEDCBA)
SYN2473 (AtrpRAtna, tet-aroG-serA, tet-trpEDCBA)
SYN2476 (AtrpRAtna, tet-trpEDCBA)

FIG. 47

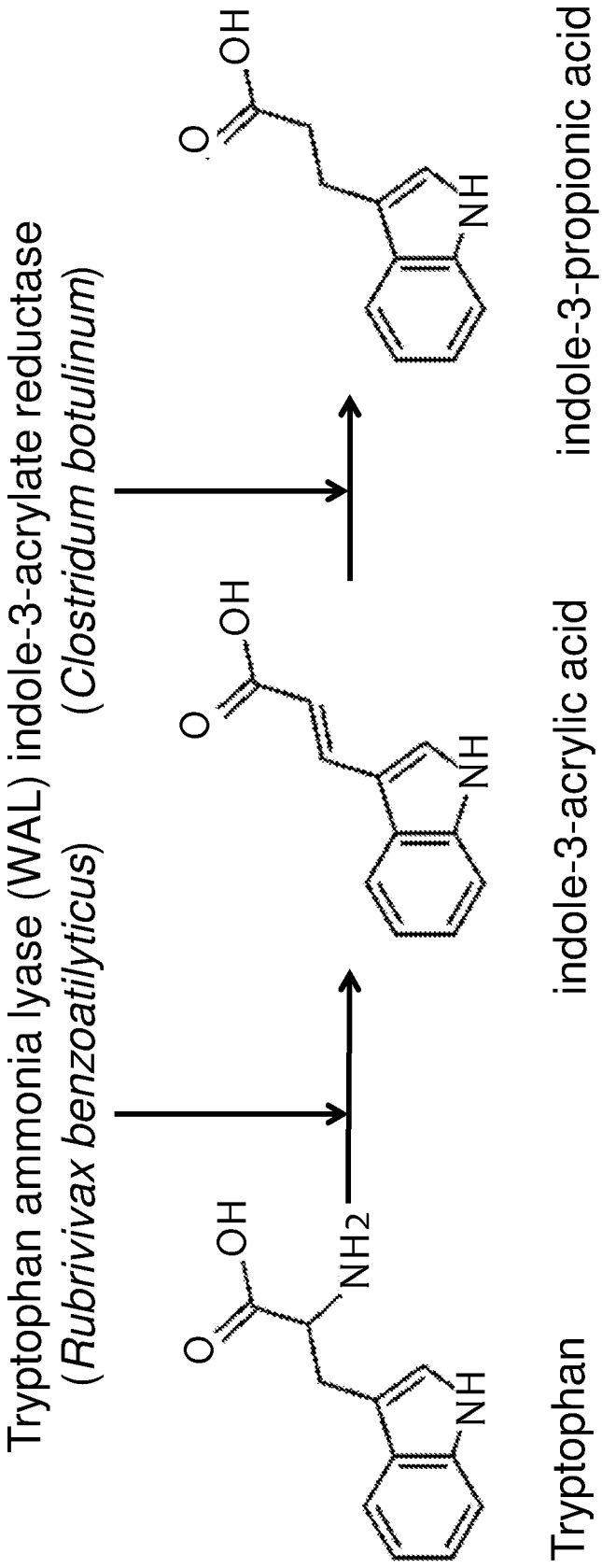
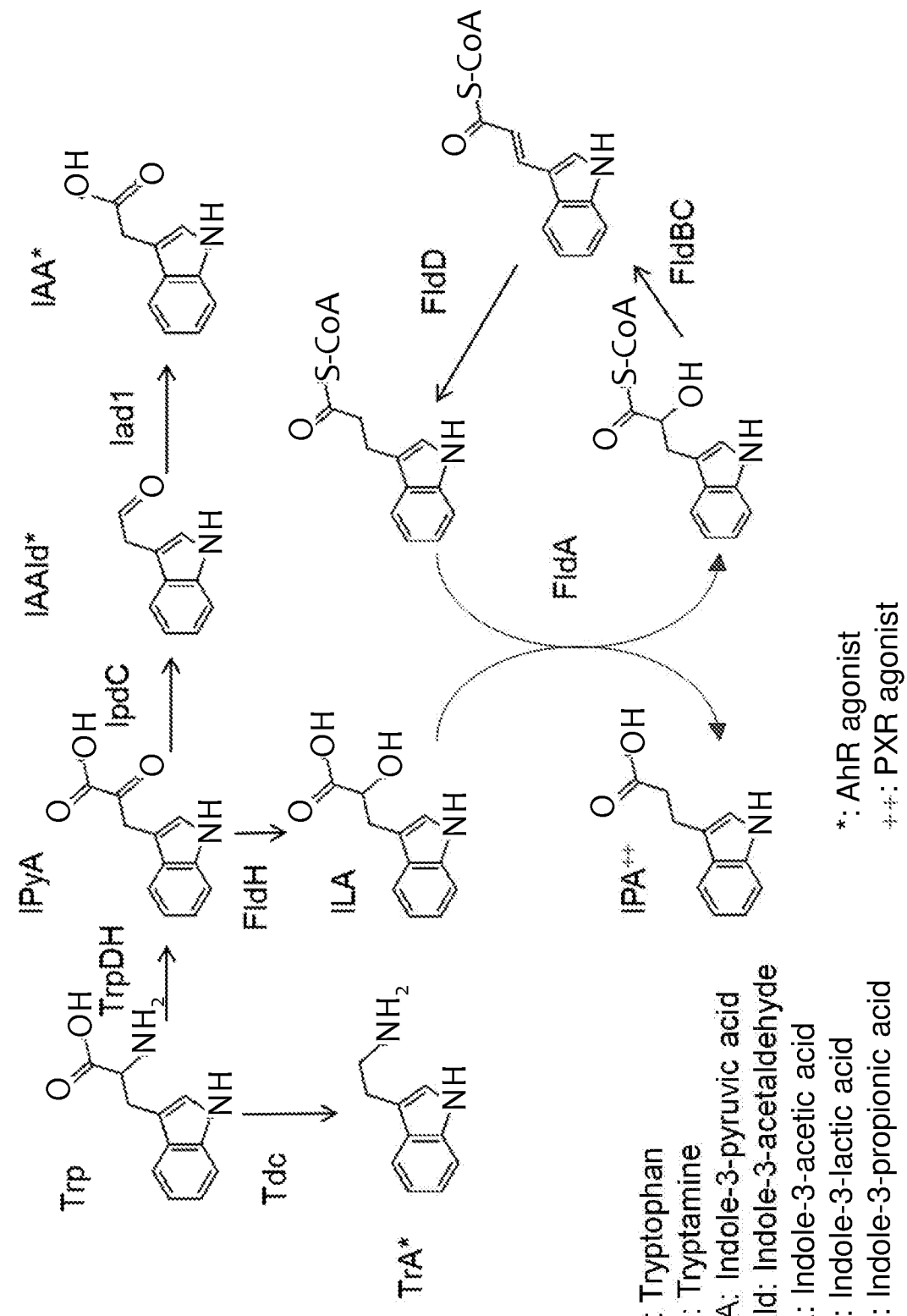

Tryptophan ammonia lyase (WAL) indole-3-acrylate reductase
(Rubrivivax benzoatilyticus) (Clostridium botulinum)
OH
O
NH2
NH
Tryptophan
indole-3-acrylic acid
indole-3-propionic acid

FIG. 48


Trp
TrpDH
TrA*
Tdc
IPyA
IpdC
IAAld*
Iad1
IAA*
FldH
ILA
FldD
FldA
FldBC
IPA++
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
*: AhR agonist
++: PXR agonist

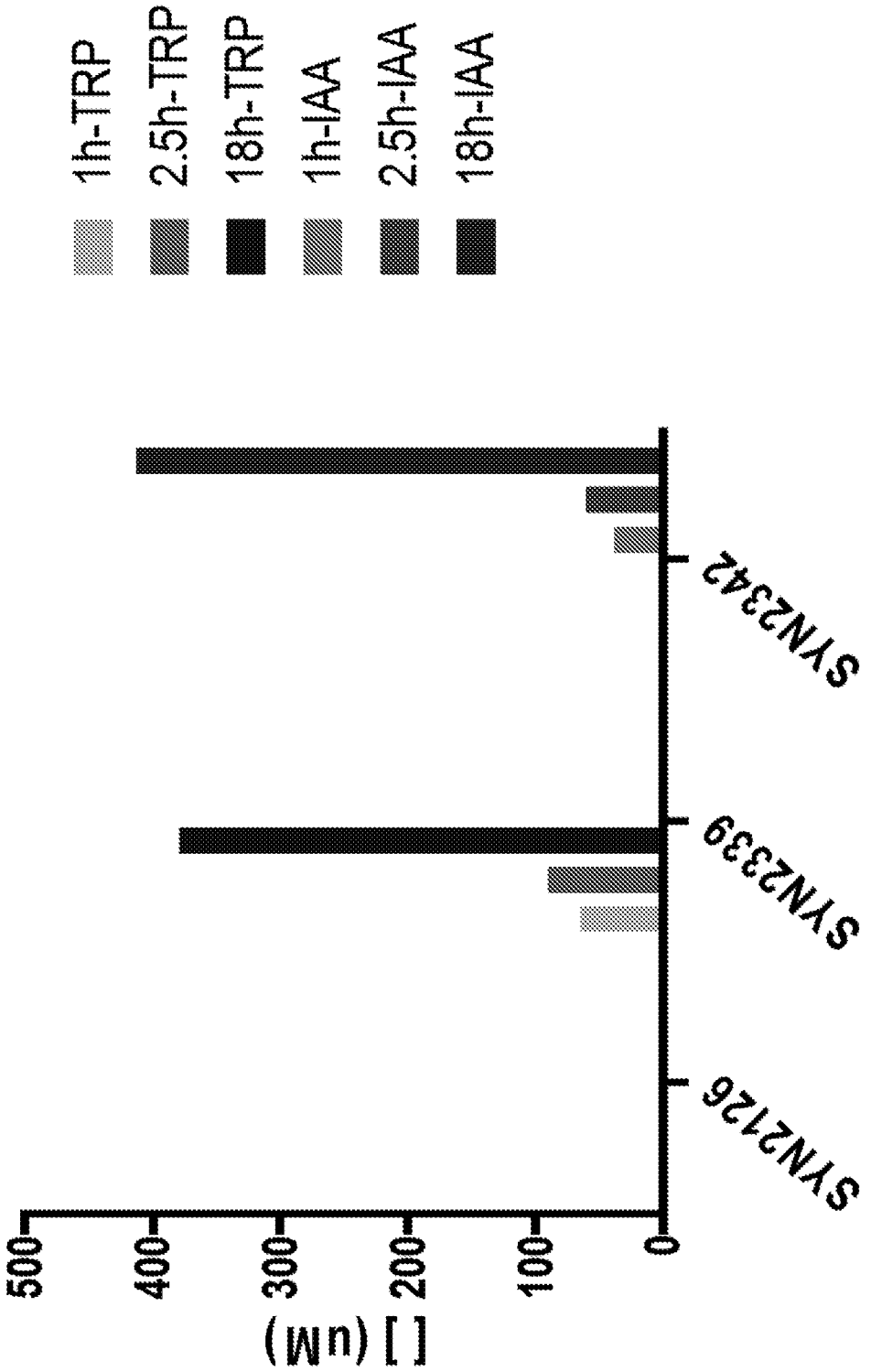

1h-TRP
2.5h-TRP
18h-TRP
1h-IAA
2.5h-IAA
18h-IAA
SYN2342
SYN2339
SYN2126
0
100
200
300
400
500
[ ] (uM)

FIG. 49

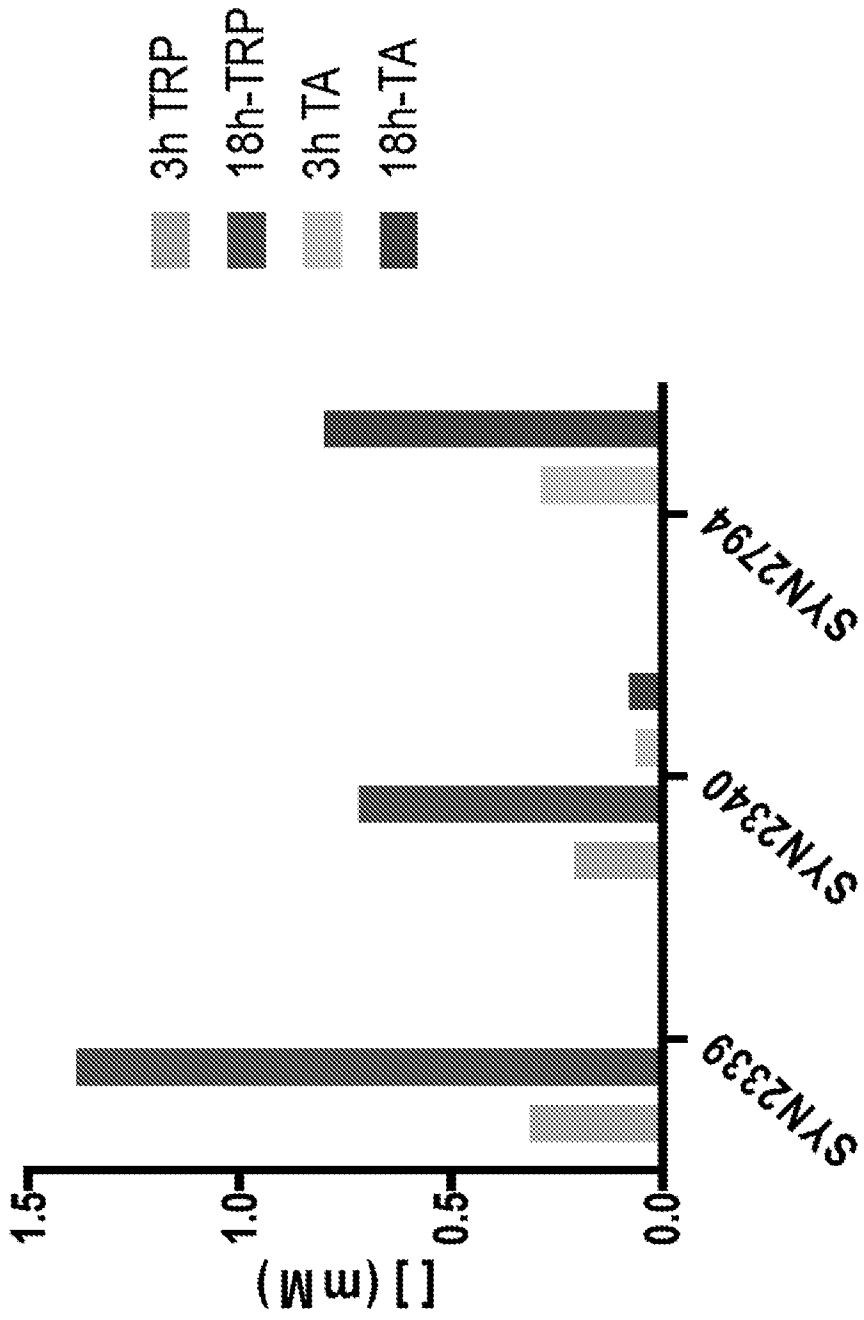

3h TRP
18h-TRP
3h TA
18h-TA
SYN2794
SYN2340
SYN2339
1.5
1.0
0.5
0.0
[ ] (mM)

FIG. 50

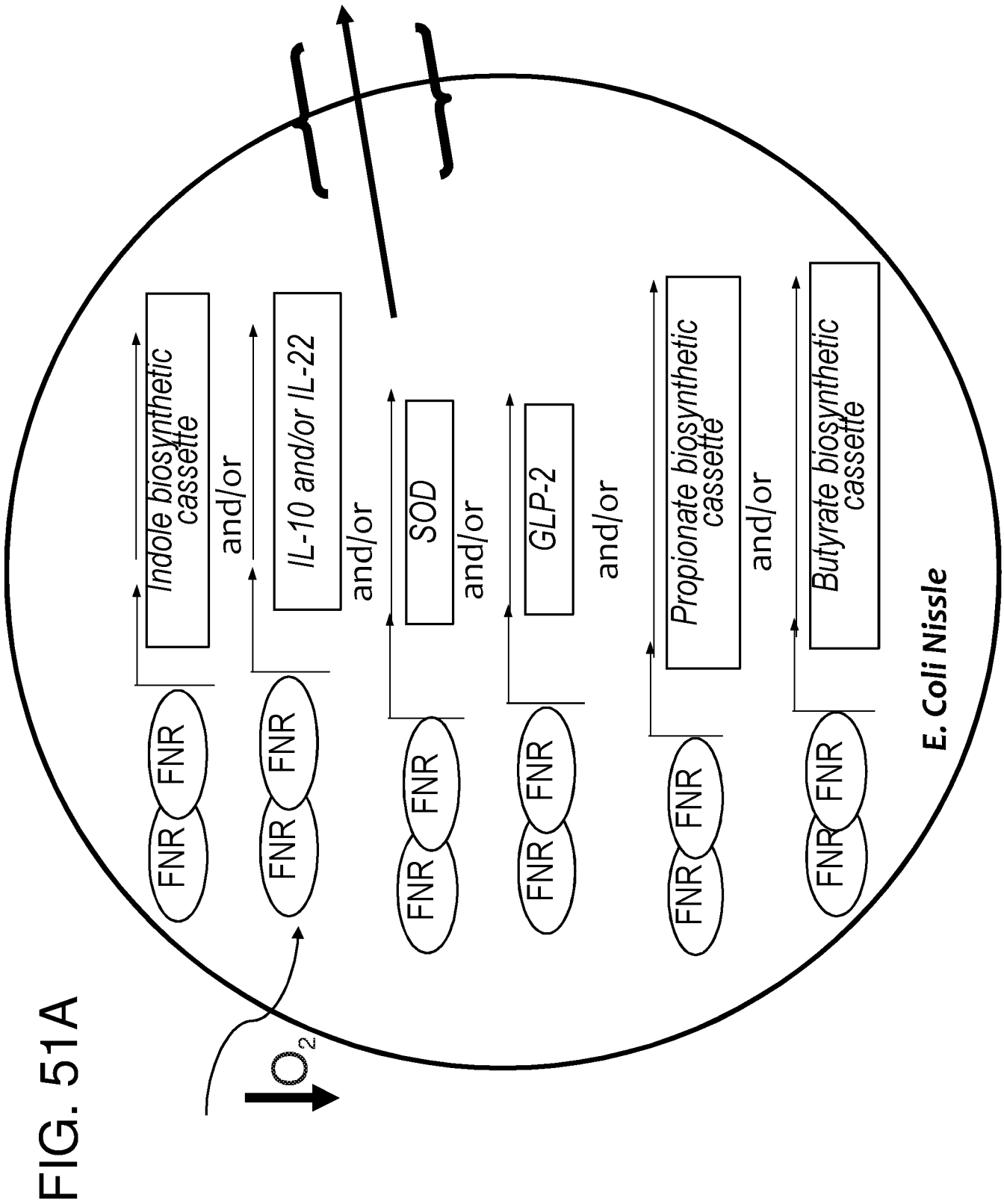

E. Coli Nissle
FNR
FNR
FNR
FNR
Indole biosynthetic cassette
and/or
IL-10 and/or IL-22
and/or
FNR
FNR
FNR
FNR
SOD
and/or
GLP-2
and/or
FNR
FNR
FNR
FNR
Propionate biosynthetic cassette
and/or
Butyrate biosynthetic cassette
FNR
FNR
FNR
FNR
O_2

FIG. 51A

FIG. 51B

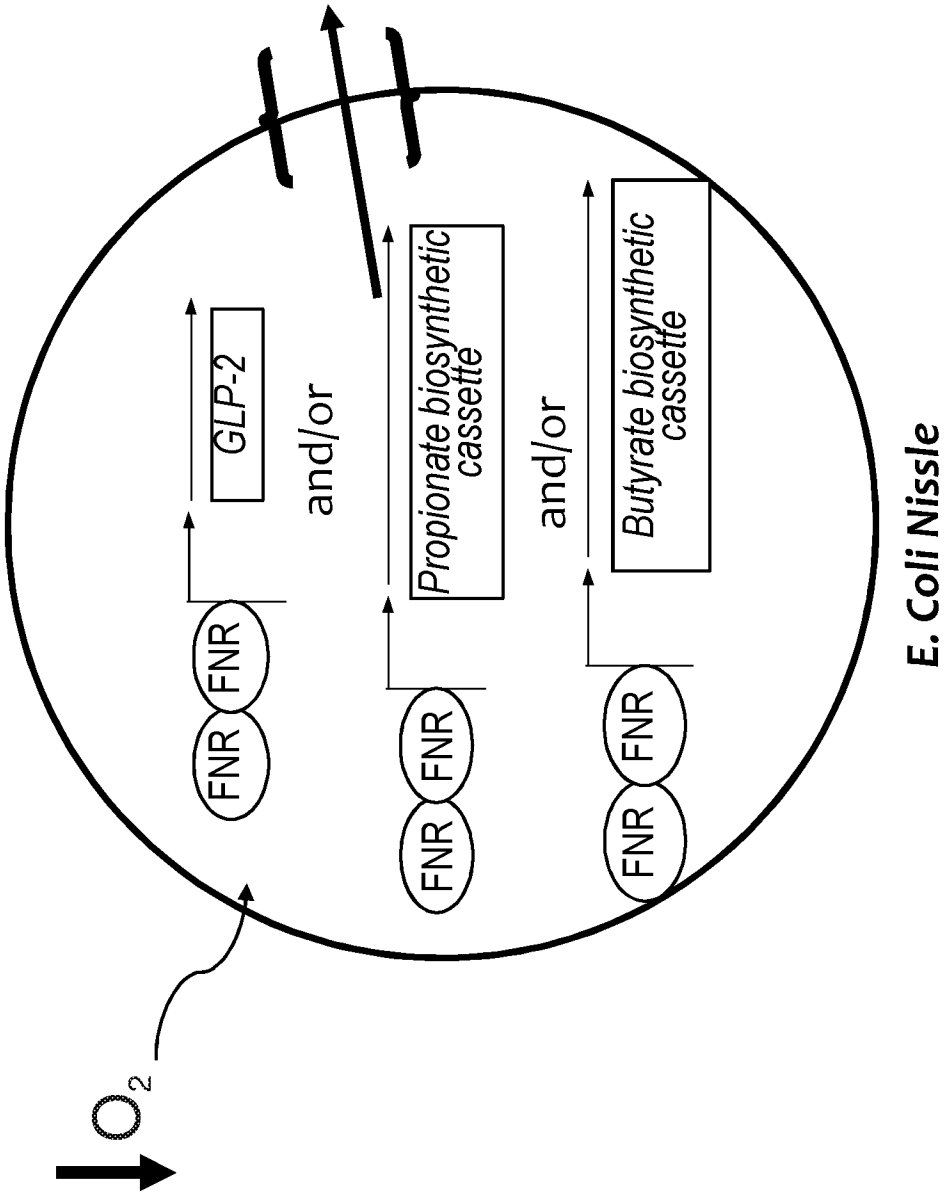

O_2
FNR
FNR
GLP-2
and/or
FNR
FNR
Propionate biosynthetic cassette
and/or
FNR
FNR
Butyrate biosynthetic cassette
E. Coli Nissle

