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(54) Titre : BACTERIES MODIFIEES POUR TRAITER LES TROUBLES IMPLIQUANT LE CATABOLISME DU PROPIONATE

(54) Title: BACTERIA ENGINEERED TO TREAT DISORDERS INVOLVING PROPIONATE CATABOLISM

(57) Abrégé/Abstract:
The present disclosure provides engineered bacterial cells comprising a heterologous gene encoding a propionate catabolism enzyme. In another aspect, the engineered bacterial cells further comprise at least one heterologous gene encoding a transporter of propionate or a kill switch. The disclosure further provides pharmaceutical compositions comprising the engineered bacteria, and methods for treating disorders involving the catabolism of propionate, such as Propionic Acidemia and Methylmalonic Acidemia, using the pharmaceutical compositions.

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(54) **Title**: BACTERIA ENGINEERED TO TREAT DISORDERS INVOLVING PROPIONATE CATABOLISM

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CONTENANT LES PAGES 1 À 236

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JUMBO APPLICATIONS/PATENTS

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BACTERIA ENGINEERED TO TREAT DISORDERS INVOLVING PROPIONATE CATABOLISM

Related Applications

[01] This application claims priority to U.S. Provisional Patent Application No. 62/199,445, filed on July 31, 2015, PCT Application No. PCT/US2016/032565, filed May 13, 2016, U.S. Provisional Patent Application No. 62/336,338, filed May 13, 2016, U.S. Provisional Patent Application No. 62/341,320, filed May 25, 2016, and PCT Application No. PCT/US2016/037098, filed June 10, 2016, the entire contents of each of which are expressly incorporated herein by reference.

Background

[02] In healthy subjects, the human body converts certain amino acids, such as isoleucine, valine, threonine, and methionine, as well as odd chain fatty acids, into propionyl CoA to create energy (**FIG. 4**). The enzyme propionyl CoA carboxylase (PCC) then converts propionyl CoA to methylmalonyl CoA, and the methylmalonyl CoA mutase (MUT) enzyme then converts methylmalonyl CoA into succinyl CoA, which enters the citric acid cycle and glucogenesis.

[03] Enzyme deficiencies or mutations which lead to the toxic accumulation of propionyl CoA or methylmalonyl CoA result in the development of disorders associated with propionate catabolism, such as Propionic Acidemia (PA) and Methylmalonyl Acidemia (MMA). Severe nutritional deficiencies of Vitamin B12 can also result in MMA (Higginbottom et al., M. Engl. J. Med., 299(7):317-323, 1978). In these diseases, propionic acid or methylmalonic acid can build up in the blood stream, leading to damage of the brain, heart, and liver (**FIG. 3** and **FIG. 4**). Clinical manifestations of the disease vary depending on the degree of enzyme deficiency and include seizures, vomiting, lethargy, hypotonia, encephalopathy, developmental delay, failure to thrive, and secondary hyperammonemia (Deodato et al., Methylmalonic and propionic aciduria, *Am. J. Med. Genet. C. Semin. Med. Genet,* 142(2):104-112, 2006).

[04] Currently available treatments for disorders involving propionate catabolism are inadequate for the long-term management of the disorders and have severe limitations. A low protein diet, with micronutrient and vitamin supplementation, as necessary, is the widely accepted long-term disease management strategy for many such disorders (Saudubray *et al.*,

Inborn Metabolic Diseases, Diagnosis, and Treatment, 2012). Supplementation with L-carnitine, as well as antibiotic therapy to remove intestinal propiogenic flora is also often utilized. However, dietary-intake restrictions can be particularly problematic since protein is required for metabolic activities (Baumgartner *et al., Orphanet. J. Rare Dis.*, 9(130):1-36, 2014). Thus, even with proper monitoring and patient compliance, dietary restrictions result in a high incidence of mental retardation (Baumgartner *et al.*, 2014). Liver transplantation has recently been considered for PA and MMA subjects (Li *et al., Liver Transpl.*, 2015). However, the limited availability of donor organs, the costs associated with the transplantation itself, and the undesirable effects associated with continued immunosuppressant therapy limit the practicality of liver transplantation for treatment of disorders involving the catabolism of propionate. Therefore, there is significant unmet need for effective, reliable, and/or long-term treatment for disorders involving the catabolism of propionate.

Summary

[05] The present disclosure provides engineered bacterial cells, pharmaceutical compositions thereof, and methods of modulating and treating disorders involving the catabolism of propionate. Specifically, the engineered bacteria disclosed herein have been constructed to comprise genetic circuits composed of, for example, one or more propionate catabolism genes to treat the disease, as well as other optional circuitry designed to ensure the safety and non-colonization of a subject that is administered the engineered bacteria, such as, for example, auxotrophies, kill switches, and combinations thereof. These engineered bacteria are safe and well tolerated and augment the innate activities of the subject's microbiome to achieve a therapeutic effect.

[06] In some embodiments, the disclosure provides a bacterial cell that has been genetically engineered to comprise one or more genes, gene cassettes, and/or synthetic circuits encoding a propionate catabolism enzyme or propionate catabolism pathway, and is capable of metabolizing propionate and/or other metabolites, such as propionyl CoA, methylmalonate, and/or methylmalonyl CoA. Thus, the genetically engineered bacterial cells and pharmaceutical compositions comprising the bacterial cells may be used to treat and/or prevent diseases associated with propionate catabolism, such as propionic acidemia (PA) and methylmalonic acidemia (MMA).

[07] In some embodiments, the disclosure provides a bacterial cell that has been engineered to comprise gene sequence(s) encoding one or more propionate catabolism

enzyme(s). In some embodiments, the disclosure provides a bacterial cell has been engineered to comprise gene sequence(s) encoding one or more propionate catabolism enzyme(s) and is capable of reducing the level of propionate and/or other metabolites, for example, methylmalonate, propionyl CoA and/or methylmalonyl CoA. In some embodiments, the disclosure provides a bacterial cell has been engineered to comprise gene sequence(s) encoding one or more propionate catabolism enzyme(s) that is operably linked to an inducible promoter. In some embodiments, the disclosure provides a bacterial cell has been engineered to comprise gene sequence(s) encoding one or more propionate catabolism enzyme(s) that is operably linked to an inducible promoter that is induced under low oxygen and/or anaerobic conditions, e.g., such as those conditions found in the mammalian gut. In some embodiments, the disclosure provides a bacterial cell has been engineered to comprise gene sequence(s) encoding one or more propionate catabolism enzyme(s) that is operably linked to an inducible promoter that is induced by environmental signals and/or conditions found in the mammalian gut (e.g., induced by metabolites or biomolecules found in the mammalian gut). In some embodiments, the disclosure provides a bacterial cell has been engineered to comprise gene sequence(s) encoding one or more propionate catabolism enzyme(s) and is capable of reducing the level of propionate and/or other metabolites, for example, methylmalonate, propionyl CoA and/or methylmalonyl CoA in low-oxygen environments, *e.g.*, the gut. In some embodiments, the bacterial cell has been genetically engineered to comprise one or more circuits encoding one or more propionate catabolism enzyme(s) and is capable of processing and reducing levels of propionate, methylmalonate, propionyl CoA and/or methylmalonyl CoA, e.g., in low-oxygen environments, *e.g.*, the gut. In some embodiments, the bacterial cell of the disclosure has also been genetically engineered to comprise gene sequence(s) encoding one or more transporter(s) of propionate. Thus, the genetically engineered bacterial cells and pharmaceutical compositions comprising the bacterial cells of the disclosure may be used to convert excess propionic acid, propionyl CoA, and/or methylmalonyl CoA into non-toxic molecules in order to treat and/or prevent conditions associated with disorders involving the catabolism of propionate, such as Propionic Acidemia or Methylmalonic Acidemia.

Brief Description of the Drawings

[08] **FIG. 1** depicts schematics of exemplary synthetic biotics of the disclosure for the treatment of propionic acidemia and/or methylmalonic acidemia and/or disorders characterized by propionic acidemia and/or methylmalonic acidemia. **FIG. 1A** depicts a

schematic of an exemplary synthetic biotic of the disclosure comprising a gene cassette expressing the prpE, phaB, phaC, and phaA genes under the control of an inducible promoter. PrpE, PhaB, PhaC, and PhaA are capable of catabolizing propionate or propionyl CoA and/or methylmalonic acid or methylmalonyl CoA into P(HV-co-HB). Protein lysine acyltransferase is deleted to prevent inactivation of PrpE. **FIG. 1B** depicts a schematic of an exemplary synthetic biotic of the disclosure comprising a gene cassette expressing prpE, accA, pccB, mmcE, mutA and mutB as two polycistronic messages from two inducible promoters. PrpE, accA, pccB, mmcE, mutA and mutB are capable of catabolizing propionate or propionyl CoA and/or methylmalonic acid or methylmalonyl CoA into succinate, which can be utilized through the TCA cycle or exported from the cell. Protein lysine acyltransferase (pka) is deleted to prevent inactivation of PrpE.

[09] **FIG. 2** depicts various branched chain amino acid (BCAA) degradative pathways and the metabolites and associated diseases relating to BCAA metabolism.

[010] **FIG. 3** depicts the cause and symptoms of a disease associated with propionate catabolism, such as Propionic Acidemia (PA) and Methylmalonic Acidemia (MMA), which result from genetic defects in propionyl-CoA carboxylase or methylmalonyl-CoA mutase.

[011] **FIG. 4** depicts the differences between healthy (normal) human subjects, and subjects having a disease associated with propionate catabolism, such as propionic acidemia (PA).

[012] **FIG. 5** depicts schematics of the major pathway (**FIG. 5A**) and minor pathways (**FIG. 5B**) of propionate catabolism in healthy human subjects. Briefly, propionyl CoA is carboxylated to D-methylmalonyl CoA by the enzyme Propionyl CoA Carboxylase (PCC), which is isomerized to L-methylmalonyl CoA. A vitamin B_{12}-dependent enzyme, Methylmalonyl CoA Mutase (MUT) then catalyzes the rearrangement of L-methylmalonyl CoA to succinyl CoA, which is then incorporated into the citric acid cycle. Minor propionate catabolism pathways also exist and are present in subjects having diseases associated with propionate catabolism, such as PA, but these pathways are insufficient to counterbalance the lack of the major pathway. **FIG. 5C** depicts a schematic showing the metabolic relationship between PA and MMA. **FIG. 5D** depicts enzyme and other deficiencies in PA and MMA.

[013] **FIG. 6** depicts a graph showing propionic acidemia biomarkers in PCCAA138T hypomorph mouse model as compared to a WT FVB mouse. **FIG. 6A, FIG. 6B, and FIG. 6C** depict graphs showing detection of blood biomarkers;

propionylcarnitine/acetylcarnitine ratio (**FIG. 6A**), propionate concentration (**FIG. 6B**), and 2-methylcitrate (**FIG. 6C**). **FIG. 6D, FIG. 6E, and FIG. 6F** depict graphs showing the detection of urine biomarkers; propionyl-glycine (**FIG. 6D**), Tigylglycine (**FIG. 6E**), and 2-methylcitrate (**FIG. 6F**).

[014] **FIG. 7A, FIG. 7B, FIG. 7C** and **FIG. 7D** depict bar graphs showing the levels of endogenous (**FIG. 7A** and **FIG. 7B**) and radiolabeled propionic acid (**FIG. 7C** and **FIG. 7D**) in blood, small intestine and large intestine at various time points post subcutaneous administration of isotopic propionic acid in C57BL/6J (**FIG. 7A** and **FIG. 7C**) and PCCAA138T mice (**FIG. 7B** and **FIG. 7D**). Isotopic propionic acid is seen at very low levels in the blood, small intestine, and cecum within 30 min, indicating that enterorecirculation of propionic acid is occurring.

[015] **FIG. 8** depicts potential pathways that may be engineered into the bacteria in order to consume propionic acid and/or methylmalonic acid into inert end products. **FIG. 8A** depicts a schematic of propionate catabolism, resulting in an inert product. **FIG. 8B, FIG. 8C** and **FIG.8D** depict schematics of three exemplary pathways, which can be utilized for propionate or methylmalonic acid catalysis. The methylmalonyl-CoA (human) pathway and the 2-methylcitrate pathway produce succinate. In some embodiments, a succinate exporter can also be expressed in the engineered bacteria. In another embodiment, the polyhydroxyalkanoate pathway can be designed and utilized, resulting in the production of polyhydroxyalkanotes in the engineered bacteria. These pathways serve as a framework for the designed propionate catabolism pathway circuits disclosed herein. **FIG. 8D** depicts a schematic showing a rearranged version of **FIG. 8C**, showing predictions for the fate of the carbon from propionic acid. For the PHA pathway, the carbon is stored as PHA polymers in the cell. In the MMCA pathway, propionate is consumed via the TCA cycle (releasing the carbon as CO2) or succinate is exported.

[016] **FIG. 9** depicts schematics showing the activation of propionate to propionyl CoA. **FIG. 9A** shows a schematic of propionate activation through PrpE. PrpE converts propionate and free CoA to propionyl-CoA in an irreversible, ATP-dependent manner, releasing AMP and PPi (pyrophosphate). PrpE can be inactivated by posttranslational modification of the active site lysine. Protein lysine acetyltransferase (Pka) in E. coli carries out the propionylation of PrpE. The enzyme CobB depropionylates PrpE-Pr, making the inactivation reversible. By simply deleting the *pka* gene, the PrpE inactivation is eliminated altogether. In some embodiments of the disclosure, the genetically engineered bacteria

comprise Δpka to prevent inactivation of PrpE and to increase activity through the downstream catabolic pathways. **FIG. 9B** shows a schematic of propionate activation through pct. Pct converts propionate and acetyl-CoA to propionyl-CoA and acetate in a reversible reaction.

[017] **FIG. 10** depicts a schematic of the polyhydroxyalkanoate pathway (**FIG. 10A**) and chemical structures of the polymers produced from propionate through the PHA pathway (**FIG. 10B**) and an exemplary circuit design for the engineered bacteria of the disclosure (**FIG. 10C**). The PHA pathway is a heterologous bacterial pathway used for carbon storage as polymers. In the circuit, the prpE, phaB, phaC, and phaA genes are expressed under the control of an inducible promoter. PrpE, PhaB, PhaC, and PhaA are capable of catabolizing propionate or propionyl CoA into polyhydroxybutyrate, polyhydroxyvalerate, or P(HV-co-HB). Specifically, PrpE, a propionate-CoA ligase, converts propionate to propionyl CoA. PhaA, a beta-ketothiolase, then converts propionyl CoA to 3-keto-valeryl-CoA or converts acetyl-CoA to acetoacetyl-CoA. PhaB, an acetoacetyl-CoA reductase, then converts acetoacetyl-CoA into 3-hydroxy-butyryl-CoA or 3-keto-valeryl-CoA to 3-hydroxy-valeryl-CoA. PhaC, a polyhydroxyalkanoate synthase converts 3-hydroxy-butyryl-CoA into polyhydroxybutyrate or 3-hydroxy-valeryl-CoA to polyhydroxyvalerate or converts polyhydroxybutyrate and polyhydroxyvalerate to P(HV-co-HB). In some embodiments, the phaBCA genes are from Acinetobacter sp RA3849 and are codon-optimized for E. coli. In some embodiments, the E. coli Nissle prpE gene and the codon-optimized phaBCA genes are under the control of an aTc-inducible promoter in a single operon.

[018] **FIG. 11** depicts a schematic of the gene organization of an exemplary construct, comprising a prpE-phaBCA gene cassette under the control of a tetracycline inducible promoter sequence, on a ~10-copy, kanamycin-resistant plasmid.

[019] **FIG. 12** depicts a graph showing propionate concentrations over time in samples comprising genetically engineered bacteria expressing the polyhydroxyalkanoate (PHA) pathway on a ~10-copy plasmid, as compared to wild type Nissle controls, in the presence and absence of the inducer molecule. Bacteria were induced with ATC (or left uninduced), and then grown in culture medium supplemented to an OD600 of 2.0. Samples were harvested by centrifugation and resuspended in M9 minimal media. The activity of resuspended samples was measured by inoculating samples into M9 minimal media supplemented with glucose and sodium propionate (3 mM) to an OD600 of 1.0. Samples were removed at 0 hrs, 1.5, 3, and 4.5 hrs post-inoculation, and propionate concentrations

were determined by mass spectrometry. The graph depicts propionate consumption by the polyhydroxylalkanoate circuit design for the engineered bacteria (SYN-PHA) in the induced as compared to wild type Nissle. Propionate assay was initiated with ~10^9 cfu/mlpre-induced bacteria and the propionate consumption rate was ~1.4 umol hr-1 per 10^9 cells.

[020] **FIG. 13** depicts graphs showing propionate (**FIG. 13A**), acetate (**FIG. 13B**) and butyrate (**FIG. 13C**) concentrations over time in samples comprising genetically engineered bacteria expressing the polyhydroxyalkanoate (PHA) pathway on a ~10 copy plasmid (SYN-PHA), as compared to wild type Nissle controls, both in the presence of the inducer molecule. The PHA assay was performed in a mixture of short chain fatty acids to mimic the colon ratios (propionate:acetate:butyrate, approximately 6:10:4). Bacteria were induced with ATC (or left uninduced), and then grown in culture medium supplemented to an OD600 of 2.0. Samples were harvested by centrifugation and resuspended in M9 minimal media. The activity of resuspended samples was measured by inoculating samples into M9 minimal media supplemented with glucose and sodium propionate (6 mM), acetate (10 mM), and butyrate (4 mM) to an OD600 of 1.0. Samples were removed at 0 hrs, 1.5, 3, and 4.5 hrs post-inoculation, and propionate concentrations were determined by mass spectrometry. The data show that propionate consumption rate is consistent in the presence or absence of acetate and butyrate, and that the PHA pathway does not significantly affect acetate and butyrate concentrations.

[021] **FIG. 14** depicts graphs showing propionate concentrations over time in samples comprising genetically engineered bacteria expressing an inducible polyhydroxyalkanoate (PHA) cassette (ptet-prpE-phaBCA) on a ~10 copy plasmid (SYN-PHA), in the presence of the inducer molecule. These strains were further supplemented with an second plasmid (~15-copies) expressing one of the genes, i.e., prpE (**FIG. 14A**), phaB (**FIG. 14B**), phaC (**FIG. 14C**), and phaB (**FIG. 14D**), under the control of an inducible promoter, i.e., an arabinose inducible promoter. In this assay, either the prpE-phaBCA operon alone, or both the prpE-phaBCA plasmid and the arabinose inducible plasmid carrying the second copy of one of the operon genes were induced. Wild type Nissle was included for reference. Bacteria were induced with ATC or ATC and arabinose (or left uninduced), and then grown in culture medium supplemented to an OD600 of 2.0. Samples were harvested by centrifugation and resuspended in M9 minimal media. The activity of resuspended samples was measured by inoculating samples into M9 minimal media supplemented with glucose and sodium propionate (3 mM) to an OD600 of 1.0. Samples were removed at 0 hrs, 1.5, 3,

and 4.5 hrs post-inoculation, and propionate concentrations were determined by mass spectrometry. The graph shows that the rate of propionate consumption is increased most significantly when more *phaC* is expressed, suggesting that the pathway is improved by increasing the PhaC levels from the original prpE-phaBCA plasmid. This can for example be accomplished by increasing the translation rate by employing a stronger ribosome binding site in front of the phaC gene. Alternatively, an additional copy of the gene may be added to the same or an additional circuit. In some embodiments, the genetically engineered bacteria comprise a prpE-phaBCA operon, in which PhaC levels are increased through the utilization of a strong ribosome binding site (RBS). In some embodiments, the genetically engineered bacteria comprising a prpE-phaBCA operon further comprise an additional copy of phaC.

[022] **FIG. 15** depicts schematics of the methylmalonyl-CoA pathway and exemplary methylmalonylCoA circuit designs. **FIG. 15A** depicts a schematic showing PrpE reaction and by the methylmalonylCoA pathway, in which the products of the *prpE, pccB, accA1, mmcE, mutA,* and *mutB* genes convert propionate into succinate, and which can be used for circuit design. The methylmalonyl-CoA pathway carries out reactions homologous to those in the mammalian pathway and the pathway is assembled from heterologous bacterial enzymes. In one embodiment, genes *accA* (from *Streptomyces coelicolor*), *pccB* (from *Streptomyces coelicolor*), *mmcE* (from *Propionibacterium freudenreichii*), and mutAB (from *Propionibacterium freudenreichii*) were used and codon-optimized for expression in *E. coli* Nissle. **FIG. 15B** depicts a schematic showing an exemplary circuit design of the disclosure, in which the genetically engineered bacteria comprise a gene cassette comprising the *prpE, pccB, accA1, mmcE, mutA,* and *mutB* genes under the control of an inducible promoter, e.g., a aTc-inducible promoter. **FIG. 15C** depicts a schematic showing an exemplary circuit design of the disclosure, in which the genetically engineered bacteria comprise a cassette comprising *prpE, pccB, accA1,* under the control of a first inducible promoter, e.g., Ptet (aTc inducible) and a second cassette comprising *mmcE and mutAB* under the control of a second inducible promoter, e.g., Para (arabinose inducible). Induction of the pathway requires the addition of aTc and arabinose. In either circuit (**FIG. 15B** or **FIG. 15C**), a succinate exporter may also be expressed in the engineered bacteria.

[023] **FIG. 16** depicts schematics of the gene organization of exemplary constructs. **FIG. 16A** depicts a schematic of the gene organization of an exemplary construct, comprising a mmcE-mutA-mutB gene cassette under the control of an arabinose inducible promoter sequence, on a ~15-copy, ampicillin-resistant plasmid. **FIG. 16B** depicts a

schematic of the gene organization of an exemplary construct, comprising a prpE-accA-pccB gene cassette under the control of a tetracycline inducible promoter sequence, on a ~10-copy, kanamycin-resistant plasmid.

[024] **FIG. 17** depicts schematics of the MMCA pathway combined with a succinate exporter and related exemplary genetic circuits and synthetic biotics. **FIG. 17A** depicts a schematic of propionate and/or methylmalonic acid catabolism through the MMCA pathway. The resulting succinate can be metabolized through the TCA cycle or removed from the bacterial cell through an exporter. Exemplary exporters include *sucE1* succinate exporter (e.g., from *Corynebacterium glutamicum*) and/or the native Nissle succinate exporter *dcuC*. **FIG. 17B** depicts an exemplary circuit or gene cassette for the expression of the *sucE1* succinate exporter (e.g., from *Corynebacterium glutamicum*) under the control of an inducible promoter, e.g., an arabinose-inducible promoter. This construct can either be expressed in the synthetic biotic on a plasmid, or it can be integrated into the genome. For example, a knock-in of the construct, which deletes the araBA genes and part of the araD gene, can be performed, which eliminates metabolism of arabinose by *E. coli*. **FIG. 17C** depicts a schematic of an exemplary synthetic biotic of the disclosure comprising a gene cassette expressing the prpE, phaB, phaC, and phaA genes under the control of an inducible promoter. The synthetic biotic further comprises a gene cassette expressing the sucE1 gene under the control of an inducible promoter. **FIG. 17D** depicts a schematic of a construct comprising the *sucE1* succinate exporter (from *Corynebacterium glutamicum*. **FIG. 17E** depicts a schematic of a construct comprising the E. coli dcuC succinate transporter. **FIG. 17F** depicts a schematic of a construct comprising or comprising both *sucE1* and dcuC transporters.

[025] **FIG. 18** depicts a graph showing propionate concentrations over time in samples comprising genetically engineered bacteria expressing the methylmalonyl-CoA pathway circuit (SYN-MMCA) or a polyhydroxylalkanoate pathway circuit (SYN-PHA) on a ~10- and ~15-copy plasmids as compared to wild type Nissle controls, in the presence of the inducer molecule. Bacteria were induced ATC or ATC and arabinose (or left uninduced), and then grown in culture medium supplemented to an OD600 of 2.0. Samples were harvested by centrifugation and resuspended in M9 minimal media. The activity of resuspended samples was measured by inoculating samples into M9 minimal media supplemented with glucose and sodium propionate (3 mM) to an OD600 of 1.0. Samples were removed at were removed at 0 hrs, 1.5, 3, 4.5, and 18 hrs post-inoculation, cells were removed, and propionate

concentrations were determined by mass spectrometry. The graph depicts propionate consumption by the methylmalonyl-CoA pathway or a polyhydroxylalkanoate circuit design for the engineered bacteria in the induced as compared to wild type Nissle. Propionate assay was initiated with ~109 cfu/mlpre-induced bacteria and the propionate consumption rate was ~ 3.8 μmol/hr/10^9 bacteria in the strain expressing the methylmalonyl-CoA pathway circuit.

[026] **FIG. 19** depicts one example of a normal pathway for the catabolism of propionate via the methylcitrate cycle in bacteria, for example, *E. coli*. Briefly, PrpE, a Propionate-CoA ligase, converts propionate to propionyl CoA. PrpC, a 2-methylcitrate synthetase, then converts propionyl CoA to 2-methylcitrate. PrpD, a 2-methylcitrate dehydrogenase, then converts 2-methylcitrate into 2-methyisocitrate, and PrpB, a 2-methylisocitrate lyase, converts 2-methyisocitrate into succinate and pyruvate.

[027] **FIG. 20** depicts schematics of the 2-methylcitrate cycle in bacteria, e.g., E. coli, (**FIG. 20A**) and a schematics of an exemplary circuit design for the engineered bacteria (**FIG. 20B**). In the circuit, the prpB, prpC, prpD, and prpE genes are expressed under the control of an inducible promoter in order to produce succinate and pyruvate. In some embodiments, a succinate exporter may also be expressed in the engineered bacteria. **FIG. 20C** depicts a schematic of the gene organization of an exemplary construct, comprising a prpBCDE gene cassette under the control of a tetracycline inducible promoter sequence, on a ~10-copy, kanamycin-resistant plasmid.

[028] **FIG. 21** depicts schematics of exemplary synthetic biotics of the disclosure for the treatment of propionic acidemia and/or methylmalonic acidemia and/or disorders characterized by propionic acidemia and/or methylmalonic acidemia. **FIG. 21A** depicts a schematic of an exemplary synthetic biotic of the disclosure comprising a gene cassette expressing the prpE, phaB, phaC, and phaA genes under the control of an inducible promoter. PrpE, PhaB, PhaC, and PhaA are capable of catabolizing propionate or propionyl CoA and/or methylmalonic acid or methylmalonyl CoA into P(HV-co-HB). Protein lysine acyltransferase (pka) is deleted to prevent inactivation of PrpE. In certain embodiments, the prpE-phaBCA circuit is further modified by adding a strong RBS upstream of the phaC translation start site. In other embodiments, synthetic biotic comprised multiple copies of the PhaC gene. In some embodiments, the PhaC gene is located immediately distal to the promoter, as the rst genes in the cassette, to ensure the greatest number of transcripts. T7 polymerase may produce incomplete polycistronic transcripts (prematurely terminated). **FIG. 21B** depicts a schematic of a synthetic biotic of **FIG. 1A** or **FIG 21A,** with the addition of a ThyA auxotrophy. **FIG.**

21C depicts the synthetic biotic of **FIG. 1B**, with the additional of a ThyA auxotrophy. **FIG. 21D** depicts a schematic of an exemplary synthetic biotic of the disclosure comprising a gene cassette expressing prpE, accA, pccB, mmcE, mutA and mutB as two polycistronic messages from two inducible promoters. PrpE, accA, pccB, mmcE, mutA and mutB are capable of catabolizing propionate or propionyl CoA and/or methylmalonic acid or methylmalonyl CoA into succinate, which can be utilized through the TCA cycle or exported from the cell. Protein lysine acyltransferase (pka) is deleted to prevent inactivation of PrpE. In some embodiments, the synthetic biotic comprises a SucE1 and/or dcuC exporter cassette, as described herein. **FIG. 21E** depicts a schematic of a synthetic biotic comprising one or more of two different gene cassettes for propionate catabolism (PHA and MMCA pathway cassettes). **FIG. 21F** depicts a schematic of an exemplary synthetic biotic of the disclosure comprising a gene cassette expressing prpE, accA, pccB, mmcE, mutA and mutB as two polycistronic messages from two inducible promoters in combination with MatB. Protein lysine acyltransferase (pka) is deleted to prevent inactivation of PrpE. In some embodiments, the synthetic biotic comprises a SucE1 and/or dcuC exporter cassette, as described herein. **FIG. 21G** depicts a schematic of a synthetic biotic comprising one or more of two different gene cassettes for propionate catabolism (PHA and MMCA pathway cassettes) in combination with MatB.

[029] **FIG. 22** 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 ParaBAD promoter (ParaBAD), which induces expression of the Tet repressor (TetR) and an anti-toxin. The anti-toxin builds up in the recombinant bacterial cell, while TetR prevents expression of a toxin (which is under the control of a promoter having a TetR binding site). However, when arabinose is not present, both the anti-toxin and TetR are not expressed. Since TetR is not present to repress expression of the toxin, the toxin is expressed and kills the cell. **FIG. 22** 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.

[030] **FIG. 23** 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.

[031] **FIG. 24** depicts another non-limiting embodiment of the disclosure, wherein the expression of a heterologous gene is activated by an exogenous environmental signal. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the araBAD promoter, which induces expression of the Tet repressor (TetR) and an anti-toxin. The anti-toxin builds up in the recombinant bacterial cell, while TetR prevents expression of a toxin (which is under the control of a promoter having a TetR binding site). However, when arabinose is not present, both the anti-toxin and TetR are not expressed. Since TetR is not present to repress expression of the toxin, the toxin is expressed and kills the cell. The araC gene is either under the control of a constitutive promoter or an inducible promoter (e.g., AraC promoter) in this circuit.

[032] **FIG. 25** depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, and the natural kinetics of the recombinase create a time delay in expression of the toxin, allowing the heterologous gene to be fully expressed. Once the toxin is expressed, it kills the cell.

[033] **FIG. 26** depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, and the natural kinetics of the recombinase create a time delay in expression of the toxin, allowing the heterologous gene to be fully expressed. Once the toxin is expressed, it kills the cell.

[034] **FIG. 27** depicts another non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips at least one excision enzyme into an activated conformation. The at least one excision enzyme then excises one or more essential genes, leading to senescence, and eventual cell death. The natural kinetics of the recombinase and excision genes cause a time delay, the kinetics of which can be altered and optimized depending on the number and choice of essential genes to be excised, allowing cell death to occur within a matter of hours or days. The presence of multiple nested recombinases can be used to further control the timing of cell death.

[035] **FIG. 28** depicts a schematic of one non-limiting embodiment of the disclosure, in which the genetically engineered bacteria produces equal amount of a Hok toxin and a short-lived Sok anti-toxin. When the cell loses the plasmid, the anti-toxin decays, and the cell dies. In the upper panel, the cell produces equal amounts of toxin and anti-toxin and is stable. In the center panel, the cell loses the plasmid and anti-toxin begins to decay. In the lower panel, the anti-toxin decays completely, and the cell dies.

[036] **FIG. 29** depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and a first recombinase from an inducible promoter or inducible promoters. The recombinase then flips a second recombinase from an inverted orientation to an active conformation. The activated second recombinase flips the toxin gene into an activated conformation, and the natural kinetics of the recombinase create a time delay in expression of the toxin, allowing the heterologous gene to be fully expressed. Once the toxin is expressed, it kills the cell.

[037] **FIG. 30** depicts an example of a genetically engineered bacteria that comprises a plasmid that has been modified to create a host-plasmid mutual dependency, such as the GeneGuard system described in more detail herein.

[038] **FIG. 31** depicts an exemplary schematic of the E. coli 1917 Nissle chromosome comprising multiple mechanisms of action (MoAs). A single synthetic biotic may have multiple mechanisms of action (MOAs) based on the insertion of multiple copies of the same synthetic circuit or the insertion of different synthetic circuits at different sites in a bacterial chromosome.

[039] **FIG. 32** depicts a map of integration sites within the E. coli Nissle chromosome. These sites indicate regions where circuit components may be inserted into the chromosome without interfering with essential gene expression. Backslashes (/) are used to show that the insertion will occur between divergently or convergently expressed genes. Insertions within biosynthetic genes, such as thyA, can be useful for creating nutrient auxotrophies. In some embodiments, an individual circuit component is inserted into more than one of the indicated sites.

[040] **FIG. 33** depicts three bacterial strains which constitutively express red fluorescent protein (RFP). In strains 1-3, the rfp gene was inserted into different sites in the bacterial chromosome, and resulted in varying degrees of brightness under fluorescent light. Unmodified E. coli Nissle (strain 4) is non-fluorescent.

[041] **FIG. 34** depicts a graph of Nissle residence in vivo. Streptomycin-resistant Nissle was administered to mice via oral gavage without antibiotic pre-treatment. Fecal pellets from six total mice were monitored post-administration to determine the amount of administered Nissle still residing within the mouse gastrointestinal tract. The bars represent the number of bacteria administered to the mice. The line represents the number of Nissle recovered from the fecal samples each day for 10 consecutive days.

[042] **FIG. 35** depicts a bar graph of residence over time for streptomycin resistant Nissle in various compartments of the intestinal tract at 1, 4, 8, 12, 24, and 30 hours post gavage. Mice were treated with approximately 10^9 CFU, and at each timepoint, animals (n=4) were euthanized, and intestine, cecum, and colon were removed. The small intestine was cut into three sections, and the large intestine and colon each into two sections. Intestinal effluents gathered and CFUs in each compartment were determined by serial dilution plating.

[043] **FIG. 36** 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.

[044] **FIG. 37** 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.

[045] **FIG. 38** 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.

[046] **FIG. 39** depicts a schematic of the outer and inner membranes of a gram-negative bacterium, and several deletion targets for generating a leaky or destabilized outer membrane, thereby facilitating the translocation of a therapeutic polypeptides to the extracellular space, e.g., therapeutic polypeptides of eukaryotic origin containing disulphide bonds. Deactivating mutations of one or more genes encoding a protein that tethers the outer membrane to the peptidoglycan skeleton, e.g., lpp, ompC, ompA, ompF, tolA, tolB, pal, and/or one or more genes encoding a periplasmic protease, e.g., degS, degP, nlpI, generates a leaky phenotype. Combinations of mutations may synergistically enhance the leaky phenotype.

[047] **FIG. 40** depicts a modified type 3 secretion system (T3SS) to allow the bacteria to inject secreted therapeutic proteins into the gut lumen. An inducible promoter (small arrow, top), e.g. a FNR-inducible promoter, drives expression of the T3 secretion system gene cassette (3 large arrows, top) that produces the apparatus that secretes tagged

peptides out of the cell. An inducible promoter (small arrow, bottom), e.g. a FNR-inducible promoter, drives expression of a regulatory factor, e.g. T7 polymerase, that then activates the expression of the tagged therapeutic peptide (hexagons).

[048] **FIG. 41** depicts β-galactosidase levels in samples comprising bacteria harboring a low-copy plasmid expressing lacZ from an FNR-responsive promoter selected from the exemplary FNR promoters. Different FNR-responsive promoters were used to create a library of anaerobic-inducible reporters with a variety of expression levels and dynamic ranges. These promoters included strong ribosome binding sites. Bacterial cultures were grown in either aerobic (+O2) or anaerobic conditions (-O2). Samples were removed at 4 hrs and the promoter activity based on β-galactosidase levels was analyzed by performing standard β-galactosidase colorimetric assays.

[049] **FIG. 42** depicts a schematic representation of the *lacZ* gene under the control of an exemplary FNR promoter (P_{fnrS}). *LacZ* encodes the β-galactosidase enzyme and is a common reporter gene in bacteria. **FIG. 42B** depicts FNR promoter activity as a function of β-galactosidase activity in SYN340. SYN340, an engineered bacterial strain harboring a low-copy *fnrS-lacZ* fusion gene, was grown in the presence or absence of oxygen. Values for standard β-galactosidase colorimetric assays are expressed in Miller units (Miller, 1972). These data suggest that the *fnrS* promoter begins to drive high-level gene expression within 1 hr under anaerobic conditions. **FIG. 42C** depicts the growth of bacterial cell cultures expressing *lacZ* over time, both in the presence and absence of oxygen.

[050] **FIG. 43** depicts ATC (**FIG. 43A**) or nitric oxide-inducible (**FIG. 43B**) reporter constructs. These constructs, when induced by their cognate inducer, lead to expression of GFP. Nissle cells harboring plasmids with either the control, ATC-inducible P_{tet}-GFP reporter construct or the nitric oxide inducible P_{nsrR}-GFP reporter construct induced across a range of concentrations. Promoter activity is expressed as relative florescence units. **FIG. 43C** depicts a schematic of the constructs. **FIG. 43D** depicts a dot blot of bacteria harboring a plasmid expressing NsrR under control of a constitutive promoter and the reporter gene *gfp* (green fluorescent protein) under control of an NsrR-inducible promoter. DSS-treated mice serve as exemplary models for HE. As in HE subjects, the guts of mice are damaged by supplementing drinking water with 2-3% dextran sodium sulfate (DSS). Chemiluminescent is shown for NsrR-regulated promoters induced in DSS-treated mice.

[051] **FIG. 44** depicts the prpR propionate-responsive inducible promoter. The sequence for one propionate-responsive promoter is also disclosed herein as (SEQ ID NO:70).

[052] **FIG. 45** depicts a schematic of a wild-type clbA construct and a clbA knock-out construct.

[053] **FIG. 46** depicts a schematic of non-limiting processes for designing and producing the genetically engineered bacteria of the present disclosure. The step of "defining" comprises 1. Identification of diverse candidate approaches based on microbial physiology and disease biology; 2. Use of bioinformatics to determine candidate metabolic pathways; the use of prospective tools to determine performance targets required of optimized engineered synthetic biotics. The step of "designing" comprises the use of 1. Cutting-edge DNA assembly to enable combinatorial testing of pathway organization; 2. Mathematical models to predict pathway efficiency; 3. Internal stable of proprietary switches and parts to permit control and tuning of engineered circuits. The step of "Building" comprises 1. Building core structures "chassies" 2. Stably integrating engineered circuits into optimal chromosomal locations for efficient expression; 3. Employing unique functional assays to assess genetic circuit fidelity and activity. The step of "integrating" comprises 1. Use of chromosomal markers, which enable monitoring of synthetic biotic localization and transit times in animal models; 2. Leveraging expert microbiome network and bioinformatics support to expand understanding of how specific disease states affect GI microbial flora and the behaviors of synthetic biotics in that environment; 3. Activating process development research and optimization in-house during the discovery phase, enabling rapid and seamless transition of development candidates to pre-clinical progression; Drawing upon extensive experience in specialized disease animal model refinement, which supports prudent, high quality testing of candidate synthetic biotics.

[054] **FIG. 47A**, **FIG. 47B**, **FIG. 47C**, **FIG. 47D**, and **FIG. 47E** depict a schematic of non-limiting manufacturing processes for upstream and downstream production of the genetically engineered bacteria of the present disclosure. **FIG. 47A** depicts the parameters for starter culture 1 (SC1): loop full – glycerol stock, duration overnight, temperature 37° C, shaking at 250 rpm. **FIG. 47B** depicts the parameters for starter culture 2 (SC2): 1/100 dilution from SC1, duration 1.5 hours, temperature 37° C, shaking at 250 rpm. **FIG. 47C** depicts the parameters for the production bioreactor: inoculum – SC2, temperature 37° C, pH set point 7.00, pH dead band 0.05, dissolved oxygen set point 50%, dissolved oxygen cascade

agitation/gas FLO, agitation limits 300-1200 rpm, gas FLO limits 0.5-20 standard liters per minute, duration 24 hours. **FIG. 47D** depicts the parameters for harvest: centrifugation at speed 4000 rpm and duration 30 minutes, wash 1X 10% glycerol/PBS, centrifugation, re-suspension 10% glycerol/PBS. **FIG. 47E** depicts the parameters for vial fill/storage: 1-2 mL aliquots, -80° C.

Detailed Description

[055] The present disclosure provides engineered bacterial cells, pharmaceutical compositions thereof, and methods of modulating and treating disorders associated with propionate catabolism, such as propionic acidemia, methylmalonic acidemia, or vitamin B_{12} deficiency. Specifically, the engineered bacteria disclosed herein have been constructed to comprise genetic circuits composed of, for example, at least one propionate catabolism enzyme. In some embodiments, the engineered bacteria additionally comprise optional circuitry to ensure the safety and non-colonization of the subject that is administered the engineered bacteria, such as auxotrophies, kill switches, *etc.* These engineered bacteria are safe and well tolerated and augment the innate activities of the subject's microbiome to achieve a therapeutic effect.

[056] 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.

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

[058] As used herein, the term "recombinant microorganism" refers to a microorganism, e.g., bacterial or viral cell, or bacteria or virus, that has been genetically modified from its native state. Thus, a "recombinant bacterial cell" or "recombinant bacteria" refers to a bacterial cell or bacteria that have been genetically modified from their native state. For instance, a recombinant bacterial cell may have nucleotide insertions, nucleotide deletions, nucleotide rearrangements, and nucleotide modifications introduced into their DNA. These genetic modifications may be present in the chromosome of the bacteria or bacterial cell, or on a plasmid in the bacteria or bacterial cell. Recombinant bacterial cells disclosed herein may comprise exogenous nucleotide sequences on plasmids. Alternatively, recombinant bacterial cells may comprise exogenous nucleotide sequences stably incorporated into their chromosome.

[059] A "programmed microorganism" or "engineered microorganism" refers to a microorganism, e.g., bacterial or viral cell, or bacteria or virus, that has been genetically modified from its native state to perform a specific function, e.g., to metabolize propionate and/or one or more of its metabolites. In certain embodiments, the programmed or engineered microorganism has been modified to express one or more proteins, for example, one or more proteins that have a therapeutic activity or serve a therapeutic purpose. The programmed or engineered microorganism may additionally have the ability to stop growing or to destroy itself once the protein(s) of interest have been expressed.

[060] A "programmed bacterial cell" or "engineered bacterial cell" is a bacterial cell that has been genetically modified from its native state. In certain embodiments, the programmed or engineered bacterial cell has been modified from its native state to perform a specific function, for example, to express one or more proteins, for example, one or more proteins that have a therapeutic activity or serve a therapeutic purpose, e.g., to metabolize a propionate and/or one or more of its metabolites. The programmed or engineered bacterial cell may additionally have the ability to stop growing or to destroy itself once the protein(s) of interest have been expressed. For instance, an engineered bacterial cell may have nucleotide insertions, nucleotide deletions, nucleotide rearrangements, and nucleotide modifications introduced into their DNA. These genetic modifications may be present in the chromosome of the bacteria or bacterial cell, or on a plasmid in the bacteria or bacterial cell. Engineered bacterial cells disclosed herein may comprise exogenous nucleotide sequences on plasmids. Alternatively, engineered bacterial cells may comprise exogenous nucleotide sequences stably incorporated into their chromosome.

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

[062] As used herein, the term "gene sequence" is meant to refer to a genetic sequence, e.g., a nucleic acid sequence. The gene sequence or genetic sequence is meant to include a complete gene sequence or a partial gene sequence. The gene sequence or genetic sequence is meant to include sequence that encodes a protein or polypeptide and is also meant to include genetic sequence that does not encode a protein or polypeptide, e.g., a regulatory sequence, leader sequence, signal sequence, or other non-protein coding sequence.

[063] As used herein, a "heterologous" gene or "heterologous sequence" refers to a nucleotide sequence that is not normally found in a given cell in nature. As used herein, a "heterologous sequence" encompasses a nucleic acid sequence that is exogenously introduced into a given cell and can be a native sequence (naturally found or expressed in the cell) or non-native sequence (not naturally found or expressed in the cell) and can be a natural or wild-type sequence or a variant, non-natural, or synthetic sequence. "Heterologous gene" includes a native gene, or fragment thereof, that has been introduced into the host cell in a form that is different from the corresponding native gene. For example, a heterologous gene

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

[064] As used herein, a "non-native" nucleic acid sequence refers to a nucleic acid sequence not normally present in a microorganism, e.g., an extra copy of an endogenous sequence, or a heterologous sequence such as a sequence from a different species, strain, or substrain of bacteria or virus, or a sequence that is modified and/or mutated as compared to the unmodified sequence from bacteria or virus of the same subtype. In some embodiments, the non-native nucleic acid sequence is a synthetic, non-naturally occurring sequence (see, e.g., Purcell et al., 2013). The non-native nucleic acid sequence may be a regulatory region, a promoter, a gene, and/or one or more genes in gene cassette. In some embodiments, "non-native" refers to two or more nucleic acid sequences that are not found in the same relationship to each other in nature. The non-native nucleic acid sequence may be present on a plasmid or chromosome. In some embodiments, the genetically engineered microorganism of the disclosure comprises a gene that is operably linked to a promoter that is not associated with said gene in nature. For example, in some embodiments, the genetically engineered bacteria disclosed herein comprise a gene that is operably linked to a directly or indirectly inducible promoter that is not associated with said gene in nature, e.g., an FNR responsive promoter (or other promoter disclosed herein) operably linked to a gene encoding a propionate catabolism enzyme. In some embodiments, the genetically engineered virus of the disclosure comprises a gene that is operably linked to a directly or indirectly inducible promoter that is not associated with said gene in nature, e.g., a promoter operably linked to a gene encoding a propionate catabolism enzyme.

[065] As used herein, the term "coding region" refers to a nucleotide sequence that codes for a specific amino acid sequence. The term "regulatory sequence" refers to a nucleotide sequence located upstream (5' non-coding sequences), within, or downstream (3'

non-coding sequences) of a coding sequence, and which influences the transcription, RNA processing, RNA stability, or translation of the associated coding sequence. Examples of regulatory sequences include, but are not limited to, promoters, translation leader sequences, effector binding sites, signal sequences, and stem-loop structures. In one embodiment, the regulatory sequence comprises a promoter, e.g., an FNR responsive promoter or other promoter disclosed herein.

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

[067] As used herein, a "gene cassette" or "operon" encoding a propionate catabolism pathway refers to the two or more genes that are required to catabolize propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA into an inert end-product, *e.g.*, succinate or polyhydroxyalkanotes. 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. Each gene or gene cassette may be present on a plasmid or bacterial chromosome. In addition, multiple copies of any gene, gene cassette, or regulatory region may be present in the bacterium, wherein one or more copies of the gene, gene cassette, or regulatory region may be mutated or otherwise altered as described herein. In some embodiments, the genetically engineered bacteria are engineered to comprise multiple copies of the same gene, gene cassette, or regulatory region in order to enhance copy number or to comprise multiple different components of a gene cassette performing multiple different functions.

[068] "Operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. A regulatory element is operably linked with a coding sequence when it is capable of affecting the expression of the gene coding sequence, regardless of the distance between the regulatory element and the coding sequence. More specifically, operably linked refers to a nucleic acid sequence, *e.g.*, a gene encoding a propionate catabolism enzyme, that is joined to a regulatory sequence in a manner which allows expression of the nucleic acid sequence, *e.g.*, the gene encoding the propionate catabolism enzyme. In other words, the regulatory sequence acts in *cis*. In one embodiment, a gene may be "directly linked" to a regulatory sequence in a manner which allows expression of the gene. In another embodiment, a gene may be "indirectly linked" to a regulatory sequence in a manner which allows expression of the gene. In one embodiment, two or more genes may be directly or indirectly linked to a regulatory sequence in a manner which allows expression of the two or more genes.

A regulatory region or sequence is a nucleic acid that can direct transcription of a gene of interest and may comprise promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, promoter control elements, protein binding sequences, 5′ and 3′ untranslated regions, transcriptional start sites, termination sequences, polyadenylation sequences, and introns.

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

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

[070] "Constitutive promoter" refers to a promoter that is capable of facilitating continuous transcription of a coding sequence or gene under its control and/or to which it is operably linked. Constitutive promoters and variants are well known in the art and include, but are not limited to, BBa_J23100, a constitutive *Escherichia coli* σ^S promoter (*e.g.*, an

osmY promoter (International Genetically Engineered Machine (iGEM) Registry of Standard Biological Parts Name BBa_J45992; BBa_J45993)), a constitutive *Escherichia coli* σ^{32} promoter (*e.g.*, htpG heat shock promoter (BBa_J45504)), a constitutive *Escherichia coli* σ^{70} promoter (*e.g.*, lacq promoter (BBa_J54200; BBa_J56015), *E. coli* CreABCD phosphate sensing operon promoter (BBa_J64951), GlnRS promoter (BBa_K088007), lacZ promoter (BBa_K119000; BBa_K119001); M13K07 gene I promoter (BBa_M13101); M13K07 gene II promoter (BBa_M13102), M13K07 gene III promoter (BBa_M13103), M13K07 gene IV promoter (BBa_M13104), M13K07 gene V promoter (BBa_M13105), M13K07 gene VI promoter (BBa_M13106), M13K07 gene VIII promoter (BBa_M13108), M13110 (BBa_M13110)), a constitutive *Bacillus subtilis* σ^{A} promoter (*e.g.*, promoter veg (BBa_K143013), promoter 43 (BBa_K143013), P_{liaG} (BBa_K823000), P_{lepA} (BBa_K823002), P_{veg} (BBa_K823003)), a constitutive *Bacillus subtilis* σ^{B} promoter (*e.g.*, promoter ctc (BBa_K143010), promoter gsiB (BBa_K143011)), a *Salmonella* promoter (*e.g.*, Pspv2 from *Salmonella* (BBa_K112706), Pspv from *Salmonella* (BBa_K112707)), a bacteriophage T7 promoter (*e.g.*, T7 promoter (BBa_I712074; BBa_I719005; BBa_J34814; BBa_J64997; BBa_K113010; BBa_K113011; BBa_K113012; BBa_R0085; BBa_R0180; BBa_R0181; BBa_R0182; BBa_R0183; BBa_Z0251; BBa_Z0252; BBa_Z0253)), and a bacteriophage SP6 promoter (*e.g.*, SP6 promoter (BBa_J64998)).

[071] An "inducible promoter" refers to a regulatory region that is operably linked to one or more genes, wherein expression of the gene(s) is increased in the presence of an inducer of said regulatory region. An "inducible promoter" refers to a promoter that initiates increased levels of transcription of the coding sequence or gene under its control in response to a stimulus or an exogenous environmental condition. A "directly inducible promoter" refers to a regulatory region, wherein the regulatory region is operably linked to a gene encoding a protein or polypeptide, where, in the presence of an inducer of said regulatory region, the protein or polypeptide is expressed. An "indirectly inducible promoter" refers to a regulatory system comprising two or more regulatory regions, for example, a first regulatory region that is operably linked to a first gene encoding a first protein, polypeptide, or factor, *e.g.*, a transcriptional regulator, which is capable of regulating a second regulatory region that is operably linked to a second gene, the second regulatory region may be activated or repressed, thereby activating or repressing expression of the second gene. Both a directly inducible promoter and an indirectly inducible promoter are encompassed by "inducible promoter." Exemplary inducible promoters described herein include oxygen level-dependent

promoters (*e.g.*, FNR-inducible promoter), promoters induced by inflammation or an inflammatory response (RNS, ROS promoters), and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose and tetracycline. Examples of inducible promoters include, but are not limited to, an FNR responsive promoter, a P_{araC} promoter, a P_{araBAD} promoter, and a P_{TetR} promoter, each of which are described in more detail herein. Examples of other inducible promoters are provided herein below.

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

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

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

[075] The term "genetic modification," as used herein, refers to any genetic change. Exemplary genetic modifications include those that increase, decrease, or abolish the expression of a gene, including, for example, modifications of native chromosomal or extrachromosomal genetic material. Exemplary genetic modifications also include the introduction of at least one plasmid, modification, mutation, base deletion, base addition, base substitution, and/or codon modification of chromosomal or extrachromosomal genetic sequence(s), gene over-expression, gene amplification, gene suppression, promoter modification or substitution, gene addition (either single or multi-copy), antisense expression or suppression, or any other change to the genetic elements of a host cell, whether the change produces a change in phenotype or not. Genetic modification can include the introduction of a plasmid, *e.g.*, a plasmid comprising a propionate catabolism enzyme operably linked to a

promoter, into a bacterial cell. Genetic modification can also involve a targeted replacement in the chromosome, *e.g.*, to replace a native gene promoter with an inducible promoter, regulated promoter, strong promoter, or constitutive promoter. Genetic modification can also involve gene amplification, *e.g.*, introduction of at least one additional copy of a native gene into the chromosome of the cell. Alternatively, chromosomal genetic modification can involve a genetic mutation.

[076] As used herein, the term "genetic mutation" refers to a change or changes in a nucleotide sequence of a gene or related regulatory region that alters the nucleotide sequence as compared to its native or wild-type sequence. Mutations include, for example, substitutions, additions, and deletions, in whole or in part, within the wild-type sequence. Such substitutions, additions, or deletions can be single nucleotide changes (*e.g.*, one or more point mutations), or can be two or more nucleotide changes, which may result in substantial changes to the sequence. Mutations can occur within the coding region of the gene as well as within the non-coding and regulatory sequence of the gene. The term "genetic mutation" is intended to include silent and conservative mutations within a coding region as well as changes which alter the amino acid sequence of the polypeptide encoded by the gene. A genetic mutation in a gene coding sequence may, for example, increase, decrease, or otherwise alter the activity (*e.g.*, enzymatic activity) of the gene's polypeptide product. A genetic mutation in a regulatory sequence may increase, decrease, or otherwise alter the expression of sequences operably linked to the altered regulatory sequence.

[077] Specifically, the term "genetic modification that increases import of propionate into the bacterial cell" refers to a genetic modification that increases the uptake rate or increases the uptake quantity of propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA or metabolites thereof, into the cytosol of the bacterial cell, as compared to the uptake rate or uptake quantity of the propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA into the cytosol of a bacterial cell not having said modification, *e.g.*, a wild-type bacterial cell. In one embodiment, a engineered bacterial cell having a genetic modification that increases import of propionate into the bacterial cell refers to a bacterial cell comprising a heterologous gene encoding a transporter of propionate. In one embodiment, a recombinant bacterial cell having a genetic modification that increases import of propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA and/or their metabolites from the bacterial cell comprises a genetic mutation in a native gene. In another embodiment, a recombinant bacterial cell having a genetic modification that increases import of a propionate

and/or its metabolites from the bacterial cell comprises a genetic mutation in a native promoter, which increases or activates transcription of the gene which increases import of propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA and/or their metabolites. In another embodiment, a recombinant bacterial cell having a genetic modification that increases import of p propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA and/or their metabolites from the bacterial cell comprises a genetic mutation leading to overexpression of an activator of an importer (transporter) of propionate and/or its metabolites. In another embodiment, a recombinant bacterial cell having a genetic modification that increases import of propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA and/or their metabolites from the bacterial cell comprises a genetic mutation which increases or activates translation of the gene encoding the transporter (importer).

[078] Moreover, the term "genetic modification that increases import of a propionate and/or its metabolites into the bacterial cell" refers to a genetic modification that increases the uptake rate or increases the uptake quantity of a propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA and/or their metabolites into the cytosol of the bacterial cell, as compared to the uptake rate or uptake quantity of propionate and/or its metabolites into the cytosol of a bacterial cell not having said modification, e.g., a wild-type bacterial cell. In some embodiments, an engineered bacterial cell having a genetic modification that increases import of propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA and/or their metabolites into the bacterial cell refers to a bacterial cell comprising heterologous gene sequence (native or non-native) encoding one or more importer(s) (transporter(s)) of propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA and/or their metabolites. In some embodiments, the genetically engineered bacteria comprising genetic modification that increases import of propionate and one or more of its metabolites into the bacterial cell comprise gene sequence(s) encoding a propionate transporter or other amino acid transporter that transports one or more propionate metabolites into the bacterial cell, for example a transporter that is capable of transporting methylmalonic acid into a bacterial cell. The transporter can be any transporter that assists or allows import of propionate and/or metabolites thereof into the cell. In certain embodiments, the propionate transporter is one of MctC, PutP_6, or any other propionate transporters described herein. In certain embodiments, the engineered bacterial cell contains gene sequences encoding MctC, PutP_6, or any other propionate transporters described herein. In some embodiments, the engineered

bacteria comprise more than one copy of gene sequence encoding a propionate transporter. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding more than one propionate transporter, e.g., two or more different propionate transporters.

[079] The term "propionate," as used herein, refers to C2H5COO-. Propionate is the conjugate base of propionic acid. The term "propionic acid," as used herein, refers to a carboxylic acid with the chemical formula CH3CH2COOH. Propionate is converted to propionyl coenzyme A ("propionyl CoA") as a first step in the catabolism of carboxylic acids. Propionate and propionyl CoA exist in an equilibrium. In humans and other vertebrates, propionyl CoA is carboxylated to D-methylmalonyl CoA by the enzyme Propionyl CoA Carboxylase (PCC) with the help of biotin (vitamin B7), which is isomerized to L-methylmalonyl CoA (see **FIG. 5**). As used herein, the term "methylmalonyl CoA" refers to the thioester consisting of coenzyme A linked to methylmalonic acid. A vitamin B12-dependent enzyme, Methylmalonyl CoA Mutase (MUT) then catalyzes the rearrangement of L-methylmalonyl CoA to succinyl CoA, which is then incorporated into the citric acid cycle.

[080] As used herein, the term "propionate binding protein" refers to a protein which can bind to propionate and/or one or more propionate metabolites, including, but not limited to, methylmalonate and/or methylmalonic acid.

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

[082] As used herein, the term "propionate transporter" refers to a polypeptide which functions to transport propionate and/or one or more of its metabolites, including, but not limited to, methylmalonate and/or methylmalonic acid into the bacterial cell.

[083] As used herein the terms "methylmalonic acid" and "methylmalonate" are used interchangeably. As used herein, the terms "propionate" and "propionic acid" are used interchangeably.

[084] As used herein, the phrase "propionate and/or its metabolites" or "propionate and/or one or more of its metabolites", includes any metabolite of propionate, such as any of the metabolites described herein, and also includes propionyl CoA, methylmalonic acid, or methylmalonyl CoA.

[085] "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.

[086] "Non-pathogenic bacteria" refer to bacteria that are not capable of causing disease or harmful responses in a host. In some embodiments, non-pathogenic bacteria are commensal bacteria. Examples of non-pathogenic bacteria include, but are not limited to Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Clostridium, Enterococcus, Escherichia coli, Lactobacillus, Lactococcus, Saccharomyces, and Staphylococcus, e.g., Bacillus coagulans, Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium butyricum, Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis, and Saccharomyces boulardii (Sonnenborn et al., 2009; Dinleyici et al., 2014; U.S. Patent No. 6,835,376; U.S. Patent No. 6,203,797; U.S. Patent No. 5,589,168; U.S. Patent No. 7,731,976). Naturally pathogenic bacteria may be genetically engineered to provide reduce or eliminate pathogenicity.

[087] As used herein, the term "treat" and its cognates refer to an amelioration of a disease, 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, 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. As used herein, "prevent" and its cognates refer to delaying the onset or reducing the risk of acquiring a given disease.

[088] Those in need of treatment may include individuals already having a particular medical disease, as well as those at risk of having, or who may ultimately acquire the disease. The need for treatment is assessed, for example, by the presence of one or more risk factors associated with the development of a disease, the presence or progression of a disease, or likely receptiveness to treatment of a subject having the disease. Diseases associated with the catabolism of propionate, e.g., Propionic Acidemia (PA) or Methylmalonic Acidemia (MMA), may be caused by inborn genetic mutations for which there are no known cures. Diseases can also be secondary to other conditions, e.g., liver diseases. Treating diseases involving the catabolism of propionate, such as PA or MMA, may encompass reducing normal levels of propionate, propionic acid, propionyl CoA, methylmalonic acid, and/or methylmalonyl CoA, reducing excess levels of propionate, propionic acid, propionyl CoA, methylmalonic acid, and/or methylmalonyl CoA, or eliminating propionate, propionic acid, propionyl CoA, methylmalonic acid, and/or methylmalonyl CoA, and does not necessarily encompass the elimination of the underlying disease.

[089] As used herein, the term "catabolism" refers to the conversion of an odd-chain fatty acid, cholesterol, or branched chain amino acid, such as methionine, threonine, isoleucine, or valine, into its corresponding propionyl CoA, methylmalonyl CoA, or succinyl CoA. In one embodiment, "abnormal catabolism" refers to a decrease in the rate or the level of conversion of an odd-chain fatty acid, cholesterol, or branched chain amino acid into its corresponding propionyl CoA, methylmalonyl CoA, or succinyl CoA, leading to the build-up of propionyl CoA or methylmalonyl CoA in the blood or the brain of a subject. In one embodiment, build-up of propionyl CoA or methylmalonyl CoA in the blood or the brain of a subject becomes toxic and leads to the development of a disease or disorder associated with the abnormal catabolism of propionate in the subject. "Catabolism" e.g., "Propionate catabolism", also refers to the breakdown of propionate and/or methylmalonic acid to one or more of its breakdown products as described herein.

[090] In one embodiment, a "disorder involving the catabolism of propionate" is a disease or disorder involving the abnormal catabolism of propionate, propionyl CoA, methylmalonic acid, or methylmalonyl CoA. As used herein, the term "disorder involving the abnormal catabolism of propionate" refers to a disease or disorder wherein the catabolism of propionate, propionyl CoA, methylmalonic acid, and/or methylmalonyl CoA is abnormal. In one embodiment, "abnormal catabolism" refers to a decrease in the rate or the level of conversion of propionyl CoA into methylmalonyl CoA, or a decrease in the rate or the level

of conversion of methylmalonyl CoA into succinyl CoA, leading to the build-up of propionate, propionyl CoA, methylmalonic acid, and/or methylmalonyl CoA in the blood or the brain of a subject. In one embodiment, build-up of the propionate, propionyl CoA, methylmalonic acid, and/or methylmalonyl CoA in the blood or the brain of a subject becomes toxic and leads to the development of a disease or disorder associated with the abnormal catabolism of propionate in the subject. In one embodiment, the disorder involving the abnormal catabolism of propionate is Propionic Acidemia or Methylmalonic Acidemia.

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

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

[092] Examples of oxygen level-dependent transcription factors include, but are not limited to, FNR (fumarate and nitrate reductase), ANR, and DNR. Corresponding FNR-responsive promoters, ANR (anaerobic nitrate respiration)-responsive promoters, and DNR (dissimilatory nitrate respiration regulator)-responsive promoters are known in the art (see, e.g., Castiglione et al., 2009; Eiglmeier et al., 1989; Galimand et al., 1991; Hasegawa et al., 1998; Hoeren et al., 1993; Salmon et al., 2003), and non-limiting examples are shown in Table 1.

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

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

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

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

[095] In some embodiments, the exogenous environmental condition(s) and/or signal(s) stimulates the activity of an inducible promoter. In some embodiments, the exogenous environmental condition(s) and/or signal(s) that serves to activate the inducible promoter is not naturally present within the gut of a mammal. In some embodiments, the inducible promoter is stimulated by a molecule or metabolite that is administered in combination with the pharmaceutical composition of the disclosure, for example, tetracycline, arabinose, or any biological molecule that serves to activate an inducible promoter. In some embodiments, the exogenous environmental condition(s) and/or signal(s) is added to culture media comprising a recombinant bacterial cell of the disclosure. In some embodiments, the exogenous environmental condition that serves to activate the inducible promoter is naturally present within the gut of a mammal (for example, low oxygen or anaerobic conditions, or biological molecules involved in an inflammatory response). In some embodiments, the loss of exposure to an exogenous environmental condition (for example, *in vivo*) inhibits the activity of an inducible promoter, as the exogenous environmental condition is not present to induce the promoter (for example, an aerobic environment outside the gut). "Gut" refers to the organs, glands, tracts, and systems that are responsible for the transfer and digestion of food, absorption of nutrients, and excretion of waste. In humans, the gut comprises the gastrointestinal (GI) tract, which starts at the mouth and ends at the anus, and additionally comprises the esophagus, stomach, small intestine, and large intestine. The gut also comprises accessory organs and glands, such as the spleen, liver, gallbladder, and pancreas. The upper gastrointestinal tract comprises the esophagus, stomach, and duodenum of the small intestine. The lower gastrointestinal tract comprises the remainder of the small intestine, i.e., the jejunum and ileum, and all of the large intestine, i.e.,

the cecum, colon, rectum, and anal canal. Bacteria can be found throughout the gut, e.g., in the gastrointestinal tract, and particularly in the intestines.

[096] "Microorganism" refers to an organism or microbe of microscopic, submicroscopic, or ultramicroscopic size that typically consists of a single cell. Examples of microorganisms include bacteria, viruses, parasites, fungi, certain algae, and protozoa. In some aspects, the microorganism is engineered ("engineered microorganism") to produce one or more therapeutic molecules, e.g., lysosomal enzyme(s). In certain embodiments, the engineered microorganism is an engineered bacterium. In certain embodiments, the engineered microorganism is an engineered virus.

[097] "Non-pathogenic bacteria" refer to bacteria that are not capable of causing disease or harmful responses in a host. In some embodiments, non-pathogenic bacteria are Gram-negative bacteria. In some embodiments, non-pathogenic bacteria are Gram-positive bacteria. In some embodiments, non-pathogenic bacteria do not contain lipopolysaccharides (LPS). In some embodiments, non-pathogenic bacteria are commensal bacteria. Examples of non-pathogenic bacteria include, but are not limited to certain strains belonging to the genus *Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Clostridium, Enterococcus, Escherichia coli, Lactobacillus, Lactococcus,* Saccharomyces, and *Staphylococcus, e.g., Bacillus coagulans, Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium butyricum, Enterococcus faecium, Escherichia coli, Escherichia coli Nissle, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis* and *Saccharomyces boulardii* (Sonnenborn et al., 2009; Dinleyici et al., 2014; U.S. Patent No. 6,835,376; U.S. Patent No. 6,203,797; U.S. Patent No. 5,589,168; U.S. Patent No. 7,731,976). Non-pathogenic bacteria also include commensal bacteria, which are present in the indigenous microbiota of the gut. In one embodiment, the disclosure further includes non-pathogenic *Saccharomyces*, such as *Saccharomyces boulardii*. Naturally pathogenic bacteria may be genetically engineered to reduce or eliminate pathogenicity.

[098] "Probiotic" is used to refer to live, non-pathogenic microorganisms, *e.g.*, bacteria, which can confer health benefits to a host organism that contains an appropriate amount of the microorganism. In some embodiments, the host organism is a mammal. In some embodiments, the host organism is a human. In some embodiments, the probiotic

bacteria are Gram-negative bacteria. In some embodiments, the probiotic bacteria are Gram-positive bacteria. Some species, strains, and/or subtypes of non-pathogenic bacteria are currently recognized as probiotic bacteria. Examples of probiotic bacteria include, but are not limited to, certain strains belonging to the genus *Bifidobacteria*, *Escherichia Coli*, *Lactobacillus,* and *Saccharomyces e.g., Bifidobacterium bifidum, Enterococcus faecium, Escherichia coli* strain Nissle, *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus paracasei*, and *Lactobacillus plantarum,* and *Saccharomyces boulardii* (Dinleyici et al., 2014; U.S. Patent No. 5,589,168; U.S. Patent No. 6,203,797; U.S. Patent 6,835,376). The probiotic may be a variant or a mutant strain of bacterium (Arthur *et al.,* 2012; Cuevas-Ramos *et al.,* 2010; Olier *et al.,* 2012; Nougayrede *et al.,* 2006). Non-pathogenic bacteria may be genetically engineered to enhance or improve desired biological properties, *e.g.,* survivability. Non-pathogenic bacteria may be genetically engineered to provide probiotic properties. Probiotic bacteria may be genetically engineered to enhance or improve probiotic properties.

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

[0100] As used herein, the terms "modulate" and "treat" and their cognates refer to an amelioration of a disease, disorder, and/or condition, or at least one discernible symptom thereof. In another embodiment, "modulate" and "treat" refer to an amelioration of at least one measurable physical parameter, not necessarily discernible by the patient. In another embodiment, "modulate" and "treat" refer to inhibiting the progression of a disease, disorder, and/or condition, either physically (e.g., stabilization of a discernible symptom), physiologically (e.g., stabilization of a physical parameter), or both. In another embodiment, "modulate" and "treat" refer to slowing the progression or reversing the progression of a disease, disorder, and/or condition. As used herein, "prevent" and its cognates refer to delaying the onset or reducing the risk of acquiring a given disease, disorder and/or condition or a symptom associated with such disease, disorder, and/or condition.

[0101] Those in need of treatment may include individuals already having a particular medical disease, as well as those at risk of having, or who may ultimately acquire the disease. The need for treatment is assessed, for example, by the presence of one or more risk factors associated with the development of a disease, the presence or progression of a disease, or likely receptiveness to treatment of a subject having the disease. Diseases associated with the catabolism of propionate and/or one or more of its metabolites, *e.g.*, Propionic Acidemia and/or Methylmalonic Acidemia, may be caused by inborn genetic mutations for which there are no known cures. Diseases can also be secondary to other conditions. Treating diseases involving the catabolism of propionate and methylmalonate, *e.g.*, Propionic Acidemia and/or Methylmalonic Acidemia, may encompass reducing normal levels of propionate and/or one or more of its metabolites, reducing excess levels of propionate and/or one or more of its metabolites, or eliminating of propionate and/or one or more of its metabolites and does not necessarily encompass the elimination of the underlying disease.

[0102] As used herein, "payload" refers to one or more molecules of interest to be produced by a genetically engineered microorganism, such as a bacterium or a virus. In some embodiments, the payload is a therapeutic payload, e.g., a propionate catabolic enzyme or a propionate transporter polypeptide. In some embodiments, the payload is a regulatory molecule, e.g., a transcriptional regulator such as FNR. In some embodiments, the payload comprises a regulatory element, such as a promoter or a repressor. In some embodiments, the payload comprises an inducible promoter, such as from FNRS. In some embodiments, the payload comprises a repressor element, such as a kill switch. In some embodiments, the payload comprises an antibiotic resistance gene or genes. In some embodiments, the payload is encoded by a gene, multiple genes, gene cassette, or an operon. In alternate embodiments, the payload is produced by a biosynthetic or biochemical pathway, wherein the biosynthetic or biochemical pathway may optionally be endogenous to the microorganism. In alternate embodiments, the payload is produced by a biosynthetic or biochemical pathway, wherein the biosynthetic or biochemical pathway is not endogenous to the microorganism. In some embodiments, the genetically engineered microorganism comprises two or more payloads.

[0103] As used herein, the term "polypeptide" includes "polypeptide" as well as "polypeptides," and refers to a molecule composed of amino acid monomers linearly linked by amide bonds (i.e., peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, "peptides," "dipeptides," "tripeptides, "oligopeptides," "protein," "amino acid chain," or any

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

[0104] An "isolated" polypeptide or a fragment, variant, or derivative thereof refers to a polypeptide that is not in its natural milieu. No particular level of purification is required. Recombinantly produced polypeptides and proteins expressed in host cells, including but not limited to bacterial or mammalian cells, are considered isolated for purposed of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique. Recombinant peptides, polypeptides or proteins refer to peptides, polypeptides or proteins produced by recombinant DNA techniques, i.e. produced from cells, microbial or mammalian, transformed by an exogenous recombinant DNA expression construct encoding the polypeptide. Proteins or peptides expressed in most bacterial cultures will typically be free of glycan. Fragments, derivatives, analogs or variants of the foregoing polypeptides, and any combination thereof are also included as polypeptides. The terms "fragment," "variant," "derivative" and "analog" include polypeptides having an amino acid sequence sufficiently similar to the amino acid sequence of the original peptide and include any polypeptides, which retain at least one or

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

[0105] Polypeptides also include fusion proteins. As used herein, the term "variant" includes a fusion protein, which comprises a sequence of the original peptide or sufficiently similar to the original peptide. As used herein, the term "fusion protein" refers to a chimeric protein comprising amino acid sequences of two or more different proteins. Typically, fusion proteins result from well known in vitro recombination techniques. Fusion proteins may have a similar structural function (but not necessarily to the same extent), and/or similar regulatory function (but not necessarily to the same extent), and/or similar biochemical function (but not necessarily to the same extent) and/or immunological activity (but not necessarily to the same extent) as the individual original proteins which are the components of the fusion proteins. "Derivatives" include but are not limited to peptides, which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. "Similarity" between two peptides is determined by comparing the amino acid sequence of one peptide to the sequence of a second peptide. An amino acid of one peptide is similar to the corresponding amino acid of a second peptide if it is identical or a conservative amino acid substitution. Conservative substitutions include those described in Dayhoff, M. O., ed., The Atlas of Protein Sequence and Structure 5, National Biomedical Research Foundation, Washington, D.C. (1978), and in Argos, EMBO J. 8 (1989), 779-785. For example, amino acids belonging to one of the following groups represent conservative changes or substitutions: -Ala, Pro, Gly, Gln, Asn, Ser, Thr; -Cys, Ser, Tyr, Thr; -Val, Ile, Leu, Met, Ala, Phe; -Lys, Arg, His; -Phe, Tyr, Trp, His; and -Asp, Glu.

[0106] As used herein, the term "sufficiently similar" means a first amino acid sequence that contains a sufficient or minimum number of identical or equivalent amino acid residues relative to a second amino acid sequence such that the first and second amino acid sequences have a common structural domain and/or common functional activity. For example, amino acid sequences that comprise a common structural domain that is at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at

least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100%, identical are defined herein as sufficiently similar. Preferably, variants will be sufficiently similar to the amino acid sequence of the peptides of the invention. Such variants generally retain the functional activity of the peptides of the present invention. Variants include peptides that differ in amino acid sequence from the native and wt peptide, respectively, by way of one or more amino acid deletion(s), addition(s), and/or substitution(s). These may be naturally occurring variants as well as artificially designed ones.

[0107] As used herein the term "linker", "linker peptide" or "peptide linkers" or "linker" refers to synthetic or non-native or non-naturally-occurring amino acid sequences that connect or link two polypeptide sequences, e.g., that link two polypeptide domains. As used herein the term "synthetic" refers to amino acid sequences that are not naturally occurring. Exemplary linkers are described herein. Additional exemplary linkers are provided in US 20140079701, the contents of which are herein incorporated by reference in its entirety.

[0108] As used herein the term "codon-optimized" refers to the modification of codons in the gene or coding regions of a nucleic acid molecule to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the nucleic acid molecule. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of the host organism. A "codon-optimized sequence" refers to a sequence, which was modified from an existing coding sequence, or designed, for example, to improve translation in an expression host cell or organism of a transcript RNA molecule transcribed from the coding sequence, or to improve transcription of a coding sequence. Codon optimization includes, but is not limited to, processes including selecting codons for the coding sequence to suit the codon preference of the expression host organism. Many organisms display a bias or preference for use of particular codons to code for insertion of a particular amino acid in a growing polypeptide chain. Codon preference or codon bias, differences in codon usage between organisms, is allowed by the degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, inter alia, the

properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

[0109] As used herein, the terms "secretion system" or "secretion protein" refers to a native or non-native secretion mechanism capable of secreting or exporting a biomolecule, e.g., polypeptide from the microbial, e.g., bacterial cytoplasm. The secretion system may comprise a single protein or may comprise two or more proteins assembled in a complex e.g. HlyBD. Non-limiting examples of secretion systems for gram negative bacteria include the modified type III flagellar, type I (e.g., hemolysin secretion system), type II, type IV, type V, type VI, and type VII secretion systems, resistance-nodulation-division (RND) multi-drug efflux pumps, various single membrane secretion systems. Non-liming examples of secretion systems for gram positive bacteria include Sec and TAT secretion systems. In some embodiments, the polypeptide to be secreted include a "secretion tag" of either RNA or peptide origin to direct the polypeptide to specific secretion systems. In some embodiments, the secretion system is able to remove this tag before secreting the polypeptide from the engineered bacteria. For example, in Type V auto-secretion-mediated secretion the N-terminal peptide secretion tag is removed upon translocation of the "passenger" peptide from the cytoplasm into the periplasmic compartment by the native Sec system. Further, once the auto-secretor is translocated across the outer membrane the C-terminal secretion tag can be removed by either an autocatalytic or protease-catalyzed e.g., OmpT cleavage thereby releasing the lysosomal enzyme(s) into the extracellular milieu. In some embodiments, the secretion system involves the generation of a "leaky" or de-stabilized outer membrane, which may be accomplished by deleting or mutagenizing genes responsible for tethering the outer membrane to the rigid peptidoglycan skeleton, including for example, lpp, ompC, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl. Lpp functions as the primary 'staple' of the bacterial cell wall to the peptidoglycan. TolA-PAL and OmpA complexes function similarly to Lpp and are other deletion targets to generate a leaky phenotype. Additionally, leaky phenotypes have been observed when periplasmic proteases, such as *degS*, *degP* or *nlpI*, are deactivated. Thus, in some embodiments, the engineered bacteria have one or more deleted or mutated membrane genes, e.g., selected from lpp, ompA, ompA, ompF, tolA, tolB, and pal genes. In some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes, e.g., selected from degS, degP, and nlpl. In some embodiments,

the engineered bacteria have one or more deleted or mutated gene(s), selected from lpp, ompA, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl genes.

[0110] As used herein a "pharmaceutical composition" refers to a preparation of bacterial cells with other components such as a physiologically suitable carrier and/or excipient.

[0111] 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.

[0112] The term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples include, but are not limited to, calcium bicarbonate, sodium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20.

[0113] The terms “therapeutically effective dose” and “therapeutically effective amount” are used to refer to an amount of a compound that results in prevention, delay of onset of symptoms, or amelioration of symptoms of a disease. 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 the disease. 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.

[0114] As used herein, the term "bacteriostatic” or "cytostatic” refers to a molecule or protein which is capable of arresting, retarding, or inhibiting the growth, division, multiplication or replication of engineered bacterial cell of the disclosure.

[0115] As used herein, the term “bactericidal” refers to a molecule or protein which is capable of killing the engineered bacterial cell of the disclosure.

[0116] As used herein, the term “toxin” refers to a protein, enzyme, or polypeptide fragment thereof, or other molecule which is capable of arresting, retarding, or inhibiting the growth, division, multiplication or replication of the engineered bacterial cell of the disclosure, or which is capable of killing the engineered bacterial cell of the disclosure. The term “toxin” is intended to include bacteriostatic proteins and bactericidal proteins. The term “toxin” is intended to include, but not limited to, lytic proteins, bacteriocins (*e.g.*, microcins

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

[0117] The articles "a" and "an," as used herein, should be understood to mean "at least one," unless clearly indicated to the contrary.

[0118] 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.

[0119] Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Bacterial Strains

[0120] The disclosure provides a bacterial cell that comprises at least one heterologous gene encoding a propionate catabolism enzyme. In some embodiments, the bacterial cell is a non-pathogenic bacterial cell. In some embodiments, the bacterial cell is a commensal bacterial cell. In some embodiments, the bacterial cell is a probiotic bacterial cell.

[0121] In certain embodiments, the bacterial cell is selected from the group consisting of a Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Clostridium butyricum, Clostridium scindens, Escherichia coli, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus reuteri, Lactococcus lactis, and Oxalobacter formigenes bacterial cell. In one embodiment, the bacterial cell is a Bacteroides fragilis bacterial cell. In one embodiment, the bacterial cell is a Bacteroides thetaiotaomicron bacterial cell. In one embodiment, the bacterial cell is a Bacteroides subtilis bacterial cell. In

one embodiment, the bacterial cell is a Bifidobacterium animalis bacterial cell. In one embodiment, the bacterial cell is a Bifidobacterium bifidum bacterial cell. In one embodiment, the bacterial cell is a Bifidobacterium infantis bacterial cell. In one embodiment, the bacterial cell is a Bifidobacterium lactis bacterial cell. In one embodiment, the bacterial cell is a Clostridium butyricum bacterial cell. In one embodiment, the bacterial cell is a Clostridium scindens bacterial cell. In one embodiment, the bacterial cell is an Escherichia coli bacterial cell. In one embodiment, the bacterial cell is a Lactobacillus acidophilus bacterial cell. In one embodiment, the bacterial cell is a Lactobacillus plantarum bacterial cell. In one embodiment, the bacterial cell is a Lactobacillus reuteri bacterial cell. In one embodiment, the bacterial cell is a Lactococcus lactis bacterial cell. In one embodiment, the bacterial cell is a Oxalobacter formigenes bacterial cell. In another embodiment, the bacterial cell does not include Oxalobacter formigenes.

[0122] In one embodiment, the bacterial cell is a Gram positive bacterial cell. In another embodiment, the bacterial cell is a Gram negative bacterial cell.

[0123] In some embodiments, the bacterial cell is *Escherichia coli* strain Nissle 1917 (*E. coli* Nissle), a Gram-negative bacterium of the *Enterobacteriaceae* family that has evolved into one of the best characterized probiotics (Ukena *et al.*, 2007). The strain is characterized by its complete harmlessness (Schultz, 2008), and has GRAS (generally recognized as safe) status (Reister *et al.*, 2014, emphasis added). Genomic sequencing confirmed that *E. coli* Nissle lacks prominent virulence factors (*e.g.*, *E. coli* α-hemolysin, P-fimbrial adhesins) (Schultz, 2008), and *E. coli* Nissle does not carry pathogenic adhesion factors and does not produce any enterotoxins or cytotoxins, it is not invasive, not uropathogenic (Sonnenborn *et al.*, 2009). As early as in 1917, *E. coli* Nissle was packaged into medicinal capsules, called Mutaflor, for therapeutic use. It is commonly accepted that *E. coli* Nissle's therapeutic efficacy and safety have convincingly been proven (Ukena *et al.*, 2007).

[0124] In one embodiment, the engineered bacterial cell does not colonize the subject.

[0125] One of ordinary skill in the art would appreciate that the genetic modifications disclosed herein may be adapted for other species, strains, and subtypes of bacteria. Furthermore, genes from one or more different species can be introduced into one another, *e.g.*, a gene from *Lactobacillus plantarum* or *Methanobrevibacter smithii 3142* can be expressed in *Escherichia coli*.

[0126] In some embodiments, the bacterial cell is a genetically engineered bacterial cell. In another embodiment, the bacterial cell is an engineered bacterial cell. In some embodiments, the disclosure comprises a colony of bacterial cells.

[0127] In another aspect, the disclosure provides an engineered bacterial culture which comprises engineered bacterial cells.

[0128] In some embodiments of the above described genetically engineered bacteria, the gene or gene cassette(s) are present on a plasmid in the bacterium and operatively linked on the plasmid to the promoter that is induced under low-oxygen or anaerobic conditions. In other embodiments, the gene or gene cassette(s) is present in the bacterial chromosome and is operatively linked in the chromosome to the promoter that is induced under low-oxygen or anaerobic conditions.

[0129] In some embodiments, the genetically engineered bacteria is an auxotroph or a conditional auxotroph. In one embodiment, the genetically engineered bacteria is an auxotroph selected from a *cysE, glnA, ilvD, leuB, lysA, serA, metA, glyA, hisB, ilvA, pheA, proA, thrC, trpC, tyrA, thyA, uraA, dapA, dapB, dapD, dapE, dapF, flhD, metB, metC, proAB, and thi1* auxotroph. In some embodiments, the engineered bacteria have more than one auxotrophy, for example, they may be a Δ*thyA* and Δ*dapA auxotroph*.

[0130] In some embodiments, the genetically engineered bacteria further comprise a kill-switch circuit, such as any of the kill-switch circuits provided herein. For example, in some embodiments, the genetically engineered bacteria further comprise one or more genes encoding one or more recombinase(s) under the control of an inducible promoter, and an inverted toxin sequence. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding one or more recombinase(s) under the control of an inducible promoter and one or more inverted excision genes, wherein the excision gene(s) encode an enzyme that deletes an essential gene. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding a toxin under the control of a promoter having a TetR repressor binding site and a gene encoding the TetR under the control of an inducible promoter that is induced by arabinose, such as P_{araBAD}. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin.

[0131] In some embodiments, the genetically engineered bacteria are an auxotroph and further comprises a kill-switch circuit, such as any of the kill-switch circuits described herein.

[0132] In some embodiments of the above described genetically engineered bacteria, the gene or gene cassette(s) are present on a plasmid in the bacterium and operatively linked on the plasmid to the promoter that is induced under low-oxygen or anaerobic conditions. In other embodiments, the gene or gene cassette(s) are present in the bacterial chromosome and is operatively linked in the chromosome to the promoter that is induced under low-oxygen or anaerobic conditions.

[0133] In one aspect, the disclosure provides an engineered bacterial culture which reduces levels of propionate, propionyl CoA, and/or methylmalonyl CoA in the media of the culture. In one embodiment, the levels of the propionate, propionyl CoA, and/or methylmalonyl CoA are reduced by about 50%, about 75%, or about 100% in the media of the cell culture. In another embodiment, the levels of the propionate, propionyl CoA, and/or methylmalonyl CoA are reduced by about two-fold, three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold in the media of the cell culture. In one embodiment, the levels of the propionate, propionyl CoA, and/or methylmalonyl CoA are reduced below the limit of detection in the media of the cell culture.

[0134] The genetically engineered microorganisms, or programmed microorganisms, such as genetically engineered bacteria of the disclosure are capable of producing one or more enzymes for metabolizing propionate and/or metabolizing one or more propionate metabolite(s). Non-limiting examples of such enzymes and propionate metabolic pathways are described herein. For example, propionate metabolic pathways include, but are not limited to, one or more of the polyhydroxyalkanoate (PHA), methylmalonyl-CoA (MMCA), and 2-methylcitrate (2MC) pathways, e.g., as described herein. In some aspects, the disclosure provides a bacterial cell that comprises one or more heterologous gene sequence(s) and/or gene cassette(s) encoding one or more propionate catabolism enzyme(s) or other protein(s) that results in a decrease in levels of propionate and/or certain propionate metabolites, e.g., methylmalonate.

[0135] In certain embodiments, the genetically engineered bacteria are obligate anaerobic bacteria. In certain embodiments, the genetically engineered bacteria are facultative anaerobic bacteria. In certain embodiments, the genetically engineered bacteria are aerobic bacteria. In some embodiments, the genetically engineered bacteria are Gram-

positive bacteria. In some embodiments, the genetically engineered bacteria are Gram-positive bacteria and lack LPS. In some embodiments, the genetically engineered bacteria are Gram-negative bacteria. In some embodiments, the genetically engineered bacteria are Gram-positive and obligate anaerobic bacteria. In some embodiments, the genetically engineered bacteria are Gram-positive and facultative anaerobic bacteria. In some embodiments, the genetically engineered bacteria are non-pathogenic bacteria. In some embodiments, the genetically engineered bacteria are commensal bacteria. In some embodiments, the genetically engineered bacteria are probiotic bacteria. In some embodiments, the genetically engineered bacteria are naturally pathogenic bacteria that are modified or mutated to reduce or eliminate pathogenicity. Exemplary bacteria include, but are not limited to, Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Caulobacter, Clostridium, Enterococcus, Escherichia coli, Lactobacillus, Lactococcus, Listeria, Mycobacterium, Saccharomyces, Salmonella, Staphylococcus, Streptococcus, Vibrio, Bacillus coagulans, Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium breve UCC2003, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium acetobutylicum, Clostridium butyricum, Clostridium butyricum M-55, Clostridium cochlearum, Clostridium felsineum, Clostridium histolyticum, Clostridium multifermentans, Clostridium novyi-NT, Clostridium paraputrificum, Clostridium pasteureanum, Clostridium pectinovorum, Clostridium perfringens, Clostridium roseum, Clostridium sporogenes, Clostridium tertium, Clostridium tetani, Clostridium tyrobutyricum, Corynebacterium parvum, Escherichia coli MG1655, Escherichia coli Nissle 1917, Listeria monocytogenes, Mycobacterium bovis, Salmonella choleraesuis, Salmonella typhimurium, and Vibrio cholera. In certain embodiments, the genetically engineered bacteria are selected from the group consisting of Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis, and Saccharomyces boulardii. In certain embodiments, the genetically engineered bacteria are selected from *Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides subtilis, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Clostridium butyricum, Escherichia coli, Escherichia coli Nissle, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus reuteri,* and *Lactococcus lactis* bacterial cell. In one embodiment, the bacterial cell is a *Bacteroides fragilis* bacterial cell. In one embodiment, the bacterial cell

is a *Bacteroides thetaiotaomicron* bacterial cell. In one embodiment, the bacterial cell is a *Bacteroides subtilis* bacterial cell. In one embodiment, the bacterial cell is a *Bifidobacterium bifidum* bacterial cell. In one embodiment, the bacterial cell is a *Bifidobacterium infantis* bacterial cell. In one embodiment, the bacterial cell is a *Bifidobacterium lactis* bacterial cell. In one embodiment, the bacterial cell is a *Clostridium butyricum* bacterial cell. In one embodiment, the bacterial cell is an *Escherichia coli* bacterial cell. In one embodiment, the bacterial cell is a *Lactobacillus acidophilus* bacterial cell. In one embodiment, the bacterial cell is a *Lactobacillus plantarum* bacterial cell. In one embodiment, the bacterial cell is a *Lactobacillus reuteri* bacterial cell. In one embodiment, the bacterial cell is a *Lactococcus lactis* bacterial cell.

[0136] In some embodiments, the genetically engineered bacteria are Escherichia coli strain Nissle 1917 (E. coli Nissle), a Gram-negative bacterium of the Enterobacteriaceae family that has evolved into one of the best characterized probiotics (Ukena et al., 2007). The strain is characterized by its complete harmlessness (Schultz, 2008), and has GRAS (generally recognized as safe) status (Reister et al., 2014, emphasis added). Genomic sequencing confirmed that E. coli Nissle lacks prominent virulence factors (e.g., E. coli α-hemolysin, P-fimbrial adhesins) (Schultz, 2008). In addition, it has been shown that E. coli Nissle does not carry pathogenic adhesion factors, does not produce any enterotoxins or cytotoxins, is not invasive, and not uropathogenic (Sonnenborn et al., 2009). As early as in 1917, E. coli Nissle was packaged into medicinal capsules, called Mutaflor, for therapeutic use. E. coli Nissle has since been used to treat ulcerative colitis in humans in vivo (Rembacken et al., 1999), to treat inflammatory bowel disease, Crohn's disease, and pouchitis in humans in vivo (Schultz, 2008), and to inhibit enteroinvasive Salmonella, Legionella, Yersinia, and Shigella in vitro (Altenhoefer et al., 2004). It is commonly accepted that E. coli Nissle's therapeutic efficacy and safety have convincingly been proven (Ukena et al., 2007).

[0137] One of ordinary skill in the art would appreciate that the genetic modifications disclosed herein may be adapted for other species, strains, and subtypes of bacteria. Furthermore, genes from one or more different species can be introduced into one another, *e.g.*, the phaBCA genes from *Acinetobacter sp* RA3849, the accA gene from *Streptopmyces coelicolor*, *pccB* gene from *Streptopmyces coelicolor*, *mmcE* gene from *Propionibcterium freudenreichii* or the *mutAB* genes from *Propionibcterium freudenreichii,* or *matB*, derived from *Rhodopseudomonas palustris*, can be expressed in *Escherichia coli*. In some

embodiments, the genes are codon optimized, e.g., for expression in *E. coli*. In one embodiment, the recombinant bacterial cell does not colonize the subject having the disorder. Unmodified E. coli Nissle and the genetically engineered bacteria of the invention may be destroyed, e.g., by defense factors in the gut or blood serum (Sonnenborn et al., 2009). In some embodiments, the residence time is calculated for a human subject. In some embodiments, residence time in vivo is calculated for the genetically engineered bacteria of the invention.

[0138] In some embodiments, the bacterial cell is a genetically engineered bacterial cell. In another embodiment, the bacterial cell is a recombinant bacterial cell. In some embodiments, the disclosure comprises a colony of bacterial cells disclosed herein.

[0139] In another aspect, the disclosure provides a recombinant bacterial culture which comprises bacterial cells disclosed herein. In one aspect, the disclosure provides a recombinant bacterial culture which reduces levels of propionate in the media of the culture. In one embodiment, the levels of propionate and/or one or more of its metabolites are reduced by about 50%, about 75%, or about 100% in the media of the cell culture. In another embodiment, the levels of propionate and/or one or more of its metabolites, are reduced by about two-fold, three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold in the media of the cell culture. In one embodiment, the levels of propionate and/or one or more of its metabolites are reduced below the limit of detection in the media of the cell culture.

[0140] In some embodiments of the above described genetically engineered bacteria, the gene encoding a propionate catabolism enzyme is present on a plasmid in the bacterium and operatively linked on the plasmid to a promoter that is induced under low-oxygen or anaerobic conditions, such as any of the promoters disclosed herein. In other embodiments, the gene encoding a propionate catabolism enzyme is present in the bacterial chromosome and is operatively linked in the chromosome to the promoter that is induced under low-oxygen or anaerobic conditions, such as any of the promoters disclosed herein. In some embodiments of the above described genetically engineered bacteria, the gene encoding a propionate catabolism enzyme is present on a plasmid in the bacterium and operatively linked on the plasmid to the promoter that is induced under inflammatory conditions, such as any of the promoters disclosed herein. In other embodiments, the gene encoding a propionate catabolism enzyme is present in the bacterial chromosome and is operatively linked in the

chromosome to the promoter that is induced under inflammatory conditions, such as any of the promoters disclosed herein.

[0141] In some embodiments, the genetically engineered bacteria comprising gene sequence encoding a propionate catabolism enzyme is an auxotroph. In one embodiment, the genetically engineered bacteria are an auxotroph selected from a cysE, glnA, ilvD, leuB, lysA, serA, metA, glyA, hisB, ilvA, pheA, proA, thrC, trpC, tyrA, thyA, uraA, dapA, dapB, dapD, dapE, dapF, flhD, metB, metC, proAB, and thi1 auxotroph. In some embodiments, the engineered bacteria have more than one auxotrophy, for example, they may be a ΔthyA and ΔdapA auxotroph. In some embodiments, the genetically engineered bacteria comprising gene sequence encoding a propionate catabolism enzyme lacks functional ilvC gene sequence, e.g., is a ilvC auxotroph. IlvC encodes keto acid reductoisomerase, which enzyme is required for propionate synthesis. Knock out of ilvC creates an auxotroph and requires the bacterial cell to import isoleucine and valine to survive.

[0142] In some embodiments, the genetically engineered bacteria comprising gene sequence encoding a propionate catabolism enzyme further comprise gene sequence(s) encoding a propionate transporter into the bacterial cell. In certain embodiments, the propionate transporter is MctC, PutP_6, or any other propionate transporters described herein. In certain embodiments, the bacterial cell contains gene sequence encoding MctC, PutP_6, or any other propionate transporters described herein.

[0143] In some embodiments, the genetically engineered bacteria comprising gene sequence encoding a propionate catabolism enzyme further comprise gene sequence(s) encoding a secretion protein or protein complex for secreting a biomolecule, such as any of the secretion systems disclosed herein.

[0144] In some embodiments, the genetically engineered bacteria comprising gene sequence encoding a propionate catabolism enzyme further comprise gene sequence(s) encoding one or more antibiotic gene(s), such as any of the antibiotic genes disclosed herein.

[0145] In some embodiments, the genetically engineered bacteria comprising a propionate catabolism enzyme further comprise a kill-switch circuit, such as any of the kill-switch circuits provided herein. For example, in some embodiments, the genetically engineered bacteria further comprise one or more genes encoding one or more recombinase(s) under the control of an inducible promoter, and an inverted toxin sequence. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin. In some embodiments, the engineered bacteria further comprise

one or more genes encoding one or more recombinase(s) under the control of an inducible promoter and one or more inverted excision genes, wherein the excision gene(s) encode an enzyme that deletes an essential gene. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding a toxin under the control of a promoter having a TetR repressor binding site and a gene encoding the TetR under the control of an inducible promoter that is induced by arabinose, such as ParaBAD. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an antitoxin.

[0146] In some embodiments, the genetically engineered bacteria are an auxotroph comprising gene sequence encoding a propionate catabolism enzyme and further comprises a kill-switch circuit, such as any of the kill-switch circuits described herein.

[0147] In some embodiments of the above described genetically engineered bacteria, the gene encoding a propionate catabolism enzyme is present on a plasmid in the bacterium. In some embodiments, the gene encoding a propionate catabolism enzyme is present in the bacterial chromosome. In some embodiments, the gene sequence(s) encoding a propionate transporter, e.g., MctC, PutP_6, or any other propionate transporters described herein, is present on a plasmid in the bacterium. In some embodiments, the gene sequence(s) encoding a propionate transporter, e.g., MctC, PutP_6, or any other propionate transporters described herein, is present in the bacterial chromosome. In some embodiments, the gene sequence encoding a secretion protein or protein complex for secreting a biomolecule, such as any of the secretion systems disclosed herein, is present on a plasmid in the bacterium. In some embodiments, the gene sequence encoding a secretion protein or protein complex for secreting a biomolecule, such as any of the secretion systems disclosed herein, is present in the bacterial chromosome. In some embodiments, the gene sequence(s) encoding an antibiotic resistance gene is present on a plasmid in the bacterium. In some embodiments, the gene sequence(s) encoding an antibiotic resistance gene is present in the bacterial chromosome.

Inducible Promoters

[0148] In some embodiments, the bacterial cell comprises a stably maintained plasmid or chromosome carrying the gene encoding the propionate catabolism enzyme such that the propionate catabolism enzyme can be expressed in the host cell, and the host cell is capable of survival and/or growth in vitro, e.g., in medium, and/or in vivo, e.g., in the gut. In

some embodiments, bacterial cell comprises two or more distinct propionate catabolism enzymes. In some embodiments, the genetically engineered bacteria comprise multiple copies of the same propionate catabolism enzyme gene. In some embodiments, the genetically engineered bacteria comprise multiple copies of different propionate catabolism enzyme genes. In some embodiments, the gene encoding the propionate catabolism enzyme is present on a plasmid and operably linked to a directly or indirectly inducible promoter. In some embodiments, the gene encoding the propionate catabolism enzyme is present on a plasmid and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the gene encoding the propionate catabolism enzyme is present on a chromosome and operably linked to a directly or indirectly inducible promoter. In some embodiments, the gene encoding the propionate catabolism enzyme is present in the chromosome and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the gene encoding the propionate catabolism enzyme is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline or arabinose.

[0149] In some embodiments, the bacterial cell comprises a stably maintained plasmid or chromosome carrying the at least one gene encoding a transporter of propionate and/or one or more metabolites thereof, such that the transporter, can be expressed in the host cell, and the host cell is capable of survival and/or growth in vitro, e.g., in medium, and/or in vivo, e.g., in the gut. In some embodiments, bacterial cell comprises two or more distinct copies of the at least one gene encoding a propionate transporter. In some embodiments, the genetically engineered bacteria comprise multiple copies of the same at least one gene encoding a propionate transporter. In some embodiments, the at least one gene encoding a transporter of propionate, is present on a plasmid and operably linked to a directly or indirectly inducible promoter. In some embodiments, the at least one gene encoding a propionate transporter, is present on a plasmid and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the at least one gene encoding a propionate transporter, is present on a chromosome and operably linked to a directly or indirectly inducible promoter. In some embodiments, the at least one gene encoding a propionate transporter, is present in the chromosome and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the at least one gene encoding a transporter propionate and/or methylmalonate, is present on a

plasmid and operably linked to a promoter that is induced by exposure to tetracycline or arabinose.

[0150] In some embodiments, the promoter that is operably linked to the gene encoding the propionate catabolism enzyme and the promoter that is operably linked to the gene encoding the propionate transporter, is directly induced by exogenous environmental conditions. In some embodiments, the promoter that is operably linked to the gene encoding the propionate catabolism enzyme and the promoter that is operably linked to the gene encoding the propionate transporter, is indirectly induced by exogenous environmental conditions. In some embodiments, the promoter is directly or indirectly induced by exogenous environmental conditions specific to the gut of a mammal. In some embodiments, the promoter is directly or indirectly induced by exogenous environmental conditions specific to the small intestine of a mammal. In some embodiments, the promoter is directly or indirectly induced by low-oxygen or anaerobic conditions such as the environment of the mammalian gut. In some embodiments, the promoter is directly or indirectly induced by molecules or metabolites that are specific to the gut of a mammal, *e.g.*, propionate. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the bacterial cell.

[0151] In some embodiments, the bacterial cell comprises a stably maintained plasmid or chromosome carrying the at least one gene encoding a propionate binding protein, such that the propionate binding protein, can be expressed in the host cell, and the host cell is capable of survival and/or growth *in vitro*, *e.g.,* in medium, and/or *in vivo*, *e.g.,* in the gut. In some embodiments, bacterial cell comprises two or more distinct copies of the at least one gene encoding a propionate binding protein. In some embodiments, the genetically engineered bacteria comprise multiple copies of the same at least one gene encoding a propionate binding protein. In some embodiments, the at least one gene encoding a propionate binding protein is present on a plasmid and operably linked to a directly or indirectly inducible promoter. In some embodiments, the at least one gene encoding a propionate binding protein, is present on a plasmid and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the at least one gene encoding a propionate binding protein, is present on a chromosome and operably linked to a directly or indirectly inducible promoter. In some embodiments, the at least one gene encoding a propionate binding protein, is present in the chromosome and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments,

the at least one gene encoding a propionate binding protein, is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline or arabinose.

[0152] In some embodiments, the promoter that is operably linked to the gene encoding the propionate catabolism enzyme and the promoter that is operably linked to the gene encoding the propionate binding protein, is directly induced by exogenous environmental conditions. In some embodiments, the promoter that is operably linked to the gene encoding the propionate catabolism enzyme and the promoter that is operably linked to the gene encoding the propionate binding protein, is indirectly induced by exogenous environmental conditions. In some embodiments, the promoter is directly or indirectly induced by exogenous environmental conditions specific to the gut of a mammal. In some embodiments, the promoter is directly or indirectly induced by exogenous environmental conditions specific to the small intestine of a mammal. In some embodiments, the promoter is directly or indirectly induced by low-oxygen or anaerobic conditions such as the environment of the mammalian gut. In some embodiments, the promoter is directly or indirectly induced by molecules or metabolites that are specific to the gut of a mammal, *e.g.*, propionate. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the bacterial cell.

FNR dependent regulation

[0153] In certain embodiments, the bacterial cell comprises a gene encoding a propionate catabolism enzyme is expressed under the control of the fumarate and nitrate reductase regulator (FNR) promoter. In certain embodiments, the bacterial cell comprises at least one gene encoding a propionate transporter is expressed under the control of the fumarate and nitrate reductase regulator (FNR) promoter. In certain embodiments, the bacterial cell comprises at least one gene encoding a propionate binding protein is expressed under the control of the fumarate and nitrate reductase regulator (FNR) 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.

[0154] FNR responsive promoters include, but are not limited to, the FNR responsive promoters listed in the chart, below. Underlined sequences are predicted ribosome binding sites, and bolded sequences are restriction sites used for cloning.

