(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(10) International Publication Number
WO 2016/183531 A1

(43) International Publication Date
17 November 2016 (17.11.2016)

(51) International Patent Classification:
C07K 14/195 (2006.01) *C12N 9/88* (2006.01)
A61K 35/74 (2015.01) *C12N 5/10* (2006.01)

(21) International Application Number:
PCT/US2016/032562

(22) International Filing Date:
13 May 2016 (13.05.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/161,137 13 May 2015 (13.05.2015) US
62/256,052 16 November 2015 (16.11.2015) US

(71) Applicant: **SYNLOGIC, INC.** [US/US]; 200 Sidney Street, Suite 320, Cambridge, MA 02139 (US).

(72) Inventors: **FALB, Dean**; 180 Lake Street, Sherborn, MA 01770 (US). **ISABELLA, Vincent, M.**; 465 Putnam Avenue, Unit 1, Cambrige, MA 02139 (US). **KOTULA, Jonathan, W.**; 345 Washington Street, Somerville, MA 02143 (US). **MILLER, Paul, F.**; 39 Emerald Glen Lane, Salem, CT 06420 (US). **ROWE, Sarah**; 9 Kingman Road, Somerville, MA 02143 (US). **MILLET, Yves**; 15 Frederick Street, Newton, MA 02460 (US).

(74) Agents: **McDONELL, Leslie, A.** et al.; Finnegan, Henderson, Farabow, Garrett & Dunner, LLP, 901 New York Avenue, NW, Washington, DC 20001 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2016/183531 A1

(54) Title: BACTERIA ENGINEERED TO REDUCE HYPERPHENYLALANINEMIA

(57) Abstract: Genetically engineered bacteria, pharmaceutical compositions thereof, and methods of modulating and treating diseases associated with hyperphenylalaninemia are disclosed.

BACTERIA ENGINEERED TO REDUCE HYPERPHENYLALANINEMIA

[001] The present application claims the benefit of priority to U.S. Provisional Patent Application No. 62/161,137, filed May 13, 2015, and U.S. Provisional Patent Application No. 62/256,052, filed November 16, 2015, the contents of which are hereby incorporated by reference herein in their entirety.

[002] his disclosure relates to compositions and therapeutic methods for reducing hyperphenylalaninemia. In certain aspects, the disclosure relates to genetically engineered bacteria that are capable of reducing hyperphenylalaninemia in a mammal. In certain aspects, the compositions and methods disclosed herein may be used for treating diseases associated with hyperphenylalaninemia, e.g., phenylketonuria.

[003] Phenylalanine is an essential amino acid primarily found in dietary protein. Typically, a small amount is utilized for protein synthesis, and the remainder is hydroxylated to tyrosine in an enzymatic pathway that requires phenylalanine hydroxylase (PAH) and the cofactor tetrahydrobiopterin. Hyperphenylalaninemia is a group of diseases associated with excess levels of phenylalanine, which can be toxic and cause brain damage. Primary hyperphenylalaninemia is caused by deficiencies in PAH activity that result from mutations in the PAH gene and/or a block in cofactor metabolism.

[004] Phenylketonuria (PKU) is a severe form of hyperphenylalaninemia caused by mutations in the PAH gene. PKU is an autosomal recessive genetic disease that ranks as the most common inborn error of metabolism worldwide (1 in 3,000 births), and affects approximately 13,000 patients in the United States. More than 400 different PAH gene mutations have been identified (Hoeks et al., 2009). Current PKU therapies require substantially modified diets consisting of protein restriction. Treatment from birth generally reduces brain damage and mental retardation (Hoeks et al., 2009; Sarkissian et al., 1999). However, the protein-restricted diet must be carefully monitored, and essential amino acids as well as vitamins must be supplemented in the diet. Furthermore, access to low protein foods is a challenge as they are more costly than their higher protein, nonmodified counterparts (Vockley et al., 2014).

[005] In children with PKU, growth retardation is common on a low-phenylalanine diet (Dobbelaere et al., 2003). In adulthood, new problems such as

osteoporosis, maternal PKU, and vitamin deficiencies may occur (Hoeks et al., 2009). Excess levels of phenylalanine in the blood, which can freely penetrate the blood-brain barrier, can also lead to neurological impairment, behavioral problems (e.g., irritability, fatigue), and/or physical symptoms (e.g., convulsions, skin rashes, musty body odor). International guidelines recommend lifelong dietary phenylalanine restriction, which is widely regarded as difficult and unrealistic (Sarkissian et al., 1999), and "continued efforts are needed to overcome the biggest challenge to living with PKU – lifelong adherence to the low-phe diet" (Macleod et al., 2010).

[006] In a subset of patients with residual PAH activity, oral administration of the cofactor tetrahydrobiopterin (also referred to as THB, BH4, Kuvan, or sapropterin) may be used together with dietary restriction to lower blood phenylalanine levels. However, cofactor therapy is costly and only suitable for mild forms of phenylketonuria. The annual cost of Kuvan, for example, may be as much as $57,000 per patient. Additionally, the side effects of Kuvan can include gastritis and severe allergic reactions (e.g., wheezing, lightheadedness, nausea, flushing of the skin).

[007] The enzyme phenylalanine ammonia lyase (PAL) is capable of metabolizing phenylalanine to non-toxic levels of ammonia and transcinnamic acid. Unlike PAH, PAL does not require THB cofactor activity in order to metabolize phenylalanine. Studies of oral enzyme therapy using PAL have been conducted, but "human and even the animal studies were not continued because PAL was not available in sufficient amounts at reasonable cost" (Sarkissian et al., 1999). A pegylated form of recombinant PAL (PEG-PAL) is also in development as an injectable form of treatment. However, most subjects dosed with PEG-PAL have suffered from injection site reactions and/or developed antibodies to this therapeutic enzyme (Longo et al., 2014). Thus, there is significant unmet need for effective, reliable, and/or long-term treatment for diseases associated with hyperphenylalaninemia, including PKU.

[008] L-amino acid deaminase (LAAD) catalyzes oxidative deamination of phenylalanine to generate phenylpyruvate, and trace amounts of ammonia and hydrogen peroxide. Phenylpyruvic acid (PPA) is widely used in the pharmaceutical, food, and chemical industries, and PPA is the starting material for the synthesis of D-phenylalanine, a raw intermediate in the production of many chiral drugs and food additives. LAAD has therefore been studied in the context of industrial PPA production

(Hou et al. 2015, Appl Microbiol Biotechnol. 2015 Oct;99(20):8391-402; "Production of phenylpyruvic acid from L-phenylalanine using an L-amino acid deaminase from Proteus mirabilis: comparison of enzymatic and whole-cell biotransformation approaches"). Phenylpyruvate is unable to cross the blood brain barrier (Steele, Fed Proc. 1986 Jun;45(7):2060-4; "Blood-brain barrier transport of the alpha-keto acid analogs of amino acids.," indicating that this conversion is useful in controlling the neurological phenotypes of PKU.

[009] In some embodiments, the disclosure provides genetically engineered bacteria that encode and express a phenylalanine metabolizing enzyme (PME). In some embodiments, the disclosure provides genetically engineered bacteria that encode and express phenylalanine ammonia lyase and/or phenylalanine hydroxylase and/or L-aminoacid deaminase and are capable of reducing hyperphenylalaninemia.

[010] In certain embodiments, the genetically engineered bacteria are non-pathogenic and may be introduced into the gut in order to reduce toxic levels of phenylalanine. In certain embodiments, the phenylalanine ammonia lyase and/or phenylalanine hydroxylase and/or L-aminoacid deaminase is stably produced by the genetically engineered bacteria, and/or the genetically engineered bacteria are stably maintained in vivo and/or in vitro. In certain embodiments, the genetically engineered bacteria further comprise a phenylalanine transporter gene to increase their uptake of phenylalanine. The invention also provides pharmaceutical compositions comprising the genetically engineered bacteria, and methods of modulating and treating disorders associated with hyperphenylalaninemia.

Brief Description of the Figures

[011] **Fig. 1** depicts a synthetic biotic for treating phenylketonuria (PKU) and disorders characterized by hyperphenylalaninemia.

[012] **Fig. 2A** depicts a schematic of phenylalanine hydroxylase action in phenylketonuria (PKU). **Fig. 2B** depicts a schematic of phenylalanine hydroxylase (PAH) action. **Fig. 2C** depicts a schematic of phenylalanine ammonia lyase (PAL) action. **Fig. 2D** depicts a schematic of L-amino acid deaminase (LAAD; e.g., from Proteus mirabilis) action.

[013] **Fig. 3** depicts a synthetic biotic for treating phenylketonuria (PKU) and disorders characterized by hyperphenylalaninemia.

[014] **Fig. 4** depicts a synthetic biotic for treating phenylketonuria (PKU) and disorders characterized by hyperphenylalaninemia.

[015] **Fig. 5** depicts a synthetic biotic for treating phenylketonuria (PKU) and disorders characterized by hyperphenylalaninemia.

[016] **Fig. 6** depicts the gene organization of an exemplary construct comprising a gene encoding PAL3 and a Tet promoter sequence on a high-copy plasmid e.g., as comprised in SYN-PKU202, SYN-PKU303.

[017] **Fig. 7** depicts the gene organization of an exemplary construct comprising a gene encoding PAL3 and an FNR promoter sequence on a low-copy plasmid, e.g., as comprised in SYN-PKU304, SYN-PKU307, SYN-PKU305, SYN-PKU306.

[018] **Fig. 8** depicts the gene organization of an exemplary construct comprising a gene encoding PAL3 and a Tet promoter sequence on a low-copy plasmid, e.g., SYN-PKU302, SYN-PKU201.

[019] **Fig. 9** depicts the gene organization of an exemplary construct, e.g., comprised in SYN-PKU401, comprising a cloned LAAD gene under the control of a Tet promoter sequence and a Tet repressor gene.

[020] **Fig. 10** depicts a schematic representation of the construction of a *pheP* knock-in strain, wherein recombineering is used to insert a second copy of *pheP* into the Nissle *lacZ* gene.

[021] **Fig. 11** depicts the gene organization of an exemplary construct comprising a gene encoding PheP, a gene encoding TetR, and a tet promoter sequence for chromosomal insertion e.g., as for example comprised in SYN-PKU203, SYN-PKU401, SYN-PKU402, SYN-PKU302, and SYN-PKU303.

[022] **Fig. 12** depicts the gene organization of an exemplary construct, comprising a cloned PAL3 gene under the control of an FNR promoter sequence, on a low-copy, kanamycin-resistant plasmid (pSC101 origin of replication, **(Fig 12A)**. Under anaerobic conditions, PAL3 degrades phenylalanine to non-toxic trans-

cinnamate. **Fig. 12B** depicts an additional copy of the endogenous E. coli high affinity phenylalanine transporter, pheP, driven by the PfnrS promoter and inserted into the lacZ locus on the Nissle chromosome.

[023] **Fig. 13** depicts schematic diagrams of non-limiting embodiments of the disclosure. **Fig. 13A** depicts phenylalanine degradation components integrated into the E. coli Nissle chromosome. In some embodiments, engineered plasmid-free bacterial strains are used to prevent plasmid conjugation in vivo. In some embodiments, multiple insertions of the PAL gene result in increased copy number and/or increased phenylalanine degradation activity. In some embodiments, a copy of the endogenous E. coli high affinity phenylalanine transporter, pheP, is driven by the PfnrS promoter and is inserted into the lacZ locus. **Fig. 13B** depicts a schematic diagram of one non-limiting embodiment of the disclosure, wherein the E. coli Nissle chromosome is engineered to contain four copies of PfnrS-PAL inserted at four different insertion sites across the genome (malE/K, yicS/nepI, agaI/rsmI, and cea), and one copy of a phenylalanine transporter gene inserted at a different insertion site (lacZ). In this embodiment, the PAL gene is PAL3 derived from *P. luminescens*, and the phenylalanine transporter gene is pheP derived from E. coli. In one embodiment, the strain is SYN-PKU511. **Fig. 13C** depicts a schematic diagram of one preferred embodiment of the disclosure, wherein the E. coli Nissle chromosome is engineered to contain five copies of PAL under the control of an oxygen level-dependent promoter (e.g., PfnrS-PAL3) inserted at different integration sites on the chromosome (malE/K, yicS/nepI, malP/T, agaI/rsmI, and cea), and one copy of a phenylalanine transporter gene under the control of an oxygen level-dependent promoter (e.g., PfnrS-pheP) inserted at a different integration site on the chromosome (lacZ). The genome is further engineered to include a thyA auxotrophy, in which the thyA gene is deleted and/or replaced with an unrelated gene, as well as a kanamycin resistance gene.

[024] **Fig. 14** depicts the gene organization of a non-limiting exemplary construct comprising a gene encoding araC and a gene encoding LAAD from Proteus mirabilis and an arabinose inducible promoter (ParaBAD) sequence for chromosomal insertion into the endogenous arabinose operon for chromosomal integration, e.g., as comprised in SYN-PKU705.

[025] **Fig. 15A** depicts phenylalanine concentrations in samples comprising bacteria expressing *PAL1* or on low-copy (LC; SYN-PKU101) or high-copy (HC; SYN-PKU102) plasmids or *PAL3* on low-copy (LC; SYN-PKU201) or high-copy (HC; SYN-PKU202) plasmids, induced with anhydrous tetracycline (ATC), and then grown in culture medium supplemented with 4 mM (660,000 ng/mL) of phenylalanine. Samples were removed at 0 hrs, 4 hrs, and 23 hrs. Phenylalanine concentrations were determined by mass spectrometry**.** **Fig. 15B** depicts cinnamate levels in samples at 4 hrs and 23 hrs post-induction. In *PAL3*-expressing strains, the *PAL3* gene is derived from *Photorhabdus luminescens*, an enterobacterium in the same taxonomic subdivision as *Escherichia coli*.

[026] **Fig. 16A** depicts phenylalanine concentrations in samples comprising bacteria expressing *PAL1* or *PAL3* on low-copy (LC) or high-copy (HC) plasmids, or further comprising a copy of *pheP* driven by the Tet promoter integrated into the chromosome. Bacteria were induced with ATC, and then grown in culture medium supplemented with 4 mM (660,000 ng/mL) of phenylalanine to an OD_{600} of 2.0. Samples were removed at 0 hrs, 2 hrs, and 4 hrs post-induction and phenylalanine concentrations were determined by mass spectrometry. Notably, the additional copy of *pheP* permitted the degradation of phenylalanine (4 mM) in 4 hrs. **Fig. 16B** depicts cinnamate levels in samples at 2 hrs and 4 hrs post-induction. In some embodiments, cinnamate may be used as an alternative biomarker for strain activity. *PheP* overexpression improves phenylalanine metabolism in engineered bacteria. Strains analyzed in this data set are SYN-PKU101, SYN-PKU102, SYN-PKU202, SYN-PKU201, SYN-PKU401, SYN-PKU402, SYN-PKU203, SYN-PKU302, SYN-PKU303.

[027] **Figs. 17A** and **17B** depict the state of one non-limiting embodiment of the *PAL* construct under non-inducing **(Fig. 17A)** and inducing **(Fig. 17B)** conditions. **Fig. 17A** depicts relatively low PAL and PheP production under aerobic conditions due to oxygen (O_2) preventing FNR from dimerizing and activating *PAL* and/or *pheP* gene expression**.** **Fig. 17B** depicts up-regulated PAL and PheP production under anaerobic conditions due to FNR dimerizing and inducing FNR promoter-mediated expression of *PAL* and *pheP* (squiggle above “PAL” and “pheP”). Arrows adjacent to a single rectangle, or a cluster of rectangles, depict the promoter responsible for driving

transcription (in the direction of the arrow) of such gene(s). Arrows above each rectangle depict the expression product of each gene.

[028] **Fig. 18** depicts β-galactosidase levels in samples comprising bacteria harboring a low-copy plasmid expressing *lacZ* from an FNR-responsive promoter selected from the exemplary FNR promoters shown **Table 3** (Pfnr1-5). Different FNR-responsive promoters were used to create a library of anaerobic-inducible reporters with a variety of expression levels and dynamic ranges. These promoters included strong ribosome binding sites. Bacterial cultures were grown in either aerobic (+O_2) or anaerobic conditions (-O_2). Samples were removed at 4 hrs and the promoter activity based on β-galactosidase levels was analyzed by performing standard β-galactosidase colorimetric assays.

[029] **Fig. 19A** 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. 19B** depicts FNR promoter activity as a function of β-galactosidase activity in SYN-PKU304. SYN-PKU304, 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. 19C** depicts the growth of bacterial cell cultures expressing *lacZ* over time, both in the presence and absence of oxygen.

[030] **Fig. 20A** and **20B** depict phenylalanine levels produced under aerobic (**Fig. 20A**) or anaerobic conditions (**Fig. 20B**) in samples of wild-type Nissle, samples of bacteria comprising a low-copy plasmid expressing *PAL3* from the Tet promoter or exemplary FNR promoters, or further comprising a copy of *pheP* driven by the Tet promoter and integrated into the chromosome. Samples were incubated in culture medium supplemented with ATC and 4 mM (660,000 ng/mL) of phenylalanine. Samples were removed at 0 hrs, 2 hrs, 4 hrs, and 24 hrs. Phenylalanine concentration was determined by mass spectrometry. These data suggest that the FNR-responsive *fnrS* promoter is as effective at activating *PAL3* expression as a tetracycline-inducible promoter under anaerobic conditions.

[031] **Fig. 21** depicts phenylalanine concentrations in cultures of synthetic probiotic strains, with and without an additional copy of *pheP* inserted on the chromosome. After 1.5 hrs of growth, cultures were placed in Coy anaerobic chamber supplying 90% N_2, 5% CO_2, and 5% H_2. After 4 hrs of induction, bacteria were resuspended in assay buffer containing 4 mM phenylalanine. Aliquots were removed from cell assays every 30 min for 3 hrs for phenylalanine quantification by mass spectrometry. Phenylalanine degradation rates in strains comprising an additional copy of *pheP* (SYN-PKU304 and SYN-PKU305; left) were higher than strains lacking an additional copy of *pheP* (SYN-PKU308 and SYN-PKU307; right).

[032] **Fig 22** depicts trans-cinnamate concentrations (PAL activity) for strains comprising single PAL3 insertions at various locations on the chromosome.

[033] **Fig. 23** depicts trans-cinnamate concentrations (PAL activity) for strains comprising multiple PAL3 insertions at various locations on the chromosome.

[034] **Fig. 24** depicts phenylalanine concentrations in cultures of synthetic probiotic strain SYN-PKU511 over time. After 2.5 hrs of growth, cultures were placed in Coy anaerobic chamber supplying 90% N2, 5% CO2, and 5% H2. After 3.5 hrs of induction in phenylalanine containing medium, whole cell extracts were prepared every 30 min for 3 hrs and phenylalanine was quantified by mass spectrometry. SYN-PKU511 comprises 5 integrated copies of an anaerobically (FNR) controlled gene encoding phenylalanine ammonia lyase (PAL) at 5 chromosomal locations and an anaerobically controlled gene encoding a high affinity Phe transporter (pheP) integrated in the lacZ locus.

[035] **Fig. 25A** and **25B** depict phenylalanine concentrations in cultures of a synthetic probiotic strain, SYN-PKU401, which comprises a high copy pUC57-plasmid with LAAD driven by a Tet inducible promoter, cells were grown in flasks shaking at 37 C, and induced with TCA at early log phase for a duration of 2 hours. Cells were spun down and re-suspended in assay buffer containing phenylalanine. Cells were measured at various cell concentrations and at varying oxygen levels. Cells were either incubated aerobically (1 ml) in a 14 ml culture tube, shaking at 250 rpm. For microaerobic conditions, cells (1 ml) were incubated in a 1.7 ml conical tube without shaking. Cells were incubated anaerobically in a Coy anaerobic chamber supplying 90% N2, 5% CO2, and 5% H2. Aliquots were removed from cell assays every 30 min for 2

hrs for phenylalanine quantification by mass spectrometry. **Fig. 25A** depicts phenylalanine concentrations under aerobic conditions using two cell densities. A and B are duplicates under the same experimental conditions. The activity in aerobic conditions is ~50umol/hr./1e9cells. **Fig. 25B** depicts phenylalanine concentrations of aerobically, microaerobically, or anaerobically grown cells.

[036] **Fig. 26A** shows phenylalanine concentrations before and after feeding in an *in vivo* mouse model of PKU. At the beginning of the study, homozygous BTBR-Pah^{enu2} mice were given water supplemented with 100 micrograms/mL ATC and 5% sucrose. Mice were fasted by removing chow overnight (10 hrs), and blood samples were collected by mandibular bleeding the next morning in order to determine baseline phenylalanine levels. Mice were given chow again, gavaged with 100 microliters ($5x10^9$ CFU) of bacteria (SYN-PKU302 or control Nissle) after 1 hr., and allowed to feed for another 2 hrs. Serum phenylalanine concentrations were determined 2 hrs post-gavage. **Fig. 26B** shows the percent (%) change in blood phenylalanine concentrations before and after feeding as a male or female group average ($p < 0.01$).

[037] **Figs. 27A** and **27B** depict blood phenylalanine concentrations relative to baseline following subcutaneous phenylalanine challenge in an *in vivo* mouse model of PKU. Mice were orally gavaged with 200 µL of H_2O (n=30), SYN-PKU901 (n=33), or SYN-PKU303 (n=34) at 30 and 90 minutes post-phenylalanine injection (0.1 mg/gram of average group body weight). **Figs. 27A** and **27B** show blood phenylalanine concentrations at 2 hrs and 4 hrs post-phenylalanine injection, respectively. These data indicate that oral administration of the engineered probiotic strain SYN-PKU303 significantly reduces blood phenylalanine levels in mice, compared to mice administered mock treatment (H_2O) or the parental strain (SYN-PKU901) (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.00001$). SYN-PKU303 is capable of intercepting enterorecirculating phenylalanine.

[038] **Fig. 28** depicts blood phenylalanine concentrations relative to baseline following subcutaneous phenylalanine challenge in an *in vivo* mouse model of PKU. Mice were orally gavaged with 200 µL of H_2O (n=30), SYN-PKU901 (n=33), SYN-PKU303 (n=34), or SYN-PKU304 (n=34) at 30 and 90 minutes post-phenylalanine injection (0.1 mg/gram of average group body weight). Figs. 23A and 23B Blood phenylalanine concentrations post phenylalanine injection indicate that SYN-PKU304

(low copy plasmid containing fnrS-PAL) is at least as effective as SYN-PKU303 (high copy plasmid containing Tet-PAL) in reducing circulating Phe levels in the enterorecirculation model.

[039] **Figs. 29A** and **29B** depict blood phenylalanine concentrations relative to baseline following subcutaneous phenylalanine challenge in an in vivo mouse model of PKU. Mice were orally gavaged with H2O, SYN-PKU901, SYN-PKU303, or SYN-PKU304 at 30 and 90 minutes post-phenylalanine injection (0.1 mg/gram of average group body weight). **Figs. 29A** and **29B** show blood phenylalanine concentrations at 2 hrs and 4 hrs post-phenylalanine injection, respectively. These data indicate that oral administration of engineered probiotic strains SYN-PKU303 and SYN-PKU304 significantly reduces blood phenylalanine levels in mice compared to mice administered mock treatment (H2O) or the parental strain (SYN-PKU901) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). **Figs. 29C** and **29D** depict scatter plots of the data shown in **Figs. 29A** and **29B**.

[040] **Figs. 30A** and **30B** depict blood phenylalanine concentrations relative to baseline following subcutaneous phenylalanine challenge in an in vivo mouse model of PKU. Mice were orally gavaged with 200 μL of H2O (n=12), 200 μL of SYN-PKU901 (n=12), or 100, 200, or 400 μL of SYN-PKU304 (n=12 in each dose group) at 30 and 90 minutes post-phenylalanine injection (0.1 mg/gram of average group body weight). **Figs. 30A** and **30B** show a dose-dependent decrease in blood phenylalanine levels in SYN-PKU304-treated mice compared to mice administered mock treatment (H2O) or the parental strain (SYN-PKU901) (* 30% decrease; $p < 0.05$). This experiment represents one of eight studies of this same design, and each one shows that SYN-PKU304 is capable of intercepting enterorecirculating phenylalanine.

[041] **Figs. 31A** and **31B** depicts a schematic of PKU specific and PAL specific phenylalanine metabolites. **Fig. 31A** depicts a schematic of the conversion of phenylalanine to phenylpyruvic acid and phenyllactic acid in the absence of functional PAH. **Fig. 31B** depicts a schematic of the conversion of phenylalanine to trans-cinnamic acid by PAL3, which is further metabolized to hippuric acid by liver enzymes. These metabolites can be detected by mass spectrometry as described in Examples 24-26 or by other means.

[042] **Figs. 32A**, **32B**, **32C**, **32D**, **32E**, and **32F** depict blood phenylalanine concentrations relative to baseline and concentrations of phenylalanine (**Fig. 32A**), and absolute values of phenylalanine and PKU specific and PAL specific metabolites (**Figs. 32B, 32C, 32D, 32E**, and **32F**) following subcutaneous phenylalanine challenge in an in vivo mouse model of PKU. Mice were orally gavaged with a total of 800 µL of H2O (n= 12), SYN-PKU901 (n= 12), or 800 µL of SYN-PKU304 (n= 12) (2.9e10 cfu/mouse) at 30 and 90 minutes post-phenylalanine injection. **Fig. 32A** depicts blood phenylalanine concentrations relative to baseline; total metabolic activity for SYN-PKU304 was calculated as 81.2umol/hr. and the total reduction in Δphe was 45% relative to SYN-PKU901 (P<0.05). **Fig. 32B** depicts the blood phenylalanine concentration at 0 and 4 hours post phenylalanine injection. **Fig. 32C** depicts the blood phenylpyruvate concentration at 0 and 4 hours post phenylalanine injection. **Fig. 32D** depicts the blood phenyllactate concentration at 0 and 4 hours post phenylalanine injection. **Fig. 32E** depicts the blood t-cinnamic acid concentration at 0 and 4 hours post phenylalanine injection. **Fig. 32F** depicts the blood hippuric acid concentration at 0 and 4 hours post phenylalanine injection.

[043] **Figs. 33A, 33B, 33C, 33D, 33E**, and **33F** depict blood phenylalanine concentrations relative to baseline and concentrations of phenylalanine (**Fig. 33A**), and absolute values of phenylalanine and PKU specific and PAL specific metabolites (**Figs. 33B, 33C, 33D, 33E**, and **33F**) following subcutaneous phenylalanine challenge in an in vivo mouse model of PKU. Mice were orally gavaged with a total of 800 µL of H2O (n=9]), SYN-PKU801 (n=12), or 800 µL of SYN-PKU517 (n=12) (3.6e10 cfu/mouse) at 30 and 90 minutes post-phenylalanine injection. **Fig. 33A** depicts blood phenylalanine concentrations relative to baseline; total metabolic activity for SYN-PKU517 was calculated as 39.6umol/hr. and the total reduction in Δphe was 17% relative to SYN-PKU801 (P<0.05). **Fig. 33B** depicts the blood phenylalanine concentration at 0 and 4 hours post phenylalanine injection. **Fig. 33C** depicts the blood phenylpyruvate concentration at 0 and 4 hours post phenylalanine injection. **Fig. 33D** depicts the blood phenyllactate concentration at 0 and 4 hours post phenylalanine injection**. Fig. 33E** depicts the blood t-cinnamic acid concentration at 0 and 4 hours post phenylalanine injection. **Fig. 33F** depicts the blood hippuric acid concentration at 0 and 4 hours post phenylalanine injection.

[044] **Figs. 34A, 34B, 34C, 34D, 34E**, and **34F** depict blood phenylalanine concentrations relative to baseline and concentrations of phenylalanine (**Fig. 34A**), and absolute values of phenylalanine and PKU specific and PAL specific metabolites (**Figs. 34B, 34C, 34D, 34E**, and **34F**) following subcutaneous phenylalanine challenge in an in vivo mouse model of PKU. Mice were orally gavaged with a total of 800 µL of H2O (n=12), SYN-PKU901 (n=12), or 800 µL of SYN-PKU705 (n=12) (3.6e10 cfu/mouse) at 30 and 90 minutes post-phenylalanine injection. **Fig. 34A** depicts blood phenylalanine concentrations relative to baseline; total metabolic activity for SYN-PKU705 was calculated as 133.2 umol/hr. and the total reduction in Δphe was 30% relative to SYN-PKU901 (P<0.05). **Fig. 34B** depicts the blood phenylalanine concentration at 0 and 4 hours post phenylalanine injection. **Fig. 34C** depicts the blood phenylpyruvate concentration at 0 and 4 hours post phenylalanine injection. **Fig. 34D** depicts the blood phenyllactate concentration at 0 and 4 hours post phenylalanine injection**. Fig. 34E** depicts the blood t-cinnamic acid concentration at 0 and 4 hours post phenylalanine injection. **Fig. 34F** depicts the blood hippuric acid concentration at 0 and 4 hours post phenylalanine injection.

[045] **Fig. 35** depicts phenylalanine and 2 toxic analogs, p-fluoro-DL-phenylalanine, and o-fluoro-DL-phenylalanine, which are useful for an untargeted approach to select PAL enzymes with increased activity. P-fluoro-DL-phenylalanine, and o-fluoro-DL-phenylalanine are incorporated into cellular protein in the place of phenylalanine, resulting in cell death. Since these compounds are readily taken up by PheP, and can act as a substrate for PAL as shown below, they can be employed in genetic selection and screening for the identification of strains with improved Phe consumption activity. Mutations allowing more efficient PAL metabolism may prevent the incorporation of the phenylalanine analog into cellular protein, therefore allowing growth under higher concentrations of the analog.

[046] **Fig. 36** depicts a map of exemplary integration sites within the *E. coli* 1917 Nissle chromosome. These sites indicate regions where circuit components may be inserted into the chromosome without interfering with essential gene expression. Backslashes (/) are used to show that the insertion will occur between divergently or convergently expressed genes. Insertions within biosynthetic genes, such as *thyA*, can

be useful for creating nutrient auxotrophies. In some embodiments, an individual circuit component is inserted into more than one of the indicated sites.

[047] **Fig. 37** depicts three bacterial strains which constitutively express red fluorescent protein (RFP). In strains 1-3, the *rfp* gene has been inserted into different sites within the bacterial chromosome, and results in varying degrees of brightness under fluorescent light. Unmodified *E. coli* Nissle (strain 4) is non-fluorescent.

[048] **Fig. 38** depicts a graph of Nissle residence *in vivo*. Streptomycin-resistant Nissle was administered to mice via oral gavage without antibiotic pre-treatment. Fecal pellets from 6 total mice were monitored post-administration to determine the amount of administered Nissle still residing within the mouse gastrointestinal tract. The bars represent the number of bacteria administered to the mice. The line represents the number of Nissle recovered from the fecal samples each day for 10 consecutive days.

[049] **Figs. 39A** and **39B** depict phenylalanine concentrations in SYN-PKU302 cultures over time. After 1.5 hrs of growth, ATC was added to cultures of SYN-PKU302, and SYN-PKU304 cultures were placed in Coy anaerobic chamber supplying 90% N_2, 5% CO_2, and 5% H_2. After 4 hrs of induction, bacteria were resuspended in assay buffer containing 4 mM phenylalanine and at different pH (pH range 7.25-2.25). Aliquots were removed from cell assays every 30 min for 2 hrs for phenylalanine quantification by mass spectrometry. Phenylalanine degradation rates decreased as pH of the assay buffer decreased in both strains, SYN-PKU302 (**Fig. 39A**) and SYN-PKU304 **(Fig. 39B)**.

[050] **Fig. 40** depicts an exemplary schematic of the E. coli 1917 Nissle chromosome comprising multiple mechanisms of action (MoAs).

[051] **Fig. 41** depicts the gene organization of an exemplary construct in which the *PAL3* and *pheP* genes are co-transcribed under the control of an exemplary FNR promoter (P_{fnrS}).

[052] **Figs. 42A** and **42B** depict the gene organization of an exemplary construct in which the *Int5* recombinase gene is operably linked to an exemplary FNR promoter (P_{fnrS}), and the *PAL3* gene is operably linked to a strong constitutive promoter. **Fig. 42A** depicts a schematic diagram of the *PAL3* gene, flanked by Int5 sites, in the

OFF orientation (3' to 5'). When *Int5* gene expression is activated under anaerobic conditions, recombinatorial flipping of *PAL3* to the ON orientation (5' to 3'; **Fig. 42B**) leads to the production of PAL3 and to phenylalanine metabolism. Any strong constitutive promoter sequence may be used.

[053] **Figs. 43A, 43B,** and **43C** depict the gene organization of an exemplary construct in which the *Int5* recombinase gene is operably linked to an FNR promoter (P_{fnrS}), and the gene encoding T7 RNA polymerase is flanked by recombinase sites and operably linked to a strong constitutive promoter. **Fig. 43A** depicts a schematic diagram of the T7 RNA polymerase gene, flanked by Int5 sites, in the OFF orientation. When *Int5* gene expression is activated under anaerobic conditions, the T7 RNA polymerase gene is flipped to the ON orientation (**Fig. 43B**). In engineered bacterial strains comprising a copy of *PAL3* under the control of a T7-driven promoter (P_{T7}; **Fig. 43C**), T7 RNA polymerase expression leads to the production of PAL3 and to phenylalanine metabolism.

[054] **Figs. 44A, 44B, and 44C** depict the gene organization of an exemplary construct in which the *Int5* recombinase gene is operably linked to an ParaBAD promoter (P_{araBAD}), and the gene encoding T7 RNA polymerase is flanked by recombinase sites and operably linked to a strong constitutive promoter. **Fig. 43A** depicts a schematic diagram of the T7 RNA polymerase gene, flanked by Int5 sites, in the OFF orientation. When *Int5* gene expression is activated under anaerobic conditions, the T7 RNA polymerase gene is flipped to the ON orientation (**Fig. 43B**). In engineered bacterial strains comprising a copy of *PAL3* under the control of a T7-driven promoter (P_{T7}; **Fig. 43C**), T7 RNA polymerase expression leads to the production of PAL3 and to phenylalanine metabolism.

[055] **Fig. 45A** depicts a schematic of a recombinase-based switch to activate *PAL3* expression using different inducible promoters and ribosome binding sites. Recombinase expression causes recombinatorial flipping of the *PAL3* gene to the ON orientation, leading to the production of PAL3 and to the degradation of phenylalanine. In some embodiments, recombinase-based switches are tuned to respond to specific levels of an inducer. **Fig. 45B** depicts the relationship between the concentration of an inducer and the percentage of *PAL3*-containing constructs in the ON orientation. The shaded area shows the predicted efficacy range of the inducer *in vivo*.

[056] **Fig. 46A** 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 (P_{araBAD}), which induces expression of the Tet repressor (TetR) and an anti-toxin. The anti-toxin builds up in the recombinant bacterial cell, while TetR prevents expression of a toxin (which is under the control of a promoter having a TetR binding site). However, when arabinose is not present, both the anti-toxin and TetR are not expressed. Since TetR is not present to repress expression of the toxin, the toxin is expressed and kills the cell. **Fig. 46A** 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.

[057] **Fig. 46B** 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.

[058] **Fig. 46C** 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.

[059] **Fig. 47** depicts the use of GeneGuards as an engineered safety component. All engineered DNA is present on a plasmid which can be conditionally destroyed. *See, e.g.*, Wright et al., 2015.

[060] **Fig. 48A** depicts a schematic diagram of a wild-type *clbA* construct. **Fig. 48B** depicts a schematic diagram of a *clbA* knockout construct.

[061] **Fig. 49** depicts exemplary sequences of a wild-type *clbA* construct and a *clbA* knockout construct.

[062] **Fig. 50** depicts a schematic of a secretion system based on the flagellar type III secretion in which a modified 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 so that the intracellularly expressed chimeric peptide can be mobilized across the inner and outer membranes into the surrounding host environment.

[063] **Fig. 51** 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 auto-secreter. 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 threaded

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.

[064] **Fig. 52** 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 secreter; 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.

[065] **Fig. 53** depicts a schematic of the outer and inner membranes of a gram-negative bacterium, and several deletion targets for generating a leaky or destabilized outer membrane, thereby facilitating the translocation of a therapeutic polypeptides to the extracellular space, e.g., therapeutic polypeptides of eukaryotic origin containing disulphide bonds. Deactivating mutations of one or more genes encoding a protein that tethers the outer membrane to the peptidoglycan skeleton, e.g., lpp, ompC, ompA, ompF, tolA, tolB, pal, and/or one or more genes encoding a periplasmic protease, e.g., degS, degP, nlpl, generates a leaky phenotype. Combinations of mutations may synergistically enhance the leaky phenotype.

[066] **Fig. 54** depicts a schematic of non-limiting processes for designing and producing the genetically engineered bacteria of the present disclosure.

[067] **Fig. 55** depicts a schematic of non-limiting manufacturing processes for upstream and downstream production of the genetically engineered bacteria of the present disclosure. **Figs. 55A, B, C, D,** and **E** depict a schematic of non-limiting manufacturing processes for upstream and downstream production of the genetically engineered bacteria of the present disclosure. **Fig. 55A** depicts the parameters for starter culture 1 (SC1): loop full – glycerol stock, duration overnight, temperature 37° C, shaking at 250 rpm. **Fig. 55B** 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. 55C** 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. 55D** 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. 55E** depicts the parameters for vial fill/storage: 1-2 mL aliquots, -80° C.

Description of Embodiments

[068] The present disclosure includes genetically engineered bacteria, pharmaceutical compositions thereof, and methods of modulating and treating disorders associated with hyperphenylalaninemia. In some embodiments, the genetically engineered bacteria comprise a gene encoding non-native phenylalanine ammonia lyase (PAL) and are capable of processing and reducing phenylalanine in a mammal. Thus, the genetically engineered bacteria and pharmaceutical compositions comprising those bacteria may be used to metabolize phenylalanine in the body into non-toxic molecules in order to treat and/or prevent conditions associated with hyperphenylalaninemia, including PKU. In certain aspects, the compositions comprising the genetically engineered bacteria may be used in the methods of the disclosure to treat and/or prevent disorders associated with hyperphenylalaninemia.

[069] 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.

[070] "Hyperphenylalaninemia," "hyperphenylalaninemic," and "excess phenylalanine" are used interchangeably herein to refer to increased or abnormally high concentrations of phenylalanine in the body. In some embodiments, a diagnostic signal of hyperphenylalaninemia is a blood phenylalanine level of at least 2 mg/dL, at least 4 mg/dL, at least 6 mg/dL, at least 8 mg/dL, at least 10 mg/dL, at least 12 mg/dL, at least 14 mg/dL, at least 16 mg/dL, at least 18 mg/dL, at least 20 mg/dL, or at least 25 mg/dL. As used herein, diseases associated with hyperphenylalaninemia include, but are not limited to, phenylketonuria, classical or typical phenylketonuria, atypical phenylketonuria, permanent mild hyperphenylalaninemia, nonphenylketonuric

hyperphenylalaninemia, phenylalanine hydroxylase deficiency, cofactor deficiency, dihydropteridine reductase deficiency, tetrahydropterin synthase deficiency, and Segawa's disease. Affected individuals can suffer progressive and irreversible neurological deficits, mental retardation, encephalopathy, epilepsy, eczema, reduced growth, microcephaly, tremor, limb spasticity, and/or hypopigmentation (Leonard 2006). Hyperphenylalaninemia can also be secondary to other conditions, *e.g.*, liver diseases.

[071] "Phenylalanine ammonia lyase" and "PAL" are used to refer to a phenylalanine metabolizing enzyme (PME) that converts or processes phenylalanine to trans-cinnamic acid and ammonia. Trans-cinnamic acid has low toxicity and is converted by liver enzymes in mammals to hippuric acid, which is secreted in the urine. PAL may be substituted for the enzyme PAH to metabolize excess phenylalanine. PAL enzyme activity does not require THB cofactor activity. In some embodiments, PAL is encoded by a *PAL* gene derived from a prokaryotic species. In alternate embodiments, PAL is encoded by a *PAL* gene derived from a eukaryotic species. In some embodiments, PAL is encoded by a *PAL* gene derived from a bacterial species, including but not limited to, *Achromobacter xylosoxidans, Pseudomonas aeruginosa, Photorhabdus luminescens, Anabaena variabilis,* and *Agrobacterium tumefaciens*. In some embodiments, PAL is encoded by a *PAL* gene derived from *Anabaena variabilis* and referred to as "PAL1" herein (Moffitt et al., 2007). In some embodiments, PAL is encoded by a *PAL* gene derived from *Photorhabdus luminescens* and referred to as "PAL3" herein (Williams et al., 2005). In some embodiments, PAL is encoded by a *PAL* gene derived from a yeast species, *e.g.*, *Rhodosporidium toruloides* (Gilbert et al., 1985). In some embodiments, PAL is encoded by a *PAL* gene derived from a plant species, *e.g.*, *Arabidopsis thaliana* (Wanner et al., 1995). Any suitable nucleotide and amino acid sequences of PAL, or functional fragments thereof, may be used.

[072] "Phenylalanine hydroxylase" and "PAH" are used to refer to an enzyme that catalyzes the hydroxylation of the aromatic side chain of phenylalanine to create tyrosine in the human body in conjunction with the cofactor tetrahydrobiopterin. The human gene encoding PAH is located on the long (q) arm of chromosome 12 between positions 22 and 24.2. The amino acid sequence of PAH is highly conserved among mammals. Nucleic acid sequences for human and mammalian *PAH* are well known and

widely available. The full-length human cDNA sequence for *PAH* was reported in 1985 (Kwok et al. 1985). Active fragments of *PAH* are also well known (*e.g.*, Kobe et al. 1997).

[073] "L-Aminoacid Deaminase" and "LAAD" are used to refer to an enzyme that catalyzes the stereospecific oxidative deamination of L-amino acids to generate their respective keto acids, ammonia, and hydrogen peroxide. For example, LAAD catalyzes the conversion of phenylalanine to phenylpyruvate. Multiple LAAD enzymes are known in the art, many of which are derived from bacteria, such as Proteus, Providencia, and Morganella, or venom. LAAD is characterized by fast reaction rate of phenylalanine degradation (Hou et al., Appl Microbiol Technol. 2015 Oct;99(20):8391-402; "Production of phenylpyruvic acid from L-phenylalanine using an L-amino acid deaminase from Proteus mirabilis: comparison of enzymatic and whole-cell biotransformation approaches"). Most eukaryotic and prokaryotic L-amino acid deaminases are extracellular; however, Proteus species LAAD are localized to the plasma membrane (inner membrane), facing outward into the periplasmic space, in which the enzymatic activity resides. As a consequence of this localization, phenylalanine transport through the inner membrane into the cytoplasm is not required for Proteus LAAD mediated phenylalanine degradation. Phenylalanine is readily taken up through the outer membrane into the periplasm without a transporter, eliminating the need for a transporter to improve substrate availability.

[074] In some embodiments, the genetically engineered bacteria comprise a LAAD gene derived from a bacterial species, including but not limited to, Proteus, Providencia, and Morganella bacteria. In some embodiments, the bacterial species is Proteus mirabilis. In some embodiments, the bacterial species is Proteus vulgaris. In some embodiments, the LAAD encoded by the genetically engineered bacteria is localized to the plasma membrane, facing into the periplasmic space and with the catalytic activity occurring in the periplasmic space.

[075] "Phenylalanine metabolizing enzyme" or "PME" are used to refer to an enzyme which is able to degrade phenylalanine. Any phenylalanine metabolizing enzyme known in the art may be encoded by the genetically engineered bacteria. PMEs include, but are not limited to, phenylalanine hydroxylase (PAH), phenylalanine

ammonia lyase (PAL), aminotransferase, L-amino acid deaminase (L-AAD), and phenylalanine dehydrogenases.

[076] Reactions with phenylalanine hydroxylases, phenylalanine dehydrogenases or aminotransferases require cofactors, while L-AAD and PAL do not require any additional cofactors. In some embodiments, the PME encoded by the genetically engineered bacteria requires a cofactor. In some embodiments, this cofactor is provided concurrently or sequentially with the administration of the genetically engineered bacteria. In other embodiments, the genetically engineered bacteria can produce the cofactor. In some embodiments, the genetically engineered bacteria encode a phenylalanine hydroxylase. In some embodiments, the genetically engineered bacteria encode a phenylalanine dehydrogenase. In some embodiments, the genetically engineered bacteria encode an aminotransferase. In some embodiments, the PME encoded by the genetically engineered bacteria does not require a cofactor. Without wishing to be bound by theory, the lack of need for a cofactor means that the rate of phenylalanine degradation by the enzyme is dependent on the availability of the substrate and is not limited by the availability of the cofactor. In some embodiments, the PME produced by the genetically engineered bacteria is PAL. In some embodiments, the PME produced by the genetically engineered bacteria is LAAD. In some embodiments, the genetically engineered bacteria encode combinations of PMEs.

[077] In some embodiments, the catalytic activity of the PME is dependent on oxygen levels. In some embodiments, the PME is catalytically active under microaerobic conditions. As a non-limiting example, LAAD catalytic activity is dependent on oxygen. In some embodiments, LAAD is active under low oxygen conditions, such as microaerobic conditions. In some embodiments, of the invention, the PME functions at very low levels of oxygen or in the absence of oxygen, e.g. as found in the colon. As a non-limiting example, PAL activity is not dependent on the presence of oxygen.

[078] In certain embodiments, new or improved PMEs can be identified according to methods known in the art or described herein, and are encoded by the genetically engineered bacteria. In some embodiments, the enzyme encoded by the genetically engineered bacteria is a wild type enzyme isolated from a viral, prokaryotic or eukaryotic organism. In some embodiments, the enzyme sequence has been further

modified or mutated to increase one or more specific properties of the enzyme, such as stability or catalytic activity.

[079] "Phenylalanine metabolite" refers to a metabolite that is generated as a result of the degradation of phenylalanine. The metabolite may be generated directly from phenylalanine, by the enzyme using phenylalanine as a substrate, or indirectly by a different enzyme downstream in the metabolic pathway, which acts on a phenylalanine metabolite substrate. In some embodiments, phenylalanine metabolites are produced by the genetically engineered bacteria encoding a PME.

[080] In some embodiments, the phenylalanine metabolite results directly or indirectly from PAH activity, e.g., from PAH produced by the genetically engineered bacteria. In some embodiments, the metabolite is tyrosine. In some embodiments, the phenylalanine metabolite accumulates in the blood or the urine of a PKU patient, due to defective PAH activity. Non-limiting examples of such PKU metabolites are phenylpyruvic acid and phenyl-lactic acid. Other examples include phenylacetate, phenylethylamine, and phenylacetyl glutamine.

[081] In some embodiments, the phenylalanine metabolite results directly or indirectly from PAL action, e.g., from PAL produced by the genetically engineered bacteria. Non-limiting examples of such PAL metabolites are trans-cinnamic acid and hippuric acid. In some embodiments, the phenylalanine metabolite results directly or indirectly from LAAD action, e.g., from LAAD produced by the genetically engineered bacteria. Examples of such LAAD metabolites are phenylpyruvate and phenyllactic acid.

[082] "Phenylalanine transporter" is used to refer to a membrane transport protein that is capable of transporting phenylalanine into bacterial cells (*see, e.g.*, Pi et al., 1991). In *Escherichia coli*, the *pheP* gene encodes a high affinity phenylalanine-specific permease responsible for phenylalanine transport (Pi et al., 1998). In some embodiments, the phenylalanine transporter is encoded by a *pheP* gene derived from a bacterial species, including but not limited to, *Acinetobacter calcoaceticus, Salmonella enterica,* and *Escherichia coli.* Other phenylalanine transporters include Aageneral amino acid permease, encoded by the aroP gene, transports three aromatic amino acids, including phenylalanine, with high affinity, and is thought, together with PheP, responsible for the lion share of phenylalanine import. Additionally, a low level of

phenylalanine transport activity has been traced to the activity of the LIV-I/LS system, which is a branched-chain amino acid transporter consisting of two periplasmic binding proteins, the LIV-binding protein (LIV-I system) and LS-binding protein (LS system), and membrane components, LivHMGF. In some embodiments, the phenylalanine transporter is encoded by a *aroP* gene derived from a bacterial species. In some embodiments, the phenylalanine transporter is encoded by LIV-binding protein and LS-binding protein and LivHMGF genes derived from a bacterial species. In some embodiments, the genetically engineered bacteria comprise more than one type of phenylalanine transporter, selected from pheP, aroP, and the LIV-I/LS system.

[083] “Phenylalanine” and “Phe” are used to refer to an amino acid with the formula $C_6H_5CH_2CH(NH_2)COOH$. Phenylalanine is a precursor for tyrosine, dopamine, norepinephrine, and epinephrine. L-phenylalanine is an essential amino acid and the form of phenylalanine primarily found in dietary protein; the stereoisomer D-phenylalanine is found is lower amounts in dietary protein; DL-phenylalanine is a combination of both forms. Phenylalanine may refer to one or more of L-phenylalanine, D-phenylalanine, and DL-phenylalanine.

[084] “Operably linked” refers a nucleic acid sequence, *e.g.*, a gene encoding PAL, that is joined to a regulatory region sequence in a manner which allows expression of the nucleic acid sequence, *e.g.*, acts in *cis*. A regulatory region is a nucleic acid that can direct transcription of a gene of interest and may comprise promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, promoter control elements, protein binding sequences, 5′ and 3′ untranslated regions, transcriptional start sites, termination sequences, polyadenylation sequences, and introns.

[085] 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.

[086] A “directly inducible promoter” refers to a regulatory region, wherein the regulatory region is operably linked to a gene encoding a phenylalanine-metabolizing enzyme, *e.g.*, *PAL*; in the presence of an inducer of said regulatory region, the phenylalanine-metabolizing enzyme is expressed. An “indirectly inducible promoter” refers to a regulatory system comprising two or more regulatory regions, for example, a

first regulatory region that is operably linked to a gene encoding a first molecule, *e.g.*, a transcriptional regulator, which is capable of regulating a second regulatory region that is operably linked to a gene encoding a phenylalanine-metabolizing enzyme. In the presence of an inducer of the first regulatory region, the second regulatory region may be activated or repressed, thereby activating or repressing expression of the phenylalanine-metabolizing enzyme. Both a directly inducible promoter and an indirectly inducible promoter are encompassed by "inducible promoter."

[087] "Exogenous environmental conditions" refer to settings or circumstances under which the promoter described above is directly or indirectly induced. 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, exogenous environmental conditions refer to the presence of molecules or metabolites that are specific to the mammalian gut in a healthy or disease state, *e.g.*, propionate. In some embodiments, the exogenous environmental conditions are low-oxygen, microaerobic, or anaerobic conditions, such as the environment of the mammalian gut.

[088] "Exogenous environmental condition(s)" refer to setting(s) or circumstance(s) under which the promoter described herein is 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 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 comprises 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.

[089] 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.

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

[091] 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*

[092] As used herein, a "non-native" nucleic acid sequence refers to a nucleic acid sequence not normally present in a bacterium, *e.g.*, an extra copy of an endogenous sequence, or a heterologous sequence such as a sequence from a different species, strain, or substrain of bacteria, or a sequence that is modified and/or mutated as compared to the unmodified sequence from bacteria of the same subtype. In some embodiments, the non-native nucleic acid sequence is a synthetic, non-naturally occurring sequence (*see, e.g.,* Purcell et al., 2013). The non-native nucleic acid sequence may be a regulatory region, a promoter, a gene, and/or one or more genes in a gene cassette. In some embodiments, "non-native" refers to two or more nucleic acid sequences that are not found in the same relationship to each other in nature. The non-native nucleic acid sequence may be present on a plasmid or chromosome. In addition, multiple copies of any regulatory region, promoter, gene, and/or gene cassette may be present in the bacterium, wherein one or more copies of the regulatory region, promoter, gene, and/or gene cassette may be mutated or otherwise altered as described herein. In some embodiments, the genetically engineered bacteria are engineered to comprise multiple copies of the same regulatory region, promoter, gene, and/or gene cassette in order to enhance copy number or to comprise multiple different components of a gene cassette performing multiple different functions. In some embodiments, the genetically engineered bacteria of the invention comprise a gene encoding a phenylalanine-metabolizing enzyme that is operably linked to a directly or indirectly inducible

promoter that is not associated with said gene in nature, *e.g.*, an FNR promoter operably linked to a gene encoding PAL or a ParaBAD promoter operably linked to LAAD.

[093] "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)), a bacteriophage SP6 promoter (*e.g.*, SP6 promoter (BBa_J64998)), and functional fragments thereof.

[094] "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.

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

[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 or proteins of interest. In certain aspects, the microorganism is engineered to take up and catabolize certain metabolites or other compounds from its environment, e.g., the gut. In certain aspects, the microorganism is engineered to synthesize certain beneficial metabolites or other compounds (synthetic or naturally occurring) and release them into its environment. In certain embodiments, the engineered microorganism is an engineered bacterium. In certain embodiments, the engineered microorganism is an engineered virus.

[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 are commensal bacteria, which are present in the indigenous microbiota of the gut. Examples of non-pathogenic bacteria include, but are not limited to, *Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Clostridium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Saccharomyces,* and *Staphylococcus, e.g., Bacillus*

coagulans, *Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium bifidum*, *Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium butyricum, Enterococcus faecium, Escherichia coli, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei*, *Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis,* and *Saccharomyces boulardii* (Sonnenborn et al., 2009; Dinleyici et al., 2014; U.S. Patent No. 6,835,376; U.S. Patent No. 6,203,797; U.S. Patent No. 5,589,168; U.S. Patent No. 7,731,976). Naturally pathogenic bacteria may be genetically engineered to provide 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. Some species, strains, and/or subtypes of non-pathogenic bacteria are currently recognized as probiotic. Examples of probiotic bacteria include, but are not limited to, *Bifidobacteria*, *Escherichia*, *Lactobacillus,* and *Saccharomyces, e.g.*, *Bifidobacterium bifidum, Enterococcus faecium, Escherichia coli, Escherichia coli* strain Nissle, *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Saccharomyces boulardii* (Dinleyici et al., 2014; U.S. Patent No. 5,589,168; U.S. Patent No. 6,203,797; U.S. Patent 6,835,376). The probiotic may be a variant or a mutant strain of bacterium (Arthur et al., 2012; Cuevas-Ramos et al., 2010; Olier et al., 2012; Nougayrede et al., 2006). Non-pathogenic bacteria may be genetically engineered to enhance or improve desired biological properties, *e.g.*, survivability. Non-pathogenic bacteria may be genetically engineered to provide probiotic properties. Probiotic bacteria may be genetically engineered to enhance or improve probiotic properties.

[099] 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 *PAL* gene, which is incorporated into the host genome or propagated on a self-replicating extra-chromosomal plasmid, such that the non-native genetic material is retained, expressed, and/or propagated. The stable bacterium is capable of survival and/or growth *in vitro*,

e.g., in medium, and/or *in vivo*, *e.g.*, in the gut. For example, the stable bacterium may be a genetically modified bacterium comprising a *PAL* gene, in which the plasmid or chromosome carrying the *PAL* gene is stably maintained in the host cell, such that *PAL* can be expressed in the host cell, and the host cell is capable of survival and/or growth *in vitro* and/or *in vivo*. In some embodiments, copy number affects the stability of expression of the non-native genetic material, *e.g.*, a *PAL* gene or a *PAH* gene. In some embodiments, copy number affects the level of expression of the non-native genetic material, *e.g.*, a *PAL* gene or a *PAH* gene.

[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. Primary hyperphenylalaninemia, *e.g.*, PKU, is caused by inborn genetic mutations for which there are no known cures. Hyperphenylalaninemia can also be secondary to other conditions, *e.g.*, liver diseases. Treating hyperphenylalaninemia may encompass reducing or eliminating excess phenylalanine and/or associated symptoms, and does not necessarily encompass the elimination of the underlying disease.

[0102] As used herein a "pharmaceutical composition" refers to a preparation of genetically engineered bacteria of the invention with other components such as a physiologically suitable carrier and/or excipient.

[0103] 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.

[0104] The term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples include, but are not limited to, calcium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20.

[0105] The terms “therapeutically effective dose” and “therapeutically effective amount” are used to refer to an amount of a compound that results in prevention, delay of onset of symptoms, or amelioration of symptoms of a condition, *e.g.*, hyperphenylalaninemia. 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 a disease or condition associated with excess phenylalanine levels. 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.

[0106] 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 “dipeptide” refers to a peptide of two linked amino acids. The term “tripeptide” refers to a peptide of three linked amino acids. 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.

[0107] 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.

[0108] 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.

[0109] 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.

[0110] 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.

[0111] As used herein the term "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.

[0112] 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.

[0113] As used herein, the terms “secretion system” or “secretion protein” refers to a native or non-native secretion mechanism capable of secreting or exporting the protein(s) of interest or therapeutic protein(s) 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 proteins of interest include a “secretion tag” of either RNA or peptide origin to direct the protein(s) of interest or therapeutic protein(s) to specific secretion systems. In some embodiments, the secretion system is able to remove this tag before secreting the protein(s) of interest 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 protein(s) of interest into the extracellular milieu.]]

[0114] As used herein, the term “transporter” is meant to refer to a mechanism, e.g., protein or proteins, for importing a molecule, e.g., amino acid, toxin, metabolite, substrate, etc. into the microorganism from the extracellular milieu.

[0115] The articles “a” and “an,” as used herein, should be understood to mean “at least one,” unless clearly indicated to the contrary.

[0116] The phrase “and/or,” when used between elements in a list, is intended to mean either (1) that only a single listed element is present, or (2) that more than one element of the list is present. For example, “A, B, and/or C” indicates that the selection

may be A alone; B alone; C alone; A and B; A and C; B and C; or A, B, and C. The phrase "and/or" may be used interchangeably with "at least one of" or "one or more of" the elements in a list.

Bacteria

[0117] The genetically engineered bacteria of the invention are capable of reducing excess phenylalanine. 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. In some embodiments, non-pathogenic bacteria are Gram-negative bacteria. In some embodiments, non-pathogenic bacteria are Gram-positive bacteria. Exemplary bacteria include, but are not limited to, *Bacillus, Bacteroides, Bifidobacterium*, *Brevibacteria, Clostridium, Enterococcus, Escherichia coli, Lactobacillus, Lactococcus, Saccharomyces,* and *Staphylococcus, e.g.*, *Bacillus coagulans*, *Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium bifidum*, *Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium butyricum, Enterococcus faecium*, *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei*, *Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis,* and *Saccharomyces boulardii.* In certain embodiments, the genetically engineered bacteria are selected from the group consisting of *Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides subtilis, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Clostridium butyricum, Escherichia coli* Nissle, *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus reuteri,* and *Lactococcus lactis.*

[0118] In some embodiments, the genetically engineered bacteria are *Escherichia coli* strain Nissle 1917 (*E. coli* Nissle), a Gram-negative bacterium of the *Enterobacteriaceae* family that has evolved into one of the best characterized probiotics (Ukena et al., 2007). The strain is characterized by its complete harmlessness (Schultz, 2008), and has GRAS (generally recognized as safe) status (Reister et al., 2014, emphasis added). Genomic sequencing confirmed that *E. coli* Nissle lacks prominent

virulence factors (*e.g.*, *E. coli* α-hemolysin, P-fimbrial adhesins) (Schultz, 2008). In addition, it has been shown that *E. coli* Nissle does not carry pathogenic adhesion factors, does not produce any enterotoxins or cytotoxins, is not invasive, and is not uropathogenic (Sonnenborn et al., 2009). As early as in 1917, *E. coli* Nissle was packaged into medicinal capsules, called Mutaflor, for therapeutic use. It is commonly accepted that *E. coli* Nissle's therapeutic efficacy and safety have convincingly been proven (Ukena et al., 2007).

[0119] 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 *PAL* gene from *Rhodosporidium toruloides* can be expressed in *Escherichia coli* (Sarkissian et al., 1999), and it is known that prokaryotic and eukaryotic phenylalanine ammonia lyases share sequence homology (Xiang and Moore, 2005).

[0120] Unmodified *E. coli* Nissle and the genetically engineered bacteria of the invention may be destroyed, *e.g.*, by defense factors in the gut or blood serum (Sonnenborn et al., 2009) or by activation of a kill switch, several hours or days after administration. Thus, the genetically engineered bacteria may require continued administration. In some embodiments, the residence time is calculated for a human subject. Residence time *in vivo* may be calculated for the genetically engineered bacteria of the invention (*see, e.g.*, **Fig. 38**).

[0121] In some embodiments, the genetically engineered bacteria of the invention comprise a gene encoding PAL, wherein the *PAL* gene is operably linked to a directly or indirectly inducible promoter. In some embodiments, the bacteria comprise a non-native *PAL* gene. In some embodiments, the bacteria comprise additional copies of a native *PAL* gene. In some embodiments, the promoter is not associated with the *PAL* gene in nature. In some embodiments, the genetically engineered bacteria of the invention comprise a gene encoding PAH, wherein the *PAH* gene is operably linked to a directly or indirectly inducible promoter. In some embodiments, the bacteria comprise a non-native *PAH* gene. In some embodiments, the bacteria comprise additional copies of a native *PAH* gene. In some embodiments, the promoter is not associated with the *PAH* gene in nature.

[0122] The genetically engineered bacteria further comprise a gene encoding a phenylalanine transporter (PheP). In certain embodiments, the bacteria comprise additional copies of a native gene encoding a phenylalanine transporter, wherein the phenylalanine transporter gene is operably linked to a directly or indirectly inducible promoter. In alternate embodiments, the bacteria comprise a gene encoding a non-native phenylalanine transporter, wherein the phenylalanine transporter gene is operably linked to a directly or indirectly inducible promoter. Both embodiments are encompassed by the term "non-native" phenylalanine transporter. In some embodiments, the promoter is not associated with the *pheP* gene in nature. In some embodiments, the same promoter controls expression of PheP and PAL or PAH.

[0123] In some embodiments, the promoter that is operably linked to *PAL*, *PAH*, and/or *pheP* is directly or 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 the presence of 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 exposure to tetracycline. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention.

Reducing hyperphenylalaninemia

[0124] The genetically engineered bacteria of the invention comprise a gene encoding a phenylalanine-metabolizing enzyme (PME) and are capable of reducing hyperphenylalaninemia.

[0125] Examples of phenylalanine metabolizing enzymes include, but are not limited to, phenylalanine hydroxylase (PAH), phenylalanine ammonia lyase (PAL), aminotransferases, L-amino acid deaminase (L-AAD), and phenylalanine dehydrogenases. Reactions with phenylalanine hydroxylases, phenylalanine dehydrogenases or aminotransferases require cofactors, while L-AAD and PAL do not

require any extra cofactor. Without wishing to be bound by theory, the lack of need for a cofactor means that phenylalanine degradation by the enzyme encoded by the genetically engineered bacteria is dependent on the availability of the substrate and is not limited by the availability of the cofactor.

[0126] Phenylalanine ammonia lyase (PAL; EC 4.3.1.24) is an enzyme that catalyzes a reaction converting L-phenylalanine to ammonia and trans-cinnamic acid. Phenylalanine ammonia lyase is specific for L-Phe, and to a lesser extent, L-Tyrosine. The reaction catalyzed by PAL is the spontaneous, non-oxidative deamination of L-phenylalanine to yield trans-cinnamic acid and ammonia. Unlike the mammalian enzyme (PAH), PAL is a monomer and requires no cofactors (MacDonald et al., Biochem Cell Biol 2007;85:273-82. A modern view of phenylalanine ammonia lyase). In micro-organisms, it has a catabolic role, allowing them to utilize L-phenylalanine (L-Phe) as a sole source of carbon and nitrogen. In one embodiment, the genetically engineered bacteria of the invention comprise a *PAL* gene. PAL is capable of converting phenylalanine to non-toxic levels of transcinnamic acid and ammonia. Trans-cinnamic acid (TCA) can further be converted to TCA metabolites benzoic and hippuric acids (Sarkissian et al., J Mass Spectrom. 2007 Jun;42(6):811-7; Quantitation of phenylalanine and its trans-cinnamic, benzoic and hippuric acid metabolites in biological fluids in a single GC-MS analysis). PAL enzyme activity does not require THB cofactor activity.

[0127] In some embodiments, PAL is encoded by a *PAL* gene derived from a bacterial species, including but not limited to, *Achromobacter xylosoxidans*, *Pseudomonas aeruginosa*, *Photorhabdus luminescens*, *Anabaena variabilis*, and *Agrobacterium tumefaciens*. In some embodiments, the bacterial species is *Photorhabdus luminescens*. In some embodiments, the bacterial species is *Anabaena variabilis*. In some embodiments, PAL is encoded by a *PAL* gene derived from a eukaryotic species, *e.g.*, a yeast species, a plant species. Multiple distinct PAL proteins are known in the art. The genetically engineered bacteria convert more phenylalanine when the *PAL* gene is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the genetically engineered bacteria comprising *PAL* may be used to metabolize phenylalanine in the body into non-toxic molecules in order to treat conditions associated with hyperphenylalaninemia, including PKU. In some

embodiments, the genetically engineered bacteria express *Anabaena variabilis* PAL ("PAL1"). In some embodiments, the genetically engineered bacteria express *Photorhabdus luminescens* PAL ("PAL3"). Non-limiting examples of PAL sequences of interest are shown in **Table 2**.

[0128] LAAD catalyzes the stereospecific oxidative, i.e., oxygen consuming, deamination of L-amino acids to α-keto acids along with the production of ammonia and hydrogen peroxide via an imino acid intermediate. L-AADs are found in snake venoms, and in many bacteria (Bifulco et al. 2013), specifically in the cytomembranes of the Proteus, Providencia, and Morganella bacteria. L-AADs (EC 1.4.3.2) are flavoenzymes with a dimeric structure. Each subunit contains a non-covalently-bound flavin adenine dinucleotide (FAD) cofactor) and do not require any external cofactors. Proteus mirabilis contains two types of L-AADs (Duerre and Chakrabarty 1975). One has broad substrate specificity and catalyzes the oxidation of aliphatic and aromatic L-amino acids to keto acids, typically L-phenylalanine (GenBank: U35383.1) (Baek et al., Journal of Basic Microbiology 2011, 51, 129–135; "Expression and characterization of a second L-amino acid deaminase isolated from Proteus mirabilis in Escherichia coli"). The other type acts mainly on basic L-amino acids (GenBank: EU669819.1). LAADs from bacterial, fungal, and plant sources appear to be involved in the utilization of L-amino acids (i.e., ammonia produced by the enzymatic activity) as a nitrogen source. Most eukaryotic and prokaryotic L-amino acid deaminases are extracellularly secreted, with the exception of from Proteus species LAADs, which are membrane-bound. In Proteus mirabilis, L-AADs have been reported to be located in the plasma membrane, facing outward into the periplasmic space, in which the enzymatic activity resides (Pelmont J et al., (1972) "L-amino acid oxidases of Proteus mirabilis: general properties" Biochimie 54: 1359-1374).

[0129] In one embodiment, the genetically engineered bacteria of the invention comprise a *LAAD* gene. LAAD is capable of converting phenylalanine to non-toxic levels of phenylpyruvate, which can also further be degraded, e.g., by liver enzymes, to phenyllactate. Phenylpyruvate cannot cross the blood brain barrier, which allows LAAD to reduce the levels of phenylalanine in the brain without allowing the accumulation of another potentially toxic metabolite. In some embodiments, LAAD is encoded by a LAAD gene derived from a bacterial species, including but not limited to,

Proteus, Providencia, and Morganella bacteria. In some embodiments, the bacterial species is *Proteus mirabilis*. In some embodiments, the bacterial species is *Proteus vulgaris*. In some embodiments, the genetically engineered bacteria express *Proteus mirabilis* LAAD enzyme GenBank: U35383.1. Non-limiting examples of LAAD sequences of interest are shown in **Table 2**. In some embodiments, the LAAD enzyme is derived from snake venom. According to the invention, genetically engineered bacteria convert more phenylalanine when the *LAAD* gene is expressed than unmodified bacteria of the same bacterial subtype under the same conditions. Thus, the genetically engineered bacteria comprising *LAAD* may be used to metabolize phenylalanine in the body into non-toxic molecules in order to treat conditions associated with hyperphenylalaninemia, including PKU.

[0130] In some embodiments, the genetically engineered bacteria encode a wild type enzyme as it occurs in nature. In some embodiments, the genetically engineered bacteria encode an enzyme which comprises mutations relative to the wild type sequence. In some embodiments, the mutations increase stability of the enzyme. In some embodiments, the mutations increase the catalytic activity of the enzyme. In some embodiments, the genetically engineered bacteria comprise a gene encoding one or more of the proteins listed in **Table 2**. In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding one or more of the polypeptides comprising sequence of any of SEQ ID Nos: 1-8. In some embodiments, the genetically engineered bacteria comprise gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID Nos: 1-8. In some embodiments, the genetically engineered bacteria encode one or more enzymes from **Table 2**, which comprise a mutation. In some embodiments, the genetically engineered bacteria comprise a gene encoding wild type PAH. In some embodiments, the genetically engineered bacteria encode a mutated PAH with increased stability and/or activity. In some embodiments, the genetically engineered bacteria comprise a gene encoding wild type PAL. In some embodiments, the genetically engineered bacteria encode a mutated PAL with increased stability and/or activity. In some embodiments, the genetically engineered bacteria comprise a gene encoding wild type LAAD. In some embodiments, the genetically engineered bacteria encode a

mutated LAAD with increased stability and/or activity. Methods for screening for enzymes with desirable properties are known in the art and described herein.

Table 2. Sequences of Phenylalanine Metabolizing Enzymes

Description	Sequence	SEQ ID NO
Phenylalanine ammonia-lyase (Anabaena variabilis) Acc. No.: Q3M5Z3.1	MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTIN DVARVARNGTLVSLTNNTDILQGIQASCDYINNAV ESGEPIYGVTSGFGGMANVAISREQASELQTNLVW FLKTGAGNKLPLADVRAAMLLRANSHMRGASGIR LELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYI TGSLIGLDPSFKVDFNGKEMDAPTALRQLNLSPLTL LPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAM GVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAAD QMISLLANSQLVRDELDGKHDYRDHELIQDRYSLR CLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQ ASYHGGNFLGQYVGMGMDHLRYYIGLLAKHLDV QIALLASPEFSNGLPPSLLGNRERKVNMGLKGLQIC GNSIMPLLTFYGNSIADRFPTHAEQFNQNINSQGYT SATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKT GHYDARACLSPATERLYSAVRHVVGQKPTSDRPYI WNDNEQGLDEHIARISADIAAGGVIVQAVQDILPCL H	SEQ ID NO: 1
histidine ammonia-lyase [Anabaena variabilis ATCC 29413] (Acc. NO: ABA23593.1)	MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTIN DVARVARNGTLVSLTNNTDILQGIQASCDYINNAV ESGEPIYGVTSGFGGMANVAISREQASELQTNLVW FLKTGAGNKLPLADVRAAMLLRANSHMRGASGIR LELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYI TGSLIGLDPSFKVDFNGKEMDAPTALRQLNLSPLTL LPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAM GVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAAD QMISLLANSQLVRDELDGKHDYRDHELIQDRYSLR CLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQ ASYHGGNFLGQYVGMGMDHLRYYIGLLAKHLDV QIALLASPEFSNGLPPSLLGNRERKVNMGLKGLQIC GNSIMPLLTFYGNSIADRFPTHAEQFNQNINSQGYT SATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKT GHYDARACLSPATERLYSAVRHVVGQKPTSDRPYI WNDNEQGLDEHIARISADIAAGGVIVQAVQDILPCL H	SEQ ID NO: 2
histidine ammonia-lyase [Photorhabdus luminescens] (WP_011146484)	MKAKDVQPTIIINKNGLISLEDIYDIAIKQKKVEISTE ITELLTHGREKLEEKLNSGEVIYGINTGFGGNANLV VPFEKIAEHQQNLLTFLSAGTGDYMSKPCIKASQFT MLLSVCKGWSATRPIVAQAIVDHINHDIVPLVPRYG SVGASGDLIPLSYIARALCGIGKVYYMGAEIDAAEA IKRAGLTPLSLKAKEGLALINGTRVMSGISAITVIKL EKLFKASISAIALAVEALLASHEHYDARIQQVKNHP GQNAVASALRNLLAGSTQVNLLSGVKEQANKACR	SEQ ID NO: 3

	HQEITQLNDTLQEVYSIRCAPQVLGIVPESLATARKI LEREVISANDNPLIDPENGDVLHGGNFMGQYVART MDALKLDIALIANHLHAIVALMMDNRFSRGLPNSL SPTPGMYQGFKGVQLSQTALVAAIRHDCAASGIHT LATEQYNQDIVSLGLHAAQDVLEMEQKLRNIVSMT ILVVCQAIHLRGNISEIAPETAKFYHAVREISSPLITD RALDEDIIRIADAIINDQLPLPEIMLEE	
Histidine ammonia lyase (Photorhabdus luminescens) Acc. NO: CAE15566	MKQLTIYPGKLTLDELRQVYLQPVKITLDSQIFPAIE RSVECVNAILAENRTAYGINTGFGLLASTRIEEDNL EKLQRSLVVSHAAGVGKALDDNMTRLIMVLKINSL SRGYSGIRLAVIQALIALVNAEIYPHIPCKGSVGASG DLAPLAHMSLLLLGEGQARYQGEWLPAKEALAKA NLQPITLAAKEGLALLNGTQVSTAFALRGLFEAEDL LAAAIVCGSLSVEAALGSRKPFDARVHVVRGQQGQ IDVAALYRHVLEESSELSDSHINCPKVQDPYSLRCQ PQVMGACLTQLRHAADVILTEANAVSDNPLVFAEQ GEVISGGNFHAEPVAMASDNLALVLAEIGALSERRI ALLMDSHMSQLPPFLVENGGVNSGFMIAQVTAAAL ASENKALAHPASVDSLPTSANQEDHVSMAPAAGRR LWEMAENTRGILAIEWLSACQGIDFRNGLKSSPILE EARVILRAKVDYYDQDRFFAPDIDAAVKLLAEQHL SSLLPSGQILQRKNNR	SEQ ID NO: 4
amino acid deaminase (Proteus mirabilis) Acc. No: ACD36582	MAISRRKFILGGTVVAVAAGAGVLTPMLTREGRFV PGTPRHGFVEGTGGPLPKQDDVVVIGAGILGIMTAI NLAERGLSVTIVEKGNIAGEQSSRFYGQAISYKMPD ETFLLHHLGKHRWREMNAKVGIDTTYRTQGRVEV PLDEEDLENVRKWIDAKSKDVGSDIPFRTKMIEGAE LKQRLRGATTDWKIAGFEEDSGSFDPEVATFVMAE YAKKMGIKIFTNCAARGLETQAGVISDVVTEKGPIK TSRVVVAGGVGSRLFMQNLNVDVPTLPAYQSQQLI SAAPNAPGGNVALPGGIFFRDQADGTYATSPRVIVA PVVKESFTYGYKYLPLLALPDFPVHISLNEQLINSFM QSTHWDLNEESPFEKYRDMTALPDLPELNASLEKL KKEFPAFKESTLIDQWSGAMAIAPDENPIISDVKEYP GLVINTATGWGMTESPVSAEITADLLLGKKPVLDA KPFSLYRF	SEQ ID NO: 5
amino acid deaminase [Proteus mirabilis HI4320]) Acc. No.: AAA86752.1	MNISRRKLLLGVGAAGVLAGGAALVPMVRRDGKF VEAKSRASFVEGTQGALPKEADVVIIGAGIQGIMTA INLAERGMSVTILEKGQIAGEQSGRAYSQIISYQTSP EIFPLHHYGKILWRGMNEKIGADTSYRTQGRVEAL ADEKALDKAQAWIKTAKEAAGFDTPLNTRIIKGEE LSNRLVGAQTPWTVAAFEEDSGSVDPETGTPALAR YAKQIGVKIYTNCAVRGIETAGGKISDVVSEKGAIK TSQVVLAGGIWSRLFMGNMGIDIPTLNVYLSQQRV SGVPGAPRGNVHLPNGIHFREQADGTYAVAPRIFTS SIVKDSFLLGPKFMHLLGGGELPLEFSIGEDLFNSFK MPTSWNLDEKTPFEQFRVATATQNTQHLDAVFQR MKTEFPVFEKSEVVERWGAVVSPTFDELPIISEVKE YPGLVINTATVWGMTEGPAAGEVTADIVMGKKPVI	SEQ ID NO: 6

	DPTPFSLDRFKK	
L-AAD from *Proteus vulgaris; (Acc. NO: BAA90864)*	MAISRRKFIIGGTVVAVAAGAGILTPMLTREGRFVP GTPRHGFVEGTEGALPKQADVVVVGAGILGIMTAI NLVERGLSVVIVEKGNIAGEQSSRFYGQAISYKMPD ETFLLHHLGKHRWREMNAKVGIDTTYRTQGRVEV PLDEEDLVNVRKWIDERSKNVGSDIPFKTRIIEGAEL NQRLRGATTDWKIAGFEEDSGSFDPEVATFVMAEY AKKMGVRIYTQCAARGLETQAGVISDVVTEKGAIK TSQVVVAGGVWSRLFMQNLNVDVPTLPAYQSQQL ISGSPTAPGGNVALPGGIFFREQADGTYATSPRVIVA PVVKESFTYGYKYLPLLALPDFPVHISLNEQLINSFM QSTHWNLDEVSPFEQFRNMTALPDLPELNASLEKL KAEFPAFKESKLIDQWSGAMAIAPDENPIISEVKEYP GLVINTATGWGMTESPVSAELTADLLLGKKPVLDP KPFSLYRF	SEQ ID NO: 7
Phenylalanine hydroxylase [Homo sapiens] (Acc. No. AAH26251]	MSTAVLENPGLGRKLSDFGQETSYIEDNCNQNGAIS LIFSLKEEVGALAKVLRLFEENDVNLTHIESRPSRLK KDEYEFFTHLDKRSLPALTNIIKILRHDIGATVHELS RDKKKDTVPWFPRTIQELDRFANQILSYGAELDAD HPGFKDPVYRARRKQFADIAYNYRHGQPIPRVEYM EEGKKTWGTVFKTLKSLYKTHACYEYNHIFPLLEK YCGFHEDNIPQLEDVSQFLQTCTGFRLRPVAGLLSS RDFLGGLAFRVFHCTQYIRHGSKPMYTPEPDICHEL LGHVPLFSDRSFAQFSQEIGLASLGAPDEYIEKLATI YWFTVEFGLCKQGDSIKAYGAGLLSSFGELQYCLS EKPKLLPLELEKTAIQNYTVTEFQPLYYVAESFNDA KEKVRNFAATIPRPFSVRYDPYTQRIEVLDNTQQLK ILADSINSEIGILCSALQKIK	SEQ ID NO: 8

[0131] The PME, e.g., PAL, LAAD, or PAH, gene may be present on a plasmid or chromosome in the genetically engineered bacteria. In some embodiments, the PME gene is expressed under the control of a constitutive promoter. In some embodiments, the PME gene is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions, as described herein. In some embodiments, the PME gene is expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions, such as in the presence of molecules or metabolites specific to the gut of a mammal. In one embodiment, the PME gene is expressed under the control of a promoter that is directly or indirectly induced by low-oxygen, microaerobic, or anaerobic conditions, wherein expression of the PME gene, e.g., the PAL gene, is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut.