FIG. 51C

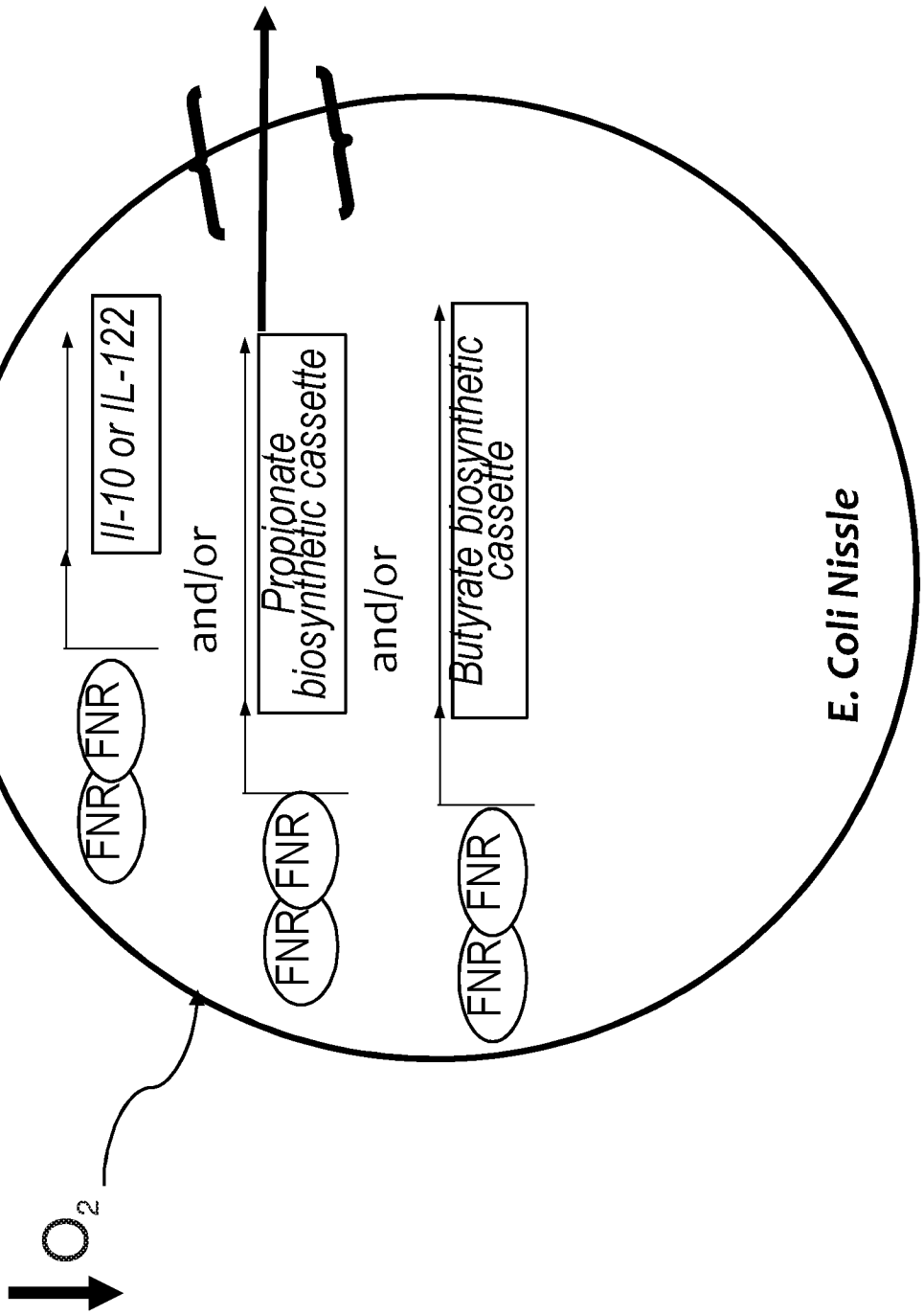

E. Coli Nissle
Il-10 or IL-122
and/or
Propionate biosynthetic cassette
and/or
Butyrate biosynthetic cassette
FNR
FNR
FNR
FNR
FNR
FNR
O2

FIG. 51D

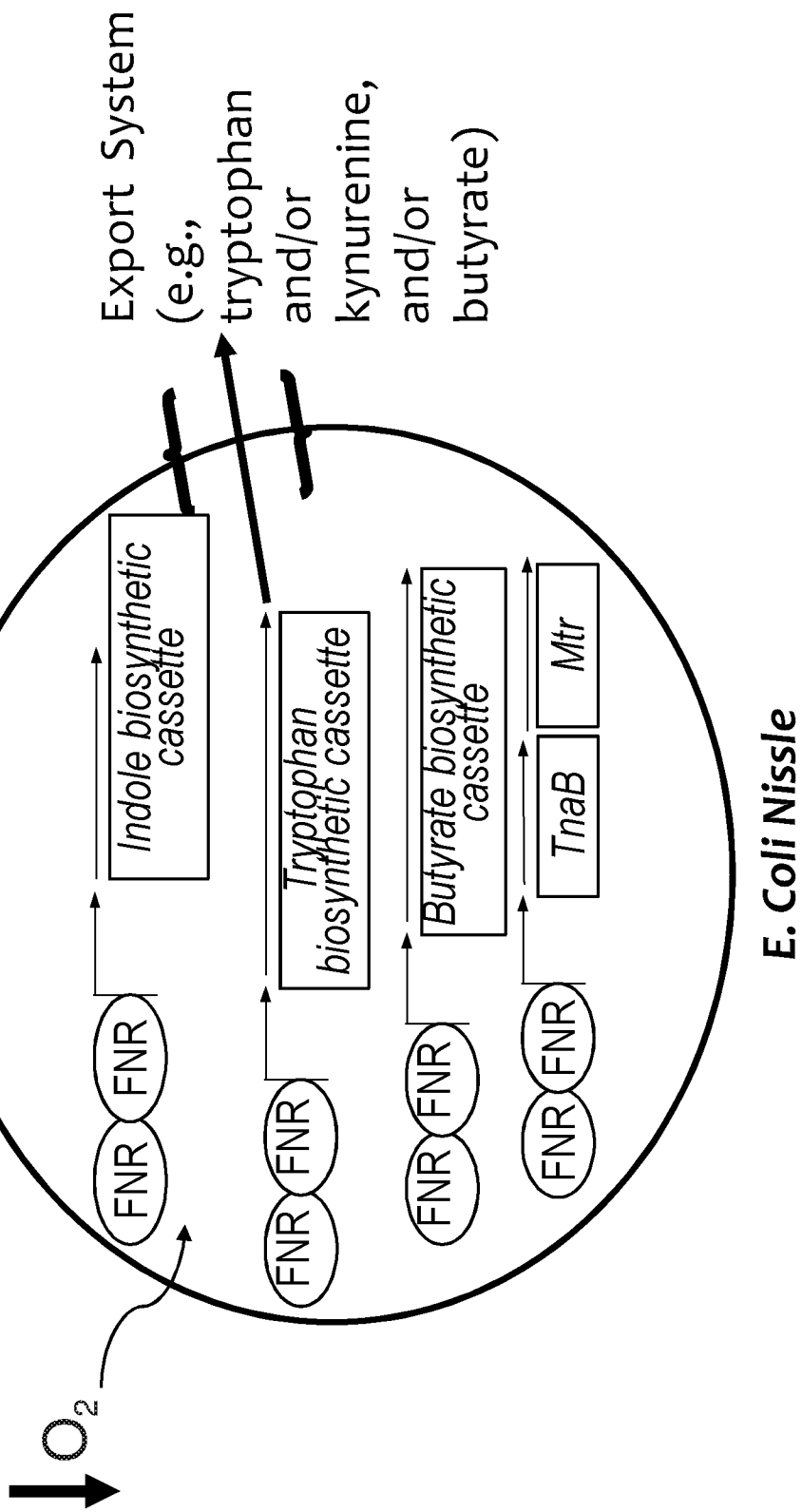

Export System (e.g., tryptophan and/or kynurenine, and/or butyrate)
Indole biosynthetic cassette
Tryptophan biosynthetic cassette
Butyrate biosynthetic cassette
TnaB
Mtr
FNR
O_2
E. Coli Nissle

FIG. 51E

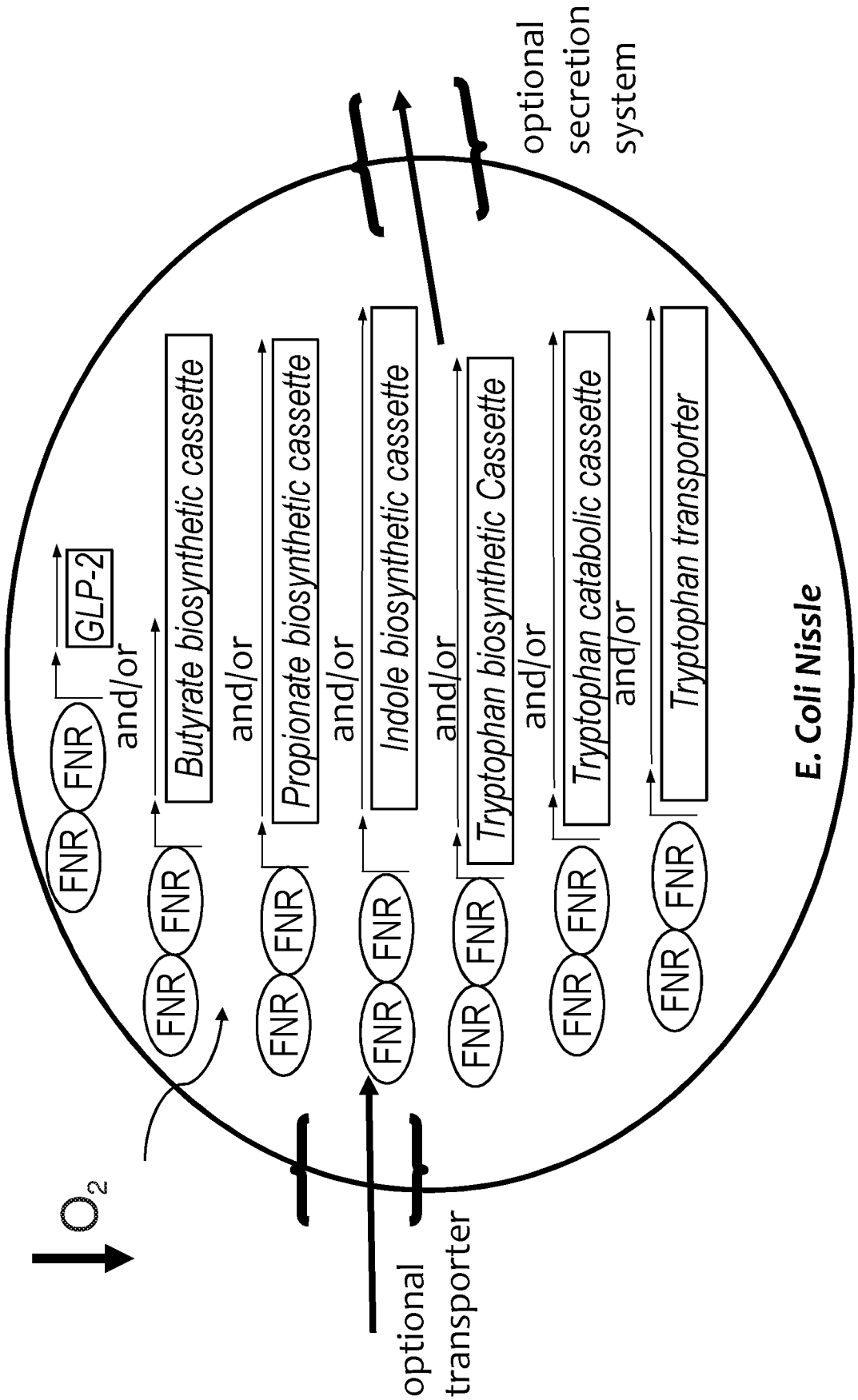
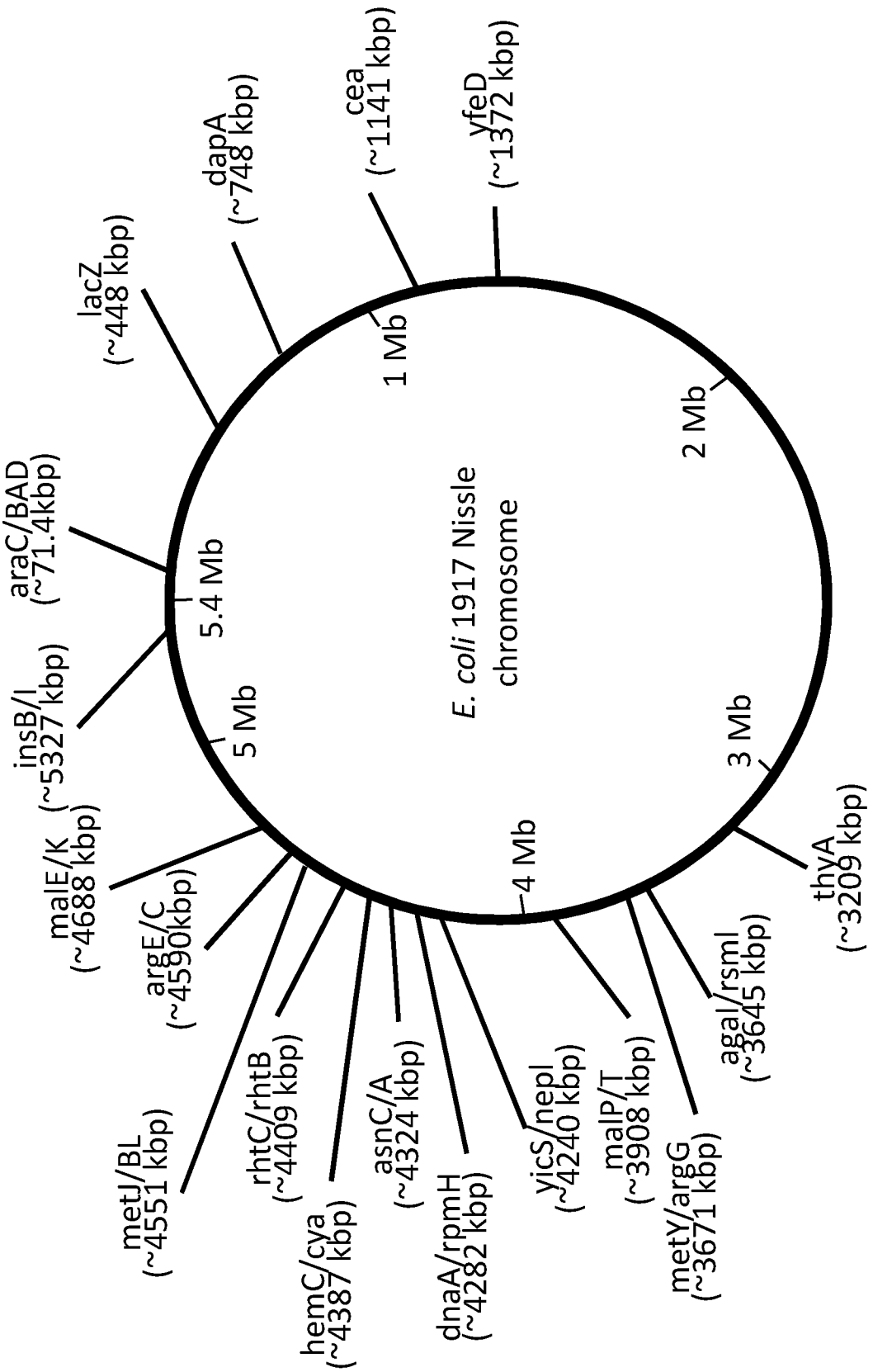

optional secretion system
E. Coli Nissle
optional transporter
O_2
FNR
FNR
GLP-2
and/or
Butyrate biosynthetic cassette
Propionate biosynthetic cassette
Indole biosynthetic cassette
Tryptophan biosynthetic Cassette
Tryptophan catabolic cassette
Tryptophan transporter

FIG. 52


E. coli 1917 Nissle chromosome
1 Mb
2 Mb
3 Mb
4 Mb
5 Mb
5.4 Mb
thyA (~3209 kbp)
agaI/rsmI (~3645 kbp)
metY/argG (~3671 kbp)
malP/T (~3908 kbp)
yicS/nepI (~4240 kbp)
dnaA/rpmH (~4282 kbp)
asnC/A (~4324 kbp)
hemC/cya (~4387 kbp)
rhtC/rhtB (~4409 kbp)
argE/C (~4590kbp)
malE/K (~4688 kbp)
metJ/BL (~4551 kbp)
insB/I (~5327 kbp)
araC/BAD (~71.4kbp)
lacZ (~448 kbp)
dapA (~748 kbp)
cea (~1141 kbp)
yfeD (~1372 kbp)

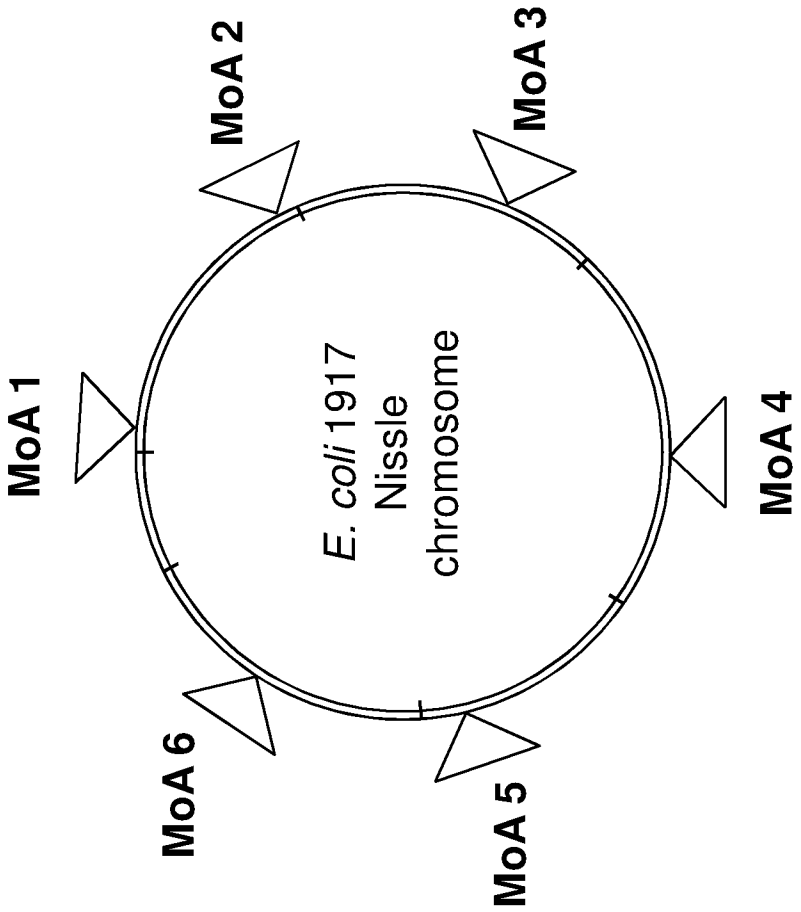


FIG. 53

FIG. 54A

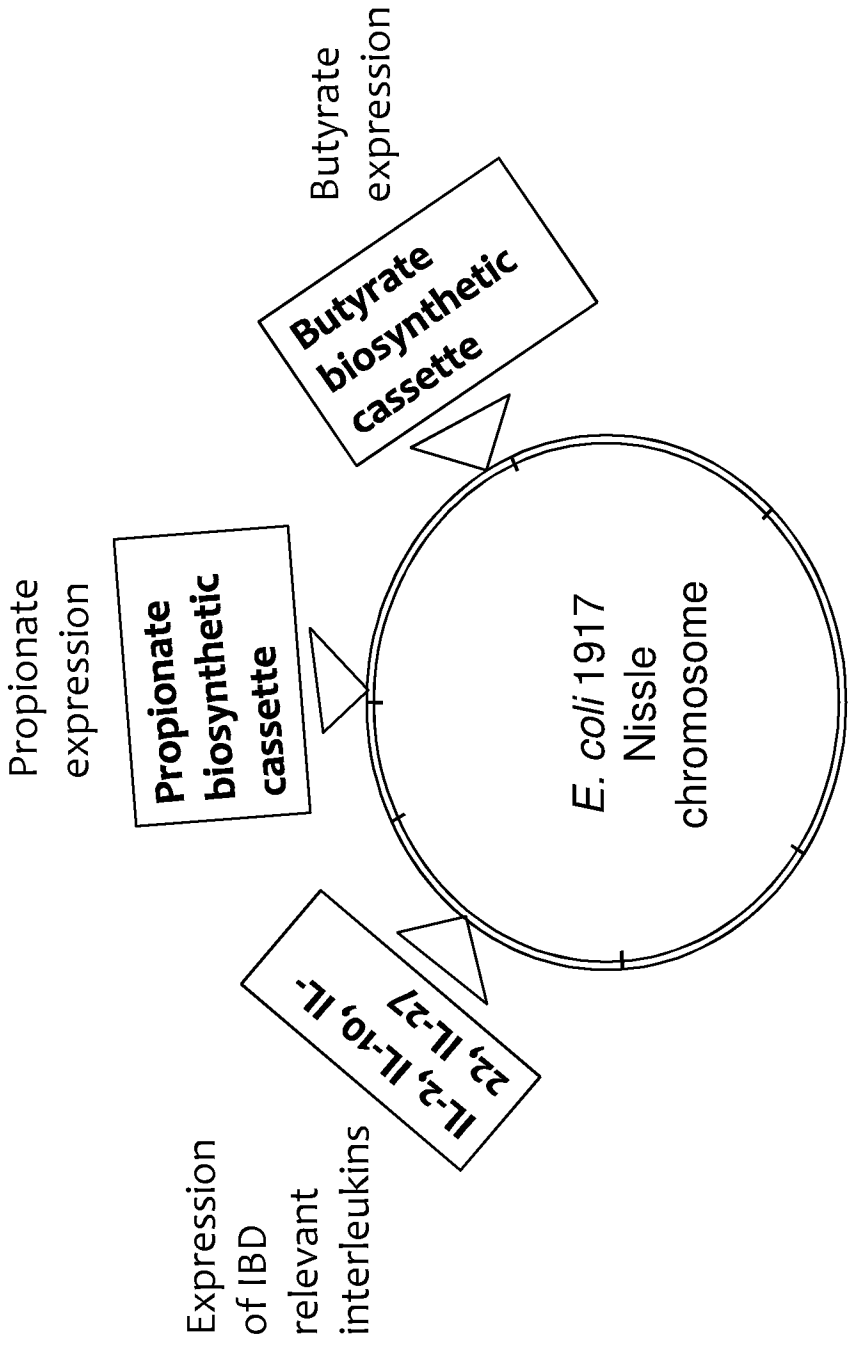
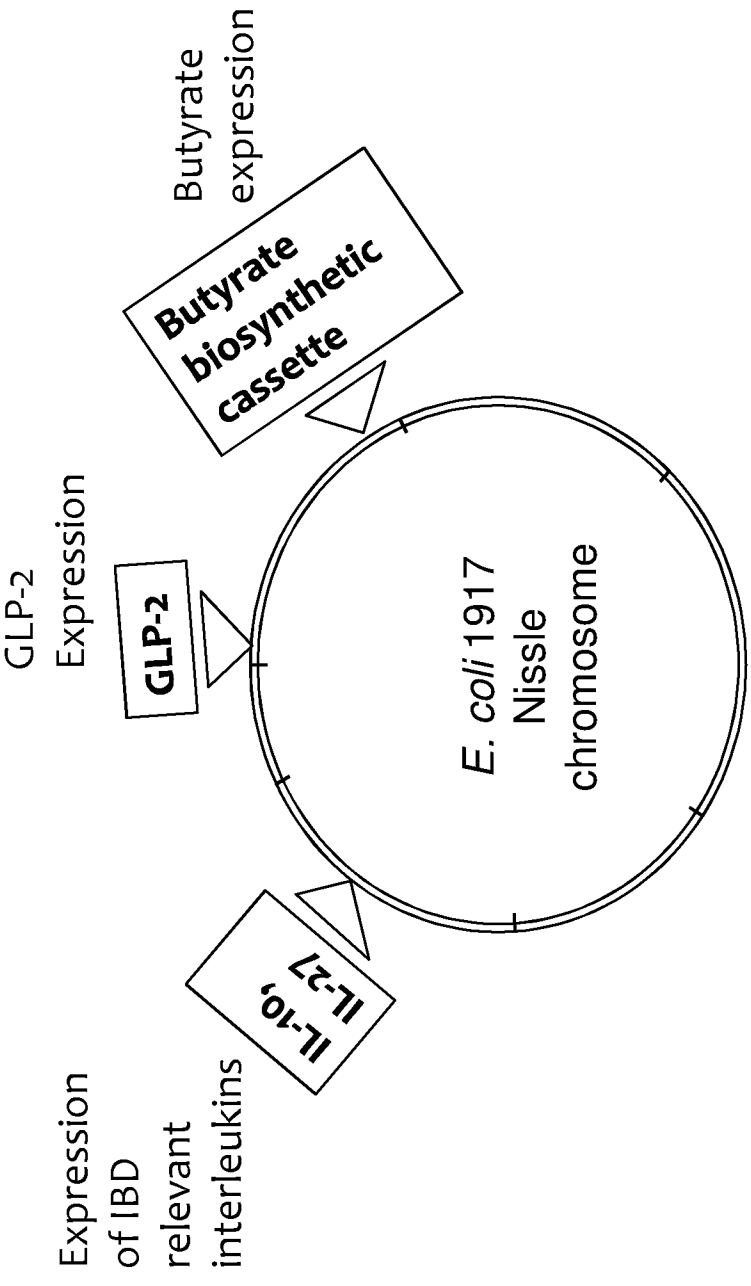

