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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 May 2017 (26.05.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/087580 A1

(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/US2016/062369

(22) International Filing Date:
16 November 2016 (16.11.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/256,052 16 November 2015 (16.11.2015) US
PCT/US2016/032562 13 May 2016 (13.05.2016) US

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(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, DJ, 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, KW, 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))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2017/087580 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. 62/256,052, filed November 16, 2015, and PCT/US2016/032562, filed May 13, 2016, the contents of which are hereby incorporated by reference herein in their entirety.

[002] This 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). A buildup of phenylalanine (phe) in the blood can cause profound damage to the central nervous system in children and adults. If untreated in newborns, PKU can cause irreversible brain damage. Treatment for PKU currently involves complete exclusion of phenylalanine from the diet. Most natural sources of protein contain phenylalanine which is an essential amino acid and necessary for growth. In patients with PKU, this means that they rely on medical foods and phe-free protein supplements together with amino acid supplements to provide just enough phenylalanine for growth. This diet is difficult for patients and has an impact on quality of life.

[005] As discussed, 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).

[006] 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).

[007] 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).

[008] 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. There is an unmet need for a treatment that will control blood Phe levels in patients while allowing consumption of more natural protein.

Summary

[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] 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. 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.

[011] 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 (PAL) and/or phenylalanine hydroxylase

(PAH) and/or L-aminoacid deaminase (L-AAD) and are capable of reducing hyperphenylalaninemia.

[012] 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. In some embodiments, the engineered bacteria further comprise a gene encoding a phenylalanine transporter, e.g., PheP. In some embodiments, the engineered bacteria may also comprise a gene encoding L-AAD. The engineered bacteria may also contain one or more gene sequences relating to bio-safety and/or bio-containment, e.g., a kill-switch, gene guard system, and/or auxotrophy. In some embodiments, the engineered bacteria may contain an antibiotic resistance gene. The expression of any these gene sequence(s) may be regulated using a variety of promoter systems, such as any of the promoter systems disclosed herein, which promoter system may involve use of the same promoter to regulate one or more different genes, may involve use of a different copy of the same promoter to regulate different genes, and/or may involve the use of different promoters used in combination to regulate the expression of different genes. The use of different regulatory or promoter systems to control gene expression provides flexibility (e.g., the ability to differentially control gene expression under different environmental conditions and/or the ability to differentially control gene expression temporally) and also provides the ability to "fine-tune" gene expression, any or all of which regulation may serve to optimize gene expression and/or growth of the bacteria.

[013] 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.

[014] The invention also provides methods of measuring and monitoring phenylalanine ammonia lyase activity, and methods of measuring and monitoring therapeutic activity of the genetically engineered bacteria expressing phenylalanine ammonia lyase.

Brief Description of the Figures

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

[016] **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.

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

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

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

[020] **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.

[021] **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.

[022] **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.

[023] **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.

[024] **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.

[025] **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.

[026] **Fig. 12A** 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). Under anaerobic and/or low oxygen 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.

[027] **Figs. 13A-C** depict 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 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.

[028] **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.

[029] **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*.

[030] **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* enhanced 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.

[031] **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.

[032] **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/low oxygen conditions 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.

[033] **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-PKU904. SYN-PKU904, 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

and/or low oxygen conditions . **Fig. 19C** depicts the growth of bacterial cell cultures expressing *lacZ* over time, both in the presence and absence of oxygen.

[034] **Figs. 20A** and **20B** depict phenylalanine levels produced under aerobic (**Fig. 20A**) or anaerobic and/or low oxygen 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.

[035] **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).

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

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

[038] **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.

[039] **Figs. 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 incubated aerobically (1 ml) in a 14 ml culture tube, shaking at 250 rpm, incubated under microaerobic conditions, 1 ml cells incubated in a 1.7 ml conical tube without shaking, or 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.

[040] **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 ($p < 0.01$).

[041] **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.

[042] **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). 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.

[043] **Figs. 29A-D** 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**.

[044] **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.

[045] **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.

[046] **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.

[047] **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.

[048] **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.

[049] **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.

[050] **Fig. 36** depicts a bar graph showing the rate of transcinnamic acid produced using phenylalanine or various phenylalanine-containing peptides as substrate. The results indicate that PKU strains were capable of degrading Phe rapidly even in the form of di- and tri-peptides and that the genetically engineered bacteria could be administered with food, as dietary protein will be broken down into di- and tri-peptides and would be available as a bacterial substrate.

[051] **Fig. 37** depicts a bar graph showing the effect of pheP, various copy numbers of PAL, and the further addition of LAAD on the rate of phenylalanine degradation *in vitro*. Results demonstrate that increasing the copy number of PAL increases the rate of phenylalanine degradation. Addition of the high affinity transporter pheP abrogates the transport limitation, allowing greater PAL activity. The transporter copy number does not increase rate (PAL, and not transport (pheP), is limiting). In the presence of oxygen, LAAD can degrade Phe at an extremely high rate.

[052] **Fig. 38A, Fig. 38B,** and **Fig. 38C** depict bar graphs showing measurements for characterization of the phenylalanine enterorecirculation model. PKU mice were maintained on Phe-free chow and were injected with phenylalanine subcutaneously (0.1mg/kg body weight) at T=0. Blood was sampled at indicated timepoints to determine the kinetics of serum Phe post injection. The Whisker plots in **Fig. 38A** and **Fig. 38B** show distribution of mouse blood Phe levels, both overall Phe levels (**Fig. 38A**) and change in Phe levels from T0 (**Fig. 38B**). As seen in **Fig. 38C**, Phe levels are stably elevated over at least a 6 hour period.

[053] **Fig. 39** depicts a graph showing the total labeled phenylalanine concentrations upon subcutaneous 13C-Phe challenge measured to determine extent of recirculation in the PKU (*enu2*) mouse model. All mice were kept on Phe-free chow with low Phe water until injection at T0 with 0.1mg/kg 13C6-Phe. Blood and intestinal effluents were taken at 0, 20 min, and 2 hours and phenylalanine concentrations were

determined by LC-MS. Enterorecirculation of labeled 13C-Phe was confirmed to occur. Additionally, the concentration of unlabeled Phe was also determined, and a high level of pre-existing (unlabeled) phenylalanine was detected in the small intestine (data not shown).

[054] **Fig. 40A, Fig. 40B**, and **Fig. 40C** depict graphs showing amino acid content in various compartments in in wild type and enu2-/- mice. **Fig. 40A** depicts graphs showing measurements of amino acid content in the blood. **Fig. 40B** depicts graphs showing measurements of amino acid content in the small intestine. **Fig. 40C** depicts graphs showing measurements of amino acid content in the large intestine. Phe levels were high in *enu2-/-* blood, as expected. No other major differences were observed between WT and *enu2-/-* mice.

[055] **Fig. 41A** and **Fig. 41B** depict graphs showing the absolute phenylalanine concentrations (**Fig. 41A**) and the change in phenylalanine relative to baseline (**Fig. 41B)** upon gavage with a strain comprising low copy PfnrS-PAL, chromosomal PfnrS-pheP (SYN-PKU305) in a fast dietary model of PKU. In this model, Enu2 mice kept on regular chow until time of dosing. Chow is taken away at T0. Mice dosed every hour for 3 hrs (4 doses total; 0,1,2, 3hr) and bled on the 4th hour to determine serum Phe concentration. Probiotic (SYN-PKU305)-treated mice show more rapid decrease in serum Phe.

[056] **Fig. 42A** and **Fig. 42B** depict graphs showing the effect of administration of SYN-PKU305 (comprising Low Copy pSC101-PfnrS-PAL3, and a chromosomal lacZ::PfnrS-pheP) with gelatin as a carrier in drinking water (5e9cells/ml) post Phe challenge. Enu2 mice were kept on regular chow throughout the experiment.

[057] **Fig. 43A** and **Fig. 43B** depict a table (**Fig.43A**) and a line graph (**Fig. 43B**) showing that *in vitro* and *in vitro* activity correlate, but then hit a maximum of *in vivo* activity at approximately 50% decrease in deltaPhe.

[058] **Fig. 44A, Fig. 44B**, **Fig. 44C**, and **Fig. 44D** depict bar graphs of transcinnamic acid (TCA) and hippuric acid concentrations in blood (**Fig. 44A** and **Fig. 44B**) and in urine (**Fig. 44C** and **Fig. 44D**) following subcutaneous phenylalanine challenge in an *in vivo* mouse model of PKU. Mice were orally gavaged with a total of 750 μL of H2O (n= 12), SYN-PKU901 (streptomycin resistant Nissle; n= 12; 3X250 ul;

1X10e11 cfu/mouse total over 3 gavages), or 750 μL of SYN-PKU706 (comprising 2 chromosomal insertions of PAL (2XfnrS-PAL (malEK, malPT)), and 2 chromosomal insertions of pheP (2XfnrS-pheP (lacZ, HA1/2)) and one chromosomal of LAAD (Para::LAAD) and dapA auxotrophy and chloramphenicol resistance; n= 12; 3X250 ul; 1X10e11 cfu/mouse total over 3 gavages) at 1, 2 and 3h post-phenylalanine injection. **Fig. 44A** depicts serum TCA concentrations at 0 and 4 hours post phenylalanine injection. **Fig. 44B** depicts the serum hippuric acid concentration at 0 and 4 hours post phenylalanine injection. **Fig. 44C** depicts the urine TCA concentration at 4 hours post phenylalanine injection. **Fig. 32D** depicts the urine hippuric acid concentration at 4 hours post phenylalanine injection. Low levels of TCA were present in both urine and serum. Lower levels of hippuric acid were detected in serum. Highest levels were detected for hippuric acid in urine, indicating that the majority of TCA generated by the bacteria is converted to hippuric acid in the liver and is excreted in the urine. Similar levels of metabolites measured in urine when administering other efficacious PKU strains.

[059] **Fig. 45A** and **Fig. 45B** depict the amount of TCA (**Fig. 45A**) and hippuric acid (**Fig. 45B**) recovered upon oral gavage of 0.0125, 0.025, 0.05, or 0.1 mg/g TCA at 4 hours after gavage. PKU mice were orally gavaged with gavaged with 0.0125, 0.025, 0.05, or 0.1 mg/g TCA (3 mice per cage, 2 cages per group). Urine, feces, and blood were collected at 4 hours post gavage and analyzed for TCA and its major breakdown product, hippuric acid. Insignificant amounts of TCA and hippurate were detected detected in blood and feces (data not shown). A nearly full recovery of TCA in the form of hippurate was observed. These data indicate that 1mol of hippurate found in the urine equals 1mol of Phe converted to TCA in the small intestine in a PKU mouse upon administration of a PKU strain.

[060] **Fig. 46** depicts a graph showing the kinetics of conversion of TCA to hippuric acid over a time course of 6 hours in PKU mice (enu2-/-) post Phe challenge. Animals were transferred to metabolic cages (3 mice per cage, 2 cages per group) and urine samples were collected at 1, 2, 3, 4, 5, 6 hours post TCA dose. TCA is converted to hippurate and excreted in the urine by 4 hours.

[061] **Fig. 47A**, **Fig. 47B**, **Fig. 47C**, **Fig. 47D, Fig. 47E, and Fig. 47F** depict schematic diagrams of non-limiting embodiments of genetically engineered bacterial

chromosomes of the disclosure. **Fig. 47A** depicts a schematic diagram of the gene organization of an exemplary bacterial chromosome comprising phenylalanine degradation components integrated into the E. coli Nissle chromosome. SYN-PKU707 comprises three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). SYN-PKU707 further comprises one copy of the mutated FNR transcription factor FNRS24Y (Para::FNRS24Y). **Fig. 47B** depicts a schematic diagram of the gene organization of an exemplary bacterial chromosome, e.g., as seen in strain SYN-PKU708. The bacterial chromosome comprises three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). The bacterial chromosome further comprises one copy of the mutated FNR transcription factor FNRS24Y (Para::FNRS24Y) and one copy of LAAD inserted at the same insertion site (the arabinose operon), which is transcribed as a bicistronic message from the endogenous arabinose promoter**.** The bacterial chromosome further comprises a dapA auxotrophy (deltaDapA). **Fig. 47C** depicts a schematic diagram of the gene organization of an exemplary bacterial chromosome, e.g., as seen in SYN-PKU709. The bacterial chromosome comprises three insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). The bacterial chromosome further comprises one copy of the LAAD inserted into the arabinose operon with expression driven by the native Para promoter (Para::LAAD). The genome is further engineered to include a dapA auxotrophy, in which the dapA gene is deleted. **Fig. 47D** depicts a schematic diagram of the gene organization of an exemplary bacterial chromosome, e.g., as seen in SYN-PKU710. The bacterial chromosome comprises three insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). The bacterial chromosome further comprises one copy of the LAAD inserted into the arabinose operon with expression driven by the native Para promoter (Para::LAAD). The bacterial chromosome further comprises two copies of IPTG inducible PAL3 (2XLacIPAL, exo/cea and rhtC/rhtB), a dapA auxotrophy and is cured of all antibiotic resistances.

[062] **Fig. 47E** depicts a schematic diagram of the gene organization of an exemplary bacterial chromosome, e.g., as seen in SYN-PKU712. SYN-PKU712 essentially corresponds to SYN-PKU707 with a dapA auxotrophy. The bacterial chromosome comprises three copies of fnrSPAL3, two copies of fnrSpheP integrated

into the chromosome and with knocked in Para-FNRS24Y and deltadapA (DAP auxotrophy). SYN-PKU712 essentially corresponds to SYN-PKU707 with a dapA auxotrophy. **Fig. 47F** depicts a schematic diagram of the gene organization of an exemplary bacterial chromosome, e.g., as seen in SYN-PKU711. SYN-PKU711 essentially corresponds to SYN-PKU708 without a dapA auxotrophy. The bacterial chromosome comprises 3 copies of -fnrSPAL3, two copies of fnrSpheP integrated into the chromosome and knocked in Para- FNRS24Y-LAAD.

[063] **Fig. 48A** and **Fig. 48B** depict the gene organization of exemplary constructs. **Fig. 48A** depicts a construct comprising FNRS24Y driven by the arabinose inducible promoter and araC in reverse direction. SYN-PKU707 and SYN-PKU712 are examples of strains which include such a construct (integrated at the arabinose operon). Exemplary sequences include SEQ ID NO: 64. **Fig. 48B** depicts a construct in which FNRS24Y and LAAD are expressed as a bicistronic message from an arabinose inducible promoter. AraC is transcribed in the reverse orientation. SYN-PKU708 is an example of a strain which includes such a construct (integrated at the arabinose operon). Exemplary sequences include SEQ ID NO: 73. In some embodiments, FNRS24Y expression (from a construct shown in **Fig. 48A** or **Fig. 48B**) is induced as part of an oxygen bypass switch as shown in **Fig. 50**. In some embodiments, the constructs of **Fig. 48A** and/or **Fig. 48B** are used in combination with low oxygen-inducible PAL3, pheP, and/or LAAD constructs. In some embodiments, the constructs are located on a plasmid, e.g., a low copy or a high copy plasmid. In some embodiments, the constructs are located on a plasmid component of a biosafety system. In some embodiments, the constructs are integrated into the bacterial chromosome at one or more locations. In a non-limiting example, the constructs of **Fig. 48A** or **Fig. 48B** are knocked into the E coli arabinose operon and are driven by the endogenous arabinose promoter.

[064] **Fig. 49** depicts the gene organization of an exemplary construct comprising LacI in reverse orientation, and a IPTG inducible promoter driving the expression of PAL3. SYN-PKU710 is non-limiting example of a strain comprising such a construct. In SYN-PKU710, *lac*PAL is inserted at the *exo/cea* locus. Exemplary sequence includes SEQ ID NO: 74. In some embodiments, this construct is useful for pre-induction and pre-loading of a therapeutic strain prior to *in vivo* administration under aerobic conditions and in the presence of inducer, e.g., IPTG. In some

embodiments, this construct is used alone. In some embodiments, the construct is used in combination with other constitutive or inducible PAL3 constructs, e.g., low oxygen, arabinose or IPTG inducible constructs. In some embodiments, the construct is used in combination with a low-oxygen inducible construct which is active in an *in vivo* setting.

[065] In some embodiments, the construct is located on a plasmid, e.g., a low copy or a high copy plasmid. In some embodiments, the construct is located on a plasmid component of a biosafety system. In some embodiments, the construct is integrated into the bacterial chromosome at one or more locations. In some embodiments, the construct is used in combination with a PheP construct, which can either be provided on a plasmid or is integrated into the bacterial chromosome at one or more locations. PheP expression may be constitutive or driven by an inducible promoter, e.g., low-oxygen, arabinose, or IPTG. In some embodiments, the construct is used in combination with a LAAD expression construct.

[066] In some embodiments, the constructs PAL3 sequences which are the original sequence from Photorhabdus chemiluminescens. In some embodiments, the PAL3 sequences are codon optimized for expression in E coli. In some embodiments, the construct is located on a plasmid, e.g., a low or high copy plasmid. In some embodiments, the construct is employed in a biosafety system, such as the system shown in **Fig. 61A**, **Fig. 61B**, **Fig. 61C**, and **Fig. 61D**. In some embodiments, the construct is integrated into the genome at one or more locations described herein.

[067] **Fig. 50** depicts a "Oxygen bypass switch" useful for aerobic pre-induction of a strain comprising one or more PME(s) and/or Phe transporters under the control of a low oxygen FNR promoter *in vitro* in a culture vessel (e.g., flask, fermenter or other vessel, e.g., used during with cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture). In some embodiments, it is desirable to pre-load a strain with active PME, e.g., PAL3 and/or LAAD prior to administration. This can be done by pre-inducing the expression of these enzymes as the strains are propagated, (e.g., in flasks, fermenters or other appropriate vesicles) and are prepared for *in vivo* administration. In some embodiments, strains are induced under anaerobic and/or low oxygen conditions, e.g. to induce FNR promoter activity and drive expression of one or more PME(s) and /or Phe transporters. In some embodiments, it is desirable to prepare, pre-load and pre-induce the strains under aerobic or microaerobic

conditions. This allows more efficient growth and, in some cases, reduces the build-up of toxic metabolites.

[068] FNRS24Y is a mutated form of FNR which is more resistant to inactivation by oxygen, and therefore can activate FNR promoters under aerobic conditions (see e.g., Jervis AJ, The O2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe-4S] to [2Fe-2S] conversion, Proc Natl Acad Sci U S A. 2009 Mar 24;106(12):4659-64, the contents of which is herein incorporated by reference in its entirety). The O2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe-4S] to [2Fe-2S] conversion, Proc Natl Acad Sci U S A. 2009 Mar 24;106(12):4659-64, the contents of which is herein incorporated by reference in its entirety). In this oxygen bypass system, FNRS24Y is induced by addition of arabinose and then drives the expression PAL3, pheP by binding and activating the FNR promoter under aerobic conditions. Thus, strains can be grown, produced or manufactured efficiently under aerobic conditions, while being effectively pre-induced and pre-loaded, as the system takes advantage of the strong FNR promoter resulting in of high levels of expression of PAL and PheP. This system does not interfere with or compromise *in vivo* activation, since the mutated FNRS24Y is no longer expressed in the absence of arabinose, and wild type FNR then binds to the FNR promoter and drives expression of PAL3 and PheP *in vivo*.

[069] This system can also be used to drive the expression of LAAD from an FNR promoter during strain production (not shown). In other embodiments described herein, LAAD expression can also be induced aerobically, e.g., by arabinose. As such LAAD and FNRS24Y can in some embodiments be induced simultaneously. In some embodiments, FNRS24Y and LAAD are transcribed as a bicistronic message whose expression is driven by an arabinose promoter. In some embodiments, FNRS24Y is knocked into the arabinose operon, allowing expression to be driven from the endogenous Para promoter. In some embodiments, FNRS24Y-LAAD is knocked into the arabinose operon, allowing expression to be driven from the endogenous Para promoter.

[070] In some embodiments, a LacI promoter and IPTG induction are used in this system (in lieu of Para and arabinose induction). In some embodiments, a rhamnose

inducible promoter is used in this system. In some embodiments, a temperature sensitive promoter is used to drive expression of FNRS24Y.

[071] **Fig. 51** depicts a bar graph showing *in vitro* PAL activity of SYN-PKU707 (3XP$_{fnrS}$-PAL3; 2XP$_{fnrS}$*pheP*; P$_{ara}$-*fnr*^{S24Y}) as measured by the rate of transcinnamic acid (TCA) produced. Cells were induced aerobically or anaerobically in the presence or absence of arabinose. Cultures were grown in 10 ml, 20 ml, or 30 ml flasks. Arabinose-induced expression of fnrS24Y results in high level activity under aerobic conditions in 10 ml, 20 ml, or 30 ml flasks. Additionally, activation in the absence of arabinose under anaerobic conditions is maintained. These results indicate that this strain is efficiently pre-induced under aerobic conditions prior to *in vivo* administration. These results also provide an indication that anaerobic activation without arabinose, e.g., "*in vivo*" activation, is likely conserved in this strain.

[072] **Fig. 52** depicts a bar graph showing blood phenylalanine concentrations relative to baseline following subcutaneous phenylalanine challenge in an *in vivo* mouse model of PKU. Mice were orally gavaged three times with a total of 750 µL of H2O (n= 9), SYN-PKU901 (n= 9), or 800 µL of SYN-PKU707 (n= 9) (1X10e11 cfu/mouse) at 1, 2, and 3 hours post-phenylalanine injection and blood and urine was collected 4 hours post injection. **Fig. 52** depicts blood phenylalanine concentrations relative to baseline; total metabolic activity for SYN-PKU707 was calculated as 269umol/hr and the total reduction in Δphe was = 49% ($P<0.05$) relative to SYN-PKU901 ($P<0.05$).

[073] **Fig. 53** depicts a bar graph showing absolute values of hippurate recovered from the urine following subcutaneous phenylalanine challenge in an *in vivo* mouse model of PKU. Mice were orally gavaged three times with a total of 750 µL of H2O (n= 9), SYN-PKU901 (n= 9), or 800 µL of SYN-PKU707 (n= 9) (1X10e11 cfu/mouse) at 1, 2, and 3 hours post-phenylalanine injection and blood and urine was collected 4 hours post injection. **Fig. 53** depicts the urine hippurate concentration at 4 hours post phenylalanine injection. These results indicate that approximately 15-20% of injected phenylalanine is converted to hippurate. Phenylalanine is converted to TCA in the small intestine, which is then converted into hippurate in the liver.

[074] **Fig. 54A** and **Fig. 54B** depict graphs showing recovery of absolute amounts non-labeled (**Fig. 54A**) and labeled (**Fig. 54B**) hippurate in the urine post SC injection with radio-labeled phenylalanine in mice gavaged 3 times with SYN-PKU707.

Mice in metabolic cages (3mice/cage; 3cages/group) were gavaged 3X with SYN1780 following SC injection of heavy Phe (1, 2, and 3h post injection).

[075] **Fig. 55** depicts a graph showing hippuric acid recovered in urine following single dose PKU strain SYN-PKU707 at T0, 2, 4, 6, and 8 hours post Phe challenge. Different cell numbers were gavaged (in a single gavage) to mice as indicated. A dose-dependent increase in hippurate recovered in the urine of mice was observed.

[076] **Fig. 56** depicts a graph showing hippurate recovery in urine, and comparing kinetics of the generation of hippuric acid from phenylalanine through the gavage of live cells to the generation of hippuric acid derived from pure TCA gavage. With pure TCA, a rapid decrease in hippurate recovery is observed in the urine within the first 15 minutes post collection start. In the case of the cells, hippurate recovery is sustained for at least the first 30 min after collection starts and has a decreasing downward slope. These results indicate that the cells remain in the small intestine producing TCA over a useful amount of time.

[077] **Fig. 57A** and **Fig. 57B** depict bar graphs showing the change in phenylalanine over baseline in blood (Fig. 57A) and the absolute levels of hippuric acid in urine (Fig. 57B) at 4 hours post phenylalanine challenge in PKU mice gavaged with SYN-PKU708 at the indicated doses. SYN-PKU708 was efficacious in reducing blood phenylalanine and hippurate was excreted in a dose dependent manner in the cages of mice treated with SYN-PKU708, indicating that the cells were active *in vivo*.

[078] **Fig. 58A** and **Fig. 58B** depict graphs showing transit time of bacteria (SYN1780) following single gavage of ~3e10 cfu. Mice were gavaged once with bacteria (approximately 3X10e10 CFU). At each timepoint (15, 30, 45, and 60 minutes post-gavage), animals (n=4) were euthanized, and the small intestine was removed and cut into three equal pieces, and flushed. Intestinal effluents were processed for serial dilution plating to determine bacterial numbers.

[079] **Fig. 59A** and **Fig. 59B** depict blood phenylalanine concentrations relative to baseline at 4 hours post SC phenylalanine injection (**Fig. 59A**) and absolute values of hippuric acid up to 4 hours post SC phenylalanine injection (**Fig. 59B**), comparing strains SYN-PKU710 and SYN-PKU708. Mice were administered single

dose of phenylalanine by subcutaneous injection at 0.1 mg per gram body weight. At 1, 2 and 3h post Phe challenge, the bacteria (or water) were administered to mice by oral gavage (300 ul/dose, total of 3Xe10 cfu/mouse). The percentage decrease in deltaPhe SYN-PKU710 and SYN-PKU708 were calculated to be 29% and 40%, respectively.

[080] **Fig. 60** depicts a bar graph showing *in vitro* PAL activity in strains in which PAL expression is under the control of various inducible promoters (arabinose, IPTG(LacI), rhamnose, Tet, temperature (CI857) as measured by the rate of TCA accumulation. SYN-PKU707 is shown as a benchmark control.

[081] **Figs. 61A-61D** depict schematics of non-limiting examples of the gene organization of plasmids, which function as a component of a biosafety system (**Fig. 61A and Fig. 61B**), which also contains a chromosomal component (shown in **Fig. 61C and Fig. 61D**). The Biosafety Plasmid System Vector comprises Kid Toxin and R6K minimal ori, dapA **(Fig. 61A)** and thyA **(Fig. 61B)** and promoter elements driving expression of these components. In a non-limiting example, the plasmid comprises SEQ ID NO: 81. In a non-limiting example, the plasmid comprises SEQ ID NO: 82. In some embodiments, bla is knocked out and replaced with one or more constructs described herein, in which PAL3 and/or PheP and/or LAAD are expressed from an inducible or constitutive promoter. **Fig. 61C** and **Fig. 61D** depict schematics of the gene organization of the chromosomal component of a biosafety system. **Fig. 61C** depicts a construct comprising low copy Rep (Pi) and Kis antitoxin, in which transcription of Pi (Rep), which is required for the replication of the plasmid component of the system, is driven by a low copy RBS containing promoter. In some embodiments, the construct comprises SEQ ID NO: 89. **Fig. 61D** depicts a construct comprising a medium-copy Rep (Pi) and Kis antitoxin, in which transcription of Pi (Rep), which is required for the replication of the plasmid component of the system, is driven by a medium copy RBS containing promoter. In some embodiments, the construct comprises SEQ ID NO: 90. If the plasmid containing the functional DapA is used (as shown in **Fig. 61A**), then the chromosomal constructs shown in **Fig. 61C** and **Fig. 61D** are knocked into the DapA locus. If the plasmid containing the functional ThyA is used (as shown in **Fig. 61B**), then the chromosomal constructs shown in **Fig. 61C** and **Fig. 61D** are knocked into the ThyA locus. In this system, the bacteria comprising the chromosomal construct and a

knocked out dapA or thyA gene can grow in the absence of dap or thymidine only in the presence of the plasmid.

[082] **Figs. 62A-C** depict schematics of non-limiting examples of PAL constructs. Fig 62A depicts a schematic of a non-;imiting example of the organization of a construct for PAL expression under the control a lambda CI inducible promoter. The construct also provides the coding sequence of a mutant of CI, CI857, which is a temperature sensitive mutant of CI. The temperature sensitive CI repressor mutant, CI857, binds tightly at 30 degrees C but is unable to bind (repress) at temperatures of 37 C and above. In some embodiments, the construct comprises SEQ ID NO: 101. In some embodiments, this construct is used alone. In some embodiments, the temperature sensitive construct is used in combination with other constitutive or inducible PAL3 constructs, e.g., low oxygen, arabinose, rhamnose, or IPTG inducible constructs. In some embodiments, the construct allows pre-induction and pre-loading of PAL3 and/or PheP and/or LAAD prior to *in vivo* administration. In some embodiments, the construct provides *in vivo* activity. In some embodiments, the construct is located on a plasmid, e.g., a low copy or a high copy plasmid. In some embodiments, the construct is located on a plasmid component of a biosafety system. In some embodiments, the construct is integrated into the bacterial chromosome at one or more locations. In some embodiments, the construct is used in combination with a PheP construct, which can either be provided on a plasmid or is integrated into the bacterial chromosome at one or more locations. PheP expression may be constitutive or driven by an inducible promoter, e.g., low-oxygen, arabinose, rhamnose, or temperature sensitive. In some embodiments, the construct is used in combination with a LAAD expression construct. In some embodiments, a temperature sensitive system can be used to set up a conditional auxotrophy. In a a strain comprising deltaThyA or deltaDapA, a dapA or thyA gene can be introduced into the strain under the control of a thermoregulated promoter system. The strain can grow in the absence of Thy and Dap only at the permissive temperature, e.g., 37 C (and not lower).

[083] **Fig. 62B** depicts a schematic of a non-limiting example of the organization of a construct for PAL expression under the control of a rhamnose inducible promoter. For the application of the rhamnose expression system it is not necessary to express the regulatory proteins in larger quantities, because the amounts

expressed from the chromosome are sufficient to activate transcription even on multi-copy plasmids. Therefore, only the rhaP BAD promoter is cloned upstream of the gene that is to be expressed. In some embodiments, this construct is used alone. In some embodiments, the rhamnose inducible construct is used in combination with other constitutive or inducible PAL3 constructs, e.g., low oxygen, arabinose, temperature sensitive, or IPTG inducible constructs. In some embodiments, the construct allows pre-induction and pre-loading of PAL3 and/or PheP and/or LAAD prior to *in vivo* administration. In a non-limiting example, the construct is useful for pre-induction and is combined with low-oxygen inducible constructs. In some embodiments, the construct is located on a plasmid, e.g., a low copy or a high copy plasmid. In some embodiments, the construct is located on a plasmid component of a biosafety system. In some embodiments, the construct is integrated into the bacterial chromosome at one or more locations. In some embodiments, the construct is used in combination with a PheP construct, which can either be provided on a plasmid or is integrated into the bacterial chromosome at one or more locations. PheP expression may be constitutive or driven by an inducible promoter, e.g., low-oxygen, arabinose, rhamnose, or temperature sensitive. In some embodiments, the construct is used in combination with a LAAD expression construct.

[084] **Fig. 62C** depicts a schematic of a non-limiting example of the organization of a construct for PAL expression under the control of an arabinose inducible promoter. The arabinose inducible PAL3 construct comprises AraC (in reverse orientation), a region comprising an Arabinose inducible promoter, and PAL3. In some embodiments, this construct is used alone. In some embodiments, the rhamnose inducible construct is used in combination with other constitutive or inducible PAL3 constructs, e.g., low oxygen, arabinose, temperature sensitive, or IPTG inducible constructs. In some embodiments, the construct allows pre-induction and pre-loading of PAL3 and/or PheP and/or LAAD prior to *in vivo* administration. In a non-limiting example, the construct is useful for pre-induction and is combined with low-oxygen inducible constructs. In some embodiments, the construct is located on a plasmid, e.g., a low copy or a high copy plasmid. In some embodiments, the construct is located on a plasmid component of a biosafety system. In some embodiments, the construct is integrated into the bacterial chromosome at one or more locations. In some embodiments, the construct is used in combination with a PheP construct, which can

either be provided on a plasmid or is integrated into the bacterial chromosome at one or more locations. PheP expression may be constitutive or driven by an inducible promoter, e.g., low-oxygen, arabinose, rhamnose, or temperature sensitive. In some embodiments, the construct is used in combination with a LAAD expression construct.

[085] **Fig. 63A** depicts a schematic of the gene organization of a PssB promoter. The ssB gene product protects ssDNA from degradation; SSB interacts directly with numerous enzymes of DNA metabolism and is believed to have a central role in organizing the nucleoprotein complexes and processes involved in DNA replication (and replication restart), recombination and repair. The PssB promoter was cloned in front of a LacZ reporter and beta-galactosidase activity was measured. **Fig. 63B** depicts a bar graph showing the reporter gene activity for the PssB promoter under aerobic and anaerobic conditions. Briefly, cells were grown aerobically overnight, then diluted 1:100 and split into two different tubes. One tube was placed in the anaerobic chamber, and the other was kept in aerobic conditions for the length of the experiment. At specific times, the cells were analyzed for promoter induction. The Pssb promoter is active under aerobic conditions, and shuts off under anaerobic conditions. This promoter can be used to express a gene of interest under aerobic conditions. This promoter can also be used to tightly control the expression of a gene product such that it is only expressed under anaerobic and/or low oxygen conditions. In this case, the oxygen induced PssB promoter induces the expression of a repressor, which represses the expression of a gene of interest. Thus, the gene of interest is only expressed in the absence of the repressor, i.e., under anaerobic and/or low oxygen conditions. This strategy has the advantage of an additional level of control for improved fine-tuning and tighter control. In one non-limiting example, this strategy can be used to control expression of thyA and/or dapA, e.g., to make a conditional auxotroph. The chromosomal copy of dapA or ThyA is knocked out. Under anaerobic and/or low oxygen conditions, dapA or thyA -as the case may be- are expressed, and the strain can grow in the absence of dap or thymidine. Under aerobic conditions, dapA or thyA expression is shut off, and the strain cannot grow in the absence of dap or thymidine. Such a strategy can, for example be employed to allow survival of bacteria under anaerobic and/or low oxygen conditions, e.g., the gut, but prevent survival under aerobic conditions (biosafety switch).

[086] **Fig. 64A** depicts a strategy to fine-tune the expression of a Para-PAL construct by using a ribosome binding site optimization strategy. Bioinformatics tools for optimization of RBS are known in the art. In one strategy, arabinose controlled PAL and pheP can be integrated into the chromosome to provide for efficient aerobic growth and pre-induction of the strain (e.g., in flasks, fermenters or other appropriate vesicles), while integrated versions of P_{fnrS}-PAL and PheP are maintained to allow for strong *in vivo* induction. **Fig. 64B** depicts a strategy allow the expression of a PAL and PheP under aerobic conditions through the arabinose inducible expression of FNRS24Y. By using a ribosome binding site optimization strategy, the levels of **Fnr^{S24Y}** expression can be fine-tuned, e.g., under optimal inducing conditions (adequate amounts of arabinose for full induction). Fine-tuning is accomplished by selection of an appropriate RBS with the appropriate translation initiation rate. Bioinformatics tools for optimization of RBS are known in the art.

[087] **Fig. 65A, Fig. 65B, Fig. 65C** and **Fig. 65D B** depict schematics the exemplary gene organization of various expression constructs described herein. These constructs can be used alone or in combination with other PAL3 and/or PheP and/or LAAD expressing constructs. **Fig. 65A**. depicts a schematic of a non-limiting example of the organization of an IPTG and low oxygen inducible construct comprising LacI in reverse orientation, a region comprising a promoter, which contains a LacO site and a FNR binding site. The promoter drives the expression of two open reading frames encoding PAL3 and third open reading frame encoding of PheP; this construct can be transcribed as a tricistronic message. In a non-limiting example, the construct comprises SEQ ID NO: 95. In some embodiments, this construct is useful for pre-induction under anaerobic and/or low oxygen conditions through the activation of the FNR promoter. In some embodiments, this construct is useful *in vivo* activation under anaerobic and/or low oxygen conditions, as they are found in certain areas of the gut, through the activation of the FNR promoter.

[088] **Fig. 65B** depicts a schematic of a non-limiting example of the organization of an IPTG and low oxygen inducible construct, comprising LacI in reverse orientation, a region comprising a promoter, which contains LacO, and a FNR binding site. The construct also comprises two open reading frames encoding PAL3. This construct is transcribed as a bicistronic message. In some embodiments, the

construct is combined with another construct expressing PheP from another plasmid. In some embodiments, the construct is combined with a construct expressing PheP, which is integrated into the bacterial chromosome at one or more locations. In a non-limiting example, the construct comprises SEQ ID NO: 97. In some embodiments, this construct is useful for pre-induction under anaerobic and/or low oxygen conditions through the activation of the FNR promoter. In some embodiments, this construct is useful *in vivo* activation under anaerobic and/or low oxygen conditions, as they are found in certain areas of the gut, through the activation of the FNR promoter.

[089] **Fig. 65C** depicts a schematic of a non-limiting example of the organization of an IPTG inducible construct, comprising LacI in reverse orientation, a region comprising a promoter, which comprises LacO. The construct also comprises a region containing two open reading frames encoding PAL3 and third open reading frame encoding of PheP. This construct can be transcribed as a tricistronic message. In a non-limiting example, the construct comprises SEQ ID NO: 96.

[090] **Fig. 65D** depicts a schematic of a non-limiting example of the organization of an IPTG inducible construct, comprising LacI in reverse orientation, a region comprising a LacO containing promoter. The construct also contains a region containing two open reading frames encoding PAL3. This construct can be transcribed as a bicistronic message. In a non-limiting example, the construct comprises SEQ ID NO: 98. In some embodiments, the construct is combined with another construct expressing PheP from another plasmid. In some embodiments, the construct is combined with a construct expressing PheP, which is integrated into the bacterial chromosome at one or more locations. In some embodiments, the constructs shown in **Fig. 65A, Fig. 65B, Fig. 65C** and/or **Fig. 65D** comprise PAL3 sequences which are the original sequence from Photorhabdus chemiluminescens. In some embodiments, the PAL3 sequences are codon optimized for expression in E coli. In some embodiments, codon optimization is used as an additional control to fine tune, i.e., to up- or downregulate, PAL3 levels expressed from the construct. In some embodiments, the construct is located on a plasmid, e.g., a low or high copy plasmid. In some embodiments, the construct is employed in a biosafety system, such as the system shown in **Fig. 61**. In some embodiments, the construct is integrated into the genome at one or more locations described herein.

[091] **Fig. 66** 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.

[092] **Fig. 67** 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.

[093] **Fig. 68** 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.

[094] **Fig. 69** depicts a bar graph of residence over time for streptomycin resistant Nissle in various compartments of the intestinal tract at 1, 4, 8, 12, 24, and 30 hours post gavage. Mice were treated with approximately 109 CFU, and at each timepoint, animals (n=4) were euthanized, and intestine, cecum, and colon were removed. The small intestine was cut into three sections, and the large intestine and colon each into two sections. Intestinal effluents gathered and CFUs in each compartment were determined by serial dilution plating.

[095] **Figs. 70A** and **70B** 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. 70A**) and SYN-PKU304 (**Fig. 70B**).

[096] **Fig. 71** depicts an exemplary schematic of the E. coli 1917 Nissle chromosome comprising multiple mechanisms of action (MoAs).

[097] **Fig. 72** 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}).

[098] **Figs. 73A and 73B** 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. 73A** 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 and/or low oxygen conditions, recombinatorial flipping of *PAL3* to the ON orientation (5' to 3'; **Fig. 73B**) leads to the production of PAL3 and to phenylalanine metabolism. Any strong constitutive promoter sequence may be used.

[099] **Figs. 74A, 74B,** and **74C** 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. 74A** 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 and/or low oxygen conditions, the T7 RNA polymerase gene is flipped to the ON orientation (**Fig. 74B**). In engineered bacterial strains comprising a copy of *PAL3* under the control of a T7-driven promoter (P_{T7}; **Fig. 74C**), T7 RNA polymerase expression leads to the production of PAL3 and to phenylalanine metabolism.

[0100] **Figs. 75A and 75B** 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. 75A** shows an exemplary construct in which the *Int5* recombinase gene is operably linked to an ParaBAD promoter (P_{araBAD}). **Fig. 75B** 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 and/or low oxygen conditions, the T7 RNA polymerase gene is flipped to the ON orientation. In engineered bacterial strains comprising a copy of *PAL3* under the control of a T7-driven promoter, T7 RNA polymerase expression leads to the production of PAL3 and to phenylalanine metabolism.

[0101] **Fig. 76A** 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. 76B** 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*.

[0102] **Figs. 77A-77C** depict other non-limiting embodiments of the disclosure, wherein the expression of a heterologous gene is activated by an exogenous environmental signal. **Fig. 77A** depicts an embodiment of heterologous gene expression in which, 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. 77A** 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.

[0103] **Fig. 77B** 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.

[0104] **Fig. 77C** 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.

[0105] **Fig. 78** depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, and the natural kinetics of the recombinase create a time delay in expression of the toxin, allowing the heterologous gene to be fully expressed. Once the toxin is expressed, it kills the cell.

[0106] **Fig. 79** depicts another non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene, an anti-toxin, and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips a toxin gene into an activated conformation, but the presence of the accumulated anti-toxin suppresses the activity of the toxin. Once the exogenous environmental condition or cue(s) is no longer present, expression of the anti-toxin is turned off. The toxin is constitutively expressed, continues to accumulate, and kills the bacterial cell.

[0107] **Fig. 80** depicts another non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and at least one recombinase from an inducible promoter or inducible promoters. The recombinase then flips at least one excision enzyme into an activated conformation. The at least one excision enzyme then excises one or more essential genes, leading to senescence, and eventual cell death. The natural kinetics of the recombinase and excision genes cause a time delay, the kinetics of which can be altered and optimized depending on the number and choice of essential genes to be excised, allowing cell death to occur within a matter of hours or days. The presence of multiple nested recombinases can be used to further control the timing of cell death.

[0108] **Fig. 81** depicts one non-limiting embodiment of the disclosure, where an exogenous environmental condition or one or more environmental signals activates expression of a heterologous gene and a first recombinase from an inducible promoter or inducible promoters. The recombinase then flips a second recombinase from an inverted orientation to an active conformation. The activated second recombinase flips the toxin gene into an activated conformation, and the natural kinetics of the recombinase create a time delay in expression of the toxin, allowing the heterologous gene to be fully expressed. Once the toxin is expressed, it kills the cell.

[0109] **Fig. 82** depicts a one non-limiting embodiment of the disclosure, which comprises a plasmid stability system with a plasmid that produces both a short-lived anti-toxin and a long-lived toxin. When the cell loses the plasmid, the anti-toxin is no longer produced, and the toxin kills the cell. In one embodiment, the genetically engineered bacteria produce an equal amount of a Hok toxin and a short-lived Sok

antitoxin. In the upper panel, the cell produces equal amounts of toxin and anti-toxin and is stable. In the center panel, the cell loses the plasmid and anti-toxin begins to decay. In the lower panel, the anti-toxin decays completely, and the cell dies.

[0110] **Fig. 83** 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.

[0111] **Fig. 84A** depicts a schematic diagram of a wild-type *clbA* construct. **Fig. 84B** depicts a schematic diagram of a *clbA* knockout construct.

[0112] **Fig. 85** depicts a schematic of a secretion system based on the flagellar type III secretion in which an incomplete flagellum is used to secrete a therapeutic peptide of interest (star) by recombinantly fusing the peptide to an N-terminal flagellar secretion signal of a native flagellar component so that the intracellularly expressed chimeric peptide can be mobilized across the inner and outer membranes into the surrounding host environment.

[0113] **Fig. 86** depicts a schematic of a type V secretion system for the extracellular production of recombinant proteins in which a therapeutic peptide (star) can be fused to an N-terminal secretion signal, a linker and the beta-domain of an autotransporter. In this system, the N-terminal signal sequence directs the protein to the SecA-YEG machinery which moves the protein across the inner membrane into the periplasm, followed by subsequent cleavage of the signal sequence. The beta-domain is recruited to the Bam complex where the beta-domain is folded and inserted into the outer membrane as a beta-barrel structure. The therapeutic peptide is then thread through the hollow pore of the beta-barrel structure ahead of the linker sequence. The therapeutic peptide is freed from the linker system by an autocatalytic cleavage or by targeting of a membrane-associated peptidase (scissors) to a complementary protease cut site in the linker.

[0114] **Fig. 87** depicts a schematic of a type I secretion system, which translocates a passenger peptide directly from the cytoplasm to the extracellular space using HlyB (an ATP-binding cassette transporter); HlyD (a membrane fusion protein); and TolC (an outer membrane protein) which form a channel through both the inner and outer membranes. The secretion signal-containing C-terminal portion of HlyA is fused

to the C-terminal portion of a therapeutic peptide (star) to mediate secretion of this peptide.

[0115] **Fig. 88** 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.

[0116] **Fig. 89** depicts a modified type 3 secretion system (T3SS) to allow the bacteria to inject secreted therapeutic proteins into the gut lumen. An inducible promoter (small arrow, top), e.g. a FNR-inducible promoter, drives expression of the T3 secretion system gene cassette (3 large arrows, top) that produces the apparatus that secretes tagged peptides out of the cell. An inducible promoter (small arrow, bottom), e.g. a FNR-inducible promoter, drives expression of a regulatory factor, e.g. T7 polymerase, that then activates the expression of the tagged therapeutic peptide (hexagons).

[0117] **Fig. 90A, Fig. 90B**, and **Fig. 90C** depict schematics of the gene organization of exemplary circuits of the disclosure for the expression of therapeutic polypeptides, which are secreted using components of the flagellar type III secretion system. A therapeutic polypeptide of interest, such as, PAL and/or LAAD, is assembled behind a fliC-5'UTR, and is driven by the native fliC and/or fliD promoter (**Fig. 90A** and **Fig. 90B**) or a Tet-inducible promoter (**Fig. 90C**). In alternate embodiments, an inducible promoter such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose can be used. The therapeutic polypeptide of interest is either expressed from a plasmid (e.g., a medium copy plasmid) or integrated into fliC loci (thereby deleting all or a portion of fliC and/or fliD). Optionally, an N terminal part of FliC is included in the construct, as shown in **Fig. 90B** and **Fig. 90C**.

[0118] **Fig. 91A and Fig. 91B** depict schematics of the gene organization of exemplary circuits of the disclosure for the expression of therapeutic polypeptides, which are secreted via a diffusible outer membrane (DOM) system. The therapeutic polypeptide of interest is fused to a prototypical N-terminal Sec-dependent secretion signal or Tat-dependent secretion signal, which is cleaved upon secretion into the periplasmic space. Exemplary secretion tags include sec-dependent PhoA, OmpF, OmpA, cvaC, and Tat-dependent tags (TorA, FdnG, DmsA). In certain embodiments, the genetically engineered bacteria comprise deletions in one or more of lpp, pal, tolA, and/or nlpI. Optionally, periplasmic proteases are also deleted, including, but not limited to, degP and ompT, e.g., to increase stability of the polypeptide in the periplasm. A FRT-KanR-FRT cassette is used for downstream integration. Expression is driven by a Tet promoter (**Fig. 91A**) or an inducible promoter, such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter, **Fig. 91B**), and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose.

[0119] **Fig. 92** depicts a schematic of a design-build-test cycle. Steps are as follows: 1: Define the disease pathway; 2. Identify target metabolites; 3. Design genetic circuits; 4. Build synthetic biotic; 5. Activate circuit *in vivo*; 6. Characterize circuit activation kinetics; 7. Optimize *in vitro* productivity to disease threshold; 8. Test optimize circuit in animal disease model; 9. Assimilate into the microbiome; 10. Develop understanding of *in vivo* PK and dosing regimen.

[0120] **Figs. 93A, 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. 93A** depicts the parameters for starter culture 1 (SC1): loop full – glycerol stock, duration overnight, temperature 37° C, shaking at 250 rpm. **Fig. 93B** 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. 93C** 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. 93D** 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. 93E** depicts the parameters for vial fill/storage: 1-2 mL aliquots, -80° C.

Description of Embodiments

[0121] The present disclosure includes, *inter alia*, 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. In some embodiments, the engineered bacteria further comprise a gene encoding a phenylalanine transporter. In some embodiments, the engineered bacteria may also comprise a gene encoding L-AAD. The engineered bacteria may also contain one or more gene sequences relating to bio-safety and/or bio-containment, e.g., a kil-switch, gene guard system, and/or auxotrophy. The expression of these gene sequence(s) may be regulated using a variety of promoter systems, such as any of the promoter systems disclosed herein, which promoter may be the same promoter to regulate one or more different genes, may be a different copy of the same promoter to regulate different genes, or may involve the use of different promoters used in combination to regulate the expression of different genes. The use of different regulatory or promoter systems to control gene expression provides flexibility (e.g., the ability to differentially control gene expression under different environmental conditions and/or the ability to differentially control gene expression temporally) and also provides the ability to "fine-tune" gene expression, any or all of which regulation may serve to optimize gene expression and/or growth of the bacteria. 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.

[0122] 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.

[0123] "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.

[0124] "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.

[0125] "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).

[0126] "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.

[0127] 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.

[0128] "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.

[0129] 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.

[0130] 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.

[0131] 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.

[0132] "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.

[0133] 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.

[0134] 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.

[0135] "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.

[0136] "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.

[0137] "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.

[0138] 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.

[0139] 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."

[0140] "Exogenous environmental condition(s)" or "environmental conditions" refer to settings or circumstances under which the promoter described herein is directly or indirectly induced. The phrase 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 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 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.

[0141] As used herein, "exogenous environmental conditions" or "environmental conditions" also refers to settings or circumstances or environmental conditions external to the engineered microorganism, which relate to *in vitro* culture conditions of the microorganism. "Exogenous environmental conditions" may also refer to the conditions during growth, production, and manufacture of the organism. Such conditions include aerobic culture conditions, anaerobic culture conditions, low oxygen culture conditions and other conditions under set oxygen concentrations. Such conditions also include the presence of a chemical and/or nutritional inducer, such as tetracycline, arabinose, IPTG, rhamnose, and the like in the culture medium. Such conditions also include the temperatures at which the microorganisms are grown prior to *in vivo* administration. For example, using certain promoter systems, certain temperatures are permissive to expression of a payload, while other temperatures are non-permissive. Oxygen levels, temperature and media composition influence such exogenous environmental conditions. Such conditions affect proliferation rate, rate of induction of the PME (e.g., PAL or LAAD), rate of induction of the transporter (e.g., PheP) and/or other regulators (e.g., FNRS24Y), and overall viability and metabolic activity of the strain during strain production.

[0142] 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.

[0143] 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**.

[0144] 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 and/or low oxygen conditions by the global transcriptional regulator FNR that is naturally found in Nissle. Under anaerobic and/or low oxygen 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*

[0145] 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.

[0146] "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.

[0147] "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.

[0148] In some embodiments, the genetically engineered bacteria are active (e.g., express one or more PMEs) in the gut. In some embodiments, the genetically engineered bacteria are active (e.g., express one or more PMEs) in the large intestine. In some embodiments, the genetically engineered bacteria are active (e.g., express one or more PMEs) in the small intestine. In some embodiments, the genetically engineered bacteria are active in the small intestine and in the large intestine. Without wishing to be

bound by theory, phenylalanine degradation may be every effective in the small intestine, because amino acid absorption, e.g., phenylalanine absorption, occurs in the small intestine. Through the prevention or reduction of phenylalanine uptake into the blood, increased levels and resulting Phe toxicity can be avoided. Additionally, extensive enterorecirculation of amino acids between the intestine and the body may allow the removal of systemic phenylalanine in PKU (e.g., described by Chang et al., in a rat model of PKU (Chang et al., A new theory of enterorecirculation of amino acids and its use for depleting unwanted amino acids using oral enzyme-artificial cells, as in removing phenylalanine in phenylketonuria; Artif Cells Blood Substit Immobil Biotechnol. 1995;23(1):1-21)). Phenylalanine from the blood circulates into the small intestine (see, e.g., **Fig. 39**) and can be cleared by bacteria which are active at this location. In some embodiments, the genetically engineered bacteria transit through the small intestine. In some embodiments, the genetically engineered bacteria have increased residence time in the small intestine. In some embodiments, the genetically engineered bacteria colonize the small intestine. In some embodiments, the genetically engineered bacteria do not colonize the small intestine. In some embodiments, the genetically engineered bacteria have increased residence time in the gut. In some embodiments, the genetically engineered bacteria colonize the small intestigutne. In some embodiments, the genetically engineered bacteria do not colonize the gut.

[0149] As used herein, the term "low oxygen" is meant to refer to a level, amount, or concentration of oxygen (O_2) that is lower than the level, amount, or concentration of oxygen that is present in the atmosphere (e.g., <21% O_2; <160 torr O_2)). Thus, the term "low oxygen condition or conditions" or "low oxygen environment" refers to conditions or environments containing lower levels of oxygen than are present in the atmosphere. In some embodiments, the term "low oxygen" is meant to refer to the level, amount, or concentration of oxygen (O_2) found in a mammalian gut, e.g., lumen, stomach, small intestine, duodenum, jejunum, ileum, large intestine, cecum, colon, distal sigmoid colon, rectum, and anal canal. In some embodiments, the term "low oxygen" is meant to refer to a level, amount, or concentration of O_2 that is 0-60 mmHg O_2 (0-60 torr O_2) (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45,46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, and 60 mmHg O_2), including any and all incremental fraction(s) thereof (e.g., 0.2 mmHg, 0.5 mmHg

O_2, 0.75 mmHg O_2, 1.25 mmHg O_2, 2.175 mmHg O_2, 3.45 mmHg O_2, 3.75 mmHg O_2, 4.5 mmHg O_2, 6.8 mmHg O_2, 11.35 mmHg O2, 46.3 mmHg O_2, 58.75 mmHg, etc., which exemplary fractions are listed here for illustrative purposes and not meant to be limiting in any way). In some embodiments, "low oxygen" refers to about 60 mmHg O_2 or less (e.g., 0 to about 60 mmHg O_2). The term "low oxygen" may also refer to a range of O_2 levels, amounts, or concentrations between 0-60 mmHg O_2 (inclusive), e.g., 0-5 mmHg O_2, < 1.5 mmHg O_2, 6-10 mmHg, < 8 mmHg, 47-60 mmHg, etc. which listed exemplary ranges are listed here for illustrative purposes and not meant to be limiting in any way. See, for example, Albenberg et al., Gastroenterology, 147(5): 1055-1063 (2014); Bergofsky et al., J Clin. Invest., 41(11): 1971- 1980 (1962); Crompton et al., J Exp. Biol., 43: 473-478 (1965); He et al., PNAS (USA), 96: 4586-4591 (1999); McKeown, Br. J. Radiol., 87:20130676 (2014) (doi: 10.1259/brj.20130676), each of which discusses the oxygen levels found in the mammalian gut of various species and each of which are incorportated by reference herewith in their entireties. In some embodiments, the term "low oxygen" is meant to refer to the level, amount, or concentration of oxygen (O_2) found in a mammalian organ or tissue other than the gut, e.g., urogenital tract, tumor tissue, etc. in which oxygen is present at a reduced level, e.g., at a hypoxic or anoxic level. In some embodiments, "low oxygen" is meant to refer to the level, amount, or concentration of oxygen (O_2) present in partially aerobic, semi aerobic, microaerobic, nanoaerobic, microoxic, hypoxic, anoxic, and/or anaerobic conditions. For example, Table A summarizes the amount of oxygen present in various organs and tissues. In some embodiments, the level, amount, or concentration of oxygen (O_2) is expressed as the amount of dissolved oxygen ("DO") which refers to the level of free, non-compound oxygen (O_2) present in liquids and is typically reported in milligrams per liter (mg/L), parts per million (ppm; 1mg/L = 1 ppm), or in micromoles (umole) (1 umole O_2 = 0.022391 mg/L O_2). Fondriest Environmental, Inc., "Dissolved Oxygen", Fundamentals of Environmental Measurements, 19 Nov 2013, www.fondriest.com/environmental-measurements/parameters/water-quality/dissolved-oxygen/>. In some embodiments, the term "low oxygen" is meant to refer to a level, amount, or concentration of oxygen (O_2) that is about 6.0 mg/L DO or less, e.g., 6.0 mg/L, 5.0 mg/L, 4.0 mg/L, 3.0 mg/L, 2.0 mg/L, 1.0 mg/L, or 0 mg/L, and any fraction therein, e.g., 3.25 mg/L, 2.5 mg/L, 1.75 mg/L, 1.5 mg/L, 1.25 mg/L, 0.9 mg/L, 0.8 mg/L, 0.7 mg/L, 0.6 mg/L, 0.5 mg/L, 0.4 mg/L, 0.3 mg/L, 0.2 mg/L and 0.1 mg/L DO,

which exemplary fractions are listed here for illustrative purposes and not meant to be limiting in any way. The level of oxygen in a liquid or solution may also be reported as a percentage of air saturation or as a percentage of oxygen saturation (the ratio of the concentration of dissolved oxygen (O_2) in the solution to the maximum amount of oxygen that will dissolve in the solution at a certain temperature, pressure, and salinity under stable equilibrium). Well-aerated solutions (e.g., solutions subjected to mixing and/or stirring) without oxygen producers or consumers are 100% air saturated. In some embodiments, the term "low oxygen" is meant to refer to 40% air saturation or less, e.g., 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, and 0% air saturation, including any and all incremental fraction(s) thereof (e.g., 30.25%, 22.70%, 15.5%, 7.7%, 5.0%, 2.8%, 2.0%, 1.65%, 1.0%, 0.9%, 0.8%, 0.75%, 0.68%, 0.5%. 0.44%, 0.3%, 0.25%, 0.2%, 0.1%, 0.08%, 0.075%, 0.058%, 0.04%. 0.032%, 0.025%, 0.01%, etc.) and any range of air saturation levels between 0-40%, inclusive (e.g., 0-5%, 0.05 – 0.1%, 0.1-0.2%, 0.1-0.5%, 0.5 – 2.0%, 0-10%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, etc.). The exemplary fractions and ranges listed here are for illustrative purposes and not meant to be limiting in any way. In some embodiments, the term "low oxygen" is meant to refer to 9% O_2 saturation or less, e.g., 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0%, O_2 saturation, including any and all incremental fraction(s) thereof (e.g., 6.5%, 5.0%, 2.2%, 1.7%, 1.4%, 0.9%, 0.8%, 0.75%, 0.68%, 0.5%. 0.44%, 0.3%, 0.25%, 0.2%, 0.1%, 0.08%, 0.075%, 0.058%, 0.04%. 0.032%, 0.025%, 0.01%, etc.) and any range of O_2 saturation levels between 0-9%, inclusive (e.g., 0-5%, 0.05 – 0.1%, 0.1-0.2%, 0.1-0.5%, 0.5 – 2.0%, 0-8%, 5-7%, 0.3-4.2% O_2, etc.). The exemplary fractions and ranges listed here are for illustrative purposes and not meant to be limiting in any way.

Table A.

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

colon	**<2torr**
Lumen of cecum	**<1 torr**
tumor	**<32 torr (most tumors are <15 torr)**

[0150] As used herein, the term "gene" or "gene sequence" is meant to refer to a genetic sequence, e.g., a nucleic acid sequence. The gene, gene sequence or genetic sequence is meant to include a complete gene sequence or a partial gene sequence. The gene, 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.

[0151] "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, yeast, 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.

[0152] "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.

[0153] "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.

[0154] 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.

[0155] 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.

[0156] 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.

[0157] 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.

[0158] 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.

[0159] 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.

[0160] 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.

[0161] 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.

[0162] 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.

[0163] 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.

[0164] 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.

[0165] 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.

[0166] 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. The term "codon-optimized" refers to the modification of codons in the gene or coding regions of a nucleic acid molecule to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the nucleic acid molecule. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of the host organism. A "codon-optimized sequence" refers to a sequence, which was modified from an existing coding sequence, or designed, for example, to improve translation in an expression host cell or organism of a transcript RNA molecule transcribed from the coding sequence, or to improve transcription of a coding sequence. In some embodiments, the improvement of transcription and/or translation involves increasing the level of transcription and/or translation. In some embodiments, the improvement of transcription and/or translation involves decreasing the level of

transcription and/or translation. In some embodiments, codon optimization is used to fine-tune the levels of expression from a construct of interest, e.g., PAL3 levels and/or PheP levels. Codon optimization includes, but is not limited to, processes including selecting codons for the coding sequence to suit the codon preference of the expression host organism. Many organisms display a bias or preference for use of particular codons to code for insertion of a particular amino acid in a growing polypeptide chain. Codon preference or codon bias, differences in codon usage between organisms, is allowed by the degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent, inter alia, on 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.

[0167] 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.]]

[0168] 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. For example, a phenylalanine transporter such as PheP imports phenylalanine into the microorganism.

[0169] The articles "a" and "an," as used herein, should be understood to mean "at least one," unless clearly indicated to the contrary.

[0170] 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

[0171] 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.*

[0172] 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).

[0173] 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).

[0174] 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. 68**).

[0175] 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 promoter is any one or more of the promoters disclosed herein.

[0176] 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. In some embodiments, the promoter is any one or more of the promoters disclosed herein.

[0177] In some embodiments, the genetically engineered bacteria of the invention comprise a gene encoding LAAD, wherein the *LAAD* gene is operably linked to a directly or indirectly inducible promoter. In some embodiments, the bacteria comprise a non-native *LAAD* gene. In some embodiments, the bacteria comprise additional copies of a native *LAAD* gene. In some embodiments, the promoter is not associated with the *LAAD* gene in nature. In some embodiments, the promoter is any one or more of the promoters disclosed herein.

[0178] In some embodiments, 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 and/or PAH and/or L-AAD. In some embodiments, the promoter that controls expression of PheP differs from the promoter that controls expression of PAL and/or PAH and/or L-AAD. In some embodiments, the promoter that controls the expression of PheP is any one or more of the promoters disclosed herein.

[0179] In some embodiments, the promoter that is operably linked to *PAL*, *PAH*, LAAD, 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 exogenous environmental conditions specific to the large intestine of a mammal. In some embodiments, the promoter is directly or indirectly induced by low-oxygen or anaerobic and/or low oxygen 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 exposure to arabinose. In some embodiments, the promoter is directly or indirectly induced by exposure to IPTG. In some embodiments, the promoter is directly or indirectly induced by exposure to rhamnose or other chemical and/or nutritional inducer known in the art. In some embodiments, the promoter is directly or indirectly regulated by the exogenous environmental temperature. In some embodiments, the promoter is directly or indirectly induced by exposure to IPTG or other lacI binding compound. In some embodiments, the promoter is directly or indirectly induced by exposure to rhamnose. In some embodiments, the promoter is directly or indirectly induced by increase in temperature. In some embodiments, the promoter is directly or indirectly induced by decrease in temperature. 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. Such a molecule may be tetracycline or IPTG or arabinose or other chemical and/or nutritional inducer known in the art.

[0180] In some embodiments, the promoter is directly or indirectly induced by conditions in a culture vessel (e.g., a flask or a fermenter or other appropriate culture vessel), in which the strain is grown or maintained prior to *in vivo* administration. Non-limiting examples of such conditions which are provided during culture of the strain prior to *in vivo* administration include low oxygen, anaerobic, microaerobic, or aerobic conditions, other defined oxygen levels (such as those exemplified below), presence of arabinose, presence of IPTG, rhamnose or other chemical and/or nutritional inducers described herein or known in the art. In some embodiments, the conditions in a culture vessel are set at certain oxygen levels, e.g, between 1% and 10% oxygen, between 10% and 20% oxygen, between 20% and 30% oxygen, between 30% and 40% oxygen, between 40% and 50% oxygen, between 60% and 70% oxygen, between 70% and 80% oxygen, between 80% and 90% oxygen, between 90% and100% oxygen, and other levels of oxygen as described herein, at which point the promoter is directly or indirectly induced.

Reducing hyperphenylalaninemia

[0181] The genetically engineered bacteria of the invention comprise a gene encoding a phenylalanine-metabolizing enzyme (PME). In some embodiments, the genetically engineered bacteria comprise a gene encoding a phenylalanine-metabolizing enzyme (PME) and are capable of reducing hyperphenylalaninemia.

[0182] 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.

[0183] In some embodiments, the engineered bacteria comprise gene sequence encoding one or more phenylalanine hydroxylase (PAH) polypeptides. In some embodiments, the engineered bacteria comprise gene sequence encoding one or more phenylalanine ammonia lyase (PAL) polypeptides. 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.

[0184] 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**.

[0185] In some embodiments, the engineered bacteria comprise gene sequence encoding one or more LAAD polypeptides. In some embodiments, the engineered bacteria comprise gene sequence encoding one or more PAL polypeptides and one or more LAAD polypeptides. 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).

[0186] 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.

[0187] 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 HQEITQLNDTLQEVYSIRCAPQVLGIVPESLATARKI LEREVISANDNPLIDPENGDVLHGGNFMGQYVART MDALKLDIALIANHLHAIVALMMDNRFSRGLPNSL	SEQ ID NO: 3

	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 DPTPFSLDRFKK	SEQ ID NO: 6
L-AAD from *Proteus*	MAISRRKFIIGGTVVAVAAGAGILTPMLTREGRFVP GTPRHGFVEGTEGALPKQADVVVVGAGILGIMTAI	SEQ ID NO: 7

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

[0188] The PME, e.g., PAL, LAAD, or PAH, gene(s) may be present on a plasmid or chromosome in the genetically engineered bacteria. In some embodiments, the PME gene sequence(s) are expressed under the control of one or more constitutive promoter(s). 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.

[0189] In some embodiments, the genetically engineered bacteria comprise gene sequence encoding one or more PAL polypeptide sequence(s). In some

embodiments, the engineered bacteria comprise gene sequence encoding one or more PAL polypeptide sequence(s) in which the gene sequene(s) is directly or indirectly induced by low-oxygen or anaerobic conditions, such as the mammalian gut. In some embodiments, the engineered bacteria comprise gene sequence encoding one or more LAAD polypeptides. In some embodiments, the engineered bacteria comprise gene sequence encoding one or more LAAD polypeptides, in which the gene sequence(s) is directly or indirectly induced by oxygenated, low oxygen, or microaerobic conditions, such as conditions found in the proximal intestine, including but not limited to the stomach, duodenum, and ileum. In other embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more PME polypeptide sequences(s) in which the gene sequene(s) 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 one or more PME gene sequences(s) which are directly or indirectly induced by an environmental factor that is not naturally present in a mammalian gut, e.g., arabinose or IPTG. In other embodiments, the genetically engineered bacteria encode one or more PME gene sequences(s) which are directly or indirectly induced by an environmental factor that is naturally present in a mammalian gut under inflammatory conditions. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more PAL polypeptides and gene sequence(s) encoding one or more L-AAD polypeptides in which the gene sequences are under the control of the same promoter or a different copy of the same promoter, which is directly or indirectly induced by exogenous environmental conditions, such as any of the environmental conditions discussed herein and such as any of the promoters discussed herein. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more PAL polypeptides and gene sequence(s) encoding one or more L-AAD polypeptides in which the gene sequences are under the control of a different promoter, which is directly or indirectly induced by exogenous environmental conditions, such as any of the environmental conditions discussed herein and such as any of the promoters discussed herein. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more PAL polypeptides and gene sequence(s) encoding one or more L-AAD polypeptides in which the gene sequences are under the control of a constitutive promoter. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more PAL polypeptides

and gene sequence(s) encoding one or more L-AAD polypeptides in which the PAL gene sequences are under the control of a constitutive promoter and the LAAD gene sequence(s) are under the control of an inducible promoter. In some embodiments, the engineered bacteria comprise gene sequence(s) encoding one or more PAL polypeptides and gene sequence(s) encoding one or more L-AAD polypeptides in which the LAAD gene sequences are under the control of a constitutive promoter and the PAL gene sequence(s) are under the control of an inducible promoter. In any of these embodiments, the bacteria may further comprise gene sequence encoding one or more Phe transporter polypeptides, which gene sequence(s) may be under the control of a constitutive or inducible promoter and may be the same or differnet promoter from the promoter controlling the Pal and/or LAAD gene sequence(s).

[0190] In other embodiments, the engineered bacteria encode one or more PME gene sequence(s) which are directly or indirectly induced prior to *in vivo* administration during bacterial cell culture; i.e., one or more PME gene sequence(s) are expressed under the control of an inducible promoter that is responsive to specific molecules or metabolites, temperature, oxygen levels or other parameters provided in the culture of the bacterium as it is grown in a flask, fermenter, or other culture vessel. In some embodiments, the engineered bacteria encode one or more PME gene sequence(s) which are directly or indirectly induced prior to *in vivo* administration during bacterial cell culture; wherein the one or more PME gene sequence(s) are expressed under low oxygen or anaerobic conditions. In some embodiments, the engineered bacteria encode one or more PME gene sequence(s) which are directly or indirectly induced prior to *in vivo* administration during bacterial cell culture; wherein the one or more PME gene sequence(s) are expressed under aerobic conditions. In some embodiments, the engineered bacteria encode one or more PME gene sequence(s) which are directly or indirectly induced prior to *in vivo* administration during bacterial cell culture; wherein the one or more PME gene sequence(s) are expressed under microaerobic conditions. In some embodiments, the engineered bacteria encode one or more PME gene sequence(s) which are directly or indirectly induced prior to *in vivo* administration during bacterial cell culture; wherein the one or more PME gene sequence(s) are expressed in the presence of arabinose. In some embodiments, the engineered bacteria encode one or more PME gene sequence(s) which are directly or

indirectly induced prior to *in vivo* administration during bacterial cell culture; wherein the one or more PME gene sequence(s) are expressed in the presence of IPTG.

[0191] 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.

[0192] In certain embodiments, the genetically engineered bacteria comprise one or more gene PME gene sequence(s), 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 and/or low oxygen 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.

[0193] 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).

[0194] 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.

[0195] 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).

[0196] In some embodiments, genetically engineered bacteria comprise one or more nucleic acid sequence(s) which are 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	ATCCCCATCACTCTTGATGGAGATCAATTCCCCAAGCTGCTAGA GCGTTACCTTGCCCTTAAACATTAGCAATGTCGATTTATCAGAG GGCCGACAGGCTCCCACAGGAGAAAACCG
SEQ ID NO: 10	CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTT GCCCTTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGG CTCCCACAGGAGAAAACCG
nirB1 SEQ ID NO: 11	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTA CATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATACCCCTTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGT AATAGAAAAGAAATCGAGGCAAAA
nirB2 SEQ ID NO: 12	CGGCCCGATCGTTGAACATAGCGGTCCGCAGGCGGCACTGCTTA CAGCAAACGGTCTGTACGCTGTCGTCTTTGTGATGTGCTTCCTGT TAGGTTTCGTCAGCCGTCACCGTCAGCATAACACCCTGACCTCT CATTAATTGCTCATGCCGGACGGCACTATCGTCGTCCGGCCTTTT CCTCTCTTCCCCCGCTACGTGCATCTATTTCTATAAACCCGCTCA TTTTGTCTATTTTTTGCACAAACATGAAATATCAGACAATTCCGT GACTTAAGAAAATTTATACAAATCAGCAATATACCCATTAAGGA GTATATAAAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCT GAATCGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAA Aatgtttgtttaactttaagaaggagatatacat
nirB3 SEQ ID NO: 13	GTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGGAC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTG CATCTATTTCTATAAACCCGCTCATTTTGTCTATTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATACCCATTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGT AATAGAAAAGAAATCGAGGCAAAA
ydfZ SEQ ID NO: 14	ATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTT ATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAA ACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCCGT TACGTGGGCTTCGACTGTAAATCAGAAAGGAGAAAACACCT

nirB+RBS SEQ ID NO: 15	GTCAGCATAACACCCTGACCTCTCATTAATTGTTCATGCCGGGC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTA CATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATACCCCTTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAA**GGATCC**CTCT AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
ydfZ+RBS SEQ ID NO: 16	CATTTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACT TATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAA ACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCC**GG ATCC**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CAT
fnrS1 SEQ ID NO: 17	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAA ATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAA ACGCCGTAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTC AGGGCAATATCTCTCTT**GGATCC**CTCTAGAAATAATTTTGTTTA ACTTTAAGAAGGAGATATACAT
fnrS2 SEQ ID NO: 18	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAA ATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAA ACGCCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTC AGGGCAATATCTCTCTTGGATCCAAAGTGAACTCTAGAAATAAT TTTGTTTAACTTTAAGAAGGAGATATACAT
nirB+crp SEQ ID NO: 19	TCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTCAGCCGTCACC GTCAGCATAACACCCTGACCTCTCATTAATTGCTCATGCCGGAC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTG CATCTATTTCTATAAACCCGCTCATTTTGTCTATTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATACCCATTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGaaatgtgat ctagttcacatttGCGGTAATAGAAAAGAAATCGAGGCAAAA*atgtttgtttaac tttaagaaggagatatacat*
fnrS+crp SEQ ID NO: 20	AGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTAA ATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAA ACGCCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTC AGGGCAATATCTCTCaaatgtgatctagttcacattt*ttgtttaactttaagaaggagatatac at*

[0197] In other embodiments, one or more PME(s), e.g., *PAL*, are expressed under the control of an oxygen level-dependent promoter fused to a binding site for a transcriptional activator, *e.g.*, CRP. CRP (cyclic AMP receptor protein or catabolite

activator protein or CAP) plays a major regulatory role in bacteria by repressing genes responsible for the uptake, metabolism, and assimilation of less favorable carbon sources when rapidly metabolizable carbohydrates, such as glucose, are present (Wu et al., 2015). This preference for glucose has been termed glucose repression, as well as carbon catabolite repression (Deutscher, 2008; Görke and Stülke, 2008). In some embodiments, 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*.

[0198] In another embodiment, one or more PME(s), 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.

[0199] In some embodiments, LAAD is under the control of an FNR promoter. In some embodiments, LAAD is under the control of promoter that not the FNR promoter. LAAD requires oxygen to catalyze the degradation of phenylalanine to phenylpyruvate. Therefore, it is desirable to induce LAAD expression under oxygenated, or even in reduced oxygen conditions where it be active (**Fig. 25**).

[0200] In some embodiments, one or more PME(s), e.g., *PAL* and/or LAAD, are 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, expression of one or more PME genes is under the control of a propionate-inducible promoter. In a more

specific embodiment, PME gene expression is under the control of one or more propionate-inducible promoter(s) 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. In one embodiment, PAL expression is under the control of the P_{araBAD} promoter. In one embodiment, PAL expression occurs under aerobic or microaerobic conditions. In one embodiment, PAL expression occurs under anaerobic or low oxygen conditions and LADD expression occurs under aerobic or microaerobic conditions. In one embodiment, PAL expression occurs under anaerobic or low oxygen conditions and LADD expression is under the control of the P_{araBAD} promoter.

[0201] In some embodiments, the one or more PME genes, e.g., *PAL* and/or *LAAD* gene are expressed under the control of a promoter that is induced by exposure to a chemical and/or nutritional inducer. In some embodiments, the one or more PME genes, e.g., *PAL* and/or *LAAD* gene are expressed under the control of a promoter that is induced by exposure to tetracycline. In some embodiments, the one or more PME genes, e.g., *PAL* and/or *LAAD* gene are expressed under the control of a promoter that is induced by exposure to arabinose. In some embodiments, the one or more PME genes, e.g., *PAL* and/or *LAAD* gene are expressed under the control of a promoter that is induced by exposure to IPTG or other LacI inducer. In some embodiments, the one or more PME genes, e.g., *PAL* and/or *LAAD* gene are expressed under the control of a promoter that is induced by exposure to rhamnose. In some embodiments, the one or more PME genes, e.g., *PAL* and/or *LAAD* gene are expressed under the control of a promoter that is induced by exposure to teracycline. In some embodiments, more than one PME gene is expressed, e.g., PAL and LAAD gene, and each gene is expressed

under the control of different promoters, such as any of the promoters discussed in this paragraph and elsewhere herein.

[0202] In some embodiments, the one or more PME genes, e.g., *PAL and/or LAAD* gene are expressed under the control of a promoter that is induced by a change in temperature from a non-permissive temperature to a permissive temperature. 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. Bioinformatics tools for the fine tuning and optimization of RBS are known in the art.

[0203] In any of the embodiments described herein above (and elsewhere herein), the engineered bacteria may additionally comprise gene sequence encoding one or more Phe transporter(s), which gene sequence(s) maye be under the control of any of the promoters discussed herein.

[0204] 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. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to arabinose. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to IPTG or another LacI inducer. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a

promoter that is induced by exposure to rhamnose. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the *PAL* gene is present on a plasmid and operably linked to a promoter that is induced by change in temperature from a non-permissive temperature to a permissive temperature. In some embodiments, the *PAL* gene is present on a chromosome and operably linked to a promoter that is induced by exposure to arabinose. In some embodiments, the *PAL* gene is present on a chromosome and operably linked to a promoter that is induced by exposure to IPTG or another LacI inducer. In some embodiments, the *PAL* gene is present on a chromosome and operably linked to a promoter that is induced by exposure to rhamnose. In some embodiments, the *PAL* gene is present on a chromosome and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the *PAL* gene is present on a chromosome and operably linked to a promoter that is induced by change in temperature from a non-permissive temperature to a permissive temperature.

[0205] 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 or tetracycline. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to arabinose. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to IPTG or another LacI inducer. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a

promoter that is induced by exposure to rhamnose. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a promoter that is induced by change in temperature from a non-permissive temperature to a permissive temperature. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a constitutive promoter. In some embodiments, the *LAAD* gene is present on a plasmid and operably linked to a promoter that is induced by exposure to tetracycline. In some embodiments, the *LAAD* gene is present on a chromosome and operably linked to a promoter that is induced by exposure to arabinose. In some embodiments, the *LAAD* gene is present on a chromosome and operably linked to a promoter that is induced by exposure to IPTG or another LacI inducer. In some embodiments, the *LAAD* gene is present on a chromosome and operably linked to a promoter that is induced by exposure to rhamnose. In some embodiments, the *LAAD* gene is present on a chromosome and operably linked to a promoter that is induced by change in temperature from a non-permissive temperature to a permissive temperature. In some embodiments, the *LAAD* gene is present on a chromosome and operably linked to a constitutive promoter.

[0206] In any of these embodiments of bacteria comprising PME gene(s), e.g., PAL, PAH, and/or LAAD, the bacteria may further comprise gene sequence encoding one or more Phe transporters, which Phe transporter gene sequence(s) may be present on a plasmid or chromosome, which may be the same or a different plasmid or chromosome from the location of the PME gene. The Phe transporter gene sequence(s) may be under the control of the same or a different promoter from the PMR gene sequence(s).

[0207] 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 or PAH*, 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.

[0208] 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 or PAH*, 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 or PAH*, 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).

[0209] 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.

[0210] 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. 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, and increase hippuric acid in the urine 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 to hippurate. In embodiments using genetically modified forms of these bacteria, PAL-mediated processing of phenylalanine will be appreciable under exogenous environmental conditions.

[0211] In some embodiments, the genetically engineered bacteria of the invention produce PAL under exogenous environmental conditions, such as under bacterial culture conditions *in vitro*, and increase transcinnamic acid in the media 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. 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. As shown herein, TCA is rapidly converted to hippuric acid *in vivo*, and hippuric acid subsequently accumulates in the urine. Therefore, hippurate, in blood and in particular in the urine, may be an even better biomarker for phenylalanine degradation *in vivo*. In some embodiments, *PAL* expression is measured by methods known in the art, e.g., measurement of blood phenylalanine levels. Hippuric acid may be measured according to methods described herein in the Examples, and methods known in the art.

[0212] 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.

[0213] In some embodiments, the genetically engineered bacteria of the invention produce more than one PME, e.g., PAL, PAH, and/or LAAD, under

exogenous environmental conditions, such as in vivo or under bacterial culture conditions *in vitro*, and reduce blood phenylalanine and/or increase transcinnamic acid in the media 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. In any of these embodiments, the bacteris amy further comprise gene sequence(s) encoding one or more Phe transporter polypeptides.

[0214] In some embodiments, one or more PME(s), e.g., *PAL, LAAD, and/or PAH,* are 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, one or more PME(s), e.g., *PAL, LAAD, and/or PAH,* are 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(s), e.g., *PAL, LAAD, and/or PAH* gene(s) 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.

[0215] 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.

[0216] In some embodiments, the PME(s), e.g., *PAL, LAAD*, and /or *PAH,* gene(s) are 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. 66**). 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 than one copy of the same PME gene or more than one copy of different PME genes.

[0217] 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)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCC TTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAG GAGAAAACCGATGAAAAACGCGTCAACCGTATCGGAAGATACTGCGTCGA ATCAAGAGCCGACGCTTCATCGCGGATTACATAACCGTCATATTCAACTGAT TGCGTTGGGTGGCGCAATTGGTACTGGTCTGTTTCTTGGCATTGGCCCGGCG ATTCAGATGGCGGGTCCGGCTGTATTGCTGGGCTACGGCGTCGCCGGGATC

Nucleotide sequences of FNR promoter-PheP construct (SEQ ID NO: 21)
ATCGCTTTCCTGATTATGCGCCAGCTTGGCGAAATGGTGGTTGAGGAGCCGG TATCCGGTTCATTTGCCCACTTTGCCTATAAATACTGGGGACCGTTTGCGGG CTTCCTCTCTGGCTGGAACTACTGGGTAATGTTCGTGCTGGTGGGAATGGCA GAGCTGACCGCTGCGGGCATCTATATGCAGTACTGGTTCCCGGATGTTCCAA CGTGGATTTGGGCTGCCGCCTTCTTTATTATCATCAACGCCGTTAACCTGGT GAACGTGCGCTTATATGGCGAAACCGAGTTCTGGTTTGCGTTGATTAAAGTG CTGGCAATCATCGGTATGATCGGCTTTGGCCTGTGGCTGCTGTTTTCTGGTC ACGGCGGCGAGAAAGCCAGTATCGACAACCTCTGGCGCTACGGTGGTTTCT TCGCCACCGGCTGGAATGGGCTGATTTTGTCGCTGGCGGTAATTATGTTCTC CTTCGGCGGTCTGGAGCTGATTGGGATTACTGCCGCTGAAGCGCGCGATCCG GAAAAAAGCATTCCAAAAGCGGTAAATCAGGTGGTGTATCGCATCCTGCTG TTTTACATCGGTTCACTGGTGGTTTTACTGGCGCTCTATCCGTGGGTGGAAG TGAAATCCAACAGTAGCCCGTTTGTGATGATTTTCCATAATCTCGACAGCAA CGTGGTAGCTTCTGCGCTGAACTTCGTCATTCTGGTAGCATCGCTGTCAGTG TATAACAGCGGGGTTTACTCTAACAGCCGCATGCTGTTTGGCCTTTCTGTGC AGGGTAATGCGCCGAAGTTTTTGACTCGCGTCAGCCGTCGCGGTGTGCCGAT TAACTCGCTGATGCTTTCCGGAGCGATCACTTCGCTGGTGGTGTTAATCAAC TATCTGCTGCCGCAAAAAGCGTTTGGTCTGCTGATGGCGCTGGTGGTAGCAA CGCTGCTGTTGAACTGGATTATGATCTGTCTGGCGCATCTGCGTTTTCGTGC AGCGATGCGACGTCAGGGGCGTGAAACACAGTTTAAGGCGCTGCTCTATCC GTTCGGCAACTATCTCTGCATTGCCTTCCTCGGCATGATTTTGCTGCTGATGT GCACGATGGATGATATGCGCTTGTCAGCGATCCTGCTGCCGGTGTGGATTGT ATTCCTGTTTATGGCATTTAAAACGCTGCGTCGGAAATAA

Table 5

Nucleotide sequences of FNR promoter-PAL1 construct, high-copy (SEQ ID NO: 22)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCC TTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAG GAGAAAACCGATGAAAACACTATCACAGGCCCAATCTAAAACTTCTTCACA GCAATTCAGCTTTACCGGGAACTCGTCTGCGAATGTAATTATCGGCAATCAA AAGCTGACCATTAATGATGTAGCTCGCGTTGCCCGGAATGGCACTTTGGTGT CACTGACGAACAATACCGACATTCTGCAAGGTATTCAAGCTAGCTGCGATT ATATCAATAACGCCGTTGAATCTGGCGAGCCAATCTACGGGGTAACAAGCG GTTTTGGTGGGATGGCGAACGTTGCCATTAGCCGTGAACAGGCGAGCGAAC TTCAGACCAACCTCGTTTGGTTCCTAAAGACAGGAGCTGGTAATAAGTTACC TCTGGCTGACGTAAGAGCCGCGATGCTGCTTCGCGCTAATAGTCACATGCGC GGCGCCAGTGGTATCCGTCTTGAGCTTATCAAGAGGATGGAAATCTTCCTCA ACGCGGGTGTCACACCATATGTTTATGAGTTTGGTAGTATCGGAGCCAGTGG TGATCTTGTTCCCCTGAGTTATATTACGGGTTCATTGATTGGTTTAGACCCGT CCTTTAAAGTGGATTTTAACGGGAAAGAAATGGACGCCCCGACCGCTTTAC GACAGCTTAATCTGAGCCCACTTACTTTGCTCCCTAAAGAAGGTCTTGCCAT GATGAATGGCACCTCTGTGATGACTGGAATTGCCGCGAATTGTGTGTATGAC ACGCAGATCCTAACGGCCATTGCCATGGGTGTTCACGCGTTGGACATTCAAG

Nucleotide sequences of FNR promoter-PAL1 construct, high-copy (SEQ ID NO: 22)
CCCTGAATGGTACAAACCAGTCGTTTCATCCGTTTATCCATAATTCAAAACC CCATCCGGGACAGCTTTGGGCTGCTGATCAGATGATCTCACTCCTGGCCAAT AGTCAACTGGTTCGGGACGAGCTCGACGGCAAACATGATTATCGCGATCAT GAGCTCATCCAGGACCGGTATTCACTTCGTTGTCTCCCACAATACCTGGGGC CTATCGTTGATGGTATATCTCAAATTGCGAAGCAAATTGAAATTGAGATCAA TAGCGTAACCGACAACCCGCTTATCGATGTTGATAATCAGGCCTCTTATCAC GGTGGCAATTTTCTGGGCCAGTATGTTGGTATGGGGATGGATCACCTGCGGT ACTATATTGGGCTTCTGGCTAAACATCTTGATGTGCAGATTGCCTTATTAGC TTCACCAGAATTTTCAAATGGACTGCCGCCATCATTGCTCGGTAACAGAGAA AGGAAAGTAAATATGGGCCTTAAGGGCCTTCAGATATGTGGTAACTCAATC ATGCCCCTCCTGACCTTTTATGGGAACTCAATTGCTGATCGTTTTCCGACAC ATGCTGAACAGTTTAACCAAAACATTAACTCACAGGGCTATACATCCGCGA CGTTAGCGCGTCGGTCCGTGGATATCTTCCAGAATTATGTTGCTATCGCTCT GATGTTCGGCGTACAGGCCGTTGATTTGCGCACTTATAAAAAAACCGGTCAC TACGATGCTCGGGCTTGCCTGTCGCCTGCCACCGAGCGGCTTTATAGCGCCG TACGTCATGTTGTGGGTCAGAAACCGACGTCGGACCGCCCCTATATTTGGAA TGATAATGAACAAGGGCTGGATGAACACATCGCCCGGATATCTGCCGATAT TGCCGCCGGAGGTGTCATCGTCCAGGCGGTACAAGACATACTTCCTTGCCTG CATTAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTA TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGC CTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCC AACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCT CACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAG AACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGC GTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAAT CGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG GCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT TACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGG GCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCA GCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTA TCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTG ATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAG CAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTA CGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCA TGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAG TTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAA TGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCA TCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCA GATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGT CCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTA

Nucleotide sequences of FNR promoter-PAL1 construct, high-copy (SEQ ID NO: 22)
GAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTAC AGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGT TCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCG GTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGT TATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCC GTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAAT AGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATA CCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTC GGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTA ACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAA GCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA GAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC TGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT ATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCT GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCG GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGG GCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGC GCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGG CCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTT

Table 6

Nucleotide sequences of FNR promoter-PAL3 construct, high-copy (SEQ ID NO: 23)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCC **TTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAG** **GAGAAAACCG**ATGAAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAA AAATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAAAAA AAAGTAGAAATATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAA AAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAATCAATACA GGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAG CATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTC CAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAA GGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTA ATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGG TGATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAA GTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCA GGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACG GCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGAAA AACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTT GCATCTCATGAACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTG

Nucleotide sequences of FNR promoter-PAL3 construct, high-copy (SEQ ID NO: 23)
GTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGC AGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCA TCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAATTCGC TGTGCACCACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGA AAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCCATTGATAGATCC AGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGGCAATATGTCGC CCGAACAATGGATGCATTAAAACTGGATATTGCTTTAATTGCCAATCATCTT CACGCCATTGTGGCTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTA ATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAACT TTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGATTGTGCTGCATCAGGT ATTCATACCCTCGCCACAGAACAATACAATCAAGATATTGTCAGTTTAGGTC TGCATGCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGT TTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATT AGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGCGAAATC AGTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCATTG CGGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAGA ATAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATC CGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCC CGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAA CGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCA CTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTC AAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA ACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATC GACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG GCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT TACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGG GCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCA GCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTA TCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTG ATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAG CAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTA CGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCA TGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAG TTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAA TGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCA TCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCA GATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGT CCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTA GAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTAC AGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGT

Nucleotide sequences of FNR promoter-PAL3 construct, high-copy (SEQ ID NO: 23)
TCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCG GTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGT TATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCC GTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAAT AGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATA CCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTC GGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTA ACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAA GCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA GAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC TGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT ATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCT GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCG GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGG GCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGC GCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGG CCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTT

Table 7

Nucleotide sequences of Tet promoter-PAL1 construct, high-copy (SEQ ID NO: 24)
CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAACA CTATCACAGGCCCAATCTAAAACTTCTTCACAGCAATTCAGCTTTACCGGGA ACTCGTCTGCGAATGTAATTATCGGCAATCAAAAGCTGACCATTAATGATGT AGCTCGCGTTGCCCGGAATGGCACTTTGGTGTCACTGACGAACAATACCGA CATTCTGCAAGGTATTCAAGCTAGCTGCGATTATATCAATAACGCCGTTGAA TCTGGCGAGCCAATCTACGGGGTAACAAGCGGTTTTGGTGGGATGGCGAAC GTTGCCATTAGCCGTGAACAGGCGAGCGAACTTCAGACCAACCTCGTTTGGT TCCTAAAGACAGGAGCTGGTAATAAGTTACCTCTGGCTGACGTAAGAGCCG CGATGCTGCTTCGCGCTAATAGTCACATGCGCGGCGCCAGTGGTATCCGTCT TGAGCTTATCAAGAGGATGGAAATCTTCCTCAACGCGGGTGTCACACCATAT GTTTATGAGTTTGGTAGTATCGGAGCCAGTGGTGATCTTGTTCCCCTGAGTT ATATTACGGGTTCATTGATTGGTTTAGACCCGTCCTTTAAAGTGGATTTTAA CGGGAAAGAAATGGACGCCCCGACCGCTTTACGACAGCTTAATCTGAGCCC ACTTACTTTGCTCCCTAAAGAAGGTCTTGCCATGATGAATGGCACCTCTGTG ATGACTGGAATTGCCGCGAATTGTGTGTATGACACGCAGATCCTAACGGCC ATTGCCATGGGTGTTCACGCGTTGGACATTCAAGCCCTGAATGGTACAAACC AGTCGTTTCATCCGTTTATCCATAATTCAAAACCCCATCCGGGACAGCTTTG GGCTGCTGATCAGATGATCTCACTCCTGGCCAATAGTCAACTGGTTCGGGAC GAGCTCGACGGCAAACATGATTATCGCGATCATGAGCTCATCCAGGACCGG

Nucleotide sequences of Tet promoter-PAL1 construct, high-copy (SEQ ID NO: 24)
TATTCACTTCGTTGTCTCCCACAATACCTGGGGCCTATCGTTGATGGTATATC TCAAATTGCGAAGCAAATTGAAATTGAGATCAATAGCGTAACCGACAACCC GCTTATCGATGTTGATAATCAGGCCTCTTATCACGGTGGCAATTTTCTGGGC CAGTATGTTGGTATGGGGATGGATCACCTGCGGTACTATATTGGGCTTCTGG CTAAACATCTTGATGTGCAGATTGCCTTATTAGCTTCACCAGAATTTTCAAA TGGACTGCCGCCATCATTGCTCGGTAACAGAGAAAGGAAAGTAAATATGGG CCTTAAGGGCCTTCAGATATGTGGTAACTCAATCATGCCCCTCCTGACCTTT TATGGGAACTCAATTGCTGATCGTTTTCCGACACATGCTGAACAGTTTAACC AAAACATTAACTCACAGGGCTATACATCCGCGACGTTAGCGCGTCGGTCCG TGGATATCTTCCAGAATTATGTTGCTATCGCTCTGATGTTCGGCGTACAGGC CGTTGATTTGCGCACTTATAAAAAAACCGGTCACTACGATGCTCGGGCTTGC CTGTCGCCTGCCACCGAGCGGCTTTATAGCGCCGTACGTCATGTTGTGGGTC AGAAACCGACGTCGGACCGCCCCTATATTTGGAATGATAATGAACAAGGGC TGGATGAACACATCGCCCGGATATCTGCCGATATTGCCGCCGGAGGTGTCAT CGTCCAGGCGGTACAAGACATACTTCCTTGCCTGCATTAAGCTTGGCGTAAT CATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGT GAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGA AACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGC GGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTC GGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACG GTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAG GCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAA GCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC GCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGT ATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACC CCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCC AACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGG ATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGG CCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGA AGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAA CCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAG AAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCT CAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAA AGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCT AAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGA GGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCC CCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTG CTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAAT AAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATC CGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCG CCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGT CACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAG GCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGT

Nucleotide sequences of Tet promoter-PAL1 construct, high-copy (SEQ ID NO: 24)
CCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTA TGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCT GTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGA CCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC AGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCT CAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACC CAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAA ACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATG TTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTT ATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAAC CATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTT CGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCC CGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCC GTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATG CGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATAC CGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCA GGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTAC GCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGC CAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGTTAA GACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGCATATGATCAATTCAAG GCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAATAATTCGATAGC TTGTCGTAATAATGGCGGCATACTATCAGTAGTAGGTGTTTCCCTTTCTTCTT TAGCGACTTGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAAATGCCC CACAGCGCTGAGTGCATATAATGCATTCTCTAGTGAAAAACCTTGTTGGCAT AAAAAGGCTAATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAGGCCGTG TACCTAAATGTACTTTTGCTCCATCGCGATGACTTAGTAAAGCACATCTAAA ACTTTTAGCGTTATTACGTAAAAAATCTTGCCAGCTTTCCCCTTCTAAAGGG CAAAAGTGAGTATGGTGCCTATCTAACATCTCAATGGCTAAGGCGTCGAGC AAAGCCCGCTTATTTTTTACATGCCAATACAATGTAGGCTGCTCTACACCTA GCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCGACCTCATTAAG CAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAATCTAGACATCATTA ATTCCTAATTTTT**GTTGACACTCTATCATTGATAGAGTTATTTTACCACTC** **CCTATCAGTGATAGAGAA**AAGTGAA

Table 8

Nucleotide sequences of Tet promoter-PAL3, high-copy construct (SEQ ID NO: 25)
CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAGCT AAAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCTTTGG AAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAAATATCAACGG AGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAAAATTAA ATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGAATGCCA

Nucleotide sequences of Tet promoter-PAL3, high-copy construct (SEQ ID NO: 25)
ATTTAGTTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAAC TTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGTATTAAAGCGT CACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACCAGACC AATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGACATTGTTCCTCTG GTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTA TATTGCACGAGCATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAA ATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAA AAGCCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAA TCAGTGCAATCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAATTTC TGCGATTGCCCTTGCTGTTGAAGCATTACTTGCATCTCATGAACATTATGAT GCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGT GCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGGGG TTAAAGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCAACTAA ATGATACCTTACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGG TATAGTGCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGAACGGGAAGT TATCTCAGCTAATGATAATCCATTGATAGATCCAGAAAATGGCGATGTTCTA CACGGTGGAAATTTTATGGGGCAATATGTCGCCCGAACAATGGATGCATTA AAACTGGATATTGCTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCTTAT GATGGATAACCGTTTCTCTCGTGGATTACCTAATTCACTGAGTCCGACACCC GGCATGTATCAAGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTG CTGCAATTCGCCATGATTGTGCTGCATCAGGTATTCATACCCTCGCCACAGA ACAATACAATCAAGATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGATGTT TTAGAGATGGAGCAGAAATTACGCAATATTGTTTCAATGACAATTCTGGTAG TTTGTCAGGCCATTCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAAC TGCTAAATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGAT CGTGCGTTGGATGAAGATATAATCCGCATTGCGGATGCAATTATTAATGATC AACTTCCTCTGCCAGAAATCATGCTGGAAGAATAAGCTTGGCGTAATCATG GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGC TAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACC TGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT TGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTA TCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCA GCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAG GCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTG GCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTC CCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCT TTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCT CAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCC GTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACC CGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTA GCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA ACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCC AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCAC CGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAA

Nucleotide sequences of Tet promoter-PAL3, high-copy construct (SEQ ID NO: 25)
AAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGT GGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGA TCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAG TATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGT CGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGC AATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGC CTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCA GTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCAC GCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCG AGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCT CCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGG CAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTG ACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG AGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGA ACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAA GGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAA CTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACA GGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTG AATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATT GTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAG GGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCA TTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCG TCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCG GAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGT CAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCG GCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCG CACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGG CTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCC AGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAG GGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGTTAAGAC CCACTTTCACATTTAAGTTGTTTTTCTAATCCGCATATGATCAATTCAAGGCC GAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAATAATTCGATAGCTTGT CGTAATAATGGCGGCATACTATCAGTAGTAGGTGTTTCCCTTTCTTCTTTAGC GACTTGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAAATGCCCCACA GCGCTGAGTGCATATAATGCATTCTCTAGTGAAAAACCTTGTTGGCATAAAA AGGCTAATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAGGCCGTGTACCT AAATGTACTTTTGCTCCATCGCGATGACTTAGTAAAGCACATCTAAAACTTT TAGCGTTATTACGTAAAAAATCTTGCCAGCTTTCCCCTTCTAAAGGGCAAAA GTGAGTATGGTGCCTATCTAACATCTCAATGGCTAAGGCGTCGAGCAAAGC CCGCTTATTTTTTACATGCCAATACAATGTAGGCTGCTCTACACCTAGCTTCT GGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCGACCTCATTAAGCAGCTC TAATGCGCTGTTAATCACTTTACTTTTATCTAATCTAGACATCATTAATTCCT AATTTTT**GTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTAT** **CAGTGATAGAGAA**AAGTGAA

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

Table 9

Nucleotide sequences of FNR promoter-PAL1 construct, low-copy (SEQ ID NO: 26)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCC TTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAG GAGAAAACCGATGAAAACACTATCACAGGCCCAATCTAAAACTTCTTCACA GCAATTCAGCTTTACCGGGAACTCGTCTGCGAATGTAATTATCGGCAATCAA AAGCTGACCATTAATGATGTAGCTCGCGTTGCCCGGAATGGCACTTTGGTGT CACTGACGAACAATACCGACATTCTGCAAGGTATTCAAGCTAGCTGCGATT ATATCAATAACGCCGTTGAATCTGGCGAGCCAATCTACGGGGTAACAAGCG GTTTTGGTGGGATGGCGAACGTTGCCATTAGCCGTGAACAGGCGAGCGAAC TTCAGACCAACCTCGTTTGGTTCCTAAAGACAGGAGCTGGTAATAAGTTACC TCTGGCTGACGTAAGAGCCGCGATGCTGCTTCGCGCTAATAGTCACATGCGC GGCGCCAGTGGTATCCGTCTTGAGCTTATCAAGAGGATGGAAATCTTCCTCA ACGCGGGTGTCACACCATATGTTTATGAGTTTGGTAGTATCGGAGCCAGTGG TGATCTTGTTCCCCTGAGTTATATTACGGGTTCATTGATTGGTTTAGACCCGT CCTTTAAAGTGGATTTTAACGGGAAAGAAATGGACGCCCCGACCGCTTTAC GACAGCTTAATCTGAGCCCACTTACTTTGCTCCCTAAAGAAGGTCTTGCCAT GATGAATGGCACCTCTGTGATGACTGGAATTGCCGCGAATTGTGTGTATGAC ACGCAGATCCTAACGGCCATTGCCATGGGTGTTCACGCGTTGGACATTCAAG CCCTGAATGGTACAAACCAGTCGTTTCATCCGTTTATCCATAATTCAAAACC CCATCCGGGACAGCTTTGGGCTGCTGATCAGATGATCTCACTCCTGGCCAAT AGTCAACTGGTTCGGGACGAGCTCGACGGCAAACATGATTATCGCGATCAT GAGCTCATCCAGGACCGGTATTCACTTCGTTGTCTCCCACAATACCTGGGGC CTATCGTTGATGGTATATCTCAAATTGCGAAGCAAATTGAAATTGAGATCAA TAGCGTAACCGACAACCCGCTTATCGATGTTGATAATCAGGCCTCTTATCAC GGTGGCAATTTTCTGGGCCAGTATGTTGGTATGGGGATGGATCACCTGCGGT ACTATATTGGGCTTCTGGCTAAACATCTTGATGTGCAGATTGCCTTATTAGC TTCACCAGAATTTTCAAATGGACTGCCGCCATCATTGCTCGGTAACAGAGAA AGGAAAGTAAATATGGGCCTTAAGGGCCTTCAGATATGTGGTAACTCAATC ATGCCCCTCCTGACCTTTTATGGGAACTCAATTGCTGATCGTTTTCCGACAC ATGCTGAACAGTTTAACCAAAACATTAACTCACAGGGCTATACATCCGCGA CGTTAGCGCGTCGGTCCGTGGATATCTTCCAGAATTATGTTGCTATCGCTCT GATGTTCGGCGTACAGGCCGTTGATTTGCGCACTTATAAAAAAACCGGTCAC TACGATGCTCGGGCTTGCCTGTCGCCTGCCACCGAGCGGCTTTATAGCGCCG TACGTCATGTTGTGGGTCAGAAACCGACGTCGGACCGCCCCTATATTTGGAA TGATAATGAACAAGGGCTGGATGAACACATCGCCCGGATATCTGCCGATAT TGCCGCCGGAGGTGTCATCGTCCAGGCGGTACAAGACATACTTCCTTGCCTG CATTAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTA TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGC CTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCC

Nucleotide sequences of FNR promoter-PAL1 construct, low-copy (SEQ ID NO: 26)
AACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCT
CACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAGTACGGGTTTTGCTGCCCGCAAACGGGCTGTTCTGGTGTT
GCTAGTTTGTTATCAGAATCGCAGATCCGGCTTCAGGTTTGCCGGCTGAAAG
CGCTATTTCTTCCAGAATTGCCATGATTTTTTCCCCACGGGAGGCGTCACTG
GCTCCCGTGTTGTCGGCAGCTTTGATTCGATAAGCAGCATCGCCTGTTTCAG
GCTGTCTATGTGTGACTGTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATT
TCATGTTCTAGTTGCTTTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTAC
ATGCTGTTCATCTGTTACATTGTCGATCTGTTCATGGTGAACAGCTTTAAATG
CACCAAAAACTCGTAAAAGCTCTGATGTATCTATCTTTTTTACACCGTTTTCA
TCTGTGCATATGGACAGTTTTCCCTTTGATATCTAACGGTGAACAGTTGTTCT
ACTTTTGTTTGTTAGTCTTGATGCTTCACTGATAGATACAAGAGCCATAAGA
ACCTCAGATCCTTCCGTATTTAGCCAGTATGTTCTCTAGTGTGGTTCGTTGTT
TTTGCGTGAGCCATGAGAACGAACCATTGAGATCATGCTTACTTTGCATGTC
ACTCAAAAATTTTGCCTCAAAACTGGTGAGCTGAATTTTTGCAGTTAAAGCA
TCGTGTAGTGTTTTTCTTAGTCCGTTACGTAGGTAGGAATCTGATGTAATGG
TTGTTGGTATTTTGTCACCATTCATTTTTATCTGGTTGTTCTCAAGTTCGGTTA
CGAGATCCATTTGTCTATCTAGTTCAACTTGGAAAATCAACGTATCAGTCGG
GCGGCCTCGCTTATCAACCACCAATTTCATATTGCTGTAAGTGTTTAAATCTT
TACTTATTGGTTTCAAAACCCATTGGTTAAGCCTTTTAAACTCATGGTAGTTA
TTTTCAAGCATTAACATGAACTTAAATTCATCAAGGCTAATCTCTATATTTG
CCTTGTGAGTTTTCTTTTGTGTTAGTTCTTTTAATAACCACTCATAAATCCTC
ATAGAGTATTTGTTTTCAAAAGACTTAACATGTTCCAGATTATATTTTATGA
ATTTTTTTAACTGGAAAAGATAAGGCAATATCTCTTCACTAAAAACTAATTC
TAATTTTTCGCTTGAGAACTTGGCATAGTTTGTCCACTGGAAAATCTCAAAG
CCTTTAACCAAAGGATTCCTGATTTCCACAGTTCTCGTCATCAGCTCTCTGGT
TGCTTTAGCTAATACACCATAAGCATTTTCCCTACTGATGTTCATCATCTGAG
CGTATTGGTTATAAGTGAACGATACCGTCCGTTCTTTCCTTGTAGGGTTTTCA
ATCGTGGGGTTGAGTAGTGCCACACAGCATAAAATTAGCTTGGTTTCATGCT
CCGTTAAGTCATAGCGACTAATCGCTAGTTCATTTGCTTTGAAAACAACTAA
TTCAGACATACATCTCAATTGGTCTAGGTGATTTTAATCACTATACCAATTG
AGATGGGCTAGTCAATGATAATTACTAGTCCTTTTCCTTTGAGTTGTGGGTA
TCTGTAAATTCTGCTAGACCTTTGCTGGAAAACTTGTAAATTCTGCTAGACC
CTCTGTAAATTCCGCTAGACCTTTGTGTGTTTTTTTTGTTTATATTCAAGTGG
TTATAATTTATAGAATAAAGAAAGAATAAAAAAAGATAAAAAGAATAGATC
CCAGCCCTGTGTATAACTCACTACTTTAGTCAGTTCCGCAGTATTACAAAAG
GATGTCGCAAACGCTGTTTGCTCCTCTACAAAACAGACCTTAAAACCCTAAA
GGCTTAAGTAGCACCCTCGCAAGCTCGGGCAAATCGCTGAATATTCCTTTTG
TCTCCGACCATCAGGCACCTGAGTCGCTGTCTTTTTCGTGACATTCAGTTCGC
TGCGCTCACGGCTCTGGCAGTGAATGGGGGTAAATGGCACTACAGGCGCCT
TTTATGGATTCATGCAAGGAAACTACCCATAATACAAGAAAAGCCCGTCAC
GGGCTTCTCAGGGCGTTTTATGGCGGGTCTGCTATGTGGTGCTATCTGACTT
TTTGCTGTTCAGCAGTTCCTGCCCTCTGATTTTCCAGTCTGACCACTTCGGAT
TATCCCGTGACAGGTCATTCAGACTGGCTAATGCACCCAGTAAGGCAGCGG
TATCATCAACAGGCTTACCCGTCTTACTGTCTTTTCTACGGGGTCTGACGCTC
AGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAA

Nucleotide sequences of FNR promoter-PAL1 construct, low-copy (SEQ ID NO: 26)
GGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTA AAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAG GCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCC CGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT GCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATA AACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCC GCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC CAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTC ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTC CTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTAT GGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCT GTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGA CCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC AGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCT CAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACC CAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAA ACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATG TTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTT ATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAAC CATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTT CGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCC CGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCC GTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATG CGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATAC CGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCA GGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTAC GCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGC CAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCG

Table 10

Nucleotide sequences of FNR promoter-PAL3 construct, low-copy (SEQ ID NO: 27)
CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTTGCCC **TTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCCACAG** **GAGAAAACCG**ATGAAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAA AAATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAAAAA AAAGTAGAAATATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAA AAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAATCAATACA GGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAG CATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTC CAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAA GGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTA

Nucleotide sequences of FNR promoter-PAL3 construct, low-copy (SEQ ID NO: 27)
ATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGG TGATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAA GTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCA GGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACG GCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGAAA AACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTT GCATCTCATGAACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTG GTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGC AGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCA TCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAATTCGC TGTGCACCACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGA AAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCCATTGATAGATCC AGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGGCAATATGTCGC CCGAACAATGGATGCATTAAAACTGGATATTGCTTTAATTGCCAATCATCTT CACGCCATTGTGGCTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTA ATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAACT TTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGATTGTGCTGCATCAGGT ATTCATACCCTCGCCACAGAACAATACAATCAAGATATTGTCAGTTTAGGTC TGCATGCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGT TTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATT AGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGCGAAATC AGTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCATTG CGGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAGA ATAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATC CGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCC CGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAA CGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCA CTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTC AAAGGCGGTAGTACGGGTTTTGCTGCCCGCAAACGGGCTGTTCTGGTGTTGC TAGTTTGTTATCAGAATCGCAGATCCGGCTTCAGGTTTGCCGGCTGAAAGCG CTATTTCTTCCAGAATTGCCATGATTTTTTCCCCACGGGAGGCGTCACTGGCT CCCGTGTTGTCGGCAGCTTTGATTCGATAAGCAGCATCGCCTGTTTCAGGCT GTCTATGTGTGACTGTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTCA TGTTCTAGTTGCTTTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTACATG CTGTTCATCTGTTACATTGTCGATCTGTTCATGGTGAACAGCTTTAAATGCAC CAAAAACTCGTAAAAGCTCTGATGTATCTATCTTTTTTACACCGTTTTCATCT GTGCATATGGACAGTTTTCCCTTTGATATCTAACGGTGAACAGTTGTTCTAC TTTTGTTTGTTAGTCTTGATGCTTCACTGATAGATACAAGAGCCATAAGAAC CTCAGATCCTTCCGTATTTAGCCAGTATGTTCTCTAGTGTGGTTCGTTGTTTT TGCGTGAGCCATGAGAACGAACCATTGAGATCATGCTTACTTTGCATGTCAC TCAAAAATTTTGCCTCAAAACTGGTGAGCTGAATTTTTGCAGTTAAAGCATC GTGTAGTGTTTTTCTTAGTCCGTTACGTAGGTAGGAATCTGATGTAATGGTT GTTGGTATTTTGTCACCATTCATTTTTATCTGGTTGTTCTCAAGTTCGGTTAC GAGATCCATTTGTCTATCTAGTTCAACTTGGAAAATCAACGTATCAGTCGGG CGGCCTCGCTTATCAACCACCAATTTCATATTGCTGTAAGTGTTTAAATCTTT

Nucleotide sequences of FNR promoter-PAL3 construct, low-copy (SEQ ID NO: 27)
ACTTATTGGTTTCAAAACCCATTGGTTAAGCCTTTTAAACTCATGGTAGTTAT
TTTCAAGCATTAACATGAACTTAAATTCATCAAGGCTAATCTCTATATTTGC
CTTGTGAGTTTTCTTTTGTGTTAGTTCTTTTAATAACCACTCATAAATCCTCA
TAGAGTATTTGTTTTCAAAAGACTTAACATGTTCCAGATTATATTTTATGAAT
TTTTTTAACTGGAAAAGATAAGGCAATATCTCTTCACTAAAAACTAATTCTA
ATTTTTCGCTTGAGAACTTGGCATAGTTTGTCCACTGGAAAATCTCAAAGCC
TTTAACCAAAGGATTCCTGATTTCCACAGTTCTCGTCATCAGCTCTCTGGTTG
CTTTAGCTAATACACCATAAGCATTTTCCCTACTGATGTTCATCATCTGAGC
GTATTGGTTATAAGTGAACGATACCGTCCGTTCTTTCCTTGTAGGGTTTTCAA
TCGTGGGGTTGAGTAGTGCCACACAGCATAAAATTAGCTTGGTTTCATGCTC
CGTTAAGTCATAGCGACTAATCGCTAGTTCATTTGCTTTGAAAACAACTAAT
TCAGACATACATCTCAATTGGTCTAGGTGATTTTAATCACTATACCAATTGA
GATGGGCTAGTCAATGATAATTACTAGTCCTTTTCCTTTGAGTTGTGGGTAT
CTGTAAATTCTGCTAGACCTTTGCTGGAAAACTTGTAAATTCTGCTAGACCC
TCTGTAAATTCCGCTAGACCTTTGTGTGTTTTTTTTGTTTATATTCAAGTGGT
TATAATTTATAGAATAAAGAAAGAATAAAAAAAGATAAAAAGAATAGATC
CCAGCCCTGTGTATAACTCACTACTTTAGTCAGTTCCGCAGTATTACAAAAG
GATGTCGCAAACGCTGTTTGCTCCTCTACAAAACAGACCTTAAAACCCTAAA
GGCTTAAGTAGCACCCTCGCAAGCTCGGGCAAATCGCTGAATATTCCTTTTG
TCTCCGACCATCAGGCACCTGAGTCGCTGTCTTTTTCGTGACATTCAGTTCGC
TGCGCTCACGGCTCTGGCAGTGAATGGGGGTAAATGGCACTACAGGCGCCT
TTTATGGATTCATGCAAGGAAACTACCCATAATACAAGAAAAGCCCGTCAC
GGGCTTCTCAGGGCGTTTTATGGCGGGTCTGCTATGTGGTGCTATCTGACTT
TTTGCTGTTCAGCAGTTCCTGCCCTCTGATTTTCCAGTCTGACCACTTCGGAT
TATCCCGTGACAGGTCATTCAGACTGGCTAATGCACCCAGTAAGGCAGCGG
TATCATCAACAGGCTTACCCGTCTTACTGTCTTTTCTACGGGGTCTGACGCTC
AGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAA
GGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTA
AAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAG
GCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCC
CGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT
GCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATA
AACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCC
GCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC
CAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTC
ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG
CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTC
CTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTAT
GGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCT
GTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGA
CCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC
AGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCT
CAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACC
CAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAA
ACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATG
TTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTT

Nucleotide sequences of FNR promoter-PAL3 construct, low-copy (SEQ ID NO: 27)
ATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAAC CATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTT CGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCC CGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCC GTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATG CGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATAC CGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCA GGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTAC GCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGC CAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCG

Table 11

Nucleotide sequences of Tet promoter-PAL1 construct, low-copy (SEQ ID NO: 28)
ACCACTCCCTATCAGTGATAGAGAAAAGTGAACTCTAGAAATAATTTTGT TTAACTTTAAGAAGGAGATATACATATGAAAACACTATCACAGGCCCAATC TAAAACTTCTTCACAGCAATTCAGCTTTACCGGGAACTCGTCTGCGAATGTA ATTATCGGCAATCAAAAGCTGACCATTAATGATGTAGCTCGCGTTGCCCGGA ATGGCACTTTGGTGTCACTGACGAACAATACCGACATTCTGCAAGGTATTCA AGCTAGCTGCGATTATATCAATAACGCCGTTGAATCTGGCGAGCCAATCTAC GGGGTAACAAGCGGTTTTGGTGGGATGGCGAACGTTGCCATTAGCCGTGAA CAGGCGAGCGAACTTCAGACCAACCTCGTTTGGTTCCTAAAGACAGGAGCT GGTAATAAGTTACCTCTGGCTGACGTAAGAGCCGCGATGCTGCTTCGCGCTA ATAGTCACATGCGCGGCGCCAGTGGTATCCGTCTTGAGCTTATCAAGAGGAT GGAAATCTTCCTCAACGCGGGTGTCACACCATATGTTTATGAGTTTGGTAGT ATCGGAGCCAGTGGTGATCTTGTTCCCCTGAGTTATATTACGGGTTCATTGA TTGGTTTAGACCCGTCCTTTAAAGTGGATTTTAACGGGAAAGAAATGGACGC CCCGACCGCTTTACGACAGCTTAATCTGAGCCCACTTACTTTGCTCCCTAAA GAAGGTCTTGCCATGATGAATGGCACCTCTGTGATGACTGGAATTGCCGCG AATTGTGTGTATGACACGCAGATCCTAACGGCCATTGCCATGGGTGTTCACG CGTTGGACATTCAAGCCCTGAATGGTACAAACCAGTCGTTTCATCCGTTTAT CCATAATTCAAAACCCCATCCGGGACAGCTTTGGGCTGCTGATCAGATGATC TCACTCCTGGCCAATAGTCAACTGGTTCGGGACGAGCTCGACGGCAAACAT GATTATCGCGATCATGAGCTCATCCAGGACCGGTATTCACTTCGTTGTCTCC CACAATACCTGGGGCCTATCGTTGATGGTATATCTCAAATTGCGAAGCAAAT TGAAATTGAGATCAATAGCGTAACCGACAACCCGCTTATCGATGTTGATAAT CAGGCCTCTTATCACGGTGGCAATTTTCTGGGCCAGTATGTTGGTATGGGGA TGGATCACCTGCGGTACTATATTGGGCTTCTGGCTAAACATCTTGATGTGCA GATTGCCTTATTAGCTTCACCAGAATTTTCAAATGGACTGCCGCCATCATTG CTCGGTAACAGAGAAAGGAAAGTAAATATGGGCCTTAAGGGCCTTCAGATA TGTGGTAACTCAATCATGCCCCTCCTGACCTTTTATGGGAACTCAATTGCTG ATCGTTTTCCGACACATGCTGAACAGTTTAACCAAAACATTAACTCACAGGG CTATACATCCGCGACGTTAGCGCGTCGGTCCGTGGATATCTTCCAGAATTAT GTTGCTATCGCTCTGATGTTCGGCGTACAGGCCGTTGATTTGCGCACTTATA

Nucleotide sequences of Tet promoter-PAL1 construct, low-copy (SEQ ID NO: 28)

AAAAAACCGGTCACTACGATGCTCGGGCTTGCCTGTCGCCTGCCACCGAGC
GGCTTTATAGCGCCGTACGTCATGTTGTGGGTCAGAAACCGACGTCGGACC
GCCCCTATATTTGGAATGATAATGAACAAGGGCTGGATGAACACATCGCCC
GGATATCTGCCGATATTGCCGCCGGAGGTGTCATCGTCCAGGCGGTACAAG
ACATACTTCCTTGCCTGCATTAAGCTTGGCGTAATCATGGTCATAGCTGTTTC
CTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAG
CATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAAT
TGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTG
CATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGC
TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCG
AGCGGTATCAGCTCACTCAAAGGCGGTAGTACGGGTTTTGCTGCCCGCAAA
CGGGCTGTTCTGGTGTTGCTAGTTTGTTATCAGAATCGCAGATCCGGCTTCA
GGTTTGCCGGCTGAAAGCGCTATTTCTTCCAGAATTGCCATGATTTTTTCCCC
ACGGGAGGCGTCACTGGCTCCCGTGTTGTCGGCAGCTTTGATTCGATAAGCA
GCATCGCCTGTTTCAGGCTGTCTATGTGTGACTGTTGAGCTGTAACAAGTTG
TCTCAGGTGTTCAATTTCATGTTCTAGTTGCTTTGTTTTACTGGTTTCACCTGT
TCTATTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGATCTGTTCATGGT
GAACAGCTTTAAATGCACCAAAAACTCGTAAAAGCTCTGATGTATCTATCTT
TTTTACACCGTTTTCATCTGTGCATATGGACAGTTTTCCCTTTGATATCTAAC
GGTGAACAGTTGTTCTACTTTTGTTTGTTAGTCTTGATGCTTCACTGATAGAT
ACAAGAGCCATAAGAACCTCAGATCCTTCCGTATTTAGCCAGTATGTTCTCT
AGTGTGGTTCGTTGTTTTTGCGTGAGCCATGAGAACGAACCATTGAGATCAT
GCTTACTTTGCATGTCACTCAAAAATTTTGCCTCAAAACTGGTGAGCTGAAT
TTTTGCAGTTAAAGCATCGTGTAGTGTTTTTCTTAGTCCGTTACGTAGGTAGG
AATCTGATGTAATGGTTGTTGGTATTTTGTCACCATTCATTTTTATCTGGTTG
TTCTCAAGTTCGGTTACGAGATCCATTTGTCTATCTAGTTCAACTTGGAAAA
TCAACGTATCAGTCGGGCGGCCTCGCTTATCAACCACCAATTTCATATTGCT
GTAAGTGTTTAAATCTTTACTTATTGGTTTCAAAACCCATTGGTTAAGCCTTT
TAAACTCATGGTAGTTATTTTCAAGCATTAACATGAACTTAAATTCATCAAG
GCTAATCTCTATATTTGCCTTGTGAGTTTTCTTTTGTGTTAGTTCTTTTAATAA
CCACTCATAAATCCTCATAGAGTATTTGTTTTCAAAAGACTTAACATGTTCC
AGATTATATTTTATGAATTTTTTTAACTGGAAAAGATAAGGCAATATCTCTT
CACTAAAAACTAATTCTAATTTTTCGCTTGAGAACTTGGCATAGTTTGTCCA
CTGGAAAATCTCAAAGCCTTTAACCAAAGGATTCCTGATTTCCACAGTTCTC
GTCATCAGCTCTCTGGTTGCTTTAGCTAATACACCATAAGCATTTTCCCTACT
GATGTTCATCATCTGAGCGTATTGGTTATAAGTGAACGATACCGTCCGTTCT
TTCCTTGTAGGGTTTTCAATCGTGGGGTTGAGTAGTGCCACACAGCATAAAA
TTAGCTTGGTTTCATGCTCCGTTAAGTCATAGCGACTAATCGCTAGTTCATTT
GCTTTGAAAACAACTAATTCAGACATACATCTCAATTGGTCTAGGTGATTTT
AATCACTATACCAATTGAGATGGGCTAGTCAATGATAATTACTAGTCCTTTT
CCTTTGAGTTGTGGGTATCTGTAAATTCTGCTAGACCTTTGCTGGAAAACTT
GTAAATTCTGCTAGACCCTCTGTAAATTCCGCTAGACCTTTGTGTGTTTTTTT
TGTTTATATTCAAGTGGTTATAATTTATAGAATAAAGAAAGAATAAAAAAA
GATAAAAAGAATAGATCCCAGCCCTGTGTATAACTCACTACTTTAGTCAGTT
CCGCAGTATTACAAAAGGATGTCGCAAACGCTGTTTGCTCCTCTACAAAACA
GACCTTAAAACCCTAAAGGCTTAAGTAGCACCCTCGCAAGCTCGGGCAAAT
CGCTGAATATTCCTTTTGTCTCCGACCATCAGGCACCTGAGTCGCTGTCTTTT

Nucleotide sequences of Tet promoter-PAL1 construct, low-copy (SEQ ID NO: 28)
TCGTGACATTCAGTTCGCTGCGCTCACGGCTCTGGCAGTGAATGGGGGTAAA
TGGCACTACAGGCGCCTTTTATGGATTCATGCAAGGAAACTACCCATAATAC
AAGAAAAGCCCGTCACGGGCTTCTCAGGGCGTTTTATGGCGGGTCTGCTATG
TGGTGCTATCTGACTTTTTGCTGTTCAGCAGTTCCTGCCCTCTGATTTTCCAG
TCTGACCACTTCGGATTATCCCGTGACAGGTCATTCAGACTGGCTAATGCAC
CCAGTAAGGCAGCGGTATCATCAACAGGCTTACCCGTCTTACTGTCTTTTCT
ACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTC
ATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAA
GTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCA
ATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCC
ATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTAC
CATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCC
AGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTG
GTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGC
TAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCT
ACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCG
GTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG
CGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGT
GTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGA
ATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTT
CGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTA
ACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT
CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG
GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAA
GCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA
GAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC
TGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT
ATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCT
GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCG
GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGG
GCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA
TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGC
GCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGG
CCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT
TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGG
CCAGTGAATTCGTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGC
ATATGATCAATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGAT
CAAATAATTCGATAGCTTGTCGTAATAATGGCGGCATACTATCAGTAGTAGG
TGTTTCCCTTTCTTCTTTAGCGACTTGATGCTCTTGATCTTCCAATACGCAAC
CTAAAGTAAAATGCCCCACAGCGCTGAGTGCATATAATGCATTCTCTAGTGA
AAAACCTTGTTGGCATAAAAAGGCTAATTGATTTTCGAGAGTTTCATACTGT
TTTTCTGTAGGCCGTGTACCTAAATGTACTTTTGCTCCATCGCGATGACTTAG
TAAAGCACATCTAAAACTTTTAGCGTTATTACGTAAAAAATCTTGCCAGCTT
TCCCCTTCTAAAGGGCAAAAGTGAGTATGGTGCCTATCTAACATCTCAATGG
CTAAGGCGTCGAGCAAAGCCCGCTTATTTTTTACATGCCAATACAATGTAGG

Nucleotide sequences of Tet promoter-PAL1 construct, low-copy (SEQ ID NO: 28)
CTGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGATT CCGACCTCATTAAGCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAA TCTAGACATCATTAATTCCTAATTTTT**GTTGACACTCTATCATTGATAGAG TTATTTT**

Table 12

Nucleotide sequences of Tet promoter-PAL3 construct, low-copy (SEQ ID NO: 29)
ACCACTCCCTATCAGTGATAGAGAAAAGTGAACTCTAGAAATAATTTTGT TTAACTTTAAGAAGGAGATATACATATGAAAGCTAAAGATGTTCAGCCAAC CATTATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTATGACATT GCGATAAAACAAAAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTTG ACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATA TATGGAATCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTG AGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTAC TGGGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATTTACTATGTTA CTTTCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAA TTGTTGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCA GTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGAGCATTAT GTGGTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAG CAATTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTC TTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGT CATTAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCT GTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAAG TAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATT GGCAGGTTCAACGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAA TAAAGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATACCTTACAGGA AGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAATCT TTAGCTACCGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGAT AATCCATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTA TGGGGCAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATATTGCTTT AATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATAACCGTTTCT CTCGTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTT TAAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGAT TGTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAATACAATCAAGATA TTGTCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCAGAA ATTACGCAATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCAT CTTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATG CAGTACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGA TATAATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCTGCCAGAA ATCATGCTGGAAGAATAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGT GTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCAT AAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGC GTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCAT TAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCT TCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAG

Nucleotide sequences of Tet promoter-PAL3 construct, low-copy (SEQ ID NO: 29)
CGGTATCAGCTCACTCAAAGGCGGTAGTACGGGTTTTGCTGCCCGCAAACG
GGCTGTTCTGGTGTTGCTAGTTTGTTATCAGAATCGCAGATCCGGCTTCAGG
TTTGCCGGCTGAAAGCGCTATTTCTTCCAGAATTGCCATGATTTTTTCCCCAC
GGGAGGCGTCACTGGCTCCCGTGTTGTCGGCAGCTTTGATTCGATAAGCAGC
ATCGCCTGTTTCAGGCTGTCTATGTGTGACTGTTGAGCTGTAACAAGTTGTC
TCAGGTGTTCAATTTCATGTTCTAGTTGCTTTGTTTTACTGGTTTCACCTGTTC
TATTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGATCTGTTCATGGTGA
ACAGCTTTAAATGCACCAAAAACTCGTAAAAGCTCTGATGTATCTATCTTTT
TTACACCGTTTTCATCTGTGCATATGGACAGTTTTCCCTTTGATATCTAACGG
TGAACAGTTGTTCTACTTTTGTTTGTTAGTCTTGATGCTTCACTGATAGATAC
AAGAGCCATAAGAACCTCAGATCCTTCCGTATTTAGCCAGTATGTTCTCTAG
TGTGGTTCGTTGTTTTTGCGTGAGCCATGAGAACGAACCATTGAGATCATGC
TTACTTTGCATGTCACTCAAAAATTTTGCCTCAAAACTGGTGAGCTGAATTT
TTGCAGTTAAAGCATCGTGTAGTGTTTTTCTTAGTCCGTTACGTAGGTAGGA
ATCTGATGTAATGGTTGTTGGTATTTTGTCACCATTCATTTTTATCTGGTTGT
TCTCAAGTTCGGTTACGAGATCCATTTGTCTATCTAGTTCAACTTGGAAAAT
CAACGTATCAGTCGGGCGGCCTCGCTTATCAACCACCAATTTCATATTGCTG
TAAGTGTTTAAATCTTTACTTATTGGTTTCAAAACCCATTGGTTAAGCCTTTT
AAACTCATGGTAGTTATTTTCAAGCATTAACATGAACTTAAATTCATCAAGG
CTAATCTCTATATTTGCCTTGTGAGTTTTCTTTTGTGTTAGTTCTTTTAATAAC
CACTCATAAATCCTCATAGAGTATTTGTTTTCAAAAGACTTAACATGTTCCA
GATTATATTTTATGAATTTTTTTAACTGGAAAAGATAAGGCAATATCTCTTC
ACTAAAAACTAATTCTAATTTTTCGCTTGAGAACTTGGCATAGTTTGTCCAC
TGGAAAATCTCAAAGCCTTTAACCAAAGGATTCCTGATTTCCACAGTTCTCG
TCATCAGCTCTCTGGTTGCTTTAGCTAATACACCATAAGCATTTTCCCTACTG
ATGTTCATCATCTGAGCGTATTGGTTATAAGTGAACGATACCGTCCGTTCTT
TCCTTGTAGGGTTTTCAATCGTGGGGTTGAGTAGTGCCACACAGCATAAAAT
TAGCTTGGTTTCATGCTCCGTTAAGTCATAGCGACTAATCGCTAGTTCATTTG
CTTTGAAAACAACTAATTCAGACATACATCTCAATTGGTCTAGGTGATTTTA
ATCACTATACCAATTGAGATGGGCTAGTCAATGATAATTACTAGTCCTTTTC
CTTTGAGTTGTGGGTATCTGTAAATTCTGCTAGACCTTTGCTGGAAAACTTG
TAAATTCTGCTAGACCCTCTGTAAATTCCGCTAGACCTTTGTGTGTTTTTTTT
GTTTATATTCAAGTGGTTATAATTTATAGAATAAAGAAAGAATAAAAAAAG
ATAAAAAGAATAGATCCCAGCCCTGTGTATAACTCACTACTTTAGTCAGTTC
CGCAGTATTACAAAAGGATGTCGCAAACGCTGTTTGCTCCTCTACAAAACA
GACCTTAAAACCCTAAAGGCTTAAGTAGCACCCTCGCAAGCTCGGGCAAAT
CGCTGAATATTCCTTTTGTCTCCGACCATCAGGCACCTGAGTCGCTGTCTTTT
TCGTGACATTCAGTTCGCTGCGCTCACGGCTCTGGCAGTGAATGGGGGTAAA
TGGCACTACAGGCGCCTTTTATGGATTCATGCAAGGAAACTACCCATAATAC
AAGAAAAGCCCGTCACGGGCTTCTCAGGGCGTTTTATGGCGGGTCTGCTATG
TGGTGCTATCTGACTTTTTGCTGTTCAGCAGTTCCTGCCCTCTGATTTTCCAG
TCTGACCACTTCGGATTATCCCGTGACAGGTCATTCAGACTGGCTAATGCAC
CCAGTAAGGCAGCGGTATCATCAACAGGCTTACCCGTCTTACTGTCTTTTCT
ACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTC
ATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAA
GTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCA
ATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCC

Nucleotide sequences of Tet promoter-PAL3 construct, low-copy (SEQ ID NO: 29)
ATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTAC CATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCC AGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTG GTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGC TAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCT ACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCG GTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG CGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGT GTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGA ATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTT CGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTA ACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAA GCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA GAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC TGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT ATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCT GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCG GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGG GCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGC GCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGG CCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGG CCAGTGAATTCGTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGC ATATGATCAATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGAT CAAATAATTCGATAGCTTGTCGTAATAATGGCGGCATACTATCAGTAGTAGG TGTTTCCCTTTCTTCTTTAGCGACTTGATGCTCTTGATCTTCCAATACGCAAC CTAAAGTAAAATGCCCCACAGCGCTGAGTGCATATAATGCATTCTCTAGTGA AAAACCTTGTTGGCATAAAAAGGCTAATTGATTTTCGAGAGTTTCATACTGT TTTTCTGTAGGCCGTGTACCTAAATGTACTTTTGCTCCATCGCGATGACTTAG TAAAGCACATCTAAAACTTTTAGCGTTATTACGTAAAAAATCTTGCCAGCTT TCCCCTTCTAAAGGGCAAAAGTGAGTATGGTGCCTATCTAACATCTCAATGG CTAAGGCGTCGAGCAAAGCCCGCTTATTTTTTACATGCCAATACAATGTAGG CTGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGATT CCGACCTCATTAAGCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAA TCTAGACATCATTAATTCCTAATTTTT**GTTGACACTCTATCATTGATAGAG TTATTTT**

Table 13

Nucleotide sequences of TetR-PheP construct, low-copy (SEQ ID NO: 30)
ccagtgaattcgttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataagaaggctgg ctctgcaccttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttcttctttag cgacttgatgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaa aaaccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtacttttgctcca tcgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatcttgccagctttccccttctaaagggcaaaag tgagtatggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgtaggctg ctctacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcact ttacttttatctaatctagacatcattaattcctaatttttt**gttgacactctatcattgatagagttattttaccactccctatcagtg atagagaa**aagtgaactctagaaataattttgtttaactttaagaaggagatatacatATGAAAAACGCGTCAA CCGTATCGGAAGATACTGCGTCGAATCAAGAGCCGACGCTTCATCGCGGAT TACATAACCGTCATATTCAACTGATTGCGTTGGGTGGCGCAATTGGTACTGG TCTGTTTCTTGGCATTGGCCCGGCGATTCAGATGGCGGGTCCGGCTGTATTG CTGGGCTACGGCGTCGCCGGGATCATCGCTTTCCTGATTATGCGCCAGCTTG GCGAAATGGTGGTTGAGGAGCCGGTATCCGGTTCATTTGCCCACTTTGCCTA TAAATACTGGGGACCGTTTGCGGGCTTCCTCTCTGGCTGGAACTACTGGGTA ATGTTCGTGCTGGTGGGAATGGCAGAGCTGACCGCTGCGGGCATCTATATG CAGTACTGGTTCCCGGATGTTCCAACGTGGATTTGGGCTGCCGCCTTCTTTA TTATCATCAACGCCGTTAACCTGGTGAACGTGCGCTTATATGGCGAAACCGA GTTCTGGTTTGCGTTGATTAAAGTGCTGGCAATCATCGGTATGATCGGCTTT GGCCTGTGGCTGCTGTTTTCTGGTCACGGCGGCGAGAAAGCCAGTATCGAC AACCTCTGGCGCTACGGTGGTTTCTTCGCCACCGGCTGGAATGGGCTGATTT TGTCGCTGGCGGTAATTATGTTCTCCTTCGGCGGTCTGGAGCTGATTGGGAT TACTGCCGCTGAAGCGCGCGATCCGGAAAAAAGCATTCCAAAAGCGGTAAA TCAGGTGGTGTATCGCATCCTGCTGTTTTACATCGGTTCACTGGTGGTTTTAC TGGCGCTCTATCCGTGGGTGGAAGTGAAATCCAACAGTAGCCCGTTTGTGAT GATTTTCCATAATCTCGACAGCAACGTGGTAGCTTCTGCGCTGAACTTCGTC ATTCTGGTAGCATCGCTGTCAGTGTATAACAGCGGGGTTTACTCTAACAGCC GCATGCTGTTTGGCCTTTCTGTGCAGGGTAATGCGCCGAAGTTTTTGACTCG CGTCAGCCGTCGCGGTGTGCCGATTAACTCGCTGATGCTTTCCGGAGCGATC ACTTCGCTGGTGGTGTTAATCAACTATCTGCTGCCGCAAAAAGCGTTTGGTC TGCTGATGGCGCTGGTGGTAGCAACGCTGCTGTTGAACTGGATTATGATCTG TCTGGCGCATCTGCGTTTTCGTGCAGCGATGCGACGTCAGGGGCGTGAAAC ACAGTTTAAGGCGCTGCTCTATCCGTTCGGCAACTATCTCTGCATTGCCTTCC TCGGCATGATTTTGCTGCTGATGTGCACGATGGATGATATGCGCTTGTCAGC GATCCTGCTGCCGGTGTGGATTGTATTCCTGTTTATGGCATTTAAAACGCTG CGTCGGAAATAA

[0218] 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

[0219] 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.

