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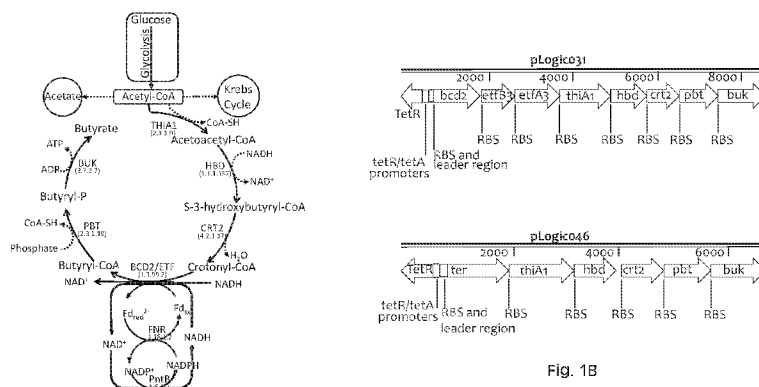


Fig. 1A

Fig. 1B

(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.

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

[01] The instant application hereby incorporates by reference U.S. Provisional Application No. 62/127,097, filed 3/2/2015; U.S. Application No. 62/248,814, filed 10/30/2015; U.S. Provisional Application No. 62/256,042, filed 11/16/2015; U.S. Provisional Application No. 62/291,461, filed 2/4/2016; U.S. Provisional Application No. 62/127, 131 filed 3/2/2015; U.S. Provisional Application No. 62/248,825, filed 10/30/2015; U.S. Provisional Application 62/256,044, dated 11/16/2015; U.S. Provisional Application No. 62/291,470, filed 2/4/2016; U.S. Provisional Application 62/184,770, filed 6/25/2015; U.S. Provisional Application No. 62/248,805, filed 10/30/2015; U.S. Provisional Application No. 62/256,048, filed 11/16/2015; U.S. Provisional Application No. 62/291,468, filed 2/4/2016; U.S. Application No. 14/998,376, dated 12/22/2015, the entire contents of each of which are expressly incorporated herein by reference in their respective entireties.

[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.

[08] The genetically engineered bacteria disclosed herein are capable of producing therapeutic anti-inflammation and/or gut barrier enhancer molecules. 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. Thus, in some embodiments, the genetically engineered bacteria of the invention 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. The genetically

engineered bacteria of the invention produce their therapeutic effect only in inducing environments such as the gut, thereby lowering the safety issues associated with systemic exposure.

Brief Description of the Figures

[010] Fig. 1 depicts a schematic of the eight-gene pathway from *c. difficile* for butyrate production. pLogic031 comprises the eight-gene pathway from *c. difficile*, *bcd2-etfB3-etfA3-thiAl-hbd-crt2-pbt-buk*, synthesized under the control of Tet-inducible promoters (pBR322 backbone). pLogic046 replaces the BCD/EFT complex, a potential rate-limiting step, with single gene from *Treponema denticola*, *ter* (trans-enoyl-2-reductase), and comprises *ter-thiAl-hbd-crt2-pbt-buk*.

[Oil] Fig. 2 depicts a schematic of a butyrate production pathway in which the circled genes (*buk* and *pbt*) may be deleted and replaced with *tesB*, which cleaves the CoA from butyryl-CoA.

[012] Fig. 3 depicts the gene organization of an exemplary recombinant bacterium of the invention and its derepression in the presence of nitric oxide (NO). In the upper panel, in the absence of NO, the NsrR transcription factor (gray circle, "NsrR") binds to and represses a corresponding regulatory region. Therefore, none of the butyrate biosynthesis enzymes (*bcd2*, *etfB3*, *etfA3*, *thiAl*, *hbd*, *crt2*, *pbt*, *buk*; black boxes) is expressed. In the lower panel, in the presence of NO, the NsrR transcription factor interacts with NO, and no longer binds to or represses the regulatory sequence. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

[013] Fig. 4 depicts the gene organization of another exemplary recombinant bacterium of the invention and its derepression in the presence of NO. In the upper panel, in the absence of NO, the NsrR transcription factor (gray circle, "NsrR") binds to and represses a corresponding regulatory region. Therefore, none of the butyrate biosynthesis enzymes (*ter*, *thiAl*, *hbd*, *crt2*, *pbt*, *buk*; black boxes) is expressed. In the lower panel, in the presence of NO, the NsrR transcription factor interacts with NO, and no longer binds to or represses the regulatory sequence. This leads to expression of the

butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

[014] Fig. 5 depicts the gene organization of an exemplary recombinant bacterium of the invention and its induction in the presence of H_2O_2 . In the upper panel, in the absence of H_2O_2 , the OxyR transcription factor (gray circle, "OxyR") binds to, but does not induce, the *oxyS* promoter. Therefore, none of the butyrate biosynthesis enzymes (*bcd2*, *etfB3*, *etfA3*, *thiA1*, *hbd*, *crt2*, *pbt*, *buk*; black boxes) is expressed. In the lower panel, in the presence of H_2O_2 , the OxyR transcription factor interacts with H_2O_2 and is then capable of inducing the *oxyS* promoter. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

[015] Fig. 6 depicts the gene organization of another exemplary recombinant bacterium of the invention and its induction in the presence of H_2O_2 . In the upper panel, in the absence of H_2O_2 , the OxyR transcription factor (gray circle, "OxyR") binds to, but does not induce, the *oxyS* promoter. Therefore, none of the butyrate biosynthesis enzymes (*ter*, *thiA1*, *hbd*, *crt2*, *pbt*, *buk*; black boxes) is expressed. In the lower panel, in the presence of H_2O_2 , the OxyR transcription factor interacts with H_2O_2 and is then capable of inducing the *oxyS* promoter. This leads to expression of the butyrate biosynthesis enzymes (indicated by gray arrows and black squiggles) and ultimately to the production of butyrate.

[016] Fig. 7 depicts the gene organization of an exemplary recombinant bacterium of the invention and its induction under low-oxygen conditions. In the upper panel, relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the butyrate biosynthesis enzymes (*bcd2*, *etfB3*, *etfA3*, *thiA1*, *hbd*, *crt2*, *pbt*, and *buk*; black boxes) is expressed. In the lower panel, increased butyrate production under low-oxygen conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate.

[017] Fig. 8 depicts the gene organization of an exemplary recombinant bacterium of the invention and its induction under low-oxygen conditions. In the upper panel, relatively low butyrate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the butyrate biosynthesis enzymes (*ter*, *thiA1*, *hbd*, *crt2*, *pbt*, and *buk*; black boxes) is expressed. In the lower panel, increased butyrate production under low-oxygen conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the butyrate biosynthesis enzymes, which leads to the production of butyrate.

[018] Fig. 9 depicts the gene organization of an exemplary recombinant bacterium of the invention and its induction under low-oxygen conditions. In the upper panel, relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*pet*, *lcdA*, *lcdB*, *lcdC*, *ettA*, *acrB*, *acrC*; black boxes) is expressed. In the lower panel, increased propionate production under low-oxygen conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the propionate biosynthesis enzymes, which leads to the production of propionate.

[019] Fig. 10 depicts an exemplary propionate biosynthesis gene cassette.

[020] Fig. 11 depicts the gene organization of an exemplary recombinant bacterium of the invention and its induction under low-oxygen conditions. In the upper panel, relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*thrA*, *thrB*, *thrC*, *ilvA*, *aceE*, *aceF*, *lpd*; black boxes) is expressed. In the lower panel, increased propionate production under low-oxygen conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and

inducing expression of the propionate biosynthesis enzymes, which leads to the production of propionate.

[021] Fig. 12 depicts an exemplary propionate biosynthesis gene cassette.

[022] Fig. 13 depicts the gene organization of an exemplary recombinant bacterium of the invention and its induction under low-oxygen conditions. In the upper panel, relatively low propionate production under aerobic conditions in which oxygen (O_2) prevents (indicated by "X") FNR (grey boxed "FNR") from dimerizing and activating the FNR-responsive promoter ("FNR promoter"). Therefore, none of the propionate biosynthesis enzymes (*thrA*, *thrB*, *thrC*, *ilvA*, *aceE*, *aceF*, *lpd*, *tesB*; black boxes) is expressed. In the lower panel, increased propionate production under low-oxygen conditions due to FNR dimerizing (two grey boxed "FNR"s), binding to the FNR-responsive promoter, and inducing expression of the propionate biosynthesis enzymes, which leads to the production of propionate.

[023] Fig. 14 depicts an exemplary propionate biosynthesis gene cassette.

[024] Fig. 15 depicts a schematic of a butyrate gene cassette, pLogic031 comprising the eight-gene butyrate cassette.

[025] Fig. 16 depicts a schematic of a butyrate gene cassette, pLogic046 comprising the *ter* substitution (oval).

[026] Fig. 17 depicts a linear schematic of a butyrate gene cassette, pLogic046.

[027] Fig. 18 depicts a graph of butyrate production. pLOGIC031 (bcd)/+02 is Nissle containing plasmid pLOGIC031 grown aerobically. pLOGIC046 (ter)/+02 is Nissle containing plasmid pLOGIC046 grown aerobically. pLOGIC031 (bcd)/-02 is Nissle containing plasmid pLOGIC031 grown anaerobically. pLOGIC046 (ter)/-02 is Nissle containing plasmid pLOGIC046 grown anaerobically. The *ter* construct results in higher butyrate production.

[028] Fig. 19 depicts a graph of butyrate production using *E. coli* BW25113 butyrate-producing circuits comprising a *nuoB* gene deletion, which results in greater levels of butyrate production as compared to a wild-type parent control. *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.

[029] Fig. 20 depicts a schematic of pLogic046-tesB, in which buk and pbt are deleted and tesB substituted.

[030] Fig. 21 depicts a linear schematic of a butyrate gene cassette, pLogic046-delta.ptb-buk-tesB+.

[031] Fig. 22 depicts butyrate production using pLOGIC046 (a Nissle strain comprising plasmid pLOGIC046, an ATC-inducible *ter*-comprising butyrate construct) and pLOGIC046-delta.pbt-buk/tesB+ (a Nissle strain comprising plasmid pLOGIC046-delta pbt.buk/tesB+, an ATC-inducible *ter*-comprising butyrate construct with a deletion in the pbt-buk genes and their replacement with the tesB gene). The *tesB* construct results in greater butyrate production.

[032] Fig. 23 depicts a schematic of a butyrate gene cassette, ydfZ-butyrate, comprising the *ter* substitution.

[033] Fig. 24 depicts SYN363 in the presence and absence of glucose and oxygen *in vitro*. SYN363 comprises a butyrate gene cassette comprising the *ter-thiAl-hbd-crt2-tesB* genes under the control of a *ydfZ* promoter.

[034] Fig. 25 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.

[035] Fig. 26 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.

[036] Fig. 27 depicts levels of mouse lipocalin 2 and calprotectin quantified by ELISA using the fecal samples in an *in vivo* model of IBD. SYN363 reduces inflammation and/or protects gut barrier function as compared to control SYN94.

[037] Fig. 28 depicts ATC or nitric oxide-inducible reporter constructs. These constructs, when induced by their cognate inducer, lead to expression of GFP. Nissle cells harboring plasmids with either the control, ATC-inducible P_{tet} -GFP reporter

construct or the nitric oxide inducible P_{nsrR} -GFP reporter construct induced across a range of concentrations. Promoter activity is expressed as relative fluorescence units.

[038] Fig. 29 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. IBD is induced in mice by supplementing drinking water with 2-3% dextran sodium sulfate (DSS).

Chemiluminescent is shown for NsrR-regulated promoters induced in DSS-treated mice.

[039] Fig. 30 depicts the construction and gene organization of an exemplary plasmid comprising a gene encoding NsrR, a regulatory sequence from *norB*, and a butyrogenic gene cassette (pLogic031-nsrR-norB-butyrate construct).

[040] Fig. 31 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).

[041] Fig. 32 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).

[042] Fig. 33 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).

[043] Fig. 34 depicts the construction and gene organization of an exemplary plasmid comprising an *oxyS* promoter and butyrogenic gene cassette (pLogic031-*oxyS*-butyrogenic gene cassette).

[044] Fig. 35 depicts the construction and gene organization of another exemplary plasmid comprising an *oxyS* promoter and butyrogenic gene cassette (pLogic046-*oxyS*-butyrogenic gene cassette).

[045] Fig. 36 depicts a schematic of an *E. coli* that is genetically engineered to express the essential gene *tnaB*, 5-methyltetrahydrofolate-homocysteine

methyln transferase (*mtr*), tryptophan transporter, and the enzymes IDO and TDO to convert tryptophan into kynurenine.

[046] Fig. 37 depicts a schematic of an *E. coli* that is genetically engineered to express interleukin under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[047] Fig. 38 depicts a schematic of an *E. coli* that is genetically engineered to express SOD under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[048] Fig. 39 depicts a schematic of an *E. coli* that is genetically engineered to express GLP-2 under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[049] Fig. 40 depicts a schematic of an *E. coli* that is genetically engineered to express a propionate gene cassette under the control of a FNR-responsive promoter.

[050] Fig. 41 depicts a schematic of an *E. coli* that is genetically engineered to express butyrate under the control of a FNR-responsive promoter.

[051] Fig. 42 depicts a schematic of an *E. coli* that is genetically engineered to express kynurenine, interleukin, SOD, GLP-2, a propionate gene cassette, and a butyrate gene cassette under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[052] Fig. 43 depicts a schematic of an *E. coli* that is genetically engineered to express interleukin, SOD, GLP-2, a propionate gene cassette, and a butyrate gene cassette under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[053] Fig. 44 depicts a schematic of an *E. coli* that is genetically engineered to express SOD, a propionate gene cassette, and a butyrate gene cassette under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[054] Fig. 45 depicts a schematic of an *E. coli* that is genetically engineered to express interleukin, a propionate gene cassette, and a butyrate gene cassette under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[055] Fig. 46 depicts a schematic of an *E. coli* that is genetically engineered to express interleukin-10 (IL-10), a propionate gene cassette, and a butyrate gene cassette under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[056] Fig. 47 depicts a schematic of an *E. coli* that is genetically engineered to express IL-2, IL-10, a propionate gene cassette, and a butyrate gene cassette under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[057] Fig. 48 depicts a schematic of an *E. coli* that is genetically engineered to express IL-2, IL-10, a propionate gene cassette, a butyrate gene cassette, and SOD under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[058] Fig. 49 depicts a schematic of an *E. coli* that is genetically engineered to express IL-2, IL-10, a propionate gene cassette, a butyrate gene cassette, SOD, and GLP-2 under the control of a FNR-responsive promoter and further comprising a TAT secretion system.

[059] Fig. 50 depicts a map of exemplary integration sites within the *E. coli* 1917 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.

[060] Fig. 51 depicts an exemplary schematic of the *E. coli* 1917 Nissle chromosome comprising multiple mechanisms of action (MoAs).

[061] Fig. 52 depicts an exemplary schematic of the *E. coli* 1917 Nissle chromosome comprising multiple mechanisms of action for producing IL-2, IL-10, IL-22, IL-27, propionate, and butyrate.

[062] Fig. 53 depicts an exemplary schematic of the *E. coli* 1917 Nissle chromosome comprising multiple mechanisms of action for producing IL-10, IL-27, GLP-2, and butyrate.

[063] Fig. 54 depicts an exemplary schematic of the *E. coli* 1917 Nissle chromosome comprising multiple mechanisms of action for producing GLP-2, IL-10, IL-22, SOD, butyrate, and propionate.

[064] Fig. 55 depicts an exemplary schematic of the *E. coli* 1917 Nissle chromosome comprising multiple mechanisms of action for producing GLP-2, IL-2, IL-10, IL-22, IL-27, SOD, butyrate, and propionate.

[065] Fig. 56 depicts a table illustrating the survival of various amino acid auxotrophs in the mouse gut, as detected 24 hours and 48 hours post-gavage. These auxotrophs were generated using BW25113, a non-Nissle strain of *E. coli*.

[066] Fig. 57 depicts a schematic of a repression-based kill switch. In a toxin-based system, the AraC transcription factor is activated in the presence of arabinose and induces expression of TetR and an anti-toxin. TetR prevents the expression of the toxin. When arabinose is removed, TetR and the anti-toxin do not get made and the toxin is produced which kills the cell. In an essential gene-based system, the AraC transcription factor is activated in the presence of arabinose and induces expression of an essential gene.

[067] Fig. 58 depicts another non-limiting embodiment of the disclosure, wherein the expression of a heterologous gene is activated by an exogenous environmental signal, e.g., low-oxygen conditions. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the araBAD promoter, which induces expression of TetR (tet repressor) and an anti-toxin. The anti-toxin builds up in the recombinant bacterial cell, while TetR prevents expression of a toxin (which is under the control of a promoter having a TetR binding site). However, when arabinose is not present, both the anti-toxin and TetR are not expressed. Since TetR is not present to repress expression of the toxin, the toxin is expressed and kills the cell. **Fig. 58** 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.

[068] Fig. 59 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.

[069] Fig. 60 depicts a schematic of a repression-based kill switch in which the AraC transcription factor is activated in the presence of arabinose and induces expression of TetR and an anti-toxin. TetR prevents the expression of the toxin. When arabinose is removed, TetR and the anti-toxin do not get made and the toxin is produced which kills the cell.

[070] Fig. 61 depicts another non-limiting embodiment of the disclosure, wherein the expression of a heterologous gene is activated by an exogenous environmental signal. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the araBAD promoter, which induces expression of TetR (tet repressor) and an anti-toxin. The anti-toxin builds up in the recombinant bacterial cell, while TetR prevents expression of a toxin (which is under the control of a promoter having a TetR binding site). However, when arabinose is not present, both the anti-toxin and TetR are not

expressed. Since TetR is not present to repress expression of the toxin, the toxin is expressed and kills the cell. The *araC* gene is under the control of a constitutive promoter in this circuit.

[071] Fig. 62 depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition, e.g., low-oxygen conditions, or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, and the natural kinetics of the recombinase create a time delay in expression of the toxin, allowing the heterologous gene to be fully expressed. Once the toxin is expressed, it kills the cell.

[072] Fig. 63 depicts another non-limiting embodiment of the disclosure, where an exogenous environmental condition, e.g., low-oxygen conditions, or one or more environmental signals activates expression of a heterologous gene, an anti-toxin, and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, but the presence of the accumulated anti-toxin suppresses the activity of the toxin. Once the exogenous environmental condition or cue(s) is no longer present, expression of the anti-toxin is turned off. The toxin is constitutively expressed, continues to accumulate, and kills the bacterial cell.

[073] Fig. 64 depicts another non-limiting embodiment of the disclosure, where an exogenous environmental condition, e.g., low-oxygen conditions, or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips at least one excision enzyme into an activated conformation. The at least one excision enzyme then excises one or more essential genes, leading to senescence, and eventual cell death. The natural kinetics of the recombinase and excision genes cause a time delay, the kinetics of which can be altered and optimized depending on the number and choice of essential genes to be excised, allowing cell death to occur within a matter of hours or days. The presence of multiple nested recombinases can be used to further control the timing of cell death.

[074] Fig. 65 depicts a schematic of an activation-based kill switch, in which P_i is any inducible promoter, e.g., a FNR-responsive promoter. When the therapeutic is induced, the anti-toxin and recombinases are turned on, which results in the toxin being 'flipped' to the ON position after 4-6 hours, which results in a build-up of anti-toxin before the toxin is expressed. In absence of the inducing signal, only toxin is made and the cell dies.

[075] Fig. 66 depicts a one non-limiting embodiment of the disclosure, in which the genetically engineered bacteria produces equal amount of a Hok toxin and a short-lived Sok anti-toxin. When the cell loses the plasmid, the anti-toxin decays, and the cell dies. In the upper panel, the cell produces equal amounts of toxin and anti-toxin and is stable. In the center panel, the cell loses the plasmid and anti-toxin begins to decay. In the lower panel, the anti-toxin decays completely, and the cell dies.

[076] Fig. 67 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., 2015.

[077] Fig. 68 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-responsive 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-responsive promoter, drives expression of a regulatory factor, e.g. T7 polymerase, that then activates the expression of the tagged therapeutic peptide (hexagons).

[078] Fig. 69 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.

[079] Fig. 70 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.

[080] Fig. 71 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.

[081] Fig. 72 depicts a schematic diagram of a wild-type clbA construct (upper panel) and a schematic diagram of a clbA knockout construct (lower panel).

[082] Fig. 73 depicts exemplary sequences of a wild-type clbA construct and a clbA knockout construct.

[083] Fig. 74 depicts a schematic for inflammatory bowel disease (IBD) therapies that target pro-inflammatory neutrophils and macrophages and regulatory T cells (Treg), restore epithelial barrier integrity, and maintain mucosal barrier function. Decreasing the pro-inflammatory action of neutrophils and macrophages and increasing Treg restores epithelial barrier integrity and the mucosal barrier.

[084] Fig. 75 depicts a schematic of non-limiting processes for designing and producing the genetically engineered bacteria of the present disclosure: identifying diverse candidate approaches based on microbial physiology and disease

biology, using bioinformatics to determine candidate metabolic pathways, prospective tools to determine performance targets required of optimized engineered synthetic biotics (A); cutting-edge DNA assembly to enable combinatorial testing of pathway organization, mathematical models to predict pathway efficiency, internal stable of proprietary switches and parts to permit control and tuning of engineered circuits (B); building core structures ("chassies"), stably integrating engineered circuits into optimal chromosomal locations for efficient expression, employing unique functional assays to assess genetic circuit fidelity and activity (C); chromosomal markers enabling monitoring of synthetic biotic localization and transit times in animal models, expert microbiome network and bioinformatics support expanding understanding of how specific disease states affect GI microbial flora and the behaviors of synthetic biotics in that environment, activating process development research and optimization in-house during the discovery phase enables rapid and seamless transition of development candidates to pre-clinical progression, extensive experience in specialized disease animal model refinement supports prudent, high quality testing of candidate synthetic biotics (D).

[085] Fig. 76 depicts a schematic of non-limiting manufacturing processes for upstream and downstream production of the genetically engineered bacteria of the present disclosure. **A** depicts the parameters for starter culture 1 (SCI): loop full - glycerol stock, duration overnight, temperature 37° C, shaking at 250 rpm. **B** depicts the parameters for starter culture 2 (SC2): 1/100 dilution from SCI, duration 1.5 hours, temperature 37° C, shaking at 250 rpm. **C** 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. **D** depicts the parameters for harvest: centrifugation at speed 4000 rpm and duration 30 minutes, wash 1 X 10% glycerol/PBS, centrifugation, re-suspension 10% glycerol/PBS. **E** depicts the parameters for vial fill/storage: 1-2 mL aliquots, -80° C.

Description of Embodiments

[086] 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). 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.

[087] 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.

[088] 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.

[089] 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.

[090] 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 osteomyelitis (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 Neuropsychiatry 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.

[091] 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), kynurenine, GLP-2, GLP-1, IL-10, IL-27, TGF- β 1, TGF- β 2, N-acylphosphatidylethanolamines (NAPEs), elafin (also called peptidase inhibitor 3 and SKALP), trefoil factor, melatonin, PGD₂, and kynurenic acid, 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-1 β , IL-6, IL-8, IL-17, and/or chemokines, e.g., CXCL-8 and CCL2. 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 *P13* 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.

[092] As used herein, 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- β 1, TGF- β 2, 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.

[093] 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-inflammation and/or gut barrier function enhancer molecule, e.g., butyrate, propionate, and acetate. 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.

[094] As used herein, "butyrogenic gene cassette" and "butyrate biosynthesis gene cassette" 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*, and these endogenous butyrate biosynthesis pathways may be a source of genes for the genetically engineered bacteria of the invention. 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-enoyl-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*. Alternatively, addition of the *tesB* gene from *Escherichia Coli* is capable of functionally replacing *pbt* and *buk* genes from *Peptoclostridium difficile*. Thus, a butyrogenic gene cassette may comprise *thiA1*, *hbd*, and *crt2* from *Peptoclostridium difficile*, *ter* from *Treponema denticola*, and *tesS* from *Escherichia Coli*, for example, *thiA1* from *Peptoclostridium difficile* strain 630, *hbd* and *crt2* from *Peptoclostridium difficile* strain 1296, *ter* from *Treponema denticola* and *tesB* from *Escherichia Coli*. The butyrogenic gene cassette may comprise genes for the aerobic biosynthesis of butyrate and/or genes for the anaerobic or microaerobic biosynthesis of butyrate. One or more of the butyrate biosynthesis genes may be functionally replaced or modified, e.g., codon optimized. Exemplary butyrate gene cassettes are shown in Figs. 1, 3, 4, 5, 6, 7, and 8.

[095] As used herein, "propionate gene cassette" and "propionate biosynthesis gene cassette" refer 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*, and these endogenous propionate biosynthesis pathways may be a source of genes for the genetically engineered bacteria of the invention. 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., *pet*, *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 (Hetzl et al., 2003, Selmer et al., 2002). In alternate embodiments, the propionate gene cassette comprises pyruvate pathway propionate biosynthesis genes

(see, e.g., Tseng et al., 2012), e.g., *thrA^{fbr}*, *thrB*, *thrC*, *ilvA^{fbr}*, *oceE*, *oceF*, and *lpd*, which encode homoserine dehydrogenase 1, homoserine kinase, L-threonine synthase, L-threonine dehydratase, pyruvate dehydrogenase, dihydrolipoamide acetyltransferase, and dihydrolipoyl dehydrogenase, respectively. In some embodiments, the propionate gene cassette further comprises *tesB*, which encodes acyl-CoA thioesterase. 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. Exemplary propionic gene cassettes are shown in Figs. 9, 11, and 13.

[096] As used herein, "acetate gene cassette" and "acetate biosynthesis gene cassette" 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, 2008). Unmodified bacteria that are capable of producing acetate via an endogenous acetate biosynthesis pathway may be a source of acetate biosynthesis genes for the genetically engineered bacteria of the invention. 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 CO₂ + H₂ 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. Examples of acetate gene cassettes are described herein.

[097] Each gene sequence and/or gene cassette may be present on a plasmid or bacterial chromosome. In embodiments in which the engineered bacteria comprise one or more gene sequence(s) and one or more gene cassettes, the gene sequence(s) may be present on one or more plasmids and the gene cassette(s) may be present in the bacterial chromosome, and vice versa. 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. In some embodiments, the genetically engineered bacteria are engineered to comprise multiple different components of a gene cassette performing multiple different functions. In some embodiments, the genetically engineered bacteria are engineered to comprise one or more copies of different genes, gene cassettes, or regulatory regions to produce engineered bacteria that express more than one therapeutic molecule and/or perform more than one function.

[098] Each gene or gene cassette may be operably linked to an inducible promoter, e.g., an FNR-responsive promoter, an ROS-responsive promoter, and/or an RNS-responsive promoter. 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.

[099] As used herein, a "directly inducible promoter" refers to a regulatory region, wherein the regulatory region is operably linked to a gene or a gene cassette encoding a biosynthetic pathway for producing an anti-inflammation and/or gut barrier function enhancer molecule, e.g. butyrate. In the presence of an inducer of said regulatory region, an anti-inflammation and/or gut barrier function enhancer molecule 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 gene encoding a first molecule, e.g., a transcription factor, which is capable of regulating a second regulatory region that is operably linked to a gene or a gene cassette encoding a biosynthetic pathway for producing an anti-inflammation and/or gut barrier function enhancer molecule, e.g. butyrate (or other anti-inflammation and/or gut barrier function enhancer molecule). In the presence of an inducer of the first regulatory region, the second regulatory region may be activated or repressed, thereby activating or repressing production of butyrate (or other anti-inflammation and/or gut barrier function enhancer molecule). Both a directly inducible promoter and an indirectly inducible promoter are encompassed by "inducible promoter."

[0100] As used herein, "operably linked" refers a nucleic acid sequence, e.g., a gene or gene cassette for producing an anti-inflammation and/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 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.

[0101] As used herein, "exogenous environmental conditions" refer to settings or circumstances under which the promoter described herein is directly or indirectly induced. The phrase "exogenous environmental conditions" is meant to refer to the environmental conditions external to the bacteria, but endogenous or native to a mammalian subject. 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 a bacterial 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 condition is an environment in which ROS is present. In some embodiments, the exogenous environmental condition is an environment in which RNS is present.

[0102] In some embodiments, the exogenous environmental conditions are low-oxygen or anaerobic conditions such as the environment of the mammalian gut. In some embodiments, exogenous environmental conditions refer to the presence of molecules or metabolites that are specific to the mammalian gut in a healthy or disease state, e.g., propionate. In some embodiments, the gene or gene cassette for producing a therapeutic molecule is operably linked to an oxygen level-dependent promoter. 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. As used herein, 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.

[0103] In some embodiments, the gene or gene cassette for producing a therapeutic molecule is operably linked to an oxygen level-dependent regulatory region such that the therapeutic molecule is expressed in low-oxygen, microaerobic, or anaerobic conditions. For example, the oxygen level-dependent regulatory region is operably linked to a butyrogenic or other gene cassette or gene sequence(s) (e.g., any of the genes described herein); in low-oxygen conditions, the oxygen level-dependent regulatory region is activated by a corresponding oxygen level-sensing transcription factor, thereby driving expression of the butyrogenic or other gene cassette or gene sequence(s). Examples of oxygen level-dependent transcription factors include, but are not limited to, FNR, ANR, and DNR. Corresponding FNR-responsive promoters, ANR-responsive promoters, and DNR-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 1.

Table 1. Examples of transcription factors and responsive genes and regulatory regions

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

[0104] 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 include, but are not limited to, nitric oxide (NO), peroxynitrite or peroxynitrite anion (ONOO^-), nitrogen dioxide ($\cdot\text{NO}_2$), dinitrogen trioxide (N_2O_3), peroxynitrous acid (ONOOH), and nitroperoxycarbonate (ONOOCO_2^-) (unpaired electrons denoted by $\cdot$). 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.

[0105] 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 gene cassette, e.g., a butyrogenic or other gene

cassette or gene sequence(s), e.g., any of the genes 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 or gene cassette. Thus, RNS induces expression of the gene or gene cassette.

[0106] 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 gene cassette, e.g., a butyrogenic or other gene cassette or 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 gene cassette.

[0107] 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 cassette. Thus, RNS represses expression of the gene or gene cassette.

[0108] 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 2**.

Table 2. 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	<i>norB, aniA, nsrR, hmpA, ytfE, ygbA, hcp, hcr, nrfA, aox</i>
NorR	NO	<i>norVW, norR</i>
DNR	NO	<i>norCB, nir, nor, nos</i>

[0109] 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 include, but are not limited to, hydrogen peroxide (H_2O_2), organic peroxide ($ROOH$), hydroxyl ion (OH^-), hydroxyl radical ($\cdot OH$), superoxide or superoxide anion ($\cdot O_2^-$), singlet oxygen ($^1O_2$), ozone (O_3), carbonate radical, peroxide or peroxy radical ($\cdot O_2^{\cdot}$), hypochlorous acid ($HOCl$), hypochlorite ion (OCl^-), sodium hypochlorite ($NaOCl$), nitric oxide ($NO\cdot$), and peroxyxynitrite or peroxyxynitrite anion ($ONOO^-$) (unpaired electrons denoted by $\cdot$). 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).

[0110] 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 cassette, e.g., a butyrogenic gene cassette. 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 cassette. Thus, ROS induces expression of the gene or gene cassette.

[0111] 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 gene cassette, e.g., a butyrogenic or other gene cassette or gene sequence(s) described herein. 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.

[0112] 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 cassette. 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 cassette. Thus, ROS represses expression of the gene or gene cassette.

[0113] 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 3**.

Table 3. 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 ₂ O ₂	<i>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</i>
PerR	H ₂ O ₂	<i>katA; ahpCF; mrgA; zoaA; fur; hemAXCDBL; srfA</i>
OhrR	Organic peroxides NaOCl	<i>ohrA</i>
SoxR	•O ₂ ⁻ NO• (also capable of sensing H ₂ O ₂)	<i>soxS</i>
RosR	H ₂ O ₂	<i>rbtT; tnp16a; rluC1; tnp5a; mscL; tnp2d; phoD; tnp15b; pstA; tnp5b; xylC; gabD1; rluC2; cgtS9; azlC; narKGHJI; rosR</i>

[0114] 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, 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.

[0115] As used herein, a "non-native" nucleic acid sequence refers to a nucleic acid sequence not normally present in a bacterium, 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 a sequence that is modified and/or mutated as compared to the unmodified sequence from bacteria 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 addition, multiple copies of any regulatory region, promoter, gene, and/or gene cassette may be present in the bacterium, wherein one or more copies of the regulatory region, promoter, gene, and/or gene cassette 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 regulatory region, promoter, gene, and/or gene cassette in order to enhance copy number or to comprise multiple different components of a gene cassette performing multiple different functions or to comprise one or more copies of different regulatory regions, promoters, genes, and/or gene cassette to produce engineered bacteria that express more than one therapeutic molecule and/or perform more than one function.

[0116] In some embodiments, the genetically engineered bacteria of the invention comprise a gene cassette that is operably linked to a directly or indirectly inducible promoter that is not associated with said gene cassette in nature, e.g., a FNR-responsive promoter operably linked to a butyrogenic gene cassette.

[0117] "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 functional variants are well known in the art and include, but are not limited to, BBa_J23100, a constitutive *Escherichia coli* σ^S promoter (e.g., an osmY promoter (International Genetically Engineered Machine (iGEM) Registry of Standard Biological Parts Name BBa_J45992; BBa_J45993)), a constitutive *Escherichia coli* σ^{32} promoter (e.g., htpG heat shock promoter (BBa_J45504)), a constitutive *Escherichia coli* σ^{70} promoter (e.g., lacq promoter (BBa_J54200; BBa_J56015), *E. coli* CreABCD phosphate sensing operon promoter (BBa_J64951), GlnRS promoter (BBa_K088007), lacZ promoter (BBa_K119000; BBa_K119001); M13K07 gene I promoter (BBa_M13101); M13K07 gene II promoter (BBa_M13102), M13K07 gene III promoter (BBa_M13103), M13K07 gene IV promoter (BBa_M13104), M13K07 gene V promoter (BBa_M13105), M13K07 gene VI promoter (BBa_M13106), M13K07 gene VIII promoter (BBa_M13108), M13110 (BBa_M13110)), a constitutive *Bacillus subtilis* σ^A promoter (e.g., promoter veg (BBa_K143013), promoter 43 (BBa_K143013), P_{laG} (BBa_K823000), P_{lepA} (BBa_K823002), P_{veg} (BBa_K823003)), a constitutive *Bacillus subtilis* σ^B promoter (e.g., promoter etc (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)).

[0118] "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.

[0119] "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 are commensal bacteria, which are present in the indigenous microbiota of the gut. Examples of non-pathogenic bacteria include, but are not limited to *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Brevibacteria*, *Clostridium*, *Enterococcus*, *Escherichia*, *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*, *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. Naturally pathogenic bacteria may be genetically engineered to reduce or eliminate pathogenicity.

[0120] "Probiotic" is used to refer to live, non-pathogenic microorganisms, e.g., bacteria, which can confer health benefits to a host organism that contains an appropriate amount of the microorganism. In some embodiments, the host organism is a mammal. In some embodiments, the host organism is a human. Some species, strains, and/or subtypes of non-pathogenic bacteria are currently recognized as probiotic. Examples of probiotic bacteria include, but are not limited to, *Bifidobacteria*, *Escherichia*, *Lactobacillus*, and *Saccharomyces*, e.g., *Bifidobacterium bifidum*, *Enterococcus faecium*, *Escherichia coli*, *Escherichia coli* strain Nissle, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Saccharomyces boulardii* (Dinleyici et al., 2014; U.S. Patent No. 5,589,168; U.S. Patent No. 6,203,797; U.S. Patent 6,835,376). The probiotic may be a variant or a mutant strain of bacterium (Arthur et al., 2012; Cuevas-Ramos et al., 2010; Olier et al., 2012; Nougayrede et al., 2006). Non-pathogenic bacteria may be genetically engineered to enhance or improve desired biological properties, e.g., survivability. Non-pathogenic bacteria may be genetically engineered to provide probiotic properties. Probiotic bacteria may be genetically engineered to enhance or improve probiotic properties.

[0121] As used herein, "stably maintained", "stably expressed" or "stable" bacterium is used to refer to a bacterial host cell carrying non-native genetic material, e.g., a butyrogenic or other gene cassette or gene sequence(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/or 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 modified bacterium comprising a butyrogenic or other gene cassette or gene sequence(s), in which the plasmid or chromosome carrying the butyrogenic or other gene cassette or gene sequence(s) is stably maintained in the host cell, such that the gene cassette or gene sequence(s) can be expressed in the host cell, and the host cell 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.

[0122] As used herein, the term "treat" and its cognates refer to an amelioration of a disease or disorder, or at least one discernible symptom thereof. In another embodiment, "treat" refers to an amelioration of at least one measurable physical parameter, not necessarily discernible by the patient. In another embodiment, "treat" refers to inhibiting the progression of a disease or disorder, either physically (e.g., stabilization of a discernible symptom), physiologically (e.g., stabilization of a physical parameter), or both. In another embodiment, "treat" refers to slowing the progression or reversing the progression of a disease or disorder. As used herein, "prevent" and its cognates refer to delaying the onset or reducing the risk of acquiring a given disease or disorder.

[0123] 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 or disorder. In some instances, the "initial colonization of the newborn intestine is particularly relevant to the proper development of the host's immune and metabolic functions and to determine disease risk in early and later life" (Sanz et al., 2015). In some embodiments, early intervention (e.g., prenatal, perinatal, neonatal) using the genetically engineered bacteria of the invention may be sufficient to prevent or delay the onset of the disease or disorder.

[0124] As used herein a "pharmaceutical composition" refers to a preparation of genetically engineered bacteria of the invention with other components such as a physiologically suitable carrier and/or excipient.

[0125] The phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be used interchangeably refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered bacterial compound. An adjuvant is included under these phrases.

[0126] 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, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20.

[0127] 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. 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 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.

[0128] The articles "a" and "an," as used herein, should be understood to mean "at least one," unless clearly indicated to the contrary.

[0129] 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.

Bacteria

[0130] The genetically engineered bacteria of the invention are capable of producing a one or more non-native anti-inflammation and/or gut barrier function

enhancer molecule(s). In some embodiments, the genetically engineered bacteria are naturally 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. In some embodiments, non-pathogenic bacteria are Gram-negative bacteria. In some embodiments, non-pathogenic bacteria are Gram-positive bacteria. Exemplary bacteria include, but are not limited to *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Brevibacteria*, *Clostridium*, *Enterococcus*, *Escherichia coli*, *Lactobacillus*, *Lactococcus*, *Saccharomyces*, and *Staphylococcus*, e.g., *Bacillus coagulans*, *Bacillus subtilis*, *Bacteroides fragilis*, *Bacteroides subtilis*, *Bacteroides thetaiotaomicron*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Clostridium butyricum*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactococcus lactis*, *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*, *Pseudobutyrvibrio*, and *Ruminococcus*. In certain embodiments, the genetically engineered bacteria are selected from the group consisting of *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides subtilis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium lactis*, *Clostridium butyricum*, *Escherichia coli* Nissle, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Lactococcus lactis*. 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* ATCC49875, *C. beijerinckii*, *C. populeti* ATCC35295, *C. tyrobutyricum* JM1, *C. tyrobutyricum* cIP 1-776, *C. tyrobutyricum* ATCC25755, *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).

[0131] 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 is 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.

[0132] 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 clostridia l butyrogenic pathway genes are widespread in the genome-sequenced Clostridia and related species (Aboul naga et al.,

2013). Furthermore, genes from one or more different species of bacteria can be introduced into one another, e.g., the butyrogenic genes from *Peptoclostridium difficile* have been expressed in *Escherichia coli* (Aboulnaga et al., 2013).

[0133] 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.

Anti-inflammation and/or gut barrier function enhancer molecules

[0134] 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 embodiments, the two or more gene sequences are sequences encoding different genes. In some embodiments, 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 embodiments, 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 embodiments, 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, IL-27, TGF- β I, TGF- β 2, N-acylphosphatidylethanolamines (NAPEs), elafin (also known as peptidase inhibitor 3 or SKALP), trefoil factor, melatonin, PGD₂, kynurenic acid, and kynurenine. 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.

[0135] 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.

[0136] 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. 51** 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.

[0137] In some embodiments, the genetically engineered bacteria of the invention comprise one or more butyrogenic gene cassette(s) and are capable of producing butyrate. The genetically engineered bacteria may include any suitable set of butyrogenic genes (see, e.g., **Table 4**). Unmodified bacteria comprising butyrate biosynthesis genes are known and include, but are not limited to, *Peptoclostridium*, *Clostridium*, *Fusobacterium*, *Butyrivibrio*, *Eubacterium*, and *Treponema*, and these endogenous butyrate 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 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 under inducing conditions. *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 under inducing conditions. 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.

[0138] 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-enoyl-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 are capable of producing butyrate in low-oxygen conditions (see, e.g., Table 4). 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, 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 under inducing conditions. Alternatively, the *tesB* gene from *Escherichia coli* is capable of functionally replacing *pbt* and *buk* genes from *Peptoclostridium difficile*. Thus, in some embodiments, a butyrogenic gene cassette may comprise *thiA1*, *hbd* and *crt2* from *Peptoclostridium difficile*, *ter* from *Treponema denticola* and *tesB* from *E. coli*. In some embodiments, one or more of the butyrate biosynthesis genes may be functionally replaced or modified, e.g., codon optimized. In some embodiments, the butyrogenic gene cassette comprises genes for the aerobic biosynthesis of butyrate and/or genes for the anaerobic or microaerobic biosynthesis of 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 low-oxygen conditions. 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. Exemplary butyrate gene cassettes are shown in Figs. 1, 3, 4, 5, 6, 7, and 8.

[0139] In some embodiments, the genetically engineered bacteria are capable of expressing the butyrate biosynthesis cassette and producing butyrate under inducing conditions. The genes may be codon-optimized, and translational and transcriptional elements may be added. **Table 4** depicts the nucleic acid sequences of exemplary genes in the butyrate biosynthesis gene cassette.

[0140] In some embodiments, the genetically engineered bacteria comprise the nucleic acid sequence of any one of SEQ ID NOs: 1-10 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 any one of SEQ ID NOs: 1-10 or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that encodes a polypeptide of any one of SEQ ID NOs: 11-20 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 any one of SEQ ID NOs: 1-10 or a functional fragment thereof. In some embodiments, genetically engineered bacteria comprise a nucleic acid 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 nucleic acid sequence that encodes a polypeptide of any one of SEQ ID NOs: 11-20 or a functional fragment thereof.

Table 4

Gene sequence	01234567890123456789012345678901234567890123456789
<i>bcd2</i> (SEQ ID NO: 1)	ATGGATTAAATTCTAAAAAATATCAGATGCTTAAAGAGCTATATGTAAG CTTCGCTGAAAATGAAGTTAAACCTTTAGCAACAGAACTTGATGAAGAAG AAAGATTTCCCTTATGAAACAGTGGAAAAAATGGCAAAGCAGGAATGATG GGTATACCATATCCAAAAGAATATGGTGGAGAAGGTGGAGACACTGTAGG ATATATAATGGCAGTTGAAGAATTGTCTAGAGTTTGTGGTACTACAGGAG TTATATTATCAGCTCATACATCTCTGGCTCATGGCCTATATATCAATAT GGTAATGAAGAACAAAAACAAAAATTCTTAAGACCACTAGCAAGTGGAGA AAAATTAGGAGCATTTGGTCTTACTGAGCCTAATGCTGGTACAGATGCGT CTGGCCAACAACAACTGCTGTTTTAGACGGGGATGAATACATACTTAAT GGCTCAAAAATATTTATAACAAACGCAATAGCTGGTGACATATATGTAGT

Gene sequence	0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
	AATGGCAATGACTGATAAATCTAAGGGGAACAAAGGAATATCAGCATTTA TAGTTGAAAAAGGAACTCCTGGGTTTAGCTTTGGAGTTAAAGAAAAGAAA ATGGGTATAAGAGGTTTCAGCTACGAGTGAATTAATATTTGAGGATTGCAG AATACCTAAAGAAAATTTACTTGGAAAAGAAGGTCAAGGATTTAAGATAG CAATGTCTACTCT TGATGGTGGTAGAAT TGGTATAGCTGCACAAGCT TTA GGTTTAGCACAAGGTGCTCTTGATGAAACTGTAAATAATGTAAAAGAAAAG AGTACAATTTGGTAGACCATTATCAAAATTCCAAAATACACAATTTCCAAT TAGCTGATATGGAAGTTAAGGTACAAGCGGCTAGACACCTTGTATAT CAA GCAGCTATAAATAAAGACTTAGGAAAACCTTATGGAGTAGAAGCAGCAAT GGCAAAATTAATTTGCAGCTGAAACAGCTATGGAAGTTACTACAAAAGCTG TACAACCTTCATGGAGGATATGGATACACTCGTGACTATCCAGTAGAAAGA ATGATGAGAGATGCTAAGATAACTGAAATATATGAAGGAAGTAGTGAAGT TCAAAGAATGGTTATTTTCAGGAAAACCTATTAAATAG
<i>etfB3</i> (SEQ ID NO: 2)	ATGAATATAGTCGTTTGTATAAAAACAAGTTCCAGATACAACAGAAGTTAA ACTAGATCCTAATACAGGTACTTTAATTAGAGATGGAGTACCAAGTATAA TAAACCCTGATGATAAAGCAGGTTTAGAAGAAGCTATAAAATTAAAAGAA GAAATGGGTGCTCATGTAAGTGT TATAACAATGGGACCTCCTCAAGCAGA TATGGCT TAAAAGAAAGCTTTAGCAATGGGTGCAGATAGAGGTATAT TAT TAACAGATAGAGCATTTCGCGGTGCT GATACTTGGGCAACTTCATCAGCA TTAGCAGGAGCATTAAAAAATATAGATTTTGATATTATAATAGCTGGAAG ACAGGCGATAGATGGAGATACTGCACAAGTTGGACCTCAAATAGCTGAAC ATTTAAATCTTCCATCAATAACATATGCTGAAGAAATAAAAAGTGAAGGT GAATATGTATTAGTAAAAAGACAATTTGAAGATTGTTGCCAT GACTTAAA AGTTAAAATGCCATGCCT TATAACAAC TCTTAAAGATATGAACACACCAA GATACATGAAAGTTGGAAGAATATATGATGCTTTTCGAAAATGATGTAGTA GAAACATGGACTGTAAAAGATATAGAAGTTGACCCTTCTAAT TTAGGTCT TAAAGGTTCTCCAAC TAGTGTAT TTAAATCATTTACAAAATCAGTTAAAC CAGCTGGTACAATATACAATGAAGATGCGAAAACATCAGCTGGAATTATC ATAGATAAATTAAAAGAGAAGTATATCATATAA
<i>etfA3</i> (SEQ ID NO: 3)	ATGGGTAAACGTTTTAGTAGTAATAGAACAAGAGAAAATGTAATTCAAAC TGT TTCT TTAGAATTACTAGGAAAGGCTACAGAAATAGCAAAAGATTATG ATACAAAAGTTTCTGCAT TACTTTTAGGTAGTAAGGTAGAAGGTTTAATA GATACAT TAGCACA CTATGGTGCAGATGAGGTAATAGTAGTAGATGATGA AGCT TTAGCAGTGTATACAAC TGAACCATATACAAAAGCAGCTTATGAAG CAATAAAAGCAGCTGACCCTATAGT TGTAT TATTTGGTGCAACTTCAATA GGTAGAGATTTAGCGCCTAGAGTTTCTGCTAGAATACATACAGGTCTTAC TGCTGACTGTACAGGTCTTGCAGTAGCTGAAGATACAAAATTATTATTAA TGACAAGACCTGCCT TTGGTGGAATAATAATGGCAACAATAGTTTGTAAA GATTTTCAGACCTCAAATGTCTACAGTTAGACCAGGGGTTATGAAGAAAAA TGAACCTGATGAACTAAAGAAGCTGTAATTAACCGTTTCAAGGTAGAAT TTAATGATGCT GATAAATTAGTTCAAGTTGTACAAGTAATAAAAAGAAGCT AAAAACAAGTTAAAATAGAAGATGCTAAGATATTAGTTTCTGCTGGACG TGGAATGGGTGGAAAAGAAAACCTTAGACATACTTTATGAATTAGCTGAAA TTATAGGTGGAGAAGTTTCTGGT TCTCGTGCCACTATAGATGCAGGT TGG TTAGATAAAGCAAGACAAGTTGGTCAAACCTGGTAAAACGTGAAGACCAGA CCTTTATATAGCAT GTGGTATAT CTGGAGCAATACAACATATAGCTGGTA TGGAAGATGCTGAGTTTATAGTTGCTATAAATAAAAATCCAGAAGCTCCA

Gene sequence	0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
	ATATTTAAATATGCTGATGT TGGTATAGT TGGAGATGTT CATAAAGTGCT TCCAGAACTTATCAGTCAGTTAAGTGTTGCAAAAGAAAAAGGTGAAGTTT TAGCTAACTAA
<i>thiA1</i> (SEQ ID NO: 4)	ATGAGAGAAGTAGTAATTGCCAGTGCAGCTAGAACAGCAGTAGGAAGTTT TGGAGGAGCATTTAAATCAGTTTCAGCGGTAGAGTTAGGGGTAACAGCAG CTAAAGAAGCTATAAAAAAGAGCTAACATAACTCCAGATATGATAGATGAA TCTCTTTTAGGGGGAGTACTTACAGCAGGTCTTGGACAAAATATAGCAAG ACAAATAGCAT TAGGAGCAGGAATACCAGTAGAAAAACCAGCTATGACTA TAAATATAGT TTGTGGT TCTGGAT TAAGATCTGT TTCAATGGCATCTCAA CTTATAGCAT TAGGTGATGCTGATATAATGTTAGTTGGTGGAGCTGAAAA CATGAGTATGTCTCCT TATTTAGTACCAAGTGCGAGATATGGTGCAAGAA TGGGTGATGCTGCT TTTGTTGATTCAATGATAAAAGATGGAT TATCAGAC ATATTTAATAAC TATCACATGGGTATTACTGCTGAAAACATAGCAGAGCA ATGGAATATAACTAGAGAAGAACAAGATGAATTAGCTCTTGCAAGTCAAA ATAAAGCTGAAAAAGCTCAAGCTGAAGGAAAATTTGATGAAGAAATAGTT CCTGT TGT TATAAAAGGAAGAAAAGGTGACACTGTAGTAGATAAAGATGA ATATATTAAGCCTGGCACTACAATGGAGAACTTGCTAAGTTAAGACCTG CATTTAAAAAAGATGGAACAGTTACTGCTGGTAATGCATCAGGAATAAAT GATGGTGCTGCTATGTAGTAGTAATGGCTAAAGAAAAAGCTGAAGAAGT AGGAATAGAGCCTCT TGCAACTATAGT TTCT TATGGAACAGCTGGTGT TG ACCCTAAAATAATGGGATATGGACCAGTTCCAGCAACTAAAAAAGCTTTA GAAGCTGCTAATATGACTATTGAAGATATAGATTTAGTTGAAGCTAATGA GGCATTGCTGCCCAATCTGTAGCTGTAATAAGAGACTTAAATATAGATA TGAATAAAGTTAATGTTAATGGTGGAGCAATAGCTATAGGACATCCAATA GGATGCTCAGGAGCAAGAATACTTACTACACTTTTATATGAAATGAAGAG AAGAGATGCTAAAAGTGGTCT TGCTACACT TTGTATAGGCGGTGGAATGG GAAGTACTTTAATAGTTAAGAGATAG
<i>hbd</i> (SEQ ID NO: 5)	ATGAAATTAGCTGTAATAGGTAGTGGAAGTATGGGAAGTGGTAT TGTACA AACTTTTGCAAGTTGTGGACATGATGTATGT TTAAAGAGTAGAACTCAAG GTGCTATAGATAAATGTTTAGCTTTAT TAGATAAAAAATTTAACTAAGTTA GTTACTAAGGGAAAAATGGATGAAGCTACAAAAGCAGAAATATTAAGTCA TGTTAGTTCAACTACTAAT TATGAAGATTTAAAAGATATGGATTTAATAA TAGAAGCATCTGTAGAAGACATGAATATAAAGAAAGATGTTTTCAAGTTA CTAGATGAATTATGTAAAGAAGATACTATCTTGGAACAAATACTTCATC ATTATCTATAACAGAAATAGCTTCT TCTAC TAAGCGCCAGATAAAGTTA TAGGAATGCATTTCTTTAATCCAGTTCCTATGATGAAATTAGTTGAAGTT ATAAGTGGTCAGTTAACATCAAAAAGTTACTTTTGATACAGTATTTGAATT ATCTAAGAGTATCAATAAAGTACCAGTAGATGTATCTGAATCTCCTGGAT TTGTAGTAAATAGAATACTTATACCTAT GATAAATGAAGCTGT TGGTATA TATGCAGATGGTGTGCAAGTAAAGAAGAAATAGATGAAGCTATGAAATT AGGAGCAAACCATCCAATGGGACCACTAGCATTAGGTGATTTAATCGGAT TAGATGTTGTTTAGCTATAAT GAACGTTTTATATACTGAATTTGGAGAT

Gene sequence	0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
	ACTAAATATAGACCTCATCCACTTTTAGCTAAAATGGT TAGAGCTAAT CA ATTAGGAAGAAAACTAAGATAGGATTCTATGATTATAATAATAA
<i>crt2</i> (SEQ ID NO: 6)	ATGAGTACAAGTGATGTTAAAGTTTATGAGAATGTAGCTGT TGAAGTAGA TGGAATAATATGTACAGTGAAAATGAATAGACCTAAAGCCCT TAATG CAA TAAATTCAAAGACTTTAGAAGAAGCTTTATGAAGTATTTGTAGATATTAAT AATGATGAACTATTGATGTTGTAATAT TGACAGGGGAAGGAAAGGCATT TGTAGCTGGAGCAGATATTGCATACATGAAAGATTAGATGCTGTAGCTG CTAAAGATTTTAGTATCTTAGGAGCAAAAGCTTTTGGAGAAATAGAAAAT AGTAAAAAAGTAGTGATAGCTGCTGTAAACGGAT TTGCT TTAGGTGGAGG ATGTGAAC TTGCAATGGCATGTGATATAAGAAT TGCAT CTGCTAAAGCTA AATTTGGTCAGCCAGAAGTAAC TCTTGGATAAATCCAGGATATGGAGGA ACTCAAAGGCTTACAAGATTGGTTGGAATGGCAAAAGCAAAAGAATTAAT CTTTACAGGTCAAGTTATAAAAGCTGATGAAGCTGAAAAAATAGGGCTAG TAAATAGAGTCGTTGAGCCAGACATTTTAATAGAAGAAGTTGAGAAATTA GCTAAGATAATAGCTAAAAATGCTCAGCTTGCAGTTAGATACTCTAAAGA AGCAATACAAC TTGGTGCT CAACTGATA TAAATACTGGAATAGATATAG AATCTAATTTATTTGGTCTTTGTTTTTCAAC TAAAGACCAAAAAGAAGGA ATGTCAGCTTTCGTTGAAAAGAGAGAAGCTAAC TTTATAAAAAGGGTAA
<i>pbt</i> (SEQ ID NO: 7)	ATGAGAAGTTTGAAGAAGTAAT TAAGTTTGCAAAAGAAAGAGGACCTAA AATATATCAGTAGCATGT TGC CAAGATAAAAGAAGTTTAAATGGCAGTTG AAATGGCTAGAAAAGAAAAAATAGCAAATGCCATTTTAGTAGGAGATATA GAAAAGACTAAAGAAATTGCAAAAAGCATAGACATGGATATCGAAAATTA TGAAGTATAGATATAAAAGAT TTAGCAGAAGCATCTCTAAAATCTGTTG AAT TAGTTTTACAAGGAAAAGCCGACATGGTAAT GAAAGGCTTAGTAGAC ACATCAATAATACTAAAAGCAGTTTTAAATAAAGAAGTAGGTCTTAGAAC TGGAATGTATTAAGTCACGTAGCAGTATTTGATG TAGAGGGATATGATA GATTATTTTTCGTAAC TGACGCAGCTATGAACTTAGCTCCT GATACAAAT ACTAAAAAGCAAATCATAGAAAATGCTTGCACAGTAGCACATTCATTAGA TATAAGTGAACCAAAAGTTGCTGCAATATGCGCAAAAGAAAAAGTAAATC CAAAAATGAAAGATACAGTTGAAGCTAAAGAACTAGAAGAAATGTATGAA AGAGGAGAAATCAAAGGTTGTATGGT TGGTGGGCCT TTTGCAAT TGATAA TGCAGTATCTTTAGAAGCAGCTAAACATAAAGGTATAAATCATCCTGTAG CAGGACGAGCTGATATATTAT TAGCCCCAGATATTGAAGGTGGTAACATA TTATATAAAGC TTTGGTAT TCTTCTCAAAATCAAAAAATGCAGGAGTTAT AGTTGGGGCTAAAGCACCAATAATATTAAC TCTAGAGCAGACAGTGAAG AACTAAACTAACTCAATAGCTTTAGGTGTTTTAAT GGCAGCAAAGGCA TAA
<i>buk</i> (SEQ ID NO: 8)	ATGAGCAAAATATTTAAATCTTAACAATAAATCCTGGT TCGACATCAAC TAAATAGCTGTATTTGATAATGAGGATTTAGTATTTGAAAAAAGCTTTAA GACATTCCTCAGAAGAAATAGGAAAATATGAGAAGGTGTCTGACCAATTT GAATTTTCGTAAACAAGTAATAGAAGAAGCTCTAAAAGAAGGTGGAGTAAA AACATCTGAATTAGATGCTGTAGTAGGTAGAGGAGGACTTCTTAAACCTA TAAAAGGTGGTACTTATTCAGTAAGTGCTGCTATGATTGAAGATTTAAAA GTGGGAGTTTTAGGAGAACACGCTTCAAACCTAGGTGGAATAATAGCAAA

Gene sequence	01234567890123456789012345678901234567890123456789
	ACAAATAGGTGAAGAAGTAAATGTTTCCTTCATACATAGTAGACCCTGTTG TTGTAGATGAATTAGAAGATGTTGCTAGAATTTCTGGTATGCCTGAAATA AGTAGAGCAAGTGTAGTACATGCTTTAAATCAAAGGCAATAGCAAGAAG ATATGCTAGAGAAATAACAAGAAATATGAAGATAAAATCTTATAGTTG CACATGCGGTGGAGGAGTTTCTGTGGAGCTCATAAAAATGGTAAATAG GTAGATGTTGCAAACGCATTAGATGGAGAAGGACCTTTCTCTCAGAAAAG AAGTGGTGGACTACCAGTAGGTGCATAGTAAAAATGTGCTTTAGTGGAA AATATAC TCAAGATGAAATAAAAAGAAAATAAAAGGTAATGGCGGACTA GTTGCATACCTAAACAC TAATGATGCTAGAGAAGTTGAAGAAAGAATTGA AGCTGGTGATGAAAAAGCTAAATTAGTATATGAAGCTATGGCATATCAAA TCTCTAAAGAAATAGGAGCTAGTGCTGCAGTTCTTAAGGGAGATGTAAAA GCAATAT TATTAAC TGGT GGAATCGCATATCAAAAAATGTTTACAGAAAT GATTGCAGATAGAGTTAAATTTATAGCAGATGTAAAGTTTATCCAGGTG AAGATGAAATGATTGCATTAGCTCAAGGTGGACTTAGAGTTTAACTGGT GAAGAAGAGGCTCAAGTTTATGATAACTAA
<i>ter</i> (SEQ ID NO: 9)	ATGATCGTAAAACCTATGGTACGCAACAATATCTGCCTGAACGCCCATCC TCA GGGCTGCAAGAAGGGAGTGAAGATCAGAT TGAATA TACCAAGAAAC GCATTACCGCAGAAGTCAAAGCTGGCGCAAAAGCTCCAAAAAACGT TCTG GTGCT TGGCTGCTCAAATGGT TACGGCCTGGCGAGCCGCATTACTGCTGC GTTCGGATACGGGGCTGCGACCATCGGCGTGTCTTTGAAAAAGCGGGTT CAGAAAC CAAATATGGTACACCGGGATGGTACAATAATTTGGCATTTGAT GAAGCGGCAAAACGCGAGGGTCTTTATAGCGTGACGATCGACGGCGATGC GTTTTCAGACGAGATCAAGGCCCAAGTAAT TGAAGAACCAAAAAAAG GTATCAAATTTGATCTGATCGTATACAGCTTGGCCA GCCCA GTACGTA CT GATCCTGATACAGGTATCATGCA CAAAAGCGTTTTGAAACCTTTGAAAA AACGTTCA CAGGCAAAACAGTAGATCCGTTTACTGGCGAGCTGAAGGAAA TCTCCGCGGAACCAGCAAATGACGAGGAAGCAGCCGCCACTGTTAAAGT T ATGGGGGGTGAAGATTGGGAACGT TGGATTAAGCAGCTGTCTGAAGGAAGG CCTCT TAGAAGAAGGCTGTAT TACCT TGGCCTATAGT TATAT TGGCCCTG AAGCTA CCAAGCTTTGTACCGTAAAGGCA CAAT CGGCAAGGCCAAAGAA CACCTGGAGGCCACAGCA CACCGTCTCAACAAAGAGAAC CCGTCAAT CCG TGCCTTCGTGAGCGTGAATAAAGGCCTGGTAACCCGCGCAAGCGCCGTAA TCCCGGTAATCCCTCTGTATCTCGCCAGCT TGT TCAAAGTAATGAAAGAG AAGGGCAATCATGAAGGT TGTAT TGAACAGATCACGCGTCTGTACGCCGA GCGCCTGTACCGTAAAGATGGTACAAT TCCAGTTGATGAGGAAAATCGCA TTCGCAT TGTGATTGGGAGTTAGAAGAAGACGTCCAGAAAGCGGTATCC GCGT TGTATGGAGAAAGTCACGGGTGAAAACGCAGAATCTCTCACTGACT T AGCGGGGTACCGCCATGATTTCT TAGCTAGTAACGGCT TGTATGTAGAAG GTATTAAT TATGAAGCGGAAGT TGAACGCTTCGACCGTATCTGA
<i>tesB</i> (SEQ ID NO: 10)	ATGAGTCAGGCGCTAAAAAAT TACTGACATTGTTAAAT CTGGAAAAAAT TGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTAGGTTTACGCCAGG TGT TGGCGGCCAGGTCGTGGGTGAGGCCTTGTATGCTGCAAAAGAGACC GTCCCTGAAGAGCGGCTGGTACATTCGT TCCACAGCTACT TTCT TCGCCC TGGCGATAGTAAGAAGCCGAT TAT TTATGATGTCGAAACGCTGCGTGACG GTAACAGCT TCAGCGCCCGCCGGGTGCTGCTAT TCAAAACGGCAAAACCG ATTTTTTATATGACTGCCTCT TCCAGGCACCAGAAGCGGGTTTCGAACA TCAAAAAACAATGCCGTCCGCGCCAGCGCCTGATGGCCTCCCT TCGGAAA

Gene sequence	0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
	CGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCAGTGCTGAAAGAT AAATTCATCTGCGATCGTCCGCTGGAAGTCCGTCGGTGGAGTTTCATAA CCCACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTGGATCCGCG CAAATGGTAGCGTGCCGGATGACCTGCGCGTTCATCAGTATCTGCTCGGT TACGCTTCTGATCTTAACCTCCTGCCGGTAGCTCTACAGCCGCACGGCAT CGGTTTTCTCGAACCGGGGATTGAGATTGCCACCATTGACCATTCCATGT GGTTCCATCGCCGTTTAATTTGAATGAATGGCTGCTGTATAGCGTGGAG AGCACCTCGGCGTCCAGCGCACGTGGCTTTGTGCGCGGTGAGTTTTATAC CCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGGAAGGGGTGATGCGTA ATCACAAT TAA

Table 5

Amino acid sequence	0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
<i>bcd2</i> (SEQ ID NO: 11)	MDLNSKKYQMLKELYVSFAENEVKPLATELDEEERFPYETVEKMAKAGMM GIPYPKEYGGEGGDTVGYIMAVEELSRVCGTTGVILSAHTSLGSWPIYQY GNEEQKQKFLRPLASGEKLGAFGLTEPNAGTDASGQQTAVLDGDEYILN GSKI FITNAIAGDI Y _{11A} /MAMTDKSKGNKGISAFIVEKGTGPGFSFGVKEKK MGIRGSATSELI FEDCRIPKENLLGKEGQGFKIAMSTLDGGRIGIAAQUAL GLAQGALDETVKYVKERVQFGRPLSKFQNTQFQLADMEVKVQAARHLVYQ AAINKDLGKPYGVEAAMAKLFAAETAMEVTTKAVQLHGGYGYTRDYPVER MMRDAKITEIYEGTSEVQRMVISGKLLK
<i>etfB3</i> (SEQ ID NO: 12)	MNIWCIKQVPDTEVKLDPNTGTLIRDGVPSIINPDDKAGLEEAIKLKE EMGAHVTVITMGPPQADMALKEALAMGADRGILLTDRAFAGADTWATSSA LAGALKNIDFDI I IAGRQAIDGDTAQVGPQIAEHLNLP I ITYAEI KTEG EYVLVKRQFEDCCHDLKVKMPCLITTLKDMNTPRYMKVGRIYDAFENDW ETWTVKDIEVDPSNLGLKGSPTS SVFKSFTKSVKPAGTI YNEDAKTSAGI I IDKLKEYI I
<i>etfA3</i> (SEQ ID NO: 13)	MGNVLWIEQRENVITQTVSLELLGKATEIAKDYDTKVSALLLGSKVEGLI DTLAHYGADEVIWDDEALAVYTTEPYTKAAYEAIKAADPIWLFGATSI GRDLAPRVSARIHTGLTADCTGLAVAEDTKLLLMTRPAFGGNIMATIVCK DFRPQMS TVRPGVMKKNE PDE TKEA _V INRFKVE FNDADKLVQWQV IKEA KKQVK IEDAK ILVSAGRGMGGKENLD ILYE LAE IIGGEVSGS RA _T IDAGW LDKARQVGQTGKTVRPDLIACGISGAIQHIAGMEDAEFIVAINKNPEAP IFKYADVGVGDVHKVLP ELISQLSVAKEKGEVLN
<i>thiA1</i> (SEQ ID NO: 14)	MREWIASAARTAVGSFGGAFKSVSAVELGVTAAKEAIKRANITPDMIDE SLLGGVLTAGLGQNIARQIALGAGIPVEKPAMTINIVCGSGLRSVSMASQ LIALGDADIMLVGGAENMSMSPYLVPSARYGARMGDAAFVDSMIKDGLSD IFNNYHMGITAENIAEQWNITREEQDELALASQNKAEKAQAEGKFDEEIV PWIKGRKGD _T WDKDEYIKPGTTMEKLAKLRPAFKKDGTVTASNASGIN DGAAMLVMAKEKAEELGIEPLATIVSYGTAGVDPKIMGYGPVPATKKAL

Amino acid sequence	01234567890123456789012345678901234567890123456789
	EAANMT IED IDLVEANEAFAAQS VAVI RDLNI DMNKVNVNGGAI A IGHP I GCSGARILTTLLYEMKRRDAKTGLATLCIGGGMGTTLIVKR
<i>hbd</i> (SEQ ID NO: 15)	MKLAVIGSGTMGSGIVQTFASCGHDVCLKSRTQGAIDKCLALLDKNLTKL VTKGKMDEATKAEILSHVSSTTNYEDLKMDLI IEASVEDMNIKKDVFKL LDELCKEDTILATNTSSLSITEIASSTKRDPKVIGMHFFNPVPMMLVEV ISGQLTSKVTFDTVFELSKSINKVPVDVSESPGFWNRLIPMINEAVGI YADGVASKEE IDEAMKLGANHPMGPLALGDL IGLDWLAIMNVL YTE FGD TKYRPHPLAKMVRANQLGRKTKIGFYDYNK
<i>crt2</i> (SEQ ID NO: 16)	MSTSDVKVYENVAVEVDGNICTVKMNRPKALNAINSKTLEELYEVFVDIN NDETIDWILTGEGKAFVAGADIAYMKDLDAVAAKDFSILGAKAFGEIEN SKKWIAAVNGFALGGGCELAMACDIRIASAKAKFGQPEVTLGITPGYGG TQRLTRLVGMAKAKELIFTGQVIKADEAEKIGLVNRWEPDILIEVEKL AKI IAKNAQLAVRYSKEAIQLGAQTDINTGIDIESNLFGLCFSTKDQKEG MSAFVEKREANFIKG
<i>pbt</i> (SEQ ID NO: 17)	MRS FEEVIKFAKERGPKT ISVACCQDKEVLMAVEMARKEKIANAI LVGDI EKTKE IAKS IDMD IENYE L ID IKDLAEAS LKSVE LVS QGKADMVMKGLVD TSIILKAVLNKEVGLRTGNVLSHVAVFDVEGYDRLFFVTDAAMNLAPDTN TKKQI IENACTVAHSLDISEPKVAAICAKEKVNPKMKDTVEAKELEEMYE RGE IKGCMVGGP FAI DNAVS LEAAKHKG INHPVAGRAD ILLAPD IEGGNI LYKALVFFFSKSNAGVIVGAKAPI ILTSRADSEETKLNS IALGVLMAAKA
<i>buk</i> (SEQ ID NO: 18)	MSKI FKILTINPGSTSTKIAVFDNEDLVFEKTLRHSSEEIGKYEKVSDQF EFRKQVIEEALKEGGVKTSELDAWGRGGLLKPIKGGTYSVSAAMIEDLK VGVLGEGHASNLGGI IAKQIGEEVNPVPSYIVDP\AA/DELEDVARISGMPEI SRASWHALNQKAIARRYAREINKKYEDINLIVAHMGGGVSVAHKNNGKI VDVANALDGEGPFSPERSGGLPVGALVKMCFSGKYTQDEIKKKIKGNGGL VAYLNTNDAREVEERIEAGDEKAKLVYEAMAYQISKEIGASAAVLKGDVK AILLTGGIAYSKMFTEMIADRVKFIADVKKVYPGEDEMIALAQGGRLVLTG EEEAQVYDN
<i>ter</i> (SEQ ID NO: 19)	M I V K P M V R N N I C L N A H P Q G C K K G V E D Q I E Y T K K R I T A E V K A G A K A P K N V L V L G C S N G Y G L A S R I T A A F G Y G A A T I G V S F E K A G S E T K Y G T P G W Y N N L A F D E A A K R E G L Y S V T I D G D A F S D E I K A Q V I E E A K K K G I K F D L I V Y S L A S P V R T D P D T G I M H K S V L K P F G K T F T G K T V D P F T G E L K E I S A E P A N D E E A A T V K V M G G E D W E R W I K Q L S K E G L L E E G C I T L A Y S Y I G P E A T Q A L Y R K G T I G K A K E H L E A T A H R L N K E N P S I R A F V S V N K G L V T R A S A V I P V I P L Y L A S L F K \ / M K E K G N H E G C I E Q I T R L Y A E R L Y R K D G T I P V D E E N R I R I D D W E L E E D V Q K A V S A L M E K V T G E N A E S L T D L A G Y R H D F L A S N G F D V E G I N Y E A E V E R F D R I
<i>tesB</i> (SEQ ID NO: 20)	MSQALKNLLTLLNLEKIEEGLFRGQSEDGLRQVFGGQWGQALYAAKET VPEERLVHSFHSYFLRPGDSKKPI IYDVETLRDGNFSARRVAAIQNGKP IFYMTASFQAPEAGFEHQKTMPAPDGLPSETQIAQSLAHLPPVLKD KFICDRPLEVRPVE FHNPLKGHVAEPHRQVWIRANGSVPDLLRVHQYLLG

Amino acid sequence	01234567890123456789012345678901234567890123456789
	YASDLNFLPVALQPHGIGFLEPGIQIATIDHSMWFHRPFNLNEWLLYSVE STSASSARG FVRGE FYT QDGVLVAS TVQE GVMRHNH

[0141] In some embodiments, the genetically engineered bacteria of the invention comprise a propionate gene cassette and are capable of producing propionate. The genetically engineered bacteria may express any suitable set of propionate biosynthesis genes (*see, e.g., Table 6*). 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*, and these endogenous propionate 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 propionate biosynthesis genes from a different species, strain, or substrain of bacteria. In some embodiments, the genetically engineered bacteria comprise the genes *pet*, *led*, and *aer* from *Clostridium propionicum*. In some embodiments, the genetically engineered bacteria comprise acrylate pathway genes for propionate biosynthesis, *e.g., pet*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*. In alternate embodiments, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, *e.g., thrA^{fb'}*, *thrB*, *thrC*, *ilvA^{fb'}*, *oceE*, *aceF*, and *lpd*, and optionally further comprise *tesB*. The genes may be codon-optimized, and translational and transcriptional elements may be added. **Table 6** depicts the nucleic acid sequences of exemplary genes in the propionate biosynthesis gene cassette.

[0142] In some embodiments, the genetically engineered bacteria comprise the nucleic acid sequence of any one of SEQ ID NOs: 21-34 and 10 or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a nucleic acid sequence that encodes a polypeptide of any one of SEQ ID NOs: 35-48 and 20 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 any one of SEQ ID NOs: 21-34 and 10 or a functional fragment thereof. In some

embodiments, genetically engineered bacteria comprise a nucleic acid 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 nucleic acid sequence that encodes a polypeptide of any one of SEQ ID NOS: 35-48 and 20 or a functional fragment thereof.