Butyrate expression
Butyrate biosynthetic cassette
Propionate expression
Propionate biosynthetic cassette
E. coli 1917 Nissle chromosome
Expression of IBD relevant interleukins
IL-2, IL-10, IL-22, IL-27

FIG. 54B


Butyrate expression
Butyrate biosynthetic cassette
GLP-2 Expression
GLP-2
E. coli 1917 Nissle chromosome
Expression of IBD relevant interleukins
IL-10, IL-27

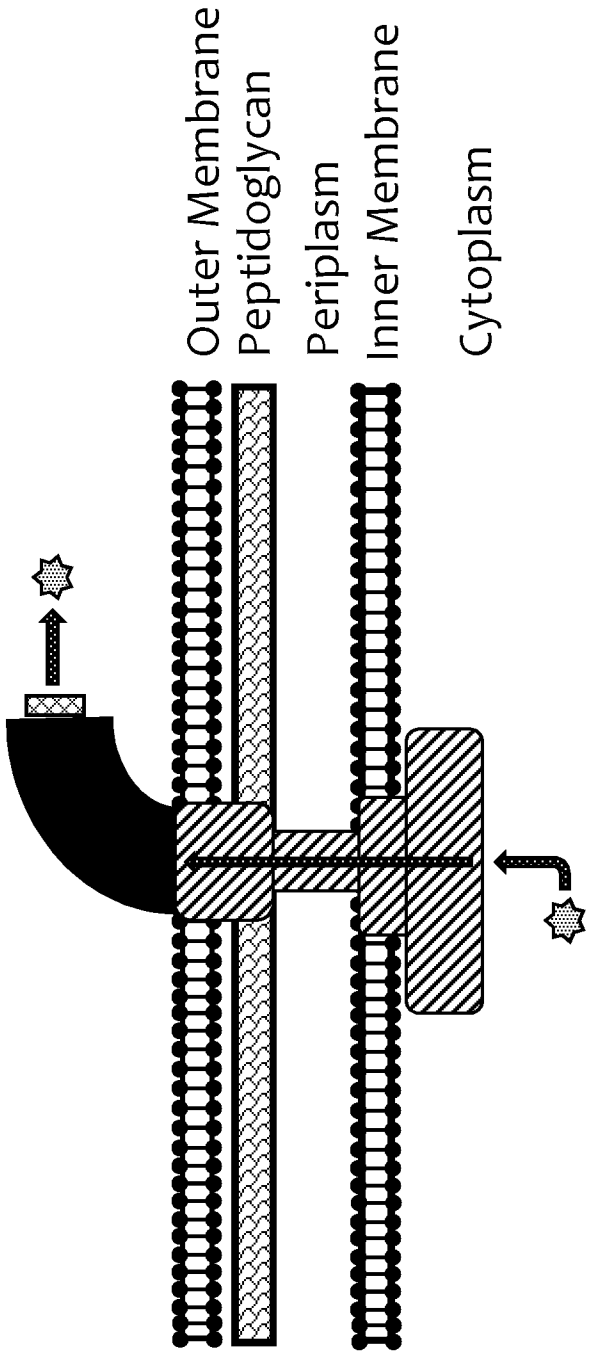

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm

FIG. 55

FIG. 56

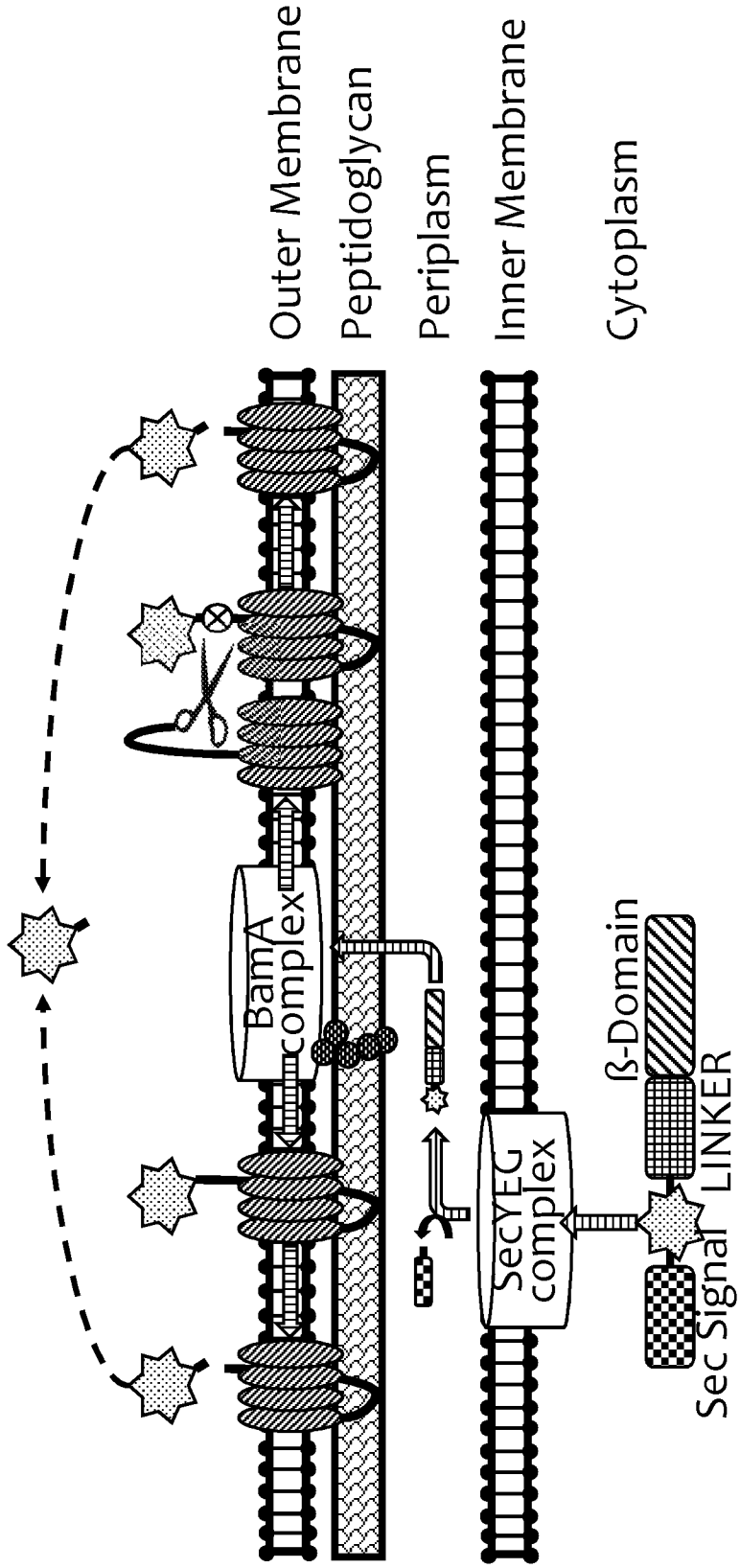

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
BamA complex
SecYEG complex
ß-Domain
LINKER
Sec Signal

FIG. 57

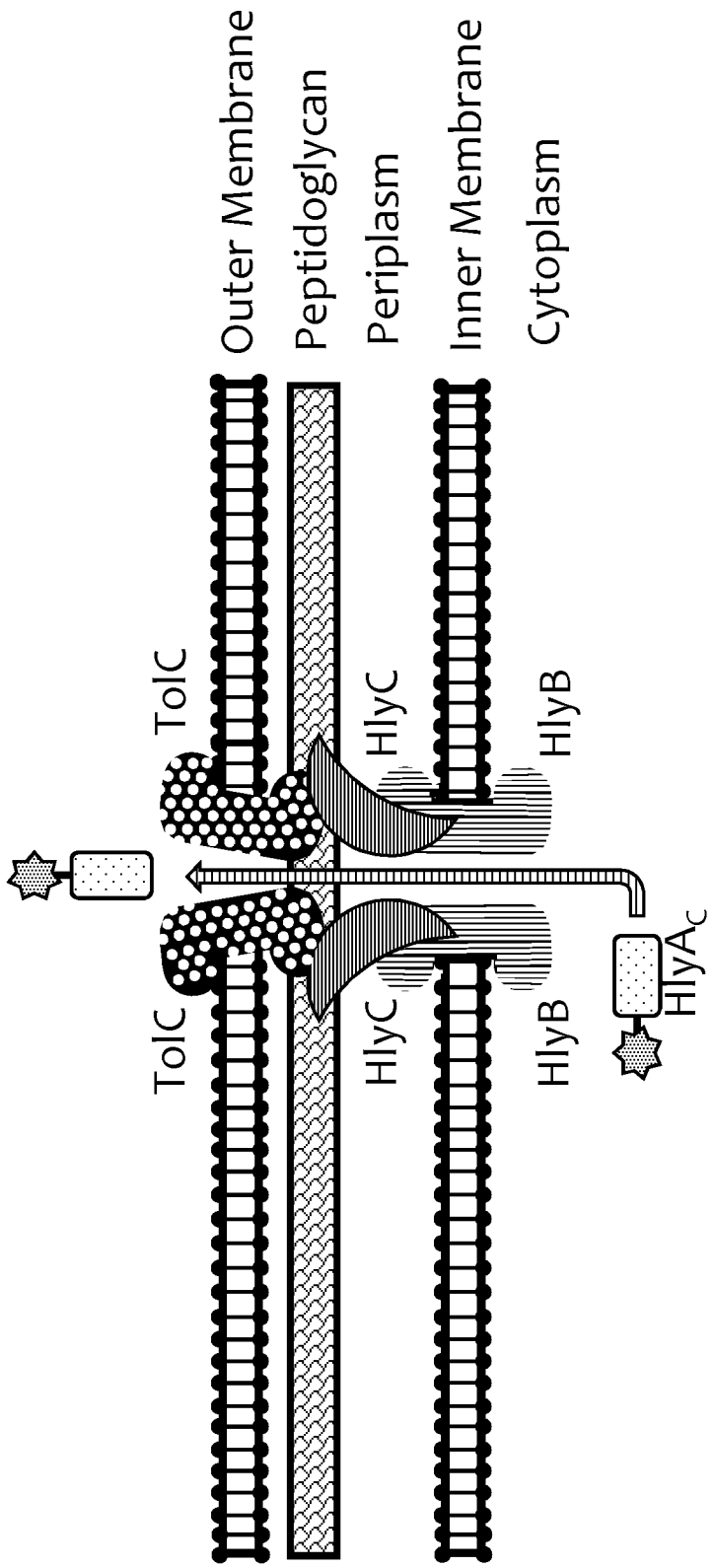
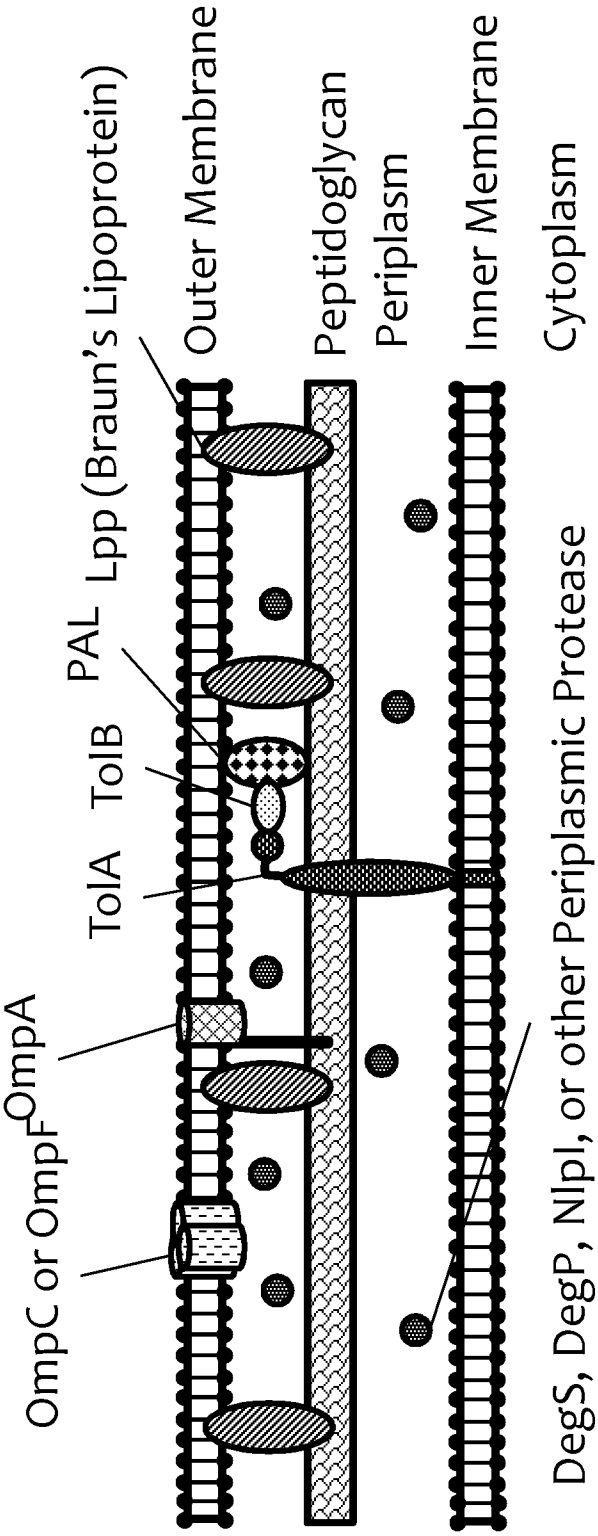

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
TolC
HlyC
HlyB
TolC
HlyC
HlyB
HlyA$_C$

FIG. 58


OmpC or OmpF
OmpA
TolA
TolB
PAL
Lpp (Braun's Lipoprotein)
Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
DegS, DegP, NlpI, or other Periplasmic Protease

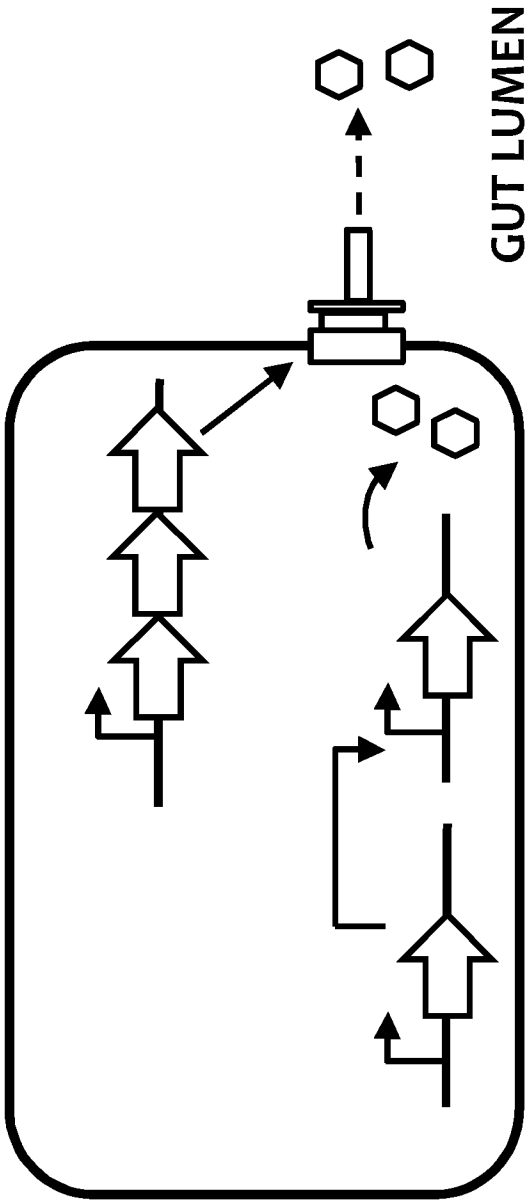


FIG. 59

FIG. 60A

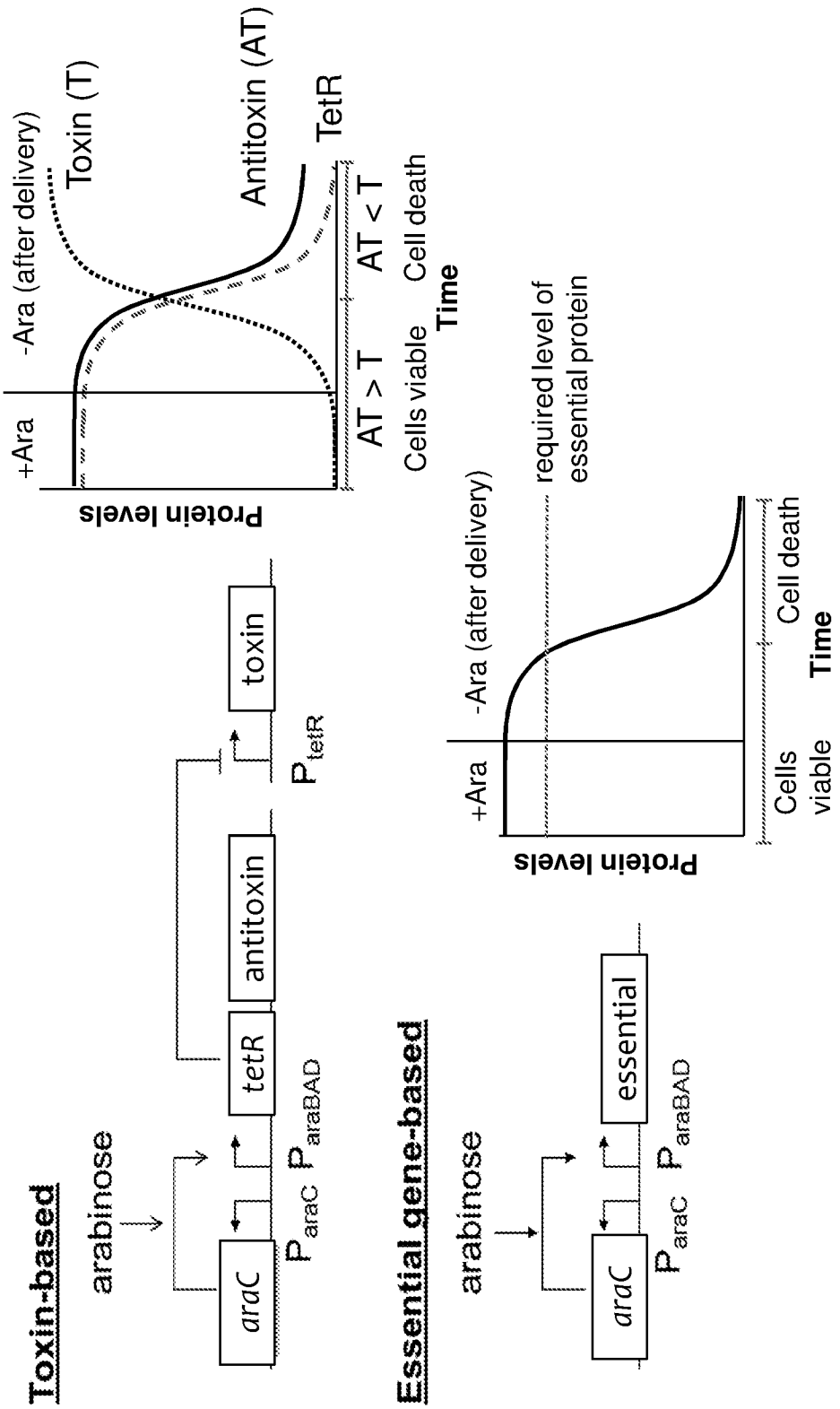

Toxin-based
arabinose
araC
tetR
antitoxin
toxin
P araC
P araBAD
P tetR
+Ara
-Ara (after delivery)
Toxin (T)
Antitoxin (AT)
TetR
Protein levels
AT > T
AT < T
Cells viable
Cell death
Time
Essential gene-based
arabinose
araC
essential
P araC
P araBAD
+Ara
-Ara (after delivery)
required level of essential protein
Protein levels
Cells viable
Cell death
Time

FIG. 60B

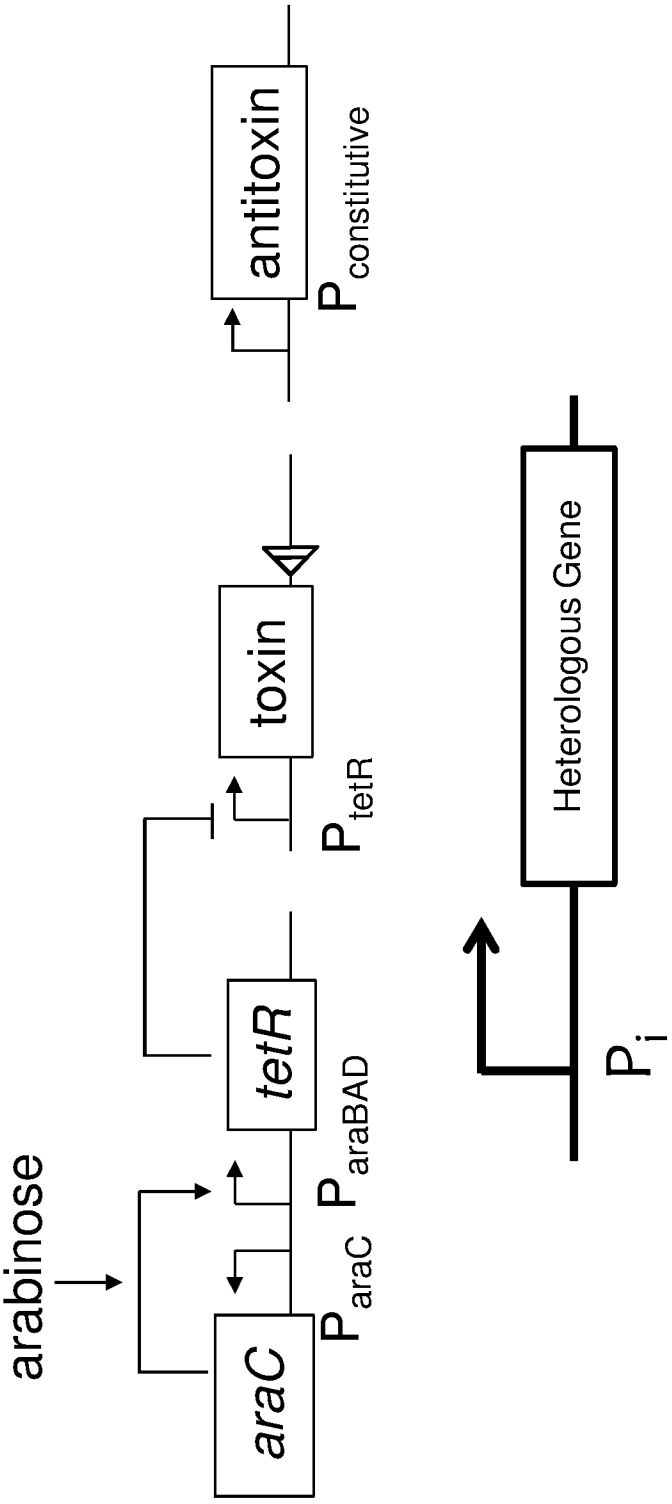

arabinose
araC
P araC
tetR
P araBAD
toxin
P tetR
antitoxin
P constitutive
Heterologous Gene
P i