Table 2 FNR responsive promoters

FNR Responsive Promoter	Sequence
SEQ ID NO: 1	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCACTATCGTCGTCCGGCCT TTTCCTCTCTTACTCTGCTACGTACATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACA AACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCCTTA AGGAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGG CGGTAATAGAAAAGAAATCGAGGCAAAA
SEQ ID NO: 2	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATGGCTCATGCATGCATCAAA AAAGATGTGAGCTTGATCAAAAACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCCG TTACGTGGGCTTCGACTGTAAATCAGAAAGGAGAAAACACCT
SEQ ID NO: 3	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCACTATCGTCGTCCGGCCT TTTCCTCTCTTACTCTGCTACGTACATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACA AACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCCTTA AGGAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTAA**GGATCC** CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
SEQ ID NO: 4	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATGGCTCATGCATGCATCAA AAAAGATGTGAGCTTGATCAAAAACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCC **GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
SEQ ID NO: 5	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGTTGTAACAAAAGCAAT TTTTCCGGCTGTCTGTATACAAAAACGCCGTAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCA TTCAGGGCAATATCTCTCTT**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CAT
SEQ ID NO: 6	ATCCCCATCACTCTTGATGGAGATCAATTCCCCAAGCTGCTAGAGCGTTACCTTGCCCTTAAACAT TAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGAGAAAACCG
SEQ ID NO: 7	CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCTTAAACATTAGCAATGT CGATTTATCAGAGGGCCGACAGGCTCCCACAGGAGAAAACCG

Table 3. FNR Promoter Sequences

SEQ ID NO	FNR-responsive regulatory region Sequence
nirB1 SEQ ID NO: 8	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCACT ATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATCTATTTCT ATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGAAATATCAGAC AATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCCTTAAG GAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAAT CGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA
nirB2 SEQ ID NO: 9	CGGCCCGATCGTTGAACATAGCGGTCCGCAGGCGGCACTGCTTACAGCAA ACGGTCTGTACGCTGTCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTC AGCCGTCACCGTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCC GGACGGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGC ATCTATTTCTATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGA AATATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATAT ACCCATTAAGGAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGG GTTGCTGAATCGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA atgtttgtttaactttaagaaggagatatacat

nirB3 SEQ ID NO: 10	GTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGGCACT ATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTATTTCT ATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGAAATATCAGAC AATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCATTAAG GAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAAT CGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA
ydfZ SEQ ID NO: 11	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATGGC TCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACAAAAAATATT TCACTCGACAGGAGTATTTATATTGCGCCCGTTACGTGGGCTTCGACTGT AAATCAGAAAGGAGAAAACACCT
nirB+RBS SEQ ID NO: 12	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCACT ATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATCTATTTCT ATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGAAATATCAGAC AATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCCTTAAG GAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAAT CGTTAA**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TACAT
ydfZ+RBS SEQ ID NO: 13	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATGG CTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACAAAAAATAT TTCACTCGACAGGAGTATTTATATTGCGCCC**GGATCC**CTCTAGAAATAAT TTTGTTTAACTTTAAGAAGGAGATATACAT
fnrS1 SEQ ID NO: 14	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGTAAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCTT **GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
fnrS2 SEQ ID NO: 15	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGCAAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCTT GGATCCAAAGTGAACTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA TATACAT
nirB+crp SEQ ID NO: 16	TCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTCAGCCGTCACCGTCAG CATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGGCACTATCGT CGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTATTTCTATAAA CCCGCTCATTTTGTCTATTTTTTGCACAAACATGAAATATCAGACAATTC CGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCATTAAGGAGTA TATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTA AGGTAGaaatgtgatctagttcacatttGCGGTAATAGAAAAGAAATCGA GGCAAAA*atgtttgtttaactttaagaaggagatatacat*

fnrS+crp SEQ ID NO: 17	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGCAAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCaa atgtgatctagttcacatttttgtttaactttaagaaggagatatacat

[0155] In one embodiment, the FNR responsive promoter comprises SEQ ID NO: 1. In another embodiment, the FNR responsive promoter comprises SEQ ID NO: 2. In another embodiment, the FNR responsive promoter comprises SEQ ID NO: 3. In another embodiment, the FNR responsive promoter comprises SEQ ID NO: 4. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 5. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 6. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 7. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 8. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 9. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 10. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 11. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 12. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 13. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 14. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 15. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 16. In yet another embodiment, the FNR responsive promoter comprises SEQ ID NO: 17.

[0156] In other embodiments, the FNR responsive promoter has at least about 80% identity with a nucleic acid sequence encoding any of SEQ ID NOs:1-17. In other embodiments, the FNR responsive promoter has at least about 85% identity with a nucleic acid sequence encoding any of SEQ ID NOs:1-17. In other embodiments, the FNR responsive promoter has at least about 90% identity with a nucleic acid sequence encoding any of SEQ ID NOs:1-17. In other embodiments, the FNR responsive promoter has at least about 95% identity with a nucleic acid sequence encoding any of SEQ ID NOs:1-17. In other embodiments, the FNR responsive promoter has at least about 96%, 97%, 98%, or 99% identity with a nucleic acid sequence encoding any of SEQ ID NOs:1-17. Accordingly, in some embodiments, the FNR responsive promoter has at least about 80%, 81%, 82%, 83%,

84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with a nucleic acid sequence encoding any of SEQ ID NOs:1-43.

[0157] In some embodiments, multiple distinct FNR nucleic acid sequences are inserted in the genetically engineered bacteria. In alternate embodiments, the genetically engineered bacteria comprise a gene encoding a propionate catabolism enzyme disclosed herein which is expressed under the control of an alternate oxygen level-dependent promoter, *e.g.*, DNR (Trunk et al., 2010) or ANR (Ray et al., 1997). In alternate embodiments, the genetically engineered bacteria comprise at least one gene encoding a propionate transporter which is expressed under the control of an alternate oxygen level-dependent promoter, *e.g.*, DNR (Trunk et al., 2010) or ANR (Ray et al., 1997). In alternate embodiments, the genetically engineered bacteria comprise at least one gene encoding a propionate binding protein which is expressed under the control of an alternate oxygen level-dependent promoter, *e.g.*, DNR (Trunk et al., 2010) or ANR (Ray et al., 1997). In these embodiments, catabolism of propionate and/or its metabolites is particularly activated in a low-oxygen or anaerobic environment, such as in the gut. In some embodiments, gene expression is further optimized by methods known in the art, *e.g.*, by optimizing ribosomal binding sites and/or increasing mRNA stability. In one embodiment, the mammalian gut is a human mammalian gut.

[0158] In some embodiments, the bacterial cell comprises an oxygen-level dependent transcriptional regulator, *e.g.*, FNR, ANR, or DNR, and corresponding promoter from a different bacterial species. The heterologous oxygen-level dependent transcriptional regulator and promoter increase the transcription of genes operably linked to said promoter, *e.g.*, the gene encoding the propionate catabolism enzyme, and/or the at least one gene encoding a propionate transporter, and/or the at least one gene encoding a propionate binding protein in a low-oxygen or anaerobic environment, as compared to the native gene(s) and promoter in the bacteria under the same conditions. In certain embodiments, the non-native oxygen-level dependent transcriptional regulator is an FNR protein from *N. gonorrhoeae* (*see, e.g.*, Isabella et al., 2011). In some embodiments, the corresponding wild-type transcriptional regulator is left intact and retains wild-type activity. In alternate embodiments, the corresponding wild-type transcriptional regulator is deleted or mutated to reduce or eliminate wild-type activity.

[0159] In some embodiments, the genetically engineered bacteria comprise a wild-type oxygen-level dependent transcriptional regulator, e.g., FNR, ANR, or DNR, and

corresponding promoter that is mutated relative to the wild-type promoter from bacteria of the same subtype. The mutated promoter enhances binding to the wild-type transcriptional regulator and increases the transcription of genes operably linked to said promoter, e.g., the gene encoding the propionate catabolism enzyme, and/or the at least one gene encoding a propionate transporter and/or the at least one gene encoding a propionate binding protein in a low-oxygen or anaerobic environment, as compared to the wild-type promoter under the same conditions. In some embodiments, the genetically engineered bacteria comprise a wild-type oxygen-level dependent promoter, *e.g.*, FNR, ANR, or DNR promoter, and corresponding transcriptional regulator that is mutated relative to the wild-type transcriptional regulator from bacteria of the same subtype. The mutated transcriptional regulator enhances binding to the wild-type promoter and increases the transcription of genes operably linked to said promoter, *e.g.*, the gene encoding the propionate catabolism enzyme, and/or the at least one gene encoding a propionate transporter, and/or the at least one gene encoding a propionate binding protein in a low-oxygen or anaerobic environment, as compared to the wild-type transcriptional regulator under the same conditions. In certain embodiments, the mutant oxygen-level dependent transcriptional regulator is an FNR protein comprising amino acid substitutions that enhance dimerization and FNR activity (*see, e.g.*, Moore et al., 2006).

[0160] In some embodiments, the bacterial cells disclosed herein comprise multiple copies of the endogenous gene encoding the oxygen level-sensing transcriptional regulator, *e.g.*, the *FNR* gene. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator is present on a plasmid. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the propionate catabolism enzyme are present on different plasmids. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the propionate catabolism enzyme and/or the at least one gene encoding a propionate transporter and/or the at least one gene encoding a propionate binding protein are present on different plasmids. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the propionate catabolism enzyme and/or the at least one gene encoding a transporter of a propionate and/or the at least one gene encoding a propionate binding protein are present on the same plasmid.

[0161] In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator is present on a chromosome. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the gene

encoding the propionate catabolism enzyme and/or the at least one gene encoding a propionate transporter and/or the at least one gene encoding a propionate binding protein are present on different chromosomes. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding the propionate catabolism enzyme and/or the at least one gene encoding a propionate transporter and/or the at least one gene encoding a propionate binding protein are present on the same chromosome. In some instances, it may be advantageous to express the oxygen level-sensing transcriptional regulator under the control of an inducible promoter in order to enhance expression stability. In some embodiments, expression of the transcriptional regulator is controlled by a different promoter than the promoter that controls expression of the gene encoding the propionate catabolism enzyme and/or the transporter of propionate and /or metabolites thereof and/or the propionate binding protein. In some embodiments, expression of the transcriptional regulator is controlled by the same promoter that controls expression of the propionate catabolism enzyme and/or the transporter of propionate and /or metabolites thereof, and/or the propionate binding protein. In some embodiments, the transcriptional regulator and the propionate catabolism enzyme are divergently transcribed from a promoter region.

RNS dependent regulation

[0162] In some embodiments, the genetically engineered bacteria comprise a gene encoding a propionate catabolism enzyme that is expressed under the control of an inducible promoter. In some embodiments, the genetically engineered bacterium that expresses a propionate catabolism enzyme and/or a transporter of propionate and /or metabolites thereof and/or propionate binding protein is under the control of a promoter that is activated by inflammatory conditions. In one embodiment, the gene for producing the propionate catabolism enzyme and/or a transporter of propionate and /or metabolites thereof and/or propionate binding protein is expressed under the control of an inflammatory-dependent promoter that is activated in inflammatory environments, e.g., a reactive nitrogen species or RNS promoter.

[0163] As used herein, "reactive nitrogen species" and "RNS" are used interchangeably to refer to highly active molecules, ions, and/or radicals derived from molecular nitrogen. RNS can cause deleterious cellular effects such as nitrosative stress. RNS includes, but is not limited to, nitric oxide (NO•), peroxynitrite or peroxynitrite anion (ONOO-), nitrogen dioxide (•NO2), dinitrogen trioxide (N2O3), peroxynitrous acid (ONOOH), and nitroperoxycarbonate (ONOOCO2-) (unpaired electrons denoted by •).

Bacteria have evolved transcription factors that are capable of sensing RNS levels. Different RNS signaling pathways are triggered by different RNS levels and occur with different kinetics.

[0164] As used herein, "RNS-inducible regulatory region" refers to a nucleic acid sequence to which one or more RNS-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression; in the presence of RNS, the transcription factor binds to and/or activates the regulatory region. In some embodiments, the RNS-inducible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor senses RNS and subsequently binds to the RNS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the RNS-inducible regulatory region in the absence of RNS; in the presence of RNS, the transcription factor undergoes a conformational change, thereby activating downstream gene expression. The RNS-inducible regulatory region may be operatively linked to a gene or genes, e.g., a propionate catabolism enzyme gene sequence(s), e.g., any of the amino acid catabolism enzymes described herein. For example, in the presence of RNS, a transcription factor senses RNS and activates a corresponding RNS-inducible regulatory region, thereby driving expression of an operatively linked gene sequence. Thus, RNS induces expression of the gene or gene sequences.

[0165] As used herein, "RNS-derepressible regulatory region" refers to a nucleic acid sequence to which one or more RNS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene expression; in the presence of RNS, the transcription factor does not bind to and does not repress the regulatory region. In some embodiments, the RNS-derepressible regulatory region comprises a promoter sequence. The RNS-derepressible regulatory region may be operatively linked to a gene or genes, e.g., propionate catabolism enzyme gene sequence(s), propionate transporter sequence(s), propionate binding protein(s). For example, in the presence of RNS, a transcription factor senses RNS and no longer binds to and/or represses the regulatory region, thereby derepressing an operatively linked gene sequence or gene cassette. Thus, RNS derepresses expression of the gene or genes.

[0166] As used herein, "RNS-repressible regulatory region" refers to a nucleic acid sequence to which one or more RNS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene

expression; in the presence of RNS, the transcription factor binds to and represses the regulatory region. In some embodiments, the RNS-repressible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor that senses RNS is capable of binding to a regulatory region that overlaps with part of the promoter sequence. In alternate embodiments, the transcription factor that senses RNS is capable of binding to a regulatory region that is upstream or downstream of the promoter sequence. The RNS-repressible regulatory region may be operatively linked to a gene sequence or gene cassette. For example, in the presence of RNS, a transcription factor senses RNS and binds to a corresponding RNS-repressible regulatory region, thereby blocking expression of an operatively linked gene sequence or gene sequences. Thus, RNS represses expression of the gene or gene sequences.

[0167] 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 4.

Table 4. Examples of RNS-sensing transcription factors and RNS-responsive genes

RNS-sensing transcription factor:	**Primarily capable of sensing:**	**Examples of responsive genes, promoters, and/or regulatory regions:**
NsrR	NO	*norB, aniA, nsrR, hmpA, ytfE, ygbA, hcp, hcr, nrfA, aox*
NorR	NO	*norVW, norR*
DNR	NO	*norCB, nir, nor, nos*

[0168] In some embodiments, the genetically engineered bacteria of the invention comprise a tunable regulatory region that is directly or indirectly controlled by a transcription factor that is capable of sensing at least one reactive nitrogen species. The tunable regulatory region is operatively linked to a gene or genes capable of directly or indirectly driving the expression of an amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein, thus controlling expression of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein relative to RNS levels. For example, the

tunable regulatory region is a RNS-inducible regulatory region, and the payload is an amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein, such as any of the amino acid catabolism enzymes, propionate transporters, and propionate binding proteins provided herein; when RNS is present, e.g., in an inflamed tissue, a RNS-sensing transcription factor binds to and/or activates the regulatory region and drives expression of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene or genes. Subsequently, when inflammation is ameliorated, RNS levels are reduced, and production of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is decreased or eliminated.

[0169] In some embodiments, the tunable regulatory region is a RNS-inducible regulatory region; in the presence of RNS, a transcription factor senses RNS and activates the RNS-inducible regulatory region, thereby driving expression of an operatively linked gene or genes. In some embodiments, the transcription factor senses RNS and subsequently binds to the RNS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the RNS-inducible regulatory region in the absence of RNS; when the transcription factor senses RNS, it undergoes a conformational change, thereby inducing downstream gene expression.

[0170] 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 genes, e.g., one or more propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene sequence(s). In the presence of RNS, a NorR transcription factor senses RNS and activates to the norVW regulatory region, thereby driving expression of the operatively linked gene(s) and producing the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein.

[0171] 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; Table 1). In certain embodiments, the genetically engineered bacteria of the invention comprise a RNS-inducible regulatory region from norCB that is operatively linked to a gene or gene cassette, e.g., a butyrogenic gene cassette. In the presence of RNS, a DNR transcription factor senses RNS and activates to the norCB regulatory region, thereby driving expression of the operatively linked gene or genes and producing one or more amino acid catabolism enzymes. In some embodiments, the DNR is Pseudomonas aeruginosa DNR.

[0172] 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.

[0173] 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 genes, e.g., a propionate catabolism enzyme gene or genes. In the presence of RNS, an NsrR transcription factor senses RNS and no longer binds to the norB regulatory region, thereby derepressing the operatively linked propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene or genes and producing the encoding an amino acid catabolism enzyme(s).

[0174] In some embodiments, it is advantageous for the genetically engineered bacteria to express a RNS-sensing transcription factor that does not regulate the expression of a significant number of native genes in the bacteria. In some embodiments, the genetically engineered bacterium of the invention expresses a RNS-sensing transcription factor from a

different species, strain, or substrain of bacteria, wherein the transcription factor does not bind to regulatory sequences in the genetically engineered bacterium of the invention. In some embodiments, the genetically engineered bacterium of the invention is Escherichia coli, and the RNS-sensing transcription factor is NsrR, e.g., from is Neisseria gonorrhoeae, wherein the Escherichia coli does not comprise binding sites for said NsrR. In some embodiments, the heterologous transcription factor minimizes or eliminates off-target effects on endogenous regulatory regions and genes in the genetically engineered bacteria.

[0175] 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.

[0176] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express an amino acid catabolism enzyme. The two repressor activation regulatory circuit comprises a first RNS-sensing repressor and a second repressor, which is operatively linked to a gene or gene cassette, e.g., encoding an amino acid catabolism enzyme. In one aspect of these embodiments, the RNS-sensing repressor inhibits transcription of the second repressor, which inhibits the transcription of the gene or gene cassette. Examples of second repressors useful in these embodiments include, but are not limited to, TetR, C1, and LexA. In the absence of binding by the first repressor (which occurs in the absence of RNS), the second repressor is transcribed, which represses expression of the gene or genes. In the presence of binding by the first repressor (which occurs in the presence of RNS), expression of the second repressor is repressed, and the gene or genes, e.g., a propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene or genes is expressed.

[0177] 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).

[0178] 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.

[0179] 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.

[0180] 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.

[0181] 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.

[0182] 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 propionate catabolism enzyme 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 propionate catabolism enzyme 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 propionate catabolism enzyme, propionate transporter, and/or propionate binding protein in the presence of RNS.

[0183] 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, 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.

[0184] In some embodiments, any of the gene(s) of the present disclosure may be integrated into the bacterial chromosome at one or more integration sites. For example, one or more copies of a propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene(s) may be integrated into the bacterial chromosome. Having multiple copies of the gene or gen(s) integrated into the chromosome allows for greater production of the amino acid catabolism enzyme(s) and also permits fine-tuning of the level of expression. Alternatively, different circuits described herein, such as any of the secretion or exporter circuits, in addition to the therapeutic gene(s) or gene cassette(s) could be integrated into the bacterial chromosome at one or more different integration sites to perform multiple different functions.

ROS-dependent regulation

[0185] In some embodiments, the genetically engineered bacteria comprise a gene for producing a propionate catabolism enzyme, propionate transporter, and/or propionate binding protein that is expressed under the control of an inducible promoter. In some embodiments, the genetically engineered bacterium that expresses a propionate catabolism enzyme, propionate transporter, and/or propionate binding protein under the control of a promoter that is activated by conditions of cellular damage. In one embodiment, the gene for producing the propionate catabolism enzyme is expressed under the control of a cellular damaged-dependent promoter that is activated in environments in which there is cellular or tissue damage, e.g., a reactive oxygen species or ROS promoter.

[0186] As used herein, "reactive oxygen species" and "ROS" are used interchangeably to refer to highly active molecules, ions, and/or radicals derived from molecular oxygen. ROS can be produced as byproducts of aerobic respiration or metal-catalyzed oxidation and may cause deleterious cellular effects such as oxidative damage. ROS includes, but is not limited to, hydrogen peroxide (H2O2), organic peroxide (ROOH),

hydroxyl ion (OH-), hydroxyl radical (•OH), superoxide or superoxide anion (•O2-), singlet oxygen (1O2), ozone (O3), carbonate radical, peroxide or peroxyl radical (•O2-2), hypochlorous acid (HOCl), hypochlorite ion (OCl-), sodium hypochlorite (NaOCl), nitric oxide (NO•), and peroxynitrite or peroxynitrite anion (ONOO-) (unpaired electrons denoted by •). Bacteria have evolved transcription factors that are capable of sensing ROS levels. Different ROS signaling pathways are triggered by different ROS levels and occur with different kinetics (Marinho et al., 2014).

[0187] As used herein, "ROS-inducible regulatory region" refers to a nucleic acid sequence to which one or more ROS-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression; in the presence of ROS, the transcription factor binds to and/or activates the regulatory region. In some embodiments, the ROS-inducible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor senses ROS and subsequently binds to the ROS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the ROS-inducible regulatory region in the absence of ROS; in the presence of ROS, the transcription factor undergoes a conformational change, thereby activating downstream gene expression. The ROS-inducible regulatory region may be operatively linked to a gene sequence or gene sequence, e.g., a sequence or sequences encoding one or more amino acid catabolism enzyme(s). For example, in the presence of ROS, a transcription factor, e.g., OxyR, senses ROS and activates a corresponding ROS-inducible regulatory region, thereby driving expression of an operatively linked gene sequence or gene sequences. Thus, ROS induces expression of the gene or genes.

[0188] As used herein, "ROS-derepressible regulatory region" refers to a nucleic acid sequence to which one or more ROS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene expression; in the presence of ROS, the transcription factor does not bind to and does not repress the regulatory region. In some embodiments, the ROS-derepressible regulatory region comprises a promoter sequence. The ROS-derepressible regulatory region may be operatively linked to a gene or genes, e.g., one or more genes encoding one or more amino acid catabolism enzyme(s). For example, in the presence of ROS, a transcription factor, e.g., OhrR, senses ROS and no longer binds to and/or represses the regulatory region, thereby

derepressing an operatively linked gene sequence or gene cassette. Thus, ROS derepresses expression of the gene or gene cassette.

[0189] As used herein, "ROS-repressible regulatory region" refers to a nucleic acid sequence to which one or more ROS-sensing transcription factors is capable of binding, wherein the binding of the corresponding transcription factor represses downstream gene expression; in the presence of ROS, the transcription factor binds to and represses the regulatory region. In some embodiments, the ROS-repressible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor that senses ROS is capable of binding to a regulatory region that overlaps with part of the promoter sequence. In alternate embodiments, the transcription factor that senses ROS is capable of binding to a regulatory region that is upstream or downstream of the promoter sequence. The ROS-repressible regulatory region may be operatively linked to a gene sequence or gene sequences. For example, in the presence of ROS, a transcription factor, e.g., PerR, senses ROS and binds to a corresponding ROS-repressible regulatory region, thereby blocking expression of an operatively linked gene sequence or gene sequences. Thus, ROS represses expression of the gene or genes.

[0190] 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 5**.

Table 5. Examples of ROS-sensing transcription factors and ROS-responsive genes

ROS-sensing transcription factor:	**Primarily capable of sensing:**	**Examples of responsive genes, promoters, and/or regulatory regions:**
OxyR	H_2O_2	*ahpC; ahpF; dps; dsbG; fhuF; flu; fur; gor; grxA; hemH; katG; oxyS; sufA; sufB; sufC; sufD; sufE; sufS; trxC; uxuA; yaaA; yaeH; yaiA; ybjM; ydcH; ydeN; ygaQ; yljA; ytfK*
PerR	H_2O_2	*katA; ahpCF; mrgA; zoaA; fur; hemAXCDBL; srfA*
OhrR	Organic peroxides	*ohrA*

ROS-sensing transcription factor:	Primarily capable of sensing:	Examples of responsive genes, promoters, and/or regulatory regions:
	NaOCl	
SoxR	$\bullet O_2^-$ NO• (also capable of sensing H_2O_2)	*soxS*
RosR	H_2O_2	*rbtT; tnp16a; rluC1; tnp5a; mscL; tnp2d; phoD; tnp15b; pstA; tnp5b; xylC; gabD1; rluC2; cgtS9; azlC; narKGHJI; rosR*

[0191] In some embodiments, the genetically engineered bacteria comprise a tunable regulatory region that is directly or indirectly controlled by a transcription factor that is capable of sensing at least one reactive oxygen species. The tunable regulatory region is operatively linked to a gene or gene cassette capable of directly or indirectly driving the expression of an amino acid catabolism enzyme, thus controlling expression of the propionate catabolism enzyme relative to ROS levels. For example, the tunable regulatory region is a ROS-inducible regulatory region, and the molecule is an amino acid catabolism enzyme; when ROS is present, e.g., in an inflamed tissue, a ROS-sensing transcription factor binds to and/or activates the regulatory region and drives expression of the gene sequence for the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein thereby producing the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein. Subsequently, when inflammation is ameliorated, ROS levels are reduced, and production of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is decreased or eliminated.

[0192] 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.

[0193] In some embodiments, the tunable regulatory region is a ROS-inducible regulatory region, and the transcription factor that senses ROS is OxyR. OxyR "functions

primarily as a global regulator of the peroxide stress response" and is capable of regulating dozens of genes, e.g., "genes involved in H2O2 detoxification (katE, ahpCF), heme biosynthesis (hemH), reductant supply (grxA, gor, trxC), thiol-disulfide isomerization (dsbG), Fe-S center repair (sufA-E, sufS), iron binding (yaaA), repression of iron import systems (fur)" and "OxyS, a small regulatory RNA" (Dubbs et al., 2012). The genetically engineered bacteria may comprise any suitable ROS-responsive regulatory region from a gene that is activated by OxyR. Genes that are capable of being activated by OxyR are known in the art (see, e.g., Zheng et al., 2001; Dubbs et al., 2012; 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, e.g., a propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene. In the presence of ROS, e.g., H2O2, an OxyR transcription factor senses ROS and activates to the oxyS regulatory region, thereby driving expression of the operatively linked propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene and producing the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein. 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.

[0194] In alternate embodiments, the tunable regulatory region is a ROS-inducible regulatory region, and the corresponding transcription factor that senses ROS is SoxR. When SoxR is "activated by oxidation of its [2Fe–2S] cluster, it increases the synthesis of SoxS, which then activates its target gene expression" (Koo et al., 2003). "SoxR is known to respond primarily to superoxide and nitric oxide" (Koo et al., 2003), and is also capable of responding to H2O2. The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is activated by SoxR. Genes that are capable of being activated by SoxR are known in the art (see, e.g., Koo et al., 2003; 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, e.g., an amino acid catabolism enzyme. In the presence of ROS, the SoxR transcription factor senses ROS and activates the soxS regulatory region, thereby driving expression of the operatively linked propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene and producing an amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein.

[0195] 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.

[0196] 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 propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene. In the presence of ROS, e.g., NaOCl, an OhrR transcription factor senses ROS and no longer binds to the ohrA regulatory region, thereby derepressing the operatively linked propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene and producing the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein.

[0197] 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).

[0198] In some embodiments, the tunable regulatory region is a ROS-derepressible regulatory region, and the corresponding transcription factor that senses ROS is RosR. RosR

is "a MarR-type transcriptional regulator" that binds to an "18-bp inverted repeat with the consensus sequence TTGTTGAYRYRTCAACWA" and is "reversibly inhibited by the oxidant H2O2" (Bussmann et al., 2010). RosR is capable of repressing numerous genes and putative genes, including but not limited to "a putative polyisoprenoid-binding protein (cg1322, gene upstream of and divergent from rosR), a sensory histidine kinase (cgtS9), a putative transcriptional regulator of the Crp/FNR family (cg3291), a protein of the glutathione S-transferase family (cg1426), two putative FMN reductases (cg1150 and cg1850), and four putative monooxygenases (cg0823, cg1848, cg2329, and cg3084)" (Bussmann et al., 2010). The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is repressed by RosR. Genes that are capable of being repressed by RosR are known in the art (see, e.g., Bussmann et al., 2010; 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., an amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein. In the presence of ROS, e.g., H2O2, a RosR transcription factor senses ROS and no longer binds to the cgtS9 regulatory region, thereby derepressing the operatively linked propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene and producing the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein.

[0199] 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.

[0200] 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.

[0201] In some embodiments, the tunable regulatory region is a ROS-repressible regulatory region, and the transcription factor that senses ROS is PerR. In Bacillus subtilis, PerR "when bound to DNA, represses the genes coding for proteins involved in the oxidative stress response (katA, ahpC, and mrgA), metal homeostasis (hemAXCDBL, fur, and zoaA) and its own synthesis (perR)" (Marinho et al., 2014). PerR is a "global regulator that responds primarily to H2O2" (Dubbs et al., 2012) and "interacts with DNA at the per box, a specific palindromic consensus sequence (TTATAATNATTATAA) residing within and near the promoter sequences of PerR-controlled genes" (Marinho et al., 2014). PerR is capable of binding a regulatory region that "overlaps part of the promoter or is immediately downstream from it" (Dubbs et al., 2012). The genetically engineered bacteria of the invention may comprise any suitable ROS-responsive regulatory region from a gene that is repressed by PerR. Genes that are capable of being repressed by PerR are known in the art (see, e.g., Dubbs et al., 2012; Table 1).

[0202] In these embodiments, the genetically engineered bacteria may comprise a two repressor activation regulatory circuit, which is used to express an amino acid catabolism enzyme. 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., an amino acid catabolism enzyme. In one aspect of these embodiments, the ROS-sensing repressor inhibits transcription of the second repressor, which inhibits the transcription of the gene or gene cassette. Examples of second repressors useful in these embodiments include, but are not limited to, TetR, C1, and LexA. In some embodiments, the ROS-sensing repressor is PerR. In some embodiments, the second repressor is TetR. In this embodiment, a PerR-repressible regulatory region drives expression of TetR, and a TetR-repressible regulatory region drives expression of the gene or gene cassette, e.g., an amino acid catabolism enzyme. 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., an amino acid catabolism enzyme. 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., an amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein is expressed.

[0203] 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.

[0204] 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.

[0205] Nucleic acid sequences of several exemplary OxyR-regulated regulatory regions are shown in Table 6. 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: 18, 19, 20, or 21, or a functional fragment thereof.

Table 6. Nucleotide sequences of exemplary OxyR-regulated regulatory regions

Regulatory sequence	**01234567890123456789012345678901234567890123456789**
katG (SEQ ID NO: 18)	TGTGGCTTTTATGAAAATCACACAGTGATCACAAATTTTAAACA GAGCACAAAATGCTGCCTCGAAATGAGGGCGGGAAAATAAGGT TATCAGCCTTGTTTTCTCCCTCATTACTTGAAGGATATGAAGCTA AAACCCTTTTTTATAAAGCATTTGTCCGAATTCGGACATAATCA AAAAAGCTTAATTAAGATCAATTTGATCTACATCTCTTTAACCA ACAATAT**GTAAGATCTCAACTATC**GCATC**CGTGGATTAATTC AATT**ATAACTTCTCTCTAACGCTGTGTATCGTAACGGTAACACT GTAGAGGGGAGCACATTGATGCGAATTCATTAAAGAGGAGAAA GGTACC
dps (SEQ ID NO: 19)	TTCCGAAAATTCCTGGCGAGCAGATAAATAAGAATTGTTCTTAT CAATATATCTAACTCATTGAATCTTTATTAGTTTTGTTTTTCA**CG CTTGTTACCACTATT**AGTGT**GATAGGAACAGCCAGAA**TAGCG GAACACATAGCCGGTGCTATACTTAATCTCGTTAATTACTGGGA CATAACATCAAGAGGATATGAAATTCGAATTCATTAAAGAGGA GAAAGGTACC
ahpC (SEQ ID NO: 20)	GCTTAGATCAGGTGATTGCCCTTTGTTTATGAGGGTGTTGTAATC CATGTCGTTGTTGCATTTGTAAGGGCAACACCTCAGCCTGCAGG CAGGCACTGAAGATACCAAAGGGTAGTTCAGATTACACGGTCA CCTGGAAAGGGGGCCATTTTACTTTTTATCGCCGCTGGCGGTGC AAAGTTCACAAAGTTGTCTTACGAAGGTT**GTAAGGTAAAACTT ATC**GATTT**GATAATGGAAACGCATT**AGCCGAATCGGCAAAAAT TGGTTACCTTACATCTCATCGAAAACACGGAGGAAGTATAGATG CGAATTCATTAAAGAGGAGAAAGGTACC
oxyS (SEQ ID NO: 21)	CTCGAGTTCATTATCCATCCTCCATCGCCAC**GATAGTTCATGGC GATA**GGTAG**AATAGCAATGAACGATT**ATCCCTATCAAGCATTC TGACTGATAATTGCTCACACGAATTCATTAAAGAGGAGAAAGGT ACC

[0206] 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.

[0207] 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.

[0208] 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.

[0209] 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.

[0210] 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 propionate catabolism enzyme, propionate transporter, and/or propionate binding protein 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 propionate catabolism enzyme, propionate transporter, and/or propionate binding protein 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 propionate catabolism enzyme in the presence of ROS.

[0211] In some embodiments, the gene or gene cassette for producing the propionate catabolism enzyme 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 propionate catabolism enzyme 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 propionate catabolism enzyme is present on a chromosome and operably linked to a promoter that is induced by exposure to tetracycline or arabinose. In some embodiments, the gene or gene cassette for producing the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline or arabinose. 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.

[0212] In some embodiments, the genetically engineered bacteria may comprise multiple copies of the gene(s) capable of producing an amino acid catabolism enzyme(s), propionate transporter(s), and/or propionate binding protein(s). In some embodiments, the gene(s) capable of producing an amino acid catabolism enzyme(s), propionate transporter(s), and/or propionate binding protein(s) is present on a plasmid and operatively linked to a ROS-responsive regulatory region. In some embodiments, the gene(s) capable of producing a propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is present in a chromosome and operatively linked to a ROS-responsive regulatory region.

[0213] Thus, in some embodiments, the genetically engineered bacteria or genetically engineered virus produce one or more amino acid catabolism enzymes 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.

[0214] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying a gene for producing an amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein such that the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein can be expressed in the host cell, and the host cell is capable of survival and/or growth in vitro, e.g., in medium, and/or in vivo. In some embodiments, a bacterium may comprise multiple copies of the gene encoding the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein. In some embodiments, the gene encoding the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is expressed on a low-copy plasmid. In some embodiments, the low-copy plasmid may be useful for increasing stability of expression. In some embodiments, the low-copy plasmid may be useful for decreasing leaky expression under non-inducing conditions. In some embodiments, the gene encoding the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein. In some embodiments, the gene encoding the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is expressed on a chromosome.

[0215] In some embodiments, the bacteria are genetically engineered to include multiple mechanisms of action (MOAs), e.g., circuits producing multiple copies of the same product (e.g., to enhance copy number) or circuits performing multiple different functions. For example, the genetically engineered bacteria may include four copies of the gene encoding a particular propionate catabolism enzyme, propionate transporter, and/or propionate binding protein inserted at four different insertion sites. Alternatively, the genetically engineered bacteria may include three copies of the gene encoding a particular propionate catabolism enzyme, propionate transporter, and/or propionate binding protein inserted at three different insertion sites and three copies of the gene encoding a different propionate catabolism enzyme, propionate transporter, and/or propionate binding protein inserted at three different insertion sites.

[0216] In some embodiments, under conditions where the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein is expressed, the genetically engineered bacteria of the disclosure produce at least about 1.5-fold, at least about 2-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, at least about 600-fold, at least about 700-fold, at least about 800-fold, at least about 900-fold, at least about 1,000-fold, or at least about 1,500-fold more of the amino acid catabolism enzyme, propionate transporter, and/or propionate binding protein and/or transcript of the gene(s) in the operon as compared to unmodified bacteria of the same subtype under the same conditions.

[0217] In some embodiments, quantitative PCR (qPCR) is used to amplify, detect, and/or quantify mRNA expression levels of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene(s). Primers specific for propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene(s) may be designed and used to detect mRNA in a sample according to methods known in the art. In some embodiments, a fluorophore is added to a sample reaction mixture that may contain propionate catabolism enzymemRNA, and a thermal cycler is used to illuminate the sample reaction mixture with a specific wavelength of light and detect the subsequent emission by the fluorophore. The reaction mixture is heated and cooled to predetermined temperatures for predetermined time periods. In certain embodiments, the heating and cooling is repeated for a predetermined number of cycles. In some embodiments, the reaction mixture is heated and cooled to 90-100° C, 60-70° C, and 30-50° C for a predetermined number of cycles. In a

certain embodiment, the reaction mixture is heated and cooled to 93-97° C, 55-65° C, and 35-45° C for a predetermined number of cycles. In some embodiments, the accumulating amplicon is quantified after each cycle of the qPCR. The number of cycles at which fluorescence exceeds the threshold is the threshold cycle (CT). At least one CT result for each sample is generated, and the CT result(s) may be used to determine mRNA expression levels of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene(s).

[0218] In some embodiments, quantitative PCR (qPCR) is used to amplify, detect, and/or quantify mRNA expression levels of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene(s). Primers specific for propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene(s) may be designed and used to detect mRNA in a sample according to methods known in the art. In some embodiments, a fluorophore is added to a sample reaction mixture that may contain propionate catabolism enzyme, propionate transporter, and/or propionate binding protein mRNA, and a thermal cycler is used to illuminate the sample reaction mixture with a specific wavelength of light and detect the subsequent emission by the fluorophore. The reaction mixture is heated and cooled to predetermined temperatures for predetermined time periods. In certain embodiments, the heating and cooling is repeated for a predetermined number of cycles. In some embodiments, the reaction mixture is heated and cooled to 90-100° C, 60-70° C, and 30-50° C for a predetermined number of cycles. In a certain embodiment, the reaction mixture is heated and cooled to 93-97° C, 55-65° C, and 35-45° C for a predetermined number of cycles. In some embodiments, the accumulating amplicon is quantified after each cycle of the qPCR. The number of cycles at which fluorescence exceeds the threshold is the threshold cycle (CT). At least one CT result for each sample is generated, and the CT result(s) may be used to determine mRNA expression levels of the propionate catabolism enzyme, propionate transporter, and/or propionate binding protein gene(s).

[0219] In other embodiments, the inducible promoter is a propionate responsive promoter. For example, the prpR promoter is a propionate responsive promoter. In one embodiment, the propionate responsive promoter comprises SEQ ID NO: 70.

Propionate Catabolism Enzymes and Propionate Catabolism Genes and Gene Cassettes

[0220] As used herein, the term "propionate catabolism gene," "propionate catabolism gene cassette," "propionate catabolism cassette", or "propionate catabolism operon" refers to a gene or set of genes capable of catabolizing propionate, and/or a metabolite thereof, and/or methylmalonic acid, an/or a metabolite thereof, in a biosynthetic pathway.

[0221] As used herein, the term "propionate catabolism enzyme" or "propionate catabolic or catabolism enzyme" or "propionate metabolic enzyme" refers to any enzyme that is capable of metabolizing propionate and/or a metabolite thereof. The term "propionate catabolism enzyme" or "propionate catabolic or catabolism enzyme" or "propionate metabolic enzyme" refers to any enzyme that is capable of metabolizing methylmalonic acid and/or a metabolite thereof. For example, the term "propionate catabolism enzyme" or "propionate catabolic or catabolism enzyme" or "propionate metabolic enzyme" refers to any enzyme that is capable of metabolizing propionate, propionyl-CoA, methylmalonic acid, and/or methylmalonylCoA. For example, the term "propionate catabolism enzyme" or "propionate catabolic or catabolism enzyme" or "propionate metabolic enzyme" refers to any enzyme that is capable of reducing accumulated propionate and/or methylmalonic acid and/or propionylCoA and/or methylmalonylCoA or that can lessen, ameliorate, or prevent one or more propionate and/or methylmalonic acid diseases or disease symptoms. Examples of propionate and/or methylmalonic acid metabolic enzymes include, but are not limited to, propionyl CoA carboxylase (PCC), methylmalonyl CoA mutase (MUT), propionyl-CoA synthetase (PrpE), 2-methylisocitrate lyase (PrpB), 2-methylcitrate synthase (prpC), 2-methylcitrate dehydratase (PrpD), propionyl-CoA carboxylase (pccB), Acetyl-/propionyl-coenzyme A carboxylase (accA1), Methylmalonyl-CoA epimerase (mmcE), methylmalonyl-CoA mutase (mutA, and mutB), Acetoacetyl-CoA reductase (phaB), Polyhydroxyalkanoic acid (PHA) synthases, e.g., encoded by phaC, and 3-ketothiolase (phaA), pct, and malonyl-coenzyme A (malonyl-CoA) synthetase (*matB*).

[0222] Functional deficiencies in these proteins result in the accumulation of propionate and/or methylmalonic acid or one or more of their metabolites in cells and tissues. Propionate catabolism enzymes of the present disclosure include both wild-type or modified propionate catabolism enzymes and can be produced using recombinant and synthetic methods or purified from nature sources. Propionate catabolism enzymes include full-length

polypeptides and functional fragments thereof, as well as homologs and variants thereof. Propionate catabolism enzymes include polypeptides that have been modified from the wild-type sequence, including, for example, polypeptides having one or more amino acid deletions, insertions, and/or substitutions and may include, for example, fusion polypeptides and polypeptides having additional sequence, e.g., regulatory peptide sequence, linker peptide sequence, and other peptide sequence.

[0223] As used herein, the term "propionate catabolism enzyme" refers to an enzyme involved in the catabolism of propionate or propionyl CoA and or methylmalonic acid or methylmalonylCoA to a non-toxic molecule, such as its corresponding methylmalonyl CoA molecule, corresponding succinyl CoA molecule, succinate, or polyhydroxyalkanotes; or the catabolism of methylmalonyl CoA to non-toxic molecule, such as its corresponding succinyl CoA molecule. Enzymes involved in the catabolism of propionate are well known to those of skill in the art.

[0224] In humans, the major pathway for metabolizing propionyl-CoA involves the enzyme propionyl CoA carboxylase (PCC), which converts propionyl CoA to methylmalonyl CoA, and the methylmalonyl CoA mutase (MUT) enzyme then converts methylmalonyl CoA into succinylCoA (see, e.g., **FIG. 5**). Enzyme deficiencies or mutations which lead to the toxic accumulation of propionyl CoA or methylmalonyl CoA result in the development of disorders associated with propionate catabolism, such as PA and MMA, and severe nutritional deficiencies of Vitamin B_{12} can also result in MMA (Higginbottom *et al., M. Engl. J. Med.,* 299(7):317-323, 1978). Other minor pathways are present in humans, but these pathways are insufficient to compensate for the absence of or mutations in the major pathway for propionyl CoA metabolism (see, e.g., **FIG. 5**). Thus, in some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionyl CoA carboxylase (PCC). In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionyl CoA carboxylase (PCC) and one or more copies of methylmalonyl CoA mutase (MUT).

[0225] For propionic acid to be consumed by any of the pathways or circuits of the present disclosure, it must first be activated to propionyl-CoA. This activation can be catalyzed by either propionyl-CoA synthetase (PrpE) or propionate CoA transferase (Pct). Thus, in some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionyl-CoA synthetase (PrpE). In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionate

CoA transferase (Pct). In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionyl-CoA synthetase (PrpE) and one or more copies of propionyl CoA carboxylase (PCC). In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionyl-CoA synthetase (PrpE), one or more copies of propionyl CoA carboxylase (PCC) and one or more copies of methylmalonyl CoA mutase (MUT). In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionate CoA transferase (Pct) and one or more copies of propionyl CoA carboxylase (PCC). In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more copies of propionate CoA transferase (Pct), one or more copies of propionyl CoA carboxylase (PCC) and one or more copies of methylmalonyl CoA mutase (MUT).

[0226] PrpE converts propionate and free CoA to propionyl-CoA in an irreversible, ATP-dependent manner, releasing AMP and PPi (pyrophosphate). PrpE can be inactivated by posttranslational modification of the active site lysine, e.g., as shown in **FIG. 9A**. Protein lysine acetyltransferase (Pka) in E. coli carries out the propionylation of PrpE. The enzyme CobB depropionylates PrpEPr making the inactivation reversible. However, the inactivation pathway can be eliminated entirely through the deletion of the pka gene. In any of the embodiments described herein and elsewhere in the specification, the genetically engineered bacteria comprise a deletion of pka (Δpka) to prevent the inactivation of PrpE. In some embodiments, the deletion of pka results in greater activity of PrpE and downstream catabolic enzymes.

[0227] Pct converts propionate and acetyl-CoA to propionyl-CoA and acetate in a reversible reaction. In some embodiments, the genetically engineered bacteria comprise a gene encoding Pct for the generation of propionylCoA from propionate, e.g., as shown in **FIG. 9B**. In some embodiments, the genetically engineered bacteria comprise Pct in combination with or as a component of one or more of PHA and/or MMCA and/or 2MC pathway cassette(s).

[0228] In bacteria, PrpB, PrpC, and PrpD are capable of converting propionyl CoA into succinate and pyruvate, and PrpB, PrpC, PrpD, and PrpE are capable of converting propionate into succinate and pyruvate. Specifically, PrpE, a propionate-CoA ligase, converts propionate to propionyl CoA. PrpC, a 2-methylcitrate synthetase, then converts propionyl CoA to 2-methylcitrate. PrpD, a 2-methylcitrate dehydrogenase, then converts 2-methylcitrate into 2-methyisocitrate, and PrpB, a 2-methylisocitrate lyase, converts 2-

methyisocitrate into succinate and pyruvate (see **FIG. 19**). Thus, in some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more of the following: PrpB, PrpC, and PrpD. In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more of the following: PrpB, PrpC, PrpD, and PrpE. In some embodiments, the engineered bacterium comprises two or more copies of a gene encoding any of the following: PrpB, PrpC, and PrpD, and combinations thereof. In some embodiments, the engineered bacterium comprises two or more copies of a gene encoding any of the following: PrpB, PrpC, PrpD, and PrpE, and combinations thereof.

[0229] In another bacterial pathway, the polyhydroxyalkanoate pathway, propionate is converted to propionyl-CoA by PrpE. Propionyl-CoA is then converted to 3-keto-valeryl-CoA by PhaA, which is then converted to 3-hydroxy-valeryl-CoA by PhaB. Finally, PhaC converts 3-hydroxy-valeryl-CoA to PHV (see **FIG. 10**). Thus, in some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more of the following: PrpE, PhaA, and PhaB.

The disclosure encompasses the design of genetic circuits which mimic the functional activities of the human methylmalonyl-CoA pathway in order to catabolize propionate to treat diseases associated with propionate catabolism. For example, a circuit can be designed to express *prpE, pccB, accA1, mmcE, mutA,* and *mutB* (**FIG. 15**). In this circuit, PrpE converts propionate to propionyl-CoA, which is then converted to D-methylmalonyl-CoA by PccB and AccA1. D-methylmalonyl-CoA is then converted to L-methylmalonyl-CoA by MmcE, and MutA and MutB convert L-methylmalonyl CoA to succinyl-CoA. Alternatively, these genes can be split up into two circuits, i.e., prpE-accA1-pccB and mmcE-mutA-mutB, as indicated in **FIG. 15**. Thus, in some embodiments, the engineered bacterium comprises gene sequence(s) selected from: *prpE, pccB, accA1, mmcE, mutA,* and *mutB*. In some embodiments, the engineered bacterium comprises gene sequence(s) encoding one or more of the following: PrpE, PccB, AccA1, MmcE, MutA, and MutB. In another embodiment, the disclosure encompasses the design of genetic circuits which constitute the 2-methylcitrate cycle pathway in bacteria, such as the *prpBCDE* circuit (**FIG. 20**) or the polyhydroxyalkanoate pathway, such as the *prpE, phaB, phaC, phaA* genes (**FIG. 10C**) in order to catabolize propionate to treat diseases associated with propionate catabolism.

[0230] The disclosure encompasses the design of genetic circuits which comprise MatB. Malonyl-coenzyme A (malonyl-CoA) synthetase (MatB) belongs to the AMP-forming acyl-CoA synthetase protein family. These enzymes catalyze the conversion of organic acids

to acyl-CoA thioesters via a ping-pong mechanism, in which ATP and the organic acid are first converted to acyl-AMP with the release of pyrophosphate, followed by coenzyme A binding, displacement of AMP, and release of the acyl-CoA product (see, e.g., Crosby et al., Structure-Guided Expansion of the Substrate Range of Methylmalonyl Coenzyme A Synthetase (MatB) of Rhodopseudomonas palustris; Appl. Environ. Microbiol. September 2012 vol. 78 no. 18 6619-6629, and references therein). MatB converts malonate to malonyl-CoA in two steps according to this mechanism via a malonyl-AMP intermediate, and similarly also converts methylmalonate to methylmalonyl-CoA.

[0231] A genetic circuit comprising MatB is useful in the treatment of methylmalonic acidemia, allowing accumulated methylmalonic acid to be converted into methylmalonylCoA. Once converted to methylmalonylCoA, catabolism can proceed along the MMCA pathway (e.g., through mmcE, mutA, and mutB). Alternatively, methylmalonylCoA can be converted to propionylCoA. This reaction may be catalyzed by the AccA1/PccB complex, which is encoded by a genetic circuit of the disclosure. The AccA1/pccB complex catalyzes the reversible conversion of propionylCoA to methylmalonylCoA, as described herein. Once methylmalonylCoA is converted to propionylCoA, any of the propionate catabolism enzymes encoded by the genetic circuits described herein, e.g., PHA, MMCA, and/or 2MC circuits, are suitable for further catalysis, resulting in an inert product. Thus, in any of the embodiments described herein and elsewhere in the specification, the engineered bacterium may further comprise gene sequence(s) encoding MatB.

[0232] In some embodiments of the disclosure, one or more gene(s) or gene cassette(s) comprise MatB, e.g., MatB derived from Rhodopseudomonas palustris. In some embodiments of the disclosure, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) comprising MatB, e.g., MatB derived from Rhodopseudomonas palustris. In a non-limiting example, genetically engineered bacteria comprising one or more gene(s) or gene cassettes comprising MatB are suitable for the treatment of methylmalonic acidemia or methylmalonic acidemia and propionic acidemia.

[0233] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding MatB and one or more MMCA gene cassettes as described herein. In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding MatB and one or more MMCA gene(s) or MMCA gene cassette(s) as described herein. In some embodiments, MatB is driven by a separate

promoter and is on a separate plasmid or chromosomal integration site. In some embodiments, MatB part of an operon comprising one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes described herein.

[0234] In some embodiments, the genetically engineered bacteria encode one or more of MatB, mmcE, mutA, and mutB. In some embodiments, the genetically engineered bacteria encode MatB, mmcE, mutA, and mutB. In some embodiments, a genetic circuit encoded by the genetically engineered bacteria comprises MatB, mmcE, mutA, and mutB.

[0235] In some embodiments, the genetically engineered bacteria encode one or more of MatB, Acc1A, and PccB. In some embodiments, the genetically engineered bacteria encode MatB, Acc1A, and PccB. In some embodiments, a genetic circuit encoded by the genetically engineered bacteria comprises MatB, Acc1A, and PccB. In some embodiments, the genetically engineered bacteria encode MatB, Acc1A, and PccB, and mmcE, mutA and mutB. In some embodiments, the genetically engineered bacteria encode MatB, Acc1A, and PccB, and mmcE, mutA and mutB and further prpE. In some embodiments, the genetically engineered bacteria encode MatB, Acc1A, and PccB, and mmcE, mutA and mutB, and further encode a PHA and/or 2MC pathway circuit, and may or may not further comprise prpE. These genes may be organized in one or more gen cassettes, as described herein. Non-limiting examples of genetically engineered bacteria comprising one or more gene(s) or gene cassettes and comprising exemplary operons or gene cassette(s) are depicted in **FIG. 21G** and **FIG. 21F**. In other non-limiting examples, the one or more gene cassettes may be organized as follows; MatB-mmcE-mutA-mutB; MatB-Acc1A-PccB and mmcE-mutA-mutB, alone or in combination with PPHA and/or 2MC pathway cassettes; PrpE-MatB-Acc1A-PccB and mmcE-mutA-mutB, alone or in combination with PPHA and/or 2MC pathway cassettes.

[0236] In one embodiment, expression of the propionate catabolism gene cassette increases the rate of propionate, propionyl CoA, and/or methylmalonyl CoA catabolism in the cell. In one embodiment, expression of the propionate catabolism gene cassette decreases the level of propionate in the cell. In another embodiment, expression of the propionate catabolism gene cassette decreases the level of propionic acid in the cell. In one embodiment, expression of the propionate catabolism gene cassette decreases the level of propionyl CoA in the cell. In one embodiment, expression of the propionate catabolism gene cassette decreases the level of methylmalonyl CoA in the cell. In one embodiment, expression of the propionate catabolism gene cassette decreases the level of methylmalonic acid in the cell.

[0237] In another embodiment, expression of the propionate catabolism gene cassette increases the level of methylmalonyl CoA in the cell as compared to the level of its corresponding propionyl CoA in the cell. In another embodiment, expression of the propionate catabolism gene cassette increases the level of succinate in the cell as compared to the level of its corresponding methylmalonyl CoA in the cell. In one embodiment, expression of the propionate catabolism gene cassette decreases the level of the propionate, propionyl CoA, and/or methylmalonyl CoA as compared to the level of succinate or succinyl CoA in the cell. In one embodiment, expression of the propionate catabolism gene cassette increases the level of succinate or succinyl CoA in the cell as compared to the level of the propionate, propionyl CoA, and/or methylmalonyl CoA in the cell.

[0238] Enzymes involved in the catabolism of propionate may be expressed or modified in the bacteria in order to enhance catabolism of propionate. Specifically, when the heterologous propionate catabolism gene or gene cassette is expressed in the engineered bacterial cells, the bacterial cells convert more propionate and/or propionyl CoA into methylmalonyl CoA, or convert more methylmalonyl CoA into succinate or succinyl CoA when the gene or gene cassette is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the genetically engineered bacteria expressing a heterologous propionate catabolism gene or gene cassette can catabolize propionate, propionyl CoA, and/or methylmalonyl CoA to treat diseases associated with catabolism of propionate, such as Propionic Acidemia (PA) and Methylmalonic Acidemia (MMA).

[0239] In some embodiments, the expression of the propionate catabolism gene cassette decreases the levels of one or more propionic acidemia and/or methylmalonic acidemia biomarkers. In some embodiments, the propionate catabolism gene cassette expressed by the genetically engineered bacteria decreases the levels of one or more propionic acidemia and/or methylmalonic acidemia biomarkers. In one embodiment, expression of the propionate catabolism gene cassette decreases the propionylcarnitine to acetylcarnitine ratio in the blood and/or the urine, e.g., in a mammalian subject with elevated levels of propionate and/or methylmalonate. In one embodiment, expression of the propionate catabolism gene cassette decreases levels of 2-methylcitrate in the blood and/or in the urine, e.g., in a mammalian subject with elevated levels of propionate and/or methylmalonate. In one embodiment, expression of the propionate catabolism gene cassette decreases levels of propionylglycine in the blood and/or in the urine, e.g., in a mammalian subject with elevated levels of propionate and/or methylmalonate. In one embodiment,

expression of the propionate catabolism gene cassette decreases levels of tiglyglycine in the blood and/or in the urine, e.g., in a mammalian subject with elevated levels of propionate and/or methylmalonate.

[0240] In one embodiment, the bacterial cell comprises at least one heterologous gene encoding at least one propionate catabolism enzyme. In one embodiment, the bacterial cell comprises at least one heterologous gene encoding a transporter of propionate and at least one heterologous gene encoding at least one propionate catabolism enzyme.

[0241] In one embodiment, the engineered bacterial cell comprises at least one heterologous gene or gene cassette encoding at least one propionate catabolism enzyme. In some embodiments, the disclosure provides a bacterial cell that comprises at least one heterologous gene or gene cassette encoding at least one propionate catabolism enzyme operably linked to a first promoter. In one embodiment, the bacterial cell comprises at least one gene or gene cassette encoding at least one propionate catabolism enzyme from a different organism, *e.g.,* a different species of bacteria. In another embodiment, the bacterial cell comprises more than one copy of a native gene or gene cassette encoding one or more propionate catabolism enzyme(s). In yet another embodiment, the bacterial cell comprises at least one native gene or gene cassette encoding at least one native propionate catabolism enzyme, as well as at least one copy of at least one gene or gene cassette encoding one or more propionate catabolism enzyme(s) from a different organism, *e.g.,* a different species of bacteria. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of a gene or gene cassette encoding one or more propionate catabolism enzyme(s). In one embodiment, the bacterial cell comprises multiple copies of a gene or gene cassette encoding one or more propionate catabolism enzyme(s). In one embodiment, a gene cassette may comprise one or more native and one or more non-native or heterologous genes.

[0242] Multiple distinct propionate catabolism enzymes are known in the art. In some embodiments, the propionate catabolism enzyme is encoded by at least one gene encoding at least one propionate catabolism enzyme derived from a bacterial species. In some embodiments, a propionate catabolism enzyme is encoded by one or more gene(s) or gene cassettes encoding a propionate catabolism enzyme derived from a non-bacterial species. In some embodiments, a propionate catabolism enzyme is encoded by a gene derived from a eukaryotic species, *e.g.,* a yeast species or a plant species. In one embodiment, a propionate catabolism enzyme is encoded by a gene derived from a human. In one embodiment, the at least one gene encoding the at least one propionate catabolism

enzyme is derived from an organism of the genus or species that includes, but is not limited to, *Acetinobacter, Azospirillum, Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Burkholderia, Citrobacter, Clostridium, Corynebacterium, Cronobacter, Enterobacter, Enterococcus, Erwinia, Helicobacter, Klebsiella, Lactobacillus, Lactococcus, Leishmania, Listeria, Macrococcus, Mycobacterium, Nakamurella, Nasonia, Nostoc, Pantoea, Pectobacterium, Pseudomonas, Psychrobacter, Ralstonia, Saccharomyces, Salmonella, Sarcina, Serratia, Staphylococcus, and Yersinia, e.g., Acetinobacter radioresistens, Acetinobacter baumannii, Acetinobacter calcoaceticus, Azospirillum brasilense, Bacillus anthracis, Bacillus cereus, Bacillus coagulans, Bacillus megaterium,Bacillus subtilis, Bacillus thuringiensis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Burkholderia xenovorans, Citrobacter youngae, Citrobacter koseri, Citrobacter rodentium, Clostridium acetobutylicum, Clostridium butyricum, Corynebacterium aurimucosum, Corynebacterium kroppenstedtii, Corynebacterium striatum, Cronobacter sakazakii, Cronobacter turicensis, Enterobacter cloacae, Enterobacter cancerogenus, Enterococcus faecium, Erwinia amylovara, Erwinia pyrifoliae, Erwinia tasmaniensis, Helicobacter mustelae, Klebsiella pneumonia, Klebsiella variicola, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis, Leishmania infantum, Leishmania major, Leishmania brazilensis, Listeria grayi, Macrococcus caseolyticus, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium kansasii, Mycobacterium leprae, Mycobacterium marinum, Mycobacterium smegmatis, Mycobacterium tuberculosis, Mycobacterium ulcerans, Nakamurella multipartita, Nasonia vitipennis, Nostoc punctiforme, Pantoea ananatis, Pantoea agglomerans, Pectobacterium atrosepticum, Pectobacterium carotovorum, Pseudomonas aeruginosa, Psychrobacter articus, Psychrobacter cryohalolentis, Ralstonia eutropha, Saccharomyces boulardii, Salmonella enterica, Sarcina ventriculi, Serratia odorifera, Serratia proteamaculans, Staphylococcus aerus, Staphylococcus capitis, Staphylococcys carnosus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus lugdunensis, Staphylococcus saprophyticus, Staphylococcus warneri, Yersinia enterocolitica, Yersinia mollaretii, Yersinia kristensenii, Yersinia rohdei, and Yersinia aldovae.*

[0243] In some embodiments, the gene encoding prpE is derived from *E. coli*. In some embodiments, the gene encoding accA1 is derived from *Streptopmyces coelicolor*. In some embodiments, the gene encoding pccB is derived from *E. coli*. In some embodiments, the gene encoding mmcE is derived from *Propionibcterium freudenreichii*. In some embodiments, the gene encoding mutA is derived from *Propionibcterium freudenreichii*. In some embodiments, the gene encoding mutB is derived from *Propionibcterium freudenreichii*. In some embodiments, the gene encoding prpB is derived from *E. coli*. In some embodiments, the gene encoding prpC is derived from *E. coli*. In some embodiments, the gene encoding prpD is derived from E. coli. In some embodiments, the gene encoding phaB is derived from *Acinetobacter sp* RA3849. In some embodiments, the gene encoding phaC is derived from *Acinetobacter sp* RA3849. In some embodiments, the gene encoding phaA is derived from *Acinetobacter sp* RA3849.

[0244] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme has been codon-optimized for use in the engineered bacterial cell. In one embodiment, the at least one gene or gene cassette encoding the one or more propionate catabolism enzyme(s) has been codon-optimized for use in *Escherichia coli*. When the at least one gene encoding the at least one propionate catabolism enzyme is expressed in the engineered bacterial cells, the bacterial cells catabolize more propionate or propionyl CoA than unmodified bacteria of the same bacterial subtype under the same conditions (*e.g.*, culture or environmental conditions). Thus, the genetically engineered bacteria comprising at least one heterologous gene or gene cassette encoding one or more propionate catabolism enzyme(s) may be used to catabolize excess propionate, propionic acid, and/or propionyl CoA to treat a disease associated with the catabolism of propionate, such as Propionic Acidemia, Methylmalonic Acidemia, or a vitamin B_{12} deficiency.

[0245] The present disclosure further comprises genes and gene cassettes encoding functional fragments of a propionate catabolism enzyme or functional variants of a propionate catabolism enzyme(s). As used herein, the term "functional fragment thereof" or "functional variant thereof" of a propionate catabolism enzyme relates to an element having qualitative biological activity in common with the wild-type propionate catabolism enzyme from which the fragment or variant was derived. For example, a functional fragment or a functional variant of a mutated propionate catabolism enzyme is one which retains essentially the same ability to catabolize propionyl CoA and/or methylmalonyl CoA as the propionate catabolism enzyme from which the functional fragment or functional variant was derived.

For example, a polypeptide having propionate catabolism enzyme activity may be truncated at the N-terminus or C-terminus and the retention of propionate catabolism enzyme activity assessed using assays known to those of skill in the art, including the exemplary assays provided herein. In one embodiment, the engineered bacterial cell comprises a heterologous gene encoding a propionate catabolism enzyme functional variant. In another embodiment, the engineered bacterial cell comprises a heterologous gene or gene cassette encoding a propionate catabolism enzyme functional fragment.

[0246] Assays for testing the activity of a propionate catabolism enzyme, a propionate catabolism enzyme functional variant, or a propionate catabolism enzyme functional fragment are well known to one of ordinary skill in the art. For example, propionate catabolism can be assessed by expressing the protein, functional variant, or fragment thereof, in an engineered bacterial cell that lacks endogenous propionate catabolism enzyme activity. In another example, propionate can be supplemented in the media, and engineered bacterial strains can be compared with corresponding wild type strains with respect to propionate depletion from the media, as described herein. Propionate levels can be assessed using mass spectrometry or gas chromatography. For example, samples can be injected into a Perkin Elmer Autosystem XL Gas Chromatograph containing a Supelco packed column, and the analysis can be performed according to manufacturing instructions (see, for example, Supelco I (1998) Analyzing fatty acids by packed column gas chromatography, Bulletin 856B:2014). Alternatively, propionate levels can be determined using high-pressure liquid chromatography (HPLC). For example, a computer-controlled Waters HPLC system equipped with a model 600 quaternary solvent delivery system, and a model 996 photodiode array detector, and components of a sample can be resolved with an Aminex HPX-87H (300 by 7.8 mm) organic acid analysis column (Bio-Rad Laboratories) (see, for example, Palacios *et al.,* 2003, *J. Bacteriol.,* 185(9):2802-2810).

[0247] In mammals, levels of certain propionate byproducts or metabolites, e.g., propionylcarnitine/acetylcarnitine ratios, 2-methyl-citrate, propionylglycine, and/or tiglyglycine, can be measured in addition to propionate levels by mass spec as described herein.

[0248] As used herein, the term "percent (%) sequence identity" or "percent (%) identity," also including "homology," is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference sequences after aligning the sequences and introducing gaps, if

necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman, 1981, Ads App. Math. 2, 482, by means of the local homology algorithm of Neddleman and Wunsch, 1970, J. Mol. Biol. 48, 443, by means of the similarity search method of Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85, 2444, or by means of computer programs which use these algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

[0249] The present disclosure encompasses genes encoding a propionate catabolism enzyme comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein. Amino acid sequences that are substantially the same as the sequences described herein include sequences comprising conservative amino acid substitutions, as well as amino acid deletions and/or insertions. A conservative amino acid substitution refers to the replacement of a first amino acid by a second amino acid that has chemical and/or physical properties (*e.g.*, charge, structure, polarity, hydrophobicity/hydrophilicity) that are similar to those of the first amino acid. Conservative substitutions include replacement of one amino acid by another within the following groups: lysine (K), arginine (R) and histidine (H); aspartate (D) and glutamate (E); asparagine (N), glutamine (Q), serine (S), threonine (T), tyrosine (Y), K, R, H, D and E; alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), tryptophan (W), methionine (M), cysteine (C) and glycine (G); F, W and Y; C, S and T. Similarly contemplated is replacing a basic amino acid with another basic amino acid (*e.g.*, replacement among Lys, Arg, His), replacing an acidic amino acid with another acidic amino acid (*e.g.*, replacement among Asp and Glu), replacing a neutral amino acid with another neutral amino acid (*e.g.*, replacement among Ala, Gly, Ser, Met, Thr, Leu, Ile, Asn, Gln, Phe, Cys, Pro, Trp, Tyr, Val).

[0250] In some embodiments, the gene(s) or gene cassette(s) encoding propionate catabolism enzyme(s) are mutagenized; mutants exhibiting increased activity are selected; and the mutagenized gene(s) or mutagenized gene cassettes) encoding the propionate catabolism enzyme(s) are isolated and inserted into the bacterial cell. In one embodiment, spontaneous mutants that arise that allow bacteria to grow on propionate as the sole carbon source can be screened for and selected. The gene(s) comprising the modifications described herein may be present on a plasmid or chromosome.

[0251] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpE*. *prpE* encodes PrpE, a propionate-CoA ligase. Accordingly, in one embodiment, the *prpE* gene has at least about 80% identity with SEQ ID NO: 25. In another embodiment, the *prpE* gene has at least about 80% identity with SEQ ID NO: 73. Accordingly, in one embodiment, the *prpE* gene has at least about 90% identity with SEQ ID NO: 25. In another embodiment, the *prpE* gene has at least about 90% identity with SEQ ID NO: 73. Accordingly, in one embodiment, the *prpE* gene has at least about 95% identity with SEQ ID NO: 25. In another embodiment, the *prpE* gene has at least about 95% identity with SEQ ID NO: 73. Accordingly, in one embodiment, the *prpE* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 25. In another embodiment, the *prpE* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 73. In another embodiment, the *prpE* gene comprises the sequence of SEQ ID NO: 25. In another embodiment, the *prpE* gene comprises the sequence of SEQ ID NO: 73. In yet another embodiment the *prpE* gene consists of the sequence of SEQ ID NO: 25. In another embodiment, the *prpE* gene consists of the sequence of SEQ ID NO: 73.

[0252] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpC*. *prpC* encodes PrpC, a 2-methylcitrate synthetase. Accordingly, in one embodiment, the *prpC* gene has at least about 80% identity with SEQ ID NO: 57. In another embodiment, the *prpC* gene has at least about 80% identity with SEQ ID NO:76. Accordingly, in one embodiment, the *prpC* gene has at least about 90% identity with SEQ ID NO: 57. In another embodiment, the *prpC* gene has at least about 90% identity with SEQ ID NO: 76. Accordingly, in one embodiment, the *prpC* gene has at least about 95% identity with SEQ ID NO: 57. In another embodiment, the *prpC* gene has at least about 95% identity with SEQ ID NO: 76. Accordingly, in one embodiment, the *prpC* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 57. In another embodiment, the *prpC* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 76. In another embodiment, the *prpC* gene comprises the sequence of SEQ ID NO: 57. In another embodiment, the *prpC* gene comprises the sequence of SEQ ID NO: 76. In yet another embodiment the *prpC* gene consists of the sequence of SEQ ID NO: 57. In another embodiment, the *prpC* gene consists of the sequence of SEQ ID NO: 76.

[0253] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpD*. *prpD* encodes PrpD, a 2-methylcitrate dehydrogenase. Accordingly, in one embodiment, the *prpD* gene has at least about 80% identity with SEQ ID NO: 58. In another embodiment, the *prpD* gene has at least about 80% identity with SEQ ID NO: 79. Accordingly, in one embodiment, the *prpD* gene has at least about 90% identity with SEQ ID NO: 58. In another embodiment, the *prpD* gene has at least about 90% identity with SEQ ID NO: 79. Accordingly, in one embodiment, the *prpD* gene has at least about 95% identity with SEQ ID NO: 58. In another embodiment, the *prpD* gene has at least about 95% identity with SEQ ID NO: 79. Accordingly, in one embodiment, the *prpD* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 58. In another embodiment, the *prpD* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 79. In another embodiment, the *prpD* gene comprises the sequence of SEQ ID NO: 58. In another embodiment, the *prpD* gene comprises the sequence of SEQ ID NO: 79. In yet another embodiment the *prpD* gene consists of the sequence of SEQ ID NO: 58. In another embodiment, the *prpD* gene consists of the sequence of SEQ ID NO: 79.

[0254] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpB*. *prpB* encodes PrpB, a 2-methylisocitrate lyase. Accordingly, in one embodiment, the *prpB* gene has at least about 80% identity with SEQ ID NO: 56. In another embodiment, the *prpB* gene has at least about 80% identity with SEQ ID NO: 82. Accordingly, in one embodiment, the *prpB* gene has at least about 90% identity with SEQ ID NO: 56. In another embodiment, the *prpB* gene has at least about 90% identity with SEQ ID NO: 82. Accordingly, in one embodiment, the *prpB* gene has at least about 95% identity with SEQ ID NO: 56. In another embodiment, the *prpB* gene has at least about 95% identity with SEQ ID NO: 82. Accordingly, in one embodiment, the *prpB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 56. In another embodiment, the *prpB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 82. In another embodiment, the *prpB* gene comprises the sequence of SEQ ID NO: 56. In another embodiment, the *prpB* gene comprises the sequence of SEQ ID NO: 82. In yet another embodiment the *prpB* gene consists of the sequence of SEQ ID NO: 56. In another embodiment, the *prpB* gene consists of the sequence of SEQ ID NO: 82.

[0255] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *phaB*. *phaB* encodes PhaB, a acetoacetyl-CoA reductase. Accordingly, in one embodiment, the *phaB* gene has at least about 80% identity with SEQ ID NO: 26. In one embodiment, the *phaB* gene has at least about 90% identity with SEQ ID NO: 26. In another embodiment, the *phaB* gene has at least about 95% identity with SEQ ID NO: 26. Accordingly, in one embodiment, the *phaB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 26. In another embodiment, the *phaB* gene comprises SEQ ID NO: 26. In yet another embodiment the *phaB* gene consists of SEQ ID NO: 26.

[0256] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *phaC*. *phaC* encodes PhaC, a polyhydroxyalkanoate synthase. Accordingly, in one embodiment, the *phaC* gene has at least about 80% identity SEQ ID NO: 27. In one embodiment, the *phaC* gene has at least about 90% identity with SEQ ID NO: 27. In another embodiment, the *phaC* gene has at least about 95% identity with SEQ ID NO: 27. Accordingly, in one embodiment, the *phaC* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 27. In another embodiment, the *phaC* gene comprises SEQ ID NO: 27. In yet another embodiment the *phaC* gene consists of SEQ ID NO: 27.

[0257] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *phaA*. *phaA* encodes PhaA, a beta-ketothiolase. Accordingly, in one embodiment, the *phaA* gene has at least about 80% identity with a sequence which encodes SEQ ID NO: 28. In one embodiment, the *phaA* gene has at least about 90% identity with a sequence which encodes SEQ ID NO: 28. In another embodiment, the *phaA* gene has at least about 95% identity with a sequence which encodes SEQ ID NO: 28. Accordingly, in one embodiment, the *phaA* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with a sequence which encodes SEQ ID NO: 28. In another embodiment, the *phaA* gene comprises a sequence which encodes SEQ ID NO: 28. In yet another embodiment the *phaA* gene consists of a sequence which encodes SEQ ID NO: 28.

[0258] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *pccB*. *pccB* encodes PccB, a propionyl CoA carboxylase. Accordingly, in one embodiment, the *pccB* gene has at least about 80% identity with SEQ ID NO: 39. In one embodiment, the *pccB* gene has at least about 90% identity with SEQ ID NO:

39. In one embodiment, the *pccB* gene has at least about 95% identity with SEQ ID NO: 39. In one embodiment, the *pccB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 39. In another embodiment, the *pccB* gene comprises the sequence of SEQ ID NO: 39. In yet another embodiment, the *pccB* gene consists of the sequence of SEQ ID NO: 39.

[0259] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *pccB*. Accordingly, in one embodiment, the *pccB* gene has at least about 80% identity with SEQ ID NO: 96. In one embodiment, the *pccB* gene has at least about 90% identity with SEQ ID NO: 96. In one embodiment, the *pccB* gene has at least about 95% identity with SEQ ID NO: 96. In one embodiment, the *pccB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 96. In another embodiment, the *pccB* gene comprises the sequence of SEQ ID NO: 96. In yet another embodiment, the *pccB* gene consists of the sequence of SEQ ID NO: 96.

[0260] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *accA1*. *accA1* encodes AccA1, an acetyl CoA carboxylase. Accordingly, in one embodiment, the *accA1* gene has at least about 80% identity with SEQ ID NO: 38. In one embodiment, the *accA1* gene has at least about 90% identity with SEQ ID NO: 38. In one embodiment, the *accA1* gene has at least about 95% identity with SEQ ID NO: 38. In one embodiment, the *accA1* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 38. In another embodiment, the *accA1* gene comprises the sequence of SEQ ID NO: 38. In yet another embodiment, the *accA1* gene consists of the sequence of SEQ ID NO: 38.

[0261] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *accA1*. *accA1* encodes AccA1, an acetyl CoA carboxylase. Accordingly, in one embodiment, the *accA1* gene has at least about 80% identity with SEQ ID NO: 104. In one embodiment, the *accA1* gene has at least about 90% identity with SEQ ID NO: 104. In one embodiment, the *accA1* gene has at least about 95% identity with SEQ ID NO: 104. In one embodiment, the *accA1* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 104. In another embodiment, the *accA1* gene comprises the sequence of SEQ ID NO: 104. In yet another embodiment, the *accA1* gene consists of the sequence of SEQ ID NO: 104.

[0262] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *mmcE*. *mmcE* encodes MmcE, a methylmalonyl-CoA mutase. Accordingly, in one embodiment, the *mmcE* gene has at least about 80% identity with SEQ ID NO: 32. In one embodiment, the *mmcE* gene has at least about 90% identity with SEQ ID NO: 32. In one embodiment, the *mmcE* gene has at least about 95% identity with SEQ ID NO: 32. In one embodiment, the *mmcE* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 32. In another embodiment, the *mmcE* gene comprises the sequence of SEQ ID NO: 32. In yet another embodiment, the *mmcE* gene consists of the sequence of SEQ ID NO: 32.

[0263] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *mmcE*. Accordingly, in one embodiment, the *mmcE* gene has at least about 80% identity with SEQ ID NO: 106. In one embodiment, the *mmcE* gene has at least about 90% identity with SEQ ID NO: 106. In one embodiment, the *mmcE* gene has at least about 95% identity with SEQ ID NO: 106. In one embodiment, the *mmcE* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 106. In another embodiment, the *mmcE* gene comprises the sequence of SEQ ID NO: 106. In yet another embodiment, the *mmcE* gene consists of the sequence of SEQ ID NO: 106.

[0264] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *mutA*. *mutA* encodes MutA, a methylmalonyl-CoA mutase small subunit. Accordingly, in one embodiment, the *mutA* gene has at least about 80% identity with SEQ ID NO: 33. In one embodiment, the *mutA* gene has at least about 90% identity with SEQ ID NO: 33. In one embodiment, the *mutA* gene has at least about 95% identity with SEQ ID NO: 33. In one embodiment, the *mutA* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 33. In another embodiment, the *mutA* gene comprises the sequence of SEQ ID NO: 33. In yet another embodiment, the *mutA* gene consists of the sequence of SEQ ID NO: 33.

[0265] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *mutA*. Accordingly, in one embodiment, the *mutA* gene has at least about 80% identity with SEQ ID NO: 110. In one embodiment, the *mutA* gene has at least about 90% identity with SEQ ID NO: 110. In one embodiment, the *mutA* gene has at least about 95% identity with SEQ ID NO: 110. In one embodiment, the *mutA* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or

99% identity with SEQ ID NO: 110. In another embodiment, the *mutA* gene comprises the sequence of SEQ ID NO: 110. In yet another embodiment, the *mutA* gene consists of the sequence of SEQ ID NO: 110.

[0266] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *mutB*. *mutB* encodes MutB, a methylmalonyl-CoA mutase large subunit. Accordingly, in one embodiment, the *mutB* gene has at least about 80% identity with SEQ ID NO: 34. In one embodiment, the *mutB* gene has at least about 90% identity with SEQ ID NO: 34. In one embodiment, the *mutB* gene has at least about 95% identity with SEQ ID NO: 34. In one embodiment, the *mutB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 34. In another embodiment, the *mutB* gene comprises the sequence of SEQ ID NO: 34. In yet another embodiment, the *mutB* gene consists of the sequence of SEQ ID NO: 34.

[0267] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *mutB*. *mutB* encodes MutB, a methylmalonyl-CoA mutase large subunit. Accordingly, in one embodiment, the *mutB* gene has at least about 80% identity with SEQ ID NO: 112. In one embodiment, the *mutB* gene has at least about 90% identity with SEQ ID NO: 112. In one embodiment, the *mutB* gene has at least about 95% identity with SEQ ID NO: 112. In one embodiment, the *mutB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 112. In another embodiment, the *mutB* gene comprises the sequence of SEQ ID NO: 112. In yet another embodiment, the *mutB* gene consists of the sequence of SEQ ID NO: 112.

[0268] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpE*. In one embodiment, the at least one propionate catabolism enzyme is prpE. In one embodiment, prpE has at least about 80% identity with SEQ ID NO: 71. In one embodiment, prpE has at least about 90% identity with SEQ ID NO: 71. In another embodiment, prpE has at least about 95% identity with SEQ ID NO: 71. Accordingly, in one embodiment, the prpE has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 71. In another embodiment, the prpE comprises a sequence which encodes SEQ ID NO: 71. In yet another embodiment, prpE consists of a sequence which encodes SEQ ID NO: 71.

[0269] In one embodiment, the at least one propionate catabolism enzyme is phaA. Accordingly, in one embodiment, phaB has at least about 80% identity with SEQ ID NO:

137. In one embodiment, phaA has at least about 90% identity with SEQ ID NO: 175. In another embodiment, phaA has at least about 95% identity with SEQ ID NO: 137. Accordingly, in one embodiment, phaA has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 137. In another embodiment, phaA comprises a sequence which encodes SEQ ID NO: 137. In yet another embodiment phaA consists of a sequence which encodes SEQ ID NO: 137.

[0270] In one embodiment, the at least one propionate catabolism enzyme is phaB. Accordingly, in one embodiment, phaB has at least about 80% identity with SEQ ID NO: 135. In one embodiment, phaB has at least about 90% identity with SEQ ID NO: 135. In another embodiment, phaB has at least about 95% identity with SEQ ID NO: 135. Accordingly, in one embodiment, phaB has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 135. In another embodiment, phaB comprises a sequence which encodes SEQ ID NO: 135. In yet another embodiment phaB consists of a sequence which encodes SEQ ID NO: 135.

[0271] In one embodiment, the at least one propionate catabolism enzyme is phaC. Accordingly, in one embodiment, phaC has at least about 80% identity with SEQ ID NO: 136. In one embodiment, phaC has at least about 90% identity with SEQ ID NO: 136. In another embodiment, phaC has at least about 95% identity with SEQ ID NO: 136. Accordingly, in one embodiment, phaC has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 136. In another embodiment, phaC comprises a sequence which encodes SEQ ID NO: 136. In yet another embodiment phaC consists of a sequence which encodes SEQ ID NO: 136.

[0272] In one embodiment, the at least one propionate catabolism enzyme is mmcE. Accordingly, in one embodiment, mmcE has at least about 80% identity with SEQ ID NO: 132. In one embodiment, mmcE has at least about 90% identity with SEQ ID NO: 132. In another embodiment, mmcE has at least about 95% identity with SEQ ID NO: 132. Accordingly, in one embodiment, mmcE has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 132. In another embodiment, mmcE comprises a sequence which encodes SEQ ID NO: 132. In yet another embodiment mmcE consists of a sequence which encodes SEQ ID NO: 132.

[0273] In one embodiment, the at least one propionate catabolism enzyme is mutA. Accordingly, in one embodiment, mutA has at least about 80% identity with SEQ ID NO: 133. In one embodiment, mutA has at least about 90% identity with SEQ ID NO: 133. In

another embodiment, mutA has at least about 95% identity with SEQ ID NO: 133. Accordingly, in one embodiment, mutA has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 133. In another embodiment, mutA comprises a sequence which encodes SEQ ID NO: 133. In yet another embodiment mutA consists of a sequence which encodes SEQ ID NO: 133.

[0274] In one embodiment, the at least one propionate catabolism enzyme is mutB. Accordingly, in one embodiment, mutB has at least about 80% identity with SEQ ID NO: 134. In one embodiment, mutB has at least about 90% identity with SEQ ID NO: 134. In another embodiment, mutB has at least about 95% identity with SEQ ID NO: 134. Accordingly, in one embodiment, mutB has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 134. In another embodiment, mutB comprises a sequence which encodes SEQ ID NO: 134. In yet another embodiment mutB consists of a sequence which encodes SEQ ID NO: 134.

[0275] In one embodiment, the at least one propionate catabolism enzyme is accA. Accordingly, in one embodiment, accA has at least about 80% identity with SEQ ID NO: 130. In one embodiment, accA has at least about 90% identity with SEQ ID NO: 130. In another embodiment, accA has at least about 95% identity with SEQ ID NO: 130. Accordingly, in one embodiment, accA has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 130. In another embodiment, accA comprises a sequence which encodes SEQ ID NO: 130. In yet another embodiment the accA consists of a sequence which encodes SEQ ID NO: 130.

[0276] In one embodiment, the at least one propionate catabolism enzyme is pccB. Accordingly, in one embodiment, pccB has at least about 80% identity with SEQ ID NO: 131. In one embodiment, pccB has at least about 90% identity with SEQ ID NO: 131. In another embodiment, pccB has at least about 95% identity with SEQ ID NO: 131. Accordingly, in one embodiment, pccB has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 131. In another embodiment, pccB comprises a sequence which encodes SEQ ID NO: 131. In yet another embodiment, pccB consists of a sequence which encodes SEQ ID NO: 131.

[0277] In one embodiment, the at least one propionate catabolism enzyme is prpC. Accordingly, in one embodiment, prpC has at least about 80% identity with SEQ ID NO: 74. In one embodiment, prpC has at least about 90% identity with SEQ ID NO: 74. In another embodiment, prpC has at least about 95% identity with SEQ ID NO: 74. Accordingly, in one

embodiment, prpC has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 74. In another embodiment, prpC comprises a sequence which encodes SEQ ID NO: 74. In yet another embodiment, prpC consists of a sequence which encodes SEQ ID NO: 74.

[0278] In one embodiment, the at least one propionate catabolism enzyme is prpD. Accordingly, in one embodiment, prpD has at least about 80% identity with SEQ ID NO: 77. In one embodiment, prpD has at least about 90% identity with SEQ ID NO: 77. In another embodiment, prpD has at least about 95% identity with SEQ ID NO: 77. Accordingly, in one embodiment, prpD has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 77. In another embodiment, prpD comprises a sequence which encodes SEQ ID NO: 77. In yet another embodiment, prpD consists of a sequence which encodes SEQ ID NO: 77.

[0279] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is MatB. MatB encodes Malonyl-coenzyme A (malonyl-CoA) synthetase (MatB). Accordingly, in one embodiment, the MatB gene has at least about 80% identity with SEQ ID NO: 141. Accordingly, in one embodiment, the MatB gene has at least about 90% identity with SEQ ID NO: 141. Accordingly, in one embodiment, the MatB gene has at least about 95% identity with SEQ ID NO: 141. Accordingly, in one embodiment, the MatB gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 141. In another embodiment, the MatB gene comprises the sequence of SEQ ID NO: 141. In yet another embodiment the MatB gene consists of the sequence of SEQ ID NO: 141.

[0280] In one embodiment, the at least one propionate catabolism enzyme is matB. Accordingly, in one embodiment, matB has at least about 89% identity with SEQ ID NO: 140. In one embodiment, matB has at least about 90% identity with SEQ ID NO: 140. In another embodiment, matB has at least about 95% identity with SEQ ID NO: 140. Accordingly, in one embodiment, matB has at least about 85%, 86%, 89%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 140. In another embodiment, matB comprises a sequence which encodes SEQ ID NO: 140. In yet another embodiment, matB consists of a sequence which encodes SEQ ID NO: 140.

[0281] In one embodiment, the at least one propionate catabolism enzyme is prpB. Accordingly, in one embodiment, prpB has at least about 80% identity with SEQ ID NO: 80. In one embodiment, prpB has at least about 90% identity with SEQ ID NO: 80. In another

embodiment, prpB has at least about 95% identity with SEQ ID NO: 80. Accordingly, in one embodiment, prpB has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 80. In another embodiment, prpB comprises a sequence which encodes SEQ ID NO: 80. In yet another embodiment, prpB consists of a sequence which encodes SEQ ID NO: 80.

[0282] In one embodiment, any combination of propionate catabolism enzymes that effectively reduce the level of propionate and/or a metabolite thereof can be used. In one embodiment, any combination of propionate catabolism enzymes that effectively reduce levels of propionate, propionyl CoA, and/or methylmalonyl CoA in a subject can be used. In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpBCD*. In another embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpBCDE*. Using all four heterologous genes, for example, *prpBCDE*, is not necessary but allows excess propionate to be converted into succinate and pyruvate, feeding the Krebs cycle and benefiting the bacteria by increasing their growth. In another embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpE, pccB, accA1, mmcE, mutA,* and *mutB*. In another embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpE, pccB,* and *accA1* under the control of a first inducible promoter, and *mmcE, mutA,* and *mutB* under the control of a second inducible promoter. In another embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is *prpE, phaB, phaC,* and *phaA*.

[0283] In one embodiment, the propionate catabolism gene cassette comprises *prpBCD*. Accordingly, in one embodiment, the *prpBCD* operon has at least about 80% identity with SEQ ID NO: 138. In another embodiment, the *prpBCD* operon has at least about 80% identity with SEQ ID NO: 83 OR SEQ ID NO: 84. Accordingly, in one embodiment, the *prpBCD* operon has at least about 90% identity with SEQ ID NO: 138. In another embodiment, the *prpBCD* operon has at least about 90% identity with SEQ ID NO: 83 OR SEQ ID NO: 84. Accordingly, in one embodiment, the *prpBCD* operon has at least about 95% identity with SEQ ID NO: 138. In another embodiment, the *prpBCD* operon has at least about 95% identity with SEQ ID NO: 83 OR SEQ ID NO: 84. Accordingly, in one embodiment, the *prpBCD* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 138. In another embodiment, the *prpBCD* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 83 OR SEQ ID

NO: 84. In another embodiment, the *prpBCD* operon comprises the sequence of SEQ ID NO: 138. In another embodiment, the *prpBCD* operon comprises the sequence of SEQ ID NO: 83 OR SEQ ID NO: 84. In yet another embodiment the *prpBCD* operon consists of the sequence of SEQ ID NO: 138. In another embodiment, the *prpBCD* operon consists of the sequence of SEQ ID NO: 83 OR SEQ ID NO: 84.

[0284] In one embodiment, the propionate catabolism gene cassette comprises *prpBCDE*. Accordingly, in one embodiment, the *prpBCDE* operon has at least about 80% identity with SEQ ID NO: 55. In another embodiment, the *prpBCDE* operon has at least about 80% identity with SEQ ID NO: 93 or SEQ ID NO: 94. Accordingly, in one embodiment, the *prpBCDE* operon has at least about 90% identity with SEQ ID NO: 55. In another embodiment, the *prpBCDE* operon has at least about 90% identity with SEQ ID NO: 93 or SEQ ID NO: 94. Accordingly, in one embodiment, the *prpBCDE* operon has at least about 95% identity with SEQ ID NO: 55. In another embodiment, the *prpBCDE* operon has at least about 95% identity with SEQ ID NO: 93 or SEQ ID NO: 94. Accordingly, in one embodiment, the *prpBCDE* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 55. In another embodiment, the *prpBCDE* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 93 or SEQ ID NO: 94. In another embodiment, the *prpBCDE* operon comprises the sequence of SEQ ID NO: 55. In another embodiment, the *prpBCDE* operon comprises the sequence of SEQ ID NO: 93 or SEQ ID NO: 94. In yet another embodiment the *prpBCDE* operon consists of the sequence of SEQ ID NO: 55. In another embodiment, the *prpBCDE* operon consists of the sequence of SEQ ID NO: 93 or SEQ ID NO: 94.

[0285] In one embodiment, the propionate catabolism gene cassette comprises *phaBCA*. Accordingly, in one embodiment, the *phaBCA* operon has at least about 80% identity with SEQ ID NO: 139. In one embodiment, the *phaBCA* operon has at least about 90% identity with SEQ ID NO: 139. In one embodiment, the *phaBCA* operon has at least about 95% identity with SEQ ID NO: 139. In one embodiment, the *phaBCA* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 139. In another embodiment, the *phaBCA* operon comprises the sequence of SEQ ID NO: 139. In another embodiment, the *phaBCA* operon consists of the sequence of SEQ ID NO: 139. In one embodiment, the propionate catabolism gene cassette comprises *prpE* and *phaBCA*.

[0286] In one embodiment, the propionate catabolism gene cassette comprises *phaBCA*. Accordingly, in one embodiment, the *phaBCA* operon has at least about 80% identity with SEQ ID NO: 102. In one embodiment, the *phaBCA* operon has at least about 90% identity with SEQ ID NO: 102. In one embodiment, the *phaBCA* operon has at least about 95% identity with SEQ ID NO: 102. In one embodiment, the *phaBCA* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 102. In another embodiment, the *phaBCA* operon comprises the sequence of SEQ ID NO: 102. In another embodiment, the *phaBCA* operon consists of the sequence of SEQ ID NO: 102. In one embodiment, the propionate catabolism gene cassette comprises *prpE* and *phaBCA*.

[0287] In one embodiment, the propionate catabolism gene cassette comprises *prpE-phaBCA*. Accordingly, in one embodiment, the *prpE-phaBCA* operon has at least about 80% identity with SEQ ID NO: 24. In one embodiment, the *prpE-phaBCA* operon has at least about 90% identity with SEQ ID NO: 24. In one embodiment, the *prpE-phaBCA* operon has at least about 95% identity with SEQ ID NO: 24. In one embodiment, the *prpE-phaBCA* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 24. In another embodiment, the *prpE-phaBCA* operon comprises the sequence of SEQ ID NO: 24. In another embodiment, the *prpE-phaBCA* operon consists of the sequence of SEQ ID NO: 24.

[0288] In one embodiment, the propionate catabolism gene cassette comprises *prpE, pccB, accA1, mmcE, mutA,* and *mutB*. Accordingly, in one embodiment, the *prpE-pccB-accA1-mmcE-mutA-mutB* operon has at least about 80% identity with a combination of SEQ ID NO: 37 and 31. In one embodiment, the *prpE-pccB-accA1-mmcE-mutA-mutB* operon has at least about 90% identity with a combination of SEQ ID NO: 37 and 31. In one embodiment, the *prpE-pccB-accA1-mmcE-mutA-mutB* operon has at least about 95% identity with a combination of SEQ ID NO: 37 and 31. In one embodiment, the *prpE-pccB-accA1-mmcE-mutA-mutB* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with a combination of SEQ ID NO: 37 and 31. In another embodiment, the *prpE-pccB-accA1-mmcE-mutA-mutB* operon comprises the sequence of a combination of SEQ ID NO: 37 and 31. In another embodiment, the *prpE-pccB-accA1-mmcE-mutA-mutB* operon consists of the sequence of a combination of SEQ ID NO: 37 and 31.

[0289] In one embodiment, the propionate catabolism gene cassette comprises *prpE, pccB,* and *accA1*. Accordingly, in one embodiment, the *prpE-pccB-accA1* operon has at least about 80% identity with SEQ ID NO: 37. In one embodiment, the *prpE-pccB-accA1* operon has at least about 90% identity with SEQ ID NO: 37. In one embodiment, the *prpE-pccB-accA1* operon has at least about 95% identity with SEQ ID NO: 37. In one embodiment, the *prpE-pccB-accA1* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 37. In another embodiment, the *prpE-pccB-accA1* operon comprises the sequence of SEQ ID NO: 37. In another embodiment, the *prpE-pccB-accA1* operon consists of the sequence of SEQ ID NO: 37.

[0290] In one embodiment, the propionate catabolism gene cassette comprises *mmcE, mutA,* and *mutB*. Accordingly, in one embodiment, the *mmcE-mutA-mutB* operon has at least about 80% identity with a combination of SEQ ID NO:31. In one embodiment, the *mmcE-mutA-mutB* operon has at least about 90% identity with a combination of SEQ ID NO: 31. In one embodiment, the *-mmcE-mutA-mutB* operon has at least about 95% identity with a combination of SEQ ID NO: 31. In one embodiment, the *mmcE-mutA-mutB* operon has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with a combination of SEQ ID NO: 31. In another embodiment, the *mmcE-mutA-mutB* operon comprises the sequence of a combination of SEQ ID NO: 31. In another embodiment, the *mmcE-mutA-mutB* operon consists of the sequence of a combination of SEQ ID NO: 31.

[0291] In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is directly operably linked to a first promoter. In another embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is indirectly operably linked to a first promoter. In one embodiment, the promoter is not operably linked with the at least one gene encoding the propionate catabolism enzyme in nature.

[0292] In some embodiments, the at least one gene encoding the at least one propionate catabolism enzyme is expressed under the control of a constitutive promoter. In another embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is expressed under the control of an inducible promoter. In some embodiments, the at least one gene encoding the at least one propionate catabolism enzyme is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the at least one gene encoding the at least one propionate

catabolism enzyme is expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the at least one gene encoding the at least one propionate catabolism enzyme is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. Inducible promoters are described in more detail *infra*.

[0293] The at least one gene encoding the at least one propionate catabolism enzyme may be present on a plasmid or chromosome in the bacterial cell. In one embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is located on a plasmid in the bacterial cell. In another embodiment, the at least one gene encoding the at least one propionate catabolism enzyme is located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding the at least one propionate catabolism enzyme is located in the chromosome of the bacterial cell, and at least one gene encoding at least one propionate catabolism enzyme from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding the at least one propionate catabolism enzyme is located on a plasmid in the bacterial cell, and at least one gene encoding the at least one propionate catabolism enzyme from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding the at least one propionate catabolism enzyme is located in the chromosome of the bacterial cell, and at least one gene encoding the at least one propionate catabolism enzyme from a different species of bacteria is located in the chromosome of the bacterial cell.

[0294] In some embodiments, the at least one gene encoding the at least one propionate catabolism enzyme is expressed on a low-copy plasmid. In some embodiments, the at least one gene encoding the at least one propionate catabolism enzyme is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of the at least one propionate catabolism enzyme, thereby increasing the catabolism of propionate, propionic acid, propionyl CoA, methylmalonic acid, and/or methylmalonyl CoA.

[0295] In some embodiments, a engineered bacterial cell comprising at least one gene encoding at least one propionate catabolism enzyme expressed on a high-copy plasmid does not increase propionate catabolism or decrease propionate, propionyl CoA, and/or methylmalonyl CoA levels as compared to a engineered bacterial cell comprising the same gene expressed on a low-copy plasmid in the absence of a heterologous importer of

propionate and additional copies of a native importer of propionate. It has been surprisingly discovered that in some embodiments, the rate-limiting step of propionate catabolism is *not* expression of a propionate catabolism enzyme, but rather availability of propionate or propionyl CoA. Thus, in some embodiments, it may be advantageous to increase propionate transport into the cell, thereby enhancing propionate catabolism. Furthermore, in some embodiments that incorporate a transporter of propionate into the engineered bacterial cell, there may be additional advantages to using a low-copy plasmid comprising the at least one gene encoding the at least one propionate catabolism enzyme in conjunction in order to enhance the stability of expression of the propionate catabolism enzyme, while maintaining high propionate catabolism and to reduce negative selection pressure on the transformed bacterium. In alternate embodiments, the importer of propionate is used in conjunction with a high-copy plasmid.

[0296] Deacylation of propionylated PrpE ($PrpE^{Pr}$) by CobB, a NAD-dependent deacylase, allows bacterial cells to catabolize propionate. Thus, in one embodiment, when the engineered bacterial cell expresses a heterologous PrpE enzyme, the engineered bacterial cell may further comprise a heterologous *cobB* gene (SEQ ID NO:114). In one embodiment, the *cobB* gene has at least about 80% identity with SEQ ID NO: 114. Accordingly, in one embodiment, the *cobB* gene has at least about 90% identity with SEQ ID NO: 114. Accordingly, in one embodiment, the *cobB* gene has at least about 95% identity with SEQ ID NO: 114. Accordingly, in one embodiment, the *cobB* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 114. In another embodiment, the *cobB* gene comprises the sequence of SEQ ID NO: 114. In yet another embodiment the *cobB* gene consists of the sequence of SEQ ID NO: 114.

[0297] In one embodiment, the at least one propionate catabolism enzyme is CobB. Accordingly, in one embodiment, CobB has at least about 113% identity with SEQ ID NO: 113. In one embodiment, CobB has at least about 90% identity with SEQ ID NO: 113. In another embodiment, CobB has at least about 95% identity with SEQ ID NO: 113. Accordingly, in one embodiment, CobB has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 113. In another embodiment, CobB comprises a sequence which encodes SEQ ID NO: 113. In yet another embodiment, CobB consists of a sequence which encodes SEQ ID NO: 113.

[0298] In another embodiment, the engineered bacterial cell comprising a heterologous *cobB* gene further comprises a genetic modification in the *pka* gene. Pka, a protein lysine acetyltransferase, renders PrpE in the propionylated form ($PrpE^{Pr}$) unable to metabolize propionate. Therefore, genetic modification of the *pka* gene (SEQ ID NO: 116) which renders it functionally inactive enhances the ability of the bacterial cells to catabolize propionate.

Transporter (Importer) of Propionate

[0299] The uptake of propionate into bacterial cells typically occurs via passive diffusion (see, for example, Kell *et al.,* 1981, *Biochem. Biophys. Res. Commun.,* 9981-9988). However, the active import of propionate is also mediated by proteins well known to those of skill in the art. For example, a bacterial transport system for the update of propionate in *Corynebacterium glutamicum* named MctC (monocarboxylic acid transporter) is known (see, for example, Jolkver *et al.,* 2009, *J. Bacteriol.,* 191(3):940-948). The putP_6 propionate transporter from *Virgibacillus* species (UniProt A0A024QGU1) has also been identified.

[0300] Propionate transporters, *e.g.,* propionate importers, may be expressed or modified in the bacteria in order to enhance propionate transport into the cell. Specifically, when the transporter (importer) of propionate is expressed in the engineered bacterial cells, the bacterial cells import more propionate into the cell when the transporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the genetically engineered bacteria comprising a heterologous gene encoding a transporter of propionate may be used to import propionate into the bacteria so that any gene encoding a propionate catabolism enzyme expressed in the organism can be used to treat diseases associated with the catabolism of propionate, such as organic acidurias (including PA and MMA) and vitamin B_{12} deficiencies. In one embodiment, the bacterial cell comprises a heterologous gene encoding transporter of propionate. In one embodiment, the bacterial cell comprises a heterologous gene encoding a transporter of propionate and at least one heterologous gene encoding at least one propionate catabolism enzyme.

[0301] Thus, in some embodiments, the disclosure provides a bacterial cell that comprises at least one heterologous gene encoding a propionate catabolism enzyme operably linked to a first promoter and at least one heterologous gene encoding a propionate transporter. In some embodiments, the disclosure provides a bacterial cell that comprises at least one heterologous gene encoding a transporter of propionate operably linked to the first

promoter. In another embodiment, the disclosure provides a bacterial cell that comprises at least one heterologous gene encoding at least one propionate catabolism enzyme operably linked to a first promoter and at least one heterologous gene encoding of propionate operably linked to a second promoter. In one embodiment, the first promoter and the second promoter are separate copies of the same promoter. In another embodiment, the first promoter and the second promoter are different promoters.

[0302] In one embodiment, the bacterial cell comprises at least one gene encoding a transporter of propionate from a different organism, *e.g.,* a different species of bacteria. In one embodiment, the bacterial cell comprises at least one native gene encoding a transporter of propionate. In some embodiments, the at least one native gene encoding a transporter of propionate is not modified. In another embodiment, the bacterial cell comprises more than one copy of at least one native gene encoding a transporter of propionate. In yet another embodiment, the bacterial cell comprises a copy of at least one gene encoding a native importer of propionate, as well as at least one copy of at least one heterologous gene encoding a transporter of propionate from a different bacterial species. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of the at least one heterologous gene encoding a transporter of propionate. In one embodiment, the bacterial cell comprises multiple copies of the at least one heterologous gene encoding a transporter of propionate.

[0303] In some embodiments, the importer of propionate is encoded by a transporter of propionate gene derived from a bacterial genus or species, including but not limited to, *Bacillus, Campylobacter, Clostridium, Corynebacterium, Escherichia, Lactobacillus, Pseudomonas, Salmonella, Staphylococcus, Bacillus subtilis, Campylobacter jejuni, Clostridium perfringens, Escherichia coli, Lactobacillus delbrueckii, Pseudomonas aeruginosa, Salmonella typhimurium, Virgibacillus,* or *Staphylococcus aureus*. In some embodiments, the bacteria are a *Virgibacillus*. In some embodiments, the bacterial is a *Corynebacterium*. In one embodiment, the bacteria are *C. glutamicum*. In another embodiment, the bacteria are *C. diphtheria*. In another embodiment, the bacteria are *C. efficiens*. In another embodiment, the bacteria are *S. coelicolor*. In another embodiment, the bacteria are *M. smegmatis*. In another embodiment, the bacteria are *N. farcinica*. In another embodiment, the bacteria are *E. coli*. In another embodiment, the bacteria are *B. subtilis*.

[0304] The present disclosure further comprises genes encoding functional fragments of a transporter of propionate or functional variants of a transporter of propionate. As used

herein, the term "functional fragment thereof" or "functional variant thereof" of a transporter of propionate relates to an element having qualitative biological activity in common with the wild-type importer of propionate from which the fragment or variant was derived. For example, a functional fragment or a functional variant of a mutated importer of propionate protein is one which retains essentially the same ability to import propionate into the bacterial cell as does the importer protein from which the functional fragment or functional variant was derived. In one embodiment, the engineered bacterial cell comprises at least one heterologous gene encoding a functional fragment of a transporter of propionate. In another embodiment, the engineered bacterial cell comprises at least one heterologous gene encoding a functional variant of a transporter of propionate.