Table 6

Gene sequence	01234567890123456789012345678901234567890123456789
<i>pct</i> SEQ ID NO: 21	ATGCGCAAAGTGCCGATTATCACGGCTGACGAGGCCGCAAACTGATCAA GGACGGCGACACCGTGACAAC TAGCGGCTTTGTGGGTAAACGCGATCCCTG AGGCCCTTGACCGTG CAGTCGAAAAGCGTTTCCTGGAACGGGCGAACCG AAGAACATTACTTATGTATATTGCGGCAGTCAGGGCAATCGCGACGGTCG TGGCGCAGAACATTTTCGCGCATGAAGGCCTGCTGAAACGTTATATCGCTG GCCATTGGGCGACCGTCCCGGCGTTAGGGAAAATGGCCATGGAGAATAAA ATGGAGGCCTACAATGTCTCTCAGGGCGCCTTGTGTCATCTCTTTCGCGA TATTGCGAGCCATAAACCGGGTGTGTTACGAAAGTAGGAATCGGCACCT TCATTGATCCACGTAACGGTGGTGGGAAGGTCAACGATATTACCAAGGAA GATATCGTAGAACTGGTGGAAATTAAAGGGCAGGAATACCTGTTTTATCC GCGGTTCCCGATCCATGTCTCGCGCTGATTCTGTGGCACCTATGCGGACGAGA GTGGTAACATCACCTTTGAAAAAGAGGTAGCGCCTTTGGAAGGGACTTCT GTCTGTCAAGCGGTGAAGAACTCGGGTGGCATTGTCTGTGGTTCAGGTTGA GCGTGTCTGTCAAAGCAGGCACGCTGGATCCGCGCCATGTGAAAGTTCGGG GTATCTATGTAGATTACGTAGTCGTCTCGCGGATCCGGAGGACCATCAACAG TCCCTTGACTGCGAATATGATCCTGCCCTTAGTGAGAGCACCGTCGTCC GGAGGTGGTGGGTGAACCACTGCCTTTATCCGCGAAGAAAGTCATCGGCC GCCGTGGCGCGATTGAGCTCGAGAAAGACGTTGCAGTGAACCTTGGGGTA GGTGCACCTGAGTATGTGGCCTCCGTGGCCGATGAAGAAGGCATTGTGGA TTTTATGACTCTCACAGCGGAGTCCGGCGCTATCGGTGGCGTTCCAGCCG GCGGTGTTCGCTTTGGGGCGAGCTACAATGCTGACGCCTTGATCGACCAG GGCTACCAATTTGATTATTACGACGGTGGGGTCTGGATCTTTGTTACCT GGGTTTAGCTGAATGCGACGAAAAGGGTAATATCAATGTTAGCCGCTTCG GTCCTCGTATCGCTGGGTGCGGCGGATTCATTAACATTACCCAAAACACG CCGAAAGTCTTCTTTTGTGGGACCTTTACAGCCGGGGGGCTGAAAGTGAA AATTGAAGATGGTAAGGTGATTATCGTTTCAGGAAGGGAAACAGAAGAAAT TCCTTAAGGCAGTGGAGCAAATCACCTTTAATGGAGACGTGGCCTTAGCG AACAAGCAACAAGTTACCTACATCACGGAGCGTTGCGTCTTCCTCCTCAA AGAAGACGGTTTACACCTTTTCGGAAATCGCGCCAGGCATCGATCTGCAGA CCCAGATTTTGGATGTTATGGACTTTGCCCGGATCATTGATCGTGACGCA AACGGGCAGATTAACTGATGGACGCGGCGTTATTTCGCAGAAGGGCTGAT GGGCTTGAAAGAAATGAAGTCTTAA

Gene sequence	01234567890123456789012345678901234567890123456789
<i>IcdA</i> SEQ ID NO: 22	ATGAGCTTAACCCAAGGCATGAAAGCTAAACAACCTGTTAGCATACTTTCA GGGTAAAGCCGATCAGGATGCACGTGAAGCGAAAGCCCGCGGTGAGCTGG TCTGCTGGTCGGCGTCAGTCGCGCCGCGGAAT TTTGCGTAACAATGGGC ATTGCCATGATCTACCCGGAGACTCATGCAGCGGGCATCGGTGCCCCGAA AGGTGCGATGGACATGCTGGAAGT TGCAGACCGCAAAGGCTACAACGTGG ATTGT TGT TCCTACGGCCGTGTAAATATGGGT TACATGGAATGT TAAAA GAAGCCGCCATCACGGGCGTCAAGCCGGAAGT TTTGGT TAAT TCCCCTGC TGCTGACGT TCCGCT TCCCGAT TTGGTGAT TACGTGTAATAATATCTGTA ACACGCTGCTGAAATGGTACGAAACCTTAGCAGCAGAACTCGATATTCTT TGCATCGTGATCGACGTACCGT TTAATCATACCATGCCGAT TCCGGAATA TGCCAAGGCCTACATCGCGGACCAGT TCCGCAATGCAAT TTCTCAGCTGG AAGTTATTTGTGGCCGTCCGT TCGATTGGAAGAAATTTAAGGAGGTCAAA GATCAGACCCAGCGTAGCGTATACCACTGGAACCGCATTGCCGAGATGGC GAAATACAAGCCTAGCCCGCTGAACGGCT TCGATCTGT TCAAT TACATGG CGT TAATCGTGGCGTGCCGCAGCCTGGAT TATGCAGAAATTACCT TAAAA GCGT TCGCGGACGAATTAGAAGAGAATTTGAAGGCGGGTATCTACGCCT T TAAAGGTGCGGAAAAAACGCGCTTTCAATGGGAAGGTATCGCGGTGTGGC CACATTTAGGTCACACGTTTAAATCTATGAAGAATCTGAATTTCGATTATG ACCGGTACGGCATAACCCGCCCT TTGGGACCTGCACTATGACGCTAACGA CGAATCTATGCACTCTATGGCT GAAGCGTACACCCGTAT TTATAT TAATA CTTGTCTGCAGAACAAAGTAGAGGTCTCTGCT TGGGATCATGAAAAAGGC CAGGTGGATGGTACCGTATAT CATCTGAATCGCAGCTGCAAACT GATGAG TTTCCTGAACGTGGAACGGCTGAAATTATTAAAGAGAAGAAGGTCTTC CTTACGTCTCCAT TGATGGCGATCAGACCGATCCTCGCGT TTTTCTCCG GCCAGTTTGATACCCGTGT TCAGGCCCTGGT TGAGATGATGGAGGCCAA TATGGCGGCAGCGGAATAA
<i>IcdB</i> SEQ ID NO: 23	ATGTCACGCGTGGAGGCAATCCTGTGCGCAGCTGAAAGATGTGCGCCGCGAA TCCGAAAAAAGCCATGGATGACTATAAAGCTGAAACAGGTAAGGGCGCGG TTGGTATCATGCCGATCTACAGCCCCGAAGAAATGGTACACGCCGCTGGC TATTTGCCGATGGGAATCTGGGGCGCCAGGGCAAAACGAT TAGTAAAGC GCGCACCTATCTGCCTGCT TTTGCCTGCAGCGTAATGCAGCAGGT TATGG AAT TACAGTGCGAGGGCGCGTATGATGACCTGTCCGCAGT TATTTTATAGC GTACCGTGCGACACTCTCAAATGTCT TAGCCAGAAATGGAAAGGTACGTC CCCAGTGAT TGTAT TTACGCATCCGCAGAACCGCGGAT TAGAAGCGGCGA ACCAATTCT TGGT TACCGAGTAT GAACTGGTAAAAGCACAAC TGGAAATCA GTCTCTGGGTGTGAAAAT TTCAAACGCCGCCCTGGAAAAT TCGATTGCAAT TTATAACGAGAATCGTGCCGTGATGCGTGAGT TCGTGAAAGTGGCAGCGG ACTATCCTCAAGTCAT TGACGCAGTGAGCCGCCACGCGGT TTTTAAAGCG CGCCAGTTTATGCT TAAGGAAAAACATAACCGCACTTGTGAAAGAACTGAT CGCTGAGATTAAAGCAACGCCAGTCCAGCCGTGGGACGGAAAAAAGGTTG TAGTGACGGGCATTCTGT TGAACCGAATGAGTTATTAGATATCTTTAAT GAGTTTAAAGATCGCGATTGTTGATGATGATTTAGCGCAGGAAAGCCGTCA GATCCGTGT TGACGT TCTGGACGGAGAAGGCGGACCGCTCTACCGTATGG CTAAAGCGTGCGCAGCAAATGTATGGCTGCTCGCTGGCAACCGACACCAAG AAGGGTCGCGGCCGTATGT TAATTAACAAAACGATTTCAGACCGGTGCGGA CGCTATCGTAGT TGCAATGATGAAGTTTTCGACCCAGAAGAATGGGATT ATCCGGTAATGTACCGTGAATTTGAAGAAAAAGGGTCAAATCACTTATG

Gene sequence	01234567890123456789012345678901234567890123456789
	ATTGAGGTGGATCAGGAAGTATCGTCT TTCGAACAGATTAAAACCCGTCT GCAGTCATTCGTCGAAATGCT TTAA
<i>IcdC</i> SEQ ID NO: 24	ATGTATACCT TGGGGATTGATGTCGGT TCTGCCTCTAGTAAAGCGGTGAT TCTGAAAGATGGAAAAGATATGTGCGTGCCGAGGT TGTCCAAGTCGGTA CCGGCTCCTCGGGTCCCCAACGCGCACTGGACAAAGCCT TTGAAGTCTCT GGCTTAAAAAAGGAAGACATCAGCTACACAGTAGCTACGGGCTATGGGCG CTTCAATTTTAGCGACGCGGATAAACAGATTTTCGAAATTAGCTGTCATG CCAAAGGCATTTATTTCT TAGTACCAACTGCGCGCACTATTATTGACATT GGCGGCCAAGATGCGAAAGCCATCCGCCTGGACGACAAGGGGGGTATTAA GCAATTCTTCATGAATGATAAATGCGCGGCGGGCACGGGGCGT TTCCTGG AAGTCATGGCTCGCGTACT TGAAACCACCCTGGATGAAATGGCTGAACTG GATGAACAGGCGACTGACACCGCTCCCAT TTCAAGCACCTGCACGGT TTT CGCCGAAAGCGAAGTAATTAGCCAATTGAGCAATGGTGTCTCACGCAACA ACATCAT TAAAGGTGTCCATCTGAGCGT TCGGTCACGTGCGTGTGGTCTG GCGTATCGCGGCGGT TTGGAGAAAGATGTTGT TATGACAGGTGGCGTGGC AAAAAATGCAGGGGTGGTGC GCGCGGTGGCGGGCGT TCTGAAGACCGATG TTATCGT TGCTCCGAATCCTCAGACGACCGGTGCACTGGGGGCAGCGCTG TATGCT TATGAGGCCGCCAGAAGAAGTA
<i>etfA</i> SEQ ID NO: 25	ATGGCCT TCAATAGCGCAGATAT TAATTCTTTCCGCGATAT TTGGGTGTT TTGTGAACAGCGTGAGGGCAAACCTGATTAACACCGATTTCGAATTAATTA GCGAAGGTCGTAAACTGGCTGACGAACGCGGAAGCAAACCTGGT TGGAATT TTGCTGGGGCACGAAGTTGAAGAAATCGCAAAGAATTAGGCGGCTATGG TGCGGACAAGGTAATTGTGTGCGATCATCCGGAAC TAAATTTTACACTA CGGATGCTTATGCCAAAGT TTTATGTGACGTCGTGATGGAAGAGAAACCG GAGGTAATTTTGATCGGTGCCACCAACAT TGGCCGTGATCTCGGACCGCG TTGTGCTGCACGCT TGCACACGGGGCTGACGGCTGAT TGCACGCACCTGG ATAT TGATAT GAATAAATATGTGGACTTTCTTAGCACCAGTAGCACCTTG GATATCTCGTCGAT GACTTTCCCTATG GAAGATACAAACCTTAAAATGAC GCGCCCTGCAT TTGGCGGACATCTGATGGCAACGATCAT TTGTCCACGCT TCCGTCCCTGTATGAGCACAGTGCGCCCCGGAGTGATGAAGAAAGCGGAG TTCTCGCAGGAGATGGCGCAAGCATGTCAAGTAGTGACCCGTCACGTAAA TTTGTCCGAT GAAGACCTTAAAACCTAAAGTAATTAATATCGTGAAGGAAA CGAAAAAGATTGTGGATCTGATCGGCGCAGAAAT TAT TGTGTCAGT TGGT CGTGGTATCTCGAAAGATGTCCAAGGTGGAAT TGCACCTGGCTGAAAAACT TGCGGACGCATTTGGTAACGGTGTGCTGGGCGGCTCGCGCGCAGTGAT TG ATTCCGGCTGGT TACCTGCGGATCATCAGGT TGGACAAACCGGTAAGACC GTGCACCCGAAAGTCTACGTGGCGCTGGGTAT TAGTGGGGCTATCCAGCA TAAGGCTGGGATGCAAGACTCTGAACTGATCATTGCCGTCAACAAAGACG AAACGGCGCCTATCT TCGACTGCGCCGAT TATGGCATCACCGGTGAT TTA TTTAAAATCGTACCGATGATGATCGACGCGATCAAAGAGGGTAAAAACGC ATGA

Gene sequence	01234567890123456789012345678901234567890123456789
<i>acrB</i> SEQ ID NO: 26	ATGCGCATCTATGTGTGTGTGAAACAAGTCCCAGATACGAGCGGCAAGGT GGCCGT TAACCTGATGGGACCCT TAACCGTGCCTCAATGGCAGCGAT TA TTAAC CCGGACGATATGTCCGCGATC GAACAGGCA T TAAAC TGAAAGAT GAAACCGGATGCCAGGTTACGGCGCTTACGATGGGTCCTCCTCCTGCCGA GGGCATGTTGCGCGAAATTAT TGCAATGGGGGCCGACGATGGTGTGCTGA TTTCGGCCCCGTGAATT TGGGGGGTCCGATACCTTCGCAACCAGTCAAAT T ATTAGCGCGGCAAT CCA TAAAT TAGGCTTAAG CAAT GAAGACA TGATCTT TTGCGGTTCGTCAGGCCATTGACGGTGATACGGCCCAAGTCGGCCCTCAAA TTGCCGAAAACTGAGCATCCCACAGGTAACCTATGGCGCAGGAATCAAA AAATCTGGTGAT TTAGTGCTGGTGAAGCGTATGT TGGAGGATGGTTATAT GATGATCGAAGTCGAAACTCCATGTCTGAT TACCTGCAT TCA GGATAAAG CGGTAAAC CACGT TACA TGACTCTCAAC GGTAT TATG GAAT GCTACTCC AAGCCGCTCCTCGT TCTCGAT TACGAAGCACTGAAAGATGAACCGCTGAT CGAACTTGATACCAT TGGGCTTAAAGGCTCCCCGACGAATATCT T TAAAT CGT TTACGCCGCCTCAGAAAGGCGTTGGTGTGATGCTCCAAGGCACCGAT AAG GAAAAAG TCGAGGATCTGGTGGATAAG CTGATGCAGAAAC ATGT CAT CTAA
<i>acrC</i> SEQ ID NO: 27	ATGT TCT TACT GAAGATTAAAAAGAAC GTATGAAACGCA TGGACTTTAG TTTAACGCGTGAACAGGAGATGT TAAAAAACTGGCGCGTCAGT TTGCTG AGATCGAGCTGGAACCGGTGGCCGAAGAGATTGATCGTGAGCACGT TTTT CCTGCAGAAAACT TTAAGAAGATGGCGGAAAT TGGCT TAACCGGCAT TGG TATCCCGAAAGAAT TTGGTGGCTCCGGTGGAGGCACCCTGGAGAAGGTCA TTGCCGTGTCAGAAT TCGGCAAAAAGTGTATGGCCTCAGCT TCCAT TTTA AGCATTCATCTTATCGCGCCGCA GGCAAT CTACAAAT ATGGGACCAAAGA ACAGAAAGAGACGTACCTGCCGCGTCT TACCAAAGGTGGTGAACCTGGGCG CCTTTGCGCTGACAGAACCAACGCCGGAAGCGATGCCGGCGCGGTAAAA ACGACCGCGATTCTGGACA GCCAGACAAAC GAGTACGTGCTGAATGGCAC CAAATGCT TTATCAGCGGGGGCGGGCGCGCGGGTGTCT TGTAAT TTTTG CGCT TACTGAACCGAAAAAAGGTCTGAAAGGGATGAGCGCGAT TATCGTG GAGAAAGGGACCCCGGGCTTCAGCATCGGCAAGGTGGAGAGCAAGATGGG GATCGCAGGTTTCGAAACCGCGGAACTTATCT TCGAAGAT TGTCGCGTTC CGGCTGCCAACCTTTTAGGTAAAGAAGGCAAAGGCT T TAAAT TGCTATG GAAGCCCTGGATGGCGCCCGTATTGGCGTGGGCGCTCAAGCAATCGGAAT TGCCGAGGGGGCGATCGACCTGAGTGTGAAGTACGTTACGAGCGCATTTC AAT TTGGTAAACCGATCGCGAATCTGCAGGGAAT TCAATGGTATATCGCG GATATGGCGACCAAAACCGCCGCGGCACGCGCACTTGT TGAGTTTGCGAGC GTATCT TGAAGACGCGGGTAAACCGTTCACAAAGGAATCTGCTATGTGCA AGCTGAACGCCTCCGAAAACGCGCGT TTTGTGACAAAT TTAGCTCTGCAG ATTACGGGGGT TACGGTTATATGAAAGAT TATCCGT TAGAGCGTATGTA TCGCGATGCTAAGAT TACGGAAAT TTACGAGGGGACA TCAGAAAT CCATA AGGTGGTGAT TGCGCGTGAAGTAATGAAACGCTAA
<i>thrA^{fbr}</i> SEQ ID NO: 28	ATGCGAGTGT TGAAGTTCGGCGGTACATCAGTGGCAAATGCAGAACGT TT TCTGCGTGT TGCCGATAT TCTGGAAGCAATGCCAGGCAGGGGCAGGTGG CCACCGTCTCTCTGCCCCCGCCAAAATCACCACCACTGGTGGCGATG ATTGAAAAAAC CAT TAGCGGCCA GGATGCTTTA CCAAT ATCA GCGATGC CGAACGTAT TTTTGCCGAAC TTTGACGGGACTCGCCGCCGCCAGCCGG GGT TCCCGCTGGCGCAATTGAAAAC T TCGTCGATCAGGAAT TTGCCCAA

Gene sequence	01234567890123456789012345678901234567890123456789
	ATAAAACATGTCCTGCATGGCAT TAGT TTTGT TGGGGCAGTGCCCGGATAG CATCAACGCTGCGCTGAT TTGCCGTGGCGAGAAAATGTCGATCGCCAT TA TGGCCGGCGTATTAGAAGCGCGCGGTCAACAACGTTACTGT TATCGATCCG GTCGAAAAACTGCTGGCAGTGGGGCATTACCTCGAATCTACCGTCGATAT TGCTGAGTCCACCCGCCGTAT TGCGGCAAGCCGCATTCCGGCTGATCACA TGGTGCTGATGGCAGGTT TCACCGCCGGTAATGAAAAAGGCGAACTGGTG GTGCT TGGACGCAACGGT TCCGACTACTCTGCTGCGGTGCTGGCTGCCTG TTTACGCGCCGATTGT TGCGAGATT TGGACGGACGT TGACGGGGTCTATA CCTGCGACCCGCGTCAGGTGCCCGATGCGAGGTTGT TGAAGTCGATGTCC TACCAGGAAGCGATGGAGCTTTCCTACT TCGGCGCTAAAGT TCT TCACCC CCGCACCATTACCCCCATCGCCCAGT TCCAGATCCCT TGCCTGAT TAAAA ATACCGGAAATCCTCAAGCACCAGGTACGCTCAT TGGTGCCAGCCGTGAT GAAGACGAAT TACCGGTCAAGGGCAT TTCCAAT CTGAA TAACA TGGCAAT GTTCAGCGT TTTCTGGTCCGGGGATGAAAGGGATGGTCGGCATGGCGGGCG GCGTCT TTTGCAGCGATGTCACGCGCCCCGTAT TCCGTGGTGCTGAT TACG CAAT CATCTTCCGAATACAGCAT CAGTTTCTGCGT TCCA CAAAG CGACTG TGTGCGAGCTGAACGGGCAATGCAGGAAGAGTTCTACCTGGAAGTGAAG AAGGCT TACTGGAGCCGCTGGCAGTGACGGAACGGCTGGCCATTATCTCG GTGGTAGGTGATGGTATGCGCACCT TGCGTGGGATCTCGGCGAAAT TCT T TGCCGCACTGGCCCGCGCCAATATCAACAT TGTCGCCAT TGCTCAGAGAT CT TCTGAACGCTCAATCTCTGTCGTGGTAAATAACGATGATGCGACCACT GGCGTGCGCGT TACTCATCAGATGCTGT TCAATACCGATCAGGT TATCGA AGTGT TTTGTGAT TGGCGTCGGTGGCGT TGGCGGTGCGCTGCTGGAGCAAC TGAAGCGTCAGCAAAGCTGGCTGAAGAATAAACATATCGACT TACGTGTC TGCGGTGT TGCCAACTCGAAGGCTCTGCTCACCAATGTACATGGCCT TAA TCTGGAAAACTGGCAGGAAGAACTGGCGCAAGCCAAAGAGCCGTTTAATC TCGGGCGCT TAAT TCGCCTCGTGAAAGAATATCATCTGCTGAACCCGGTC ATTGT TGAAGTCACT TCCAGCCAGGCAAGTGGCGGATCAATATGCCGACT T CCTGCGCGAAGGT TCCACGT TGTCACGCCGAACAAAAAGGCCAACACCT CGTCGATGGATTACTACCATCAGT TGCCT TATGCGGCGGAAAAATCGCGG CGTAAAT TCCTCTATGACACCAACGT TGGGGCTGGATTACCGGTTAT TGA GAACCTGCAAAATCTGCTCAATGCAGGTGATGAAT TGATGAAGT TCTCCG GCATTCT TTTCTGGT TCGCT TTTCT TATATCT TCGGCAAGT TAGACGAAGGC ATGAGT TTTCTCCGAGGCGACACGCTGGCGCGGGAAATGGGTTATACCGA ACCGGACCCGCGAGATGATCT TTTCTGGTATGGATGTGGCGCGTAAACTAT TGAT TCTCGCTCGTGAAACGGGACGTGAACTGGAGCTGGCGGATAT TGAA ATTGAACCTGTGCTGCCCGCAGAGT TTAACGCCGAGGGTATGTTGCCGC TTTTATGGCGAATCTGTCACAACTCGACGATCTCT TTTGCCGCGCGCGTGG CGAAGGCCCGTGATGAAGGAAAAGTTTTGCGCTATGT TGGCAATAT TGAT GAAGATGGCGTCTGCCGCGTGAAGATTGCCGAAGTGGATGGTAATGATCC GCTGT TCAAAGTGAAAAATGGCGAAAACGCCCTGGCCT TCTATAGCCACT ATTATCAGCCGCTGCCGT TGGTACTGCGCGGATATGGTGCGGGCAATGAC GT TACAGCTGCCGGTGTCT TTTGCTGATCTGCTACGTACCCTCTCATGGAA GTTAGGAGTCTGA

Gene sequence	01234567890123456789012345678901234567890123456789
<i>thrB</i> SEQ ID NO: 29	ATGGT TAAAGTTTATGCCCCGGCT TCCAGTGCCAATATGAGCGTCGGGT T TGATGTGCTCGGGGCGGCGGTGACACCTGT TGATGGTGCAT TGCTCGGAG ATGTAGTCACGGT TGAGGCGGCAGAGACATTCAAGTCTCAACAACCTCGGA CGCTTTGCCGATAAGCTGCCGTCAGAACACGGGAAAATATCGT TTATCA GTGCTGGGAGCGTTTTTGCCAGGAAGTGGGTAAGCAAATTCAGTGGCGA TGACCCTGGAAGAATATGCCGATCGGT TCGGGCT TAGGCTCCAGTGCC TGTTCGGTGGTCGCGGCGCTGATGGCGATGAATGAACACTGCGGCAAGCC GCTTAATGACACTCGT TTGCTGGCT TTGATGGGCGAGCTGGAAGGCCGTA TCTCCGGCAGCAT TCAT TACGACAACGTGGCACCCTGT TTTCTCGGTGGT ATGCAGTTGATGATCGAAGAAAACGACATCATCAGCCAGCAAGTGCCAGG GTTTGATGAGTGGCTGTGGGTGCTGGCGTATCCGGGGAT TAAAGTCTCGA CGGCAGAAGCCAGGGCTATTTTACCGGCGCAGTATCGCCGCCAGGAT TGC ATTGCGCACGGGCGACATCTGGCAGGCT TCAT TCACGCCTGCTAT TCCCG TCAGCCTGAGCT TGCCGCGAAGCTGATGAAAGATGT TATCGCTGAACCCT ACCGTGAACGGT TACTGCCAGGCT TCCGGCAGGCGCGGCAGGCGGTTCGCG GAAATCGGCGCGGTAGCGAGCGGTATCTCCGGCTCCGGCCCCGACCT TGT T CGCTCTGTGTGACAAGCCGGAACCGCCAGCGCGT TGCCGACTGGT TGG GTAAGAACTACCTGCAAAATCAGGAAGGTTTTGT TCATAT TTGCCGGCTG GATACGGCGGGCGCACGAGTACTGGAAACTAA
<i>thrC</i> SEQ ID NO: 30	ATGAAACTCTACAATCTGAAAGATCACAACGAGCAGGTCAGCTTTGCGCA AGCCGTAACCCAGGGGT TGGGCAAAAATCAGGGGCTGTTTTTTCCGCACG ACCTGCCGGAAT TCAGCCTGACTGAAAT TGATGAGATGCTGAAGCTGGAT TTTGTACCCCGCAGTGCGAAGATCCTCTCGGCGT TTATTGGTGAAGTAAAT CCCACAGGAAATCCTGGAAGAGCGCGTTCGCGCGGCGCTTTGCCT TCCCGG CTCCGGTCGCCAATGT TGAAAGCGATGTCGGTTGTCTGGAAT TGT TCCAC GGGCCAACGCTGGCATT TAAAGATTTTCGGCGGTTCGCT TTATGGCACAAAT GCTGACCCATAT TGCGGGTGATAAGCCAGTGACCAT TCTGACCGCGACCT CCGGTGATACCGGAGCGGCAGTGGCTCATGCT TTCTACGGT TTACCGAAT GTGAAAGTGGT TATCCTCTATCCACGAGGCAAAATCAGTCCACTGCAAGA AAAAGTGT TCTGTACAT TGGGCGGCAATATCGAAAGTGT TGCCATCGACG GCGATTTGATGCCTGTCAGGCGCTGGTGAAGCAGGCGT TTGATGATGAA GAACTGAAAGTGGCGCTAGGGTTAAAGTTCGGCTAACTCGAT TAACATCAG CCGTTTGCTGGCGCAGATTTGCTACTACT TTGAAGCTGT TGCGCAGCTGC CGCAGGAGACGCGCAACCAGCTGGTTGTCTCGGTGCCAAGCGGAAACT TC GGCGATTTGACGGCGGGTCTGCTGGCGAAGTCACTCGGTCTGCCGGTGAA ACGT TTTAT TGCTGCGACCAACGTGAACGATACCGTGCCACGT TTCCTGC ACGACGGTCAGTGGTCAACCAAGCGACTCAGGCGACGT TATCCAACGCG ATGGACGTGAGTCAGCCGAACAAGTGGCCGCGTGTGGAAGAGT TGT TCCG CCGCAAAATCTGGCAACTGAAAGAGCTGGGT TATGCAGCCGTGGATGATG AAACCACGCAACAGACAATGCGTGAGTTAAAAGAACTGGGCTACACTTCG GAGCCGCACGCTGCCGTAGCT TATCGTGCCTGCGTGATCAGT TGAATCC AGGCGAATATGGCT TGT TCCTCGGCACCGCGCATCCGGCGAAAT TAAAG AGAGCGTGGAAGCGATTCTCGGTGAAACGT TGGATCTGCCAAAAGAGCTG GCAGAACGTGCTGATTTACCCT TGCT TTCACATAATCTGCCCGCCGAT TT TGCTGCGT TCGGTAAAT TGATGATGAATCATCAGTAA

Gene sequence	0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
<i>HvAf^{br}</i> SEQ ID NO: 31	ATGAGTGAAACATACGTGTCTGAGAAAAGTCCAGGAGTGATGGCTAGCGG AGCGGAGCTGATTCGTGCCGCCGACATTCAAACGGCGCAGGCACGAATTT CCTCCGTCAT TGCACCAACTCCAT TGCAGTATTGCCCTCGTCT TTCTGAG GAAACCGGAGCGGAAATCTACCTTAAGCGTGAGGATCTGCAGGATGTTCTG TTCCTACAAGATCCGCGGTGCGCTGAACTCTGGAGCGCAGCTCACCCAAG AGCAGCGCGATGCAGGTATCGTTGCCGCATCTGCAGGTAACCATGCCCAG GGCGTGGCCTATGTGTGCAAGTCCT TGGGCGTTCAGGGACGCATCTATGT TCCTGTGCAGACTCCAAAGCAAAAGCGTGACCGCATCATGGT TCACGGCG GAGAGTTTGTCTCCT TGGTGGTCACTGGCAATAACT TCGACGAAGCATCG GCTGCAGCGCATGAAGATGCAGAGCGCACCGGGCGCAACGCTGATCGAGCC TTTCGATGCTCGCAACACCGTCATCGGTTCAGGGCACCGTGGCTGCTGAGA TCTTGTCGCAGCTGACTTCCATGGGCAAGAGTGCAGATCACGTGATGGTT CCAGTCGGCGGTGGCGGACTTCTTGCAGGTGTGGTCAGCTACATGGCTGA TATGGCACCTCGCACTGCGATCGT TGGTATCGAACCAGCGGGAGCAGCAT CCATGCAGGCTGCATTGCACAATGGTGGACCAATCACT TTGGAGACTGTT GATCCCTTTGTGGACGGCGCAGCAGTCAAACGTGTCTGGAGATCTCAACTA CACCATCGTGGAGAAGAACCAGGGTCGCGTGCACATGATGAGCGCGACCG AGGGCGCTGTGTGTACTGAGATGCTCGATCT TTACCAAAACGAAGGCATC ATCGCGGAGCCTGCTGGCGCGCTGTCTATCGCTGGGT TGAAGGAAATGTC CTTTGCACCTGGTTCTGCAGTGGTGTGCATCATCTCTGGTGGCAACAACG ATGTGCTGCGT TATGCGGAAATCGCTGAGCGCTCCT TGGTGCACCGCGGT TTGAAGCACTACT TCTTGGTGAACCTCCCGCAAAAGCCTGGTCACTTGCG TCACTTCCTGGAAGATATCCTGGGACCGGATGATGACATCACGCTGT TTG AGTACCTCAAGCGCAACAACCGTGAGACCGGTACTGCGT TGGTGGGTATT CACTTGAGTGAAGCATCAGGATTGGATTCTTTGCTGGAACGTATGGAGGA ATCGGCAAT TGATTCCCGTCGCCTCGAGCCGGGCACCCCTGAGTACGAAT ACTTGACCTAA
<i>aceE</i> SEQ ID NO: 32	ATGTCAGAACGT TTCCCAAATGACGTGGATCCGATCGAAACTCGCGACTG GCTCCAGGCGATCGAATCGGTTCATCCGTGAAGAAGGTGT TGAGCGTGCTC AGTATCTGATCGACCAACTGCT TGCTGAAGCCCAGCAAGGCGGTGTAAAC GTAGCCGCAAGGCACAGGTATCAGCAAC TACATCAACAACCATCCCCGTTGA AGAACAACCGGAGTATCCGGGTAATCTGGAACGGAACGCCGTAT TCGTT CAGCTATCCGCTGGAACGCCATCATGACGGTGCTGCGTGCGTCGAAAAAA GACCTCGAACTGGGCGGCCATATGGCGTCCTTCCAGTCT TCCGCAACCAT TTATGATGTGTGCT TTAACCACT TCTTCCGTGCACGCAACGAGCAGGATG GCGGCGACCTGGTTTACTTCCAGGGCCACATCTCCCCGGGCGTGTACGCT CGTGCTTTCTGGAAGGTCTGCTGACTCAGGAGCAGCTGGATAACT TCCG TCAGGAAGTTCACGGCAATGGCCTCTCT TCCTATCCGCACCCGAAACTGA TGCCGGAATTCTGGCAGTTCCCGACCGTATCTATGGGTCTGGGTCCGAT T GGTGCTATTTACCAGGCTAAAT TCCTGAAATATCTGGAACACCGTGGCCT GAAAGATACCTCTAAACAAACCGT TTACGCGTTCCCTCGGTGACGGTGAAA TGGACGAACCGGAATCCAAAGGTGCGATCACCATCGCTACCCGTGAAAAA CTGGATAACCTGGTCT TCGTTATCAACTGTAACCTGCAGCGTCT TGACGG CCCGGTCACCGGTAACGGCAAGATCATCAACGAACTGGAAGGCATCT TCG AAGGTGCTGGCTGGAACGTGATCAAAGTGATGTGGGGTAGCCGT TGGGAT GAACTGCTGCGTAAGGATACCAGCGGTAAACTGATCCAGCTGATGAACGA AACCGT TGACGGCGACTACCAGACCTTCAAATCGAAAGATGGTGCCTACG

Gene sequence	01234567890123456789012345678901234567890123456789
	TTCGTGAACACT TCT TCGGTAAATATCCTGAAACCGCAGCACTGGT TGCA GACTGGACTGACGAGCAGATCTGGGCACTGAACCGTGGTGGTCACGATCC GAAGAAAAT CTACGCTGCA TTCAAGAAAGCGCAGGAAACCAAAGGCAAAG CGACAGTAATCCT TGCTCATACCAT TAAAGGT TACGGCATGGGCGACGCG GCTGAAGGTAAAAAC ATCGCGCA CCA GGT TAAGAAAAT GAACA TGGACGG TGTGCGTCATATCCGCGACCGT TTCAATGTGCCGGTGTCTGATGCAGATA TCGAAAACTGCCGTACATCACCT TCCCGGAAGGTTCTGAAGAGCATACC TATCTGCACGCTCAGCGTCAGAACTGCACGGT TATCTGCCAAGCCGTCA GCCGAAC TACCGAGAAGCT TGAGCTGCCGAGCCTGCAAGACT TCGGCG CGCTGT TGAAGAGCAGAGCAAAGAGATCTCTACCACTATCGCT TTCGT T CGTGCTCTGAACGTGATGCTGAAGAACAAGTCGATCAAAGATCGTCTGGT ACCGATCATCGCCGACGAAGCGCGTACT TTCGGTATGGAAGGTCTGT TCC GTCAGAT TGGTA TTTA CA GCCC GAAC GGT CA GCA GTA CA CCCC GCA GGAC CGCGAGCA GGT TGCT TA CTA TAAAGAAGAC GAGAAAGGT CAGAT TCTGCA GGAAGGGATCAACGAGCTGGGCGCAGGTTGT TCCTGGCTGGCAGCGGCGA CCTCTTA CAGCA CCAACAAT CTGCCGATGATCCCGT TCTACA TCTA TTAC TCGATGT TCGGCT TCCAGCGTAT TGGCGATCTGTGCTGGGCGGCTGGCGA CCAGCAAGCGCGTGGCT TCCTGATCGGCGGTACT TCCGGTCGTACCACCC TGAACGGCGAAGGTCTGCAGCAC GAAGA TGGTCACAGCCACAT TCAGTCG CTGACTATCCCGAACTGTATCTCT TACGACCCGGCTTACGCT TACGAAGT TGCTGTCATCATGCATGACGGTCTGGAGCGTATGTACGGTGAAAAACAAG AGAAC GTTTA CTA CTA CA TCA CTA CGCT GAAC GAAAAC TA CCA CA TGCCG GCAATGCCGGAAGGTGCTGAGGAAGGTATCCGTAAAGGTATCTACAACT CGAACTAT TGAAGGTAGCAAAGGTAAAGT TCAGCTGCTCGGCTCCGGT T CTATCCTGCGTCACGTCCGTGAAGCAGCTGAGATCCTGGCGAAAGAT TAC GGCGTAGGTTCTGACGT TTATAGCGTGACCTCCT TCACCGAGCTGGCGCG TGATGGTCAGGAT TGTGAACGCTGGAACATGCTGCACCCGCTGGAACTC CGCGCGT TCCGTATATCGCTCAGGTGATGAACGACGCTCCGGCAGTGGA TCTACCGACTATATGAACTGT TCGCTGAGCAGGTCCGTACT TACGTACC GGCTGACGACTACCGCGTACTGGGTACTGATGGCT TCGGTCGT TCCGACA GCCGTGAGAACCTGCGTCACCACT TCGAAGT TGATGCT TCT TATGTCGTG GT TCGGCGCTGGGCGAACTGGCTAAACGTGGCGAAATCGATAAGAAAGT GGTTGCTGACGCAAT CGCCAAAT TCAACA TCGAT GCAGAT AAAG TTAAC C CGCGTCTGGCGTAA
<i>aceF</i> SEQ ID NO: 33	ATGGCTATCGAAATCAAAGTACCGGACATCGGGGCTGATGAAGT TGAAT CACCGAGATCCTGGTCAAAGTGGGCGACAAAGT TGAAGCCGAACAGTCGC TGATACCGTAGAAGGCGACAAAGCCTCTATGGAAGT TCCGTCTCCGCAG GCGGGTATCGTTAAAGAGATCAAAGTCTCTGT TGGCGATAAAACCCAGAC CGGCGCACTGATTATGAT TTTTCGAT TCCGCCGACGGTGCAGCAGACGCTG CACCTGCTCAGGCA GAAGA GAAGAAAGAAG CAGCTCCGGCAGCAGCACCA GCGGCTGCGGCGGCAAAAGACGT TAACGT TCCGGATATCGGCAGCGACGA AGT TGAAGTGACCGAAATCCTGGTGAAAGT TGGCGATAAAGT TGAAGCTG AACAGTCGCTGATACCGTAGAAGGCGACAAGGCT TCTATGGAAGT TCCG GCTCCGT TTGCTGGCACCGTGAAAGAGATCAAAGTGAACGTGGGTGACAA AGTGTCTACCGGCTCGCTGAT TATGGTCT TCGAAGTCGCGGGTGAAGCAG GCGCGGCGAGCTCCGGCCGCTAAACAGGAAGCAGCTCCGGCAGCGGCCCT GCACCAGCGGCTGGCGTGAAAGAAGTTAACGT TCCGGATATCGGCGGTGA

Gene sequence	01234567890123456789012345678901234567890123456789
	CGAAGTTGAAGTGACTGAAGTGATGGTGAAAGTGGGCGACAAAGT TGCCG CTGAACAGTCACTGATCACCGTAGAAGGCGACAAAGCTTCTATG GAAGTT CCGGCGCCGT TTGCAGGCGTCGTGAAGGAACTGAAAGTCAACGT TGGCGA TAAAGTGAAAAGTGGCTCGCTGAT TATGATCT TCGAAGT TGAAGGCGCAG CGCCTGCGGCAGCTCCTGCGAAACAGGAAGCGGCAGCGCCGGCACCGGCA GCAAAAGCTGAAGCCCCGGCAGCAGCACCAGCTGCGAAAGCGGAAGGCAA ATCTGAAT TTGCTGAAAACGACGCT TATGT TCACGCGACTCCGCTGATCC GCCGTCTGGCACGCGAGTTTGGTGT TAACCT TGCGAAAGTGAAGGGCACT GGCCGTAAAGGTCGTATCCTGCGCGAAGACGT TCAGGCT TACGTGAAAGA AGCTATCAAACGTGCAGAAGCAGCTCCGGCAGCGACTGGCGGTGGTATCC CTGGCATGCTGCCGTGGCCGAAGGTGGACTTCAGCAAGT TTGGTGAAATC GAAGAAGTGGAAGTGGGCCGCATCCAGAAAATCTCTGGTGCGAACCTGAG CCGTAACTGGGTAATGATCCCGCATGT TACTCACT TCGACAAAACCGATA TCA CCGAGTTGGAAGCGT TCCGTAAAC A GCA GAAC GAAGAAG CGGCGAAA CGTAAGCTGGATGTGAAGATCACCCCGGT TGTCT TCATCATGAAAGCCGT TGCTGCAGCTCT TGAGCAGATGCCTCGCT TCAATAGT TCGCTGTCGGAAG ACGGTCAGCGTCTGACCCTGAAGAAATACATCAACATCGGTGTGGCGGTG GATACCCCGAACGGTCTGGT TGT TCCGGTAT TCAAAGACGTCAACAAGAA AGGCATCATCGAGCTGTCTCGCGAGCTGATGACTAT TTCTAAGAAAGCGC GTGACGGTAAGCTGACTGCGGGCGAAATGCAGGGCGGTTGCT TCACCATC TCCAGCATCGGCGGCCTGGGTACTACCCACT TCGCGCCGATTGTGAACGC GCCGGAAGTGGCTATCCTCGGCGTTTCCAAGTCCGCGATGGAGCCGGTGT GGAATGGTAAAGAGT TCGTGCCGCGTCTGATGCTGCCGAT TTCTCTCTCC TTCGACCACCGCGTGATCGACGGTGCTGATGGTGCCCGTTTCAT TACCAT CAT TAACAACACGCTGTCTGACAT TCGCCGTCTGGTGATGTAA
<i>lpd</i> SEQ ID NO: 34	ATGAGTACTGAAATCAAAACTCAGGTCGTGGTACT TGGGGCAGGCCCGCG AGGT TACTCCGCTGCCT TCCGT TCGCTGAT TTAGGTCTGAAACCGTAA TCGTAGAACGT TACAACACCCT TGGCGGTGT TTGCCTGAACGTGCGCTGT ATCCCT TCTAAAGCACTGCTGCACGTAGCAAAAGT TATCGAAGAAGCCAA AGCGCTGGCTGAACACGGTATCGTCT TCGGCGAACCGAAAACCGATATCG ACAAGAT TCGTACCTG GAAAGAGAAAGT GATCAAT CA GCTGACCGGTGGT CTGGCTGGTATGGCGAAAGGCCGCAAAGTCAAAGTGGTCAACGGTCTGGG TAAAT TCACCGGGGCTAACACCCTGGAAGT TGAAGGTGAGAACGGCAAAA CCGTGATCAACT TCGACAACGCGATCAT TGCAGCGGGT TCTCGCCCGATC CAACTGCCGT TTAT TCCGCATGAAGATCCGCGTATCTGGGACTCCACTGA CGCGCTGGAAGTGAAGAAGTACCAGAACGCCTGCTGGTAATGGGTGGCG GTATCATCGGTCTGGAATGGGCACCGT TTACCACGCGCTGGGTTACAG ATTGACGTGGT TGAATGT TCGACCAGGT TATCCCGGCAGCTGACAAAGA CATCGTTAAAG TCTTCA CCAAG CGTA TCA GCAAGAAAT TCAAC CTGATGC TGGAACCAAAGT TACCGCCGT TGAAGCGAAAGAAGACGGCAT TTATGTG ACGATGGAAGGCAAAAAAGCACCCGCTGAACCGCAGCGT TACGACGCCGT GCTGGTAGCGATTGGTCGTGTGCCGAACGGTAAAAACCTCGACGCAGGCA AAGCAGGCGTGGAAGTTGACGACCGTGGTTTCATCCGCGT TGACAAACAG CTGCGTACCAACGTACCGCACATCT TTGCTATCGGCGATATCGTCGGTCA ACCGATGCTGGCACACAAAGGTGT TCACGAAGGTCACGT TGCCGCTGAAG TTATCGCCGGTAAGAAACACTACT TCGATCCGAAAGT TATCCCGTCCATC GCCTATACCAAACCAGAAGT TGCATGGGTGGGTCTGACTGAGAAAGAAGC

Gene sequence	01234567890123456789012345678901234567890123456789
	GAAAGAGAAAGGCATCAGCTATGAAACCGCCACCTTCCCGTGGGCTGCT T CTGGTCGTGCTATCGCT TCCGACTGCGCAGACGGTATGACCAAGCTGAT T TTCGACAAAGAATCTCACCGTGTGATCGGTGGTGCGAT TGTCGGTACTAA CGGCGGCGAGCTGCTGGGTGAAATCGGCCTGGCAATCGAAATGGGT TGTG ATGCTGAAGACATCGCACTGACCATCCACGCGCACCCGACTCTGCACGAG TCTGTGGGCCTGGCGGCAGAAGTGT TCGAAGGTAGCATTACCGACCTGCC GAACCCGAAAGCGAAGAAGAAGTAA
tesB SEQ ID NO: 10	ATGAGTCAGGCGCTAAAAAATTTACTGACATTGT TAAATCTGGAAAAAAT TGAGGAAGGACTCTTTCGCGGCCAGAGTGAAGATTTAGGTTTACGCCAGG TGT TTGGCGGCCAGGTCGTGGGTGAGGCCT TGTATGCTGCAAAAGAGACC GTCCCTGAAGAGCGGCTGGTACAT TCGT TTCACAGCTACT TTCT TCGCCC TGGCGATAGTAAGAAGCCGATTATTTATGATGTCGAAACGCTGCGTGACG GTAACAGCTTCAGCGCCCGCCGGGT TGCTGCTAT TCAAAACGGCAAACCG ATTTTTTATATGACTGCCTCT TTCCAGGCACCAGAAGCGGGTTTCGAACA TCAAAAAACAATGCCGTCCGCGCCAGCGCCTGATGGCCTCCCT TCGGAAA CGCAAATCGCCCAATCGCTGGCGCACCTGCTGCCGCCAGTGCTGAAAGAT AAATTCATCTGCGATCGTCCGCTGGAAGTCCGTCCGGTGGAGT TTCATAA CCCCTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTGGATCCGCG CAAATGGTAGCGTGCCGGATGACCTGCGCGT TCATCAGTATCTGCTCGGT TACGCTTCTGATCT TAACTTCCTGCCGGTAGCTCTACAGCCGCACGGCAT CGGT TTTCTCGAACCAGGGGAT TCAGATTGCCACCAT TGACCAT TCCATGT GGT TCCATCGCCCGT TTAATTTGAATGAATGGCTGCTGTATAGCGTGGAG AGCACCTCGGCGTCCAGCGCACGTGGCT TTGTGCGCGGTGAGT TTTATAC CCAAGACGGCGTACTGGT TGCCTCGACCGT TCAGGAAGGGGTGATGCGTA ATCACAATTAA

Table 7

Amino acid sequence	01234567890123456789012345678901234567890123456789
pct SEQ ID NO: 35	MRKVP I I TADEAAKLIKDGDTVTTSGFVGNAI PEALDRAVEKR FLETGEP KNITYVYCGSQGNRDGRGAEHFAHEGLLKRYIAGHWATVPALGKMAMENK MEAYNVSQGALCHLFRDIASHKPGVF TKVGIGTFIDPRNNGGKVN DITKE DIVELVEIKGQEYLFYPAFPIHVALIRGTYADESGNITFEKEVAPLEGTS VCQAVKNSGGIVVVQVERVVKAGTLDPRHV KVP GIYVDYVVADPEDHQQ SLDCEYDPALSGEHRREPVVGEPLPLSAKKVIGRRGAIELEKDVA VNLGV GAPEYVASVADEEGIVDFMTLTAESGAIGGVPAGGVRF GASYNADALIDQ GYQFDYYDGGGLDLCYLGLAECDEKGNINVS RFGPRIAGCGGFINITQNT PKVFFCGTFTAGGLKV KIEDGKVI IVQEGKQKKFLKAVEQITFNGDVALA NKQQVTYITERCVFLLKEDGLHLSEIAPGIDLQTQILDVMDFAPIIDRDA NGQIKLMDAALFAEGLMGLKEMKS

Amino acid sequence	01234567890123456789012345678901234567890123456789
<i>lcdA</i> SEQ ID NO: 36	MSLTQGMKAKQLLAYFQGKADQDAREAKARGELVCWSASVAPPEFCVTMG IAMI YPETHAAGIGARKGAMDMLEVADRKGYNVDCCSYGRVNMGYMECLK EAAI TGVKPEVLVNS PAADVPLPDLVI TCNN ICNTLLKWEYENLAAELDI P CIVIDVPFNHTMPIPEYAKAYIADQFRNAISQLEVICGRPFDWKKFKEVK DQTQRSVYHWNRIAEMAKYKPSPLNGFDLFNYMALIVACRSLDYAEITFK AFADELEENLKAGI YAFKGAEKTRFQWEGIAVWPHLGHTFKSMKNLNS IM TGTAYPALWDLHYDANDESMHSMAEAYTRI YINTCLQNKVEVLLGIMEKG QVDGTVYHLNRSCKLMSFLNVETAEI IKEKNGLPYVS IDGDQTDPRVFSP AQFDTRVQAL VEMMEANMAAAE
<i>lcdB</i> SEQ ID NO: 37	MSRVEAI LSQLKDVAANPKKAMDD YKAE TGKGAVG IMP IYSPEEMVHAAG YLPNGIWAQGKTISKARTYLPAFACSVMQVMELQCEGAYDDLSAVIFS VPCDTLKCLSQKWKGTSPIVVFTHPQNRGLEAANQFLVTEYELVKAQLES VLGVKISNAALENS IAI YNENRAVMREFVKVAADYPQVIDAVSRHAVFKA RQFMLKEKHTALVKELIAEIKATPVQPWDGKK\AA/ TGILLEPNELLDI FN EFKIAIVDDDLAQESRQIRVDVLDGEGGPLYRMAKAWQQMYGCSLATDTK KGRGRML INKT IQTGADAIWAMMKFCDPEEWDYPVMYRE FEEKGVKS LM IEVDQEVSSFEQIKTRLQSFVEML
<i>lcdC</i> SEQ ID NO: 38	MYTLGIDVGSAS SKAVI LKDGKD IVAAE VVQVGTGSSGPQRALDKAFEVS GLKKEDISYTVATGYGRFNFSADKQISEISCHAKGIYFLVPTARTI IDI GGQDAKAIRLDDKGGIKQFFMNDKCAAGTGRFLEV MARVLETTLDEMAEL DEQATDTAPISSTCTVFAESEVISQLSNGVSRNNI IKGVHLSVASRACGL AYRGGLEKD VMTGGVAKNAGWRAVAGVLKTDVIVAPNPQT TGALGAAL YAYEAAQKK
<i>etfA</i> SEQ ID NO: 39	MAFNSADINSFRDIWFCEQREGKLINTDFELISEGRKLADERGSKLVGI LLGHEVEEIAKELGGYGADKVIVCDHPELKFYTTDAYAKVLCDVVMEKP EVILIGATNIGRDLGPRCAARLHTGLTADCTHLDIDMNKYVDFLSTSSTL DISSMT FPMEDTNLKMTRPAFGGHLMT 11CPRFRPCMS TVRPGVMKKAE FSQEMAQACQWTRHVNLSDEDLKT VINIVKETKKIVDLIGAEIIVSVG RGISKDVQGGIALAEKLADAFNGWGGSRAVIDSGWL PADHQVQGTGKT VHPKVYVALG ISGAI QHKAGMQDS EL 11AVNKDE TAP IFDCADYG ITGDL FK IVPMM IDAI KEGKNA
<i>acrB</i> SEQ ID NO: 40	MRI YVCVKQVPDTSQKVAVNPDGTLNRSMAAI INPDDMSAIEQALKLKD ETGCQVTALTMGPPPAEGLREI IAMGADDGVLISAREFGGSDTFATSQI ISAAIHLGLSNEDMI FCGRQAIDGDTAQVGPQIAEKLS IPQVTYAGAIK KSGDLVLVKRMLEDGYMMIEVETPCLITCIQDKAVKPRYMTLNGIMECYS KPLLVL DYALKDEPLIELDTIGLKGSPNTI FKSFTPPQKGVGVMLQGT KEKVEDLV DKLMLQKHVI

Amino acid sequence	01234567890123456789012345678901234567890123456789
<i>acrC</i> SEQ ID NO: 41	MFLKIKKERMKRMDFSLTREQEMLKKLARQFAEIELEPVAAEEIDREHVF PAENFKKMAE IGLTG IGIPKE FGGS GGGTLEKVI AVSE FGKKCMASAS IL SIHLIAPQAI YKYGTKEQKETYLPRLTKGGELGAFALTEPNAGSDAGAVK TTAILDSQTNEYVLNGTKCFISGGGRAGVLVI FALTEPKKGLKGMSAI IV EKGTPGFS IGKVESKMGIAGSETAELI FEDCRVPAANLLGKEGKGFKIAM EALDGARIGVGAQAIGIAEGAILDSVKYVHERIQFGKPIANLQGIQWYIA DMATKTAARALVEFAAYLEDAGKPFTKESAMCKLNASENARFVTNLALQ IHGGYGYMKDYPLERMYRDAKITEIYEGTSEIHKWIAREVMKR
<i>thrA^{fbr}</i> SEQ ID NO: 42	MRVLKFGGTSVANAERFLRVADILESNAQQQVATVLSAPAKITNHLVAM IEKTISGQDALPNISDAERI FAELLTGLAAQPGFPLAQLKTFVDQEFQAQ IKHVLHGISLLGQCPDS INAALICRGEKMS IAIMAGVLEARGHNVTVIDP VEKLLAVGH YLE STVDIAESTRR IAAS RIPADHMLMAG FTAGNEKGE LV VLGRNGSDYSAAVLAACLRADCCEIWDVDGVYTC DPRQVPDARLLKSMS YQEAMELSYFGAKVLHPRTITPIAQFQIPCLIKNTGNPQAPGTLIGASRD EDEL PVKGI SNLNNMAMFSVSGPGMKGMVGMAARVFAAMSRARI SWLI T QSSSEYS ISFCVPQSDCVRAERAMQEEFYELKEGLLEPLAVTERLAI IS WGDGMRTL RGISAKFFAALARANINIVAI AQRSSERS ISVWNDDATT GVRVTHQMLFNTDQVIEVFVIGVGGVGGALLEQLKRQQSWLKNKHIDLRV CGVANSKALLTNVHGLNLENWQEELAQAKEPFNLGRLIRLVKEYHLLNPV IVDCTSSQAVADQYADFLREGFHWTPNKKANTSSMDYYHQLRYAAEKSR RKFLYDTNVGAGLPVIENTLQNLNAGDELMKFSGILSGSLSYI FGKLDEG MSFSEATTLAREMGYTEPDRDDL SGMDVARKLLILARETGRELELADIE IEPVL PAE FNAE GDVAAFMANL SQLDDL FAARVAKARDE GKVLRYVGN ID EDGVCRVKIAEVDGNDPLFKVKNGENALAFYSHYYQPLPLVLRGYGAGND VTAAGVFADLLRTL SWKLGV
<i>thrB</i> SEQ ID NO: 43	MVKVYAPASSANMSVGFDVLGA AVTPVDGALLGDWTV EAAETFS LNNLG RFADKLPSEP RENIVYQCWERFCQELGKQIPVAMTLEKNMPIGSGLGSSA CSWAALMAMNEHCGKPLNDTRLLALMGELEGRISGS IHYDNVAPCFLGG MQLMIEENDI ISQQVPGFDEWLWVLAYPG IKVS TAEARAI LPAQYRRQDC IAHGRHLAGFIHACYSRQPELA AKLMKD VIAEPYRERLLPGFRQARQAVA EIGAVASGISGSGPTLFALCDKPETAQRVADWL GKNYLQNQEGFVHICRL DTAGARVLEN
<i>thrC</i> SEQ ID NO: 44	MKLYNLKDHNEQVSFAQAVTQGLGKNQGLFFPHDLPEFSLTEIDEMLKLD FVTRSAKILSAFIGDEIPQEILEERVRAAFAFPAPVANVESDVGCELELFH GPTLAFKDFGGRFMAQMLTHIAGDKPVTILTATSGDTGA AVAHAFYGLPN VKWILYPRGKISPLQEKL FCTLG GNIETVAIDGDFDACQALVKQAFDDE ELKVALGLNSANS INISRLLAQICYFEAVAQLPQETRNLWSVPSGNF GDLTAGLLAKSLGLPVKRFIAATNVNDTVPRFLHDGQWSPKATQATLSNA MDVSQPNNWPRVEELFRRKIWQLKELGYAAVDDETTQQTRELKELGYTS EPHA AVAYRALRDQLNPGEYGLFLGTAHPAKFKESVEAILGETLDLPKEL AERADLPLLSHNL PADFAALRKLMMNHQ

Amino acid sequence	01234567890123456789012345678901234567890123456789
<i>Hva^{fbr}</i> SEQ ID NO: 45	MSETYVSEKSPGVMASGAELIRAADIQTAQARISSVIAPTPLQYCPRLSE ETGAEI YLKREDLQDVRSYKIRGALNSGAQLTQEQRDAGIVAASAGNHAQ GVAYVCKS LGVQGR IYVPVQT PKQKRDR IMVHGGE FVS LVVTGNNFDEAS AAAHEDAERTGATLIEPFDARNTVIGQGTVA AEILS QL TSMGKSADHVMV PVGGGGLLAGWS YMADMAPRTA IVG IE PAGAASMQAALHNGGP ITLETV DPFVDGAAVKRVGDLNYTIVEKNQGRVHMMSATEGAVCTEMLDLYQNEGI IAEPAGAL SIAGLKEMSFAPGSAWC 11SGGNNDV LRYAE IAERSLVHRG LKHYFLVNFPPKPGQLRHFLLEDILGPDDITLFEYLKRNNRETGTALVGI HLSEASGLDSL LERMEESAIDSRRLEPGTPEYEYLT
<i>aceE</i> SEQ ID NO: 46	MSERFPNDDVDPIETRDWLQAIESVIREEGVERAQYLIDQLLAEARKGGVN VAAGTGISNYINTIPVEEQPEYPGNLELERRIRSAIRWNAIMTVLRASKK DLELGGHMASFQSSATI YDVCFNHFFRARN EQDGGDLVYFQGHISPGVYA RAFLEGRLTQEQLDNFRQEVHGNGLSSYPHPKLMPEFWQFPTVSMGLGPI GAI YQAKFLKYLEHRGLKDTSKQTVYAFLGDGEMDEPESKGAITIATREK LDNLV FVINCNLQRLDGPVTGNKI INELEGIFEGAGWNVIKVMWGSRW ELLRKDTSGKLIQLMNETVDGDYQTFKSKDGAYVREHFFGKYPETAALVA DWTDEQIWALNRGGHDPKKI YAAFKKAQETKGKATVILAHTIKGYGMGDA AEGKNIAHQVKKMMDGVRHIRDRFNVPVSDADIEKLPIYITFPEGSEEHT YLHAQRQKLHG YLPSRQPNFTEKLELPSLQDFGALLEEQSKEISTTIAFV RALNVMLKNKS IKDRLVPI IADEARTFGMEGLFRQIGI YSPNGQQYTPQD REQVAYYKEDEKGQILQEGINELGAGCSWLA AATSYSTNNLPMIPFYIYY SMFGFQRIGDLCWAAGDQQARGFLIGGTSGRTTLNGEGLQHEDGHSHIQS LTIPNCISYDPAYAYEVAVIMHDGLERMYGEKQENVYIYI TTLNENYHMP AMPEGAE EGIRKGI YKLETIEGSKGKVQLLGSGS ILRHVREAAEILAKDY GVGSDVYSVTSFTELARDGQDCERWNMLHPLETTPRPYIAQVMNDAPAVA STDYMKLFAEQVRTYVPADDYRVLGTDGFGRSDSRENLRHHFEVDASYW VAALGELAKRGE IDKKWADAIKFNIDADKVNPRLA
<i>aceF</i> SEQ ID NO: 47	MAI E IKVPDI GADEVE ITE ILVKVGDKVEAEQS LITVEGDKASMEVPS PQ AGIVKE IKVSVGDKTQTGAL IM IFDSADGAADAAPAAQAE EKKEAAPAAAP AAAAAKDVNVPD IGSDEVEVTE ILVKVGDKVEAEQS LITVEGDKASMEVP APFAGTVKE IKVNVGDKVS TGSLIMVFEVAGEAGAAAPAAKQEAAPAAAP APAAGVKEVNVPDIGGDEVEVTEVMVKVGDKVAAEQSLITVEGDKASMEV PAP FAGWKE LKVNVDGKVKT GSLIM IFEVEGAAPAAAPAKQEAAPAPA AKAEAPAAAPAAKAE GKSE FAENDAYVHAT PLIRRLARE FGVNLAKVKGT GRKGRILREDVQAYVKEAIKRAEAPAAATGGGIPGMLPWPKVDFSKFGEI EEVELGRI QKI SGANLSRNWVMI PHVTHFDKTDI TELEAFRKQQNEEAAK RKLDVKITPWFIMKAVAAALEQMPRFNSSLS SEDGQRLTLKKYINIGVAV DTPNGLWPVKDVNKKGI IELSRELMTISKKARDGKLTAGEMQGGCFTI SSI GGLGT THFAP IVNAPEVAI LGVSKSAME PVWNGKE FVPRMLP ISLS FDHRVIDGADGARFITI INNTLSDIRRLVM

Amino acid sequence	01234567890123456789012345678901234567890123456789
<i>lpd</i> SEQ ID NO: 48	MSTEIKTQWVLGAGPAGYSAAFRCADLGLETVIVERYNTLGGVCLNVGC IPSKALLHVAKVIEEAKALAEHGIVFGEPKTDIDKIRTWKEKVINQLTGG LAGMAKGRKVKWNLGKFTGANTLEVEGENGKTVINFDNAIIAAGSRPI QLPFIPHEDPRIWDSTDALELKEVPERLLVMGGGIIGLEMGTVYHALGSQ IDWEMFDQVIPAADKDIVKVFTKRISKKFNLMLETKVTAVEAKEDGIYV TMEGKKAPAEPQRYDAVLVAIGRVPNGKNLDAGKAGVEVDDRGFIRVDKQ LRTNVPHI FAIGDIVGQPM LAHKG VHEGHVAAEVIAGKKHYFDPKVIPS I AYTKPEVAWVGLTEKEAKEKGISYETATFPWAASGRAIASDCADGMTKLI FDKESHRVIGGAIVGTNGGELLGEIGLAIEMGCDAEDIALTIHAHPTLHE SVGLAAEVFEFS ITDLPNPKAKKK
<i>tesB</i> SEQ ID NO: 20	MSQALKNLLTLLNLEKIEEGLFRGQSEDLGLRQVFGGQWGQALYAAKET VPEERLVHSFHSYFLRPGDSKKPI IYDVETLRDGNFSARRVAAIQNGKP IFYMTASFQAPEAGFEHQKTMP SAPAPDGLPSETQIAQSLAHLPPVLKD KFICDRPLEVRPVE FHNPLKGHVAEPHRQVWIRANGSV PDDL RVH QYLLG YASDLNFLPVALQPHGIGFLEPGIQIATIDHSMWFHRPFNLNEWLLYSVE STSA SSARG FVRGE FYT QDGV LVAS TVQE GVMRNHN

[0143] 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. 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. 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 under inducing conditions. 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 under inducing conditions. In some embodiments, the genetically engineered bacteria are capable of expressing the propionate biosynthesis cassette and producing propionate under inducing conditions.

[0144] 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. 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 *Acetivibrio*, *Acetoanaerobium*, *Acetohalobium*, *Acetonema*, *Balutia*, *Butyrivibrio*, *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.

[0145] 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. 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: 49 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.

IL-10 (SEQ ID NO: 49):

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ATG AGC CCG GGA GAG GGA ACT CAA AGC GAG AAG AGC TGC ACA GAT ITT CC
A GGTAAT CTT CCA AAT ATG CTT CGT GAC TIG CGT GAG GCT TIC TCI CGCGT
G AAA ACC TTT TTT GAG ATG AAG GAT CAG TTA GAT AAT CTG CTG CTG AAA
GAA ICG CTT CTTGAG GAC TTC AAG GGA TAT CTG GGA TGT CAG GCG TTATCT
GAG ATG ATI CAG TTT TAT TTG GAA GAA GTT ATG CCC CAG GCT GAG AAT CA
A GAC CCT GAC ATC AAA GCGCAT GTG AAT AGC CTG GGC GAG AAT CTGAAG AC
A CTG CGC CTG CGT CTT CGC CGC TGI CAC CGI TIT CTG CCT TGC GAA AAT
AAG AGT AAG GCC GTT GAG CAA GTG AAAAAT GCT TTC AAC AAG TIACAA GAA
AAA GGG ATT TAG AAA GCT ATG TCT GAG TTT GAC ATT TTC ATT AAT TAG AT
T GAG GCC TAG ATG ACT ATG AAG ATT CGC AAT

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[0146] 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: 50 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: 50 or a functional fragment thereof. 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.

SEQ ID NO: 50
MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM
LTFKFYMPKK ATELKHLQCL EEELKPLEEV LNLAQSKNFH
LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN
RWITFCQSII STLT

[0147] 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: 51 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: 51 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.

SEQ ID NO: 51				
MAALQKSVSS	FLMGTLATSC	LLLLALLVQG	GAAAPISSHC	RLDKSNFQQP
YITNRTFMLA	KEASLADNNT	DVRLIGEKLF	HGVSMSERCY	LMKQVLNFTL
EEVLFPQSDR	FQPYMQEWP	FLARLSNRLS	TCHIEGDDLH	IQRNVQKLKD
TVKKLGESGE	IKAIGELDLL	FMSLRNACI		

[0148] 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: 52 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: 52 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.

SEQ ID NO: 52				
MGQTAGDLGW	RLSLLLLPLL	LVQAGVWGFP	RPPGRPQLSL	QELRREFTVS
LHLARKLLSE	VRGQHRFAE	SHLPGVNLYL	LPLGEQLPDV	SLTFQAWRR
SDPERLCFIS	TTLQPFHALL	GGLGTQGRWT	NMERMLWAM	RLDLRDLQRH
LRFQVLAAGF	NLPEEEEEEE	EEEEERKGL	LPGALGSALQ	GPAQVSWPQL
LSTYRLLHSL	ELVLSRAVRE	LLLLSKAGHS	VWPLGFPTLS	PQP

[0149] 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: 52 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: 53 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.

SEQ ID NO: 53
MATKAVCVLK GDGPVQGI IN FEQKESNGPV KVGSIKGLT EGLHGFHVHE FGDNTAGCTS AGPHFNPLSR KHGGPKDEER HVGDLGNVTA DKDGVADVSI EDSVISLSGD HCIIGRTLW HEKADDLGKG GNEESTKTGN AGSRLACGVI GIAQ

[0150] 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- γ . 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: 54 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: 54 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.

SEQ ID NO: 54
HADGSFSDEMNTILDNLAARDFINWLIQTKITD

[0151] 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.

[0152] 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 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.

[0153] 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.

[0154] 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-1 β , IL-6, IL-8, IL-17, IL-18, IL-21, IL-23, IL-26, IL-32, Arachidonic acid, prostaglandins (e.g., PGE₂), PGI₂, serotonin, thromboxanes (e.g., TXA₂), leukotrienes (e.g., LTB₄), heparanase, 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 8. In some embodiments, the genetically engineered bacteria are capable of inhibiting, removing, degrading, and/or metabolizing one or more inflammatory molecules.

Table 8

Metabolites	Related bacteria	Potential biological functions
Bile acids: cholate, hyocholate, deoxycholate, chenodeoxycholate, a-muricholate, b-muricholate, w-muricholate, taurocholate, glycocholate, taurochenodeoxycholate, glycochenodeoxycholate, taurocholate, tauro-a-muricholate, tauro-b-muricholate, lithocholate, ursodeoxycholate, hyodeoxycholate, glycodeoxycholate	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-	Clostridium difficile, F. prausnitzii, Bifidobacterium, Subdoligranulum,	Detoxification of xenobiotics; indicate gut microbial composition and activity; utilize polyphenols. Urinary hippuric acid may be a biomarker of hypertension and

hydroxyhippuric acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxyphenylpropionate, 4-hydroxyphenylpropionate, 3-hydroxycinnamate, 4-methylphenol, tyrosine, phenylalanine, 4-cresol, 4-cresyl sulfate, 4-cresyl glucuronide, 4-hydroxyphenylacetate	Lactobacillus	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, indoleacetyl glycine (IAG), indole, indoxyl sulfate, indoles-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 intestine-brain-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, α-ketoisovalerate, creatine, creatinine, endocannabinoids, 2-arachidonoylglycerol (2-AG), N-arachidonoyl ethanolamide, LPS	Bacteroides, Pseudobutyrvibrio, Ruminococcus, Faecalibacterium	Direct or indirect synthesis or utilization of compounds or modulation of linked pathways including endocannabinoid system.

[0155] 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., PGE₂.

[0156] 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.

[0157] 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).

[0158] 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.

[0159] 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.

[0160] 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.

Inducible regulatory regions

Oxygen level-dependent regulation

[0161] The genetically engineered bacteria of the invention comprise a promoter that is directly or indirectly induced by exogenous environmental conditions. In some embodiments, a gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule is operably linked to an oxygen level-dependent promoter or regulatory region comprising said promoter. In some embodiments, the gene or gene cassette is operably linked to an oxygen level-dependent promoter such that the therapeutic molecule 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 therapeutic molecule.

[0162] In certain embodiments, the genetically engineered bacteria comprise a gene or a gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule expressed under the control of a fumarate and nitrate reductase regulator (FNR)-responsive promoter, an anaerobic regulation of arginine deiminase and nitrate reduction (ANR)-responsive promoter, or a dissimilatory nitrate respiration regulator (DNR)-responsive promoter, which are capable of being regulated by the transcription factors FNR, ANR, or DNR, respectively.

[0163] In certain embodiments, the genetically engineered bacteria comprise a 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. In some embodiments, multiple distinct FNR nucleic acid sequences are inserted in the genetically engineered bacteria.

[0164] In alternate embodiments, the promoter is an alternate oxygen level-dependent promoter, e.g., DNR (Trunk et al., 2010) or ANR (Ray et al., 1997). In *P. aeruginosa*, the anaerobic regulation of ANR is "required for the expression of physiological functions which are inducible under oxygen-limiting or anaerobic conditions" (Sawers, 1991; Winteler et al., 1996). *P. aeruginosa* ANR is homologous with *E. coli* FNR, and "the consensus FNR site (TTGAT—ATCAA) was recognized efficiently by ANR and FNR" (Winteler et al., 1996). Like FNR, in the anaerobic state, ANR activates numerous genes responsible for adapting to anaerobic growth. In the aerobic state, ANR is inactive. *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas syringae*, and *Pseudomonas mendocina* all have functional analogs of ANR (Zimmermann et al., 1991). Promoters that are regulated by ANR are known in the art, e.g., the promoter of the *arcDABC* operon (see, e.g., Hasegawa et al., 1998).

[0165] The FNR family also includes the dissimilatory nitrate respiration regulator (DNR) (Arai et al., 1995), a transcription factor which 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). 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.

[0166] 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 gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule. Non-limiting FNR promoter sequences are provided in **Table 9**. In some embodiments, the genetically engineered bacteria of the invention comprise one or more of: SEQ ID NO: 55, SEQ ID NO: 56, *nirBI* promoter (SEQ ID NO: 57), *nirB2* promoter (SEQ ID NO: 58), *nirB3* promoter (SEQ ID NO: 59), *ydfZ* promoter (SEQ ID NO: 60), *nirB* promoter fused to a strong ribosome binding site (SEQ ID NO: 61), *ydfZ* promoter fused to a strong ribosome binding site (SEQ ID NO: 62), *fnrS*, an anaerobically induced small RNA gene (*fnrS1* promoter SEQ ID NO: 63 or *fnrS2* promoter SEQ ID NO: 64), *nirB* promoter fused to a *crp* binding site (SEQ ID NO: 65), and *nrS* fused to a *crp* binding site (SEQ ID NO: 66). 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: 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, or 66, or a functional fragment thereof.

Table 9

FNR-responsive regulatory region	12345678901234567890123456789012345678901234567890
SEQ ID NO: 55	ATCCCCATCACTCTTGATGGAGATCAATTCCCCAAGCTGCTAGAGCGTTA CCTTGCCCTTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCT CCCACAGGAGAAAACCG
SEQ ID NO: 56	CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCT TAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGA GAAAACCG

<i>nirB1</i> SEQ ID NO: 57	GTCAGCATAACACCCTGACCTCTCATTAATTGTTTCATGCCGGGCGGCACT ATCGTCGTCCGGCCTTTTCTCTCTTACTCTGCTACGTACATCTATTTCT ATAAATCCGTTCAATTTGTCTGTTTTTGCACAAACATGAAATATCAGAC AAT TCCGTGAC TTAAGAAAAT TTATACAAAT CAGCAATA TACCCCTTAAG GAG TATA TAAAG GTGAAT TTGAT TTACAT CAATAAGCGGGGT TGCTGAAT CGTTAAG GTAGGCGGTAATAGAAAAGAAAT <u>CGAGGCAAAA</u>
<i>nirB2</i> SEQ ID NO: 58	CGGCCCGATCGTTGAACATAGCGGTCCGCAGGCGGCACTGCTTACAGCAA ACGGTCTGTACGCTGTCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTC AGCCGTCACCGTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCC GGACGGCACTATCGTCGTCCGGCCTTTTCTCTCTTCCCCGCTACGTGC ATCTATTTCTATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGA AATA TCAGACAAT TCCGTGAC TTAAGAAAAT TTATACAAAT CAGCAATA T ACCCAT TAAGGAGTATATAAAGGTGAAT TTGAT TTACATCAATAAGCGGG GTTGCTGAATCGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA atgtttgtttaactttaagaaggagatatatacat
<i>nirB3</i> SEQ ID NO: 59	GTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGGCACT ATCGTCGTCCGGCCTTTTCTCTCTTCCCCGCTACGTGCATCTATTTCT A TAAAC CCGCTCATTTTGTCTATTTTTTG CACAAACA TGAAATA TCAGAC AAT TCCGTGAC TTAAGAAAAT TTATACAAAT CAGCAATA TACCCATTAAG GAG TATA TAAAG GTGAAT TTGAT TTACAT CAATAAGCGGGGT TGCTGAAT CGTTAAG GTAGGCGGTAATAGAAAAGAAAT CGAGGCAAAA
<i>ydfZ</i> SEQ ID NO: 60	ATTTCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCGACTTATGGC TCAT GCAT GCAT CAAAAAAGAT GTGAGC TTGAT CAAAAACAAAAAATAT T TCACTCGACAGGAGTATTTATATTGCGCCCGTTACGTGGGCTTCGACTGT AAAT <u>CAGAAAG GAGAAAACACCT</u>
<i>nirB+RBS</i> SEQ ID NO: 61	GTCAGCATAACACCCTGACCTCTCATTAATTGTTTCATGCCGGGCGGCACT ATCGTCGTCCGGCCTTTTCTCTCTTACTCTGCTACGTACATCTATTTCT A TAAAT CCGTTCAAT TTGTCTGTTTTTGCACAAACA TGAAATA TCAGAC AAT TCCGTGAC TTAAGAAAAT TTATACAAAT CAGCAATA TACCCCTTAAG GAG TATA TAAAG GTGAAT TTGAT TTACAT CAATAAGCGGGGT TGCTGAAT CGTTAAGGATCCCTCTAGAAAT AAT TTTGTTTAACTTTAAGAAGGAGATA <u>TACAT</u>
<i>ydfZ+RBS</i> SEQ ID NO: 62	CATTTCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCGACTTATGG CTCAT GCAT GCAT CAAAAAAGAT GTGAGC TTGAT CAAAAACAAAAAATAT TCACTCGACAGGAGTATTTATATTGCGCCC <u>GGATCCCTCTAGAAAT AAT</u> <u>TTTGTTTAACTTTAAGAAGGAGATA TACA T</u>

<i>fnrS1</i> SEQ ID NO: 63	AGTTGTTCTTATTGGTGGTGTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAAC AAAAG CAATTTTTCCGGCTGTCTGTATACAAAAAC GCCGTAAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCTT GGATCCCTCTAGAAATAAT TTTGTTTAAC TTTAAGAAG GAGAT A T A C A T
<i>fnrS2</i> SEQ ID NO: 64	AGTTGTTCTTATTGGTGGTGTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAAC AAAAG CAATTTTTCCGGCTGTCTGTATACAAAAACGCCGC AAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCTT GGAT CCAAAG TGAAC TCTAGAAATAAT TTTGTTTAAC TTTAAGAAG GAGA TATACAT
<i>nirB+crp</i> SEQ ID NO: 65	TCGTCTTTGTGATGTGCTTCCTGTTAGGTTTTCGTCAGCCGTCACCGTCAG CATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGGCACTATCGT CGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTATTTCTATAAA CCCGCTCAT TTTGTCTAT TTTTGCACAAAC ATGAAAT ATCAGACAAT TC CGTGAC TTAAGAAAAT TTA TACAAAT CAGCAAT ATACCA TTAAG GAG TA TATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTA AGGTAGaaatgtgatctagttcacatttGCGTAATAGAAAAGAAATCGA <i>GGCAAAAatgtttgtttaactttaagaaggagatatacat</i>
<i>fnrS+crp</i> SEQ ID NO: 66	AGTTGTTCTTATTGGTGGTGTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAAC AAAAG CAATTTTTCCGGCTGTCTGTATACAAAAACGCCGC AAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCaa atgtgatctagttcacattt <i>tttgtttaactt taagaaggagatatacat</i>

[0167] In other embodiments, the gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule is 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; Gorke and Stulke, 2008). In some embodiments, the gene or gene cassette for producing an anti-inflammation and/or gut barrier function enhancer molecule is controlled by an oxygen level-dependent promoter fused to a CRP binding site. In some embodiments, the gene or gene cassette for producing an anti-

inflammation and/or gut barrier function enhancer molecule is 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 anti-inflammation and/or gut barrier function enhancer molecule 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 an anti-inflammation and/or gut barrier function enhancer molecule is not expressed under anaerobic conditions when sufficient amounts of glucose are present, e.g., by adding glucose to growth media *in vitro*.