FIG. 60C

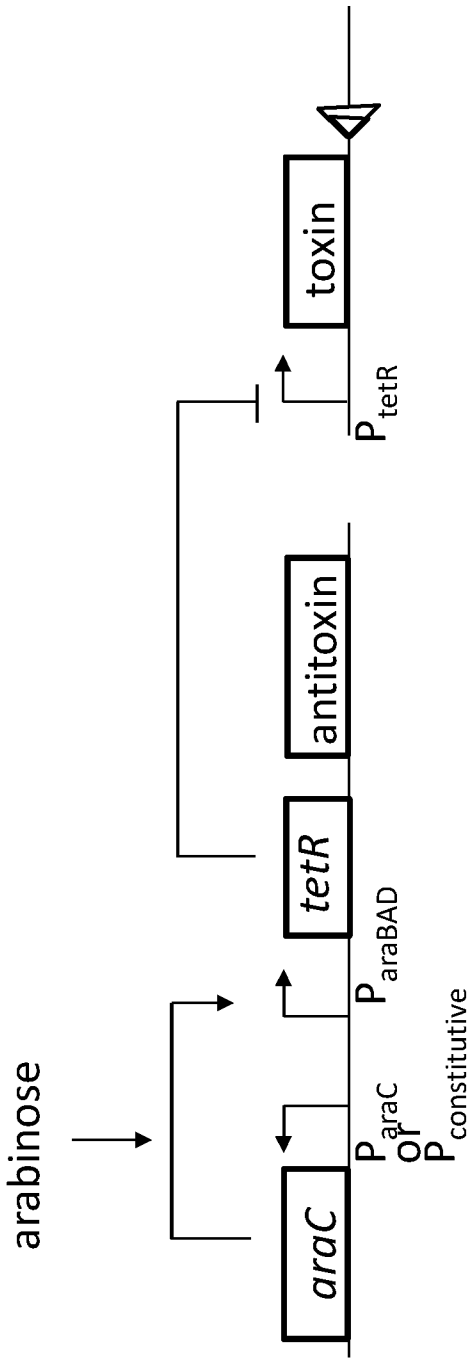

arabinose
araC
P araC or P constitutive
tetR
P araBAD
antitoxin
toxin
P tetR

FIG. 61

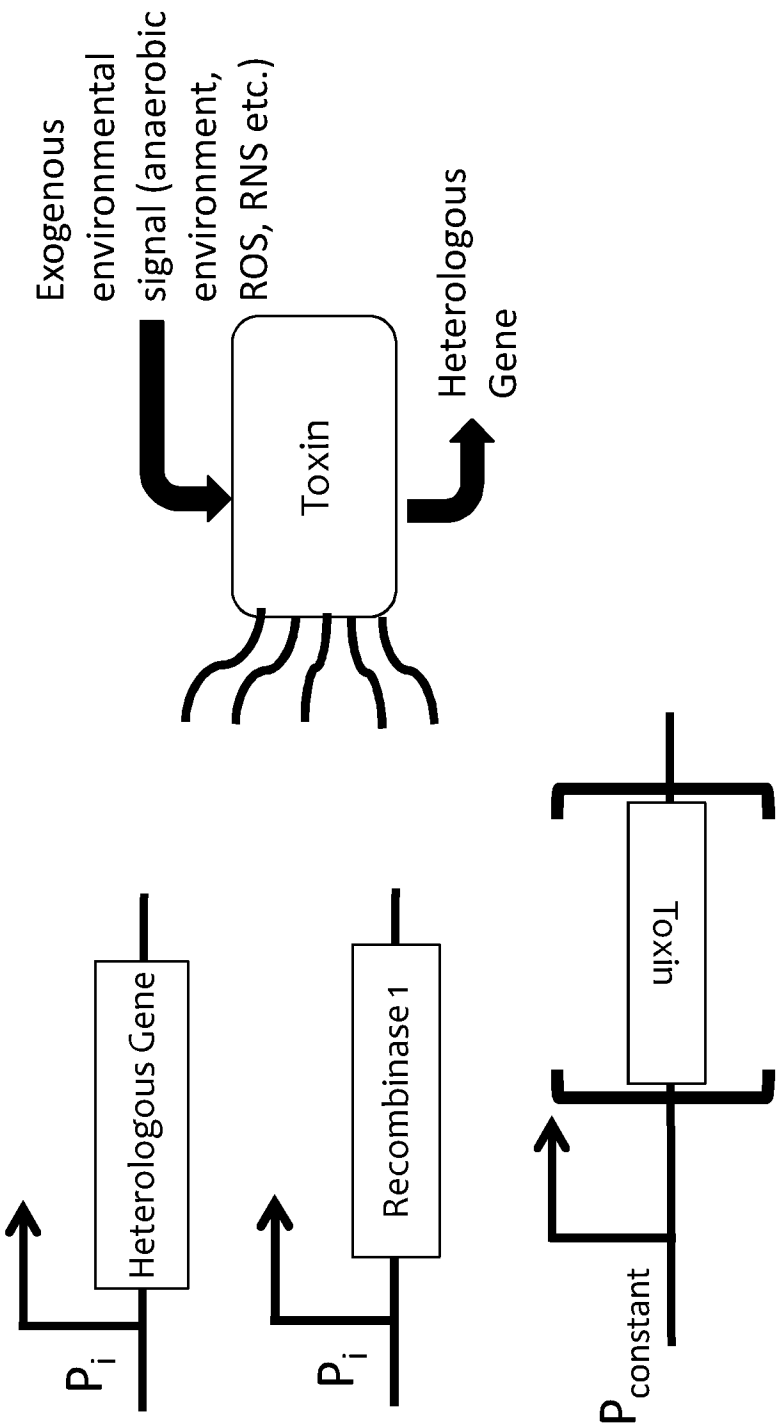

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
Heterologous Gene
P_i
Recombinase 1
P_i
Toxin
$P_{constant}$

FIG. 62

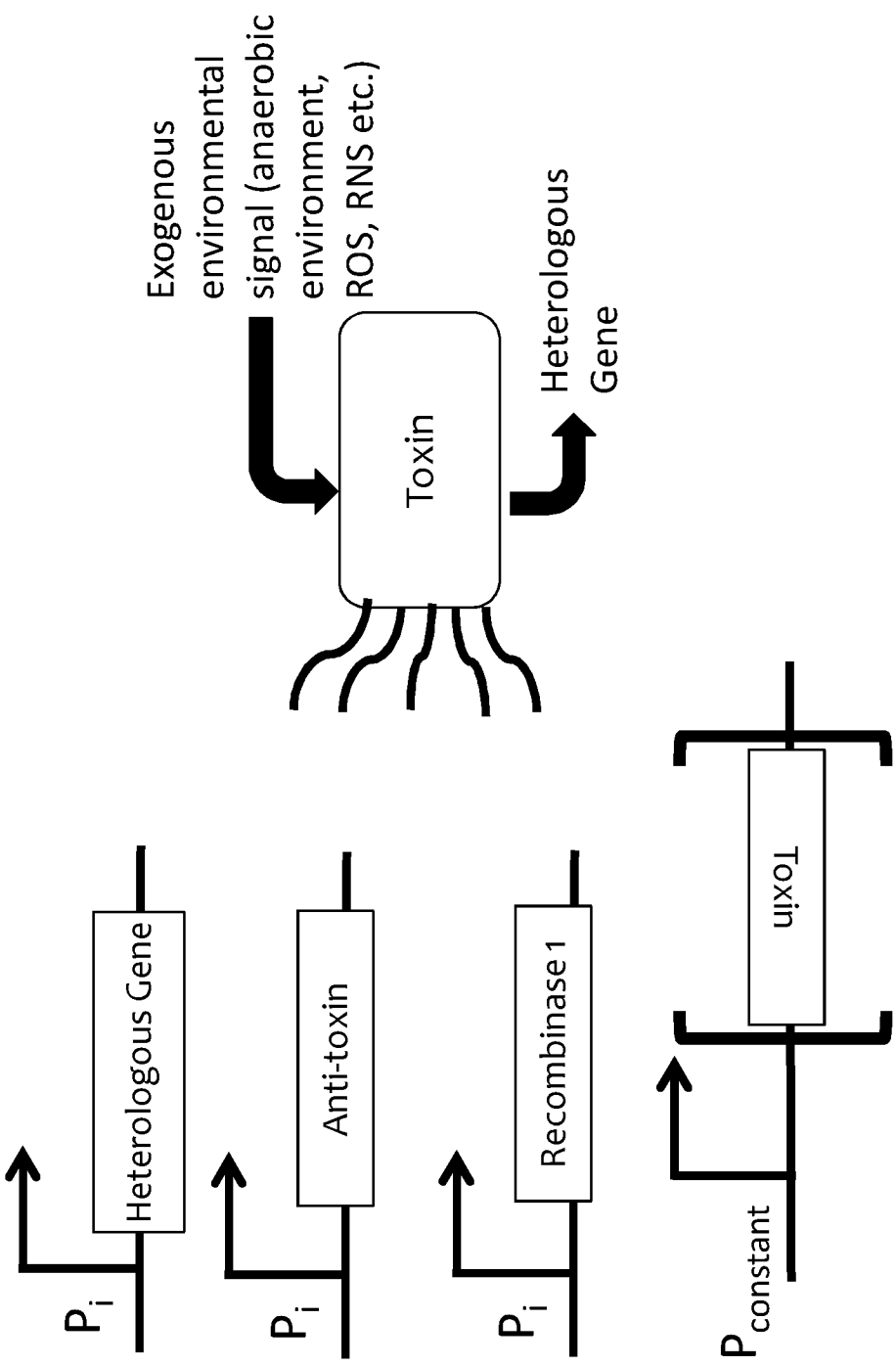

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
Heterologous Gene
Anti-toxin
Recombinase 1
Toxin
P_i
P_i
P_i
P_constant

FIG. 63

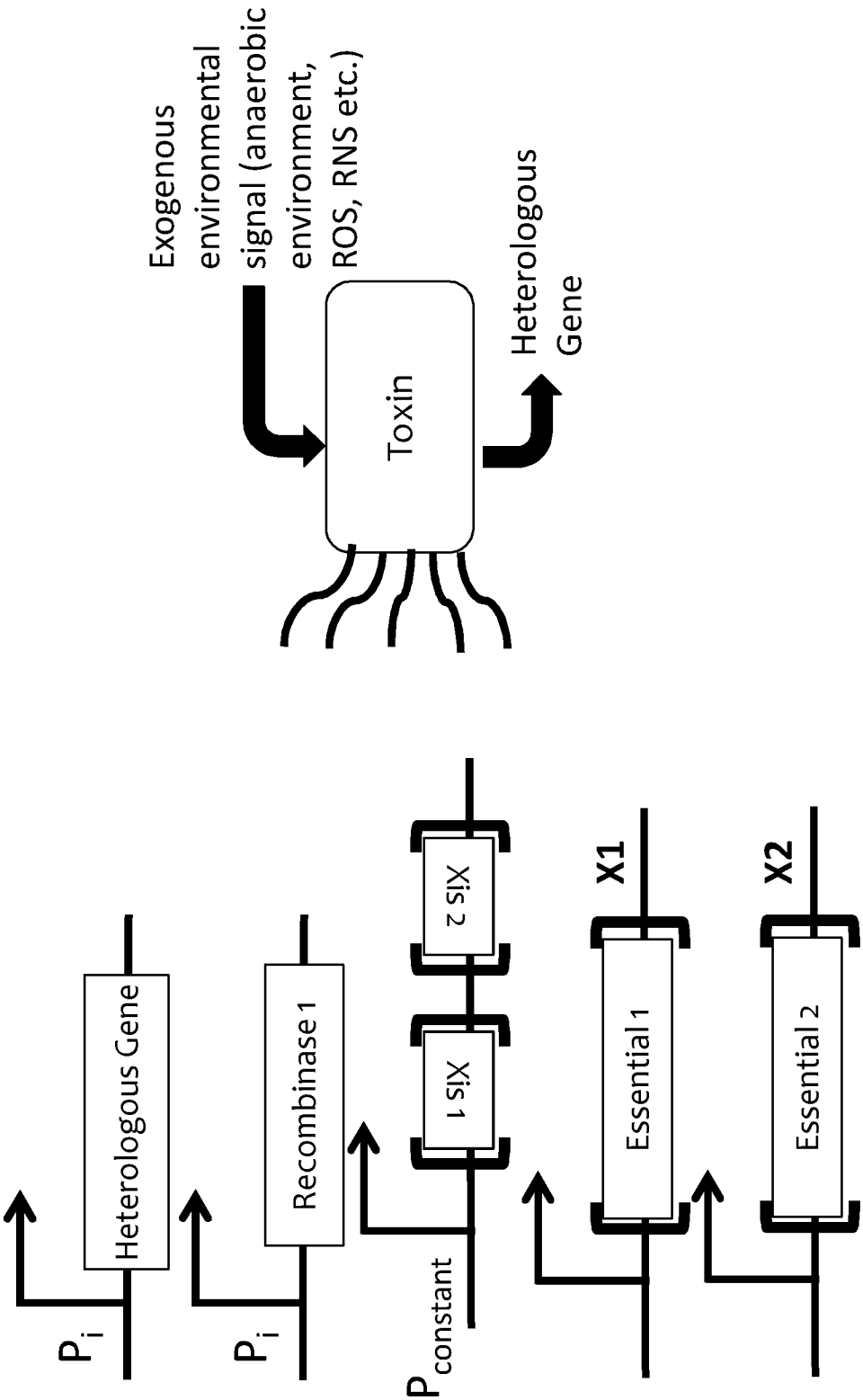

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
Heterologous Gene
Recombinase 1
Xis 1
Xis 2
X1
X2
Essential 1
Essential 2
P_i
P_i
P_constant

FIG. 64

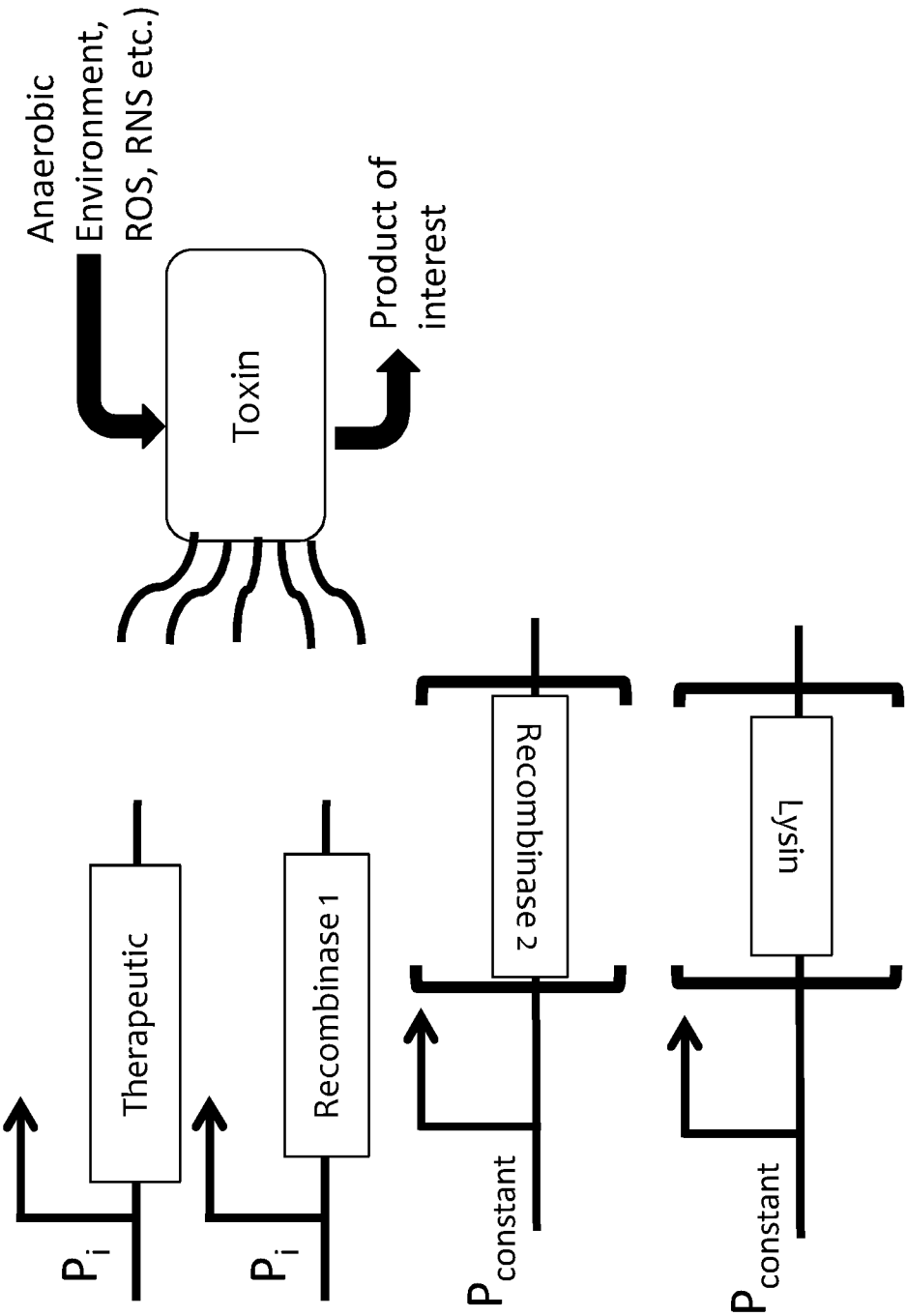

Anaerobic Environment, ROS, RNS etc.)
Toxin
Product of interest
Therapeutic
Recombinase 1
Recombinase 2
Lysin
P_i
P_i
$P_{constant}$
$P_{constant}$

FIG. 65

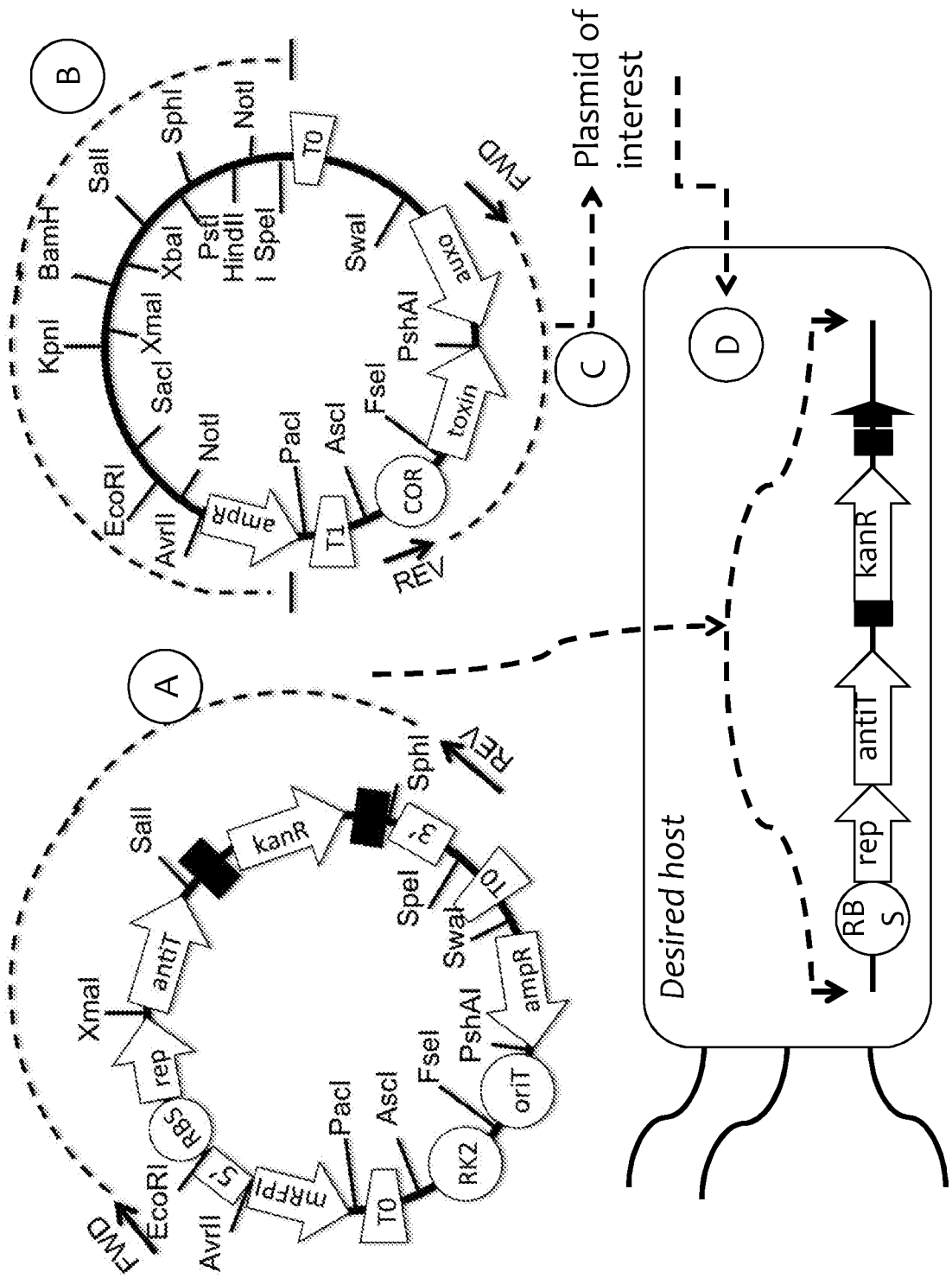

A
B
C
D
Plasmid of interest
Desired host
FWD
REV
EcoRI
AvrII
RBS
rep
XmaI
antiT
SalI
kanR
SphI
3'
SpeI
T0
SwaI
ampR
oriT
PshAI
FseI
AscI
PacI
mRFPI
5'
RK2
T1
NotI
ampR
SacI
XmaI
KpnI
BamH
SalI
SphI
NotI
XbaI
PstI
HindIII
I SpeI
T0
SwaI
oxyR
PshAI
toxin
FseI
AscI
COR
PacI
T1
NotI
ampR
AvrII
EcoRI
RBS
rep
antiT
kanR

FIG. 66

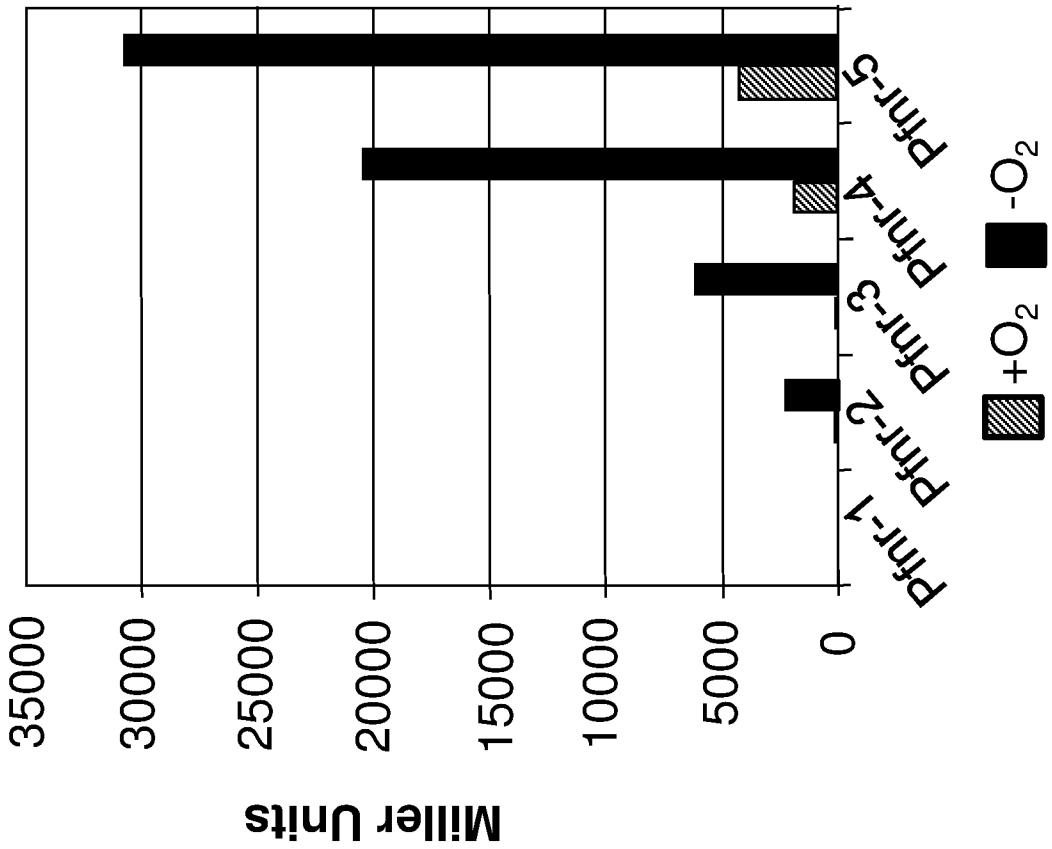

Miller Units
35000
30000
25000
20000
15000
10000
5000
0
Pfnr-1
Pfnr-2
Pfnr-3
Pfnr-4
Pfnr-5
-O2
+O2