[0305] Assays for testing the activity of a transporter of propionate, a transporter of propionate functional variant, or a transporter of propionate functional fragment are well known to one of ordinary skill in the art. For example, propionate import can be assessed by expressing the protein, functional variant, or fragment thereof, in an engineered bacterial cell that lacks an endogenous propionate importer. Propionate import can also be assessed using mass spectrometry. Propionate import can also be expressed using gas chromatography. For example, samples can be injected into a Perkin Elmer Autosystem XL Gas Chromatograph containing a Supelco packed column, and the analysis can be performed according to manufacturing instructions (see, for example, Supelco I (1998) Analyzing fatty acids by packed column gas chromatography, Bulletin 856B:2014). Alternatively, samples can be analyzed for propionate import using high-pressure liquid chromatography (HPLC). For example, a computer-controlled Waters HPLC system equipped with a model 600 quaternary solvent delivery system, and a model 996 photodiode array detector, and components of the sample can be resolved with an Aminex HPX-87H (300 by 7.8 mm) organic acid analysis column (Bio-Rad Laboratories) (see, for example, Palacios *et al.,* 2003, *J. Bacteriol.,* 185(9):2802-2810).

[0306] In one embodiment, the genes encoding the importer of propionate have been codon-optimized for use in the host organism. In one embodiment, the genes encoding the importer of propionate have been codon-optimized for use in *Escherichia coli.*

[0307] The present disclosure also encompasses genes encoding a transporter of propionate comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein. Amino acid sequences that are substantially the same as the

sequences described herein include sequences comprising conservative amino acid substitutions, as well as amino acid deletions and/or insertions.

[0308] In some embodiments, the at least one gene encoding a transporter of propionate is mutagenized; mutants exhibiting increased propionate transport are selected; and the mutagenized at least one gene encoding a transporter of propionate is isolated and inserted into the bacterial cell. In some embodiments, the at least one gene encoding a transporter of propionate is mutagenized; mutants exhibiting decreased propionate transport are selected; and the mutagenized at least one gene encoding a transporter of propionate is isolated and inserted into the bacterial cell. The importer modifications described herein may be present on a plasmid or chromosome.

[0309] In one embodiment, the propionate importer is MctC. In one embodiment, the *mctC* gene has at least about 80% identity to SEQ ID NO: 88. Accordingly, in one embodiment, the *mctC* gene has at least about 90% identity to SEQ ID NO: 88. Accordingly, in one embodiment, the *mctC* gene has at least about 95% identity to SEQ ID NO: 88. Accordingly, in one embodiment, the *mctC* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 88. In another embodiment, the *mctC* gene comprises the sequence of SEQ ID NO: 88. In yet another embodiment the *mctC* gene consists of the sequence of SEQ ID NO: 88.

[0310] In one embodiment, the at least one propionate catabolism enzyme is MctC. Accordingly, in one embodiment, MctC has at least about 87% identity with SEQ ID NO: 87. In one embodiment, MctC has at least about 90% identity with SEQ ID NO: 87. In another embodiment, MctC has at least about 95% identity with SEQ ID NO: 87. Accordingly, in one embodiment, MctC has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 87. In another embodiment, MctC comprises a sequence which encodes SEQ ID NO: 87. In yet another embodiment, MctC consists of a sequence which encodes SEQ ID NO: 87.

[0311] In another embodiment, the propionate importer is PutP_6. In one embodiment, the *putP_6* gene has at least about 80% identity to SEQ ID NO: 90. Accordingly, in one embodiment, the *putP_6* gene has at least about 90% identity to SEQ ID NO: 90. Accordingly, in one embodiment, the *putP_6* gene has at least about 95% identity to SEQ ID NO: 90. Accordingly, in one embodiment, the *putP_6* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 90. In another embodiment, the *putP_6* gene comprises the sequence of SEQ

ID NO: 90. In yet another embodiment the *putP_6* gene consists of the sequence of SEQ ID NO: 90.

[0312] In one embodiment, the at least one propionate catabolism enzyme is PutP_6. Accordingly, in one embodiment, PutP_6 has at least about 89% identity with SEQ ID NO: 89. In one embodiment, PutP_6 has at least about 90% identity with SEQ ID NO: 89. In another embodiment, PutP_6 has at least about 95% identity with SEQ ID NO: 89. Accordingly, in one embodiment, PutP_6 has at least about 85%, 86%, 89%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 89. In another embodiment, PutP_6 comprises a sequence which encodes SEQ ID NO: 89. In yet another embodiment, PutP_6 consists of a sequence which encodes SEQ ID NO: 89.

[0313] Other propionate importer genes are known to those of ordinary skill in the art. See, for example, Jolker *et al., J. Bacteriol.,* 2009, 191(3):940-948. In one embodiment, the propionate importer comprises the *mctBC* genes from *C. glutamicum*. In another embodiment, the propionate importer comprises the *dip0780 and dip0791* genes from *C. diphtheria*. In another embodiment, the propionate importer comprises the *ce0909* and *ce0910* genes from *C. efficiens*. In another embodiment, the propionate importer comprises the *ce1091* and *ce1092* genes from *C. efficiens*. In another embodiment, the propionate importer comprises the *sco1822* and *sco1823* genes from *S. coelicolor*. In another embodiment, the propionate importer comprises the *sco1218* and *sco1219* genes from *S. coelicolor*. In another embodiment, the propionate importer comprises the *ce1091* and *sco5827* genes from *S. coelicolor*. In another embodiment, the propionate importer comprises the *m_5160, m_5161, m_5165,* and *m_5166* genes from *M. smegmatis*. In another embodiment, the propionate importer comprises the *nfa 17930, nfa 17940, nfa 17950,* and *nfa 17960* genes from *N. farcinica*. In another embodiment, the propionate importer comprises the *actP* and *yjcH* genes from *E. coli*. In another embodiment, the propionate importer comprises the *ywcB* and *ywcA* genes from *B. subtilis*.

[0314] In some embodiments, the bacterial cell comprises at least one heterologous gene encoding at least one propionate catabolism enzyme operably linked to a first promoter and at least one heterologous gene encoding a transporter of propionate. In some embodiments, the at least one heterologous gene encoding a transporter of propionate is operably linked to the first promoter. In other embodiments, the at least one heterologous gene encoding a transporter of propionate is operably linked to a second promoter. In one embodiment, the at least one gene encoding a transporter of propionate is directly operably

linked to the second promoter. In another embodiment, the at least one gene encoding a transporter of propionate is indirectly operably linked to the second promoter.

[0315] In some embodiments, expression of at least one gene encoding a transporter of propionate is controlled by a different promoter than the promoter that controls expression of the at least one gene encoding the at least one propionate catabolism enzyme. In some embodiments, expression of the at least one gene encoding a transporter of propionate is controlled by the same promoter that controls expression of the at least one propionate catabolism enzyme. In some embodiments, at least one gene encoding a transporter of propionate and the propionate catabolism enzyme are divergently transcribed from a promoter region. In some embodiments, expression of each of genes encoding the at least one gene encoding a transporter of propionate and the at least one gene encoding the at least one propionate catabolism enzyme is controlled by different promoters.

[0316] In one embodiment, the promoter is not operably linked with the at least one gene encoding a transporter of propionate in nature. In some embodiments, the at least one gene encoding the importer of propionate is controlled by its native promoter. In some embodiments, the at least one gene encoding the importer of propionate is controlled by an inducible promoter. In some embodiments, the at least one gene encoding the importer of propionate is controlled by a promoter that is stronger than its native promoter. In some embodiments, the at least one gene encoding the importer of propionate is controlled by a constitutive promoter.

[0317] In another embodiment, the promoter is an inducible promoter. Inducible promoters are described in more detail *infra*.

[0318] In one embodiment, the at least one gene encoding a transporter of propionate is located on a plasmid in the bacterial cell. In another embodiment, the at least one gene encoding a transporter of propionate is located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding a transporter of propionate is located in the chromosome of the bacterial cell, and a copy of at least one gene encoding a transporter of propionate from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding a transporter of a propionate is located on a plasmid in the bacterial cell, and a copy of at least one gene encoding a transporter of propionate from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding a transporter of propionate is located in the

chromosome of the bacterial cell, and a copy of the at least one gene encoding a transporter of propionate from a different species of bacteria is located in the chromosome of the bacterial cell.

[0319] In some embodiments, the at least one native gene encoding the importer in the bacterial cell is not modified, and one or more additional copies of the native importer are inserted into the genome. In one embodiment, the one or more additional copies of the native importer that is inserted into the genome are under the control of the same inducible promoter that controls expression of the at least one gene encoding the propionate catabolism enzyme, *e.g.*, the FNR responsive promoter, or a different inducible promoter than the one that controls expression of the at least one propionate catabolism enzyme, or a constitutive promoter. In alternate embodiments, the at least one native gene encoding the importer is not modified, and one or more additional copies of the importer from a different bacterial species is inserted into the genome of the bacterial cell. In one embodiment, the one or more additional copies of the importer inserted into the genome of the bacterial cell are under the control of the same inducible promoter that controls expression of the at least one gene encoding the propionate catabolism enzyme, *e.g.*, the FNR responsive promoter, or a different inducible promoter than the one that controls expression of the at least one gene encoding the at least one propionate catabolism enzyme, or a constitutive promoter.

[0320] In one embodiment, when the importer of propionate is expressed in the engineered bacterial cells, the bacterial cells import 10% more propionate into the bacterial cell when the importer is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In another embodiment, when the importer of propionate is expressed in the engineered bacterial cells, the bacterial cells import 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% more propionate into the bacterial cell when the importer is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the importer of propionate is expressed in the engineered bacterial cells, the bacterial cells import two-fold more propionate into the cell when the importer is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the importer of propionate is expressed in the engineered bacterial cells, the bacterial cells import three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold more propionate into the cell when the importer is expressed than unmodified bacteria of the same bacterial subtype under the same conditions.

Exporters of Succinate

[0321] Succinate export in bacteria is normally active under anaerobic conditions. The export of succinate is mediated by proteins well known to those of skill in the art. For example, a succinate exporter in *Corynebacterium glutamicum* is known as SucE1. SucE1 is a membrane protein belonging to the aspartate: alanine exchanger (AAE) family (see, for example, Fukui *et al.*, 2011, *J. Bacteriol.*, 154(1):25-34). The DcuC succinate exporter from *E. coli* has also been identified (see, for example, Cheng *et al.*, 2013, *J. Biomed. Res. Int.*, 2013:ID 538790).

[0322] Succinate transporters, *e.g.*, succinate exporters, may be expressed or modified in the bacteria in order to enhance succinate export out of the cell. Specifically, when the exporter of succinate is expressed in the engineered bacterial cells, the bacterial cells export more succinate outside of the cell when the exporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In one embodiment, the bacterial cell comprises a heterologous gene encoding an exporter of succinate. In one embodiment, the bacterial cell comprises a heterologous gene encoding an exporter of succinate and at least one heterologous gene or gene cassette encoding at least one propionate catabolism enzyme.

[0323] Thus, in some embodiments, the disclosure provides a bacterial cell that comprises at least one heterologous gene or gene cassette encoding a propionate catabolism enzyme or enzymes operably linked to a first promoter and at least one heterologous gene encoding an exporter of succinate. In some embodiments, the at least one heterologous gene encoding an exporter of succinate is operably linked to the first promoter. In another embodiment, the at least one heterologous gene encoding the at least one propionate catabolism enzyme operably is linked to a first promoter, and the heterologous gene encoding an exporter of succinate is operably linked to a second promoter. In one embodiment, the first promoter and the second promoter are separate copies of the same promoter. In another embodiment, the first promoter and the second promoter are different promoters.

[0324] In one embodiment, the bacterial cell comprises at least one gene encoding an exporter of succinate from a different organism, *e.g.*, a different species of bacteria. In one embodiment, the bacterial cell comprises at least one native gene encoding an exporter of succinate. In some embodiments, the at least one native gene encoding an exporter of succinate is not modified. In another embodiment, the bacterial cell comprises more than one copy of at least one native gene encoding an exporter of succinate. In yet another embodiment, the bacterial cell comprises a copy of at least one gene encoding a native

exporter of succinate, as well as at least one copy of at least one heterologous gene encoding an exporter of succinate from a different bacterial species. In one embodiment, the bacterial cell comprises at least one, two, three, four, five, or six copies of the at least one heterologous genes encoding an exporter of succinate. In one embodiment, the bacterial cell comprises multiple copies of the at least one heterologous gene encoding an exporter of succinate.

[0325] In some embodiments, the exporter of succinate is encoded by an exporter of succinate gene derived from a bacterial genus or species, including but not limited to, *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, and Mannheimia succiniciproducens, Escherichia coli, Corynebacterium glutamicum, Salmonella typhimurium, Klebsiella pneumoniae, Serratia plymuthica, Enterobacter cloacae, Bacillus subtilis, Bacillus anthracis, bacillus lichenformis, and Saccharomyces cerevisiae.* In some embodiments, the exporter of succinate is derived from *Corynebacterium.* In one embodiment, the exporter of succinate is derived from *C. glutamicum.* In another embodiment, the exporter of succinate is from *Vibrio cholerae.* In another embodiment, the exporter of succinate is from *E. coli.* In another embodiment, the exporter of succinate is from *Bacillus subtilis.*

[0326] The present disclosure further comprises genes encoding functional fragments of an exporter of succinate or functional variants of an exporter of succinate. As used herein, the term "functional fragment thereof" or "functional variant thereof" of an exporter of succinate relates to an element having qualitative biological activity in common with the wild-type exporter of succinate from which the fragment or variant was derived. For example, a functional fragment or a functional variant of a mutated exporter of succinate protein is one which retains essentially the same ability to import succinate into the bacterial cell as does the exporter protein from which the functional fragment or functional variant was derived. In one embodiment, the engineered bacterial cell comprises at least one heterologous gene encoding a functional fragment of an exporter of succinate. In another embodiment, the engineered bacterial cell comprises at least one heterologous gene encoding a functional variant of an exporter of succinate.

[0327] In some embodiments, the genetically engineered bacteria further comprise a mutation or deletion in one or more succinate importers, e.g., Dct, DctC, ybhI or ydjN. In some embodiments, succinate dehydrogenase (SUCDH) may be mutated or deleted. Without wishing to be bound by theory, such mutations may decrease intracellular succinate concentrations and increase the flux through propionate catabolism pathways.

[0328] Assays for testing the activity of an exporter of succinate, an exporter of succinate functional variant, or an exporter of succinate functional fragment are well known to one of ordinary skill in the art. For example, succinate export can be assessed by expressing the protein, functional variant, or fragment thereof, in an engineered bacterial cell that lacks an endogenous succinate exporter and assessing succinate levels in the media after expression of the protein. Methods for measuring succinate export are well known to one of ordinary skill in the art. For example, see Fukui *et al., J. Biotechnol.,* 154(1):25-34, 2011.

[0329] In one embodiment, the genes encoding the exporter of succinate have been codon-optimized for use in the host organism. In one embodiment, the genes encoding the exporter of succinate have been codon-optimized for use in *Escherichia coli.*

[0330] The present disclosure also encompasses genes encoding an exporter of succinate comprising amino acids in its sequence that are substantially the same as an amino acid sequence described herein. Amino acid sequences that are substantially the same as the sequences described herein include sequences comprising conservative amino acid substitutions, as well as amino acid deletions and/or insertions.

[0331] In some embodiments, the at least one gene encoding an exporter of succinate is mutagenized; mutants exhibiting increased succinate transport are selected; and the mutagenized at least one gene encoding an exporter of succinate is isolated and inserted into the bacterial cell. In some embodiments, the at least one gene encoding an exporter of succinate is mutagenized; mutants exhibiting decreased succinate transport are selected; and the mutagenized at least one gene encoding an exporter of succinate is isolated and inserted into the bacterial cell. The exporter modifications described herein may be present on a plasmid or chromosome.

[0332] In one embodiment, the succinate exporter is DcuC. In one embodiment, the *dcuC* gene has at least about 80% identity to SEQ ID NO: 49. Accordingly, in one embodiment, the *dcuC* gene has at least about 90% identity to SEQ ID NO: 49. Accordingly, in one embodiment, the *dcuC* gene has at least about 95% identity to SEQ ID NO: 49. Accordingly, in one embodiment, the *dcuC* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 49. In another embodiment, the *dcuC* gene comprises the sequence of SEQ ID NO: 49. In yet another embodiment the *dcuC* gene consists of the sequence of SEQ ID NO:70.

[0333] In one embodiment, the at least one propionate catabolism enzyme is DcuC. Accordingly, in one embodiment, DcuC has at least about 89% identity with SEQ ID NO:

129. In one embodiment, DcuC has at least about 90% identity with SEQ ID NO: 129. In another embodiment, DcuC has at least about 95% identity with SEQ ID NO: 129. Accordingly, in one embodiment, DcuC has at least about 85%, 86%, 89%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 129. In another embodiment, DcuC comprises a sequence which encodes SEQ ID NO: 129. In yet another embodiment, DcuC consists of a sequence which encodes SEQ ID NO: 129.

[0334] In one embodiment, the succinate exporter is DcuC. In one embodiment, the dcuC gene has at least about 80% identity to SEQ ID NO: 118. Accordingly, in one embodiment, the dcuC gene has at least about 90% identity to SEQ ID NO: 118. Accordingly, in one embodiment, the dcuC gene has at least about 95% identity to SEQ ID NO: 118. Accordingly, in one embodiment, the dcuC gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 118. In another embodiment, the dcuC gene comprises the sequence of SEQ ID NO: 118. In yet another embodiment the *dcuC* gene consists of the sequence of SEQ ID NO: 118.

[0335] In one embodiment, the at least one propionate catabolism enzyme is DcuC. Accordingly, in one embodiment, DcuC has at least about 89% identity with SEQ ID NO: 117. In one embodiment, DcuC has at least about 90% identity with SEQ ID NO: 117. In another embodiment, DcuC has at least about 95% identity with SEQ ID NO: 117. Accordingly, in one embodiment, DcuC has at least about 85%, 86%, 89%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 117. In another embodiment, DcuC comprises a sequence which encodes SEQ ID NO: 117. In yet another embodiment, DcuC consists of a sequence which encodes SEQ ID NO: 117.

[0336] In another embodiment, the succinate exporter is SucE1. In one embodiment, the *sucE1* gene has at least about 80% identity to SEQ ID NO: 46. Accordingly, in one embodiment, the *sucE1* gene has at least about 90% identity to SEQ ID NO: 46. Accordingly, in one embodiment, the *sucE1* gene has at least about 95% identity to SEQ ID NO: 46. Accordingly, in one embodiment, the *sucE1* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 46. In another embodiment, the *sucE1* gene comprises the sequence of SEQ ID NO: 46. In yet another embodiment the *sucE1* gene consists of the sequence of SEQ ID NO: 46.

[0337] In another embodiment, the succinate exporter is SucE1. In one embodiment, the *sucE1* gene has at least about 80% identity to SEQ ID NO: 120. Accordingly, in one embodiment, the *sucE1* gene has at least about 90% identity to SEQ ID NO: 120.

Accordingly, in one embodiment, the *sucE1* gene has at least about 95% identity to SEQ ID NO: 120. Accordingly, in one embodiment, the *sucE1* gene has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 120. In another embodiment, the *sucE1* gene comprises the sequence of SEQ ID NO: 120. In yet another embodiment the *sucE1* gene consists of the sequence of SEQ ID NO: 120.

[0338] In one embodiment, the at least one succinate exporter is sucE1. Accordingly, in one embodiment, sucE1 has at least about 89% identity with SEQ ID NO: 128. In one embodiment, sucE1 has at least about 90% identity with SEQ ID NO: 128. In another embodiment, sucE1 has at least about 95% identity with SEQ ID NO: 128. Accordingly, in one embodiment, sucE1 has at least about 85%, 86%, 89%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 128. In another embodiment, sucE1 comprises a sequence which encodes SEQ ID NO: 128. In yet another embodiment, sucE1 consists of a sequence which encodes SEQ ID NO: 128. In another embodiment, the sucE1 has at least about 89% identity with SEQ ID NO: 119. In one embodiment, sucE1 has at least about 90% identity with SEQ ID NO: 119. In another embodiment, sucE1 has at least about 95% identity with SEQ ID NO: 119. Accordingly, in one embodiment, sucE1 has at least about 85%, 86%, 89%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 119. In another embodiment, sucE1 comprises a sequence which encodes SEQ ID NO: 119. In yet another embodiment, sucE1 consists of a sequence which encodes SEQ ID NO: 119.

[0339] In some embodiments, the bacterial cell comprises at least one heterologous gene encoding at least one propionate catabolism enzyme operably linked to a first promoter and at least one heterologous gene encoding an exporter of succinate. In some embodiments, the at least one heterologous gene encoding an exporter of succinate is operably linked to the first promoter. In other embodiments, the at least one heterologous gene encoding an exporter of succinate is operably linked to a second promoter. In one embodiment, the at least one gene encoding an exporter of succinate is directly operably linked to the second promoter. In another embodiment, the at least one gene encoding an exporter of succinate is indirectly operably linked to the second promoter.

[0340] In some embodiments, expression of at least one gene encoding an exporter of succinate is controlled by a different promoter than the promoter that controls expression of the at least one gene encoding the at least one propionate catabolism enzyme. In some

embodiments, expression of the at least one gene encoding an exporter of succinate is controlled by the same promoter that controls expression of the at least one propionate catabolism enzyme. In some embodiments, at least one gene encoding an exporter of succinate and the propionate catabolism enzyme are divergently transcribed from a promoter region. In some embodiments, expression of each of genes encoding the at least one gene encoding an exporter of succinate and the at least one gene encoding the at least one propionate catabolism enzyme is controlled by different promoters.

[0341] In one embodiment, the promoter is not operably linked with the at least one gene encoding an exporter of succinate in nature. In some embodiments, the at least one gene encoding the exporter of succinate is controlled by its native promoter. In some embodiments, the at least one gene encoding the exporter of succinate is controlled by an inducible promoter. In some embodiments, the at least one gene encoding the exporter of succinate is controlled by a promoter that is stronger than its native promoter. In some embodiments, the at least one gene encoding the exporter of succinate is controlled by a constitutive promoter.

[0342] In another embodiment, the promoter is an inducible promoter. Inducible promoters are described in more detail *infra*.

[0343] In one embodiment, the at least one gene encoding an exporter of succinate is located on a plasmid in the bacterial cell. In another embodiment, the at least one gene encoding an exporter of succinate is located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding an exporter of succinate is located in the chromosome of the bacterial cell, and a copy of at least one gene encoding an exporter of succinate from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding an exporter of a succinate is located on a plasmid in the bacterial cell, and a copy of at least one gene encoding an exporter of succinate from a different species of bacteria is located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the at least one gene encoding an exporter of succinate is located in the chromosome of the bacterial cell, and a copy of the at least one gene encoding an exporter of succinate from a different species of bacteria is located in the chromosome of the bacterial cell.

[0344] In some embodiments, the at least one native gene encoding the exporter in the bacterial cell is not modified, and one or more additional copies of the native exporter are inserted into the genome. In one embodiment, the one or more additional copies of the native

exporter that is inserted into the genome are under the control of the same inducible promoter that controls expression of the at least one gene encoding the propionate catabolism enzyme, *e.g.*, the FNR responsive promoter, or a different inducible promoter than the one that controls expression of the at least one propionate catabolism enzyme, or a constitutive promoter. In alternate embodiments, the at least one native gene encoding the exporter is not modified, and one or more additional copies of the exporter from a different bacterial species is inserted into the genome of the bacterial cell. In one embodiment, the one or more additional copies of the exporter inserted into the genome of the bacterial cell are under the control of the same inducible promoter that controls expression of the at least one gene encoding the propionate catabolism enzyme, *e.g.*, the FNR responsive promoter, or a different inducible promoter than the one that controls expression of the at least one gene encoding the at least one propionate catabolism enzyme, or a constitutive promoter.

[0345] In one embodiment, when the exporter of succinate is expressed in the engineered bacterial cells, the bacterial cells export 10% more succinate out of the bacterial cell when the exporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In another embodiment, when the exporter of succinate is expressed in the engineered bacterial cells, the bacterial cells export 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% more succinate out of the bacterial cell when the exporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the exporter of succinate is expressed in the engineered bacterial cells, the bacterial cells export two-fold more succinate out of the cell when the exporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. In yet another embodiment, when the exporter of succinate is expressed in the engineered bacterial cells, the bacterial cells export three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold more succinate out of the cell when the exporter is expressed than unmodified bacteria of the same bacterial subtype under the same conditions.

Essential Genes and Auxotrophs

[0346] As used herein, the term "essential gene" refers to a gene which is necessary to for cell growth and/or survival. Bacterial essential genes are well known to one of ordinary skill in the art, and can be identified by directed deletion of genes and/or random mutagenesis and screening (see, for example, Zhang and Lin, 2009, DEG 5.0, a database of

essential genes in both prokaryotes and eukaryotes, Nucl. Acids Res., 37: D455-D458 and Gerdes *et al.,* Essential genes on metabolic maps, Curr. Opin. Biotechnol., 17(5):448-456, the entire contents of each of which are expressly incorporated herein by reference).

[0347] An "essential gene" may be dependent on the circumstances and environment in which an organism lives. For example, a mutation of, modification of, or excision of an essential gene may result in the engineered bacteria of the disclosure becoming an auxotroph, *e.g.,* the bacteria may be an auxotroph depending on the environmental conditions (a conditional auxotroph). An auxotrophic modification is intended to cause bacteria to die in the absence of an exogenously added nutrient essential for survival or growth because they lack the gene(s) necessary to produce that essential nutrient.

[0348] 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 an oligonucleotide synthesis gene, for example, *thyA*. In another embodiment, the essential gene is a cell wall synthesis gene, for example, *dapA*. In yet another embodiment, the essential gene is an amino acid gene, for example, *serA* or *MetA*. Any gene required for cell survival and/or growth may be targeted, including but not limited to, *cysE, glnA, ilvD, leuB, lysA, serA, metA, glyA, hisB, ilvA, pheA, proA, thrC, trpC, tyrA, thyA, uraA, dapA, dapB, dapD, dapE, dapF, flhD, metB, metC, proAB,* and *thi1*, as long as the corresponding wild-type gene product is not produced in the bacteria.

[0349] **Table 7** lists depicts exemplary bacterial genes which may be disrupted or deleted to produce an auxotrophic strain. These include, but are not limited to, genes required for oligonucleotide synthesis, amino acid synthesis, and cell wall synthesis.

Table 7. Non-limiting Examples of Bacterial Genes Useful for Generation of an Auxotroph

Amino Acid	Oligonucleotide	Cell Wall
cysE	thyA	dapA
glnA	uraA	dapB
ilvD		dapD
leuB		dapE

lysA		dapF
serA		
metA		
glyA		
hisB		
ilvA		
pheA		
proA		
thrC		
trpC		
tyrA		

[0350] **Table 8** shows the survival of various amino acid auxotrophs in the mouse gut, as detected 24 hrs and 48 hrs post-gavage. These auxotrophs were generated using BW25113, a non-Nissle strain of E. coli.

Table 8. Survival of amino acid auxotrophs in the mouse gut

Gene	AA Auxotroph	Pre-Gavage	24 hours	48 hours
argA	**Arginine**	Present	Present	Absent
cysE	**Cysteine**	Present	Present	Absent
glnA	**Glutamine**	Present	Present	Absent
glyA	**Glycine**	Present	Present	Absent
hisB	**Histidine**	Present	Present	Present
ilvA	**Isoleucine**	Present	Present	Absent
leuB	**Leucine**	Present	Present	Absent
lysA	**Lysine**	Present	Present	Absent
metA	**Methionine**	Present	Present	Present
pheA	**Phenylalanine**	Present	Present	Present
proA	**Proline**	Present	Present	Absent
serA	**Serine**	Present	Present	Present
thrC	**Threonine**	Present	Present	Present
trpC	**Tryptophan**	Present	Present	Present
tyrA	**Tyrosine**	Present	Present	Present
ilvD	**Valine/Isoleucine/Leucine**	Present	Present	Absent

thyA	**Thiamine**	Present	Absent	Absent
uraA	**Uracil**	Present	Absent	Absent
flhD	**FlhD**	Present	Present	Present

[0351] 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).

[0352] Diaminopimelic acid (DAP) is an amino acid synthetized within the lysine biosynthetic pathway and is required for bacterial cell wall growth (Meadow *et al.,* 1959; Clarkson *et al.,* 1971). In some embodiments, any of the genetically engineered bacteria described herein is a *dapD* auxotroph in which *dapD* is deleted and/or replaced with an unrelated gene. A *dapD* auxotroph can grow only when sufficient amounts of DAP are present, *e.g.*, by adding DAP to growth media in vitro. Without sufficient amounts of DAP, the *dapD* auxotroph dies. In some embodiments, the auxotrophic modification is used to ensure that the bacterial cell does not survive in the absence of the auxotrophic gene product (*e.g.,* outside of the gut).

[0353] In other embodiments, the genetically engineered bacterium of the present disclosure is a *uraA* auxotroph in which *uraA* is deleted and/or replaced with an unrelated gene. The *uraA* gene codes for UraA, a membrane-bound transporter that facilitates the uptake and subsequent metabolism of the pyrimidine uracil (Andersen *et al.,* 1995). A *uraA* auxotroph can grow only when sufficient amounts of uracil are present, *e.g.,* by adding uracil to growth media *in vitro*. Without sufficient amounts of uracil, the *uraA* auxotroph dies. In some embodiments, auxotrophic modifications are used to ensure that the bacteria do not survive in the absence of the auxotrophic gene product (*e.g.,* outside of the gut).

[0354] In complex communities, it is possible for bacteria to share DNA. In very rare circumstances, an auxotrophic bacterial strain may receive DNA from a non-auxotrophic strain, which repairs the genomic deletion and permanently rescues the auxotroph. Therefore, engineering a bacterial strain with more than one auxotroph may greatly decrease the probability that DNA transfer will occur enough times to rescue the auxotrophy. In some embodiments, the genetically engineered bacteria comprise a deletion or mutation in two or more genes required for cell survival and/or growth.

[0355] Other examples of essential genes include, but are not limited to yhbV, yagG, hemB, secD, secF, ribD, ribE, thiL, dxs, ispA, dnaX, adk, hemH, lpxH, cysS, fold, rplT, infC, thrS, nadE, gapA, yeaZ, aspS, argS, pgsA, yefM, metG, folE, yejM, gyrA, nrdA, nrdB, folC, accD, fabB, gltX, ligA, zipA, dapE, dapA, der, hisS, ispG, suhB, tadA, acpS, era, rnc, ftsB, eno, pyrG, chpR, lgt, fbaA, pgk, yqgD, metK, yqgF, plsC, ygiT, pare, ribB, cca, ygjD, tdcF, yraL, yihA, ftsN, murI, murB, birA, secE, nusG, rplJ, rplL, rpoB, rpoC, ubiA, plsB, lexA, dnaB, ssb, alsK, groS, psd, orn, yjeE, rpsR, chpS, ppa, valS, yjgP, yjgQ, dnaC, ribF, lspA, ispH, dapB, folA, imp, yabQ, ftsL, ftsI, murE, murF, mraY, murD, ftsW, murG, murC, ftsQ, ftsA, ftsZ, lpxC, secM, secA, can, folK, hemL, yadR, dapD, map, rpsB, infB ,nusA, ftsH, obgE, rpmA, rplU, ispB, murA, yrbB, yrbK, yhbN, rpsI, rplM, degS, mreD, mreC, mreB, accB, accC, yrdC, def, fmt, rplQ, rpoA, rpsD, rpsK, rpsM, entD, mrdB, mrdA, nadD, hlepB, rpoE, pssA, yfiO, rplS, trmD, rpsP, ffh, grpE, yfjB, csrA, ispF, ispD, rplW, rplD, rplC, rpsJ, fusA, rpsG, rpsL, trpS, yrfF, asd, rpoH, ftsX, ftsE, ftsY, frr, dxr, ispU, rfaK, kdtA, coaD, rpmB, dfp, dut, gmk, spot, gyrB, dnaN, dnaA, rpmH, rnpA, yidC, tnaB, glmS, glmU, wzyE, hemD, hemC, yigP, ubiB, ubiD, hemG, secY, rplO, rpmD, rpsE, rplR, rplF, rpsH, rpsN, rplE, rplX, rplN, rpsQ, rpmC, rplP, rpsC, rplV, rpsS, rplB, cdsA, yaeL, yaeT, lpxD, fabZ, lpxA, lpxB, dnaE, accA, tilS, proS, yafF, tsf, pyrH, olA, rlpB, leuS, lnt, glnS, fldA, cydA, infA, cydC, ftsK, lolA, serS, rpsA, msbA, lpxK, kdsB, mukF, mukE, mukB, asnS, fabA, mviN, rne, yceQ, fabD, fabG, acpP, tmk, holB, lolC, lolD, lolE, purB, ymfK, minE, mind, pth, rsA, ispE, lolB, hemA, prfA, prmC, kdsA, topA, ribA, fabI, racR, dicA, ydfB, tyrS, ribC, ydiL, pheT, pheS, yhhQ, bcsB, glyQ, yibJ, and gpsA. Other essential genes are known to those of ordinary skill in the art.

[0356] 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).

[0357] 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.

[0358] In some embodiments, the genetically engineered bacterium is complemented by a ligand. In some embodiments, the ligand is selected from the group consisting of benzothiazole, indole, 2-aminobenzothiazole, indole-3-butyric acid, indole-3-acetic acid, and L-histidine methyl ester. For example, bacterial cells comprising mutations in metG (E45Q, N47R, I49G, and A51C) are complemented by benzothiazole, indole, 2-aminobenzothiazole, indole-3-butyric acid, indole-3-acetic acid or L-histidine methyl ester. Bacterial cells comprising mutations in dnaN (H191N, R240C, I317S, F319V, L340T, V347I, and S345C) are complemented by benzothiazole, indole or 2-aminobenzothiazole. Bacterial cells comprising mutations in pheS (F125G, P183T, P184A, R186A, and I188L) are complemented by benzothiazole or 2-aminobenzothiazole. Bacterial cells comprising mutations in tyrS (L36V, C38A, and F40G) are complemented by benzothiazole or 2-aminobenzothiazole. Bacterial cells comprising mutations in adk (I4L, L5I and L6G) are complemented by benzothiazole or indole.

[0359] 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).

[0360] In some embodiments, the genetically engineered bacterium is a conditional auxotroph whose essential gene(s) is replaced using the arabinose system described herein.

[0361] In some embodiments, the genetically engineered bacterium of the disclosure is an auxotroph and also comprises kill-switch circuitry, such as any of the kill-switch components and systems described herein. For example, the engineered bacteria may comprise a deletion or mutation in an essential gene required for cell survival and/or growth, for example, in a DNA synthesis gene, for example, *thyA*, cell wall synthesis gene, for example, *dapA* and/or an amino acid gene, for example, *serA* or *MetA* and may also comprise a toxin gene that is regulated by one or more transcriptional activators that are expressed in response to an environmental condition(s) and/or signal(s) (such as the described arabinose system) or regulated by one or more recombinases that are expressed upon sensing an exogenous environmental condition(s) and/or signal(s) (such as the recombinase systems described herein). Other embodiments are described in Wright *et al.*, "GeneGuard: A Modular Plasmid System Designed for Biosafety," ACS Synthetic Biology (2015) 4: 307-16, the entire contents of which are expressly incorporated herein by reference). In some embodiments, the genetically engineered bacterium of the disclosure is an auxotroph and also comprises kill-switch circuitry, such as any of the kill-switch components and systems described herein, as well as another biosecurity system, such a conditional origin of replication (see Wright *et al., supra*).

Genetic Regulatory Circuits

[0362] 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 a propionate catabolism enzyme, propionate transporter, and/or propionate binding protein or

rescue an auxotroph. In certain embodiments, the invention provides methods for selecting genetically engineered bacteria that produce one or more genes of interest.

[0363] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a payload and a T7 polymerase-regulated genetic regulatory circuit. For example, the genetically engineered bacteria comprise a first gene encoding a T7 polymerase, wherein the first gene is operably linked to a fumarate and nitrate reductase regulator (FNR)-responsive promoter; a second gene or gene cassette for producing a payload, 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 payload 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 payload 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.

[0364] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a payload 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 payload 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 payload is not expressed. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, thereby inducing expression of mf-lon protease. The mf-lon protease recognizes the mf-lon degradation signal and degrades the tetR, and the payload is expressed.

[0365] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a payload 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 payload 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 payload 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 payload is expressed.

[0366] Examples of repressors useful in these embodiments include, but are not limited to, ArgR, TetR, ArsR, AscG, LacI, CscR, DeoR, DgoR, FruR, GalR, GatR, CI, LexA, RafR, QacR, and PtxS (US20030166191).

[0367] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a payload 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 payload. 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 payload. The regulatory RNA is capable of eliminating the mRNA hairpin and inducing payload 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 payload 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 payload is expressed.

[0368] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a payload 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 payload, 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 payload 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 payload is expressed.

[0369] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a payload 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 payload 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 payload remains in the 3' to 5' orientation, and no functional payload is produced. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, the recombinase is expressed, the payload is reverted to the 5' to 3' orientation, and functional payload is produced.

[0370] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a payload 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 payload 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 payload. 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 payload 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 payload is expressed.

Kill Switches

[0371] In some embodiments, the genetically engineered bacteria also comprise a kill switch (*see, e.g.*, U.S. Provisional Application Nos. 62/183,935 and 62/263,329, each of which are expressly incorporated herein by reference in their entireties). The kill switch is intended to actively kill engineered microbes in response to external stimuli. As opposed to an auxotrophic mutation where bacteria die because they lack an essential nutrient for survival, the kill switch is triggered by a particular factor in the environment that induces the production of toxic molecules within the microbe that cause cell death.

[0372] Bacteria engineered with kill switches have been engineered for *in vitro* research purposes, *e.g.*, to limit the spread of a biofuel-producing microorganism outside of a laboratory environment. Bacteria engineered for *in vivo* administration to treat a disease or disorder may also be programmed to die at a specific time after the expression and delivery of a heterologous gene, genes or gene cassette(s), for example, a therapeutic gene(s) or after the subject has experienced the therapeutic effect. For example, in some embodiments, the kill switch is activated to kill the bacteria after a period of time following expression of the propionate catabolism enzyme cassette(s) and/or gene(s) present in the engineered bacteria. In some embodiments, the kill switch is activated in a delayed fashion following expression of the heterologous gene(s) or gene cassette(s), for example, after the production of the corresponding protein(s) or molecule(s). Alternatively, the bacteria may be engineered to die after the bacteria has spread outside of a disease site. Specifically, it may be useful to prevent long-term colonization of subjects by the microorganism, spread of the microorganism outside the area of interest (for example, outside the gut) within the subject, or spread of the microorganism outside of the subject into the environment (for example, spread to the environment through the stool of the subject).

[0373] Examples of such toxins that can be used in kill-switches include, but are not limited to, bacteriocins, lysins, and other molecules that cause cell death by lysing cell membranes, degrading cellular DNA, or other mechanisms. Such toxins can be used individually or in combination. The switches that control their production can be based on,

for example, transcriptional activation (toggle switches; *see, e.g.,* Gardner *et al.,* 2000), translation (riboregulators), or DNA recombination (recombinase-based switches), and can sense environmental stimuli such as anaerobiosis or reactive oxygen species. These switches can be activated by a single environmental factor or may require several activators in AND, OR, NAND and NOR logic configurations to induce cell death. For example, an AND riboregulator switch is activated by tetracycline, isopropyl β-D-1-thiogalactopyranoside (IPTG), and arabinose to induce the expression of lysins, which permeabilize the cell membrane and kill the cell. IPTG induces the expression of the endolysin and holin mRNAs, which are then derepressed by the addition of arabinose and tetracycline. All three inducers must be present to cause cell death. Examples of kill switches are known in the art (Callura *et al.,* 2010). In some embodiments, the kill switch is activated to kill the bacteria after a period of time following oxygen level-dependent expression of a heterologous gene(s) or gene cassette(s). In some embodiments, the kill switch is activated in a delayed fashion following oxygen level-dependent expression of a heterologous gene(s) or gene cassette(s).

[0374] Kill-switches can be designed such that a toxin is produced in response to an environmental condition or external signal (*e.g.,* the bacteria is killed in response to an external cue; *i.e.,* an activation-based kill switch) or, alternatively designed such that a toxin is produced once an environmental condition no longer exists or an external signal is ceased (*i.e.,* a repression-based kill switch).

[0375] Thus, in some embodiments, the genetically engineered bacteria of the disclosure are further programmed to die after sensing an exogenous environmental signal, for example, in a low oxygen environment. In some embodiments, the genetically engineered bacteria of the present disclosure comprise one or more genes encoding one or more recombinase(s), whose expression is induced in response to an environmental condition or signal and causes one or more recombination events that ultimately leads to the expression of a toxin which kills the cell. In some embodiments, the at least one recombination event is the flipping of an inverted heterologous gene encoding a bacterial toxin which is then constitutively expressed after it is flipped by the first recombinase. In one embodiment, constitutive expression of the bacterial toxin kills the genetically engineered bacterium. In these types of kill-switch systems once the engineered bacterial cell senses the exogenous environmental condition and expresses the heterologous gene of interest, the engineered bacterial cell is no longer viable.

[0376] 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.

[0377] 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.

[0378] In one embodiment, the at least one recombination event is flipping of an inverted heterologous gene encoding a second recombinase by a first recombinase, followed by flipping of an inverted heterologous gene encoding a third recombinase by the second recombinase, followed by flipping of an inverted heterologous gene encoding a bacterial toxin by the third recombinase. Accordingly, in one embodiment, the disclosure provides at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 recombinases that can be used serially.

[0379] In one embodiment, the at least one recombination event is flipping of an inverted heterologous gene encoding a first excision enzyme by a first recombinase. In one embodiment, the inverted heterologous gene encoding the first excision enzyme is located between a first forward recombinase recognition sequence and a first reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the first excision enzyme is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the first excision enzyme excises a first essential gene. In one embodiment, the programmed engineered bacterial cell is not viable after the first essential gene is excised.

[0380] In one embodiment, the first recombinase further flips an inverted heterologous gene encoding a second excision enzyme. In one embodiment, the wherein the inverted heterologous gene encoding the second excision enzyme is located between a second forward recombinase recognition sequence and a second reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the second excision enzyme is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the genetically engineered bacterium dies or is no longer viable when the first essential gene and the second essential gene are both excised. In one embodiment, the genetically engineered bacterium dies or is no longer viable when either the first essential gene is excised or the second essential gene is excised by the first recombinase.

[0381] In one embodiment, the first excision enzyme is Xis1. In one embodiment, the first excision enzyme is Xis2. In one embodiment, the first excision enzyme is Xis1, and the second excision enzyme is Xis2.

[0382] 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.

[0383] In any of these embodiment, the recombinase can be a recombinase selected from the group consisting of: BxbI, PhiC31, TP901, BxbI, PhiC31, TP901, HK022, HP1, R4, Int1, Int2, Int3, Int4, Int5, Int6, Int7, Int8, Int9, Int10, Int11, Int12, Int13, Int14, Int15, Int16, Int17, Int18, Int19, Int20, Int21, Int22, Int23, Int24, Int25, Int26, Int27, Int28, Int29, Int30, Int31, Int32, Int33, and Int34, or a biologically active fragment thereof.

[0384] 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) are described herein. The disclosure provides engineered bacterial cells which express one or more heterologous gene(s) upon sensing arabinose or other sugar in the exogenous environment. In this aspect, the engineered bacterial cells contain the *araC* gene, which encodes the AraC transcription factor, as well as one or more genes under the control of the *araBAD* promoter. In the absence of arabinose, the AraC transcription factor adopts a conformation that represses transcription of genes under the control of the *araBAD* promoter. In the presence of arabinose, the AraC transcription factor undergoes a conformational change that allows it to bind to and activate the *araBAD* promoter, which induces expression of the desired gene, for example tetR, which represses expression of a toxin gene. In this embodiment, the 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.

[0385] Thus, in some embodiments in which one or more heterologous gene(s) are expressed upon sensing arabinose in the exogenous environment, the one or more heterologous genes are directly or indirectly under the control of the *araBAD* promoter. In some embodiments, the expressed heterologous gene is selected from one or more of the following: a heterologous therapeutic gene, a heterologous gene encoding an antitoxin, a heterologous gene encoding a repressor protein or polypeptide, for example, a TetR

repressor, a heterologous gene encoding an essential protein not found in the bacterial cell, and/or a heterologous encoding a regulatory protein or polypeptide.

[0386] Arabinose inducible promoters are known in the art, including P_{ara}, P_{araB}, P_{araC}, and P_{araBAD}. In one embodiment, the arabinose inducible promoter is from *E. coli*. In some embodiments, the P_{araC} promoter and the P_{araBAD} promoter operate as a bidirectional promoter, with the P_{araBAD} promoter controlling expression of a heterologous gene(s) in one direction, and the P_{araC} (in close proximity to, and on the opposite strand from the P_{araBAD} promoter), controlling expression of a heterologous gene(s) in the other direction. In the presence of arabinose, transcription of both heterologous genes from both promoters is induced. However, in the absence of arabinose, transcription of both heterologous genes from both promoters is not induced.

[0387] In one exemplary embodiment of the disclosure, the engineered bacteria of the present disclosure contains a kill-switch having at least the following sequences: a P_{araBAD} promoter operably linked to a heterologous gene encoding a Tetracycline Repressor Protein (TetR), a P_{araC} promoter operably linked to a heterologous gene encoding AraC transcription factor, and a heterologous gene encoding a bacterial toxin operably linked to a promoter which is repressed by the Tetracycline Repressor Protein (P_{TetR}). In the presence of arabinose, the AraC transcription factor activates the P_{araBAD} promoter, which activates transcription of the TetR protein which, in turn, represses transcription of the toxin. In the absence of arabinose, however, AraC suppresses transcription from the P_{araBAD} promoter and no TetR protein is expressed. In this case, expression of the heterologous toxin gene is activated, and the toxin is expressed. The toxin builds up in the engineered bacterial cell, and the engineered bacterial cell is killed. In one embodiment, the *araC* gene encoding the AraC transcription factor is under the control of a constitutive promoter and is therefore constitutively expressed.

[0388] In one embodiment of the disclosure, the engineered bacterial cell further comprises an antitoxin under the control of a constitutive promoter. In this situation, in the presence of arabinose, the toxin is not expressed due to repression by TetR protein, and the antitoxin protein builds-up in the cell. However, in the absence of arabinose, TetR protein is not expressed, and expression of the toxin is induced. The toxin begins to build-up within the engineered bacterial cell. The engineered bacterial cell is no longer viable once the toxin protein is present at either equal or greater amounts than that of the anti-toxin protein in the cell, and the engineered bacterial cell will be killed by the toxin.

[0389] In another embodiment of the disclosure, the engineered bacterial cell further comprises an antitoxin under the control of the P_{araBAD} promoter. In this situation, in the presence of arabinose, TetR and the anti-toxin are expressed, the anti-toxin builds up in the cell, and the toxin is not expressed due to repression by TetR protein. However, in the absence of arabinose, both the TetR protein and the anti-toxin are not expressed, and expression of the toxin is induced. The toxin begins to build-up within the engineered bacterial cell. The engineered bacterial cell is no longer viable once the toxin protein is expressed, and the engineered bacterial cell will be killed by the toxin.

[0390] In another exemplary embodiment of the disclosure, the engineered bacteria of the present disclosure contains a kill-switch having at least the following sequences: a P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the engineered bacterial cell (and required for survival), and a P_{araC} promoter operably linked to a heterologous gene encoding AraC transcription factor. In the presence of arabinose, the AraC transcription factor activates the P_{araBAD} promoter, which activates transcription of the heterologous gene encoding the essential polypeptide, allowing the engineered bacterial cell to survive. In the absence of arabinose, however, AraC suppresses transcription from the P_{araBAD} promoter and the essential protein required for survival is not expressed. In this case, the engineered bacterial cell dies in the absence of arabinose. In some embodiments, the sequence of P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the engineered bacterial cell can be present in the bacterial cell in conjunction with the TetR/toxin kill-switch system described directly above. In some embodiments, the sequence of P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the engineered bacterial cell can be present in the bacterial cell in conjunction with the TetR/toxin/anto-toxin kill-switch system described directly above.

[0391] 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.

[0392] In some embodiments, the engineered bacteria of the present disclosure, for example, bacteria described herein may further comprise the gene(s) encoding the components of any of the above-described kill-switch circuits.

[0393] In any of the above-described embodiments, the bacterial toxin is selected from the group consisting of a lysin, Hok, Fst, TisB, LdrD, Kid, SymE, MazF, FlmA, Ibs, XCV2162, dinJ, CcdB, MazF, ParE, YafO, Zeta, hicB, relB, yhaV, yoeB, chpBK, hipA, microcin B, microcin B17, microcin C, microcin C7-C51, microcin J25, microcin ColV, microcin 24, microcin L, microcin D93, microcin L, microcin E492, microcin H47, microcin I47, microcin M, colicin A, colicin E1, colicin K, colicin N, colicin U, colicin B, colicin Ia, colicin Ib, colicin 5, colicin10, colicin S4, colicin Y, colicin E2, colicin E7, colicin E8, colicin E9, colicin E3, colicin E4, colicin E6; colicin E5, colicin D, colicin M, and cloacin DF13, or a biologically active fragment thereof.

[0394] In any of the above-described embodiments, the anti-toxin is selected from the group consisting of an anti-lysin, Sok, RNAII, IstR, RdlD, Kis, SymR, MazE, FlmB, Sib, ptaRNA1, yafQ, CcdA, MazE, ParD, yafN, Epsilon, HicA, relE, prlF, yefM, chpBI, hipB, MccE, $MccE^{CTD}$, MccF, Cai, ImmE1, Cki, Cni, Cui, Cbi, Iia, Imm, Cfi, Im10, Csi, Cyi, Im2, Im7, Im8, Im9, Im3, Im4, ImmE6, cloacin immunity protein (Cim), ImmE5, ImmD, and Cmi, or a biologically active fragment thereof.

[0395] 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.

[0396] In one embodiment, the method further comprises administering a second engineered bacterial cell to the subject, wherein the second engineered bacterial cell comprises a heterologous reporter gene operably linked to an inducible promoter that is directly or indirectly induced by an exogenous environmental condition. In one embodiment, the heterologous reporter gene is a fluorescence gene. In one embodiment, the fluorescence gene encodes a green fluorescence protein (GFP). In another embodiment, the method further comprises administering a second engineered bacterial cell to the subject, wherein the second engineered bacterial cell expresses a *lacZ* reporter construct that cleaves a substrate to produce a small molecule that can be detected in urine (see, for example, Danio *et al.*, *Science Translational Medicine,* 7(289):1-12, 2015, the entire contents of which are expressly incorporated herein by reference).

Isolated Plasmids

[0397] In other embodiments, the disclosure provides an isolated plasmid comprising a first nucleic acid encoding a first payload operably linked to a first inducible promoter, and a second nucleic acid encoding a second payload operably linked to a second inducible promoter. In other embodiments, the disclosure provides an isolated plasmid further comprising a third nucleic acid encoding a third payload operably linked to a third inducible promoter. In other embodiments, the disclosure provides a plasmid comprising four, five, six, or more nucleic acids encoding four, five, six, or more payloads operably linked to inducible promoters. In any of the embodiments described here, the first, second, third, fourth, fifth, sixth, etc "payload(s)" can be a propionate catabolism enzyme, a propionate transporter, a propionate binding protein, or other sequence described herein. In one embodiment, the nucleic acid encoding the first payload and the nucleic acid encoding the second payload are operably linked to the first inducible promoter. In one embodiment, the nucleic acid encoding the first payload is operably linked to a first inducible promoter and the nucleic acid encoding the second payload is operably linked to a second inducible promoter. In one embodiment, the first inducible promoter and the second inducible promoter are separate copies of the same inducible promoter. In another embodiment, the first inducible promoter and the second inducible promoter are different inducible promoters. In other embodiments comprising a third nucleic acid, the nucleic acid encoding the third payload and the nucleic acid encoding the first and second payloads are all operably linked to the same inducible promoter. In other embodiments, the nucleic acid encoding the first payload is operably linked to a first inducible promoter, the nucleic acid encoding the second payload is operably linked to a second inducible promoter, and the nucleic acid encoding the third payload is operably linked to a third inducible promoter. In some embodiments, the first, second, and third inducible promoters are separate copies of the same inducible promoter. In other embodiments, the first inducible promoter, the second inducible promoter, and the third inducible promoter are different inducible promoters. In some embodiments, the first promoter, the second promoter, and the optional third promoter, or the first promoter and the second promoter and the optional third promoter, are each directly or indirectly induced by low-oxygen or anaerobic conditions. In other embodiments, the first promoter, the second promoter, and the optional third promoter, or the first promoter and the second promoter and the optional third promoter, are each a fumarate and nitrate reduction regulator (FNR) responsive promoter. In other embodiments, the first promoter, the second promoter, and the

optional third promoter, or the first promoter and the second promoter and the optional third promoter are each a ROS-inducible regulatory region. In other embodiments, the first promoter, the second promoter, and the optional third promoter, or the first promoter and the second promoter and the optional third promoter are each a RNS-inducible regulatory region.

[0398] In some embodiments, the heterologous gene encoding a propionate catabolism enzyme is operably linked to a constitutive promoter. In one embodiment, the constitutive promoter is a lac promoter. In another embodiment, the constitutive promoter is a tet promoter. In another embodiment, the constitutive promoter is a constitutive Escherichia coli σ32 promoter. In another embodiment, the constitutive promoter is a constitutive Escherichia coli σ70 promoter. In another embodiment, the constitutive promoter is a constitutive Bacillus subtilis σA promoter. In another embodiment, the constitutive promoter is a constitutive Bacillus subtilis σB promoter. In another embodiment, the constitutive promoter is a Salmonella promoter. In other embodiments, the constitutive promoter is a bacteriophage T7 promoter. In other embodiments, the constitutive promoter is and a bacteriophage SP6 promoter. In any of the above-described embodiments, the plasmid further comprises a heterologous gene encoding a propionate transporter, a propionate binding protein, and/or a kill switch construct, which may be operably linked to a constitutive promoter or an inducible promoter.

[0399] In some embodiments, the isolated plasmid comprises at least one heterologous propionate catabolism enzyme gene operably linked to a first inducible promoter; a heterologous gene encoding a TetR protein operably linked to a ParaBAD promoter, a heterologous gene encoding AraC operably linked to a ParaC promoter, a heterologous gene encoding an antitoxin operably linked to a constitutive promoter, and a heterologous gene encoding a toxin operably linked to a PTetR promoter. In another embodiment, the isolated plasmid comprises at least one heterologous gene encoding a propionate catabolism enzyme operably linked to a first inducible promoter; a heterologous gene encoding a TetR protein and an anti-toxin operably linked to a ParaBAD promoter, a heterologous gene encoding AraC operably linked to a ParaC promoter, and a heterologous gene encoding a toxin operably linked to a PTetR promoter.

[0400] In some embodiments, a first nucleic acid encoding a propionate catabolism enzyme comprises a prpE and/or a Pha gene. In other embodiments, a first nucleic acid encoding a propionate catabolism enzyme is a Pha gene or a Pha operon, e.g. prpE-phaB-phaC-phaA. In some embodiments, the prpE gene or Pha gene or Pha operon is coexpressed

with an additional propionate catabolism gene or gene cassette, e.g. a MMCA cassette and/or a 2MC cassette described herein. In other embodiments, a gene encoding a succinate exporter, e.g., SucE1 and/or DcuC, is further expressed. In other embodiments, a propionate importer is further expressed.

[0401] In some embodiments, a first nucleic acid encoding a propionate catabolism enzyme comprises a prpE and/or a MMCA pathway gene. In other embodiments, a first nucleic acid encoding a propionate catabolism enzyme is a prpE and/or a MMCA pathway gene or a MMCA pathway operon, e.g. prpE-accA1-pccB-mmcE-mutA-mutB or prpE-accA1-pccB or mmcE-mutA-mutB. In some embodiments, the prpE and/or a MMCA pathway gene or a MMCA pathway operon is coexpressed with an additional propionate catabolism gene or gene cassette, e.g. a Pha cassette and/or a 2MC cassette described herein. In other embodiments, a gene encoding a succinate exporter, e.g., SucE1 and/or DcuC, is further expressed. In other embodiments, a propionate importer is further expressed.

[0402] In some embodiments, a first nucleic acid encoding a propionate catabolism enzyme comprises a prpE and/or a 2MC pathway gene. In other embodiments, a first nucleic acid encoding a propionate catabolism enzyme is a prpE and/or a 2MC pathway gene or a 2MC pathway operon, e.g. prpB-prpC-prpD-prpE or prpB-prpC-prpD. In some embodiments, the prpE and/or a 2MC pathway gene or a 2MC pathway operon is coexpressed with an additional propionate catabolism gene or gene cassette, e.g. a Pha cassette and/or a MMCA cassette described herein. In other embodiments, a gene encoding a succinate exporter, e.g., SucE1 and/or DcuC, is further expressed. In other embodiments, a propionate importer is further expressed.

[0403] In one embodiment, the plasmid is a high-copy plasmid. In another embodiment, the plasmid is a low-copy plasmid.

[0404] In another aspect, the disclosure provides a recombinant bacterial cell comprising an isolated plasmid described herein. In another embodiment, the disclosure provides a pharmaceutical composition comprising the recombinant bacterial cell.

[0405] In one embodiment, the bacterial cell further comprises a genetic mutation in an endogenous gene encoding a lysine acetyltransferase, e.g. pka, which propionylates and inactivates prpE. In another embodiment, the bacterial cell further comprises a genetic mutation which reduces export of propionate and/or its metabolites from the bacterial cell.

[0406] In one embodiment, the bacterial cell further comprises a genetic mutation in an endogenous gene encoding a propionate biosynthesis gene, wherein the genetic mutation reduces biosynthesis of propionate and one or more of its metabolites in the bacterial cell.

Multiple Mechanisms of Action

[0407] In some embodiments, the bacteria are genetically engineered to include multiple mechanisms of action (MOAs), *e.g.,* circuits producing multiple copies of the same product (*e.g.,* to enhance copy number) or circuits performing multiple different functions. Examples of insertion sites include, but are not limited to, *malE/K, insB/I, araC/BAD, lacZ, dapA, cea,* and other shown in **FIG. 32**. For example, the genetically engineered bacteria may include four copies of a propionate catabolism gene or propionate catabolism gene cassette, or four copies of a propionate catabolism gene inserted at four different insertion sites, *e.g., malE/K, insB/I, araC/BAD,* and *lacZ.* Alternatively, the genetically engineered bacteria may include one or more copies of a propionate catabolism gene or gene cassette inserted at one or more different insertion sites, *e.g., malE/K, insB/I,* and *lacZ,* one or more copies of a propionate catabolism gene or gene cassette inserted at one or more different insertion sites, *e.g., dapA, cea,* and *araC/BAD* and/or one or more copies of a propionate catabolism gene or gene cassette inserted at one or more different insertion sites.

[0408] In some embodiments, the genetically engineered bacteria comprise one or more of: (1) one or more gene(s) and/or gene cassettes encoding one or more propionate catabolism enzyme(s), in wild type or in a mutated form (for increased stability or metabolic activity); (2) one or more gene(s) and/or gene cassette(s) encoding one or more transporter(s) for uptake of propionate and/or one or more of its metabolites, including methylmalonic acid, in wild type or in mutated form (for increased stability or metabolic activity); (3) one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzyme(s) for secretion and extracellular degradation of propionate and/or one or more of its metabolites, (4) one or more gene(s) or gene cassette(s) encoding one or more components of secretion machinery, as described herein (5) one or more auxotrophies, e.g., deltaThyA; (6) one or more gene(s) or gene cassette(s) encoding one or more antibiotic resistance(s), including but not limited to, kanamycin or chloramphenicol resistance; (7) one or more modifications that increase succinate export from the bacterial cell; (8) one or modifications that reduce succinate import into the bacterial cell; (9) mutations/deletions in genes, as described herein,

e.g., pka, succinate importers or propionate exporters (10) mutations/deletions in genes of the endogenous propionate synthesis pathway.

[0409] In some embodiments, the genetically engineered bacteria comprise two or more different pathway cassettes or operons comprising propionate catabolism enzymes. In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes. In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding one or more propionate catabolism enzymes selected from PrpE, AccA1, PccB, MmcE, MutA, and MutB, and combinations thereof. In some embodiments, the genetically engineered bacteria comprise gene sequence(s) comprising two or more copies of any genes selected from prpE, accA1, pccB, mmcE, mutA, and mutB. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more propionate catabolism enzymes selected from PrpE, PhaB, PhaC, and PhaA, and combinations thereof. In some embodiments, the genetically engineered bacteria comprise gene sequence(s) comprising two or more copies of any genes selected from prpE, phaB, phaC, and phaA. In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more propionate catabolism enzymes selected from PrpB, PrpC, PrpD, and PrpE, and combinations thereof. In some embodiments, the genetically engineered bacteria comprise gene sequence(s) comprising two or more copies of any genes selected from prpB-prpC, prpD, and prpE. Non-limiting examples of combinations include genetically engineered bacteria comprising one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in combination with one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA). In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in combination with one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE). In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB), one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA). In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA). In another non-limiting example of combinations, the

genetically engineered bacteria comprise one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB).

[0410] Non-limiting examples of combinations include genetically engineered bacteria comprising one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in combination with one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and in combination with one or more cassettes comprising *matB*. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in combination with one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE) and in combination with one or more cassettes comprising *matB*. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB), one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and in combination with one or more cassettes comprising *matB*. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and in combination with one or more cassettes comprising *MatB*. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) and in combination with one or more cassettes comprising *matB*. Any of the combinations described above comprising *matB* may or may not comprise *prpE, e.g.*, may comprise *matB* in lieu of *prpE*.

[0411] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes and one or more gene(s) or gene cassette(s) encoding one or more propionate transporters (importers), such as any of the propionate transporters described herein and otherwise known in the art.

[0412] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes and

one or more gene(s) or gene cassette(s) encoding one or more succinate exporters, e.g. SucE1 and/or dcuC. Non-limiting examples of combinations include genetically engineered bacteria comprising one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in combination with one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and one or more gene(s) or gene cassette(s) encoding one or more succinate exporters, e.g. SucE1 and/or dcuC. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in combination with one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE) and one or more gene(s) or gene cassette(s) encoding one or more succinate exporters, e.g. SucE1 and/or dcuC. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB), one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and one or more gene(s) or gene cassette(s) encoding one or more succinate exporters, e.g. SucE1 and/or dcuC. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and one or more gene(s) or gene cassette(s) encoding one or more succinate exporters, e.g. SucE1 and/or dcuC. In another non-limiting example of combinations, the genetically engineered bacteria comprise one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), and one or more MMCA pathway operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) and one or more gene(s) or gene cassette(s) encoding one or more succinate exporters, e.g. SucE1 and/or dcuC. In other non-limiting examples, the genetically engineered bacteria comprising one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes and one or more gene(s) or gene cassette(s) encoding one or more succinate exporters, e.g. SucE1 and/or dcuC, e.g., as described supra, may comprise one or more gene(s) or gene cassette(s) comprising *matB* or *matB* may be substituted in lieu of *prpE*. In any of the embodiments, the engineered bacterium may also comprise gene sequence(s) encoding one or more propionate transporters.

[0413] In some embodiments, the genetically engineered bacteria comprise one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes and

one or more genetic modifications that reduce or decrease succinate import into the bacterial cell, such as any of the genetic modifications described herein and otherwise known in the art. The engineered bacterium may further comprise gene sequence(s) encoding one or more propionate transporters. The engineered bacterium may further comprise gene sequence encoding one or more succinate exporters. Thus, in some embodiments the engineered bacterium comprises one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes, one or more genetic modifications that reduce or decrease succinate import into the bacterial cell, and gene sequence(s) encoding one or more propionate transporters. In some embodiments, the engineered bacterium comprises one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes, one or more genetic modifications that reduce or decrease succinate import into the bacterial cell, and gene sequence(s) encoding one or more succinate exporters. In some embodiments, the engineered bacterium comprises one or more gene(s) or gene cassette(s) encoding one or more propionate catabolism enzymes, one or more genetic modifications that reduce or decrease succinate import into the bacterial cell, gene sequence(s) encoding one or more propionate transporters, and gene sequence(s) encoding one or more succinate exporters.

[0414] In some embodiments, certain catalytic steps are rate limiting and in such a case it may be beneficial to add additional copies of one or more gene(s) encoding one or more rate limiting enzyme(s). In a non-limiting example, the genetically engineered bacteria may encode one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and one or more additional gene(s) or gene cassette(s) encoding one or more of phaA. In a non-limiting example, the genetically engineered bacteria may one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA) and one or more additional gene(s) or gene cassette(s) encoding one or more of prpE and/or phaB and/or phaC and/or phaA.

[0415] In a non-limiting example, the genetically engineered bacteria may encode one or more MMCA pathway operon(s) e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) and one or more additional gene(s) or gene cassette(s) encoding one or more of prpE and/or accA1 and/or opccB and/or mmcE and/or mutA and/or mutB. In another non-limiting example, the genetically engineered bacteria may one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE) and one or more additional gene(s) or gene cassette(s) encoding prpB and/or prpC and/or prpD and/or prpE).

[0416] In some embodiments, each gene from a propionate catabolism pathway described herein, e.g., PHA, MMCA, and/or 2MC, can be expressed individually, each under

control of a separate (same or different) promoter. For example, one or more of prpE and/or phaB and/or phaC and/or phaA can be expressed individually, each under control of a separate (same or different) promoter. For example, one or more of prpE and/or accA1 and/or opccB and/or mmcE and/or mutA and/or mutB can be expressed individually, each under control of a separate (same or different) promoter. For example, one or more of prpB and/or prpC and/or prpD and/or prpE can be expressed individually, each under control of a separate (same or different) promoter. In some embodiments, each gene from a propionate catabolism pathway described herein, e.g., a matB comprising pathway (e.g., matA, mmcE, mutA and mutB, and/or MatB, Acc1A, and PccB, (e.g., with PrpE)) can be expressed individually, each under control of a separate (same or different) promoter.

[0417] In certain embodiments, the order of the genes within a gene cassette can be modified, e.g., to increase or decrease levels of a particular gene within a cassette. In a non-limiting example, the genetically engineered bacteria may encode one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA), in phaC comes first or phaB comes first, or prpE comes first or phaA comes first. In a non-limiting example, the genetically engineered bacteria may encode one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA), in which that phaC comes second or phaB comes second, or prpE comes second or phaA comes second. In a non-limiting example, the genetically engineered bacteria may encode one or more PHA pathway operon(s) (e.g., prpE-phaB-phaC-phaA), in which phaC comes third or phaB comes third, or prpE comes third or phaA comes third.

[0418] In a non-limiting example, the genetically engineered bacteria may encode one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), in which prpB comes first or prpC comes first or prpD comes first or prpE comes first. In a non-limiting example, the genetically engineered bacteria may encode one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), in which prpB comes second or prpC comes second or prpD comes second or prpE comes second. In a non-limiting example, the genetically engineered bacteria may encode one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), in which prpB comes third or prpC comes third or prpD comes third or prpE comes third. In a non-limiting example, the genetically engineered bacteria may encode one or more 2MC pathway operon(s) (e.g., prpB-prpC-prpD-prpE), in which prpB comes fourth or prpC comes fourth or prpD comes fourth or prpE comes fourth.

[0419] In a non-limiting example, the genetically engineered bacteria may encode one or more MMCA operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB

and mmcE-mutA-mutB) in which prpE comes first or accA1 comes first or pccB comes first or mmcE comes first or mutA comes first or mutB comes first. In a non-limiting example, the genetically engineered bacteria may encode one or more MMCA operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in which prpE comes second or accA1 comes second or pccB comes second or mmcE comes second or mutA comes second or mutB comes second. In a non-limiting example, the genetically engineered bacteria may encode one or more MMCA operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in which prpE comes third or accA1 comes third or pccB comes third or mmcE comes third or mutA comes third or mutB comes third. In a non-limiting example, the genetically engineered bacteria may encode one or more MMCA operon(s) (e.g., prpE-accA1-pccB-mmcE-mutA-mutB, or prpE-accA1-pccB and mmcE-mutA-mutB) in which prpE comes fourth, fifth or sixth or accA1 comes fourth, fifth or sixth or pccB comes fourth, fifth or sixth or mmcE comes fourth, fifth or sixth or mutA comes fourth, fifth or sixth or mutB comes fourth, fifth or sixth. In some embodiments, matB comes first, second, third, fourth, fifth, or sixth in a gene cassette comprising *matB*.

[0420] In any of the embodiments described in this section or elsewhere in the specification, any one or more the genes can be operably linked to a directly or indirectly inducible promoter, such as any of the promoters described herein, e.g., induced by low oxygen or anaerobic conditions, such as those found in the mammalian gut.

[0421] In certain embodiments, ribosome binding sites, e.g., stronger or weaker ribosome binding sites can be used to modulate (increase or decrease) the levels of expression of a propionate catabolism enzyme within a cassette.

[0422] In some embodiments, the genetically engineered bacteria further comprise mutations or deletions, e.g., in pka, succinate importers or propionate exporters, and an auxotrophy.

Host-Plasmid Mutual Dependency

[0423] In some embodiments, the genetically engineered bacteria also comprise a plasmid that has been modified to create a host-plasmid mutual dependency. In certain embodiments, the mutually dependent host-plasmid platform is GeneGuard (Wright *et al.*, 2015). In some embodiments, the GeneGuard plasmid comprises (i) a conditional origin of replication, in which the requisite replication initiator protein is provided *in trans*; (ii) an auxotrophic modification that is rescued by the host via genomic translocation and is also

compatible for use in rich media; and/or (iii) a nucleic acid sequence which encodes a broad-spectrum toxin. The toxin gene may be used to select against plasmid spread by making the plasmid DNA itself disadvantageous for strains not expressing the anti-toxin (*e.g.*, a wild-type bacterium). In some embodiments, the GeneGuard plasmid is stable for at least one-hundred generations without antibiotic selection. In some embodiments, the GeneGuard plasmid does not disrupt growth of the host. The GeneGuard plasmid is used to greatly reduce unintentional plasmid propagation in the genetically engineered bacteria described herein.

[0424] 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.

[0425] In some embodiments, the vector comprises a conditional origin of replication. In some embodiments, the conditional origin of replication is a R6K or ColE2-P9. In embodiments where the plasmid comprises the conditional origin of replication R6K, the host cell expresses the replication initiator protein π. In embodiments where the plasmid comprises the conditional origin or replication ColE2, the host cell expresses the replication initiator protein RepA. It is understood by those of skill in the art that the expression of the replication initiator protein may be regulated so that a desired expression level of the protein is achieved in the host cell to thereby control the replication of the plasmid. For example, in some embodiments, the expression of the gene encoding the replication initiator protein may be placed under the control of a strong, moderate, or weak promoter to regulate the expression of the protein.

[0426] In some embodiments, the vector comprises a gene encoding a protein required for complementation of a host cell auxotrophy, preferably a rich-media compatible auxotrophy. In some embodiments, the host cell is auxotrophic for thymidine (Δ*thyA*), and the vector comprises the thymidylate synthase (*thyA*) gene. In some embodiments, the host cell is auxotrophic for diaminopimelic acid (Δ*dapA*) and the vector comprises the 4-hydroxy-tetrahydrodipicolinate synthase (*dapA*) gene. It is understood by those of skill in the art that

the expression of the gene encoding a protein required for complementation of the host cell auxotrophy may be regulated so that a desired expression level of the protein is achieved in the host cell.

[0427] In some embodiments, the vector comprises a toxin gene. In some embodiments, the host cell comprises an anti-toxin gene encoding and/or required for the expression of an anti-toxin. In some embodiments, the toxin is Zeta and the anti-toxin is Epsilon. In some embodiments, the toxin is Kid, and the anti-toxin is Kis. In preferred embodiments, the toxin is bacteriostatic. Any of the toxin/antitoxin pairs described herein may be used in the vector systems of the present disclosure. It is understood by those of skill in the art that the expression of the gene encoding the toxin may be regulated using art known methods to prevent the expression levels of the toxin from being deleterious to a host cell that expresses the anti-toxin. For example, in some embodiments, the gene encoding the toxin may be regulated by a moderate promoter. In other embodiments, the gene encoding the toxin may be cloned adjacent to ribosomal binding site of interest to regulate the expression of the gene at desired levels (see, *e.g.*, Wright *et al.* (2015)).

Integration

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

[02] For example, **FIG. 32** depicts a map of integration sites within the *E. coli* Nissle chromosome. **FIG. 33** depicts three bacterial strains wherein the RFP gene has been successfully integrated into the bacterial chromosome at an integration site.

Secretion

[0428] 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 propionate catabolism enzyme

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.

[0429] In Gram-negative bacteria, secretion machineries may span one or both of the inner and outer membranes. In some embodiments, the genetically engineered bacteria further comprise a non-native double membrane-spanning secretion system. Double membrane-spanning secretion systems include, but are not limited to, the type I secretion system (T1SS), the type II secretion system (T2SS), the type III secretion system (T3SS), the type IV secretion system (T4SS), the type VI secretion system (T6SS), and the resistance-nodulation-division (RND) family of multi-drug efflux pumps (Pugsley 1993; Gerlach et al., 2007; Collinson et al., 2015; Costa et al., 2015; Reeves et al., 2015; WO2014138324A1, incorporated herein by reference). Examples of such secretion systems are shown in Fig. 36-38. 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).

[0430] 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, Bivrio, 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 propionate catabolism enzyme from the bacterial cytoplasm. In some embodiments, the secreted molecule, such as a heterologous protein or peptide, e.g., a propionate catabolism

enzyme, comprises a type III secretion sequence that allows the propionate catabolism enzyme to be secreted from the bacteria.

[0431] In some embodiments, a flagellar type III secretion pathway is used to secrete the molecule of interest, e.g., a propionate catabolism enzyme. In some embodiments, an incomplete flagellum is used to secrete a therapeutic peptide of interest by recombinantly fusing the peptide to an N-terminal flagellar secretion signal of a native flagellar component. In this manner, the intracellularly expressed chimeric peptide can be mobilized across the inner and outer membranes into the surrounding host environment.

[0432] In some embodiments, a Type V Autotransporter Secretion System is used to secrete the molecule of interest, e.g., therapeutic peptide. 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. 37, 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 thread 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 complimentary protease cut site in the linker. Thus, in some embodiments, the secreted molecule, such as a heterologous protein or peptide, e.g., a propionate catabolism enzyme, 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.

[0433] In some embodiments, a Hemolysin-based Secretion System is used to secrete the molecule of interest, e.g., therapeutic peptide. Type I Secretion systems offer the advantage of translocating their passenger peptide directly from the cytoplasm to the extracellular space, obviating the two-step process of other secretion types. Fig. 38 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 peptide of the present disclosure, the secretion signal-containing C-terminal portion of HlyA is fused to the C-terminal portion of a therapeutic peptide (star) to mediate secretion of this peptide.

[0434] In alternate embodiments, the genetically engineered bacteria further comprise a non-native single membrane-spanning secretion system. Single membrane-spanning transporters may act as a component of a secretion system, or may export substrates independently. Such transporters include, but are not limited to, ATP-binding cassette translocases, flagellum/virulence-related translocases, conjugation-related translocases, the general secretory system (e.g., the SecYEG complex in E. coli), the accessory secretory system in mycobacteria and several types of Gram-positive bacteria (e.g., Bacillus anthracis, Lactobacillus johnsonii, Corynebacterium glutamicum, Streptococcus gordonii, Staphylococcus aureus), and the twin-arginine translocation (TAT) system (Saier, 2006; Rigel and Braunstein, 2008; Albiniak et al., 2013). It is known that the general secretory and TAT systems can both export substrates with cleavable N-terminal signal peptides into the periplasm, and have been explored in the context of biopharmaceutical production. The TAT system may offer particular advantages, however, in that it is able to transport folded substrates, thus eliminating the potential for premature or incorrect folding. In certain embodiments, the genetically engineered bacteria comprise a TAT or a TAT-like system and are capable of secreting the propionate catabolism enzyme from the bacterial cytoplasm. One of ordinary skill in the art would appreciate that the secretion systems disclosed herein may be modified to act in different species, strains, and subtypes of bacteria, and/or adapted to deliver different payloads.

[0435] In order to translocate a protein, e.g., therapeutic polypeptide, to the extracellular space, the polypeptide must first be translated intracellularly, mobilized across the inner membrane and finally mobilized across the outer membrane. Many effector proteins (e.g., therapeutic polypeptides) – particularly those of eukaryotic origin – contain disulphide bonds to stabilize the tertiary and quaternary structures. While these bonds are capable of correctly forming in the oxidizing periplasmic compartment with the help of periplasmic chaperones, in order to translocate the polypeptide across the outer membrane the disulphide bonds must be reduced and the protein unfolded again.

[0436] One way to secrete properly folded proteins in gram-negative bacteria– particularly those requiring disulphide bonds – is to target the periplasm in bacteria with a

destabilized outer membrane. In this manner, the protein is mobilized into the oxidizing environment and allowed to fold properly. In contrast to orchestrated extracellular secretion systems, the protein is then able to escape the periplasmic space in a correctly folded form by membrane leakage. These "leaky" gram-negative mutants are therefore capable of secreting bioactive, properly disulphide-bonded polypeptides. In some embodiments, the genetically engineered bacteria have a "leaky" or de-stabilized outer membrane. Destabilizing the bacterial outer membrane to induce leakiness can be accomplished by deleting or mutagenizing genes responsible for tethering the outer membrane to the rigid peptidoglycan skeleton, including for example, lpp, ompC, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpI. Lpp is the most abundant polypeptide in the bacterial cell existing at ~500,000 copies per cell and functions as the primary 'staple' of the bacterial cell wall to the peptidoglycan. [[1.]]Silhavy, T. J., Kahne, D. & Walker, S. The bacterial cell envelope. *Cold Spring Harb Perspect Biol* **2,** a000414 (2010). TolA-PAL and OmpA complexes function similarly to Lpp and are other deletion targets to generate a leaky phenotype. Additionally, leaky phenotypes have been observed when periplasmic proteases are deactivated. The periplasm is very densely packed with protein and therefore encode several periplasmic proteins to facilitate protein turnover. Removal of periplasmic proteases such as *degS*, *degP* or *nlpI* can induce leaky phenotypes by promoting an excessive build-up of periplasmic protein. Mutation of the proteases can also preserve the effector polypeptide by preventing targeted degradation by these proteases. Moreover, a combination of these mutations may synergistically enhance the leaky phenotype of the cell without major sacrifices in cell viability. Thus, in some embodiments, the engineered bacteria have one or more deleted or mutated membrane genes. In some embodiments, the engineered bacteria have a deleted or mutated lpp gene. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from ompA, ompA, and ompF genes. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from tolA, tolB, and pal genes. In some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes. In some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes selected from degS, degP, and nlpI. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from lpp, ompA, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpI genes.

[0437] To minimize disturbances to cell viability, the leaky phenotype can be made inducible by placing one or more membrane or periplasmic protease genes, e.g., selected

from lpp, ompA, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl, under the control of an inducible promoterFor example, expression of *lpp* or other cell wall stability protein or periplasmic protease can be repressed in conditions where the therapeutic polypeptide needs to be delivered (secreted). For instance, under inducing conditions a transcriptional repressor protein or a designed antisense RNA can be expressed which reduces transcription or translation of a target membrane or periplasmic protease gene. Conversely, overexpression of certain peptides can result in a destabilized phenotype, e.g., over expression of colicins or the third topological domain of TolA, which peptide overexpression can be induced in conditions in which the therapeutic polypeptide needs to be delivered (secreted). These sorts of strategies would decouple the fragile, leaky phenotypes from biomass production. Thus, in some embodiments, the engineered bacteria have one or more membrane and/or periplasmic protease genes under the control of an inducible promoter.

[0438] **Tables 9 and 10:** The tables below lists secretion systems for Gram positive bacteria and Gram negative bacteria.

Table 9. Secretion systems for gram positive bacteria

Bacterial Strain	Relevant Secretion System
C. novyi-NT (Gram+)	Sec pathway Twin- arginine (TAT) pathway
C. butryicum (Gram+)	Sec pathway Twin- arginine (TAT) pathway
Listeria monocytogenes (Gram +)	Sec pathway Twin- arginine (TAT) pathway

Table 10. Secretion Systems for Gram negative bacteria

Protein secretary pathways (SP) in gram-negative bacteria and their descendants							
Type (Abbreviation)	Name	TC#[2]	Bacteria	Archaea	Eukarya	# Proteins/System	Energy Source
IMPS – Gram-negative bacterial inner membrane channel-forming translocases							
ABC (SIP)	ATP binding cassette translocase	3.A.1	+	+	+	3-4	ATP

SEC (IISP)	General secretory translocase	3.A.5	+	+	+	~12	GTP OR ATP + PMF
Fla/Path (IIISP)	Flagellum/virulence-related translocase	3.A.6	+	-	-	>10	ATP
Conj (IVSP)	Conjugation-related translocase	3.A.7	+	-	-	>10	ATP
Tat (IISP)	Twin-arginine targeting translocase	2.A.64	+	+	+ (chloroplasts)	2-4	PMF
Oxa1 (YidC)	Cytochrome oxidase biogenesis family	2.A.9	+	+	+ (mitochondria chloroplasts)	1	None or PMF
MscL	Large conductance mechanosensitive channel family	1.A.22	+	+	+	1	None
Holins	Holin functional superfamily	1.E.1•21	+	-	-	1	None
Eukaryotic Organelles							
MPT	Mitochondrial protein translocase	3.A.B	-	-	+ (mitochondrial)	>20	ATP
CEPT	Chloroplast envelope protein translocase	3.A.9	(+)	-	+ (chloroplasts)	≥3	GTP
Bcl-2	Eukaryotic Bcl-2 family (programmed cell death)	1.A.21	-	-	+	1?	None
Gram-negative bacterial outer membrane channel-forming translocases							
MTB (IISP)	Main terminal branch of the general secretory translocase	3.A.15	+[b]	-	-	~14	ATP; PMF

FUP AT-1	Fimbrial usher protein Autotransporter-1	1.B.11 1.B.12	+[b] +[b]	-	- -	1 1	None None
AT-2 OMF (ISP)	Autotransporter-2	1.B.40 1.B.17	+[b] +[b]	-	- +(?)	1 1	None None
TPS Secretin (IISP and IISP)		1.B.20 1.B.22	+ +[b]	-	+ -	1 1	None None
OmpIP	Outer membrane insertion porin	1.B.33	+	-	+ (mitochondria; chloroplasts)	≥4	None ?

[0439] The above tables for gram positive and gram negative bacteria list secretion systems that can be used to secrete polypeptides and other propionate catabolism enzyme from the engineered bacteria, which are reviewed in Milton H. Saier, Jr. Microbe / Volume 1, Number 9, 2006 "Protein Secretion Systems in Gram-Negative Bacteria Gram-negative bacteria possess many protein secretion-membrane insertion systems that apparently evolved independently", the contents of which is herein incorporated by reference in its entirety.

[0440] In some embodiments, one or more propionate catabolic enzymes described herein are secreted. In some embodiments, the one or more propionate catabolic enzymes described herein are further modified to improve secretion efficiency, decreased susceptibility to proteases, stability, and/or half-life. In some embodiments, PrpE is secreted, alone or in combination other propionate catabolic enzymes, e.g., with one or more of accA1, pccB, mmcE, mutA, and mutB and/or one or more of prpB, prpC, prpD, and/or one or more of phaB, phaC, phaA. In some embodiments, one or more of accA1, pccB, mmcE, mutA, mutB are secreted. In some embodiments, one or more of prpB, prpC, prpD are secreted. In some embodiments, one or more of phaB, phaC, phaA are secreted.

[0441] Alternatively, any of the enzymes expressed by the genes described herein, e.g., in **FIG. 9, FIG. 10, FIG. 15,** and **FIG. 20** may be combined.

In Vivo Models

[0442] The engineered bacteria may be evaluated *in vivo, e.g.,* in an animal model. Any suitable animal model of a disease or condition associated with catabolism of propionate

may be used. For example, a hypomorphic mouse model of propionic acidemia as described by Guenzel *et al.* can be used (see, for example, Guenzel *et al.,* 2013, *Molecular. Ther.,* 21(7):1316-1323). This *PCCA-/-* knock-out mouse lacks Pcca protein and accumulates high levels of propionylcarnitine and methyl citrate and dies within 36 hours of birth. However, the hypomorphic mouse of *PCCA-/-* (A138T) survives with elevated levels of propionic acidemia and hence it is a great model to use. Intravenous injections of adeno-associated virus 2/8 (AAV8) vectors to these hypomorphic mice reduced propionylcarnitine and methyl citrate and mediated long lasting effects. A *PCCA-/-* knock-out mouse model can also be used (see, for example, Miyazaki *et al.,* 2001, *J. Biol. Chem.,* 276:35995-35999). A mouse model of Methylmalonic Acidemia has also been described by Peters *et al.* (see, for example, Peters *et al.,* 2012, *PLoS ONE*, 7(7): e40609).

[0443] Alternatively, mouse model of methylmalonic acidemia has been generated by targeted deletion of a critical exon in the murine methylmalonyl-CoA mutase (Mut) gene (VENDITTI CP, et al/. Genetic and genomic systems to study methylmalonic acidemia (MMA) Mol Genet Metab. 2005; 84:207–208). The Mut–/– mice display early neonatal lethality and faithfully replicate the severe phenotype of affected humans and display early neonatal lethality. Studies in the Mut–/– mice have demonstrated progressive hepatic pathology and massive accumulation of methylmalonic acid in the liver near the time of death. This model has been extensively used to examine the effectiveness of rAAVs in the treatment of MMA. For example, a serotype 9 rAAV expressing the Mut cDNA effectively rescued the Mut-/- mice from lethality, conferred long-term survival, markedly improved metabolism and resulted in striking preservation of renal function and histology (Senac et al., Gene therapy in a murine model of Methylmalonic Acidemia (MMA) using rAAV9 mediated gene delivery; Gene Ther. 2012 Apr; 19(4): 385–391). Another Mut (-/-) mouse has been described by Peters et al. (Peters et al., A knock-out mouse model for methylmalonic aciduria resulting in neonatal lethality; J Biol Chem. 2003 Dec 26;278(52):52909-13 and also Peters *et al.,* 2012, *PLoS ONE*, 7(7): e40609).

[0444] The engineered bacterial cells may administered to the animal, *e.g.,* by oral gavage, and treatment efficacy is determined, *e.g.,* by measuring blood levels of propionylcarnitine, acetylcarnitine, and/or methylcitrate before and after treatment (see, for example, Guenzel *et al.,* 2013). The animal may be sacrificed, and tissue samples may be collected and analyzed. A decrease in blood levels of propionylcarnitine, acetylcarnitine,

and/or methylcitrate after treatment indicates that the engineered bacteria are effective for treating the disease.

Methods of Screening

Generation of Bacterial Strains with Enhance Ability to Transport Metabolites or Biomolecules

[0445] Due to their ease of culture, short generation times, very high population densities and small genomes, microbes can be evolved to unique phenotypes in abbreviated timescales. Adaptive laboratory evolution (ALE) is the process of passaging microbes under selective pressure to evolve a strain with a preferred phenotype. Most commonly, this is applied to increase utilization of carbon/energy sources or adapting a strain to environmental stresses (e.g., temperature, pH), whereby mutant strains more capable of growth on the carbon substrate or under stress will outcompete the less adapted strains in the population and will eventually come to dominate the population.

[0446] This same process can be extended to any essential metabolite by creating an auxotroph. An auxotroph is a strain incapable of synthesizing an essential metabolite and must therefore have the metabolite provided in the media to grow. In this scenario, by making an auxotroph and passaging it on decreasing amounts of the metabolite, the resulting dominant strains should be more capable of obtaining and incorporating this essential metabolite or biomolecule.

[0447] For example, if the biosynthetic pathway for producing a certain metabolite or biomolecule is disrupted a strain capable of high-affinity capture of said metabolite or biomolecule can be evolved via ALE. First, the strain is grown in varying concentrations of the auxotrophic amino acid or metabolite, until a minimum concentration to support growth is established. The strain is then passaged at that concentration, and diluted into lowering concentrations of the metabolite or biomolecule at regular intervals. Over time, cells that are most competitive for the metabolite or biomolecule – at growth-limiting concentrations – will come to dominate the population. These strains will likely have mutations in their metabolite-transporters resulting in increased ability to import the essential and limiting metabolite or biomolecule.

[0448] Similarly, by using an auxotroph that cannot use an upstream metabolite to form a certain metabolite or biomolecule, a strain can be evolved that not only can more efficiently imports the upstream metabolite, but also converts the metabolite into the essential downstream metabolite. These strains will also evolve mutations to increase import of the

upstream metabolite, but may also contain mutations which increase expression or reaction kinetics of downstream enzymes, or that reduce competitive substrate utilization pathways.

[0449] A metabolite innate to the microbe can be made essential via mutational auxotrophy and selection applied with growth-limiting supplementation of the endogenous metabolite. However, phenotypes capable of consuming non-native compounds can be evolved by tying their consumption to the production of an essential compound. For example, if a gene from a different organism is isolated which can produce an essential compound or a precursor to an essential compound, this gene can be recombinantly introduced and expressed in the heterologous host. This new host strain will now have the ability to synthesize an essential nutrient from a previously non-metabolizable substrate.

[0450] Hereby, a similar ALE process can be applied by creating an auxotroph incapable of converting an immediately downstream metabolite and selecting in growth-limiting amounts of the non-native compound with concurrent expression of the recombinant enzyme. This will result in mutations in the transport of the non-native substrate, expression and activity of the heterologous enzyme and expression and activity of downstream native enzymes. It should be emphasized that the key requirement in this process is the ability to tether the consumption of the non-native metabolite to the production of a metabolite essential to growth.

[0451] Once the basis of the selection mechanism is established and minimum levels of supplementation have been established, the actual ALE experimentation can proceed. Throughout this process several parameters must be vigilantly monitored. It is important that the cultures are maintained in an exponential growth phase and not allowed to reach saturation/stationary phase. This means that growth rates must be check during each passaging and subsequent dilutions adjusted accordingly. If growth rate improves to such a degree that dilutions become large, then the concentration of auxotrophic supplementation should be decreased such that growth rate is slowed, selection pressure is increased and dilutions are not so severe as to heavily bias subpopulations during passaging. In addition, at regular intervals cells should be diluted, grown on solid media and individual clones tested to confirm growth rate phenotypes observed in the ALE cultures.

[0452] Predicting when to halt the stop the ALE experiment also requires vigilance. As the success of directing evolution is tied directly to the number of mutations "screened" throughout the experiment and mutations are generally a function of errors during DNA replication, the cumulative cell divisions (CCD) acts as a proxy for total mutants which have

been screened. Previous studies have shown that beneficial phenotypes for growth on different carbon sources can be isolated in about 1011.2 CCD1. This rate can be accelerated by the addition of chemical mutagens to the cultures – such as N-methyl-N-nitro-N-nitrosoguanidine (NTG) – which causes increased DNA replication errors. However, when continued passaging leads to marginal or no improvement in growth rate the population has converged to some fitness maximum and the ALE experiment can be halted.

[0453] At the conclusion of the ALE experiment, the cells should be diluted, isolated on solid media and assayed for growth phenotypes matching that of the culture flask. Best performers from those selected are then prepped for genomic DNA and sent for whole genome sequencing. Sequencing with reveal mutations occurring around the genome capable of providing improved phenotypes, but will also contain silent mutations (those which provide no benefit but do not detract from desired phenotype). In cultures evolved in the presence of NTG or other chemical mutagen, there will be significantly more silent, background mutations. If satisfied with the best performing strain in its current state, the user can proceed to application with that strain. Otherwise the contributing mutations can be deconvoluted from the evolved strain by reintroducing the mutations to the parent strain by genome engineering techniques. See Lee, D.-H., Feist, A. M., Barrett, C. L. & Palsson, B. Ø. Cumulative Number of Cell Divisions as a Meaningful Timescale for Adaptive Laboratory Evolution of Escherichia coli. PLoS ONE 6, e26172 (2011).

[0454] Similar methods can be used to generate E. coli Nissle mutants that consume or import propionate and or one or more of its metabolites.

Pharmaceutical Compositions and Formulations

[0455] Pharmaceutical compositions comprising the genetically engineered bacteria described herein may be used to treat, manage, ameliorate, and/or prevent disorders associated with propionate catabolism. 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.

[0456] In certain embodiments, the pharmaceutical composition comprises one species, strain, or subtype of bacteria that are engineered to comprise the genetic modifications described herein, *e.g.*, to express at least one propionate catabolism gene or gene cassette. In alternate embodiments, the pharmaceutical composition comprises two or more species, strains, and/or subtypes of bacteria that are each engineered to comprise the

genetic modifications described herein, *e.g.*, to express at least one propionate catabolism gene(s) or gene cassette(s).

[0457] 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, granulates, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. Appropriate formulation depends on the route of administration.

[0458] 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.

[0459] The composition may be administered before, during, or following a meal. In one embodiment, the pharmaceutical composition is administered before the subject eats a meal. In one embodiment, the pharmaceutical composition is administered currently with a meal. In one embodiment, the pharmaceutical composition is administered after the subject eats a meal.

[0460] The genetically engineered bacteria may be formulated into pharmaceutical compositions comprising one or more pharmaceutically acceptable carriers, thickeners, diluents, buffers, buffering agents, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds, and other pharmaceutically

acceptable carriers or agents. For example, the pharmaceutical composition may include, but is not limited to, the addition of calcium bicarbonate, sodium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20. In some embodiments, the genetically engineered bacteria may be formulated in a solution of sodium bicarbonate, *e.g.*, 1 molar solution of sodium bicarbonate (to buffer an acidic cellular environment, such as the stomach, for example). The genetically engineered bacteria may be administered and formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric; phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium; calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. The genetically engineered bacteria disclosed herein may be administered topically and formulated in the form of an ointment, cream, transdermal patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form well-known to one of skill in the art. *See, e.g.,* "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA. In an embodiment, for non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity greater than water are employed. Suitable formulations include, but are not limited to, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, etc., which may be sterilized or mixed with auxiliary agents (*e.g.,* preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, *e.g.,* osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (*e.g.,* a gaseous propellant, such as freon) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms. Examples of such additional ingredients are well known in the art. In one embodiment, the pharmaceutical composition comprising the engineered bacteria may be formulated as a hygiene product. For example, the hygiene product may be an antibacterial formulation, or a fermentation product such as a fermentation broth. Hygiene products may be, for example, shampoos, conditioners, creams, pastes, lotions, and lip balms.

[0461] 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.

[0462] Tablets or capsules can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.,* pregelatinized maize starch, polyvinylpyrrolidone, hydroxypropyl methylcellulose, carboxymethylcellulose, polyethylene glycol, sucrose, glucose, sorbitol, starch, gum, kaolin, and tragacanth); fillers (*e.g., l*actose, microcrystalline cellulose, or calcium hydrogen phosphate); lubricants (*e.g.,* calcium, aluminum, zinc, stearic acid, polyethylene glycol, sodium lauryl sulfate, starch, sodium benzoate, L-leucine, magnesium stearate, talc, or silica); disintegrants (*e.g.,* starch, potato starch, sodium starch glycolate, sugars, cellulose derivatives, silica powders); or wetting agents (*e.g.,* sodium lauryl sulphate). The tablets may be coated by methods well known in the art. A coating shell may be present, and common membranes include, but are not limited to, polylactide, polyglycolic acid, polyanhydride, other biodegradable polymers, alginate-polylysine-alginate (APA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroymethylacrylate-methyl methacrylate (HEMA-MMA), multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitrile/sodium methallylsulfonate (AN-69), polyethylene glycol/poly pentamethylcyclopentasiloxane/polydimethylsiloxane (PEG/PD5/PDMS), poly N,N-dimethyl acrylamide (PDMAAm), siliceous encapsulates, cellulose sulphate/sodium alginate/polymethylene-co-guanidine (CS/A/PMCG), cellulose acetate phthalate, calcium alginate, k-carrageenan-locust bean gum gel beads, gellan-xanthan beads, poly(lactide-co-glycolides), carrageenan, starch poly-anhydrides, starch polymethacrylates, polyamino acids, and enteric coating polymers.

[0463] In some embodiments, the genetically engineered bacteria are enterically coated for release into the gut or a particular region of the gut, for example, the large

intestine. The typical pH profile from the stomach to the colon is about 1-4 (stomach), 5.5-6 (duodenum), 7.3-8.0 (ileum), and 5.5-6.5 (colon). In some diseases, the pH profile may be modified. In some embodiments, the coating is degraded in specific pH environments in order to specify the site of release. In some embodiments, at least two coatings are used. In some embodiments, the outside coating and the inside coating are degraded at different pH levels.

[0464] In some embodiments, enteric coating materials may be used, in one or more coating layers (e.g., outer, inner and/o intermediate coating layers). Enteric coated polymers remain unionized at low pH, and therefore remain insoluble. But as the pH increases in the gastrointestinal tract, the acidic functional groups are capable of ionization, and the polymer swells or becomes soluble in the intestinal fluid.

[0465] Materials used for enteric coatings include Cellulose acetate phthalate (CAP), Poly(methacrylic acid-co-methyl methacrylate), Cellulose acetate trimellitate (CAT), Poly(vinyl acetate phthalate) (PVAP) and Hydroxypropyl methylcellulose phthalate (HPMCP), fatty acids, waxes, Shellac (esters of aleurtic acid), plastics and plant fibers. Additionally, Zein, Aqua-Zein (an aqueous zein formulation containing no alcohol), amylose starch and starch derivatives, and dextrins (e.g., maltodextrin) are also used. Other known enteric coatings include ethylcellulose, methylcellulose, hydroxypropyl methylcellulose, amylose acetate phthalate, cellulose acetate phthalate, hydroxyl propyl methyl cellulose phthalate, an ethylacrylate, and a methylmethacrylate.

[0466] Coating polymers also may comprise one or more of, phthalate derivatives, CAT, HPMCAS, polyacrylic acid derivatives, copolymers comprising acrylic acid and at least one acrylic acid ester, Eudragit™ S (poly(methacrylic acid, methyl methacrylate)1:2); Eudragit L100™ S (poly(methacrylic acid, methyl methacrylate)1:1); Eudragit L30D™, (poly(methacrylic acid, ethyl acrylate)1:1); and (Eudragit L100-55) (poly(methacrylic acid, ethyl acrylate)1:1) (Eudragit™ L is an anionic polymer synthesized from methacrylic acid and methacrylic acid methyl ester), polymethyl methacrylate blended with acrylic acid and acrylic ester copolymers, alginic acid, ammonia alginate, sodium, potassium, magnesium or calcium alginate, vinyl acetate copolymers, polyvinyl acetate 30D (30% dispersion in water), a neutral methacrylic ester comprising poly(dimethylaminoethylacrylate) ("Eudragit E™), a copolymer of methylmethacrylate and ethylacrylate with trimethylammonioethyl methacrylate chloride, a copolymer of methylmethacrylate and ethylacrylate, Zein, shellac, gums, or polysaccharides, or a combination thereof.

[0467] Coating layers may also include polymers which contain Hydroxypropylmethylcellulose (HPMC), Hydroxypropylethylcellulose (HPEC), Hydroxypropylcellulose (HPC), hydroxypropylethylcellulose (HPEC), hydroxymethylpropylcellulose (HMPC), ethylhydroxyethylcellulose (EHEC) (Ethulose), hydroxyethylmethylcellulose (HEMC), hydroxymethylethylcellulose (HMEC), propylhydroxyethylcellulose (PHEC), methylhydroxyethylcellulose (M H EC), hydrophobically modified hydroxyethylcellulose (NEXTON), carboxymethyl hydroxyethylcellulose (CMHEC), Methylcellulose, Ethylcellulose, water soluble vinyl acetate copolymers, gums, polysaccharides such as alginic acid and alginates such as ammonia alginate, sodium alginate, potassium alginate, acid phthalate of carbohydrates, amylose acetate phthalate, cellulose acetate phthalate (CAP), cellulose ester phthalates, cellulose ether phthalates, hydroxypropylcellulose phthalate (HPCP), hydroxypropylethylcellulose phthalate (HPECP), hydroxyproplymethylcellulose phthalate (HPMCP), hydroxyproplymethylcellulose acetate succinate (HPMCAS).

[0468] 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.

[0469] 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.

[0470] 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.

[0471] 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.

[0472] 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.

[0473] In another embodiment, the pharmaceutical composition comprising the engineered bacteria may be a comestible product, for example, a food product. In one embodiment, the food product is milk, concentrated milk, fermented milk (yogurt, sour milk, frozen yogurt, lactic acid bacteria-fermented beverages), milk powder, ice cream, cream cheeses, dry cheeses, soybean milk, fermented soybean milk, vegetable-fruit juices, fruit juices, sports drinks, confectionery, candies, infant foods (such as infant cakes), nutritional food products, animal feeds, or dietary supplements. In one embodiment, the food product is a fermented food, such as a fermented dairy product. In one embodiment, the fermented

dairy product is yogurt. In another embodiment, the fermented dairy product is cheese, milk, cream, ice cream, milk shake, or kefir. In another embodiment, the engineered bacteria are combined in a preparation containing other live bacterial cells intended to serve as probiotics. In another embodiment, the food product is a beverage. In one embodiment, the beverage is a fruit juice-based beverage or a beverage containing plant or herbal extracts. In another embodiment, the food product is a jelly or a pudding. Other food products suitable for administration of the engineered bacteria are well known in the art. For example, see U.S. 2015/0359894 and US 2015/0238545, the entire contents of each of which are expressly incorporated herein by reference. In yet another embodiment, the pharmaceutical composition is injected into, sprayed onto, or sprinkled onto a food product, such as bread, yogurt, or cheese.

[0474] In some embodiments, the composition is formulated for intraintestinal administration, intrajejunal administration, intraduodenal administration, intraileal administration, gastric shunt administration, or intracolic administration, via nanoparticles, nanocapsules, microcapsules, or microtablets, which are enterically coated or uncoated. The pharmaceutical compositions may also be formulated in rectal compositions such as suppositories or retention enemas, using, *e.g.,* conventional suppository bases such as cocoa butter or other glycerides. The compositions may be suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain suspending, stabilizing and/or dispersing agents.

[0475] 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.

[0476] 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).

[0477] 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.

[0478] 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.

[0479] In other embodiments, the composition can be delivered in a controlled release or sustained release system. In one embodiment, a pump may be used to achieve controlled or sustained release. In another embodiment, polymeric materials can be used to achieve controlled or sustained release of the therapies of the present disclosure (see *e.g.*, U.S. Patent No. 5,989,463). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N- vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. The polymer used in a sustained release formulation may be inert, free of leachable impurities, stable on storage, sterile, and biodegradable. In some embodiments, a controlled or sustained release system can be placed in proximity of the prophylactic or therapeutic target, thus requiring only a fraction of the systemic dose. Any suitable technique known to one of skill in the art may be used.