[0220] 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.

[0221] 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.

[0222] 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.

[0223] 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.

[0224] 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.

[0225] 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.

[0226] In other embodiments, the gene(s) encoding the one or more Phe transporter(s) may be located on a plasmid or in the chromosome and the gene expression may be regulated by any of the promoters disclosed herein, which may be the same or different from the promoters regulating the PME gene(s).

[0227] 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).

[0228] 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.

[0229] In other embodiments, the genetically engineered bacteria comprise AroP and PheP, under the control of the same or different inducible or constitutive promoters.

[0230] 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. 66**). 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.

[0231] In some embodiments, the genetically engineered bacteria encode one or more Phe transporter(s) which are directly or indirectly pre-induced prior to *in vivo* administration, e.g., are expressed under the control of an inducible promoter that is responsive to specific molecules or metabolites in the provided in the culture of the bacterium in a flask, fermenter, or other culture vessel, during production of the strain prior to *in vivo* administration.

[0232] In other embodiments, the genetically engineered bacteria encode one or more Phe transporter(s) which are directly or indirectly induced *in vivo* administration, e.g., are expressed under the control of an inducible promoter that is responsive conditions or to specific molecules or metabolites in the exogenous *in vivo* environment, e.g., the gut. In some embodiments, the promoter is induced by gut specific molecules, or low oxygen conditions. In some embodiments, the bacterial strains are administered in combination with a chemical and/or nutritional inducer.

[0233] 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.

Oxygen Level Independent Inducible Promoters

[0234] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are inducible through an arabinose inducible system. The genes of arabinose metabolism are organized in one operon, AraBAD, which is controlled by the PAraBAD promoter. The PAraBAD (or Para) promoter suitably fulfills the criteria of inducible expression systems. PAraBAD displays tighter control of payload gene expression than many other systems, likely due to the dual regulatory role of AraC, which functions both as an inducer and as a repressor. Additionally, the level of ParaBAD-based expression can be modulated over a wide range of L-arabinose concentrations to fine-tune levels of expression of the payload. However, the cell population exposed to sub-saturating L-arabinose concentrations is divided into two subpopulations of induced and uninduced cells, which is determined by the differences between individual cells in the availability of L-arabinose transporter (Zhang et al., Development and Application of an Arabinose-Inducible Expression System by Facilitating Inducer Uptake in Corynebacterium glutamicum; Appl. Environ. Microbiol. August 2012 vol. 78 no. 16 5831-5838). Alternatively, inducible expression from the ParaBad can be controlled or fine-tuned through the optimization of the ribosome binding site (RBS), as described herein. An exemplary construct is depicted in **Fig. 62C** (construct for PAL expression under the control of a arabinose inducible promoter).

[0235] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more arabinose inducible promoter(s). In one embodiment, expression of PAL is driven directly or indirectly by an arabinose inducible promoter. In one embodiment, expression of PheP is driven directly or indirectly by an arabinose inducible promoter. In one embodiment, expression of LAAD is driven directly or indirectly by an arabinose inducible promoter. In one embodiment, expression of FNRS24Y, is driven directly or indirectly by an arabinose inducible promoter.

[0236] In another embodiment, one or more arabinose inducible promoter(s) drive the expression of one or more bicistronic message(s). Bicistronic messages induced by arabinose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s),

e.g., FNRS24Y. Non-limiting examples of such bicistronic messages include, in either orientation, PAL and PheP, PAL and LAAD, PAL and FNRS24Y, PheP and LAAD, PheP and FNRS24Y, LAAD and FNRS24Y. Bicistronic messages also include two transcripts of the same gene, e.g., PAL-PAL, LAAD-LAAD, PheP-PheP and/or FNRSY24S-FNRSY24S. Non-limiting examples of bicistronic messages are described herein and include Para-FNRS24Y-LAAD, e.g., as comprised in SEQ ID NO: 73.

[0237] In one embodiment, one or more arabinose inducible promoter(s) drive the expression of tri-cistronic messages. Tri-cistronic messages induced by arabinose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y. Such tri-cistronic messages may include for example (1) transcription of three messages of the same gene; (2) one message of a first gene, and two messages of a second gene; or (3) transcription of three messages of three different genes. Any combination of one or more PME(s), e.g., PAL and/or LAAD, and/or PheP may be contained in a tri-cistronic message. Non-limiting examples of tri-cistronic messages are described herein and include PAL-PAL-PheP, e.g., as comprised in SEQ ID NO: 95 (in bold).

[0238] In one embodiment, one or more arabinose inducible promoter(s) drive the expression of multi-cistronic messages. Multi-cistronic messages induced by arabinose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y. Such multi-cistronic messages may include for example (arranged on the DNA like beads on a string) (1) transcription of several messages of one and the same gene; (2) one or more message of a first gene, and one or more messages of a second gene; or (3) transcription of one or more messages of from one or more different genes. Any combination of one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y, genes may be contained in a multi-cistronic message.

[0239] In some embodiments, the arabinose inducible promoter is useful for or induced during *in vivo* expression of the one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y. In some embodiments, expression of one or more PME(s) and/or Phe transporter(s),

e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more arabinose inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, e.g., arabinose

[0240] In some embodiments, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more arabinose inducible promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the arabinose inducible promoter(s) are induced in culture, e.g., grown in a flask, a fermenter or another appropriate culture vessel. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with the payload prior to administration, e.g., arabinose. In some embodiments, the cultures, which are induced by arabinose, are grown arerobically. In some embodiments, the cultures, which are induced by arabinose, are grown anaerobically.

[0241] In one embodiment, the arabinose inducible promoter drives the expression of a construct comprising one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, jointly with a second promoter, e.g., a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the arabinose inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (i.e., during preparation of the culture in a flask, fermenter or other appropriate culture vessel, e.g., arabinose and IPTG). In another non-limiting example, the first inducing conditions may be culture conditions, e.g., including arabinose presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more arabinose promoters drive expression of one or more PME(s), e.g., PAL and/or LAAD, and/or

Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0242] In some embodiments, the arabinose inducible promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the arabinose inducible promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0243] In some embodiments, one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y are knocked into the arabinose operon and are driven by the native arabinose inducible promoter In some embodiments, FNRS24Y is knocked into the arabinose operon and are driven by the native arabinose inducible promoter. In some embodiments, FNRS24Y-LAAD is knocked into the arabinose operon and are driven by the native arabinose inducible promoter.

[0244] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 67. In some embodiments, the arabinose inducible construct further comprises a gene encoding AraC, which is divergently transcribed from the same promoter as the one or more one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 66. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%,

95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of SEQ ID NO: 66.

[0245] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are inducible through a rhamnose inducible system. The genes rhaBAD are organized in one operon which is controlled by the rhaP BAD promoter. The rhaP BAD promoter is regulated by two activators, RhaS and RhaR, and the corresponding genes belong to one transcription unit which divergently transcribed in the opposite direction of rhaBAD. In the presence of L-rhamnose, RhaR binds to the rhaP RS promoter and activates the production of RhaR and RhaS. RhaS together with L-rhamnose then bind to the rhaP BAD and the rhaP T promoter and activate the transcription of the structural genes. In contrast to the arabinose system, in which AraC is provided and divergently transcribed in the gene sequence(s), it is not necessary to express the regulatory proteins in larger quantities in the rhamnose expression system because the amounts expressed from the chromosome are sufficient to activate transcription even on multi-copy plasmids. Therefore, only the rhaP BAD promoter is cloned upstream of the gene that is to be expressed. Full induction of rhaBAD transcription also requires binding of the CRP-cAMP complex, which is a key regulator of catabolite repression. Alternatively, inducible expression from the rhaBAD can be controlled or fine-tuned through the optimization of the ribosome binding site (RBS), as described herein. An exemplary construct is depicted in **Fig. 62B (**construct for PAL expression under the control of a rhamnose inducible promoter**)**.

[0246] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more rhamnose inducible promoter(s). In one embodiment, expression of PAL is driven directly or indirectly by a rhamnose inducible promoter. In one embodiment, expression of PheP is driven directly or indirectly by a rhamnose inducible promoter. In one embodiment, expression of LAAD is driven directly or indirectly by a rhamnose inducible promoter. In one embodiment, expression of FNRS24Y, is driven directly or indirectly by a rhamnose inducible promoter. In a non-limiting example, PAL expression is driven by a construct comprising include rhamnose inducible PAL, e.g., as comprised in SEQ ID NO: 106.

[0247] In another embodiment, one or more rhamnose inducible promoter(s) drive the expression of one or more bicistronic message(s). Bicistronic messages induced by rhamnose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction.

[0248] In one embodiment, one or more rhamnose inducible promoter(s) drive the expression of tri-cistronic messages. Tri-cistronic messages induced by rhamnose may include one or more PME(s), e.g., PAL and/or LAAD and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y. Tricistronic messages induced by rhamnose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction.

[0249] In one embodiment, one or more rhamnose inducible promoter(s) drive the expression of multi-cistronic messages. Multi-cistronic messages induced by rhamnose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y. Multi-cistronic messages induced by rhamnose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction.

[0250] In some embodiments, the rhamnose inducible promoter is useful for or induced during *in vivo* expression of the one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y. In some embodiments, expression of one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y, is driven directly or indirectly by one or more rhamnose inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, e.g., rhamnose

[0251] In some embodiments, expression of one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y, is driven directly or indirectly by one or more rhamnose inducible

promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the rhamnose inducible promoter(s) are induced in culture, e.g., grown in a flask, a fermenter or another appropriate culture vessel. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with the payload prior to administration, e.g., rhamnose. In some embodiments, the cultures, which are induced by rhamnose, are grown arerobically. In some embodiments, the cultures, which are induced by rhamnose, are grown anaerobically.

[0252] In one embodiment, the rhamnose inducible promoter drives the expression of a construct comprising one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y, jointly with a second promoter, e.g., a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the rhamnose inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (i.e., during preparation of the culture in a flask, fermenter or other appropriate culture vessel, e.g., rhamnose and arabinose). In another non-limiting example, the first inducing conditions may be culture conditions, e.g., including rhamnose presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more rhamnose promoters drive expression of one or more PME(s) and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0253] In some embodiments, the rhamnose inducible promoter drives the expression of one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y, from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In

some embodiments, the rhamnose inducible promoter drives the expression of one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y, from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0254] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 107.

[0255] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are inducible through an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible system or other compound which induced transcription from the Lac Promoter. IPTG is a molecular mimic of allolactose, a lactose metabolite that activates transcription of the lac operon. In contrast to allolactose, the sulfur atom in IPTG creates a non-hydrolyzable chemical blond, which prevents the degradation of IPTG, allowing the concentration to remain constant. IPTG binds to the lac repressor and releases the tetrameric repressor (lacI) from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon. Since IPTG is not metabolized by E. coli, its concentration stays constant and the rate of expression of Lac promoter-controlled is tightly controlled, both *in vivo* and *in vitro*. IPTG intake is independent on the action of lactose permease, since other transport pathways are also involved. Inducible expression from the PLac can be controlled or fine-tuned through the optimization of the ribosome binding site (RBS), as described herein. Other compounds which inactivate LacI, can be used instead of IPTG in a similar manner.

[0256] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more IPTG inducible promoter(s). In one embodiment, expression of PAL is driven directly or indirectly by an IPTG inducible promoter. In one embodiment, expression of PheP is driven directly or indirectly by an IPTG inducible promoter. In one embodiment, expression of LAAD is driven directly or indirectly by an IPTG inducible promoter. In one embodiment, expression of FNRS24Y, is driven directly or indirectly by an IPTG inducible promoter.

Non-limiting examples of a construct comprising PAL under the control of an IPTG inducible promoter e.g., as comprised in SEQ ID NO: 74 (see, e.g., **Fig. 60)**.

[0257] In another embodiment, one or more IPTG inducible promoter(s) drive the expression of one or more bicistronic message(s). Bicistronic messages induced by IPTG may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In one embodiment, one or more IPTG inducible promoter(s) drive the expression of tri-cistronic messages. Tri-cistronic messages induced by IPTG may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In one embodiment, one or more IPTG inducible promoter(s) drive the expression of multi-cistronic messages. Multi-cistronic messages induced by IPTG may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction.

[0258] In some embodiments, the IPTG inducible promoter is useful for or induced during *in vivo* expression of the one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y. In some embodiments, expression of one or more PME(s) and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more IPTG inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, e.g., IPTG.

[0259] In some embodiments, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more IPTG inducible promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the IPTG inducible promoter(s) are induced in culture, e.g., grown in a flask, a fermenter or another appropriate culture vessel. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the

bacterium with the payload prior to administration, e.g., IPTG. In some embodiments, the cultures, which are induced by IPTG, are grown arerobically. In some embodiments, the cultures, which are induced by IPTG, are grown anaerobically.

[0260] In one embodiment, the IPTG inducible promoter drives the expression of a construct comprising one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, jointly with a second promoter, e.g., a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the IPTG inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (i.e., during preparation of the culture in a flask, fermenter or other appropriate culture vessel, e.g., arabinose and IPTG). In another non-limiting example, the first inducing conditions may be culture conditions, e.g., including IPTG presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more IPTG inducible promoters drive expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0261] In some embodiments, the IPTG inducible promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the IPTG inducible promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0262] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any

of the sequences of SEQ ID NO:76. In some embodiments, the IPTG inducible construct further comprises a gene encoding lacI, which is divergently transcribed from the same promoter as the one or more one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 75. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of SEQ ID NO: 75.

[0263] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are inducible through a tetracycline inducible system. The initial system Gossen and Bujard (Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Gossen M & Bujard H.*PNAS*, 1992 Jun 15;89(12):5547-51) developed is known as tetracycline off: in the presence of tetracycline, expression from a tet-inducible promoter is reduced. Tetracycline-controlled transactivator (tTA) was created by fusing tetR with the C-terminal domain of VP16 (virion protein 16) from herpes simplex virus.In the absence of tetracycline, the tetR portion of tTA will bind tetO sequences in the tet promoter, and the activation domain promotes expression. In the presence of tetracycline, tetracycline binds to tetR, precluding tTA from binding to the tetO sequences. Next, a reverse Tet repressor (rTetR), was developed which created a reliance on the presence of tetracycline for induction, rather than repression. The new transactivator rtTA (reverse tetracycline-controlled transactivator) was created by fusing rTetR with VP16. The tetracycline on system is also known as the rtTA-dependent system.

[0264] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP , and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more tetracycline inducible promoter(s). In one embodiment, expression of PAL is driven directly or indirectly by a tetracycline inducible promoter. In one embodiment, expression of PheP is driven

directly or indirectly by a tetracycline inducible promoter. In one embodiment, expression of LAAD is driven directly or indirectly by a tetracycline inducible promoter. In one embodiment, expression of FNRS24Y, is driven directly or indirectly by a tetracycline inducible promoter.

[0265] In another embodiment, one or more tetracycline inducible promoter(s) drive the expression of one or more bicistronic message(s). Bicistronic messages induced by tetracycline may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In one embodiment, one or more tetracycline inducible promoter(s) drive the expression of tri-cistronic messages. Tri-cistronic messages induced by tetracycline may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In one embodiment, one or more tetracycline inducible promoter(s) drive the expression of multi-cistronic messages. Multi-cistronic messages induced by tetracycline may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction.

[0266] In some embodiments, the tetracycline inducible promoter is useful for or induced during *in vivo* expression of the one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y. In some embodiments, expression of one or more PME(s) and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more tetracycline inducible promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, e.g., tetracycline

[0267] In some embodiments, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more tetracycline inducible promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, the tetracycline inducible promoter(s) are

induced in culture, e.g., grown in a flask, a fermenter or another appropriate culture vessel. In some embodiments, the promoter is directly or indirectly induced by a molecule that is added to in the bacterial culture to induce expression and pre-load the bacterium with the payload prior to administration, e.g., tetracycline. In some embodiments, the cultures, which are induced by tetracycline, are grown arerobically. In some embodiments, the cultures, which are induced by tetracycline, are grown anaerobically.

[0268] In one embodiment, the tetracycline inducible promoter drives the expression of a construct comprising one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, jointly with a second promoter, e.g., a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the tetracycline inducible promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (i.e., during preparation of the culture in a flask, fermenter or other appropriate culture vessel, e.g., tetracycline and IPTG). In another non-limiting example, the first inducing conditions may be culture conditions, e.g., including tetracycline presence, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more tetracycline promoters drive expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0269] In some embodiments, the tetracycline inducible promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the tetracycline inducible promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or

transcriptional regulator(s), e.g., FNRS24Y, from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0270] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the bolded sequences of SEQ ID NO: 39 (tet promoter is in bold). In some embodiments, the tetracycline inducible construct further comprises a gene encoding AraC, which is divergently transcribed from the same promoter as the one or more one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 39 in italics (Tet repressor is in italics). In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of SEQ ID NO: 39 in italics (Tet repressor is in italics).

[0271] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) whose expression is controlled by a temperature sensitive mechanism. Thermoregulators are advantageous because of strong transcriptional control without the use of external chemicals or specialized media (see, e.g., Nemani et al., Magnetic nanoparticle hyperthermia induced cytosine deaminase expression in microencapsulated E. coli for enzyme-prodrug therapy; J Biotechnol. 2015 Jun 10; 203: 32–40, and references therein). Thermoregulated protein expression using the mutant cI857 repressor and the pL and/or pR phage λ promoters have been used to engineer recombinant bacterial strains. The gene of interest cloned downstream of the λ promoters can then be efficiently regulated by the mutant thermolabile cI857 repressor of bacteriophage λ. At temperatures below 37 °C, cI857 binds to the oL or oR regions of the pR promoter and blocks transcription by RNA polymerase. At higher temperatures, the functional cI857 dimer is destabilized, binding to the oL or oR DNA sequences is abrogated, and mRNA transcription is initiated. An exemplary construct is depicted in

Fig. 62A. Inducible expression from the ParaBad can be controlled or further fine-tuned through the optimization of the ribosome binding site (RBS), as described herein.

[0272] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more thermoregulated promoter(s). In one embodiment, expression of PAL is driven directly or indirectly by a thermoregulated promoter. In one embodiment, expression of PheP is driven directly or indirectly by a thermoregulated promoter. In one embodiment, expression of LAAD is driven directly or indirectly by a thermoregulated promoter. In one embodiment, expression of FNRS24Y, is driven directly or indirectly by a thermoregulated promoter. Non-limiting examples of a construct in which PAL expression is induced at 37 C and 42 C and uninduced at lower temperatures, is comprised in SEQ ID NO: 101 (see, e.g., **Fig. 60** and **Fig. 62A**).

[0273] In another embodiment, one or more thermoregulated promoter(s) drive the expression of one or more bicistronic message(s). Bicistronic messages induced by temperature, e.g., 37 C and./or 42 C, may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In one embodiment, one or more thermoregulated promoter(s) drive the expression of tri-cistronic messages. Tri-cistronic messages induced by temperature, e.g., 37 C and./or 42 C, may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In one embodiment, one or more thermoregulated promoter(s) drive the expression of multi-cistronic messages. Multi-cistronic messages induced by thermoregulation may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction.

[0274] In some embodiments, the thermoregulated promoter is useful for or induced during *in vivo* expression of the one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y. In some embodiments, expression of one or more PME(s) and/or Phe transporter(s),

e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more thermoregulated promoter(s) *in vivo*. In some embodiments, the promoter is directly or indirectly induced by a molecule that is co-administered with the genetically engineered bacteria of the invention, e.g., temperature.

[0275] In some embodiments, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more thermoregulated promoter(s) during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, it may be advantageous to shup off production of the one or more PME(s) and/or PheP. This can be done in a thermoregulated system by growing the strain at lower temperatures, e.g., 30 C. Expression can then be induced by elevating the temperature to 37 C and/or 42 C. In some embodiments, the thermoregulated promoter(s) are induced in culture, e.g., grown in a flask, a fermenter or another appropriate culture vessel. In some embodiments, the cultures, which are induced by temperatures between 37 C and 42 C, are grown arerobically. In some embodiments, the cultures, which are induced by induced by temperatures between 37 C and 42 C, are grown anaerobically.

[0276] In one embodiment, the thermoregulated promoter drives the expression of a construct comprising one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, jointly with a second promoter, e.g., a second constitutive or inducible promoter. In some embodiments, two promoters are positioned proximally to the construct and drive its expression, wherein the thermoregulated promoter drives expression under a first set of exogenous conditions, and the second promoter drives the expression under a second set of exogenous conditions. In a non-limiting example, the first and second conditions may be two sequential culture conditions (i.e., during preparation of the culture in a flask, fermenter or other appropriate culture vessel, e.g., thermoregulation and arabinose). In another non-limiting example, the first inducing conditions may be culture conditions, e.g., permissive temperature, and the second inducing conditions may be *in vivo* conditions. Such *in vivo* conditions include low-oxygen, microaerobic, or anaerobic conditions, presence of gut metabolites, and/or metabolites administered in combination with the bacterial strain. In some embodiments, the one or more

thermoregulated promoters drive expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, in combination with the FNR promoter driving the expression of the same gene sequence(s).

[0277] In some embodiments, the thermoregulated promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the thermoregulated promoter drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0278] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 103. In some embodiments, the thermoregulated construct further comprises a gene encoding mutant cI857 repressor, which is divergently transcribed from the same promoter as the one or more one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more regulators, e.g., FNRS24Y. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 102. In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding a polypeptide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with the polypeptide encoded by any of the sequences of SEQ ID NO: 102.

[0279] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) which are indirectly inducible through a system driven by the PssB promoter. The Pssb promoter is active under aerobic conditions, and shuts off under anaerobic conditions.

[0280] This promoter can be used to express a gene of interest under aerobic conditions. This promoter can also be used to tightly control the expression of a gene product such that it is only expressed under anaerobic conditions. In this case, the oxygen induced PssB promoter induces the expression of a repressor, which represses the expression of a gene of interest. As a result, the gene of interest is only expressed in the absence of the repressor, i.e., under anaerobic conditions. This strategy has the advantage of an additional level of control for improved fine-tuning and tighter control. **Fig. 63A** depicts a schematic of the gene organization of a PssB promoter.

[0281] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP , and/or transcriptional regulator(s), e.g., FNRS24Y, is indirectly regulated by a repressor expressed under the control of one or more PssB promoter(s). In one embodiment, expression of PAL is indirectly regulated by a repressor expressed under the control of one or more PssB promoter(s). In one embodiment, expression of PheP is indirectly regulated by a repressor expressed under the control of one or more PssB promoter(s). In one embodiment, expression of LAAD is indirectly regulated by a repressor expressed under the control of one or more PssB promoter(s). In one embodiment, expression of FNRS24Y, is indirectly regulated by a repressor expressed under the control of one or more PssB promoter(s).

[0282] In another embodiment, induction of the RssB promoter(s) drives the expression of one or more bicistronic message(s). Bicistronic messages induced may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In another embodiment, induction of the RssB promoter(s) drives the expression of one or more tri-cistronic messages. Tri-cistronic messages induced may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction. In one embodiment, induction of the RssB promoter(s) indirectly drives the expression of one or more of multi-cistronic messages. Multi-cistronic messages induced by arabinose may include one or more PME(s), e.g. PAL or LAAD, and/or one or more Phe transporter(s) e.g., PheP, and/or one or more

transcriptional regulator(s), e.g., FNRS24Y and are the same as those described above for arabinose induction.

[0283] In some embodiments, induction of the RssB promoter(s) indirectly drives the *in vivo* expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y. In some embodiments, induction of the RssB promoter(s) indirectly drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y during *in vitro* growth, preparation, or manufacturing of the strain prior to *in vivo* administration. In some embodiments, conditions for induction of the RssB promoter(s) are provided in culture, e.g., in a flask, a fermenter or another appropriate culture vessel.

[0284] In some embodiments, the PssB promoter indirectly drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a low-copy plasmid or a high copy plasmid or a biosafety system plasmid described herein. In some embodiments, the PssB promoter indirectly drives the expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP and/or transcriptional regulator(s), e.g., FNRS24Y, from a construct which is integrated into the bacterial chromosome. Exemplary insertion sites are described herein.

[0285] In another non-limiting example, this strategy can be used to control expression of thyA and/or dapA, e.g., to make a conditional auxotroph. The chromosomal copy of dapA or ThyA is knocked out. Under anaerobic conditions, dapA or thyA -as the case may be- are expressed, and the strain can grow in the absence of dap or thymidine. Under aerobic conditions, dapA or thyA expression is shut off, and the strain cannot grow in the absence of dap or thymidine. Such a strategy can, for example be employed to allow survival of bacteria under anaerobic conditions, e.g., the gut, but prevent survival under aerobic conditions (biosafety switch). In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,96%, 97%, 98%, or 99% identity with any of the sequences of SEQ ID NO: 117.

[0286] In addition to promoters that are induced in response to oxygen levels, the PME gene(s) and/or Phe transporter gene(s) can be regulated by promoters that are induced in response to inflammatory conditions, such as in presence of reactive nitrogen species or in the presence of reactive oxygen species. Examples of such inducible promoters are found in co-pending, co-owned International Application PCT/US2016/050836, filed 09/08/2016.

Induction of PMEs and/or Phe Transporters During Strain Culture

[0287] In some embodiments, it is desirable to pre-induce PME expression and PME activity prior to administration to a subject. That is, in some embodiments, it is desirable to pre-induce PME expression and PME activity in culture prior to their activation in vivo. In such situations, the strains are pre-loaded with active PME (PAL3 and/or LAAD) and/or Phe transporter, e.g., PheP. In such instances, the genetically engineered bacteria of the invention express one or more PME(s) and/or Phe transporter(s), e.g., PheP, under conditions provided in bacterial culture during with cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture prior to administration *in vivo*. Such culture conditions can be provided in a flask, fermenter or other appropriate culture vessel, e.g., used during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. As used herein, the term "bacterial culture" or bacterial cell culture" or "culture" refers to bacterial cells or microorganisms, which are maintained or grown *in vitro* during several production processes, including cell growth, cell expansion, recovery, purification, fermentation, and/or manufacture. As used herein, the term "fermentation" refers to the growth, expansion, and maintenance of bacteria under defined conditions. Fermentation may occur under a number of cell culture conditions, including anaerobic or low oxygen or oxygenated conditions, in the presence of inducers, nutrients, at defined temperatures, and the like.

[0288] Culture conditions are selected to achieve a high yield and high viability of bacterial cells, while maintaining optimal activity of the cells. A number of cell culture parameters are monitored and adjusted to achieve optimal activity, high yield and high viability, including oxygen levels (e.g., low oxygen, microaerobic, aerobic), temperature of the medium, and nutrients, chemical inducers and other components provided in the medium. In some embodiments, phenylalanine is added to

the media, e.g., to boost cell health. Without wishing to be bound by theory, addition of phenylalanine to the medium may prevent bacteria from catabolizing endogenously produced phenylalanine required for cell growth.

[0289] In some embodiments, the one or more PME(s) and or Phe transporter(s) are directly or indirectly induced, while the strains is grown up for *in vivo* administration. Without wishing to be bound by theory, pre-induction may boost *in vivo* activity. This is particularly important in proximal regions of the gut which are reached first by the bacteria, e.g., the small intestine. If the bacterial residence time in this compartment is relatively short, the bacteria may pass through the small intestine without reaching full *in vivo* induction capacity. In contrast, if a strain is pre-induced and preloaded, the strains are already fully active, allowing for greater activity more quickly as the bacteria reach the intestine. Ergo, no transit time is "wasted", in which the strain is not optimally active. As the bacteria continue to move through the intestine, *in vivo* induction occurs under environmental conditions of the gut (e.g., low oxygen, or in the presence of gut metabolites).

[0290] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more PAL gene sequence(s) is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more PheP gene sequence(s) is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more LAAD gene sequence(s) is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation,

and/or manufacture. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s) is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture.

[0291] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is driven from the same promoter as a multicistronic message. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is driven from the same promoter as two or more separate messages. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is driven from the one or more different promoters.

[0292] In some embodiments, the strains are administered without any pre-induction protocols during strain growth prior to *in vivo* administration.

Anaerobic induction

[0293] In some embodiments, cells are induced under anaerobic or low oxygen conditions in culture. In such instances, cells are grown (e.g., for 1.5 to 3 hours) until they have reached a certain OD, e.g., ODs within the range of 0.1 to 10, indicating a certain density e.g., ranging from 1X10^8 to 1X10^11, and exponential growth and are then switched to anaerobic or low oxygen conditions for approximately 3 to 5 hours. In some embodiments, strains are induced under anaerobic or low oxygen conditions, e.g. to induce FNR promoter activity and drive expression of one or more PME(s) and /or Phe transporters under the control of one or more FNR promoters.

[0294] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PAL gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PheP gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth,

cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of one or more LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic or low oxygen conditions.

[0295] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the same promoter in the form of a multicistronic message under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the same promoter as two or more separate messages under anaerobic or low oxygen conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the one or more different promoters under anaerobic or low oxygen conditions.

[0296] Without wishing to be bound by theory, strains that comprise one or more PME(s) and/or PheP transporter(s) and/or transcriptional regulator(s) under the control of an FNR promoter, may allow expression of PME(s) and/or PheP transporter(s) and/or transcriptional regulator(s) from these promoters *in vitro*, under anaerobic or low oxygen culture conditions, and *in vivo*, under the low oxygen conditions found in the gut.

[0297] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers can be induced under anaerobic or low oxygen conditions in the presence of the chemical and/or nutritional inducer. In particular, strains may comprise a combination of gene sequence(s), some of which are under control of FNR promoters and others which are under control of promoters induced by chemical and/or nutritional inducers. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of one or more FNR promoter(s) and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of one or more FNR promoter(s), and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more constitutive promoter(s) described herein. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of an FNR promoter and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more thermoregulated promoter(s) described herein.

[0298] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s),

e.g., FNRS24Y, is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PAL gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, the

chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one embodiment, the chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0299] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or more separate messages under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promoters under anaerobic and/or low oxygen conditions.

[0300] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers, under anaerobic or low oxygen conditions. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers. In some embodiments, the strains comprise gene sequence(s) under the control of a a third inducible promoter, e.g., an anaerobic/low oxygen promoter, e.g., FNR promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a chemically induced promoter or a low oxygen promoter and others which are under control of a second inducible

promoter, e.g. a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a FNR promoter and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a chemically induced and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of an FNR promoter and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria strains further comprise PME and or Phe transporter sequence(s) under the control of one or more constitutive promoter(s) active under low oxygen conditions.

Aerobic induction

[0301] In some embodiments, it is desirable to prepare, pre-load and pre-induce the strains under aerobic conditions. This allows more efficient growth and viability, and, in some cases, reduces the build-up of toxic metabolites. In such instances, cells are grown (e.g., for 1.5 to 3 hours) until they have reached a certain OD, e.g., ODs within the range of 0.1 to 10, indicating a certain density e.g., ranging from 1X10^8 to 1X10^11, and exponential growth and are then induced through the addition of the inducer or through other means, such as shift to a permissive temperature, for approximately 3 to 5 hours.

[0302] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art can be induced under aerobic conditions in the presence of the chemical and/or nutritional inducer during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression

of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PAL gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions.

[0303] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message under aerobic conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or more separate messages under aerobic conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promoters under aerobic conditions.

[0304] In one embodiment, the chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one embodiment, the chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0305] In some embodiments, promoters regulated by temperature are induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PAL gene sequence(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PheP gene sequence(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery,

purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more LAAD gene sequence(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under aerobic conditions.

[0306] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is driven from the same promoter in the form of a multicistronic message under aerobic conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is driven directly or indirectly by one or more thermoregulated promoter(s)and is driven from the same promoter as two or more separate messages under aerobic conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is driven directly or indirectly by one or more thermoregulated promoter(s) and is driven from the one or more different promoters under aerobic conditions.

[0307] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced under aerobic conditions. In some embodiments, a strain comprises three or more different promoters which are induced under aerobic culture conditions.

[0308] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g. a chemically inducible promoter, and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter under aerobic culture conditions. In some embodiments two or more chemically induced promoter gene sequence(s) are combined with a thermoregulated construct described herein. In one embodiment, the chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one embodiment, the chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0309] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a FNR promoter and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a chemically induced and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of an FNR promoter and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional

inducer(s), including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria strains further comprise PME and or Phe transporter sequence(s) under the control of one or more constitutive promoter(s) active under aerobic conditions.

[0310] In some embodiments, genetically engineered strains comprise PAL, LAAD, and PheP gene sequence(s) which are induced under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0311] In some embodiments, genetically engineered strains comprise PAL gene sequence(s), which are arabinose inducible under aerobic culture conditions. In some embodiments, such genetically engineered strains further comprise LAAD gene sequences which are arabinose inducible under aerobic culture conditions. In some embodiments, such strains further comprise PheP gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0312] In some embodiments, genetically engineered strains comprise PAL gene sequence(s), which are IPTG inducible under aerobic culture conditions. In some embodiments, such genetically engineered strains further comprise LAAD gene sequences which are IPTG inducible under aerobic culture conditions. In some embodiments, such strains further comprise PheP gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0313] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are arabinose inducible under aerobic culture conditions. In

some embodiments, such a strain further comprises PAL gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0314] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0315] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0316] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some

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

[0317] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0318] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0319] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0320] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are IPTG

inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0321] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0322] In some embodiments, genetically engineered strains comprise LAAD gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PAL gene sequence(s) which are arabinose inducible under aerobic culture conditions. In some embodiments, such a strain further comprises PheP gene sequence(s) which are IPTG inducible under aerobic culture conditions. In some embodiments, these strains further comprise FNR inducible gene PME and/or Phe transporter sequence(s) for *in vivo* activation in the gut. In some embodiments, these strains do not further comprise FNR inducible gene sequence(s) for *in vivo* activation in the gut.

[0323] As evident from the above non-limiting examples, genetically engineered strains comprise inducible PME and or Phe transporter gene sequence(s) which can be induced numerous combinations. For example, rhamnose or tetracycline can be used as an inducer with the appropriate promoters in addition or in lieu of arabinose and/or IPTG or with thermoregulation. Additionally, such bacterial strains can also be induced with the chemical and/or nutritional inducers under anaerobic conditions.

Microaerobic Induction

[0324] In some embodiments, viability, growth, and activity are optimized by pre-inducing the bacterial strain under microaerobic conditions. In some embodiments, microaerobic conditions are best suited to "strike a balance" between optimal growth, activity and viability conditions and optimal conditions for induction; in particular, if the expression of the one or more PME(s) and/or Phe transporter(s) are driven by a anaerobic and/or low oxygen promoter, e.g., a FNR promoter. In such instances, cells are grown (e.g., for 1.5 to 3 hours) until they have reached a certain OD, e.g., ODs within the range of 0.1 to 10, indicating a certain density e.g., ranging from 1X10^8 to 1X10^11, and exponential growth and are then induced through the addition of the inducer or through other means, such as shift to at a permissive temperature, for approximately 3 to 5 hours.

[0325] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PAL gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PheP gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery,

purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions.

[0326] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the same promoter in the form of a multicistronic message under microaerobic conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the same promoter as two or more separate messages under microaerobic conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the one or more different promoters under microaerobic conditions.

[0327] Without wishing to be bound by theory, strains that comprise one or more PME(s) and/or PheP transporter(s) and/or transcriptional regulator(s) under the control of an FNR promoter, may allow expression of PME(s) and/or PheP transporter(s) and/or transcriptional regulator(s) from these promoters *in vitro*, under microaerobic culture conditions, and *in vivo*, under the low oxygen conditions found in the gut.

[0328] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers can be induced under microaerobic conditions in the presence of the chemical and/or nutritional inducer. In particular, strains may comprise a combination of gene sequence(s), some of which are under control of FNR promoters and others which are under control of promoters induced by chemical and/or nutritional inducers. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene

sequence(s) and /or transcriptional regulator gene sequence(s) under the control of one or more FNR promoter(s) and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of one or more FNR promoter(s), and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more constitutive promoter(s) described herein. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of an FNR promoter and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more thermoregulated promoter(s) described herein.

[0329] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PAL gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion,

fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under microaerobic conditions. In one embodiment, the chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one embodiment, the chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0330] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message under microaerobic conditions. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or more separate messages under microaerobic conditions. In one embodiment, expression of

one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promoters under microaerobic conditions.

[0331] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers, under microaerobic conditions. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers. In some embodiments, the strains comprise gene sequence(s) under the control of a a third inducible promoter, e.g., an anaerobic/low oxygen promoter or microaerobic promoter, e.g., FNR promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a chemically induced promoter or a low oxygen or microaerobic promoter and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a FNR promoter and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a chemically induced and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of an FNR promoter and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria

strains further comprise PME and or Phe transporter sequence(s) under the control of one or more constitutive promoter(s) active under low oxygen conditions.

Induction of Strains using Phasing, Pulsing and/or Cycling

[0332] In some embodiments, cycling, phasing, or pulsing techniques are emplyed during cell growth, expansion, recovery, purification, fermentation, and/or manufacture to efficienty induce and grow the strains prior to *in vivo* administration. This method is used to "strike a balance" between optimal growth, activity, cell health, and viability conditions and optimal conditions for induction; in particular, if growth, cell health or viability are negatively affected under inducing conditions. In such instances, cells are grown (e.g., for 1.5 to 3 hours) in a first phase or cycle until they have reached a certain OD, e.g., ODs within the range of 0.1 to 10, indicating a certain density e.g., ranging from 1X10^8 to 1X10^11,and are then induced through the addition of the inducer or through other means, such as shift to a permissive temperature (if a promoter is thermoregulated), or change in oxygen levels (e.g., reduction of oxygen level in the case of induction of an FNR promoter driven construct) for approximately 3 to 5 hours. In a second phase or cycle, conditions are brought back to the original conditions which support optimal growth, cell health and viability. Alternatively, if a chemical and/or nutritional inducer is used, then the culture can be spiked with a second dose of the inducer in the second phase or cycle.

[0333] In some embodiments, two cycles of optimal conditions and inducing conditions are employed (i.e, growth, induction, recovery and growth, induction). In some embodiments, three cycles of optimal conditions and inducing conditions are employed. In some embodiments, four or more cycles of optimal conditions and inducing conditions are employed. In a non-liming example, such cycling and/or phasing is used for induction under anaerobic and/or low oxygen conditions (e.g., induction of FNR promoters). In one embodiment, cells are grown to the optimal density and then induced under anaerobic and/or low oxygen conditions. Before growth and/or viability are negatively impacteddue to stressful induction conditions, , cells are returned to oxygenated conditions to recover, after which they are then returned to inducing anaerobic and/or low oxygen conditions for a second time. In some embodiments, these cycles are repeated as needed.

[0334] In some embodiments, growing cultures are spiked once with the chemical and/or nutritional inducer. In some embodiments, growing cultures are spiked twice with the chemical and/or nutritional inducer. In some embodiments, growing cultures are spiked three or more times with the chemical and/or nutritional inducer. In a non-limiting example, cells are first grown under optimal growth conditions up to a certain density, e.g., for 1.5 to 3 hour) to reached an of 0.1 to 10, until the cells are at a density ranging from 1X10^8 to 1X10^11. Then the chemical inducer, e.g., arabinose or IPTG, is added to the culture. After 3 to 5 hours, an additional dose of the inducer is added to re-initiate the induction. Spiking can be repeated as needed.

[0335] In some embodiments, phasing or cycling changes in temperature in the culture. In another embodiment, adjustment of temperature may be used to improve the activity of a PME. For example, lowering the temperature during culture may improve the proper folding of the PME. In such instances, cells are first grown at a temperature optimal for growth (e.g., 37 C). In some embodiments, the cells are then induced, e.g., by a chemical inducer, to express the PME. Concurrently or after a set amount of induction time, the temperature in the media is lowered, e.g., between 25 and 35 C, to allow improved folding of the expressed PME, e.g., PAL.

[0336] In some embodiments, PME(s) and Phe transporters are under the control of different inducible promoters, for example two different chemical inducers. In other embodiments, the PME is induced under low oxygen conditions or microaerobic conditions and the Phe transporter is induced by a chemical inducer. In other embodiments, the Phe transporter is induced under low oxygen or microaerobic conditions and the PME is induced by a chemical inducer, e.g, arabinose or IPTG. For optimal results, it is desirable to induce the Phe transporter first or concurrently with the PME. This in some cases may allow for more efficient uptake of Phe, and, as a result, continuouscell growth is maintained. In a non-limiting example, PheP can be induced under microaerobic conditions for 1 to 2 hours before the PME is induced by another inducer, such as arabinose, e.g, for another 3 to 5 hours. In a non-limiting example, PheP and PME, e.g., PAL and LAAD are induced concurrently with arabinose. In another non-limiting example, PheP is induced microaerobically, PAL is induced with IPTG, and lastly, LAAD are induced with arabinose.

[0337] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture by using phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PAL gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PheP gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s) is under the control of one or more FNR promoter(s) and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture through the employment of phasing or cycling or pulsing or spiking techniques.

[0338] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the same promoter in the form of a multicistronic message through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the same promoter as two or more separate messages through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more FNR promoter(s) and is driven from the one or more different promoters through the employment of phasing or cycling or pulsing or spiking techniques.

[0339] Without wishing to be bound by theory, strains that comprise one or more PME(s) and/or PheP transporter(s) and/or transcriptional regulator(s) under the control of an FNR promoter, may allow expression of PME(s) and/or PheP transporter(s) and/or transcriptional regulator(s) from these promoters *in vitro*, under microaerobic culture conditions, and *in vivo*, under the low oxygen conditions found in the gut.

[0340] In some embodiments, promoters inducible by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers can be induced through the employment of phasing or cycling or pulsing or spiking techniques in the presence of the chemical and/or nutritional inducer. In particular, strains may comprise a combination of gene sequence(s), some of which are under control of FNR promoters and others which are under control of promoters induced by chemical and/or nutritional inducers. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of one or more FNR promoter(s) and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s), including, but not limited to, arabinose, IPTG, rhamnose, tetracycline, and/or other

chemical and/or nutritional inducers described herein or known in the art. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of one or more FNR promoter(s), and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more constitutive promoter(s) described herein and are induced through the employment of phasing or cycling or pulsing or spiking techniques. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of an FNR promoter and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more thermoregulated promoter(s) described herein, and are induced through the employment of phasing or cycling or pulsing or spiking techniques.

[0341] Any of the strains described herein can be grown through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), e.g., FNRS24Y, is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PAL gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen

conditions. In one embodiment, expression of one or more PAL and PheP gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PAL and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PheP and LAAD gene sequence(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, expression of one or more PheP and LAAD and PAL gene sequence(s), is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is induced during cell growth, cell expansion, fermentation, recovery, purification, formulation, and/or manufacture under anaerobic and/or low oxygen conditions. In one embodiment, the chemical and/or nutritional inducer is arabinose and the promoter is inducible by arabinose. In one embodiment, the chemical and/or nutritional inducer is IPTG and the promoter is inducible by IPTG. In one embodiment, the chemical and/or nutritional inducer is rhamnose and the promoter is inducible by rhamnose. In one embodiment, the chemical and/or nutritional inducer is tetracycline and the promoter is inducible by tetracycline.

[0342] In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter in the form of a multicistronic message and which are induced through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the same promoter as two or

more separate messages and is grown through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, expression of one or more PME(s), e.g., PAL and/or LAAD, and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s) is under the control of one or more promoter(s) regulated by chemical and/or nutritional inducers and is driven from the one or more different promotersal of which are induced through the employment of phasing or cycling or pulsing or spiking techniques.

[0343] In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers, through the employment of phasing or cycling or pulsing or spiking techniques. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter and others which are under control of a second inducible promoter, both induced by chemical and/or nutritional inducers through the employment of phasing or cycling or pulsing or spiking techniques. In some embodiments, the strains comprise gene sequence(s) under the control of a a third inducible promoter, e.g., an anaerobic/low oxygen promoter, e.g., FNR promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a chemically induced promoter or a low oxygen promoter and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a FNR promoter and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In one embodiment, strains may comprise a combination of gene sequence(s), some of which are under control of a first inducible promoter, e.g., a chemically induced and others which are under control of a second inducible promoter, e.g. a temperature sensitive promoter. In some embodiments, strains may comprise one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of an FNR promoter and one or more PME gene sequence(s) and/or Phe transporter gene sequence(s) and /or transcriptional regulator gene sequence(s) under the control of a one or more promoter(s) which are induced by a one or more chemical and/or nutritional inducer(s),

including, but not limited to, by arabinose, IPTG, rhamnose, tetracycline, and/or other chemical and/or nutritional inducers described herein or known in the art. Additionally the strains may comprise a construct which is under thermoregulatory control. In some embodiments, the bacteria strains further comprise PME and or Phe transporter sequence(s) under the control of one or more constitutive promoter(s) active under low oxygen conditions. Any of the strains described in these embodiments may be induced through the employment of phasing or cycling or pulsing or spiking techniques.

Aerobic induction of the FNR promoter

[0344] [066] FNRS24Y is a mutated form of FNR which is more resistant to inactivation by oxygen, and therefore can activate FNR promoters under aerobic conditions (see e.g., Jervis AJ The O2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe-4S] to [2Fe-2S] conversion, Proc Natl Acad Sci U S A. 2009 Mar 24;106(12):4659-64, the contents of which is herein incorporated by reference in its entirety). In some embodiments, oxygen bypass system shown and described in **Fig. 50** is used. In this oxygen bypass system, FNRS24Y is induced by addition of arabinose and then drives the expression PAL3, pheP by binding and activating the FNR promoter under aerobic conditions. Thus, strains can be grown, produced or manufactured efficiently under aerobic conditions, while being effectively pre-induced and pre-loaded, as the system takes advantage of the strong FNR promoter resulting in of high levels of expression of PAL and PheP. This system does not interfere with or compromise *in vivo* activation, since the mutated FNRS24Y is no longer expressed in the absence of arabinose, and wild type FNR then binds to the FNR promoter and drives expression of PAL3 and PheP.

[0345] [067] In some embodiments, FNRS24Y is expressed during aerobic culture growth and induces FNR-PAL. In some embodiments, FNRS24Y is expressed during culture growth and induces FNR-pheP. In some embodiments, FNRS24Y is expressed during culture growth and induces FNR-LAAD. In other embodiments, FNRS24Y is expressed during culture growth and induces FNR-PAL and FNR-PheP. In other embodiments, FNRS24Y is expressed during culture growth and induces FNR-PAL and FNR-LAAD. In other embodiments, FNRS24Y is expressed during culture growth and induces FNR-LAAD and FNR-PheP. In other embodiments, FNRS24Y is

expressed during culture growth and induces FNR-PAL and FNR-PheP and FNR-LAAD.

[0346] In other embodiments described herein, LAAD expression can also be induced aerobically, e.g., by arabinose. In a non-limiting example, LAAD and FNRS24Y can in some embodiments be induced simultaneously, e.g., from an arabinose inducible promoter. In some embodiments, FNRS24Y and LAAD are transcribed as a bicistronic message whose expression is driven by an arabinose promoter. In some embodiments, FNRS24Y is knocked into the arabinose operon, allowing expression to be driven from the endogenous Para promoter. In some embodiments, FNRS24Y-LAAD is knocked into the arabinose operon, allowing expression to be driven from the endogenous Para promoter.

[0347] [068] In some embodiments, a LacI promoter and IPTG induction are used in this system (in lieu of Para and arabinose induction). In some embodiments, a rhamnose inducible promoter is used in this system. In some embodiments, a temperature sensitive promoter is used to drive expression of FNRS24Y.

Measurement of Pre-induction

[0348] In some embodiments, such culture conditions, in which expression of the PME(s) and or PheP are induced result in the reduction of phenylalanine in the culture 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, or as compared to the baseline levels. In some embodiments, such culture conditions, in which expression of the PME(s) and or PheP are induced result in the production of transcinnamic acid (TCA) in the culture 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, or as compared to the baseline levels.

[0349] In some embodiments, cinnamate accumulation in the bacterial cultures is measured by methods known in the art and described herein. Cinnamate production is directly correlated with phenylalanine degradation, and in some embodiments, cinnamate may be used as an indicator for strain activity during strain growth, production and manufacture. Measurement of a reduction in phenylalanine and or the production of TCA therefore may be used to measure and monitor, and fine tune the induction of a therapeutic strain prior to administration *in vivo*.

Multiple Mechanisms of Action

[0350] 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. 66**. 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.*

[0351] In some embodiments, the genetically engineered bacteria comprise one or more gene sequence(s) encoding one or more of (1) PAL, PAH, and/or LAAD for degradation of phenylalanine, in wild type or in a mutated form (for increased stability or metabolic activity) (2) transporter PheP and/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 and/or deltadapA (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) (9) one or more biosafety systems constructs and/or kill switches

[0352] In some embodiments, under conditions where the gene sequence(s) for producing the payload(s), e.g., the PME(s), Phe tranporter(s), and/or transcriptional regulator(s) are expressed, the genetically engineered bacteria of the disclosure both degrade phenylalanine and generate TCA at levels at least about 1.5-fold, at least about 2-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, at least about 600-fold, at least about 700-fold, at least about 800-fold, at least about 900-fold, at least about 1,000-fold, or at least about 1,500-fold greater as compared to unmodified bacteria of the same subtype under the same conditions.

[0353] In some embodiments, under conditions where the gene sequence(s) for producing the payload(s), e.g., the PME(s), Phe tranporter(s), and/or transcriptional regulator(s) are expressed, the genetically engineered bacteria of the disclosure both degrade phenylalanine and generate hippurate at levels at least about 1.5-fold, at least about 2-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 30-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, at least about 600-fold, at least about 700-fold, at least about 800-fold, at least about 900-fold, at least about 1,000-fold, or at least about 1,500-fold greater as compared to unmodified bacteria of the same subtype under the same conditions.

[0354] In some embodiments, the gene sequence(s) encoding the PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y_are expressed under the control of a constitutive promoter. In another embodiment, the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y are expressed under the control of an inducible promoter. In some embodiments, the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are expressed under the control of a promoter that is directly or indirectly induced by exogenous environmental conditions. In one embodiment, the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP,

and/or other regulatory proteins, e.g., FNRS24Y, are expressed under the control of a promoter that is directly or indirectly induced by low-oxygen or anaerobic conditions, wherein expression of the gene sequence(s) encoding the PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. Exemplary inducible promoters described herein include oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter) and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose, tetracycline, IPTG, rhamnose, and other chemical and/or nutritional inducers. In some embodiments, such inducible promoters described herein, are induced under *in vitro* culture conditions, as strains are prepared prior to *in vivo* administration, as described herein. Examples of inducible promoters include, but are not limited to, an FNR responsive promoter, a P_{araBAD} promoter, and a P_{TetR} promoter, Plac promoter, the rhaP BAD (rhamnose) promtoer, each of which are described in more detail herein. Inducible promoters are described in more detail *infra*.

[0355] The at least one gene encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, may be present on a plasmid or chromosome in the bacterial cell. In one embodiment, the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are located on a plasmid in the bacterial cell. In another embodiment, the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are located in the chromosome of the bacterial cell. In yet another embodiment, a native copy of the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are located in the chromosome of the bacterial cell, and one or more gene(s) encoding one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are located on a plasmid in the bacterial cell. In yet another embodiment, a native copy of the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are located on a plasmid in the bacterial cell, and at least one gene encoding the at least one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, from a different species of bacteria are located on a plasmid in the

bacterial cell. In yet another embodiment, a native copy of the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are located in the chromosome of the bacterial cell, and the one or more gene(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, from a different species of bacteria are located in the chromosome of the bacterial cell. In some embodiments, the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are expressed on a low-copy plasmid. In some embodiments, the gene sequence(s) encoding the one or more PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y, are expressed on a high-copy plasmid. In some embodiments, the high-copy plasmid may be useful for increasing expression of the at least one PME(s) and/or Phe transporters, e.g., PheP, and/or other regulatory proteins, e.g., FNRS24Y.

[0356] In some embodiments, the genetically engineered bacteria described above further comprise one or more of the modifications, mutations, and/or deletions in endogenous genes described herein.

[0357] In one embodiment, the genetically engineered bacterial strain comprises three chromosomal insertions of FNR driven PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K), e.g., SEQ ID NO: 38) and two copies of FNR driven pheP (2XfnrS-pheP (lacZ, agaI/rsmI), e.g., SEQ ID NO: 62). The strain further comprises one copy of the mutated FNR transcription factor FNRS24Y knocked into the arabinose operon with expression driven by the native Para promoter (Para::FNRS24Y, e.g., SEQ ID NO: 64). In one embodiment, the genetically engineered bacterial strain is SYN-PKU707 (e.g., as depicted in **Fig. 47A**).

[0358] In one embodiment, the genetically engineered bacterial strain comprises three chromosomal insertions of FNR driven PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K), e.g., SEQ ID NO: 38) and two copies of FNR driven pheP (2XfnrS-pheP (lacZ, agaI/rsmI), e.g., SEQ ID NO: 62). The strain further comprises one copy of the mutated FNR transcription factor FNRS24Y knocked into the arabinose operon with expression driven by the native Para promoter (Para::FNRS24Y e.g., SEQ ID NO: 64). The genome is further engineered to include a dapA auxotrophy, in which the dapA

gene is deleted. In one embodiment, the genetically engineered bacterial strain is SYN-PKU712 (e.g., as depicted in **Fig. 47E**).

[0359] In one embodiment, the genetically engineered bacterial strain comprises a bacterial chromosome with three chromosomal insertions of FNR driven PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K), e.g., SEQ ID NO: 38) and two copies of FNR driven pheP (2XfnrS-pheP (lacZ, agaI/rsmI), e.g., SEQ ID NO: 62). The strain further comprises one copy of the mutated FNR transcription factor FNRS24Y knocked into the arabinose operon with expression driven by the native Para promoter and one copy of LAAD inserted at the same insertion site (Para::FNRS24Y-LAAD, e.g., SEQ ID NO: 73), which is transcribed as a bicistronic message from the endogenous arabinose promoter. The genome is further engineered to include a dapA auxotrophy, in which the dapA gene is deleted. In one embodiment, the genetically engineered bacterial strain is SYN-PKU708 (e.g., as depicted in **Fig. 47B**).

[0360] In one embodiment, the genetically engineered bacterial strain comprises a bacterial chromosome with three chromosomal insertions of FNR driven PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K), e.g., SEQ ID NO: 38) and two copies of FNR driven pheP (2XfnrS-pheP (lacZ, agaI/rsmI), e.g., SEQ ID NO: 62). The strain further comprises one copy of the mutated FNR transcription factor FNRS24Y knocked into the arabinose operon with expression driven by the native Para promoter and one copy of LAAD inserted at the same insertion site (Para::FNRS24Y-LAAD, e.g., SEQ ID NO: 73), which is transcribed as a bicistronic message from the endogenous arabinose promoter. In one embodiment, the genetically engineered bacterial strain is SYN-PKU711 (e.g., as depicted in **Fig. 47F**).

[0361] In one embodiment, the genetically engineered bacterial strain comprises a bacterial chromosome comprising three chromosomal insertions of FNR driven PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K), e.g., SEQ ID NO: 38) and two copies of FNR driven pheP (2XfnrS-pheP (lacZ, agaI/rsmI), e.g., SEQ ID NO: 62). The strain further comprises one copy of LAAD knocked into the arabinose operon with expression driven by the native Para promoter (Para::LAAD, e.g., SEQ ID NO: 40). The genome is further engineered to include a dapA auxotrophy, in which the dapA gene is deleted. In one embodiment, the genetically engineered bacterial strain is SYN-PKU709 (e.g., as depicted in **Fig. 47C**).

[0362] In one embodiment, the genetically engineered bacterial strain comprises a bacterial chromosome comprising three chromosomal insertions of FNR driven PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K), e.g., SEQ ID NO: 38) and two copies of FNR driven pheP (2XfnrS-pheP (lacZ, agaI/rsmI), e.g., SEQ ID NO: 62). The strain further comprises one copy of the LAAD knocked into the arabinose operon with expression driven by the native Para promoter (Para::LAAD, e.g., SEQ ID NO: 40). SYN-PKU710 further comprises two copies of IPTG inducible PAL3 (2XLacIPAL, exo/cea and rhtC/rhtB, e.g., SEQ ID NO: 74), a dapA auxotrophy and is cured of all antibiotic resistances. In one embodiment, the genetically engineered bacterial strain is SYN-PKU710 (e.g., as depicted in **Fig. 47D**).

[0363] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1001.

[0364] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS;dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL, e.g., SEQ ID NO:98). In one embodiment, the strain is SYN-PKU1002.

[0365] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1003.

[0366] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL , e.g., SEQ ID NO: 98). In one embodiment, the strain is SYN-PKU1004.

[0367] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1005.

[0368] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL, e.g., SEQ ID NO: 98). In one embodiment, the strain is SYN-PKU1006.

[0369] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1007.

[0370] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive

promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL, e.g., SEQ ID NO: 98). In one embodiment, the strain is SYN-PKU1008.

[0371] In one embodiment, the genetically engineered bacterium a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1009.

[0372] In one embodiment, the genetically engineered bacterium a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS;dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL , e.g., SEQ ID NO: 98). In one embodiment, the strain is SYN-PKU1010.

[0373] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1011.

[0374] In one embodiment, the genetically engineered bacterium a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL, e.g., SEQ ID NO: 98). In one embodiment, the strain is SYN-PKU1012

[0375] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig.**

61B, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1013

[0376] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL, e.g., SEQ ID NO: 98). In one embodiment, the strain is SYN-PKU1014.

[0377] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL, e.g., SEQ ID NO: 97). In one embodiment, the strain is SYN-PKU1015.

[0378] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL, e.g., SEQ ID NO: 98). In one embodiment, the strain is SYN-PKU1016.

[0379] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1017.

[0380] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome

(low copy RBS;dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1018.

[0381] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1019.

[0382] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1020.

[0383] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1021.

[0384] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome

(low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1022.

[0385] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1023.

[0386] In one embodiment, the genetically engineered bacterium comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1024.

[0387] In one embodiment, the genetically engineered bacterium a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1025.

[0388] In one embodiment, the genetically engineered bacterium a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS;dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that

the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1026.

[0389] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1027.

[0390] In one embodiment, the genetically engineered bacterium a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1028.

[0391] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1029.

[0392] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1030.

[0393] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome

(medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65A (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 95**)**. In one embodiment, the strain is SYN-PKU1032.

[0394] In one embodiment, the genetically engineered bacterium comprises a construct shown in **Fig. 61D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65C (**LacI Fnrs-Ptac-PAL-PAL-PheP, e.g., SEQ ID NO: 96**)**. In one embodiment, the strain is SYN-PKU1032.

[0395] 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.

[0396] 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.

[0397] 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/or transporters may be comprised on low or high copy plasmids.

[0398] 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/or transporters may be comprised on low or high copy plasmids.

[0399] 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).

[0400] 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).

[0401] 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).

[0402] 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).

[0403] 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.

[0404] 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.

[0405] 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.

[0406] 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.

[0407] 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.

[0408] 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.

[0409] **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*
SYN-PKU713	*LacZ::PfnrS-PAL3::pheP*
SYN-PKU706	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::LAAD, ΔdapA::cm*
SYN-PKU707	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y::cm*
SYN-PKU708	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y-LAAD; ΔdapA*
SYN-PKU-709	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para-LAAD; ΔdapA*
SYN-PKU-710	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::LAAD; exo/cea:: LacIPAL3; rhtC/rhtB::LacIPAL3; ΔdapA*
SYN-PKU711	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y-LAAD;*
SYN-PKU-712	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y; ΔDapA*
SYN-PKU-714	*lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP*

Secretion

[0410] In some embodiments, the genetically engineered bacteria further comprise a native secretion mechanism or non-native secretion mechanism that is capable of secreting a molecule from the bacterial cytoplasm in the extracellular environment. Many bacteria have evolved sophisticated secretion systems to transport substrates across the bacterial cell envelope. Substrates, such as small molecules, proteins, and DNA, may be released into the extracellular space or periplasm (such as

the gut lumen or other space), injected into a target cell, or associated with the bacterial membrane.

[0411] 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. Membrane-spanning secretion systems include, but are not limited to, the type I secretion system (T1SS), the type II secretion system (T2SS), the type III secretion system (T3SS), the type IV secretion system (T4SS), the type VI secretion system (T6SS), and the resistance-nodulation-division (RND) family of multi-drug efflux pumps (Pugsley 1993; Gerlach et al., 2007; Collinson et al., 2015; Costa et al., 2015; Reeves et al., 2015; WO2014138324A1, incorporated herein by reference). Examples of such secretion systems are shown in **Fig. 94, 95, 96, 97, 98, 99, and 100**. Mycobacteria, which have a Gram-negative-like cell envelope, may also encode a type VII secretion system (T7SS) (Stanley et al., 2003). With the exception of the T2SS, double membrane-spanning secretions generally transport substrates from the bacterial cytoplasm directly into the extracellular space or into the target cell. In contrast, the T2SS and secretion systems that span only the outer membrane may use a two-step mechanism, wherein substrates are first translocated to the periplasm by inner membrane-spanning transporters, and then transferred to the outer membrane or secreted into the extracellular space. Outer membrane-spanning secretion systems include, but are not limited to, the type V secretion or autotransporter system or autosecreter system (T5SS), the curli secretion system, and the chaperone-usher pathway for pili assembly (Saier, 2006; Costa et al., 2015).

[0412] 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 molecule of interest from the bacterial

cytoplasm. In some embodiments, the secreted molecule, such as a heterologous protein or peptide comprises a type III secretion sequence that allows the molecule of interest to be secreted from the bacteria.

[0413] In some embodiments, a flagellar type III secretion pathway is used to secrete the molecule of interest. In some embodiments, an incomplete flagellum is used to secrete a therapeutic peptide of interest by recombinantly fusing the peptide to an N-terminal flagellar secretion signal of a native flagellar component. In this manner, the intracellularly expressed chimeric peptide can be mobilized across the inner and outer membranes into the surrounding host environment. For example, a modified flagellar type III secretion apparatus in which untranslated DNA fragment upstream of the gene fliC (encoding flagellin), e.g., a 173-bp region, is fused to the gene encoding the polypeptide of interest can be used to secrete heterologous polypeptides (See, e.g., Majander et al., Extracellular secretion of polypeptides using a modified Escherichia coli flagellar secretion apparatus. Nat Biotechnol. 2005 Apr;23(4):475-81). In some cases, the untranslated region from the fliC loci, may not be sufficient to mediate translocation of the passenger peptide through the flagella. Here it may be necessary to extend the N-terminal signal into the amino acid coding sequence of FliC, for example using the 173 bp of untranslated region along with the first 20 amino acids of FliC (see, e.g., Duan et al., Secretion of Insulinotropic Proteins by Commensal Bacteria: Rewiring the Gut To Treat Diabetes, Appl. Environ. Microbiol. December 2008 vol. 74 no. 23 7437-7438).

[0414] In some embodiments, a Type V Autotransporter Secretion System is used to secrete the molecule of interest, e.g., therapeutic peptide. Due to the simplicity of the machinery and capacity to handle relatively large protein fluxes, the Type V secretion system is attractive for the extracellular production of recombinant proteins. As shown in **Fig. 94**, a therapeutic peptide (star) can be fused to an N-terminal secretion signal, a linker, and the beta-domain of an autotransporter. The N-terminal, Sec-dependent signal sequence directs the protein to the SecA-YEG machinery which moves the protein across the inner membrane into the periplasm, followed by subsequent cleavage of the signal sequence. The Beta-domain is recruited to the Bam complex ('Beta-barrel assembly machinery') where the beta-domain is folded and inserted into the outer membrane as a beta-barrel structure. The therapeutic peptide is

threaded through the hollow pore of the beta-barrel structure ahead of the linker sequence. Once exposed to the extracellular environment, the therapeutic peptide can be freed from the linker system by an autocatalytic cleavage (left side of Bam complex) or by targeting of a membrane-associated peptidase (black scissors; right side of Bam complex) to a complimentary protease cut site in the linker. Thus, in some embodiments, the secreted molecule, such as a heterologous protein or peptide 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.

[0415] In some embodiments, a Hemolysin-based Secretion System is used to secrete the molecule of interest, e.g., therapeutic peptide. Type I Secretion systems offer the advantage of translocating their passenger peptide directly from the cytoplasm to the extracellular space, obviating the two-step process of other secretion types. **Fig. 96** 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.

[0416] In alternate embodiments, the genetically engineered bacteria further comprise a non-native single membrane-spanning secretion system. Single membrane-spanning transporters may act as a component of a secretion system, or may export substrates independently. Such transporters include, but are not limited to, ATP-binding cassette translocases, flagellum/virulence-related translocases, conjugation-related translocases, the general secretory system (e.g., the SecYEG complex in E. coli), the accessory secretory system in mycobacteria and several types of Gram-positive bacteria (e.g., Bacillus anthracis, Lactobacillus johnsonii, Corynebacterium glutamicum, Streptococcus gordonii, Staphylococcus aureus), and the twin-arginine translocation (TAT) system (Saier, 2006; Rigel and Braunstein, 2008; Albiniak et al., 2013). It is known that the general secretory and TAT systems can both export substrates with cleavable N-terminal signal peptides into the periplasm, and have been explored in the context of biopharmaceutical production. The TAT system may offer particular

advantages, however, in that it is able to transport folded substrates, thus eliminating the potential for premature or incorrect folding. In certain embodiments, the genetically engineered bacteria comprise a TAT or a TAT-like system and are capable of secreting the molecule of interest from the bacterial cytoplasm. One of ordinary skill in the art would appreciate that the secretion systems disclosed herein may be modified to act in different species, strains, and subtypes of bacteria, and/or adapted to deliver different payloads.

[0417] In order to translocate a protein, e.g., therapeutic polypeptide, to the extracellular space, the polypeptide must first be translated intracellularly, mobilized across the inner membrane and finally mobilized across the outer membrane. Many effector proteins (e.g., therapeutic polypeptides) – particularly those of eukaryotic origin – contain disulphide bonds to stabilize the tertiary and quaternary structures. While these bonds are capable of correctly forming in the oxidizing periplasmic compartment with the help of periplasmic chaperones, in order to translocate the polypeptide across the outer membrane the disulphide bonds must be reduced and the protein unfolded again.

[0418] One way to secrete properly folded proteins in gram-negative bacteria– particularly those requiring disulphide bonds – is to target the reducing-environment periplasm in conjunction with a destabilizing outer membrane. In this manner, the protein is mobilized into the oxidizing environment and allowed to fold properly. In contrast to orchestrated extracellular secretion systems, the protein is then able to escape the periplasmic space in a correctly folded form by membrane leakage. These "leaky" gram-negative mutants are therefore capable of secreting bioactive, properly disulphide-bonded polypeptides. In some embodiments, the genetically engineered bacteria have a "leaky" or de-stabilized outer membrane. Destabilizing the bacterial outer membrane to induce leakiness can be accomplished by deleting or mutagenizing genes responsible for tethering the outer membrane to the rigid peptidoglycan skeleton, including for example, lpp, ompC, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl. Lpp is the most abundant polypeptide in the bacterial cell existing at ~500,000 copies per cell and functions as the primary 'staple' of the bacterial cell wall to the peptidoglycan. 1. Silhavy, T. J., Kahne, D. & Walker, S. The bacterial cell envelope. Cold Spring Harb Perspect Biol 2, a000414 (2010). TolA-PAL and OmpA complexes

function similarly to Lpp and are other deletion targets to generate a leaky phenotype. Additionally, leaky phenotypes have been observed when periplasmic proteases are inactivated. The periplasm is very densely packed with protein and therefore encode several periplasmic proteins to facilitate protein turnover. Removal of periplasmic proteases such as degS, degP or nlpI can induce leaky phenotypes by promoting an excessive build-up of periplasmic protein. Mutation of the proteases can also preserve the effector polypeptide by preventing targeted degradation by these proteases. Moreover, a combination of these mutations may synergistically enhance the leaky phenotype of the cell without major sacrifices in cell viability. Thus, in some embodiments, the engineered bacteria have one or more deleted or mutated membrane genes. In some embodiments, the engineered bacteria have a deleted or mutated lpp gene. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from ompA, ompA, and ompF genes. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from tolA, tolB, and pal genes. in some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes. In some embodiments, the engineered bacteria have one or more deleted or mutated periplasmic protease genes selected from degS, degP, and nlpl. In some embodiments, the engineered bacteria have one or more deleted or mutated gene(s), selected from lpp, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl genes.

[0419] To minimize disturbances to cell viability, the leaky phenotype can be made inducible by placing one or more membrane or periplasmic protease genes, e.g., selected from lpp, ompA, ompF, tolA, tolB, pal, degS, degP, and nlpl, under the control of an inducible promoter. For example, expression of lpp or other cell wall stability protein or periplasmic protease can be repressed in conditions where the therapeutic polypeptide needs to be delivered (secreted). For instance, under inducing conditions a transcriptional repressor protein or a designed antisense RNA can be expressed which reduces transcription or translation of a target membrane or periplasmic protease gene. Conversely, overexpression of certain peptides can result in a destabilized phenotype, e.g., over expression of colicins or the third topological domain of TolA, which peptide overexpression can be induced in conditions in which the therapeutic polypeptide needs to be delivered (secreted). These sorts of strategies would decouple the fragile, leaky phenotypes from biomass production. Thus, in some embodiments, the engineered

bacteria have one or more membrane and/or periplasmic protease genes under the control of an inducible promoter.

[0420] **Table 15A** and **Table 15B** 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 15A. 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 15B. 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		1.B.2	+	-	+	1	None

Secretin (IISP and IISP)		0 1.B.2 2	+[b]		-	1	None
OmpIP	Outer membrane insertion porin	1.B.3 3	+	-	+ (mitochon dria; chloroplast s)	≥4	None ?

[0421] The above tables for gram positive and gram negative bacteria list secretion systems that can be used to secrete polypeptides and other molecules from the engineered bacteria, which are reviewed in Milton H. Saier, Jr. Microbe / Volume 1, Number 9, 2006 "Protein Secretion Systems in Gram-Negative Bacteria Gram-negative bacteria possess many protein secretion-membrane insertion systems that apparently evolved independently", the contents of which is herein incorporated by reference in its entirety.

[0422] Any of the secretion systems described herein may according to the disclosure be employed to secrete the proteins of interest. Non-limiting examples of proteins of interest include PME, e.g., PAH, PAL and/or LAAD as described herein. These polypeptides may be mutated to increase stability, resistance to protease digestion, and/or activity.

Table 16. Comparison of Secretion systems for secretion of polypeptide from engineered bacteria

Secretion System	**Tag**	**Cleavage**	**Advantages**	**Other features**
Modified Type III (flagellar)	mRNA (or N-terminal)	No cleavage necessary	- No peptide tag - Endogenous	May not be as suited for larger proteins Deletion of flagellar genes
Type V autotransport	N- and C-terminal	Yes	Large proteins Endogenous Cleavable	2-step secretion
Type I	C-terminal	No		Tag; Exogenous Machinery
Diffusible Outer Membrane (DOM)	N-terminal	Yes	Disulfide bond formation	May affect cell fragility/survivability/ growth/yield

[0423] 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.

[0424] 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 Sarkissian 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.

[0425] 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 and/or low oxygen 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.

[0426] 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.

[0427] 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.

[0428] 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.

[0429] 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.

[0430] In some embodiments, the therapeutic polypeptides of interest are secreted using components of the flagellar type III secretion system. In a non-limiting example, such a therapeutic polypeptide of interest, such as, the PME, e.g., LAAD,

PAH, and or PAL, is assembled behind a fliC-5'UTR (e.g., 173-bp untranslated region from the fliC loci), and is driven by the native promoter. In other embodiments, the expression of the therapeutic peptide of interested secreted using components of the flagellar type III secretion system is driven by a Tet-inducible promoter. In alternate embodiments, an inducible promoter such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), and promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose is used. In some embodiments, the therapeutic polypeptide of interest is expressed from a plasmid (e.g., a medium copy plasmid). In some embodiments, the therapeutic polypeptide of interest is expressed from a construct which is integrated into fliC locus (thereby deleting fliC), where it is driven by the native FliC promoter. In some embodiments, an N terminal part of FliC (e.g., the first 20 amino acids of FliC) is included in the construct, to further increase secretion efficiency.

[0431] In some embodiments, the therapeutic polypeptides of interest such as, the PME, e.g., LAAD, PAH, and or PAL, are secreted using via a diffusible outer membrane (DOM) system. In some embodiments, the therapeutic polypeptide of interest is fused to a N-terminal Sec-dependent secretion signal. Non-limiting examples of such N-terminal Sec-dependent secretion signals include PhoA, OmpF, OmpA, and cvaC. In alternate embodiments, the therapeutic polypeptide of interest is fused to a Tat-dependent secretion signal. Exemplary Tat-dependent tags include TorA, FdnG, and DmsA. In some embodiments, expression of the secretion-tagged therapeutic protein is driven by a tet promoter or an inducible promoter, such as oxygen level-dependent promoters (*e.g.*, FNR-inducible promoter), or by promoters induced by a metabolite that may or may not be naturally present (e.g., can be exogenously added) in the gut, e.g., arabinose. In some embodiments, the secretion-tagged therapeutic polypeptide of interest is expressed from a plasmid (e.g., a medium copy plasmid). In other embodiments, the therapeutic polypeptide of interest is expressed from a construct which is integrated into the bacterial chromosome, e.g., at one or more of the integration sites described herein**.** In certain embodiments, the genetically engineered bacteria comprise deletions or mutations in one or more of the outer membrane and/or periplasmic proteins. Non-limiting examples of such proteins, one or more of which may be deleted or mutated, include lpp, pal, tolA, and/or nlpI. In some embodiments, lpp is deleted or mutated. In some embodiments, pal is deleted or mutated. In some

embodiments, tolA is deleted or mutated. In other embodiments, nlpI is deleted or mutated. In yet other embodiments, certain periplasmic proteases are deleted or mutated, e.g., to increase stability of the polypeptide in the periplasm. Non-limiting examples of such proteases include degP and ompT. In some embodiments, degP is deleted or mutated. In some embodiments, ompT is deleted or mutated. In some embodiments, degP and ompT are deleted or mutated.

[0432] In some embodiments, the therapeutic polypeptides of interest, e.g. the PME, e.g., LAAD, PAH, and or PAL, are secreted via a Type V Auto-secreter (*pic* Protein) Secretion. In some embodiments, the therapeutic protein of interest is expressed as a fusion protein with the native Nissle auto-secreter E. coli_01635 (where the original passenger protein is replaced with the therapeutic polypeptides of interest.

[0433] In some embodiments, the therapeutic polypeptides of interest, e.g., the PME, e.g., LAAD, PAH, and or PAL, are secreted via Type I Hemolysin Secretion. In one embodiment, therapeutic polypeptide of interest is expressed as fusion protein with the 53 amino acids of the C terminus of alpha-hemolysin (hlyA) of *E. coli* CFT073.

Oxygen consuming enzymes

[0434] LAAD catalytic activity is dependent on oxygen, and therefore may not be active in anaerobic and/or low oxygen environments in the intestine, e.g., the colon. Oxygen is present in more proximal compartments of the GI tract.

[0435] 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)

[0436] 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. In some embodiments, PAL is expressed under the conditions found in the small intestine, e.g. under the control of an FNR promoter, constitutive promoter, or a different inducible promoter described herein. In some embodiments, PAL and/or LAAD are pre-induced prior to *in vivo* administration, and are expressed and active in the proximal part of the intestine. In some embodiments, PAL and/or LAAD are pre-induced (aerobically or anaerobically, or with or without chemical and/or nutritional inducer, as described herein) prior to *in vivo* administration, and are expressed and active in the distal part of the intestine.

[0437] 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.

[0438] 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

[0439] 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).

[0440] 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		

[0441] **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

[0442] 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).

[0443] 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).

[0444] 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).

[0445] 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.

[0446] 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.

[0447] 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).

[0448] 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.

[0449] 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.

[0450] 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).

[0451] In some embodiments, the genetically engineered bacterium is a conditional auxotroph whose essential gene(s) is replaced using the arabinose system shown in **Figs. 85-86.**

[0452] 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). In one embodiment, a genetically engineered bacterium, comprises one or more biosafety constructs integrated into the bacterial chromosome in combination with one or more biosafety plasmid(s). In some embodiments, the plasmid comprises a conditional origin of replication (COR), for which the plasmid replication initiator protein is provided in trans, i.e., is encoded by the chromosomally integrated biosafety construct. In some embodiments, the chromosomally integrated construct is further introduced into the host such that an auxotrophy results (e.g., dapA or thyA auxotrophy), which in turn is complemented by a gene product expressed from the biosafety plasmid construct. In some embodiments, the biosafety plasmid further encodes a broad-spectrum toxin (e.g., Kis), while the integrated biosafety construct encodes an anti-toxin (e.g., anti-Kis), permitting propagation of the plasmid in the bacterial cell containing both constructs. Without wishing to be bound by theory, this mechanism functions to select against plasmid spread by making the plasmid DNA itself disadvantageous to maintain by a wild-type bacterium. A non-limiting example of such a biosafety system is shown in **Fig. 61A, Fig. 61B, Fig. 61C**, and **Fig. 61D**.

[0453] In some embodiments, the genetically engineered bacteria comprise a chromosomally inserted biosafety construct nucleic acid sequence (to be combined with a plasmid based biosafety construct) 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: 81, 82, 83, 84, 85, or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a chromosomally inserted biosafety construct nucleic acid sequence (to be combined with a plasmid based biosafety construct) encoding a polypeptide sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the polypeptide sequence of SEQ ID NO: 86, 87, 88, or a functional fragment thereof.

[0454] In some embodiments, the genetically engineered bacteria comprise a chromosome based biosafety construct nucleic acid sequence (to be combined with a plasmid based biosafety construct) 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: 89, 90, 91, 92, 93, 94 or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a chromosome based biosafety construct nucleic acid sequence (to be combined with a plasmid based biosafety construct) encoding a polypeptide sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the polypeptide sequence encoded by the DNA sequence of SEQ ID NO: 89, 90, 91, 92, 93, 94 or a functional fragment thereof.

[0455] In some embodiments, the genetically engineered bacteria comprise a plasmid based biosafety construct nucleic acid sequence (to be combined with a chromosome based biosafety construct) that comprises a payload construct for the catabolism of phenylalanine. In some embodiments, the plasmid based construct comprises one or more copies of PAL. In some embodiments, the plasmid based construct comprises one or more copies of PheP. In some embodiments, the plasmid based construct comprises one or more copies of LAAD. In some embodiments, the plasmid based construct comprises one or more copies of PAL and one or more copies of PheP. In some embodiments, the plasmid based construct comprises one or more copies of PAL and one or more copies of LAAD. In some embodiments, the plasmid based construct comprises one or more copies of LAAD and one or more copies of PheP. In some embodiments, the plasmid based construct comprises one or more copies of PAL and one or more copies of PheP and one or more copies of LAAD. In some embodiments, the phenylalanine catabolizing plasmid payload(s) (i.e., PAL, PheP, and/or LAAD) are under the control of one or more constitutive or inducible promoter(s) as described herein (e.g., low oxygen, arabinose, IPTG inducible, or a combination thereof). In some embodiments, the promoter is useful for pre-induction. In some embodiments, the promoter is useful for *in vivo* activation. In some embodiments, the promoter is useful for pre-induction and *in vivo* activity. In some embodiments, the construct comprises two or more promoters, some of which are useful for pre0induction, and some of which are useful for *in vivo* activity.

[0456] In some embodiments, the genetically engineered bacteria comprise a plasmid based biosafety construct payload nucleic acid sequence 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: 36, 37, 74, 95, 96, 98, 99, 100, 113 or a functional fragment thereof. In some embodiments, the genetically engineered bacteria comprise a plasmid based biosafety construct payload nucleic acid sequence encoding a polypeptide which is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% homologous to the polypeptide encoded by the DNA sequence of SEQ ID NO: 36, 37, 74, 95, 96, 98, 99, 100, 113 or a functional fragment thereof.

[0457] 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.

Genetic Regulatory Circuits

[0458] 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.

[0459] 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.

[0460] 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.

[0461] 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.

[0462] 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).

[0463] 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.

[0464] 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.

[0465] 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. 82**).

[0466] 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. 83**).

[0467] 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

[0468] 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.

[0469] 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

[0470] 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.

[0471] 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).

[0472] 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.

[0473] 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.

[0474] 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.

[0475] 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.

[0476] 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.

[0477] 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.

[0478] 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.

[0479] 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.

[0480] 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.

[0481] 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.

[0482] 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.

[0483] 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.

[0484] 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.

[0485] 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.

[0486] 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.

[0487] 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.

[0488] 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.

[0489] 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.

[0490] 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.

[0491] 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.

[0492] 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.

[0493] 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.

[0494] 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.

[0495] 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.

[0496] 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

[0497] 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.

[0498] 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.

[0499] 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.

[0500] 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.

[0501] 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.

[0502] 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.

[0503] 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.

[0504] 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.

[0505] 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.

[0506] 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.

[0507] 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.

[0508] 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.

[0509] 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.

[0510] 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.

[0511] 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.

[0512] 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.

[0513] 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).

[0514] 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.

[0515] 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.

[0516] 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.

[0517] 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.

[0518] 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.

[0519] 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

[0520] Another aspect of the disclosure provides methods of treating a disease associated with hyperphenylalaninemia or symptom(s) associated with hyperphenylalaninemia. In some embodiments, the disclosure provides a method for treating a disease associated with hyperphenylalaninemia or symptom(s) associated with hyperphenylalaninemia comprising administering to a subject in need thereof a composition comprising an engineered bacteria disclosed herein. In some embodiments, the disclosure provides a method for treating a disease associated with hyperphenylalaninemia or symptom(s) associated with hyperphenylalaninemia comprising administering to a subject in need thereof a composition comprising an engineered bacteria comprising gene sequence encoding one or PMEs, e.g., PAH and/or PAH, and/or LAAD. In some embodiments, the disclosure provides a method for treating a disease associated with hyperphenylalaninemia or symptom(s) associated with hyperphenylalaninemia comprising administering to a subject in need thereof a composition comprising an engineered bacteria comprising gene sequence encoding one or more PMEs, e.g., PAH and/or PAH, and/or LAAD and gene sequence encoding one or more Phe transporters. In some embodiments, the disclosure provides a method for treating a disease associated with hyperphenylalaninemia or symptom(s) associated with hyperphenylalaninemia comprising administering to a subject in need thereof a composition comprising an engineered bacteria comprising gene sequence encoding one or more PMEs, e.g., PAH and/or PAH, and/or LAAD and optionally gene sequence

encoding one or more Phe transporters, wherein the gene sequence(s) encoding the one or more PMES are under the control of an inducible promoter, such as any of the inducible promoters disclosed herein. In some embodiments, the disclosure provides a method for treating a disease associated with hyperphenylalaninemia or symptom(s) associated with hyperphenylalaninemia comprising administering to a subject in need thereof a composition comprising an engineered bacteria comprising gene sequence encoding one or more PMEs, e.g., PAH and/or PAH, and/or LAAD and optionally gene sequence encoding one or more Phe transporters, wherein the gene sequence(s) encoding the one or more PMES are under the control of an inducible promoter, and the gene sequence encoding the one or more Phe transporters are under the control of an inducible promoter, such as any of the inducible promoters disclosed herein. The gene sequence(s) may be under the control of the same or different inducible promoters. In some embodiments, one or more of the gene sequence encoding the one or more PMEs, e.g., PAH and/or PAH, and/or LAAD are under the control of constitutive promoter. In some embodiments, one or more of the gene sequence encoding the one or more Phe transporters are under the control of constitutive promoter. In other embodiments, the bacteria may comprise one or more of the following: one or more auxotrophies, one or more kill-switches, gene guard components, and/or antibiotic resistance.

[0521] 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.

[0522] 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.

[0523] 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.

[0524] 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.

[0525] 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.

[0526] Hippurate 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 described herein may include administration of the compositions of the invention to reduce phenylalanine and resulting in increased levels of hippurate production. In some embodiments, the methods may include administration of the compositions of the invention to reduce phenylalanine to undetectable levels in a subject, and concurrently and proportionately increase hippurate levels, e.g., in the urine. In some embodiments, the methods may include administration of the compositions of the invention, leading to

an increase hippurate concentrations to more than about 1%, 2%, 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or up to 99% or up to 100% of the subject's urine hippurate levels prior to treatment.

[0527] In some embodiments, the activity of genetically engineered bacteria expressing PAL (e.g., phenylalanine degrading activity) can be detected in the urine of a mammalian subject, e.g., an animal model or a human, by measuring the amounts of hippurate produced and the rate of its accumulation. Hippurate is a PAL specific breakdown product, and is normally present in human urine at low concentrations. It is the end product of metabolism of phenylalanine via the PAL pathway. Phenylalanine ammonia lyase mediates the conversion of phenylalanine to cinnamate. When cinnamate is produced in the gut, is absorbed and quickly converted to hippurate in the liver and excreted in the liver (Hoskins JA and Gray Phenylalanine ammonia lyase in the management of phenylketonuria: the relationship between ingested cinnamate and urinary hippurate in humans. J Res Commun Chem Pathol Pharmacol. 1982 Feb;35(2):275-82). Phenylalainine is converted to hippurate in a 1:1 ratio, i.e., 1 mole of Phe is converted into 1 mol of hippurate. Thus, changes in urinary hippurate levels can be used as a non-invasive measure of the effect of therapies that utilize this mechanism.

[0528] Hippuric acid thus has the potential to function as a biomarker allowing monitoring of dietary adherence and treatment effect in patients receiving PAL-based regimens. It can be used as an adjunct to measurement of blood Phe levels in the management of patients and because it is a urinary biomarker, it can have advantages particularly in children to adjust protein intake- which can be challenging as needs vary based on growth.

[0529] In some embodiments, the methods of the present disclosure increase the hippurate levels in the urine of a subject by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more as compared to levels in an untreated or control subject. In some embodiments, the increase is measured by comparing the hippurate level in a subject before and after administration of the pharmaceutical composition of the disclosure.

[0530] In this section, the term "PAL-based drug" refers to any drug, polypeptide, biologic, or treatment regimen that has PAL activity, for example, PEG-PAL, Kuvan, a composition comprising a bacteria of the present disclosure, e.g.,

bacteria encoding PAL and optionally PheP transporter. In some embodiments, the disclosure provides a method for measuring PAL activity in vivo by administering to a subject, e.g., a mammalian subject, a PAL-based drug and measuring the amount of hippurate produced in the subject as a measure of PAL activity. In some embodiments, the disclosure provides a method for monitoring the therapeutic activity of a PAL-based drug by administering to a subject, e.g., a mammalian subject, the PAL-based drug and measuring the amount of hippurate produced in the subject as a measure of PAL therapeutic activity. In some embodiments, the disclosure provides a method for adjusting the dosage of a PAL-based drug by administering to a subject, e.g., a mammalian subject, the PAL-based drug, measuring the amount of hippurate produced in the subject to determine PAL activity, and adjusting (e.g., increasing or decreasing) the dosage of the drug to increase or decrease the PAL activity in the subject. In some embodiments, the disclosure provides a method for adjusting the protein intake and/or diet of a subject having hyperphenylalanemia comprising administering to the subject a PAL-based drug, measuring the amount of hippurate produced in the subject, and adjusting (e.g., increasing or decreasing) the protein intake or otherwise adjusting the diet of the subject to increase or decrease the PAL activity in the subject. In some embodiments, the disclosure provides a method for confirming adherence to a protein intake and/or diet regimen of a subject having hyperphenylalanemia comprising administering to the subject a PAL-based drug, measuring the amount of hippurate produced in the subject, and measuring PAL activity in the subject.

[0531] In some embodiments of the methods disclosed herein, both blood phenylalanine levels and urine hippurate levels are monitored in a subject. In some embodiments, blood phenylalanine and hippurate in the urine are measured at multiple time points, to determine the rate of phenylalanine breakdown. In some embodiments, hippurate levels in the urine are used evaluate PAL activity or strain activity in animal models.

[0532] In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used to the strain prove mechanism of action. In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used as a tool to

differentiate between PAL and LAAD activity in a strain, and allow to determine the contribution of each enzyme to the overall strain activity.

[0533] [095] In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used evaluate safety in animal models and human subjects. In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used in the evaluation of dose-response and optimal regimen for the desired pharmacologic effect and safety. In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used as surrogate endpoint for efficacy and/or toxicity. In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used to predict patients' response to a regimen comprising a therapeutic strain. In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used for the identification of certain patient populations that are more likely to respond to the drug therapy. In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used to avoid specific adverse events. In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are useful for patient selection.

[0534] In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used as one method for adjusting protein intake/diet of PKU patient on a regimen which includes the administration of a therapeutic PKU strain expressing PAL.

[0535] In some embodiments, measurement of urine levels of hippuric acid, alone or in combination with blood phenylalanine measurements, is used to measure and/or monitor the activity of recombinant PAL. In some embodiments, measurement of urine levels of hippuric acid is used to measure and/or monitor the activity of recombinant pegylated PAL (Peg-PAL). In some embodiments, measurement of urine levels of hippuric acid, alone or in combination with blood phenylalanine measurements, is used to measure and/or monitor the activity of recombinant PAL administered in combination with a therapeutic strain as described herein.

[0536] In some embodiments, hippuric acid measurements in the urine, alone or in combination with blood phenylalanine measurements, are used in combination with other biomarkers, e.g., clinical safety biomarkers. Non-limiting examples of such safety markers include physical examination, vital signs, and electrocardiogram (ECG). Other non-limiting examples include liver safety tests known in the art, e.g., serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and bilirubin. Such biosafety markers also include renal safety tests, e.g., those known in the art, e.g., blood urea nitrogen (BUN), serum creatinine, glomerular filtration rate (GFR), creatinine clearance, serum electrolytes (sodium, potassium, chloride, and bicarbonate), and complete urine analysis (color, pH, specific gravity, glucose, proteins, ketone bodies, and microscopic exam for blood, leukocytes, casts), as well as Cystatin-c, β 2-microglobulin, uric acid, clusterin, N-acetyl-beta-dglucosaminidase, neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-β-dglucosaminidase (NAG), and kidney injury molecule-1 (KIM-1). Other non-limiting examples include Hematology safety biomarkers known in the art, e.g., Complete blood count, total hemoglobin, hematocrit, red cell count, mean red cell volume, mean cell hemoglobin, red cell distribution width%, mean cell hemoglobin concentration, total white cell count, differential white cell count (Neutrophils, lymphocytes, basophils, esinophils, and monocytes), and platelets. Other no-liming examples include bone safety markers known in the art, e.g., Serum calcium and inorganic phosphates. Other non-limiting examples include basic metabolic safety biomarkers known in the art, e.g., blood glucose, triglycerides (TG), total cholesterol, low density lipoprotein cholesterol (LDLc), and high density lipoprotein cholesterol (HDL-c). Other specific safety biomarkers known in the art include, e.g., serum immunoglobulin levels, C-reactive protein (CRP), fibrinogen, thyroid stimulating hormone (TSH), thyroxine, testosterone, insulin, lactate dehydrogenase (LDH), creatine kinase (CK) and its isoenzymes, cardiac troponin (cTn), and methemoglobin.

[0537] In some embodiments, the activity of 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.

[0538] 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.

[0539] In some embodiments, quantitative PCR (qPCR) is used to amplify, detect, and/or quantify mRNA expression levels of the gene, gene(s), or gene cassettes for producing the payloads, e.g., PME(s) and/or PheP. Primers may be designed and used to detect mRNA in a sample according to methods known in the art. In some embodiments, a fluorophore is added to a sample reaction mixture that may contain payload RNA, and a thermal cycler is used to illuminate the sample reaction mixture with a specific wavelength of light and detect the subsequent emission by the fluorophore. The reaction mixture is heated and cooled to predetermined temperatures for predetermined time periods. In certain embodiments, the heating and cooling is repeated for a predetermined number of cycles. In some embodiments, the reaction mixture is heated and cooled to 90-100° C, 60-70° C, and 30-50° C for a predetermined number of cycles. In a certain embodiment, the reaction mixture is heated and cooled to 93-97° C, 55-65° C, and 35-45° C for a predetermined number of cycles. In some embodiments, the accumulating amplicon is quantified after each cycle of the qPCR. The number of cycles at which fluorescence exceeds the threshold is the threshold cycle (CT). At least one CT result for each sample is generated, and the CT result(s) may be used to determine mRNA expression levels of the payload(s).

[0540] In some embodiments, quantitative PCR (qPCR) is used to amplify, detect, and/or quantify mRNA expression levels of the payload(s). Primers may be designed and used to detect mRNA in a sample according to methods known in the art. In some embodiments, a fluorophore is added to a sample reaction mixture that may contain payload, payloads, e.g., PME(s) and/or PheP and/or FNRS24Y, mRNA, and a thermal cycler is used to illuminate the sample reaction mixture with a specific wavelength of light and detect the subsequent emission by the fluorophore. The reaction mixture is heated and cooled to predetermined temperatures for predetermined time periods. In certain embodiments, the heating and cooling is repeated for a predetermined number of cycles. In some embodiments, the reaction mixture is heated and cooled to 90-100° C, 60-70° C, and 30-50° C for a predetermined number of cycles. In a certain embodiment, the reaction mixture is heated and cooled to 93-97° C, 55-65° C, and 35-45° C for a predetermined number of cycles. In some embodiments, the accumulating amplicon is quantified after each cycle of the qPCR. The number of cycles at which fluorescence exceeds the threshold is the threshold cycle (CT). At least one CT result for each sample is generated, and the CT result(s) may be used to determine mRNA expression levels of the payload(s) e.g., PME(s) and/or PheP and/or FNRS24Y.

[0541] 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. 68**. In alternate embodiments, the genetically engineered bacteria are not destroyed within hours or days after administration and may propagate and colonize the gut.

[0542] 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).

[0543] 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.

[0544] 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.

[0545] 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.

[0546] 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.

[0547] In some embodiments, the one or more PME administered is PAL. In some embodiments, PAL is modified as described in Sarkissian 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.

[0548] 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.

[0549] 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.

[0550] 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.

[0551] 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.

[0552] In some embodiments, the PME, e.g., PAH, PAL, and/or LAAD is formulated with aprotinin, e.g., 40 mg/ml aprotinin.

[0553] 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.

[0554] 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.

[0555] 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.

[0556] 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.

[0557] 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 ($\mu mol/10^9$ 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 ($\mu mol/10^9$ 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 ($\mu mol/10^9$ 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

[0558] 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.

[0559] 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.

[0560] 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.

[0561] 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.

[0562] 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.

[0563] 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*

[0564] 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.

[0565] The bacteria can be administered to the animal as a bolus, once or more per day, once or more per week, e.g., as described in the examples. Alternatively, a more chronic administration may be desirable, e.g., by providing bacteria in the drinking water. The bacteria can be provided in the drinking water with 0.125% gelatin as a carrier to keep the bacteria in suspension, e.g., as described in the Examples (Example 44 and 45), and as described in the art (for example in Chen et al., Incorporation of therapeutically modified bacteria into gut microbiota inhibits obesity J Clin Invest. 2014;124(8):3391–3406).

[0566] 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.

Screening Methods

[0567] 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

[0568] 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.

[0569] 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.

[0570] 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.

[0571] 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.

[0572] 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.

[0573] 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.

[0574] 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.

[0575] 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).

[0576] In some embodiments, the ALE method can be used to identify genetically engineered bacteria with improved phenylalanine uptake.

Specific Screen to improve PME activity

[0577] 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.

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Examples

[0578] 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

[0579] 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*

[0580] 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

[0581] 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.*

[0582] 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.

[0583] 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

[0584] 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

[0585] To determine the effect of the phenylalanine transporter on phenylalanine degradation,

[0586] 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.

[0587] 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.

[0588] 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 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 a high-copy *PAL*-expressing plasmid.

Example 6. FNR promoter activity

[0589] 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.**

[0590] **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.

[0591] **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.

[0592] **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.

[0593] **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.

[0594] **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.

[0595] **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.

[0596] **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.

[0597] **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**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatcgtcgtccggccttttcctc tcttactctgctacgtacatctatttctataaatccgttcaatttgtctgtttttttgcacaaacatgaaatatcagacaattcc gtgacttaagaaaatttatacaaatcagcaatatacccctaaggagtatataaaggtgaatttgatttacatcaataag cggggttgctgaatcgttaaggtaggcggtaatagaaaagaaatcgaggcaaaaATGagcaaagtcagactcgc aattat**GGATCCTCTGGCCGTCGTATTACAACGTCGTGACTGGGAAAACCCTGG CGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGCGT AATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTG AATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAA AGCTGGCTGGAGTGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAA ACTGGCAGATGCACGGTTACGATGCGCCTATCTACACCAACGTGACCTATCC CATTACGGTCAATCCGCCGTTTGTTCCCGCGGAGAATCCGACAGGTTGTTAC TCGCTCACATTTAATATTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGA ATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCT GGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGCGC ATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAG TGACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTT CCGTGACGTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAA GTTACCACTCTCTTTAATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAG TTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGGTGACGGTTTCTTTGTG GCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAAT TATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTT GAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTG GTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGAC GTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGC AAGCCGTTGCTGATTCGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATG GTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGC AGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTG GTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATT GAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGG CTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAAT CACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCT AATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGG TACAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCC

Nucleotide sequences of Pfnr1-lacZ construct, low-copy (SEQ ID NO: 31)
CGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAAT GGTCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCT TTGCGAATATGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATAC TGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACT GGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGG CTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAA CGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAGCAAA ACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTG ACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGG TGGCACTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTG GCCCGCAAGGTAAGCAGTTGATTGAACTGCCTGAACTGCCGCAGCCGGAGA GCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCAACCAAACGCGACCG CATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTCTGGCGG AAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAACTGAC CACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTT AACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAA CTGCTGACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACA TTGGCGTAAGTGAAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCT GGAAGGCGGCGGGCCATTACCAGGCCGAAGCGGCGTTGTTGCAGTGCACGG CAGATACACTTGCCGACGCGGTGCTGATTACAACCGCCCACGCGTGGCAGC ATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGGC ACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGC ATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGG TAAACTGGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTG CAGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTA CGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCGAATTGAATTA TGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCGCTACAG CCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGA AGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGA CTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTAC CATTACCAGTTGGTCTGGTGTCAAAAATAA

Table 22

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 32)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctcatgcatgcatcaaaaaag atgtgagcttgatcaaaaacaaaaaatatttcactcgacaggagtatttatattgcgcccgttacgtgggcttcgactgt aaatc**agaaaggagaaaacacct**ATGacgacctacgatcg**GGATCCTCTGGCCGTCGTATTAC AACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCGGC ACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCG CCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTT CCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAC GCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGC CTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCC

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 32)
CGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGATGAAAGC
TGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGT
TTCATCTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGCCGTTT
GCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTC
GCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAGGAT
ATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGA
CCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGATGATTTCAG
CCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTGCGCGATGA
ACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGG
CACCGCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGTTATGCCGAT
CGCGTCACACTACGCCTGAACGTTGAAAATCCGGAACTGTGGAGCGCCGAA
ATCCCGAATCTCTATCGTGCAGTGGTTGAACTGCACACCGCCGACGGCACGC
TGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCGAGGTGCGGATTGAAA
ATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCG
TCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGT
GCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTC
GCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGCTACGGCCTG
TATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCAATGAAT
CGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGAGCGAACGCGTAACG
CGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTG
GGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATC
AAATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCGGAGCCGAC
ACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGGATGAAGAC
CAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCTGC
CTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGCGATGGGTA
ACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAGTACCCCCG
TTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATAT
GATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACG
CCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGC
CGCATCCGGCGCTGACGGAAGCAAAACACCAACAGCAGTATTTCCAGTTCC
GTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTTCCGTCATAG
CGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAGCCGCTGGC
AAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAGTTGATTGA
ACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGGCTAACGGT
ACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGACACATCAG
CGCCTGGCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACACTCCCCTC
CGCGTCCCACGCCATCCCTCAACTGACCACCAGCGGAACGGATTTTTGCATC
GAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTTCAC
AGATGTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGCGCGATCAGT
TCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCA
TTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGG
CCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGACGCGGTGC
TGATTACAACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCA
GCCGGAAAACCTACCGGATTGATGGGCACGGTGAGATGGTCATCAATGTGG
ATGTTGCGGTGGCAAGCGATACACCGCATCCGGCGCGGATTGGCCTGACCT
GCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTCGGCCTGGGGCCGC
AAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCTGGGATCT

Nucleotide sequences of Pfnr2-lacZ construct, low-copy (SEQ ID NO: 32)
GCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTG CGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGAC TTCCAGTTCAACATCAGCCGCTACAGCCAACAACAACTGATGGAAACCAGC CATCGCCATCTGCTGCACGCGGAAGAAGGCACATGGCTGAATATCGACGGT TTCCATATGGGGATTGGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCG GAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAAA AATAA

Table 23

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 33)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatcgtcgtccggccttttcctc tcttactctgctacgtacatctatttctataaatccgttcaatttgtctgtttttgcacaaacatgaaatatcagacaattcc gtgacttaagaaaatttatacaaatcagcaatatacccctaaggagtatataaaggtgaatttgatttacatcaataag cggggttgctgaatcgttaa**GGATCC**ctctagaaataattttgtttaactttaagaaggagatatacat**ATG**AC TATGATTACGGATTCTCTGGCCGTCGTATTACAACGTCGTGACTGGGAAAAC CCTGGCGTTACCCAACTTAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCT GGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCA GCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGC CGGAAAGCTGGCTGGAGTGCGATCTTCCTGACGCCGATACTGTCGTCGTCCC CTCAAACTGGCAGATGCACGGTTACGATGCGCCTATCTACACCAACGTGAC CTATCCCATTACGGTCAATCCGCCGTTTGTTCCCGCGGAGAATCCGACAGGT TGTTACTCGCTCACATTTAATATTGATGAAAGCTGGCTACAGGAAGGCCAGA CGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGG GCGCTGGGTCGGTTACGGCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTG AGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGC TGGAGTGACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGC ATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACCACGCAAATCAGCGATT TCCAAGTTACCACTCTCTTTAATGATGATTTCAGCCGCGCGGTACTGGAGGC AGAAGTTCAGATGTACGGCGAGCTGCGCGATGAACTGCGGGTGACGGTTTC TTTGTGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGG TGAAATTATCGATGAGCGTGGCGGTTATGCCGATCGCGTCACACTACGCCTG AACGTTGAAAATCCGGAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGT GCAGTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCC TGCGACGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGA ACGGCAAGCCGTTGCTGATTCGCGGCGTTAACCGTCACGAGCATCATCCTCT GCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGAT GAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCG CTGTGGTACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCC AATATTGAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGATGATCCG CGCTGGCTACCCGCGATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGAT CGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCAC GGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCC GCCCGGTACAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTA TTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCC

Nucleotide sequences of Pfnr3-lacZ construct, low-copy (SEQ ID NO: 33)
GAAATGGTCCATCAAAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCT GATCCTTTGCGAATATGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCT AAATACTGGCAGGCGTTTCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCT GGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGT GGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTG TATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGA AGCAAAACACCAACAGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATC GAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACT GGATGGTGGCACTGGATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGG ATGTTGGCCCGCAAGGTAAGCAGTTGATTGAACTGCCTGAACTGCCGCAGC CGGAGAGCGCCGGACAACTCTGGCTAACGGTACGCGTAGTGCAACCAAACG CGACCGCATGGTCAGAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTC TGGCGGAAAACCTCAGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCA ACTGACCACCAGCGGAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTG GCAATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAA AAACAACTGCTGACCCCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGAT AACGACATTGGCGTAAGTGAAGCGACCCGCATTGACCCTAACGCCTGGGTC GAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCGGCGTTGTTGCAG TGCACGGCAGATACACTTGCCGACGCGGTGCTGATTACAACCGCCCACGCG TGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATT GATGGGCACGGTGAGATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGAT ACACCGCATCCGGCGCGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCA GAGCGGGTAAACTGGCTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGC CTTACTGCAGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGACATGTATA CCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCGAAT TGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCC GCTACAGCCAACAACAACTGATGGAAACCAGCCATCGCCATCTGCTGCACG CGGAAGAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTG GCGACGACTCCTGGAGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCG GTCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAA

Table 24

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 34)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctcatgcatgcatcaaaaaag** **atgtgagcttgatcaaaaacaaaaaatatttcactcgacaggagtatttatattgcgccc**GGATCC**ctctagaaat** **aattttgtttaactttaagaaggagatatacatATG**ACTATGATTACGGATTCTCTGGCCGTCG TATTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCT TGCGGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCAC CGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCC TGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTT CCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACG ATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTT TGTTCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTTAATATTGAT GAAAGCTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAAC

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 34)
TCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTCGGTTACGGCCAGGAC
AGCCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAA
ACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAG
ATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCA
TAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCTTTAATGAT
GATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACGGCGAGCTG
CGCGATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAACGCAGGTC
GCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGCGGT
TATGCCGATCGCGTCACACTACGCCTGAACGTTGAAAATCCGGAACTGTGG
AGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGCACACCGCC
GACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCCGCGAGGTG
CGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGCG
GCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATGGATGAGCA
GACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGT
GCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGACCGC
TACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTG
CCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCGATGAGCGAA
CGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATC
TGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTAT
CGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGTATGAAGGCGGCG
GAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGG
ATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCAAAAAATGGC
TTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATATGCCCACGC
GATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGCGTTTCGTCAG
TACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGA
TTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTG
GCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGA
CCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAACAGCAGTATTT
CCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGAATACCTGTTC
CGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTGGATGGCAAG
CCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAAGGTAAGCAG
TTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGACAACTCTGG
CTAACGGTACGCGTAGTGCAACCAAACGCGACCGCATGGTCAGAAGCCGGA
CACATCAGCGCCTGGCAGCAATGGCGTCTGGCGGAAAACCTCAGCGTGACA
CTCCCCTCCGCGTCCCACGCCATCCCTCAACTGACCACCAGCGGAACGGATT
TTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTT
TCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTGACCCCGCTGCGC
GATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTAAGTGAAGCG
ACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCAT
TACCAGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACACTTGCCGAC
GCGGTGCTGATTACAACCGCCCACGCGTGGCAGCATCAGGGGAAAACCTTA
TTTATCAGCCGGAAAACCTACCGGATTGATGGGCACGGTGAGATGGTCATC
AATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCCGGCGCGGATTGGC
CTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGGCTCGGCCTG
GGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTTTTGACCGCT
GGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAA
CGGTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCG

Nucleotide sequences of Pfnr4-lacZ construct, low-copy (SEQ ID NO: 34)
CGGCGACTTCCAGTTCAACATCAGCCGCTACAGCCAACAACAACTGATGGA AACCAGCCATCGCCATCTGCTGCACGCGGAAGAAGGCACATGGCTGAATAT CGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGCCCGTCAGT ATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGG TGTCAAAAATAA

Table 25

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 35)
GGTACC**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgtaacaaaagcaatttttccgg ctgtctgtatacaaaaacgccgtaaagtttgagcgaagtcaataaactctctacccattcagggcaatatctctctt**GG ATCC**ctctagaaataattttgtttaactttaagaaggagatatacatATG**CTATGATTACGGATTCTC TGGCCGTCGTATTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACT TAATCGCCTTGCGGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAG GCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGG CGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAG TGCGATCTTCCTGACGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGC ACGGTTACGATGCGCCTATCTACACCAACGTGACCTATCCCATTACGGTCAA TCCGCCGTTTGTTCCCGCGGAGAATCCGACAGGTTGTTACTCGCTCACATTT AATATTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGAT GGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTCGGTTACG GCCAGGACAGCCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGC CGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTA TCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCG TTGCTGCATAAACCGACCACGCAAATCAGCGATTTCCAAGTTACCACTCTCT TTAATGATGATTTCAGCCGCGCGGTACTGGAGGCAGAAGTTCAGATGTACG GCGAGCTGCGCGATGAACTGCGGGTGACGGTTTCTTTGTGGCAGGGTGAAA CGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGATGAGC GTGGCGGTTATGCCGATCGCGTCACACTACGCCTGAACGTTGAAAATCCGG AACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCAGTGGTTGAACTGC ACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGACGTCGGTTTCC GCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGC TGATTCGCGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCAT GGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTT TAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTG TGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCAC GGCATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCCGCG ATGAGCGAACGCGTAACGCGGATGGTGCAGCGCGATCGTAATCACCCGAGT GTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGAC GCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTACAGTATG AAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACG CGCGCGTGGATGAAGACCAGCCCTTCCCGGCGGTGCCGAAATGGTCCATCA AAAAATGGCTTTCGCTGCCTGGAGAAATGCGCCCGCTGATCCTTTGCGAATA TGCCCACGCGATGGGTAACAGTCTTGGCGGCTTCGCTAAATACTGGCAGGC GTTTCGTCAGTACCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGAT

Nucleotide sequences of Pfnrs-lacZ construct, low-copy (SEQ ID NO: 35)
CAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGC GGTGATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGG TCTTTGCCGACCGCACGCCGCATCCGGCGCTGACGGAAGCAAAACACCAAC AGCAGTATTTCCAGTTCCGTTTATCCGGGCGAACCATCGAAGTGACCAGCGA ATACCTGTTCCGTCATAGCGATAACGAGTTCCTGCACTGGATGGTGGCACTG GATGGCAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGGCCCGCAA GGTAAGCAGTTGATTGAACTGCCTGAACTGCCGCAGCCGGAGAGCGCCGGA CAACTCTGGCTAACGGTACGCGTAGTGCAACCAAACGCGACCGCATGGTCA GAAGCCGGACACATCAGCGCCTGGCAGCAATGGCGTCTGGCGGAAAACCTC AGCGTGACACTCCCCTCCGCGTCCCACGCCATCCCTCAACTGACCACCAGCG GAACGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGCCA GTCAGGCTTTCTTTCACAGATGTGGATTGGCGATGAAAAACAACTGCTGACC CCGCTGCGCGATCAGTTCACCCGTGCGCCGCTGGATAACGACATTGGCGTA AGTGAAGCGACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCG GCGGGCCATTACCAGGCCGAAGCGGCGTTGTTGCAGTGCACGGCAGATACA CTTGCCGACGCGGTGCTGATTACAACCGCCCACGCGTGGCAGCATCAGGGG AAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGGCACGGTGAG ATGGTCATCAATGTGGATGTTGCGGTGGCAAGCGATACACCGCATCCGGCG CGGATTGGCCTGACCTGCCAGCTGGCGCAGGTCTCAGAGCGGGTAAACTGG CTCGGCCTGGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCAGCCTGTT TTGACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCC GAGCGAAAACGGTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCACA CCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCGCTACAGCCAACAACA ACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGAAGGCACATG GCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAG CCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAG TTGGTCTGGTGTCAAAAATAA

Table 26

Nucleotide sequences of Pfnr3-PAL3 construct, low-copy (SEQ ID NO: 36)
GGTACC**gtcagcataacaccctgacctctcattaattgttcatgccgggcggcactatcgtcgtccggccttttcctc** **tcttactctgctacgtacatctatttctataaatccgttcaatttgtctgtttttttgcacaaacatgaaatatcagacaattcc** **gtgacttaagaaaatttatacaaatcagcaatatacccctttaaggagtatataaaggtgaatttgatttacatcaataag** **cggggttgctgaatcgttaa**GGATCC**ctctagaaataattttgtttaactttaagaaggagatatacat**ATGAA AGCTAAAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCT TTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAAATATCA ACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAAAA TTAAATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGAAT GCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGT TAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGTATTAAA GCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACCA GACCAATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGACATTGTTCC TCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTAT CTTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTATTATATGGGCGC

Nucleotide sequences of Pfnr3-PAL3 construct, low-copy (SEQ ID NO: 36)
AGAAATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTATC GTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTC AGGAATCAGTGCAATCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTC AATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCATCTCATGAACATT ATGATGCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGG CAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATC TGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCA ACTAAATGATACCTTACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGTA TTAGGTATAGTGCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGAACGG GAAGTTATCTCAGCTAATGATAATCCATTGATAGATCCAGAAAATGGCGAT GTTCTACACGGTGGAAATTTTATGGGGCAATATGTCGCCCGAACAATGGAT GCATTAAAACTGGATATTGCTTTAATTGCCAATCATCTTCACGCCATTGTGG CTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTAATTCACTGAGTCCG ACACCCGGCATGTATCAAGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTT TAGTTGCTGCAATTCGCCATGATTGTGCTGCATCAGGTATTCATACCCTCGC CACAGAACAATACAATCAAGATATTGTCAGTTTAGGTCTGCATGCCGCTCAA GATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGTTTCAATGACAATTC TGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATTAGTGAAATTGCGCC TGAAACTGCTAAATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTGATC ACTGATCGTGCGTTGGATGAAGATATAATCCGCATTGCGGATGCAATTATTA ATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAGAATAA

Table 27

Nucleotide sequences of Pfnr4-PAL3 construct, low-copy (SEQ ID NO: 37)
GGTACC**catttcctctcatcccatccggggtgagagtcttttcccccgacttatggctcatgcatgcatcaaaaaag atgtgagcttgatcaaaaacaaaaaatatttcactcgacaggagtatttatattgcgcccGGATCC<u>ctctagaaat aattttgtttaactttaagaaggagatatacat</u>ATG**AAAGCTAAAGATGTTCAGCCAACCATT ATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGA TAAAACAAAAAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTTGACGC ATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATG GAATCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGA AAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGG GGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACTT TCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTG TTGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTG GGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTG GTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAA TTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTG CTCTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCAT TAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTT GAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAAGTAA AAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGC AGGTTCAACGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAA AGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTT

Nucleotide sequences of Pfnr4-PAL3 construct, low-copy (SEQ ID NO: 37)
TATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAATCTTTAG CTACCGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATC CATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGG GGCAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATATTGCTTTAAT TGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATAACCGTTTCTCTC GTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAA AGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGATTGT GCTGCATCAGGTATTCATACCCTCGCCACAGAACAATACAATCAAGATATTG TCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATT ACGCAATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCATTCATCTTC GCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGT ACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGATATA ATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAATCA TGCTGGAAGAATAA

Table 28

Nucleotide sequences of PfnrS-PAL3 construct, low-copy (SEQ ID NO: 38)
GGTACC**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgtaacaaaagcaatttttccgg ctgtctgtatacaaaaacgccgtaaagtttgagcgaagtcaataaactctctacccattcagggcaatatctctctt**GG ATCC**ctctagaaataattttgtttaactttaagaaggagatatacatATG**AAAGCTAAAGATGTTCA GCCAACCATTATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTAT GACATTGCGATAAAACAAAAAAAAGTAGAAATATCAACGGAGATCACTGA ACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGA GGTTATATATGGAATCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTG CCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCTTTCTG CTGGTACTGGGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATTTAC TATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCT CAAGCAATTGTTGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTA TGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGA GCATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTG CTGAAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAG AAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAA TCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGC CCTTGCTGTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATT CAACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGT AATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGGGGTTAAAGAAC AAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATACCTT ACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCA GAATCTTTAGCTACCGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTA ATGATAATCCATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAA ATTTTATGGGGCAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATAT TGCTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATAACC GTTTCTCTCGTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCA AGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGC

Nucleotide sequences of PfnrS-PAL3 construct, low-copy (SEQ ID NO: 38)
CATGATTGTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAATACAATC AAGATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGA GCAGAAATTACGCAATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCC ATTCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAAATTTT ACCATGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGA TGAAGATATAATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCTG CCAGAAATCATGCTGGAAGAATAA

[0598] 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

[0599] 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*

[0600] 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

[0601] 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.

[0602] 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.

[0603] 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

[0604] 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.

[0605] 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-	**0.47**

	PKU523	

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

[0606] 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.

[0607] 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

[0608] 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.

[0609] 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.

[0610] 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.

[0611] **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.

[0612] 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)
*Ttaagacccactttcacatttaagttgtttttctaatccgcatatgatcaattcaaggccgaataagaaggctggctctgcacc ttggtgatcaaataattcgatagcttgtcgtaataatggcggcatactatcagtagtaggtgtttccctttcttctttagcgacttg atgctcttgatcttccaatacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaa ccttgttggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtacttttgctccat cgcgatgacttagtaaagcacatctaaaacttttagcgttattacgtaaaaaatcttgccagctttccccttctaaagggcaa aagtgagtatggtgcctatctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatgt aggctgctctacacctagcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgct gttaatcactttacttttatctaatctagacat*cattaattcctaatttttt**gttgacactctatcattgatagagttatttttaccactc cctatcagtgatagagaa**aagtgaa*ctctagaaataattttgtttaactttaagaaggagatatacat*atgaacatttcaagg agaaagctacttttaggtgttggtgctgcgggcgttttagcaggtggtgcggctttagttccaatggttcgccgtgacggcaaatt tgtggaagctaaatcaagagcatcatttgttgaaggtacgcaaggggctcttcctaaagaagcagatgtagtgattattggtgcc ggtattcaagggatcatgaccgctattaaccttgctgaacgtggtatgagtgtcactatcttagaaaagggtcagattgccggtg agcaatcaggccgtgcatacagccaaattattagttaccaaacatcgccagaaatcttcccattacaccattatgggaaaatatta tggcgtggcatgaatgagaaaattggtgcggataccagttatcgtactcaaggtcgtgtagaagcgctggcagatgaaaaagc attagataaagctcaagcgtggatcaaaacagctaaagaagcggcaggttttgatacaccattaaatactcgcatcattaaaggt gaagagctatcaaatcgcttagtcggtgctcaaacgccatggactgttgctgcatttgaagaagattcaggctctgttgatcctga aacaggcacacctgcactcgctcgttatgccaaacaaatcggtgtgaaaatttataccaactgtgcagtaagaggtattgaaact gcgggtggtaaaatctctgatgtggtgagtgagaaaggggcgattaaaacgtctcaagttgtactcgctgggggtatctggtcg cgtttatttatgggcaatatgggtattgatatcccaacgctcaatgtatatctatcacaacaacgtgtctcaggggttcctggtgca ccacgtggtaatgtgcatttacctaatggtattcatttccgcgaacaagcggatggtacttatgccgttgcaccacgtatctttacg agttcaatagtcaaagatagcttcctgctagggcctaaatttatgcacttattaggtggcggagagttaccgttggaattctctattg gtgaagatctatttaattcatttaaaatgccgacctcttggaatttagatgaaaaaacaccattcgaacaattccgagttgccacgg caacacaaaatacgcaacacttagatgctgttttccaaagaatgaaaacagaattcccagtatttgaaaaatcagaagttgttgaa cgttggggtgccgttgtgagtccaacatttgatgaattacctatcatttctgaggtcaaagaatacccaggcttagtgattaacac ggcaacagtgtggggtatgacagaaggcccggcagcgggtgaagtgaccgctgatattgtcatgggcaagaaacctgttatt gatccaacgccgtttagtttggatcgttttaagaagtaa*

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)

Nucleotide sequences of AraC-ARABAD promoter-LAAD construct (SEQ ID NO: 40)
Ttattcacaacctgccctaaactcgctcggactcgccccggtgcatttttaaatactcgcgagaaatagagttgatcgtcaaaa ccgacattgcgaccgacggtggcgataggcatccgggtggtgctcaaaagcagcttcgcctgactgatgcgctggtcctcgc gccagcttaatacgctaatccctaactgctggcggaacaaatgcgacagacgcgacggcgacaggcagacatgctgtgcga cgctggcgatatcaaaattactgtctgccaggtgatcgctgatgtactgacaagcctcgcgtacccgattatccatcggtggatg gagcgactcgttaatcgcttccatgcgccgcagtaacaattgctcaagcagatttatcgccagcaattccgaatagcgcccttcc ccttgtccggcattaatgatttgcccaaacaggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaaccggtattg gcaaatatcgacggccagttaagccattcatgccagtaggcgcgcggacgaaagtaaacccactggtgataccattcgtgag cctccggatgacgaccgtagtgatgaatctctccaggcgggaacagcaaaatatcacccggtcggcagacaaattctcgtcc ctgatttttcaccaccccctgaccgcgaatggtgagattgagaatataacctttcattcccagcggtcggtcgataaaaaaatcg agataaccgttggcctcaatcggcgttaaacccgccaccagatgggcgttaaacgagtatcccggcagcaggggatcattttg cgcttcagccatactttcatactcccgccattcagagaagaaaccaattgtccatattgcatcagacattgccgtcactgcgtctt ttactggctcttctcgctaacccaaccggtaaccccgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaa aacgcgtaacaaaagtgtctataatcacggcagaaaagtccacattgattatttgcacggcgtcacactttgctatgccatagcat ttttatccataagattagcggatccagcctgacgctttttttcgcaactctctactgtttctccatAc*ctctagaaataattttgtttaac* *tttaagaaggagatatacatatgaacatttcaaggagaaagctacttttaggtgttggtgctgcgggcgttttagcaggtgg* *tgcggctttagttccaatggttcgccgtgacggcaaatttgtggaagctaaatcaagagcatcatttgttgaaggtacgcaa* *ggggctcttcctaaagaagcagatgtagtgattattggtgccggtattcaagggatcatgaccgctattaaccttgctgaac* *gtggtatgagtgtcactatcttagaaaagggtcagattgccggtgagcaatcaggccgtgcatacagccaaattattagtta* *ccaaacatcgccagaaatcttcccattacaccattatgggaaaatattatggcgtggcatgaatgagaaaattggtgcgga* *taccagttatcgtactcaaggtcgtgtagaagcgctggcagatgaaaaagcattagataaagctcaagcgtggatcaaaa* *cagctaaagaagcggcaggttttgatacaccattaaatactcgcatcattaaaggtgaagagctatcaaatcgcttagtcg* *gtgctcaaacgccatggactgttgctgcatttgaagaagattcaggctctgttgatcctgaaacaggcacacctgcactcgc* *tcgttatgccaaacaaatcggtgtgaaaatttataccaactgtgcagtaagaggtattgaaactgcgggtggtaaaatctct* *gatgtggtgagtgagaaaggggcgattaaaacgtctcaagttgtactcgctgggggtatctggtcgcgtttatttatgggca* *atatgggtattgatatcccaacgctcaatgtatatctatcacaacaacgtgtctcaggggttcctggtgcaccacgtggtaat* *gtgcatttacctaatggtattcatttccgcgaacaagcggatggtacttatgccgttgcaccacgtatctttacgagttcaatag* *tcaaagatagcttcctgctagggcctaaatttatgcacttattaggtggcggagagttaccgttggaattctctattggtgaag* *atctatttaattcatttaaaatgccgacctcttggaatttagatgaaaaaacaccattcgaacaattccgagttgccacggca* *acacaaaatacgcaacacttagatgctgttttccaaagaatgaaaacagaattcccagtatttgaaaaatcagaagttgtt* *gaacgttggggtgccgttgtgagtccaacatttgatgaattacctatcatttctgaggtcaaagaatacccaggcttagtgatt* *aacacggcaacagtgtggggtatgacagaaggcccggcagcgggtgaagtgaccgctgatattgtcatgggcaagaa* *acctgttattgatccaacgccgtttagtttggatcgttttaagaagtaa*

Example 13. Efficacy of PAL-expressing Bacteria in aMouse Model Of PKU

[0613] 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.

[0614] 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.

[0615] **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

[0616] 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.

[0617] 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.

[0618] **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

[0619] 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.

[0620] 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.

[0621] **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)

[0622] 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*).

[0623] 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.

[0624] 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.

[0625] 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.

[0626] 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)

[0627] 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.

[0628] 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.

[0629] 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.

[0630] 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.

[0631] 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)

[0632] 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.

[0633] 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

[0634] 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.

[0635] 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%).

[0636] 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

[0637] 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.

[0638] 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*

[0639] 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. 70**, phenylalanine degradation rates decreased as pH of the assay buffer decreased in both strains, SYN-PKU302 (**Fig. 70A**) and SYN-PKU304 (**Fig. 70B**).

Example 21. Degradation of Dipeptides and Tripeptides

[0640] 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 **Fig. 36** and indicate that PKU strains were capable of degrading Phe rapidly even in the form of di- and tri-peptides.

Example 22. Engineering Bacterial Strains Using Chromosomal Insertions

[0641] 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**.