[0132] In one embodiment, the genetically engineered bacteria encode a PAL gene which is directly or indirectly induced by low-oxygen or anaerobic conditions, such as the mammalian gut. In one embodiment, the genetically engineered bacteria encode a LAAD gene which is directly or indirectly induced by oxygenated, low oxygen, or microaerobic conditions, such as conditions found in the the proximal intestine, including but not limited to the stomach, duodenum, and ileum. In other embodiments, the genetically engineered bacteria encode a PME gene which is directly or indirectly induced by an environmental factor that is naturally present in a mammalian gut. In other embodiments, the genetically engineered bacteria encode a PME gene which is directly or indirectly induced by an environmental factor that is not naturally present in a mammalian gut, e.g., arabinose. In other embodiments, the genetically engineered bacteria encode a PME gene which is directly or indirectly induced by an environmental factor that is naturally present in a mammalian gut under inflammatory conditions.

[0133] Bacteria have evolved transcription factors that are capable of sensing oxygen levels. Different signaling pathways may be triggered by different oxygen levels and occur with different kinetics. An oxygen level-dependent promoter is a nucleic acid sequence to which one or more oxygen level-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression. In one embodiment, the PME gene is expressed under the control of an oxygen level-dependent promoter. In a more specific aspect, the *PAL* gene is under the control of an oxygen level-dependent promoter that is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut.

[0134] In certain embodiments, the genetically engineered bacteria comprise a PME, e.g., *PAL*, 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. 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 PME, e.g., *PAL*, expressed under the control of an alternate oxygen level-dependent promoter, *e.g.*, an ANR promoter (Ray et al., 1997), a DNR promoter (Trunk et al., 2010). In some embodiments, phenylalanine metabolism is particularly activated in a low-oxygen or anaerobic environment, such as in the gut.

[0135] In *P. aeruginosa,* the anaerobic regulation of arginine deiminase and nitrate reduction (ANR) transcriptional regulator is "required for the expression of physiological functions which are inducible under oxygen-limiting or anaerobic conditions" (Winteler et al., 1996; Sawers 1991). *P. aeruginosa* ANR is homologous with *E. coli* FNR, and "the consensus FNR site (TTGAT----ATCAA) was recognized efficiently by ANR and FNR" (Winteler et al., 1996). Like FNR, in the anaerobic state, ANR activates numerous genes responsible for adapting to anaerobic growth. In the aerobic state, ANR is inactive. *Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas syringae*, and *Pseudomonas mendocina* all have functional analogs of ANR (Zimmermann et al., 1991). Promoters that are regulated by ANR are known in the art, *e.g.*, the promoter of the arcDABC operon (*see, e.g.,* Hasegawa et al., 1998).

[0136] The FNR family also includes the dissimilatory nitrate respiration regulator (DNR) (Arai et al., 1995), a transcriptional regulator which is required in conjunction with ANR for "anaerobic nitrate respiration of *Pseudomonas aeruginosa*" (Hasegawa et al., 1998). For certain genes, the FNR-binding motifs "are probably recognized only by DNR" (Hasegawa et al., 1998). Any suitable transcriptional regulator that is controlled by exogenous environmental conditions and corresponding regulatory region may be used. Non-limiting examples include ArcA/B, ResD/E, NreA/B/C, and AirSR, and others are known in the art.

[0137] FNR promoter sequences are known in the art, and any suitable FNR promoter sequence(s) may be used in the genetically engineered bacteria of the invention. Any suitable FNR promoter(s) may be combined with any suitable *PAL*. Non-limiting FNR promoter sequences are provided in **Table 3**, and non-limiting *PAL* sequences are also provided herein. In some embodiments, the genetically engineered bacteria of the invention comprise one or more of: SEQ ID NO: 9, SEQ ID NO: 10, *nirB1* promoter (SEQ ID NO: 11), *nirB2* promoter (SEQ ID NO: 12), *nirB3* promoter (SEQ ID NO: 13), *ydfZ* promoter (SEQ ID NO: 14), *nirB* promoter fused to a strong

ribosome binding site (SEQ ID NO: 15), *ydfZ* promoter fused to a strong ribosome binding site (SEQ ID NO: 16), *fnrS*, an anaerobically induced small RNA gene (*fnrS1* promoter SEQ ID NO: 9 or *fnrS2* promoter SEQ ID NO: 17), *nirB* promoter fused to a *crp* binding site (SEQ ID NO: 18), and *fnrS* fused to a *crp* binding site (SEQ ID NO: 19).

[0138] 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: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 or a functional fragment thereof.

Table 3. FNR Sequences

SEQ ID NO	FNR-responsive regulatory region Sequence
SEQ ID NO: 9	ATCCCCATCACTCTTGATGGAGATCAATTCCCCAAGCTGCTAGAGCGTTA CCTTGCCCTTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCT CCCACAGGAGAAAACCG
SEQ ID NO: 10	CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCT TAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGA GAAAACCG
nirB1 SEQ ID NO: 11	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCACT ATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATCTATTTCT ATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGAAATATCAGAC AATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCCTTAAG GAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAAT CGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA
nirB2 SEQ ID NO: 12	CGGCCCGATCGTTGAACATAGCGGTCCGCAGGCGGCACTGCTTACAGCAA ACGGTCTGTACGCTGTCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTC AGCCGTCACCGTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCC GGACGGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGC ATCTATTTCTATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGA AATATCAGACAATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATAT ACCCATTAAGGAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGG GTTGCTGAATCGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA atgtttgtttaactttaagaaggagatatacat

nirB3 SEQ ID NO: 13	GTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGGCACT ATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTATTTCT ATAAACCCGCTCATTTTGTCTATTTTTTGCACAAACATGAAATATCAGAC AATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCATTAAG GAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAAT CGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA
ydfZ SEQ ID NO: 14	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATGGC TCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACAAAAAATATT TCACTCGACAGGAGTATTTATATTGCGCCCGTTACGTGGGCTTCGACTGT AAATCAGAAAGGAGAAAACACCT
nirB+RBS SEQ ID NO: 15	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGCGGCACT ATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTACATCTATTTCT ATAAATCCGTTCAATTTGTCTGTTTTTTGCACAAACATGAAATATCAGAC AATTCCGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCCTTAAG GAGTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAAT CGTTAA**GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TACAT
ydfZ+RBS SEQ ID NO: 16	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTTATGG CTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAAACAAAAAATAT TTCACTCGACAGGAGTATTTATATTGCGCCC**GGATCC**CTCTAGAAATAAT TTTGTTTAACTTTAAGAAGGAGATATACAT
fnrS1 SEQ ID NO: 17	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGTAAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCTT **GGATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
fnrS2 SEQ ID NO: 18	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGCAAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCTT GGATCCAAAGTGAACTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA TATACAT
nirB+crp SEQ ID NO: 19	TCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTCAGCCGTCACCGTCAG CATAACACCCTGACCTCTCATTAATTGCTCATGCCGGACGGCACTATCGT CGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTATTTCTATAAA CCCGCTCATTTTGTCTATTTTTTGCACAAACATGAAATATCAGACAATTC CGTGACTTAAGAAAATTTATACAAATCAGCAATATACCCATTAAGGAGTA TATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTA AGGTAGaaatgtgatctagttcacatttGCGGTAATAGAAAAGAAATCGA GGCAAAA*atgtttgtttaactttaagaaggagatatacat*

fnrS+crp SEQ ID NO: 20	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGT TGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGCAAAG TTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCaa atgtgatctagttcacatttt*ttgtttaactttaagaaggagatatacat*

[0139] In other embodiments, a PME, e.g., *PAL*, is expressed under the control of an oxygen level-dependent promoter fused to a binding site for a transcriptional activator, *e.g.*, CRP. CRP (cyclic AMP receptor protein or catabolite activator protein or CAP) plays a major regulatory role in bacteria by repressing genes responsible for the uptake, metabolism, and assimilation of less favorable carbon sources when rapidly metabolizable carbohydrates, such as glucose, are present (Wu et al., 2015). This preference for glucose has been termed glucose repression, as well as carbon catabolite repression (Deutscher, 2008; Görke and Stülke, 2008). In some embodiments, PME, e.g., *PAL*, expression is controlled by an oxygen level-dependent promoter fused to a CRP binding site. In some embodiments, *PAL* expression is controlled by an FNR promoter fused to a CRP binding site. In these embodiments, cyclic AMP binds to CRP when no glucose is present in the environment. This binding causes a conformational change in CRP, and allows CRP to bind tightly to its binding site. CRP binding then activates transcription of the PME gene, e.g., *PAL* gene, by recruiting RNA polymerase to the FNR promoter via direct protein-protein interactions. In the presence of glucose, cyclic AMP does not bind to CRP and a PME, e.g., *PAL*, gene transcription is repressed. In some embodiments, an oxygen level-dependent promoter (*e.g.*, an FNR promoter) fused to a binding site for a transcriptional activator is used to ensure that a PME, e.g., *PAL*, is not expressed under anaerobic conditions when sufficient amounts of glucose are present, *e.g.*, by adding glucose to growth media *in vitro*.

[0140] In another embodiment, a PME, e.g., *LAAD*, is expressed under the control of an inducible promoter fused to a binding site for a transcriptional activator, *e.g.*, CRP, such that expression is repressed in the presence of glucose.

[0141] In some embodiments, LAAD is not under the control of an FNRs promoter. LAAD requires oxygen to catalyze the degradation of phenylalanine to phenylpyruvate. Therefore, it would not be desirable to induce LAAD expression under strictly anaerobic conditions where it would be minimally active (**Fig. 25**).

[0142] In some embodiments, a PME, e.g., *PAL* or LAAD, is expressed under the control of an inducible promoter that is responsive to specific molecules or metabolites in the environment, *e.g.*, the mammalian gut. For example, the short-chain fatty acid propionate is a major microbial fermentation metabolite localized to the gut (Hosseini et al., 2011). In one embodiment, *PAL* gene expression is under the control of a propionate-inducible promoter. In a more specific embodiment, PME gene expression is under the control of a propionate-inducible promoter that is activated by the presence of propionate in the mammalian gut. Any molecule or metabolite found in the mammalian gut, in a healthy and/or disease state, may be used to induce PME gene expression. Non-limiting examples include propionate, bilirubin, aspartate aminotransferase, alanine aminotransferase, blood coagulation factors II, VII, IX, and X, alkaline phosphatase, gamma glutamyl transferase, hepatitis antigens and antibodies, alpha fetoprotein, anti-mitochondrial, smooth muscle, and anti-nuclear antibodies, iron, transferrin, ferritin, copper, ceruloplasmin, ammonia, and manganese. In alternate embodiments, PME, e.g., PAL and/or LAAD, gene expression is under the control of a P_{araBAD} promoter, which is activated in the presence of the sugar arabinose. In one embodiment, LAAD expression is under the control of the P_{araBAD} promoter. In one embodiment, expression of LAAD occurs under aerobic or microaerobic conditions.

[0143] In some embodiments, the *PAL* gene is expressed under the control of a promoter that is induced by exposure to tetracycline. In some embodiments, gene expression is further optimized by methods known in the art, *e.g.*, by optimizing ribosomal binding sites, manipulating transcriptional regulators, and/or increasing mRNA stability.

[0144] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying the *PAL* gene, such that *PAL* 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, the genetically engineered bacteria comprise two or more distinct *PAL* genes. In some embodiments, the genetically engineered bacteria comprise multiple copies of the same *PAL* gene. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a directly or indirectly inducible promoter. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a promoter that is induced under low-

oxygen or anaerobic conditions. In some embodiments, the *PAL* gene is present on a chromosome and operably linked to a directly or indirectly inducible promoter. In some embodiments, the *PAL* gene is present in the chromosome and operably linked to a promoter that is induced under low-oxygen or anaerobic conditions. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline.

[0145] In some embodiments, the genetically engineered bacteria comprise a stably maintained plasmid or chromosome carrying the *LAAD* gene, such that *LAAD* 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, the genetically engineered bacteria comprise two or more distinct *LAAD* genes. In some embodiments, the genetically engineered bacteria comprise multiple copies of the same *LAAD* gene. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a directly or indirectly inducible promoter. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a promoter that is inducible, e.g., by arabinose or tetracycline. In some embodiments, the *LAAD* gene is present on a chromosome and operably linked to a directly or indirectly inducible promoter. In some embodiments, the *LAAD* gene is present in the chromosome and operably linked to a promoter that is induced, e.g., by arabinose. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline.

[0146] In some embodiments, the genetically engineered bacteria comprise an oxygen-level dependent transcriptional regulator, *e.g.*, FNR, ANR, or DNR, and corresponding promoter from a different bacterial species. The non-native oxygen-level dependent transcriptional regulator and promoter increase the transcription of genes operably linked to said promoter, *e.g.*, *PAL*, in a low-oxygen or anaerobic environment, as compared to the native transcriptional regulator 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.

[0147] 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.*, *PAL*, 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.*, *PAL*, 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).

[0148] In some embodiments, the genetically engineered bacteria of the invention 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 PAL are present on different plasmids. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding PAL are present on the same plasmid. 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 PAL are present on different chromosomes. In some embodiments, the gene encoding the oxygen level-sensing transcriptional regulator and the gene encoding PAL 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 phenylalanine-metabolizing enzyme. In some embodiments, expression of the transcriptional regulator is controlled by the same promoter that controls expression of the phenylalanine-metabolizing enzyme. In some embodiments, the transcriptional regulator and the phenylalanine-metabolizing enzyme are divergently transcribed from a promoter region.

[0149] In some embodiments, the genetically engineered bacteria of the invention produce PAL under exogenous environmental conditions, such as the low-oxygen environment of the mammalian gut, to reduce blood phenylalanine 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. Certain unmodified bacteria will not have appreciable levels of phenylalanine processing. In embodiments using genetically modified forms of these bacteria, PAL-mediated processing of phenylalanine will be appreciable under exogenous environmental conditions. Phenylalanine may be measured by methods known in the art, *e.g.*, blood sampling and mass spectrometry. In some embodiments, cinnamate is measured by methods known in the art to assess PAL activity. Cinnamate production is directly correlated with phenylalanine degradation, and in some embodiments, that cinnamate may be used as an alternative biomarker for strain activity **(Fig. 16B)**. Cinnamate can be further degraded to hippuric acid by liver enzymes; both can be measured as described in Example 24-26. In some embodiments, *PAL* expression is measured by methods known in the art to assess PAL activity.

[0150] In some embodiments, the genetically engineered bacteria of the invention produce LAAD, to reduce blood phenylalanine 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. Certain unmodified bacteria will not have appreciable levels of phenylalanine processing. In embodiments using genetically modified forms of these bacteria, LAAD-mediated processing of phenylalanine will be appreciable under exogenous environmental conditions. Phenylalanine may be measured by methods known in the art, *e.g.*, blood sampling and mass spectrometry. Pyruvic acid and phenylpyruvate, the LAAD generated degradation products can be measured using masspectrometry as described in Examples 24-26, and can be used as an additional readout of LAAD activity.

[0151] In some embodiments, the PME, e.g., *PAL, LAAD, and/or PAH,* 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 PME, e.g., *PAL, LAAD, and/or PAH,* is expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing the PME, e.g., *PAL, LAAD, and/or PAH,* expression, thereby increasing the metabolism of phenylalanine and reducing hyperphenylalaninemia. In some embodiments, a genetically engineered bacterium comprising a the PME, e.g., *PAL, LAAD, and/or PAH,* expressed on a high-copy plasmid does not increase phenylalanine metabolism or decrease phenylalanine levels as compared to a genetically engineered bacterium comprising the same PME, e.g., *PAL, LAAD, and/or PAH,* expressed on a low-copy plasmid in the absence of heterologous *pheP* and additional copies of a native *pheP*. Genetically engineered bacteria comprising the same the PME gene, e.g., *PAL, LAAD, and/or PAH* gene on high and low copy plasmids were generated. For example, either *PAL1* or *PAL3* on a high-copy plasmid and a low-copy plasmid were generated, and each metabolized and reduced phenylalanine to similar levels (**Fig. 15**). Thus, in some embodiments, the rate-limiting step of phenylalanine metabolism is phenylalanine availability (*see, e.g.,* **Fig. 16**). In these embodiments, it may be advantageous to increase phenylalanine transport into the cell, thereby enhancing phenylalanine metabolism. In conjunction with *pheP*, even low-copy PAL plasmids are capable of almost completely eliminating Phe from a test sample (*see, e.g.*, **Fig. 16A**). Furthermore, in some embodiments, that incorporate *pheP*, there may be additional advantages to using a low-copy PAL-expressing plasmid in conjunction in order to

enhance the stability of *PAL* expression while maintaining high phenylalanine metabolism, and to reduce negative selection pressure on the transformed bacterium. In alternate embodiments, the phenylalanine transporter is used in conjunction with the high-copy plasmid.

[0152] In some embodiments, a transporter may not increase phenylalanine degradation. For example, *Proteus mirabilis* LAAD is localized to the plasma membrane, with the enzymatic catalysis occurring in the periplasm. Phenylalanine can readily traverse the outer membrane without the need of a transporter. Therefore, in embodiments, in which the genetically engineered bacteria express LAAD, a transporter may not be needed or improve phenylalanine metabolism.

[0153] In some embodiments, the PME, e.g., *PAL, LAAD*, and /or *PAH,* gene is expressed on a chromosome. In some embodiments, expression from the chromosome may be useful for increasing stability of expression of the PME. In some embodiments, the PME gene, e.g., *PAL, LAAD*, and /or *PAH* gene(s), is integrated into the bacterial chromosome at one or more integration sites in the genetically engineered bacteria. In some embodiments, the PME gene, e.g., *PAL, LAAD*, and /or *PAH* gene(s) is inserted into the bacterial genome at one or more of the following insertion sites in *E. coli* Nissle: *malE/K, insB/I, araC/BAD, lacZ, agal/rsml, thyA, and malP/T.* Any suitable insertion site may be used (*see, e.g.,* **Fig. 36**). The insertion site may be anywhere in the genome, *e.g.*, in a gene required for survival and/or growth, such as *thyA* (to create an auxotroph); in an active area of the genome, such as near the site of genome replication; and/or in between divergent promoters in order to reduce the risk of unintended transcription, such as between AraB and AraC of the arabinose operon. In some embodiments, more than one copy, e.g., two, three, four, five, six, seven, eight, nine, ten or more copies of the PME gene, e.g., PAL, PAH, and/or LAAD is integrated into the bacterial chromosome at one or more integration sites in the genetically engineered bacteria. The more than one copy of a PME gene may be more then one copy of the same PME gene or more than one copy of different PME genes.

[0154] Exemplary constructs are shown in **4-13** below. **Table 4** shows the sequence of an exemplary construct comprising a gene encoding PheP and an FNR promoter sequence for chromosomal insertion (SEQ ID NO: 21), with the *pheP* sequence underlined and the FNR promoter sequence bolded. **Table 5** shows the

sequence of an exemplary construct comprising a gene encoding PAL1 and an FNR promoter sequence on a high-copy plasmid (SEQ ID NO: 22), with the *PAL1* sequence underlined and the FNR promoter sequence bolded. **Table 6** shows the sequence of an exemplary construct comprising a gene encoding PAL3 and an FNR promoter sequence on a high-copy plasmid (SEQ ID NO: 23), with the *PAL3* sequence underlined and the FNR promoter sequence bolded. **Table 7** shows the sequence of an exemplary construct comprising a gene encoding PAL1 and a Tet promoter sequence on a high-copy plasmid (SEQ ID NO: 24), with the *PAL1* sequence underlined and the Tet promoter sequence bolded. **Table 8** shows the sequence of an exemplary construct comprising a gene encoding PAL3 and a Tet promoter sequence on a high-copy plasmid (SEQ ID NO: 25), with the *PAL3* sequence underlined and the Tet promoter sequence bolded. **Table 9** shows the sequence of an exemplary construct comprising a gene encoding PAL1 and an FNR promoter sequence on a low-copy plasmid (SEQ ID NO: 26), with the *PAL1* sequence underlined and the FNR promoter sequence bolded. **Table 10** shows the sequence of an exemplary construct comprising a gene encoding PAL3 and an FNR promoter sequence on a low-copy plasmid (SEQ ID NO: 27), with the *PAL3* sequence underlined and the FNR promoter sequence bolded. **Table 11** shows the sequence of an exemplary construct comprising a gene encoding PAL1 and a Tet promoter sequence on a low-copy plasmid (SEQ ID NO: 28), with the *PAL1* sequence underlined and the Tet promoter sequence bolded. **Table 12** shows the sequence of an exemplary construct comprising a gene encoding PAL3 and a Tet promoter sequence on a low-copy plasmid (SEQ ID NO: 29), with the *PAL3* sequence underlined and the Tet promoter sequence bolded. **Table 13** shows the sequence of an exemplary construct comprising a gene encoding PheP, a gene coding TetR, and a Tet promoter sequence for chromosomal insertion (SEQ ID NO: 30), with the *pheP* sequence underlined, the TetR sequence boxed, and the FNR promoter sequence bolded.

Table 4

Nucleotide sequences of FNR promoter-PheP construct (SEQ ID NO: 21)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCTTAAACATTA **GCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGAGAAAACCG**ATGAAAAACG CGTCAACCGTATCGGAAGATACTGCGTCGAATCAAGAGCCGACGCTTCATCGCGGATTA CATAACCGTCATATTCAACTGATTGCGTTGGGTGGCGCAATTGGTACTGGTCTGTTTCT TGGCATTGGCCCGGCGATTCAGATGGCGGGTCCGGCTGTATTGCTGGGCTACGGCGTCG CCGGGATCATCGCTTTCCTGATTATGCGCCAGCTTGGCGAAATGGTGGTTGAGGAGCCG

Nucleotide sequences of FNR promoter-PheP construct (SEQ ID NO: 21)
GTATCCGGTTCATTTGCCCACTTTGCCTATAAATACTGGGGACCGTTTGCGGCTTCCT CTCTGGCTGGAACTACTGGGTAATGTTCGTGCTGGTGGGAATGGCAGAGCTGACCGCTG CGGGCATCTATATGCAGTACTGGTTCCCGGATGTTCCAACGTGGATTTGGGCTGCCGCC TTCTTTATTATCATCAACGCCGTTAACCTGGTGAACGTGCGCTTATATGGCGAAACCGA GTTCTGGTTTGCGTTGATTAAAGTGCTGGCAATCATCGGTATGATCGGCTTTGGCCTGT GGCTGCTGTTTTCTGGTCACGGCGGCGAGAAAGCCAGTATCGACAACCTCTGGCGCTAC GGTGGTTTCTTCGCCACCGGCTGGAATGGGCTGATTTTGTCGCTGGCGGTAATTATGTT CTCCTTCGGCGGTCTGGAGCTGATTGGGATTACTGCCGCTGAAGCGCGCGATCCGGAAA AAAGCATTCCAAAAGCGGTAAATCAGGTGGTGTATCGCATCCTGCTGTTTTACATCGGT TCACTGGTGGTTTTACTGGCGCTCTATCCGTGGGTGGAAGTGAAATCCAACAGTAGCCC GTTTGTGATGATTTTCCATAATCTCGACAGCAACGTGGTAGCTTCTGCGCTGAACTTCG TCATTCTGGTAGCATCGCTGTCAGTGTATAACAGCGGGGTTTACTCTAACAGCCGCATG CTGTTTGGCCTTTCTGTGCAGGGTAATGCGCCGAAGTTTTTGACTCGCGTCAGCCGTCG CGGTGTGCCGATTAACTCGCTGATGCTTTCCGGAGCGATCACTTCGCTGGTGGTGTTAA TCAACTATCTGCTGCCGCAAAAAGCGTTTGGTCTGCTGATGGCGCTGGTGGTAGCAACG CTGCTGTTGAACTGGATTATGATCTGTCTGGCGCATCTGCGTTTTCGTGCAGCGATGCG ACGTCAGGGGCGTGAAACACAGTTTAAGGCGCTGCTCTATCCGTTCGGCAACTATCTCT GCATTGCCTTCCTCGGCATGATTTTGCTGCTGATGTGCACGATGGATGATATGCGCTTG TCAGCGATCCTGCTGCCGGTGTGGATTGTATTCCTGTTTATGGCATTTAAAACGCTGCG TCGGAAATAA

Table 5

Nucleotide sequences of FNR promoter-PAL1 construct, high-copy (SEQ ID NO: 22)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCTTAAACATTA **GCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGAGAAAACCG**ATGAAAACAC TATCACAGGCCCAATCTAAAACTTCTTCACAGCAATTCAGCTTTACCGGGAACTCGTCT GCGAATGTAATTATCGGCAATCAAAAGCTGACCATTAATGATGTAGCTCGCGTTGCCCG GAATGGCACTTTGGTGTCACTGACGAACAATACCGACATTCTGCAAGGTATTCAAGCTA GCTGCGATTATATCAATAACGCCGTTGAATCTGGCGAGCCAATCTACGGGGTAACAAGC GGTTTTGGTGGGATGGCGAACGTTGCCATTAGCCGTGAACAGGCGAGCGAACTTCAGAC CAACCTCGTTTGGTTCCTAAAGACAGGAGCTGGTAATAAGTTACCTCTGGCTGACGTAA GAGCCGCGATGCTGCTTCGCGCTAATAGTCACATGCGCGGCGCCAGTGGTATCCGTCTT GAGCTTATCAAGAGGATGGAAATCTTCCTCAACGCGGGTGTCACACCATATGTTTATGA GTTTGGTAGTATCGGAGCCAGTGGTGATCTTGTTCCCCTGAGTTATATTACGGGTTCAT TGATTGGTTTAGACCCGTCCTTTAAAGTGGATTTTAACGGGAAAGAAATGGACGCCCCG ACCGCTTTACGACAGCTTAATCTGAGCCCACTTACTTTGCTCCCTAAAGAAGGTCTTGC CATGATGAATGGCACCTCTGTGATGACTGGAATTGCCGCGAATTGTGTGTATGACACGC AGATCCTAACGGCCATTGCCATGGGTGTTCACGCGTTGGACATTCAAGCCCTGAATGGT ACAAACCAGTCGTTTCATCCGTTTATCCATAATTCAAAACCCCATCCGGGACAGCTTTG GGCTGCTGATCAGATGATCTCACTCCTGGCCAATAGTCAACTGGTTCGGGACGAGCTCG ACGGCAAACATGATTATCGCGATCATGAGCTCATCCAGGACCGGTATTCACTTCGTTGT CTCCCACAATACCTGGGGCCTATCGTTGATGGTATATCTCAAATTGCGAAGCAAATTGA AATTGAGATCAATAGCGTAACCGACAACCCGCTTATCGATGTTGATAATCAGGCCTCTT ATCACGGTGGCAATTTTCTGGGCCAGTATGTTGGTATGGGGATGGATCACCTGCGGTAC

Nucleotide sequences of FNR promoter-PAL1 construct, high-copy (SEQ ID NO: 22)
TATATTGGGCTTCTGGCTAAACATCTTGATGTGCAGATTGCCTTATTAGCTTCACCAGA
ATTTTCAAATGGACTGCCGCCATCATTGCTCGGTAACAGAGAAAGGAAAGTAAATATGG
GCCTTAAGGGCCTTCAGATATGTGGTAACTCAATCATGCCCCTCCTGACCTTTTATGGG
AACTCAATTGCTGATCGTTTTCCGACACATGCTGAACAGTTTAACCAAAACATTAACTC
ACAGGGCTATACATCCGCGACGTTAGCGCGTCGGTCCGTGGATATCTTCCAGAATTATG
TTGCTATCGCTCTGATGTTCGGCGTACAGGCCGTTGATTTGCGCACTTATAAAAAAACC
GGTCACTACGATGCTCGGGCTTGCCTGTCGCCTGCCACCGAGCGGCTTTATAGCGCCGT
ACGTCATGTTGTGGGTCAGAAACCGACGTCGGACCGCCCCTATATTTGGAATGATAATG
AACAAGGGCTGGATGAACACATCGCCCGGATATCTGCCGATATTGCCGCCGGAGGTGTC
ATCGTCCAGGCGGTACAAGACATACTTCCTTGCCTGCATTAAGCTTGGCGTAATCATGG
TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGC
CGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGA
ATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCT
CACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGG
CGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAA
GGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCT
CCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGA
CAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTT
CCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCT
TTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGG
GCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGT
CTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAG
GATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACT
ACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTC
GGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTT
TTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGA
TCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTC
ATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAA
ATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTG
AGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTC
GTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACC
GCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGG
CCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGC
CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGC
TACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCC
AACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTC
GGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGC
AGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTG
AGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCG
GCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGG
AAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGA
TGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCT
GGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAA
ATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATT
GTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCG
CGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATT

Nucleotide sequences of FNR promoter-PAL1 construct, high-copy (SEQ ID NO: 22)
AACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACG GTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTG GCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAA TACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTG CGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAA AGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGAC GTT

Table 6

Nucleotide sequences of FNR promoter-PAL3 construct, high-copy (SEQ ID NO: 23)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCTTAAACATTA **GCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGAGAAAACCG**ATGAAAGCTA AAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATC TATGACATTGCGATAAAACAAAAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTT GACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAA TCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAG CATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACC TTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAA CCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGACATTGTTCCTCTG GTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGC ACGAGCATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTG AAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCT CTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGA AAAACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCAT CTCATGAACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCG GTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGG GGTTAAAGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATA CCTTACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAA TCTTTAGCTACCGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCC ATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGGCAATATG TCGCCCGAACAATGGATGCATTAAAACTGGATATTGCTTTAATTGCCAATCATCTTCAC GCCATTGTGGCTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTAATTCACTGAG TCCGACACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAG TTGCTGCAATTCGCCATGATTGTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAA TACAATCAAGATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGA GCAGAAATTACGCAATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATC TTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGC GAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCATTGC GGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAGAATAAGCTT GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCAC ACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAA CTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTT

Nucleotide sequences of FNR promoter-PAL3 construct, high-copy (SEQ ID NO: 23)
CCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCA GCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAA CATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGT TTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGG TGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGT GCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGG GAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATC CGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAG CCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAG TGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAA GCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTG GTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAA GAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTA AGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAA AATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAA TGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGC CTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTG CTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAG CCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTC TATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACG TTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTC AGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGC GGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCAC TCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTT TCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAG TTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAG TGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTG AGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTT CACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAA GGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATT TATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACA AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTA TTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGT TTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTG TCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCG GGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCAT ATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTC GCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTAC GCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTT TCCCAGTCACGACGTT

Table 7

Nucleotide sequences of Tet promoter-PAL1 construct, high-copy (SEQ ID NO: 24)

Nucleotide sequences of Tet promoter-PAL1 construct, high-copy (SEQ ID NO: 24)
CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAACACTATCAC AGGCCCAATCTAAAACTTCTTCACAGCAATTCAGCTTTACCGGGAACTCGTCTGCGAAT GTAATTATCGGCAATCAAAAGCTGACCATTAATGATGTAGCTCGCGTTGCCCGGAATGG CACTTTGGTGTCACTGACGAACAATACCGACATTCTGCAAGGTATTCAAGCTAGCTGCG ATTATATCAATAACGCCGTTGAATCTGGCGAGCCAATCTACGGGGTAACAAGCGGTTTT GGTGGGATGGCGAACGTTGCCATTAGCCGTGAACAGGCGAGCGAACTTCAGACCAACCT CGTTTGGTTCCTAAAGACAGGAGCTGGTAATAAGTTACCTCTGGCTGACGTAAGAGCCG CGATGCTGCTTCGCGCTAATAGTCACATGCGCGGCGCCAGTGGTATCCGTCTTGAGCTT ATCAAGAGGATGGAAATCTTCCTCAACGCGGGTGTCACACCATATGTTTATGAGTTTGG TAGTATCGGAGCCAGTGGTGATCTTGTTCCCCTGAGTTATATTACGGGTTCATTGATTG GTTTAGACCCGTCCTTTAAAGTGGATTTTAACGGGAAAGAAATGGACGCCCCGACCGCT TTACGACAGCTTAATCTGAGCCCACTTACTTTGCTCCCTAAAGAAGGTCTTGCCATGAT GAATGGCACCTCTGTGATGACTGGAATTGCCGCGAATTGTGTGTATGACACGCAGATCC TAACGGCCATTGCCATGGGTGTTCACGCGTTGGACATTCAAGCCCTGAATGGTACAAAC CAGTCGTTTCATCCGTTTATCCATAATTCAAAACCCCATCCGGGACAGCTTTGGGCTGC TGATCAGATGATCTCACTCCTGGCCAATAGTCAACTGGTTCGGGACGAGCTCGACGGCA AACATGATTATCGCGATCATGAGCTCATCCAGGACCGGTATTCACTTCGTTGTCTCCCA CAATACCTGGGGCCTATCGTTGATGGTATATCTCAAATTGCGAAGCAAATTGAAATTGA GATCAATAGCGTAACCGACAACCCGCTTATCGATGTTGATAATCAGGCCTCTTATCACG GTGGCAATTTTCTGGGCCAGTATGTTGGTATGGGGATGGATCACCTGCGGTACTATATT GGGCTTCTGGCTAAACATCTTGATGTGCAGATTGCCTTATTAGCTTCACCAGAATTTTC AAATGGACTGCCGCCATCATTGCTCGGTAACAGAGAAAGGAAAGTAAATATGGGCCTTA AGGGCCTTCAGATATGTGGTAACTCAATCATGCCCCTCCTGACCTTTTATGGGAACTCA ATTGCTGATCGTTTTCCGACACATGCTGAACAGTTTAACCAAAACATTAACTCACAGGG CTATACATCCGCGACGTTAGCGCGTCGGTCCGTGGATATCTTCCAGAATTATGTTGCTA TCGCTCTGATGTTCGGCGTACAGGCCGTTGATTTGCGCACTTATAAAAAAACCGGTCAC TACGATGCTCGGGCTTGCCTGTCGCCTGCCACCGAGCGGCTTTATAGCGCCGTACGTCA TGTTGTGGGTCAGAAACCGACGTCGGACCGCCCCTATATTTGGAATGATAATGAACAAG GGCTGGATGAACACATCGCCCGGATATCTGCCGATATTGCCGCCGGAGGTGTCATCGTC CAGGCGGTACAAGACATACTTCCTTGCCTGCATTAAGCTTGGCGTAATCATGGTCATAG CTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAG CATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGC GCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGA CTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAA TACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCC CCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACC CTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCA TAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTG TGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAG TCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAG CAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCT ACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAA AGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGT TTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTT

Nucleotide sequences of Tet promoter-PAL1 construct, high-copy (SEQ ID NO: 24)
CTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGA TTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAAT CTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCAC CTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAG ATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGA CCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGC GCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGG CATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGAT CAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCT CCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACT GCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACT CAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCA ATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACG TTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAAC CCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGA GCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTG AATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCA TGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACA TTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTA TAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAA ACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGG AGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAA CTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGC ACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAAC TGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTA AAACGACGGCCAGTGAATTCGTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATC CGCATATGATCAATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAAT AATTCGATAGCTTGTCGTAATAATGGCGGCATACTATCAGTAGTAGGTGTTTCCCTTTC TTCTTTAGCGACTTGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAAATGCCCCA CAGCGCTGAGTGCATATAATGCATTCTCTAGTGAAAAACCTTGTTGGCATAAAAAGGCT AATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAGGCCGTGTACCTAAATGTACTTT TGCTCCATCGCGATGACTTAGTAAAGCACATCTAAAACTTTTAGCGTTATTACGTAAAA AATCTTGCCAGCTTTCCCCTTCTAAAGGGCAAAAGTGAGTATGGTGCCTATCTAACATC TCAATGGCTAAGGCGTCGAGCAAAGCCCGCTTATTTTTTACATGCCAATACAATGTAGG CTGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCGACCT CATTAAGCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAATCTAGACATCATT AATTCCTAATTTTT**GTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCA** **GTGATAGAGAA**AAGTGAA

Table 8

Nucleotide sequences of Tet promoter-PAL3, high-copy construct (SEQ ID NO: 25)

Nucleotide sequences of Tet promoter-PAL3, high-copy construct (SEQ ID NO: 25)
CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAGCTAAAGATG TTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTATGAC ATTGCGATAAAACAAAAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTTGACGCA TGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAATCAATA CAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAGCATCAG CAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGTAT TAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACCAGAC CAATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGACATTGTTCCTCTGGTTCCT CGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGAGC ATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAA TTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCTGATT AACGGCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGAAAAACT ATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCATCTCATG AACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGGCA AGTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGGGGTTAA AGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATACCTTAC AGGAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAATCTTTA GCTACCGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCCATTGAT AGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGGCAATATGTCGCCC GAACAATGGATGCATTAAAACTGGATATTGCTTTAATTGCCAATCATCTTCACGCCATT GTGGCTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTAATTCACTGAGTCCGAC ACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTG CAATTCGCCATGATTGTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAATACAAT CAAGATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCAGAA ATTACGCAATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATCTTCGCG GCAATATTAGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGCGAAATC AGTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCATTGCGGATGC AATTATTAATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAGAATAAGCTTGGCGTA ATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACA TTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCA TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTT CCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTG AGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGA AACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTC TCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCG TGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCC AAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGG CCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCG GTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGAT CCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGAT TTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAA

Nucleotide sequences of Tet promoter-PAL3, high-copy construct (SEQ ID NO: 25)
GTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTA ATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACT CCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAA TGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCC GGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAA TTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTG CCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCC GGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAG CTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGG TTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTG ACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTC TTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCA TCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCC AGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAG CGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGA CACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAG GGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGG GGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCA TGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGT GATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTA AGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTC GGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGG TGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATT CAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGC TGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAG TCACGACGTTGTAAAACGACGGCCAGTGAATTCGTTAAGACCCACTTTCACATTTAAGT TGTTTTTCTAATCCGCATATGATCAATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACC TTGGTGATCAAATAATTCGATAGCTTGTCGTAATAATGGCGGCATACTATCAGTAGTAG GTGTTTCCCTTTCTTCTTTAGCGACTTGATGCTCTTGATCTTCCAATACGCAACCTAAA GTAAAATGCCCCACAGCGCTGAGTGCATATAATGCATTCTCTAGTGAAAAACCTTGTTG GCATAAAAAGGCTAATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAGGCCGTGTAC CTAAATGTACTTTTGCTCCATCGCGATGACTTAGTAAAGCACATCTAAAACTTTTAGCG TTATTACGTAAAAAATCTTGCCAGCTTTCCCCTTCTAAAGGGCAAAAGTGAGTATGGTG CCTATCTAACATCTCAATGGCTAAGGCGTCGAGCAAAGCCCGCTTATTTTTTACATGCC AATACAATGTAGGCTGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCT TCGATTCCGACCTCATTAAGCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAA TCTAGACATCATTAATTCCTAATTTTT**GTTGACACTCTATCATTGATAGAGTTATTTTA** **CCACTCCCTATCAGTGATAGAGAA**AAGTGAA

Table 9

Nucleotide sequences of FNR promoter-PAL1 construct, low-copy (SEQ ID NO: 26)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCTTAAACATTA **GCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGAGAAAACCG**ATGAAAACAC

Nucleotide sequences of FNR promoter-PAL1 construct, low-copy (SEQ ID NO: 26)
TATCACAGGCCCAATCTAAAACTTCTTCACAGCAATTCAGCTTTACCGGAACTCGTCT GCGAATGTAATTATCGGCAATCAAAAGCTGACCATTAATGATGTAGCTCGCGTTGCCCG GAATGGCACTTTGGTGTCACTGACGAACAATACCGACATTCTGCAAGGTATTCAAGCTA GCTGCGATTATATCAATAACGCCGTTGAATCTGGCGAGCCAATCTACGGGGTAACAAGC GGTTTTGGTGGGATGGCGAACGTTGCCATTAGCCGTGAACAGGCGAGCGAACTTCAGAC CAACCTCGTTTGGTTCCTAAAGACAGGAGCTGGTAATAAGTTACCTCTGGCTGACGTAA GAGCCGCGATGCTGCTTCGCGCTAATAGTCACATGCGCGGCGCCAGTGGTATCCGTCTT GAGCTTATCAAGAGGATGGAAATCTTCCTCAACGCGGGTGTCACACCATATGTTTATGA GTTTGGTAGTATCGGAGCCAGTGGTGATCTTGTTCCCCTGAGTTATATTACGGGTTCAT TGATTGGTTTAGACCCGTCCTTTAAAGTGGATTTTAACGGGAAAGAAATGGACGCCCCG ACCGCTTTACGACAGCTTAATCTGAGCCCACTTACTTTGCTCCCTAAAGAAGGTCTTGC CATGATGAATGGCACCTCTGTGATGACTGGAATTGCCGCGAATTGTGTGTATGACACGC AGATCCTAACGGCCATTGCCATGGGTGTTCACGCGTTGGACATTCAAGCCCTGAATGGT ACAAACCAGTCGTTTCATCCGTTTATCCATAATTCAAAACCCCATCCGGGACAGCTTTG GGCTGCTGATCAGATGATCTCACTCCTGGCCAATAGTCAACTGGTTCGGGACGAGCTCG ACGGCAAACATGATTATCGCGATCATGAGCTCATCCAGGACCGGTATTCACTTCGTTGT CTCCCACAATACCTGGGGCCTATCGTTGATGGTATATCTCAAATTGCGAAGCAAATTGA AATTGAGATCAATAGCGTAACCGACAACCCGCTTATCGATGTTGATAATCAGGCCTCTT ATCACGGTGGCAATTTTCTGGGCCAGTATGTTGGTATGGGGATGGATCACCTGCGGTAC TATATTGGGCTTCTGGCTAAACATCTTGATGTGCAGATTGCCTTATTAGCTTCACCAGA ATTTTCAAATGGACTGCCGCCATCATTGCTCGGTAACAGAGAAAGGAAAGTAAATATGG GCCTTAAGGGCCTTCAGATATGTGGTAACTCAATCATGCCCCTCCTGACCTTTTATGGG AACTCAATTGCTGATCGTTTTCCGACACATGCTGAACAGTTTAACCAAAACATTAACTC ACAGGGCTATACATCCGCGACGTTAGCGCGTCGGTCCGTGGATATCTTCCAGAATTATG TTGCTATCGCTCTGATGTTCGGCGTACAGGCCGTTGATTTGCGCACTTATAAAAAAACC GGTCACTACGATGCTCGGGCTTGCCTGTCGCCTGCCACCGAGCGGCTTTATAGCGCCGT ACGTCATGTTGTGGGTCAGAAACCGACGTCGGACCGCCCCTATATTTGGAATGATAATG AACAAGGGCTGGATGAACACATCGCCCGGATATCTGCCGATATTGCCGCCGGAGGTGTC ATCGTCCAGGCGGTACAAGACATACTTCCTTGCCTGCATTAAGCTTGGCGTAATCATGG TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGC CGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGA ATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCT CACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGG CGGTAGTACGGGTTTTGCTGCCCGCAAACGGGCTGTTCTGGTGTTGCTAGTTTGTTATC AGAATCGCAGATCCGGCTTCAGGTTTGCCGGCTGAAAGCGCTATTTCTTCCAGAATTGC CATGATTTTTTCCCCACGGGAGGCGTCACTGGCTCCCGTGTTGTCGGCAGCTTTGATTC GATAAGCAGCATCGCCTGTTTCAGGCTGTCTATGTGTGACTGTTGAGCTGTAACAAGTT GTCTCAGGTGTTCAATTTCATGTTCTAGTTGCTTTGTTTTACTGGTTTCACCTGTTCTA TTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGATCTGTTCATGGTGAACAGCTTT AAATGCACCAAAAACTCGTAAAAGCTCTGATGTATCTATCTTTTTTACACCGTTTTCAT CTGTGCATATGGACAGTTTTCCCTTTGATATCTAACGGTGAACAGTTGTTCTACTTTTG TTTGTTAGTCTTGATGCTTCACTGATAGATACAAGAGCCATAAGAACCTCAGATCCTTC CGTATTTAGCCAGTATGTTCTCTAGTGTGGTTCGTTGTTTTTGCGTGAGCCATGAGAAC GAACCATTGAGATCATGCTTACTTTGCATGTCACTCAAAAATTTTGCCTCAAAACTGGT GAGCTGAATTTTTGCAGTTAAAGCATCGTGTAGTGTTTTTCTTAGTCCGTTACGTAGGT AGGAATCTGATGTAATGGTTGTTGGTATTTTGTCACCATTCATTTTTATCTGGTTGTTC

Nucleotide sequences of FNR promoter-PAL1 construct, low-copy (SEQ ID NO: 26)
TCAAGTTCGGTTACGAGATCCATTTGTCTATCTAGTTCAACTTGGAAAATCAACGTATC AGTCGGGCGGCCTCGCTTATCAACCACCAATTTCATATTGCTGTAAGTGTTTAAATCTT TACTTATTGGTTTCAAAACCCATTGGTTAAGCCTTTTAAACTCATGGTAGTTATTTTCA AGCATTAACATGAACTTAAATTCATCAAGGCTAATCTCTATATTTGCCTTGTGAGTTTT CTTTTGTGTTAGTTCTTTTAATAACCACTCATAAATCCTCATAGAGTATTTGTTTTCAA AAGACTTAACATGTTCCAGATTATATTTTATGAATTTTTTTAACTGGAAAAGATAAGGC AATATCTCTTCACTAAAAACTAATTCTAATTTTTCGCTTGAGAACTTGGCATAGTTTGT CCACTGGAAAATCTCAAAGCCTTTAACCAAAGGATTCCTGATTTCCACAGTTCTCGTCA TCAGCTCTCTGGTTGCTTTAGCTAATACACCATAAGCATTTTCCCTACTGATGTTCATC ATCTGAGCGTATTGGTTATAAGTGAACGATACCGTCCGTTCTTTCCTTGTAGGGTTTTC AATCGTGGGGTTGAGTAGTGCCACACAGCATAAAATTAGCTTGGTTTCATGCTCCGTTA AGTCATAGCGACTAATCGCTAGTTCATTTGCTTTGAAAACAACTAATTCAGACATACAT CTCAATTGGTCTAGGTGATTTTAATCACTATACCAATTGAGATGGGCTAGTCAATGATA ATTACTAGTCCTTTTCCTTTGAGTTGTGGGTATCTGTAAATTCTGCTAGACCTTTGCTG GAAAACTTGTAAATTCTGCTAGACCCTCTGTAAATTCCGCTAGACCTTTGTGTGTTTTT TTTGTTTATATTCAAGTGGTTATAATTTATAGAATAAAGAAAGAATAAAAAAAGATAAA AAGAATAGATCCCAGCCCTGTGTATAACTCACTACTTTAGTCAGTTCCGCAGTATTACA AAAGGATGTCGCAAACGCTGTTTGCTCCTCTACAAAACAGACCTTAAAACCCTAAAGGC TTAAGTAGCACCCTCGCAAGCTCGGGCAAATCGCTGAATATTCCTTTTGTCTCCGACCA TCAGGCACCTGAGTCGCTGTCTTTTTCGTGACATTCAGTTCGCTGCGCTCACGGCTCTG GCAGTGAATGGGGGTAAATGGCACTACAGGCGCCTTTTATGGATTCATGCAAGGAAACT ACCCATAATACAAGAAAAGCCCGTCACGGGCTTCTCAGGGCGTTTTATGGCGGGTCTGC TATGTGGTGCTATCTGACTTTTTGCTGTTCAGCAGTTCCTGCCCTCTGATTTTCCAGTC TGACCACTTCGGATTATCCCGTGACAGGTCATTCAGACTGGCTAATGCACCCAGTAAGG CAGCGGTATCATCAACAGGCTTACCCGTCTTACTGTCTTTTCTACGGGGTCTGACGCTC AGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTC ACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTA AACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTC TATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAG GGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCC AGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAA CTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCG CCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTC GTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGAT CCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGT AAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGT CATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAG AATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGATAATACCGCG CCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACT CTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACT GATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA AATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCT TTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTG AATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCA CCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCAC GAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGC TCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAG

Nucleotide sequences of FNR promoter-PAL1 construct, low-copy (SEQ ID NO: 26)
GGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCA GATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAA AATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCG GTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATT AAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAAT TCG

Table 10

Nucleotide sequences of FNR promoter-PAL3 construct, low-copy (SEQ ID NO: 27)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCCTTAAACATTA **GCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAGGAGAAAACCG**ATGAAAGCTA AAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATC TATGACATTGCGATAAAACAAAAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTT GACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAA TCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAG CATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACC TTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAA CCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGACATTGTTCCTCTG GTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGC ACGAGCATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTG AAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCT CTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGA AAAACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCAT CTCATGAACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCG GTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGG GGTTAAAGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATA CCTTACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAA TCTTTAGCTACCGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCC ATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGGCAATATG TCGCCCGAACAATGGATGCATTAAAACTGGATATTGCTTTAATTGCCAATCATCTTCAC GCCATTGTGGCTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTAATTCACTGAG TCCGACACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAG TTGCTGCAATTCGCCATGATTGTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAA TACAATCAAGATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGA GCAGAAATTACGCAATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATC TTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGC GAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCATTGC GGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAGAATAAGCTT GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCAC ACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAA CTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTT CCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCA GCTCACTCAAAGGCGGTAGTACGGGTTTTGCTGCCCGCAAACGGGCTGTTCTGGTGTTG

Nucleotide sequences of FNR promoter-PAL3 construct, low-copy (SEQ ID NO: 27)

CTAGTTTGTTATCAGAATCGCAGATCCGGCTTCAGGTTTGCCGGCTGAAAGCGCTATTT
CTTCCAGAATTGCCATGATTTTTTCCCCACGGGAGGCGTCACTGGCTCCCGTGTTGTCG
GCAGCTTTGATTCGATAAGCAGCATCGCCTGTTTCAGGCTGTCTATGTGTGACTGTTGA
GCTGTAACAAGTTGTCTCAGGTGTTCAATTTCATGTTCTAGTTGCTTTGTTTTACTGGT
TTCACCTGTTCTATTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGATCTGTTCAT
GGTGAACAGCTTTAAATGCACCAAAAACTCGTAAAAGCTCTGATGTATCTATCTTTTTT
ACACCGTTTTCATCTGTGCATATGGACAGTTTTCCCTTTGATATCTAACGGTGAACAGT
TGTTCTACTTTTGTTTGTTAGTCTTGATGCTTCACTGATAGATACAAGAGCCATAAGAA
CCTCAGATCCTTCCGTATTTAGCCAGTATGTTCTCTAGTGTGGTTCGTTGTTTTTGCGT
GAGCCATGAGAACGAACCATTGAGATCATGCTTACTTTGCATGTCACTCAAAAATTTTG
CCTCAAAACTGGTGAGCTGAATTTTTGCAGTTAAAGCATCGTGTAGTGTTTTTCTTAGT
CCGTTACGTAGGTAGGAATCTGATGTAATGGTTGTTGGTATTTTGTCACCATTCATTTT
TATCTGGTTGTTCTCAAGTTCGGTTACGAGATCCATTTGTCTATCTAGTTCAACTTGGA
AAATCAACGTATCAGTCGGGCGGCCTCGCTTATCAACCACCAATTTCATATTGCTGTAA
GTGTTTAAATCTTTACTTATTGGTTTCAAAACCCATTGGTTAAGCCTTTTAAACTCATG
GTAGTTATTTTCAAGCATTAACATGAACTTAAATTCATCAAGGCTAATCTCTATATTTG
CCTTGTGAGTTTTCTTTTGTGTTAGTTCTTTTAATAACCACTCATAAATCCTCATAGAG
TATTTGTTTTCAAAAGACTTAACATGTTCCAGATTATATTTTATGAATTTTTTTAACTG
GAAAAGATAAGGCAATATCTCTTCACTAAAAACTAATTCTAATTTTTCGCTTGAGAACT
TGGCATAGTTTGTCCACTGGAAAATCTCAAAGCCTTTAACCAAAGGATTCCTGATTTCC
ACAGTTCTCGTCATCAGCTCTCTGGTTGCTTTAGCTAATACACCATAAGCATTTTCCCT
ACTGATGTTCATCATCTGAGCGTATTGGTTATAAGTGAACGATACCGTCCGTTCTTTCC
TTGTAGGGTTTTCAATCGTGGGGTTGAGTAGTGCCACACAGCATAAAATTAGCTTGGTT
TCATGCTCCGTTAAGTCATAGCGACTAATCGCTAGTTCATTTGCTTTGAAAACAACTAA
TTCAGACATACATCTCAATTGGTCTAGGTGATTTTAATCACTATACCAATTGAGATGGG
CTAGTCAATGATAATTACTAGTCCTTTTCCTTTGAGTTGTGGGTATCTGTAAATTCTGC
TAGACCTTTGCTGGAAAACTTGTAAATTCTGCTAGACCCTCTGTAAATTCCGCTAGACC
TTTGTGTGTTTTTTTTGTTTATATTCAAGTGGTTATAATTTATAGAATAAAGAAAGAAT
AAAAAAAGATAAAAAGAATAGATCCCAGCCCTGTGTATAACTCACTACTTTAGTCAGTT
CCGCAGTATTACAAAAGGATGTCGCAAACGCTGTTTGCTCCTCTACAAAACAGACCTTA
AAACCCTAAAGGCTTAAGTAGCACCCTCGCAAGCTCGGGCAAATCGCTGAATATTCCTT
TTGTCTCCGACCATCAGGCACCTGAGTCGCTGTCTTTTTCGTGACATTCAGTTCGCTGC
GCTCACGGCTCTGGCAGTGAATGGGGGTAAATGGCACTACAGGCGCCTTTTATGGATTC
ATGCAAGGAAACTACCCATAATACAAGAAAAGCCCGTCACGGGCTTCTCAGGGCGTTTT
ATGGCGGGTCTGCTATGTGGTGCTATCTGACTTTTTGCTGTTCAGCAGTTCCTGCCCTC
TGATTTTCCAGTCTGACCACTTCGGATTATCCCGTGACAGGTCATTCAGACTGGCTAAT
GCACCCAGTAAGGCAGCGGTATCATCAACAGGCTTACCCGTCTTACTGTCTTTTCTACG
GGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATC
AAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAA
GTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATC
TCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAAC
TACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCAC
GCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGA
AGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAG
AGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCG
TGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG
CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGAT

Nucleotide sequences of FNR promoter-PAL3 construct, low-copy (SEQ ID NO: 27)
CGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATA
ATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACC
AAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACG
GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTT
CGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACT
CGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAA
AACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATAC
TCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGC
GGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCC
CCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAA
ATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTC
TGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAG
ACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATG
CGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGA
TGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTG
GGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTG
CTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACG
ACGGCCAGTGAATTCG

Table 11

Nucleotide sequences of Tet promoter-PAL1 construct, low-copy (SEQ ID NO: 28)
ACCACTCCCTATCAGTGATAGAGAAAAGTGAACTCTAGAAATAATTTTGTTTAACTTTA
AGAAGGAGATATACATATGAAAACACTATCACAGGCCCAATCTAAAACTTCTTCACAGC
AATTCAGCTTTACCGGGAACTCGTCTGCGAATGTAATTATCGGCAATCAAAAGCTGACC
ATTAATGATGTAGCTCGCGTTGCCCGGAATGGCACTTTGGTGTCACTGACGAACAATAC
CGACATTCTGCAAGGTATTCAAGCTAGCTGCGATTATATCAATAACGCCGTTGAATCTG
GCGAGCCAATCTACGGGGTAACAAGCGGTTTTGGTGGGATGGCGAACGTTGCCATTAGC
CGTGAACAGGCGAGCGAACTTCAGACCAACCTCGTTTGGTTCCTAAAGACAGGAGCTGG
TAATAAGTTACCTCTGGCTGACGTAAGAGCCGCGATGCTGCTTCGCGCTAATAGTCACA
TGCGCGGCGCCAGTGGTATCCGTCTTGAGCTTATCAAGAGGATGGAAATCTTCCTCAAC
GCGGGTGTCACACCATATGTTTATGAGTTTGGTAGTATCGGAGCCAGTGGTGATCTTGT
TCCCCTGAGTTATATTACGGGTTCATTGATTGGTTTAGACCCGTCCTTTAAAGTGGATT
TTAACGGGAAAGAAATGGACGCCCCGACCGCTTTACGACAGCTTAATCTGAGCCCACTT
ACTTTGCTCCCTAAAGAAGGTCTTGCCATGATGAATGGCACCTCTGTGATGACTGGAAT
TGCCGCGAATTGTGTGTATGACACGCAGATCCTAACGGCCATTGCCATGGGTGTTCACG
CGTTGGACATTCAAGCCCTGAATGGTACAAACCAGTCGTTTCATCCGTTTATCCATAAT
TCAAAACCCCATCCGGGACAGCTTTGGGCTGCTGATCAGATGATCTCACTCCTGGCCAA
TAGTCAACTGGTTCGGGACGAGCTCGACGGCAAACATGATTATCGCGATCATGAGCTCA
TCCAGGACCGGTATTCACTTCGTTGTCTCCCACAATACCTGGGGCCTATCGTTGATGGT
ATATCTCAAATTGCGAAGCAAATTGAAATTGAGATCAATAGCGTAACCGACAACCCGCT
TATCGATGTTGATAATCAGGCCTCTTATCACGGTGGCAATTTTCTGGGCCAGTATGTTG
GTATGGGGATGGATCACCTGCGGTACTATATTGGGCTTCTGGCTAAACATCTTGATGTG
CAGATTGCCTTATTAGCTTCACCAGAATTTTCAAATGGACTGCCGCCATCATTGCTCGG
TAACAGAGAAAGGAAAGTAAATATGGGCCTTAAGGGCCTTCAGATATGTGGTAACTCAA
TCATGCCCCTCCTGACCTTTTATGGGAACTCAATTGCTGATCGTTTTCCGACACATGCT

Nucleotide sequences of Tet promoter-PAL1 construct, low-copy (SEQ ID NO: 28)
GAACAGTTTAACCAAAACATTAACTCACAGGGCTATACATCCGCGACGTTAGCGCGTCG GTCCGTGGATATCTTCCAGAATTATGTTGCTATCGCTCTGATGTTCGGCGTACAGGCCG TTGATTTGCGCACTTATAAAAAAACCGGTCACTACGATGCTCGGGCTTGCCTGTCGCCT GCCACCGAGCGGCTTTATAGCGCCGTACGTCATGTTGTGGGTCAGAAACCGACGTCGGA CCGCCCCTATATTTGGAATGATAATGAACAAGGGCTGGATGAACACATCGCCCGGATAT CTGCCGATATTGCCGCCGGAGGTGTCATCGTCCAGGCGGTACAAGACATACTTCCTTGC CTGCATTAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG CTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTA ATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA ACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGT ATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCG GCGAGCGGTATCAGCTCACTCAAAGGCGGTAGTACGGGTTTTGCTGCCCGCAAACGGGC TGTTCTGGTGTTGCTAGTTTGTTATCAGAATCGCAGATCCGGCTTCAGGTTTGCCGGCT GAAAGCGCTATTTCTTCCAGAATTGCCATGATTTTTTCCCCACGGGAGGCGTCACTGGC TCCCGTGTTGTCGGCAGCTTTGATTCGATAAGCAGCATCGCCTGTTTCAGGCTGTCTAT GTGTGACTGTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTCATGTTCTAGTTGCT TTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTACATGCTGTTCATCTGTTACATTG TCGATCTGTTCATGGTGAACAGCTTTAAATGCACCAAAAACTCGTAAAAGCTCTGATGT ATCTATCTTTTTTACACCGTTTTCATCTGTGCATATGGACAGTTTTCCCTTTGATATCT AACGGTGAACAGTTGTTCTACTTTTGTTTGTTAGTCTTGATGCTTCACTGATAGATACA AGAGCCATAAGAACCTCAGATCCTTCCGTATTTAGCCAGTATGTTCTCTAGTGTGGTTC GTTGTTTTTGCGTGAGCCATGAGAACGAACCATTGAGATCATGCTTACTTTGCATGTCA CTCAAAAATTTTGCCTCAAAACTGGTGAGCTGAATTTTTGCAGTTAAAGCATCGTGTAG TGTTTTTCTTAGTCCGTTACGTAGGTAGGAATCTGATGTAATGGTTGTTGGTATTTTGT CACCATTCATTTTTATCTGGTTGTTCTCAAGTTCGGTTACGAGATCCATTTGTCTATCT AGTTCAACTTGGAAAATCAACGTATCAGTCGGGCGGCCTCGCTTATCAACCACCAATTT CATATTGCTGTAAGTGTTTAAATCTTTACTTATTGGTTTCAAAACCCATTGGTTAAGCC TTTTAAACTCATGGTAGTTATTTTCAAGCATTAACATGAACTTAAATTCATCAAGGCTA ATCTCTATATTTGCCTTGTGAGTTTTCTTTTGTGTTAGTTCTTTTAATAACCACTCATA AATCCTCATAGAGTATTTGTTTTCAAAAGACTTAACATGTTCCAGATTATATTTTATGA ATTTTTTTAACTGGAAAAGATAAGGCAATATCTCTTCACTAAAAACTAATTCTAATTTT TCGCTTGAGAACTTGGCATAGTTTGTCCACTGGAAAATCTCAAAGCCTTTAACCAAAGG ATTCCTGATTTCCACAGTTCTCGTCATCAGCTCTCTGGTTGCTTTAGCTAATACACCAT AAGCATTTTCCCTACTGATGTTCATCATCTGAGCGTATTGGTTATAAGTGAACGATACC GTCCGTTCTTTCCTTGTAGGGTTTTCAATCGTGGGGTTGAGTAGTGCCACACAGCATAA AATTAGCTTGGTTTCATGCTCCGTTAAGTCATAGCGACTAATCGCTAGTTCATTTGCTT TGAAAACAACTAATTCAGACATACATCTCAATTGGTCTAGGTGATTTTAATCACTATAC CAATTGAGATGGGCTAGTCAATGATAATTACTAGTCCTTTTCCTTTGAGTTGTGGGTAT CTGTAAATTCTGCTAGACCTTTGCTGGAAAACTTGTAAATTCTGCTAGACCCTCTGTAA ATTCCGCTAGACCTTTGTGTGTTTTTTTTGTTTATATTCAAGTGGTTATAATTTATAGA ATAAAGAAAGAATAAAAAAAGATAAAAAGAATAGATCCCAGCCCTGTGTATAACTCACT ACTTTAGTCAGTTCCGCAGTATTACAAAAGGATGTCGCAAACGCTGTTTGCTCCTCTAC AAAACAGACCTTAAAACCCTAAAGGCTTAAGTAGCACCCTCGCAAGCTCGGGCAAATCG CTGAATATTCCTTTTGTCTCCGACCATCAGGCACCTGAGTCGCTGTCTTTTTCGTGACA TTCAGTTCGCTGCGCTCACGGCTCTGGCAGTGAATGGGGGTAAATGGCACTACAGGCGC CTTTTATGGATTCATGCAAGGAAACTACCCATAATACAAGAAAAGCCCGTCACGGCTT CTCAGGGCGTTTTATGGCGGGTCTGCTATGTGGTGCTATCTGACTTTTTGCTGTTCAGC AGTTCCTGCCCTCTGATTTTCCAGTCTGACCACTTCGGATTATCCCGTGACAGGTCATT

Nucleotide sequences of Tet promoter-PAL1 construct, low-copy (SEQ ID NO: 28)
CAGACTGGCTAATGCACCCAGTAAGGCAGCGGTATCATCAACAGGCTTACCCGTCTTAC TGTCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGG TCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTT AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAG TGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCG TCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATA CCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAG GGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTT GCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATT GCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTC CCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCT TCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATG GCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGG TGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCC CGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATT GGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTC GATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGG AAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTA TTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTC CGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACA TTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGA CGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGG ATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGC TGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGA AATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGC TGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCG AAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACG ACGTTGTAAAACGACGGCCAGTGAATTCGTTAAGACCCACTTTCACATTTAAGTTGTTT TTCTAATCCGCATATGATCAATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGT GATCAAATAATTCGATAGCTTGTCGTAATAATGGCGGCATACTATCAGTAGTAGGTGTT TCCCTTTCTTCTTTAGCGACTTGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAA ATGCCCCACAGCGCTGAGTGCATATAATGCATTCTCTAGTGAAAAACCTTGTTGGCATA AAAAGGCTAATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAGGCCGTGTACCTAAA TGTACTTTTGCTCCATCGCGATGACTTAGTAAAGCACATCTAAAACTTTTAGCGTTATT ACGTAAAAAATCTTGCCAGCTTTCCCCTTCTAAAGGGCAAAAGTGAGTATGGTGCCTAT CTAACATCTCAATGGCTAAGGCGTCGAGCAAAGCCCGCTTATTTTTTACATGCCAATAC AATGTAGGCTGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGAT TCCGACCTCATTAAGCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAATCTAG ACATCATTAATTCCTAATTTTT**GTTGACACTCTATCATTGATAGAGTTATTTT**

Table 12

Nucleotide sequences of Tet promoter-PAL3 construct, low-copy (SEQ ID NO: 29)
ACCACTCCCTATCAGTGATAGAGAAAAGTGAACTCTAGAAATAATTTTGTTTAACTTTA AGAAGGAGATATACAT<u>ATGAAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAAAA</u> <u>ATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAA</u>

Nucleotide sequences of Tet promoter-PAL3 construct, low-copy (SEQ ID NO: 29)
ATATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAAAATT
AAATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGAATGCCAATTTAG
TTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCTTTCTGCT
GGTACTGGGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACT
TTCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATC
ATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGT
GATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTATTA
TATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTAT
CGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAATC
AGTGCAATCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGC
CCTTGCTGTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAAG
TAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGT
TCAACGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCA
TCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAATTCGCTGTGCAC
CACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGAACGG
GAAGTTATCTCAGCTAATGATAATCCATTGATAGATCCAGAAAATGGCGATGTTCTACA
CGGTGGAAATTTTATGGGGCAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATA
TTGCTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATAACCGTTTC
TCTCGTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAAGG
CGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGATTGTGCTGCATCAG
GTATTCATACCCTCGCCACAGAACAATACAATCAAGATATTGTCAGTTTAGGTCTGCAT
GCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGTTTCAATGACAAT
TCTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAA
CTGCTAAATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCG
TTGGATGAAGATATAATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCTGCC
AGAAATCATGCTGGAAGAATAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG
AAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAG
CCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAG
AGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAGTACGGGTTTTGCTG
CCCGCAAACGGGCTGTTCTGGTGTTGCTAGTTTGTTATCAGAATCGCAGATCCGGCTTC
AGGTTTGCCGGCTGAAAGCGCTATTTCTTCCAGAATTGCCATGATTTTTTCCCCACGGG
AGGCGTCACTGGCTCCCGTGTTGTCGGCAGCTTTGATTCGATAAGCAGCATCGCCTGTT
TCAGGCTGTCTATGTGTGACTGTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTCA
TGTTCTAGTTGCTTTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTACATGCTGTTC
ATCTGTTACATTGTCGATCTGTTCATGGTGAACAGCTTTAAATGCACCAAAAACTCGTA
AAAGCTCTGATGTATCTATCTTTTTTACACCGTTTTCATCTGTGCATATGGACAGTTTT
CCCTTTGATATCTAACGGTGAACAGTTGTTCTACTTTTGTTTGTTAGTCTTGATGCTTC
ACTGATAGATACAAGAGCCATAAGAACCTCAGATCCTTCCGTATTTAGCCAGTATGTTC
TCTAGTGTGGTTCGTTGTTTTTGCGTGAGCCATGAGAACGAACCATTGAGATCATGCTT
ACTTTGCATGTCACTCAAAAATTTTGCCTCAAAACTGGTGAGCTGAATTTTTGCAGTTA
AAGCATCGTGTAGTGTTTTTCTTAGTCCGTTACGTAGGTAGGAATCTGATGTAATGGTT
GTTGGTATTTTGTCACCATTCATTTTTATCTGGTTGTTCTCAAGTTCGGTTACGAGATC
CATTTGTCTATCTAGTTCAACTTGGAAAATCAACGTATCAGTCGGGCGGCCTCGCTTAT
CAACCACCAATTTCATATTGCTGTAAGTGTTTAAATCTTTACTTATTGGTTTCAAAACC
CATTGGTTAAGCCTTTTAAACTCATGGTAGTTATTTTCAAGCATTAACATGAACTTAAA
TTCATCAAGGCTAATCTCTATATTTGCCTTGTGAGTTTCTTTTGTGTTAGTTCTTTTA

Nucleotide sequences of Tet promoter-PAL3 construct, low-copy (SEQ ID NO: 29)
ATAACCACTCATAAATCCTCATAGAGTATTTGTTTTCAAAAGACTTAACATGTTCCAGA TTATATTTTATGAATTTTTTTAACTGGAAAAGATAAGGCAATATCTCTTCACTAAAAAC TAATTCTAATTTTTCGCTTGAGAACTTGGCATAGTTTGTCCACTGGAAAATCTCAAAGC CTTTAACCAAAGGATTCCTGATTTCCACAGTTCTCGTCATCAGCTCTCTGGTTGCTTTA GCTAATACACCATAAGCATTTTCCCTACTGATGTTCATCATCTGAGCGTATTGGTTATA AGTGAACGATACCGTCCGTTCTTTCCTTGTAGGGTTTTCAATCGTGGGGTTGAGTAGTG CCACACAGCATAAAATTAGCTTGGTTTCATGCTCCGTTAAGTCATAGCGACTAATCGCT AGTTCATTTGCTTTGAAAACAACTAATTCAGACATACATCTCAATTGGTCTAGGTGATT TTAATCACTATACCAATTGAGATGGGCTAGTCAATGATAATTACTAGTCCTTTTCCTTT GAGTTGTGGGTATCTGTAAATTCTGCTAGACCTTTGCTGGAAAACTTGTAAATTCTGCT AGACCCTCTGTAAATTCCGCTAGACCTTTGTGTGTTTTTTTTGTTTATATTCAAGTGGT TATAATTTATAGAATAAAGAAAGAATAAAAAAAGATAAAAAGAATAGATCCCAGCCCTG TGTATAACTCACTACTTTAGTCAGTTCCGCAGTATTACAAAAGGATGTCGCAAACGCTG TTTGCTCCTCTACAAAACAGACCTTAAAACCCTAAAGGCTTAAGTAGCACCCTCGCAAG CTCGGGCAAATCGCTGAATATTCCTTTTGTCTCCGACCATCAGGCACCTGAGTCGCTGT CTTTTTCGTGACATTCAGTTCGCTGCGCTCACGGCTCTGGCAGTGAATGGGGGTAAATG GCACTACAGGCGCCTTTTATGGATTCATGCAAGGAAACTACCCATAATACAAGAAAAGC CCGTCACGGGCTTCTCAGGGCGTTTTATGGCGGGTCTGCTATGTGGTGCTATCTGACTT TTTGCTGTTCAGCAGTTCCTGCCCTCTGATTTTCCAGTCTGACCACTTCGGATTATCCC GTGACAGGTCATTCAGACTGGCTAATGCACCCAGTAAGGCAGCGGTATCATCAACAGGC TTACCCGTCTTACTGTCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGT TAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTA AAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACC AATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTT GCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAG TGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACC AGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAG TCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAA CGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCAT TCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAA GCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATC ACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCT TTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG AGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAA AGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGT TGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACT TTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAAT AAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCA TTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAA CAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCAT TATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGC GTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCT TGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGG CGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACC ATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCAT TCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATT ACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGT TTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGTTAAGACCCACTTTCAC

Nucleotide sequences of Tet promoter-PAL3 construct, low-copy (SEQ ID NO: 29)
ATTTAAGTTGTTTTTCTAATCCGCATATGATCAATTCAAGGCCGAATAAGAAGGCTGGC TCTGCACCTTGGTGATCAAATAATTCGATAGCTTGTCGTAATAATGGCGGCATACTATC AGTAGTAGGTGTTTCCCTTTCTTCTTTAGCGACTTGATGCTCTTGATCTTCCAATACGC AACCTAAAGTAAAATGCCCCACAGCGCTGAGTGCATATAATGCATTCTCTAGTGAAAAA CCTTGTTGGCATAAAAAGGCTAATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAGG CCGTGTACCTAAATGTACTTTTGCTCCATCGCGATGACTTAGTAAAGCACATCTAAAAC TTTTAGCGTTATTACGTAAAAAATCTTGCCAGCTTTCCCCTTCTAAAGGGCAAAAGTGA GTATGGTGCCTATCTAACATCTCAATGGCTAAGGCGTCGAGCAAAGCCCGCTTATTTTT TACATGCCAATACAATGTAGGCTGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTG TTAAACCTTCGATTCCGACCTCATTAAGCAGCTCTAATGCGCTGTTAATCACTTTACTT TTATCTAATCTAGACATCATTAATTCCTAATTTTT**GTTGACACTCTATCATTGATAGAG** **TTATTTT**

Table 13

Nucleotide sequences of TetR-PheP construct, low-copy (SEQ ID NO: 30)
ccagtgaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatg atcaattcaaggccgaataagaaggctggctctgcaccttggtgatcaaataattcga tagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttcttcttt agcgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcg ctgagtgcatataatgcattctctagtgaaaaaccttgttggcataaaaaggctaatt gattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtacttttgc tccatcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaa tcttgccagctttccccttctaaagggcaaaagtgagtatggtgcctatctaacatct caatggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtagg ctgctctacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacc tcattaagcagctctaatgcgctgttaatcactttacttttatctaatctagacatca ttaattcctaattttt**gttgacactctatcattgatagagttattttaccactccctat** **cagtgatagagaa**aagtgaactctagaaataattttgtttaactttaagaaggagatat acatATGAAAAACGCGTCAACCGTATCGGAAGATACTGCGTCGAATCAAGAGCCGACGC TTCATCGCGGATTACATAACCGTCATATTCAACTGATTGCGTTGGGTGGCGCAATTGGT ACTGGTCTGTTTCTTGGCATTGGCCCGGCGATTCAGATGGCGGGTCCGGCTGTATTGCT GGGCTACGGCGTCGCCGGGATCATCGCTTTCCTGATTATGCGCCAGCTTGGCGAAATGG TGGTTGAGGAGCCGGTATCCGGTTCATTTGCCCACTTTGCCTATAAATACTGGGGACCG TTTGCGGGCTTCCTCTCTGGCTGGAACTACTGGGTAATGTTCGTGCTGGTGGGAATGGC AGAGCTGACCGCTGCGGGCATCTATATGCAGTACTGGTTCCCGGATGTTCCAACGTGGA TTTGGGCTGCCGCCTTCTTTATTATCATCAACGCCGTTAACCTGGTGAACGTGCGCTTA TATGGCGAAACCGAGTTCTGGTTTGCGTTGATTAAAGTGCTGGCAATCATCGGTATGAT CGGCTTTGGCCTGTGGCTGCTGTTTTCTGGTCACGGCGGCGAGAAAGCCAGTATCGACA ACCTCTGGCGCTACGGTGGTTTCTTCGCCACCGGCTGGAATGGGCTGATTTTGTCGCTG GCGGTAATTATGTTCTCCTTCGGCGGTCTGGAGCTGATTGGGATTACTGCCGCTGAAGC GCGCGATCCGGAAAAAAGCATTCCAAAAGCGGTAAATCAGGTGGTGTATCGCATCCTGC TGTTTTACATCGGTTCACTGGTGGTTTTACTGGCGCTCTATCCGTGGGTGGAAGTGAAA TCCAACAGTAGCCCGTTTGTGATGATTTTCCATAATCTCGACAGCAACGTGGTAGCTTC TGCGCTGAACTTCGTCATTCTGGTAGCATCGCTGTCAGTGTATAACAGCGGGGTTTACT

Nucleotide sequences of TetR-PheP construct, low-copy (SEQ ID NO: 30)
CTAACAGCCGCATGCTGTTTGGCCTTTCTGTGCAGGGTAATGCGCCGAAGTTTTTGACT CGCGTCAGCCGTCGCGGTGTGCCGATTAACTCGCTGATGCTTTCCGGAGCGATCACTTC GCTGGTGGTGTTAATCAACTATCTGCTGCCGCAAAAAGCGTTTGGTCTGCTGATGGCGC TGGTGGTAGCAACGCTGCTGTTGAACTGGATTATGATCTGTCTGGCGCATCTGCGTTTT CGTGCAGCGATGCGACGTCAGGGGCGTGAAACACAGTTTAAGGCGCTGCTCTATCCGTT CGGCAACTATCTCTGCATTGCCTTCCTCGGCATGATTTTGCTGCTGATGTGCACGATGG ATGATATGCGCTTGTCAGCGATCCTGCTGCCGGTGTGGATTGTATTCCTGTTTATGGCA TTTAAAACGCTGCGTCGGAAATAA

[0155] In some embodiments, the genetically engineered bacteria contain gene sequence(s) comprising one or more sequence(s) of any of SEQ ID Nos: 21-30. In some embodiments, the genetically engineered bacteria contain gene sequence(s) comprising one or more sequence(s) having at least 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID Nos: 21-30.