[0480] Dosage regimens may be adjusted to provide a therapeutic response. Dosing can depend on several factors, including severity and responsiveness of the disease, route of administration, time course of treatment (days to months to years), and time to amelioration of the disease. For example, a single bolus may be administered at one time, several divided

doses may be administered over a predetermined period of time, or the dose may be reduced or increased as indicated by the therapeutic situation. The specification for the dosage is dictated by the unique characteristics of the active compound and the particular therapeutic effect to be achieved. Dosage values may vary with the type and severity of the condition to be alleviated. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgment of the treating clinician.

Toxicity and therapeutic efficacy of compounds provided herein can be determined by standard pharmaceutical procedures in cell culture or animal models. For example, LD_{50}, ED_{50}, EC_{50}, and IC_{50} may be determined, and the dose ratio between toxic and therapeutic effects (LD_{50}/ED_{50}) may be calculated as the therapeutic index. Compositions that exhibit toxic side effects may be used, with careful modifications to minimize potential damage to reduce side effects. Dosing may be estimated initially from cell culture assays and animal models. The data obtained from *in vitro* and *in vivo* assays and animal studies can be used in formulating a range of dosage for use in humans. The ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachet indicating the quantity of active agent. If the mode of administration is by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0481] 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

[0482] Another aspect of the disclosure provides methods of treating a disease associated with catabolism of propionate in a subject, or symptom(s) associated with the disease associated with the catabolism of propionate in a subject. In one embodiment, the disorder involving the catabolism of propionate is a metabolic disorder involving the abnormal catabolism of propionate. Metabolic diseases associated with abnormal catabolism of propionate include propionic acidemia (PA) and methylmalonic acidemia (MMA), as well as severe nutritional vitamin B_{12} deficiencies. In one embodiment, the disease associated with abnormal catabolism of propionate is propionic acidemia. In one embodiment, the disease associated with abnormal catabolism of propionate is methylmalonic acidemia. In another embodiment, the disease associated with abnormal catabolism of propionate is a vitamin B_{12} deficiency.

[0483] In one embodiment, the disease is propionic acidemia. Propionic acidemia, also known as propionyl-CoA carboxylase deficiency, PROP, PCC deficiency, ketotic hyperglycinemia, ketotic glycinemia, and hyper glycinemia with ketoacidosis and leukopenia, is an autosomal recessive disorder caused by impaired activity of Propionyl CoA carboxylase (PCC; EC 6.4.1.3). PCC is responsible for converting propionyl CoA into methylmalonyl CoA. Patients with PA are unable to properly process propionyl CoA, which can lead to the toxic accumulation of propionyl CoA and propionic acid in the blood, cerebrospinal fluid and tissues. Clinical manifestations of the disease vary depending on the degree of enzyme deficiency and include seizures, vomiting, lethargy, hypotonia, encephalopathy, developmental delay, failure to thrive, and secondary hyperammonemia (Deodato *et al.*, Methylmalonic and propionic aciduria, *Am. J. Med. Genet. C. Semin. Med. Genet,* 142(2):104-112, 2006).

[0484] Propionyl CoA Carboxylase (PCC) is a dodecameric enzyme comprised of alpha and beta subunits. The alpha subunit of PCC (also called PCCA; NM_000282) comprises the biotin carboxylase and biotin carboxyl carrier protein domains, while the beta subunit (also called PCCB; NM_000532) contains the carboxyltransferase activity (Diacovich *et al., Biochemistry,* 43(44):14027-14036, 2004). Mutations in either the PPCA

or PPCB genes can lead to the development of Propionic Acidemia, and more than twenty-four mutations in genes encoding PCCA or PCCB have been identified that result in Propionic Acidemia (Perez *et al., Mol. Genet Metabol.,* 78(1):59-67, 2003), including missense mutations, nonsense mutations, point exonic mutations affecting splicing, splicing mutations, insertions and deletions.

[0485] In one embodiment, the disease is methylmalonic acidemia. Methylmalonic acidemia, also known as methylmalonic aciduria or isolated methylmalonic acidemia, is an autosomal recessive disorder caused by impaired activity of one of several genes: *MUT* (OMIM 251000), *MMAA* (OMIM 251100), *MMAB* (OMIM 251110), *MMACHC* (OMIM 27740), *MMADHC* (OMIM 277410), or *LMBRD1* (OMIM 277380). However, over sixty percent of subjects with methylmalonic acidemia have mutations in the methylmalonyl CoA mutase (*MUT*) gene. MUT is responsible for converting methylmalonyl CoA into succinyl CoA and requires a vitamin B_{12}-derived prosthetic group, adenosylcoalamin (also known as AdoCbl) to function. Upon entry into the mitochondria, the mitochondrial leader sequence at the N-terminus of MUT is cleaved, and MUT monomers then associate into homodimers. The methylmalonic aciduria type A protein, mitochondrial (also known as MMAA) aides AdoCbl loading onto MUT. Similarly, Cob(I)yrinic acid, a,c-diamind adenosyltransferase, mitochondrial (MMAB), is an enzyme that catalyzes the final step in the conversion of vitamin B_{12} into adenosylcobalamin (AdoCbl). Methylmalonic aciduria and homocystinurea type C protein, mitochondrial (also known as MMACHC) and methylmalonic aciduria and homocystinuria type D protein, mitochondrial (also known as MMADHC) encode mitochondrial proteins that are also involved in vitamin B_{12} (cobalamin) synthesis.

[0486] Patients with MMA are unable to properly process methylmalonyl CoA, which can lead to the toxic accumulation of methylmalonyl CoA and methylmalonic acid in the blood, cerebrospinal fluid and tissues. Clinical manifestations of the disease vary depending on the degree of enzyme deficiency and include seizures, vomiting, lethargy, hypotonia, encephalopathy, developmental delay, failure to thrive, and secondary hyperammonemia (Deodato *et al.,* Methylmalonic and propionic aciduria, *Am. J. Med. Genet. C. Semin. Med. Genet,* 142(2):104-112, 2006).

[0487] Because of the inability to properly breakdown amino acids completely, patients having a disease associated with catabolism of propionate accumulate different byproduct molecules in their blood and urine (Carrillo-Carrasco and Venditti, Gene Reviews. Seattle (WA): University of Washington, Seattle; 1993-2015). The abnormal levels of these

by-product molecules are used as the main diagnostic criteria for diagnosing the disorder (See, *e.g.*, Table 11).

Table 11. Breakdown Products of Propionate for Use as Biomarkers

Blood metabolite	**LC-MS/MS method**
Propionylcarnitine	Yes
Methylcitrate	Yes
Glycine	Yes
Propionate	Yes (i*n vitro* assay)
Urine metabolite	**LC-MS/MS method**
3-hydroxypropionate	No
Methylcitrate	Yes
Triglylglycine	No
Propionylglycine	No

[0488] Detectable urinary organic acids useful for diagnosis and markers include, but are not limited to, N-propionylglycine, N-tiglyglycine, 2-methyl-3-oxovaleric acid, 3-hydroxy-2-methylbutyric acid, 2 methyl-3-oxobutyric acid, 3-hydroxy-n-valeric acid, 3-oxo-n-valeric acid

[0489] Currently available treatments for Propionic Acidemia and Methylmalonic Acidemia are inadequate for the long-term management of the disease and have severe limitations (Li *et al., Liver Transplantation*, 2015). A low protein diet, with micronutrient and vitamin supplementation, as necessary, is the widely accepted long-term disease management strategy for PA and MMA (Li *et al.,* 2015). However, protein-intake restrictions can be particularly problematic and result in significant morbidity. Even with proper monitoring and patient compliance, protein dietary restrictions result in a high incidence of mental retardation and mortality (Li *et al.,* 2015). Additional non-surgical chronic management regimens include L carnitine administration, antibiotics (metronidazole), Vit B12 for select MMA responsive patients (cblA>cblB>mut (-)), and amino acid dietary formulas (isoleucine/valine, glutamine, alanine supplementation), and dialysis. Further, a few cases of PA and MMA have been treated by liver transplantation (Li *et al.,* 2015), kidney transplantation or combined liver/kidney transplantation. However, the

limited availability of donor organs, the costs associated with the transplantation itself, and the undesirable effects associated with continued immunosuppressant therapy limit the practicality of liver transplantation for treatment of disease. Therefore, there is significant unmet need for effective, reliable, and/or long-term treatment for PA and MMA.

[0490] The present disclosure surprisingly demonstrates that pharmaceutical compositions comprising the engineered bacterial cells may be used to treat metabolic diseases involving the abnormal catabolism of propionate, such as PA and MMA.

[0491] In one embodiment, the subject having PA has a mutation in a *PCCA* gene. In another embodiment, the subject having PA has a mutation in the *PCCB* gene.

[0492] In one embodiment, the subject having MMA has a mutation in the *MUT* gene. In another embodiment, the subject having MMA has a mutation in the *MMAA* gene. In another embodiment, the subject having MMA has a mutation in the *MMAB* gene. In another embodiment, the subject having MMA has a mutation in the *MMACHC* gene. In another embodiment, the subject having MMA has a mutation in the *MMADHC* gene. In another embodiment, the subject having MMA has a mutation in the *LMBRD1* gene.

[0493] In another aspect, the disclosure provides methods for decreasing the plasma level of propionate, propionyl CoA, and/or methylmalonic CoA in a subject by administering a pharmaceutical composition comprising a bacterial cell to the subject, thereby decreasing the plasma level of the propionate, propionyl CoA, and/or methylmalonic CoA in the subject. In one embodiment, the subject has a disease or disorder involving the catabolism of propionate. In one embodiment, the disorder involving the catabolism of propionate is a metabolic disorder involving the abnormal catabolism of propionate. In another embodiment, the disorder involving the catabolism of propionate is propionic acidemia. In another embodiment, the disorder involving the catabolism of propionate is methylmalonic acidemia. In another embodiment, the disorder involving the catabolism of propionate is a vitamin B_{12} deficiency.

[0494] In some embodiments, the disclosure provides methods for reducing, ameliorating, or eliminating one or more symptom(s) associated with these diseases, including but not limited to seizures, vomiting, lethargy, hypotonia, encephalopathy, developmental delay, failure to thrive, liver failure, and/or secondary hyperammonemia. In some embodiments, the disease is secondary to other conditions, *e.g.*, liver disease.

[0495] In certain embodiments, the bacterial cells are capable of catabolizing propionate, propionyl CoA and/or methylmalonyl CoA in a subject in order to treat a disease

associated with catabolism of propionate. In some embodiments, the bacterial cells are delivered simultaneously with dietary protein. In another embodiment, the bacterial cells are delivered simultaneously with L-carnitine. In some embodiments, the bacterial cells and dietary protein are delivered after a period of fasting or protein-restricted dieting. In these embodiments, a patient suffering from a disorder involving the catabolism of propionate, *e.g.*, PA or MMA, may be able to resume a substantially normal diet, or a diet that is less restrictive than a protein-free or very low-protein diet. In some embodiments, the bacterial cells may be capable of catabolizing propionate, propionyl CoA, and/or methylmalonyl CoA from additional sources, *e.g.*, the blood, in order to treat a disease associated with the catabolism of propionate. In these embodiments, the bacterial cells need not be delivered simultaneously with dietary protein, and a gradient is generated, *e.g.*, from blood to gut, and the engineered bacteria catabolize the propionate, propionyl CoA, and/or methylmalonyl CoA and reduce plasma levels of the propionate, propionyl CoA, and/or methylmalonyl CoA.

[0496] The method may comprise preparing a pharmaceutical composition with at least one genetically engineered species, strain, or subtype of bacteria described herein, and administering the pharmaceutical composition to a subject in a therapeutically effective amount. In some embodiments, the genetically engineered bacteria disclosed herein are administered orally, *e.g.*, in a liquid suspension. In some embodiments, the genetically engineered bacteria are lyophilized in a gel cap and administered orally. In some embodiments, the genetically engineered bacteria are administered via a feeding tube or gastric shunt. In some embodiments, the genetically engineered bacteria are administered rectally, *e.g.*, by enema. In some embodiments, the genetically engineered bacteria are administered topically, intraintestinally, intrajejunally, intraduodenally, intraileally, and/or intracolically.

[0497] In certain embodiments, the pharmaceutical composition described herein is administered to reduce propionate, propionyl CoA, and/or methylmalonyl CoA levels in a subject. In some embodiments, the methods of the present disclosure reduce the propionate, propionyl CoA, and/or methylmalonyl CoA levels in a subject by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or more. In another embodiment, the methods of the present disclosure reduce the propionate, propionyl CoA, and/or methylmalonyl CoA levels in a subject by at least two-fold, three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold. In some embodiments, reduction is measured by comparing the propionate, propionyl CoA, and/or methylmalonyl CoA level

in a subject before and after administration of the pharmaceutical composition. In one embodiment, the propionate, propionyl CoA, and/or methylmalonyl CoA level is reduced in the gut of the subject. In another embodiment, the propionate, propionyl CoA, and/or methylmalonyl CoA level is reduced in the blood of the subject. In another embodiment, the propionate, propionyl CoA, and/or methylmalonyl CoA level is reduced in the plasma of the subject. In another embodiment, the propionate, propionyl CoA, and/or methylmalonyl CoA level is reduced in the brain of the subject.

[0498] In one embodiment, the pharmaceutical composition described herein is administered to reduce propionate, propionyl CoA, and/or methylmalonyl CoA levels in a subject to normal levels. In another embodiment, the pharmaceutical composition described herein is administered to reduce propionate, propionyl CoA, and/or methylmalonyl CoA levels in a subject to below a normal level.

[0499] In some embodiments, the method of treating the disorder involving the catabolism of propionate, *e.g.*, PA or MMA, allows one or more symptoms of the condition or disorder to improve by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more. In some embodiments, the method of treating the disorder involving the catabolism of propionate, *e.g.*, PA or MMA, allows one or more symptoms of the condition or disorder to improve by at least about two-fold, three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, or ten-fold.

[0500] Before, during, and after the administration of the pharmaceutical composition, propionate, propionyl CoA, and/or methylmalonyl CoA levels in the subject may be measured in a biological sample, such as blood, serum, plasma, urine, peritoneal fluid, cerebrospinal fluid, fecal matter, 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 disclosure to reduce levels of the propionate, propionyl CoA, and/or methylmalonyl CoA. In some embodiments, the methods may include administration of the compositions of the disclosure to reduce the propionate, propionyl CoA, and/or methylmalonyl CoA to undetectable levels in a subject. In some embodiments, the methods may include administration of the compositions of the disclosure to reduce the propionate, propionyl CoA, and/or methylmalonyl CoA concentrations to undetectable levels, or to less than about 1%, 2%, 5%,

10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% of the subject's propionate, propionyl CoA, and/or methylmalonyl CoA levels prior to treatment.

[0501] In some embodiments, the engineered bacterial cells produce a propionate catabolism enzyme under exogenous environmental conditions, such as the low-oxygen environment of the mammalian gut, to reduce levels of propionate, propionyl CoA, and/or methylmalonyl CoA in the blood or plasma by at least about 1.5-fold, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, or at least about 50-fold as compared to unmodified bacteria of the same subtype under the same conditions.

[0502] Certain unmodified bacteria will not have appreciable levels of propionyl CoA and/or methylmalonyl CoA processing. In embodiments using genetically modified forms of these bacteria, processing of propionyl CoA and/or methylmalonyl CoA will be appreciable under exogenous environmental conditions.

[0503] Propionate, propionyl CoA, and/or methylmalonyl CoA levels may be measured by methods known in the art, *e.g.,* blood sampling and mass spectrometry as described in Guenzel *et al.,* 2013, *Molecular Ther.,* 21(7):1316-1323. In some embodiments, propionate catabolism enzyme, *e.g.,* PrpBCDE, expression is measured by methods known in the art. In another embodiment, propionate catabolism enzyme activity is measured by methods known in the art to assess PrpBCDE activity (see propionate catabolism enzyme sections, *supra*). In another embodiment, propionate catabolism enzyme activity is measured by methods known in the art to assess activity of a PHA pathway circuit described herein. In another embodiment, propionate catabolism enzyme activity is measured by methods known in the art to assess the activity of a MMCA circuit described herein.

[0504] 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 engineered bacteria may be re-administered at a therapeutically effective dose and frequency. Length of Nissle residence *in vivo* in mice can be determined. In alternate embodiments, the genetically engineered bacteria are not destroyed within hours or days after administration and may propagate and colonize the gut.

[0505] In one embodiments, the bacterial cells are administered to a subject once daily. In another embodiment, the bacterial cells are administered to a subject twice daily. In another embodiment, the bacterial cells are administered to a subject three times daily. In another embodiment, the bacterial cells are administered to a subject in combination with a meal. In another embodiment, the bacterial cells are administered to a subject prior to a meal. In another embodiment, the bacterial cells are administered to a subject after a meal. The dosage of the pharmaceutical composition and the frequency of administration may be selected based on the severity of the symptoms and the progression of the disease. The appropriate therapeutically effective dose and/or frequency of administration can be selected by a treating clinician.

[0506] The methods disclosed herein may comprise administration of a composition alone or in combination with one or more additional therapies, *e.g.*, phenylbutyrate, thiamine supplementation, L-carnitine, and/or a low-protein diet. The pharmaceutical composition may be administered alone or in combination with one or more additional therapeutic agents.

[0507] An important consideration in the selection of the one or more additional therapeutic agents is that the agent(s) should be compatible with the bacteria, *e.g.*, the agent(s) must not interfere with or kill the bacteria. In some embodiments, the pharmaceutical composition is administered with food. In alternate embodiments, the pharmaceutical composition is administered before or after eating food. The pharmaceutical composition may be administered in combination with one or more dietary modifications, *e.g.*, low-protein diet and amino acid supplementation. The dosage of the pharmaceutical composition and the frequency of administration may be selected based on the severity of the symptoms and the progression of the disorder. The appropriate therapeutically effective dose and/or frequency of administration can be selected by a treating clinician.

[0508] The methods may further comprise isolating a plasma sample from the subject prior to administration of a composition and determining the level of the propionate, propionyl CoA and/or methylmalonyl CoA in the sample. In some embodiments, the methods may further comprise isolating a plasma sample from the subject after to administration of a composition and determining the level of the propionate, propionyl CoA and/or methylmalonyl CoA in the sample.

[0509] In one embodiment, the methods further comprise comparing the level of the propionate, propionyl CoA, and/or methylmalonyl CoA in the plasma sample from the subject after administration of a composition to the subject to the plasma sample from the

subject before administration of a composition to the subject. In one embodiment, a reduced level of the propionate, propionyl CoA, and/or methylmalonyl CoA in the plasma sample from the subject after administration of a composition indicates that the plasma levels of the propionate, propionyl CoA, and/or methylmalonyl CoA are decreased, thereby treating the disorder involving the catabolism of propionate in the subject. In one embodiment, the plasma level of the propionate, propionyl CoA, and/or methylmalonyl CoA is decreased at least 10%, 20%, 30%, 40$, 50%, 60%, 70%, 80%, 90%, or 100% in the sample after administration of the pharmaceutical composition as compared to the plasma level in the sample before administration of the pharmaceutical composition. In another embodiment, the plasma level of the propionate, propionyl CoA, and/or methylmalonyl CoA is decreased at least two-fold, three-fold, four-fold, or five-fold in the sample after administration of the pharmaceutical composition as compared to the plasma level in the sample before administration of the pharmaceutical composition.

[0510] In one embodiment, the methods further comprise comparing the level of the propionate, propionyl CoA, and/or methylmalonyl CoA in the plasma sample from the subject after administration of a composition to a control level of propionate, propionyl CoA, and/or methylmalonyl CoA.

[0511] The methods may further comprise isolating a plasma sample from the subject prior to administration of a composition and determining the level of the propionate, propionyl CoA and/or methylmalonyl CoA in the sample. In some embodiments, the methods may further comprise isolating a plasma sample from the subject after to administration of a composition and determining the level of the propionate, propionyl CoA and/or methylmalonyl CoA in the sample.

[0512] In another embodiment, the methods further comprise comparing the level of methylcitrate, propionylcarnitine, and/or acetylcarnitine, and/or the propionylcarnitine to acetylcarnitine ratio in the plasma sample from the subject after administration of a composition to the subject to the plasma sample from the subject before administration of a composition to the subject. In one embodiment, a reduced level of methylcitrate, propionylcarnitine, and/or acetylcarnitine the propionylcarnitine to acetylcarnitine ratio in the plasma sample from the subject after administration of a composition indicates that the plasma levels of methylcitrate, propionylcarnitine, and/or acetylcarnitine are decreased, thereby treating the disorder involving the catabolism of propionate in the subject. In one embodiment, the plasma level of methylcitrate, propionylcarnitine, and/or acetylcarnitine,

and/or the propionylcarnitine to acetylcarnitine ratio is decreased at least 10%, 20%, 30%, 40$, 50%, 60%, 70%, 80%, 90%, or 100% in the sample after administration of the pharmaceutical composition as compared to the plasma level in the sample before administration of the pharmaceutical composition. In another embodiment, the plasma level of methylcitrate, propionylcarnitine, and/or acetylcarnitine, and/or the propionylcarnitine to acetylcarnitine ratio is decreased at least two-fold, three-fold, four-fold, or five-fold in the sample after administration of the pharmaceutical composition as compared to the plasma level in the sample before administration of the pharmaceutical composition.

[0513] In one embodiment, the methods further comprise comparing the level of methylcitrate, propionylcarnitine, and/or acetylcarnitine, and/or the propionylcarnitine to acetylcarnitine ratio in the plasma sample from the subject after administration of a composition to a control level of methylcitrate, propionylcarnitine, and/or acetylcarnitine.

Examples

[0514] The present disclosure is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references, including literature references, issued patents, and published patent applications, as cited throughout this application are hereby expressly incorporated herein by reference. It should further be understood that the contents of all the figures and tables attached hereto are also expressly incorporated herein by reference.

Development of Engineered Bacterial Cells

Example 1. Construction of Plasmids Encoding Propionate Catabolism Enzymes and Propionate Transporters (*prpBCDE* operon and *mtC gene*)

[0515] Either the *prpBCDE* operon from *E. coli* strain Nissle (SEQ ID NO: 45) or *Salmonella* (SEQ ID NO: 94) are synthesized (Genewiz), fused to the Tet promoter, cloned into the high-copy plasmid pUC57-Kan by Gibson assembly, and transformed into *E. coli* DH5α as described herein to generate the plasmid pTet-prpBCDE. The *mctC* gene of *Corynebacterium* fused to the Tet promoter (SEQ ID NO: 88) is synthesized (Genewiz) and cloned into the high-copy plasmid pUC57-Kan to generate the plasmid pTet-mctC.

[0516] In certain constructs, the *prpBCDE* operon is operably linked to a FNR-responsive promoter, which may be is further fused to a strong ribosome binding site sequence. For efficient translation, each synthetic gene in the operon was separated by a 15-

base pair ribosome binding site derived from the T7 promoter/translational start site. Each gene cassette and regulatory region construct is expressed on a high-copy plasmid, a low-copy plasmid, or a chromosome.

[0517] In certain embodiments, the construct is inserted into the bacterial genome at one or more of the following insertion sites in *E. coli* Nissle: *malE/K, araC/BAD, lacZ, thyA, malP/T.* Any suitable insertion site may be used (*see, e.g.*, **FIG. 32**). The insertion site may be anywhere in the genome, *e.g.*, in a gene required for survival and/or growth, such as *thyA* (to create an auxotroph); in an active area of the genome, such as near the site of genome replication; and/or in between divergent promoters in order to reduce the risk of unintended transcription, such as between AraB and AraC of the arabinose operon. At the site of insertion, DNA primers that are homologous to the site of insertion and to the propionate construct are designed. A linear DNA fragment containing the construct with homology to the target site is generated by PCR, and lambda red recombination is performed as described below. The resulting *E. coli* Nissle bacteria are genetically engineered to express a propionate biosynthesis cassette and produce propionate.

Example 2. Construction of Plasmids Encoding Propionate Catabolism Enzymes (PHA Pathway)

[0518] First, the E. coli Nissle prpE gene and phaBCA genes from Acinetobacter sp RA3849 were codon optimized for expression in E. coli Nissle, synthesized, and were placed under the control of an aTc-inducible promoter in a single operon in a high copy plasmid the ~10-copy plasmid p15A-Kan by Golden Gate assembly, as shown in **FIG. 10C** and **FIG. 11.** Corresponding construct sequences are listed in **Table 12.**

Table 12. prpE-PhaBCA pathway circuit sequences

Description	**Sequence**	**SEQ ID NO**
Construct comprising TetR (reverse orientation, italic) and a prpE-PhaBCA gene cassette driven by a tet	Ttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaat aagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcgg catactatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatac gcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaa ccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgt acctaaatgtacttttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgttat tacgtaaaaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatcta acatctcaatggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgta	SEQ ID NO:22

promoter (italic) (as shown in **FIG. 11**); ribosome binding sites are underlined; L3S2P11 terminator in italics and underline; his terminator in bold.	ggctgctctacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctca ttaagcagctctaatgcgctgttaatcactttacttttatctaatctagacatcat*TAATTC* *CTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTAC* *CACTCCCTATCAGTGATAGAGAAAAGTGAATAAGGCG*TAA GTTCAACAGGAGAGCATTATGTCTTTTAGCGAATTTTA TCAGCGTTCGATTAACGAACCGGAGAAGTTCTGGGCC GAGCAGGCCCGGCGTATTGACTGGCAGACGCCCTTTA CGCAAACGCTCGACCACAGCAACCCGCCGTTTGCCCG TTGGTTTTGTGAAGGCCGAACCAACTTGTGTCACAAC GCTATCGACCGCTGGCTGGAGAAACAGCCAGAGGCGC TGGCATTGATTGCCGTCTCTTCGGAAACAGAGGAAGA GCGTACCTTTACCTTCCGCCAGTTACATGACGAAGTGA ATGCGGTGGCGTCAATGCTGCGCTCACTGGGCGTGCA GCGTGGCGATCGGGTGCTGGTGTATATGCCGATGATT GCCGAAGCGCATATTACCCTGCTGGCCTGCGCGCGCA TTGGTGCTATTCACTCGGTGGTGTTTGGGGGATTTGCT TCGCACAGCGTGGCAACGCGAATTGATGACGCTAAAC CGGTGCTGATTGTCTCGGCTGATGCCGGGGCGCGCGG CGGTAAAATCATTCCGTATAAAAAATTGCTCGACGAT GCGATAAGTCAGGCACAGCATCAGCCGCGTCACGTTT TACTGGTGGATCGCGGGCTGGCGAAAATGGCGCGCGT TAGCGGGCGGGATGTCGATTTCGCGTCGTTGCGCCAT CAACACATCGGCGCGCGGGTGCCGGTGGCATGGCTGG AATCCAACGAAACCTCCTGCATTCTCTACACCTCCGGC ACGACCGGCAAACCTAAAGGTGTGCAGCGTGATGTCG GCGGATATGCGGTGGCGCTGGCGACCTCGATGGACAC CATTTTTGGCGGCAAAGCGGGCGGCGTGTTCTTTTGTG CTTCGGATATCGGCTGGGTGGTAGGGCATTCGTATATC GTTTACGCGCCGCTGCTGGCGGGGATGGCGACTATCG TTTACGAAGGATTGCCGACCTGGCCGGACTGCGGCGT GTGGTGGAAAATTGTCGAGAAATATCAGGTTAGCCGC ATGTTCTCAGCGCCGACCGCCATTCGCGTGCTGAAAA AATTCCCTACCGCTGAAATTCGCAAACACGATCTTTCG TCGCTGGAAGTGCTCTATCTGGCTGGAGAACCGCTGG ACGAGCCGACCGCCAGTTGGGTGAGCAATACGCTGGA TGTGCCGGTCATCGACAACTACTGGCAGACCGAATCC GGCTGGCCGATTATGGCGATTGCTCGCGGTCTGGATG ACAGACCGACGCGTCTGGGAAGCCCCGGCGTGCCGAT GTATGGCTATAACGTGCAGTTGCTCAATGAAGTCACC GGCGAACCGTGTGGCGTCAATGAGAAAGGGATGCTGG TAGTGGAGGGGCCATTGCCGCCAGGCTGTATTCAAAC CATCTGGGGCGACGACGACCGCTTTGTGAAGACGTAC TGGTCGCTGTTTTCCCGTCCGGTGTACGCCACTTTTGA CTGGGGCATCCGCGATGCTGACGGTTATCACTTTATTC TCGGGCGCACTGACGATGTGATTAACGTTGCCGGACA TCGGCTGGGTACGCGTGAGATTGAAGAGAGTATCTCC AGTCATCCGGGCGTTGCCGAAGTGGCGGTGGTTGGGG TGAAAGATGCGCTGAAAGGGCAGGTGGCGGTGGCGTT TGTCATTCCGAAAGAGAGCGACAGTCTGGAAGACCGT	

GAGGTGGCGCACTCGCAAGAGAAGGCGATTATGGCGC
TGGTGGACAGCCAGATTGGCAACTTTGGCCGCCCGGC
GCACGTCTGGTTTGTCTCGCAATTGCCAAAAACGCGA
TCCGGAAAAATGCTGCGCCGCACGATCCAGGCGATTT
GCGAAGGACGCGATCCTGGGGATCTGACGACCATTGA
TGATCCGGCGTCGTTGGATCAGATCCGCCAGGCGATG
GAAGAGTAGTACTGATCAAAAAGGTTAGCCTCAAGAG
GGTCATAAAAATGTCAGAGCAGAAAGTAGCTCTGGTT
ACCGGTGCGTTAGGTGGTATCGGAAGTGAGATCTGCC
GCCAGCTTGTGACCGCCGGGTACAAGATTATCGCCAC
CGTTGTTCCACGCGAAGAAGACCGCGAAAAACAATGG
TTGCAAAGTGAGGGGTTTCAAGACTCTGATGTGCGTTT
CGTATTAACAGATTTAAACAATCACGAAGCTGCGACA
GCGGCAATTCAAGAAGCGATTGCCGCCGAAGGACGCG
TTGATGTATTGGTCAACAACGCGGGGATCACGCGCGA
TGCTACATTTAAGAAAATGTCCTATGAGCAATGGTCC
CAAGTCATCGACACGAATTTAAAGACTCTTTTTACCGT
GACCCAGCCAGTATTTAATAAAATGCTTGAACAGAAG
TCTGGCCGCATCGTAAACATTAGCTCTGTCAATGGTTT
AAAAGGGCAATTTGGTCAAGCCAACTACTCGGCCTCG
AAAGCAGGGATTATCGGGTTTACTAAAGCATTGGCGC
AGGAGGGTGCTCGCTCGAACATTTGCGTCAATGTCGT
TGCTCCTGGTTACACAGCGACACCCATGGTCACAGCA
ATGCGCGAGGATGTAATTAAGTCAATCGAAGCTCAAA
TTCCCCTGCAACGTCTGGCAGCACCGGCGGAGATTGC
GGCAGCGGTTATGTATTTGGTGAGTGAACACGGTGCA
TACGTGACGGGCGAAACTTTGAGTATCAACGGCGGGC
TGTACATGCACTAAAGGTGCTTTTAGTCTAGCGCTAGA
GCAGGTACCATATTAATGAATCCAAATTCCTTTCAGTT
TAAAGAGAATATCTTACAGTTTTTCAGCGTGCACGAC
GATATTTGGAAAAAACTGCAGGAATTTTACTATGGAC
AATCGCCCATCAATGAAGCGTTGGCGCAGTTAAATAA
GGAAGACATGAGTTTATTCTTCGAGGCGTTATCAAAA
AACCCTGCTCGTATGATGGAGATGCAGTGGTCCTGGT
GGCAAGGGCAGATTCAAATTTACCAGAACGTGTTAAT
GCGTAGTGTAGCCAAGGACGTAGCCCCCTTTATCCAG
CCAGAGTCCGGAGATCGTCGCTTCAACTCGCCACTTTG
GCAAGAACATCCAAATTTTGATTTACTGAGTCAATCCT
ACTTGTTGTTTTCTCAGTTGGTTCAAAATATGGTGGAT
GTCGTTGAAGGAGTACCTGATAAGGTCCGCTATCGCA
TCCATTTCTTTACACGTCAGATGATCAATGCGTTGTCT
CCTTCTAATTTCCTGTGGACGAACCCTGAAGTAATTCA
ACAGACGGTCGCTGAACAGGGTGAGAATTTAGTACGC
GGGATGCAAGTATTTCACGATGATGTAATGAATTCGG
GTAAATATTTGAGCATCCGTATGGTAAATAGCGACAG
TTTCTCTCTTGGCAAGGACTTGGCGTATACGCCAGGAG
CCGTAGTTTTCGAGAACGACATCTTTCAGCTTCTTCAA
TACGAAGCCACAACCGAGAACGTATATCAAACCCCTA
TTCTTGTCGTACCTCCCTTCATCAACAAGTACTACGTG

CTGGACCTGCGCGAACAGAATAGCTTGGTTAATTGGC
TGCGCCAACAAGGACATACGGTGTTTTTGATGTCGTG
GCGTAACCCCAACGCAGAGCAGAAGGAGCTTACCTTC
GCTGACTTAATTACCCAAGGATCGGTAGAAGCATTAC
GTGTTATCGAAGAAATCACGGGAGAGAAAGAAGCTA
ACTGTATTGGATATTGCATCGGTGGTACACTTCTGGCT
GCTACCCAGGCATATTATGTAGCTAAACGCCTGAAAA
ATCACGTAAAGTCAGCGACTTATATGGCGACGATTAT
TGATTTTGAGAACCCCGGCTCATTGGGTGTTTTCATTA
ATGAGCCGGTCGTAAGTGGACTTGAAAACCTTAATAA
TCAACTTGGTTACTTCGACGGGCGTCAACTTGCAGTGA
CATTTTCGTTGTTGCGCGAAAACACCTTGTATTGGAAT
TATTACATCGATAATTACTTGAAGGGTAAGGAACCGT
CCGACTTTGACATCTTATACTGGAACTCGGATGGTACG
AATATCCCAGCAAAGATTCACAATTTCCTGTTACGTAA
CCTTTATCTTAACAACGAACTTATTTCTCCAAATGCCG
TCAAAGTTAATGGTGTGGGTTTAAACCTTTCGCGCGTG
AAGACTCCATCATTCTTCATTGCTACGCAGGAGGACC
ATATCGCATTGTGGGATACCTGTTTTCGCGGCGCGGAT
TACCTGGGGGGTGAGAGCACACTTGTGCTTGGGGAAA
GCGGACACGTCGCCGGCATTGTCAACCCGCCTTCTCGT
AACAAGTATGGTTGTTACACGAACGCCGCCAAGTTTG
AAAATACCAAGCAATGGCTTGACGGTGCAGAATATCA
TCCCGAAAGCTGGTGGTTACGTTGGCAGGCATGGGTC
ACGCCTTATACTGGAGAGCAGGTTCCTGCGCGTAATTT
GGGAAACGCACAGTACCCCAGTATTGAAGCGGCCCCT
GGGCGTTATGTGCTGGTAAACCTGTTTTAACGCTCACA
TACAAGCAATCTATAATTATTCACGGTATAAATGAAA
GATGTTGTTATCGTAGCCGCTAAACGCACTGCGATCG
GTTCCTTTCTGGGGAGTCTGGCTTCCCTGAGCGCCCCT
CAGTTGGGTCAGACGGCTATCCGCGCAGTTTTGGATTC
TGCAAATGTGAAACCAGAACAAGTGGACCAAGTAATT
ATGGGGAATGTGCTGACCACCGGCGTTGGGCAAAATC
CTGCTCGTCAGGCAGCAATCGCCGCTGGGATTCCTGT
ACAAGTTCCCGCCAGCACGCTTAATGTAGTGTGTGGG
TCCGGATTACGTGCCGTTCACCTGGCAGCTCAAGCCAT
CCAATGCGATGAAGCCGATATCGTCGTTGCCGGAGGT
CAAGAATCAATGTCCCAGTCTGCTCATTACATGCAGCT
TCGCAATGGCCAGAAAATGGGTAACGCACAGTTAGTC
GATTCAATGGTGGCCGACGGCTTGACCGACGCGTATA
ATCAATACCAGATGGGTATCACCGCGGAGAATATCGT
CGAAAAACTTGGTCTTAATCGTGAAGAACAAGACCAG
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AGGCTGCCGGAAAATTCAAGGATGAAATTGCGGTCGT
TTCGATTCCCCAGCGCAAAGGAGAGCCGGTCGTCTTC
GCGGAAGACGAATATATCAAGGCCAATACCTCGTTGG
AATCCTTGACGAAACTGCGTCCAGCATTCAAAAAAGA
CGGTTCTGTTACAGCCGGCAACGCATCTGGCATTAAT
GATGGGGCAGCCGCGGTCCTGATGATGTCCGCCGACA

	AAGCGGCTGAACTGGGCTTAAAGCCTTTAGCACGCAT TAAAGGTTACGCGATGTCAGGAATTGAGCCGGAAATC ATGGGACTGGGTCCTGTAGACGCCGTTAAGAAAACCC TTAATAAGGCTGGTTGGTCCTTAGACCAGGTCGATCTG ATCGAGGCCAATGAGGCTTTTGCTGCCCAAGCACTGG GAGTAGCCAAGGAGCTTGGGCTGGACCTGGACAAGGT AAATGTTAACGGAGGTGCGATCGCGCTGGGACACCCG ATCGGGGCTTCGGGTTGTCGTATCTTGGTCACGTTATT ACACGAAATGCAGCGTCGTGATGCAAAGAAGGGTATC GCCACATTGTGTGTGGGAGGTGGAATGGGGGTGGCGC TTGCCGTTGAGCGCGATTAAGGAGGTCGGATAAGGCG CTCGCGCCGCATCCGACACCGTGCGCAGATGCCTGAT GCGACGCTGACGCGTCTTATCATGCCTCGCTCTCGAGT CCCGTCAAGTCAGACGATCGCACGCCCCATGTGAACG ATTGGTAAACCCGGTG**AACGCATGAGAAAGCCCCCG** **GAAGATCACCTTCCGGGGGCTTTTTTATTGCGC***GG* *ACCAAAACGAAAAAAGACGCTCGAAAGCGTCTCTTTTCTG* *GAATTTGGTACCGAG*GCGTAATGCTCTGCCAGTGTTAC AACCAATTAACCAATTCTGAT	
Construct comprising a prpE-PhaBCA gene cassette under the control of the Ptet promoter(italic) (as shown in **FIG. 11**) ribosome binding sites are underlined ;.L3S2P11 terminator in italics and underline; his terminator in bold	*TAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTA* *TTTTACCACTCCCTATCAGTGATAGAGAAAAGTGAATAAG* *GCG*TAAGGCGTAAGTTCAACAGGAGAGCATTATGTCT TTTAGCGAATTTTATCAGCGTTCGATTAACGAACCGGA GAAGTTCTGGGCCGAGCAGGCCCGGCGTATTGACTGG CAGACGCCCTTTACGCAAACGCTCGACCACAGCAACC CGCCGTTTGCCCGTTGGTTTTGTGAAGGCCGAACCAAC TTGTGTCACAACGCTATCGACCGCTGGCTGGAGAAAC AGCCAGAGGCGCTGGCATTGATTGCCGTCTCTTCGGA AACAGAGGAAGAGCGTACCTTTACCTTCCGCCAGTTA CATGACGAAGTGAATGCGGTGGCGTCAATGCTGCGCT CACTGGGCGTGCAGCGTGGCGATCGGGTGCTGGTGTA TATGCCGATGATTGCCGAAGCGCATATTACCCTGCTG GCCTGCGCGCGCATTGGTGCTATTCACTCGGTGGTGTT TGGGGGATTTGCTTCGCACAGCGTGGCAACGCGAATT GATGACGCTAAACCGGTGCTGATTGTCTCGGCTGATG CCGGGGCGCGCGGCGGTAAAATCATTCCGTATAAAAA ATTGCTCGACGATGCGATAAGTCAGGCACAGCATCAG CCGCGTCACGTTTTACTGGTGGATCGCGGGCTGGCGA AAATGGCGCGCGTTAGCGGGCGGGATGTCGATTTCGC GTCGTTGCGCCATCAACACATCGGCGCGCGGGTGCCG GTGGCATGGCTGGAATCCAACGAAACCTCCTGCATTC TCTACACCTCCGGCACGACCGGCAAACCTAAAGGTGT GCAGCGTGATGTCGGCGGATATGCGGTGGCGCTGGCG ACCTCGATGGACACCATTTTTGGCGGCAAAGCGGGCG GCGTGTTCTTTTGTGCTTCGGATATCGGCTGGGTGGTA GGGCATTCGTATATCGTTTACGCGCCGCTGCTGGCGG GGATGGCGACTATCGTTTACGAAGGATTGCCGACCTG GCCGGACTGCGGCGTGTGGTGGAAAATTGTCGAGAAA TATCAGGTTAGCCGCATGTTCTCAGCGCCGACCGCCAT	SEQ ID NO: 23

TCGCGTGCTGAAAAAATTCCCTACCGCTGAAATTCGC
AAACACGATCTTTCGTCGCTGGAAGTGCTCTATCTGGC
TGGAGAACCGCTGGACGAGCCGACCGCCAGTTGGGTG
AGCAATACGCTGGATGTGCCGGTCATCGACAACTACT
GGCAGACCGAATCCGGCTGGCCGATTATGGCGATTGC
TCGCGGTCTGGATGACAGACCGACGCGTCTGGGAAGC
CCCGGCGTGCCGATGTATGGCTATAACGTGCAGTTGC
TCAATGAAGTCACCGGCGAACCGTGTGGCGTCAATGA
GAAAGGGATGCTGGTAGTGGAGGGGCCATTGCCGCCA
GGCTGTATTCAAACCATCTGGGGCGACGACGACCGCT
TTGTGAAGACGTACTGGTCGCTGTTTTCCCGTCCGGTG
TACGCCACTTTTGACTGGGGCATCCGCGATGCTGACG
GTTATCACTTTATTCTCGGGCGCACTGACGATGTGATT
AACGTTGCCGGACATCGGCTGGGTACGCGTGAGATTG
AAGAGAGTATCTCCAGTCATCCGGGCGTTGCCGAAGT
GGCGGTGGTTGGGGTGAAAGATGCGCTGAAAGGGCA
GGTGGCGGTGGCGTTTGTCATTCCGAAAGAGAGCGAC
AGTCTGGAAGACCGTGAGGTGGCGCACTCGCAAGAGA
AGGCGATTATGGCGCTGGTGGACAGCCAGATTGGCAA
CTTTGGCCGCCCGGCGCACGTCTGGTTTGTCTCGCAAT
TGCCAAAAACGCGATCCGGAAAAATGCTGCGCCGCAC
GATCCAGGCGATTTGCGAAGGACGCGATCCTGGGGAT
CTGACGACCATTGATGATCCGGCGTCGTTGGATCAGA
TCCGCCAGGCGATGGAAGAGTAGTACTGATCAAAAAG
GTTAGCCTCAAGAGGGTCATAAAAATGTCAGAGCAGA
AAGTAGCTCTGGTTACCGGTGCGTTAGGTGGTATCGG
AAGTGAGATCTGCCGCCAGCTTGTGACCGCCGGGTAC
AAGATTATCGCCACCGTTGTTCCACGCGAAGAAGACC
GCGAAAAACAATGGTTGCAAAGTGAGGGGTTTCAAGA
CTCTGATGTGCGTTTCGTATTAACAGATTTAAACAATC
ACGAAGCTGCGACAGCGGCAATTCAAGAAGCGATTGC
CGCCGAAGGACGCGTTGATGTATTGGTCAACAACGCG
GGGATCACGCGCGATGCTACATTTAAGAAAATGTCCT
ATGAGCAATGGTCCCAAGTCATCGACACGAATTTAAA
GACTCTTTTTACCGTGACCCAGCCAGTATTTAATAAAA
TGCTTGAACAGAAGTCTGGCCGCATCGTAAACATTAG
CTCTGTCAATGGTTTAAAAGGGCAATTTGGTCAAGCC
AACTACTCGGCCTCGAAAGCAGGGATTATCGGGTTTA
CTAAAGCATTGGCGCAGGAGGGTGCTCGCTCGAACAT
TTGCGTCAATGTCGTTGCTCCTGGTTACACAGCGACAC
CCATGGTCACAGCAATGCGCGAGGATGTAATTAAGTC
AATCGAAGCTCAAATTCCCCTGCAACGTCTGGCAGCA
CCGGCGGAGATTGCGGCAGCGGTTATGTATTTGGTGA
GTGAACACGGTGCATACGTGACGGGCGAAACTTTGAG
TATCAACGGCGGGCTGTACATGCACTAAAGGTGCTTT
TAGTCTAGCGCTAGAGCAGGTACCATATTAATGAATC
CAAATTCCTTTCAGTTTAAAGAGAATATCTTACAGTTT
TTCAGCGTGCACGACGATATTTGGAAAAAACTGCAGG
AATTTTACTATGGACAATCGCCCATCAATGAAGCGTT

GGCGCAGTTAAATAAGGAAGACATGAGTTTATTCTTC
GAGGCGTTATCAAAAAACCCTGCTCGTATGATGGAGA
TGCAGTGGTCCTGGTGGCAAGGGCAGATTCAAATTTA
CCAGAACGTGTTAATGCGTAGTGTAGCCAAGGACGTA
GCCCCCTTTATCCAGCCAGAGTCCGGAGATCGTCGCTT
CAACTCGCCACTTTGGCAAGAACATCCAAATTTTGATT
TACTGAGTCAATCCTACTTGTTGTTTTCTCAGTTGGTTC
AAAATATGGTGGATGTCGTTGAAGGAGTACCTGATAA
GGTCCGCTATCGCATCCATTTCTTTACACGTCAGATGA
TCAATGCGTTGTCTCCTTCTAATTTCCTGTGGACGAAC
CCTGAAGTAATTCAACAGACGGTCGCTGAACAGGGTG
AGAATTTAGTACGCGGGATGCAAGTATTTCACGATGA
TGTAATGAATTCGGGTAAATATTTGAGCATCCGTATG
GTAAATAGCGACAGTTTCTCTCTTGGCAAGGACTTGG
CGTATACGCCAGGAGCCGTAGTTTTCGAGAACGACAT
CTTTCAGCTTCTTCAATACGAAGCCACAACCGAGAAC
GTATATCAAACCCCTATTCTTGTCGTACCTCCCTTCAT
CAACAAGTACTACGTGCTGGACCTGCGCGAACAGAAT
AGCTTGGTTAATTGGCTGCGCCAACAAGGACATACGG
TGTTTTTGATGTCGTGGCGTAACCCCAACGCAGAGCA
GAAGGAGCTTACCTTCGCTGACTTAATTACCCAAGGA
TCGGTAGAAGCATTACGTGTTATCGAAGAAATCACGG
GAGAGAAAGAAGCTAACTGTATTGGATATTGCATCGG
TGGTACACTTCTGGCTGCTACCCAGGCATATTATGTAG
CTAAACGCCTGAAAAATCACGTAAAGTCAGCGACTTA
TATGGCGACGATTATTGATTTTGAGAACCCCGGCTCAT
TGGGTGTTTTCATTAATGAGCCGGTCGTAAGTGGACTT
GAAAACCTTAATAATCAACTTGGTTACTTCGACGGGC
GTCAACTTGCAGTGACATTTTCGTTGTTGCGCGAAAAC
ACCTTGTATTGGAATTATTACATCGATAATTACTTGAA
GGGTAAGGAACCGTCCGACTTTGACATCTTATACTGG
AACTCGGATGGTACGAATATCCCAGCAAAGATTCACA
ATTTCCTGTTACGTAACCTTTATCTTAACAACGAACTT
ATTTCTCCAAATGCCGTCAAAGTTAATGGTGTGGGTTT
AAACCTTTCGCGCGTGAAGACTCCATCATTCTTCATTG
CTACGCAGGAGGACCATATCGCATTGTGGGATACCTG
TTTTCGCGGCGCGGATTACCTGGGGGGTGAGAGCACA
CTTGTGCTTGGGGAAAGCGGACACGTCGCCGGCATTG
TCAACCCGCCTTCTCGTAACAAGTATGGTTGTTACACG
AACGCCGCCAAGTTTGAAAATACCAAGCAATGGCTTG
ACGGTGCAGAATATCATCCCGAAAGCTGGTGGTTACG
TTGGCAGGCATGGGTCACGCCTTATACTGGAGAGCAG
GTTCCTGCGCGTAATTTGGGAAACGCACAGTACCCCA
GTATTGAAGCGGCCCCTGGGCGTTATGTGCTGGTAAA
CCTGTTTTAACGCTCACATACAAGCAATCTATAATTAT
TCACGGTATAAATGAAAGATGTTGTTATCGTAGCCGC
TAAACGCACTGCGATCGGTTCCTTTCTGGGGAGTCTGG
CTTCCCTGAGCGCCCCTCAGTTGGGTCAGACGGCTATC
CGCGCAGTTTTGGATTCTGCAAATGTGAAACCAGAAC

	AAGTGGACCAAGTAATTATGGGGAATGTGCTGACCAC CGGCGTTGGGCAAAATCCTGCTCGTCAGGCAGCAATC GCCGCTGGGATTCCTGTACAAGTTCCCGCCAGCACGC TTAATGTAGTGTGTGGGTCCGGATTACGTGCCGTTCAC CTGGCAGCTCAAGCCATCCAATGCGATGAAGCCGATA TCGTCGTTGCCGGAGGTCAAGAATCAATGTCCCAGTC TGCTCATTACATGCAGCTTCGCAATGGCCAGAAAATG GGTAACGCACAGTTAGTCGATTCAATGGTGGCCGACG GCTTGACCGACGCGTATAATCAATACCAGATGGGTAT CACCGCGGAGAATATCGTCGAAAAACTTGGTCTTAAT CGTGAAGAACAAGACCAGCTTGCTCTGACAAGTCAAC AACGTGCTGCAGCAGCGCAGGCTGCCGGAAAATTCAA GGATGAAATTGCGGTCGTTTCGATTCCCCAGCGCAAA GGAGAGCCGGTCGTCTTCGCGGAAGACGAATATATCA AGGCCAATACCTCGTTGGAATCCTTGACGAAACTGCG TCCAGCATTCAAAAAAGACGGTTCTGTTACAGCCGGC AACGCATCTGGCATTAATGATGGGGCAGCCGCGGTCC TGATGATGTCCGCCGACAAAGCGGCTGAACTGGGCTT AAAGCCTTTAGCACGCATTAAAGGTTACGCGATGTCA GGAATTGAGCCGGAAATCATGGGACTGGGTCCTGTAG ACGCCGTTAAGAAAACCCTTAATAAGGCTGGTTGGTC CTTAGACCAGGTCGATCTGATCGAGGCCAATGAGGCT TTTGCTGCCCAAGCACTGGGAGTAGCCAAGGAGCTTG GGCTGGACCTGGACAAGGTAAATGTTAACGGAGGTGC GATCGCGCTGGGACACCCGATCGGGGCTTCGGGTTGT CGTATCTTGGTCACGTTATTACACGAAATGCAGCGTCG TGATGCAAAGAAGGGTATCGCCACATTGTGTGTGGGA GGTGGAATGGGGGTGGCGCTTGCCGTTGAGCGCGATT AAGGAGGTCGGATAAGGCGCTCGCGCCGCATCCGACA CCGTGCGCAGATGCCTGATGCGACGCTGACGCGTCTT ATCATGCCTCGCTCTCGAGTCCCGTCAAGTCAGACGAT CGCACGCCCCATGTGAACGATTGGTAAACCCGGTG**AA** **CGCATGAGAAAGCCCCCGGAAGATCACCTTCCGGG** **GGCTTTTTTATTGCGC***GGACCAAAACGAAAAAAGACGC* *TCGAAAGCGTCTCTTTTCTGGAATTTGGTACCGAG*GCGTA ATGCTCTGCCAGTGTTACAACCAATTAACCAATTCTGA T	
Construct comprising a prpE-PhaBCA gene cassette; (as shown in **FIG. 11**) ribosome binding sites are underlined	TAAGGCGTAAGTTCAACAGGAGAGCATTATGTCTTTT AGCGAATTTTATCAGCGTTCGATTAACGAACCGGAGA AGTTCTGGGCCGAGCAGGCCCGGCGTATTGACTGGCA GACGCCCTTTACGCAAACGCTCGACCACAGCAACCCG CCGTTTGCCCGTTGGTTTTGTGAAGGCCGAACCAACTT GTGTCACAACGCTATCGACCGCTGGCTGGAGAAACAG CCAGAGGCGCTGGCATTGATTGCCGTCTCTTCGGAAA CAGAGGAAGAGCGTACCTTTACCTTCCGCCAGTTACA TGACGAAGTGAATGCGGTGGCGTCAATGCTGCGCTCA CTGGGCGTGCAGCGTGGCGATCGGGTGCTGGTGTATA TGCCGATGATTGCCGAAGCGCATATTACCCTGCTGGC CTGCGCGCGCATTGGTGCTATTCACTCGGTGGTGTTTG	SEQ ID NO: 24

GGGGATTTGCTTCGCACAGCGTGGCAACGCGAATTGA
TGACGCTAAACCGGTGCTGATTGTCTCGGCTGATGCC
GGGGCGCGCGGCGGTAAAATCATTCCGTATAAAAAAT
TGCTCGACGATGCGATAAGTCAGGCACAGCATCAGCC
GCGTCACGTTTTACTGGTGGATCGCGGGCTGGCGAAA
ATGGCGCGCGTTAGCGGGCGGGATGTCGATTTCGCGT
CGTTGCGCCATCAACACATCGGCGCGCGGGTGCCGGT
GGCATGGCTGGAATCCAACGAAACCTCCTGCATTCTC
TACACCTCCGGCACGACCGGCAAACCTAAAGGTGTGC
AGCGTGATGTCGGCGGATATGCGGTGGCGCTGGCGAC
CTCGATGGACACCATTTTTGGCGGCAAAGCGGGCGGC
GTGTTCTTTTGTGCTTCGGATATCGGCTGGGTGGTAGG
GCATTCGTATATCGTTTACGCGCCGCTGCTGGCGGGG
ATGGCGACTATCGTTTACGAAGGATTGCCGACCTGGC
CGGACTGCGGCGTGTGGTGGAAAATTGTCGAGAAATA
TCAGGTTAGCCGCATGTTCTCAGCGCCGACCGCCATTC
GCGTGCTGAAAAAATTCCCTACCGCTGAAATTCGCAA
ACACGATCTTTCGTCGCTGGAAGTGCTCTATCTGGCTG
GAGAACCGCTGGACGAGCCGACCGCCAGTTGGGTGAG
CAATACGCTGGATGTGCCGGTCATCGACAACTACTGG
CAGACCGAATCCGGCTGGCCGATTATGGCGATTGCTC
GCGGTCTGGATGACAGACCGACGCGTCTGGGAAGCCC
CGGCGTGCCGATGTATGGCTATAACGTGCAGTTGCTC
AATGAAGTCACCGGCGAACCGTGTGGCGTCAATGAGA
AAGGGATGCTGGTAGTGGAGGGGCCATTGCCGCCAGG
CTGTATTCAAACCATCTGGGGCGACGACGACCGCTTT
GTGAAGACGTACTGGTCGCTGTTTTCCCGTCCGGTGTA
CGCCACTTTTGACTGGGGCATCCGCGATGCTGACGGTT
ATCACTTTATTCTCGGGCGCACTGACGATGTGATTAAC
GTTGCCGGACATCGGCTGGGTACGCGTGAGATTGAAG
AGAGTATCTCCAGTCATCCGGGCGTTGCCGAAGTGGC
GGTGGTTGGGGTGAAAGATGCGCTGAAAGGGCAGGT
GGCGGTGGCGTTTGTCATTCCGAAAGAGAGCGACAGT
CTGGAAGACCGTGAGGTGGCGCACTCGCAAGAGAAG
GCGATTATGGCGCTGGTGGACAGCCAGATTGGCAACT
TTGGCCGCCCGGCGCACGTCTGGTTTGTCTCGCAATTG
CCAAAAACGCGATCCGGAAAAATGCTGCGCCGCACGA
TCCAGGCGATTTGCGAAGGACGCGATCCTGGGGATCT
GACGACCATTGATGATCCGGCGTCGTTGGATCAGATC
CGCCAGGCGATGGAAGAGTAGTACTGATCAAAAAGGT
TAGCCTCAAGAGGGTCATAAAAATGTCAGAGCAGAAA
GTAGCTCTGGTTACCGGTGCGTTAGGTGGTATCGGAA
GTGAGATCTGCCGCCAGCTTGTGACCGCCGGGTACAA
GATTATCGCCACCGTTGTTCCACGCGAAGAAGACCGC
GAAAAACAATGGTTGCAAAGTGAGGGGTTTCAAGACT
CTGATGTGCGTTTCGTATTAACAGATTTAAACAATCAC
GAAGCTGCGACAGCGGCAATTCAAGAAGCGATTGCCG
CCGAAGGACGCGTTGATGTATTGGTCAACAACGCGGG
GATCACGCGCGATGCTACATTTAAGAAAATGTCCTAT

GAGCAATGGTCCCAAGTCATCGACACGAATTTAAAGA
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CTTGAACAGAAGTCTGGCCGCATCGTAAACATTAGCT
CTGTCAATGGTTTAAAAGGGCAATTTGGTCAAGCCAA
CTACTCGGCCTCGAAAGCAGGGATTATCGGGTTTACT
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ATGGTCACAGCAATGCGCGAGGATGTAATTAAGTCAA
TCGAAGCTCAAATTCCCCTGCAACGTCTGGCAGCACC
GGCGGAGATTGCGGCAGCGGTTATGTATTTGGTGAGT
GAACACGGTGCATACGTGACGGGCGAAACTTTGAGTA
TCAACGGCGGGCTGTACATGCACTAAAGGTGCTTTTA
GTCTAGCGCTAGAGCAGGTACCATATTAATGAATCCA
AATTCCTTTCAGTTTAAAGAGAATATCTTACAGTTTTT
CAGCGTGCACGACGATATTTGGAAAAAACTGCAGGAA
TTTTACTATGGACAATCGCCCATCAATGAAGCGTTGGC
GCAGTTAAATAAGGAAGACATGAGTTTATTCTTCGAG
GCGTTATCAAAAAACCCTGCTCGTATGATGGAGATGC
AGTGGTCCTGGTGGCAAGGGCAGATTCAAATTTACCA
GAACGTGTTAATGCGTAGTGTAGCCAAGGACGTAGCC
CCCTTTATCCAGCCAGAGTCCGGAGATCGTCGCTTCAA
CTCGCCACTTTGGCAAGAACATCCAAATTTTGATTTAC
TGAGTCAATCCTACTTGTTGTTTTCTCAGTTGGTTCAA
AATATGGTGGATGTCGTTGAAGGAGTACCTGATAAGG
TCCGCTATCGCATCCATTTCTTTACACGTCAGATGATC
AATGCGTTGTCTCCTTCTAATTTCCTGTGGACGAACCC
TGAAGTAATTCAACAGACGGTCGCTGAACAGGGTGAG
AATTTAGTACGCGGGATGCAAGTATTTCACGATGATG
TAATGAATTCGGGTAAATATTTGAGCATCCGTATGGT
AAATAGCGACAGTTTCTCTCTTGGCAAGGACTTGGCG
TATACGCCAGGAGCCGTAGTTTTCGAGAACGACATCT
TTCAGCTTCTTCAATACGAAGCCACAACCGAGAACGT
ATATCAAACCCCTATTCTTGTCGTACCTCCCTTCATCA
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CTTGGTTAATTGGCTGCGCCAACAAGGACATACGGTG
TTTTTGATGTCGTGGCGTAACCCCAACGCAGAGCAGA
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GAGAAAGAAGCTAACTGTATTGGATATTGCATCGGTG
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GGTGTTTTCATTAATGAGCCGGTCGTAAGTGGACTTGA
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TTGTATTGGAATTATTACATCGATAATTACTTGAAGGG
TAAGGAACCGTCCGACTTTGACATCTTATACTGGAACT
CGGATGGTACGAATATCCCAGCAAAGATTCACAATTT
CCTGTTACGTAACCTTTATCTTAACAACGAACTTATTT

	CTCCAAATGCCGTCAAAGTTAATGGTGTGGGTTTAAA CCTTTCGCGCGTGAAGACTCCATCATTCTTCATTGCTA CGCAGGAGGACCATATCGCATTGTGGGATACCTGTTT TCGCGGCGCGGATTACCTGGGGGGTGAGAGCACACTT GTGCTTGGGGAAAGCGGACACGTCGCCGGCATTGTCA ACCCGCCTTCTCGTAACAAGTATGGTTGTTACACGAAC GCCGCCAAGTTTGAAAATACCAAGCAATGGCTTGACG GTGCAGAATATCATCCCGAAAGCTGGTGGTTACGTTG GCAGGCATGGGTCACGCCTTATACTGGAGAGCAGGTT CCTGCGCGTAATTTGGGAAACGCACAGTACCCCAGTA TTGAAGCGGCCCCTGGGCGTTATGTGCTGGTAAACCT GTTTTAACGCT<u>CACATACAAGCAATCTATAATTATTCA</u> <u>CGGTATAA</u>ATGAAAGATGTTGTTATCGTAGCCGCTAA ACGCACTGCGATCGGTTCCTTTCTGGGGAGTCTGGCTT CCCTGAGCGCCCCTCAGTTGGGTCAGACGGCTATCCG CGCAGTTTTGGATTCTGCAAATGTGAAACCAGAACAA GTGGACCAAGTAATTATGGGGAATGTGCTGACCACCG GCGTTGGGCAAAATCCTGCTCGTCAGGCAGCAATCGC CGCTGGGATTCCTGTACAAGTTCCCGCCAGCACGCTTA ATGTAGTGTGTGGGTCCGGATTACGTGCCGTTCACCTG GCAGCTCAAGCCATCCAATGCGATGAAGCCGATATCG TCGTTGCCGGAGGTCAAGAATCAATGTCCCAGTCTGC TCATTACATGCAGCTTCGCAATGGCCAGAAAATGGGT AACGCACAGTTAGTCGATTCAATGGTGGCCGACGGCT TGACCGACGCGTATAATCAATACCAGATGGGTATCAC CGCGGAGAATATCGTCGAAAAACTTGGTCTTAATCGT GAAGAACAAGACCAGCTTGCTCTGACAAGTCAACAAC GTGCTGCAGCAGCGCAGGCTGCCGGAAAATTCAAGGA TGAAATTGCGGTCGTTTCGATTCCCCAGCGCAAAGGA GAGCCGGTCGTCTTCGCGGAAGACGAATATATCAAGG CCAATACCTCGTTGGAATCCTTGACGAAACTGCGTCC AGCATTCAAAAAAGACGGTTCTGTTACAGCCGGCAAC GCATCTGGCATTAATGATGGGGCAGCCGCGGTCCTGA TGATGTCCGCCGACAAAGCGGCTGAACTGGGCTTAAA GCCTTTAGCACGCATTAAAGGTTACGCGATGTCAGGA ATTGAGCCGGAAATCATGGGACTGGGTCCTGTAGACG CCGTTAAGAAAACCCTTAATAAGGCTGGTTGGTCCTT AGACCAGGTCGATCTGATCGAGGCCAATGAGGCTTTT GCTGCCCAAGCACTGGGAGTAGCCAAGGAGCTTGGGC TGGACCTGGACAAGGTAAATGTTAACGGAGGTGCGAT CGCGCTGGGACACCCGATCGGGGCTTCGGGTTGTCGT ATCTTGGTCACGTTATTACACGAAATGCAGCGTCGTG ATGCAAAGAAGGGTATCGCCACATTGTGTGTGGGAGG TGGAATGGGGGTGGCGCTTGCCGTTGAGCGCGATTAA	
prpE sequence (comprised in the prpE-PhaBCA construct	ATGTCTTTTAGCGAATTTTATCAGCGTTCGATTAACGA ACCGGAGAAGTTCTGGGCCGAGCAGGCCCGGCGTATT GACTGGCAGACGCCCTTTACGCAAACGCTCGACCACA GCAACCCGCCGTTTGCCCGTTGGTTTTGTGAAGGCCGA ACCAACTTGTGTCACAACGCTATCGACCGCTGGCTGG	SEQ ID NO: 25

shown in **FIG. 11**)	AGAAACAGCCAGAGGCGCTGGCATTGATTGCCGTCTC TTCGGAAACAGAGGAAGAGCGTACCTTTACCTTCCGC CAGTTACATGACGAAGTGAATGCGGTGGCGTCAATGC TGCGCTCACTGGGCGTGCAGCGTGGCGATCGGGTGCT GGTGTATATGCCGATGATTGCCGAAGCGCATATTACC CTGCTGGCCTGCGCGCGCATTGGTGCTATTCACTCGGT GGTGTTTGGGGGATTTGCTTCGCACAGCGTGGCAACG CGAATTGATGACGCTAAACCGGTGCTGATTGTCTCGG CTGATGCCGGGGCGCGCGGCGGTAAAATCATTCCGTA TAAAAAATTGCTCGACGATGCGATAAGTCAGGCACAG CATCAGCCGCGTCACGTTTTACTGGTGGATCGCGGGCT GGCGAAAATGGCGCGCGTTAGCGGGCGGGATGTCGAT TTCGCGTCGTTGCGCCATCAACACATCGGCGCGCGGG TGCCGGTGGCATGGCTGGAATCCAACGAAACCTCCTG CATTCTCTACACCTCCGGCACGACCGGCAAACCTAAA GGTGTGCAGCGTGATGTCGGCGGATATGCGGTGGCGC TGGCGACCTCGATGGACACCATTTTTGGCGGCAAAGC GGGCGGCGTGTTCTTTTGTGCTTCGGATATCGGCTGGG TGGTAGGGCATTCGTATATCGTTTACGCGCCGCTGCTG GCGGGGATGGCGACTATCGTTTACGAAGGATTGCCGA CCTGGCCGGACTGCGGCGTGTGGTGGAAAATTGTCGA GAAATATCAGGTTAGCCGCATGTTCTCAGCGCCGACC GCCATTCGCGTGCTGAAAAAATTCCCTACCGCTGAAA TTCGCAAACACGATCTTTCGTCGCTGGAAGTGCTCTAT CTGGCTGGAGAACCGCTGGACGAGCCGACCGCCAGTT GGGTGAGCAATACGCTGGATGTGCCGGTCATCGACAA CTACTGGCAGACCGAATCCGGCTGGCCGATTATGGCG ATTGCTCGCGGTCTGGATGACAGACCGACGCGTCTGG GAAGCCCCGGCGTGCCGATGTATGGCTATAACGTGCA GTTGCTCAATGAAGTCACCGGCGAACCGTGTGGCGTC AATGAGAAAGGGATGCTGGTAGTGGAGGGGCCATTGC CGCCAGGCTGTATTCAAACCATCTGGGGCGACGACGA CCGCTTTGTGAAGACGTACTGGTCGCTGTTTTCCCGTC CGGTGTACGCCACTTTTGACTGGGGCATCCGCGATGCT GACGGTTATCACTTTATTCTCGGGCGCACTGACGATGT GATTAACGTTGCCGGACATCGGCTGGGTACGCGTGAG ATTGAAGAGAGTATCTCCAGTCATCCGGGCGTTGCCG AAGTGGCGGTGGTTGGGGTGAAAGATGCGCTGAAAG GGCAGGTGGCGGTGGCGTTTGTCATTCCGAAAGAGAG CGACAGTCTGGAAGACCGTGAGGTGGCGCACTCGCAA GAGAAGGCGATTATGGCGCTGGTGGACAGCCAGATTG GCAACTTTGGCCGCCCGGCGCACGTCTGGTTTGTCTCG CAATTGCCAAAAACGCGATCCGGAAAAATGCTGCGCC GCACGATCCAGGCGATTTGCGAAGGACGCGATCCTGG GGATCTGACGACCATTGATGATCCGGCGTCGTTGGAT CAGATCCGCCAGGCGATGGAAGAGTAG	
phaB sequence (comprised in the prpE-	ATGTCAGAGCAGAAAGTAGCTCTGGTTACCGGTGCGT TAGGTGGTATCGGAAGTGAGATCTGCCGCCAGCTTGT GACCGCCGGGTACAAGATTATCGCCACCGTTGTTCCA	SEQ ID NO:

PhaBCA construct shown in **FIG. 11**)	CGCGAAGAAGACCGCGAAAAACAATGGTTGCAAAGT GAGGGGTTTCAAGACTCTGATGTGCGTTTCGTATTAAC AGATTTAAACAATCACGAAGCTGCGACAGCGGCAATT CAAGAAGCGATTGCCGCCGAAGGACGCGTTGATGTAT TGGTCAACAACGCGGGGATCACGCGCGATGCTACATT TAAGAAAATGTCCTATGAGCAATGGTCCCAAGTCATC GACACGAATTTAAAGACTCTTTTTACCGTGACCCAGCC AGTATTTAATAAAATGCTTGAACAGAAGTCTGGCCGC ATCGTAAACATTAGCTCTGTCAATGGTTTAAAAGGGC AATTTGGTCAAGCCAACTACTCGGCCTCGAAAGCAGG GATTATCGGGTTTACTAAAGCATTGGCGCAGGAGGGT GCTCGCTCGAACATTTGCGTCAATGTCGTTGCTCCTGG TTACACAGCGACACCCATGGTCACAGCAATGCGCGAG GATGTAATTAAGTCAATCGAAGCTCAAATTCCCCTGC AACGTCTGGCAGCACCGGCGGAGATTGCGGCAGCGGT TATGTATTTGGTGAGTGAACACGGTGCATACGTGACG GGCGAAACTTTGAGTATCAACGGCGGGCTGTACATGC ACTAA	26
phaC sequence (comprised in the prpE-PhaBCA construct shown in **FIG. 11**)	ATGAATCCAAATTCCTTTCAGTTTAAAGAGAATATCTT ACAGTTTTTCAGCGTGCACGACGATATTTGGAAAAAA CTGCAGGAATTTTACTATGGACAATCGCCCATCAATG AAGCGTTGGCGCAGTTAAATAAGGAAGACATGAGTTT ATTCTTCGAGGCGTTATCAAAAAACCCTGCTCGTATGA TGGAGATGCAGTGGTCCTGGTGGCAAGGGCAGATTCA AATTTACCAGAACGTGTTAATGCGTAGTGTAGCCAAG GACGTAGCCCCCTTTATCCAGCCAGAGTCCGGAGATC GTCGCTTCAACTCGCCACTTTGGCAAGAACATCCAAA TTTTGATTTACTGAGTCAATCCTACTTGTTGTTTTCTCA GTTGGTTCAAAATATGGTGGATGTCGTTGAAGGAGTA CCTGATAAGGTCCGCTATCGCATCCATTTCTTTACACG TCAGATGATCAATGCGTTGTCTCCTTCTAATTTCCTGT GGACGAACCCTGAAGTAATTCAACAGACGGTCGCTGA ACAGGGTGAGAATTTAGTACGCGGGATGCAAGTATTT CACGATGATGTAATGAATTCGGGTAAATATTTGAGCA TCCGTATGGTAAATAGCGACAGTTTCTCTCTTGGCAAG GACTTGGCGTATACGCCAGGAGCCGTAGTTTTCGAGA ACGACATCTTTCAGCTTCTTCAATACGAAGCCACAACC GAGAACGTATATCAAACCCCTATTCTTGTCGTACCTCC CTTCATCAACAAGTACTACGTGCTGGACCTGCGCGAA CAGAATAGCTTGGTTAATTGGCTGCGCCAACAAGGAC ATACGGTGTTTTTGATGTCGTGGCGTAACCCCAACGCA GAGCAGAAGGAGCTTACCTTCGCTGACTTAATTACCC AAGGATCGGTAGAAGCATTACGTGTTATCGAAGAAAT CACGGGAGAGAAAGAAGCTAACTGTATTGGATATTGC ATCGGTGGTACACTTCTGGCTGCTACCCAGGCATATTA TGTAGCTAAACGCCTGAAAAATCACGTAAAGTCAGCG ACTTATATGGCGACGATTATTGATTTTGAGAACCCCGG CTCATTGGGTGTTTTCATTAATGAGCCGGTCGTAAGTG GACTTGAAAACCTTAATAATCAACTTGGTTACTTCGAC	SEQ ID NO: 27

	GGGCGTCAACTTGCAGTGACATTTTCGTTGTTGCGCGA AAACACCTTGTATTGGAATTATTACATCGATAATTACT TGAAGGGTAAGGAACCGTCCGACTTTGACATCTTATA CTGGAACTCGGATGGTACGAATATCCCAGCAAAGATT CACAATTTCCTGTTACGTAACCTTTATCTTAACAACGA ACTTATTTCTCCAAATGCCGTCAAAGTTAATGGTGTGG GTTTAAACCTTTCGCGCGTGAAGACTCCATCATTCTTC ATTGCTACGCAGGAGGACCATATCGCATTGTGGGATA CCTGTTTTCGCGGCGCGGATTACCTGGGGGGTGAGAG CACACTTGTGCTTGGGGAAAGCGGACACGTCGCCGGC ATTGTCAACCCGCCTTCTCGTAACAAGTATGGTTGTTA CACGAACGCCGCCAAGTTTGAAAATACCAAGCAATGG CTTGACGGTGCAGAATATCATCCCGAAAGCTGGTGGT TACGTTGGCAGGCATGGGTCACGCCTTATACTGGAGA GCAGGTTCCTGCGCGTAATTTGGGAAACGCACAGTAC CCCAGTATTGAAGCGGCCCCTGGGCGTTATGTGCTGG TAAACCTGTTTTAA	
phaA sequence (comprised in the prpE-PhaBCA construct shown in **FIG. 11**)	ATGAAAGATGTTGTTATCGTAGCCGCTAAACGCACTG CGATCGGTTCCTTTCTGGGGAGTCTGGCTTCCCTGAGC GCCCCTCAGTTGGGTCAGACGGCTATCCGCGCAGTTTT GGATTCTGCAAATGTGAAACCAGAACAAGTGGACCAA GTAATTATGGGGAATGTGCTGACCACCGGCGTTGGGC AAAATCCTGCTCGTCAGGCAGCAATCGCCGCTGGGAT TCCTGTACAAGTTCCCGCCAGCACGCTTAATGTAGTGT GTGGGTCCGGATTACGTGCCGTTCACCTGGCAGCTCA AGCCATCCAATGCGATGAAGCCGATATCGTCGTTGCC GGAGGTCAAGAATCAATGTCCCAGTCTGCTCATTACA TGCAGCTTCGCAATGGCCAGAAAATGGGTAACGCACA GTTAGTCGATTCAATGGTGGCCGACGGCTTGACCGAC GCGTATAATCAATACCAGATGGGTATCACCGCGGAGA ATATCGTCGAAAAACTTGGTCTTAATCGTGAAGAACA AGACCAGCTTGCTCTGACAAGTCAACAACGTGCTGCA GCAGCGCAGGCTGCCGGAAAATTCAAGGATGAAATTG CGGTCGTTTCGATTCCCCAGCGCAAAGGAGAGCCGGT CGTCTTCGCGGAAGACGAATATATCAAGGCCAATACC TCGTTGGAATCCTTGACGAAACTGCGTCCAGCATTCA AAAAAGACGGTTCTGTTACAGCCGGCAACGCATCTGG CATTAATGATGGGGCAGCCGCGGTCCTGATGATGTCC GCCGACAAAGCGGCTGAACTGGGCTTAAAGCCTTTAG CACGCATTAAAGGTTACGCGATGTCAGGAATTGAGCC GGAAATCATGGGACTGGGTCCTGTAGACGCCGTTAAG AAAACCCTTAATAAGGCTGGTTGGTCCTTAGACCAGG TCGATCTGATCGAGGCCAATGAGGCTTTTGCTGCCCA AGCACTGGGAGTAGCCAAGGAGCTTGGGCTGGACCTG GACAAGGTAAATGTTAACGGAGGTGCGATCGCGCTGG GACACCCGATCGGGGCTTCGGGTTGTCGTATCTTGGTC ACGTTATTACACGAAATGCAGCGTCGTGATGCAAAGA AGGGTATCGCCACATTGTGTGTGGGAGGTGGAATGGG GGTGGCGCTTGCCGTTGAGCGCGATTAA	SEQ ID NO: 28

[0519] The plasmid was transformed into *E. coli* DH5α as described herein to generate the plasmid pTet-prpE-PhaBCA.

[0520] In certain constructs, the *prpE-PhaBCA* operon is operably linked to a FNR-responsive promoter, which may be is further fused to a strong ribosome binding site sequence. For efficient translation, a 20-30 bp ribosome binding site was included for each synthetic gene in the operon. Each gene cassette and regulatory region construct is expressed on a high-copy plasmid, a low-copy plasmid, or a chromosome.

[0521] In certain embodiments, the construct is inserted into the bacterial genome at one or more of the following insertion sites in *E. coli* Nissle: *malE/K, araC/BAD, lacZ, thyA, malP/T.* Any suitable insertion site may be used (*see, e.g.,* **FIG. 32**). The insertion site may be anywhere in the genome, *e.g.*, in a gene required for survival and/or growth, such as *thyA* (to create an auxotroph); in an active area of the genome, such as near the site of genome replication; and/or in between divergent promoters in order to reduce the risk of unintended transcription, such as between AraB and AraC of the arabinose operon. At the site of insertion, DNA primers that are homologous to the site of insertion and to the propionate construct are designed. A linear DNA fragment containing the construct with homology to the target site is generated by PCR, and lambda red recombination is performed as described below. The resulting *E. coli* Nissle bacteria are genetically engineered to express a propionate biosynthesis cassette and produce propionate.

Example 3. Construction of Plasmids Encoding Propionate Catabolism Enzymes (MMCA Pathway)

[0522] The methylmalonyl-CoA pathway (MMCA) carries out reactions homologous to those in the mammalian pathway. Genes *accA* (from *Streptomyces coelicolor*), *pccB* (from *Streptomyces coelicolor*), *mmcE* (from *Propionibacterium freudenreichii*), and mutAB (from *Propionibacterium freudenreichii*) were codon-optimized for expression in *E. coli* Nissle. Two constructs were synthesized, the first with a cassette comprising *prpE, pccB, accA1,* under the control of an inducible Ptet promoter and the second with a cassette comprising *mmcE and mutAB* under the control of a second inducible promoter, Para, (as shown in **FIG 15C** and **FIG 16A** and **FIG. 16B**).

[0523] The constructs were cloned into the plasmids p15a-Kan (pTet-prpE- pccB-accA1) and an ColE1-Amp (pAra-*mmcE-mutAB)* by Golden Gate assembly, and transformed

into *E. coli* DH5α as described herein. Sequences of MMCA pathway circuits are listed in **Table 13**.

Table 13. MMCA Pathway Circuit Sequences

Description	Sequence	SEQ ID NO
Construct comprising AraC (reverse orientation, lower case) and a mmcE-mutA-mutB gene cassette under Para promoter (italics) (as shown in **FIG. 15B and FIG. 16**); ribosome binding sites are underlined ;. L3S2P11 terminator in italics; his terminator in bold; coding regions bold underlined	ttattcacaacctgccctaaactcgctcggactcgccccggtgcatttttaaa tactcgcgagaaatagagttgatcgtcaaaaccgacattgcgaccgacggt ggcgataggcatccgggtggtgctcaaaagcagcttcgcctgactgatgc gctggtcctcgcgccagcttaatacgctaatccctaactgctggcggaacaa atgcgacagacgcgacggcgacaggcagacatgctgtgcgacgctggc gatatcaaaattactgtctgccaggtgatcgctgatgtactgacaagcctcgc gtacccgattatccatcggtggatggagcgactcgttaatcgcttccatgcg ccgcagtaacaattgctcaagcagatttatcgccagcaattccgaatagcgc ccttcccccttgtccggcattaatgatttgcccaaacaggtcgctgaaatgcgg ctggtgcgcttcatccgggcgaaagaaaccggtattggcaaatatcgacgg ccagttaagccattcatgccagtaggcgcgcggacgaaagtaaacccact ggtgataccattcgtgagcctccggatgacgaccgtagtgatgaatctctcc aggcgggaacagcaaaatatcacccggtcggcagacaaattctcgtccct gatttttcaccaccccctgaccgcgaatggtgagattgagaatataacctttc attcccagcggtcggtcgataaaaaaatcgagataaccgttggcctcaatcg gcgttaaacccgccaccagatgggcgttaaacgagtatcccggcagcagg ggatcattttgcgcttcagccat*ACTTTTCATACTCCCGCCAT TCAGAGAAGAAACCAATTGTCCATATTGCATCAG ACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCT CGCTAACCCAACCGGTAACCCCGCTTATTAAAAG CATTCTGTAACAAAGCGGGACCAAAGCCATGACA AAAACGCGTAACAAAAGTGTCTATAATCACGGCA GAAAAGTCCACATTGATTATTTGCACGGCGTCAC ACTTTGCTATGCCATAGCATTTTTATCCATAAGAT TAGCGGATCCAGCCTGACGCTTTTTTTCGCAACT CTCTACTGTTTCTCCATACC*GGGAAACCACCGC GCCCAGCTTAATTTT**ATGAGTAACGAAGATT TATTCATTTGCATCGACCACGTCGCGTATG CGTGCCCGGATGCCGATGAAGCTTCTAAGT ATTACCAGGAAACATTCGGTTGGCACGAGT TGCACCGCGAAGAGAATCCAGAACAGGGC GTGGTGGAAATTATGATGGCGCCTGCTGCG AAATTGACGGAGCACATGACTCAGGTGCAA GTTATGGCGCCTTTGAACGATGAGAGTACG GTCGCGAAGTGGCTTGCGAAACACAATGG GCGTGCTGGATTGCACCACATGGCATGGCG TGTTGATGACATCGACGCAGTGTCCGCAAC ACTTCGCGAGCGCGGTGTACAGTTGCTTTA CGACGAGCCGAAACTGGGTACAGGTGGGA ATCGTATCAACTTCATGCATCCGAAATCTG GTAAAGGCGTGCTGATTGAACTGACCCAGT ACCCCAAGAATTGA**TAAAGGTTTTTCCTAAG	SEQ ID NO: 29

	ACGCTAGCGCATAAGGTCCACCAAATGTCAA GTACAGACCAAGGCACGAACCCTGCTGACA CGGATGATTTAACGCCAACCACATTATCCC TGGCTGGTGATTTCCCTAAGGCTACGGAAG AGCAGTGGGAGCGCGAGGTTGAAAAGGTG TTGAACCGTGGGCGCCCACCCGAGAAGCA GTTGACGTTTGCTGAATGTTTAAAACGTCT TACTGTGCACACAGTAGATGGCATTGACAT CGTTCCAATGTATCGCCCGAAGGATGCCCC TAAGAAACTGGGGTATCCAGGGGTTGCTCC CTTTACGCGTGGCACTACGGTTCGCAATGG GGATATGGACGCTTGGGACGTTCGCGCCCT GCACGAAGACCCTGATGAAAAATTCACGCG CAAAGCTATTCTGGAGGGGCTGGAGCGCG GCGTAACAAGTTTGCTTCTTCGTGTGGACC CTGATGCAATCGCTCCCGAACACTTAGACG AAGTGTTAAGTGACGTTTTGCTGGAAATGA CCAAGGTTGAGGTGTTTTCCCGCTATGATC AGGGAGCTGCGGCTGAAGCTCTTGTCTCGG TATATGAGCGCAGCGACAAACCGGCTAAAG ATTTGGCCTTAAATTTGGGACTGGACCCAA TCGCATTTGCTGCACTTCAGGGCACTGAGC CAGACTTGACCGTACTTGGTGATTGGGTTC GTCGTTTGGCTAAATTCAGCCCAGACTCAC GCGCTGTAACAATTGATGCTAATATTTATC ACAACGCCGGTGCAGGCGACGTTGCCGAG CTGGCCTGGGCACTTGCGACCGGAGCAGA GTACGTCCGTGCGCTGGTAGAGCAAGGATT CACCGCCACAGAGGCATTTGATACCATTAA CTTCCGTGTGACAGCGACCCATGATCAATT TTTAACGATTGCCCGCCTTCGTGCGTTACG TGAAGCGTGGGCTCGTATCGGTGAGGTATT CGGAGTAGATGAGGATAAACGTGGAGCGC GCCAGAATGCTATTACGTCCTGGCGTGAAC TGACACGCGAGGATCCCTATGTGAACATTT TACGTGGAAGTATTGCCACGTTCTCTGCGT CCGTTGGGGGCGCGGAGTCTATTACCACTT TGCCATTCACGCAGGCATTGGGCCTTCCAG AGGATGATTTTCCATTACGTATCGCACGTA ATACAGGAATTGTCTTAGCTGAGGAGGTAA ACATTGGGCGTGTAAATGACCCTGCCGGGG GGTCATACTATGTGGAGAGCTTGACTCGTT CTCTTGCAGATGCAGCATGGAAAGAGTTCC AAGAGGTTGAAAAGTTGGGTGGTATGTCTA AGGCCGTCATGACCGAACACGTCACGAAG GTTTTAGATGCTTGCAACGCAGAGCGCGCG AAGCGCTTGGCCAACCGCAAGCAACCTATT ACGGCAGTTTCCGAATTTCCGATGATTGGC GCACGCAGCATTGAGACGAAACCATTTCCG	

	GCTGCTCCGGCCCGTAAAGGGCTGGCATG GCACCGCGATTCCGAAGTCTTCGAGCAACT TATGGACCGCTCCACGTCAGTTTCAGAGCG TCCGAAAGTATTTTTAGCATGTCTTGGGAC GCGCCGCGATTTTGGAGGACGCGAAGGAT TTTCATCTCCGGTTTGGCACATTGCCGGGA TTGACACGCCTCAAGTAGAAGGTGGGACGA CTGCTGAAATCGTGGAAGCGTTCAAAAAAT CTGGGGCCCAAGTCGCCGATTTATGTTCGA GTGCCAAAGTGTATGCTCAACAAGGCTTAG AGGTGGCAAAGGCTCTGAAAGCGGCTGGG GCTAAGGCGCTGTATTTGAGCGGAGCATTT AAGGAGTTCGGAGACGATGCAGCGGAAGC CGAAAAACTTATCGACGGACGCCTTTTCAT GGGCATGGATGTCGTTGACACCCTGTCTTC CACTTTAGATATCCTTGGAGTGGCGAAGTG ATAAGCTTAAAACAATTTACATCCGGCCGGAA CTTACTATGTCTACCTTACCTCGCTTTGACA GTGTTGATTTAGGAAATGCGCCGGTCCCAG CAGATGCTGCACGTCGTTTTGAGGAACTTG CGGCGAAAGCCGGGACCGGCGAAGCCTGG GAAACTGCGGAACAAATTCCAGTAGGCACG TTGTTTAATGAAGACGTATACAAGGACATG GATTGGCTTGATACTTACGCTGGCATTCCT CCCTTCGTCCATGGTCCGTACGCTACTATG TATGCATTTCGTCCTTGGACCATTCGCCAA TATGCCGGTTTTTCGACTGCAAAGGAGTCA AACGCATTTTACCGTCGTAATTTGGCTGCA GGCCAGAAAGGTCTTAGTGTTGCTTTTGAC TTACCCACTCACCGCGGTTATGATTCCGAC AACCCCCGCGTGGCCGGAGATGTTGGTATG GCCGGTGTGGCTATCGATTCGATTTATGAC ATGCGTGAGCTGTTCGCCGGCATCCCATTA GATCAGATGAGCGTGTCGATGACAATGAAC GGTGCTGTCTTGCCGATTTTGGCTCTTTAT GTGGTTACGGCGGAGGAGCAAGGCGTGAA GCCAGAACAACTGGCGGGTACTATTCAAAA TGATATTCTGAAGGAATTTATGGTTCGTAA TACATATATTTACCCGCCGCAACCTAGTAT GCGCATTATCAGCGAGATTTTTGCATACAC ATCAGCAAACATGCCGAAGTGGAACTCCAT TAGTATCAGCGGCTATCATATGCAGGAGGC TGGAGCGACTGCGGATATCGAGATGGCGT ATACCTTAGCTGATGGAGTTGATTACATCC GTGCTGGTGAGTCAGTAGGACTTAATGTGG ACCAATTTGCTCCACGCCTGTCCTTCTTCT GGGGCATTGGTATGAACTTTTTCATGGAGG TAGCGAAGTTACGCGCTGCCCGTATGCTGT GGGCGAAGCTTGTCCACCAGTTCGGCCCGA	

	AAAACCCGAAGAGTATGTCTCTGCGCACGC ACTCTCAAACATCGGGTTGGTCTTTGACAG CTCAAGACGTATATAATAACGTTGTACGTA CATGCATCGAAGCCATGGCTGCTACTCAAG GCCATACTCAATCACTTCATACAAATTCGTT GGATGAAGCCATTGCATTGCCTACGGACTT TTCAGCCCGCATTGCCCGCAATACTCAATT ATTTCTGCAACAAGAGAGCGGGACGACTCG TGTGATCGACCCTTGGTCAGGTTCCGCATA CGTCGAAGAGTTGACTTGGGATTTAGCTCG TAAAGCCTGGGGGCATATTCAGGAGGTTGA GAAGGTGGGGGGCATGGCTAAGGCAATCG AGAAGGGGATTCCGAAGATGCGCATTGAG GAGGCAGCCGCCCGTACCCAAGCACGTATT GATTCGGGACGCCAGCCATTAATTGGGGTC AATAAATACCGTCTGGAGCACGAACCACCC CTGGATGTGTTGAAGGTAGACAATAGCACC GTGTTAGCTGAGCAAAAGGCCAAACTTGTT AAATTGCGCGCAGAACGCGACCCAGAAAA GGTCAAGGCTGCTCTGGACAAAATCACTTG GGCGGCTGGCAATCCTGATGATAAAGACCC TGATCGCAACTTATTAAAGCTGTGCATTGA TGCGGGGCGCGCGATGGCAACGGTAGGAG AGATGAGTGACGCTTTAGAGAAAGTTTTTG GGCGCTACACAGCGCAAATTCGCACTATTT CAGGAGTATATTCAAAAGAAGTCAAAAACA CTCCGGAAGTCGAGGAGGCTCGCGAACTG GTAGAAGAGTTTGAGCAGGCCGAAGGCCG TCGCCCACGTATCCTGCTGGCTAAAATGGG GCAGGACGGTCATGACCGTGGGCAAAAGG TCATCGCGACTGCATACGCCGATTTGGGAT TTGACGTGGACGTTGGCCCGTTATTCCAAA CTCCCGAGGAAACTGCTCGCCAAGCCGTCG AAGCCGATGTGCACGTAGTGGGGGTGAGC TCTCTGGCGGGAGGGCATCTTACGCTTGTG CCTGCGCTTCGCAAAGAGCTGGACAAGTTG GGTCGTCCAGATATTCTGATTACCGTAGGA GGGGTTATTCCCGAGCAGGACTTCGATGAG CTTCGTAAGGATGGCGCTGTTGAAATCTAC ACACCGGGGACGGTCATTCCAGAATCGGCT ATCTCTTTAGTTAAAAAATTGCGCGCCTCC CTGGATGCTTGATAAGGAG*CTCGGTACCAAAT TCCAGAAAAGAGACGCTTTCGAGCGTCTTTTTC GTTTTGGTCC*GCGCAATAAAAAAGCCCCCGG AAGGTGATCTTCCGGGGGCTTTCTCATGCG TT**</u>	
Construct comprising a mmcE-mutA-mutB gene cassette under the control	*ACTTTTCATACTCCCGCCATTCAGAGAAGAAACC AATTGTCCATATTGCATCAGACATTGCCGTCACTG CGTCTTTTACTGGCTCTTCTCGCTAACCCAACCG*	**SEQ ID NO: 30**

of the Para promoter (as shown in **FIG. 15B and FIG. 16**) ribosome binding sites are underlined ;.coding regions bold underlined	*GTAACCCCGCTTATTAAAAGCATTCTGTAACAAAG* *CGGGACCAAAGCCATGACAAAAACGCGTAACAAA* *AGTGTCTATAATCACGGCAGAAAAGTCCACATTG* *ATTATTTGCACGGCGTCACACTTTGCTATGCCATA* *GCATTTTTATCCATAAGATTAGCGGATCCAGCCT* *GACGCTTTTTTTCGCAACTCTCTACTGTTTCTCCA* *TACC*GGGAAACCACCGCGCCCAGCTTAATTTT **ATGAGTAACGAAGATTTATTCATTTGCATC** **GACCACGTCGCGTATGCGTGCCCGGATGCC** **GATGAAGCTTCTAAGTATTACCAGGAAACA** **TTCGGTTGGCACGAGTTGCACCGCGAAGAG** **AATCCAGAACAGGGCGTGGTGGAAATTATG** **ATGGCGCCTGCTGCGAAATTGACGGAGCAC** **ATGACTCAGGTGCAAGTTATGGCGCCTTTG** **AACGATGAGAGTACGGTCGCGAAGTGGCTT** **GCGAAACACAATGGGCGTGCTGGATTGCAC** **CACATGGCATGGCGTGTTGATGACATCGAC** **GCAGTGTCCGCAACACTTCGCGAGCGCGGT** **GTACAGTTGCTTTACGACGAGCCGAAACTG** **GGTACAGGTGGGAATCGTATCAACTTCATG** **CATCCGAAATCTGGTAAAGGCGTGCTGATT** **GAACTGACCCAGTACCCCAAGAATTGA**TAA AGGTTTTTCCTAAGACGCTAGCGCATAAGGTC CACCAA**ATGTCAAGTACAGACCAAGGCACG** **AACCCTGCTGACACGGATGATTTAACGCCA** **ACCACATTATCCCTGGCTGGTGATTTCCCT** **AAGGCTACGGAAGAGCAGTGGGAGCGCGA** **GGTTGAAAAGGTGTTGAACCGTGGGCGCC** **CACCCGAGAAGCAGTTGACGTTTGCTGAAT** **GTTTAAAACGTCTTACTGTGCACACAGTAG** **ATGGCATTGACATCGTTCCAATGTATCGCC** **CGAAGGATGCCCCTAAGAAACTGGGGTATC** **CAGGGGTTGCTCCCTTTACGCGTGGCACTA** **CGGTTCGCAATGGGGATATGGACGCTTGG** **GACGTTCGCGCCCTGCACGAAGACCCTGAT** **GAAAAATTCACGCGCAAAGCTATTCTGGAG** **GGGCTGGAGCGCGGCGTAACAAGTTTGCTT** **CTTCGTGTGGACCCTGATGCAATCGCTCCC** **GAACACTTAGACGAAGTGTTAAGTGACGTT** **TTGCTGGAAATGACCAAGGTTGAGGTGTTT** **TCCCGCTATGATCAGGGAGCTGCGGCTGAA** **GCTCTTGTCTCGGTATATGAGCGCAGCGAC** **AAACCGGCTAAAGATTTGGCCTTAAATTTG** **GGACTGGACCCAATCGCATTTGCTGCACTT** **CAGGGCACTGAGCCAGACTTGACCGTACTT** **GGTGATTGGGTTCGTCGTTTGGCTAAATTC** **AGCCCAGACTCACGCGCTGTAACAATTGAT** **GCTAATATTTATCACAACGCCGGTGCAGGC** **GACGTTGCCGAGCTGGCCTGGGCACTTGC**	

	GACCGGAGCAGAGTACGTCCGTGCGCTGG TAGAGCAAGGATTCACCGCCACAGAGGCAT TTGATACCATTAACTTCCGTGTGACAGCGA CCCATGATCAATTTTTAACGATTGCCCGCC TTCGTGCGTTACGTGAAGCGTGGGCTCGTA TCGGTGAGGTATTCGGAGTAGATGAGGATA AACGTGGAGCGCGCCAGAATGCTATTACGT CCTGGCGTGAACTGACACGCGAGGATCCCT ATGTGAACATTTTACGTGGAAGTATTGCCA CGTTCTCTGCGTCCGTTGGGGGCGCGGAGT CTATTACCACTTTGCCATTCACGCAGGCAT TGGGCCTTCCAGAGGATGATTTTCCATTAC GTATCGCACGTAATACAGGAATTGTCTTAG CTGAGGAGGTAAACATTGGGCGTGTAAATG ACCCTGCCGGGGGGTCATACTATGTGGAGA GCTTGACTCGTTCTCTTGCAGATGCAGCAT GGAAAGAGTTCCAAGAGGTTGAAAAGTTGG GTGGTATGTCTAAGGCCGTCATGACCGAAC ACGTCACGAAGGTTTTAGATGCTTGCAACG CAGAGCGCGCGAAGCGCTTGGCCAACCGC AAGCAACCTATTACGGCAGTTTCCGAATTT CCGATGATTGGCGCACGCAGCATTGAGACG AAACCATTTCCGGCTGCTCCGGCCCGTAAA GGGCTGGCATGGCACCGCGATTCCGAAGT CTTCGAGCAACTTATGGACCGCTCCACGTC AGTTTCAGAGCGTCCGAAAGTATTTTTAGC ATGTCTTGGGACGCGCCGCGATTTTGGAGG ACGCGAAGGATTTTCATCTCCGGTTTGGCA CATTGCCGGGATTGACACGCCTCAAGTAGA AGGTGGGACGACTGCTGAAATCGTGGAAG CGTTCAAAAAATCTGGGGCCCAAGTCGCCG ATTTATGTTCGAGTGCCAAAGTGTATGCTC AACAAGGCTTAGAGGTGGCAAAGGCTCTGA AAGCGGCTGGGGCTAAGGCGCTGTATTTGA GCGGAGCATTTAAGGAGTTCGGAGACGAT GCAGCGGAAGCCGAAAAACTTATCGACGG ACGCCTTTTCATGGGCATGGATGTCGTTGA CACCCTGTCTTCCACTTTAGATATCCTTGG AGTGGCGAAGTGATAAGCTTAAAACAATTTA CATCCGGCCGGAACTTACTATGTCTACCTTA CCTCGCTTTGACAGTGTTGATTTAGGAAAT GCGCCGGTCCCAGCAGATGCTGCACGTCGT TTTGAGGAACTTGCGGCGAAAGCCGGGAC CGGCGAAGCCTGGGAAACTGCGGAACAAA TTCCAGTAGGCACGTTGTTTAATGAAGACG TATACAAGGACATGGATTGGCTTGATACTT ACGCTGGCATTCCTCCCTTCGTCCATGGTC CGTACGCTACTATGTATGCATTTCGTCCTT GGACCATTCGCCAATATGCCGGTTTTTCGA	

	CTGCAAAGGAGTCAAACGCATTTTACCGTC GTAATTTGGCTGCAGGCCAGAAAGGTCTTA GTGTTGCTTTTGACTTACCCACTCACCGCG GTTATGATTCCGACAACCCCCCGCGTGGCCG GAGATGTTGGTATGGCCGGTGTGGCTATCG ATTCGATTTATGACATGCGTGAGCTGTTCG CCGGCATCCCATTAGATCAGATGAGCGTGT CGATGACAATGAACGGTGCTGTCTTGCCGA TTTTGGCTCTTTATGTGGTTACGGCGGAGG AGCAAGGCGTGAAGCCAGAACAACTGGCG GGTACTATTCAAAATGATATTCTGAAGGAA TTTATGGTTCGTAATACATATATTTACCCGC CGCAACCTAGTATGCGCATTATCAGCGAGA TTTTTGCATACACATCAGCAAACATGCCGA AGTGGAACTCCATTAGTATCAGCGGCTATC ATATGCAGGAGGCTGGAGCGACTGCGGAT ATCGAGATGGCGTATACCTTAGCTGATGGA GTTGATTACATCCGTGCTGGTGAGTCAGTA GGACTTAATGTGGACCAATTTGCTCCACGC CTGTCCTTCTTCTGGGGCATTGGTATGAAC TTTTTCATGGAGGTAGCGAAGTTACGCGCT GCCCGTATGCTGTGGGCGAAGCTTGTCCAC CAGTTCGGCCCGAAAAACCCGAAGAGTATG TCTCTGCGCACGCACTCTCAAACATCGGGT TGGTCTTTGACAGCTCAAGACGTATATAAT AACGTTGTACGTACATGCATCGAAGCCATG GCTGCTACTCAAGGCCATACTCAATCACTT CATACAAATTCGTTGGATGAAGCCATTGCA TTGCCTACGGACTTTTCAGCCCGCATTGCC CGCAATACTCAATTATTTCTGCAACAAGAG AGCGGGACGACTCGTGTGATCGACCCTTGG TCAGGTTCCGCATACGTCGAAGAGTTGACT TGGGATTTAGCTCGTAAAGCCTGGGGGCAT ATTCAGGAGGTTGAGAAGGTGGGGGGCAT GGCTAAGGCAATCGAGAAGGGGATTCCGA AGATGCGCATTGAGGAGGCAGCCGCCCGT ACCCAAGCACGTATTGATTCGGGACGCCAG CCATTAATTGGGGTCAATAAATACCGTCTG GAGCACGAACCACCCCTGGATGTGTTGAAG GTAGACAATAGCACCGTGTTAGCTGAGCAA AAGGCCAAACTTGTTAAATTGCGCGCAGAA CGCGACCCAGAAAAGGTCAAGGCTGCTCTG GACAAAATCACTTGGGCGGCTGGCAATCCT GATGATAAAGACCCTGATCGCAACTTATTA AAGCTGTGCATTGATGCGGGGCGCGCGAT GGCAACGGTAGGAGAGATGAGTGACGCTT TAGAGAAAGTTTTTGGGCGCTACACAGCGC AAATTCGCACTATTTCAGGAGTATATTCAA AAGAAGTCAAAAACACTCCGGAAGTCGAGG	