[0642] 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. 81**). **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.

[0643] 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.

[0644] 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).

[0645] 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.

[0646] 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 *malT* locus (**Fig. 82**). **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.

[0647] 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.**

83). [**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)
ggtaccAGTTGTTCTTATTGGTGGTGTTGCTTTATGGTTGCATCGTAGTA AATGGTTGTAACAAAAGCAATTTTTCCGGCTGTCTGTATACAAAAACG CCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAA TATCTCTCTTggatccaaagtgaactctagaaataattttgtttaactttaagaaggagatatacatATGAAA
GCTAAAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCT
TTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAAATATC
AACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAA
AATTAAATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGG
AATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAA
TCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGT
ATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGTCT
GCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGAC
ATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTA
ATTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTAT
TATATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTT
GACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACGGCA
CCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGAAAAA
CTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTT
GCATCTCATGAACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCC
TGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAA
CGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGT
CGTCATCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTC
AATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTAC
CGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCCAT
TGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGG

Nucleotide sequences of FNR promoter-PAL3-pheP construct (SEQ ID NO: 41)
CAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATATTGCTTTAATT GCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATAACCGTTTCTCT CGTGGATTACCTAATTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTT AAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCCATGAT TGTGCTGCATCAGGTATTCATACCCTCGCCACAGAACAATACAATCAAGA TATTGTCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCA GAAATTACGCAATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCAT TCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGCTAAATTTTA CCATGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGGA TGAAGATATAATCCGCATTGCGGATGCAATTATTAATGATCAACTTCCTCT GCCAGAAATCATGCTGGAAGAATAAaagaaggagatatacatatgAAAAACGCGTCA ACCGTATCGGAAGATACTGCGTCGAATCAAGAGCCGACGCTTCATCGCGG ATTACATAACCGTCATATTCAACTGATTGCGTTGGGTGGCGCAATTGGTAC TGGTCTGTTTCTTGGCATTGGCCCGGCGATTCAGATGGCGGGTCCGGCTGT ATTGCTGGGCTACGGCGTCGCCGGGATCATCGCTTTCCTGATTATGCGCCA GCTTGGCGAAATGGTGGTTGAGGAGCCGGTATCCGGTTCATTTGCCCACTT TGCCTATAAATACTGGGGACCGTTTGCGGGCTTCCTCTCTGGCTGGAACTA CTGGGTAATGTTCGTGCTGGTGGGAATGGCAGAGCTGACCGCTGCGGGCA TCTATATGCAGTACTGGTTCCCGGATGTTCCAACGTGGATTTGGGCTGCCG CCTTCTTTATTATCATCAACGCCGTTAACCTGGTGAACGTGCGCTTATATG GCGAAACCGAGTTCTGGTTTGCGTTGATTAAAGTGCTGGCAATCATCGGTA TGATCGGCTTTGGCCTGTGGCTGCTGTTTTCTGGTCACGGCGGCGAGAAAG CCAGTATCGACAACCTCTGGCGCTACGGTGGTTTCTTCGCCACCGGCTGGA ATGGGCTGATTTTGTCGCTGGCGGTAATTATGTTCTCCTTCGGCGGTCTGG AGCTGATTGGGATTACTGCCGCTGAAGCGCGCGATCCGGAAAAAAGCATT CCAAAAGCGGTAAATCAGGTGGTGTATCGCATCCTGCTGTTTTACATCGGT TCACTGGTGGTTTTACTGGCGCTCTATCCGTGGGTGGAAGTGAAATCCAAC AGTAGCCCGTTTGTGATGATTTTCCATAATCTCGACAGCAACGTGGTAGCT TCTGCGCTGAACTTCGTCATTCTGGTAGCATCGCTGTCAGTGTATAACAGC GGGGTTTACTCTAACAGCCGCATGCTGTTTGGCCTTTCTGTGCAGGGTAAT GCGCCGAAGTTTTTGACTCGCGTCAGCCGTCGCGGTGTGCCGATTAACTCG CTGATGCTTTCCGGAGCGATCACTTCGCTGGTGGTGTTAATCAACTATCTG CTGCCGCAAAAAGCGTTTGGTCTGCTGATGGCGCTGGTGGTAGCAACGCT GCTGTTGAACTGGATTATGATCTGTCTGGCGCATCTGCGTTTTCGTGCAGC GATGCGACGTCAGGGGCGTGAAACACAGTTTAAGGCGCTGCTCTATCCGT TCGGCAACTATCTCTGCATTGCCTTCCTCGGCATGATTTTGCTGCTGATGTG CACGATGGATGATATGCGCTTGTCAGCGATCCTGCTGCCGGTGTGGATTGT ATTCCTGTTTATGGCATTTAAAACGCTGCGTCGGAAAtaa

Table 45

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

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

atcgatgcaggtatcaataattgcattacgcatggtcggggttgcacgcagccacagttcttcataatcgctgctatcaacaat
ccagctaacatcaactgctgcgcttgcgctgctttcgctaactgcatctttggctgcctgcagggtgctcagtgcttcttgatat
gcaggggcaaaaaactgttctgccggaccatcataaacaccattctgacgatcacgcagcaggcgacccagatttttttcgg
cttcacgaactgcggcttttgcatacttttcatcttcgcttgcctgcggatgggtcagtgctgcccagcgatctgcaactgcaat
aacaaacggatcatccggttcgcttgctgctaattttgctgcccaacgaaatgcaacatattcttcaacgcttttacgtgcaaca
taggtcggtgccggacaaccacctttcacactgctacgccaacaacgataaccattaccgctatagctacagctaccaccac
aacccggacaacgcatacgaccgctcagcagatgtttgcgacgggtatcatgatcgctaccatccagcggaacaccaaca
ccatcttcacctttaacggctgcttttgcggcttcttgttcttcatcggtcaccagcggaggaccatgcataacgctaacacgttt
accttcaccgttataaaaggtcagacgacgctgtttaccatcctgacgacctgtggtctgccaacccgcatatgccggattct
gaatcatatcacgcacggtaactgcaatccacggaccaccggtcgggctcggaatttcacgggtattcattgcatgtgcggt
gcctgcatagctcagacgatcggtaaccggcagggtaaaaaccagacgggctgcttctgctttggtcagaccatcaggac
cacccgcatcttcatcatctgctgccagtttacgttcatcatattcatcaccctcttcatcactaacggtaaccagaacaacacg
cagaccatacggtgcacgggcattaacccattcaccattttcacgctgatgtgctttggtatcacgaacacgttcgctcagtttt
tctgcttcttcgcgtgcttcttctgcacgacgaatcagttcaccgcgatcacgtttattggtgctatccagaaccggacgaccg
gtatcttcatcccaaccaaacagcagacgacgaggcataccatcttccggttcgataattttcagaattgcaccggcaccacc
acgatcccaacgatccagacgataacaccacagtgcaccaacttcaccgctttccagggctttcagtgctttgctctgatcat
cacgtgctttacctttacgaaaacggcttgcgctaccaacttctttccaaacatgacgaacctgcatacccagcagtgctgca
actttacgacccagggtttcttgtgctgcaatgctaatttcttgtttacgacgctgacctgcaccatttgcacggcttttaactgctt
tgcttttacgacaaaacaggtcaatcagacctgcaggatccggaccggtttcggtggtcataccaggcatatgtatatctccttc
cttaaagttaaacaaaattatttctagagttcactttggatccAAGAGAGATATTGCCCTGAATGGGT
AGAGAGTTTATTGACTTCGCTCAAACTTTGCGGCGTTTTTGTATACAGACA
GCCGGAAAAATTGCTTTTGTTACAACCATTTACTACGATGCAACCATAAA
GCAACACCACCAATAAGAACAACTggtaccGGATATTCATATGGACCATGGC
AGCTAGCCCTGCAGGGTGCAC TCAGAAAATTATTTTAAATTTCCTCTTGT
CAGGCCGGAATAACTCCCTATAATGCGCCACCAC gagcgccggatcagggagtggac
ggcctgggagcgctacacgctgtggctgcggtcggtgcTTATTCTTCCAGCATGATTTCTGGCAG
AGGAAGTTGATCATTAATAATTGCATCCGCAATGCGGATTATATCTTCATC
CAACGCACGATCAGTGATCAAAGGAGAACTGATTTCGCGTACTGCATGGT
AAAATTTAGCAGTTTCAGGCGCAATTTCACTAATATTGCCGCGAAGATGA
ATGGCCTGACAAACTACCAGAATTGTCATTGAAACAATATTGCGTAATTTC
TGCTCCATCTCTAAAACATCTTGAGCGGCATGCAGACCTAAACTGACAAT
ATCTTGATTGTATTGTTCTGTGGCGAGGGTATGAATACCTGATGCAGCACA
ATCATGGCGAATTGCAGCAACTAAAGCGGTTTGAGAAAGTTGGACGCCTT
TAAAACCTTGATACATGCCGGGTGTCGGACTCAGTGAATTAGGTAATCCA
CGAGAGAAACGGTTATCCATCATAAGAGCCACAATGGCGTGAAGATGATT
GGCAATTAAAGCAATATCCAGTTTTAATGCATCCATTGTTCGGGCGACATA
TTGCCCCATAAAATTTCCACCGTGTAGAACATCGCCATTTTCTGGATCTAT
CAATGGATTATCATTAGCTGAGATAACTTCCCGTTCCAATATTTTCCGAGC
GGTAGCTAAAGATTCTGGCACTATACCTAATACTTGTGGTGCACAGCGAA
TTGAATAAACTTCCTGTAAGGTATCATTTAGTTGGGTAATTTCTTGATGAC
GACAAGCTTTATTGGCTTGTTCTTTAACCCCAGATAATAGATTAACCTGCG
TTGAACCTGCCAATAAATTACGCAATGCACTTGCCACCGCGTTTTGACCAG
GATGATTTTTTACTTGTTGAATCCGGGCATCATAATGTTCATGAGATGCAA
GTAATGCTTCAACAGCAAGGGCAATCGCAGAAATTGAGGCTTTAAATAGT
TTTTCCAGTTTAATGACGGTGATTGCACTGATTCCTGACATTACCCGGGTG

Nucleotide sequences of FNR promoter-Int5, rrnBUP-PAL3 construct (SEQ ID NO: 42)
CCGTTAATCAGAGCAAGACCTTCTTTGGCTTTTAACGATAATGGTGTCAAC CCTGCACGTTTAATTGCTTCAGCAGCGTCAATTTCTGCGCCCATATAATAA ACTTTGCCGATACCACATAATGCTCGTGCAATATAAGATAAAGGAATTAA ATCACCGCTTGCACCCACTGAGCCATAGCGAGGAACCAGAGGAACAATGT CATGATTAATATGATCAACAATTGCTTGAGCGACAATTGGTCTGGTTGCAG ACCAACCTTTGCAAACAGAAAGTAACATAGTAAATTGTGACGCTTTAATA CAAGGTTTGGACATATAGTCCCCAGTACCAGCAGAAAGAAAAGTTAACAG ATTTTGCTGATGCTCTGCGATTTTCTCAAATGGCACAACTAAATTGGCATT CCCTCCAAATCCTGTATTGATTCCATATATAACCTCTCCTGAATTTAATTTT TCCTCTAATTTTTCACGACCATGCGTCAAAAGTTCAGTGATCTCCGTTGAT ATTTCTACTTTTTTTTGTTTTATCGCAATGTCATAGATATCTTCCAAAGAGA TAAGGCCATTTTTATTAATAATAATGGTTGGCTGAACATCTTTAGCTTTCA Tatgtatatctccttcttaaagttaaacaaaattatttctagag**cagatcagggtgcgcaagttgtcaacgctcccaggaga gttatcgacttgcgtattaggg**

Table 46

Nucleotide sequences of FNR promoter-Int5, rrnBUP-T7 construct (SEQ ID NO: 43)
ttaggtacgggctgcccatttgattttaacgcgttcatcaccatcaaacggacgaccacgctggccttttgcaaccca aatttcatcgatgcaggtatcaataattgcattacgcatggtcggggttgcacgcagccacagttcttcataatcgctg ctatcaacaatccagctaacatcaactgctgcgcttgcgctgctttcgctaactgcatctttggctgcctgcagggtgc tcagtgcttcttgatatgcaggggcaaaaaactgttctgccggaccatcataaacaccattctgacgatcacgcagc aggcgacccagatttttttcggcttcacgaactgcggcttttgcatacttttcatcttcgcttgcctgcggatgggtcagt gctgcccagcgatctgcaactgcaataacaaacggatcatccggttcgcttgctgctaattttgctgcccaacgaaat gcaacatattcttcaacgcttttacgtgcaacataggtcggtgccggacaaccacctttcacactgctacgccaacaa cgataaccattaccgctatagctacagctaccaccacaacccggacaacgcatacgaccgctcagcagatgtttgc gacgggtatcatgatcgctaccatccagcggaacaccaacaccatcttcacctttaacggctgcttttgcggcttcttg ttcttcatcggtcaccagcggaggaccatgcataacgctaacacgtttaccttcaccgttataaaaggtcagacgac gctgtttaccatcctgacgacctgtggtctgccaacccgcatatgccggattctgaatcatatcacgcacggtaactg caatccacggaccaccggtcgggctcggaatttcacgggtattcattgcatgtgcggtgcctgcatagctcagacga tcggtaaccggcagggtaaaaaccagacgggctgcttctgctttggtcagaccatcaggaccacccgcatcttcatc atctgctgccagtttacgttcatcatattcatcaccctcttcatcactaacggtaaccagaacaacacgcagaccatac ggtgcacgggcattaacccattcaccattttcacgctgatgtgctttggtatcacgaacacgttcgctcagtttttctgc ttcttcgcgtgcttcttctgcacgacgaatcagttcaccgcgatcacgtttattggtgctatccagaaccggacgaccg gtatcttcatcccaaccaaacagcagacgacgaggcataccatcttccggttcgataattttcagaattgcaccggca ccaccacgatcccaacgatccagacgataacaccacagtgcaccaacttcaccgctttccagggctttcagtgctttg ctctgatcatcacgtgctttacctttacgaaaacggcttgcgctaccaacttctttccaaacatgacgaacctgcatac ccagcagtgctgcaactttacgacccagggtttcttgtgctgcaatgctaatttcttgtttacgacgctgacctgcacca tttgcacggcttttaactgctttgcttttacgacaaaacaggtcaatcagacctgcaggatccggaccggtttcggtgg tcataccaggcatatgtatatctccttcttaaagttaaacaaaattatttctagagttcactttggatccAAGAGAGAT ATTGCCCTGAATGGGTAGAGAGTTTATTGACTTCGCTCAAACTTTGCGGCG TTTTTGTATACAGACAGCCGGAAAAATTGCTTTTGTTACAACCATTTACTA CGATGCAACCATAAAGCAACACCACCAATAAGAACAACTggtaccGGATATT

Nucleotide sequences of FNR promoter-Int5, rrnBUP-T7 construct (SEQ ID NO: 43)
CATATGGACCATGGCAGCTAGCCCTGCAGGGTGCAC TCAGAAAATTATTT TAAATTTCCTCTTGTCAGGCCGGAATAACTCCCTATAATGCGCCACCAC **gagcgccggatcagggagtggacggcctgggagcgctacacgctgtggctgcggtcggtgc**ttacgcgaacgcgaagtccgactctaagatgtcacggaggttcaagttacctttagccggaagtgctggcattttgtccaattgagactcgtgcaactggtcagcgaactggtcgtagaaatcagccagtacatcacaagactcatatgtgtcaaccatagtttcgcgcactgctttgaacaggttcgcagcgtcagccggaatggtaccgaaggagtcgtgaatcagtgcaaaagattcgattccgtacttctcgtgtgcccacactacagtcttacgaaggtggctaccgtcttggctgtgtacaaagttaggagcgataccagactcctgtttgtgtgcatcaatctcgctatctttgttggtgttaatggtaggctgtaagcggaactgaccgaggaacatcaggttcaagcgcgtctgaataggcttcttgtattcctgccacacagggaaaccatcaggagttacccaatgcacagcgcaacgcttgcgaagaatctctccagtcttcttatctttgacctcagcagccagcagcttagcagcagacttaagccagttcattgcttcaaccgcagctaccaccgtcacgctcacagattcccaaatcagcttagccatgtatccagcagcctgattcggctgagtgaacatcagacccttgccggaatcaatagctggctgaatggtatcttccagcacttgttgacggaagccgaactctttggacccgtaagccagcgtcatgactgaacgcttagtcacactgcgagtaacaccgtaagccagccattgaccagccagtgccttagtgcccagcttgactttctcagagatttcaccagtgttctcatcggtcacggtaactacttcgttatcggtcccattgattgcgtctgcttgtagaatctcgttgactttcttagcaacaatccccgtagatgtcctgaacggtttcactaggaagcaagttaaccgcgcgaccacctacctcatctcggagcatcgcggagaagtgctggatgccagagcaagacccgtcaaacgccagcggaagggagcagttatagctcaggccgtggtgctgtaccccagcgtactcaaagcagaacgcaaggaagcagaacggagaatcttgctcagcccaccaagtgttctccagtggagacttagcgcaagccatgatgttctcgtggttttcctcaatgaacttgatgcgctcagggaacggaaccttatcgacacccgcacagtttgcaccgtggattttcagccagtagtaaccttccttaccgattggtttacctttcgccagcgtaagcagtcctttggtcatatcgttacctgcgggttgaacattgacacagcgtaaacacgaccgcgccagtccatgttgtaagggaaccagatggccttatggttagcaaacttattggcttgctcaagcatgaactcaaggctgatacggcgagacttgcgagccttgtccttgcggtacacagcagcggcagcacgtttccacgcggtgagagcctcaggattcatgtcgatgtcttccggtttcatcgggagttcttcacgctcaatcgcaggatgtcctcgaccggacaatgcttccacttggtgattacgttggcgaccgctaggactttcttgttgattttccatgcggtgtttgcgcaatgttaatcgctttgtacacctcaggcatgtaaacgtcttcgtagcgcatcagtgctttcttactgtgagtacgcaccagcgccagaggacgacgaccgttagcccaatagccaccaccagtaatgccagtccacggcttaggaggaactacgcaaggttggaacatcggagagatgccagccagcgcacctgcacgggttgcgatagcctcagcgtattcaggtgcgagttcgatagtctcagagtcttgacctactacgccagcattttggcggtgtaagctaaccattccggttgactcaatgagcatctcgatgcagcgtactcctacatgaatagagtcttccttatgccacgaagaccacgcctcgccaccgagtagacccttagagagcatgtcagcctcgacaacttgcataaatgctttcttgtagacgtgccctacgcgcttgttgagttgttcctcaacgtttttcttgaagtgcttagcttcaaggtcacggatacgaccgaagcgagcctcgtcctcaatggcccgaccgattgcgcttgctacagcctgaacggttgtattgtcagcactggttaggcaagccagagtggtcttaatggtgatgtacgctacggcttccggcttgatttcttgcaggaactggaaggctgtcgggcgcttgccgcgcttagctttcacttcctcaaaccagtcgttgatgcgtgcaatcatcttagggagtagggtagtgatgagaggcttggcggcagcgttatccgcaacctcaccagctttaagttgacgctcaaacatcttgcggaagcgtgcttcacccatctcgtaagactcatgctcaagggccaactgttcgcgagctaaacgctcaccgtaatggtcagccagagtgttgaacgggatagcagccagttcgatgtcagagaagtcgttcttagcgatgttaatcgtgttcatatgtatatctccttcttaaagttaaacaaaattatttctagag**cagatcagggtgcgcaagttgtcaacgctcccaggagagttatcgacttgcgtattaggg**

Table 47

Nucleotide sequences of T7 promoter-PAL3 construct (SEQ ID NO: 44)
taatacgactcactatagggagaaagtgaactctagaaataattttgtttaactttaagaaggagatatacat**ATGAAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCT**

Nucleotide sequences of T7 promoter-PAL3 construct (SEQ ID NO: 44)
CTTTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAAA TATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAG AGGAAAAATTAAATTCAGGAGAGGTTATATATGGAATCAATACAGGAT TTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAG CATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTAT ATGTCCAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTG TTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTG TTGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCT CAGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGAG CATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACG CTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAAAAG CCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGA ATCAGTGCAATCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCA ATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCATCTCATGAA CATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAAC GCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGT TAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCA TCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAAT TCGCTGTGCACCACAAGTATTAGGTATAGTGCCAGAATCTTTAGCTAC CGCTCGGAAAATATTGGAACGGGAAGTTATCTCAGCTAATGATAATCC ATTGATAGATCCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTAT GGGGCAATATGTCGCCCGAACAATGGATGCATTAAAACTGGATATTG CTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCTTATGATGGATA ACCGTTTCTCTCGTGGATTACCTAATTCACTGAGTCCGACACCCGGCA TGTATCAAGGTTTTAAAGGCGTCCAACTTTCTCAAACCGCTTTAGTTG CTGCAATTCGCCATGATTGTGCTGCATCAGGTATTCATACCCTCGCCA CAGAACAATACAATCAAGATATTGTCAGTTTAGGTCTGCATGCCGCTC AAGATGTTTTAGAGATGGAGCAGAAATTACGCAATATTGTTTCAATGA CAATTCTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATATTAGTG AAATTGCGCCTGAAACTGCTAAATTTTACCATGCAGTACGCGAAATCA GTTCTCCTTTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCA TTGCGGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAATCATGC TGGAAGAATAA

[0648] To construct the SYN-PKU602 strain comprising P_{ARA}-*Int5* construct, P_{T7}-*PAL3* construct, and P_{Lac}-*T7 polymerase* construct (**Fig. 84**), Gibson assembly was used essentially as described above.

[0649] **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
TTATTCACAACCTGCCCTAAACTCGCTCGGACTCGCCCCGGTGCATTTTTTAAATA

CTCGCGAGAAATAGAGTTGATCGTCAAAACCGACATTGCGACCGACGGTGGCGA
TAGGCATCCGGGTGGTGCTCAAAAGCAGCTTCGCCTGACTGATGCGCTGGTCCT
CGCGCCAGCTTAATACGCTAATCCCTAACTGCTGGCGGAACAAATGCGACAGAC
GCGACGGCGACAGGCAGACATGCTGTGCGACGCTGGCGATATCAAAATTACTGT
CTGCCAGGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCGATTATCCATCGG
TGGATGGAGCGACTCGTTAATCGCTTCCATGCGCCGCAGTAACAATTGCTCAAGC
AGATTTATCGCCAGCAATTCCGAATAGCGCCCTTCCCCTTGTCCGGCATTAATGAT
TTGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCATCCGGGCGAAAGAA
ACCGGTATTGGCAAATATCGACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCG
CGGACGAAAGTAAACCCACTGGTGATACCATTCGTGAGCCTCCGGATGACGACC
GTAGTGATGAATCTCTCCAGGCGGGAACAGCAAAATATCACCCGGTCGGCAGAC
AAATTCTCGTCCCTGATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGAGA
ATATAACCTTTCATTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAACCGTTGGC
CTCAATCGGCGTTAAACCCGCCACCAGATGGGCGTTAAACGAGTATCCCGGCAG
*CAGGGGATCATTTTGCGCTTCAGCCAT*ACTTTTCATACTCCCGCCATTCAGAGA
AGAAACCAATTGTCCATATTGCATCAGACATTGCCGTCACTGCGTCTTTTAC
TGGCTCTTCTCGCTAACCCAACCGGTAACCCCGCTTATTAAAAGCATTCTGT
AACAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTAT
AATCACGGCAGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTTGCT
ATGCCATAGCATTTTTATCCATAAGATTAGCGGATCCAGCCTGACGCTTTTT
TTCGCAACTCTCTACTGTTTCTCCATACCTCTAGAAATAATTTTGTTTAACTT
TAAGAAGGAGATATACAT**ATGCCTGGTATGACCACCGAAACCGGTCCGG**
ATCCTGCAGGTCTGATTGACCTGTTTTGTCGTAAAAGCAAAGCAGTTAA
AAGCCGTGCAAATGGTGCAGGTCAGCGTCGTAAACAAGAAATTAGCAT
TGCAGCACAAGAAACCCTGGGTCGTAAAGTTGCAGCACTGCTGGGTAT
GCAGGTTCGTCATGTTTGGAAAGAAGTTGGTAGCGCAAGCCGTTTTCGT
AAAGGTAAAGCACGTGATGATCAGAGCAAAGCACTGAAAGCCCTGGAA
AGCGGTGAAGTTGGTGCACTGTGGTGTTATCGTCTGGATCGTTGGGAT
CGTGGTGGTGCCGGTGCAATTCTGAAAATTATCGAACCGGAAGATGGT
ATGCCTCGTCGTCTGCTGTTTGGTTGGGATGAAGATACCGGTCGTCCG
GTTCTGGATAGCACCAATAAACGTGATCGCGGTGAACTGATTCGTCGTG
CAGAAGAAGCACGCGAAGAAGCAGAAAAACTGAGCGAACGTGTTCGTG
ATACCAAAGCACATCAGCGTGAAAATGGTGAATGGGTTAATGCCCGTG
CACCGTATGGTCTGCGTGTTGTTCTGGTTACCGTTAGTGATGAAGAGGG
TGATGAATATGATGAACGTAAACTGGCAGCAGATGATGAAGATGCGGG
TGGTCCTGATGGTCTGACCAAAGCAGAAGCAGCCCGTCTGGTTTTTACC
CTGCCGGTTACCGATCGTCTGAGCTATGCAGGCACCGCACATGCAATG
AATACCCGTGAAATTCCGAGCCCGACCGGTGGTCCGTGGATTGCAGTT
ACCGTGCGTGATATGATTCAGAATCCGGCATATGCGGGTTGGCAGACC
ACAGGTCGTCAGGATGGTAAACAGCGTCGTCTGACCTTTTATAACGGTG
AAGGTAAACGTGTTAGCGTTATGCATGGTCCTCCGCTGGTGACCGATG
AAGAACAAGAAGCCGCAAAAGCAGCCGTTAAAGGTGAAGATGGTGTTG
GTGTTCCGCTGGATGGTAGCGATCATGATACCCGTCGCAAACATCTGCT
GAGCGGTCGTATGCGTTGTCCGGGTTGTGGTGGTAGCTGTAGCTATAG
CGGTAATGGTTATCGTTGTTGGCGTAGCAGTGTGAAAGGTGGTTGTCC
GGCACCGACCTATGTTGCACGTAAAAGCGTTGAAGAATATGTTGCATTT
CGTTGGGCAGCAAAATTAGCAGCAAGCGAACCGGATGATCCGTTTGTT
ATTGCAGTTGCAGATCGCTGGGCAGCACTGACCCATCCGCAGGCAAGC
GAAGATGAAAAGTATGCAAAAGCCGCAGTTCGTGAAGCCGAAAAAAAT

CTGGGTCGCCTGCTGCGTGATCGTCAGAATGGTGTTTATGATGGTCCG GCAGAACAGTTTTTTGCCCCTGCATATCAAGAAGCACTGAGCACCCTGC AGGCAGCCAAAGATGCAGTTAGCGAAAGCAGCGCAAGCGCAGCAGTTG ATGTTAGCTGGATTGTTGATAGCAGCGATTATGAAGAACTGTGGCTGCG TGCAACCCCGACCATGCGTAATGCAATTATTGATACCTGCATCGATGAA ATTTGGGTTGCAAAAGGCCAGCGTGGTCGTCCGTTTGATGGTGATGAA CGCGTTAAAATCAAATGGGCAGCCCGTACCTAA

Example 23. Generation of DeltaThyA

[0650] 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.

[0651] 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	tagaactgatgcaaaaagtgctcgacgaaggcacacagaTGT GTAGGCTGGAGCTGCTTC	Round 1: binds on pKD3	**SEQ ID NO: 46**
SR38	gtttcgtaattagatagccaccggcgctttaatgcccggaCATA TGAATATCCTCCTTAG	Round 1: binds on pKD3	**SEQ ID NO: 47**
SR33	caacacgtttcctgaggaaccatgaaacagtatttagaactgatgc aaaaag	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	aagaatttaacggagggcaaaaaaaaccgacgcacactggcgtc ggc	Round 3: binds to round 2 PCR product	**SEQ ID NO: 51**

[0652] 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:

[0653] step1: 98c for 30s

[0654] step2: 98c for 10s

[0655] step3: 55c for 15s

[0656] step4: 72c for 20s

[0657] repeat step 2-4 for 30 cycles

[0658] step5: 72c for 5min

[0659] 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.

[0660] 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.

[0661] 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).

[0662] 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.

[0663] 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)

[0664] 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

[0665] 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

[0666] 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)

[0667] 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

[0668] 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

[0669] 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)

[0670] 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

[0671] 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

[0672] 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

[0673] 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	1.1	NA

	resistant, chromosomal *lacZ::*P*tet-pheP::cam*		
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::*PfnrS-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::*PfnrS-pheP*::*cam	4.9	NA
SYN-PKU601	*malPT::*P*fnrS*-INT5::kan, *rrnBUP* -[PAL3]; *lacZ::*PfnrS-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*; *agaI/rsmI::PfnrS-*	7.7	NA

	*PAL3; cea::PfnrS-PAL3; yicS/nepI::*P*fnrS-PAL3::kan; malPT::PfnrS-PAL3*; *lacZ::*PfnrS-pheP*; ΔthyA*		
SYN-PKU204	*lacZ::*PfnrS-pheP*::cam*	ND	NA
SYN-PKU512	*malEK::PfnrS-PAL3*; *agaI/rsmI::PfnrS-PAL3; cea::PfnrS-PAL3; malPT::PfnrS-PAL3*; *lacZ::*PfnrS-pheP*::cam; ΔthyA*	6.7	NA
SYN-PKU513	*malEK:: PfnrS-PAL3*; *agaI/rsmI::PfnrS-PAL3; cea::PfnrS-PAL3; lacZ::*PfnrS-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*-PfnrS-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::*PfnrS-pheP*::cam*	2.4	NA
SYN-PKU522	*cea::*P*fnrS-PAL3::kan; lacZ::*PfnrS-pheP*::cam*	ND	NA
SYN-PKU523	*malPT::*P*fnrS-PAL3::kan; lacZ::*PfnrS-pheP*::cam*	0.5	NA
SYN-PKU524	*malEK:: PfnrS-PAL3*; *malPT::PfnrS-PAL3*; *lacZ::*PfnrS-pheP	2.9	NA
SYN-PKU702	*malEK:: PfnrS-PAL3*; *lacZ::*PfnrS-pheP*; Para::LAAD*	1.5	ND
SYN-PKU703	*malEK:: PfnrS-PAL3*; *malPT::PfnrS-PAL3*; *lacZ::*PfnrS-pheP*; agaI/rsmI::PfnrS::pheP; Para::LAAD*	3.1	ND
SYN-PKU704	*malEK:: PfnrS-PAL3*; *malPT::PfnrS-PAL3*; *yicS/nepI::*P*fnrS-PAL3; lacZ::*PfnrS-pheP*; Para::LAAD*	3.5	ND
SYN-PKU705	*malEK:: PfnrS-PAL3*; *malPT::PfnrS-PAL3*; *yicS/nepI::*P*fnrS-PAL3::kan;*	3.7	ND

	*lacZ::*PfnrS-pheP*; agaI/rsmI::PfnrS::pheP Para::LAAD*		
SYN-PKU602	*malEK*:: P*T7::PAL3; Para::INT5::cam (recombinase); lacZ::*PfnrS-pheP*; malPT*::P*constitutive*::*T7 polymerase (unflipped);	2.4	NA
SYN-PKU901	*Nissle with streptomycin resistance*	NA	NA
SYN-PKU713	*LacZ::PfnrS-PAL3::pheP*	NA	NA
SYN-PKU706	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::LAAD, ΔdapA::cm*	ND	ND
SYN-PKU707	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y::cm*	4.0	NA
SYN-PKU708	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y-LAAD; ΔdapA*	4.0	44 ($^{+}O_2$),0 ($^{-}O_2$)
SYN-PKU-709	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para-LAAD; ΔdapA*	ND	NA
SYN-PKU-710	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::LAAD; exo/cea:: LacIPAL3; rhtC/rhtB::LacIPAL3; ΔdapA*	4.4	40 ($^{+}O_2$),0 ($^{-}O_2$)
SYN-PKU711	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y-LAAD;*	ND	ND
SYN-PKU-712	*malEK:: PfnrS-PAL3; malPT::PfnrS-PAL3; yicS/nepI:: PfnrS-PAL3; lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP; Para::FNRS24Y; ΔDapA*	ND	ND
SYN-PKU-714	*lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP*	ND	ND

Example 28. Screening for Improved Phe Consumption

[0674] 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.

[0675] 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.

[0676] 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)

[0677] 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.

[0678] 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:

[0679] 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).

[0680] 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).

[0681] 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.

[0682] 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.

[0683] 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

Grou	Dose	Flu	

p	Test/ Control Articles	Dose Level (cfu/Animal)	Conc. (cfu/mL)	Volume (mL/ Animal)	Bicarb. Conc. (M)	Volume (mL/ Animal)	Number of Females
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

[0684] 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. 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.

[0685] 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

[0686] 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.

[0687] 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.

[0688] 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

[0689] 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.

[0690] 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.

[0691] 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.

[0692] Residual serum is stored at-60 to -86 °C and disposed of prior to study finalization.

[0693] 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.

[0694] 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.

[0695] 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)

[0696] 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.

[0697] 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.

[0698] 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).

[0699] 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

[0700] 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

PROCEDURE	TIME POINTS
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)
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

[0701] 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 QKKVEISTEITELLTHGREKLEEKLNSG EVIYGINTGFGGNANLVVPFEKIAEHQ QNLLTFLSAGTGDYMSKPCIKASQFTM LLSVCKGWSATRPIVAQAIVDHINHDI VPLVPRYGSVGASGDLIPLSYIARALCG IGKVYYMGAEIDAAEAIKRAGLTPLSL KAKEGLALINGTRVMSGISAITVIKLEK LFKASISAIALAVEALLASHEHYDARIQ QVKNHPGQNAVASALRNLLAGSTQVN LLSGVKEQANKACRHQEITQLNDTLQE VYSIRCAPQVLGIVPESLATARKILERE VISANDNPLIDPENGDVLHGGNFMGQY VARTMDALKLDIALIANHLHAIVALM MDNRFSRGLPNSLSPTPGMYQGFKGV QLSQTALVAAIRHDCAASGIHTLATEQ YNQDIVSLGLHAAQDVLEMEQKLRNI VSMTILVVCQAIHLRGNISEIAPETAKF YHAVREISSPLITDRALDEDIIRIADAIIN DQLPLPEIMLEE lnplineiskiisaagnfdvkeeraaasllqlsgnasdfsygr nsitltasa*
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)	MNISRRKLLLGVGAAGVLAGGAALVP MVRRDGKFVEAKSRASFVEGTQGALP KEADVVIIGAGIQGIMTAINLAERGMS VTILEKGQIAGEQSGRAYSQIISYQTSPE IFPLHHYGKILWRGMNEKIGADTSYRT QGRVEALADEKALDKAQAWIKTAKEA AGFDTPLNTRIIKGEELSNRLVGAQTP WTVAAFEEDSGSVDPETGTPALARYA KQIGVKIYTNCAVRGIETAGGKISDVVS EKGAIKTSQVVLAGGIWSRLFMGNMGI DIPTLNVYLSQQRVSGVPGAPRGNVHL PNGIHFREQADGTYAVAPRIFTSSIVKD SFLLGPKFMHLLGGGELPLEFSIGEDLF NSFKMPTSWNLDEKTPFEQFRVATATQ NTQHLDAVFQRMKTEFPVFEKSEVVE RWGAVVSPTFDELPIISEVKEYPGLVIN TATVWGMTEGPAAGEVTADIVMGKK PVIDPTPFSLDRFKK lnplineiskiisaagnfdvkeeraaasllqlsgnasdfsygr

		nsitltasa
SEQ ID NO: 55	HylA secretion signal	CTTAATCCATTAATTAATGAAATCAG CAAAATCATTTCAGCTGCAGGTAATT TTGATGTTAAAGAGGAAAGAGCTGC AGCTTCTTTATTGCAGTTGTCCGGTA ATGCCAGTGATTTTTCATATGGACGG AACTCAATAACTTTGACAGCATCAGC ATAA
SEQ ID NO: 56	LAAD (bold italics) driven by ParaBAD (underlined) with C terminal HylA Secretion tag (bold)	Actttttcatactcccgccattcagagaagaaaccaattgtcca tattgcatcagacattgccgtcactgcgtcttttactggctcttct cgctaacccaaccggtaaccccgcttattaaaagcattctgta acaaagcgggaccaaagccatgacaaaaacgcgtaacaaa agtgtctataatcacggcagaaaa ***atgaacatttcaaggagaaagctacttttaggtgttggtgct gcgggcgttttagcaggtggtgcggctttagttccaatggtt cgccgtgacggcaaatttgtggaagctaaatcaagagcat catttgttgaaggtacgcaaggggctcttcctaaagaagca gatgtagtgattattggtgccggtattcaagggatcatgacc gctattaaccttgctgaacgtggtatgagtgtcactatcttag aaaagggtcagattgccggtgagcaatcaggccgtgcat acagccaaattattagttaccaaacatcgccagaaatcttc ccattacaccattatgggaaaatattatggcgtggcatgaa tgagaaaattggtgcggataccagttatcgtactcaaggtc gtgtagaagcgctggcagatgaaaaagcattagataaag ctcaagcgtggatcaaaacagctaaagaagcggcaggtt ttgatacaccattaaatactcgcatcattaaaggtgaagag ctatcaaatcgcttagtcggtgctcaaacgccatggactgtt gctgcatttgaagaagattcaggctctgttgatcctgaaaca ggcacacctgcactcgctcgttatgccaaacaaatcggtgt gaaaatttataccaactgtgcagtaagaggtattgaaactg cgggtggtaaaatctctgatgtggtgagtgagaaaggggc gattaaaacgtctcaagttgtactcgctgggggtatctggtc gcgtttatttatgggcaatatgggtattgatatcccaacgctc aatgtatatctatcacaacaacgtgtctcaggggttcctggt gcaccacgtggtaatgtgcatttacctaatggtattcatttcc gcgaacaagcggatggtacttatgccgttgcaccacgtatc tttacgagttcaatagtcaaagatagcttcctgctagggcct aaatttatgcacttattaggtggcggagagttaccgttggaa ttctctattggtgaagatctatttaattcatttaaaatgccgac ctcttggaatttagatgaaaaaacaccattcgaacaattcc gagttgccacggcaacacaaaatacgcaacacttagatg ctgttttccaaagaatgaaaacagaattcccagtatttgaa aaatcagaagttgttgaacgttggggtgccgttgtgagtcc aacatttgatgaattacctatcatttctgaggtcaaagaata cccaggcttagtgattaacacggcaacagtgtggggtatg acagaaggcccggcagcgggtgaagtgaccgctgatatt gtcatgggcaagaaacctgttattgatccaacgccgtttagt

		ttggatcgttttaagaagtaaCTTAATCCATTAATTAATGAAATCAGCAAAATCATTTCAGCTGCAGGTAATTTTGATGTTAAAGAGGAAAGAGCTGCAGCTTCTTTATTGCAGTTGTCCGGTAATGCCAGTGATTTTTCATATGGACGGAACTCAATAACTTTGACAGCATCAGCATAA
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**agttgttcttattggtggtgttgctttatggttgcatcgtagtaaatggttgtaacaaaagcaatttttccggctgtctgtatacaaaaacgccgtaaagtttgagcgaagtcaataaactctctacccattcagggcaatatctctctt**GGATCC**ctctagaaataattttgtttaactttaagaaggagatatacat**ATGAAAGCTAAAGATGTTCAGCCAACCATTATTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTATGACATTGCGATAAAACAAAAAAAAGTAGAAATATCAACGGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAATCAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTGTTGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGAATCAGTGCAATCACCGTCATTAAACTGGAAAAACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATTACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAATTACCCAACTAAATGATACCTTACAGGAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGTGCCAG

		AATCTTTAGCTACCGCTCGGAAAATA TTGGAACGGGAAGTTATCTCAGCTAA TGATAATCCATTGATAGATCCAGAAA ATGGCGATGTTCTACACGGTGGAAAT TTTATGGGGCAATATGTCGCCCGAAC AATGGATGCATTAAAACTGGATATTG CTTTAATTGCCAATCATCTTCACGCC ATTGTGGCTCTTATGATGGATAACCG TTTCTCTCGTGGATTACCTAATTCACT GAGTCCGACACCCGGCATGTATCAAG GTTTTAAAGGCGTCCAACTTTCTCAA ACCGCTTTAGTTGCTGCAATTCGCCA TGATTGTGCTGCATCAGGTATTCATA CCCTCGCCACAGAACAATACAATCAA GATATTGTCAGTTTAGGTCTGCATGC CGCTCAAGATGTTTTAGAGATGGAGC AGAAATTACGCAATATTGTTTCAATG ACAATTCTGGTAGTTTGTCAGGCCAT TCATCTTCGCGGCAATATTAGTGAAA TTGCGCCTGAAACTGCTAAATTTTAC CATGCAGTACGCGAAATCAGTTCTCC TTTGATCACTGATCGTGCGTTGGATG AAGATATAATCCGCATTGCGGATGCA ATTATTAATGATCAACTTCCTCTGCC AGAAATCATGCTGGAAGAATAA**CTT AATCCATTAATTAATGAAATCAGCA AAATCATTTCAGCTGCAGGTAATTT TGATGTTAAAGAGGAAAGAGCTGC AGCTTCTTTATTGCAGTTGTCCGGT AATGCCAGTGATTTTTCATATGGAC GGAACTCAATAACTTTGACAGCAT CAGCATAA**

Table 66 HlyB and HlyD protein sequences

SEQ ID NO: 58	HlyB protein	MDSCHKIDYGLYALEILAQYHNVSVNP EEIKHRFDTDGTGLGLTSWLLAAKSLE LKVKQVKKTIDRLNFISLPALVWREDG RHFILTKVSKEANRYLIFDLEQRNPRVL EQSEFEALYQGHIILIASRSSVTGKLAK FDFTWFIPAIIKYRKIFIETLVVSVFLQLF ALITPLFFQVVMDKVLVHRGFSTLNVI TVALSVVVVFEIILSGLRTYIFAHSTSRI DVELGAKLFRHLLALPISYFESRRVGD TVARVRELDQIRNFLTGQALTSVLDLL FSFIFFAVMWYYSPKLTLVILFSLPCYA AWSVFISPILRRRLDDKFSRNADNQSFL VESVTAINTIKAMAVSPQMTNIWDKQL AGYVAAGFKVTVLATIGQQGIQLIQKT

		VMIINLWLGAHLVISGDLSIGQLIAFNM LAGQIVAPVIRLAQIWQDFQQVGISVT RLGDVLNSPTESYHGKLALPEINGNITF RNIRFRYKPDSPVILDNINLSIKQGEVIG IVGRSGSGKSTLTKLIQRFYIPENGQVLI DGHDLALADPNWLRRQVGVVLQDNV LLNRSIIDNISLANPGMSVEKVIYAAKL AGAHDFISELREGYNTIVGEQGAGLSG GQRQRIAIARALVNNPKILIFDEATSAL DYESEHIIMRNMHKICKGRTVIIIAHRL STVKNADRIIVMEKGKIVEQGKHKELL SEPESLYSYLYQLQSD
SEQ ID NO: 59	HlyD protein	MKTWLMGFSEFLLRYKLVWSETWKIR KQLDTPVREKDENEFLPAHLELIETPVS RRPRLVAYFIMGFLVIAVILSVLGQVEI VATANGKLTLSGRSKEIKPIENSIVKEII VKEGESVRKGDVLLKLTALGAEADTL KTQSSLLQTRLEQTRYQILSRSIELNKL PELKLPDEPYFQNVSEEEVLRLTSLIKE QFSTWQNQKYQKELNLDKKRAERLTI LARINRYENLSRVEKSRLDDFRSLLHK QAIAKHAVLEQENKYVEAANELRVYK SQLEQIESEILSAKEEYQLVTQLFKNEIL DKLRQTTDNIELLTLELEKNEERQQAS VIRAPVSGKVQQLKVHTEGGVVTTAE TLMVIVPEDDTLEVTALVQNKDIGFIN VGQNAIIKVEAFPYTRYGYLVGKVKNI NLDAIEDQKLGLVFNVIVSVEENDLST GNKHIPLSSGMAVTAEIKTGMRSVISY LLSPLEESVTESLHER

Example 32: Genetically Engineered Bacteria Comprising Additional Constructs

[0702] 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)	MKNASTVSEDTASNQEPTLHRGLHNRHIQ LIALGGAIGTGLFLGIGPAIQMAGPAVLLG YGVAGIIAFLIMRQLGEMVVEEPVSGSFAH FAYKYWGPFAGFLSGWNYWVMFVLVGM AELTAAGIYMQYWFPDVPTWIWAAAFFIII	60

	NAVNLVNVRLYGETEFWFALIKVLAIIGMI GFGLWLLFSGHGGEKASIDNLWRYGGFFA TGWNGLILSLAVIMFSFGGLELIGITAAEA RDPEKSIPKAVNQVVYRILLFYIGSLVVLL ALYPWVEVKSNSSPFVMIFHNLDSNVVAS ALNFVILVASLSVYNSGVYSNSRMLFGLS VQGNAPKFLTRVSRRGVPINSLMLSGAITS LVVLINYLLPQKAFGLLMALVVATLLLNW IMICLAHLRFRAAMRRQGRETQFKALLYP FGNYLCIAFLGMILLLMCTMDDMRLSAIL LPVWIVFLFMAFKTLRRK	
aromatic amino acid transport protein AroP [Escherichia coli F11] Acc. NO: EDV65095	MEGQQHGEQLKRGLKNRHIQLIALGGAIG TGLFLGSASVIQSAGPGIILGYAIAGFIAFLI MRQLGEMVVEEPVAGSFSHFAYKYWGSF AGFASGWNYWVLYVLVAMAELTAVGKY IQFWYPEIPTWVSAAVFFVVINAINLTNVK VFGEMEFWFAIIKVIAVVAMIIFGAWLLFS GNGGPQASVSNLWDQGGFLPHGFTGLVM MMAIIMFSFGGLELVGITAAEADNPEQSIP KATNQVIYRILIFYIGSLAVLLSLMPWTRV TADTSPFVLIFHELGDTFVANALNIVVLTA ALSVYNSCVYCNSRMLFGLAQQGNAPKA LASVDKRGVPVNTILVSALVTALCVLINYL APESAFGLLMALVVSALVINWAMISLAHM KFRRAKQEQGVVTRFPALLYPLGNWVCL LFMAAVLVIMLMTPGMAISVYLIPVWLIV LGIGYLFKEKTAKAVKAH	61
FNRS promoter (bold, lower case)-PheP (upper case underlined	**GGTACCagttgttcttattggtggtgttgctttatggttgca tcgtagtaaatggttgtaacaaaagcaatttttccggctgtctgt atacaaaaacgccgtaaagtttgagcgaagtcaataaactct ctacccattcagggcaatatctctcttGGATCCctctagaa ataattttgtttaactttaagaaggagatatacat**ATGAAA AACGCGTCAACCGTATCGGAAGATACTG CGTCGAATCAAGAGCCGACGCTTCATCG CGGATTACATAACCGTCATATTCAACTG ATTGCGTTGGGTGGCGCAATTGGTACTG GTCTGTTTCTTGGCATTGGCCCGGCGATT CAGATGGCGGGTCCGGCTGTATTGCTGG GCTACGGCGTCGCCGGGATCATCGCTTT CCTGATTATGCGCCAGCTTGGCGAAATG GTGGTTGAGGAGCCGGTATCCGGTTCAT TTGCCCACTTTGCCTATAAATACTGGGG ACCGTTTGCGGGCTTCCTCTCTGGCTGGA ACTACTGGGTAATGTTCGTGCTGGTGGG AATGGCAGAGCTGACCGCTGCGGGCATC TATATGCAGT63ACTGGTTCCCGGATGTT CCAACGTGGATTTGGGCTGCCGCCTTCTT TATTATCATCAACGCCGTTAACCTGGTG AACGTGCGCTTATATGGCGAAACCGAGT	62

	TCTGGTTTGCGTTGATTAAAGTGCTGGC AATCATCGGTATGATCGGCTTTGGCCTG TGGCTGCTGTTTTCTGGTCACGGCGGCG AGAAAGCCAGTATCGACAACCTCTGGCG CTACGGTGGTTTCTTCGCCACCGGCTGG AATGGGCTGATTTTGTCGCTGGCGGTAA TTATGTTCTCCTTCGGCGGTCTGGAGCTG ATTGGGATTACTGCCGCTGAAGCGCGCG ATCCGGAAAAAAGCATTCCAAAAGCGGT AAATCAGGTGGTGTATCGCATCCTGCTG TTTTACATCGGTTCACTGGTGGTTTTACT GGCGCTCTATCCGTGGGTGGAAGTGAAA TCCAACAGTAGCCCGTTTGTGATGATTTT CCATAATCTCGACAGCAACGTGGTAGCT TCTGCGCTGAACTTCGTCATTCTGGTAGC ATCGCTGTCAGTGTATAACAGCGGGGTT TACTCTAACAGCCGCATGCTGTTTGGCCT TTCTGTGCAGGGTAATGCGCCGAAGTTT TTGACTCGCGTCAGCCGTCGCGGTGTGC CGATTAACTCGCTGATGCTTTCCGGAGC GATCACTTCGCTGGTGGTGTTAATCAAC TATCTGCTGCCGCAAAAAGCGTTTGGTC TGCTGATGGCGCTGGTGGTAGCAACGCT GCTGTTGAACTGGATTATGATCTGTCTG GCGCATCTGCGTTTTCGTGCAGCGATGC GACGTCAGGGGCGTGAAACACAGTTTAA GGCGCTGCTCTATCCGTTCGGCAACTAT CTCTGCATTGCCTTCCTCGGCATGATTTT GCTGCTGATGTGCACGATGGATGATATG CGCTTGTCAGCGATCCTGCTGCCGGTGT GGATTGTATTCCTGTTTATGGCATTTAAA ACGCTGCGTCGGAAATAA	
FNRS promoter (bold, lower case)-AroP (upper case underlined, codon optimized)	**GGTACCagttgttcttattggtggtgttgctttatggttgca tcgtagtaaatggttgtaacaaaagcaatttttccggctgtctgt atacaaaaacgccgtaaagtttgagcgaagtcaataaactct ctacccattcagggcaatatctctcttGGATCCctctagaa ataattttgtttaactttaagaaggagatatacat**ATGGAG GGGCAGCAGCATGGGGAGCAACTGAAG CGCGGGTTAAAAAATCGTCACATTCAAT TAATCGCGCTGGGCGGAGCAATTGGTAC GGGATTGTTCCTGGGTTCAGCGAGCGTC ATCCAATCGGCAGGTCCAGGGATCATCT TGGGATATGCGATCGCAGGCTTTATCGC TTTTCTTATTATGCGCCAATTAGGTGAGA TGGTGGTCGAGGAGCCTGTAGCTGGCTC CTTCTCACATTTCGCGTACAAGTATTGGG GATCCTTTGCGGGATTTGCTTCTGGTTGG AACTATTGGGTTCTTTATGTCCTGGTGGC	63

	CATGGCGGAGCTGACCGCGGTTGGAAAA TATATCCAGTTCTGGTACCCCGAGATCC CGACGTGGGTCTCAGCCGCGGTATTCTT TGTTGTTATCAATGCAATCAATTTAACCA ACGTAAAAGTATTTGGTGAAATGGAGTT CTGGTTCGCGATTATCAAAGTAATTGCC GTAGTTGCTATGATTATTTTTGGGGCATG GTTGCTTTTCTCAGGAAATGGCGGACCA CAAGCGTCGGTTTCAAACCTGTGGGATC AAGGGGGATTCCTGCCGCACGGATTTAC GGGCTTGGTGATGATGATGGCTATCATT ATGTTTTCTTTCGGTGGTCTTGAATTAGT GGGTATTACCGCAGCAGAGGCAGATAAT CCCGAACAAAGCATCCCAAAAGCTACTA ACCAAGTTATTTACCGTATCCTGATTTTT TATATTGGTTCTCTGGCAGTCCTGCTTTC CTTAATGCCCTGGACACGTGTAACGGCC GATACATCCCCTTTTGTACTTATCTTTCA CGAACTGGGAGACACGTTCGTCGCCAAT GCATTAAACATTGTTGTGCTGACAGCTG CCTTATCTGTGTATAATAGCTGCGTTTAT TGCAATTCACGTATGTTATTCGGGCTTGC TCAGCAGGGTAACGCGCCAAAGGCGTTG GCCTCAGTAGATAAGCGCGGAGTGCCTG TAAATACAATTTTGGTCAGCGCATTAGT CACGGCTCTTTGCGTTCTGATTAACTATC TGGCTCCTGAAAGCGCATTCGGATTACT TATGGCCCTGGTTGTTTCCGCCCTGGTTA TCAATTGGGCAATGATTAGTTTGGCACA TATGAAGTTCCGCCGTGCTAAACAAGAA CAAGGTGTCGTAACTCGTTTCCCTGCCTT ATTGTATCCGCTGGGGAATTGGGTATGC CTTCTTTTTATGGCCGCAGTACTGGTAAT TATGTTGATGACGCCCGGCATGGCTATT AGTGTATACCTTATTCCGGTATGGTTAAT CGTCTTGGGTATCGGCTACTTATTTAAAG AAAAAACAGCAAAAGCCGTAAAGGCTC AT	

[0703]

[0704]

[0705]

Example 33. Hippuric acid, Trans-cinnamic acid, Phenylalanine, and Phenylpyruvate Quantification in Plasma and Urine by LC-MS/MS

Sample Preparation

[0706] The following stock solutions were prepared (10mg/mL), aliquoted in 1.5mL microcentrifuge tubes (100µL) and store at -20°C: phenylalanine (0.1M HCl), sodium phenylpyruvate (0.1M HCl), trans-cinnamic acid (DMSO), and hippuric acid (DMSO). Next, 250, 100, 20, 4, 0.8, 0.16, 0.032 µg/mL of each of the standards were prepared in water.

[0707] Urine from mice was diluted 40-fold by adding 195µL water to 5µL urine. On ice, sample and standards (10µL) were pipetted into a V-bottom polypropylene 96-well plate. Then 90µL of the derivatizing solution containing 50mM of 2-hydrazinoquinoline (2-HQ), dipyridyl disulfide, and triphenylphospine in acetonitrile with 1 ug/mL of phenylalanine-d5, hippuric acid-d5, trans-cinnamic acid-d7, and phenylpyruvate-d5 were added to the final solution. The plate was heat-sealed with a ThermASeal foil and mixed well, and samples were incubated at 60°C for 1hr for derivatization and centrifuged at 4000rpm for 5min. In a round-bottom 96-well plate, 20µL of the derivatized samples were added to 180µL of 0.1% formic acid in water/ACN (140:40). The plate was again heat-sealed with a ClearASeal sheet and mixed well.

LC-MS/MS method

[0708] Derivatized metabolites were measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. **Table 67**, **Table 68,** and **Table 69** provide the summary of the LC-MS/MS method.

Table 67. LC/MS Method

Column	C18 column, 3 µm (100 x 2 mm)
Mobile Phase A	100% H2O, 0.1% Formic Acid
Mobile Phase B	100% ACN, 0.1% Formic Acid
Injection volume	0uL

Table 68. HPLC Method:

Time (min)	Flow Rate (μL/min)	A%	B%
0	500	90	10
0.5	500	90	10
2.0	500	72.5	97
4.0	500	60	97
4.01	500	10	10
4.25	500	10	10

Table 69. Tandem Mass Spectrometry:

Ion Source	HESI-II
Polarity	Positive
SRM transitions:	
Phenylalanine	307.2/186.0
Phenyld5-alanine	312.2/186
Trans-cinnamic acid	290.2/131.0
Trans-cinnamic acid-d7	297.05/138.1
Hippuric acid	321.1/160
Hippuric acid-d5	326.1/160
Phenylpyruvate	306.1/260.0
Phenylpyruvate-d5	311.1/265.1

Example 34. Effect of PAL Copy Number, pheP, and LAAD on Strain Activity

[0709] To illustrate the effect of pheP, various copy numbers of PAL, and the further addition of LAAD on the rate of phenylalanine degradation *in vitro*, strains containing different copy numbers of PAL, either in the presence or absence of pheP and LAAD were compared sided by side in an *in vitro* phenylalanine consumption assay.

[0710] The genetically engineered bacteria were grown overnight, diluted and allowed to grow for another 2.5 hours in the absence or presence of

0.1% arabinose (if the construct comprises LAAD). Cultures were then placed in Coy anaerobic chamber supplying 90% N2, 5% CO2, and 5% H2 for 4 hours in phenylalanine containing medium (4 mM phenylalanine). Whole cell extracts were prepared and phenylalanine was quantified by mass spectrometry and rates were calculated.

[0711] Results shown in **Fig. 37** demonstrate that increasing the copy number of PAL increases the rate of phenylalanine degradation. Addition of the high affinity transporter pheP abrogates the transport limitation, allowing greater PAL activity. The transporter copy number does not increase rate (PAL, and not transport (pheP), is limiting). In the presence of oxygen, LAAD can degrade Phe at an extremely high rate.

Example 35. PAL-specific Metabolite Detection in SYN-PKU706 in Blood and Urine

[0712] To evaluate levels of TCA and hippuric acid in the urine, and to assess the utility of TCA and hippuric acid measurements as an indicator of strain activity, levels of TCA and hippuric acid were measured in serum and urine in an *in vivo* mouse model (BTBR-Pahenu2 mice) following subcutaneous phenylalanine challenge. SYN-PKU706 (comprising three copies of fnrS-PAL (integrated at MalP/T, HA3/4, and MalE/K), 2 copies of fnr-PheP (integrated at HA1/2 and LacZ), and one copy of Para-LAAD (LAAD knocked into the arabinose operon (Para::LAAD)), was compared to wild type Nissle with a streptomycin resistance (SYN-PKU901) in this study.

[0713] To prepare the cells, cells were diluted 1:100 in LB (2 L), grown for 1.5 h aerobically in the presence of 0.1% arabinose, then shifted to the anaerobe chamber for 4 hours. Prior to administration, cells were concentrated and frozen (15% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice, mixed 9:1 in 1M bicarbonate. Each mouse gavaged 750 uL total, or 1X10e11 cfu/mouse total over 3 gavages.

[0714] Beginning 4 days prior to the study (i.e., Days -4-1), Pah ENU2/2 mice (~11-15 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 1, 2 and 3h post Phe challenge, the bacteria were administered to mice by oral gavage (3X250 ul; 1X10e11 cfu/mouse total over 3 gavages) as follows: SYN-PKU901 (streptomycin resistant Nissle n=12), SYN-PKU-706 (n=12). Sodium bicarbonate was added to final concentration of 100mM for both strains. H2O only was administered as control (n=12). Animals were bled and urine was collected from all animals at 4h post Phe challenge. Blood was stored on ice for LC/MS analysis.

[0715] TCA and Hippuric acid were measured as described in Example 33. As seen in **Fig. 44A, Fig. 44B, Fig. 44C, and Fig. 44D**, both serum and urine levels of TCA and Hippuric acid were increased in SYN-PKU-706 over SYN-PKU-901 as compared to the H2O controls. Similar levels of metabolites were measured in urine when administering other efficacious PKU strains. Low levels of TCA were present in both urine and serum. Lower levels of hippuric acid were detected in serum. Highest levels were detected for hippuric acid in urine, indicating that the majority of TCA generated by the bacteria is converted to hippuric acid in the liver and is excreted in the urine. These and other results described herein indicate that levels of hippuric acid in the urine can be used as an indicator or biomarker of PAL activity.

Example 36. Hippuric Acid Measurement as a Method to Measure in vivo Cell Activity

[0716] To determine whether hippuric acid detection in the urine is suitable as a measure for *in vivo* cell activity and a potential biomarker, the extent of turnover of TCA into hippuric acid was assessed by oral gavage of various concentrations of TCA in PKU mice and subsequent measurement of TCA and hippuric acid by LC/MS.

[0717] On day 1 of the study, Pah ENU2/2 mice (~8-10 weeks) were randomized into TCA challenge treatment groups as follows: Group 1: 0.1mg/g TCA (n=6); Group 2: 0.05 mg/g TCA (n=6); Group 3: 0.025mg/g TCA (n=6); Group 4: 0.0125mg/g TCA (n=6); Group 5: H2O Control (n=6). Various TCA concentrations were administered by oral gavage.

[0718] Animals were transferred to metabolic cages (3 mice per cage, 2 cages per group) and urine and feces were collected at for 4h post TCA dose. Urine and feces were transferred to appropriate tubes and store samples on ice until processed for MS analysis. **Fig. 45A** and **Fig. 45B** depict the amount of TCA (**Fig. 45A**) and hippuric acid (**Fig. 45B**) recovered upon oral gavage of 0.0125, 0.025, 0.05, or 0.1 mg/g TCA at 4 hours after gavage. Insignificant amounts of TCA and hippurate were detected in blood and feces (data not shown). A nearly full recovery of TCA in the form of hippurate was observed in the urine. As a result, 1 mol of hippurate found in the urine would equal 1 mol of Phe converted to TCA in the small intestine in a PKU mouse upon administration of a PKU strain.

[0719] Next, the kinetics of conversion of TCA to hippuric acid was assessed in a time course post pure TCA oral gavage. On day 1 of the study, Pah ENU2/2 mice (~8-10 weeks) were randomized into TCA challenge treatment groups as follows: Group 1: 0.033 mg/g TCA (n=6); Group 2: 0.1 mg/g TCA (n=6); Group 3: H2O Control (n=6). TCA concentrations were administered by oral gavage. Animals were transferred to metabolic cages (3 mice per cage, 2 cages per group) and urine samples were collected at 1, 2, 3, 4, 5, 6 hours post TCA dose. Urine was transferred to appropriate tubes and store samples on ice until processed for MS analysis. As seen in **Fig. 46**, at the high dose TCA gavage, TCA is converted to hippurate and excreted in the urine by 4 hours.

Example 37. Generation of Additional PKU Strains

[0720] The following PKU strains were generated for use in subsequent examples. Schematics are depicted in **Fig. 47A, Fig. 47B, Fig. 47C, Fig. 47D, Fig. 47E,** and **Fig. 47F.**

[0721] SYN-PKU707 comprises three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). SYN-PKU707 further comprises one copy of the mutated FNR transcription factor FNRS24Y (Para::FNRS24Y). SYN-PKU712 essentially corresponds to SYN-PKU707 with a dapA auxotrophy.

[0722] SYN-PKU708 comprises a bacterial chromosome with three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). SYN-PKU708 further comprises one copy of the mutated FNR transcription factor FNRS24Y (Para::FNRS24Y) and one copy of LAAD inserted at the same insertion site (the arabinose operon), which is transcribed as a bicistronic message from the endogenous arabinose promoter. The genome is further engineered to include a dapA auxotrophy, in which the dapA gene is deleted. SYN-PKU711 essentially corresponds to SYN-PKU708 without a dapA auxotrophy.

[0723] SYN-PKU709 comprises a bacterial chromosome comprising three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). SYN-PKU709 further comprises one copy of the LAAD inserted into the arabinose operon with expression driven by the native Para promoter (Para::LAAD). The genome is further engineered to include a dapA auxotrophy, in which the dapA gene is deleted.

[0724] SYN-PKU710 comprises a bacterial chromosome comprising three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). SYN-PKU710 further comprises one copy of the LAAD inserted into the arabinose operon with expression driven by the native Para promoter (Para::LAAD). SYN-PKU710 further comprises two copies of IPTG inducible PAL3 (2XLacIPAL, exo/cea and rhtC/rhtB), a dapA auxotrophy and is cured of all antibiotic resistances. Constructs and methods for the generation of these strains are described herein. Additional constructs needed for strain construction are generated according to methods described herein, e.g., Examples 1, 2, 22, and 23 and are shown in Table 70, Table 71, and Table 72.

Table 70. Para-FNRS24Y Sequences

Description	Sequence	SEQ ID NO
FNRS24Y (bold) driven by the arabinose inducible promoter (underlined) and araC in reverse direction (italic). RBS is underlined and bolded (see **Fig. 48A**)	*TTATTCACAACCTGCCCTAAACTCGCTCGGACTCGCCCCGGTG CATTTTTTAAATACTCGCGAGAAATAGAGTTGATCGTCAAAACC GACATTGCGACCGACGGTGGCGATAGGCATCCGGGTGGTGC TCAAAAGCAGCTTCGCCTGACTGATGCGCTGGTCCTCGCGCC AGCTTAATACGCTAATCCCTAACTGCTGGCGGAACAAATGCGA CAGACGCGACGGCGACAGGCAGACATGCTGTGCGACGCTGG CGATATCAAAATTACTGTCTGCCAGGTGATCGCTGATGTACTG ACAAGCCTCGCGTACCCGATTATCCATCGGTGGATGGAGCGA CTCGTTAATCGCTTCCATGCGCCGCAGTAACAATTGCTCAAGC AGATTTATCGCCAGCAATTCCGAATAGCGCCCTTCCCCTTGTC CGGCATTAATGATTTGCCCAAACAGGTCGCTGAAATGCGGCT GGTGCGCTTCATCCGGGCGAAAGAAACCGGTATTGGCAAATA TCGACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCGCGGAC GAAAGTAAACCCACTGGTGATACCATTCGTGAGCCTCCGGAT GACGACCGTAGTGATGAATCTCTCCAGGCGGGAACAGCAAAA TATCACCCGGTCGGCAGACAAATTCTCGTCCCTGATTTTTCAC CACCCCCTGACCGCGAATGGTGAGATTGAGAATATAACCTTTC ATTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAACCGTTGG CCTCAATCGGCGTTAAACCCGCCACCAGATGGGCGTTAAACG AGTATCCCGGCAGCAGGGGATCATTTTGCGCTTCAGCCATACT TTTCATACTCCCGCCATTCAGAGAAGAAACCAATTGTCCATATT GCAT*<u>CAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTC TCGCTAACCCAACCGGTAACCCCGCTTATTAAAAGCATTC TGTAACAAAGCGGGACCAAAGCCATGACAAAAACGCGTA ACAAAAGTGTCTATAATCACGGCAGAAAAGTCCACATTGA TTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTT TATCCATAAGATTAGCGGATCCAGCCTGACGCTTTTTTTCG CAACTCTCTACTGTTTCTCCATAC</u>**<u>CTCTAGAAATAATTTT GTTTAACTTTAAGAAGGAGATATACAT</u>ATGATCCCGGA AAAGCGAATTATACGGCGCATTCAGTCTGGCGGTTGTG CTATCCATTGCCAGGATTGCTATATCAGCCAGCTTTGC ATCCCGTTCACACTCAACGAACATGAGCTTGATCAGCT TGATAATATCATTGAGCGGAAGAAGCCTATTCAGAAAG GCCAGACGCTGTTTAAGGCTGGAGATGAACTTAAATCG CTTTATGCCATCCGCTCCGGTACGATTAAAAGTTATAC CATCACTGAGCAAGGCGACGAGCAAATCACTGGTTTCC ATTTAGCAGGCGATCTGGTGGGATTTGATGCCATCGGC AGCGGTCATCACCCGAGTTTCGCGCAGGCGCTGGAAA CCTCGATGGTATGTGAAATCCCGTTCGAAACGCTGGAC GATTTGTCTGGTAAAATGCCGAATCTGCGTCAGCAGAT GATGCGTCTGATGAGCGGTGAAATCAAAGGCGATCAG GACATGATCCTGCTGTTGTCGAAGAAAAATGCCGAGG AACGTCTGGCTGCATTCATCTACAACCTGTCCCGTCGT TTTGCCCAACGCGGCTTCTCCCCTCGTGAATTCCGCCT*	64

	GACGATGACTCGTGGTGATATCGGTAACTATCTGGGCC TGACGGTTGAAACCATCAGCCGTCTGCTGGGTCGCTTC CAGAAAAGCGGTATGCTGGCAGTCAAAGGTAAATACA TCACTATCGAAAATAACGATGCGCTGGCCCAGCTTGCT GGTCATACGCGTAACGTTGCCTGA	
FNRS24Y	ATGATCCCGGAAAAGCGAATTATACGGCGCATTCAGTCTG GCGGTTGTGCTATCCATTGCCAGGATTGCTATATCAGCCA GCTTTGCATCCCGTTCACACTCAACGAACATGAGCTTGAT CAGCTTGATAATATCATTGAGCGGAAGAAGCCTATTCAGA AAGGCCAGACGCTGTTTAAGGCTGGAGATGAACTTAAATC GCTTTATGCCATCCGCTCCGGTACGATTAAAAGTTATACC ATCACTGAGCAAGGCGACGAGCAAATCACTGGTTTCCATT TAGCAGGCGATCTGGTGGGATTTGATGCCATCGGCAGCGG TCATCACCCGAGTTTCGCGCAGGCGCTGGAAACCTCGATG GTATGTGAAATCCCGTTCGAAACGCTGGACGATTTGTCTG GTAAAATGCCGAATCTGCGTCAGCAGATGATGCGTCTGAT GAGCGGTGAAATCAAAGGCGATCAGGACATGATCCTGCT GTTGTCGAAGAAAAATGCCGAGGAACGTCTGGCTGCATTC ATCTACAACCTGTCCCGTCGTTTTGCCCAACGCGGCTTCTC CCCTCGTGAATTCCGCCTGACGATGACTCGTGGTGATATC GGTAACTATCTGGGCCTGACGGTTGAAACCATCAGCCGTC TGCTGGGTCGCTTCCAGAAAAGCGGTATGCTGGCAGTCAA AGGTAAATACATCACTATCGAAAATAACGATGCGCTGGCC CAGCTTGCTGGTCATACGCGTAACGTTGCCTGA	65
AraC (reverse orientation)	TTATTCACAACCTGCCCTAAACTCGCTCGGACTCGCCCCG GTGCATTTTTTAAATACTCGCGAGAAATAGAGTTGATCGT CAAAACCGACATTGCGACCGACGGTGGCGATAGGCATCC GGGTGGTGCTCAAAAGCAGCTTCGCCTGACTGATGCGCTG GTCCTCGCGCCAGCTTAATACGCTAATCCCTAACTGCTGG CGGAACAAATGCGACAGACGCGACGGCGACAGGCAGACA TGCTGTGCGACGCTGGCGATATCAAAATTACTGTCTGCCA GGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCGATT ATCCATCGGTGGATGGAGCGACTCGTTAATCGCTTCCATG CGCCGCAGTAACAATTGCTCAAGCAGATTTATCGCCAGCA ATTCCGAATAGCGCCCTTCCCCTTGTCCGGCATTAATGATT TGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCAT CCGGGCGAAAGAAACCGGTATTGGCAAATATCGACGGCC AGTTAAGCCATTCATGCCAGTAGGCGCGCGGACGAAAGT AAACCCACTGGTGATACCATTCGTGAGCCTCCGGATGACG ACCGTAGTGATGAATCTCTCCAGGCGGGAACAGCAAAAT ATCACCCGGTCGGCAGACAAATTCTCGTCCCTGATTTTTCA CCACCCCCTGACCGCGAATGGTGAGATTGAGAATATAACC TTTCATTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAA CCGTTGGCCTCAATCGGCGTTAAACCCGCCACCAGATGGG CGTTAAACGAGTATCCCGGCAGCAGGGGATCATTTTGCGC TTCAGCCATACTTTTCATACTCCCGCCATTCAGAGAAGAA ACCAATTGTCCATATTGCAT	66
Promoter region	CAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTCGC TAACCCAACCGGTAACCCCGCTTATTAAAAGCATTCTGTA	67

	ACAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAA AAGTGTCTATAATCACGGCAGAAAAGTCCACATTGATTAT TTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATC CATAAGATTAGCGGATCCAGCCTGACGCTTTTTTTCGCAA CTCTCTACTGTTTCTCCATACCTCTAGAAATAATTTTGTTT AACTTTAAGAAGGAGATATACAT	
RBS and Leader Region	CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CAT	68
FNRS24Y Polypeptide	MIPEKRIIRRIQSGGCAIHCQDCYISQLCIPFTLNEHELDQLDNI IERKKPIQKGQTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQI TGFHLAGDLVGFDAIGSGHHPSFAQALETSMVCEIPFETLDD LSGKMPNLRQQMMRLMSGEIKGDQDMILLLSKKNAEERLA AFIYNLSRRFAQRGFSPREFRLTMTRGDIGNYLGLTVETISRL LGRFQKSGMLAVKGKYITIENNDALAQLAGHTRNVA	69
AraC polypeptide	MQYGQLVSSLNGGSMKSMAEAQNDPLLPGYSFNAHLVAGL TPIEANGYLDFFIDRPLGMKGYILNLTIRGQGVVKNQGREFV CRPGDILLFPPGEIHHYGRHPEAHEWYHQWVYFRPRAYWHE WLNWPSIFANTGFFRPDEAHQPHFSDLFGQIINAGQGEGRYS ELLAINLLEQLLLRRMEAINESLHPPMDNRVREACQYISDHL ADSNFDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRIS QAKLLLSTTRMPIATVGRNVGFDDQLYFSRVFKKCTGASPSE FRAGCE*	70
Wild Type FNR	ATGATCCCGGAAAAGCGAATTATACGGCGCATTCAGTCTG GCGGTTGTGCTATCCATTGCCAGGATTGCACGATCAGCCA GCTTTGCATCCCGTTCACACTCAACGAACATGAGCTTGAT CAGCTTGATAATATCATTGAGCGGAAGAAGCCTATTCAGA AAGGCCAGACGCTGTTTAAGGCTGGAGATGAACTTAAATC GCTTTATGCCATCCGCTCCGGTACGATTAAAAGTTATACC ATCACTGAGCAAGGCGACGAGCAAATCACTGGTTTCCATT TAGCAGGCGATCTGGTGGGATTTGATGCCATCGGCAGCGG TCATCACCCGAGTTTCGCGCAGGCGCTGGAAACCTCGATG GTATGTGAAATCCCGTTCGAAACGCTGGACGATTTGTCTG GTAAAATGCCGAATCTGCGTCAGCAGATGATGCGTCTGAT GAGCGGTGAAATCAAAGGCGATCAGGACATGATCCTGCT GTTGTCGAAGAAAAATGCCGAGGAACGTCTGGCTGCATTC ATCTACAACCTGTCCCGTCGTTTTGCCCAACGCGGCTTCTC CCCTCGTGAATTCCGCCTGACGATGACTCGTGGTGATATC GGTAACTATCTGGGCCTGACGGTTGAAACCATCAGCCGTC TGCTGGGTCGCTTCCAGAAAAGCGGTATGCTGGCAGTCAA AGGTAAATACATCACTATCGAAAATAACGATGCGCTGGCC CAGCTTGCTGGTCATACGCGTAACGTTGCCTGA	71
Wild Type FNR polypeptide	MIPEKRIIRRIQSGGCAIHCQDCSISQLCIPFTLNEHELDQLDNI IERKKPIQKGQTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQI TGFHLAGDLVGFDAIGSGHHPSFAQALETSMVCEIPFETLDD LSGKMPNLRQQMMRLMSGEIKGDQDMILLLSKKNAEERLA AFIYNLSRRFAQRGFSPREFRLTMTRGDIGNYLGLTVETISRL LGRFQKSGMLAVKGKYITIENNDALAQLAGHTRNVA	72

[0725]

Table 71. Arabinose inducible FNRS24Y-LAAD sequence

Description	Sequence	SEQ ID NO
Construct in which FNRS24Y (Bold) and LAAD (Bold underlined) are expressed as a bicistronic message from an arabinose inducible promoter. AraC (italic) is transcribed in the reverse orientation. Promoter is underlined. RBS are in bold, italic and underline (**Fig. 48B**)	*TTATTCACAACCTGCCCTAAACTCGCTCGGACTCGCC* *CCGGTGCATTTTTTAAATACTCGCGAGAAATAGAGTT* *GATCGTCAAAACCGACATTGCGACCGACGGTGGCGA* *TAGGCATCCGGGTGGTGCTCAAAAGCAGCTTCGCCT* *GACTGATGCGCTGGTCCTCGCGCCAGCTTAATACGC* *TAATCCCTAACTGCTGGCGGAACAAATGCGACAGACG* *CGACGGCGACAGGCAGACATGCTGTGCGACGCTGG* *CGATATCAAAATTACTGTCTGCCAGGTGATCGCTGAT* *GTACTGACAAGCCTCGCGTACCCGATTATCCATCGGT* *GGATGGAGCGACTCGTTAATCGCTTCCATGCGCCGC* *AGTAACAATTGCTCAAGCAGATTTATCGCCAGCAATTC* *CGAATAGCGCCCTTCCCCTTGTCCGGCATTAATGATT* *TGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCGCT* *TCATCCGGGCGAAAGAAACCGGTATTGGCAAATATCG* *ACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCGCG* *GACGAAAGTAAACCCACTGGTGATACCATTCGTGAGC* *CTCCGGATGACGACCGTAGTGATGAATCTCTCCAGG* *CGGGAACAGCAAAATATCACCCGGTCGGCAGACAAA* *TTCTCGTCC*CTGATTTTTCACCACCCCCTGACCGCG AATGGTGAGATTGAGAATATAACCTTTCATTCCCA GCGGTCGGTCGATAAAAAAATCGAGATAACCGTT GGCCTCAATCGGCGTTAAACCCGCCACCAGATGG GCGTTAAACGAGTATCCCGGCAGCAGGGGATCAT TTTGCGCTTCAGCCATACTTTTCATACTCCCGCCAT TCAGAGAAGAAACCAATTGTCCATATTGCATCAG ACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTC GCTAACCCAACCGGTAACCCCGCTTATTAAAAGC ATTCTGTAAC***AAAGCGGGACCAAAGCCATGACAAA*** ***AACGCGTAACAAAAGTG***TCTATAATCACGGCAGAA AAGTCCACATTGATTATTTGCACGGCGTCACACTT TGCTATGCCATAGCATTTTTATCCATAAGATTAGC GGATCCAGCCTGACGCTTTTTTTCGCAACTCTCTA CTGTTTCTCCATACCTCTAGAAATAATTTTGTTTAA CTTTAAGAAGGAGATATACAT**ATGATCCCGGAAA** **AGCGAATTATACGGCGCATTCAGTCTGGCGGT** **TGTGCTATCCATTGCCAGGATTGCTATATCAGC** **CAGCTTTGCATCCCGTTCACACTCAACGAACAT** **GAGCTTGATCAGCTTGATAATATCATTGAGCGG** **AAGAAGCCTATTCAGAAAGGCCAGACGCTGTT** **TAAGGCTGGAGATGAACTTAAATCGCTTTATGC** **CATCCGCTCCGGTACGATTAAAAGTTATACCAT** **CACTGAGCAAGGCGACGAGCAAATCACTGGTT** **TCCATTTAGCAGGCGATCTGGTGGGATTTGATG** **CCATCGGCAGCGGTCATCACCCGAGTTTCGCG**	73

	CAGGCGCTGGAAACCTCGATGGTATGTGAAAT CCCGTTCGAAACGCTGGACGATTTGTCTGGTAA AATGCCGAATCTGCGTCAGCAGATGATGCGTC TGATGAGCGGTGAAATCAAAGGCGATCAGGAC ATGATCCTGCTGTTGTCGAAGAAAAATGCCGA GGAACGTCTGGCTGCATTCATCTACAACCTGTC CCGTCGTTTTGCCCAACGCGGCTTCTCCCCTCG TGAATTCCGCCTGACGATGACTCGTGGTGATAT CGGTAACTATCTGGGCCTGACGGTTGAAACCA TCAGCCGTCTGCTGGGTCGCTTCCAGAAAAGC GGTATGCTGGCAGTCAAAGGTAAATACATCACT ATCGAAAATAACGATGCGCTGGCCCAGCTTGC TGGTCATACGCGTAACGTTGCCTGA *AAGAAGGAGATATACAT*ATGAACATTTCAAGGAG AAAGCTACTTTTAGGTGTTGGTGCTGCGGGCG TTTTAGCAGGTGGTGCGGCTTTAGTTCCAATGG TTCGCCGTGACGGCAAATTTGTGGAAGCTAAAT CAAGAGCATCATTTGTTGAAGGTACGCAAGGG GCTCTTCCTAAAGAAGCAGATGTAGTGATTATT GGTGCCGGTATTCAAGGGATCATGACCGCTAT TAACCTTGCTGAACGTGGTATGAGTGTCACTAT CTTAGAAAAGGGTCAGATTGCCGGTGAGCAAT CAGGCCGTGCATACAGCCAAATTATTAGTTACC AAACATCGCCAGAAATCTTCCCATTACACCATT ATGGGAAAATATTATGGCGTGGCATGAATGAG AAAATTGGTGCGGATACCAGTTATCGTACTCAA GGTCGTGTAGAAGCGCTGGCAGATGAAAAAGC ATTAGATAAAGCTCAAGCGTGGATCAAAACAG CTAAAGAAGCGGCAGGTTTTGATACACCATTAA ATACTCGCATCATTAAAGGTGAAGAGCTATCAA ATCGCTTAGTCGGTGCTCAAACGCCATGGACT GTTGCTGCATTTGAAGAAGATTCAGGCTCTGTT GATCCTGAAACAGGCACACCTGCACTCGCTCG TTATGCCAAACAAATCGGTGTGAAAATTTATAC CAACTGTGCAGTAAGAGGTATTGAAACTGCGG GTGGTAAAATCTCTGATGTGGTGAGTGAGAAA GGGGCGATTAAAACGTCTCAAGTTGTACTCGCT GGGGGTATCTGGTCGCGTTTATTTATGGGCAAT ATGGGTATTGATATCCCAACGCTCAATGTATAT CTATCACAACAACGTGTCTCAGGGGTTCCTGGT GCACCACGTGGTAATGTGCATTTACCTAATGGT ATTCATTTCCGCGAACAAGCGGATGGTACTTAT GCCGTTGCACCACGTATCTTTACGAGTTCAATA GTCAAAGATAGCTTCCTGCTAGGGCCTAAATTT ATGCACTTATTAGGTGGCGGAGAGTTACCGTT GGAATTCTCTATTGGTGAAGATCTATTTAATTC ATTTAAAATGCCGACCTCTTGGAATTTAGATGA AAAAACACCATTCGAACAATTCCGAGTTGCCAC GGCAACACAAAATACGCAACACTTAGATGCTGT	

	TTTCCAAAGAATGAAAACAGAATTCCCAGTATT TGAAAAATCAGAAGTTGTTGAACGTTGGGGTG CCGTTGTGAGTCCAACATTTGATGAATTACCTA TCATTTCTGAGGTCAAAGAATACCCAGGCTTAG TGATTAACACGGCAACAGTGTGGGGTATGACA GAAGGCCCGGCAGCGGGTGAAGTGACCGCTGA TATTGTCATGGGCAAGAAACCTGTTATTGATCC AACGCCGTTTAGTTTGGATCGTTTTAAGAAGTA A	

Table 72. Inducible Lac-PAL3 construct sequences

[0726]

Sequence	**Description**	**SEQ ID NO**
Construct sequence comprising LacI in reverse orientation (underlined), tac promoter sequence (italic underlined), lacO (bold underlined), RBS and leader region (bold), PAL3 (italic) (see **Fig. 49**)	TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG CCAGCTGCATTAATGAATCGGCCAACGCGCGGGG AGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTT TTTCTTTTCACCAGTGAGACTGGCAACAGCTGATT GCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGC AAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAA AATCCTGTTTGATGGTGGTTAACGGCGGGATATAA CATGAGCTATCTTCGGTATCGTCGTATCCCACTAC CGAGATATCCGCACCAACGCGCAGCCCGGACTCG GTAATGGCGCGCATTGCGCCCAGCGCCATCTGATC GTTGGCAACCAGCATCGCAGTGGGAACGATGCCC TCATTCAGCATTTGCATGGTTTGTTGAAAACCGGA CATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCG GCTGAATTTGATTGCGAGTGAGATATTTATGCCAG CCAGCCAGACGCAGACGCGCCGAGACAGAACTTA ATGGGCCCGCTAACAGCGCGATTTGCTGGTGACC CAATGCGACCAGATGCTCCACGCCCAGTCGCGTA CCGTCCTCATGGGAGAAAATAATACTGTTGATGG GTGTCTGGTCAGAGACATCAAGAAATAACGCCGG AACATTAGTGCAGGCAGCTTCCACAGCAATGGCA TCCTGGTCATCCAGCGGATAGTTAATGATCAGCCC ACTGACGCGTTGCGCGAGAAGATTGTGCACCGCC GCTTTACAGGCTTCGACGCCGCTTCGTTCTACCAT CGACACCACCACGCTGGCACCCAGTTGATCGGCG CGAGATTTAATCGCCGCGACAATTTGCGACGGCG CGTGCAGGGCCAGACTGGAGGTGGCAACGCCAAT CAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCA CGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCC GCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTG GCTGGCCTGGTTCACCACGCGGGAAACGGTCTGA	74

	TAAGAGACACCGGCATACTCTGCGACATCGTATA ACGTTACTGGTTTCATATTCACCACCCTGAATTGA CTCTCTTCCGGGCGCTATCATGCCATACCGCGAAA GGTTTTGCGCCATTCGATGGCGCGCCGCTTCGTCA GGCCACATAGCTTTCTTGTTCTGATCGGAACGATC GTTGGCTGTGT*TGACAATTAATCATCGGCTCGTATAA* *TGT*GT**GGAATTGTGAGCGCTCACAATT**AGCTGTC ACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAG GACGAAACAGCCTCTACAAATAATTTTGTTTAAAA CAACACCCACTAAGATAACTCTAGAAATAATTTTG TTTAACTTTAAGAAGGAGATATACATATGAAAGC TAAAGATGTTCAGCCAACCATTATTATTAATAAAA ATGGCCTTATCTCTTTGGAAGATATCTATGACATT GCGATAAAACAAAAAAAAGTAGAAATATCAACG GAGATCACTGAACTTTTGACGCATGGTCGTGAAA AATTAGAGGAAAAATTAAATTCAGGAGAGGTTAT ATATGGAATCAATACAGGATTTGGAGGGAATGCC AATTTAGTTGTGCCATTTGAGAAAATCGCAGAGC ATCAGCAAAATCTGTTAACTTTTCTTTCTGCTGGT ACTGGGGACTATATGTCCAAACCTTGTATTAAAGC GTCACAATTTACTATGTTACTTTCTGTTTGCAAAG GTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCA ATTGTTGATCATATTAATCATGACATTGTTCCTCT GGTTCCTCGCTATGGCTCAGTGGGTGCAAGCGGTG ATTTAATTCCTTTATCTTATATTGCACGAGCATTAT GTGGTATCGGCAAAGTTTATTATATGGGCGCAGA AATTGACGCTGCTGAAGCAATTAAACGTGCAGGG TTGACACCATTATCGTTAAAAGCCAAAGAAGGTC TTGCTCTGATTAACGGCACCCGGGTAATGTCAGGA ATCAGTGCAATCACCGTCATTAAACTGGAAAAAC TATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCT GTTGAAGCATTACTTGCATCTCATGAACATTATGA TGCCCGGATTCAACAAGTAAAAAATCATCCTGGT CAAAACGCGGTGGCAAGTGCATTGCGTAATTTATT GGCAGGTTCAACGCAGGTTAATCTATTATCTGGGG TTAAAGAACAAGCCAATAAAGCTTGTCGTCATCA AGAAATTACCCAACTAAATGATACCTTACAGGAA GTTTATTCAATTCGCTGTGCACCACAAGTATTAGG TATAGTGCCAGAATCTTTAGCTACCGCTCGGAAAA TATTGGAACGGGAAGTTATCTCAGCTAATGATAAT CCATTGATAGATCCAGAAAATGGCGATGTTCTAC ACGGTGGAAATTTTATGGGGCAATATGTCGCCCG AACAATGGATGCATTAAAACTGGATATTGCTTTAA TTGCCAATCATCTTCACGCCATTGTGGCTCTTATG ATGGATAACCGTTTCTCTCGTGGATTACCTAATTC ACTGAGTCCGACACCCGGCATGTATCAAGGTTTTA AAGGCGTCCAACTTTCTCAAACCGCTTTAGTTGCT GCAATTCGCCATGATTGTGCTGCATCAGGTATTCA TACCCTCGCCACAGAACAATACAATCAAGATATT	

	GTCAGTTTAGGTCTGCATGCCGCTCAAGATGTTTT AGAGATGGAGCAGAAATTACGCAATATTGTTTCA ATGACAATTCTGGTAGTTTGTCAGGCCATTCATCT TCGCGGCAATATTAGTGAAATTGCGCCTGAAACT GCTAAATTTTACCATGCAGTACGCGAAATCAGTTC TCCTTTGATCACTGATCGTGCGTTGGATGAAGATA TAATCCGCATTGCGGATGCAATTATTAATGATCAA CTTCCTCTGCCAGAAATCATGCTGGAAGAATAA	
LacI (in reverse orientation)	TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG CCAGCTGCATTAATGAATCGGCCAACGCGCGGGG AGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTT TTTCTTTTCACCAGTGAGACTGGCAACAGCTGATT GCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGC AAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAA AATCCTGTTTGATGGTGGTTAACGGCGGGATATAA CATGAGCTATCTTCGGTATCGTCGTATCCCACTAC CGAGATATCCGCACCAACGCGCAGCCCGGACTCG GTAATGGCGCGCATTGCGCCCAGCGCCATCTGATC GTTGGCAACCAGCATCGCAGTGGGAACGATGCCC TCATTCAGCATTTGCATGGTTTGTTGAAAACCGGA CATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCG GCTGAATTTGATTGCGAGTGAGATATTTATGCCAG CCAGCCAGACGCAGACGCGCCGAGACAGAACTTA ATGGGCCCGCTAACAGCGCGATTTGCTGGTGACC CAATGCGACCAGATGCTCCACGCCCAGTCGCGTA CCGTCCTCATGGGAGAAAATAATACTGTTGATGG GTGTCTGGTCAGAGACATCAAGAAATAACGCCGG AACATTAGTGCAGGCAGCTTCCACAGCAATGGCA TCCTGGTCATCCAGCGGATAGTTAATGATCAGCCC ACTGACGCGTTGCGCGAGAAGATTGTGCACCGCC GCTTTACAGGCTTCGACGCCGCTTCGTTCTACCAT CGACACCACCACGCTGGCACCCAGTTGATCGGCG CGAGATTTAATCGCCGCGACAATTTGCGACGGCG CGTGCAGGGCCAGACTGGAGGTGGCAACGCCAAT CAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCA CGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCC GCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTG GCTGGCCTGGTTCACCACGCGGGAAACGGTCTGA TAAGAGACACCGGCATACTCTGCGACATCGTATA ACGTTACTGGTTTCAT	75
Promoter region	ATTCACCACCCTGAATTGACTCTCTTCCGGGCGCT ATCATGCCATACCGCGAAAGGTTTTGCGCCATTCG ATGGCGCGCCGCTTCGTCAGGCCACATAGCTTTCT TGTTCTGATCGGAACGATCGTTGGCTGTGTTGACA ATTAATCATCGGCTCGTATAATGTGTGGAATTGTG AGCGCTCACAATTAGCTGTCACCGGATGTGCTTTC CGGTCTGATGAGTCCGTGAGGACGAAACAGCCTC TACAAATAATTTTGTTTAAAACAACACCCACTAAG ATAACTCTAGAAATAATTTTGTTTAACTTTAAGAA	76

	GGAGATATACAT	
Tac promoter	TGACAATTAATCATCGGCTCGTATAATGT	77
LacO	GGAATTGTGAGCGCTCACAATT	78
RBS and leader region	TGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAAC AGCCTCTA	79
LacI polypeptide sequence	MKPVTLYDVAEYAGVSYQTVSRVVNQASHVSAKT REKVEAAMAELNYIPNRVAQQLAGKQSLLIGVATSS LALHAPSQIVAAIKSRADQLGASVVVSMVERSGVEA CKAAVHNLLAQRVSGLIINYPLDDQDAIAVEAACTN VPALFLDVSDQTPINSIIFSHEDGTRLGVEHLVALGH QQIALLAGPLSSVSARLRLAGWHKYLTRNQIQPIAER EGDWSAMSGFQQTMQMLNEGIVPTAMLVANDQM ALGAMRAITESGLRVGADISVVGYDDTEDSSCYIPPL TTIKQDFRLLGQTSVDRLLQLSQGQAVKGNQLLPVS LVKRKTTLAPNTQTASPRALADSLMQLARQVSRLES GQ	80

[0727]

Example 38. Analysis of blood Phe Levels and Metabolic Conversion to Hippurate Following Administration of PKU Strain SYN-PKU-707

[0728] The ability of engineered probiotic strain SYN-PKU-707 to convert phenylalanine to hippurate was assessed. Strain SYN-PKU-707 comprises three copies of PAL driven by the FNR promoter (inserted into the chromosome at the malE/K, yicS/nepl, an dmalP/T loci), and two copies of pheP driven by the FNR promoter (inserted into the chromosome at the LacZ and agaI/rsmI loci), and the mutant FNRS24Y (see, e.g., **Fig. 47A**).

[0729] Cultures (1:100 back-dilutions from overnight cultures) were grown to early log phase for 1.5h before the addition of L-arabinose at 0.15% final concentration for induction. Cultures were induced for 4 hours (aerobically). . Prior to administration, cells were concentrated 200X and frozen (10% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice, and mixed 9:1 in 1M bicarbonate. Each mouse was gavaged 750uL total, or 1X10e11 cfu/mouse.

[0730] Beginning 4 days prior to the study (i.e., Days -4-1), Pah ENU2/2 mice (~11-15 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 according to weight as follows: Group 1: SYN-PKU901 (n=9); Group 2: SYN-PKU-707 (n=9); Group 3: H2O Control

(n=9). Blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. 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 1, 2 and 3h post Phe challenge, the bacteria (or water) were administered to mice by oral gavage (3X250 ul; 1X10e11 total bacteria). Sodium bicarbonate was added to final concentration of 100mM for both strains. Animals were bled and urine was collected from all animals up to 4h post Phe challenge. All treatment groups were bled at 4h post phenylalanine challenge. Blood was stored on ice for LC/MS analysis. Results in **Fig. 52 and Fig. 53** show that SYN-PKU707 was efficacious in reducing blood phenylalanine and that hippurate can be found specifically excreted in the cages of mice treated with SYN-PKU707, indicating that the cells are active *in vivo*.

[0731] **Fig. 52** depicts blood phenylalanine concentrations relative to baseline; total metabolic activity for SYN-PKU707 was calculated as 269umol/hr and the total reduction in Δphe was = 49% ($P<0.05$) relative to SYN-PKU901 ($P<0.05$). Fig. 53 depicts the urine hippurate concentration at 4 hours post phenylalanine injection. In Fig. 53, levels for each cage (three mice per cage) are shown separately and numbered 1-3 for each strain. These results indicate that approximately 15-20% of injected phenylalanine is converted to hippurate. Phenylalanine is converted to TCA in the small intestine, which is then converted into hippurate in the liver.

Example 39. Hippuric acid Recovered in Urine Following a Single Dose PKU Strain SYN-PKU707

[0732] To determine how cell numbers gavaged in a single gavage affect recovery of hippuric acid in the urine, PKU mice were gavaged with a single dose of SYN-PKU707 at various doses and hippuric acid levels were monitored over a period of 6 hours post-gavage. Strain SYN-PKU-707 comprises three copies of PAL driven by the FNR promoter (inserted into the chromosome at the malE/K, yicS/nepl, an dmalP/T loci), and two copies of pheP driven by the FNR promoter (inserted into the chromosome at the LacZ and agaI/rsmI loci), and the mutant FNRS24Y (see, e.g., **Fig. 47A**).

[0733] To prepare the cells, cells were diluted 1:100 in LB (2 L), grown for 1.5 h aerobically, then induced aerobically in the presence of 0.15% arabinose for 4 hours. Prior to administration, cells were concentrated 200X and frozen (10% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice. Each mouse was gavaged 750 uL total, at 3e10, 6e6, 1.2e9, and 2.4e8 cfu/mouse.

[0734] Briefly, beginning 4 days prior to the study, Pah ENU2/2 mice (~11-15 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 according to weight into groups as follows: Group 1: SYN-PKU-707 (n=6); Group 2: H2O Control (n=6). Blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. 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 1, 2 and 3h post Phe challenge, the bacteria were administered to mice by oral gavage (3X250 ul) at various doses (3e10, 6e9, 1.2e9, and 2.4e8). Sodium bicarbonate was added to final concentration of 100mM for both strains. Urine was collected from all animals up to at 1, 2, 3, 4, 5, and 6 hours post phenylalanine challenge, and total amounts of hippuric acid recovered at each time point was determined by LC/MS. **Fig. 55** shows the absolute amount of hippuric acid recovered in urine over the 6 hour time frame, as determined by mass spectroscopy. A dose-dependent increase in hippurate recovered in the urine of mice was observed.

Example 40. Hippurate Recovery in Urine: Live Cells vs. Pure TCA Gavage

[0735] To assess duration of strain activity and transit time, the kinetics of recovery of hippuric acid in the urine between live cells and pure TCA was compared. Pure TCA gavaged orally is taken up rapidly through the small intestine into the blood, converted to hippurate in the liver, and excreted in the urine. Conversely, PAL must first convert phenylalanine to TCA, before it can be taken up and converted to hippurate. Therefore, a more delayed accumulation of hippuric acid is expected, and therefore, this side by side comparison may be useful in the probing of the transit time of gavaged cells. PKU mice were

gavaged with a single dose of water, TCA, or SYN-PKU707 and hippuric acid levels were monitored over a period of 1.5 hours post-gavage.

[0736] To prepare the cells, cells were diluted 1:100 in LB (2 L), grown for 1.5 h aerobically, then induced aerobically in the presence of 0.15% arabinose for 4 hours. Prior to administration, cells were concentrated 200X and frozen (10% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice. Each mouse was gavaged 300 uL total, at 3e10 CFU cfu/mouse.

[0737] Briefly, beginning 4 days prior to the study, Pah ENU2/2 mice (~11-15 weeks of age) were maintained on normal chow and water. On Day 1, mice were randomized into treatment groups according to weight into groups as follows: Group 1: SYN-PKU-707 (3e10 CFU; n=6); Group 2: TCA (0.033mg/g; n=6); Group 3: H2O Control (n=6). The bacteria, TCA or H2O were administered to mice by oral gavage (in 300 ul). Sodium bicarbonate was added to final concentration of 100mM for both strains. Urine was collected from all animals up to at 15, 30, 45, 60, 90, and 120 minutes post gavage, and total amounts of hippuric acid recovered at each time point was determined by LC/MS. **Fig. 56** shows the absolute amount of hippuric acid recovered in urine at various time points over the 1.5 hour time frame, as determined by mass spectroscopy. The slope of the lines can be used as an indicator of transit time of the bacteria. With pure TCA, a rapid decrease in hippurate recovery in the urine within the first 15 minutes after collection starts is observed. For genetically engineered cells, hippurate recovery is sustained for at least the first 30 min after collection starts and has a decreasing downward slope. These results indicate that the cells remain in the small intestine producing TCA over a useful amount of time. Consistent with this interpretation, SYN-PKU-707 was shown to lead to decrease in blood phenylalanine in other studies described herein.

Example 41. Protein Challenge and the Effects of SYN-PKU707, SYN-PKU711 and SYNPKU712 on Blood and Urine Parameters and SITZMARKS® Gastrointestinal Transit in Cynomolgus Monkeys (Non-GLP)

[0738] Responses in cynomolgus monkeys to PKU strain administration with a protein challenge after multiple doses (Phase 1), and SITZMARKS® gastrointestinal transit are evaluated.

[0739] Cynomolgus monkeys are selected because this species is closely related, both phylogenetically and physiologically, to humans and is a species commonly used for nonclinical toxicity evaluations.

[0740] 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. Urine and plasma measurements are conducted and fecal samples are examined for bacterial load.

[0741] The following three strains are compared in the study: 1. SYN-PKU712 (comprising 3X-fnrSPAL3, 2X-fnrSpheP with knocked in Para-FNRS24Y-deltadapA (DAP auxotroph); 2. SYN-PKU707 – without LAAD (3X-fnrSPAL3, 2X-fnrSpheP with knocked in Para- FNRS24Y); 3. SYN-PKU711 – with LAAD (3X-fnrSPAL3, 2X-fnrSpheP with knocked in Para-FNRS24Y-LAAD (LAAD positive)).

Animals

[0742] Purpose bred cynomolgus monkeys (Macaca fascicularis; male; 12 Naïve animals, 3 to 6 kg (at initial physical exam); 3 to 8 years (at initial physical exam)) are used in the study. Twelve animals are used 10 of which are used for dosing. Selected animals are being examined by veterinary staff and only animals that meet facility health criteria and that are considered healthy are approved by a veterinarian for use on study.

[0743] Animals are housed in a temperature- and humidity-controlled environment. The targeted range of temperature and relative humidity is between 18 and 29 °C and 30 and 70%, respectively. An automatic lighting system is set to provide a 12-hour light/dark cycle. The animals are individually

housed/co-housed/group-housed in cages that comply with the Animal Welfare Act and recommendations set forth in the Guide for the Care and Use of Laboratory Animals (National Research Council 2011) and SNBL USA SOPs. Animals within an established co-housed/group-housed unit may be introduced to a new social partner during the study in the event of separation due to necropsy or upon veterinary recommendation.

[0744] Animals are offered PMI LabDiet® Fiber-Plus® Monkey Diet 5049 biscuits twice daily. Animals are fasted as required by specific procedures (e.g., prior to blood draws for serum chemistry, urine collection). The diet is routinely analyzed for contaminants and found to be within manufacturer's specifications. Drinking Water Fresh drinking water is provided *ad libitum* to all animals. The water is routinely analyzed for contaminants. Animals are given fruits, vegetables, other dietary supplements, and cage enrichment devices throughout the course of the study.

[0745] A stratified randomization scheme incorporating body weights is used to assign animals to study groups.

[0746] Previously quarantined animals are acclimated to the study room for a minimum of 14 days prior to initiation of dosing. Acclimation phase data is collected from all animals, including spares. As needed, assigned animals are replaced with spare animals based on results generated during the acclimation phase. Spare animals are removed from the study on or after Day 1.

Test article dosing, preparation and administration

[0747] In Phase 1 of the study, animals are dosed as indicated in **Table 73.** Animals are dosed (approximately 10^{12} bacteria per animal per dose) with a fixed volume of each test article (10 mL) via nasal gastric gavage (NG) and a fixed volume of protein challenge (20 mL). A fixed volume (2 mL) of flush with dose vehicle is administered following each dose of test article. A fixed volume (2 mL) of flush with deionized water is administered following each dose of protein challenge. This administration route is consistent with the proposed route of administration in humans.

[0748] Vanilla flavored protein shake (MET-Rx RTD51 (MET-Rx), stored at room temperature) is used for the protein challenge. Phosphate buffered saline (PBS) with 0.36M Sodium Bicarbonate and 15% Glycerol) is used as a vehicle for the administration of the strains. An additional dose vehicle is prepared by combining PBS with sodium bicarbonate and glycerol to obtain a solution containing 0.36 M sodium bicarbonate and 15% glycerol.

Table 73. Dosing Schedule

Group	Dose Schedule							Dose Route	Number of Animals
	Day 1[a,b]	Day 4[a,c]	Day 9[a,d]	Day 15[a,e]	Day 19[a]	Day 24[a]	Day 29[a,f]		Males
1	Protein + SYN-707	SYN-707 + Protein	Protein + SYN-PKU707	SYN-PKU707 + Protein	SYN-PKU707	Vehicle	SYN-PKU711	NG[g]	5
2							SYN-1967		5

[a] Food made available at least 6 hours after all dosing procedures are complete
[b] Protein Challenge administered, wait at least 30 minutes, then SYN-PKU707 administered
[c] SYN-PKU707 administered, wait at least 30 minutes, then Protein Challenge administered
[d] Protein Challenge administered, wait at least 2 hours, then SYN-PKU707 administered [e] SYN-PKU707 administered, wait at least 2 hours, then Protein Challenge administered
[f] SYN-PKU711 and SYN-PKU712 administration details to be added by amendment
[g] Doses administered via nasogastric gavage (NG)

[0749] In Phase 2 of the study, animals are assigned to groups and treated as indicated in **Table** 74. Animals are dosed via oral gavage (OG). SITZMARKS capsules are used as supplied from the manufacturer/supplier to determine transit time.

i. Table 74. Group assignments

	Dose Schedule			Number of Animals	
Group	Day 33	Level	Dose Route	Males	X-Rays[b]
1	SITZMARKS®	1 Capsule	OG[a]	12	0.5, 1, 2, 4, 6 and 12 hours postdose
2					

[a] One SITZMARKS® capsule is administered via oral gavage (OG)
[b] X-rays is administered to n=2 per time point

[0750] On day 1, a protein challenge is administered first followed by a dose of SYN-PKU707 administered at least 30 minutes after the protein challenge. On day 4, SYN-PKU707 is administered first followed by a protein challenge administered at least 30 minutes after the SYN-PKU707 dose. On day 9, a Protein challenge is administered first followed by a dose of SYN-PKU707 administered at least 2 hours after the protein challenge. On day 15, SYN-PKU707 is administered first followed by a Protein challenge to administered at least 2 hours after the SYN-PKU707 dose. On day 19, only SYN-PKU707 is administered. On day 24, only vehicle is administered. On day 29, SYN-PKU711 (Group 1) and SYNPKU712 (Group 2) is administered.

[0751] On day 37, a single SITZMARKS® capsule is administered once to each animal followed by a series of X-rays to track the contents of the capsule. X-rays are from 2 animals per time point at 0.5, 1, 3, 4, 6, and 12 hours post-dose.

[0752] On each dose day, including day 24, animals are fasted overnight and food is made available again at least 6 hours following dosing. The frequency of administration in Phase 1 is to assess the effects of a protein challenge administered at different times before, and after, the test article dose. The frequency of administration in Phase 2 is once and will assess the effects of the transit time in the gastrointestinal tract following Phase 1.

Observations and Examinations

[0753] Clinical Observations: Clinical observations are performed daily beginning on the second day of acclimation for each animal. Additional clinical observations are performed, as necessary.

[0754] Food Consumption: A qualitative evaluation of food consumption is made once daily in the morning prior to feeding, for each animal beginning on the second day of acclimation. An assessment of consumption is made based on mean number of biscuits remaining from the prior feeding. Food consumption is not assessed following overnight fasting for scheduled procedures. Food consumption data is reported as normal, low, no consumption, or removed.

[0755] Body Weight: Each animal is weighed prior to the first day of dosing, and twice weekly throughout the study. Additional body weights may be taken if necessary.

Urine and Blood Collection Procedures

[0756] Specimen collection frequency is described in **Table 75.**

Table 75. Specimen collection frequency

Time Point (Study Week)	Urine	Feces	Blood
Acclimation (Week -1)	–	–	–
Acclimation (Week -2)	–	–	–
Phase 1 Dosing (Week 1)	Days 1 and 4 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Days 1 and 4 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Days 1 and 4 at pre-dose, 2, 4, and 6 hours post-dose
Phase 1 Dosing (Week 2)	Day 9 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Days 9 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Days 9 at pre-dose, 2, 4, and 6 hours post-dose
Phase 1 Dosing (Week 3)	Days 15 and 19 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Days 15 and 19 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Days **15 and** 19 at pre-dose, 2, 4, and 6 hours post-dose
Phase 1 Dosing (Week 4)	Day 24 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Day 24 at: at least -12 hours (predose) 0 to 3 hours (post-dose)[a] 3 to 6 hours (post-dose)[a] 6 to 12 hours (post-dose)[b] 12 to 24 hours (post-dose)[b]	Day 24 at pre-dose, 2, 4, and 6 hours post-dose
Phase 1 Dosing (Week 5)	–	–	–
Phase 1 Dosing (Week 6)	–	Day 36 at: One 24 hour[b] collection from Day 35 to Day 36	–

= Not applicable
Note: All times is based on dosing of test article
[a] Collections is within +/- 10 minutes
[b] Collections is with +/- 30 minutes

[0757] Urine Collection: Urine is collected on wet ice from a urine collection pan placed beneath the animal cage or from the bladder at necropsy. Urine is collected, if available, on wet ice or gel packs from a urine collection

pan placed beneath the animal cage A sample is obtained in the morning before feeding.

[0758] Total volume of each urine sample is determined and recorded. Urine samples is stored on dry ice or at -60 to -86 °C prior to analysis.

[0759] Feces Collection: Feces is collected, if available, at ambient temperature from a screen placed over the urine collection pan beneath the animal cage. Feces samples is collected into tared containers and a total weight is recorded. Samples are placed on wet ice or refrigerated gel packs following weighing. If no sample is present a fecal swab is collected.

[0760] Feces samples are transferred to a 50 mL Falcon tube containing approximately 5 mL of PBS. If more than one tube is needed samples are split into approximately equal samples. Feces swabs are transferred to a 15 mL Falcon tube containing approximately 1 mL of PBS. Feces samples are stored at -15 to -25 °C until analysis.

[0761] Blood Collection: Blood is collected from a peripheral vein of restrained, conscious animals.

[0762] Minimum Sample Volume is 0.5 mL of blood, collected in .0.5 mL K2EDTA MAP tubes. Blood samples are centrifuged at room temperature following collection to separate plasma. Plasma is removed and placed into a tube prior to storage at -60 to -86 °C. Samples may be placed on dry ice prior to analysis.

[0763] Results from this study inform on the impact of the presence of LAAD on hippurate levels and the effect of LAAD on the level plasma phenylalanine. The study also shows the impact of the dap auxotrophy on strain activity. The study also informs on the timing of dosing relative to food intake for optimal phenylalanine conversion based on urinary hippurate levels.

Example 42. *In vitro* activity of SYN-PKU709, SYN-PKU707, and SYN-PKU708

[0764] A 1:100 back-dilution from overnight culture of SYN-PKU709- as shown in **Fig. 47C** was grown to early log phase for 1.5h before moving to the anaerobic chamber for 4 hours for induction as described herein. To perform

activity assay, 1e8 cells were resuspended and incubated in assay buffer (M9 media with 0.5% glucose, 50mM Phe, and 50mM MOPS). Supernatant samples were taken over time and TCA (the product of PAL) was measured by absorbance at 290nm to determine the rate of TCA production/PAL activity.

[0765] For strains possessing FNRS24Y (SYN-PKU707 and SYN-PKU708): Cultures (1:100 back-dilutions from overnight cultures) were grown to early log phase for 1.5h before the addition of L-arabinose at 0.15% final concentration for induction. Cultures were induced for 4 hours (aerobically). To perform activity assay, 1e8 cells were resuspended and incubated in assay buffer (M9 media with 0.5% glucose, 50mM Phe, and 50mM MOPS). Supernatant samples were taken over time and TCA (the product of PAL) was measured by absorbance at 290nm to determine the rate of TCA production/PAL activity.

[0766] All cultures shared the same level of PAL activity, 4 umol TCA produced/hr/1e9cells.

Example 43. Determination of the Kinetics of Phenylalanine in the Enterocirculation Model

[0767] To characterize the phenylalanine enterocirculation model, first the kinetics of serum levels of phenylalanine post phenylalanine challenger were assessed.

[0768] On day -6, PKU (*enu2*) mice were placed on phenylalanine-free chow and water (+)Phe (0.5g/L)

[0769] On day 1, animals were bled to get T=0 (pre Phe challenge), and animals were randomized into two groups based on phenylalanine levels measured. The first treatment group (n=15) included mice for use for the 2, 6 and 24 hour post phenylalanine challenge time points; Group 2 (n=15) included mice for the 4and 8 hour post phenylalanine challenge time points.

[0770] Mice were pre-weighed to obtain the average weight for each group. Phenylalanine was dosed at a concentration equal to average group weight. Animals were dosed subcutaneously with 0.1mg/g phenylalanine. In Group 1, animals were bled at 2, 6 and 24h post Phe challenge. In Group 2,

animals were bled at 4 and 8h post Phe challenge. Whisker plots in **Fig. 38A** and **Fig. 38B** show distribution of mouse blood phenylalanine levels (both overall phenylalanine levels **(Fig. 38A)** and change in phenylalanine levels from T0 **(Fig. 38B).** Phenylalanine levels were stably elevated over at least a 6 hour period.

[0771] Next, subcutaneous 13C-Phe challenge was performed to determine extent of recirculation in our PKU (*enu2*) mice.

[0772] On day -6, PKU mice were placed on phenylalanine-free chow and water (+)Phe (0.5g/L). Animals were pre-weighed to obtain average weight for each group. On day 1, animals were bled to obtain T=0 (pre Phe challenge). Animals were randomized into three treatment groups based on phenylalanine levels measured. Groups (n=2 per group) were as follows: Group 1=0 min (no Phe challenge); Group 2 =20 min post Phe challenge time point; Group 3=2h post Phe challenge time point. Animals were dosed phenylalanine at a concentration equal to average group weight at a subcutaneous dose of 0.1mg/g Phe. Group 1 (0 min group) did not receive a phenylalanine dose. At each time point post Phe Challenge (20 min and 2h), animals were bled and euthanized, the GI tract was removed and sectioned into small and large intestines.

[0773] For the T=0 min group. organ harvest is in absence of any Phe challenge. Sections were flushed sections with ~1ml cold PBS and effluent was collected in 1ml microfuge tubes, and samples were stored on ice. Consequently, all intestinal effluents approximately 2.5X diluted in the measurements due to the intestinal PBS flush, indicating absolute levels *in vivo* are likely be higher than shown).

[0774] As seen in **Fig. 39**, isotopic Phe injected subcutaneously (SC) is seen in the small intestine within 20 min, and enterorecirculation of labeled 13C-Phe was confirmed to occur.

[0775] Next the overall amino acid levels were measured in blood, small and large intestine in wild type and PKU mice.

[0776] On day 1, animals were fasted for 1h, and bled to obtain T=0. Animals were euthanized and organs were harvested for each animal. The GI

tract was removed and sectioned into small and large intestines. Sections were flushed with ~1ml cold PBS and effluent was collected in 1ml microfuge tubes. Samples were stored on ice (blood and intestinal effluents) until LC-MS analysis. As shown in **Fig. 40**, phenylalanine levels were high in *enu2* blood, but no other major differences between WT and *enu2* mice were observed.

Example 44. Efficacy of PKU Probiotic Strain SYN-PKU305 Suspended in Gelatin in Mouse Drinking Water

[0777] The efficacy of SYN-PKU305 suspended in gelatin in mouse drinking water was assessed. On day -7, animals (Enu PKU mice) were placed on normal chow and water. *Enu*2 mice were kept on regular chow throughout experiment. On day 1, animals were randomized into treatment groups and bled to get baseline blood phenylalanine (Phe) levels (T=0). Mice were grouped as follows: Group 1: H20 (n=12); Group 2: SYN-PKU305 (n=12); For Group 2, drinking water was changed to H2O(+) SYN-PKU305 (5e9cells/ml) and 0.125% gelatin, 5% sucrose and ATC at 0.02 mg/L; to make the mixture, 20ml of SYN-PKU305 mixed into 280ml H2O(+) 0.125% gelatin, 5% sucrose and split between 2 cages of animals. On day 3, the SYN-PKU305-containing water bottle was changed. On day 4, treatment groups for d=4 post gelatin/water addition were bled for blood phenylalanine analysis. On day 5, the SYN-PKU305 -containing water bottle was changed. On day 8.all treatment groups were bled for d=8 post gelatin/water addition blood phenylalanine analysis.

[0778] To prepare the cells for this study, cells were diluted 1:100 in LB, grown for 1.5 h aerobically, then ATC was added to cultures at a concentration of 100 ng/mL and cells were grown for an additional 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 mixed 9:1 in 1M bicarbonate.

[0779] Results are shown in **Fig. 42A** and **Fig. 42B**, and show that absolute phenylalanine levels are reduced in SYN-PKU305 treated mice as compared to the water treated mice at both 4 days and the 8 days post phenylalanine challenge.

Example 45. Efficacy of PKU Probiotic Strain SYN-PKU707 Suspended in Gelatin in Mouse Drinking Water

[0780] The efficacy of SYN-PKU707 (3XP*fnrS*-PAL3; 2XP*fnrS*-*pheP;* P*ara*-*fnr*S24Y*)* suspended in gelatin in mouse drinking water, which is changed on a daily basis, is assessed. On day -7, animals (Enu PKU mice) are placed on normal chow and water. *Enu*2 mice are kept on regular chow throughout experiment. On day 1, animals are randomized into treatment groups and bled to get baseline blood phenylalanine (Phe) levels (T=0). Mice are grouped as follows: Group 1: H20 (n=12); Group 2: SYN-PKU901 (n=12; streptomycin resistant Nissle); Group 3: SYN-PKU707 (n=12). For Groups 2 and 3, drinking water is changed to H2O with and 0.125% gelatin, 5% sucrose and SYN-PKU901 (1e10cells/ml) or SYN-PKU707 (1e10cells/ml). On days 2 and 3, the SYN-PKU901 and SYN-PKU707-containing water bottles are replaced with new bottles containing fresh mixtures of the bacterial strains. On day 3, mice are moved into metabolic cages (4 cages per group, 3 mice per cage). On day 4, treatment groups for d=4 post gelatin/water addition are bled for blood phenylalanine analysis and urine is collected for hippuric acid analysis. Fecal pellets are collected to determine the bacterial fecal count.

[0781] To prepare the cells for this study, cells are grown in fermentation media to OD 0.2 and then induced for an additional 4 hours in the presence of arabinose at 0.15% final concentration before cells are concentrated and frozen (15% glycerol, 2 g/L glucose, in PBS). Cells are thawed on ice, and mixed 9:1 in 1M bicarbonate.

[0782] A second study is carried out essentially as described above, except that Enu2 mice are kept on a low Phe chow throughout the study, starting at day -7.

Example 46. Fast Dietary Model of PKU

[0783] The efficacy of SYN-PKU305 was assessed in a fast dietary model of PKU. In this model, Enu2 mice were kept on regular chow until time of dosing. Chow was taken away at T0. Mice were dosed according to the regimen described below.

[0784] To prepare the cells for this study, 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 mixed 9:1 in 1M bicarbonate.

[0785] On day -5, animals were placed on normal chow and water. On day 1, animals were randomized into treatment groups according to similar average weights between groups as follows: Group 1: H2O Control (n=12); Group 2: SYN-PKU901-200ul (n=12); Group 3: SYN-PKU305-200ul (n=12). Animals were fasted for 4 hours, and bled to obtain baseline blood phenylalanine levels (T=0 post fast). Each group was dosed by oral gavage at 0.5 and 1.5 and 2.5h post fast. Each mouse was gavaged with 600uL total, or 1X10e11 CFUs. All treatment groups were bled 2 and 4h post fast. After last bleed animals were placed on Phe-free chow and H2O(+)0.5g/L Phe.

[0786] To prepare the cells for this study, 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 mixed 9:1 in 1M bicarbonate. Results in **Fig. 41A** and **Fig. 41B** show SYN-PKU305-treated mice show more rapid decrease in serum Phe.

Example 47. Hippurate Recovery in Mice Gavaged with Labeled Phenylalanine

[0787] Hippurate recovery in mice injected with labeled phenylalanine and gavaged three times with SYN-PKU305 was assessed.

[0788] To prepare the cells for this study, cells were diluted 1:100 in LB (2 L), grown for 1.5 h aerobically, then induced aerobically in the presence of 0.15% arabinose for 4 hours. Prior to administration, cells were concentrated 200X and frozen (10% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice.

[0789] Beginning 4 days prior to the study, Pah ENU2/2 mice (~11-15 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 according to weight into groups as follows: Group 1: SYN-PKU-707 (n=6 (3XP*fnrS*-PAL3; 2XP*fnrS*-*pheP*; P*ara*-*fnr*S24Y)); Group 2: SYN-PKU901 (n=6; (streptomycin resistant Nissle)); Group 3: H2O Control (n=6). Blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. Mice were then administered single dose of radiolabeled phenylalanine by subcutaneous injection at 0.1 mg per gram body weight, according to the average group weight. At 1, 2 and 3h post Phe challenge, the bacteria were administered to mice by oral gavage (3X250 ul) at 3 doses of 3X10e10). Sodium bicarbonate was added to final concentration of 100mM for both strains. Urine was collected from all animals up to at 1, 2, 3, 4, 5, and 6 hours post phenylalanine challenge, and amounts of unlableled and radiolabeled of hippuric acid recovered at each time point were determined by LC/MS. **Fig. 54A** and **Fig. 54B** show the amount of unlabeled and labeled hippuric acid recovered in urine over the 6 hour time frame, as determined by mass spectroscopy. An increase in hippurate recovered in the urine of mice was observed. ~4.5% of the hippurate recovered in the urine was heavy, suggesting a majority of the phenylalanine degraded in this study is pre-existing in the SI and not that which is injected SC.

Example 48. *In vivo* Administration and Efficacy of SYN-PKU708 at Various Doses

[0790] The ability of engineered probiotic strain SYN-PKU-708 change levels of phenylalanine post SC injection and to convert phenylalanine to hippurate was assessed at various doses. Strain SYN-PKU-707 comprises three copies of PAL driven by the FNR promoter (inserted into the chromosome at the malE/K, yicS/nepl, an dmalP/T loci), and two copies of pheP driven by the FNR promoter (inserted into the chromosome at the LacZ and agaI/rsmI loci), and the mutant FNRS24Y-LAAD knocked into the arabinose operon, which is transcribed as a bicistronic message (see, e.g., **Fig. 47B**).

[0791] Cultures (1:100 back-dilutions from overnight cultures) were grown to early log phase for 1.5h before the addition of L-arabinose at 0.15% final concentration for induction. Cultures were induced for 4 hours (aerobically). .Prior to administration, cells were concentrated 200X and frozen

(10% glycerol, 2 g/L glucose, in PBS). Cells were thawed on ice, and mixed 9:1 in 1M bicarbonate. Each mouse was gavaged 750uL total, or 5.3X10e11, 1.8X10e11, 6X10e11, 2X10e9 cfu/mouse.

[0792] Beginning 4 days prior to the study (i.e., Days -4-1), Pah ENU2/2 mice (~11-15 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 according to weight as follows: Group 1: SYN-PKU-708 (n=9; dosing with 5.3X10e11 CFU); Group 2: SYN-PKU-708 (n=6; dosing with 1.8X10e11 CFU); Group3: SYN-PKU-708 (n=6; dosing with 6X10e11 CFU); Group 4: SYN-PKU-708 (n=6; dosing with 2X10e9 CFU); Group 5: H2O Control (n=6).

[0793] Animals were transferred to metabolic cages (3 mice per cage, 3 cages per group) and blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. 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 1, 2 and 3h post Phe challenge, the bacteria (or water) were administered to mice by oral gavage (3X250 ul; 5.3X10e11, 1.8X10e11, 6X10e11, 2X10e9 cfu/mouse). Sodium bicarbonate was added to final concentration of 100mM for both strains. Animals were bled and urine was collected from all animals up to 4h post Phe challenge. All treatment groups were bled at 4h post phenylalanine challenge. Blood and urine was stored on ice for LC/MS analysis.

[0794] **Fig. 57A** depicts blood phenylalanine concentrations relative to baseline; and the total reduction in Δphe is listed in **Table 76**.

Table 76. Decrease in delta[Phe] (%)

Dose	Decrease in delta[Phe] (%)
SYN1967 5.3e10	37
SYN1967 1.8e10	46
SYN1967 6e9	24
SYN1967 2e9	26

[0795] **Fig. 57B** depicts the urine hippurate concentration at 4 hours post phenylalanine injection. Results in **Fig. 57** show that SYN-PKU708 was efficacious in reducing blood phenylalanine and that hippurate was excreted in a dose dependent manner in the cages of mice treated with SYN-PKU708, indicating that the cells were active *in vivo*.

Example 48. Transit Time of Bacteria (SYN-PKU707) following single gavage of ~3e10 cfu

[0796] Localization and intestinal residence time of SYN-UCD707 ($3XP_{fnrS}$-PAL3; $2XP_{fnrS}$*pheP*; P_{ara}-*fnr*S24Y, **Fig. 47A**) was assessed. Mice were gavaged, sacrificed at various time points, and effluents were collected from the upper, mid, and lower small intestine.

[0797] Bacterial cultures were grown overnight and pelleted. The pellets were resuspended in PBS. Mice (C57BL6/J, 10-12 weeks old) were gavaged once with 100 µL of bacteria (approximately 3X10e10 CFU). Drinking water for the mice was changed to contain 0.1 mg/mL anhydrotetracycline (ATC) and 5% sucrose for palatability. At each timepoint (15, 30, 45, and 60 minutes post-gavage), animals were euthanized, and the intestine was removed. The small intestine was cut into three equal pieces. Each section was flushed with 0.5 ml cold PBS and collected in separate 1.5 ml tubes. Intestinal effluents were placed on ice for serial dilution plating.

[0798] In order to determine the CFU of bacteria in each effluent, the effluent was serially diluted, and plated onto LB plates containing kanamycin. The plates were incubated at 37°C overnight, and colonies were counted. The amount of bacteria and residence time of SYN-UCD707 seen in each compartment is shown in **Fig. 58**.

Example 50. Activity of Inducible FNRS24Y Expressing Strain SYN-PKU707 Grown under Aerobic Conditions

[0799] The activity of SYN-PKU707 ($3XP_{fnrS}$-PAL3; $2XP_{fnrS}$*pheP*; P_{ara}-fnr^{S24Y}), a strain expressing FNRS24Y under the control of the arabinose promoter, was assessed under aerobic growth conditions and compared to the activity achieved under anaerobic conditions.

[0800] Overnight cultures of SYN-PKU707, comprising 3XP$_{fnrS}$-PAL3; 2XP$_{fnrS}$*pheP*; P$_{ara}$-*fnr*^{S24Y}were diluted 1:100, and were grown to early log phase for 1.5h. Cells were grown aerobically for an additional 4 hours in the presence or absence of the inducer arabinose at 0.15% final concentration in 10 ml, 20 ml, or 30 ml flasks. In parallel, in separate samples, the strain was also induced anaerobically for 4 hours in the presence or absence of arabinose. To perform the activity assay, 1e9 cells were resuspended and incubated in assay buffer (M9 media with 0.5% glucose, 50mM Phe, and 50mM MOPS). Supernatant samples were taken over time and TCA (the product of PAL) was measured by absorbance at 290nm to determine the rate of TCA production/PAL activity. As seen in **Fig. 51**, arabinose-induced expression of fnrS24Y results in high level activity under aerobic conditions in 10 ml, 20 ml, or 30 ml flasks. Additionally, activation in the absence of arabinose under anaerobic conditions is maintained. This indicates that this strain is efficiently pre-induced under aerobic conditions prior to *in vivo* administration. These results also provide an indication that anaerobic activation without arabinose "*in vivo*" activation is likely conserved in this strain.

Example 51. Activity Comparison of SYN2619 vs SYN1967 in the Inducible Acute PKU Model

[0801] *In vivo* activity of two strains, SYN-PKU710 (as shown in **Fig. 47D** and SYN-PKU708 (as shown in **Fig. 47A**) was compared in the Pah ENU2/2 PKU mouse model. SYN-PKU708, comprises three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two chromosomal copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). SYN-PKU708 further comprises one knocked in copy of the mutated FNR transcription factor FNRS24Y (Para::FNRS24Y) and one copy of LAAD inserted at the same insertion site (the arabinose operon), which is transcribed as a bicistronic message from the endogenous arabinose promoter. SYN-PKU708 further comprises a deltadapA (dapA auxotrophy). SYN-PKU710 comprises three chromosomal insertions of PAL3 (3X fnrS-PAL (malP/T, yicS/nepI, malE/K)) and two chromosomal copies of pheP (2XfnrS-pheP (lacZ, agaI/rsmI)). SYN-PKU710 further comprises two copies of PAL driven by the IPTG inducible

Lac-promoter (2Xlac-PAL (exo/cea and rhtC/rhtB)) and one copy of the LAAD knocked into the arabinose operon with expression driven by the native Para promoter (Para::LAAD). SYN-PKU710 is a dapA auxotroph.

[0802] Beginning 4 days prior to the study (i.e., Days -4-1), Pah ENU2/2 mice (~11-15 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 weighed to determine the average weight for each group.and were randomized into treatment groups according to weight as follows: Group 1: H2O Control (n=12); Group 2: SYN-PKU-710 (n=12); Group3: SYN-PKU-708 (n=12). Blood samples were collected by sub-mandibular skin puncture to determine baseline phenylalanine levels.

[0803] 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 1, 2 and 3h post Phe challenge, the bacteria (or water) were administered to mice by oral gavage (300 ul/dose, total of 3Xe10 cfu/mouse administered in three doses). Sodium bicarbonate was added to final concentration of 100 mM for both strains. Urine was collected from all animals up to 4h post Phe challenge. All treatment groups were bled at 4h post phenylalanine challenge. Blood and urine was stored on ice for LC/MS analysis. Blood phenylalanine concentrations relative to baseline at 4 hours post SC phenylalanine injection are shown in **Fig. 59A** The percentage decrease in dPhe SYN-PKU710 and SYN-PKU708 were calculated to be 29% and 40%, respectively. Total hippurate recovered in urine is shown in **Fig. 59B**. Negligible hippurate was recovered in mice treated with dH20.

[0804] Cells for this study were prepared in a fermenter as follows. For SYN-PKU708, a freezer vial was thawed and used to inoculate a flask culture of fermentation medium composed of glycerol, yeast extract, soytone, and buffer, and DAP (SYN-PKU708 is deltaDapA). The flask was grown overnight and used to inoculate a fermentation tank containing the same medium at 37°C, pH7, and 60% dissolved oxygen. Following a short initial growth phase, the culture was induced with 0.6 mM arabinose to turn on expression of FNRS24Y to induce FNR-driven PAL and PheP expression. Cells were concentrated by

centrifugation and resuspended in a formulation buffer comprising glycerol, sucrose, and buffer to protect the cells during freezing at <-60°C.