FIG. 67A

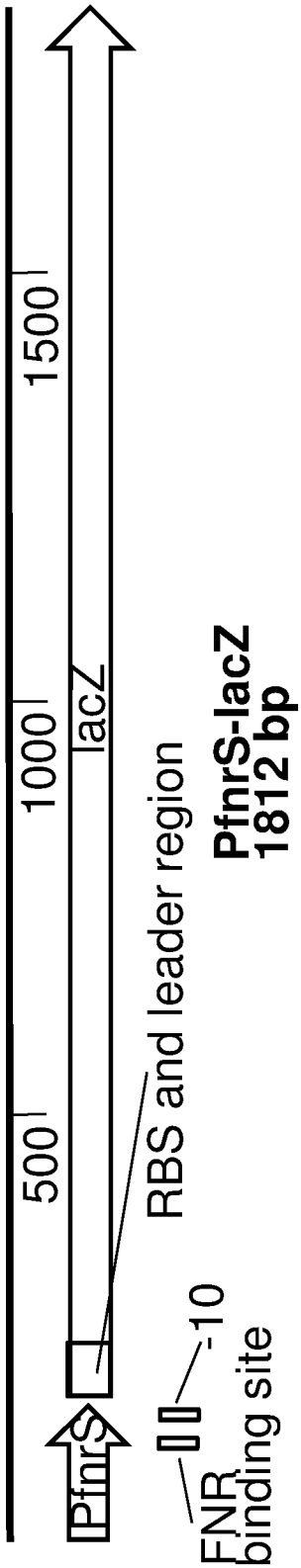


FIG. 67B

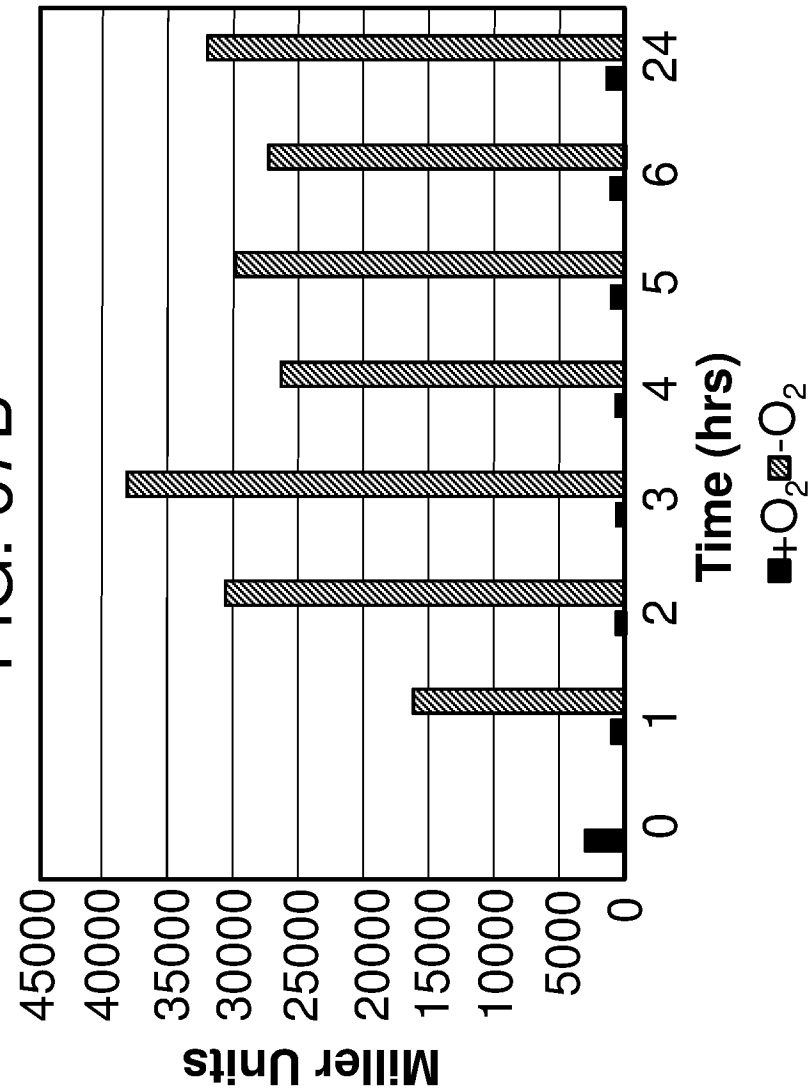


FIG. 67C

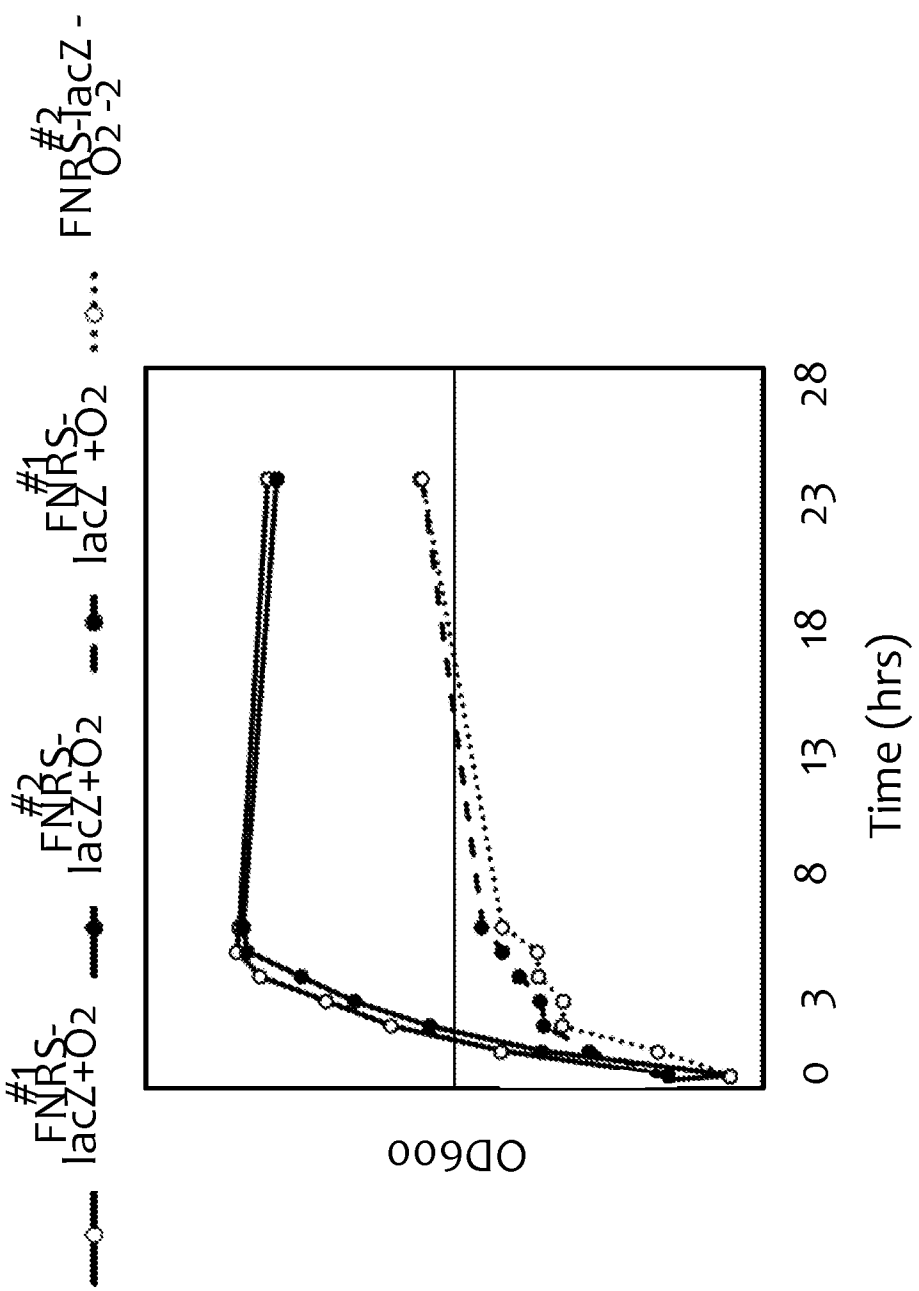

FNR#1-lacZ+O2
FNR#2-lacZ+O2
FNR#1-lacZ+O2
FNR#2-lacZ-O2-2
OD600
0
3
8
13
18
23
28
Time (hrs)

FIG. 68A

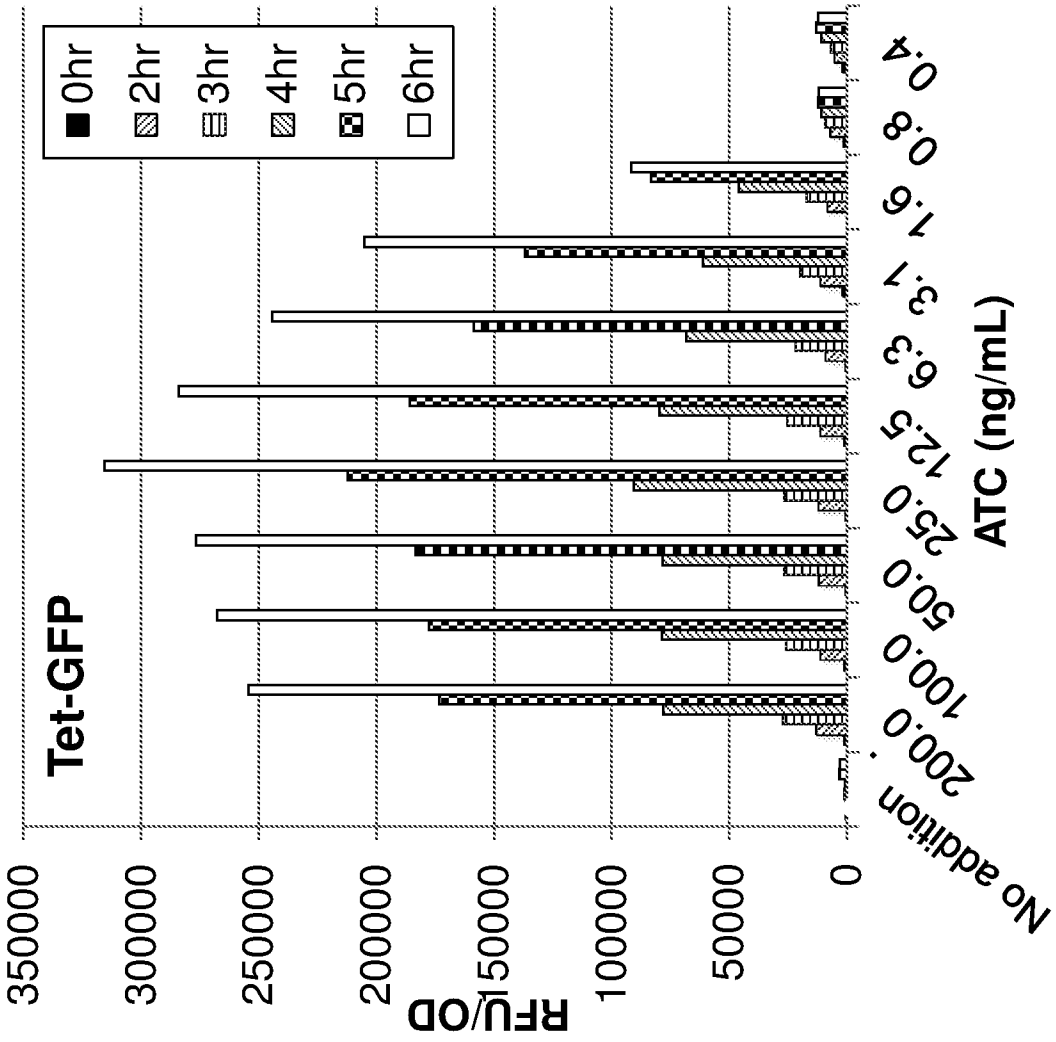
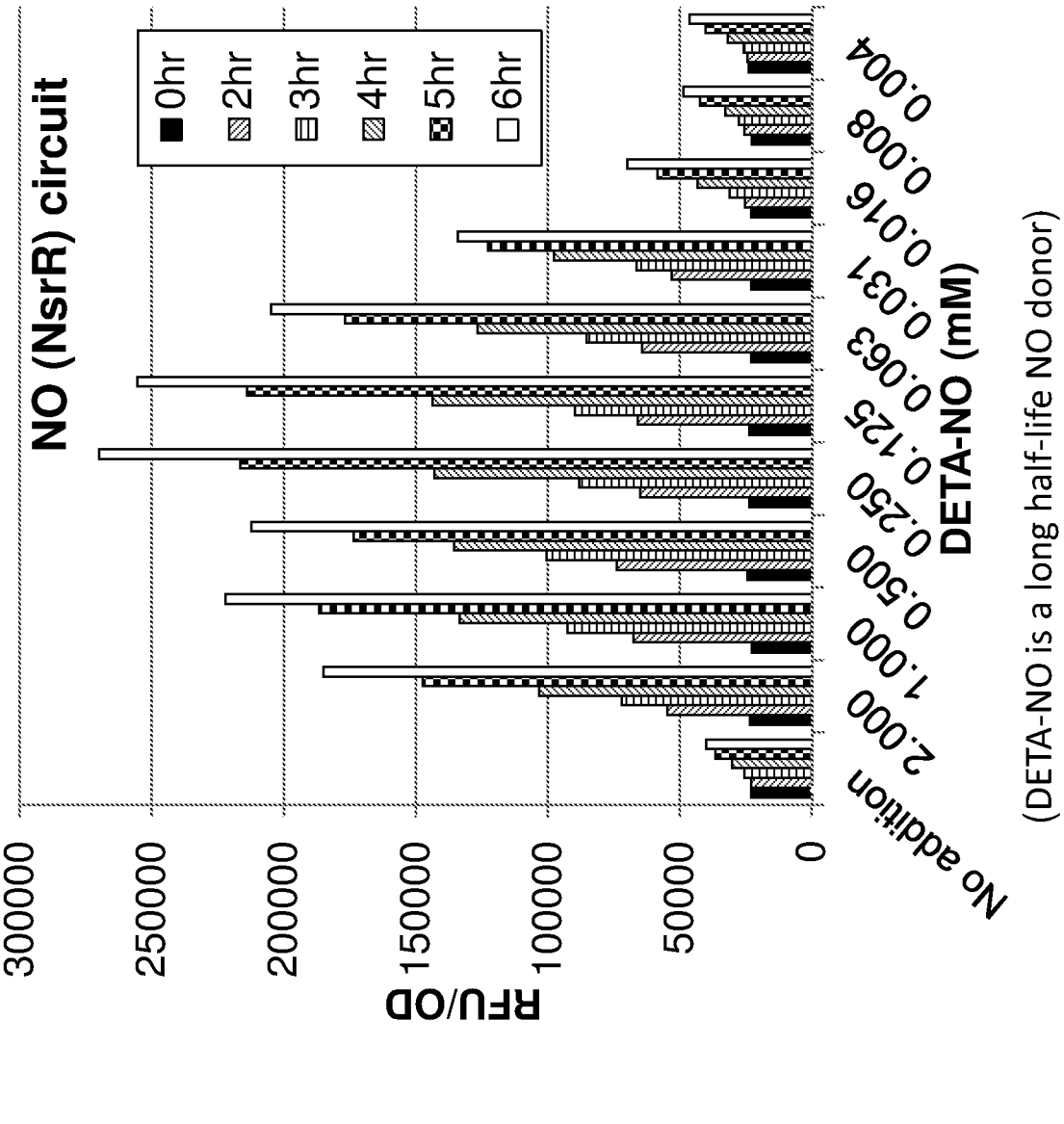

Tet-GFP
0hr
2hr
3hr
4hr
5hr
6hr
RFU/OD
350000
300000
250000
200000
150000
100000
50000
0
No addition
200.0
100.0
50.0
25.0
12.5
6.3
3.1
1.6
0.8
0.4
ATC (ng/mL)

FIG. 68B


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)

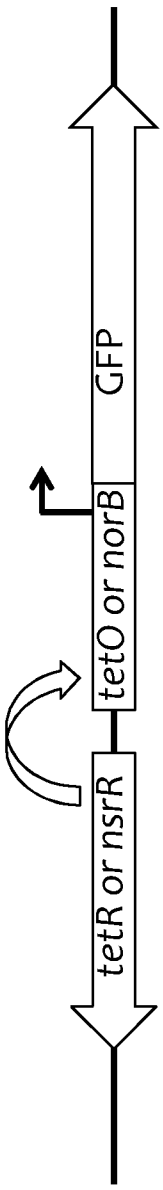


FIG. 68C

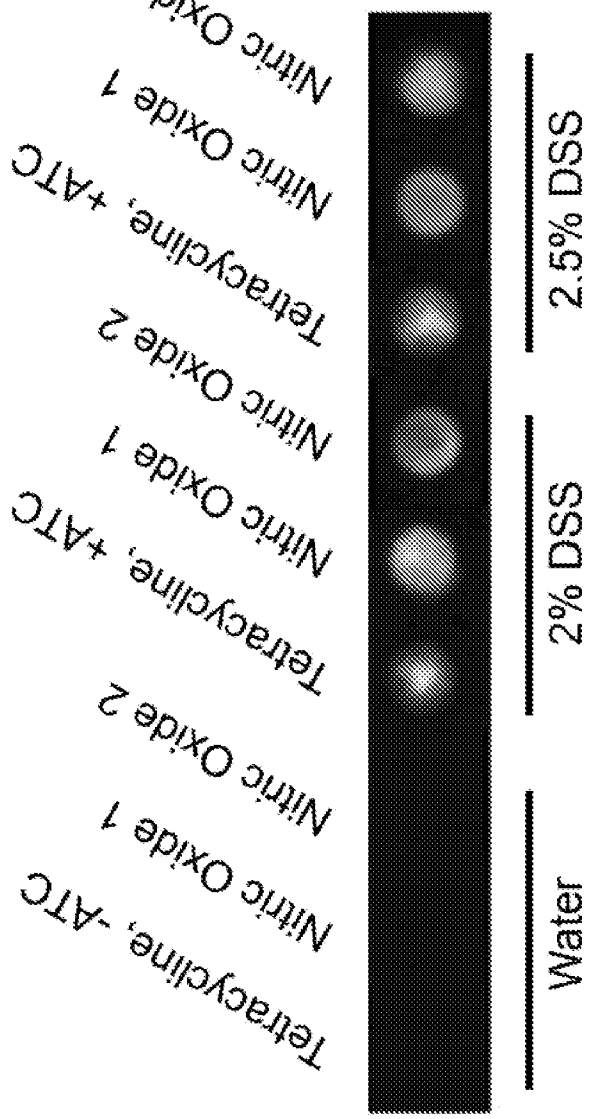

Tetracycline, -ATC
Nitric Oxide 1
Nitric Oxide 2
Tetracycline, +ATC
Nitric Oxide 1
Nitric Oxide 2
Water
2% DSS
2.5% DSS

FIG. 68D

FIG. 69

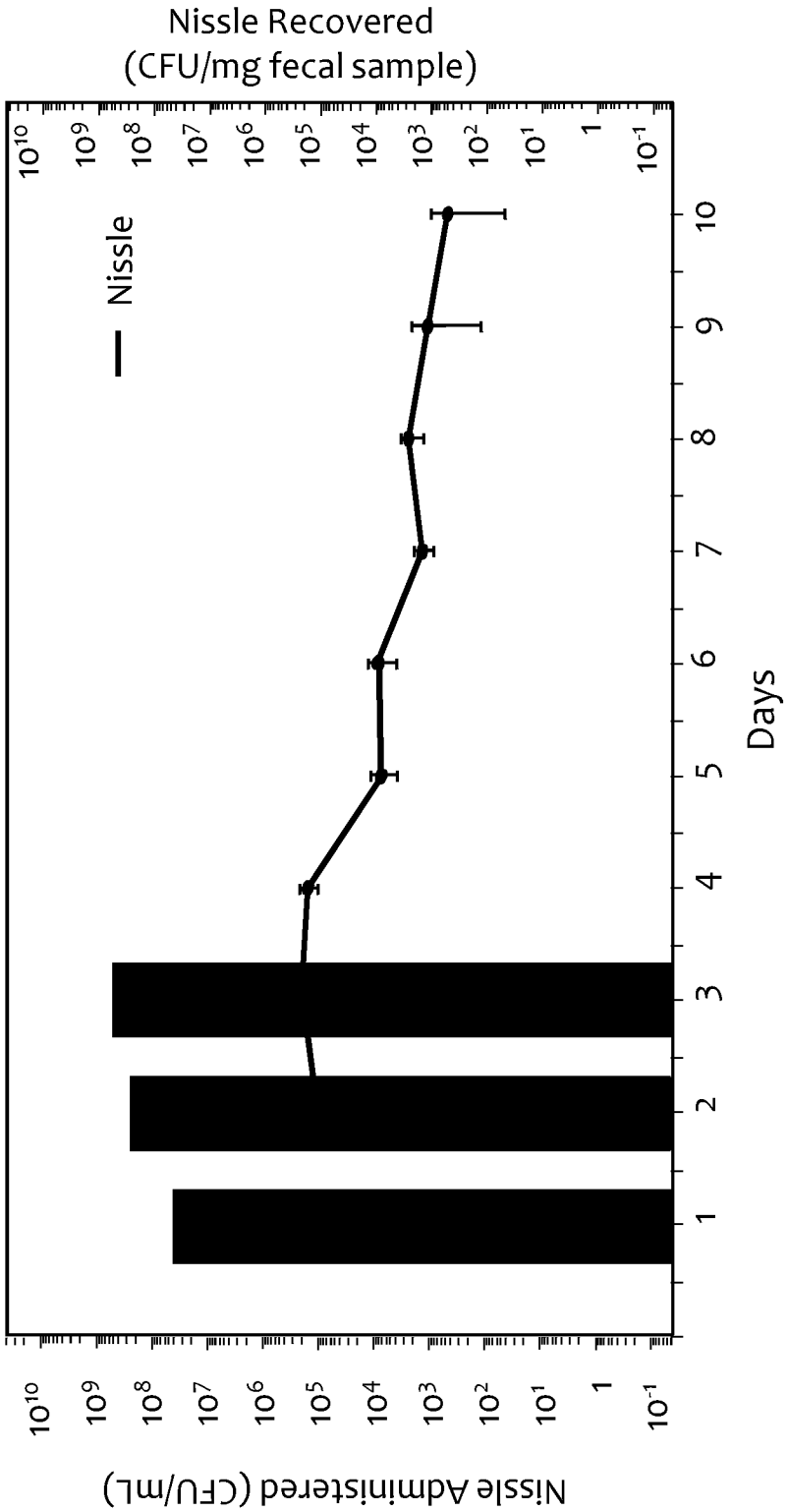

Nissle Recovered
(CFU/mg fecal sample)
Nissle
Days
Nissle Administered (CFU/mL)