	AGGCTCGCGAACTGGTAGAAGAGTTTGAGC AGGCCGAAGGCCGTCGCCCACGTATCCTGC TGGCTAAAATGGGGCAGGACGGTCATGAC CGTGGGCAAAAGGTCATCGCGACTGCATAC GCCGATTTGGGATTTGACGTGGACGTTGGC CCGTTATTCCAAACTCCCGAGGAAACTGCT CGCCAAGCCGTCGAAGCCGATGTGCACGTA GTGGGGGTGAGCTCTCTGGCGGGAGGGCA TCTTACGCTTGTGCCTGCGCTTCGCAAAGA GCTGGACAAGTTGGGTCGTCCAGATATTCT GATTACCGTAGGAGGGGTTATTCCCGAGCA GGACTTCGATGAGCTTCGTAAGGATGGCGC TGTTGAAATCTACACACCGGGGACGGTCAT TCCAGAATCGGCTATCTCTTTAGTTAAAAA ATTGCGCGCCTCCCTGGATGCT	
Construct comprising a mmcE-mutA-mutB gene cassette; (as shown in **FIG. 15B and FIG. 16)** ribosome binding sites are underlined	GGGAAACCACCGCGCCCAGCTTAATTTTATGA GTAACGAAGATTTATTCATTTGCATCGACC ACGTCGCGTATGCGTGCCCGGATGCCGATG AAGCTTCTAAGTATTACCAGGAAACATTCG GTTGGCACGAGTTGCACCGCGAAGAGAATC CAGAACAGGGCGTGGTGGAAATTATGATG GCGCCTGCTGCGAAATTGACGGAGCACATG ACTCAGGTGCAAGTTATGGCGCCTTTGAAC GATGAGAGTACGGTCGCGAAGTGGCTTGC GAAACACAATGGGCGTGCTGGATTGCACCA CATGGCATGGCGTGTTGATGACATCGACGC AGTGTCCGCAACACTTCGCGAGCGCGGTGT ACAGTTGCTTTACGACGAGCCGAAACTGGG TACAGGTGGGAATCGTATCAACTTCATGCA TCCGAAATCTGGTAAAGGCGTGCTGATTGA ACTGACCCAGTACCCCAAGAATTGATAAAG GTTTTTCCTAAGACGCTAGCGCATAAGGTCCA CCAAATGTCAAGTACAGACCAAGGCACGAA CCCTGCTGACACGGATGATTTAACGCCAAC CACATTATCCCTGGCTGGTGATTTCCCTAA GGCTACGGAAGAGCAGTGGGAGCGCGAGG TTGAAAAGGTGTTGAACCGTGGGCGCCCAC CCGAGAAGCAGTTGACGTTTGCTGAATGTT TAAAACGTCTTACTGTGCACACAGTAGATG GCATTGACATCGTTCCAATGTATCGCCCGA AGGATGCCCCTAAGAAACTGGGGTATCCAG GGGTTGCTCCCTTTACGCGTGGCACTACGG TTCGCAATGGGGATATGGACGCTTGGGACG TTCGCGCCCTGCACGAAGACCCTGATGAAA AATTCACGCGCAAAGCTATTCTGGAGGGGC TGGAGCGCGGCGTAACAAGTTTGCTTCTTC GTGTGGACCCTGATGCAATCGCTCCCGAAC ACTTAGACGAAGTGTTAAGTGACGTTTTGC TGGAAATGACCAAGGTTGAGGTGTTTTCCC	**SEQ ID NO: 31**

	GCTATGATCAGGGAGCTGCGGCTGAAGCTC TTGTCTCGGTATATGAGCGCAGCGACAAAC CGGCTAAAGATTTGGCCTTAAATTTGGGAC TGGACCCAATCGCATTTGCTGCACTTCAGG GCACTGAGCCAGACTTGACCGTACTTGGTG ATTGGGTTCGTCGTTTGGCTAAATTCAGCC CAGACTCACGCGCTGTAACAATTGATGCTA ATATTTATCACAACGCCGGTGCAGGCGACG TTGCCGAGCTGGCCTGGGCACTTGCGACCG GAGCAGAGTACGTCCGTGCGCTGGTAGAG CAAGGATTCACCGCCACAGAGGCATTTGAT ACCATTAACTTCCGTGTGACAGCGACCCAT GATCAATTTTTAACGATTGCCCGCCTTCGT GCGTTACGTGAAGCGTGGGCTCGTATCGGT GAGGTATTCGGAGTAGATGAGGATAAACGT GGAGCGCGCCAGAATGCTATTACGTCCTGG CGTGAACTGACACGCGAGGATCCCTATGTG AACATTTTACGTGGAAGTATTGCCACGTTC TCTGCGTCCGTTGGGGGCGCGGAGTCTATT ACCACTTTGCCATTCACGCAGGCATTGGGC CTTCCAGAGGATGATTTTCCATTACGTATC GCACGTAATACAGGAATTGTCTTAGCTGAG GAGGTAAACATTGGGCGTGTAAATGACCCT GCCGGGGGGTCATACTATGTGGAGAGCTT GACTCGTTCTCTTGCAGATGCAGCATGGAA AGAGTTCCAAGAGGTTGAAAAGTTGGGTGG TATGTCTAAGGCCGTCATGACCGAACACGT CACGAAGGTTTTAGATGCTTGCAACGCAGA GCGCGCGAAGCGCTTGGCCAACCGCAAGC AACCTATTACGGCAGTTTCCGAATTTCCGA TGATTGGCGCACGCAGCATTGAGACGAAAC CATTTCCGGCTGCTCCGGCCCGTAAAGGGC TGGCATGGCACCGCGATTCCGAAGTCTTCG AGCAACTTATGGACCGCTCCACGTCAGTTT CAGAGCGTCCGAAAGTATTTTTAGCATGTC TTGGGACGCGCCGCGATTTTGGAGGACGC GAAGGATTTTCATCTCCGGTTTGGCACATT GCCGGGATTGACACGCCTCAAGTAGAAGGT GGGACGACTGCTGAAATCGTGGAAGCGTTC AAAAAATCTGGGGCCCAAGTCGCCGATTTA TGTTCGAGTGCCAAAGTGTATGCTCAACAA GGCTTAGAGGTGGCAAAGGCTCTGAAAGC GGCTGGGGCTAAGGCGCTGTATTTGAGCG GAGCATTTAAGGAGTTCGGAGACGATGCAG CGGAAGCCGAAAAACTTATCGACGGACGCC TTTTCATGGGCATGGATGTCGTTGACACCC TGTCTTCCACTTTAGATATCCTTGGAGTGG CGAAGTGATAAGCTTAAAACAATTTACATCC GGCCGGAACTTACT**ATGTCTACCTTACCTCG**	

	CTTTGACAGTGTTGATTTAGGAAATGCGCC GGTCCCAGCAGATGCTGCACGTCGTTTTGA GGAACTTGCGGCGAAAGCCGGGACCGGCG AAGCCTGGGAAACTGCGGAACAAATTCCAG TAGGCACGTTGTTTAATGAAGACGTATACA AGGACATGGATTGGCTTGATACTTACGCTG GCATTCCTCCCTTCGTCCATGGTCCGTACG CTACTATGTATGCATTTCGTCCTTGGACCA TTCGCCAATATGCCGGTTTTTCGACTGCAA AGGAGTCAAACGCATTTTACCGTCGTAATT TGGCTGCAGGCCAGAAAGGTCTTAGTGTTG CTTTTGACTTACCCACTCACCGCGGTTATG ATTCCGACAACCCCCGCGTGGCCGGAGATG TTGGTATGGCCGGTGTGGCTATCGATTCGA TTTATGACATGCGTGAGCTGTTCGCCGGCA TCCCATTAGATCAGATGAGCGTGTCGATGA CAATGAACGGTGCTGTCTTGCCGATTTTGG CTCTTTATGTGGTTACGGCGGAGGAGCAAG GCGTGAAGCCAGAACAACTGGCGGGTACT ATTCAAAATGATATTCTGAAGGAATTTATG GTTCGTAATACATATATTTACCCGCCGCAA CCTAGTATGCGCATTATCAGCGAGATTTTT GCATACACATCAGCAAACATGCCGAAGTGG AACTCCATTAGTATCAGCGGCTATCATATG CAGGAGGCTGGAGCGACTGCGGATATCGA GATGGCGTATACCTTAGCTGATGGAGTTGA TTACATCCGTGCTGGTGAGTCAGTAGGACT TAATGTGGACCAATTTGCTCCACGCCTGTC CTTCTTCTGGGGCATTGGTATGAACTTTTT CATGGAGGTAGCGAAGTTACGCGCTGCCC GTATGCTGTGGGCGAAGCTTGTCCACCAGT TCGGCCCGAAAAACCCGAAGAGTATGTCTC TGCGCACGCACTCTCAAACATCGGGTTGGT CTTTGACAGCTCAAGACGTATATAATAACG TTGTACGTACATGCATCGAAGCCATGGCTG CTACTCAAGGCCATACTCAATCACTTCATA CAAATTCGTTGGATGAAGCCATTGCATTGC CTACGGACTTTTCAGCCCGCATTGCCCGCA ATACTCAATTATTTCTGCAACAAGAGAGCG GGACGACTCGTGTGATCGACCCTTGGTCAG GTTCCGCATACGTCGAAGAGTTGACTTGGG ATTTAGCTCGTAAAGCCTGGGGGCATATTC AGGAGGTTGAGAAGGTGGGGGGCATGGCT AAGGCAATCGAGAAGGGGATTCCGAAGAT GCGCATTGAGGAGGCAGCCGCCCGTACCC AAGCACGTATTGATTCGGGACGCCAGCCAT TAATTGGGGTCAATAAATACCGTCTGGAGC ACGAACCACCCCTGGATGTGTTGAAGGTAG ACAATAGCACCGTGTTAGCTGAGCAAAAGG	

	CCAAACTTGTTAAATTGCGCGCAGAACGCG ACCCAGAAAAGGTCAAGGCTGCTCTGGACA AAATCACTTGGGCGGCTGGCAATCCTGATG ATAAAGACCCTGATCGCAACTTATTAAAGC TGTGCATTGATGCGGGGCGCGCGATGGCA ACGGTAGGAGAGATGAGTGACGCTTTAGA GAAAGTTTTTGGGCGCTACACAGCGCAAAT TCGCACTATTTCAGGAGTATATTCAAAAGA AGTCAAAAACACTCCGGAAGTCGAGGAGG CTCGCGAACTGGTAGAAGAGTTTGAGCAGG CCGAAGGCCGTCGCCCACGTATCCTGCTGG CTAAAATGGGGCAGGACGGTCATGACCGT GGGCAAAAGGTCATCGCGACTGCATACGCC GATTTGGGATTTGACGTGGACGTTGGCCCG TTATTCCAAACTCCCGAGGAAACTGCTCGC CAAGCCGTCGAAGCCGATGTGCACGTAGTG GGGGTGAGCTCTCTGGCGGGAGGGCATCT TACGCTTGTGCCTGCGCTTCGCAAAGAGCT GGACAAGTTGGGTCGTCCAGATATTCTGAT TACCGTAGGAGGGGTTATTCCCGAGCAGGA CTTCGATGAGCTTCGTAAGGATGGCGCTGT TGAAATCTACACACCGGGGACGGTCATTCC AGAATCGGCTATCTCTTTAGTTAAAAAATT GCGCGCCTCCCTGGATGCT	
mmcE sequence (comprised in the mmcE-mutA-mutB construct shown in **FIG. 15B and FIG. 16**)	ATGAGTAACGAAGATTTATTCATTTGCATCGA CCACGTCGCGTATGCGTGCCCGGATGCCGATG AAGCTTCTAAGTATTACCAGGAAACATTCGGT TGGCACGAGTTGCACCGCGAAGAGAATCCAG AACAGGGCGTGGTGGAAATTATGATGGCGCC TGCTGCGAAATTGACGGAGCACATGACTCAG GTGCAAGTTATGGCGCCTTTGAACGATGAGAG TACGGTCGCGAAGTGGCTTGCGAAACACAAT GGGCGTGCTGGATTGCACCACATGGCATGGC GTGTTGATGACATCGACGCAGTGTCCGCAACA CTTCGCGAGCGCGGTGTACAGTTGCTTTACGA CGAGCCGAAACTGGGTACAGGTGGGAATCGT ATCAACTTCATGCATCCGAAATCTGGTAAAGG CGTGCTGATTGAACTGACCCAGTACCCCAAGA ATTGA	**SEQ ID NO: 32**
mutA sequence (comprised in the mmcE-mutA-mutB construct shown in **FIG. 15B and FIG. 16**)	ATGTCAAGTACAGACCAAGGCACGAACCCTG CTGACACGGATGATTTAACGCCAACCACATTA TCCCTGGCTGGTGATTTCCCTAAGGCTACGGA AGAGCAGTGGGAGCGCGAGGTTGAAAAGGTG TTGAACCGTGGGCGCCCACCCGAGAAGCAGT TGACGTTTGCTGAATGTTTAAAACGTCTTACT GTGCACACAGTAGATGGCATTGACATCGTTCC AATGTATCGCCCGAAGGATGCCCCTAAGAAA CTGGGGTATCCAGGGGTTGCTCCCTTTACGCG	**SEQ ID NO: 33**

	TGGCACTACGGTTCGCAATGGGGATATGGAC GCTTGGGACGTTCGCGCCCTGCACGAAGACCC TGATGAAAAATTCACGCGCAAAGCTATTCTGG AGGGGCTGGAGCGCGGCGTAACAAGTTTGCT TCTTCGTGTGGACCCTGATGCAATCGCTCCCG AACACTTAGACGAAGTGTTAAGTGACGTTTTG CTGGAAATGACCAAGGTTGAGGTGTTTTCCCG CTATGATCAGGGAGCTGCGGCTGAAGCTCTTG TCTCGGTATATGAGCGCAGCGACAAACCGGCT AAAGATTTGGCCTTAAATTTGGGACTGGACCC AATCGCATTTGCTGCACTTCAGGGCACTGAGC CAGACTTGACCGTACTTGGTGATTGGGTTCGT CGTTTGGCTAAATTCAGCCCAGACTCACGCGC TGTAACAATTGATGCTAATATTTATCACAACG CCGGTGCAGGCGACGTTGCCGAGCTGGCCTG GGCACTTGCGACCGGAGCAGAGTACGTCCGT GCGCTGGTAGAGCAAGGATTCACCGCCACAG AGGCATTTGATACCATTAACTTCCGTGTGACA GCGACCCATGATCAATTTTTAACGATTGCCCG CCTTCGTGCGTTACGTGAAGCGTGGGCTCGTA TCGGTGAGGTATTCGGAGTAGATGAGGATAA ACGTGGAGCGCGCCAGAATGCTATTACGTCCT GGCGTGAACTGACACGCGAGGATCCCTATGT GAACATTTTACGTGGAAGTATTGCCACGTTCT CTGCGTCCGTTGGGGGCGCGGAGTCTATTACC ACTTTGCCATTCACGCAGGCATTGGGCCTTCC AGAGGATGATTTTCCATTACGTATCGCACGTA ATACAGGAATTGTCTTAGCTGAGGAGGTAAA CATTGGGCGTGTAAATGACCCTGCCGGGGGGT CATACTATGTGGAGAGCTTGACTCGTTCTCTT GCAGATGCAGCATGGAAAGAGTTCCAAGAGG TTGAAAAGTTGGGTGGTATGTCTAAGGCCGTC ATGACCGAACACGTCACGAAGGTTTTAGATGC TTGCAACGCAGAGCGCGCGAAGCGCTTGGCC AACCGCAAGCAACCTATTACGGCAGTTTCCGA ATTTCCGATGATTGGCGCACGCAGCATTGAGA CGAAACCATTTCCGGCTGCTCCGGCCCGTAAA GGGCTGGCATGGCACCGCGATTCCGAAGTCTT CGAGCAACTTATGGACCGCTCCACGTCAGTTT CAGAGCGTCCGAAAGTATTTTTAGCATGTCTT GGGACGCGCCGCGATTTTGGAGGACGCGAAG GATTTTCATCTCCGGTTTGGCACATTGCCGGG ATTGACACGCCTCAAGTAGAAGGTGGGACGA CTGCTGAAATCGTGGAAGCGTTCAAAAAATCT GGGGCCCAAGTCGCCGATTTATGTTCGAGTGC CAAAGTGTATGCTCAACAAGGCTTAGAGGTG GCAAAGGCTCTGAAAGCGGCTGGGGCTAAGG CGCTGTATTTGAGCGGAGCATTTAAGGAGTTC GGAGACGATGCAGCGGAAGCCGAAAAACTTA	

	TCGACGGACGCCTTTTCATGGGCATGGATGTC GTTGACACCCTGTCTTCCACTTTAGATATCCTT GGAGTGGCGAAGTGA	
mutB sequence (comprised in the mmcE-mutA-mutB construct shown in **FIG. 15B and FIG. 16**)	ATGTCTACCTTACCTCGCTTTGACAGTGTTGAT TTAGGAAATGCGCCGGTCCCAGCAGATGCTGC ACGTCGTTTTGAGGAACTTGCGGCGAAAGCCG GGACCGGCGAAGCCTGGGAAACTGCGGAACA AATTCCAGTAGGCACGTTGTTTAATGAAGACG TATACAAGGACATGGATTGGCTTGATACTTAC GCTGGCATTCCTCCCTTCGTCCATGGTCCGTA CGCTACTATGTATGCATTTCGTCCTTGGACCA TTCGCCAATATGCCGGTTTTTCGACTGCAAAG GAGTCAAACGCATTTTACCGTCGTAATTTGGC TGCAGGCCAGAAAGGTCTTAGTGTTGCTTTTG ACTTACCCACTCACCGCGGTTATGATTCCGAC AACCCCCGCGTGGCCGGAGATGTTGGTATGGC CGGTGTGGCTATCGATTCGATTTATGACATGC GTGAGCTGTTCGCCGGCATCCCATTAGATCAG ATGAGCGTGTCGATGACAATGAACGGTGCTGT CTTGCCGATTTTGGCTCTTTATGTGGTTACGGC GGAGGAGCAAGGCGTGAAGCCAGAACAACTG GCGGGTACTATTCAAAATGATATTCTGAAGGA ATTTATGGTTCGTAATACATATATTTACCCGC CGCAACCTAGTATGCGCATTATCAGCGAGATT TTTGCATACACATCAGCAAACATGCCGAAGTG GAACTCCATTAGTATCAGCGGCTATCATATGC AGGAGGCTGGAGCGACTGCGGATATCGAGAT GGCGTATACCTTAGCTGATGGAGTTGATTACA TCCGTGCTGGTGAGTCAGTAGGACTTAATGTG GACCAATTTGCTCCACGCCTGTCCTTCTTCTGG GGCATTGGTATGAACTTTTTCATGGAGGTAGC GAAGTTACGCGCTGCCCGTATGCTGTGGGCGA AGCTTGTCCACCAGTTCGGCCCGAAAAACCCG AAGAGTATGTCTCTGCGCACGCACTCTCAAAC ATCGGGTTGGTCTTTGACAGCTCAAGACGTAT ATAATAACGTTGTACGTACATGCATCGAAGCC ATGGCTGCTACTCAAGGCCATACTCAATCACT TCATACAAATTCGTTGGATGAAGCCATTGCAT TGCCTACGGACTTTTCAGCCCGCATTGCCCGC AATACTCAATTATTTCTGCAACAAGAGAGCGG GACGACTCGTGTGATCGACCCTTGGTCAGGTT CCGCATACGTCGAAGAGTTGACTTGGGATTTA GCTCGTAAAGCCTGGGGGCATATTCAGGAGG TTGAGAAGGTGGGGGGCATGGCTAAGGCAAT CGAGAAGGGGATTCCGAAGATGCGCATTGAG GAGGCAGCCGCCCGTACCCAAGCACGTATTG ATTCGGGACGCCAGCCATTAATTGGGGTCAAT AAATACCGTCTGGAGCACGAACCACCCCTGG ATGTGTTGAAGGTAGACAATAGCACCGTGTTA	**SEQ ID NO: 34**

	GCTGAGCAAAAGGCCAAACTTGTTAAATTGC GCGCAGAACGCGACCCAGAAAAGGTCAAGGC TGCTCTGGACAAAATCACTTGGGCGGCTGGCA ATCCTGATGATAAAGACCCTGATCGCAACTTA TTAAAGCTGTGCATTGATGCGGGGCGCGCGAT GGCAACGGTAGGAGAGATGAGTGACGCTTTA GAGAAAGTTTTGGGCGCTACACAGCGCAAA TTCGCACTATTTCAGGAGTATATTCAAAAGAA GTCAAAAACACTCCGGAAGTCGAGGAGGCTC GCGAACTGGTAGAAGAGTTTGAGCAGGCCGA AGGCCGTCGCCCACGTATCCTGCTGGCTAAAA TGGGGCAGGACGGTCATGACCGTGGGCAAAA GGTCATCGCGACTGCATACGCCGATTTGGGAT TTGACGTGGACGTTGGCCCGTTATTCCAAACT CCCGAGGAAACTGCTCGCCAAGCCGTCGAAG CCGATGTGCACGTAGTGGGGGTGAGCTCTCTG GCGGGAGGGCATCTTACGCTTGTGCCTGCGCT TCGCAAAGAGCTGGACAAGTTGGGTCGTCCA GATATTCTGATTACCGTAGGAGGGGTTATTCC CGAGCAGGACTTCGATGAGCTTCGTAAGGAT GGCGCTGTTGAAATCTACACACCGGGGACGG TCATTCCAGAATCGGCTATCTCTTTAGTTAAA AAATTGCGCGCCTCCCTGGATGCT	
Construct comprising TetR (reverse orientation, lowercase) and prpE-accA-pccB gene cassette driven by tet inducible promoter (italics) (as shown in **FIG. 15B and FIG. 16**); ribosome binding sites are underlined ;coding sequences bold and underlined	Ttaagacccactttcacatttaagttgttttctaatccgcatatgatcaattcaa ggccgaataagaaggctggctctgcaccttggtgatcaaataattcgatagc ttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttcttctttag cgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccaca gcgctgagtgcatataatgcattctctagtgaaaaaccttgttggcataaaaa ggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaat gtactttgctccatcgcgatgacttagtaaagcacatctaaaacttttagcgtt attacgtaaaaaatcttgccagctttccccttctaaagggcaaaagtgagtat ggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttattt tttacatgccaatacaatgtaggctgctctacacctagcttctgggcgagttta cgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgtta atcactttacttttatctaatctagacatcat*TAATTCCTAATTTTTG* *TTGACACTCTATCATTGATAGAGTTATTTTACCAC* *TCCCTATCAGTGATAGAGAAAAGTGAA*T*AAGGCG* *TAAGTTCAACAGGAGAGCATT*TAAGGCGTAAGT TCAACAGGAGAGCATT**ATGTCTTTTAGCGAA** **TTTTATCAGCGTTCGATTAACGAACCGGAG** **AAGTTCTGGGCCGAGCAGGCCCGGCGTATT** **GACTGGCAGACGCCCTTTACGCAAACGCTC** **GACCACAGCAACCCGCCGTTTGCCCGTTGG** **TTTTGTGAAGGCCGAACCAACTTGTGTCAC** **AACGCTATCGACCGCTGGCTGGAGAAACAG** **CCAGAGGCGCTGGCATTGATTGCCGTCTCT** **TCGGAAACAGAGGAAGAGCGTACCTTTACC** **TTCCGCCAGTTACATGACGAAGTGAATGCG** **GTGGCGTCAATGCTGCGCTCACTGGGCGTG**	**SEQ ID NO: 35**

	CAGCGTGGCGATCGGGTGCTGGTGTATATG CCGATGATTGCCGAAGCGCATATTACCCTG CTGGCCTGCGCGCGCATTGGTGCTATTCAC TCGGTGGTGTTTGGGGGATTTGCTTCGCAC AGCGTGGCAACGCGAATTGATGACGCTAAA CCGGTGCTGATTGTCTCGGCTGATGCCGGG GCGCGCGGCGGTAAAATCATTCCGTATAAA AAATTGCTCGACGATGCGATAAGTCAGGCA CAGCATCAGCCGCGTCACGTTTTACTGGTG GATCGCGGGCTGGCGAAAATGGCGCGCGT TAGCGGGCGGGATGTCGATTTCGCGTCGTT GCGCCATCAACACATCGGCGCGCGGGTGC CGGTGGCATGGCTGGAATCCAACGAAACCT CCTGCATTCTCTACACCTCCGGCACGACCG GCAAACCTAAAGGTGTGCAGCGTGATGTCG GCGGATATGCGGTGGCGCTGGCGACCTCG ATGGACACCATTTTTGGCGGCAAAGCGGGC GGCGTGTTCTTTTGTGCTTCGGATATCGGC TGGGTGGTAGGGCATTCGTATATCGTTTAC GCGCCGCTGCTGGCGGGGATGGCGACTAT CGTTTACGAAGGATTGCCGACCTGGCCGGA CTGCGGCGTGTGGTGGAAAATTGTCGAGAA ATATCAGGTTAGCCGCATGTTCTCAGCGCC GACCGCCATTCGCGTGCTGAAAAAATTCCC TACCGCTGAAATTCGCAAACACGATCTTTC GTCGCTGGAAGTGCTCTATCTGGCTGGAGA ACCGCTGGACGAGCCGACCGCCAGTTGGG TGAGCAATACGCTGGATGTGCCGGTCATCG ACAACTACTGGCAGACCGAATCCGGCTGGC CGATTATGGCGATTGCTCGCGGTCTGGATG ACAGACCGACGCGTCTGGGAAGCCCCGGC GTGCCGATGTATGGCTATAACGTGCAGTTG CTCAATGAAGTCACCGGCGAACCGTGTGGC GTCAATGAGAAAGGGATGCTGGTAGTGGA GGGGCCATTGCCGCCAGGCTGTATTCAAAC CATCTGGGGCGACGACGACCGCTTTGTGAA GACGTACTGGTCGCTGTTTTCCCGTCCGGT GTACGCCACTTTTGACTGGGGCATCCGCGA TGCTGACGGTTATCACTTTATTCTCGGGCG CACTGACGATGTGATTAACGTTGCCGGACA TCGGCTGGGTACGCGTGAGATTGAAGAGA GTATCTCCAGTCATCCGGGCGTTGCCGAAG TGGCGGTGGTTGGGGTGAAAGATGCGCTG AAAGGGCAGGTGGCGGTGGCGTTTGTCATT CCGAAAGAGAGCGACAGTCTGGAAGACCG TGAGGTGGCGCACTCGCAAGAGAAGGCGA TTATGGCGCTGGTGGACAGCCAGATTGGCA ACTTTGGCCGCCCGGCGCACGTCTGGTTTG TCTCGCAATTGCCAAAAACGCGATCCGGAA	

	AAATGCTGCGCCGCACGATCCAGGCGATTT **GCGAAGGACGCGATCCTGGGGATCTGACG** **ACCATTGATGATCCGGCGTCGTTGGATCAG** **ATCCGCCAGGCGATGGAAGAGTAG**TACTAG ATTCAATATAGAGTAAAAGAGGTAAGAGTAT CCA**TGCGTAAAGTTCTGATCGCTAATCGTG** **GAGAAATTGCTGTACGTGTAGCACGTGCAT** **GTCGTGATGCGGGAATCGCATCAGTAGCCG** **TATACGCGGACCCGGATCGTGACGCGTTGC** **ATGTGCGCGCGGCGGACGAAGCATTTGCA** **CTGGGTGGTGATACGCCTGCAACATCTTAC** **TTAGACATCGCCAAGGTGTTAAAGGCTGCA** **CGTGAGAGTGGTGCAGACGCCATTCATCCC** **GGTTACGGCTTTTTAAGTGAAAATGCCGAG** **TTCGCGCAGGCCGTGTTAGATGCGGGTCTT** **ATCTGGATCGGACCACCGCCCCATGCAATC** **CGCGATCGTGGGGAAAAAGTTGCAGCTCG** **CCATATTGCCCAGCGTGCTGGGGCGCCGCT** **GGTTGCGGGCACCCCTGACCCGGTTTCTGG** **TGCTGACGAAGTCGTCGCCTTCGCGAAAGA** **GCATGGACTGCCGATCGCGATTAAGGCTGC** **TTTTGGAGGCGGTGGTCGTGGTTTAAAGGT** **TGCCCGTACATTGGAAGAAGTGCCCGAGTT** **ATATGACTCCGCCGTGCGTGAAGCTGTGGC** **GGCATTCGGACGTGGCGAATGTTTCGTGGA** **GCGCTATTTAGACAAACCGCGTCATGTAGA** **AACCCAGTGCTTGGCAGATACTCACGGTAA** **TGTAGTTGTGGTTTCTACTCGCGACTGTTC** **GTTACAGCGTCGTCATCAGAAACTGGTAGA** **GGAGGCACCCGCCCCGTTTTTAAGCGAAGC** **TCAGACAGAGCAACTGTACTCCTCCTCCAA** **GGCTATTCTTAAGGAAGCTGGGTATGGTGG** **AGCGGGAACCGTTGAGTTTTTAGTAGGTAT** **GGATGGTACTATCTTCTTCTTGGAGGTCAA** **TACCCGCCTGCAGGTGGAGCACCCTGTGAC** **CGAAGAAGTCGCAGGGATCGACCTGGTCC** **GTGAAATGTTCCGCATTGCAGATGGCGAGG** **AGCTGGGGTACGACGATCCAGCCCTTCGCG** **GCCACTCGTTCGAATTTCGCATCAATGGGG** **AGGACCCAGGTCGTGGTTTTTTGCCCGCAC** **CTGGTACGGTTACGCTTTTTGATGCTCCGA** **CCGGACCCGGAGTCCGCCTGGATGCCGGG** **GTTGAGTCAGGTTCCGTAATCGGACCGGCA** **TGGGACTCACTGCTGGCTAAACTTATCGTT** **ACCGGGCGTACACGTGCCGAGGCGCTTCA** **GCGCGCAGCCCGCGCCTTAGATGAATTTAC** **GGTTGAGGGCATGGCAACCGCGATCCCTTT** **CCATCGCACAGTAGTACGCGATCCAGCATT** **CGCTCCTGAGCTTACCGGGTCAACGGACCC**	

	ATTCACCGTTCATACACGCTGGATTGAAAC TGAATTTGTCAACGAAATTAAGCCTTTTAC CACCCCTGCCGACACGGAGACAGATGAAG AGTCTGGGCGCGAGACAGTGGTAGTCGAG GTCGGTGGGAAACGCTTAGAGGTAAGTCTT CCGTCCAGCCTGGGAATGTCGTTGGCCCGT ACCGGCCTTGCCGCGGGGGCCCGCCCCAA ACGCCGCGCGGCCAAGAAGTCAGGCCCTG CAGCATCGGGTGATACACTGGCATCTCCTA TGCAAGGTACGATCGTAAAGATCGCCGTGG AAGAGGGACAAGAAGTACAGGAGGGAGATCT GATTGTGGTTCTTGAAGCTATGAAGATGGAAC AGCCACTTAATGCCCACCGTTCGGGAACCATT AAGGGGCTTACTGCTGAAGTAGGTGCTTCACT GACGTCGGGCGCCGCTATCTGTGAAATCAAG GATTGATAACGCTAACGAAAAAGTTAAATAC AGGAACAAGAGAACATATGTCGGAGCCCGA GGAACAGCAGCCAGATATCCACACGACAGC GGGCAAGTTAGCTGATCTTCGTCGCCGCAT CGAAGAGGCAACGCACGCCGGTTCTGCGC GCGCGGTGGAGAAACAGCACGCGAAGGGT AAACTTACGGCTCGTGAGCGTATCGATTTG TTGCTGGACGAAGGGTCTTTTGTAGAGCTT GATGAGTTTGCGCGTCACCGTTCGACGAAT TTCGGACTGGATGCCAACCGTCCATATGGA GATGGAGTGGTGACTGGCTATGGAACTGTT GACGGACGTCCGGTTGCCGTCTTTTCGCAA GACTTTACGGTCTTTGGGGGCGCTCTGGGG GAAGTATACGGGCAAAAAATTGTGAAGGTC ATGGATTTCGCTCTTAAGACCGGGTGTCCC GTCGTGGGTATTAATGACTCAGGTGGGGCA CGCATTCAAGAGGGTGTAGCAAGTCTGGGC GCGTATGGAGAGATTTTCCGTCGCAATACG CACGCGTCGGGCGTGATCCCTCAGATTTCG CTTGTAGTTGGCCCATGCGCAGGGGGAGCT GTGTACTCTCCAGCTATTACTGACTTTACG GTAATGGTCGACCAAACATCGCATATGTTT ATCACCGGACCCGATGTGATTAAGACAGTG ACAGGGGAGGATGTGGGTTTTGAGGAACTT GGTGGTGCGCGTACGCACAACAGTACGTCT GGGGTTGCCCATCATATGGCTGGGGATGA GAAAGACGCTGTGGAGTATGTTAAGCAATT ATTGAGTTATTTGCCGTCGAACAATTTAAG TGAGCCTCCGGCGTTTCCTGAAGAGGCTGA TTTAGCCGTTACGGACGAAGATGCGGAATT AGATACAATTGTGCCGGATTCGGCTAACCA ACCCTATGATATGCATTCTGTAATCGAGCA TGTCCTTGACGATGCGGAATTTTTCGAGAC TCAACCGTTGTTTGCCCCCAACATCCTGAC	

	CGGCTTTGGTCGCGTTGAAGGCCGTCCGGT **GGGTATCGTGGCGAATCAGCCGATGCAGTT** **TGCTGGATGCTTAGATATCACTGCCTCAGA** **AAAAGCTGCTCGTTTCGTTCGCACTTGCGA** **CGCTTTCAACGTCCCTGTGCTTACGTTTGT** **AGACGTCCCCGGGTTTTTACCGGGCGTAGA** **TCAGGAGCATGACGGGATCATCCGCCGCG** **GTGCGAAGTTGATTTTTGCCTATGCAGAAG** **CGACCGTGCCGTTGATCACAGTAATCACGC** **GCAAAGCCTTCGGAGGTGCGTATGACGTAA** **TGGGCTCAAAACACCTTGGCGCTGACCTTA** **ATCTGGCATGGCCCACGGCCCAAATCGCTG** **TAATGGGCGCTCAAGGTGCTGTAAACATCC** **TTCATCGTCGTACGATTGCAGATGCGGGGG** **ACGATGCGGAAGCCACGCGCGCCCGTTTAA** **TTCAAGAGTACGAGGATGCTTTATTAAATC** **CCTATACTGCGGCTGAGCGCGGGTATGTAG** **ACGCGGTCATCATGCCCTCAGATACTCGCC** **GTCATATCGTACGTGGTTTACGCCAATTAC** **GCACCAAGCGCGAGTCTTTACCCCCGAAAA** **AGCACGGGAACATTCCCCTT** TGAGGAGGTCGGATAAGGCGCTCGCGCCGCA TCCGACACCGTGCGCAGATGCCTGATGCGACG CTGACGCGTCTTATCATGCCTCGCTCTCGAGT CCCGTCAAGTCAGCGTAATGCTCTGCCAGTGT TACAACCAATTAACCAATTCTGAT	
Construct comprising a prpE-accA-pccB gene cassette under the control of the Ptet promoter (as shown in **FIG. 15B and FIG. 16**) ribosome binding sites are underlined ;. L3S2P11 terminator in italics; his terminator in bold ; coding sequences bold and underlined	*TAATTCCTAATTTTTGTTGACACTCTATCATTGATA* *GAGTTATTTTACCACTCCCTATCAGTGATAGAGAA* *AAGTGAATAAGGCGTAAGTTCAACAGGAGAGCAT* *T*TAAGGCGTAAGTTCAACAGGAGAGCATTA**T** **GTCTTTTAGCGAATTTTATCAGCGTTCGATT** **AACGAACCGGAGAAGTTCTGGGCCGAGCA** **GGCCCGGCGTATTGACTGGCAGACGCCCTT** **TACGCAAACGCTCGACCACAGCAACCCGCC** **GTTTGCCCGTTGGTTTTGTGAAGGCCGAAC** **CAACTTGTGTCACAACGCTATCGACCGCTG** **GCTGGAGAAACAGCCAGAGGCGCTGGCAT** **TGATTGCCGTCTCTTCGGAAACAGAGGAAG** **AGCGTACCTTTACCTTCCGCCAGTTACATG** **ACGAAGTGAATGCGGTGGCGTCAATGCTGC** **GCTCACTGGGCGTGCAGCGTGGCGATCGG** **GTGCTGGTGTATATGCCGATGATTGCCGAA** **GCGCATATTACCCTGCTGGCCTGCGCGCGC** **ATTGGTGCTATTCACTCGGTGGTGTTTGGG** **GGATTTGCTTCGCACAGCGTGGCAACGCGA** **ATTGATGACGCTAAACCGGTGCTGATTGTC** **TCGGCTGATGCCGGGGCGCGCGGCGGTAA** **AATCATTCCGTATAAAAAATTGCTCGACGA** **TGCGATAAGTCAGGCACAGCATCAGCCGCG**	**SEQ ID NO: 36**

	TCACGTTTTACTGGTGGATCGCGGGCTGGC **GAAAATGGCGCGCGTTAGCGGGCGGGATG** **TCGATTTCGCGTCGTTGCGCCATCAACACA** **TCGGCGCGCGGGTGCCGGTGGCATGGCTG** **GAATCCAACGAAACCTCCTGCATTCTCTAC** **ACCTCCGGCACGACCGGCAAACCTAAAGGT** **GTGCAGCGTGATGTCGGCGGATATGCGGT** **GGCGCTGGCGACCTCGATGGACACCATTTT** **TGGCGGCAAAGCGGGCGGCGTGTTCTTTTG** **TGCTTCGGATATCGGCTGGGTGGTAGGGCA** **TTCGTATATCGTTTACGCGCCGCTGCTGGC** **GGGGATGGCGACTATCGTTTACGAAGGATT** **GCCGACCTGGCCGGACTGCGGCGTGTGGT** **GGAAAATTGTCGAGAAATATCAGGTTAGCC** **GCATGTTCTCAGCGCCGACCGCCATTCGCG** **TGCTGAAAAAATTCCCTACCGCTGAAATTC** **GCAAACACGATCTTTCGTCGCTGGAAGTGC** **TCTATCTGGCTGGAGAACCGCTGGACGAGC** **CGACCGCCAGTTGGGTGAGCAATACGCTG** **GATGTGCCGGTCATCGACAACTACTGGCAG** **ACCGAATCCGGCTGGCCGATTATGGCGATT** **GCTCGCGGTCTGGATGACAGACCGACGCG** **TCTGGGAAGCCCCGGCGTGCCGATGTATG** **GCTATAACGTGCAGTTGCTCAATGAAGTCA** **CCGGCGAACCGTGTGGCGTCAATGAGAAA** **GGGATGCTGGTAGTGGAGGGGCCATTGCC** **GCCAGGCTGTATTCAAACCATCTGGGGCGA** **CGACGACCGCTTTGTGAAGACGTACTGGTC** **GCTGTTTTCCCGTCCGGTGTACGCCACTTT** **TGACTGGGGCATCCGCGATGCTGACGGTTA** **TCACTTTATTCTCGGGCGCACTGACGATGT** **GATTAACGTTGCCGGACATCGGCTGGGTAC** **GCGTGAGATTGAAGAGAGTATCTCCAGTCA** **TCCGGGCGTTGCCGAAGTGGCGGTGGTTG** **GGGTGAAAGATGCGCTGAAAGGGCAGGTG** **GCGGTGGCGTTTGTCATTCCGAAAGAGAGC** **GACAGTCTGGAAGACCGTGAGGTGGCGCA** **CTCGCAAGAGAAGGCGATTATGGCGCTGGT** **GGACAGCCAGATTGGCAACTTTGGCCGCCC** **GGCGCACGTCTGGTTTGTCTCGCAATTGCC** **AAAAACGCGATCCGGAAAAATGCTGCGCCG** **CACGATCCAGGCGATTTGCGAAGGACGCG** **ATCCTGGGGATCTGACGACCATTGATGATC** **CGGCGTCGTTGGATCAGATCCGCCAGGCG** **ATGGAAGAGTAG**TACTAGATTCAATATAGAG TAAAAGAGGTAAGAGTATCCA**TGCGTAAAGT** **TCTGATCGCTAATCGTGGAGAAATTGCTGT** **ACGTGTAGCACGTGCATGTCGTGATGCGGG** **AATCGCATCAGTAGCCGTATACGCGGACCC**	

	GGATCGTGACGCGTTGCATGTGCGCGCGG CGGACGAAGCATTTGCACTGGGTGGTGATA CGCCTGCAACATCTTACTTAGACATCGCCA AGGTGTTAAAGGCTGCACGTGAGAGTGGT GCAGACGCCATTCATCCCGGTTACGGCTTT TTAAGTGAAAATGCCGAGTTCGCGCAGGCC GTGTTAGATGCGGGTCTTATCTGGATCGGA CCACCGCCCCATGCAATCCGCGATCGTGGG GAAAAAGTTGCAGCTCGCCATATTGCCCAG CGTGCTGGGGCGCCGCTGGTTGCGGGCAC CCCTGACCCGGTTTCTGGTGCTGACGAAGT CGTCGCCTTCGCGAAAGAGCATGGACTGCC GATCGCGATTAAGGCTGCTTTTGGAGGCGG TGGTCGTGGTTTAAAGGTTGCCCGTACATT GGAAGAAGTGCCCGAGTTATATGACTCCGC CGTGCGTGAAGCTGTGGCGGCATTCGGAC GTGGCGAATGTTTCGTGGAGCGCTATTTAG ACAAACCGCGTCATGTAGAAACCCAGTGCT TGGCAGATACTCACGGTAATGTAGTTGTGG TTTCTACTCGCGACTGTTCGTTACAGCGTC GTCATCAGAAACTGGTAGAGGAGGCACCC GCCCCGTTTTTAAGCGAAGCTCAGACAGAG CAACTGTACTCCTCCTCCAAGGCTATTCTT AAGGAAGCTGGGTATGGTGGAGCGGGAAC CGTTGAGTTTTTAGTAGGTATGGATGGTAC TATCTTCTTCTTGGAGGTCAATACCCGCCT GCAGGTGGAGCACCCTGTGACCGAAGAAG TCGCAGGGATCGACCTGGTCCGTGAAATGT TCCGCATTGCAGATGGCGAGGAGCTGGGG TACGACGATCCAGCCCTTCGCGGCCACTCG TTCGAATTTCGCATCAATGGGGAGGACCCA GGTCGTGGTTTTTTGCCCGCACCTGGTACG GTTACGCTTTTTGATGCTCCGACCGGACCC GGAGTCCGCCTGGATGCCGGGGTTGAGTC AGGTTCCGTAATCGGACCGGCATGGGACTC ACTGCTGGCTAAACTTATCGTTACCGGGCG TACACGTGCCGAGGCGCTTCAGCGCGCAG CCCGCGCCTTAGATGAATTTACGGTTGAGG GCATGGCAACCGCGATCCCTTTCCATCGCA CAGTAGTACGCGATCCAGCATTCGCTCCTG AGCTTACCGGGTCAACGGACCCATTCACCG TTCATACACGCTGGATTGAAACTGAATTTG TCAACGAAATTAAGCCTTTTACCACCCCTG CCGACACGGAGACAGATGAAGAGTCTGGG CGCGAGACAGTGGTAGTCGAGGTCGGTGG GAAACGCTTAGAGGTAAGTCTTCCGTCCAG CCTGGGAATGTCGTTGGCCCGTACCGGCCT TGCCGCGGGGGCCCGCCCCAAACGCCGCG CGGCCAAGAAGTCAGGCCCTGCAGCATCG	

	GGTGATACACTGGCATCTCCTATGCAAGGT **ACGATCGTAAAGATCGCCGTGGA**AGAGGGA CAAGAAGTACAGGAGGGAGATCTGATTGTGG TTCTTGAAGCTATGAAGATGGAACAGCCACTT AATGCCCACCGTTCGGGAACCATTAAGGGGCT TACTGCTGAAGTAGGTGCTTCACTGACGTCGG GCGCCGCTATCTGTGAAATCAAGGATTGATAA CGCTAACGAAAAAGTTAAATACAGGAACAAG AGAACATATGTCGGAGCCCGAGGAACAGCA GCCAGATATCCACACGACAGCGGGCAAGTT AGCTGATCTTCGTCGCCGCATCGAAGAGGC AACGCACGCCGGTTCTGCGCGCGCGGTGG AGAAACAGCACGCGAAGGGTAAACTTACG GCTCGTGAGCGTATCGATTTGTTGCTGGAC GAAGGGTCTTTTGTAGAGCTTGATGAGTTT GCGCGTCACCGTTCGACGAATTTCGGACTG GATGCCAACCGTCCATATGGAGATGGAGTG GTGACTGGCTATGGAACTGTTGACGGACGT CCGGTTGCCGTCTTTTCGCAAGACTTTACG GTCTTTGGGGGCGCTCTGGGGGAAGTATAC GGGCAAAAAATTGTGAAGGTCATGGATTTC GCTCTTAAGACCGGGTGTCCCGTCGTGGGT ATTAATGACTCAGGTGGGGCACGCATTCAA GAGGGTGTAGCAAGTCTGGGCGCGTATGG AGAGATTTTCCGTCGCAATACGCACGCGTC GGGCGTGATCCCTCAGATTTCGCTTGTAGT TGGCCCATGCGCAGGGGGAGCTGTGTACT CTCCAGCTATTACTGACTTTACGGTAATGG TCGACCAAACATCGCATATGTTTATCACCG GACCCGATGTGATTAAGACAGTGACAGGG GAGGATGTGGGTTTTGAGGAACTTGGTGGT GCGCGTACGCACAACAGTACGTCTGGGGTT GCCCATCATATGGCTGGGGATGAGAAAGAC GCTGTGGAGTATGTTAAGCAATTATTGAGT TATTTGCCGTCGAACAATTTAAGTGAGCCT CCGGCGTTTCCTGAAGAGGCTGATTTAGCC GTTACGGACGAAGATGCGGAATTAGATACA ATTGTGCCGGATTCGGCTAACCAACCCTAT GATATGCATTCTGTAATCGAGCATGTCCTT GACGATGCGGAATTTTTCGAGACTCAACCG TTGTTTGCCCCCAACATCCTGACCGGCTTT GGTCGCGTTGAAGGCCGTCCGGTGGGTAT CGTGGCGAATCAGCCGATGCAGTTTGCTGG ATGCTTAGATATCACTGCCTCAGAAAAAGC TGCTCGTTTCGTTCGCACTTGCGACGCTTT CAACGTCCCTGTGCTTACGTTTGTAGACGT CCCCGGGTTTTTACCGGGCGTAGATCAGGA GCATGACGGGATCATCCGCCGCGGTGCGA AGTTGATTTTTGCCTATGCAGAAGCGACCG	

	TGCCGTTGATCACAGTAATCACGCGCAAAG CCTTCGGAGGTGCGTATGACGTAATGGGCT CAAAACACCTTGGCGCTGACCTTAATCTGG CATGGCCCACGGCCCAAATCGCTGTAATGG GCGCTCAAGGTGCTGTAAACATCCTTCATC GTCGTACGATTGCAGATGCGGGGGACGAT GCGGAAGCCACGCGCGCCCGTTTAATTCAA GAGTACGAGGATGCTTTATTAAATCCCTAT ACTGCGGCTGAGCGCGGGTATGTAGACGC GGTCATCATGCCCTCAGATACTCGCCGTCA TATCGTACGTGGTTTACGCCAATTACGCAC CAAGCGCGAGTCTTTACCCCCGAAAAAGCA CGGGAACATTCCCCTT	
Construct comprising a prpE-accA-pccB gene cassette; (as shown in **FIG. 15B and FIG. 16)** ribosome binding sites are underlined; coding sequences bold and underlined	TAAGGCGTAAGTTCAACAGGAGAGCATTATG **TCTTTTAGCGAATTTTATCAGCGTTCGATTA ACGAACCGGAGAAGTTCTGGGCCGAGCAG GCCCGGCGTATTGACTGGCAGACGCCCTTT ACGCAAACGCTCGACCACAGCAACCCGCCG TTTGCCCGTTGGTTTTGTGAAGGCCGAACC AACTTGTGTCACAACGCTATCGACCGCTGG CTGGAGAAACAGCCAGAGGCGCTGGCATT GATTGCCGTCTCTTCGGAAACAGAGGAAGA GCGTACCTTTACCTTCCGCCAGTTACATGA CGAAGTGAATGCGGTGGCGTCAATGCTGC GCTCACTGGGCGTGCAGCGTGGCGATCGG GTGCTGGTGTATATGCCGATGATTGCCGAA GCGCATATTACCCTGCTGGCCTGCGCGCGC ATTGGTGCTATTCACTCGGTGGTGTTTGGG GGATTTGCTTCGCACAGCGTGGCAACGCGA ATTGATGACGCTAAACCGGTGCTGATTGTC TCGGCTGATGCCGGGGCGCGCGGCGGTAA AATCATTCCGTATAAAAAATTGCTCGACGA TGCGATAAGTCAGGCACAGCATCAGCCGCG TCACGTTTTACTGGTGGATCGCGGGCTGGC GAAAATGGCGCGCGTTAGCGGGCGGGATG TCGATTTCGCGTCGTTGCGCCATCAACACA TCGGCGCGCGGGTGCCGGTGGCATGGCTG GAATCCAACGAAACCTCCTGCATTCTCTAC ACCTCCGGCACGACCGGCAAACCTAAAGGT GTGCAGCGTGATGTCGGCGGATATGCGGT GGCGCTGGCGACCTCGATGGACACCATTTT TGGCGGCAAAGCGGGCGGCGTGTTCTTTTG TGCTTCGGATATCGGCTGGGTGGTAGGGCA TTCGTATATCGTTTACGCGCCGCTGCTGGC GGGGATGGCGACTATCGTTTACGAAGGATT GCCGACCTGGCCGGACTGCGGCGTGTGGT GGAAAATTGTCGAGAAATATCAGGTTAGCC GCATGTTCTCAGCGCCGACCGCCATTCGCG TGCTGAAAAAATTCCCTACCGCTGAAATTC**	**SEQ ID NO: 37**

	GCAAACACGATCTTTCGTCGCTGGAAGTGC TCTATCTGGCTGGAGAACCGCTGGACGAGC CGACCGCCAGTTGGGTGAGCAATACGCTG GATGTGCCGGTCATCGACAACTACTGGCAG ACCGAATCCGGCTGGCCGATTATGGCGATT GCTCGCGGTCTGGATGACAGACCGACGCG TCTGGGAAGCCCCGGCGTGCCGATGTATG GCTATAACGTGCAGTTGCTCAATGAAGTCA CCGGCGAACCGTGTGGCGTCAATGAGAAA GGGATGCTGGTAGTGGAGGGGCCATTGCC GCCAGGCTGTATTCAAACCATCTGGGGCGA CGACGACCGCTTTGTGAAGACGTACTGGTC GCTGTTTTCCCGTCCGGTGTACGCCACTTT TGACTGGGGCATCCGCGATGCTGACGGTTA TCACTTTATTCTCGGGCGCACTGACGATGT GATTAACGTTGCCGGACATCGGCTGGGTAC GCGTGAGATTGAAGAGAGTATCTCCAGTCA TCCGGGCGTTGCCGAAGTGGCGGTGGTTG GGGTGAAAGATGCGCTGAAAGGGCAGGTG GCGGTGGCGTTTGTCATTCCGAAAGAGAGC GACAGTCTGGAAGACCGTGAGGTGGCGCA CTCGCAAGAGAAGGCGATTATGGCGCTGGT GGACAGCCAGATTGGCAACTTTGGCCGCCC GGCGCACGTCTGGTTTGTCTCGCAATTGCC AAAAACGCGATCCGGAAAAATGCTGCGCCG CACGATCCAGGCGATTTGCGAAGGACGCG ATCCTGGGGATCTGACGACCATTGATGATC CGGCGTCGTTGGATCAGATCCGCCAGGCG ATGGAAGAGTAGTACTAGATTCAATATAGAG TAAAAGAGGTAAGAGTATCCATGCGTAAAGT TCTGATCGCTAATCGTGGAGAAATTGCTGT ACGTGTAGCACGTGCATGTCGTGATGCGGG AATCGCATCAGTAGCCGTATACGCGGACCC GGATCGTGACGCGTTGCATGTGCGCGCGG CGGACGAAGCATTTGCACTGGGTGGTGATA CGCCTGCAACATCTTACTTAGACATCGCCA AGGTGTTAAAGGCTGCACGTGAGAGTGGT GCAGACGCCATTCATCCCGGTTACGGCTTT TTAAGTGAAAATGCCGAGTTCGCGCAGGCC GTGTTAGATGCGGGTCTTATCTGGATCGGA CCACCGCCCCATGCAATCCGCGATCGTGGG GAAAAAGTTGCAGCTCGCCATATTGCCCAG CGTGCTGGGGCGCCGCTGGTTGCGGGCAC CCCTGACCCGGTTTCTGGTGCTGACGAAGT CGTCGCCTTCGCGAAAGAGCATGGACTGCC GATCGCGATTAAGGCTGCTTTTGGAGGCGG TGGTCGTGGTTTAAAGGTTGCCCGTACATT GGAAGAAGTGCCCGAGTTATATGACTCCGC CGTGCGTGAAGCTGTGGCGGCATTCGGAC	

	GTGGCGAATGTTTCGTGGAGCGCTATTTAG **ACAAACCGCGTCATGTAGAAACCCAGTGCT** **TGGCAGATACTCACGGTAATGTAGTTGTGG** **TTTCTACTCGCGACTGTTCGTTACAGCGTC** **GTCATCAGAAACTGGTAGAGGAGGCACCC** **GCCCCGTTTTTAAGCGAAGCTCAGACAGAG** **CAACTGTACTCCTCCTCCAAGGCTATTCTT** **AAGGAAGCTGGGTATGGTGGAGCGGGAAC** **CGTTGAGTTTTTAGTAGGTATGGATGGTAC** **TATCTTCTTCTTGGAGGTCAATACCCGCCT** **GCAGGTGGAGCACCCTGTGACCGAAGAAG** **TCGCAGGGATCGACCTGGTCCGTGAAATGT** **TCCGCATTGCAGATGGCGAGGAGCTGGGG** **TACGACGATCCAGCCCTTCGCGGCCACTCG** **TTCGAATTTCGCATCAATGGGGAGGACCCA** **GGTCGTGGTTTTTTGCCCGCACCTGGTACG** **GTTACGCTTTTTGATGCTCCGACCGGACCC** **GGAGTCCGCCTGGATGCCGGGGTTGAGTC** **AGGTTCCGTAATCGGACCGGCATGGGACTC** **ACTGCTGGCTAAACTTATCGTTACCGGGCG** **TACACGTGCCGAGGCGCTTCAGCGCGCAG** **CCCGCGCCTTAGATGAATTTACGGTTGAGG** **GCATGGCAACCGCGATCCCTTTCCATCGCA** **CAGTAGTACGCGATCCAGCATTCGCTCCTG** **AGCTTACCGGGTCAACGGACCCATTCACCG** **TTCATACACGCTGGATTGAAACTGAATTTG** **TCAACGAAATTAAGCCTTTTACCACCCCTG** **CCGACACGGAGACAGATGAAGAGTCTGGG** **CGCGAGACAGTGGTAGTCGAGGTCGGTGG** **GAAACGCTTAGAGGTAAGTCTTCCGTCCAG** **CCTGGGAATGTCGTTGGCCCGTACCGGCCT** **TGCCGCGGGGGCCCGCCCCAAACGCCGCG** **CGGCCAAGAAGTCAGGCCCTGCAGCATCG** **GGTGATACACTGGCATCTCCTATGCAAGGT** **ACGATCGTAAAGATCGCCGTGGA** AGAGGGACAAGAAGTACAGGAGGGAGATCTG ATTGTGGTTCTTGAAGCTATGAAGATGGAACA GCCACTTAATGCCCACCGTTCGGGAACCATTA AGGGGCTTACTGCTGAAGTAGGTGCTTCACTG ACGTCGGGCGCCGCTATCTGTGAAATCAAGG ATTGATAACGCTAACGAAAAAGTTAAATACA GGAACAAGAGAACA**TGTCGGAGCCCGAG** **GAACAGCAGCCAGATATCCACACGACAGCG** **GGCAAGTTAGCTGATCTTCGTCGCCGCATC** **GAAGAGGCAACGCACGCCGGTTCTGCGCG** **CGCGGTGGAGAAACAGCACGCGAAGGGTA** **AACTTACGGCTCGTGAGCGTATCGATTTGT** **TGCTGGACGAAGGGTCTTTTGTAGAGCTTG** **ATGAGTTTGCGCGTCACCGTTCGACGAATT**	

	TCGGACTGGATGCCAACCGTCCATATGGAG ATGGAGTGGTGACTGGCTATGGAACTGTTG ACGGACGTCCGGTTGCCGTCTTTTCGCAAG ACTTTACGGTCTTTGGGGGCGCTCTGGGGG AAGTATACGGGCAAAAAATTGTGAAGGTCA TGGATTTCGCTCTTAAGACCGGGTGTCCCG TCGTGGGTATTAATGACTCAGGTGGGGCAC GCATTCAAGAGGGTGTAGCAAGTCTGGGC GCGTATGGAGAGATTTTCCGTCGCAATACG CACGCGTCGGGCGTGATCCCTCAGATTTCG CTTGTAGTTGGCCCATGCGCAGGGGGAGCT GTGTACTCTCCAGCTATTACTGACTTTACG GTAATGGTCGACCAAACATCGCATATGTTT ATCACCGGACCCGATGTGATTAAGACAGTG ACAGGGGAGGATGTGGGTTTTGAGGAACTT GGTGGTGCGCGTACGCACAACAGTACGTCT GGGGTTGCCCATCATATGGCTGGGGATGA GAAAGACGCTGTGGAGTATGTTAAGCAATT ATTGAGTTATTTGCCGTCGAACAATTTAAG TGAGCCTCCGGCGTTTCCTGAAGAGGCTGA TTTAGCCGTTACGGACGAAGATGCGGAATT AGATACAATTGTGCCGGATTCGGCTAACCA ACCCTATGATATGCATTCTGTAATCGAGCA TGTCCTTGACGATGCGGAATTTTTCGAGAC TCAACCGTTGTTTGCCCCCAACATCCTGAC CGGCTTTGGTCGCGTTGAAGGCCGTCCGGT GGGTATCGTGGCGAATCAGCCGATGCAGTT TGCTGGATGCTTAGATATCACTGCCTCAGA AAAAGCTGCTCGTTTCGTTCGCACTTGCGA CGCTTTCAACGTCCCTGTGCTTACGTTTGT AGACGTCCCCGGGTTTTTACCGGGCGTAGA TCAGGAGCATGACGGGATCATCCGCCGCG GTGCGAAGTTGATTTTTGCCTATGCAGAAG CGACCGTGCCGTTGATCACAGTAATCACGC GCAAAGCCTTCGGAGGTGCGTATGACGTAA TGGGCTCAAAACACCTTGGCGCTGACCTTA ATCTGGCATGGCCCACGGCCCAAATCGCTG TAATGGGCGCTCAAGGTGCTGTAAACATCC TTCATCGTCGTACGATTGCAGATGCGGGGG ACGATGCGGAAGCCACGCGCGCCCGTTTAA TTCAAGAGTACGAGGATGCTTTATTAAATC CCTATACTGCGGCTGAGCGCGGGTATGTAG ACGCGGTCATCATGCCCTCAGATACTCGCC GTCATATCGTACGTGGTTTACGCCAATTAC GCACCAAGCGCGAGTCTTTACCCCCGAAAA AGCACGGGAACATTCCCCTT	
prpE sequence (comprised in the prpE-accA-pccB construct shown in **FIG.**	ATGTCTTTTAGCGAATTTTATCAGCGTTCGATT AACGAACCGGAGAAGTTCTGGGCCGAGCAGG CCCGGCGTATTGACTGGCAGACGCCCTTTACG	**SEQ ID NO: 25**

15B and FIG. 16)	CAAACGCTCGACCACAGCAACCCGCCGTTTGC CCGTTGGTTTTGTGAAGGCCGAACCAACTTGT GTCACAACGCTATCGACCGCTGGCTGGAGAA ACAGCCAGAGGCGCTGGCATTGATTGCCGTCT CTTCGGAAACAGAGGAAGAGCGTACCTTTAC CTTCCGCCAGTTACATGACGAAGTGAATGCGG TGGCGTCAATGCTGCGCTCACTGGGCGTGCAG CGTGGCGATCGGGTGCTGGTGTATATGCCGAT GATTGCCGAAGCGCATATTACCCTGCTGGCCT GCGCGCGCATTGGTGCTATTCACTCGGTGGTG TTTGGGGGATTTGCTTCGCACAGCGTGGCAAC GCGAATTGATGACGCTAAACCGGTGCTGATTG TCTCGGCTGATGCCGGGGCGCGCGGCGGTAA AATCATTCCGTATAAAAAATTGCTCGACGATG CGATAAGTCAGGCACAGCATCAGCCGCGTCA CGTTTTACTGGTGGATCGCGGGCTGGCGAAAA TGGCGCGCGTTAGCGGGCGGGATGTCGATTTC GCGTCGTTGCGCCATCAACACATCGGCGCGCG GGTGCCGGTGGCATGGCTGGAATCCAACGAA ACCTCCTGCATTCTCTACACCTCCGGCACGAC CGGCAAACCTAAAGGTGTGCAGCGTGATGTC GGCGGATATGCGGTGGCGCTGGCGACCTCGA TGGACACCATTTTTGGCGGCAAAGCGGGCGG CGTGTTCTTTTGTGCTTCGGATATCGGCTGGGT GGTAGGGCATTCGTATATCGTTTACGCGCCGC TGCTGGCGGGGATGGCGACTATCGTTTACGAA GGATTGCCGACCTGGCCGGACTGCGGCGTGTG GTGGAAAATTGTCGAGAAATATCAGGTTAGC CGCATGTTCTCAGCGCCGACCGCCATTCGCGT GCTGAAAAAATTCCCTACCGCTGAAATTCGCA AACACGATCTTTCGTCGCTGGAAGTGCTCTAT CTGGCTGGAGAACCGCTGGACGAGCCGACCG CCAGTTGGGTGAGCAATACGCTGGATGTGCCG GTCATCGACAACTACTGGCAGACCGAATCCG GCTGGCCGATTATGGCGATTGCTCGCGGTCTG GATGACAGACCGACGCGTCTGGGAAGCCCCG GCGTGCCGATGTATGGCTATAACGTGCAGTTG CTCAATGAAGTCACCGGCGAACCGTGTGGCGT CAATGAGAAAGGGATGCTGGTAGTGGAGGGG CCATTGCCGCCAGGCTGTATTCAAACCATCTG GGGCGACGACGACCGCTTTGTGAAGACGTAC TGGTCGCTGTTTTCCCGTCCGGTGTACGCCAC TTTTGACTGGGGCATCCGCGATGCTGACGGTT ATCACTTTATTCTCGGGCGCACTGACGATGTG ATTAACGTTGCCGGACATCGGCTGGGTACGCG TGAGATTGAAGAGAGTATCTCCAGTCATCCGG GCGTTGCCGAAGTGGCGGTGGTTGGGGTGAA AGATGCGCTGAAAGGGCAGGTGGCGGTGGCG TTTGTCATTCCGAAAGAGAGCGACAGTCTGGA	

	AGACCGTGAGGTGGCGCACTCGCAAGAGAAG GCGATTATGGCGCTGGTGGACAGCCAGATTG GCAACTTTGGCCGCCCGGCGCACGTCTGGTTT GTCTCGCAATTGCCAAAAACGCGATCCGGAA AAATGCTGCGCCGCACGATCCAGGCGATTTGC GAAGGACGCGATCCTGGGGATCTGACGACCA TTGATGATCCGGCGTCGTTGGATCAGATCCGC CAGGCGATGGAAGAGTAG	
accA sequence (comprised in the prpE-accA-pccB construct shown in **FIG. 15B and FIG. 16**)	ATGCGTAAAGTTCTGATCGCTAATCGTGGAGA AATTGCTGTACGTGTAGCACGTGCATGTCGTG ATGCGGGAATCGCATCAGTAGCCGTATACGC GGACCCGGATCGTGACGCGTTGCATGTGCGCG CGGCGGACGAAGCATTTGCACTGGGTGGTGA TACGCCTGCAACATCTTACTTAGACATCGCCA AGGTGTTAAAGGCTGCACGTGAGAGTGGTGC AGACGCCATTCATCCCGGTTACGGCTTTTTAA GTGAAAATGCCGAGTTCGCGCAGGCCGTGTTA GATGCGGGTCTTATCTGGATCGGACCACCGCC CCATGCAATCCGCGATCGTGGGGAAAAAGTT GCAGCTCGCCATATTGCCCAGCGTGCTGGGGC GCCGCTGGTTGCGGGCACCCCTGACCCGGTTT CTGGTGCTGACGAAGTCGTCGCCTTCGCGAAA GAGCATGGACTGCCGATCGCGATTAAGGCTG CTTTTGGAGGCGGTGGTCGTGGTTTAAAGGTT GCCCGTACATTGGAAGAAGTGCCCGAGTTATA TGACTCCGCCGTGCGTGAAGCTGTGGCGGCAT TCGGACGTGGCGAATGTTTCGTGGAGCGCTAT TTAGACAAACCGCGTCATGTAGAAACCCAGT GCTTGGCAGATACTCACGGTAATGTAGTTGTG GTTTCTACTCGCGACTGTTCGTTACAGCGTCG TCATCAGAAACTGGTAGAGGAGGCACCCGCC CCGTTTTTAAGCGAAGCTCAGACAGAGCAACT GTACTCCTCCTCCAAGGCTATTCTTAAGGAAG CTGGGTATGGTGGAGCGGGAACCGTTGAGTTT TTAGTAGGTATGGATGGTACTATCTTCTTCTTG GAGGTCAATACCCGCCTGCAGGTGGAGCACC CTGTGACCGAAGAAGTCGCAGGGATCGACCT GGTCCGTGAAATGTTCCGCATTGCAGATGGCG AGGAGCTGGGGTACGACGATCCAGCCCTTCG CGGCCACTCGTTCGAATTTCGCATCAATGGGG AGGACCCAGGTCGTGGTTTTTTGCCCGCACCT GGTACGGTTACGCTTTTTGATGCTCCGACCGG ACCCGGAGTCCGCCTGGATGCCGGGGTTGAGT CAGGTTCCGTAATCGGACCGGCATGGGACTCA CTGCTGGCTAAACTTATCGTTACCGGGCGTAC ACGTGCCGAGGCGCTTCAGCGCGCAGCCCGC GCCTTAGATGAATTTACGGTTGAGGGCATGGC AACCGCGATCCCTTTCCATCGCACAGTAGTAC GCGATCCAGCATTCGCTCCTGAGCTTACCGGG	**SEQ ID NO: 38**

	TCAACGGACCCATTCACCGTTCATACACGCTG GATTGAAACTGAATTTGTCAACGAAATTAAGC CTTTTACCACCCCTGCCGACACGGAGACAGAT GAAGAGTCTGGGCGCGAGACAGTGGTAGTCG AGGTCGGTGGGAAACGCTTAGAGGTAAGTCT TCCGTCCAGCCTGGGAATGTCGTTGGCCCGTA CCGGCCTTGCCGCGGGGGCCCGCCCCAAACG CCGCGCGGCCAAGAAGTCAGGCCCTGCAGCA TCGGGTGATACACTGGCATCTCCTATGCAAGG TACGATCGTAAAGATCGCCGTGGAAGAGGGA CAAGAAGTACAGGAGGGAGATCTGATTGTGG TTCTTGAAGCTATGAAGATGGAACAGCCACTT AATGCCCACCGTTCGGGAACCATTAAGGGGCT TACTGCTGAAGTAGGTGCTTCACTGACGTCGG GCGCCGCTATCTGTGAAATCAAGGATTG	
pccB sequence (comprised in the prpE-accA-pccB construct shown in **FIG. 15B and FIG. 16**)	ATGTCGGAGCCCGAGGAACAGCAGCCAGATA TCCACACGACAGCGGGCAAGTTAGCTGATCTT CGTCGCCGCATCGAAGAGGCAACGCACGCCG GTTCTGCGCGCGCGGTGGAGAAACAGCACGC GAAGGGTAAACTTACGGCTCGTGAGCGTATC GATTTGTTGCTGGACGAAGGGTCTTTTGTAGA GCTTGATGAGTTTGCGCGTCACCGTTCGACGA ATTTCGGACTGGATGCCAACCGTCCATATGGA GATGGAGTGGTGACTGGCTATGGAACTGTTGA CGGACGTCCGGTTGCCGTCTTTTCGCAAGACT TTACGGTCTTTGGGGGCGCTCTGGGGGAAGTA TACGGGCAAAAAATTGTGAAGGTCATGGATTT CGCTCTTAAGACCGGGTGTCCCGTCGTGGGTA TTAATGACTCAGGTGGGGCACGCATTCAAGA GGGTGTAGCAAGTCTGGGCGCGTATGGAGAG ATTTTCCGTCGCAATACGCACGCGTCGGGCGT GATCCCTCAGATTTCGCTTGTAGTTGGCCCAT GCGCAGGGGGAGCTGTGTACTCTCCAGCTATT ACTGACTTTACGGTAATGGTCGACCAAACATC GCATATGTTTATCACCGGACCCGATGTGATTA AGACAGTGACAGGGGAGGATGTGGGTTTTGA GGAACTTGGTGGTGCGCGTACGCACAACAGT ACGTCTGGGGTTGCCCATCATATGGCTGGGGA TGAGAAAGACGCTGTGGAGTATGTTAAGCAA TTATTGAGTTATTTGCCGTCGAACAATTTAAG TGAGCCTCCGGCGTTTCCTGAAGAGGCTGATT TAGCCGTTACGGACGAAGATGCGGAATTAGA TACAATTGTGCCGGATTCGGCTAACCAACCCT ATGATATGCATTCTGTAATCGAGCATGTCCTT GACGATGCGGAATTTTTCGAGACTCAACCGTT GTTTGCCCCCAACATCCTGACCGGCTTTGGTC GCGTTGAAGGCCGTCCGGTGGGTATCGTGGCG AATCAGCCGATGCAGTTTGCTGGATGCTTAGA TATCACTGCCTCAGAAAAAGCTGCTCGTTTCG	**SEQ ID NO: 39**

	TTCGCACTTGCGACGCTTTCAACGTCCCTGTG CTTACGTTTGTAGACGTCCCCGGGTTTTTACC GGGCGTAGATCAGGAGCATGACGGGATCATC CGCCGCGGTGCGAAGTTGATTTTTGCCTATGC AGAAGCGACCGTGCCGTTGATCACAGTAATC ACGCGCAAAGCCTTCGGAGGTGCGTATGACG TAATGGGCTCAAAACACCTTGGCGCTGACCTT AATCTGGCATGGCCCACGGCCCAAATCGCTGT AATGGGCGCTCAAGGTGCTGTAAACATCCTTC ATCGTCGTACGATTGCAGATGCGGGGGACGA TGCGGAAGCCACGCGCGCCCGTTTAATTCAAG AGTACGAGGATGCTTTATTAAATCCCTATACT GCGGCTGAGCGCGGGTATGTAGACGCGGTCA TCATGCCCTCAGATACTCGCCGTCATATCGTA CGTGGTTTACGCCAATTACGCACCAAGCGCGA GTCTTTACCCCCGAAAAAGCACGGGAACATTC CCCTTTG	

[0524] In certain constructs, the prpE-pccB-accA1 and mmcE-mutAB cassettes are operably linked to a FNR-responsive promoter, which may be is further fused to a strong ribosome binding site sequence. For efficient translation, a 15-base pair ribosome binding site was designed for each synthetic gene in the operon. Each gene cassette and regulatory region construct is expressed on a high-copy plasmid, a low-copy plasmid, or a chromosome.

[0525] In certain embodiments, the construct is inserted into the bacterial genome at one or more of the following insertion sites in *E. coli* Nissle: *malE/K, araC/BAD, lacZ, thyA, malP/T.* Any suitable insertion site may be used (*see, e.g.,* **FIG. 32**). The insertion site may be anywhere in the genome, *e.g.*, in a gene required for survival and/or growth, such as *thyA* (to create an auxotroph); in an active area of the genome, such as near the site of genome replication; and/or in between divergent promoters in order to reduce the risk of unintended transcription, such as between AraB and AraC of the arabinose operon. At the site of insertion, DNA primers that are homologous to the site of insertion and to the propionate construct are designed. A linear DNA fragment containing the construct with homology to the target site is generated by PCR, and lambda red recombination is performed as described below. The resulting *E. coli* Nissle bacteria are genetically engineered to express a propionate biosynthesis cassette and produce propionate.

Example 4. Generation of Engineered Bacteria Comprising a transporter of Propionate and/or a Propionate Catabolism Enzyme

[0526] The pTet-prpE-PhaBCA plasmids (and other plasmids described herein) are transformed into E. coli Nissle, DH5α, or PIR1. All tubes, solutions, and cuvettes are pre-chilled to 4° C. An overnight culture of E. coli (Nissle, DH5α or PIR1) is diluted 1:100 in 4 mL of LB and grown until it reaches an OD600 of 0.4-0.6. 1mL of the culture is then centrifuged at 13,000 rpm for 1 min in a 1.5mL microcentrifuge tube and the supernatant is removed. The cells are then washed three times in pre-chilled 10% glycerol and resuspended in 40uL pre-chilled 10% glycerol. The electroporator is set to 1.8kV. 1uL of a pTet-prpE-PhaBCA miniprep is added to the cells, mixed by pipetting, and pipetted into a sterile, chilled 1mm cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. 500uL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 37° C for 1 hr. The cells are spread out on an LB plate containing 50ug/mL Kanamycin for pTet-prpBCDE and pTet-mctC.

[0527] In alternate embodiments, the pTet-prpE-PhaBCA cassettes or pFNR-prpE-PhaBCA cassettes may be inserted into the Nissle genome through homologous recombination (Genewiz, Cambridge, MA).

[0528] To create a vector capable of integrating the synthesized the pTet-prpE-PhaBCA or pFNR-prpE-PhaBCA cassettes into the chromosome, Gibson assembly is first used to add 1000bp sequences of DNA homologous to the a Nissle locues, e.g., the lacZ locus into the R6K origin plasmid pKD3. This targets DNA cloned between these homology arms to be integrated into the locus, e.g., the lacZ locus in the Nissle genome. Gibson assembly is 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 prpE-PhaBCA cassettes between them. This PCR fragment is used to transform electrocompetent Nissle-pKD46, a strain that contains a temperature-sensitive plasmid encoding the lambda red recombinase genes. After transformation, cells are 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

Example 5. Lambda Red Recombination

[0529] Lambda red recombination is used to make chromosomal modifications, e.g., to express one or more prpE-PhaBCA cassette(s) (or other cassettes described herein) in E.

coli Nissle. Lambda red is a procedure using recombination enzymes from a bacteriophage lambda to insert a piece of custom DNA into the chromosome of E. coli. A pKD46 plasmid is transformed into the E. coli Nissle host strain. E. coli Nissle cells are grown overnight in LB media. The overnight culture is diluted 1:100 in 5 mL of LB media and grown until it reaches an OD600 of 0.4-0.6. All tubes, solutions, and cuvettes are pre-chilled to 4° C. The E. coli cells are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 1 mL of 4° C water. The E. coli are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.5 mL of 4° C water. The E. coli are centrifuged at 2,000 rpm for 5 min. at 4° C, the supernatant is removed, and the cells are resuspended in 0.1 mL of 4° C water. The electroporator is set to 2.5 kV. 1 ng of pKD46 plasmid DNA is added to the E. coli cells, mixed by pipetting, and pipetted into a sterile, chilled cuvette. The dry cuvette is placed into the sample chamber, and the electric pulse is applied. 1 mL of room-temperature SOC media is immediately added, and the mixture is transferred to a culture tube and incubated at 30° C for 1 hr. The cells are spread out on a selective media plate and incubated overnight at 30° C.

[0530] DNA sequences comprising the desired prpE-PhaBCA cassette(s) shown above are ordered from a gene synthesis company. The lambda enzymes are used to insert this construct into the genome of E. coli Nissle through homologous recombination. The construct is inserted into a specific site in the genome of E. coli Nissle based on its DNA sequence. To insert the construct into a specific site, the homologous DNA sequence flanking the construct is identified, and includes approximately 50 bases on either side of the sequence. The homologous sequences are ordered as part of the synthesized gene. Alternatively, the homologous sequences may be added by PCR. The construct includes an antibiotic resistance marker that may be removed by recombination. The resulting construct comprises approximately 50 bases of homology upstream, a kanamycin resistance marker that can be removed by recombination, the prpE-PhaBCA cassette(s), and approximately 50 bases of homology downstream.

Example 6. Establishment of Propionic Acidemia Biomarkers in the PCCAA138T Hypomorph Mouse Model

[0531] For in vivo studies, PCCAA138T hypomorph mice were obtained for use as a model for propionic acidemia. First, biomarkers for propionic acidemia were established.

[0532] PCCAA138T mice and FVB (parental) controls (10-12 weeks old) were kept on normal chow. Blood and urine were collected and were assayed for known biomarkers of propionic acidemia. In blood, the propionylcarnitine/acetylcarnitine ratio, propionate concentration, and 2-methylcitrate concentration were determined by mass spectrometry as described herein. Results are shown in **FIG. 6A-FIG. 6C**. For urine, propionyl-glycine, Tigylglycine, and 2-methylcitrate were measured by LC-MS/MS as described herein, and results are shown in **FIG. 6D-FIG. 6F**.

Example 7. Enterorecirculation of Propionic Acid in the PCCAA138T Hypomorph Mouse Model

[0533] To determine whether propionate undergoes enterorecirculation, in a similar manner as has been hypothesized and shown for amino acids (see e.g., Chang et al., A new theory of enterorecirculation of amino acids and its use for depleting unwanted amino acids using oral enzyme-artificial cells, as in removing phenylalanine in phenylketonuria; Artif Cells Blood Substit Immobil Biotechnol. 1995;23(1):1-21), levels of enteroconversion of labeled propionate from the bloodstream were measured in various compartments of the gut using the PCCAA138T mouse model.

[0534] All PCCAA138T mice (10-12 weeks old) were kept on normal chow until 0.1mg/g isotopic propionic acid was administered at T0 by subcutaneous injection.

[0535] At each timepoint (0, 30 min, 1h and 2h post-SC injection), animals [[(n=X)]] were euthanized, and blood, small intestine, large intestine and cecum, were removed and collected. Each intestinal section was flushed with 0.5 ml cold PBS and collected in separate 1.5 ml tubes. The cecum was harvested, contents were squeezed out, and flushed with 0.5 ml cold PBS and collected in a 1.5 ml tube. Blood was collected by mandibular bleeding. Concentrations of endogenous and radiolabeled propionate in the blood, intestinal compartments, and cecum were measured by LC-MS/MS as described herein. As shown in **FIG. 7A-FIG. 7D**, isotopic propionic acid injected SC is seen at very low levels in the blood, small intestine, and cecum within 30 min, indicating that propionate has circulated from blood into the intestinal compartments in the PA/MMA animal model.

Example 8. Bacterial Contribution to PA Biomarkers

[0536] Experiments with antibiotic-treated PA patients suggest that bacterial metabolism in the gut contributes ~30% of the propionate. The bacterial contribution to levels

of PA biomarkers are evaluated by measuring the effects of an antibiotic treatment which significantly reduces the microbiota population (>99.9%) in the $PCCA^{A138T}$ model.

[0537] PCCAA138T mice are kept on normal chow until Day 1 of the study. On day 1, plasma, urine, fecal samples are taken and, antibiotics supplemented in water of half of the mice (Ampicilln (1g/L), Vancomycin (0.5 g/L), Neomycin (1 g/L), Metronidazole (1 g/L)) On D8, plasma, urine, fecal samples (n=4) are taken and metabolite levels quantified by LC-MS/MS as described herein. Bacterial levels are quantified by qPCR using primers which amplify DNA from Nissle and total bacteria. Metabolites (propionate, propionlylcarnitine/acetylcarnitine ratio; propionylcarnitine, 2-methylcitrate, acetylcarnitine, are quantified by LC-MS/MS as described herein.

Example 9. Polyhydroxyalkanoate (PHA) Pathway Propionate Consumption Assay

[0538] PHA pathway is a heterologous bacterial pathway used for carbon storage as polymers, and was assessed for its ability to consume propionate.

[0539] As described herein, the E. coli Nissle prpE gene and phaBCA genes from Acinetobacter sp RA3849 (codon optimized for expression in E. coli Nissle) were placed under the control of an aTc-inducible promoter in a single operon in a high copy plasmid, as shown in **FIG. 10C** and **FIG. 11.** Corresponding construct sequences are listed in **Table 12** in **Example 2.** Next, the rate of propionate consumption of genetically engineered bacteria comprising the prpE-phaBCA circuit was assessed in vitro.

[0540] Cultures of *E. coli* Nissle transformed with the plasmid comprising the prpE-phaBCA circuit driven by the tet promoter and cultures of wild type control Nissle were grown overnight and then diluted 1:200 in LB. ATC was added to the cultures of the strain containing the prpE-phaBCA construct plasmid at a concentration of 100 ng/mL to induce expression of the prpE and phaBCA genes. Then, the cells were grown with shaking at 250 rpm. After 2 hrs of incubation, cells were pelleted down, washed, and resuspended in 1mL M9 medium supplemented with glucose (0.2%) and propionate (2-8 mM) at a concentration of ~10^9 cfu/ml bacteria. Aliquots were collected at 0 hrs, 1.5 hr, 3 hrs, and 4.5 hrs for propionate quantification as described herein. As shown in **FIG. 12**, the genetically engineered bacteria expressing prpE and phaBCA genes driven by the tet promoter are more efficient at removing propionate than wild type Nissle or the uninduced engineered strain. The catabolic rate was calculated to be 0.396-1.4 umol hr^{-1} per 10^9 cells.

Example 10. PHA Pathway Performance with Mixed Organic Acids

[0541] To determine whether acetate or butyrate (which are abundant in the colon) may have an effect on propionate consumption through the PHA pathway, the PHA assay was performed in a mixture of short chain fatty acids to mimic the colon ratios (propionate: acetate: butyrate, approximately 6:10:4).

[0542] Cultures of *E. coli* Nissle transformed with the plasmid comprising the prpE-phaBCA circuit driven by the tet promoter (as described in Example 9) and wild type control Nissle were grown overnight and then diluted 1:200 in LB. ATC was added to the cultures of the strain containing the prpE-phaBCA construct plasmid and the wild type Nissle cultures and cells were incubated for two hours. Cells were spun down and resuspended in as described in Example 9 in 1mL M9 medium supplemented with glucose (0.2%) and propionate (6 mM), butyrate (4 MM), and acetate (10 mM) at a concentration of ~10^9 cfu/ml bacteria. Aliquots were collected at 0 hrs, 1.5 hrs, 3 hrs, and 4.5 hrs for propionate quantification via LC-MS/MS as described herein. As shown in **FIG. 13A**, the genetically engineered bacteria expressing the tet-prpE and phaBCA gene cassette reduced the concentration of propionate compared to the wild type Nissle at a rate similar to the rate observed in the absence of acetate and butyrate in Example 9. The catabolic rate was calculated to be 0.396 -1.4 umol hr-1 per 109 cells.

[0543] Also, the genetically engineered bacteria did not affect acetate or butyrate levels as compared to wild type Nissle (**FIG. 13B** and **FIG. 13C**), indicating that the PHA pathway does not significantly affect acetate and butyrate concentrations.

Example 11. Optimization of the PHA Pathway

[0544] To optimize the PHA pathway and to determine the rate-limiting step in the pathway, the base strain expressing the aTc-inducible prpE-phaBCA operon was supplemented with a second plasmid expressing a construct containing one of the operon genes under the control of an arabinose inducible promoter, as shown in **FIG. 14A-FIG. 14D**. **Table 14** lists the construct sequences from the additional plasmids.

[0545] In this assay, either the prpE-phaBCA operon alone, or both the prpE-phaBCA plasmid and the arabinose inducible plasmid carrying the additional copy of one of the genes in the pathway were induced to assess whether additional expression of any of the genes could increase propionate consumption. Wild type Nissle was included for reference.

Table 14. PHA Pathway Sequences – Additional Plasmid Constructs

Description	Sequence	SEQ ID NO
araC-Para-phaA (araC: lower case; RBS underlined; phaA: italics; L3S2P11 terminator: underlined bold; his terminator: bold)	ttattcacaacctgccctaaactcgctcggactcgccccggtgcatttttaaatactc gcgagaaatagagttgatcgtcaaaaccgacattgcgaccgacggtggcgatag gcatccgggtggtgctcaaaagcagcttcgcctgactgatgcgctggtcctcgcg ccagcttaatacgctaatccctaactgctggcggaacaaatgcgacagacgcgac ggcgacaggcagacatgctgtgcgacgctggcgatatcaaaattactgtctgcca ggtgatcgctgatgtactgacaagcctcgcgtacccgattatccatcggtggatgg agcgactcgttaatcgcttccatgcgccgcagtaacaattgctcaagcagatttatc gccagcaattccgaatagcgcccttcccccttgtccggcattaatgatttgcccaaac aggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaaccggtattgg caaatatcgacggccagttaagccattcatgccagtaggcgcgcggacgaaagta aacccactggtgataccattcgtgagcctccggatgacgaccgtagtgatgaatct ctccaggcgggaacagcaaaatatcacccggtcggcagacaaattctcgtccctg atttttcaccaccccctgaccgcgaatggtgagattgagaatataacctttcattccc agcggtcggtcgataaaaaaatcgagataaccgttggcctcaatcggcgttaaacc cgccaccagatgggcgttaaacgagtatcccggcagcaggggatcattttgcgctt cagccatacttttcatactcccgccattcagagaagaaaccaattgtccatattgcat CAGACATTGCCGTCACTGCGTCTTTTACTGGCTCT TCTCGCTAACCCAACCGGTAACCCCGCTTATTAAA AGCATTCTGTAACAAAGCGGGACCAAAGCCATGA CAAAAACGCGTAACAAAAGTGTCTATAATCACGG CAGAAAAGTCCACATTGATTATTTGCACGGCGTCA CACTTTGCTATGCCATAGCATTTTTATCCATAAGA TTAGCGGATCCAGCCTGACGCTTTTTTTCGCAACT CTCTACTGTTTCTCCATACCATATTCATAGAAAGA ATACTAAGAGAGGTCAGA*ATGAAAGATGTTGTTATC GTAGCCGCTAAACGCACTGCGATCGGTTCCTTTCTGG GGAGTCTGGCTTCCCTGAGCGCCCCTCAGTTGGGTC AGACGGCTATCCGCGCAGTTTTGGATTCTGCAAATGT GAAACCAGAACAAGTGGACCAAGTAATTATGGGGAAT GTGCTGACCACCGGCGTTGGGCAAAATCCTGCTCGT CAGGCAGCAATCGCCGCTGGGATTCCTGTACAAGTT CCCGCCAGCACGCTTAATGTAGTGTGTGGGTCCGGA TTACGTGCCGTTCACCTGGCAGCTCAAGCCATCCAAT GCGATGAAGCCGATATCGTCGTTGCCGGAGGTCAAG AATCAATGTCCCAGTCTGCTCATTACATGCAGCTTCG CAATGGCCAGAAAATGGGTAACGCACAGTTAGTCGAT TCAATGGTGGCCGACGGCTTGACCGACGCGTATAAT CAATACCAGATGGGTATCACCGCGGAGAATATCGTCG AAAAACTTGGTCTTAATCGTGAAGAACAAGACCAGCT TGCTCTGACAAGTCAACAACGTGCTGCAGCAGCGCA GGCTGCCGGAAAATTCAAGGATGAAATTGCGGTCGTT TCGATTCCCCAGCGCAAAGGAGAGCCGGTCGTCTTC GCGGAAGACGAATATATCAAGGCCAATACCTCGTTGG AATCCTTGACGAAACTGCGTCCAGCATTCAAAAAAGA CGGTTCTGTTACAGCCGGCAACGCATCTGGCATTAAT	SEQ ID NO: 40

	GATGGGGCAGCCGCGGTCCTGATGATGTCCGCCGAC *AAAGCGGCTGAACTGGGCTTAAAGCCTTTAGCACGCA* *TTAAAGGTTACGCGATGTCAGGAATTGAGCCGGAAAT* *CATGGGACTGGGTCCTGTAGACGCCGTTAAGAAAAC* *CCTTAATAAGGCTGGTTGGTCCTTAGACCAGGTCGAT* *CTGATCGAGGCCAATGAGGCTTTGCTGCCCAAGCA* *CTGGGAGTAGCCAAGGAGCTTGGGCTGGACCTGGAC* *AAGGTAAATGTTAACGGAGGTGCGATCGCGCTGGGA* *CACCCGATCGGGGCTTCGGGTTGTCGTATCTTGGTC* *ACGTTATTACACGAAATGCAGCGTCGTGATGCAAAGA* *AGGGTATCGCCACATTGTGTGTGGGAGGTGGAATGG* *GGGTGGCGCTTGCCGTTGAGCGCGATTAA*GGAG**CT** **CGGTACCAAATTCCAGAAAAGAGACGCTTTCG** **AGCGTCTTTTTTCGTTTTGGTCC**GCGCAATAAA **AAAGCCCCCGGAAGGTGATCTTCCGGGGGCTT** **TCTCATGCGTT**	
araC-Para-phaB (araC: lower case; RBS underlined; phaB: italics; L3S2P11 terminator: underlined bold; his terminator: bold)	Ttattcacaacctgccctaaactcgctcggactcgccccggtgcatttttaaatact cgcgagaaatagagttgatcgtcaaaaccgacattgcgaccgacggtggcgata ggcatccgggtggtgctcaaaagcagcttcgcctgactgatgcgctggtcctcgc gccagcttaatacgctaatccctaactgctggcggaacaaatgcgacagacgcga cggcgacaggcagacatgctgtgcgacgctggcgatatcaaaattactgtctgcc aggtgatcgctgatgtactgacaagcctcgcgtacccgattatccatcggtggatg gagcgactcgttaatcgcttccatgcgccgcagtaacaattgctcaagcagatttat cgccagcaattccgaatagcgcccttcccccttgtccggcattaatgatttgcccaaa caggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaaccggtattg gcaaatatcgacggccagttaagccattcatgccagtaggcgcgcggacgaaagt aaacccactggtgataccattcgtgagcctccggatgacgaccgtagtgatgaatc tctccaggcgggaacagcaaaatatcacccggtcggcagacaaattctcgtccct gatttttcaccacccccctgaccgcgaatggtgagattgagaatataacctttcattcc cagcggtcggtcgataaaaaaatcgagataaccgttggcctcaatcggcgttaaac ccgccaccagatgggcgttaaacgagtatcccggcagcaggggatcattttgcgc ttcagccatacttttcatactcccgccattcagagaagaaaccaattgtccatattgca tCAGACATTGCCGTCACTGCGTCTTTTACTGGCTCT TCTCGCTAACCCAACCGGTAACCCCGCTTATTAAA AGCATTCTGTAACAAAGCGGGACCAAAGCCATGA CAAAAACGCGTAACAAAAGTGTCTATAATCACGG CAGAAAAGTCCACATTGATTATTTGCACGGCGTCA CACTTTGCTATGCCATAGCATTTTTATCCATAAGA TTAGCGGATCCAGCCTGACGCTTTTTTTCGCAACT CTCTACTGTTTCTCCATAcc<u>GCTAGAACTAGATCTA</u> <u>GAGTAATAAGGAGGAAGGA</u>*ATGTCAGAGCAGAAAG* *TAGCTCTGGTTACCGGTGCGTTAGGTGGTATCGGAA* *GTGAGATCTGCCGCCAGCTTGTGACCGCCGGGTACA* *AGATTATCGCCACCGTTGTTCCACGCGAAGAAGACCG* *CGAAAAACAATGGTTGCAAAGTGAGGGGTTTCAAGAC* *TCTGATGTGCGTTTCGTATTAACAGATTTAAACAATCA* *CGAAGCTGCGACAGCGGCAATTCAAGAAGCGATTGC* *CGCCGAAGGACGCGTTGATGTATTGGTCAACAACGC* *GGGGATCACGCGCGATGCTACATTTAAGAAAATGTCC*	SEQ ID NO: 41

	TATGAGCAATGGTCCCAAGTCATCGACACGAATTTAA *AGACTCTTTTTACCGTGACCCAGCCAGTATTTAATAAA* *ATGCTTGAACAGAAGTCTGGCCGCATCGTAAACATTA* *GCTCTGTCAATGGTTTAAAAGGGCAATTTGGTCAAGC* *CAACTACTCGGCCTCGAAAGCAGGGATTATCGGGTTT* *ACTAAAGCATTGGCGCAGGAGGGTGCTCGCTCGAAC* *ATTTGCGTCAATGTCGTTGCTCCTGGTTACACAGCGA* *CACCCATGGTCACAGCAATGCGCGAGGATGTAATTAA* *GTCAATCGAAGCTCAAATTCCCCTGCAACGTCTGGCA* *GCACCGGCGGAGATTGCGGCAGCGGTTATGTATTTG* *GTGAGTGAACACGGTGCATACGTGACGGGCGAAACT* *TTGAGTATCAACGGCGGGCTGTACATGCACTAA*GGA G**CTCGGTACCAAATTCCAGAAAAGAGACGCTTT** **CGAGCGTCTTTTTTCGTTTTGGTCC**GCGCAATA **AAAAAGCCCCCGGAAGGTGATCTTCCGGGGGC** **TTTCTCATGCGTT**	
acaC-Para-phaC (araC: lower case; RBS underlined; phaC: italics; L3S2P11 terminator: underlined bold; his terminator: bold)	Ttattcacaacctgccctaaactcgctcggactcgccccggtgcatttttaaatact cgcgagaaatagagttgatcgtcaaaaccgacattgcgaccgacggtggcgata ggcatccgggtggtgctcaaaagcagcttcgcctgactgatgcgctggtcctcgc gccagcttaatacgctaatccctaactgctggcggaacaaatgcgacagacgcga cggcgacaggcagacatgctgtgcgacgctggcgatatcaaaattactgtctgcc aggtgatcgctgatgtactgacaagcctcgcgtacccgattatccatcggtggatg gagcgactcgttaatcgcttccatgcgccgcagtaacaattgctcaagcagatttat cgccagcaattccgaatagcgcccttcccccttgtccggcattaatgatttgcccaaa caggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaaccggtattg gcaaatatcgacggccagttaagccattcatgccagtaggcgcgcggacgaaagt aaacccactggtgataccattcgtgagcctccggatgacgaccgtagtgatgaatc tctccaggcgggaacagcaaaatatcacccggtcggcagacaaattctcgtccct gatttttcaccacccccctgaccgcgaatggtgagattgagaatataacctttcattcc cagcggtcggtcgataaaaaaatcgagataaccgttggcctcaatcggcgttaaac ccgccaccagatgggcgttaaacgagtatcccggcagcaggggatcattttgcgc ttcagccatacttttcatactcccgccattcagagaagaaaccaattgtccatattgca tCAGACATTGCCGTCACTGCGTCTTTTACTGGCTCT TCTCGCTAACCCAACCGGTAACCCCGCTTATTAAA AGCATTCTGTAACAAAGCGGGACCAAAGCCATGA CAAAAACGCGTAACAAAAGTGTCTATAATCACGG CAGAAAAGTCCACATTGATTATTTGCACGGCGTCA CACTTTGCTATGCCATAGCATTTTTATCCATAAGA TTAGCGGATCCAGCCTGACGCTTTTTTTCGCAACT CTCTACTGTTTCTCCATACC<u>ACTATTATTTAATATA</u> <u>CGACATCAGGAGGTTCCA</u>*ATGAATCCAAATTCCTTT* *CAGTTTAAAGAGAATATCTTACAGTTTTTCAGCGTGCA* *CGACGATATTTGGAAAAAACTGCAGGAATTTTACTATG* *GACAATCGCCCATCAATGAAGCGTTGGCGCAGTTAAA* *TAAGGAAGACATGAGTTTATTCTTCGAGGCGTTATCAA* *AAAACCCTGCTCGTATGATGGAGATGCAGTGGTCCTG* *GTGGCAAGGGCAGATTCAAATTTACCAGAACGTGTTA* *ATGCGTAGTGTAGCCAAGGACGTAGCCCCCTTTATCC* *AGCCAGAGTCCGGAGATCGTCGCTTCAACTCGCCAC*	SEQ ID NO: 42

	*TTTGGCAAGAACATCCAAATTTTGATTTACTGAGTCAA TCCTACTTGTTGTTTTCTCAGTTGGTTCAAAATATGGT GGATGTCGTTGAAGGAGTACCTGATAAGGTCCGCTAT CGCATCCATTTCTTTACACGTCAGATGATCAATGCGTT GTCTCCTTCTAATTTCCTGTGGACGAACCCTGAAGTA ATTCAACAGACGGTCGCTGAACAGGGTGAGAATTTAG TACGCGGGATGCAAGTATTTCACGATGATGTAATGAA TTCGGGTAAATATTTGAGCATCCGTATGGTAAATAGC GACAGTTTCTCTCTTGGCAAGGACTTGGCGTATACGC CAGGAGCCGTAGTTTTCGAGAACGACATCTTTCAGCT TCTTCAATACGAAGCCACAACCGAGAACGTATATCAA ACCCCTATTCTTGTCGTACCTCCCTTCATCAACAAGTA CTACGTGCTGGACCTGCGCGAACAGAATAGCTTGGTT AATTGGCTGCGCCAACAAGGACATACGGTGTTTTTGA TGTCGTGGCGTAACCCCAACGCAGAGCAGAAGGAGC TTACCTTCGCTGACTTAATTACCCAAGGATCGGTAGA AGCATTACGTGTTATCGAAGAAATCACGGGAGAGAAA GAAGCTAACTGTATTGGATATTGCATCGGTGGTACAC TTCTGGCTGCTACCCAGGCATATTATGTAGCTAAACG CCTGAAAAATCACGTAAAGTCAGCGACTTATATGGCG ACGATTATTGATTTTGAGAACCCCGGCTCATTGGGTG TTTTCATTAATGAGCCGGTCGTAAGTGGACTTGAAAA CCTTAATAATCAACTTGGTTACTTCGACGGGCGTCAA CTTGCAGTGACATTTTCGTTGTTGCGCGAAAACACCT TGTATTGGAATTATTACATCGATAATTACTTGAAGGGT AAGGAACCGTCCGACTTTGACATCTTATACTGGAACT CGGATGGTACGAATATCCCAGCAAAGATTCACAATTT CCTGTTACGTAACCTTTATCTTAACAACGAACTTATTT CTCCAAATGCCGTCAAAGTTAATGGTGTGGGTTTAAA CCTTTCGCGCGTGAAGACTCCATCATTCTTCATTGCTA CGCAGGAGGACCATATCGCATTGTGGGATACCTGTTT TCGCGGCGCGGATTACCTGGGGGGTGAGAGCACACT TGTGCTTGGGGAAAGCGGACACGTCGCCGGCATTGT CAACCCGCCTTCTCGTAACAAGTATGGTTGTTACACG AACGCCGCCAAGTTTGAAAATACCAAGCAATGGCTTG ACGGTGCAGAATATCATCCCGAAAGCTGGTGGTTACG TTGGCAGGCATGGGTCACGCCTTATACTGGAGAGCA GGTTCCTGCGCGTAATTTGGGAAACGCACAGTACCC CAGTATTGAAGCGGCCCCTGGGCGTTATGTGCTGGT AAACCTGTTTTAA*GGAG**CTCGGTACCAAATTCCAG AAAAGAGACGCTTTCGAGCGTCTTTTTTCGTTT TGGTCC**GCGCAATAAAAAAGCCCCCGGAAGGT GATCTTCCGGGGGCTTTCTCATGCGTT	
AraC-pAra-PrpE (AraC: Lower Case; RBS Underlined; PrpE: Italics; L3s2p11 Terminator:	ttattcacaacctgccctaaactcgctcggactcgccccggtgcatttttaaatactc gcgagaaatagagttgatcgtcaaaaccgacattgcgaccgacggtggcgatag gcatccgggtggtgctcaaaagcagcttcgcctgactgatgcgctggtcctcgcg ccagcttaatacgctaatccctaactgctggcggaacaaatgcgacagacgcgac ggcgacaggcagacatgctgtgcgacgctggcgatatcaaaattactgtctgcca ggtgatcgctgatgtactgacaagcctcgcgtacccgattatccatcggtggatgg	SEQ ID NO: 43

Underlined; His Terminator: Bold)	agcgactcgttaatcgcttccatgcgccgcagtaacaattgctcaagcagatttatc gccagcaattccgaatagcgcccttcccccttgtccggcattaatgatttgcccaaac aggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaaccggtattgg caaatatcgacggccagttaagccattcatgccagtaggcgcgcggacgaaagta aacccactggtgataccattcgtgagcctccggatgacgaccgtagtgatgaatct ctccaggcgggaacagcaaaatatcacccggtcggcagacaaattctcgtccctg atttttcaccaccccctgaccgcgaatggtgagattgagaatataacctttcattccc agcggtcggtcgataaaaaaatcgagataaccgttggcctcaatcggcgttaaacc cgccaccagatgggcgttaaacgagtatcccggcagcaggggatcattttgcgctt cagccatacttttcatactcccgccattcagagaagaaaccaattgtccatattgcat CAGACATTGCCGTCACTGCGTCTTTTACTGGCTCT TCTCGCTAACCCAACCGGTAACCCCGCTTATTAAA AGCATTCTGTAACAAAGCGGGACCAAAGCCATGA CAAAAACGCGTAACAAAAGTGTCTATAATCACGG CAGAAAAGTCCACATTGATTATTTGCACGGCGTCA CACTTTGCTATGCCATAGCATTTTTATCCATAAGA TTAGCGGATCCAGCCTGACGCTTTTTTTCGCAACT CTCTACTGTTTCTCCATACCAGATTTAAAGTAAGG CCAGGGAATAA*ATGTCTTTTAGCGAATTTTATCAGCG* *TTCGATTAACGAACCGGAGAAGTTCTGGGCCGAGCA* *GGCCCGGCGTATTGACTGGCAGACGCCCTTTACGCA* *AACGCTCGACCACAGCAACCCGCCGTTTGCCCGTTG* *GTTTTGTGAAGGCCGAACCAACTTGTGTCACAACGCT* *ATCGACCGCTGGCTGGAGAAACAGCCAGAGGCGCTG* *GCATTGATTGCCGTCTCTTCGGAAACAGAGGAAGAGC* *GTACCTTTACCTTCCGCCAGTTACATGACGAAGTGAA* *TGCGGTGGCGTCAATGCTGCGCTCACTGGGCGTGCA* *GCGTGGCGATCGGGTGCTGGTGTATATGCCGATGAT* *TGCCGAAGCGCATATTACCCTGCTGGCCTGCGCGCG* *CATTGGTGCTATTCACTCGGTGGTGTTTGGGGGATTT* *GCTTCGCACAGCGTGGCAACGCGAATTGATGACGCT* *AAACCGGTGCTGATTGTCTCGGCTGATGCCGGGGCG* *CGCGGCGGTAAAATCATTCCGTATAAAAAATTGCTCG* *ACGATGCGATAAGTCAGGCACAGCATCAGCCGCGTC* *ACGTTTTACTGGTGGATCGCGGGCTGGCGAAAATGG* *CGCGCGTTAGCGGGCGGGATGTCGATTTCGCGTCGT* *TGCGCCATCAACACATCGGCGCGCGGGTGCCGGTG* *GCATGGCTGGAATCCAACGAAACCTCCTGCATTCTCT* *ACACCTCCGGCACGACCGGCAAACCTAAAGGTGTGC* *AGCGTGATGTCGGCGGATATGCGGTGGCGCTGGCG* *ACCTCGATGGACACCATTTTTGGCGGCAAAGCGGGC* *GGCGTGTTCTTTTGTGCTTCGGATATCGGCTGGGTGG* *TAGGGCATTCGTATATCGTTTACGCGCCGCTGCTGGC* *GGGGATGGCGACTATCGTTTACGAAGGATTGCCGAC* *CTGGCCGGACTGCGGCGTGTGGTGGAAAATTGTCGA* *GAAATATCAGGTTAGCCGCATGTTCTCAGCGCCGACC* *GCCATTCGCGTGCTGAAAAAATTCCCTACCGCTGAAA* *TTCGCAAACACGATCTTTCGTCGCTGGAAGTGCTCTA* *TCTGGCTGGAGAACCGCTGGACGAGCCGACCGCCA*	

	GTTGGGTGAGCAATACGCTGGATGTGCCGGTCATCG *ACAACTACTGGCAGACCGAATCCGGCTGGCCGATTAT* *GGCGATTGCTCGCGGTCTGGATGACAGACCGACGCG* *TCTGGGAAGCCCCGGCGTGCCGATGTATGGCTATAA* *CGTGCAGTTGCTCAATGAAGTCACCGGCGAACCGTG* *TGGCGTCAATGAGAAAGGGATGCTGGTAGTGGAGGG* *GCCATTGCCGCCAGGCTGTATTCAAACCATCTGGGG* *CGACGACGACCGCTTTGTGAAGACGTACTGGTCGCT* *GTTTTCCCGTCCGGTGTACGCCACTTTTGACTGGGGC* *ATCCGCGATGCTGACGGTTATCACTTTATTCTCGGGC* *GCACTGACGATGTGATTAACGTTGCCGGACATCGGCT* *GGGTACGCGTGAGATTGAAGAGAGTATCTCCAGTCAT* *CCGGGCGTTGCCGAAGTGGCGGTGGTTGGGGTGAA* *AGATGCGCTGAAAGGGCAGGTGGCGGTGGCGTTTGT* *CATTCCGAAAGAGAGCGACAGTCTGGAAGACCGTGA* *GGTGGCGCACTCGCAAGAGAAGGCGATTATGGCGCT* *GGTGGACAGCCAGATTGGCAACTTTGGCCGCCCGGC* *GCACGTCTGGTTTGTCTCGCAATTGCCAAAAACGCGA* *TCCGGAAAAATGCTGCGCCGCACGATCCAGGCGATT* *TGCGAAGGACGCGATCCTGGGGATCTGACGACCATT* *GATGATCCGGCGTCGTTGGATCAGATCCGCCAGGCG* *ATGGAAGAGTAG*GGAG**CTCGGTACCAAATTCCAG** **AAAAGAGACGCTTTCGAGCGTCTTTTTTCGTTT** **TGGTCCGCGCAATAAAAAAGCCCCCGGAAGGT** **GATCTTCCGGGGGCTTTCTCATGCGTT**	

[0546] Cultures of *E. coli* Nissle transformed with the plasmid comprising the tet-prpE-phaBCA circuit and the second plasmid (containing one of pAra-prpE or pAra-phaB or pAra-phaC or pAra-phaA) were grown overnight and then diluted 1:200 in LB. Wild type control Nissle cultures were also grown as a reference. ATC (100 ng/mL) was added to induce the tet- prpE-phaBCA construct gene cassette. In half of the cultures of the four strains containing the tet-prpE-phaBCA circuit, arabinose was added at a concentration of 10 mM to induce the second plasmid. Cells were grown with shaking at 250 rpm. After 2 hrs of incubation, cells were pelleted down, washed, and resuspended in 1mL M9 medium 0.5% glucose 8 mM propionate added at a concentration of ~10^9 cfu/ml bacteria. Aliquots were collected at 0 hrs, 1hrs, 2 hrs, 3 hrs, 4 hrs, and 5 hrs for propionate quantification by LC-MS/MS. As shown in **FIG. 14A-FIG. 14D**, the rate of propionate consumption is increased most significantly when more *phaC* is expressed, suggesting that the pathway is improved by increasing the PhaC levels from the original prpE-phaBCA plasmid.