[0805] For SYN-PKU710, a freezer vial was and used thawed to inoculate a flask culture of fermentation medium composed of glycerol, yeast extract, soytone, buffer, and DAP (SYN-PKU708 is deltaDapA). The flask was grown overnight and used to inoculate a fermentation tank containing the same medium at 37°C, pH7, and 30% dissolved oxygen. Following a short initial growth phase, the culture was induced with 1mM IPTG to turn on expression of the Plac promoters controlling expression of PAL. LAAD expression was not induced in this study. Following 5 hours of activation, the cells were concentrated by centrifugation and resuspended in a formulation buffer (comprising glycerol, sucrose, and buffer) to protect the cells during freezing at <-60°C.

Example 52. Assessment of *in vitro* and in vivo activity of Biosafety System Containing Strain

[0806] The activity of the following strains are tested:

[0807] SYN-PKU1001 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL)

[0808] SYN-PKU1002 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS;dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0809] SYN-PKU1003 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL).

[0810] SYN-PKU1004 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0811] SYN-PKU1005 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL)

[0812] SYN-PKU1006 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0813] SYN-PKU1007 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-

Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL).

[0814] SYN-PKU1008 comprises two chromosomal copies of pheP (lacZ::PfnrS-pheP, agaI/rsmI::PfnrS-pheP) and a construct shown in **Fig. 61D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0815] SYN-PKU1009 a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL)

[0816] SYN-PKU1010 a construct shown in **Fig. 61C** knocked into the dapA locus on the bacterial chromosome (low copy RBS;dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61A**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0817] SYN-PKU1011 comprises a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL).

[0818] SYN-PKU1012 a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; dapA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in Fig. 61A, except

that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0819] SYN-PKU1013 comprises a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL)

[0820] SYN-PKU1014 comprises a construct shown in **Fig. 61C** knocked into the thyA locus on the bacterial chromosome (low copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0821] SYN-PKU1015 comprises a construct shown in **Fig. 61D** knocked into the dapA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65B** (LacI Fnrs-Ptac-PAL-PAL).

[0822] SYN-PKU1016 comprises a construct shown in **Fig. 61D** knocked into the thyA locus on the bacterial chromosome (medium copy RBS; thyA::constitutive prom1 (BBA_J26100)-Pi(R6K)-constitutive promoter 2(P1)-Kis antitoxin). The strain further comprises a plasmid shown in **Fig. 61B**, except that the bla gene is replaced with the construct of **Fig. 65D** (lacI –Ptac-PAL-PAL).

[0823] Cells are grown overnight in LB and diluted 1:100. After 1.5 hrs of growth, Cells are grown for 4 hours in the presence of 1 mM IPTG 1mM to turn on expression of the Plac promoters controlling expression of PAL (and in some cases PheP). Bacteria are spun down and are resuspended in assay buffer containing 50 mM phenylalanine. Aliquots are removed from cell assays every

20 min for 1.5 hrs for trans-cinnamate quantification by absorbance at 290 nm. In another study, the same constructs used above are employed, with the strain further comprising chromosomally integrated Para-LAAD, which is induced in parallel with PLacI. In another study the same constructs as above are employed except that the strains further comprise chromosomally integrated Para-FNRS24Y. In another study the same constructs as above are employed except that the strains further comprise chromosomally integrated Para-FNRS24Y-LAAD.

[0824] Sequences for the construction of these constructs are shown in **Table 77**, **Table 78**, and **Table 79**. In some embodiments, the PAL3 used in the above strains is codon optimized. In other embodiments, the original PAL3 sequence from Photorhabdus chemiluminescens as described herein is used in any of the constructs described above and in **Fig. 65**.

[0825] *In vivo* studies are conducted as described in Example 51, Example 44 and elsewhere herein.

Table 77. Biosafety System Constructs and Sequence Components

Description	**Sequence**	**SEQ ID NO**
Biosafety Plasmid System Component – dapA Biosafety Plasmid System Vector sequences, comprising dapA, Kid Toxin and R6K minimal ori, and promoter elements driving expression of these components, as shown in **Fig. 61A**	ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAG GGTTATTGTCTCATGAGCGGATACATATTTGAATGT ATTTAGAAAAATAAACAAATAGGGGAATTAAAAAA AAGCCCGCTCATTAGGCGGGCTACTACCTAGGCCG CGGCCGCGCGAATTCGAGCTCGGTACCCGGGGATC CTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGG CCGCGTCGTGACTGGGAAAACCCTGGCGACTAGTC TTGGACTCCTGTTGATAGATCCAGTAATGACCTCAG AACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCC GCCGGGCGTTTTTTATTGGTGAGAATCCAGGGGTCC CCAATAATTACGATTTAAATCACAGCAAACACCAC GTCGGCCCTATCAGCTGCGTGCTTTCTATGAGTCGT TGCTGCATAACTTGACAATTAACATCCGGCTCGTAG GGTTTGTGGAGGGCCCAAGTTCACTTAAAAAGGAG ATCAACAATGAAAGCAATTTTCGTACTGAAACATCT TAATCATGCTGGGGAGGGTTTCTAATGTTCACGGGA AGTATTGTCGCGATTGTTACTCCGATGGATGAAAAA GGTAATGTCTGTCGGGCTAGCTTGAAAAAACTGATT GATTATCATGTCGCCAGCGGTACTTCGGCGATCGTT TCTGTTGGCACCACTGGCGAGTCCGCTACCTTAAAT	81

	CATGACGAACATGCTGATGTGGTGATGATGACGCT GGATCTGGCTGATGGGCGCATTCCGGTAATTGCCGG GACCGGCGCTAACGCTACTGCGGAAGCCATTAGCC TGACGCAGCGCTTCAATGACAGTGGTATCGTCGGCT GCCTGACGGTAACCCCTTACTACAATCGTCCGTCGC AAGAAGGTTTGTATCAGCATTTCAAAGCCATCGCTG AGCATACTGACCTGCCGCAAATTCTGTATAATGTGC CGTCCCGTACTGGCTGCGATCTGCTCCCGGAAACGG TGGGCCGTCTGGCGAAAGTAAAAAATATTATCGGA ATCAAAGAGGCAACAGGGAACTTAACGCGTGTAAA CCAGATCAAAGAGCTGGTTTCAGATGATTTTGTTCT GCTGAGCGGCGATGATGCGAGCGCGCTGGACTTCA TGCAATTGGGCGGTCATGGGGTTATTTCCGTTACGG CTAACGTCGCAGCGCGTGATATGGCCCAGATGTGC AAACTGGCAGCAGAAGGGCATTTTGCCGAGGCACG CGTTATTAATCAGCGTCTGATGCCATTACACAACAA ACTATTTGTCGAACCCAATCCAATCCCGGTGAAATG GGCATGTAAGGAACTGGGTCTTGTGGCGACCGATA CGCTGCGCCTGCCAATGACACCAATCACCGACAGT GGCCGTGAGACGGTCAGAGCGGCGCTTAAACATGC CGGTTTGCTGTAAGACTTTTGTCAGGTTCCTACTGT GACGACTACCACCGATAGACTGGAGTGTTGCTGCG AAAAAACCCCGCCGAAGCGGGGTTTTTTGCGAGAA GTCACCACGATTGTGCTTTACACGGAGTAGTCGGCA GTTCCTTAAGTCAGAATAGTGGACAGGCGGCCAAG AACTTCGTTCATGATAGTCTCCGGAACCCGTTCGAG TCGTTTTCCGCCCCGTGCTTTCATATCAATTGTCCGG GGTTGATCGCAACGTACAACACCTGTGGTACGTATG CCAACACCATCCAACGACACCGCAAAGCCGGCAGT GCGGGCAAAATTGCCTCCGCTGGTTACGGGCACAA CAACAGGCAGGCGGGTCACGCGATTAAAGGCCGCC GGTGTGACAATCAGCACCGGCCGCGTTCCCTGCTGC TCATGACCTGCGGTAGGATCAAGCGAGACAAGCCA GATTTCCCCTCTTTCCATCTAGTATAACTATTGTTTC TCTAGTAACATTTATTGTACAACACGAGCCCATTTT TGTCAAATAAATTTTAAATTATATCAACGTTAATAA GACGTTGTCAATAAAATTATTTTGACAAAATTGGCC GGCCGGCGCGCCGATCTGAAGATCAGCAGTTCAAC CTGTTGATAGTACGTACTAAGCTCTCATGTTTCACG TACTAAGCTCTCATGTTTAACGTACTAAGCTCTCAT GTTTAACGAACTAAACCCTCATGGCTAACGTACTAA GCTCTCATGGCTAACGTACTAAGCTCTCATGTTTCA CGTACTAAGCTCTCATGTTTGAACAATAAAATTAAT ATAAATCAGCAACTTAAATAGCCTCTAAGGTTTTAA GTTTTATAAGAAAAAAAAGAATATATAAGGCTTTT AAAGCCTTTAAGGTTTAACGGTTGTGGACAACAAG CCAGGGATGTAACGCACTGAGAAGCCCTTAGAGCC TCTCAAAGCAATTTTGAGTGACACAGGAACACTTA ACGGCTGACATGGGGCGCGCCCAGCTGTCTAGGGC	

	GGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGAC AAACAACAGATAAAACGAAAGGCCCAGTCTTTCGA CTGAGCCTTTCGTTTTATTTGATGCCT	
Biosafety Plasmid System Component – ThyA Biosafety Plasmid System Vector sequences, comprising ThyA, Kid Toxin and R6K minimal ori, and promoter elements driving expression of these components, as shown in **Fig. 61B**	ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAG GGTTATTGTCTCATGAGCGGATACATATTTGAATGT ATTTAGAAAAATAAACAAATAGGGGAATTAAAAAA AAGCCCGCTCATTAGGCGGGCTACTACCTAGGCCG CGGCCGCGCGAATTCGAGCTCGGTACCCGGGGATC CTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGG CCGCGTCGTGACTGGGAAAACCCTGGCGACTAGTC TTGGACTCCTGTTGATAGATCCAGTAATGACCTCAG AACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCC GCCGGGCGTTTTTTATTGGTGAGAATCCAGGGGTCC CCAATAATTACGATTTAAATCACAGCAAACACCAC GTCGGCCCTATCAGCTGCGTGCTTTCTATGAGTCGT TGCTGCATAACTTGACAATTAATCATCCGGCTCGTA GGGTTTGTGGAGGGCCCAAGTTCACTTAAAAAGGA GATCAACAATGAAAGCAATTTTCGTACTGAAACAT CTTAATCATGCTGGGGAGGGTTTCTAATGAAACAGT ATTTAGAACTGATGCAAAAAGTGCTCGACGAAGGC ACACAGAAAAACGACCGTACCGGAACCGGAACGCT TTCCATTTTTGGTCATCAGATGCGTTTTAACCTGCA AGATGGATTCCCGCTGGTGACAACTAAACGTTGCC ACCTGCGTTCCATCATCCATGAACTGCTGTGGTTTC TTCAGGGCGACACTAACATTGCTTATCTACACGAAA ACAATGTCACCATCTGGGACGAATGGGCCGATGAA AACGGCGACCTCGGGCCAGTGTATGGTAAACAGTG GCGTGCCTGGCCAACGCCAGATGGTCGTCATATTGA CCAGATCACTACGGTACTGAACCAGCTGAAAAACG ACCCGGATTCGCGCCGCATTATTGTTTCAGCGTGGA ACGTAGGCGAACTGGATAAAATGGCGCTGGCACCG TGCCATGCATTCTTCCAGTTCTATGTGGCAGACGGC AAACTCTCTTGCCAGCTTTATCAGCGCTCCTGTGAC GTCTTCCTCGGCCTGCCGTTCAACATTGCCAGCTAC GCGTTATTGGTGCATATGATGGCGCAGCAGTGCGAT CTGGAAGTGGGTGATTTTGTCTGGACCGGTGGCGAC ACGCATCTGTACAGCAACCATATGGATCAAACTCAT CTGCAATTAAGCCGCGAACCGCGTCCGCTGCCGAA GTTGATTATCAAACGTAAACCCGAATCCATCTTCGA CTACCGTTTCGAAGACTTTGAGATTGAAGGCTACGA TCCGCATCCGGGCATTAAAGCGCCGGTGGCTATCTA AGACTTTTGTCAGGTTCCTACTGTGACGACTACCAC CGATAGACTGGAGTGTTGCTGCGAAAAAACCCCGC CGAAGCGGGGTTTTTTGCGAGAAGTCACCACGATT GTGCTTTACACGGAGTAGTCGGCAGTTCCTTAAGTC AGAATAGTGGACAGGCGGCCAAGAACTTCGTTCAT GATAGTCTCCGGAACCCGTTCGAGTCGTTTTCCGCC CCGTGCTTTCATATCAATTGTCCGGGGTTGATCGCA ACGTACAACACCTGTGGTACGTATGCCAACACCATC	82

	CAACGACACCGCAAAGCCGGCAGTGCGGGCAAAAT TGCCTCCGCTGGTTACGGGCACAACAACAGGCAGG CGGGTCACGCGATTAAAGGCCGCCGGTGTGACAAT CAGCACCGGCCGCGTTCCCTGCTGCTCATGACCTGC GGTAGGATCAAGCGAGACAAGCCAGATTTCCCCTC TTTCCATCTAGTATAACTATTGTTTCTCTAGTAACAT TTATTGTACAACACGAGCCCATTTTTGTCAAATAAA TTTTAAATTATATCAACGTTAATAAGACGTTGTCAA TAAAATTATTTTGACAAAATTGGCCGGCCGGCGCGC CGATCTGAAGATCAGCAGTTCAACCTGTTGATAGTA CGTACTAAGCTCTCATGTTTCACGTACTAAGCTCTC ATGTTTAACGTACTAAGCTCTCATGTTTAACGAACT AAACCCTCATGGCTAACGTACTAAGCTCTCATGGCT AACGTACTAAGCTCTCATGTTTCACGTACTAAGCTC TCATGTTTGAACAATAAAATTAATATAAATCAGCAA CTTAAATAGCCTCTAAGGTTTTAAGTTTTATAAGAA AAAAAAGAATATATAAGGCTTTTAAAGCCTTTAAG GTTTAACGGTTGTGGACAACAAGCCAGGGATGTAA CGCACTGAGAAGCCCTTAGAGCCTCTCAAAGCAAT TTTGAGTGACACAGGAACACTTAACGGCTGACATG GGGCGCGCCCAGCTGTCTAGGGCGGCGGATTTGTC CTACTCAGGAGAGCGTTCACCGACAAACAACAGAT AAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCG TTTTATTTGATGCCT	
Kid toxin (reverse orientation)	TTAAGTCAGAATAGTGGACAGGCGGCCAAGAACTT CGTTCATGATAGTCTCCGGAACCCGTTCGAGTCGTT TTCCGCCCCGTGCTTTCATATCAATTGTCCGGGGTT GATCGCAACGTACAACACCTGTGGTACGTATGCCA ACACCATCCAACGACACCGCAAAGCCGGCAGTGCG GGCAAAATTGCCTCCGCTGGTTACGGGCACAACAA CAGGCAGGCGGGTCACGCGATTAAAGGCCGCCGGT GTGACAATCAGCACCGGCCGCGTTCCCTGCTGCTCA TGACCTGCGGTAGGATCAAGCGAGACAAGCCAGAT TTCCCCTCTTTCCAT	83
dapA	ATGTTCACGGGAAGTATTGTCGCGATTGTTACTCCG ATGGATGAAAAAGGTAATGTCTGTCGGGCTAGCTT GAAAAAACTGATTGATTATCATGTCGCCAGCGGTA CTTCGGCGATCGTTTCTGTTGGCACCACTGGCGAGT CCGCTACCTTAAATCATGACGAACATGCTGATGTGG TGATGATGACGCTGGATCTGGCTGATGGGCGCATTC CGGTAATTGCCGGGACCGGCGCTAACGCTACTGCG GAAGCCATTAGCCTGACGCAGCGCTTCAATGACAG TGGTATCGTCGGCTGCCTGACGGTAACCCCTTACTA CAATCGTCCGTCGCAAGAAGGTTTGTATCAGCATTT CAAAGCCATCGCTGAGCATACTGACCTGCCGCAAA TTCTGTATAATGTGCCGTCCCGTACTGGCTGCGATC TGCTCCCGGAAACGGTGGGCCGTCTGGCGAAAGTA AAAAATATTATCGGAATCAAAGAGGCAACAGGGAA CTTAACGCGTGTAAACCAGATCAAAGAGCTGGTTTC	84

	AGATGATTTTGTTCTGCTGAGCGGCGATGATGCGAG CGCGCTGGACTTCATGCAATTGGGCGGTCATGGGGT TATTTCCGTTACGGCTAACGTCGCAGCGCGTGATAT GGCCCAGATGTGCAAACTGGCAGCAGAAGGGCATT TTGCCGAGGCACGCGTTATTAATCAGCGTCTGATGC CATTACACAACAAACTATTTGTCGAACCCAATCCAA TCCCGGTGAAATGGGCATGTAAGGAACTGGGTCTT GTGGCGACCGATACGCTGCGCCTGCCAATGACACC AATCACCGACAGTGGCCGTGAGACGGTCAGAGCGG CGCTTAAACATGCCGGTTTGCTGTAA	
thyA	ATGAAACAGTATTTAGAACTGATGCAAAAAGTGCT CGACGAAGGCACACAGAAAAACGACCGTACCGGA ACCGGAACGCTTTCCATTTTTGGTCATCAGATGCGT TTTAACCTGCAAGATGGATTCCCGCTGGTGACAACT AAACGTTGCCACCTGCGTTCCATCATCCATGAACTG CTGTGGTTTCTTCAGGGCGACACTAACATTGCTTAT CTACACGAAAACAATGTCACCATCTGGGACGAATG GGCCGATGAAAACGGCGACCTCGGGCCAGTGTATG GTAAACAGTGGCGTGCCTGGCCAACGCCAGATGGT CGTCATATTGACCAGATCACTACGGTACTGAACCAG CTGAAAAACGACCCGGATTCGCGCCGCATTATTGTT TCAGCGTGGAACGTAGGCGAACTGGATAAAATGGC GCTGGCACCGTGCCATGCATTCTTCCAGTTCTATGT GGCAGACGGCAAACTCTCTTGCCAGCTTTATCAGCG CTCCTGTGACGTCTTCCTCGGCCTGCCGTTCAACAT TGCCAGCTACGCGTTATTGGTGCATATGATGGCGCA GCAGTGCGATCTGGAAGTGGGTGATTTTGTCTGGAC CGGTGGCGACACGCATCTGTACAGCAACCATATGG ATCAAACTCATCTGCAATTAAGCCGCGAACCGCGTC CGCTGCCGAAGTTGATTATCAAACGTAAACCCGAA TCCATCTTCGACTACCGTTTCGAAGACTTTGAGATT GAAGGCTACGATCCGCATCCGGGCATTAAAGCGCC GGTGGCTATCTAA	85
Kid toxin polypeptide	MERGEIWLVSLDPTAGHEQQGTRPVLIVTPAAFNRVT RLPVVVPVTSGGNFARTAGFAVSLDGVGIRTTGVVRC DQPRTIDMKARGGKRLERVPETIMNEVLGRLSTILT*	86
dapA polypeptide	MFTGSIVAIVTPMDEKGNVCRASLKKLIDYHVASGTS AIVSVGTTGESATLNHDEHADVVMMTLDLADGRIPVI AGTGANATAEAISLTQRFNDSGIVGCLTVTPYYNRPS QEGLYQHFKAIAEHTDLPQILYNVPSRTGCDLLPETVG RLAKVKNIIGIKEATGNLTRVNQIKELVSDDFVLLSGD DASALDFMQLGGHGVISVTANVAARDMAQMCKLAA EGHFAEARVINQRLMPLHNKLFVEPNPIPVKWACKEL GLVATDTLRLPMTPITDSGRETVRAALKHAGLL	87
ThyA polypeptide	MKQYLELMQKVLDEGTQKNDRTGTGTLSIFGHQMRF NLQDGFPLVTTKRCHLRSIIHELLWFLQGDTNIAYLHE NNVTIWDEWADENGDLGPVYGKQWRAWPTPDGRHI DQITTVLNQLKNDPDSRRIIVSAWNVGELDKMALAPC HAFFQFYVADGKLSCQLYQRSCDVFLGLPFNIASYAL	88

	LVHMMAQQCDLEVGDFVWTGGDTHLYSNHMDQTH LQLSREPRPLPKLIIKRKPESIFDYRFEDFEIEGYDPHPG IKAPVAI*	

Table 78. Chromosomally Inserted Biosafety System Constructs

Description	Sequence	SEQ ID NO
Biosafety Chromosomal Construct - low copy Rep (Pi) and Kis antitoxin (as shown in **Fig. 61C)**	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGGAT CTGCTGGAACAGGTGGTGAGACTCAAGGTCATGATGGA CGTGAACAAAAAAACGAAAATTCGCCACCGAAACGAGC TAAATCACACCCTGGCTCAACTTCCTTTGCCCGCAAAGC GAGTGATGTATATGGCGCTTGCTCCCATTGATAGCAAAG AACCTCTTGAACGAGGGCGAGTTTTCAAAATTAGGGCTG AAGACCTTGCAGCGCTCGCCAAAATCACCCCATCGCTTG CTTATCGACAATTAAAAGAGGGTGGTAAATTACTTGGTG CCAGCAAAATTTCGCTAAGAGGGGATGATATCATTGCTT TAGCTAAAGAGCTTAACCTGCTCTTTACTGCTAAAAACT CCCCTGAAGAGTTAGACCTTAACATTATTGAGTGGATAG CTTATTCAAATGATGAAGGATACTTGTCTTTAAAATTCA CCAGAACCATAGAACCATATATCTCTAGCCTTATTGGGA AAAAAAATAAATTCACAACGCAATTGTTAACGGCAAGC TTACGCTTAAGTAGCCAGTATTCATCTTCTCTTTATCAAC TTATCAGGAAGCATTACTCTAATTTTAAGAAGAAAAATT ATTTTATTATTTCCGTTGATGAGTTAAAGGAAGAGTTAA TAGCTTATACTTTTGATAAAGATGGAAATATTGAGTACA AATACCCTGACTTTCCTATTTTTAAAAGGGATGTGTTAA ATAAAGCCATTGCTGAAATTAAAAAGAAAACAGAAATA TCGTTTGTTGGCTTCACTGTTCATGAAAAAGAAGGAAGA AAAATTAGTAAGCTGAAGTTCGAATTTGTCGTTGATGAA GATGAATTTTCTGGCGATAAAGATGATGAAGCTTTTTTT ATGAATTTATCTGAAGCTGATGCAGCTTTTCTCAAGGTA TTTGATGAAACCGTACCTCCCAAAAAAGCTAAGGGGTGA GGATCTCCAGGCATCAAATAAAACGAAAGGCTCAGTCG AAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGA ACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTG GGCCTTTCTGCGTTTATACCCGGGAAAAAGAGTATTGAC TtaaagtctaacctataggTATAATGTGTGGAGACCAGAGGTAAGG AGGTAACAACCATGCGAGTGTTGAAGAAACATCTTAATC ATGCTAAGGAGGTTTTCTAATGCATACCACCCGACTGAA GAGGGTTGGCGGCTCAGTTATGCTGACCGTCCCACCGGC ACTGCTGAATGCGCTGTCTCTGGGCACAGATAATGAAGT TGGCATGGTCATTGATAATGGCCGGCTGATTGTTGAGCC GTACAGACGCCCGCAATATTCACTGGCTGAGCTACTGGC ACAGTGTGATCCGAATGCTGAAATATCAGCTGAAGAAC GAGAATGGCTGGATGCACCGGCGACTGGTCAGGAGGAA ATCTGA	89

Biosafety Chromosomal Construct - medium copy Rep (Pi) and Kis antitoxin (as shown in **Fig. 61D)**	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGGAT CTTCCGGAAGACTAGGTGAGACTCAAGGTCATGATGGAC GTGAACAAAAAAACGAAAATTCGCCACCGAAACGAGCT AAATCACACCCTGGCTCAACTTCCTTTGCCCGCAAAGCG AGTGATGTATATGGCGCTTGCTCCCATTGATAGCAAAGA ACCTCTTGAACGAGGGCGAGTTTTCAAAATTAGGGCTGA AGACCTTGCAGCGCTCGCCAAAATCACCCCATCGCTTGC TTATCGACAATTAAAAGAGGGTGGTAAATTACTTGGTGC CAGCAAAATTTCGCTAAGAGGGGATGATATCATTGCTTT AGCTAAAGAGCTTAACCTGCTCTTTACTGCTAAAAACTC CCCTGAAGAGTTAGACCTTAACATTATTGAGTGGATAGC TTATTCAAATGATGAAGGATACTTGTCTTTAAAATTCAC CAGAACCATAGAACCATATATCTCTAGCCTTATTGGGAA AAAAAATAAATTCACAACGCAATTGTTAACGGCAAGCTT ACGCTTAAGTAGCCAGTATTCATCTTCTCTTTATCAACTT ATCAGGAAGCATTACTCTAATTTTAAGAAGAAAAATTAT TTTATTATTTCCGTTGATGAGTTAAAGGAAGAGTTAATA GCTTATACTTTTGATAAAGATGGAAATATTGAGTACAAA TACCCTGACTTTCCTATTTTTAAAAGGGATGTGTTAAATA AAGCCATTGCTGAAATTAAAAAGAAAACAGAAATATCG TTTGTTGGCTTCACTGTTCATGAAAAAGAAGGAAGAAAA ATTAGTAAGCTGAAGTTCGAATTTGTCGTTGATGAAGAT GAATTTTCTGGCGATAAAGATGATGAAGCTTTTTTTATG AATTTATCTGAAGCTGATGCAGCTTTTCTCAAGGTATTTG ATGAAACCGTACCTCCCAAAAAAGCTAAGGGGTGAGGA TCTCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAA GACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACG CTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGC CTTTCTGCGTTTATACCCGGGAAAAAGAGTATTGACTtaaa gtctaacctataggTATAATGTGTGGAGACCAGAGGTAAGGAGG TAACAACCATGCGAGTGTTGAAGAAACATCTTAATCATG CTAAGGAGGTTTTCTAATGCATACCACCCGACTGAAGAG GGTTGGCGGCTCAGTTATGCTGACCGTCCCACCGGCACT GCTGAATGCGCTGTCTCTGGGCACAGATAATGAAGTTGG CATGGTCATTGATAATGGCCGGCTGATTGTTGAGCCGTA CAGACGCCCGCAATATTCACTGGCTGAGCTACTGGCACA GTGTGATCCGAATGCTGAAATATCAGCTGAAGAACGAG AATGGCTGGATGCACCGGCGACTGGTCAGGAGGAAATC TGA	90
Rep (Pi)	TGAGACTCAAGGTCATGATGGACGTGAACAAAAAAACG AAAATTCGCCACCGAAACGAGCTAAATCACACCCTGGCT CAACTTCCTTTGCCCGCAAAGCGAGTGATGTATATGGCG CTTGCTCCCATTGATAGCAAAGAACCTCTTGAACGAGGG CGAGTTTTCAAAATTAGGGCTGAAGACCTTGCAGCGCTC GCCAAAATCACCCCATCGCTTGCTTATCGACAATTAAAA GAGGGTGGTAAATTACTTGGTGCCAGCAAAATTTCGCTA AGAGGGGATGATATCATTGCTTTAGCTAAAGAGCTTAAC CTGCTCTTTACTGCTAAAAACTCCCCTGAAGAGTTAGAC CTTAACATTATTGAGTGGATAGCTTATTCAAATGATGAA	91

	GGATACTTGTCTTTAAAATTCACCAGAACCATAGAACCA TATATCTCTAGCCTTATTGGGAAAAAAAATAAATTCACA ACGCAATTGTTAACGGCAAGCTTACGCTTAAGTAGCCAG TATTCATCTTCTCTTTATCAACTTATCAGGAAGCATTACT CTAATTTTAAGAAGAAAAATTATTTTATTATTTCCGTTGA TGAGTTAAAGGAAGAGTTAATAGCTTATACTTTTGATAA AGATGGAAATATTGAGTACAAATACCCTGACTTTCCTAT TTTTAAAAGGGATGTGTTAAATAAAGCCATTGCTGAAAT TAAAAAGAAAACAGAAATATCGTTTGTTGGCTTCACTGT TCATGAAAAAGAAGGAAGAAAAATTAGTAAGCTGAAGT TCGAATTTGTCGTTGATGAAGATGAATTTTCTGGCGATA AAGATGATGAAGCTTTTTTTATGAATTTATCTGAAGCTG ATGCAGCTTTTCTCAAGGTATTTGATGAAACCGTACCTC CCAAAAAAGCTAAGGGGTGA	
Kis antitoxin	CATACCACCCGACTGAAGAGGGTTGGCGGCTCAGTTATG CTGACCGTCCCACCGGCACTGCTGAATGCGCTGTCTCTG GGCACAGATAATGAAGTTGGCATGGTCATTGATAATGGC CGGCTGATTGTTGAGCCGTACAGACGCCCGCAATATTCA CTGGCTGAGCTACTGGCACAGTGTGATCCGAATGCTGAA ATATCAGCTGAAGAACGAGAATGGCTGGATGCACCGGC GACTGGTCAGGAGGAAATCTGA	92
RBS (low copy)	GCTGGAACAGGTGG	93
RBS (medium copy)	TCCGGAAGACTAGG	94

Table 79. Inducible PAL Constructs

Description	Sequence	SEQ ID NO
IPTG and low oxygen inducible constructs as shown in **Fig. 65A**, comprising LacI in reverse orientation, underlined), a region comprising a promoter (italic), LacO (italic bold), and a FNR binding site (italic underline), and two open reading frames encoding PAL3 (non-native codon altered sequence 1 and no-	TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAG GCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTT CACCAGTGAGACTGGCAACAGCTGATTGCCCTTCACC GCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACG CTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGG TGGTTAACGGCGGGATATAACATGAGCTATCTTCGGT ATCGTCGTATCCCACTACCGAGATATCCGCACCAACG CGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCC AGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGG GAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTG AAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCC GCTATCGGCTGAATTTGATTGCGAGTGAGATATTTAT GCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAAC TTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACC CAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCG TCCTCATGGGAGAAAATAATACTGTTGATGGGTGTCT GGTCAGAGACATCAAGAAATAACGCCGGAACATTAG	95

native codon altered sequence 2, both bold) and third open reading frame encoding of PheP; RBS sequences are underlined, italic and bold; This construct can be transcribed as a tricistronic message.	TGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATC CAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGC GCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGA CGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC ACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACA ATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTG GCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTT GTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGC CATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAA CGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTG ATAAGAGACACCGGCATACTCTGCGACATCGTATAA CGTTACTGGTTTCAT*ATTCACCACCCTGAATTGACTCTC* *TTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCG* *CCATTCGATGGTGTCTGTATACAAAAACGCCGTAAAGTTT* *GAGCGAAGTCAATAAACTCTCTACCCATTCAGGGCAATAT* *CTCTCTTCGTCAGGCCACATAGCTTTCTTGTTCTGATCGG* *AACGATCGTTGGCTGTGTTGACAATTAATCATCGGCTCGT* *ATAATGTGT**GGAATTGTGAGCGCTCACAATT**AGCTGTCA* *CCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGA* *AACAGCCTCTACAAATAATTTTGTTTAAAACAACACCCACT* *AAGATAA**CTCTAGAAATAATTTTGTTTAACTTTAAGAAG*** ***GAGATATACAT**ATGAAAGCAAAAGATGTTCAGCCA* **ACCATTATTATTAATAAAAATGGCCTTATCTCTTTG** **GAAGATATCTATGACATTGCGATAAAACAGAAAAA** **AGTAGAAATATCAACGGAGATCACTGAGCTTTTGA** **CGCATGGTCGTGAAAAATTAGAGGAAAAATTAAAT** **TCAGGAGAGGTTATATATGGAATCAATACAGGATT** **TGGAGGGAATGCCAATTTAGTTGTGCCATTTGAGA** **AGATTGCAGAGCACCAGCAAAACCTGTTAACTTTT** **CTTTCTGCTGGTACTGGGGACTATATGTCCAAGCC** **TTGTATTAAAGCGTCACAATTTACTATGTTACTTTC** **TGTTTGCAAAGGTTGGTCTGCAACCAGACCAATTG** **TCGCTCAGGCGATTGTTGATCATATTAACCATGAC** **ATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGGG** **TGCATCGGGTGATTTAATTCCTTTATCTTATATTG** **CACGCGCTTTATGTGGTATCGGCAAAGTTTATTAT** **ATGGGTGCAGAAATTGACGCTGCTGAAGCAATTA** **AGCGTGCAGGGTTGACACCATTATCGTTAAAAGCC** **AAAGAAGGTCTTGCTCTGATTAACGGCACCCGGG** **TAATGTCAGGAATCAGTGCAATCACCGTCATTAAA** **CTGGAAAAACTATTTAAAGCCTCAATTTCTGCGAT** **TGCCCTTGCTGTGGAAGCATTACTTGCGTCTCACG** **AACATTATGATGCCCGGATTCAACAAGTTAAGAAC** **CATCCTGGTCAGAATGCGGTGGCATCAGCATTGC** **GTAATTTATTGGCAGGTTCAACGCAGGTTAATCTA** **CTGTCTGGGGTTAAAGAGCAGGCGAATAAAGCTT** **GTCGTCATCAGGAGATTACCCAACTCAATGATACC** **TTACAGGAAGTTTATTCAATTCGCTGTGCACCACA** **GGTATTAGGTATAGTGCCAGAATCTTTAGCTACTG**	

	CTCGGAAGATATTGGAACGGGAAGTTATCTCAGCT AATGATAATCCATTGATAGATCCAGAGAATGGCGA TGTGCTACACGGTGGAAACTTTATGGGGCAATATG TCGCCCGAACAATGGATGCATTAAAACTGGATATT GCTTTGATTGCCAATCATCTTCACGCCATTGTGGC TCTTATGATGGATAACCGTTTCTCTCGTGGATTAC CTAATTCACTGAGTCCGACACCCGGCATGTACCAA GGTTTTAAAGGCGTCCAACTTTCTCAAACGGCTTT AGTTGCAGCGATTCGCCATGATTGTGCTGCATCAG GTATTCATACCCTCGCAACAGAACAGTACAATCAG GATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGA TGTTTTAGAGATGGAGCAGAAATTACGCAATATTG TTTCAATGACAATTCTGGTAGTTTGTCAGGCCATT CATCTTCGCGGCAATATTAGTGAAATTGCGCCTGA AACTGCTAAATTTTACCATGCAGTACGCGAAATCA GTTCTCCTTTGATCACTGATCGTGCGTTGGATGAA GATATAATCCGCATTGCGGATGCAATTATTAATGA TCAACTTCCTCTGCCAGAAATCATGCTGGAAGAAT GA*CCCAGATAAAACGCAAGGAGGTTCT*ATGAAGGCC AAAGATGTACAGCCGACCATCATCATTAACAAAAA CGGTTTGATTAGCCTGGAAGACATCTATGATATCG CCATTAAGCAGAAAAAGGTTGAAATCTCCACGGAA ATTACAGAACTGTTGACTCACGGTCGCGAAAAACT GGAGGAAAAACTGAACAGCGGTGAAGTTATTTAC GGTATCAACACTGGCTTTGGTGGTAACGCTAACCT TGTTGTGCCGTTCGAAAAGATTGCCGAACACCAGC AGAACCTGCTCACCTTCCTGTCTGCGGGTACAGGC GACTATATGTCCAAACCGTGTATCAAAGCGTCTCA GTTTACAATGCTGCTGTCTGTGTGCAAAGGTTGGT CCGCCACGCGGCCTATTGTAGCGCAAGCAATCGT CGATCACATCAACCATGATATCGTTCCGCTGGTGC CTCGTTACGGCAGCGTGGGCGCATCTGGTGATCT GATCCCGCTGTCGTACATTGCTCGCGCTCTGTGCG GTATTGGCAAGGTGTACTACATGGGCGCGGAAAT CGACGCCGCCGAGGCAATCAAACGTGCGGGCCTG ACTCCGTTATCTCTGAAAGCGAAGGAAGGTCTGG CTCTGATCAACGGCACGCGTGTAATGTCTGGCATC TCCGCCATTACCGTGATTAAACTGGAAAAACTGTT CAAAGCTTCCATCTCCGCGATCGCATTGGCGGTCG AGGCGTTGCTGGCATCCCACGAACACTACGATGC CCGCATTCAACAGGTTAAAAACCATCCGGGTCAGA ACGCGGTTGCATCCGCACTTCGCAACTTGCTGGC GGGTTCTACTCAGGTGAATCTGCTGTCAGGTGTTA AGGAACAGGCAAACAAAGCGTGTCGTCACCAGGA AATCACTCAGCTGAACGACACCCTGCAGGAAGTAT ACTCCATCCGTTGCGCACCGCAAGTGCTGGGCATT GTACCGGAAAGCCTGGCAACCGCACGTAAAATCC TGGAACGTGAGGTAATTTCGGCCAACGATAATCC GTTGATCGATCCAGAGAATGGCGACGTACTGCAC	

	GGCGGGAACTTTATGGGCCAGTACGTTGCTCGCA **CTATGGACGCGCTGAAACTCGATATTGCTCTGATT** **GCCAACCATCTCCACGCGATCGTTGCACTGATGAT** **GGACAATCGTTTCAGTCGCGGTCTGCCGAACAGC** **CTGTCCCCGACTCCGGGTATGTATCAGGGCTTTAA** **AGGTGTGCAGCTGTCCCAAACGGCTCTGGTTGCG** **GCGATTCGTCATGATTGCGCCGCGAGCGGCATCC** **ATACCTTAGCGACTGAACAGTATAACCAGGACATC** **GTTAGCCTGGGTTTGCATGCGGCGCAGGACGTTC** **TAGAAATGGAACAGAAACTGCGTAACATCGTATCC** **ATGACTATTCTGGTTGTTTGCCAGGCAATCCACCT** **GCGCGGCAACATCAGTGAAATCGCGCCAGAAACC** **GCGAAATTCTACCACGCGGTTCGTGAAATTTCCTC** **ACCGCTGATCACCGATCGTGCTCTTGACGAAGATA** **TCATCCGCATCGCGGATGCGATCATTAATGACCAG** **CTGCCGCTGCCGGAAATTATGCTGGAAGAGTAA*AC*** ***CCTAAGGTATTTACTAAACCGAAGGAGTACAAT*ATGA** **AAAACGCGTCAACCGTATCGGAAGATACTGCGTC** **GAATCAAGAGCCGACGCTTCATCGCGGATTACATA** **ACCGTCATATTCAACTGATTGCGTTGGGTGGCGCA** **ATTGGTACTGGTCTGTTTCTTGGCATTGGCCCGGC** **GATTCAGATGGCGGGTCCGGCTGTATTGCTGGGC** **TACGGCGTCGCCGGGATCATCGCTTTCCTGATTAT** **GCGCCAGCTTGGCGAAATGGTGGTTGAGGAGCCG** **GTATCCGGTTCATTTGCCCACTTTGCCTATAAATA** **CTGGGGACCGTTTGCGGGCTTCCTCTCTGGCTGG** **AACTACTGGGTAATGTTCGTGCTGGTGGGAATGG** **CAGAGCTGACCGCTGCGGGCATCTATATGCAGTA** **CTGGTTCCCGGATGTTCCAACGTGGATTTGGGCTG** **CCGCCTTCTTTATTATCATCAACGCCGTTAACCTG** **GTGAACGTGCGCTTATATGGCGAAACCGAGTTCT** **GGTTTGCGTTGATTAAAGTGCTGGCAATCATCGGT** **ATGATCGGCTTTGGCCTGTGGCTGCTGTTTTCTGG** **TCACGGCGGCGAGAAAGCCAGTATCGACAACCTC** **TGGCGCTACGGTGGTTTCTTCGCCACCGGCTGGA** **ATGGGCTGATTTTGTCGCTGGCGGTAATTATGTTC** **TCCTTCGGCGGTCTGGAGCTGATTGGGATTACTGC** **CGCTGAAGCGCGCGATCCGGAAAAAAGCATTCCA** **AAAGCGGTAAATCAGGTGGTGTATCGCATCCTGCT** **GTTTTACATCGGTTCACTGGTGGTTTTACTGGCGC** **TCTATCCGTGGGTGGAAGTGAAATCCAACAGTAG** **CCCGTTTGTGATGATTTTCCATAATCTCGACAGCA** **ACGTGGTAGCTTCTGCGCTGAACTTCGTCATTCTG** **GTAGCATCGCTGTCAGTGTATAACAGCGGGGTTTA** **CTCTAACAGCCGCATGCTGTTTGGCCTTTCTGTGC** **AGGGTAATGCGCCGAAGTTTTTGACTCGCGTCAG** **CCGTCGCGGTGTGCCGATTAACTCGCTGATGCTTT** **CCGGAGCGATCACTTCGCTGGTGGTGTTAATCAAC** **TATCTGCTGCCGCAAAAAGCGTTTGGTCTGCTGAT**	

	GGCGCTGGTGGTAGCAACGCTGCTGTTGAACTGG ATTATGATCTGTCTGGCGCATCTGCGTTTTCGTGC AGCGATGCGACGTCAGGGGCGTGAAACACAGTTT AAGGCGCTGCTCTATCCGTTCGGCAACTATCTCTG CATTGCCTTCCTCGGCATGATTTTGCTGCTGATGT GCACGATGGATGATATGCGCTTGTCAGCGATCCT GCTGCCGGTGTGGATTGTATTCCTGTTTATGGCAT TTAAAACGCTGCGTCGGAAAtaa	
IPTG inducible constructs as shown in **Fig. 65C**, comprising LacI in reverse orientation, underlined), a region comprising a promoter (italic), LacO (italic bold), and two open reading frames encoding PAL3 (non-native codon altered sequence 1 and no-native codon altered sequence 2, both bold) and third open reading frame encoding of PheP; RBS sequences are underlined, italic and bold; This construct can be transcribed as a tricistronic message.	TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAG GCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTT CACCAGTGAGACTGGCAACAGCTGATTGCCCTTCACC GCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACG CTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGG TGGTTAACGGCGGGATATAACATGAGCTATCTTCGGT ATCGTCGTATCCCACTACCGAGATATCCGCACCAACG CGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCC AGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGG GAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTG AAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCC GCTATCGGCTGAATTTGATTGCGAGTGAGATATTTAT GCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAAC TTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACC CAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCG TCCTCATGGGAGAAAATAATACTGTTGATGGGTGTCT GGTCAGAGACATCAAGAAATAACGCCGGAACATTAG TGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATC CAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGC GCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGA CGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC ACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACA ATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTG GCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTT GTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGC CATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAA CGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTG ATAAGAGACACCGGCATACTCTGCGACATCGTATAA CGTTACTGGTTTCAT*ATTCACCACCCTGAATTGACTCTC TTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCG CCATTCGATGGCGCGCCGCTTCG**TCAGGCCACATAGCT TTCTTGT**TCTGATCGGAACGATCGTTGGCTGTGTTGACA ATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGC TCACAATTAGCTGTCACCGGATGTGCTTTCCGGTCTGAT GAGTCCGTGAGGACGAAACAGCCTCTACAAATAATTTTGT TTAAAACAACACCCACTAAGATAA**CTCTAGAAATAATTTT GTTTAACTTTAAGAAGGAGATATACAT**ATGAAAGCAA AAGATGTTCAGCCAACCATTATTATTAATAAAAAT GGCCTTATCTCTTTGGAAGATATCTATGACATTGC GATAAAACAGAAAAAAGTAGAAATATCAACGGAG*	96

	ATCACTGAGCTTTTGACGCATGGTCGTGAAAAATT AGAGGAAAAATTAAATTCAGGAGAGGTTATATATG GAATCAATACAGGATTTGGAGGGAATGCCAATTTA GTTGTGCCATTTGAGAAGATTGCAGAGCACCAGC AAAACCTGTTAACTTTTCTTTCTGCTGGTACTGGG GACTATATGTCCAAGCCTTGTATTAAAGCGTCACA ATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGT CTGCAACCAGACCAATTGTCGCTCAGGCGATTGTT GATCATATTAACCATGACATTGTTCCTCTGGTTCC TCGCTATGGCTCAGTGGGTGCATCGGGTGATTTAA TTCCTTTATCTTATATTGCACGCGCTTTATGTGGT ATCGGCAAAGTTTATTATATGGGTGCAGAAATTGA CGCTGCTGAAGCAATTAAGCGTGCAGGGTTGACA CCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCT GATTAACGGCACCCGGGTAATGTCAGGAATCAGT GCAATCACCGTCATTAAACTGGAAAAACTATTTAA AGCCTCAATTTCTGCGATTGCCCTTGCTGTGGAAG CATTACTTGCGTCTCACGAACATTATGATGCCCGG ATTCAACAAGTTAAGAACCATCCTGGTCAGAATGC GGTGGCATCAGCATTGCGTAATTTATTGGCAGGTT CAACGCAGGTTAATCTACTGTCTGGGGTTAAAGAG CAGGCGAATAAAGCTTGTCGTCATCAGGAGATTAC CCAACTCAATGATACCTTACAGGAAGTTTATTCAA TTCGCTGTGCACCACAGGTATTAGGTATAGTGCCA GAATCTTTAGCTACTGCTCGGAAGATATTGGAACG GGAAGTTATCTCAGCTAATGATAATCCATTGATAG ATCCAGAGAATGGCGATGTGCTACACGGTGGAAA CTTTATGGGGCAATATGTCGCCCGAACAATGGATG CATTAAAACTGGATATTGCTTTGATTGCCAATCAT CTTCACGCCATTGTGGCTCTTATGATGGATAACCG TTTCTCTCGTGGATTACCTAATTCACTGAGTCCGA CACCCGGCATGTACCAAGGTTTTAAAGGCGTCCAA CTTTCTCAAACGGCTTTAGTTGCAGCGATTCGCCA TGATTGTGCTGCATCAGGTATTCATACCCTCGCAA CAGAACAGTACAATCAGGATATTGTCAGTTTAGGT CTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCA GAAATTACGCAATATTGTTTCAATGACAATTCTGG TAGTTTGTCAGGCCATTCATCTTCGCGGCAATATT AGTGAAATTGCGCCTGAAACTGCTAAATTTTACCA TGCAGTACGCGAAATCAGTTCTCCTTTGATCACTG ATCGTGCGTTGGATGAAGATATAATCCGCATTGCG GATGCAATTATTAATGATCAACTTCCTCTGCCAGA AATCATGCTGGAAGAATGA*CCCAGATAAAACGCAAG* *GAGGTTCT*ATGAAGGCCAAAGATGTACAGCCGACC ATCATCATTAACAAAAACGGTTTGATTAGCCTGGA AGACATCTATGATATCGCCATTAAGCAGAAAAAGG TTGAAATCTCCACGGAAATTACAGAACTGTTGACT CACGGTCGCGAAAAACTGGAGGAAAAACTGAACA GCGGTGAAGTTATTTACGGTATCAACACTGGCTTT	

	GGTGGTAACGCTAACCTTGTTGTGCCGTTCGAAAA GATTGCCGAACACCAGCAGAACCTGCTCACCTTCC TGTCTGCGGGTACAGGCGACTATATGTCCAAACC GTGTATCAAAGCGTCTCAGTTTACAATGCTGCTGT CTGTGTGCAAAGGTTGGTCCGCCACGCGGCCTAT TGTAGCGCAAGCAATCGTCGATCACATCAACCATG ATATCGTTCCGCTGGTGCCTCGTTACGGCAGCGTG GGCGCATCTGGTGATCTGATCCCGCTGTCGTACAT TGCTCGCGCTCTGTGCGGTATTGGCAAGGTGTACT ACATGGGCGCGGAAATCGACGCCGCCGAGGCAAT CAAACGTGCGGGCCTGACTCCGTTATCTCTGAAAG CGAAGGAAGGTCTGGCTCTGATCAACGGCACGCG TGTAATGTCTGGCATCTCCGCCATTACCGTGATTA AACTGGAAAAACTGTTCAAAGCTTCCATCTCCGCG ATCGCATTGGCGGTCGAGGCGTTGCTGGCATCCC ACGAACACTACGATGCCCGCATTCAACAGGTTAAA AACCATCCGGGTCAGAACGCGGTTGCATCCGCAC TTCGCAACTTGCTGGCGGGTTCTACTCAGGTGAAT CTGCTGTCAGGTGTTAAGGAACAGGCAAACAAAG CGTGTCGTCACCAGGAAATCACTCAGCTGAACGA CACCCTGCAGGAAGTATACTCCATCCGTTGCGCAC CGCAAGTGCTGGGCATTGTACCGGAAAGCCTGGC AACCGCACGTAAAATCCTGGAACGTGAGGTAATTT CGGCCAACGATAATCCGTTGATCGATCCAGAGAAT GGCGACGTACTGCACGGCGGGAACTTTATGGGCC AGTACGTTGCTCGCACTATGGACGCGCTGAAACTC GATATTGCTCTGATTGCCAACCATCTCCACGCGAT CGTTGCACTGATGATGGACAATCGTTTCAGTCGCG GTCTGCCGAACAGCCTGTCCCCGACTCCGGGTAT GTATCAGGGCTTTAAAGGTGTGCAGCTGTCCCAAA CGGCTCTGGTTGCGGCGATTCGTCATGATTGCGC CGCGAGCGGCATCCATACCTTAGCGACTGAACAG TATAACCAGGACATCGTTAGCCTGGGTTTGCATGC GGCGCAGGACGTTCTAGAAATGGAACAGAAACTG CGTAACATCGTATCCATGACTATTCTGGTTGTTTG CCAGGCAATCCACCTGCGCGGCAACATCAGTGAA ATCGCGCCAGAAACCGCGAAATTCTACCACGCGG TTCGTGAAATTTCCTCACCGCTGATCACCGATCGT GCTCTTGACGAAGATATCATCCGCATCGCGGATGC GATCATTAATGACCAGCTGCCGCTGCCGGAAATTA TGCTGGAAGAGTAAACCCTAAGGTATTTACTAAAC CGAAGGAGTACAATATGAAAAACGCGTCAACCGT ATCGGAAGATACTGCGTCGAATCAAGAGCCGACG CTTCATCGCGGATTACATAACCGTCATATTCAACT GATTGCGTTGGGTGGCGCAATTGGTACTGGTCTG TTTCTTGGCATTGGCCCGGCGATTCAGATGGCGG GTCCGGCTGTATTGCTGGGCTACGGCGTCGCCGG GATCATCGCTTTCCTGATTATGCGCCAGCTTGGCG AAATGGTGGTTGAGGAGCCGGTATCCGGTTCATTT	

	GCCCACTTTGCCTATAAATACTGGGGACCGTTTGC GGGCTTCCTCTCTGGCTGGAACTACTGGGTAATGT TCGTGCTGGTGGGAATGGCAGAGCTGACCGCTGC GGGCATCTATATGCAGTACTGGTTCCCGGATGTTC CAACGTGGATTTGGGCTGCCGCCTTCTTTATTATC ATCAACGCCGTTAACCTGGTGAACGTGCGCTTATA TGGCGAAACCGAGTTCTGGTTTGCGTTGATTAAAG TGCTGGCAATCATCGGTATGATCGGCTTTGGCCTG TGGCTGCTGTTTTCTGGTCACGGCGGCGAGAAAG CCAGTATCGACAACCTCTGGCGCTACGGTGGTTTC TTCGCCACCGGCTGGAATGGGCTGATTTTGTCGCT GGCGGTAATTATGTTCTCCTTCGGCGGTCTGGAGC TGATTGGGATTACTGCCGCTGAAGCGCGCGATCC GGAAAAAAGCATTCCAAAAGCGGTAAATCAGGTG GTGTATCGCATCCTGCTGTTTTACATCGGTTCACT GGTGGTTTTACTGGCGCTCTATCCGTGGGTGGAA GTGAAATCCAACAGTAGCCCGTTTGTGATGATTTT CCATAATCTCGACAGCAACGTGGTAGCTTCTGCGC TGAACTTCGTCATTCTGGTAGCATCGCTGTCAGTG TATAACAGCGGGGTTTACTCTAACAGCCGCATGCT GTTTGGCCTTTCTGTGCAGGGTAATGCGCCGAAGT TTTTGACTCGCGTCAGCCGTCGCGGTGTGCCGATT AACTCGCTGATGCTTTCCGGAGCGATCACTTCGCT GGTGGTGTTAATCAACTATCTGCTGCCGCAAAAAG CGTTTGGTCTGCTGATGGCGCTGGTGGTAGCAAC GCTGCTGTTGAACTGGATTATGATCTGTCTGGCGC ATCTGCGTTTTCGTGCAGCGATGCGACGTCAGGG GCGTGAAACACAGTTTAAGGCGCTGCTCTATCCGT TCGGCAACTATCTCTGCATTGCCTTCCTCGGCATG ATTTTGCTGCTGATGTGCACGATGGATGATATGCG CTTGTCAGCGATCCTGCTGCCGGTGTGGATTGTAT TCCTGTTTATGGCATTTAAAACGCTGCGTCGGAAA TAA	
IPTG and low oxygen inducible constructs as shown in **Fig. 65B**, comprising LacI in reverse orientation, underlined), a region comprising a promoter (italic), LacO (italic bold), and a FNR binding site (italic underline), and two open reading frames encoding PAL3 (non-native	TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAG GCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTT CACCAGTGAGACTGGCAACAGCTGATTGCCCTTCACC GCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACG CTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGG TGGTTAACGGCGGGATATAACATGAGCTATCTTCGGT ATCGTCGTATCCCACTACCGAGATATCCGCACCAACG CGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCC AGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGG GAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTG AAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCC GCTATCGGCTGAATTTGATTGCGAGTGAGATATTTAT GCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAAC TTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACC CAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCG	97

codon altered sequence 1 and non-native codon altered sequence 2, both bold); RBS sequences are underlined, italic and bold; This construct can be transcribed as a bicistronic message.	TCCTCATGGGAGAAAATAATACTGTTGATGGGTGTCT GGTCAGAGACATCAAGAAATAACGCCGGAACATTAG TGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATC CAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGC GCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGA CGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC ACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACA ATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTG GCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTT GTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGC CATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAA CGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTG ATAAGAGACACCGGCATACTCTGCGACATCGTATAA CGTTACTGGTTTCAT*ATTCACCACCCTGAATTGACTCTC* *TTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCG* *CCATTCGATGGTGTCTGTATACAAAAACGCCGTAAAG*TTT *GAGCGAAGTCAA**TAAACTCTCTACCCATTCAGGGCAATAT*** *CTCTCTTCG***TCAGGCCACATAGCTTTCTTGT***TCTGATCG* *GAACGATCGTTGGCTGTGTTGACAATTAATCATCGGCTC* *GTATAATGTGTGGAATTGTGAGCGCTCACAATTAGCTGTC* *ACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACG* *AAACAGCCTCTACAAATAATTTTGTTTAAAACAACACCCAC* *TAAGATAA**CTCTAGAAATAATTTTGTTTAACTTTAAGAA*** ***GGAGATATACAT*****ATGAAAGCAAAAGATGTTCAGCC** **AACCATTATTATTAATAAAAATGGCCTTATCTCTTT** **GGAAGATATCTATGACATTGCGATAAAACAGAAAA** **AAGTAGAAATATCAACGGAGATCACTGAGCTTTTG** **ACGCATGGTCGTGAAAAATTAGAGGAAAAATTAAA** **TTCAGGAGAGGTTATATATGGAATCAATACAGGAT** **TTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAG** **AAGATTGCAGAGCACCAGCAAAACCTGTTAACTTT** **TCTTTCTGCTGGTACTGGGGACTATATGTCCAAGC** **CTTGTATTAAAGCGTCACAATTTACTATGTTACTTT** **CTGTTTGCAAAGGTTGGTCTGCAACCAGACCAATT** **GTCGCTCAGGCGATTGTTGATCATATTAACCATGA** **CATTGTTCCTCTGGTTCCTCGCTATGGCTCAGTGG** **GTGCATCGGGTGATTTAATTCCTTTATCTTATATT** **GCACGCGCTTTATGTGGTATCGGCAAAGTTTATTA** **TATGGGTGCAGAAATTGACGCTGCTGAAGCAATTA** **AGCGTGCAGGGTTGACACCATTATCGTTAAAAGCC** **AAAGAAGGTCTTGCTCTGATTAACGGCACCCGGG** **TAATGTCAGGAATCAGTGCAATCACCGTCATTAAA** **CTGGAAAAACTATTTAAAGCCTCAATTTCTGCGAT** **TGCCCTTGCTGTGGAAGCATTACTTGCGTCTCACG** **AACATTATGATGCCCGGATTCAACAAGTTAAGAAC** **CATCCTGGTCAGAATGCGGTGGCATCAGCATTGC** **GTAATTTATTGGCAGGTTCAACGCAGGTTAATCTA** **CTGTCTGGGGTTAAAGAGCAGGCGAATAAAGCTT** **GTCGTCATCAGGAGATTACCCAACTCAATGATACC**	

	TTACAGGAAGTTTATTCAATTCGCTGTGCACCACA GGTATTAGGTATAGTGCCAGAATCTTTAGCTACTG CTCGGAAGATATTGGAACGGGAAGTTATCTCAGCT AATGATAATCCATTGATAGATCCAGAGAATGGCGA TGTGCTACACGGTGGAAACTTTATGGGGCAATATG TCGCCCGAACAATGGATGCATTAAAACTGGATATT GCTTTGATTGCCAATCATCTTCACGCCATTGTGGC TCTTATGATGGATAACCGTTTCTCTCGTGGATTAC CTAATTCACTGAGTCCGACACCCGGCATGTACCAA GGTTTTAAAGGCGTCCAACTTTCTCAAACGGCTTT AGTTGCAGCGATTCGCCATGATTGTGCTGCATCAG GTATTCATACCCTCGCAACAGAACAGTACAATCAG GATATTGTCAGTTTAGGTCTGCATGCCGCTCAAGA TGTTTTAGAGATGGAGCAGAAATTACGCAATATTG TTTCAATGACAATTCTGGTAGTTTGTCAGGCCATT CATCTTCGCGGCAATATTAGTGAAATTGCGCCTGA AACTGCTAAATTTTACCATGCAGTACGCGAAATCA GTTCTCCTTTGATCACTGATCGTGCGTTGGATGAA GATATAATCCGCATTGCGGATGCAATTATTAATGA TCAACTTCCTCTGCCAGAAATCATGCTGGAAGAAT GA*CCCAGATAAAACGCAAGGAGGTTCT*ATGAAGGCC AAAGATGTACAGCCGACCATCATCATTAACAAAAA CGGTTTGATTAGCCTGGAAGACATCTATGATATCG CCATTAAGCAGAAAAAGGTTGAAATCTCCACGGAA ATTACAGAACTGTTGACTCACGGTCGCGAAAAACT GGAGGAAAAACTGAACAGCGGTGAAGTTATTTAC GGTATCAACACTGGCTTTGGTGGTAACGCTAACCT TGTTGTGCCGTTCGAAAAGATTGCCGAACACCAGC AGAACCTGCTCACCTTCCTGTCTGCGGGTACAGGC GACTATATGTCCAAACCGTGTATCAAAGCGTCTCA GTTTACAATGCTGCTGTCTGTGTGCAAAGGTTGGT CCGCCACGCGGCCTATTGTAGCGCAAGCAATCGT CGATCACATCAACCATGATATCGTTCCGCTGGTGC CTCGTTACGGCAGCGTGGGCGCATCTGGTGATCT GATCCCGCTGTCGTACATTGCTCGCGCTCTGTGCG GTATTGGCAAGGTGTACTACATGGGCGCGGAAAT CGACGCCGCCGAGGCAATCAAACGTGCGGGCCTG ACTCCGTTATCTCTGAAAGCGAAGGAAGGTCTGG CTCTGATCAACGGCACGCGTGTAATGTCTGGCATC TCCGCCATTACCGTGATTAAACTGGAAAAACTGTT CAAAGCTTCCATCTCCGCGATCGCATTGGCGGTCG AGGCGTTGCTGGCATCCCACGAACACTACGATGC CCGCATTCAACAGGTTAAAAACCATCCGGGTCAGA ACGCGGTTGCATCCGCACTTCGCAACTTGCTGGC GGGTTCTACTCAGGTGAATCTGCTGTCAGGTGTTA AGGAACAGGCAAACAAAGCGTGTCGTCACCAGGA AATCACTCAGCTGAACGACACCCTGCAGGAAGTAT ACTCCATCCGTTGCGCACCGCAAGTGCTGGGCATT GTACCGGAAAGCCTGGCAACCGCACGTAAAATCC	

	TGGAACGTGAGGTAATTTCGGCCAACGATAATCC GTTGATCGATCCAGAGAATGGCGACGTACTGCAC GGCGGGAACTTTATGGGCCAGTACGTTGCTCGCA CTATGGACGCGCTGAAACTCGATATTGCTCTGATT GCCAACCATCTCCACGCGATCGTTGCACTGATGAT GGACAATCGTTTCAGTCGCGGTCTGCCGAACAGC CTGTCCCCGACTCCGGGTATGTATCAGGGCTTTAA AGGTGTGCAGCTGTCCCAAACGGCTCTGGTTGCG GCGATTCGTCATGATTGCGCCGCGAGCGGCATCC ATACCTTAGCGACTGAACAGTATAACCAGGACATC GTTAGCCTGGGTTTGCATGCGGCGCAGGACGTTC TAGAAATGGAACAGAAACTGCGTAACATCGTATCC ATGACTATTCTGGTTGTTTGCCAGGCAATCCACCT GCGCGGCAACATCAGTGAAATCGCGCCAGAAACC GCGAAATTCTACCACGCGGTTCGTGAAATTTCCTC ACCGCTGATCACCGATCGTGCTCTTGACGAAGATA TCATCCGCATCGCGGATGCGATCATTAATGACCAG CTGCCGCTGCCGGAAATTATGCTGGAAGAGTAA	
IPTG inducible constructs as shown in **Fig. 65D**, comprising LacI in reverse orientation, underlined), a region comprising a promoter (italic), LacO (italic bold), and two open reading frames encoding PAL3 (non-native codon altered sequence 1 and no-native codon altered sequence 2, both bold); RBS sequences are underlined, italic and bold; This construct can be transcribed as a bicistronic message.	TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAG GCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTT CACCAGTGAGACTGGCAACAGCTGATTGCCCTTCACC GCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACG CTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGG TGGTTAACGGCGGGATATAACATGAGCTATCTTCGGT ATCGTCGTATCCCACTACCGAGATATCCGCACCAACG CGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCC AGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGG GAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTG AAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCC GCTATCGGCTGAATTTGATTGCGAGTGAGATATTTAT GCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAAC TTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACC CAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCG TCCTCATGGGAGAAAATAATACTGTTGATGGGTGTCT GGTCAGAGACATCAAGAAATAACGCCGGAACATTAG TGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATC CAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGC GCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGA CGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC ACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACA ATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTG GCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTT GTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGC CATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAA CGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTG ATAAGAGACACCGGCATACTCTGCGACATCGTATAA CGTTACTGGTTTCAT*ATTCACCACCCTGAATTGACTCTC TTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCG*	98

	*CCATTCGATGGCGCGCCGCTTCGTCAGGCCACATAGCTT TCTTGTTCTGATCGGAACGATCGTTGGCTGTGTTGACAAT TAATCATCGGCTCGTATAATGTGT**GGAATTGTGAGCGCT CACAATT**AGCTGTCACCGGATGTGCTTTCCGGTCTGATG AGTCCGTGAGGACGAAACAGCCTCTACAAATAATTTTGTT TAAAACAACACCCACTAAGATAA**CTCTAGAAATAATTTT GTTTAACTTTAAGAAGGAGATATACAT**ATGAAAGCAA AAGATGTTCAGCCAACCATTATTATTAATAAAAAT GGCCTTATCTCTTTGGAAGATATCTATGACATTGC GATAAAACAGAAAAAAGTAGAAATATCAACGGAG ATCACTGAGCTTTTGACGCATGGTCGTGAAAAATT AGAGGAAAAATTAAATTCAGGAGAGGTTATATATG GAATCAATACAGGATTTGGAGGGAATGCCAATTTA GTTGTGCCATTTGAGAAGATTGCAGAGCACCAGC AAAACCTGTTAACTTTTCTTTCTGCTGGTACTGGG GACTATATGTCCAAGCCTTGTATTAAAGCGTCACA ATTTACTATGTTACTTTCTGTTTGCAAAGGTTGGT CTGCAACCAGACCAATTGTCGCTCAGGCGATTGTT GATCATATTAACCATGACATTGTTCCTCTGGTTCC TCGCTATGGCTCAGTGGGTGCATCGGGTGATTTAA TTCCTTTATCTTATATTGCACGCGCTTTATGTGGT ATCGGCAAAGTTTATTATATGGGTGCAGAAATTGA CGCTGCTGAAGCAATTAAGCGTGCAGGGTTGACA CCATTATCGTTAAAAGCCAAAGAAGGTCTTGCTCT GATTAACGGCACCCGGGTAATGTCAGGAATCAGT GCAATCACCGTCATTAAACTGGAAAAACTATTTAA AGCCTCAATTTCTGCGATTGCCCTTGCTGTGGAAG CATTACTTGCGTCTCACGAACATTATGATGCCCGG ATTCAACAAGTTAAGAACCATCCTGGTCAGAATGC GGTGGCATCAGCATTGCGTAATTTATTGGCAGGTT CAACGCAGGTTAATCTACTGTCTGGGGTTAAAGAG CAGGCGAATAAAGCTTGTCGTCATCAGGAGATTAC CCAACTCAATGATACCTTACAGGAAGTTTATTCAA TTCGCTGTGCACCACAGGTATTAGGTATAGTGCCA GAATCTTTAGCTACTGCTCGGAAGATATTGGAACG GGAAGTTATCTCAGCTAATGATAATCCATTGATAG ATCCAGAGAATGGCGATGTGCTACACGGTGGAAA CTTTATGGGGCAATATGTCGCCCGAACAATGGATG CATTAAAACTGGATATTGCTTTGATTGCCAATCAT CTTCACGCCATTGTGGCTCTTATGATGGATAACCG TTTCTCTCGTGGATTACCTAATTCACTGAGTCCGA CACCCGGCATGTACCAAGGTTTTAAAGGCGTCCAA CTTTCTCAAACGGCTTTAGTTGCAGCGATTCGCCA TGATTGTGCTGCATCAGGTATTCATACCCTCGCAA CAGAACAGTACAATCAGGATATTGTCAGTTTAGGT CTGCATGCCGCTCAAGATGTTTTAGAGATGGAGCA GAAATTACGCAATATTGTTTCAATGACAATTCTGG TAGTTTGTCAGGCCATTCATCTTCGCGGCAATATT AGTGAAATTGCGCCTGAAACTGCTAAATTTTACCA*	

	TGCAGTACGCGAAATCAGTTCTCCTTTGATCACTG ATCGTGCGTTGGATGAAGATATAATCCGCATTGCG GATGCAATTATTAATGATCAACTTCCTCTGCCAGA AATCATGCTGGAAGAATGA*CCCAGATAAAACGCAAG* *GAGGTTCT*ATGAAGGCCAAAGATGTACAGCCGACC ATCATCATTAACAAAAACGGTTTGATTAGCCTGGA AGACATCTATGATATCGCCATTAAGCAGAAAAAGG TTGAAATCTCCACGGAAATTACAGAACTGTTGACT CACGGTCGCGAAAAACTGGAGGAAAAACTGAACA GCGGTGAAGTTATTTACGGTATCAACACTGGCTTT GGTGGTAACGCTAACCTTGTTGTGCCGTTCGAAAA GATTGCCGAACACCAGCAGAACCTGCTCACCTTCC TGTCTGCGGGTACAGGCGACTATATGTCCAAACC GTGTATCAAAGCGTCTCAGTTTACAATGCTGCTGT CTGTGTGCAAAGGTTGGTCCGCCACGCGGCCTAT TGTAGCGCAAGCAATCGTCGATCACATCAACCATG ATATCGTTCCGCTGGTGCCTCGTTACGGCAGCGTG GGCGCATCTGGTGATCTGATCCCGCTGTCGTACAT TGCTCGCGCTCTGTGCGGTATTGGCAAGGTGTACT ACATGGGCGCGGAAATCGACGCCGCCGAGGCAAT CAAACGTGCGGGCCTGACTCCGTTATCTCTGAAAG CGAAGGAAGGTCTGGCTCTGATCAACGGCACGCG TGTAATGTCTGGCATCTCCGCCATTACCGTGATTA AACTGGAAAAACTGTTCAAAGCTTCCATCTCCGCG ATCGCATTGGCGGTCGAGGCGTTGCTGGCATCCC ACGAACACTACGATGCCCGCATTCAACAGGTTAAA AACCATCCGGGTCAGAACGCGGTTGCATCCGCAC TTCGCAACTTGCTGGCGGGTTCTACTCAGGTGAAT CTGCTGTCAGGTGTTAAGGAACAGGCAAACAAAG CGTGTCGTCACCAGGAAATCACTCAGCTGAACGA CACCCTGCAGGAAGTATACTCCATCCGTTGCGCAC CGCAAGTGCTGGGCATTGTACCGGAAAGCCTGGC AACCGCACGTAAAATCCTGGAACGTGAGGTAATTT CGGCCAACGATAATCCGTTGATCGATCCAGAGAAT GGCGACGTACTGCACGGCGGGAACTTTATGGGCC AGTACGTTGCTCGCACTATGGACGCGCTGAAACTC GATATTGCTCTGATTGCCAACCATCTCCACGCGAT CGTTGCACTGATGATGGACAATCGTTTCAGTCGCG GTCTGCCGAACAGCCTGTCCCCGACTCCGGGTAT GTATCAGGGCTTTAAAGGTGTGCAGCTGTCCCAAA CGGCTCTGGTTGCGGCGATTCGTCATGATTGCGC CGCGAGCGGCATCCATACCTTAGCGACTGAACAG TATAACCAGGACATCGTTAGCCTGGGTTTGCATGC GGCGCAGGACGTTCTAGAAATGGAACAGAAACTG CGTAACATCGTATCCATGACTATTCTGGTTGTTTG CCAGGCAATCCACCTGCGCGGCAACATCAGTGAA ATCGCGCCAGAAACCGCGAAATTCTACCACGCGG TTCGTGAAATTTCCTCACCGCTGATCACCGATCGT GCTCTTGACGAAGATATCATCCGCATCGCGGATGC	

	GATCATTAATGACCAGCTGCCGCTGCCGGAAATTA TGCTGGAAGAGTAA	
Codon optimized PAL3 sequence 1	ATGAAAGCAAAAGATGTTCAGCCAACCATTATTATTA ATAAAAATGGCCTTATCTCTTTGGAAGATATCTATGA CATTGCGATAAAACAGAAAAAAGTAGAAATATCAAC GGAGATCACTGAGCTTTTGACGCATGGTCGTGAAAA ATTAGAGGAAAAATTAAATTCAGGAGAGGTTATATA TGGAATCAATACAGGATTTGGAGGGAATGCCAATTT AGTTGTGCCATTTGAGAAGATTGCAGAGCACCAGCA AAACCTGTTAACTTTTCTTTCTGCTGGTACTGGGGACT ATATGTCCAAGCCTTGTATTAAAGCGTCACAATTTAC TATGTTACTTTCTGTTTGCAAAGGTTGGTCTGCAACC AGACCAATTGTCGCTCAGGCGATTGTTGATCATATTA ACCATGACATTGTTCCTCTGGTTCCTCGCTATGGCTCA GTGGGTGCATCGGGTGATTTAATTCCTTTATCTTATAT TGCACGCGCTTTATGTGGTATCGGCAAAGTTTATTAT ATGGGTGCAGAAATTGACGCTGCTGAAGCAATTAAG CGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAA GAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGT CAGGAATCAGTGCAATCACCGTCATTAAACTGGAAA AACTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCT GTGGAAGCATTACTTGCGTCTCACGAACATTATGATG CCCGGATTCAACAAGTTAAGAACCATCCTGGTCAGA ATGCGGTGGCATCAGCATTGCGTAATTTATTGGCAGG TTCAACGCAGGTTAATCTACTGTCTGGGGTTAAAGAG CAGGCGAATAAAGCTTGTCGTCATCAGGAGATTACCC AACTCAATGATACCTTACAGGAAGTTTATTCAATTCG CTGTGCACCACAGGTATTAGGTATAGTGCCAGAATCT TTAGCTACTGCTCGGAAGATATTGGAACGGGAAGTTA TCTCAGCTAATGATAATCCATTGATAGATCCAGAGAA TGGCGATGTGCTACACGGTGGAAACTTTATGGGGCA ATATGTCGCCCGAACAATGGATGCATTAAAACTGGAT ATTGCTTTGATTGCCAATCATCTTCACGCCATTGTGGC TCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTA ATTCACTGAGTCCGACACCCGGCATGTACCAAGGTTT TAAAGGCGTCCAACTTTCTCAAACGGCTTTAGTTGCA GCGATTCGCCATGATTGTGCTGCATCAGGTATTCATA CCCTCGCAACAGAACAGTACAATCAGGATATTGTCA GTTTAGGTCTGCATGCCGCTCAAGATGTTTTAGAGAT GGAGCAGAAATTACGCAATATTGTTTCAATGACAATT CTGGTAGTTTGTCAGGCCATTCATCTTCGCGGCAATA TTAGTGAAATTGCGCCTGAAACTGCTAAATTTTACCA TGCAGTACGCGAAATCAGTTCTCCTTTGATCACTGAT CGTGCGTTGGATGAAGATATAATCCGCATTGCGGATG CAATTATTAATGATCAACTTCCTCTGCCAGAAATCAT GCTGGAAGAATGA	99
Codon optimized PAL3 sequence 2	ATGAAGGCCAAAGATGTACAGCCGACCATCATCATT AACAAAAACGGTTTGATTAGCCTGGAAGACATCTAT GATATCGCCATTAAGCAGAAAAAGGTTGAAATCTCC	100

	ACGGAAATTACAGAACTGTTGACTCACGGTCGCGAA AAACTGGAGGAAAAACTGAACAGCGGTGAAGTTATT TACGGTATCAACACTGGCTTTGGTGGTAACGCTAACC TTGTTGTGCCGTTCGAAAAGATTGCCGAACACCAGCA GAACCTGCTCACCTTCCTGTCTGCGGGTACAGGCGAC TATATGTCCAAACCGTGTATCAAAGCGTCTCAGTTTA CAATGCTGCTGTCTGTGTGCAAAGGTTGGTCCGCCAC GCGGCCTATTGTAGCGCAAGCAATCGTCGATCACATC AACCATGATATCGTTCCGCTGGTGCCTCGTTACGGCA GCGTGGGCGCATCTGGTGATCTGATCCCGCTGTCGTA CATTGCTCGCGCTCTGTGCGGTATTGGCAAGGTGTAC TACATGGGCGCGGAAATCGACGCCGCCGAGGCAATC AAACGTGCGGGCCTGACTCCGTTATCTCTGAAAGCGA AGGAAGGTCTGGCTCTGATCAACGGCACGCGTGTAA TGTCTGGCATCTCCGCCATTACCGTGATTAAACTGGA AAAACTGTTCAAAGCTTCCATCTCCGCGATCGCATTG GCGGTCGAGGCGTTGCTGGCATCCCACGAACACTAC GATGCCCGCATTCAACAGGTTAAAAACCATCCGGGTC AGAACGCGGTTGCATCCGCACTTCGCAACTTGCTGGC GGGTTCTACTCAGGTGAATCTGCTGTCAGGTGTTAAG GAACAGGCAAACAAAGCGTGTCGTCACCAGGAAATC ACTCAGCTGAACGACACCCTGCAGGAAGTATACTCC ATCCGTTGCGCACCGCAAGTGCTGGGCATTGTACCGG AAAGCCTGGCAACCGCACGTAAAATCCTGGAACGTG AGGTAATTTCGGCCAACGATAATCCGTTGATCGATCC AGAGAATGGCGACGTACTGCACGGCGGGAACTTTAT GGGCCAGTACGTTGCTCGCACTATGGACGCGCTGAA ACTCGATATTGCTCTGATTGCCAACCATCTCCACGCG ATCGTTGCACTGATGATGGACAATCGTTTCAGTCGCG GTCTGCCGAACAGCCTGTCCCCGACTCCGGGTATGTA TCAGGGCTTTAAAGGTGTGCAGCTGTCCCAAACGGCT CTGGTTGCGGCGATTCGTCATGATTGCGCCGCGAGCG GCATCCATACCTTAGCGACTGAACAGTATAACCAGG ACATCGTTAGCCTGGGTTTGCATGCGGCGCAGGACGT TCTAGAAATGGAACAGAAACTGCGTAACATCGTATC CATGACTATTCTGGTTGTTTGCCAGGCAATCCACCTG CGCGGCAACATCAGTGAAATCGCGCCAGAAACCGCG AAATTCTACCACGCGGTTCGTGAAATTTCCTCACCGC TGATCACCGATCGTGCTCTTGACGAAGATATCATCCG CATCGCGGATGCGATCATTAATGACCAGCTGCCGCTG CCGGAAATTATGCTGGAAGAGTAA	

Example 53. Comparison of *in vitro* PAL Activity from Various Inducible Promoters

[0826] *In vitro* PAL activity in strains in which PAL expression is under the control of various inducible promoters (arabinose, IPTG (LacI), rhamnose,

Tet, temperature (CI857) is measured by the rate of TCA accumulation. In this study, SYN-PKU707 is shown as a benchmark control. Strains were induced with 0.1% arabinose 1mM IPTG or rhamnose, or tetracycline as described herein. Ci857 was induced at 37 C and 42 C. Cells were resuspended in assay buffer and TCA levels were measured at various time points to determine the activity as described herein. Results are shown in **Fig. 60.** Constructs used in this study are described in **Table 80, Table 81,** and **Table 82.** LacI-PAL and Tet-PAL is shown elsewhere herein (e.g., **Table 72**). Constructs are shown in **Fig. 49**, **Fig. 62A, Fig. 62B,** and **Fig. 62C.**

Table 80. Construct for PAL expression under the control of mutant cI857 repressor sensitive promoters

Description	**SEQUENCE**	**SEQ ID NO**
Temperature sensitive promoter construct comprising mutant cI857 repressor (reverse orientation, underlined bold) and region comprising the CI857-inducible promoter (underlined italic) driving the expression of PAL3 (regular font); RBS and leader region in underlined italic and bold (see **Fig. 62A**)	**TCAGCCAAACGTCTCTTCAGGCCACTGAC TAGCGATAACTTTCCCCACAACGGAACAA CTCTCATTGCATGGGATCATTGGGTACTG TGGGTTTAGTGGTTGTAAAAACACCTGAC CGCTATCCCTGATCAGTTTCTTGAAGGTA AACTCATCACCCCCAAGTCTGGCTATGCA GAAATCACCTGGCTCAACAGCCTGCTCAG GGTCAACGAGAATTAACATTCCGTCAGGA AAGCTTGGCTTGGAGCCTGTTGGTGCGGT CATGGAATTACCTTCAACCTCAAGCCAGA ATGCAGAATCACTGGCTTTTTTGGTTGTG CTTACCCATCTCTCCGCATCACCTTTGGT AAAGGTTCTAAGCTTAGGTGAGAACATCC CTGCCTGAACATGAGAAAAAACAGGGTAC TCATACTCACTTCTAAGTGACGGCTGCAT ACTAACCGCTTCATACATCTCGTAGATTT CTCTGGCGATTGAAGGGCTAAATTCTTCA ACGCTAACTTTGAGAATTTTTGTAAGCAA TGCGGCGTTATAAGCATTTAATGCATTGA TGCCATTAAATAAAGCACCAACGCCTGAC TGCCCCATCCCCATCTTGTCTGCGACAGA TTCCTGGGATAAGCCAAGTTCATTTTTCT TTTTTTCATAAATTGCTTTAAGGCGACGT GCGTCCTCAAGCTGCTCTTGTGTTAATGG TTTCTTTTTTGTGCTCA**T*ACGTTAAATCTATC ACCGCAAGGGATAAATATCTAACACCGTGCGTG TTGACTATTTTACCTCTGGCGGTGATAATGGTT GCATAGCTGTCACCGGATGTGCTTTCCGGTCT GATGAGTCCGTGAGGACGAAACAGCCTCTACA AATAATTTTGTTTAAAACAACACCCACTAAGATA*	101

	ACTCTAGAAATAATTTTGTTTAACTTTAAGAA GGAGATATACATATGAAAGCTAAAGATGTTC AGCCAACCATTATTATTAATAAAAATGGCCT TATCTCTTTGGAAGATATCTATGACATTGCG ATAAAACAAAAAAAAGTAGAAATATCAACG GAGATCACTGAACTTTTGACGCATGGTCGTG AAAAATTAGAGGAAAAATTAAATTCAGGAG AGGTTATATATGGAATCAATACAGGATTTGG AGGGAATGCCAATTTAGTTGTGCCATTTGAG AAAATCGCAGAGCATCAGCAAAATCTGTTA ACTTTTCTTTCTGCTGGTACTGGGGACTATAT GTCCAAACCTTGTATTAAAGCGTCACAATTT ACTATGTTACTTTCTGTTTGCAAAGGTTGGT CTGCAACCAGACCAATTGTCGCTCAAGCAAT TGTTGATCATATTAATCATGACATTGTTCCTC TGGTTCCTCGCTATGGCTCAGTGGGTGCAAG CGGTGATTTAATTCCTTTATCTTATATTGCAC GAGCATTATGTGGTATCGGCAAAGTTTATTA TATGGGCGCAGAAATTGACGCTGCTGAAGC AATTAAACGTGCAGGGTTGACACCATTATCG TTAAAAGCCAAAGAAGGTCTTGCTCTGATTA ACGGCACCCGGGTAATGTCAGGAATCAGTG CAATCACCGTCATTAAACTGGAAAAACTATT TAAAGCCTCAATTTCTGCGATTGCCCTTGCT GTTGAAGCATTACTTGCATCTCATGAACATT ATGATGCCCGGATTCAACAAGTAAAAAATC ATCCTGGTCAAAACGCGGTGGCAAGTGCATT GCGTAATTTATTGGCAGGTTCAACGCAGGTT AATCTATTATCTGGGGTTAAAGAACAAGCCA ATAAAGCTTGTCGTCATCAAGAAATTACCCA ACTAAATGATACCTTACAGGAAGTTTATTCA ATTCGCTGTGCACCACAAGTATTAGGTATAG TGCCAGAATCTTTAGCTACCGCTCGGAAAAT ATTGGAACGGGAAGTTATCTCAGCTAATGAT AATCCATTGATAGATCCAGAAAATGGCGAT GTTCTACACGGTGGAAATTTTATGGGGCAAT ATGTCGCCCGAACAATGGATGCATTAAAACT GGATATTGCTTTAATTGCCAATCATCTTCAC GCCATTGTGGCTCTTATGATGGATAACCGTT TCTCTCGTGGATTACCTAATTCACTGAGTCC GACACCCGGCATGTATCAAGGTTTTAAAGGC GTCCAACTTTCTCAAACCGCTTTAGTTGCTG CAATTCGCCATGATTGTGCTGCATCAGGTAT TCATACCCTCGCCACAGAACAATACAATCAA GATATTGTCAGTTTAGGTCTGCATGCCGCTC AAGATGTTTTAGAGATGGAGCAGAAATTAC GCAATATTGTTTCAATGACAATTCTGGTAGT TTGTCAGGCCATTCATCTTCGCGGCAATATT AGTGAAATTGCGCCTGAAACTGCTAAATTTT	

	ACCATGCAGTACGCGAAATCAGTTCTCCTTT GATCACTGATCGTGCGTTGGATGAAGATATA ATCCGCATTGCGGATGCAATTATTAATGATC AACTTCCTCTGCCAGAAATCATGCTGGAAGA ATAA	
mutant cI857 repressor	TCAGCCAAACGTCTCTTCAGGCCACTGACTA GCGATAACTTTCCCCACAACGGAACAACTCT CATTGCATGGGATCATTGGGTACTGTGGGTT TAGTGGTTGTAAAAACACCTGACCGCTATCC CTGATCAGTTTCTTGAAGGTAAACTCATCAC CCCCAAGTCTGGCTATGCAGAAATCACCTGG CTCAACAGCCTGCTCAGGGTCAACGAGAATT AACATTCCGTCAGGAAAGCTTGGCTTGGAGC CTGTTGGTGCGGTCATGGAATTACCTTCAAC CTCAAGCCAGAATGCAGAATCACTGGCTTTT TTGGTTGTGCTTACCCATCTCTCCGCATCACC TTTGGTAAAGGTTCTAAGCTTAGGTGAGAAC ATCCCTGCCTGAACATGAGAAAAAACAGGG TACTCATACTCACTTCTAAGTGACGGCTGCA TACTAACCGCTTCATACATCTCGTAGATTTC TCTGGCGATTGAAGGGCTAAATTCTTCAACG CTAACTTTGAGAATTTTTGTAAGCAATGCGG CGTTATAAGCATTTAATGCATTGATGCCATT AAATAAAGCACCAACGCCTGACTGCCCCAT CCCCATCTTGTCTGCGACAGATTCCTGGGAT AAGCCAAGTTCATTTTTCTTTTTTTCATAAAT TGCTTTAAGGCGACGTGCGTCCTCAAGCTGC TCTTGTGTTAATGGTTTCTTTTTTGTGCTCAT	102
Region comprising Temperature sensitive promoter	ACGTTAAATCTATCACCGCAAGGGATAAAT ATCTAACACCGTGCGTGTTGACTATTTTACC TCTGGCGGTGATAATGGTTGCATAGCTGTCA CCGGATGTGCTTTCCGGTCTGATGAGTCCGT GAGGACGAAACAGCCTCTACAAATAATTTT GTTTAAAACAACACCCACTAAGATAACTCTA GAAATAATTTTGTTTAACTTTAAGAAGGAGA TATACAT	103
RBS and leader region	CTCTAGAAATAATTTTGTTTAACTTTAAGAA GGAGATATACAT	104
mutant cI857 repressor polypeptide sequence	MSTKKKPLTQEQLEDARRLKAIYEKKKNELGL SQESVADKMGMGQSGVGALFNGINALNAYN AALLTKILKVSVEEFSPSIAREIYEMYEAVSMQ PSLRSEYEYPVFSHVQAGMFSPKLRTFTKGDA ERWVSTTKKASDSAFWLEVEGNSMTAPTGSK PSFPDGMLILVDPEQAVEPGDFCIARLGGDEFT FKKLIRDSGQVFLQPLNPQYPMIPCNESCSVVG KVIASQWPEETFG	105

Table 81. Construct for PAL Expression under the Control of Rhamnose Sensitive Promoters

Description	Sequence	SEQ ID NO
PAL3 driven by Rhamnose-sensitive promoter (italic); CRP-cAMP binding sites are italic underlined; Rha S binding sites are bold italic; RBS leader region is bold italic underlined (**Fig. 62B**)	*CGGTGAGCATCACATCACCACAATTCAGCAAATTGTGA ACATCATCACGTTC**ATCTTTCCCTGGTTGCC**AATGGCC CATTTTCCT**GTCAGTAACGAGAAGGT**CGCGAATCAGG CGCTTTTTAGACTGGTCGTAATGAAATTCAGCTGTCACC GGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGA AACAGCCTCTACAAATAATTTTGTTTAAAACAACACCCA CTAAGATAA**CTCTAGAAATAATTTTGTTTAACTTTAAG AAGGAGATATACAT**ATGAAAGCTAAAGATGTTCAGC* CAACCATTATTATTAATAAAAATGGCCTTATCTCTT TGGAAGATATCTATGACATTGCGATAAAACAAAAA AAAGTAGAAATATCAACGGAGATCACTGAACTTTT GACGCATGGTCGTGAAAAATTAGAGGAAAAATTAA ATTCAGGAGAGGTTATATATGGAATCAATACAGGA TTTGGAGGGAATGCCAATTTAGTTGTGCCATTTGAG AAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTT CTTTCTGCTGGTACTGGGGACTATATGTCCAAACCT TGTATTAAAGCGTCACAATTTACTATGTTACTTTCT GTTTGCAAAGGTTGGTCTGCAACCAGACCAATTGTC GCTCAAGCAATTGTTGATCATATTAATCATGACATT GTTCCTCTGGTTCCTCGCTATGGCTCAGTGGGTGCA AGCGGTGATTTAATTCCTTTATCTTATATTGCACGA GCATTATGTGGTATCGGCAAAGTTTATTATATGGGC GCAGAAATTGACGCTGCTGAAGCAATTAAACGTGC AGGGTTGACACCATTATCGTTAAAAGCCAAAGAAG GTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAG GAATCAGTGCAATCACCGTCATTAAACTGGAAAAA CTATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCT GTTGAAGCATTACTTGCATCTCATGAACATTATGAT GCCCGGATTCAACAAGTAAAAAATCATCCTGGTCA AAACGCGGTGGCAAGTGCATTGCGTAATTTATTGGC AGGTTCAACGCAGGTTAATCTATTATCTGGGGTTAA AGAACAAGCCAATAAAGCTTGTCGTCATCAAGAAA TTACCCAACTAAATGATACCTTACAGGAAGTTTATT CAATTCGCTGTGCACCACAAGTATTAGGTATAGTGC CAGAATCTTTAGCTACCGCTCGGAAAATATTGGAAC GGGAAGTTATCTCAGCTAATGATAATCCATTGATAG ATCCAGAAAATGGCGATGTTCTACACGGTGGAAAT TTTATGGGGCAATATGTCGCCCGAACAATGGATGC ATTAAAACTGGATATTGCTTTAATTGCCAATCATCT TCACGCCATTGTGGCTCTTATGATGGATAACCGTTT CTCTCGTGGATTACCTAATTCACTGAGTCCGACACC CGGCATGTATCAAGGTTTTAAAGGCGTCCAACTTTC TCAAACCGCTTTAGTTGCTGCAATTCGCCATGATTG TGCTGCATCAGGTATTCATACCCTCGCCACAGAACA	106

	ATACAATCAAGATATTGTCAGTTTAGGTCTGCATGC CGCTCAAGATGTTTTAGAGATGGAGCAGAAATTAC GCAATATTGTTTCAATGACAATTCTGGTAGTTTGTC AGGCCATTCATCTTCGCGGCAATATTAGTGAAATTG CGCCTGAAACTGCTAAATTTTACCATGCAGTACGCG AAATCAGTTCTCCTTTGATCACTGATCGTGCGTTGG ATGAAGATATAATCCGCATTGCGGATGCAATTATTA ATGATCAACTTCCTCTGCCAGAAATCATGCTGGAAG AATAA	
Region comprising rhamnose inducible promoter	CGGTGAGCATCACATCACCACAATTCAGCAAATTGT GAACATCATCACGTTCATCTTTCCCTGGTTGCCAAT GGCCCATTTTCCTGTCAGTAACGAGAAGGTCGCGA ATCAGGCGCTTTTTAGACTGGTCGTAATGAAATTCA GCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCC GTGAGGACGAAACAGCCTCTACAAATAATTTTGTTT AAAACAACACCCACTAAGATAACTCTAGAAATAAT TTTGTTTAACTTTAAGAAGGAGATATACAT	107
RBS and leader region	CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA TATACAT	108
CRP-cAMP binding site 1	GTCAGTAACGAGAAGGT	109
CRP-cAMP binding site 2	AATTGTGAACATCATCACGTTC	110
RhaS binding site 1	ATCTTTCCCTGGTTGCC	111
RhaS binding site 2	GTCAGTAACGAGAAGGT	112

Table 82. Arabinose Inducible PAL3 Constructs and PssB promoter

Description	**Sequence**	**SEQ ID NO**
Arabinose inducible PAL3 construct comprising AraC (in reverse orientation, underlined), a region comprising an Arabinose inducible promoter (italic), and PAL3. RBS and leader region in	TTATTCACAACCTGCCCTAAACTCGCTCGGACTCGCCCCG GTGCATTTTTTAAATACTCGCGAGAAATAGAGTTGATCGT CAAAACCGACATTGCGACCGACGGTGGCGATAGGCATCC GGGTGGTGCTCAAAAGCAGCTTCGCCTGACTGATGCGCT GGTCCTCGCGCCAGCTTAATACGCTAATCCCTAACTGCTG GCGGAACAAATGCGACAGACGCGACGGCGACAGGCAGA CATGCTGTGCGACGCTGGCGATATCAAAATTACTGTCTGC CAGGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCG ATTATCCATCGGTGGATGGAGCGACTCGTTAATCGCTTCC ATGCGCCGCAGTAACAATTGCTCAAGCAGATTTATCGCC AGCAATTCCGAATAGCGCCCTTCCCCTTGTCCGGCATTAA TGATTTGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCG CTTCATCCGGGCGAAAGAAACCGGTATTGGCAAATATCG ACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCGCGGAC GAAAGTAAACCCACTGGTGATACCATTCGTGAGCCTCCG GATGACGACCGTAGTGATGAATCTCTCCAGGCGGGAACA	113

underlined and italic (**Fig. 62C**)	GCAAAATATCACCCGGTCGGCAGACAAATTCTCGTCCCT GATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGAG AATATAACCTTTCATTCCCAGCGGTCGGTCGATAAAAAA ATCGAGATAACCGTTGGCCTCAATCGGCGTTAAACCCGC CACCAGATGGGCGTTAAACGAGTATCCCGGCAGCAGGGG ATCATTTTGCGCTTCAGCCATACTTTTCATACTCCCGCCAT TCAGAGAAGAAACCAATTGTCCATATTGCAT *CAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTCGCTA* *ACCCAACCGGTAACCCCGCTTATTAAAAGCATTCTGTAACAAA* *GCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCT* *ATAATCACGGCAGAAAAGTCCACATTGATTATTTGCACGGCGT* *CACACTTTGCTATGCCATAGCATTTTTATCCATAAGATTAGCG* *GATCCAGCCTGACGCTTTTTTTCGCAACTCTCTACTGTTTCTC* *CATACCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATAT* *ACAT*ATGAAAGCTAAAGATGTTCAGCCAACCATTATTATT AATAAAAATGGCCTTATCTCTTTGGAAGATATCTATGACA TTGCGATAAAACAAAAAAAAGTAGAAATATCAACGGAG ATCACTGAACTTTTGACGCATGGTCGTGAAAAATTAGAG GAAAAATTAAATTCAGGAGAGGTTATATATGGAATCAAT ACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCCATTTG AGAAAATCGCAGAGCATCAGCAAAATCTGTTAACTTTTCT TTCTGCTGGTACTGGGGACTATATGTCCAAACCTTGTATT AAAGCGTCACAATTTACTATGTTACTTTCTGTTTGCAAAG GTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCAATTGT TGATCATATTAATCATGACATTGTTCCTCTGGTTCCTCGCT ATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCTTTATC TTATATTGCACGAGCATTATGTGGTATCGGCAAAGTTTAT TATATGGGCGCAGAAATTGACGCTGCTGAAGCAATTAAA CGTGCAGGGTTGACACCATTATCGTTAAAAGCCAAAGAA GGTCTTGCTCTGATTAACGGCACCCGGGTAATGTCAGGA ATCAGTGCAATCACCGTCATTAAACTGGAAAAACTATTTA AAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAAGCATT ACTTGCATCTCATGAACATTATGATGCCCGGATTCAACAA GTAAAAAATCATCCTGGTCAAAACGCGGTGGCAAGTGCA TTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAATCTAT TATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGTCGTC ATCAAGAAATTACCCAACTAAATGATACCTTACAGGAAG TTTATTCAATTCGCTGTGCACCACAAGTATTAGGTATAGT GCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGAACG GGAAGTTATCTCAGCTAATGATAATCCATTGATAGATCCA GAAAATGGCGATGTTCTACACGGTGGAAATTTTATGGGG CAATATGTCGCCCGAACAATGGATGCATTAAAACTGGAT ATTGCTTTAATTGCCAATCATCTTCACGCCATTGTGGCTCT TATGATGGATAACCGTTTCTCTCGTGGATTACCTAATTCA CTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAAGGC GTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTCGCC ATGATTGTGCTGCATCAGGTATTCATACCCTCGCCACAGA ACAATACAATCAAGATATTGTCAGTTTAGGTCTGCATGCC GCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGCAAT	

	ATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCATTC ATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAACTGC TAAATTTTACCATGCAGTACGCGAAATCAGTTCTCCTTTG ATCACTGATCGTGCGTTGGATGAAGATATAATCCGCATTG CGGATGCAATTATTAATGATCAACTTCCTCTGCCAGAAAT CATGCTGGAAGAATAA	
Arabinose inducible PAL3 construct comprising AraC (codon optimized in reverse orientation, underlined), a region comprising an Arabinose inducible promoter (italic), and PAL3. RBS and leader region in underlined and italic; araC binding sites in italic and bold, CRP binding site lower case italic and bold	TTACTCGCAACCCGCACGGAACTCGGATGGGGACGCGCC CGTGCATTTTTTGAAAACGCGGGAGAAGTACAGCTGGTC ATCGAAGCCAACGTTACGACCGACGGTCGCGATCGGCAT GCGGGTGGTAGACAGCAGCAGCTTAGCCTGGCTGATGCG CTGGTCTTCACGCCAAGACAGCACGCTAATACCAAGCTG CTGACGGAACAGGTGGGACAGACGGCTCGGACTCAGGCA CACGTGCTGTGCAACAGACGCAATATCAAAGTTGCTATC CGCCAGATGGTCGCTGATGTACTGGCAAGCCTCGCGCAC GCGGTTGTCCATCGGTGGGTGGAGACTTTCGTTGATCGCT TCCATACGACGCAGCAGCAGCTGTTCCAGCAGGTTGATC GCCAGCAGTTCGCTGTAGCGGCCTTCACCCTGACCGGCAT TGATAATCTGACCGAACAGATCGCTGAAGTGCGGCTGGT GAGCTTCATCCGGGCGGAAAAAACCAGTGTTTGCAAAGA TAGACGGCCAGTTCAGCCACTCATGCCAGTAAGCACGAG GGCGGAAGTATACCCACTGGTGATACCATTCATGCGCCTC CGGATGACGGCCGTAGTGATGGATTTCACCCGGCGGAAA TAACAGAATATCACCAGGACGACAAACGAATTCGCGACC CTGGTTTTTCACAACACCCTGGCCGCGAATAGTCAGGTTG AGAATGTAACCTTTCATACCCAGTGGACGGTCGATGAAG AAATCCAGGTAGCCGTTAGCCTCGATCGGGGTCAGACCC GCCACAAGGTGTGCATTGAAGGAATAGCCCGGCAGCAGC GGATCGTTCTGTGCTTCTGCCAT*CTTAGTAAGCTCCTTTTTC* *CTTAGTGTTCTCCTGCTAGCACAATCCCTAGGACTGAGCTAG* *CTGTCAAAGAAAAGAAAAAACACCCGTTAGGGTGTTTTTAGTT* *AGATCAGAGAA**GAAACCAATTGTCCATA**TTGCATCAGACATT* *GCCGTCACTGCGTCTTTTACTGGCTCTTCTCGCTAACCCAAC* *CGGTAACCCCGCTTATTAAAAGCATTCTGTAACAAAGCGGGA* *CCAAAGCCATGACAAAAACGCGT**AACAAAAGTGTCTATA**ATC* *AC**GGCAGAAAAGTCCACA**TTGAttatttgcacggcgtcacacttTGCTAT* *GCCA**TAGCATTTTTATCCATA**AGAT**TAGCGGATCCAGCCTG*** ***A**CGCTTTTTTTCGCAACTCTCTACTGTTTCTCCATACCCGAGC* *TGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGAC* *GAAACAGCCTCTACAAATAATTTTGTTTAAAACAACACCCACT* *AAGATAA**CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGA*** ***GATATACAT***ATGAAAGCTAAAGATGTTCAGCCAACCATTA TTATTAATAAAAATGGCCTTATCTCTTTGGAAGATATCTA TGACATTGCGATAAAACAAAAAAAAGTAGAAATATCAAC GGAGATCACTGAACTTTTGACGCATGGTCGTGAAAAATT AGAGGAAAAATTAAATTCAGGAGAGGTTATATATGGAAT CAATACAGGATTTGGAGGGAATGCCAATTTAGTTGTGCC ATTTGAGAAAATCGCAGAGCATCAGCAAAATCTGTTAAC TTTTCTTTCTGCTGGTACTGGGGACTATATGTCCAAACCTT	114

	GTATTAAAGCGTCACAATTTACTATGTTACTTTCTGTTTGC AAAGGTTGGTCTGCAACCAGACCAATTGTCGCTCAAGCA ATTGTTGATCATATTAATCATGACATTGTTCCTCTGGTTCC TCGCTATGGCTCAGTGGGTGCAAGCGGTGATTTAATTCCT TTATCTTATATTGCACGAGCATTATGTGGTATCGGCAAAG TTTATTATATGGGCGCAGAAATTGACGCTGCTGAAGCAAT TAAACGTGCAGGGTTGACACCATTATCGTTAAAAGCCAA AGAAGGTCTTGCTCTGATTAACGGCACCCGGGTAATGTC AGGAATCAGTGCAATCACCGTCATTAAACTGGAAAAACT ATTTAAAGCCTCAATTTCTGCGATTGCCCTTGCTGTTGAA GCATTACTTGCATCTCATGAACATTATGATGCCCGGATTC AACAAGTAAAAAATCATCCTGGTCAAAACGCGGTGGCAA GTGCATTGCGTAATTTATTGGCAGGTTCAACGCAGGTTAA TCTATTATCTGGGGTTAAAGAACAAGCCAATAAAGCTTGT CGTCATCAAGAAATTACCCAACTAAATGATACCTTACAG GAAGTTTATTCAATTCGCTGTGCACCACAAGTATTAGGTA TAGTGCCAGAATCTTTAGCTACCGCTCGGAAAATATTGGA ACGGGAAGTTATCTCAGCTAATGATAATCCATTGATAGAT CCAGAAAATGGCGATGTTCTACACGGTGGAAATTTTATG GGGCAATATGTCGCCCGAACAATGGATGCATTAAAACTG GATATTGCTTTAATTGCCAATCATCTTCACGCCATTGTGG CTCTTATGATGGATAACCGTTTCTCTCGTGGATTACCTAA TTCACTGAGTCCGACACCCGGCATGTATCAAGGTTTTAAA GGCGTCCAACTTTCTCAAACCGCTTTAGTTGCTGCAATTC GCCATGATTGTGCTGCATCAGGTATTCATACCCTCGCCAC AGAACAATACAATCAAGATATTGTCAGTTTAGGTCTGCAT GCCGCTCAAGATGTTTTAGAGATGGAGCAGAAATTACGC AATATTGTTTCAATGACAATTCTGGTAGTTTGTCAGGCCA TTCATCTTCGCGGCAATATTAGTGAAATTGCGCCTGAAAC TGCTAAATTTTACCATGCAGTACGCGAAATCAGTTCTCCT TTGATCACTGATCGTGCGTTGGATGAAGATATAATCCGCA TTGCGGATGCAATTATTAATGATCAACTTCCTCTGCCAGA AATCATGCTGGAAGAATAA	
Codon optimized AraC (reverse orientation)	TTACTCGCAACCCGCACGGAACTCGGATGGGGACGCGCC CGTGCATTTTTTGAAAACGCGGGAGAAGTACAGCTGGTC ATCGAAGCCAACGTTACGACCGACGGTCGCGATCGGCAT GCGGGTGGTAGACAGCAGCAGCTTAGCCTGGCTGATGCG CTGGTCTTCACGCCAAGACAGCACGCTAATACCAAGCTG CTGACGGAACAGGTGGGACAGACGGCTCGGACTCAGGCA CACGTGCTGTGCAACAGACGCAATATCAAAGTTGCTATC CGCCAGATGGTCGCTGATGTACTGGCAAGCCTCGCGCAC GCGGTTGTCCATCGGTGGGTGGAGACTTTCGTTGATCGCT TCCATACGACGCAGCAGCAGCTGTTCCAGCAGGTTGATC GCCAGCAGTTCGCTGTAGCGGCCTTCACCCTGACCGGCAT TGATAATCTGACCGAACAGATCGCTGAAGTGCGGCTGGT GAGCTTCATCCGGGCGGAAAAAACCAGTGTTTGCAAAGA TAGACGGCCAGTTCAGCCACTCATGCCAGTAAGCACGAG GGCGGAAGTATACCCACTGGTGATACCATTCATGCGCCTC CGGATGACGGCCGTAGTGATGGATTTCACCCGGCGGAAA	115

	TAACAGAATATCACCAGGACGACAAACGAATTCGCGACC CTGGTTTTTCACAACACCCTGGCCGCGAATAGTCAGGTTG AGAATGTAACCTTTCATACCCAGTGGACGGTCGATGAAG AAATCCAGGTAGCCGTTAGCCTCGATCGGGGTCAGACCC GCCACAAGGTGTGCATTGAAGGAATAGCCCGGCAGCAGC GGATCGTTCTGTGCTTCTGCCAT	
Arabinose inducible promoter	CTTAGTAAGCTCCTTTTTCCTTAGTGTTCTCCTGCTAGCAC AATCCCTAGGACTGAGCTAGCTGTCAAAGAAAAGAAAAA ACACCCGTTAGGGTGTTTTTAGTTAGATCAGAGAAGAAA CCAATTGTCCATATTGCATCAGACATTGCCGTCACTGCGT CTTTTACTGGCTCTTCTCGCTAACCCAACCGGTAACCCCG CTTATTAAAAGCATTCTGTAACAAAGCGGGACCAAAGCC ATGACAAAAACGCGTAACAAAAGTGTCTATAATCACGGC AGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTT GCTATGCCATAGCATTTTTATCCATAAGATTAGCGGATCC AGCCTGACGCTTTTTTTCGCAACTCTCTACTGTTTCTCCAT ACCCGAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGT CCGTGAGGACGAAACAGCCTCTACAAATAATTTTGTTTAA AACAACACCCACTAAGATAA	116
PssB promoter	TCACCTTTCCCGGATTAAACGCTTTTTTGCCCGGTGGCAT GGTGCTACCGGCGATCACAAACGGTTAATTATGACACAA ATTGACCTGAATGAATATACAGTATTGGAATGCATTACCC GGAGTGTTGTGTAACAATGTCTGGCCAGGTTTGTTTCCCG GAACCGAGGTCACAACATAGTAAAAGCGCTATTGGTAAT GGTACAATCGCGCGTTTACACTTATTC	117

Example 54. Strain Activity

[0827] The activity of SYN-707, SYN-PKU710, and SYN-PKU708 was measured *in vitro*. Results are reported in Table 83.

[0828] For SYN-PKU710, cells were grown to OD 0.2 in fermentation media and induced by addition of 1 mM IPTG for one hour. Then, was added arabinose at 0.009 % final concentration. Cells were induced for another for 4 hours. For SYN-PKU708 and SYN-PKU707, cells were grown to OD 0.2 in fermentation media and induced by addition of arabinose at 0.15% final concentration for 4 hours. In the presence of oxygen (shaking), high levels of activity are observed which are not limited by oxygen, glucose, pH or substrate. Under microaerobic conditions (static incubation), LAAD activity is dependent on oxygen. Results are reported in **Table 83.**

Table 83. Strain Activity Table

	PAL activity (umol/he/1e9 cfu)		**LAAD activity (umol/he/1e9 cfu)**		**Total Phe degradation (umol/he/1e9 cfu)**	
	static (micro)	+O2	static (micro)	+O2	static (micro)	+O2
SYN-PKU707	**4.09** ± 0.53	**4.37** ± 0.69	0	0	**4.1**	**4.4**
SYN-PKU710	**1.83** ± 0.13	**2.41** ± 0.24	**7.56** ± 0.93	**31.23** ± 4.94	**9.9**	**33.7**
SYN-PKU708	NT	**3.67** ± 0.27	**8.11** ± 1.22	**47.81** ± 3.41	**NT**	**51.5**

Example 55. Wild Type clbA and clbA knock out

Table 84. wild Type clbA and clbA knock out

Wild-type clbA (SEQ ID NO: 118)	caaatatcacataatcttaacatatcaataaacacagtaaagtttcatgtgaaaaacatcaaacataaaata caagctcggaatacgaatcacgctatacacattgctaacaggaatgagattatctaaatgaggattgatat attaattggacatactagtttttttcatcaaaccagtagagataacttccttcactatctcaatgaggaagaaa taaaacgctatgatcagtttcattttgtgagtgataaagaactctatattttaagccgtatcctgctcaaaaca gcactaaaaagatatcaacctgatgtctcattacaatcatggcaatttagtacgtgcaaatatggcaaacc atttatagtttttcctcagttggcaaaaaagatttttttttaacctttcccatactatagatacagtagccgttgct attagttctcactgcgagcttggtgtcgatattgaacaaataagagatttagacaactcttatctgaatatca gtcagcatttttttactccacaggaagctactaacatagtttcacttcctcgttatgaaggtcaattacttttttg gaaaatgtggacgctcaaagaagcttacatcaaatatcgaggtaaaggcctatctttaggactggattgt attgaatttcatttaacaaataaaaaactaacttcaaaatatagaggttcacctgtttatttctctcaatggaaa atatgtaactcatttctcgcattagcctctccactcatcacccctaaaataactattgagctatttcctatgca gtcccaactttatcaccacgactatcagctaattcattcgtcaaatgggcagaattgaatcgccacggata atctagacacttctgagccgtcgataatattgattttcatattccgtcggtggtgtaagtatcccgcataatc gtgccattcacatttag
clbA knockout (SEQ ID NO: 119)	ggatgggggggaaacatggataagttcaaagaaaaaaacccgttatctctgcgtgaaagacaagtattgc gcatgctggcacaaggtgatgagtactctcaaatatcacataatcttaacatatcaataaacacagtaaag tttcatgtgaaaaacatcaaacataaaatacaagctcggaatacgaatcacgctatacacattgctaacag gaatgagattatctaaatgaggattgaTGTGTAGGCTGGAGCTGCTTCGAAGTT CCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCG GAATAGGAACTAAGGAGGATATTCATATGtcgtcaaatgggcagaattgaa tcgccacggataatctagacacttctgagccgtcgataatattgattttcatattccgtcggtgg

WHAT IS CLAIMED:

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;
   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; and
   c) one or more gene(s) encoding a mutated fumarate and nitrate reductase regulator (FNR) wherein the gene(s) encoding mutated FNR is operably linked to a directly or indirectly inducible promoter that is not associated with the FNR 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 mutated FNR can activate an FNR promoter under oxygenated conditions.

4. The genetically engineered bacterium of claim 3, wherein the mutated FNR is FNRS24Y.

5. The genetically engineered bacterium of any of claims 1 - 4, 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.

6. The genetically engineered bacterium of any of claims 1 - 4, 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.

7. The genetically engineered bacterium of any one of claims 2-6, wherein the gene(s) encoding a LAAD is operably linked to a different promoter from the promoter operably linked to the gene(s) encoding a PAL and the promoter operably linked to the gene(s) encoding a phenylalanine transporter.

8. The genetically engineered bacterium of any one of claims 2-7, wherein the promoter operably linked to the gene(s) encoding a PAL, the promoter operably linked to the gene(s) encoding a phenylalanine transporter, and the promoter operably linked to the gene(s) encoding a LAAD are directly or indirectly induced by exogenous environmental conditions.

9. The genetically engineered bacterium of any one of claims 1-8, 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 directly or indirectly induced by exogenous environmental conditions found in the gut of a mammal.

10. The genetically engineered bacterium of claim 9, 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 directly or indirectly induced by exogenous environmental conditions found in the small intestine of a mammal.

11. The genetically engineered bacterium of claim any one of claims 1-10, 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 directly or indirectly induced under low-oxygen or anaerobic conditions.

12. The genetically engineered bacterium of claim 11, 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 is an FNR-responsive promoter.

13. The genetically engineered bacterium of any one of claims 2-12, 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.

14. The genetically engineered bacterium of any one of claims 2-12, 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.

15. The genetically engineered bacterium of claim 14, wherein the gene encoding a LAAD is under the control of a promoter that is directly or indirectly induced by the presence of a chemical and/or nutritional inducer selected from arabinose, IPTG, tetracycline, and rhamnose.

16. The genetically engineered bacterium of any of claims 1-15, wherein the gene(s) encoding mutated FNR is under the control of a promoter that is directly or indirectly induced by the presence of a chemical and/or nutritional inducer selected from arabinose, IPTG, tetracycline, and rhamnose.

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

18. The genetically engineered bacterium of any one of claims 1-16, wherein the gene(s) encoding a phenylalanine transporter is located on a plasmid in the bacterium.

19. The genetically engineered bacterium of any one of claims 1-18, wherein the gene(s) encoding a PAL is located on a plasmid in the bacterium.

20. The genetically engineered bacterium of any one of claims 1-18, wherein the gene(s) encoding a PAL is located on a chromosome in the bacterium.

21. The genetically engineered bacterium of any one of claims 1-20, wherein the gene(s) encoding mutated FNR is located on a plasmid in the bacterium.

22. The genetically engineered bacterium of any one of claims 1-20, wherein the gene(s) encoding mutated FNR is located on a chromosome in the bacterium.

23. The genetically engineered bacterium of any one of claims 2-22, wherein the gene(s) encoding LAAD is located on a plasmid in the bacterium.

24. The genetically engineered bacterium of any one of claims 2-22, wherein the gene(s) encoding LAAD is located on a chromosome in the bacterium.

25. The genetically engineered bacterium of any of claims 2-24, wherein the gene(s) encoding mutated FNR and the gene(s) encoding LAAD are under the control of the same promoter.

26. The genetically engineered bacterium of claim 25, wherein the promoter is directly or indirectly induced by the presence of a chemical and/or nutritional inducer selected from arabinose, IPTG, tetracycline, and rhamnose.

27. A genetically engineered bacterium comprising:
    a) two or more gene(s) encoding a phenylalanine ammonia lyase (PAL), wherein the gene(s) encoding a PAL are 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;

c) wherein at least one of the gene(s) encoding PAL is operably linked to a directly or indirectly inducible promoter that is induced by a chemical and/or nutritional inducer.

28. The genetically engineered bacterium of claim 27, wherein the chemical and/or nutritional inducer is selected from arabinose, IPTG, tetracycline, and rhamnose.

29. The genetically engineered bacterium of claim 27 or claim 28, wherein at least a second gene(s) encoding PAL is operably linked to a directly or indirectly inducible promoter that is induced by exogenous environmental conditions found in the gut of a mammal.

30. The genetically engineered bacterium of claim 29, wherein the promoter operably linked to the at least second gene encoding a PAL is directly or indirectly induced by exogenous environmental conditions found in the small intestine of a mammal.

31. The genetically engineered bacterium of claim 30, wherein the promoter operably linked to the at least second gene encoding a PAL is directly or indirectly induced under low-oxygen or anaerobic conditions.

32. The genetically engineered bacterium of claim 11, wherein the promoter operably linked to the at least second gene(s) encoding a PAL and the promoter operably linked to the gene(s) encoding a phenylalanine transporter is an FNR-responsive promoter.

33. The genetically engineered bacterium of any one of claims 1-32, wherein the PAL is from *Anabaena* variabilis (PAL1) or from *Photorhabdus luminescens* (PAL3).

34. The genetically engineered bacterium of any one of claims 1-33, wherein the phenylalanine transporter is PheP.

35. The genetically engineered bacterium of any one of claims 1-34, wherein the bacterium is a probiotic bacterium.

36. The genetically engineered bacterium of claim 35, wherein the bacterium is selected from the group consisting of *Bacteroides, Bifidobacterium, Clostridium, Escherichia, Lactobacillus*, and *Lactococcus*.

37. The genetically engineered bacterium of claim 36, wherein the bacterium is *Escherichia coli* strain Nissle.

38. The genetically engineered bacterium of any one of claims 1-37, wherein the bacterium is an auxotroph in a gene that is complemented when the bacterium is present in a mammalian gut.

39. The genetically engineered bacterium of claim 38, wherein mammalian gut is a human gut.

40. The genetically engineered bacterium of claim 38 or 39, wherein the bacterium is an auxotroph in diaminopimelic acid or an enzyme in the thymidine biosynthetic pathway.

41. The genetically engineered bacterium of any one of claims 1-40, 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.

42. A pharmaceutically acceptable composition comprising the bacterium of any one of claims 1-41; and a pharmaceutically acceptable carrier.

43. The composition of claim 42 formulated for oral administration.

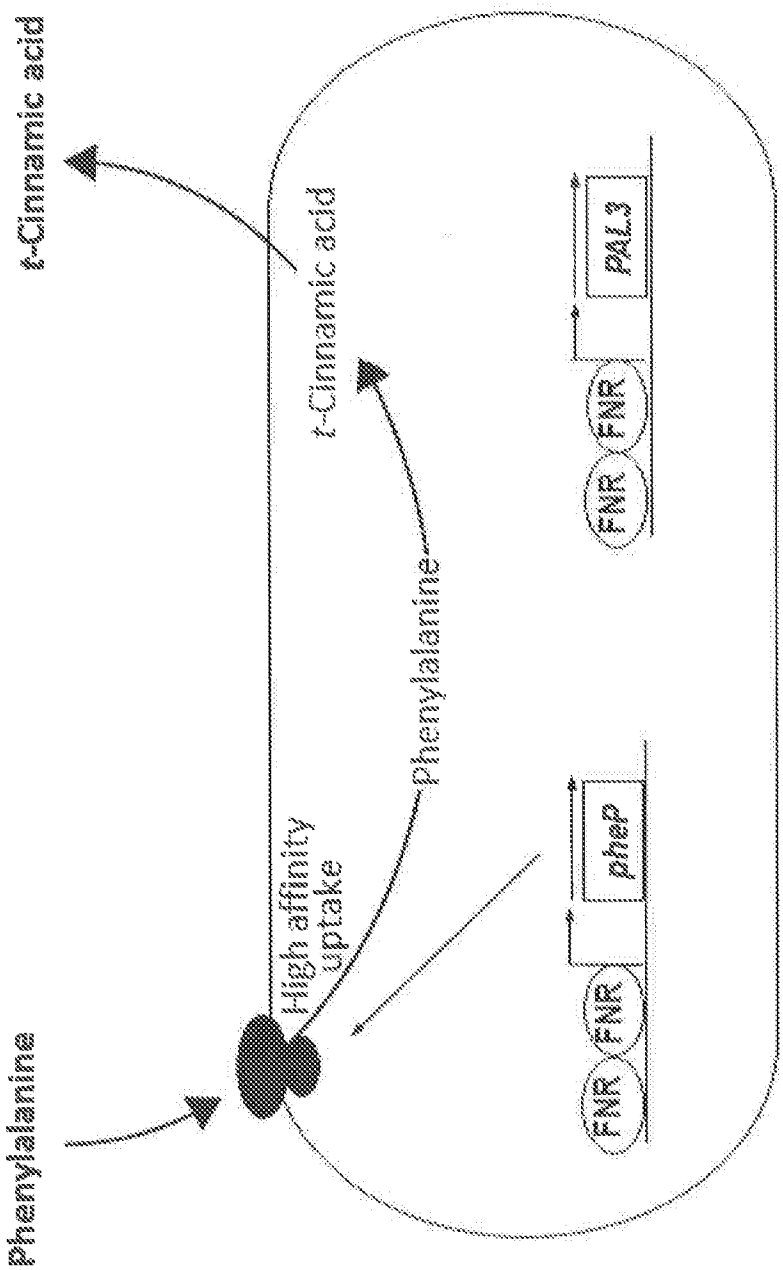
44. A method of reducing hyperphenylalaninemia or treating a disease associated with hyperphenylalaninemia comprising the step of administering to a subject in need thereof, the composition of claim 42 or 43.

45. The method of claim 44, 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.

46. A method for measuring PAL activity in a subject, the method comprising administering a PAL-based drug to the subject and subsequently measuring the amount of hippurate produced in urine of the subject.

47. A method for monitoring the therapeutic activity of a PAL-based drug in a subject, the method comprising administering the PAL-based drug to the subject and subsequently measuring the amount of hippurate produced in the urine of the subject.

48. A method for adjusting the dosage of a PAL-based drug administered to a subject, the method comprising administering the PAL-based drug to a subject, measuring the amount of hippurate produced in the urine of the subject, and increasing or decreasing the dosage of the drug to increase or decrease the PAL activity in the subject.