Phenylalanine Transport

[0156] Each of *PAL1* and *PAL3* was expressed on a high-copy plasmid and a low-copy plasmid in genetically engineered E. coli Nissle. Surprisingly, each construct metabolized and reduced phenylalanine to similar levels (**Fig. 15**), and the rate-limiting step of phenylalanine metabolism was phenylalanine availability (**Fig. 16**). Thus, in some embodiments, it is advantageous to increase phenylalanine transport into the cell, thereby enhancing phenylalanine metabolism. Unexpectedly, even low-copy PAL plasmids are capable of almost completely eliminating Phe from a test sample when expressed in conjunction with *pheP* (**Fig. 16A**). Furthermore, there may be additional advantages to using a low-copy PAL-expressing plasmid in conjunction with *pheP* in order to enhance the stability of *PAL* expression while maintaining high phenylalanine metabolism, and to reduce negative selection pressure on the transformed bacterium. In alternate embodiments, the phenylalanine transporter is used in conjunction with the high-copy plasmid.

[0157] The genetically engineered bacteria further comprise a gene encoding a phenylalanine transporter. Phenylalanine transporters may be expressed or modified in the genetically engineered bacteria of the invention in order to enhance phenylalanine transport into the cell.

[0158] PheP is a membrane transport protein that is capable of transporting phenylalanine into bacterial cells (*see, e.g.*, Pi et al., 1991). In some embodiments, the native *pheP* gene in the genetically modified bacteria of the invention is not modified. In some embodiments, the genetically engineered bacteria of the invention comprise multiple copies of the native *pheP* gene. In some embodiments, the genetically engineered bacteria of the invention comprise multiple copies of a non-native *pheP* gene. In some embodiments, the genetically engineered bacteria of the invention comprise a *pheP* 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 embodiments, expression of the *pheP* gene is controlled by a different promoter than the promoter that controls expression of the gene encoding the phenylalanine-metabolizing enzyme and/or the transcriptional regulator. In some embodiments, expression of the *pheP* gene is controlled by the same promoter that controls expression of the phenylalanine-metabolizing enzyme and/or the transcriptional regulator. In some embodiments, the *pheP* gene and the phenylalanine-metabolizing enzyme and/or the transcriptional regulator are divergently transcribed from a promoter region. In some embodiments, expression of each of the genes encoding PheP, the phenylalanine-metabolizing enzyme, and the transcriptional regulator is controlled by a different promoter. In some embodiments, expression of the genes encoding PheP, the phenylalanine-metabolizing enzyme, and the transcriptional regulator is controlled by the same promoter.

[0159] In some embodiments, the native *pheP* gene in the genetically modified bacteria is not modified, and one or more additional copies of the native *pheP* gene are inserted into the genome under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of *PAL*, or a constitutive promoter. In alternate embodiments, the native *pheP* gene is not modified, and a copy of a non-native *pheP* gene from a different bacterial species is inserted into the genome under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of *PAL*, or a constitutive promoter.

[0160] In some embodiments, the native *pheP* gene in the genetically modified bacteria is not modified, and one or more additional copies of the native *pheP* gene are present in the bacteria on a plasmid and under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of the PME, or a constitutive promoter. In alternate embodiments, the native *pheP* gene is not modified, and a copy of a non-native *pheP* gene from a different bacterial species is present in the bacteria on a plasmid and under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of *PAL,* or a constitutive promoter.

[0161] In some embodiments, the native *pheP* gene is mutagenized, mutants exhibiting increased phenylalanine transport are selected, and the mutagenized *pheP* gene is isolated and inserted into the genetically engineered bacteria (*see, e.g.,* Pi et al., 1996; Pi et al., 1998). The phenylalanine transporter modifications described herein may be present on a plasmid or chromosome.

[0162] In some embodiments, the genetically engineered bacterium is *E. coli* Nissle, and the native *pheP* gene in *E. coli* Nissle is not modified; one or more additional copies the native *E. coli* Nissle *pheP* genes are inserted into the *E. coli* Nissle genome under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of *PAL,* or a constitutive promoter. In an alternate embodiment, the native *pheP* gene in *E. coli* Nissle is not modified, and a copy of a non-native *pheP* gene from a different bacterium is inserted into the *E. coli* Nissle genome under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of *PAL,* or a constitutive promoter. In some embodiments, the genetically engineered bacterium is *E. coli* Nissle, and the native *pheP* gene in *E. coli* Nissle is not modified; one or more additional copies the native *E. coli* Nissle *pheP* genes are present in the bacterium on a plasmid and under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of *PAL*, or a constitutive promoter. In an alternate embodiment, the native *pheP* gene in *E. coli* Nissle is not modified, and a copy of a non-native *pheP* gene

from a different bacterium, are present in the bacterium on a plasmid and under the control of the same inducible promoter that controls expression of *PAL*, *e.g.*, the FNR promoter, or a different inducible promoter than the one that controls expression of *PAL,* or a constitutive promoter.

[0163] It has been reported that Escherichia coli has five distinct transport systems (AroP, Mtr, PheP, TnaB, and TyrP) for the accumulation of aromatic amino acids. A general amino acid permease, encoded by the aroP gene, transports three aromatic amino acids, including phenylalanine, with high affinity, and is thought, together with PheP, responsible for the lion share of phenylalanine import. Additionally, a low level of accumulation of phenylalanine was observed in an aromatic amino acid transporter-deficient E. coli strain (ΔaroP ΔpheP Δmtr Δtna ΔtyrP), and was traced to the activity of the LIV-I/LS system, which is a branched-chain amino acid transporter consisting of two periplasmic binding proteins, the LIV-binding protein (LIV-I system) and LS-binding protein (LS system), and membrane components, LivHMGF (Koyanagi et al., and references therein; Identification of the LIV-I/LS System as the Third Phenylalanine Transporter in Escherichia coli K-12).

[0164] In some embodiments, the genetically engineered bacteria comprise an aroP gene. In some embodiments, the genetically engineered bacterium is *E. coli* Nissle, and the native *aroP* gene in *E. coli* Nissle is not modified; one or more additional copies of the native *E. coli* Nissle *aroP* genes are present in the bacterium on a plasmid or in the chromosome and under the control of the same inducible promoter that controls expression of the PME, *e.g.*, the FNR promoter, or the araBAD promoter, a different inducible promoter than the one that controls expression of the PME, or a constitutive promoter. In an alternate embodiment, the native *aroP* gene in *E. coli* Nissle is not modified, and a copy of a non-native *aroP* gene from a different bacterium, are present in the bacterium on a plasmid or in the chromosome and under the control of the same inducible promoter that controls expression of the PME, *e.g.*, the FNR promoter or the AraBAD promoter, or a different inducible promoter than the one that controls expression of the PME, or a constitutive promoter.

[0165] In other embodiments, the genetically engineered bacteria comprise AroP and PheP, under the control of the same or different inducible or constitutive promoters.

[0166] In some embodiments, the *pheP* gene is expressed on a chromosome. In some embodiments, expression from the chromosome may be useful for increasing stability of expression of *pheP*. In some embodiments, the *pheP* gene is integrated into the bacterial chromosome at one or more integration sites in the genetically engineered bacteria. In some embodiments, the *pheP* gene is inserted into the bacterial genome at one or more of the following insertion sites in *E. coli* Nissle: *malE/K, insB/I, araC/BAD, lacZ, agal/rsml, thyA, and malP/T.* Any suitable insertion site may be used (*see, e.g.,* **Fig. 36**). 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.

[0167] In some embodiments, the genetically engineered bacterium comprises multiple mechanisms of action and/or one or more auxotrophies. In certain embodiments, the bacteria are genetically engineered to comprise five copies of *PAL* under the control of an oxygen level-dependent promoter (*e.g.*, P_{fnrS}-*PAL3*) inserted at different integration sites on the chromosome (*e.g.*, *malE/K, yicS/nepI, malP/T, agaI/rsmI,* and *cea*), and one copy of a phenylalanine transporter gene under the control of an oxygen level-dependent promoter (*e.g.*, P_{fnrS}-*pheP*) inserted at a different integration site on the chromosome (*e.g.*, *lacZ*). In a more specific aspect, the bacteria are genetically engineered to further include a kanamycin resistance gene, and a *thyA* auxotrophy, in which the *thyA* gene is deleted and/or replaced with an unrelated gene.

Multiple Mechanisms of Action

[0168] 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, yicS/nepI, insB/I, araC/BAD, lacZ, agal/rsml, thyA, malP/T, dapA, and cea,* and others shown in **Fig. 36**. For example, the genetically engineered bacteria may include four copies of *PAL* inserted at four different insertion sites, *e.g.*, *malE/K, insB/I, araC/BAD,* and *lacZ.* The genetically engineered bacteria may also include four copies of *PAL* inserted at four different insertion sites, *e.g.*, *malE/K, yicS/nepI, agaI/rsmI,* and *cea*, and one copy of a

phenylalanine transporter gene inserted at a different insertion site, *e.g.*, *lacZ* (**Fig. 13B**). Alternatively, the genetically engineered bacteria may include three copies of *PAL* inserted at three different insertion sites, *e.g.*, *malE/K, insB/I,* and *lacZ*, and three copies of a phenylalanine transporter gene inserted at three different insertion sites, *e.g.*, *dapA, cea,* and *araC/BAD*.

[0169] In some embodiments, the genetically engineered bacteria comprise one or more of (1) PAL, PAH, LAAD for degradation of phenylalanine, in wild type or in a mutated form (for increased stability or metabolic activity) (2) transporter PheP or AroP for uptake of phenylalanine, in wild type or in mutated form (for increased stability or metabolic activity) (3) PAL, PAH, LAAD, and/or PheP for secretion and extracellular phenylalanine degradation, (4) components of secretion machinery, as described herein (5) Auxotrophy, e.g., deltaThyA (6) antibiotic resistance, including but not limited to, kanamycin or chloramphenicol resistance (7) mutations/deletions in genes involved in oxygen metabolism, as described herein and (8) mutations/deletions in genes of the endogenous Nissle phenylalanine synthesis pathway (e.g., delta PheA for Phe auxotrophy).

[0170] In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, (e.g., under the control of a Pfnr promoter) and one or more copies of PAL1 (e.g. under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, (e.g., under the control of a Pfnr promoter) and one or more copies of PAL1 (e.g. under the control of a Pfnr promoter); and further comprises one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, (e.g., under the control of a Pfnr promoter) and one or more copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, (e.g., under the control of a Pfnr promoter) and one or more copies of LAAD (e.g., under the control of the ParaBAD promoter); and further comprises one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, (e.g., under the control of a Pfnr promoter) and one or more copies of PAH. In one embodiment, the genetically

engineered bacteria comprise one or more copies of PAL3, (e.g., under the control of a Pfnr promoter) and one or more copies of PAH; and further comprises one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL1, (e.g., under the control of a Pfnr promoter) and one or more copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL1, (e.g., under the control of a Pfnr promoter) and one or more copies of LAAD (e.g., under the control of the ParaBAD promoter); and further comprises one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL1 (e.g., under the control of a Pfnr promoter) and one or more copies of PAH. In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL1 (e.g., under the control of a Pfnr promoter) and one or more copies of PAH; and further comprises one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAH and one or more copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAH and one or more copies of LAAD (e.g., under the control of the ParaBAD promoter); and further comprises one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). PMEs and transporters may be integrated into any of the insertion sites described herein.

[0171] In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of LAAD (e.g., under the control of the ParaBAD promoter), and one or more copies of PAH. In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of LAAD (e.g., under the control of the ParaBAD promoter), and one or more copies of PAH; and further comprise one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of LAAD (e.g.,

under the control of the ParaBAD promoter), and one or more copies of PAL1 (e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of LAAD (e.g., under the control of the ParaBAD promoter), and one or more copies of PAL1 (e.g., under the control of a Pfnr promoter); and further comprise one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of PAL1 (e.g., under the control of a Pfnr promoter), and one or more copies of PAH. In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of PAL1 (e.g., under the control of a Pfnr promoter), and one or more copies of PAH; and further comprise one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of LAAD (e.g., under the control of the ParaBAD promoter), one or more copies of PAH, and one or more copies of PAL1 (e.g., under the control of an Pfnr promoter). In one embodiment, the genetically engineered bacteria comprise one or more copies of LAAD (e.g., under the control of the ParaBAD promoter), one or more copies of PAH, and one or more copies of PAL1 (e.g., under the control of an Pfnr promoter); and further comprise one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). PMEs and/or transporters may be integrated into any of the insertion sites described herein. Alternatively, PMEs and/or transporters may be comprised on low or high copy plasmids. PMEs and/or transporters may be integrated into any of the insertion sites described herein in combination with PMEs and/or transporters that are comprised on low or high copy plasmids.

[0172] In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of PAL1, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of LAAD (e.g., under the control of the ParaBAD promoter), and one or more copies of PAH. In one embodiment, the genetically engineered bacteria comprise one or more copies of PAL3, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of

PAL1, e.g. (e.g., under the control of a Pfnr promoter), one or more copies of LAAD (e.g., under the control of the ParaBAD promoter), and one or more copies of PAH; and further comprise one or more copies of a phenylalanine transporter (e.g., PheP and/or AroP, e.g., under the control of a Pfnr promoter). PMEs and transporters may be integrated into any of the insertion sites described herein. Alternatively, PMEs and/ortransporters may be comprised on low or high copy plasmids.

[0173] In one embodiment, the genetically engineered bacteria comprise one copy of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise one copy of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise one copy of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise one copy of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter). PMEs and transporters may be integrated into any of the insertion sites described herein. Alternatively, located PMEs and/ortransporters may be comprised on low or high copy plasmids.

[0174] In one embodiment, the genetically engineered bacteria comprise two copies of PAL (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise two copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise two copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control

of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise two copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter).

[0175] In one embodiment, the genetically engineered bacteria comprise three copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise three copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise three copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise three copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise three copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter), three copies of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise three copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter), three copies of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter).

[0176] In one embodiment, the genetically engineered bacteria comprise four copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise four copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter).

In one embodiment, the genetically engineered bacteria comprise four copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise four copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter).

[0177] In one embodiment, the genetically engineered bacteria comprise five copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise five copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and one copy of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise five copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) one copy of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise five copies of PAL, (e.g., PAL1 or PAL3, e.g., under the control of a Pfnr promoter) two copies of PheP (e.g., under the control of a Pfnr promoter), and two copies of LAAD (e.g., under the control of the ParaBAD promoter).

[0178] In one embodiment, the genetically engineered bacteria comprise one or more PMEs for metabolizing phenylalanine in combination with one or more PMEs for secretion. In one embodiment, the genetically engineered bacteria comprise one or more PMEs for metabolizing phenylalanine and a phenylalanine transporter in combination with one or more PMEs for secretion. In one embodiment, the genetically engineered bacteria comprise one or more PMEs for metabolizing phenylalanine and a phenylalanine transporter in combination with one or more PMEs for secretion, and also include an auxotrophy and/or an antibiotic resistance. Secretion systems described herein are utilized to secrete the PMEs in the genetically engineered bacteria with multiple mechanisms of action.

[0179] In one embodiment, the genetically engineered bacteria comprise two additional copies of PheP (in addition to the wild type gene). This provides redundancy, in case one of the PheP genes acquires a mutation. In one embodiment, the PheP genes are inserted at lacZ and agal/rsml. In one embodiment, the two copies of PheP are under the control of the PfnrS promoter. In one embodiment, the genetically engineered bacteria comprise three copies of PAL3. In one embodiment, the genetically engineered bacteria comprise three copies of PAL3, inserted at malEK, malPT, yicS/nepl. In one embodiment, the expression of the three copies of PAL3 is under the control of the PfnrS promoter. In one embodiment, the genetically engineered bacteria comprise one or more copies of LAAD. In one embodiment, the genetically engineered bacteria comprise one copy of LAAD, inserted in the arabinose operon. In one embodiment, LAAD is under the control of the endogenous ParaBAD promoter. In one embodiment, the genetically engineered bacteria comprise an auxotrophy, e.g., deltaThyA. In one embodiment, the genetically engineered bacteria comprise an antibiotic resistance. In one embodiment the genetically engineered bacteria comprise an antibiotic resistance and an auxotrophy, e.g., deltaThyA. In one embodiment, the genetically engineered bacteria do not comprise an auxotrophy, e.g., deltaThyA. In one embodiment, the genetically engineered bacteria do not comprise an antibiotic resistance. In one embodiment the genetically engineered bacteria comprise neither an antibiotic resistance nor an auxotrophy, e.g., deltaThyA.

[0180] In one embodiment, the genetically engineered bacteria comprise three copies of PAL, e.g., PAL3, 2 copies of PheP (in addition to the endogenous PheP), and one copy of LAAD. In one embodiment, the genetically engineered bacteria comprise three copies of PAL, e.g., PAL3, 2 copies of PheP (in addition to the endogenous PheP), and one copy of LAAD, and an auxotrophy, e.g., delta ThyA. In one embodiment, the genetically engineered bacteria comprise three copies of PAL, 2 copies of PheP (in addition to the endogenous PheP), and one copy of LAAD, and an antibiotic resistance gene. In one embodiment, the genetically engineered bacteria comprise three copies of PAL, 2 copies of PheP (in addition to the endogenous PheP), and one copy of LAAD, and an antibiotic resistance gene and an auxotrophy, e.g., delta ThyA.

[0181] In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter), 2 copies of PheP (each under

control of a PfnrS promoter), and one copy of LAAD (under the control of the endogenous ParaBAD promoter). In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter), 2 copies of PheP (each under control of a PfnrS promoter), and one copy of LAAD (under the control of the endogenous ParaBAD promoter), and an antibiotic resistance. In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter), 2 copies of PheP (each under control of a PfnrS promoter), and one copy of LAAD (under the control of the endogenous ParaBAD promoter), and an auxotrophy, e.g., delta ThyA. In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter), 2 copies of PheP (each under control of a PfnrS promoter), and one copy of LAAD (under the control of the endogenous ParaBAD promoter), and an antibiotic resistance and an auxotrophy, e.g., deltaThyA.

[0182] In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter and inserted at the malEK, malPT, and yicS/nepl sites), 2 copies of PheP (each under control of a PfnrS promoter and inserted at the LacZ and agal/rsml sites), and one copy of LAAD (under the control of the endogenous ParaBAD promoter, and inserted in the endogenous arabinose operon). In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter and inserted at the malEK, malPT, and yicS/nepl sites), 2 copies of PheP (each under control of a PfnrS promoter and inserted at the LacZ and agal/rsml sites), and one copy of LAAD (under the control of the endogenous ParaBAD promoter, and inserted in the endogenous arabinose operon), and further comprise an antibiotic resistance. In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter and inserted at the malEK, malPT, and yicS/nepl sites), 2 copies of PheP (each under control of a PfnrS promoter and inserted at the LacZ and agal/rsml sites), and one copy of LAAD (under the control of the endogenous ParaBAD promoter, and inserted in the endogenous arabinose operon) and further comprise an auxotrophy, e.g., deltaThyA. In one embodiment, the genetically engineered bacteria comprise three copies of PAL (each under control of a PfnrS promoter and inserted at the malEK, malPT, and yicS/nepl sites), 2 copies of PheP (each under control of a PfnrS promoter and inserted at the LacZ and agal/rsml sites), and one copy of LAAD (under the control of the

endogenous ParaBAD promoter, and inserted in the endogenous arabinose operon), and further comprise an antibiotic resistance and an auxotrophy, e.g., deltaThyA.

[0183] In one embodiment, the genetically engineered bacteria are SYN-PKU705. In one embodiment, SYN-PKU705 further comprises an antibiotic resistance. In one embodiment, SYN-PKU705 further comprises an auxotrophy, e.g., deltaThyA. In one embodiment, SYN-PKU705 further comprises an antibiotic resistance and auxotrophy, e.g., deltaThyA.

[0184] **Table 14** contains non-limiting examples of the genetically engineered bacteria of the disclosure. In certain embodiments, the genetically engineered bacteria of Table 14 further contain a PME for secretion.

Table 14. Non-limiting Examples of Embodiments of the Disclosure

Strain Name	Genotype
Plasmid -based strains	
SYN-PKU101	Low copy pSC101-P*tet*::*PAL1*, ampicillin resistant
SYN-PKU102	High copy pColE1-P*tet*::*PAL1*, ampicillin resistant,
SYN-PKU201	Low copy pSC101-P*tet*::*PAL3*, ampicillin resistant
SYN-PKU202	High copy pColE1-P*tet*::*PAL3*, ampicillin resistant,
SYN-PKU203	*lacZ::*P*tet-pheP*::*cam*
SYN-PKU401	Low copy pSC101-P*tet*::*PAL1*, ampicillin resistant, chromosomal *lacZ::*P*tet-pheP*::*cam*
SYN-PKU402	High copy pColE1-P*tet*::*PAL1*, ampicillin resistant, chromosomal *lacZ::*P*tet-pheP*::*cam*
SYN-PKU302	Low Copy pSC101-P*tet*::*PAL3,* ampicillin resistant; chromosomal *lacZ::*P*tet-pheP*::*cam*
SYN-PKU303	High copy pColE1-P*tet*::*PAL3*, ampicillin resistant, chromosomal *lacZ::*P*tet-pheP*::*cam*
SYN-PKU304	Low Copy pSC101-*PfnrS*::*PAL3,* ampicillin resistant; chromosomal *lacZ*::*PfnrS-pheP::cam*
SYN-PKU305	Low Copy pSC101-*PfnrS*::*PAL3*, kanamycin resistant; chromosomal *lacZ*::P*fnrS-pheP::cam*
SYN-PKU306	Low Copy pSC101-*PfnrS*::*PAL3*, kanamycin resistant; *thyA*
SYN-PKU307	Low Copy pSC101-*PfnrS*::*PAL3,* ampicillin resistant;
SYN-PKU308	Low Copy pSC101-*PfnrS*::*PAL3,* kanamycin resistant;
SYN-PKU401	High Copy pUC57-P*tet*::*LAAD;* kanamycin

	resistant
Integrated strains	
SYN-PKU501	*malPT:: PfnrS::PAL3::kan*
SYN-PKU502	*malPT:: PfnrS::PAL3::kan*; bicistronic *lacZ:: PfnrS::PAL3-pheP::cam*
SYN-PKU503	*malEK::PfnrS::PAL3::cam*
SYN-PKU504	*agaI/rsmI::PfnrS::PAL3*
SYN-PKU505	*cea::PfnrS::PAL3*
SYN-PKU506	*malEK::PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3*
SYN-PKU507	*malEK:: PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; lacZ::*P*fnr-pheP::cam*
SYN-PKU508	*malEK::PfnrS::PAL3; pheA* auxotroph
SYN-PKU509	*malEK::PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; lacZ::*P*fnr-pheP::*cam
SYN-PKU601	*malPT::*P*fnrS*-INT5::kan, *rrnBUP* -[PAL3]; *lacZ::*P*fnr-pheP::cam* (recombinase based strain)
SYN-PKU510	*malEK::PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3;*
SYN-PKU511	*malEK:: PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; yicS/nepI::*P*fnrS-PAL3::kan; malPT::PfnrS::*PAL3; *lacZ::*P*fnr-pheP; ΔthyA*
SYN-PKU204	*lacZ::*P*fnr-pheP::cam*
SYN-PKU512	*malEK::PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; malPT::PfnrS::*PAL3; *lacZ::*P*fnr-pheP::cam; ΔthyA*
SYN-PKU513	*malEK:: PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; lacZ::*P*fnr-pheP; ΔthyA*
SYN-PKU514	*malEK:: PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; malPT::PfnrS::*PAL3; *ΔthyA*
SYN-PKU515	*malEK:: PfnrS::PAL3; agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; ΔthyA*
SYN-PKU516	*agaI/rsmI::PfnrS::PAL3::kan*
SYN-PKU517	*malEK:: PfnrS::PAL3::*cam; *malPT::PfnrS::*PAL3::kan; *lacZ::*P*fnrS-pheP; ΔthyA*
SYN-PKU518	*malEK-*P*fnrS::PAL3::cam; PfnrS::pheP::kan*
SYN-PKU519	*ParaBC-PAL3::cam; PfnrS-pheP::kan*
SYN-PKU520	*agaI/rsmI::PfnrS::PAL3::kan*; P*fnrS-PheP::cam*
SYN-PKU801	*ΔargR; thyA::cam*
SYN-PKU701	*ParaBC-LAAD::cam; malEK-*P*fnrS-PAL3; malPT::PfnrS*-PAL3::kan; *PfnrS-pheP*
SYN-PKU521	*yicS/nepI::*P*fnrS-PAL3::kan; lacZ::*P*fnr-pheP::cam*
SYN-PKU522	*cea::*P*fnrS-PAL3::kan; lacZ::*P*fnr-pheP::cam*
SYN-PKU523	*malPT::*P*fnrS-PAL3::kan; lacZ::*P*fnr-pheP::cam*

SYN-PKU524	*malEK*:: *PfnrS*::*PAL3*; *malPT*::*PfnrS*::PAL3; *lacZ*::P*fnr-pheP*
SYN-PKU702	*malEK*:: *PfnrS*::*PAL3*; *lacZ*::P*fnr-pheP; Para::LAAD*
SYN-PKU703	*malEK*:: *PfnrS*::*PAL3*; *malPT*::*PfnrS*::PAL3; *lacZ*::P*fnr-pheP; agaI/rsmI*::*PfnrS*::*pheP; Para::LAAD*
SYN-PKU704	*malEK*:: *PfnrS*::*PAL3*; *malPT*::*PfnrS*::PAL3; *yicS/nepI*::P*fnrS-PAL3; lacZ*::P*fnr-pheP; Para::LAAD*
SYN-PKU705	*malEK*:: *PfnrS*::*PAL3*; *malPT*::*PfnrS*::PAL3; *yicS/nepI*::P*fnrS-PAL3::kan; lacZ*::P*fnr-pheP; agaI/rsmI*::*PfnrS*::*pheP Para::LAAD*
SYN-PKU602	*malEK*:: P*T7*::*PAL3; Para::INT5::cam (recombinase); lacZ*::P*fnr-pheP; malPT*::P*constitutive*::T7 polymerase (unflipped);
SYN-PKU901	*Nissle with streptomycin resistance*

Secretion

[0185] 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 protein(s) of interest or therapeutic protein(s), e.g., PAH, PAL or LAAD, 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.

[0186] In Gram-negative bacteria, secretion machineries may span one or both of the inner and outer membranes. In some embodiments, the genetically engineered bacteria further comprise a non-native double membrane-spanning secretion system. Double membrane-spanning secretion systems include, but are not limited to, the type I secretion system (T1SS), the type II secretion system (T2SS), the type III secretion system (T3SS), the type IV secretion system (T4SS), the type VI secretion system (T6SS), and the resistance-nodulation-division (RND) family of multi-drug efflux pumps (Pugsley 1993; Gerlach et al., 2007; Collinson et al., 2015; Costa et al., 2015; Reeves et al., 2015; WO2014138324A1, incorporated herein by reference). Examples

of such secretion systems are shown in Figures 3-6. 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).

[0187] 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 protein(s) of interest or therapeutic protein(s) from the bacterial cytoplasm. In some embodiments, the secreted molecule, such as a heterologous protein or peptide, e.g., the protein of interest or therapeutic protein e.g., PAH, PAL or LAAD, comprises a type III secretion sequence that allows the protein(s) of interest or therapeutic protein(s) to be secreted from the bacteria.

[0188] In some embodiments, a flagellar type III secretion pathway is used to secrete the molecule of interest, e.g., PAH, PAL or LAAD. In some embodiments, an incomplete flagellum is used to secrete a therapeutic peptide of interest, e.g., PAH, PAL or LAAD, 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.

[0189] In some embodiments, a Type V Autotransporter Secretion System is used to secrete the therapeutic peptide, e.g., PAH, PAL or LAAD. 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 Figure 10, 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, e.g., PAH, PAL or LAAD, is threaded through the hollow pore of the beta-barrel structure ahead of the linker sequence. Once exposed to the extracellular environment, the therapeutic peptide, e.g., PAH, PAL or LAAD, 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., the protein of interest or therapeutic protein, 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.

[0190] In some embodiments, a Hemolysin-based Secretion System is used to secrete the molecule of interest, e.g., e.g., PAH, PAL or LAAD. 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. Figure 11 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.

[0191] In alternate embodiments, the genetically engineered bacteria further comprise a non-native single membrane-spanning secretion system. Single membrane-spanning exporters may act as a component of a secretion system, or may export substrates independently. Such exporters 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 protein(s) of interest or therapeutic protein(s), e.g., PAH, PAL or LAAD, 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.

[0192] In order to translocate a protein, e.g., therapeutic polypeptide, e.g., PAH, PAL or LAAD, 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.

[0193] One way to secrete properly folded proteins in gram-negative bacteria– particularly those requiring disulphide bonds – is to target the periplasm in a bacterium 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 nlpl. Lpp is the most abundant polypeptide in the bacterial cell existing at ~500,000 copies per cell and functions as the primary ‘staple’ of the bacterial cell wall to the peptidoglycan. 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 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.

[0194] 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 promoter. For example, expression of *lpp* or other cell wall stability protein or periplasmic protease can be repressed in conditions where the therapeutic polypeptide needs to be delivered (secreted). For instance, under inducing conditions a transcriptional repressor protein or a designed antisense RNA can be expressed which reduces transcription or translation of a target membrane or periplasmic protease gene. Conversely, overexpression of certain peptides can result in a destabilized phenotype, e.g., overexpression of colicins or the third topological domain of TolA, wherein peptide overexpression can be induced in conditions in which the therapeutic polypeptide needs to be delivered (secreted). These sorts of strategies would decouple the fragile, leaky phenotypes from biomass production. Thus, in some embodiments, the engineered bacteria have one or more membrane and/or periplasmic protease genes under the control of an inducible promoter.

[0195] **Table 15** and **Table 16** list secretion systems for Gram positive bacteria and Gram negative bacteria. These can be used to secrete polypeptides, proteins of interest or therapeutic protein(s) 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.

Table 15. 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 16. 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?

[0196] In some embodiments, the genetically engineered bacterial comprise a native or non-native secretion system described herein for the secretion of a PME, e.g., PAH, PAL and/or LAAD. In some embodiments, the secretion system is selected from 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, a single membrane secretion system, Sec and, TAT secretion systems.

[0197] In some embodiments, the PMEs secreted by the genetically engineered bacteria are modified to increase resistance to proteases. For example, in some embodiments, the one or more PME administered is modified as described in Sakissian et al., 2011, Mol Genet Metab. 2011 Nov; 104(3): 249–254, the contents of which is herein incorporated by reference in its entirety. In some embodiments, the secreted PAL is Av-p.C503S/p.C565S/p.F18A PAL. In some embodiments, the secreted PAL is PEG-Av-p.C503S/p.C565S/p.F18A PAL.

[0198] In some embodiments, the one or more PMEs for secretion are under the control of an inducible promoter, as described herein. In one example, the one or more PMEs are under the control of the FNR promoter and are produced and secreted under anaerobic conditions. In some embodiments, the PMEs for secretion are under the control of the ParaBAD promoter. In some embodiments, the PMEs for secretion are under the control of a constitutive promoter.

[0199] In some embodiments in which the one or more PMEs are secreted or exported from the microorganism, the engineered microorganism comprises gene sequence(s) that includes a secretion tag. In some embodiments, the PME(s) include a “secretion tag” of either RNA or peptide origin to direct the PME(s) to specific secretion systems. For example, a secretion tag for the Type I Hemolysin secretion

system is encoded in the C-terminal 53 amino acids of the alpha hemolysin protein (HlyA). HlyA secretion signal.

[0200] HlyB inserts into inner membrane to form a pore, HlyD aligns HlyB with TolC (outer membrane pore) thereby forming a channel through inner and outer membrane. The C-terminal secretion tag can be removed by either an autocatalytic or protease-catalyzed e.g., OmpT cleavage thereby releasing the PME(s) into the extracellular milieu.

[0201] The Type V Auto-secretion System utilizes an N-terminal Sec-dependent peptide tag (inner membrane) and C-terminal tag (outer-membrane). This uses Sec-system to get from cytoplasm to periplasm. C-terminal tag then inserts into the outer membrane forming a pore through which the "passenger protein" threads through. Once across the outer membrane, the passenger (anti-cancer molecule) is released from the membrane-embedded C-terminal tag by either an autocatalytic, intein-like mechanism or via a membrane-bound protease (I.e., OmpT). The N-terminal tag is removed by the Sec system. Thus, in some embodiments, the secretion system is able to remove this tag before secreting the PME(s), e.g., PAL, PAH, and/or LAAD from the engineered bacteria. In the Type V auto-secretion-mediated secretion the N-terminal peptide secretion tag is removed upon translocation of the "passenger" peptide from the cytoplasm into the periplasmic compartment by the native Sec system. Further, once the auto-secretor is translocated across the outer membrane the C-terminal secretion tag can be removed by either an autocatalytic or protease-catalyzed e.g., OmpT cleavage thereby releasing the anti-cancer molecule(s) into the extracellular milieu.

[0202] In the Flagellar modified Type III Secretion, the tag is encoded in 5'untranslated region of the mRNA and thus there is no peptide tag to cleave/remove. This modified system does not contain the "syringe" portion and instead uses the basal body of the flagella structure as the pore to translocate across both membranes and out through the forming flagella. If the fliC/fliD genes (encoding the flagella "tail"/whip) are disrupted the flagella cannot fully form and this promotes overall secretion. In some embodiments, the tail portion can be removed entirely. In the Type III traditional secretion system, the basal body closely resembles the flagella, however, instead of a "tail"/whip, the traditional T3SS has a syringe to inject the passenger proteins into host cells. The secretion tag is encoded by an N-terminal peptide (lengths vary and there are

several different tags, see PCT/US14/020972). The N-terminal tag is not removed from the polypeptides in this secretion system.

[0203] In some embodiments the PME contains expressed as fusion protein with the 53 amino acids of the C termini of alpha-hemolysin (hlyA) of E. coli CFT073 (C terminal secretion tag).

Oxygen consuming enzymes

[0204] LAAD catalytic activity is dependent on oxygen, and therefore may not be active in anaerobic environments in the intestine, e.g., the colon. Oxygen is present in more proximal compartments of the GI tract.

[0205] The oxygen tension as measured in healthy mice is shown in **Table 17**. He et al., Proc Natl Acad Sci U S A. 1999 Apr 13;96(8):4586-91; “Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral EPR imaging”, the contents of which is herein incorporated by reference in its entirety. A marked oxygen gradient from the proximal to the distal GI tract. As noted by He et al., the observed oxygen gradient seen along the GI tract can be explained by a combination of processes. Without wishing to be bound by theory, food, when swallowed, is initially equilibrated with the oxygen tension of ambient room air. On passage to the stomach and later the small intestine, the oxygen levels may fall as oxygen diffuses across the mucosal membrane. A gradual process of equilibration with the capillary levels of oxygen (i.e., 5–10 torr; ref. 9) may occur. On passage to the colon, with its heavy bacterial colonization, further decreases in oxygenation occur. Finally, the lumen of the distal colon displays marked hypoxia, as expected, based on the abundance of anaerobic bacteria at this site.

Table 17. Oxygen Tension in Gastrointestinal Tract Compartments

Compartment	Oxygen Tension
Ambient Air	159 Torr
stomach	~60 torr
duodenum and first part of jejunum	(~30 torr); ~20% oxygen in ambient air
ileum	(~10 torr); ~6% oxygen in ambient air
colon	(<2torr)

[0206] As shown in **Fig. 25B**, LAAD activity is retained in microaerobic conditions, albeit at lower levels than under aerobic conditions (**Fig. 25A** and **Fig.25B**).

LAAD therefore may be active in the more proximal areas of the intestine, such as stomach, duodenum, jejunum, and ileum. It is contemplated as part of this disclosure that LAAD expressed by the genetically engineered bacteria may advantageously be active in a different compartment than PAL, which may be expressed in the colon if under the control of an FNR promoter. In one embodiment, the genetically engineered bacteria express two enzymes, which have different oxygen requirements and/or are induced under different oxygen conditions, such that an PME is expressed and active throughout the entire gastrointestinal system. For example, the first enzyme, e.g., LAAD, which is dependent on the presence of oxygen, is expressed in one or more of stomach, duodenum and ileum under the control of a constitutive or inducible promoter (such as ParaBAD), and the second enzyme, e.g., PAL, is expressed in the colon under the control of an FNR promoter.

[0207] Several strategies can be employed to further increase LAAD activity under oxygen limiting conditions. For example, the activity of other enzymes that consume large amounts of oxygen can be reduced or extinguished. One such enzyme is NADH dehydrogenase. E coli has two NADH dehydrogenases; *nuo* and *ndh2*, and is has been shown that knock out of both of these enzymes reduces oxygen consumption by 80%. In some embodiments, additional measures are taken to conserve limiting oxygen, i.e., to allow LAAD to function under lower exogenous oxygen conditions in the genetically engineered bacteria expressing LAAD. In some embodiments, the genetically engineered bacteria further comprise a mutation in one or more genes involved in oxygen consumption. In some embodiments, one or both E. coli NADH dehydrogenases are knocked out. In some embodiments, the knocked out NADH dehydrogenase is *nuo*. In some embodiments the knocked out NADH dehydrogenase is *ndh2*. In some embodiments *nuo* and *ndh2* are knocked out. Other enzymes involved in E. coli oxygen metabolism may also be knocked out, including enzymes in the respiratory chain, such as cydB (a subunit of high affinity terminal oxidase), cydD (an enzyme required to make cytochrome D), and cyoABC (subunits of low affinity cytochrome oxidase). In some embodiments, the genetically engineered bacteria harbor a knock out mutation/deletion in one more genes selected from cydB, cydD, and cyoABC.

[0208] In one embodiment, the one or more PME encoded by the genetically engineered bacteria are expressed and show activity in the stomach. In one embodiment, the one or more PME encoded by the genetically engineered bacteria are expressed and show activity in the duodenum. In one embodiment, the one or more PME encoded by the genetically engineered bacteria are expressed and show activity in the jejunum. In one embodiment, the one or more PME encoded by the genetically engineered bacteria are expressed and show activity in the ileum. In one embodiment, the one or more PME encoded by the genetically engineered bacteria are expressed and show activity in the colon.

Essential Genes and Auxotrophs

[0209] As used herein, the term "essential gene" refers to a gene that is necessary for cell growth and/or survival. Bacterial essential genes are well known to one of ordinary skill in the art, and can be identified by directed deletion of genes and/or random mutagenesis and screening (*see, e.g.,* Zhang and Lin, "DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes," Nucl Acids Res, 2009;37:D455-D458 and Gerdes et al., "Essential genes on metabolic maps," Curr Opin Biotechnol, 2006;17(5):448-456, the entire contents of each of which are expressly incorporated herein by reference).

[0210] An "essential gene" may be dependent on the circumstances and environment in which an organism lives. For example, a mutation of, modification of, or excision of an essential gene may result in the genetically engineered bacteria of the disclosure becoming an auxotroph. An auxotrophic modification is intended to cause bacteria to die in the absence of an exogenously added nutrient essential for survival or growth because they lack the gene(s) necessary to produce that essential nutrient. In some embodiments, any of the genetically engineered bacteria described herein also comprise a deletion or mutation in a gene required for cell survival and/or growth. In one embodiment, the essential gene is a DNA synthesis gene, for example, *thyA*. In another embodiment, the essential gene is a cell wall synthesis gene, for example, *dapA*. In yet another embodiment, the essential gene is an amino acid gene, for example, *serA* or *MetA*. Any gene required for cell survival and/or growth may be targeted, including but not limited to, *cysE, glnA, ilvD, leuB, lysA, serA, metA, glyA, hisB, ilvA, pheA, proA, thrC, trpC, tyrA, thyA, uraA, dapA, dapB, dapD, dapE, dapF, flhD, metB, metC, proAB,*

and *thi1*, as long as the corresponding wild-type gene product is not produced in the bacteria. **Table 18** lists 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 18. 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		

[0211] **Table 19** 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 19. 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

[0212] 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).

[0213] 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,* or

in the presence of high DAP levels found naturally in the human gut *in vivo*. 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).

[0214] 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,* or in the presence of high uracil levels found naturally in the human gut *in vivo*. 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).

[0215] In complex communities, it is possible for bacteria to share DNA. In very rare circumstances, an auxotrophic bacterial strain may receive DNA from a non-auxotrophic strain, which repairs the genomic deletion and permanently rescues the auxotroph. Therefore, engineering a bacterial strain with more than one auxotroph may greatly decrease the probability that DNA transfer will occur enough times to rescue the auxotrophy. In some embodiments, the genetically engineered bacteria of the invention comprise a deletion or mutation in two or more genes required for cell survival and/or growth.

[0216] 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.

[0217] 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 Synth Biol 2015;4(12):1279-1286, the entire contents of which are expressly incorporated herein by reference).

[0218] 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.

[0219] 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.

[0220] 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).

[0221] In some embodiments, the genetically engineered bacterium is a conditional auxotroph whose essential gene(s) is replaced using the arabinose system shown in **FIGS. 43-47.**

[0222] In some embodiments, the genetically engineered bacterium of the disclosure is an auxotroph and also comprises kill switch circuitry, such as any of the kill switch components and systems described herein. For example, the genetically engineered bacteria may comprise a deletion or mutation in an essential gene required for cell survival and/or growth, for example, in a DNA synthesis gene, for example, *thyA*, cell wall synthesis gene, for example, *dapA* and/or an amino acid gene, for example, *serA* or *MetA* and may also comprise a toxin gene that is regulated by one or more transcriptional activators that are expressed in response to an environmental condition(s) and/or signal(s) (such as the described arabinose system) or regulated by one or more recombinases that are expressed upon sensing an exogenous environmental condition(s) and/or signal(s) (such as the recombinase systems described herein). Other embodiments are described in Wright et al., "GeneGuard: A Modular Plasmid System Designed for Biosafety," ACS Synth Biol, 2015;4(3):307-316, the entire contents of which are expressly incorporated herein by reference). In some embodiments, the genetically engineered bacterium of the disclosure is an auxotroph and also comprises kill switch circuitry, such as any of the kill switch components and systems described herein, as well as another biosecurity system, such a conditional origin of replication (Wright et al., 2015).

[0223] The addition of a Phe-auxotrophy may also have utility for increasing the rate of phenylalanine degradation. For example, the deletion of the pheA gene confers phenylalanine auxotrophy. By turning off endogenous bacterial phenylalanine production, this may drive increased uptake from the environment and also result in increased degradation of phenylalanine taken up from the environment.

<u>Genetic Regulatory Circuits</u>

[0224] 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 phenylalanine-metabolizing enzyme or rescue an auxotroph. In certain embodiments, the invention provides methods for selecting genetically engineered bacteria that produce one or more genes of interest.

[0225] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) 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 an FNR-responsive promoter; a second gene or gene cassette for producing a phenylalanine-metabolizing enzyme, 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 phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme 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.

[0226] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) 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 an FNR-responsive promoter; a second gene or gene cassette for producing a phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme is not expressed. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, thereby inducing expression of the mf-lon protease. The mf-lon protease recognizes the mf-lon degradation signal and degrades the TetR, and the phenylalanine-metabolizing enzyme is expressed.

[0227] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) 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 an FNR-responsive promoter; a second gene or gene cassette for producing a phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme is expressed.

[0228] 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).

[0229] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) 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 an FNR-responsive promoter, and a second gene or gene cassette for producing a phenylalanine-metabolizing enzyme. 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 phenylalanine-metabolizing enzyme. The regulatory RNA is capable of eliminating the mRNA hairpin and inducing translation via the ribosomal binding site. In the presence of oxygen, FNR does not bind the FNR-responsive promoter, the regulatory RNA is not expressed, and the

mRNA hairpin prevents the phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme is expressed.

[0230] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) 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 an FNR-responsive promoter; a second gene or gene cassette for producing a phenylalanine-metabolizing enzyme, 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 phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme is expressed.

[0231] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) 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 an FNR-responsive promoter, and a second gene or gene cassette for producing a phenylalanine-metabolizing enzyme operably linked to a constitutive promoter. The second gene or gene cassette is inverted in orientation (3' to 5') and flanked by recombinase binding sites, and the recombinase is capable of binding to the recombinase binding sites to induce expression of the second gene or gene cassette by reverting its orientation (5' to 3'). In the

presence of oxygen, FNR does not bind the FNR-responsive promoter, the recombinase is not expressed, the gene or gene cassette remains in the 3' to 5' orientation, and no functional phenylalanine-metabolizing enzyme is produced. In the absence of oxygen, FNR dimerizes and binds the FNR-responsive promoter, the recombinase is expressed, the gene or gene cassette is reverted to the 5' to 3' orientation, and a functional phenylalanine-metabolizing enzyme is produced (*see, e.g.*, **Fig. 42**).

[0232] In some embodiments, the invention provides genetically engineered bacteria comprising a gene or gene cassette for producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) 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 an FNR-responsive promoter; a second gene or gene cassette for producing a phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme. 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 phenylalanine-metabolizing enzyme 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 phenylalanine-metabolizing enzyme is expressed (*see, e.g.*, **Fig. 43**).

[0233] Synthetic gene circuits expressed on plasmids may function well in the short term but lose ability and/or function in the long term (Danino et al., 2015). In some embodiments, the genetically engineered bacteria comprise stable circuits for expressing genes of interest over prolonged periods. In some embodiments, the genetically engineered bacteria are capable of producing a phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) and further comprise a toxin-anti-toxin system that simultaneously produces a toxin (hok) and a short-lived anti-toxin (sok), wherein loss of the plasmid causes the cell to be killed by the long-lived toxin (Danino et al., 2015). In

some embodiments, the genetically engineered bacteria further comprise *alp7* from *B. subtilis* plasmid pL20 and produces filaments that are capable of pushing plasmids to the poles of the cells in order to ensure equal segregation during cell division (Danino et al., 2015).

Host-Plasmid Mutual Dependency

[0234] In some embodiments, the genetically engineered bacteria of the invention also comprise a plasmid that has been modified to create a host-plasmid mutual dependency. In certain embodiments, the mutually dependent host-plasmid platform is GeneGuard (Wright et al., 2015). In some embodiments, the GeneGuard plasmid comprises (i) a conditional origin of replication, in which the requisite replication initiator protein is provided in *trans*; (ii) an auxotrophic modification that is rescued by the host via genomic translocation and is also compatible for use in rich media; and/or (iii) a nucleic acid sequence which encodes a broad-spectrum toxin. The toxin gene may be used to select against plasmid spread by making the plasmid DNA itself disadvantageous for strains not expressing the anti-toxin (*e.g.*, a wild-type bacterium). In some embodiments, the GeneGuard plasmid is stable for at least 100 generations without antibiotic selection. In some embodiments, the GeneGuard plasmid does not disrupt growth of the host. The GeneGuard plasmid is used to greatly reduce unintentional plasmid propagation in the genetically engineered bacteria of the invention.

[0235] 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.

Kill Switch

[0236] In some embodiments, the genetically engineered bacteria of the invention also comprise a kill switch (*see, e.g.*, U.S. Provisional Application Nos. 62/183,935 and 62/263,329, incorporated herein by reference in their entireties). The kill switch is intended to actively kill genetically engineered bacteria in response to external stimuli. As opposed to an auxotrophic mutation where bacteria die because they lack an essential nutrient for survival, the kill switch is triggered by a particular factor in the environment that induces the production of toxic molecules within the microbe that cause cell death.

[0237] Bacteria comprising kill switches have been engineered for *in vitro* research purposes, *e.g.*, to limit the spread of a biofuel-producing microorganism outside of a laboratory environment. Bacteria engineered for *in vivo* administration to treat a disease may also be programmed to die at a specific time after the expression and delivery of a heterologous gene or genes, for example, a phenylalanine-metabolizing enzyme, 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 oxygen level-dependent expression of the phenylalanine-metabolizing enzyme (*e.g.*, PAL or PAH) and/or the phenylalanine transporter gene. In some embodiments, the kill switch is activated in a delayed fashion following oxygen level-dependent expression of the phenylalanine-metabolizing enzyme and/or phenylalanine transporter gene. Alternatively, the bacteria may be engineered to die after the bacterium has spread outside of a disease site. Specifically, it may be useful to prevent long-term colonization of subjects by the microorganism, spread of the microorganism outside the area of interest (for example, outside the gut) within the subject, or spread of the microorganism outside of the subject into the environment (for example, spread to the environment through the stool of the subject). Examples of such toxins that can be used in kill switches include, but are not limited to, bacteriocins, lysins, and other molecules that cause cell death by lysing cell membranes, degrading cellular DNA, or other mechanisms. Such toxins can be used individually or in combination. The switches that control their production can be based on, for example, transcriptional activation (toggle switches; *see, e.g.*, Gardner et al., 2000), translation (riboregulators), or DNA recombination (recombinase-based switches), and can sense environmental

stimuli such as anaerobiosis or reactive oxygen species. These switches can be activated by a single environmental factor or may require several activators in AND, OR, NAND and NOR logic configurations to induce cell death. For example, an AND riboregulator switch is activated by tetracycline, isopropyl β-D-1-thiogalactopyranoside (IPTG), and arabinose to induce the expression of lysins, which permeabilize the cell membrane and kill the cell. IPTG induces the expression of the endolysin and holin mRNAs, which are then derepressed by the addition of arabinose and tetracycline. All three inducers must be present to cause cell death. Examples of kill switches are known in the art (Callura et al., 2010).

[0238] Kill switches can be designed such that a toxin is produced in response to an environmental condition or external signal (*e.g.*, the bacteria is killed in response to an external cue) or, alternatively designed such that a toxin is produced once an environmental condition no longer exists or an external signal is ceased.

[0239] 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 recombinant bacterial cell is no longer viable.

[0240] 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.

[0241] 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.

[0242] 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.

[0243] In one embodiment, the at least one recombination event is flipping of an inverted heterologous gene encoding a first excision enzyme by a first recombinase. In one embodiment, the inverted heterologous gene encoding the first excision enzyme is located between a first forward recombinase recognition sequence and a first reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the first excision enzyme is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the first excision enzyme excises a first essential gene. In one embodiment, the programmed recombinant bacterial cell is not viable after the first essential gene is excised.

[0244] In one embodiment, the first recombinase further flips an inverted heterologous gene encoding a second excision enzyme. In one embodiment, the inverted heterologous gene encoding the second excision enzyme is located between a second forward recombinase recognition sequence and a second reverse recombinase recognition sequence. In one embodiment, the heterologous gene encoding the second excision enzyme is constitutively expressed after it is flipped by the first recombinase. In one embodiment, the genetically engineered bacterium dies or is no longer viable when the first essential gene and the second essential gene are both excised. In one embodiment, the genetically engineered bacterium dies or is no longer viable when either the first essential gene is excised or the second essential gene is excised by the first recombinase.

[0245] 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.

[0246] 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.

[0247] 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 (*i.e.*, not produced) and then produced once the environmental condition or external signal is no longer present. Such kill switches are called repression-based kill switches and represent systems in which the bacterial cells are viable only in the presence of an external factor or signal, such as arabinose or other sugar. Exemplary kill switch designs in which the toxin is repressed in the presence of an external factor or signal (and activated once the external signal is removed) is shown in **Figs. 43-47**. The disclosure provides recombinant bacterial cells which express one or more heterologous gene(s) upon sensing arabinose or other sugar in the exogenous environment. In this aspect, the recombinant bacterial cells contain the *araC* gene, which encodes the AraC transcription factor, as well as one or more genes under the control of the araBAD promoter (ParaBAD). 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.

[0248] 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 anti-

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

[0249] Arabinose inducible promoters are known in the art, including P_{ara}, P_{araB}, P_{araC}, and ParaBAD. 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.

[0250] In one exemplary embodiment of the disclosure, the genetically engineered bacteria of the present disclosure contain a kill switch having at least the following sequences: a P_{araBAD} promoter operably linked to a heterologous gene encoding a tetracycline repressor (TetR) protein, a P_{araC} promoter operably linked to a heterologous gene encoding the AraC transcription factor, and a heterologous gene encoding a bacterial toxin operably linked to a promoter which is repressed by the TetR protein. In the presence of arabinose, the AraC transcription factor activates the P_{araBAD} promoter, which activates transcription of the TetR protein which, in turn, represses transcription of the toxin. In the absence of arabinose, however, AraC suppresses transcription from the P_{araBAD} promoter and no TetR protein is expressed. In this case, expression of the heterologous toxin gene is activated, and the toxin is expressed. The toxin builds up in the recombinant bacterial cell, and the recombinant bacterial cell is killed. In one embodiment, the *araC* gene encoding the AraC transcription factor is under the control of a constitutive promoter and is therefore constitutively expressed.

[0251] In one embodiment of the disclosure, the genetically engineered bacterium further comprises an anti-toxin under the control of a constitutive promoter. In this situation, in the presence of arabinose, the toxin is not expressed due to repression by TetR protein, and the anti-toxin protein builds-up in the cell. However, in the absence of arabinose, TetR protein is not expressed, and expression of the toxin is induced. The toxin begins to build-up within the recombinant bacterial cell. The

recombinant bacterial cell is no longer viable once the toxin protein is present at either equal or greater amounts than that of the anti-toxin protein in the cell, and the recombinant bacterial cell will be killed by the toxin.

[0252] In another embodiment of the disclosure, the genetically engineered bacterium further comprises an anti-toxin under the control of the P_{araBAD} promoter. In this situation, in the presence of arabinose, TetR and the anti-toxin are expressed, the anti-toxin builds up in the cell, and the toxin is not expressed due to repression by TetR protein. However, in the absence of arabinose, both the TetR protein and the anti-toxin are not expressed, and expression of the toxin is induced. The toxin begins to build-up within the recombinant bacterial cell. The recombinant bacterial cell is no longer viable once the toxin protein is expressed, and the recombinant bacterial cell will be killed by the toxin.

[0253] In another exemplary embodiment of the disclosure, the genetically engineered bacteria of the present disclosure contain a kill switch having at least the following sequences: a P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the recombinant bacterial cell (and required for survival), and a P_{araC} promoter operably linked to a heterologous gene encoding the AraC transcription factor. In the presence of arabinose, the AraC transcription factor activates the P_{araBAD} promoter, which activates transcription of the heterologous gene encoding the essential polypeptide, allowing the recombinant bacterial cell to survive. In the absence of arabinose, however, AraC suppresses transcription from the P_{araBAD} promoter and the essential protein required for survival is not expressed. In this case, the recombinant bacterial cell dies in the absence of arabinose. In some embodiments, the sequence of P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the recombinant bacterial cell can be present in the bacterial cell in conjunction with the TetR/toxin kill switch system described directly above. In some embodiments, the sequence of P_{araBAD} promoter operably linked to a heterologous gene encoding an essential polypeptide not found in the recombinant bacterial cell can be present in the bacterial cell in conjunction with the TetR/toxin/anti-toxin kill switch system described directly above.

[0254] 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.

[0255] In some embodiments, the engineered bacteria of the present disclosure further comprise the gene(s) encoding the components of any of the above-described kill switch circuits.

[0256] 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.

[0257] 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.

[0258] 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.

[0259] In some embodiments, the genetically engineered bacterium provided herein is an auxotroph. In one embodiment, the genetically engineered bacterium is an auxotroph selected from a *cysE, glnA, ilvD, leuB, lysA, serA, metA, glyA, hisB, ilvA, pheA, proA, thrC, trpC, tyrA, thyA, uraA, dapA, dapB, dapD, dapE, dapF, flhD, metB, metC, proAB,* and *thi1* auxotroph. In some embodiments, the engineered bacteria have more than one auxotrophy, for example, they may be a Δ*thyA* and Δ*dapA* auxotroph.

[0260] In some embodiments, the genetically engineered bacterium provided herein further comprises a kill switch circuit, such as any of the kill switch circuits provided herein. For example, in some embodiments, the genetically engineered bacteria further comprise one or more genes encoding one or more recombinase(s) under the control of an inducible promoter and an inverted toxin sequence. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an anti-toxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding one or more recombinase(s) under the control of an inducible promoter and one or more inverted excision genes, wherein the excision gene(s) encode an enzyme that deletes an essential gene. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an anti-toxin. In some embodiments, the engineered bacteria further comprise one or more genes encoding a toxin under the control of a promoter having a TetR repressor binding site and a gene encoding the TetR under the control of an inducible promoter that is induced by arabinose, such as P_{araBAD}. In some embodiments, the genetically engineered bacteria further comprise one or more genes encoding an anti-toxin.

[0261] In some embodiments, the genetically engineered bacterium is an auxotroph comprising a gene encoding a phenylalanine-metabolizing enzyme and further comprises a kill switch circuit, such as any of the kill switch circuits described herein.

[0262] In some embodiments, of the above described genetically engineered bacteria, the gene or gene cassette for producing the phenylalanine-metabolizing enzyme is 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 for producing the phenylalanine-metabolizing 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.

Pharmaceutical Compositions and Formulations

[0263] Pharmaceutical compositions comprising the genetically engineered bacteria of the invention may be used to treat, manage, ameliorate, and/or prevent diseases associated with hyperphenylalaninemia, *e.g.*, PKU. Pharmaceutical compositions of the invention comprising one or more genetically engineered bacteria,

alone or in combination with prophylactic agents, therapeutic agents, and/or and pharmaceutically acceptable carriers are provided. In certain embodiments, the pharmaceutical composition comprises one species, strain, or subtype of bacteria that are engineered to comprise the genetic modifications described herein. 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.

[0264] The pharmaceutical compositions described herein may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into compositions for pharmaceutical use. Methods of formulating pharmaceutical compositions are known in the art (*see, e.g.,* "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA). In some embodiments, the pharmaceutical compositions are subjected to tabletting, lyophilizing, direct compression, conventional mixing, dissolving, granulating, levigating, emulsifying, encapsulating, entrapping, or spray drying to form tablets, granulates, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. Appropriate formulation depends on the route of administration.

[0265] The genetically engineered bacteria described herein may be formulated into pharmaceutical compositions in any suitable dosage form (*e.g.,* liquids, capsules, sachet, hard capsules, soft capsules, tablets, enteric coated tablets, suspension powders, granules, or matrix sustained release formations for oral administration) and for any suitable type of administration (*e.g.,* oral, topical, injectable, immediate-release, pulsatile-release, delayed-release, or sustained release). Suitable dosage amounts for the genetically engineered bacteria may range from about 10^5 to 10^{12} bacteria, *e.g.*, approximately 10^5 bacteria, approximately 10^6 bacteria, approximately 10^7 bacteria, approximately 10^8 bacteria, approximately 10^9 bacteria, approximately 10^{10} bacteria, approximately 10^{11} bacteria, or approximately 10^{11} bacteria. The composition may be administered once or more daily, weekly, or monthly. The composition may be administered before, during, or following a meal. In one embodiment, the pharmaceutical composition is administered before the subject eats a meal. In one

embodiment, the pharmaceutical composition is administered currently with a meal. In one embodiment, the pharmaceutical composition is administered after the subject eats a meal.

[0266] The genetically engineered bacteria may be formulated into pharmaceutical compositions comprising one or more pharmaceutically acceptable carriers, thickeners, diluents, buffers, buffering agents, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers or agents. For example, the pharmaceutical composition may include, but is not limited to, the addition of calcium bicarbonate, sodium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20. In some embodiments, the genetically engineered bacteria of the invention may be formulated in a solution of sodium bicarbonate, *e.g.*, 1 molar solution of sodium bicarbonate (to buffer an acidic cellular environment, such as the stomach, for example). The genetically engineered bacteria may be administered and formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0267] The genetically engineered bacteria disclosed herein may be administered topically and formulated in the form of an ointment, cream, transdermal patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form well-known to one of skill in the art. *See, e.g.,* "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA. In an embodiment, for non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity greater than water are employed. Suitable formulations include, but are not limited to, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, etc., which may be sterilized or mixed with auxiliary agents (*e.g.,* preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, *e.g.,* osmotic

pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (*e.g.*, a gaseous propellant, such as freon) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms. Examples of such additional ingredients are well known in the art. In one embodiment, the pharmaceutical composition comprising the recombinant bacteria of the invention may be formulated as a hygiene product. For example, the hygiene product may be an antibacterial formulation, or a fermentation product such as a fermentation broth. Hygiene products may be, for example, shampoos, conditioners, creams, pastes, lotions, and lip balms.

[0268] 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.

[0269] Tablets or capsules can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone, hydroxypropyl methylcellulose, carboxymethylcellulose, polyethylene glycol, sucrose, glucose, sorbitol, starch, gum, kaolin, and tragacanth); fillers (*e.g.*, *l*actose, microcrystalline cellulose, or calcium hydrogen phosphate); lubricants (*e.g.*, calcium, aluminum, zinc, stearic acid, polyethylene glycol, sodium lauryl sulfate, starch, sodium benzoate, L-leucine, magnesium stearate, talc, or silica); disintegrants (*e.g.*, starch, potato starch, sodium starch glycolate, sugars, cellulose derivatives, silica powders); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art.

A coating shell may be present, and common membranes include, but are not limited to, polylactide, polyglycolic acid, polyanhydride, other biodegradable polymers, alginate-polylysine-alginate (APA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroymethylacrylate-methyl methacrylate (HEMA-MMA), multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitrile/sodium methallylsulfonate (AN-69), polyethylene glycol/poly pentamethylcyclopentasiloxane/polydimethylsiloxane (PEG/PD5/PDMS), poly N,N-dimethyl acrylamide (PDMAAm), siliceous encapsulates, cellulose sulphate/sodium alginate/polymethylene-co-guanidine (CS/A/PMCG), cellulose acetate phthalate, calcium alginate, k-carrageenan-locust bean gum gel beads, gellan-xanthan beads, poly(lactide-co-glycolides), carrageenan, starch poly-anhydrides, starch polymethacrylates, polyamino acids, and enteric coating polymers.

[0270] 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.

[0271] 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.

[0272] 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., 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.

[0273] 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.

[0274] 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.

[0275] 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.

[0276] In another embodiment, the pharmaceutical composition comprising the recombinant bacteria of the invention may be a comestible product, for example, a food product. In one embodiment, the food product is milk, concentrated milk, fermented milk (yogurt, sour milk, frozen yogurt, lactic acid bacteria-fermented beverages), milk powder, ice cream, cream cheeses, dry cheeses, soybean milk, fermented soybean milk, vegetable-fruit juices, fruit juices, sports drinks, confectionery, candies, infant foods (such as infant cakes), nutritional food products, animal feeds, or dietary supplements. In one embodiment, the food product is a fermented food, such as a fermented dairy product. In one embodiment, the fermented dairy product is yogurt. In another embodiment, the fermented dairy product is cheese, milk, cream, ice cream, milk shake, or kefir. In another embodiment, the recombinant bacteria of the invention are combined in a preparation containing other live bacterial cells intended to serve as probiotics. In another embodiment, the food product is a beverage. In one embodiment, the beverage is a fruit juice-based beverage or a beverage containing plant or herbal extracts. In another embodiment, the food product is a jelly or a pudding. Other food products suitable for administration of the recombinant bacteria of the invention are well known in the art. *See, e.g.,* US 2015/0359894 and US 2015/0238545, the entire contents of each of which are expressly incorporated herein by reference. In yet another embodiment, the pharmaceutical composition of the invention is injected into, sprayed onto, or sprinkled onto a food product, such as bread, yogurt, or cheese.

[0277] 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.

[0278] 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.

[0279] 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).

[0280] 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.

[0281] 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.

[0282] 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.

[0283] 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.

[0284] 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.

[0285] 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

[0286] Another aspect of the invention provides methods of treating a disease associated with hyperphenylalaninemia or symptom(s) associated with hyperphenylalaninemia. In some embodiments, the disease is selected from the group consisting of: phenylketonuria, classical or typical phenylketonuria, atypical phenylketonuria, permanent mild hyperphenylalaninemia, nonphenylketonuric

hyperphenylalaninemia, phenylalanine hydroxylase deficiency, cofactor deficiency, dihydropteridine reductase deficiency, tetrahydropterin synthase deficiency, and Segawa's disease. In some embodiments, hyperphenylalaninemia is secondary to other conditions, *e.g.*, liver diseases. In some embodiments, the invention provides methods for reducing, ameliorating, or eliminating one or more symptom(s) associated with these diseases, including but not limited to neurological deficits, mental retardation, encephalopathy, epilepsy, eczema, reduced growth, microcephaly, tremor, limb spasticity, and/or hypopigmentation. In some embodiments, the subject to be treated is a human patient.

[0287] In certain embodiments, the genetically engineered bacteria are capable of metabolizing phenylalanine in the diet in order to treat a disease or disorder associated with hyperphenylalaninemia, *e.g.*, PKU. In some embodiments, the genetically engineered bacteria are delivered simultaneously with dietary protein. In other embodiments, the genetically engineered bacteria are not delivered simulaneously with dietary protein. Studies have shown that pancreatic and other glandular secretions into the intestine contain high levels of proteins, enzymes, and polypeptides, and that the amino acids produced as a result of their catabolism are reabsorbed back into the blood in a process known as "enterorecirculation" (Chang, 2007; Sarkissian et al., 1999). Thus, high intestinal levels of phenylalanine may be partially independent of food intake, and are available for breakdown by PAL. In some embodiments, the genetically engineered bacteria and dietary protein are delivered after a period of fasting or phenylalanine-restricted dieting. In these embodiments, a patient suffering from hyperphenylalaninemia may be able to resume a substantially normal diet, or a diet that is less restrictive than a phenylalanine-free diet. In some embodiments, the genetically engineered bacteria may be capable of metabolizing phenylalanine from additional sources, *e.g.*, the blood, in order to treat a disease associated with hyperphenylalaninemia, *e.g.*, PKU. In these embodiments, the genetically engineered bacteria need not be delivered simultaneously with dietary protein, and a phenylalanine gradient is generated, *e.g.*, from blood to gut, and the genetically engineered bacteria metabolize phenylalanine and reduce phenylalaninemia.

[0288] The method may comprise preparing a pharmaceutical composition with at least one genetically engineered species, strain, or subtype of bacteria described

herein, and administering the pharmaceutical composition to a subject in a therapeutically effective amount. In some embodiments, the genetically engineered bacteria of the invention are administered orally, *e.g.*, in a liquid suspension. In some embodiments, the genetically engineered bacteria of the invention are lyophilized in a gel cap and administered orally. In some embodiments, the genetically engineered bacteria of the invention are administered via a feeding tube or gastric shunt. In some embodiments, the genetically engineered bacteria of the invention are administered rectally, *e.g.*, by enema. In some embodiments, the genetically engineered bacteria of the invention are administered topically, intraintestinally, intrajejunally, intraduodenally, intraileally, and/or intracolically.

[0289] In certain embodiments, the pharmaceutical composition described herein is administered to reduce phenylalanine levels in a subject. In some embodiments, the methods of the present disclosure reduce the phenylalanine levels in a subject by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or more as compared to levels in an untreated or control subject. In some embodiments, reduction is measured by comparing the phenylalanine level in a subject before and after administration of the pharmaceutical composition. In some embodiments, the method of treating or ameliorating hyperphenylalaninemia 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.

[0290] Before, during, and after the administration of the pharmaceutical composition, phenylalanine 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 invention to reduce phenylalanine. In some embodiments, the methods may include administration of the compositions of the invention to reduce phenylalanine to undetectable levels in a subject. In some embodiments, the methods may include administration of the compositions of the invention to reduce phenylalanine concentrations to undetectable

levels, or to less than about 1%, 2%, 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, or 80% of the subject's phenylalanine levels prior to treatment.

[0291] In certain embodiments, the genetically engineered bacteria are *E. coli* Nissle. The genetically engineered bacteria may be destroyed, *e.g.*, by defense factors in the gut or blood serum (Sonnenborn et al., 2009) or by activation of a kill switch, several hours or days after administration. Thus, the pharmaceutical composition comprising the genetically engineered bacteria may be re-administered at a therapeutically effective dose and frequency. Length of Nissle residence *in vivo* in mice is shown in **Fig. 38**. In alternate embodiments, the genetically engineered bacteria are not destroyed within hours or days after administration and may propagate and colonize the gut.

[0292] The methods of the invention may comprise administration of the pharmaceutical composition alone or in combination with one or more additional therapeutic agents. In some embodiments, the pharmaceutical composition is administered in conjunction with the cofactor tetrahydrobiopterin (*e.g.*, Kuvan/sapropterin), large neutral amino acids (*e.g.*, tyrosine, tryptophan), glycomacropeptides, a probiotic (*e.g.*, VSL3), an enzyme (*e.g.*, pegylated-PAL), and/or other agents used in the treatment of phenylketonuria (Al Hafid and Christodoulou, 2015).