FIG. 70

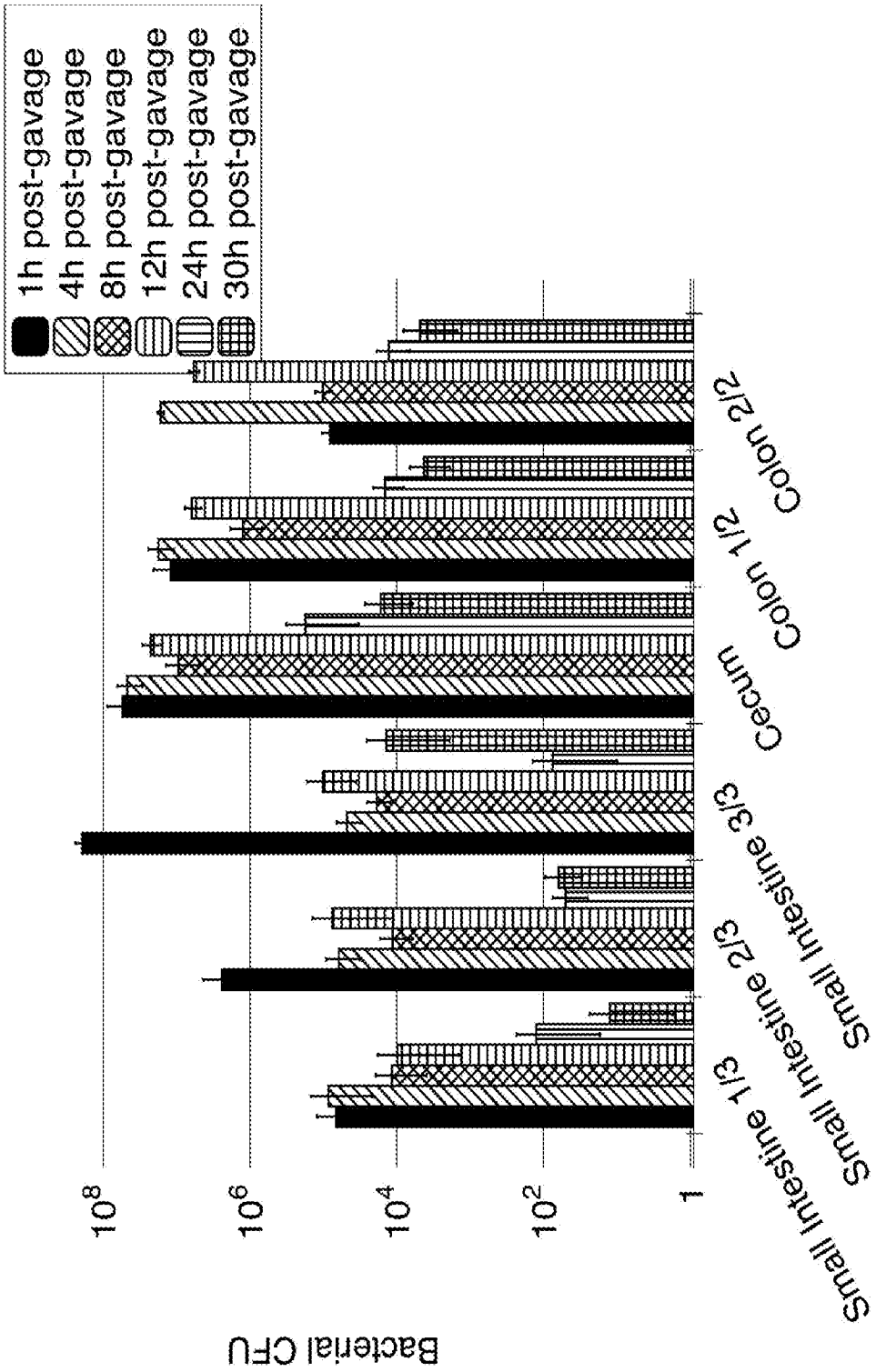
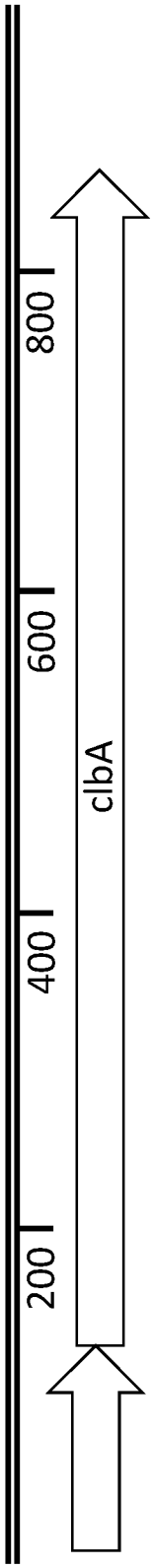
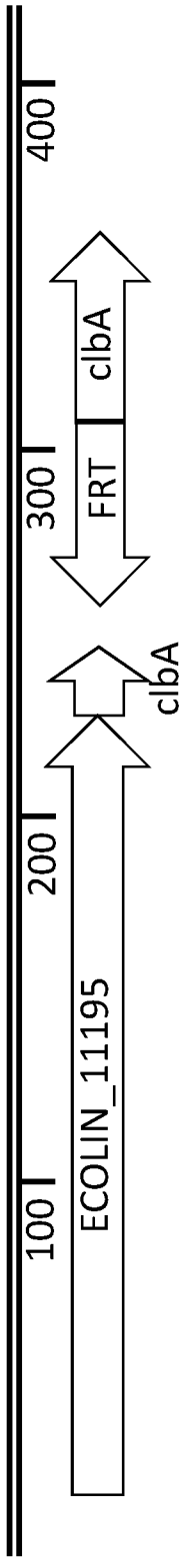

1h post-gavage
4h post-gavage
8h post-gavage
12h post-gavage
24h post-gavage
30h post-gavage
Bacterial CFU
10^8
10^6
10^4
10^2
1
Small Intestine 1/3
Small Intestine 2/3
Small Intestine 3/3
Cecum
Colon 1/2
Colon 2/2

FIG. 71A



clbA
967 bp

FIG. 71B



clbA KO
424 bp

FIG. 72

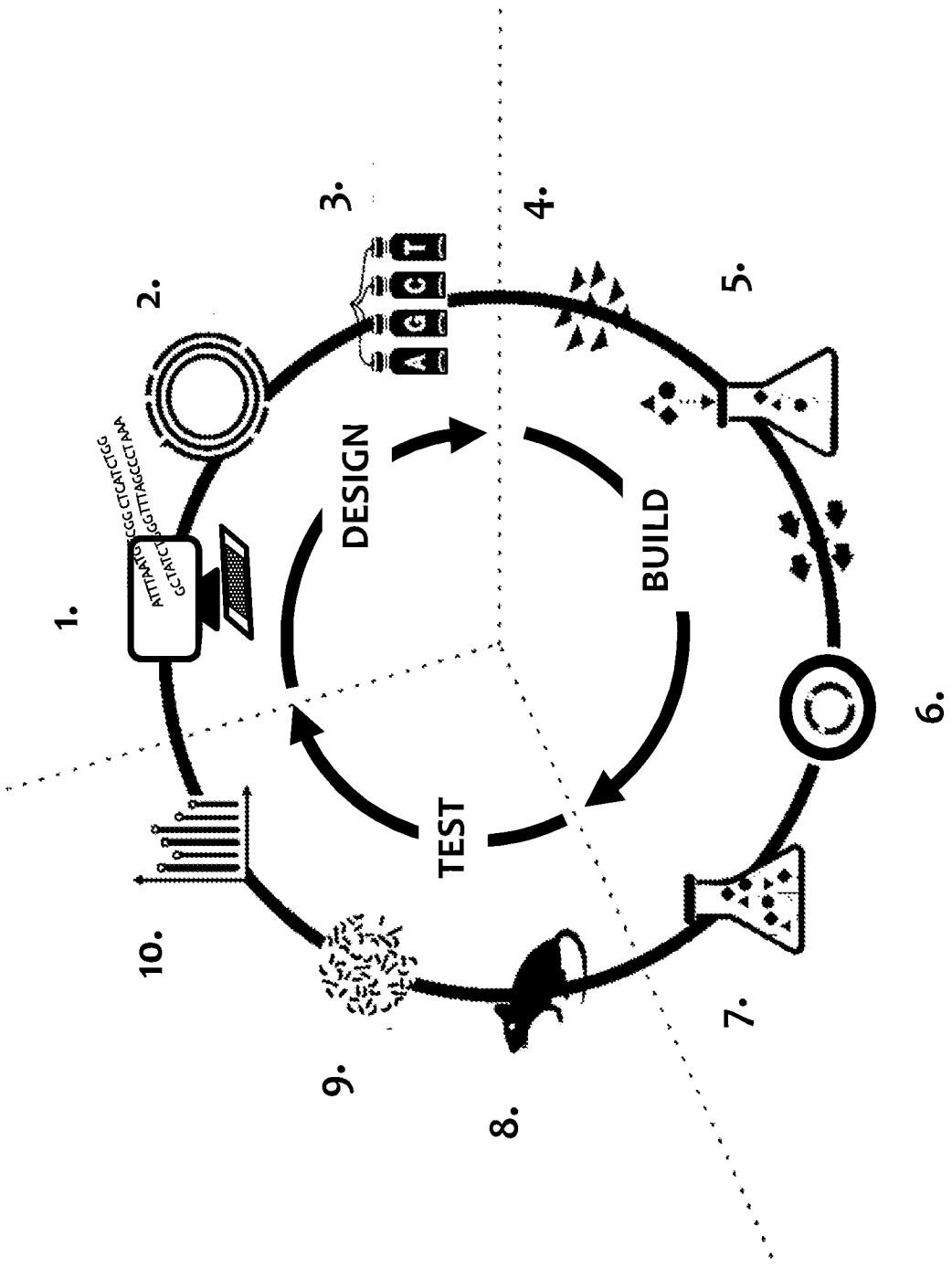
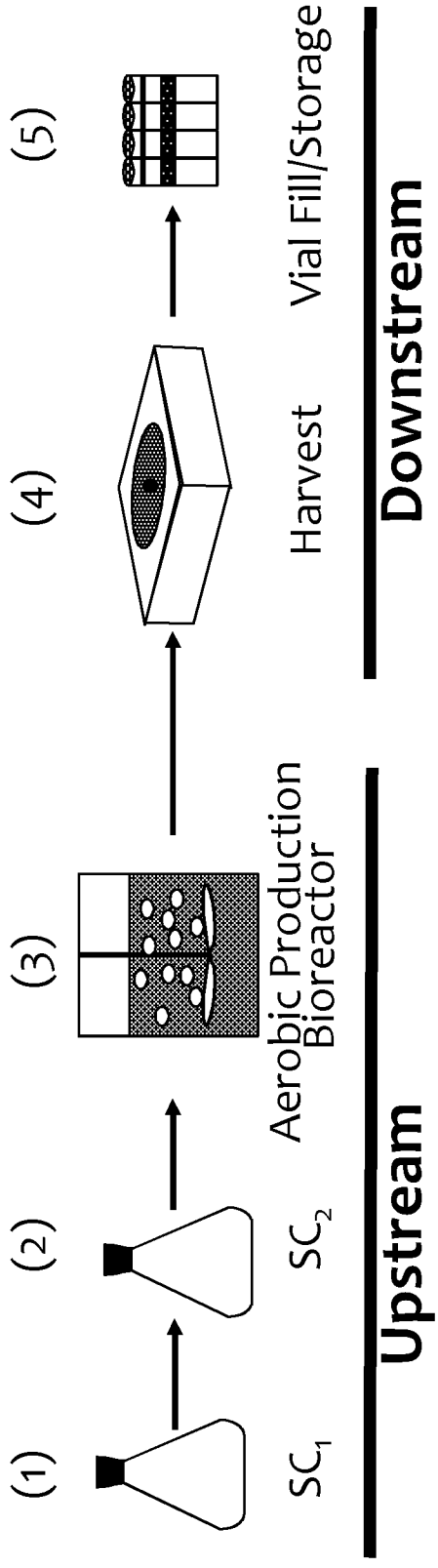


FIG. 73


(1)
(2)
(3)
(4)
(5)
SC_1
SC_2
Aerobic Production Bioreactor
Harvest
Vial Fill/Storage
Upstream
Downstream

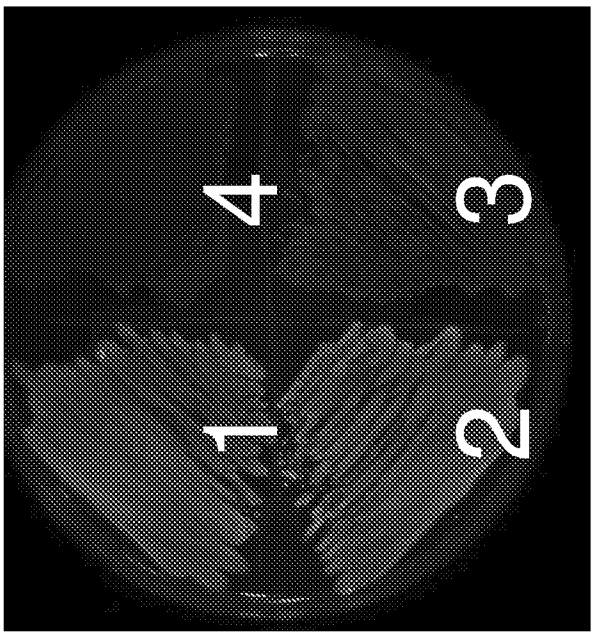


FIG. 74

Brightness of constitutive RFP integrated in three locations:

1. AraB/C
2. MalE/K
3. MetY/ArgG
4. Nissle (non-fluorescent)

FIG. 75A

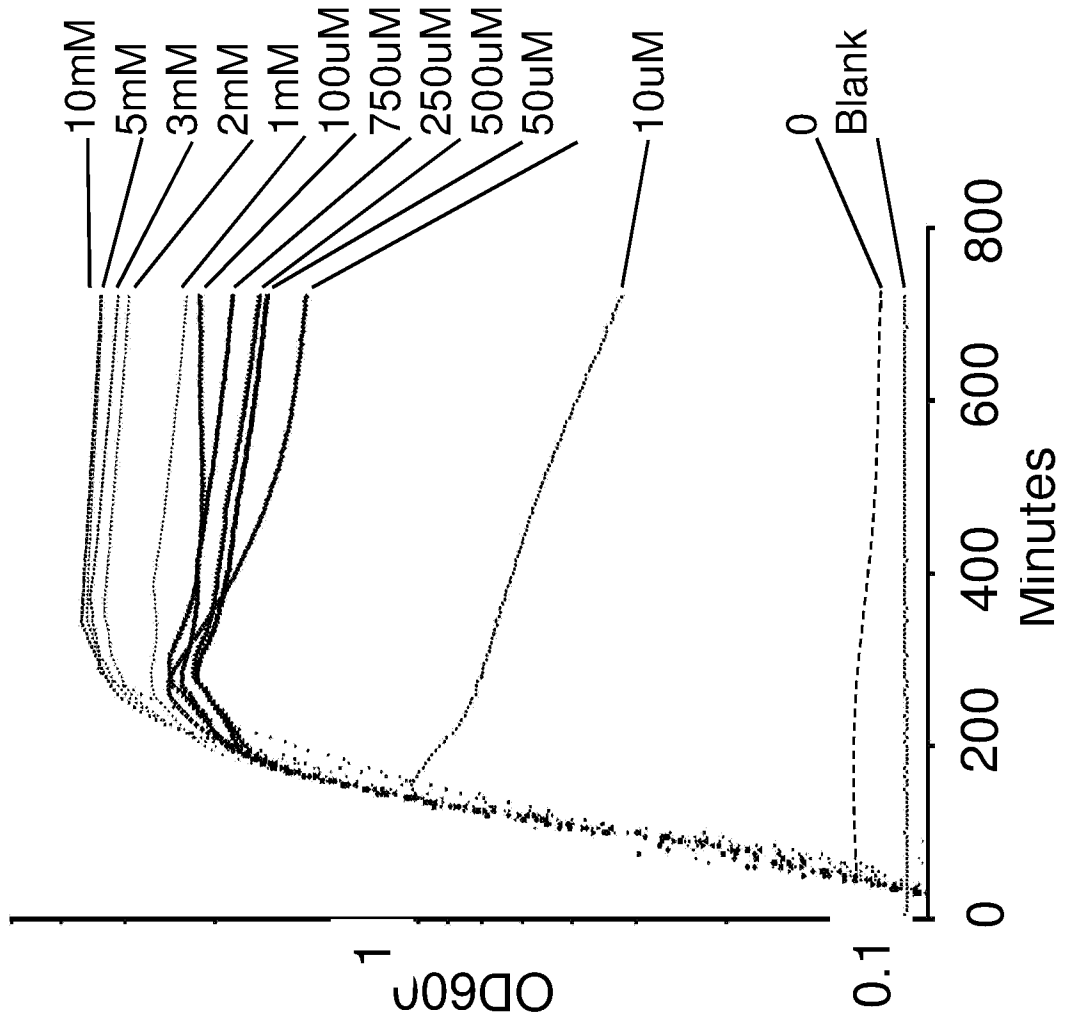

10mM
5mM
3mM
2mM
1mM
100uM
750uM
250uM
500uM
50uM
10uM
0
Blank
OD60n
1
0.1
0
200
400
600
800
Minutes

FIG. 75B

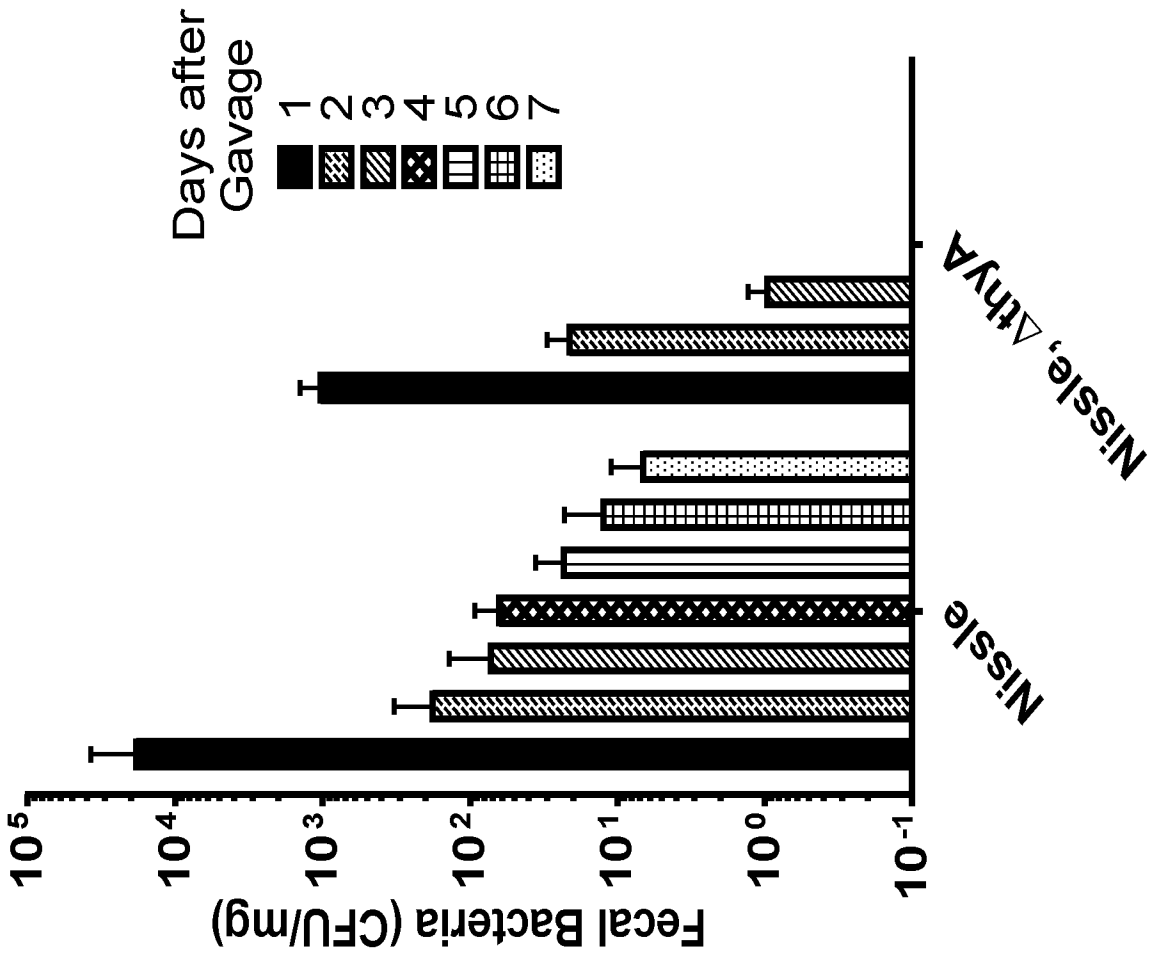

Days after Gavage
1
2
3
4
5
6
7
Fecal Bacteria (CFU/mg)
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
Nissle
Nissle, ΔthyA

FIG. 76

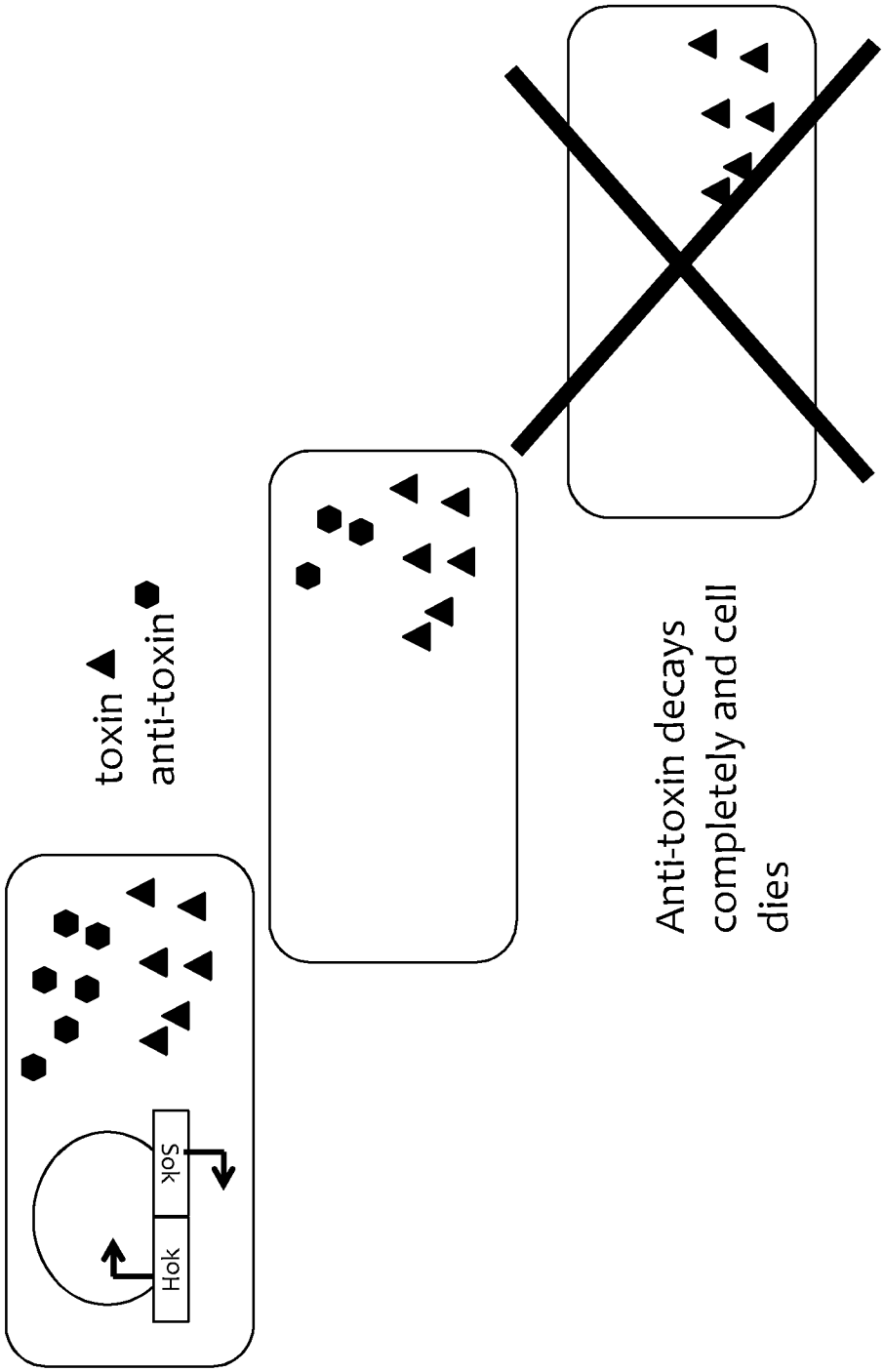

toxin
anti-toxin
Hok
Sok
Anti-toxin decays completely and cell dies

FIG. 77A

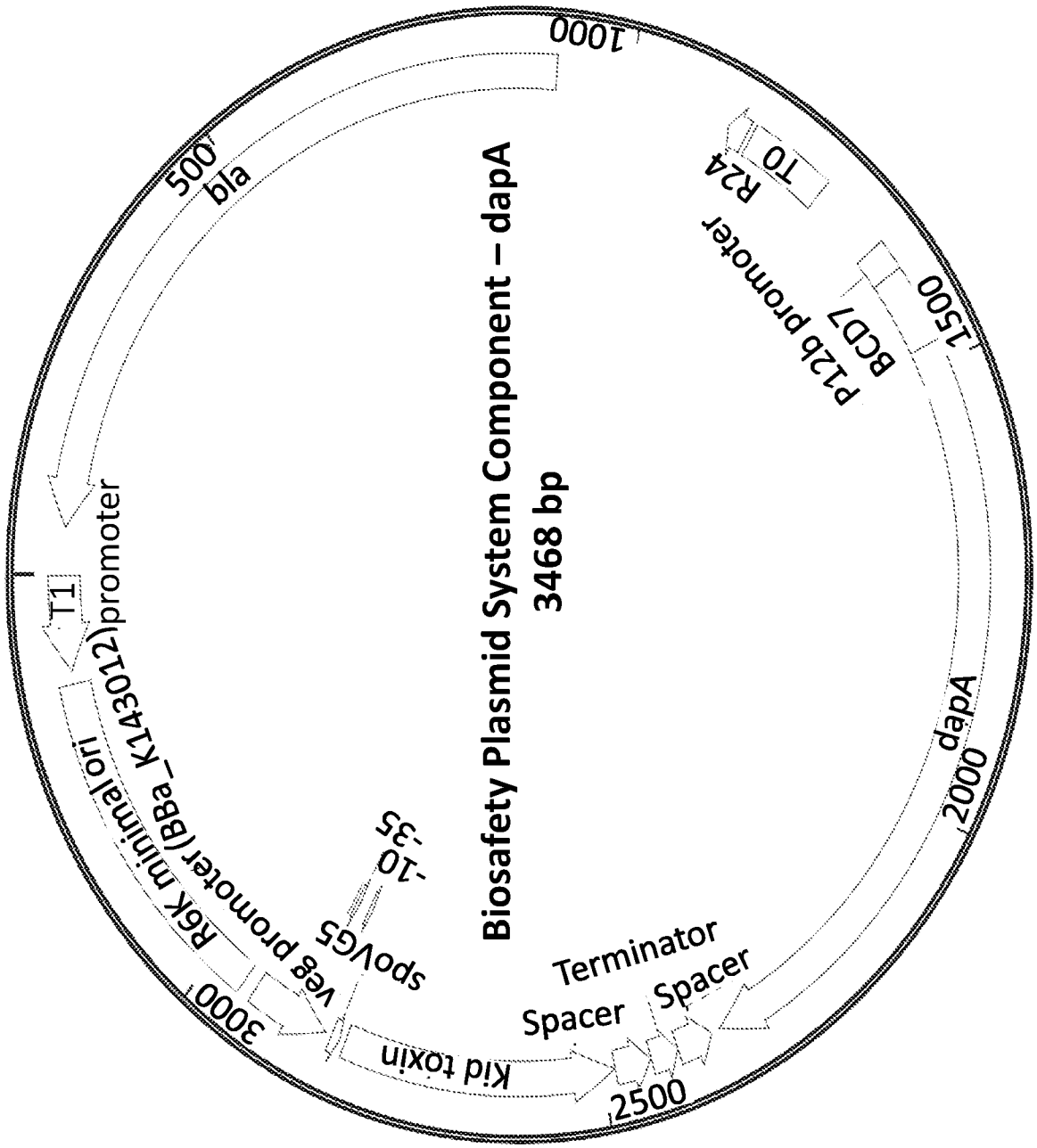

Biosafety Plasmid System Component – dapA
3468 bp
1000
500
bla
T0
R2a
p12b promoter
BCD7
1500
dapA
2000
T1
promoter
R6K minimal ori
vgb promoter (BBa_K143012)
-10
-35
spoVG5
3000
Kid toxin
Spacer
Terminator
Spacer
2500