[0547] In certain embodiments, the prpE-phaBCA circuit is further modified by adding a strong RBS upstream of the phaC translation start site. In certain embodiments, the

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 236

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 236

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

Claims

1. A bacterium comprising gene sequence(s) encoding one or more propionate catabolism enzyme(s) operably linked to a directly or indirectly inducible promoter that is not associated with the propionate catabolism enzyme gene in nature.

2. The bacterium of claim 1, wherein the bacterium further comprises gene sequence(s) encoding one or more transporter(s) of propionate operably linked to a promoter that is not associated with the transporter gene in nature.

3. The bacterium of claim 1 or claim 2, wherein the bacterium further comprises gene sequence(s) encoding one or more exporter(s) of succinate operably linked to a promoter that is not associated with the transporter gene in nature.

4. The bacterium of any one of claims 1-3, wherein the bacterium further comprises a genetic modification that reduces the import of succinate into the bacterium.

5. The bacterium any one of claims 2-4, wherein the promoter is a directly or indirectly inducible promoter.

6. The bacterium of any one of claims 1-5, wherein the bacterium further comprises a genetic modification that reduces endogenous biosynthesis of propionate in the bacterium.

7. The bacterium of any of claims 2-6, wherein the promoter operably linked to the gene sequence(s) encoding a propionate catabolism enzyme and the promoter operably linked to the gene sequence(s) encoding a transporter of propionate are separate copies of the same promoter.

8. The bacterium of any of claims 2-6, wherein the promoter operably linked to the gene sequence(s) encoding a propionate catabolism enzyme and the promoter operably

linked to the gene sequence(s) encoding a transporter of propionate are the same copy of the same promoter.

9. The bacterium of any of claims 2-6, wherein the promoter operably linked to the gene sequence(s) encoding a propionate catabolism enzyme and the promoter operably linked to the gene sequence(s) encoding a transporter of propionate are different promoters.

10. The bacterium of any one of claims 1-9, wherein the promoter operably linked to the gene sequence(s) encoding a propionate catabolism enzyme is directly or indirectly induced by exogenous environmental conditions found in the mammalian gut.

11. The bacterium of claim any one of claims 1-10, wherein the promoter operably linked to the gene sequence(s) encoding a propionate catabolism enzyme is directly or indirectly induced under low-oxygen or anaerobic conditions.

12. The bacterium of claim any one of claims 1-11, wherein the promoter operably linked to the gene sequence(s) encoding a propionate catabolism enzyme is selected from the group consisting of an FNR-responsive promoter, an ANR-responsive promoter, and a DNR-responsive promoter.

13. The bacterium of claim any one of claims 1-12, wherein the promoter operably linked to the gene sequence(s) encoding a propionate catabolism enzyme is an FNRS promoter.

14. The bacterium of any one of claims 2-13, wherein the promoter operably linked to the gene sequence(s) encoding a transporter of propionate is directly or indirectly induced by exogenous environmental conditions found in the mammalian gut.

15. The bacterium of claim any one of claims 2-14, wherein the promoter operably linked to the gene sequence(s) encoding a transporter of propionate is directly or indirectly induced under low-oxygen or anaerobic conditions.

16. The bacterium of claim any one of claims 2-15, wherein the promoter operably linked to the gene sequence(s) encoding a transporter of propionate is selected from the group consisting of an FNR-responsive promoter, an ANR-responsive promoter, and a DNR-responsive promoter.

17. The bacterium of any one of claims 1-16, wherein the gene sequence(s) encoding a propionate catabolism enzyme is located on a chromosome in the bacterium.

18. The bacterium of any one of claims 1-17, wherein the gene sequence(s) encoding a propionate catabolism enzyme is located on a plasmid in the bacterium.

19. The bacterium of any one of claims 1-18, wherein the bacterium comprises gene sequence(s) encoding one or more propionate catabolism enzyme(s) that convert propionate to succinate.

20. The bacterium of any one of claims 1-19, wherein the bacterium comprises gene sequence(s) encoding one or more propionate catabolism enzyme(s) selected from prpE, pccB, accA1, mmcE, mutA, and mutB.

21. The bacterium of any one of claims 1-20, wherein the gene sequence(s) encoding one or more propionate catabolism enzyme(s) are present in a single gene cassette.

22. The bacterium of any one of claims 1-21, wherein the bacterium comprises at least two gene sequence(s) encoding one or more propionate catabolism enzyme(s) and wherein the gene sequences are present in two or more separate gene cassettes.

23. The bacterium of claim 22, wherein the gene sequence(s) encoding one or more propionate catabolism enzyme(s) are present in a first gene cassette, operably linked to a first promoter and present in a second gene cassette, operably linked to a second promoter.

24. The bacterium of claim 23, wherein the first promoter and the second promoter are inducible promoters.

25. The bacterium of claim 23 or claim 24, wherein the first promoter and the second promoter are different promoters.

26. The bacterium of claim 23 or claim 24, wherein the first promoter and the second promoter are separate copies of the same promoter.

27. The bacterium of any of claims 23-26, wherein the first gene cassette comprises prpE, pccB, and accA1 and the second gene cassette comprises mmcE, mutA, and mutB.

28. The bacterium of claim 27, wherein the gene sequence(s) encoding prpE has at least 90% identity to SEQ ID NO: 25.

29. The bacterium of claim 27, wherein the gene sequence(s) encoding pccB has at least 90% identity to SEQ ID NO: 39.

30. The bacterium of claim 27, wherein the gene sequence(s) encoding accA1 has at least 90% identity to SEQ ID NO: 38.

31. The bacterium of claim 27, wherein the gene sequence(s) encoding mmcE has at least 90% identity to SEQ ID NO: 32.

32. The bacterium of claim 27, wherein the gene sequence(s) encoding mutA has at least 90% identity to SEQ ID NO: 33.

33. The bacterium of claim 27, wherein the gene sequence(s) encoding mutB has at least 90% identity to SEQ ID NO: 34.

34. The bacterium of any of claims 1-26, wherein the bacterium comprises one or more gene sequence(s) encoding one or more propionate catabolism enzyme(s) that convert propionate to polyhydroxyalkanoate.

35. The bacterium of claim 34, wherein the bacterium comprises one or more gene sequence(s) encoding prpE, phaB, phaC, and phaA.

36. The bacterium of claim 35, wherein the gene sequence(s) encoding prpE has at least 90% identity to SEQ ID NO: 25.

37. The bacterium of claim 35, wherein the gene sequence(s) encoding phaB has at least 90% identity to a sequence encoding SEQ ID NO: 26.

38. The bacterium of claim 35, wherein the gene sequence(s) encoding phaC has at least 90% identity to a sequence encoding SEQ ID NO: 27.

39. The bacterium of claim 35, wherein the gene sequence(s) encoding phaA has at least 90% identity to a sequence encoding SEQ ID NO: 28.

40. The bacterium of any of claims 1-26, wherein the bacterium comprises gene sequence(s) encoding one or more propionate catabolism enzyme(s) that convert propionate to pyruvate and succinate.

41. The bacterium of claim 40, wherein the one or more gene sequence(s) encode prpB, a prpC, and prpD.

42. The bacterium of claim 40 or claim 41, wherein the one or more gene sequence(s) encode prpE.

43. The bacterium of claim 40 or claim 41, wherein the gene sequence(s) encoding prpE has at least 90% identity to SEQ ID NO: 25.

44. The bacterium of claim 40 or claim 41, wherein the one or more gene sequence(s) encoding prpC has at least 90% identity to SEQ ID NO: 57.

45. The bacterium of claim 40 or claim 41, wherein the one or more gene sequence(s) encoding prpD has at least 90% identity to SEQ ID NO: 58.

46. The bacterium of claim 40 or claim 41, wherein the one or more gene sequence(s) encoding prpB has at least 90% identity to SEQ ID NO: 56.

47. The bacterium of any of claims 1-46 wherein the one or more gene sequence(s) encoding one or more propionate catabolism enzyme(s) comprise one or more gene(s) encoding one or more propionate catabolism enzyme(s) located on a plasmid in the bacterial cell.

48. The bacterium of claims 1-47 wherein the one or more gene sequence(s) encoding one or more propionate catabolism enzyme(s) comprise one or more gene(s) encoding one or more propionate catabolism enzyme(s) located on a chromosome in the bacterial cell.

49. The bacterium of any of claims 3-48, wherein the gene sequence(s) encoding the succinate exporter encodes dcuC.

50. The bacterium of claim 49, wherein the gene sequence(s) encoding dcuC is at least about 90% identity to the sequence of SEQ ID NO: 49.

51. The bacterium of any of claims 3-48, wherein the gene sequence(s) encoding the succinate exporter encodes sucE1.

52. The bacterium of claim 51, wherein the gene sequence(s) encoding sucE1 has at least about 90% identity to the sequence of SEQ ID NO: 46.

53. The bacterium of any one of claims 1-52, wherein the engineered bacterial cell further comprises a genetic modification that increases activity of the at least one heterologous gene encoding the at least one propionate catabolism enzyme.

54. The bacterium of any one of claims 1-53, wherein the engineered bacterial cell further comprises a genetic modification that increases activity of prpE.

55. The bacterium of any one of claims 1-54, wherein the engineered bacterial cell further comprises a genetic modification in pka.

56. The bacterium of any one of claims 1-55, wherein the bacterium is a probiotic bacterial cell.

57. The bacterium of any one of claims 1-56, wherein the bacterium is a member of a genus selected from the group consisting of *Bacteroides*, *Bifidobacterium, Clostridium, Escherichia, Lactobacillus* and *Lactococcus*.

58. The bacterium of any one of claims 1-57, wherein the bacterium is of the genus *Escherichia*.

59. The bacterium of any one of claims 1-58, wherein the engineered bacterial cell is of the species *Escherichia coli* strain *Nissle*.

60. The bacterium of any one of claims 1-59, wherein the engineered bacterial cell is an auxotroph in a gene that is complemented when the engineered bacterial cell is present in a mammalian gut.

61. The bacterium of claim 60, wherein the mammalian gut is a human gut.

62. The bacterium of claim 60 or claim 61, wherein the engineered bacterial cell is an auxotroph in diaminopimelic acid or an enzyme in the thymine biosynthetic pathway.

63. The bacterium of claims 1-62, wherein the engineered bacterial cell is further engineered to harbor a gene encoding a substance that is toxic to the bacterium, wherein the gene is under the control of a promoter is directly or indirectly induced by an environmental condition not naturally present in the mammalian gut.

64. A pharmaceutical composition comprising the bacterium in any of claims 1-63, and a pharmaceutically acceptable carrier.

65. The pharmaceutical composition of claim 64 formulated for oral administration.

66. A method for reducing the levels of propionate, methylmalonate and their byproduct molecules in a subject and/or treating a disease or disorder involving the catabolism of propionate in a subject, the method comprising administering a pharmaceutical composition of claim 64 or claim 65.

67. The method of claims 66, wherein the disorder involving the catabolism of propionate is an organic acidemia.

68. The method of claim 67, wherein the organic acidemia is propionic acidemia (PA).

69. The method of claim 67, wherein the organic acidemia is methylmalonic acidemia (MMA).

70. The method of claim 66, wherein the disorder involving the catabolism of propionate is a vitamin B_{12} deficiency.

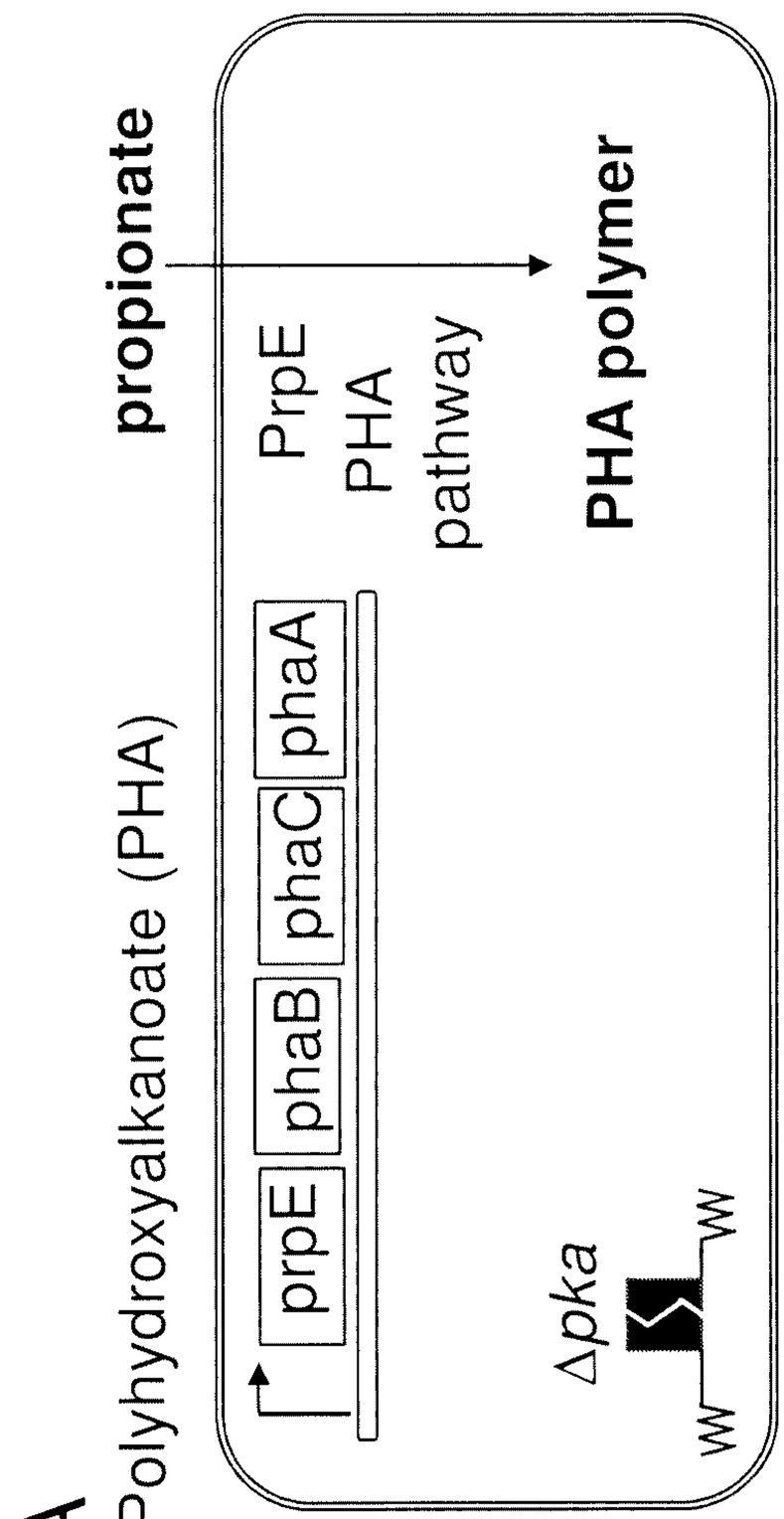

Polyhydroxyalkanoate (PHA)
propionate
PrpE
PHA
pathway
PHA polymer
prpE
phaB
phaC
phaA
Δpka

FIG. 1A

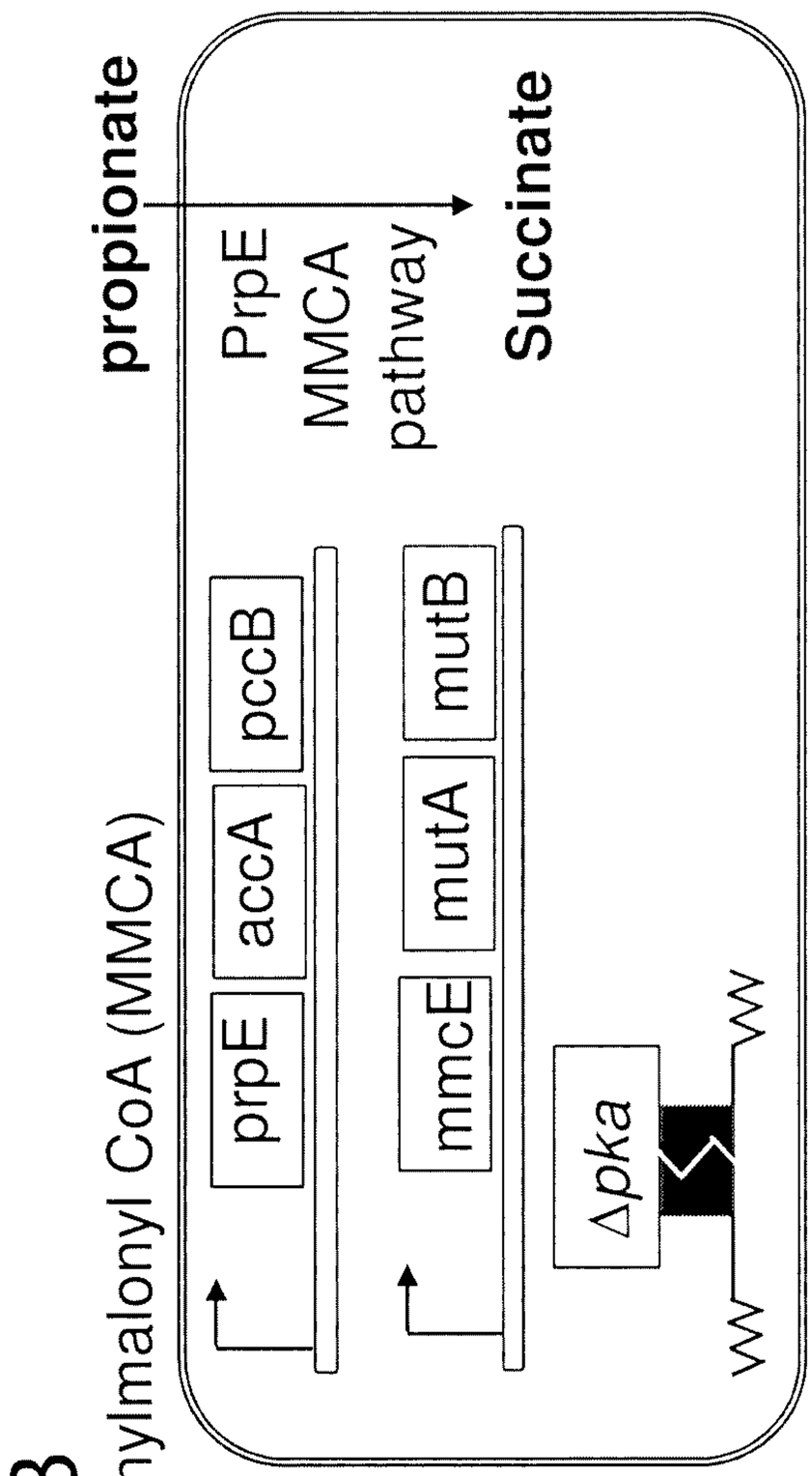
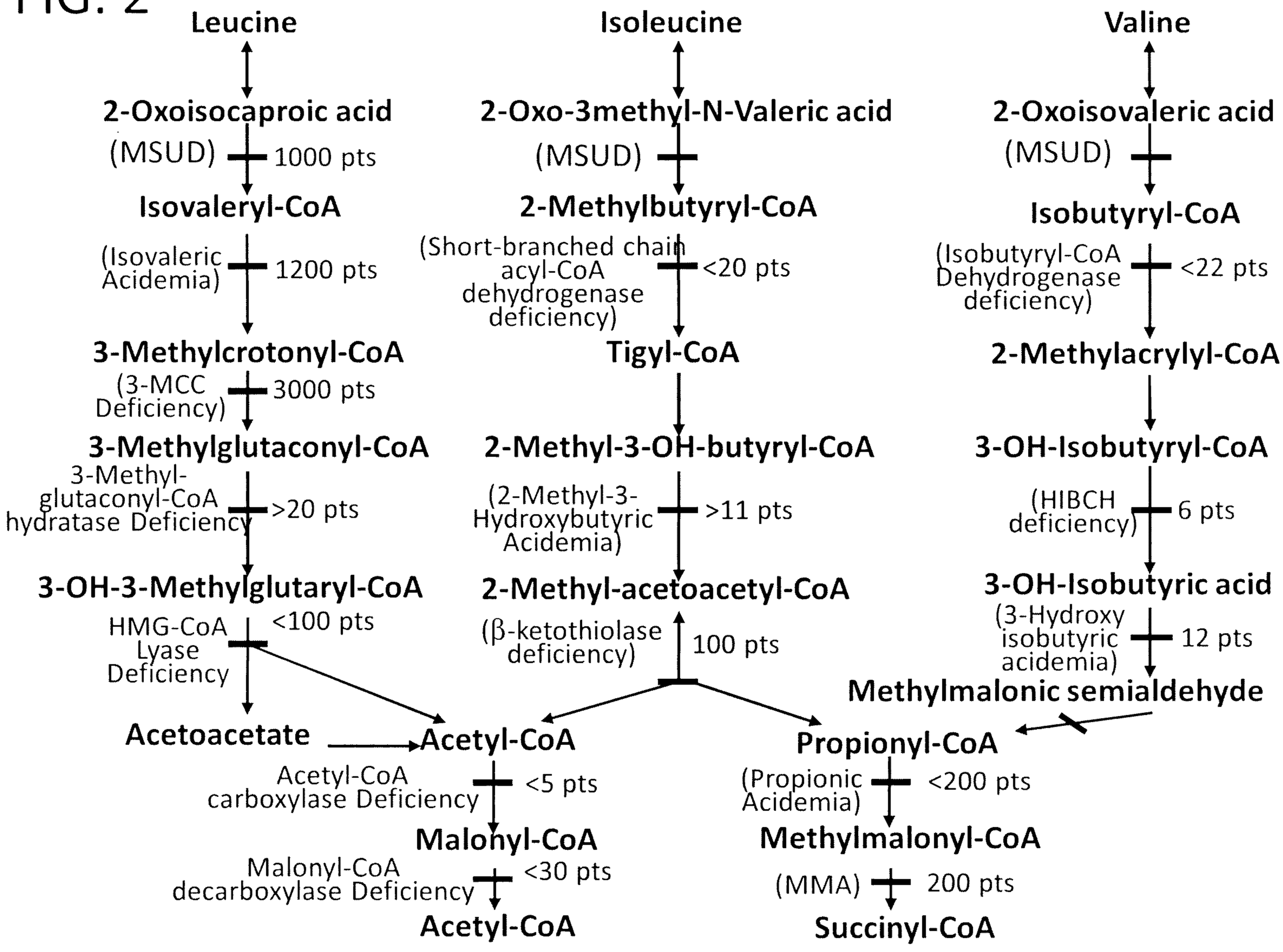

Methylmalonyl CoA (MMCA)
propionate
PrpE
MMCA
pathway
Succinate
prpE
accA
pccB
mmcE
mutA
mutB
Δpka

FIG. 1B

FIG. 2



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PCT/US 2016/044 922 – 02.06.2017

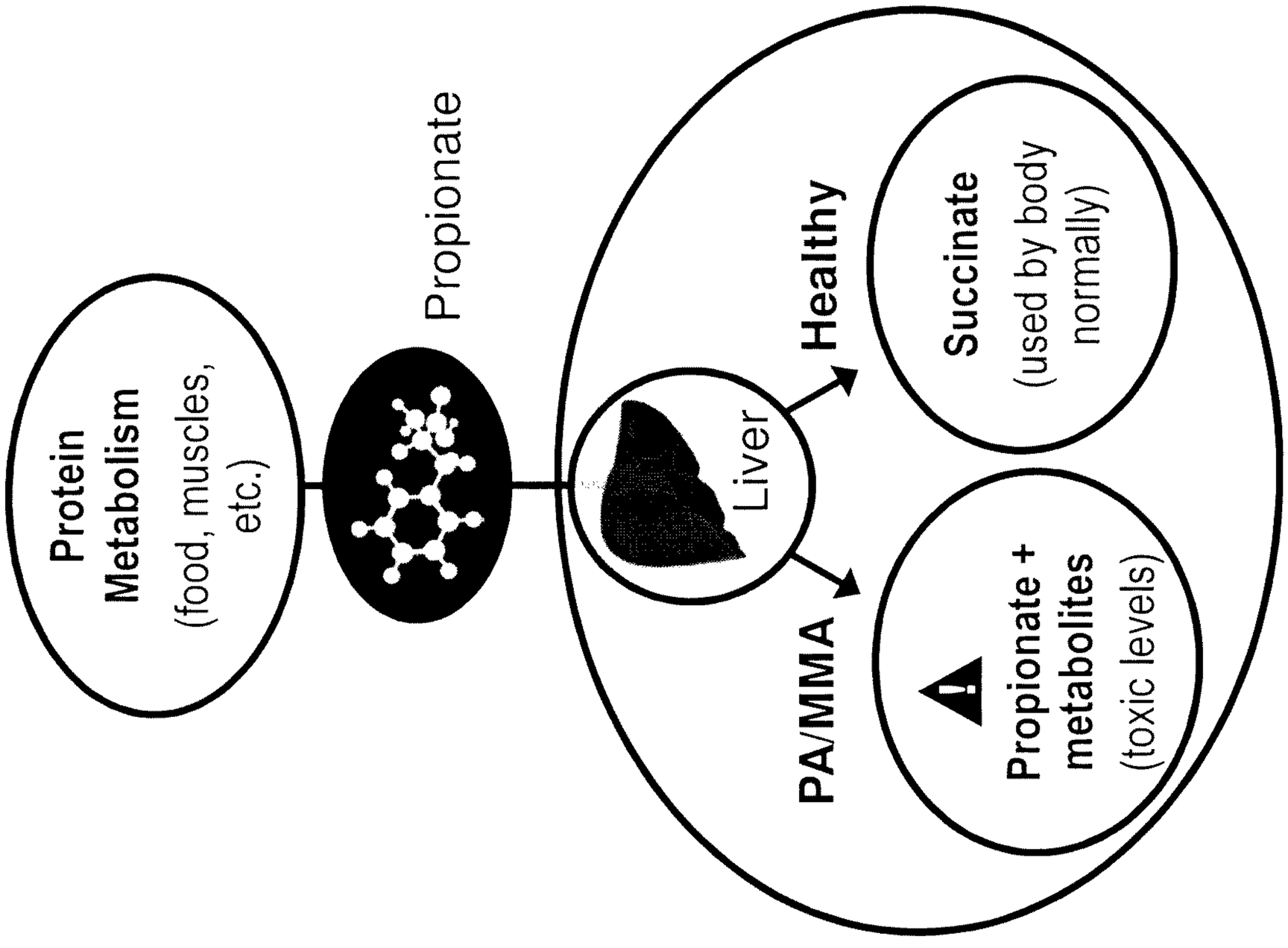

Protein Metabolism
(food, muscles, etc.)
Propionate
Liver
Healthy
Succinate
(used by body normally)
PA/MMA
Propionate + metabolites
(toxic levels)

FIG. 3

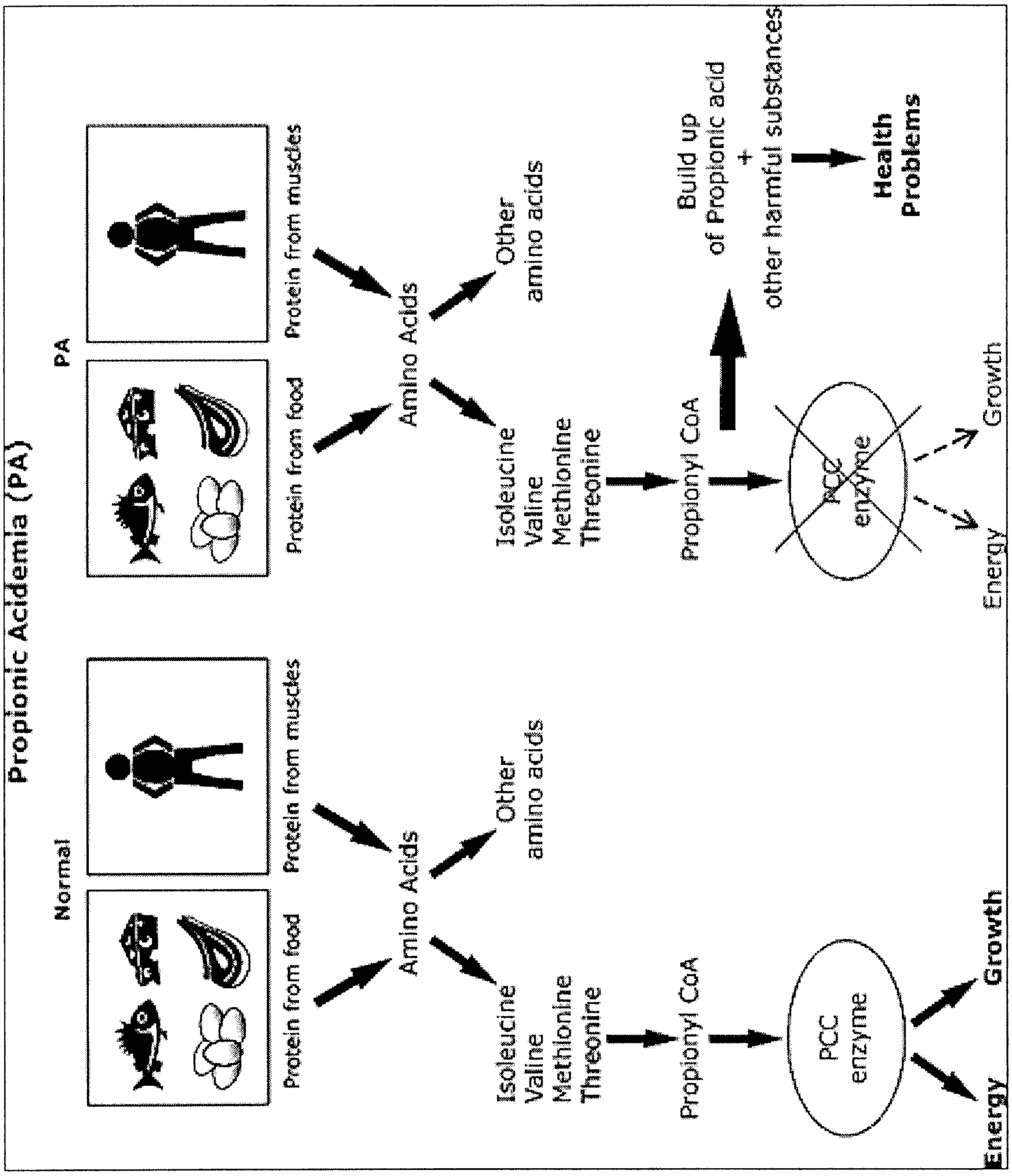

Propionic Acidemia (PA)
PA
Protein from food
Protein from muscles
Amino Acids
Other amino acids
Isoleucine
Valine
Methionine
Threonine
Propionyl CoA
Build up of Propionic acid
+
other harmful substances
Health Problems
PCC enzyme
Energy
Growth
Normal
Protein from food
Protein from muscles
Amino Acids
Other amino acids
Isoleucine
Valine
Methionine
Threonine
Propionyl CoA
PCC enzyme
Energy
Growth

FIG. 4

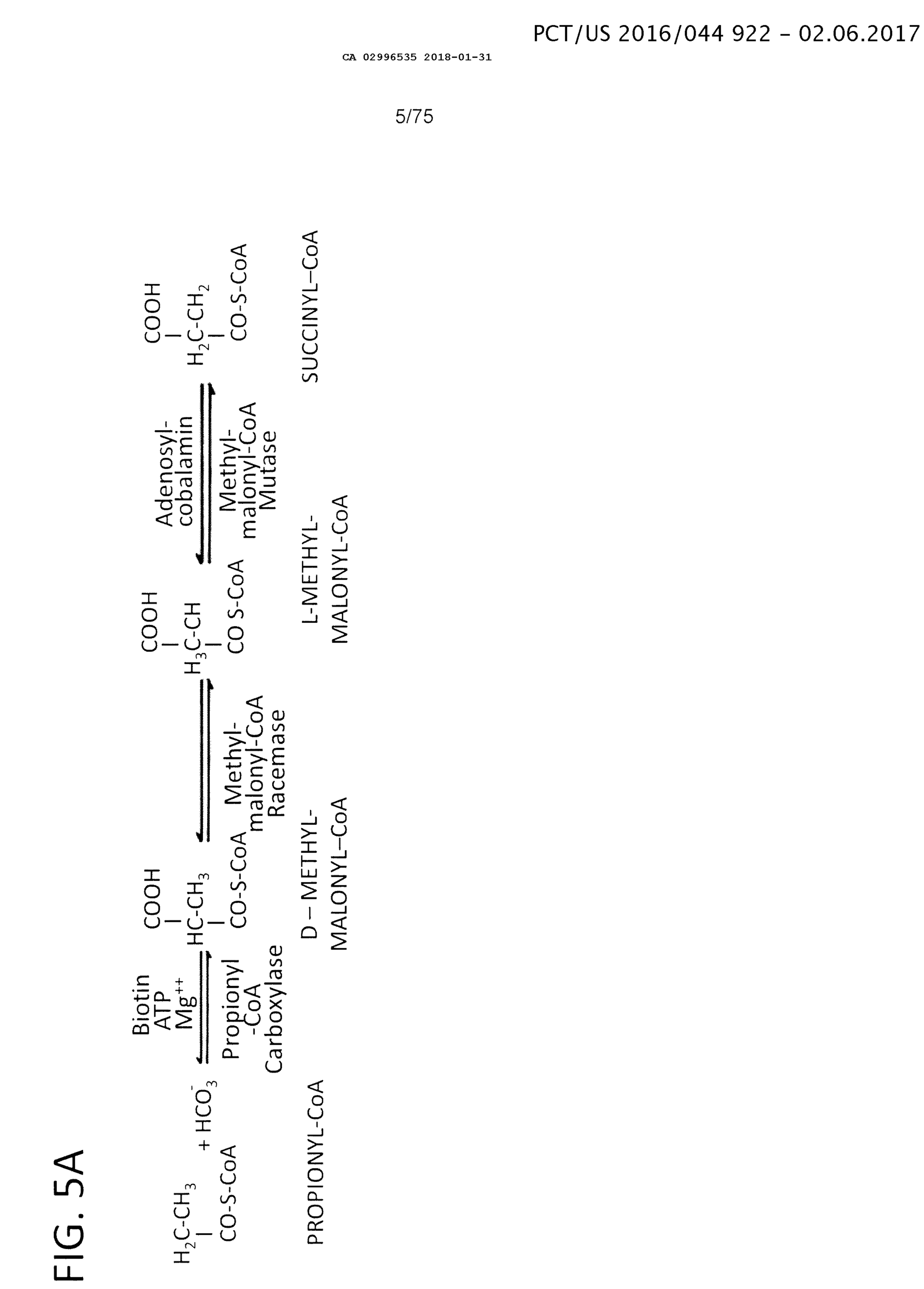

COOH
H2C-CH2
CO-S-CoA
SUCCINYL–CoA
Adenosyl-cobalamin
Methyl-malonyl-CoA Mutase
L-METHYL-MALONYL-CoA
COOH
H3C-CH
CO S-CoA
CO3-
Biotin
ATP
Mg++
Propionyl-CoA Carboxylase
CoA
D – METHYL-MALONYL–CoA
Methyl-malonyl-CoA Racemase
H2C-CH3
CO-S-CoA
+ H
PROPIONYL-

FIG. 5A

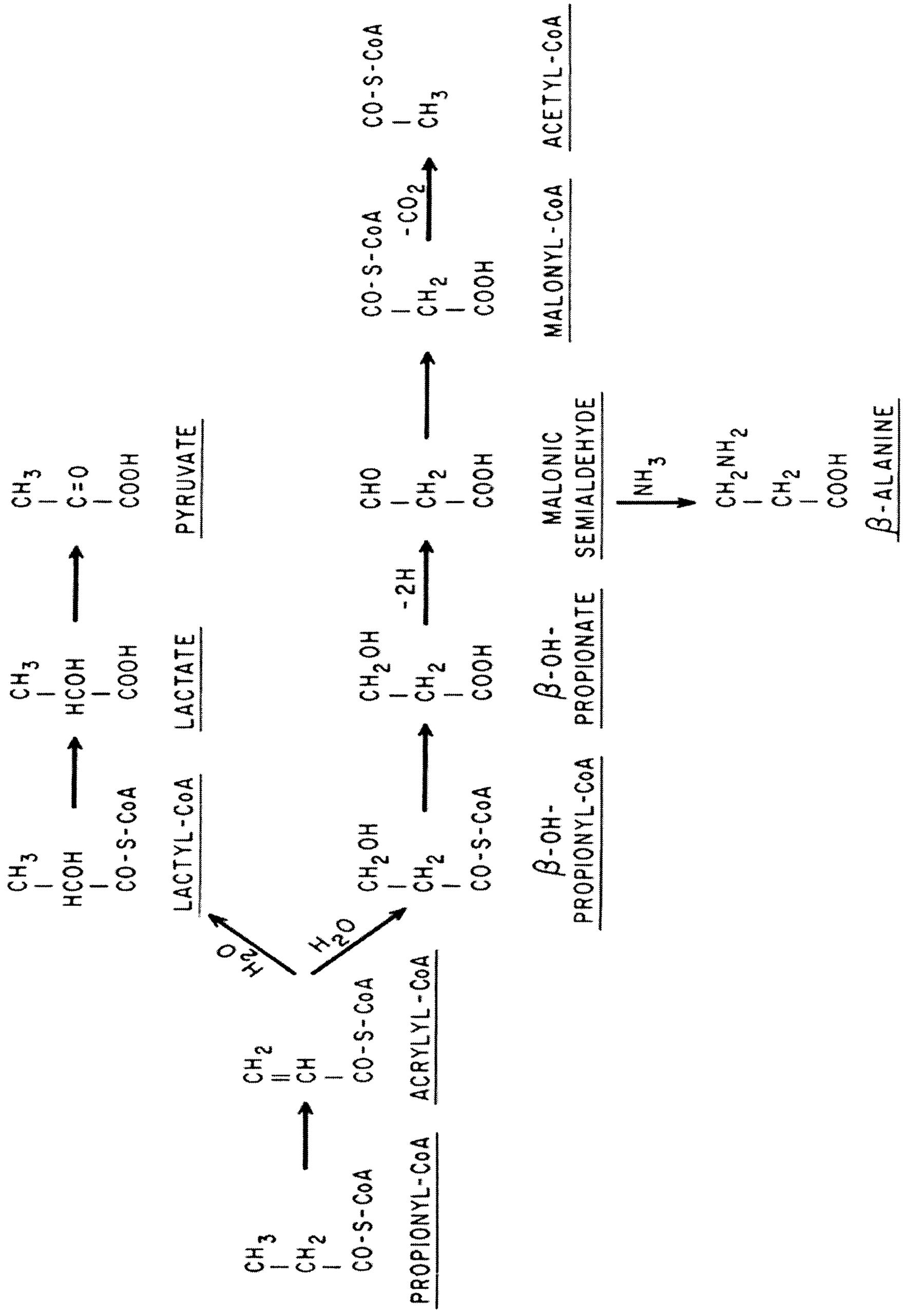

CO-S-CoA
CH3
ACETYL-CoA
CH3
HCOH
COOH
LACTATE
CH3
C=O
COOH
PYRUVATE
CO-S-CoA
-CO2
CH2
COOH
MALONYL-CoA
CHO
CH2
COOH
MALONIC SEMIALDEHYDE
CH2OH
CH2
COOH
β-OH-PROPIONATE
NH3
CH2NH2
CH2
COOH
β-ALANINE
CH3
HCOH
CO-S-CoA
LACTYL-CoA
O2
H2O
CH2OH
CH2
CO-S-CoA
β-OH-PROPIONYL-CoA
CH2
CH
CO-S-CoA
ACRYLYL-CoA
CH3
CH2
CO-S-CoA
PROPIONYL-CoA

FIG. 5B

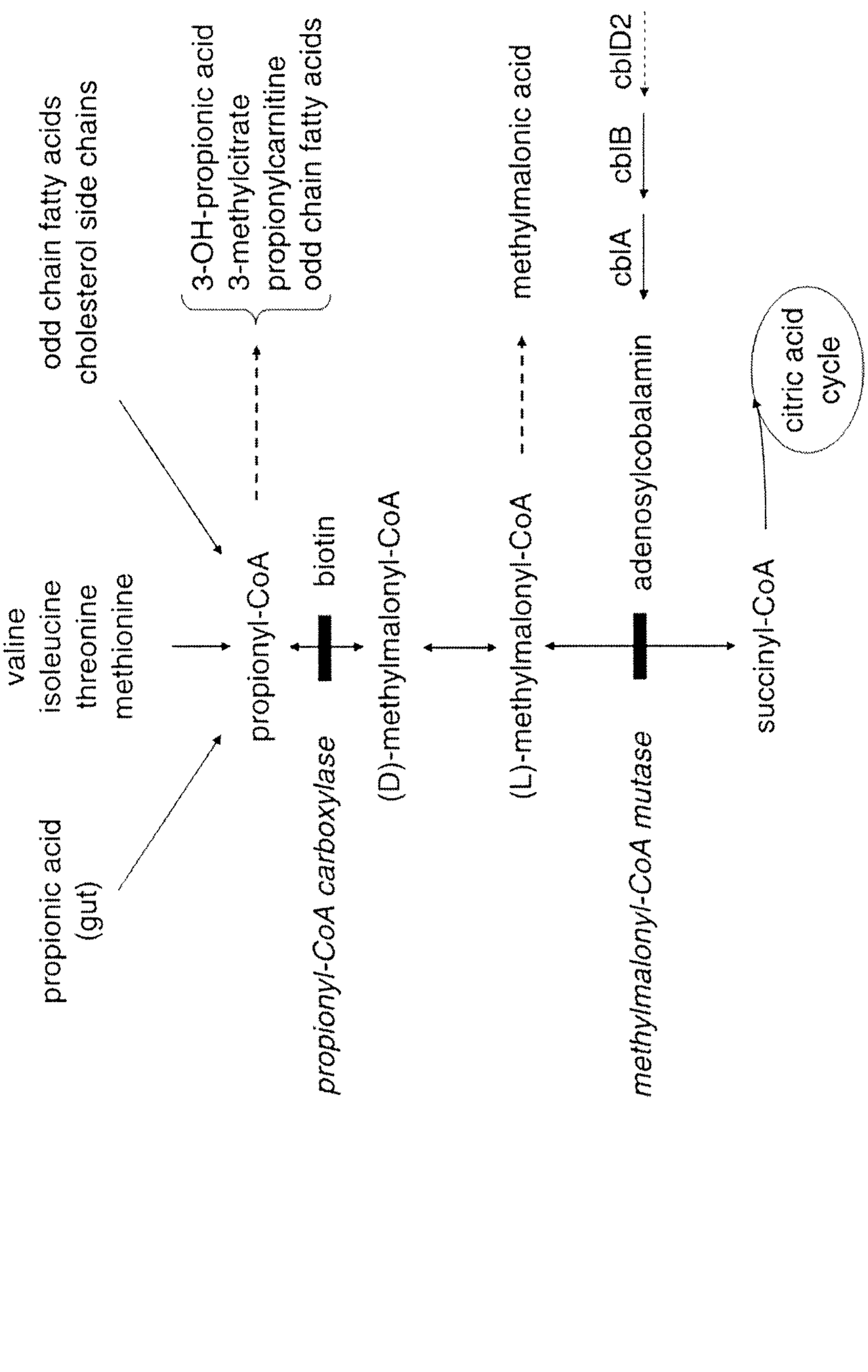

odd chain fatty acids
cholesterol side chains
3-OH-propionic acid
3-methylcitrate
propionylcarnitine
odd chain fatty acids
methylmalonic acid
cblA
cblB
cblD2
ylcobalamin
citric acid cycle
valine
isoleucine
threonine
methionine
propionic acid (gut)
propionyl-CoA
biotin
propionyl-CoA carboxylase
(D)-methylmalonyl-CoA
(L)-methylmalonyl-CoA
adenosyl
methylmalonyl-CoA mutase
succinyl-CoA

FIG. 5C

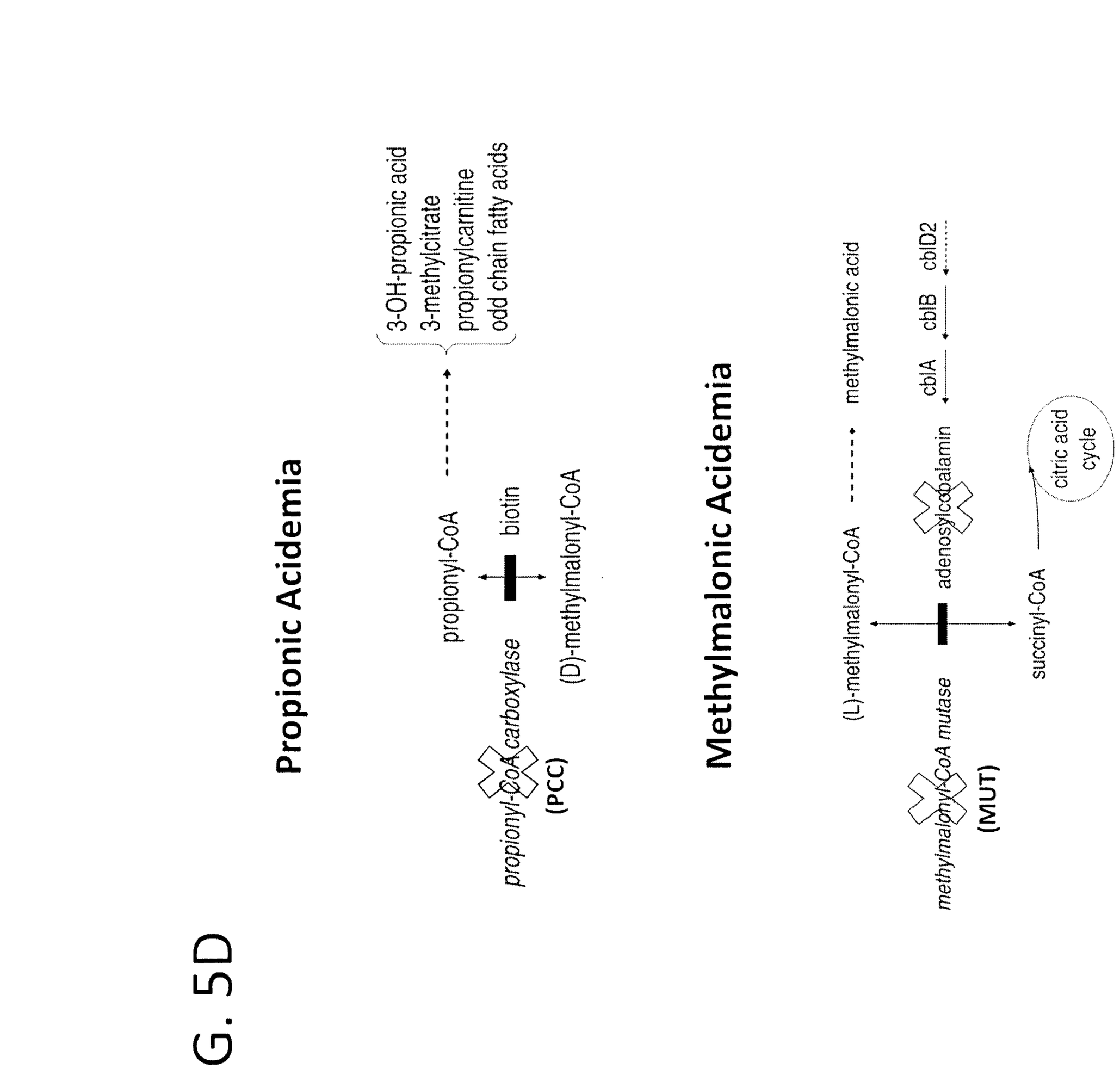

Propionic Acidemia
propionyl-CoA
propionyl-CoA carboxylase
(PCC)
biotin
(D)-methylmalonyl-CoA
3-OH-propionic acid
3-methylcitrate
propionylcarnitine
odd chain fatty acids
Methylmalonic Acidemia
(L)-methylmalonyl-CoA
methylmalonyl-CoA mutase
(MUT)
adenosylcobalamin
succinyl-CoA
citric acid cycle
methylmalonic acid
cblA
cblB
cblD2

FIG. 5D

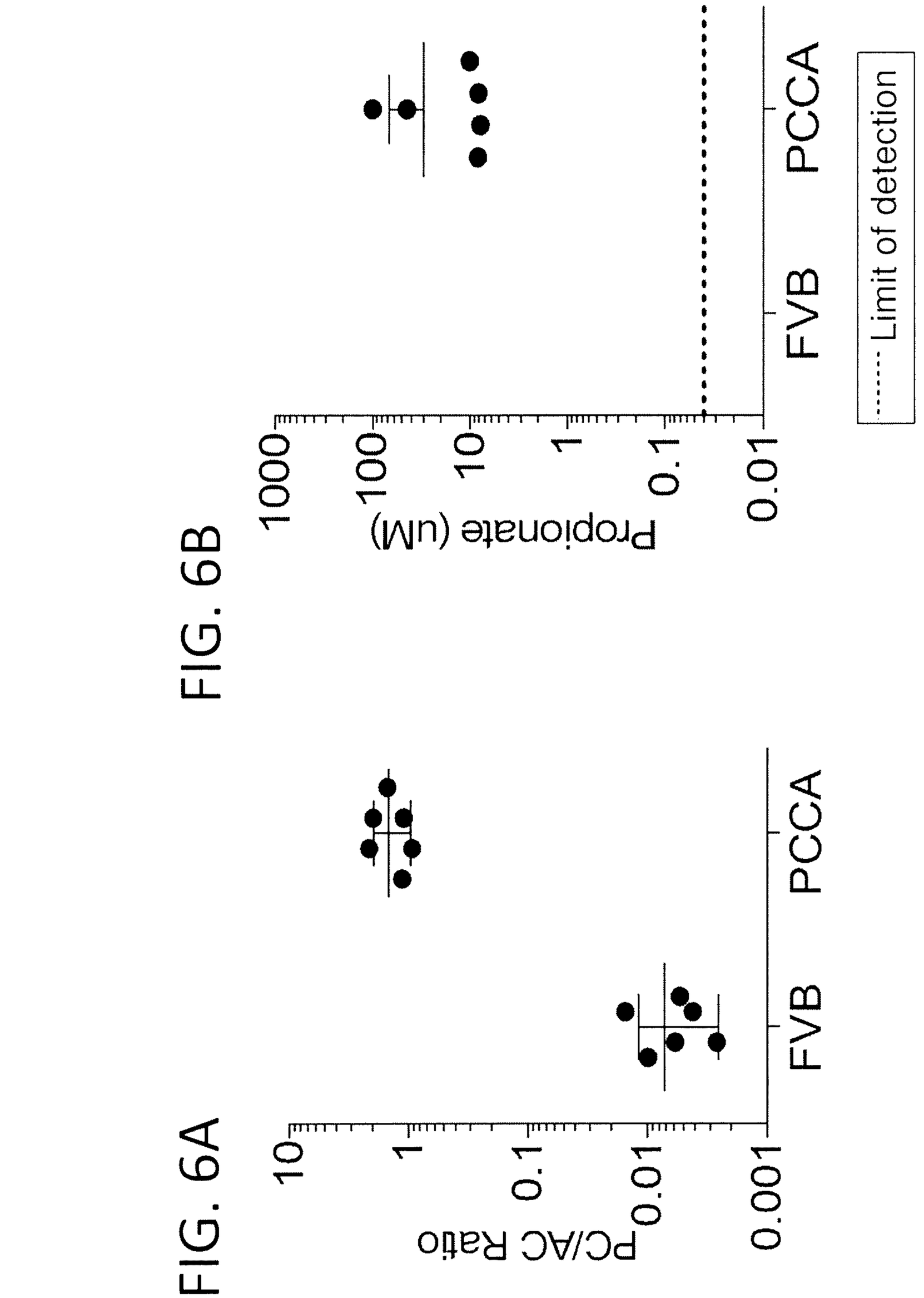

6A
PC/AC Ratio
10
1
0.1
0.01
0.001
FVB
PCCA
6B
Propionate (uM)
1000
100
10
1
0.1
0.01
FVB
PCCA
Limit of detection

FIG. 6A

FIG. 6B

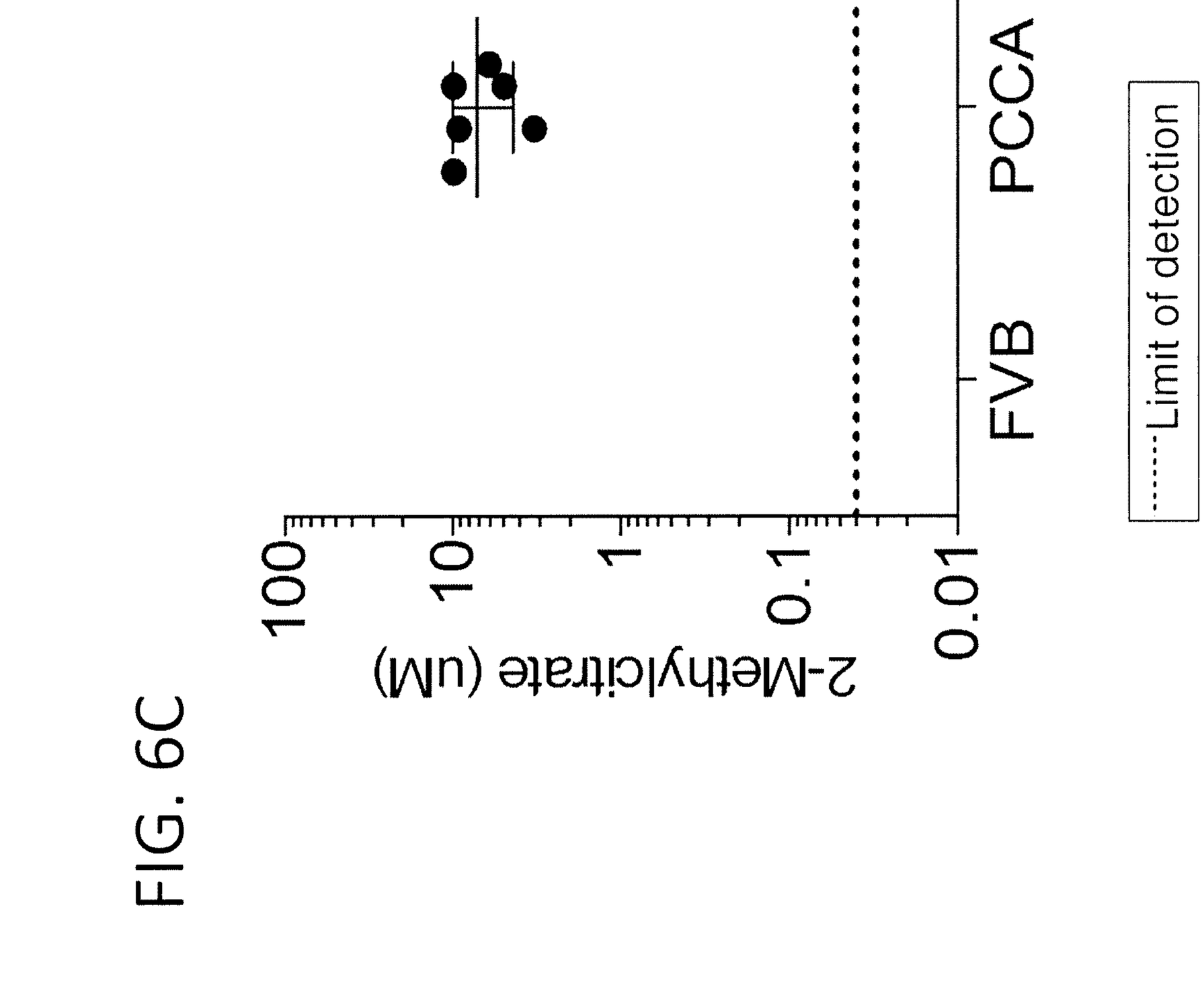

2-Methylcitrate (uM)
100
10
1
0.1
0.01
FVB
PCCA
Limit of detection

FIG. 6C

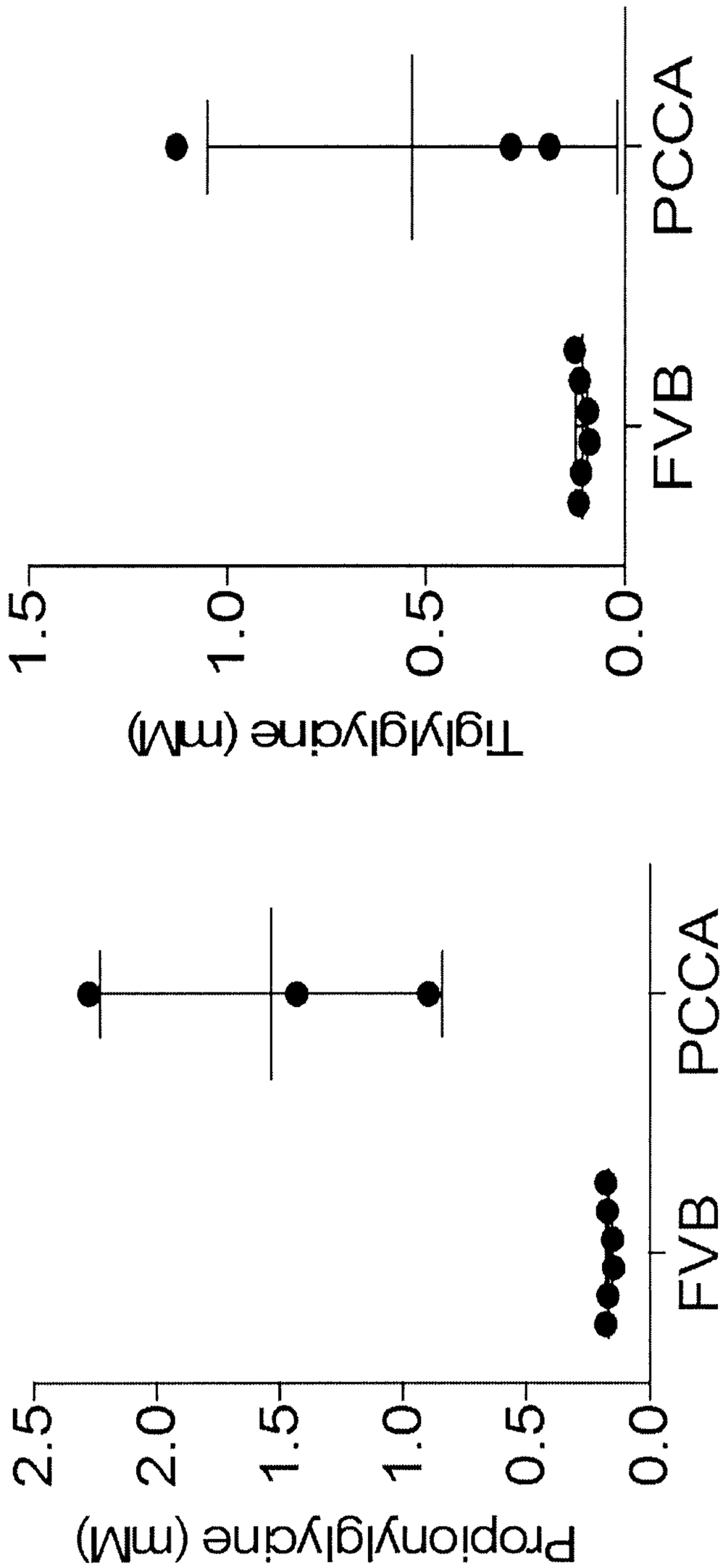

Triglycine (mM)
1.5
1.0
0.5
0.0
FVB
PCCA

FIG. 6E

Propionylglycine (mM)
2.5
2.0
1.5
1.0
0.5
0.0
FVB
PCCA

FIG. 6D

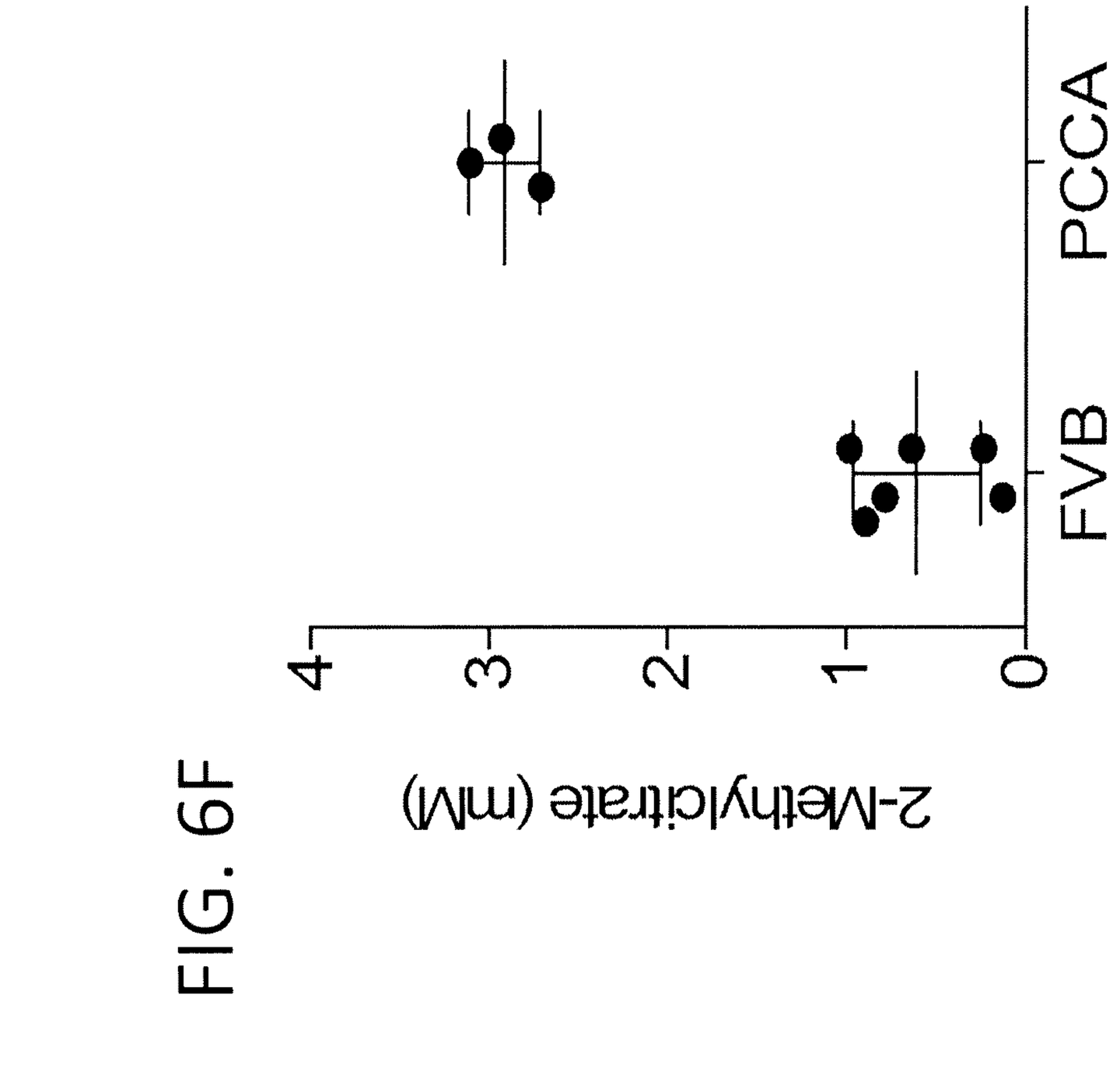


FIG. 6F

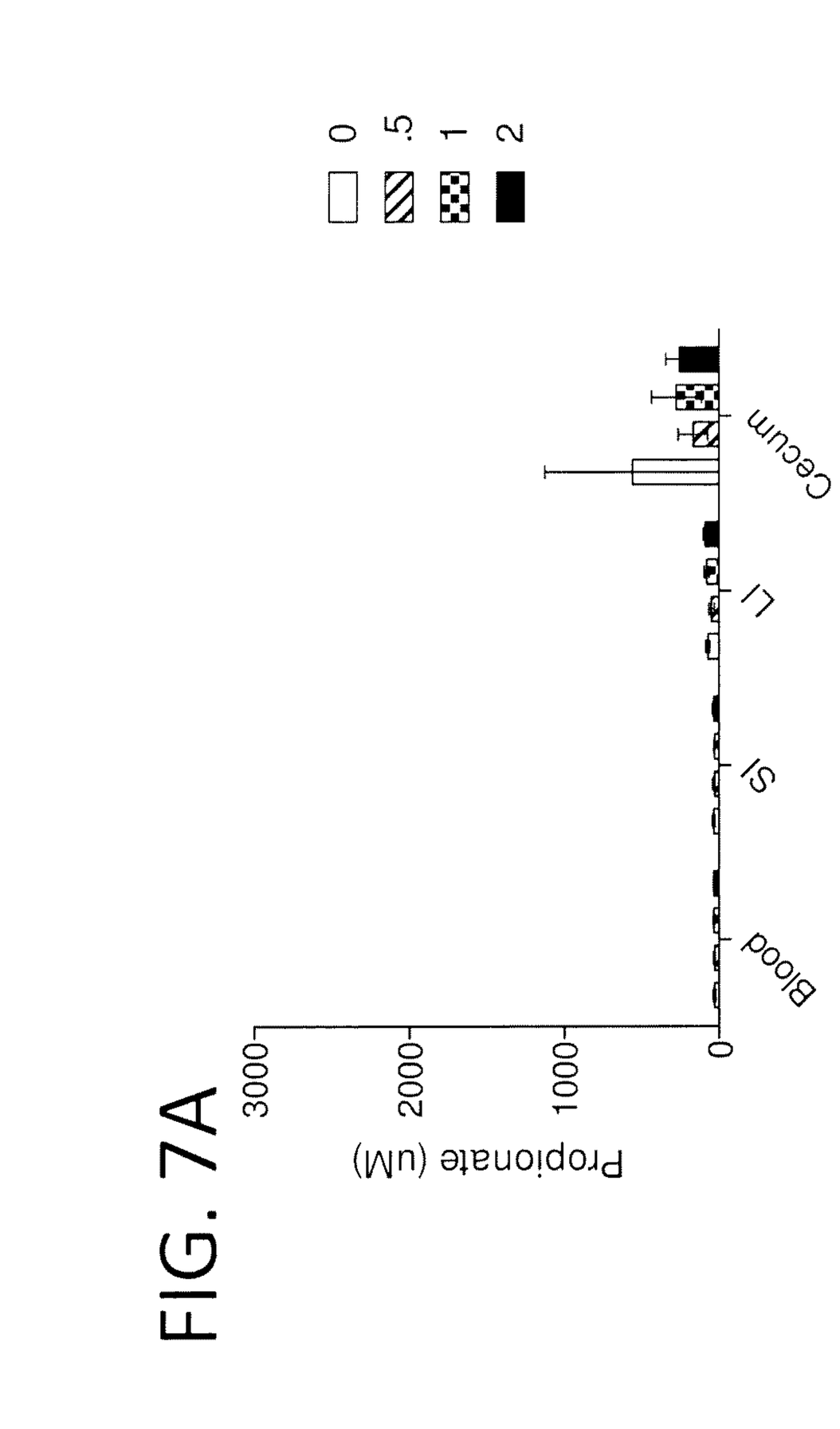

0
.5
1
2
Cecum
LI
SI
Blood
Propionate (uM)
3000
2000
1000
0

FIG. 7A

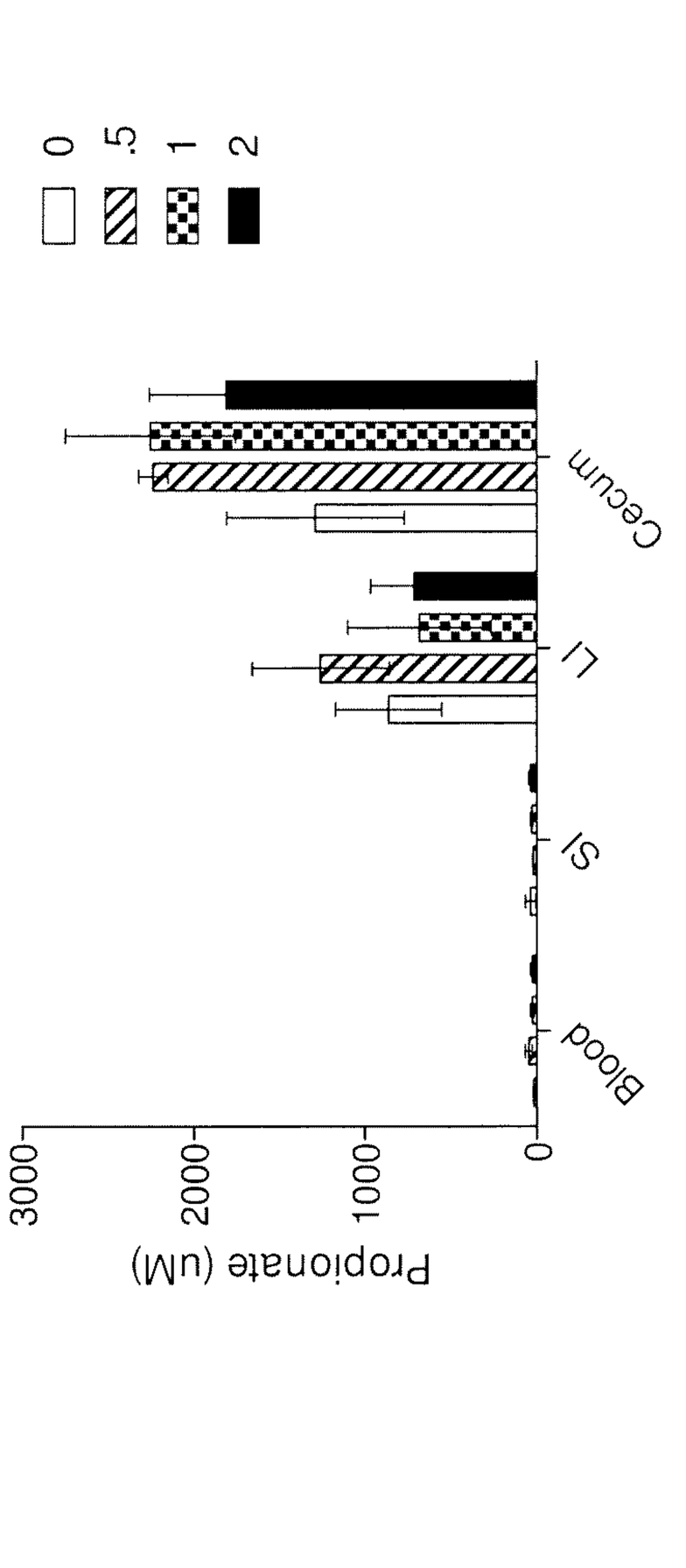

0
.5
1
2
Cecum
LI
SI
Blood
Propionate (uM)
3000
2000
1000
0

FIG. 7B

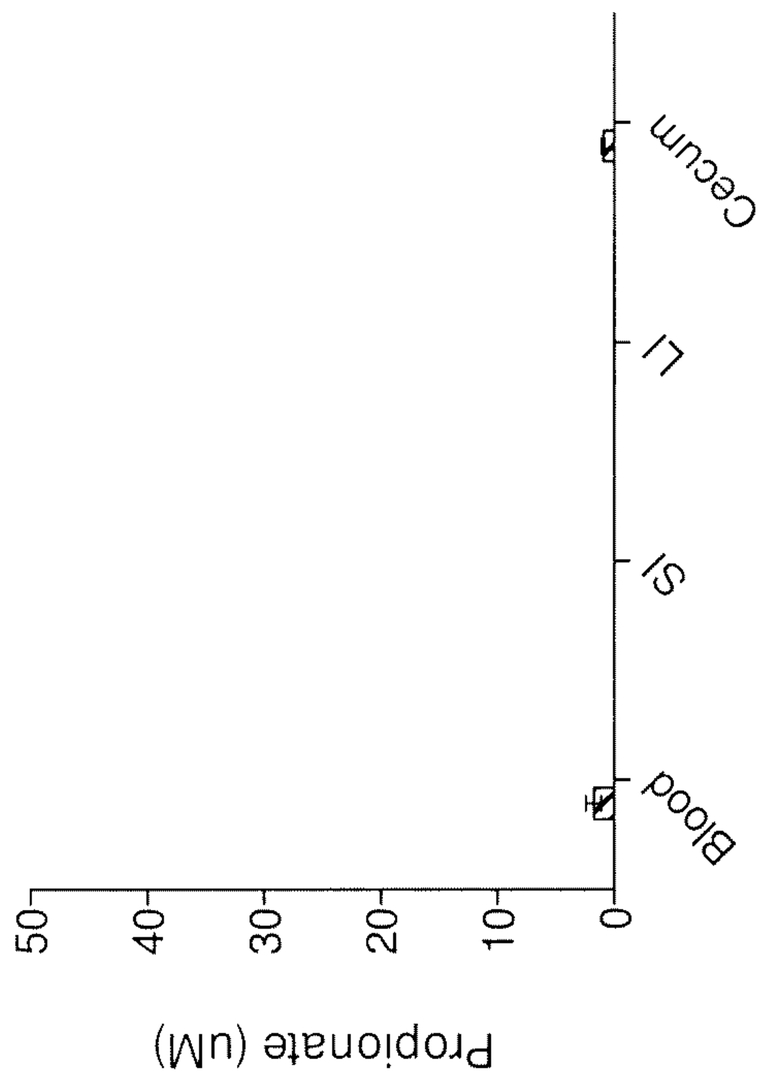

0
.5
1
2
C13 Propionate PCCA
Cecum
LI
SI
Blood
Propionate (uM)
0
10
20
30
40
50

FIG. 7C

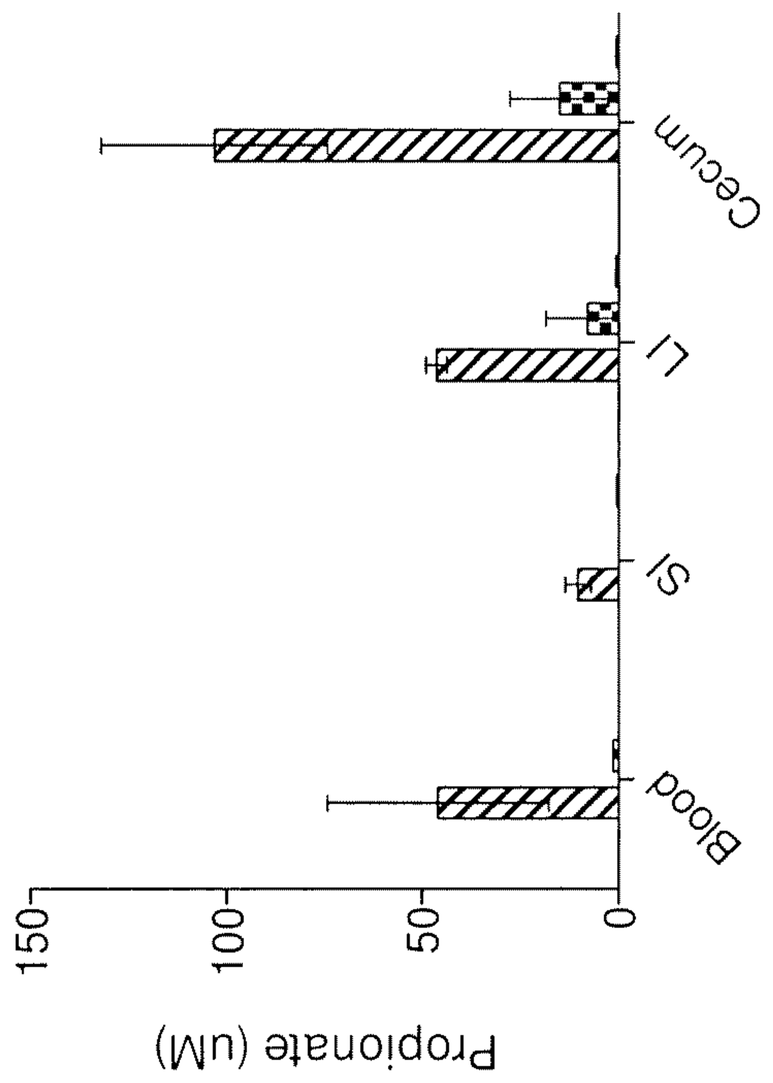

0
.5
1
2
C13 Propionate PCCA
Cecum
LI
SI
Blood
Propionate (uM)
0
50
100
150

FIG. 7D

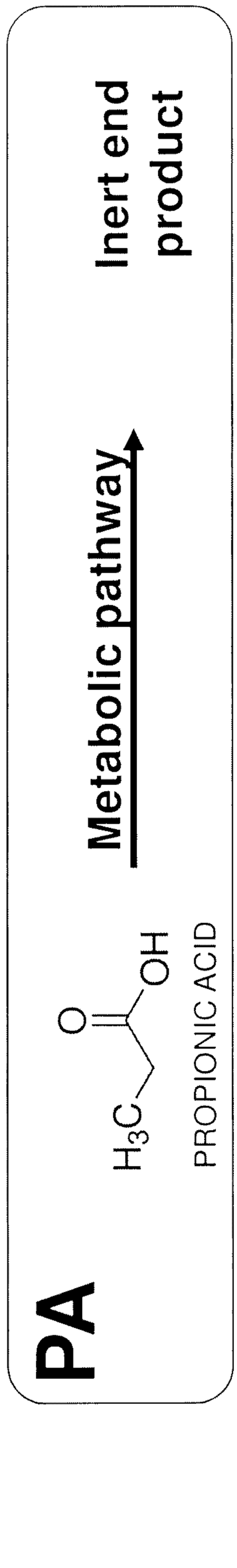

PA
Metabolic pathway
Inert end product
PROPIONIC ACID
H3C
O
OH

FIG. 8A

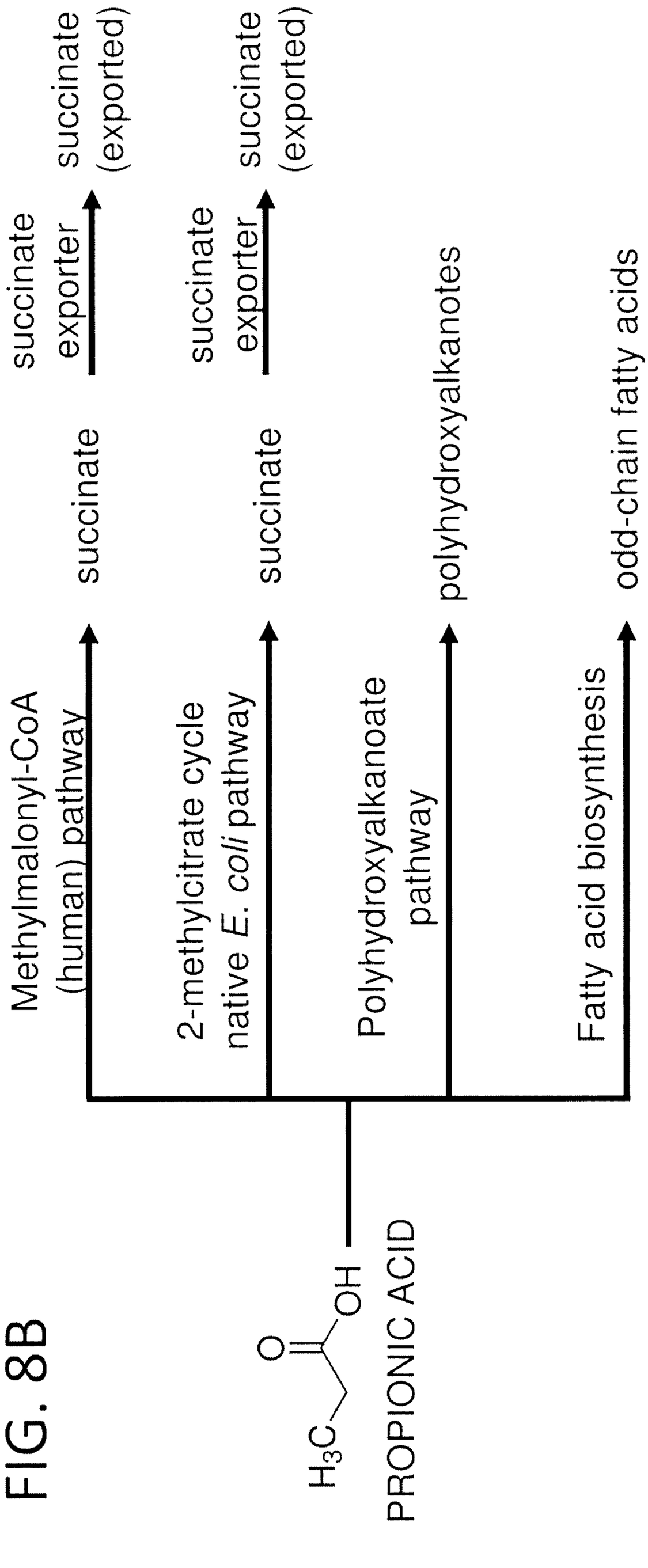

Methylmalonyl-CoA (human) pathway
succinate
succinate exporter
succinate (exported)
2-methylcitrate cycle native E. coli pathway
succinate
succinate exporter
succinate (exported)
Polyhydroxyalkanoate pathway
polyhydroxyalkanotes
Fatty acid biosynthesis
odd-chain fatty acids
PROPIONIC ACID
H3C
O
OH

FIG. 8B

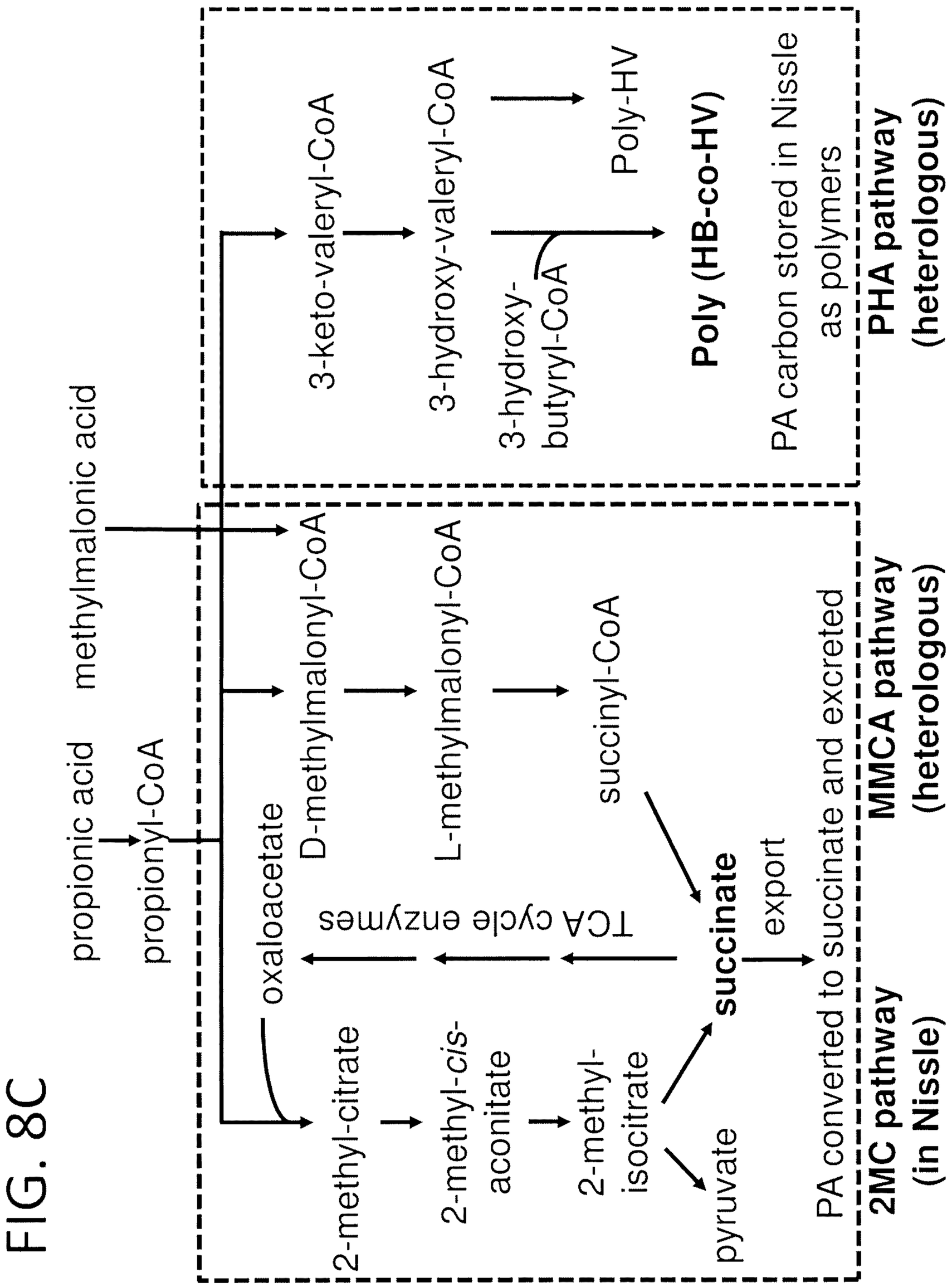

propionic acid
methylmalonic acid
propionyl-CoA
oxaloacetate
D-methylmalonyl-CoA
L-methylmalonyl-CoA
succinyl-CoA
TCA cycle enzymes
2-methyl-citrate
2-methyl-cis-aconitate
2-methyl-isocitrate
succinate
export
pyruvate
PA converted to succinate and excreted
2MC pathway (in Nissle)
MMCA pathway (heterologous)
3-keto-valeryl-CoA
3-hydroxy-valeryl-CoA
3-hydroxy-butyryl-CoA
Poly-HV
Poly (HB-co-HV)
PA carbon stored in Nissle as polymers
PHA pathway (heterologous)

FIG. 8C

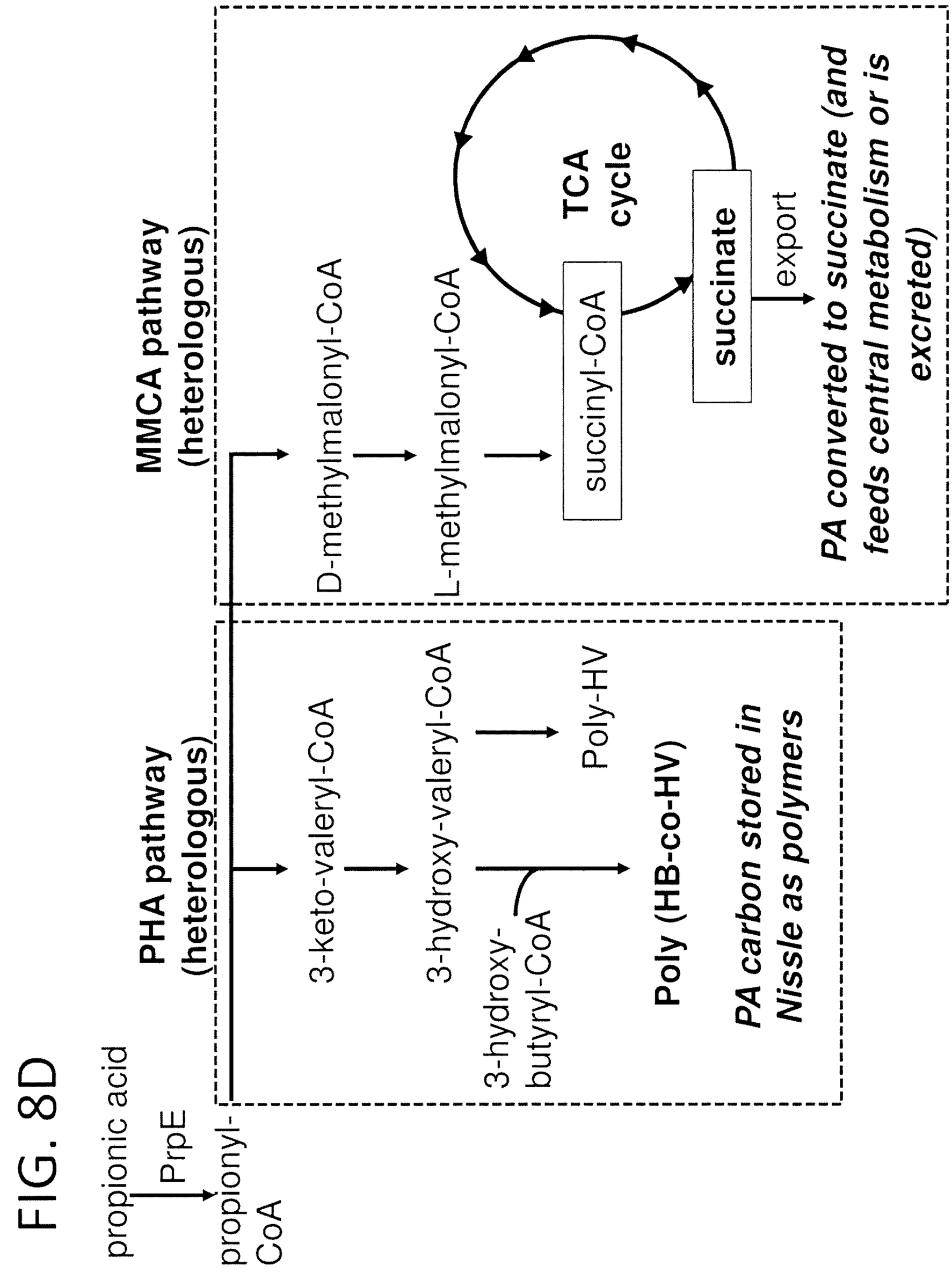

propionic acid
PrpE
propionyl-CoA
MMCA pathway
(heterologous)
D-methylmalonyl-CoA
L-methylmalonyl-CoA
succinyl-CoA
TCA cycle
succinate
export
PA converted to succinate (and feeds central meteted or is excreted)
PHA pathway
(heterologous)
3-keto-valeryl-CoA
3-hydroxy-valeryl-CoA
3-hydroxy-butyryl-CoA
Poly-HV
Poly (HB-co-HV)
PA carbon stored in Nissle as polymers

FIG. 8D

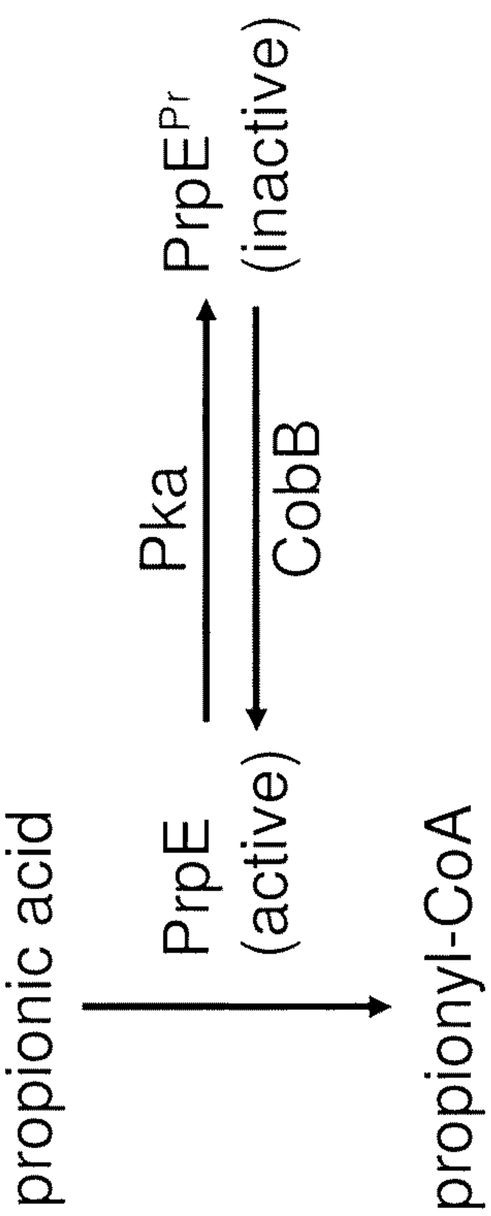

propionic acid
PrpE
(active)
propionyl-CoA

FIG. 9A

Pka
PrpE^{Pr}
(inactive)
CobB

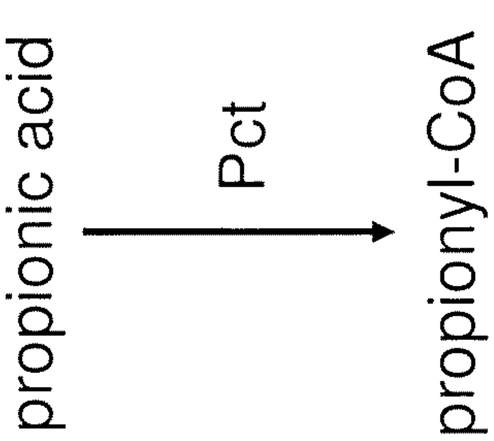

propionic acid
Pct
propionyl-CoA

FIG. 9B

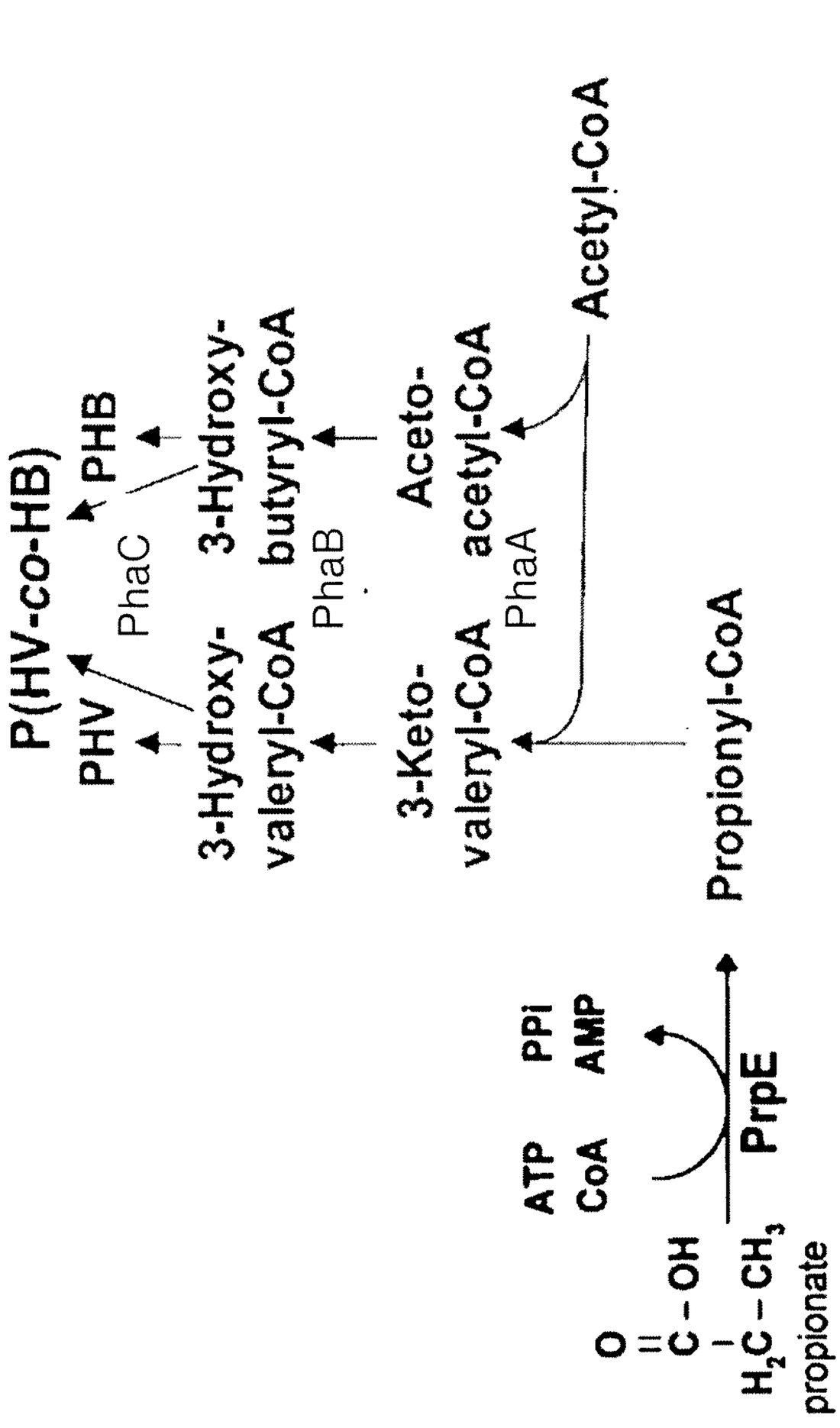

P(HV-co-HB)
PHB
PhaC
3-Hydroxy-
butyryl-CoA
PhaB
Aceto-
acetyl-CoA
PhaA
Acetyl-CoA
PHV
PhaC
3-Hydroxy-
valeryl-CoA
PhaB
3-Keto-
valeryl-CoA
PhaA
Propionyl-CoA
ATP
CoA
PPi
AMP
PrpE
O
C – OH
H2C – CH3
propionate