[0168] 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 transcription factor and/or promoter may increase the production of the anti-inflammation and/or gut barrier enhancer molecule in low-oxygen conditions, as compared to the native transcription factor and promoter in the bacteria under the same conditions. In certain embodiments, the non-native oxygen level-dependent transcription factor is a FNR protein from *N. gonorrhoeae* (see, e.g., Isabella et al., 2011). In some embodiments, the corresponding wild-type transcription factor is deleted or mutated to reduce or eliminate wild-type activity. In alternate embodiments, the corresponding wild-type transcription factor is left intact and retains wild-type activity. In some embodiments, the heterologous transcription factor minimizes or eliminates off-

target effects on endogenous regulatory regions and genes in the genetically engineered bacteria.

[0169] In some embodiments, the genetically engineered bacteria comprise a wild-type gene encoding an oxygen level-dependent transcription factor, such as FNR, ANR or DNR, and a corresponding promoter that is mutated relative to the wild-type promoter from bacteria of the same subtype. The mutated promoter increases the production of an anti-inflammation and/or gut barrier enhancer molecule in low-oxygen conditions, 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., a FNR-, ANR- or DNR-responsive promoter, and a corresponding transcription factor 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 anti-inflammation and/or gut barrier enhancer molecule in low-oxygen conditions, as compared to the wild-type transcription factor under the same conditions. In certain embodiments, the mutant oxygen level-dependent transcription factor is a 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 transcription factor and corresponding promoter are mutated relative to the wild-type sequences from bacteria of the same subtype in order to increase expression of the anti-inflammation and/or gut barrier enhancer molecule in low-oxygen conditions.

[0170] In some embodiments, the genetically engineered bacteria of the invention comprise a gene encoding an oxygen level-sensing transcription factor, e.g., FNR, ANR or DNR, that is controlled by its native promoter, an inducible promoter, a promoter that is stronger than the native promoter, e.g., a *GlnRS* promoter, a *P(Bla)* promoter, or a constitutive promoter. In some instances, it may be advantageous to express the oxygen level-dependent transcription factor under the control of an inducible promoter in order to enhance expression stability. In some embodiments, expression of the oxygen level-dependent transcription factor is controlled by a different promoter than the promoter that controls expression of the therapeutic molecule. In some embodiments, expression of the oxygen level-dependent transcription factor is

controlled by the same promoter that controls expression of the therapeutic molecule. In some embodiments, the oxygen level-dependent transcription factor and therapeutic molecule are divergently transcribed from a promoter region.

[0171] In some embodiments, the genetically engineered bacteria of the invention comprise multiple copies of the endogenous gene encoding the oxygen level-sensing transcription factor, e.g., the *fnr* gene. In some embodiments, the gene encoding the oxygen level-sensing transcription factor is present on a plasmid. In some embodiments, the gene encoding the oxygen level-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 oxygen level-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 oxygen level-sensing transcription factor is present on a chromosome. In some embodiments, the gene encoding the oxygen level-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 oxygen level-sensing transcription factor and the gene or gene cassette for producing the therapeutic molecule are present on the same chromosome.

[0172] In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operably linked to a promoter that is induced by low-oxygen conditions. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present in the chromosome and operably linked to a promoter that is induced by low-oxygen conditions. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a chromosome and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, expression is further optimized by methods known

in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability.

[0173] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule, such that the gene(s) or gene cassette(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, a bacterium may comprise multiple copies of the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhance molecule. In some embodiments, the gene or gene cassette 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 or gene cassette 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 is expressed on a chromosome.

[0174] In some embodiments, the genetically engineered bacteria may comprise multiple copies of the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule. In some embodiments, the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operably linked to an oxygen level-dependent promoter. In some embodiments, the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule is present in a chromosome and operably linked to an oxygen level-dependent promoter.

[0175] In some embodiments, the genetically engineered bacteria of the invention produce at least one anti-inflammation and/or gut barrier enhancer molecule in low-oxygen conditions 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).

[0176] 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 an anti-inflammation and/or gut barrier enhancer molecule in low-oxygen conditions than unmodified bacteria of the same subtype under the same conditions. Certain unmodified bacteria will not have detectable levels of the anti-inflammation and/or gut barrier enhancer molecule. In embodiments using genetically modified forms of these bacteria, the anti-inflammation and/or gut barrier enhancer molecule will be detectable in low-oxygen conditions.

[0177] In certain 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 bacteria l cells of the invention are harvested and lysed to measure butyrate production. In alternate embodiments, butyrate production is measured in the bacteria l 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 μ M/OD, at least about 10 μ M/OD, at least about 100 μ M/OD, at least about 500 μ M/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.

[0178] In certain embodiments, the anti-inflammation and/or gut barrier enhancer 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 bacteria l cells of the invention are harvested and lysed to measure propionate production. In alternate embodiments, propionate production is measured in the bacteria l cell medium. In some embodiments, the genetically engineered bacteria produce at least about 1 μ M, at least about 10 μ M, at least about 100 μ M, at least about 500 μ M, 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.

RNS-dependent regulation

[0179] 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 gene cassette capable of directly or indirectly driving the expression of an anti-inflammation and/or gut barrier function enhancer molecule, thus controlling expression of the molecule relative to RNS

levels. For example, the tunable regulatory region is a RNS-inducible regulatory region, and the molecule is butyrate; 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 butyrogenic gene cassette, thereby producing butyrate, which exerts anti-inflammation and/or gut barrier enhancing effects. Subsequently, when inflammation is ameliorated, RNS levels are reduced, and butyrate production is decreased or eliminated.

[0180] 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 gene cassette. 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.

[0181] 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; Table 1). 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 gene cassette, e.g., a butyrogenic gene cassette. 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 butyrogenic gene cassette and producing butyrate.

[0182] 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 butyrogenic gene cassette and producing butyrate. In some embodiments, the DNR is *Pseudomonas aeruginosa* DNR.

[0183] 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.

[0184] 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; Table 1). 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 gene cassette, e.g., a butyrogenic gene cassette. 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 butyrogenic gene cassette and producing butyrate.

[0185] 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 *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.

[0186] 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.

[0187] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express an anti-inflammation and/or gut barrier function enhancer molecule. The two repressor activation regulatory circuit comprises a first RNS-sensing repressor and a second repressor, which is operatively linked to a gene or gene cassette, e.g., a butyrogenic gene cassette. 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, CI, 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 gene cassette, e.g., a butyrogenic gene cassette. 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 gene cassette, e.g., a butyrogenic gene cassette, is expressed.

[0188] 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).

[0189] 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.

[0190] 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.

[0191] 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.

[0192] 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.

[0193] 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 anti-inflammation and/or gut barrier enhancer molecule 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 anti-inflammation and/or gut barrier enhancer molecule 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 anti-inflammation and/or gut barrier enhancer molecule in the presence of RNS.

[0194] Nucleic acid sequences of exemplary RNS-regulated constructs comprising a gene encoding NsrR and a *norB* promoter are shown in **Table 10** and **Table 11**. **Table 10** 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: 67). The sequence encoding NsrR is **underlined and bolded**, and the NsrR binding site, i.e., a regulatory

region of *norB* is boxed. **Table 11** 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: 68). The sequence encoding NsrR is underlined and bolded, and the NsrR binding site, i.e., a regulatory region of *norB* is boxed. Nucleic acid sequences of tetracycline-regulated constructs comprising a *tet* promoter are shown in **Table 12** and **Table 13**. **Table 12** 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: 69). The sequence encoding TetR is underlined, and the overlapping *tetR/tetA* promoters are boxed. **Table 13** 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: 70). The sequence encoding TetR is underlined, and the overlapping *tetR/tetA* promoters are boxed. 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: 67, 68, 69, or 70, or a functional fragment thereof.

Table 10

Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 67)

11a11atcgacccgcaatcgggattttcgattcataaagcaggctcgtaggtcggcttgtt
gagcaggctcttgcagcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgcc
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ctcgaccagctgcatcggttcgaggtggcggacgaccgcgccgatattgatgcgttcggg
cggcgcgccagcctcagcccgcgcctttcccgctacgctgtgcaagaaccgccttt
gaccagcgcggttaaccactttcatcaaatggcttttgaaatgccgtaggtcgaggcgat
ggtggcgatattgaccagcgcgctcgctcggttgacggcggtgtagatgaggacgcgcagccc
gtagtcgggtatgttgggtcagatacat acaacctccttaqtacatqcaaaattatttcta
gagcaacatacagccggaagcataaaagtgtaaagcctggggtgcctaatagttgagtt
gaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatgggtgttga
caattaatcatcggtctgtataatgtataacattcatattttgtgaattttaaaccttag
aaataattttgtttaactttaagaaggagatatacatatggatttaaattctaaaaata
tcagatgcttaaagagctatatgtaagcttcgctgaaaatgaagttaaacccttagcaac
agaacttgatgaagaagaagatttccttatgaaacagtggaaaaaatggcaaaagcagg
aatgatgggtataccatatccaaaagaatatggtggagaagggtggagacactgtaggata
tataatggcagttgaagaattgtctagagtttggtggtactacaggagttatattatcagc
tcatacatctcttggtcatggcctatatatcaatatggtaatgaagaacaaaaacaaa
attcttaagaccactagcaagtggagaaaaattaggaqcatttgggtcttactgaqcctaa

Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 67)

tgctggtacagatgcgtctggccaacaaacaactgctggttttagacggggatgaatacat
acttaatggctcaaaaatatttataacaaacgcaatagctggtgacatatatgtagtaat
ggcaatgactgataaatctaagggaacaaaggaatatcagcatttatagttgaaaagg
aactcctgggttttagcttttgaggttaaagaaaagaaaatgggtataagaggttcagctac
gagtgaattaatatttgaggattgcagaatacctaagaaaatttacttggaaaagaagg
tcaaggatttaagatagcaatgtctactcttgatggtggtagaattggtatagctgcaca
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aaaaccttatggagtagaagcagcaatggcaaaattatttgcagctgaaacagctatgga
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atttgaagattggttgcctgacttaaaagttaaaatgccatgccttataacaactcttaa
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tgtagtagaaacatggactgtaaaagatatagaagttgaccttctaatttaggtcttaa
aggttctccaactagtgtattttaaatcatttacaaaatcagttaaaccagctgggtacaat
atacaatgaagatgcgaaaacatcagctggaattatcatagataaattaaaagagaagta
tatcatataataagaaggagatatacatatgggtaacgttttagtagtaatagaacaaag
agaaaatgtaattcaaactgtttctttagaattactaggaagggtacagaaatagcaaa
agattatgatacaaaaagtttctgcattacttttaggtagtaaggtagaaggtttaataga
tacattagcacactatggtgcagatgaggtaatagtagtagatgatgaagcttttagcagt
gtatacaactgaaccatatacaaaagcagcttatgaagcaataaaagcagctgacctat
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aatacatacagggtct tact get gactgtacagggtcttgcagtagctgaagatacaaaatt
attattaatgacaagacctgccttttggtggaaatataatggcaacaatagtttgtaaaga
tttcagacctcaaatgtctacagtttagaccagggttatgaagaaaaatgaacctgatga
aactaaagaagctgtaattaaaccgtttcaaggtagaatttaatatgatgctgataaattagt
tcaagttgtacaagtaataaaaagaagctaaaaaacaagttaaaatagaagatgctaagat
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agctgaaattatagggtggagaagtttctggttctcgtgccactatagatgcagggttggtt
agataaagcaagacaagttggtcaaactggtaaaactgtaagaccagacctttatatagc
atgtggtatatctggagcaatacaacatatagctggtatggaagatgctgagtttatagt
tgctataaataaaaatccagaagctccaatatttaaatatgctgatgttggtatagttgg
agatgttcataaagtgttccagaacttatcagtcagtttaagtgttgcaaaagaaaagg
tgaagtttttagctaactaataagaaggagatatacatatgagagaagtagtaattgccag
tgcagctagaacagcagtaggaagttttggaggagcatttaaatcagtttcagcggtaga
gttaggggtaacagcagctaaagaagctataaaaagagctaacataactccagatatgat
agatgaatctcttttagggggagttacttacagcagggtcttggacaaaatatagcaagaca

Nucleotide sequences of pLogic031-nsrR-norB-butyrate construct (SEQ ID NO: 67)

aatagcattaggagcaggaataaccagtagaaaaaccagctatgactataaatatagtttg
 tggttctggattaagatctgtttcaatggcatctcaacttatagcattagggtgatgctga
 tataatgttagttgggtggagctgaaaacatgagtatgtctccttatttagtaccaagtgc
 gagatatgggtgcaagaatgggtgatgctgcttttgttgattcaatgataaaagatggatt
 atcagacatatttaataactatcacatgggtattactgctgaaaacatagcagagcaatg
 gaatataactagagaagaacaagatgaattagctcttgcaagtcaaaataaagctgaaaa
 agctcaagctgaaggaaaatttgatgaagaaatagttcctgttggtataaaaaggaagaaa
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 aataaatgatgggtgctgctatgttagtagtaatggctaaagaaaaagctgaagaactagg
 aatagagcctcttgcaactatagtttcttatggaacagctgggtgttgaccctaaaataat
 gggatatggaccagttccagcaactaaaaaagctttagaagctgctaatatgactattga
 agatatagatttagttgaagctaattgaggcatttgctgccaatctgtagctgtaataag
 agacttaaatatagatatgaataaagttaatgttaatgggtggagcaatagctataggaca
 tccaataggatgctcaggagcaagaatacttactacacttttatatgaaatgaagagaag
 agatgctaaaactgggtcttgctacactttgtataggcggtggaatgggaactactttaat
 agttaagagatagtaagaaggagatatacatatgaaattagctgtaataggttagtggaac
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 cttggcaacaaatacttcatcattatctataacagaaatagcttcttctactaagcgccc
 agataaagttataggaatgcatttctttaatccagttcctatgatgaaattagttgaagt
 tataagtgggtcagttaacatcaaaagttacttttgatacagtatttgaattatctaagag
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 tatacctatgataaatgaagctgtttggtatatatgcagatgggtgttgcaagtaagaaga
 aatagatgaagctatgaaattaggagcaaacatccaatgggaccactagcattaggtga
 ttaaatcggattagatgtttgttttagctataatgaacgttttatatactgaatttgga
 tactaaatatagacctcatccacttttagctaaaatgggttagagctaataataggaag
 aaaaactaagataggattctatgattataataaataataagaaggagatatacatatgag
 tacaagtgatgttaagtttatgagaatgtagctgttgaaagtagatggaaatatatgtac
 agtgaaaatgaatagacctaaagcccttaatgcaataaattcaaagactttagaagaact
 ttatgaagtattttgtagatattaataatgatgaaactattgatgttgtaatatgtgacagg
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 agctgctaaagatttttagtatcttaggagcaaaagcttttgagaaatagaaaatagtaa
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 ggcatgtgatataagaattgcatctgctaaagctaaatttggtcagccagaagtaactct
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 ttcaactaaagacccaaaaagaaggaatgtcagctttcggtgaaaagagagaagctaactt
 tataaaagggttaataagaaggagatatacatatgagaagttttgaagaagtaattaagtt
 tgcaaaagaaaggagacctaaaactatatcagtagcatgttgccaagataaagaagtttt
 aatggcagttgaaatggctagaaaagaaaaaatagcaaatgccatttttagtaggagatat

Nucleotide sequences of pLogic031-nsrR-norB-butyrat construct (SEQ ID NO: 67)
agaaaagactaaagaaattgcaaaaagcatagacatggatatcgaaaattatgaactgat agatataaaaagatttagcagaagcatctctaaaatctgttgaattagtttcacaaggaaa agccgacatggtaatgaaaggcttagtagacacatcaataataactaaaagcagttttaaa taaagaagtaggtcttagaactggaaatgtattaaagtcacgtagcagtagttgatgtaga gggatatgatagattatcttctgtaactgacgcagctatgaacttagctcctgatacaaa tactaaaaagcaaatcatagaaaatgcttgcacagtagcacattcattagatataagtga accaaaagttgctgcaatatgcgcaaaaagaaaaagtaaatccaaaaatgaaagatacagt tgaagctaaagaactagaagaaatgtatgaaagaggagaaatcaaagggtgtatgggtgg tgggccttttgcaattgataatgcagtagcttttagaagcagctaaacataaagggtataaa tcatcctgtagcaggacgagctgatataattattagccccagatatgtgaagggtggtaacat attatataaaagcttttggtattcttctcaaaaatcaaaaaatgcaggagttatagttggggc taaagcaccaataatattaacttctagagcagacagtgaaagaaactaaactaaactcaat agcttttaggtgttttaatggcagcaaaaggcataataagaaggagatatacatatgagcaa aatattttaaactcttaacaataaatcctgggttcgacatcaactaaaatagctgtatttga taatgaggatttagtatttgaaaaaactttaagacattcttcagaagaaataggaaaata tgagaagggtgtctgaccaatttgaatttcgtaaacaagtaatagaagaagctctaaaaga aggtggagtaaaaacatctgaattagatgctgttagtaggtagaggaggacttcttaaacc tataaaagggtggtacttattcagtaagtgtgctatgattgaagattttaaaggtgggagt tttaggagaacacgcttcaaacctagggtggaataatagcaaaacaaatagggtgaagaagt aatgttctctcatacatagtagaccctgttgtttagatgaattagaagatgttgcttag aatttctgggtatgcctgaaataagtagagcaagtgtagtacatgctttaaataaaaaggc aatagcaagaagatatgctagagaaataaacaagaaatatgaagatataaatcttatagt tgcacacatgggtggaggagtttctgttggagctcataaaaatggtaaaatagtagatgt tgcaaacgcattagatggagaaggaccttctctccagaaagaagtgggtggactaccagt aggtgcatttagtaaaaatgtgcttttagtggaataataactcaagatgaaattaaaaagaa aataaaaggtaatggcggactagttgcatactttaaactaatgatgctagagaagttga agaaagaattgaagctgggtgatgaaaaagctaaatttagtatatgaagctatggcatatca aatctctaaagaataaggagctagtgtgcagttcttaaggagatgtaaaagcaatatt attaaactgggtggaatcgcatattcaaaaatgtttacagaaatgattgcagatagagttaa atttatagcagatgtaaaagtttatccagggtgaagatgaaatgattgcattagctcaagg tggacttagagttttaactgggtgaagaagggtcaagtttatgataactaataa

Table 11

Nucleotide sequences of pLogic046-nsrR-norB-butyrat construct (SEQ ID NO: 68)
<u>11a11atcgaccgcaatcgggattttcgattcataaagcaggctcgtaggctcggttgtt</u> <u>gagcaggctcttgacgcgtgaaaccgtccagatagctgaaaaacgacttcattgcaccgcc</u> <u>gagtatgcccgtcagccggcaggacggcgtaatcaggcattcggtgttcgggcccataca</u> <u>ctcgaccagctgcatcggttcgaggtggcggacgaccgcgcgatattgatgcgttcggg</u> <u>cggcgcgccagcctcagcccgcgcctttcccgcgtacgctgtgcaagaaccgccttt</u> <u>gaccagcgcggttaaccactttcatcaaatggcttttggaatgccgtaggctcgaggcgat</u> <u>ggtggcgatattgaccagcgcgctcgctcgttgacggcggtgtagatgaggacgcgcagccc</u> <u>gtagtcggtatgttgggtcagatacatacaacctccttagtacatgcaaaattatttcta</u> <u>gagcaacatacagccggaagcataaagtgtaaagcctgggggtgcctaataaggttgagt</u> <u>gaggaattataacaggaagaaatattcctcatagcgttgtaattcctctatgggtgttga</u>

Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 68)

caattaatcatcggctcgtataatgtataacattcatattttgtgaattttaactctag
aaataattttgtttaactttaagaaggagatatacatatgatcgtaaacctatggtacg
caacaatatctgcctgaacgcccattcctcagggctgcaagaaggagtggaagatcagat
tgaatataccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaa
cgttctgggtget tggct get caaatggttacggcctggcgagccgcat tact get gcgt
cggatacggggctgcgaccatcggcgtgtcctttgaaaaagcgggttcagaaaccaaata
tggtacaccgggatggtacaataatttggcattttgatgaagcggcaaacgcgagggctt
ttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaaggcccaggtaattga
ggaagccaaaaaaaagggtatcaaatttgatctgatcgtatacagcttggccagcccagt
acgtactgatcctgatacaggtatcatgcacaaaagcgttttgaaaccctttgaaaaac
gttcacaggcaaaacagtagatccgtttactggcgagctgaaggaaatctccgcggaacc
agcaaatgacgaggaagcagccgccactgttaaagttatggggggtgaagattgggaacg
ttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggccta
tagttatattggccctgaagctacccaagctttgtaccgtaaaggcacaatcggcaaggc
caaagaacacctggaggccacagcacaccgtctcaacaaagagaaccgtcaatccgtgc
cttcgtgagcgtgaataaaggcctggtaaccgcgcaagcgcgtaatcccggtaatccc
tctgtatctcgccagcttgttcaaagtaatgaaagagaagggaatcatgaagggtgtat
tgaacagatcacgcgtctgtacgccgagcgcctgtaccgtaaagatggtacaattccagt
tgatgaggaaaatcgcattcgcattgatgattgggagttagaagaagacgtccagaaagc
ggtatccgcgttgatggagaaagtcacgggtgaaaacgcagaatctctcactgacttagc
ggggtaccgccatgatttcttagctagtaacggctttgatgtagaagggtattaattatga
agcgggaagttgaacgcttcgaccgtatctgataagaaggagatatacatatgagagaagt
agtaattgccagtgacgtagaagcagtaggaagttttggaggagcatttaaatcagt
ttcagcggtagagttaggggtaacagcagctaaagaagctataaaaagagctaacataac
tccagatatgatagatgaatctcttttagggggagtacttacagcagggtcttggacaaaa
tatagcaagacaaatagcattaggagcaggaataaccagtagaaaaaccagctatgactat
aatatagtttgtgggttctggattaagatctgtttcaatggcatctcaacttatagcatt
agggtgatgctgatataatgttagttgggtggagctgaaaacatgagtatgtctccttattt
agtaccaagtgcgagatatggtgcaagaatgggtgatgctgcttttgttgattcaatgat
aaaagatggattatcagacataatttaataactatcacatgggtattactgctgaaaacat
agcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaa
taaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttggtat
aaaaggaagaaaagggtgacactgtagtagataaagatgaatatattaagcctggcactac
aatggagaaactt get aagttaagacctgcatttaaaaagatggaacagt tact get gg
taatgcatcaggaataaatgatgggtgctgctatgttagtagtaatggctaaagaaaaagc
tgaagaactaggaatagagcctcttgcaactatagtttcttatggaacagctgggtgttga
ccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaa
tatgactattgaagatatagatttagttgaagctaattgaggcatttgctgccaatctgt
agctgtaataagagacttaatatagatatgaataaagttaatgttaatgggtggagcaat
agctataggacatccaataggatgctcaggagcaagaatacttactacacttttatatga
aatgaagagaagagatgctaaaactgggtcttgctacactttgtataggcgggtggaatggg
aactacttttaatagtttaagagatagtaagaaggagatatacatatgaattagctgtaat
aggtagtggaactatgggaagtggtattgtacaaacttttgcaagttgtggacatgatgt
atgttttaagagtagaactcaagggtgctatagataaatgttttagctttattagataaaaa
tttaactaagttagttactaagggaataatggatgaagctacaaaagcagaaatattaag
tcatgttagttcaactactaattatgaagatttaaaagatatggatttaataatagaagc
atctgtagaagacatgaatataaagaaagatgttttcaagttactagatgaattatgtaa

Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 68)

agaagatactatcttggcaacaatacttcatcattatctataacagaaatagcttcttc
 tactaagcgcccagataaagttataggaatgcatttctttaatccagttcctatgatgaa
 attagttgaagttataagtggtcagttaacatcaaaagttacttttgatacagtatattga
 attatctaagagtatcaataaagtaccagtagatgtatctgaatctcctggatttgtagt
 aaatagaatacttatacctatgataaatgaagctgttgggtatataatgcagatgggtgtgc
 aagtaaagaagaaatagatgaagctatgaaattaggagcaaaccatccaatgggaccact
 agcattaggtgatttaatcggattagatgttgttttagctataatgaacgttttatatac
 tgaatttgagatactaaatatagacctcatccacttttagctaaaatggtagagctaa
 tcaattaggaagaaaaactaagataggattctatgattataataaataaagaaggaga
 tatacatatgagtacaagtgtgttaaagtttatgagaatgtagctgttgaagtagatgg
 aaatatatgtacagtgaataatgaatagacctaaagcccttaatgcaataaattcaaagac
 tttagaagaactttatgaagtatttgtagatattaataatgatgaaactattgatgttgt
 aatattgacaggggaaggaaaggcattttgtagctggagcagatattgcatacatgaaaga
 tttagatgctgtagctgctaaagatttttagtatcttaggagcaaaagcttttgagaaat
 agaaaatagtaaaaaagtagtgatagctgctgtaaacggatttgcttttaggtggaggatg
 tgaacttgcaatggcatgtgatataagaattgcatctgctaaagctaaatttggtcagcc
 agaagtaactcttgggaataactccaggatatggaggaactcaaaggcttacaagattgggt
 tggaatggcaaaagcaaaagaattaatctttacaggtcaagttataaaaagctgatgaagc
 tgaaaaaatagggctagtaaatagagtcggtgagccagacattttaatagaagaagttga
 gaaattagctaagataatagctaaaaatgctcagcttgtagttagatactctaaagaagc
 aatacaacttgggtgctcaaactgatataaatactggaatagatatagaatctaatttatt
 tggctctttgtttttcaactaaagacaaaaagaaggaatgtcagctttcggtgaaaagag
 agaagctaactttataaaaagggttaataagaaggagatatatacatatgagaagttttgaaga
 agtaattagtttgcaaaagaagaggacctaaaactatatcagtagcatgttgccaaga
 taaagaagttttaatggcagttgaaatggctagaaaaagaaaaaatagcaaatgccatttt
 agtaggagatatagaaaagactaaagaaattgcaaaaagcatagacatggatatcgaaaa
 ttatgaactgatagatatataaagatttagcagaagcatctctaaaatctgttgaaatagt
 ttcacaaggaaaagccgacatggtaatgaaaggcttagtagacacatcaataatactaaa
 agcagtttttaataaagaagtaggtcttagaactggaaatgtattaagtcacgtagcagt
 atttgatgtagagggatatgatagattattttctgtaactgacgcagctatgaacttagc
 tctgatacaataactaaaaagcaaatcatagaaaatgcttgacagtagcacattcatt
 agatataagtgaacccaaaagttgctgcaatatgcgcaaaagaaaaagtaaatccaaaaat
 gaaagatacagttgaagctaaagaactagaagaaatgtatgaaagaggagaaatcaaagg
 ttgatgtggttgggtgggaccttttgcaattgataatgcagtatctttagaagcagctaaaca
 taaagggtataaatcatcctgtagcaggacgagctgatataattattagccccagatattga
 aggtggtaacatattatataaagctttgggtattcttctcaaaatcaaaaaatgcaggagt
 tatagttggggctaaagcaccaataatattaacttctagagcagacagtgaaagaaactaa
 actaaactcaatagcttttaggtgttttaatggcagcaaaggcataataagaaggagatat
 acatatgagcaaaaatatttaaaatcttaacaataaatcctgggttcgacatcaactaaaat
 agctgtattttgataatgaggatttagtatttgaaaaaactttaagacattcttcagaaga
 aatagggaaaatatgagaaggtgtctgaccaatttgaaatttcgtaacaagtaatagaaga
 agctctaaaagaaggtggagtaaaaacatctgaattagatgctgtagtaggttagaggagg
 acttcttaaacctataaaaggtgggtacttattcagtaagtgtgctatgattgaagattt
 aaaagtgggagttttaggagaacacgcttcaaaccctagggtggaataatagcaaaacaat
 aggtgaagaagtaaatgttccttcatacatagtagaccctgttgtttagatgaattaga
 agatgttgctagaatttctgggtatgcctgaaataagtagagcaagtgtagtacatgcttt
 aatcaaaaggcaatagcaagaagatatgctagagaaataaacaagaaatatgaagatat

Nucleotide sequences of pLogic046-nsrR-norB-butyrate construct (SEQ ID NO: 68)

aaatcttatagttgcacacatgggtggaggagtttctgttggagctcataaaaaatggtaa
 aatagtagatgttgcacacgcattagatggagaaggacctttctctccagaaagaagtgg
 tggactaccagtaggtgcattagtaaaaatgtgcttttagtggaataataactcaagatga
 aattaaaaagaaaataaaaaggtaatggcggactagttgcatacttaaacactaatgatgc
 tagagaagttgaagaaagaattgaagctgggtgatgaaaaagctaaattagtatatgaagc
 tatggcatatcaaatctctaaagaaataggagctagtgtgctgcagttcttaagggagatgt
 aaaagcaatattattaactgggtggaatcgcatattcaaaaatgtttacagaaatgattgc
 agatagagttaaatttatagcagatgtaaaagtttatccagggtgaagatgaaatgattgc
 attagctcaagggtggacttagagttttaactgggtgaagaagagggtcaagtttatgataa
 ctaataa

Table 12**Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 69)**

1	gtaaaacgac	ggccagtgaa	<u>ttcgttaaga</u>	<u>cccactttca</u>	<u>catttaagtt</u>
<u>gtttttctaa</u>					
61	<u>tccgcatatg</u>	<u>atcaattcaa</u>	<u>ggccgaataa</u>	<u>gaaggctggc</u>	<u>tctgcacctt</u>
<u>ggtgatcaaa</u>					
121	<u>taattcgata</u>	<u>gcttgtcgta</u>	<u>ataatggcgg</u>	<u>catactatca</u>	<u>gtagtaggtg</u>
<u>tttccctttc</u>					
181	<u>ttcttttagcg</u>	<u>acttgatgct</u>	<u>cttgatcttc</u>	<u>caatacgcaa</u>	<u>cctaaagtaa</u>
<u>aatgccccac</u>					
241	<u>agcgctgagt</u>	<u>gcatataatg</u>	<u>cattctctag</u>	<u>tgaaaaacct</u>	<u>tgttggcata</u>
<u>aaaaggctaa</u>					
301	<u>ttgattttcg</u>	<u>agagtttcat</u>	<u>actgtttttc</u>	<u>tgtaggccgt</u>	<u>gtacctaaat</u>
<u>gtacttttgc</u>					
361	<u>tccatcgcgga</u>	<u>tgacttagta</u>	<u>aagcacatct</u>	<u>aaaactttta</u>	<u>gcgttattac</u>
<u>gtaaaaaatc</u>					
421	<u>ttgccagctt</u>	<u>tccccttcta</u>	<u>aagggcaaaa</u>	<u>gtgagtatgg</u>	<u>tgccatatcta</u>
<u>acatctcaat</u>					
481	<u>ggctaaggcg</u>	<u>tcgagcaaag</u>	<u>cccgttatt</u>	<u>ttttacatgc</u>	<u>caatacaatg</u>
<u>taggctgctc</u>					
541	<u>tacacctagc</u>	<u>ttctgggcga</u>	<u>gtttacgggt</u>	<u>tgttaaacct</u>	<u>tcgattccga</u>
<u>cctcattaag</u>					
601	<u>cagctctaata</u>	<u>gcgctgttaa</u>	<u>tcactttact</u>	<u>tttatctaata</u>	<u>ctagacatca</u>
<u>ttaattccta</u>					
661	<u>atTTTT</u>	<u>gttg</u>	<u>acactctatc</u>	<u>attgatagag</u>	<u>ttatTTTacc</u>
<u>actccctatq</u>					
<u>agtgatagag)</u>					
721	<u>aa</u>	<u>laagtgaac</u>	<u>tctagaaata</u>	<u>atTTTgttta</u>	<u>actttaagaa</u>
<u>ggagatatac</u>					
<u>atatggattt</u>					
781	<u>aaattctaaa</u>	<u>aaatatcaga</u>	<u>tgcttaaaga</u>	<u>gctatatgta</u>	<u>agcttcgctg</u>
<u>aaaatgaagt</u>					
841	<u>taaaccttta</u>	<u>gcaacagaac</u>	<u>ttgatgaaga</u>	<u>agaaagattt</u>	<u>ccttatgaaa</u>
<u>cagtggaaaa</u>					
901	<u>aatggcaaaa</u>	<u>gcaggaatga</u>	<u>tgggtatacc</u>	<u>atatccaaaa</u>	<u>gaatatgggtg</u>
<u>gagaagggtg</u>					
961	<u>agacactgta</u>	<u>ggatatataa</u>	<u>tggcagttga</u>	<u>agaattgtct</u>	<u>agagtttgtg</u>
<u>gtactacagg</u>					

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 69)					
1021	agttatatta	tcagctcata	catctcttgg	ctcatggcct	atatatcaat
atggtaatga	1081	agaacaaaaa	caaaaattct	taagaccact	agcaagtgga
gagcatttgg	1141	tcttactgag	cctaattgctg	gtacagatgc	gtctggccaa
ctgtttttaga	1201	cggggatgaa	tacatactta	atggctcaaa	aatatttata
tagctggtga	1261	catatatgta	gtaatggcaa	tgactgataa	atctaagggg
tatcagcatt	1321	tatagttgaa	aaaggaactc	ctgggttttag	ctttggagtt
aaatgggtat	1381	aagaggttca	gctacgagtg	aattaatatt	tgaggattgc
aagaaaattt	1441	acttgaaaaa	gaaggtcaag	gatttaagat	agcaatgtct
gtggtagaat	1501	tggtatagct	gcacaagctt	taggttttagc	acaaggtgct
ctgttaaata	1561	tgtaaaagaa	agagtacaat	ttggtagacc	attatcaaaa
cacaattcca	1621	attagctgat	atggaagtta	aggtacaagc	ggctagacac
aagcagctat	1681	aaataaagac	ttaggaaaac	cttatggagt	agaagcagca
tatttgcagc	1741	tgaacagct	atggaagtta	ctacaaaagc	tgtacaactt
atggatacac	1801	tcgtgactat	ccagtagaaa	gaatgatgag	agatgctaag
tatatgaagg	1861	aactagttaa	gttcaaagaa	tggttatttc	aggaaaacta
aagaaggaga	1921	tatacatatg	gaggaaggat	ttatgaatat	agtcgtttgt
ttccagatac	1981	aacagaagtt	aaactagatc	ctaatacagg	tactttaatt
taccaagtat	2041	aataaacctt	gatgataaag	caggttttaga	agaagctata
aagaaatggg	2101	tgctcatgta	actgtttataa	caatgggacc	tcctcaagca
taaaagaagc	2161	tttagcaatg	ggtgcagata	gaggtatatt	attaacagat
cgggtgctga	2221	tacttgggca	acttcatcag	cattagcagg	agcattaaaa
ttgatattat	2281	aatagctgga	agacaggcga	tagatggaga	tactgcacaa
aaatagctga	2341	acattttaat	cttccatcaa	taacatatgc	tgaagaaata
gtgaatatgt	2401	attagtaaaa	agacaatttg	aagattgttg	ccatgactta
tgccatgcct	2461	tataacaact	cttaaagata	tgaacacacc	aagatacatg
gaatatatga	2521	tgctttcgaa	aatgatgtag	tagaaacatg	gactgtaaaa
ttgacccttc					gatatagaag

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 69)					
2581	taatttaggt	cttaaagggt	ctccaactag	tgtattttaa	tcatttacaa
aatcagttaa					
2641	accagctggt	acaatataca	atgaagatgc	gaaaacatca	gctggaatta
tcatagataa					
2701	attaaaagag	aagtatatca	tataataaga	aggagatata	catatgggta
acgttttagt					
2761	agtaatagaa	caaagagaaa	atgtaattca	aactgtttct	ttagaattac
taggaaaggc					
2821	tacagaaata	gcaaaagatt	atgatacaaa	agtttctgca	ttacttttag
gtagtaagg					
2881	agaaggttta	atagatacat	tagcacacta	tgggtgcagat	gaggtaatag
tagtagatga					
2941	tgaagcttta	gcagtgtata	caactgaacc	atatacaaaa	gcagcttatg
aagcaataaa					
3001	agcagctgac	cctatagttg	tattatttgg	tgcaacttca	ataggtagag
atttagcgcc					
3061	tagagtttct	gctagaatac	atacaggctc	tactgctgac	tgtacaggtc
ttgcagtagc					
3121	tgaagataca	aaattattat	taatgacaag	acctgccttt	gggtggaata
taatggcaac					
3181	aatagtttgt	aaagatttca	gacctcaaat	gtctacagtt	agaccagggg
ttatgaagaa					
3241	aatgaacct	gatgaaacta	aagaagctgt	aattaaccgt	ttcaaggtag
aatttaatga					
3301	tgctgataaa	ttagttcaag	ttgtacaagt	aataaaaaga	gctaaaaaac
aagttaaaat					
3361	agaagatgct	aagatattag	tttctgctgg	acgtggaatg	gggtgaaaag
aaaacttaga					
3421	catactttat	gaattagctg	aaattatagg	tggagaagtt	tctggttctc
gtgccactat					
3481	agatgcaggt	tggttagata	aagcaagaca	agttggtcaa	actggtaaaa
ctgtaagacc					
3541	agacctttat	atagcatgtg	gtatatctgg	agcaatacaa	catatagctg
gtatggaaga					
3601	tgctgagttt	atagttgcta	taaataaaaa	tccagaagct	ccaatattta
aatatgctga					
3661	tgttggtata	gttgagatg	ttcataaagt	gcttccagaa	cttatcagtc
agttaagtgt					
3721	tgcaaaaaga	aaaggtgaag	ttttagctaa	ctaataagaa	ggagatatac
atatgagaga					
3781	agtagtaatt	gccagtgcag	ctagaacagc	agtaggaagt	tttggaggag
catttaaadc					
3841	agtttcagcg	gtagagttag	gggtaacagc	agctaaagaa	gctataaaaa
gagctaacad					
3901	aactccagat	atgatagatg	aatctctttt	agggggagta	cttacagcag
gtcttggaac					
3961	aaatatagca	agacaaatag	cattaggagc	aggaatacca	gtagaaaaac
cagctatgac					
4021	tataaatata	gtttgtgggt	ctggattaag	atctgtttca	atggcatctc
aacttatagc					
4081	attaggtgat	gctgatataa	tgttagttgg	tggagctgaa	aacatgagta
tgtctcctta					

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 69)					
4141	tttagtacca	agtgcgagat	atgggtgcaag	aatgggtgat	gctgcttttg
4201	gataaaagat	ggattatcag	acatatTTaa	taactatcac	atgggtatta
4261	catagcagag	caatggaata	taactagaga	agaacaagat	gaattagctc
4321	aaataaagct	gaaaaagctc	aagctgaagg	aaaatttgat	gaagaaatag
4381	tataaaagga	agaaaagggtg	acactgtagt	agataaagat	gaatatatta
4441	tacaatggag	aaacttgcta	agttaagacc	tgcatTTaaa	aaagatggaa
4501	tggtaatgca	tcaggaataa	atgatgggtgc	tgctatgtta	gtagtaatgg
4561	agctgaagaa	ctaggaatag	agcctcttgc	aactatagtt	tcttatggaa
4621	tgaccctaaa	ataatgggat	atggaccagt	tccagcaact	aaaaaagctt
4681	taatatgact	attgaagata	tagatttagt	tgaagcta	gaggcatttg
4741	tgtagctgta	ataagagact	taaatataga	tatgaataaa	gttaatgtta
4801	aatagctata	ggacatccaa	taggatgctc	aggagcaaga	atacttacta
4861	tgaaatgaag	agaagagala	ctaaaactgg	tcttgctaca	ctttgtatag
4921	gggaactact	ttaatagtl	agagatagta	agaaggagat	atacatatga
4981	aataggtagt	ggaactatc	gaagtgggtat	tgtacaaact	tttgcaagtt
5041	tgtatgttta	aagagtagia	ctcaagggtgc	tatagataaa	tgtttagctt
5101	aaatttaact	aagttagtl	ctaagggaaa	aatggatgaa	gctacaaaag
5161	aagtcatggt	agttcaad	ctaattatga	agatttaaaa	gatatggatt
5221	agcatctgta	gaagacatc	atataaagaa	agatgttttc	aagttactag
5281	taaagaagat	actatcttc	caacaaatac	ttcatcatta	tctataacag
5341	ttctactaag	cgcccagala	aagttatagg	aatgcatttc	tttaatccag
5401	gaaattagtt	gaagttatia	gtgggtcagtt	aacatcaaaa	gttacttttg
5461	tgaattatct	aagagtatc	ataaagtacc	agtagatgta	tctgaatctc
5521	agtaaataga	atacttati	ctatgataaa	tgaagctggt	ggatatatatg
5581	tgcaagtaaa	gaagaaati	atgaagctat	gaaattagga	gcaaaccatc
5641	actagcatta	ggtgatttia	tcggattaga	tgttgtttta	gctataatga
	acgtttttata				

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 69)					
5701	tactgaattt	ggagatacta	aatatagacc	tcatccactt	ttagctaaaa
5761	taatcaatta	ggaagaaaaa	ctaagatagg	attctatgat	tataataaat
5821	agatatacat	atgagtacaa	gtgatgttaa	agtttatgag	aatgtagctg
5881	tggaaatata	tgtacagtga	aaatgaatag	acctaaagcc	cttaatgcaa
5941	gacttttagaa	gaactttatg	aagtatttgt	agatattaat	aatgatgaaa
6001	tgtaatattg	acaggggaag	gaaaggcatt	tgtagctgga	gcagatattg
6061	agatttagat	gctgtagctg	ctaaagattt	tagtatctta	ggagcaaaaag
6121	aatagaaaat	agtaaaaaag	tagtgatagc	tgctgtaaac	ggatttgctt
6181	atgtgaactt	gcaatggcat	gtgatataag	aattgcatct	gctaaagcta
6241	gccagaagta	actcttgga	taactccagg	atatggagga	actcaaaggc
6301	ggttggaatg	gcaaaagcaa	aagaattaat	ctttacaggt	caagttataa
6361	agctgaaaaa	atagggctag	taaatagagt	cgttgagcca	gacattttta
6421	tgagaaatta	gctaagataa	tagctaaaaa	tgctcagctt	gcagttagat
6481	agcaatacaa	cttggtgctc	aaactgatat	aaatactgga	atagatatag
6541	atttggtctt	tgtttttcaa	ctaaagacca	aaaagaagga	atgtcagctt
6601	gagagaagct	aactttataa	aagggttaata	agaaggagat	atacatatga
6661	agaagtaatt	aagtttgcaa	aagaaagagg	acctaaaact	atatcagtag
6721	agataaagaa	gttttaaatg	cagttgaaat	ggctagaaaa	gaaaaaatag
6781	tttagtagga	gatatagaaa	agactaaaga	aattgcaaaa	agcatagaca
6841	aaattatgaa	ctgatagata	taaaagattt	agcagaagca	tctctaaaat
6901	agtttcacaa	ggaaaagccg	acatggtaat	gaaaggctta	gtagacacat
6961	aaaagcagtt	ttaaataaag	aagtaggtct	tagaactgga	aatgtattaa
7021	agtatttgat	gtagagggat	atgatagatt	atttttcgta	actgacgcag
7081	agctcctgat	acaataacta	aaaagcaa	catagaaaat	gcttgacacag
7141	attagatata	agtgaaccaa	aagttgctgc	aatatgcgca	aaagaaaaag
7201	aatgaaagat	acagttgaag	ctaaagaact	agaagaaatg	tatgaaagag
	gagaaatcaa				

Nucleotide sequences of pLogic031-tet-butyrat construct (SEQ ID NO: 69)					
7261	aggttgatg	gttggtgggc	cttttgcaat	tgataatgca	gtatctttag
aagcagctaa					
7321	acataaaggt	ataaatcatc	ctgtagcagg	acgagctgat	atattattag
ccccagatat					
7381	tgaaggtggt	aacatattat	ataaagcttt	ggtattcttc	tcaaaatcaa
aaaatgcagg					
7441	agttataglt	ggggctaaag	caccaataat	attaacttct	agagcagaca
gtgaagaaac					
7501	taaactaa;ac	tcaatagctt	taggtgtttt	aatggcagca	aaggcataat
aagaaggaga					
7561	tatacatal;g	agcaaaatat	ttaaaatctt	aacaataaat	cctggttcga
catcaactaa					
7621	aatagctgl;ta	tttgataatg	aggatttagt	atttgaaaaa	actttaagac
attcttcaga					
7681	agaaatagc;ja	aaatatgaga	aggtgtctga	ccaatttgaa	tttcgtaaac
aagtaataga					
7741	agaagctd ;ta	aaagaagggtg	gagtaaaaac	atctgaatta	gatgctgtag
taggtagagg					
7801	aggacttd ;t	aaacctataa	aagggtgtac	ttattcagta	agtgtgtcta
tgattgaaga					
7861	tttaaaagl;g	ggagtttttag	gagaacacgc	ttcaaacctta	ggtggaataa
tagcaaaaca					
7921	aataggtgi;aa	gaagtaaattg	ttccttcata	catagtagac	cctgtttgttg
tagatgaatt					
7981	agaagatgl;t	gctagaattt	ctggtatgcc	tgaaataagt	agagcaagtg
tagtacatgc					
8041	tttaaatci;aa	aaggcaatag	caagaagata	tgctagagaa	ataaacaaga
aatatgaaga					
8101	tataaatd ;t	atagttgcac	acatgggtgg	aggagtttct	gttggagctc
ataaaaatgg					
8161	taaaatagl;ta	gatgttgcaa	acgcattaga	tggagaagga	cctttctctc
cagaaagaag					
8221	tggtggacta	ccagtaggtg	cattagtaaa	aatgtgcttt	agtggaaaat
atactcaaga					
8281	tgaaattaaa	aagaaaataa	aaggtaatgg	cggactagtt	gcatacttaa
acactaatga					
8341	tgctagagaa	gttgaagaaa	gaattgaagc	tggtgatgaa	aaagctaaat
tagtatatga					
8401	agctatggca	tatcaaattct	ctaaagaaat	aggagctagt	gctgcagttc
ttaagggaga					
8461	tgtaaaagca	atattattaa	ctggtggaat	cgcattattca	aaaatgttta
cagaaatgat					
8521	tgcatagata	gttaaatttta	tagcagatgt	aaaagtttat	ccaggtgaag
atgaaatgat					
8581	tgcattagct	caaggtggac	ttagagtttt	aactggtgaa	gaagaggctc
aagtttatga					
8641	taactaataa				

Table 13

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 70)					
1	gtaaaacgac	ggccagtgaa	<u>ttcgttaaga</u>	<u>cccactttca</u>	<u>catttaagtt</u>
	<u>gtttttctaa</u>				
61	<u>tccgcatatg</u>	<u>atcaattcaa</u>	<u>ggccgaataa</u>	<u>gaaggctggc</u>	<u>tctgcacctt</u>
	<u>ggtgatcaaa</u>				
121	<u>taattcgata</u>	<u>gcttgtcgta</u>	<u>ataatggcgg</u>	<u>catactatca</u>	<u>gtagtaggtg</u>
	<u>tttccctttc</u>				
181	<u>ttcttttagcg</u>	<u>acttgatgct</u>	<u>cttgatcttc</u>	<u>caatacgcaa</u>	<u>cctaaagtaa</u>
	<u>aatgccccac</u>				
241	<u>agcgctgagt</u>	<u>gcatataatg</u>	<u>cattctctag</u>	<u>tgaaaaacct</u>	<u>tgttggcata</u>
	<u>aaaaggctaa</u>				
301	<u>ttgattttcg</u>	<u>agagtttcat</u>	<u>actgtttttc</u>	<u>tgtaggccgt</u>	<u>gtacctaaat</u>
	<u>gtacttttgc</u>				
361	<u>tccatcgcca</u>	<u>tgacttagta</u>	<u>aagcacatct</u>	<u>aaaactttta</u>	<u>gcgttattac</u>
	<u>gtaaaaaatc</u>				
421	<u>ttgccagctt</u>	<u>tccccttcta</u>	<u>aagggcaaaa</u>	<u>gtgagtatgg</u>	<u>tgccatatcta</u>
	<u>acatctcaat</u>				
481	<u>ggctaaggcg</u>	<u>tcgagcaaag</u>	<u>cccgttatt</u>	<u>ttttacatgc</u>	<u>caatacaatg</u>
	<u>taggctgctc</u>				
541	<u>tacacctagc</u>	<u>ttctgggcga</u>	<u>gtttacgggt</u>	<u>tgtaaacct</u>	<u>tcgattccga</u>
	<u>cctcattaag</u>				
601	<u>cagctctaata</u>	<u>gcgctgttaa</u>	<u>tcactttact</u>	<u>tttatctaata</u>	<u>ctagacatca</u>
	<u>ttaattccta</u>				
661	<u>atttttjgttg</u>	<u>acactctatc</u>	<u>attgatagag</u>	<u>ttattttacc</u>	<u>actccctatq</u>
	<u>agtgatagag</u>				
721	<u>aaaagtgaac</u>	<u>tctagaaata</u>	<u>attttgttta</u>	<u>actttaagaa</u>	<u>ggagatatac</u>
	<u>atatgatcgt</u>				
781	<u>aaaacctatg</u>	<u>gtacgcaaca</u>	<u>atatctgcct</u>	<u>gaacgcccac</u>	<u>cctcaggggt</u>
	<u>gcaagaaggg</u>				
841	<u>agtggaagat</u>	<u>cagattgaat</u>	<u>ataccaagaa</u>	<u>acgcattacc</u>	<u>gcagaagtca</u>
	<u>aagctggcgc</u>				
901	<u>aaaagctcca</u>	<u>aaaaacgttc</u>	<u>tggtgcttgg</u>	<u>ctgctcaaat</u>	<u>ggttacggcc</u>
	<u>tggcgagccg</u>				
961	<u>cattactgct</u>	<u>gcgttcggat</u>	<u>acggggctgc</u>	<u>gaccatcggc</u>	<u>gtgtcctttg</u>
	<u>aaaaagcggg</u>				
1021	<u>ttcagaaaacc</u>	<u>aaatatggta</u>	<u>caccgggatg</u>	<u>gtacaataat</u>	<u>ttggcatttg</u>
	<u>atgaagcggc</u>				
1081	<u>aaaacgcgag</u>	<u>ggtctttata</u>	<u>gcgtgacgat</u>	<u>cgacggcgat</u>	<u>gcgttttcag</u>
	<u>acgagatcaa</u>				
1141	<u>ggcccaggtg</u>	<u>attgaggaag</u>	<u>ccaaaaaaa</u>	<u>aggtatcaaa</u>	<u>tttgatctga</u>
	<u>tcgtatacag</u>				
1201	<u>cttggccagc</u>	<u>ccagtacgta</u>	<u>ctgatcctga</u>	<u>tacaggtatc</u>	<u>atgcacaaaa</u>
	<u>gcgttttgaa</u>				
1261	<u>cccttttgga</u>	<u>aaaacgttca</u>	<u>caggcaaaac</u>	<u>agtagatccg</u>	<u>tttactggcg</u>
	<u>agctgaagga</u>				
1321	<u>atctccgcg</u>	<u>gaaccagcaa</u>	<u>atgacgagga</u>	<u>agcagccgcc</u>	<u>actgttaaaag</u>
	<u>ttatgggggg</u>				
1381	<u>tgaagattgg</u>	<u>gaacgttgga</u>	<u>ttaagcagct</u>	<u>gtcgaaggaa</u>	<u>ggcctcttag</u>
	<u>aagaaggctg</u>				
1441	<u>tattaccttg</u>	<u>gcctatagtt</u>	<u>atattggccc</u>	<u>tgaagctacc</u>	<u>caagctttgt</u>
	<u>accgtaaagg</u>				
1501	<u>cacaatcggc</u>	<u>aaggccaaag</u>	<u>aacacctgga</u>	<u>ggccacagca</u>	<u>caccgtctca</u>
	<u>acaagagaaa</u>				
1561	<u>cccgctcaatc</u>	<u>cgtgccttcg</u>	<u>tgagcgtgaa</u>	<u>taaaggcctg</u>	<u>gtaacccgcg</u>
	<u>caagcgccgt</u>				
1621	<u>aatcccggta</u>	<u>atccctctgt</u>	<u>atctcgccag</u>	<u>cttgttcaaa</u>	<u>gtaatgaaaag</u>

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 70)					
agaagggcaa					
1681	tcatgaaggt	tgtattgaac	agatcacgcg	tctgtacgcc	gagcgcctgt
accgtaaaga					
1741	tggtacaatt	ccagttgatg	aggaaaaatcg	cattcgcatt	gatgattggg
agttagaaga					
1801	agacgtccag	aaagcgggat	ccgcgttgat	ggagaaagtc	acgggtgaaa
acgcagaatc					
1861	tctcactgac	ttagcggggg	accgccatga	tttcttagct	agtaacggct
ttgatgtaga					
1921	aggtattaat	tatgaagcgg	aagttgaacg	cttcgaccgt	atctgataag
aaggagatat					
1981	acatatgaga	gaagtagtaa	ttgccagtgc	agctagaaca	gcagtaggaa
gttttgagg					
2041	agcatttaaa	tcagtttcag	cggtagagtt	aggggtaaca	gcagctaaag
aagctataaa					
2101	aagagctaac	ataactccag	atatgataga	tgaatctctt	ttagggggag
tacttacagc					
2161	aggtcttgga	caaaatatag	caagacaaat	agcattagga	gcaggaatac
cagtagaaaa					
2221	accagctatg	actataaata	tagtttggtg	ttctggatta	agatctgttt
caatggcatc					
2281	tcaacttata	gcattaggtg	atgctgatat	aatgtagtt	ggtaggagctg
aaaacatgag					
2341	tatgtctcct	tatttagtac	caagtgcgag	atatggtgca	agaatgggtg
atgctgcttt					
2401	tggtgattca	atgataaaag	atggattatc	agacatattt	aataactatc
acatgggtat					
2461	tactgctgaa	aacatagcag	agcaatggaa	tataactaga	gaagaacaag
atgaattagc					
2521	tcttgcaagt	caaaataaag	ctgaaaaagc	tcaagctgaa	ggaaaatttg
atgaagaaat					
2581	agttcctggt	gttataaaag	gaagaaaagg	tgacactgta	gtagataaag
atgaatatat					
2641	taagcctggc	actacaatgg	agaaacttgc	taagttaaga	cctgcattta
aaaaagatgg					
2701	aacagttact	gctggtaatg	catcaggaat	aaatgatggt	gctgctatgt
tagtagtaat					
2761	ggctaaagaa	aaagctgaag	aactaggaat	agagcctctt	gcaactatag
tttcttatgg					
2821	aacagctggg	gttgacccta	aaataatggg	atatggacca	gttcagcaa
ctaaaaaagc					
2881	tttagaagct	gctaatatga	ctattgaaga	tatagattta	gttgaagcta
atgaggcatt					
2941	tgctgcccaa	tctgtagctg	taataagaga	cttaaataata	gatatgaata
aagttaatgt					
3001	taatggtgga	gcaatagcta	taggacatcc	aataggatgc	tcaggagcaa
gaatacttac					
3061	tacactttta	tatgaaatga	agagaagaga	tgctaaaact	ggctcttgcta
cactttgtat					
3121	aggcgggtgga	atgggaacta	ctttaatagt	taagagatag	taagaaggag
atatacatat					
3181	gaaattagct	gtaataggta	gtggaactat	gggaagtggg	attgtacaaa
cttttgcaag					
3241	ttgtggacat	gatgtatggt	taaagagtag	aactcaaggt	gctatagata
aatgttttagc					
3301	tttattagat	aaaaatttaa	ctaagttagt	tactaaggga	aaaatggatg
aagctacaaa					

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 70)					
3361	agcagaaata	ttaagtcattg	ttagttcaac	tactaattat	gaagatttaa
aagatatgga					
3421	tttaataata	gaagcatctg	tagaagacat	gaatataaag	aaagatgttt
tcaagttact					
3481	agatgaatta	tgtaaagaag	atactatctt	ggcaacaaat	acttcatcat
tatctataac					
3541	agaaatagct	tcttctacta	agcgcccaga	taaagttata	ggaatgcatt
tctttaatcc					
3601	agttcctatg	atgaaattag	ttgaagttat	aagtggtcag	ttaacatcaa
aagttacttt					
3661	tgatacagta	tttgaattat	ctaagagtat	caataaagta	ccagtagatg
tatctgaatc					
3721	tcctggattt	gtagtaaata	gaatacttat	acctatgata	aatgaagctg
ttggtatata					
3781	tgcataggt	gttgcaagta	aagaagaaat	agatgaagct	atgaaattag
gagcaaacca					
3841	tccaatggga	ccactagcat	taggtgattt	aatcggatta	gatgttgttt
tagctataat					
3901	gaacgtttta	tatactgaat	ttggagatac	taaatataga	cctcatccac
ttttagctaa					
3961	aatggttaga	gctaataaat	taggaagaaa	aactaagata	ggattctatg
attataataa					
4021	ataataagaa	ggagatatac	atatgagtac	aagtgatgtt	aaagtttatg
agaatgtagc					
4081	tggtgaagta	gatggaaata	tatgtacagt	gaaaatgaat	agacctaaag
cccttaatgc					
4141	aataaattca	aagacttttag	aagaacttta	tgaagtattt	gtagatatta
ataatgatga					
4201	aactattgat	gttgtaatat	tgacagggga	aggaaaggca	ttttagctg
gagcagatat					
4261	tgcatacatg	aaagatttag	atgctgtagc	tgctaaagat	tttagtatct
taggagcaaa					
4321	agcttttgga	gaaatagaaa	atagtaaaaa	agtagtgata	gctgctgtaa
acggatttgc					
4381	tttaggtgga	ggatgtgaac	ttgcaatggc	atgtgatata	agaattgcat
ctgctaaagc					
4441	taaatttggt	cagccagaag	taactcttgg	aataactcca	ggatatggag
gaactcaaag					
4501	gcttacaaga	ttggttgga	tggaacaaagc	aaaagaatta	atctttacag
gtcaagttat					
4561	aaaagctgat	gaagctgaaa	aaatagggct	agtaaataga	gtcgttgagc
cagacatttt					
4621	aatagaagaa	gttgagaaat	tagctaagat	aatagctaaa	aatgctcagc
ttgcagtttag					
4681	atactctaaa	gaagcaatac	aacttggtgc	tcaaactgat	ataaatactg
gaatagatat					
4741	agaatctaata	ttatttggtc	tttgtttttc	aactaaagac	caaaaagaag
gaatgtcagc					
4801	tttcgttgaa	aagagagaag	ctaactttat	aaaagggtaa	taagaaggag
atatacatat					
4861	gagaagtttt	gaagaagtaa	ttaagtttgc	aaaagaaaga	ggacctaaaa
ctatatcagt					
4921	agcatgttgc	caagataaag	aagttttaat	ggcagttgaa	atggctagaa
aagaaaaaat					
4981	agcaaatgcc	atttttagtag	gagatataga	aaagactaaa	gaaattgcaa
aaagcataga					
5041	catggatatc	gaaaattatg	aactgataga	tataaaagat	ttagcagaag

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 70)					
catctctaaa					
5101	atctgttgaa	ttagtttcac	aaggaaaagc	cgacatggta	atgaaaggct
tagtagacac					
5161	atcaataata	ctaaaagcag	ttttaataaa	agaagtaggt	cttagaactg
gaaatgtatt					
5221	aagtcacgta	gcagtatttg	atgtagaggg	atatgataga	ttatTTTTcg
taactgacgc					
5281	agctatgaac	ttagctcctg	atacaaatac	taaaaagcaa	atcatagaaa
atgcttgacac					
5341	agtagcacat	tcattagata	taagtgaacc	aaaagttgct	gcaatatgcg
caaaagaaaa					
5401	agtaaatacca	aaaatgaaag	atacagttga	agctaaagaa	ctagaagaaa
tgtatgaaag					
5461	aggagaaatc	aaaggttgta	tgggttggtg	gccttttgca	attgataatg
cagtatcttt					
5521	agaagcagct	aaacataaag	gtataaatca	tcctgtagca	ggacgagctg
atatattatt					
5581	agccccagat	attgaagggtg	gtaacatatt	atataaagct	ttggtattct
tctcaaaatc					
5641	aaaaaatgca	ggagttatag	ttggggctaa	agcaccaata	atattaactt
ctagagcaga					
5701	cagtgaagaa	actaaactaa	actcaatagc	tttaggtggt	ttaatggcag
caaaggcata					
5761	ataagaagga	gatatacata	tgagcaaaat	atttaaaatc	ttaacaataa
atcctgggttc					
5821	gacatcaact	aaaatagctg	tatttgataa	tgaggattta	gtatttgaaa
aaactttaag					
5881	acattcttca	gaagaaatag	gaaaatatga	gaaggtgtct	gaccaatttg
aatttcgtaa					
5941	acaagtaata	gaagaagctc	taaaagaagg	tggagtaaaa	acatctgaat
tagatgctgt					
6001	agtaggtaga	ggaggacttc	ttaaacctat	aaaagggtgt	acttattcag
taagtgtctgc					
6061	tatgattgaa	gatttaaaag	tgggagtttt	aggagaacac	gcttcaaacc
taggtggaat					
6121	aatagcaaaa	caaatagggtg	aagaagtaaa	tgttccttca	tacatagtag
accctgttgt					
6181	tgtagatgaa	ttagaagatg	ttgctagaat	ttctggtatg	cctgaaataa
gtagagcaag					
6241	tgtagtacat	gctttaaatc	aaaaggcaat	agcaagaaga	tatgctagag
aaataaacia					
6301	gaaatatgaa	gatataaatc	ttatagttgc	acacatgggt	ggaggagttt
ctgttgagac					
6361	tcataaaaaat	ggtaaaatag	tagatgttgc	aaacgcatta	gatggagaag
gacctttctc					
6421	tccagaaaga	agtgggtggac	taccagtagg	tgcattagta	aaaatgtgct
ttagtggaag					
6481	atatactcaa	gatgaaatta	aaaagaaaat	aaaaggtaat	ggcggactag
ttgcatactt					
6541	aaacactaat	gatgctagag	aagttgaaga	aagaattgaa	gctggtgatg
aaaaagctaa					
6601	attagtatat	gaagctatgg	catatcaaat	ctctaaagaa	ataggagcta
gtgctgcagt					
6661	tcttaaggga	gatgtaaaag	caatattatt	aactggtgga	atcgcatatt
caaaaatggt					
6721	tacagaaatg	attgcagata	gagttaaatt	tatagcagat	gtaaaagttt
atccagggtga					

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 70)					
6781	agatgaaatg	attgcattag	ctcaaggtgg	acttagagtt	ttaactggtg
aagaagaggc					
6841	tcaagtttat	gataactaat	aa		

[0195] In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operably linked to a promoter that is induced by RNS. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present in the chromosome and operably linked to a promoter that is induced by RNS. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a chromosome and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability.

[0196] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule, such that the gene(s) or gene cassette(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, a bacterium may comprise multiple copies of the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhance molecule. In some embodiments, gene or gene cassette 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 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 is expressed on a chromosome.

[0197] In some embodiments, the genetically engineered bacteria may comprise multiple copies of the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule. In some embodiments, the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operatively linked to a RNS-responsive regulatory region. In some embodiments, the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule is present in a chromosome and operatively linked to a RNS-responsive regulatory region.

[0198] In some embodiments, any of the gene(s) or gene cassette(s) of the present disclosure may be integrated into the bacterial chromosome at one or more integration sites. For example, one or more copies of the butyrogenic gene cassette may be integrated into the bacterial chromosome. Having multiple copies of the butyrogenic gene cassette integrated into the chromosome allows for greater production of the butyrate and also permits fine-tuning of the level of expression. Alternatively, different circuits described herein, such as any of the kill-switch circuits, in addition to the therapeutic gene(s) or gene cassette(s) could be integrated into the bacterial chromosome at one or more different integration sites to perform multiple different functions.

[0199] In some embodiments, the genetically engineered bacteria of the invention produce at least one anti-inflammation and/or gut barrier enhancer molecule 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 (MesoScience, Cayman Chemical, Qiagen).

[0200] 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 an anti-inflammation and/or gut barrier enhancer molecule 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 anti-inflammation and/or gut barrier enhancer molecule. In embodiments using genetically modified forms of these bacteria, the anti-inflammation and/or gut barrier enhancer molecule will be detectable in the presence of RNS.

[0201] In certain 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 μ M/OD, at least about 10

$\mu\text{M}/\text{OD}$, at least about 100 $\mu\text{M}/\text{OD}$, at least about 500 $\mu\text{M}/\text{OD}$, 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 RNS.

ROS-dependent regulation

[0202] 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 oxygen species. The tunable regulatory region is operatively linked to a gene or gene cassette capable of directly or indirectly driving the expression of an anti-inflammation and/or gut barrier function enhancer molecule, thus controlling expression of the molecule relative to ROS levels. For example, the tunable regulatory region is a ROS-inducible regulatory region, and the molecule is butyrate; 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 butyrogenic gene cassette, thereby producing butyrate, which exerts anti-inflammation and/or gut barrier enhancing effects. Subsequently, when inflammation is ameliorated, ROS levels are reduced, and butyrate production is decreased or eliminated.

[0203] 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.

[0204] 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 H_2O_2 detoxification (*katE*, *ohpCF*), 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 of the invention 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; Table 1). 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 or gene cassette, e.g., a butyrogenic gene cassette. In the presence of ROS, e.g., H_2O_2 , an OxyR transcription factor senses ROS and activates the *oxyS* regulatory region, thereby driving expression of the operatively linked butyrogenic gene cassette and producing butyrate. 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*.

[0205] 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 H_2O_2 . 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; Table 1). 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 or gene cassette, e.g., a butyrogenic gene cassette. In the presence of ROS, the SoxR transcription factor senses ROS and activates the *soxS* regulatory region, thereby driving expression of the operatively linked butyrogenic gene cassette and producing butyrate.

[0206] 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.

[0207] 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; Table 1). 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 butyrogenic gene cassette. 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 butyrogenic gene cassette and producing butyrate.

[0208] 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).

[0209] 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 H_2O_2 " (Bussmann et al., 2010). RosR is capable of repressing numerous genes and putative genes, including but not limited to "a putative polyisoprenoid-binding protein (cgl322, 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 (cgl426), two putative FMN reductases (cgl150 and cgl850), and four putative monooxygenases (cg0823, cgl848, 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; Table 1). 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 butyrogenic gene cassette. In the presence of ROS, e.g., H_2O_2 , a RosR transcription factor senses ROS and no longer binds to the *cgtS9* regulatory region, thereby derepressing the operatively linked butyrogenic gene cassette and producing butyrate.

[0210] 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.

[0211] 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.

[0212] 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 *zooA*) 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; Table 1).

[0213] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express an anti-inflammation and/or gut barrier function enhancer molecule. The two repressor activation regulatory circuit comprises a first ROS-sensing repressor, e.g., PerR, and a

second repressor, e.g., TetR, which is operatively linked to a gene or gene cassette, e.g., a butyrogenic gene cassette. 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, Cl, 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 butyrogenic gene cassette. 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 butyrogenic gene cassette. 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 butyrogenic gene cassette, is expressed.

[0214] 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; Table 1). 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.

[0215] 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.

[0216] Nucleic acid sequences of several exemplary OxyR-regulated regulatory regions are shown in **Table 14**. 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: 71, 72, 73, or 74, or a functional fragment thereof.

Table 14: Nucleotide sequences of exemplary OxyR-regulated regulatory regions

Regulatory sequence	01234567890123456789012345678901234567890123456789

Regulatory sequence	01234567890123456789012345678901234567890123456789
<i>kotG</i> (SEQ ID NO: 71)	TGTGGCTTTTATGAAAATCACACAGTGATCACAAATTTTAAACAGAGCAC AAAATGCTGCCTCGAAATGAGGGCGGGAAAATAAGGTATCAGCCTTGT TTCTCCCTCATTACTTGAAGGATATGAAGCTAAACCTTTTTTATAAAG CATTTGTCCGAATTCGGACATAATCAAAAAAGCTTAATTAAGATCAATTT GATCTACATCTCTTTAACCAACAATATGTAAGATCTCAACTATCGCATCC <u>GTGGATTAATTCAATTATAACTTCTCTCTAACGCTGTGTATCGTAACGGT</u> AACACTGTAGAGGGGAGCACATTTGATGCGAATTCATTAAAGAGGAGAAAG GTACC
<i>dps</i> (SEQ ID NO: 72)	TTCCGAAAATTCCTGGCGAGCAGATAAATAAGAATTGTTCTTATCAATAT ATCTAACTCATTGAATCTTTATAGTTTTGTTTTTCACGCTTGTTACCAC <u>TATTAGTGTGATAGGAACAGCCAGAATAGCGGAACACATAGCCGGTGCTA</u> <u>TACTTAATCTCGTTAATTACTGGGACATAACATCAAGAGGATATGAAATT</u> CGAATTCATTAAAGAGGAGAAAGGTACC
<i>ahpC</i> (SEQ ID NO: 73)	GCTTAGATCAGGTGATTGCCCTTTGTTTATGAGGGTGTGTAATCCATGT CGTTGTTGCATTTGTAAGGGCAACACCTCAGCCTGCAGGCAGGCACTGAA GATACCAAAGGGTAGTTTACAGATTACACGGTCACTGGAAAGGGGGCCATT TTACTTTTATCGCCGCTGGCGGTGCAAAGTTACAAAGTTGTCTTACGA AGGTTGTAAGGTAAAACTTATCGATTGATAATGGAAACGCATTAGCCGA <u>ATCGGCAAAAATGGTTACCTTACATCTCATCGAAAACACGGAGGAAGTA</u> TAGATGCGAATTCATTAAAGAGGAGAAAGGTACC
<i>oxyS</i> (SEQ ID NO: 74)	CTCGAGTTCATTATCCATCCTCCATCGCCACGATAGTTTCATGGCGATAGG <u>TAGAATAGCAATGAACGATTATCCCTATCAAGCATTCTGACTGATAATTG</u> <u>CTCACACGAATTCATTAAAGAGGAGAAAGGTACC</u>

[0217] 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.

[0218] 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.

[0219] 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.

[0220] 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.

[0221] 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 anti-inflammation and/or gut barrier enhancer molecule 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 anti-inflammation and/or gut barrier enhancer molecule 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 anti-inflammation and/or gut barrier enhancer molecule in the presence of ROS.

[0222] Nucleic acid sequences of exemplary ROS-regulated constructs comprising an *oxyS* promoter are shown in **Table 15** and **Table 16**. The nucleic acid sequence of an exemplary construct encoding OxyR is shown in **Table 17**. Nucleic acid sequences of tetracycline-regulated constructs comprising a *tet* promoter are shown in **Table 18** and **Table 19**. **Table 15** 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: 75). **Table 16** 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: 76). **Table 17** depicts the nucleic acid sequence of an exemplary construct encoding OxyR (pZA22-*oxyR* construct; SEQ ID NO: 77). **Table 18** 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 19 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.