[0293] In some embodiments, the genetically engineered bacteria are administered in combination with one or more recombinantly produced PME enzymes, e.g. recombinant PAL, LAAD or PAH. In some embodiments, the recombinant enzymes are further formulated for improved stability and/or delivery. In some embodiments, the one or more PME enzyme administered in combination with the genetically engineered bacteria is peggylated. In some embodiments, the one or more PME enzyme administered in combination with the genetically engineered bacteria is delivered as a fusion protein. A non-limiting example of such a fusion protein is a fusion between a PME and a transduction domain for uptake into cells. A non-limiting example of such transduction domain or cell penetrating peptide is the TAT peptide. In some embodiments, the one or more PME enzyme administered in combination with the genetically engineered bacteria is formulated in a nanoparticle. A non-limiting example of such a nanoparticle is a dextran sulfate/chitosan PME nanoparticle. In some

embodiments, the one or more PME enzyme administered in combination with the genetically engineered bacteria is delivered as a PME microsphere. A non-limiting example of such a microsphere is a barium alginate PME microsphere. In some embodiments, the one or more PME enzyme administered in combination with the genetically engineered bacteria is delivered as amorphous silica PME particles.

[0294] In some embodiments, the genetically engineered bacteria are administered in combination with PAL. In some embodiments, the genetically engineered bacteria are administered in combination with PAH. In some embodiments, the genetically engineered bacteria are administered in combination with LAAD. In some embodiments, the genetically engineered bacteria are administered in combination with PAL and PAH. In some embodiments, the genetically engineered bacteria are administered in combination with PAL and LAAD. In some embodiments, the genetically engineered bacteria are administered in combination with PAH and LAAD. In some embodiments, the genetically engineered bacteria are administered in combination with PAL, PAH, and LAAD.

[0295] In some embodiments, the genetically engineered bacteria are administered in combination with pegylated PAL. In some embodiments, the genetically engineered bacteria are administered in combination with pegylated PAH. In some embodiments, the genetically engineered bacteria are administered in combination with pegylated LAAD. In some embodiments, the genetically engineered bacteria are administered in combination with a PAL fusion protein, e.g., a cell penetrating peptide. In some embodiments, the genetically engineered bacteria are administered in combination with a PAH fusion protein, e.g., a cell penetrating peptide. In some embodiments, the genetically engineered bacteria are administered in combination with a LAAD fusion protein, e.g., a cell penetrating peptide. In some embodiments, the genetically engineered bacteria are administered in combination with PAL-nanoparticles. In some embodiments, the genetically engineered bacteria are administered in combination with PAH-nanoparticles. In some embodiments, the genetically engineered bacteria are administered in combination with LAAD nanoparticles. In some embodiments, the genetically engineered bacteria are administered in combination with PAL-microspheres. In some embodiments, the genetically engineered bacteria are administered in combination with PAH-

microspheres. In some embodiments, the genetically engineered bacteria are administered in combination with LAAD-microspheres. In some embodiments, the genetically engineered bacteria are administered in combination with PAL-silica particles. In some embodiments, the genetically engineered bacteria are administered in combination with PAH-silica particles. In some embodiments, the genetically engineered bacteria are administered in combination with LAAD-silica particles.

[0296] In some embodiments, a recombinant enzyme replacement therapy or substitution therapy, e.g. PAL, PAH, and/or LAAD is administered without the genetically engineered bacteria.

[0297] In some embodiments, the one or more PME administered is PAL. In some embodiments, PAL is modified as described in Sakissian et al., 2011, Mol Genet Metab. 2011 Nov; 104(3): 249–254, the contents of which is herein incorporated by reference in its entirety. In some embodiments, the PAL is Av-p.C503S/p.C565S/p.F18A PAL. In some embodiments, the PAL is PEG-Av-p.C503S/p.C565S/p.F18A PAL.

[0298] In some embodiments, the PAL is PEGylated. In one embodiment, the pegylated PAL is from *Anabaena variabilis*. In one embodiment, the pegylated PAL is from *Photorhabdus luminescens*. In some embodiments, the one or more PME administered is PAH. In one embodiment, PAH is human PAH. In some embodiments, the one or more PME administered is LAAD. In one embodiment, the LAAD protein administered is derived from *Proteus mirabilis*. In some embodiments, the one or more PME administered in combination with PAL and PAH. In some embodiments, the one or more PME administered is PAL and LAAD. In some embodiments, the one or more PME administered is PAH and LAAD. In some embodiments, the one or more PME administered is PAL, PAH, and LAAD.

[0299] In some embodiments, the recombinant enzymes are further formulated for improved stability and/or delivery. In some embodiments, the one or more PME enzyme administered is peggylated. In some embodiments, the one or more PME enzyme administered is delivered as a fusion protein. A non-limiting example of such a fusion protein is a fusion between a PME and a transduction domain for uptake into cells. A non-limiting example of such transduction domain or cell penetrating peptide is the TAT peptide. In some embodiments, the one or more PME enzyme administered is

formulated in a nanoparticle. A non-limiting example of such a nanoparticle is a dextran sulfate/chitosan PME nanoparticle. In some embodiments, the one or more PME enzyme administered is delivered as a PME microsphere. A non-limiting example of such a microsphere is a barium alginate PME microsphere. In some embodiments, the one or more PME enzyme administered is delivered as amorphous silica PME particles.

[0300] In some embodiments, pegylated PAL is administered. In some embodiments, pegylated LAAD is administered. In some embodiments peggylated LAAD from *Proteus mirabilis* is administered. In some embodiments, pegylated PAH is administered.

[0301] In one embodiment, a PAL fusion protein, e.g., with a cell penetrating peptide, is administered. In one embodiment, a LAAD fusion protein, e.g., with a cell penetrating peptide, is administered. In one embodiment, a PAH fusion protein, e.g., with a cell penetrating peptide, is administered. In some embodiments, PAL-nanoparticles are administered. In some embodiments, PAH-nanoparticles are administered. In some embodiments, LAAD-nanoparticles are administered. In some embodiments, PAL-microspheres are administered. In some embodiments, PAH-microspheres are administered. In some embodiments, LAAD-microspheres are administered. In some embodiments, PAL-silica particles are administered. In some embodiments, PAH-silica particles are administered. In some embodiments, LAAD-silica particles are administered.

[0302] In some embodiments the PME, e.g., PAH, PAL, and/or LAAD is formulated with aprotinin, e.g., 40 mg/ml aprotinin.

[0303] In some embodiments the PMEs are delivered as gene therapy. In some embodiments, a CRISPR technology is used. In some embodiments a gene therapy vector is used to deliver the one or more PME, e.g., PAL, LAAD, and/or PAH. Gene therapy vectors are known in the art and include, but are not limited to, retroviral vectors, adenoviral vectors, adeno-associated viral vectors. Alternatively, formulated or naked PME gene DNA or RNA can be delivered.

[0304] An important consideration in the selection of the one or more additional therapeutic agents is that the agent(s) should be compatible with the genetically engineered bacteria of the invention, *e.g.*, the agent(s) must not 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-phenylalanine diet. 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.

[0305] The methods of the invention also include kits comprising the pharmaceutical composition described herein. The kit can include one or more other elements including, but not limited to: instructions for use; other reagents, *e.g.*, a label, an additional therapeutic agent; devices or materials for measuring phenylalanine levels, or levels of other molecules or metabolites associated with hyperphenylalaninemia, in a subject; devices or other materials for preparing the pharmaceutical composition of the invention for administration; and devices or other materials for administration to a subject. Instructions for use can include guidance for therapeutic application, such as suggested dosages and/or modes of administration, *e.g.*, in a patient with hyperphenylalaninemia. The kit can further contain at least one additional therapeutic agent, and/or one or more additional genetically engineered bacterial strains of the invention, formulated as appropriate, in one or more separate pharmaceutical preparations.

[0306] In some embodiments, the kit is used for administration of the pharmaceutical composition to a subject. In some embodiments, the kit is used for administration of the pharmaceutical composition, alone or in combination with one or more additional therapeutic agents, to a subject. In some embodiments, the kit is used for measuring phenylalanine levels (*e.g.*, blood phenylalanine levels) in a subject before, during, or after administration of the pharmaceutical composition to the subject. In certain embodiments, the kit is used for administration and/or re-administration of the pharmaceutical composition, alone or in combination with one or more additional therapeutic agents, when blood phenylalanine levels are increased or abnormally high. In some embodiments, a diagnostic signal of hyperphenylalaninemia is a blood phenylalanine level of at least 2 mg/dL, at least 4 mg/dL, at least 6 mg/dL, at least 8

mg/dL, at least 10 mg/dL, at least 12 mg/dL, at least 14 mg/dL, at least 16 mg/dL, at least 18 mg/dL, at least 20 mg/dL, or at least 25 mg/dL.

[0307] Table 20 shows non-limiting examples of target degradation rates, based on levels of phenylalanine on average in classical PKU patients.

Table 20. Target Degradation Rates

Age (years)	0-6 months	7-12 months	1-3	4-8	9-13	14-18 (M)	14-18 (F)	>18 (M)	>18 (F)
RDA Protein (g/d)	9.1	11	13	19	34	52	46	56	46
Daily PHE (mg)- Healthy subject (1g protein= 47mg PHE)	428	517	611	893	1598	2444	2162	2632	2162
Daily PHE tolerance (mg) (Classical PKU)	250	250	250	250	250	250	250	250	250
Target Reduction (mg)	178	267	361	643	1348	2194	1912	2382	1912
Target Reduction (mmol)	1.08	1.62	2.19	3.89	8.16	13.28	11.57	14.42	11.57
Target degradation rate (µmol/10^9 CFUs/hr) (based on 3.10^{11} CFUs/day dose) assuming all dose functioning for 24 hours	0.15	0.22	0.3	0.54	1.13	1.84	1.61	2	1.61

Target degradation rate 2 hrs transit time (μmol/10^9 CFUs/hr) ass uming 2 hour transit time per dose	0.6	0.9	1.21	2.16	4.53	7.38	6.43	8.01	6.43
Target degradation rate 6 hrs transit time (μmol/10^9 CFUs/hr) assuming 6 hour transit time per dose	0.2	0.3	0.4	0.72	1.51	2.46	2.14	2.67	2.14

[0308] In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.15 to about 8.01 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.15 to about 2 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.6 to about 8.01 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.2 to about 2.67 μmol/10^9 CFUs/hr.

[0309] In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.15 to about 0.6 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.22 to about 0.9 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.3 to about 1.21 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.54 to about 2.16 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 1.13 to about 4.53 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 1.84 to about 7.38

μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 1.61 to about 6.43 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 2 to about 8.01 μmol/10^9 CFUs/hr.

[0310] In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 0.1 to about 1 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 1 to about 2 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 2 to about 3 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 3 to about 4 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 4 to about 5 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 5 to about 6 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 6 to about 7 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of about 7 to about 8 μmol/10^9 CFUs/hr.

[0311] In some embodiments, the genetically engineered bacteria achieve a target reduction rate of less than 0.15 μmol/10^9 CFUs/hr. In some embodiments, the genetically engineered bacteria achieve a target degradation rate of greater than 8.01 μmol/10^9 CFUs/hr.

[0312] In some embodiments, the genetically engineered bacteria achieve a target reduction of between about 178 mg and 2382 mg. In some embodiments, the genetically engineered bacteria achieve a target reduction of 1.08 mmol to 14.42 mmol. In some embodiments, the reduction is less than 1.08 mmol. In some embodiments, the reduction is greater than 14.42 mmol.

[0313] In some embodiments, target reduction and target degradation rates are based on classical PKU phenylalanine levels. In some embodiments, the target reduction and target degradation rates are based on phenylalanine levels observed in mild PKU. In some embodiments, target reduction and target degradation rates are based on phenylalanine levels observed in mild hyperphenylalaninemia.

Treatment *In Vivo*

[0314] The genetically engineered bacteria of the invention may be evaluated *in vivo, e.g.*, in an animal model. Any suitable animal model of a disease or condition associated with hyperphenylalaninemia may be used (*see, e.g.,* Sarkissian et al., 1999). In some embodiments, the animal model is a mouse model of PKU. In certain embodiments, the mouse model of PKU is an PAH mutant BTBR mouse (BTBR-Pah^{enu2}, Jackson Laboratories). In these embodiments, the mouse model contains a chemically (ENU)-induced homozygous missense mutation (T835C) in exon 7 of the *Pah* gene, which results in a phenylalanine to serine substitution at amino acid 263 (F263S). This residue is located in the active site of the PAH enzyme, as shown by crystal structure analysis, and results in the complete loss of PAH activity. On normal diets, these mutant mice demonstrate a 10- to 20-fold increase in serum phenylalanine levels compared to unaffected controls. The genetically engineered bacteria of the invention may be administered to the animal, *e.g.*, by oral gavage, and treatment efficacy is determined, *e.g.*, by measuring blood phenylalanine and/or cinnamate before and after treatment. In animal models, it is noted that residence time of the genetically engineered bacteria within the GI tract may be shorter than residence time in humans. The animal may be sacrificed, and tissue samples may be collected and analyzed.

[0315] In some embodiments, pharmacokinetics and pharmacodynamic studies may be conducted in non-human primates to determine any potential toxicities arising from administration of the genetically engineered bacteria. the pharmacokinetics and pharmacodynamics of the genetically engineered bacteria. Non-limiting examples of such studies are described in Examples 30 and 31.

[0316] In some embodiments, the genetically engineered bacteria expressing LAAD can be specifically detected in the feces and differentiated from other E. coli strains. A Phenylalanine Deaminase Test “Phenylalanine Agar Slant” can be used for this purpose. Phenylalanine agar used to determine whether the microbe can use phenylalanine and convert it to phenyl pyruvate. When the test chemicals are added to the tube containing the sample on the phenylalanine agar, phenylpyruvate is converted to a green compound, indicating a positive test. Wild type E. coli does not produce phenylpyruvate, since they do not encode an enzyme, which can produce phenylpyruvate from phenylalanine, allowing differentiation from other E coli strains.

The genetically engineered bacteria can be differentiated from other bacterial species which are able to produce phenylpyruvate by PCR-based tests known in the art. For example, species specific sequences can be amplified. For example, universal PCR that amplifies conserved regions in various bacteria is ideal to detect any pathogen in screening of specimens. For this purpose, the conserved region of the 16S rRNA gene can be used as a target gene for the universal PCR; the 16S rRNA gene contains species-specific regions by which a large number of bacterial species can be differentiated.

[0317] In some embodiments, the Phenylalanine Deaminase Test can be used to detect the genetically engineered bacteria in a feces sample. In some embodiments, PCR-based tests can be conducted to differentiate the genetically engineered bacteria from other bacterial species.

Screening Methods

[0318] In some embodiments, of the disclosure a genetically engineered strain may be improved upon by using screening and selection methods, e.g., to increase PME enzymatic activity or to increase the ability of a strain to take up phenylalanine. In some embodiments, the screen serves to generate a bacterial strain with improved PME activity. In some embodiments, the screen serves to generate a bacterial strain which has improved phenylalanine uptake ability. In some embodiments, the screen may identify a bacterial strain with both improved PME activity and enhanced substrate import. Non-limiting examples of methods of screening which can be used are described herein.

Generation of Bacterial Strains with Enhance Ability to Transport Biomolecules

[0319] 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.

[0320] 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.

[0321] For example, if the biosynthetic pathway for producing an amino acid is disrupted a strain capable of high-affinity capture of said amino acid can be evolved via ALE. First, the strain is grown in varying concentrations of the auxotrophic amino acid, until a minimum concentration to support growth is established. The strain is then passaged at that concentration, and diluted into lowering concentrations of the amino acid at regular intervals. Over time, cells that are most competitive for the amino acid – at growth-limiting concentrations – will come to dominate the population. These strains will likely have mutations in their amino acid-transporters resulting in increased ability to import the essential and limiting amino acid.

[0322] Similarly, by using an auxotroph that cannot use an upstream metabolite to form an amino acid, a strain can be evolved that not only can more efficiently import the upstream metabolite, but also convert the metabolite into the essential downstream metabolite. These strains will also evolve mutations to increase import of the upstream metabolite, but may also contain mutations which increase expression or reaction kinetics of downstream enzymes, or that reduce competitive substrate utilization pathways.

[0323] In the previous examples, a metabolite innate to the microbe was made essential via mutational auxotrophy and selection was applied with growth-limiting supplementation of the endogenous metabolite. However, phenotypes capable of consuming non-native compounds can be evolved by tying their consumption to the production of an essential compound. For example, if a gene from a different organism is isolated which can produce an essential compound or a precursor to an essential compound this gene can be recombinantly introduced and expressed in the heterologous host. This new host strain will now have the ability to synthesize an essential nutrient from a previously non-metabolizable substrate. Hereby, a similar ALE process can be applied by creating an auxotroph incapable of converting an immediately downstream

metabolite and selecting in growth-limiting amounts of the non-native compound with concurrent expression of the recombinant enzyme. This will result in mutations in the transport of the non-native substrate, expression and activity of the heterologous enzyme and expression and activity of downstream native enzymes. It should be emphasized that the key requirement in this process is the ability to tether the consumption of the non-native metabolite to the production of a metabolite essential to growth.

[0324] 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.

[0325] Predicting when to halt the stop the ALE experiment also requires vigilance. As the success of directing evolution is tied directly to the number of mutations “screened” throughout the experiment and mutations are generally a function of errors during DNA replication, the cumulative cell divisions (CCD) acts as a proxy for total mutants which have been screened. Previous studies have shown that beneficial phenotypes for growth on different carbon sources can be isolated in about $10^{11.2}$ CCD[1]. This rate can be accelerated by the addition of chemical mutagens to the cultures – such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) – which causes increased DNA replication errors. However, when continued passaging leads to marginal or no improvement in growth rate the population has converged to some fitness maximum and the ALE experiment can be halted.

[0326] 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).

[0327] In some embodiments, the ALE method can be used to identify genetically engineered bacteria with improved phenylalanine uptake.

Specific Screen to improve PME activity

[0328] Screens using genetic selection are conducted to improve phenylalanine consumption in the genetically engineered bacteria. Toxic phenylalanine analogs exert their mechanism of action (MOA) by being incorporated into cellular protein, causing cell death. These compounds, such as paralog p-fluoro-DL-phenylalanine and ortholog o-fluoro-DL-phenylalanine have utility in an untargeted approach to select PAL enzymes with increased activity. Assuming that these toxic compounds can be metabolized by PAL into a non-toxic metabolite, rather than being incorporated into cellular protein, genetically engineered bacteria which have improved phenylalanine degradation activity can tolerate higher levels of these compounds, and can be screened for and selected on this basis.

References

Al Hafid N, Christodoulou J. Phenylketonuria: a review of current and future treatments. Transl Pediatr. 2015 Oct;4(4):304-317. PMID: 26835392;

Altenhoefer et al. The probiotic Escherichia coli strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. FEMS Immunol Med Microbiol. 2004 Apr 9;40(3):223-229. PMID: 15039098;

Andersen et al. Uracil uptake in Escherichia coli K-12: isolation of uraA mutants and cloning of the gene. J Bacteriol. 1995 Apr;177(8):2008-2013. PMID: 7721693;
Arai et al. Expression of the nir and nor genes for denitrification of Pseudomonas aeruginosa requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. FEBS Lett. 1995 Aug 28;371(1):73-76. PMID: 7664887;
Arthur et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science. 2012 Oct 5;338(6103):120-123. PMID: 22903521;
Callura et al. Tracking, Tuning and terminating microbial physiology using synthetic riboregulators. Proc Natl Acad Sci USA. 2010;27(36):15898-15903. PMID: 20713708;
Castiglione et al. The transcription factor DNR from Pseudomonas aeruginosa specifically requires nitric oxide and haem for the activation of a target promoter in Escherichia coli. Microbiology. 2009 Sep;155(Pt 9):2838-2844. PMID: 19477902;
Chang, ed. (2007) “Use of Enzyme Artificial Cells for Genetic Enzyme Defects.” In Artificial Cells: Biotechnology, Nanomedicine, Regenerative Medicine, Blood Substitutes, Bioencapsulation, and Cell/Stem Cell Therapy. World Scientific Publishing, pp. 147-159;
Clarkson et al. Diaminopimelic acid and lysine auxotrophs of Pseudomonas aeruginosa 8602. J Gen Microbiol. 1971 May;66(2):161-169. PMID: 4999073;
Cuevas-Ramos et al. Escherichia coli induces DNA damage in vivo and triggers genomic instability in mammalian cells. Proc Natl Acad Sci USA. 2010 Jun 22;107(25):11537-11542. PMID: 20534522;
Danino et al. Programmable probiotics for detection of cancer in urine. Sci Transl Med. 2015 May 27;7(289):289ra84. PMID: 26019220;
Deutscher. The mechanisms of carbon catabolite repression in bacteria. Curr Opin Microbiol. 2008 Apr;11(2):87-93. PMID: 18359269;
Dinleyici et al. Saccharomyces boulardii CNCM I-745 in different clinical conditions. Expert Opin Biol Ther. 2014 Nov;14(11):1593-1609. PMID: 24995675;
Dobbelaere et al. Evaluation of nutritional status and pathophysiology of growth retardation in patients with phenylketonuria. J Inherit Metab Dis. 2003;26(1):1-11. PMID: 12872834;
Eiglmeier et al. Molecular genetic analysis of FNR-dependent promoters. Mol Microbiol. 1989 Jul;3(7):869-878. PMID: 2677602;
Estrem et al. Identification of an UP element consensus sequence for bacterial promoters. Proc Natl Acad Sci USA. 1998 Aug 18;95(17):9761-9766. PMID: 9707549;
Galimand et al. Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in Pseudomonas aeruginosa. J Bacteriol. 1991 Mar;173(5):1598-1606. PMID: 1900277;
Gardner et al. Construction of a genetic toggle switch in Escherichia coli. Nature. 2000;403:339-342. PMID: 10659857;
Gerdes et al. Essential genes on metabolic maps. Curr Opin Biotechnol. 2006 Oct;17(5):448-456. PMID: 16978855;
Gilbert et al. Molecular cloning of the phenylalanine ammonia lyase gene from Rhodosporidium toruloides in Escherichia coli K-12. J Bacteriol. 1985 Jan;161(1):314-320. PMID: 2981805;
Görke B, Stülke J. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol. 2008 Aug;6(8):613-624. PMID: 18628769;

Hasegawa et al. Activation of a consensus FNR-dependent promoter by DNR of Pseudomonas aeruginosa in response to nitrite. FEMS Microbiol Lett. 1998 Sep 15;166(2):213-217. PMID: 9770276;

Hoeks et al. Adult issues in phenylketonuria. Neth J Med. 2009 Jan;67(1):2-7. PMID: 19155540;

Hoeren et al. Sequence and expression of the gene encoding the respiratory nitrous-oxide reductase from Paracoccus denitrificans. Eur J Biochem. 1993 Nov 15;218(1):49-57. PMID: 8243476;

Hosseini et al. Propionate as a health-promoting microbial metabolite in the human gut. Nutr Rev. 2011 May;69(5):245-258. PMID: 21521227;

Isabella et al. Deep sequencing-based analysis of the anaerobic stimulon in Neisseria gonorrhoeae. BMC Genomics. 2011 Jan 20;12:51. PMID: 21251255;

Ivanovska et al. Pediatric drug formulations: a review of challenges and progress. Pediatrics. 2014 Aug;134(2):361-372. PMID: 25022739;Kobe et al. Regulation and crystallization of phosphorylated and dephosphorylated forms of truncated dimeric phenylalanine hydroxylase. Protein Sci. 1997 Jun; 6(6):1352-1357. PMID: 9194198;

Kwok et al. Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase. Biochemistry 1985 Jan 29; 24(3):556-561. PMID: 2986678;

Leonard JV (2006). Disorders of the urea cycle and related enzymes. *Inborn Metabolic Diseases*, 4th ed (pp. 263-272). Springer Medizin Verlag Heidelberg;

Longo et al. Phase 1 Trial of Subcutaneous rAvPAL-PEG in Subjects with Phenylketonuria. Lancet. 2014 Jul 5; 384(9937):37–44;

Lopez G, Anderson JC. Synthetic Auxotrophs with Ligand-Dependent Essential Genes for a BL21(DE3) Biosafety Strain. ACS Synth Biol. 2015 Dec 18;4(12):1279-1286. PMID: 26072987;

Macleod et al. Nutritional Management of Phenylketonuria. Ann Nestle Eng. 2010 Jun;68(2):58-69. PMID: 22475869;

Meadow et al. Biosynthesis of diaminopimelic acid and lysine in Escherichia coli. Biochem J. 1959 Jul;72(3):396-400. PMID: 16748796;

Miller (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY;

Moffitt et al. Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization. Biochemistry. 2007 Jan 30;46(4):1004-1012. PMID: 17240984;

Moore et al. Regulation of FNR dimerization by subunit charge repulsion. J Biol Chem. 2006 Nov 3;281(44):33268-33275. PMID: 16959764;

Nougayrede et al. Escherichia coli induces DNA double-strand breaks in eukaryotic cells. Science. 2006 Aug 11;313(5788):848-51. PMID: 16902142;

Olier et al. Genotoxicity of Escherichia coli Nissle 1917 strain cannot be dissociated from its probiotic activity. Gut Microbes. 2012 Nov-Dec;3(6):501-509. PMID: 22895085;

Pi et al. Cloning and sequencing of the pheP gene, which encodes the phenylalanine-specific transport system of Escherichia coli. J Bacteriol. 1991 Jun;173(12):3622-3629. PMID: 1711024;

Pi et al. Topology of the phenylalanine-specific permease of Escherichia coli. J Bacteriol. 1996 May;178(9):2650-2655. PMID: 8626334;

Pi et al. Functional consequences of changing proline residues in the phenylalanine-specific permease of Escherichia coli. J Bacteriol. 1998 Nov;180(21):5515-5519. PMID: 9791098;

Purcell et al. Towards a whole-cell modeling approach for synthetic biology. Chaos. 2013 Jun;23(2):025112. PMID: 23822510;

Ray et al. The effects of mutation of the anr gene on the aerobic respiratory chain of Pseudomonas aeruginosa. FEMS Microbiol Lett. 1997 Nov 15;156(2):227-232. PMID: 9513270;

Reister et al. Complete genome sequence of the Gram-negative probiotic Escherichia coli strain Nissle 1917. J Biotechnol. 2014 Oct 10;187:106-107. PMID: 25093936;

Rembacken et al. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet. 1999 Aug 21;354(9179):635-639. PMID: 10466665;

Remington's Pharmaceutical Sciences (2012), 22nd ed. Mack Publishing Co, Easton, PA.

Salmon et al. Global gene expression profiling in Escherichia coli K12. The effects of oxygen availability and FNR. J Biol Chem. 2003 Aug 8;278(32):29837-29855. PMID: 12754220;

Sarkissian et al. A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase. Proc Natl Acad Sci USA. 1999 Mar 2;96(5):2339-2344. PMID: 10051643;

Sat et al. The Escherichia coli mazEF suicide module mediates thymineless death. J Bacteriol. 2003 Mar;185(6):1803-1807. PMID: 12618443;

Sawers. Identification and molecular characterization of a transcriptional regulator from Pseudomonas aeruginosa PAO1 exhibiting structural and functional similarity to the FNR protein of Escherichia coli. Mol Microbiol. 1991 Jun;5(6):1469-1481. PMID: 1787797;

Schultz. Clinical use of E. coli Nissle 1917 in inflammatory bowel disease. Inflamm Bowel Dis. 2008 Jul;14(7):1012-1018. PMID: 18240278;

Sonnenborn et al. The non-pathogenic *Escherichia coli* strain Nissle 1917 – features of a versatile probiotic. Microbial Ecology in Health and Disease. 2009;21:122-158;

Trunk et al. Anaerobic adaptation in Pseudomonas aeruginosa: definition of the Anr and Dnr regulons. Environ Microbiol. 2010 Jun;12(6):1719-1733. PMID: 20553552;

Ukena et al. Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One. 2007 Dec 12;2(12):e1308. PMID: 18074031;

Unden et al. Alternative respiratory pathways of Escherichia coli: energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta. 1997 Jul 4;1320(3):217-234. PMID: 9230919;

Vockley et al. Phenylalanine hydroxylase deficiency: diagnosis and management guideline. Genet Med. 2014 Feb;16(2):188-200. PMID: 24385074;

Wanner et al. The phenylalanine ammonia-lyase gene family in Arabidopsis thaliana. Plant Mol Biol. 1995 Jan;27(2):327-338. PMID: 7888622;

Williams et al. The gene stlA encodes a phenylalanine ammonia-lyase that is involved in the production of a stilbene antibiotic in Photorhabdus luminescens TT01. Microbiology. 2005 Aug;151(Pt 8):2543-2550. PMID: 16079333.

Winteler et al. The homologous regulators ANR of Pseudomonas aeruginosa and FNR of Escherichia coli have overlapping but distinct specificities for anaerobically inducible promoters. Microbiology. 1996 Mar;142 (Pt 3):685-693. PMID: 8868444;

Wright et al. GeneGuard: A Modular Plasmid System Designed for Biosafety. ACS Synth Biol. 2015 Mar 20;4(3):307-316. PMID: 24847673;
Wu et al. Direct regulation of the natural competence regulator gene tfoX by cyclic AMP (cAMP) and cAMP receptor protein in Vibrios. Sci Rep. 2015 Oct 7;5:14921. PMID: 26442598;
Xiang L, Moore BS. Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. J Bacteriol. 2005 Jun;187(12):4286-4289. PMID: 15937191;
Zhang R, Lin Y. DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes. Nucleic Acids Res. 2009 Jan;37(Database issue):D455-D458. PMID: 18974178;
Zimmermann et al. Anaerobic growth and cyanide synthesis of Pseudomonas aeruginosa depend on anr, a regulatory gene homologous with fnr of Escherichia coli. Mol Microbiol. 1991 Jun;5(6):1483-1490. PMID: 1787798.

Examples

[0329] The following examples provide illustrative embodiments of the disclosure. One of ordinary skill in the art will recognize the numerous modifications and variations that may be performed without altering the spirit or scope of the disclosure. Such modifications and variations are encompassed within the scope of the disclosure. The Examples do not in any way limit the disclosure.

Example 1. Construction of PAL plasmids

[0330] To facilitate inducible production of PAL in *Escherichia coli* Nissle, the *PAL* gene of *Anabaena variabilis* ("PAL1") or *Photorhabdus luminescens* ("PAL3"), as well as transcriptional and translational elements, were synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. The *PAL* gene was placed under the control of an inducible promoter. Low-copy and high-copy plasmids were generated for each of *PAL1* and *PAL3* under the control of an inducible FNR promoter or a Tet promoter. Exemplary FNR promoters are shown in **Table 3**. Organization and nucleotide sequences of these constructs are shown in **Figs. 6-9**. However, as noted above, other promoters may be used to drive expression of the *PAL* gene, other *PAL* genes may be used, and other phenylalanine metabolism-regulating genes may be used.

Example 2. Transforming E. *coli*

[0331] Each of the plasmids described herein was transformed into *E. coli* Nissle for the studies described herein according to the following steps. All tubes, solutions, and cuvettes were pre-chilled to 4 °C. An overnight culture of *E. coli* Nissle

was diluted 1:100 in 5 mL of lysogeny broth (LB) containing ampicillin and grown until it reached an OD_{600} of 0.4-0.6. The *E. coli* cells were then centrifuged at 2,000 rpm for 5 min at 4 °C, the supernatant was removed, and the cells were resuspended in 1 mL of 4 °C water. The *E. coli* were again centrifuged at 2,000 rpm for 5 min at 4 °C, the supernatant was removed, and the cells were resuspended in 0.5 mL of 4 °C water. The *E. coli* were again centrifuged at 2,000 rpm for 5 min at 4 °C, the supernatant was removed, and the cells were finally resuspended in 0.1 mL of 4 °C water. The electroporator was set to 2.5 kV. Plasmid (0.5 µg) was added to the cells, mixed by pipetting, and pipetted into a sterile, chilled cuvette. The dry cuvette was placed into the sample chamber, and the electric pulse was applied. One mL of room-temperature SOC media was added immediately, and the mixture was transferred to a culture tube and incubated at 37 °C for 1 hr. The cells were spread out on an LB plate containing ampicillin and incubated overnight.

Example 3. Comparison of Phenylalanine Metabolism between High-copy and Low copy plasmids expressing PAL1 and PAL2

[0332] Genetically engineered bacteria comprising the same *PAL* gene, either *PAL3* on a low-copy plasmid or high copy plasmid (SYN-PKU101 and SYN-PKU102) or PAL3 on a low-copy plasmid or a high copy plasmid (SYN-PKU201 and SYN-PKU202) were assayed for phenylalanine metabolism *in vitro.*

[0333] Engineered bacteria were induced with anhydrous tetracycline (ATC), and then grown in culture medium supplemented with 4 mM (660,000 ng/mL) of phenylalanine for 2 hours. Samples were removed at 0 hrs, 4 hrs, and 23 hrs, and phenylalanine (**Fig. 15A**) and trans-cinnamic acid(TCA) (**Fig. 15B**) concentrations were determined by mass spectrometry as described in Examples 24-26.

[0334] High copy plasmids and low copy plasmid strains were found to metabolize and reduce phenylalanine to similar levels (**Fig. 15**). A greater reduction in phenylalanine levels and increase in TCA levels was observed in the strains expressing PAL3.

Example 4. Phenylalanine transporter - Integration of PheP into the bacterial chromosome

[0335] In some embodiments, it may be advantageous to increase phenylalanine transport into the cell, thereby enhancing phenylalanine metabolism. Therefore, a second copy of the native high affinity phenylalanine transporter, PheP, driven by an inducible promoter, was inserted into the Nissle genome through homologous recombination. Organization of the construct is shown in **Fig. 11**. The *pheP* gene was placed downstream of the P_{tet} promoter, and the tetracycline repressor, TetR, was divergently transcribed (*see, e.g.*, **Fig. 11**). This sequence was synthesized by Genewiz (Cambridge, MA). To create a vector capable of integrating the synthesized TetR-PheP construct into the chromosome, Gibson assembly was first used to add 1000 bp sequences of DNA homologous to the Nissle *lacZ* locus into the R6K origin plasmid pKD3. This targets DNA cloned between these homology arms to be integrated into the *lacZ* locus in the Nissle genome **(Fig. 10)**. Gibson assembly was used to clone the TetR-PheP 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 *pheP* sequence between them. This PCR fragment was used to transform electrocompetent Nissle-pKD46, a strain that contains a temperature-sensitive plasmid encoding the lambda red recombinase genes. After transformation, cells were grown for 2 hrs before plating on chloramphenicol at 20 µg/mL at 37 °C. Growth at 37 °C cures the pKD46 plasmid. Transformants containing anhydrous tetracycline (ATC)-inducible *pheP* were lac-minus (lac-) and chloramphenicol resistant.

Example 5. Effect of the Phenylalanine transporter on phenylalanine degradation

[0336] To determine the effect of the phenylalanine transporter on phenylalanine degradation,

[0337] phenylalanine degradation and trans-cinnamate accumulation achieved by genetically engineered bacteria expressing PAL1 or PAL3 on low-copy (LC) or high-copy (HC) plasmids in the presence or absence of a copy of pheP driven by the Tet promoter integrated into the chromosome was assessed.

[0338] For *in vitro* studies, all incubations were performed at 37 °C. Cultures of *E. coli* Nissle transformed with a plasmid comprising the *PAL* gene driven by the Tet promoter were grown overnight and then diluted 1:100 in LB. The cells were grown with shaking (200 rpm) to early log phase. Anhydrous tetracycline (ATC) was added to cultures at a concentration of 100 ng/mL to induce expression of *PAL*, and bacteria were grown for another 2 hrs. Bacteria were then pelleted, washed, and resuspended in minimal media, and supplemented with 4 mM phenylalanine. Aliquots were removed at 0 hrs, 2 hrs, and 4 hrs for phenylalanine quantification (**Fig. 16A**), and at 2 hrs and 4 hrs for cinnamate quantification (**Fig. 16B**), by mass spectrometry, as described in Examples 24-26. As shown in **Fig. 16**, expression of *pheP* in conjunction with *PAL* significantly enhances the degradation of phenylalanine as compared to *PAL* alone or *pheP* alone. Notably, the additional copy of pheP permitted the complete degradation of phenylalanine (4 mM) in 4 hrs (**Fig. 16A**). **Fig. 16B** depicts cinnamate levels in samples at 2 hrs and 4 hrs post-induction. Since cinnamate production is directly correlated with phenylalanine degradation, these data suggest that phenylalanine disappearance is due to phenylalanine catabolism, and that cinnamate may be used as an alternative biomarker for strain activity. PheP overexpression improves phenylalanine metabolism in engineered bacteria.

[0339] In conclusion, in conjunction with *pheP*, even low-copy *PAL*-expressing plasmids are capable of almost completely eliminating phenylalanine from a test sample (**Figs. 16A and 16B**). Furthermore, without wishing to be bound by theory, in some embodiments, that incorporate *pheP*, there may be additional advantages to using a low-copy *PAL*-expressing plasmid in conjunction in order to enhance the stability of *PAL* expression while maintaining high phenylalanine metabolism, and to reduce negative selection pressure on the transformed bacterium. In alternate embodiments, the phenylalanine transporter is used in conjunction with a high-copy *PAL*-expressing plasmid.

Example 6. FNR promoter activity

[0340] In order to measure the promoter activity of different FNR promoters, the *lacZ* gene, as well as transcriptional and translational elements, were synthesized (Gen9, Cambridge, MA) and cloned into vector pBR322. The *lacZ* gene was placed under the control of any of the exemplary FNR promoter sequences disclosed in **Table 3**. The

nucleotide sequences of these constructs are shown in **Tables 21-28** (SEQ ID NOs 31-38). However, as noted above, the *lacZ* gene may be driven by other inducible promoters in order to analyze activities of those promoters, and other genes may be used in place of the *lacZ* gene as a readout for promoter activity. Alternatively, beta-galactosidase may be used as a reporter, exemplary results are shown in **Fig. 18.**

[0341] **Table 21** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr1} (SEQ ID NO: 3). The construct comprises a translational fusion of the Nissle *nirB1* gene and the *lacZ* gene, in which the translational fusions are fused in frame to the 8th codon of the *lacZ* coding region. The P_{fnr1} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[0342] **Table 22** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr2} (SEQ ID NO: 6). The construct comprises a translational fusion of the Nissle *ydfZ* gene and the *lacZ* gene, in which the translational fusions are fused in frame to the 8th codon of the *lacZ* coding region. The P_{fnr2} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[0343] **Table 23** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr3} (SEQ ID NO: 7). The construct comprises a transcriptional fusion of the Nissle *nirB* gene and the *lacZ* gene, in which the transcriptional fusions use only the promoter region fused to a strong ribosomal binding site. The P_{fnr3} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[0344] **Table 24** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, P_{fnr4} (SEQ ID NO: 8). The construct comprises a transcriptional fusion of the Nissle *ydfZ* gene and the

lacZ gene. The P_{fnr4} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[0345] **Table 25** shows the nucleotide sequence of an exemplary construct comprising a gene encoding lacZ, and an exemplary FNR promoter, PfnrS (SEQ ID NO: 9). The construct comprises a transcriptional fusion of the anaerobically induced small RNA gene, *fnrS1*, fused to *lacZ*. The P_{fnrs} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *lacZ* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[0346] **Table 26** shows the nucleotide sequence of an exemplary construct comprising a gene encoding PAL3, and an exemplary FNR promoter, P_{fnr3} (SEQ ID NO: 7). The construct comprises a transcriptional fusion of the Nissle *nirB* gene and the *PAL3* gene, in which the transcriptional fusions use only the promoter region fused to a strong ribosomal binding site. The P_{fnr3} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *PAL3* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[0347] **Table 27** shows the nucleotide sequences of an exemplary construct comprising a gene encoding PAL3, and an exemplary FNR promoter, P_{fnr4} (SEQ ID NO: 8). The construct comprises a transcriptional fusion of the Nissle *ydfZ* gene and the *PAL3* gene. The P_{fnr4} sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *PAL3* sequence is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

[0348] **Table 28** shows the nucleotide sequences of an exemplary construct comprising a gene encoding PAL3, and an exemplary FNR promoter, P_{fnrs} (SEQ ID NO: 9). The construct comprises a transcriptional fusion of the anaerobically induced small RNA gene, *fnrS1*, fused to *PAL3*. The Pfnrs sequence is **bolded** lower case, and the predicted ribosome binding site within the promoter is underlined. The *PAL3* sequence

is underlined upper case. ATG site is **bolded** upper case, and the cloning sites used to synthesize the construct are shown in regular upper case.

Table 21

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 31)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatc** **gtcgtccggccttttcctctcttactctgctacgtacatctatttctataaatccgttc** **aatttgtctgtttttgcacaaacatgaaatatcagacaattccgtgacttagaaaat** **ttatacaaatcagcaatatacccttaaggagtatataaaggtgaatttgatttacatc** **aataagcggggttgctgaatcgttaaggtaggcggtaatagaaaagaaatcgaggcaaa** **aATGagcaaagtcagactcgcaattat**GGATCCTCTGGCCGTCGTATTACAACGTCGTG ACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCC AGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCT GAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGC TGGAGTGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCAC GGTTACGATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTT TGTTCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCT GGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTG TGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGA CCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGA GTGACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGAC GTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTT TAATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGC GCGATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGC ACCGCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCAC ACTACGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATC GTGCAGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGAC GTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTT GCTGATTCGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATG AGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGC TGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGCTACGGCCTGTA TGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCAATGAATCGTCTGACCG ATGATCCGCGCTGGCTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGAT CGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAA TCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGTATG AAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTG GATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCT GCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTAACAGTC TTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGGCGGC TTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTG GTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACG GTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAG CAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTT CCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAGCCGCTGG CAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACTGCCT GAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCAACC

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 31)
AAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTCTGG CGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAACTGACCACC AGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTC AGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGCGCG ATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCATT GACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCGGC GTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACCGCCCACG CGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGG CACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCCGGC GCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTCGGCC TGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCTGGGAT CTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTG CGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACA TCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCG GAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTC CTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGT TGGTCTGGTGTCAAAAATAA

Table 22

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 32)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctc** **atgcatgcatcaaaaaagatgtgagcttgatcaaaaacaaaaaatatttcactcgacag** **gagtatttatattgcgcccgttacgtgggcttcgactgtaaatcagaaaggagaaaaca** **cctATGacgacctacgatcg**GGATCCTCTGGCCGTCGTATTACAACGTCGTGACTGGGA AAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGC GTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGC GAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTG CGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACG ATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCC GCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACA GGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCA ACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGC GCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGG CAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGT TGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGAT GATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGA ACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGC CTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGC CTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGT GGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTT TCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATT CGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCAGAC GATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGC ATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTG

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 32)
GATGAAGCCAATATTGAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCC GCGCTGGCTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATC ACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGAC GCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGG CGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGGATGAAG ACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCTGCCTGGA GAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTAACAGTCTTGGCGG CTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCT GGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCT TACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGT CTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAGCAGTATT TCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTTCCGTCAT AGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAGCCGCTGGCAAGCGG TGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACTGCCTGAACTGC CGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCAACCAAACGCG ACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTCTGGCGGAAAA CCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAACTGACCACCAGCGGAA CGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTT CTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGCGCGATCAGTT CACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCATTGACCCTA ACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCGGCGTTGTTG CAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACCGCCCACGCGTGGCA GCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGCACGGTG AGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCCGGCGCGGATT GGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTCGGCCTGGGGCC GCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCTGGGATCTGCCAT TGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACG CGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCG CTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGAAG GCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGC CCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTG GTGTCAAAAATAA

Table 23

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 33)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatc** **gtcgtccggccttttcctctcttactctgctacgtacatctatttctataaatccgttc** **aatttgtctgtttttgcacaaacatgaaatatcagacaattccgtgacttaagaaaat** **ttatacaaatcagcaatataccccttaaggagtatataaaggtgaatttgatttacatc** **aataagcggggttgctgaatcgttaa**GGATCC**ctctagaaataattttgtttaacttta** **agaaggagatatacatATG**ACTATGATTACGGATTCTCTGGCCGTCGTATTACAACGTC GTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTC GCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAG CCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCT GGCTGGAGTGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATG

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 33)
CACGGTTACGATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCC GTTTGTTCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAA GCTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCAT CTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATT TGACCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCT GGAGTGACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGT GACGTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCT CTTTAATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGC TGCGCGATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGC GGCACCGCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGT CACACTACGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCT ATCGTGCAGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGC GACGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCC GTTGCTGATTCGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGG ATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTG CGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGCTACGGCCT GTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCAATGAATCGTCTGA CCGATGATCCGCGCTGGCTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGC GATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGC TAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGT ATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGC GTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTC GCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTAACA GTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGGC GGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCC GTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGA ACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAA CAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCT GTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAGCCGC TGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACTG CCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCA ACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTC TGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAACTGACC ACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCA GTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGC GCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGC ATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGC GGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACCGCCC ACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGAT GGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCC GGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTCG GCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCTGG GATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCG CTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCA ACATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCAC GCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGA CTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACC AGTTGGTCTGGTGTCAAAAATAA

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 33)

Table 24

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 34)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctc** **atgcatgcatcaaaaaagatgtgagcttgatcaaaaacaaaaaatatttcactcgacag** **gagtatttatattgcgccc**GGATCC**ctctagaaataattttgtttaactttaagaagga** **gatatacatATG**ACTATGATTACGGATTCTCTGGCCGTCGTATTACAACGTCGTGACTG GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCT GGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAAT GGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGA GTGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTT ACGATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTT CCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCT ACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGT GCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTG AGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGA CGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCT CGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAAT GATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGA TGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCG CGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTA CGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGC AGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCG GTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTG ATTCGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCA GACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTT CGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTG GTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGA TCCGCGCTGGCTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTA ATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCAC GACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGG CGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGGATG AAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCTGCCT GGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTAACAGTCTTGG CGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGGCGGCTTCG TCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCG GCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCT GGTCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAGCAGT ATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTTCCGT CATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAGCCGCTGGCAAG CGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACTGCCTGAAC TGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCAACCAAAC GCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTCTGGCGGA AAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAACTGACCACCAGCG GAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGC

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 34)
TTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGCGCGATCA GTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCATTGACC CTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCGGCGTTG TTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACCGCCCACGCGTG GCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGCACG GTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCCGGCGCGG ATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTCGGCCTGGG GCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCTGGGATCTGC CATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGG ACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAG CCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAG AAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGG AGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGT CTGGTGTCAAAAATAA

Table 25

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 35)
GGTACC**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgt** **aacaaaagcaatttttccggctgtctgtatacaaaaacgccgtaaagtttgagcgaagt** **caataaactctctacccattcagggcaatatctctctt**GGATCC**ctctagaaataattt** **tgtttaactttaagaaggagatatacatATG**CTATGATTACGGATTCTCTGGCCGTCGT ATTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGCAC ATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAA CAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGT GCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAA ACTGGCAGATGCACGGTTACGATGCGCCTATCTACACCAACGTGACCTATCCCATTACG GTCAATCCGCCGTTTGTTCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAA TATTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACT CGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTG CCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGAT GGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCG GCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAA GTTACCACTCTCTTTAATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGAT GTACGGCGAGCTGCGCGATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGC AGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTAT GCCGATCGCGTCACACTACGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAAAT CCCGAATCTCTATCGTGCAGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAG CAGAAGCCTGCGACGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTG AACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGG TCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACT TTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGAC CGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCAAT GAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGAGCGAACGCGTAACGCGGA TGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCA GGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCG

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 35)
CCCGGTACAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGA TGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAA AAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGC GATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCC GTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAA AACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCA GTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAG CAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACC AGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGA TGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGT TGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGTA CGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCA GCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCC CTCAACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAA TTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCT GACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTG AAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTAC CAGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGCTGAT TACAACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCT ACCGGATTGATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGAT ACACCGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGT AAACTGGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTT TTGACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAA AACGGTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGA CTTCCAGTTCAACATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCC ATCTGCTGCACGCGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATT GGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCG CTACCATTACCAGTTGGTCTGGTGTCAAAAATAA

Table 26

Nucleotide sequences of Pfnr3-PAL3 construct, low-copy (SEQ ID NO: 36)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatc** **gtcgtccggccttttcctctcttactctgctacgtacatctatttctataaatccgttc** **aatttgtctgtttttttgcacaaacatgaaatatcagacaattccgtgacttaagaaaat** **ttatacaaatcagcaatataccccttaaggagtatataaaggtgaatttgatttacatc** **aataagcggggttgctgaatcgttaa**GGATCC**ctctagaaataattttgtttaacttta** **agaaggagatatacatATG**AAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAAAA ATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAA ATATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAAAATT AAATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGAATGCCAATTTAG TTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCTTTCTGCT GGTACTGGGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACT TTCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATC ATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGT GATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTATTA

Nucleotide sequences of Pfnr3-PAL3 construct, low-copy (SEQ ID NO: 36)
TATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTAT CGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAATC AGTGCAATCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGC CCTTGCTGTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAAG TAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGT TCAACGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCA TCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAATTCGCTGTGCAC CACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGAACGG GAAGTTATCTCAGCTAATGATAATCCATTGATAGATCCAGAAAATGGCGATGTTCTACA CGGTGGAAATTTTATGGGGCAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATA TTGCTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATAACCGTTTC TCTCGTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAAGG CGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGATTGTGCTGCATCAG GTATTCATACCCTCGCCACAGAACAATACAATCAAGATATTGTCAGTTTAGGTCTGCAT GCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGTTTCAATGACAAT TCTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAA CTGCTAAATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCG TTGGATGAAGATATAATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCTGCC AGAAATCATGCTGGAAGAATAA

Table 27

Nucleotide sequences of Pfnr4-PAL3 construct, low-copy (SEQ ID NO: 37)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctc** **atgcatgcatcaaaaaagatgtgagcttgatcaaaaacaaaaaatatttcactcgacag** **gagtatttatattgcgccc**GGATCC**ctctagaaataattttgtttaactttaagaagga** **gatatacatATG**AAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCT TATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAAATATCAA CGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCA GGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCC ATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTG GGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTT TGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTAA TCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAA TTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTATTATATGGGC GCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAAA AGCCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAA TCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCT GTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAAGTAAAAAA TCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGC AGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCATCAAGAA ATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGT ATTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGAACGGGAAGTTA TCTCAGCTAATGATAATCCATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGA AATTTTATGGGGCAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATATTGCTTT AATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATAACCGTTTCTCTCGTG

Nucleotide sequences of Pfnr4-PAL3 construct, low-copy (SEQ ID NO: 37)
GATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAA CTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGATTGTGCTGCATCAGGTATTCA TACCCTCGCCACAGAACAATACAATCAAGATATTGTCAGTTTAGGTCTGCATGCCGCTC AAGATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGTTTCAATGACAATTCTGGTA GTTTGTCAGGCCATTCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAA ATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGATG AAGATATAATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAATC ATGCTGGAAGAATAA

Table 28

Nucleotide sequences of PfnrS-PAL3 construct, low-copy (SEQ ID NO: 38)
GGTACC**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgt** **aacaaaagcaatttttccggctgtctgtatacaaaaacgccgtaaagtttgagcgaagt** **caataaactctctacccattcagggcaatatctctctt**GGATCC**ctctagaaataattt** **tgtttaactttaagaaggagatatacatATG**AAAGCTAAAGATGTTCAGCCAACCATTA TTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAA AAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATT AGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGA ATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAACT TTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATT TACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAG CAATTGTTGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTG GGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGG CAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCAGGGT TGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTA ATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAAT TTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCC GGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAAT TTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAA AGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAA TTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGAAA ATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCCATTGATAGATCCAGAAAATGG CGATGTTCTACACGGTGGAAATTTTATGGGGCAATATGTCGCCCGAACAATGGATGCAT TAAAACTGGATATTGCTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATG GATAACCGTTTCTCTCGTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCA AGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGATT GTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAATACAATCAAGATATTGTCAGT TTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGT TTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATTAGTGAAA TTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTGATC ACTGATCGTGCGTTGGATGAAGATATAATCCGCATTGCGGATGCAATTATTAATGATCA ACTTCCTCTGCCAGAAATCATGCTGGAAGAATAA

[0349] Each of the plasmids was transformed into *E. coli* Nissle, as described above. Cultures of transformed *E. coli* Nissle were grown overnight and then diluted 1:200 in LB. The cells were grown with shaking at 250 rpm either aerobically or anaerobically in a Coy anaerobic chamber supplied with 90% N_2, 5% CO_2, and 5% H_2. After 4-6 hrs of incubation, samples were collected, and promoter activity was analyzed by performing β-galactosidase assays (Miller, 1972). As shown in **Fig. 20**, the activities of the FNR promoters were greatly enhanced under anaerobic conditions compared to aerobic conditions.

Example 7. Measuring the activity of an FNR promoter

[0350] To determine the kinetics of FNR promoter-driven gene expression, *E. coli* strains harboring a low-copy *fnrS-lacZ* fusion gene (**Fig. 19A**) were grown aerobically with shaking at 250 rpm. Cultures were split after 1 hr., and then incubated either aerobically or anaerobically in a Coy anaerobic chamber (supplying 90% N_2, 5% CO_2, and 5%H_2) at 37 °C. Promoter activity was measured as a function of β-galactosidase activity using a standard colorimetric assay (Miller, 1972). **Fig. 19B** demonstrates that the *fnrS* promoter begins to drive high-level gene expression within 1 hr. under anaerobic conditions. Growth curves of bacterial cell cultures expressing *lacZ* are shown in **Fig. 19C**, both in the presence and absence of oxygen.

Example 8. Production of PAL from FNR promoter in recombinant *E. coli*

[0351] Cultures of *E. coli* Nissle transformed with a plasmid comprising the *PAL* gene driven by any of the exemplary FNR promoters were grown overnight and then diluted 1:200 in LB. The bacterial cells may further comprise the *pheP* gene driven by the Tet promoter and incorporated into the chromosome. ATC was added to cultures at a concentration of 100 ng/mL to induce expression of *pheP*, and the cells were grown with shaking at 250 rpm either aerobically or anaerobically in a Coy anaerobic chamber supplied with 90% N_2, 5% CO_2, and 5% H_2. After 4 hrs of incubation, cells were pelleted down, washed, and resuspended in M9 minimal medium supplemented with 0.5% glucose and 4 mM phenylalanine. Aliquots were collected at 0 hrs, 2 hrs, 4 hrs, and 24 hrs for phenylalanine quantification (**Fig. 20**). As shown in **Fig. 20B**, the genetically engineered bacteria expressing *PAL3* driven by the FNR promoter are more efficient at removing phenylalanine from culture medium under anaerobic

conditions, compared to aerobic conditions (**Fig. 20A**). The expression of *pheP* in conjunction with *PAL3* further decreased levels of phenylalanine.

Example 9. Phenylalanine degradation in recombinant *E. coli* with and without *pheP* overexpression

[0352] The SYN-PKU304 and SYN-PKU305 strains contain low-copy plasmids harboring the *PAL3* gene, and a copy of *pheP* integrated at the *lacZ* locus. The SYN-PKU308 and SYN-PKU307 strains also contain low-copy plasmids harboring the *PAL3* gene, but lack a copy of *pheP* integrated at the *lacZ* locus. In all four strains, expression of *PAL3* and *pheP* (when applicable) is controlled by an oxygen level-dependent promoter.

[0353] To determine rates of phenylalanine degradation in engineered *E. coli* Nissle with and without *pheP* on the chromosome, overnight cultures of SYN-PKU304 and SYN-PKU307 were diluted 1:100 in LB containing ampicillin, and overnight cultures of SYN-PKU308 and SYN-PKU305 were diluted 1:100 in LB containing kanamycin. All strains were grown for 1.5 hrs before cultures were placed in a Coy anaerobic chamber supplying 90% N_2, 5% CO_2, and 5% H_2. After 4 hrs of induction, bacteria were pelleted, washed in PBS, and resuspended in 1 mL of assay buffer. Assay buffer contained M9 minimal media supplemented with 0.5% glucose, 8.4% sodium bicarbonate, and 4 mM of phenylalanine.

[0354] For the activity assay, starting counts of colony-forming units (cfu) were quantified using serial dilution and plating. Aliquots were removed from each cell assay every 30 min for 3 hrs for phenylalanine quantification by mass spectrometry. Specifically, 150 µL of bacterial cells were pelleted and the supernatant was harvested for LC-MS analysis, with assay media without cells used as the zero-time point. **Fig. 21** shows the observed phenylalanine degradation for strains with *pheP* on the chromosome (SYN-PKU304 and SYN-PKU305; left), as well as strains lacking *pheP* on the chromosome (SYN-PKU308 and SYN-PKU307; right). These data show that *pheP* overexpression is important in order to increase rates of phenylalanine degradation in synthetic probiotics.

Example 10. Activity of Strains with single and multiple chromosomal PAL3 insertions

[0355] To assess the effect of insertion site and number of insertions on the activity of the genetically engineered bacteria, in vitro activity of strains with different single insertions of PAL3 at various chromosomal locations and with multiple PAL3 insertions was measured.

[0356] Cells were grown overnight in LB and diluted 1:100. After 1.5 hrs of growth, cultures were placed in Coy anaerobic chamber supplying 90% N_2, 5% CO_2, and 5% H_2. After 4 hrs of induction, bacteria were resuspended in assay buffer containing 50 mM phenylalanine. Aliquots were removed from cell assays every 20 min for 1.5 hrs for trans-cinnamate quantification by absorbance at 290 nm. Results are shown in **Fig. 22 and 23** and **Table 39** and **Table 40. Fig 22** depicts trans-cinnamate concentrations (PAL activity) for strains comprising single PAL3 insertions at various locations on the chromosome. **Fig. 23** depicts trans-cinnamate concentrations (PAL activity) for strains comprising multiple PAL3 insertions at various locations on the chromosome.

Table 39. Activity of various strains comprising a single PAL3 chromosomal insertion at various sites

Insertion:	Strain:	rate (umol/hr./1e9 cells):
agaI/rsmI	SYN-PKU520	**1.97**
yicS/nepI	SYN-PKU521	**2.44**
cea	SYN-PKU522	**ND**
malEK	SYN-PKU518	**1.66**
malPT	SYN-PKU523	**0.47**

Table 40. In vitro activity of various strains comprising one or more chromosomal PAL3 insertions

Genotypes:	Strain	Rate (umol/hr./1e9 cells)
agaI:PAL, cea:PAL, matPT:PAL, malEK:PAL, lacZ:pheP, thyA-	SYN-PKU512	**6.76**
agaI:PAL, yicS:PAL, cea:PAL, matPT:PAL, malEK:PAL, lacZ:pheP, thyA-	SYN-PKU511	**7.65**
malPT:PAL, malEK:PAL, lacZ:pheP	SYN-PKU524	**2.89**
malEK:PAL, lacZ:pheP, ara-LAAD	SYN-PKU702	**1.53**
malPT:PAL, malEK:PAL, lacZ:pheP, ara-LAAD	SYN-PKU701	**2.65**
malPT:PAL, malEK:PAL, lacZ:pheP, agaI:pheP, ara-LAAD	SYN-PKU703	**3.14**
yicS:PAL, malPT:PAL, malEK:PAL lacZ:pheP, ara-LAAD	SYN-PKU704	**3.47**
yicS:PAL, malPT:PAL, malEK:PAL, lacZ:pheP, agaI:pheP, ara-LAAD	SYN-PKU705	**3.74**

Example 11. Activity of a strain with five chromosomal copies of PAL3

[0357] The activity of a strain SYN-PKU511, a strain comprising five integrated copies of an anaerobically (FNR) controlled PAL3 and an anaerobically controlled pheP integrated in the lacZ locus, was assessed.

[0358] The genetically engineered bacteria were grown overnight, diluted and allowed to grow for another 2.5 hours. Cultures were then placed in Coy anaerobic chamber supplying 90% N2, 5% CO2, and 5% H2. After 3.5 hrs of induction in phenylalanine containing medium (4 mM phenylalanine), whole cell extracts were prepared every 30 min for 3 hrs and phenylalanine was quantified by mass spectrometry. Results are shown in **Fig. 24**. The in vitro activity of the cells was 8umol/hr./1e9 cells. Phenylalanine levels drop to about half of the original levels after 2 hours.

Example 12. Activity of a Strain expressing LAAD

[0359] To assess whether LAAD expression can be used as an alternative, additional or complementary phenylalanine degradation means to PAL3, the ability of

genetically engineered strain SYN-PKU401, which contains a high copy plasmid expressing LAAD driven by a Tet-inducible promoter, was measured at various cell concentrations and at varying oxygen levels.

[0360] Overnight cultures of SYN-PKU401 were diluted 1:100 and grown to early log phase before induction with ATC (100 ng/ml) for 2 hours. Cells were spun down and incubated as follows.

[0361] Cells (1 ml) were incubated aerobically in a 14 ml culture tube, shaking at 250 rpm (Fig 25 A and B). For microaerobic conditions, cells (1 ml) were incubated in a 1.7 ml conical tube without shaking. Cells were incubated anaerobically in a Coy anaerobic chamber supplying 90% N2, 5% CO2, and 5% H2 (Fig.25B). Aliquots were removed from cell assays every 30 min for 2 hrs for phenylalanine quantification by mass spectrometry, and results are shown in **Fig. 25A and 25B. Fig. 25A** shows cell concentration dependent aerobic activity. The activity in aerobic conditions is ~50umol/hr./1e9cells, and some activity is retained under microaerobic conditions, which may allow for activity in environments with oxygen concentrations less than ambient air. The activity of SYN-PKU401 under microaerobic conditions is comparable to SYN-PKU304 under anaerobic conditions, however, activity seems to be dependent on cell density.

[0362] **Table 41** and **Table 42** contain LAAD constructs of interest. **Table 41** shows the sequence of an exemplary construct comprising a gene encoding LAAD from Proteus mirabilis and a Tet repressor gene and a Tet promoter sequence and RBS and leader region, on a plasmid SEQ ID NO: 39, with the *LAAD* sequence underlined the TetR sequence in italics and the Tet promoter sequence bolded and the RBS and leader region underlined and italics. **Table 42** shows the sequence of an exemplary construct comprising a gene encoding araC and a gene encoding LAAD from Proteus mirabilis and an arabinose inducible promoter (ParaBAD) sequence for chromosomal insertion into the endogenous arabinose operon (SEQ ID NO: 40), with the *araC* sequence underlined and the ParaBAD promoter sequence bolded and the LAAD sequence in italics and the RBS and leader region underlined and in italics.

[0363] 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: 20-42, or a functional fragment thereof.

Table 41. LAAD driven by a Tet inducible promoter on a plasmid

Nucleotide sequences of TetR-LAAD plasmid construct (SEQ ID NO: 39)
Ttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggc *cgaataagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcgtaat* *aatggcggcatactatcagtagtaggtgtttccctttcttctttagcgacttgatgctc* *ttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatg* *cattctctagtgaaaaaccttgttggcataaaaaggctaattgattttcgagagtttca* *tactgtttttctgtaggccgtgtacctaaatgtacttttgctccatcgcgatgacttag* *taaagcacatctaaaacttttagcgttattacgtaaaaaatcttgccagctttcccctt* *ctaaagggcaaaagtgagtatggtgcctatctaacatctcaatggctaaggcgtcgagc* *aaagcccgcttattttttacatgccaatacaatgtaggctgctctacacctagcttctg* *ggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgc* *tgttaatcactttacttttatctaatctagacat*cattaattcctaatttttt**gttgaca** **ctctatcattgatagagttattttaccactccctatcagtgatagagaa**aagtgaa*ctc* *tagaaataattttgtttaactttaagaaggagatatacat*atgaacatttcaaggagaa agctacttttaggtgttggtgctgcgggcgttttagcaggtggtgcggctttagttcca atggttcgccgtgacggcaaatttgtggaagctaaatcaagagcatcatttgttgaagg tacgcaaggggctcttcctaaagaagcagatgtagtgattattggtgccggtattcaag ggatcatgaccgctattaaccttgctgaacgtggtatgagtgtcactatcttagaaaag ggtcagattgccggtgagcaatcaggccgtgcatacagccaaattattagttaccaaac atcgccagaaatcttcccattacaccattatgggaaaatattatggcgtggcatgaatg agaaaattggtgcggataccagttatcgtactcaaggtcgtgtagaagcgctggcagat gaaaaagcattagataaagctcaagcgtggatcaaaacagctaaagaagcggcaggttt tgatacaccattaaatactcgcatcattaaaggtgaagagctatcaaatcgcttagtcg gtgctcaaacgccatggactgttgctgcatttgaagaagattcaggctctgttgatcct gaaacaggcacacctgcactcgctcgttatgccaaacaaatcggtgtgaaaatttatac caactgtgcagtaagaggtattgaaactgcgggtggtaaaatctctgatgtggtgagtg agaaaggggcgattaaaacgtctcaagttgtactcgctgggggtatctggtcgcgttta tttatgggcaatatgggtattgatatcccaacgctcaatgtatatctatcacaacaacg tgtctcaggggttcctggtgcaccacgtggtaatgtgcatttacctaatggtattcatt tccgcgaacaagcggatggtacttatgccgttgcaccacgtatctttacgagttcaata gtcaaagatagcttcctgctagggcctaaatttatgcacttattaggtggcggagagtt accgttggaattctctattggtgaagatctatttaattcatttaaaatgccgacctctt ggaatttagatgaaaaacaccattcgaacaattccgagttgccacggcaacacaaaat acgcaacacttagatgctgttttccaaagaatgaaaacagaattcccagtatttgaaaa atcagaagttgttgaacgttggggtgccgttgtgagtccaacatttgatgaattaccta tcatttctgaggtcaaagaatacccaggcttagtgattaacacggcaacagtgtggggt atgacagaaggcccggcagcgggtgaagtgaccgctgatattgtcatgggcaagaaacc tgttattgatccaacgccgtttagtttggatcgttttaagaagtaa

Table 42. LAAD sequence driven by the AraBAD promoter for insertion into the Ara operon

Nucleotide sequences of AraC-ARABAD promoter-LAAD construct (SEQ ID NO: 40)
Ttattcacaacctgccctaaactcgctcggactcgccccggtgcatttttaaatactc gcgagaaatagagttgatcgtcaaaaccgacattgcgaccgacggtggcgataggcatc cgggtggtgctcaaaagcagcttcgcctgactgatgcgctggtcctcgcgccagcttaa tacgctaatccctaactgctggcggaacaaatgcgacagacgcgacggcgacaggcaga catgctgtgcgacgctggcgatatcaaaattactgtctgccaggtgatcgctgatgtac tgacaagcctcgcgtacccgattatccatcggtggatggagcgactcgttaatcgcttc catgcgccgcagtaacaattgctcaagcagatttatcgccagcaattccgaatagcgcc cttccccttgtccggcattaatgatttgcccaaacaggtcgctgaaatgcggctggtgc gcttcatccgggcgaaagaaaccggtattggcaaatatcgacggccagttaagccattc atgccagtaggcgcgcggacgaaagtaaacccactggtgataccattcgtgagcctccg gatgacgaccgtagtgatgaatctctccaggcgggaacagcaaaatatcacccggtcgg cagacaaattctcgtccctgatttttcaccaccccctgaccgcgaatggtgagattgag aatataacctttcattcccagcggtcggtcgataaaaaaatcgagataaccgttggcct caatcggcgttaaacccgccaccagatgggcgttaaacgagtatcccggcagcagggga tcattttgcgcttcagccatacttttcatactcccgccattcagagaagaaaccaattg tccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccc aaccggtaaccccgcttattaaaagcattctgtaacaaagcgggaccaaagccatgaca aaaacgcgtaacaaaagtgtctataatcacggcagaaaagtccacattgattatttgca cggcgtcacactttgctatgccatagcatttttatccataagattagcggatccagcct gacgctttttttcgcaactctctactgtttctccatAc*ctctagaaataattttgttta* *actttaagaaggagatatacatatgaacatttcaaggagaaagctacttttaggtgttg* *gtgctgcgggcgttttagcaggtggtgcggctttagttccaatggttcgccgtgacggc* *aaatttgtggaagctaaatcaagagcatcatttgttgaaggtacgcaagggggctcttcc* *taaagaagcagatgtagtgattattggtgccggtattcaagggatcatgaccgctatta* *accttgctgaacgtggtatgagtgtcactatcttagaaaagggtcagattgccggtgag* *caatcaggccgtgcatacagccaaattattagttaccaaacatcgccagaaatcttccc* *attacaccattatgggaaaatattatggcgtggcatgaatgagaaaattggtgcggata* *ccagttatcgtactcaaggtcgtgtagaagcgctggcagatgaaaaagcattagataaa* *gctcaagcgtggatcaaaacagctaaagaagcggcaggttttgatacaccattaaatac* *tcgcatcattaaaggtgaagagctatcaaatcgcttagtcggtgctcaaacgccatgga* *ctgttgctgcatttgaagaagattcaggctctgttgatcctgaaacaggcacacctgca* *ctcgctcgttatgccaaacaaatcggtgtgaaaatttataccaactgtgcagtaagagg* *tattgaaactgcgggtggtaaaatctctgatgtggtgagtgagaaaggggcgattaaaa* *cgtctcaagttgtactcgctgggggtatctggtcgcgtttatttatgggcaatatgggt* *attgatatcccaacgctcaatgtatatctatcacaacaacgtgtctcaggggttcctgg* *tgcaccacgtggtaatgtgcatttacctaatggtattcatttccgcgaacaagcggatg* *gtacttatgccgttgcaccacgtatctttacgagttcaatagtcaaagatagcttcctg* *ctagggcctaaatttatgcacttattaggtggcggagagttaccgttggaattctctat* *tggtgaagatctatttaattcatttaaaatgccgacctcttggaatttagatgaaaaaa* *caccattcgaacaattccgagttgccacggcaacacaaaatacgcaacacttagatgct*

Nucleotide sequences of AraC-ARABAD promoter-LAAD construct (SEQ ID NO: 40)
gttttccaaagaatgaaaacagaattcccagtatttgaaaaatcagaagttgttgaacg ttggggtgccgttgtgagtccaacatttgatgaattacctatcatttctgaggtcaaag aatacccaggcttagtgattaacacggcaacagtgtggggtatgacagaaggcccggca gcgggtgaagtgaccgctgatattgtcatgggcaagaaacctgttattgatccaacgcc gtttagtttggatcgttttaagaagtaa

Example 13. Efficacy of PAL-expressing bacteria in a mouse model of PKU

[0364] For *in vivo* studies, BTBR-*Pah*^{enu2} mice were obtained from Jackson Laboratory and bred to homozygosity for use as a model of PKU. Bacteria harboring a low-copy pSC101 origin plasmid expressing *PAL3* from the Tet promoter, as well as a copy of *pheP* driven by the Tet promoter integrated into the genome (SYN-PKU302), were grown. SYN-PKU1 was induced by ATC for 2 hrs prior to administration. Bacteria were resuspended in phosphate buffered saline (PBS) and 10^9 ATC-induced SYN-PKU302 or control Nissle bacteria were administered to mice by oral gavage.

[0365] At the beginning of the study, mice were given water that was supplemented with 100 micrograms/mL ATC and 5% sucrose. Mice were fasted by removing chow overnight (10 hrs), and blood samples were collected by mandibular bleeding the next morning in order to determine baseline phenylalanine levels. Blood samples were collected in heparinized tubes and spun at 2G for 20 min to produce plasma, which was then removed and stored at -80° C. Mice were given chow again, and were gavaged after 1 hr. with 100 μL ($5x10^9$ CFU) of bacteria that had previously been induced for 2 hrs with ATC. Mice were put back on chow for 2 hrs. Plasma samples were prepared as described above.

[0366] **Fig. 26A** shows phenylalanine levels before and after feeding, and **Fig. 26B** shows the percent (%) change in blood phenylalanine levels before and after feeding as a male or female group average ($p < 0.01$). As shown in **Fig. 26**, PKU mice treated with SYN-PKU1 exhibit a significantly reduced post-feeding rise in serum phenylalanine levels compared to controls.

Example 14. Efficacy of *PAL*-expressing bacteria following subcutaneous phenylalanine challenge

[0367] Streptomycin-resistant *E. coli* Nissle (SYN-PKU901) was grown from frozen stocks to a density of 10^{10} cells/mL. Bacteria containing a copy of *pheP* under the control of a Tet promoter integrated into the *lacZ* locus, as well as a high-copy plasmid expressing *PAL3* under the control of a Tet promoter (SYN-PKU303) were grown to an A_{600} of 0.25 and then induced by ATC (100 ng/mL) for 4 hrs. Bacteria were centrifuged, washed, and resuspended in bicarbonate buffer at density of $1x10^{10}$ cells/mL before freezing at -80 °C.

[0368] Beginning at least 3 days prior to the study (i.e., Days -6 to -3), homozygous BTBR-Pah^{enu2} mice (approx. 6-12 weeks of age) were maintained on phenylalanine-free chow and water that was supplemented with 0.5 grams/L phenylalanine. On Day 1, mice were randomized into treatment groups and blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. Mice were also weighed to determine the average weight for each group. Mice were then administered single dose of phenylalanine by subcutaneous injection at 0.1 mg per gram body weight, according to the average group weight. At 30 and 90 min post-injection, 200 µL of H_2O (n=30), SYN-PKU901 (n=33), or SYN-PKU303 (n=34) were administered to mice by oral gavage. Blood samples were collected at 2 hrs and 4 hrs following phenylalanine challenge, and phenylalanine levels in the blood were measured using mass spectrometry.

[0369] **Fig. 27** shows phenylalanine blood concentrations relative to baseline concentrations at 2 hrs (**Fig. 27A**) and 4 hrs (**Fig. 27B**) post-phenylalanine injection. These data suggest that subcutaneous injection of phenylalanine causes hyperphenylalanemia in homozygous *enu2/enu2* mice, and that oral administration of SYN-PKU303 significantly reduces blood phenylalanine levels following phenylalanine challenge, compared to control groups ($p < 0.00001$ at 4 hrs). Moreover, these results confirm that the orally-administered engineered bacteria, and not the non-engineered Nissle parent, can significantly impact blood-phenylalanine levels independent of dietary exposure. Thus, a PKU-specific probiotic may not need to be co-administered in conjunction with diet.

Example 15. Dose-response activity of PAL-expressing bacteria on systemic phenylalanine

[0370] Streptomycin-resistant *E. coli* Nissle (SYN-PKU901) were grown from frozen stocks to a density of 10^{10} cells/mL. Bacteria containing a copy of *pheP* under the control of a P_{fnrS} promoter integrated into the *lacZ* locus, as well as a low-copy plasmid expressing *PAL3* under the control of a P_{fnrS} promoter (SYN-PKU304) were grown to an A_{600} of 0.25 and then induced anaerobically by purging the bacterial fermenter with nitrogen for 4 hrs. Bacteria were centrifuged, washed, and resuspended in bicarbonate buffer at density of $5x10^9$ cells/mL before freezing at -80 °C.

[0371] Beginning at least 3 days prior to the study (*i.e.*, Days -6 to -3), mice were maintained on phenylalanine-free chow and water that was supplemented with 0.5 grams/L phenylalanine. On Day 1, mice were randomized into treatment groups and blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. Mice were also weighed to determine the average weight for each group. Mice were then administered single dose of phenylalanine by subcutaneous injection at 0.1 mg per gram body weight, according to the average group weight. At 30 and 90 min post-injection, 200 μL of H_2O (n=12), 200 μL of SYN-PKU901 (n=12), or 100 μL, 200 μL, or 400 μL of SYN-PKU304 (n=12 in each dose group) were administered to mice by oral gavage. Blood samples were collected at 2 hrs and 4 hrs following phenylalanine challenge, and phenylalanine levels in the blood were measured using mass spectrometry.

[0372] **Fig. 30** shows phenylalanine blood concentrations relative to baseline concentrations post-phenylalanine injection. These data demonstrate a dose-dependent decrease in blood phenylalanine levels in SYN-PKU304-treated mice compared to mock treatment (H_2O) or administration of the parental strain (SYN-PKU901), following subcutaneous injection of phenylalanine (* 30% decrease; $p < 0.05$).

Example 16. Phenylalanine degradation activity in vivo (PAL)

[0373] To compare the correlation between in vivo and in vitro phenylalanine activity, SYN-PKU304(containing a low copy plasmin expressing PAL3 with a chromosomal insertion of PfnrS-pheP at the LacZ locus, was compared to SYN-PKU901, a control Nissle strain with streptomycin resistance in vivo).

[0374] Beginning at least 3 days prior to the study (i.e., Days -6 to -3), homozygous BTBR-*Pah*^{enu2} mice (approx. 6-12 weeks of age) were maintained on phenylalanine-free chow and water that was supplemented with 0.5 grams/L phenylalanine. On Day 1, mice were randomized into treatment groups and blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. Mice were also weighed to determine the average weight for each group. Mice were then administered single dose of phenylalanine by subcutaneous injection at 0.1 mg per gram body weight, according to the average group weight. At 30 and 90 min post-injection, the bacteria were administered to mice by oral gavage.

[0375] To prepare the cells, cells were diluted 1:100 in LB (2 L), grown for 1.5 h aerobically, then shifted to the anaerobe chamber for 4 hours. Prior to administration, cells were concentrated 200X and frozen (15% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice, and 4e10 cfu/mL and mixed 9:1 in 1M bicarbonate. Each mouse gavaged 800uL total, or 2.9e10 cfu/mouse.

[0376] Blood samples were collected at 2 hrs and 4 hrs following phenylalanine challenge, and phenylalanine levels in the blood were measured using mass spectrometry, and the change in Phenylalanine concentration per hour was calculated. Results are shown in **Fig. 32**. The total metabolic activity measured was 81.2 umol/hr. and the total reduction in change in phenylalanine was 45% ($P<0.05$). These same cells showed an in vitro activity of 2.8 umol/hr./1e9 cells.

[0377] Additionally, various metabolites were measured to determine whether secondary metabolites can be used as an additional parameter to assess the rate of phenylalanine consumption of the engineered bacteria. When PAH activity is reduced in PKU, the accumulated phenylalanine is converted into PKU specific metabolites phenylpyruvate, which can be further converted into phenyllactic acid. In the presence of the genetically engineered bacteria, phenylalanine is converted by PAL to PAL specific metabolites trans-cinnamic acid, which then can be further converted by liver enzymes to hippuric acid (**Fig. 32**). Blood samples were analyzed for phenylpyruvate, phenyllactate, trans-cinnamic acid, and hippuric acid as described in Example 24-26. Results are shown in **Fig. 32C, 32D, 32E**, and **32F** and are consistent with the phenylalanine degradation shown in **Fig. 32A** and **32B**. For SYN-PKU304, PAL specific metabolites are detected at 4 hours, and moreover, lower levels of PKU specific

metabolites are observed as compared to SYN-PKU901, indicating that PAL phenylalanine degradation may cause a shift away from PKU specific metabolites in favor or PAL specific metabolites.

Example 17. Phenylalanine degradation activity in vivo (PAL)

[0378] SYN-PKU517 (comprising 2 chromosomal insertions of PAL (2XfnrS-PAL (malEK, malPT)), and a chromosomal insertion of pheP (fnrS-pheP (lacZ)), thyA auxotrophy (kan/cm)) was compared to SYN-PKU901.

[0379] Mice were maintained, fed, and administered phenylalanine as described above. To prepare the bacterial cells for gavage, cells were diluted 1:100 in LB (2 L), grown for 1.5 h aerobically, then shifted to the anaerobe chamber for 4 hours. Prior to administration, cells were concentrated 200X and frozen (15% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice, and 4e10 cfu/mL was mixed 9:1 in 1M bicarbonate. Each mouse gavaged 800uL total, or 3.6e10 cfu/mouse.

[0380] As described above, blood samples were collected, and the change in phenylalanine concentration as compared to baseline was calculated. Results are shown in **Fig. 33A** and **33B**. The total metabolic activity measured was 39.6 umol/hr. and the total reduction in change in phenylalanine was 17% ($P<0.05$). These same cells showed an in vitro activity of 1.1 umol/hr./1e9 cells.

[0381] Absolute levels of phenylalanine and of PKU and PAL metabolites are shown in **Fig. 33C**, **33D, 33E**, and **33F** and are consistent with the phenylalanine degradation shown in **Fig. 33A** and **33B**. For SYN-PKU517, PAL specific metabolites were detected at 4 hours, and moreover, lower levels of PKU specific metabolites were observed as compared to SYN-PKU901, indicating that PAL phenylalanine degradation may cause a shift away from PKU specific metabolites in favor or PAL specific metabolites.

[0382] In some embodiments, urine is collected at predetermined time points, and analyzed for phenylalanine levels and levels of PAL and PKU metabolites.

Example 18. Phenylalanine degradation activity in vivo (PAL)

[0383] SYN-PKU705 (comprising 3 chromosomal insertions of PAL (3XfnrS-PAL (malEK, malPT, yicS/nepl)), and 2 chromosomal insertions of pheP (2XfnrS-pheP

(lacZ, agal/rsml)), and LAAD (driven by the ParaBAD promoter integrated within the endogenous arabinose operon) was compared to SYN-PKU901.

[0384] Mice were maintained, fed, and administered phenylalanine as described above. To prepare the bacterial cells for gavage, cells were diluted 1:100 in LB (2 L), grown for 1.5 h aerobically, then shifted to the anaerobe chamber for 4 hours. Prior to administration, cells were concentrated 200X and frozen (15% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice, and 5e10 cfu/mL was mixed 9:1 in 1M bicarbonate. Each mouse gavaged 800uL total, or 3.6e10 cfu/mouse. Note: Though this strain contains the LAAD gene, it was not induced in this study

[0385] As described above, blood samples were collected, and the change in phenylalanine concentration as compared to baseline was calculated. Results are shown in **Fig. 34A**. The total metabolic activity measured was 133.2 umol/hr. and the total reduction in change in phenylalanine was 30% ($P<0.05$). These same cells showed an in vitro activity of 3.7umol/hr./1e9 cells.

[0386] Absolute levels of phenylalanine and of PKU and PAL metabolites are shown in **Fig. 34C**, **34D, 34E**, and **34F** and are consistent with the phenylalanine degradation shown in **Fig. 34A** and **34B**. PAL specific metabolites were detected at 4 hours, and moreover, lower levels of PKU specific metabolites were observed as compared to SYN-PKU901, indicating that PAL phenylalanine degradation may cause a shift away from PKU specific metabolites in favor or PAL specific metabolites. total metabolic activity measured activity was greater than the total metabolic activity measured of the PAL3 plasmid-based strain SYN-PKU304 and the total reduction in phenylalanine approached that of SYN-PKU304 (30% as compared to 45%).

[0387] In some embodiments, urine is collected at predetermined time points, and analyzed for phenylalanine levels and levels of PAL and PKU metabolites.

Example 19. Phenylalanine degradation activity in vivo (PAL) LAAD

[0388] The suitability of *P. proteus* LAAD for phenylalanine degradation by the genetically engineered bacteria is further assessed in vivo. Bacterial strain SYN-PKU401 (comprising a high copy plasmid comprising LAAD driven by a Tet-inducible promoter is compared to SYN-PKU901.

[0389] Mice are maintained, fed, and administered phenylalanine as described above. To prepare the bacterial cells for gavage, cells are diluted 1:100 in LB (2 L), grown for 1.5 h aerobically, then ATC is added and the cells are grown for another 2 hours. Prior to administration, cells are concentrated 200X and frozen for storage. Cells are thawed on ice, and resuspended. Cells are mixed 9:1 in 1M bicarbonate. Each mouse is gavaged four times with 800uL total volume, or with a total of bacteria ranging from 2 $X10^9$ to $1X10^{10}$. Blood samples are collected from the mice described in the previous examples and are analyzed for phenylalanine, phenylpyruvate, phenyllactate, trans-cinnamic acid, and hippuric acid levels. Total reduction in phenylalanine and total metabolic activity are calculated.

Example 20. Effect of pH on phenylalanine degradation in recombinant *E. coli*

[0390] To determine whether the rates of phenylalanine degradation in SYN-PKU304 and SYN-PKU302 are affected by low pH, overnight cultures of both strains were diluted 1:100 in LB and grown with shaking (250 rpm) at 37 °C. After 1.5 hrs of growth, ATC (100 ng/mL) was added to cultures of SYN-PKU302, and SYN-PKU304 cultures were placed in a Coy anaerobic chamber (supplying 90% N_2, 5% CO_2, and 5% H_2). After 4 hrs of induction, bacteria were pelleted, washed in PBS, and resuspended in assay buffer (M9 minimal media with 0.5% glucose, 8.4% sodium bicarbonate, and 4 mM Phe) to a concentration of 5e9 cells/mL. Assay buffer was prepared with incrementally decreasing values of pH, ranging from 7.25-2.25, using 1M HCl. Aliquots were removed from the cell assay every 30 min for 2 hrs for phenylalanine quantification by mass spectrometry. As shown in **Fig. 39**, phenylalanine degradation rates decreased as pH of the assay buffer decreased in both strains, SYN-PKU302 (**FIG. 39A**) and SYN-PKU304 (**FIG. 39B**).

Example 21. Degradation of Dipeptides and Tripeptides

[0391] Overnight strains of SYN-PKU304, and SYN-PKU705 were diluted 1:100 and grown to early log before shifting to anaerobic conditions for induction of PAL and pheP. One culture of SYN-PKU705 was also induced with arabinose to induce the LAAD protein. The focus of this study was to determine if PKU strains could degrade Phe when sequestered in the form of di and tripeptides. After strain

induction Cells were spun down and resuspended in assay buffer containing M9 minimal media, 0.5% glucose, 50mM MOPS, and 50mM of Phe or Phe-containing di- or tri- peptide. Supernatant samples were removed every 20 minutes for a total of 80 minutes, and supernatant was analyzed on a UV-Vis spectrophotometer to measure absorbance at 290nm (the absorption peak for trans-cinnamic acid). Results are shown in **Table 43** indicated that PKU strains were capable of degrading Phe rapidly even in the form of di- and tri-peptides.

Table 43. Dipeptide and Tripeptide Degradation Rates

	Rate (umol TCA produced/hr./1e9 cfu)					
	Phe	**Phe-Val**	**Phe-Ala**	**Gly-Phe**	**Phe-Pro**	**Phe-Gly-Gly**
SYN-PKU304	4.1	3.9	3.5	1.7	1.1	2.0
SYN-PKU705	6.9	5.8	5.0	4.1	1.3	4.5
SYN-PKU705 +ara	4.8	5.8	4.2	2.0	1.4	3.3

Example 22. Engineering bacterial strains using chromosomal insertions

[0392] Bacterial strains, in which the *pheP* and/or *PAL3* genes are integrated directly into the *E. coli* Nissle genome under the control of an FNR-responsive promoter, were constructed. The methods described below may be used for engineering bacterial strains comprising chromosomal insertions (*e.g.*, SYN-PKU902 and/or any of the integrated strains listed in **Table 14**.

[0393] The SYN-PKU902 strain (*lacZ::*P_{fnrS}-*PAL3-pheP*) contains a copy of *PAL3* and a copy of *pheP* integrated at the *lacZ* locus, with both genes operatively linked to a single *fnrS* promoter and co-transcribed in a bicistronic message (**Fig. 41**). **Table 21** shows the sequence of an exemplary construct in which the *PAL3* and *pheP* genes are co-transcribed under the control of an exemplary FNR promoter (SEQ ID NO: 31), with the FNR promoter sequence **bolded**, the *PAL3* sequence boxed, the *pheP* sequence underlined, and ribosomal binding sites highlighted.

[0394] To create a vector capable of integrating the P_{fnrS}-*PAL3-pheP* sequence into the chromosome, Gibson assembly was used to add 1000 bp sequences of DNA homologous to the Nissle *lacZ* locus to both sides of a flippase recombination target (FRT) site-flanked chloramphenicol resistance (cm^R) cassette on a knock-in knock-out

(KIKO) plasmid. Gibson assembly was then used to clone the P_{fnrS}-*PAL3-pheP* DNA sequence between these homology arms, adjacent to the FRT-*cm*^{R}-FRT site. Successful insertion of the fragment was validated by sequencing. PCR was used to amplify the entire *lacZ*::FRT-*cm*^{R}-FRT*::* P_{fnrS}-*PAL3-pheP::lacZ* region. This knock-in PCR fragment was used to transform an electrocompetent Nissle strain that contains a temperature-sensitive plasmid encoding the lambda red recombinase genes. After transformation, cells were grown for 2 hrs at 37 °C. Growth at 37 °C cured the temperature-sensitive plasmid. Transformants with successful chromosomal integration of the fragment were selected on chloramphenicol at 20 μg/mL.

[0395] The SYN-PKU501 strain (*malPT::*P_{fnrS}-*PAL3*, *lacZ*::P_{fnrS}-*pheP*) contains a copy of *PAL3* integrated at the *malP*/*T* locus, and a copy of *pheP* integrated at the *lacZ* locus, with both genes operatively linked to separate *fnrS* promoters (*see* **Table 28**; SEQ ID NO: 38). The SYN-PKU502 strain (*malPT::*P_{fnrS}-PAL3, *lacZ::*P_{fnrS}-PAL3-*pheP*) contains a copy of *PAL3* integrated at the *malP*/*T* locus under the control of an *fnrS* promoter (*see* **Table 28**; SEQ ID NO: 38), as well as a *PAL3-pheP* construct integrated at the *lacZ* locus, wherein both genes at the *lacZ* locus are operatively linked to a single *fnrS* promoter and co-transcribed in a bicistronic message (*see* **Table 21**; SEQ ID NO: 31).

[0396] To create a vector capable of integrating the P_{fnrS}-*PAL3* sequence (SEQ ID NO: 38) into the *E. coli* Nissle chromosome in SYN-PKU501 and SYN-PKU502, Gibson assembly was used to add 1000 bp sequences of DNA homologous to the Nissle *malP* and *malT* loci on either side of an FRT site-flanked kanamycin resistance (*kn*^{R}) cassette on a KIKO plasmid. Gibson assembly was then used to clone the P_{fnrS}-*PAL3* DNA sequence between these homology arms, adjacent to the FRT-*kn*^{R}-FRT site. Successful insertion of the fragment was validated by sequencing. PCR was used to amplify the entire *malP*::FRT-*kn*^{R}-FRT:: P_{fnrS}-*PAL3*::*malT* region. This knock-in PCR fragment was used to transform an electrocompetent Nissle strain already containing P_{fnrS}-*pheP* or bicistronic P_{fnrS}-*PAL3-pheP* in the *lacZ* locus, and expressing the lambda red recombinase genes. After transformation, cells were grown for 2 hrs at 37 °C. Transformants with successful integration of the fragment were selected on kanamycin at 50 μg/mL. These same methods may be used to create a vector capable of integrating

the P_{fnrS}-*PAL3* sequence (SEQ ID NO: 38) at the *malE/K* insertion site in SYN-PKU506 and SYN-PKU507.

[0397] In some embodiments, recombinase-based switches may be used to activate *PAL3* expression. The SYN-PKU601 strain (*malPT::*P_{fnrS}-*Int5*, *rrnBUP-PAL3*; *lacZ*::P_{fnrS}-*pheP*) contains the Int5 recombinase operably linked to a P_{fnrS} promoter, as well as a copy of *PAL3* under the control of a strong constitutive promoter, integrated at the *mal/T* locus (**Fig. 42**). **Table 45** shows the sequence of an exemplary P_{fnrS}-*Int5*, *rrnBUP-PAL3* construct (SEQ ID NO: 42), wherein P_{fnrS}, *Int5*, and *PAL3* are in reverse orientation. The *Int5* sequence is **bolded**, the P_{fnrS} sequence is boxed, the *PAL3* sequence is underlined, and recombinase sites are **bolded** and underlined. Ribosomal binding sites are highlighted, and the *rrnBUP* constitutive promoter sequence is boxed. The UP element-containing *E. coli rrnBUP* promoter was selected to yield high *PAL3* expression (Estrem et al., 1998), although any strong promoter may be used. SYN-PKU601 also contains a copy of *pheP* integrated at the *lacZ* locus.

[0398] To construct the SYN-PKU601 strain, the P_{fnrS}-driven *Int5* gene and the *rrnBUP*-driven, recombinase site-flanked *PAL3* gene sequences were synthesized by Genewiz (Cambridge, MA). Gibson assembly was used to add 1000 bp sequences of DNA homologous to the Nissle *malP* and *malT* loci on either side of the P_{fnrS}-*Int5*, *rrnBUP-PAL3* DNA sequence and to clone this sequence between the homology arms. Successful insertion of the fragment into a KIKO plasmid was validated by sequencing. PCR was used to amplify the entire P_{fnrS}-*Int5*, *rrnBUP-PAL3* region. This knock-in PCR fragment was used to transform an electrocompetent Nissle strain already containing P_{fnrS}-*pheP* in the *lacZ* locus, and expressing the lambda red recombinase genes. After transformation, cells were grown for 2 hrs at 37 °C. Transformants with successful integration of the P_{fnrS}-*PAL3* fragment at the *malPT* intergenic region were selected on kanamycin at 50 μg/mL. This strategy may also be used to construct a recombinase-based strain requiring T7 polymerase activity for *PAL3* expression (**Fig. 43**). [**Table 46** shows the sequence of an exemplary P_{fnrS}-*Int5*, *rrnBUP-T7* construct (SEQ ID NO: 43), wherein P_{fnrS}, *Int5*, and the T7 polymerase gene are in reverse orientation. The *Int5* sequence is **bolded**, the P_{fnrS} sequence is boxed, the T7 polymerase sequence is underlined, and recombinase sites are **bolded** and underlined. Ribosomal binding sites are highlighted, and the *rrnBUP* constitutive promoter

sequence is boxed. **Table 44** shows the sequence of an exemplary P_{T7}-*PAL3* construct, with the P_{T7} sequence highlighted, the ribosome binding site underlined, and the *PAL3* sequence **bolded**.

Table 44

Nucleotide sequences of FNR promoter-PAL3-pheP construct (SEQ ID NO: 41)
ggtaccAGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAAATGGTT GTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACGCCGCAAAGTTTGAGCG AAGTCAATAAACTCTCTACCCATTCAGGGCAATATCTCTCTTggatccaaagtgaac tctagaaataattttgtttaactttaagaaggagatatacatATGAAAGCTAAAGAT GTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTAT GACATTGCGATAAAACAAAAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTTG ACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGA ATCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCA GAGCATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTCC AAACCTTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGG TCTGCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGACATT GTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTA TCTTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAA ATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCC AAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAATC ACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCT GTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAAGTAAAA AATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCA ACGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCAT CAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAATTCGCTGTGCA CCACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGAA CGGGAAGTTATCTCAGCTAATGATAATCCATTGATAGATCCAGAAAATGGCGATGTT CTACACGGTGGAAATTTTATGGGGCAATATGTCGCCCGAACAATGGATGCATTAAAA CTGGATATTGCTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGAT AACCGTTTCTCTCGTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCAA GGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGAT TGTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAATACAATCAAGATATTGTC AGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGCAAT ATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATT AGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGCGAAATCAGTTCT CCTTTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCATTGCGGATGCAATT ATTAATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAGAATAAaagaaggagata tacatatgAAAAACGCGTCAACCGTATCGGAAGATACTGCGTCGAATCAAGAGCCGA CGCTTCATCGCGGATTACATAACCGTCATATTCAACTGATTGCGTTGGGTGGCGCAA TTGGTACTGGTCTGTTTCTTGGCATTGGCCCGGCGATTCAGATGGCGGGTCCGGCTG TATTGCTGGGCTACGGCGTCGCCGGGATCATCGCTTTCCTGATTATGCGCCAGCTTG

Nucleotide sequences of FNR promoter-PAL3-pheP construct (SEQ ID NO: 41)
GCGAAATGGTGGTTGAGGAGCCGGTATCCGGTTCATTTGCCCACTTTGCCTATAAAT ACTGGGGACCGTTTGCGGGCTTCCTCTCTGGCTGGAACTACTGGGTAATGTTCGTGC TGGTGGGAATGGCAGAGCTGACCGCTGCGGGCATCTATATGCAGTACTGGTTCCCGG ATGTTCCAACGTGGATTTGGGCTGCCGCCTTCTTTATTATCATCAACGCCGTTAACC TGGTGAACGTGCGCTTATATGGCGAAACCGAGTTCTGGTTTGCGTTGATTAAAGTGC TGGCAATCATCGGTATGATCGGCTTTGGCCTGTGGCTGCTGTTTTCTGGTCACGGCG GCGAGAAAGCCAGTATCGACAACCTCTGGCGCTACGGTGGTTTCTTCGCCACCGGCT GGAATGGGCTGATTTTGTCGCTGGCGGTAATTATGTTCTCCTTCGGCGGTCTGGAGC TGATTGGGATTACTGCCGCTGAAGCGCGCGATCCGGAAAAAAGCATTCCAAAAGCGG TAAATCAGGTGGTGTATCGCATCCTGCTGTTTTACATCGGTTCACTGGTGGTTTTAC TGGCGCTCTATCCGTGGGTGGAAGTGAAATCCAACAGTAGCCCGTTTGTGATGATTT TCCATAATCTCGACAGCAACGTGGTAGCTTCTGCGCTGAACTTCGTCATTCTGGTAG CATCGCTGTCAGTGTATAACAGCGGGGTTTACTCTAACAGCCGCATGCTGTTTGGCC TTTCTGTGCAGGGTAATGCGCCGAAGTTTTTGACTCGCGTCAGCCGTCGCGGTGTGC CGATTAACTCGCTGATGCTTTCCGGAGCGATCACTTCGCTGGTGGTGTTAATCAACT ATCTGCTGCCGCAAAAAGCGTTTGGTCTGCTGATGGCGCTGGTGGTAGCAACGCTGC TGTTGAACTGGATTATGATCTGTCTGGCGCATCTGCGTTTTCGTGCAGCGATGCGAC GTCAGGGGCGTGAAACACAGTTTAAGGCGCTGCTCTATCCGTTCGGCAACTATCTCT GCATTGCCTTCCTCGGCATGATTTTGCTGCTGATGTGCACGATGGATGATATGCGCT TGTCAGCGATCCTGCTGCCGGTGTGGATTGTATTCCTGTTTATGGCATTTAAAACGC TGCGTCGGAAAtaa

Table 45

Nucleotide sequences of FNR promoter-Int5, rrnBUP-PAL3 construct (SEQ ID NO: 42)
ttaggtacgggctgcccatttgattttaacgcgttcatcaccatcaaacggacgacc acgctggccttttgcaacccaaatttcatcgatgcaggtatcaataattgcattacg catggtcggggttgcacgcagccacagttcttcataatcgctgctatcaacaatcca gctaacatcaactgctgcgcttgcgctgctttcgctaactgcatctttggctgcctg cagggtgctcagtgcttcttgatatgcaggggcaaaaaactgttctgccggaccatc ataaacaccattctgacgatcacgcagcaggcgacccagatttttttcggcttcacg aactgcggcttttgcatacttttcatcttcgcttgcctgcggatgggtcagtgctgc ccagcgatctgcaactgcaataacaaacggatcatccggttcgcttgctgctaattt tgctgcccaacgaaatgcaacatattcttcaacgcttttacgtgcaacataggtcgg tgccggacaaccacctttcacactgctacgccaacaacgataaccattaccgctata gctacagctaccaccacaacccggacaacgcatacgaccgctcagcagatgtttgcg acgggtatcatgatcgctaccatccagcggaacaccaacaccatcttcacctttaac ggctgcttttgcggcttcttgttcttcatcggtcaccagcggaggaccatgcataac gctaacacgtttaccttcaccgttataaaaggtcagacgacgctgtttaccatcctg acgacctgtggtctgccaacccgcatatgccggattctgaatcatatcacgcacggt aactgcaatccacggaccaccggtcgggctcggaatttcacgggtattcattgcatg tgcggtgcctgcatagctcagacgatcggtaaccggcagggtaaaaaccagacgggc tgcttctgctttggtcagaccatcaggaccacccgcatcttcatcatctgctgccag tttacgttcatcatattcatcaccctcttcatcactaacggtaaccagaacaacacg

Nucleotide sequences of FNR promoter-Int5, rrnBUP-PAL3 construct (SEQ ID NO: 42)
cagaccatacggtgcacgggcattaacccattcaccattttcacgctgatgtgcttt ggtatcacgaacacgttcgctcagtttttctgcttcttcgcgtgcttcttctgcacg acgaatcagttcaccgcgatcacgtttattggtgctatccagaaccggacgaccggt atcttcatcccaaccaaacagcagacgacgaggcataccatcttccggttcgataat tttcagaattgcaccggcaccaccacgatcccaacgatccagacgataacaccacag tgcaccaacttcaccgctttccagggctttcagtgctttgctctgatcatcacgtgc tttacctttacgaaaacggcttgcgctaccaacttctttccaaacatgacgaacctg catacccagcagtgctgcaactttacgacccagggtttcttgtgctgcaatgctaat ttcttgtttacgacgctgacctgcaccatttgcacggcttttaactgctttgctttt acgacaaaacaggtcaatcagacctgcaggatccggaccggtttcggtggtcatacc aggcatatgtatatctccttcttaaagttaaacaaaattatttctagagttcacttt ggatccAAGAGAGATATTGCCCTGAATGGGTAGAGAGTTTATTGACTTCGCTCAAAC TTTGCGGCGTTTTTGTATACAGACAGCCGGAAAAATTGCTTTTGTTACAACCATTTA CTACGATGCAACCATAAAGCAACACCACCAATAAGAACAACTggtaccGGATATTCA TATGGACCATGGCAGCTAGCCCTGCAGGGTGCAC TCAGAAAATTATTTTAAATTTC CTCTTGTCAGGCCGGAATAACTCCCTATAATGCGCCACCAC gagcgccggatcagg gagtggacggcctgggagcgctacacgctgtggctgcggtcggtgcTTATTCTTCCA GCATGATTTCTGGCAGAGGAAGTTGATCATTAATAATTGCATCCGCAATGCGGATTA TATCTTCATCCAACGCACGATCAGTGATCAAAGGAGAACTGATTTCGCGTACTGCAT GGTAAAATTTAGCAGTTTCAGGCGCAATTTCACTAATATTGCCGCGAAGATGAATGG CCTGACAAACTACCAGAATTGTCATTGAAACAATATTGCGTAATTTCTGCTCCATCT CTAAAACATCTTGAGCGGCATGCAGACCTAAACTGACAATATCTTGATTGTATTGTT CTGTGGCGAGGGTATGAATACCTGATGCAGCACAATCATGGCGAATTGCAGCAACTA AAGCGGTTTGAGAAAGTTGGACGCCTTTAAAACCTTGATACATGCCGGGTGTCGGAC TCAGTGAATTAGGTAATCCACGAGAGAAACGGTTATCCATCATAAGAGCCACAATGG CGTGAAGATGATTGGCAATTAAAGCAATATCCAGTTTTAATGCATCCATTGTTCGGG CGACATATTGCCCCATAAAATTTCCACCGTGTAGAACATCGCCATTTTCTGGATCTA TCAATGGATTATCATTAGCTGAGATAACTTCCCGTTCCAATATTTTCCGAGCGGTAG CTAAAGATTCTGGCACTATACCTAATACTTGTGGTGCACAGCGAATTGAATAAACTT CCTGTAAGGTATCATTTAGTTGGGTAATTTCTTGATGACGACAAGCTTTATTGGCTT GTTCTTTAACCCCAGATAATAGATTAACCTGCGTTGAACCTGCCAATAAATTACGCA ATGCACTTGCCACCGCGTTTTGACCAGGATGATTTTTTACTTGTTGAATCCGGGCAT CATAATGTTCATGAGATGCAAGTAATGCTTCAACAGCAAGGGCAATCGCAGAAATTG AGGCTTTAAATAGTTTTTCCAGTTTAATGACGGTGATTGCACTGATTCCTGACATTA CCCGGGTGCCGTTAATCAGAGCAAGACCTTCTTTGGCTTTTAACGATAATGGTGTCA ACCCTGCACGTTTAATTGCTTCAGCAGCGTCAATTTCTGCGCCCATATAATAAACTT TGCCGATACCACATAATGCTCGTGCAATATAAGATAAAGGAATTAAATCACCGCTTG CACCCACTGAGCCATAGCGAGGAACCAGAGGAACAATGTCATGATTAATATGATCAA CAATTGCTTGAGCGACAATTGGTCTGGTTGCAGACCAACCTTTGCAAACAGAAAGTA ACATAGTAAATTGTGACGCTTTAATACAAGGTTTGGACATATAGTCCCCAGTACCAG CAGAAAGAAAAGTTAACAGATTTTGCTGATGCTCTGCGATTTTCTCAAATGGCACAA CTAAATTGGCATTCCCTCCAAATCCTGTATTGATTCCATATATAACCTCTCCTGAAT TTAATTTTTCCTCTAATTTTTCACGACCATGCGTCAAAAGTTCAGTGATCTCCGTTG ATATTTCTACTTTTTTTTGTTTTATCGCAATGTCATAGATATCTTCCAAAGAGATAA GGCCATTTTTATTAATAATAATGGTTGGCTGAACATCTTTAGCTTTCATatgtatat ctccttcttaaagttaaacaaaattatttctagag**cagatcagggtgcgcaagttgt**

Nucleotide sequences of FNR promoter-Int5, rrnBUP-PAL3 construct (SEQ ID NO: 42)
caacgctcccaggagagttatcgacttgcgtattaggg

Table 46

Nucleotide sequences of FNR promoter-Int5, rrnBUP-T7 construct (SEQ ID NO: 43)
ttaggtacgggctgcccatttgattttaacgcgttcatcaccatcaaacggacgacc acgctggccttttgcaacccaaatttcatcgatgcaggtatcaataattgcattacg catggtcggggttgcacgcagccacagttcttcataatcgctgctatcaacaatcca gctaacatcaactgctgcgcttgcgctgctttcgctaactgcatctttggctgcctg cagggtgctcagtgcttcttgatatgcaggggcaaaaaactgttctgccggaccatc ataaacaccattctgacgatcacgcagcaggcgacccagatttttttcggcttcacg aactgcggcttttgcatacttttcatcttcgcttgcctgcggatgggtcagtgctgc ccagcgatctgcaactgcaataacaaacggatcatccggttcgcttgctgctaattt tgctgcccaacgaaatgcaacatattcttcaacgcttttacgtgcaacataggtcgg tgccggacaaccacctttcacactgctacgccaacaacgataaccattaccgctata gctacagctaccaccacaacccggacaacgcatacgaccgctcagcagatgtttgcg acgggtatcatgatcgctaccatccagcggaacaccaacaccatcttcacctttaac ggctgcttttgcggcttcttgttcttcatcggtcaccagcggaggaccatgcataac gctaacacgtttaccttcaccgttataaaaggtcagacgacgctgtttaccatcctg acgacctgtggtctgccaacccgcatatgccggattctgaatcatatcacgcacggt aactgcaatccacggaccaccggtcgggctcggaatttcacgggtattcattgcatg tgcggtgcctgcatagctcagacgatcggtaaccggcagggtaaaaaccagacgggc tgcttctgctttggtcagaccatcaggaccacccgcatcttcatcatctgctgccag tttacgttcatcatattcatcaccctcttcatcactaacggtaaccagaacaacacg cagaccatacggtgcacgggcattaacccattcaccattttcacgctgatgtgcttt ggtatcacgaacacgttcgctcagtttttctgcttcttcgcgtgcttcttctgcacg acgaatcagttcaccgcgatcacgtttattggtgctatccagaaccggacgaccggt atcttcatcccaaccaaacagcagacgacgaggcataccatcttccggttcgataat tttcagaattgcaccggcaccaccacgatcccaacgatccagacgataacaccacag tgcaccaacttcaccgctttccagggctttcagtgctttgctctgatcatcacgtgc tttacctttacgaaaacggcttgcgctaccaacttctttccaaacatgacgaacctg catacccagcagtgctgcaactttacgacccagggtttcttgtgctgcaatgctaat ttcttgtttacgacgctgacctgcaccatttgcacggcttttaactgctttgctttt acgacaaaacaggtcaatcagacctgcaggatccggaccggtttcggtggtcatacc aggcatatgtatatctccttcttaaagttaaacaaaattatttctagagttcacttt ggatccAAGAGAGATATTGCCCTGAATGGGTAGAGAGTTTATTGACTTCGCTCAAAC TTTGCGGCGTTTTTGTATACAGACAGCCGGAAAAATTGCTTTTGTTACAACCATTTA CTACGATGCAACCATAAAGCAACACCACCAATAAGAACAACTggtaccGGATATTCA TATGGACCATGGCAGCTAGCCCTGCAGGGTGCAC TCAGAAAATTATTTTAAATTTC CTCTTGTCAGGCCGGAATAACTCCCTATAATGCGCCACCAC gagcgccggatcagg gagtggacggcctgggagcgctacacgctgtggctgcggtcggtgcttacgcgaacg cgaagtccgactctaagatgtcacggaggttcaagttacctttagccggaagtgctg

Nucleotide sequences of FNR promoter-Int5, rrnBUP-T7 construct (SEQ ID NO: 43)

gcattttgtccaattgagactcgtgcaactggtcagcgaactggtcgtagaaatcag
ccagtacatcacaagactcatatgtgtcaaccatagtttcgcgcactgctttgaaca
ggttcgcagcgtcagccggaatggtaccgaaggagtcgtgaatcagtgcaaaagatt
cgattccgtacttctcgtgtgcccacactacagtcttacgaaggtggctaccgtctt
ggctgtgtacaaagttaggagcgataccagactcctgtttgtgtgcatcaatctcgc
tatctttgttggtgttaatggtaggctgtaagcggaactgaccgaggaacatcaggt
tcaagcgcgtctgaataggcttcttgtattcctgccacacagggaaaccatcaggag
ttacccaatgcacagcgcaacgcttgcgaagaatctctccagtcttcttatctttga
cctcagcagccagcagcttagcagcagacttaagccagttcattgcttcaaccgcag
ctaccaccgtcacgctcacagattcccaaatcagcttagccatgtatccagcagcct
gattcggctgagtgaacatcagacccttgccggaatcaatagctggctgaatggtat
cttccagcacttgttgacggaagccgaactctttggacccgtaagccagcgtcatga
ctgaacgcttagtcacactgcgagtaacaccgtaagccagccattgaccagccagtg
ccttagtgcccagcttgactttctcagagatttcaccagtgttctcatcggtcacgg
taactacttcgttatcggtcccattgattgcgtctgcttgtagaatctcgttgactt
tcttagcaacaatcccgtagatgtcctgaacggtttcactaggaagcaagttaaccg
cgcgaccacctacctcatctcggagcatcgcggagaagtgctggatgccagagcaag
acccgtcaaacgccagcggaagggagcagttatagctcaggccgtggtgctgtaccc
cagcgtactcaaagcagaacgcaaggaagcagaacggagaatcttgctcagcccacc
aagtgttctccagtggagacttagcgcaagccatgatgttctcgtggttttcctcaa
tgaacttgatgcgctcagggaacggaaccttatcgacacccgcacagtttgcaccgt
ggattttcagccagtagtaaccttccttaccgattggtttacctttcgccagcgtaa
gcagtcctttggtcatatcgttaccttgcgggttgaacattgacacagcgtaaacac
gaccgcgccagtccatgttgtaagggaaccagatggccttatggttagcaaacttat
tggcttgctcaagcatgaactcaaggctgatacggcgagacttgcgagccttgtcct
tgcggtacacagcagcggcagcacgtttccacgcggtgagagcctcaggattcatgt
cgatgtcttccggtttcatcgggagttcttcacgctcaatcgcagggatgtcctcga
ccggacaatgcttccacttggtgattacgttggcgaccgctaggactttcttgttga
ttttccatgcggtgttttgcgcaatgttaatcgctttgtacacctcaggcatgtaaa
cgtcttcgtagcgcatcagtgctttcttactgtgagtacgcaccagcgccagaggac
gacgaccgttagcccaatagccaccaccagtaatgccagtccacggcttaggaggaa
ctacgcaaggttggaacatcggagagatgccagccagcgcacctgcacggttgcga
tagcctcagcgtattcaggtgcgagttcgatagtctcagagtcttgacctactacgc
cagcattttggcggtgtaagctaaccattccggttgactcaatgagcatctcgatgc
agcgtactcctacatgaatagagtcttccttatgccacgaagaccacgcctcgccac
cgagtagacccttagagagcatgtcagcctcgacaacttgcataaatgctttcttgt
agacgtgccctacgcgcttgttgagttgttcctcaacgtttttcttgaagtgcttag
cttcaaggtcacggatacgaccgaagcgagcctcgtcctcaatggcccgaccgattg
cgcttgctacagcctgaacggttgtattgtcagcactggttaggcaagccagagtgg
tcttaatggtgatgtacgctacggcttccggcttgatttcttgcaggaactggaagg
ctgtcgggcgcttgccgcgcttagctttcacttcctcaaaccagtcgttgatgcgtg
caatcatcttagggagtagggtagtgatgagaggcttggcggcagcgttatccgcaa
cctcaccagctttaagttgacgctcaaacatcttgcggaagcgtgcttcacccatct
cgtaagactcatgctcaagggccaactgttcgcgagctaaacgctcaccgtaatggt
cagccagagtgttgaacgggatagcagccagttcgatgtcagagaagtcgttcttag
cgatgttaatcgtgttcatatgtatatctccttcttaaagttaaacaaaattatttc
tagag**cagatcagggtgcgcaagttgtcaacgctcccaggagagttatcgacttgcg**

Nucleotide sequences of FNR promoter-Int5, rrnBUP-T7 construct (SEQ ID NO: 43)
tattaggg

Table 47

Nucleotide sequences of T7 promoter-PAL3 construct (SEQ ID NO: 44)
taatacgactcactatagggagaaagtgaactctagaaataattttgtttaacttta agaaggagatatacatATGAAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAA AAATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGT AGAAATATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGA AAAATTAAATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGAATGC CAATTTAGTTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTT TCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATT TACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCA AGCAATTGTTGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTC AGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTGG TATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACG TGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACGG CACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGAAAAACTATT TAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCATCTCATGA ACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGGC AAGTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGGGGT TAAAGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATAC CTTACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGA ATCTTTAGCTACCGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGATAA TCCATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGGCA ATATGTCGCCCGAACAATGGATGCATTAAAACTGGATATTGCTTTAATTGCCAATCA TCTTCACGCCATTGTGGCTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTAA TTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAACTTTCTCA AACCGCTTTAGTTGCTGCAATTCGCCATGATTGTGCTGCATCAGGTATTCATACCCT CGCCACAGAACAATACAATCAAGATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGA TGTTTTAGAGATGGAGCAGAAATTACGCAATATTGTTTCAATGACAATTCTGGTAGT TTGTCAGGCCATTCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAA ATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGA TGAAGATATAATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCTGCCAGA AATCATGCTGGAAGAATAA

[0399] To construct the SYN-PKU602 strain comprising P_{ARA}-*Int5* construct, P_{T7}-*PAL3* construct, and P_{Lac}-*T7 polymerase* construct (Fig. 44), Gibson assembly was used essentially as described above.

[0400] **Table 48** shows the sequence of an exemplary P_{ARA}-*Int5* construct (SEQ ID NO: 45), for integration at the Ara locus. The *Int5* sequence is bolded, the P_{ara} sequence containing TSS and RBS sites is underlined, and AraC sequence is *in italics*.

Table 48

Nucleotide Sequence of P_{ARA}-*Int5* construct; SEQ ID NO: 45
TTATTCACAACCTGCCCTAAACTCGCTCGGACTCGCCCCGGTGCATTTTTTAAATACTC
GCGAGAAATAGAGTTGATCGTCAAAACCGACATTGCGACCGACGGTGGCGATAGGCATC
CGGGTGGTGCTCAAAAGCAGCTTCGCCTGACTGATGCGCTGGTCCTCGCGCCAGCTTAA
TACGCTAATCCCTAACTGCTGGCGGAACAAATGCGACAGACGCGACGGCGACAGGCAGA
CATGCTGTGCGACGCTGGCGATATCAAAATTACTGTCTGCCAGGTGATCGCTGATGTAC
TGACAAGCCTCGCGTACCCGATTATCCATCGGTGGATGGAGCGACTCGTTAATCGCTTC
CATGCGCCGCAGTAACAATTGCTCAAGCAGATTTATCGCCAGCAATTCCGAATAGCGCC
CTTCCCCTTGTCCGGCATTAATGATTTGCCCAAACAGGTCGCTGAAATGCGGCTGGTGC
GCTTCATCCGGGCGAAAGAAACCGGTATTGGCAAATATCGACGGCCAGTTAAGCCATTC
ATGCCAGTAGGCGCGCGGACGAAAGTAAACCCACTGGTGATACCATTCGTGAGCCTCCG
GATGACGACCGTAGTGATGAATCTCTCCAGGCGGGAACAGCAAAATATCACCCGGTCGG
CAGACAAATTCTCGTCCCTGATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGAG
AATATAACCTTTCATTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAACCGTTGGCCT
CAATCGGCGTTAAACCCGCCACCAGATGGGCGTTAAACGAGTATCCCGGCAGCAGGGGA
*TCATTTTGCGCTTCAGCCAT*ACTTTTCATACTCCCGCCATTCAGAGAAGAAACCAATTG
TCCATATTGCATCAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTCGCTAACCC
AACCGGTAACCCCGCTTATTAAAAGCATTCTGTAACAAAGCGGGACCAAAGCCATGACA
AAAACGCGTAACAAAAGTGTCTATAATCACGGCAGAAAAGTCCACATTGATTATTTGCA
CGGCGTCACACTTTGCTATGCCATAGCATTTTTATCCATAAGATTAGCGGATCCAGCCT
GACGCTTTTTTTCGCAACTCTCTACTGTTTCTCCATACCTCTAGAAATAATTTTGTTTA
ACTTTAAGAAGGAGATATACAT**ATGCCTGGTATGACCACCGAAACCGGTCCGGATCCTG**
CAGGTCTGATTGACCTGTTTTGTCGTAAAAGCAAAGCAGTTAAAAGCCGTGCAAATGGT
GCAGGTCAGCGTCGTAAACAAGAAATTAGCATTGCAGCACAAGAAACCCTGGGTCGTAA
AGTTGCAGCACTGCTGGGTATGCAGGTTCGTCATGTTTGGAAAGAAGTTGGTAGCGCAA
GCCGTTTTCGTAAAGGTAAAGCACGTGATGATCAGAGCAAAGCACTGAAAGCCCTGGAA
AGCGGTGAAGTTGGTGCACTGTGGTGTTATCGTCTGGATCGTTGGGATCGTGGTGGTGC
CGGTGCAATTCTGAAAATTATCGAACCGGAAGATGGTATGCCTCGTCGTCTGCTGTTTG
GTTGGGATGAAGATACCGGTCGTCCGGTTCTGGATAGCACCAATAAACGTGATCGCGGT
GAACTGATTCGTCGTGCAGAAGAAGCACGCGAAGAAGCAGAAAAACTGAGCGAACGTGT
TCGTGATACCAAAGCACATCAGCGTGAAAATGGTGAATGGGTTAATGCCCGTGCACCGT
ATGGTCTGCGTGTTGTTCTGGTTACCGTTAGTGATGAAGAGGGTGATGAATATGATGAA
CGTAAACTGGCAGCAGATGATGAAGATGCGGGTGGTCCTGATGGTCTGACCAAAGCAGA
AGCAGCCCGTCTGGTTTTTACCCTGCCGGTTACCGATCGTCTGAGCTATGCAGGCACCG
CACATGCAATGAATACCCGTGAAATTCCGAGCCCGACCGGTGGTCCGTGGATTGCAGTT
ACCGTGCGTGATATGATTCAGAATCCGGCATATGCGGGTTGGCAGACCACAGGTCGTCA
GGATGGTAAACAGCGTCGTCTGACCTTTTATAACGGTGAAGGTAAACGTGTTAGCGTTA
TGCATGGTCCTCCGCTGGTGACCGATGAAGAACAAGAAGCCGCAAAAGCAGCCGTTAAA
GGTGAAGATGGTGTTGGTGTTCCGCTGGATGGTAGCGATCATGATACCCGTCGCAAACA
TCTGCTGAGCGGTCGTATGCGTTGTCCGGGTTGTGGTGGTAGCTGTAGCTATAGCGGTA
ATGGTTATCGTTGTTGGCGTAGCAGTGTGAAAGGTGGTTGTCCGGCACCGACCTATGTT
GCACGTAAAAGCGTTGAAGAATATGTTGCATTTCGTTGGGCAGCAAAATTAGCAGCAAG
CGAACCGGATGATCCGTTTGTTATTGCAGTTGCAGATCGCTGGGCAGCACTGACCCATC

CGCAGGCAAGCGAAGATGAAAAGTATGCAAAAGCCGCAGTTCGTGAAGCCGAAAAAAAT CTGGGTCGCCTGCTGCGTGATCGTCAGAATGGTGTTTATGATGGTCCGGCAGAACAGTT TTTTGCCCCTGCATATCAAGAAGCACTGAGCACCCTGCAGGCAGCCAAAGATGCAGTTA GCGAAAGCAGCGCAAGCGCAGCAGTTGATGTTAGCTGGATTGTTGATAGCAGCGATTAT GAAGAACTGTGGCTGCGTGCAACCCCGACCATGCGTAATGCAATTATTGATACCTGCAT CGATGAAATTTGGGTTGCAAAAGGCCAGCGTGGTCGTCCGTTTGATGGTGATGAACGCG TTAAAATCAAATGGGCAGCCCGTACCTAA

Example 23. Generation of DeltaThyA

[0401] An auxotrophic mutation causes bacteria to die in the absence of an exogenously added nutrient essential for survival or growth because they lack the gene(s) necessary to produce that essential nutrient. In order to generate genetically engineered bacteria with an auxotrophic modification, the *thyA*, a gene essential for oligonucleotide synthesis was deleted. Deletion of the *thyA* gene in *E. coli* Nissle yields a strain that cannot form a colony on LB plates unless they are supplemented with thymidine.

[0402] A *thyA::cam* PCR fragment was amplified using 3 rounds of PCR as follows. Sequences of the primers used at a 100um concentration are found in Table 49.

Table 49. Primer Sequences

Name	Sequence	Description	SEQ ID NO
SR36	tagaactgatgcaaaaagtgctcgacgaaggcacacagaTGTGTAGGCTGGAGCTGCTTC	Round 1: binds on pKD3	**SEQ ID NO: 46**
SR38	gtttcgtaattagatagccaccggcgctttaatgcccggaCATATGAATATCCTCCTTAG	Round 1: binds on pKD3	**SEQ ID NO: 47**
SR33	caacacgtttcctgaggaaccatgaaacagtatttagaactgatgcaaaaag	Round 2: binds to round 1 PCR product	**SEQ ID NO: 48**
SR34	cgcacactggcgtcggctctggcaggatgtttcgtaattagatagc	Round 2: binds to round 1 PCR product	**SEQ ID NO: 49**
SR43	atatcgtcgcagcccacagcaacacgtttcctgagg	Round 3: binds to round 2 PCR product	**SEQ ID NO: 50**
SR44	aagaatttaacggagggcaaaaaaaaccgacgcacactggcgtcggc	Round 3: binds to round 2 PCR product	**SEQ ID NO: 51**

[0403] For the first PCR round, 4x50ul PCR reactions containing 1ng pKD3 as template, 25ul 2xphusion, 0.2ul primer SR36 and SR38, and either 0, 0.2, 0.4 or 0.6ul

DMSO were brought up to 50 ul volume with nuclease free water and amplified under the following cycle conditions:

[0404] step1: 98c for 30s

[0405] step2: 98c for 10s

[0406] step3: 55c for 15s

[0407] step4: 72c for 20s

[0408] repeat step 2-4 for 30 cycles

[0409] step5: 72c for 5min

[0410] Subsequently, 5ul of each PCR reaction was run on an agarose gel to confirm PCR product of the appropriate size. The PCR product was purified from the remaining PCR reaction using a Zymoclean gel DNA recovery kit according to the manufacturer's instructions and eluted in 30ul nuclease free water.

[0411] For the second round of PCR, 1ul purified PCR product from round 1 was used as template, in 4x50ul PCR reactions as described above except with 0.2ul of primers SR33 and SR34. Cycle conditions were the same as noted above for the first PCR reaction. The PCR product run on an agarose gel to verify amplification, purified, and eluted in 30ul as described above.

[0412] For the third round of PCR, 1ul of purified PCR product from round 2 was used as template in 4x50ul PCR reactions as described except with primer SR43 and SR44. Cycle conditions were the same as described for rounds 1 and 2. Amplification was verified, the PCR product purified, and eluted as described above. The concentration and purity was measured using a spectrophotometer. The resulting linear DNA fragment, which contains 92 bp homologous to upstream of *thyA*, the chloramphenicol cassette flanked by frt sites, and 98 bp homologous to downstream of the *thyA* gene, was transformed into a *E. coli* Nissle 1917 strain containing pKD46 grown for recombineering. Following electroporation, 1ml SOC medium containing 3mM thymidine was added, and cells were allowed to recover at 37 C for 2h with shaking. Cells were then pelleted at 10,000xg for 1 minute, the supernatant was discarded, and the cell pellet was resuspended in 100ul LB containing 3mM thymidine and spread on LB agar plates containing 3mM thy and 20ug/ml chloramphenicol. Cells

were incubated at 37 C overnight. Colonies that appeared on LB plates were restreaked. + cam 20ug/ml + or - thy 3mM. (*thyA* auxotrophs will only grow in media supplemented with thy 3mM).

[0413] Next, the antibiotic resistance was removed with pCP20 transformation. pCP20 has the yeast Flp recombinase gene, FLP, chloramphenicol and ampicillin resistant genes, and temperature sensitive replication. Bacteria were grown in LB media containing the selecting antibiotic at 37°C until OD600 = 0.4 - 0.6. 1mL of cells were washed as follows: cells were pelleted at 16,000xg for 1 minute. The supernatant was discarded and the pellet was resuspended in 1mL ice-cold 10% glycerol. This wash step was repeated 3x times. The final pellet was resuspended in 70ul ice-cold 10% glycerol. Next, cells were electroporated with 1ng pCP20 plasmid DNA, and 1mL SOC supplemented with 3mM thymidine was immediately added to the cuvette. Cells were resuspended and transferred to a culture tube and grown at 30°C for 1hours. Cells were then pelleted at 10,000xg for 1 minute, the supernatant was discarded, and the cell pellet was resuspended in 100ul LB containing 3mM thymidine and spread on LB agar plates containing 3mM thy and 100ug/ml carbenicillin and grown at 30°C for 16-24 hours. Next, transformants were colony purified non-selectively (no antibiotics) at 42°C.

[0414] To test the colony-purified transformants, a colony was picked from the 42°C plate with a pipette tip and resuspended in 10μL LB. 3μL of the cell suspension was pipetted onto a set of 3 plates: Cam, (37°C; tests for the presence/absence of CamR gene in the genome of the host strain), Amp, (30°C, tests for the presence/absence of AmpR from the pCP20 plasmid) and LB only (desired cells that have lost the chloramphenicol cassette and the pCP20 plasmid), 37°C. Colonies were considered cured if there is no growth in neither the Cam or Amp plate, picked, and re-streaked on an LB plate to get single colonies, and grown overnight at 37°C.

Example 24. Phenylalanine quantification (dansyl-chloride derivatization)

[0415] For in vitro and in vivo assays described herein, which assess the ability of the genetically engineered bacteria to degrade phenylalanine and which require quantification of phenylalanine levels in the sample, a dansyl-chloride derivatization protocol was employed as follows.

Sample Preparation

[0416] Phenylalanine standards (1000, 500, 250, 100, 20, 4 and 0.8μg/mL in water) were prepared. On ice, 10μL of sample was pipetted into a V-bottom polypropylene 96-well plate, and 190μL of 60% acetonitrile with 1ug/mL of L-Phenyl-d_5-alanine internal standard was added. The plate was heat sealed, mixed well, and centrifuged at 4000rpm for 5min. Next, 5μL of diluted samples were added to 95μL of derivatization mix (85μL 10mM $NaHCO_3$ pH 9.7 and 10μL 10mg/mL dansyl-chloride (diluted in acetonitrile)) in a V-bottom 96-well polypropylene plate, and the plate was heat-sealed and mixed well. The samples were incubated at 60°C for 45min for derivatization and then centrifuged at 4000rpm for 5 minutes. Next, 20μL of the derivatized samples were added to 180μL of water with 0.1% formic acid in a round-bottom 96-well plate, plates were heat-sealed and mixed well.

LC-MS/MS method

[0417] Phenylalanine was measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. HPLC Method details are described in **Table 50** and **Table 51**. **Tandem Mass Spectrometry details** are described in **Table 52**.

Table 50. HPLC Method Details

Column	Luna C18(2) column, 5 μm (50 x 2.1 mm)
Mobile Phase A	100% H2O, 0.1% Formic Acid
Mobile Phase B	100% ACN, 0.1% Formic Acid
Injection volume	10uL

Table 51. HPLC Method Details

Total Time (min)	Flow Rate (μL/min)	A%	B%
0	400	90	10
0.5	400	90	10
0.6	400	10	90
2	400	10	90
2.01	400	90	10
3	400	90	10

Table 52. Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM transitions	
L-Phenylalanine	399.1/170.1
L-Phenyl-d5-alanine	404.1/170.1

Example 25 Trans-cinnamic acid quantification (trifluoroethylamine derivatization)

[0418] For in vitro and in vivo assays described herein, which assess the ability of the genetically engineered bacteria to degrade phenylalanine and which require quantification of Trans-cinnamic acid levels in the sample, a trifluoroethylamine derivatization protocol was employed as follows.

Sample preparation

[0419] Trans-cinnamic acid standard (500, 250, 100, 20, 4 and 0.8µg/mL in water) were prepared. On ice, 10µL of sample was pipetted into a V-bottom polypropylene 96-well plate. Next, 30µL of 80% acetonitrile with 2ug/mL of trans-cinnamic acid-d7 internal standard was added, and the plate was heat sealed, mixed well, and centrifuged at 4000rpm for 5 minutes. Next, 20µL of diluted samples were added to 180µL of 10mM MES pH4, 20mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), 20mM trifluoroethylamine in a round-bottom 96-well polypropylene plate. The plate was heat-sealed, mixed well, and samples were incubated at room temperature for 1 hour.

LC-MS/MS method

[0420] Trans-cinnamic acid was measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. HPLC Method details are described in **Table 53** and **Table 54**. Tandem Mass Spectrometry details are described in **Table 55**.

Table 53. HPLC Method Details

Column	Thermo Aquasil C18 column, 5 µm (50 x 2.1 mm)

Mobile Phase A	100% H2O, 0.1% Formic Acid
Mobile Phase B	100% ACN, 0.1% Formic Acid
Injection volume	10uL

Table 54. HPLC Method Details

Total Time (min)	Flow Rate (μL/min)	A%	B%
0	500	100	0
1	500	100	0
2	500	10	90
4	500	10	90
4.01	500	100	0
5	500	100	0

Table 55. Tandem Mass Spectrometry Details

Ion Source:	HESI-II
Polarity	Positive
SRM transitions	
Trans-cinnamic acid:	230.1/131.1
Trans-cinnamic acid-d7	237.1/137.2

Example 26. Phenylalanine, trans-cinnamic acid, phenylacetic acid, phenylpyruvic acid, phenyllactic acid, hippuric acid and benzoic acid quantification (2-Hydrazinoquinoline derivatization)

[0421] For in vitro and in vivo assays described herein, which assess the ability of the genetically engineered bacteria to degrade phenylalanine and which require quantification of phenylalanine, trans-cinnamic acid, phenylacetic acid, phenylpyruvic acid, phenyllactic acid, hippuric acid, and benzoic acid levels in the sample, a 2-Hydrazinoquinoline derivatization protocol was employed as follows

Sample preparation

[0422] Standard solutions containing 250, 100, 20, 4, 0.8, 0.16 and 0.032 μg/mL of each standard in water were prepared. On ice, 10μL of sample was pipetted into a V-bottom polypropylene 96-well plate, and 90μL of the derivatizing solution containing 50mM of 2-Hydrazinoquinoline (2-HQ), dipyridyl disulfide, and triphenylphospine in acetonitrile with 1ug/mL of L-Phenyl-d_5-alanine, 1ug/mL of hippuric acid-d5 and

0.25ug/mL trans-cinnamic acid-d7 internal standards was added. The plate was heat-sealed, mixed well, and samples were incubated at 60°C for 1 hour for derivatization, and then centrifuged at 4000rpm for 5min. In a round-bottom 96-well plate, 20µL of the derivatized samples were added to 180µL of water with 0.1% formic acid. Plates were heat-sealed and mixed well.

LC-MS/MS method

[0423] Metabolites derivatized by 2-HQ were measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. HPLC details are described in **Table 56** and **Table 57**. Tandem Mass Spectrometry details are described in **Table 58**.

Table 56. HPLC Method Details

Column	Luna C18(2) column, 3 µm (150 x 2.1 mm)
Mobile Phase A	100% H2O, 0.1% Formic Acid
Mobile Phase B	100% ACN, 0.1% Formic Acid
Injection volume	10 uL

Table 57. HPLC Method Details

Total Time (min)	Flow Rate (µL/min)	A%	B%
0	500	90	10
0.5	500	90	10
2	500	10	90
4	500	10	90
4.01	500	90	10
4.25	500	90	10

Table 58.Tandem Mass Spectrometry Details

Ion Source	HESI-II
Polarity	Positive
SRM transitions	
L-Phenylalanine:	307.1/186.1
L-Phenyld5-alanine	312.1/186
Trans-cinnamic acid	290.05/131.1
Trans-cinnamic acid-d7	297.05/138.1
Hippuric acid	321.1/160.1

Hippuric acid-d5	326/160
Phenylacetic acid	278.05/160.1
Phenyllactic acid	308.05/144.1
Benzoic acid	264.05/105.1
Phenylpyruvate	306.05/260.1

Example 27. Relative efficacy of chromosomal insertion and plasmid-bearing strains

[0424] To compare the rate of phenylalanine degradation between engineered bacterial strains with chromosomal insertions and those harboring plasmids, overnight cultures were diluted 1:100 in LB and grown with shaking (250 rpm) at 37 °C. After 1.5 hrs of growth, cultures were placed in a Coy anaerobic chamber supplying 90% N_2, 5% CO_2, 5% H_2. After 4 hrs of induction, bacteria were pelleted, washed in PBS, and resuspended in assay buffer (M9 minimal media with 0.5% glucose, 8.4% sodium bicarbonate, and 4 mM Phe). Rates of phenylalanine degradation (*i.e.*, disappearance from the assay solution) or cinnamate accumulation from 30 to 90 min were normalized to 1e9 cells. **Table 59** shows the normalized rates for all strains and describes genotypes and the activities of non-limiting examples of engineered plasmid-bearing strains and engineered strains comprising chromosomal insertions.

Table 59. Genotype and Activity of engineered plasmid-bearing strains and engineered strains comprising chromosomal insertions.

Strain Name	**Genotype**	**PAL Activity** (umol/hr./10^9 cells)	**LAAD activity** (umol/hr./10^9 cells)
Plasmid -based strains			
SYN-PKU101	Low copy pSC101-P*tet*::*PAL1*, ampicillin resistant	ND	NA
SYN-PKU102	High copy pColE1-P*tet*::*PAL1*, ampicillin resistant,	ND	NA
SYN-PKU201	Low copy pSC101-P*tet*::*PAL3*, ampicillin resistant	ND	NA
SYN-PKU202	High copy pColE1-P*tet*::*PAL3*, ampicillin resistant,	ND	NA
SYN-PKU203	*lacZ*::P*tet-pheP*::*cam*	0	NA
SYN-PKU401	Low copy pSC101-P*tet*::*PAL1*, ampicillin resistant, chromosomal *lacZ*::P*tet-pheP*::*cam*	1.1	NA

SYN-PKU402	High copy pColE1-P*tet*::*PAL1*, ampicillin resistant, chromosomal *lacZ*::P*tet-pheP*::*cam*	0.8	NA
SYN-PKU302	Low Copy pSC101-P*tet*::*PAL3*, ampicillin resistant; chromosomal *lacZ*::P*tet-pheP*::*cam*	2.2	NA
SYN-PKU303	High copy pColE1-P*tet*::*PAL3*, ampicillin resistant, chromosomal *lacZ*::P*tet-pheP*::*cam*	7.1	NA
SYN-PKU304	Low Copy pSC101-*PfnrS*::*PAL3*, ampicillin resistant; chromosomal *lacZ*::*PfnrS-pheP*::*cam*	3	NA
SYN-PKU305	Low Copy pSC101-*PfnrS*::*PAL3*, kanamycin resistant; chromosomal *lacZ*::P*fnrS-pheP*::*cam*	3	NA
SYN-PKU306	Low Copy pSC101-*PfnrS*::*PAL3*, kanamycin resistant; *thyA*	0.3	NA
SYN-PKU307	Low Copy pSC101-*PfnrS*::*PAL3*, ampicillin resistant;	0.3	NA
SYN-PKU308	Low Copy pSC101-*PfnrS*::*PAL3*, kanamycin resistant;	0.3	NA
SYN-PKU401	High Copy pUC57-P*tet*::*LAAD;* kanamycin resistant	NA	50 ($^{+}O_2$),0($^{-}O_2$)
Integrated strains			
SYN-PKU501	*malPT*:: *PfnrS*::*PAL3*::*kan*	0.3	NA
SYN-PKU502	*malPT*:: *PfnrS*::*PAL3*::*kan*; bicistronic *lacZ*:: *PfnrS*::*PAL3-pheP*::*cam*	ND	NA
SYN-PKU503	*malEK*::*PfnrS*::*PAL3*::*cam*	0.3	NA
SYN-PKU504	*agaI/rsmI*::*PfnrS*::*PAL3*	0.3	NA
SYN-PKU505	*cea*::*PfnrS*::*PAL3*	0.3	NA
SYN-PKU506	*malEK*::*PfnrS*::*PAL3*; *agaI/rsmI*::*PfnrS*::*PAL3; cea*::*PfnrS*::*PAL3*	0.7	NA
SYN-PKU507	*malEK*:: *PfnrS*::*PAL3*; *agaI/rsmI*::*PfnrS*::*PAL3; cea*::*PfnrS*::*PAL3*; *lacZ*::P*fnr-pheP*::*cam*	5.2	NA
SYN-PKU508	*malEK*::*PfnrS*::*PAL3*; *pheA* auxotroph	0.4	NA
SYN-PKU509	*malEK*::*PfnrS*::*PAL3*; *agaI/rsmI*::*PfnrS*::*PAL3; cea*::*PfnrS*::*PAL3; lacZ*::P*fnr-pheP*::cam	4.9	NA
SYN-PKU601	*malPT*::P*fnrS*-INT5::kan, *rrnBUP* - [PAL3]; *lacZ*::P*fnr-pheP*::*cam* (recombinase based strain)	0.9	NA
SYN-PKU510	*malEK*::*PfnrS*::*PAL3*; *agaI/rsmI*::*PfnrS*::*PAL3; cea*::*PfnrS*::*PAL3;*	0.6	NA
SYN-PKU511	*malEK*:: *PfnrS*::*PAL3*;	7.7	NA

	*agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; yicS/nepI::*P*fnrS-PAL3::kan; malPT::PfnrS::*PAL3; *lacZ::*P*fnr-pheP; ΔthyA*		
SYN-PKU204	*lacZ::*P*fnr-pheP::cam*	ND	NA
SYN-PKU512	*malEK::PfnrS::PAL3*; *agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; malPT::PfnrS::*PAL3; *lacZ::*P*fnr-pheP::cam; ΔthyA*	6.7	NA
SYN-PKU513	*malEK:: PfnrS::PAL3*; *agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; lacZ::*P*fnr-pheP; ΔthyA*	4.9	NA
SYN-PKU514	*malEK:: PfnrS::PAL3*; *agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; malPT::PfnrS::*PAL3; *ΔthyA*	0.8	NA
SYN-PKU515	*malEK:: PfnrS::PAL3*; *agaI/rsmI::PfnrS::PAL3; cea::PfnrS::PAL3; ΔthyA*	0.7	NA
SYN-PKU516	*agaI/rsmI::PfnrS::PAL3::kan*	0.3	NA
SYN-PKU517	*malEK:: PfnrS::PAL3*::cam; *malPT::PfnrS::*PAL3::kan; *lacZ::*P*fnrS-pheP; ΔthyA*	2.9	NA
SYN-PKU518	*malEK-*P*fnrS::PAL3::cam; PfnrS::pheP::kan*	1.7	NA
SYN-PKU519	*ParaBC-PAL3::cam; PfnrS-pheP::kan*	1.3	NA
SYN-PKU520	*agaI/rsmI::PfnrS::PAL3::kan*; P*fnrS-PheP::cam*	2.0	NA
SYN-PKU801	*ΔargR*; *thyA::cam*	ND	NA
SYN-PKU701	*ParaBC-LAAD::cam; malEK-*P*fnrS-PAL3; malPT::PfnrS-*PAL3::kan; *PfnrS-pheP*	2.7	28 ($^{+}O_2$),0 ($^{-}O_2$)
SYN-PKU521	*yicS/nepI::*P*fnrS-PAL3::kan; lacZ::*P*fnr-pheP::cam*	2.4	NA
SYN-PKU522	*cea::*P*fnrS-PAL3::kan; lacZ::*P*fnr-pheP::cam*	ND	NA
SYN-PKU523	*malPT::*P*fnrS-PAL3::kan; lacZ::*P*fnr-pheP::cam*	0.5	NA
SYN-PKU524	*malEK:: PfnrS::PAL3*; *malPT::PfnrS::*PAL3; *lacZ::*P*fnr-pheP*	2.9	NA
SYN-PKU702	*malEK:: PfnrS::PAL3*; *lacZ::*P*fnr-pheP; Para::LAAD*	1.5	ND
SYN-PKU703	*malEK:: PfnrS::PAL3*; *malPT::PfnrS::*PAL3; *lacZ::*P*fnr-pheP; agaI/rsmI::PfnrS::pheP; Para::LAAD*	3.1	ND
SYN-PKU704	*malEK:: PfnrS::PAL3*;	3.5	ND

	*malPT::PfnrS::*PAL3; *yicS/nepI::*P*fnrS-PAL3; lacZ::*P*fnr-pheP; Para::LAAD*		
SYN-PKU705	*malEK:: PfnrS::PAL3; malPT::PfnrS::*PAL3; *yicS/nepI::*P*fnrS-PAL3::kan; lacZ::*P*fnr-pheP; agaI/rsmI::PfnrS::pheP Para::LAAD*	3.7	ND
SYN-PKU602	*malEK::* P*T7::PAL3; Para::INT5::cam (recombinase); lacZ::*P*fnr-pheP; malPT::*P*constitutive::*T7 polymerase (unflipped);	2.4	NA
SYN-PKU901	*Nissle with streptomycin resistance*	NA	NA

Example 28. Screening for Improved Phe consumption

[0425] Screens using genetic selection are conducted to improve phenylalanine consumption in the genetically engineered bacteria. Toxic phenylalanine analogs exert their mechanism of action (MOA) by being incorporated into cellular protein, causing cell death. These compounds were evaluated for their utility in an untargeted approach to select PAL enzymes with increased activity. Assuming that these toxic compounds can be metabolized by PAL into a non-toxic metabolite, rather than being incorporated into cellular protein, genetically engineered bacteria which have improved phenylalanine degradation activity can tolerate higher levels of these compounds, and can be screened for and selected on this basis.

[0426] Various genetically engineered bacterial strains as well as control Nissle were treated with two analogs, p-fluoro-DL-minimum phenylalanine and o-fluoro-DL-phenylalanine (**Fig. 35**) at increasing concentrations. Minimum inhibitory concentration (MIC) was determined and the fold change relative to the wild type Nissle was determined. Results are shown in Table 60.

[0427] These results indicate that the para-analog appear to be taken up readily by pheP and are potentially a substrate of PAL, and that the ortholog appears to be taken up readily by pheP and is potentially a substrate of PAL. As a result, these compounds have utility for screening for PAL enzymes with greater activity.

Table 60. MIC and Fold Change Relative to WT for various strains

para-fluoro-Phe		
MIC (ug/mL)	**fold change (WT)**	**Strain**

1250	1	Wild Type Nissle
<2.4	>↓520X	SYN-PKU203 (Ptet::pheP chr.)
2500	↑2X	SYN-PKU202 (Ptet-PAL3 high copy)
19.5	↓64X	SYN-PKU302 (Ptet-PAL low copy + Ptet-pheP chr.)
39	↓32X	SYN-PKU303 (Ptet-PAL high copy + Ptet-pheP chr.)
ortho-fluoro-Phe		
MIC (ug/mL)	**fold change (WT)**	**Strain**
62.5	1	Wild Type Nissle
1	↓64X	SYN-PKU203 (Ptet::pheP chr.)
250	↑4X	SYN-PKU202 (Ptet-PAL3 high copy)
31.3	↓2X	SYN-PKU302 (Ptet-PAL low copy + Ptet-pheP chr.)
15.6	↓4X	SYN-PKU303 (Ptet-PAL high copy + Ptet-pheP chr.)

Example 29. Repeat-Dose Pharmacokinetic and Pharmacodynamic Study of Genetically Engineered Bacteria Following Daily Nasogastric Gavage Dose Administration for 28-days in Cynomolgus Monkeys (non-GLP)

[0428] To evaluate any potential toxicities arising from administration of the genetically engineered bacteria or E coli Nissle alone, the pharmacokinetics and pharmacodynamics of the genetically engineered bacteria and an E. coli Nissle are studied following daily nasogastric gavage (NG) dose administration for 28-days to female cynomolgus monkeys. Cynomolgus monkeys is selected because this species is closely related, both phylogenetically and physiologically, to humans and is a species commonly used for nonclinical toxicity evaluations. The genetically engineered bacteria are administered by nasal gastric gavage, consistent with the proposed route of administration in humans. Animals overall well-being (clinical observations), weight clinical pathology (serum chemistry, hematology, and coagulation) are tracked. Plasma is analyzed for ammonia levels, and fecal samples examined for bacterial load.

[0429] The genetically engineered strain comprises one or more copies of PAL3 integrated into the chromosome and one or more copies of PheP integrated into the chromosome, each of which are under the control of an FNRS promoter. In some embodiments, the genetically engineered strain also comprises one or more copies of

LAAD, driven by an arabinose inducible promoter, e.g., ParaBAD. In some embodiments, the strain further comprises a auxotrophy mutation, e.g., deltaThyA. In some embodiments, the genetically engineered bacteria further comprise an antibiotic resistance, e.g., kanamycin. In some embodiments, the genetically engineered bacteria do not comprise an auxotrophy mutation. In some embodiments, the genetically engineered bacteria do not comprise an antibiotic resistance.

Materials, animals and dosing regimen:

[0430] The study is conducted in compliance with nonclinical Laboratory Studies Good Laboratory Practice Regulations issued by the U.S. Food and Drug Administration (Title 21 of the Code of Federal Regulations, Part 58; effective June 20, 1979) and the OECD Principles on Good Laboratory Practice (C [97]186/Final; effective 1997). The animals are individually housed based on the recommendations set forth in the Guide for the Care and Use of Laboratory Animals (National Research Council 2011).

[0431] Animals used in the study are Female Purpose-bred, non-naive cynomolgus monkey (Macaca fascicularis) with 3 to 6 kg (at initial physical exam) 3 to 8 years (at initial physical exam) of age (SNBL USA stock, Origin: Cambodia).

[0432] For the duration of the study, animals are offered PMI LabDiet® Fiber-Plus® Monkey Diet 5049 biscuits twice daily. Animal are fasted for at least 2 hours prior to dose administration and fed within 1-hour post dose. Animals also are fasted as required by specific procedures (e.g., prior to blood draws for serum chemistry, fecal collection). The diet is routinely analyzed for contaminants and found to be within manufacturer's specifications. No contaminants are expected to be present at levels that would interfere with the outcome of the study. Food analysis records are maintained in the testing facility records.

[0433] Fresh drinking water is provided *ad libitum* to all animals. The water is routinely analyzed for contaminants. No contaminants are present at levels that would interfere with the outcome of the study. Animals are given fruits, vegetables, other dietary supplements, and cage enrichment devices throughout the course of the study.

[0434] Previously quarantined animals are acclimated to the study room for 7 days prior to initiation of dosing (day 1). The last dosing occurs on day 28. A stratified

randomization scheme incorporating body weights is used to assign animals to study groups. Animals are assigned to groups and treated as indicated in **Table 61**.

Table 61. Group Assignments

Group	Dose				Flu		Number of Females
	Test/ Control Articles	Dose Level (cfu/Animal)	Conc. (cfu/mL)	Volume (mL/ Animal)	Bicarb. Conc. (M)	Volume (mL/ Animal)	
1	Control Article	0	0	10	0.36	5	3
2	E coli Nissle	1×10^9	1×10^9	1	0.12	14	3
3	E coli Nissle	1×10^{12}	1×10^{11}	10	0.36	5	3
4	Genetically engineered bacteria	1×10^9	1×10^9	1	0.12	14	3
5	Genetically engineered bacteria	1×10^{12}	1×10^{11}	10	0.36	5	3

[0435] Nissle control and genetically engineered bacterial stocks are prepared at 1×109 cfu/mL and 1×1011 cfu/mL in 15% glycerol in 1X PBS with 2.2% glucose and 3 mM thymidine and are kept at 86 to -60 °C (see **Table 61**). PBS made in 20% glycerol with sodium bicarbonate is used as a control vehicle. Carbonate concentration is 0.36M and 0.12M for sodium bicarbonate (see table XXX). On the day of each dosing, bacteria and vehicle control are removed from the freezer and put on ice and thawed and placed on ice until dosing.

[0436] Animals are dosed at 0, 1×10^9, or 1×10^{12} cfu/animal. All animals are dosed via nasal gastric gavage (NG) followed by control/vehicle flush once daily for 28-days. The concentration of bicarbonate and volume for each group is specified in Table

YYY. Vials are inverted at least 3 times prior to drawing the dose in the syringe. The dose site and dose time (end of flush time) is recorded.

Analysis

[0437] Overall condition: Clinical observations are performed twice daily beginning on the second day of acclimation for each animal. The first observation is in the AM, prior to room cleaning. The second observation is no sooner than 4 hours after the AM observation. During the dosing phase, the second observation is performed 4 hour (±10 minutes) post dose administration. Additional clinical observations are performed, as necessary.

[0438] Weight: Each animal is weighed on Day -6, 1, 8, 15, 22, and 29 prior to the first feeding and also prior to dose administration. Additional body weights are taken as needed if necessary.

[0439] Blood Collection: Blood is collected from a peripheral vein of restrained, conscious animals. Whenever possible, blood is collected via a single draw and then divided appropriately. Specimen collection frequency is summarized in **Table 62**.

Table 62. Specimen collection frequency

Time Point	Hematology	Coagulation	Serum Chemistry	Plasma Sample (on ice)	Fecal sample (on ice)
Acclimation Week 1	1x	1x	1x	1x	1x
Dosing	Day 2 (Predose)	Day 2 (Predose)	Day 2 (Predose)	Days 2 and 7	Days 2 and 7
Dosing	Day 14 (Predose)	Day 14 (Predose)	Day 14 (Predose)	Day 14 (Predose)	Day 14-20
Dosing	–	–	–	–	Day 21-27–
Dosing	–	–	–	Day28 (Predose)	Day 28-30
Dosing Weeks	Day 30	Day 30	Day 30	Day 30	Day 35, 40

– = Not applicable

x = Number of times procedure performed within the week

[0440] Hematology: Approximately 1.3 mL of blood is tested in 2 mL K2EDTA tubes using an Advia automated analyzer. Parameters measured are White Blood Cells, Red Blood Cells, Hemoglobin, Hematocrit, Mean Corpuscular Volume,

Mean Corpuscular Hemoglobin, Mean Corpuscular Hemoglobin Concentration, Red Cell Distribution Width, Platelets, Mean Platelet Volume, Differential leukocyte count (absolute): Neutrophils Absolute Lymphocytes Absolute Monocytes Absolute Eosinophils Absolute, Basophils Absolute Reticulocyte Percent, and Reticulocyte Absolute Count.

[0441] Coagulation: Approximately 1.3 mL of blood is tested in 1.8 mL 3.2% sodium citrate tubes. The following Coagulation parameters are determined using a STACompact automated analyzer: Activated Partial Thromboplastin Time, Fibrinogen, and Prothrombin Time. Sodium citrate-treated plasma is stored at -60 to -86 °C prior to analysis and discarded after analysis.

[0442] Serum Chemistry: Animals are fasted for 4 hours prior to removal of sample. The following parameters are tested in approximately 1 mL of blood in 4 mL serum separator tubes using a AU680 analyzer: Albumin, Alkaline Phosphatase, Alanine Aminotransferase Aspartate Aminotransferase, Total Bilirubin, Calcium, Total Cholesterol, Creatine Kinase, Creatinine, Glucose, Inorganic Phosphorus, Total Protein, Triglyceride, Sodium, Potassium, Chloride Globulin, Albumin/Globulin Ratio, Blood Urea Nitrogen, and Gamma Glutamyltransferase.

[0443] Residual serum is stored at-60 to -86 °C and disposed of prior to study finalization.

[0444] Plasma Samples: Animals are fasted for 4 hours prior to removal of the sample. Blood samples are collected from the femoral vein at the target time points listed in Table YYY. After aliquotting the target volume of blood in the blood tube, approximately 0.05 mL of mineral oil is added covering the surface of blood. Tubes are not inverted and placed on a rack and wet ice. Blood sample collection dates and times were recorded. The minimum sample volume is 1 ml of blood collected in a 2 ml lithium heparin tube. Within 15 minutes of collection, the samples are centrifuged at 2 to 8 °C to obtain plasma. Plasma is transferred to a vial and stored at-60 to -86 °C. Specimens are stored on dry ice prior to analysis. Analysis of specimens is conducted using a blood ammonia analyzer instrument.

[0445] Phenylalanine, trans-cinnamic acid, and hippuric acid is measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer.

[0446] Fecal Sample Collection: Two fecal samples per animal are collected at the target time points listed in Table YYY. Sample collection dates and times are recorded. 50 mL falcon tube with approximately 5mL PBS are used as the container (If feces is liquid, no PBS is added). To get the fecal sample weight, pre- and post-sampling weight of container was taken. Samples are collected from the bottom of the cage from each animal. To get fresh and un-contaminated samples, remaining food is removed and the cage pan was cleaned and squeegeed to remove debris and/or water before the collection. Sample is put on wet ice immediately after the collection. Samples are stored at -20 to -15 °C until analysis. Analysis of specimens is conducted using a PCR analytical method.

Example 30. 4-Week Toxicity Study in Cynomolgus Monkeys with a 4-Week Recovery (GLP)

[0447] To evaluate any potential toxicities arising from administration of the genetically engineered bacteria, the pharmacokinetics and pharmacodynamics of the genetically engineered bacteria is studied following daily nasogastric gavage (NG) dose administration for 28-days to female cynomolgus monkeys under GLP conditions.

[0448] The genetically engineered strain comprises one or more copies of PAL3 integrated into the chromosome and one or more copies of PheP integrated into the chromosome, each of which are under the control of an FNRS promoter. In some embodiments, the genetically engineered strain also comprises one or more copies of LAAD, driven by and arabinose inducible promoter, e.g., ParaBAD. In some embodiments, the strains further comprise a auxotrophy mutation, e.g., deltaThyA. In some embodiments, the genetically engineered bacteria further comprise an antibiotic resistance, e.g., kanamycin. In some embodiments, the genetically engineered bacteria do not comprise an auxotrophy mutation. In some embodiments, the genetically engineered bacteria do not comprise an antibiotic resistance.

[0449] The study is conducted in compliance with nonclinical Laboratory Studies Good Laboratory Practice Regulations issued by the U.S. Food and Drug

Administration (Title 21 of the Code of Federal Regulations, Part 58; effective June 20, 1979) and the OECD Principles on Good Laboratory Practice (C[97]186/Final; effective 1997). The animals are individually housed based on the recommendations set forth in the Guide for the Care and Use of Laboratory Animals (National Research Council 2011).

[0450] Animals are administered the genetically engineered bacteria or control vehicle essentially as described in Example 29, except that all materials are manufactured under GMP standards. Dosing is tabulated in **Table 63**. Additionally, animals are acclimated for 14 days and the dosing period is daily for 28 days followed by a recovery period of 28 days. Additionally, animals are euthanized at the end of the study to conduct histological analysis.

Table 63. Dosing Period and Regimen

ACCLIMATION	14 days
TEST ARTICLE PREP	Daily
DOSING PERIOD	Daily for 28 days
RECOVERY PERIOD	28 days
REGULATIONS	FDA GLP

				NUMBER OF ANIMALS	
GROUP	TEST ARTICLE	DOSE LEVEL	DOSE ROUTE	MALES (♂)	FEMALES (♀)
1	Vehicle	0	NG	3[a]+2[b]	3[a]+2[b]
2	Genetically engineered bacteria	1x10^9	NG	3[a]	3[a]
3	Genetically engineered bacteria	1x10^10	NG	3[a]	3[a]
4	Genetically engineered bacteria	1x10^11	NG	3[a]+2[b]	3[a]+2[b]

[a]Terminal Necropsy, Day 29

[b]Recovery Necropsy, Day 56

[0451] Study Analysis is conducted as described in **Table 64**. Hematology, Coagulation, Serum Chemistry and Plasma Samples parameters are essentially as

described in Example 30, and are analyzed using the methods described in Example 30. Collection and analysis of fecal samples is essentially conducted as described in Example 30.

Table 64. Study Analysis

PROCEDURE	TIME POINTS
DOSE CONCENTRATION ANALYSIS	Day 1 and Day 28
CLINICAL OBSERVATIONS	Twice Daily (cageside observations)
FOOD CONSUMPTION	Daily (qualitative)
BODY WEIGHTS	Weekly
OPHTHALMOLOGY	Once during acclimation, Week 4, and Week 8
ECGS/HR/BP	Once during acclimation, Week 4, and Week 8
HEMATOLOGY	Twice during acclimation, Day 2 (pre-dose), Day 15 (pre-dose), Day 29, Day 42, and Day 56
COAGULATION	Twice during acclimation, Day 2 (pre-dose), Day 15 (pre-dose), Day 29, Day 42, and Day 56
SERUM CHEMISTRY	Twice during acclimation, Day 2 (pre-dose), Day 15 (pre-dose), Day 29, Day 42, and Day 56
BODY (RECTAL) TEMPERATURE	Twice during acclimation (with at least 7 days between measurements); once weekly during dosing (~6 hrs post-dose), and Weeks 5 and 8
STOOL SAMPLE COLLECTION (BACTERIAL CULTURE)	Once during acclimation, prior to dosing on Days 2, 7, and 14, Day 29, Day 33, and Week 8 Rectal/Fecal swabs are collected via cotton tip applicator; the cotton part of the swab is transferred to a tube with an appropriate broth/media and immediately put on wet ice. Fecal samples are stored at 2 to 8 °C until time of analysis.
CYTOKINE BLOOD COLLECTIONS	Once during acclimation, Days 1, 3, 7, 14 and 28 (6 hrs post-dose), and Day 56
ARCHIVE BLOOD SAMPLE COLLECTION (SAMPLE TO BE HELD FOR POSSIBLE ANALYSIS)	Once during acclimation, Days 1, 3, 7, 14 and 28 (6 hrs post-dose), and Day 56; Blood samples are processed to serum; samples are stored frozen.
NECROPSY & TISSUE COLLECTION	All animals (e.g., colon, intestine, cecum, liver, spleen)

PROCEDURE	TIME POINTS
ORGAN WEIGHTS	All animals
TISSUE COLLECTION FOR PK/PD ASSESSMENT	All animals
HISTOPATHOLOGY	All animals
STATISTICAL ANALYSIS	Comparative (Anova/Bartletts)

Example 31: Genetically engineered bacteria with HlyA Tag for Secretion of PMEs

[0452] Constructs for secretion of PMEs were generated as shown in **Table 65**. This sequences are subsequently tagged, e.g., with a HIS tag, e.g., inserted before the C terminal secretion sequence. E. coli are transformed with the constructs on a low-copy plasmid. Secreted PMEs are isolated from the media using affinity chromatography (His-Tag). PME molecular weight is confirmed by western blot. Activity of the purified enzyme is tested in an in vitro assay in a phenylalanine-containing buffer. Metabolites are measured over time as described in Examples 24-26.

Table 65. Secretion Sequences

SEQ ID NO	Description	Sequence
SEQ ID NO: 52	HylA Secretion tag	LNPLINEISKIISAAGNFDVKEERAAASL LQLSGNASDFSYGRNSITLTASA
SEQ ID NO: 53	PAL (upper case) expressed as fusion protein with the 53 amino acids of the C termini of alpha-hemolysin (hlyA) of E. coli CFT073 (lower case).	MKAKDVQPTIIINKNGLISLEDIYDIAIK QKKVEISTEITELLTHGREKLEEKLNSGE VIYGINTGFGGNANLVVPFEKIAEHQQNL LTFLSAGTGDYMSKPCIKASQFTMLLSVC KGWSATRPIVAQAIVDHINHDIVPLVPRY GSVGASGDLIPLSYIARALCGIGKVYYMG AEIDAAEAIKRAGLTPLSLKAKEGLALIN GTRVMSGISAITVIKLEKLFKASISAIAL AVEALLASHEHYDARIQQVKNHPGQNAVA SALRNLLAGSTQVNLLSGVKEQANKACRH QEITQLNDTLQEVYSIRCAPQVLGIVPES LATARKILEREVISANDNPLIDPENGDVL HGGNFMGQYVARTMDALKLDIALIANHLH AIVALMMDNRFSRGLPNSLSPTPGMYQGF KGVQLSQTALVAAIRHDCAASGIHTLATE QYNQDIVSLGLHAAQDVLEMEQKLRNIVS MTILVVCQAIHLRGNISEIAPETAKFYHA VREISSPLITDRALDEDIIRIADAIINDQ LPLPEIMLEE lnplineiskiisaagnfdvkeeraaasl

		lqlsgnasdfsygrnsitltasa*
SEQ ID NO: 54	LAAD (uppercase)expressed as fusion protein with the 53 amino acids of the C termini of alpha-hemolysin (hlyA) of E. coli CFT073 (lower case)	MNISRRKLLLGVGAAGVLAGGAALVPMVR RDGKFVEAKSRASFVEGTQGALPKEADVV IIGAGIQGIMTAINLAERGMSVTILEKGQ IAGEQSGRAYSQIISYQTSPEIFPLHHYG KILWRGMNEKIGADTSYRTQGRVEALADE KALDKAQAWIKTAKEAAGFDTPLNTRIIK GEELSNRLVGAQTPWTVAAFEEDSGSVDP ETGTPALARYAKQIGVKIYTNCAVRGIET AGGKISDVVSEKGAIKTSQVVLAGGIWSR LFMGNMGIDIPTLNVYLSQQRVSGVPGAP RGNVHLPNGIHFREQADGTYAVAPRIFTS SIVKDSFLLGPKFMHLLGGGELPLEFSIG EDLFNSFKMPTSWNLDEKTPFEQFRVATA TQNTQHLDAVFQRMKTEFPVFEKSEVVER WGAVVSPTFDELPIISEVKEYPGLVINTA TVWGMTEGPAAGEVTADIVMGKKPVIDPT PFSLDRFKK lnplineiskiisaagnfdvkeeraaasl lqlsgnasdfsygrnsitltasa
SEQ ID NO: 55	HylA secretion signal	CTTAATCCATTAATTAATGAAATCAGCAA AATCATTTCAGCTGCAGGTAATTTTGATG TTAAAGAGGAAAGAGCTGCAGCTTCTTTA TTGCAGTTGTCCGGTAATGCCAGTGATTT TTCATATGGACGGAACTCAATAACTTTGA CAGCATCAGCATAA
SEQ ID NO: 56	LAAD (bold italics) driven by ParaBAD (underlined) with C terminal HylA Secretion tag (bold)	Acttttcatactcccgccattcagagaag aaaccaattgtccatattgcatcagacat tgccgtcactgcgtcttttactggctctt ctcgctaacccaaccggtaaccccgctta ttaaaagcattctgtaacaaagcgggacc aaagccatgacaaaaacgcgtaacaaaag tgtctataatcacggcagaaaa ***atgaacatttcaaggagaaagctactttt aggtgttggtgctgcgggcgttttagcag gtggtgcggctttagttccaatggttcgc cgtgacggcaaatttgtggaagctaaatc aagagcatcatttgttgaaggtacgcaag gggctcttcctaaagaagcagatgtagtg attattggtgccggtattcaagggatcat gaccgctattaaccttgctgaacgtggta tgagtgtcactatcttagaaaagggtcag attgccggtgagcaatcaggccgtgcata cagccaaattattagttaccaaacatcgc cagaaatcttcccattacaccattatggg aaaatattatggcgtggcatgaatgagaa aattggtgcggataccagttatcgtactc aaggtcgtgtagaagcgctggcagatgaa***

		aaagcattagataaagctcaagcgtggat *caaaacagctaaagaagcggcaggttttg* *atacaccattaaatactcgcatcattaaa* *ggtgaagagctatcaaatcgcttagtcgg* *tgctcaaacgccatggactgttgctgcat* *ttgaagaagattcaggctctgttgatcct* *gaaacaggcacacctgcactcgctcgtta* *tgccaaacaaatcggtgtgaaaatttata* *ccaactgtgcagtaagaggtattgaaact* *gcgggtggtaaaatctctgatgtggtgag* *tgagaaaggggcgattaaaacgtctcaag* *ttgtactcgctggggggtatctggtcgcgt* *ttatttatgggcaatatgggtattgatat* *cccaacgctcaatgtatatctatcacaac* *aacgtgtctcaggggttcctggtgcacca* *cgtggtaatgtgcatttacctaatggtat* *tcatttccgcgaacaagcggatggtactt* *atgccgttgcaccacgtatctttacgagt* *tcaatagtcaaagatagcttcctgctagg* *gcctaaatttatgcacttattaggtggcg* *gagagttaccgttggaattctctattggt* *gaagatctatttaattcatttaaaatgcc* *gacctcttggaatttagatgaaaaaacac* *cattcgaacaattccgagttgccacggca* *acacaaaatacgcaacacttagatgctgt* *tttccaaagaatgaaaacagaattcccag* *tatttgaaaaatcagaagttgttgaacgt* *tggggtgccgttgtgagtccaacatttga* *tgaattacctatcatttctgaggtcaaag* *aatacccaggcttagtgattaacacggca* *acagtgtggggtatgacagaaggcccggc* *agcgggtgaagtgaccgctgatattgtca* *tgggcaagaaacctgttattgatccaacg* *ccgtttagtttggatcgttttaagaagta* *a**CTTAATCCATTAATTAATGAAATCAGCA*** ***AAATCATTTCAGCTGCAGGTAATTTTGAT*** ***GTTAAAGAGGAAAGAGCTGCAGCTTCTTT*** ***ATTGCAGTTGTCCGGTAATGCCAGTGATT*** ***TTTCATATGGACGGAACTCAATAACTTTG*** ***ACAGCATCAGCATAA***
SEQ ID NO: 57	PfnrS-PAL3 with C terminal secretion tag. PfnrS **(bolded lower case),** *PAL3* sequence is underlined upper case C terminal secretion tag is bold uppercase	GGTACC**agttgttcttattggtggtgttg** **ctttatggttgcatcgtagtaaatggttg** **taacaaaagcaatttttccggctgtctgt** **atacaaaaacgccgtaaagtttgagcgaa** **gtcaataaactctctacccattcagggca** **atatctctctt**GGATCC**ctctagaaataa** **ttttgtttaactttaagaaggagatatac** **at**ATGAAAGCTAAAGATGTTCAGCCAACC ATTATTATTAATAAAAATGGCCTTATCTC TTTGGAAGATATCTATGACATTGCGATAA

		AACAAAAAAAAGTAGAAATATCAACGGAG ATCACTGAACTTTTGACGCATGGTCGTGA AAAATTAGAGGAAAAATTAAATTCAGGAG AGGTTATATATGGAATCAATACAGGATTT GGAGGGAATGCCAATTTAGTTGTGCCATT TGAGAAAATCGCAGAGCATCAGCAAAATC TGTTAACTTTTCTTTCTGCTGGTACTGGG GACTATATGTCCAAACCTTGTATTAAAGC GTCACAATTTACTATGTTACTTTCTGTTT GCAAAGGTTGGTCTGCAACCAGACCAATT GTCGCTCAAGCAATTGTTGATCATATTAA TCATGACATTGTTCCTCTGGTTCCTCGCT ATGGCTCAGTGGGTGCAAGCGGTGATTTA ATTCCTTTATCTTATATTGCACGAGCATT ATGTGGTATCGGCAAAGTTTATTATATGG GCGCAGAAATTGACGCTGCTGAAGCAATT AAACGTGCAGGGTTGACACCATTATCGTT AAAAGCCAAAGAAGGTCTTGCTCTGATTA ACGGCACCCGGGTAATGTCAGGAATCAGT GCAATCACCGTCATTAAACTGGAAAAACT ATTTAAAGCCTCAATTTCTGCGATTGCCC TTGCTGTTGAAGCATTACTTGCATCTCAT GAACATTATGATGCCCGGATTCAACAAGT AAAAAATCATCCTGGTCAAAACGCGGTGG CAAGTGCATTGCGTAATTTATTGGCAGGT TCAACGCAGGTTAATCTATTATCTGGGGT TAAAGAACAAGCCAATAAAGCTTGTCGTC ATCAAGAAATTACCCAACTAAATGATACC TTACAGGAAGTTTATTCAATTCGCTGTGC ACCACAAGTATTAGGTATAGTGCCAGAAT CTTTAGCTACCGCTCGGAAAATATTGGAA CGGGAAGTTATCTCAGCTAATGATAATCC ATTGATAGATCCAGAAAATGGCGATGTTC TACACGGTGGAAATTTTATGGGGCAATAT GTCGCCCGAACAATGGATGCATTAAAACT GGATATTGCTTTAATTGCCAATCATCTTC ACGCCATTGTGGCTCTTATGATGGATAAC CGTTTCTCTCGTGGATTACCTAATTCACT GAGTCCGACACCCGGCATGTATCAAGGTT TTAAAGGCGTCCAACTTTCTCAAACCGCT TTAGTTGCTGCAATTCGCCATGATTGTGC TGCATCAGGTATTCATACCCTCGCCACAG AACAATACAATCAAGATATTGTCAGTTTA GGTCTGCATGCCGCTCAAGATGTTTTAGA GATGGAGCAGAAATTACGCAATATTGTTT CAATGACAATTCTGGTAGTTTGTCAGGCC ATTCATCTTCGCGGCAATATTAGTGAAAT TGCGCCTGAAACTGCTAAATTTTACCATG CAGTACGCGAAATCAGTTCTCCTTTGATC ACTGATCGTGCGTTGGATGAAGATATAAT

		CCGCATTGCGGATGCAATTATTAATGATC AACTTCCTCTGCCAGAAATCATGCTGGAA GAATAA**CTTAATCCATTAATTAATGAAAT CAGCAAAATCATTTCAGCTGCAGGTAATT TTGATGTTAAAGAGGAAAGAGCTGCAGCT TCTTTATTGCAGTTGTCCGGTAATGCCAG TGATTTTTCATATGGACGGAACTCAATAA CTTTGACAGCATCAGCATAA**

Table 66 HlyB and HlyD protein sequences

SEQ ID NO: 58	HlyB protein	MDSCHKIDYGLYALEILAQYHNVSVNPEE IKHRFDTDGTGLGLTSWLLAAKSLELKVK QVKKTIDRLNFISLPALVWREDGRHFILT KVSKEANRYLIFDLEQRNPRVLEQSEFEA LYQGHIILIASRSSVTGKLAKFDFTWFIP AIIKYRKIFIETLVVSVFLQLFALITPLF FQVVMDKVLVHRGFSTLNVITVALSVVVV FEIILSGLRTYIFAHSTSRIDVELGAKLF RHLLALPISYFESRRVGDTVARVRELDQI RNFLTGQALTSVLDLLFSFIFFAVMWYYS PKLTLVILFSLPCYAAWSVFISPILRRRL DDKFSRNADNQSFLVESVTAINTIKAMAV SPQMTNIWDKQLAGYVAAGFKVTVLATIG QQGIQLIQKTVMIINLWLGAHLVISGDLS IGQLIAFNMLAGQIVAPVIRLAQIWQDFQ QVGISVTRLGDVLNSPTESYHGKLALPEI NGNITFRNIRFRYKPDSPVILDNINLSIK QGEVIGIVGRSGSGKSTLTKLIQRFYIPE NGQVLIDGHDLALADPNWLRRQVGVVLQD NVLLNRSIIDNISLANPGMSVEKVIYAAK LAGAHDFISELREGYNTIVGEQGAGLSGG QRQRIAIARALVNNPKILIFDEATSALDY ESEHIIMRNMHKICKGRTVIIIAHRLSTV KNADRIIVMEKGKIVEQGKHKELLSEPES LYSYLYQLQSD
SEQ ID NO: 59	HlyD protein	MKTWLMGFSEFLLRYKLVWSETWKIRKQL DTPVREKDENEFLPAHLELIETPVSRRPR LVAYFIMGFLVIAVILSVLGQVEIVATAN GKLTLSGRSKEIKPIENSIVKEIIVKEGE SVRKGDVLLKLTALGAEADTLKTQSSLLQ TRLEQTRYQILSRSIELNKLPELKLPDEP YFQNVSEEEVLRLTSLIKEQFSTWQNQKY QKELNLDKKRAERLTILARINRYENLSRV EKSRLDDFRSLLHKQAIAKHAVLEQENKY VEAANELRVYKSQLEQIESEILSAKEEYQ LVTQLFKNEILDKLRQTTDNIELLTLELE KNEERQQASVIRAPVSGKVQQLKVHTEGG VVTTAETLMVIVPEDDTLEVTALVQNKDI GFINVGQNAIIKVEAFPYTRYGYLVGKVK

		NINLDAIEDQKLGLVFNVIVSVEENDLST GNKHIPLSSGMAVTAEIKTGMRSVISYLL SPLEESVTESLHER

Example 32: Genetically engineered bacteria comprising additional constructs

Constructs for secretion of PMEs were generated as shown in **Table 66**.

Table 66

Description	**Sequence**	**SEQ ID NO**
phenylalanine transporter [Escherichia coli str. K-12 substr. MG1655] Acc. No. NP_415108 (PheP)	MKNASTVSEDTASNQEPTLHRGLHNRHIQLIA LGGAIGTGLFLGIGPAIQMAGPAVLLGYGVAG IIAFLIMRQLGEMVVEEPVSGSFAHFAYKYWG PFAGFLSGWNYWVMFVLVGMAELTAAGIYMQY WFPDVPTWIWAAAFFIIINAVNLVNVRLYGET EFWFALIKVLAIIGMIGFGLWLLFSGHGGEKA SIDNLWRYGGFFATGWNGLILSLAVIMFSFGG LELIGITAAEARDPEKSIPKAVNQVVYRILLF YIGSLVVLLALYPWVEVKSNSSPFVMIFHNLD SNVVASALNFVILVASLSVYNSGVYSNSRMLF GLSVQGNAPKFLTRVSRRGVPINSLMLSGAIT SLVVLINYLLPQKAFGLLMALVVATLLLNWIM ICLAHLRFRAAMRRQGRETQFKALLYPFGNYL CIAFLGMILLLMCTMDDMRLSAILLPVWIVFL FMAFKTLRRK	60
aromatic amino acid transport protein AroP [Escherichia coli F11] Acc. NO: EDV65095	MEGQQHGEQLKRGLKNRHIQLIALGGAIGTGL FLGSASVIQSAGPGIILGYAIAGFIAFLIMRQ LGEMVVEEPVAGSFSHFAYKYWGSFAGFASGW NYWVLYVLVAMAELTAVGKYIQFWYPEIPTWV SAAVFFVVINAINLTNVKVFGEMEFWFAIIKV IAVVAMIIFGAWLLFSGNGGPQASVSNLWDQG GFLPHGFTGLVMMMAIIMFSFGGLELVGITAA EADNPEQSIPKATNQVIYRILIFYIGSLAVLL SLMPWTRVTADTSPFVLIFHELGDTFVANALN IVVLTAALSVYNSCVYCNSRMLFGLAQQGNAP KALASVDKRGVPVNTILVSALVTALCVLINYL APESAFGLLMALVVSALVINWAMISLAHMKFR RAKQEQGVVTRFPALLYPLGNWVCLLFMAAVL VIMLMTPGMAISVYLIPVWLIVLGIGYLFKEK TAKAVKAH	61
FNRS promoter (bold, lower case)-PheP (upper case underlined	**GGTACCagttgttcttattggtggtgttgctt tatggttgcatcgtagtaaatggttgtaacaa aagcaatttttccggctgtctgtatacaaaaa cgccgtaaagtttgagcgaagtcaataaactc tctacccattcagggcaatatctctcttGGAT CCctctagaaataattttgtttaactttaaga**	62

	aggagatatacatATGAAAAACGCGTCAACCG TATCGGAAGATACTGCGTCGAATCAAGAGCCG ACGCTTCATCGCGGATTACATAACCGTCATAT TCAACTGATTGCGTTGGGTGGCGCAATTGGTA CTGGTCTGTTTCTTGGCATTGGCCCGGCGATT CAGATGGCGGGTCCGGCTGTATTGCTGGGCTA CGGCGTCGCCGGGATCATCGCTTTCCTGATTA TGCGCCAGCTTGGCGAAATGGTGGTTGAGGAG CCGGTATCCGGTTCATTTGCCCACTTTGCCTA TAAATACTGGGGACCGTTTGCGGGCTTCCTCT CTGGCTGGAACTACTGGGTAATGTTCGTGCTG GTGGGAATGGCAGAGCTGACCGCTGCGGGCAT CTATATGCAGT63ACTGGTTCCCGGATGTTCC AACGTGGATTTGGGCTGCCGCCTTCTTTATTA TCATCAACGCCGTTAACCTGGTGAACGTGCGC TTATATGGCGAAACCGAGTTCTGGTTTGCGTT GATTAAAGTGCTGGCAATCATCGGTATGATCG GCTTTGGCCTGTGGCTGCTGTTTTCTGGTCAC GGCGGCGAGAAAGCCAGTATCGACAACCTCTG GCGCTACGGTGGTTTCTTCGCCACCGGCTGGA ATGGGCTGATTTTGTCGCTGGCGGTAATTATG TTCTCCTTCGGCGGTCTGGAGCTGATTGGGAT TACTGCCGCTGAAGCGCGCGATCCGGAAAAAA GCATTCCAAAAGCGGTAAATCAGGTGGTGTAT CGCATCCTGCTGTTTTACATCGGTTCACTGGT GGTTTTACTGGCGCTCTATCCGTGGGTGGAAG TGAAATCCAACAGTAGCCCGTTTGTGATGATT TTCCATAATCTCGACAGCAACGTGGTAGCTTC TGCGCTGAACTTCGTCATTCTGGTAGCATCGC TGTCAGTGTATAACAGCGGGGTTTACTCTAAC AGCCGCATGCTGTTTGGCCTTTCTGTGCAGGG TAATGCGCCGAAGTTTTTGACTCGCGTCAGCC GTCGCGGTGTGCCGATTAACTCGCTGATGCTT TCCGGAGCGATCACTTCGCTGGTGGTGTTAAT CAACTATCTGCTGCCGCAAAAAGCGTTTGGTC TGCTGATGGCGCTGGTGGTAGCAACGCTGCTG TTGAACTGGATTATGATCTGTCTGGCGCATCT GCGTTTTCGTGCAGCGATGCGACGTCAGGGGC GTGAAACACAGTTTAAGGCGCTGCTCTATCCG TTCGGCAACTATCTCTGCATTGCCTTCCTCGG CATGATTTTGCTGCTGATGTGCACGATGGATG ATATGCGCTTGTCAGCGATCCTGCTGCCGGTG TGGATTGTATTCCTGTTTATGGCATTTAAAAC GCTGCGTCGGAAATAA	
FNRS promoter (bold, lower case)-AroP (upper case underlined, codon optimized)	**GGTACCagttgttcttattggtggtgttgctt tatggttgcatcgtagtaaatggttgtaacaa aagcaatttttccggctgtctgtatacaaaaa cgccgtaaagtttgagcgaagtcaataaactc tctacccattcagggcaatatctctcttGGAT**	63

| | **CCctctagaaataattttgtttaactttaaga aggagatatacat**ATGGAGGGGCAGCAGCATG GGGAGCAACTGAAGCGCGGGTTAAAAAATCGT CACATTCAATTAATCGCGCTGGGCGGAGCAAT TGGTACGGGATTGTTCCTGGGTTCAGCGAGCG TCATCCAATCGGCAGGTCCAGGGATCATCTTG GGATATGCGATCGCAGGCTTTATCGCTTTTCT TATTATGCGCCAATTAGGTGAGATGGTGGTCG AGGAGCCTGTAGCTGGCTCCTTCTCACATTTC GCGTACAAGTATTGGGGATCCTTTGCGGGATT TGCTTCTGGTTGGAACTATTGGGTTCTTTATG TCCTGGTGGCCATGGCGGAGCTGACCGCGGTT GGAAAATATATCCAGTTCTGGTACCCCGAGAT CCCGACGTGGGTCTCAGCCGCGGTATTCTTTG TTGTTATCAATGCAATCAATTTAACCAACGTA AAAGTATTTGGTGAAATGGAGTTCTGGTTCGC GATTATCAAAGTAATTGCCGTAGTTGCTATGA TTATTTTTGGGGCATGGTTGCTTTTCTCAGGA AATGGCGGACCACAAGCGTCGGTTTCAAACCT GTGGGATCAAGGGGGATTCCTGCCGCACGGAT TTACGGGCTTGGTGATGATGATGGCTATCATT ATGTTTTCTTTCGGTGGTCTTGAATTAGTGGG TATTACCGCAGCAGAGGCAGATAATCCCGAAC AAAGCATCCCAAAAGCTACTAACCAAGTTATT TACCGTATCCTGATTTTTTATATTGGTTCTCT GGCAGTCCTGCTTTCCTTAATGCCCTGGACAC GTGTAACGGCCGATACATCCCCTTTTGTACTT ATCTTTCACGAACTGGGAGACACGTTCGTCGC CAATGCATTAAACATTGTTGTGCTGACAGCTG CCTTATCTGTGTATAATAGCTGCGTTTATTGC AATTCACGTATGTTATTCGGGCTTGCTCAGCA GGGTAACGCGCCAAAGGCGTTGGCCTCAGTAG ATAAGCGCGGAGTGCCTGTAAATACAATTTTG GTCAGCGCATTAGTCACGGCTCTTTGCGTTCT GATTAACTATCTGGCTCCTGAAAGCGCATTCG GATTACTTATGGCCCTGGTTGTTTCCGCCCTG GTTATCAATTGGGCAATGATTAGTTTGGCACA TATGAAGTTCCGCCGTGCTAAACAAGAACAAG GTGTCGTAACTCGTTTCCCTGCCTTATTGTAT CCGCTGGGGAATTGGGTATGCCTTCTTTTTAT GGCCGCAGTACTGGTAATTATGTTGATGACGC CCGGCATGGCTATTAGTGTATACCTTATTCCG GTATGGTTAATCGTCTTGGGTATCGGCTACTT ATTTAAAGAAAAAACAGCAAAAGCCGTAAAGG CTCAT | |

Claims

1. A genetically engineered bacterium comprising:
   a) one or more gene(s) encoding a phenylalanine ammonia lyase (PAL), wherein the gene(s) encoding a PAL is operably linked to a directly or indirectly inducible promoter that is not associated with the PAL gene in nature; and
   b) one or more gene(s) encoding a phenylalanine transporter, wherein the gene(s) encoding the phenylalanine transporter is operably linked to a directly or indirectly inducible promoter that is not associated with the phenylalanine transporter gene in nature.

2. The genetically engineered bacterium of claim 1, further comprising one or more gene(s) encoding an L-aminoacid deaminase (LAAD), wherein the gene(s) encoding LAAD is operably linked to a directly or indirectly inducible promoter that is not associated with the LAAD gene in nature.

3. The genetically engineered bacterium of claim 1 or claim 2, wherein the promoter operably linked to the gene(s) encoding a PAL and the promoter operably linked to the gene(s) encoding a phenylalanine transporter are separate copies of the same promoter.

4. The genetically engineered bacterium of claim 1 or claim 2, wherein the gene(s) encoding a PAL and the gene(s) encoding a phenylalanine transporter are operably linked to the same copy of the same promoter.

5. The genetically engineered bacterium of any one of claims 2-4, wherein the gene(s) encoding a LAAD is operably linked to a different promoter from that of the gene(s) encoding a PAL and the gene(s) encoding a phenylalanine transporter.

6. The genetically engineered bacterium of any one of claims 1-5, wherein the promoter or promoters operably linked to the gene(s) encoding a PAL, the gene(s) encoding a phenylalanine transporter, and the gene(s) encoding a LAAD are directly or indirectly induced by exogenous environmental conditions.

7. The genetically engineered bacterium of any one of claims 1-6, wherein the promoter or promoters operably linked to the gene(s) encoding a PAL and the gene(s) encoding a phenylalanine transporter are directly or indirectly induced by exogenous environmental conditions found in the small intestine of a mammal.

8. The genetically engineered bacterium of claim any one of claims 1-7, wherein the promoter or promoters operably linked to the gene(s) encoding a PAL and the gene(s) encoding a phenylalanine transporter are directly or indirectly induced under low-oxygen or anaerobic conditions.

9. The genetically engineered bacterium of any one of claims 1-8, wherein the promoter or promoters operably linked to the gene(s) encoding a PAL and the gene(s) encoding a phenylalanine transporter are selected from the group consisting of an FNR-responsive promoter, an ANR-responsive promoter, and a DNR-responsive promoter.

10. The genetically engineered bacterium of any one of claims 5-9, wherein the gene(s) encoding a PAL and the gene(s) encoding a phenylalanine transporter are operably linked to the same copy of the same promoter.

11. The genetically engineered bacterium of any one of claims 2-9, wherein the gene encoding a LAAD is under the control of a promoter that is directly or indirectly induced by an environmental factor that is naturally present in a mammalian gut.

12. The genetically engineered bacterium of any one of claims 2-9, wherein the gene encoding a LAAD is under the control of a promoter that is directly or indirectly induced by an environmental factor that is not naturally present in a mammalian gut.

13. The genetically engineered bacterium of any one of claims 1-12, wherein the gene(s) encoding a phenylalanine transporter is located on a chromosome in the bacterium.

14. The genetically engineered bacterium of any one of claims 1-12, wherein the gene(s) encoding a phenylalanine transporter is located on a plasmid in the bacterium.

15. The genetically engineered bacterium of any one of claims 1-14, wherein the gene(s) encoding a PAL is located on a plasmid in the bacterium.

16. The genetically engineered bacterium of any one of claims 1-14, wherein the gene(s) encoding a PAL is located on a chromosome in the bacterium.

17. The genetically engineered bacterium of any one of claims 1-16, wherein the PAL is from *Anabaena* variabilis (PAL1) or from *Photorhabdus luminescens* (PAL3).

18. The genetically engineered bacterium of any one of claims 1-17, wherein the phenylalanine transporter is PheP.

19. The genetically engineered bacterium of any one of claims 1-18, wherein the engineered bacterium comprises 1-5 copies of a gene(s) encoding a PAL, 1-5 copies of a gene(s) encoding a phenylalanine transporter, and optionally 1-5 copies of gene(s) encoding a LAAD.

20. The genetically engineered bacterium of any one of claims 1-19, wherein the bacterium is a probiotic bacterium.

21. The genetically engineered bacterium of claim 20, wherein the bacterium is selected from the group consisting of *Bacteroides, Bifidobacterium, Clostridium, Escherichia, Lactobacillus*, and *Lactococcus*.

22. The genetically engineered bacterium of claim 21, wherein the bacterium is *Escherichia coli* strain Nissle.

23. The genetically engineered bacterium of any one of claims 1-22, wherein the bacterium is an auxotroph in a gene that is complemented when the bacterium is present in a mammalian gut.

24. The genetically engineered bacterium of claim 23, wherein mammalian gut is a human gut.

25. The genetically engineered bacterium of claim 23 or 24, wherein the bacterium is an auxotroph in diaminopimelic acid or an enzyme in the thymidine biosynthetic pathway.

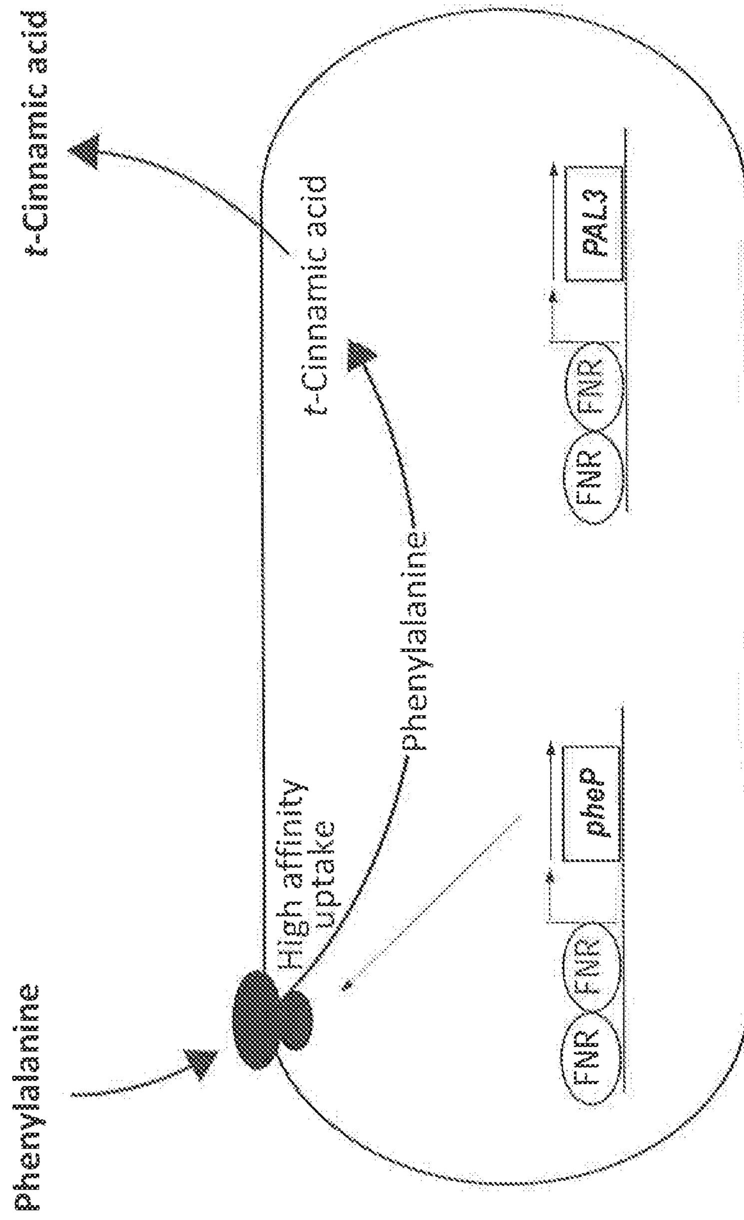
26. The genetically engineered bacterium of any one of claims 1-23, wherein the bacterium is further engineered to harbor a gene encoding a substance toxic to the bacterium, wherein the gene is under the control of a promoter that is directly or indirectly induced by an environmental factor not naturally present in a mammalian gut.

27. A pharmaceutically acceptable composition comprising the bacterium of any one of claims 1-26; and a pharmaceutically acceptable carrier.

28. The composition of claim 27 formulated for oral administration.

29. A method of reducing hyperphenylalaninemia or treating a disease associated with hyperphenylalaninemia comprising the step of administering to a subject in need thereof, the composition of claim 27 or 28.

30. The method of claim 29, wherein the disease is selected from the group consisting of: phenylketonuria, classical or typical phenylketonuria, atypical phenylketonuria, permanent mild hyperphenylalaninemia, nonphenylketonuric hyperphenylalaninemia, phenylalanine hydroxylase deficiency, cofactor deficiency, dihydropteridine reductase deficiency, tetrahydropterin synthase deficiency, Segawa’s disease, and liver disease.



PAL = Phenylalanine Ammonia Lyase
PheP =High Affinity Phenylalanine Transporter

Fig. 1

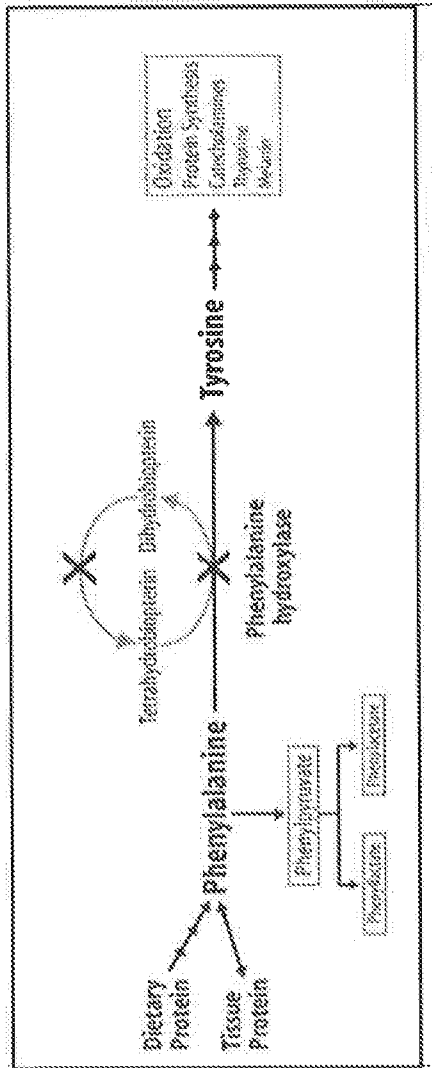


Fig. 2A

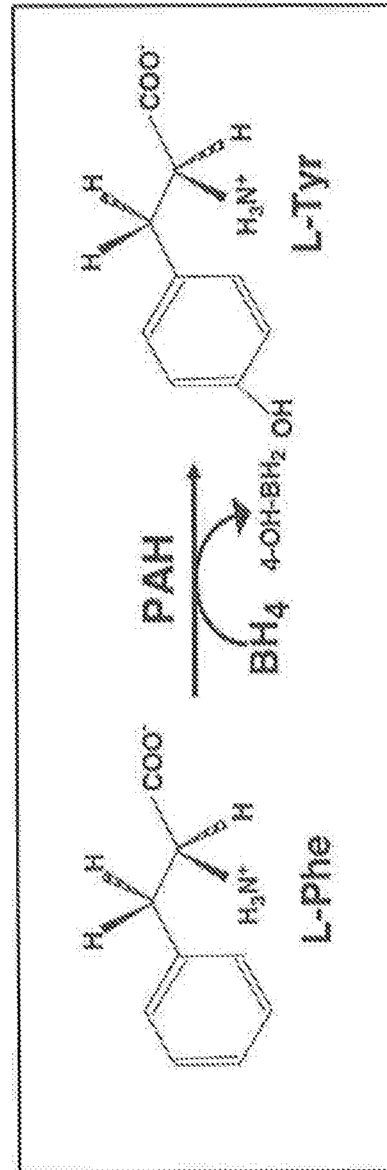


Fig. 2B

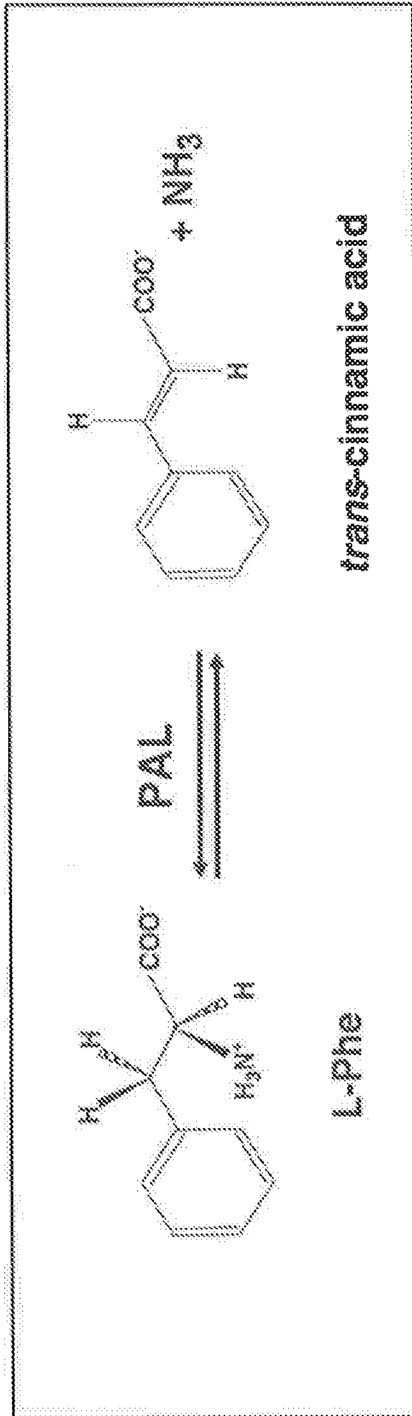


Fig. 2C

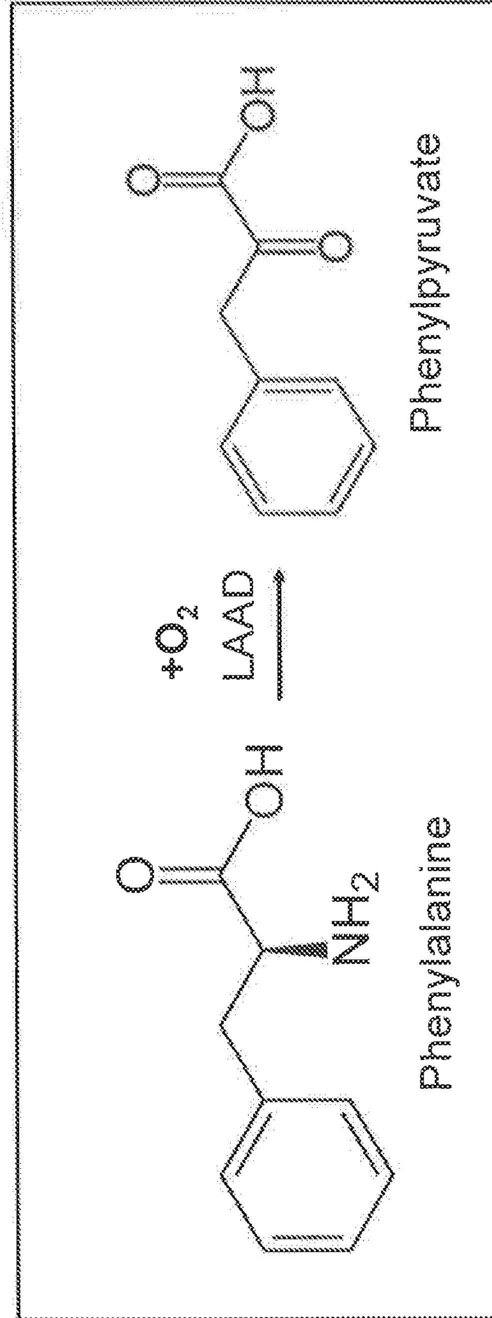
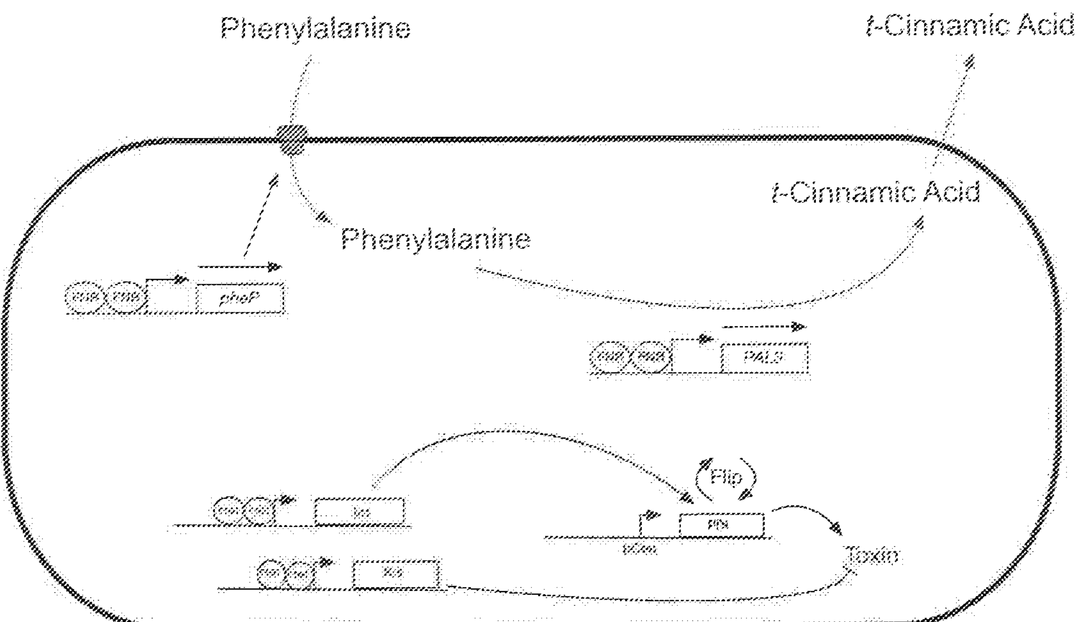


Fig. 2D



- PAL = phenylalanine ammonia lyase from an Enterobacteriaciae species
- PheP = high affinity phenylalanine transporter

Fig. 3

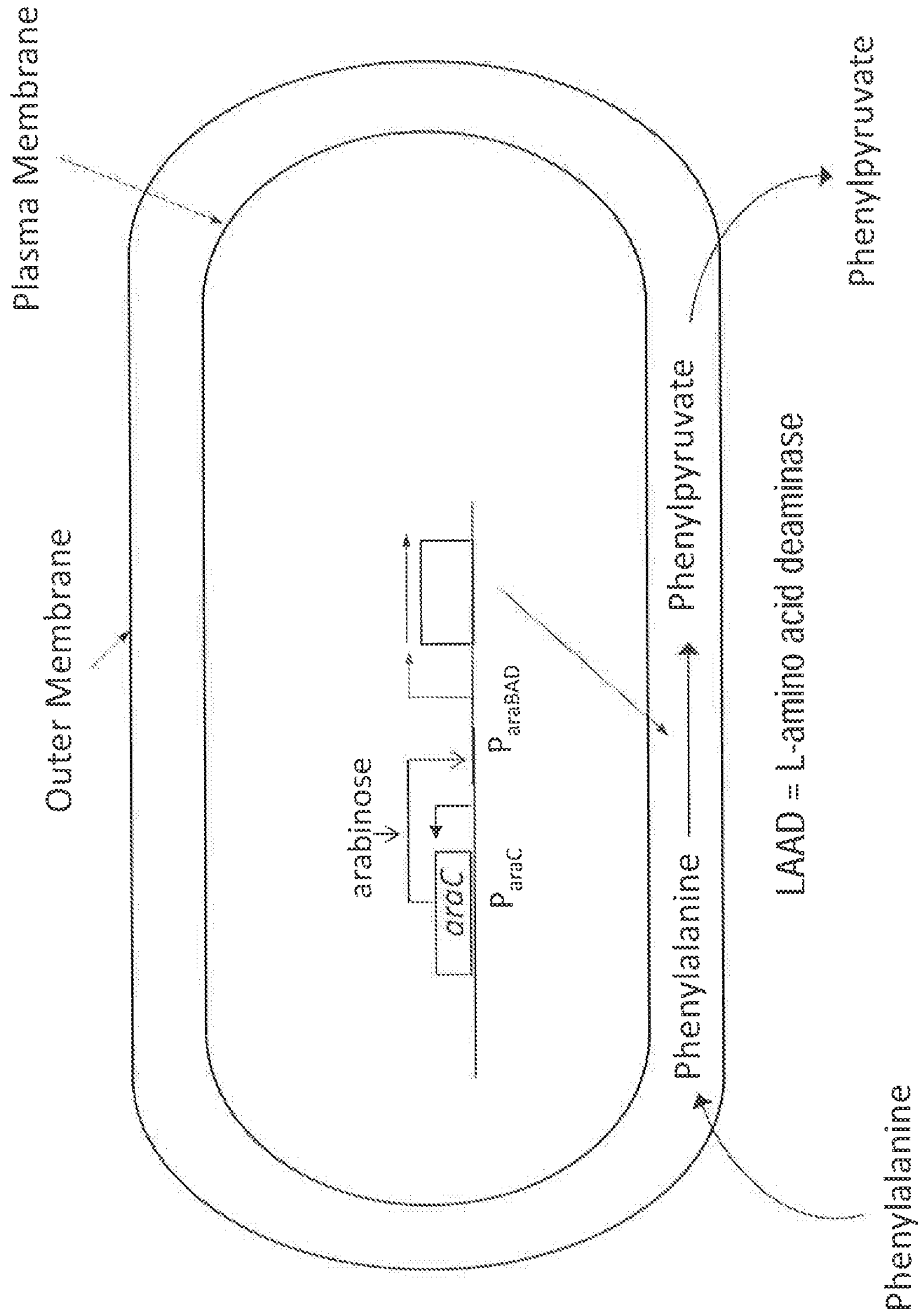


Fig. 4

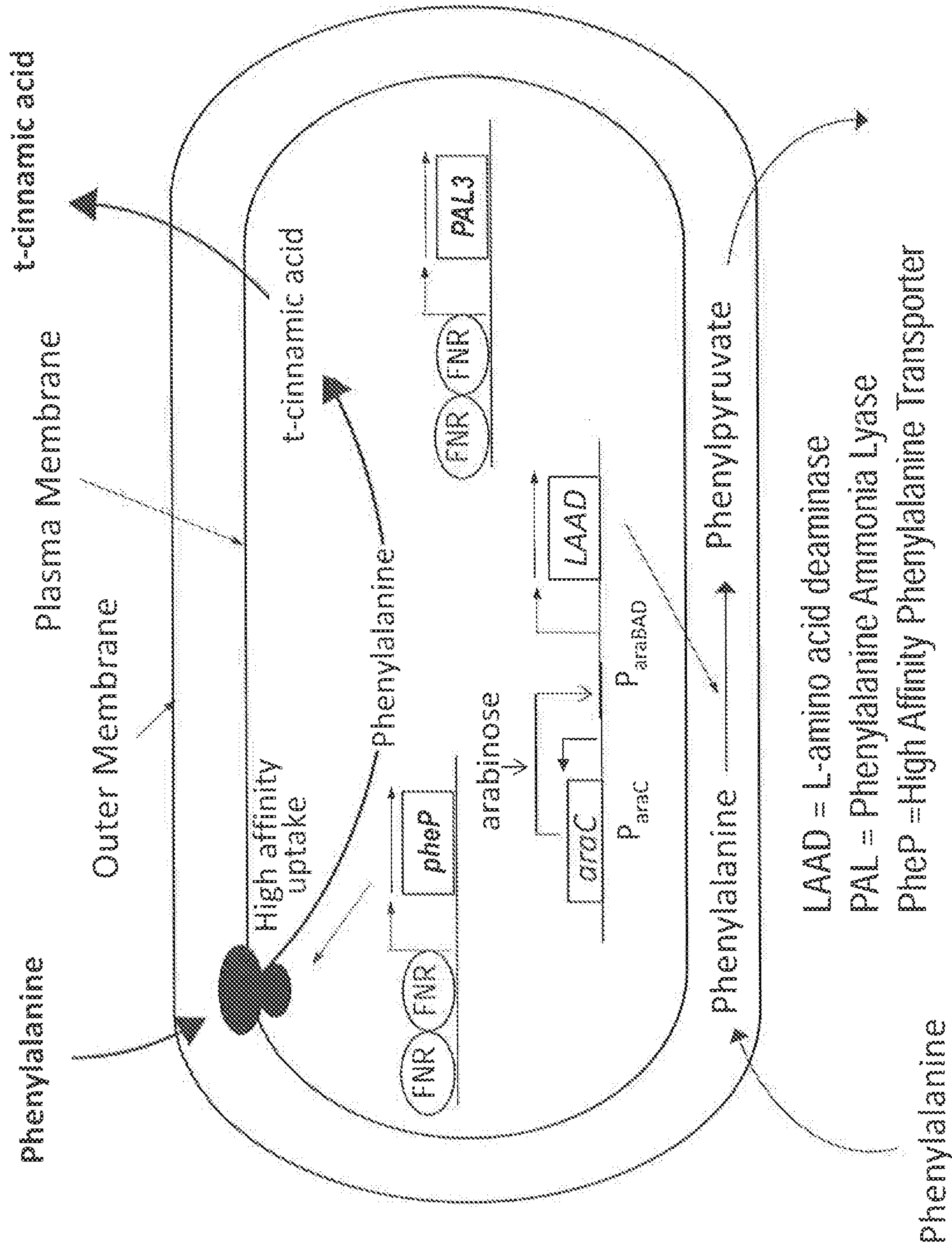


Fig. 5

Fig. 6

Fig. 7

Fig. 8

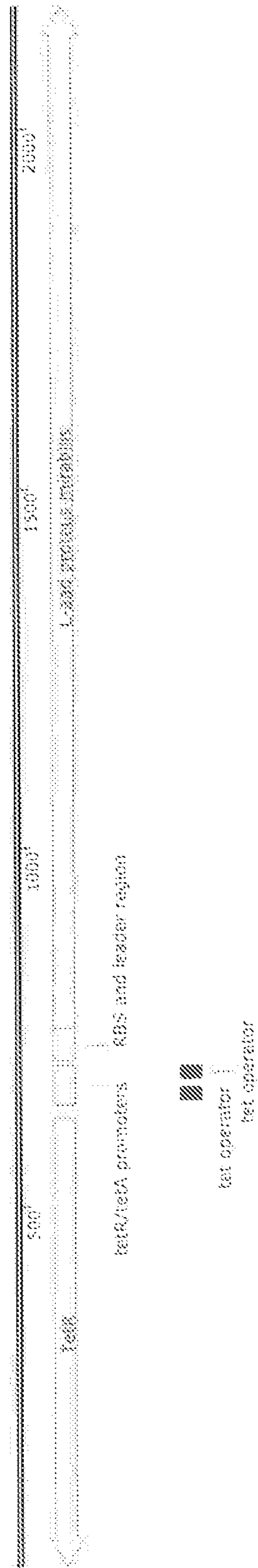


Fig. 9

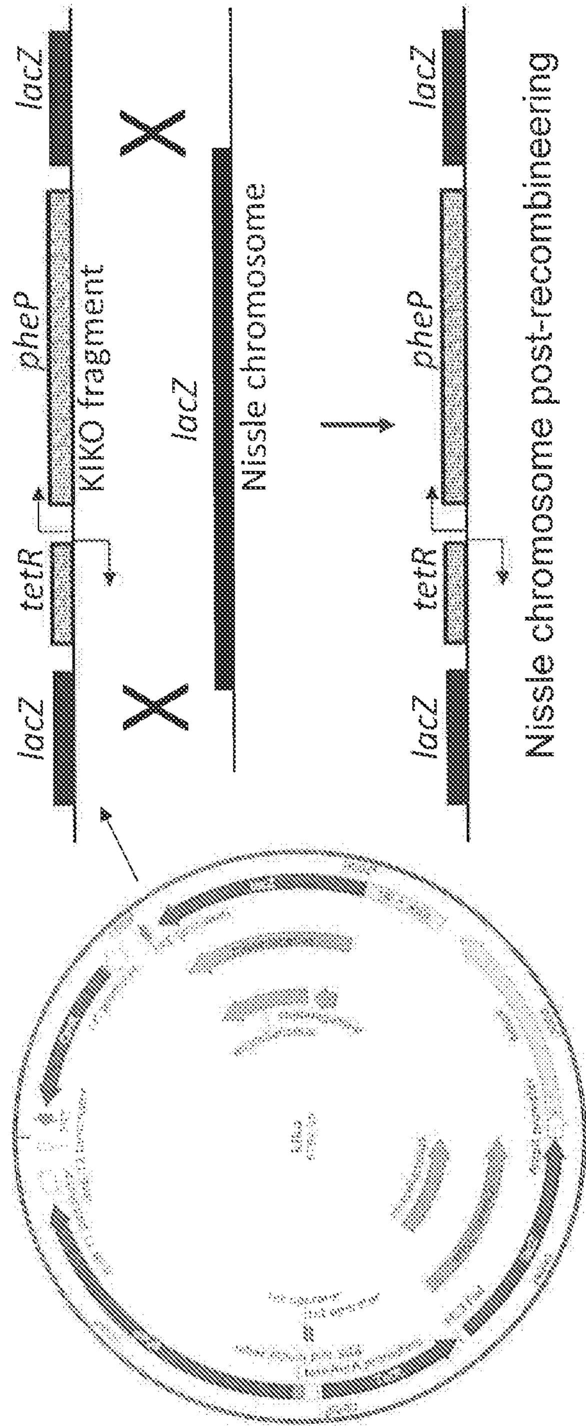


Fig. 10

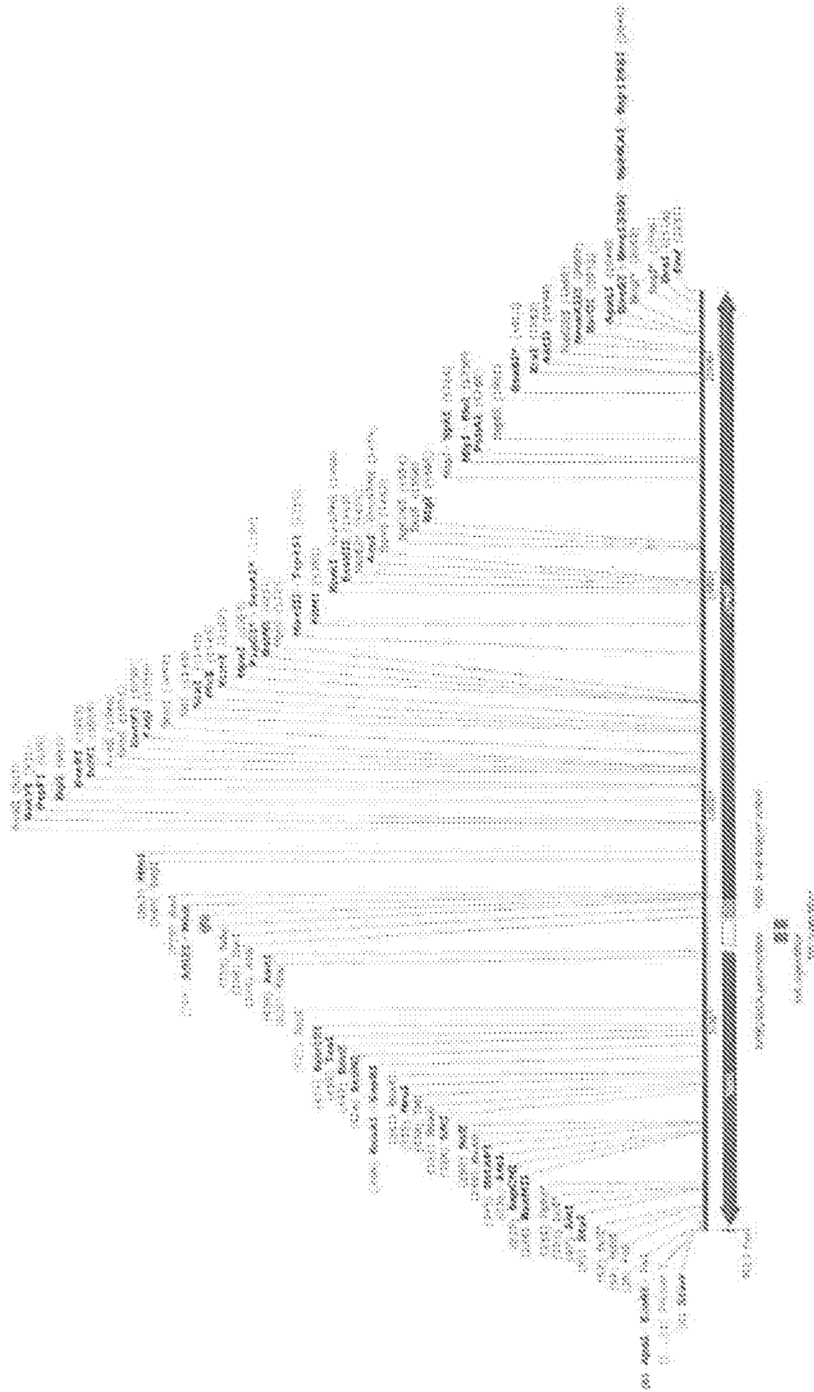


Fig. 11

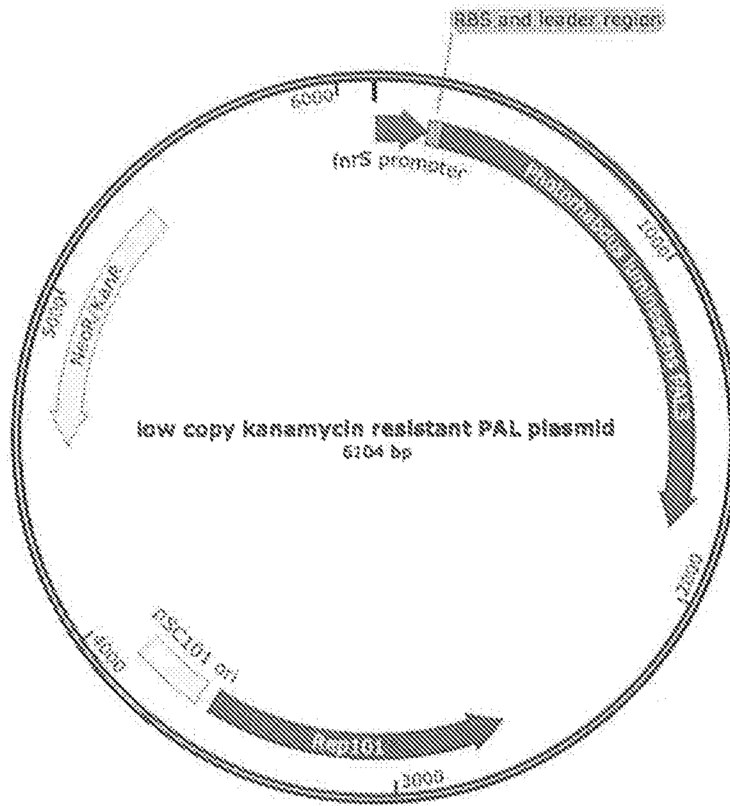


Fig. 12A

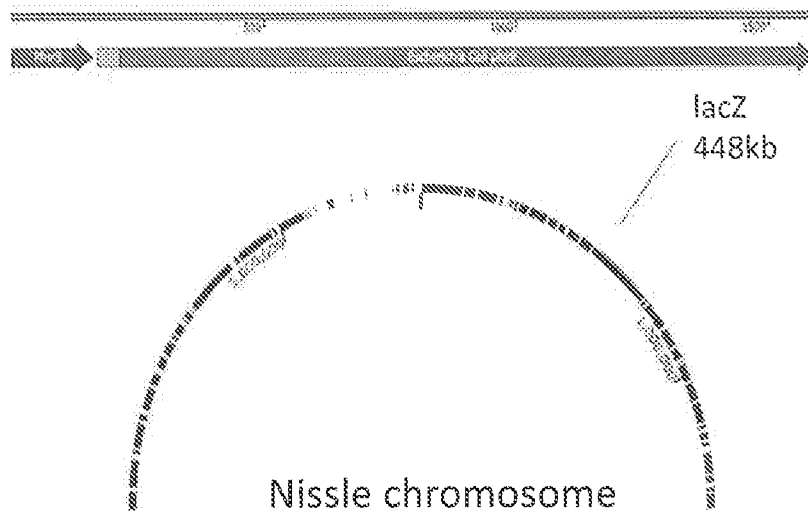


Fig. 12B

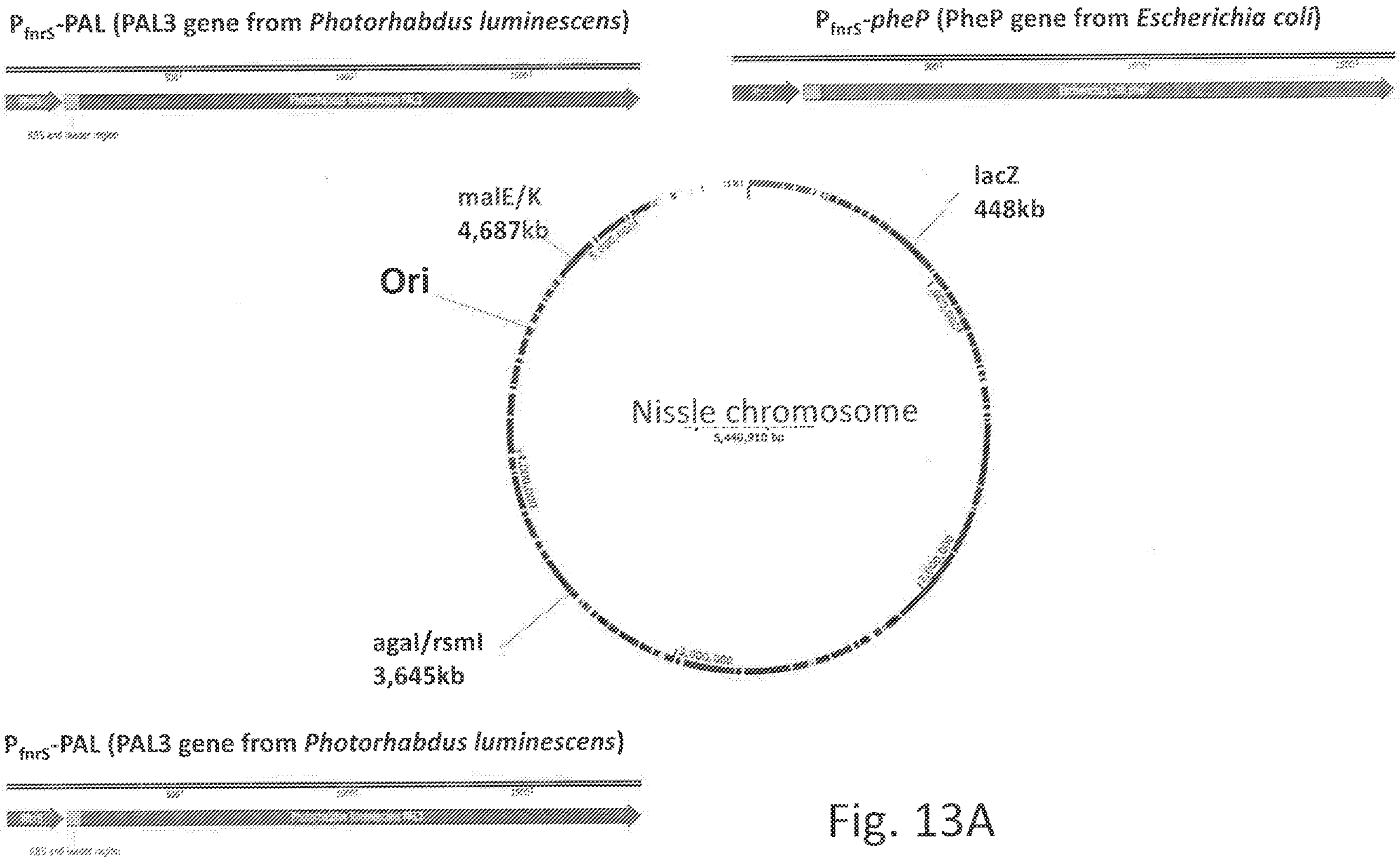


Fig. 13A

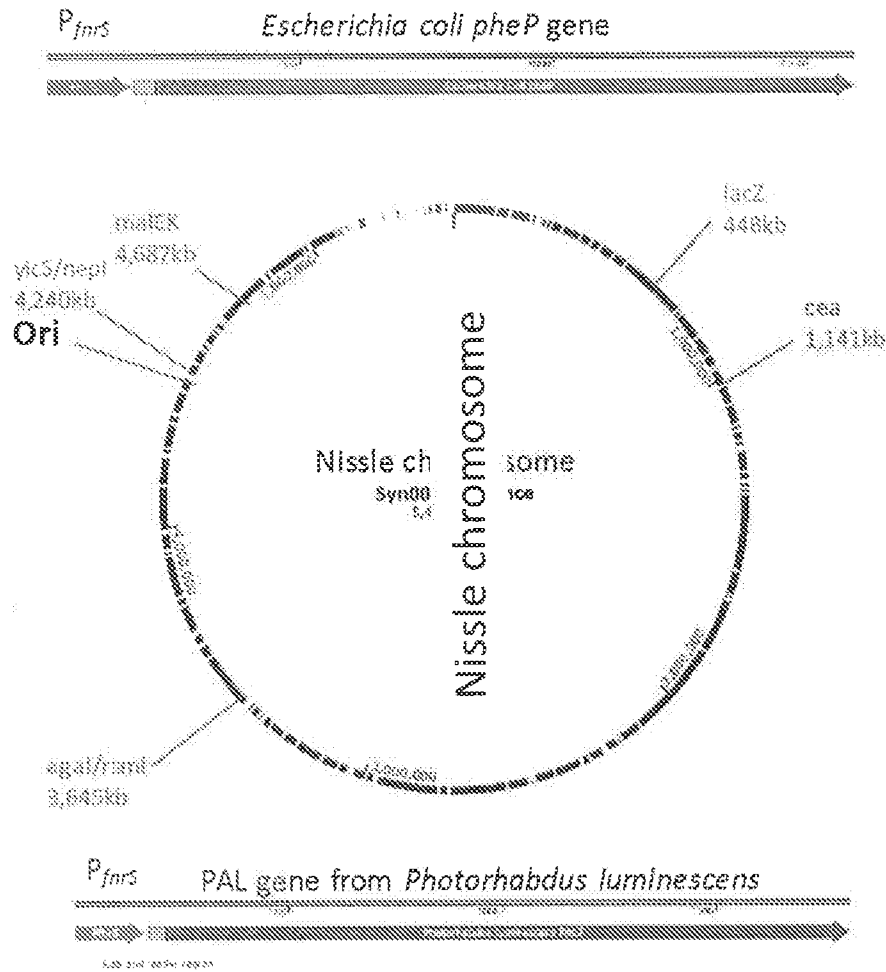


Fig. 13B

Fig. 13C

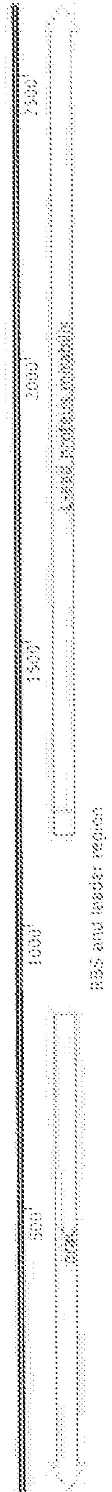


Fig. 14

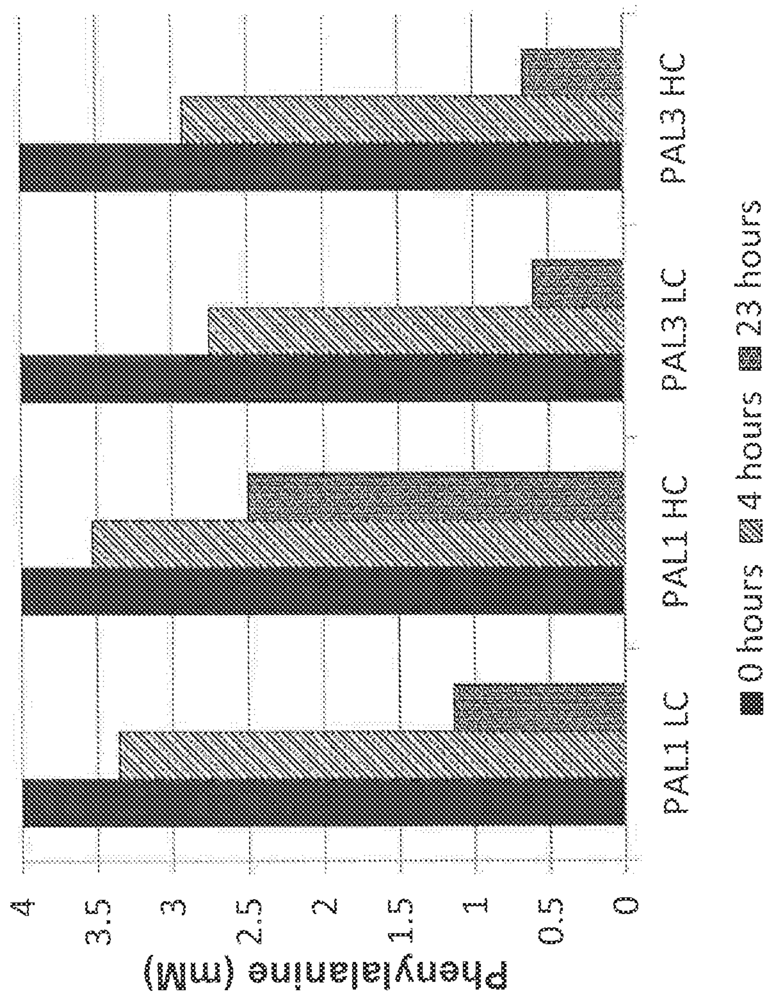


Fig. 15A

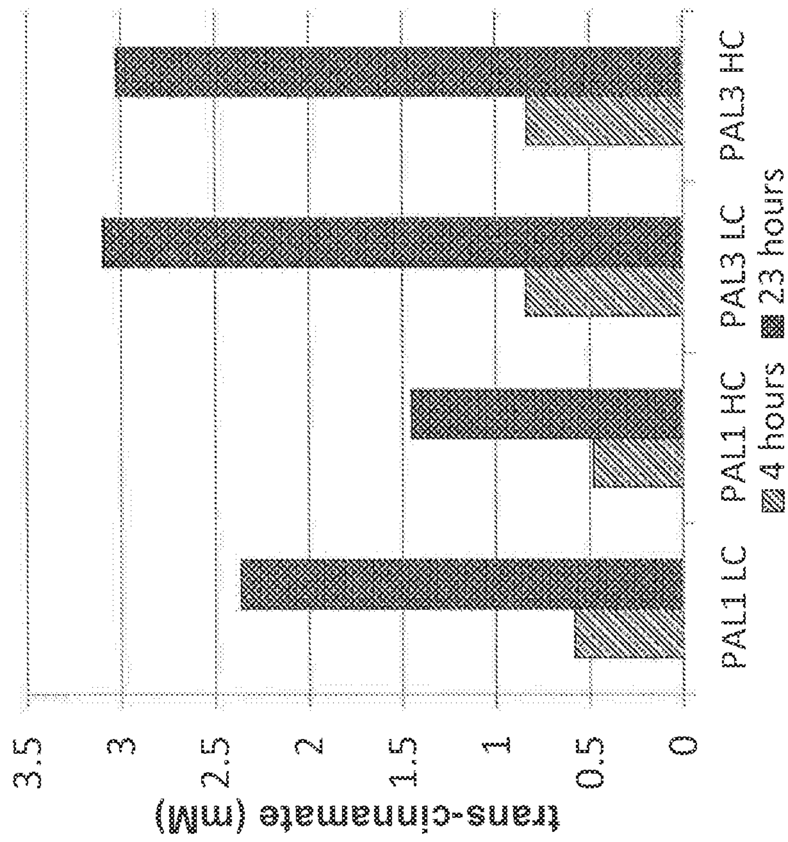


Fig. 15B

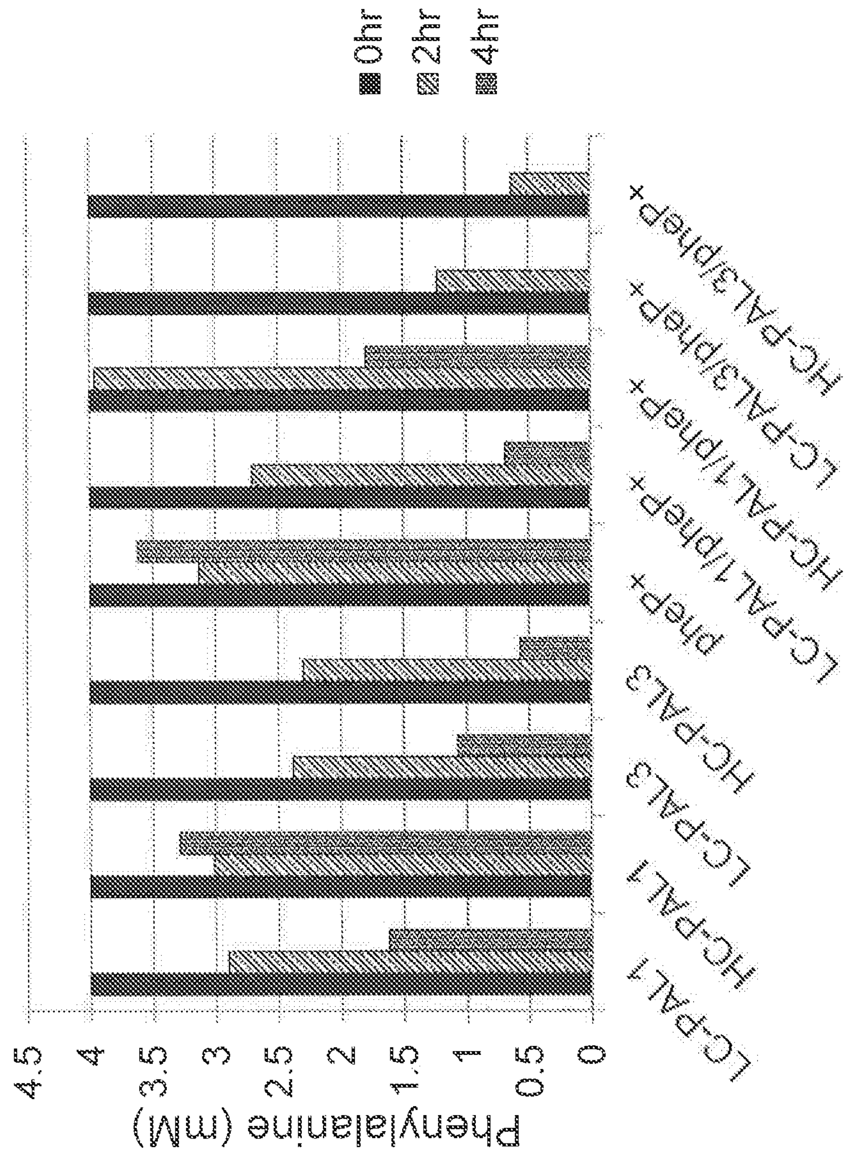


Fig. 16A

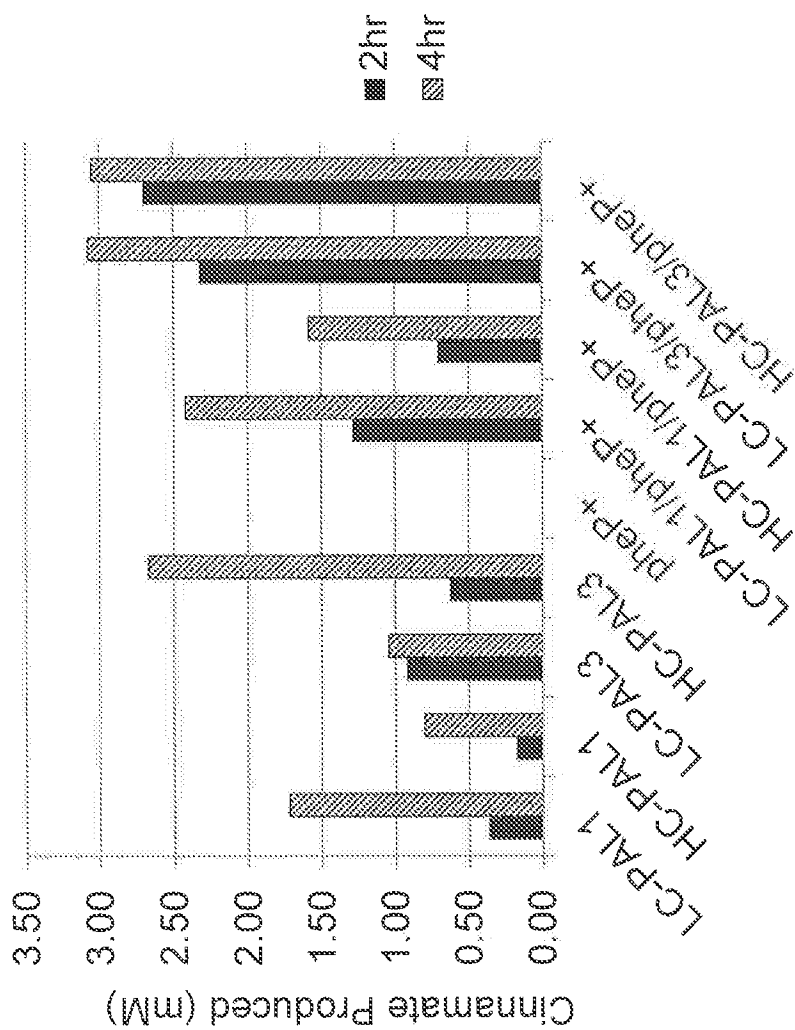


Fig. 16B

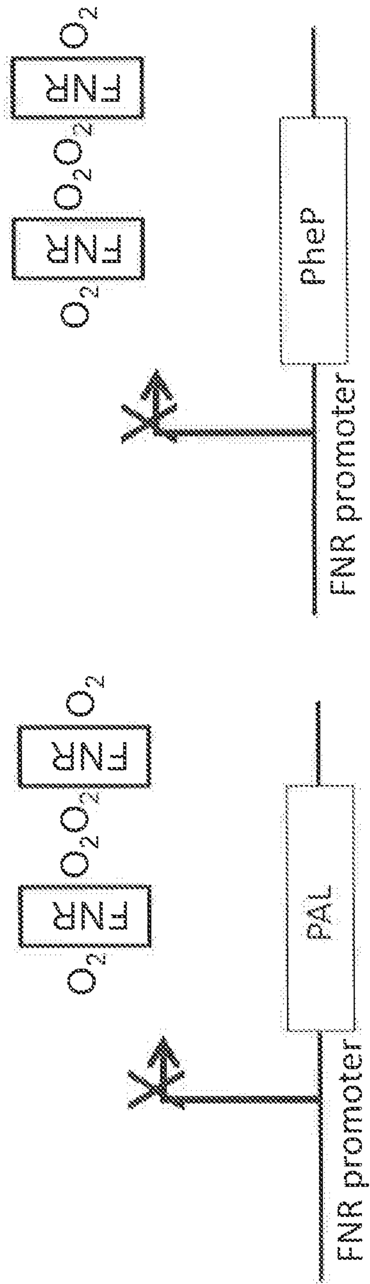


Fig. 17A

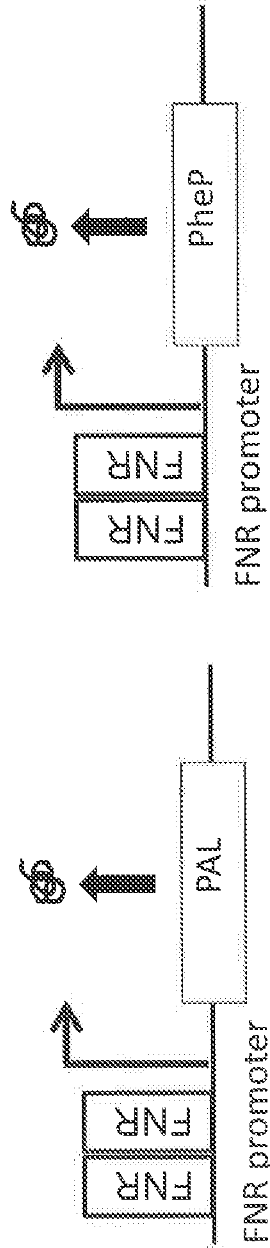


Fig. 17B

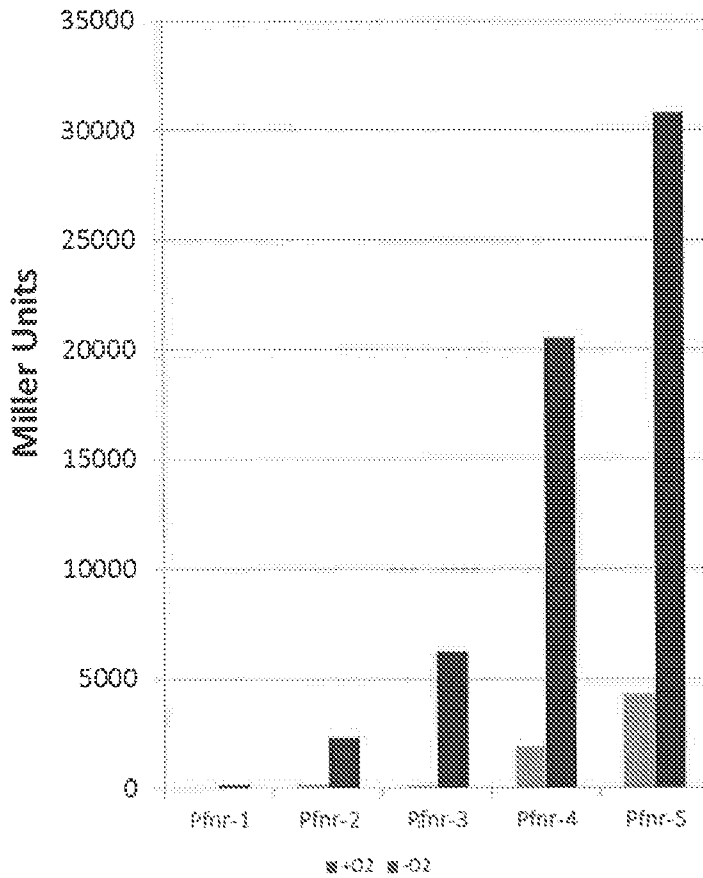


Fig. 18

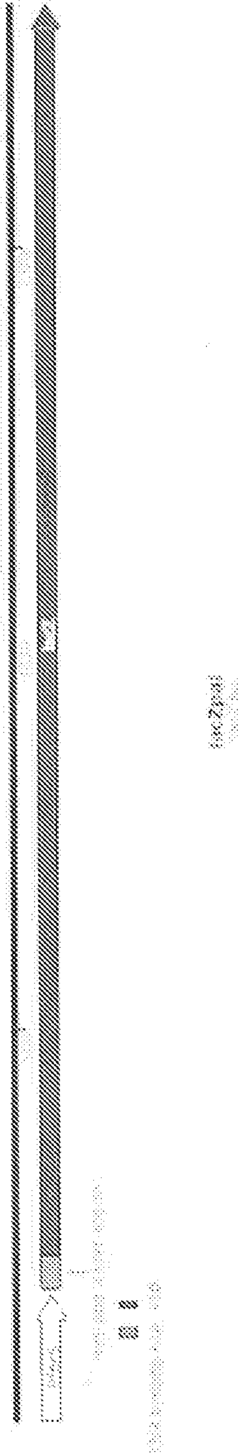


Fig. 19A

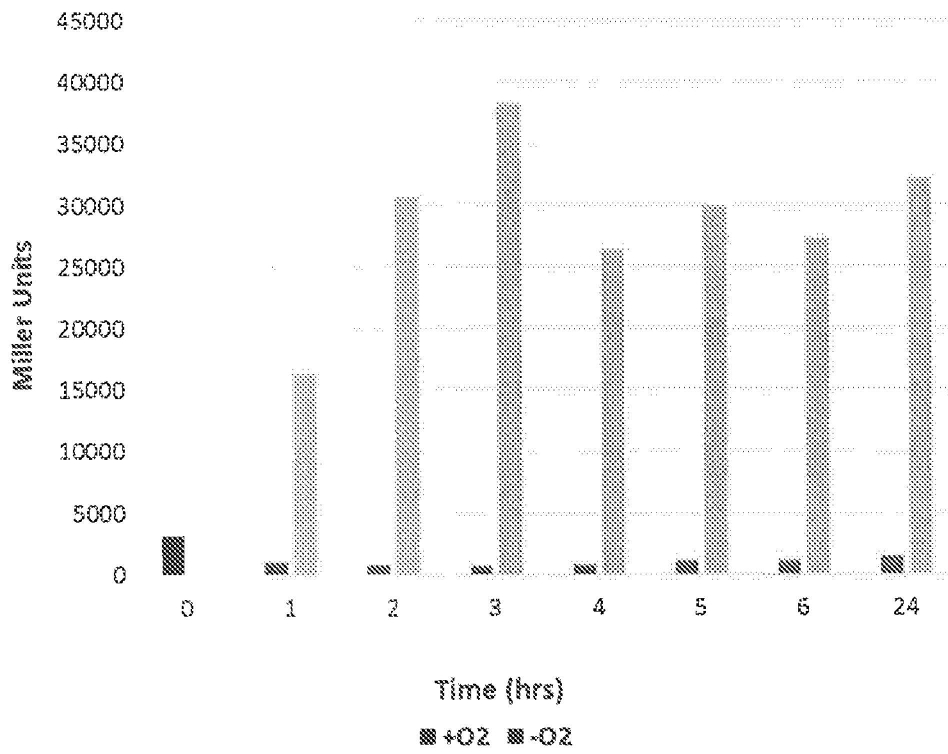


Fig. 19B

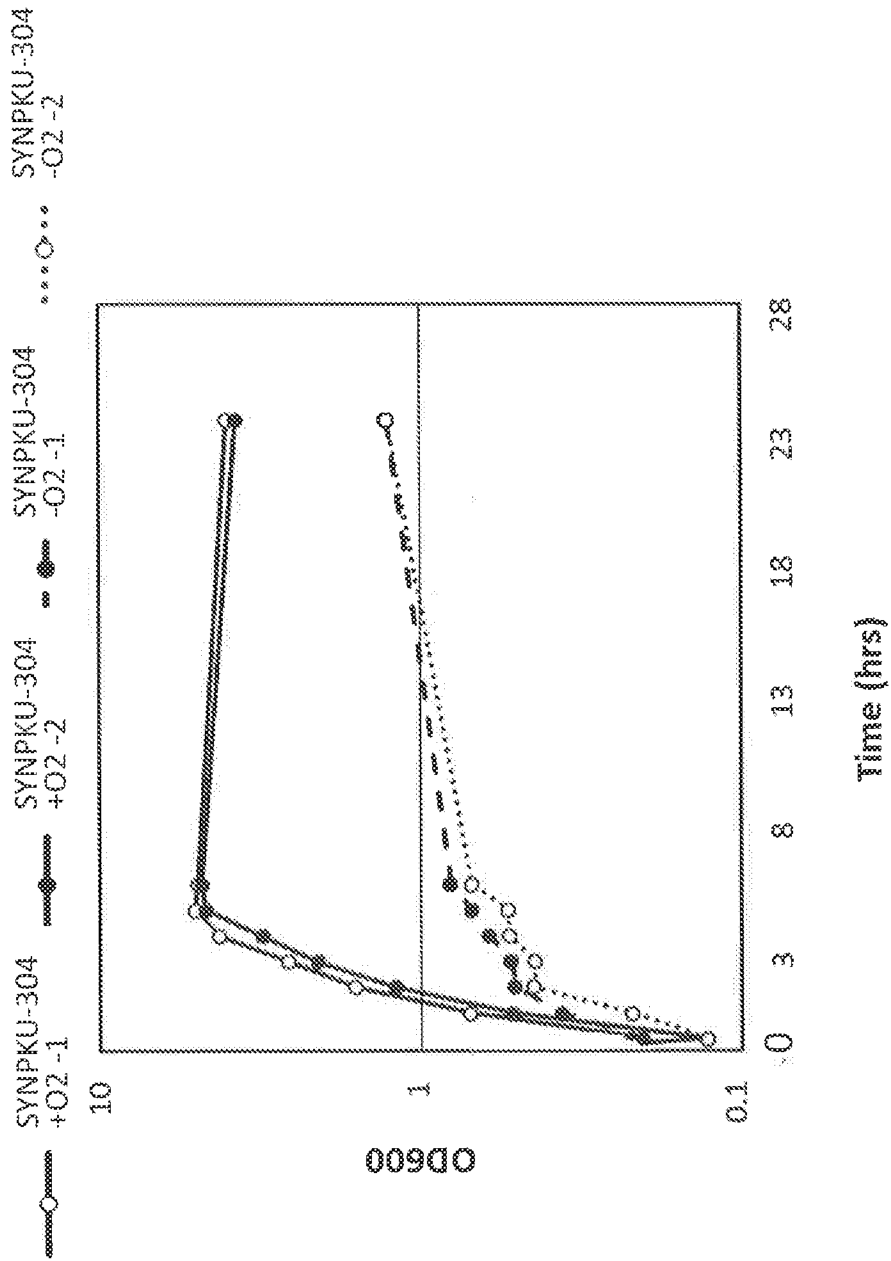


Fig. 19C

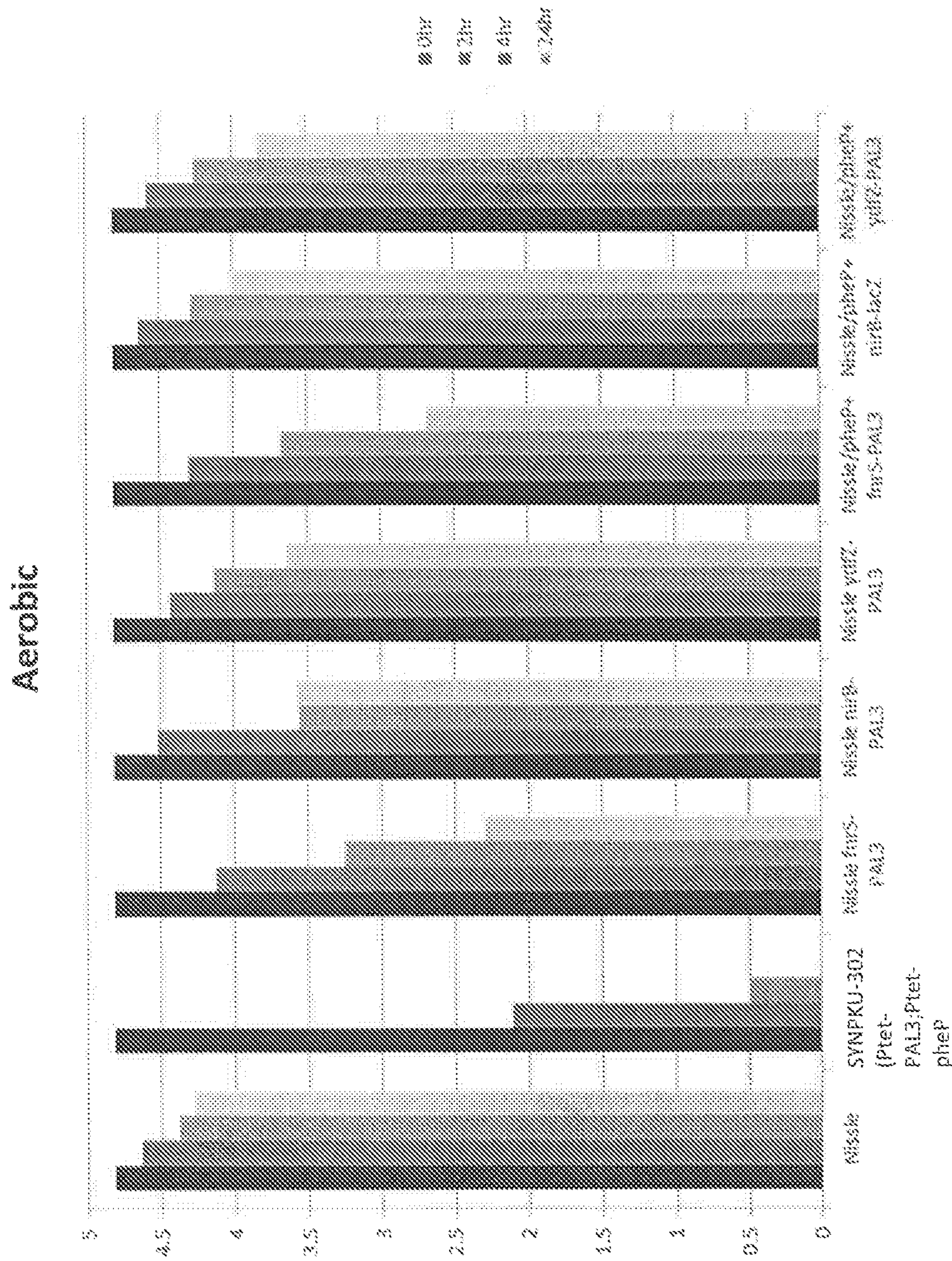


Fig. 20A

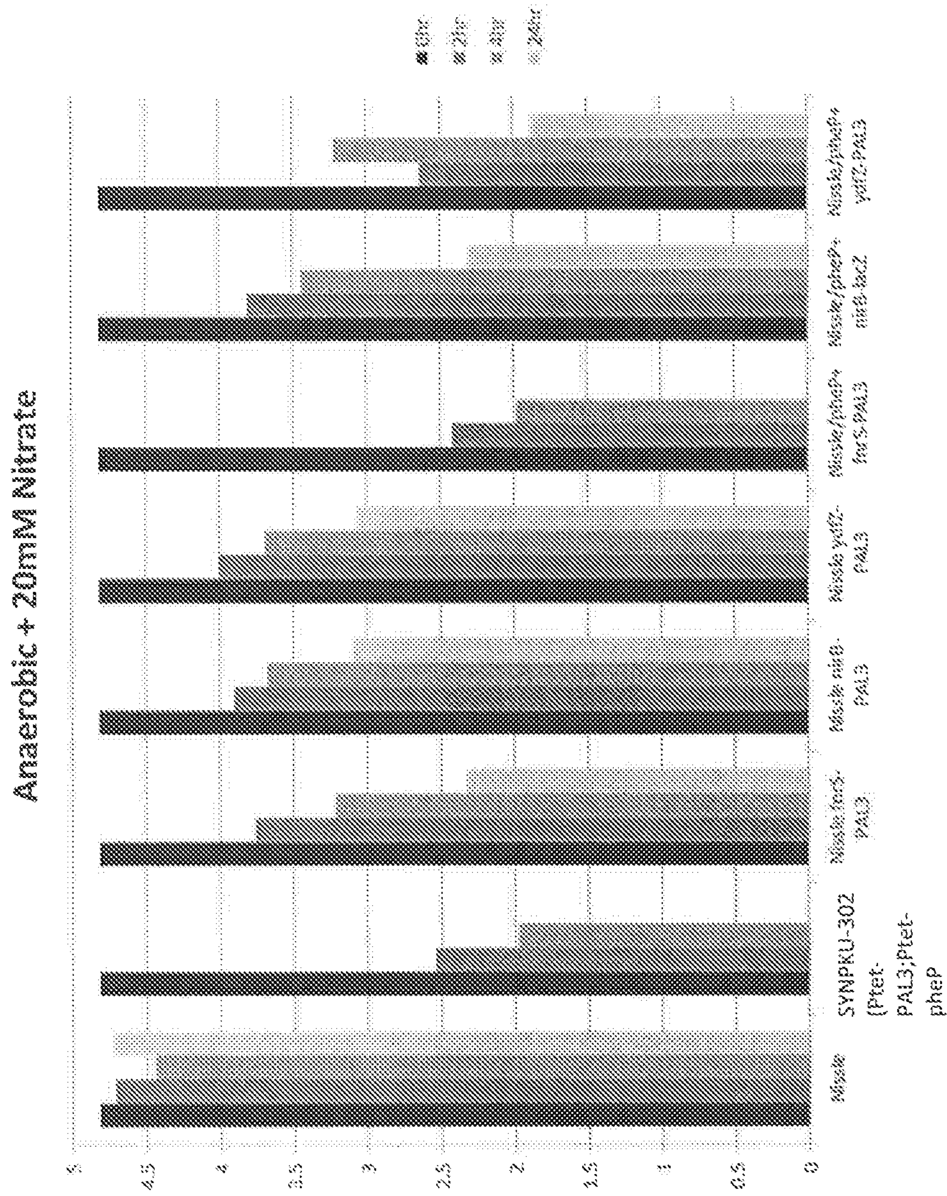


Fig. 20B

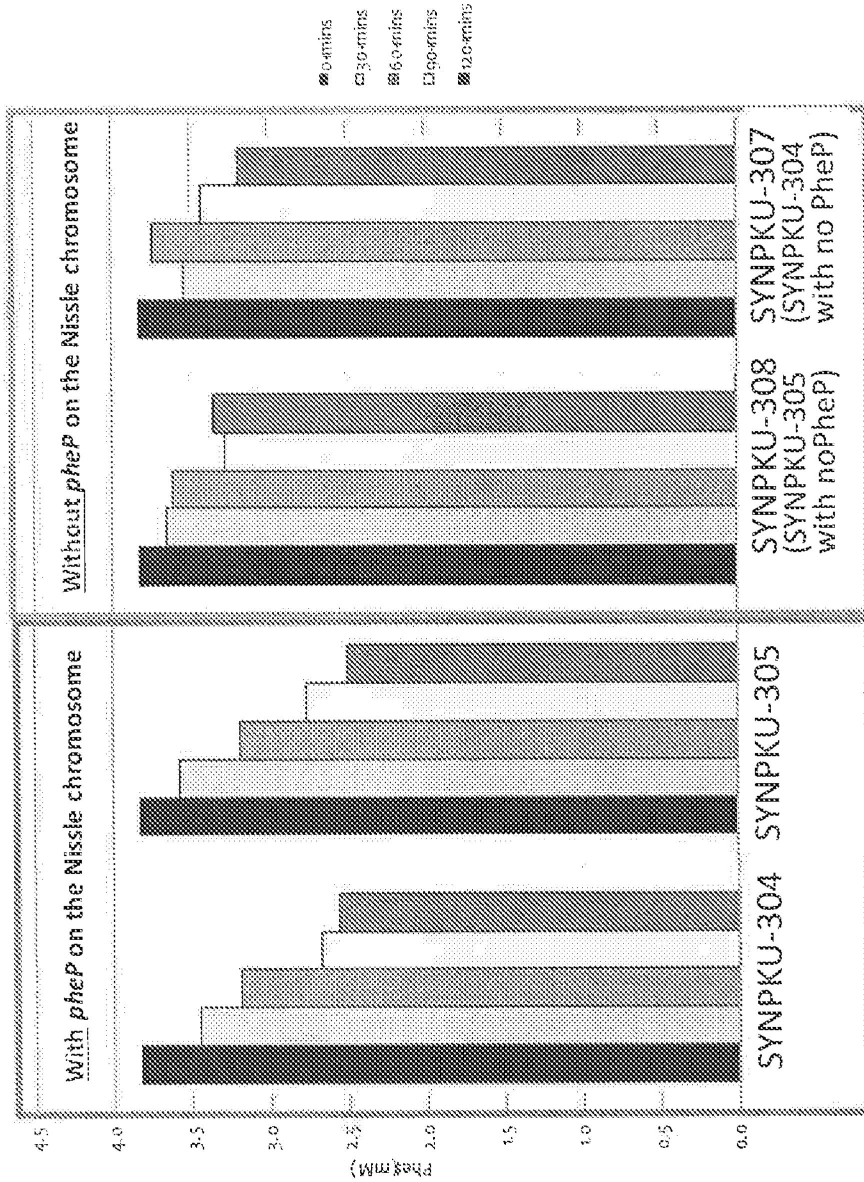


Fig. 21

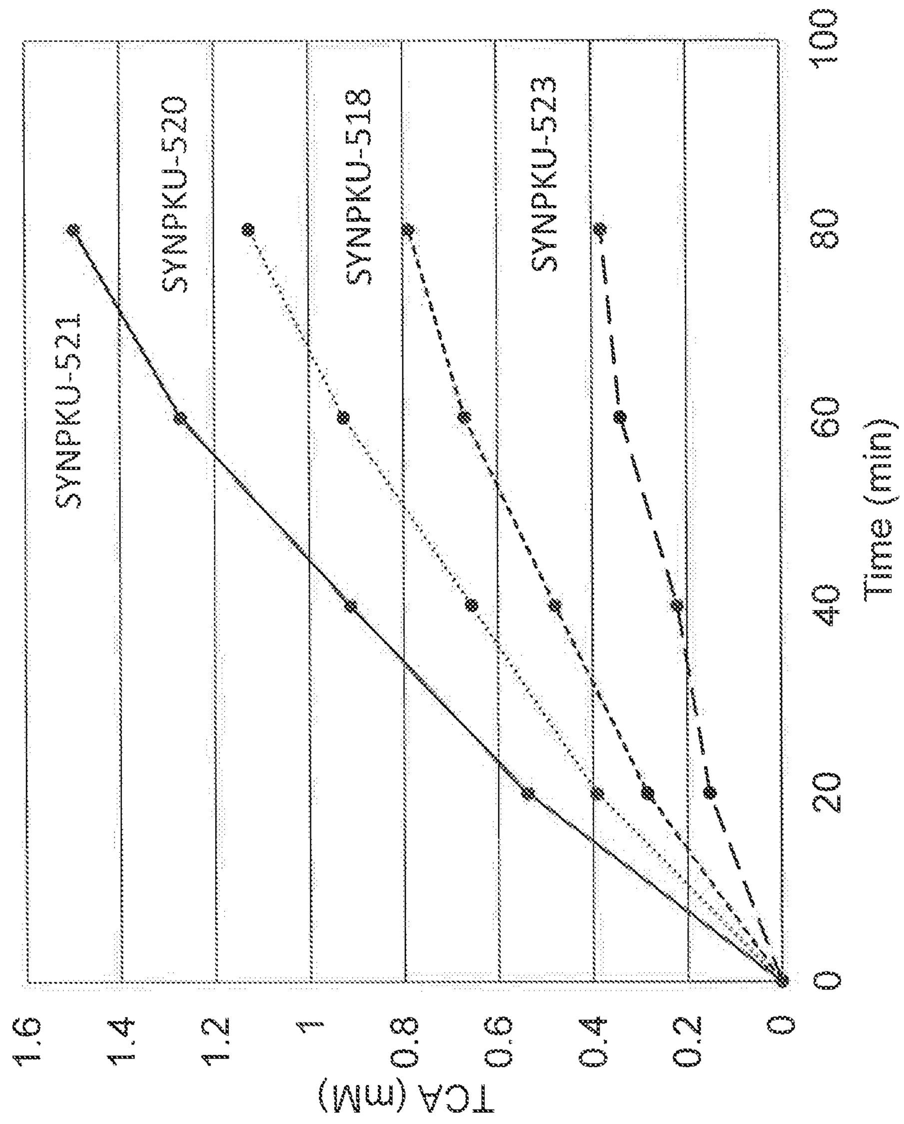


Fig. 22

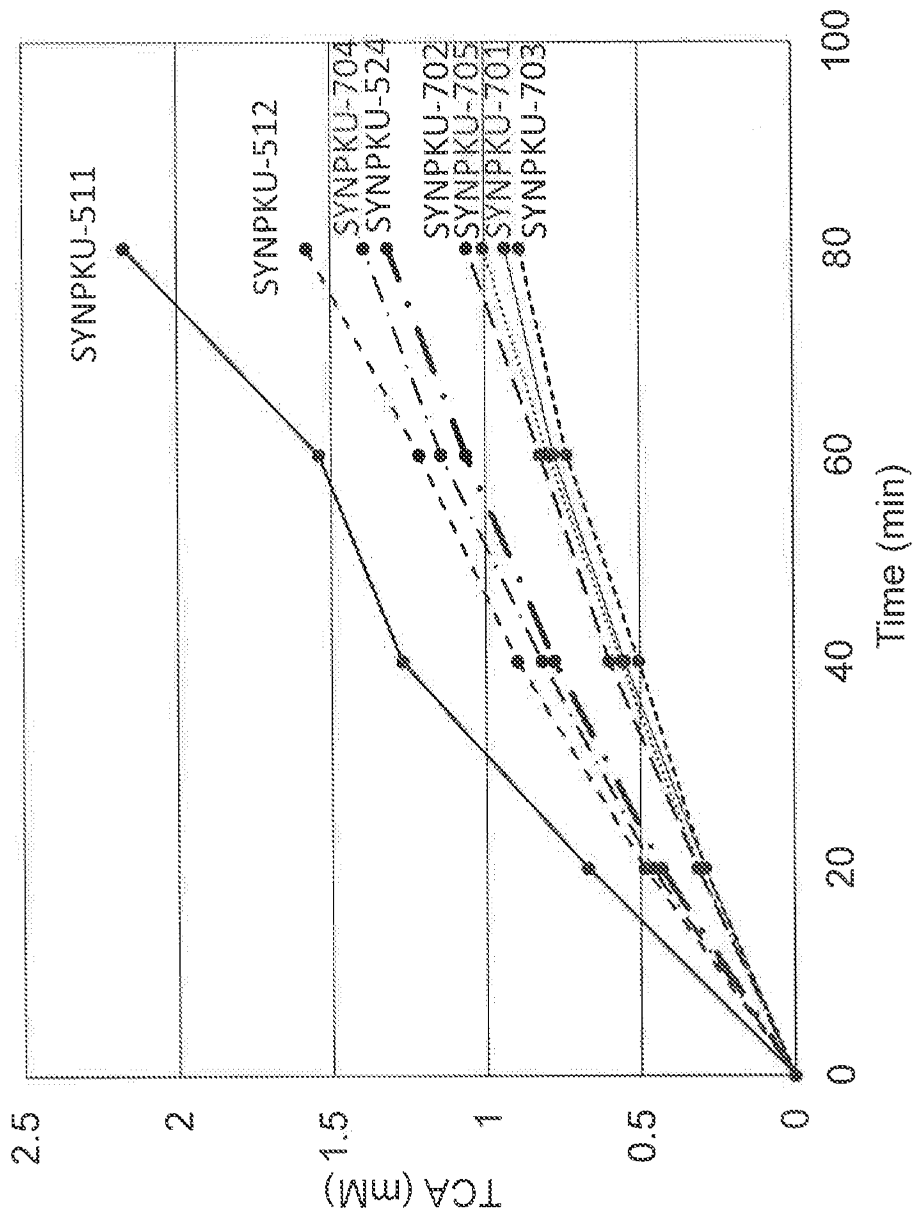
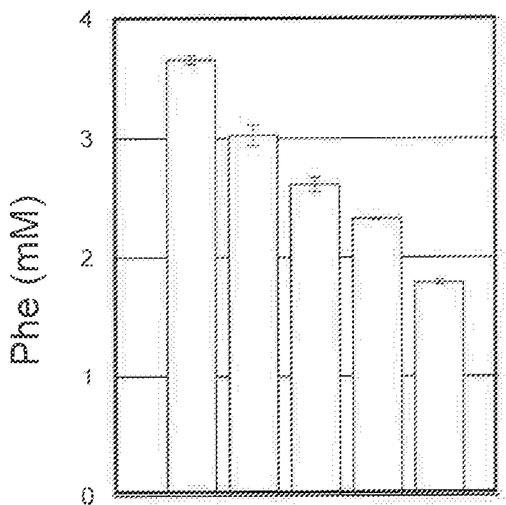


Fig. 23



- In vitro activity of cells: **8umol/hr/1e9 cells**

Fig. 24

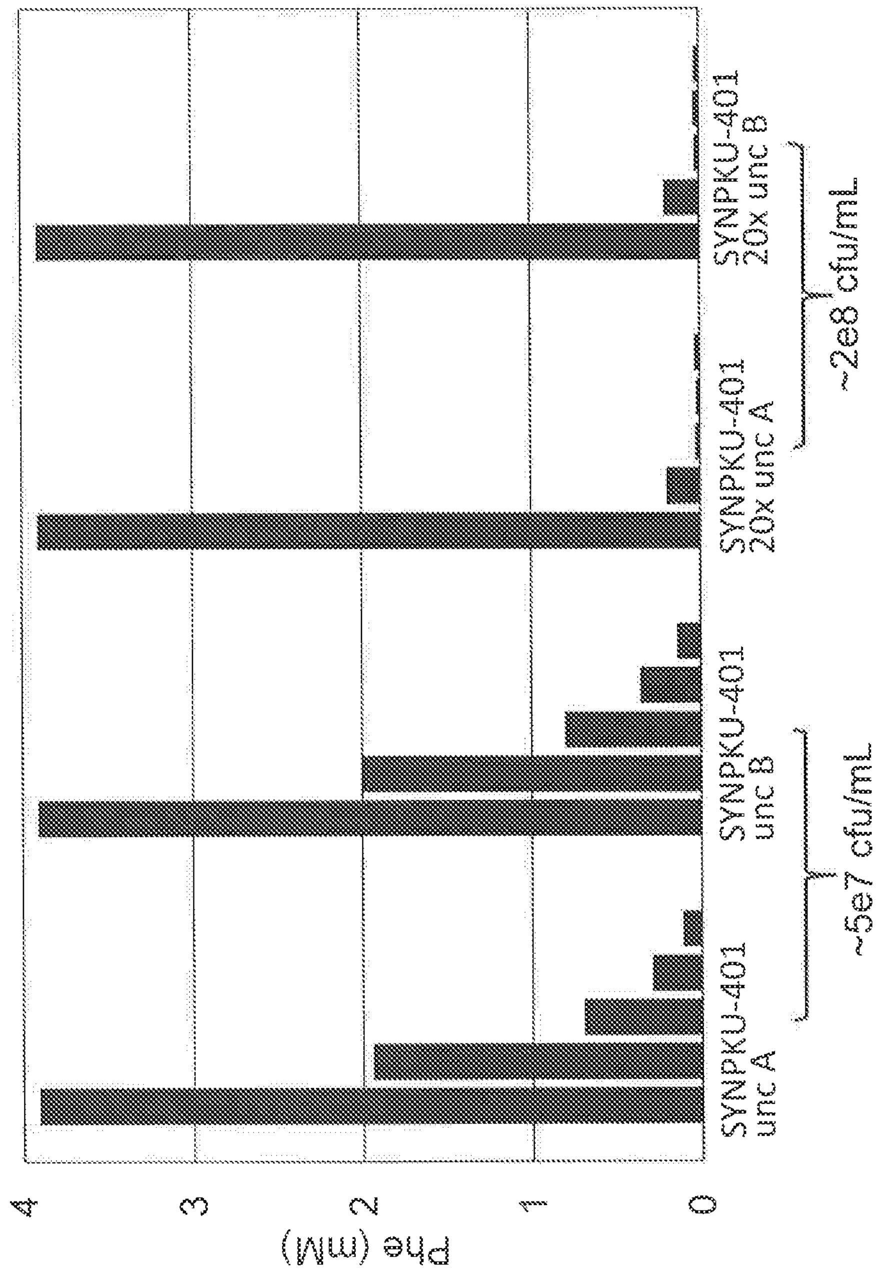


Fig. 25A

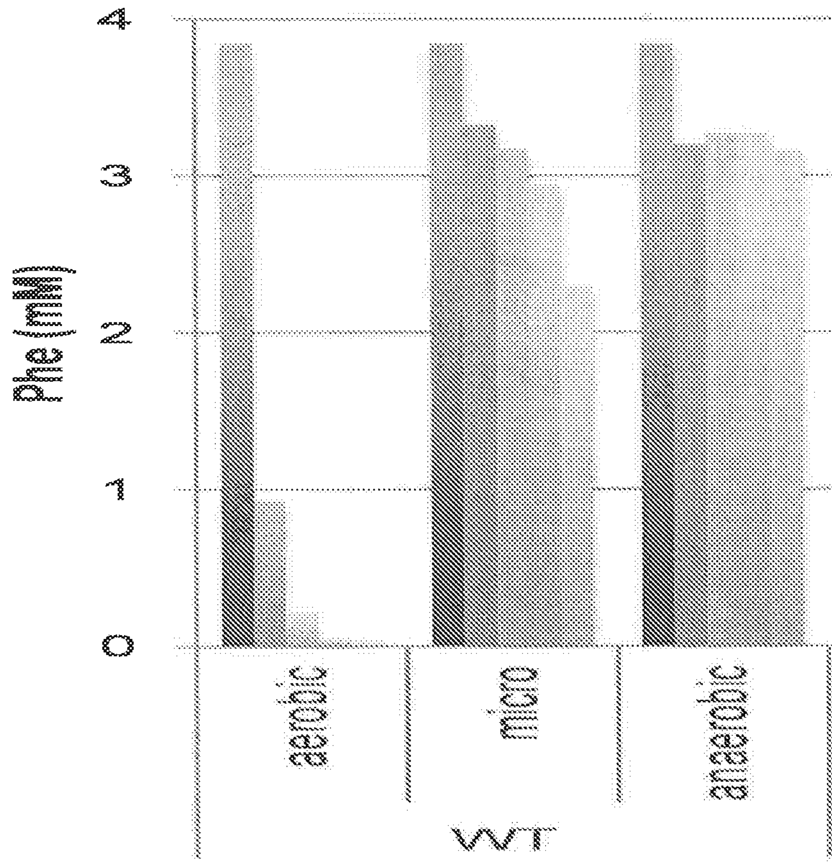


Fig. 25B

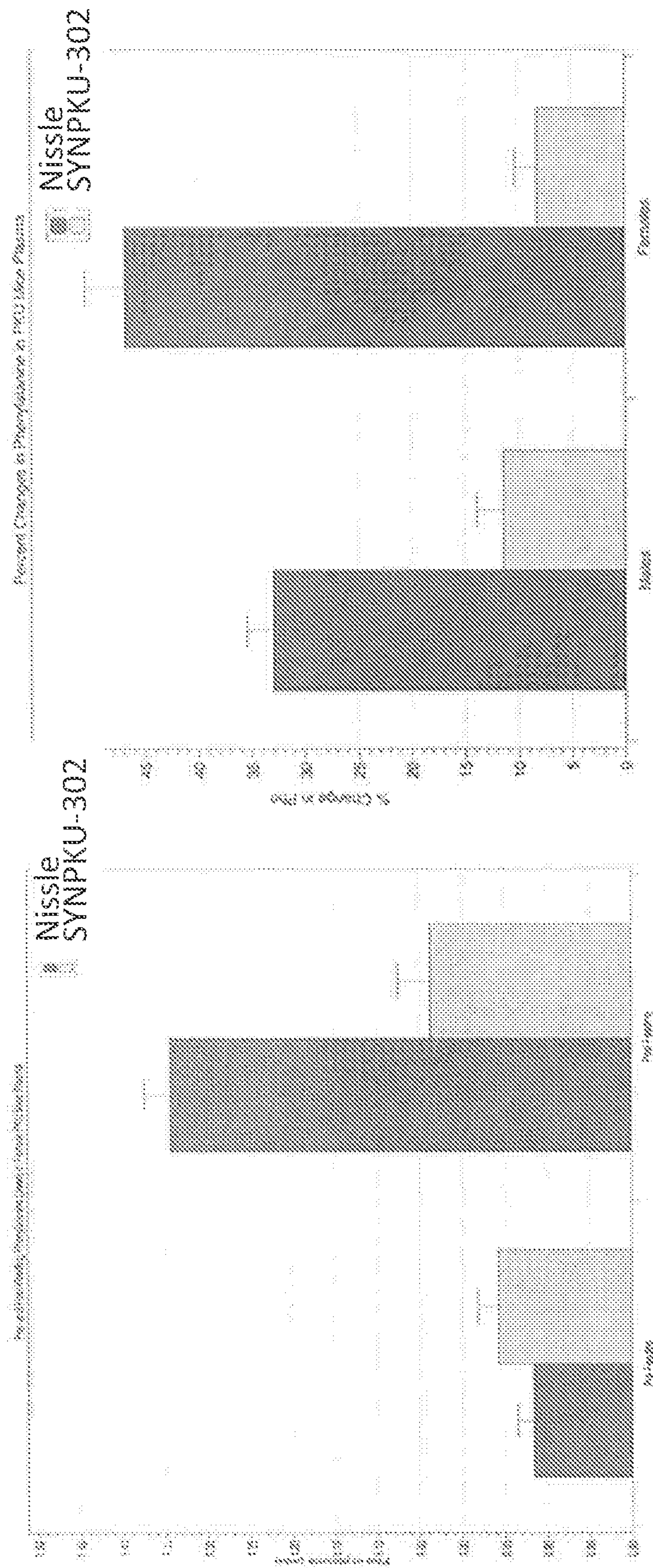


Fig. 26A

Fig. 26B

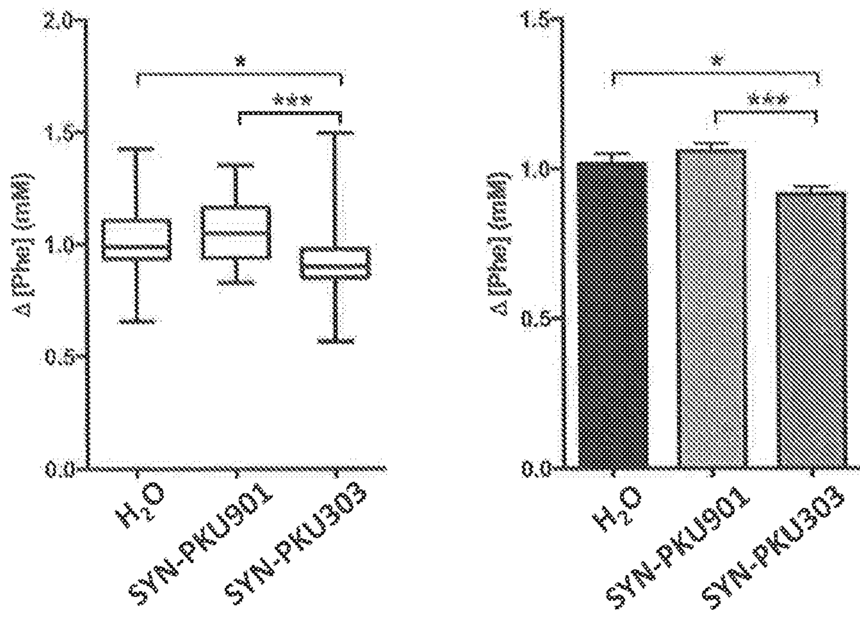


Fig. 27A

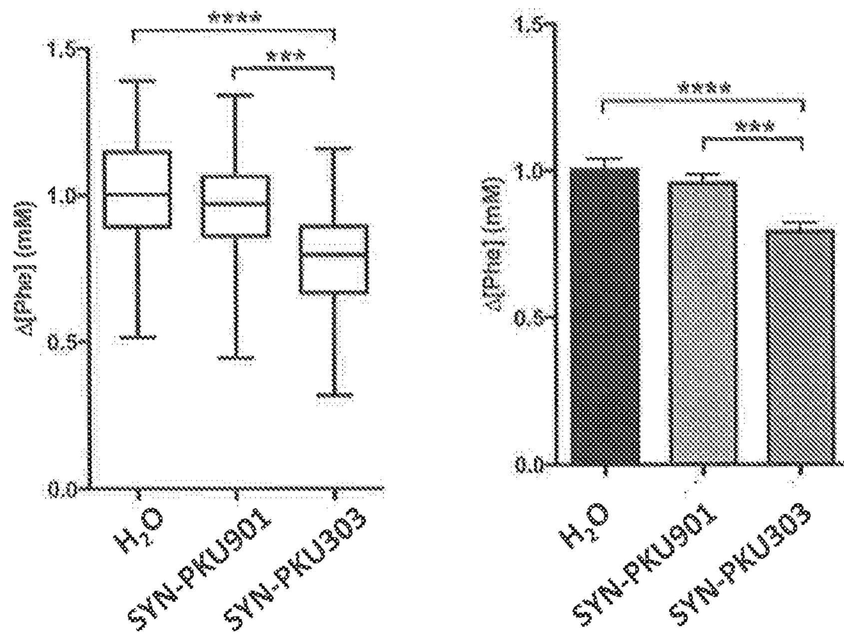


Fig. 27B

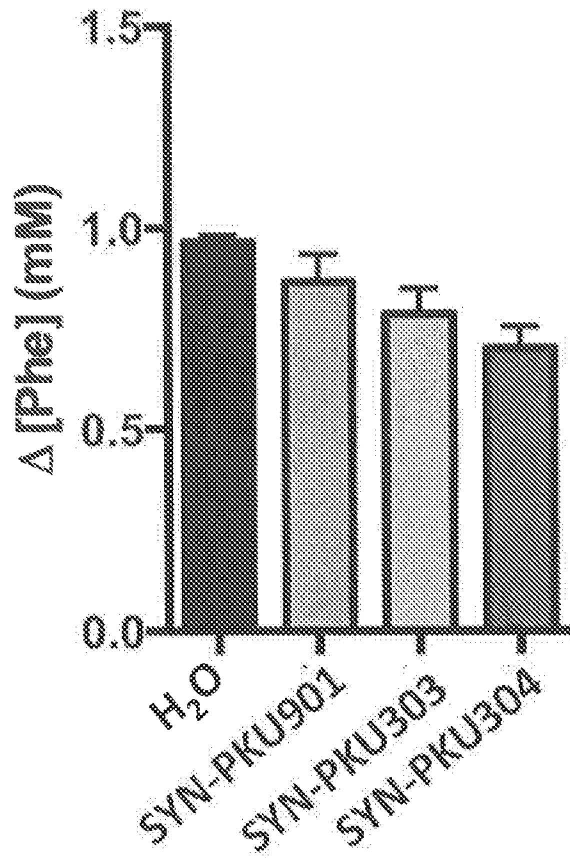


Fig. 28

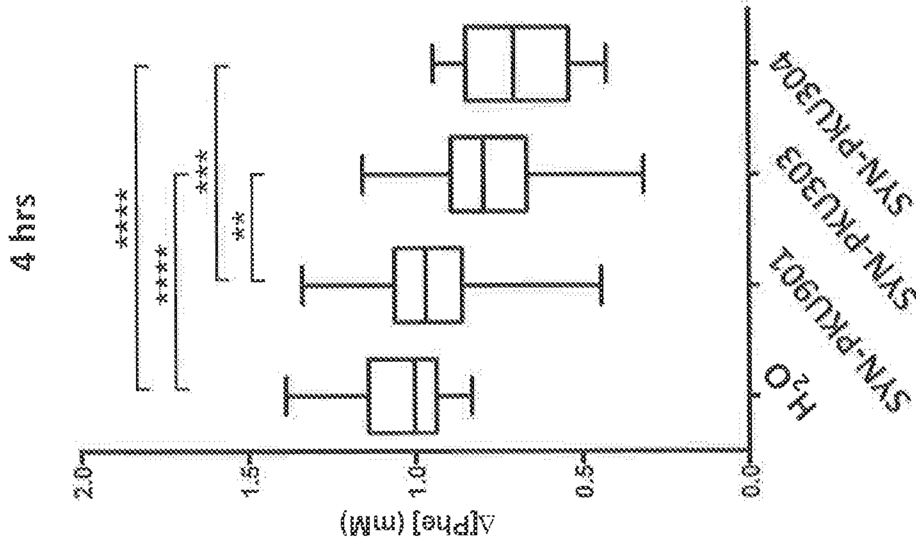


Fig. 29B

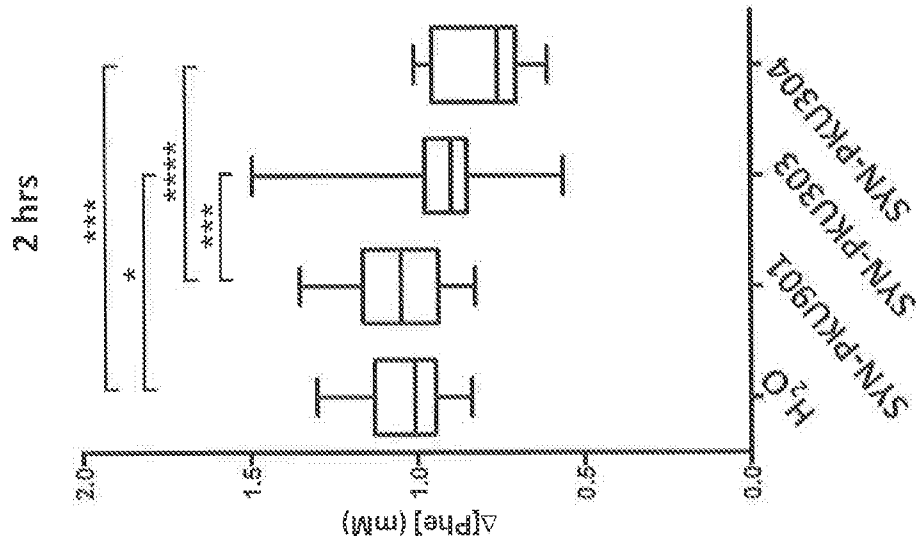


Fig. 29A

P-values: ANOVA with Tukey pairwise comparison
* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

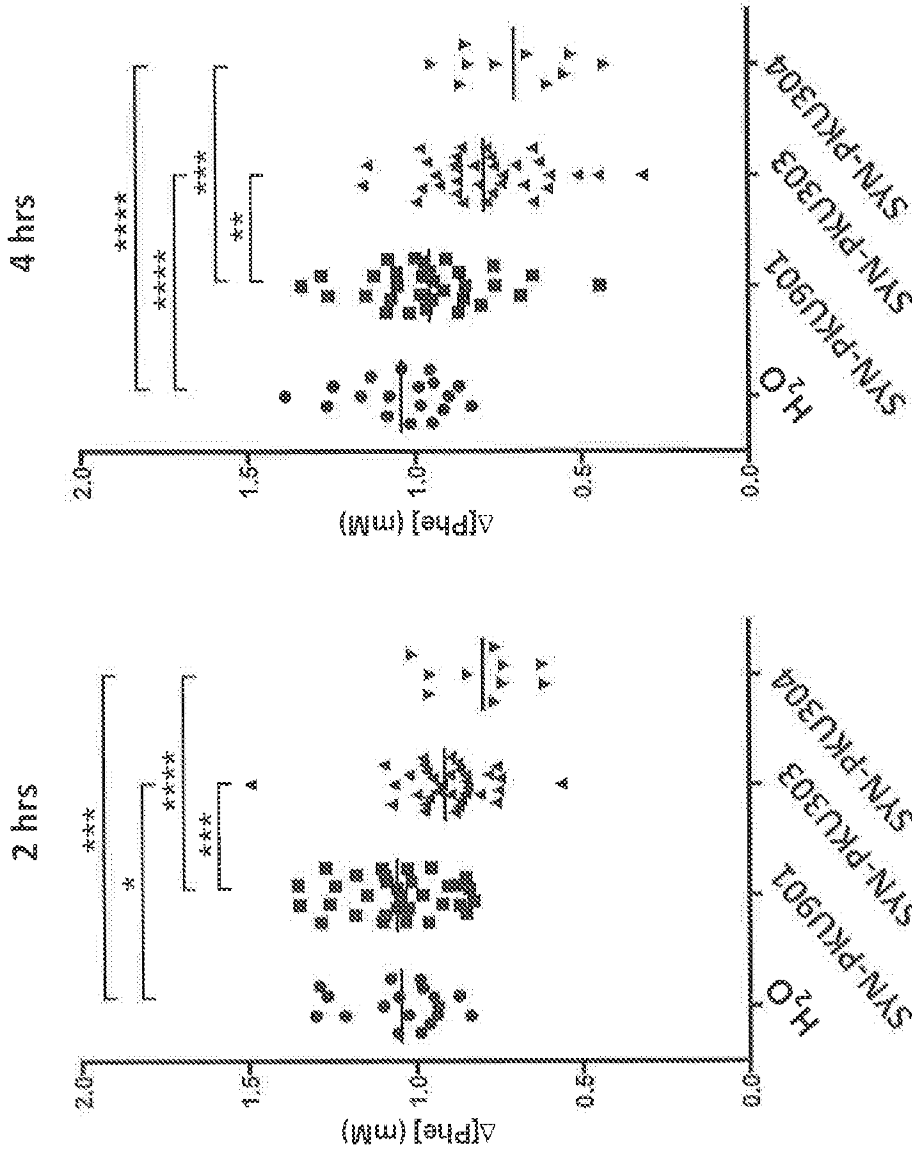


Fig. 29C

Fig. 29D

P-values: ANOVA with Tukey pairwise comparison
* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

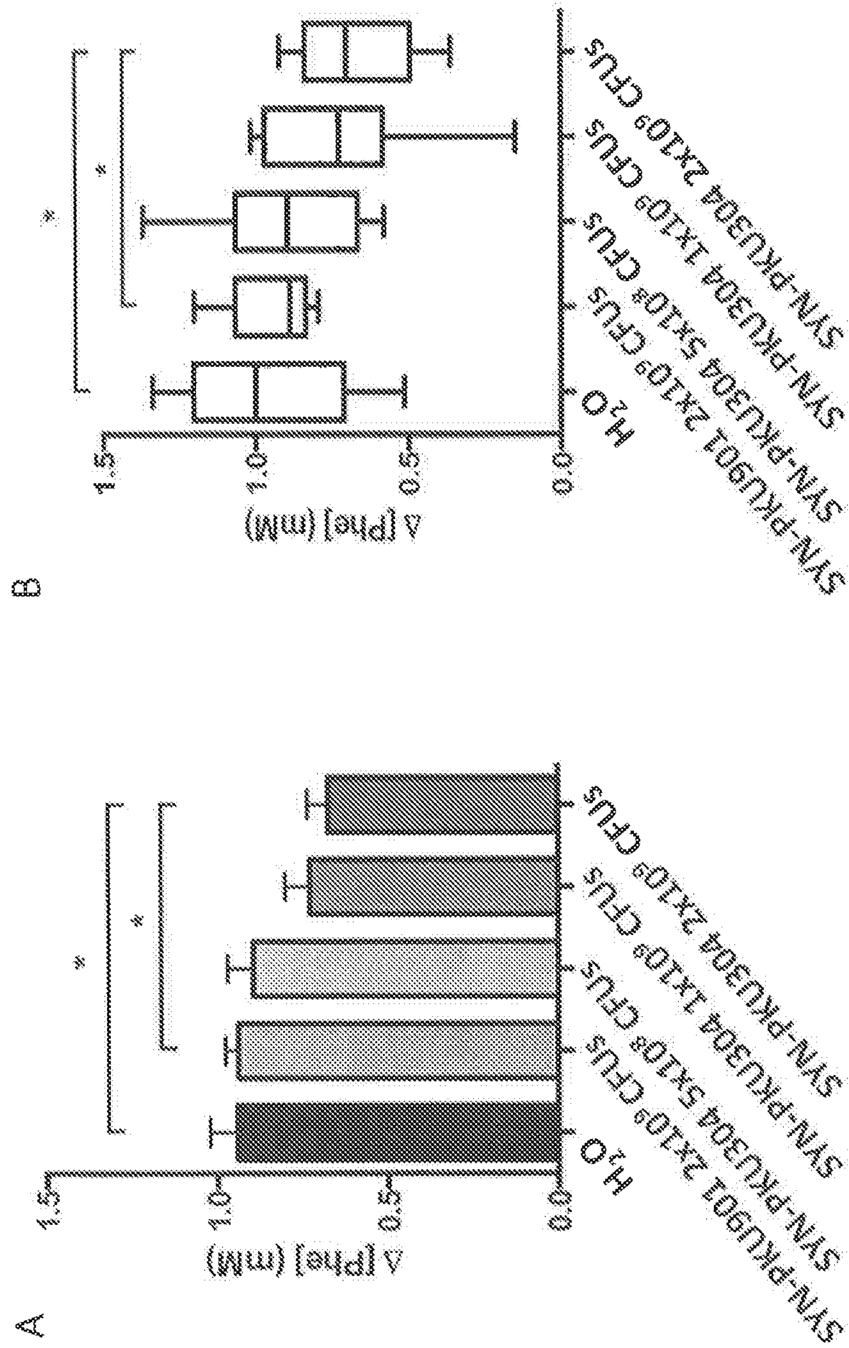


Fig. 30A

Fig. 30B

PKU specific:

Lacking
in PKU

Phenylalanine

Tyrosine

Phenylpyruvic acid

Phenyllactic acid

Fig. 31A

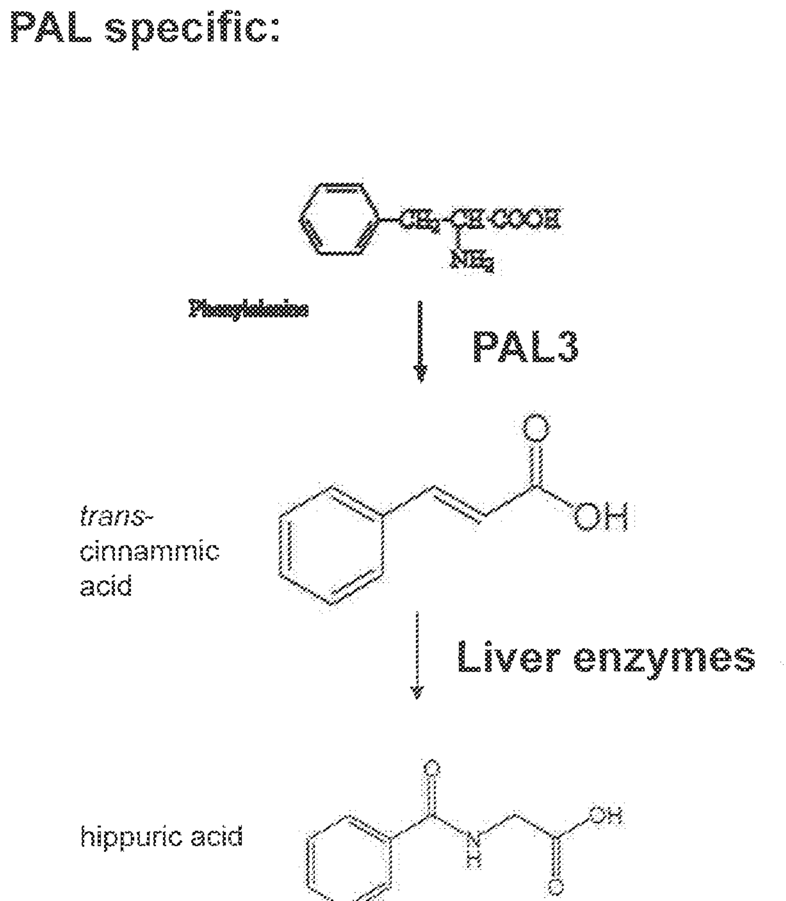


Fig. 31B

Fig. 32A

Fig. 32B

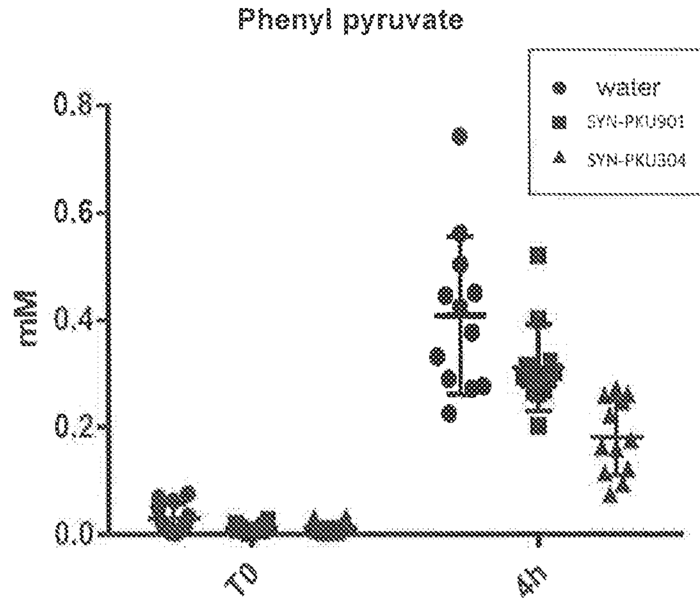


Fig.32C

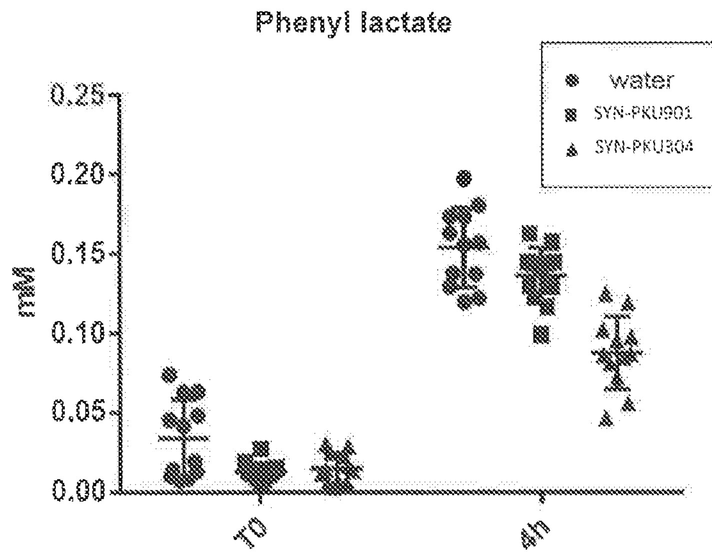


Fig. 32D

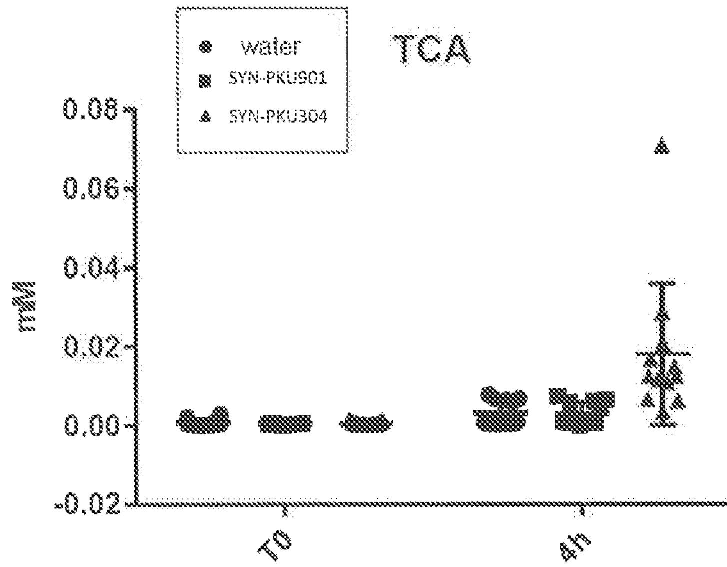


Fig. 32E

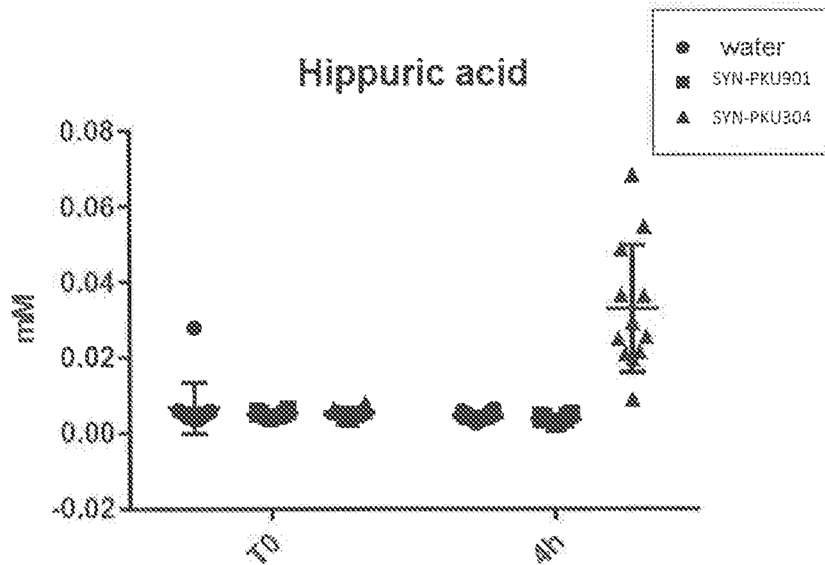


Fig. 32F

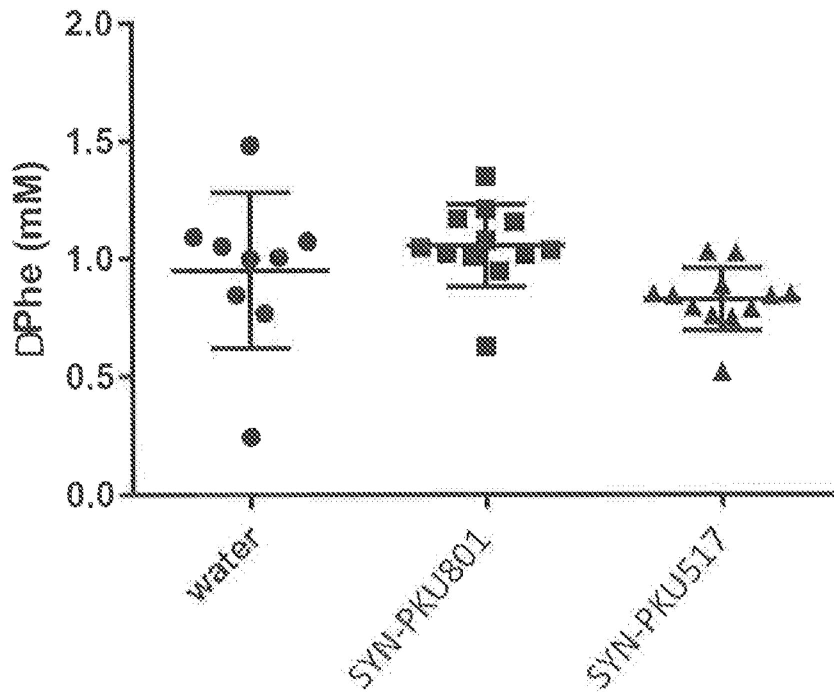


Fig. 33A

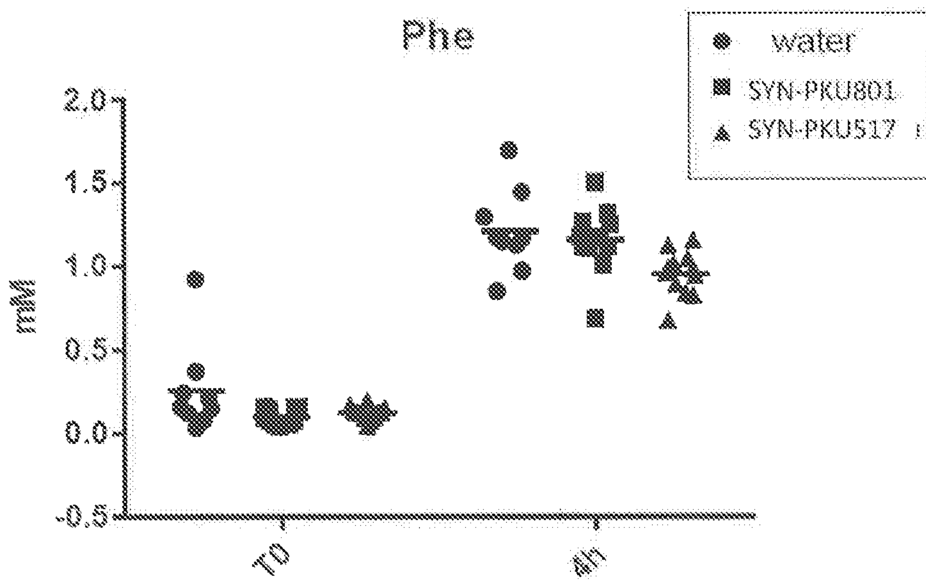


Fig. 33B

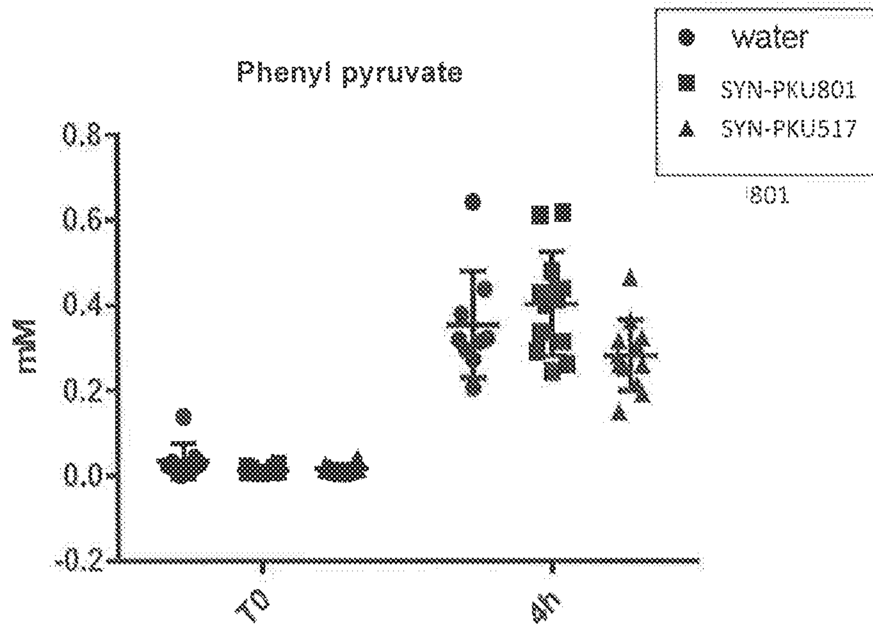


Fig. 33C

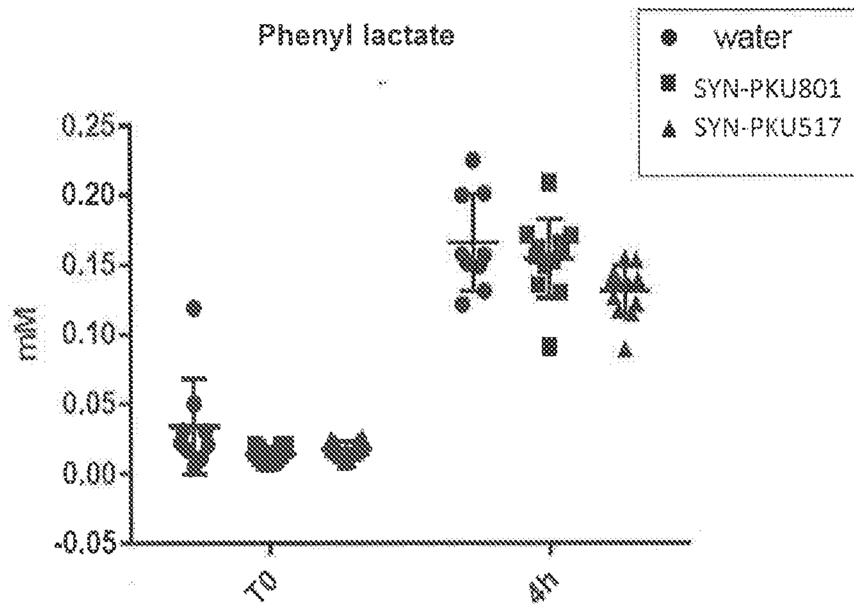


Fig. 33D

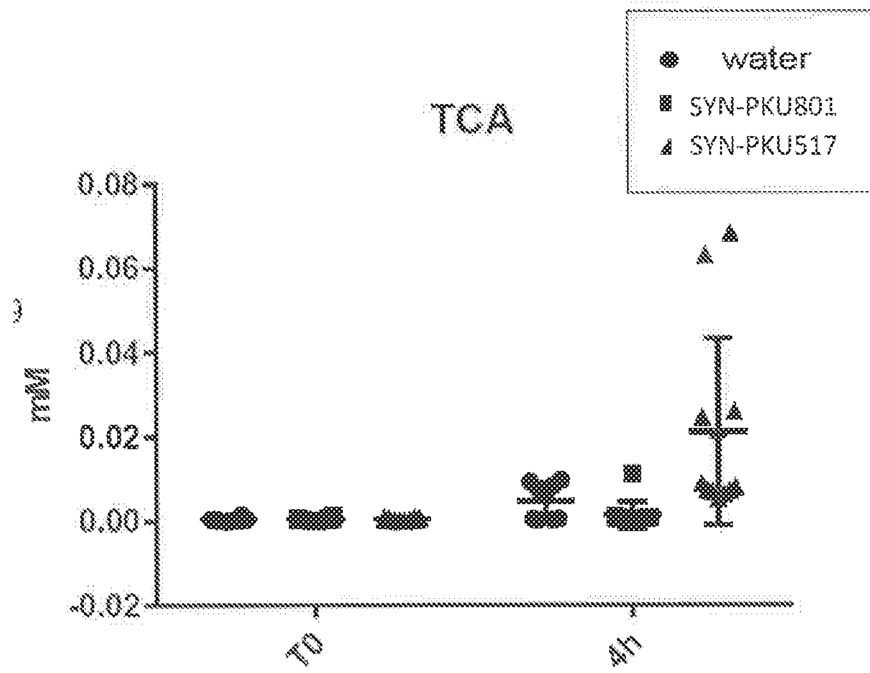


Fig. 33E

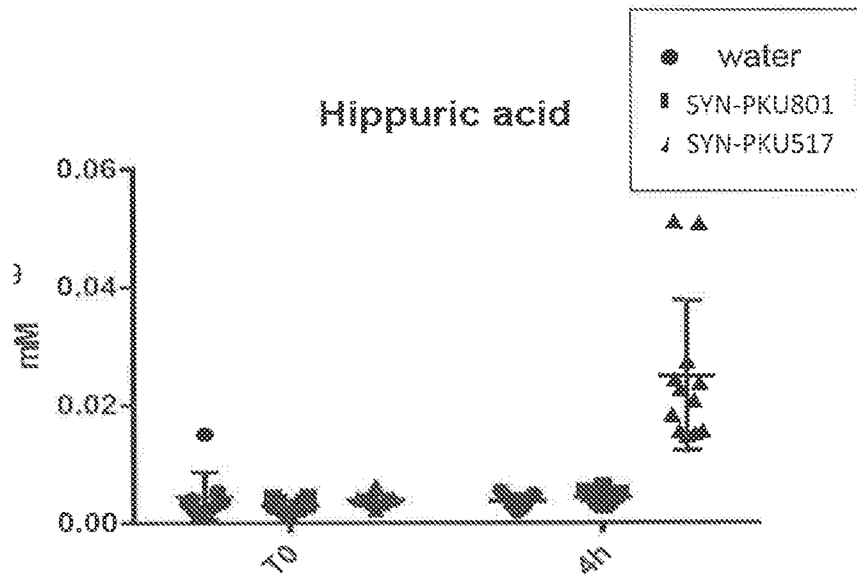


Fig. 33F

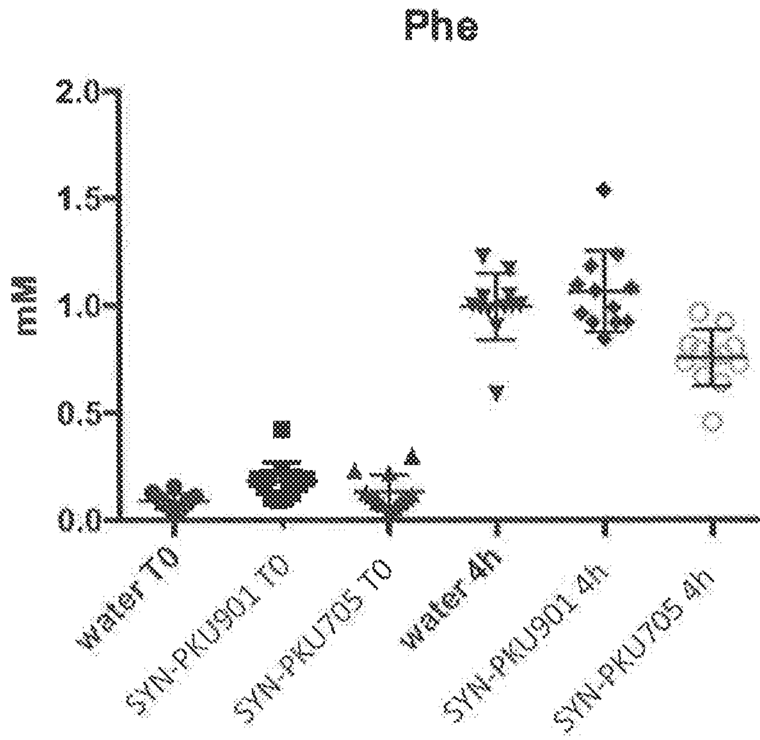


Fig. 34A

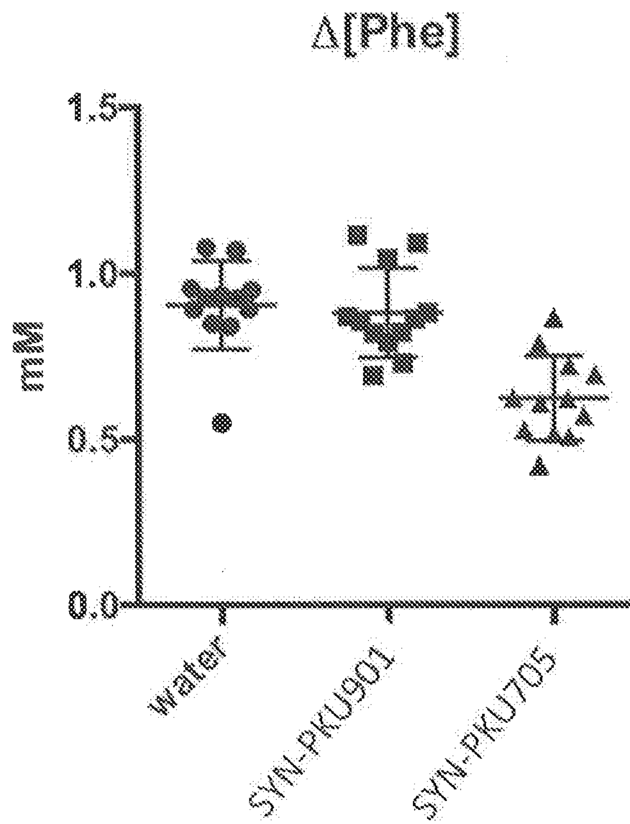


Fig. 34B

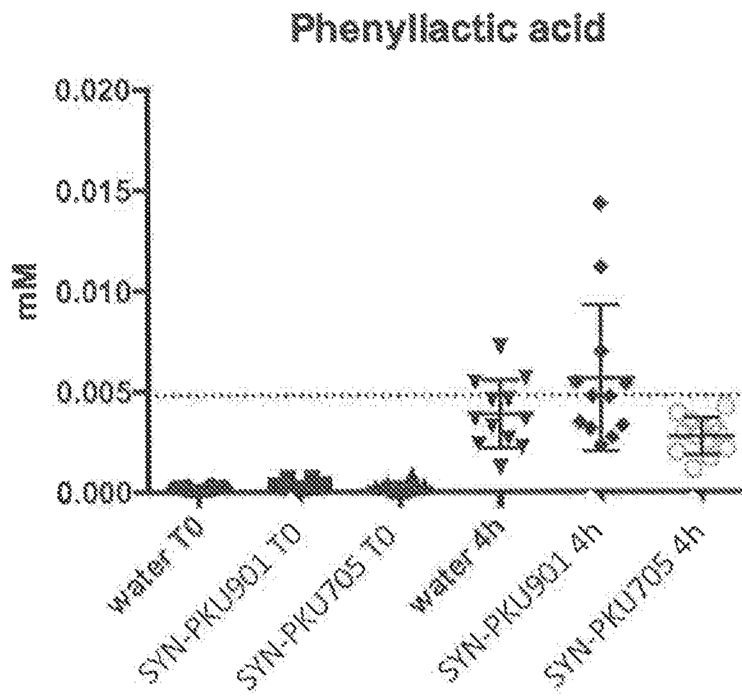


Fig. 34C

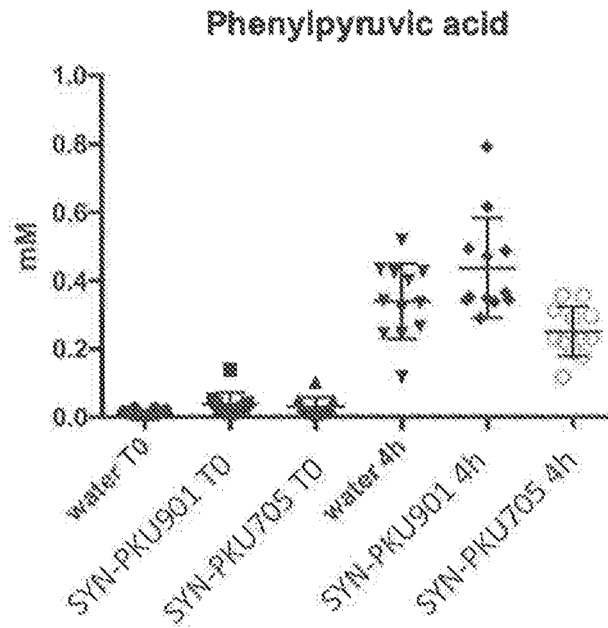


Fig. 34D

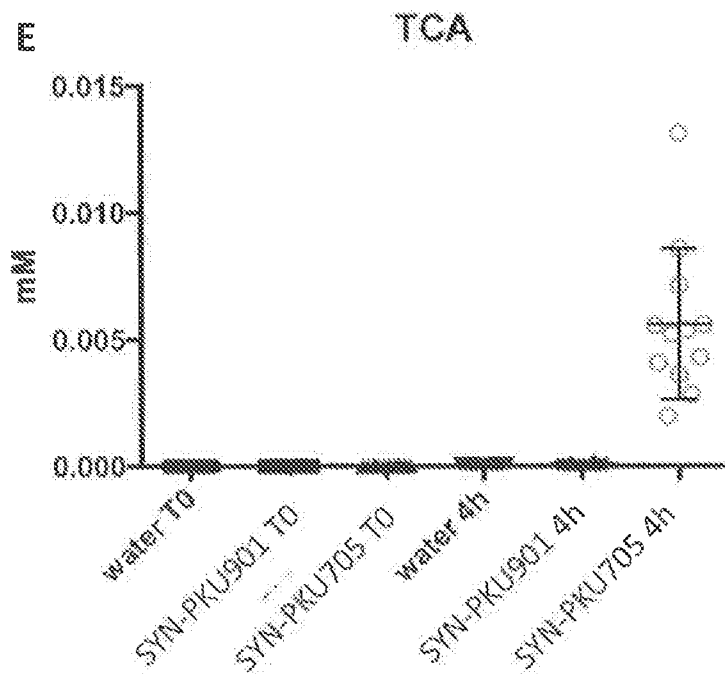


Fig. 34E

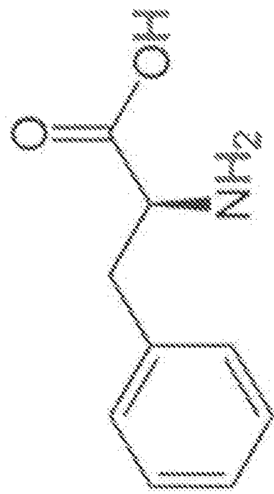
Hippuric acid

mM

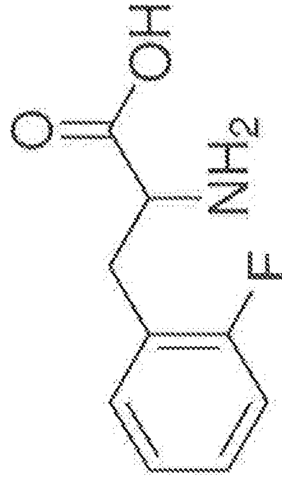
0.020
0.015
0.010
0.005
0.000

water T0
SYN-PKU901 T0
SYN-PKU705 T0
water 4h
SYN-PKU901 4h
SYN-PKU705 4h

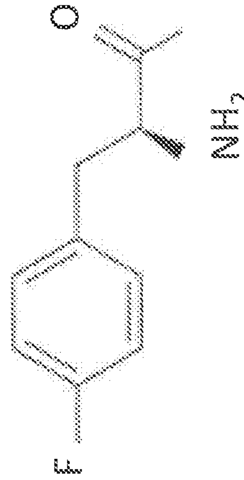
Fig. 34F



phenylalanine



o-fluoro-DL-phenylalanine



p-fluoro-DL-phenylalanine

Fig. 35

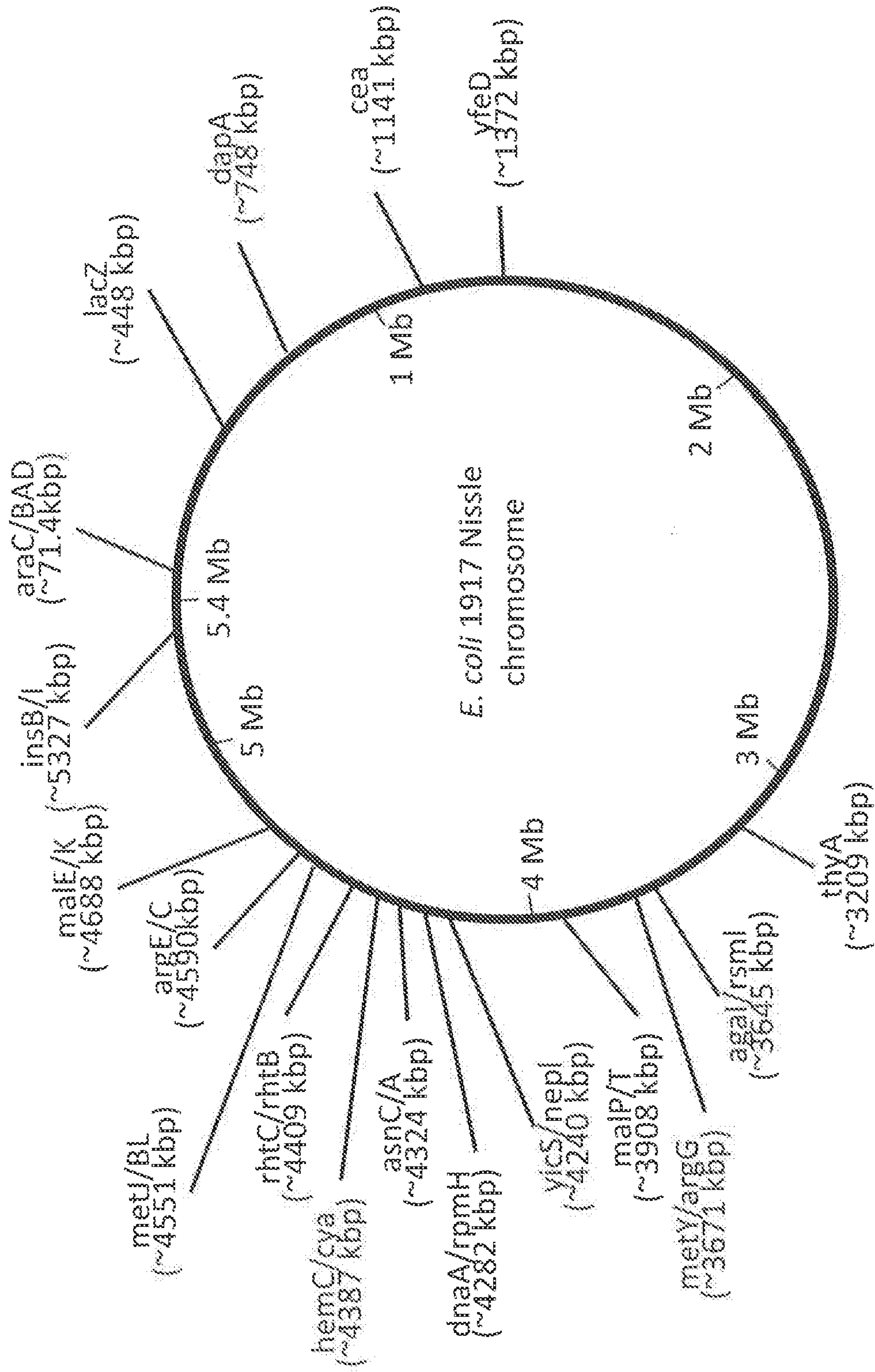


Fig. 36

Brightness of constitutive RFP integrated in three locations:

1. AraB/C
2. MalE/K
3. MetY/ArgG
4. Nissle (non-fluorescent)

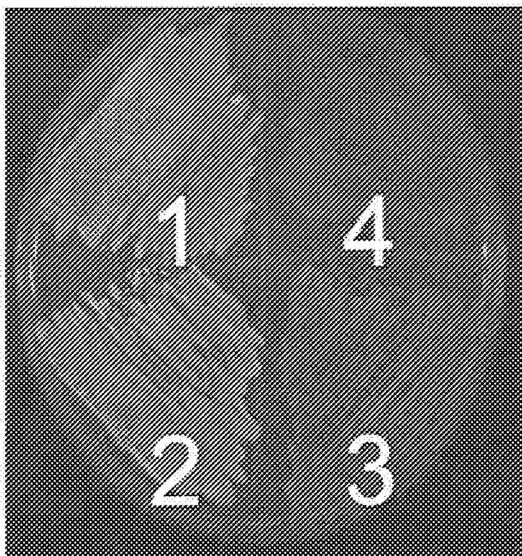


Fig. 37

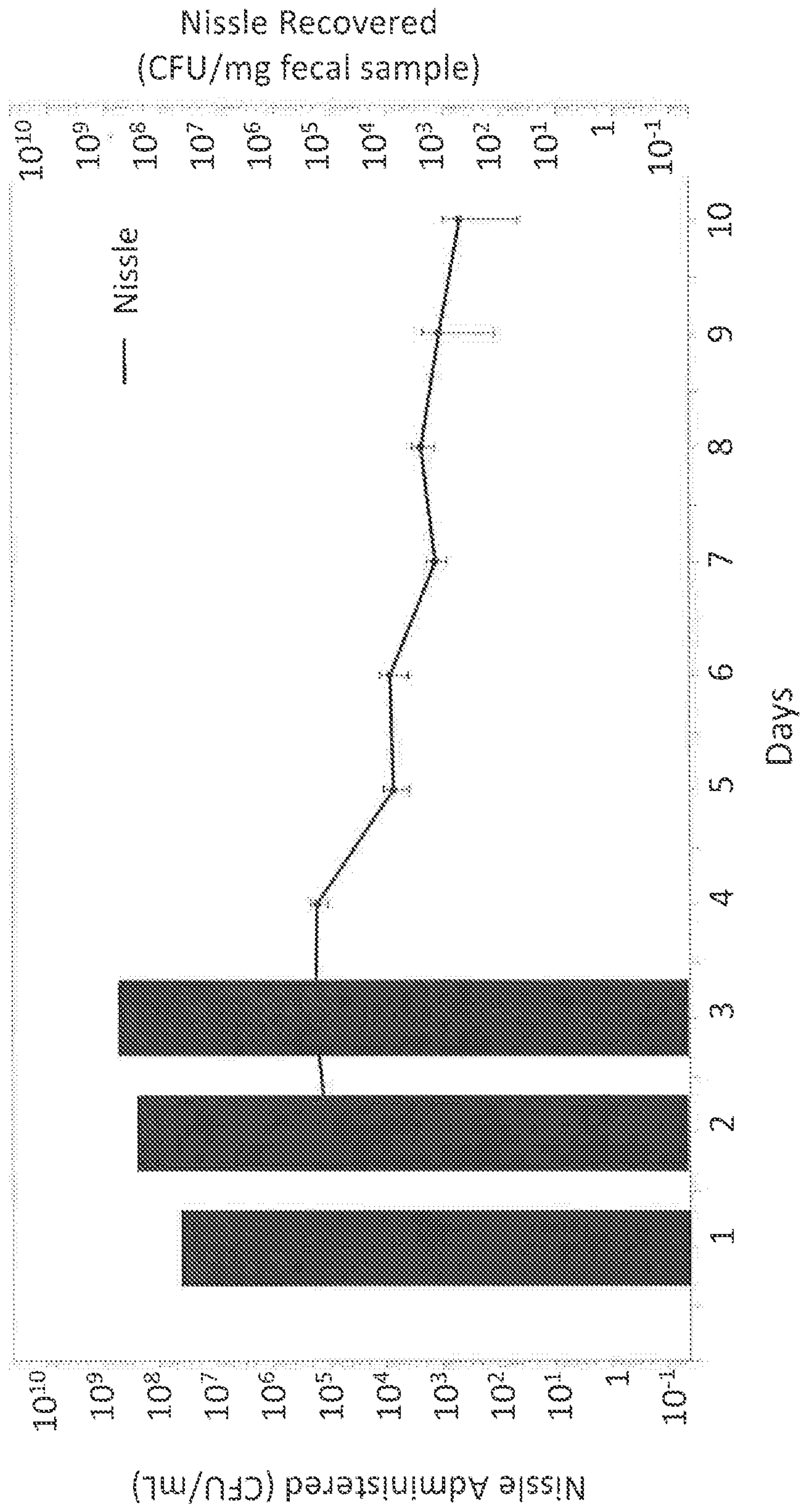


Fig. 38

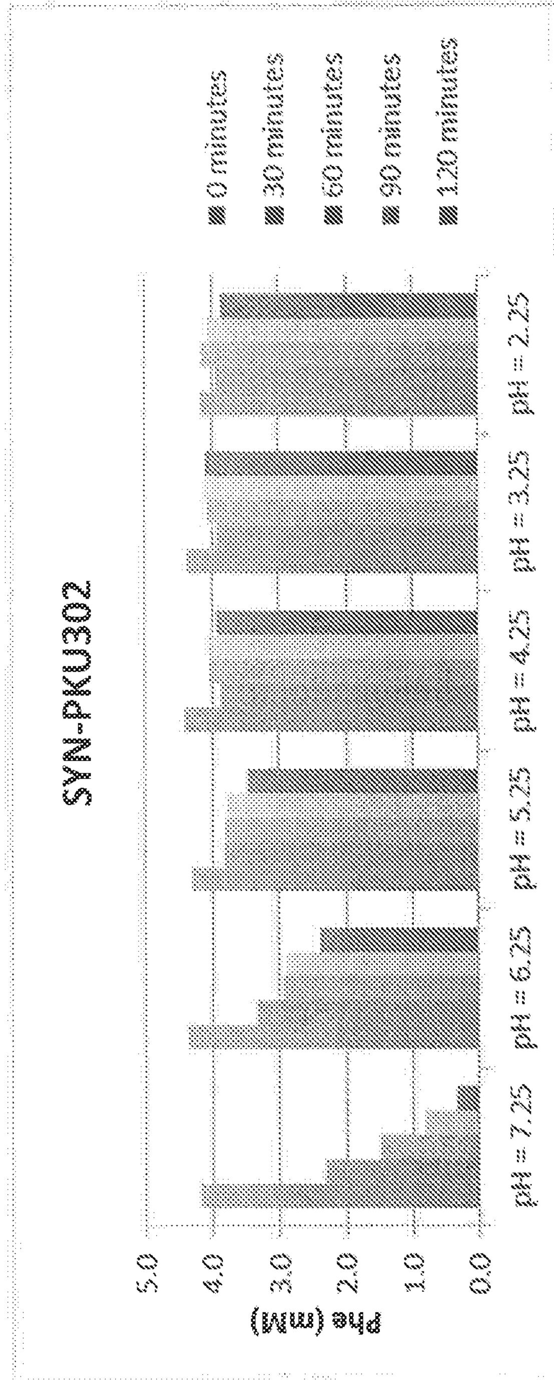


Fig. 39A

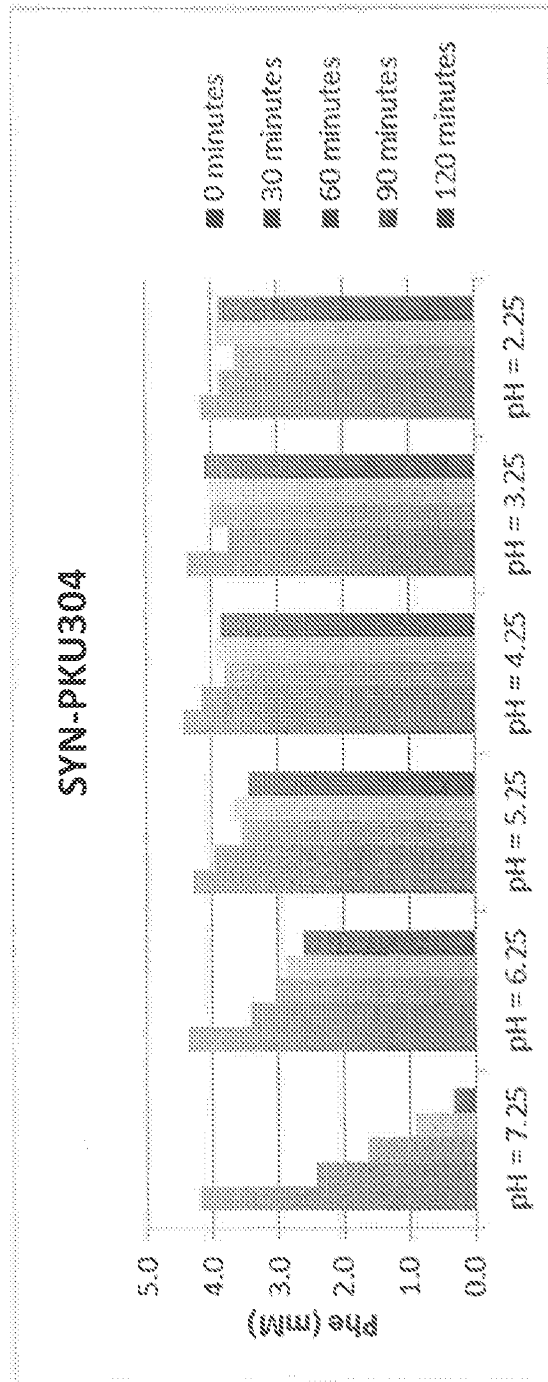


Fig. 39B

Synthetic Biotics: Single Product; Multiple MoAs

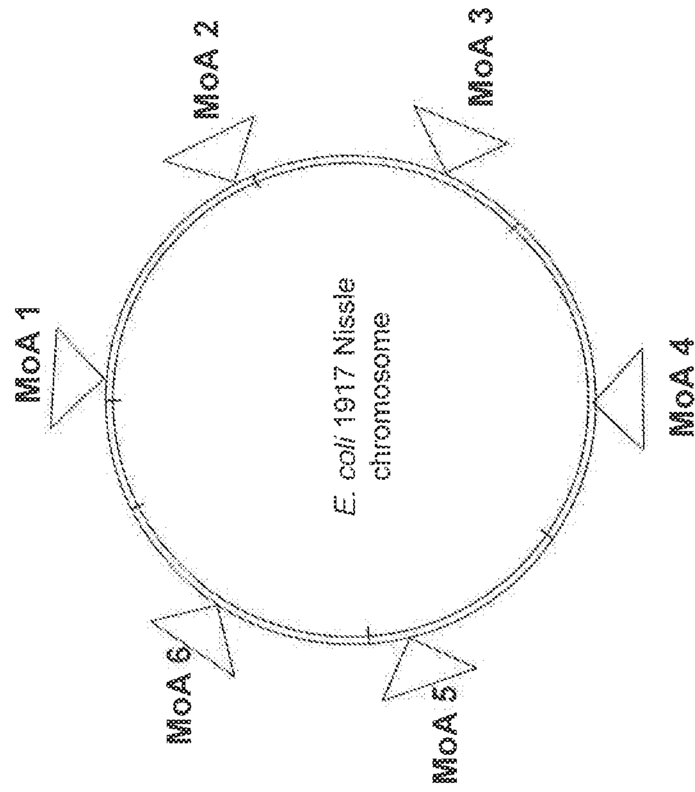


Fig. 40

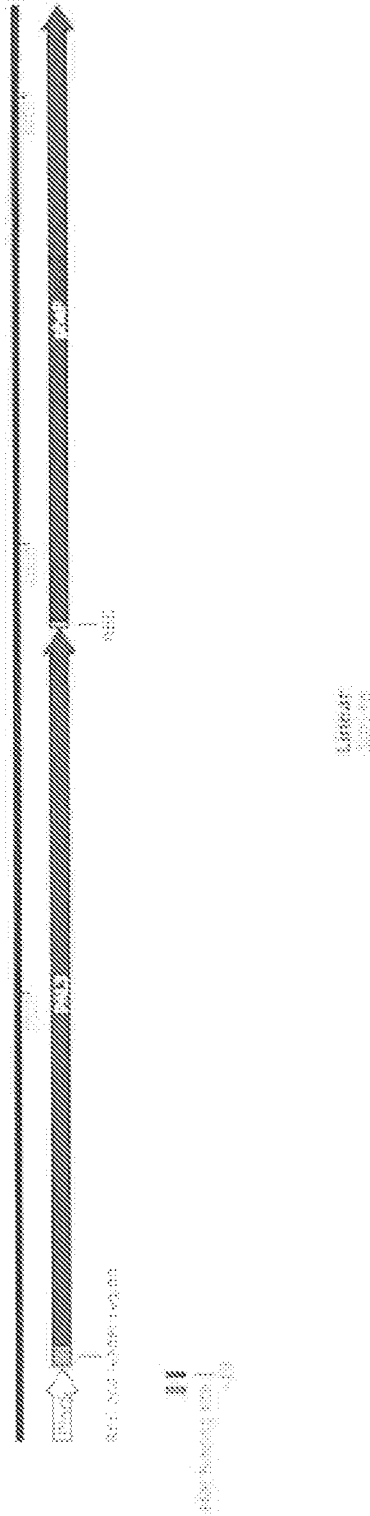


Fig. 41

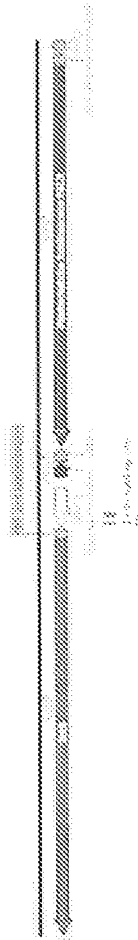


Fig. 42A

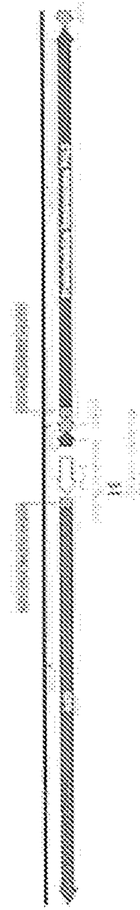


Fig. 42B

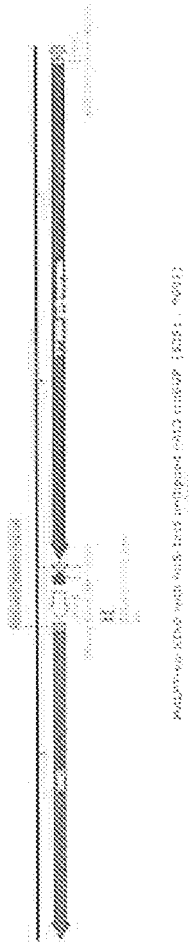


Fig. 43A

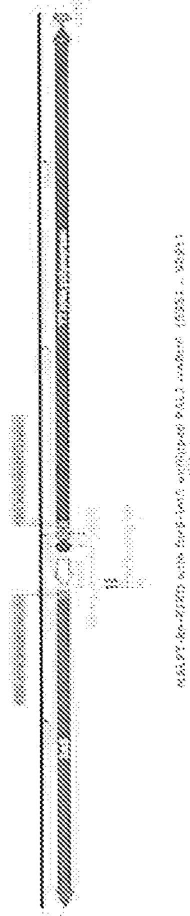


Fig. 43B

Fig. 43C

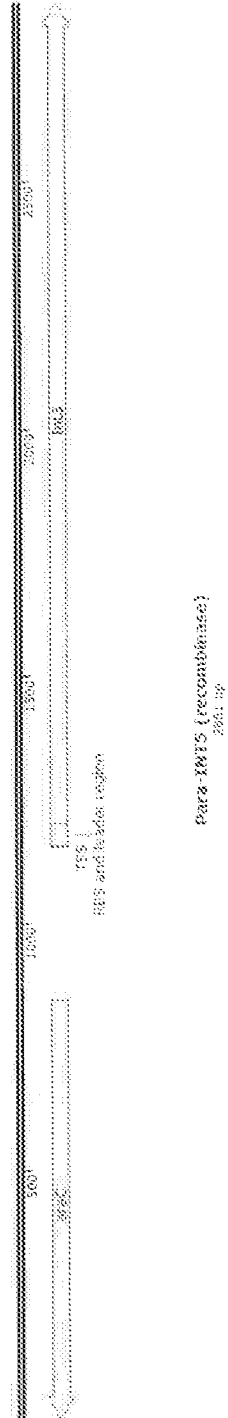


Fig. 44A

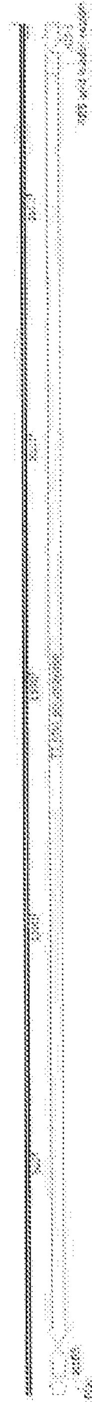


Fig. 44B

Fig. 44C

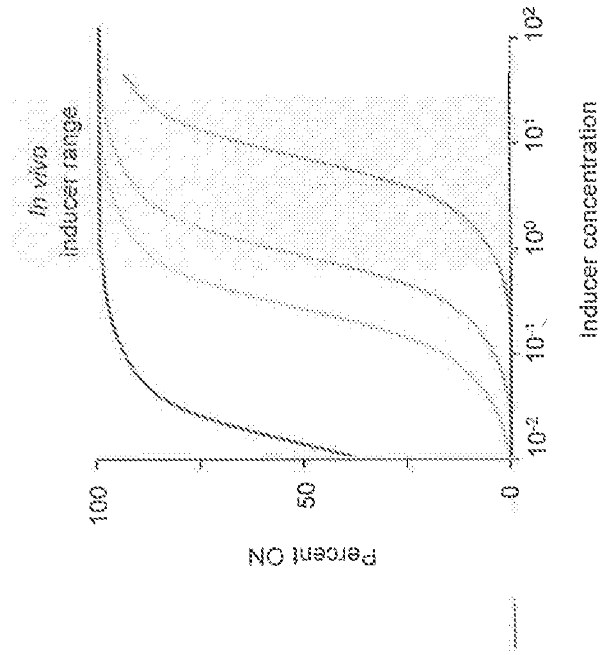


Fig. 45B

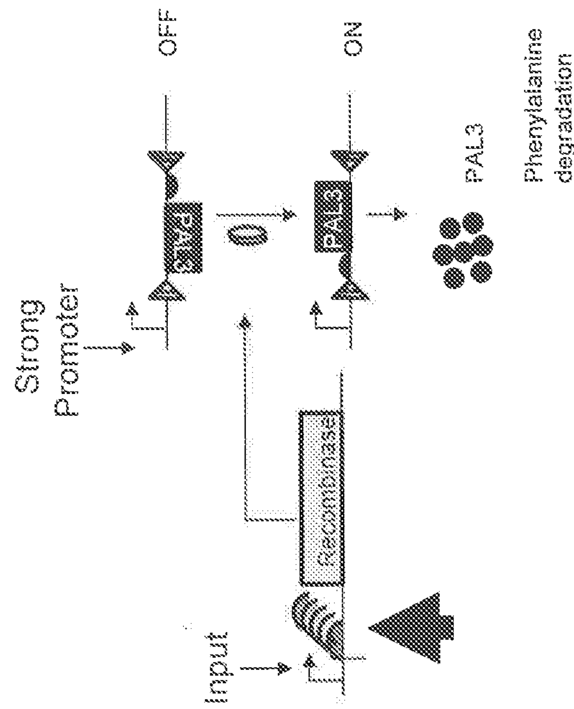


Fig. 45A

Fig. 46A

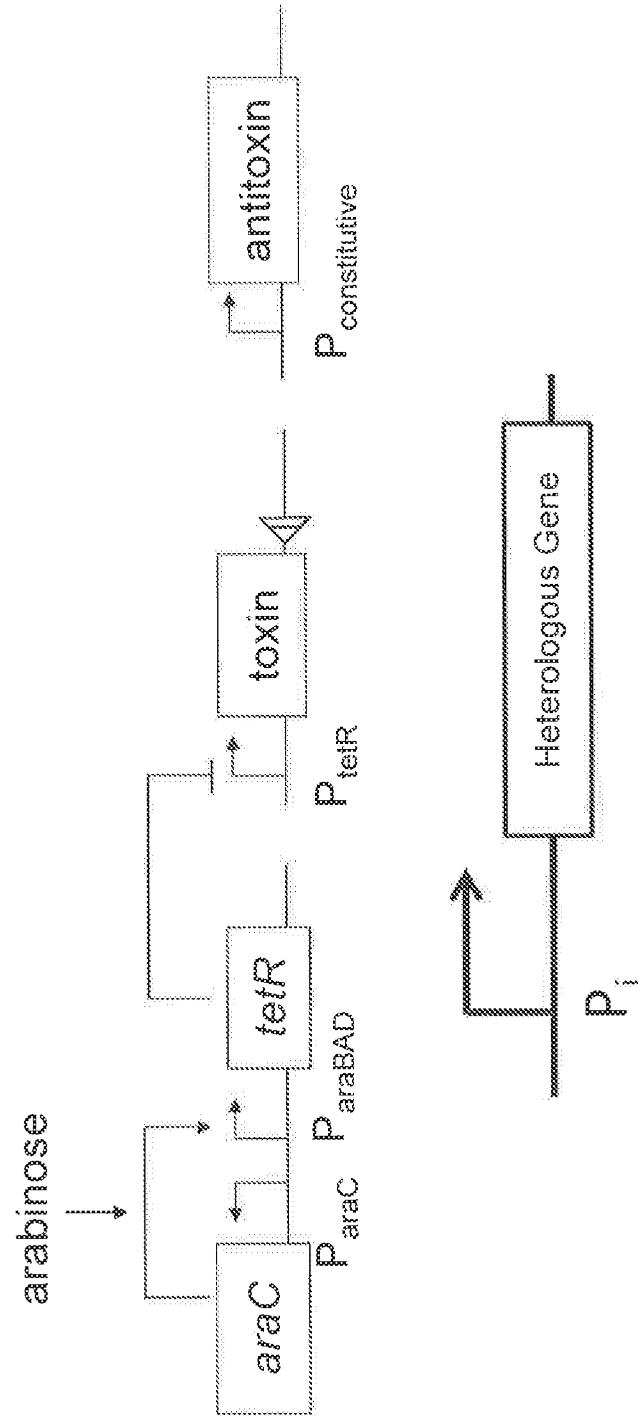


Fig. 46B

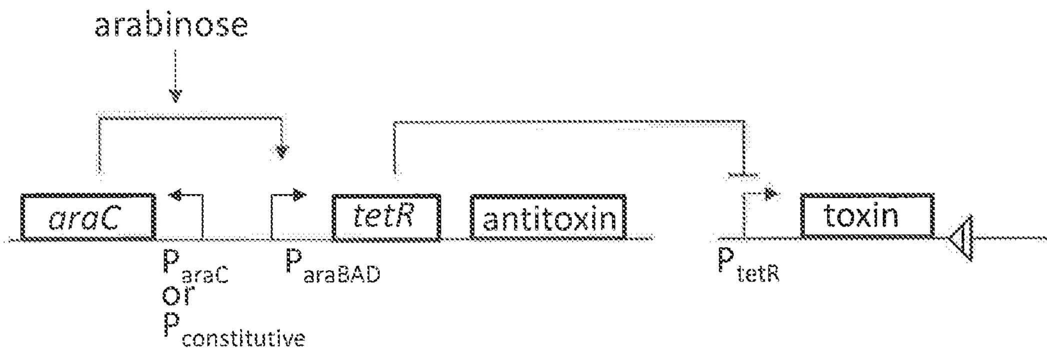


Fig. 46C

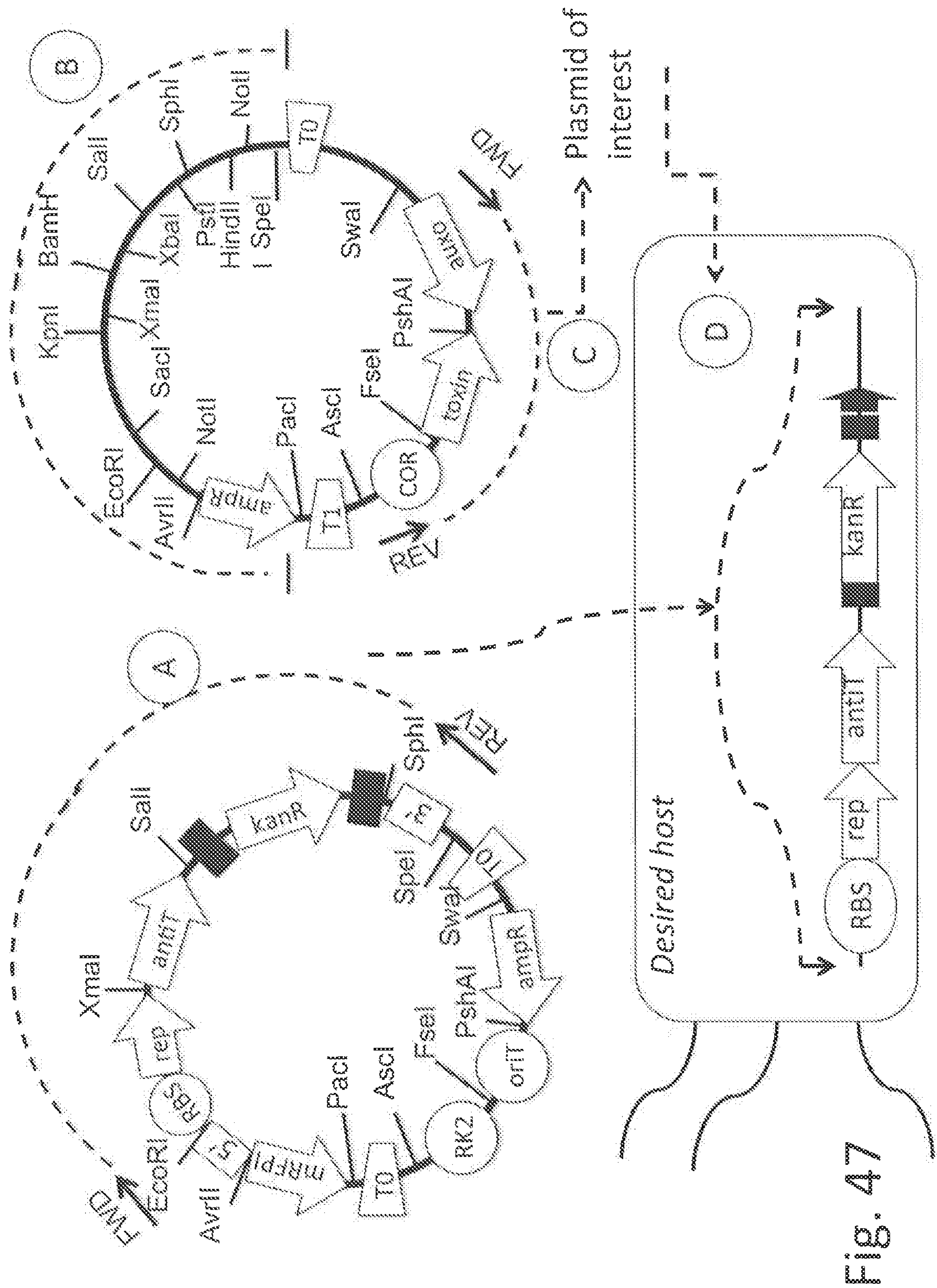


Fig. 47

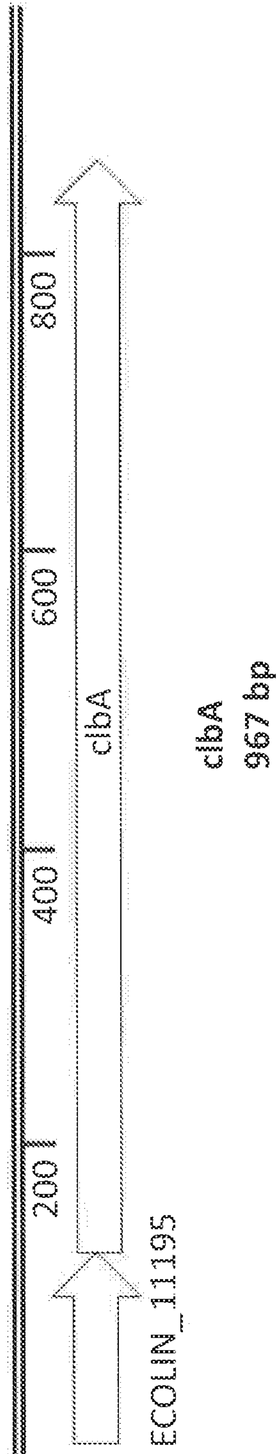


Fig. 48A

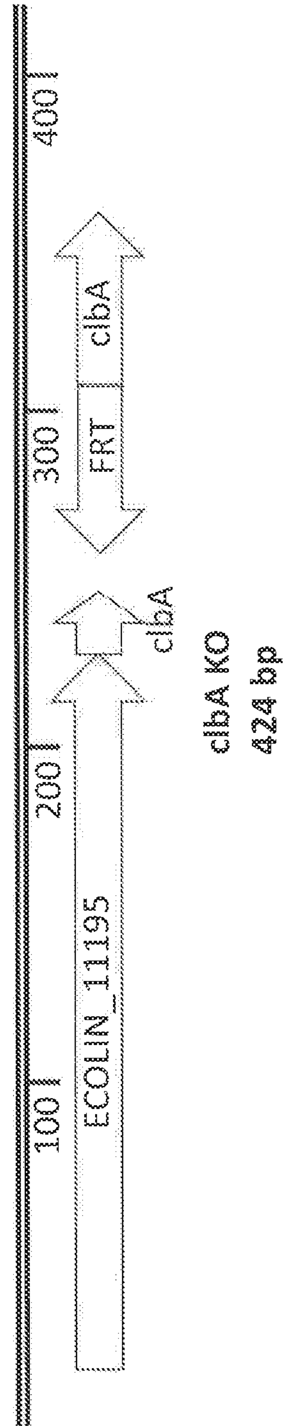


Fig.48B

Wild-type clbA (SEQ ID NO: 64)
caaatatcacataatcttaacatatcaataaacacagtaaagtttcatgtgaaaaacatcaaaca taaaatacaagctcggaatacgaatcacgctatacacattgctaacaggaatgagattatctaaa tgaggattgatatattaattggacatactagtttttttcatcaaaccagtagagataacttcctt cactatctcaatgaggaagaaataaaacgctatgatcagtttcatttgtgagtgataaagaact ctatattttaagccgtatcctgctcaaaacagcactaaaagatatcaacctgatgtctcattac aatcatggcaatttagtacgtgcaaatatggcaaaccatttatagtttttcctcagttggcaaaa aagattttttttaacctttcccatactatagatacagtagccgttgctattagttctcactgcga gcttggtgtcgatattgaacaaataagagatttagacaactcttatctgaatatcagtcagcatt tttttactccacaggaagctactaacatagtttcacttcctcgttatgaaggtcaattactttttt tggaaaatgtggacgctcaaagaagcttacatcaaatatcgaggtaaaggcctatctttaggact ggattgtattgaatttcatttaacaaataaaaaactaacttcaaaatatagaggttcacctgttt atttctctcaatggaaaatatgtaactcatttctcgcattagcctctccactcatcacccctaaa ataactattgagctatttcctatgcagtcccaactttatcaccacgactatcagctaattcattc gtcaaatgggcagaattgaatcgccacggataatctagacacttctgagccgtcgataatattga ttttcatattccgtcggtggtgtaagtatcccgcataatcgtgccattcacatttag
clbA knockout (SEQ ID NO: 65)
ggatgggggaaacatggataagttcaaagaaaaaacccgttatctctgcgtgaaagacaagta ttgcgcatgctggcacaaggtgatgagtactctcaaatatcacataatcttaacatatcaataaa cacagtaaagtttcatgtgaaaaacatcaaacataaaatacaagctcggaatacgaatcacgcta tacacattgctaacaggaatgagattatctaaatgaggattgaTGTGTAGGCTGGAGCTGCTTCG AAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCGGAATAGGAACTAAGGAG GATATTCATATGtcgtcaaatgggcagaattgaatcgccacggataatctagacacttctgagcc gtcgataatattgattttcatattccgtcggtgg

Fig. 49

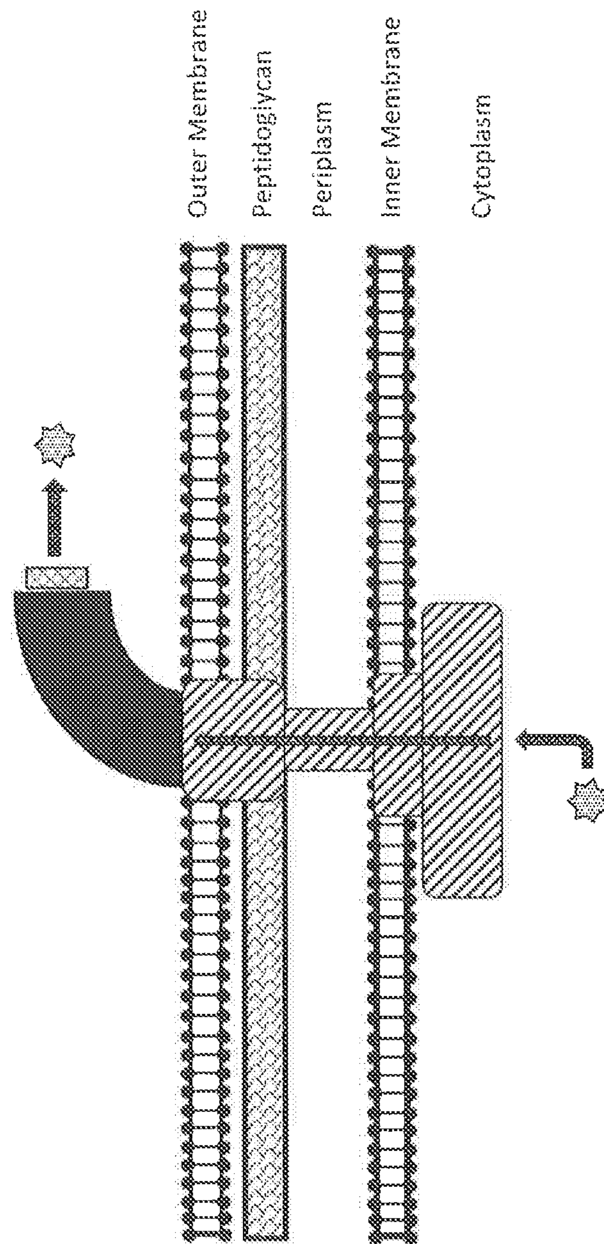


Fig. 50

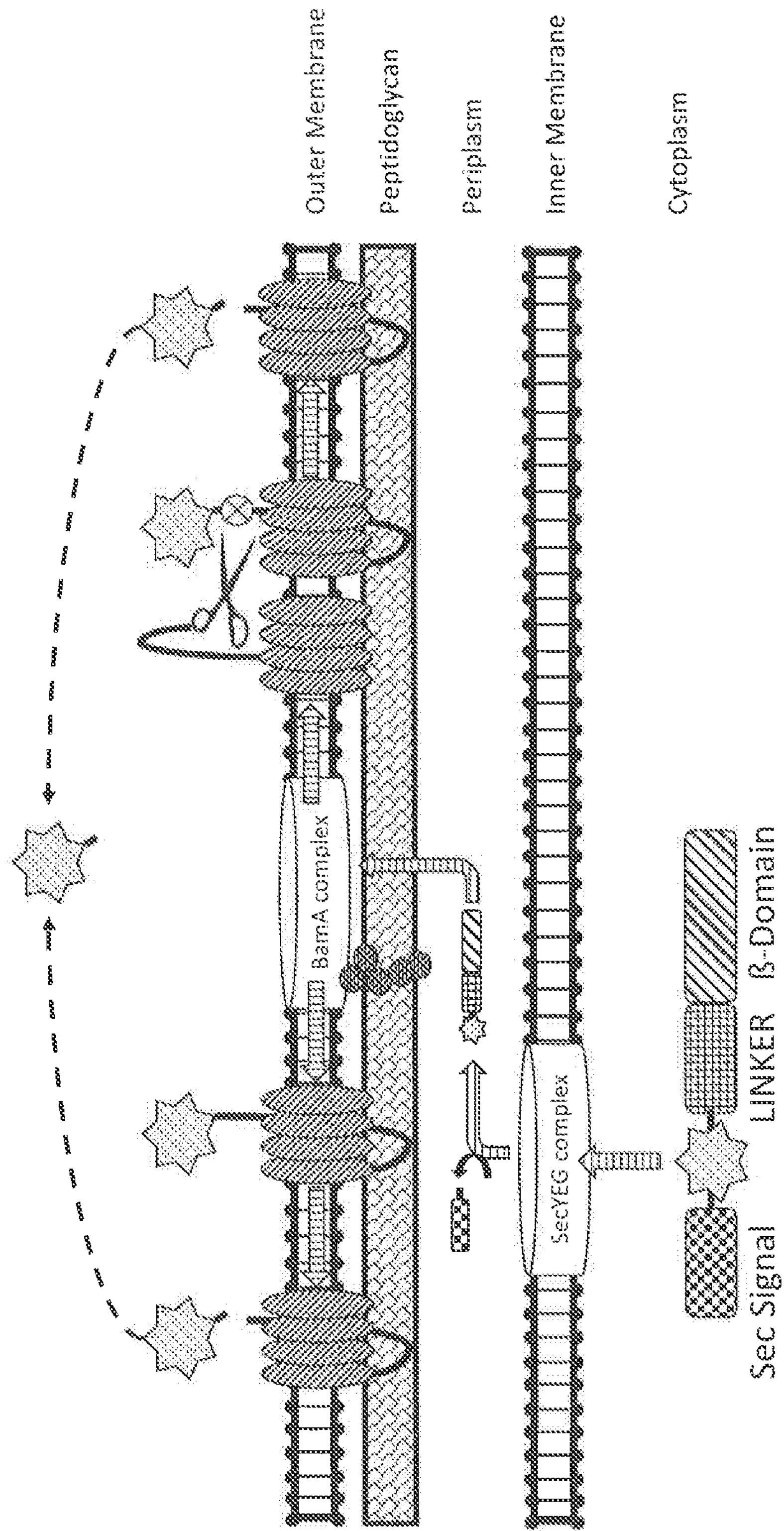


Fig. 51

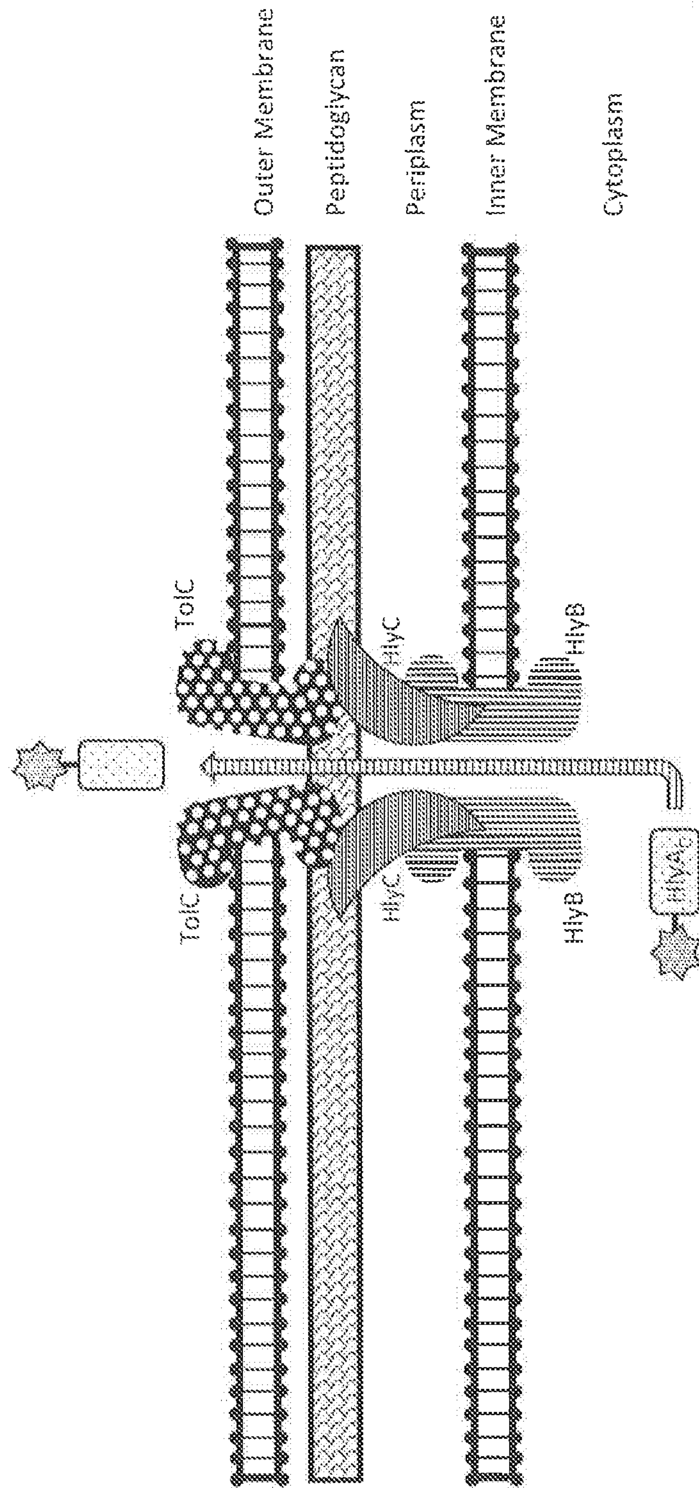


Fig. 52

Fig. 53

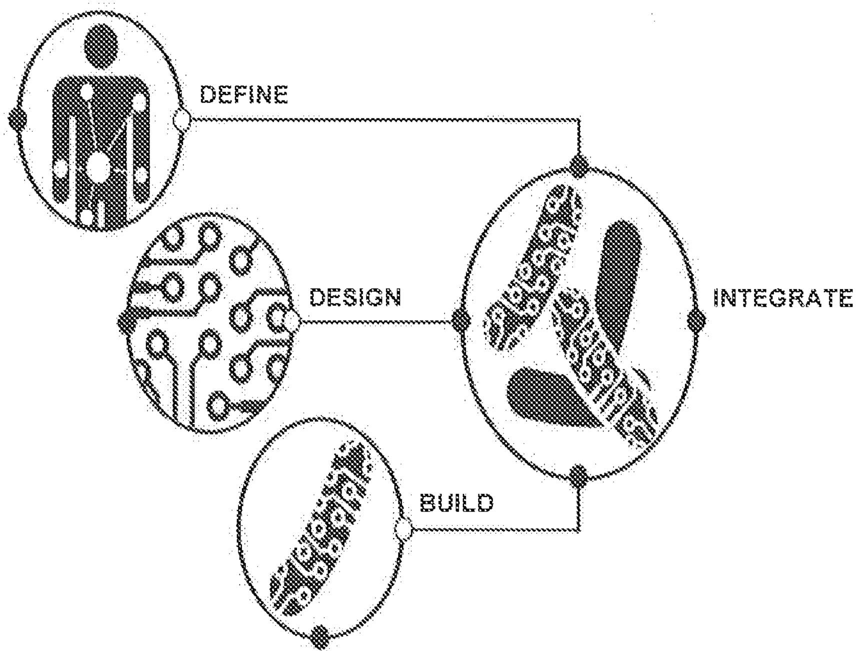


Fig. 54

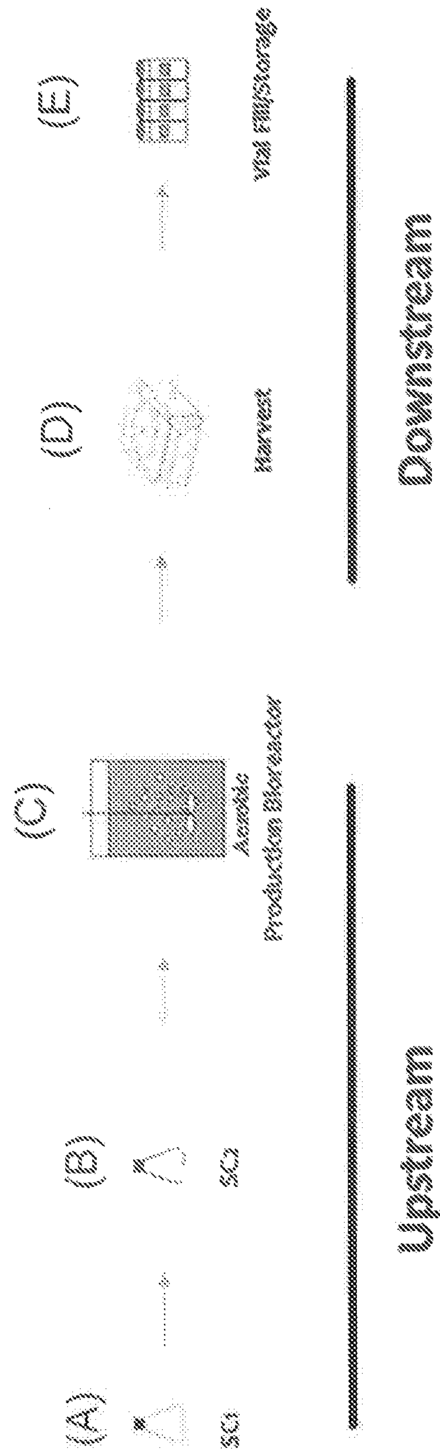


Fig. 55

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/032562

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/195 A61K35/74 C12N9/88 C12N5/10
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K C12N

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

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

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/066945 A1 (SYDNEY CHILDREN S HOSPITAL NETWORK RANDWICK & WESTMEAD [AU]) 8 May 2014 (2014-05-08) the whole document page 2, line 15 - line 27 page 5, line 11 - line 13; claims paragraph [02.5] - paragraph [02.6] -----	1-30
A	WO 2014/018832 A1 (UNIV NORTH TEXAS [US]) 30 January 2014 (2014-01-30) abstract -----	1-30
A	WO 2013/192543 A2 (PHYTOGENE INC [US]) 27 December 2013 (2013-12-27) abstract; claims 20,23 -----	1-30

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2016	22/08/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilat, Daniel

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. CHRISTODOULOU ET AL: "Abstr. 166. Enzyme substitution therapy for phenylketonuria delivered orally using a genetically modified probiotic: proof of principle.", 62ND ANNUAL MEETING OF THE AMERICAN SOCIETY OF HUMAN GENETICS, 6-10 NOVEMBER 2012, SAN FRANCISCO, vol. 62, 8 November 2012 (2012-11-08), - 10 November 2012 (2012-11-10), pages 1-1, XP055264422, US abstract -----	1-30
A	SARKISSIAN CHRISTINEH N ET AL: "A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US , vol. 96, no. 5 2 March 1999 (1999-03-02), pages 2339-2344, XP002525651, ISSN: 0027-8424, DOI: 10.1073/PNAS.96.5.2339 Retrieved from the Internet: URL:http://www.pnas.org/content/96/5/2339.full [retrieved on 2016-08-09] abstract page 2342, column 1, last paragraph -----	1-30
A	LIU JINGZHONG ET AL: "Study on a novel strategy to treatment of phenylketonuria", ARTIFICIAL CELLS, BLOOD SUBSTITUTES, AND IMMOBILIZATION BIOTECHNOLOGY, MARCEL DEKKER INC, US, vol. 30, no. 4, 1 January 2002 (2002-01-01), pages 243-257, XP009172887, ISSN: 1073-1199 abstract ----- -/--	1-30

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KANG T S ET AL: "Converting an injectable protein therapeutic into an oral form: Phenylalanine ammonia lyase for phenylketonuria", MOLECULAR GENETICS AND METABOLISM, ACADEMIC PRESS, AMSTERDAM, NL, vol. 99, no. 1, 1 January 2010 (2010-01-01), pages 4-9, XP026802464, ISSN: 1096-7192 [retrieved on 2009-09-11] abstract -----	1-30
A	CHEN X ET AL: "High-level Expression of Phenylalanine Ammonia-lyase in Lactococcus lactis via Synthesized Sequence Based on Bias Codons", CHINESE JOURNAL OF BIOTECHNOLOGY,, vol. 22, no. 2, 1 March 2006 (2006-03-01), pages 187-190, XP022857531, ISSN: 1872-2075, DOI: 10.1016/S1872-2075(06)60022-8 [retrieved on 2006-03-01] abstract -----	1-30

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/032562

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014066945 A1	08-05-2014	EP 2914274 A1	09-09-2015
		US 2015246085 A1	03-09-2015
		WO 2014066945 A1	08-05-2014
WO 2014018832 A1	30-01-2014	AU 2013295621 A1	12-03-2015
		CA 2880620 A1	30-01-2014
		EP 2877190 A1	03-06-2015
		US 2015246083 A1	03-09-2015
		WO 2014018832 A1	30-01-2014
WO 2013192543 A2	27-12-2013	CN 104487581 A	01-04-2015
		EP 2864491 A2	29-04-2015
		US 2015337336 A1	26-11-2015
		WO 2013192543 A2	27-12-2013