FIG. 10A

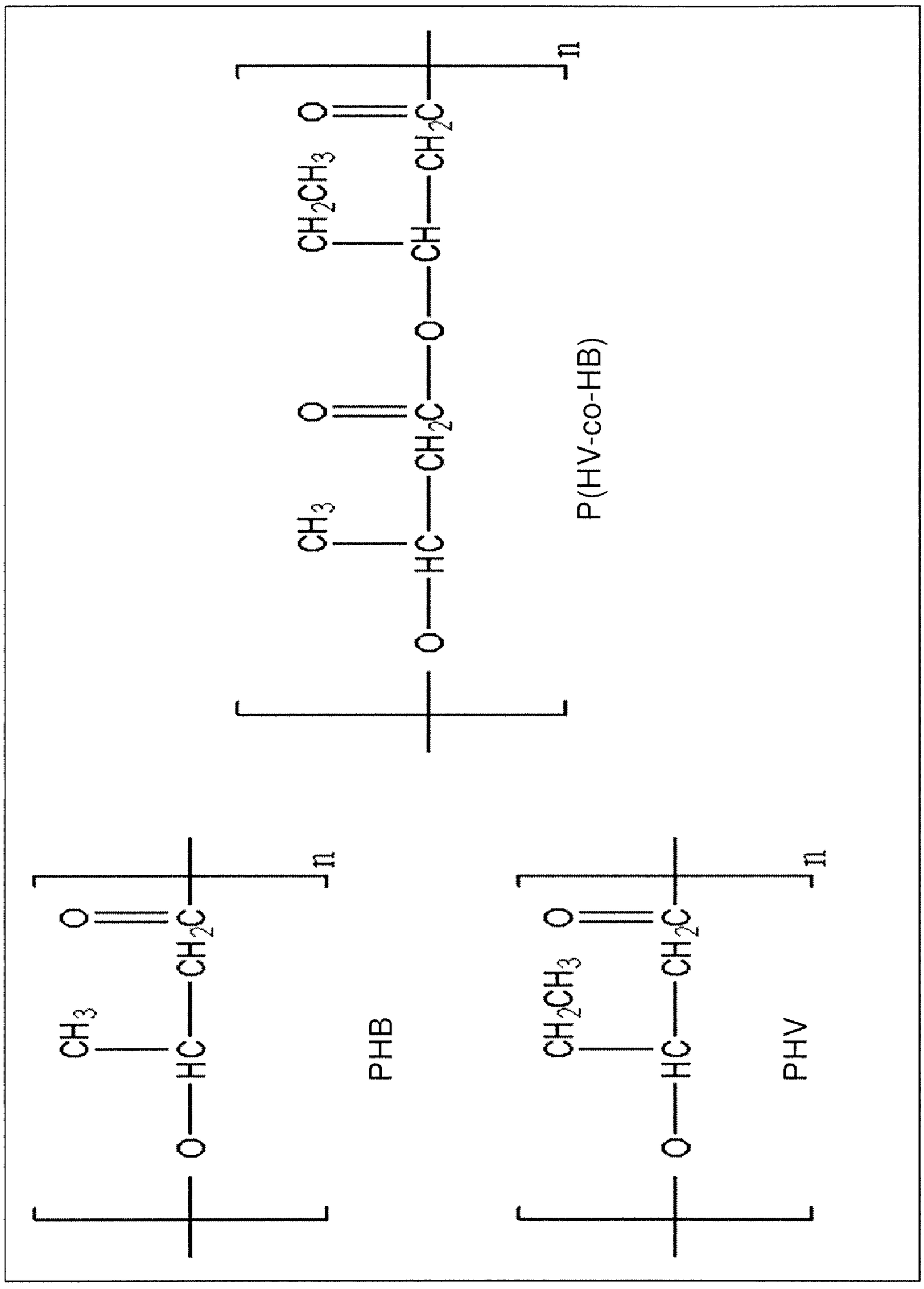


FIG. 10B


PHA pathway circuit design
aTc
tetR
P tet
prpE
phaB
phaC
phaA

FIG. 10C

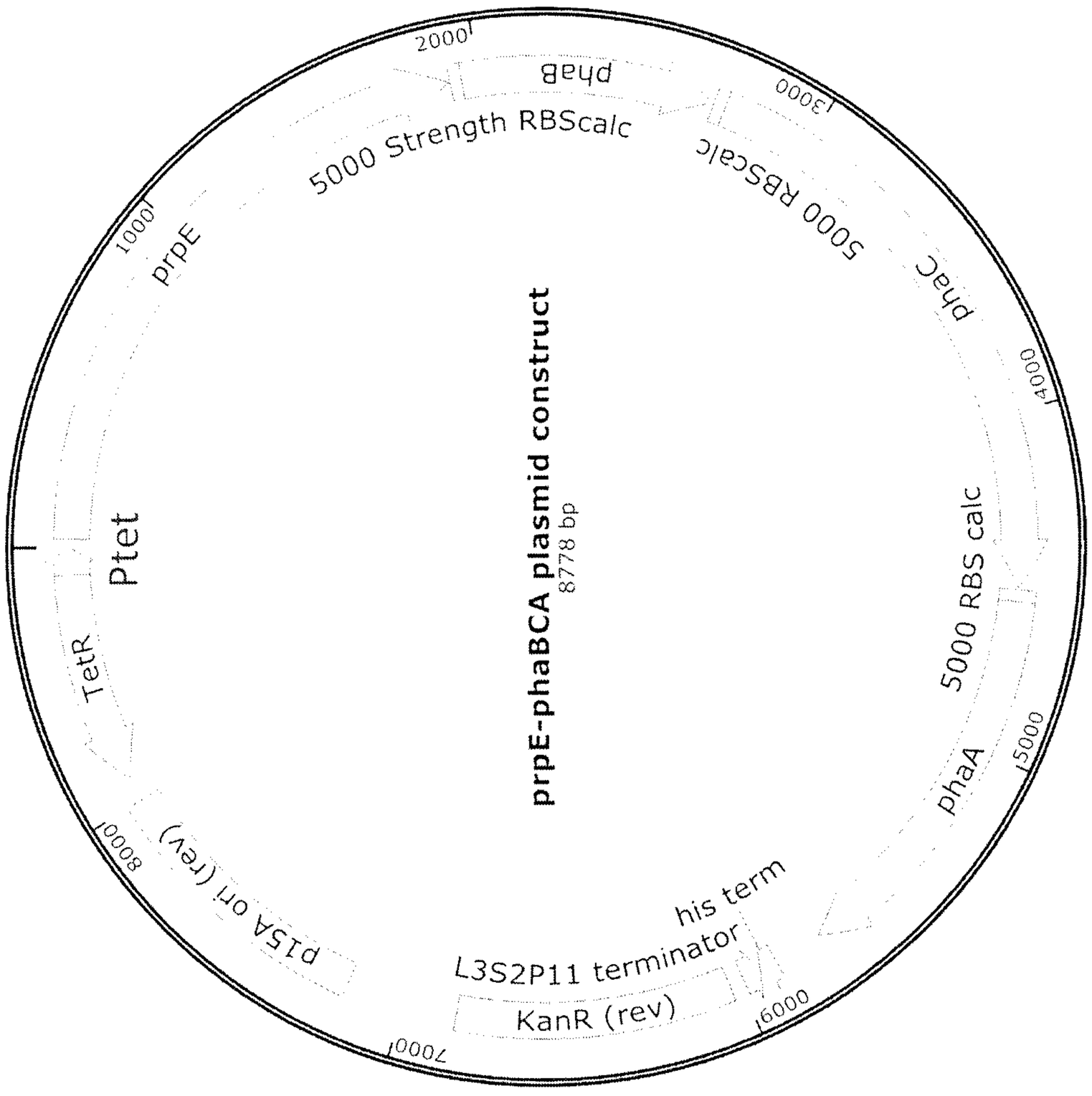

prpE-phaBCA plasmid construct
8778 bp
1000
2000
3000
4000
5000
6000
7000
8000
prpE
phaB
5000 Strength RBScalc
5000 RBScalc
phaC
5000 RBS calc
phaA
his term
L3S2P11 terminator
KanR (rev)
p15A ori (rev)
TetR
Ptet

FIG. 11

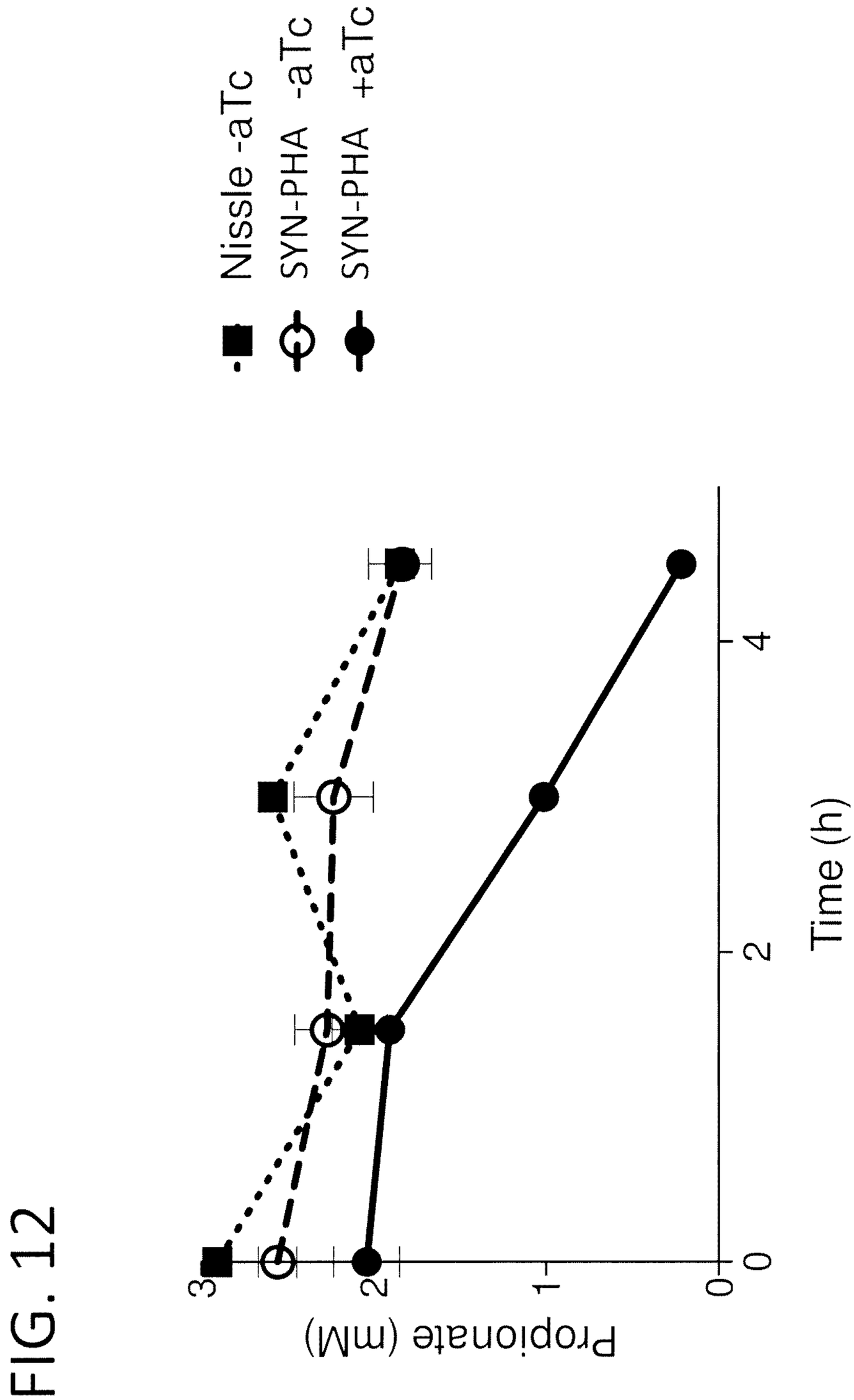


FIG. 12

FIG. 13A

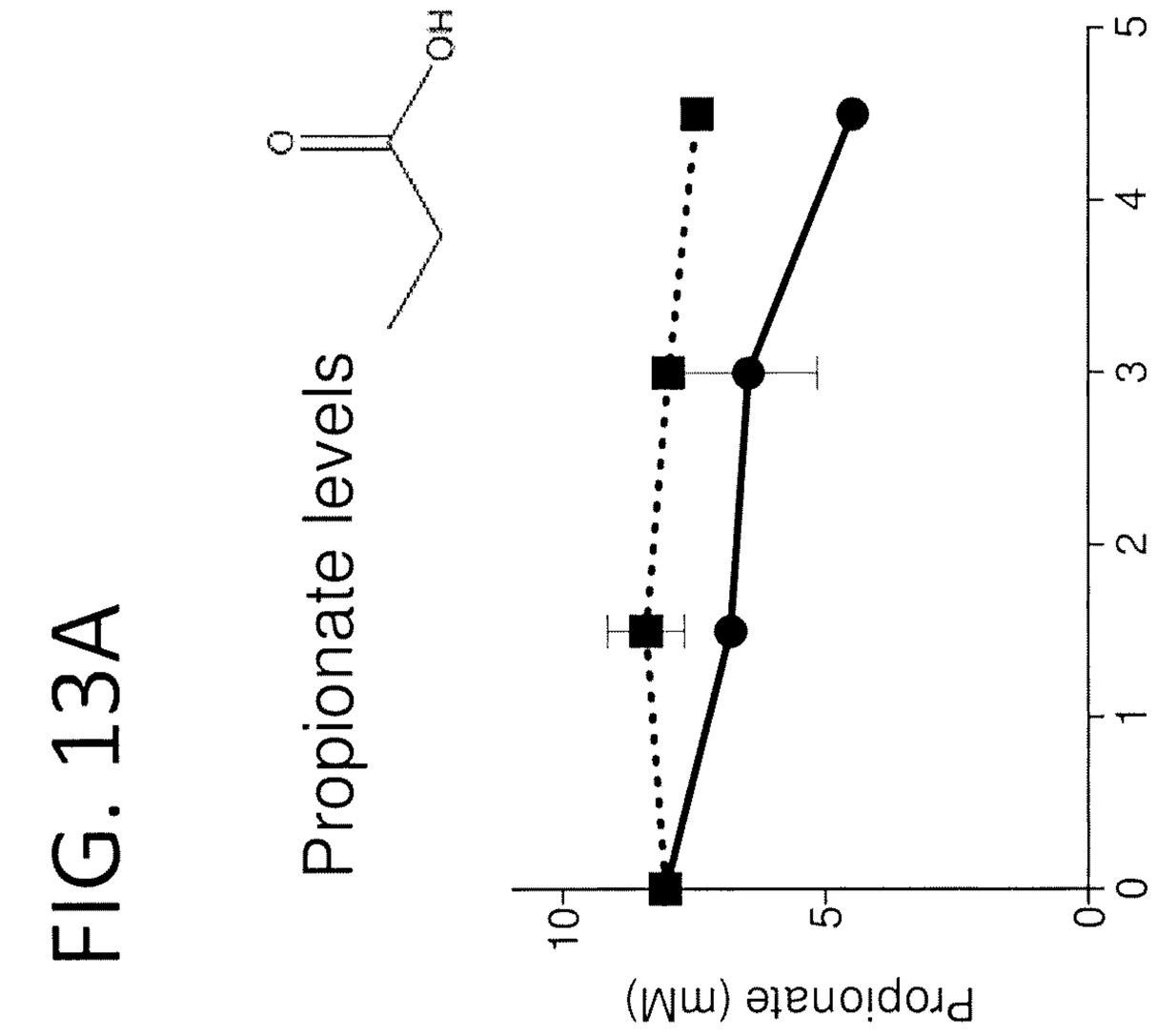
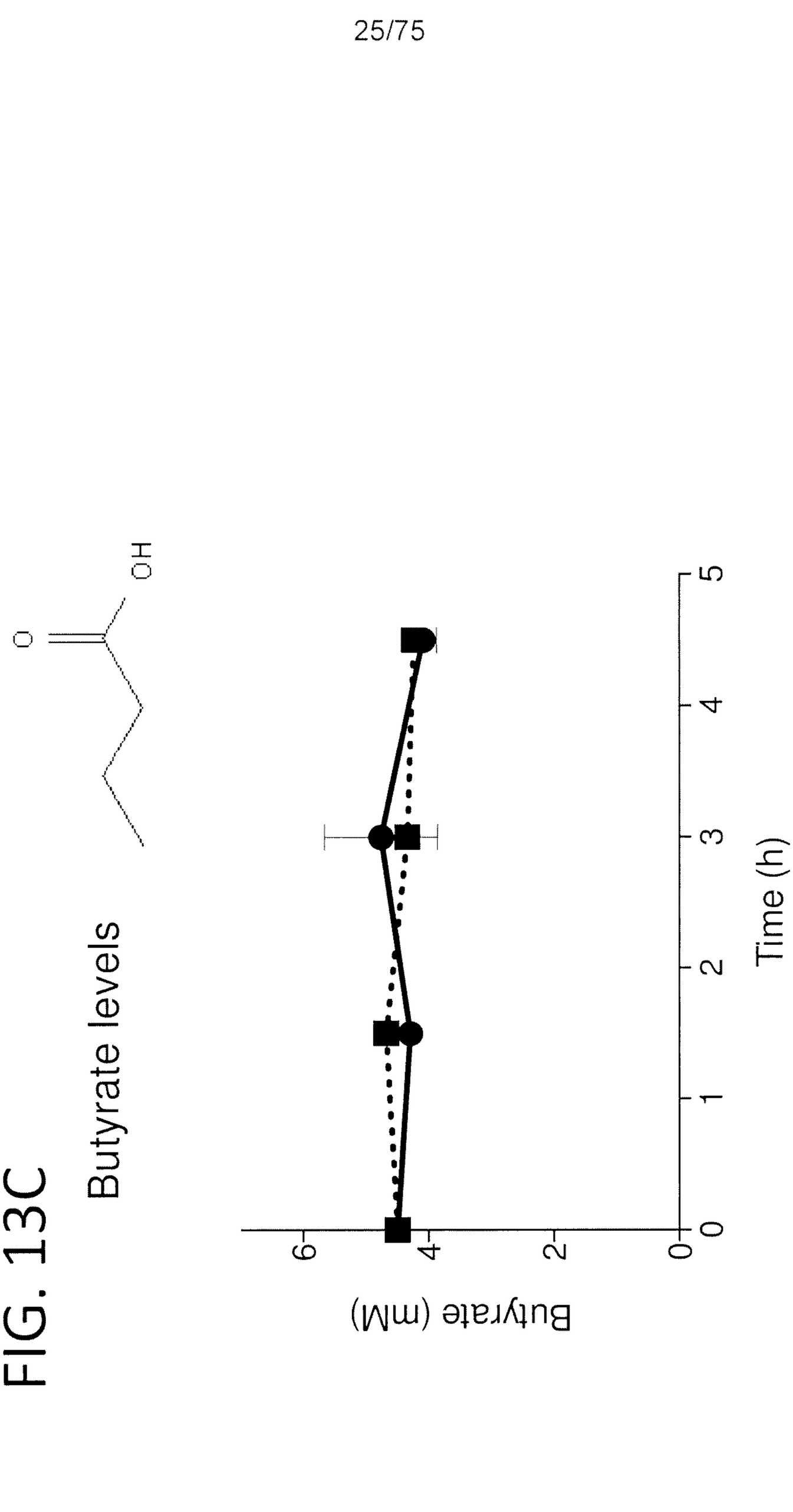
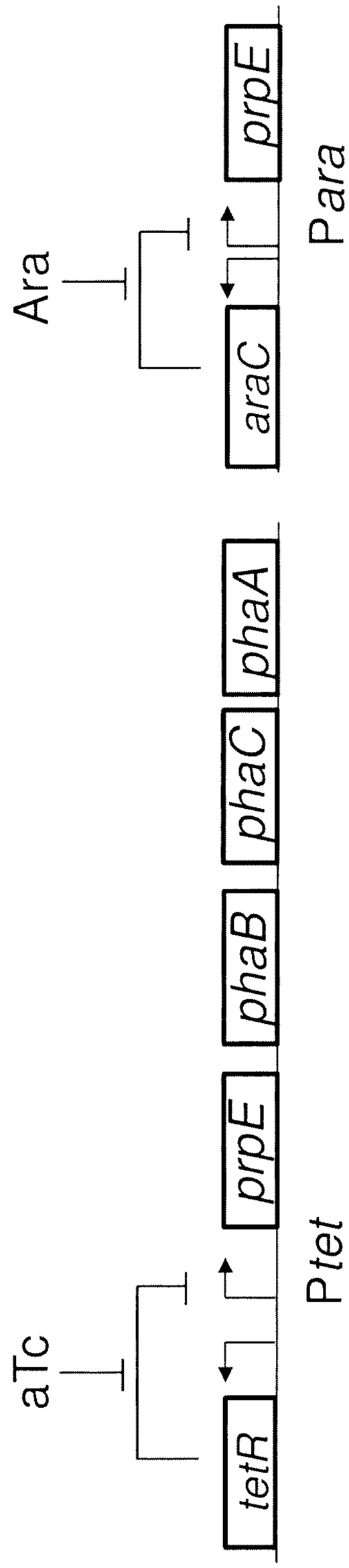


FIG. 13B

FIG. 13C


Butyrate levels
O
OH
Butyrate (mM)
0
2
4
6
0
1
2
3
4
5
Time (h)
Nissle +aTc
SYN-PHA +aTC

14A


aTc
tetR
phaC
phaA
prpE
phaB
Ptet

Ara
araC
prpE
Para


Nissle
SYN-PHA
SYN-PHA + prpE

Propionate (mM)
10
8
6
4
2
0
1
2
3
4
5
Time (h)

FIG. 14A

FIG. 14B

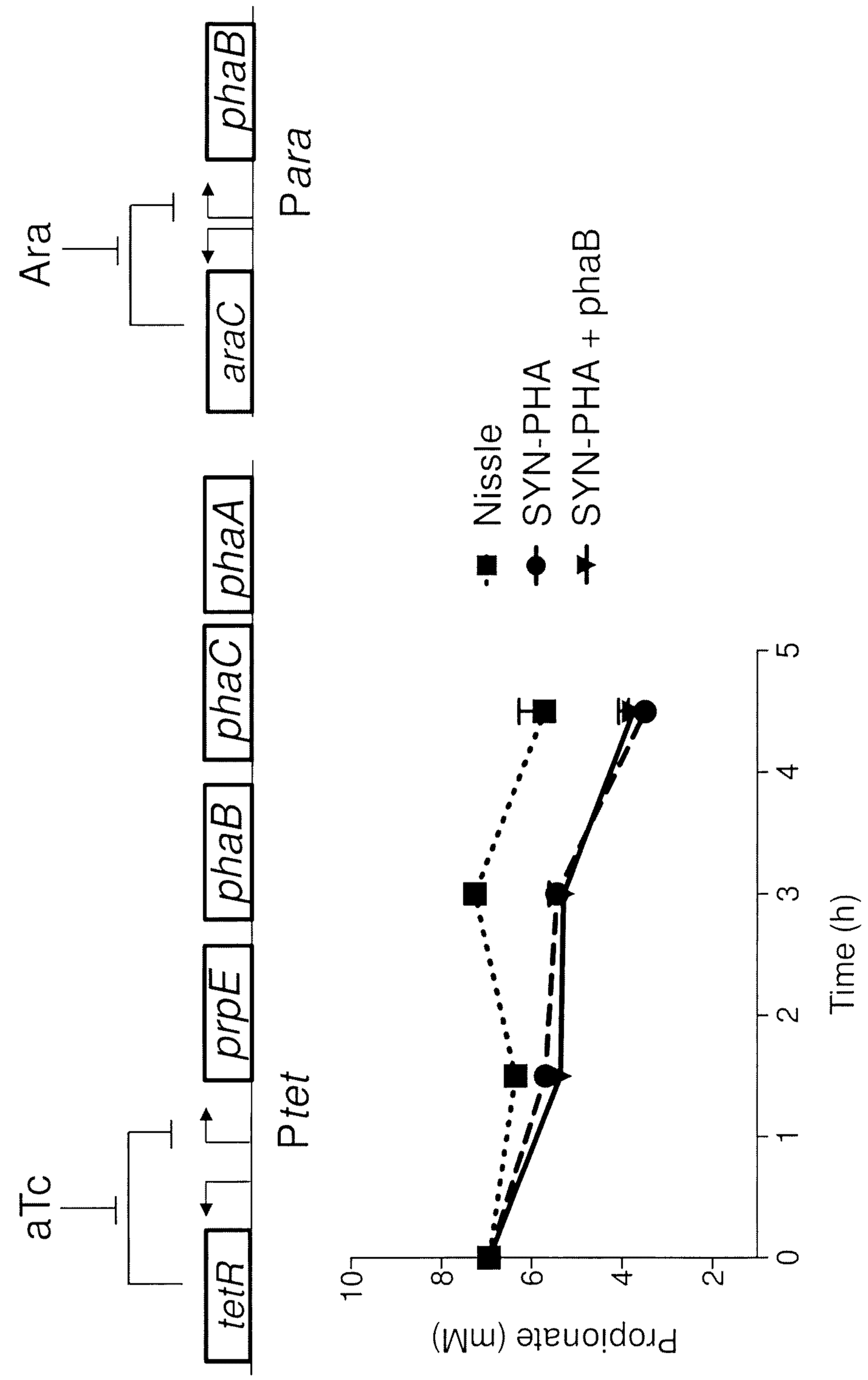
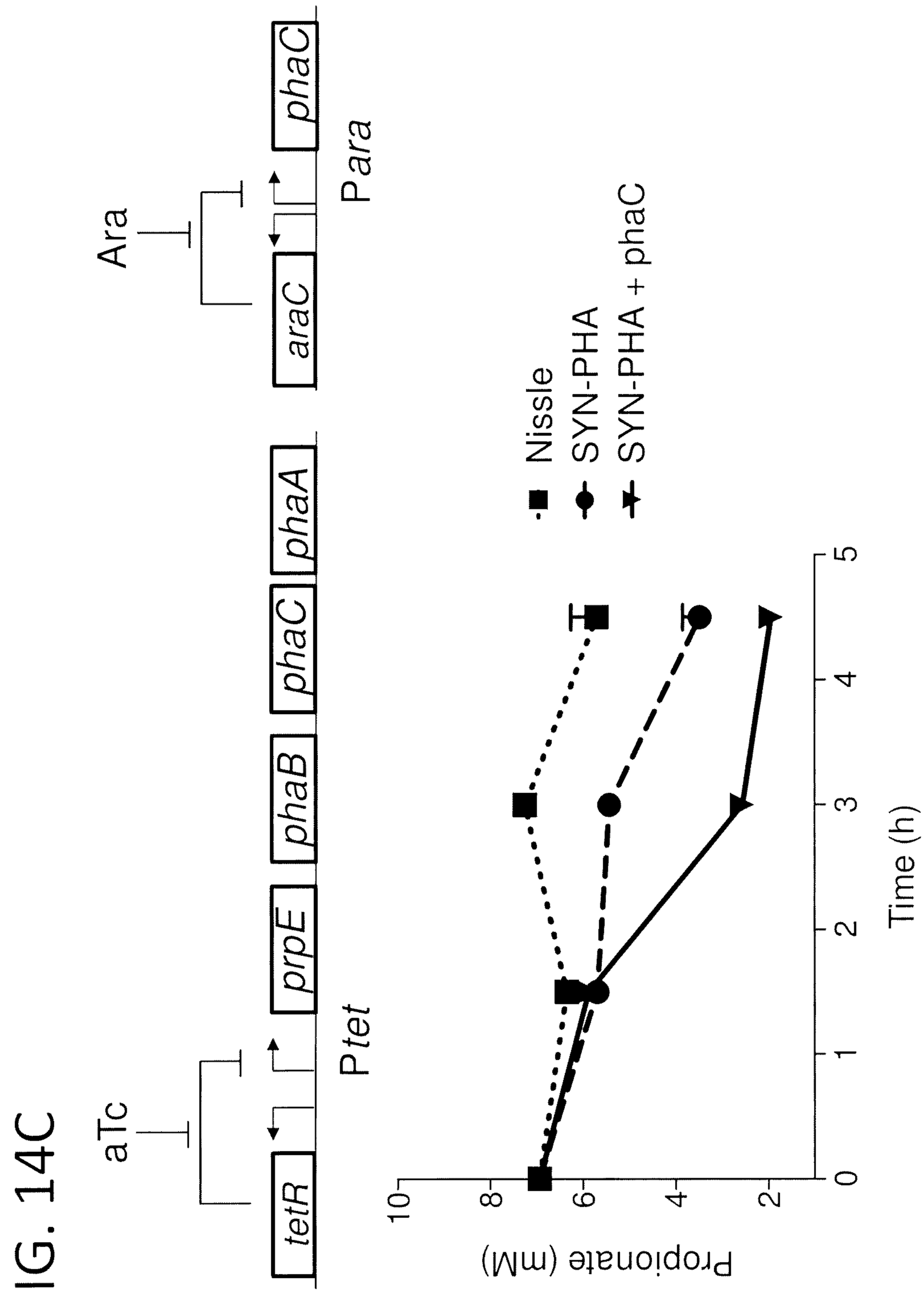

aTc
tetR
Ptet
prpE
phaB
phaC
Ara
araC
Para
phaB
Nissle
SYN-PHA
SYN-PHA + phaB
Propionate (mM)
Time (h)
0
1
2
3
4
5
2
4
6
8
10

FIG. 14C


Ara
araC
Para
phaC
phaA
phaB
aTc
tetR
prpE
Ptet
Nissle
SYN-PHA
SYN-PHA + phaC
Propionate (mM)
Time (h)
0
1
2
3
4
5
2
4
6
8
10

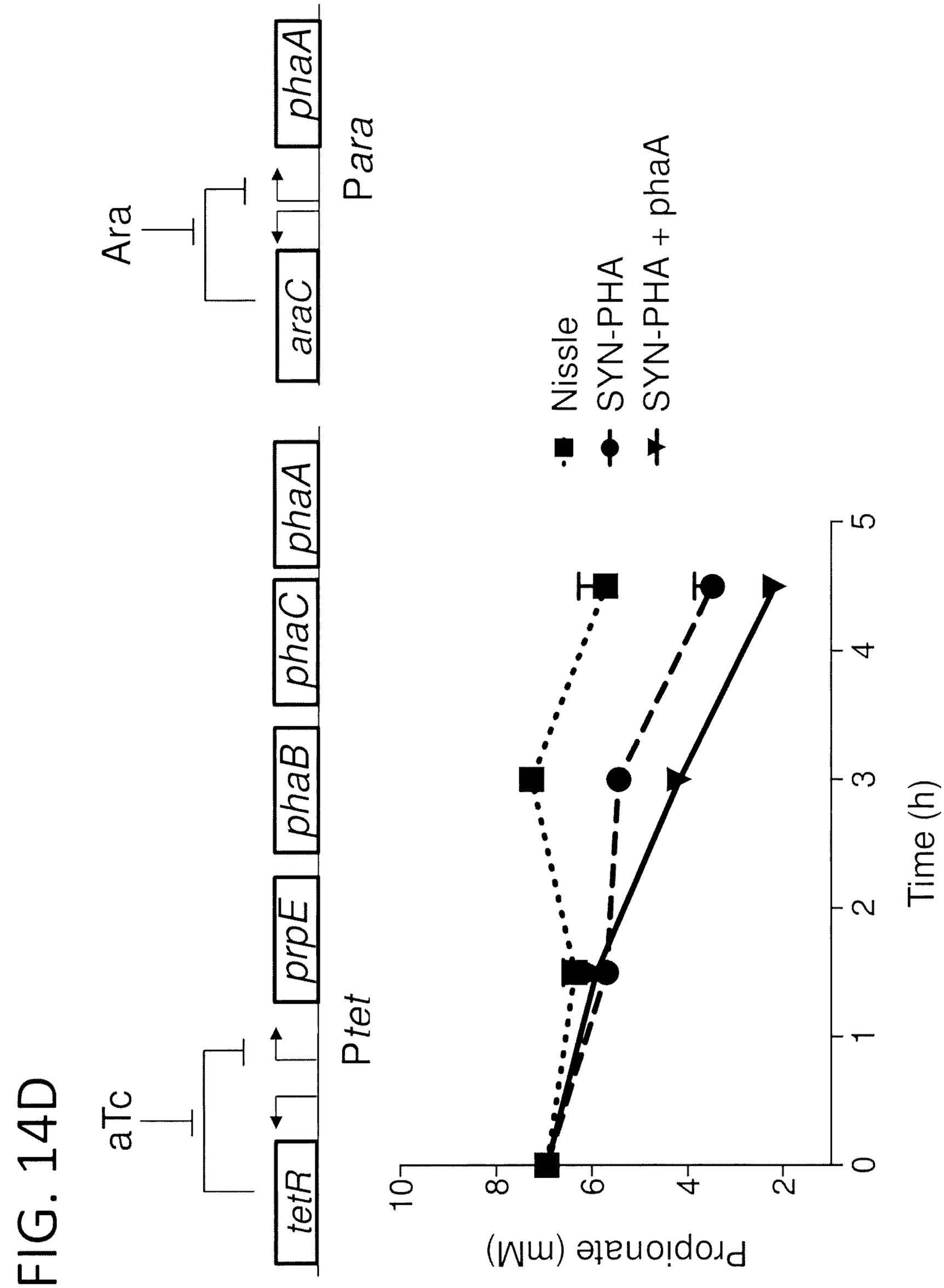
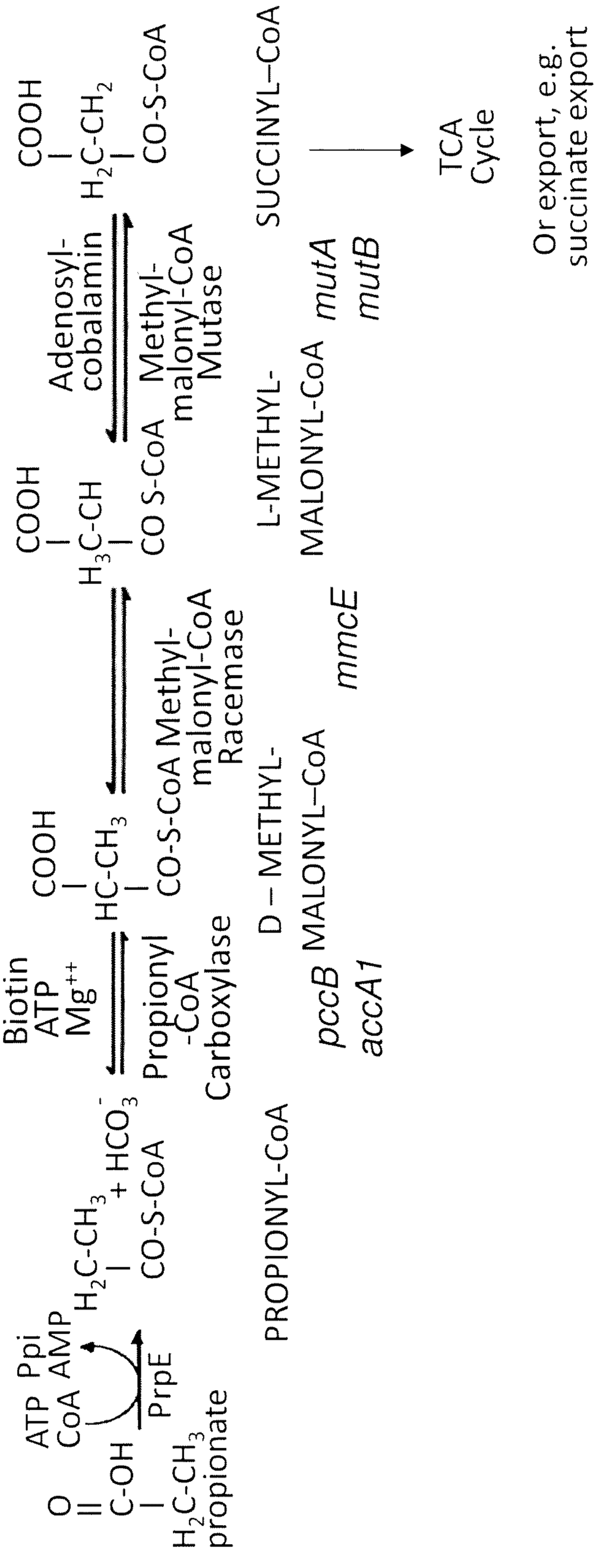

Ara
araC
Para
phaC
phaA
phaB
prpE
tetR
Ptet
aTc
Nissle
SYN-PHA
SYN-PHA + phaA
Propionate (mM)
Time (h)
0
1
2
3
4
5
2
4
6
8
10

FIG. 14D

FIG. 15A


PROPIONYL-CoA
Propionyl -CoA Carboxylase
Biotin
ATP
Mg^{++}
H_2C-CH_3
CO-S-CoA
+ HCO_3^-
D – METHYL-MALONYL-CoA
mmcE
Methyl-malonyl-CoA Racemase
L-METHYL-MALONYL-CoA
Adenosyl-cobalamin
Methyl-malonyl-CoA Mutase
mutA
mutB
SUCCINYL–CoA
TCA Cycle
Or export, e.g. succinate export
pccB MALONYL–CoA
accA1
ATP Ppi
CoA AMP
PrpE
O = C-OH
H_2C-CH_3
propionate

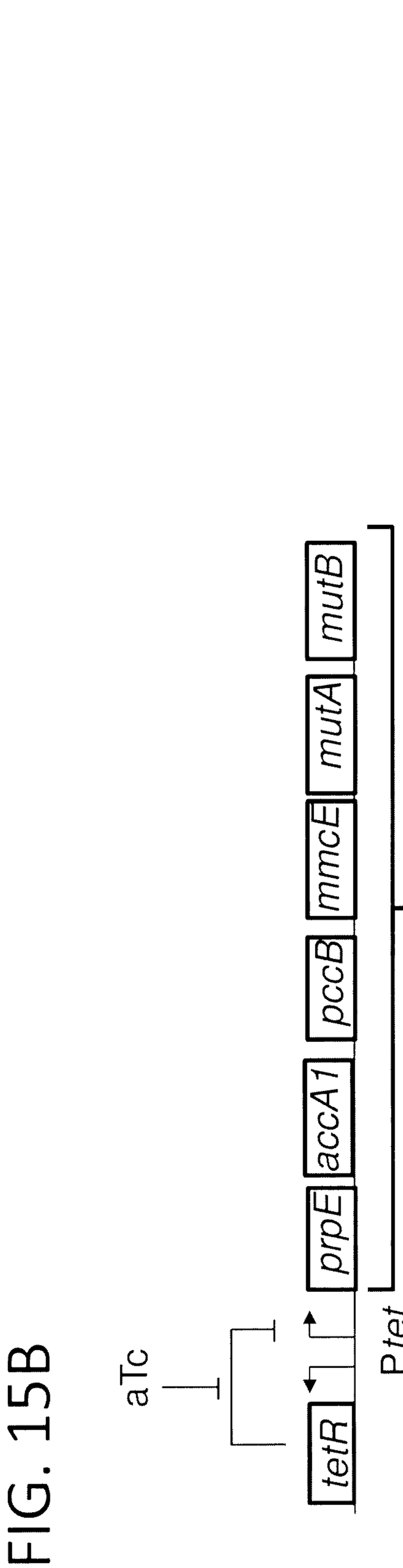


FIG. 15B

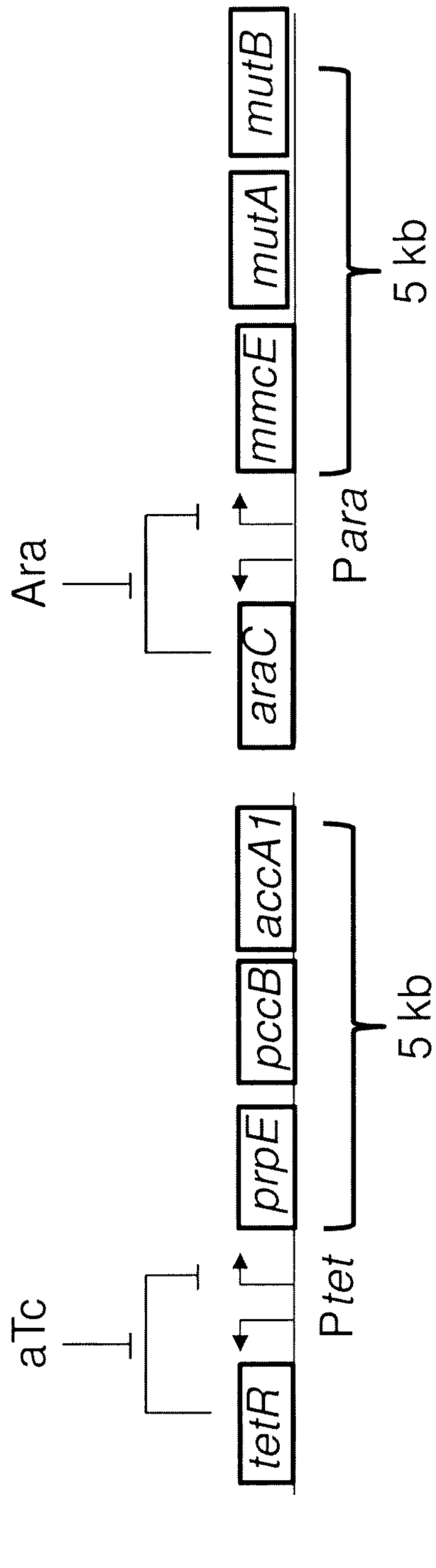


FIG. 15C

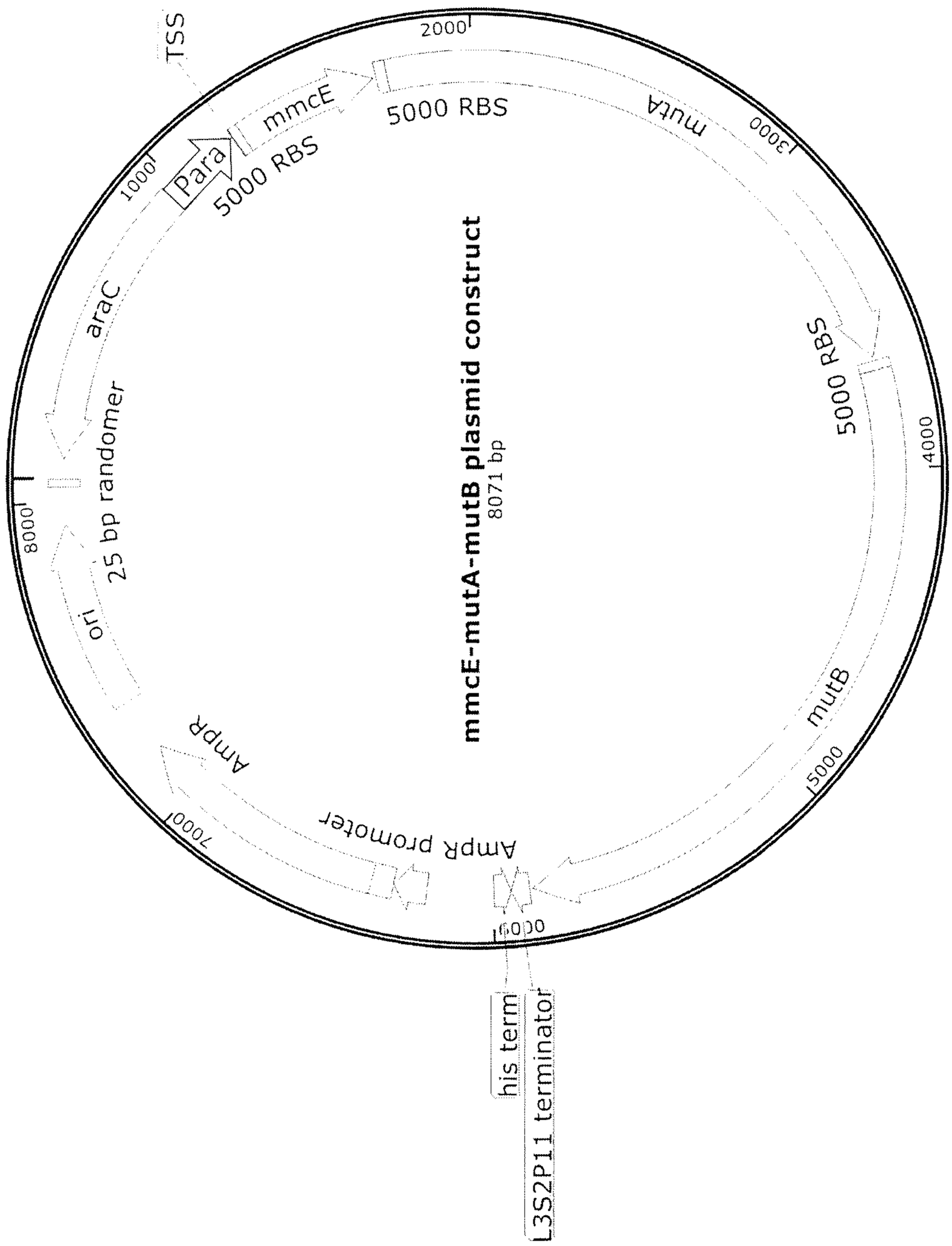

TSS
2000
mmcE
5000 RBS
mutA
3000
Para
araC
1000
5000 RBS
5000 RBS
4000
8000
25 bp randomer
ori
mmcE-mutA-mutB plasmid construct
8071 bp
AmpR
AmpR promoter
7000
mutB
6000
his term
L3S2P11 terminator
5000

FIG. 16A

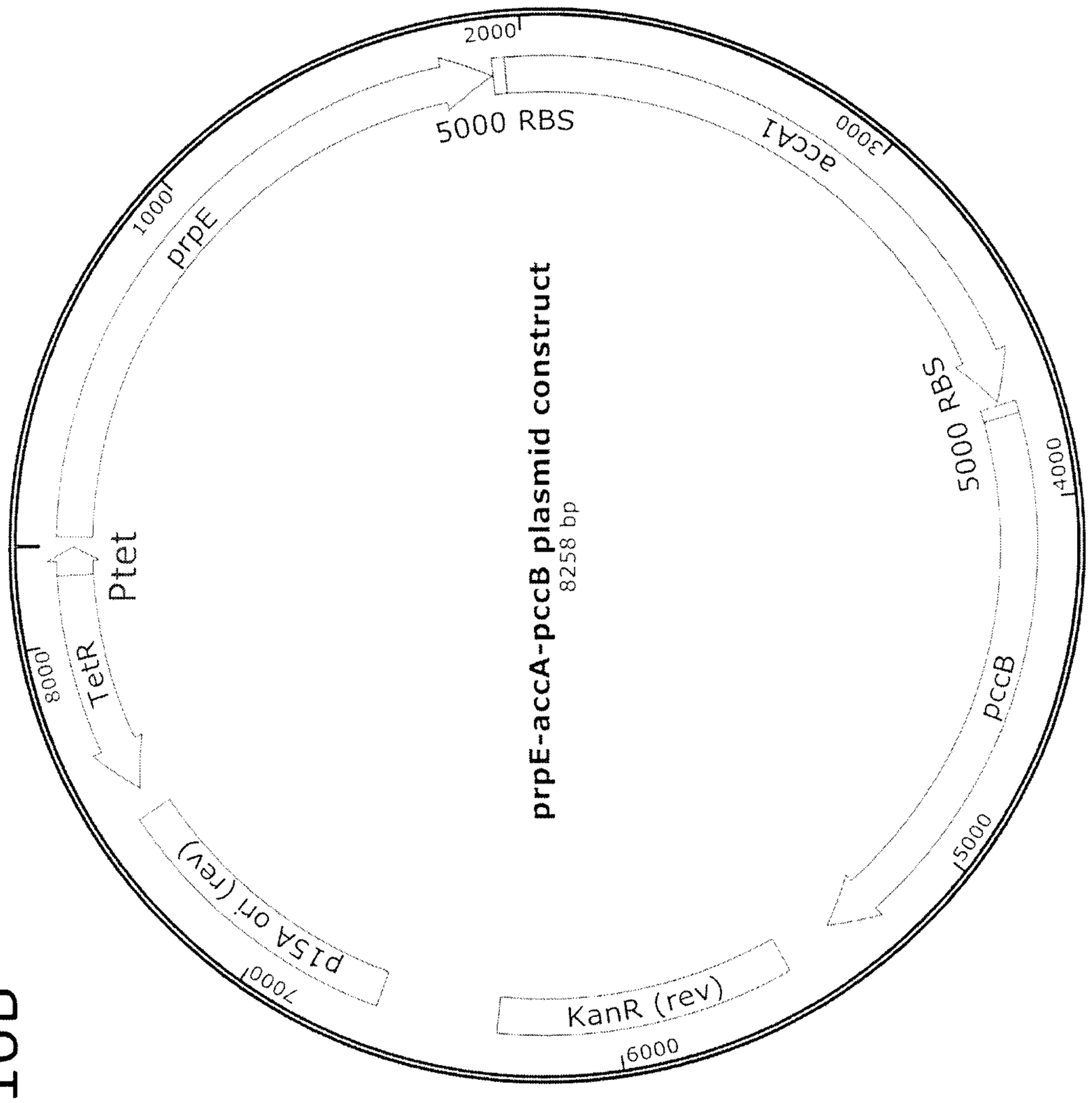

prpE-accA-pccB plasmid construct
8258 bp
1000
2000
3000
4000
5000
6000
7000
8000
prpE
5000 RBS
accA1
5000 RBS
pccB
KanR (rev)
P15A ori (rev)
TetR
Ptet

FIG. 16B

FIG. 17A

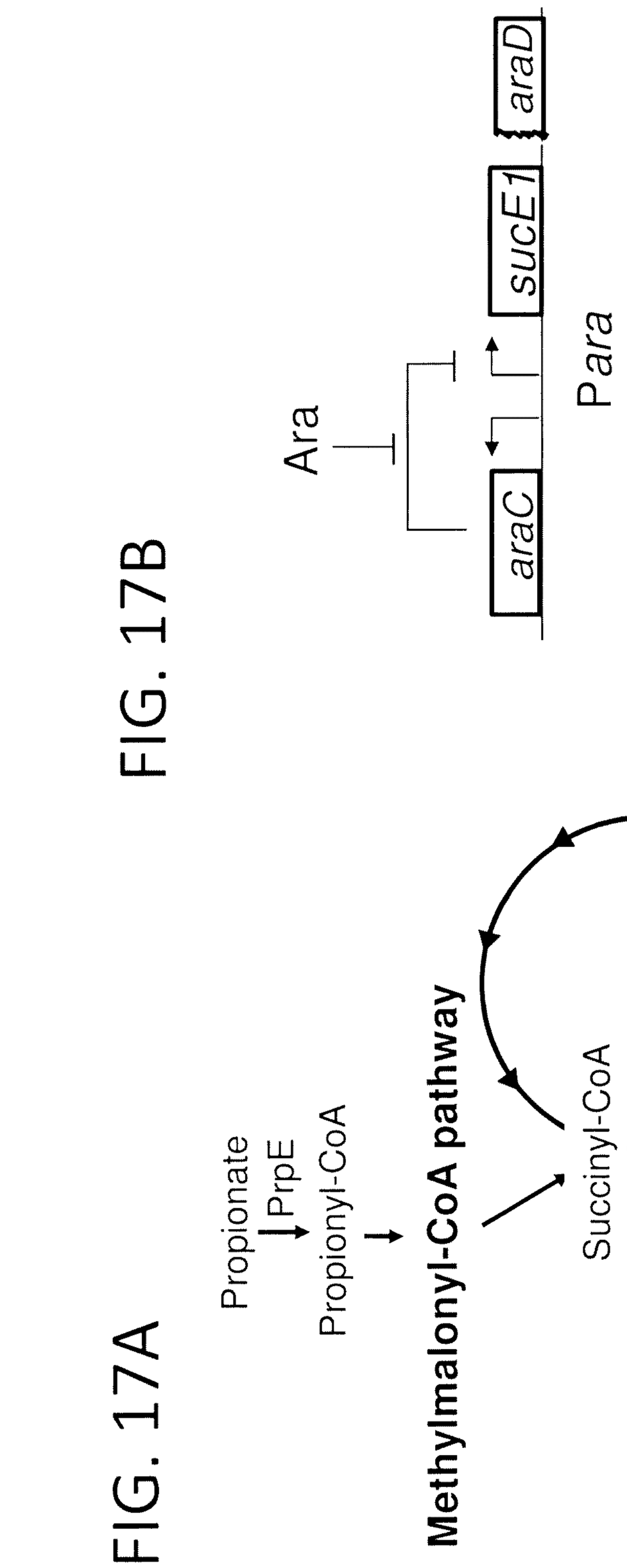


FIG. 17B

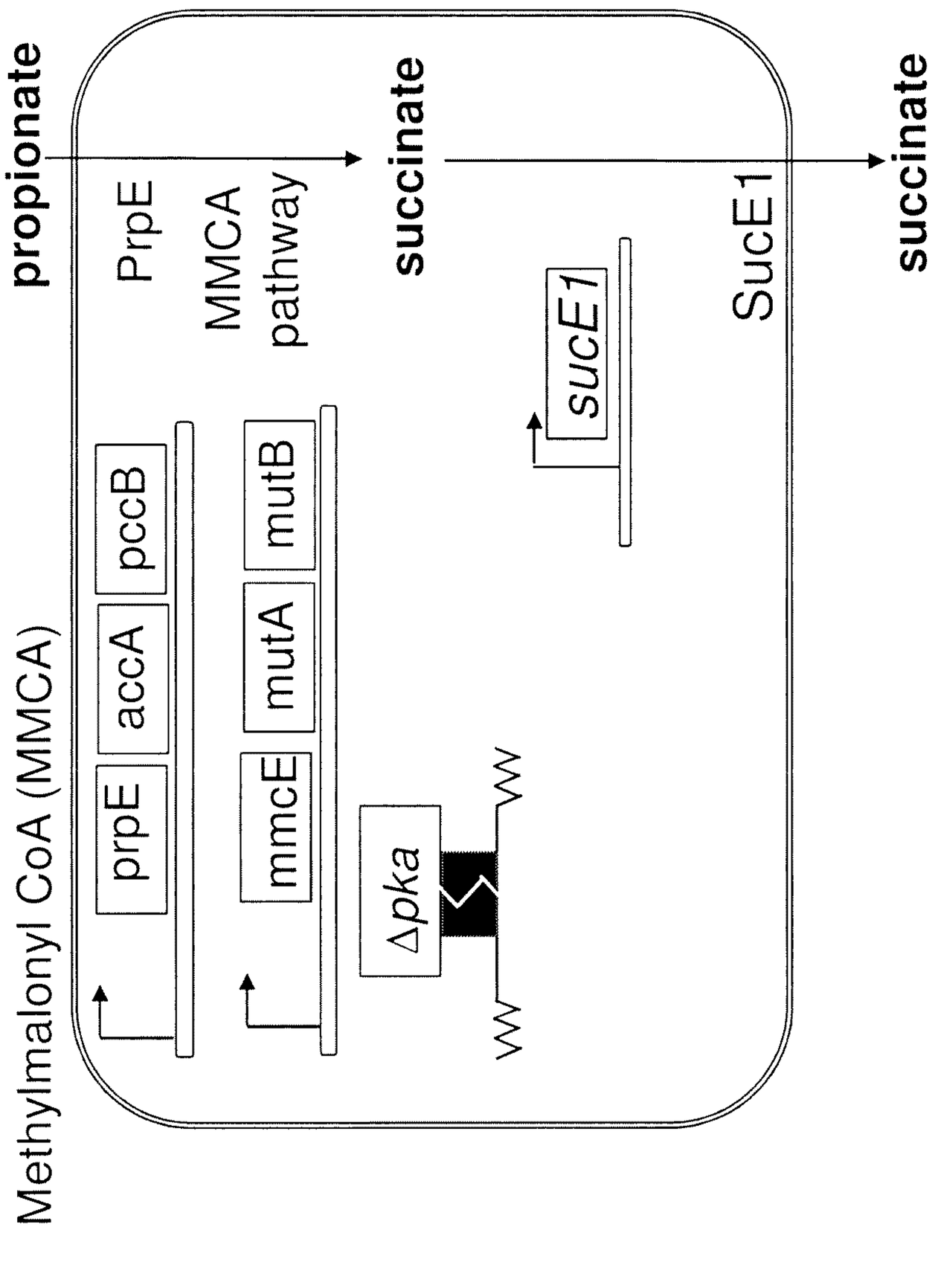

propionate
PrpE
MMCA pathway
succinate
SucE1
succinate
sucE1
pccB
mutB
Methylmalonyl CoA (MMCA)
prpE
accA
mmcE
mutA
Δpka

FIG. 17C

FIG. 17D

Para-sucE integration sequence
3614 bp

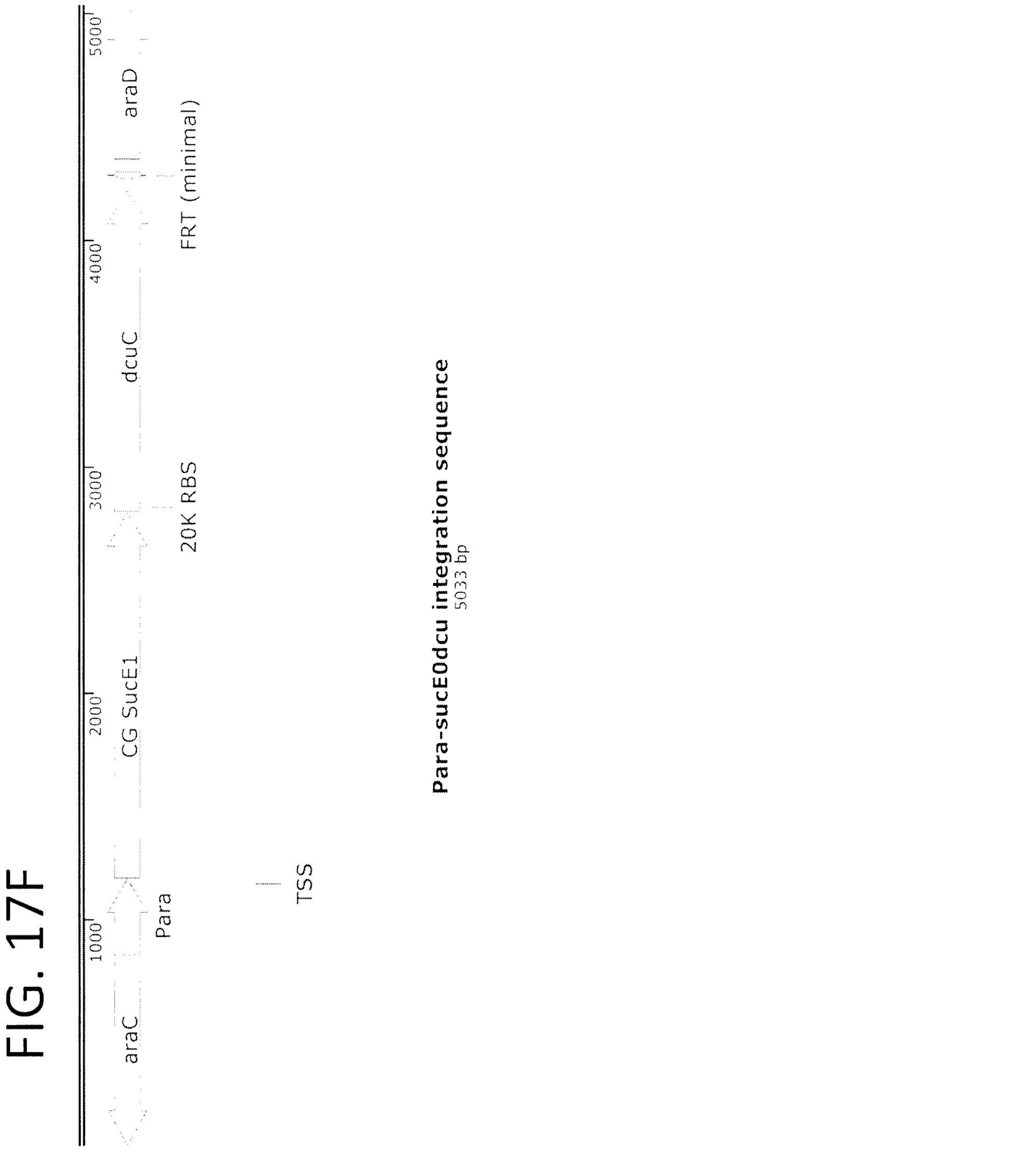
FIG. 17E

Para-dcuC integration sequence
3388 bp

REPLACEMENT SHEET

AMENDED SHEET

FIG. 17F


1000
2000
3000
4000
5000
araC
Para
TSS
CG SucE1
dcuC
20K RBS
FRT (minimal)
araD
Para-sucE0dcu integration sequence
5033 bp

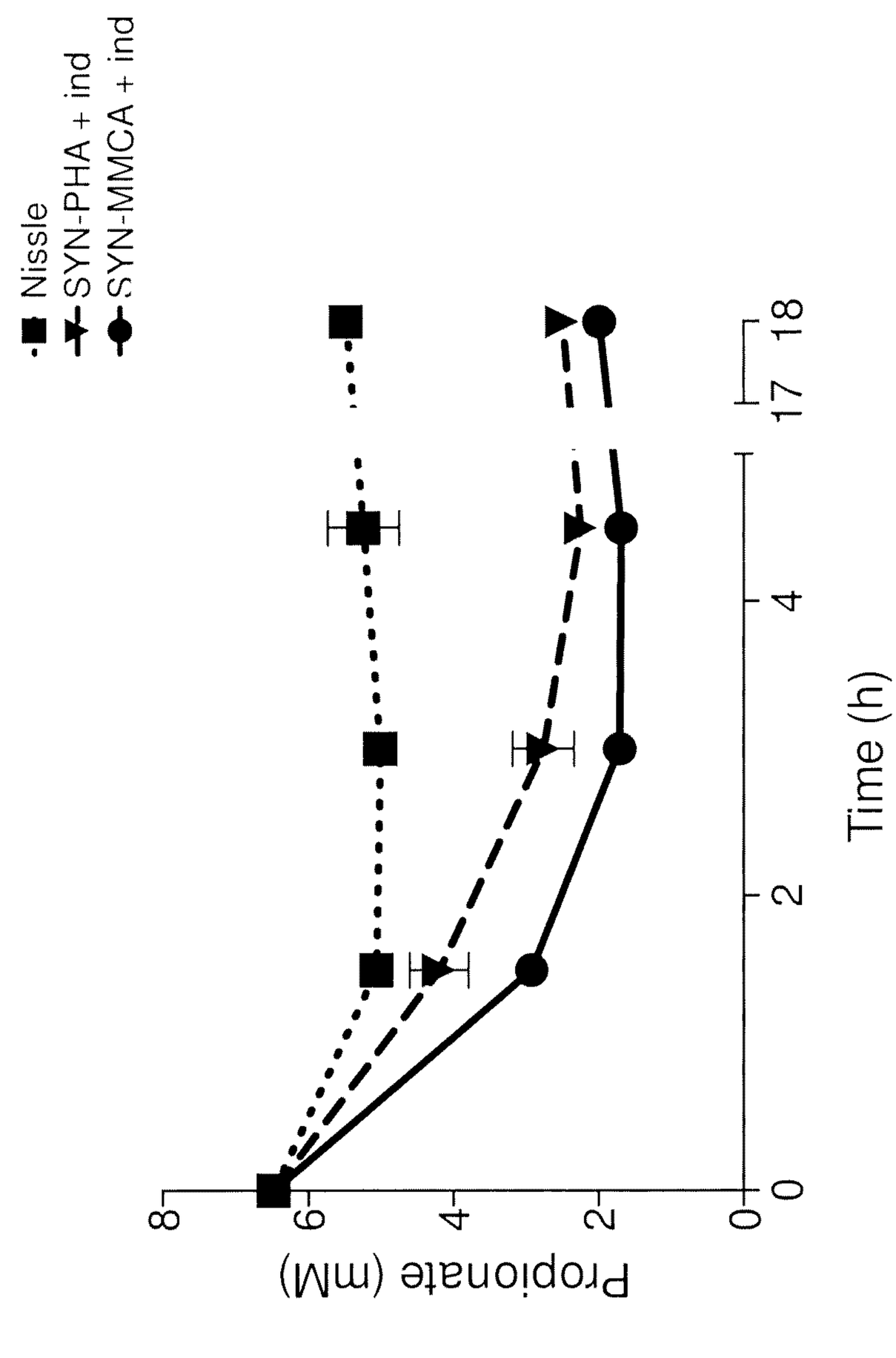

Nissle
SYN-PHA + ind
SYN-MMCA + in
Propionate (mM)
0
2
4
6
8
Time (h)
0
2
4
17
18

FIG. 18

FIG. 19

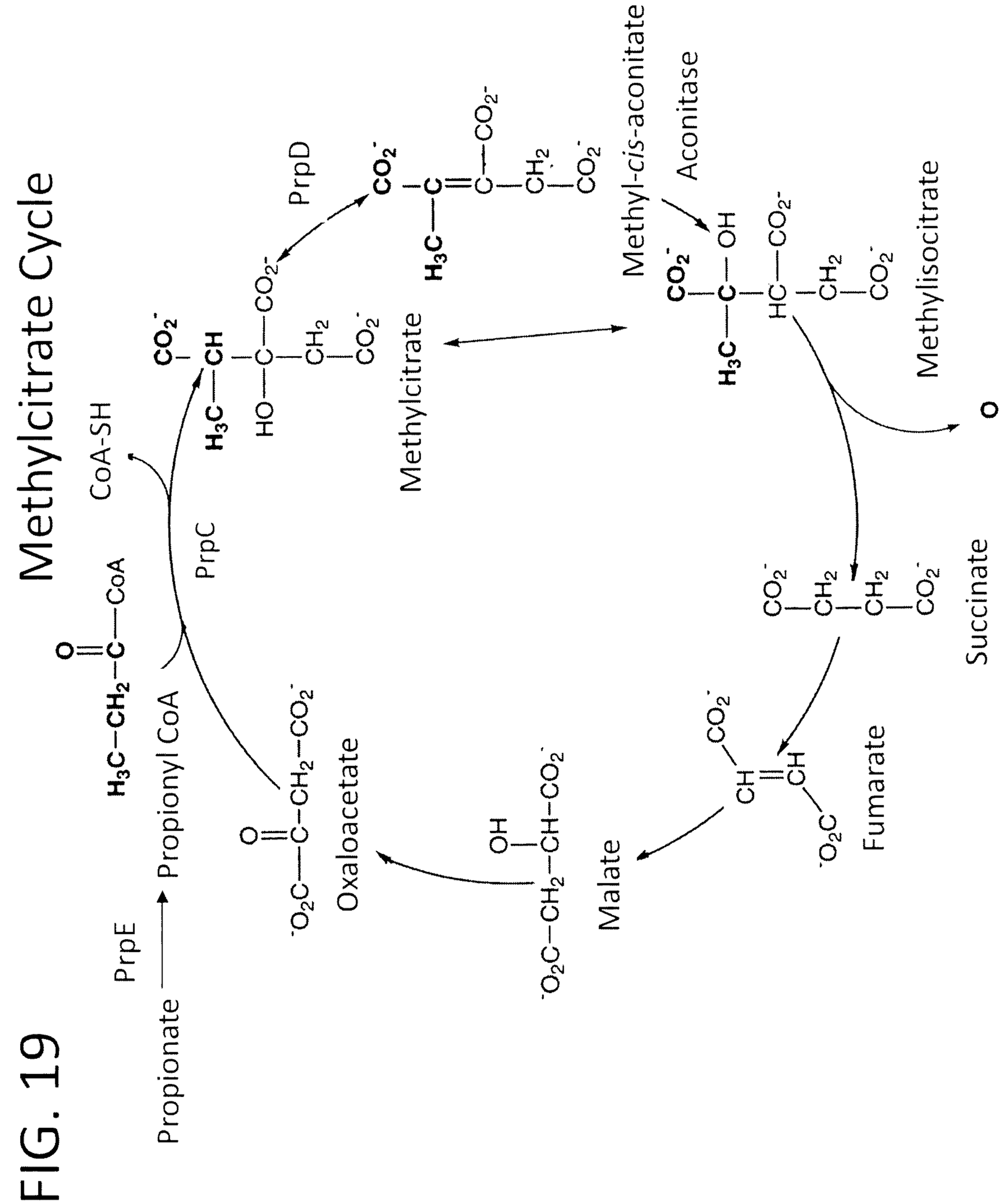

Methylcitrate Cycle
CoA-SH
PrpC
PrpD
Methylcitrate
Methyl-cis-aconitate
Aconitase
Methylisocitrate
PrpE
Propionyl CoA
Propionate
Pyruvate
Oxaloacetate
Malate
Fumarate
Succinate

FIG. 20A

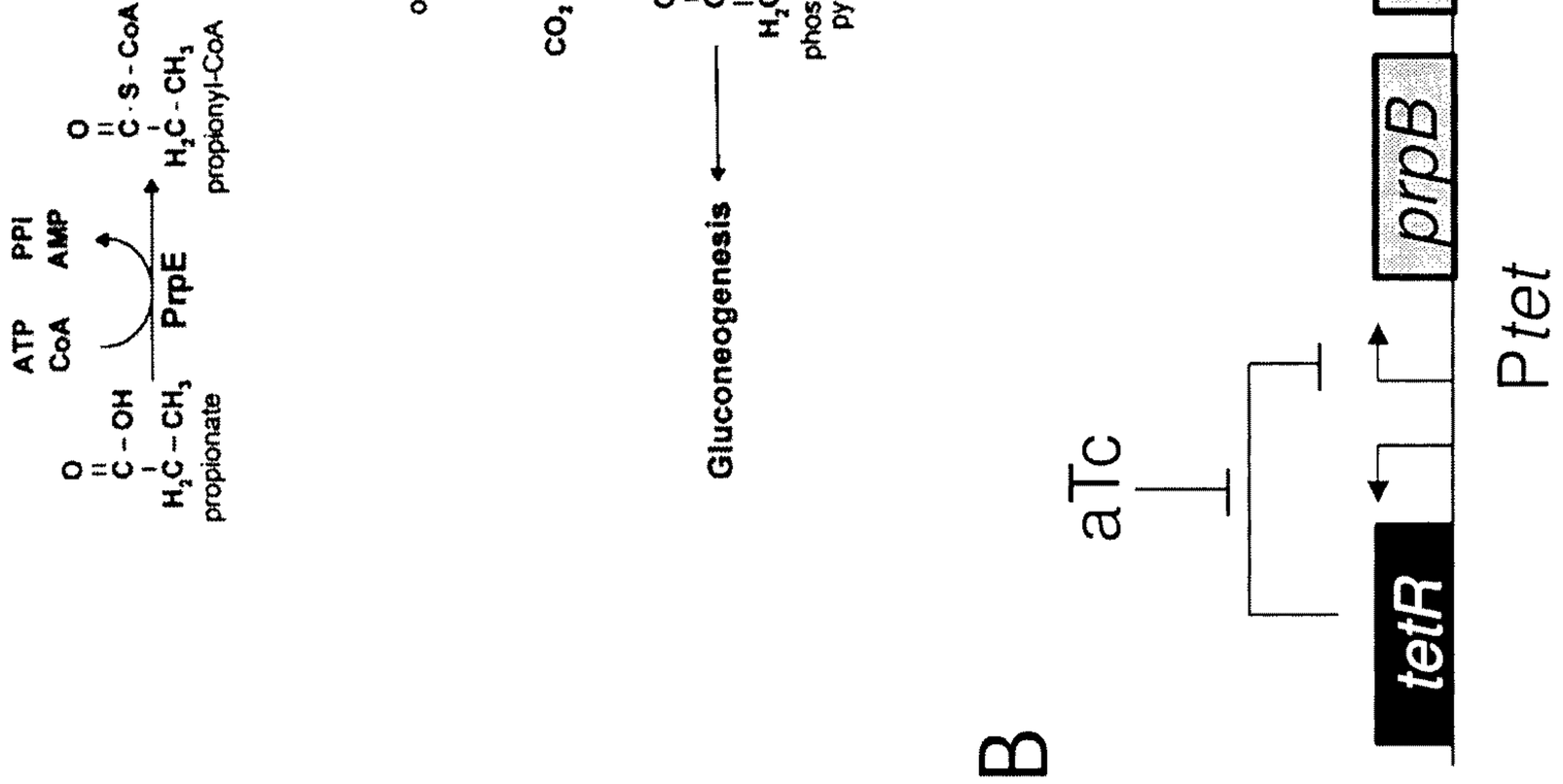


FIG. 20B

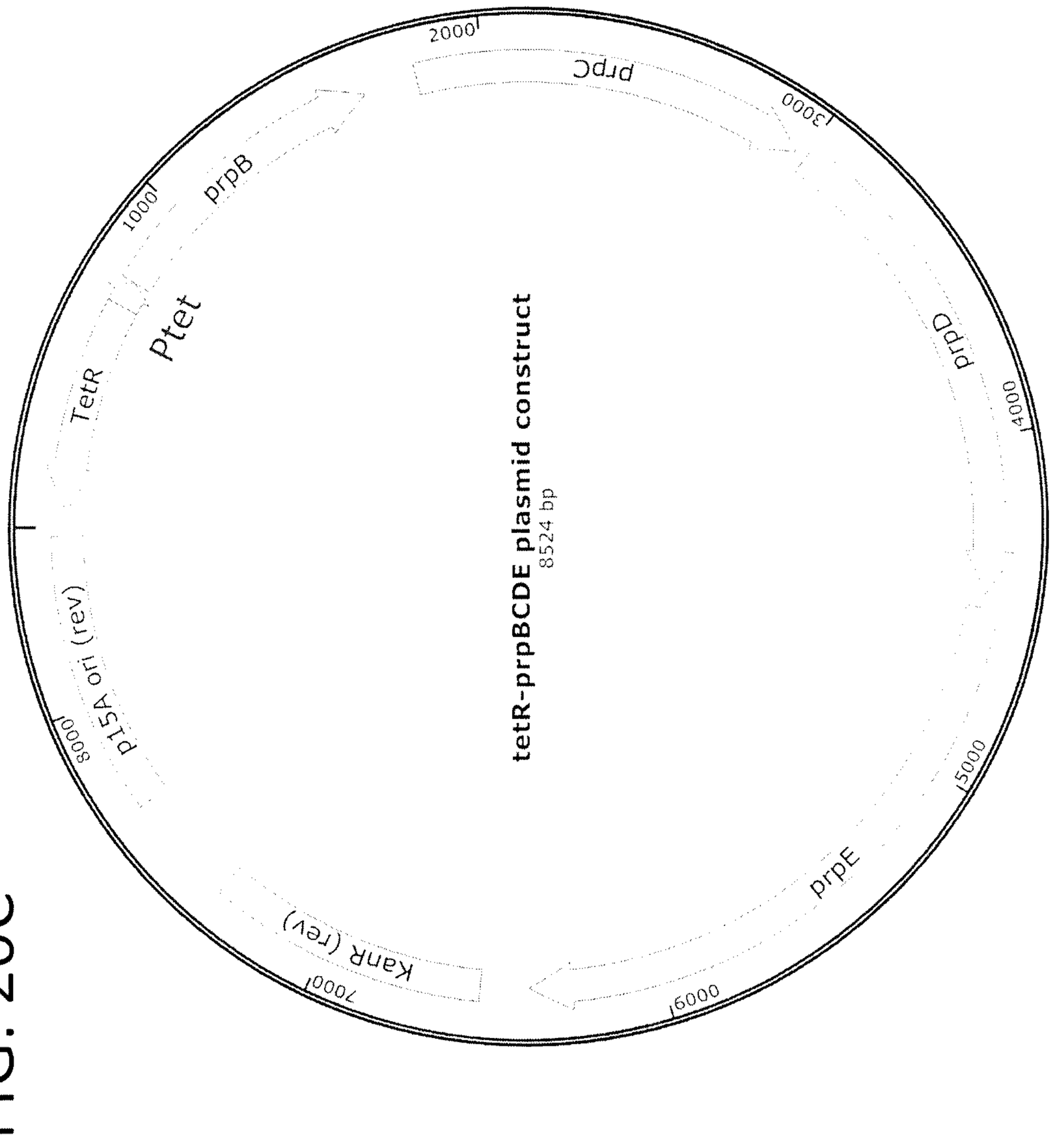

tetR-prpBCDE plasmid construct
8524 bp
TetR
Ptet
prpB
prpC
prpD
prpE
Kanr (rev)
p15A ori (rev)
1000
2000
3000
4000
5000
6000
7000
8000

FIG. 20C

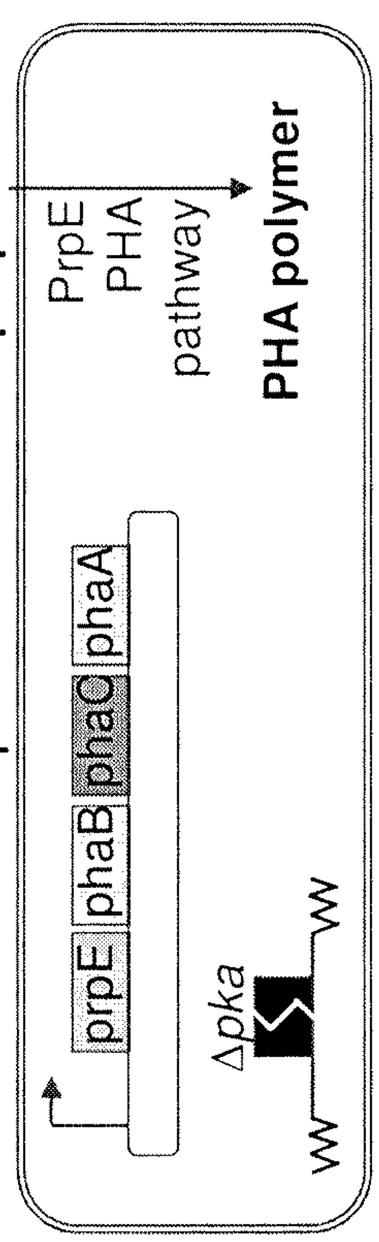


FIG. 21A . PHA increased phaC

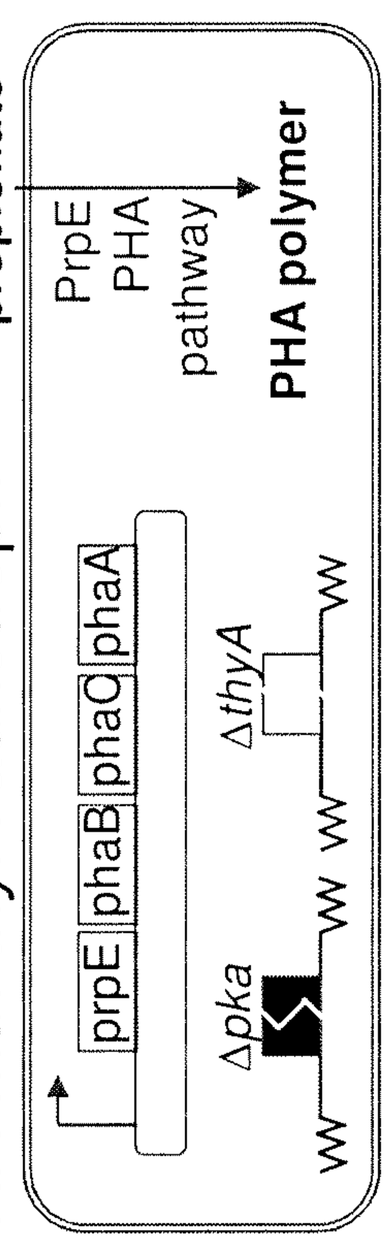


FIG. 21B PHA with *thyA* auxotroph

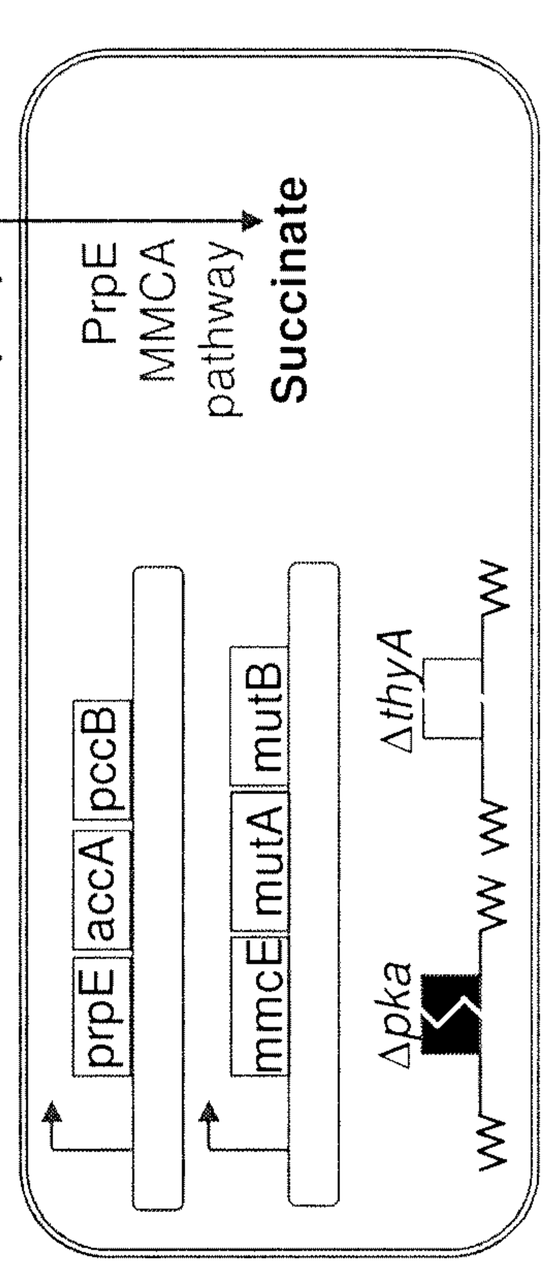


FIG. 21C MMCA with *thyA* auxotroph

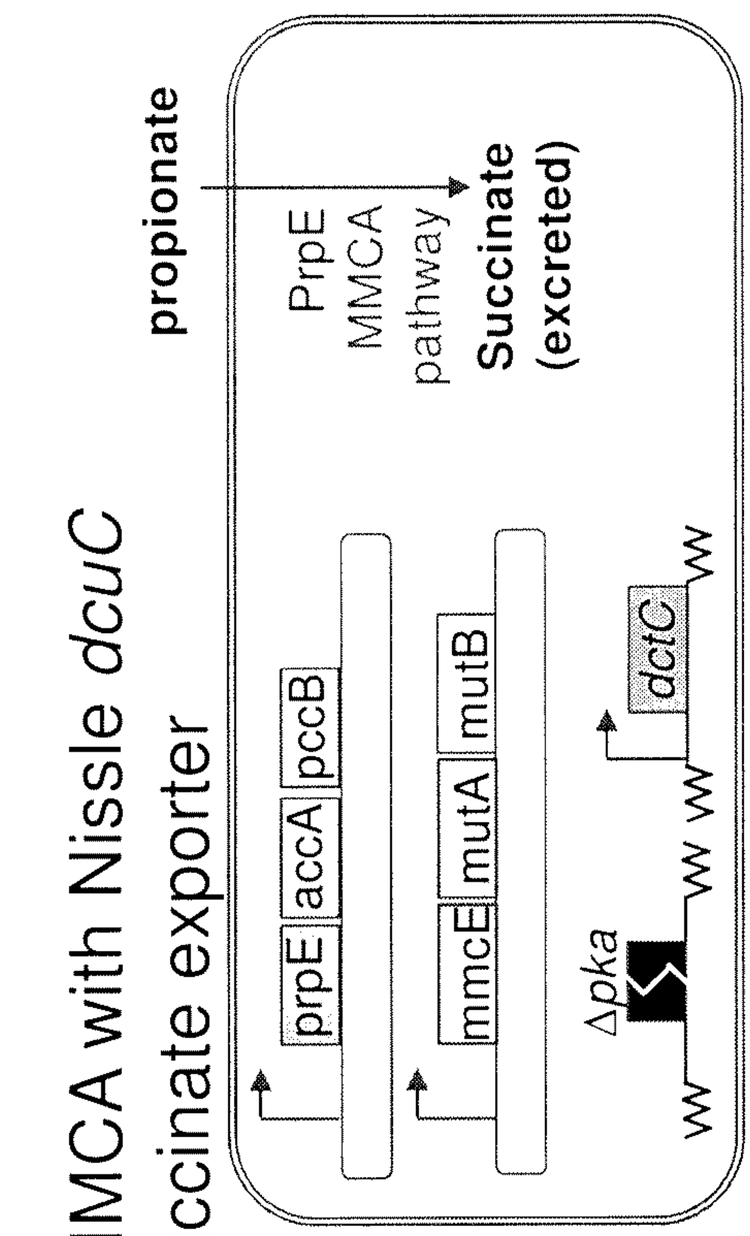


FIG. 21D

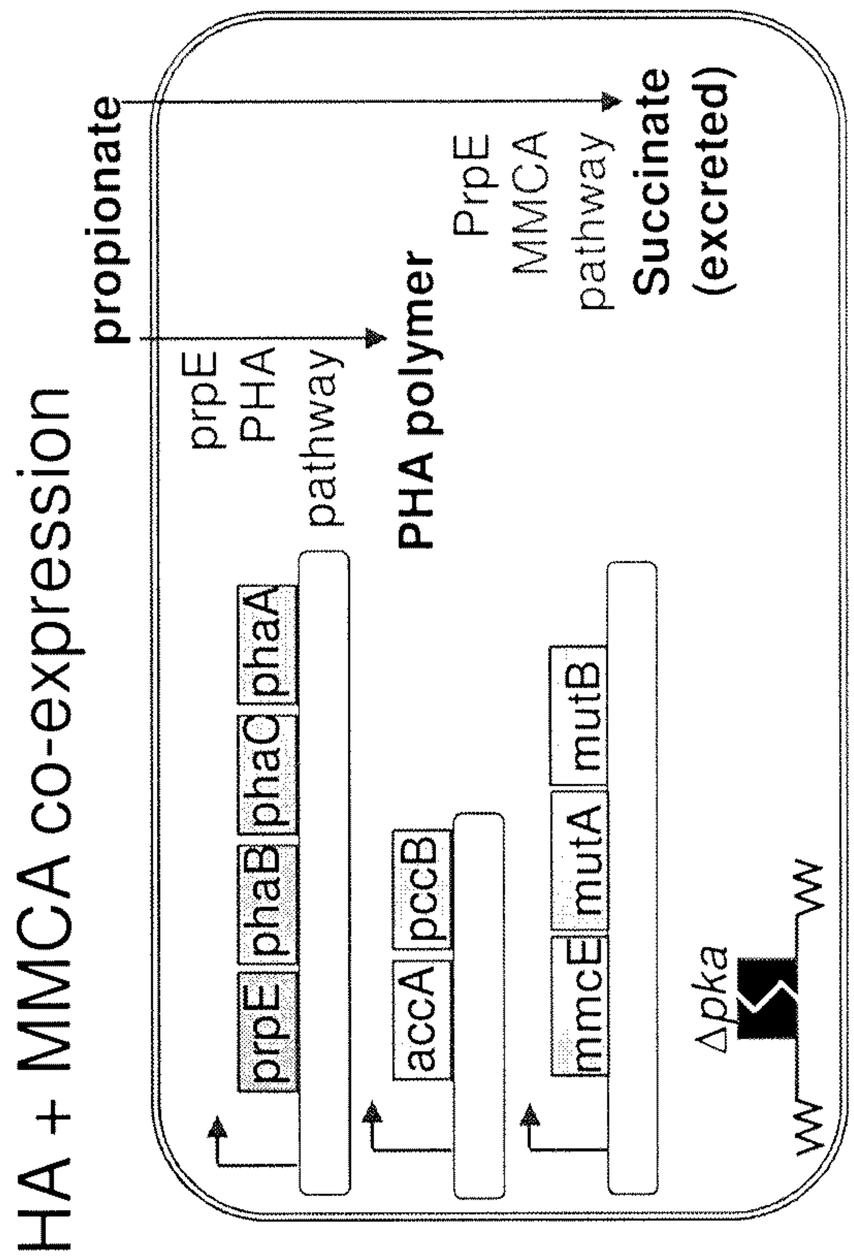


FIG. 21E

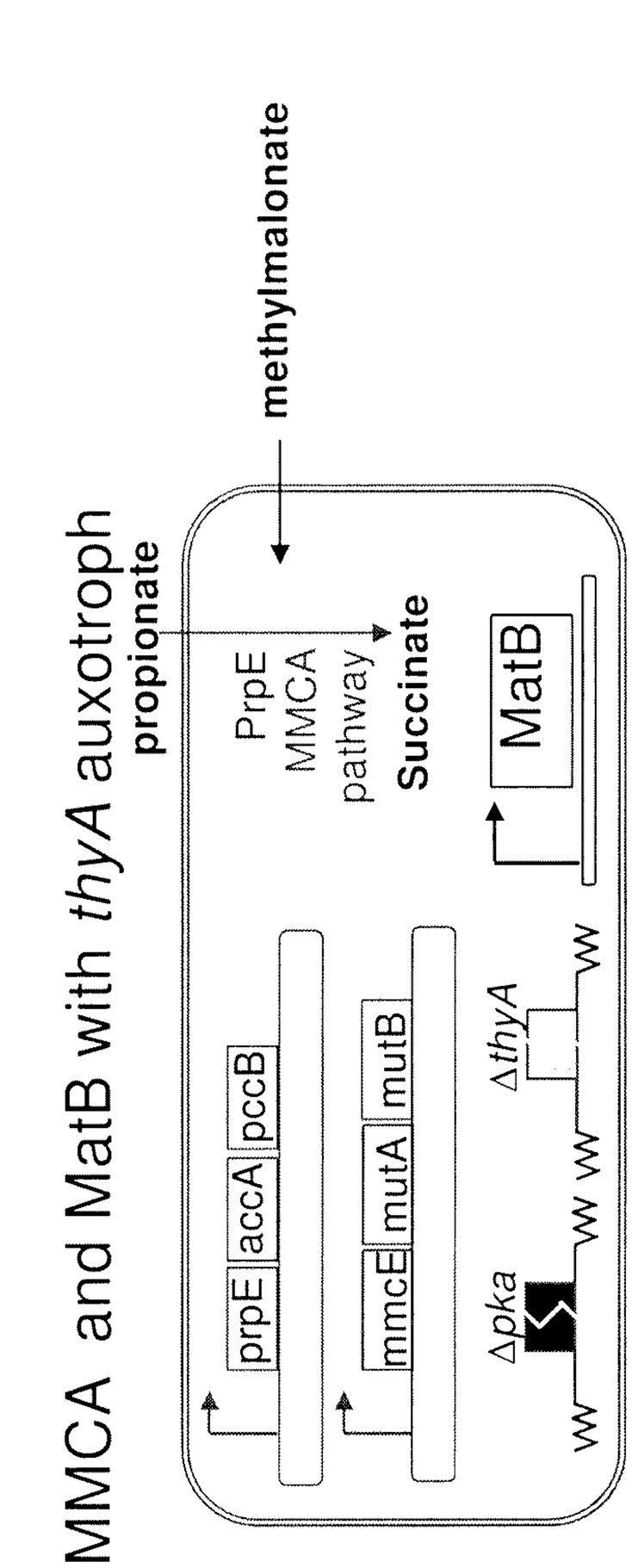


FIG. 21F

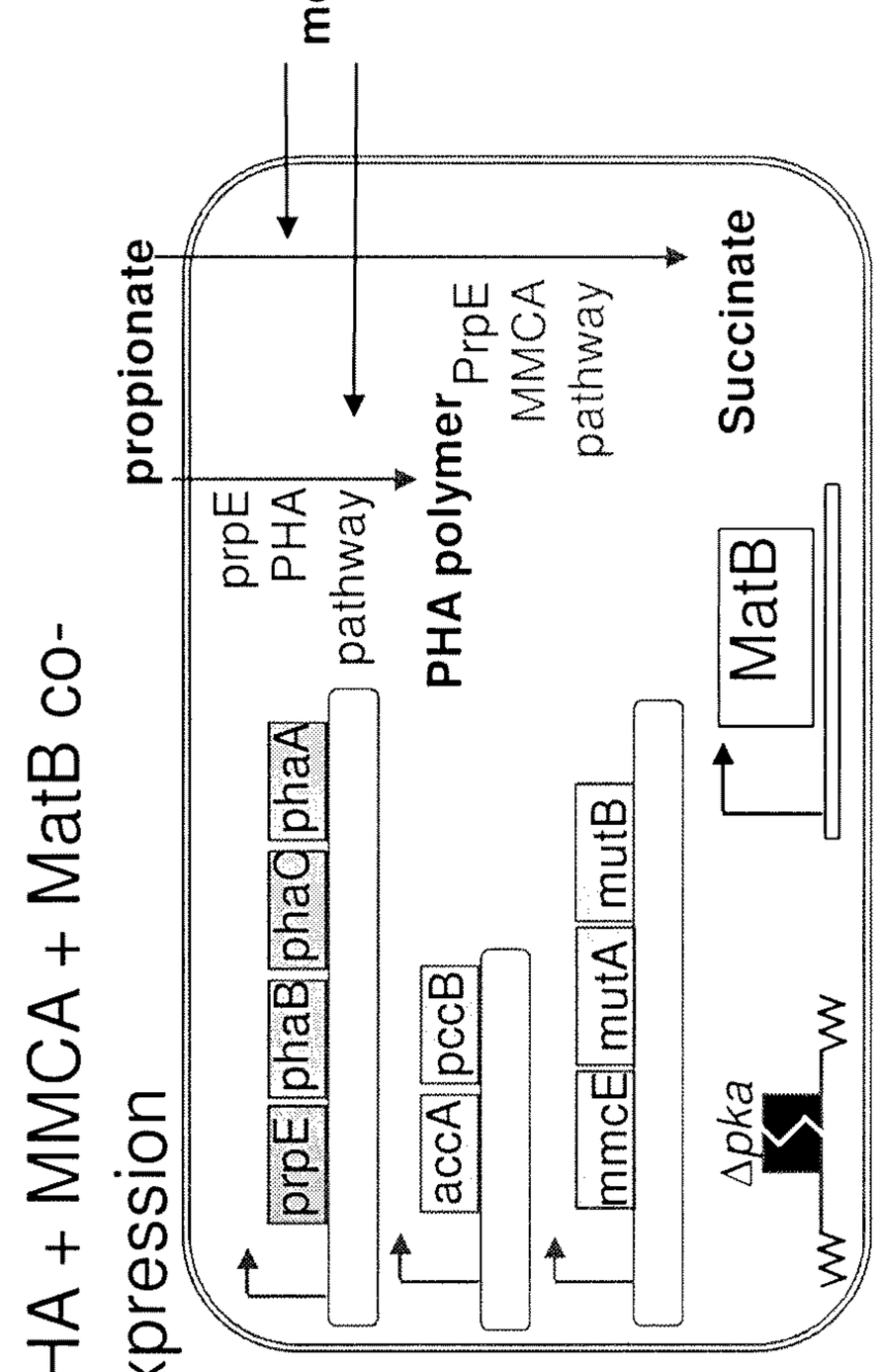
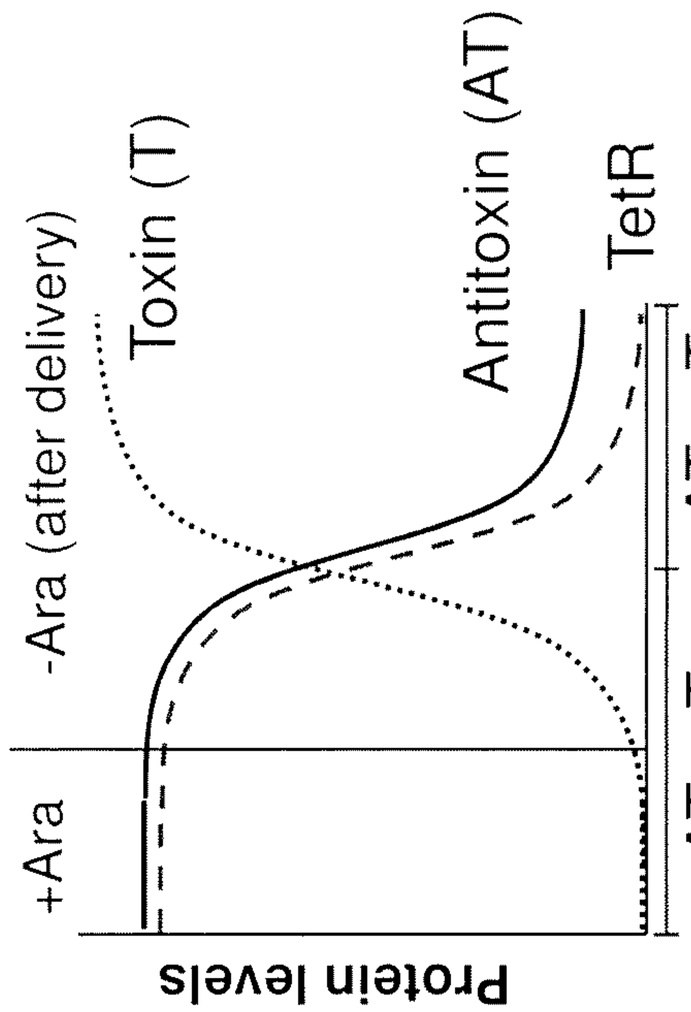
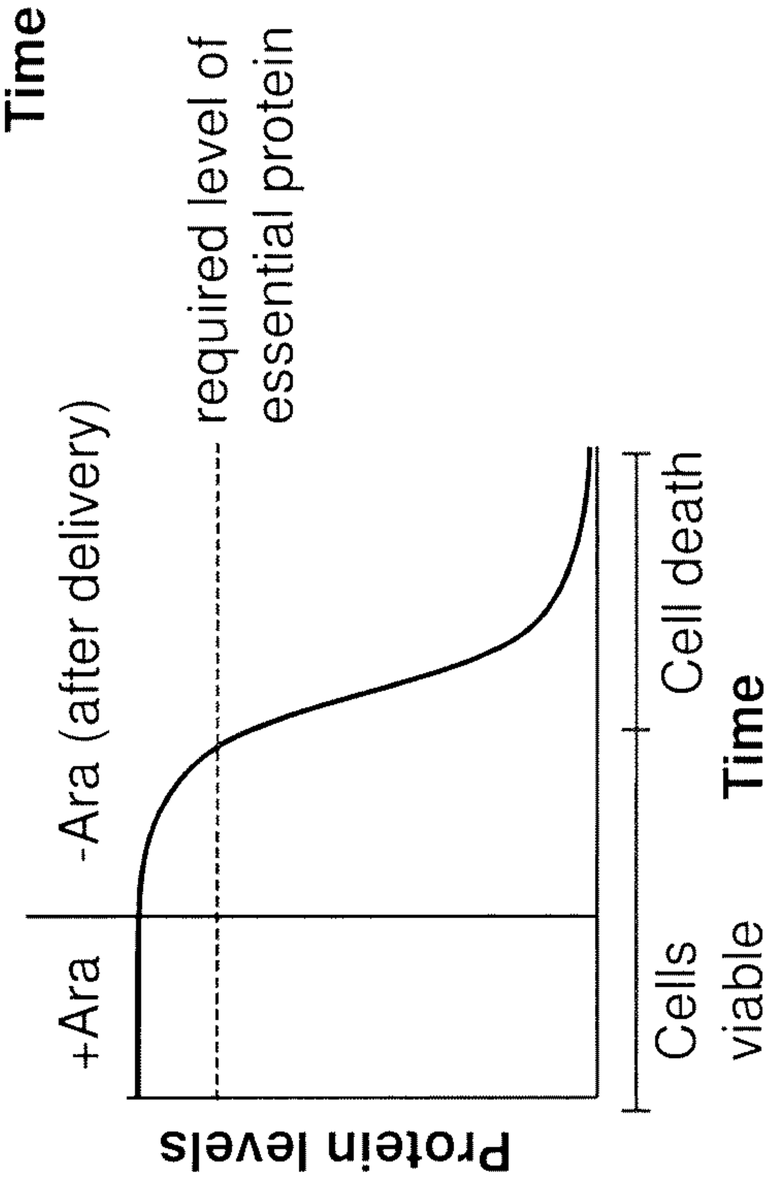
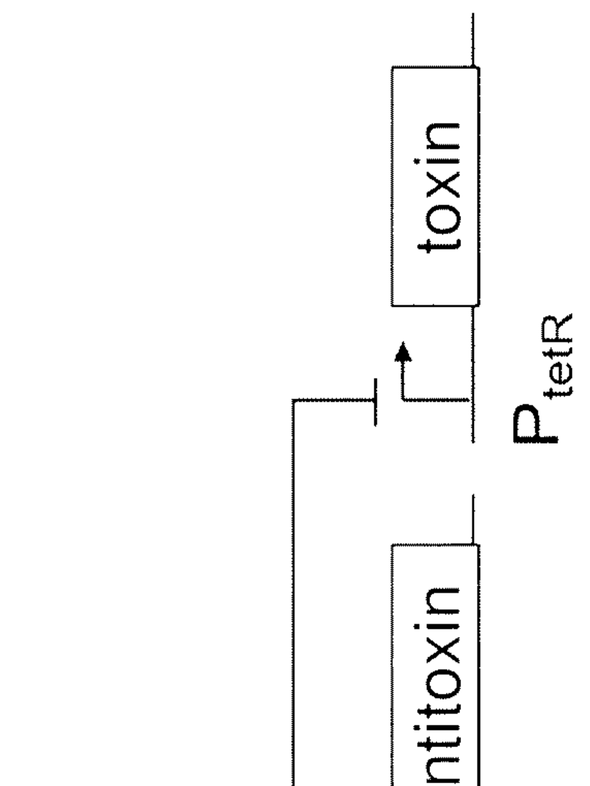


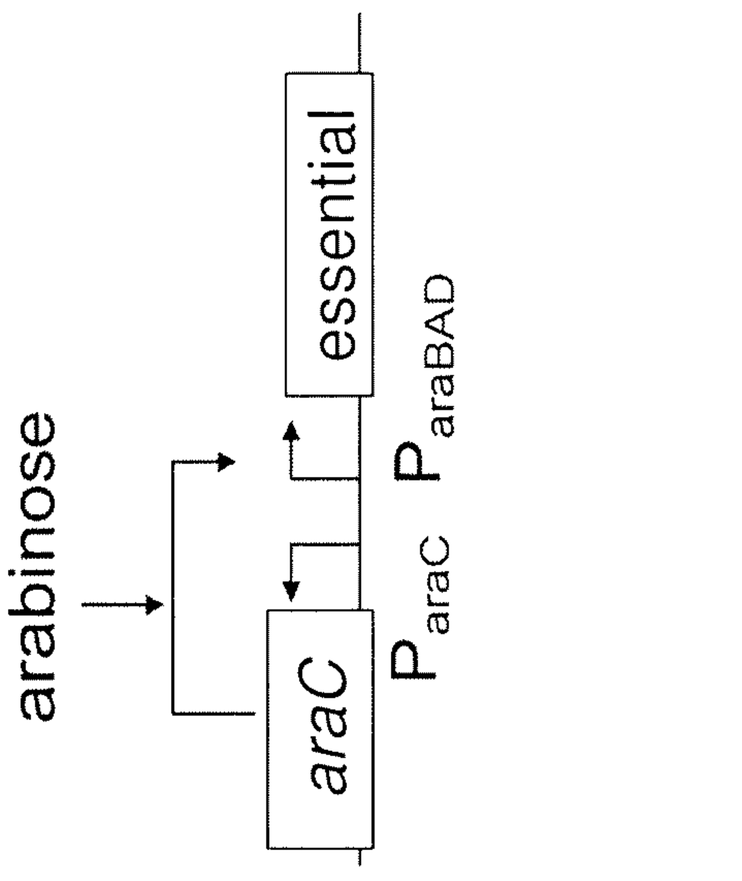
FIG. 21G

FIG. 22


Protein levels
+Ara
-Ara (after delivery)
Toxin (T)
Antitoxin (AT)
TetR
AT > T
AT < T
Cells viable
Cell death
Time


Protein levels
+Ara
-Ara (after delivery)
required level of essential protein
Cells viable
Cell death
Time