Table 15

Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 75)					
1	ctcgagttca	ttatccatcc	tccatcgcca	cgatagttca	tggcgatagg
tagaatagca					
61	atgaacgatt	atccctatca	agcattctga	ctgataattg	ctcacacgaa
ttcattaaag					
121	aggagaaagg	taccatggat	ttaaattcta	aaaaatatca	gatgcttaaa
gagctatatg					
181	taagcttcgc	tgaaaatgaa	gttaaaccct	tagcaacaga	acttgatgaa
gaagaaagat					
241	ttccttatga	aacagtggaa	aaaatggcaa	aagcaggaat	gatgggtata
ccatatccaa					
301	<u>gaagaat</u> atgg	tggaagaagg	ggagacactg	taggatatat	aatggcagtt
gaagaattgt					
361	ctagagtttg	tggtactaca	ggagttatat	tatcagctca	tacatctctt
ggctcatggc					
421	ctatatatca	atatggtaat	gaagaacaaa	aacaaaaatt	cttaagacca
ctagcaagtg					
481	gagaaaaatt	aggagcattt	ggtcttactg	agcctaatgc	tggtacagat
gcgtctggcc					
541	<u>gacaaacaac</u>	tgctgtttta	gacggggatg	aatacatact	taatggctca
aaaatatatta					
601	taacaaacgc	aatagctggg	gacatatatg	tagtaatggc	aatgactgat
aaatctaagg					
661	ggaacaaagg	aatatcagca	tttatagttg	aaaaaggaac	tcctgggttt
agctttggag					
721	ttaaagaaaa	gaaaatgggt	ataagagggt	cagctacgag	tgaattaata
tttgaggatt					
781	gcagaatacc	taaagaaaat	ttacttggaa	aagaagggtca	aggatttaag
atagcaatgt					
841	ctactcttga	tggtggtaga	attgggtatag	ctgcacaagc	tttaggttta
gcacaagggtg					
901	ctcttgatga	aactgttaaa	tatgtaaaag	aaagagtaca	atttggtaga
ccattatcaa					
961	<u>gattccaaaa</u>	tacacaattc	caattagctg	atatggaagt	taagggtacaa
gcggctagac					
1021	<u>gaccttg</u> tata	tcaagcagct	ataaataaag	acttaggaaa	accttatgga
gtagaagcag					
1081	caatggcaaa	attatttgca	gctgaaacag	ctatggaagt	tactacaaaa
gctgtacaac					
1141	ttcatggagg	atatggatac	actcgtgact	atccagtaga	aagaatgatg
agagatgcta					
1201	<u>gataactga</u>	aatatatgaa	ggaactagtg	aagttcaaag	aatgggttatt
tcaggaaaaac					

Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 75)					
1261	tattaaaata	gtaagaagga	gatatacata	tggaggaagg	atttatgaat
atagtcgttt	1321	gtataaaaca	agttccagat	acaacagaag	ttaaactaga
ggtagctttta	1381	ttagagatgg	agtaccaagt	ataataaacc	ctgatgataa
gaagaagcta	1441	taaaattaaa	agaagaaatg	ggtagctcatg	taactgttat
cctcctcaag	1501	cagatatggc	tttaaaagaa	gcttttagcaa	tgggtgcaga
ttattaacag	1561	atagagcatt	tgcgggtgct	gatacttggg	caacttcatc
ggagcattaa	1621	aaaatataga	ttttgatatt	ataatagctg	gaagacaggc
gatactgcac	1681	aagttggacc	tcaaatagct	gaacatttaa	atcttccatc
gctgaagaaa	1741	taaaaactga	aggtgaatat	gtattagtaa	aaagacaatt
tgccatgact	1801	taaaagttaa	aatgccatgc	cttataacaa	ctcttaaaga
ccaagataca	1861	tgaaagttag	aagaatatat	gatgctttcg	aaaatgatgt
tgtagctgtaa	1921	aagatataga	agttgaccct	tctaatttag	gtcttaaagg
agtgtagttta	1981	aatcatttac	aaaatcagtt	aaaccagctg	gtacaatata
gcgaaaacat	2041	cagctggaat	tatcatagat	aaattaaaag	agaagtatat
gaaggagata	2101	tacatatggg	taacgtttta	gtagtaatag	aacaaagaga
caaactgttt	2161	ctttagaatt	actaggaaag	gctacagaaa	tagcaaaaaga
aaagtttctg	2221	cattactttt	aggtagtaag	gtagaagggt	taatagatac
tatggtgcag	2281	atgaggtaat	agtagtagat	gatgaagctt	tagcagtgtg
ccatatacaa	2341	aagcagctta	tgaagcaata	aaagcagctg	accctatagt
ggtgcaactt	2401	caataggtag	agatttagcg	cctagagttt	ctgctagaat
cttactgctg	2461	actgtacagg	tcttgtagta	gctgaagata	caaaattatt
agacctgcct	2521	ttggtggaaa	tataatggca	acaatagttt	gtaaagattt
atgtctacag	2581	ttagaccagg	ggttatgaag	aaaaatgaac	ctgatgaaac
gtaattaacc	2641	gtttcaagg	agaattta	gatgctgata	aattagttca
gtaataaaaag	2701	aagctaaaaa	acaagttaaa	atagaagatg	ctaagatatt
ggacgtggaa	2761	tgggtggaaa	agaaaactta	gacatacttt	atgaattagc
ggtggagaag	2821	tttctgggtc	tcgtgccact	atagatgcag	gttggttaga
caagttgggtc	2881	aaactggtaa	aactgtaaga	ccagaccttt	atatagcatg
ggagcaatac	2941	aacatatagc	tggtatggaa	gatgctgagt	ttatagttgc
					tataaataaa

Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 75)					
aatccagaag					
3001	ctccaatatt	taaatatgct	gatgttggtta	tagttggaga	tgttcataaa
gtgcttccag					
3061	aacttatcag	tcagttaagt	gttgcaaaag	aaaaaggtga	agtttttagct
aactaataag					
3121	aaggagatat	acatatgaga	gaagtagtaa	ttgccagtgc	agctagaaca
gcagtaggaa					
3181	gttttgagg	agcattttaa	tcagtttcag	cggtagagtt	aggggtaaca
gcagctaaag					
3241	aagctataaa	aagagctaac	ataactccag	atatgataga	tgaatctctt
ttagggggag					
3301	tacttacagc	aggtcttgga	caaaatatag	caagacaaat	agcattagga
gcaggaatac					
3361	cagtagaaaa	accagctatg	actataaata	tagtttgtgg	ttctggatta
agatctgttt					
3421	caatggcatc	tcaacttata	gcattaggtg	atgctgatat	aatgttagtt
ggtggagctg					
3481	aaaacatgag	tatgtctcct	tatttagtac	caagtgcgag	atatggtgca
agaatgggtg					
3541	atgctgcttt	tgttgattca	atgataaaag	atggattatc	agacatattt
aataactatc					
3601	acatgggtat	tactgctgaa	aacatagcag	agcaatggaa	tataactaga
gaagaacaag					
3661	atgaattagc	tcttgcaagt	caaaataaag	ctgaaaaagc	tcaagctgaa
ggaaaatttg					
3721	atgaagaaat	agttcctggt	gttataaaag	gaagaaaagg	tgacactgta
gtagataaag					
3781	atgaatatat	taagcctggc	actacaatgg	agaaacttgc	taagttaaga
cctgcattta					
3841	aaaaagatgg	aacagttact	gctggtaatg	catcaggaat	aaatgatggt
gctgctatgt					
3901	tagtagtaat	ggctaaagaa	aaagctgaag	aactaggaat	agagcctctt
gcaactatag					
3961	tttcttatgg	aacagctggt	gttgacccta	aaataatggg	atatggacca
gttccagcaa					
4021	ctaaaaaagc	tttagaagct	gctaatatga	ctattgaaga	tatagattta
gttgaagcta					
4081	atgaggcatt	tgctgcccaa	tctgtagctg	taataagaga	cttaaatata
gatatgaata					
4141	aagttaatgt	taatggtgga	gcaatagcta	taggacatcc	aataggatgc
tcaggagcaa					
4201	gaatacttac	tacactttta	tatgaaatga	agagaagaga	tgctaaaact
ggtcttgcta					
4261	cactttgtat	aggcggtgga	atgggaacta	ctttaatagt	taagagatag
taagaaggag					
4321	atatacatat	gaaattagct	gtaataggta	gtggaactat	gggaagtgggt
attgtacaaa					
4381	cttttgcaag	ttgtggacat	gatgtatggt	taaagagtag	aactcaagggt
gctatagata					
4441	aatgttttagc	tttatttagat	aaaaatttaa	ctaagttagt	tactaaggga
aaaatggatg					
4501	aagctacaaa	agcagaaata	ttaagtcatg	ttagttcaac	tactaattat
gaagatttaa					
4561	aagatatgga	tttaataata	gaagcatctg	tagaagacat	gaatataaag
aaagatgttt					
4621	tcaagttact	agatgaatta	tgtaaagaag	atactatctt	ggcaacaaat
acttcatcat					

Nucleotide sequences of pLogic031-oxyS-butyrate construct (SEQ ID NO: 75)					
4681	tatctataac	agaaatagct	tcttctacta	agcgcccaga	taaagttata
ggaatgcatt					
4741	tctttaatcc	agttcctatg	atgaaattag	ttgaagttat	aagtggtcag
ttaacatcaa					
4801	aagttacttt	tgatacagta	tttgaattat	ctaagagtat	caataaagta
ccagtagatg					
4861	tatctgaatc	tcctggattt	gtagtaaata	gaatacttat	acctatgata
aatgaagctg					
4921	ttggtatata	tgacagatgg	gttgcaagta	aagaagaaat	agatgaagct
atgaaattag					
4981	gagcaaacca	tccaatggga	ccactagcat	taggtgattt	aatcggatta
gatgttgttt					
5041	tagctataat	gaacgtttta	tatactgaat	ttggagatac	taaatataga
cctcatccac					
5101	ttttagctaa	aatggttaga	gctaataaat	taggaagaaa	aactaagata
ggattctatg					
5161	attataataa	ataataagaa	ggagatatatac	atatgagtac	aagtgatgtt
aaagtttatg					
5221	agaatgtagc	tggtgaagta	gatggaaata	tatgtacagt	gaaaatgaat
agacctaaag					
5281	cccttaatgc	aataaattca	aagacttttag	aagaacttta	tgaagtattt
gtagatatta					
5341	ataatgatga	aactattgat	gttgtaatat	tgacagggga	aggaaaggca
ttttagctg					
5401	gagcagatat	tgacatacatg	aaagatttag	atgctgtagc	tgctaaagat
tttagtatct					
5461	taggagcaaa	agctttttgga	gaaatagaaa	atagtaaaaa	agtagtgata
gctgctgtaa					
5521	acggatttgc	tttaggtgga	ggatgtgaac	ttgcaatggc	atgtgatata
agaattgcat					
5581	ctgctaaagc	taaatttggg	cagccagaag	taactcttgg	aataactcca
ggatatggag					
5641	gaactcaaag	gcttacaaga	ttggttgga	tggaacaaagc	aaaagaatta
atctttacag					
5701	gtcaagttat	aaaagctgat	gaagctgaaa	aaatagggtc	agtaaataga
gtcgttgagc					
5761	cagacatttt	aatagaagaa	gttgagaaat	tagctaagat	aatagctaaa
aatgctcagc					
5821	ttgcagttag	atactctaaa	gaagcaatac	aacttggtgc	tcaaactgat
ataaatactg					
5881	gaatagatat	agaatctaata	ttatttgggc	tttgtttttc	aactaaagac
caaaaagaag					
5941	gaatgtcagc	tttcgttgaa	aagagagaag	ctaactttat	aaaagggtaa
taagaaggag					
6001	atatacatat	gagaagtttt	gaagaagtaa	ttaagtttgc	aaaagaaaga
ggacctaaaa					
6061	ctatatcagt	agcatgttgc	caagataaag	aagttttaat	ggcagttgaa
atggctagaa					
6121	aagaaaaaat	agcaaagtc	atttttagtag	gagatataga	aaagactaaa
gaaattgcaa					
6181	aaagcataga	catggatatac	gaaaattatg	aactgataga	tataaaagat
ttagcagaag					
6241	catctctaaa	atctgttgaa	ttagtttcac	aaggaaaagc	cgacatggta
atgaaaggct					
6301	tagtagacac	atcaataata	ctaaaagcag	ttttaataa	agaagtaggt
cttagaactg					
6361	gaaatgtatt	aagtcacgta	gcagtatttg	atgtagaggg	atatgataga

Nucleotide sequences of pLogic031-oxyS-butyrat construct (SEQ ID NO: 75)					
ttatttttcg					
6421 taactgacgc	agctatgaac	ttagctcctg	atacaaatac	taaaaagcaa	
atcatagaaa					
6481 atgcttgac	agtagcacat	tcattagata	taagtgaacc	aaaagttgct	
gcaatatg					
6541 caaaagaaaa	agtaaatacca	aaaatgaaag	atacagttga	agctaaagaa	
ctagaagaaa					
6601 tgtatgaaag	aggagaaatc	aaaggttgta	tggttggtgg	gccttttgca	
attgataatg					
6661 cagtatcttt	agaagcagct	aaacataaag	gtataaatca	tcctgtagca	
ggacgagctg					
6721 atatattatt	agccccagat	attgaagggtg	gtaacatatt	atataaagct	
ttggtattct					
6781 tctcaaaatc	aaaaaatgca	ggagttatag	ttggggctaa	agcaccaata	
atattaactt					
6841 ctagagcaga	cagtgaagaa	actaaactaa	actcaatagc	tttaggtgtt	
ttaatggcag					
6901 caaaggcata	ataagaagga	gatatacata	tgagcaaaat	atttaaaatc	
ttaacaataa					
6961 atcctgggtc	gacatcaact	aaaatagctg	tatttgataa	tgaggattta	
gtatttgaaa					
7021 aaactttaag	acattcttca	gaagaaatag	gaaaatatga	gaagggtgtct	
gaccaatttg					
7081 aatttcgtaa	acaagtaata	gaagaagctc	taaaagaagg	tggagtaaaa	
acatctgaat					
7141 tagatgctgt	agtaggtaga	ggaggacttc	ttaaacctat	aaaagggtggt	
acttattcag					
7201 taagtgctgc	tatgattgaa	gatttaaaag	tgggagtttt	aggagaacac	
gcttcaaacc					
7261 taggtggaat	aatagcaaaa	caaataagggtg	aagaagtaaa	tggtccttca	
tacatagtag					
7321 accctgttgt	tgtagatgaa	ttagaagatg	ttgctagaat	ttctggtatg	
cctgaaataa					
7381 gtagagcaag	tgtagtacat	gctttaaatc	aaaaggcaat	agcaagaaga	
tatgctagag					
7441 aaataaacia	gaaatatgaa	gatataaatc	ttatagttgc	acacatgggt	
ggaggagttt					
7501 ctgttgagc	tcataaaaat	ggtaaaatag	tagatgttgc	aaacgcatta	
gatggagaag					
7561 gacctttctc	tccagaaaga	agtgggtggac	taccagtagg	tgcattagta	
aaaatgtgct					
7621 ttagtgga	atataactca	gatgaaatta	aaaagaaaat	aaaaggtaat	
ggcggactag					
7681 ttgcatactt	aaacactaat	gatgctagag	aagttgaaga	aagaattgaa	
gctggtgatg					
7741 aaaaagctaa	attagtatat	gaagctatgg	catatcaaat	ctctaaagaa	
ataggagcta					
7801 gtgctgcagt	tcttaaggga	gatgtaaaag	caatattatt	aactggtgga	
atcgcatatt					
7861 caaaaatgtt	tacagaaatg	attgcagata	gagttaaatt	tatagcagat	
gtaaaagttt					
7921 atccaggtga	agatgaaatg	attgcattag	ctcaagggtg	acttagagtt	
ttaactgggtg					
7981 aagaagaggc	tcaagtttat	gataactaat	aa		

Table 16

Nucleotide sequences of pLogic046-oxyS-butyrate construct (SEQ ID NO: 76)					
1	ctcgagttca	ttatccatcc	tccatcgcca	cgatagttca	tggcgatagg
tagaatagca					
61	atgaacgatt	atccctatca	agcattctga	ctgataattg	ctcacacgaa
ttcattaaag					
121	aggagaaagg	taccatgata	gtaaaacctt	tggtagcga	caatatctgc
ctgaacgccc					
181	atcctcaggg	ctgcaagaag	ggagtggga	atcagattga	atataccaag
aaacgcatta					
241	ccgcagaagt	caaagctggc	gcaaaagctc	caaaaaacgt	tctggtgctt
ggctgctcaa					
301	atgggttacg	cctggcgagc	cgcattactg	ctgcgttcgg	atacggggct
gcgaccatcg					
361	gcgtgtcctt	tgaaaaagcg	ggttcagaaa	ccaaatatgg	tacaccggga
tgggtacaata					
421	atgttgcatc	tgatgaagcg	gcaaaacgcg	agggctctta	tagcgtgacg
atcgacggcg					
481	atgcgttttc	agacgagatc	aaggcccagg	taattgagga	agccaaaaaa
aaaggtatca					
541	aatttgatct	gatcgtatac	agcttggcca	gcccagtagc	tactgatcct
gatacaggta					
601	tcatgcacaa	aagcgttttg	aaaccctttg	gaaaaacggt	cacaggcaaa
acagtagatc					
661	cgtttactgg	cgagctgaag	gaaatctccg	cggaaccagc	aatgacgag
gaagcagccg					
721	ccactgttaa	agttatgggg	ggatgaagatt	gggaacggtg	gattaagcag
ctgtcgaagg					
781	aaggcctctt	agaagaaggc	tgtattacct	tggcctatag	ttatatgggc
cctgaagcta					
841	ccaagcttt	gtaccgtaaa	ggcacaatcg	gcaaggccaa	agaacacctg
gaggccacag					
901	cacaccgtct	caacaaagag	aaccctgcaa	tccgtgcctt	cgtgagcgtg
aataaaggcc					
961	tggtaacccg	cgcaagcgcc	gtaatcccgg	taatccctct	gtatctcgcc
agcttggtca					
1021	aagtaatgaa	agagaagggc	aatcatgaag	gttgatttga	acagatcacg
cgtctgtacg					
1081	ccgagcgcct	gtaccgtaaa	gatggtacaa	ttccagttga	tgaggaaaat
cgcattcgca					
1141	ttgatgattg	ggagttagaa	gaagacgtcc	agaaagcggg	atccgcgttg
atggagaaag					
1201	tcacgggtga	aaacgcagaa	tctctcactg	acttagcggg	gtaccgccat
gatttcttag					
1261	ctagtaacgg	ctttgatgta	gaaggtatta	attatgaagc	ggaagttgaa
cgcttcgacc					
1321	gtatctgata	agaaggagat	atacatatga	gagaagtagt	aattgccagt
gcagctagaa					
1381	cagcagtagg	aagttttgga	ggagcattta	aatcagtttc	agcggtagag
ttaggggtaa					
1441	cagcagctaa	agaagctata	aaaagagcta	acataactcc	agatatgata
gatgaatctc					
1501	ttttaggggg	agtacttaca	gcaggctctg	gacaaaatat	agcaagacaa
atagcattag					
1561	gagcaggaat	accagtagaa	aaaccagcta	tgactataaa	tatagtttgt
ggttctggat					
1621	taagatctgt	ttcaatggca	tctcaactta	tagcattagg	tgatgctgat
ataatgttag					
1681	ttggtggagc	tgaaaacatg	agtatgtctc	cttatttagt	accaagtgcg

Nucleotide sequences of pLogic046-oxyS-butyrate construct (SEQ ID NO: 76)					
agatatggtg					
1741	caagaatggg	tgatgctgct	tttgttgatt	caatgataaa	agatggatta
tcagacatat					
1801	ttaataacta	tcacatgggt	attactgctg	aaaacatagc	agagcaatgg
aataataacta					
1861	gagaagaaca	agatgaatta	gctcttgcaa	gtcaaaataa	agctgaaaaa
gctcaagctg					
1921	aaggaaaatt	tgatgaagaa	atagttcctg	ttgtttataaa	aggaagaaaa
ggtgacactg					
1981	tagtagataa	agatgaatat	attaagcctg	gcactacaat	ggagaaactt
gctaagttaa					
2041	gacctgcatt	taaaaaagat	ggaacagtta	ctgctggtaa	tgcatacagga
ataaatgatg					
2101	gtgctgctat	gttagtagta	atggctaaag	aaaaagctga	agaactagga
atagagcctc					
2161	ttgcaactat	agtttcttat	ggaacagctg	gtgttgaccc	taaaataatg
ggatatggac					
2221	cagttccagc	aactaaaaaa	gctttagaag	ctgctaatat	gactattgaa
gatatagatt					
2281	tagttgaagc	taatgaggca	tttgctgccc	aatctgtagc	tgtaataaga
gacttaaata					
2341	tagatatgaa	taaagttaat	gttaatgggtg	gagcaatagc	tataggacat
ccaataggat					
2401	gctcaggagc	aagaatactt	actacacttt	tatatgaaat	gaagagaaga
gatgctaaaa					
2461	ctggtcttgc	tacactttgt	ataggcggtg	gaatgggaac	tactttaata
gttaagagat					
2521	agtaagaagg	agatatacat	atgaaattag	ctgtaatagg	tagtggaact
atgggaagtg					
2581	gtattgtaca	aacttttgca	agttgtggac	atgatgtatg	tttaaagagt
agaactcaag					
2641	gtgctataga	taaatgttta	gctttattag	ataaaaaattt	aactaagtta
gttactaagg					
2701	gaaaaatgga	tgaagctaca	aaagcagaaa	tattaagtca	tgttagttca
actactaatt					
2761	atgaagattt	aaaagatatg	gatttaataa	tagaagcatc	tgtagaagac
atgaatataa					
2821	agaaagatgt	tttcaagtta	ctagatgaat	tatgtaaaga	agatactatc
ttggcaacaa					
2881	atacttcac	attatctata	acagaaatag	cttcttctac	taagcgccca
gataaagtta					
2941	taggaatgca	tttctttaat	ccagttccta	tgatgaaatt	agttgaagtt
ataagtggc					
3001	agttaacatc	aaaagttact	tttgatacag	tatttgaatt	atctaagagt
atcaataaag					
3061	taccagtaga	tgtatctgaa	tctcctggat	ttgtagtaaa	tagaataactt
atacctatga					
3121	taaatgaagc	tgttggtata	tatgcagatg	gtgttgcaag	taaagaagaa
atagatgaag					
3181	ctatgaaatt	aggagcaaac	catccaatgg	gaccactagc	attaggtgat
ttaatcggat					
3241	tagatgttgt	tttagctata	atgaacgttt	tatatactga	atttgagat
actaaatata					
3301	gacctcatcc	acttttagct	aaaatggtta	gagctaataca	attaggaaga
aaaactaaga					
3361	taggattcta	tgattataat	aaataataag	aaggagatat	acatatgagt
acaagtgatg					

Nucleotide sequences of pLogic046-oxyS-butyrate construct (SEQ ID NO: 76)					
3421	ttaaagttta	tgagaatgta	gctgttgaag	tagatggaaa	tatatgtaca
gtgaaaatga	3481	atagacctaa	agcccttaat	gcaataaatt	caaagacttt
tatgaagtat	3541	ttgtagatat	taataatgat	gaaactattg	atgttgtaat
gaaggaaagg	3601	catttgtagc	tgagacagat	attgcataca	tgaaagattt
gctgctaaag	3661	atttttagtat	cttaggagca	aaagcttttg	gagaaataga
aaagtagtga	3721	tagctgctgt	aaacggattt	gcttttaggtg	gaggatgtga
gcatgtgata	3781	taagaattgc	atctgctaaa	gctaaatttg	gtcagccaga
ggaataactc	3841	caggatatgg	aggaactcaa	aggcttaca	gattggttgg
gcaaaagaat	3901	taatctttac	agggtcaagtt	ataaaagctg	atgaagctga
ctagtaaata	3961	gagtcgttga	gccagacatt	ttaatagaag	aagttgagaa
ataatagcta	4021	aaaatgctca	gcttgcagtt	agatactcta	aagaagcaat
gctcaaactg	4081	atataaatac	tggaatagat	atagaatcta	atttatttgg
tcaactaaag	4141	accaaaaaga	aggaatgtca	gctttcgttg	aaaagagaga
ataaaaggg	4201	aataagaagg	agatatacat	atgagaagtt	ttgaagaagt
gcaaaagaaa	4261	gaggacctaa	aactatatca	gtagcatgtt	gccaagataa
atggcagttg	4321	aatgggctag	aaaagaaaaa	atagcaaattg	ccatttttagt
gaaaagacta	4381	aagaaattgc	aaaaagcata	gacatggata	tcgaaaatta
gatataaaaag	4441	atttagcaga	agcatctcta	aatctgtttg	aattagtttc
gccgacatgg	4501	taatgaaagg	cttagtagac	acatcaataa	tactaaaagc
aaagaagtag	4561	gtcttagaac	tggaatgta	ttaagtcacg	tagcagtatt
ggatatgata	4621	gattatTTTT	cgtaactgac	gcagctatga	acttagctcc
actaaaaagc	4681	aatcataga	aatgcttg	acagtagcac	attcattaga
ccaaaagttg	4741	ctgcaatatg	cgcaaaagaa	aaagtaaattc	caaaaatgaa
gaagctaaag	4801	aactagaaga	aatgtatgaa	agaggagaaa	tcaaaggttg
gggccttttg	4861	caattgataa	tgacgtatct	ttagaagcag	ctaaacataa
catcctgtag	4921	caggacgagc	tgatatatta	ttagccccag	atattgaagg
ttatataaag	4981	ctttggtatt	cttctcaaaa	tcaaaaaatg	caggagttat
aaagcaccaa	5041	taatattaac	ttctagagca	gacagtgaag	aaactaaact
gcttttaggtg	5101	ttttaatggc	agcaaaggca	taataagaag	gagatataca
					tatgagcaaa

Nucleotide sequences of pLogic046-oxyS-butyrat construct (SEQ ID NO: 76)						
atatttaaaa						
5161	tcttaaca	at	aaatcctggt	tcgacatcaa	ctaaaatagc	tgtatttgat
aatgaggatt						
5221	tagtattt	ga	aaaaacttta	agacattctt	cagaagaaat	aggaaaatat
gagaagggtg						
5281	ctgaccaa	tt	tgaatttcgt	aaacaagtaa	tagaagaagc	tctaaaagaa
ggtggagtaa						
5341	aaacatct	ga	attagatgct	gtagtaggta	gaggaggact	tcttaaacct
ataaaagggtg						
5401	gtacttat	tc	agtaagtgt	gctatgattg	aagatttaaa	agtgggagtt
ttaggagaac						
5461	acgcttca	aa	cctagggtga	ataatagcaa	aacaaatagg	tgaagaagta
aatgttcctt						
5521	catacata	gt	agaccctgtt	gttgtagatg	aattagaaga	tgttgctaga
atttctggta						
5581	tgcttgaa	at	aagtagagca	agtgtagtac	atgctttaaa	tcaaaaggca
atagcaagaa						
5641	gatatgct	ag	agaaataaac	aagaaatatg	aagatataaa	tcttatagtt
gcacacatgg						
5701	gtggagga	gt	ttctgttgga	gctcataaaa	atggtaaaat	agtagatgtt
gcaaacgcat						
5761	tagatgga	ga	aggacccttc	tctccagaaa	gaagtgggtg	actaccagta
ggtgcattag						
5821	taaaaatg	tg	ctttagtggga	aaatatactc	aagatgaaat	taaaaagaaa
ataaaaggta						
5881	atggcgga	ct	agttgcatac	ttaaactacta	atgatgctag	agaagttgaa
gaaagaattg						
5941	aagctggt	ga	tgaaaaagct	aaattagtat	atgaagctat	ggcatatcaa
atctctaaag						
6001	aaatagga	gc	tagtgctgca	gttcttaagg	gagatgtaaa	agcaatatta
ttaactgggtg						
6061	gaatcgca	ta	ttcaaaaatg	tttacagaaa	tgattgcaga	tagagttaaa
tttatagcag						
6121	atgtaaaa	gt	ttatccaggt	gaagatgaaa	tgattgcatt	agctcaaggt
ggacttagag						
6181	ttttaactgg	ttggaaaaggaaaaggaag	gctcaagttt	atgataacta	ataa	

Table 17

Nucleotide sequences of pZA22-oxyR constrcut (SEQ ID NO: 77)					
1	ctcgagatgc	tagcaattgt	gagcggataa	caattgacat	tgtgagcgga
taacaagata					
61	ctgagcacat	cagcaggacg	cactgacctt	aattaaaaga	attcattaaa
gaggagaaag					
121	gtaccatgaa	tattcgtgat	cttgagtacc	tggtggcatt	ggctgaacac
cgccattttc					
181	ggcgtgcggc	agattcctgc	cacgttagcc	agccgacgct	tagcgggcaa
attcgtaagc					
241	tggaaagatga	gctgggctgtg	atgttgctgg	agcggaccag	ccgtaaagtg
ttgttcaccc					
301	aggcgggaat	gctgctggtg	gatcaggcgc	gtaccgtgct	gcgtgaggtg
aaagtcctta					
361	aagagatggc	aagccagcag	ggcgagacga	tgtccggacc	gctgcacatt
ggtttgattc					
421	ccacagttgg	accgtacctg	ctaccgcata	ttatccctat	gctgcaccag

Nucleotide sequences of pZA22-oxyR constrcut (SEQ ID NO: 77)					
acctttccaa					
481	agctggaaat	gtatctgcat	gaagcacaga	cccaccagtt	actggcgcaa
ctggacagcg					
541	gcaaactcga	ttgcgtgatc	ctcgcgctgg	tgaaagagag	cgaagcattc
attgaagtgc					
601	cgttgtttga	tgagccaatg	ttgctggcta	tctatgaaga	tcacccgtgg
gcgaaccgcg					
661	aatgcgtacc	gatggccgat	ctggcagggg	aaaaactgct	gatgctggaa
gatggtcact					
721	gtttgcgcga	tcaggcaatg	ggtttctgtt	ttgaagccgg	ggcggatgaa
gatacacact					
781	tccgcgcgac	cagcctggaa	actctgcgca	acatggtggc	ggcaggtagc
gggatcactt					
841	tactgccagc	gctggctgtg	ccgccggagc	gcaaacgcga	tggggttgtt
tatctgccgt					
901	gcattaagcc	ggaaccacgc	cgcactattg	gcctggttta	tcgtcctggc
tcaccgctgc					
961	gcagccgcta	tgagcagctg	gcagaggcca	tccgcgcaag	aatggatggc
catttcgata					
1021	aagttttaaa	acaggcggtt	taaggatccc	atggtacgcg	tgctagaggc
atcaaataaaa					
1081	acgaaaggct	cagtcgaaag	actgggcctt	tcgttttatc	tgttgtttgt
cggatgaacgc					
1141	tctcctgagt	aggacaaatc	cgccgcccta	gacctagggg	atatattccg
cttcctcgct					
1201	cactgactcg	ctacgctcgg	tcgttcgact	gcggcgagcg	gaaatggctt
acgaacgggg					
1261	cggagatttc	ctggaagatg	ccaggaagat	acttaacagg	gaagtgagag
ggccgcggca					
1321	aagccgtttt	tccataggct	ccgccccct	gacaagcatc	acgaaatctg
acgctcaaatt					
1381	cagtggatggc	gaaacccgac	aggactataa	agataccagg	cgtttcccc
tggcggctcc					
1441	ctcgtgcgct	ctcctgttcc	tgcctttcgg	tttaccggtg	tcattccgct
gttatggccg					
1501	cgtttgtctc	attccacgcc	tgacactcag	ttccgggtag	gcagttcgct
ccaagctgga					
1561	ctgtatgcac	gaaccccccg	ttcagtccga	ccgctgcgcc	ttatccggta
actatcgtct					
1621	tgagtccaac	ccggaaagac	atgcaaaagc	accactggca	gcagccactg
gtaattgatt					
1681	tagaggagtt	agtcttgaag	tcatgcgccg	gttaaggcta	aactgaaagg
acaagttttg					
1741	gtgactgcgc	tcctccaagc	cagttacctc	ggttcaaaga	gttggtagct
cagagaacct					
1801	tcgaaaaacc	gccctgcaag	gcggtttttt	cgttttcaga	gcaagagatt
acgcgcagac					
1861	caaaacgatc	tcaagaagat	catcttatta	atcagataaa	atatttctag
atttcagtgc					
1921	aatttatctc	ttcaaagtga	gcacctgaag	tcagcccat	acgatataag
ttgttactag					
1981	tgcttgatt	ctcaccaata	aaaaacgccc	ggcggcaacc	gagcgttctg
aacaaatcca					
2041	gatggagttc	tgaggtcatt	actggatcta	tcaacaggag	tccaagcgag
ctctcgaacc					
2101	ccagagtccc	gctcagaaga	actcgtcaag	aaggcgatag	aaggcgatgc
gctgcgaatc					

Nucleotide sequences of pZA22-oxyR constrcut (SEQ ID NO: 77)					
2161	gggagcggcg	ataccgtaaa	gcacgaggaa	gcggtcagcc	cattcgccgc
caagctcttc	2221	agcaatatca	cgggtagcca	acgctatgtc	ctgatagcgg
ccagccggcc	2281	acagtcgatg	aatccagaaa	agcggccatt	ttccaccatg
agcaggcatc	2341	gccatgggtc	acgacgagat	cctcgccgtc	gggcatgcgc
tggcgaacag	2401	ttcggctggc	gcgagcccct	gatgtctctc	gtccagatca
caagaccggc	2461	ttccatccga	gtacgtgctc	gctcgatgcg	atgtttcgct
atgggcagg	2521	agccggatca	agcgtatgca	gccgccgc	tgcatcagcc
ctttctcggc	2581	aggagcaagg	tgagatgaca	ggagatcctg	ccccggcact
gcagccagtc	2641	ccttcccgtc	tcagtgacaa	cgtcgagcac	agctgcgcaa
tcgtggccag	2701	ccacgatagc	cgcgctgcct	cgctctgcag	ttcattcagg
ggtcgggtctt	2761	gacaaaaaga	accgggcgcc	cctgcgctga	cagccggaac
cagagcagcc	2821	gattgtctgt	tgtgccagtc	catagccgaa	tagcctctcc
ccggagaacc	2881	tgcgtgcaat	ccatcttggt	caatcatgcg	aaacgatcct
cttgatcaga	2941	tcttgatccc	ctgcgccatc	agatccttgg	cggcaagaaa
ttactttgca	3001	gggcttccca	accttaccag	agggcgcccc	agctggcaat
aagaaaccat	3061	tattatcatg	acattaacct	ataaaaatag	gcgtatcacg
gtcttcac					aggccctttc

Table 18

Nucleotide sequences of pLogic031-tet-butyrat construct (SEQ ID NO: 78)					
1	gtaaaacgac	ggccagtga	ttcgtaaga	cccactttca	catttaagtt
gtttttctaa	61	tccgcatatg	atcaattcaa	ggccgaataa	gaaggctggc
ggtgatcaaa	121	taattcgata	gcttgtcgta	ataatggcgg	catactatca
tttccctttc	181	ttcttttagc	acttgatgct	cttgatcttc	caatacgcaa
aatgccccac	241	agcgctgagt	gcatataatg	cattctctag	tgaaaaacct
aaaaggctaa	301	ttgatcttcg	agagtttcat	actgtttttc	tgtaggccgt
gtacttttgc	361	tccatcgcca	tgacttagta	aagcacatct	aaaactttta
gtaaaaaatc	421	ttgccagctt	tccccttcta	aagggaacaa	gtgagtatgg
acatctcaat	481	ggctaaggcg	tcgagcaaag	cccgtttatt	ttttacatgc
taggctgctc	541	tacacctagc	ttctgggcga	gtttacgggt	tgttaaacct
					tcgattccga

Nucleotide sequences of pLogic031-tet-butyrat construct (SEQ ID NO: 78)						
cctcattaag						
601	cagctcta	at gcgctgttaa	tcactttact	tttatcta	at ctagacatca	
taaattccta						
661	atTTTT	gttg	acactctatc	attgatagag	ttatTTTacc	actccctatq
agtgatagag						
721	aaJaagtgaac	tctagaaata	atTTTgttta	actttaagaa	ggagatatatac	
atatggattt						
781	aaattctaaa	aaatatcaga	tgcttaaaga	gctatatgta	agcttcgctg	
aaaatgaagt						
841	taaaccttta	gcaacagaac	ttgatgaaga	agaaagattt	ccttatgaaa	
cagtggaaaa						
901	aatggcaaaa	gcaggaatga	tgggtatacc	atatccaaaa	gaatatggtg	
gagaagggtg						
961	agacactgta	ggatatataa	tggcagttga	agaattgtct	agagtttgtg	
gtactacagg						
1021	agttatatta	tcagctcata	catctcttgg	ctcatggcct	atatatcaat	
atggtaatga						
1081	agaacaaaaa	caaaaattct	taagaccact	agcaagtggg	gaaaaattag	
gagcatttgg						
1141	tcttactgag	cctaattgctg	gtacagatgc	gtctggccaa	caaacaactg	
ctgttttaga						
1201	cggggatgaa	tacatactta	atggctcaaa	aatatttata	acaaacgcaa	
tagctgggtga						
1261	catatatgta	gtaatggcaa	tgactgataa	atctaagggg	aacaaggaa	
tatcagcatt						
1321	tatagttgaa	aaaggaactc	ctgggttttag	ctttggagtt	aaagaaaaga	
aatgggtat						
1381	aagaggttca	gctacgagtg	aattaatatt	tgaggattgc	agaataccta	
aagaaaattt						
1441	acttggaana	gaaggtcaag	gatttaagat	agcaatgtct	actcttgatg	
gtggtagaat						
1501	tggatatagct	gcacaagctt	taggttttagc	acaagggtgct	cttgatgaaa	
ctgttaaata						
1561	tgtaaaagaa	agagtacaat	ttggtagacc	attatcaaaa	ttccaaaata	
cacaattcca						
1621	attagctgat	atggaagtta	aggtacaagc	ggctagacac	cttgatatatc	
aagcagctat						
1681	aaataaagac	ttaggaaaac	cttatggagt	agaagcagca	atggcaaat	
tatttcagc						
1741	tgaacagct	atggaagtta	ctacaaaagc	tgtacaactt	catggaggat	
atggatacac						
1801	tcgtgactat	ccagtagaaa	gaatgatgag	agatgctaag	ataactgaaa	
tatatgaagg						
1861	aactagtga	gttcaaagaa	tggttatttc	aggaaaacta	ttaaaatagt	
aagaaggaga						
1921	tatacatatg	gaggaaggat	ttatgaatat	agtcgtttgt	ataaaacaag	
ttccagatac						
1981	aacagaagtt	aaactagatc	ctaatacagg	tactttaatt	agagatggag	
taccaagtat						
2041	aataaacct	gatgataaag	caggtttaga	agaagctata	aaattaaaag	
aagaaatggg						
2101	tgctcatgta	actgttataa	caatgggacc	tcctcaagca	gatatggctt	
taaaagaagc						
2161	tttagcaatg	ggtgcagata	gaggatatatt	attaacagat	agagcatttg	
cgggtgctga						

Nucleotide sequences of pLogic031-tet-butyrat construct (SEQ ID NO: 78)					
2221	tacttgggca	acttcatcag	cattagcagg	agcattaaaa	aatatagatt
ttgatattat	2281	aatagctgga	agacaggcga	tagatggaga	tactgcacaa
aaatagctga	2341	acatttaa	cttccatcaa	taacatatgc	tgaagaaata
gtgaatatgt	2401	attagtaaaa	agacaatttg	aagattgttg	ccatgactta
tgccatgcct	2461	tataacaact	cttaaagata	tgaacacacc	aagatacatg
gaatatatga	2521	tgctttcgaa	aatgatgtag	tagaaacatg	gactgtaaaa
ttgacccttc	2581	taatttaggt	cttaaagggt	ctccaactag	tgtattttaa
aatcagttaa	2641	accagctggg	acaatataca	atgaagatgc	gaaaacatca
tcatagataa	2701	attaaaagag	aagtatatca	tataataaga	aggagatata
acgttttagt	2761	agtaatagaa	caaagagaaa	atgtaattca	aactgtttct
taggaaaggc	2821	tacagaaata	gcaaaagatt	atgatacaaa	agtttctgca
gtagtaagggt	2881	agaagggtta	atagatacat	tagcacacta	tgggtgcagat
tagtagatga	2941	tgaagcttta	gcagtgtata	caactgaacc	atatacaaaa
aagcaataaaa	3001	agcagctgac	cctatagttg	tattatttgg	tgcaacttca
atttagcgcc	3061	tagagtttct	gctagaatac	atacagggtct	tactgctgac
ttgcagtagc	3121	tgaagataca	aaattattat	taatgacaag	acctgccttt
taatggcaac	3181	aatagtttgt	aaagatttca	gacctcaa	gtctacagtt
ttatgaagaa	3241	aatgaacct	gatgaaacta	aagaagctgt	aattaaccgt
aatttaaatga	3301	tgctgataaa	ttagttcaag	ttgtacaagt	aataaaagaa
aagttaaaaat	3361	agaagatgct	aagatattag	tttctgctgg	acgtggaatg
aaaacttaga	3421	catactttat	gaattagctg	aaattatagg	tggagaagtt
gtgccactat	3481	agatgcagg	tggtagata	aagcaagaca	agttggtcaa
ctgtaagacc	3541	agacctttat	atagcatgtg	gtatatctgg	agcaatacaa
gtatggaaga	3601	tgctgagttt	atagttgcta	taaataaaaa	tccagaagct
aatatgctga	3661	tgttggtata	gttgagatg	ttcataaagt	gcttccagaa
agttaagtgt	3721	tgcaaaagaa	aaagggtgaag	ttttagctaa	ctaataagaa
atatgagaga	3781	agtagtaatt	gccagtgacg	ctagaacagc	agtaggaagt
catttaa	3841	agtttcagcg	gtagagttag	gggtaacagc	agctaaagaa
gagcta	3901	aactccagat	atgatagatg	aatctctttt	agggggagta
					cttacagcag

Nucleotide sequences of pLogic031-tet-butyrat construct (SEQ ID NO: 78)					
gtcttggaca					
3961	aaatatagca	agacaaatag	cattaggagc	aggaatacca	gtagaaaaac
cagctatgac					
4021	tataaatata	gtttgtgggt	ctggattaag	atctgtttca	atggcatctc
aacttatagc					
4081	attaggtgat	gctgatataa	tgtagttgg	tgagagctgaa	aacatgagta
tgtctcctta					
4141	tttagtacca	agtgcgagat	atggtgcaag	aatgggtgat	gctgcttttg
ttgattcaat					
4201	gataaaagat	ggattatcag	acatatttaa	taactatcac	atgggtatta
ctgctgaaaa					
4261	catagcagag	caatggaata	taactagaga	agaacaagat	gaattagctc
ttgcaagtca					
4321	aaataaagct	gaaaaagctc	aagctgaagg	aaaatttgat	gaagaaatag
ttcctgttgt					
4381	tataaaagga	agaaaagggtg	acactgtagt	agataaagat	gaatatatta
agcctggcac					
4441	tacaatggag	aaacttgcta	agttaagacc	tgcatttaaa	aaagatggaa
cagttactgc					
4501	tggaatgca	tcaggaataa	atgatgggtgc	tgctatgtta	gtagtaatgg
ctaaagaaaa					
4561	agctgaagaa	ctaggaatag	agcctcttgc	aactatagtt	tcttatggaa
cagctgggtgt					
4621	tgaccctaaa	ataatgggat	atggaccagt	tccagcaact	aaaaaagctt
tagaagctgc					
4681	taatatgact	attgaagata	tagatttagt	tgaagctaata	gaggcatttg
ctgcccattc					
4741	tgtagctgta	ataagagact	taaatataga	tatgaataaa	gttaatgtta
atggtggagc					
4801	aatagctata	ggacatccaa	taggatgctc	aggagcaaga	atacttacta
cacttttata					
4861	tgaaatgaag	agaagagatg	ctaaaactgg	tcttgctaca	ctttgtatag
gcggtggaat					
4921	gggaactact	ttaatagtta	agagatagta	agaaggagat	atacatatga
aattagctgt					
4981	aataggtagt	ggaactatgg	gaagtgggtat	tgtacaaact	tttgcaagtt
gtggacatga					
5041	tgtatgttta	aagagtagaa	ctcaagggtgc	tatagataaa	tgtttagctt
tattagataa					
5101	aaatttaact	aagttagtta	ctaagggaaa	aatggatgaa	gctacaaaag
cagaaatatt					
5161	aagtcattgtt	agttcaacta	ctaattatga	agatttataa	gatattggatt
taataataga					
5221	agcatctgta	gaagacatga	atataaagaa	agatgttttc	aagttactag
atgaattatg					
5281	taaagaagat	actatcttgg	caacaaatac	ttcatcatta	tctataacag
aaatagcttc					
5341	ttctactaag	cgcccagata	aagttatagg	aatgcatttc	tttaatccag
ttcctatgat					
5401	gaaattagtt	gaagttataa	gtggtcagtt	aacatcaaaa	gttacttttg
atacagtatt					
5461	tgaattatct	aagagtatca	ataaagtacc	agtagatgta	tctgaatctc
ctggatttgt					
5521	agtaaataga	atacttatac	ctatgataaa	tgaagctggt	ggatatatatg
cagatgggtgt					
5581	tgcaagtaaa	gaagaaatag	atgaagctat	gaaattagga	gcaaaccatc
caatgggacc					

Nucleotide sequences of pLogic031-tet-butyrat construct (SEQ ID NO: 78)					
5641	actagcatta	ggtgatttaa	tcggattaga	tgttgtttta	gctataatga
acgtttttata					
5701	tactgaattt	ggagatacta	aatatagacc	tcatccactt	ttagctaaaa
tggtttagagc					
5761	taatcaatta	ggaagaaaaa	ctaagatagg	attctatgat	tataataaat
aataagaagg					
5821	agatatacat	atgagtacaa	gtgatgttaa	agtttatgag	aatgtagctg
ttgaagtaga					
5881	tggaaatata	tgtacagtga	aatgaatag	acctaaagcc	cttaatgcaa
taaattcaaa					
5941	gacttttagaa	gaactttatg	aagtatttgt	agatattaat	aatgatgaaa
ctattgatgt					
6001	tgtaatattg	acaggggaag	gaaaggcatt	tgtagctgga	gcagatattg
catacatgaa					
6061	agatttagat	gctgtagctg	ctaaagattt	tagtatctta	ggagcaaaag
cttttgga					
6121	aatagaaaat	agtaaaaaag	tagtgatagc	tgctgtaaac	ggatttgctt
taggtggagg					
6181	atgtgaactt	gcaatggcat	gtgatataag	aattgcatct	gctaaagcta
aatttgggtca					
6241	gccagaagta	actcttgaa	taactccagg	atatggagga	actcaaaggc
ttacaagatt					
6301	ggttggaatg	gcaaaagcaa	aagaattaat	ctttacaggt	caagttataa
aagctgatga					
6361	agctgaaaaa	atagggctag	taaatagagt	cgttgagcca	gacattttta
tagaagaagt					
6421	tgagaaatta	gctaagataa	tagctaaaaa	tgctcagctt	gcagttagat
actctaaaga					
6481	agcaatacaa	cttggtgctc	aaactgatat	aaatactgga	atagatatag
aatctaattt					
6541	atttgggtctt	tgtttttcaa	ctaaagacca	aaaagaagga	atgtcagctt
tcgttgaaaa					
6601	gagagaagct	aactttataa	aagggttaata	agaaggagat	atacatatga
gaagttttga					
6661	agaagtaatt	aagtttgcaa	aagaaagagg	acctaaaact	atatcagtag
catgttgcca					
6721	agataaagaa	gttttaaatg	cagttgaaat	ggctagaaaa	gaaaaaatag
caaatgccat					
6781	tttagtagga	gatatagaaa	agactaaaga	aattgcaaaa	agcatagaca
tggatatcga					
6841	aaattatgaa	ctgatagata	taaaagattt	agcagaagca	tctctaaaat
ctggttgaatt					
6901	agtttcacaa	ggaaaagccg	acatggtaat	gaaaggctta	gtagacacat
caataatact					
6961	aaaagcagtt	ttaaataaag	aagtaggtct	tagaactgga	aatgtattaa
gtcacgtagc					
7021	agtatttgat	gtagagggat	atgatagatt	atttttcgta	actgacgcag
ctatgaactt					
7081	agctcctgat	acaaatacta	aaaagcaa	catagaaaat	gcttgacacag
tagcacattc					
7141	attagatata	agtgaaccaa	aagttgctgc	aatatgcgca	aaagaaaaag
taaatccaaa					
7201	aatgaaagat	acagttgaag	ctaaagaact	agaagaaatg	tatgaaagag
gagaaatcaa					
7261	aggttgtatg	gttggtgggc	cttttgcaat	tgataatgca	gtatcttttag
aagcagctaa					
7321	acataaaggt	ataaatcatc	ctgtagcagg	acgagctgat	atattattag

Nucleotide sequences of pLogic031-tet-butyrate construct (SEQ ID NO: 78)					
ccccagatat					
7381	tgaaggtggt	aacatattat	ataaagcttt	ggtattcttc	tcaaaatcaa
aaaatgcagg					
7441	agttatag tt	ggggctaaag	caccaataat	attaacttct	agagcagaca
gtgaagaaac					
7501	taaactaa ac	tcaatagctt	taggtgtttt	aatggcagca	aaggcataat
aagaaggaga					
7561	tatacata tg	agcaaaatat	ttaaaatctt	aacaataaat	cctggttcga
catcaactaa					
7621	aatagctg ta	tttgataatg	aggatttagt	atttgaaaaa	actttaagac
attcttcaga					
7681	agaaatag'ga	aaatatgaga	agggtgtctga	ccaatttgaa	tttcgtaaac
aagtaataga					
7741	agaagctc ta	aaagaagggtg	gagtaaaaac	atctgaatta	gatgctgtag
taggtagagg					
7801	aggacttc tt	aaacctataa	aagggtggtac	ttattcagta	agtgtctgta
tgattgaaga					
7861	tttaaaag tg	ggagtttttag	gagaacacgc	ttcaaaccta	ggtggaataa
tagcaaaaaca					
7921	aataggtg.aa	gaagtaaagt	ttccttcata	catagtagac	cctgttggtg
tagatgaatt					
7981	agaagatg tt	gctagaattt	ctggtatgcc	tgaaataagt	agagcaagtg
tagtacatgc					
8041	tttaaatc aa	aaggcaatag	caagaagata	tgctagagaa	ataaacaaga
aatatgaaga					
8101	tataaatc tt	atagttgcac	acatgggttg	aggagtttct	gttgagctc
ataaaaatgg					
8161	taaaatag ta	gatgttgcaa	acgcattaga	tggaagaagga	cctttctctc
cagaaagaag					
8221	tggtggacta	ccagtaggtg	cattagtaaa	aatgtgcttt	agtggaaaat
atactcaaga					
8281	tgaaattaaa	aagaaaataa	aaggtaatgg	cggactagtt	gcatacttaa
acactaatga					
8341	tgctagagaa	gttgaagaaa	gaattgaagc	tggtgatgaa	aaagctaaat
tagtatatga					
8401	agctatggca	tatcaaatct	ctaaagaaat	aggagctagt	gctgcagttc
ttaagggaga					
8461	tgtaaaagca	atattattaa	ctggtggaat	cgcatattca	aaaatgttta
cagaaatgat					
8521	tcgagataga	gttaaattta	tagcagatgt	aaaagtttat	ccaggtgaag
atgaaatgat					
8581	tcgattagct	caaggtggac	ttagagtttt	aactggtgaa	gaagaggctc
aagtttatga					
8641	taactaataa				

Table 19

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 79)					
1	gtaaaacgac	ggccagtga	ttcgttaaga	cccactttca	catttaagtt
61	tccgcatatg	atcaattcaa	ggccgaataa	gaaggctggc	tctgcacctt
121	taattcgata	gcttgctgta	ataatggcgg	catactatca	gtagtaggtg
	tttccctttc				

Nucleotide sequences of pLogic046-tet-butyrate construct (SEQ ID NO: 79)					
181	ttcttttagcg	acttgatgct	cttgatcttc	caatacgcaa	cctaaagtaa
241	agcgctgagt	gcatataatg	cattctctag	tgaaaaacct	tgttggcata
301	ttgattttcg	agagtttcat	actgtttttc	tgtaggccgt	gtacctaaat
361	tccatcgcg	tgacttagta	aagcacatct	aaaactttta	gcgttattac
421	ttgccagctt	tccccttcta	aagggcaaaa	gtgagtatgg	tgcctatcta
481	ggctaaggcg	tcgagcaaag	cccgcttatt	ttttacatgc	caatacaatg
541	tacacctagc	ttctgggcga	gtttacgggt	tgttaaacct	tcgattccga
601	cagctcta	gcgctgttaa	tcactttact	tttatcta	ctagacatca
661	atTTTTgttg	acactctatc	attgatagag	ttatTTTacc	actccctatc
721	aa	laagtgaac	tctagaaata	atTTTgttta	actttaagaa
781	aaaacctatg	gtacgcaaca	atatctgcct	gaacgcccat	cctcagggct
841	agtgaagat	cagattgaat	ataccaagaa	acgcattacc	gcagaagtca
901	aaaagctcca	aaaaacgttc	tgggtgcttg	ctgctcaaat	ggttacggcc
961	cattactgct	gcgttcggat	acggggctgc	gaccatcggc	gtgtcctttg
1021	ttcagaaacc	aaatatggta	caccgggatg	gtacaataat	ttggcatttg
1081	aaaacgcgag	ggctctttata	gcgtgacgat	cgacggcgat	gcgttttcag
1141	ggcccaggta	attgaggaag	ccaaaaaaaa	aggtatcaaa	tttgatctga
1201	cttggccagc	ccagtacgta	ctgatcctga	tacaggatc	atgcacaaaa
1261	accctttgga	aaaacgttca	caggcaaaac	agtagatccg	tttactggcg
1321	aatctccg	gaaccagcaa	atgacgagga	agcagccgcc	actgttaaag
1381	tgaagattgg	gaacgttgg	ttaagcagct	gtcgaaggaa	ggcctcttag
1441	tattaccttg	gcctatagtt	atattggccc	tgaagctacc	caagctttgt
1501	cacaatcggc	aaggccaaag	aacacctgga	ggccacagca	caccgtctca
1561	cccgtcaatc	cgtgccttcg	tgagcgtgaa	taaaggcctg	gtaaccgcg
1621	aatcccggta	atccctctgt	atctcgccag	cttgttcaaa	gtaatgaaag
1681	tcatgaaggt	tgtattgaac	agatcacgcg	tctgtacgcc	gagcgctgt
1741	tgggtacaatt	ccagttgatg	aggaaaatcg	cattcgcat	gatgattggg
1801	agacgtccag	aaagcggtat	ccgcgttgat	ggagaaagtc	acgggtgaaa

Nucleotide sequences of pLogic046-tet-butyrat construct (SEQ ID NO: 79)					
acgcagaatc					
1861	tctcactgac	ttagcgggggt	accgccatga	tttcttagct	agtaacggct
ttgatgtaga					
1921	aggtattaat	tatgaagcgg	aagttgaacg	cttcgaccgt	atctgataag
aaggagatat					
1981	acatatgaga	gaagtagtaa	ttgccagtgc	agctagaaca	gcagtaggaa
gttttggagg					
2041	agcattttaa	tcagtttcag	cggtagagtt	aggggtaaca	gcagctaaag
aagctataaa					
2101	aagagctaac	ataactccag	atatgataga	tgaatctctt	ttagggggag
tacttacagc					
2161	aggtcttggg	caaaatatag	caagacaaat	agcattagga	gcaggaatac
cagtagaaaa					
2221	accagctatg	actataaata	tagtttgttg	ttctggatta	agatctgttt
caatggcatc					
2281	tcaacttata	gcattaggtg	atgctgatat	aatgttagtt	ggtggagctg
aaaacatgag					
2341	tatgtctcct	tatttagtac	caagtgcgag	atatggtgca	agaatgggtg
atgctgcttt					
2401	tggtgattca	atgataaaag	atggattatc	agacatattt	aataactatc
acatgggtat					
2461	tactgctgaa	aacatagcag	agcaatggaa	tataactaga	gaagaacaag
atgaattagc					
2521	tcttgcaagt	caaaataaag	ctgaaaaagc	tcaagctgaa	ggaaaatttg
atgaagaaat					
2581	agttcctgtt	gttataaaag	gaagaaaagg	tgacactgta	gtagataaag
atgaatatat					
2641	taagcctggc	actacaatgg	agaaacttgc	taagttaaga	cctgcattta
aaaaagatgg					
2701	aacagttact	gctggtaatg	catcaggaat	aatgatgggt	gctgctatgt
tagtagtaat					
2761	ggctaaagaa	aaagctgaag	aactaggaat	agagcctctt	gcaactatag
tttcttatgg					
2821	aacagctggg	gttgacccta	aaataatggg	atatggacca	gttccagcaa
ctaaaaaagc					
2881	tttagaagct	gctaatatga	ctattgaaga	tatagattta	gttgaagcta
atgaggcatt					
2941	tgctgcccaa	tctgtagctg	taataagaga	cttaaataata	gatatgaata
aagttaatgt					
3001	taatgggtgga	gcaatagcta	taggacatcc	aataggatgc	tcaggagcaa
gaatacttac					
3061	tacactttta	tatgaaatga	agagaagaga	tgctaaaact	ggtcttgcta
cactttgtat					
3121	aggcggtgga	atgggaacta	ctttaatagt	taagagatag	taagaaggag
atatacatat					
3181	gaaattagct	gtaataggta	gtggaactat	gggaagtggg	attgtacaaa
cttttgcaag					
3241	ttgtggacat	gatgtatggt	taaagagtag	aactcaagggt	gctatagata
aatgttttagc					
3301	tttattagat	aaaaatttaa	ctaagttagt	tactaaggga	aaaatggatg
aagctacaaa					
3361	agcagaaata	ttaagtcatg	ttagttcaac	tactaattat	gaagatttaa
aagatatgga					
3421	tttaataata	gaagcatctg	tagaagacat	gaatataaag	aaagatgttt
tcaagttact					
3481	agatgaatta	tgtaaagaag	atactatctt	ggcaacaaat	acttcatcat
tatctataac					

Nucleotide sequences of pLogic046-tet-butyrat construct (SEQ ID NO: 79)					
3541	agaaatagct	tcttctacta	agcgcccaga	taaagttata	ggaatgcatt
tctttaatcc	3601	agttcctatg	atgaaattag	ttgaagttat	aagtggtcag
aagttacttt	3661	tgatacagta	tttgaattat	ctaagagtat	caataaagta
tatctgaatc	3721	tcctggattt	gtagtaaata	gaatacttat	acctatgata
ttggtatata	3781	tgcagatggg	gttgcaagta	aagaagaaat	agatgaagct
gagcaaacca	3841	tccaatggga	ccactagcat	taggtgattt	aatcggatta
tagctataat	3901	gaacgtttta	tatactgaat	ttggagatac	taaatataga
ttttagctaa	3961	aatggttaga	gctaatacat	taggaagaaa	aactaagata
attataataa	4021	ataataagaa	ggagatatatac	atatgagtac	aagtgatgtt
agaatgtagc	4081	tggtgaagta	gatggaaata	tatgtacagt	gaaaatgaat
cccttaatgc	4141	aataaattca	aagacttttag	aagaacttta	tgaagtattt
ataatgatga	4201	aactattgat	gttgtaatat	tgacagggga	aggaaaggca
gagcagatat	4261	tgatacatg	aaagattttag	atgctgtagc	tgctaaagat
taggagcaaa	4321	agcttttggg	gaaatagaaa	atagtaaaaa	agtagtgata
acggatttgc	4381	tttaggtgga	ggatgtgaac	ttgcaatggc	atgtgatata
ctgctaaagc	4441	taaatttggg	cagccagaag	taactcttgg	aataactcca
gaactcaaag	4501	gcttacaaga	ttggttggaa	tggcaaaagc	aaaagaatta
gtcaagttat	4561	aaaagctgat	gaagctgaaa	aaatagggct	agtaaataga
cagacatttt	4621	aatagaagaa	gttgagaaat	tagctaagat	aatagctaaa
ttgcagttag	4681	atactctaaa	gaagcaatac	aacttgggtgc	tcaaactgat
gaatagatat	4741	agaatctaata	ttatttgggtc	tttgtttttc	aactaaagac
gaatgtcagc	4801	tttcgttgaa	aagagagaag	ctaactttat	aaaagggtaa
atatacatat	4861	gagaagtttt	gaagaagtaa	ttaagtttgc	aaaagaaaga
ctatatcagt	4921	agcatgttgc	caagataaag	aagttttaat	ggcagttgaa
aagaaaaaat	4981	agcaaatgcc	atttttagtag	gagatataga	aaagactaaa
aaagcataga	5041	catggatatc	gaaaattatg	aactgataga	tataaaagat
catctctaaa	5101	atctgttgaa	ttagtttcac	aaggaaaagc	cgacatggta
tagtagacac	5161	atcaataata	ctaaaagcag	ttttaataaa	agaagtaggt
gaaatgtatt	5221	aagtcacgta	gcagtatttg	atgtagaggg	atatgataga
				ttatttttcg	

Nucleotide sequences of pLogic046-tet-butyrat construct (SEQ ID NO: 79)					
taactgacgc					
5281 agctatgaac	ttagctcctg	atacaaatac	taaaaagcaa	atcatagaaa	
atgcttgacac					
5341 agtagcacat	tcattagata	taagtgaacc	aaaagttgct	gcaatatgcg	
caaaaagaaaa					
5401 agtaaatcca	aaaatgaaag	atacagttga	agctaaagaa	ctagaagaaa	
tgtatgaaag					
5461 aggagaaatc	aaagggttgta	tgggttggtgg	gccttttgca	attgataatg	
cagtatcttt					
5521 agaagcagct	aaacataaag	gtataaatca	tcctgtagca	ggacgagctg	
atatattatt					
5581 agccccagat	attgaagggtg	gtaacatatt	atataaagct	ttgggtattct	
tctcaaaatc					
5641 aaaaaatgca	ggagttatag	ttggggctaa	agcaccaata	atattaactt	
ctagagcaga					
5701 cagtgaagaa	actaaactaa	actcaatagc	tttaggtggt	ttaatggcag	
caaaggcata					
5761 ataagaagga	gatatacata	tgagcaaaat	atttaaaatc	ttaacaataa	
atcctgggttc					
5821 gacatcaact	aaaatagctg	tatttgataa	tgaggattta	gtatttgaaa	
aaactttaag					
5881 acattcttca	gaagaaatag	gaaaatatga	gaagggtgtct	gaccaatttg	
aatttcgtaa					
5941 acaagtaata	gaagaagctc	taaaagaagg	tggagtaaaa	acatctgaat	
tagatgctgt					
6001 agtaggtaga	ggaggacttc	ttaaacctat	aaaagggtgt	acttattcag	
taagtgtctgc					
6061 tatgattgaa	gatttaaaag	tgggagtttt	aggagaacac	gcttcaaacc	
taggtggaat					
6121 aatagcaaaa	caaataggtg	aagaagtaaa	tggttccttca	tacatagtag	
accctgttgt					
6181 tgtagatgaa	ttagaagatg	ttgctagaat	ttctggtatg	cctgaaataa	
gtagagcaag					
6241 tgtagtacat	gctttaaatc	aaaaggcaat	agcaagaaga	tatgctagag	
aaataaacia					
6301 gaaatatgaa	gatataaatc	ttatagttgc	acacatgggt	ggaggagttt	
ctgttgagc					
6361 tcataaaaaat	ggtaaaatag	tagatgttgc	aaacgcatta	gatggagaag	
gacctttctc					
6421 tccagaaaga	agtgggtggac	taccagtagg	tgcattagta	aaaatgtgct	
ttagtggaaa					
6481 atataactca	gatgaaatta	aaaagaaaat	aaaaggtaat	ggcggactag	
ttgcatactt					
6541 aaacactaat	gatgctagag	aagttgaaga	aagaattgaa	gctgggtgatg	
aaaaagctaa					
6601 attagtatat	gaagctatgg	catatcaa	ctctaaagaa	ataggagcta	
gtgctgcagt					
6661 tcttaaggga	gatgtaaaag	caatattatt	aactgggtgga	atcgcatatt	
caaaaatggt					
6721 tacagaaatg	attgcagata	gagttaaatt	tatagcagat	gtaaaagttt	
atccaggtga					
6781 agatgaaatg	attgcattag	ctcaagggtg	acttagagtt	ttaactggtg	
aagaagaggc					
6841 tcaagtttat	gataactaat	aa			

[0119] In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operably linked to a promoter that is induced by ROS. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule 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 anti-inflammation and/or gut barrier function enhancer molecule is present on a chromosome and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, expression is further optimized by methods known in the art, e.g., by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability.

[0120] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule, such that the gene(s) or gene cassette(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, a bacterium may comprise multiple copies of the gene or gene cassette for producing the anti-inflammation and/or gut barrier function enhance molecule. In some embodiments, gene or gene cassette 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 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 is expressed on a chromosome.

[0121] In some embodiments, the genetically engineered bacteria may comprise multiple copies of the gene(s) or gene cassette(s) capable of producing an anti-

inflammation and/or gut barrier function enhancer molecule. In some embodiments, the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule is present on a plasmid and operatively linked to a ROS-responsive regulatory region. In some embodiments, the gene(s) or gene cassette(s) capable of producing an anti-inflammation and/or gut barrier function enhancer molecule is present in a chromosome and operatively linked to a ROS-responsive regulatory region.

[0122] In some embodiments, the genetically engineered bacteria of the invention produce at least one anti-inflammation and/or gut barrier enhancer molecule in the presence of ROS 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).

[0123] 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 an anti-inflammation and/or gut barrier enhancer molecule in the presence of ROS than unmodified bacteria of the same subtype under the same conditions. Certain unmodified

bacteria will not have detectable levels of the anti-inflammation and/or gut barrier enhancer molecule. In embodiments using genetically modified forms of these bacteria, the anti-inflammation and/or gut barrier enhancer molecule will be detectable in the presence of ROS.

[0124] In certain 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 μ M/OD, at least about 10 μ M/OD, at least about 100 μ M/OD, at least about 500 μ M/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.

Multiple mechanisms of action

[0223] 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. 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. Examples of insertion sites include, but are not limited to, *malE/K*, *insB/I*, *araC/BAD*, *lacZ*, *dapA*, *cea*, and other shown in **Fig. 51**. 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 butyrate gene cassette inserted at three different insertion sites, e.g., *dapA*, *cea*, and *oroC/BAD*.

Secretion

[0224] In some embodiments, the genetically engineered bacteria further comprise a native secretion mechanism (e.g., Gram-positive bacteria) or non-native secretion mechanism (e.g., Gram-negative bacteria) that is capable of secreting the anti-inflammation and/or gut barrier enhancer molecule from the bacterial cytoplasm. 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.

[0225] 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 **Figs. 68-71**. 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 (T5SS), the curli secretion system, and the chaperone-usher pathway for pili assembly (Saier, 2006; Costa et al., 2015).

[0226] In some embodiments, the genetically engineered bacteria of the invention further comprise a type III or a type III-like secretion system (T3SS) from *Shigella*, *Salmonella*, *E. coli*, *Bifidobacterium*, *Burkholderia*, *Yersinia*, *Chlamydia*, or *Pseudomonas*. The T3SS is capable of transporting a protein from the bacterial cytoplasm to the host cytoplasm through a needle complex. The T3SS may be modified to secrete the molecule from the bacterial cytoplasm, but not inject the molecule into the host cytoplasm. Thus, the molecule is secreted into the gut lumen or other extracellular space. In some embodiments, the genetically engineered bacteria comprise said modified T3SS and are capable of secreting the anti-inflammation and/or gut barrier enhancer molecule from the bacterial cytoplasm. In some embodiments, the secreted molecule comprises a type III secretion sequence that allows the molecule to be secreted from the bacteria.

[0227] In some embodiments, a flagellar type III secretion pathway is used to secrete the anti-inflammation and/or gut barrier enhancer molecule. In some embodiments, an incomplete flagellum is used to secrete a therapeutic molecule by recombinantly fusing the molecule to an N-terminal flagellar secretion signal of a native flagellar component. In this manner, the intracellularly expressed chimeric molecule can be mobilized across the inner and outer membranes into the surrounding host environment.

[0228] In some embodiments, a type V autotransporter secretion system is used to secrete the anti-inflammation and/or gut barrier enhancer molecule. 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. 69**, 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 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 exposed to the extracellular environment, the therapeutic peptide can be freed from the linker system by an autocatalytic cleavage (left side of Bam complex) or by targeting of a membrane-associated peptidase (black scissors; right side of Bam complex) to a complementary 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.

[0229] In some embodiments, a hemolysin-based secretion system is used to secrete the anti-inflammation and/or gut barrier enhancer molecule. 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. 71** 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. Natively, this channel is used to secrete HlyA, however, to secrete the therapeutic molecule of the present disclosure, the secretion signal-containing C-terminal portion of HlyA is fused to the C-terminal portion of a therapeutic molecule (star) to mediate secretion of this molecule.

[0230] 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; Albinia 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 anti-inflammation and/or gut barrier enhancer molecule 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 effector molecules.

Essential genes and auxotrophs

[0231] 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).

[0232] 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.

[0233] 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 *thiI*, as long as the corresponding wild-type gene product is not produced in the bacteria. 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 thymidylate 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).

[0234] Diaminopimelic acid (DAP) is an amino acid synthesized 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 the *dapD* gene 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).

[0235] In other embodiments, the genetically engineered bacterium of the present disclosure is a *uraA* auxotroph in which the *uraA* gene 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). An *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).

[0236] 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.

[0237] 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*, *yal*, *yihA*, *ftsN*, *murl*, *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*, *ribE*, *lspA*,

ispH, dopB, folA, imp, yabQ, ftsL, ftsI, murE, murF, mroY, 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, rpsi, 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.

[0238] 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).

[0239] 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.

[0240] 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 *dnoN* (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.

[0241] 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).

[0242] In some embodiments, the genetically engineered bacterium is a conditional auxotroph whose essential gene(s) is replaced using the arabinose system shown in Figs. 57-61.

[0243] 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*, a 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: 307-316, 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-inflammation and/or gut barrier enhancer molecule. In some embodiments, the genetically engineered bacteria further comprise an antibiotic resistance gene.

Genetic regulatory circuits

[0244] In some embodiments, the genetically engineered bacteria comprise multi-layered genetic regulatory circuits for expressing the constructs described herein (see, e.g., U.S. Provisional Application No. 62/184,811, incorporated herein by reference in its entirety). 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.

[0245] 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.

[0246] 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.

[0247] 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.

[0248] Examples of repressors useful in these embodiments include, but are not limited to, ArgR, TetR, ArsR, AscG, LacI, CscR, DeoR, DgoR, FruR, GalR, GatR, Cl, LexA, RafR, QacR, and PtxS (US20030166191).

[0249] 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.

[0250] 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.

[0251] 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.

[0252] 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.

[0253] 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

[0254] 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.

[0255] 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.

[0256] 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 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; **Fig. 66**). 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

[0257] 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.

[0258] 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-1. 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-l-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).

[0259] 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.

[0260] 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.

[0261] 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.

[0262] 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.

[0263] 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.

[0264] 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.

[0265] 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.

[0266] 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.

[0267] In any of these embodiment, the recombinase can be a recombinase selected from the group consisting of: BxbI, PhI, TP901, BxbI, PhI, TP901, HK022, HP1, R4, IntI, 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.

[0268] 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.

[0269] 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 (*ParaBAD*). 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 gene encoding a regulatory protein or polypeptide.

[0270] Arabinose inducible promoters are known in the art, including P_{ara} , *ParaB*, *ParaC*, 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.

[0271] 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 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.

[0272] 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.

[0273] 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.

[0274] 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_{ara} 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.

[0275] 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.

[0276] 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.

[0277] 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, lbs, 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 147, microcin M, colicin A, colicin EI, colicin K, colicin N, colicin U, colicin B, colicin Ia, colicin Ib, colicin 5, colicinIO, 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.

[0278] In any of the above-described embodiments, the anti-toxin may be selected from the group consisting of an anti-lysin, Sok, RNAII, IstR, RdID, Kis, SymR, MazE, FlmB, Sib, ptaRNAI, yafQ, CcdA, MazE, ParD, yafN, Epsilon, HicA, relE, prIF, yefM, chpBI, hipB, MccE, MccE^{CTD}, MccF, Cai, ImmEI, Cki, Cni, Cui, Cbi, lia, Imm, Cfi, ImIO, Csi, Cyi, Im2, Im7, Im8, Im9, Im3, Im4, ImmE6, cloacin immunity protein (Cim), ImmE5, ImmD, and Cmi, or a biologically active fragment thereof.

[0279] 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.

[0280] 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 *thiI* auxotroph. In some embodiments, the engineered bacteria have more than one auxotrophy, for example, they may be a *MhyA* and Δ *dapA* auxotroph.

[0281] 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_{arBAD} . In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an anti-toxin.

[0282] 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.

[0283] 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 enhancer molecule is present in the bacterial chromosome and is operatively linked in the chromosome to the inducible promoter.

Mutagenesis

[0284] 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.

[0285] 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.

Pharmaceutical compositions and formulations

[0286] Pharmaceutical compositions comprising the genetically engineered bacteria described herein may be used to inhibit inflammatory mechanisms in the gut, restore and tighten gut mucosal barrier function, and/or treat or prevent autoimmune disorders. Pharmaceutical compositions comprising one or more genetically engineered bacteria, alone or in combination with prophylactic agents, therapeutic agents, and/or pharmaceutically acceptable carriers are provided. 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.

[0287] 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 tableting, lyophilizing, direct compression, conventional mixing, dissolving, granulating, levigating, emulsifying, encapsulating, entrapping, or spray drying to form tablets, granules, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. Appropriate formulation depends on the route of administration.

[0288] The genetically engineered bacteria described herein may be formulated into pharmaceutical compositions in any suitable dosage form (e.g., liquids, capsules, sachet, hard capsules, soft capsules, tablets, enteric coated tablets, suspension powders, granules, or matrix sustained release formations for oral administration) and for any suitable type of administration (e.g., oral, topical, injectable, immediate-release, pulsatile-release, delayed-release, or sustained release). Suitable dosage amounts for the genetically engineered bacteria may range from about 10^5 to 10^{12} bacteria, e.g., approximately 10^5 bacteria, approximately 10^6 bacteria, approximately 10^7 bacteria, approximately 10^8 bacteria, approximately 10^9 bacteria, approximately 10^{10} bacteria, approximately 10^{11} bacteria, or approximately 10^{11} bacteria. The composition may be administered once or more daily, weekly, or monthly. The composition may be administered before, during, or following a meal. In one embodiment, the pharmaceutical composition is administered before the subject eats a meal. In one embodiment, the pharmaceutical composition is administered concurrently with a meal. In one embodiment, the pharmaceutical composition is administered after the subject eats a meal.