FIG. 77B

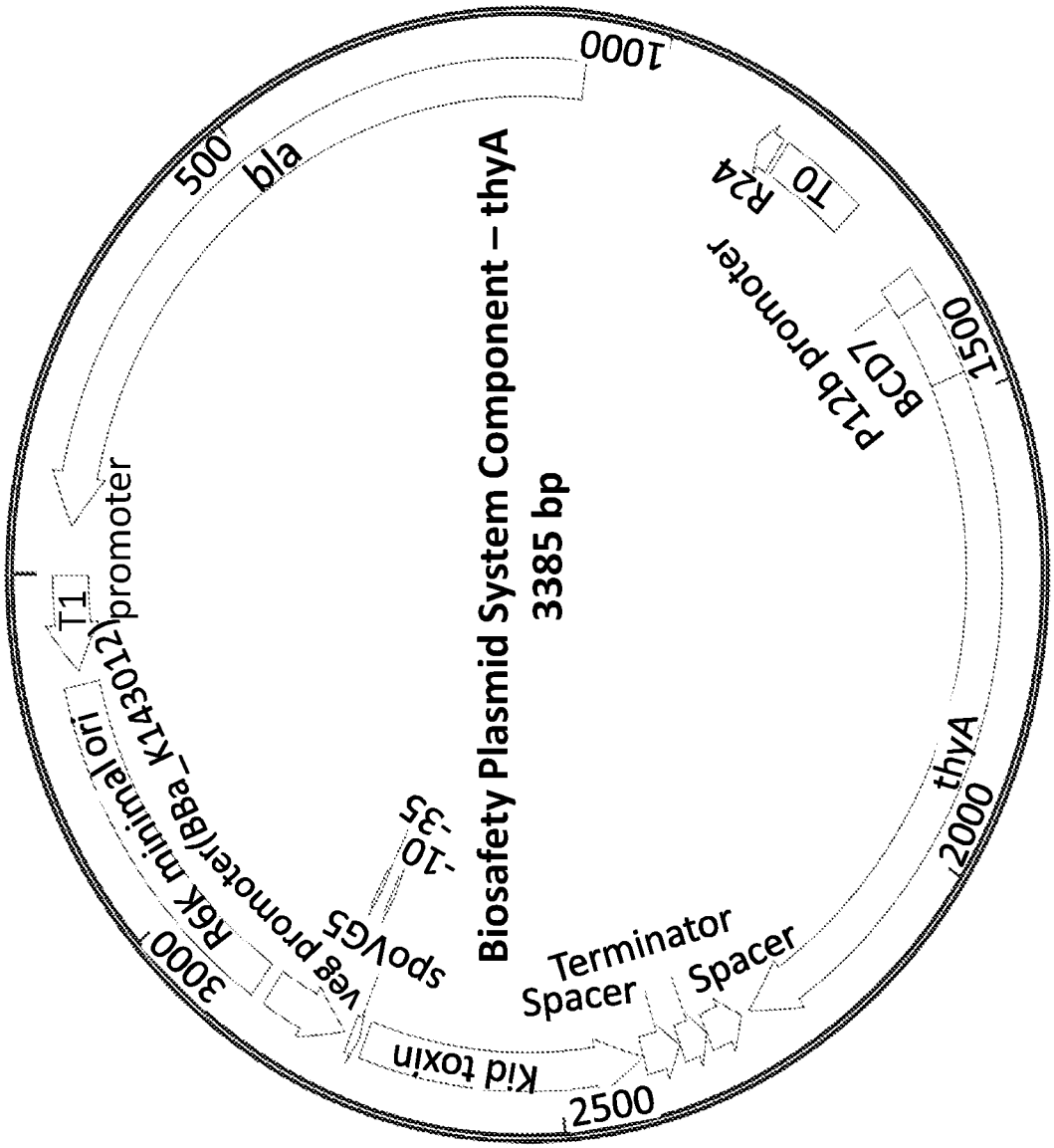
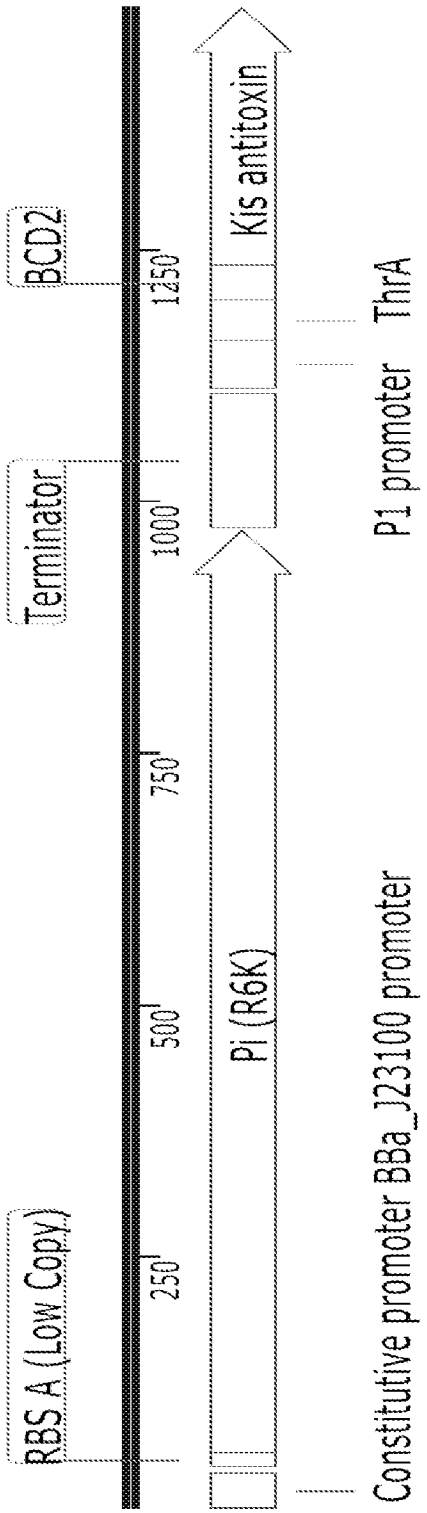
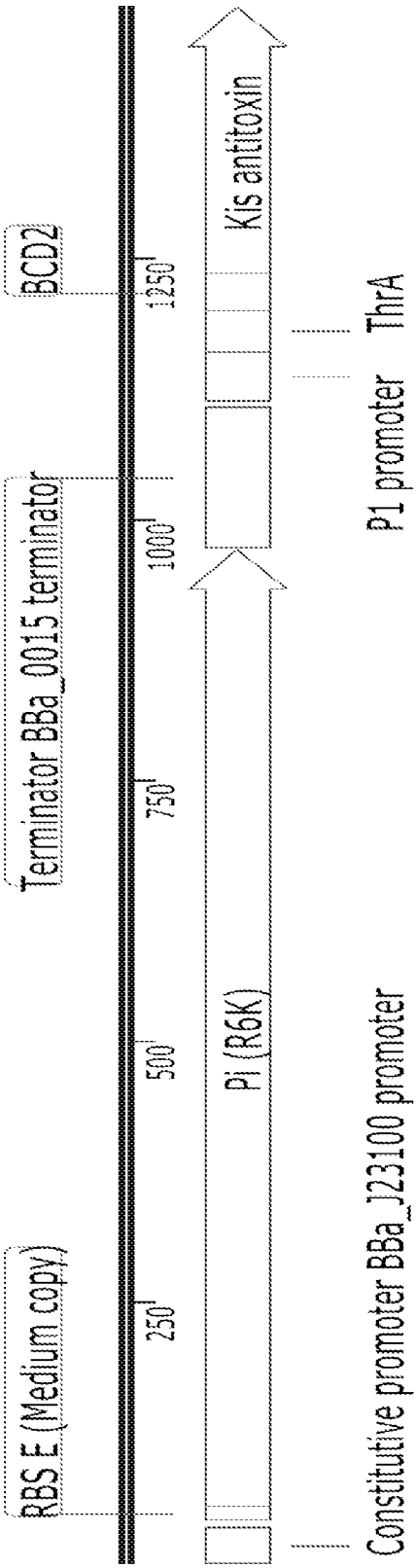

Biosafety Plasmid System Component – thyA
3385 bp
1000
500
bla
T0
R2a
P12b promoter
BCD7
1500
thyA
2000
Spacer
Terminator
Spacer
2500
kid toxin
3000
R6K minimal ori
T1
promoter
-10
-35
spoVG5
veg promoter(Bba_K143012)

FIG. 77C



Biosafety Chromosomally Integrated Construct – low copy Rep (Pi) and Kis antitoxin
1490 bp

FIG. 77D



Biosafety Chromosomally Integrated Construct – medium copy Rep (Pi) and Kis antitoxin
1490 bp

FIG. 78

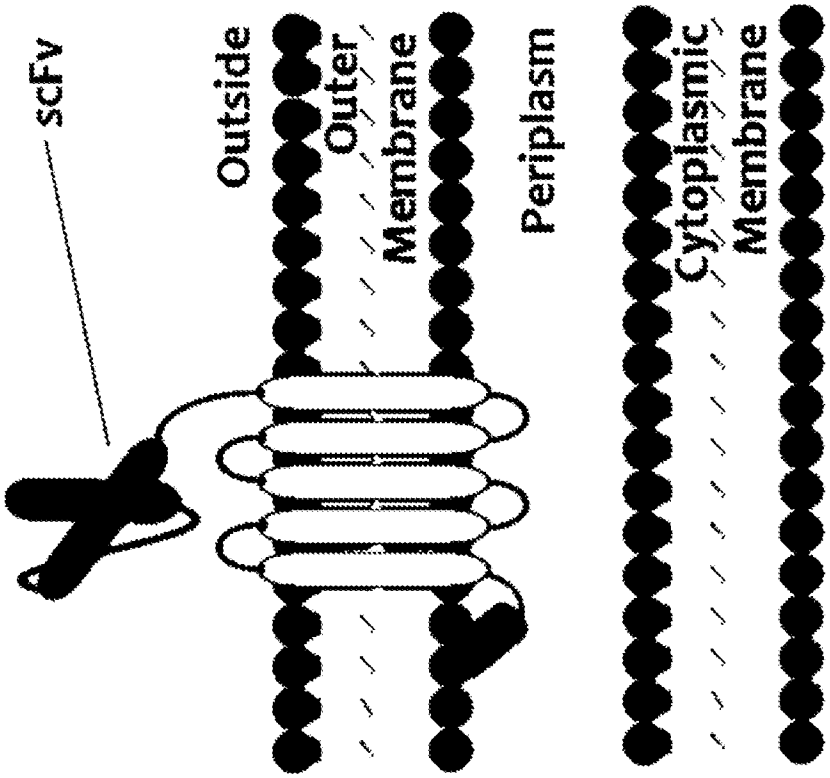

scFv
Outside
Outer
Membrane
Periplasm
Cytoplasmic
Membrane

FIG. 79

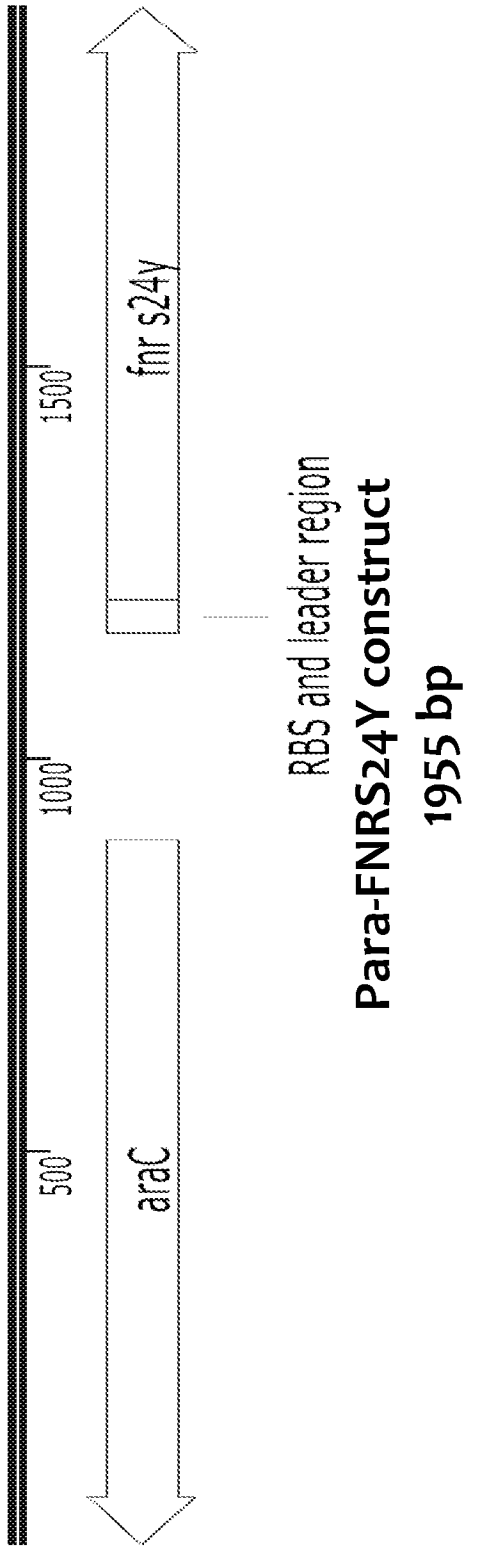

500
1000
1500
araC
fnr s24y
RBS and leader region
Para-FNRS24Y construct
1955 bp

FIG. 80A

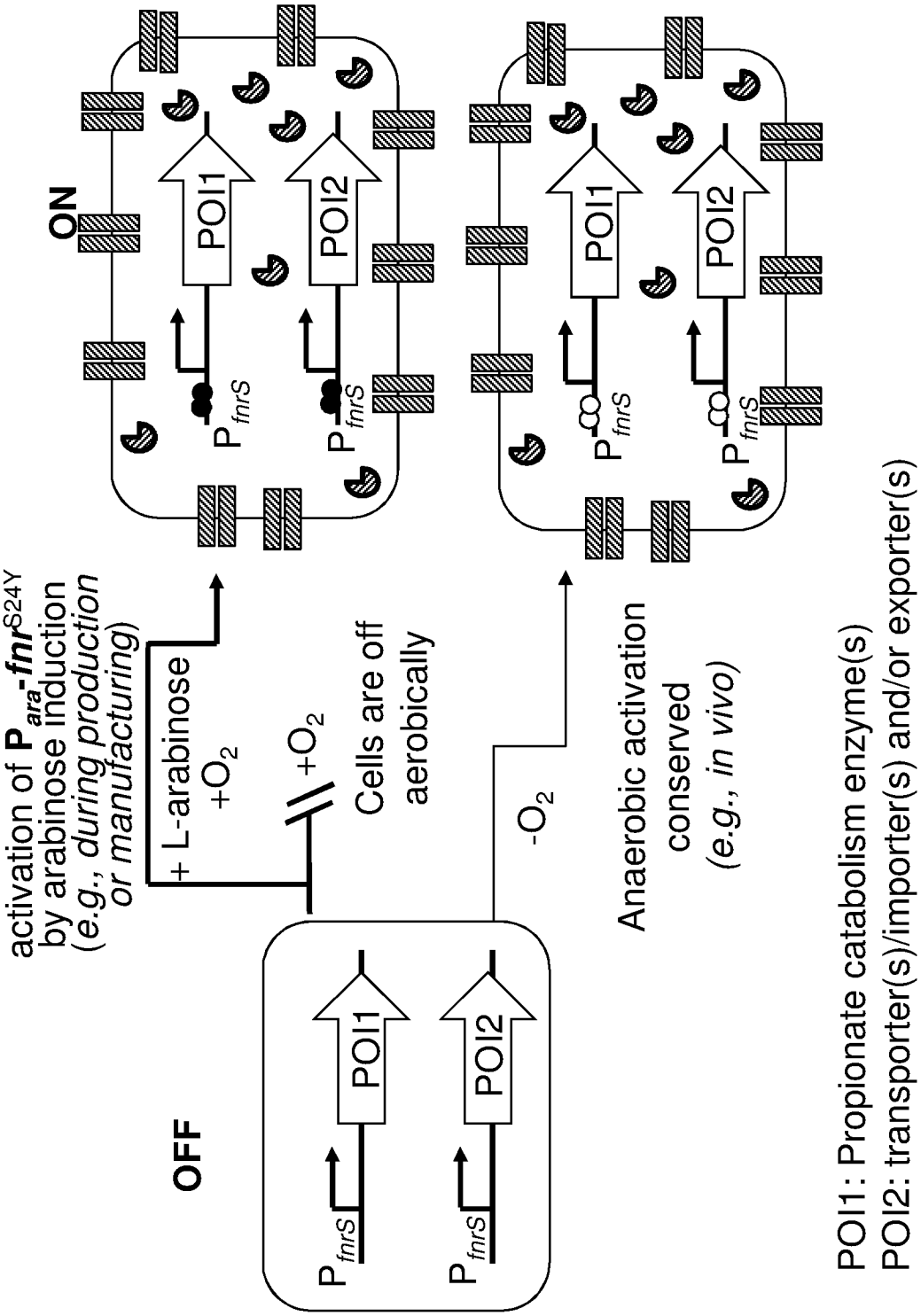

ON
POI1
POI2
P_{fnrS}
activation of P_{ara}-fnr^{S24Y} by arabinose induction (e.g., during production or manufacturing)
+ L-arabinose
$+O_2$
$+O_2$
Cells are off aerobically
$-O_2$
Anaerobic activation conserved (e.g., in vivo)
OFF
POI1: Propionate catabolism enzyme(s)
POI2: transporter(s)/importer(s) and/or exporter(s)

FIG. 80B

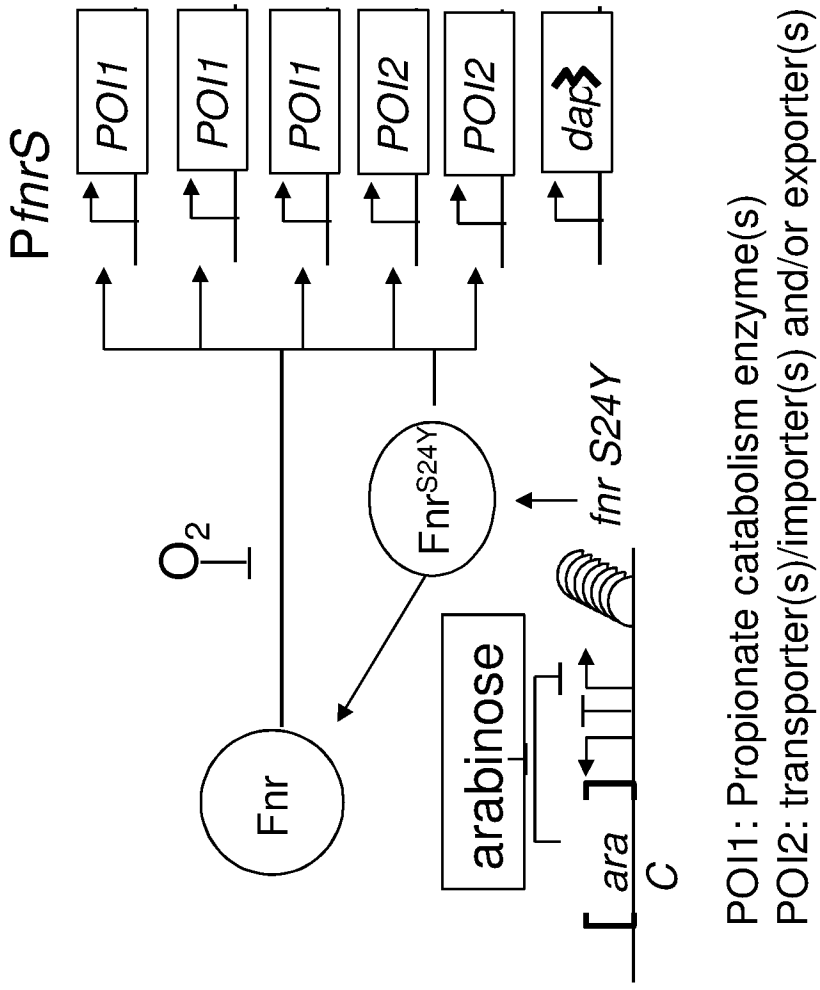

P*fnrS*
POI1
POI1
POI1
POI2
POI2
dapA
O_2
Fnr
Fnr^{S24Y}
fnr S24Y
arabinose
ara
C
POI1: Propionate catabolism enzyme(s)
POI2: transporter(s)/importer(s) and/or exporter(s)

FIG. 80C

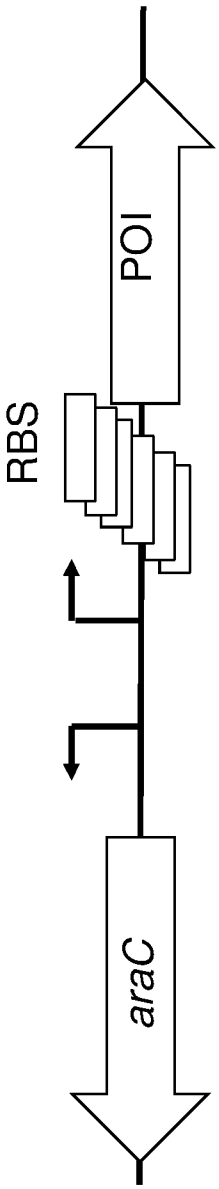


FIG. 81

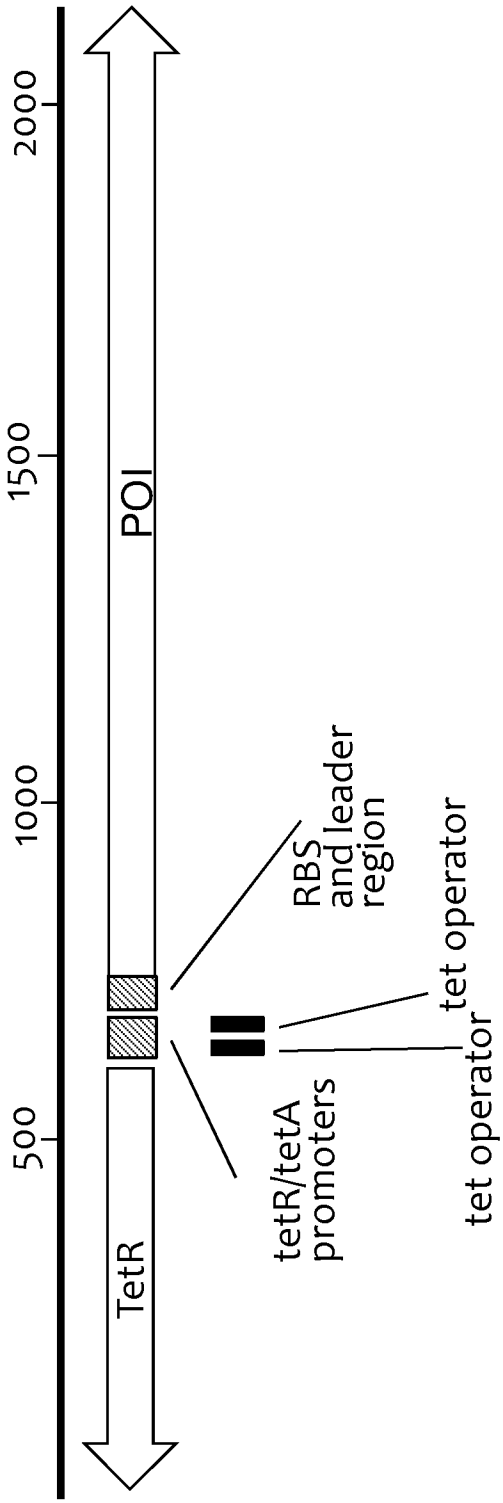

2000
1500
1000
500
POI
TetR
RBS and leader region
tet operator
tet operator
tetR/tetA promoters

FIG. 82

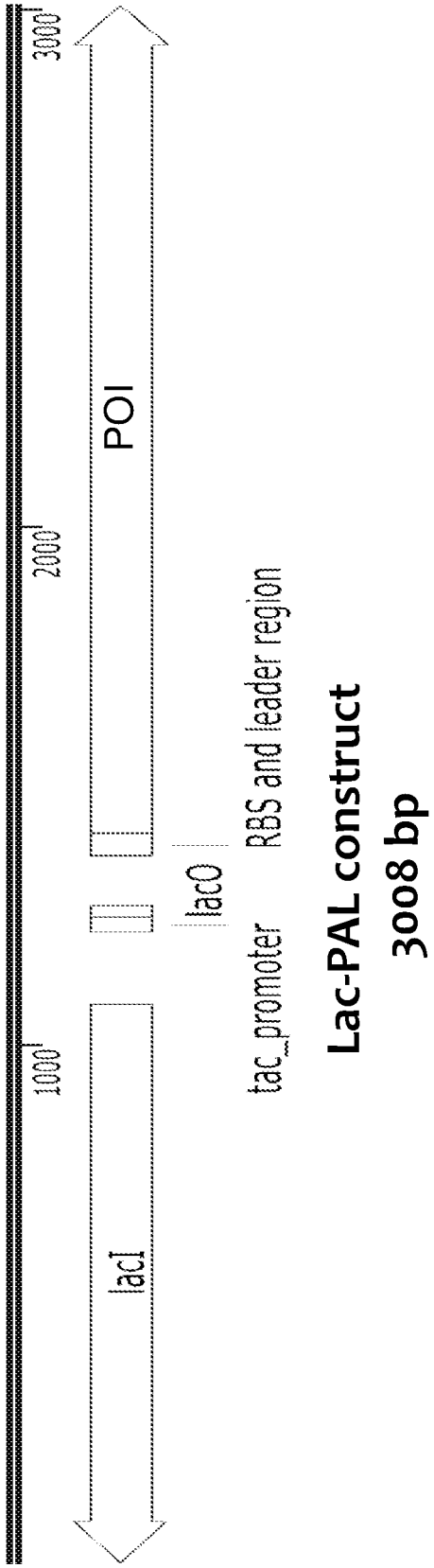
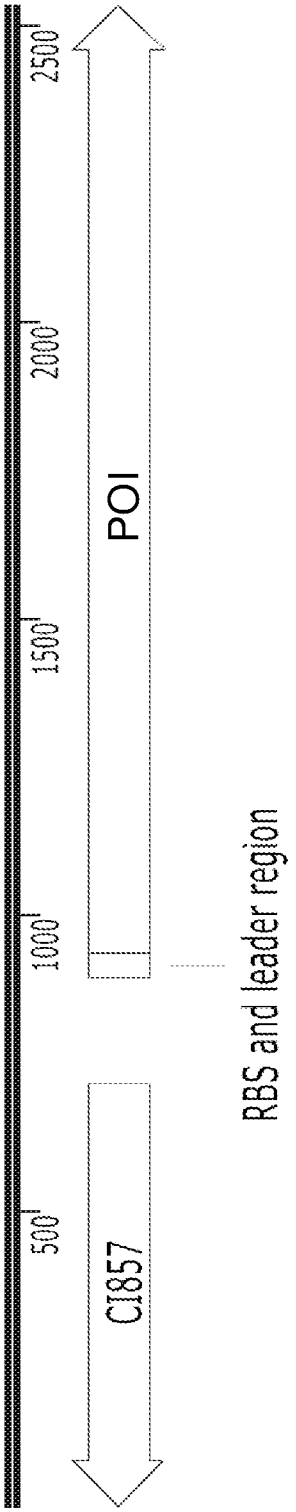

3000
2000
1000
POI
lacI
lacO
tac_promoter
RBS and leader region
Lac-PAL construct
3008 bp

FIG. 83A



Temperature-sensitive CI857 promoter – POI construct

FIG. 83B

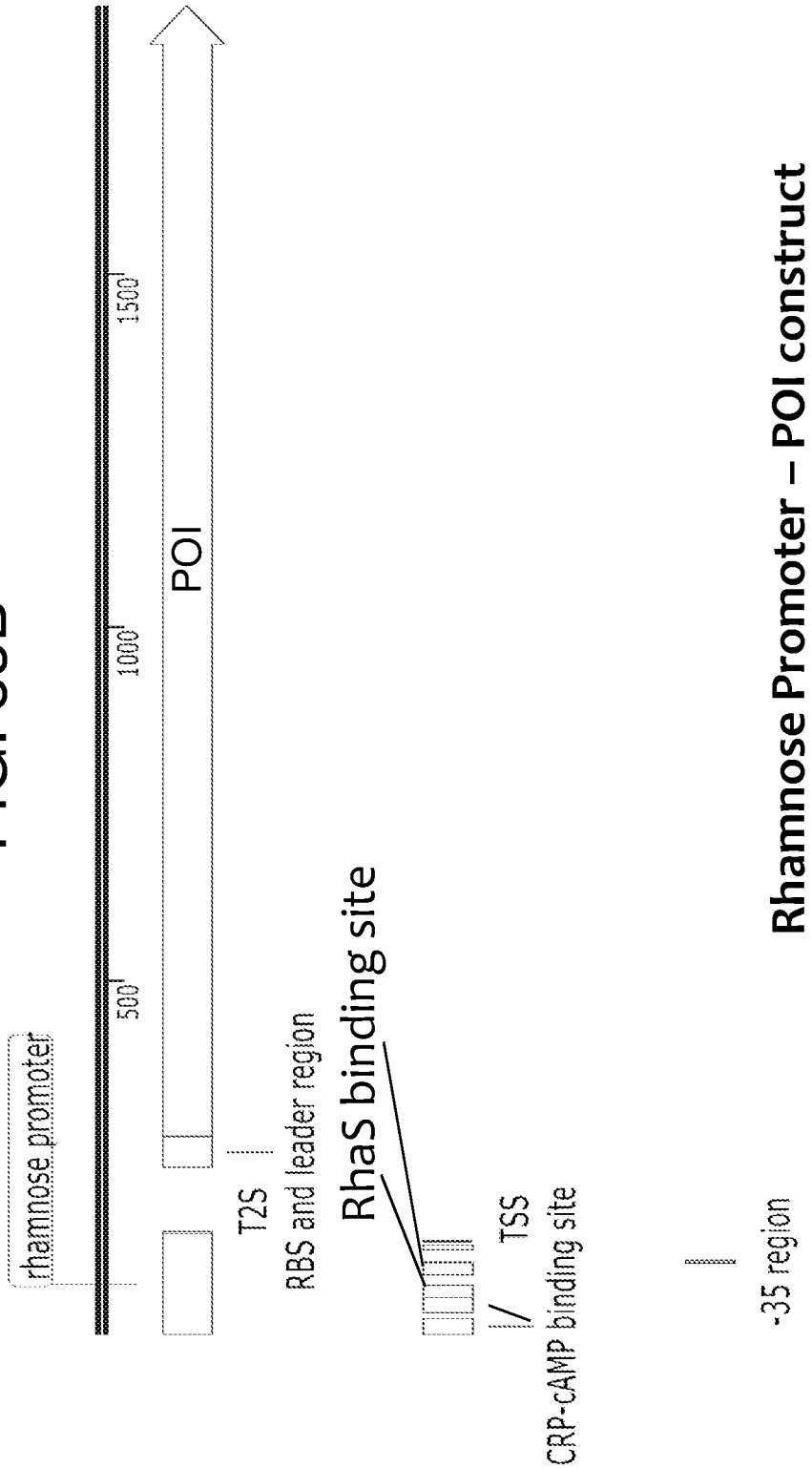

rhamnose promoter
500
1000
1500
POI
T2S
RBS and leader region
RhaS binding site
TSS
CRP-cAMP binding site
-35 region
Rhamnose Promoter – POI construct

FIG. 83C

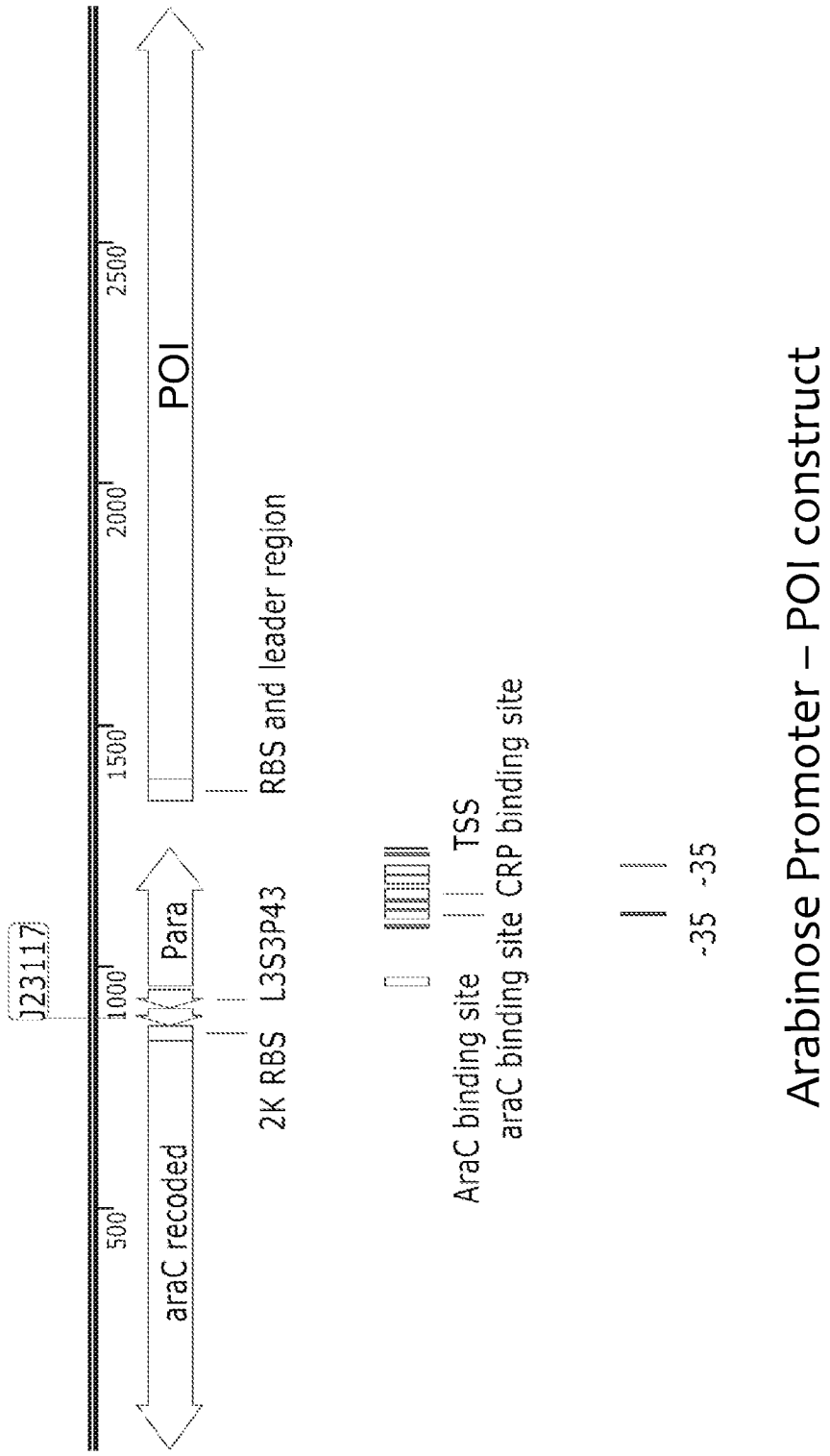

J23117
500
1000
1500
2000
2500
araC recoded
Para
POI
2K RBS
L3S3P43
RBS and leader region
AraC binding site
TSS
araC binding site
CRP binding site
-35
-35
Arabinose Promoter – POI construct
2986 bp

FIG. 84A

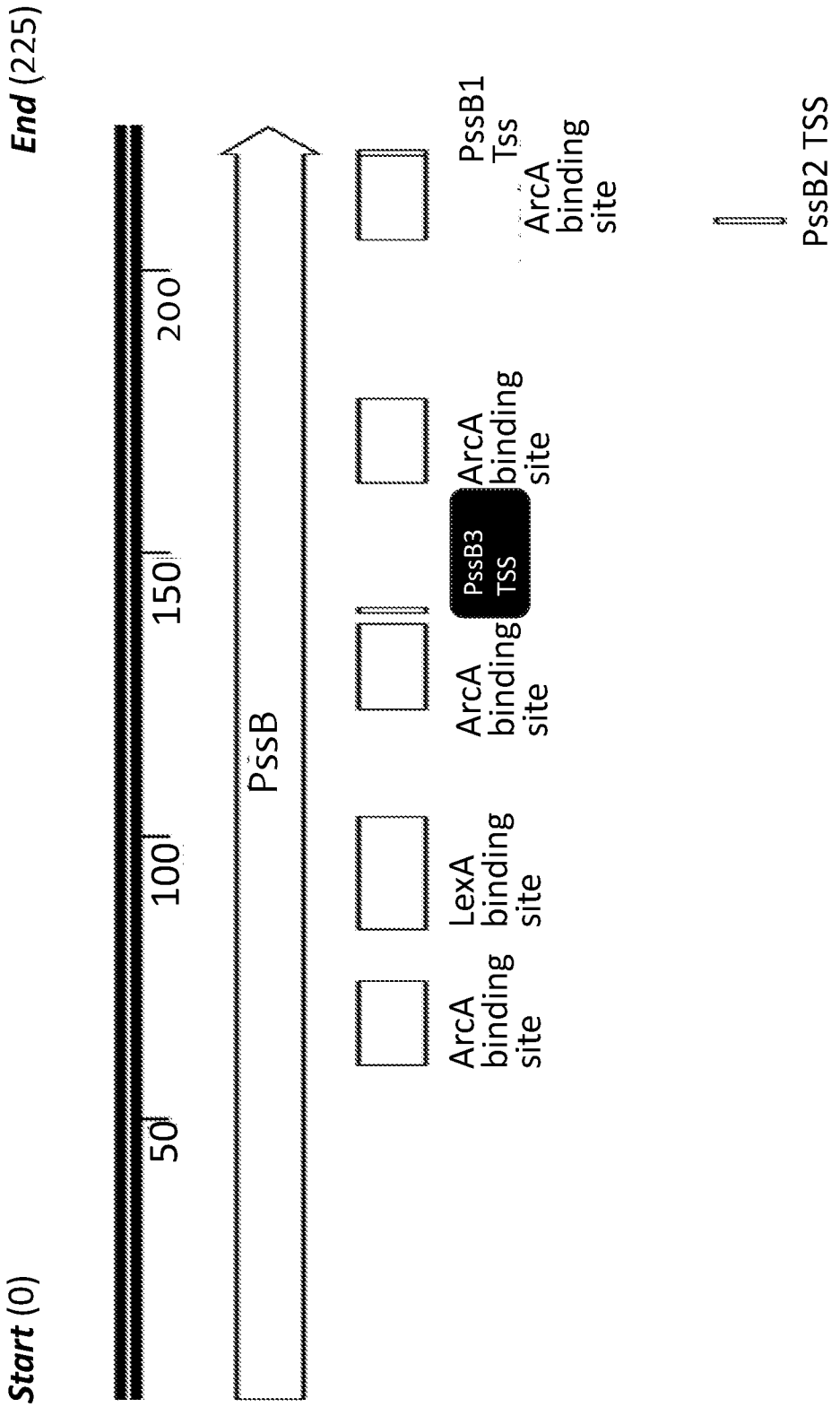

Start (0)
End (225)
50
100
150
200
PssB
ArcA binding site
LexA binding site
ArcA binding site
PssB3 TSS
ArcA binding site
PssB1 Tss
PssB2 TSS

FIG. 84B

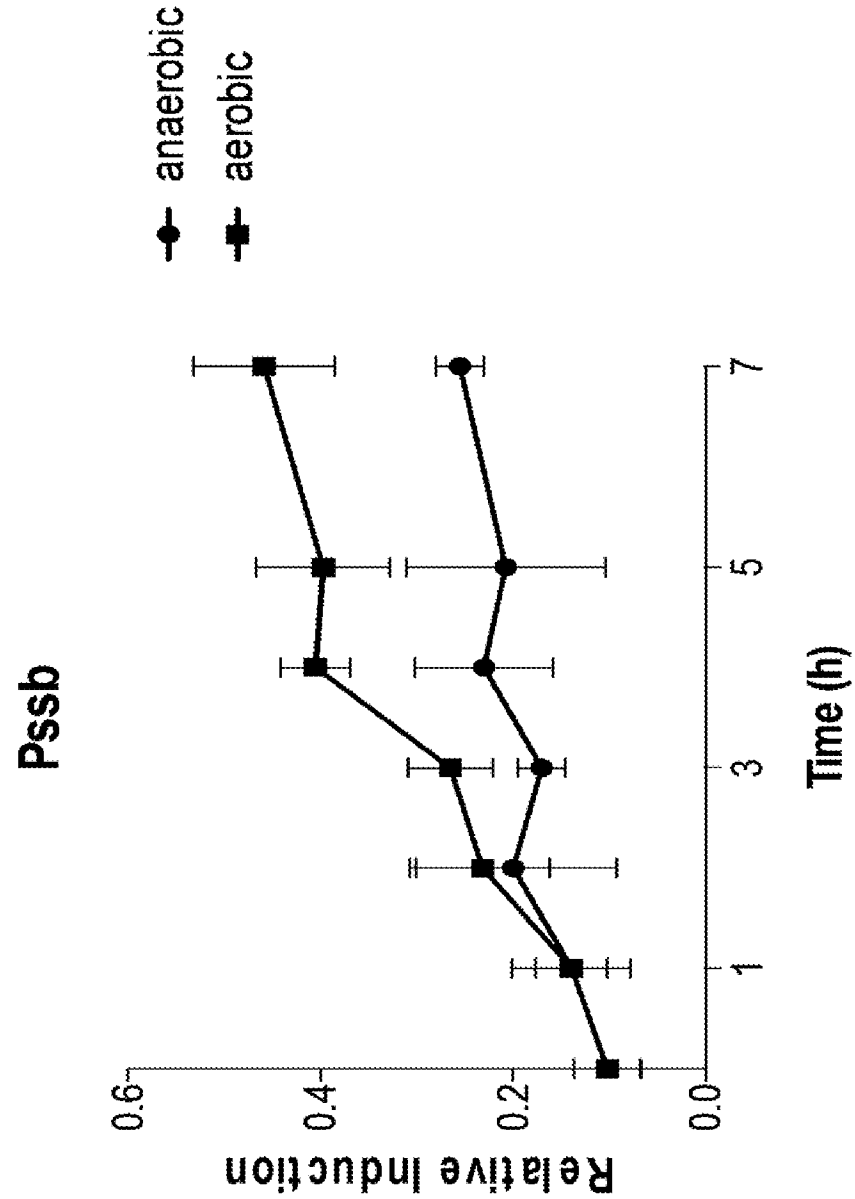
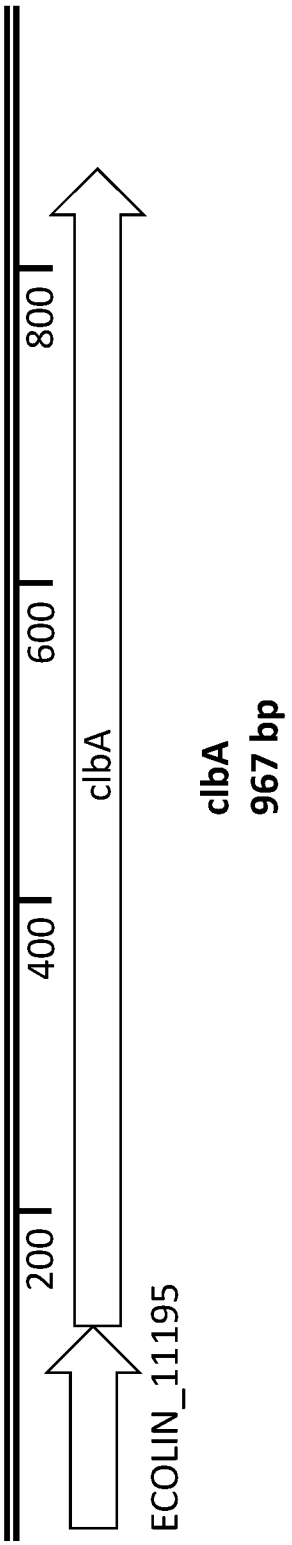
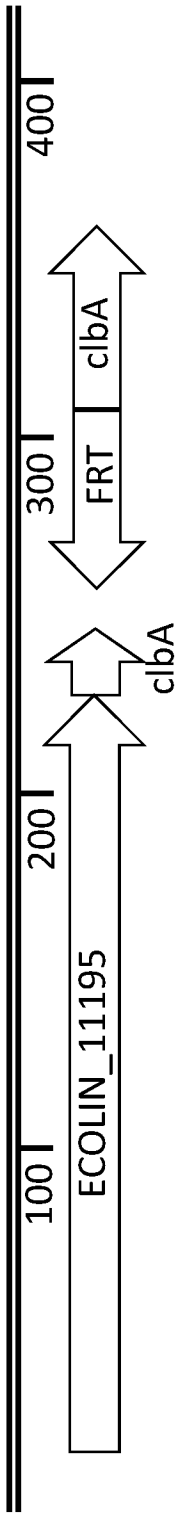

anaerobic
aerobic
Pssb
Relative Induction
0.0
0.2
0.4
0.6
1
3
5
7
Time (h)

FIG. 85A



clbA
967 bp

FIG. 85B



clbA KO
424 bp