PAL = Phenylalanine Ammonia Lyase
PheP =High Affinity Phenylalanine Transporter

Fig. 1

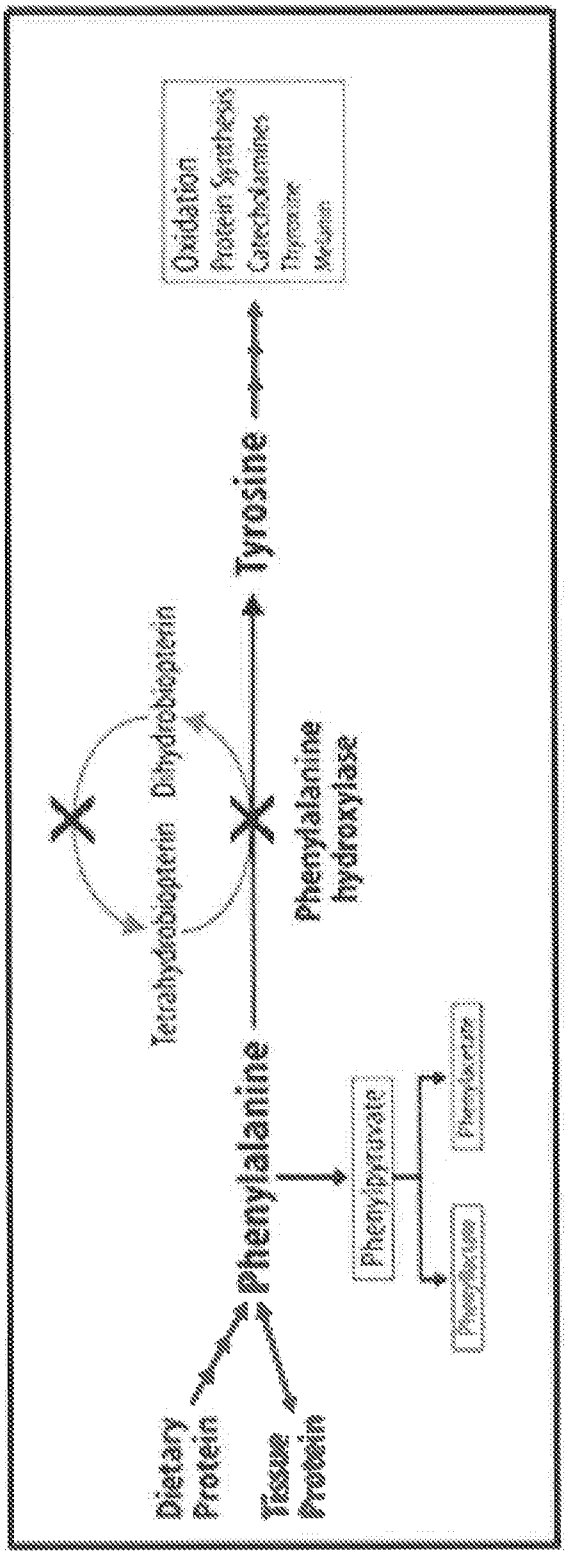

Oxidation
Protein Synthesis
Catecholamines
Thyroxine
Melanin
Tyrosine
Tetrahydrobiopterin
Dihydrobiopterin
Phenylalanine hydroxylase
Phenylalanine
Phenylpyruvate
Phenylacetate
Phenylalanine
Dietary Protein
Tissue Protein

Fig. 2A

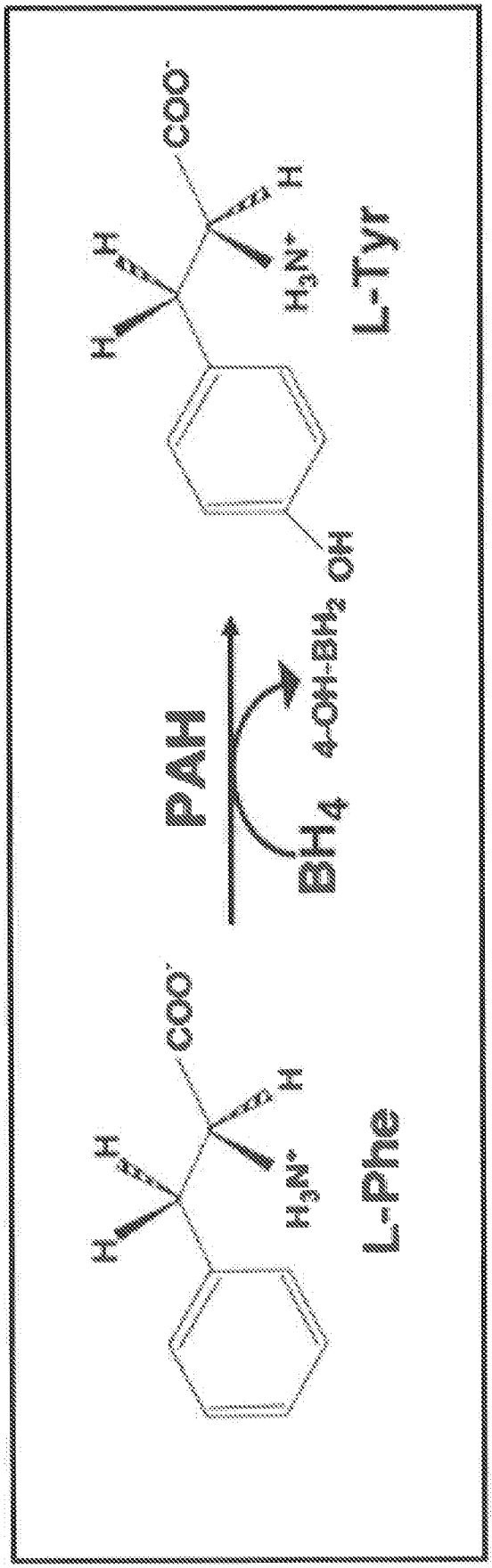


Fig. 2B

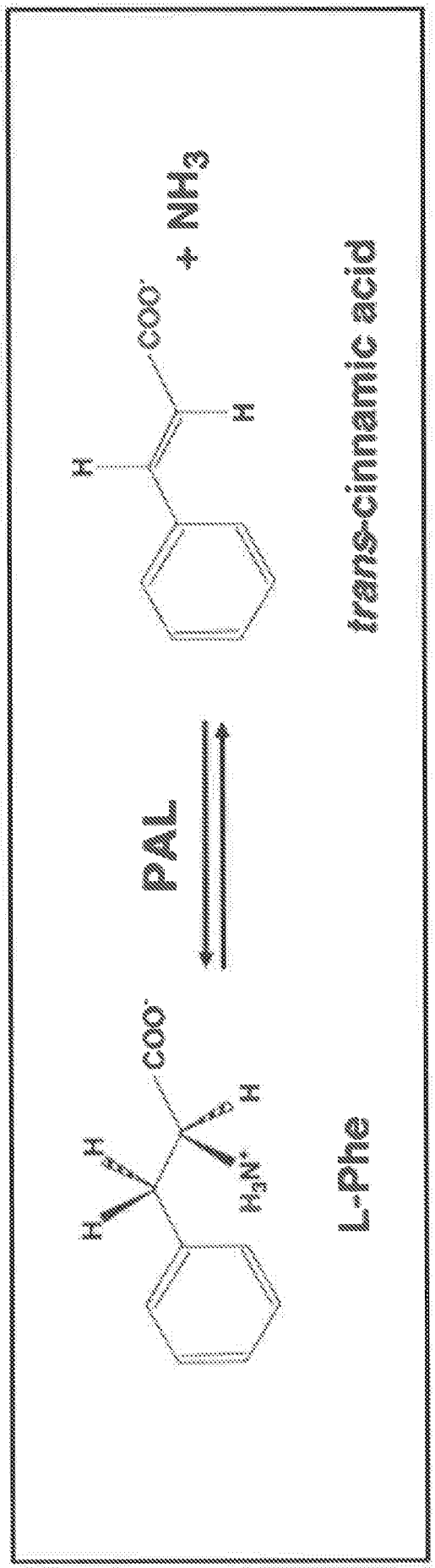


Fig. 2C

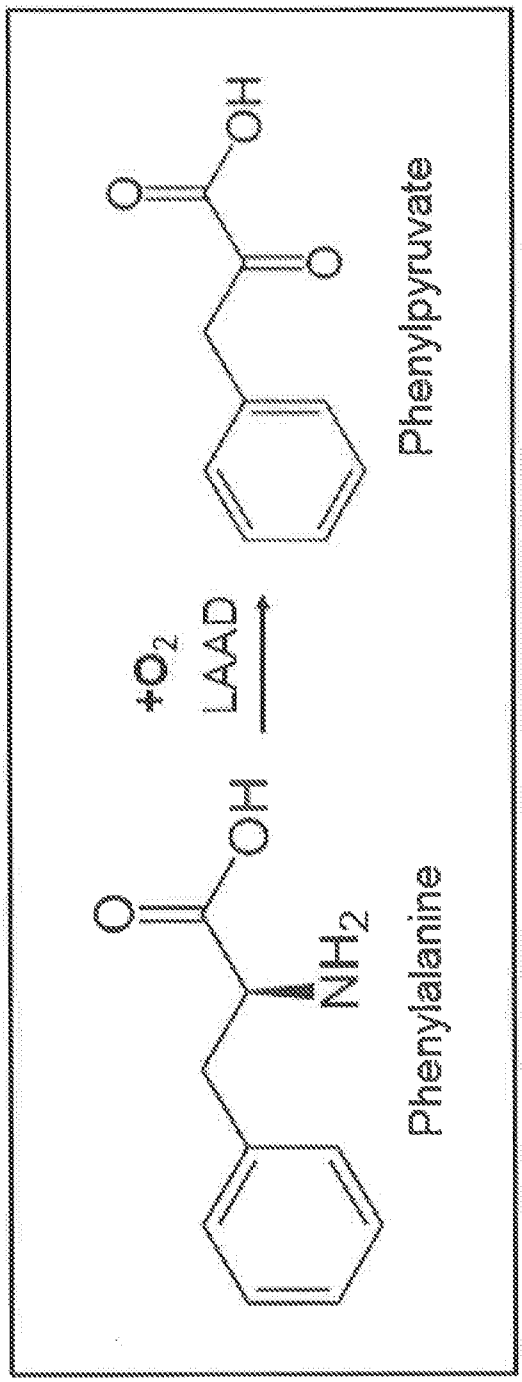
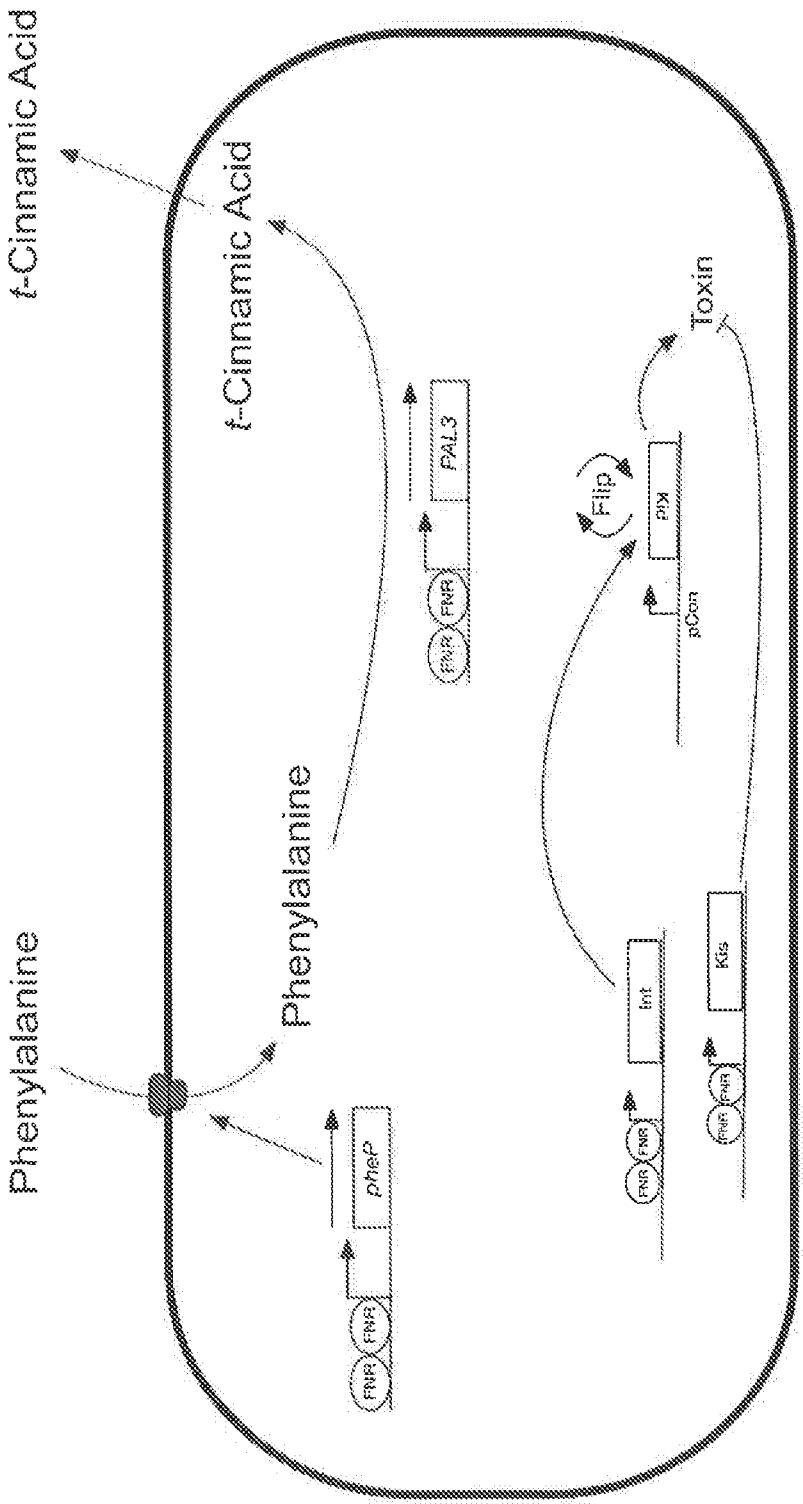


Fig. 2D



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

Fig. 3

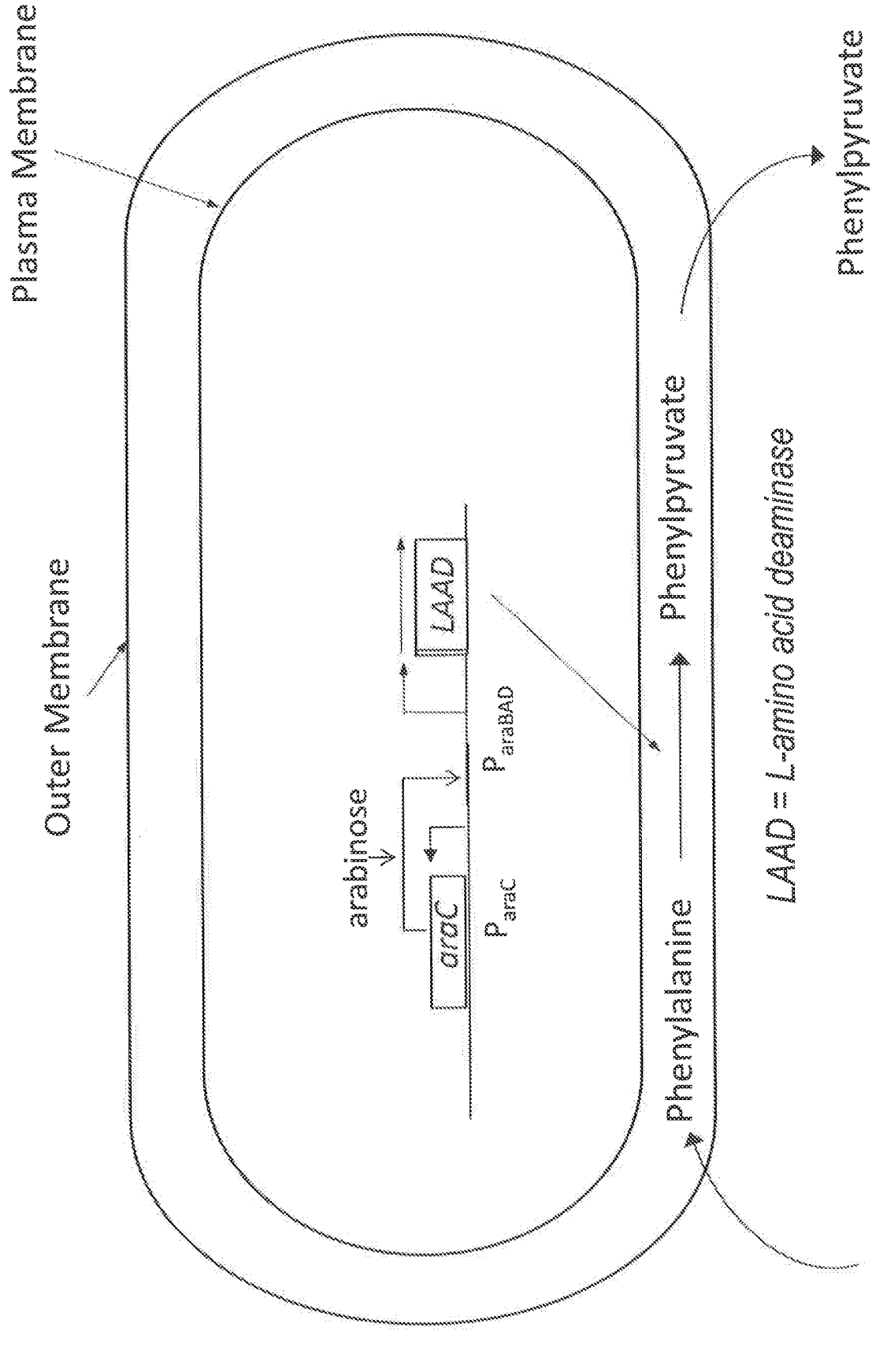

Plasma Membrane
Outer Membrane
arabinose
araC
LAAD
P_{araC}
P_{araBAD}
Phenylpyruvate
Phenylalanine
Phenylpyruvate
Phenylalanine
LAAD = L-amino acid deaminase

Fig. 4

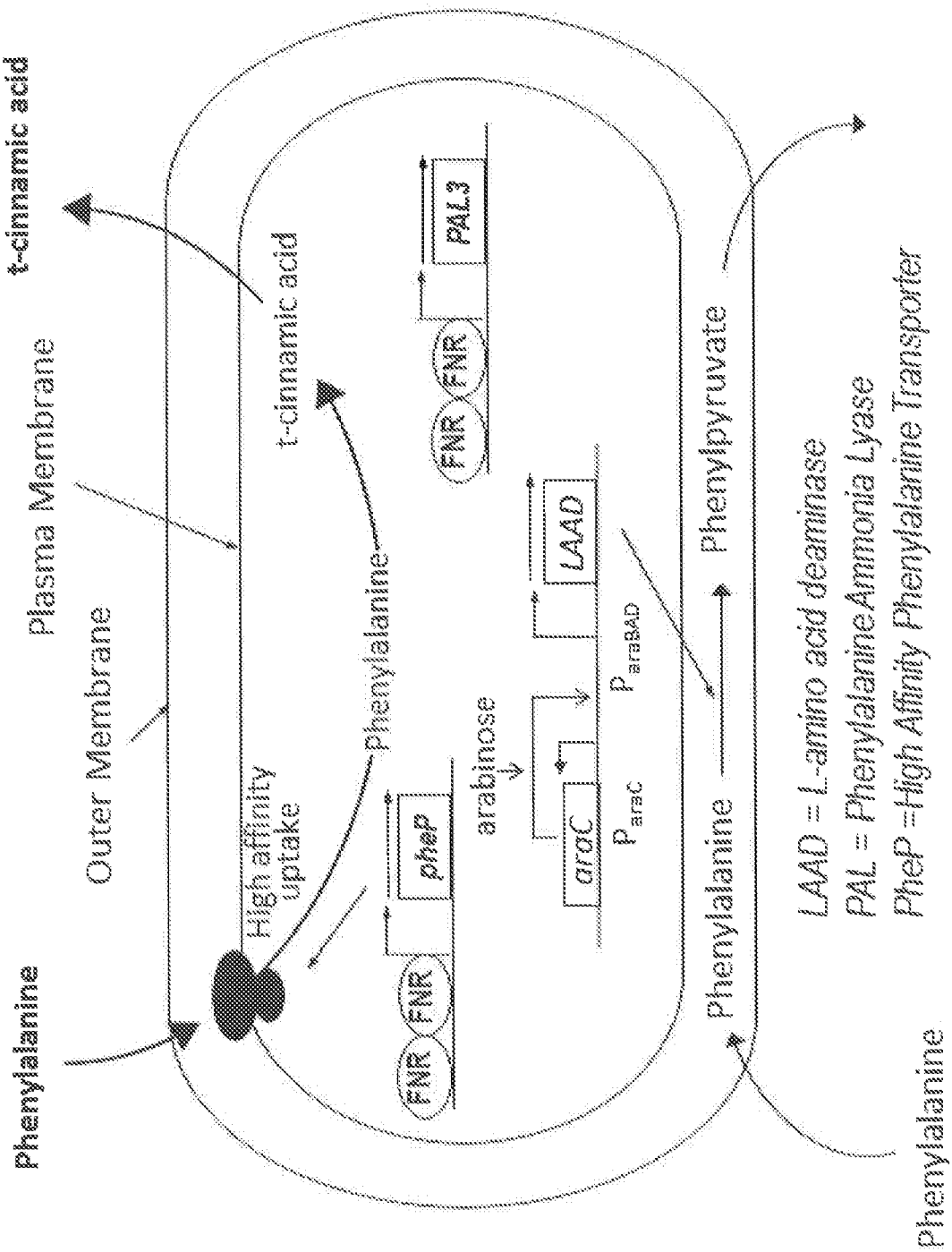

t-cinnamic acid
Plasma Membrane
Outer Membrane
Phenylalanine
High affinity uptake
t-cinnamic acid
Phenylalanine
FNR
FNR
pheP
arabinose
araC
P araC
P araBAD
LAAD
FNR
FNR
PAL3
Phenylalanine
Phenylpyruvate
Phenylalanine
LAAD = L-amino acid deaminase
PAL = PhenylalanineAmmonia Lyase
PheP =High Affinity Phenylalanine Transporter

Fig. 5

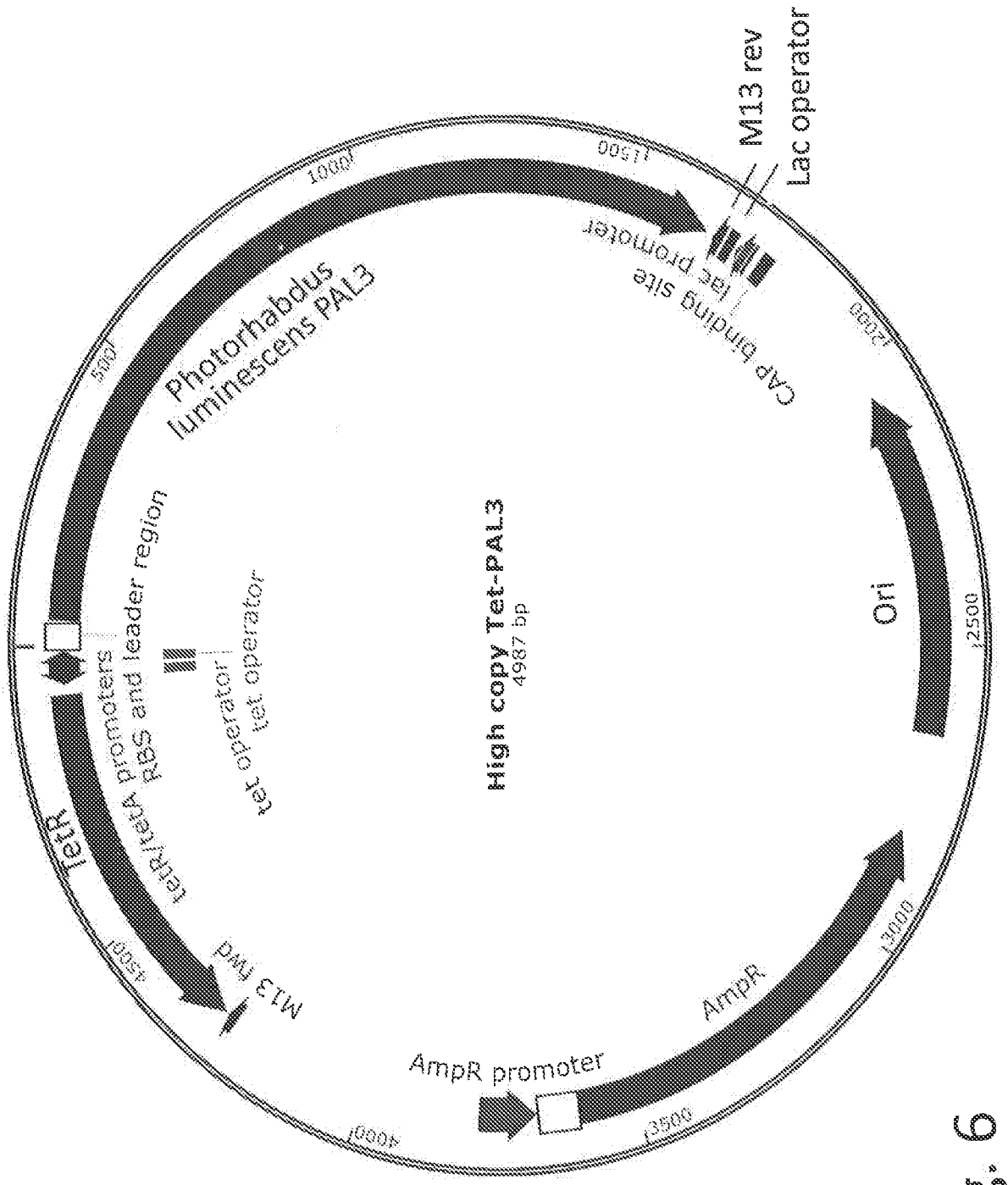
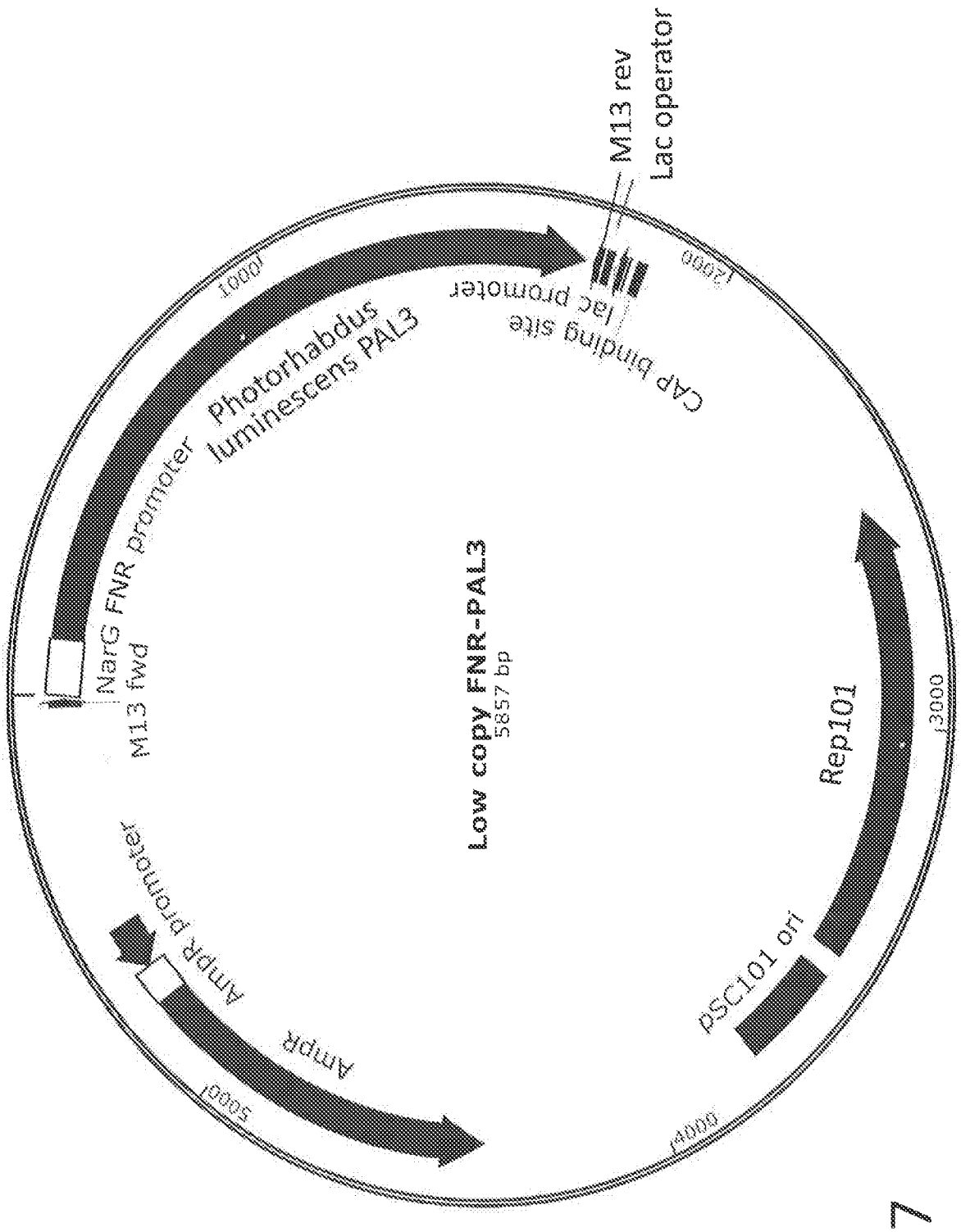

High copy Tet-PAL3
4987 bp
TetR
TetR/tetA promoters
RBS and leader region
tet operator
M13 fwd
AmpR promoter
AmpR
Ori
CAP binding site
lac promoter
M13 rev
Lac operator
Photorhabdus luminescens PAL3
500
1000
1500
2000
2500
3000
3500
4000
4500

Fig. 6


Low copy FNR-PAL3
5857 bp
M13 rev
Lac operator
CAP binding site
lac promoter
Photorhabdus luminescens PAL3
NarG FNR promoter
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AmpR promoter
AmpR
pSC101 ori
Rep101
1000
2000
3000
4000
5000

Fig. 7

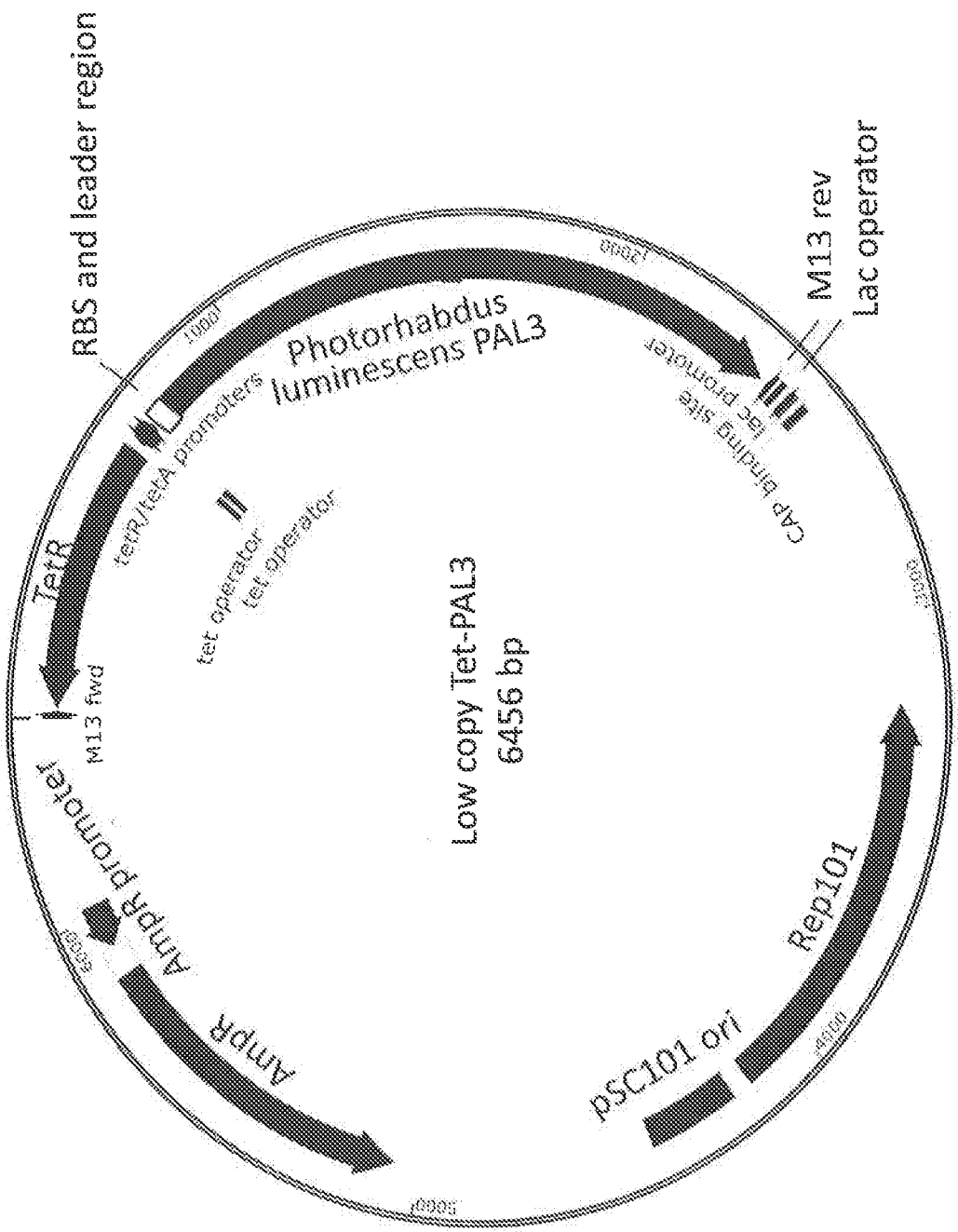

RBS and leader region
M13 rev
Lac operator
Photorhabdus luminescens PAL3
TetR
tetR/tetA promoters
tet operator
tet operator
CAP binding site
lac promoter
M13 fwd
Low copy Tet-PAL3
6456 bp
AmpR promoter
AmpR
pSC101 ori
Rep101
1000
2000
3000
4000
5000
6000

Fig. 8

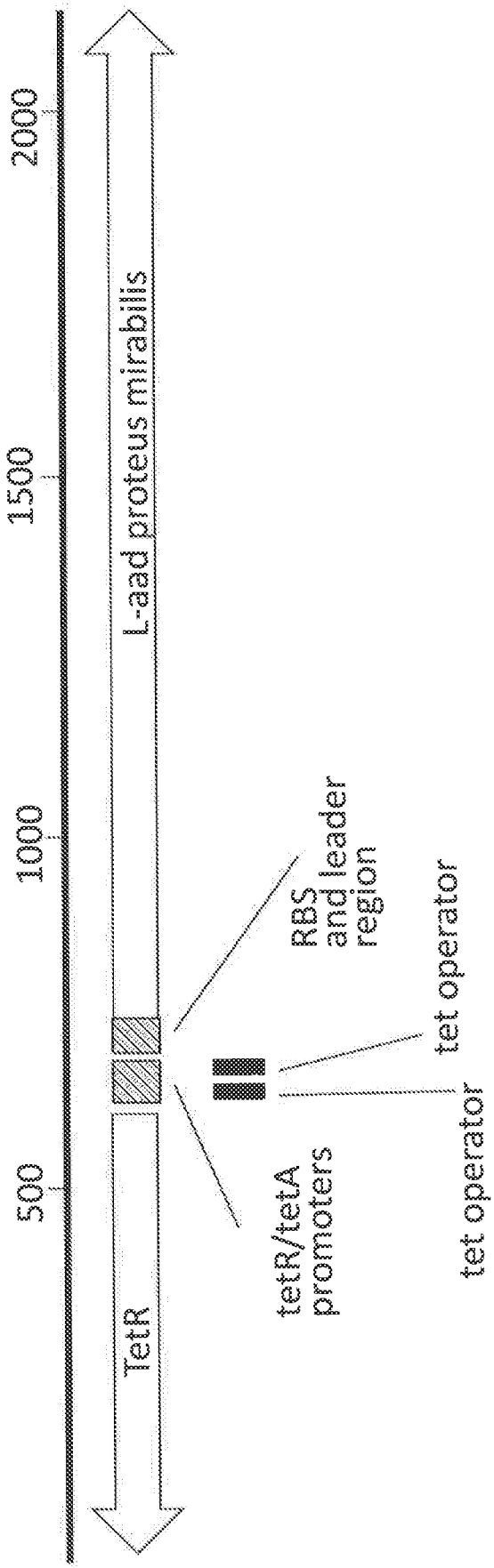

2000
1500
1000
500
L-aad proteus mirabilis
TetR
tetR/tetA promoters
RBS and leader region
tet operator
tet operator

Fig. 9

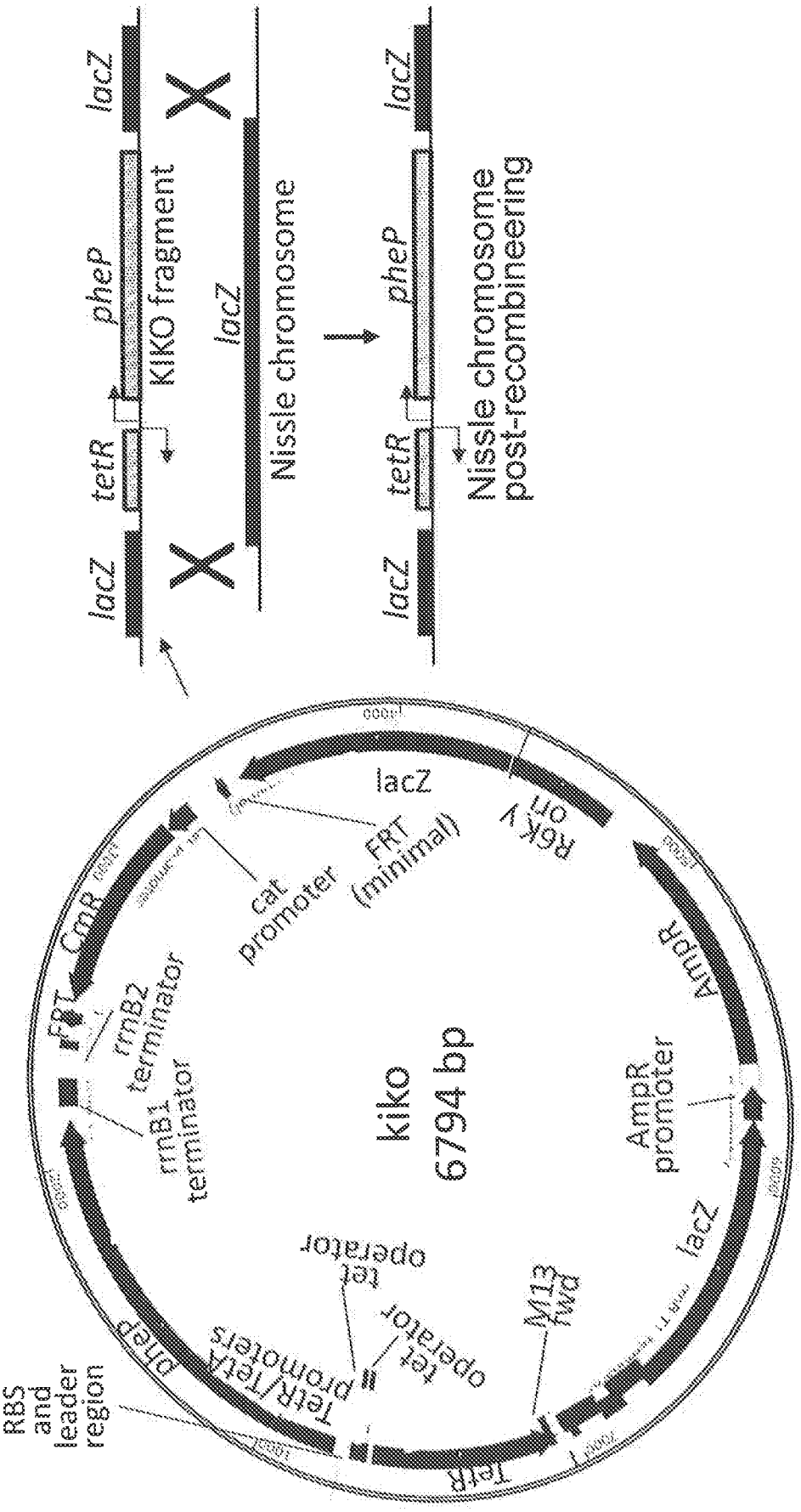

lacZ
pheP
KIKO fragment
tetR
lacZ
Nissle chromosome
lacZ
pheP
tetR
lacZ
Nissle chromosome
post-recombineering
kiko
6794 bp
lacZ
RBS
and
leader
region
pheP
TetR/TetA
promoters
tet
operator
tet
operator
M13
fwd
TetR
lacZ
AmpR
promoter
AmpR
FRT
rrnB2
terminator
rrnB1
terminator
CmR
FRT
(minimal)
cat
promoter
RBS
lacZ
RBS

Fig. 10

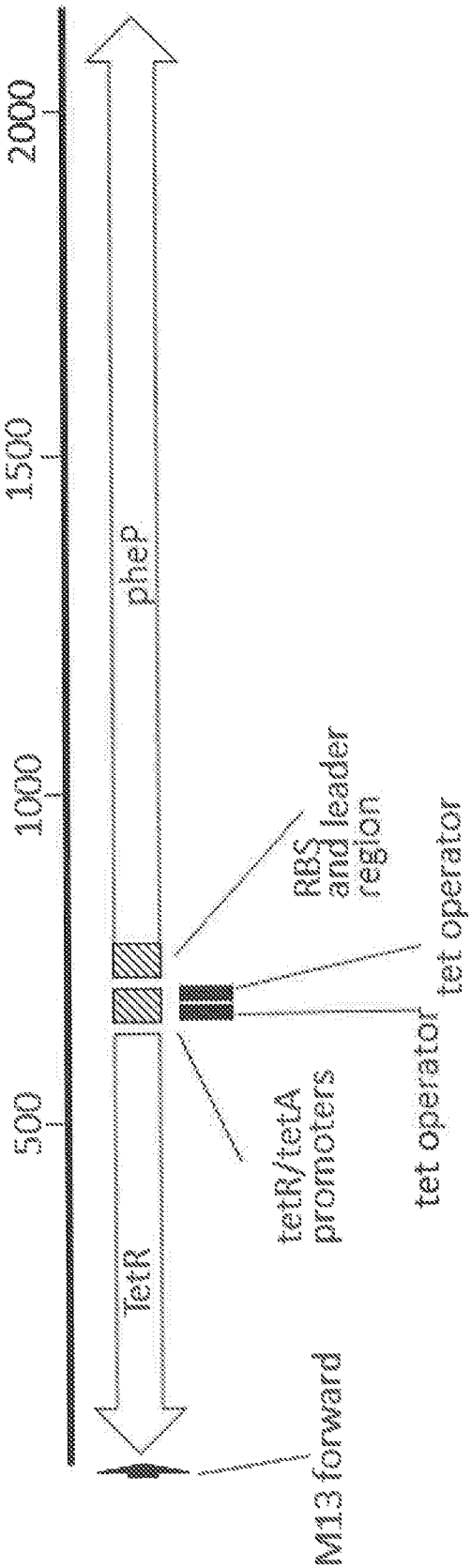

2000
1500
1000
500
phep
TetR
RBS and leader region
tetR/tetA promoters
tet operator
tet operator
M13 forward

Fig. 11

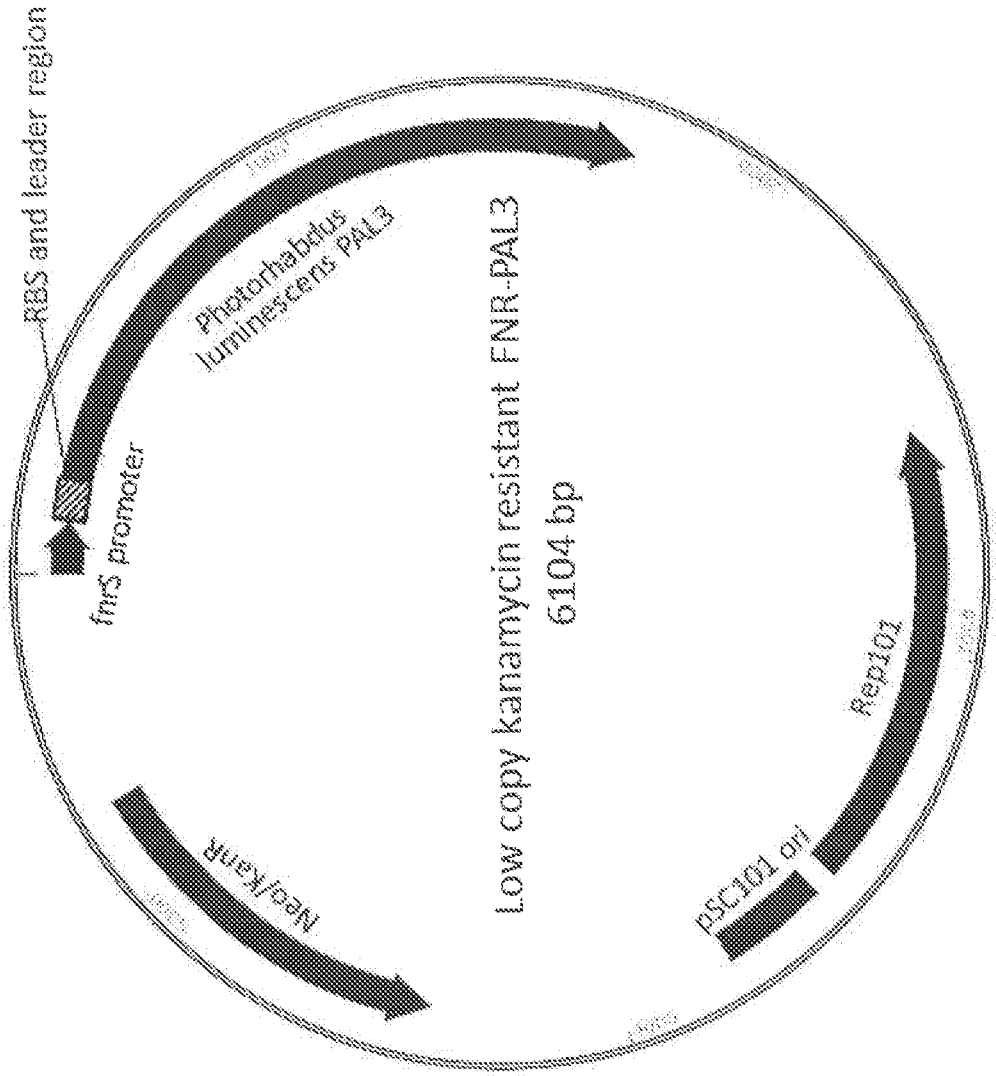

RBS and leader region
fnrS promoter
Photorhabdus luminescens PAL3
Low copy kanamycin resistant FNR-PAL3
6104 bp
Neo/KanR
pSC101 ori
Rep101

Fig. 12A

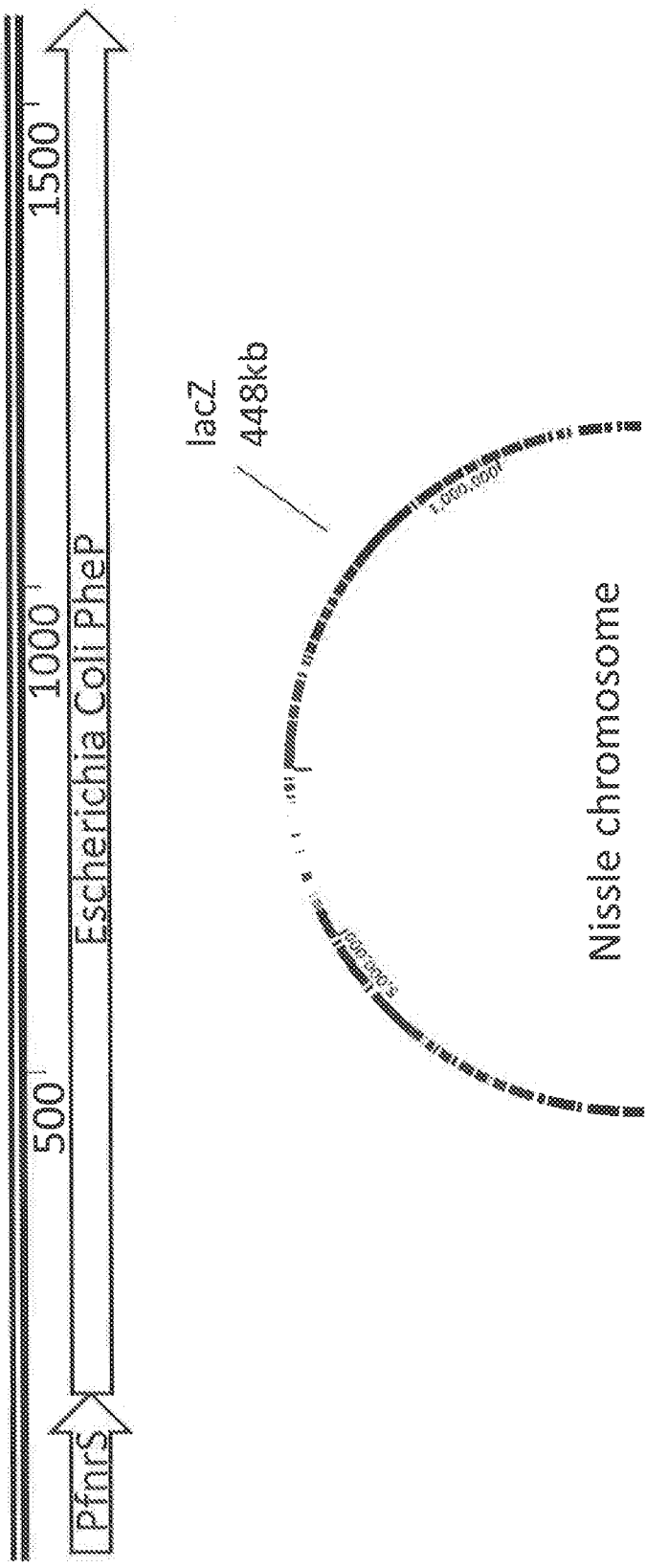

PfmrS
Escherichia Coli PheP
500
1000
1500
lacZ
448kb
Nissle chromosome

Fig. 12B

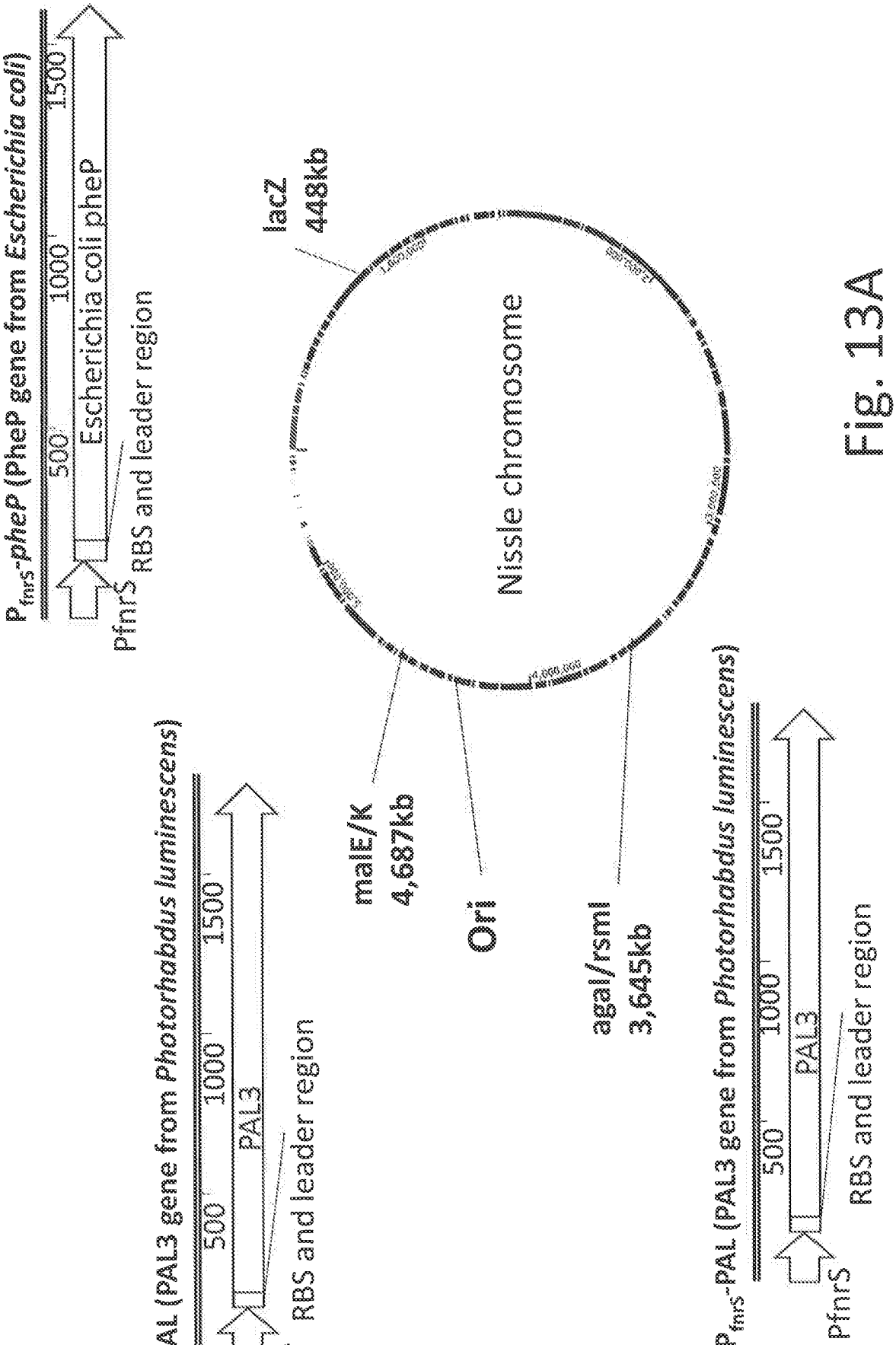

P fnrS-pheP (PheP gene from Escherichia coli)
500
1000
1500
Escherichia coli pheP
PfnrS
RBS and leader region
lacZ
448kb
Nissle chromosome
malE/K
4,687kb
Ori
agaI/rsmI
3,645kb
P fnrS-PAL (PAL3 gene from Photorhabdus luminescens)
500
1000
1500
PAL3
PfnrS
RBS and leader region
P fnrS-PAL (PAL3 gene from Photorhabdus luminescens)
500
1000
1500
PAL3
PfnrS
RBS and leader region

Fig. 13A

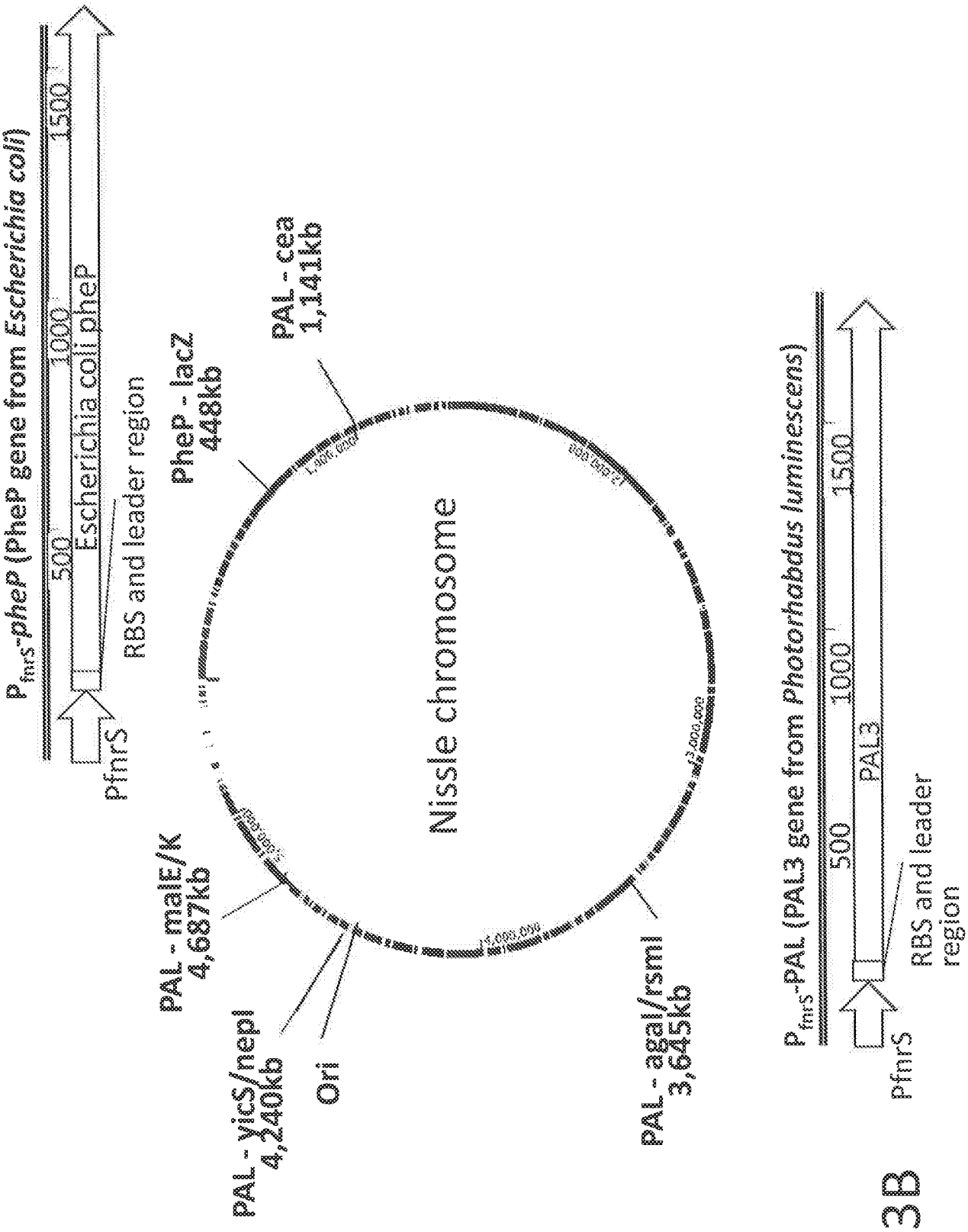

P fnrS-pheP (PheP gene from Escherichia coli)
500
1000
1500
Escherichia coli pheP
RBS and leader region
PfnrS
PheP - lacZ
448kb
PAL - cea
1,141kb
Nissle chromosome
PAL - malE/K
4,687kb
PAL - yicS/nepI
4,240kb
Ori
PAL - agaI/rsmI
3,645kb
P fnrS-PAL (PAL3 gene from Photorhabdus luminescens)
500
1000
1500
PAL3
RBS and leader region
PfnrS

Fig. 13B

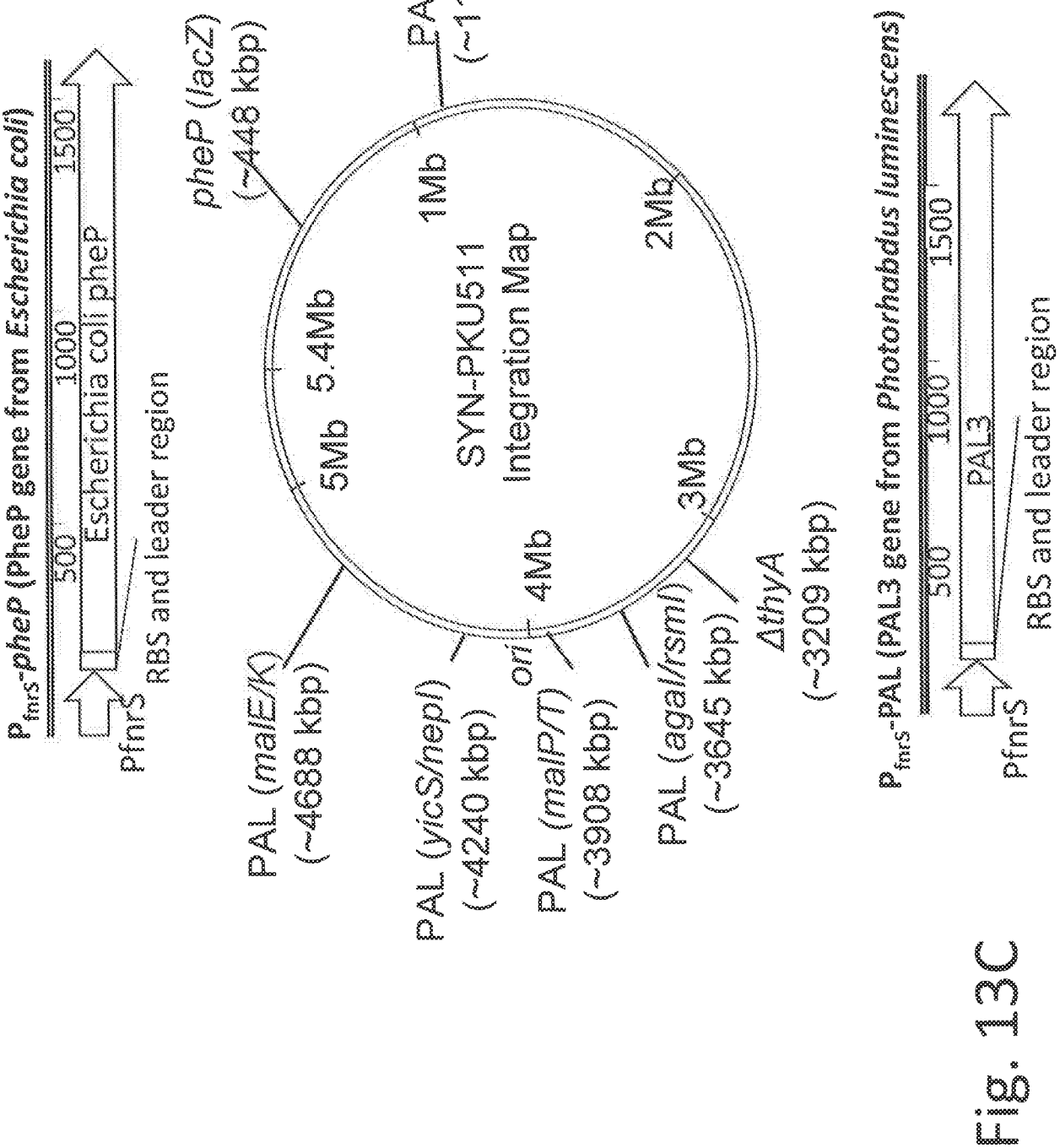

P fnrS-pheP (PheP gene from Escherichia coli)
500
1000
1500
Escherichia coli pheP
PfnrS
RBS and leader region
pheP (lacZ)
(~448 kbp)
PAL (cea)
(~1141 kbp)
1Mb
2Mb
5.4Mb
5Mb
SYN-PKU511
Integration Map
3Mb
4Mb
ori
PAL (malE/K)
(~4688 kbp)
PAL (yicS/nepI)
(~4240 kbp)
PAL (malP/T)
(~3908 kbp)
PAL (agaI/rsmI)
(~3645 kbp)
ΔthyA
(~3209 kbp)
P fnrS-PAL (PAL3 gene from Photorhabdus luminescens)
500
1000
1500
PAL3
PfnrS
RBS and leader region

Fig. 13C

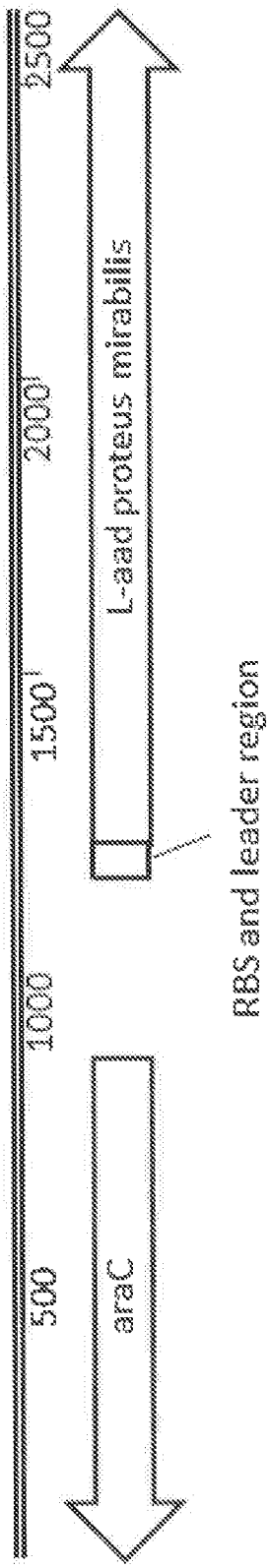

2500
2000
1500
1000
500
L-aad proteus mirabilis
RBS and leader region
araC

Fig. 14

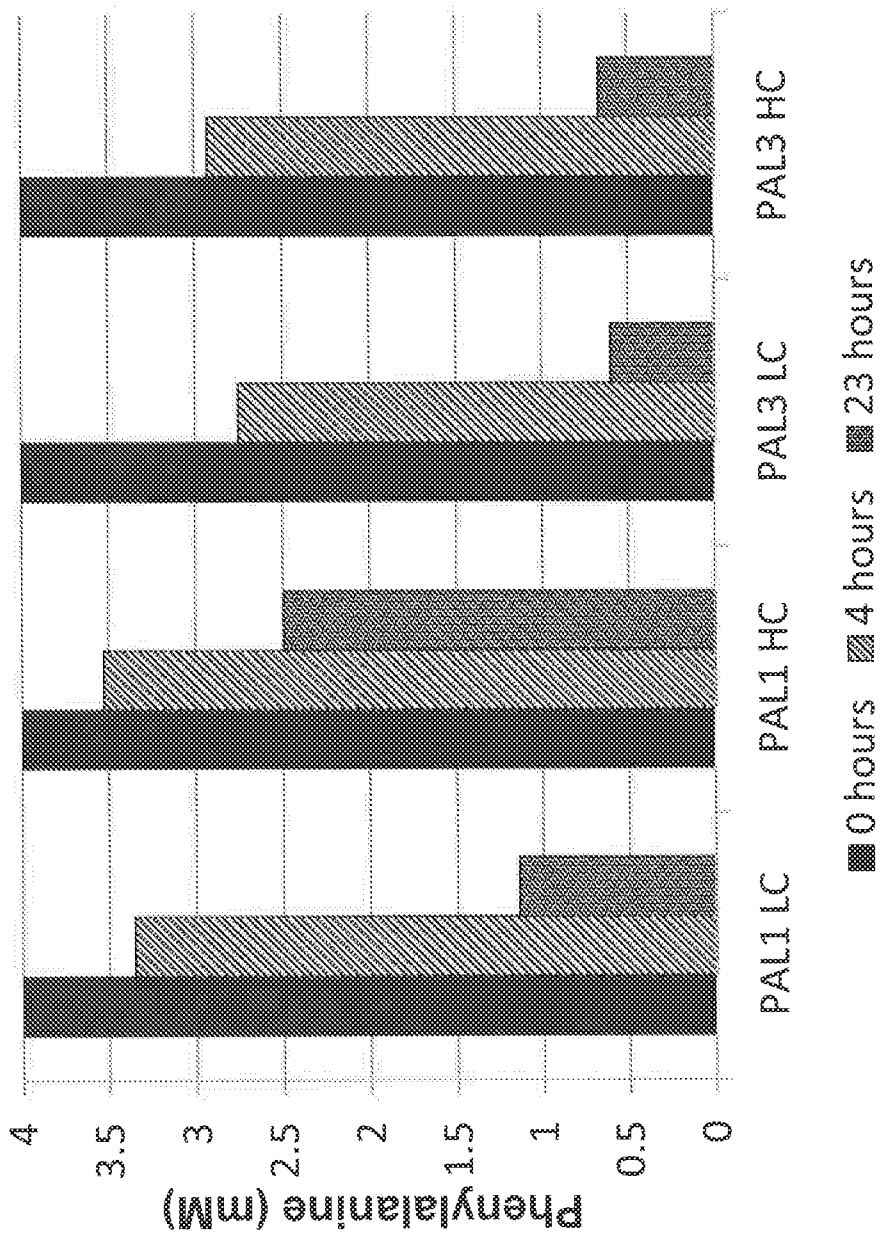

Phenylalanine (mM)
4
3.5
3
2.5
2
1.5
1
0.5
0
PAL1 LC
PAL1 HC
PAL3 LC
PAL3 HC
0 hours
4 hours
23 hours

Fig. 15A

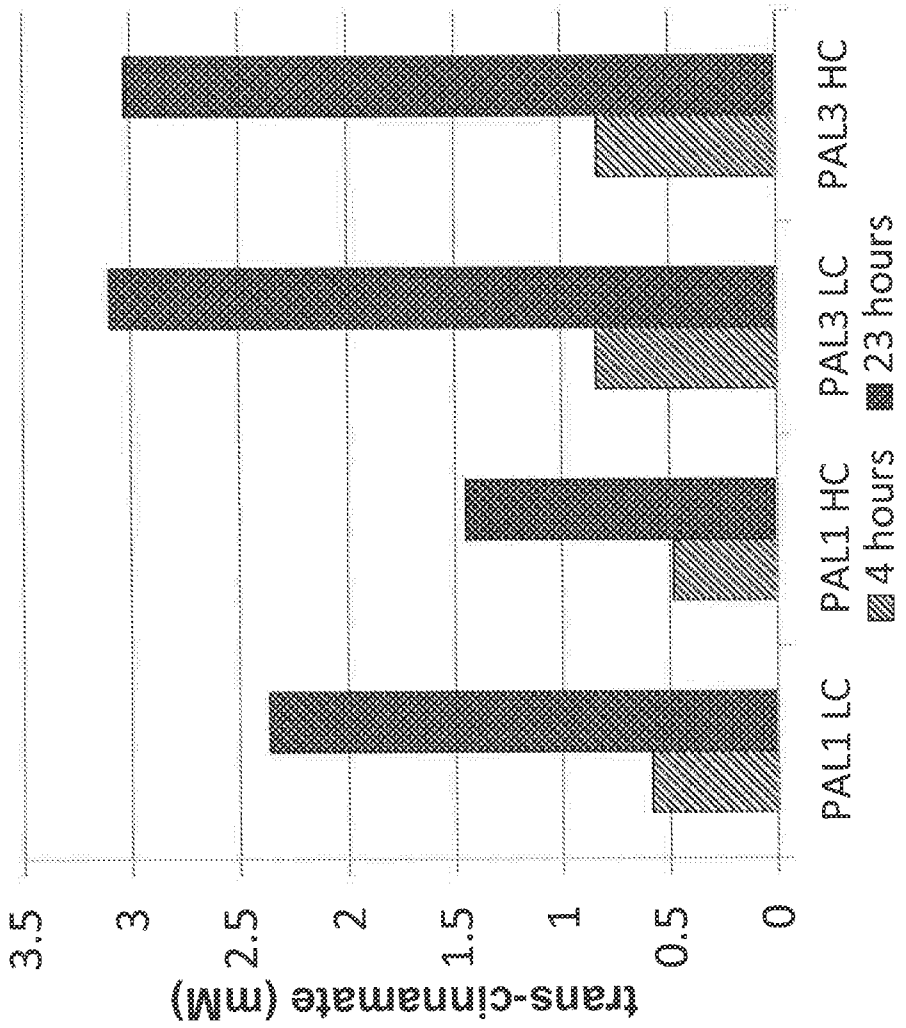

trans-cinnamate (mM)
3.5
3
2.5
2
1.5
1
0.5
0
PAL1 LC
PAL1 HC
PAL3 LC
PAL3 HC
4 hours
23 hours

Fig. 15B

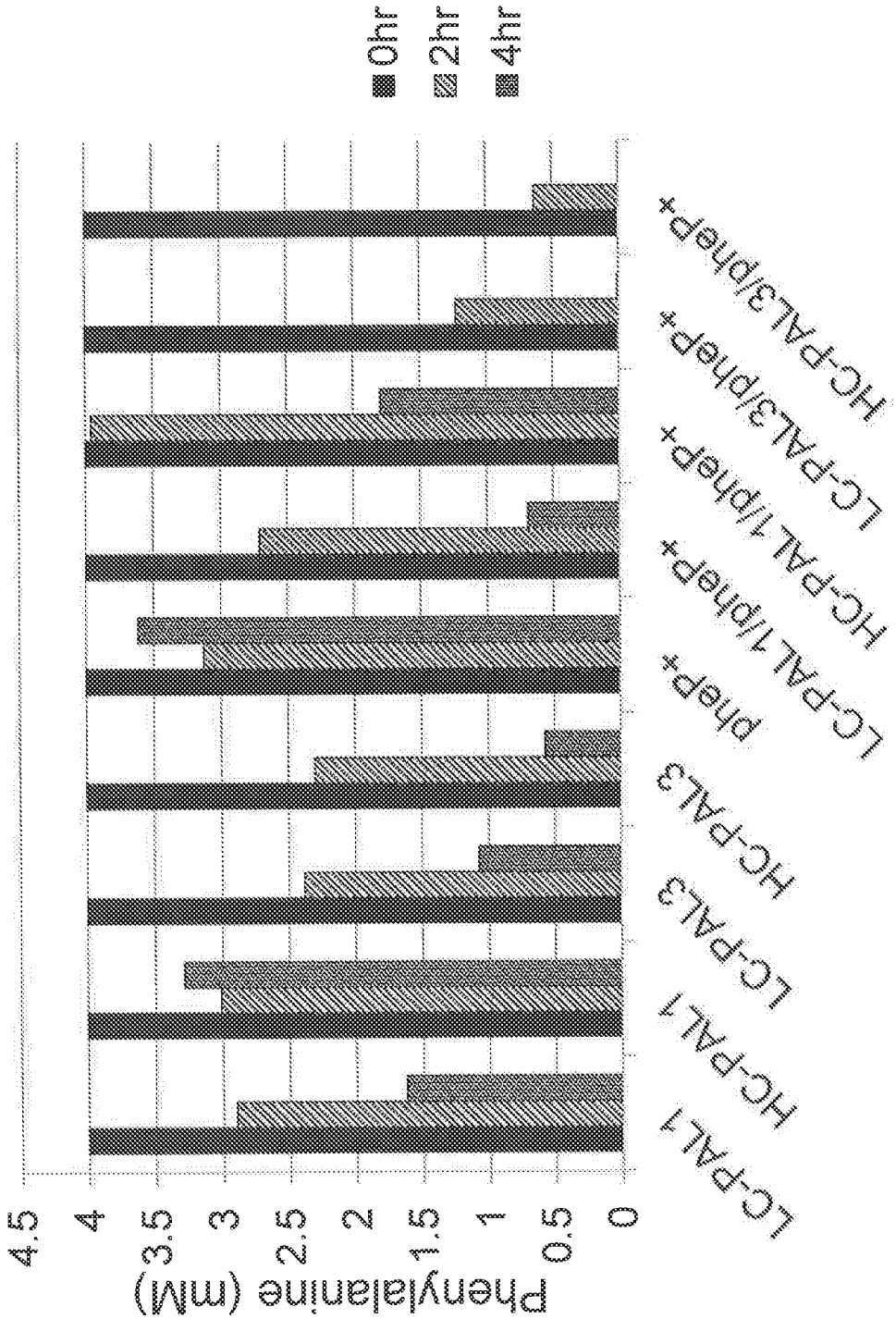

0hr
2hr
4hr
Phenylalanine (mM)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
LC-PAL1
HC-PAL1
LC-PAL3
HC-PAL3
phap+
LC-PAL1/phap+
HC-PAL1/phap+
LC-PAL3/phap+
HC-PAL3/phap+

Fig. 16A

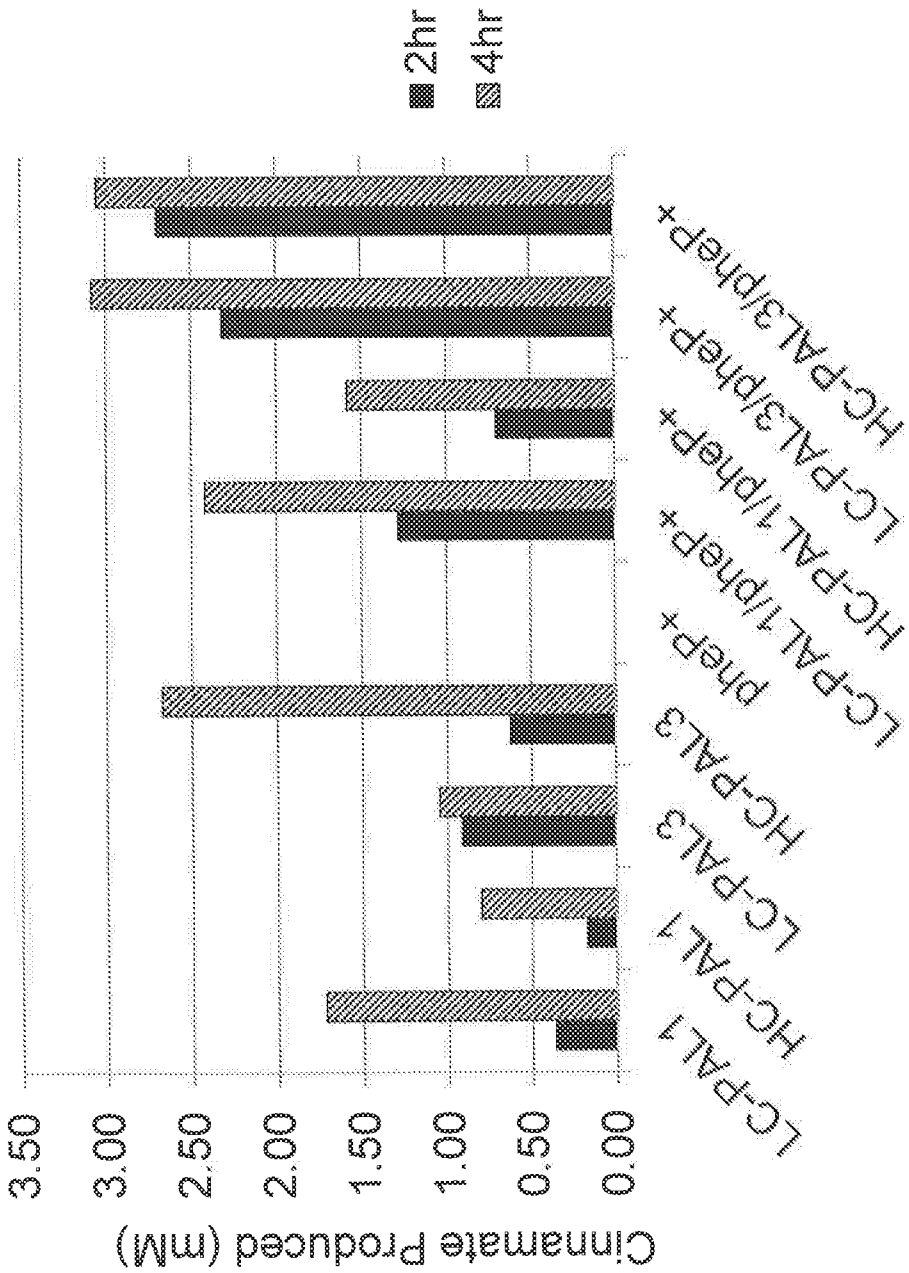

2hr
4hr
Cinnamate Produced (mM)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
LC-PAL1
HC-PAL1
LC-PAL3
HC-PAL3
phep+
LC-PAL1/phep+
HC-PAL1/phep+
LC-PAL3/phep+
HC-PAL3/phep+

Fig. 16B

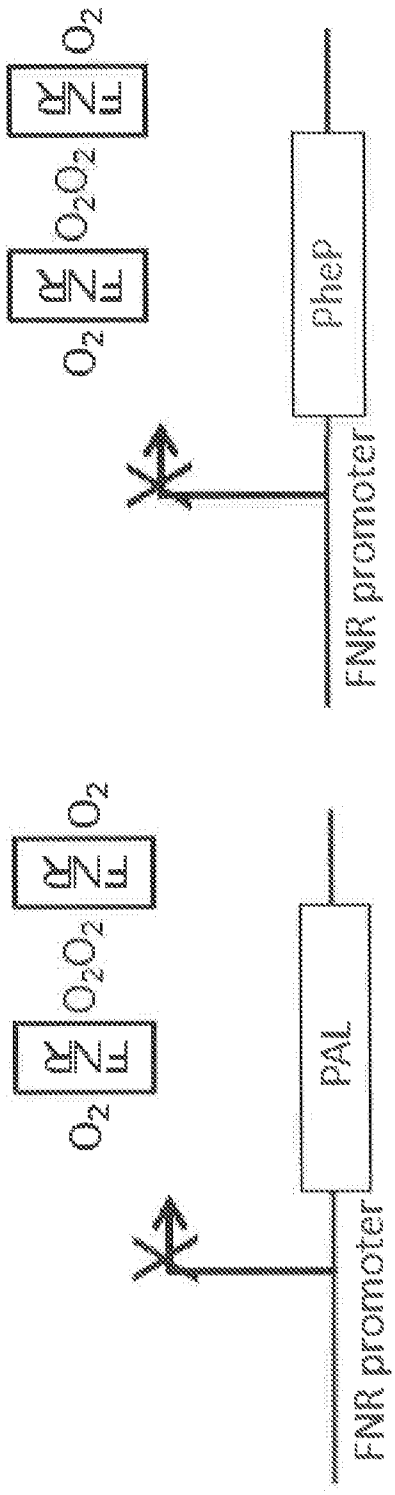

FNR
O2
FNR
O2O2
FNR
O2
FNR promoter
phaP
FNR
O2
FNR
O2O2
FNR
O2
FNR promoter
PAL

Fig. 17A

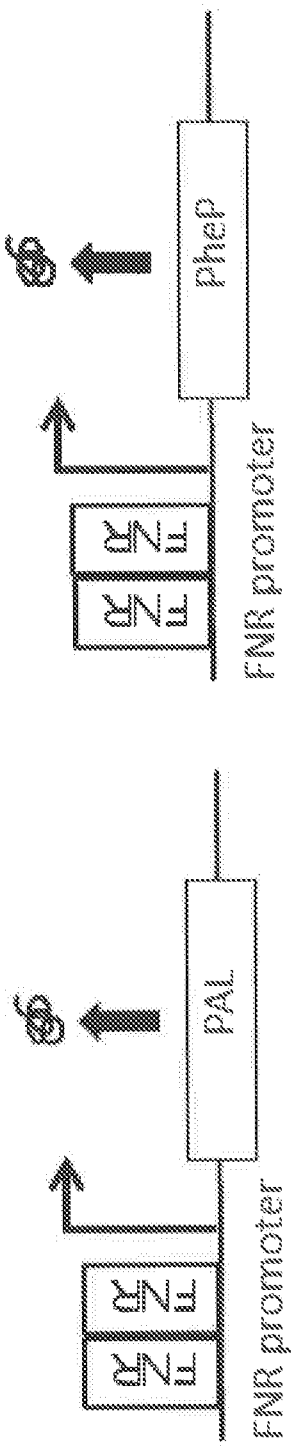


Fig. 17B

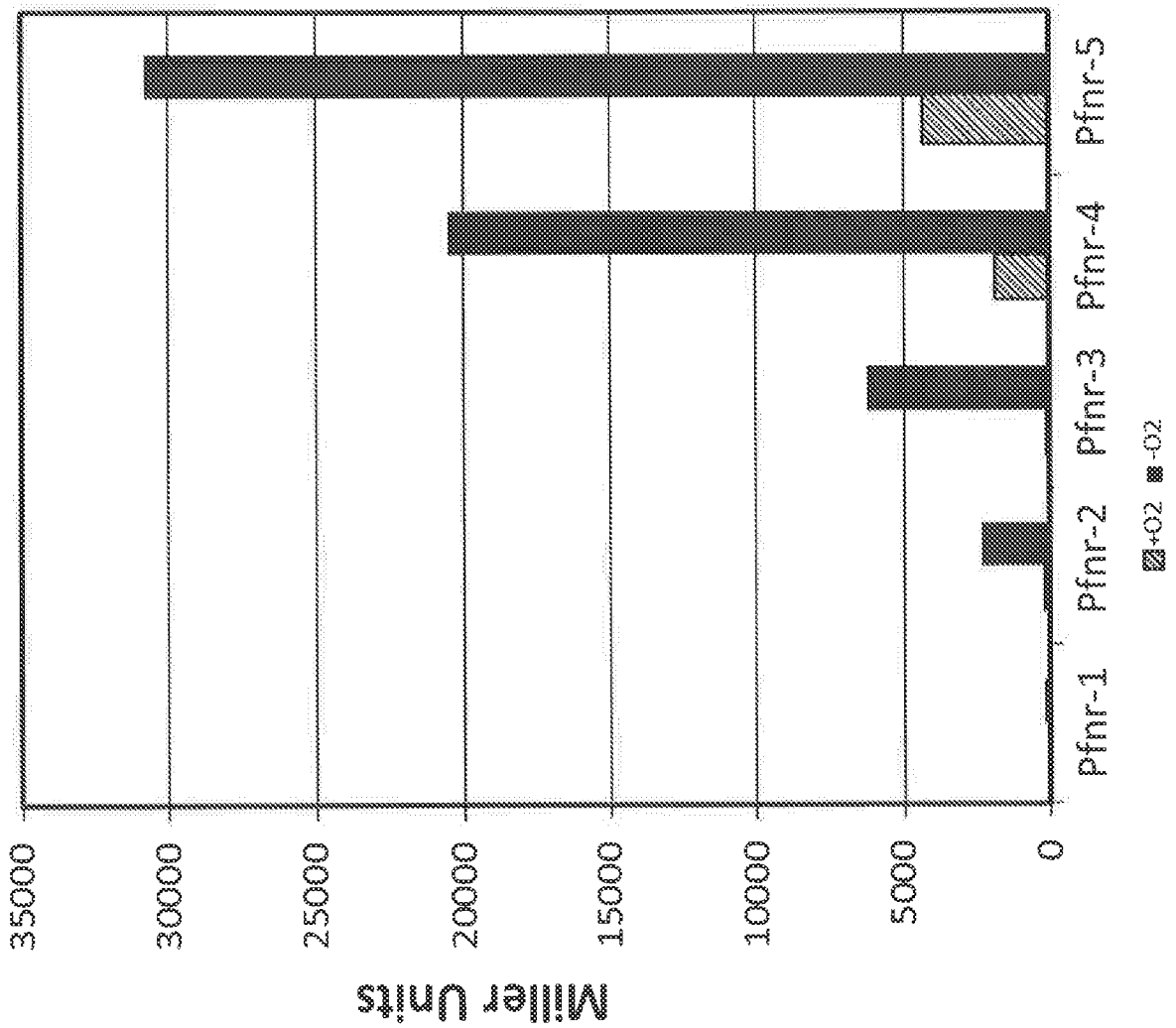
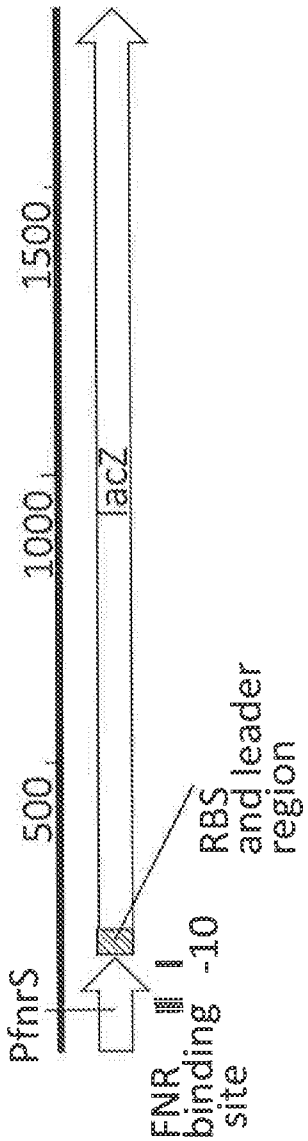

Miller Units
35000
30000
25000
20000
15000
10000
5000
0
Pfnr-1
Pfnr-2
Pfnr-3
Pfnr-4
Pfnr-5
+O2
-O2

Fig. 18



lacZpal
1812 bp

Fig. 19A

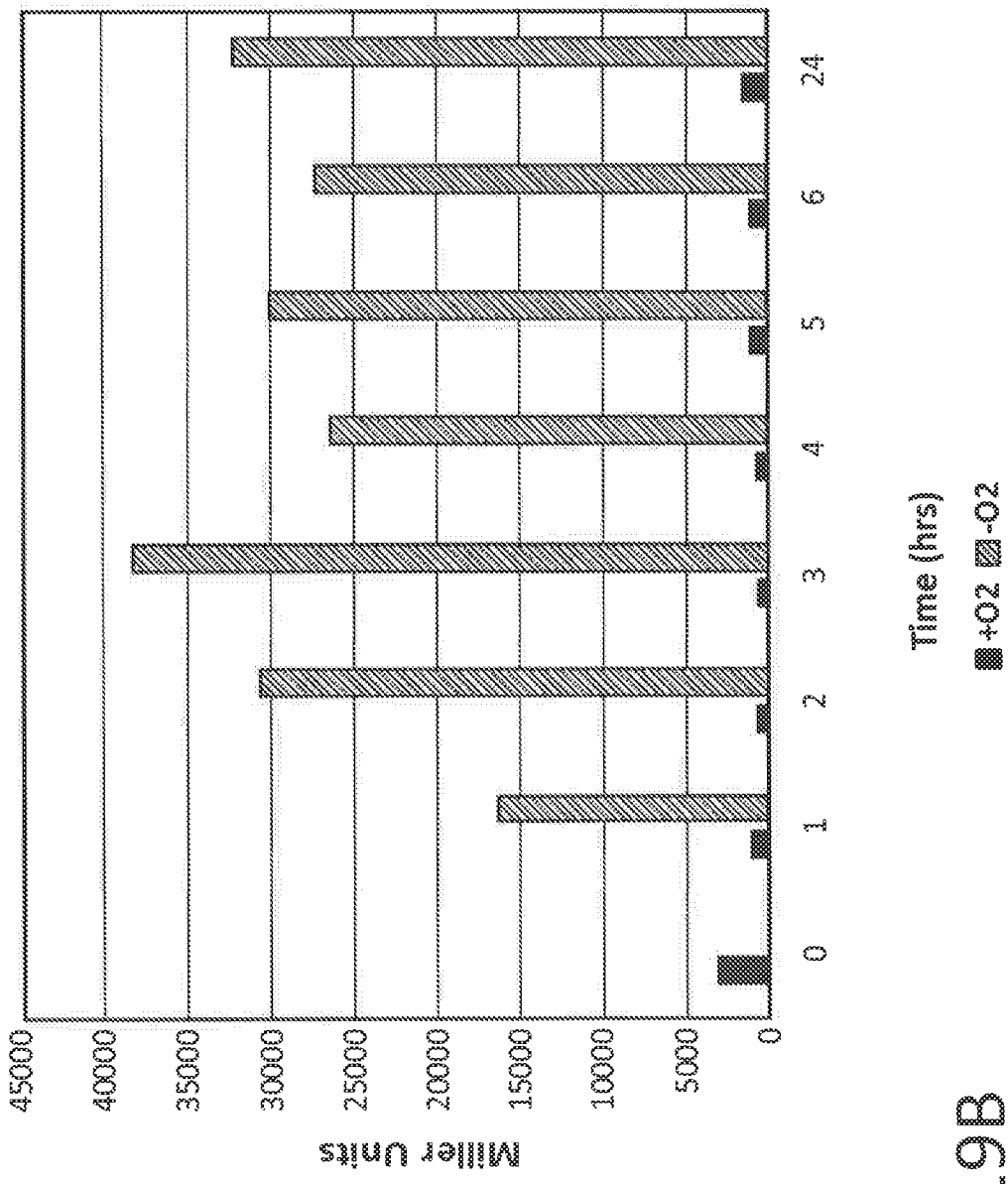

Miller Units
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
1
2
3
4
5
6
24
Time (hrs)
+O2
-O2

Fig. 19B

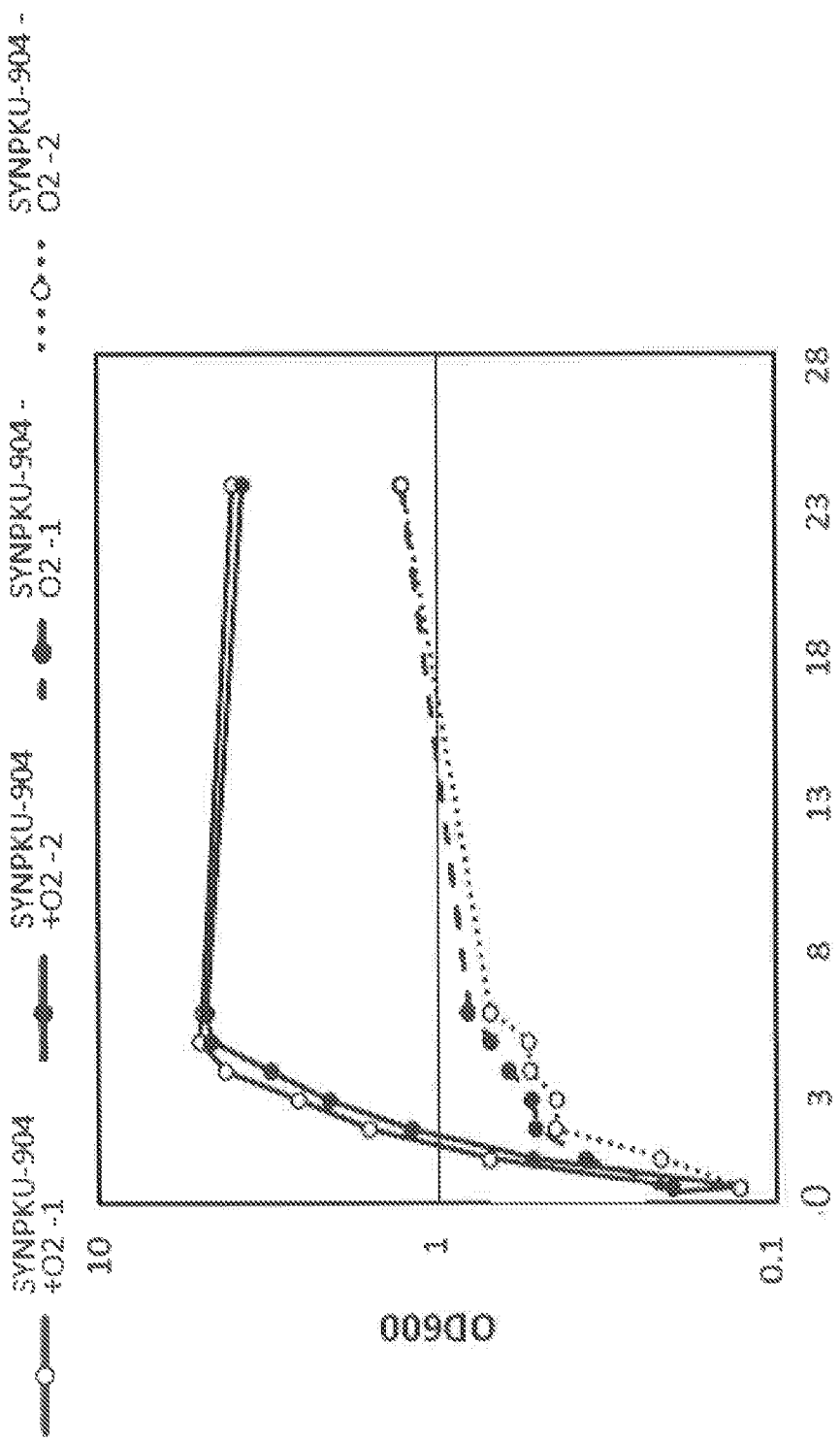

SYNPKU-904 +O2 -1
SYNPKU-904 +O2 -2
SYNPKU-904 - O2 -1
SYNPKU-904 - O2 -2
OD600
10
1
0.1
0
3
8
13
18
23
28
Time (hrs)

Fig. 19C

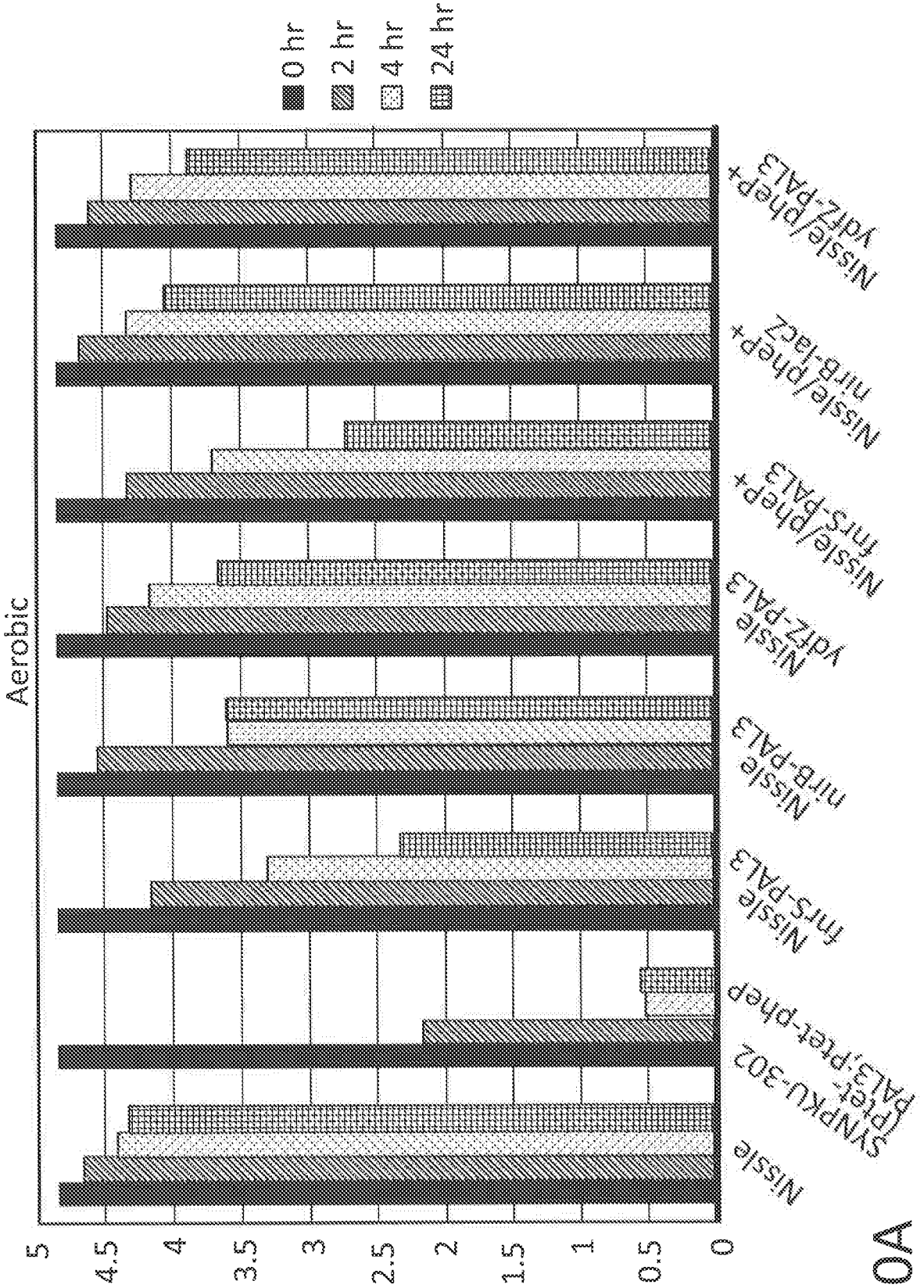

0 hr
2 hr
4 hr
24 hr
Aerobic
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Nissle
SYNPKU-302 (Pret-bAL3-Pret-pher
Nissle fnrS-pAL3
Nissle nirB-pAL3
Nissle ydfZ-pAL3
Nissle/pher+ fnrS-pAL3
Nissle/pher+ nirB-lacZ
Nissle/pher+ ydfZ-pAL3

Fig. 20A

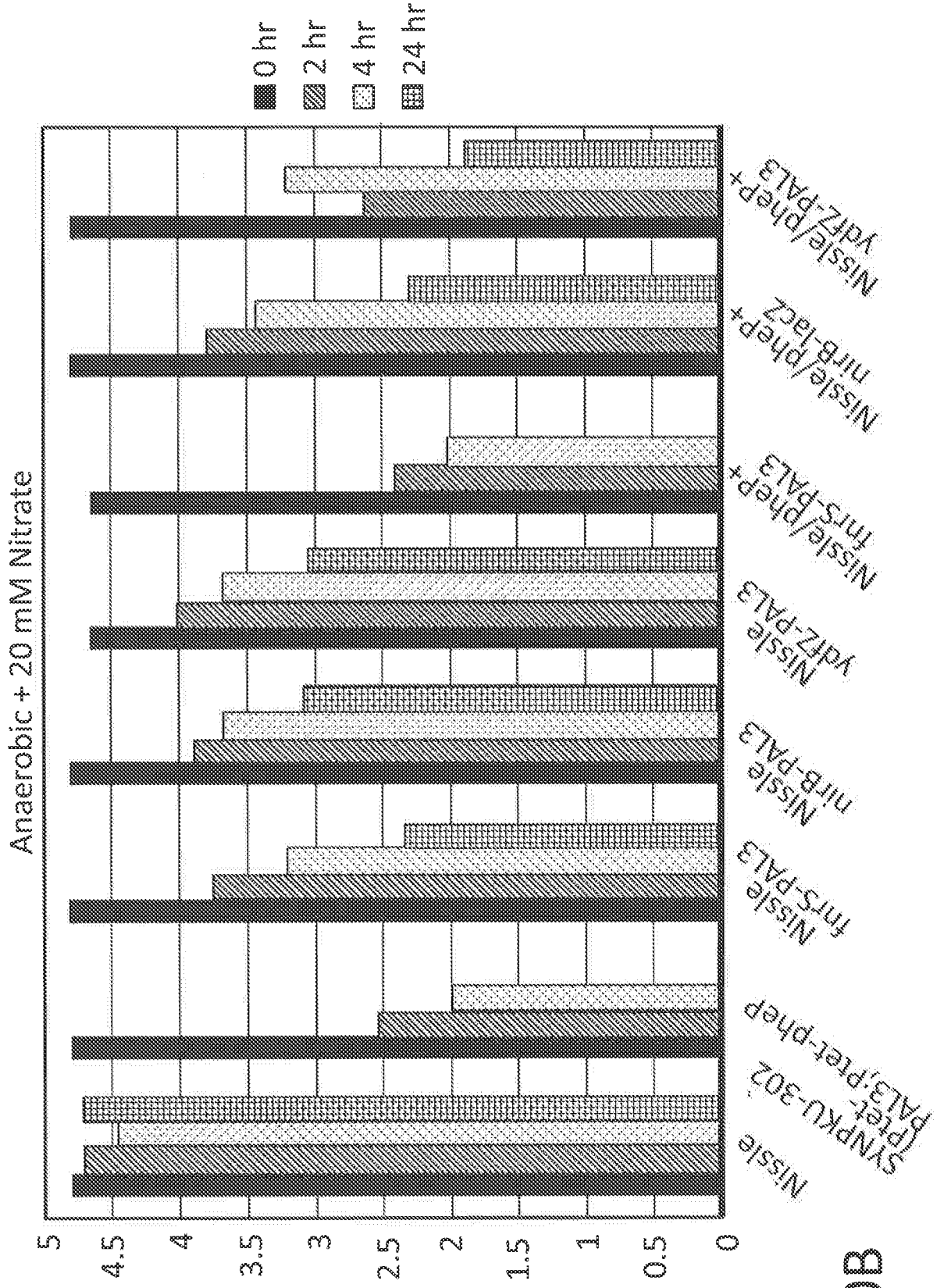

0 hr
2 hr
4 hr
24 hr
Anaerobic + 20 mM Nitrate
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Nissle
SYNPKU-302.1 pTet-BAL3.pTet-phep
Nissle fnrS-pAL3
Nissle nirB-pAL3
Nissle ydfZ-pAL3
Nissle/phep+ fnrS-pAL3
Nissle/phep+ nirB-lacZ
Nissle/phep+ ydfZ-pAL3

Fig. 20B

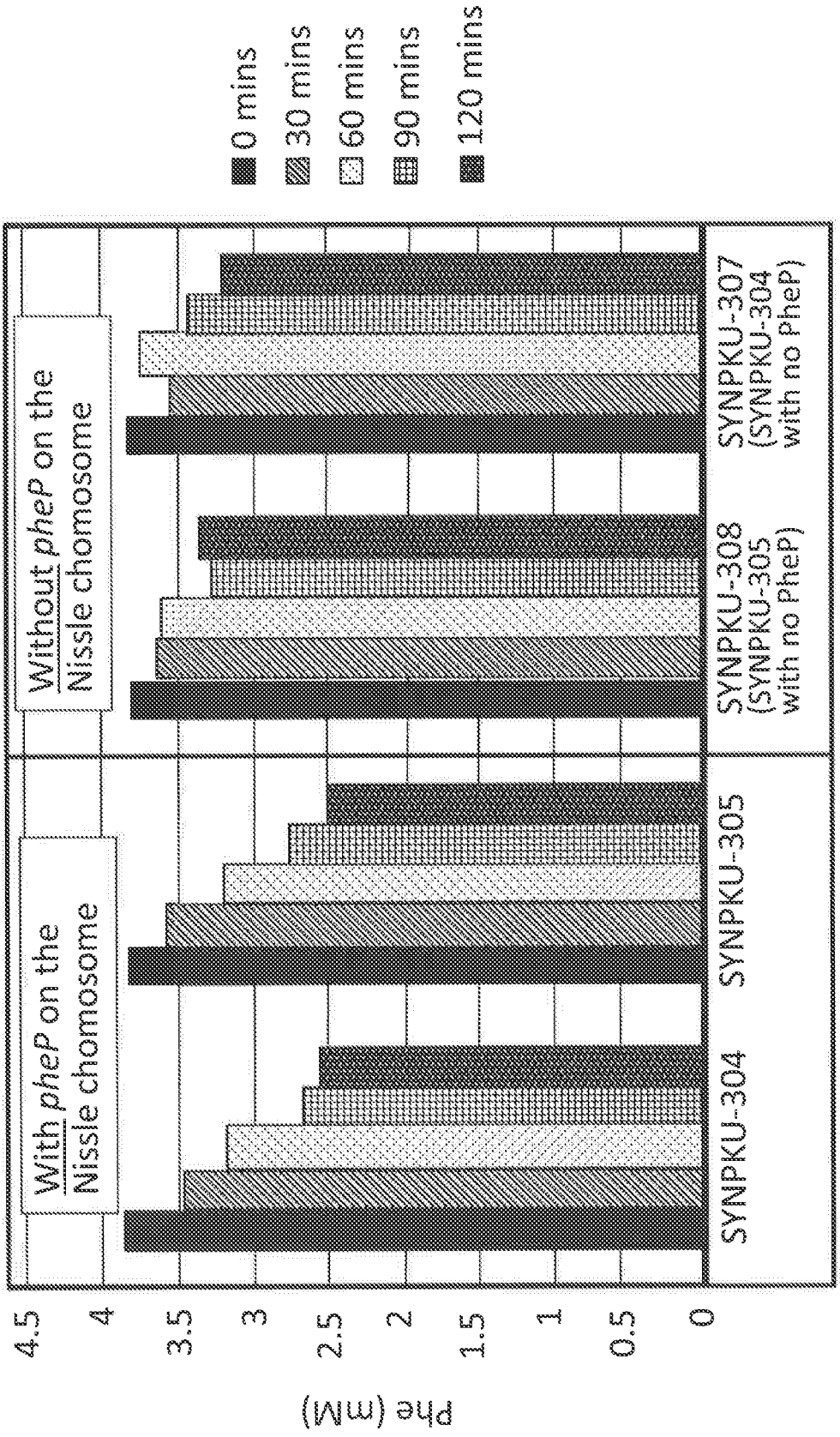

0 mins
30 mins
60 mins
90 mins
120 mins
With pheP on the Nissle chomosome
Without pheP on the Nissle chomosome
SYNPKU-304
SYNPKU-305
SYNPKU-308 (SYNPKU-305 with no PheP)
SYNPKU-307 (SYNPKU-304 with no PheP)
Phe (mM)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

Fig. 21

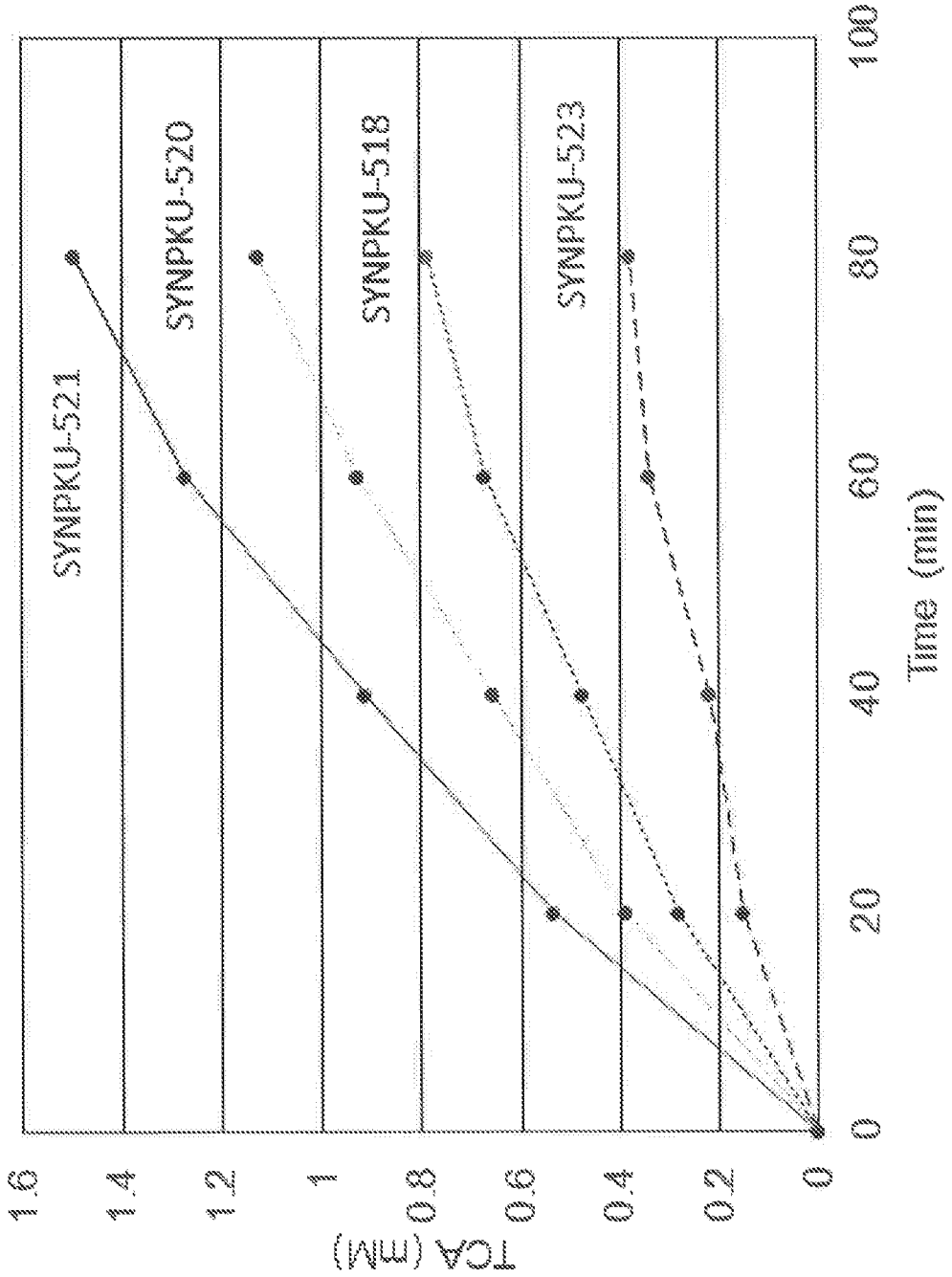

SYNPKU-521
SYNPKU-520
SYNPKU-518
SYNPKU-523
TCA (mM)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Time (min)

Fig. 22

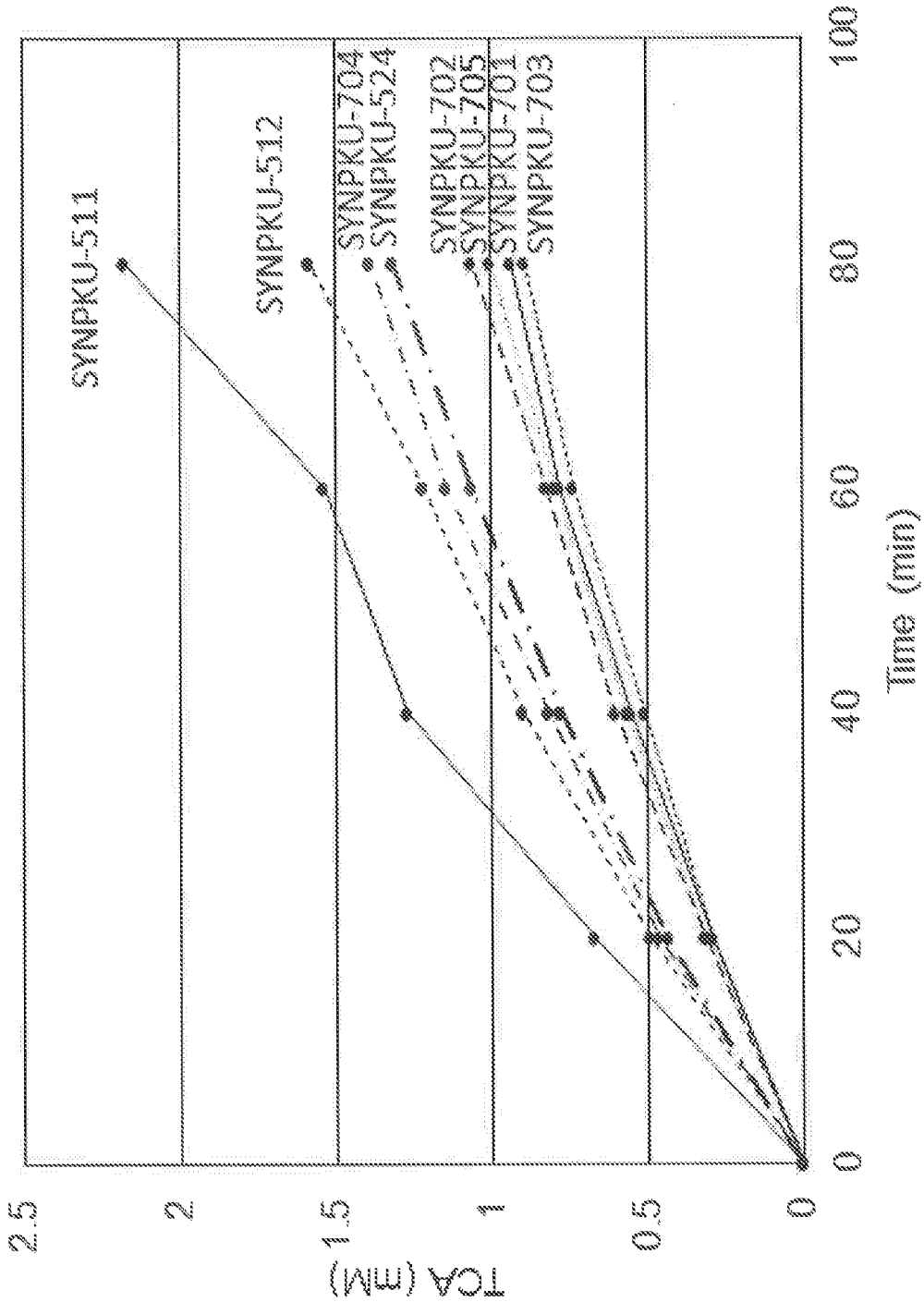
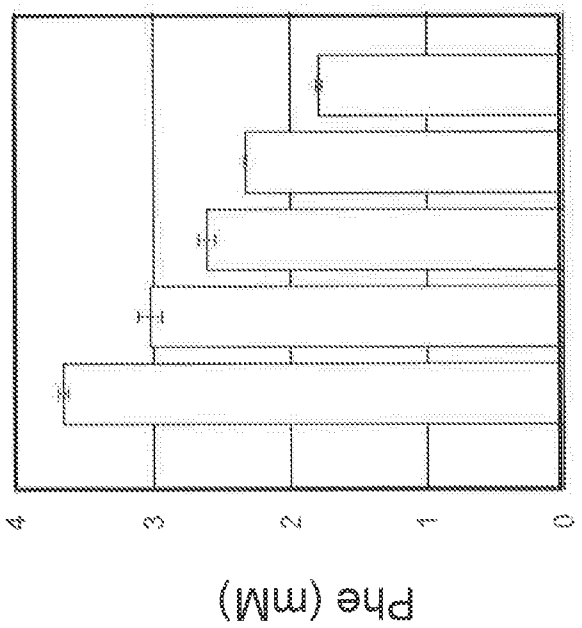

SYNPKU-511
SYNPKU-512
SYNPKU-704
SYNPKU-524
SYNPKU-702
SYNPKU-705
SYNPKU-701
SYNPKU-703
TCA (mM)
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
Time (min)

Fig. 23



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

Fig. 24

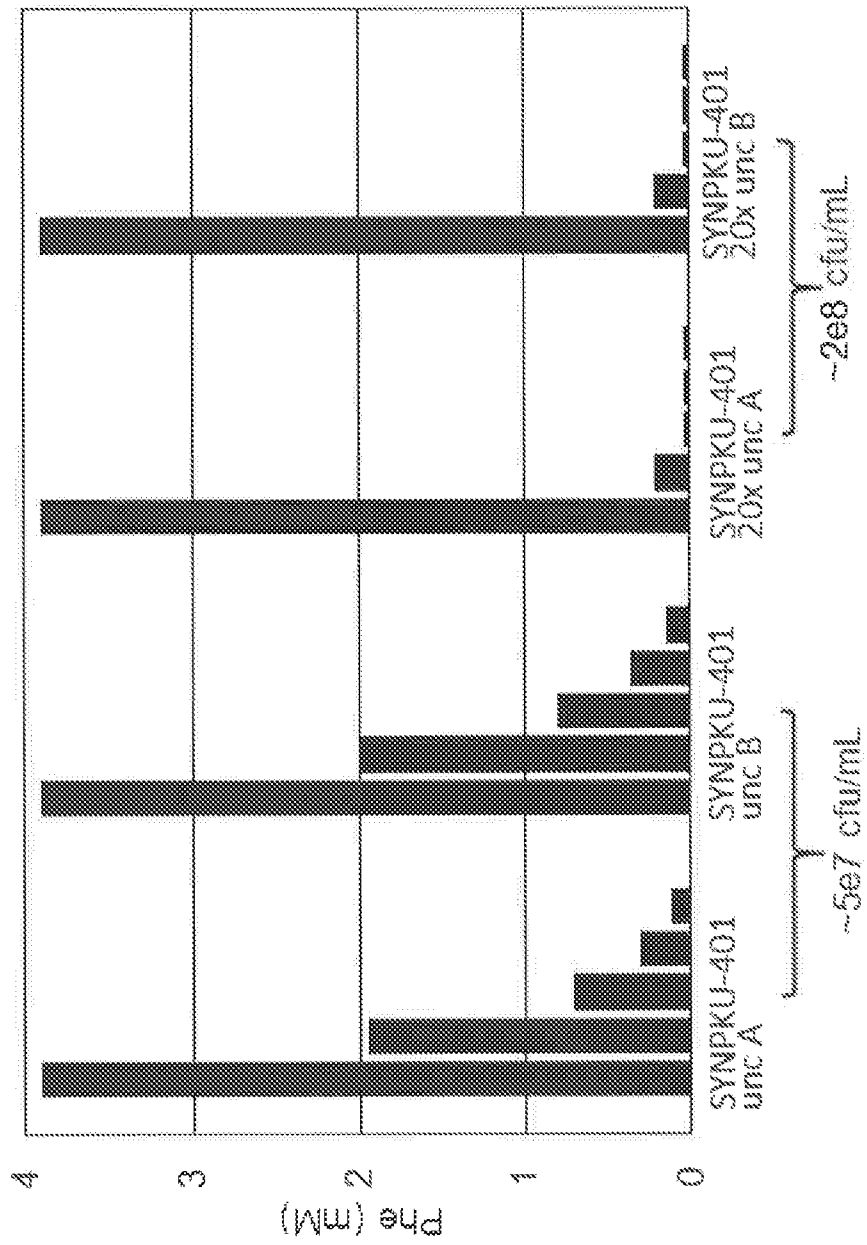

Phe (mM)
0
1
2
3
4
SYNPKU-401 unc A
SYNPKU-401 unc B
SYNPKU-401 20x unc A
SYNPKU-401 20x unc B
~5e7 cfu/mL
~2e8 cfu/mL

Fig. 25A

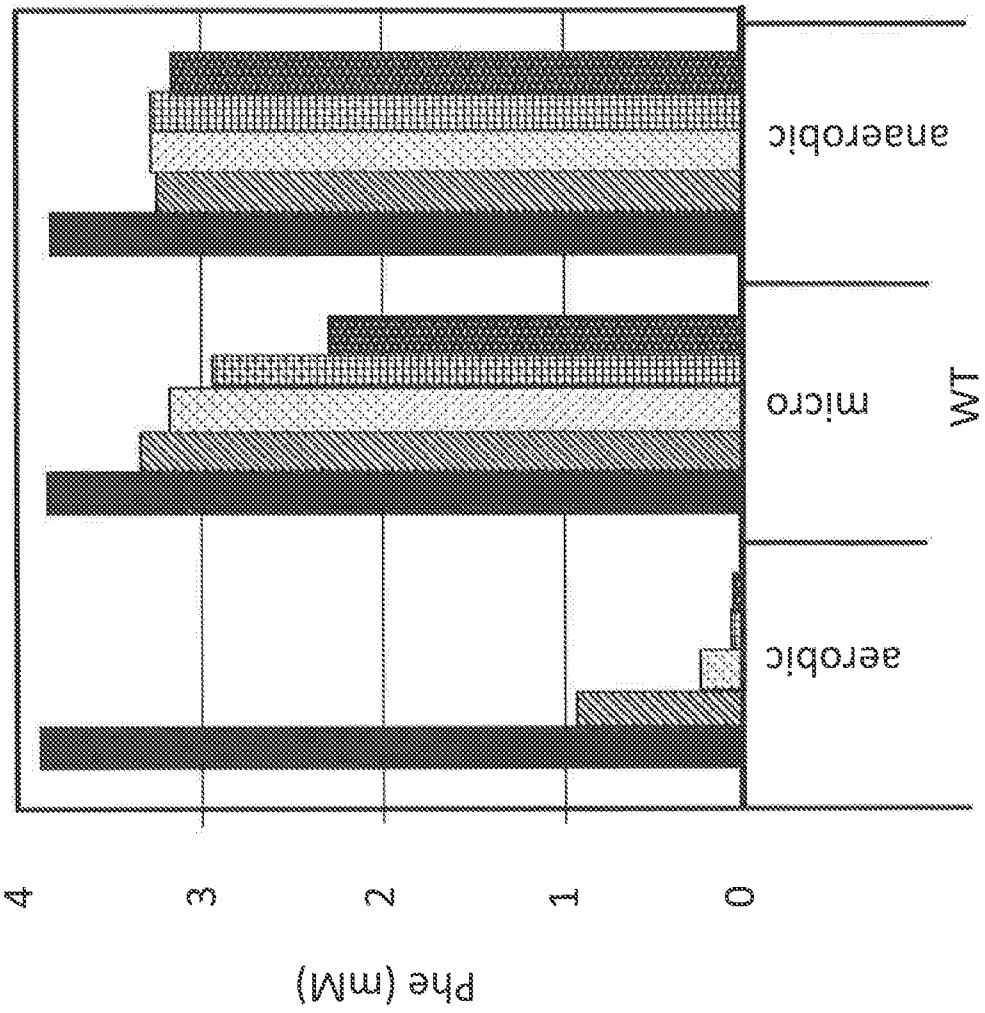


Fig. 25B

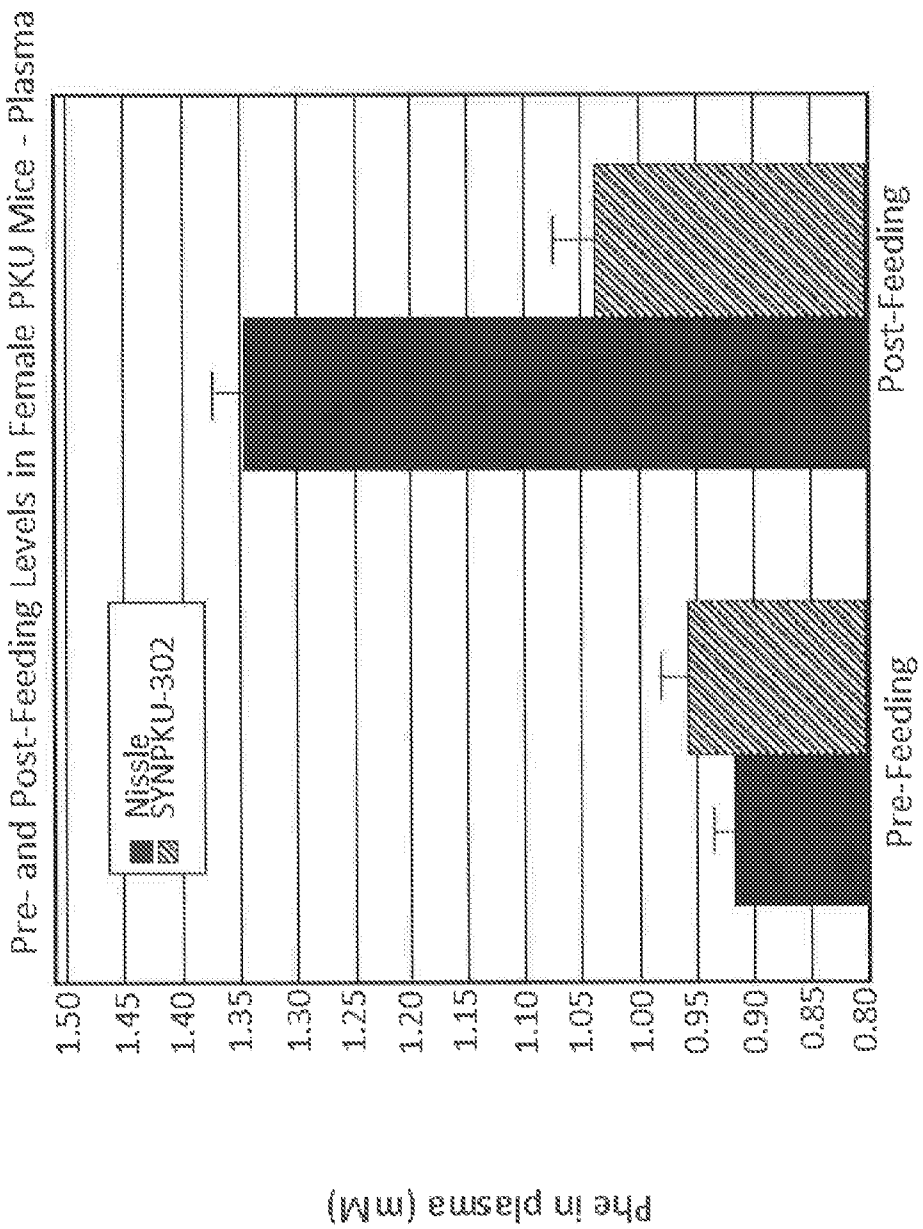

Pre- and Post-Feeding Levels in Female PKU Mice - Plasma
Nissle
SYNPKU-302
Phe in plasma (mM)
1.50
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
Pre-Feeding
Post-Feeding

Fig. 26A

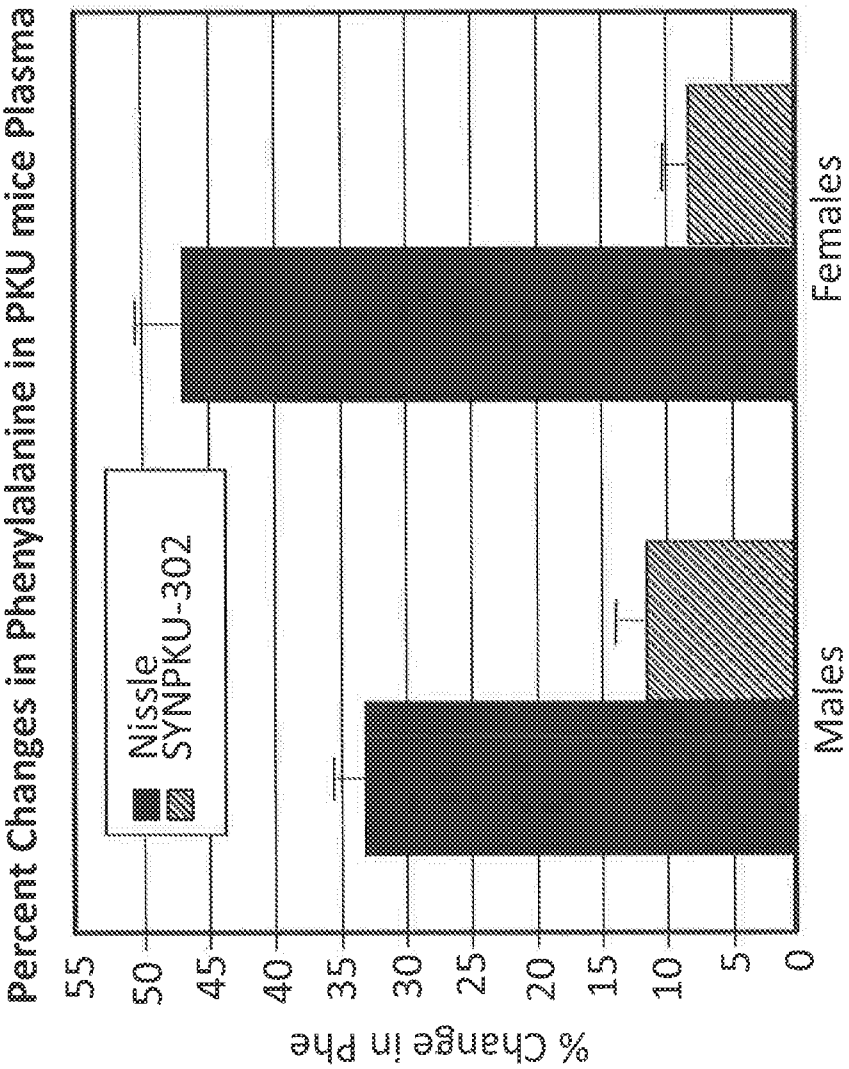

Percent Changes in Phenylalanine in PKU mice Plasma
Nissle
SYNPKU-302
% Change in Phe
0
5
10
15
20
25
30
35
40
45
50
55
Males
Females

Fig. 26B

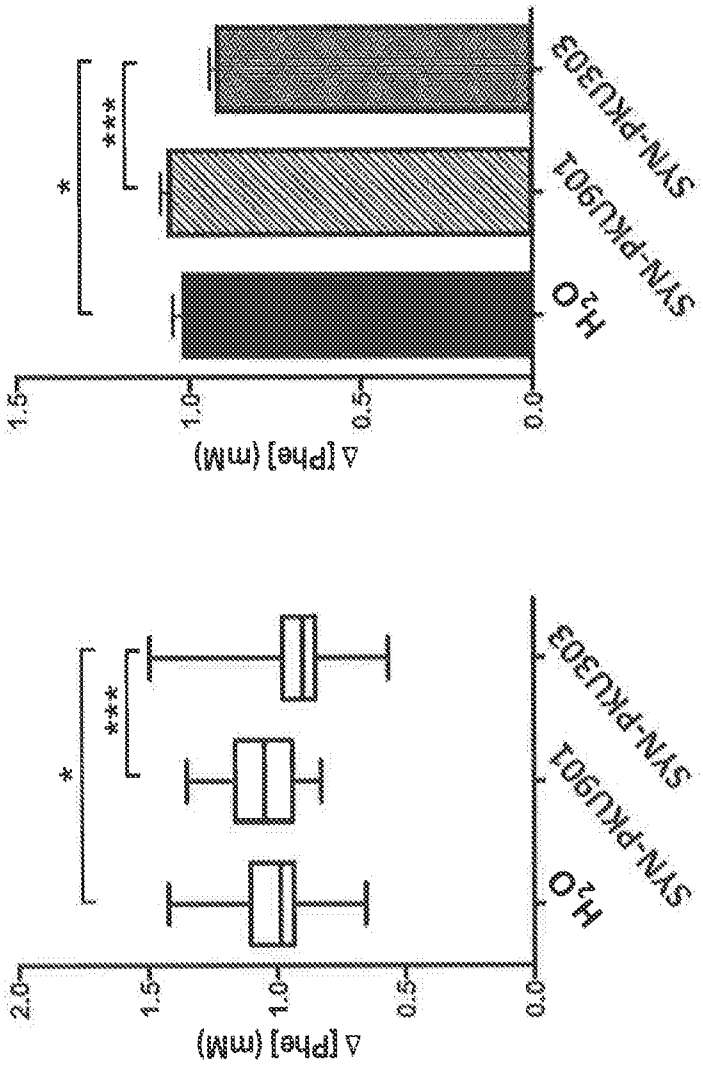


Fig. 27A

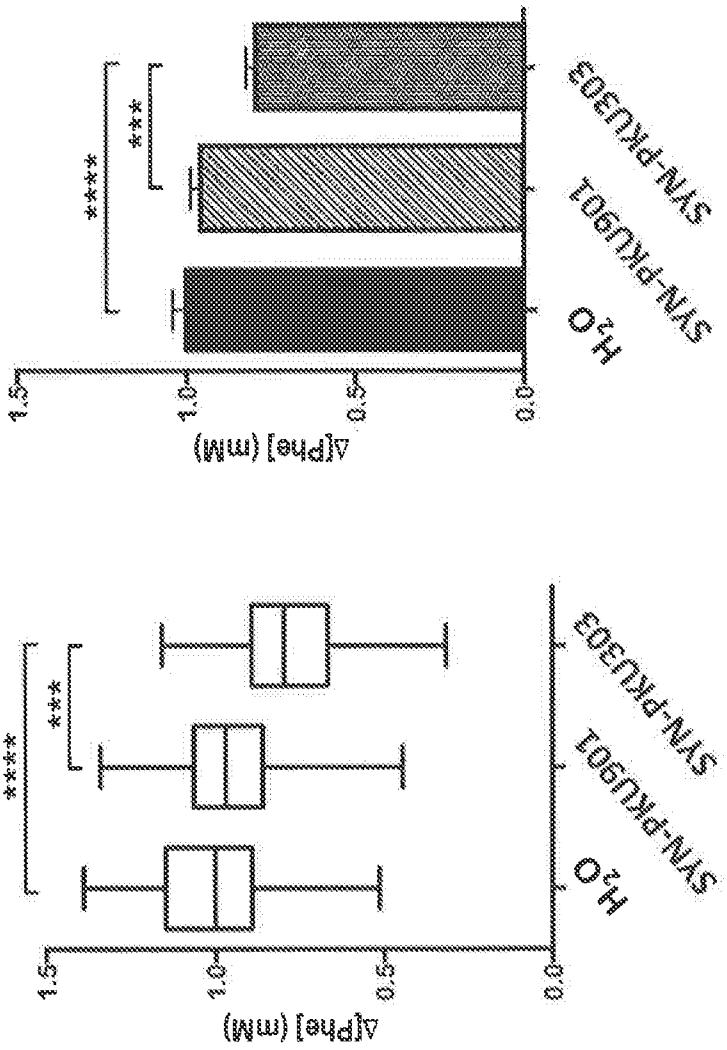

H2O
SYN-PKU901
SYN-PKU303
****
***
1.5
1.0
0.5
0.0
Δ[Phe] (mM)
H2O
SYN-PKU901
SYN-PKU303
****
***
1.5
1.0
0.5
0.0
Δ[Phe] (mM)

Fig. 27B

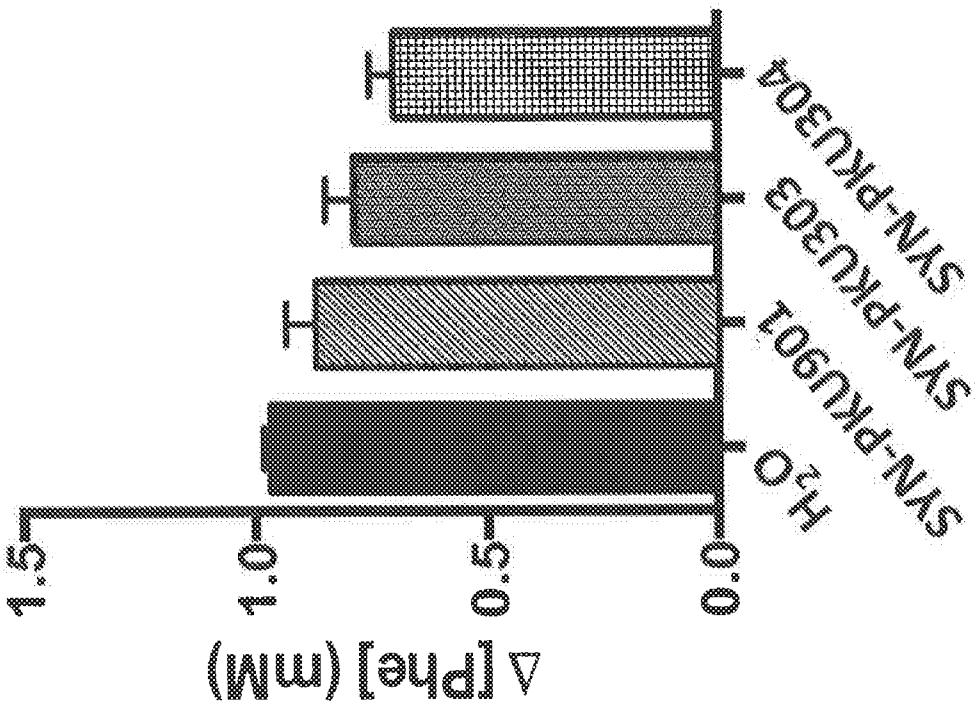
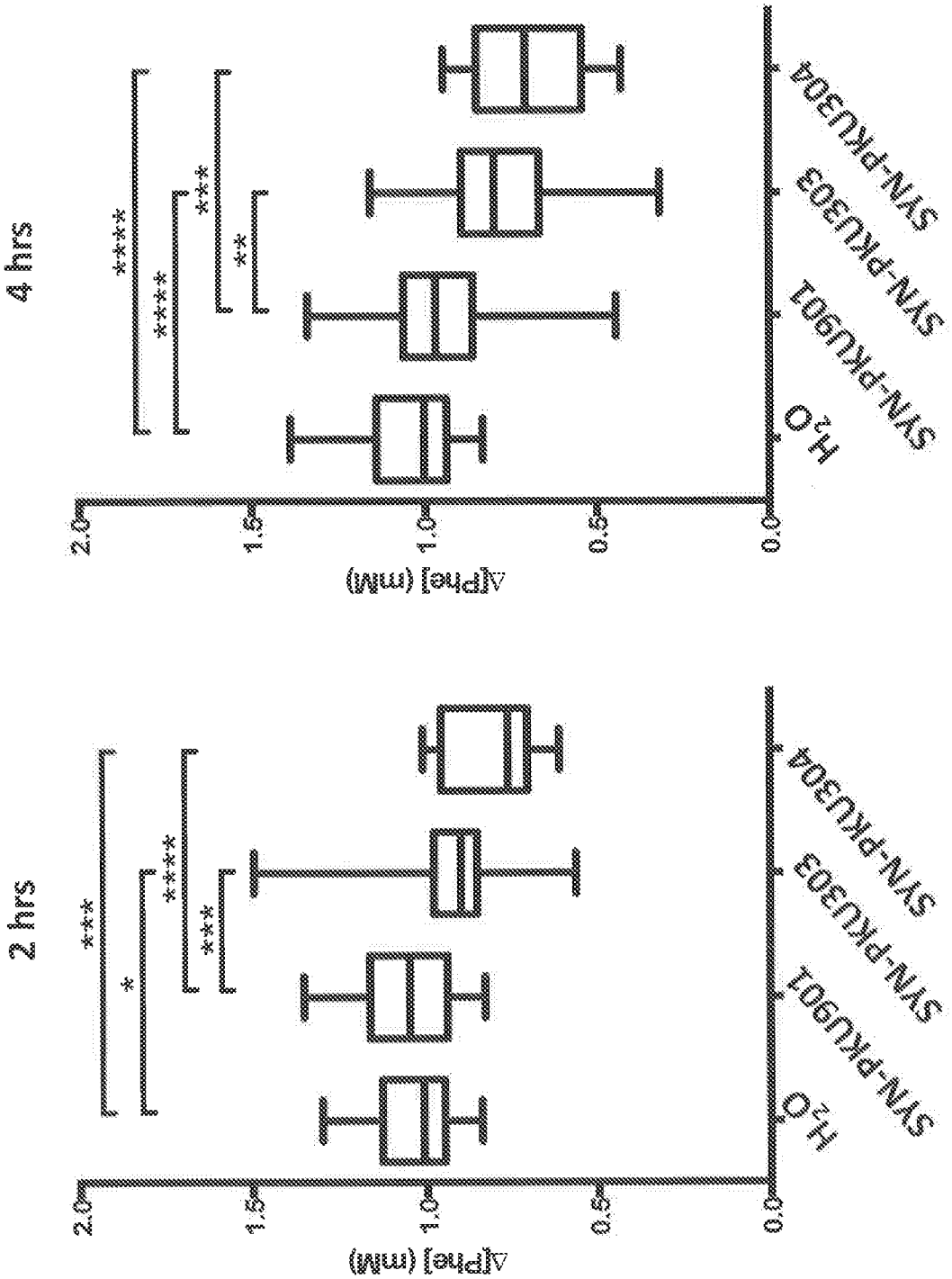