Toxin-based
arabinose
araC
toxin
tetR
antitoxin
PtetR
ParaC ParaBAD


Essential gene-based
arabinose
araC
essential
ParaC ParaBAD

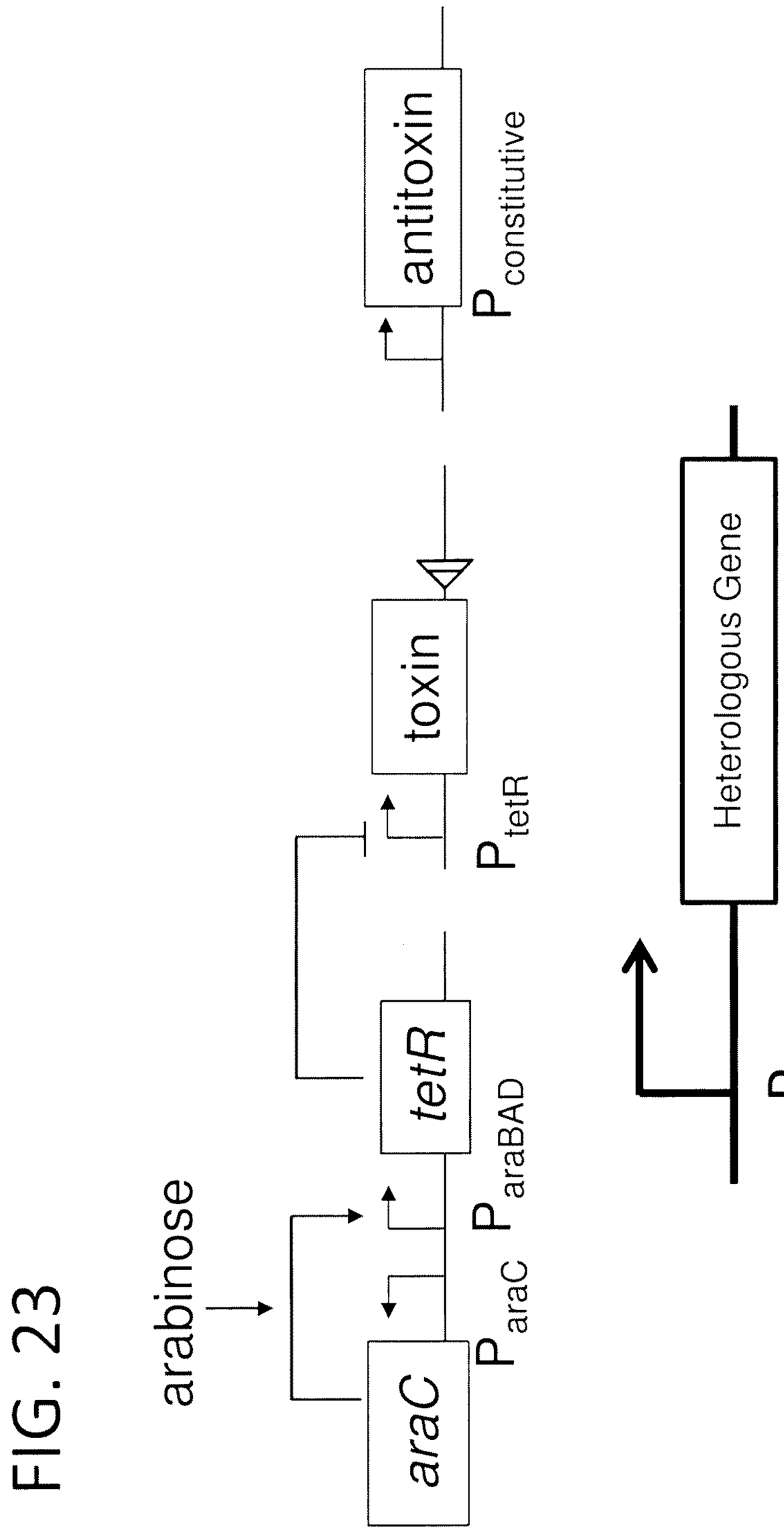

arabinose
araC
P_{araC}
tetR
P_{araBAD}
toxin
P_{tetR}
antitoxin
$P_{constitutive}$
Heterologous Gene
P_i

FIG. 23

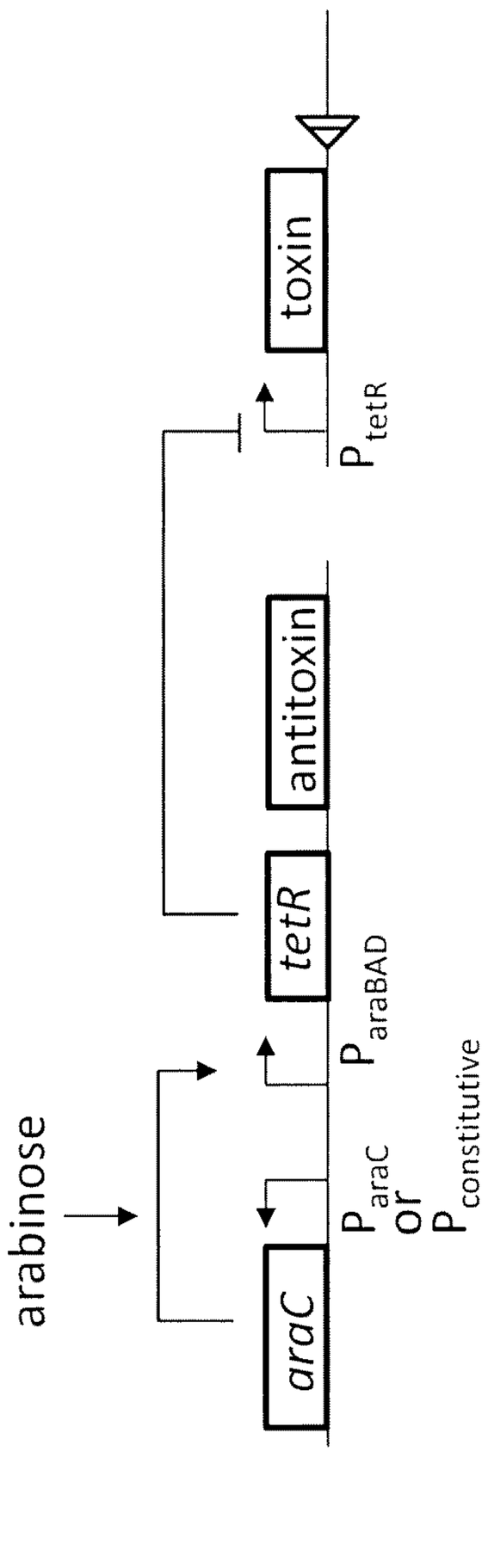
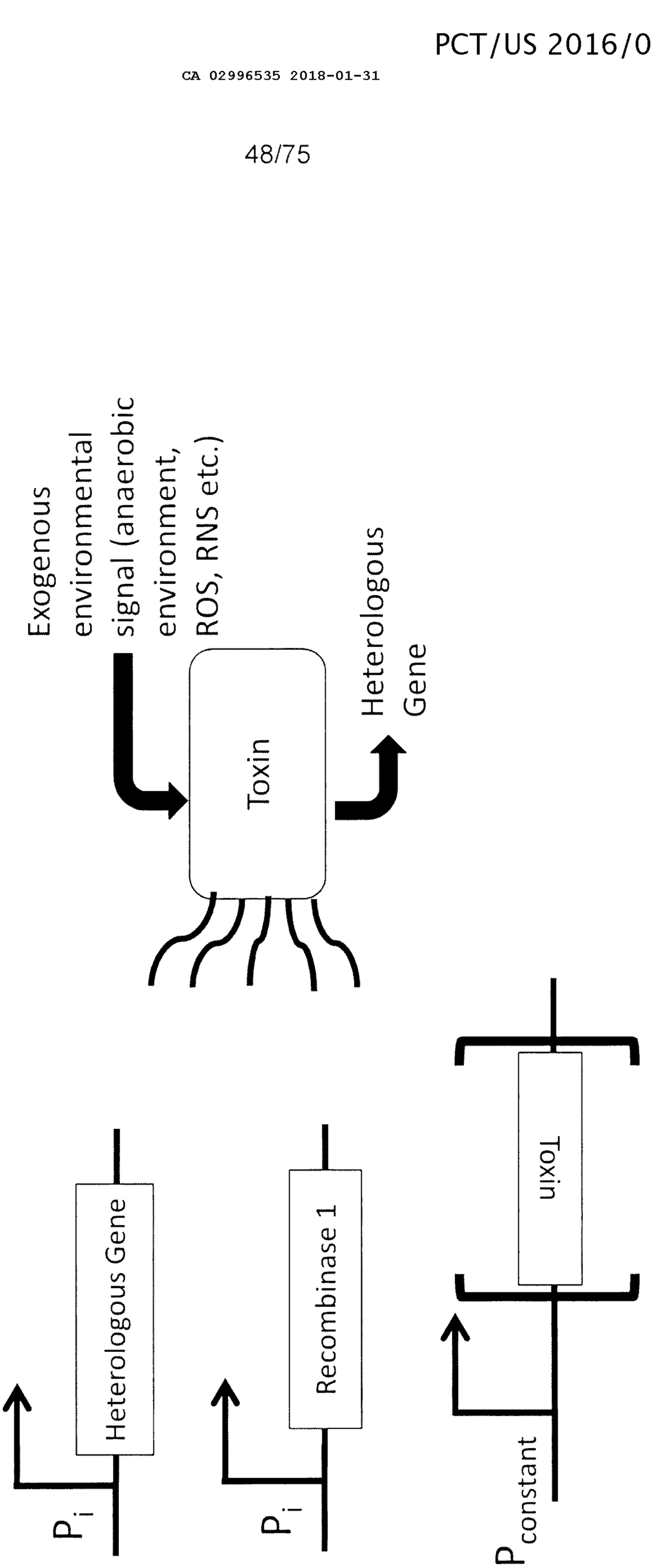

toxin
P_{tetR}
antitoxin
arabinose
araC
tetR
P_{araC} or $P_{constitutive}$
P_{araBAD}

FIG. 24

FIG. 25


Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
P_i
Heterologous Gene
P_i
Recombinase 1
$P_{constant}$
Toxin

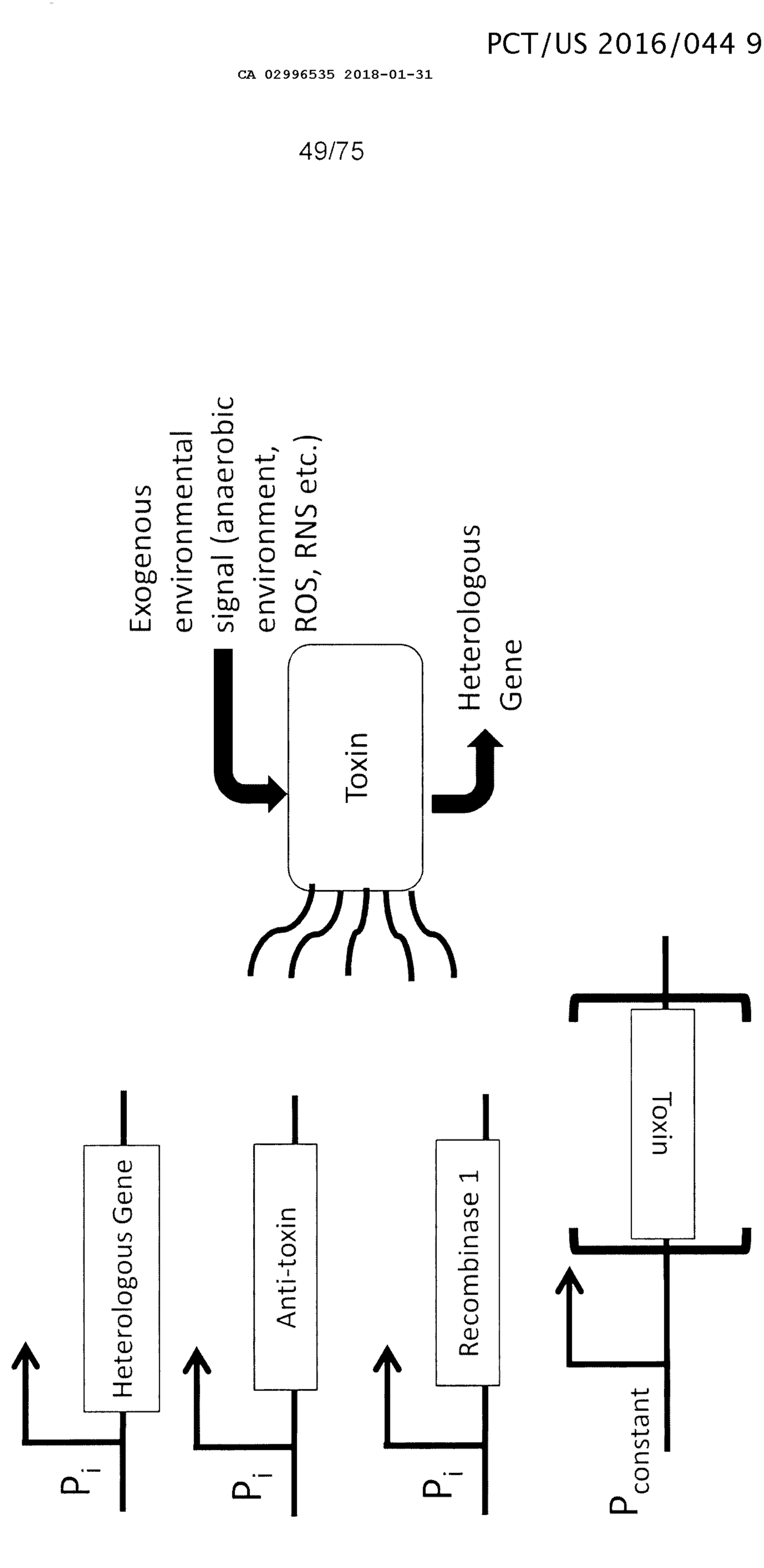

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
P_i
Heterologous Gene
Anti-toxin
P_i
Anti-toxin
Recombinase 1
P_i
Recombinase 1
Toxin
$P_{constant}$

FIG. 26

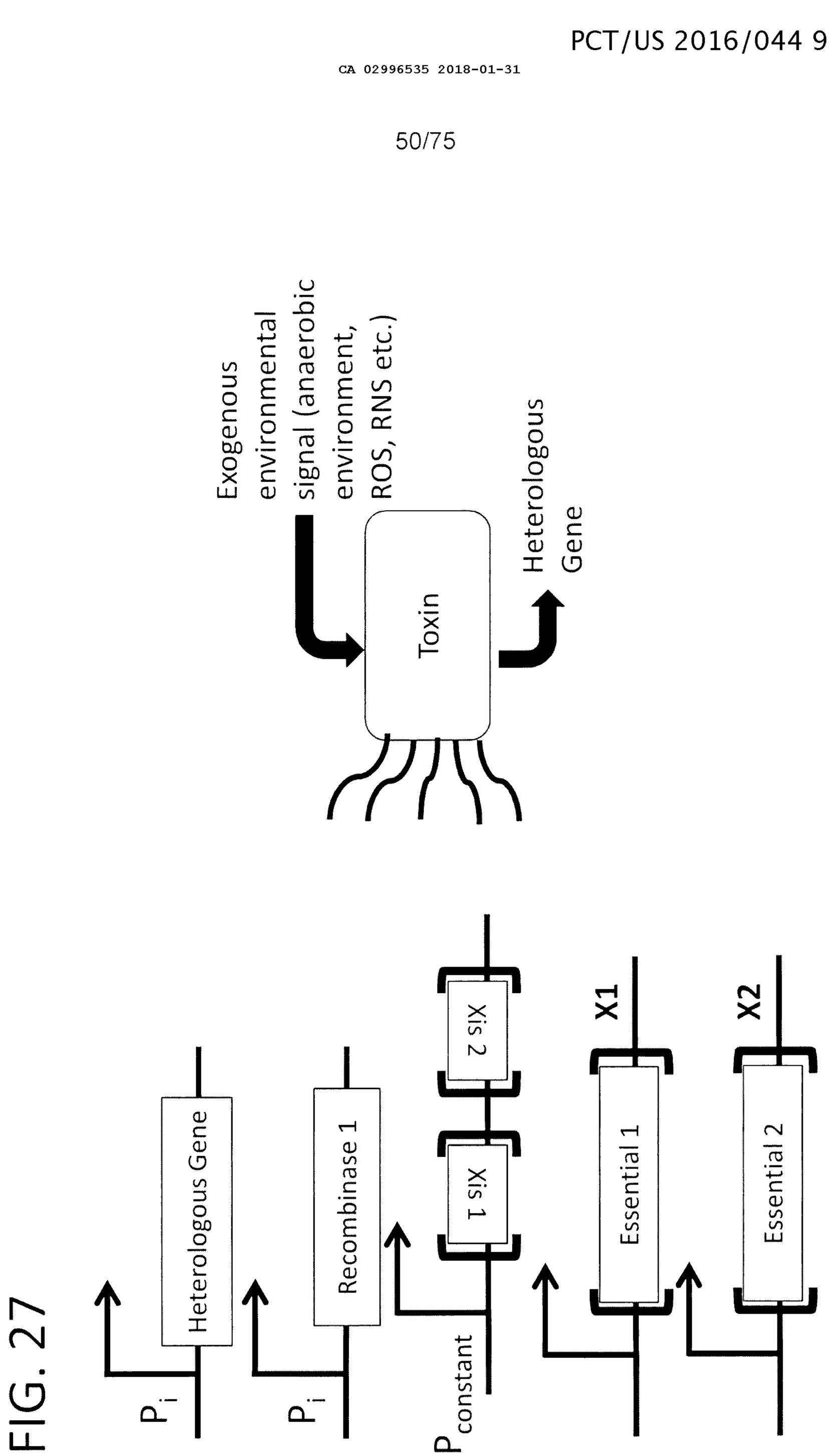

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
P_i
Heterologous Gene
P_i
Recombinase 1
$P_{constant}$
Xis 1
Xis 2
X1
Essential 1
X2
Essential 2

FIG. 27

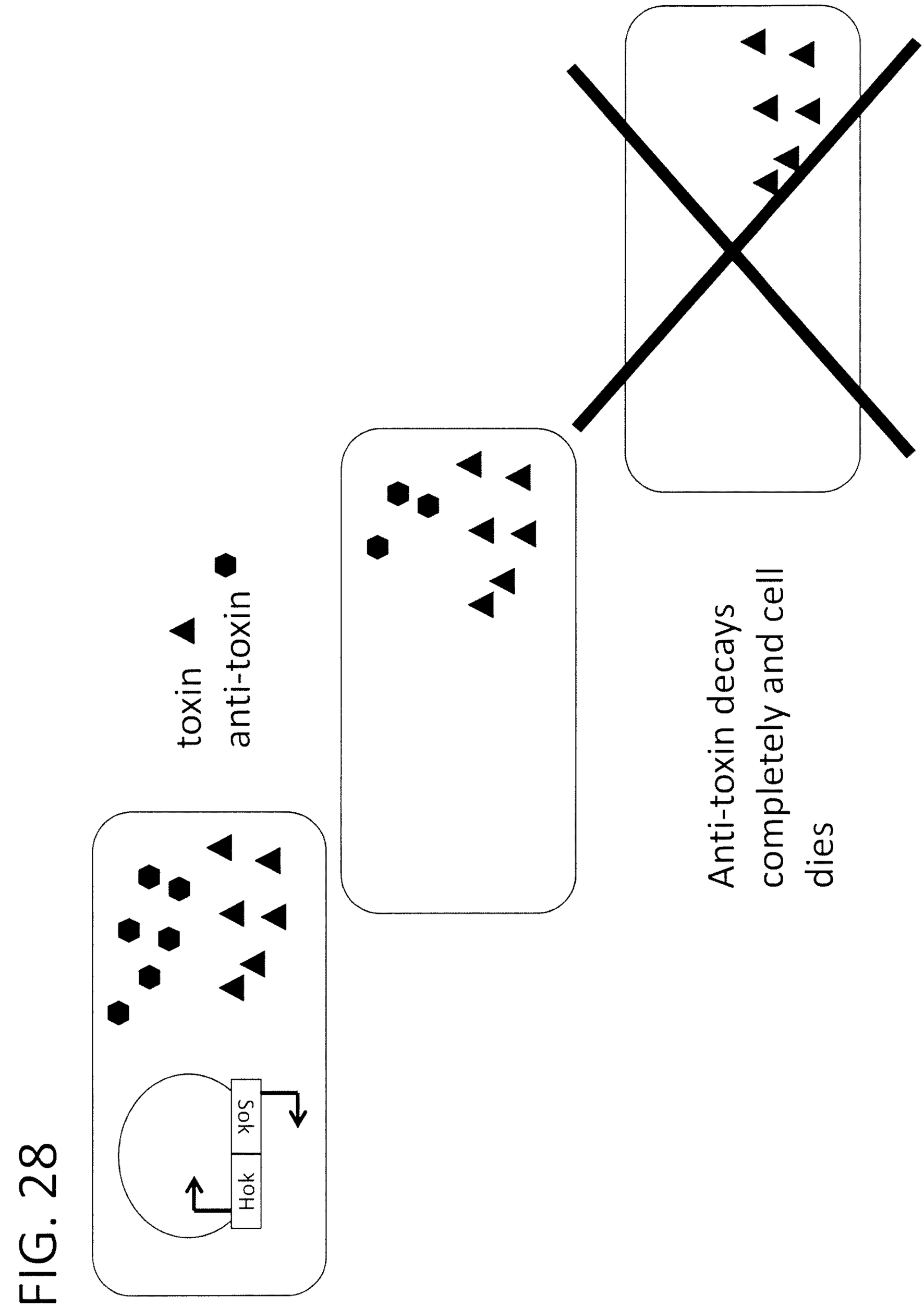

toxin
anti-toxin
Anti-toxin decays completely and cell dies
Sok
Hok

FIG. 28

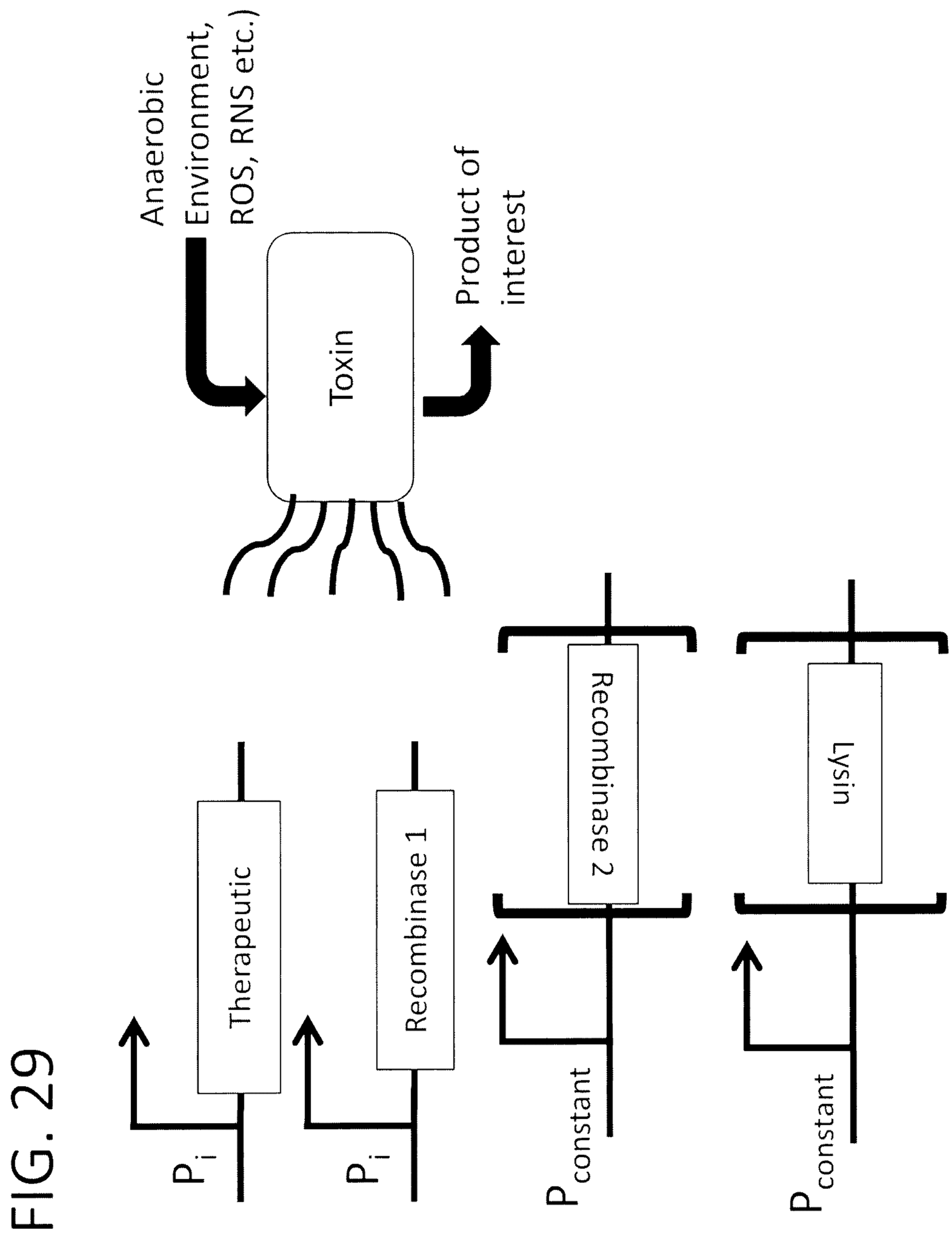

Anaerobic Environment ROS, RNS etc.)
Toxin
Product of interest
P_i
Therapeutic
P_i
Recombinase 1
$P_{constant}$
Recombinase 2
$P_{constant}$
Lysin

FIG. 29

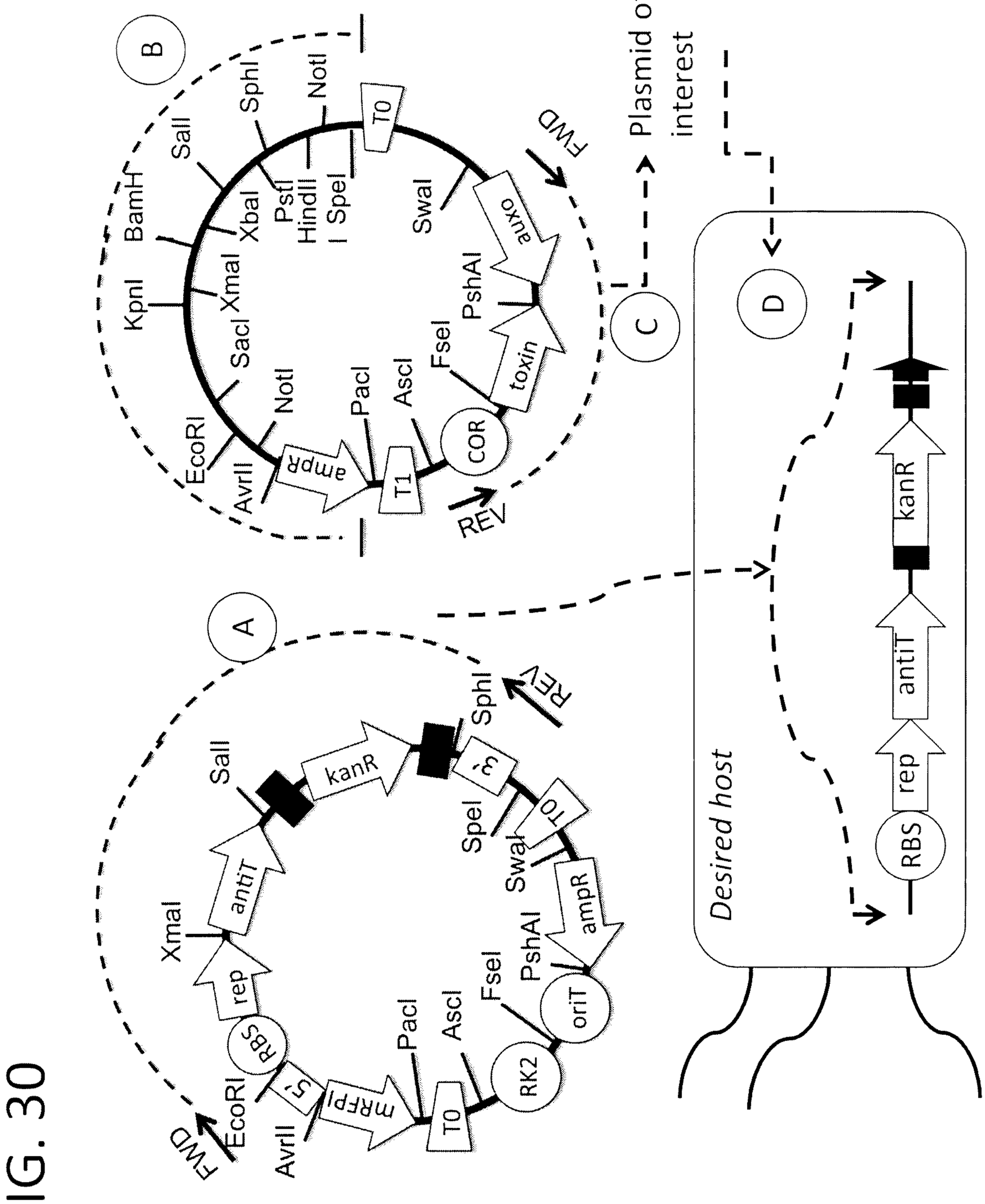

A
B
C
D
FWD
REV
EcoRI
AvrII
NotI
SacI
XmaI
KpnI
BamH
SalI
XbaI
PstI
HindIII
SpeI
SphI
NotI
T0
SwaI
oxna
PshAI
toxin
FseI
AscI
PacI
ampR
T1
COR
RBS
rep
antiT
kanR
RK2
oriT
ampR
T0
mRFPI
S1
S2
Plasmid of interest
Desired host

FIG. 30

FIG. 31

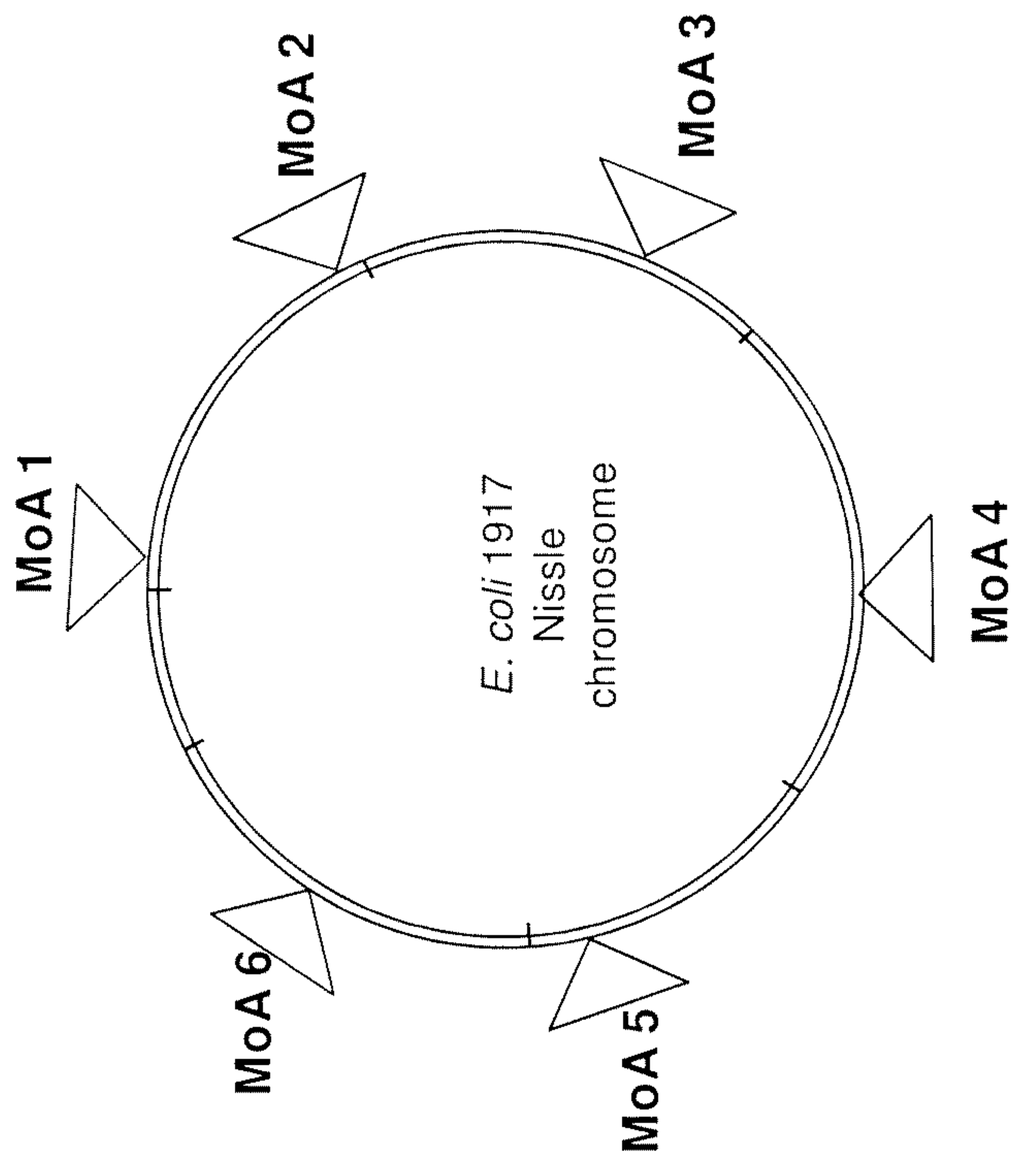
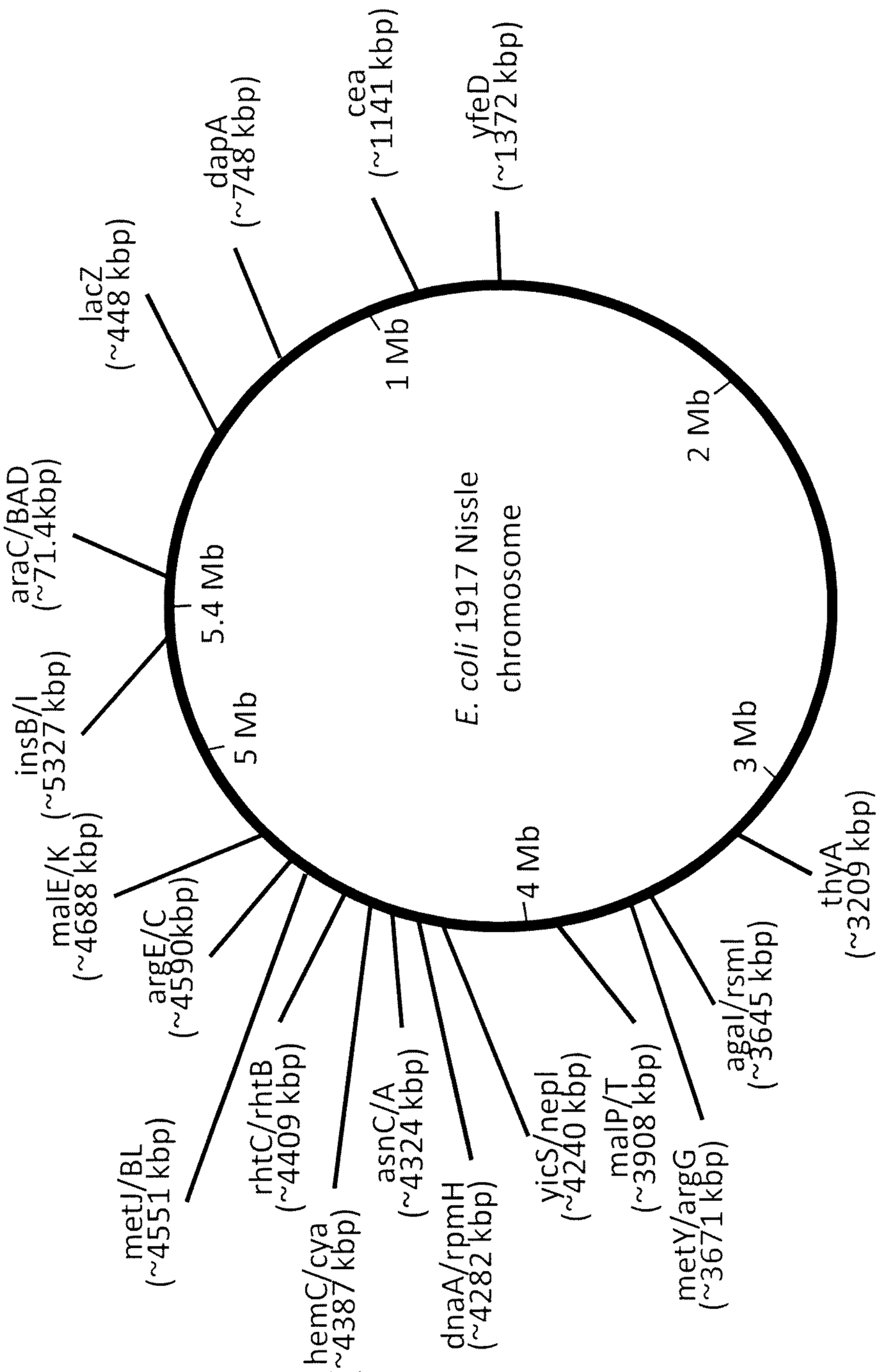


FIG. 32


araC/BAD (~71.4kbp)
lacZ (~448 kbp)
dapA (~748 kbp)
cea (~1141
yfeD (~1372 k
1 Mb
2 Mb
3 Mb
4 Mb
5 Mb
5.4 Mb
E. coli 1917 Nissle chromosome
insB/I (~5327 kbp)
malE/K (~4688 kbp)
argE/C (~4590kbp)
metJ/BL (~4551 kbp)
rhtC/rhtB (~4409 kbp)
asnC/A (~4324 kbp)
yicS/nepI (~4240 kbp)
malP/T (~3908 kbp)
agaI/rsmI (~3645 kbp)
metY/argG (~3671 kbp)
thyA (~3209 kbp)

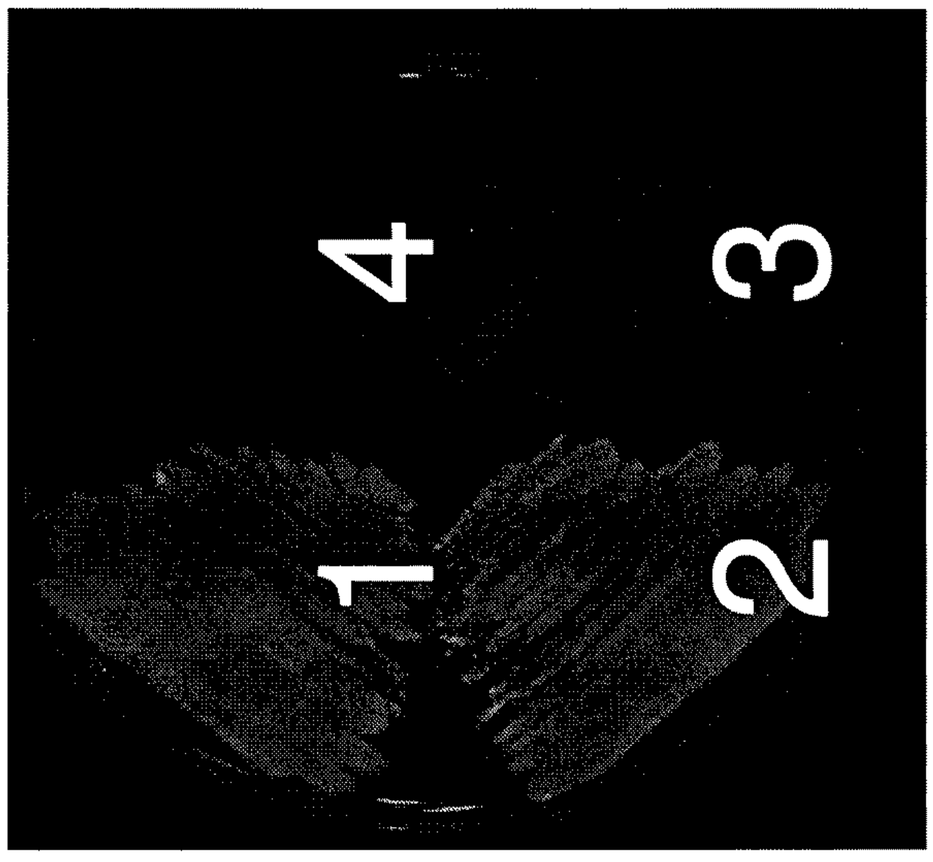


FIG. 33

Brightness of constitutive RFP integrated in three locations:
1. AraB/C
2. MalE/K
3. MetY/ArgG
4. Nissle (non-fluorescent)

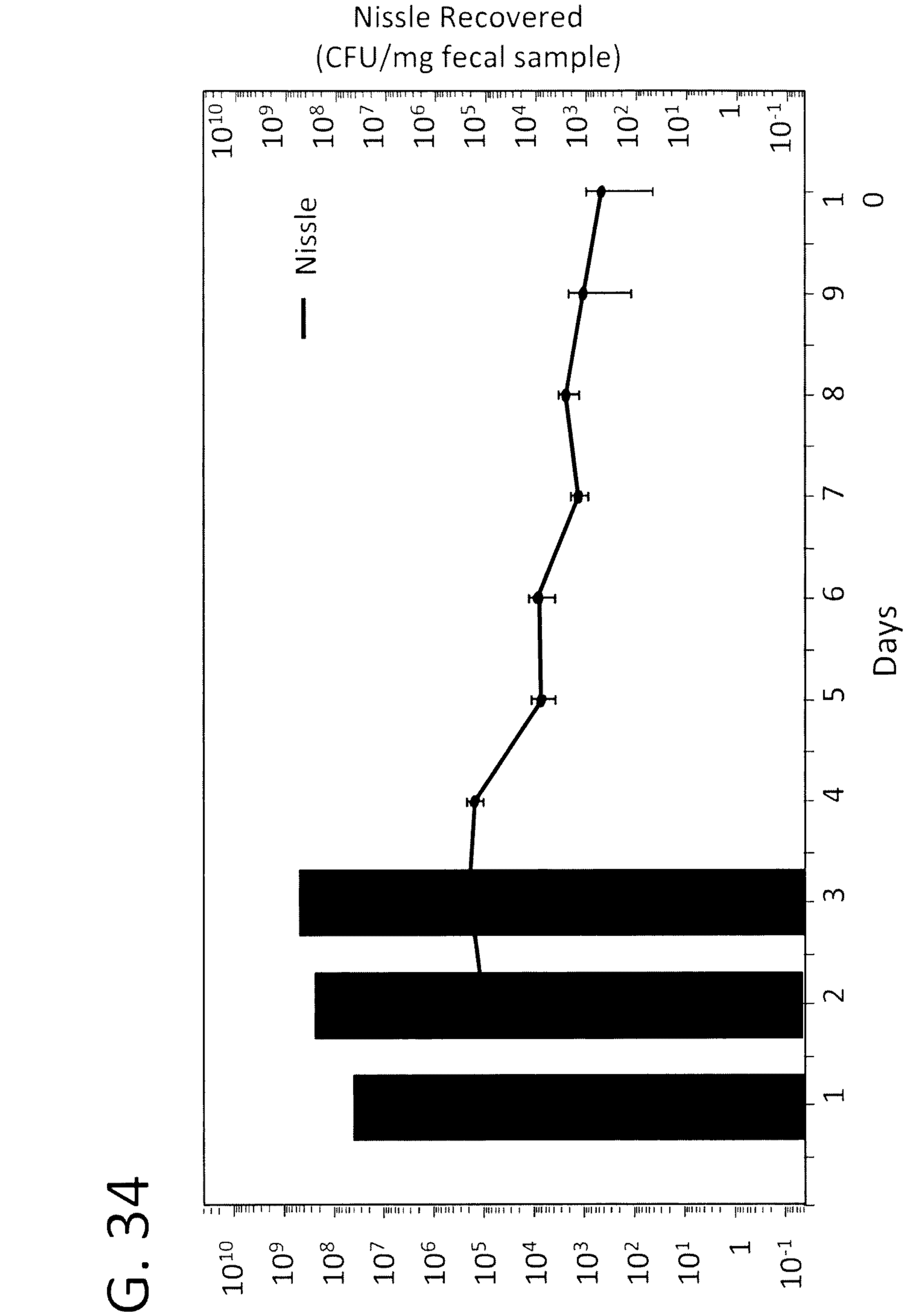

Nissle Recovered
(CFU/mg fecal sample)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
1
10^{-1}
Nissle
0
1
9
8
7
6
5
4
3
2
1
Days
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
1
10^{-1}
Nissle Administered (CFU/mL)

FIG. 34

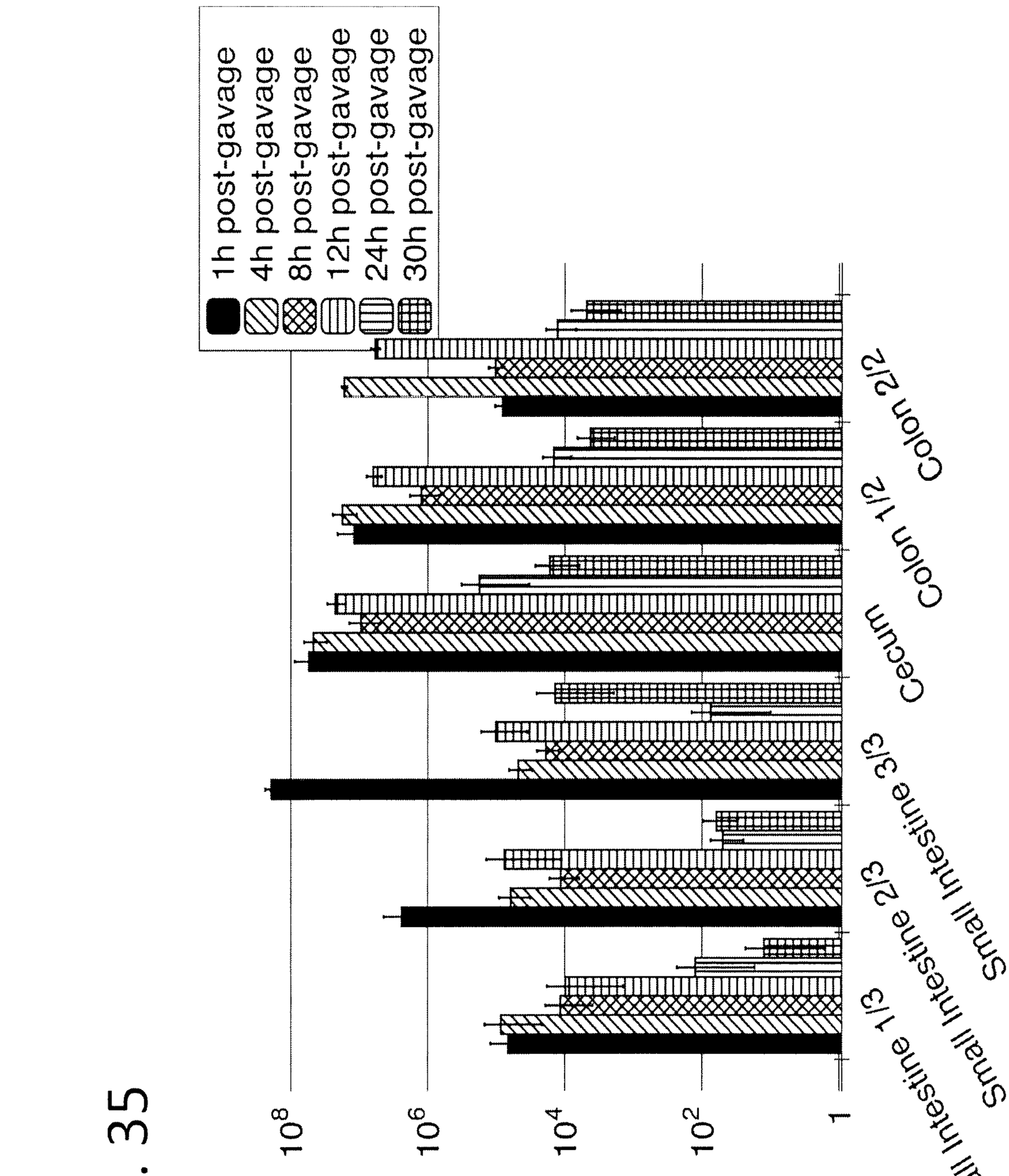

1h post-gavage
4h post-gavage
8h post-gavage
12h post-gavage
24h post-gavage
30h post-gavage
Cecum
Colon 1/2
Colon 2/2
Small Intestine 1/3
Small Intestine 2/3
Small Intestine 3/3
10^8
10^6
10^4
10^2
1
Bacterial CFU

FIG. 35

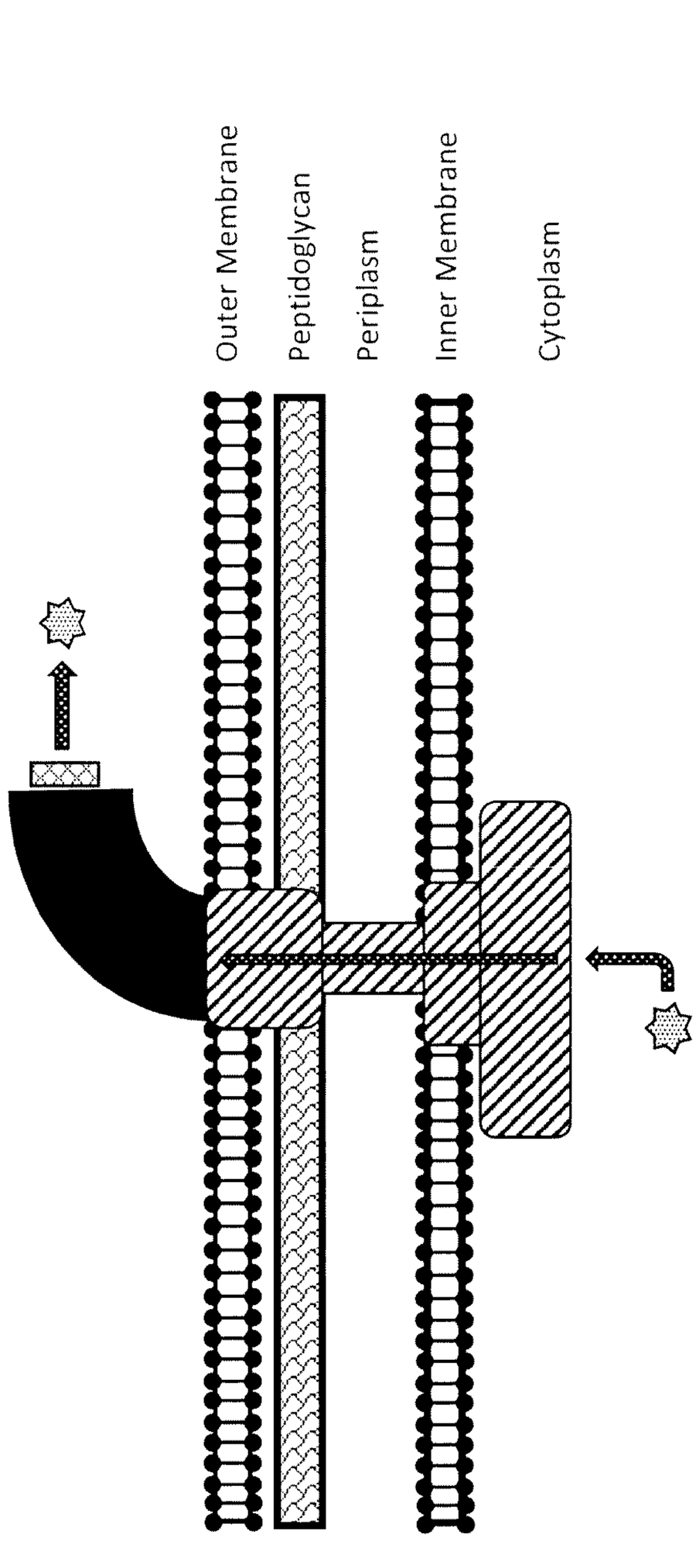

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm

FIG. 36

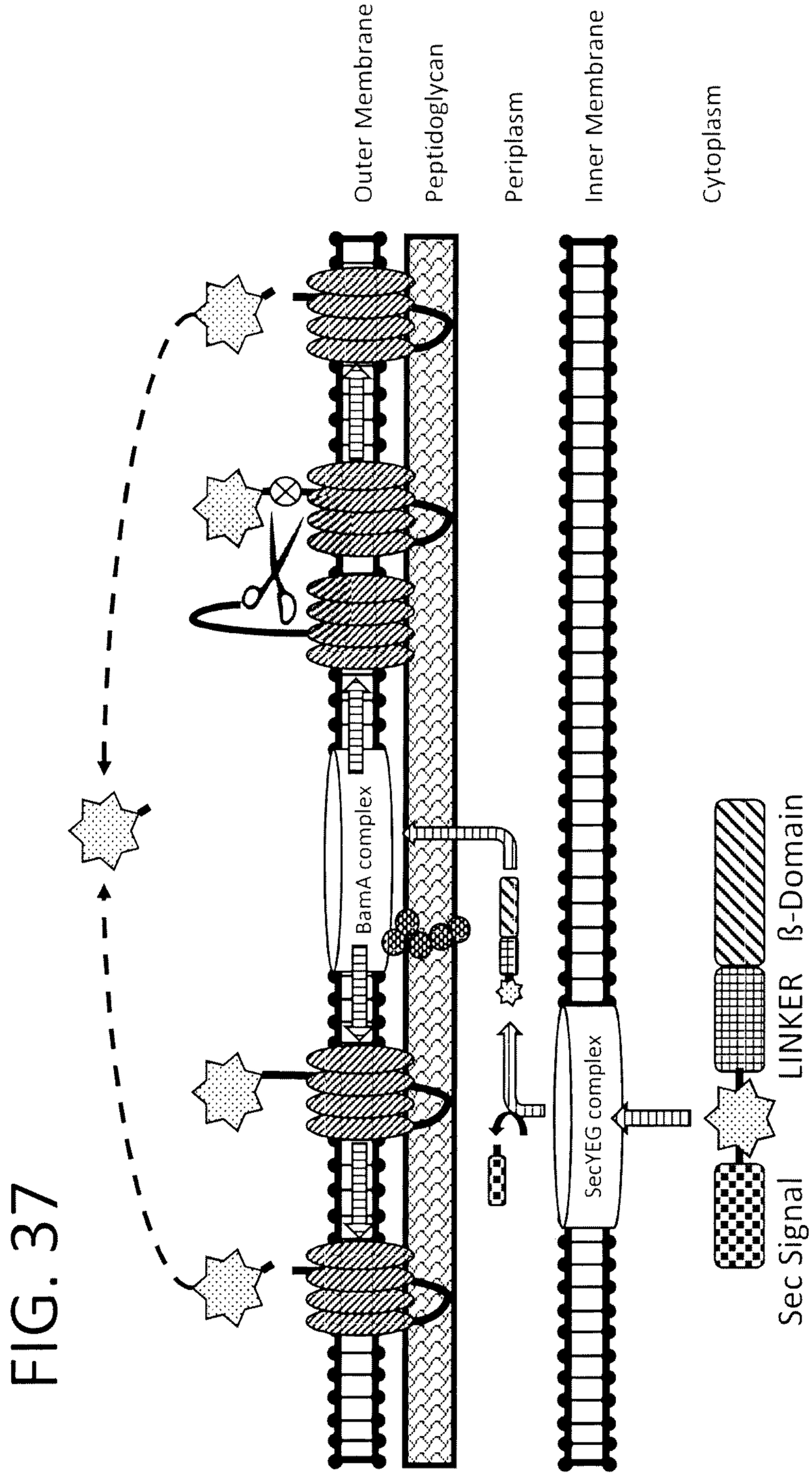

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
BamA complex
SecYEG complex
Sec Signal
LINKER
ß-Domain

FIG. 37

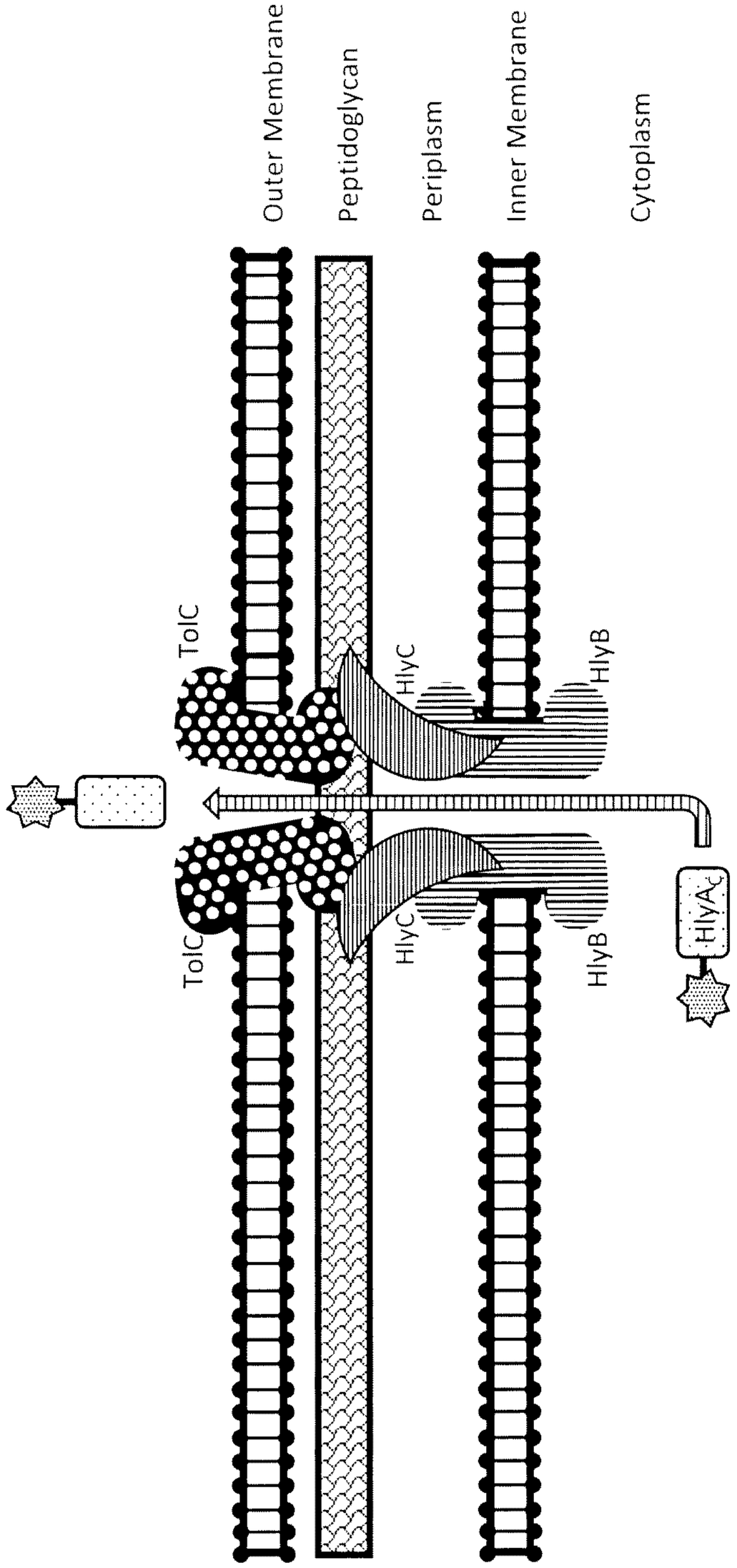

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
TolC
HlyC
HlyB
TolC
HlyC
HlyB
HlyA

FIG. 38

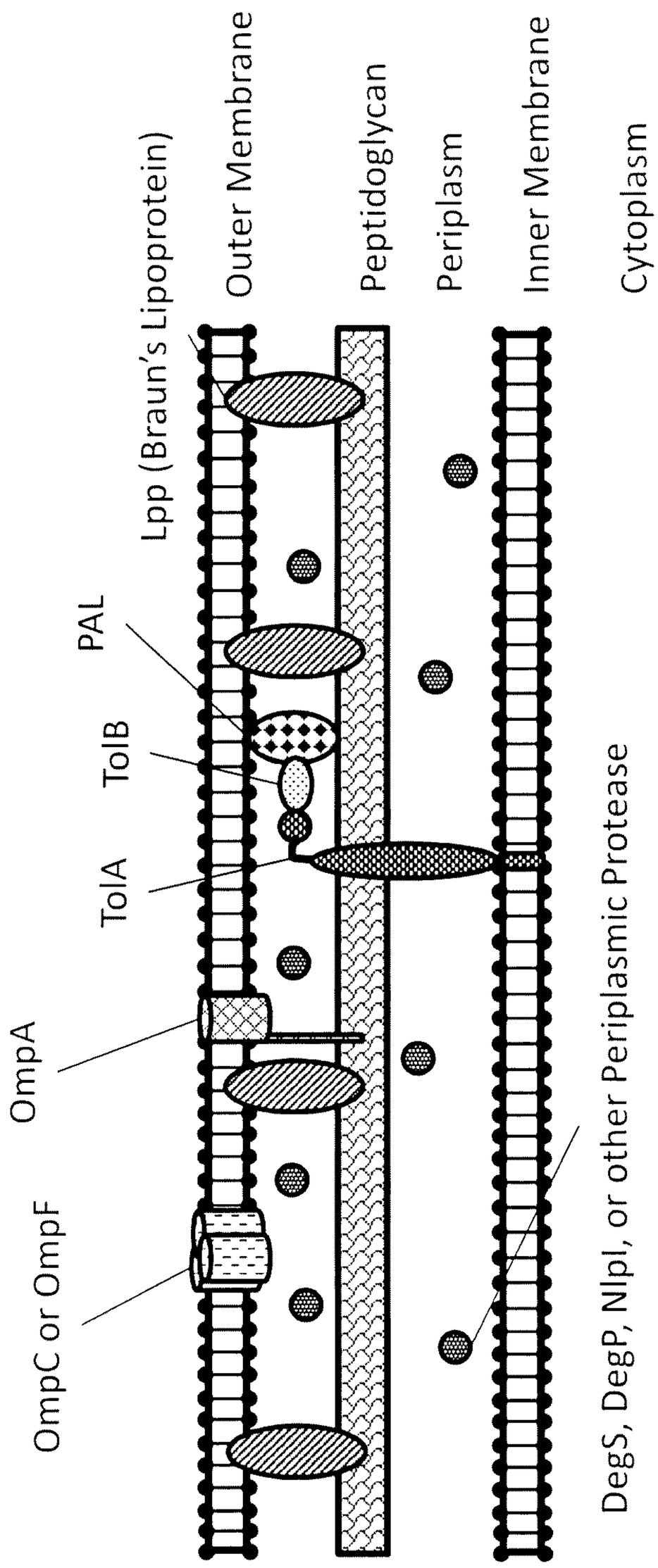

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
Lpp (Braun's Lipoprotein)
PAL
TolB
TolA
OmpA
OmpC or OmpF
DegS, DegP, NlpI, or other Periplasmic Protease

FIG. 39

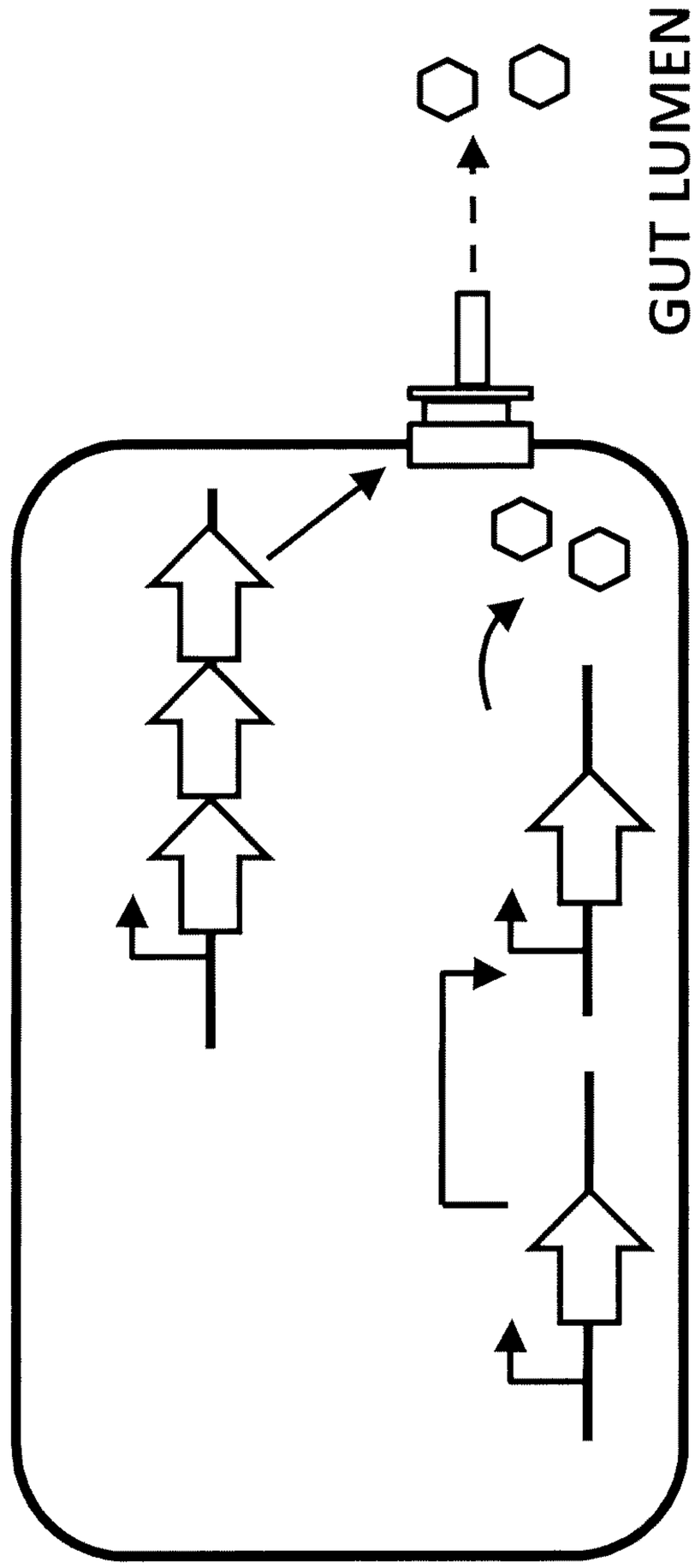


FIG. 40

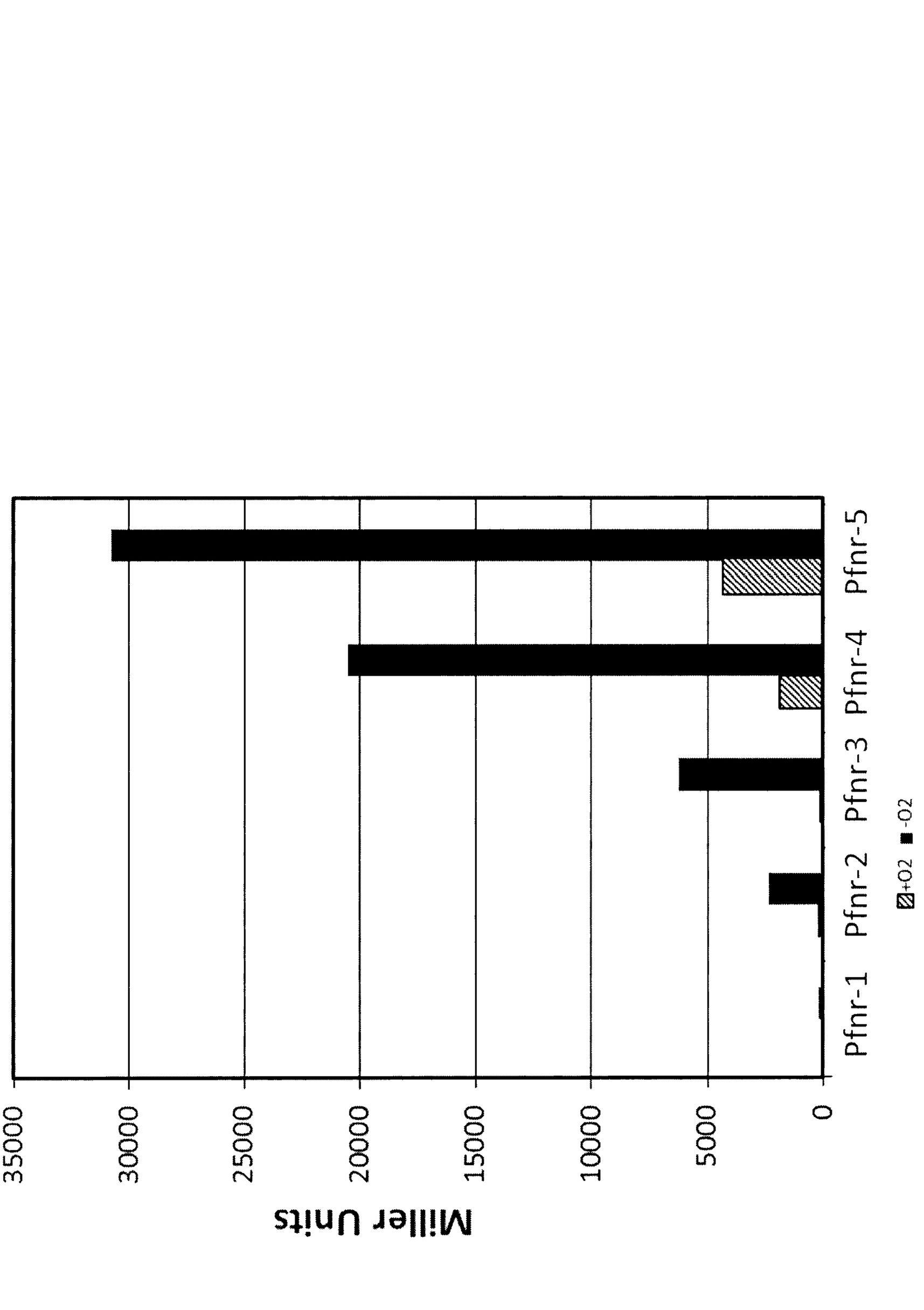

Miller Units
35000
30000
25000
20000
15000
10000
5000
0
Pfnr-1
Pfnr-2
Pfnr-3
Pfnr-4
Pfnr-5
+O2
-O2

FIG. 41

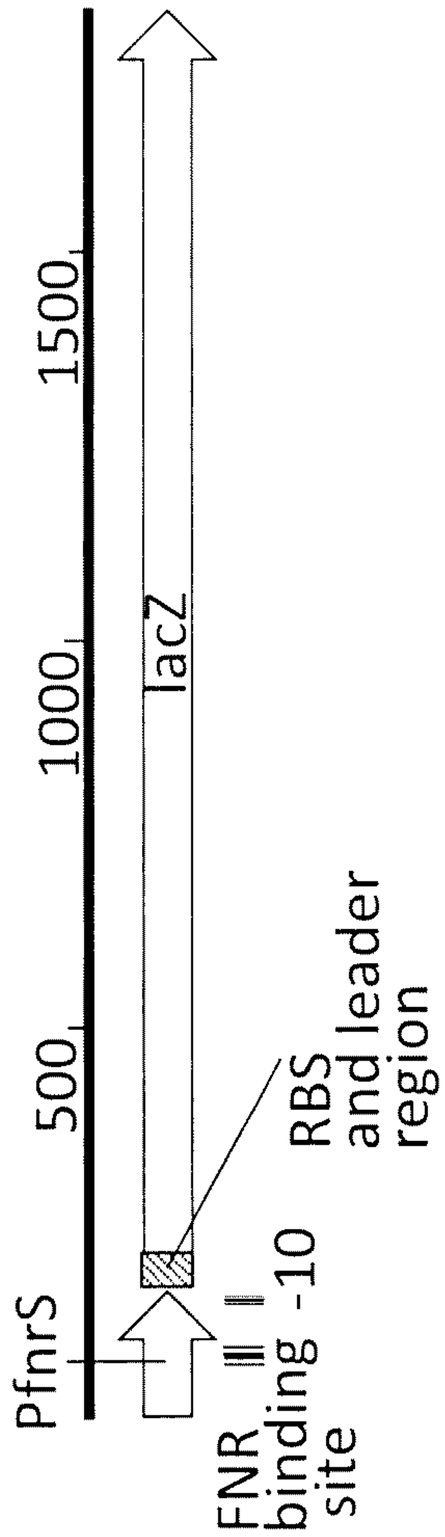

1000
1500
500
lacZ
PfnrS
FNR binding site
-10
RBS and lead region
lacZpal
1812 bp

FIG. 42A

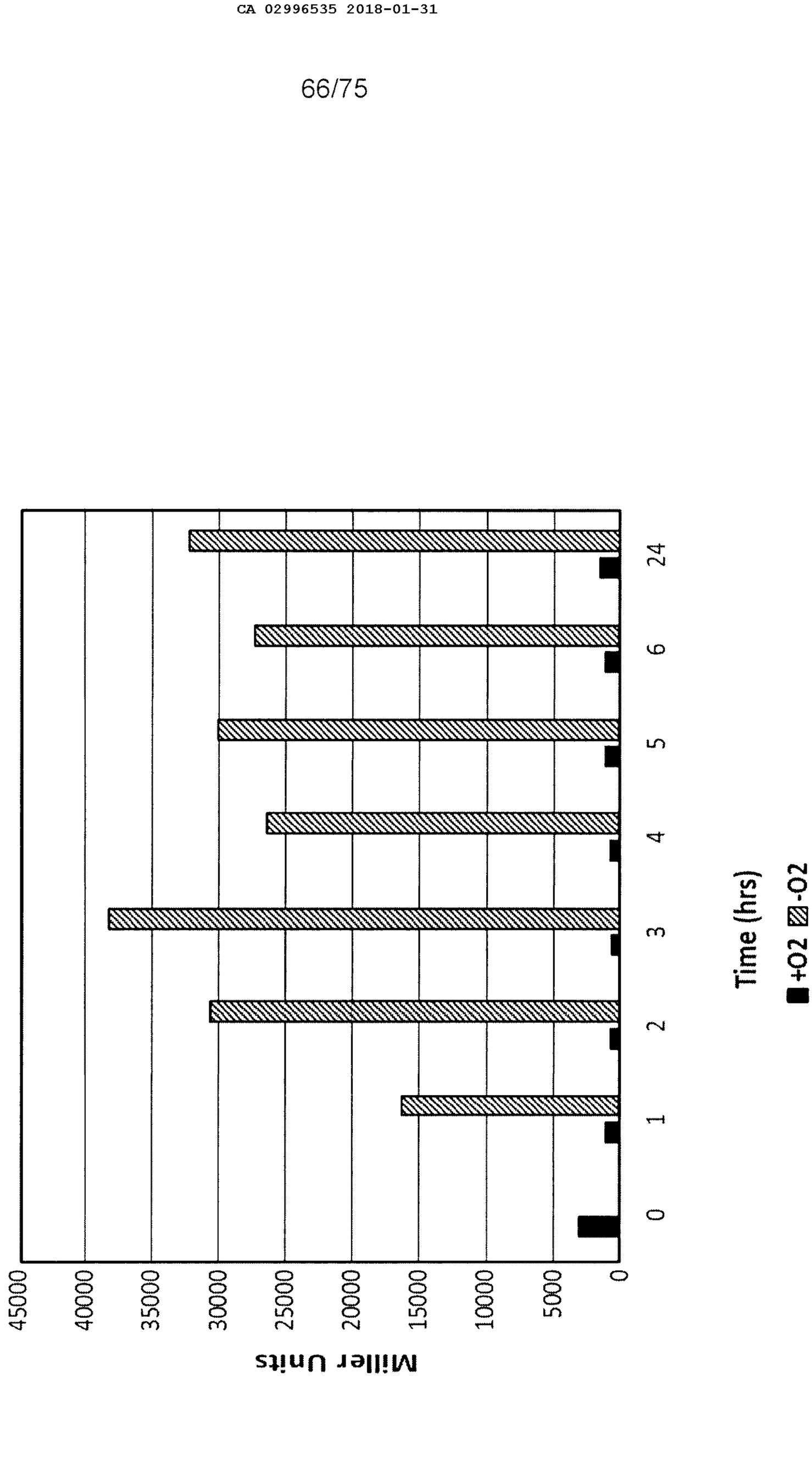

Miller Units
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
1
2
3
4
5
6
24
Time (hrs)
+O2
-O2

FIG. 42B

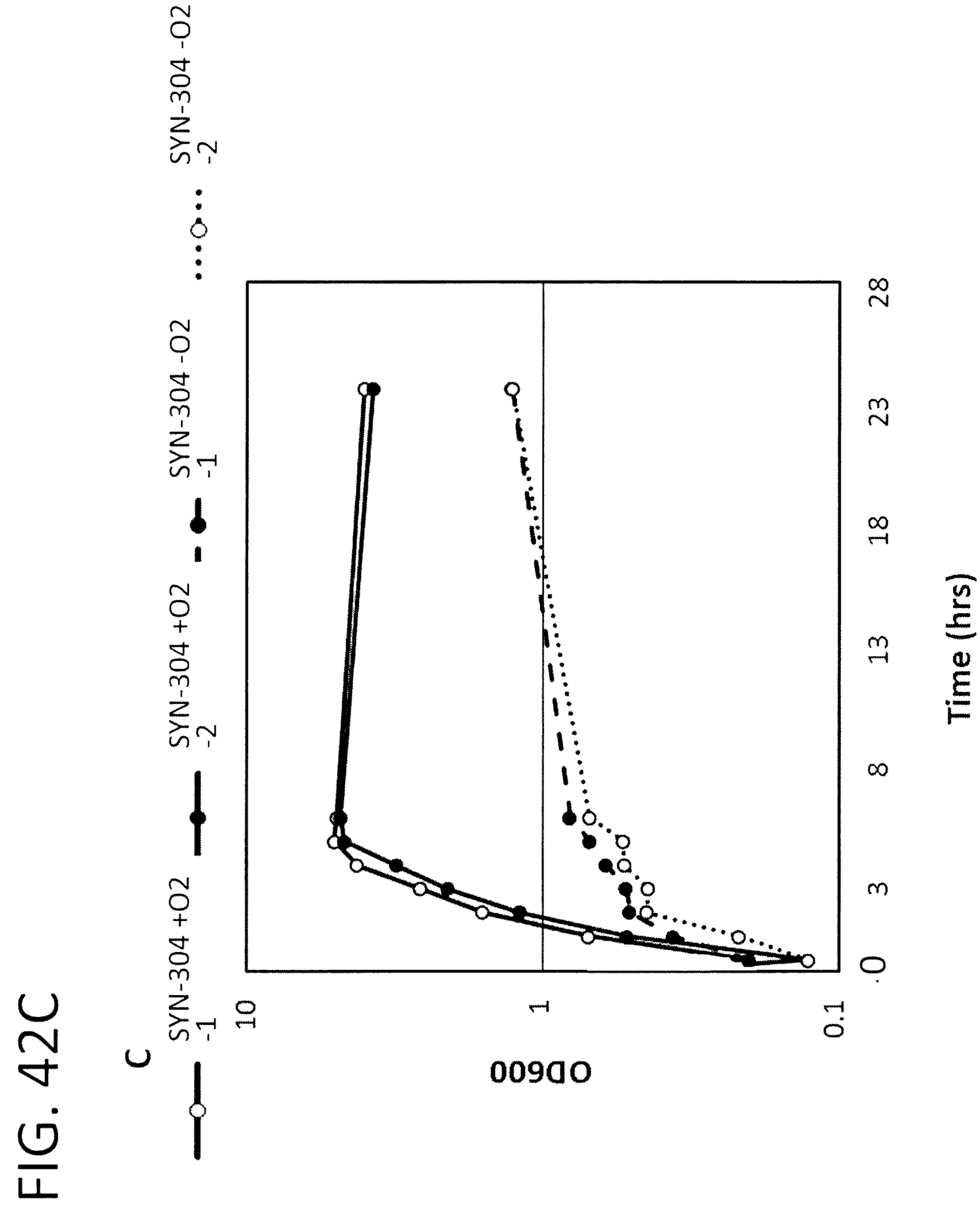

SYN-304 -O2 -1
SYN-304 -O2 -2
SYN-304 +O2 -1
SYN-304 +O2 -2
OD600
Time (hrs)
0.1
1
10
0
3
8
13
18
23
28

FIG. 42C

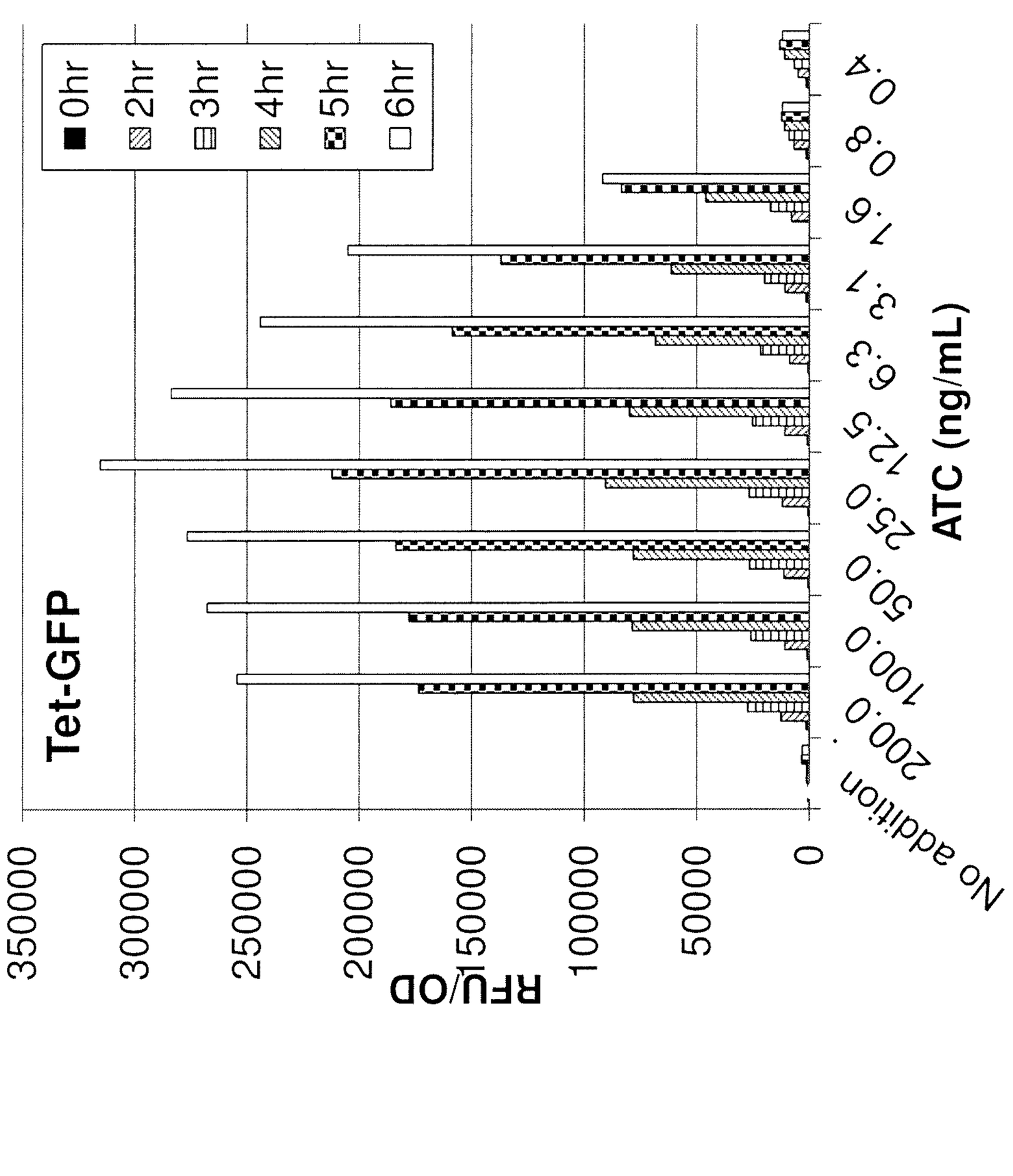

Tet-GFP
0hr
2hr
3hr
4hr
5hr
6hr
RFU/OD
350000
300000
250000
200000
150000
100000
50000
0
ATC (ng/mL)
No addition
200.0
100.0
50.0
25.0
12.5
6.3
3.1
1.6
0.8
0.4

FIG. 43A

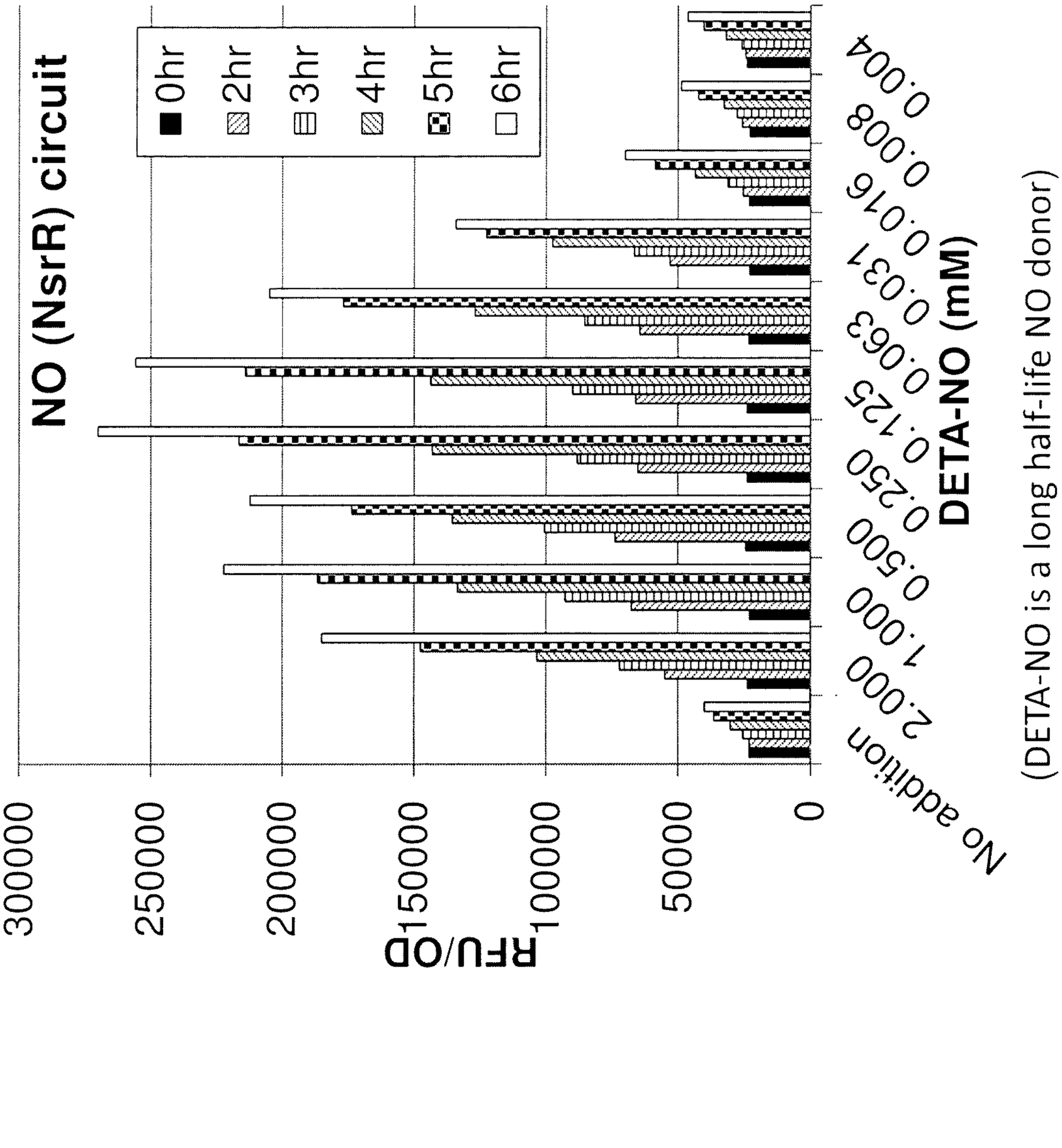

NO (NsrR) circuit
0hr
2hr
3hr
4hr
5hr
6hr
RFU/OD
300000
250000
200000
150000
100000
50000
0
No addition
2.000
1.000
0.500
0.250
0.125
0.063
0.031
0.016
0.008
0.004
DETA-NO (mM)
(DETA-NO is a long half-life NO donor)

FIG. 43B

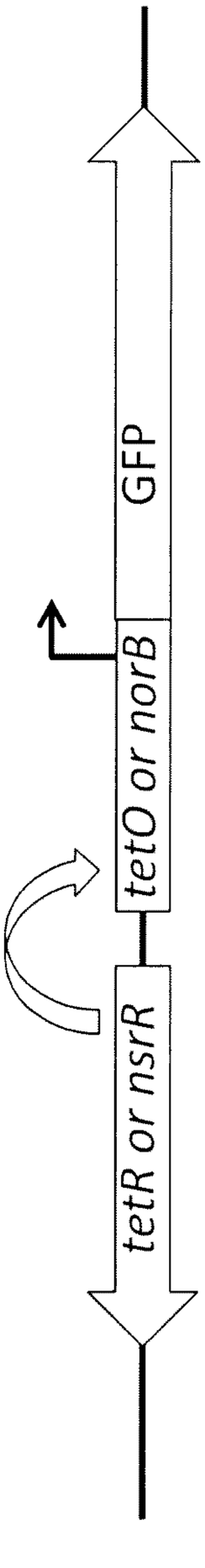


FIG. 43C

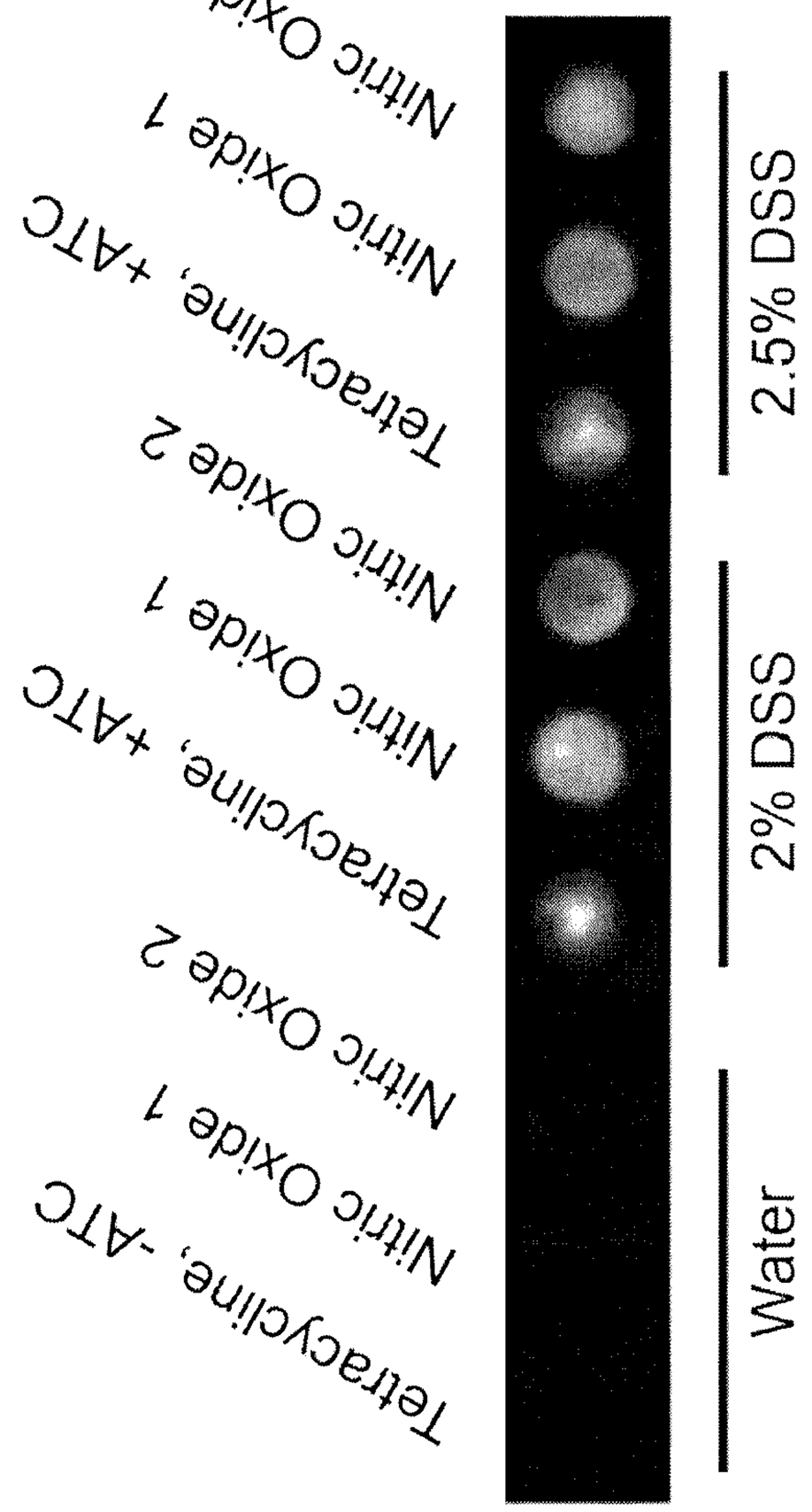

Water
2% DSS
2.5% DSS
Tetracycline, -ATC
Nitric Oxide 1
Nitric Oxide 2
Tetracycline, +ATC
Nitric Oxide 1
Nitric Oxide 2

FIG. 43D

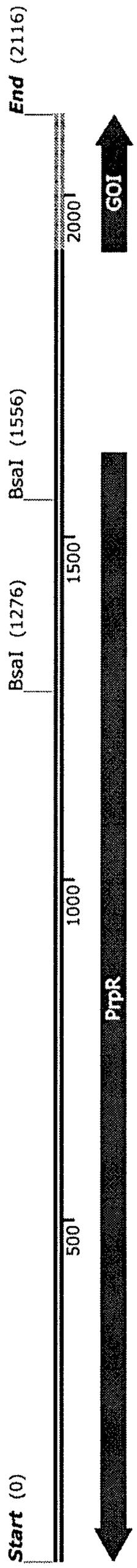

End (2116)
2000
GOI
BsaI (1556)
1500
BsaI (1276)
1000
PrpR
500
Start (0)

FIG. 44

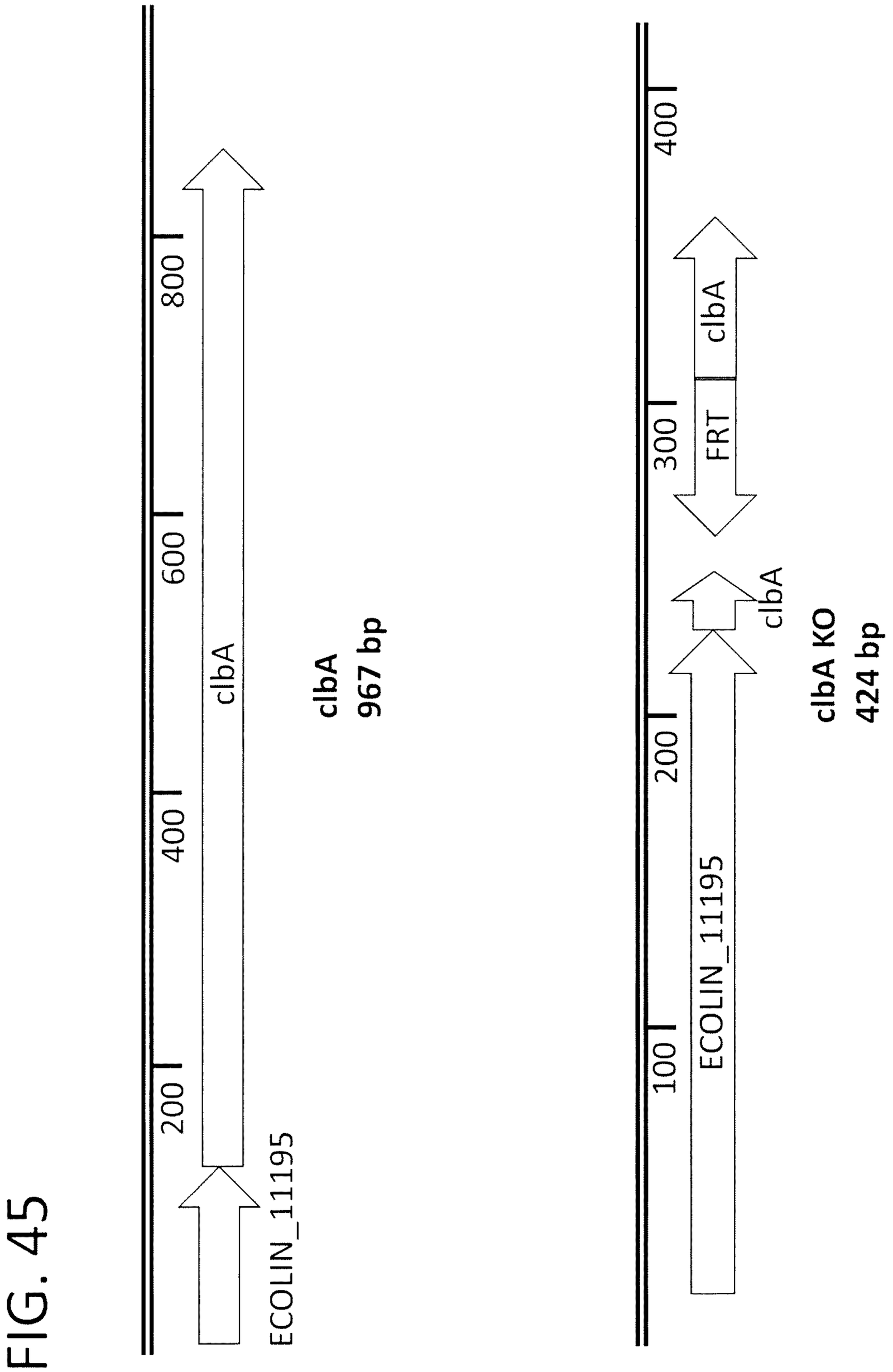

clbA
967 bp
600
800
clbA
ECOLIN_11195
200
400
clbA KO
424 bp
300
FRT
clbA
100
200
400

FIG. 45

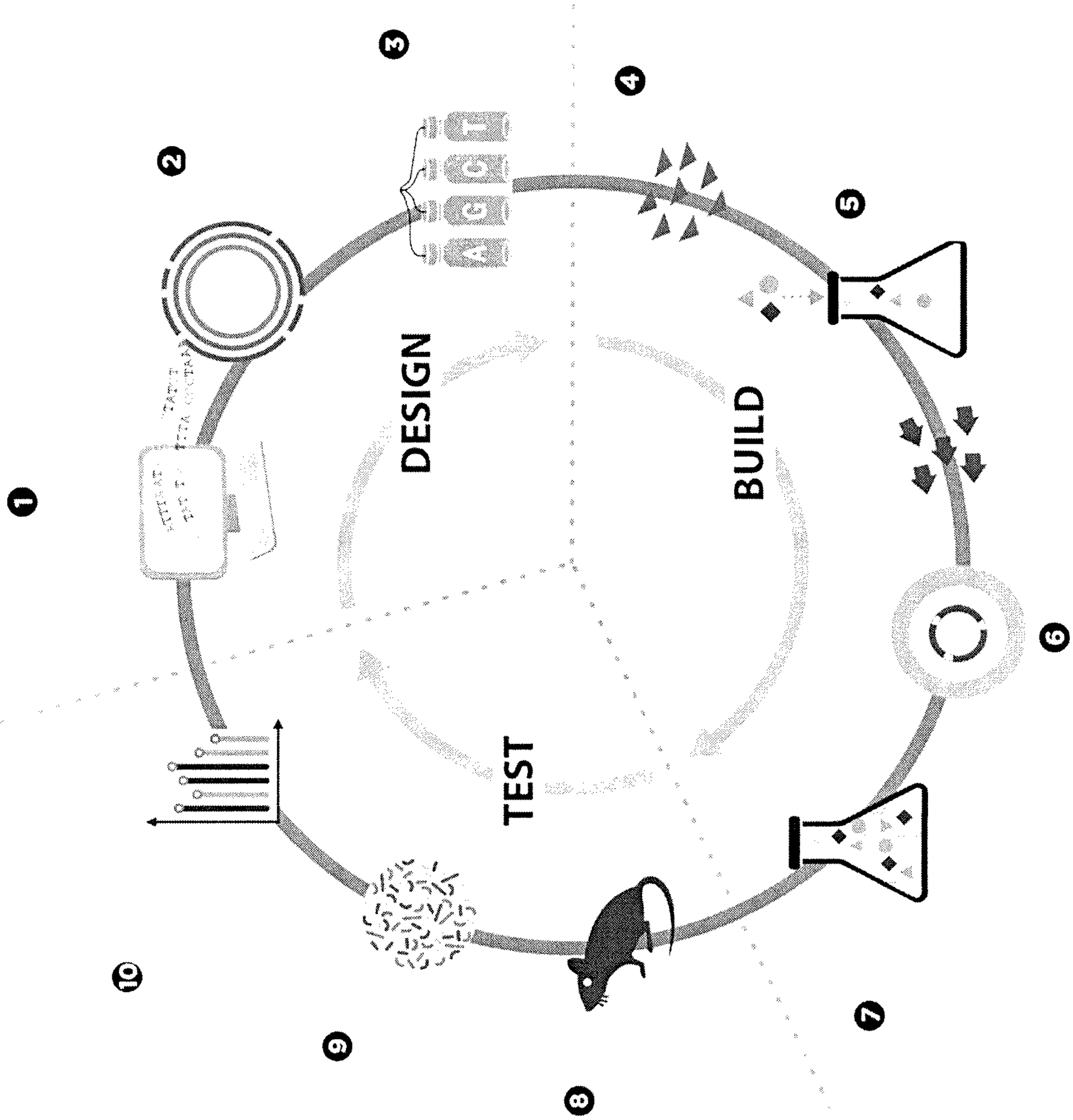


FIG. 46

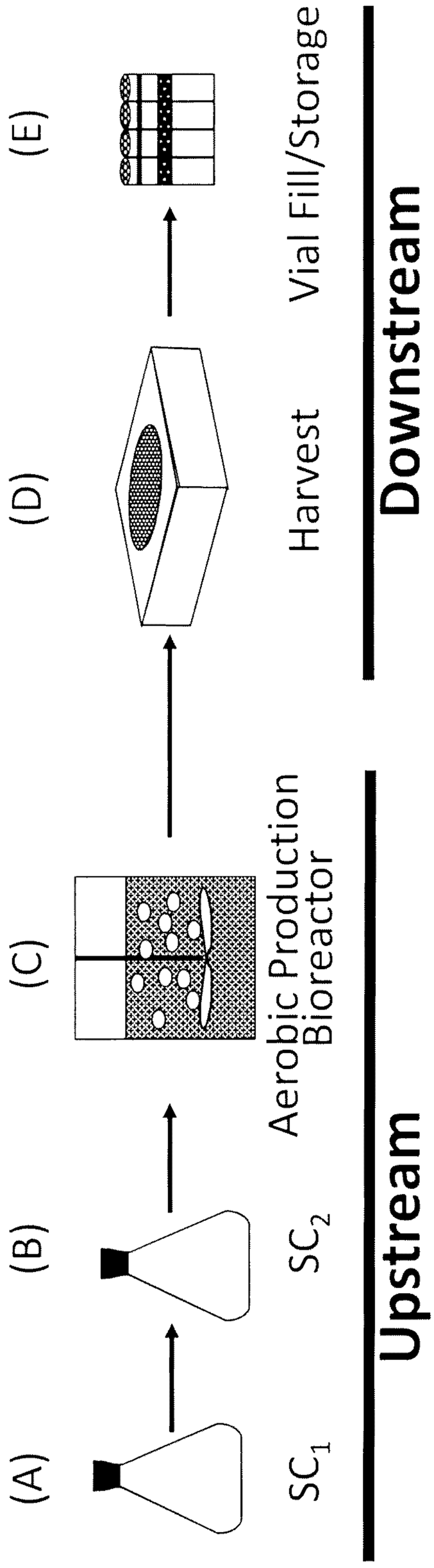

Downstream
Vial Fill/Storage
(E)
Harvest
(D)
Aerobic Production Bioreactor
(C)
SC_2
(B)
Upstream
SC_1
(A)

FIG. 47