[0289] The genetically engineered bacteria may be formulated into pharmaceutical compositions comprising one or more pharmaceutically acceptable carriers, thickeners, diluents, buffers, buffering agents, surface active agents, neutral or

cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers or agents. For example, the pharmaceutical composition may include, but is not limited to, the addition of calcium bicarbonate, sodium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20. In some embodiments, the genetically engineered bacteria 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-ethylaminoethanol, histidine, procaine, etc.

[0290] The genetically engineered bacteria disclosed herein may be administered topically and formulated in the form of an ointment, cream, transdermal patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form well known to one of skill in the art. See, e.g., "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA. In an embodiment, for non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity greater than water are employed. Suitable formulations include, but are not limited to, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, etc., which may be sterilized or mixed with auxiliary agents (e.g., preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, e.g., osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (e.g., a gaseous propellant, such as freon) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms. Examples of such additional ingredients are well known in the art. In one

embodiment, the pharmaceutical composition comprising the 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.

[0291] The genetically engineered bacteria disclosed herein may be administered orally and formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, etc. Pharmacological compositions for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients include, but are not limited to, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose compositions such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP) or polyethylene glycol (PEG). Disintegrating agents may also be added, such as cross-linked polyvinylpyrrolidone, agar, alginic acid or a salt thereof such as sodium alginate.

[0292] 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), hydroxymethylacrylate-methyl

methacrylate (HEMA-MMA), multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitrile/sodium methallylsulfonate (AN-69), polyethylene glycol/poly pentamethylcyclotrisiloxane/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.

[0293] In some embodiments, the genetically engineered bacteria are enterically coated for release into the gut or a particular region of the gut, for example, the large intestine. The typical pH profile from the stomach to the colon is about 1-4 (stomach), 5.5-6.0 (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.

[0294] Liquid preparations for oral administration may take the form of solutions, syrups, suspensions, or a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable agents such as suspending agents (e.g., sorbitol syrup, cellulose derivatives, or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring, and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated for slow release, controlled release, or sustained release of the genetically engineered bacteria described herein.

[0295] In one embodiment, the genetically engineered bacteria of the disclosure may be formulated in a composition suitable for administration to pediatric subjects. As is well known in the art, children differ from adults in many aspects, including different

rates of gastric emptying, pH, gastrointestinal permeability, etc. (Ivanovska et al., *Pediatrics*, 134(2):361-372, 2014). Moreover, pediatric formulation acceptability and preferences, such as route of administration and taste attributes, are critical for achieving acceptable pediatric compliance. Thus, in one embodiment, the composition suitable for administration to pediatric subjects may include easy-to-swallow or dissolvable dosage forms, or more palatable compositions, such as compositions with added flavors, sweeteners, or taste blockers. In one embodiment, a composition suitable for administration to pediatric subjects may also be suitable for administration to adults.

[0296] 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.

[0297] 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.

[0298] In certain embodiments, the genetically engineered bacteria may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

[0299] 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.

[0300] In some embodiments, the composition is formulated for intrainestinal 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.

[0301] The genetically engineered bacteria described herein may be administered intranasally, formulated in an aerosol form, spray, mist, or in the form of drops, and conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant (e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas). Pressurized aerosol dosage units may be determined by providing a valve to deliver a metered amount. Capsules and cartridges (e.g., of gelatin) for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0302] The genetically engineered bacteria may be administered and formulated as depot preparations. Such long acting formulations may be administered by implantation or by injection, including intravenous injection, subcutaneous injection, local injection, direct injection, or infusion. For example, the compositions may be formulated with suitable polymeric or hydrophobic materials (e.g., as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives (e.g., as a sparingly soluble salt).

[0303] 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.

[0304] 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.

[0305] 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), polyvinyl 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.

[0306] 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.

[0307] 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.

[0308] 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

[0309] 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.

[0310] 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
 osteomyelitis (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
Neuropsychiatry 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).

[0311] 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.

[0312] 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.

[0313] 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.

[0314] In certain embodiments, the genetically engineered bacteria are *E. coli* Nissle. The genetically engineered bacteria may be destroyed, e.g., by defense factors in the gut or blood serum (Sonnenborn et al., 2009) or by activation of a kill switch, several hours or days after administration. Thus, the pharmaceutical composition comprising the genetically engineered bacteria may be re-administered at a therapeutically effective dose and frequency. In alternate embodiments, the genetically engineered bacteria are not destroyed within hours or days after administration and may propagate and colonize the gut.

[0315] 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) (Triantafylidis 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 bacteria. The dosage of the pharmaceutical composition and the frequency of administration may be selected based on the severity of the symptoms and the progression of the disorder. The appropriate therapeutically effective dose and/or frequency of administration can be selected by a treating clinician.

Treatment *in vivo*

[0316] 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.

[0317] 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.

[0318] 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; Whitem 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.

[0319] 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-1 β , TNF- α , IL-6, IFN- γ and IL-10).

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Examples

[0320] 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.

Examples

[0321] 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

[0322] To facilitate inducible production of butyrate in *Escherichia coli* Nissle, the eight genes of the butyrate production pathway from *Peptoclostridium difficile* 630 (*bcd2*, *etfB3*, *etfAS*, *thiA1*, *hbd*, *crt2*, *pbt*, and *buk*; NCBI; Table 4), as well as transcriptional and translational elements, are synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. In some embodiments, the butyrate gene cassette is placed under the control of a FNR regulatory region selected from SEQ ID NOs: 55-66 (Table 9). 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 constructs, the butyrate gene cassette is placed under the control of an RNS-responsive regulatory region, e.g., *norB*, and the bacteria further comprises a gene encoding a corresponding RNS-responsive transcription factor, e.g., *nsrR* (see, e.g., Tables 10 and 11). In certain constructs, the butyrate gene cassette is placed under the control of an ROS-responsive regulatory region, e.g., *oxyS*, and the bacteria further comprises a gene encoding a corresponding ROS-responsive transcription factor, e.g., *oxyR* (see, e.g., Tables 14-17). In certain

constructs, the butyrate gene cassette is placed under the control of a tetracycline-inducible or constitutive promoter.

[0323] 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-enoyl-CoA reductase (*ter*, Table 4), 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. In certain constructs, the second butyrate gene cassette is placed under control of a FNR regulatory region as described above (Table 4). In certain constructs, the butyrate gene cassette is placed under the control of an RNS-responsive regulatory region, e.g., *norB*, and the bacteria further comprises a gene encoding a corresponding RNS-responsive transcription factor, e.g., *nsrR* (see, e.g., Tables 10 and 11). In certain constructs, the butyrate gene cassette is placed under the control of an ROS-responsive regulatory region, e.g., *oxyS*, and the bacteria further comprises a gene encoding a corresponding ROS-responsive transcription factor, e.g., *oxyR* (see, e.g., Tables 14-17). In certain constructs, the butyrate gene cassette is placed under the control of a tetracycline-inducible or constitutive promoter.

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 Fig. 2).

[0324] In one embodiment, *tesB* is placed under the control of a FNR regulatory region selected from SEQ ID NOs: 55-66 (Table 9) In an alternate embodiment, *tesB* is

placed under the control of an RNS-responsive regulatory region, e.g., *norB*, and the bacteria further comprises a gene encoding a corresponding RNS-responsive transcription factor, e.g., *nsrR* (see, e.g., Tables 10 and 11). In yet another embodiment, *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., Tables 14-17). In certain constructs, the different described butyrate gene cassettes are each placed under the control of a tetracycline-inducible or constitutive promoter. For example, genetically engineered Nissle are generated comprising a butyrate gene cassette in which the *pbt* and *buk* genes are replaced with *tesB* (SEQ ID NO: 10) expressed under the control of a nitric oxide-responsive regulatory (SEQ ID NO: 80). SEQ ID NO: 80 comprises a reverse complement of the *nsrR* repressor gene from *Neisseria gonorrhoeae* (underlined), intergenic region containing divergent promoters controlling *nsrR* and the butyrogenic gene cassette and their respective RBS (bold), and the butyrate genes (*ter-thiAl-hbd-crt2-tesB*) separated by RBS.

SEQ ID NO: 80
<u>ttattatcgacccgcaatcgggattttcgattcataaagcaggctcgtaggtcggcttggt</u> <u>gagcaggctcttgacgcgtgaaaccgtccagatacgtgaaaaacgacttcattgcaccgcc</u> <u>gagtatgcccgtagccggcaggacggcgtaatcaggcattcggtgttcggggccataca</u> <u>ctcgaccagctgcatcggttcgaggtggcgagcagccgcgcgatattgatgcgttcggg</u> <u>cggcgcgccagcctcagcccgcgcctttcccgctacgctgtgcaagaaccgccttt</u> <u>gaccagcgcggttaaccactttcatcaaatggccttttgaaatgccgtaggtcgaggcgat</u> <u>ggtggcgatattgaccagcgcgctcgctcggtgacggcggtgtagatgaggacgcgcagccc</u> <u>gtagtcgggtatgttgggtcagatacatacaacctccttagtacatgcaaaattatttcta</u> gagcaacatacagagccggaagcataaagtgtaaagcctgggggtgcctaataatgagttgagtt gaggaattataacaggaagaaatattcctcatacgcttgtaattcctctatgggtgttga caattaatcatcggtcgtataatgtataacattcatattttgtgaattttaaactctag aaataattttgtttaactttaagaaggagatatacatatgatcgtaaacctatggtacg caacaatatctgcctgaacgcccattcctcagggctgcaagaaggagtggaagatcagat tgaatataccaagaaacgcattaccgcagaagtcaaagctggcgcaaaagctccaaaaa cgttctggtgettggctgetcaaatggttacggcctggcgagccgcat tactgetgcgtt cggatacggggctgacgacctcggcggtgctcctttgaaaaagcgggttcagaaaccaaata tggtacaccgggatggtacaataatttggcatttgatgaagcggcaaaacgcgaggggtct ttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaaggcccaggttaattga ggaagccaaaaaaaagggtatcaaatttgatctgatcgatatacagcttgccagcccagt acgtactgatcctgatacaggtatcatgcacaaaagcgttttgaaaccctttgaaaaac gttcacaggcaaaacagtagatccgtttactggcgagctgaaggaaatctccgcggaacc agcaaatgacgaggaagcagccgccactgttaaagttatggggggtgaagattgggaacg ttggattaagcagctgtcgaaggaaggcctcttagaagaaggctgtattaccttggccta

tagttatattggccctgaagctacccaagctttgtaccgtaaaggcacaatcggcaaggc
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cttcgtgagcgtgaataaaggcctggtaacccgcgcaagcgccgtaatcccggtaatccc
tctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaatcatgaaggttgtat
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tgatgagggaaaatcgcatctgcattgatgattgggagttagaagaagacgtccagaaagc
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ggggtaccgccatgatttcttagctagtaacggctttgatgtagaaggatattaattatga
agcgggaagttgaacgcttcgaccgtatctgataagaaggagatatacatatgagagaagt
agtaattgccagtgacgtagaacagcagtaggaagttttggaggagcatttaaatacagt
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agcagagcaatggaatataactagagaagaacaagatgaattagctcttgcaagtcaaaa
taaagctgaaaaagctcaagctgaaggaaaatttgatgaagaaatagttcctgttggtat
aaaaggaagaaaagggtgacactgtagtagataaagatgaatatattaagcctggcactac
aatggagaaactt get aagttaagacctgcatttaaaaaagatggaacagt tact get gg
taatgcatcaggaataaatgatgggtgctgctatgttagtagtaatggctaaagaaaaagc
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ccctaaaataatgggatatggaccagttccagcaactaaaaaagctttagaagctgctaa
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agctgtaataagagacttaaatatagatatgaataaagttaatgttaatgggtggagcaat
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ttaactaagttagttactaagggaaaaatggatgaagctacaaaagcagaaatattaag
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Ttaa

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Butyrate, 11-10, 11-22, GLP-2

[0325] 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: 49). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding IL-2 (see, e.g., 50). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding IL-22 (see, e.g., 51). 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: 52). In some embodiments, the bacteria comprise a gene cassette for producing butyrate as described above, and a gene encoding SOD (see, e.g., 53). 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: 54). 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 NOs: 55-66 (Table 9). 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., Tables 10 and 11). 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., Tables 14-17). 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

[0326] 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, *pet*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*. In an alternate embodiment, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, *thrA^{fbf}*, *thrB*, *thrC*, *ilvA^{fbf}*, *aceE*, *aceF*, and *lpd*. In another alternate embodiment, the genetically engineered bacteria comprise *thrA^{fbf}*, *thrB*, *thrC*, *ilvA^{fbf}*, *aceE*, *aceF*, *lpd*, and *tesB*.

[0327] 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: 52), a gene encoding IL-22 (see, e.g., SEQ ID NO: 51), and a gene encoding IL-2 (see, e.g., SEQ ID NO: 50). 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: 55-66 (Table 9). 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., Tables 10 and 11). 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., Tables 14-17). 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, SOD, GLP-2, kynurenine

[0328] 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, *pet*, *lcdA*, *lcdB*, *lcdC*, *etfA*, *acrB*, and *acrC*. In an alternate embodiment, the genetically engineered bacteria comprise

pyruvate pathway genes for propionate biosynthesis, *thrA^{fb}*, *thrB*, *thrC*, *ilvA^{fb}*, *oceE*, *aceF*, and *lpd*. In another alternate embodiment, the genetically engineered bacteria comprise *thrA^{fb}*, *thrB*, *thrC*, *ilvA^{fb}*, *oceE*, *aceF*, *lpd*, and *tesB*.

[0329] 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-22 (see, e.g., SEQ ID NO: 51), 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 NOS: 55-66 (Table 9). 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., Tables 10 and 11). 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., Tables 14-17). 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

[0330] 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, *pet*, *lcdA*,

lcdB, *lcdC*, *etfA*, *ocrB*, and *ocrC*. In an alternate embodiment, the genetically engineered bacteria comprise pyruvate pathway genes for propionate biosynthesis, *thrA^{fb'}*, *thrB*, *thrC*, *ilvA^{fb'}*, *oceE*, *oceF*, and *lpd*. In another alternate embodiment, the genetically engineered bacteria comprise *thrA^{fb'}*, *thrB*, *thrC*, *ilvA^{fb'}*, *oceE*, *oceF*, *lpd*, and *tesB*.

[0331] 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: 52), 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 NOs: 55-66 (Table 9). 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., Tables 9 and 10). 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., Tables 14-17). In certain constructs, one or more of the genes is placed under the control of a tetracycline-inducible or constitutive promoter.

[0332] 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 below.

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		

Example 2. Transforming *E. coli*

[0333] 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 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.

[0334] In alternate embodiments, the butyrate cassette can be inserted into the Nissle genome through homologous recombination (Genewiz, Cambridge, MA).

Organization of the constructs and nucleotide sequences are provided herein. To create a vector capable of integrating the synthesized butyrate cassette construct into the chromosome, Gibson assembly was first used to add 1000bp sequences of DNA homologous to the Nissle lacZ locus into the R6K origin plasmid pKD3. This targets DNA cloned between these homology arms to be integrated into the lacZ locus in the Nissle genome. Gibson assembly was used to clone the fragment between these arms. PCR was used to amplify the region from this plasmid containing the entire sequence of the homology arms, as well as the butyrate cassette between them. This PCR fragment was used to transform electrocompetent Nissle-pKD46, a strain that contains a temperature-sensitive plasmid encoding the lambda red recombinase genes. After transformation, cells were grown out for 2 hours before plating on chloramphenicol at 20ug/mL at 37 degrees C. Growth at 37 degrees C also cures the pKD46 plasmid. Transformants containing cassette were chloramphenicol resistant and lac-minus (lac-).

Example 3. Production of Butyrate in Recombinant E. coli using tet-inducible promoter

[0335] Figures 15-17, 20 and 21 show butyrate cassettes described above under the control of a tet-inducible promoter. Production of butyrate is assessed using the methods described below in Example 4. 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-butyrate cassette in which tesB is substituted in place of pbt and buk genes. Figure 18 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).

[0336] 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. Figure 22 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.

[0337] Figure 19 shows the BW25113 strain of *E. Coli*, which is a common cloning strain and the background of the KEIO collection of *E. Coli* mutants. NuoB mutants having NuoB deletion were obtained. NuoB is a protein complex involved in the oxidation of NADH during respiratory growth (form of growth requiring electron transport). Preventing the coupling of NADH oxidation to electron transport allows an increase in the amount of NADH being used to support butyrate production. Figure 19 shows that compared with wild-type Nissle, deletion of NuoB results in greater production of butyrate.

pLOGIC046-tesB-butyrate:

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gtaaaacgacggccagtggaattcggttaagacccactttcacatttaagttgtttttctaa
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 GCCGTCCGCGCCAGCGCTGATGGCCTCCCTTCGGAAACGCAAATCGCCCAATCGCTGGC
 GCACCTGCTGCCGCCAGTGCTGAAAGATAAATTCATCTGCGATCGTCCGCTGGAAGTCCG
 TCCGGTGGAGTTTCATAACCCACTGAAAGGTCACGTCGCAGAACCACATCGTCAGGTGTG
 GATCCGCGCAAATGGTAGCGTGCCGGATGACCTGCGCGTTCATCAGTATCTGCTCGGTTA
 CGCTTCTGATCTTAACCTTCCTGCCGGTAGCTCTACAGCCGCACGGCATCGGTTTTCTCGA
 ACCGGGGATTGAGATTGCCACCATTTGACCATTTCCATGTGGTTCCATCGCCGTTTAATTT
 GAATGAATGGCTGCTGTATAGCGTGGAGAGCACCTCGGCGTCCAGCGCACGTGGCTTTGT
 GCGCGGTGAGTTTTATACCAAGACGGCGTACTGGTTGCCTCGACCGTTTCAGGAAGGGGT
 GATGCGTAAT CACAAT taa

Example 4. Production of Butyrate in Recombinant *E. coli*

[0338] Production of butyrate is assessed in *E. coli* Nissle strains containing the butyrate cassettes described above in order to determine the effect of oxygen on butyrate production. All incubations are performed at 37° C. Cultures of *E. coli* strains DH5a and Nissle transformed with the butyrate cassettes are grown overnight in LB and then diluted 1:200 into 4 mL of M9 minimal medium containing 0.5% glucose. The cells are grown with shaking (250 rpm) for 4-6 h and incubated aerobically or anaerobically in a Coy anaerobic chamber (supplying 90% N₂, 5% CO₂, 5%H₂). One mL culture aliquots are prepared in 1.5 mL capped tubes and incubated in a stationary incubator to limit culture aeration. One tube is removed at each time point (0, 1, 2, 4, and 20 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.

[0339] In an alternate embodiment, overnight bacterial cultures were diluted 1:100 into fresh LB and grown for 1.5 hrs to allow entry into early log phase. At this point, long half-life nitric oxide donor (DETA-NO; diethylenetriamine-nitric oxide adduct) was added to cultures at a final concentration of 0.3mM to induce expression from plasmid. After 2 hours of induction, cells were spun down, supernatant was discarded,

and the cells were resuspended in M9 minimal media containing 0.5% glucose. Culture supernatant was then analyzed at indicated time points to assess levels of butyrate production. Genetically engineered Nissle comprising pLogic031-nsrR-norB-butyrate operon construct; SYN133) or (pLogic046-nsrR-norB-butyrate operon construct; SYN145) produce significantly more butyrate as compared to wild-type Nissle (SYN001).

[0340] Genetically engineered Nissle were generated comprising a butyrate gene cassette in which the *pbt* and *buk* genes are replaced with *tesB* (SEQ ID NO: 24) expressed under the control of a tetracycline promoter (pLOGIC046-tesB-butyrate; SEQ ID NO: 81). SEQ ID NO: 81 comprises a reverse complement of the tetR repressor (underlined), an intergenic region containing divergent promoters controlling tetR and the butyrate operon and their respective RBS (bold), and the butyrate genes (*ter-thiA-hbd-crt2-tesB*) separated by RBS.

SEQ ID NO.: 81
<u>gtaaaacgacggccaagtgaattcgttaagaccactttcacatttaagttgtttttctaa</u> <u>tccgcataatgatcaattcaaggccgaataagaaggctggctctgcaccttgggtgatcaaa</u> <u>taattcgatagcttgtcgttaataatggcggcatactatcagtagtaggtgtttcccttc</u> <u>ttcttttagcgacttgatgctcttgatcttccaatacgaacctaaagtaaaatgccccac</u> <u>aqcgtgagtgcatataatgcattGtctagtgaaaaaccttgttggcataaaaaggctaa</u> <u>ttgattttcgagagtttcatactgtttttctgtaggccgtgtacctaataatgtacttttg</u> <u>tccatcgcgatgacttagtaaaagcacatctaaaacttttagcggttattacgtaaaaaatc</u> <u>ttgccagctttccccttctaaaggqcaaaaqtgaqtatqgtgcctatctaactct .caat</u> <u>qgctaaggcgctcgagcaaagcGcgcttattttttacatgccaatacaatgtaggctgctc</u> <u>tacacctagcttctgggaggtttacgggttgtaaaccttcgattccgacctcattaag</u> <u>cagctctaattgcgctgttaatcactttacttttatctaattctagacatcattaattccta</u> <u>atttttgttgacactctatcattgatagagttattttaccactccctatcagtgatagag</u> <u>aaaagtgaactctagaataattttgtttaactttaagaaggagatatacatatgatcgt</u> aaaacctatggtacgcaacaatatctgcctgaacgccatcctcagggctgcaagaaggg agtggaagatcagattgaatataccaagaacgcattaccgcagaagtcaaagctggcgc aaaagctccaaaaaacgttctggtgcttggtgctgctcaaatggttacggcctggcgagccg cattactgctgcttcggatacggggctgcgaccatcggcgtgtcctttgaaaaagcggg ttcagaaaccaa-atatggtacaccgggatggtacaataatttggcattttgatgaagcggc aaaacgcgaggggtctttatagcgtgacgatcgacggcgatgcgttttcagacgagatcaa ggcccaggttaattgaggaagccaaaaaaaagggtatcaaatttgatctgatcgtatacag cttggccagcccagtagctactgatcctgatacaggtatcatgcacaaaagcgttttgaa accctttgaaaaacgttcacaggcaaaacagtagatccgtttactggcgagctgaagga aatctccgcggaa.ccagcaaatacagaggaagcagccgccactgttaaagttatgggggg tgaagattgggaacgttggattaagcagctgtcgaaggaaggcctcttagaagaaggctg tattaccttggcctatagttatattggccctgaagctaccaagctttgtaccgtaaaagg cacaatcggcaaggccaaagaacacctggaggccacagcacaccgtctcaacaaagagaa cccgtcaatccgtgccttcgtgagcgtgaataaaggectggttaaccgcgcaagcgccgt aatcccggtaatccctctgtatctcgccagcttgttcaaagtaatgaaagagaagggcaa

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GCGCGGTGAGTTTTATACCAAGACGGCGTACTGGTTGCCTCGACCGTTCAGGAAGGGGT
GATGCGTAAT CACAAT taa

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[0341] Overnight bacterial cultures were diluted 1:100 into fresh LB and grown for 1.5 hrs to allow entry into early log phase. At this point, anhydrous tetracycline (ATC) was added to cultures at a final concentration of 100 ng/mL to induce expression of butyrate genes from plasmid. After 2 hours of induction, cells were spun down, supernatant was discarded, and the cells were resuspended in M9 minimal media containing 0.5% glucose. Culture supernatant was then analyzed at indicated time points to assess levels of butyrate production. Replacement of pbt and buk with tesB leads to greater levels of butyrate production.

[0342] Figure 24 shows butyrate production in strains comprising an FNR-butyrate cassette syn 363 (having the ter substitution) in the presence/absence of glucose and oxygen. Figure 24 Shows that bacteria need both glucose and anaerobic conditions for butyrate production from the FNR promoter. Cells were grown aerobically or anaerobically in media containing no glucose (LB) or in media containing glucose at 0.5% (RMC). Culture samples were taken at indicated time points and supernatant fractions were assessed for butyrate concentration using LC-MS. These data show that SYN 363 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 5. Efficacy of Butyrate-Expressing Bacteria in a Mouse Model of IBD

[0343] 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-1 β , TNF- α , IL-6, IFN- γ and IL-10).

Example 6. Generating a DSS-Induced Mouse Model of IBD

[0344] 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.

[0345] 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.

[0346] 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).

[0347] Figure 27 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). Figure 27 shows that Syn 363 (ter substitution) reduces inflammation and/or protects gut barrier as compared to Syn 94 (wildtype Nissle).

Example 7. Monitoring Disease Progression In Vivo

[0348] 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 hemocult; 1, soft stools with positive hemocult; 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.

[0349] 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 8. In Vivo Efficacy of Genetically Engineered Bacteria Following DSS Induction

[0350] 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 μ L 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.

[0351] 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-1 β , TNF- α , IL-6, IFN- γ , and IL-10), as well as myeloperoxidase activity, are measured using methods described below.

Example 9. Euthanasia Procedures for Rodent Models of IBD

[0352] Four and 24 hours prior to sacrifice, 5-bromo-2'-deoxyuridine (BrdU) (Invitrogen, Waltham, MA; Cat. No. B23151) may be intraperitoneal[^] 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 (Abeam, Cambridge, MA).

[0353] 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.

[0354] 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 10. Isolation and Preservation of Colonic Tissues

[0355] 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.

[0356] 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.

[0357] 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.

[0358] 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).

[0359] 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 11. Myeloperoxidase Activity Assay

[0360] 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.

[0361] 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.

[0362] 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 μ mol of peroxide per minute at 25 °C. This assay is used to analyze MPO activity in rodent colonic samples, particularly in DSS-induced tissues.

[0363] 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 μ L TMB (final concentration, 1.6 mM) dissolved in dimethylsulfoxide, and 70 μ L 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 12. RNA Isolation and Gene Expression Analysis

[0364] 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.

[0365] 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.

[0366] 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.

[0367] 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 13. Histology

[0368] 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.

[0369] 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 (Abeam). 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).

[0370] 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 14. Ex Vivo Culturing of Rodent Colons

[0371] 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 15. In Vivo Efficacy of Genetically Engineered Bacteria Following TNBS Induction

[0372] Apart from DSS, the genetically engineered bacteria described in 1 can also be tested in other chemically induced animal models of IBO. Non-limiting examples include those induced by oxazolone (Boirivant et al., 1998), acetic acid (MacPherson and Pfeiffer, 1978), indomethacin (Sabiou et al., 2016), sulfhydryl inhibitors (Satoh et al., 1997), and trinitrobenzene sulfonic acid (TNBS) (Gurtner et al., 2003; Segui 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 (Segui 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.

[0373] 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).

[0374] 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 16. Generating a Cell Transfer Mouse Model of IBD

[0375] 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 CD45RB^{hi} 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 CD45RB^{hi} 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

[0376] Following euthanization of G57BL/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 25×10^7 cells.

[0377] 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

[0378] 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/ 5×10^7 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.

[0379] 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 μ i 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 μ L FACS buffer. The plate is incubated on a rocking platform at 4 °C for 15 min. After two washes, cells are resuspended in 200 μ L of FACS buffer, transferred into twelve 75-mm flow tubes containing 150-200 μ L of FACS buffer, and the tubes are placed on ice.

[0380] 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 $25\text{-}50 \times 10^6$ cells/mL.

Purification of CD4⁺ CD45RB^{Hi} T Cells

[0381] Cell sorting of CD45RB^{Hi} and CD45RB^{Low} 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 CD45RB^{Hi} population is defined as the 40% of cells which exhibit the brightest CD45RB staining, whereas the CD45RB^{Low} population is defined as the 15% of cells with the dimmest CD45RB expression. Each of these populations is sorted individually, and the CD45RB^{Hi} cells are used for adoptive transfer.

Adoptive Transfer

[0382] Purified populations of CD4⁺ CD45RB^{Hi} 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 μ L PBS. Resuspended cells are transferred into an injection tube, with a maximum of 5×10^6 cells per tube, and diluted with cold PBS to a final concentration of 1×10^6 cells/mL. Injection tubes are kept on ice.

[0383] Prior to injection, recipient mice are weighed and injection tubes are gently inverted several times to mix the cells. Mixed cells (0.5 mL, $\sim 0.5 \times 10^6$ cells) are carefully drawn into a 1-mL syringe with a 26G3/8 needle attached. Cells are then intraperitoneally injected into recipient mice.

Example 17. Efficacy of Genetically Engineered Bacteria in a CD45RBHi T Cell Transfer Model

[0384] 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.

[0385] 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 μ L 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 18. Efficacy of Genetically Engineered Bacteria in a Genetic Mouse Model of IBD

[0386] 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).

[0387] 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 μ L 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.

[0388] 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., SAMPl/YitFc (Pizarro et al., 2011), dominant negative N-cadherin mutant (NCAD delta; Hermiston and Gordon, 1995), TNFAARE (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., TCR α ^{-/-} (Mombaerts et al., 1993; Sugimoto et al., 2008), WASP^{-/-} (Nguyen et al., 2007), Mdr1a^{-/-} (Wilk et al., 2005), IL-2 R α ^{-/-} (Hsu et al., 2009), Gai2^{-/-} (Ohman et al., 2002), and TRUC (Tbet^{-/-}Rag2^{-/-}; Garrett et al., 2007).

Example 19. Efficacy of Genetically Engineered Bacteria in a Transgenic Rat Model of IBD

[0389] 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 p2-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 μ L of bacteria (or vehicle) is administered by oral gavage to transgenic F344-HLA-B27 rats. Bacterial treatment is repeated once daily for 2 weeks.

[0390] 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.

[0391] 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 $\pm$ standard error.

Example 19. Butyrate-Producing Bacterial Strain Reduces Gut Inflammation in a Low-Dose DSS-Induced Mouse Model of IBD

[0392] At Day 0, 40 C57BL6 mice (8 weeks of age) were weighed and randomized into the following five treatment groups (n=8 per group): H₂O 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.

[0393] At Day 4, groups 4 and 5 were gavaged with bacteria, and then all mice were weighed. Cage water was changed to either H₂O + 0.5% DSS (groups 2, 4, and 5), or H₂O + 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.

[0394] 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).

[0395] FIG. 27 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 SYN363 produces enough butyrate to significantly reduce LCN2 concentrations, as well as gut inflammation, in a low-dose DSS-induced mouse model of IBD.

Example 20. Nitric oxide-inducible reporter constructs

[0396] ATC and nitric oxide-inducible reporter constructs were synthesized (Genewiz, Cambridge, MA). When induced by their cognate inducers, these constructs express GFP, which is detected by monitoring fluorescence in a plate reader at an excitation/emission of 395/509 nm, respectively. Nissle cells harboring plasmids with either the control, ATC-inducible Ptet-GFP reporter construct, or the nitric oxide inducible PnsrR-GFP reporter construct were first grown to early log phase (OD600 of about 0.4-0.6), at which point they were transferred to 96-well microtiter plates containing LB and two-fold decreased inducer (ATC or the long half-life NO donor, DETA-NO (Sigma)). Both ATC and NO were able to induce the expression of GFP in their respective constructs across a range of concentrations (Fig. 28); promoter activity is expressed as relative fluorescence 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.

[0397] 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 ($OD_{600} \approx 0.4-0.6$), 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 fluorescence units.

[0398] Figure 29 shows NO-GFP constructs (the dot blot) *E. coli* Nissle harboring the nitric oxide inducible NsrR-GFP reporter fusion were grown overnight in LB supplemented with kanamycin. Bacteria were then diluted 1:100 into LB containing kanamycin and grown to an optical density of 0.4-0.5 and then pelleted by centrifugation. Bacteria were resuspended in phosphate buffered saline and 100 microliters were administered by oral gavage to mice. IBD is induced in mice by supplementing drinking water with 2-3% dextran sodium sulfate for 7 days prior to bacterial gavage. At 4 hours post-gavage, mice were sacrificed and bacteria were recovered from colonic samples. Colonic contents were boiled in SOS, 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.

[0399] 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 HRP (horse radish peroxidase). Detection was visualized using Pierce chemiluminescent detection kit. Fig. 15 shows NsrR-regulated promoters are induced in DSS-treated mice, but not in untreated mice.

Claims

1. A genetically engineered bacterium comprising:
 - a) a first promoter that is induced by exogenous environmental conditions and operably linked to one or more of:
 - i. a first gene encoding a non-native anti-inflammation molecule;
 - ii. a first gene encoding a non-native gut barrier function enhancer molecule; and
 - iii. a gene cassette encoding a biosynthetic pathway, wherein the final product of the biosynthetic pathway is selected from the group consisting of an anti-inflammation molecule and a gut barrier function enhancer molecule.
2. The bacterium of claim 1, wherein the first promoter is induced under low-oxygen or anaerobic conditions.
3. The bacterium of claim 2, wherein the first promoter that is induced under low-oxygen or anaerobic conditions is a FNR-responsive promoter, an ANR-responsive promoter, or a DNR-responsive promoter.
4. The bacterium of claim 3, wherein the first promoter is a FNR-responsive promoter.
5. The bacterium of claim 1, wherein the first promoter is induced by the presence of reactive nitrogen species.
6. The bacterium of claim 1, wherein the first promoter is induced by the presence of reactive oxygen species.
7. The bacterium of any one of claims 1-6, wherein the first gene and/or gene cassette is located on a chromosome in the bacterium.

8. The bacterium of any one of claims 1-7, wherein the first gene and/or gene cassette is located on a plasmid in the bacterium.
9. The bacterium of any one of claims 1-8, wherein the anti-inflammation and/or gut barrier enhancer molecule is selected from a short-chain fatty acid, propionate, butyrate, acetate, IL-10, IL-27, TGF-R2, TGF- β I, GLP-2, NAFEs, elafin, and trefoil factor.
10. The bacterium of any one of claims 1-8, wherein the anti-inflammation and/or gut barrier enhancer molecule is selected from a scFv, antisense RNA, siRNA, and shRNA directed against a pro-inflammatory molecule selected from TNF- α , IFN- γ , IL-1 β , IL-6, IL-8, IL-17, CXCL-8, and CCL2.
11. The bacterium of any one of claims 1-10, wherein the bacterium is a probiotic bacterium.
12. The bacterium of claim 11, wherein the bacterium is selected from the group consisting of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Escherichia*, *Lactobacillus*, and *Lactococcus*.
13. The bacterium of claim 12, wherein the bacterium is *Escherichia coli* strain Nissle.
14. The bacterium of any one of claims 1-13, wherein the bacterium is an auxotroph in a gene that is complemented when the bacterium is present in a mammalian gut.
15. The bacterium of claim 14, wherein mammalian gut is a human gut.
16. The bacterium of claim 14 or 15, wherein the bacterium is an auxotroph in diaminopimelic acid or an enzyme in the thymine biosynthetic pathway.

17. The bacterium of any one of claims 1-16, wherein the bacterium is further engineered to harbor a second gene coding for a substance toxic to the bacterium, wherein the second gene is under the control of a second promoter that is directly or indirectly induced by an environmental factor not naturally present in a mammalian gut.
18. The bacterium of any one of claims 1-17, wherein the bacterium is further engineered to harbor a third gene coding for a substance toxic to the bacterium, wherein the third gene is under the control of the first promoter, and wherein the expression of the toxic substance is delayed in time as compared to the expression of the anti-inflammation molecule, the gut barrier enhancer molecule, or the gene cassette encoding the biosynthetic pathway.
19. A pharmaceutically acceptable composition comprising the bacterium of any one of claims 1-18; and a pharmaceutically acceptable carrier.
20. The composition of claim 19 formulated for oral or rectal administration.
21. 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 19 or 20.
22. 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 19 or 20.
23. The method of claim 21, 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 osteomyelitis (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.

24. The method of claim 23, 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.
25. The method of claim 22, wherein the disease or condition is selected from an inflammatory bowel disease, including Crohn's disease and ulcerative colitis, and a diarrheal disease.

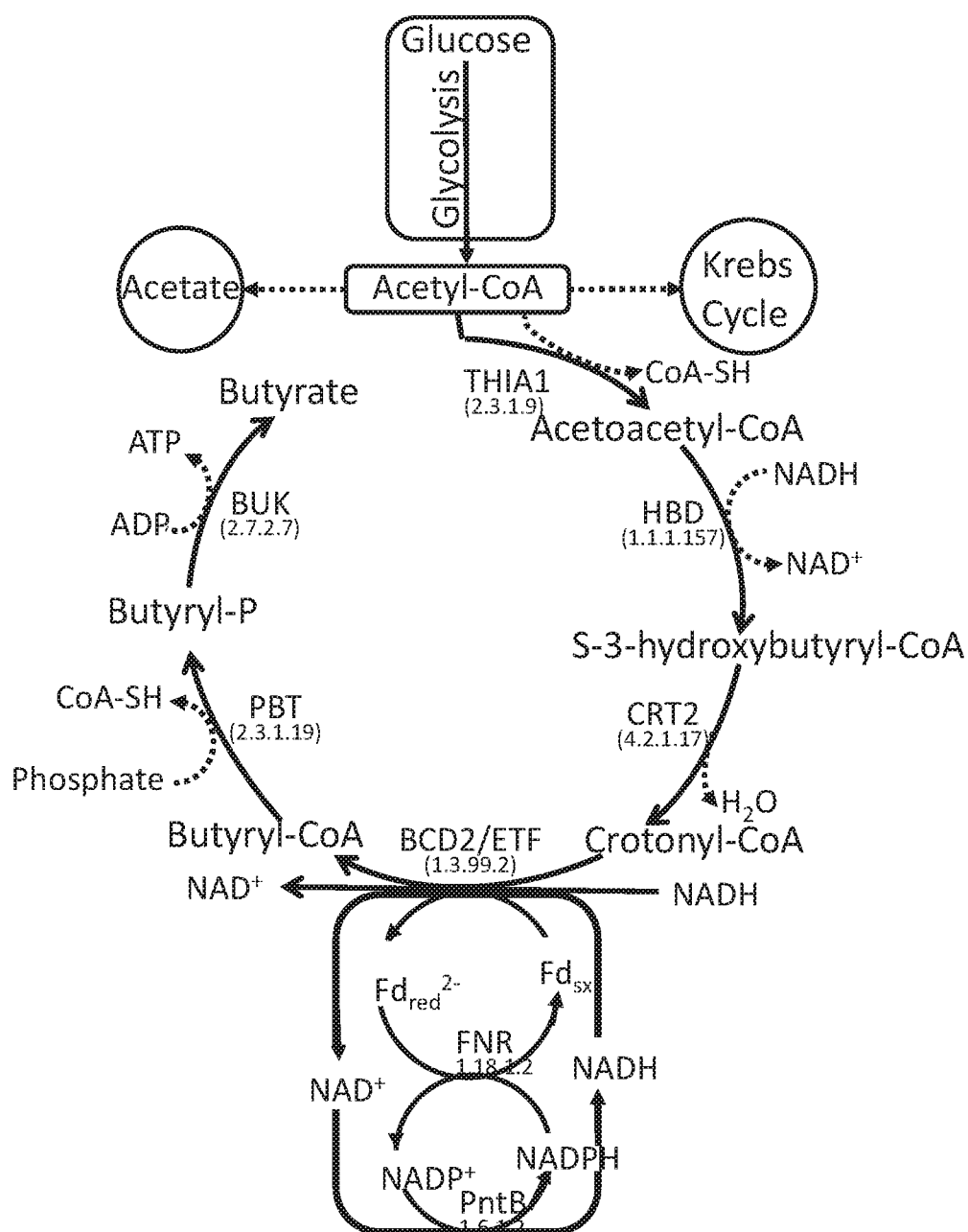


Fig. 1A

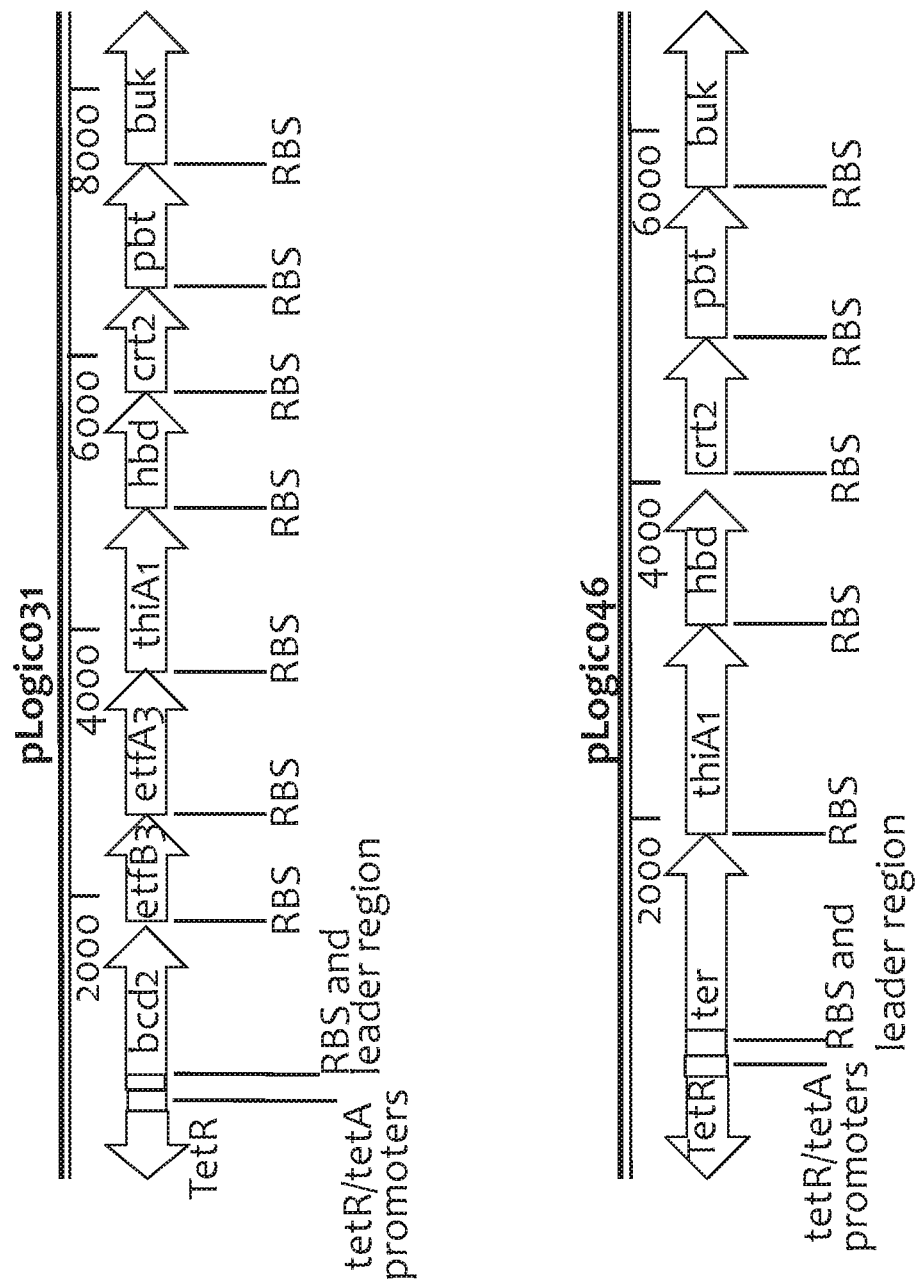


Fig. 1B

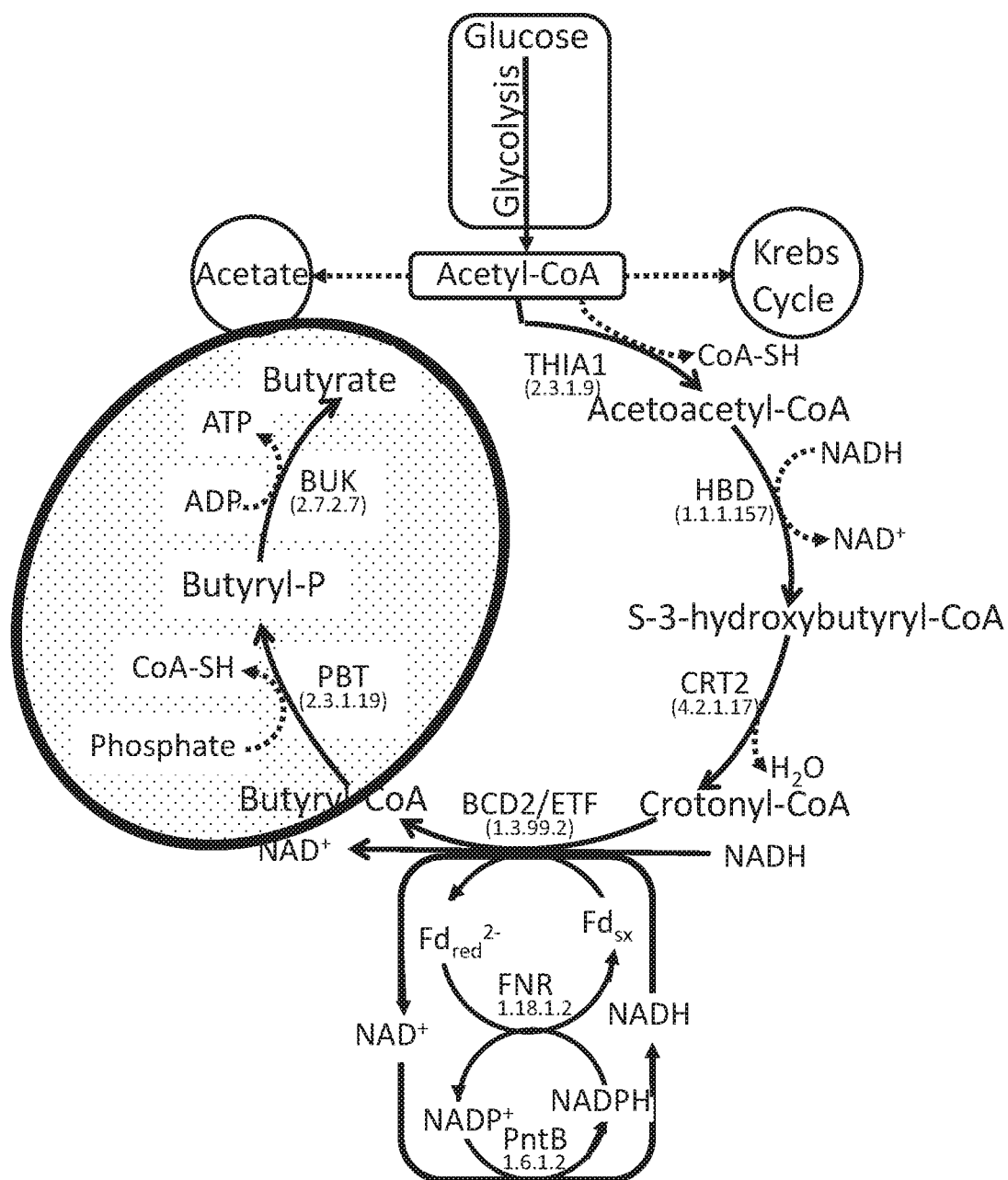
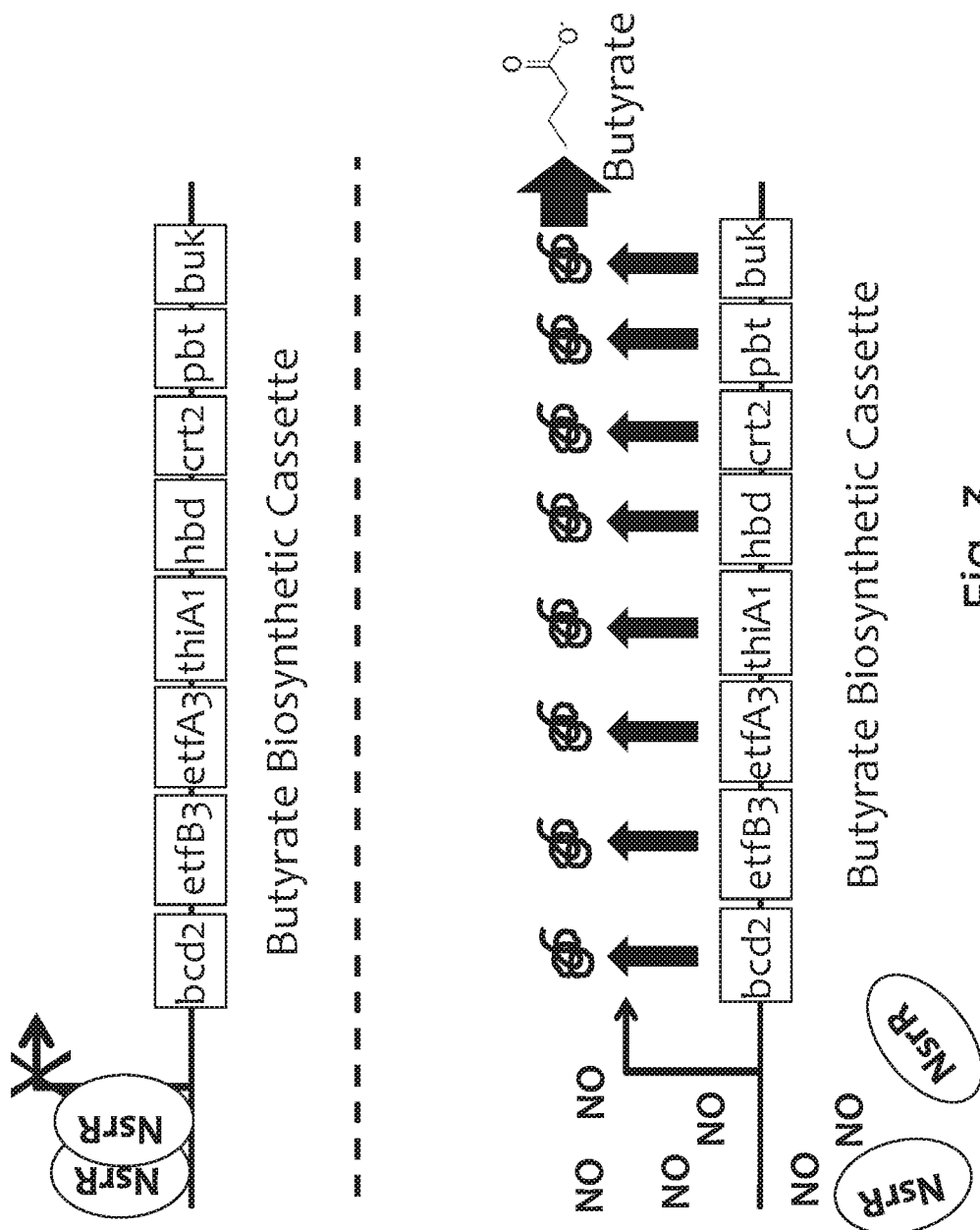