Fig. 28



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

Fig. 29A

Fig. 29B

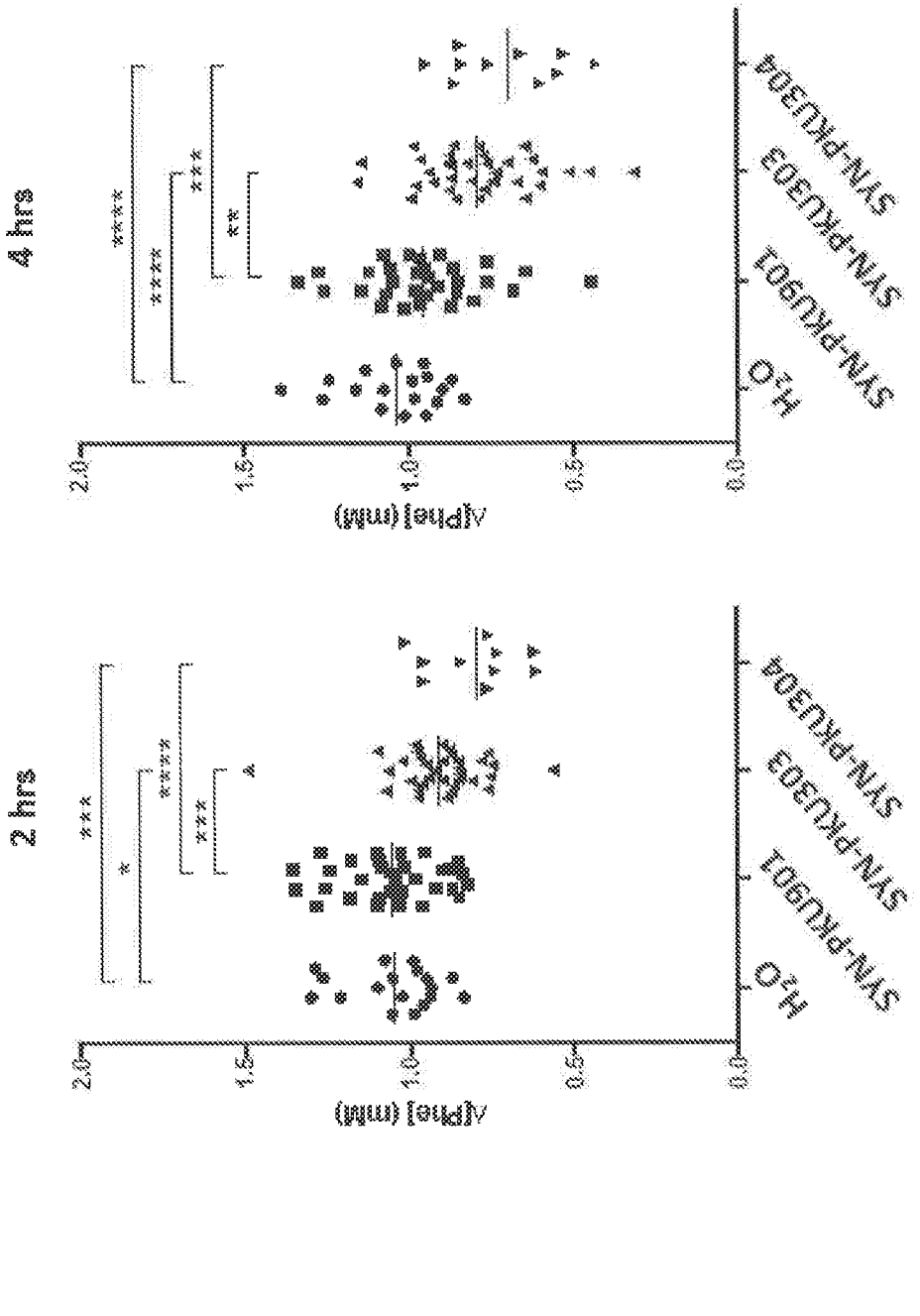


Fig. 29C

Fig. 29D

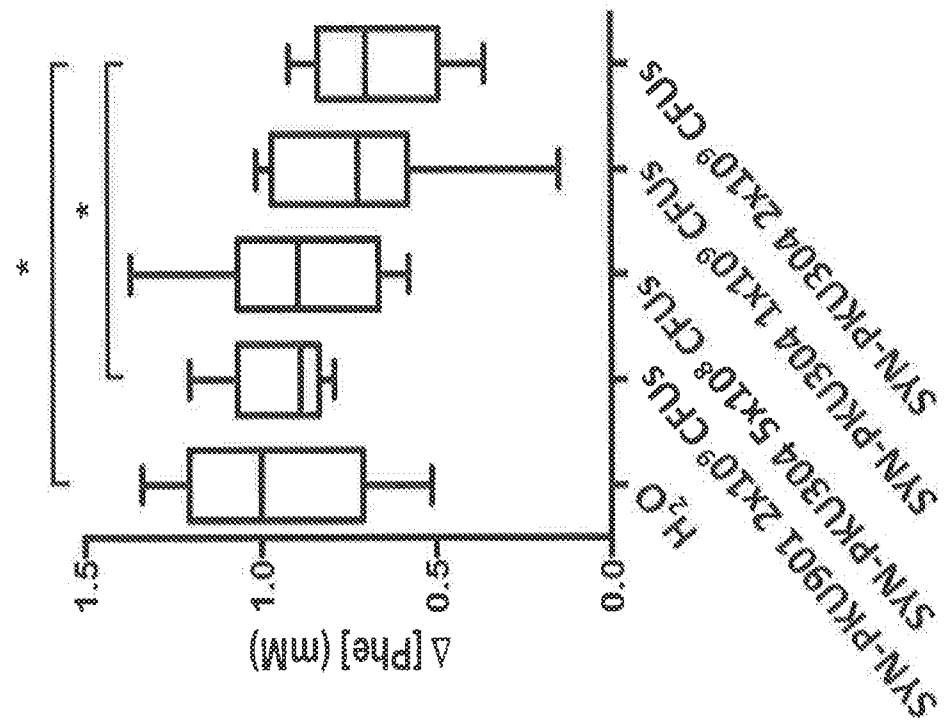


Fig. 30B

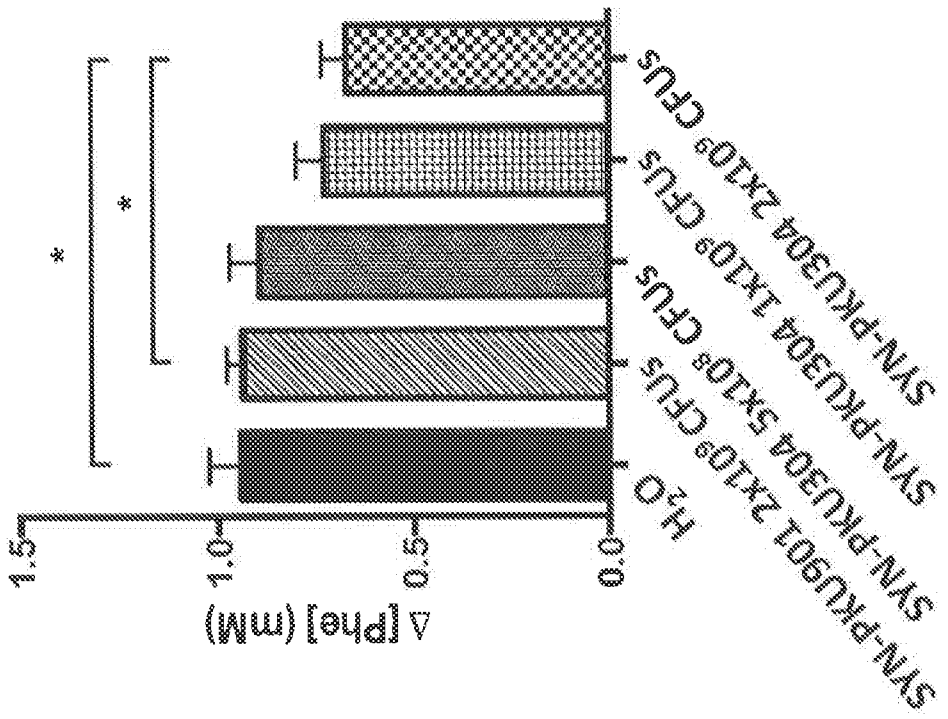


Fig. 30A

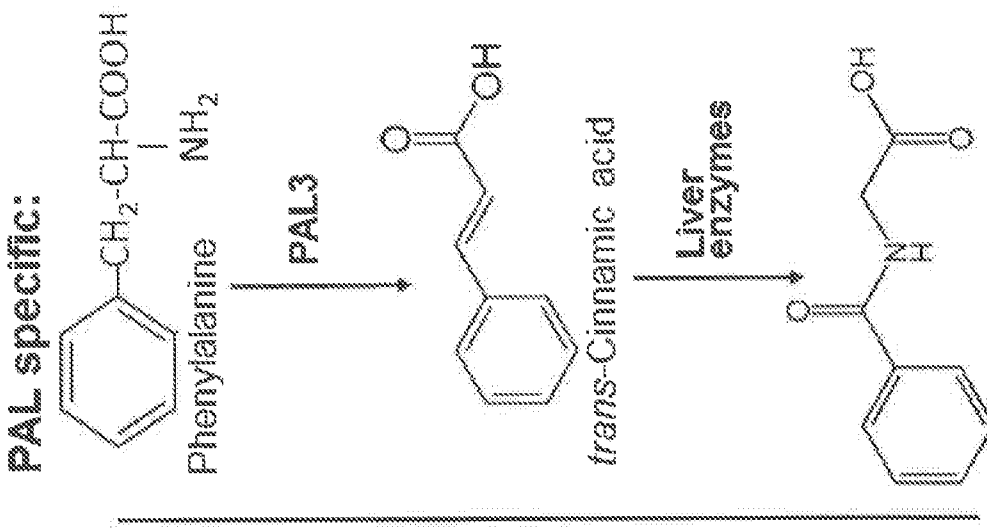


Fig. 31B

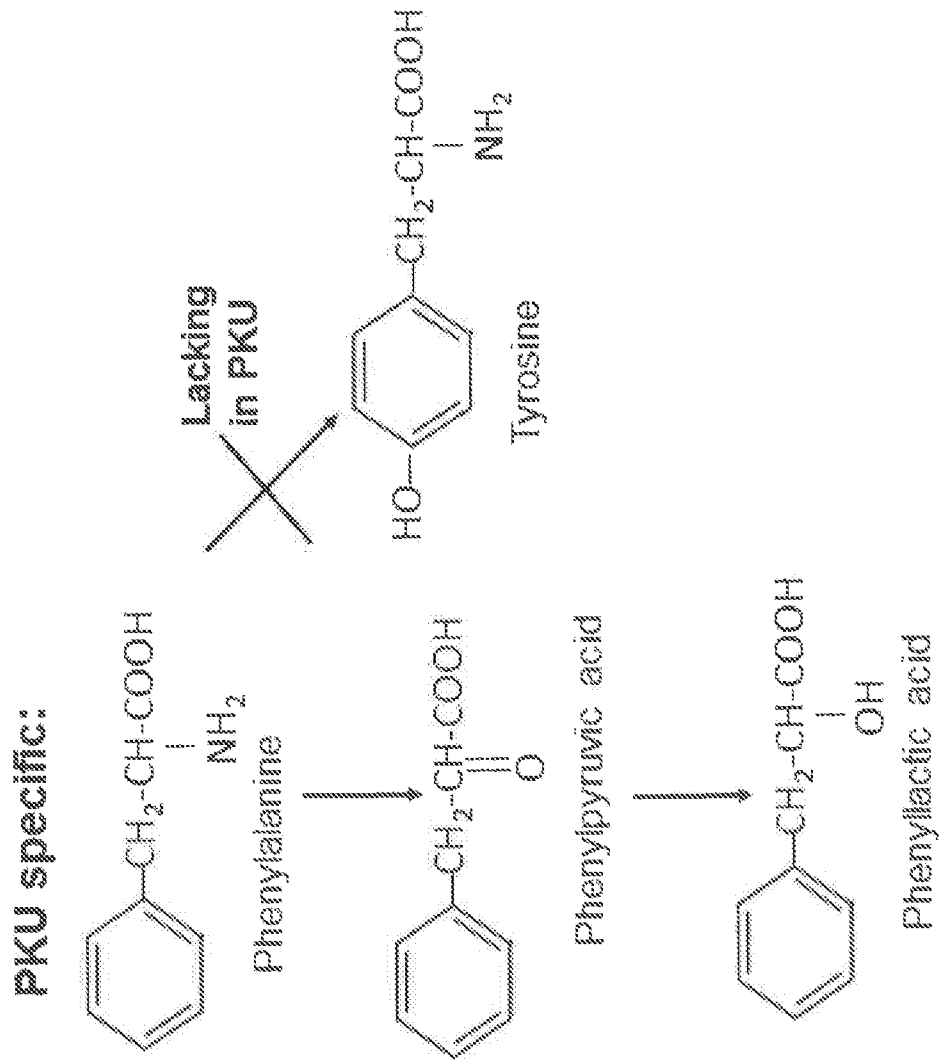


Fig. 31A

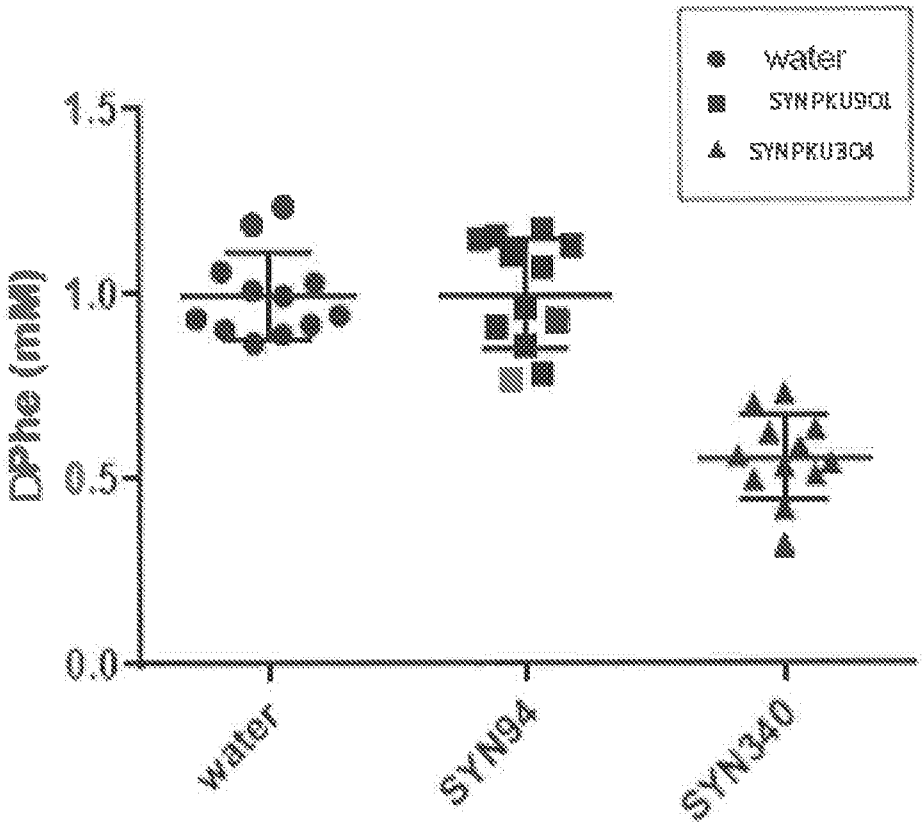

water
SYNPKU90L
SYNPKU340
ΔPhe (mM)
1.5
1.0
0.5
0.0
water
SYN94
SYN340

Fig. 32A

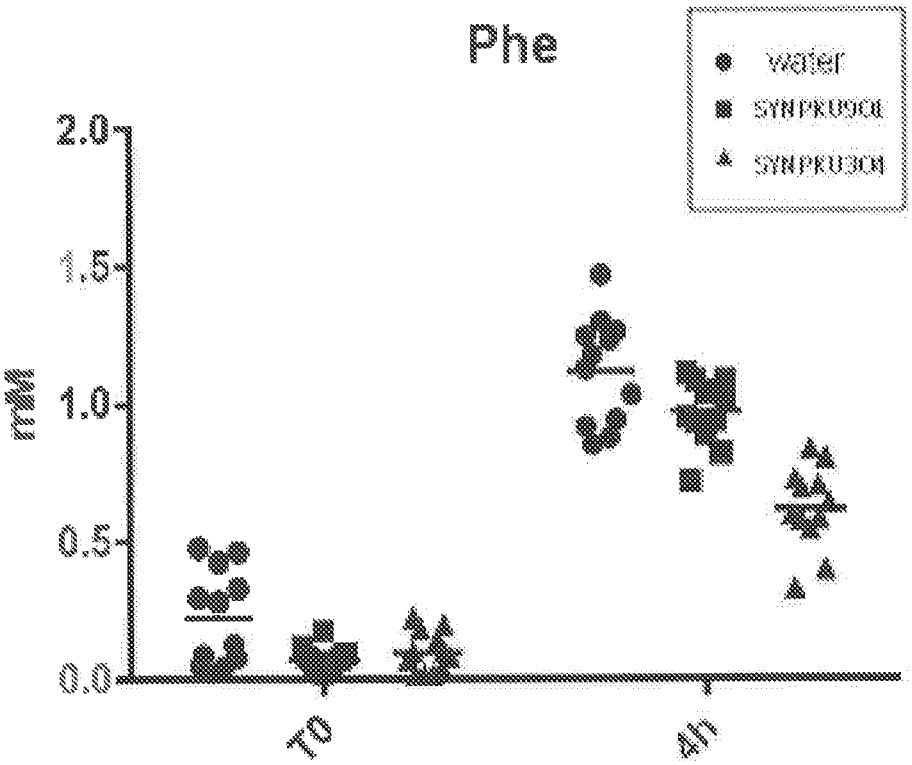


Fig. 32B

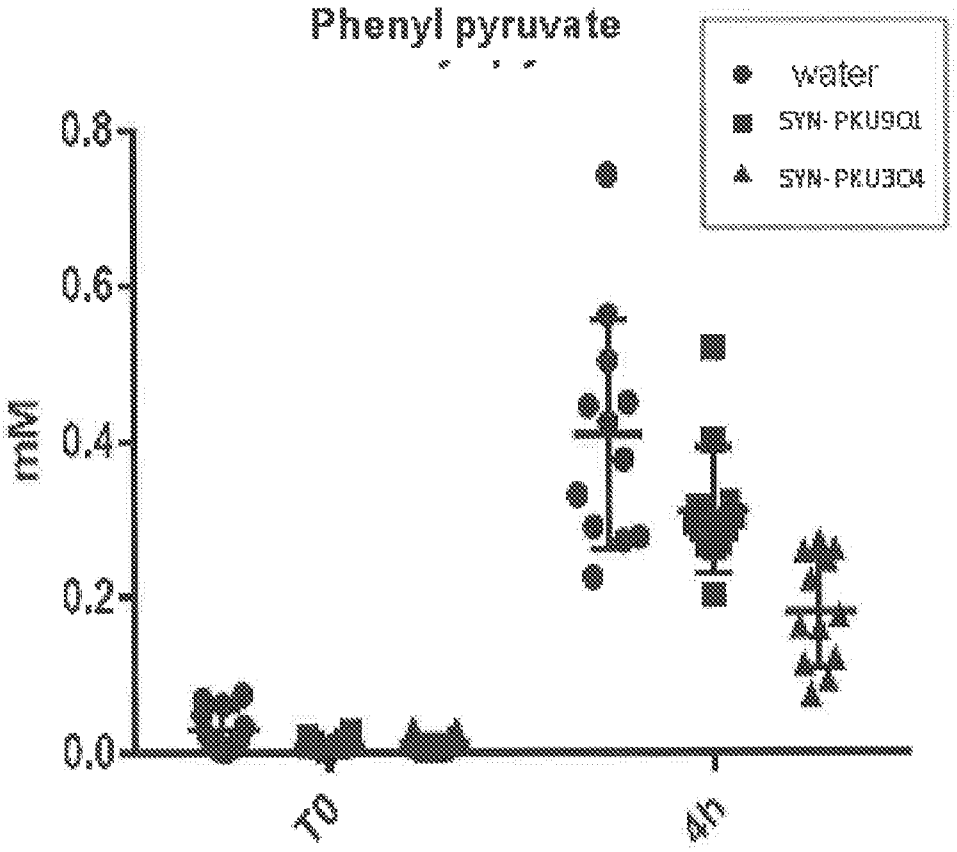

Phenyl pyruvate
water
SYN-PKU9QL
SYN-PKU3C4
0.8
0.6
0.4
0.2
0.0
mM
T0
4h

Fig. 32C

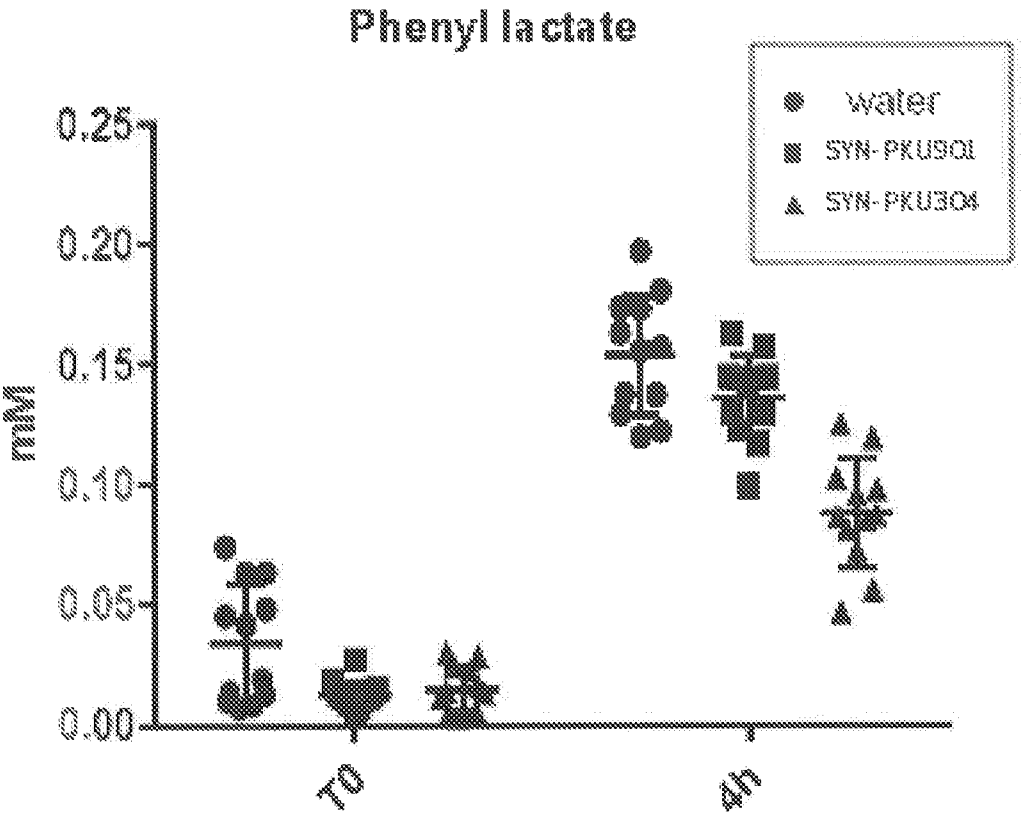

Phenyl lactate
water
SYN-PKU901
SYN-PKU304
0.25
0.20
0.15
0.10
0.05
0.00
mM
T0
4h

Fig. 32D

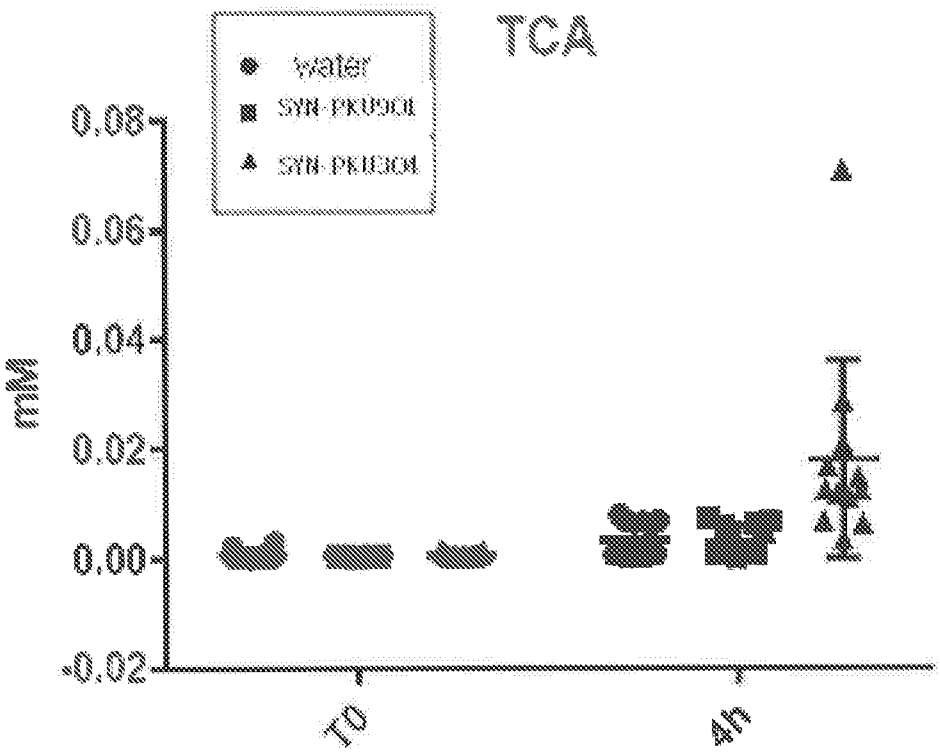

TCA
water
SYN-PKU901
SYN-PKU902
0,08
0,06
0,04
0,02
0,00
-0,02
mM
T0
4h

Fig. 32E

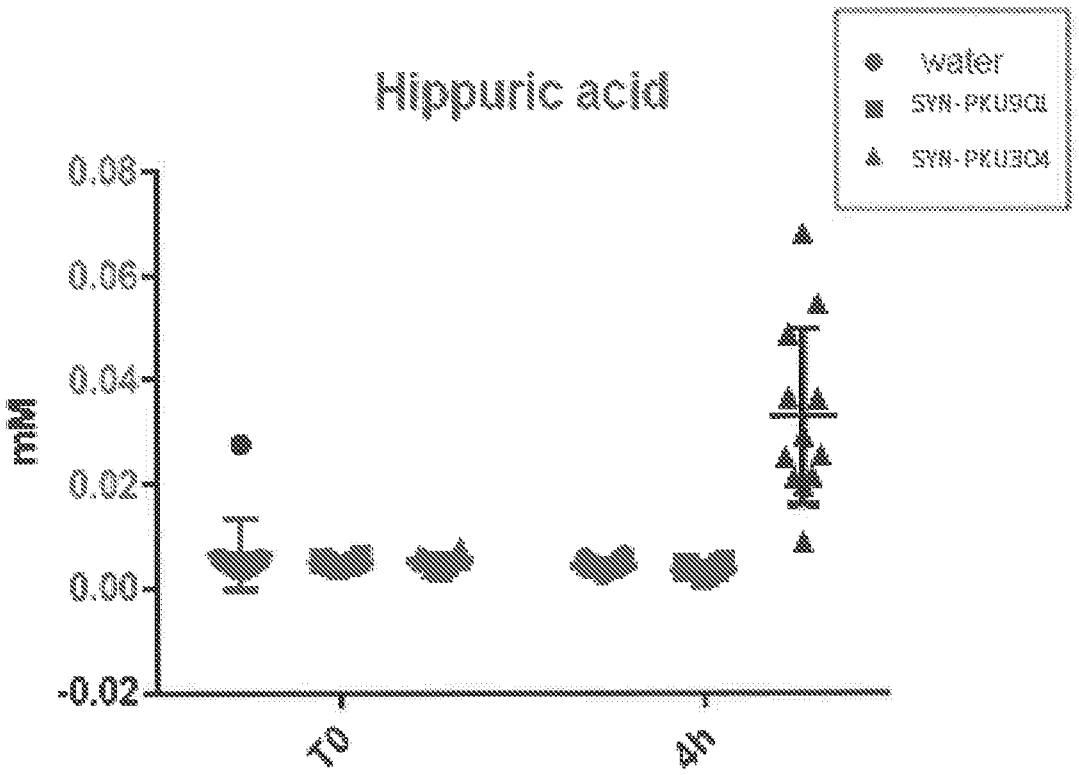

Hippuric acid
water
SYN-PKU3CB
SYN-PKU3CH
mM
0.08
0.06
0.04
0.02
0.00
-0.02
T0
Δ8

Fig. 32F

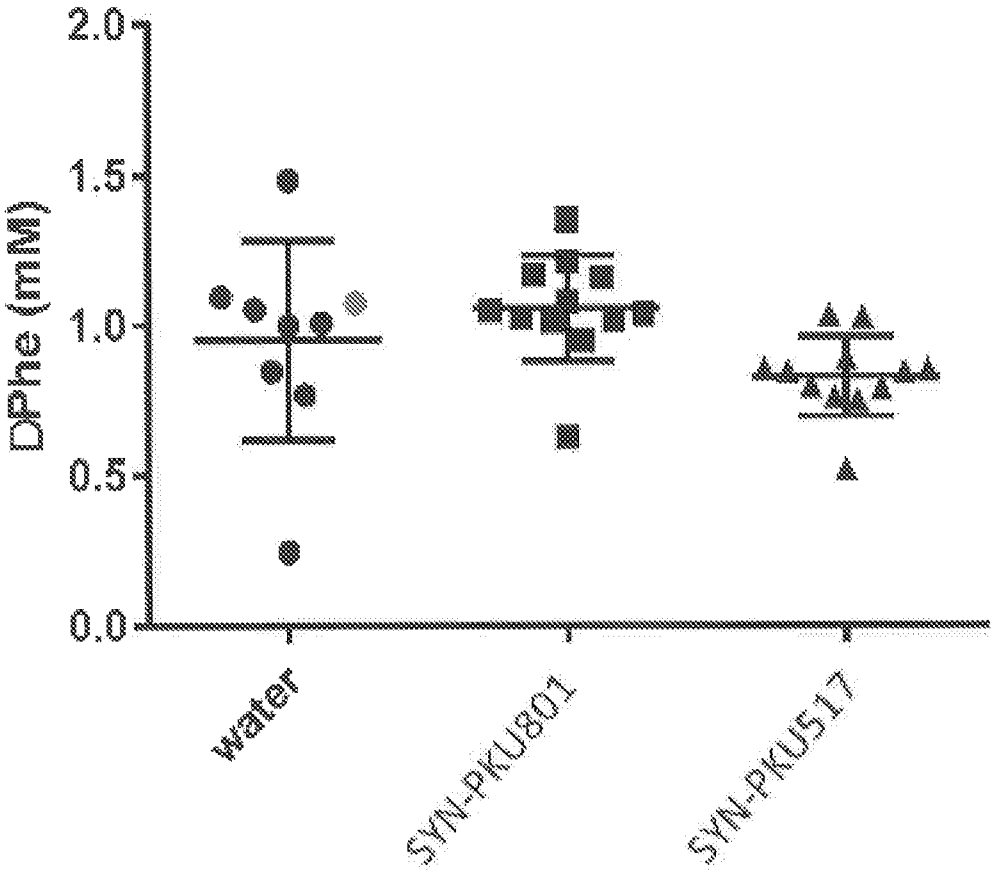


Fig. 33A

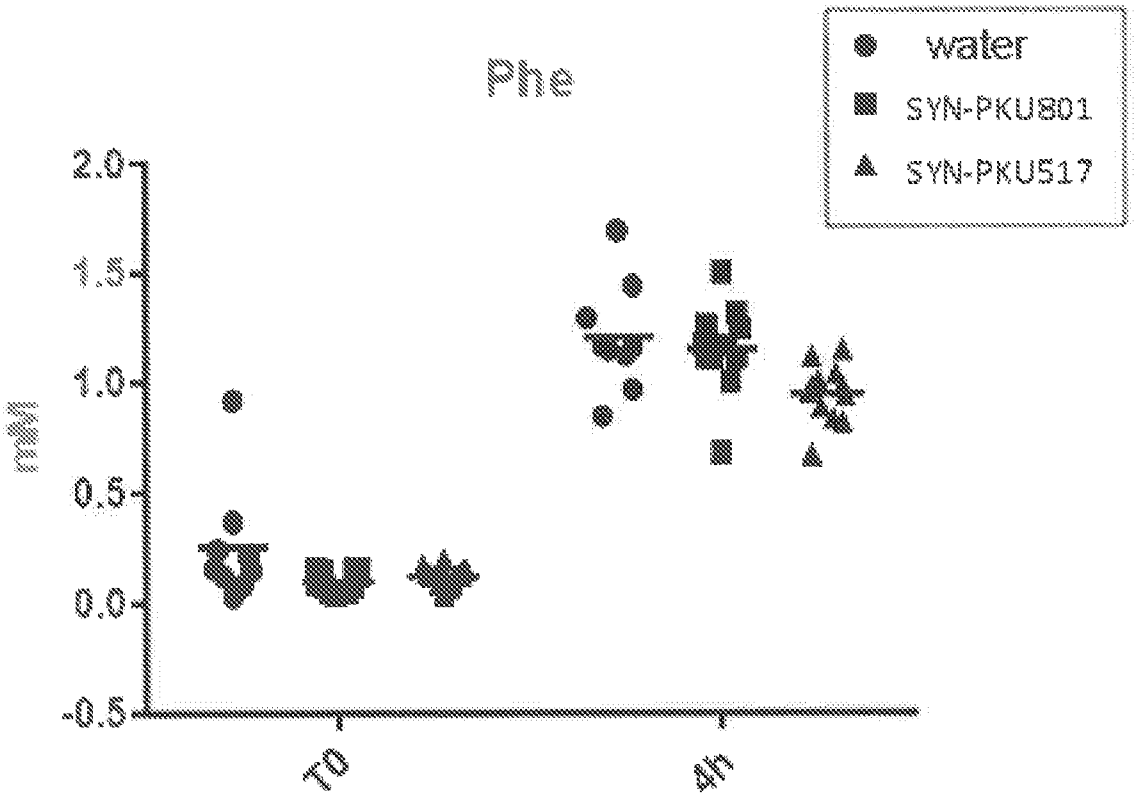


Fig. 33B

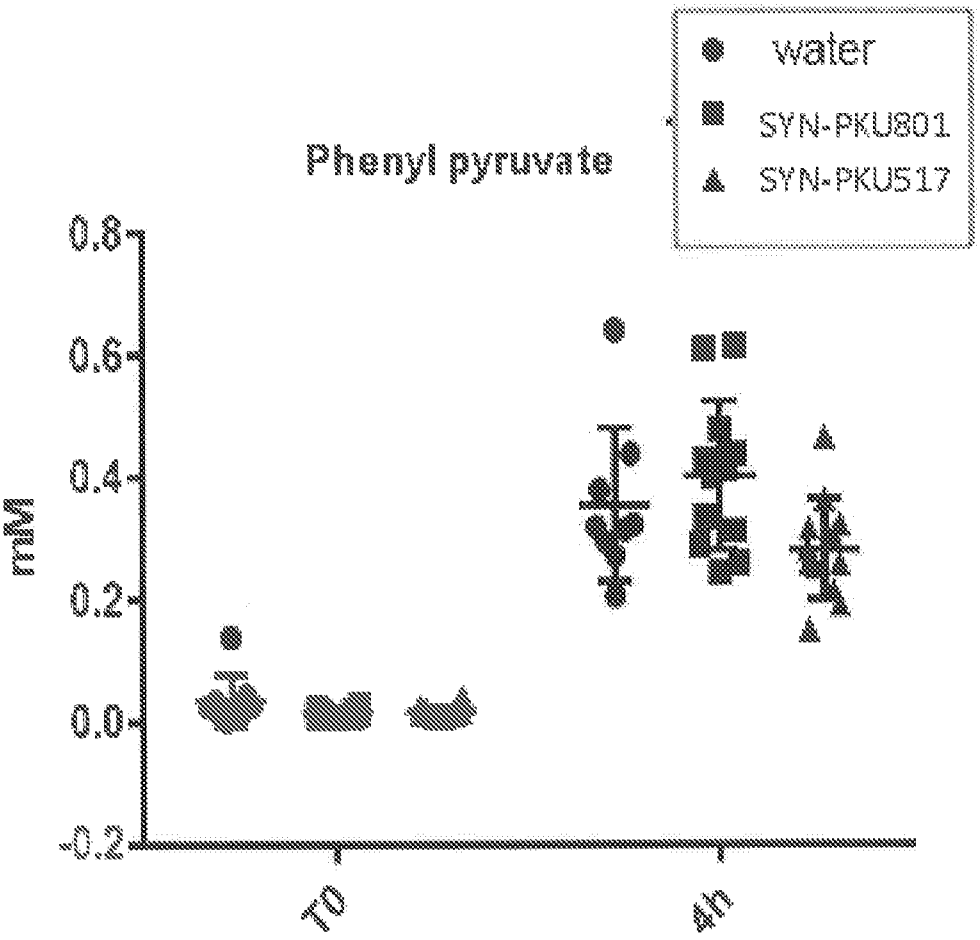

water
SYN-PKU801
SYN-PKU517
Phenyl pyruvate
0.8
0.6
0.4
0.2
0.0
-0.2
mM
T0
4h

Fig. 33C

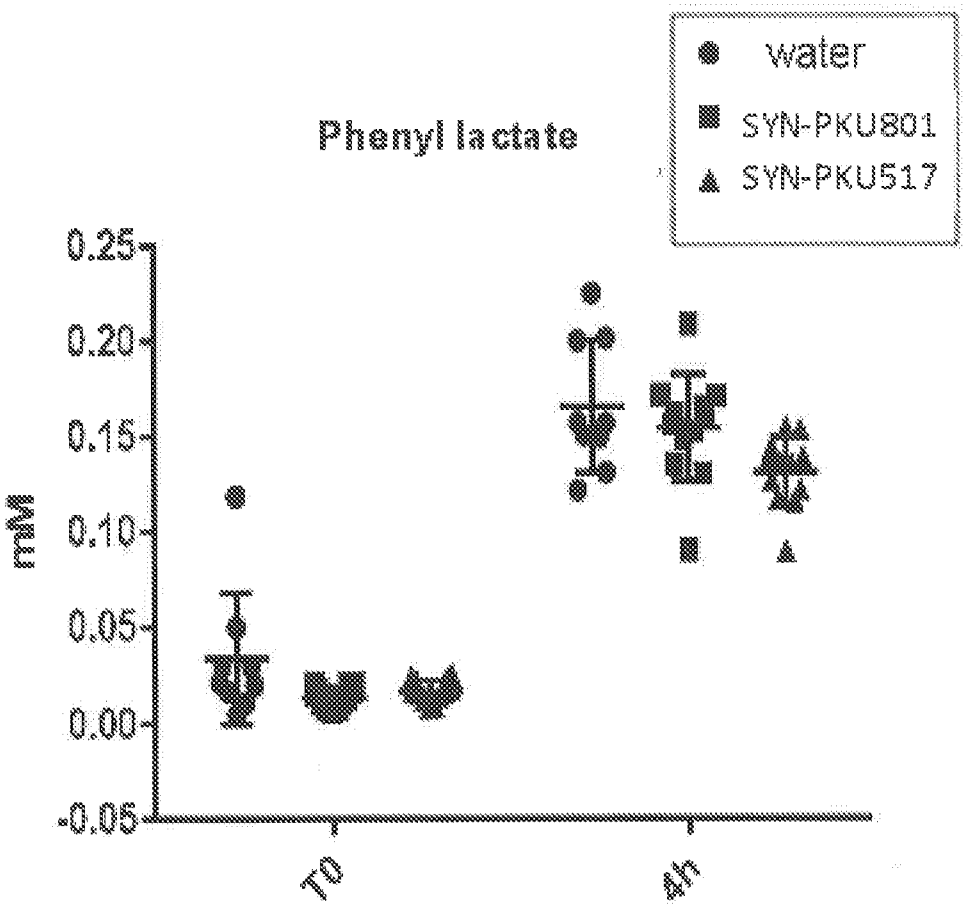

Phenyl lactate
water
SYN-PKU801
SYN-PKU517
mM
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
T0
4h

Fig. 33D

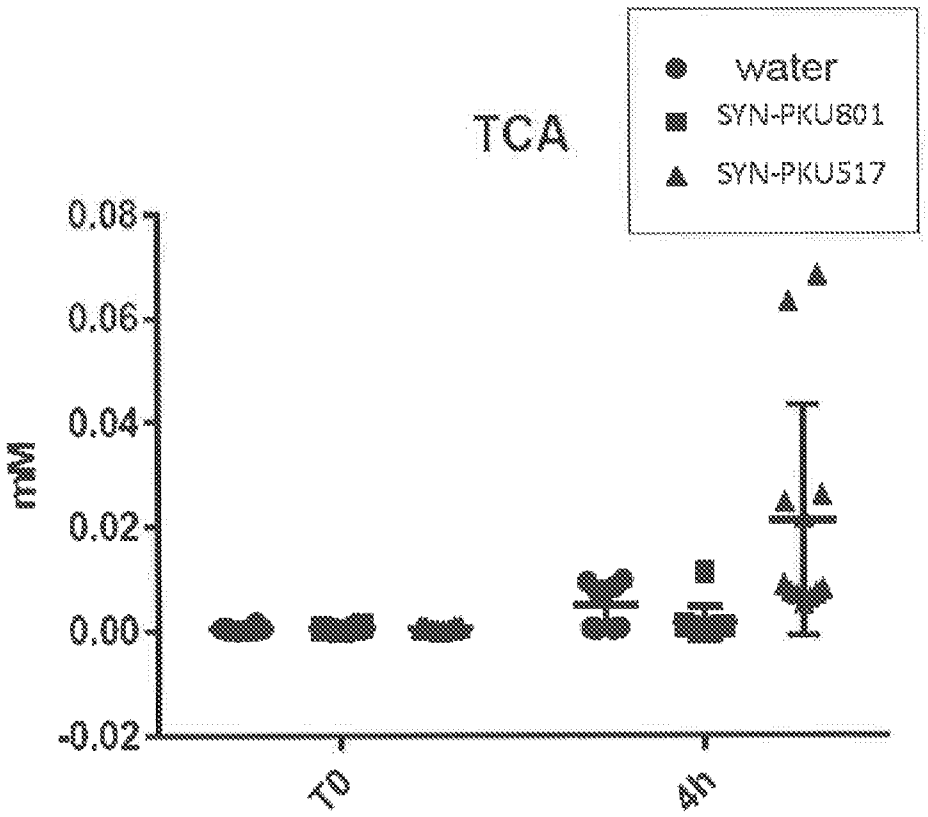

TCA
water
SYN-PKU801
SYN-PKU517
0.08
0.06
0.04
0.02
0.00
-0.02
mM
T0
4h

Fig. 33E

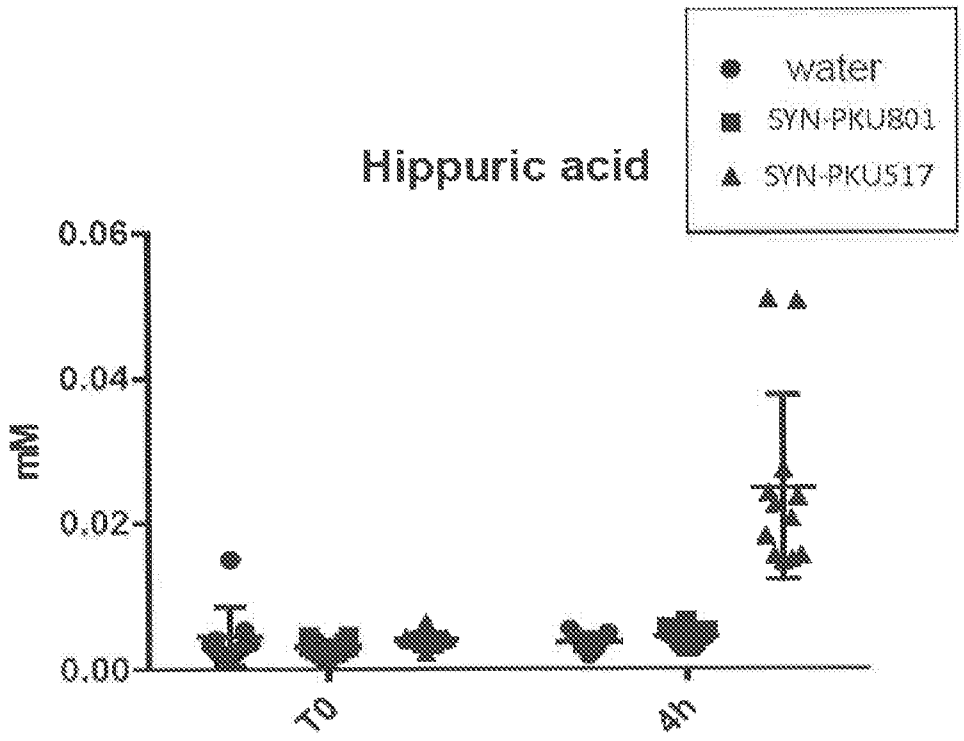

Hippuric acid
water
SYN-PKU801
SYN-PKU517
0.06
0.04
0.02
0.00
mM
T0
4h

Fig. 33F

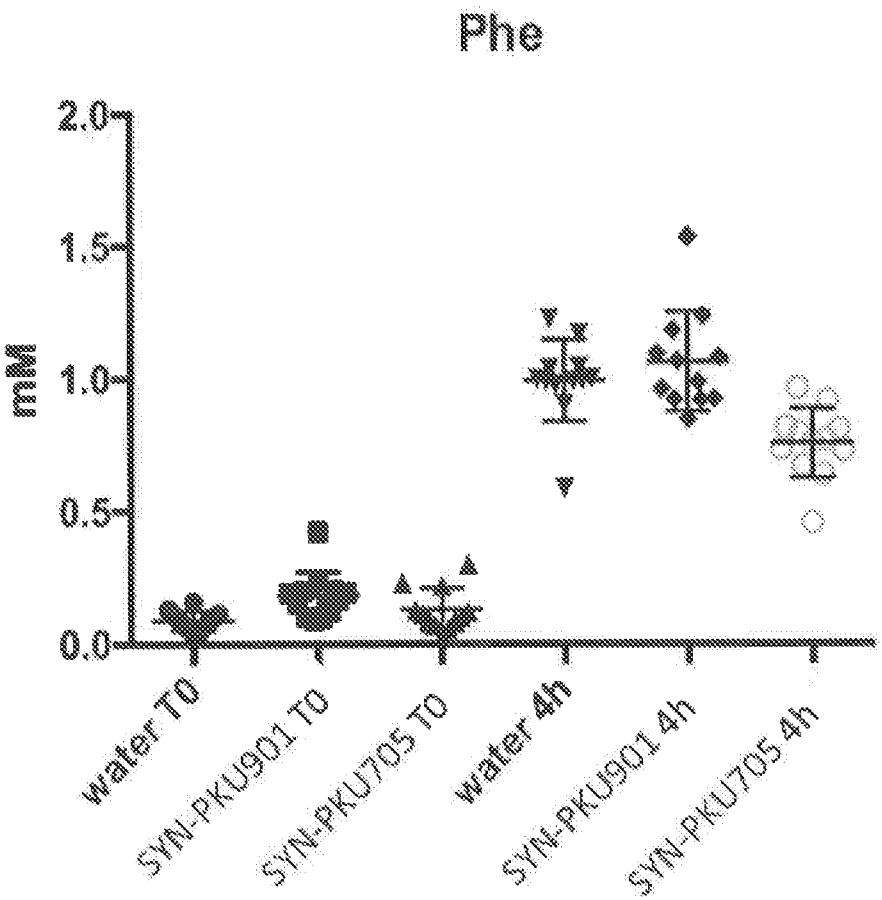

Phe
mM
2.0
1.5
1.0
0.5
0.0
water T0
SYN-PKU901 T0
SYN-PKU705 T0
water 4h
SYN-PKU901 4h
SYN-PKU705 4h

Fig. 34A

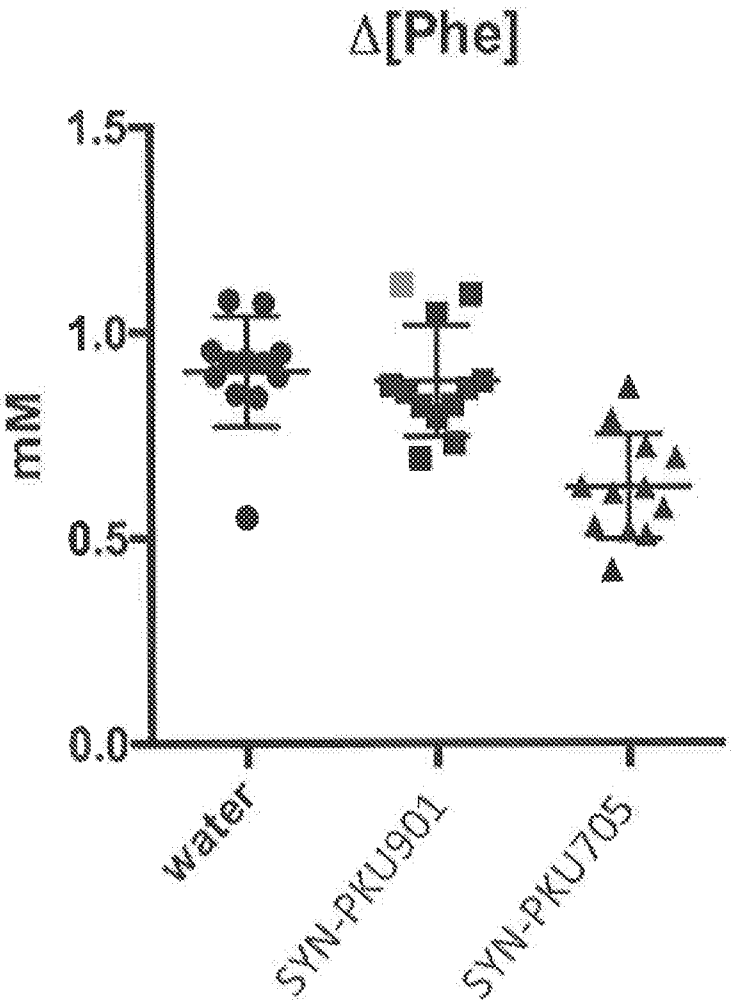


Fig. 34B

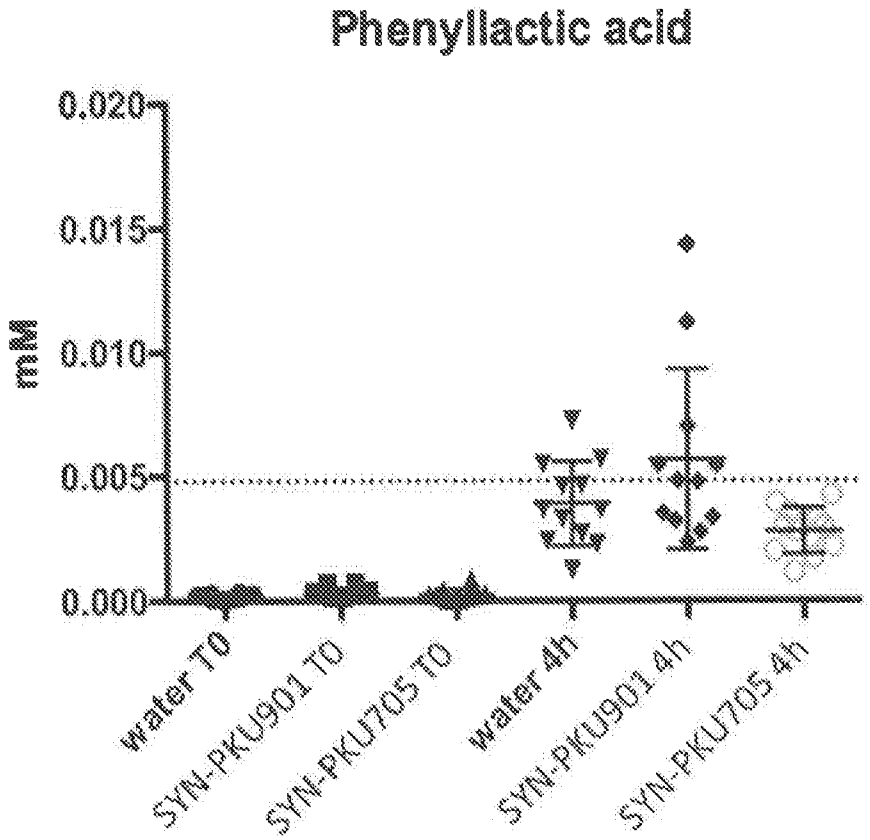

Phenyllactic acid
mM
0.020
0.015
0.010
0.005
0.000
water T0
SYN-PKU901 T0
SYN-PKU705 T0
water 4h
SYN-PKU901 4h
SYN-PKU705 4h

Fig. 34C

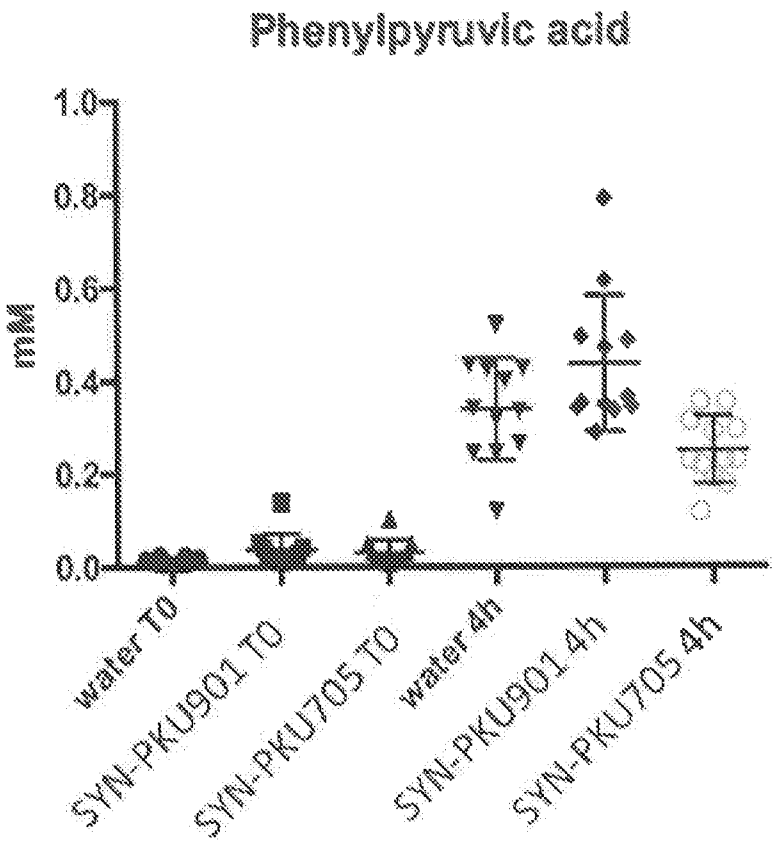

Phenylpyruvic acid
mM
1.0
0.8
0.6
0.4
0.2
0.0
water T0
SYN-PKU901 T0
SYN-PKU705 T0
water 4h
SYN-PKU901 4h
SYN-PKU705 4h

Fig. 34D

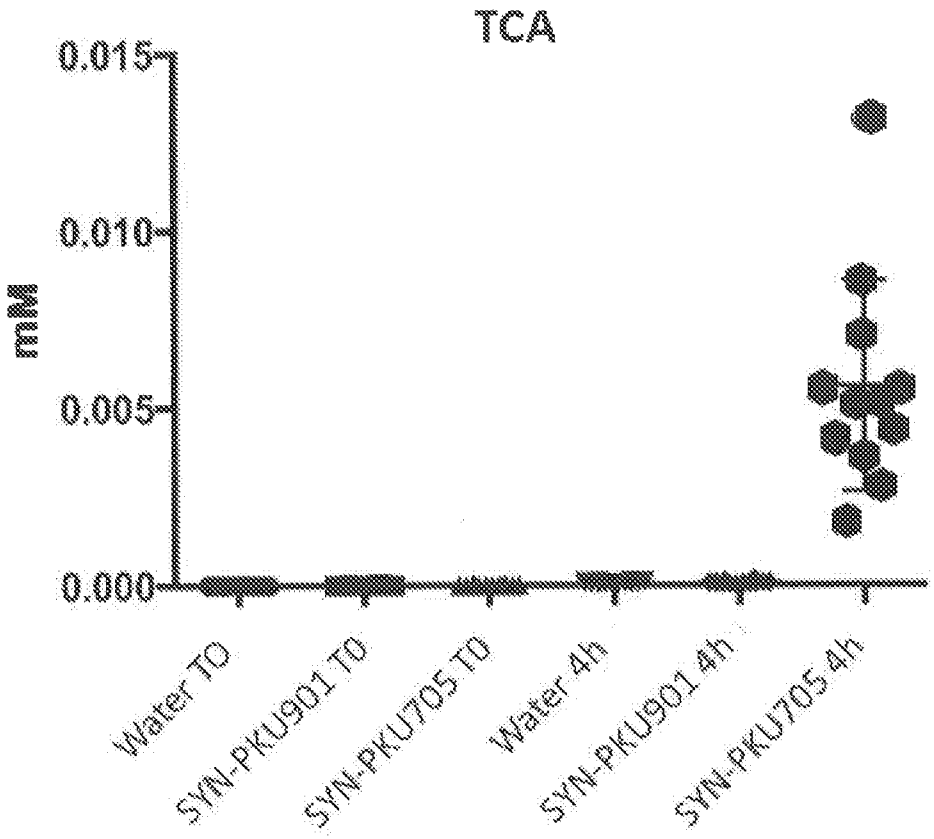

TCA
0.015
0.010
0.005
0.000
mM
Water T0
SYN-PKU901 T0
SYN-PKU705 T0
Water 4h
SYN-PKU901 4h
SYN-PKU705 4h

Fig. 34E

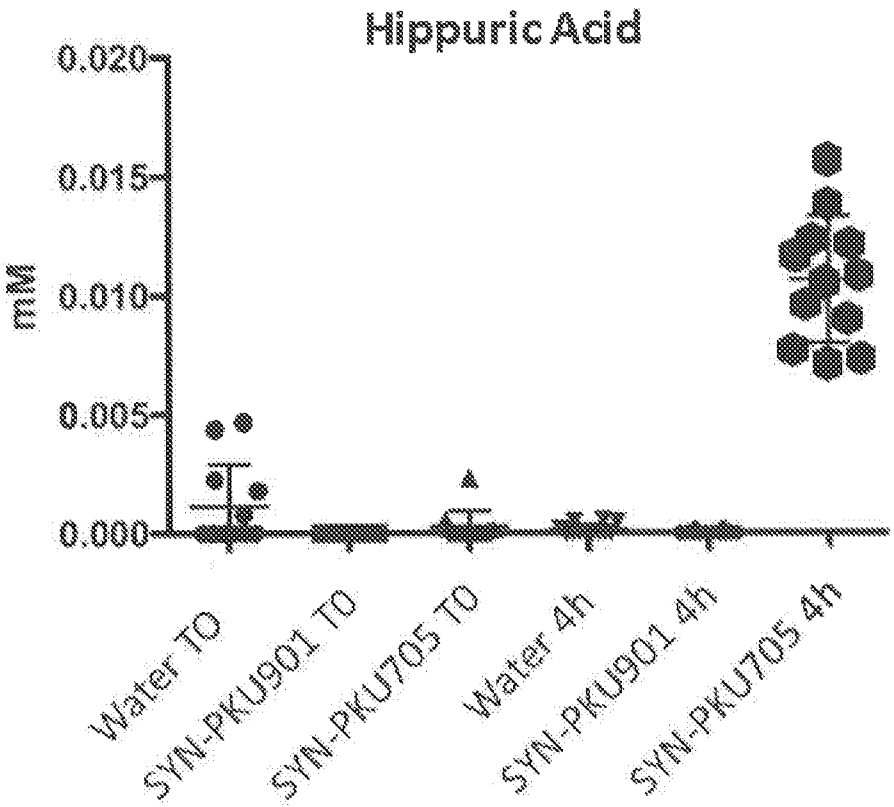

Hippuric Acid
0.020
0.015
0.010
0.005
0.000
mM
Water T0
SYN-PKU901 T0
SYN-PKU705 T0
Water 4h
SYN-PKU901 4h
SYN-PKU705 4h

Fig. 34F

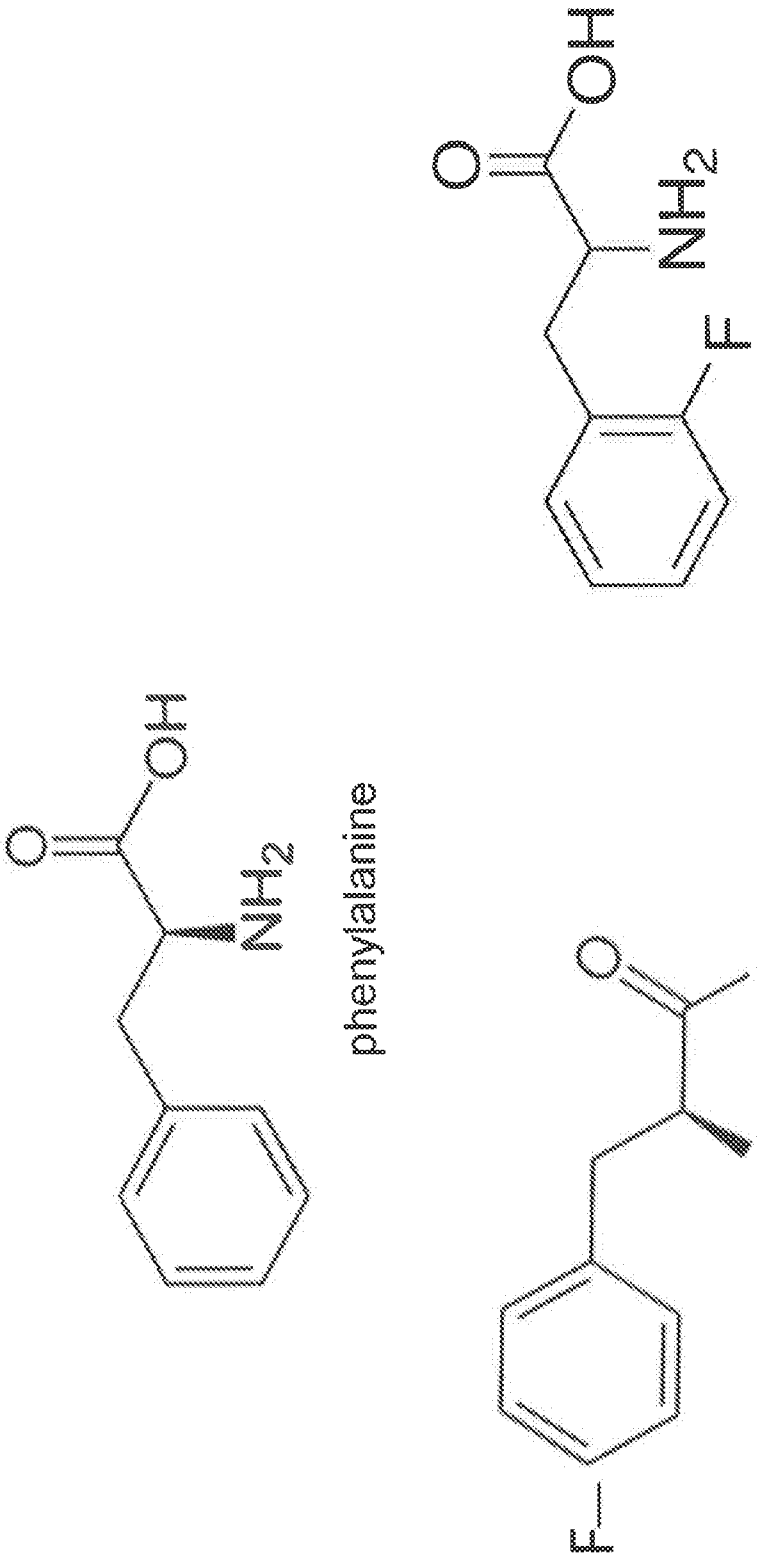

O
OH
NH2
F
o-fluoro-DL-phenylalanine
O
OH
NH2
phenylalanine
O
NH2
F
p-fluoro-DL-phenylalanine

Fig. 35

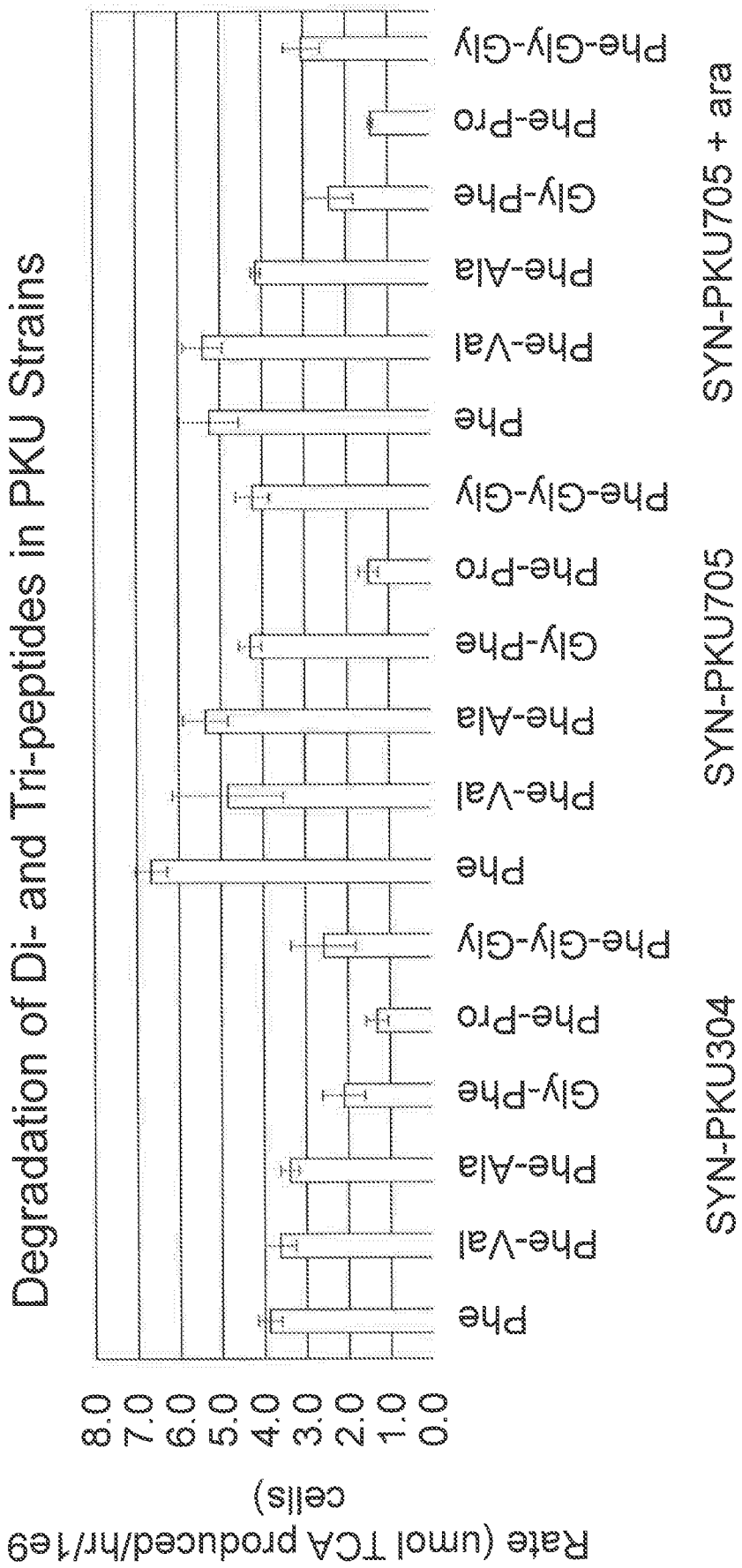
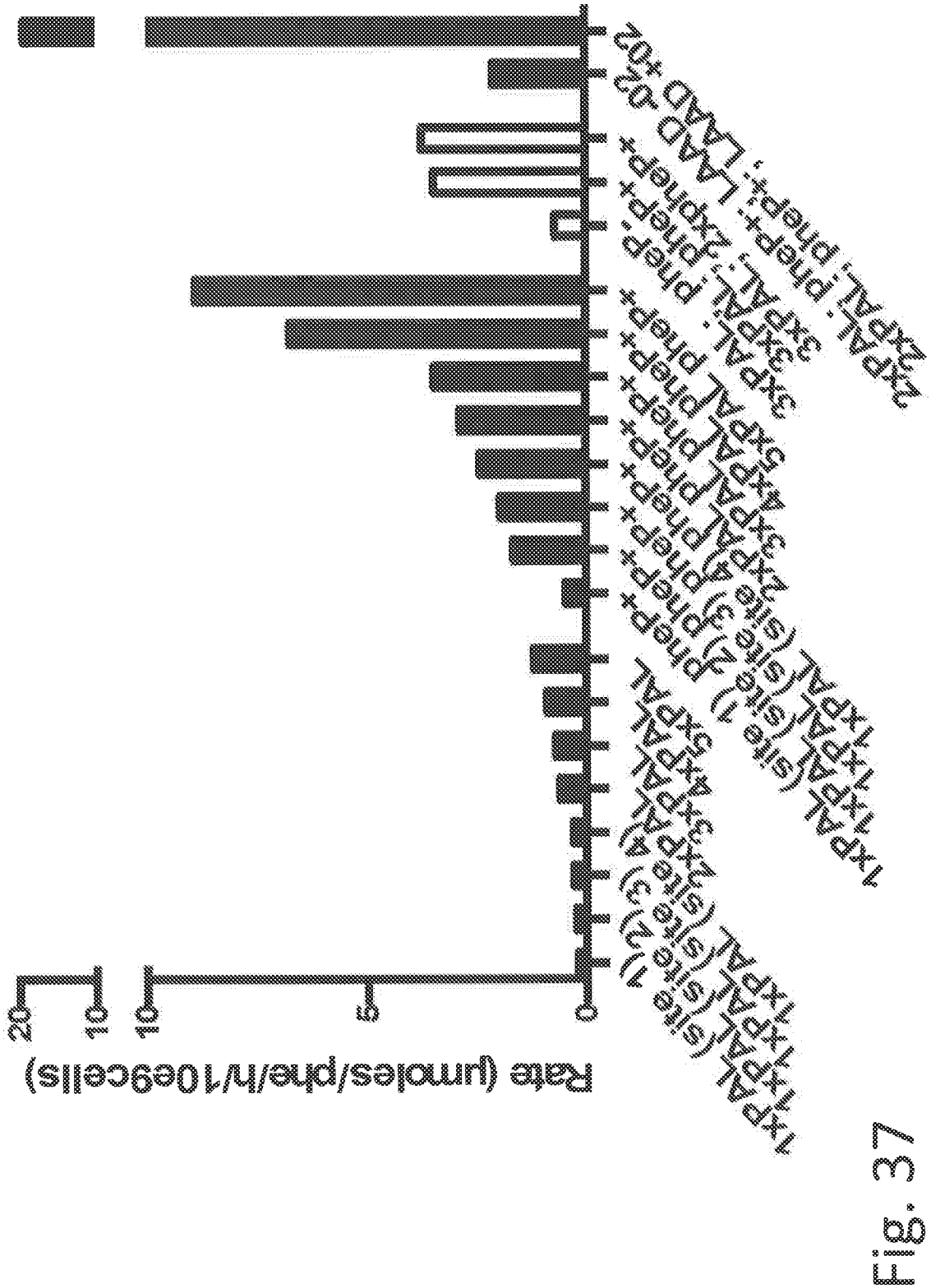

Degradation of Di- and Tri-peptides in PKU Strains
Rate (umol TCA produced/hr/1e9 cells)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Phe
Phe-Val
Phe-Ala
Gly-Phe
Phe-Pro
Phe-Gly-Gly
SYN-PKU304
Phe
Phe-Val
Phe-Ala
Gly-Phe
Phe-Pro
Phe-Gly-Gly
SYN-PKU705
Phe
Phe-Val
Phe-Ala
Gly-Phe
Phe-Pro
Phe-Gly-Gly
SYN-PKU705 + ara

Fig. 36


Rate (umoles/phe/h/10e9cells)
0
5
10
20
LAD 202
pheP+; pheP+; 3xpAL; 3xpAL; pheP+; pheP+; pheP+

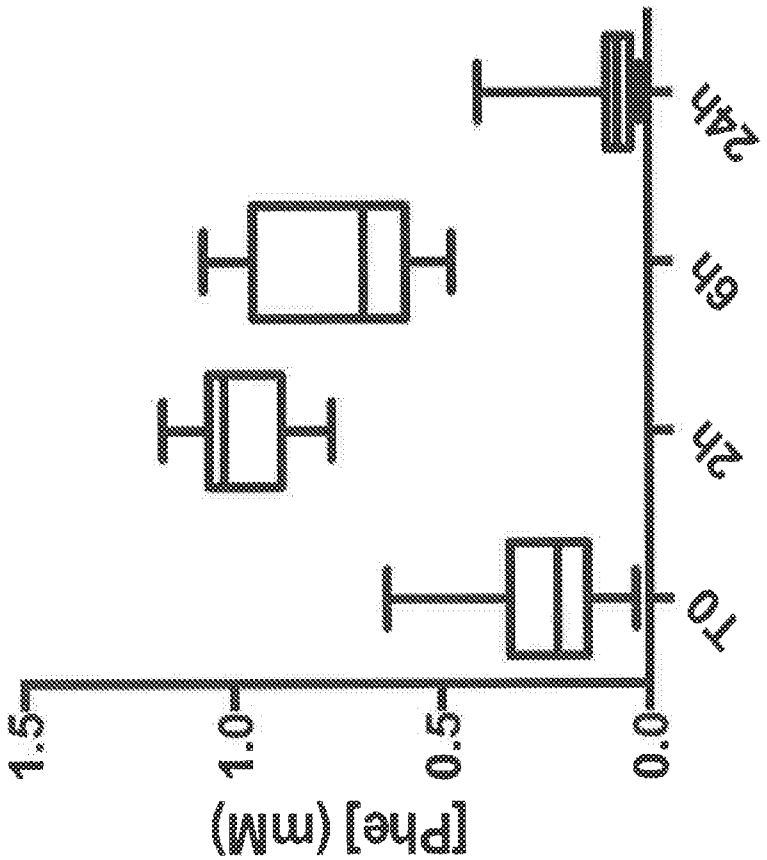


Fig. 38A

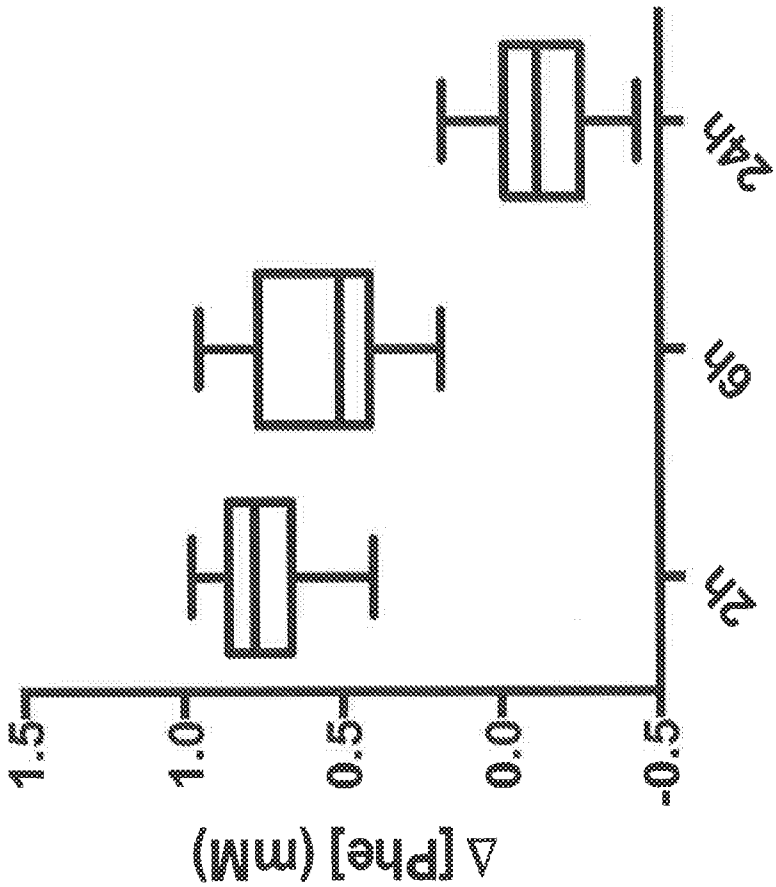


Fig. 38B

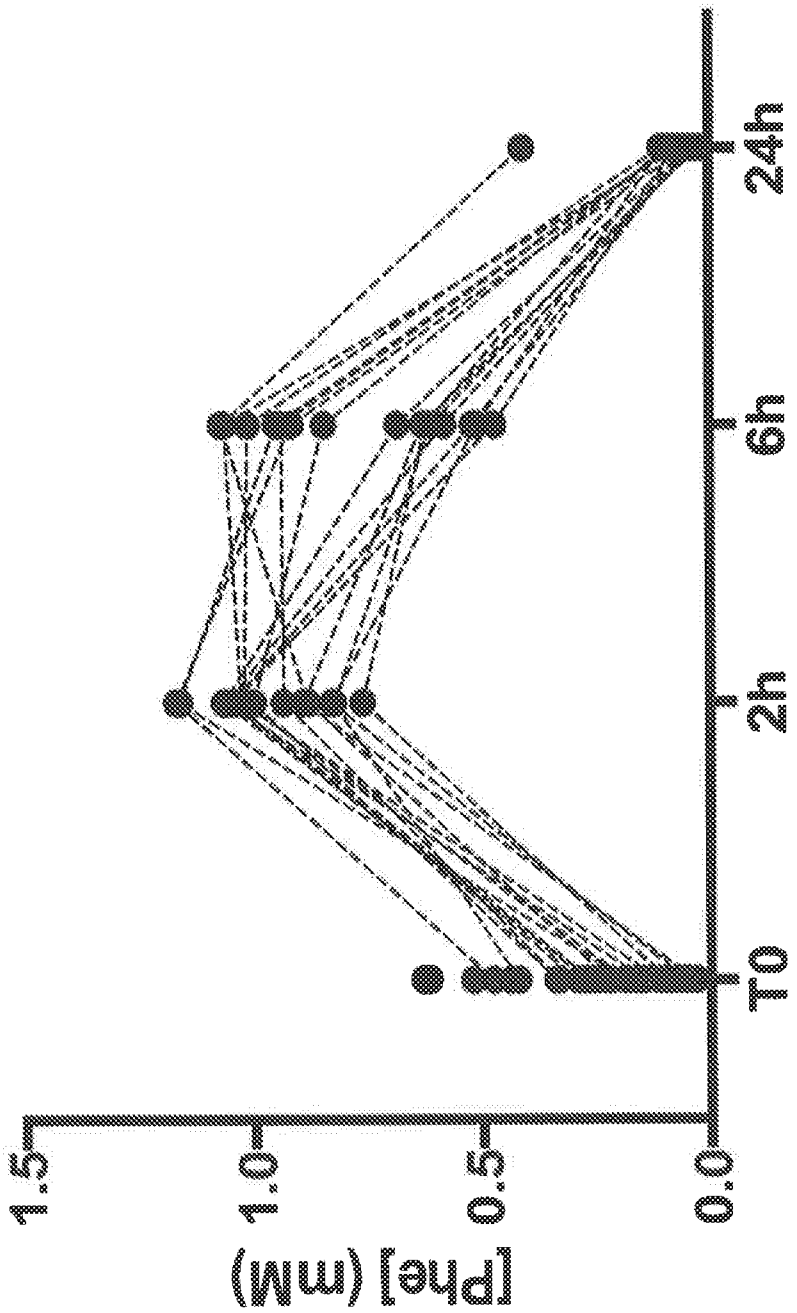


Fig. 38C

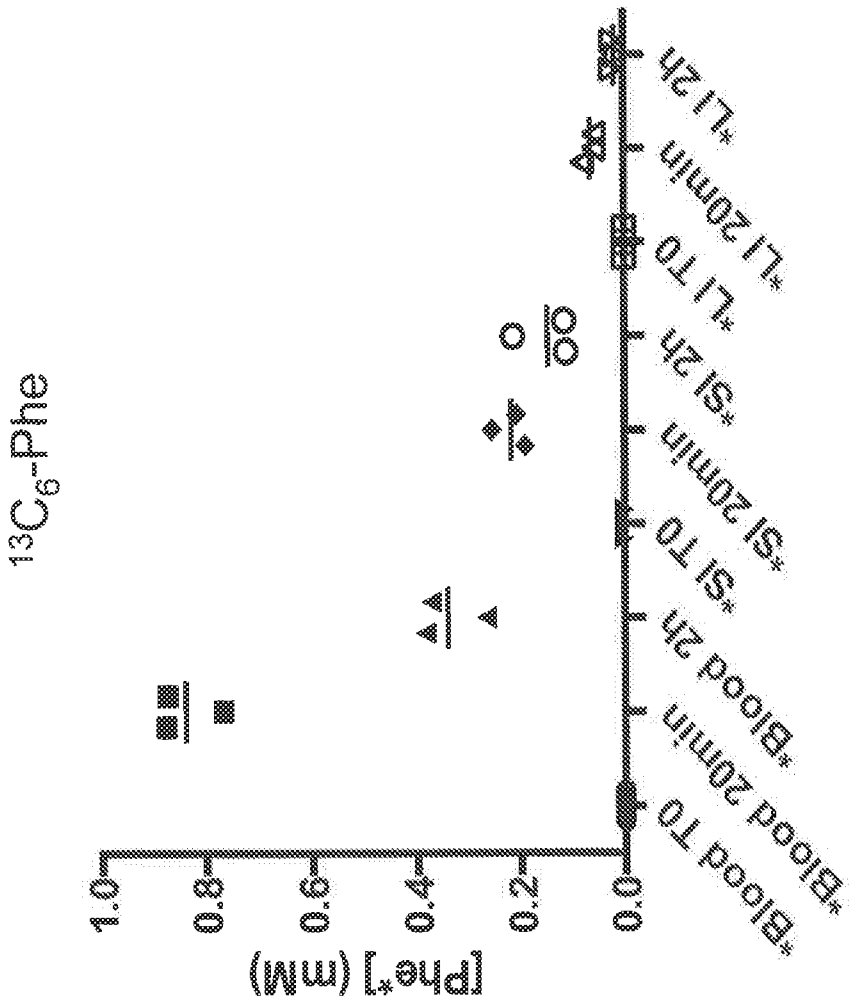

$^{13}C_6$-Phe
[Phe*] (mM)
1.0
0.8
0.6
0.4
0.2
0.0
*Blood T0
*Blood 20min
*Blood 2h
*SI T0
*SI 20min
*SI 2h
*LI T0
*LI 20min
*LI 2h

Fig. 39

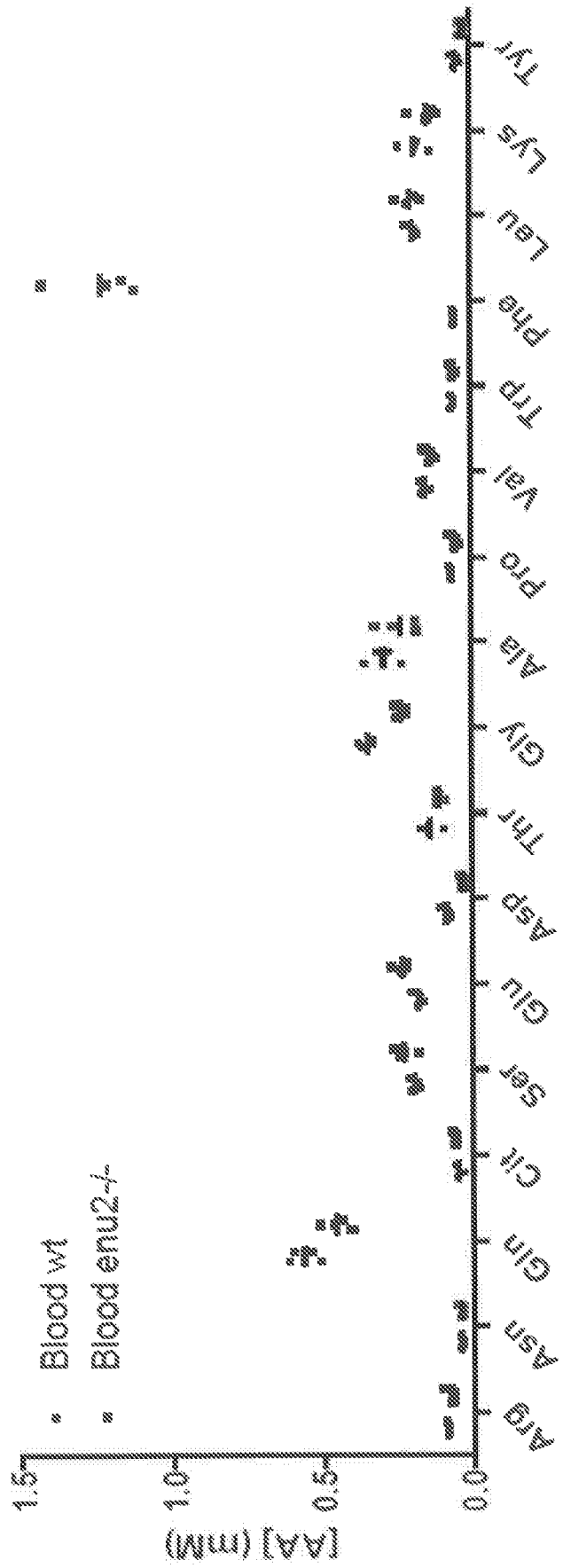

Blood wt
Blood enu2-/-
[AA] (mM)
1.5
1.0
0.5
0.0
Arg
Asn
Gln
Cit
Ser
Glu
Asp
Thr
Gly
Ala
Pro
Val
Trp
Phe
Leu
Lys
Tyr

Fig. 40A

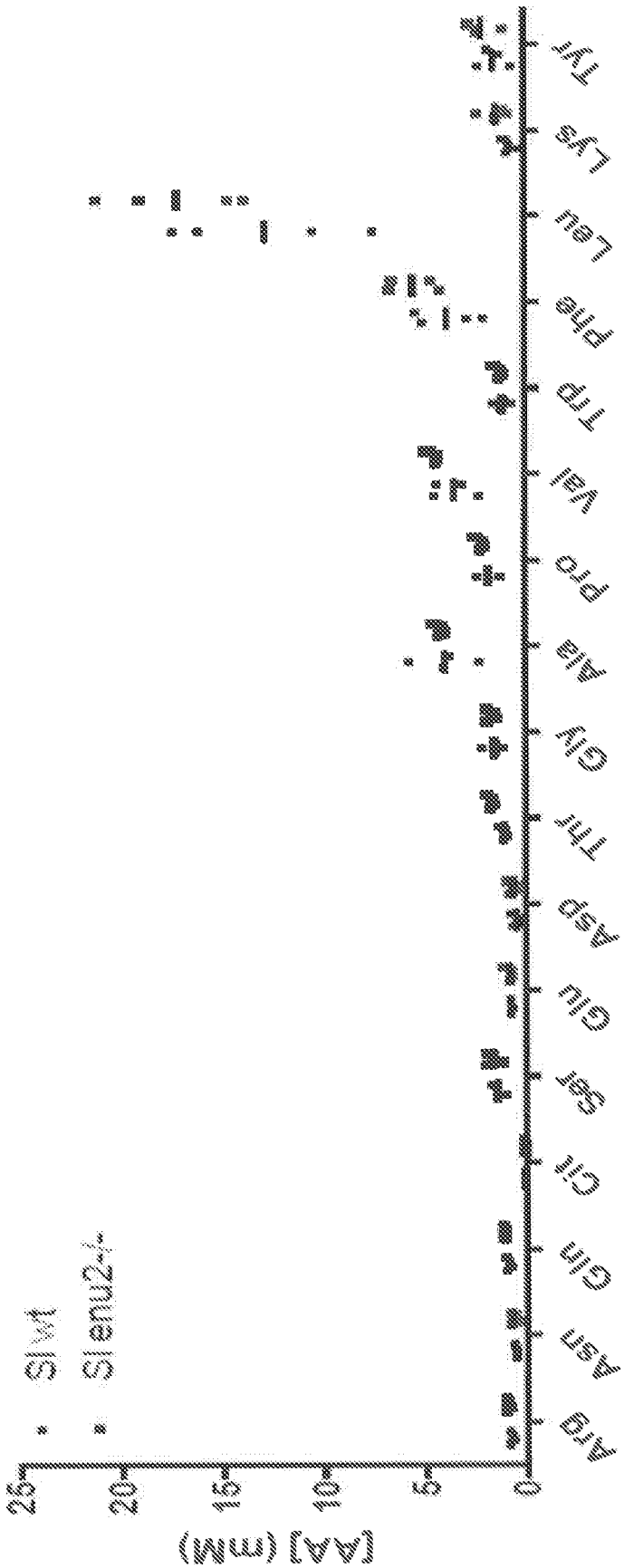

slwt
slenu24-
[AA] (mM)
25
20
15
10
5
0
Arg
Asn
Gln
Cit
Ser
Glu
Asp
Thr
Gly
Ala
Pro
Val
Trp
Phe
Leu
Lys
Tyr

Fig. 40B

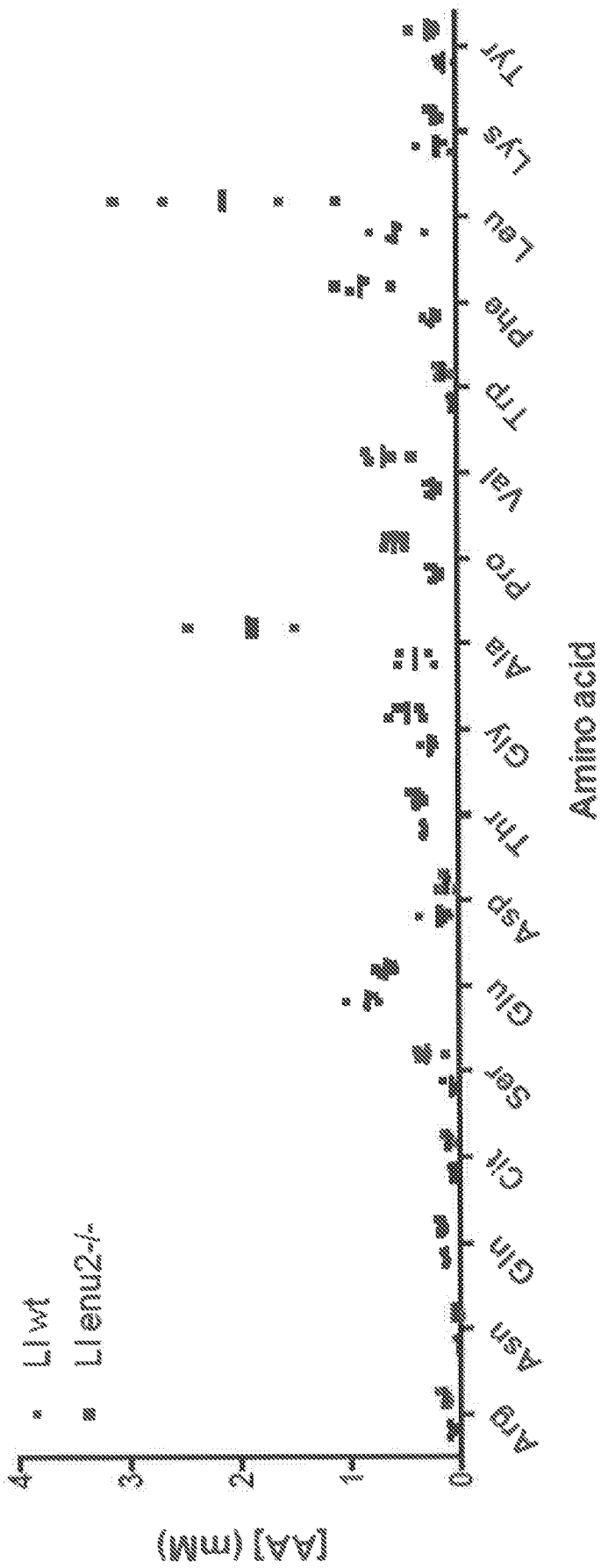

[AA] (mM)
0
1
2
3
4
Amino acid
Arg
Asn
Gln
Cit
Ser
Glu
Asp
Thr
Gly
Ala
Pro
Val
Trp
Phe
Leu
Lys
Tyr
Ll wt
Ll enu2-/-

Fig. 40C

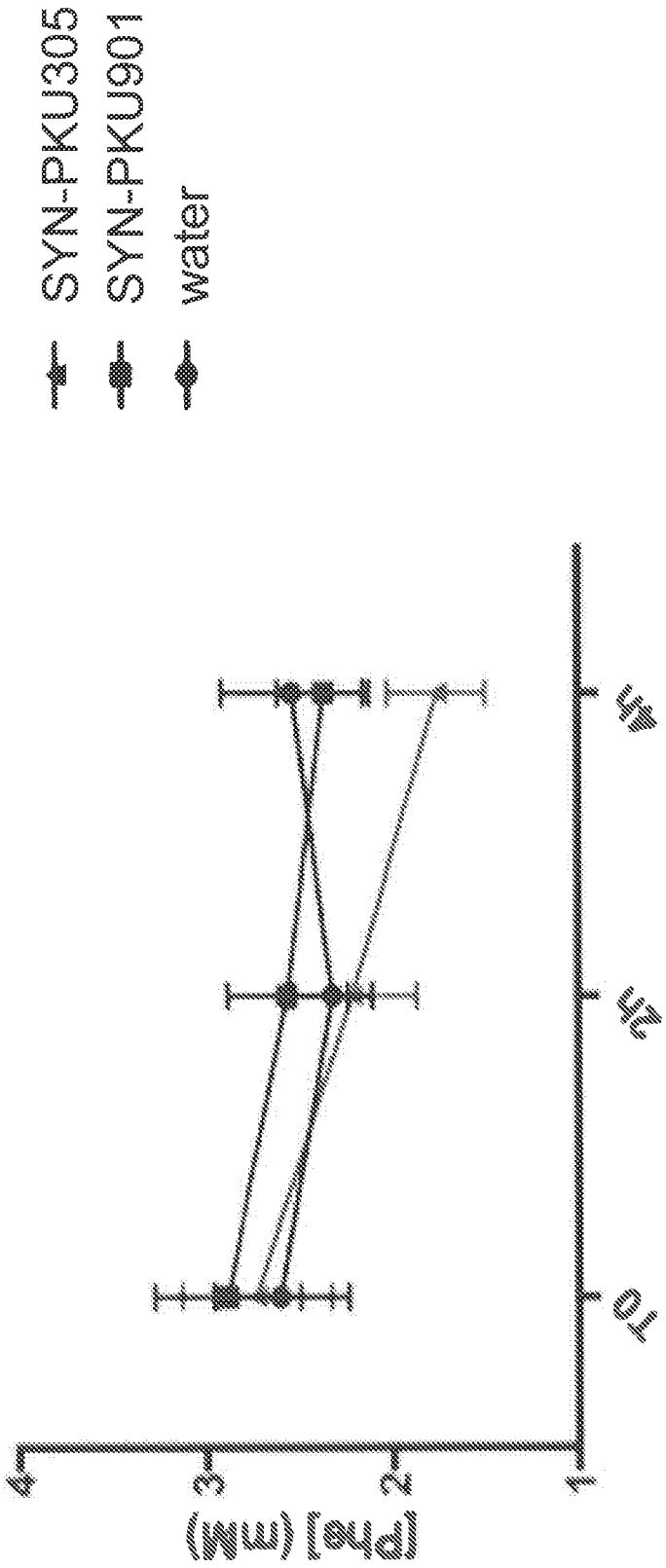


Fig. 41A

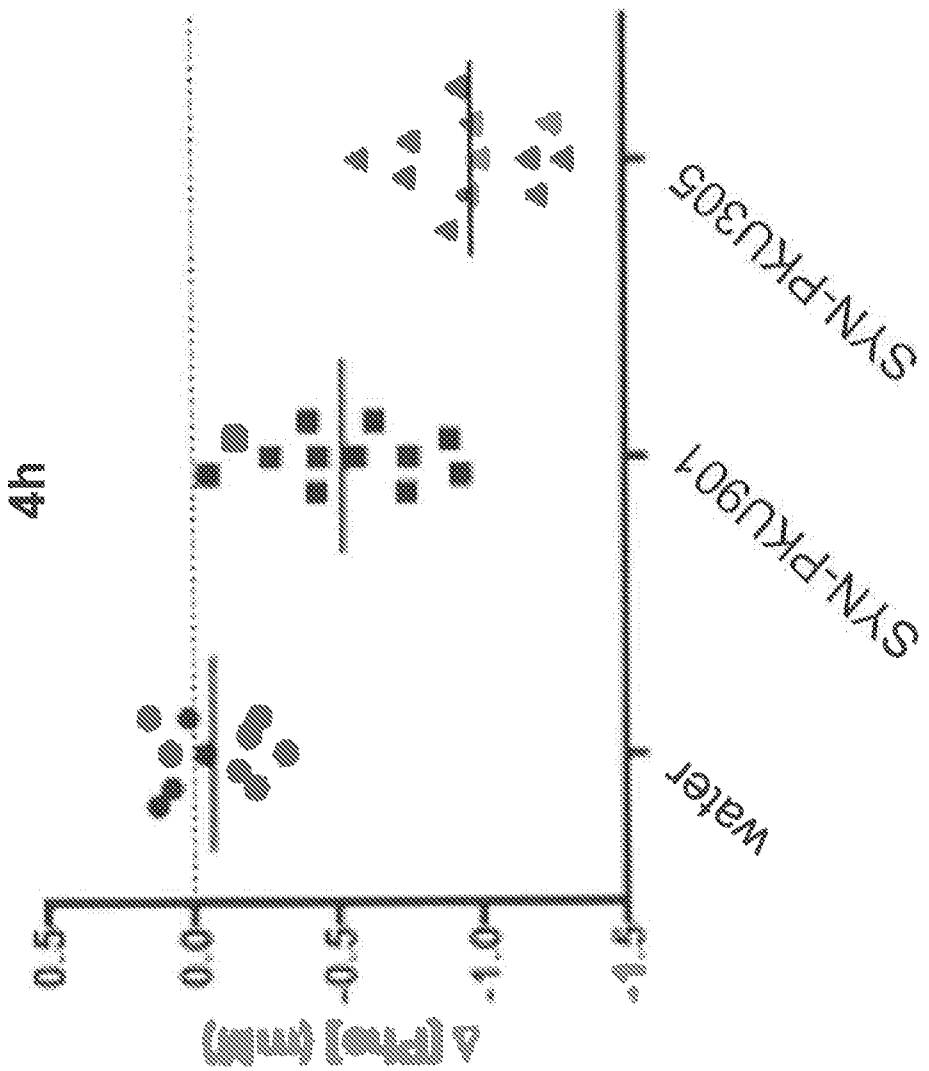


Fig. 41B

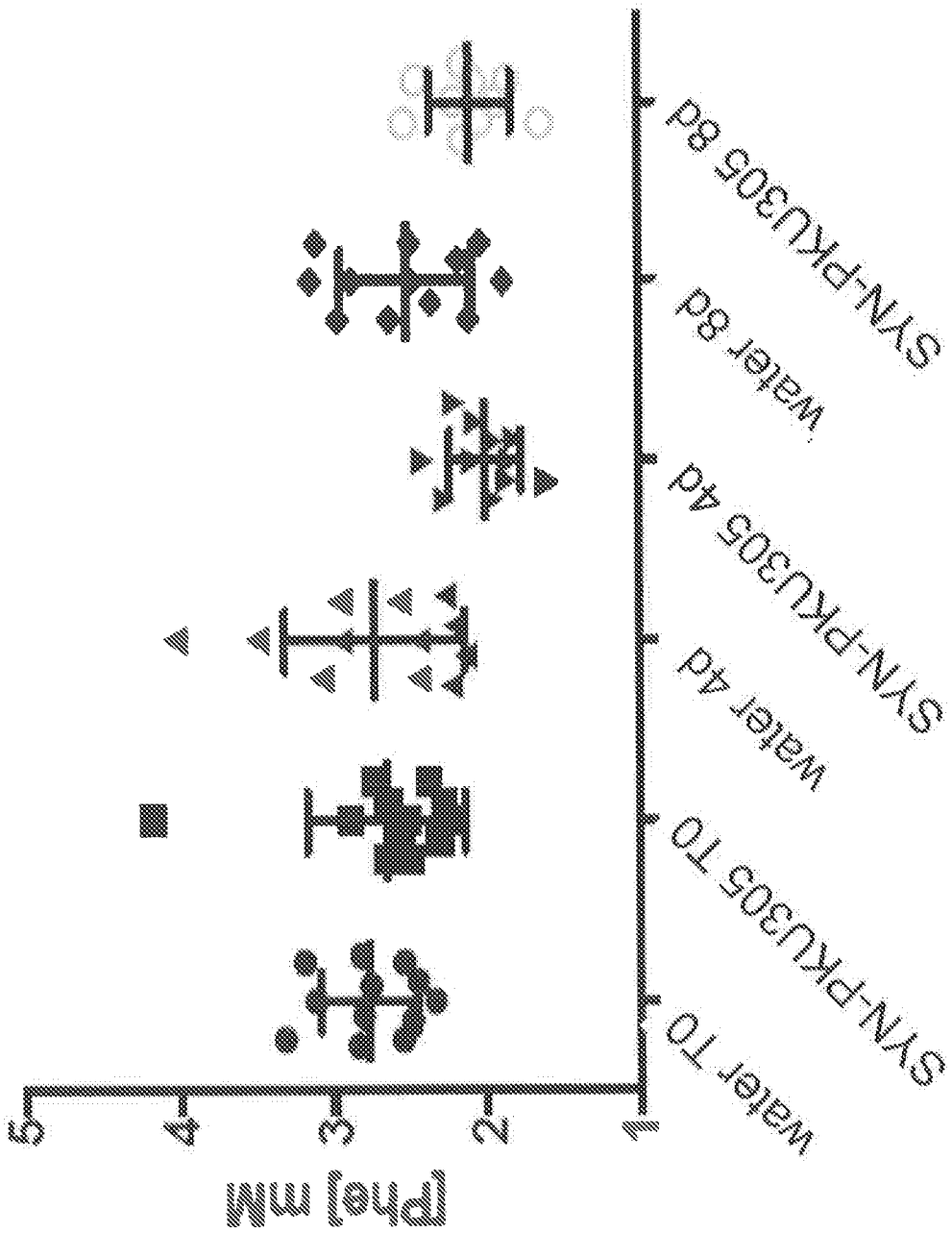

[Phe] mM
5
4
3
2
1
Water TO
SYN-PKU305 TO
Water 4d
SYN-PKU305 4d
Water 8d
SYN-PKU305 8d

Fig. 42A

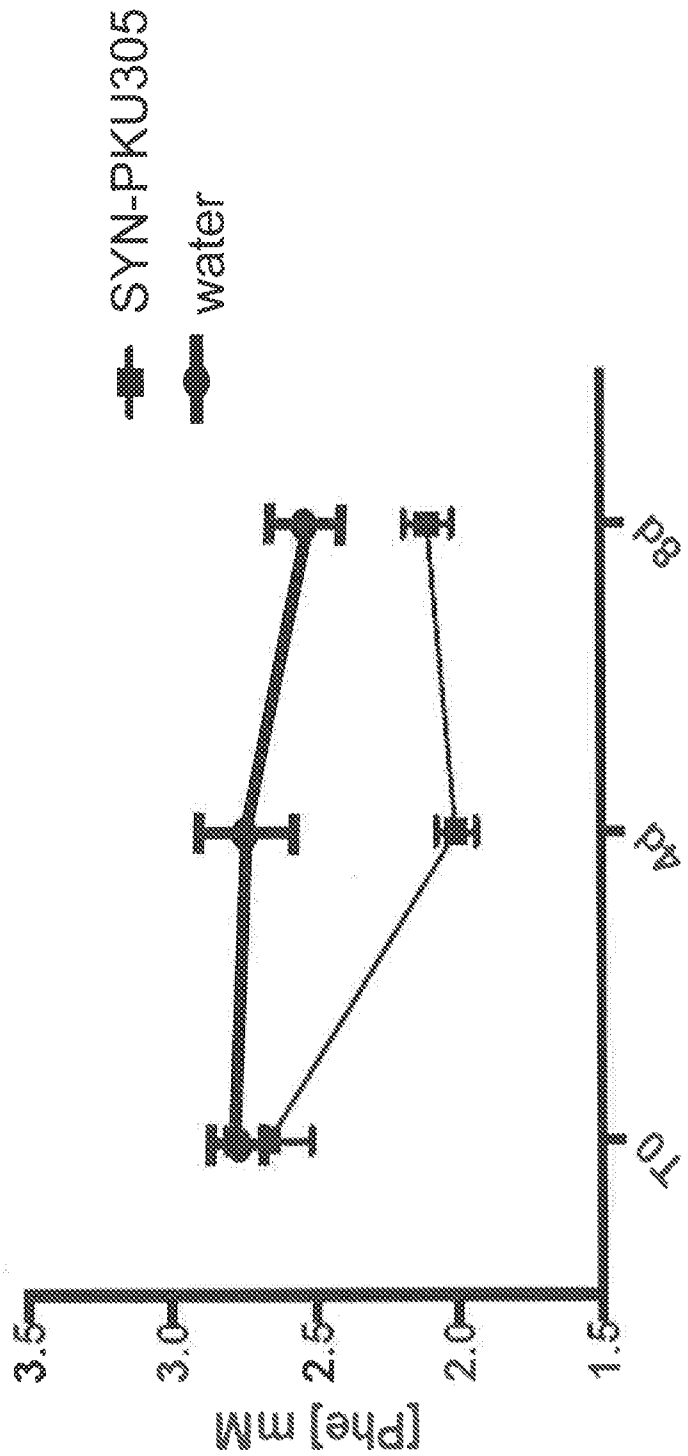


Fig. 42B

Genotype	PAL activity (umol/min)	Decrease in Δ[phe] %
5XPAL; 1XpheP; ΔthyA	18	0
5XPAL; 1XpheP; ΔthyA	32	12
2XPAL; 1XpheP	40	17
3XPAL; 2XpheP; 1XLAAD	160	31
pSC101-PAL3; 1XpheP	81	45
3XPAL; 2XpheP; 1XLAAD	150	50
3XPAL; 2XpheP; 1XLAAD	176	63
3XPAL; 2XpheP; 1XLAAD; ΔthyA	204	16
3XPAL; 2XpheP; 1XLAAD; ΔthyA	83	20
3XPAL; 2XpheP; 1XLAAD; ΔtdcA	227	41
3XPAL; 2XpheP; 1XLAAD; fnrS-LAAD	91	24
3XPAL; 2XpheP; 1XPara-fnr S24Y	506	47
3XPAL; 2XpheP; 1XPara-fnr S24Y	269	49

Fig. 43A

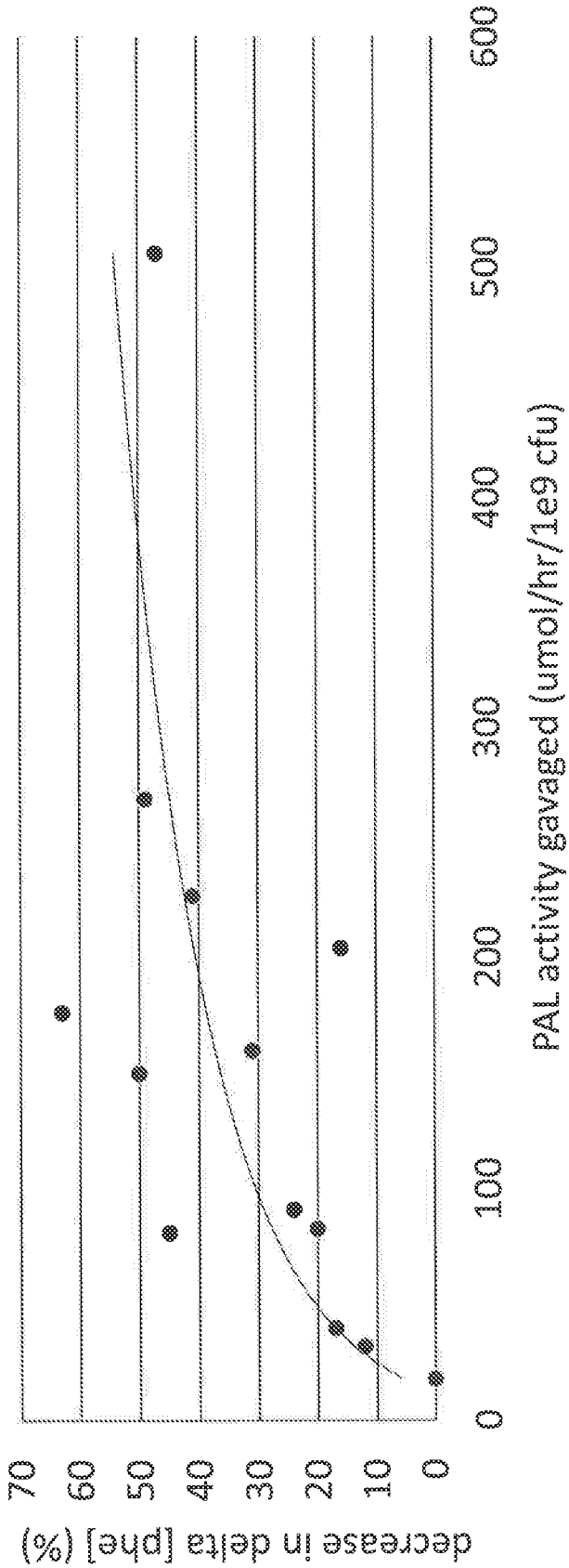

decrease in delta [phe] (%)
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
PAL activity gavaged (umol/hr/1e9 cfu)

Fig. 43B

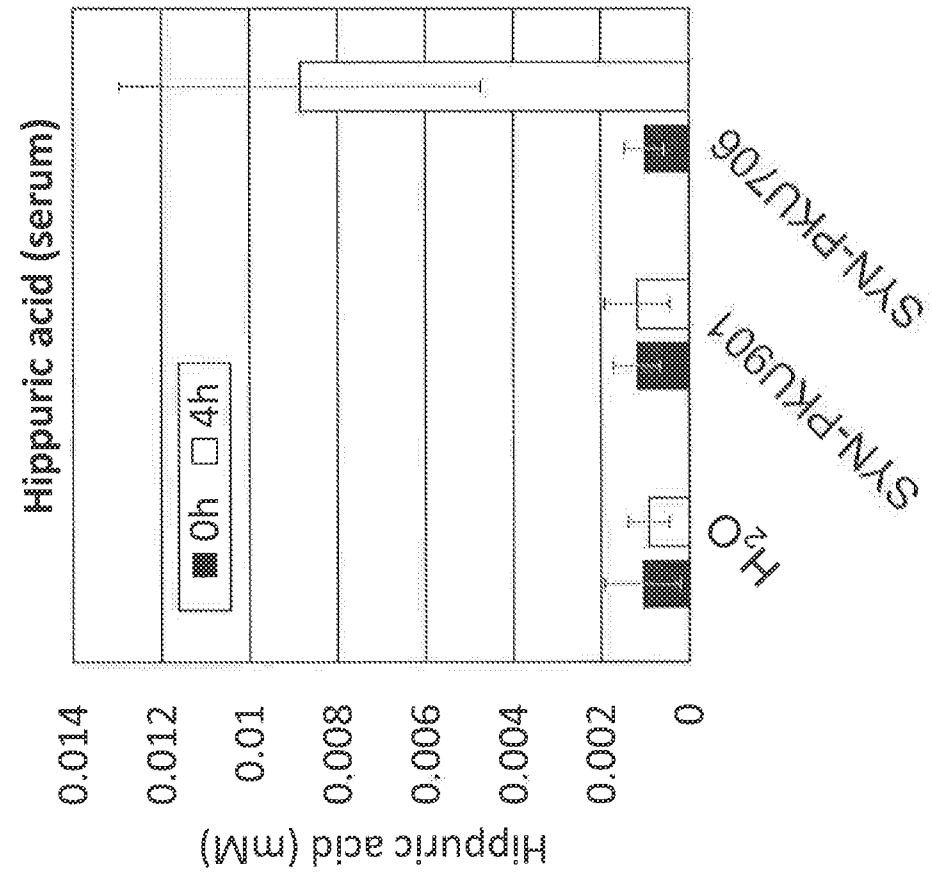


Fig. 44B

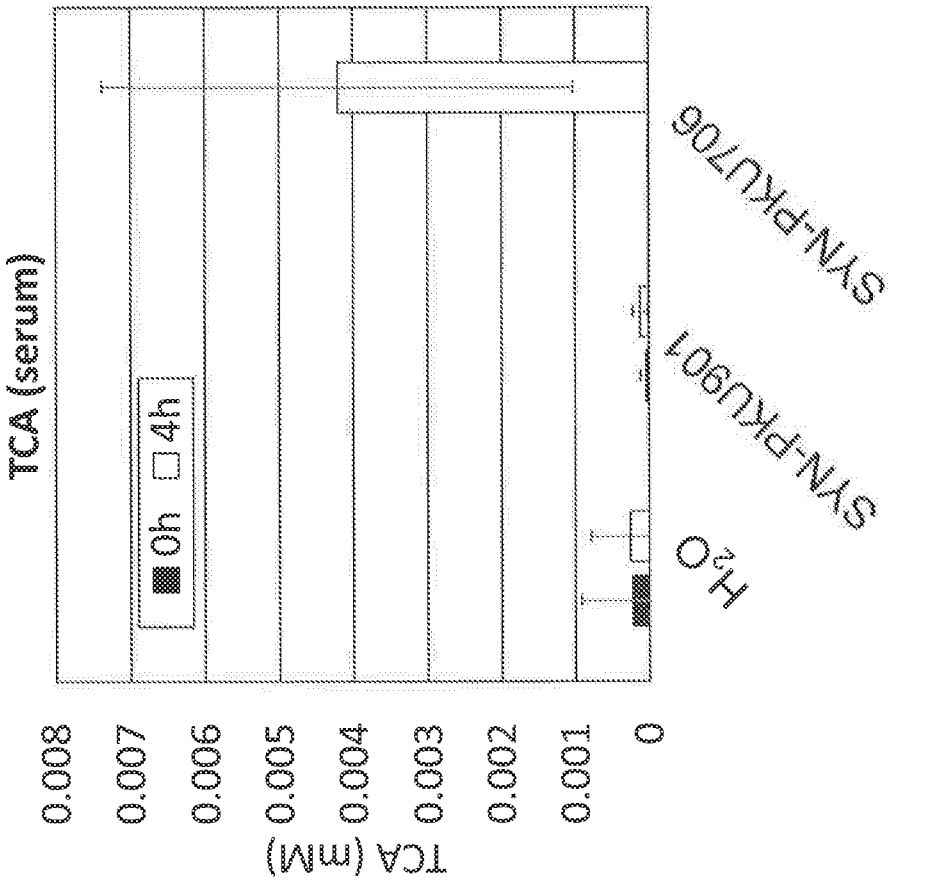


Fig. 44A

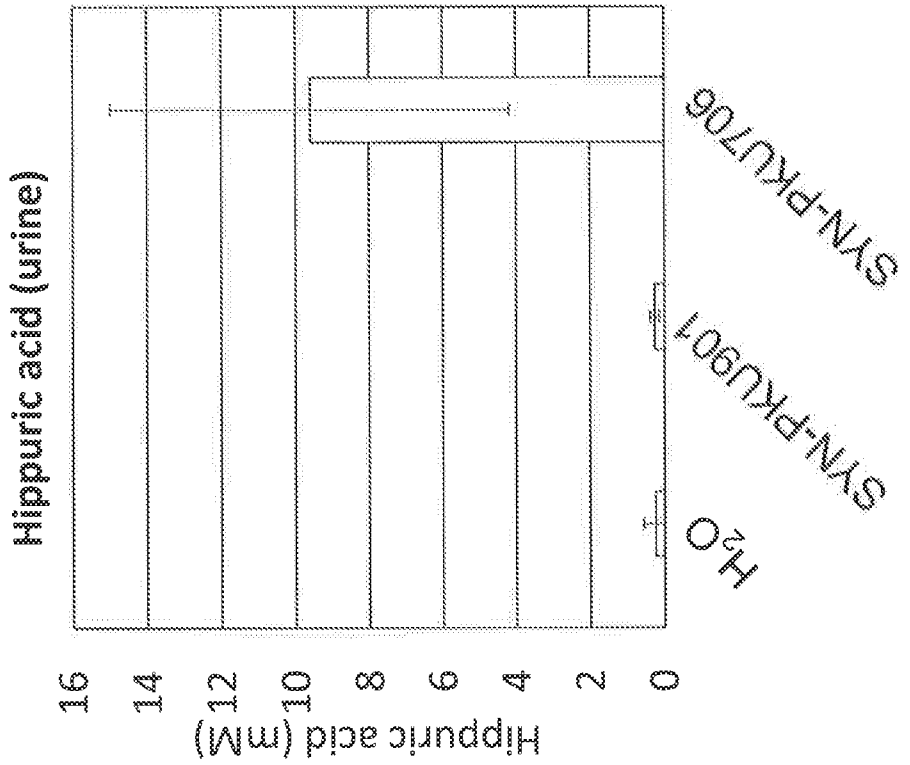


Fig. 44D

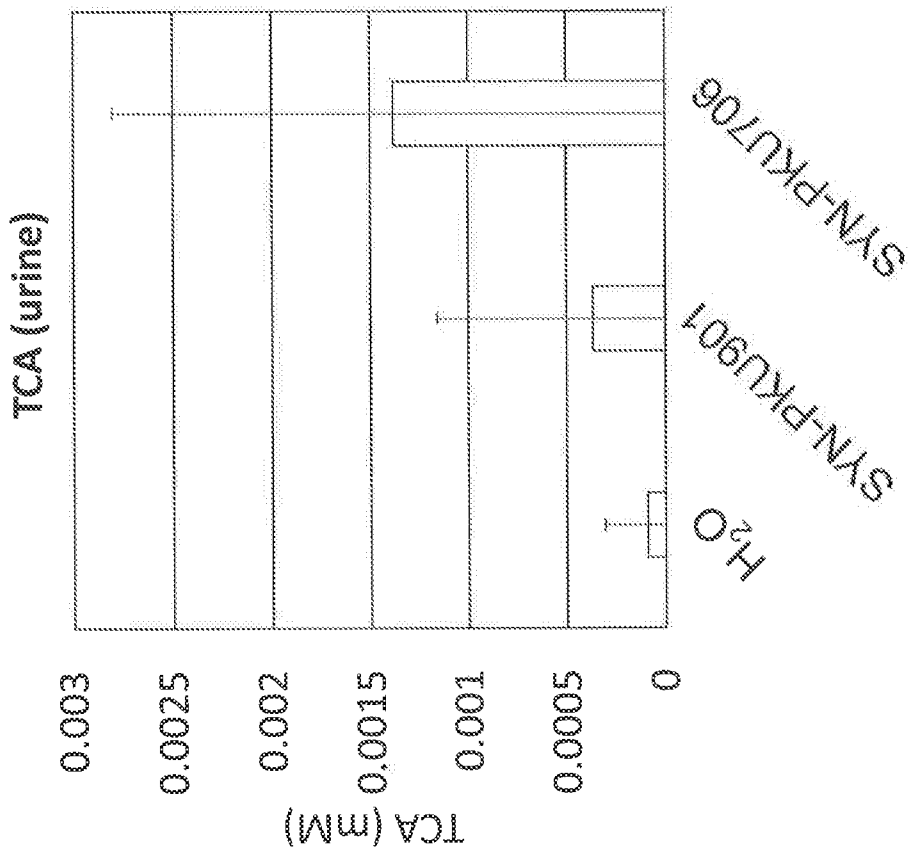


Fig. 44C

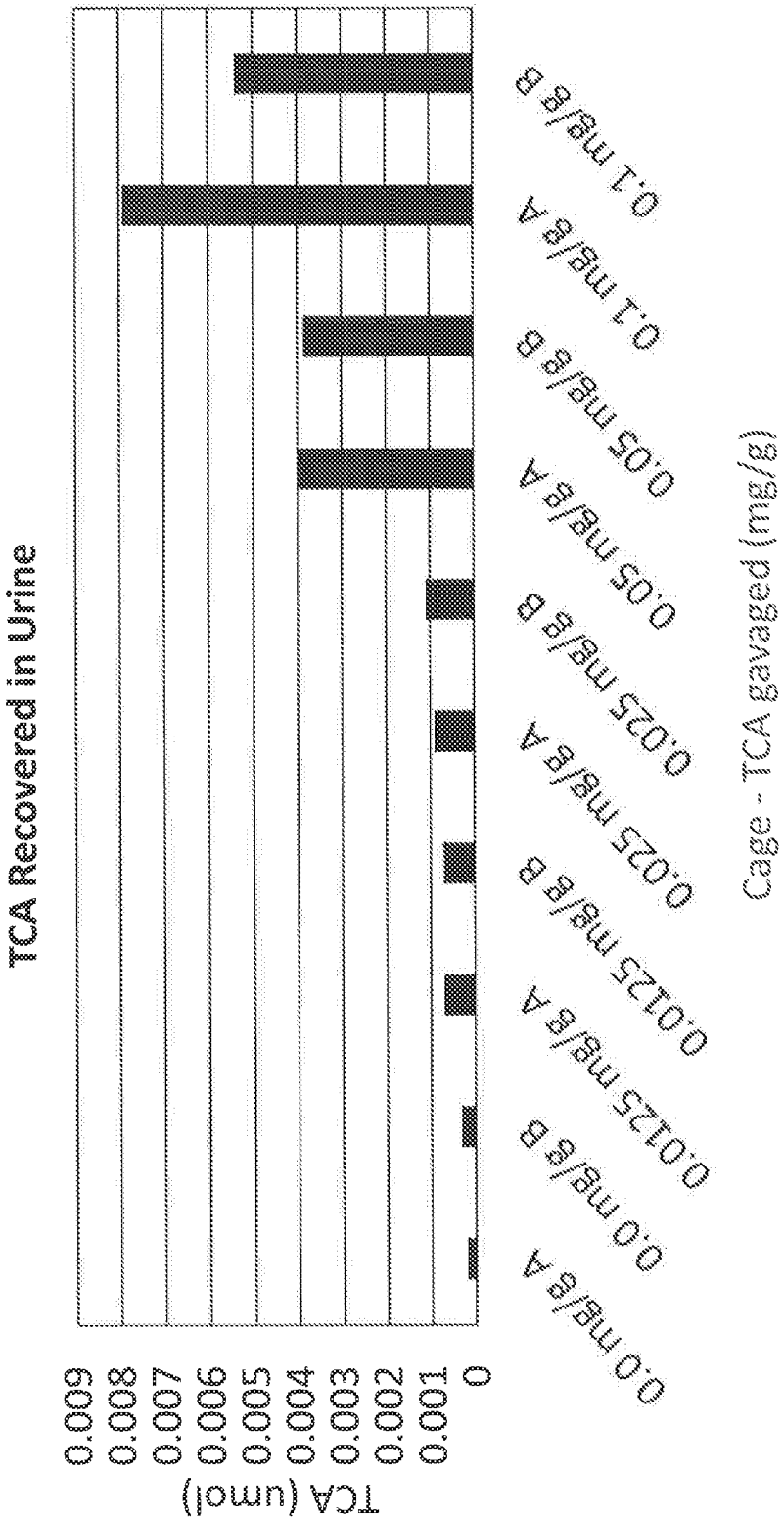

TCA Recovered in Urine
TCA (umol)
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
0.0 mg/g A
0.0 mg/g B
0.0125 mg/g A
0.0125 mg/g B
0.025 mg/g A
0.025 mg/g B
0.05 mg/g A
0.05 mg/g B
0.1 mg/g A
0.1 mg/g B
Case - TCA gavaged (mg/g)

Fig. 45A

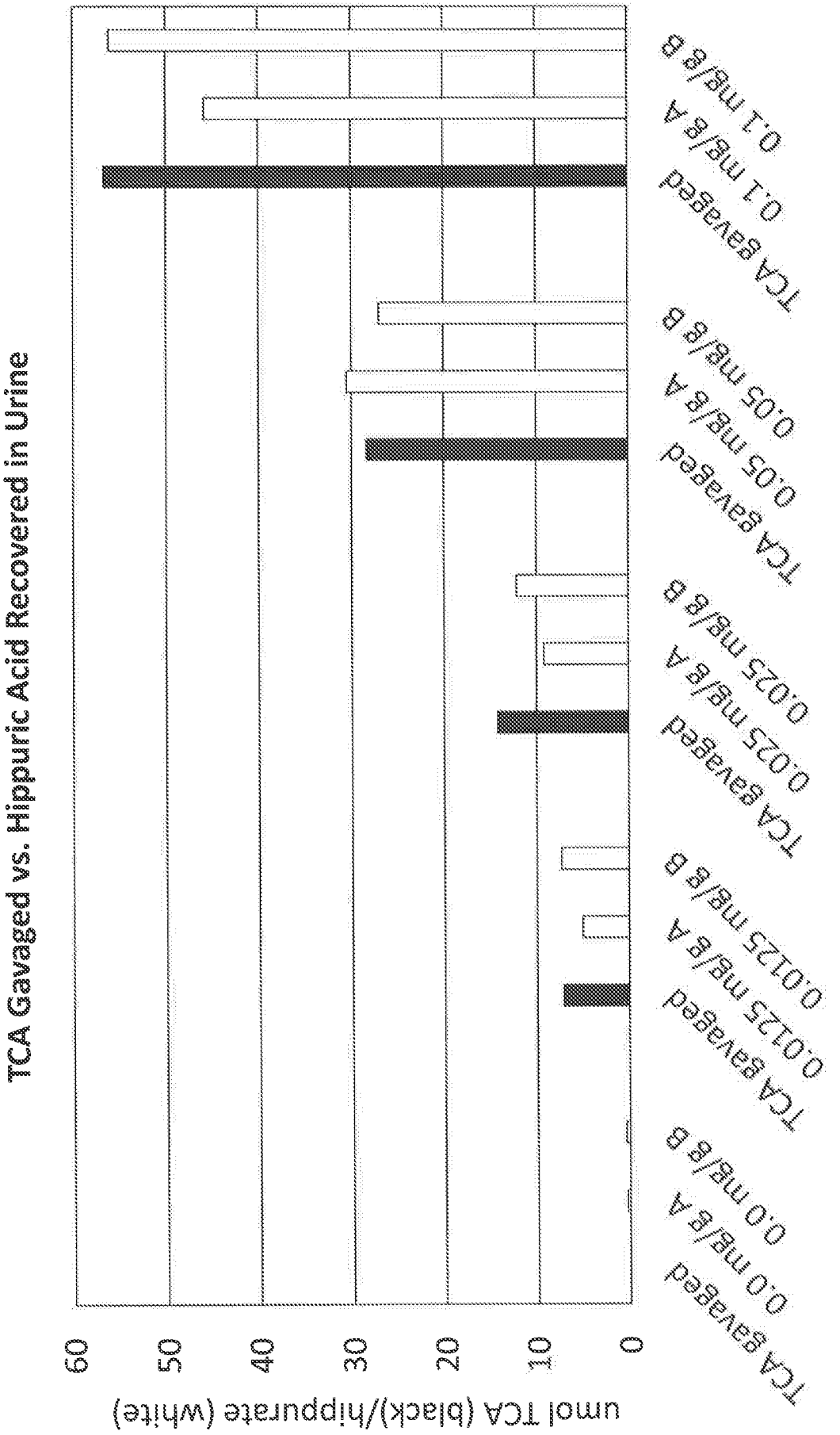

TCA Gavaged vs. Hippuric Acid Recovered in Urine
umol TCA (black)/hippurate (white)
60
50
40
30
20
10
0
TCA gavaged 0.0 mg/g A
0.0 mg/g B
TCA gavaged 0.0125 mg/g A
0.0125 mg/g B
TCA gavaged 0.025 mg/g A
0.025 mg/g B
TCA gavaged 0.05 mg/g A
0.05 mg/g B
TCA gavaged 0.1 mg/g A
0.1 mg/g B