Fig. 2



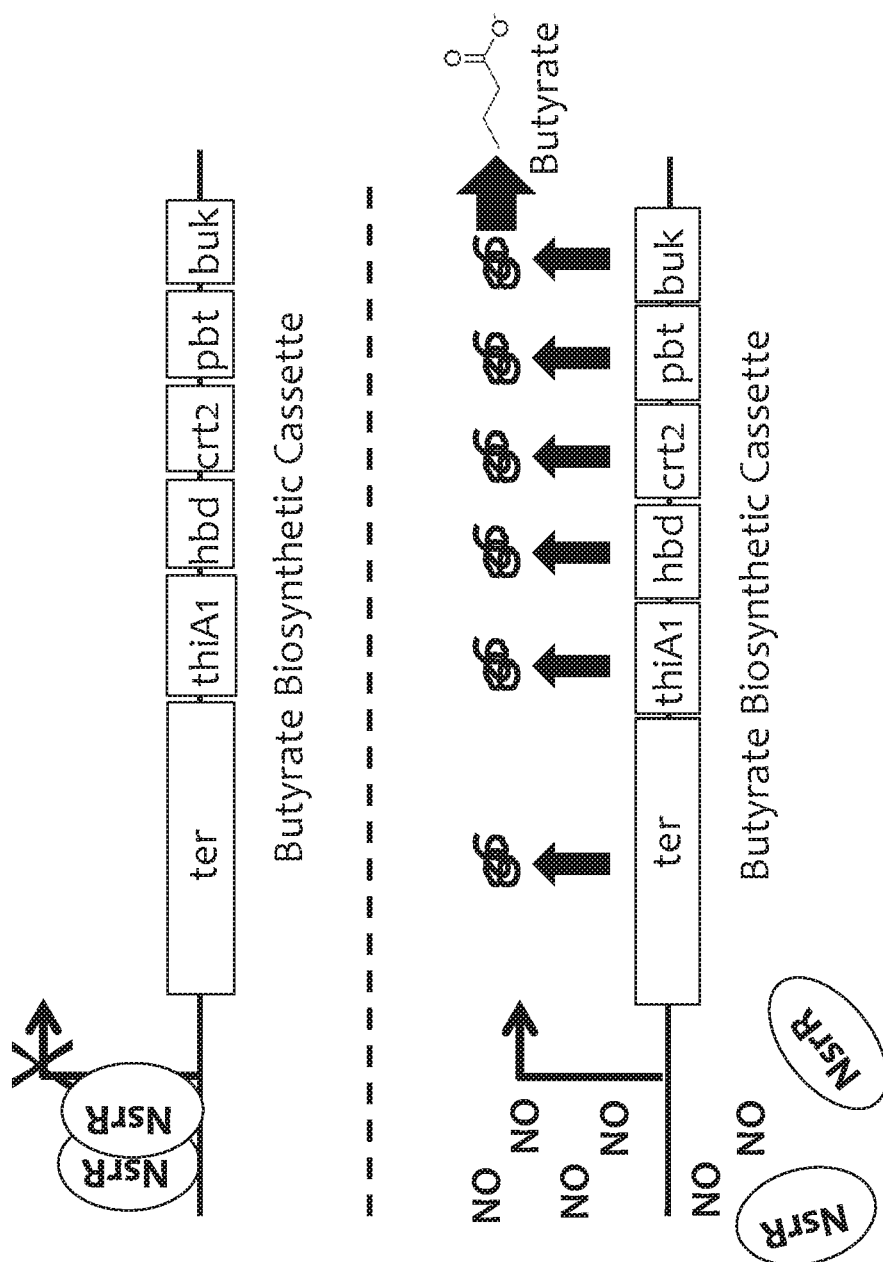


Fig. 4

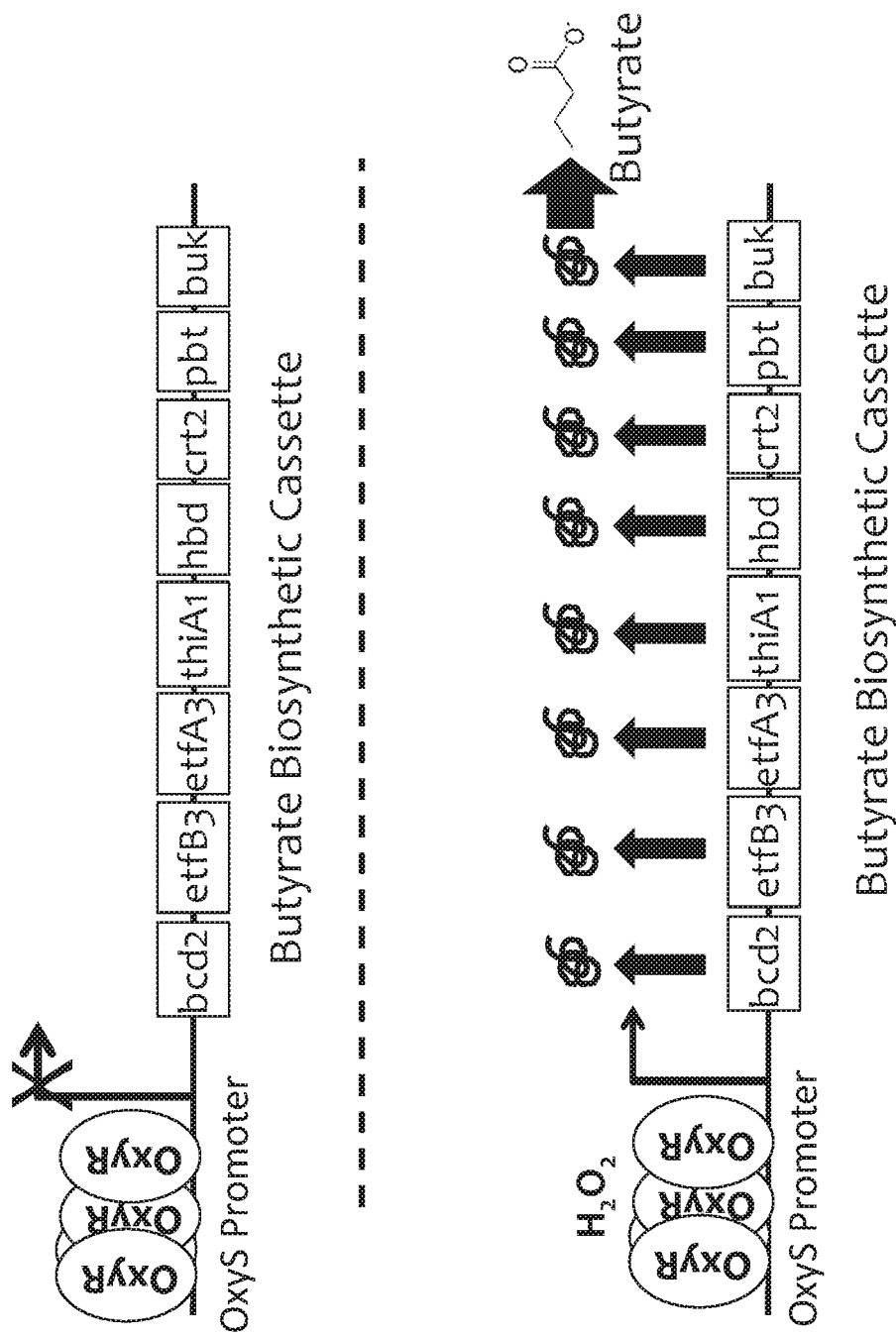


Fig. 5

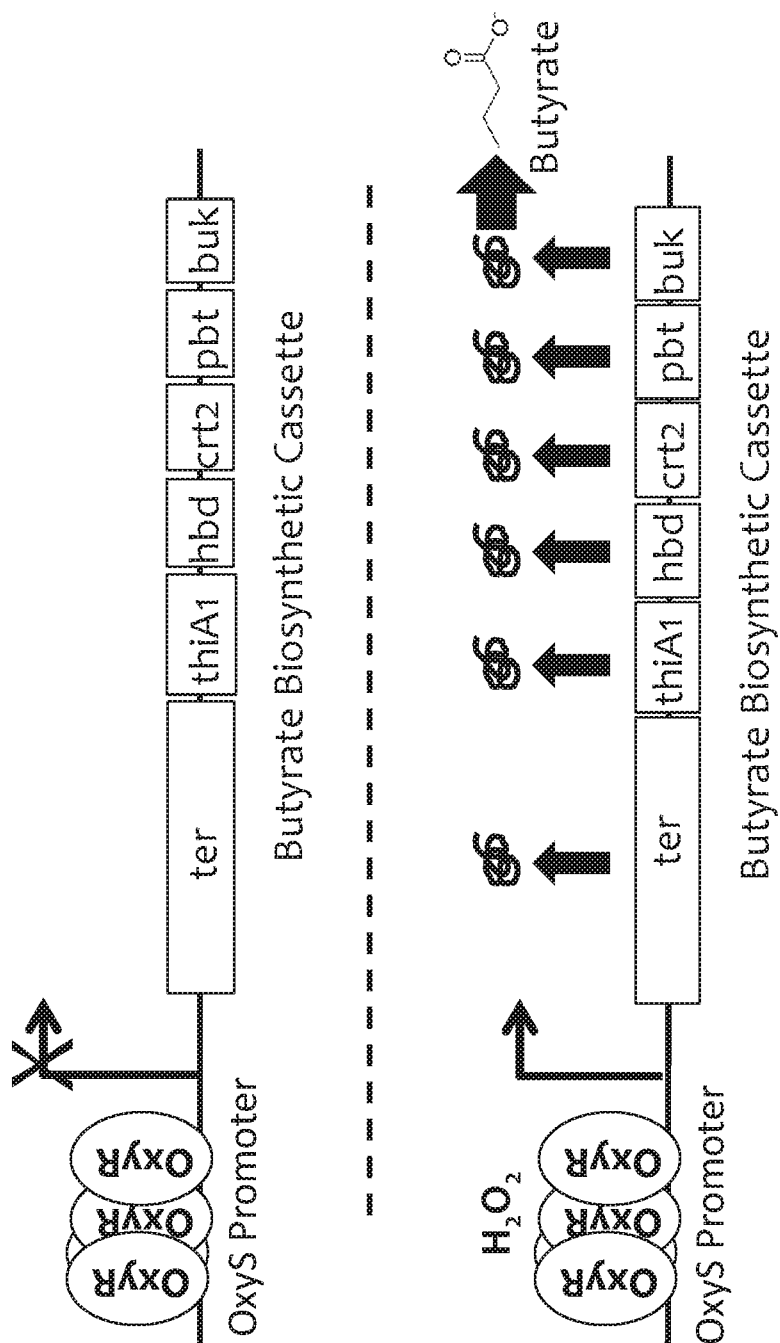


Fig. 6

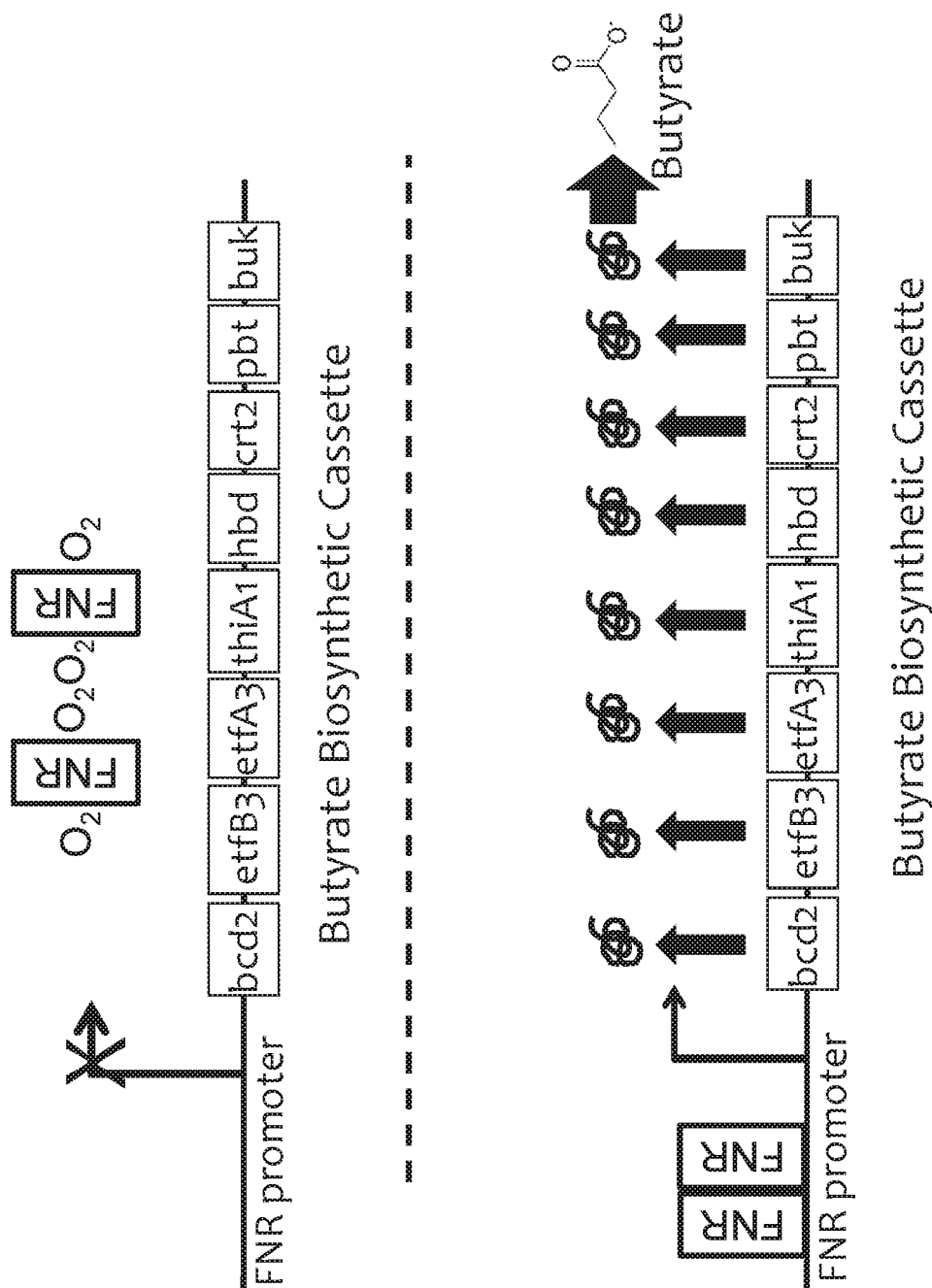


Fig. 7

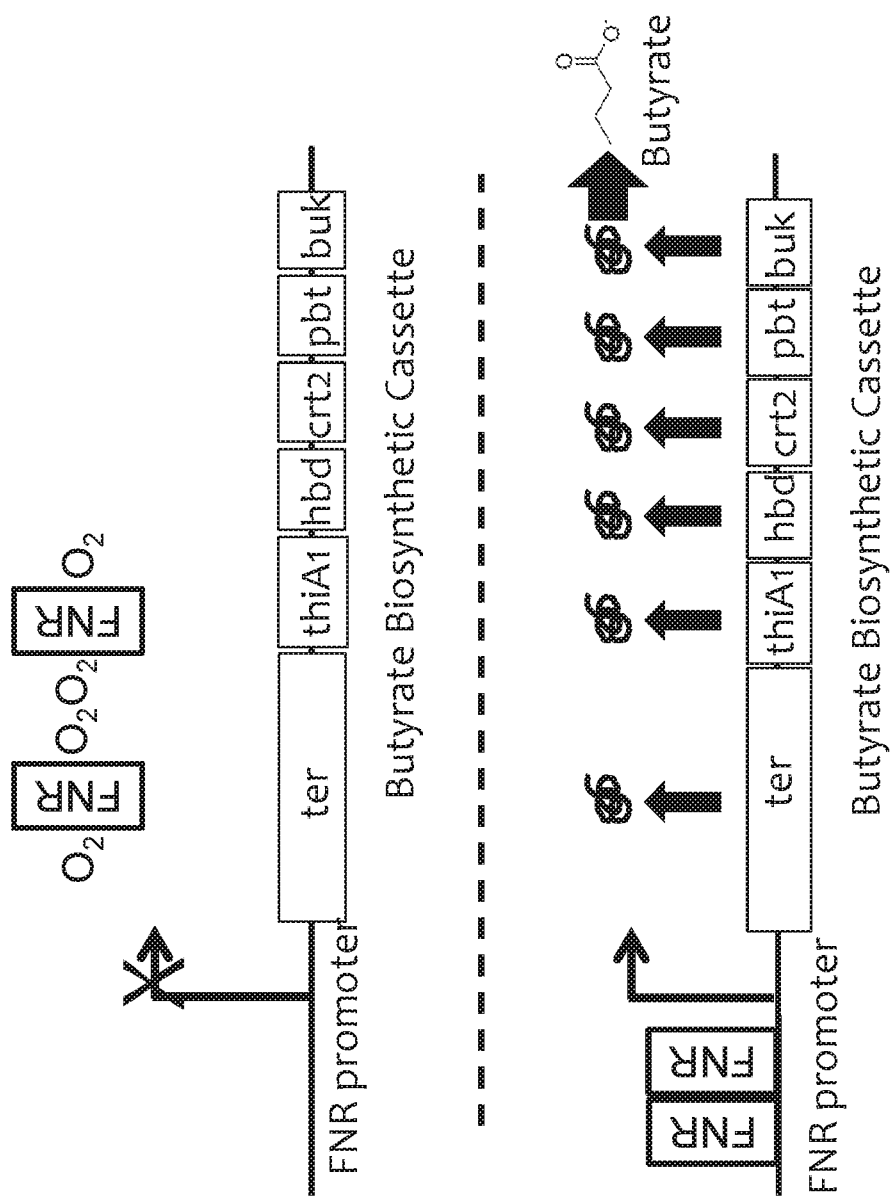


Fig. 8

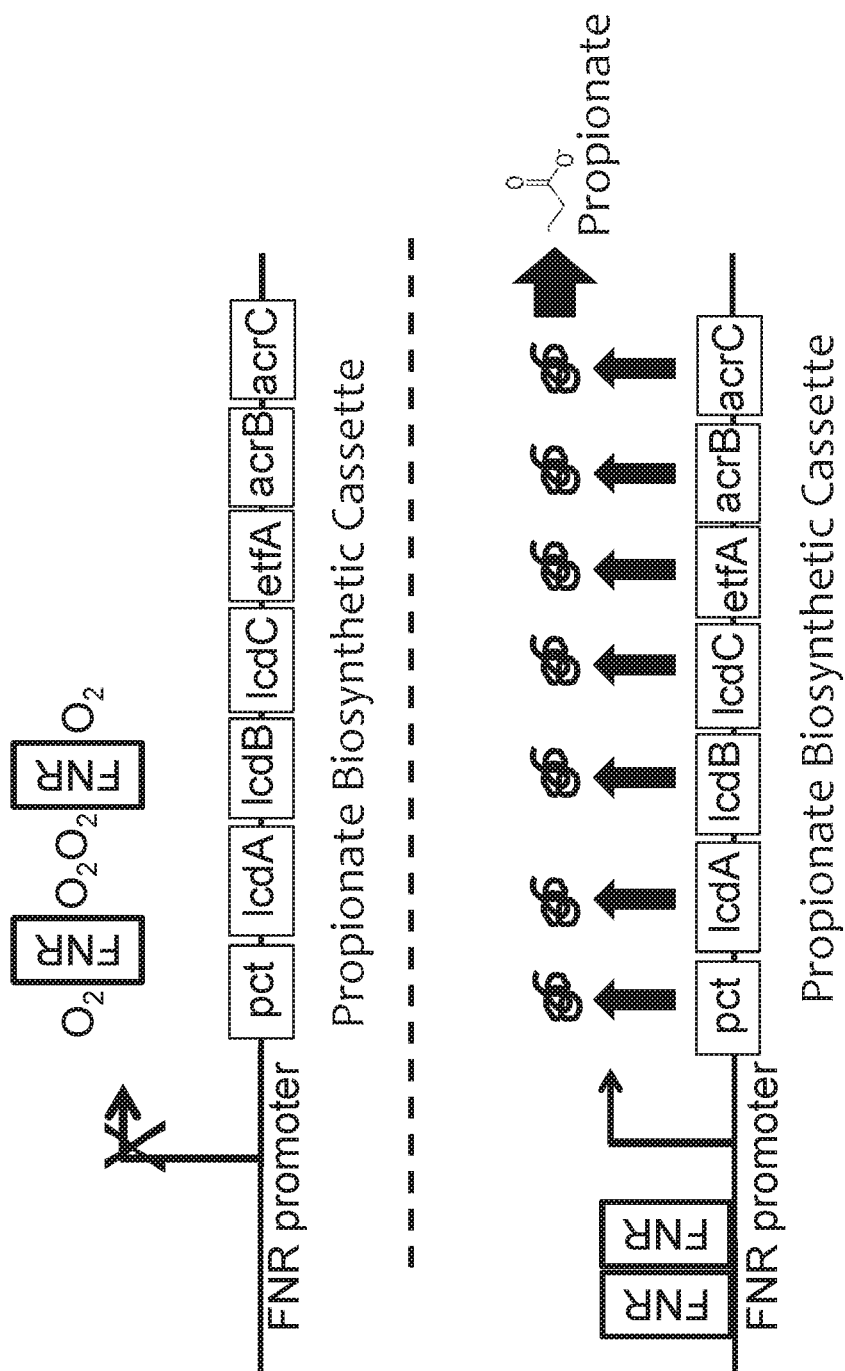


Fig. 9

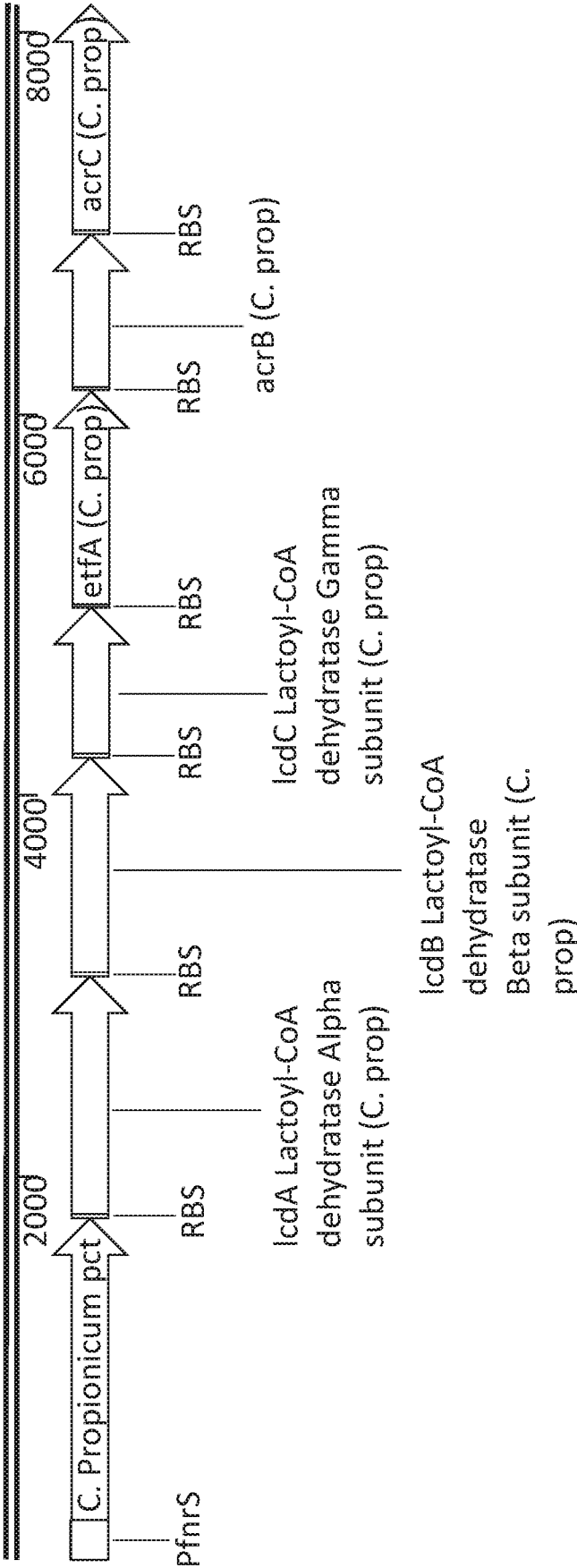


Fig. 10

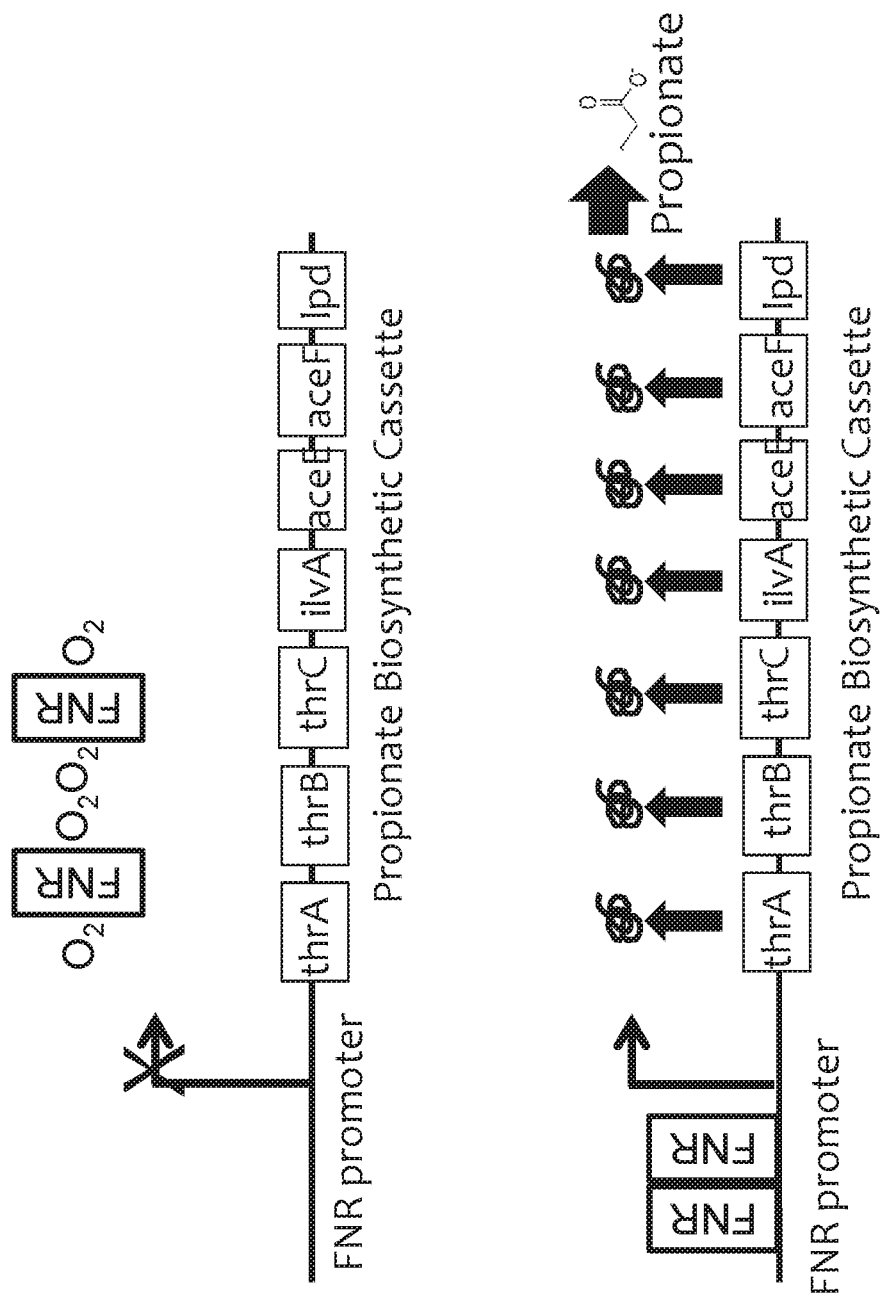


Fig. 11

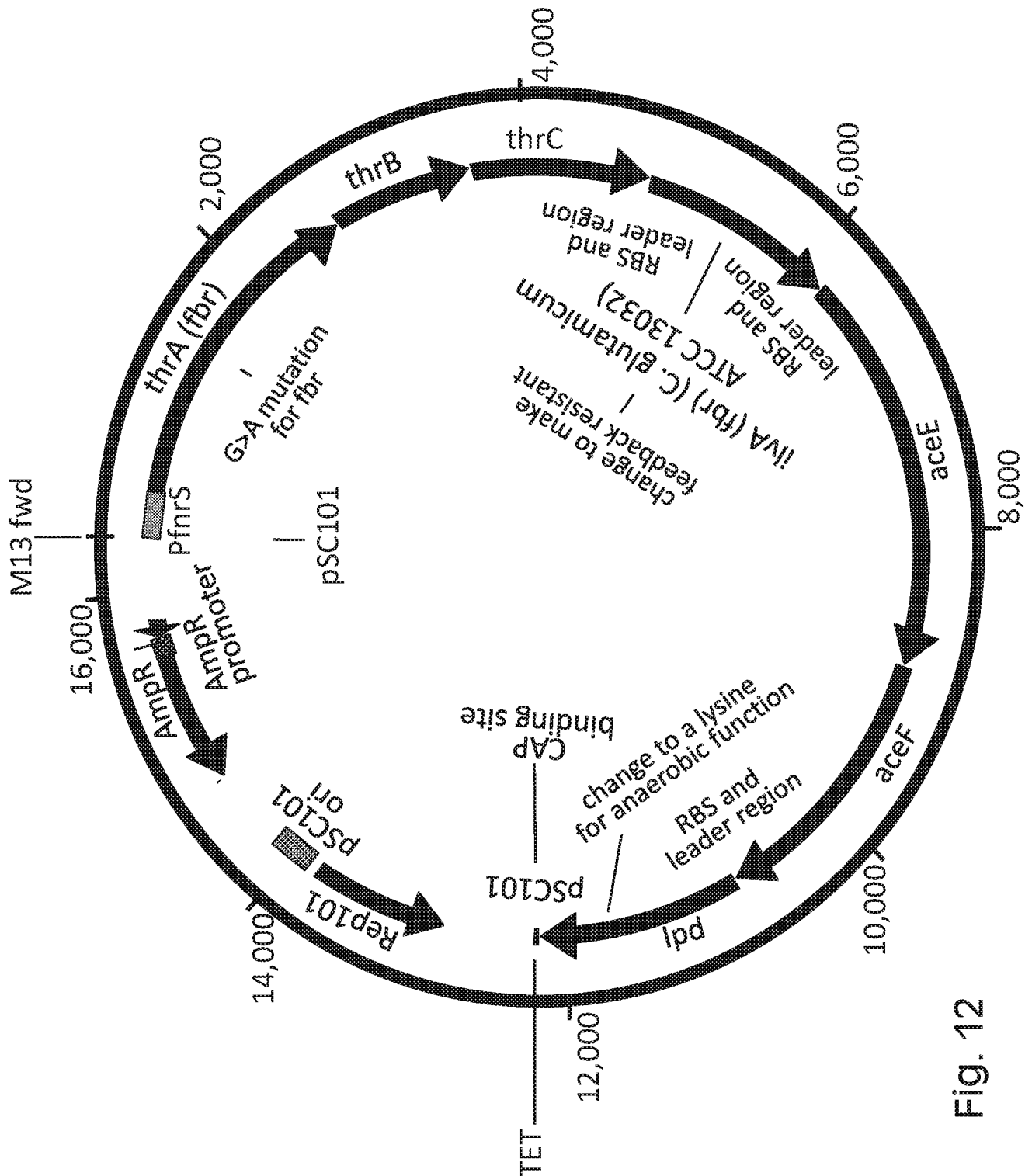


Fig. 12

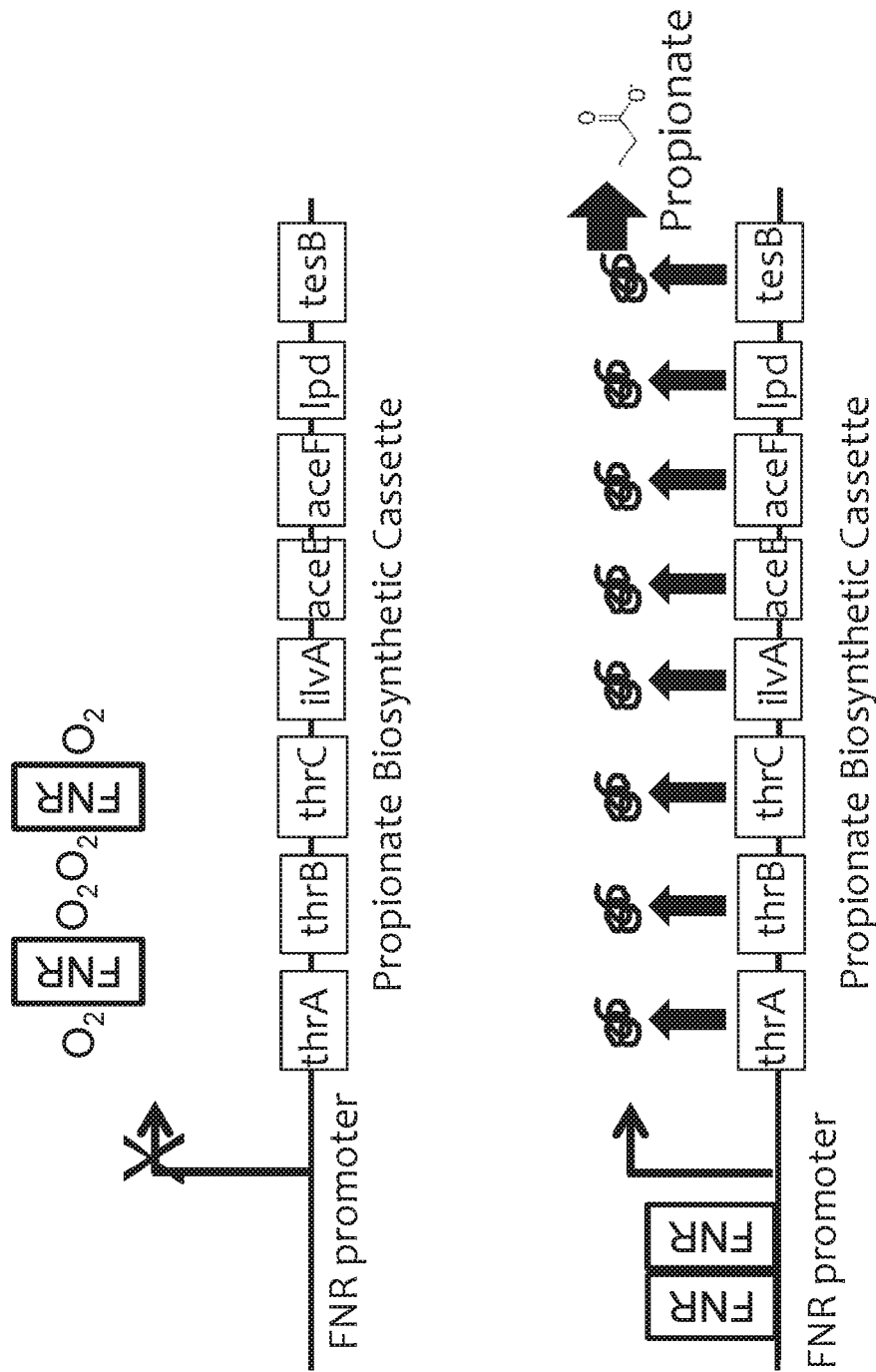
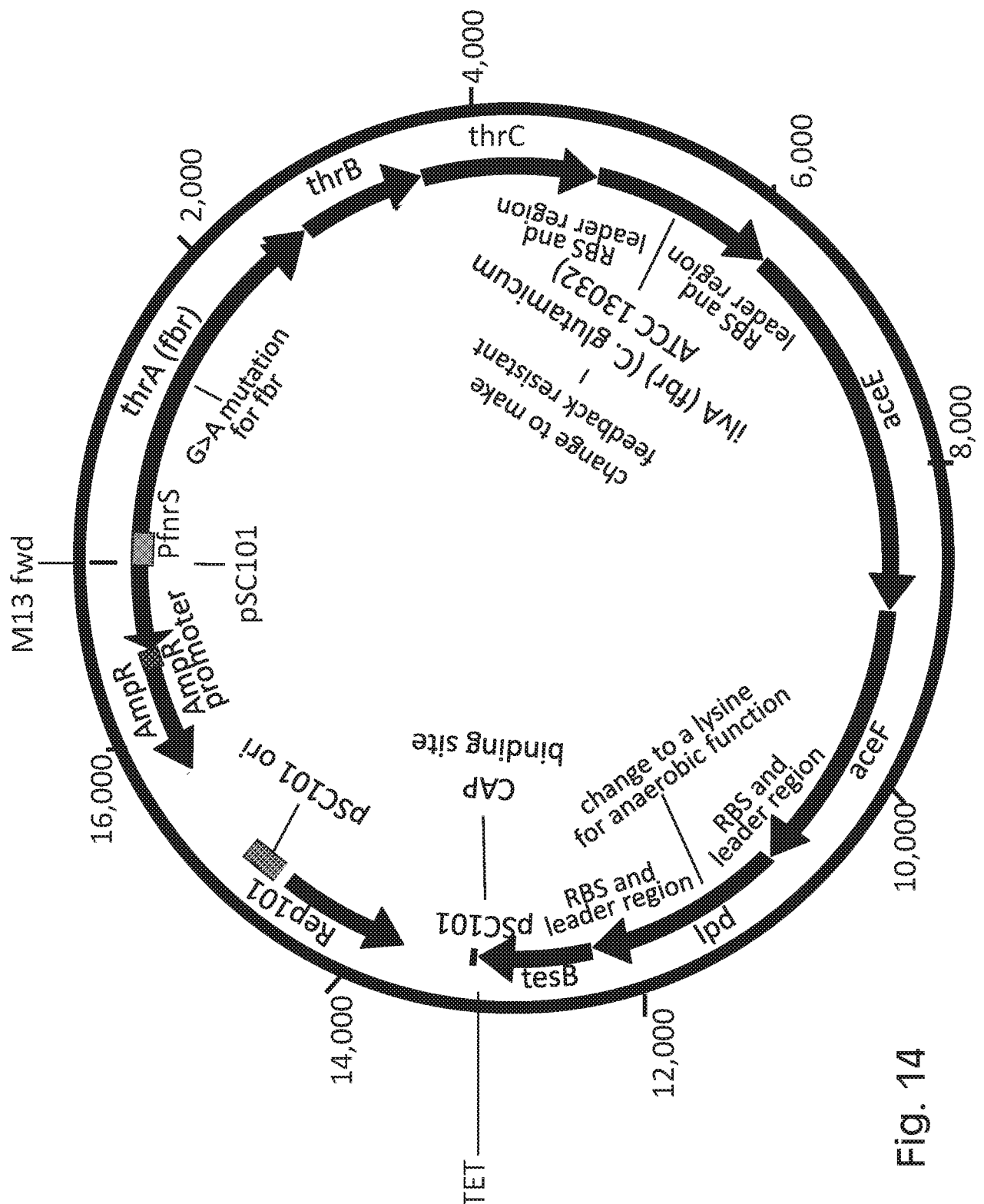


Fig. 13



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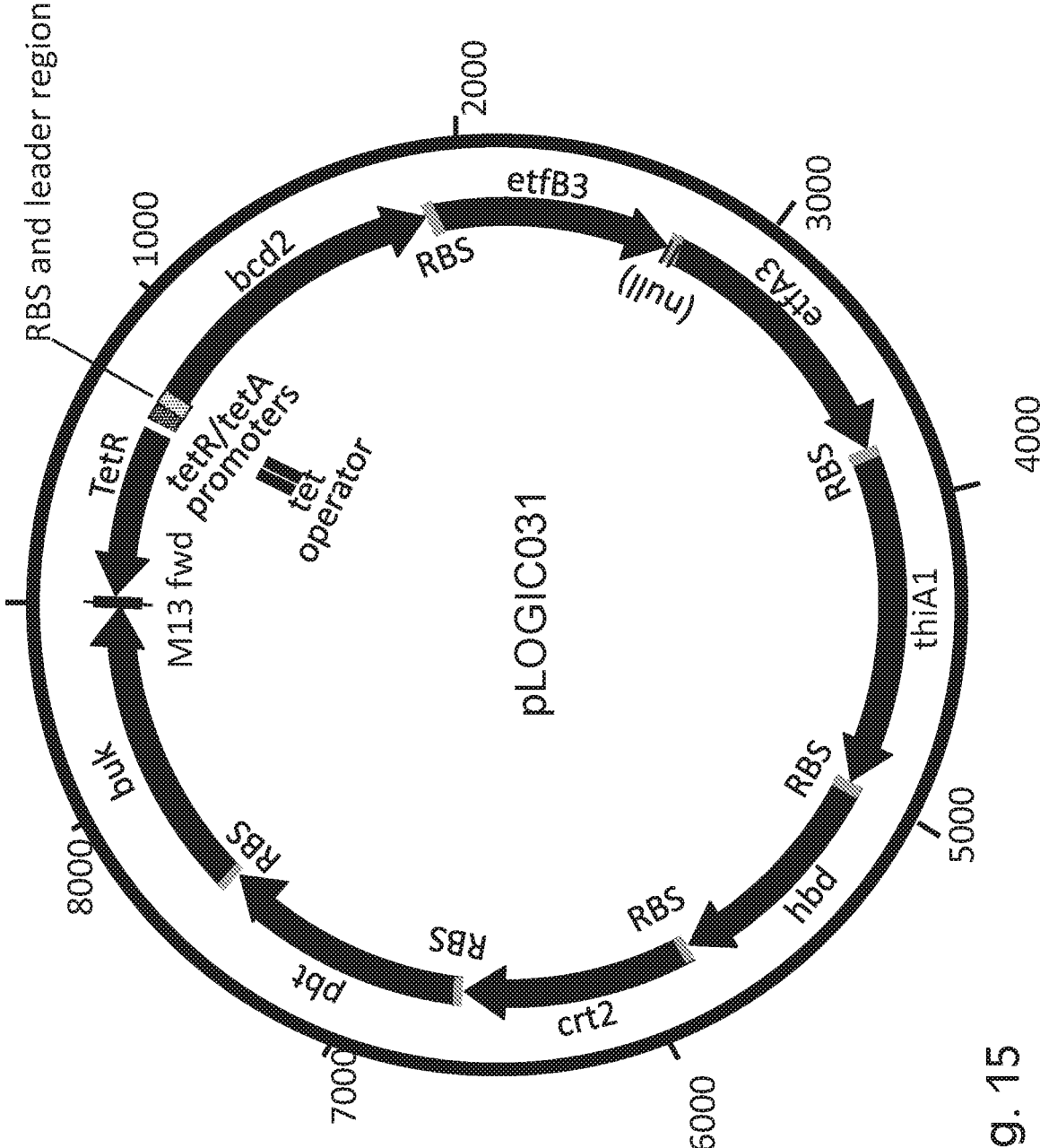


Fig. 15

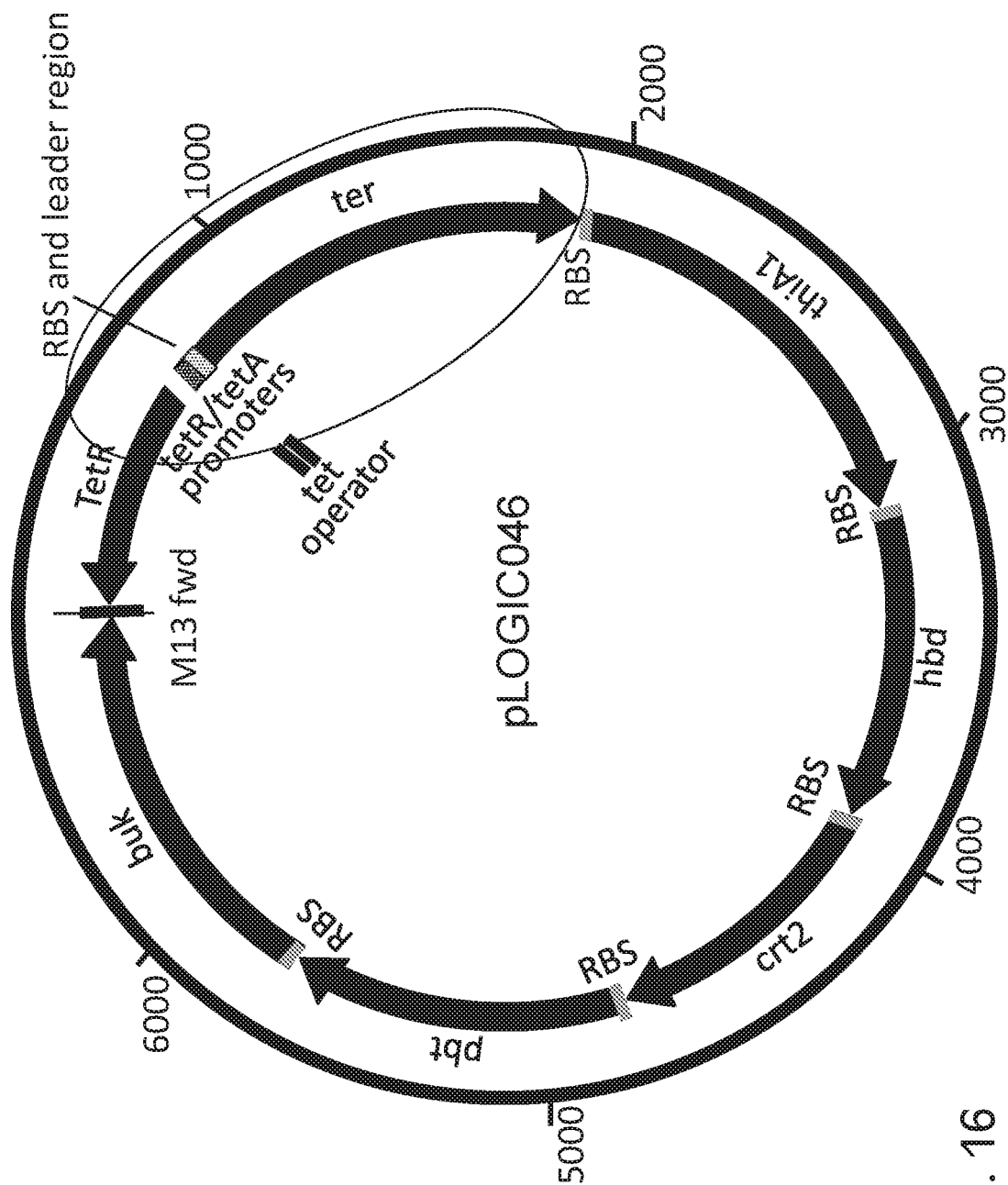


Fig. 16

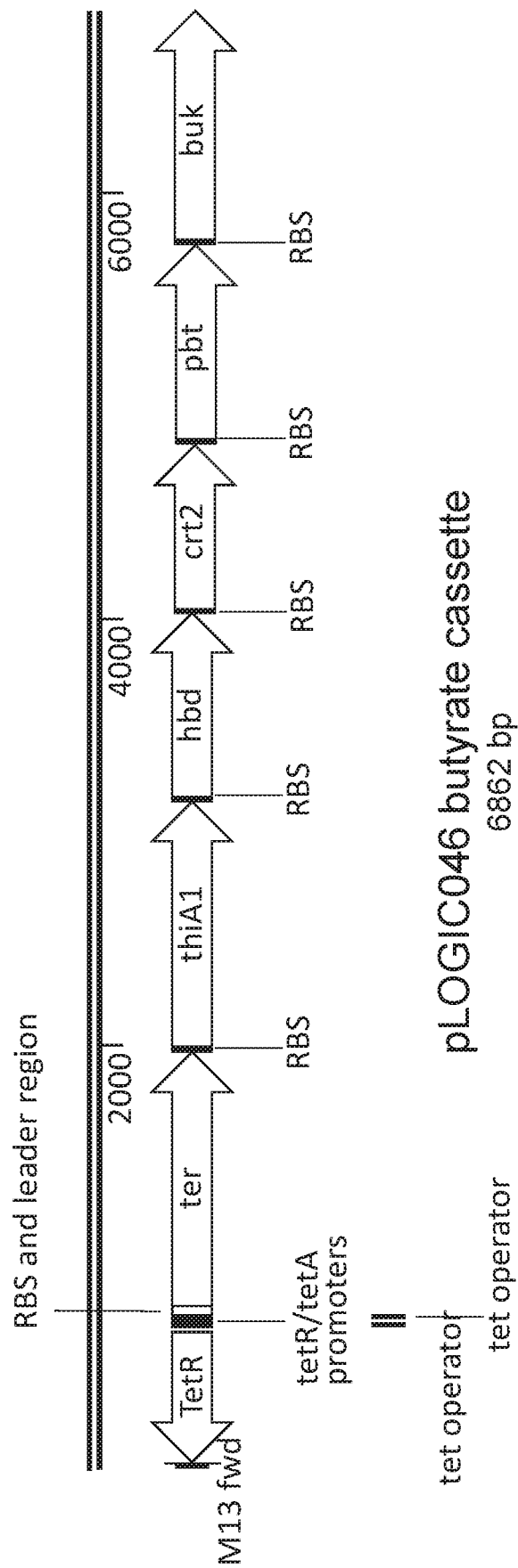


Fig. 17

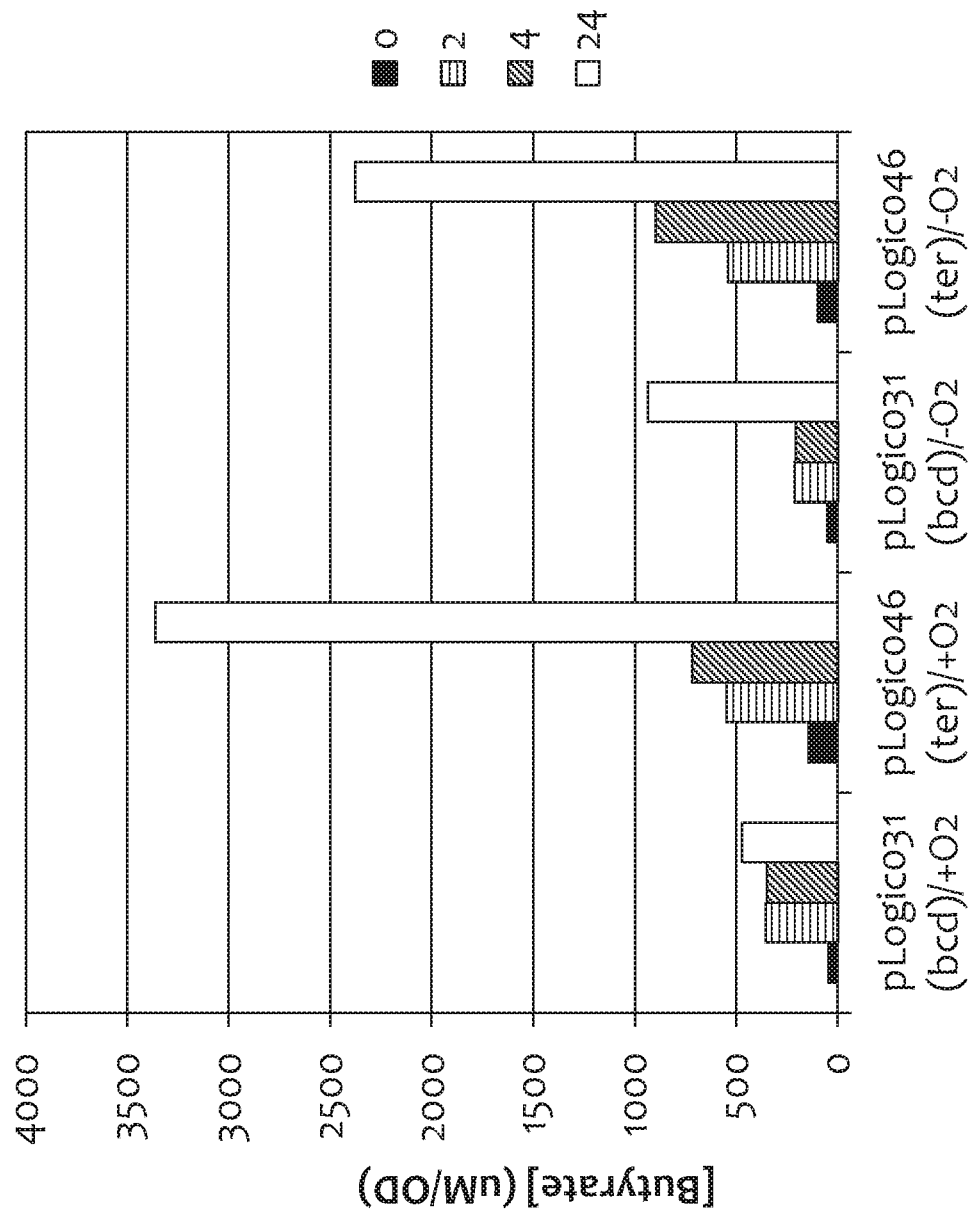
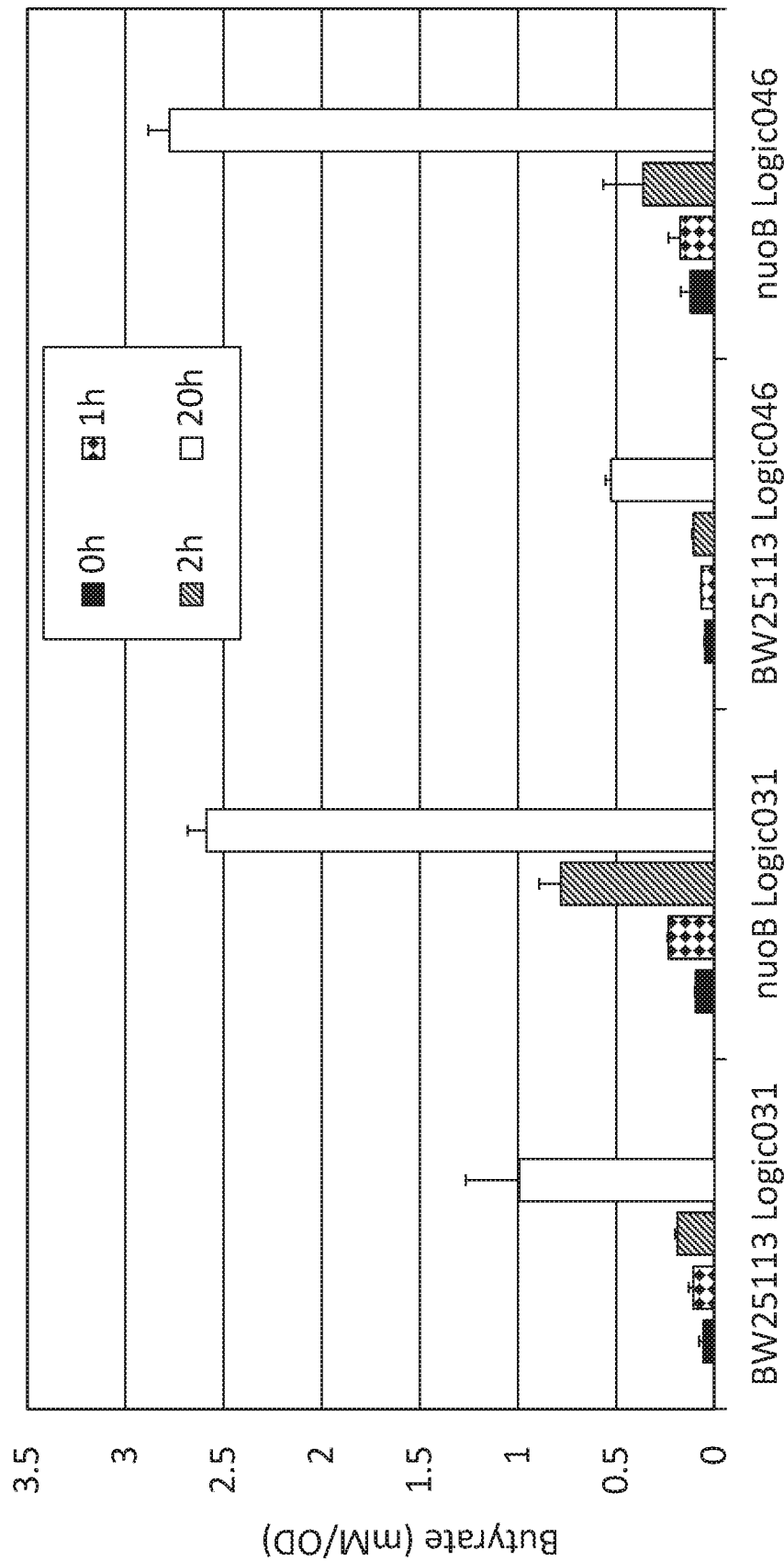


Fig. 18



Logic031 – pBR322/*bcd*-butyrate cassette
Logic046 – pBR322/*ter*-butyrate cassette

Fig. 19

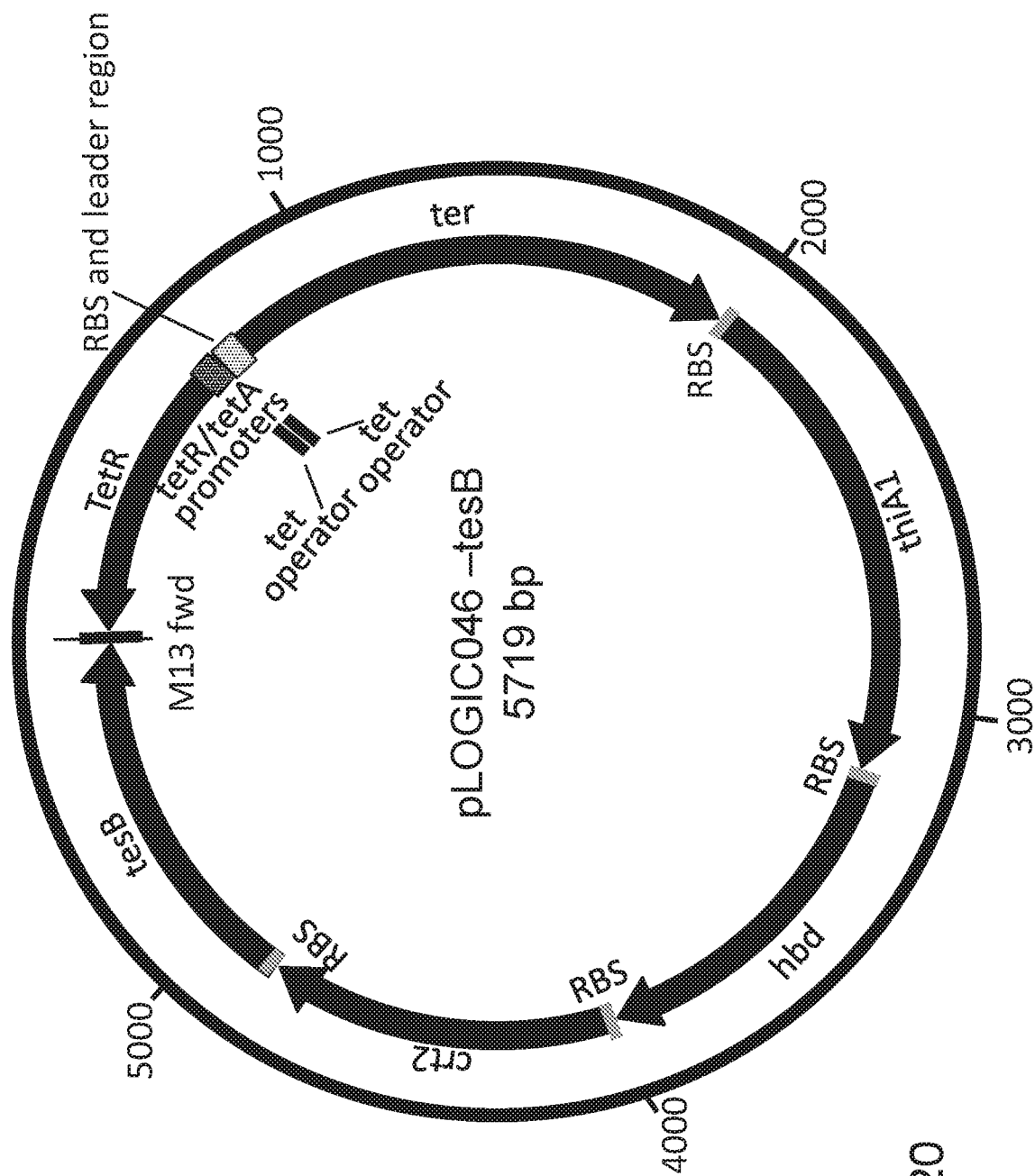


Fig. 20

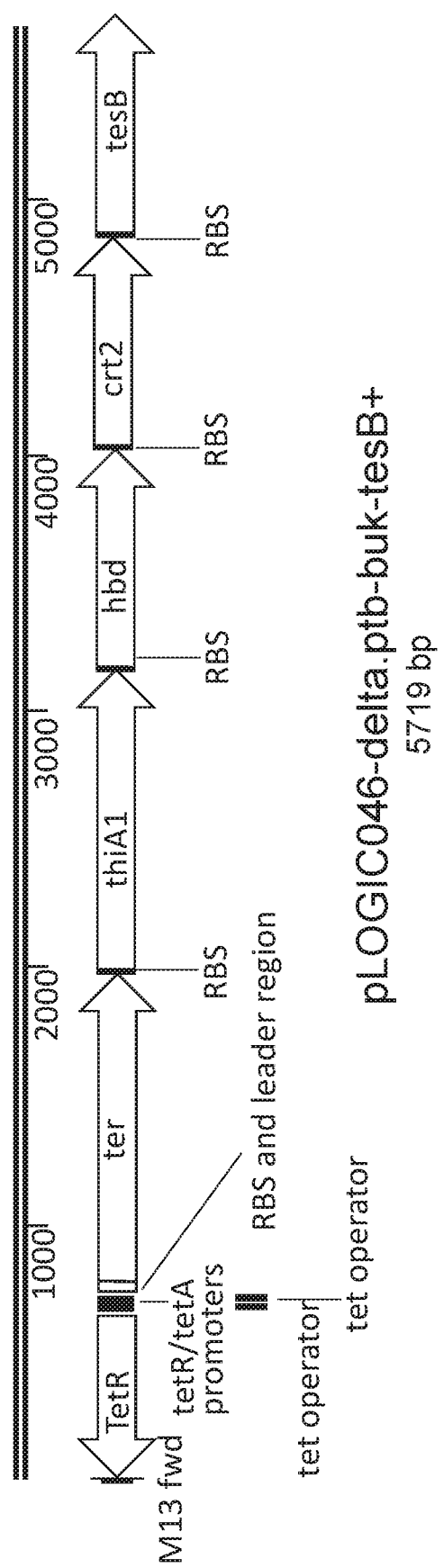


Fig. 21

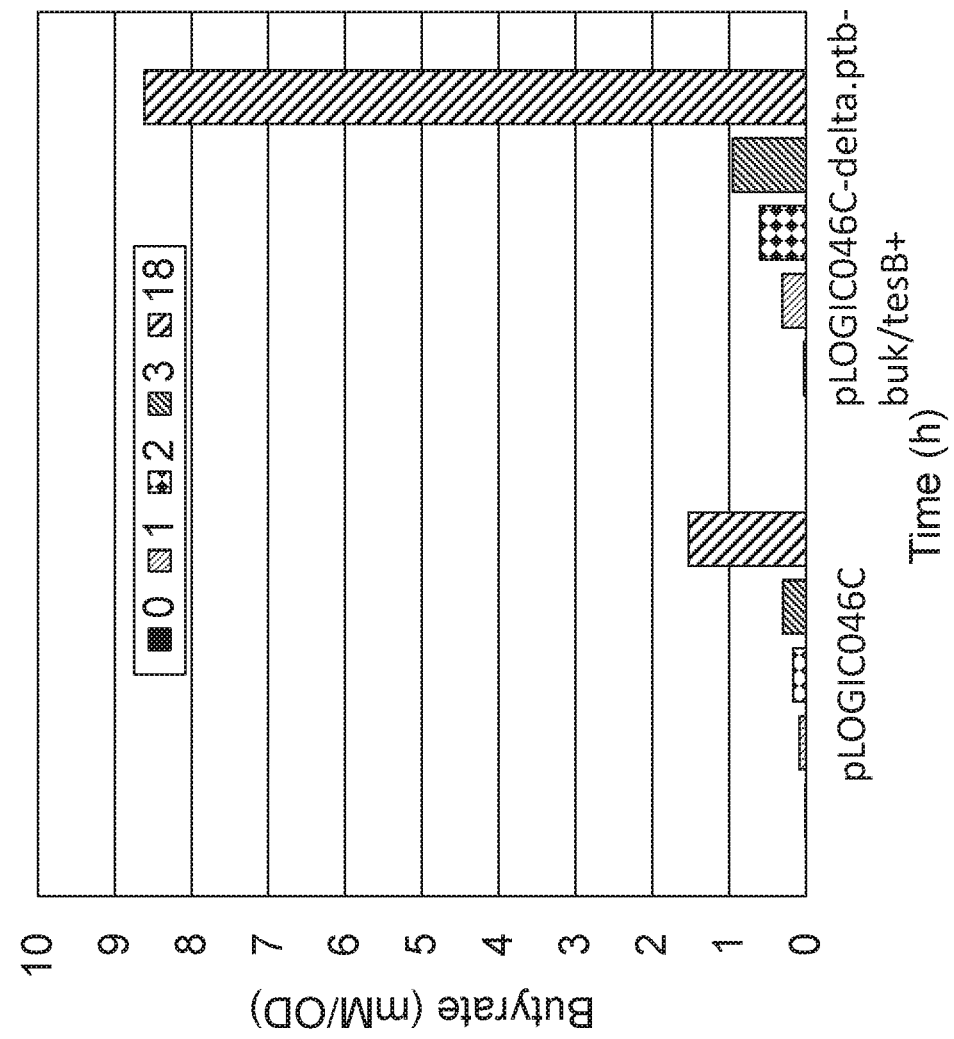


Fig. 22

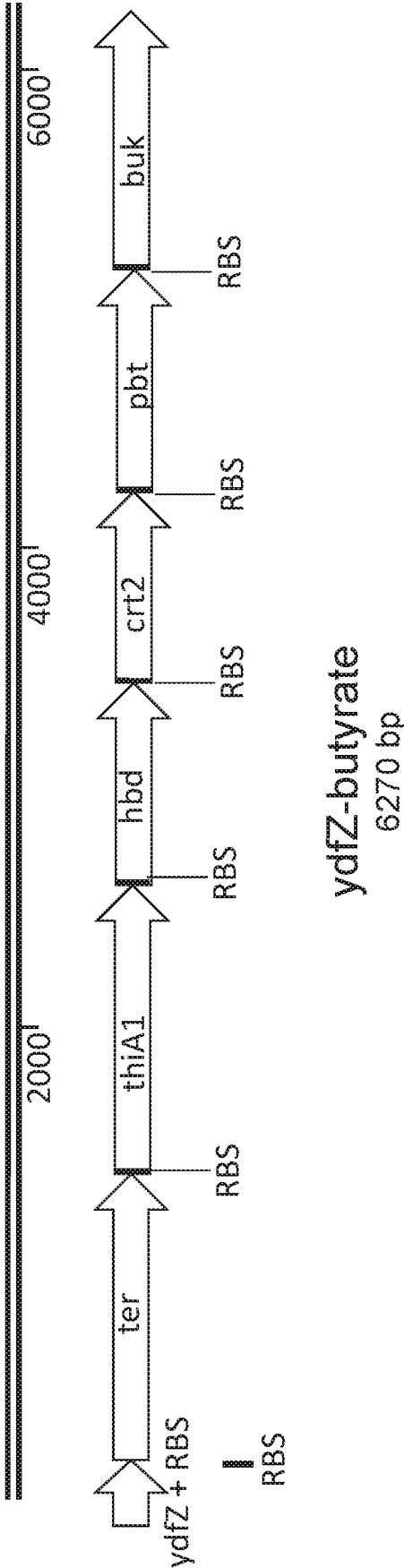


Fig. 23

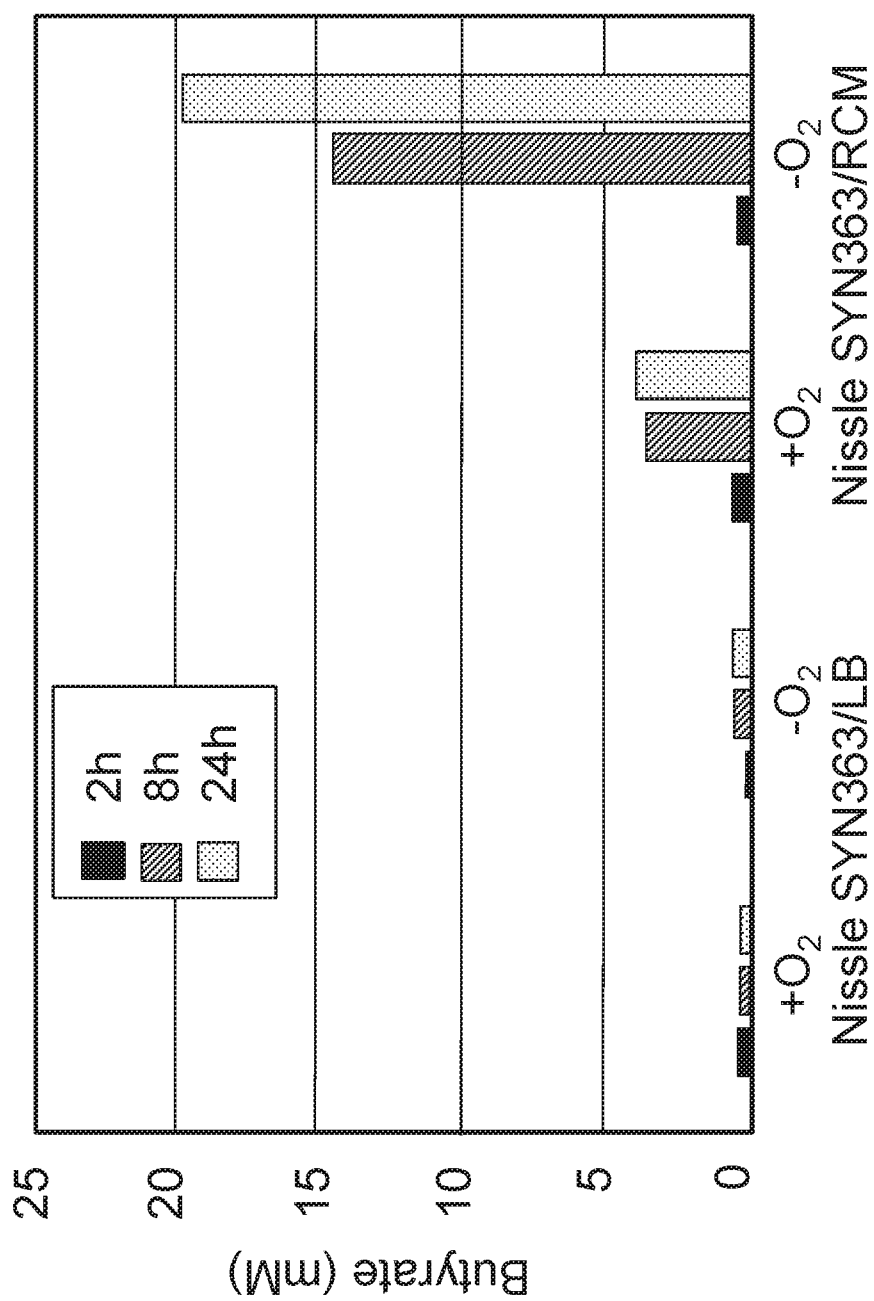


Fig. 24

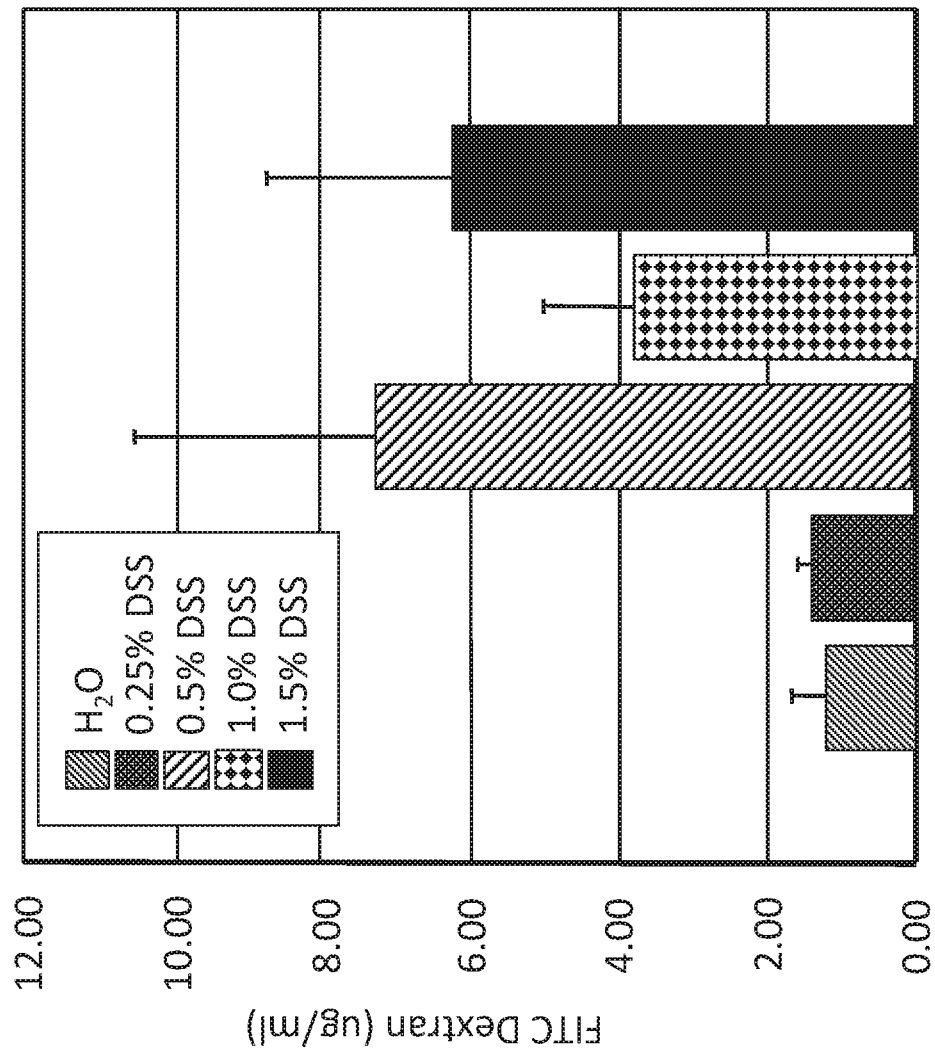


Fig. 25

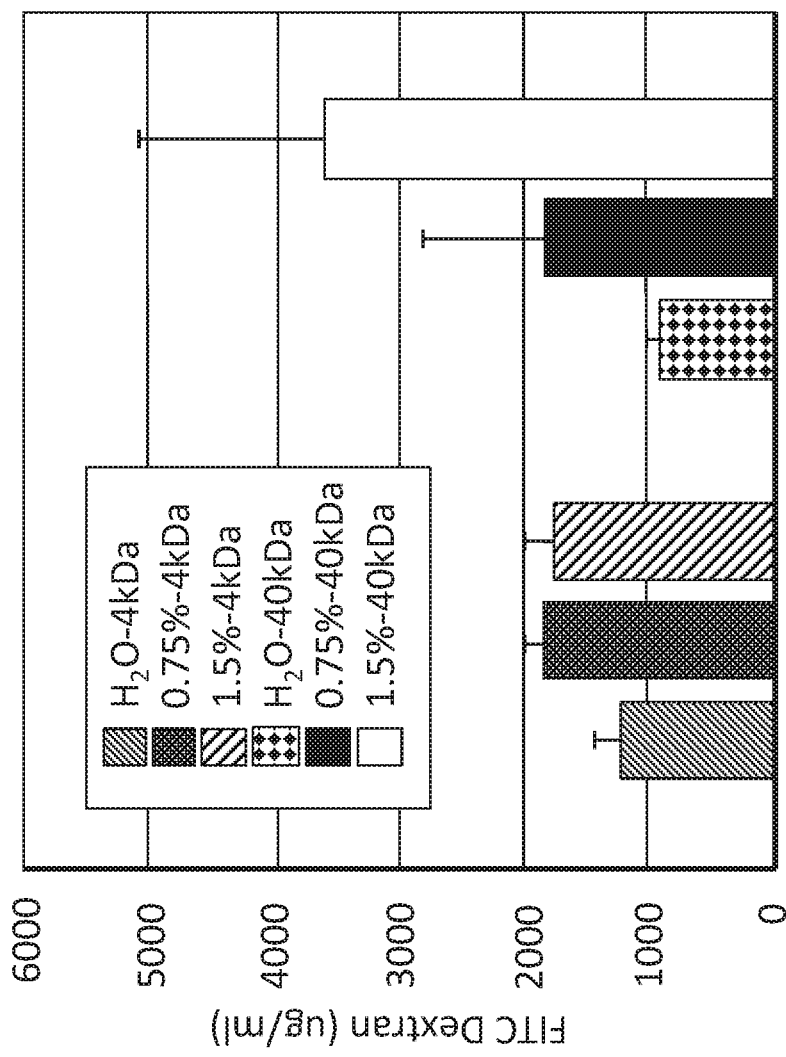
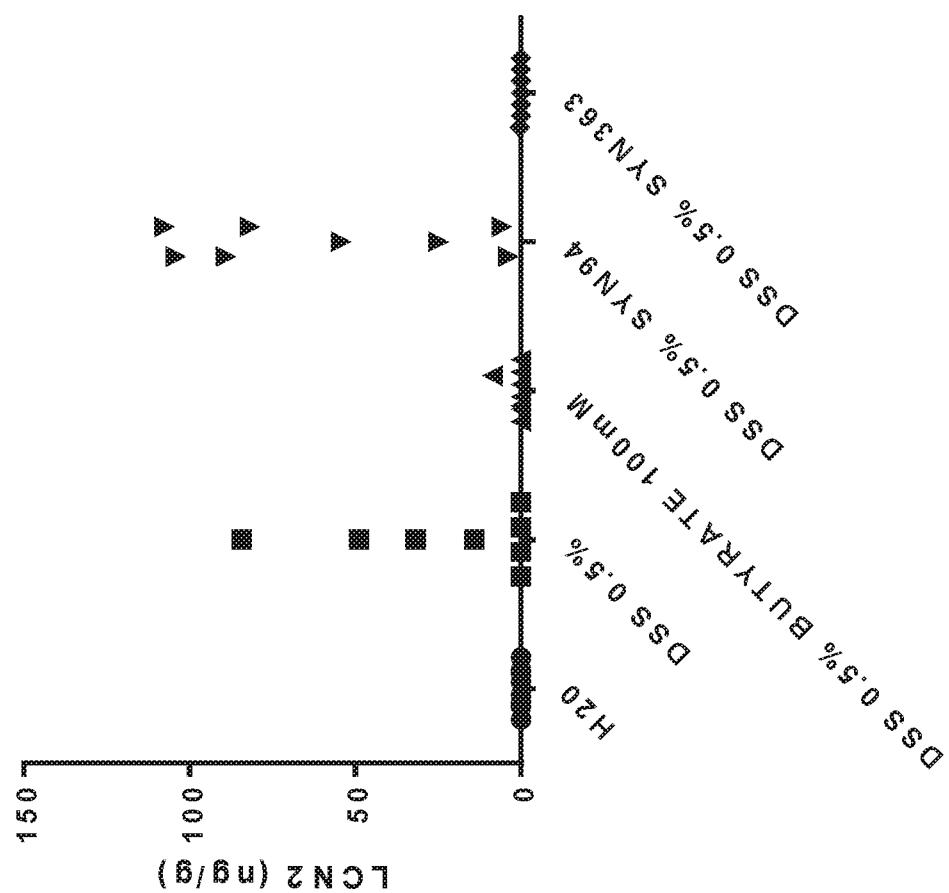


Fig. 26



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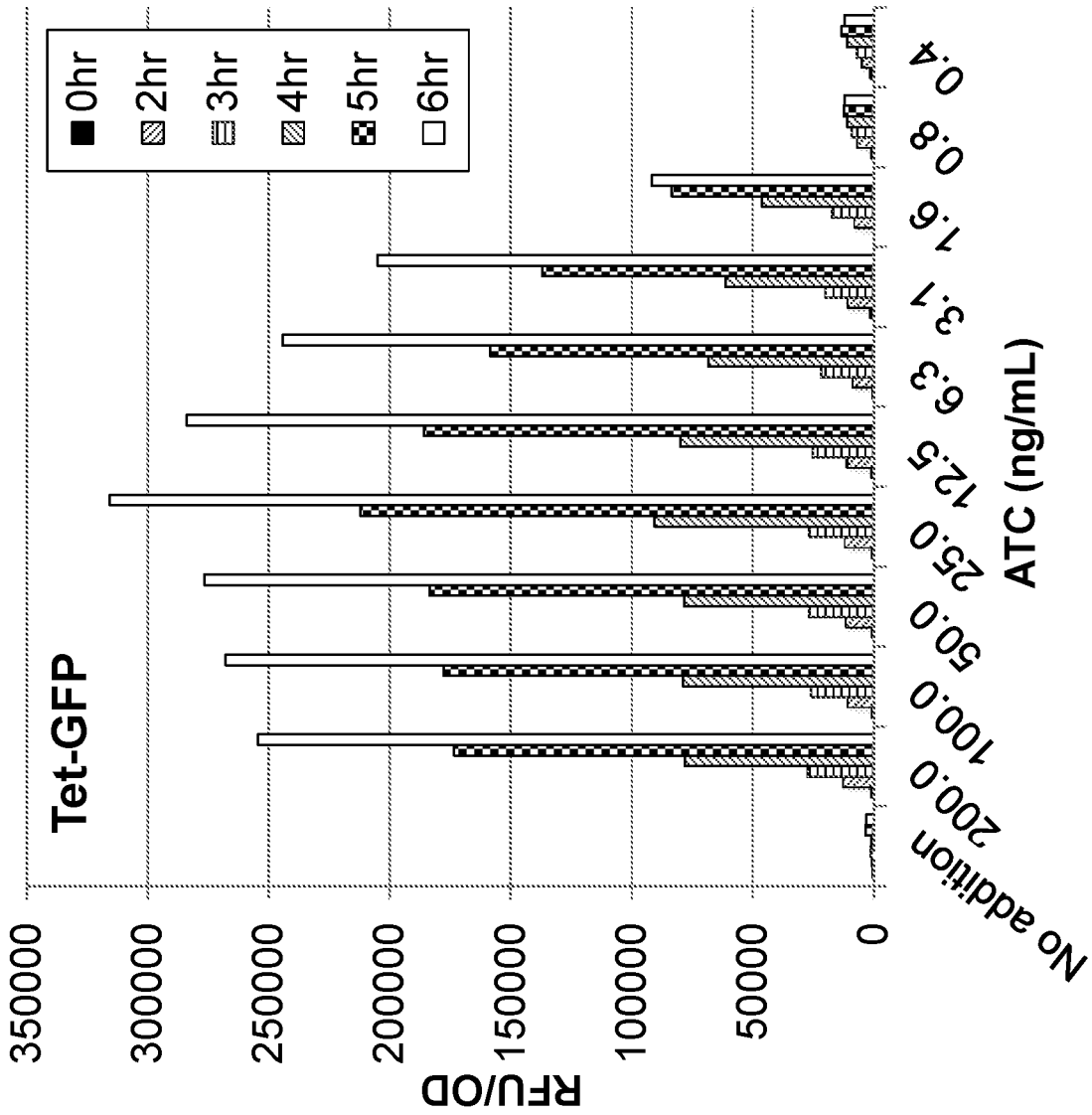
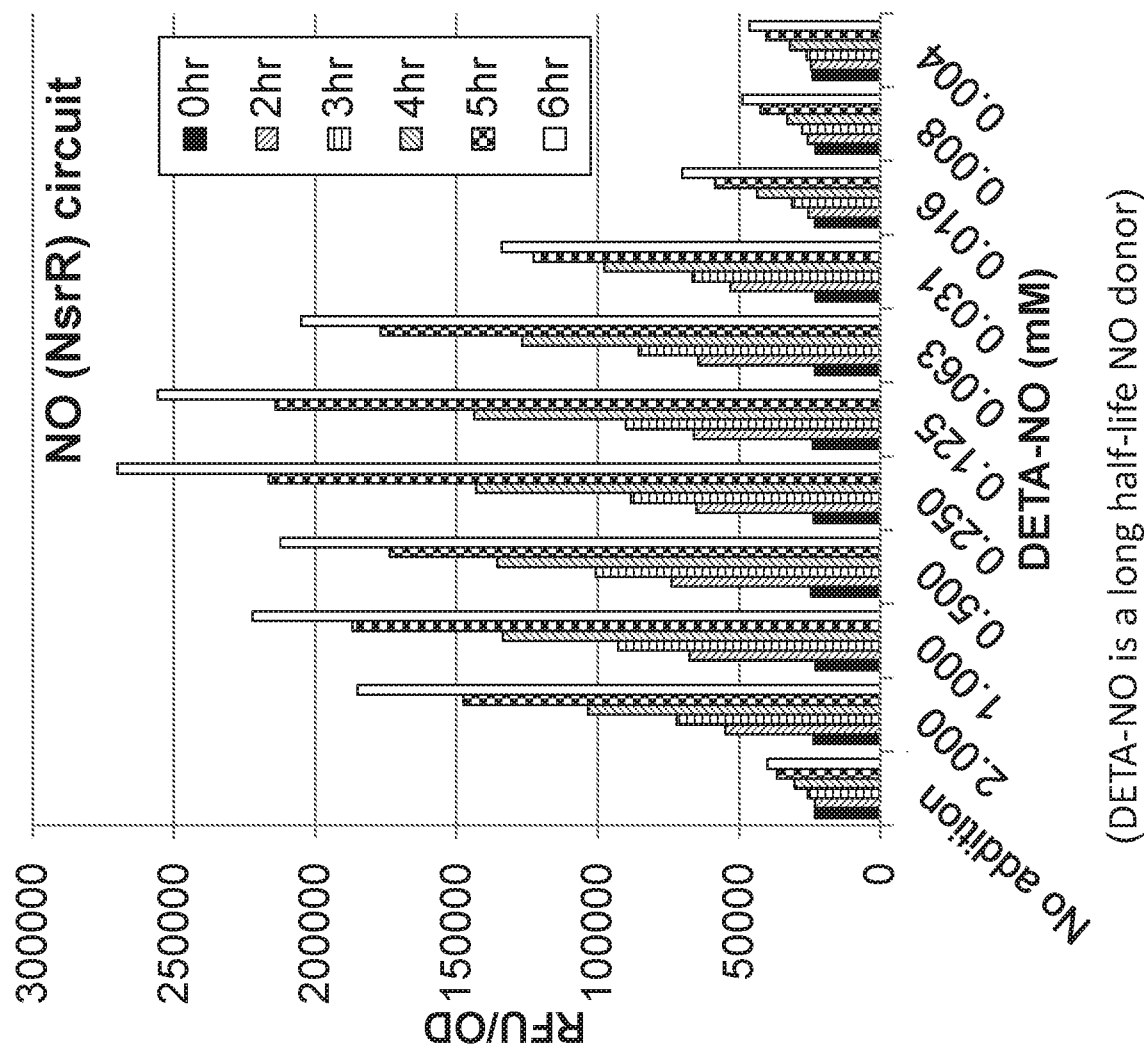


Fig. 28A



(DETA-NO is a long half-life NO donor)

Fig. 28B

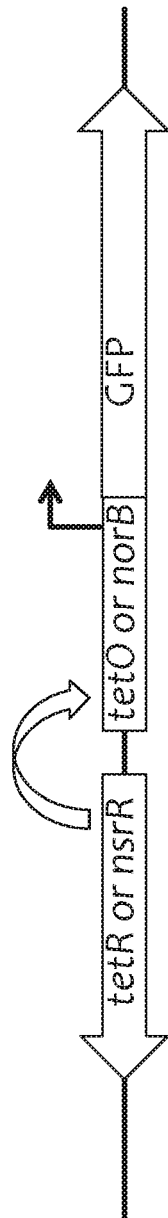


Fig. 28C

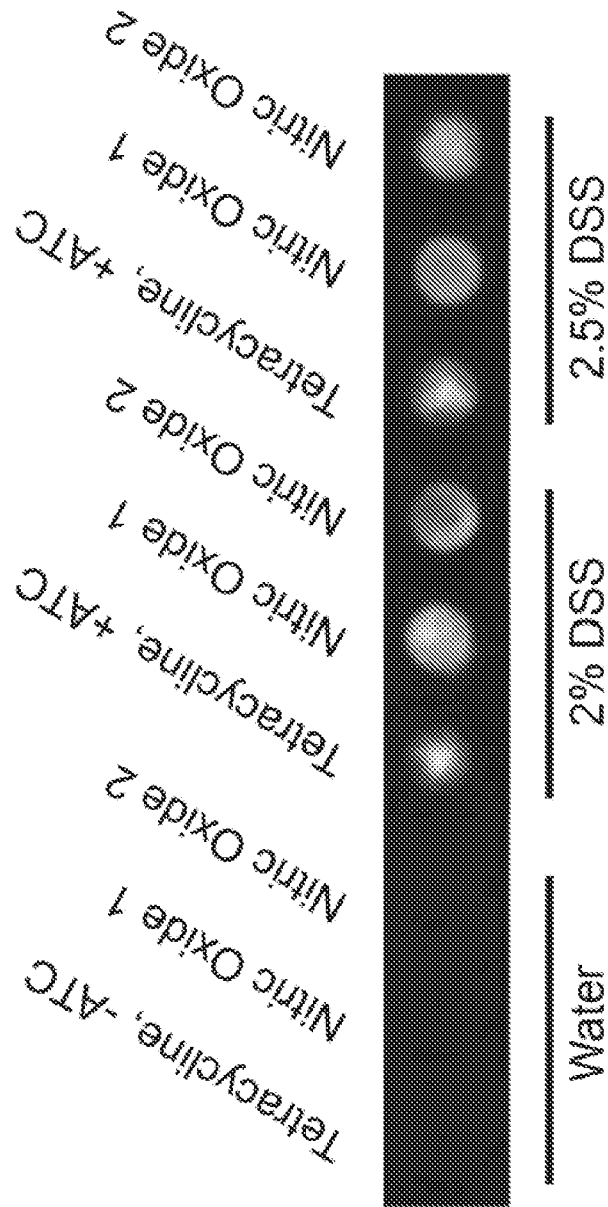


Fig. 29

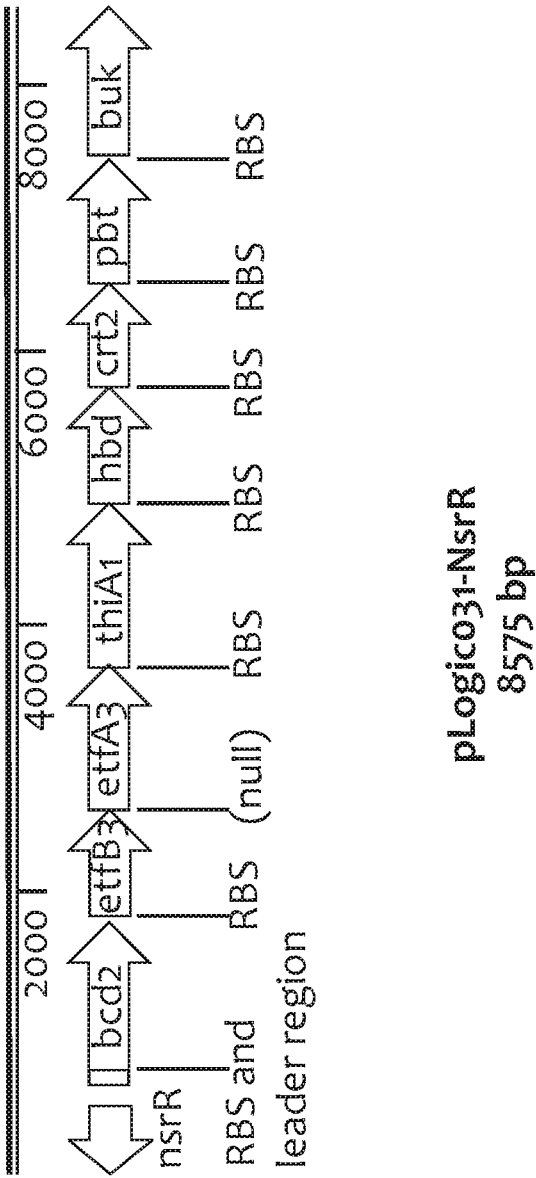


Fig. 30

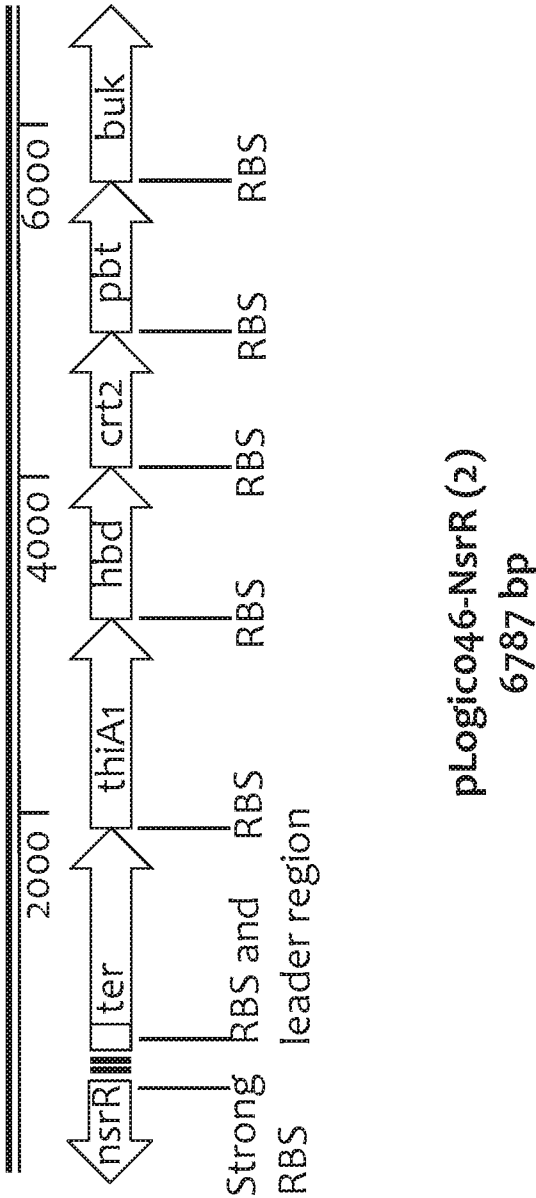


Fig. 31

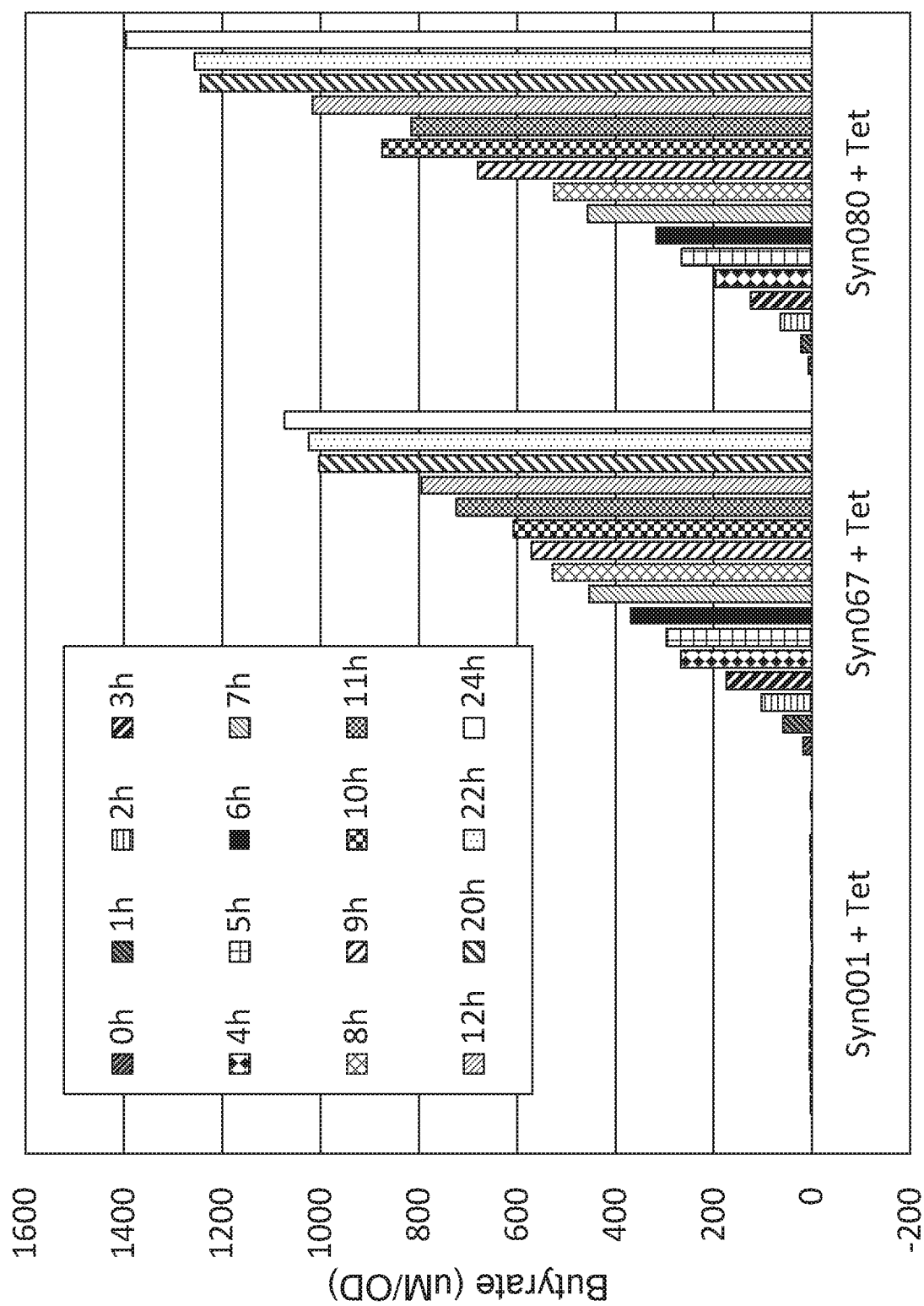


Fig. 32

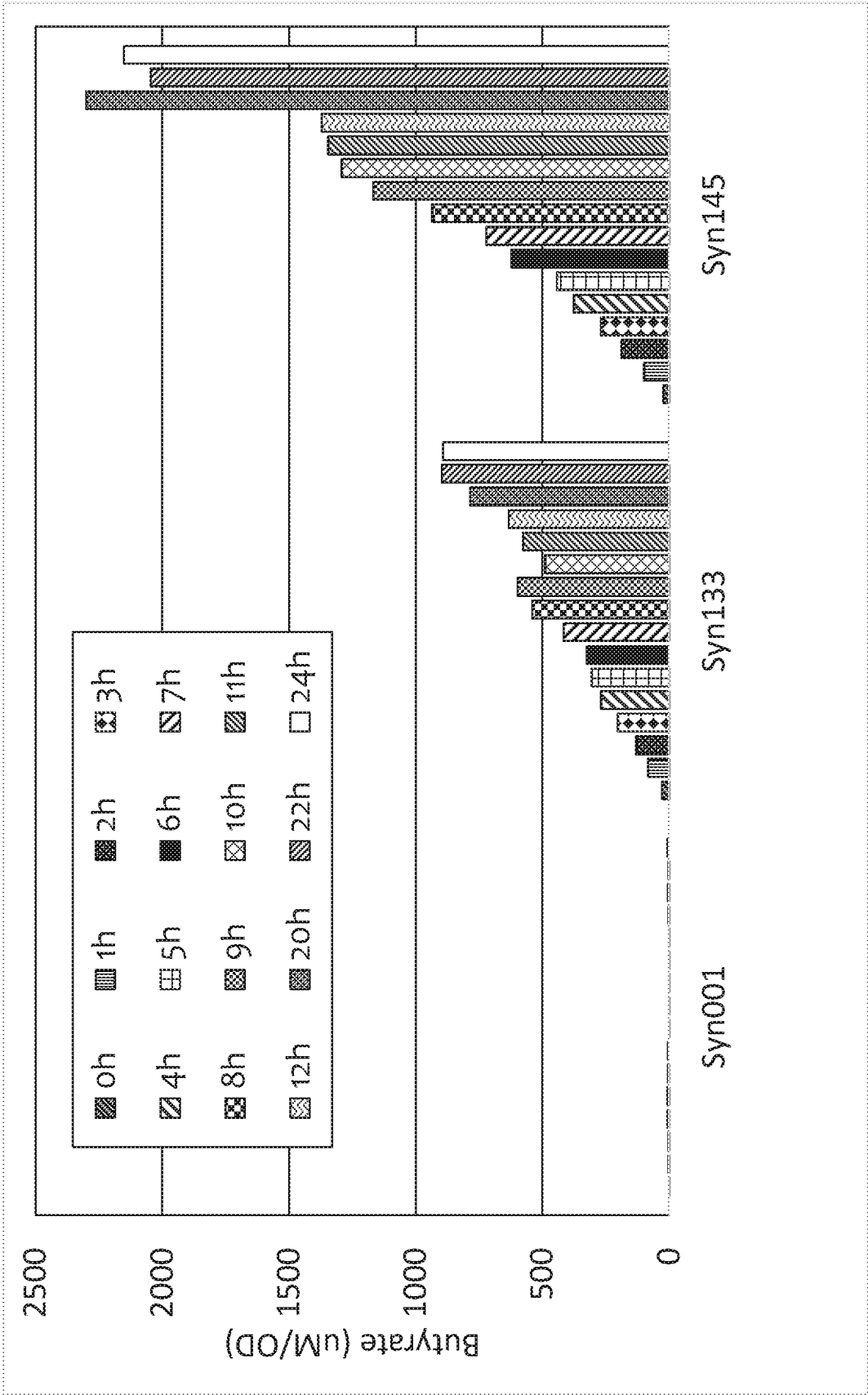


Fig. 33

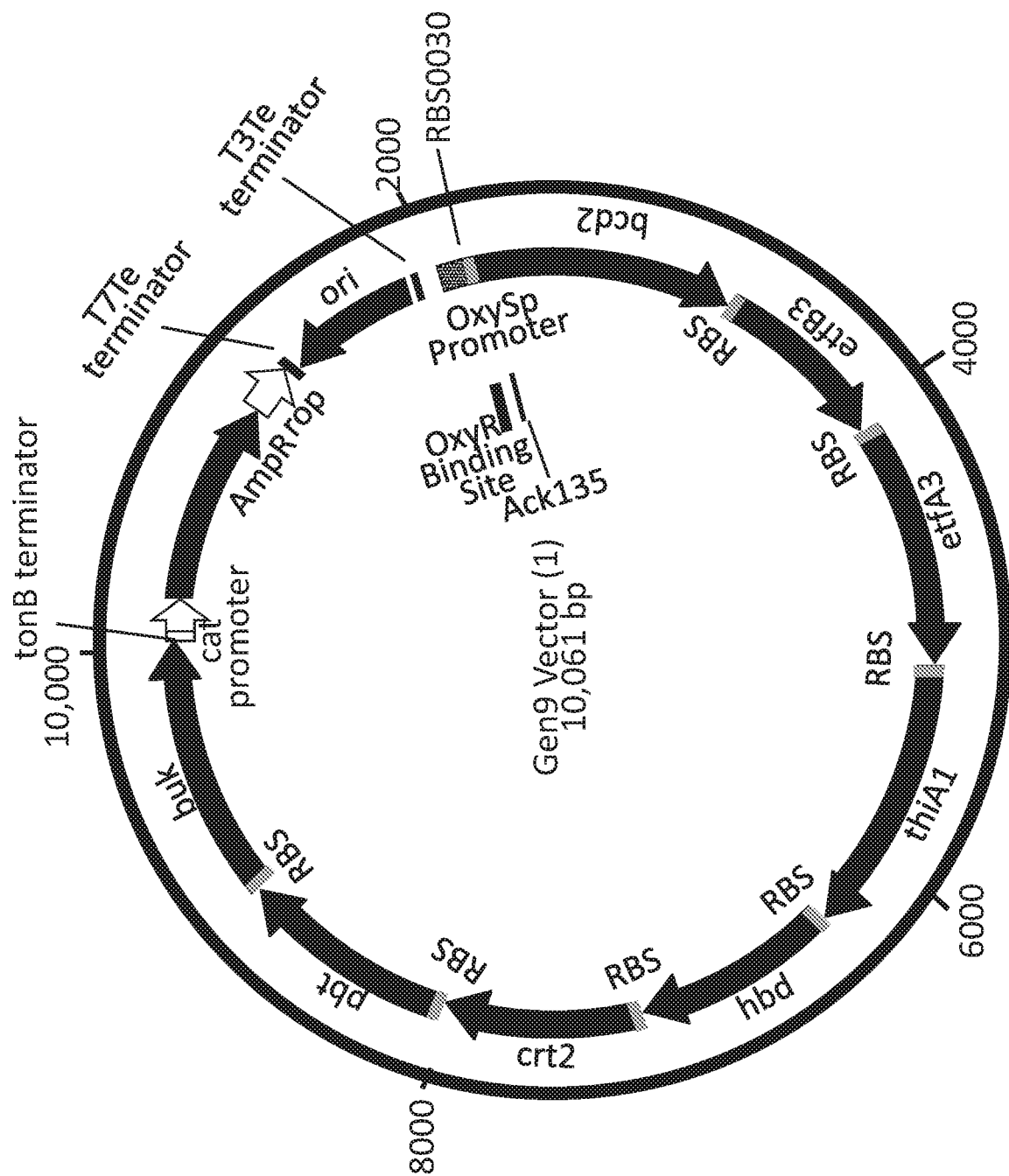


Fig. 34

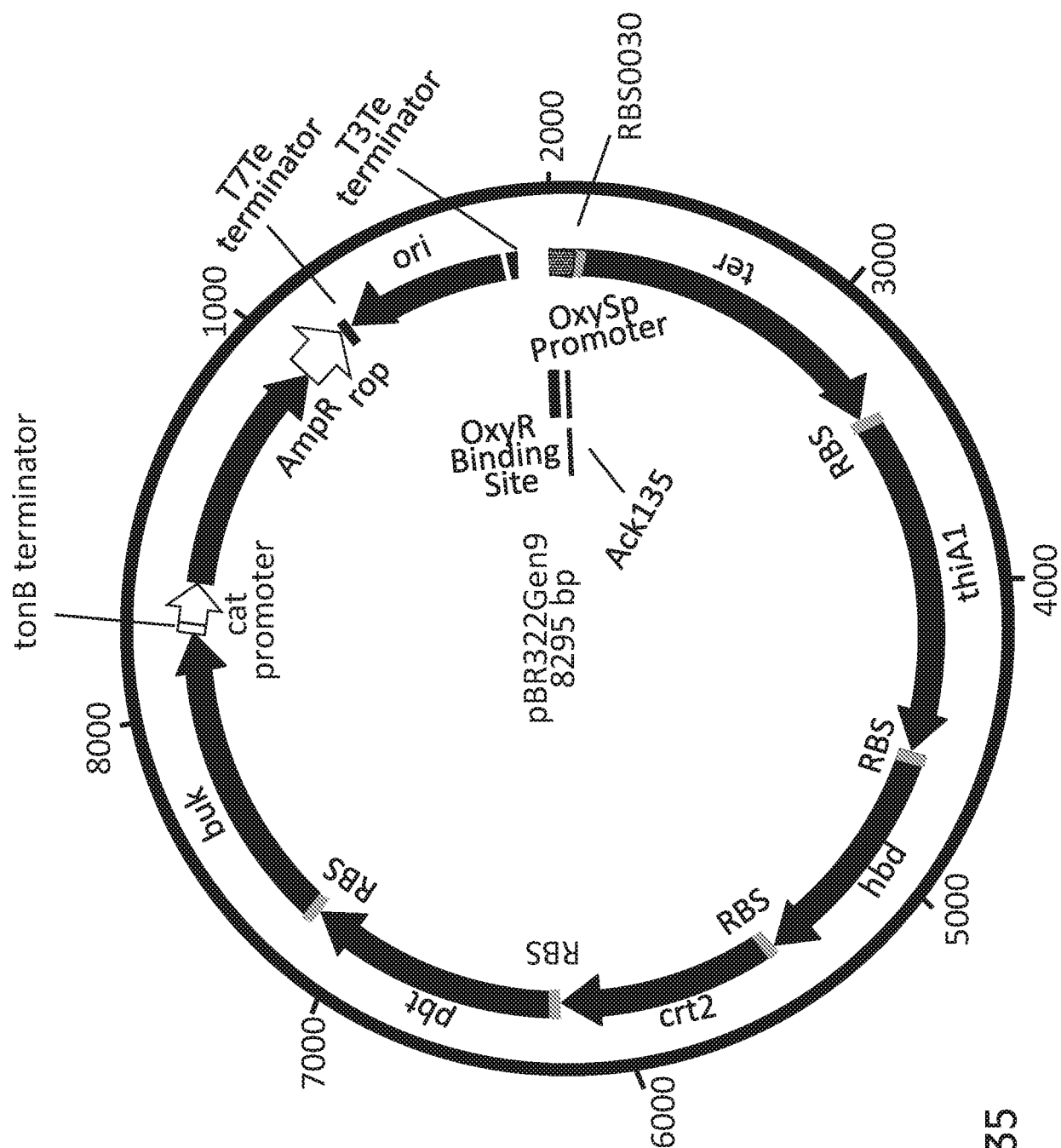


Fig. 35

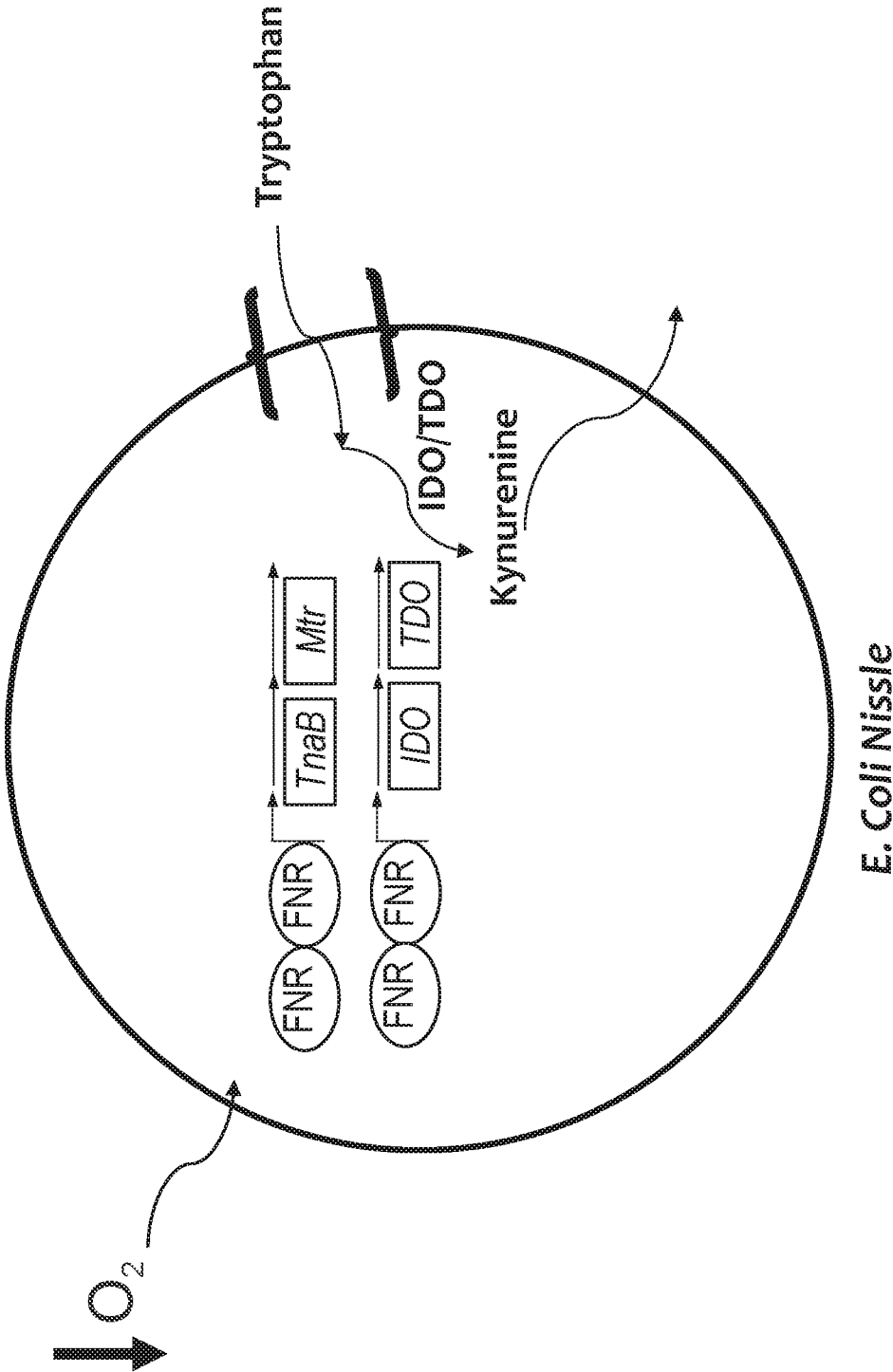


Fig. 36

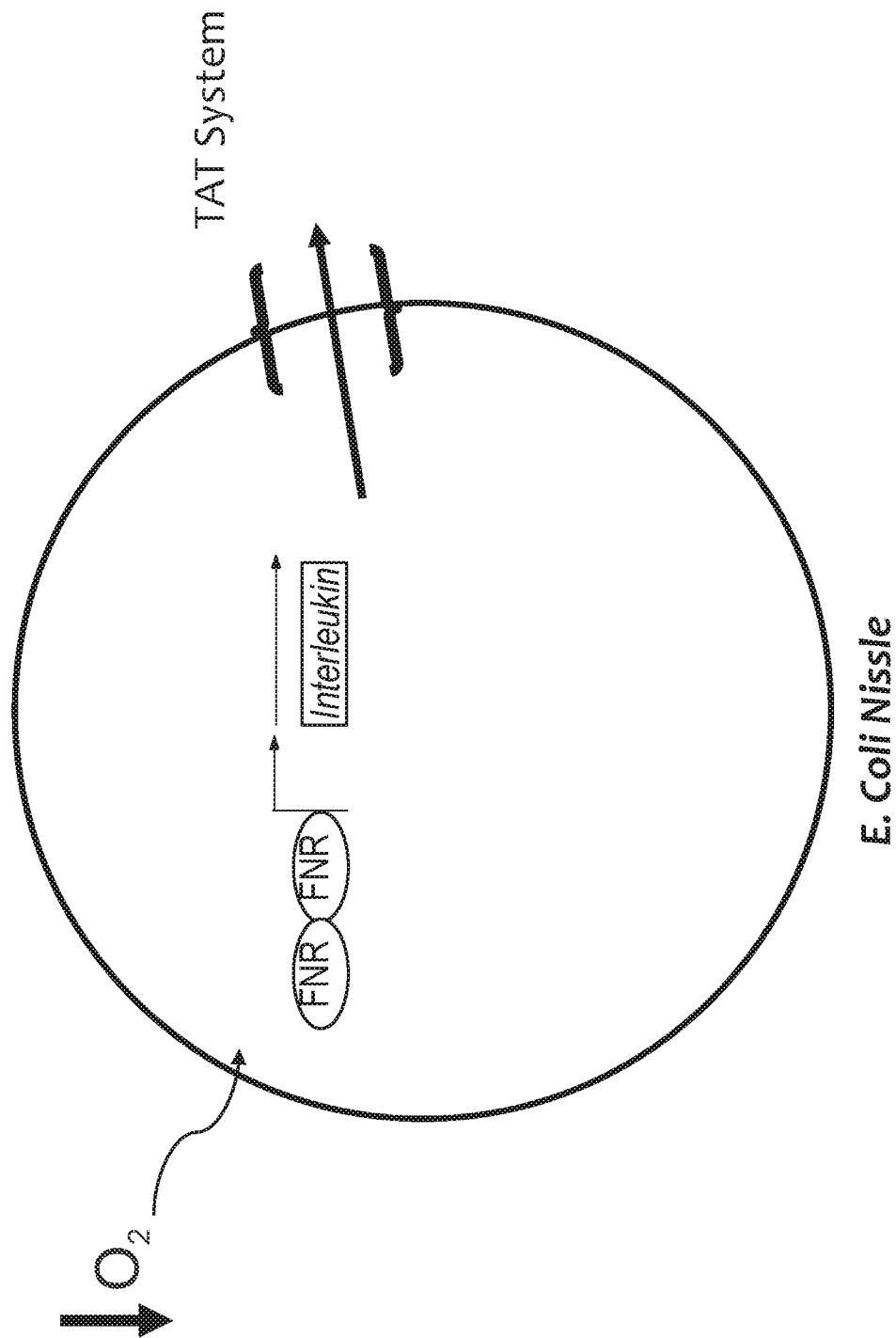


Fig. 37

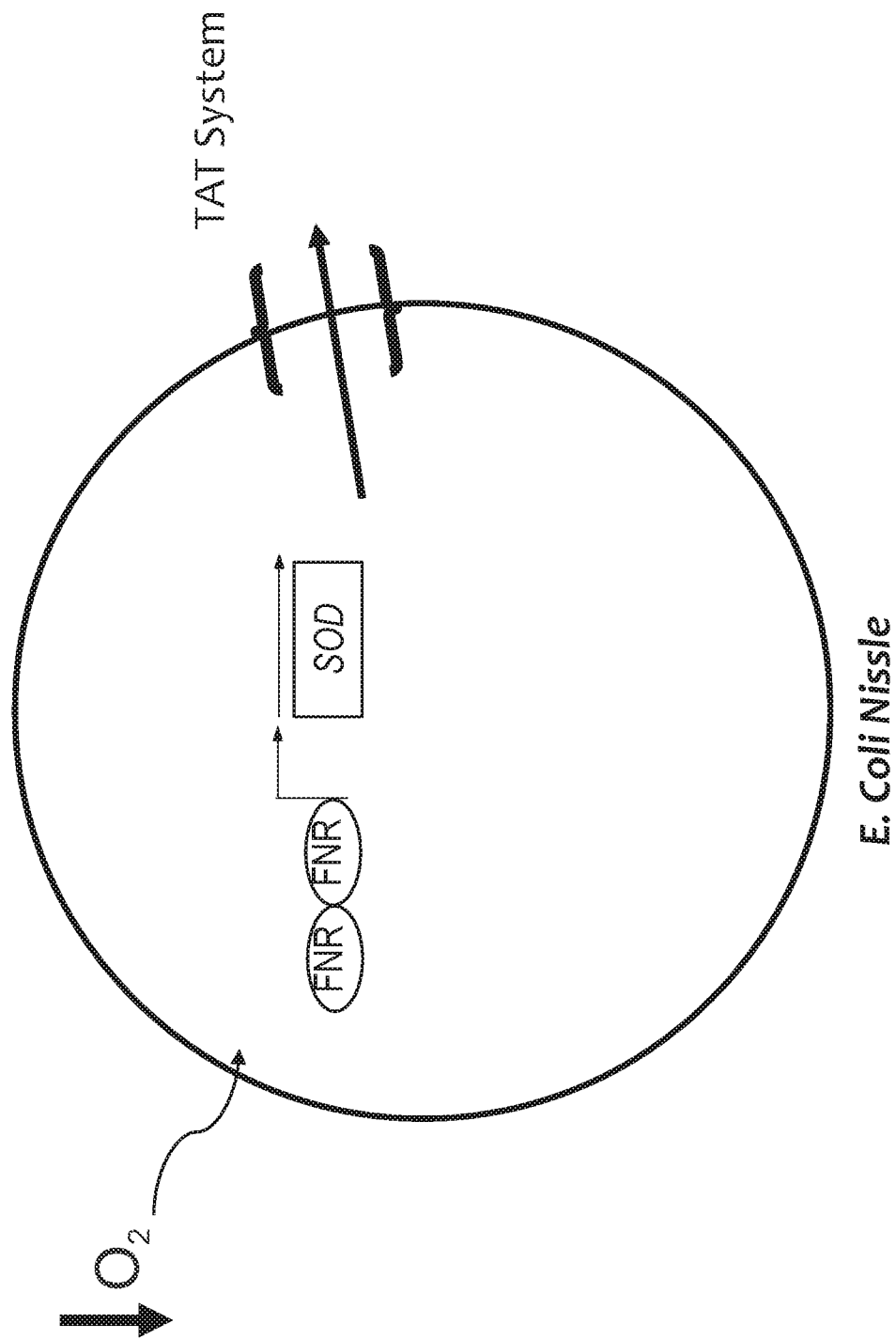


Fig. 38

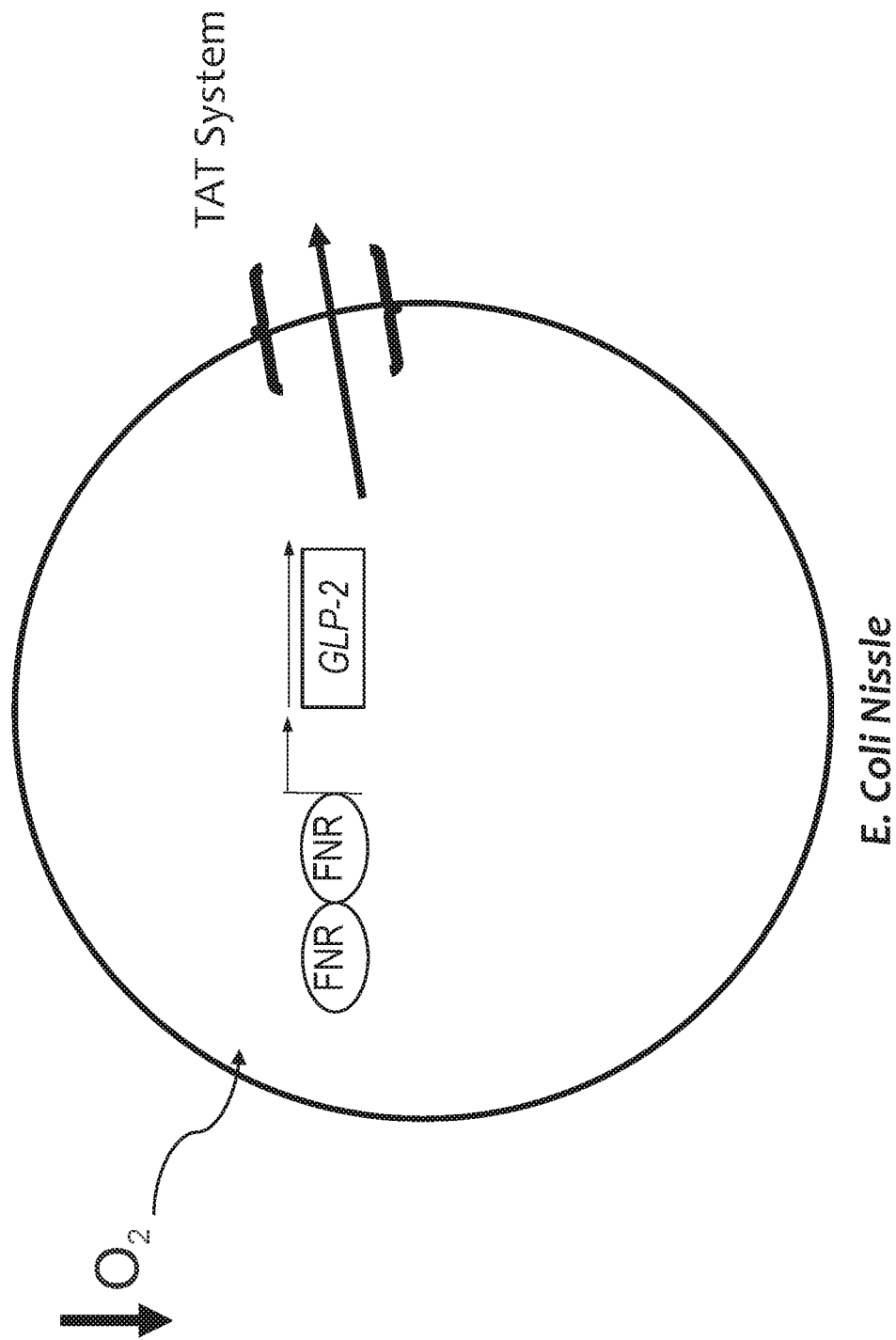


Fig. 39

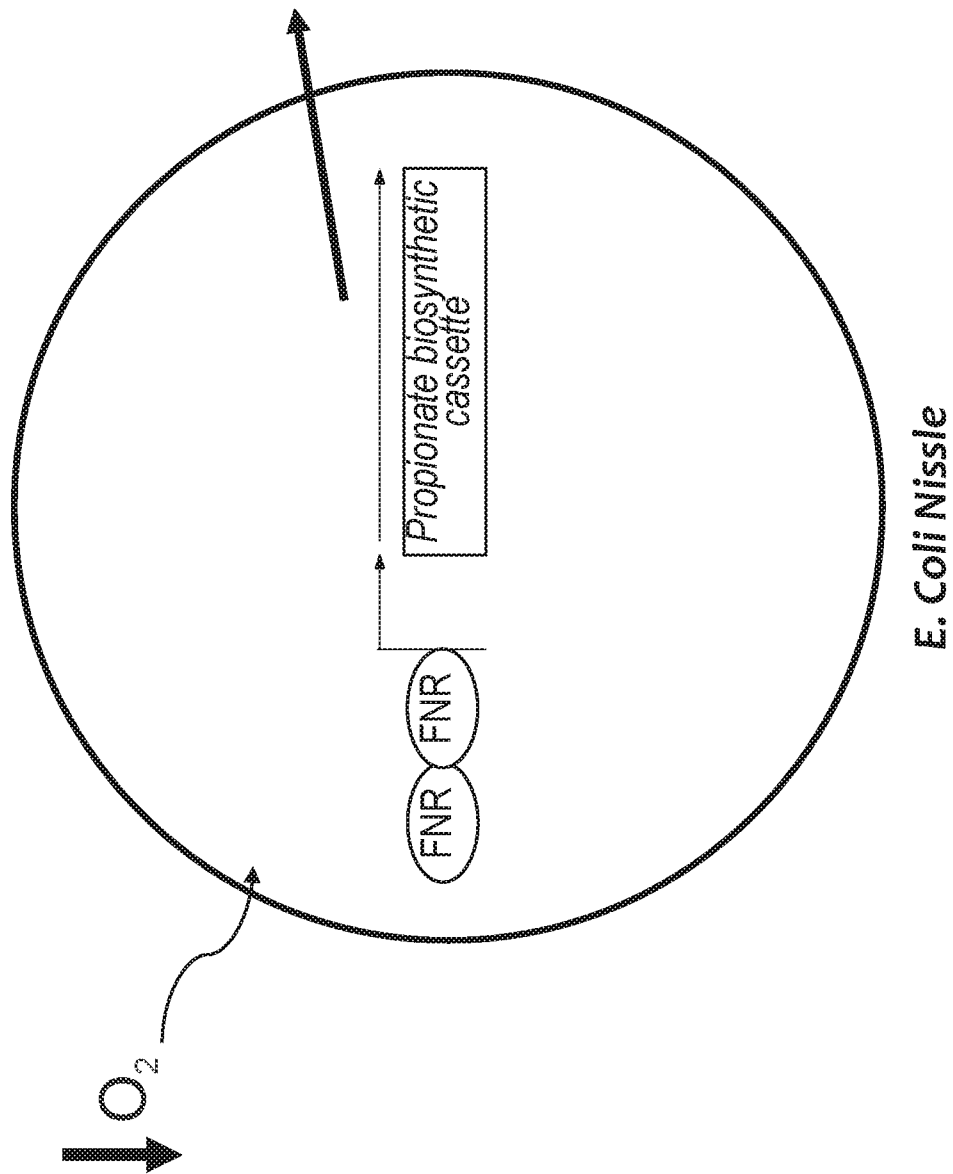


Fig. 40

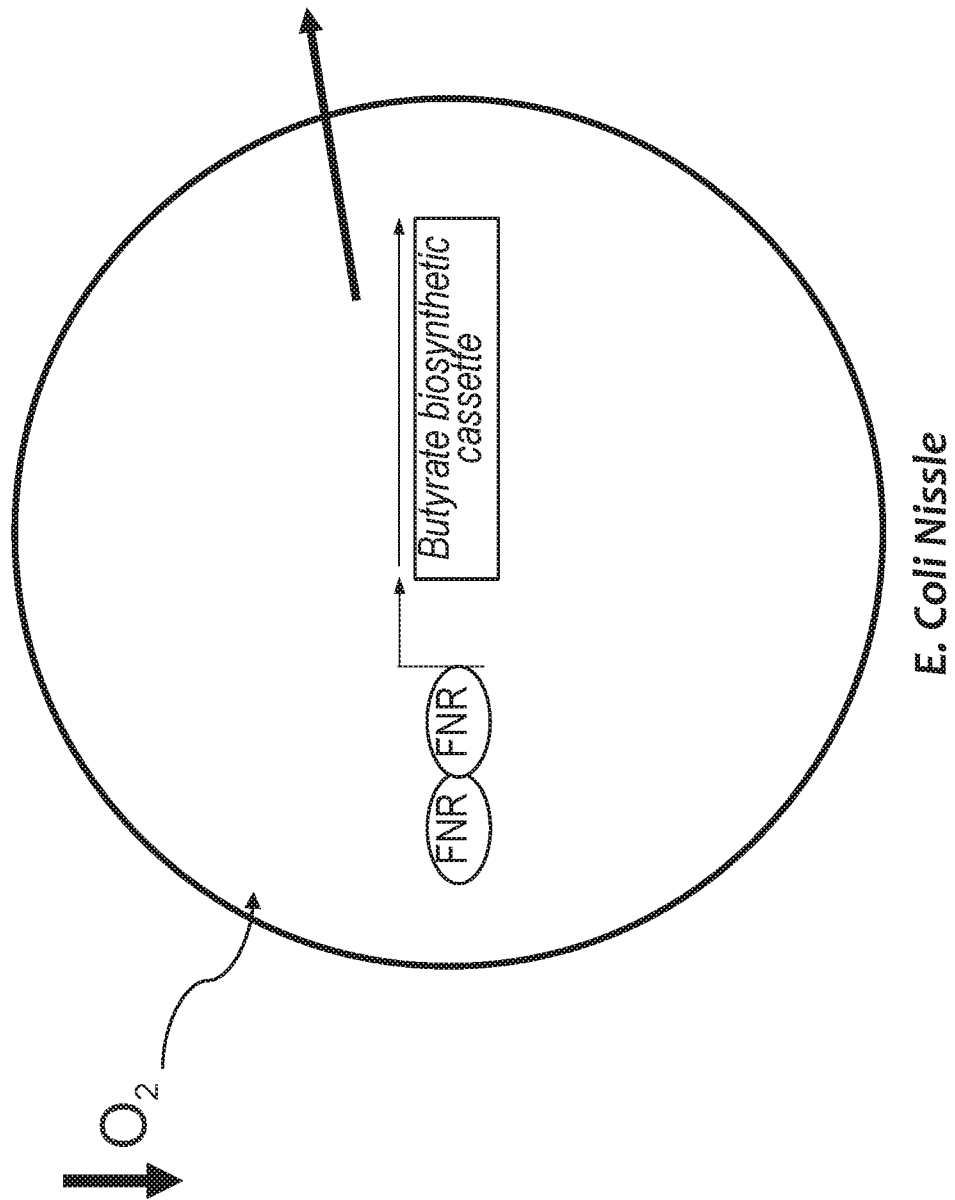


Fig. 41

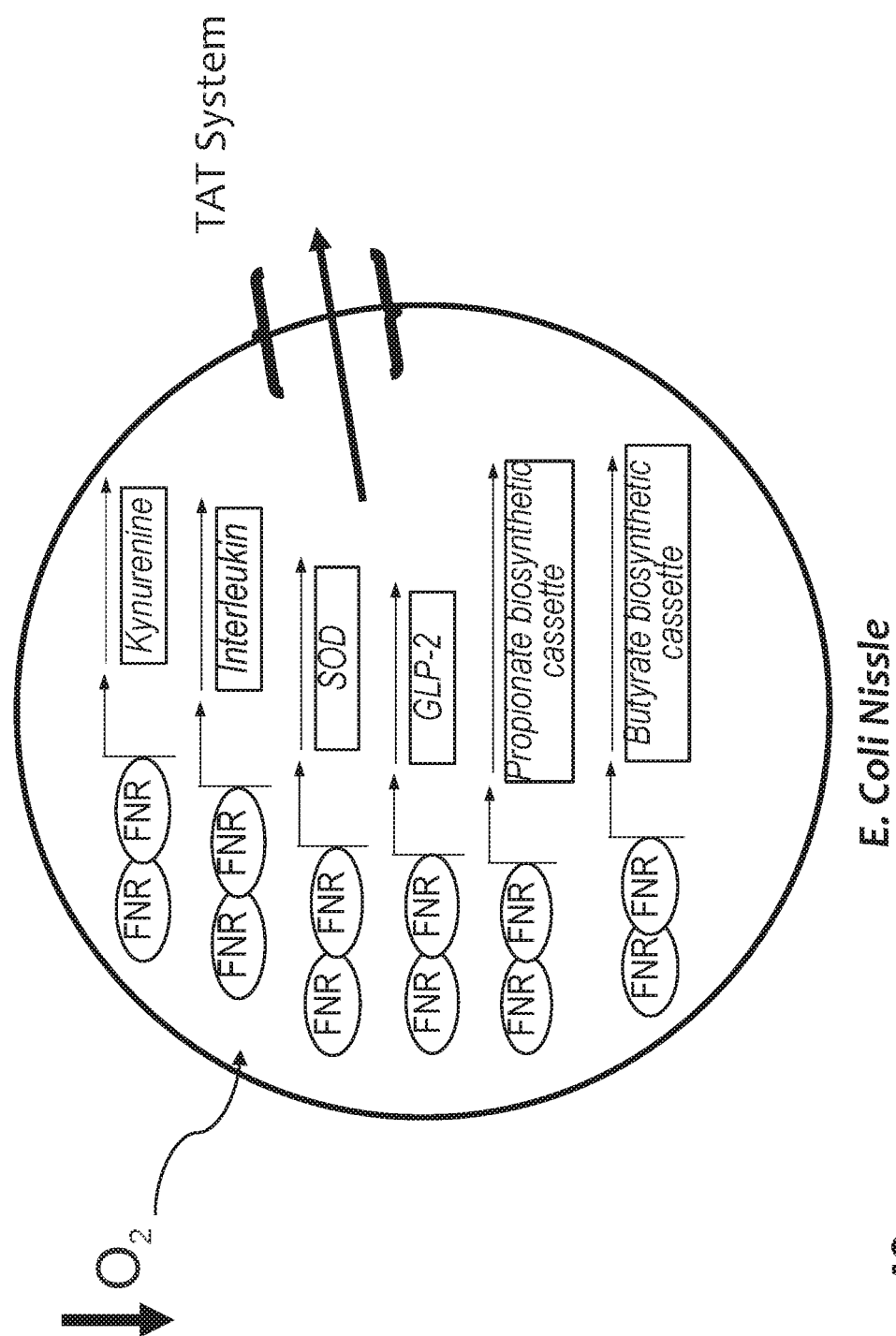
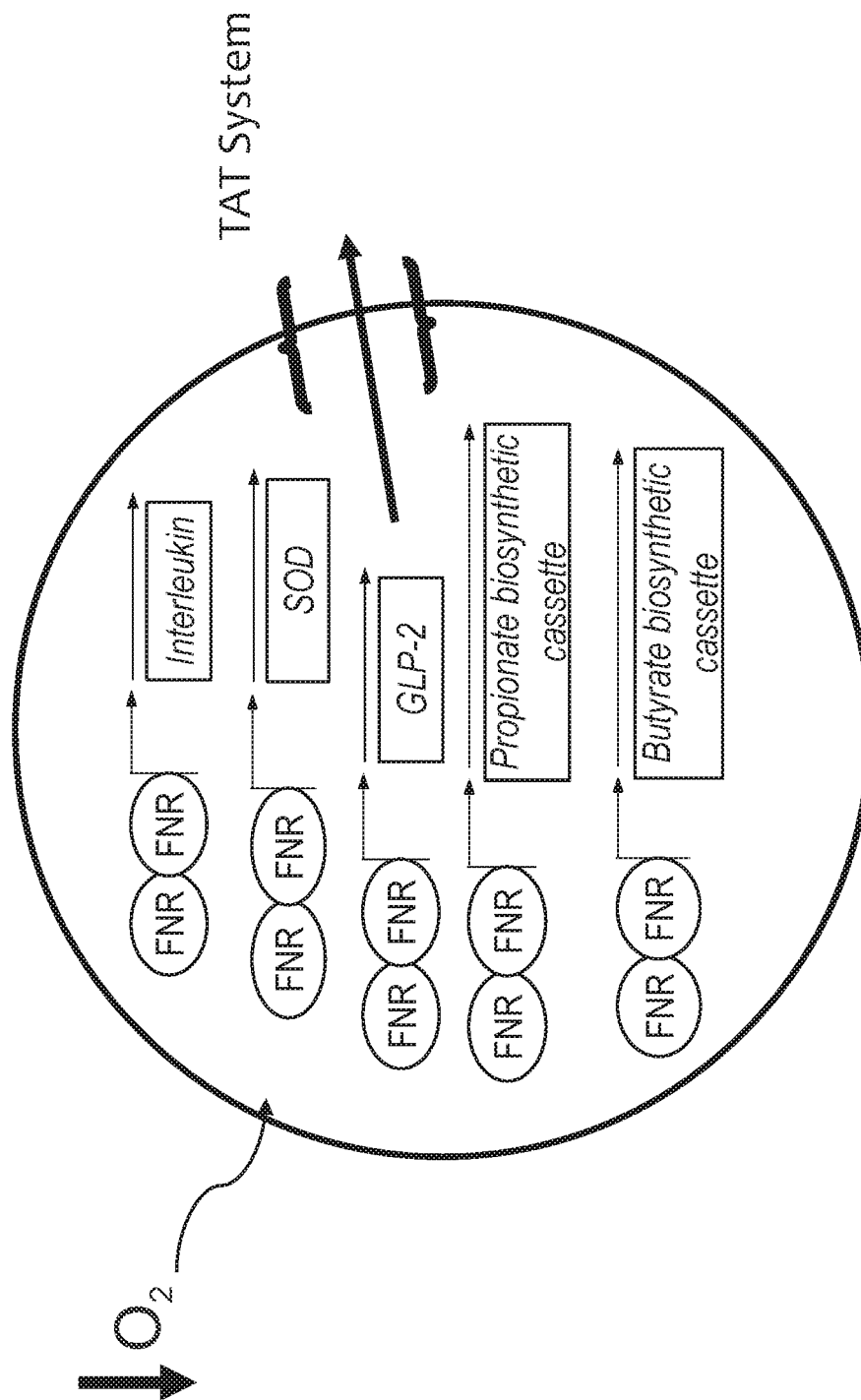


Fig. 42



E. coli Nissle

Fig. 43

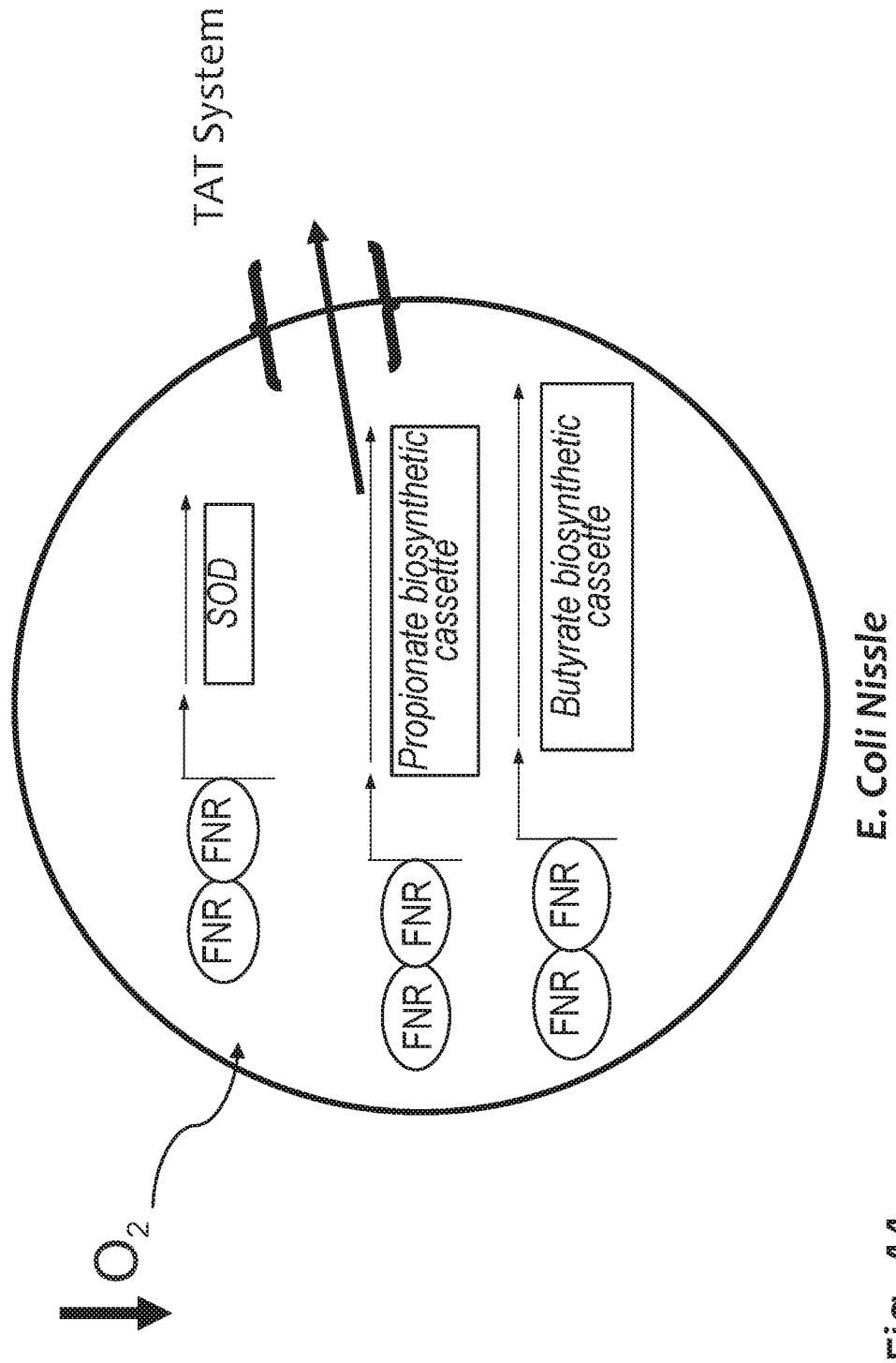
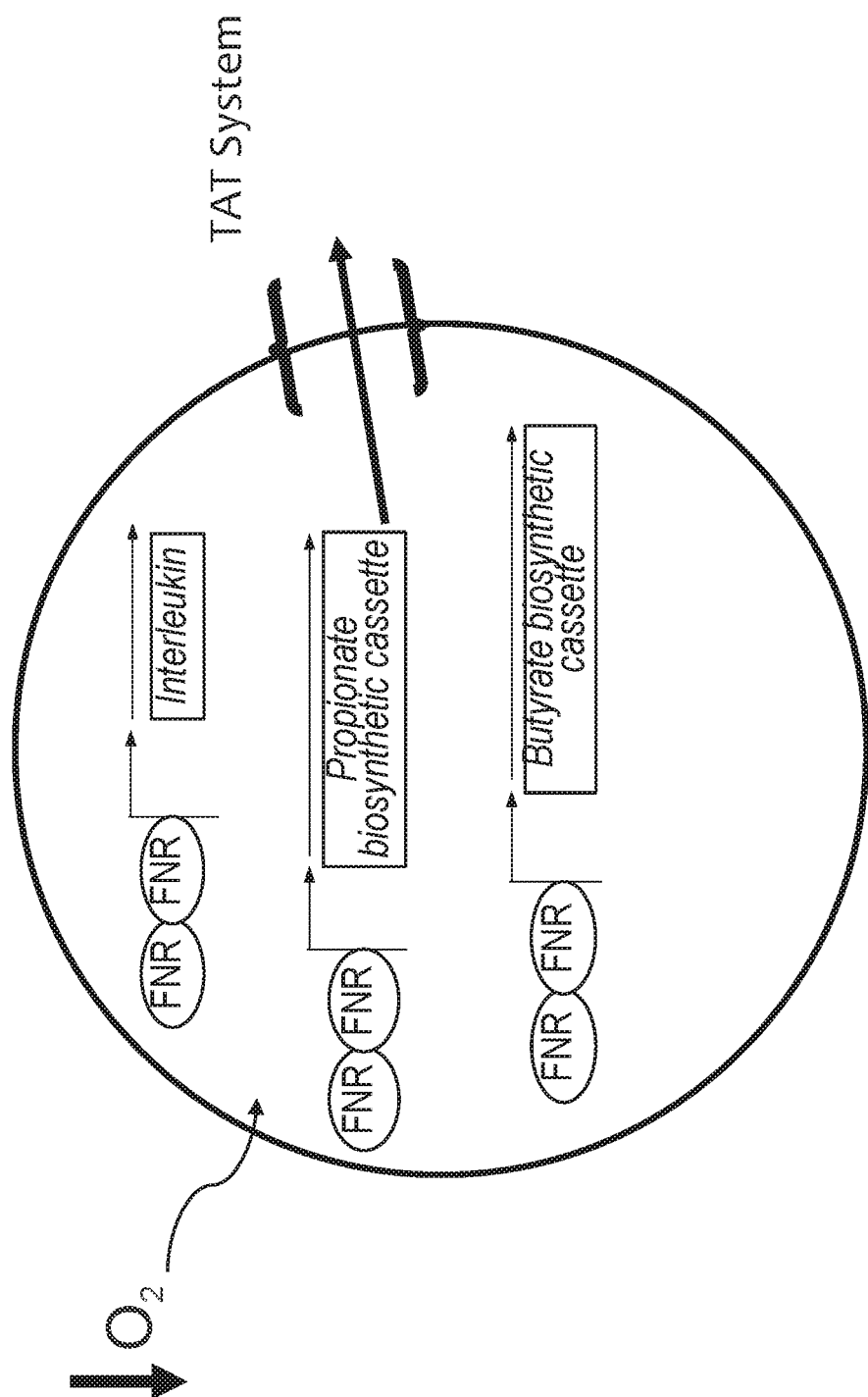
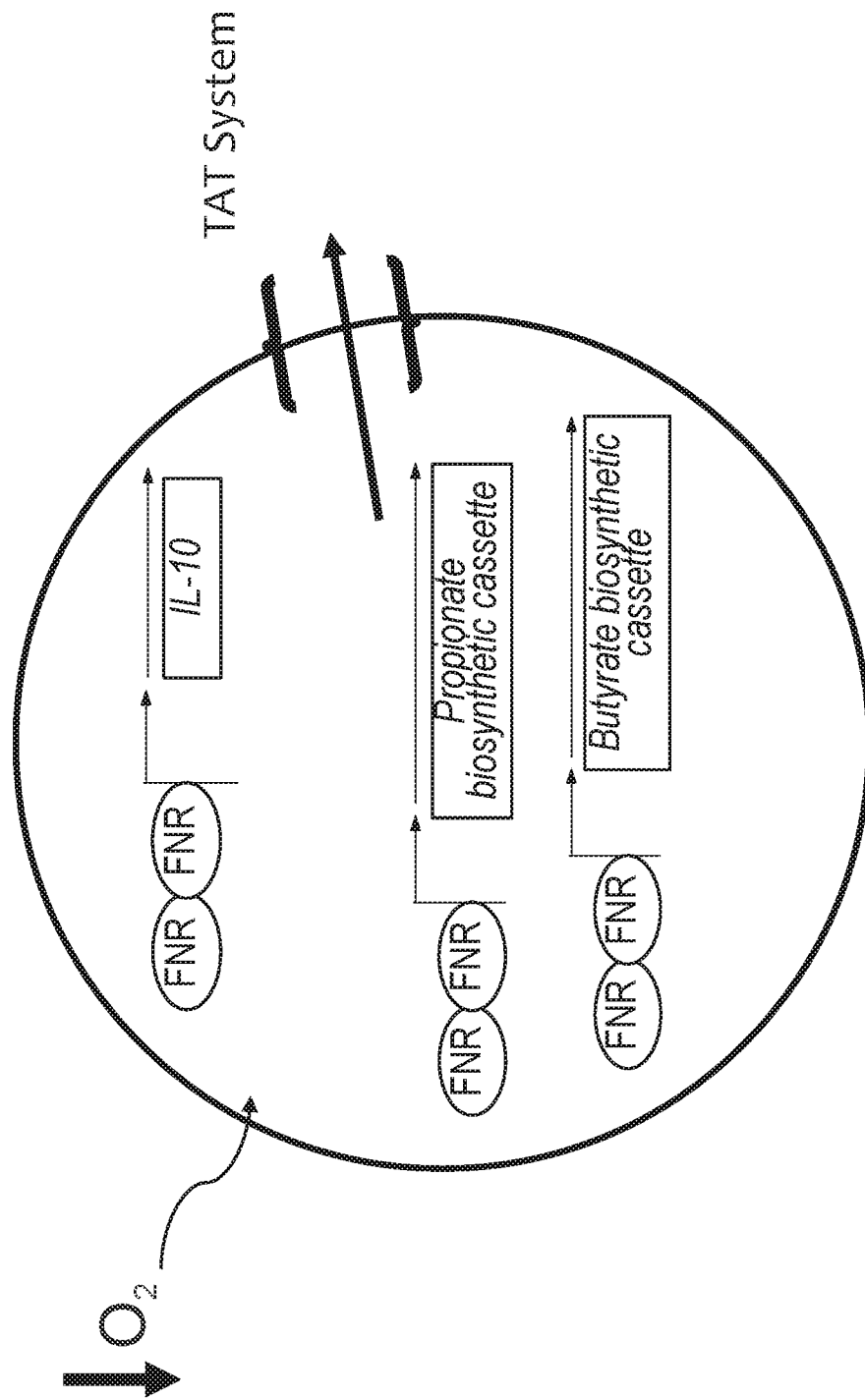


Fig. 44



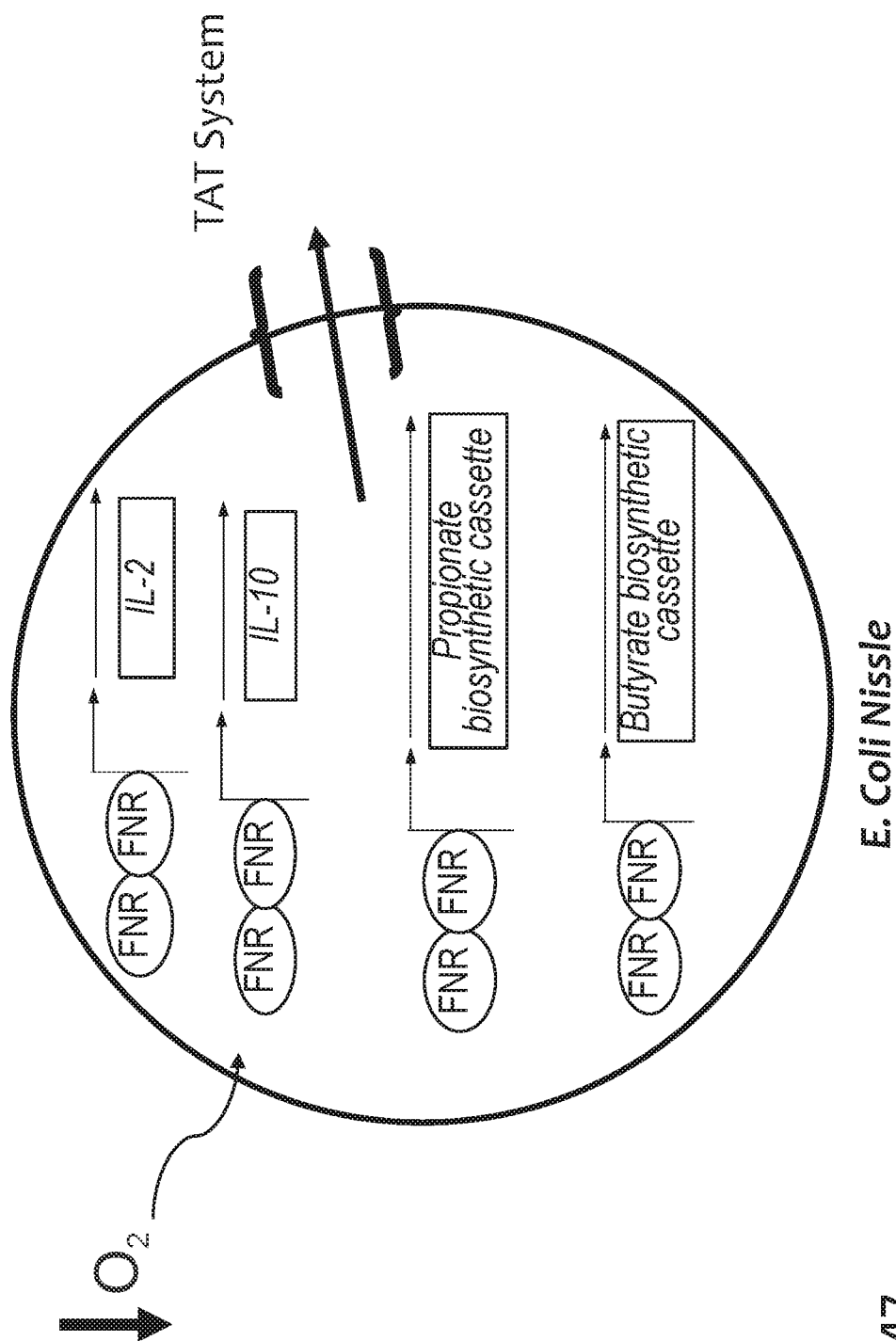
E. coli Nissle

Fig. 45



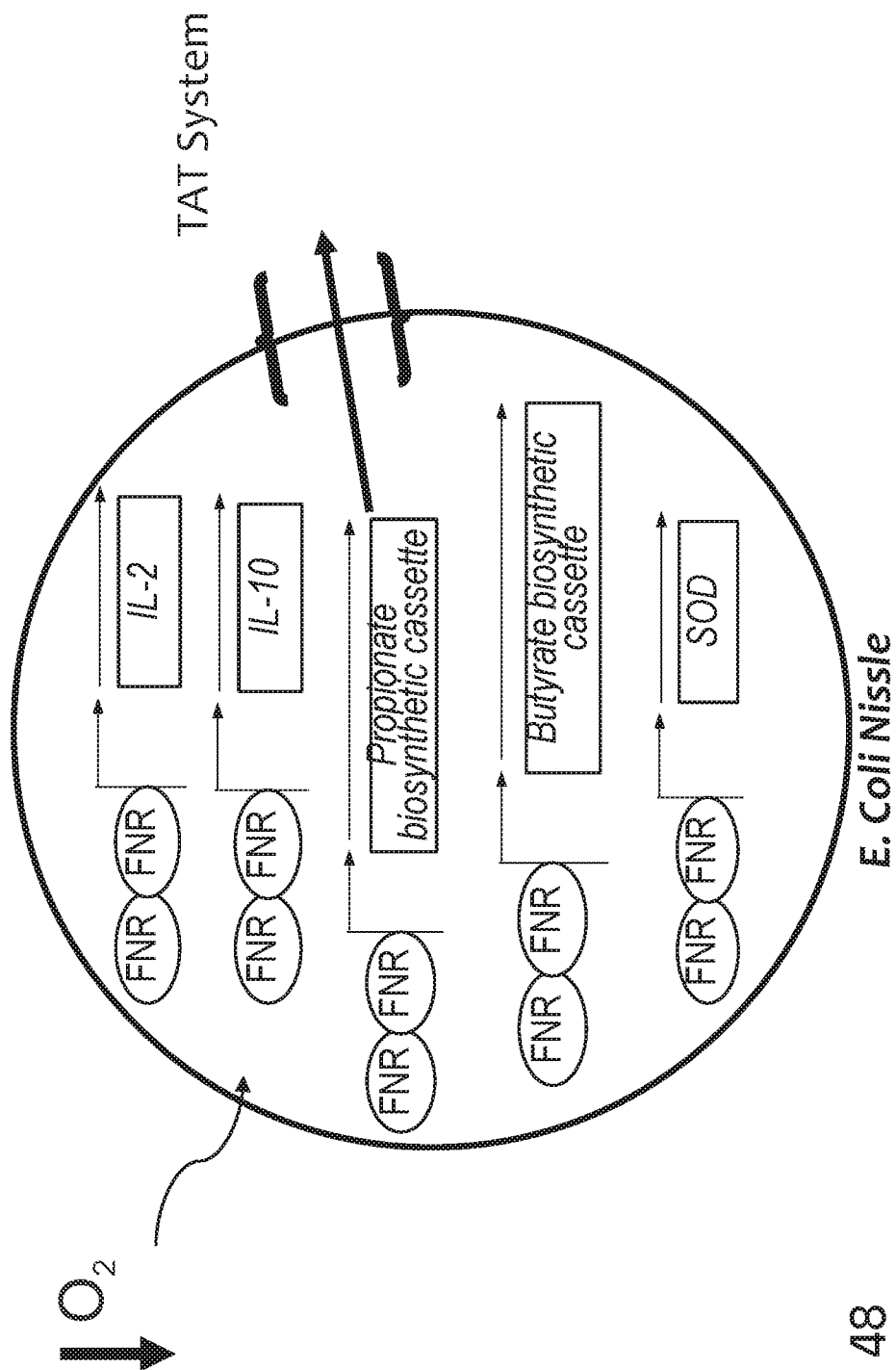
E. Coli Nissle

Fig. 46



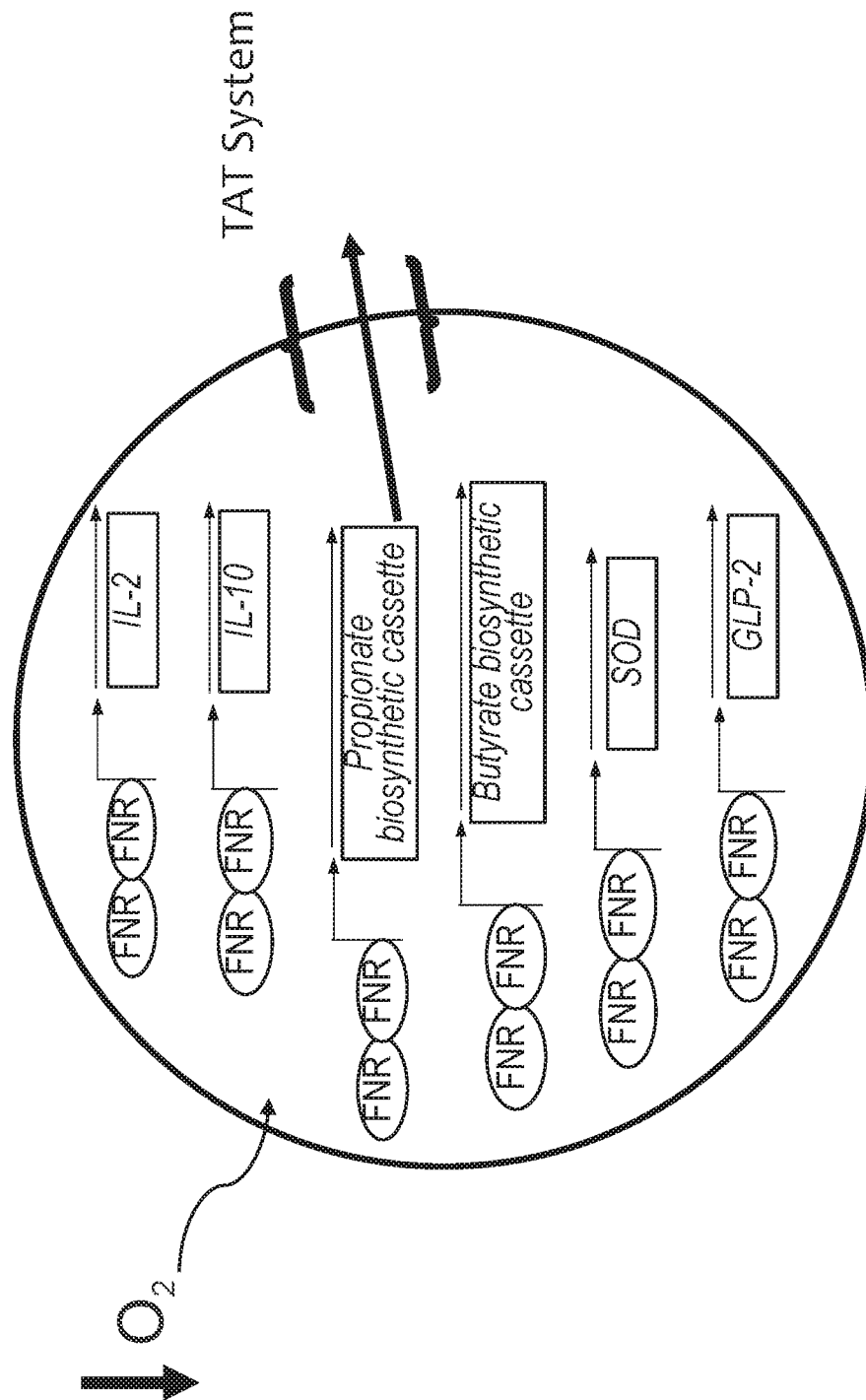
E. coli Nissle

Fig. 47



E. coli Nissle

Fig. 48



E. coli Nissle

Fig. 49

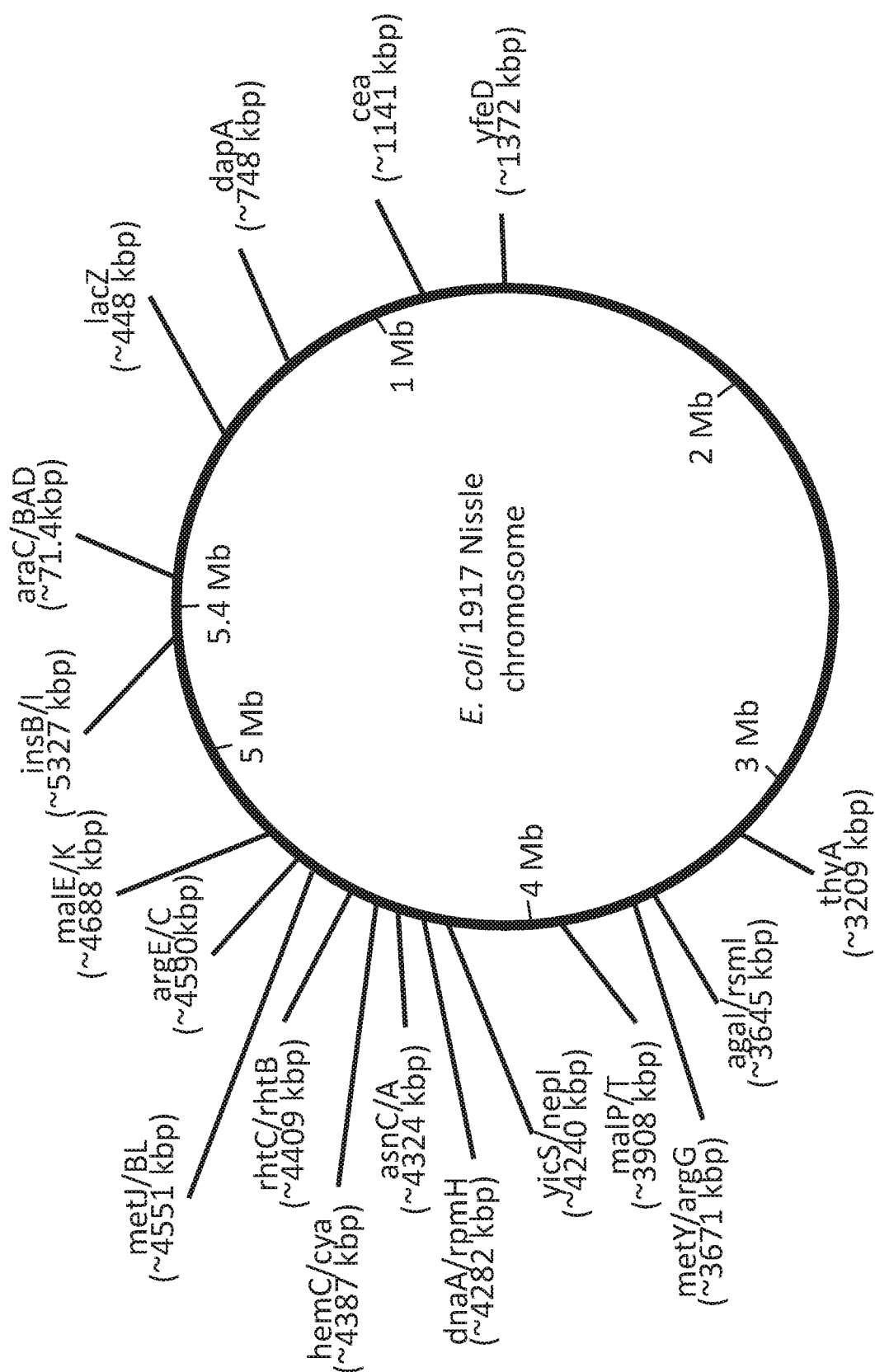


Fig. 50

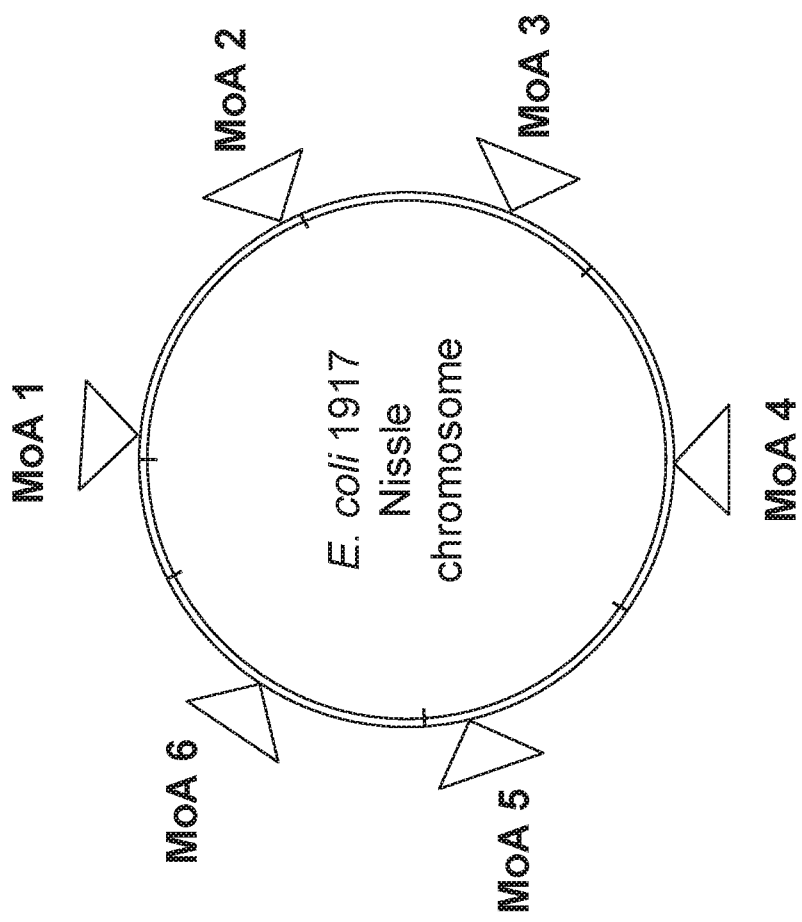


Fig. 51

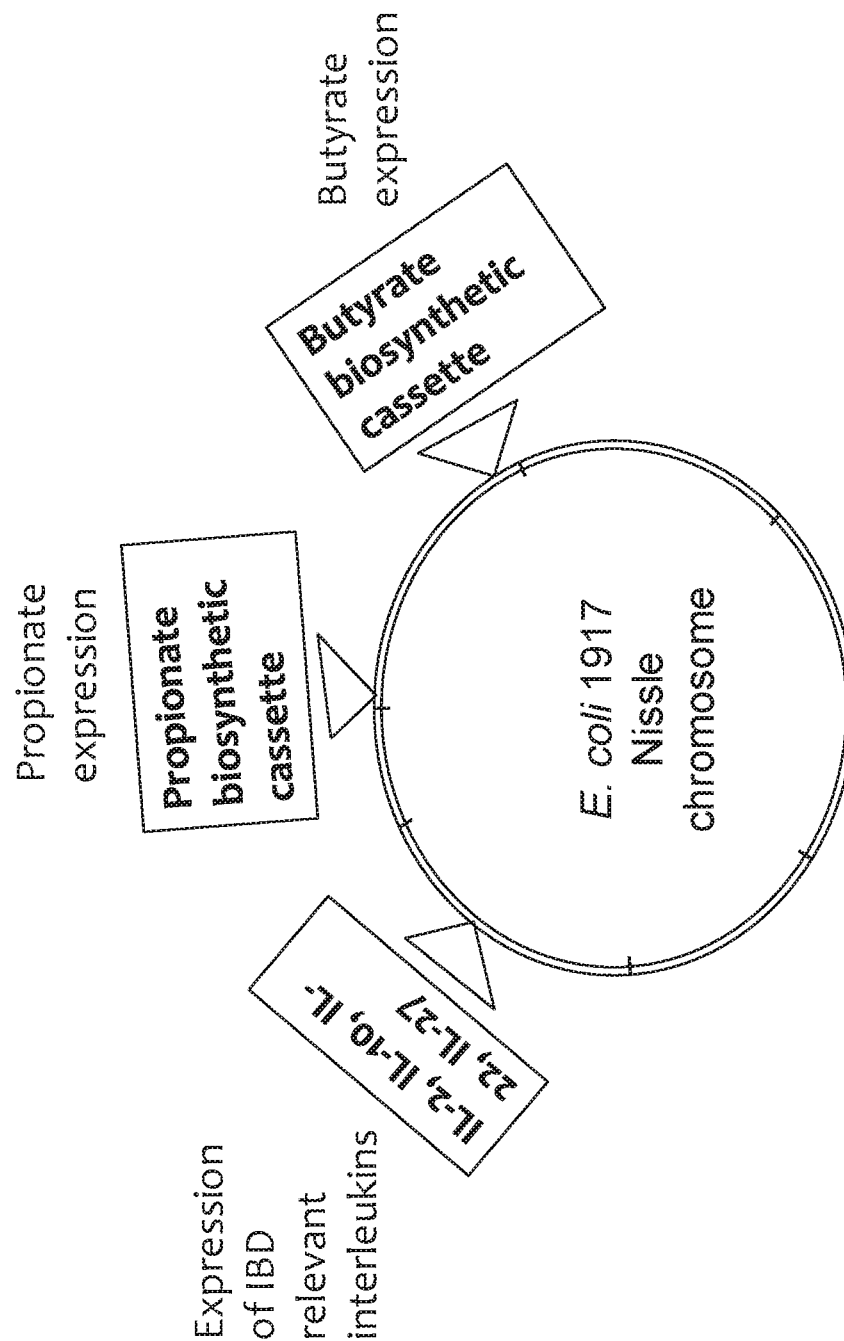


Fig. 52

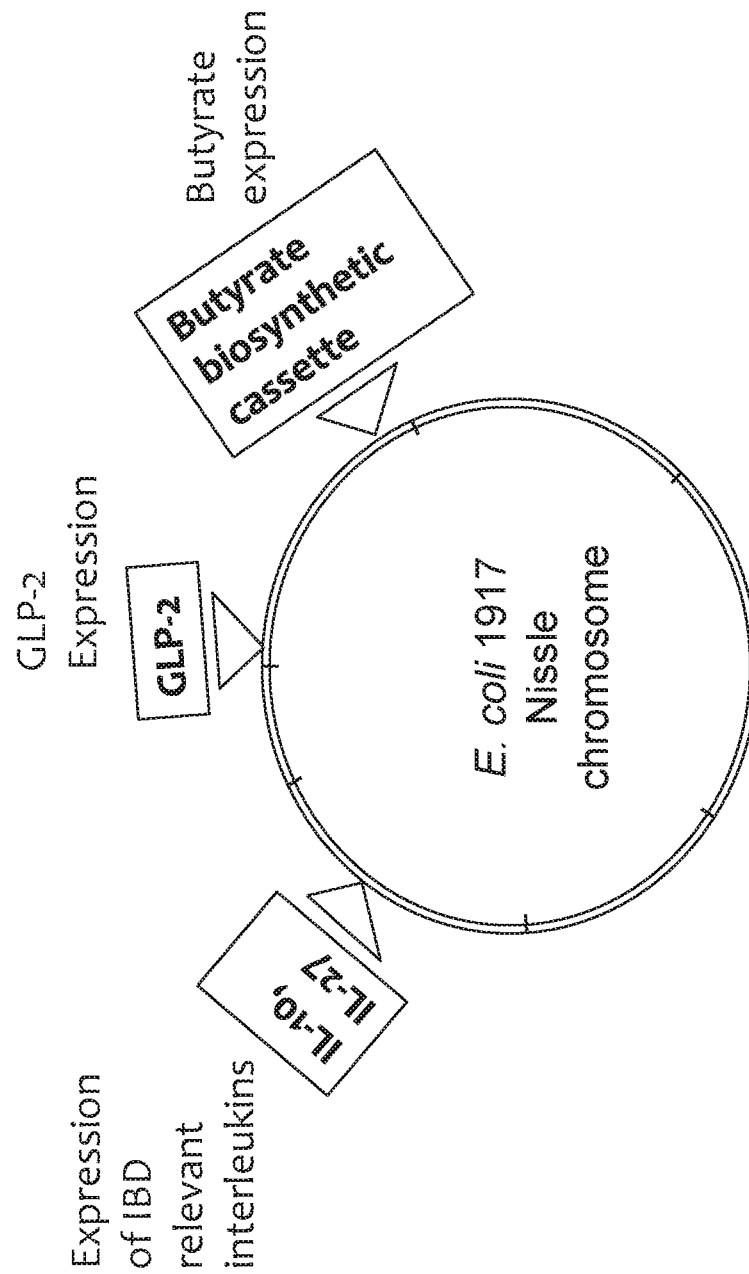


Fig. 53

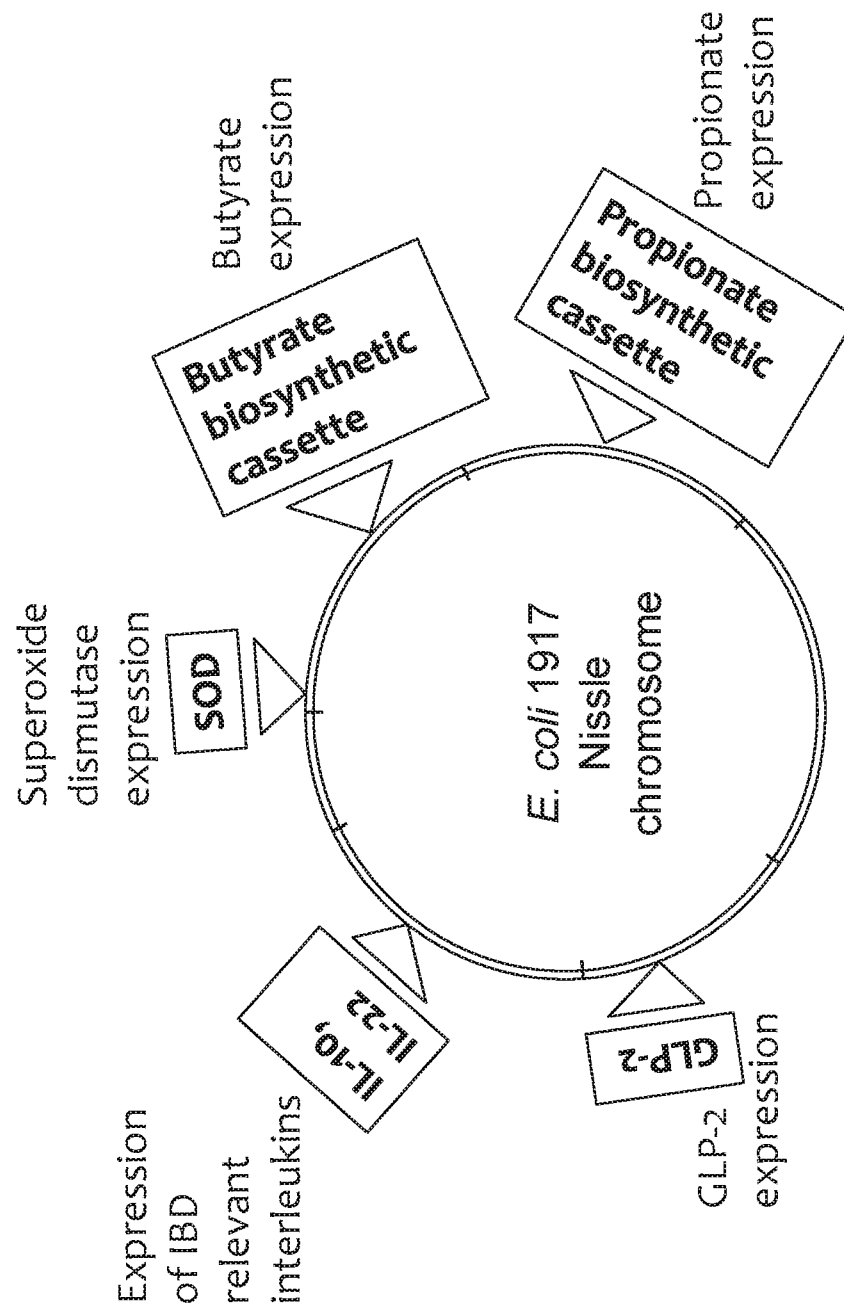


Fig. 54

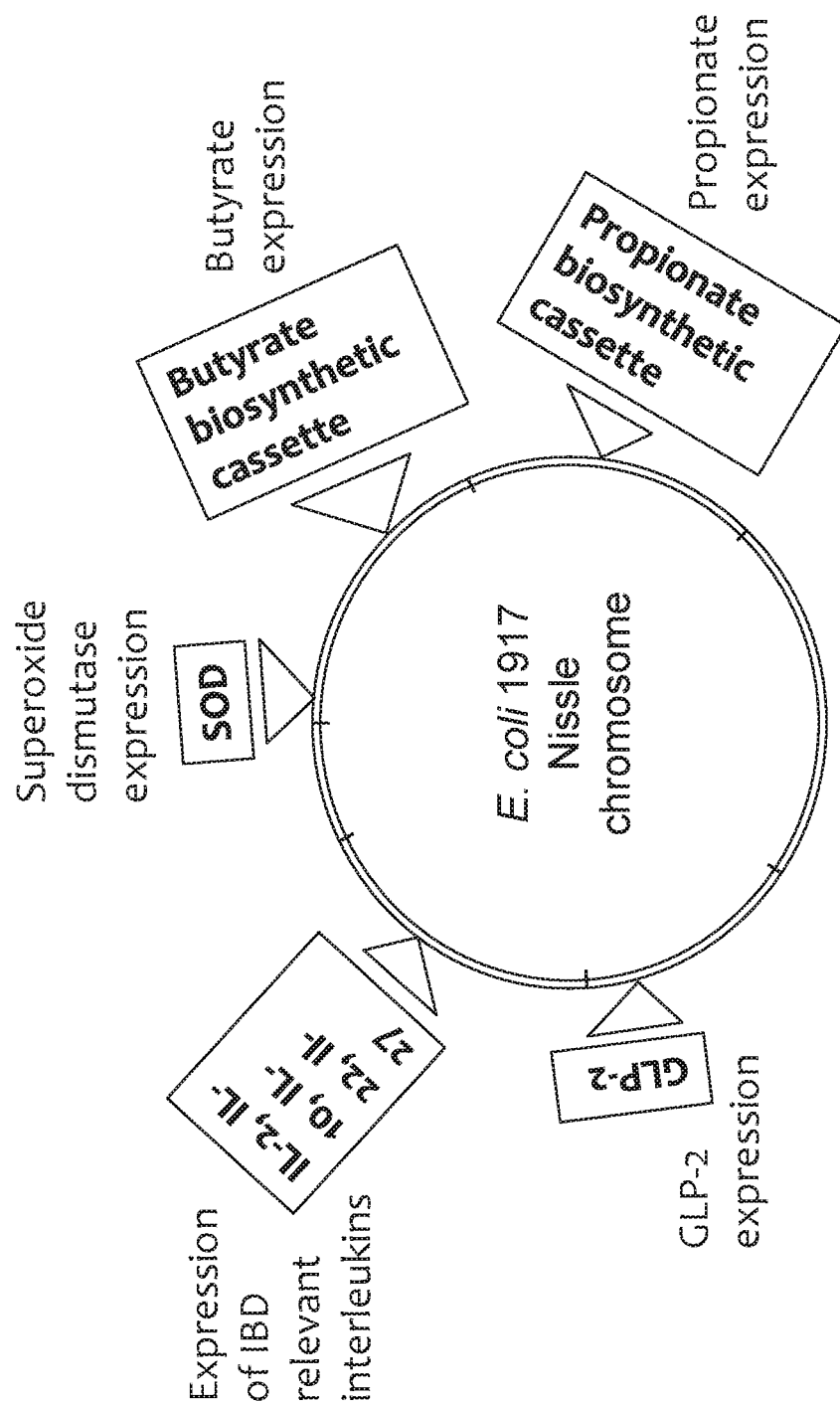


Fig. 55

Gene	argA	cysE	glnA	glyA	hisB	ilvA	leuB	lysA	metA
AA Auxotroph	Arginine	Cysteine	Glutamine	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine
Pre-Gavage									
24 hours									
48 hours									