Fig. 45B

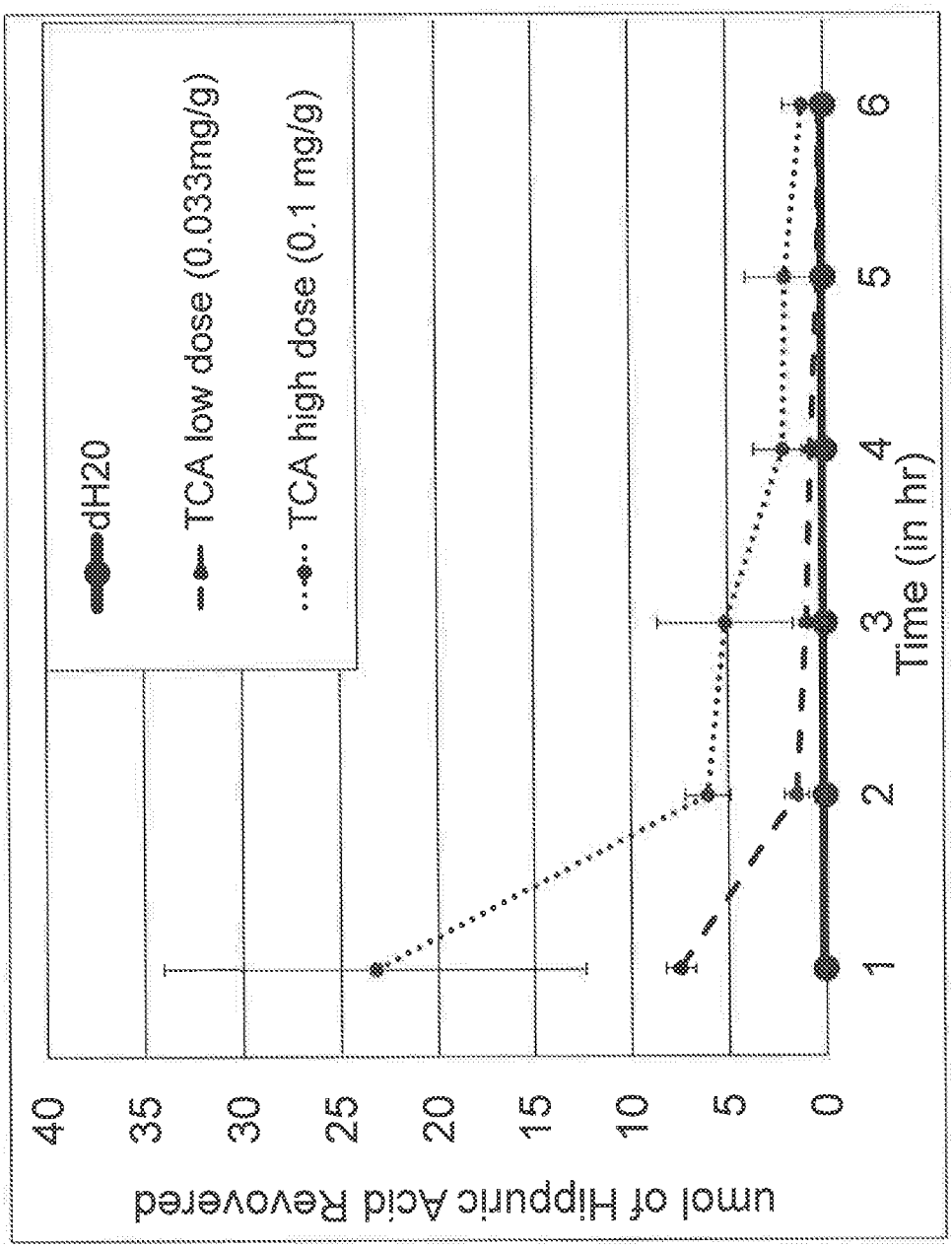

umol of Hippuric Acid Revovered
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
Time (in hr)
dH20
TCA low dose (0.033mg/g)
TCA high dose (0.1 mg/g)

Fig. 46

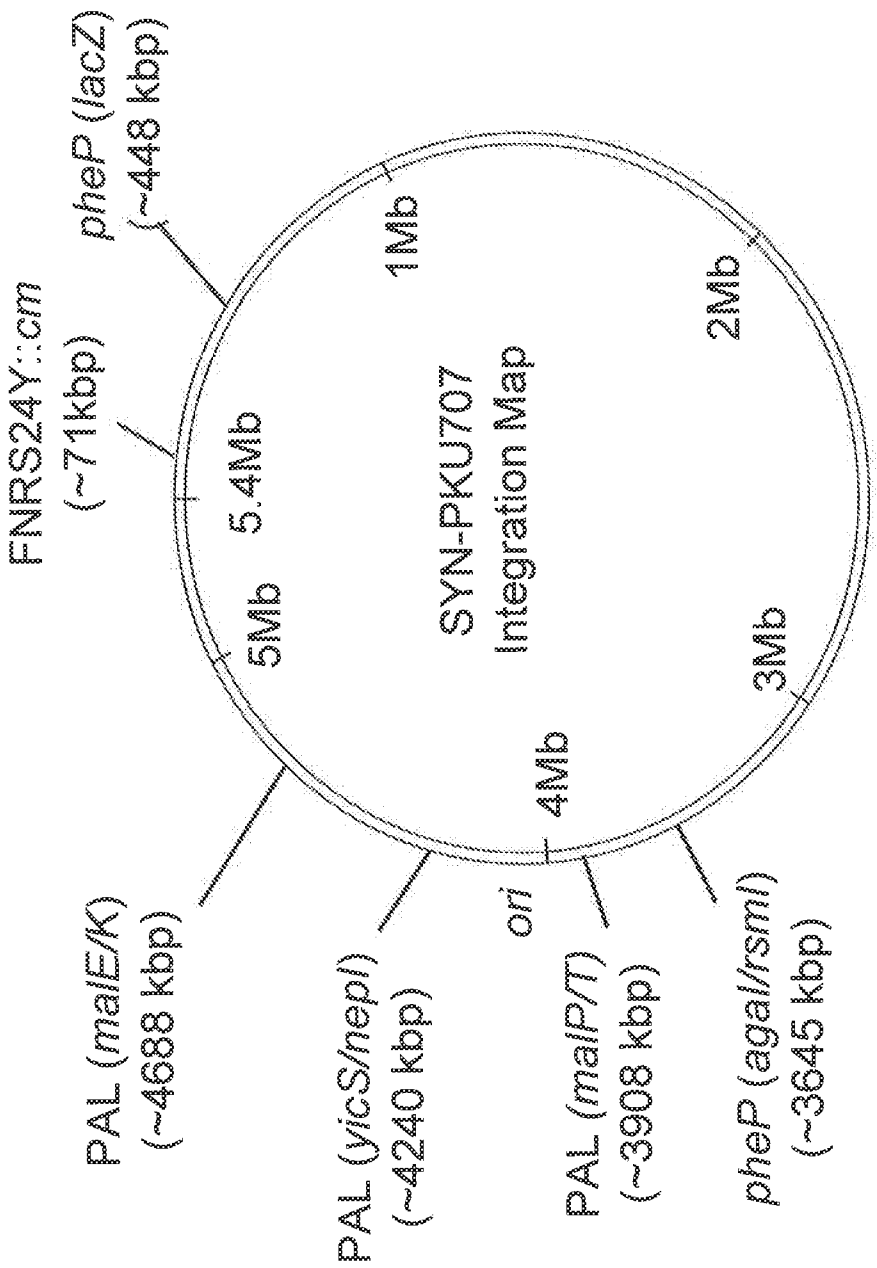

FNRS24Y::cm
(~71kbp)
pheP (lacZ)
(~448 kbp)
1Mb
2Mb
5.4Mb
5Mb
SYN-PKU707
Integration Map
3Mb
4Mb
ori
PAL (malE/K)
(~4688 kbp)
PAL (yicS/nepI)
(~4240 kbp)
PAL (malP/T)
(~3908 kbp)
pheP (agaI/rsmI)
(~3645 kbp)

Fig. 47A

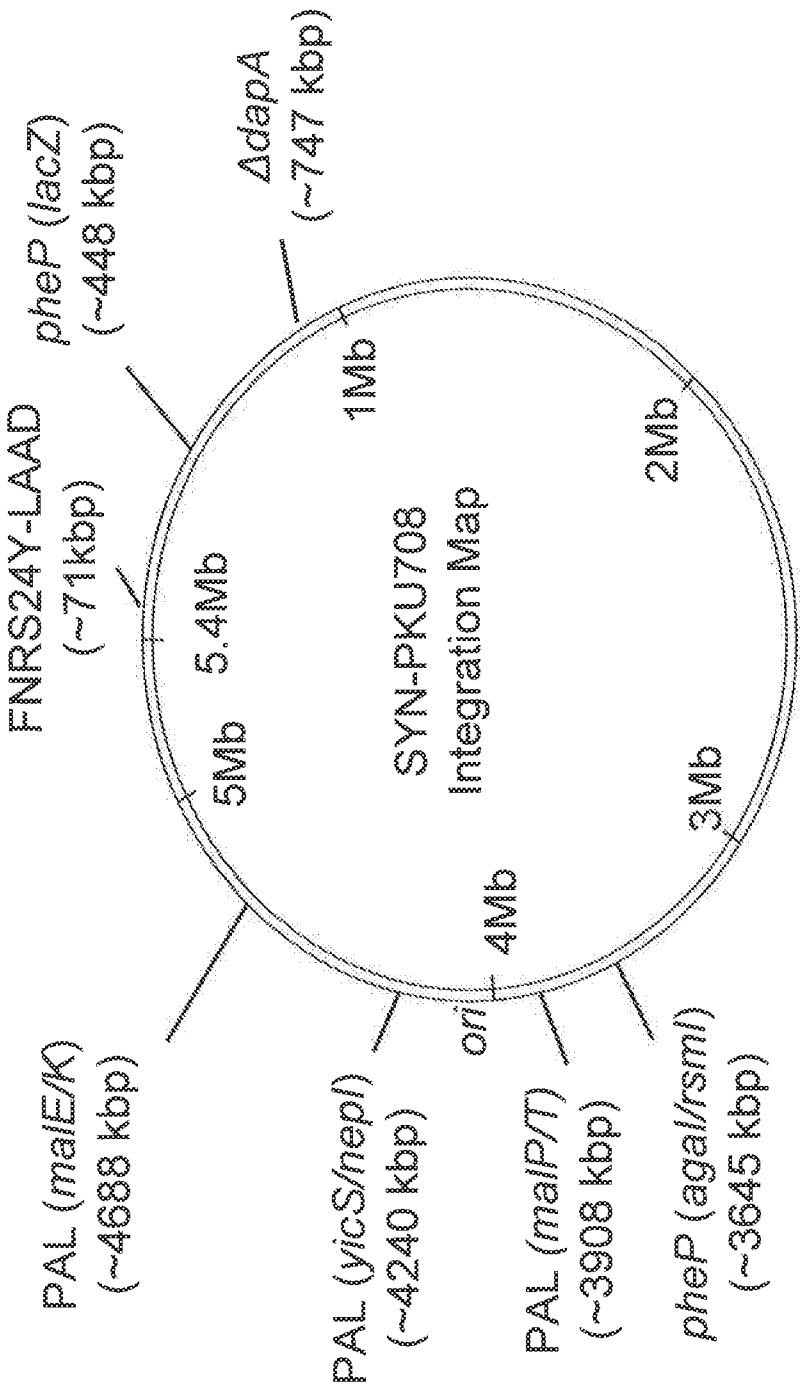

pheP (lacZ)
(~448 kbp)
ΔdapA
(~747 kbp)
FNRS24Y-LAAD
(~71kbp)
1Mb
2Mb
5.4Mb
5Mb
SYN-PKU708
Integration Map
3Mb
4Mb
ori
PAL (malE/K)
(~4688 kbp)
PAL (yicS/nepI)
(~4240 kbp)
PAL (malP/T)
(~3908 kbp)
pheP (agaI/rsmI)
(~3645 kbp)

Fig. 47B

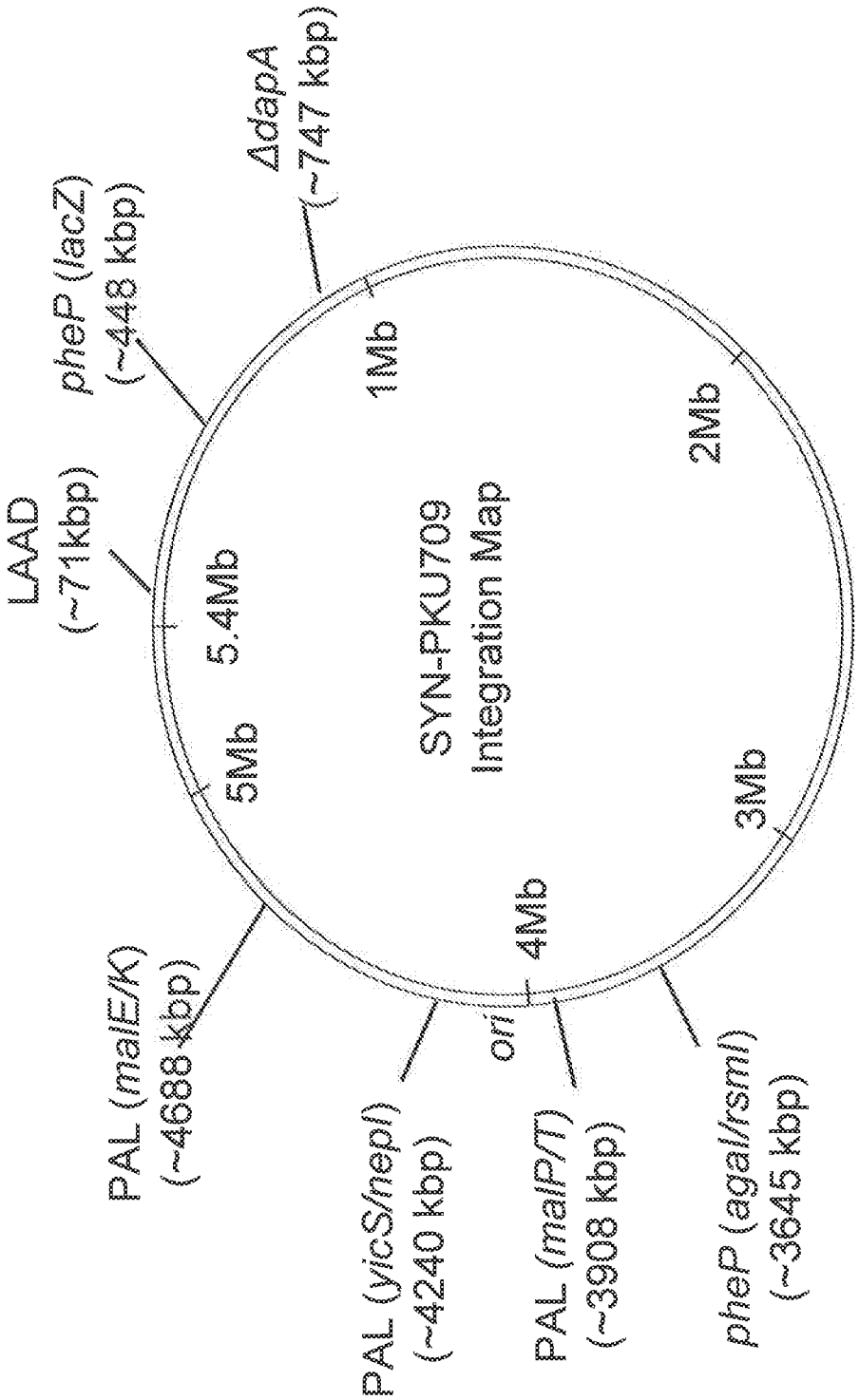

ΔdapA
(~747 kbp)
pheP (lacZ)
(~448 kbp)
LAAD
(~71kbp)
PAL (malE/K)
(~4688 kbp)
PAL (yicS/nepI)
(~4240 kbp)
PAL (malP/T)
(~3908 kbp)
pheP (agaI/rsmI)
(~3645 kbp)
1Mb
2Mb
3Mb
4Mb
5Mb
5.4Mb
ori
SYN-PKU709
Integration Map

Fig. 47C

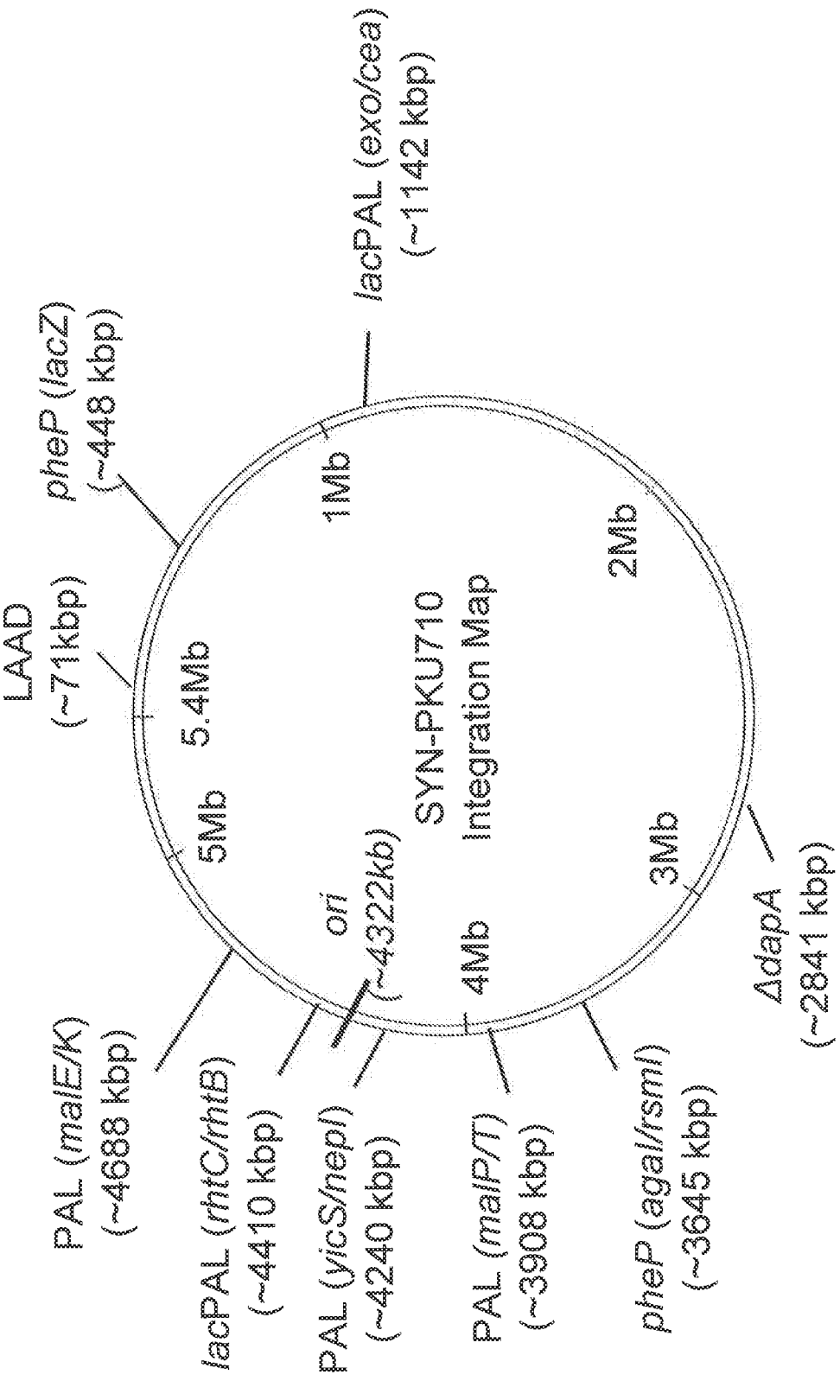

SYN-PKU710
Integration Map
1Mb
2Mb
3Mb
4Mb
5Mb
5.4Mb
ori
(~4322kb)
lacPAL (exo/cea)
(~1142 kbp)
pheP (lacZ)
(~448 kbp)
LAAD
(~71kbp)
PAL (malE/K)
(~4688 kbp)
lacPAL (rhtC/rhtB)
(~4410 kbp)
PAL (ycjS/nepI)
(~4240 kbp)
PAL (malP/T)
(~3908 kbp)
pheP (agaI/rsmI)
(~3645 kbp)
ΔdapA
(~2841 kbp)

Fig. 47D

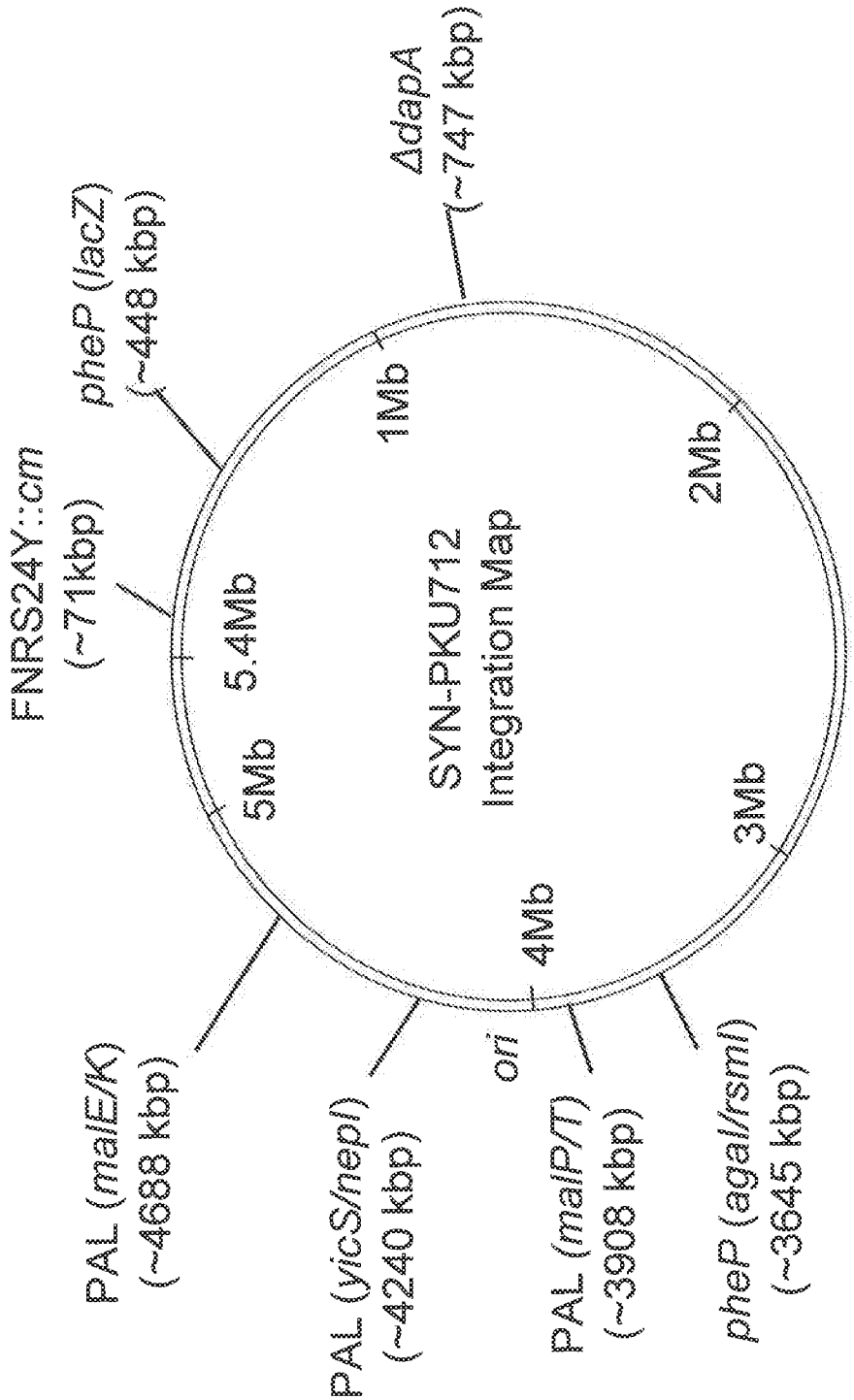

ΔdapA
(~747 kbp)
pheP (lacZ)
(~448 kbp)
FNRS24Y::cm
(~71kbp)
1Mb
2Mb
5.4Mb
5Mb
SYN-PKU712
Integration Map
3Mb
4Mb
ori
PAL (malE/K)
(~4688 kbp)
PAL (vicS/nepI)
(~4240 kbp)
PAL (malP/T)
(~3908 kbp)
pheP (agaI/rsmI)
(~3645 kbp)

Fig. 47E

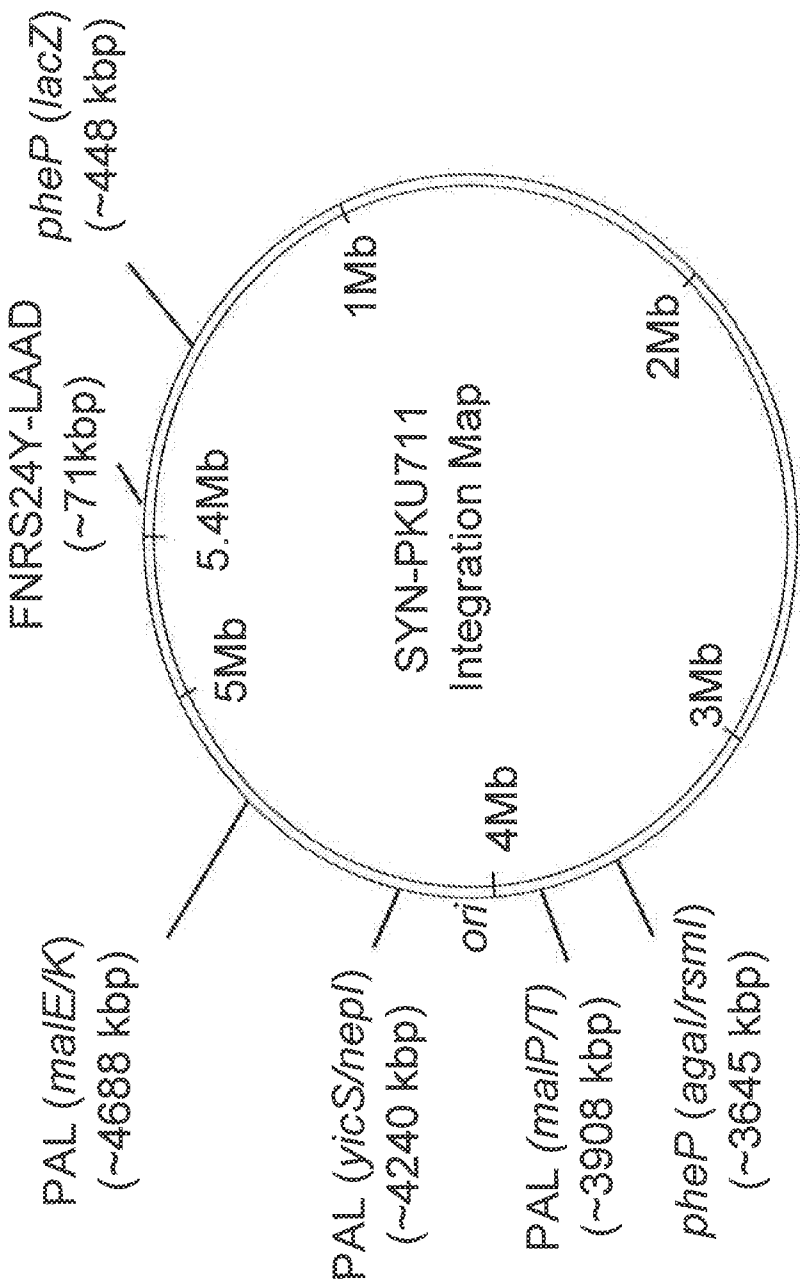

pheP (lacZ)
(~448 kbp)
FNRS24Y-LAAD
(~71kbp)
1Mb
2Mb
5.4Mb
5Mb
SYN-PKU711
Integration Map
3Mb
4Mb
ori
PAL (malE/K)
(~4688 kbp)
PAL (yicS/nepI)
(~4240 kbp)
PAL (malP/T)
(~3908 kbp)
pheP (agaI/rsmI)
(~3645 kbp)

Fig. 47F

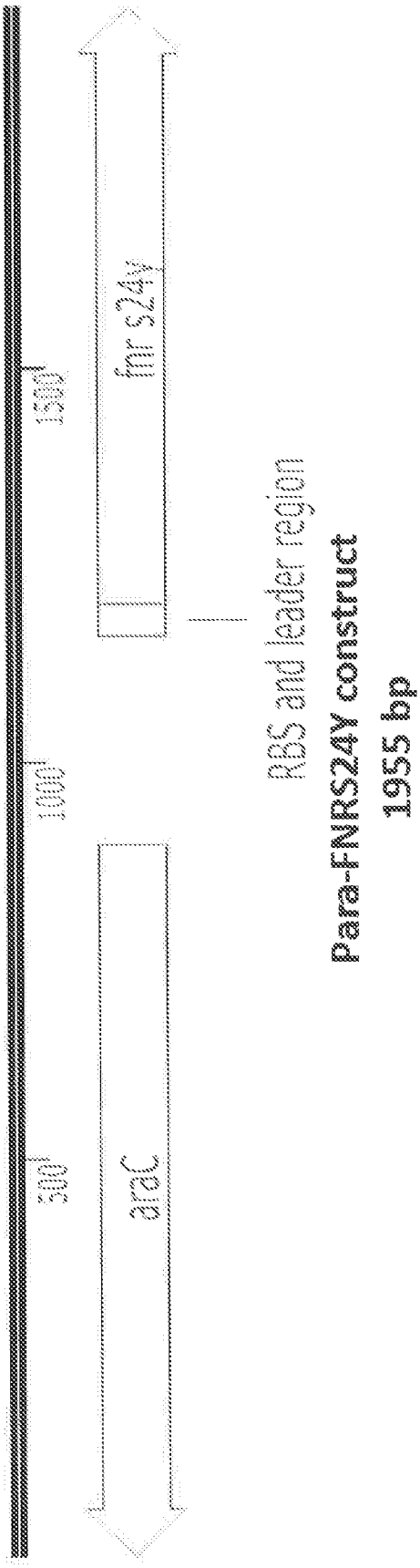

1500
1000
300
fnr s24y
araC
RBS and leader region
Para-FNRS24Y construct
1955 bp

Fig. 48A

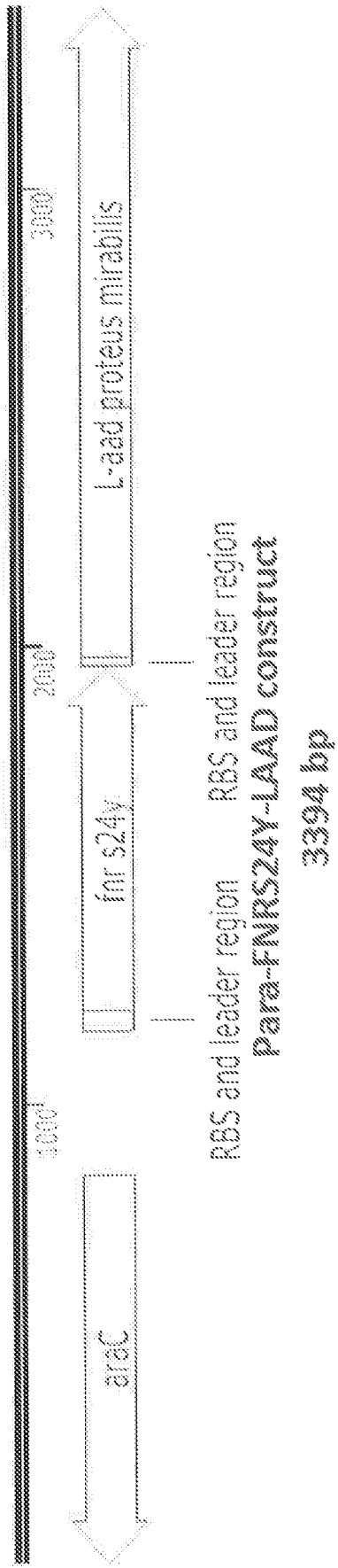
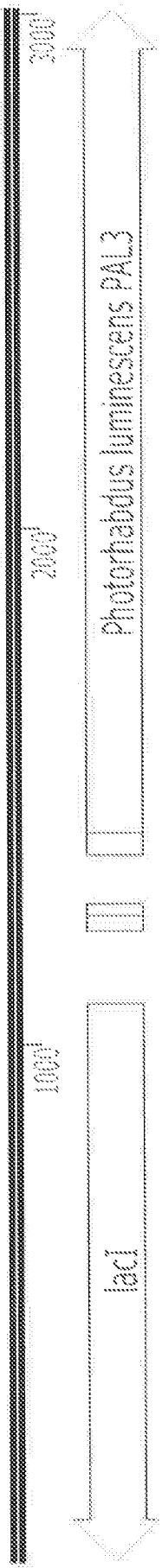

araC
fnr s24y
L-aad proteus mirabilis
1000
2000
3000
RBS and leader region
RBS and leader region
Para-FNRS24Y-LAAD construct
3394 bp

Fig. 48B



Lac-PAL construct
3008 bp

Fig. 49

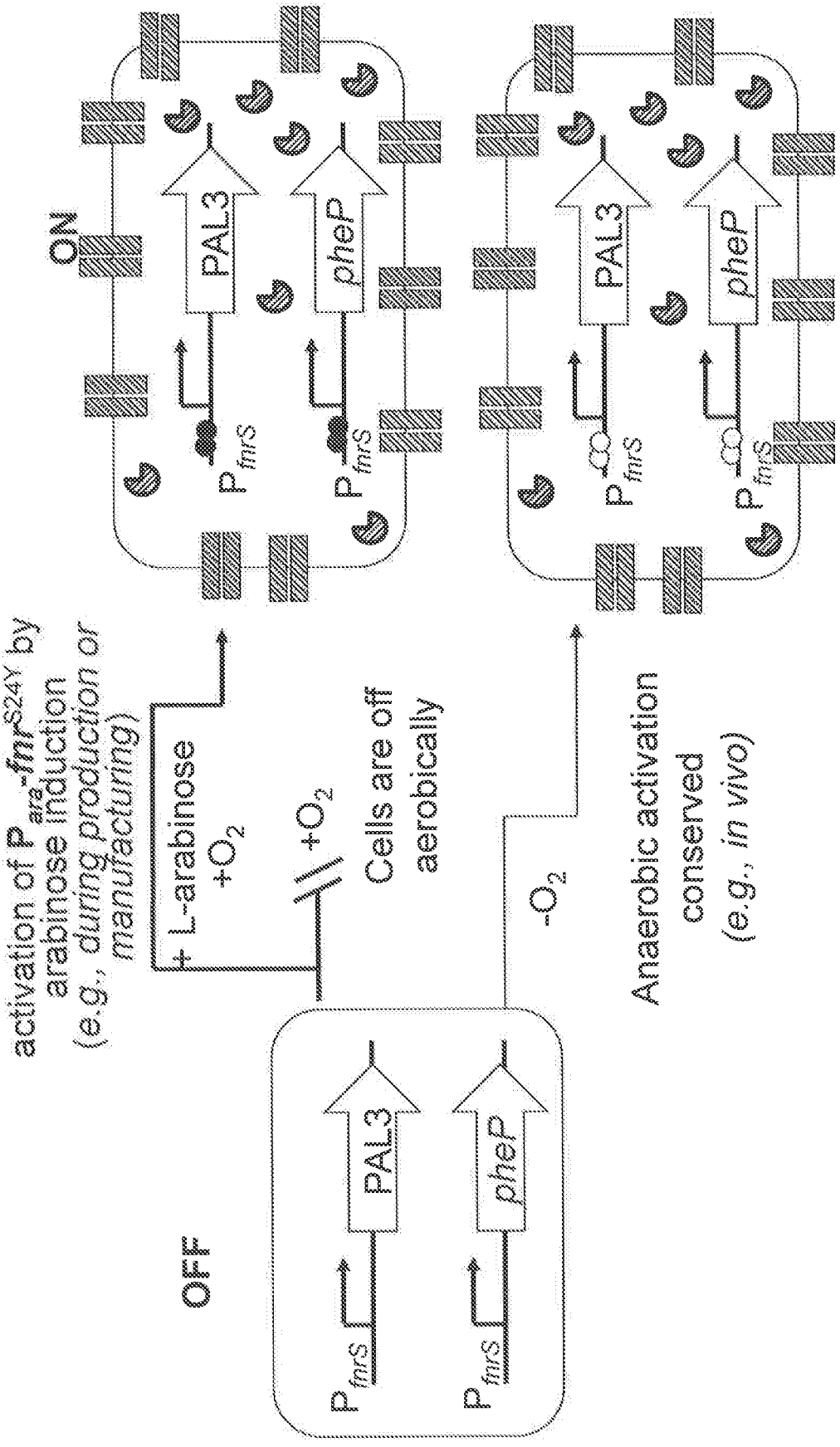

ON
PAL3
pheP
P_{fnrS}
P_{fnrS}
PAL3
pheP
P_{fnrS}
P_{fnrS}
activation of P_{ara}-fnr^{S24Y} by arabinose induction (e.g., during production or manufacturing)
+ L-arabinose
$+O_2$
$+O_2$
Cells are off aerobically
$-O_2$
Anaerobic activation conserved (e.g., in vivo)
OFF
PAL3
pheP
P_{fnrS}
P_{fnrS}

Fig. 50

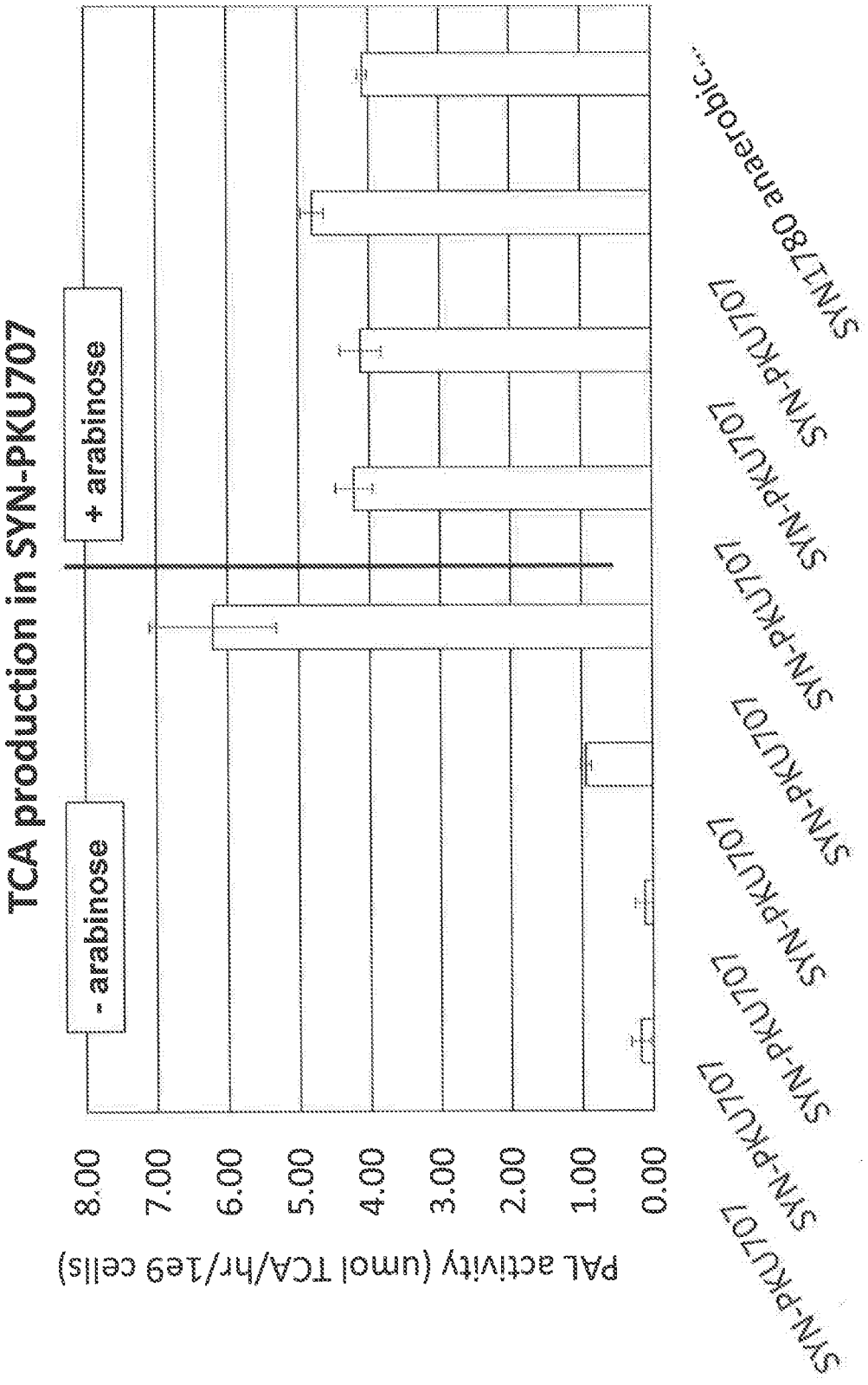

TCA production in SYN-PKU707
+ arabinose
- arabinose
PAL activity (umol TCA/hr/1e9 cells)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
SYN-PKU707
SYN-PKU707
SYN-PKU707
SYN-PKU707
SYN-PKU707
SYN-PKU707
SYN-PKU707
SYN1780 anaerobic...

Fig. 51

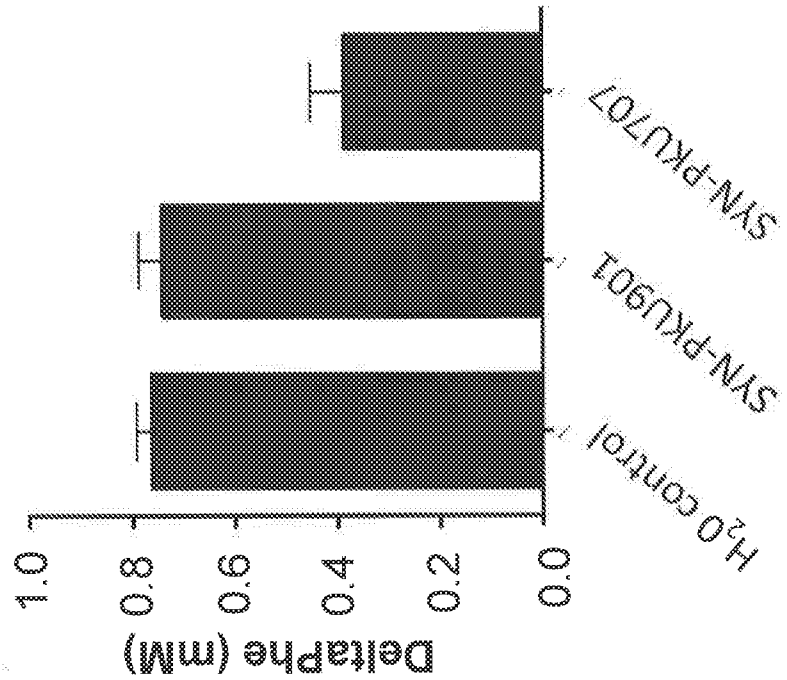

DeltaPhe (mM)
1.0
0.8
0.6
0.4
0.2
0.0
H2O control
SYN-PKU901
SYN-PKU707

Fig. 52

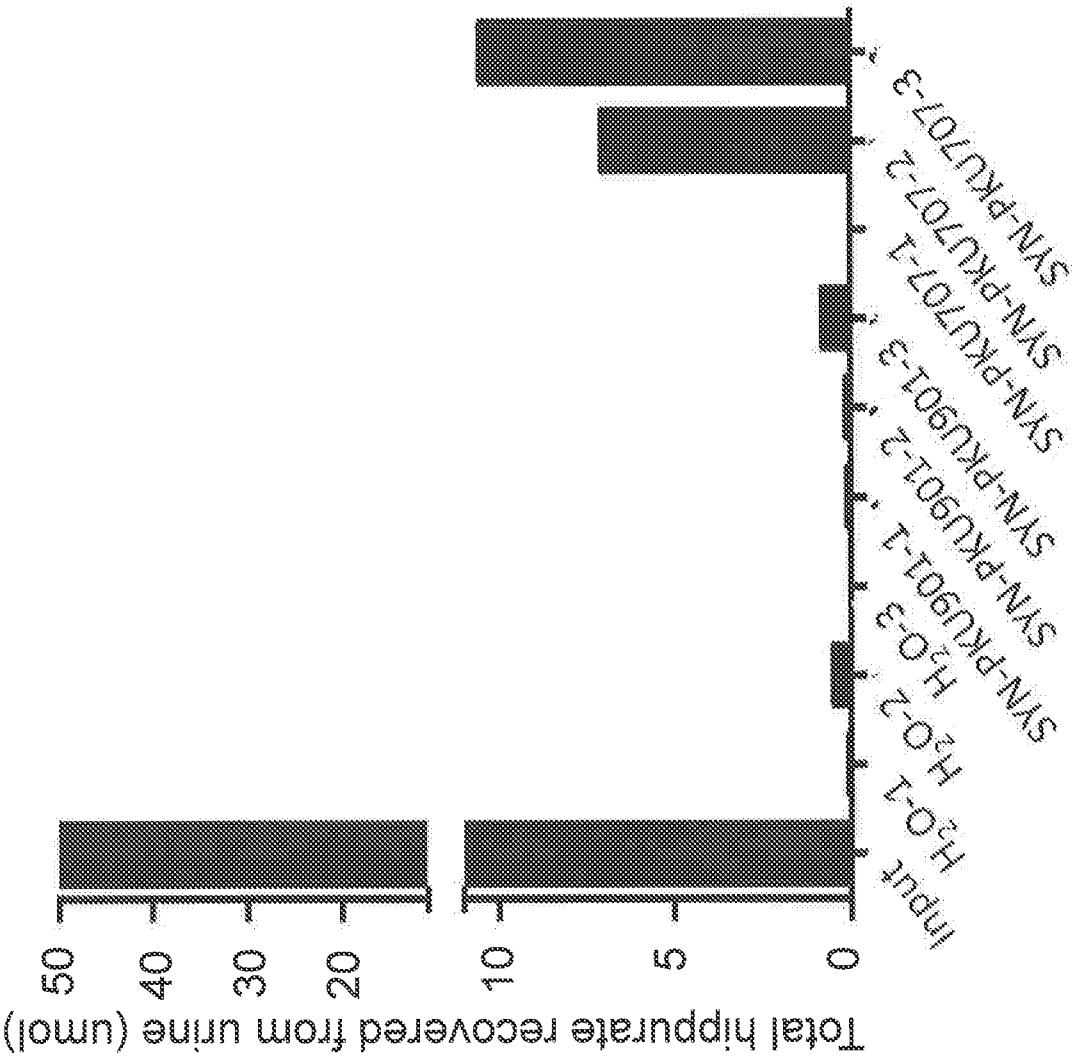

Total hippurate recovered from urine (umol)
50
40
30
20
10
5
0
Input
H2O-1
H2O-2
H2O-3
SYN-PKU901-1
SYN-PKU901-2
SYN-PKU901-3
SYN-PKU707-1
SYN-PKU707-2
SYN-PKU707-3

Fig. 53

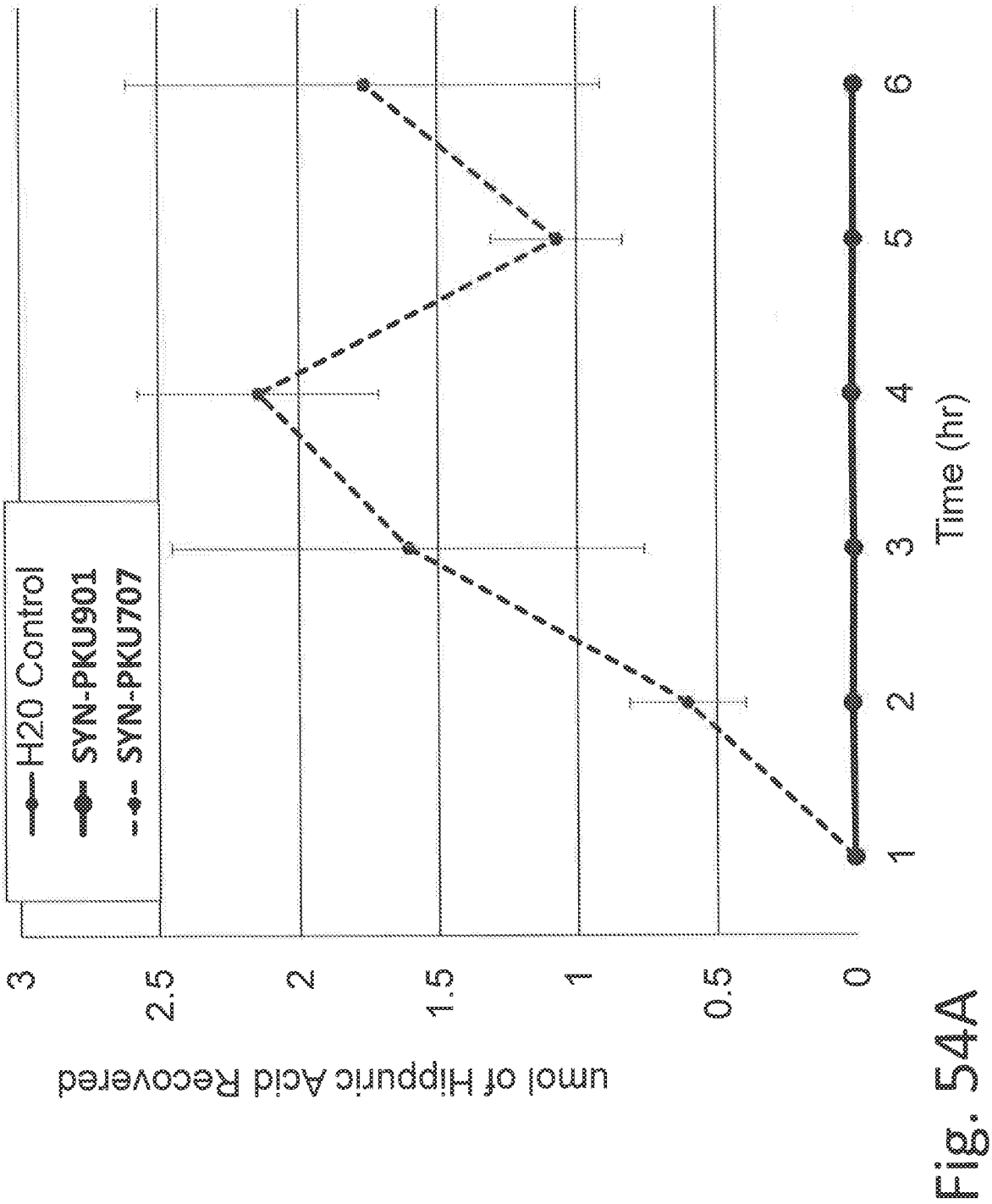

umol of Hippuric Acid Recovered
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
Time (hr)
H20 Control
SYN-PKU901
SYN-PKU707

Fig. 54A

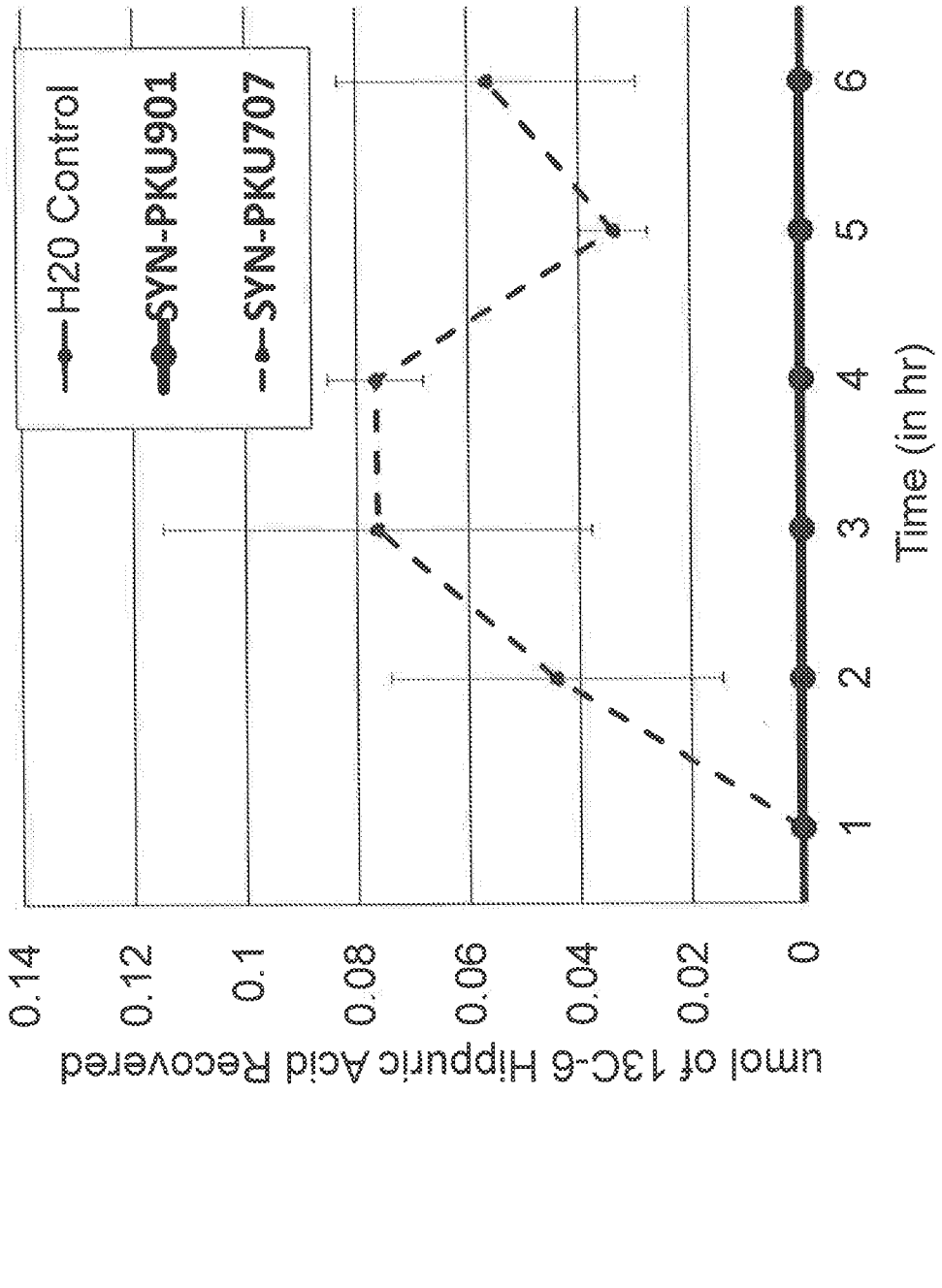

umol of 13C-6 Hippuric Acid Recovered
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6
Time (in hr)
H20 Control
SYN-PKU901
SYN-PKU707
SYN1780 – 3XP$_{fnrS}$-PAL3; 2XP$_{fnrS}$-*pheP*; P$_{ara}$-*fnrS24Y*

Fig. 54B

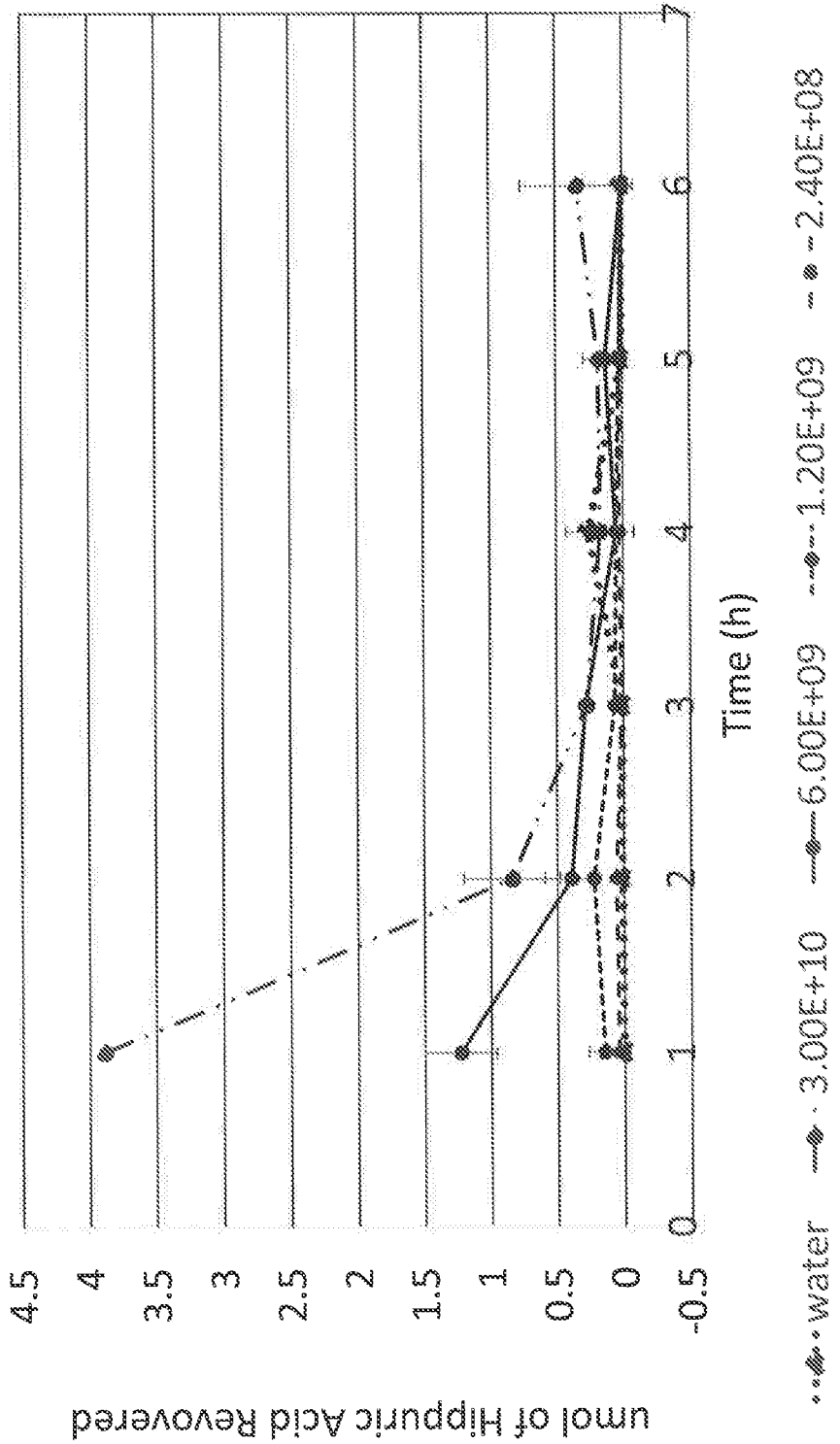

umol of Hippuric Acid Revovered
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
0
1
2
3
4
5
6
7
Time (h)
water
3.00E+10
6.00E+09
1.20E+09
2.40E+08

Fig. 55

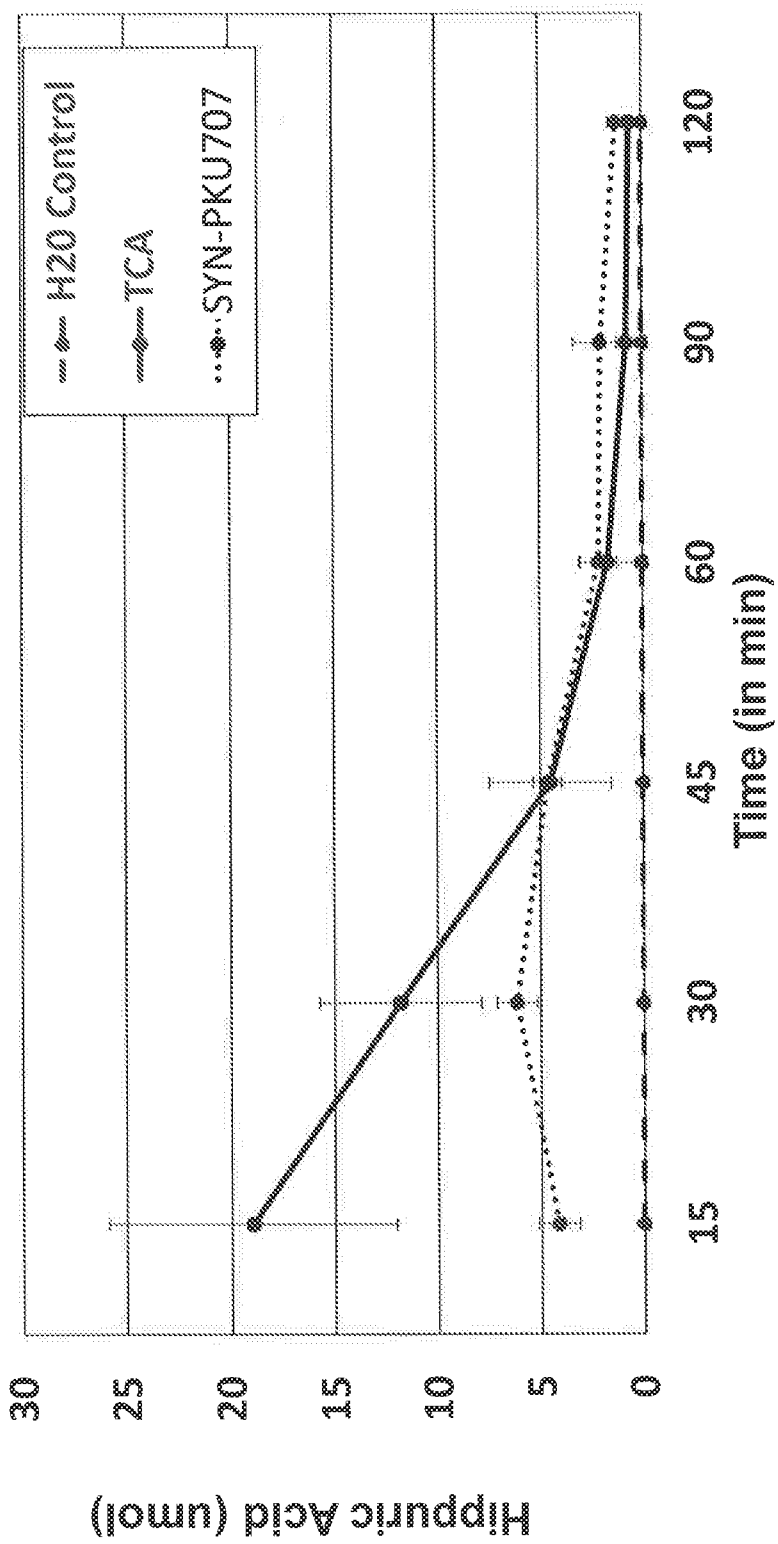
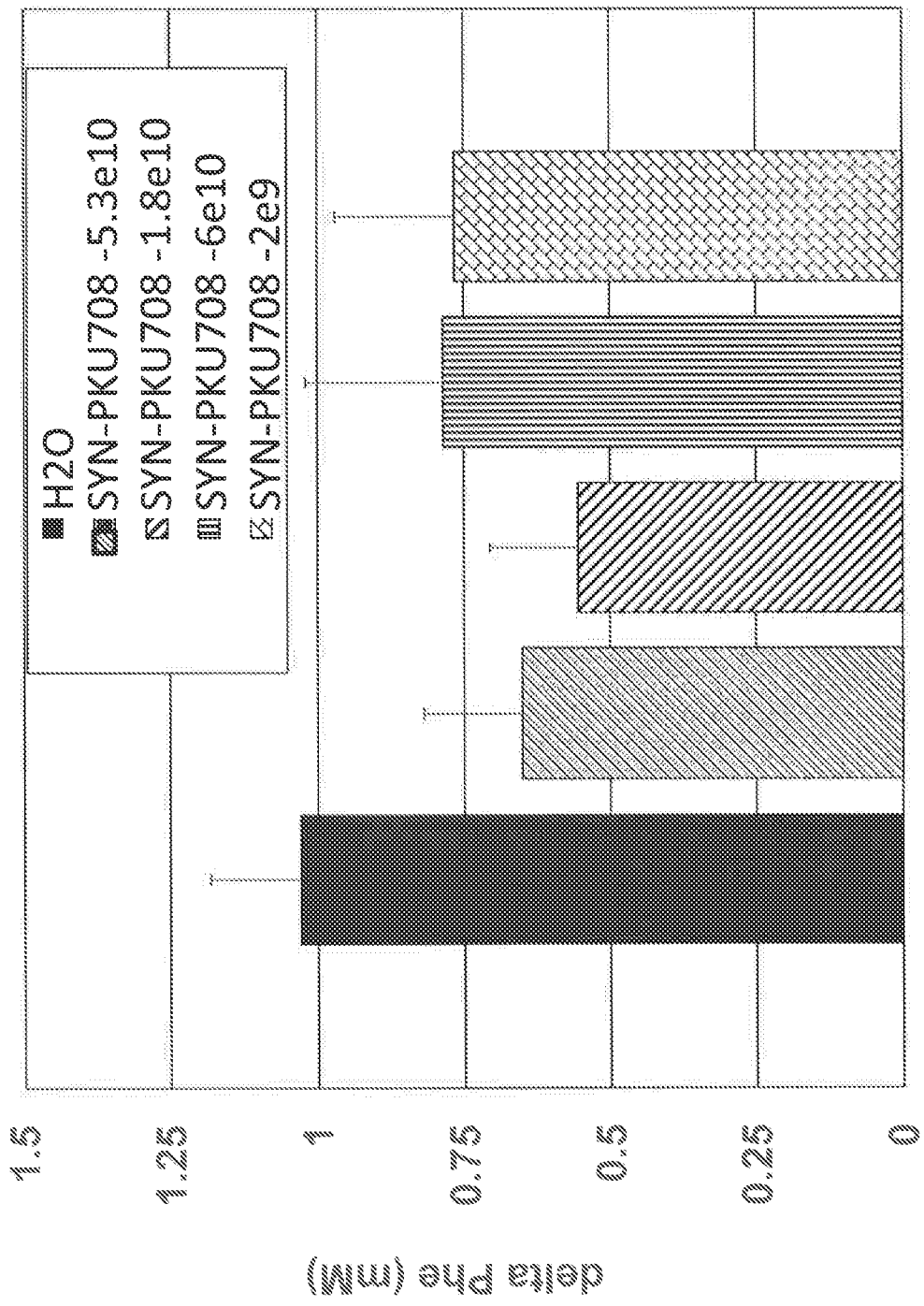

H20 Control
TCA
SYN-PKU707
Hippuric Acid (umol)
30
25
20
15
10
5
0
15
30
45
60
90
120
Time (in min)

Fig. 56



SYN1967 – 3XP$_{fnrS}$-PAL3; 2XP$_{fnrS}$*pheP*; P$_{ara}$-*fnrS24Y*-LAAD; ddapA

Fig. 57A

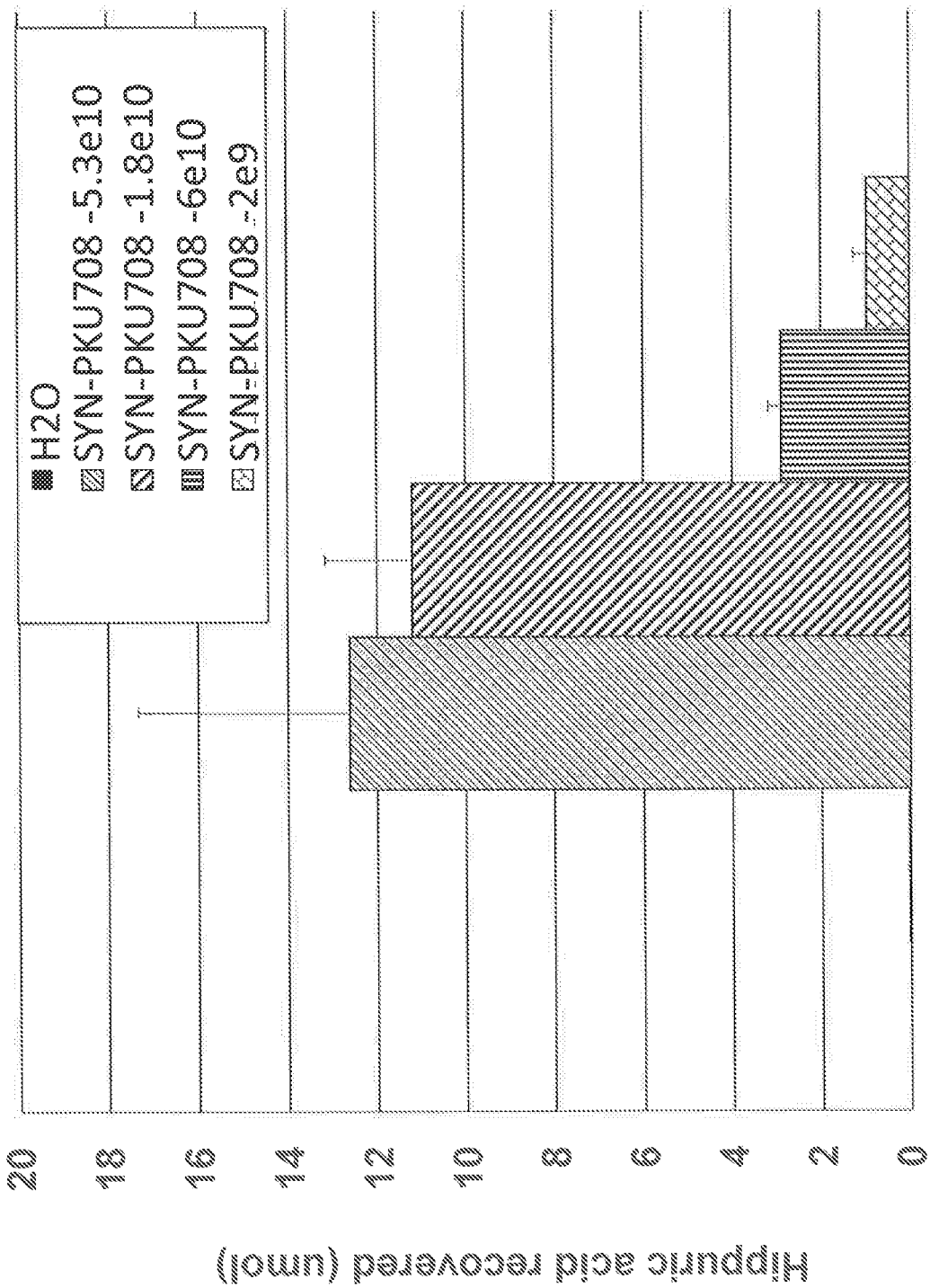

H2O
SYN-PKU708 -5.3e10
SYN-PKU708 -1.8e10
SYN-PKU708 -6e10
SYN-PKU708 -2e9
0
2
4
6
8
10
12
14
16
18
20
Hippuric acid recovered (umol)

Fig. 57B

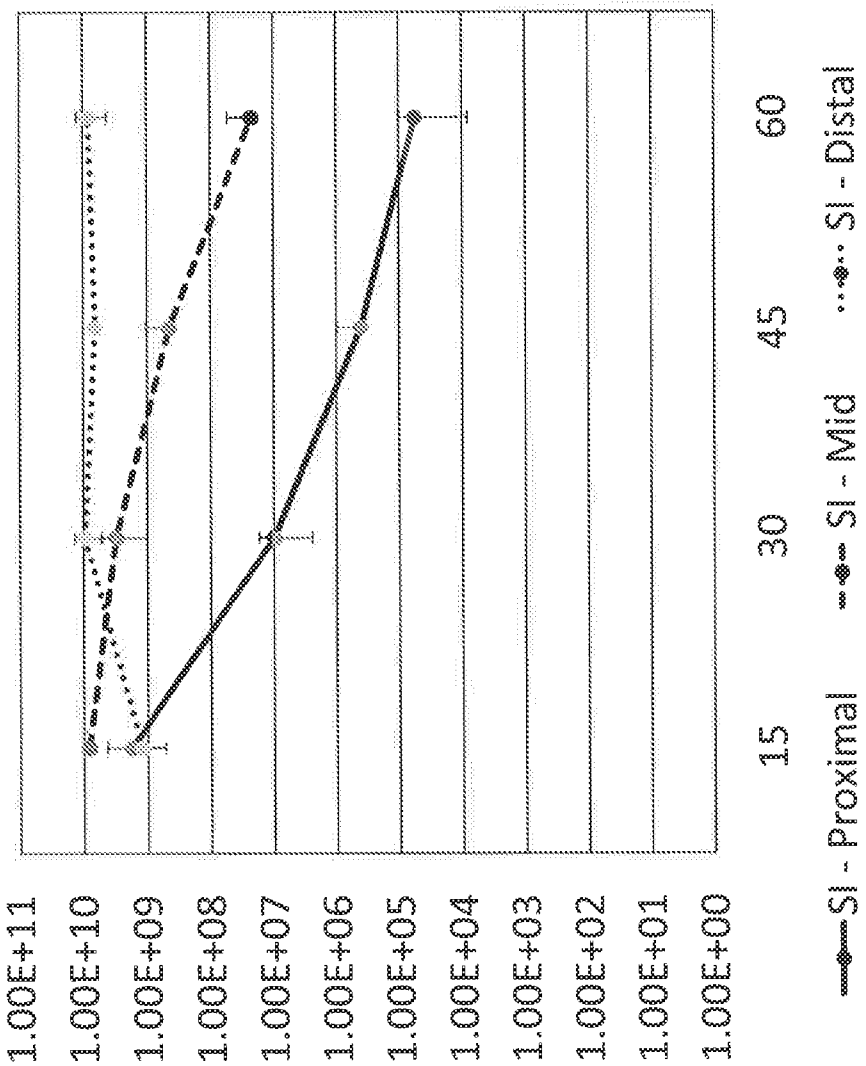

1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
15
30
45
60
SI - Proximal
SI - Mid
SI - Distal

Fig. 58A

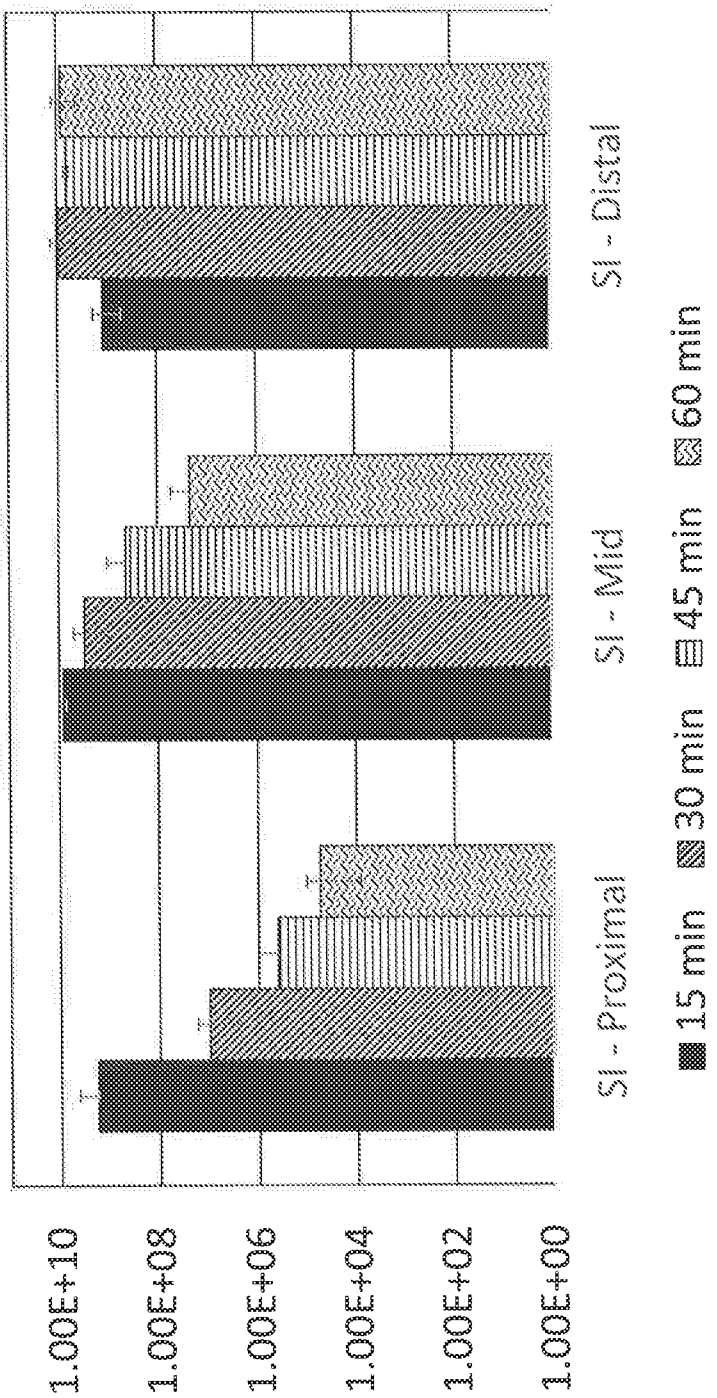

1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
SI - Proximal
SI - Mid
SI - Distal
15 min
30 min
45 min
60 min

Fig. 58B

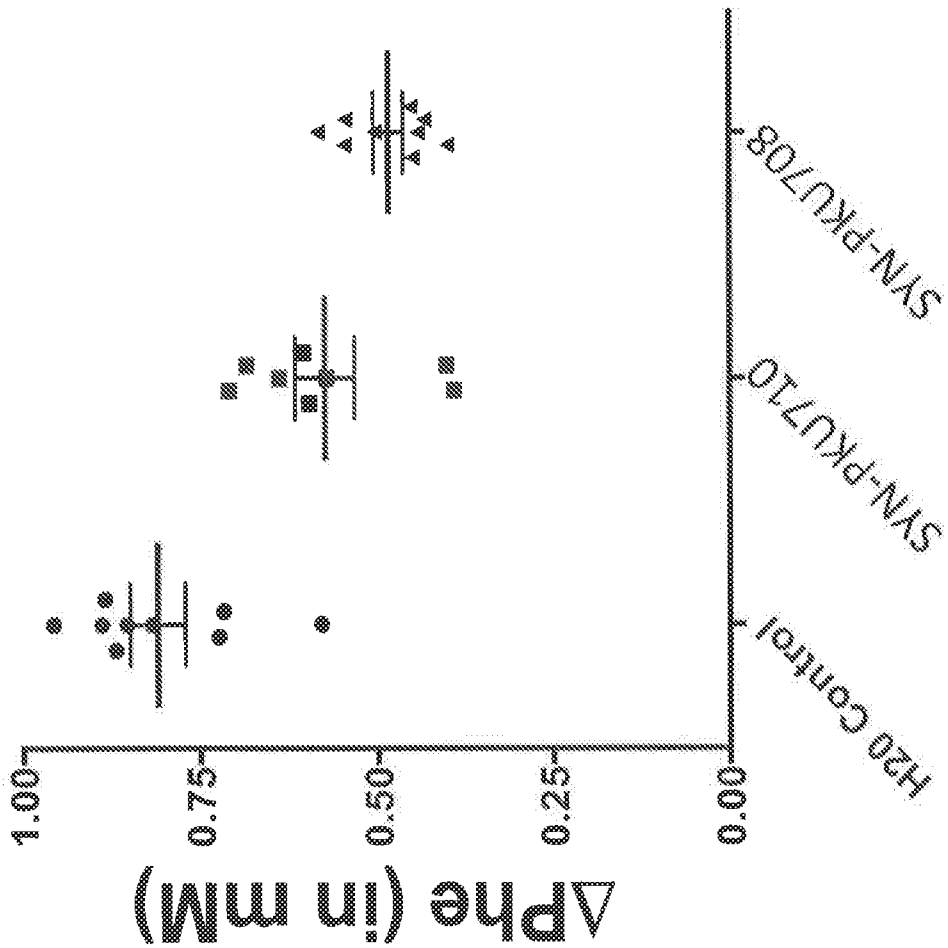

ΔPhe (in mM)
1.00
0.75
0.50
0.25
0.00
H20 Control
SVN-PKU710
SVN-PKU708

Fig. 59A

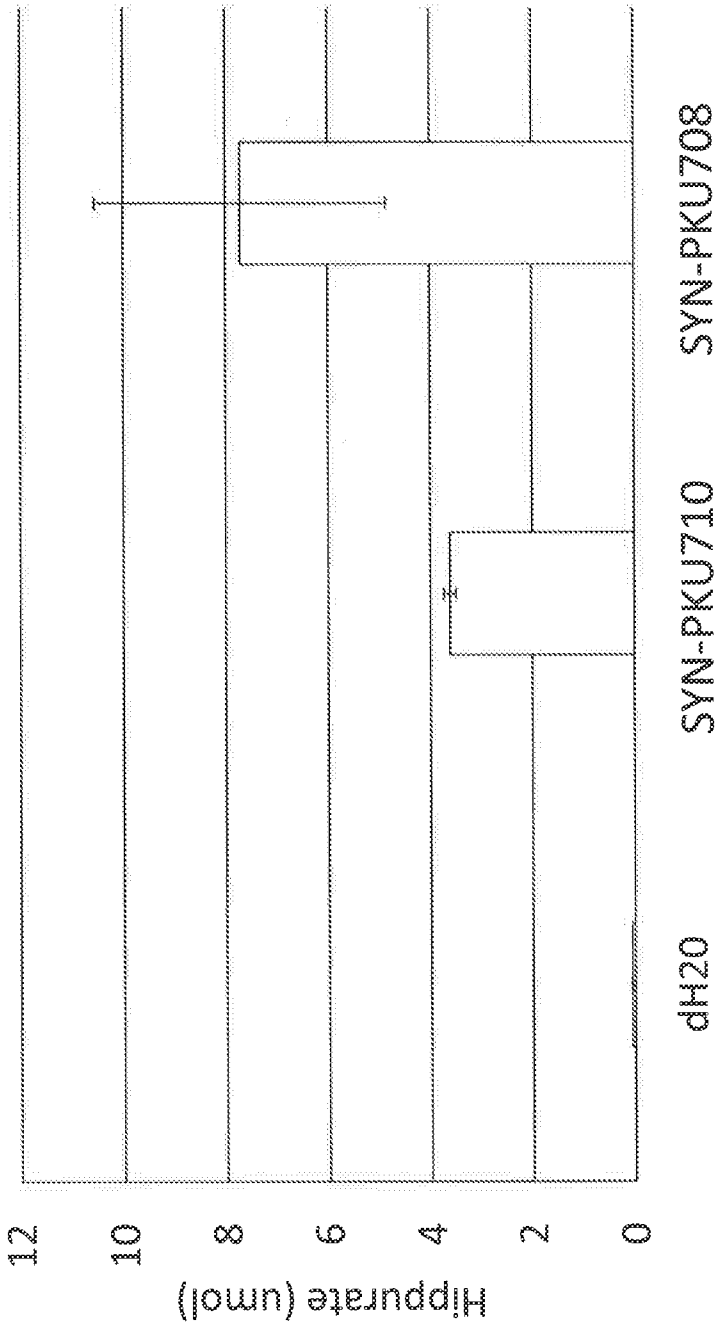


Fig. 59B

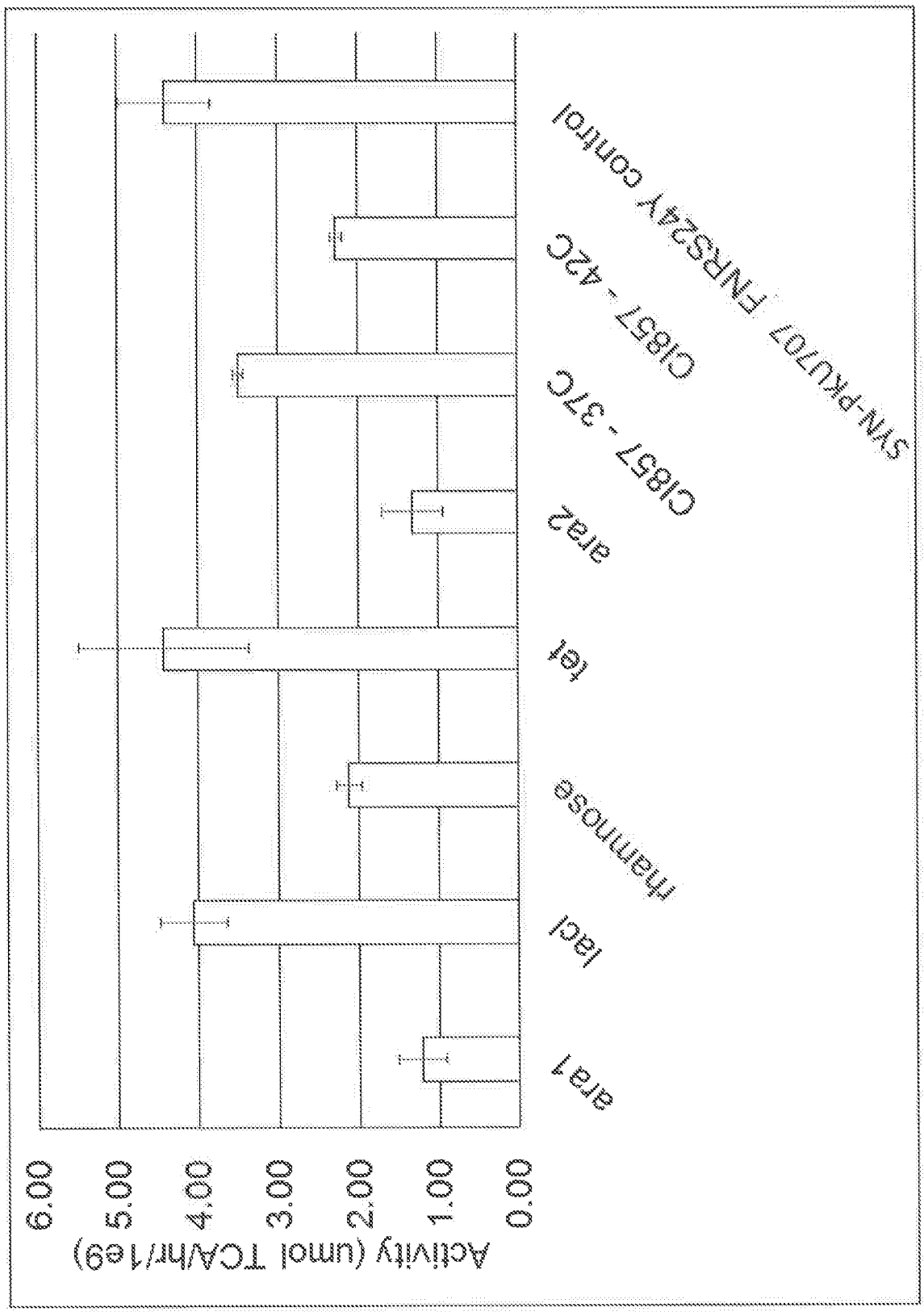

SYN-PKU707 FNR52AV control
C1857 - 42C
C1857 - 37C
ara2
tet
rhamnose
lacI
ara1
Activity (umol TCA/hr/1e9)
0.00
1.00
2.00
3.00
4.00
5.00
6.00

Fig. 60

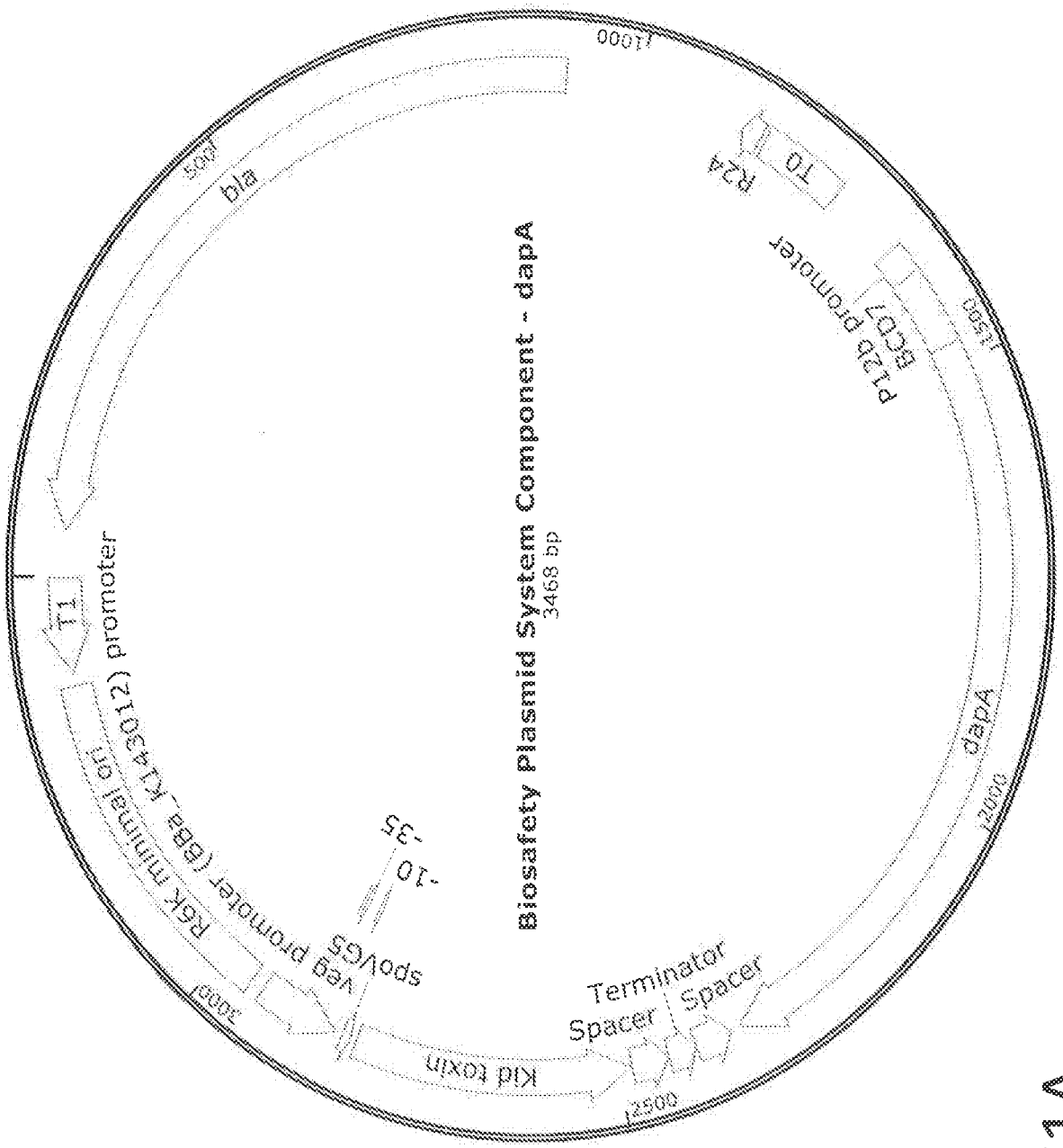

Biosafety Plasmid System Component - dapA
3468 bp
1000
500
bla
R2A
T0
P12b promoter
BcD7
1500
dapA
2000
2500
Kid toxin
Terminator
Spacer
Spacer
-10
-35
spoVG5
Veg promoter (Bba_K143012) promoter
RGK minimal ori
3000
T1
1

Fig. 61A

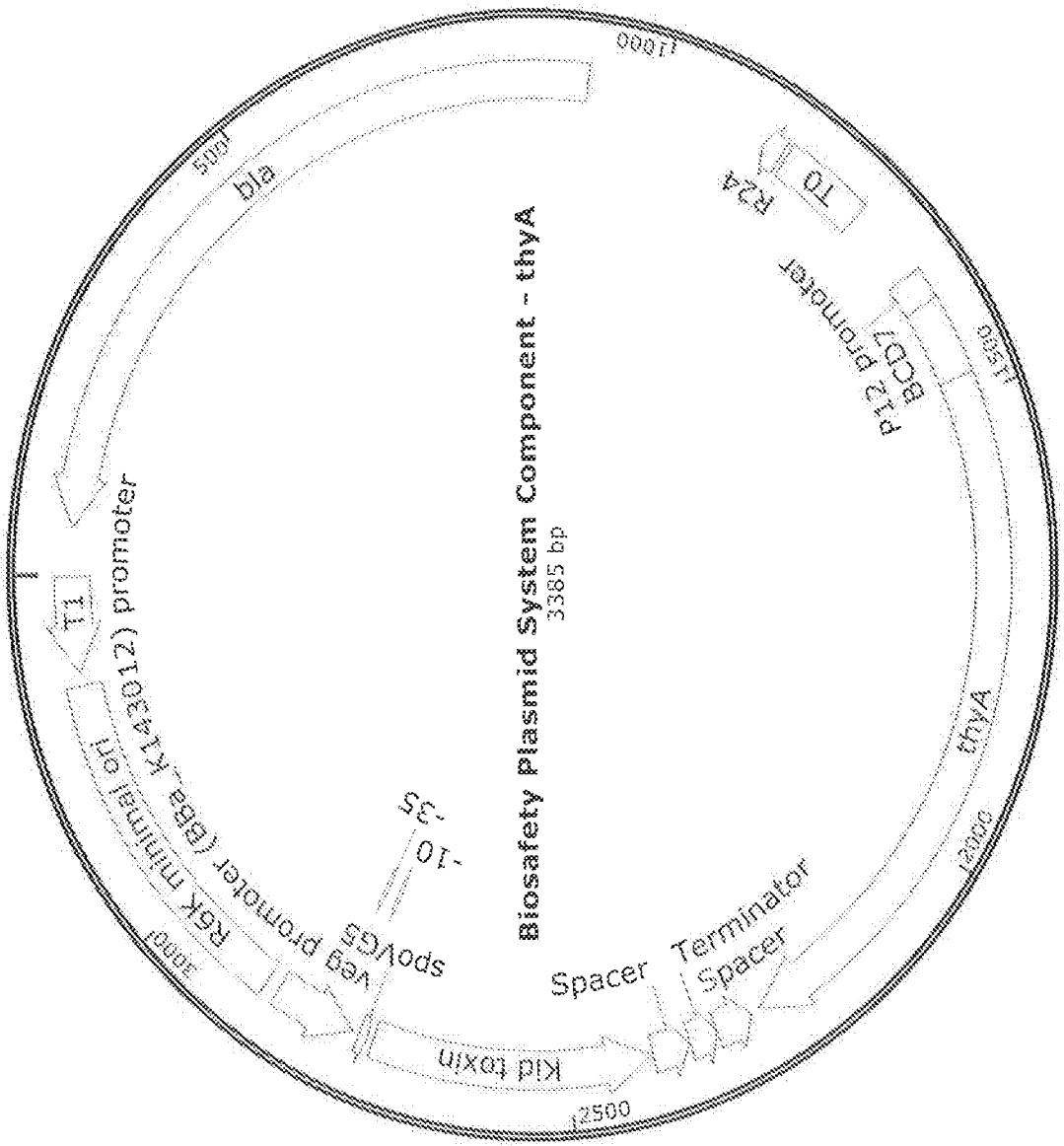
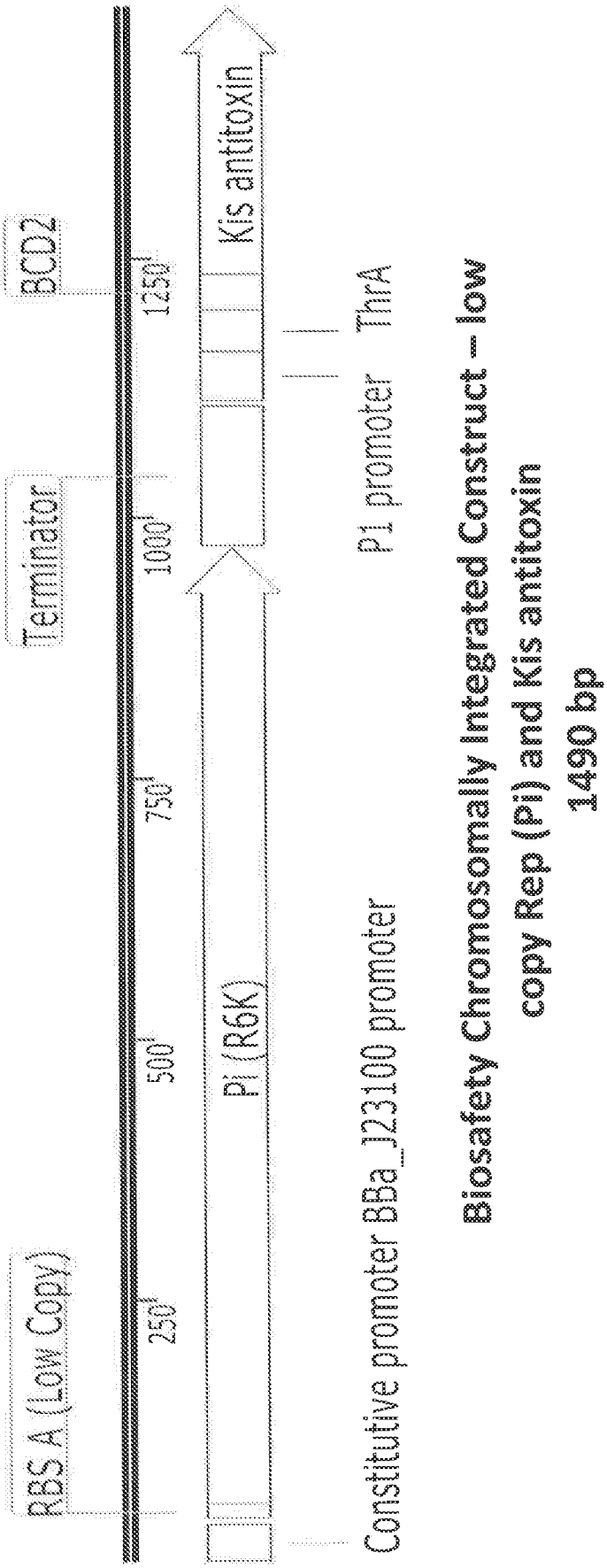
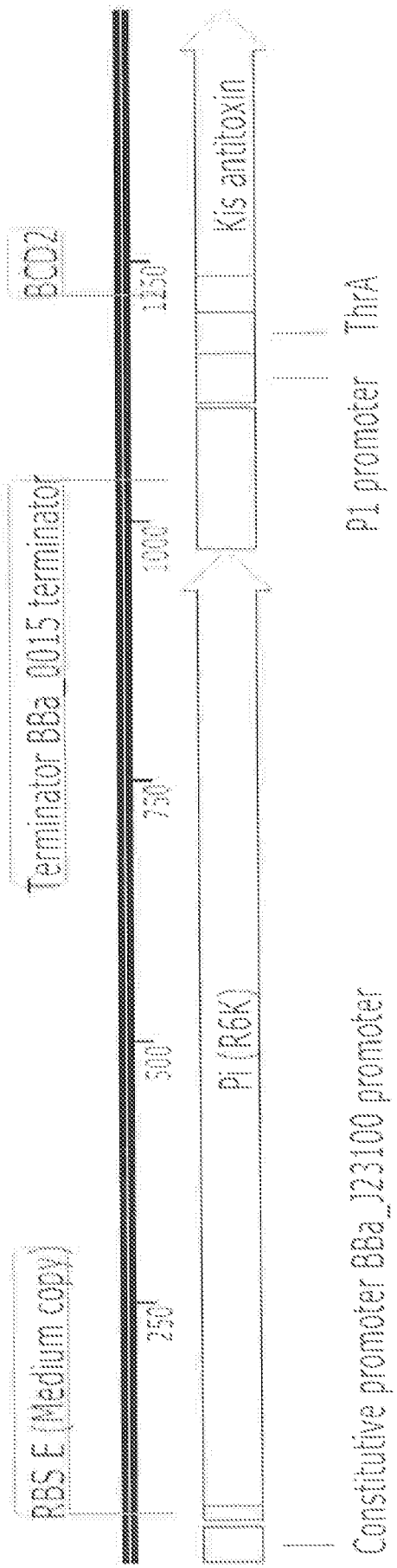

Biosafety Plasmid System Component - thyA
3385 bp
bla
500
1000
T1
RGK minimal ori
2000
RGK promoter (GGa_K1430012) promoter
-10
-35
vegpromoter
spoVGS
Kid toxin
2500
Spacer
Terminator
Spacer
thyA
2000
1500
P12 promoter
BCD7
T0
R2A

Fig. 61B



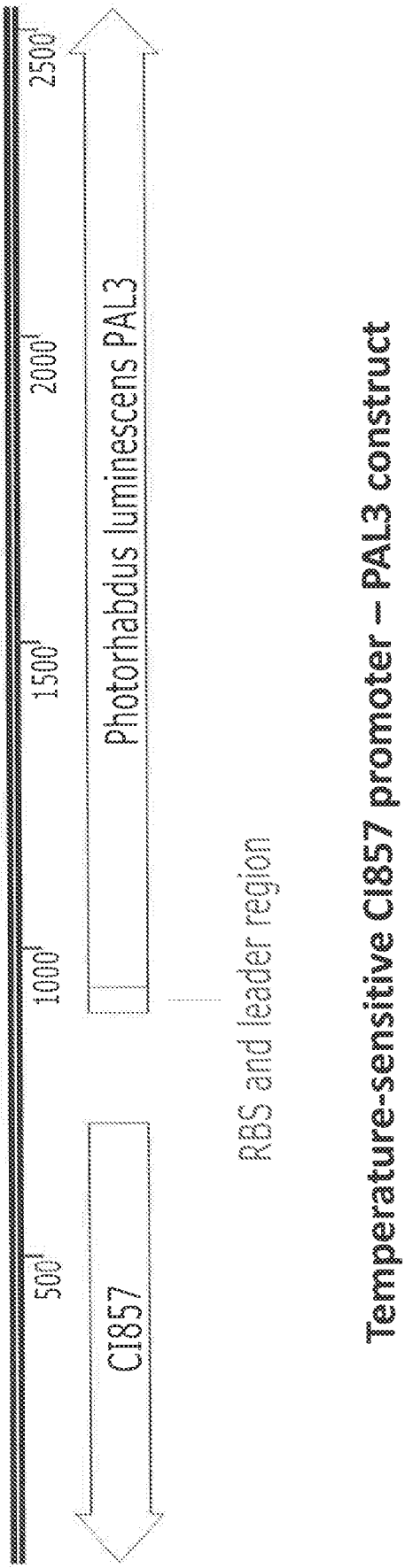
Biosafety Chromosomally Integrated Construct – low copy Rep (Pi) and Kis antitoxin
1490 bp

Fig. 61C



Biosafety Chromosomally Integrated Construct – medium copy Rep (Pi) and Kis antitoxin
1490 bp

Fig. 61D



Temperature-sensitive CI857 promoter – PAL3 construct

Fig. 62A

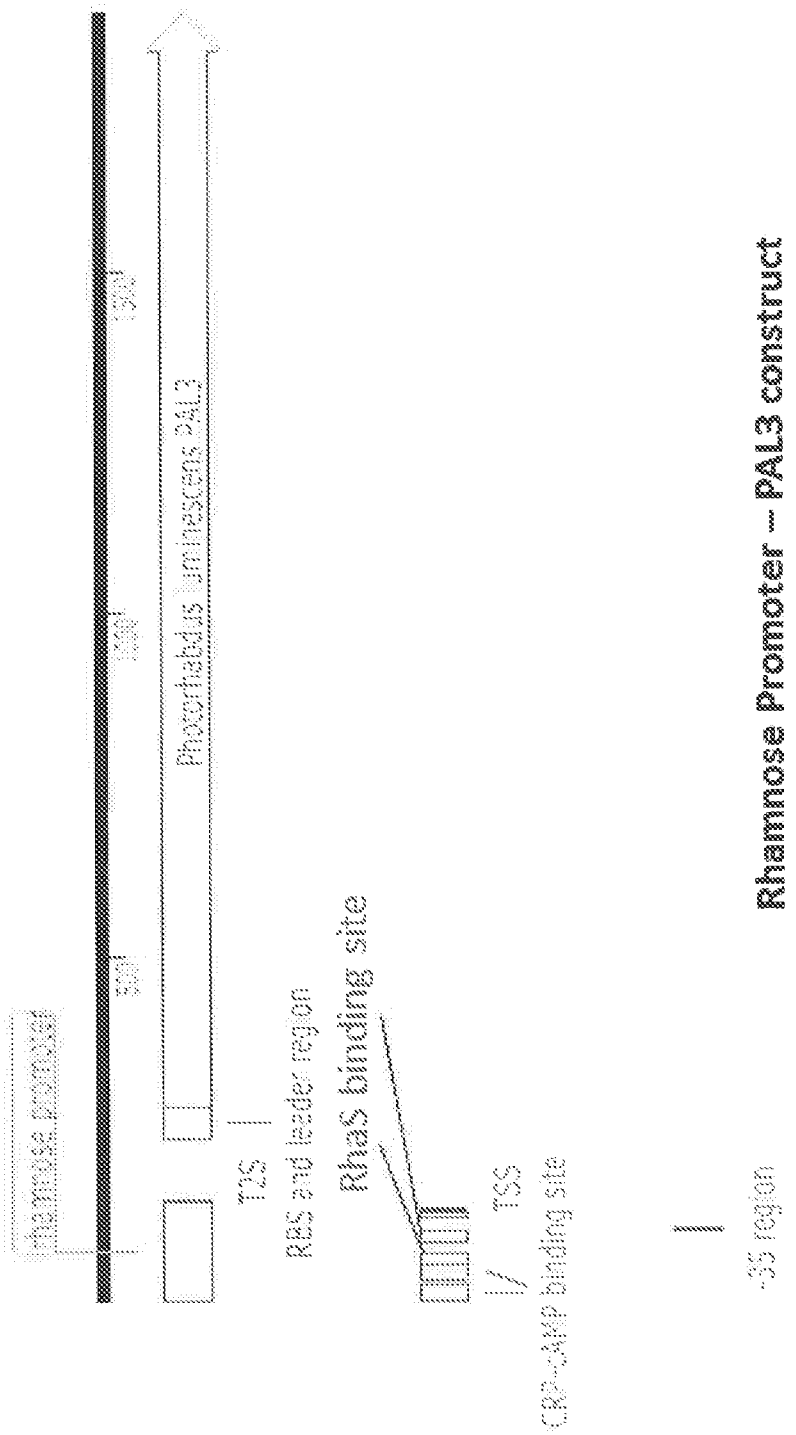

rhamnose promoter
500
400
300
200
100
Photorhabdus luminescens 2413
TSS
RBS and leader region
RhaS binding site
TSS
CRP-cAMP binding site
-35 region
Rhamnose Promoter – PAL3 construct

Fig. 62B

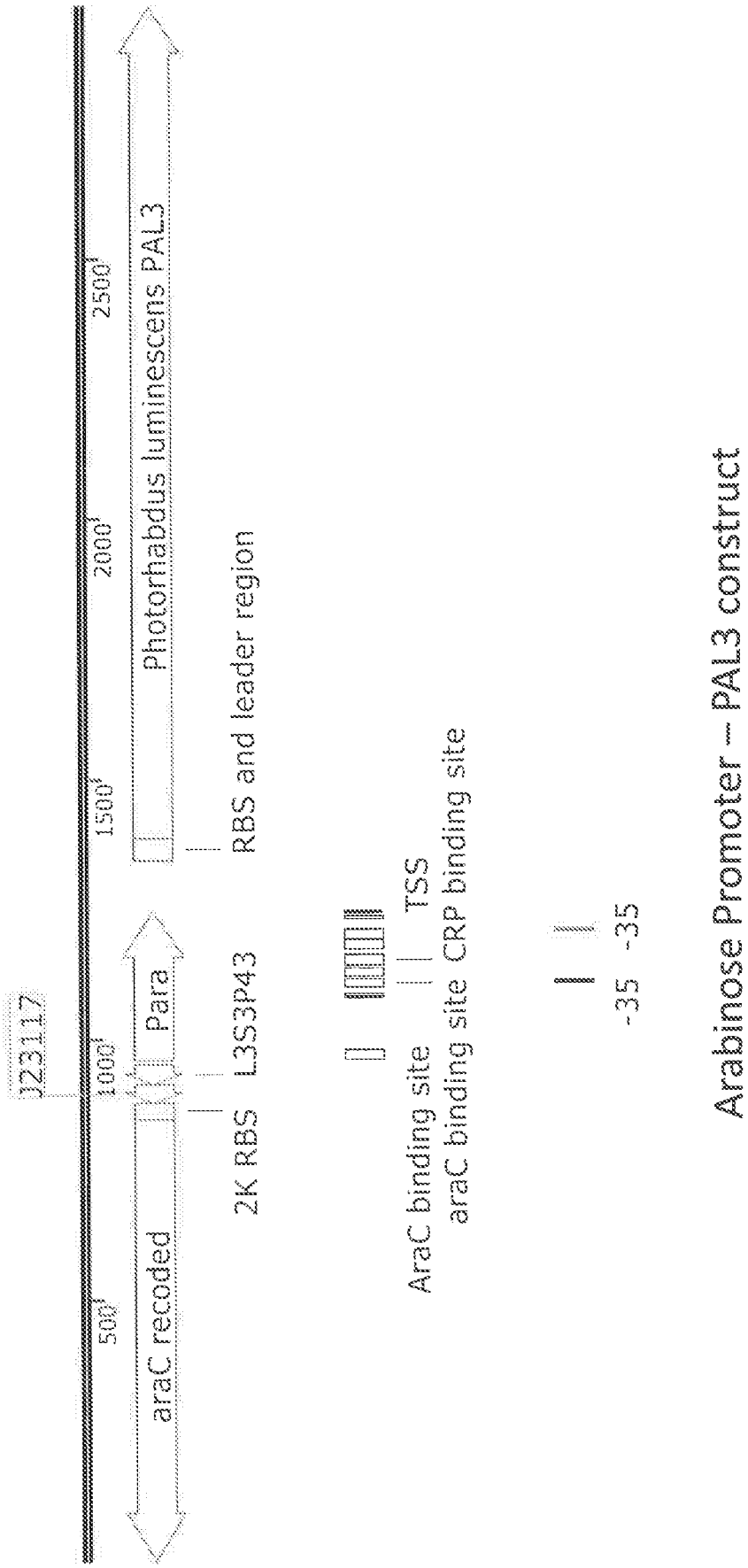

123117
500
1000
1500
2000
2500
araC recoded
Para
Photorhabdus luminescens PAL3
2K RBS
L3S3P43
RBS and leader region
AraC binding site
TSS
araC binding site
CRP binding site
-35
-35
Arabinose Promoter – PAL3 construct
2986 bp

Fig. 62C

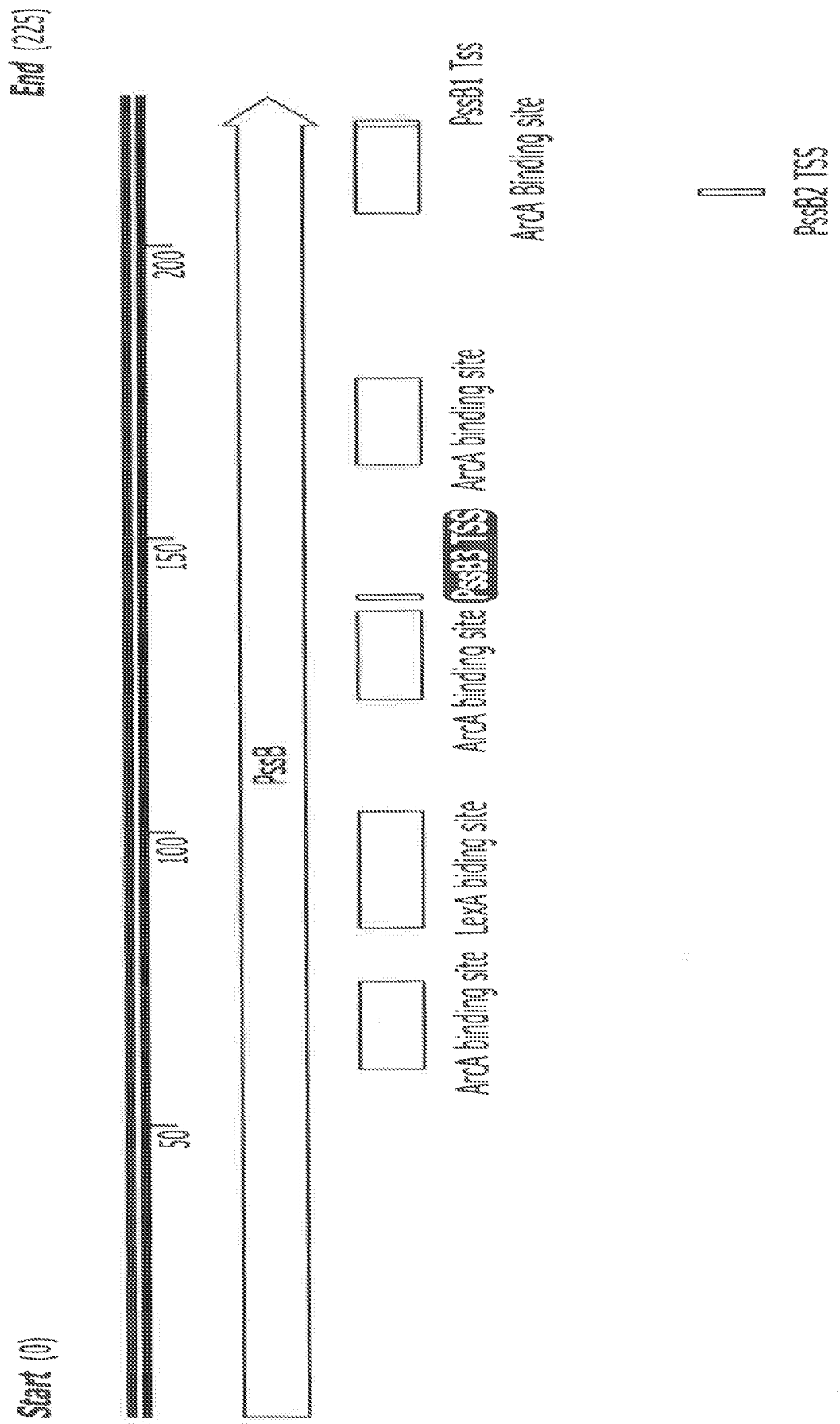

Start (0)
50
100
150
200
End (225)
Pss8
ArcA binding site
LexA binding site
ArcA binding site
Pss81 TSS
ArcA binding site
ArcA Binding site
Pss82 TSS

Fig. 63A

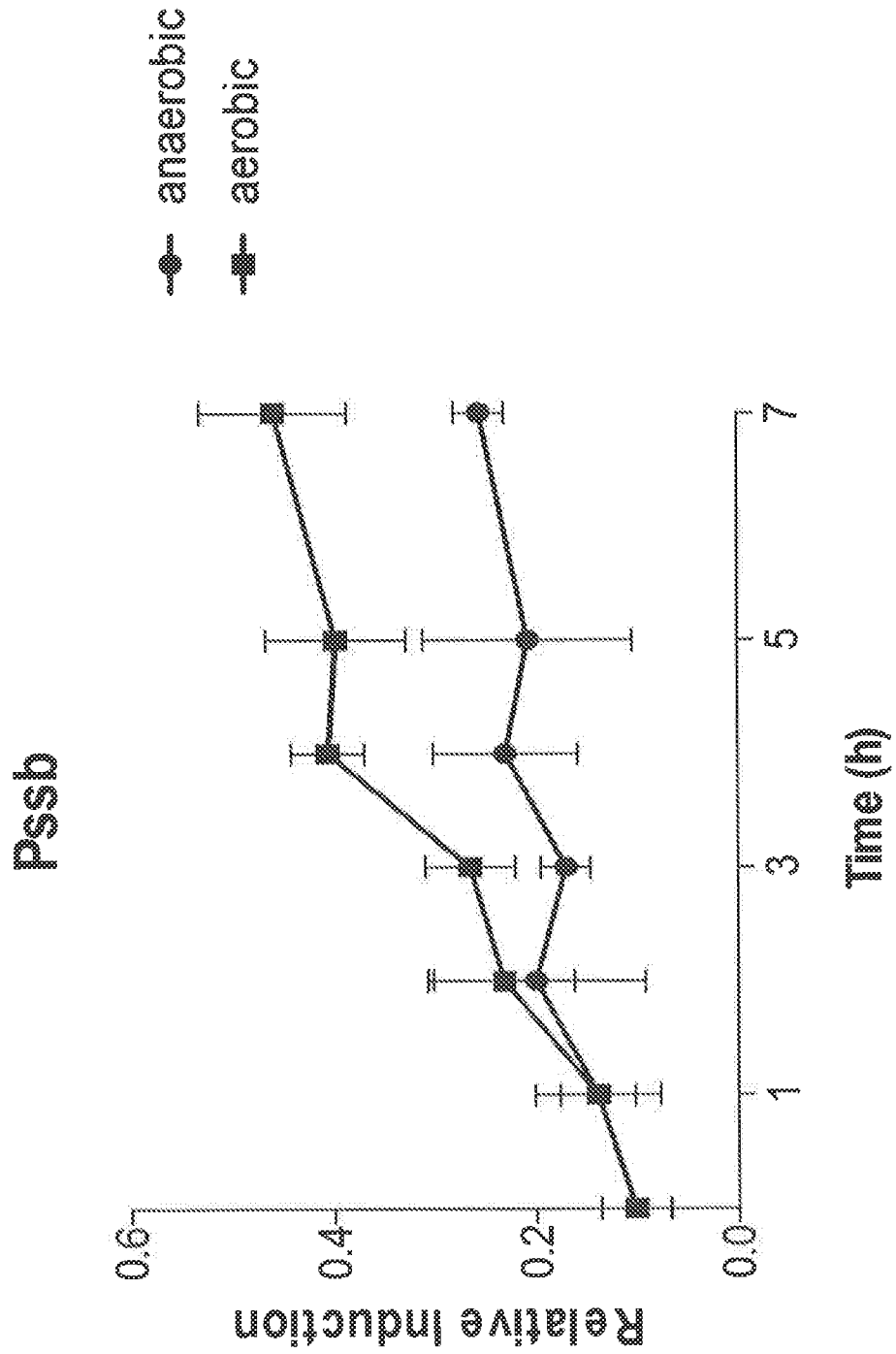

anaerobic
aerobic
Pssb
Relative induction
0.0
0.2
0.4
0.6
1
3
5
7
Time (h)

Fig. 63B

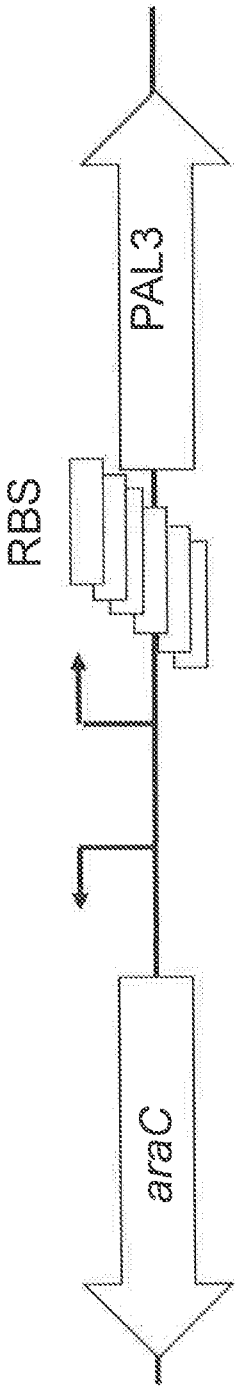


Fig. 64A

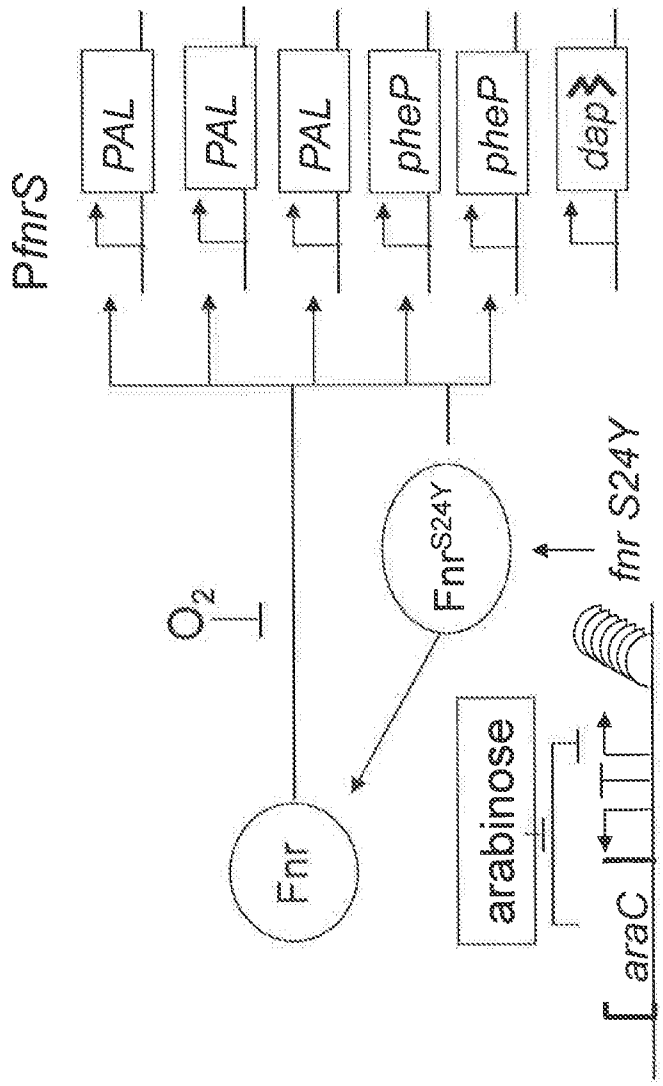
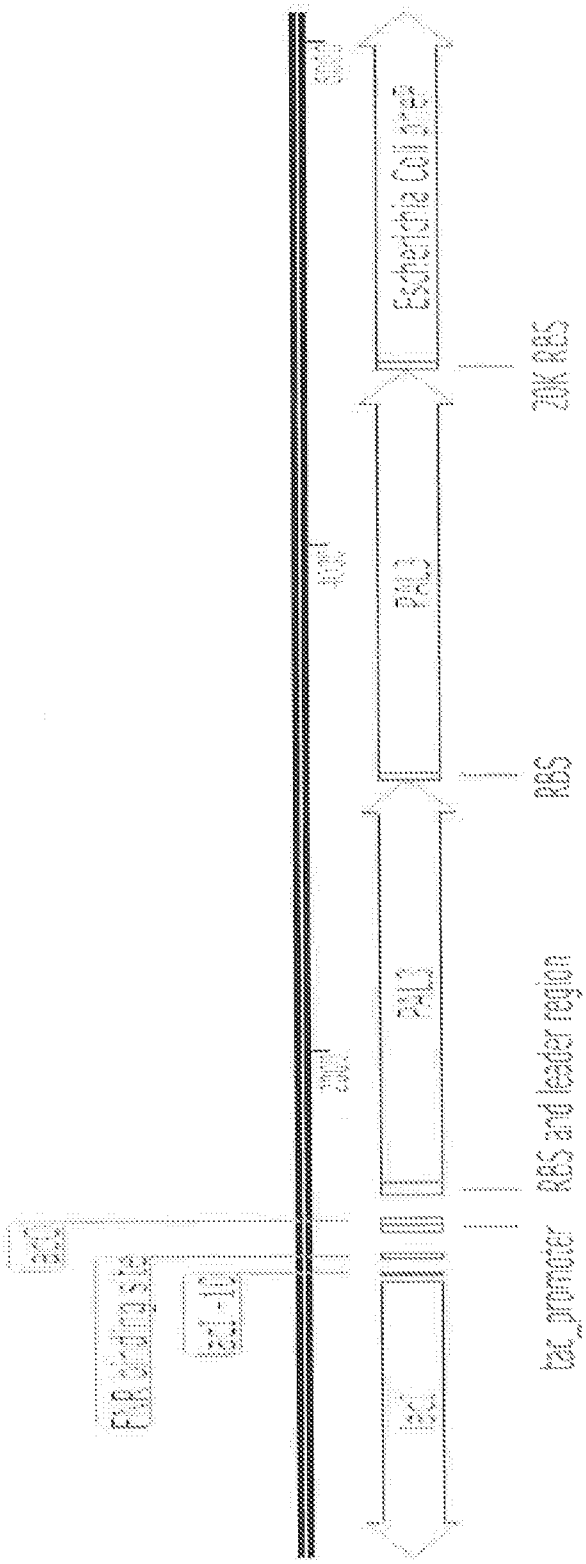
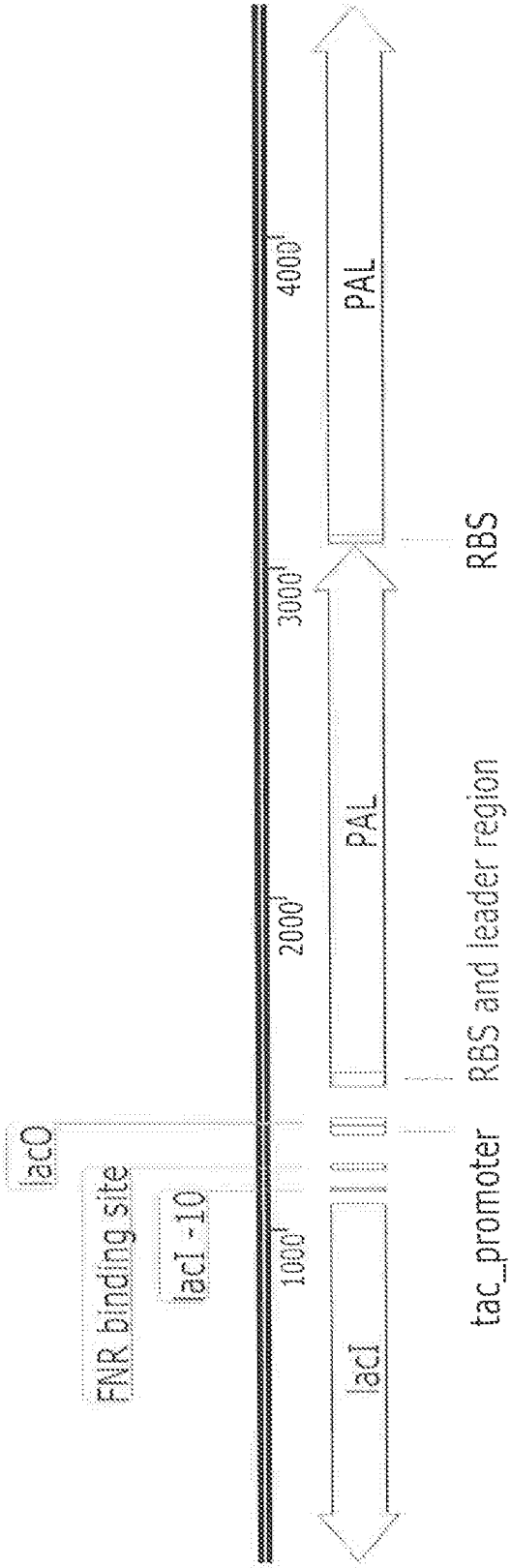

PfnrS
PAL
PAL
PAL
pheP
pheP
dapA
O2
Fnr
FnrS24Y
fnr S24Y
arabinose
araC

Fig. 64B



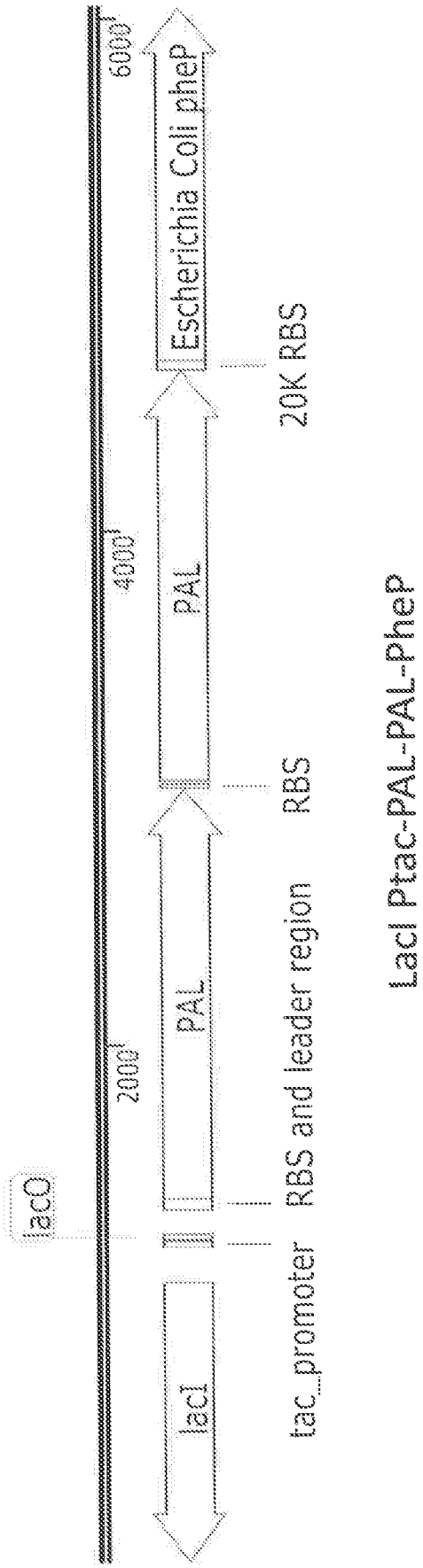
LacI FNRS Ptac-PAL-PAL-PheP
6044 bp

Fig. 65A



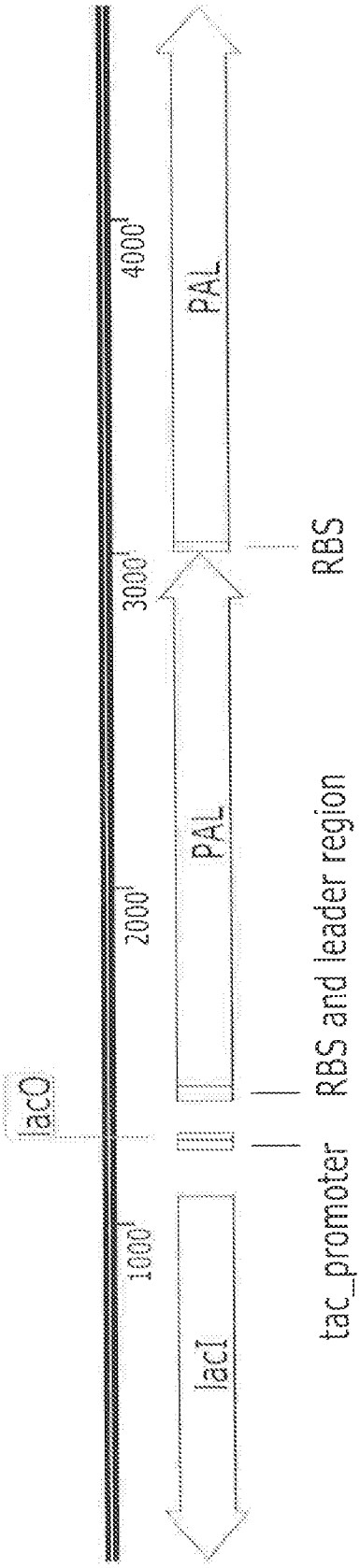
LacI Fnrs-Ptac-PAL-PAL
4698 bp

Fig. 65B



LacI Ptac-PAL-PAL-PheP
6044 bp

Fig. 65C



lacI –Ptac-PAL-PAL
4632 bp

Fig. 65D

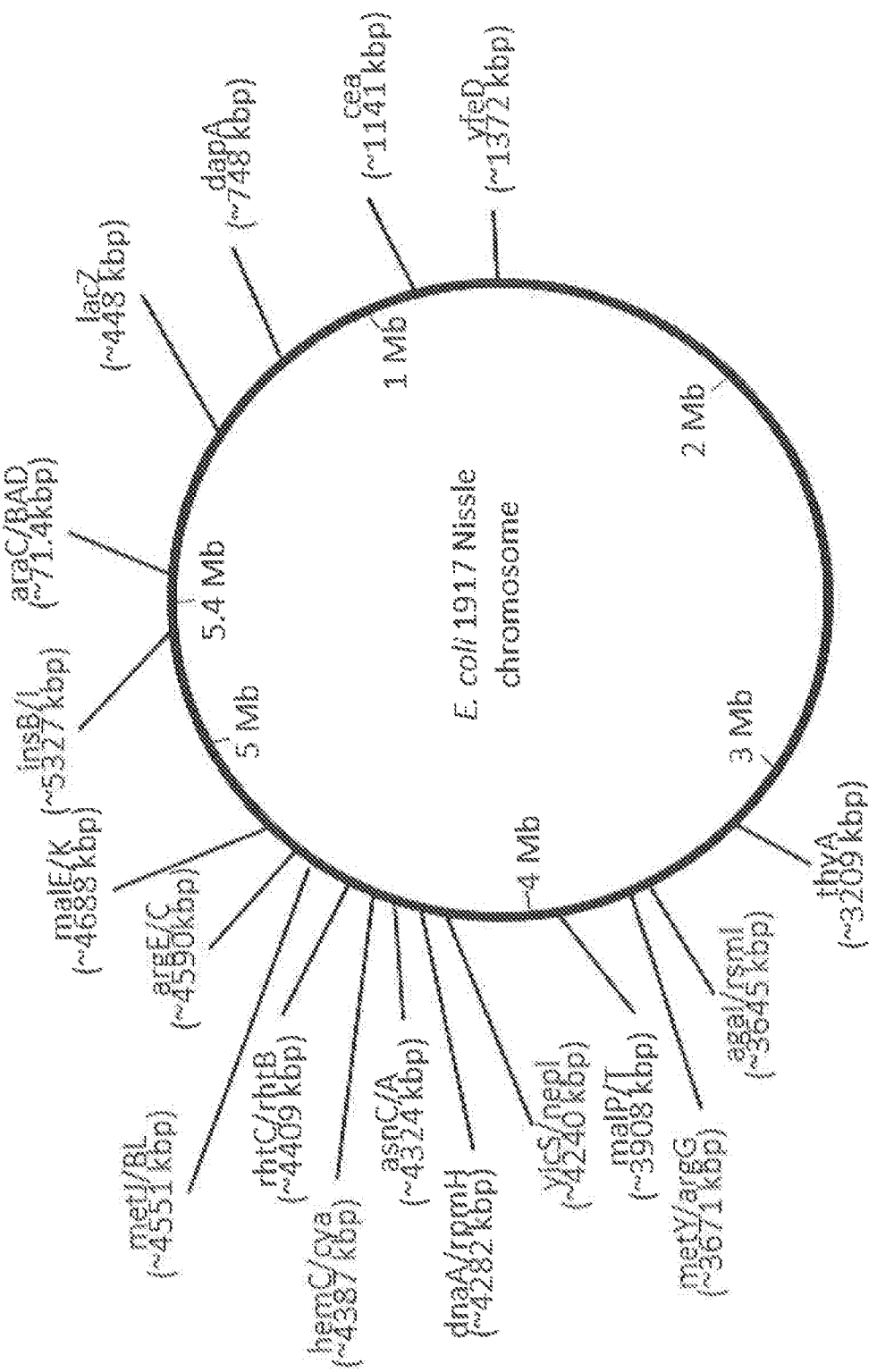

E. coli 1917 Nissle chromosome
1 Mb
2 Mb
3 Mb
4 Mb
5 Mb
5.4 Mb
lacZ (~448 kbp)
dapA (~748 kbp)
cea (~1141 kbp)
yfeD (~1372 kbp)
araC/BAD (~71.4kbp)
insB/I (~5327 kbp)
malE/K (~4683 kbp)
argE/C (~4590kbp)
rhtC/rhtB (~4409 kbp)
hemG/cyaA (~4387 kbp)
asnC/A (~4324 kbp)
dnaA/rpmH (~4282 kbp)
ylcS/nepI (~4240 kbp)
malP/T (~3908 kbp)
metY/argG (~3671 kbp)
agaI/rsmI (~3645 kbp)
thyA (~3209 kbp)
metJ/BL (~4551 kbp)

Fig. 66

Brightness of constitutive RFP integrated in three locations:

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

Fig. 67

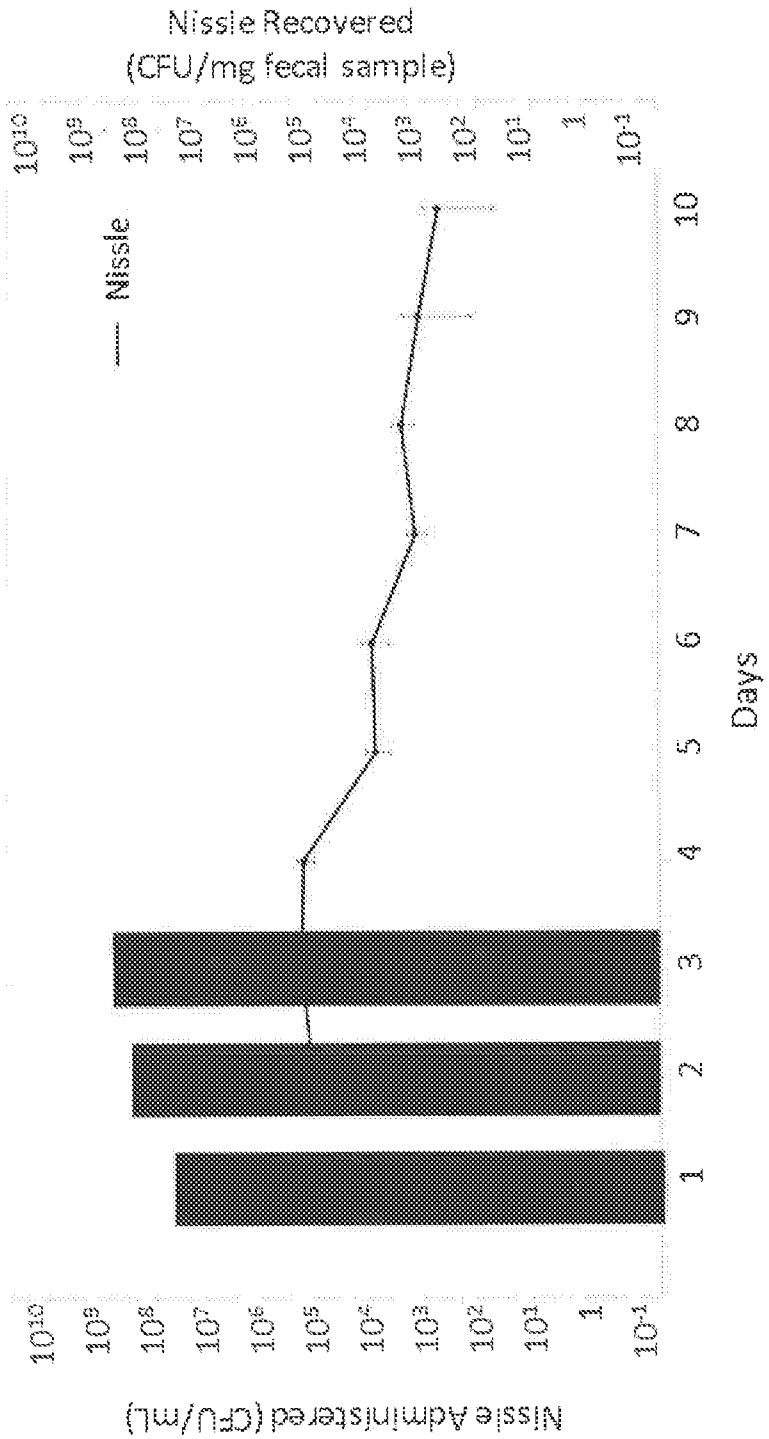

Nissle Recovered
(CFU/mg fecal sample)
Nissle
Days
Nissle Administered (CFU/mL)

Fig. 68

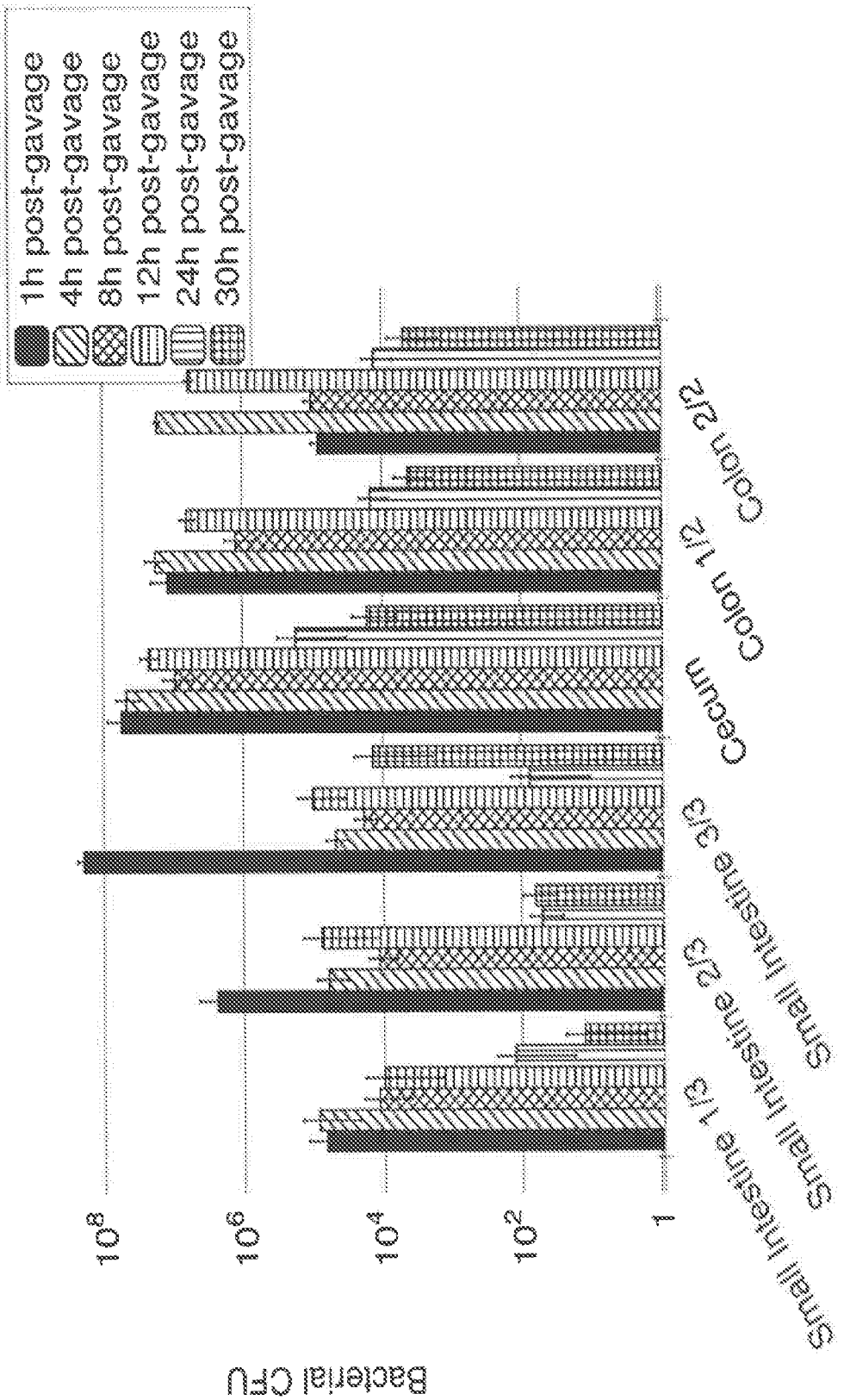

1h post-gavage
4h post-gavage
8h post-gavage
12h post-gavage
24h post-gavage
30h post-gavage
Small Intestine 1/3
Small Intestine 2/3
Small Intestine 3/3
Cecum
Colon 1/2
Colon 2/2
10^8
10^6
10^4
10^2
1
Bacterial CFU

Fig. 69

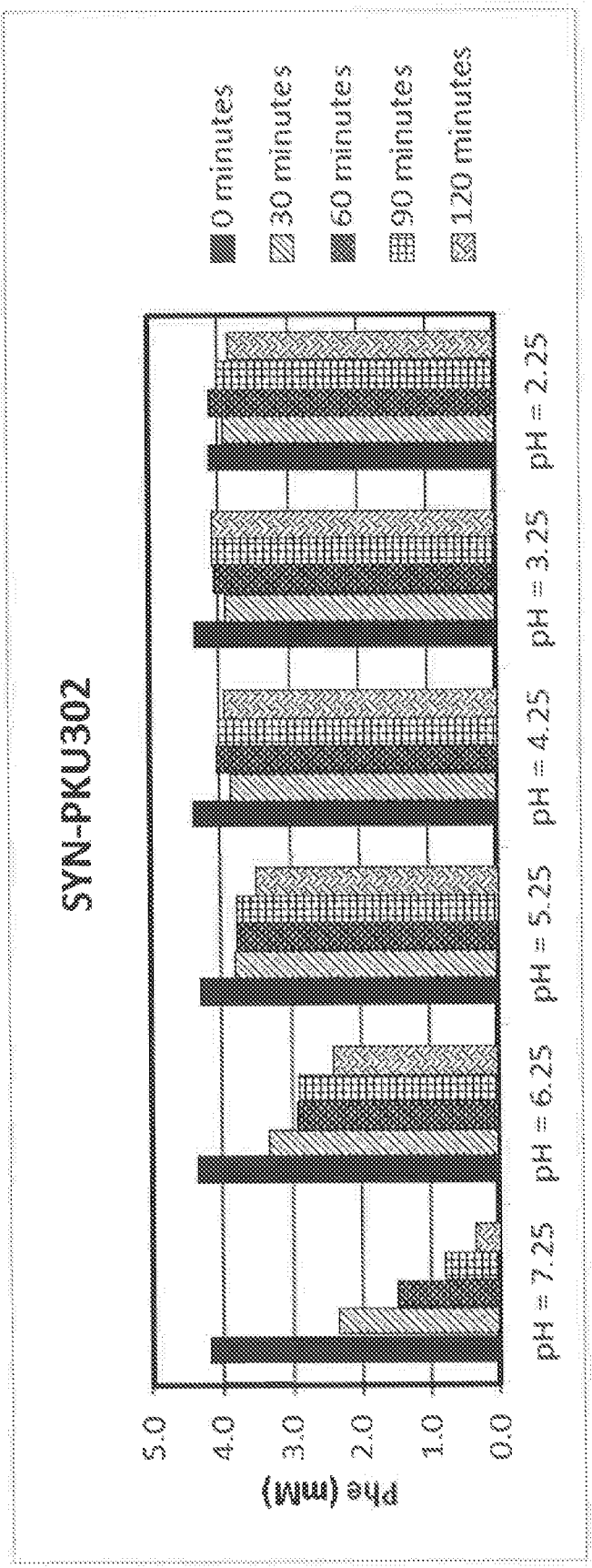

SYN-PKU302
0 minutes
30 minutes
60 minutes
90 minutes
120 minutes
Phe (mM)
5.0
4.0
3.0
2.0
1.0
0.0
pH = 7.25
pH = 6.25
pH = 5.25
pH = 4.25
pH = 3.25
pH = 2.25

Fig. 70A

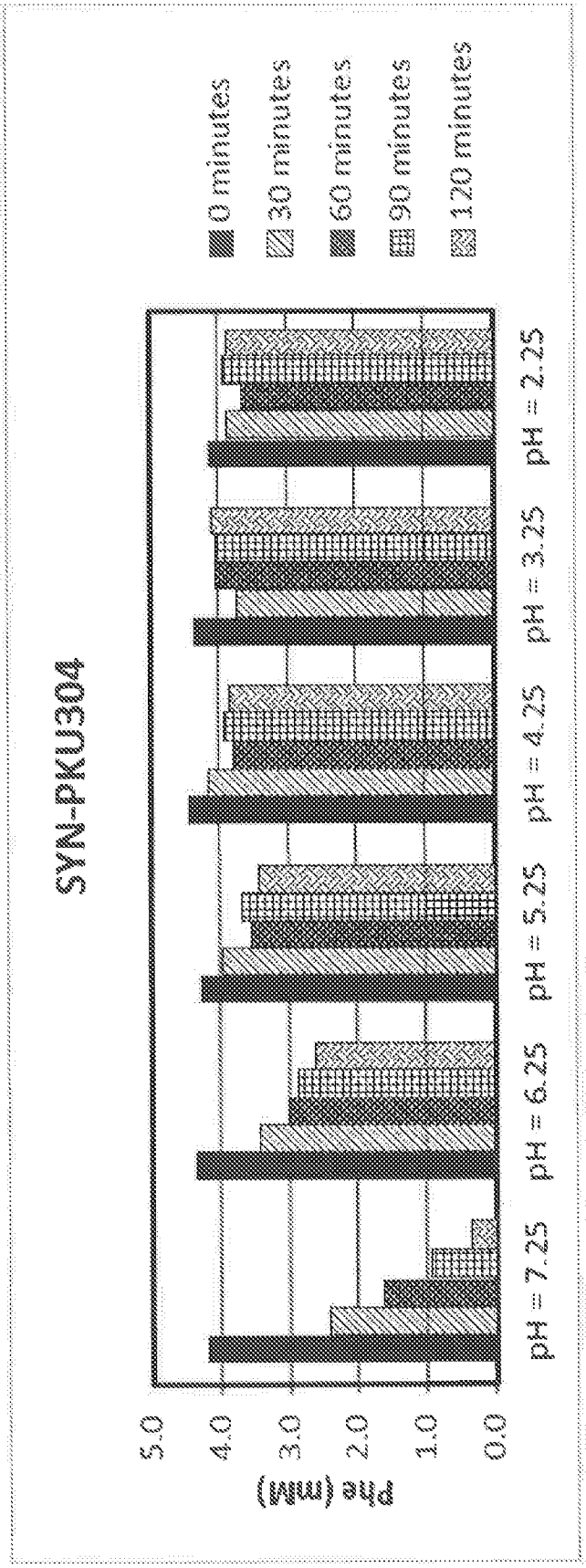

SYN-PKU304
0 minutes
30 minutes
60 minutes
90 minutes
120 minutes
Phe (mM)
5.0
4.0
3.0
2.0
1.0
0.0
pH = 7.25
pH = 6.25
pH = 5.25
pH = 4.25
pH = 3.25
pH = 2.25

Fig. 70B

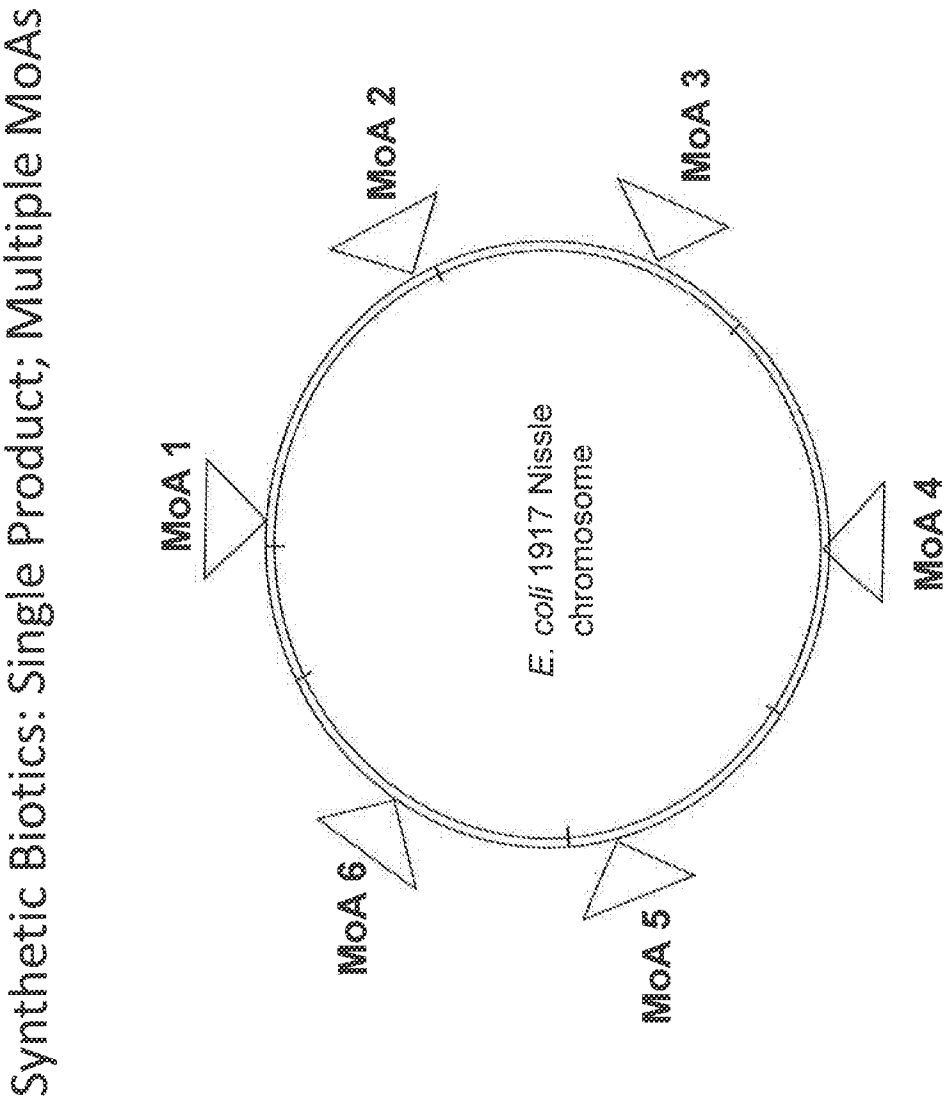

Synthetic Biotics: Single Product; Multiple MoAs
MoA 1
MoA 2
MoA 3
MoA 4
MoA 5
MoA 6
E. coli 1917 Nissle chromosome

Fig. 71

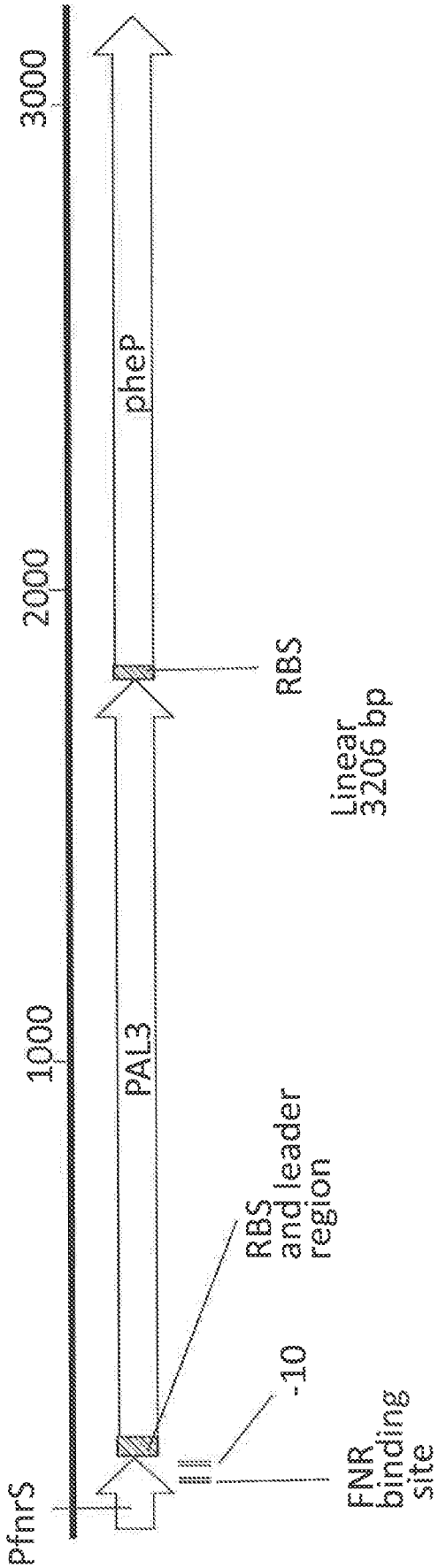

3000
2000
1000
pheP
PAL3
RBS
Linear
3206 bp
RBS
and leader
region
-10
FNR
binding
site
PfnrS

Fig. 72

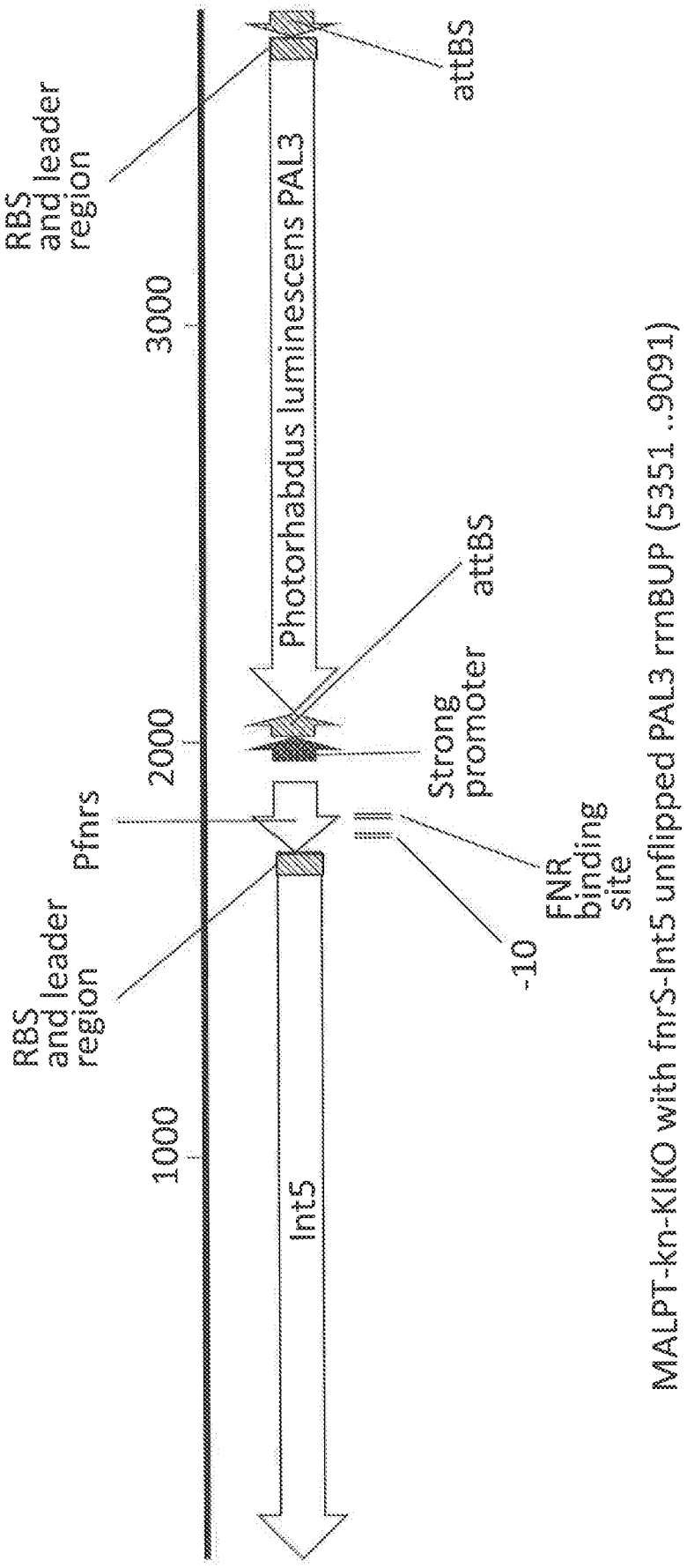

RBS and leader region
attBS
Photorhabdus luminescens PAL3
3000
attBS
Strong promoter
2000
pfnrs
FNR binding site
-10
RBS and leader region
1000
Int5
MALPT-kn-KIKO with fnrS-Int5 unflipped PAL3 rrnBUP (5351..9091)
3741 bp

Fig. 73A

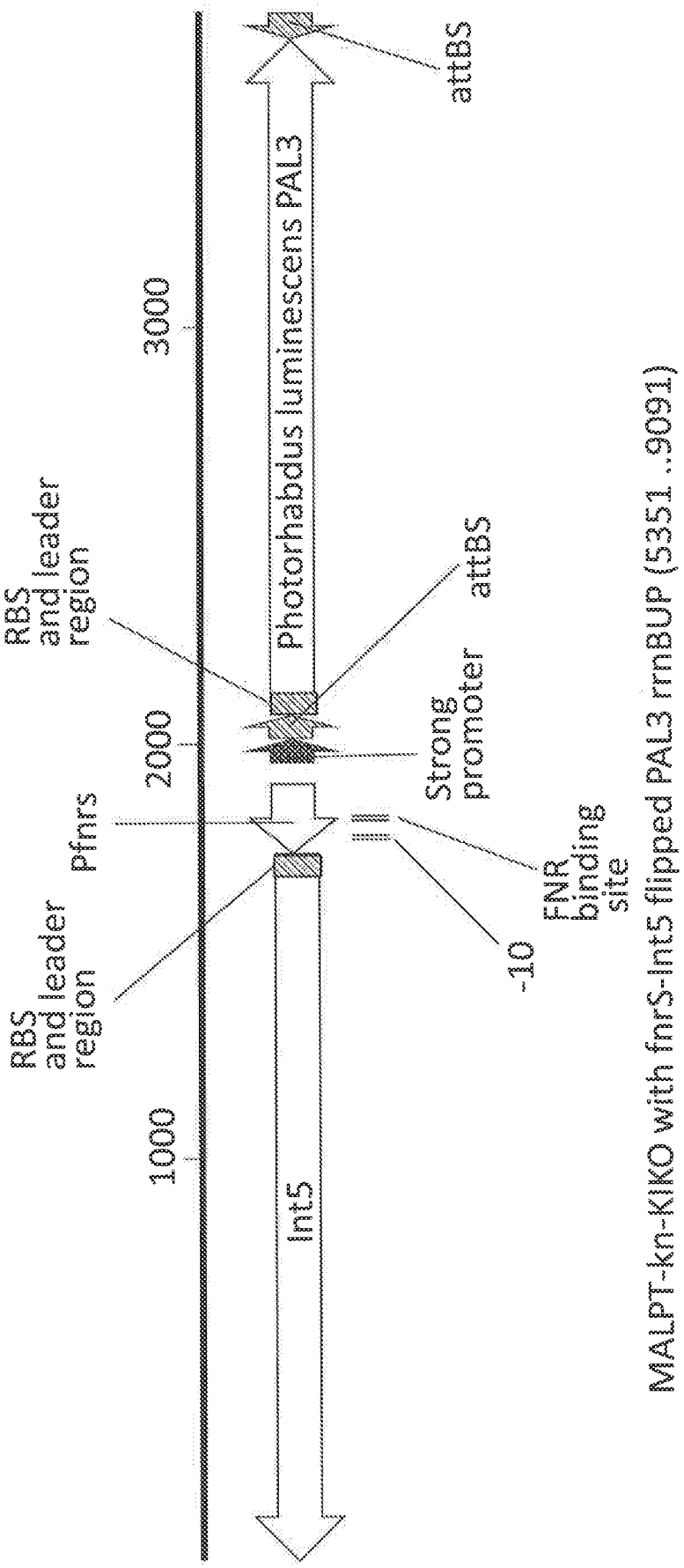

attBS
Photorhabdus luminescens PAL3
3000
RBS and leader region
attBS
Strong promoter
2000
Pfnrs
FNR binding site
-10
RBS and leader region
1000
Int5
MALPT-kn-KIKO with fnrS-Int5 flipped PAL3 rrnBUP (5351..9091)
3741 bp

Fig. 73B

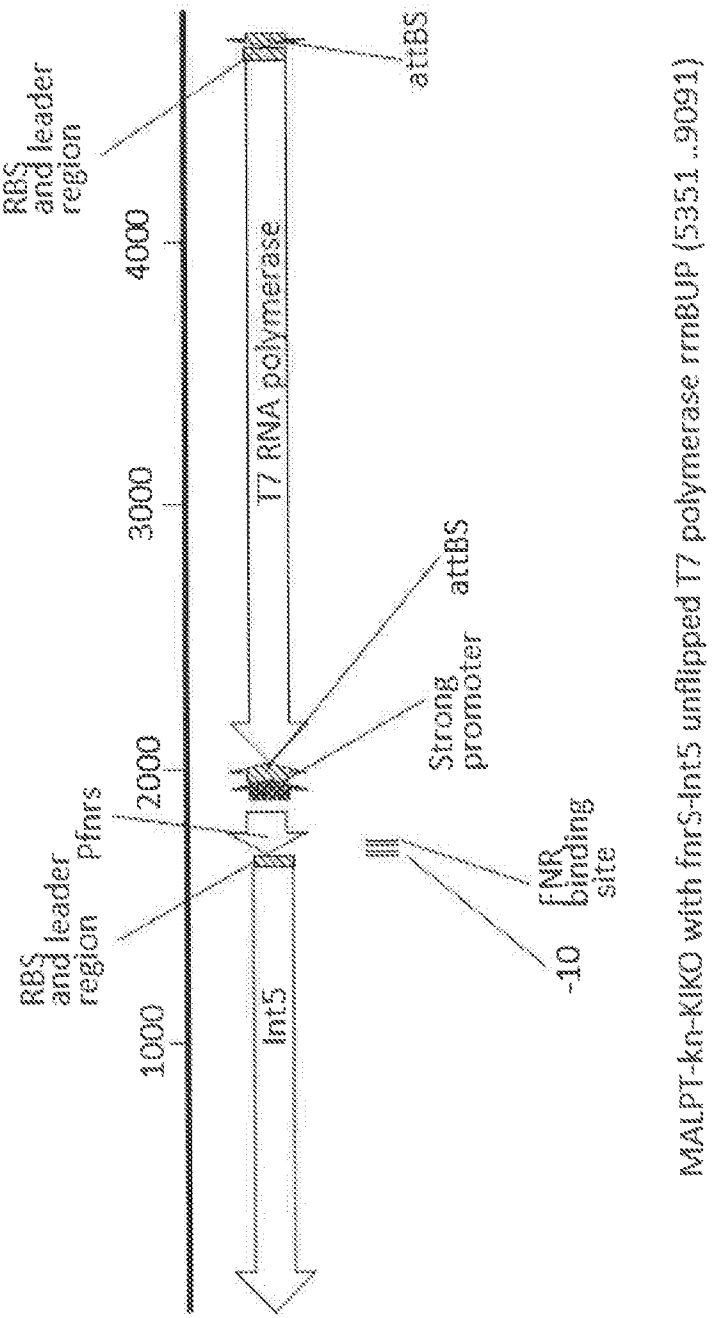
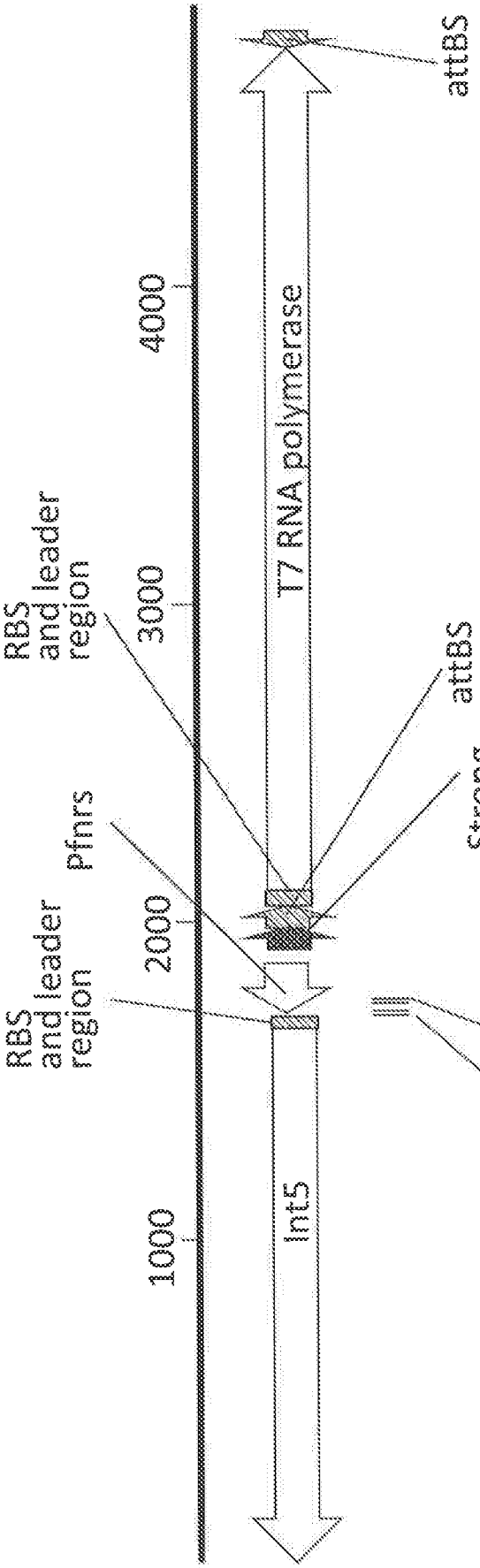

RBS and leader region
attBS
T7 RNA polymerase
4000
3000
2000
1000
attBS
Strong promoter
Pfnrs
RBS and leader region
FNR binding site
-10
Int5
MALPT-kn-KIKO with fnrS-Int5 unflipped T7 polymerase rnBUP (5351..9091)
4794 bp

Fig. 74A



MALPT-kn-KIKO with fnrS-Int5 flipped T7 polymerase rrnBUP (5351..9091)

4794 bp

Fig. 74B

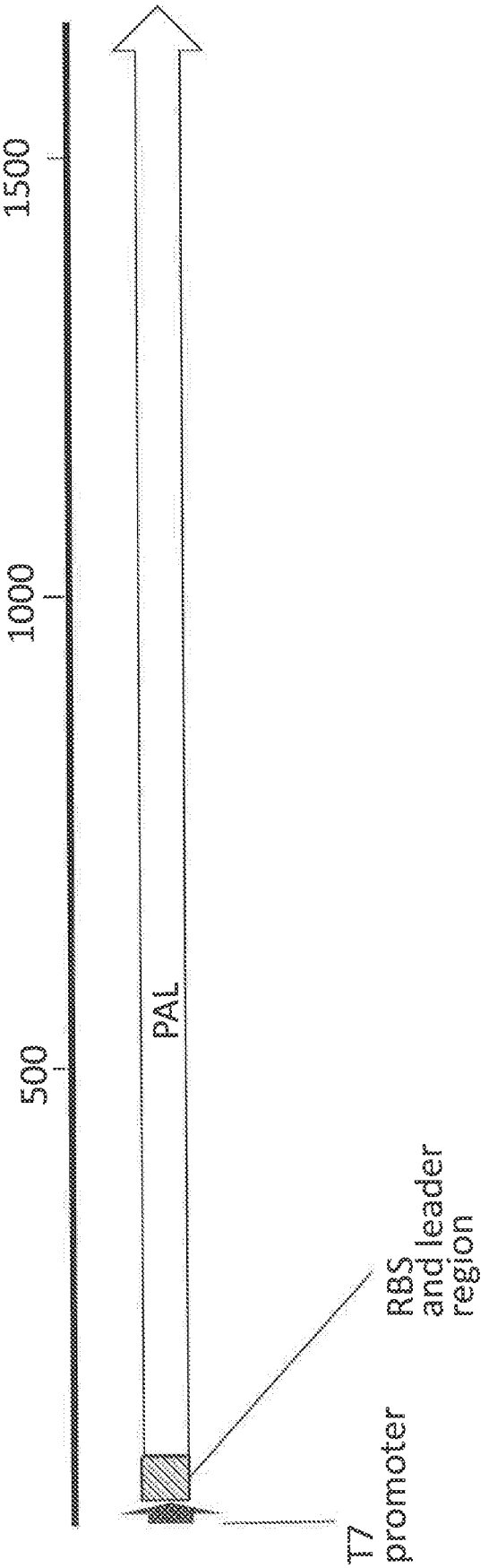


Fig. 74C

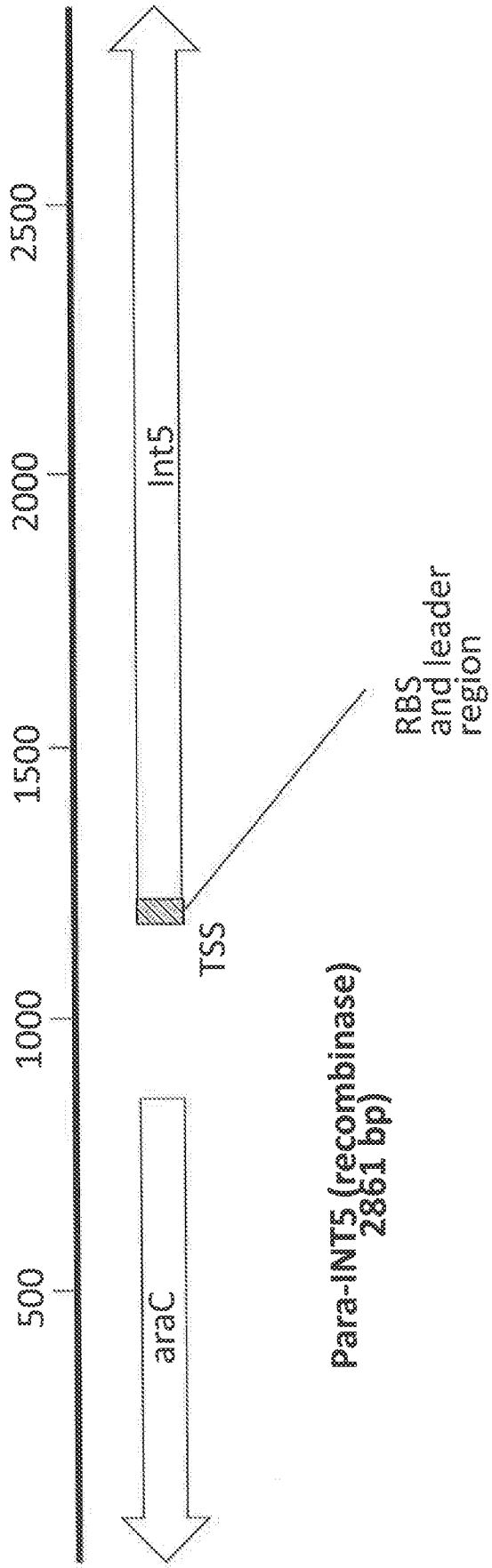

2500
2000
1500
1000
500
Int5
RBS
and leader
region
TSS
araC
Para-INT5 (recombinase)
2861 bp)

Fig. 75A

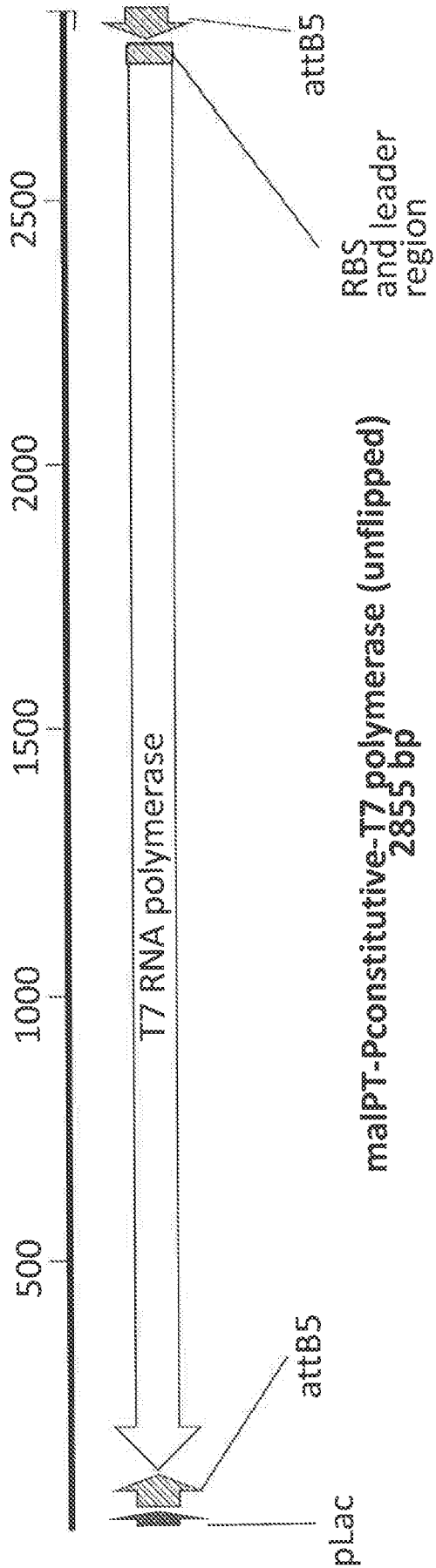

attB5
RBS and leader region
2500
2000
1500
1000
500
T7 RNA polymerase
malPT-Pconstitutive-T7 polymerase (unflipped)
2855 bp
attB5
pLac

Fig. 75B

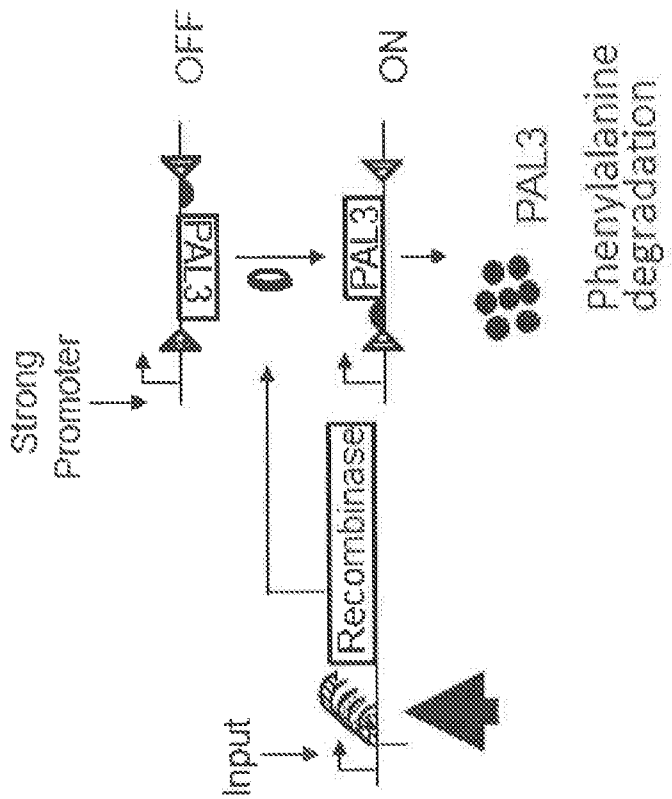

Strong Promoter
PAL3
OFF
0
PAL3
ON
PAL3
Phenylalanine degradation
Recombinase
Input

Fig. 76A

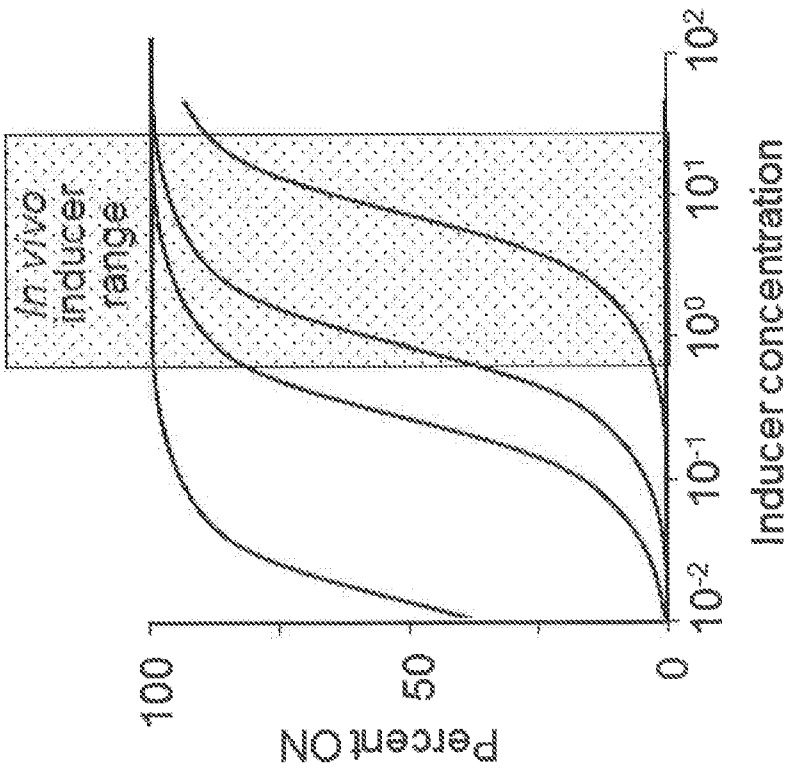

In vivo inducer range
Percent ON
100
50
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
Inducer concentration

Fig. 76B

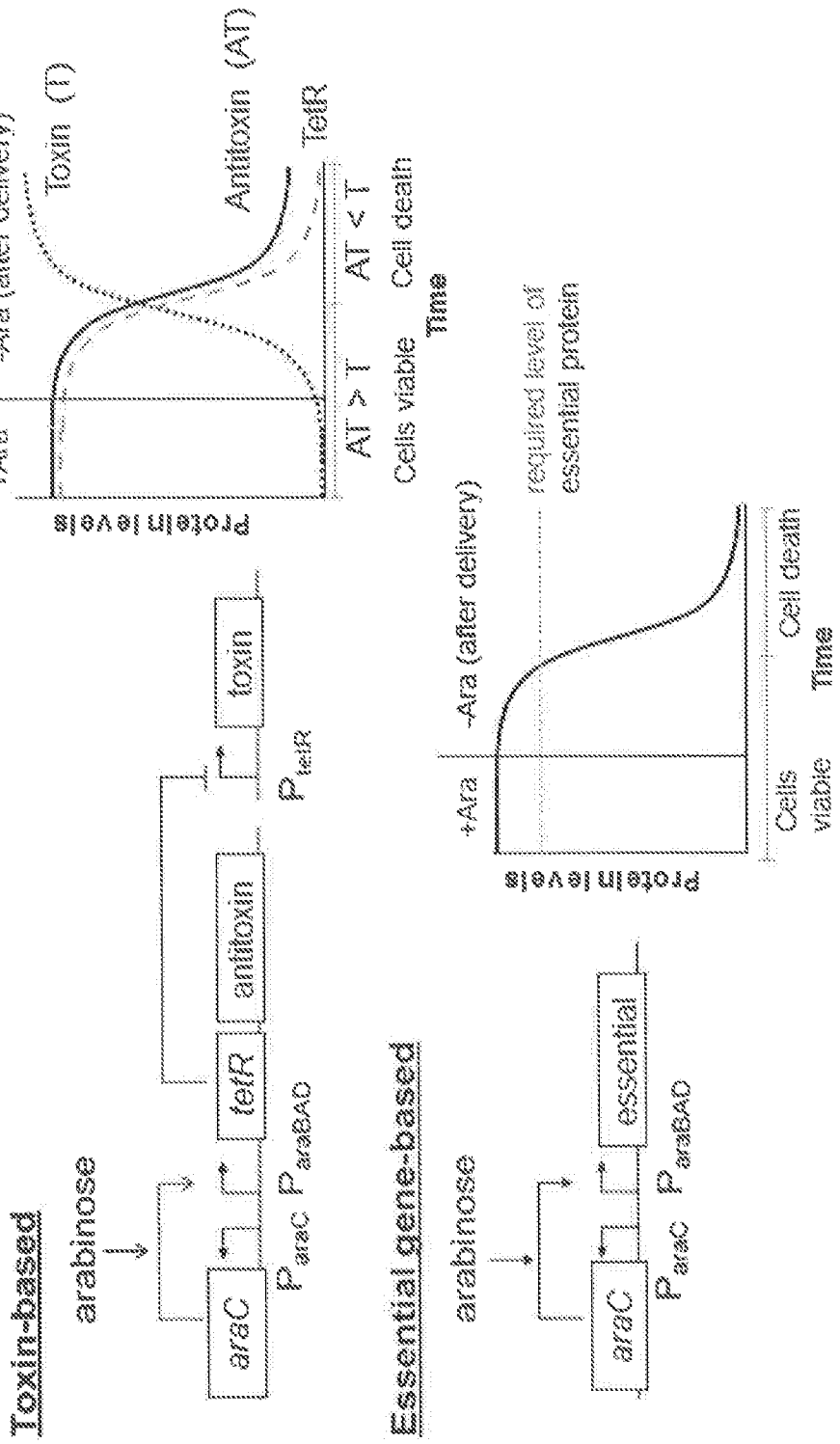

Toxin-based
arabinose
araC
P_{araC}
tetR
P_{araBAD}
antitoxin
toxin
P_{tetR}
+Ara
-Ara (after delivery)
Toxin (T)
Antitoxin (AT)
TetR
Protein levels
AT > T
AT < T
Cells viable
Cell death
Time
Essential gene-based
arabinose
araC
P_{araC}
essential
P_{araBAD}
+Ara
-Ara (after delivery)
required level of essential protein
Protein levels
Cells viable
Cell death
Time

Fig. 77A

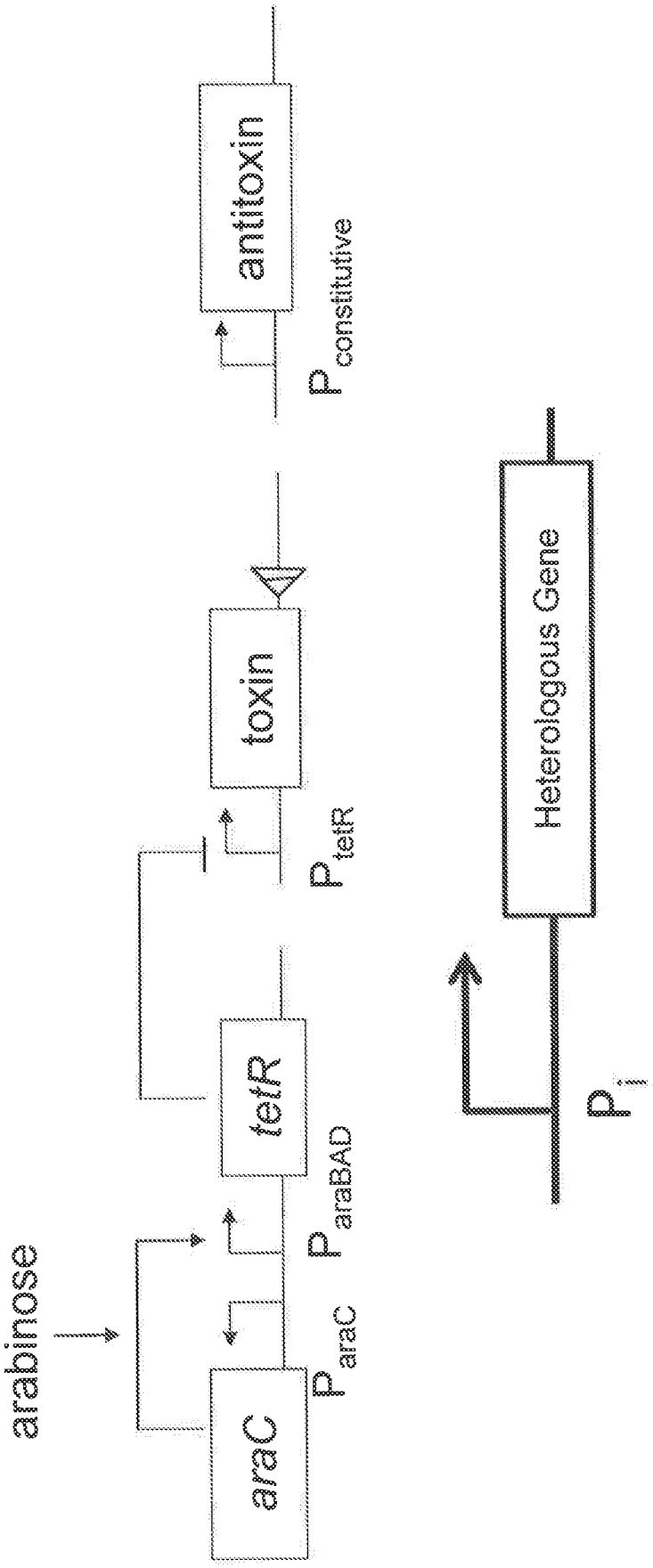

arabinose
araC
P araC
tetR
P araBAD
toxin
P tetR
antitoxin
P constitutive
Heterologous Gene
P i

Fig. 77B

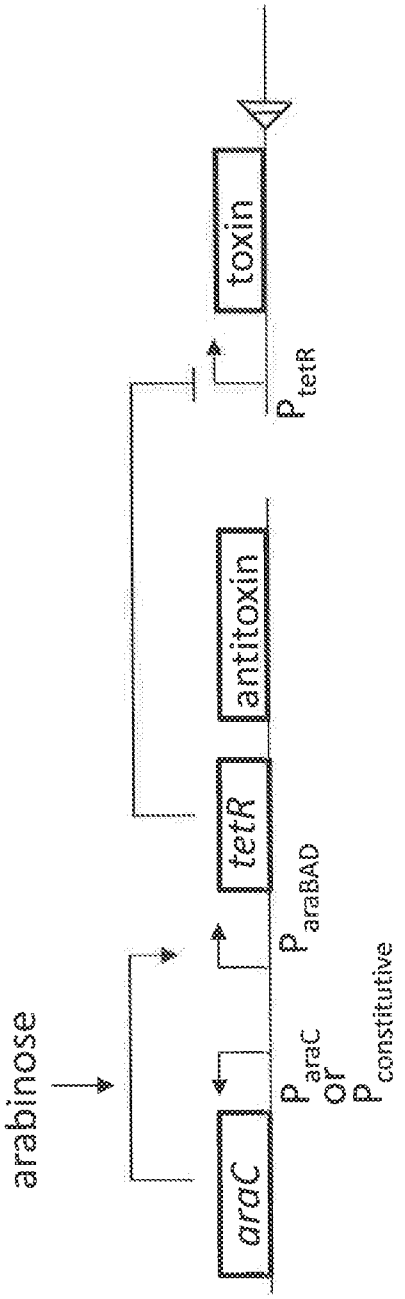

arabinose
araC
tetR
antitoxin
toxin
P araC
OR
P constitutive
P araBAD
P tetR

Fig. 77C

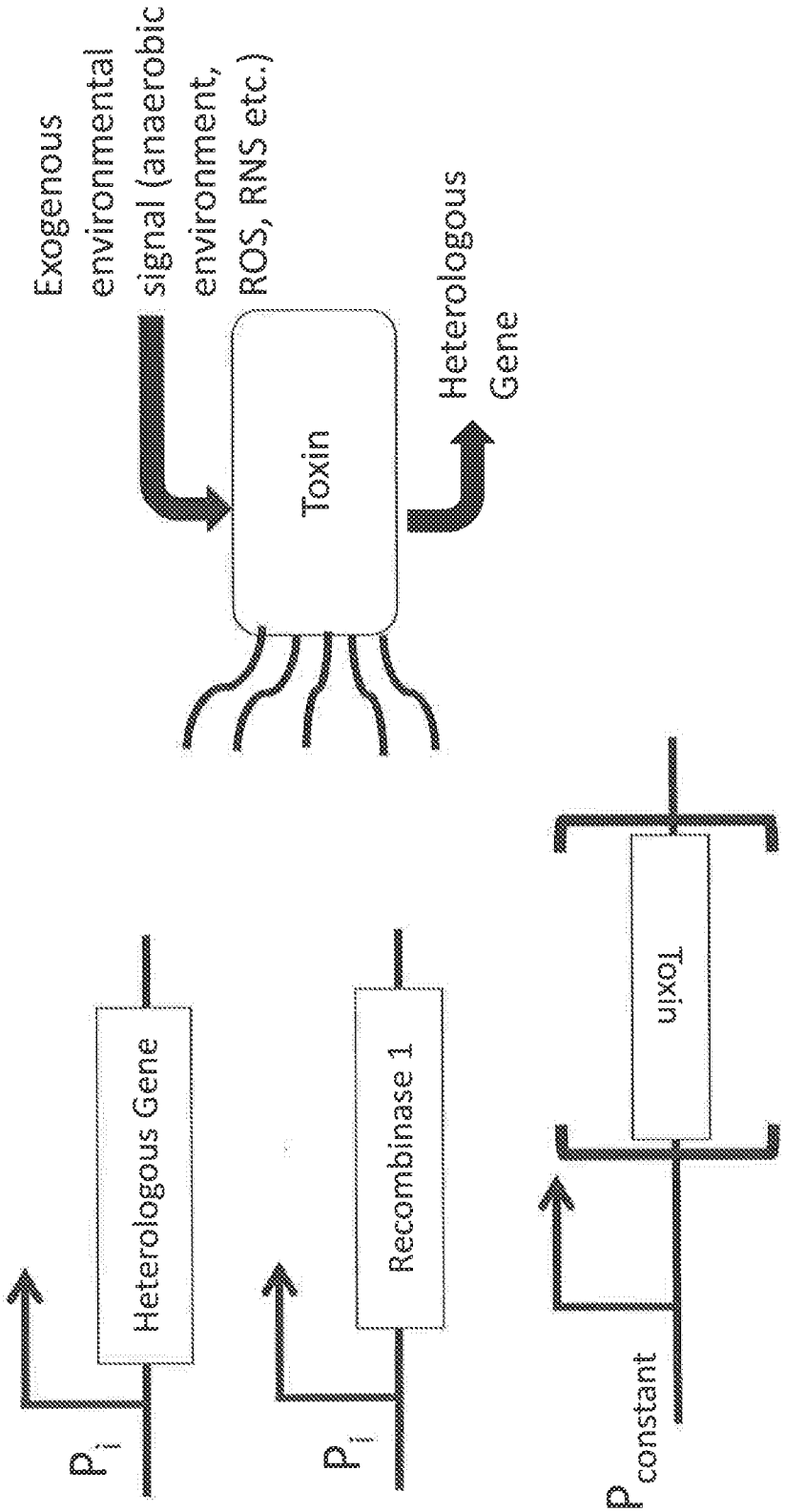

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
Heterologous Gene
Recombinase 1
Toxin
P_i
P_i
$P_{constant}$

Fig. 78

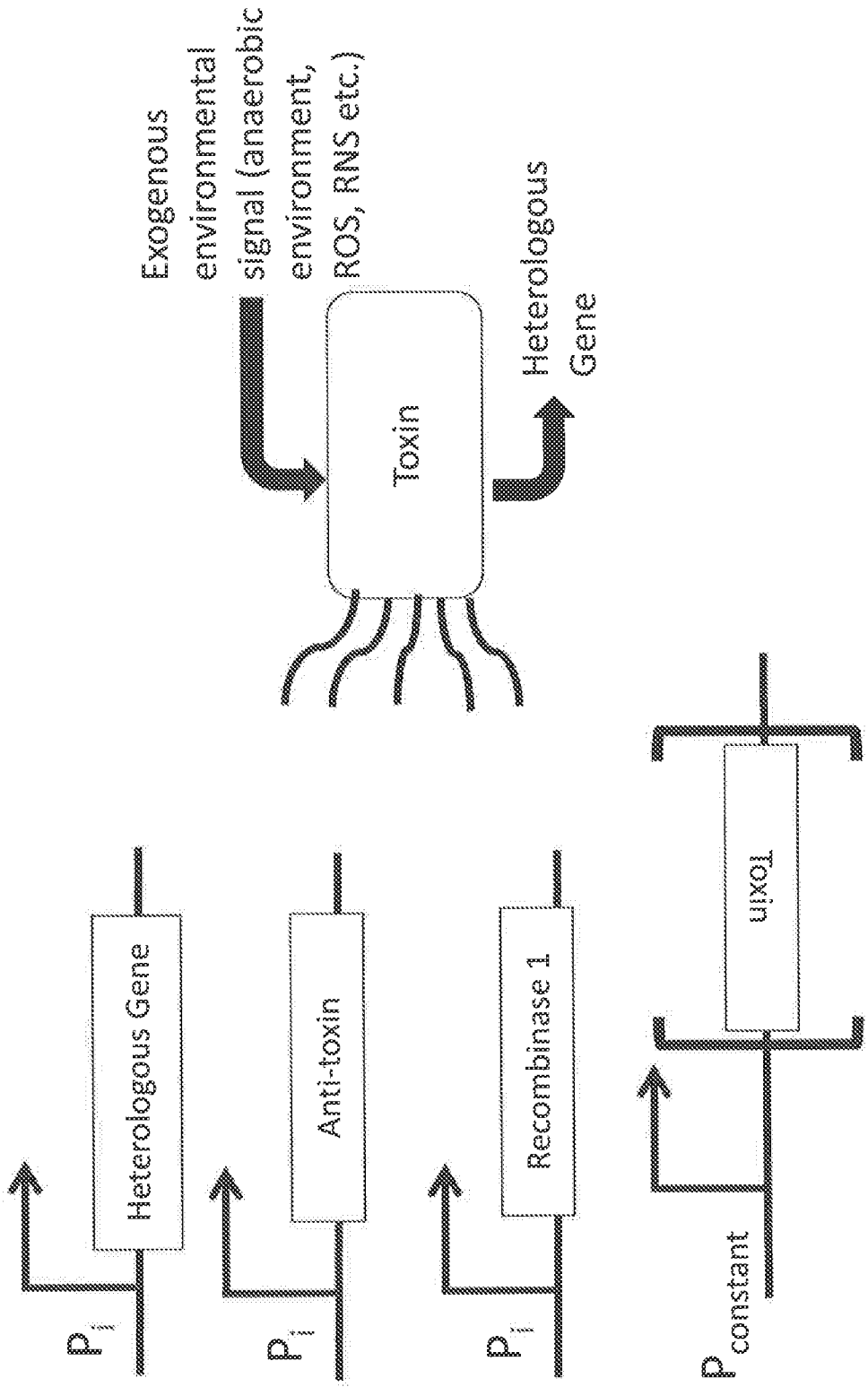

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
Heterologous Gene
Anti-toxin
Recombinase 1
Toxin
P_i
P_i
P_i
P_constant

Fig. 79

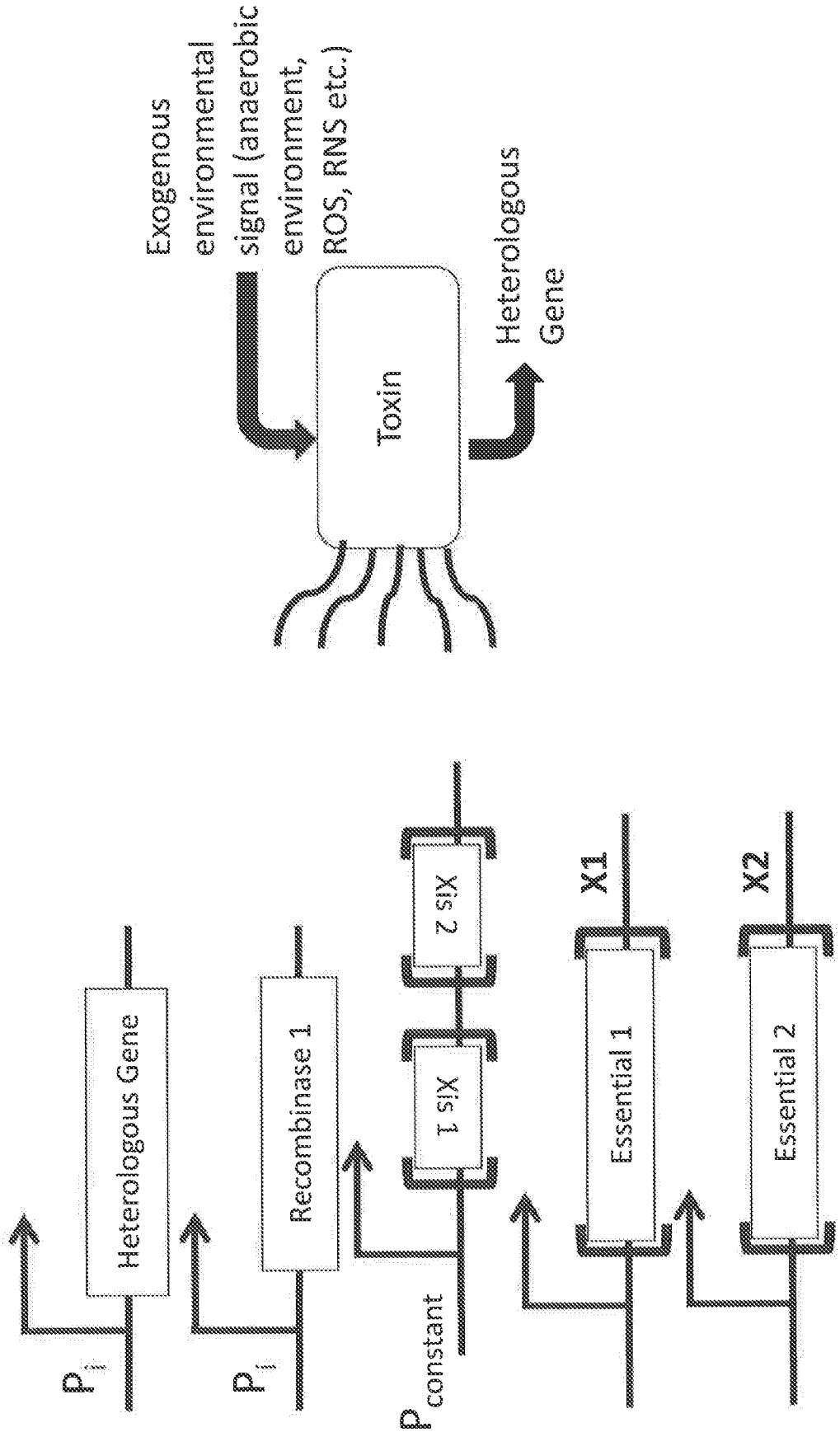

Exogenous environmental signal (anaerobic environment, ROS, RNS etc.)
Toxin
Heterologous Gene
Heterologous Gene
Recombinase 1
Xis 1
Xis 2
X1
X2
Essential 1
Essential 2
P_i
P_i
$P_{constant}$

Fig. 80

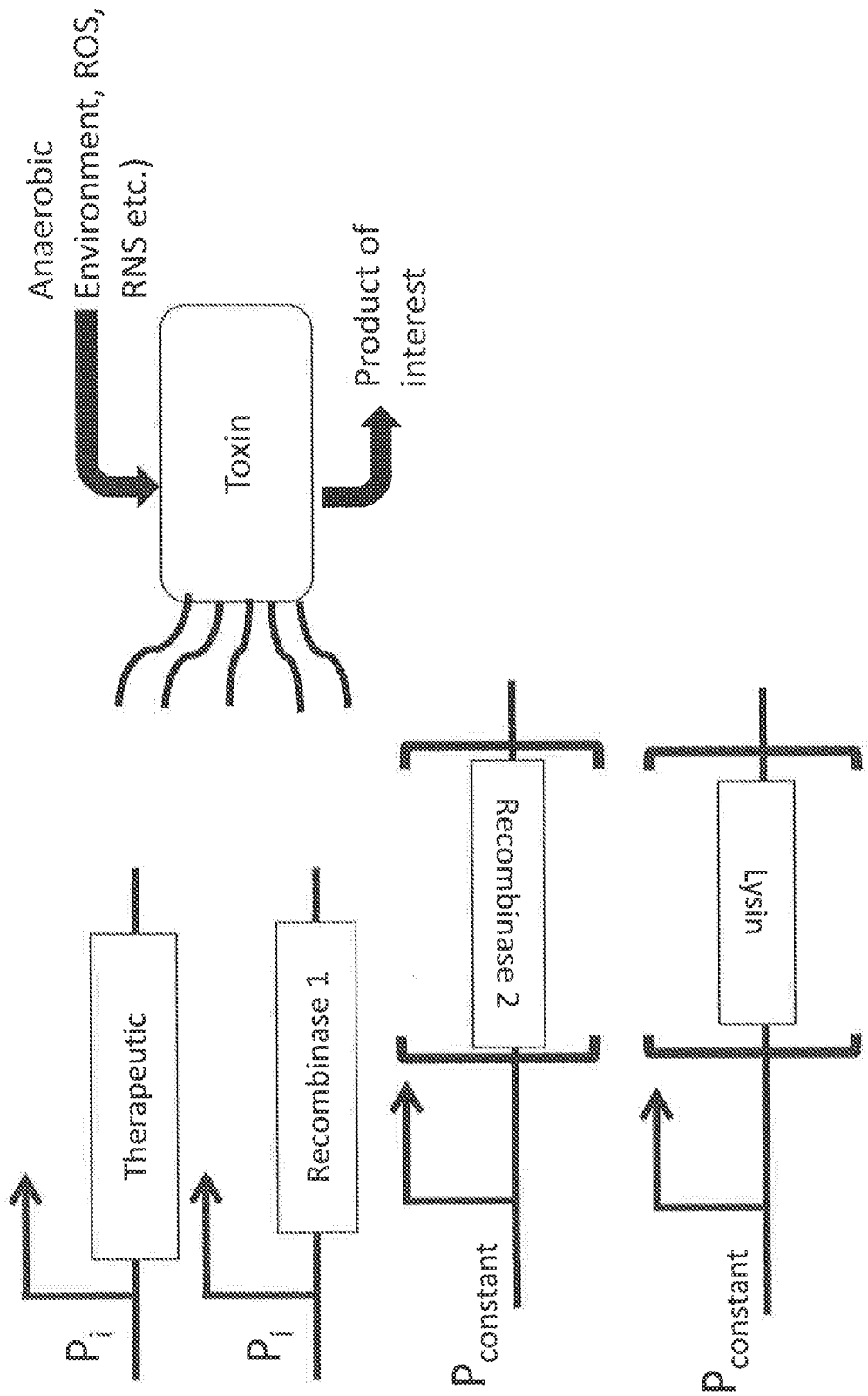

Anaerobic Environment, ROS, RNS etc.)
Toxin
Product of interest
Therapeutic
Recombinase 1
Recombinase 2
Lysin
P_i
P_i
$P_{constant}$
$P_{constant}$

Fig. 81

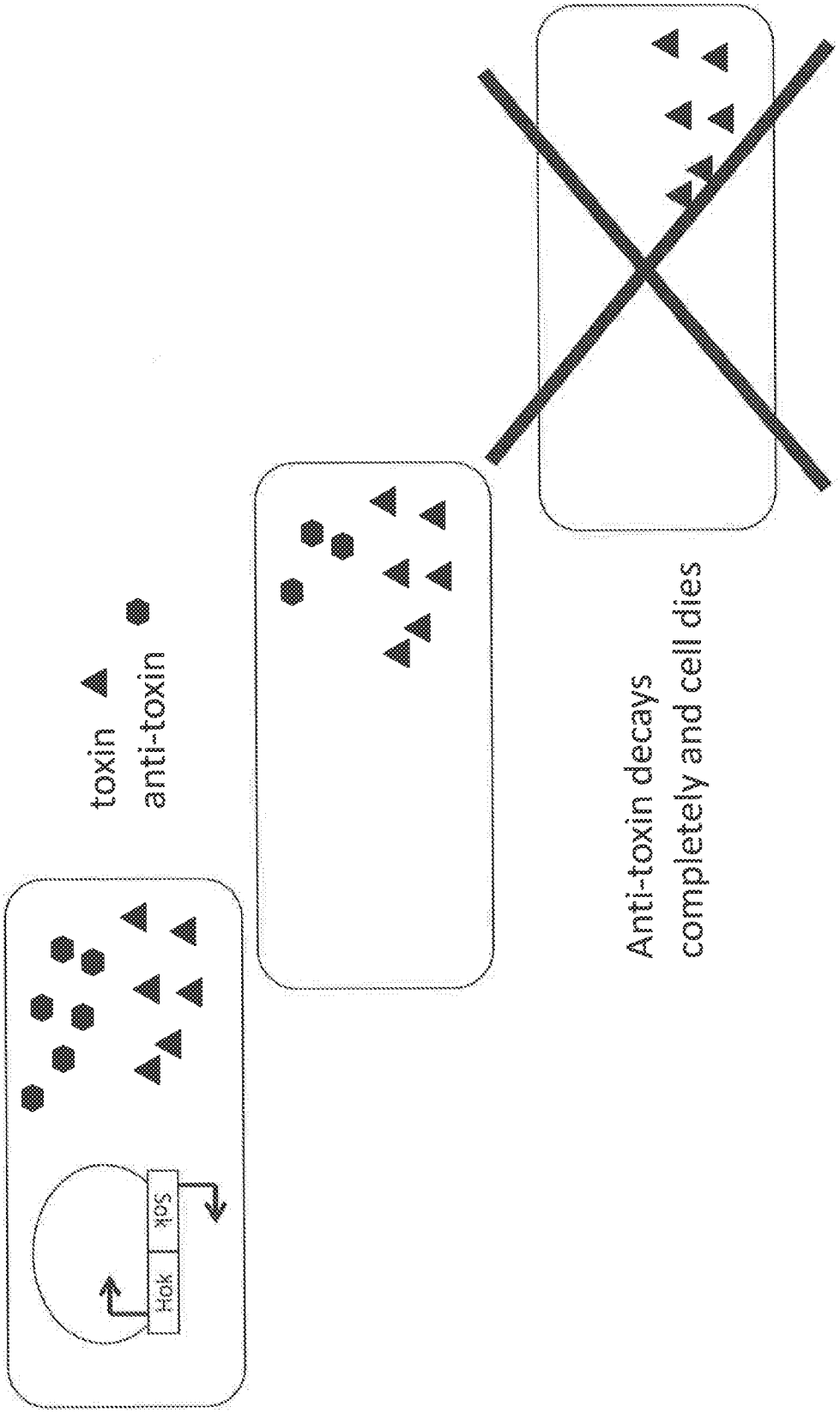

toxin
anti-toxin
Hok
sok
Anti-toxin decays
completely and cell dies

Fig. 82

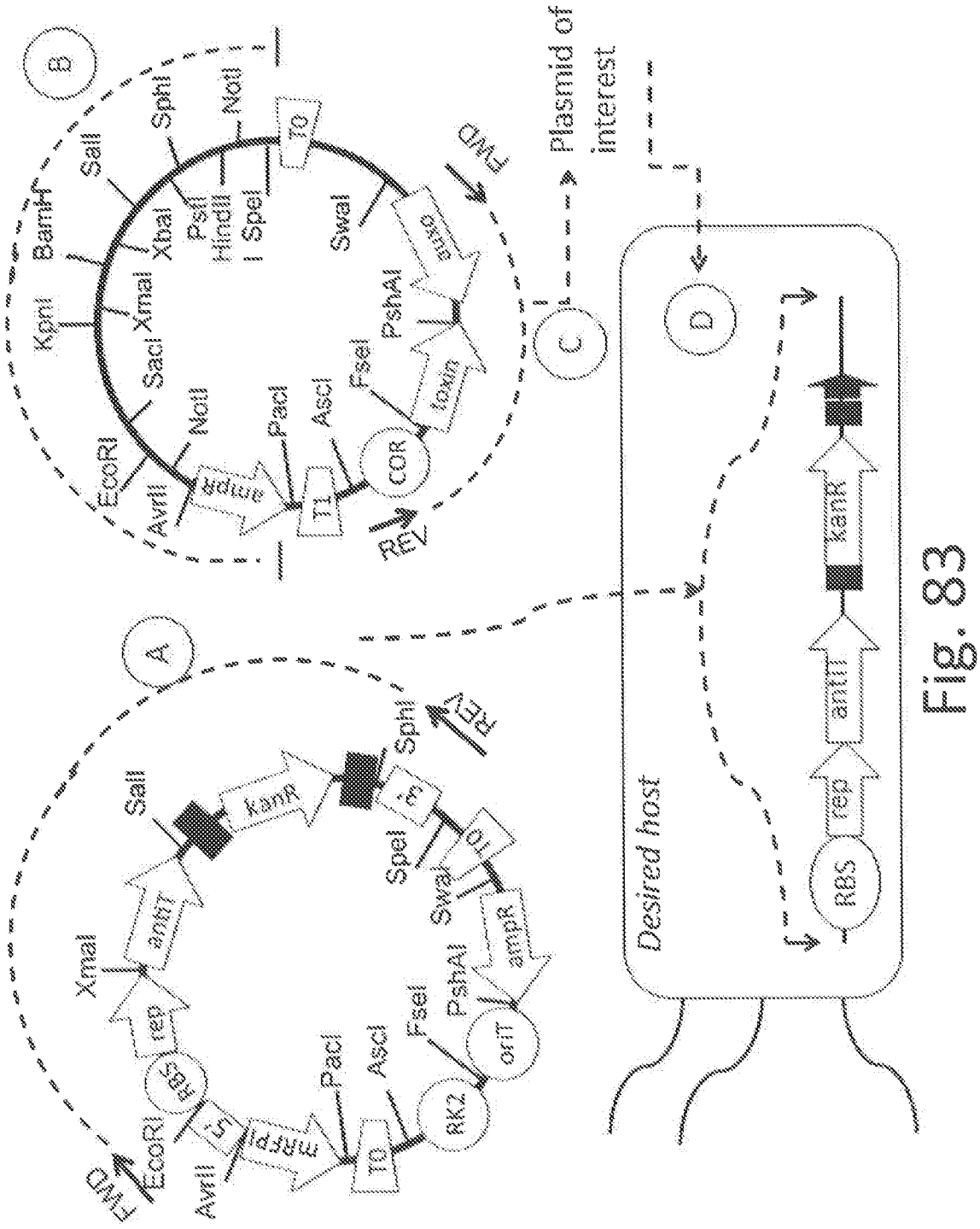

A
B
C
D
Plasmid of interest
Desired host
FWD
REV
EcoRI
AvrII
XmaI
SalI
XbaI
PstI
HindIII
SpeI
NotI
SphI
PacI
AscI
FseI
PshAI
SwaI
T0
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
RBS
rep
antiT
kanR
ampR
oriT
RK2
COR
toxin
antitoxin
araBAD
araC
3RFP1

Fig. 83

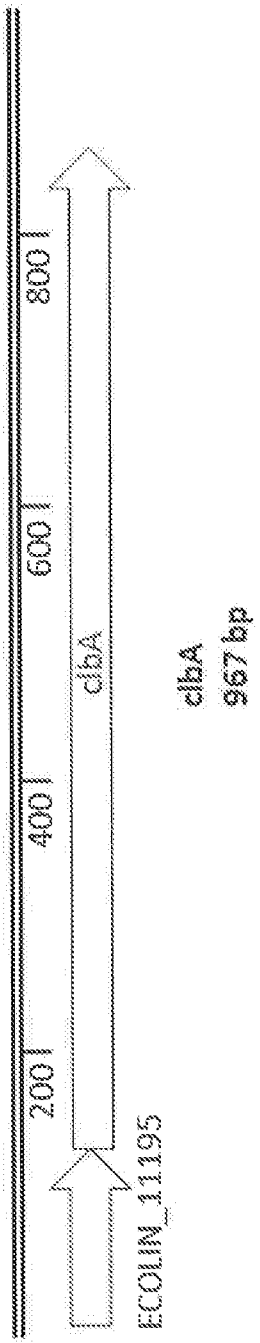


Fig. 84A

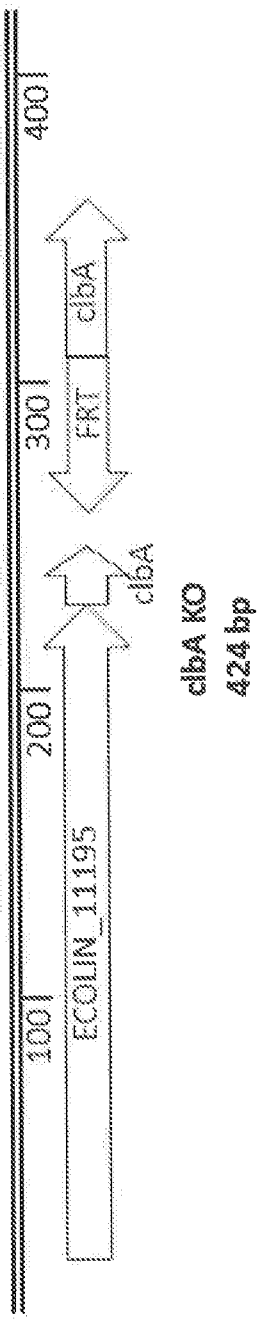


Fig. 84B

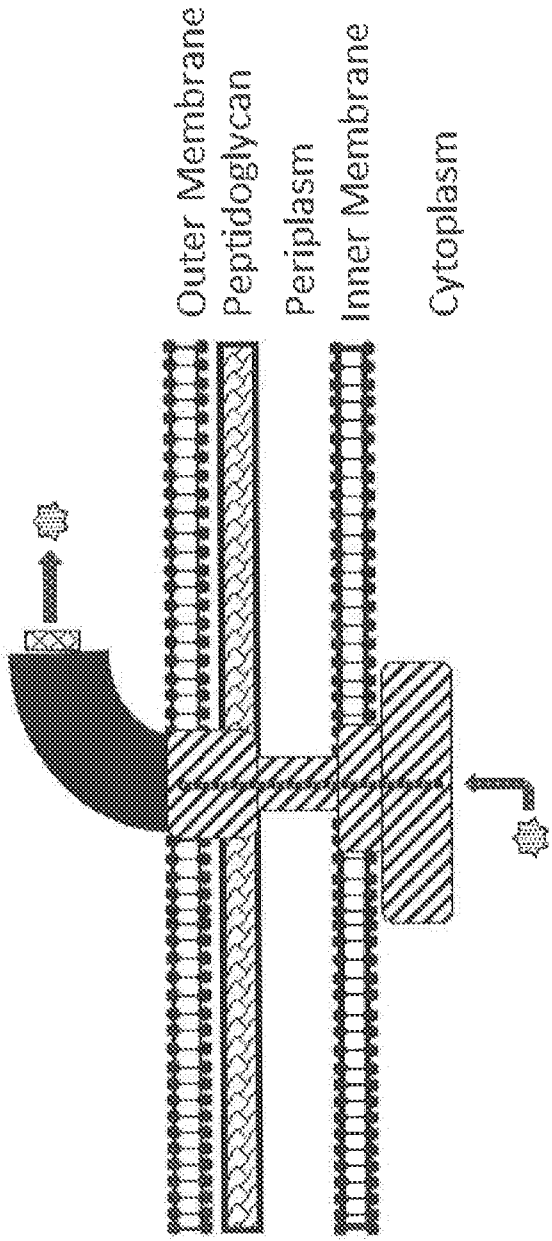

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm

Fig. 85

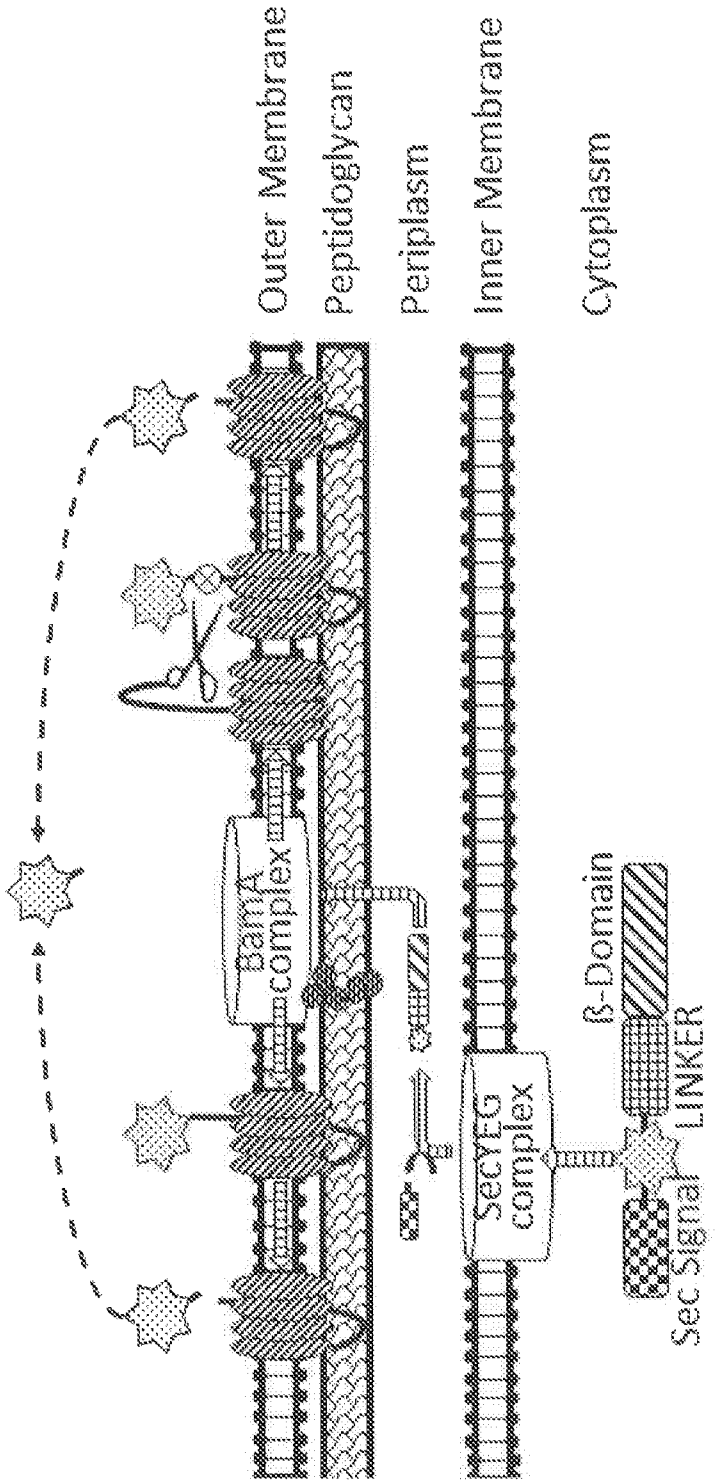

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
BamA complex
SecYEG complex
ß-Domain
LINKER
Sec Signal

Fig. 86

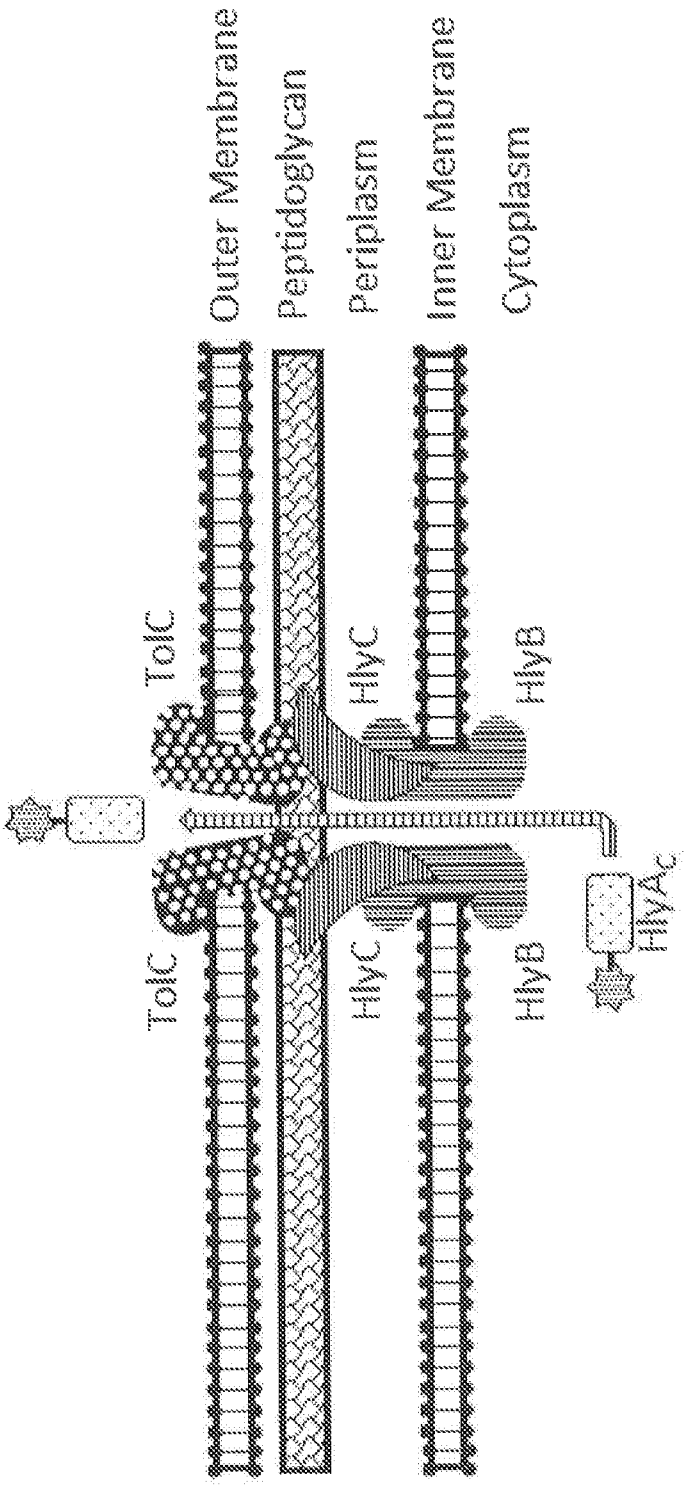

Outer Membrane
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
TolC
HlyC
HlyB
TolC
HlyC
HlyB
HlyA$_C$

Fig. 87

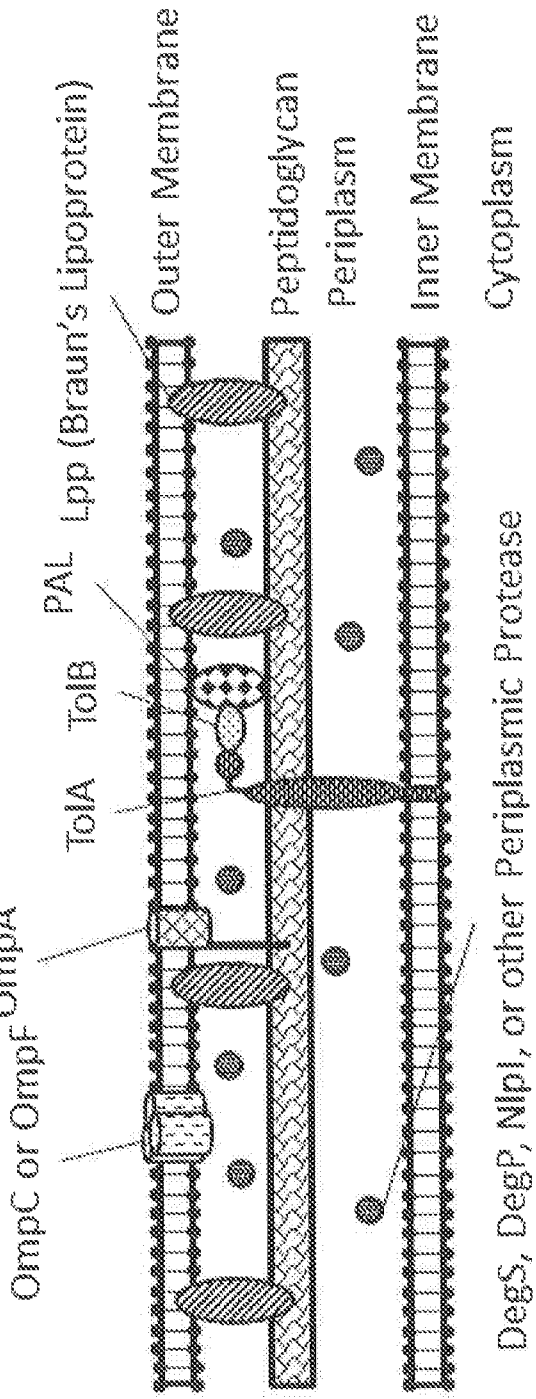

Outer Membrane
Lpp (Braun's Lipoprotein)
PAL
TolB
TolA
OmpA
OmpC or OmpF
Peptidoglycan
Periplasm
Inner Membrane
Cytoplasm
DegS, DegP, Nlpl, or other Periplasmic Protease

Fig. 88

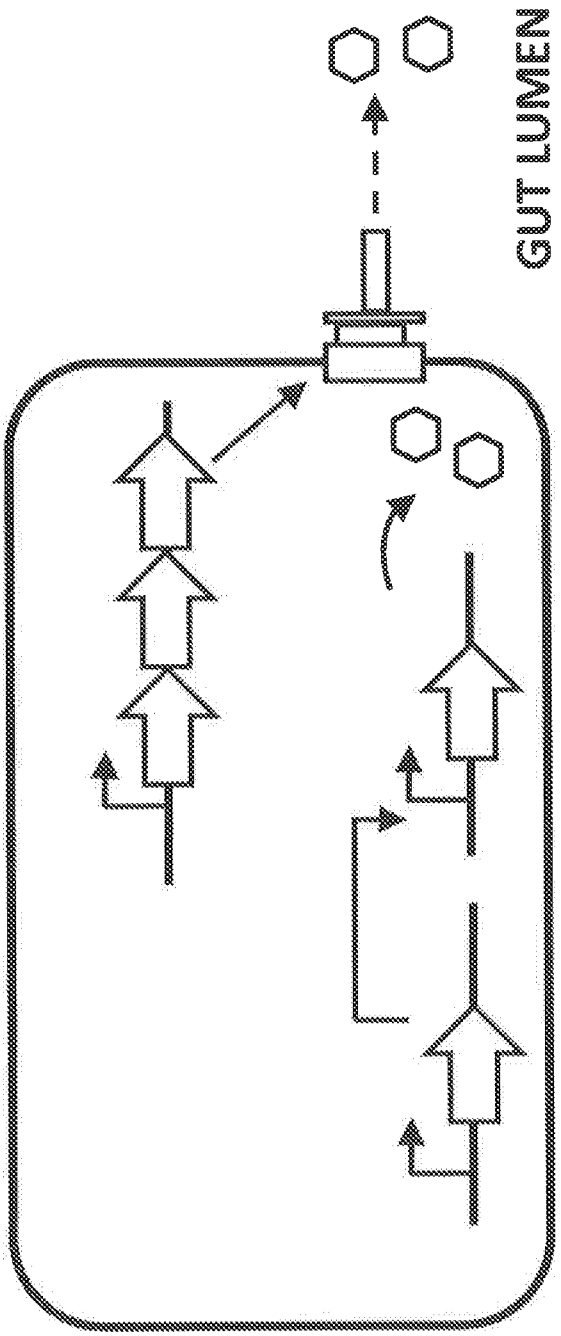


Fig. 89

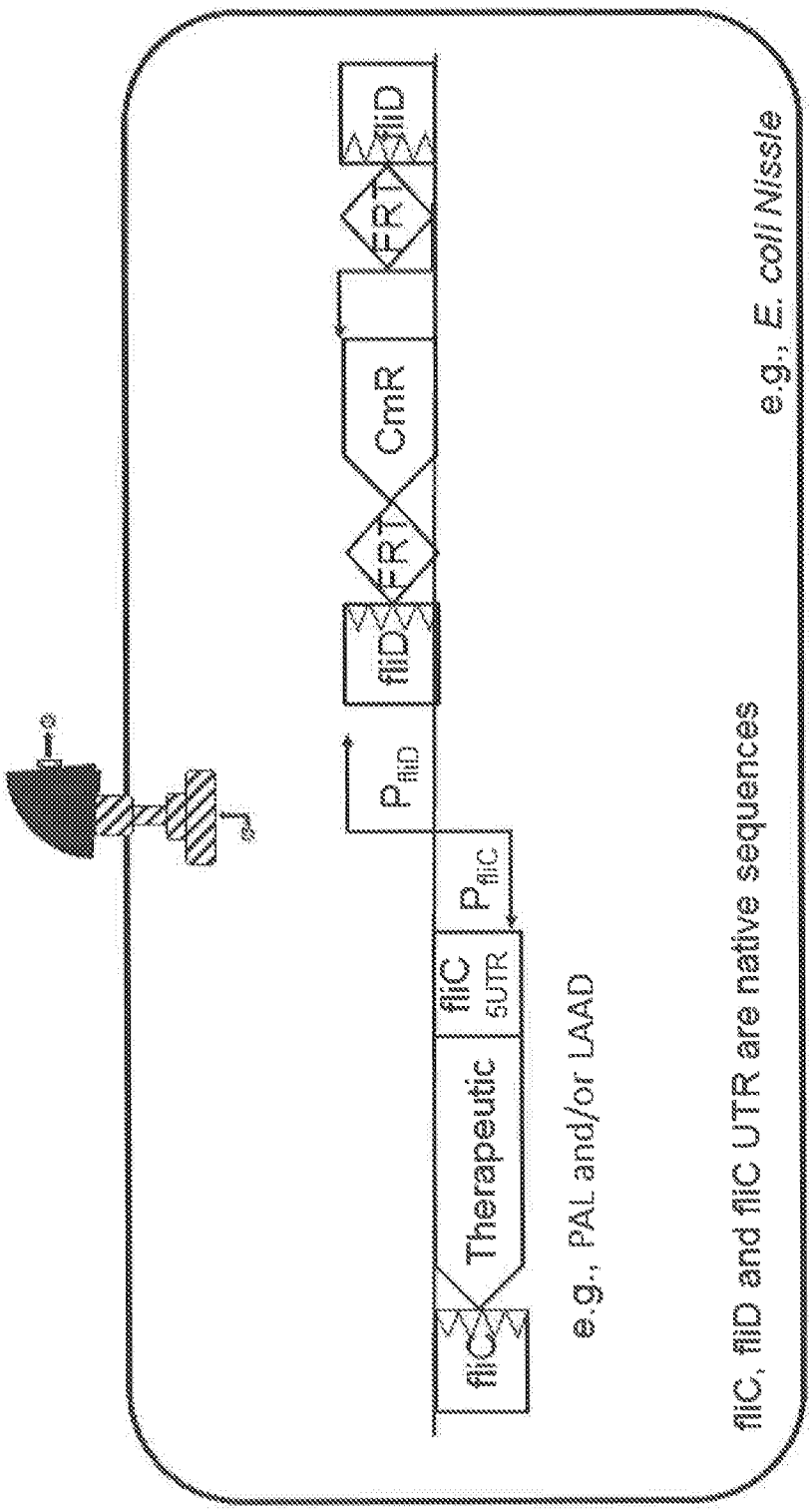

e.g., E. coli Nissle
flIC, flID and flIC UTR are native sequences
e.g., PAL and/or LAAD
flIC
Therapeutic
flIC 5UTR
P flIC
P flID
flID
FRT
CmR
FRT
flID

Fig. 90A

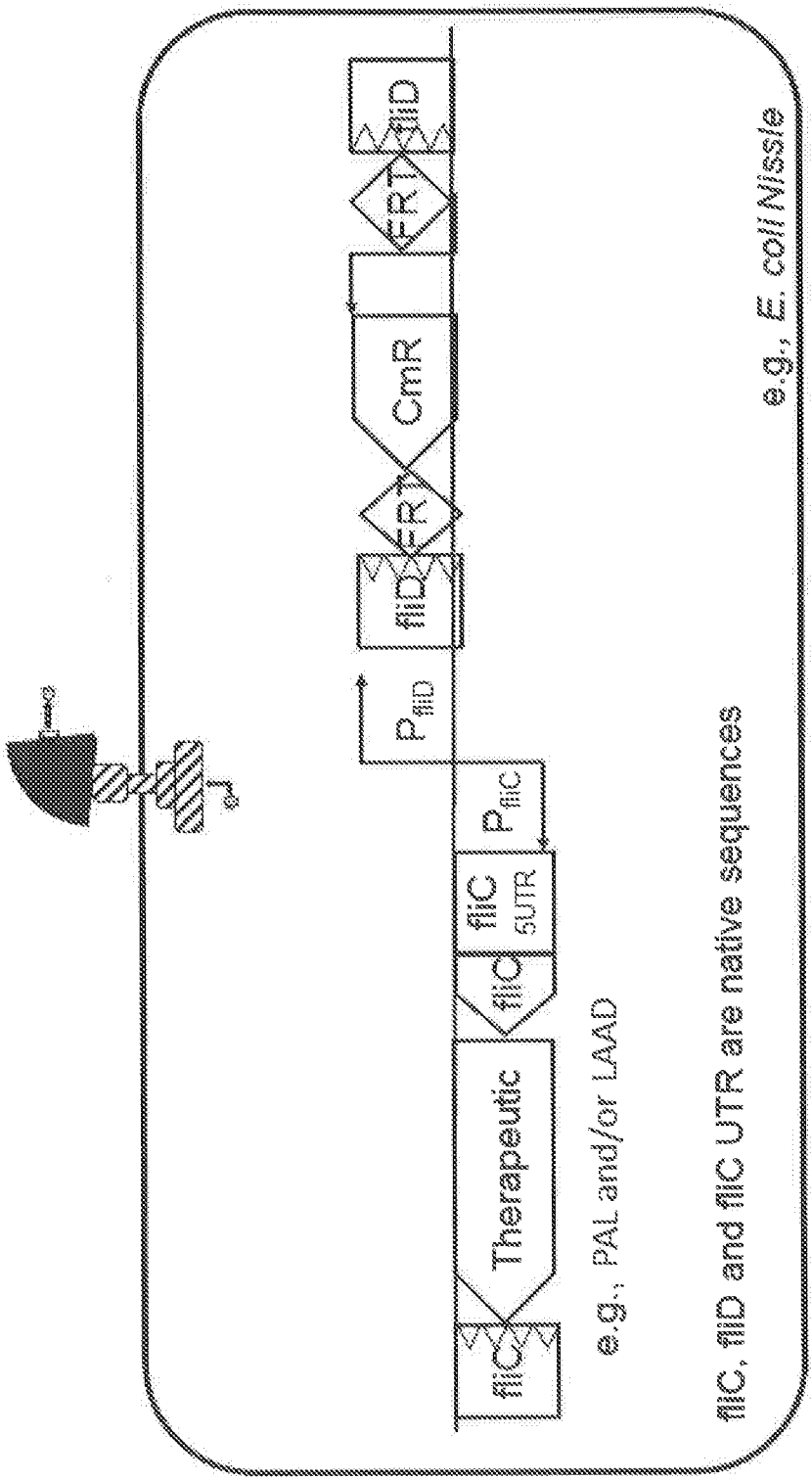

e.g., E. coli Nissle
fliC, flID and fliC UTR are native sequences
e.g., PAL and/or LAAD
fliC
Therapeutic
fliC
fliC 5UTR
PfliC
PflID
flID
FRT
CmR
FRT
flID
FRT

Fig. 90B

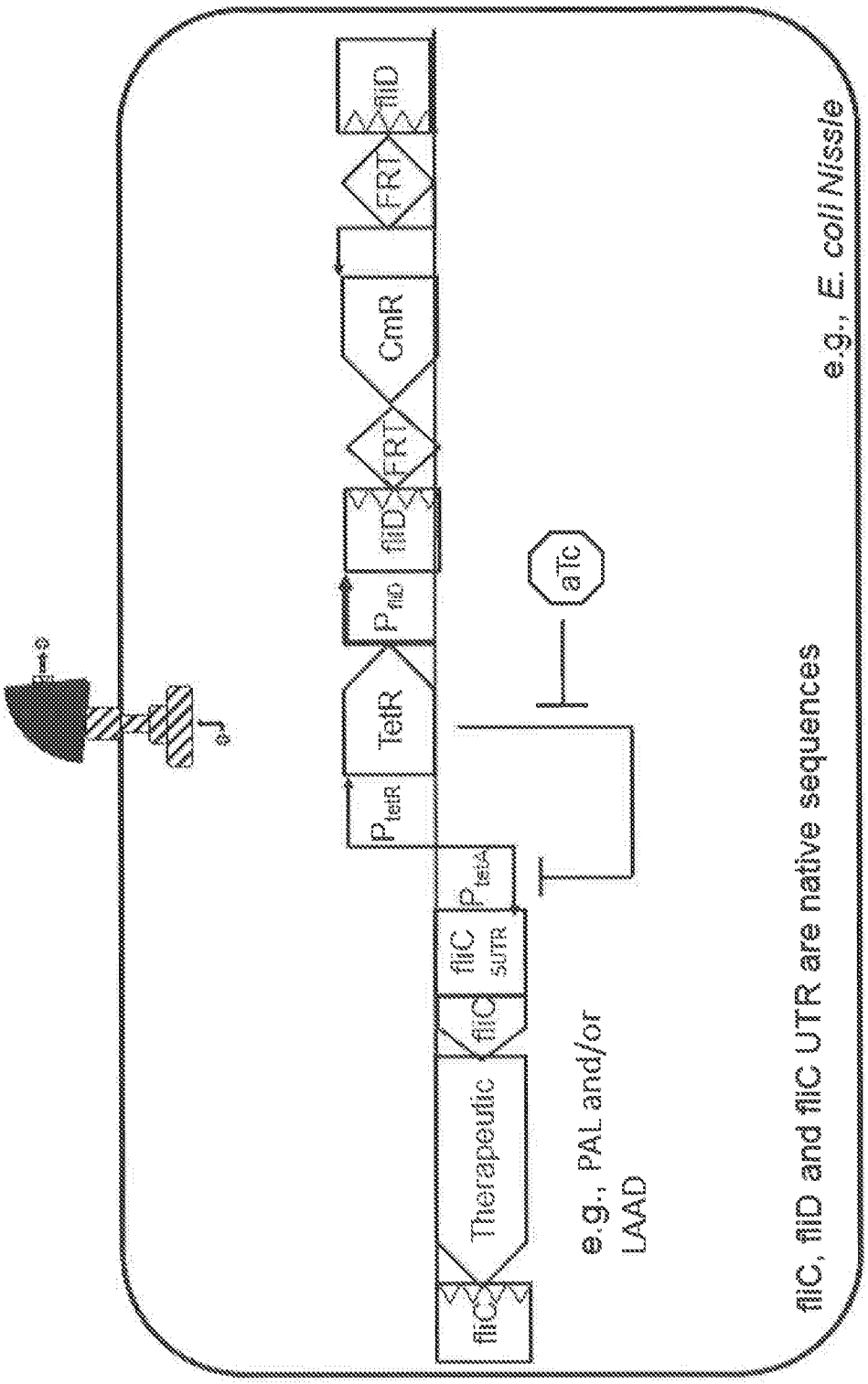

fliC, fliD and fliC UTR are native sequences
e.g., E. coli Nissle
fliC
Therapeutic
fliD
fliC 5UTR
P_{tetA}
e.g., PAL and/or LAAD
P_{tetR}
TetR
aTc
P_{fliD}
fliD
FRT
CmR
FRT
fliD
FRT

Fig. 90C

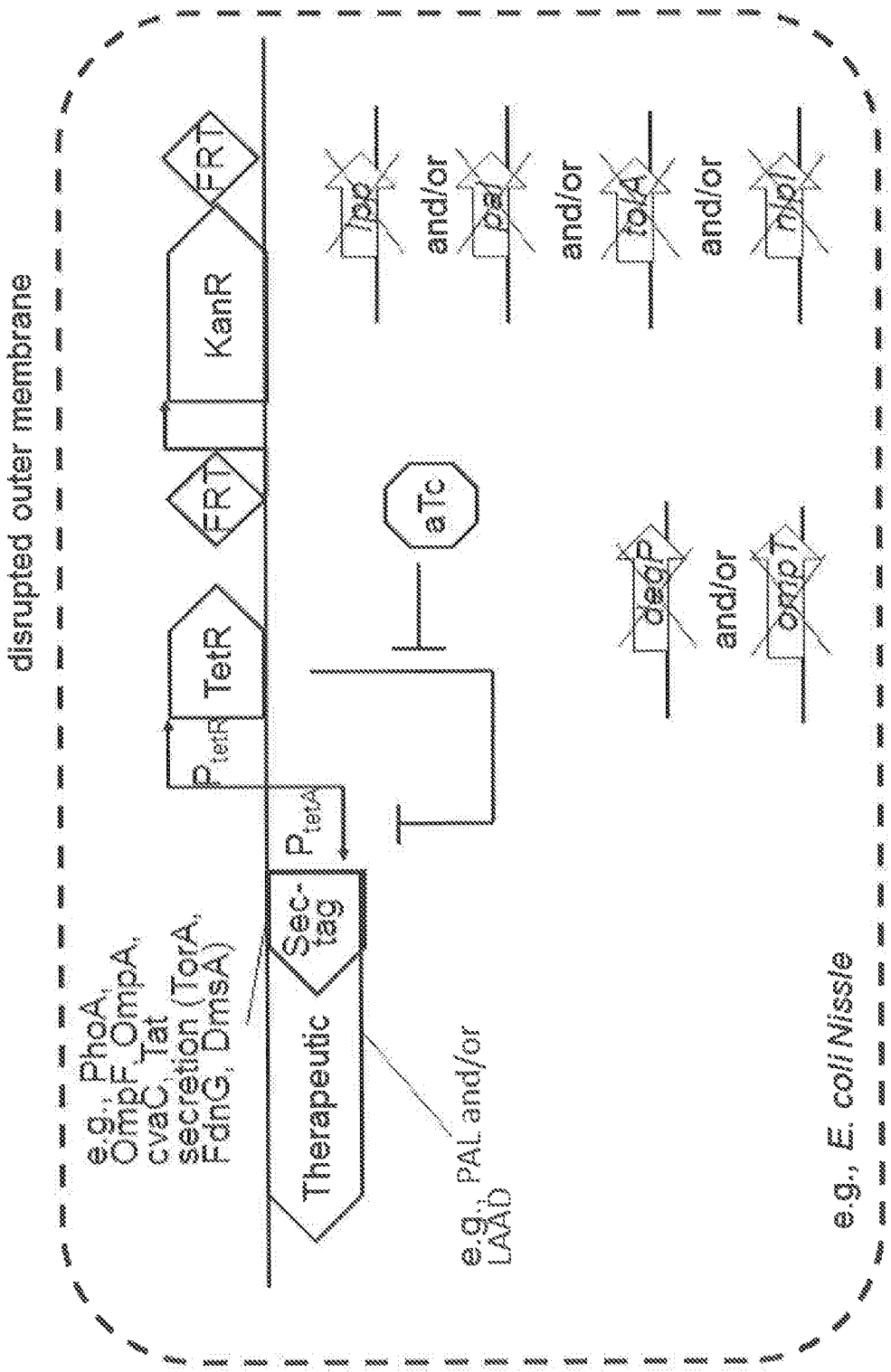

disrupted outer membrane
e.g., PhoA, OmpF, OmpA, cvaC, Tat secretion (TorA, FdnG, DmsA)
Therapeutic
Sec-tag
e.g., PAL and/or LAAD
P_{tetA}
P_{tetR}
TetR
FRT
KanR
FRT
aTc
e.g., E. coli Nissle
lpp
and/or
pal
and/or
tolA
and/or
nlpI
degP
and/or
ompT

Fig. 91A

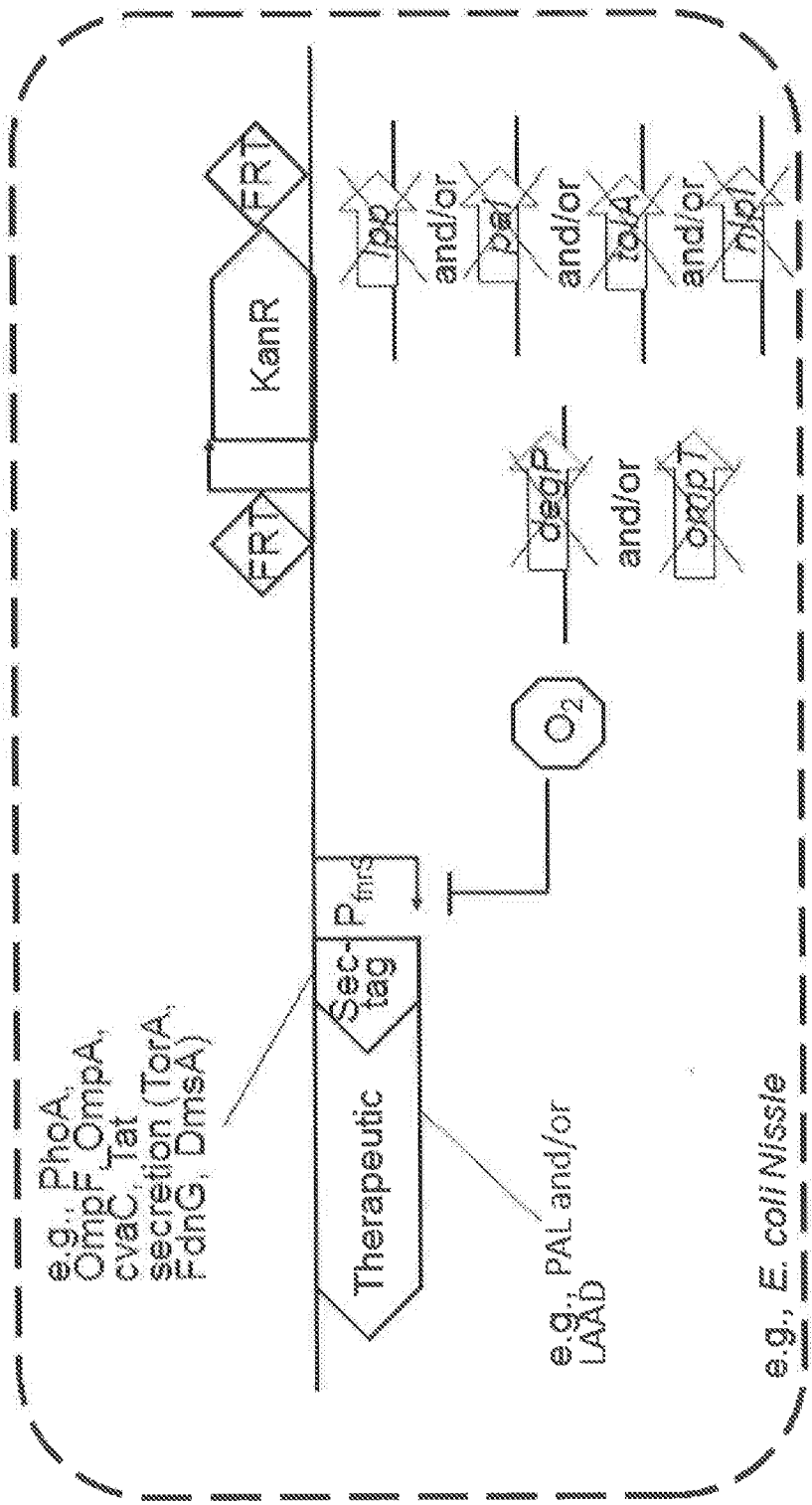

e.g., PhoA, OmpF, OmpA, cvaC, Tat secretion (TorA, FdnG, DmsA)
Sec tag
Therapeutic
P_{fnr}
O_2
e.g., PAL and/or LAAD
FRT
KanR
FRT
lpp
and/or
pal
and/or
tolA
and/or
nlpI
dcaP
and/or
ompT
e.g., E. coli Nissle

Fig. 91B

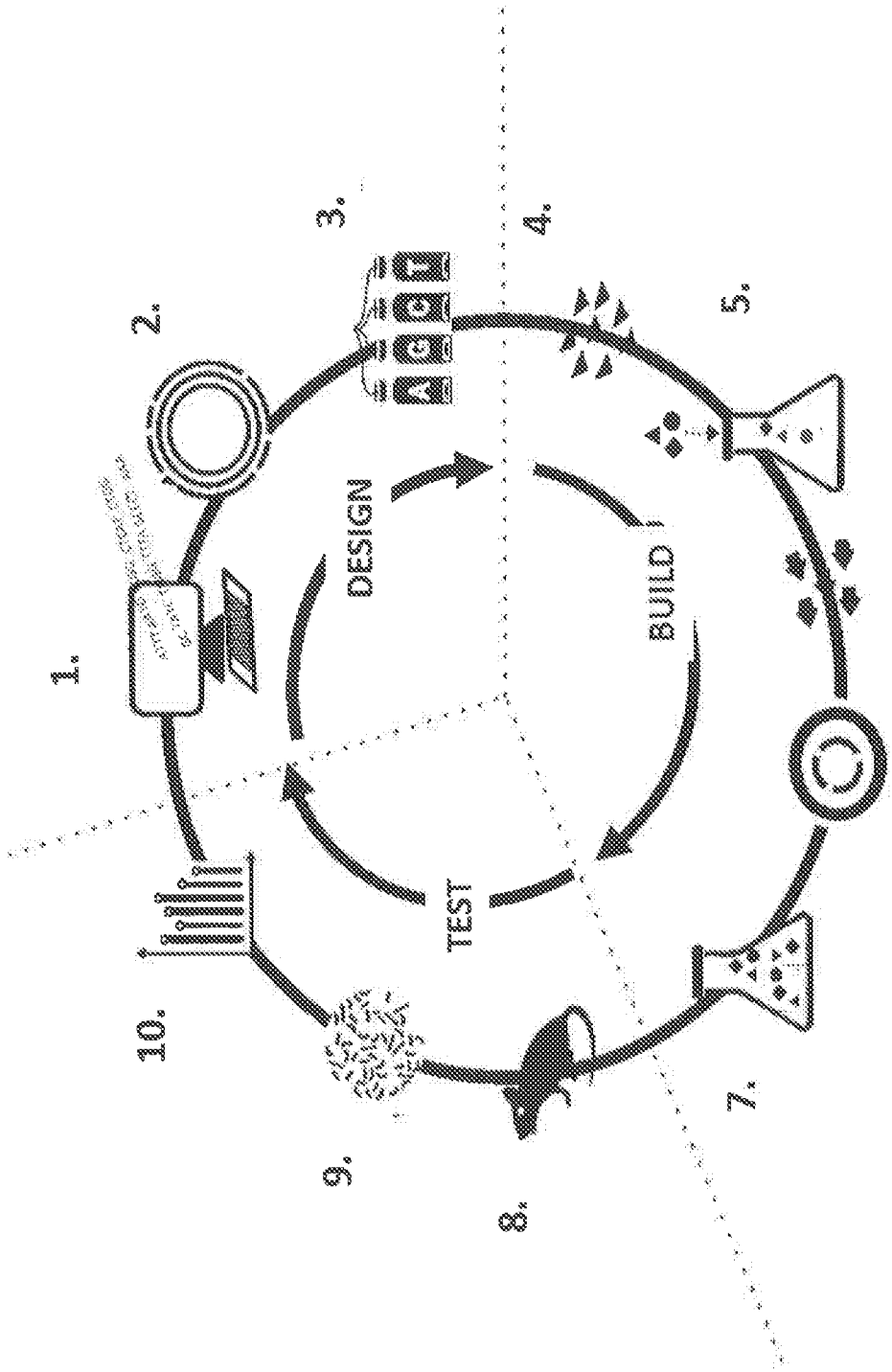


Fig. 92

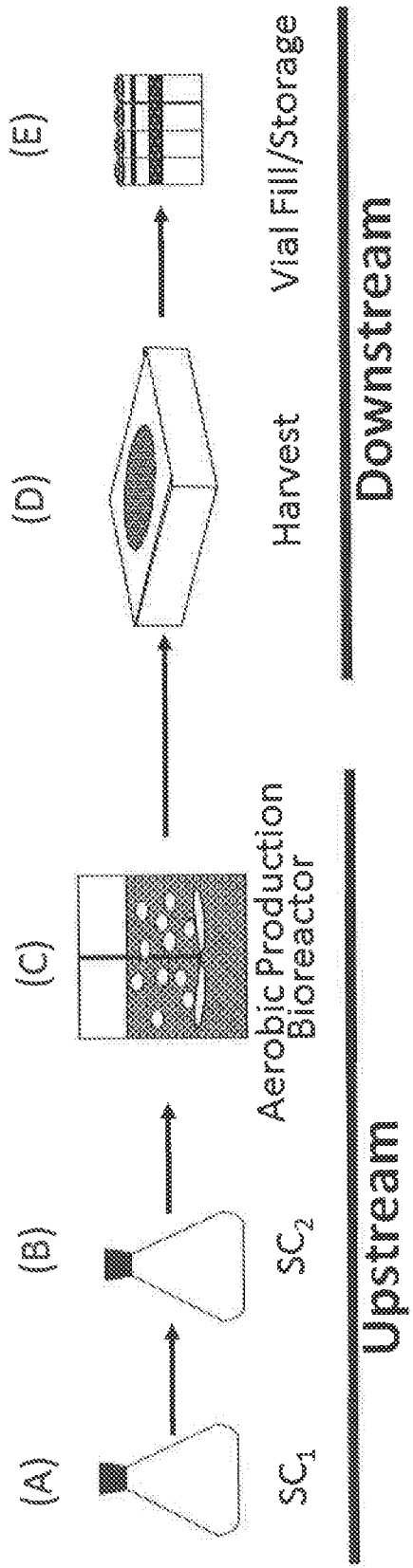

(A)
(B)
(C)
(D)
(E)
SC_1
SC_2
Aerobic Production Bioreactor
Harvest
Vial Fill/Storage
Upstream
Downstream

Fig. 93

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/062369

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.
X	CHRISTINEH N. SARKISSIAN ET AL: "Quantitation of phenylalanine and itstrans-cinnamic, benzoic and hippuric acid metabolites in biological fluids in a single GC-MS analysis", JOURNAL OF MASS SPECTROMETRY., vol. 42, no. 6, 1 June 2007 (2007-06-01), pages 811-817, XP055348903, GB ISSN: 1076-5174, DOI: 10.1002/jms.1218 figure 5; table 1 page 812 - column 2, paragraph 1 page 816, lines 11-18;30-37 - column 2 ----- -/--	46-48

[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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2017	10/03/2017

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/062369

C(Continuation). 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 paragraph [02.5] - paragraph [02.6]	1-45
A	WO 2013/192543 A2 (PHYTOGENE INC [US]) 27 December 2013 (2013-12-27) abstract; claims 20,23 paragraphs [0152], [0357]	1-45
A	WO 2009/004595 A2 (UNIV NORTHWEST [ZA]; GROBLER ANNE FREDERICA [ZA]; KOTZE ABRAHAM FREDER) 8 January 2009 (2009-01-08) abstract	1-45
E	WO 2016/183532 A1 (SYNLOGIC INC [US]) 17 November 2016 (2016-11-17) the whole document	1-48
E	WO 2016/183531 A1 (SYNLOGIC INC [US]) 17 November 2016 (2016-11-17) the whole document	1-48
E	WO 2016/210373 A2 (SYNLOGIC INC [US]) 29 December 2016 (2016-12-29) the whole document	1-48
A	NENAD BLAU ET AL: "Alternative therapies to address the unmet medical needs of patients with phenylketonuria", EXPERT OPINION ON PHARMACOTHERAPY, vol. 16, no. 6, 13 April 2015 (2015-04-13) , pages 791-800, XP055348839, LONDON, UK ISSN: 1465-6566, DOI: 10.1517/14656566.2015.1013030 abstract	46-48

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/062369

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		US 2017014457 A1	19-01-2017
		WO 2016183531 A1	17-11-2016
WO 2016210373 A2	29-12-2016	NONE	