Gene	pheA	proA	serA	thrC	trpC	tyrA	ilvD	thyA	uraA	flhD
AA Auxotroph	Phenylalanine	Proline	Serine	Threonine	Tryptophan	Tyrosine	Valine/Isoleucine/Leucine	Thiamine	Uracil	FlhD
Pre-Gavage										
24 hours										
48 hours										

Present

Absent

Fig. 56

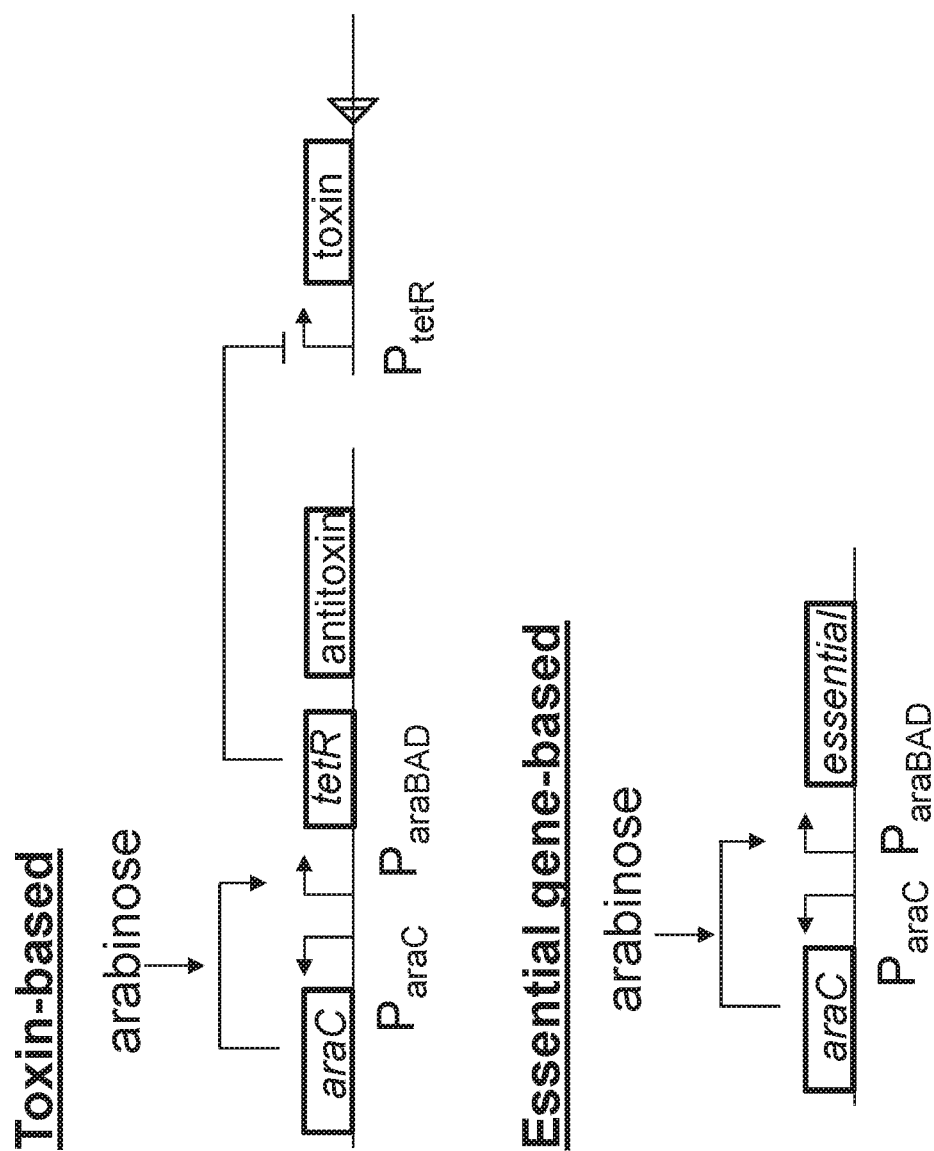
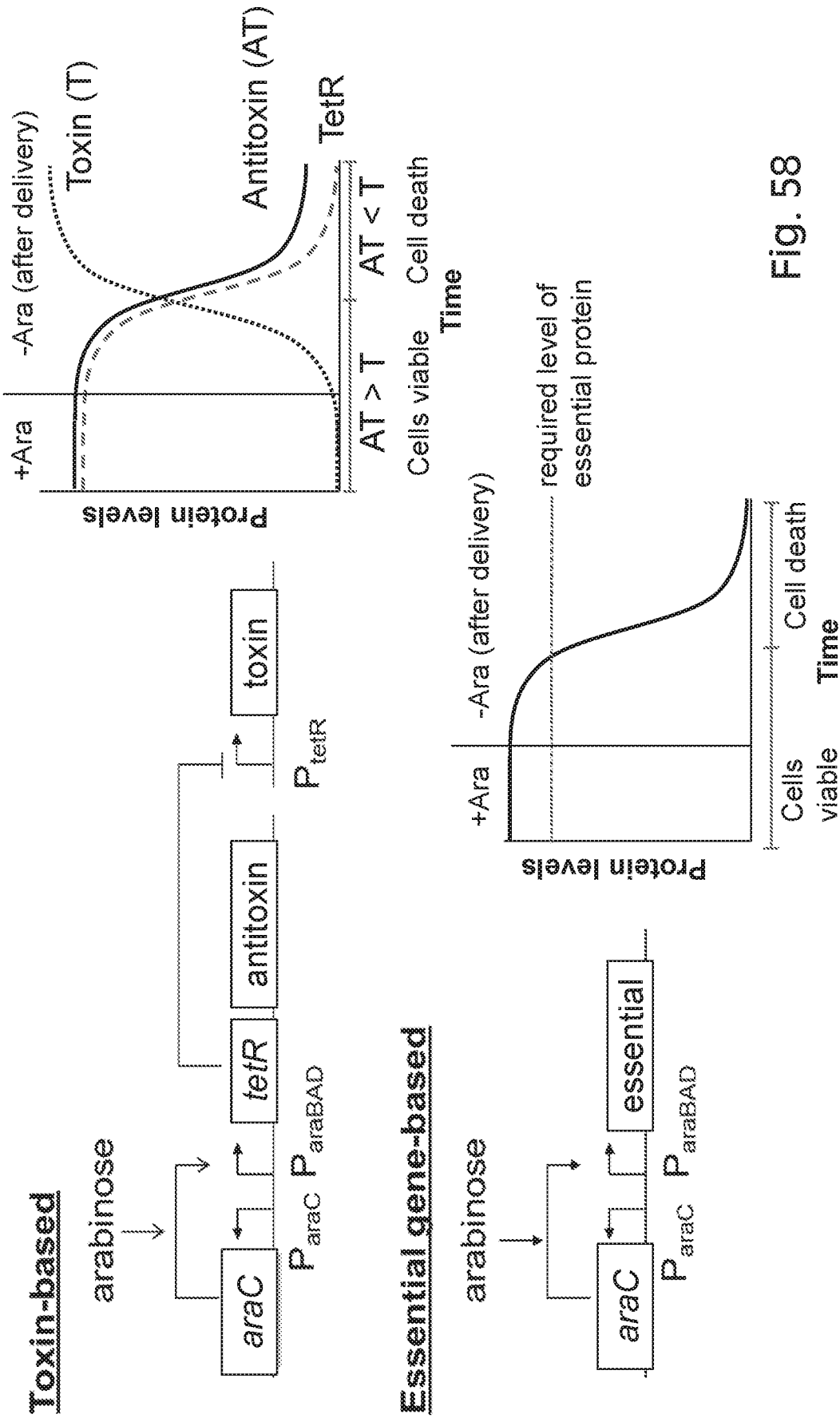


Fig. 57



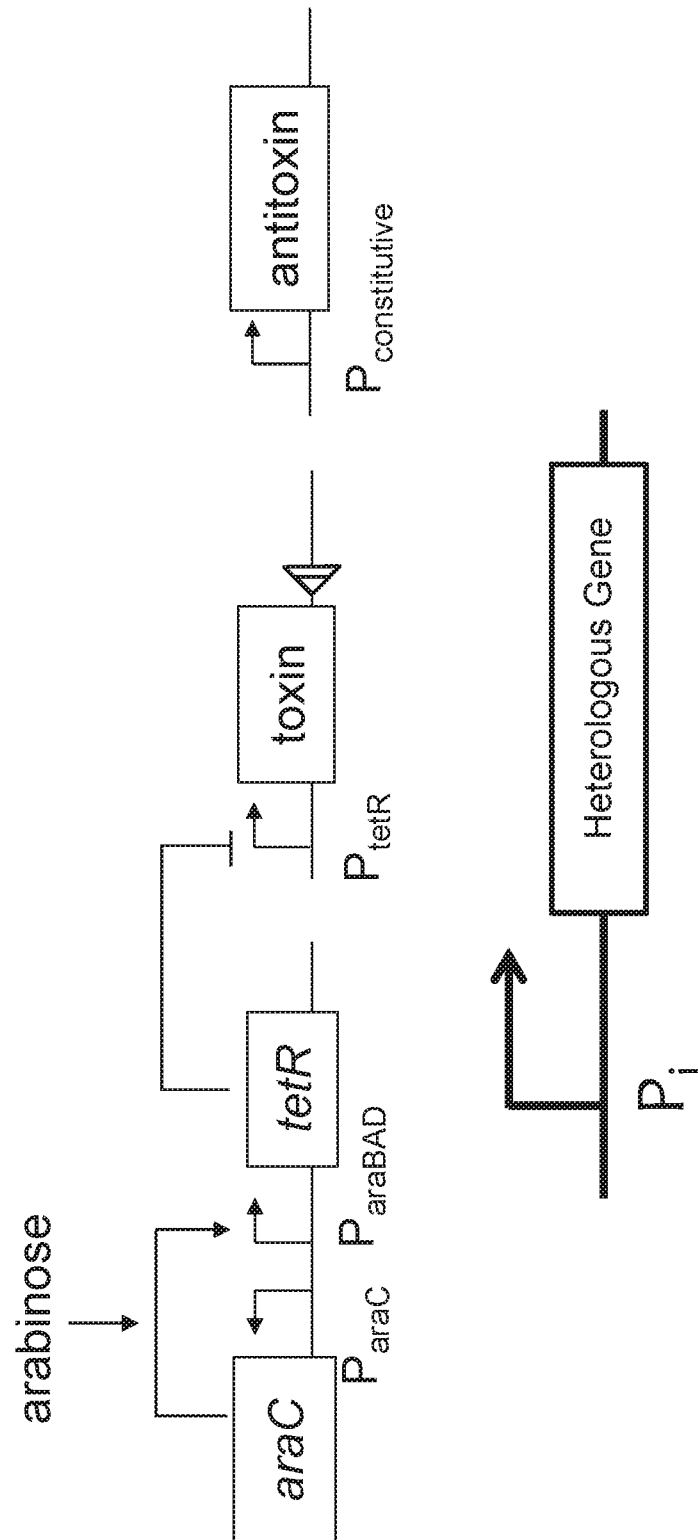


Fig. 59

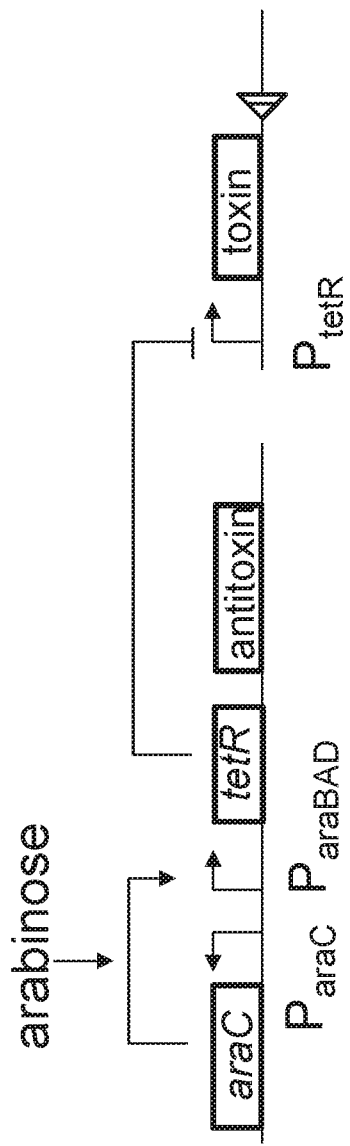


Fig. 60

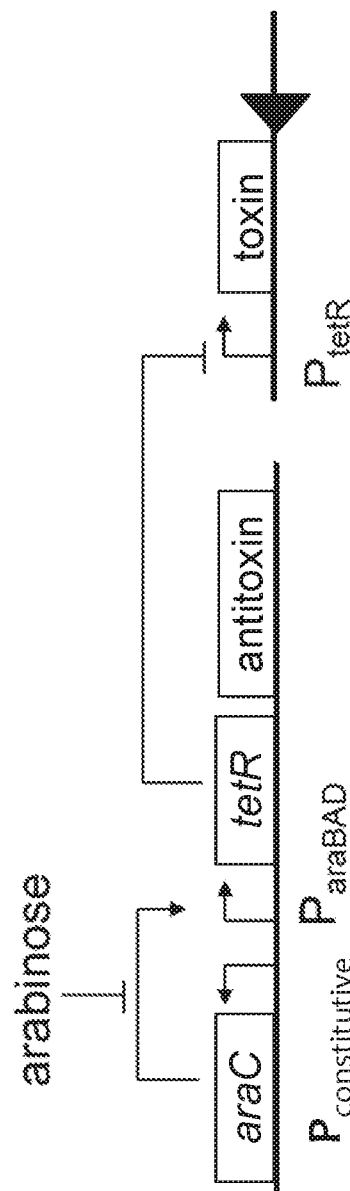


Fig. 61

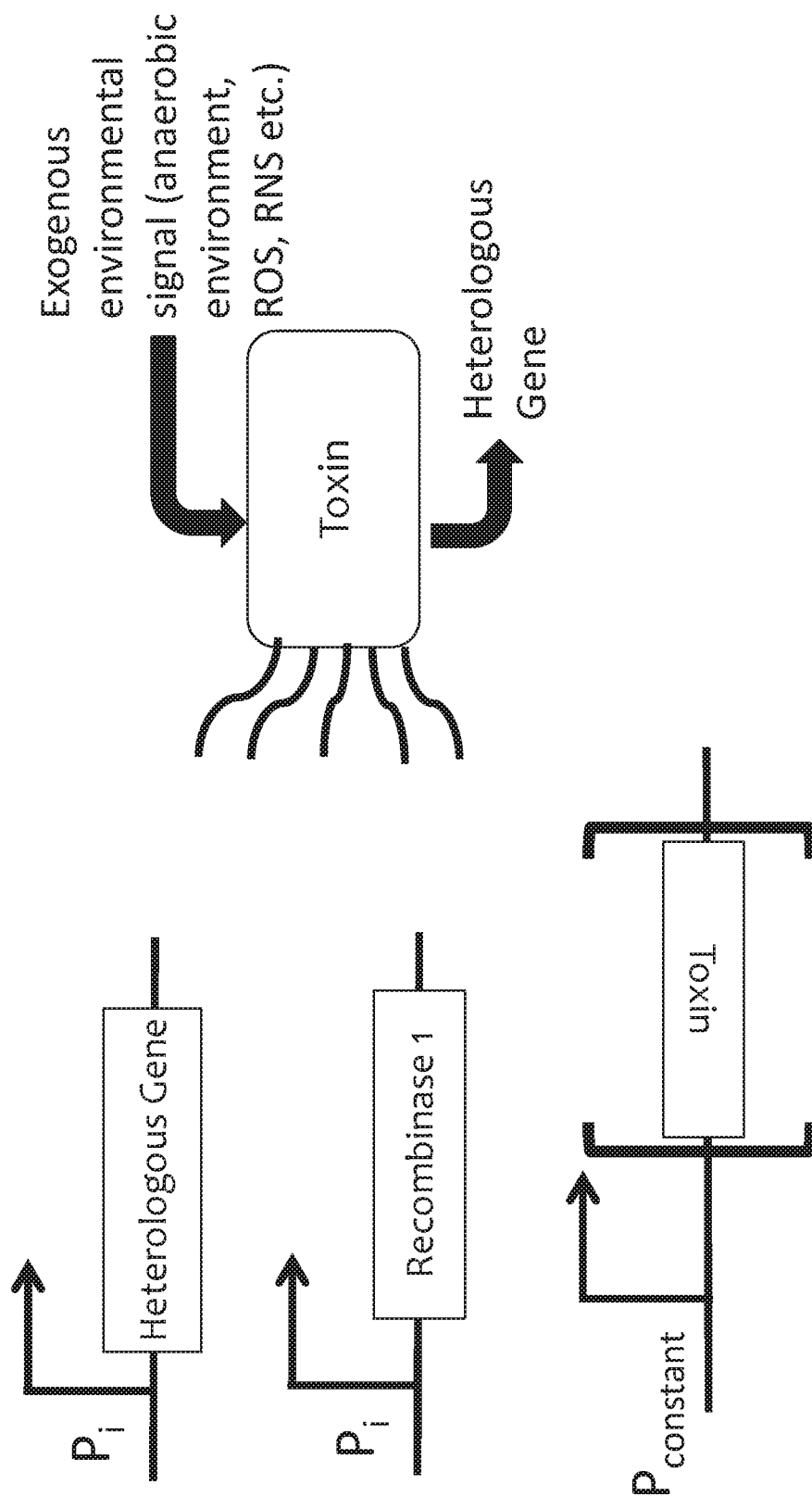


Fig. 62

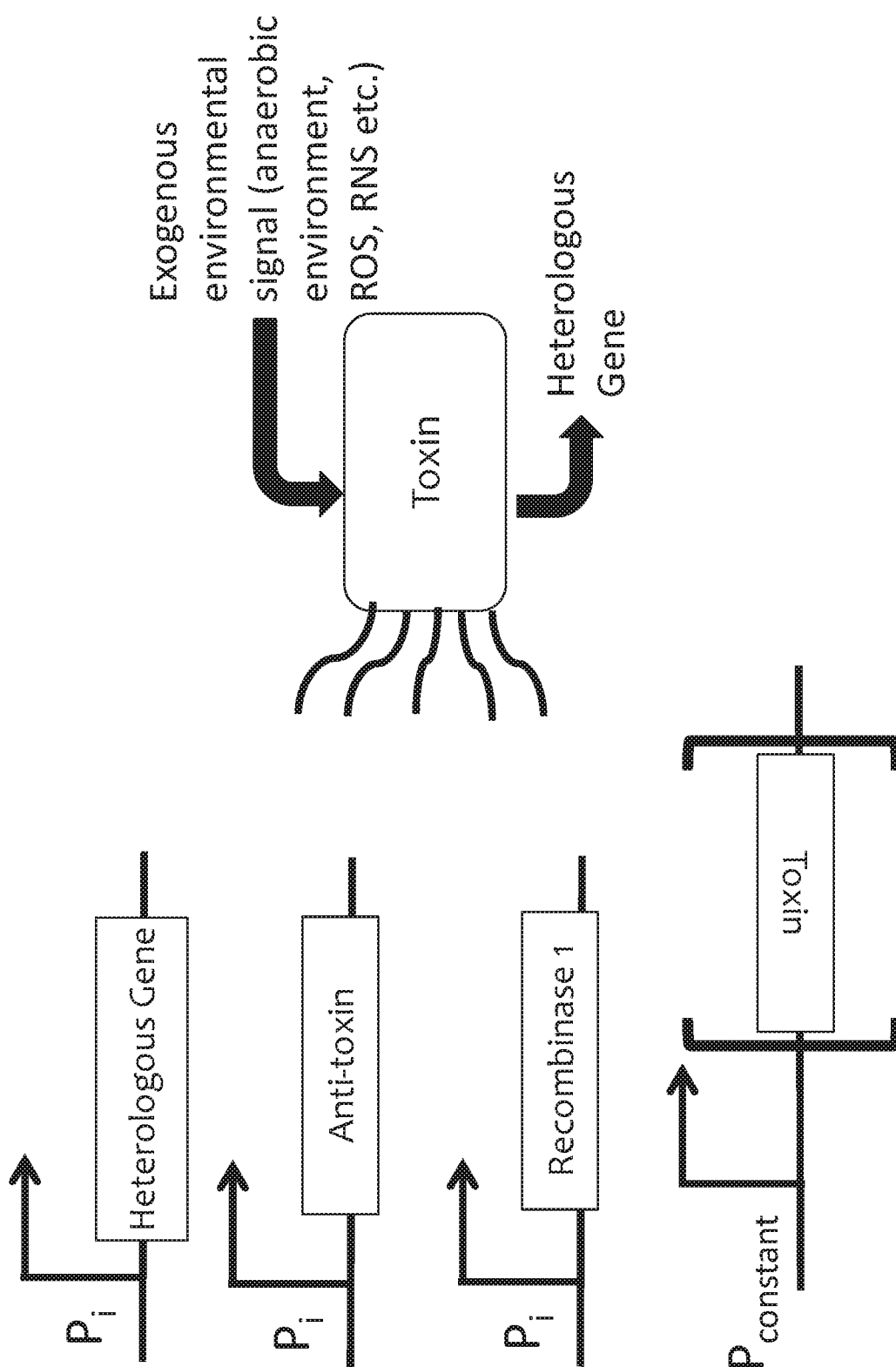


Fig. 63

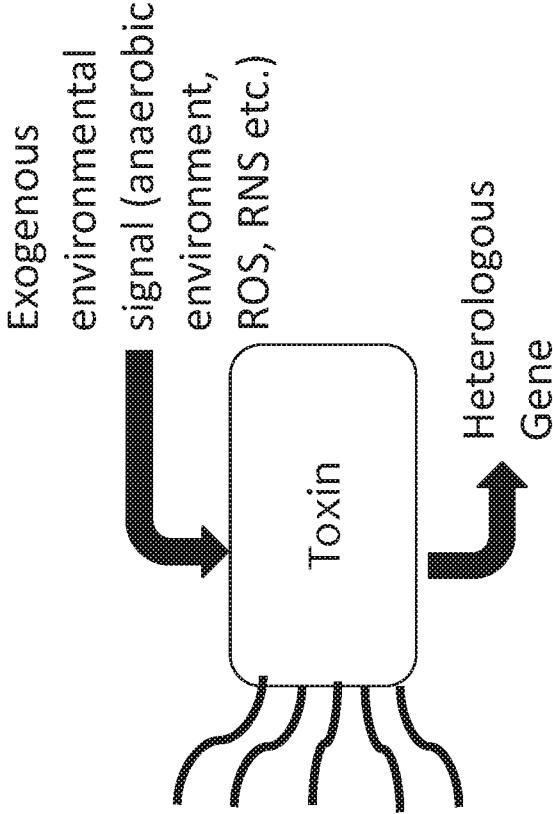
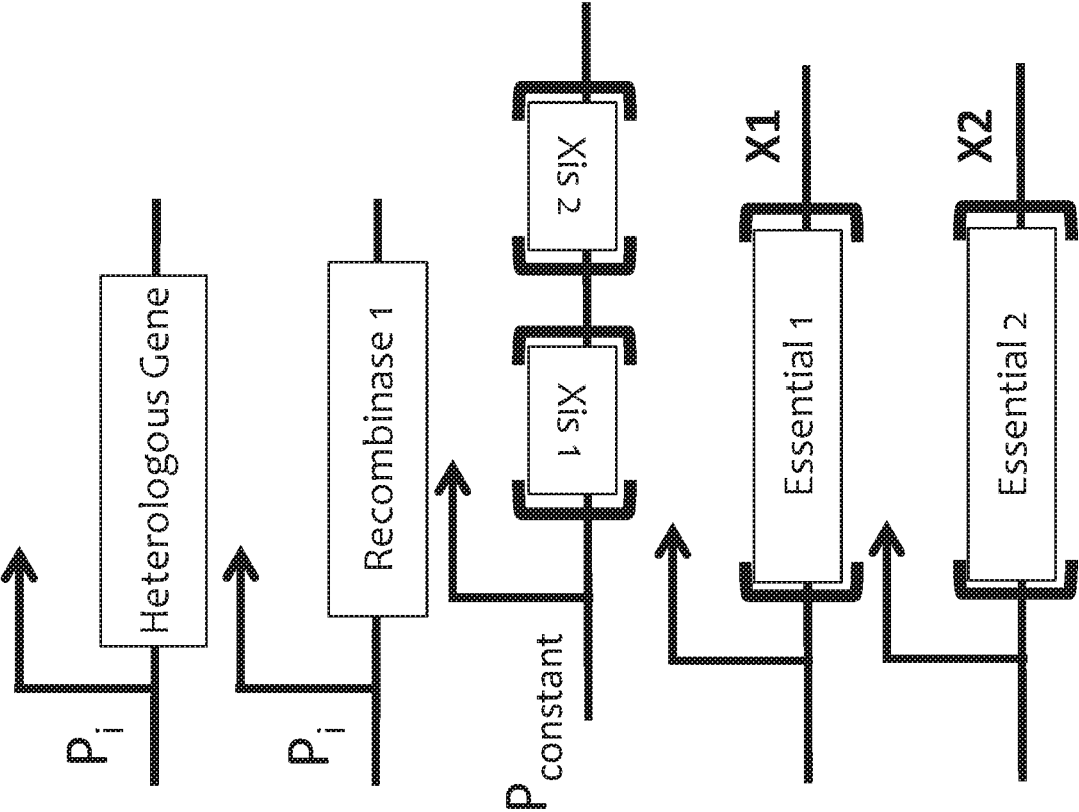


Fig. 64

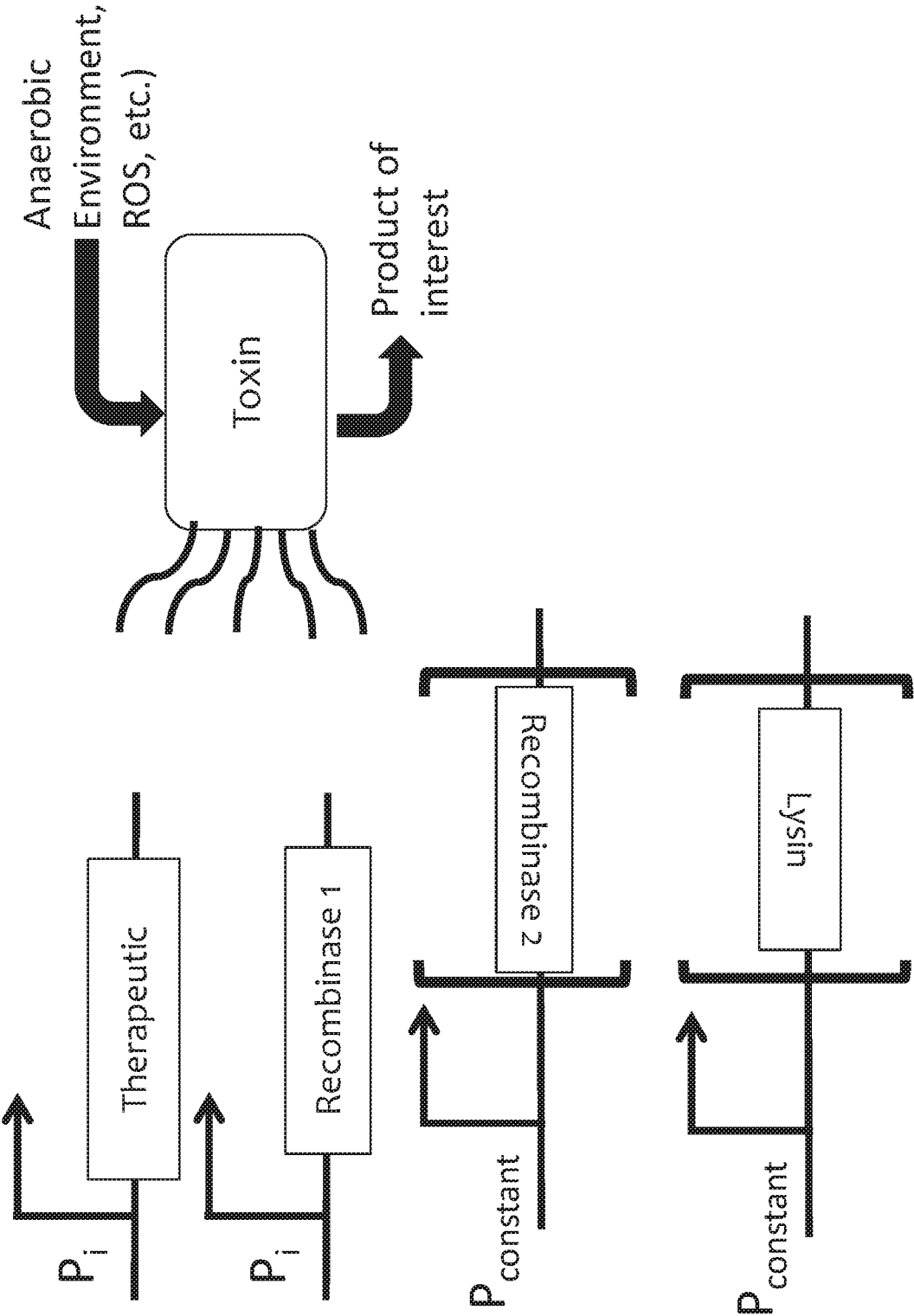


Fig. 65

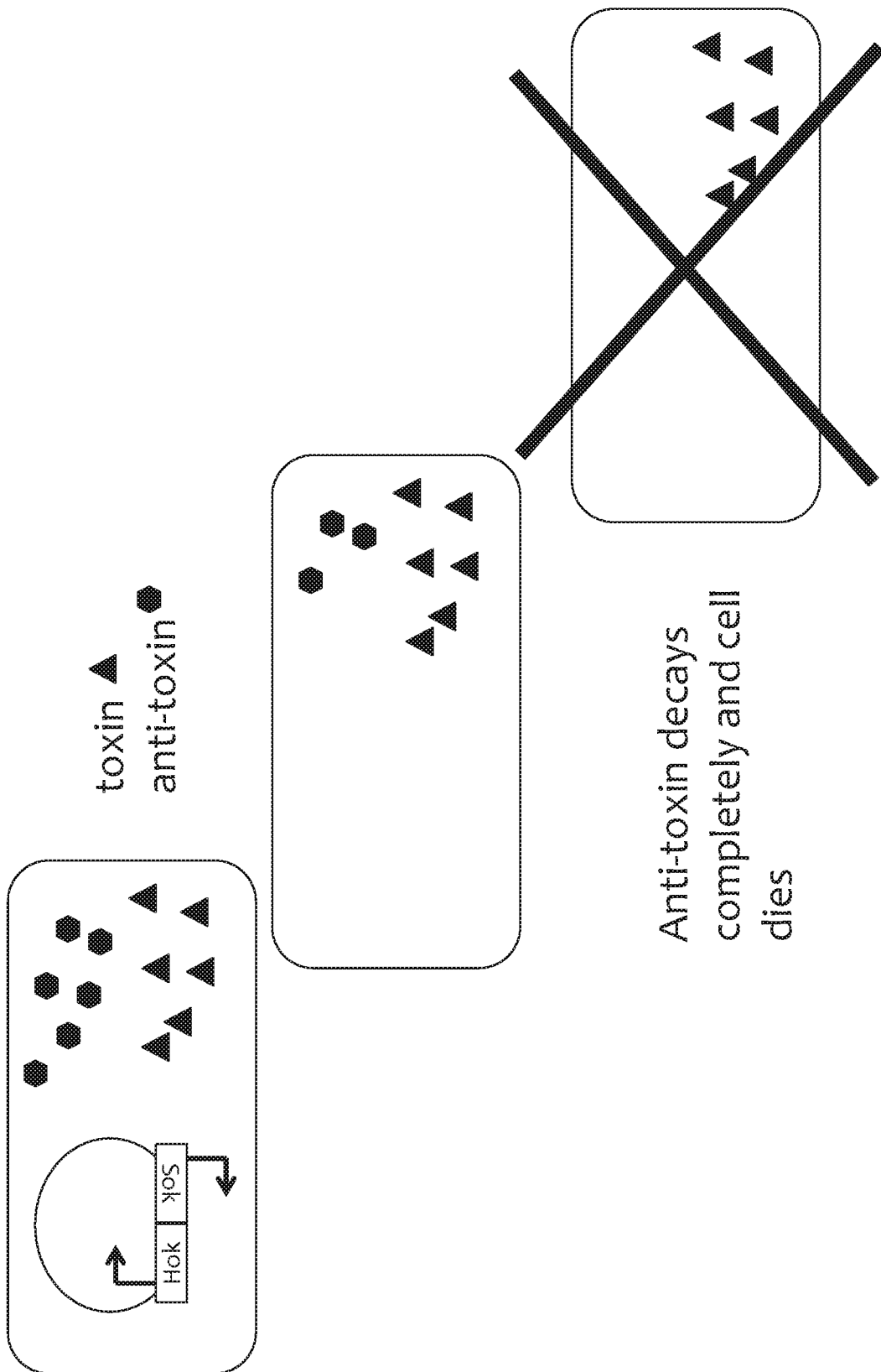


Fig. 66

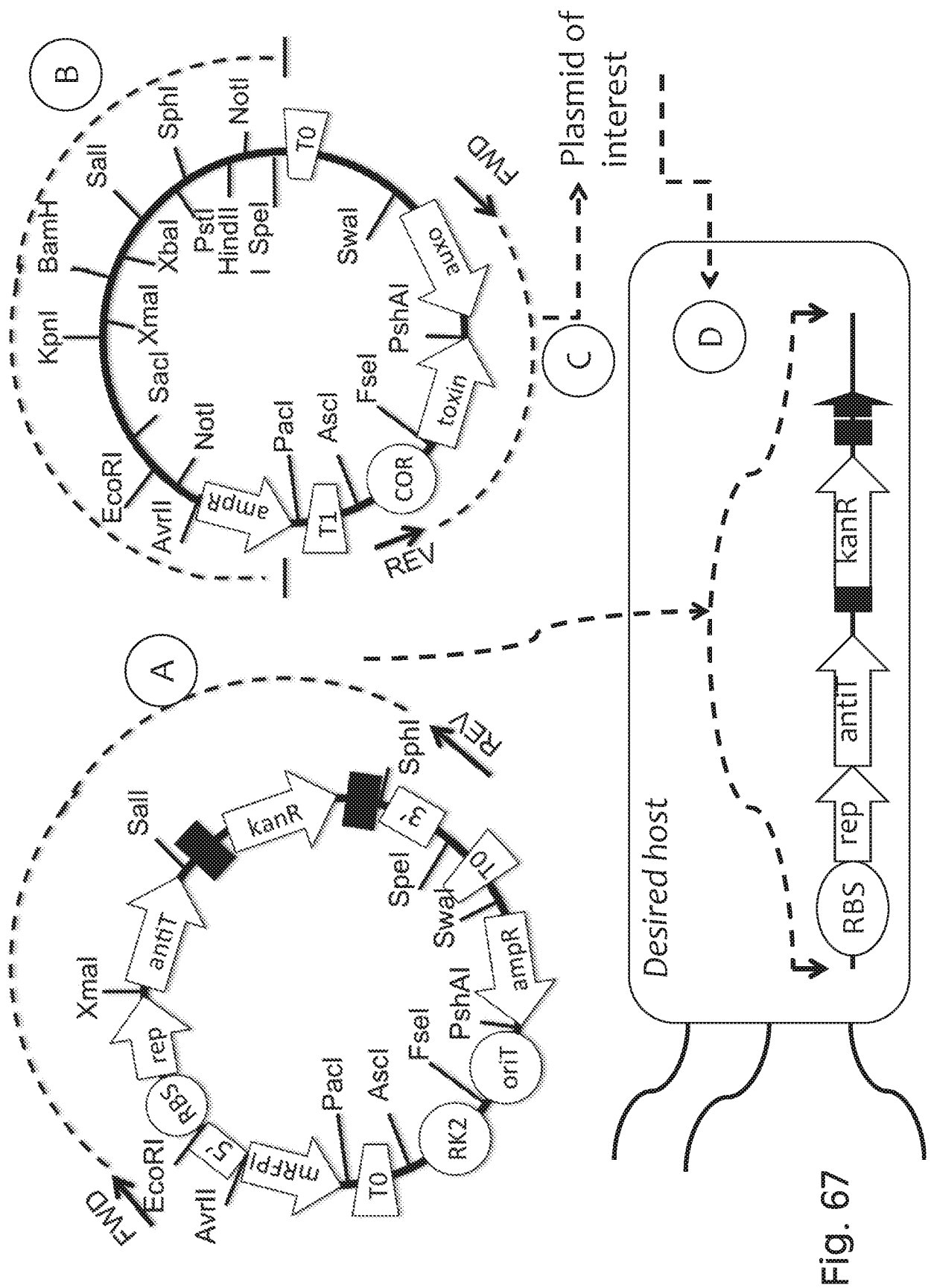


Fig. 67

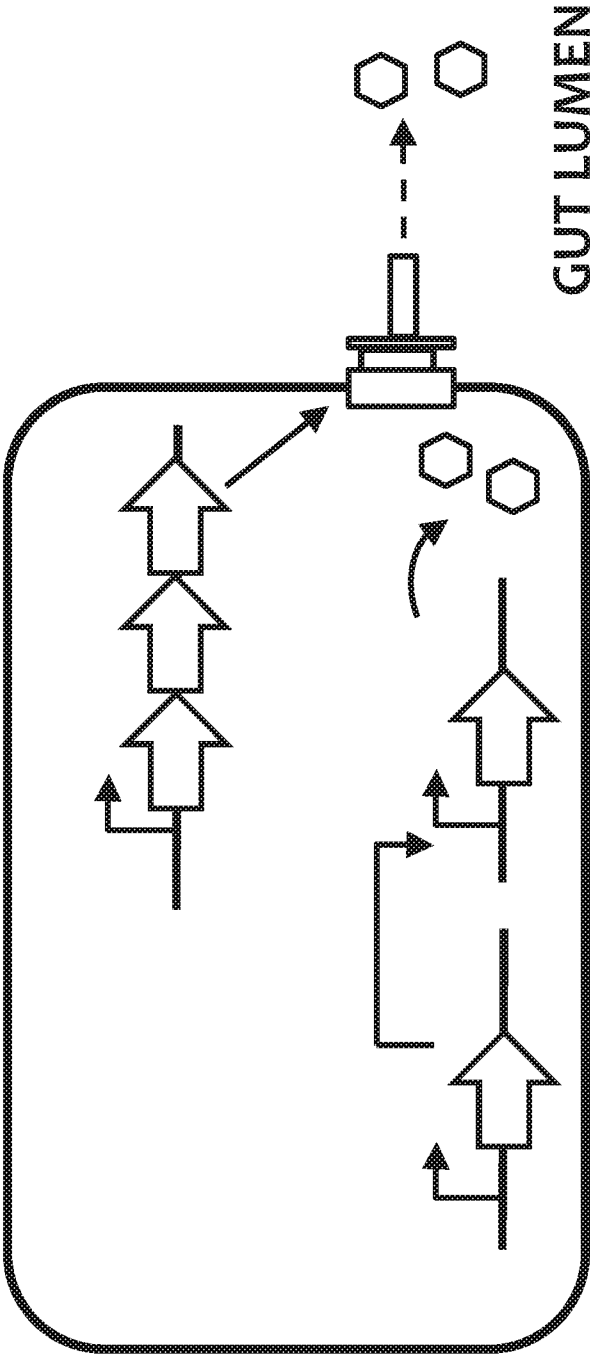


Fig. 68

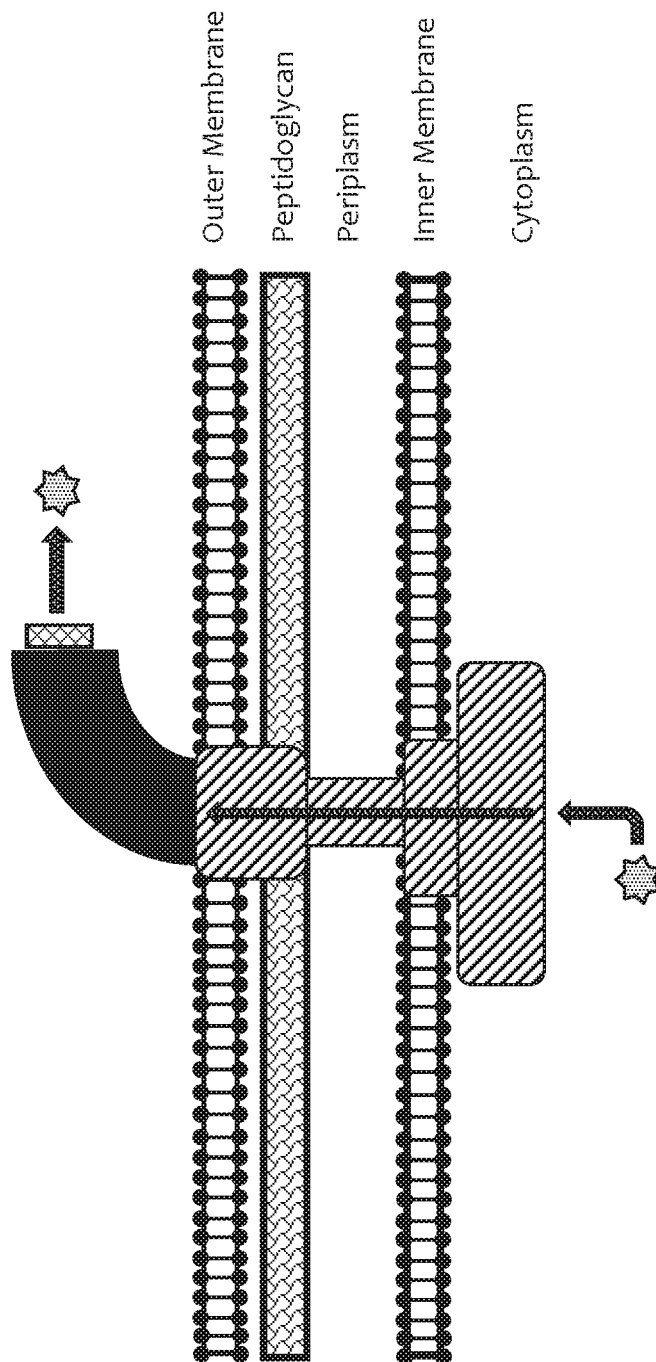


Fig. 69

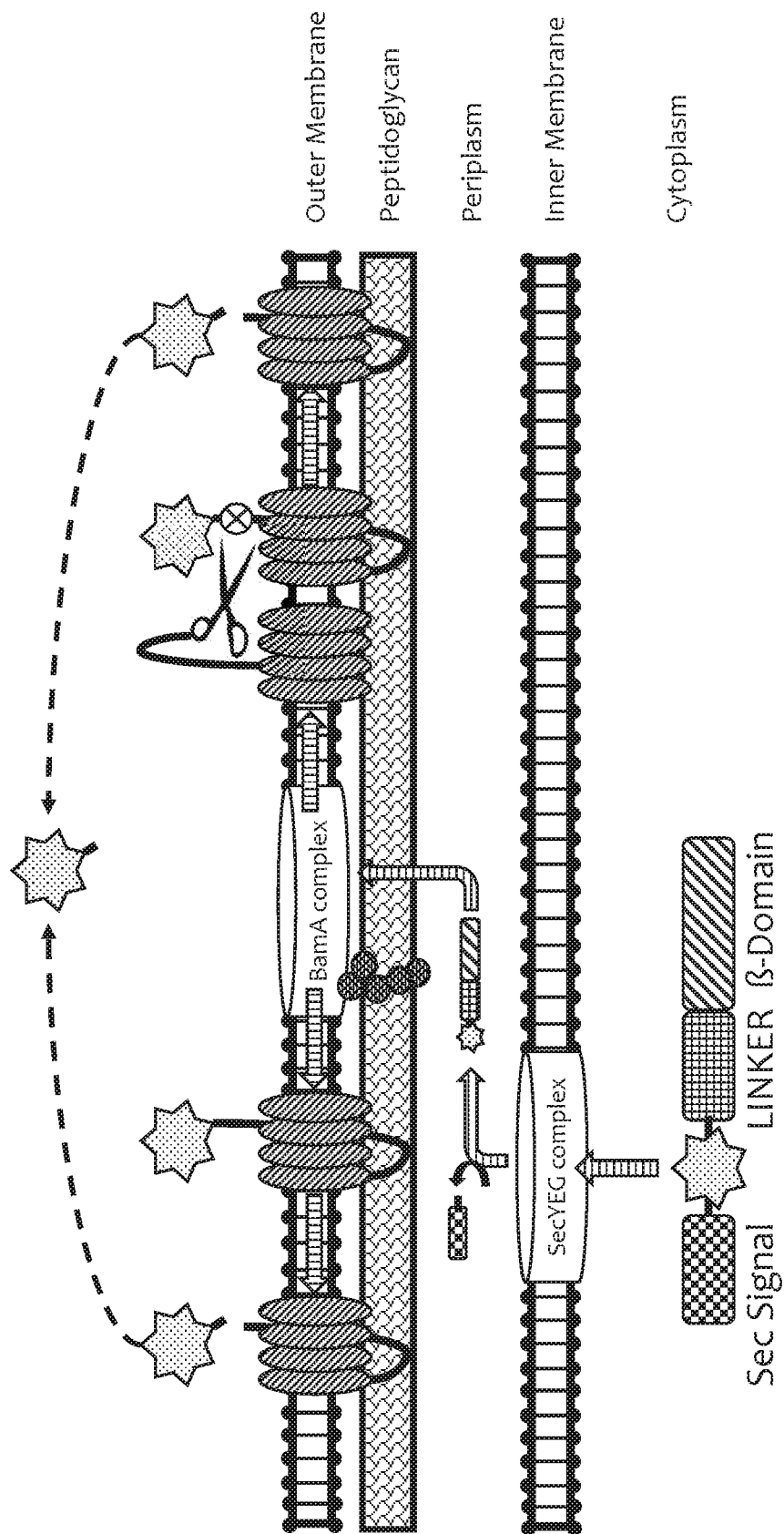


Fig. 70

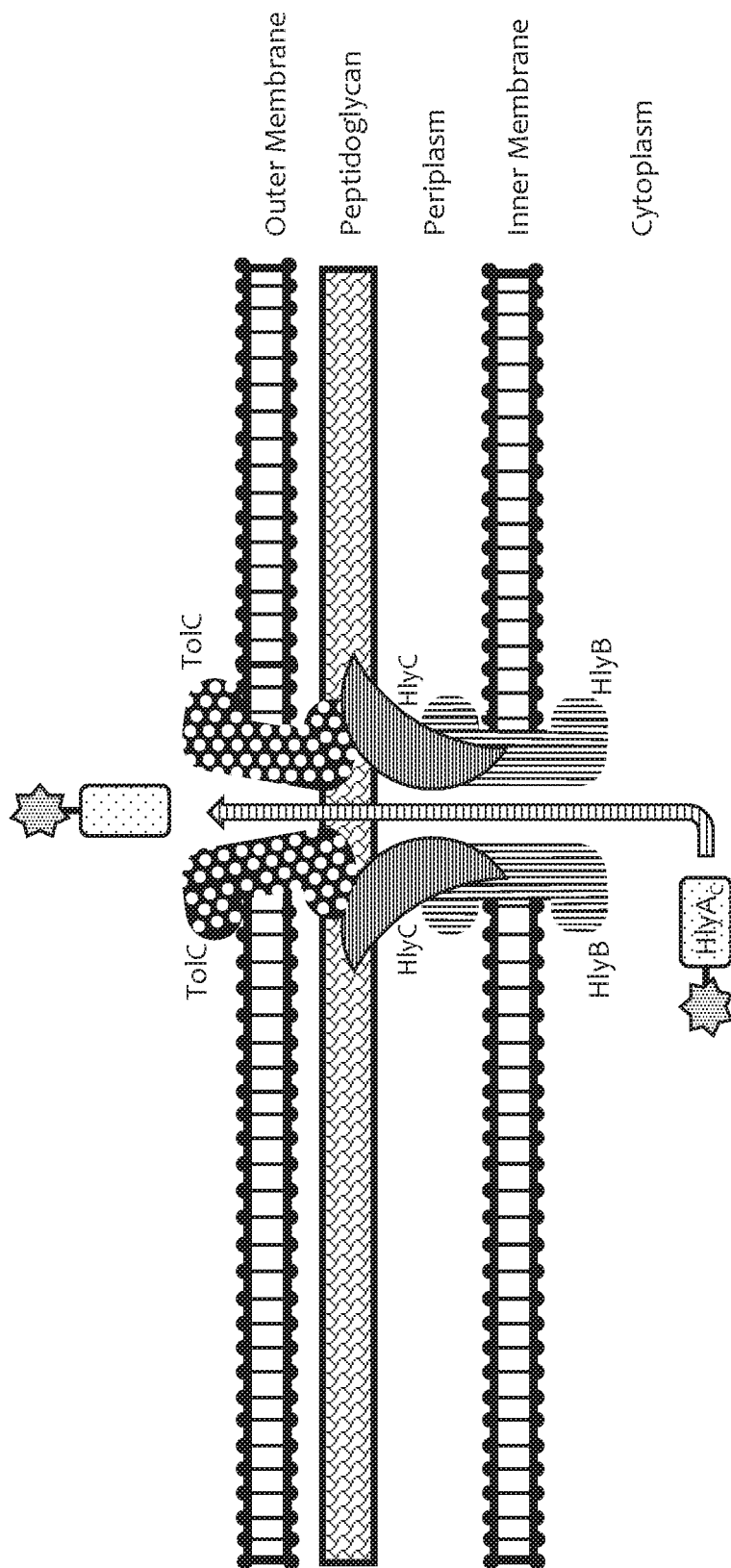


Fig. 71

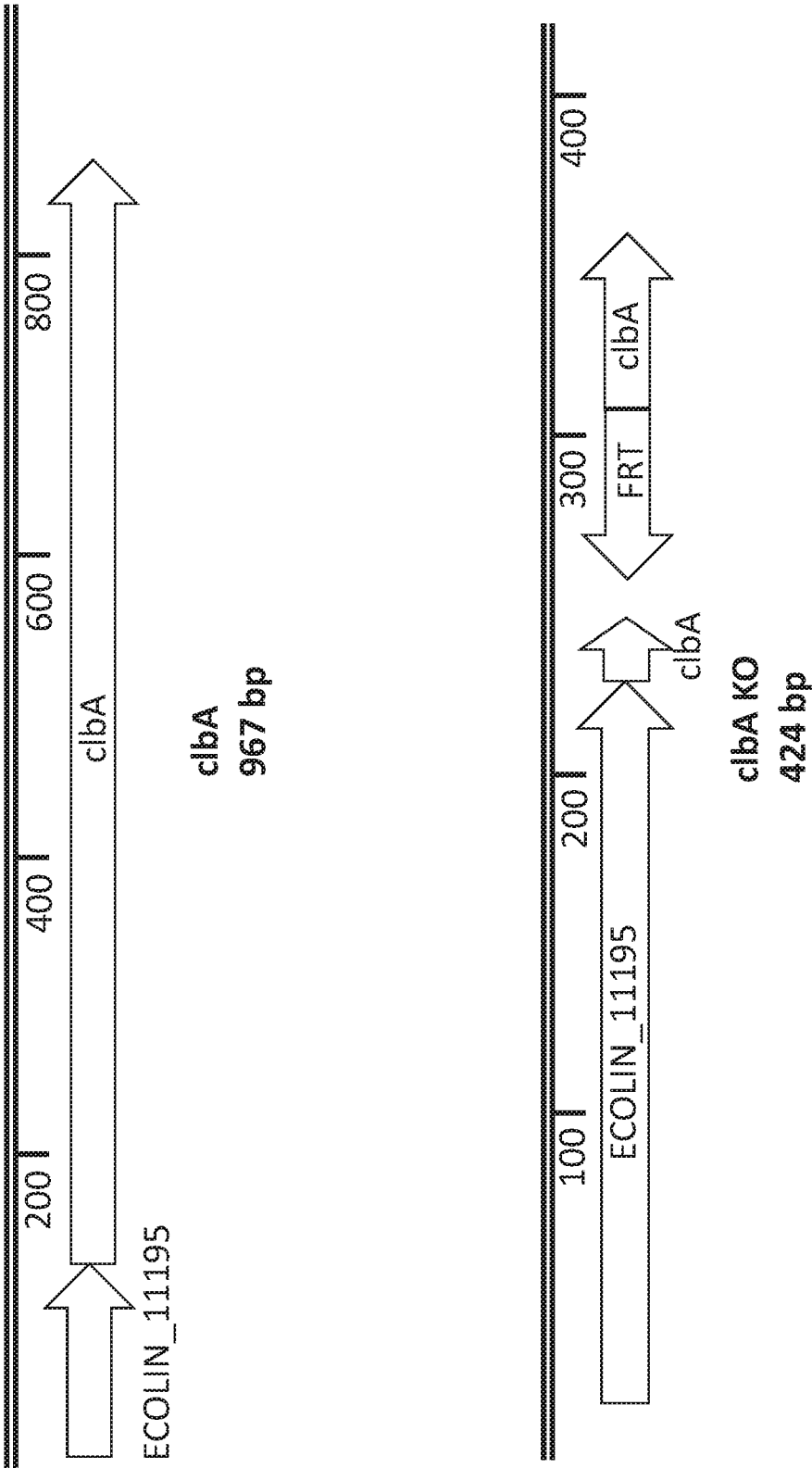


Fig. 72

Wild-type clbA (SEQ ID NO: 82)	clbA knockout (SEQ ID NO: 83)
caaatacacataatcttaacatatcaataaacaacagtaaaagtctcatgtgaaaaacatcaaac ataaaatacaagctcggaatacgaatcacgctatacacattgctaacaggaatgagattatcta aatgaggattgatatataattggacatactagttttttcatcaaacccagtagagataacttc cttcactatctcaatgaggagaataaaaacgctatgatcagttttcattttgtgagtataaaag aactctatattttaagccgtatcctgctcaaaaacagcaactaaaagatatcaacctgatgtctc attacaatcatggcaatttagtacgtgcaaatatggcaaacatttatagtttttccctcagttg gcaaaaaagatttttttaacctttcccatactatagatacagtagccgttgctattagttctc actgcgagcttgggtcgatattgaacaaataagagatttagacaacctttatctgaatatcag tcagcattttttactccacaggaagctactaacataagtttccacttccctcgttatgaaggtcaa ttacttttttgaaaaatgtggacgctcaaaagagcttacatcaaatatcgaaggtaaaggcctat ctttaggactggattgtattgaattttcattttaacaaataaaaaaactaaacttcaaaatataaggg ttccacctgtttatttctctcaatggaaaatatgtaactcatlttctcgcattagccctctccactc atcacccctaaaaataactattgagctattttcctatgcagtgcccaactttatcaccacgactatc agctaatttcattcgtcaaatgggcagaattggaatcgccacggataaatctagacacttctgagcc gtcgataaatattgattttcatattccgtcgtggtgtaagtatccgcataaatcgtgccattca catttag	ggatggggggaacatggataaagttcaaaagaaaaaaacccgttatctctgcgtgaaagacaagt attgcgcatgctggcacaaggatgatgagtactctcaaatatcacataatcttaacatatcaata aacacagtaaaagtctcatgtgaaaaacatcaaacataaaaatacaagctcgggaatcgaatcacg ctatacacattgctaacaggaatgagattatctaaatgaggattgaTGTAGGCTGGAGCTGC TTCGAAGTTCCTATACTTCTAGAGAAATAGGAAC ^{TT} CGGAATAGGAAC ^{TT} CGGAATAGGAAC ^{TA} AGGAGGATATTCATATGtcgtcaaatgggcagaattgaatcgccacggataaatctagacacttc tgagccgctcgataaatattgattttcatattccgtcgtggtg

Fig. 73

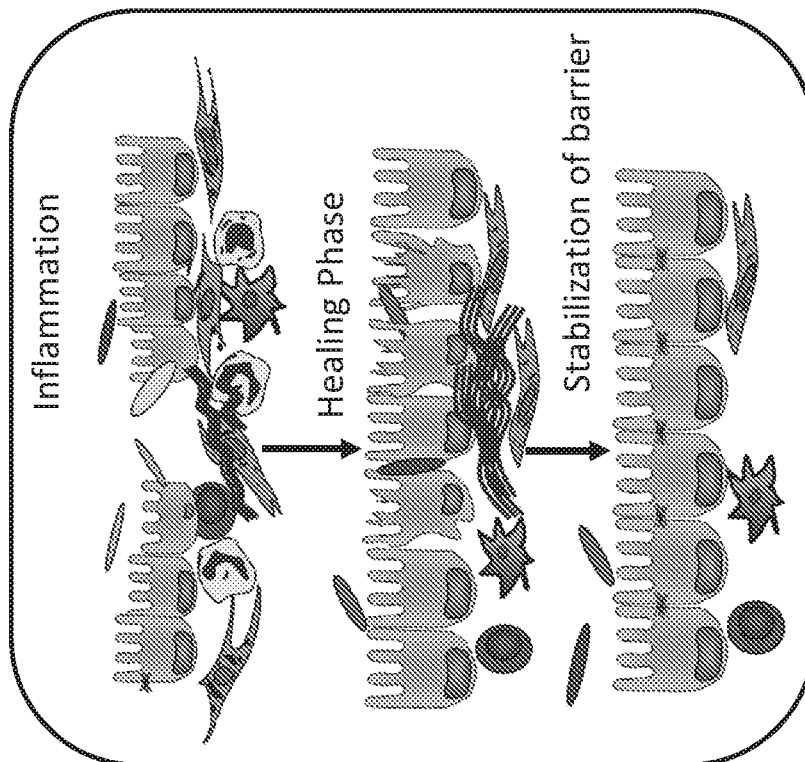


Fig. 74

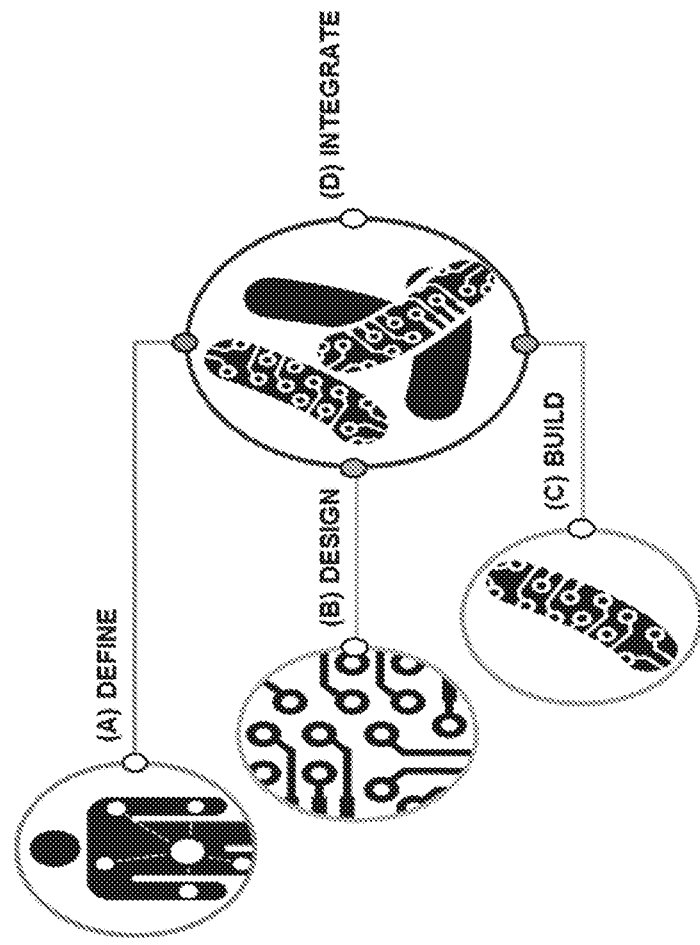


Fig. 75

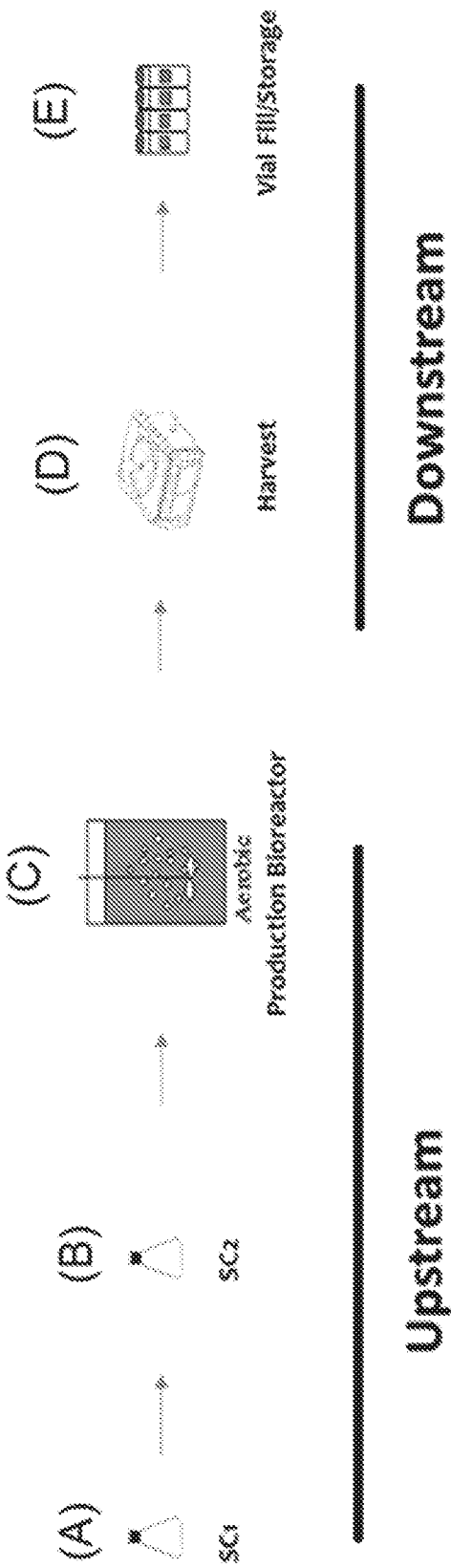


Fig. 76

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/020530

A. CLASSIFICATION OF SUBJECT MATTER
 INV. A61K35/74 C12N15/70 C12N15/74
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal , BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	wo 2006/079790 A2 (UNIV LEEDS [GB] ; CARDING SIMON [GB] ; FARRAR MARK [GB]) 3 August 2006 (2006-08-03) Whole doc., in parti cul ar pi (bottom para.); p.4, 1.18-19; p.7, 1.13; p.7, 1.23-33 . -----	I , 7-9 , II , 12 , 19-23 , 25
X	wo 2013/175358 A1 (AGRONOMIQUE INST NAT RECH [FR]) 28 November 2013 (2013-11-28) Refer to ci tati on bel ow for content ----- -/--	I , 7-9 , II , 12 , 19-23 , 25



Further documents are listed in the continuation of Box C.



See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 June 2016

Date of mailing of the international search report

24/06/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040,
 Fax: (+31-70) 340-3016

Authorized officer

Roscoe, Ri chard

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/020530

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 2015/139940 AI (BERMUDEZ HUMARAN LUIS [FR] ET AL) 21 May 2015 (2015-05-21) Note: Cited as English Equivalent of W02013175358 (French), published on 28.11.2013. Whole doc., especially para. [0009, 0013, 0035, 0055]	1,7-9 , 11, 12 , 19-23 ,25
A	----- K. SCHREIBER ET AL: "The Anaerobic Regulatory Network Required for Pseudomonas aeruginosa Nitrate Respiration", JOURNAL OF BACTERIOLOGY, vol. 189, no. 11, 1 June 2007 (2007-06-01), pages 4310-4314, XP55278698, US ISSN: 0021-9193, DOI: 10.1128/JB.00240-07 the whole document	1-25
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A	----- STEIDLER L ET AL: "Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, US, vol. 21, no. 7, 1 July 2003 (2003-07-01), pages 785-789, XP002276104, ISSN: 1087-0156, DOI: 10.1038/NBT840 the whole document -----	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2016/020530

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US201 6/020530

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☐ forming part of the international application as filed:
- ☐ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13fer1 (a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
- ☒ in the form of an Annex C/ST.25 text file (Rule 13fer1 (a)).
- ☒ on paper or in the form of an image file (Rule 13fer1 (b) and Administrative Instructions, Section 7 13).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: