

MICROORGANISMS ENGINEERED TO REDUCE
HYPERPHENYLALANINEMIA

[01] This application claims the benefit of U.S. Provisional Application No. 62/992,637, filed on March 20, 2020, and U.S. Provisional Application No. 63/017,755, filed on April 30, 2020, the contents of which are incorporated by reference in their entireties.

[02] This disclosure relates to compositions and therapeutic methods for reducing hyperphenylalaninemia. In certain aspects, the disclosure relates to genetically engineered microorganisms, e.g., 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.

[03] 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.

[04] 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.

[05] 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).

[06] 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).

[07] 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).

[08] 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.

[009] In some embodiments, the disclosure provides mutant PAL polypeptides and polynucleotides. In some embodiments, the mutant PAL exhibits increased stability and/or increased ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia compared to a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises one or more mutations at amino acid positions 92, 133, 167, 432, 470, 433, 263, 366 and/or 396 compared to a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises one or more mutations at amino acid positions S92, H133, I167, L432, V470, A433, A263, K366, and/or L396 compared to a wild type PAL, e.g., *P. luminescens* PAL.

[010] In some embodiments, the disclosure provides genetically engineered microorganisms, e.g., bacteria, that produce the mutant PAL. In some embodiments, the engineered microorganisms further comprise a gene encoding a phenylalanine transporter, e.g., PheP. In some embodiments, the engineered microorganisms may also comprise a gene encoding L-amino acid deaminase (LAAD). The engineered microorganisms may also contain one or more gene sequences relating to biosafety and/or biocontainment. The expression of any these gene sequence(s) in a gene expression system may be regulated using a suitable promoter or promoter system.

[011] In certain embodiments, the genetically engineered microorganisms are non-pathogenic and may be introduced into the gut in order to reduce toxic levels of phenylalanine. The disclosure also provides pharmaceutical compositions comprising the genetically engineered microorganisms, and methods of modulating and treating disorders associated with hyperphenylalaninemia. In some embodiments, the genetically engineered bacterium comprising the mutant PAL comprises one or more phage genome(s), wherein one or more of the phage genomes are defective, e.g., such that lytic phage is not produced.

Brief Description of the Figures

[012] **FIG. 1** depicts phenylalanine metabolism by mPAL1, mPAL2 and mPAL3 as measured by TCA.

[013] **FIG. 2** depicts phenylalanine metabolism by mPAL1, mPAL2 and mPAL3 compared to wild type PAL3.

[014] **FIGS. 3A-B** depict Michaelis-Menten graphs of wild type PAL3, mPAL1, mPAL2, and mPAL3.

[015] **FIGS. 4A-C** depict in vitro studies of mutant PAL efficacy in a cynomolgus monkey model. Activity of PAL variants was measured by assaying levels of plasma TCA and hippurate in urine.

[016] **FIGS. 5A-C** depict TCA feedback inhibition of wild type PAL3 activity as determined via whole cell and cell lysate assays.

Detailed Description

[017] The present disclosure includes, *inter alia*, mutant PAL polypeptides and polynucleotides. In some embodiments, the mutant PAL exhibits increased stability and/or increased ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia compared to a wild type PAL, e.g., *P. luminescens* PAL. The present disclosure also includes genetically engineered microorganisms comprising the mutant PAL, pharmaceutical compositions thereof, and methods of modulating and treating disorders associated with hyperphenylalaninemia, e.g., PKU.

[018] 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.

[019] “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.

[020] “Phenylalanine ammonia lyase” and “PAL” are used to refer to a 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 from or derived from a prokaryotic species. In alternate embodiments, PAL is encoded by a *PAL* gene derived from or from a eukaryotic species. In some embodiments, PAL is encoded by a *PAL* gene from or derived from a bacterial species, including but not limited to, *Achromobacter xylosoxidans*, *Pseudomonas aeruginosa*, *Photobacterium 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 *Photobacterium 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.*, *Rhodospiridium 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.

[021] As used herein, PAL encompasses wild type, naturally occurring PAL as well as mutant, non-naturally occurring PAL. As used herein, a “mutant PAL” or “PAL mutant” refers to a non-naturally occurring and/or synthetic PAL that has been modified, *e.g.*, mutagenized, compared to a wild type, naturally occurring PAL polynucleotide or polypeptide sequence. In some embodiments, the modification is a silent mutation, *e.g.*, a change in the polynucleotide sequence without a change in the corresponding polypeptide sequence. In some embodiments, the mutant PAL exhibits increased stability and/or increased ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia as compared to the wild type PAL. In some embodiments the mutant PAL is derived from *Photobacterium luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises one or more mutations at amino acid positions 92, 133, 167, 432, 470, 433, 263,

366 and/or 396 compared to a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises one or more mutations at amino acid positions S92, H133, I167, L432, V470, A433, A263, K366, and/or L396 compared to a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises one or more mutations at amino acid positions S92G, H133F, I167K, L432I, V470A, A433S, A263T, K366K (e.g., silent mutation in polynucleotide sequence), and/or L396L (e.g., silent mutation in polynucleotide sequence) compared to the positions in a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises S92G; H133M; I167K; L432I; V470A compared to the positions in a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises S92G; H133F; A433S; V470A compared to the positions in a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL polypeptide comprises S92G; H133F; A263T; K366K (e.g., silent mutation in polynucleotide sequence); L396L (e.g., silent mutation in polynucleotide sequence); V470A compared to the positions in a wild type PAL, e.g., *P. luminescens* PAL.

[022] “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).

[023] “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. In some embodiments, the genetically engineered microorganisms 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 microorganisms is localized to the plasma membrane, facing into the periplasmic space and with the catalytic activity occurring in the periplasmic space.

[024] “Phenylalanine metabolizing enzyme” or “PME” are used to refer to an enzyme which is able to degrade phenylalanine, e.g., into a non-toxic metabolite. Any phenylalanine metabolizing enzyme known in the art may be encoded by the genetically engineered microorganisms, e.g., bacteria, of the disclosure. PMEs include, but are not limited to, phenylalanine hydroxylase (PAH), phenylalanine ammonia lyase (PAL), aminotransferase, L-amino acid deaminase (LAAD), and phenylalanine dehydrogenases.

[025] Reactions with phenylalanine hydroxylases, phenylalanine dehydrogenases or aminotransferases require cofactors, while LAAD and PAL do not require any additional cofactors. In some embodiments, the PME encoded by the genetically engineered microorganisms requires a cofactor. In some embodiments, this cofactor is provided concurrently or sequentially with the administration of the genetically engineered microorganisms. In other embodiments, the genetically engineered microorganisms can produce the cofactor. In some embodiments, the genetically engineered microorganisms encode a phenylalanine hydroxylase. In some embodiments, the genetically engineered microorganisms encode a phenylalanine dehydrogenase. In some embodiments, the genetically engineered microorganisms encode an aminotransferase. 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 microorganisms is PAL. In some embodiments, the PME produced by the genetically engineered microorganisms is LAAD. In some embodiments, the genetically engineered microorganisms encode combinations of PMEs.

[026] 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, the PME functions at very low levels of oxygen or in the absence of oxygen, e.g., as found in the colon.

[027] “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. In some embodiments, the phenylalanine metabolite results directly or indirectly from PAL action, e.g., from PAL produced by the genetically engineered microorganisms. 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 microorganisms. Examples of such LAAD metabolites are phenylpyruvate and phenyllactic acid.

[028] “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 *Ageneral* 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 an *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 microorganisms comprise more than one type of phenylalanine transporter, selected from pheP, aroP, and the LIV-I/LS system. Exemplary phenylalanine transporters are known in the art, see, e.g., PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference.

[029] “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 in 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.

[030] As used herein, “gene expression system” refers to a combination of gene(s) and regulatory element(s) that enable or regulate gene expression. A gene expression system may comprise gene(s), e.g., encoding a mutant PAL polypeptide, together with one or more promoters, terminators, enhancers, insulators, silencers and other regulatory sequences to facilitate gene expression. In some embodiments, a gene expression system may comprise a gene encoding a mutant PAL and a promoter to which it is operably linked to facilitate gene expression. In some embodiment, a gene expression system may comprise multiple genes operably linked to one or more promoters to facilitate gene expression. In some embodiments, the multiple genes may be on the same plasmid or chromosome, e.g., *in cis* and operably linked to the same promoter. In some embodiments, the multiple genes may be on the different plasmid(s) or chromosome(s) and operably linked to the different promoters.

[031] “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.

[032] As used herein, a “non-native” nucleic acid sequence refers to a nucleic acid sequence not normally present in a microorganism, *e.g.*, an extra copy of an endogenous sequence, or a heterologous sequence such as a sequence from a different species, strain, or substrain of microorganism, or a sequence that is modified and/or mutated as compared to the unmodified sequence from microorganisms 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 microorganism, 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 microorganisms 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 microorganisms of the invention comprise a gene encoding a phenylalanine-metabolizing enzyme that is operably linked to an 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.

[033] 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.

[034] “Exogenous environmental condition(s)” or “environmental conditions” refer to settings or circumstances under which the promoter described herein is 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.

[035] As used herein, “exogenous environmental conditions” or “environmental conditions” also refer 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.

[036] 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. 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**. 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	<i>nirB</i> , <i>ydfZ</i> , <i>pdhR</i> , <i>focA</i> , <i>ndH</i> , <i>hlyE</i> , <i>narK</i> , <i>narX</i> , <i>narG</i> , <i>yfiD</i> , <i>tdcD</i>
ANR	<i>arcDABC</i>
DNR	<i>norb</i> , <i>norC</i>

[037] Exemplary oxygen-level dependent promoters, e.g., FNR promoters, are well known in the art and exemplary FNR promoters are provided in **Table 2**. See, e.g., PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference.

Table 2. Examples of FNR-responsive regulatory region sequences

SEQ ID NO	FNR-responsive regulatory region sequences
SEQ ID NO: 9	ATCCCCATCACTCTTGATGGAGATCAATTCCCCAAGCTGCTAGA GCGTTACCTTGCCCTTAAACATTAGCAATGTCGATTTATCAGAG GGCCGACAGGCTCCACAGGAGAAAACCG
SEQ ID NO: 10	CTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGCGTTACCTT GCCCTTAAACATTAGCAATGTCGATTTATCAGAGGGCCGACAGG CTCCACAGGAGAAAACCG
<i>nirB1</i> SEQ ID NO: 11	GTCAGCATAACACCCTGACCTCTCATTAAATTGTTTCATGCCGGGC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTA CATCTATTTCTATAAATCCGTTCAATTTGTCTGTTTTTGCACAA ACATGAAATATCAGACAATTCGGTGACTTAAGAAAATTTATACA AATCAGCAATATAACCCCTTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGT AATAGAAAAGAAATCGAGGCAAAA
<i>nirB2</i> SEQ ID NO: 12	CGGCCCGATCGTTGAACATAGCGGTCCGCAGGCGGCACTGCTTA CAGCAAACGGTCTGTACGCTGTCGTCTTTGTGATGTGCTTCCTGT TAGGTTTCGTCAGCCGTCACCGTCAGCATAACACCCTGACCTCT CATTAAATTGCTCATGCCGACGGCACTATCGTCGTCCGGCCTTTT CCTCTCTTCCCCCGCTACGTGCATCTATTTCTATAAACCCGCTCA TTTTGTCTATTTTTTGCACAAACATGAAATATCAGACAATTCGGT GACTTAAGAAAATTTATACAAATCAGCAATATAACCCATTAAGGA GTATATAAAGGTGAATTTGATTACATCAATAAGCGGGGTTGCT GAATCGTTAAGGTAGGCGGTAATAGAAAAGAAATCGAGGCAAAA <u>Aatgtttgttaacttaagaaggagatat</u>
<i>nirB3</i> SEQ ID NO: 13	GTCAGCATAACACCCTGACCTCTCATTAAATTGCTCATGCCGGAC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTG CATCTATTTCTATAAACCCTGCTATTTTGTCTATTTTTTGCACAA ACATGAAATATCAGACAATTCGGTGACTTAAGAAAATTTATACA AATCAGCAATATAACCCATTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGGCGGT AATAGAAAAGAAATCGAGGCAAAA
<i>ydfZ</i> SEQ ID NO: 14	ATTCCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCCGACTT ATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAA ACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCCGT TACGTGGGCTTCGACTGTAAATCAGAAAGGAGAAAACACCT

<i>nirB</i> +RBS SEQ ID NO: 15	GTCAGCATAACACCCTGACCTCTCATTAAATTGTTTCATGCCGGGC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTACTCTGCTACGTA CATCTATTTCTATAAAATCCGTTCAATTTGTCTGTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATAACCCCTTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGATCCCTCT <u>AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT</u>
<i>ydfZ</i> +RBS SEQ ID NO: 16	CATTTCTCTCATCCCATCCGGGGTGAGAGTCTTTTCCCCGACT TATGGCTCATGCATGCATCAAAAAAGATGTGAGCTTGATCAAAA ACAAAAAATATTTCACTCGACAGGAGTATTTATATTGCGCCCGG <u>ATCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA</u> <u>CAT</u>
<i>fmrS1</i> SEQ ID NO: 17	AGTTGTTCTTATTGGTGGTGTGCTTTATGGTTGCATCGTAGTAA ATGGTTGTAACAAAAGCAATTTTCCGGCTGTCTGTATACAAAA ACGCCGTAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTC AGGGCAATATCTCTCTTGGATCCCTCTAGAAATAATTTTGTTA <u>ACTTTAAGAAGGAGATATACAT</u>
<i>fmrS2</i> SEQ ID NO: 18	AGTTGTTCTTATTGGTGGTGTGCTTTATGGTTGCATCGTAGTAA ATGGTTGTAACAAAAGCAATTTTCCGGCTGTCTGTATACAAAA ACGCCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTC AGGGCAATATCTCTCTTGGATCCAAAGTGAAGTCTAGAAATAAT <u>TTTGTTTAACTTTAAGAAGGAGATATACAT</u>
<i>nirB</i> + <i>crp</i> SEQ ID NO: 19	TCGTCTTTGTGATGTGCTTCCTGTTAGGTTTCGTCAGCCGTCACC GTCAGCATAACACCCTGACCTCTCATTAAATTGCTCATGCCGGAC GGCACTATCGTCGTCCGGCCTTTTCCTCTCTTCCCCCGCTACGTG CATCTATTTCTATAAAACCCGCTCATTTTGTCTATTTTTTGCACAA ACATGAAATATCAGACAATTCCGTGACTTAAGAAAATTTATACA AATCAGCAATATAACCCATTAAGGAGTATATAAAGGTGAATTTGA TTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGTAGaaatgat ctagttcacatttGCGGTAATAGAAAAGAAATCGAGGCAAAAatgtttgtttaac tttaagaaggagatatatacat
<i>fmrS</i> + <i>crp</i> SEQ ID NO: 20	AGTTGTTCTTATTGGTGGTGTGCTTTATGGTTGCATCGTAGTAA ATGGTTGTAACAAAAGCAATTTTCCGGCTGTCTGTATACAAAA ACGCCGCAAAGTTTGAGCGAAGTCAATAAACTCTCTACCCATTC AGGGCAATATCTCTCaaatgatctagttcacattttgttttaactttaagaaggagatatatacat

[038] As used herein, the term “low oxygen” is meant to refer to a level, amount, or concentration of oxygen (O₂) that is lower than the level, amount, or concentration of oxygen that is present in the atmosphere (e.g., <21% O₂; <160 torr O₂). 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 (O₂) 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 O₂ that is 0-60 mmHg O₂ (0-60 torr O₂) (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 O₂), including any and all incremental fraction(s) thereof (e.g., 0.2 mmHg O₂, 0.5 mmHg O₂, 0.75 mmHg O₂, 1.25 mmHg O₂, 2.175 mmHg O₂, 3.45 mmHg O₂, 3.75 mmHg O₂, 4.5 mmHg O₂, 6.8 mmHg O₂, 11.35 mmHg O₂, 46.3 mmHg O₂, 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 O₂ or less (e.g., 0 to about 60 mmHg O₂). The term “low oxygen” may also refer to a range of O₂ levels, amounts, or concentrations between 0-60 mmHg O₂ (inclusive), e.g., 0-5 mmHg O₂, < 1.5 mmHg O₂, 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 incorporated 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 (O₂) 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 (O₂) present in partially aerobic, semi aerobic, microaerobic, nanoaerobic, microoxic, hypoxic, anoxic, and/or anaerobic conditions. Summaries of the amount of oxygen present in various organs and tissues are provided in PCT/US2016/062369, the contents of which is herein incorporated by reference in its entirety. In some embodiments, the level, amount, or concentration of

oxygen (O₂) is expressed as the amount of dissolved oxygen (“DO”) which refers to the level of free, non-compound oxygen (O₂) 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 O₂ = 0.022391 mg/L O₂). 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 (O₂) 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 (O₂) 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% O₂ saturation or less, e.g., 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0%, O₂ 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 O₂ 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% O₂, etc.). The exemplary fractions and ranges listed here are for illustrative purposes and not meant to be limiting in any way.

[039] “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.

[040] Constitutive promoters, inducible promoters, and variants thereof are well known in the art and described in PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference.

[041] “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. In some embodiments, the genetically engineered microorganisms are active (*e.g.*, express one or more PME_s) in the gut. In some embodiments, the genetically engineered microorganisms are active (*e.g.*, express one or more PME_s) in the large intestine. In some embodiments, the genetically engineered microorganisms are active (*e.g.*, express one or more PME_s) in the small intestine. In some embodiments, the genetically engineered microorganisms 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 and can be cleared by microorganisms which are active at this location. In some embodiments, the genetically engineered microorganisms transit through the small intestine. In some embodiments, the genetically engineered microorganisms have increased residence time in the small intestine. In some embodiments, the genetically engineered microorganisms colonize the small intestine. In some embodiments, the genetically engineered microorganisms do not colonize the small intestine. In some embodiments, the genetically engineered microorganisms have increased residence time in the gut. In some embodiments, the genetically engineered microorganisms colonize the gut. In some embodiments, the genetically engineered microorganisms do not colonize the gut.

[042] “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.

[043] “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*, *Brevibacterium*, *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.

[044] “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.

[045] As used herein, “stable” microorganism is used to refer to a microorganism 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, *e.g.*, under particular conditions. The stable microorganism 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 microorganisms may be a genetically modified bacterium comprising a *PAL* gene, *e.g.*, mutant *PAL*, 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.

[046] 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. 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.

[047] 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.

[048] 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.

[049] 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.

[050] 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.

[051] 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 microorganism of the current invention.

[052] The terms “phage” and “bacteriophage” are used interchangeably herein. Both terms refer to a virus that infects and replicates within a bacterium. As used herein “phage” or bacteriophage” collectively refers to prophage, lysogenic, dormant, temperate, intact, defective, cryptic, and satellite phage, phage tail bacteriocins, tailiocins, and gene transfer agents.

[053] As used therein the term “prophage” refers to the genomic material of a bacteriophage, which is integrated into a replicon of the host cell and replicates along with the host. The prophage may be able to produce phages if specifically activated. In some cases, the prophage is not able to produce phages or has never done so (i.e., defective or cryptic prophages). In some cases, prophage also refers to satellite phages. The terms “prophage” and “endogenous phage” are used interchangeably herein.

[054] “Endogenous phage” or “endogenous prophage” also refers to a phage that is present in the natural state of a bacterium (and its parental strain).

[055] As used herein the term “phage knockout” or “inactivated phage” refers to a phage which has been modified so that it can either no longer produce and/or package phage particles or it produces fewer phage particles than the wild type phage sequence. In some embodiments, the inactivated phage or phage knockout refers to the inactivation of a temperate phage in its lysogenic state, i.e., to a prophage. Such a modification refers to a mutation in the phage; such mutations include insertions, deletions (partial or complete deletion of phage genome), substitutions, inversions, at one or more positions within the phage genome, e.g., within one or more genes within the phage genome.

[056] As used herein the adjectives “phage-free”, “phage free” and “phageless” are used interchangeably to characterize a bacterium or strain which contains one or more prophages, one or more of which have been modified. The modification can result in a loss of the ability of the prophage to be induced or release phage particles. Alternatively, the modification can result in less efficient or less frequent induction or less efficient or less frequent phage release as compared to the isogenic strain without the modification. Ability to induce and release phage can be measured using a plaque assay as described herein.

[057] As used herein phage induction refers to the part of the life cycle of a lysogenic prophage, in which the lytic phage genes are activated, phage particles are produced and lysis occurs.

PAL Mutants

[058] The present disclosure provides mutant PAL polypeptides and polynucleotides encoding the same. In some embodiments, the mutant PAL is encoded by a gene derived from a prokaryotic species. In some embodiments, the mutant PAL is encoded by a gene derived from a eukaryotic species. In some embodiments, the mutant PAL is encoded by a *PAL* gene derived from a bacterial species, including but not limited

to, *Achromobacter xylosoxidans*, *Pseudomonas aeruginosa*, *Photobacterium luminescens*, *Anabaena variabilis*, and *Agrobacterium tumefaciens*. In some embodiments, the mutant PAL is encoded by a *PAL* gene derived from *Anabaena variabilis*. In some embodiments, the mutant PAL is encoded by a *PAL* gene derived from *Photobacterium luminescens*. In some embodiments, the mutant PAL is encoded by a *PAL* gene derived from a yeast species, e.g., *Rhodospiridium toruloides*. In some embodiments, the mutant PAL is encoded by a *PAL* gene derived from a plant species, e.g., *Arabidopsis thaliana*. Any suitable nucleotide and amino acid sequences of PAL, or functional fragments thereof, may be used to derive the mutant PAL. In some embodiments, the mutant PAL exhibits increased stability and/or activity compared to the wild type PAL. Non-limiting examples of PAL genes are shown in **Table 3**.

Table 3. Sequences of Exemplary Phenylalanine Metabolizing Enzymes

Description	Sequence
Phenylalanine ammonia-lyase (Anabaena variabilis) Acc. No.: Q3M5Z3.1	MKTL SQAQSKTSSQQFSFTGNSSANVIIGNQKL TINDVARVARN GTLVSLTNNTDILQGIQASCDYINNAVESGEPIYGVTSGFGGMAN VAISREQASELQTNLVWFLKTGAGNKLPLADVRAAMLLRANSH MRGASGIRLELIKRM EIFLNAGVTPYVYEFSGISASGDLVPLSYIT GSLIGLDPSFKVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMM NGTSVMTGIAANCYDQTQILTAIAMGVHALDIQALNGTNQSFHP FIHNSKPHPGQLWAADQMISLLANSQ LVRDEL DKGKHDYRDHELI QDRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQA SYHGGNFLGQYVGMGM DHLRYYIGLLAKHLDVQIALLASPEFS NGLPPSLLGNRERKVN MGLKGLQICGNSIMPLLT FYGNSIADRF THAEQFNQINNSQGYTSATLARRSVDIFQNYVAIALMFGVQAVD LRTYKKTGHYDARACLS PATERLYSAVRHVVGQKPTSDRPYIW NDNEQGLDEHIARISADIAAGGVIVQAVQDILPCLH
histidine ammonia-lyase [Anabaena variabilis ATCC 29413] (Acc. NO: ABA23593.1)	MKTL SQAQSKTSSQQFSFTGNSSANVIIGNQKL TINDVARVARN GTLVSLTNNTDILQGIQASCDYINNAVESGEPIYGVTSGFGGMAN VAISREQASELQTNLVWFLKTGAGNKLPLADVRAAMLLRANSH MRGASGIRLELIKRM EIFLNAGVTPYVYEFSGISASGDLVPLSYIT GSLIGLDPSFKVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMM NGTSVMTGIAANCYDQTQILTAIAMGVHALDIQALNGTNQSFHP FIHNSKPHPGQLWAADQMISLLANSQ LVRDEL DKGKHDYRDHELI QDRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQA SYHGGNFLGQYVGMGM DHLRYYIGLLAKHLDVQIALLASPEFS NGLPPSLLGNRERKVN MGLKGLQICGNSIMPLLT FYGNSIADRF THAEQFNQINNSQGYTSATLARRSVDIFQNYVAIALMFGVQAVD LRTYKKTGHYDARACLS PATERLYSAVRHVVGQKPTSDRPYIW NDNEQGLDEHIARISADIAAGGVIVQAVQDILPCLH
histidine ammonia-lyase [Photobacterium luminescens]	MKAKDVQPTIINKNGLISLEDIYDIAIKQKKVEISTEITELLTHGR EKLEEKLNSEVIY GINTGFGGNANLVVPFEKIAEHQQNLLTFLS AGTGDYMSKPCIKASQFTMLLSVCKGWSATRPVQAQIVDHINH DIVPLVPRYGSVGASGDLIPLSYIARALCGIGKVYYMGAEIDAAE

(WP_011146484)	AIKRAGLTPLSLKAKEGLALINGTRVMSGISAITVIKLEKLFKASI SAIALAVEALLASHEHYDARIQQVKNHPGQNAVASALRNLLAG STQVNLLSGVKEQANKACRHQEITQLNDTLQEVYSIRCAPQVLG IVPESLATARKILEREVISANDNPLIDPENGVDVLHGGMFMGQYVA RTMDALKLDIALIANHLHAIVALMMDNRFSGRLPNLSPTPGMY QGFKGVQLSQ TALVAAIRHDCAASGIHTLATEQYNQDIVSLGLH AAQDVLEMEQKLNRNIVSMTILVVCQAIHLRGNISEIAPETAKFYH AVREISSPLITDRALDEDIIRIADAIINDQLPLPEIMLEE
Histidine ammonia lyase (Photorhabdus luminescens) Acc. NO: CAE15566	MKQLTIYPGKLTLDLQVYLPVKITLDSQIFPAIERSVECVNAI LAENRTAYGINTGFGLLASTRIEEDNLEKLQSLVSHAAGVGK ALDDNMTRLIMVLKINSLSRGYSGIRLAVIQALIALVNAEIYPHIP CKGSVGASGDLAPLAHMSLLLLGEGQARYQGEWLPALAK ANLQPITLAAKEGLALLNGTQVSTAFALRGLFEADLLAAAIVC GSLVEAALGSRKPFDAHVHVVRGQQGQIDVAALYRHVLEESS ELSDSHINCPKVQDPYSLRCQPQVMGACLTQLRHAADVILTEAN AVSDNPLVFAEQGEVISGGNFHAEPVAMASDNALVLAIGALS ERRIALLMDSHMSQLPPFLVENGGVNSGMIAQVTAALASENK ALAHPASVDSLPTSANQEDHVSMAAAGRRLWEMAENTRGILA IEWLSACQGIDFRNGLKSSPILEEARVILRAKVDYDQDRFFAPD IDAAVKLLAEQHLSSLLPSGQILQRKNNR
amino acid deaminase (Proteus mirabilis) Acc. No: ACD36582	MAISRRKFILGGTVVAVAAGAGVLTPLMTREGRFVPGTPRHGFV EGTGGPLPKQDDVVIGAGILGIMTAINLAERGLSVTIVEKGNIA GEQSSRFYGQAISYKMPDETFLHHLGKHRWREMNKAVGIDTT YRTQGRVEVPLDEEDLENVRKWIDAKSKDVGS DIPFRTKMIEGA ELKQRLRGATTDWKIAGFEEDSGSFDPEVATFVMAEYAKKMGI KIFTNCAARGLETQAGVISDVVTEKGPIKTSRVVAGGVGSRLF MQNLNVDVPTLPAYQSQQQLISAAPNAPGGNVALPGGIFFRDQAD GTYATSPRVIVAPVVKESFTYGYKYLPLLALPDFPVHISLNEQLI NSFMQSTHWDLNEESPFEKYRDMTALPDLPELNASLEKLKKEFP AFKESTLIDQWSGAMAIAPDENPIISDVKEYPGLVINTATGWGM TESPVSAEITADLLLGGKPVLDKPFSLYRF
amino acid deaminase [Proteus mirabilis HI4320]) Acc. No.: AAA86752.1	MNISRRKLLLGVAAGVLAGGAALVPMVRRDGGKFVEAKSRASF VEGTQGALPKEADVVIAGAGIQQGIMTAINLAERGM SVTILEKGQI AGEQSGRAYSQIISYQTSPEIFPLHHYKILWRGMNEKIGADTSY RTQGRVEALADEKALDKAQAWIKTAKEAAGFDTPLNTRIIEGEE LSNRLVGAQTPWTVAAFEEDSGSVD PETGTPALARYAKQIGVKI YTNC AVRGIETAGGKISDVVSEKGAIKTSQVVLAGGIWSRLFMG NMGIDIPTLNVYLSQQRVSGVPGAPRG NVHLPNGIHFREQADGT YAVAPRIFTSSIVKDSFLLGPKFMHLLGGGELPLEFSIGEDLFNSF KMPTSWNLDEKTPEQFRVATATQNTQHLDV FQRMKTEFPVF EKSEVVERWGAVVSPTFDELPIISEVKEYPGLVINTATVWGMTE GPAAGEVTADIVMGKKPVIDPTPFS LDRFKK
L-AAD from <i>Proteus vulgaris</i> ; (Acc. NO: BAA90864)	MAISRRKFIIGGTVVAVAAGAGILTPMLTREGRFVPGTPRHGFVE GTEGALPKQADV VVVVAGILGIMTAINLVERGLSVVIVEKGNIA GEQSSRFYGQAISYKMPDETFLHHLGKHRWREMNKAVGIDTT YRTQGRVEVPLDEEDLVNVRKWIDERSKNVGS DIPFKTRIIEGAE LNQRLRGATTDWKIAGFEEDSGSFDPEVATFVMAEYAKKMGR IYTQCAARGLETQAGVISDVVTEKGAIKTSQVVVAGGVWSRLF MQNLNVDVPTLPAYQSQQQLISGSPTAPGGNVALPGGIFFREQAD

	GTYATSPRVIVAPVVKESFTYGYKYLPLLALPDFPVHISLNEQLI NSFMSQSTHWNLDEVSPFEQFRNMTALPDLPELNASLEKLKAEFP AFKESKLIDQWSGAMAIAPDENPIISEVKEYPGLVINTATGWGM TESPVSAELTADLLLGGKPVLDPKPFSLYRF
Phenylalanine hydroxylase [Homo sapiens] (Acc. No. AAH26251]	MSTAVLENPGLGRKLSDFGQETSYIEDNCNQNNGAISLIFSLKEEV GALAKVLRLEENDVNLTHIESRPSRLKKDEYEFFTHLDRSLPA LTNIILRHDIGATVHELSDRDKKDTVPWFPRTIQELDRFANQIL SYGAELDADHPGFKDPVYRARRKQFADIAYNRYHGOPIRVEY MEEGKKTWGTVFVKTLKSLYKTHACYEYNHIFPILLEKYCGFHED NIPQLEDVSQFLQTCTGFRLRPVAGLLSSRDFLGGLAFRVFHCTQ YIRHGSKPMYTPEDICHELLGHVPLFSDRSFAQFSQEIGLASLGA PDEYIEKLATYIWFTEVFGGLCKQGDSIKAYGAGLLSSFGEQYCL SEKPKLLPLELEKTAIQNYTVTEFQPLYYVAESFNDAKEKVRNF AATIPRPFVRYDPYTQRIEVLDTQQLKILADSINSEIGILCSALQ KIK

[059] In some embodiments, the mutant PAL is encoded by a *PAL* gene derived from wild-type *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the mutant PAL comprises mutations in one or more amino acid positions selected from 92, 133, 167, 432, 470, 433, 263, 366 and 396 compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the mutant PAL comprises mutations in one or more amino acid positions selected from S92, H133, I167, L432, V470, A433, A263, K366, and/or L396 compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the amino acid mutations are silent mutations, e.g., a change in the polynucleotide sequence without a corresponding change in the amino acid coding sequence. In some embodiments, the mutant PAL comprises mutations in one or more amino acid positions selected from S92G, H133M, H133F, I167K, L432I, V470A, A433S, A263T, K366K (e.g., silent mutation in polynucleotide sequence), and/or L396L (e.g., silent mutation in polynucleotide sequence) compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1.

[060] In some embodiments, the mutant PAL comprises mutations in one or more amino acid positions selected from S92G, H133M, I167K, L432I, and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. This mutant is referred to herein as “mPAL1” (SEQ ID NO: 2; **Table 4**).

[061] In some embodiments, the mutant PAL comprises mutations in one or more amino acid positions selected from S92G, H133F, A433S, and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1.

This mutant is referred to herein as “mPAL2” (SEQ ID NO: 3; **Table 4**).

[062] In some embodiments, the mutant PAL comprises mutations in one or more amino acid positions selected from S92G, H133F, A263T, K366K (e.g., silent mutation in polynucleotide sequence), L396L (e.g., silent mutation in polynucleotide sequence), and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. This mutant is referred to herein as “mPAL3” (SEQ ID NO: 4; **Table 4**).

Table 4. Sequences of Exemplary PAL Mutants

Name	Amino Acid Sequence
<i>Photorhabdus luminescens</i> PAL3 Wild Type (SEQ ID NO: 1)	MKAKDVQPTIIINKNGLISLEDIYDIAIKQKKVEISTEITELLTHGRE KLEEKLNLSGEVIYGINTGFGGNANLVVPFEKIAEHQQNLLTFLSAG TGDYMSKPCIKASQFTMLLSVCKGWSATRPVAAQAIVDHINHDI LVPRYGSVGASGDLIPLSYIARALCGIGKVYYMGAEIDAAEAIKRA GLTPLSLKAKEGLALINGTRVMSGISAITVIKLEKLFKASISAI EALLASHEHYDARIQQVKNHPGQNAVASALRNLLAGSTQVNLLS GVKEQANKACRHQEITQLNDTLQEVYSIRCAPQVLGIVPESLATA RKILEREVISANDNPLIDPENGVDVLHGGNFMGQYVARTMDALKLD IALIANHLHAIVALMMDNRFSGRLPNSLSPTPGMYQGFGKVQLSQ TALVAIRHDCAASGIHTLATEQYNQDIVSLGLHAAQDVLEMEQK LRNIVSMTILVVCQAIHLRGNISEIAPETAKFYHAVREISSPLITDRA LDEDIIRIADAIINDQLPLPEIMLEE
mPAL1 (SEQ ID NO: 2)	MKAKDVQPTIIINKNGLISLEDIYDIAIKQKKVEISTEITELLTHGRE KLEEKLNLSGEVIYGINTGFGGNANLVVPFEKIAEHQQNLLTFLGAG TGDYMSKPCIKASQFTMLLSVCKGWSATRPVAAQAIVDMINHDI PLVPRYGSVGASGDLIPLSYIARALCGIGKVYYMGAEIDAAEAIK RAGLTPLSLKAKEGLALINGTRVMSGISAITVIKLEKLFKASISAI AVEALLASHEHYDARIQQVKNHPGQNAVASALRNLLAGSTQVN LSGVKEQANKACRHQEITQLNDTLQEVYSIRCAPQVLGIVPESLAT ARKILEREVISANDNPLIDPENGVDVLHGGNFMGQYVARTMDALK DIALIANHLHAIVALMMDNRFSGRLPNSLSPTPGMYQGFGKVQLS QTALVAIRHDCAASGIHTIATEQYNQDIVSLGLHAAQDVLEMEQ KLRNIVSMTILVACQAIHLRGNISEIAPETAKFYHAVREISSPLITDR ALDEDIIRIADAIINDQLPLPEIMLEE
mPAL2 (SEQ ID NO: 3)	MKAKDVQPTIIINKNGLISLEDIYDIAIKQKKVEISTEITELLTHGRE KLEEKLNLSGEVIYGINTGFGGNANLVVPFEKIAEHQQNLLTFLGAG TGDYMSKPCIKASQFTMLLSVCKGWSATRPVAAQAIVDHINHDI LVPRYGSVGASGDLIPLSYIARALCGIGKVYYMGAEIDAAEAIKRA GLTPLSLKAKEGLALINGTRVMSGISAITVIKLEKLFKASISAI EALLASHEHYDARIQQVKNHPGQNAVASALRNLLAGSTQVNLLS GVKEQANKACRHQEITQLNDTLQEVYSIRCAPQVLGIVPESLATA

	RKILEREVISANDNPLIDPENGVDVLHGGNFMGQYVARTMDALKLD IALIANHLHAIVALMMDNRFSGRLPNSLSPTPGMYQGFKGVQLSQ TALVAAIRHDCAASGIHTLSTEQYNQDIVSLGLHAAQDVLEMEQK LRNIVSMTILVACQAIHLRGNISEIAPETAKFYHAVREISSPLITDRA LDEDIIRIADAIINDQLPLPEIMLEE
mPAL3 (SEQ ID NO: 4)	MKAKDVQPTIIINKNGLISLEDIYDIAIKQKKVEISTEITELLTHGRE KLEEKLNSEVIYGINTEGFGGNANLVVPFEKIAEHQQNLLTFLGAG TGDYMSKPCIKASQFTMLLSVCKGWSATRPIVAQAIVDFINHDIVP LVPRYGSVGASGDLIPLSYIARALCGIGKVYYMGAEIDAAEAIKRA GLTPLSLKAKEGLALINGTRVMSGISAITVIKLEKLFKASISAIALAV EALLASHEHYDARIQQVKNHPGQNAVASTLRNLLAGSTQVNLLS GVKEQANKACRHQEITQLNDTLQEVYSIRCAPQVLGIVPESLATA RKILEREVISANDNPLIDPENGVDVLHGGNFMGQYVARTMDALKLD IALIANHLHAIVALMMDNRFSGRLPNSLSPTPGMYQGFKGVQLSQ TALVAAIRHDCAASGIHTLSTEQYNQDIVSLGLHAAQDVLEMEQK LRNIVSMTILVACQAIHLRGNISEIAPETAKFYHAVREISSPLITDRA LDEDIIRIADAIINDQLPLPEIMLEE

[063] In some embodiments, the mutant PAL exhibits increased stability compared to wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL exhibits about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90% or more than 100% increased stability compared to wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL exhibits about two-, three-, four-, or five-fold increased stability compared to wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL exhibits increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia compared to a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL exhibits about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90% or more than 100% increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia compared to wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL exhibits about two-, three-, four-, or five-fold increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia compared to wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the mutant PAL exhibits at least a two-fold increase in activity compared to wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the mutant exhibits at least a three-fold increase in activity compared to the wild type PAL, e.g., *Photorhabdus*

luminescens PAL. In some embodiments, the mutant exhibits at least a four-fold increase in activity compared to the wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the mutant exhibits at least a five-fold increase in activity compared to the wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the increase in PAL ability to metabolize phenylalanine is measured by detecting levels of phenylalanine, hippurate and/or transcinnamic acid *in vitro* or *in vivo*.

Gene Expression Systems

[064] In some embodiments, a gene expression system comprises gene(s), e.g., encoding a mutant PAL polypeptide, together with one or more promoters, terminators, enhancers, insulators, silencers and other regulatory sequences to facilitate gene expression.

[065] In some embodiments, the present disclosure provides gene expression systems comprising one or more copies of a gene encoding PAL, e.g., mutant PAL.

[066] In some embodiments, the gene expression system comprises a mutant PAL derived from wild-type *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the gene expression system comprises a mutant PAL with mutations in one or more amino acid positions selected from 92, 133, 167, 432, 470, 433, 263, 366 and 396 compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the gene expression system comprises a mutant PAL with mutations in one or more amino acid positions selected from S92, H133, I167, L432, V470, A433, A263, K366, and/or L396 compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the gene expression system comprises a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133F, H133M, I167K, L432I, V470A, A433S, A263T, K366K (e.g., silent mutation in polynucleotide sequence), and/or L396L (e.g., silent mutation in polynucleotide sequence) compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1.

[067] In some embodiments, the gene expression system comprises a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133M, I167K, L432I, and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the gene expression system comprises a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133F, A433S, and V470A compared to positions in wild type PAL, e.g.,

Photorhabdus luminescens PAL, e.g., SEQ ID NO: 1. In some embodiments, the gene expression system comprises a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133F, A263T, K366K (e.g., silent mutation in polynucleotide sequence), L396L (e.g., silent mutation in polynucleotide sequence), and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the gene expression system comprises mPAL1. In some embodiments, the gene expression system comprises mPAL2. In some embodiments, the gene expression system comprises mPAL3.

[068] In some embodiments, the gene expression system comprises a mutant PAL and exhibits increased stability compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the gene expression system comprising the mutant PAL exhibits about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90% or more than 100% increased stability as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the gene expression system comprising the mutant PAL exhibits about two-, three-, four-, or five-fold increased stability as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the gene expression system comprises a mutant PAL and exhibits increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia compared to a suitable control, e.g. a gene expression system comprising a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the gene expression system comprising the mutant PAL exhibits about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90% or more than 100% increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the gene expression system comprising the mutant PAL exhibits about two-, three-, four-, or five-fold increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the gene expression system comprises a mutant PAL and exhibits at least a two-fold increase in activity as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the gene

expression system comprises a mutant PAL and exhibits at least a three-fold increase in activity as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the gene expression system comprises a mutant PAL and exhibits at least a four-fold increase in activity as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the gene expression system comprises a mutant PAL and exhibits at least a five-fold increase in activity as compared to a suitable control, e.g. a gene expression system comprising wild type PAL, e.g., *Photorhabdus luminescens* PAL.

[069] In some embodiments, the gene expression system further comprises additional PME(s), e.g., PAH, LAAD. Exemplary PMEs and combinations thereof are known in the art, see, e.g., PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference. In some embodiments, the gene expression system comprises a mutant PAL and a wild type PAL.

[070] In some embodiments, the gene expression system comprises one or more genes encoding a phenylalanine transporter, in addition to the one or more PMEs. 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*. Examples of 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 an *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.

[071] In some embodiments, the gene expression system comprises one or more genes encoding a transcriptional regulator, e.g., a transcription factor.

[072] In some embodiments, the one or more PME(s) and/or phenylalanine transporter and/or transcriptional regulator are operably linked to a promoter. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the one or more PME(s) and/or phenylalanine transporter and/or transcriptional regulator is operably linked to an inducible promoter. In some embodiments, the one or more PME and/or phenylalanine transporter and/or transcriptional regulator is under the control of a promoter that is induced by exogenous environmental conditions, as described herein. In some embodiments, the one or more PME(s) and/or phenylalanine transporter and/or transcriptional regulator is under the control of a promoter that is 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 one or more PME(s) and/or phenylalanine transporter and/or transcriptional regulator is expressed under the control of a promoter that is induced by low-oxygen, microaerobic, or anaerobic conditions, wherein expression of the gene, is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. In some embodiments, the promoter is an FNR, an ANR, or a DNR promoter. Non-limiting examples of FNR promoter sequences are provided in Table 2. In other embodiments, one or more PME(s) and/or phenylalanine transporter and/or transcriptional regulator 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).

[073] In alternate embodiments, the one or more PME(s) and/or phenylalanine transporter and/or transcriptional regulator 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 LAAD 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_{ParaBAD} promoter. In some embodiments, the one or more PME and/or phenylalanine transporter 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 and/or phenylalanine transporter and/or transcriptional regulator gene are expressed under the control of a promoter that is induced by exposure to tetracycline. In some embodiments, the one or more PME and/or phenylalanine transporter and/or transcriptional regulator gene are expressed under the control of a promoter that is induced by exposure to arabinose. In some embodiments the one or more PME and/or phenylalanine transporter and/or transcriptional regulator 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 and/or phenylalanine transporter and/or transcriptional regulator gene are expressed under the control of a promoter that is induced by exposure to rhamnose. In some embodiments, the one or more PME and/or phenylalanine transporter and/or transcriptional regulator gene are expressed under the control of a promoter that is induced by exposure to tetracycline. 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 herein.

[074] In some embodiments, the gene expression system comprises 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, e.g. 37–42 °C, the functional cI857 dimer is destabilized, binding to the oL or oR DNA sequences is abrogated, and mRNA transcription is initiated. Inducible expression from the ParaBad can be controlled or further fine-tuned through the optimization of the ribosome binding site (RBS), as

described herein.

[075] In one embodiment, expression of the one or more PME(s) and/or Phe transporter, e.g., PheP, and/or transcriptional regulator(s), is driven by one or more thermoregulated promoter(s). In one embodiment, expression of PAL is driven by a thermoregulated promoter. In one embodiment, expression of PheP is driven by a thermoregulated promoter. In one embodiment, expression of LAAD is driven by a thermoregulated promoter.

[076] 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 the same promoter, such as any of the promoters discussed herein. In some embodiments, the PME gene(s) and/or phenylalanine transporter gene and/or transcriptional regulator are expressed under the control of different promoters, such as any of the promoters discussed herein. In some embodiments, the PME gene(s) and/or phenylalanine transporter gene and/or transcriptional regulator are expressed under the control of the same promoter, such as any of the promoters discussed herein.

[077] In another embodiment, one or more inducible promoter(s), e.g., thermoregulated, arabinose-inducible, tet-inducible, and IPTG-inducible promoters, drive the expression of one or more bicistronic message(s). Bicistronic messages 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). In one embodiment, one or more inducible promoter(s) drive the expression of tri-cistronic messages. Tri-cistronic messages 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). In one embodiment, one or more inducible promoter(s) drive the expression of multi-cistronic messages. Multi-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).

[078] In some embodiments, the gene expression system may also comprise one or more gene sequences relating to biosafety and/or biocontainment, e.g., a kill-switch, gene guard system, and/or auxotrophy. The expression of these gene sequence(s) may be regulated using the promoters or promoter systems described herein. The 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 microorganism. Examples and combinations are known in the art, see, e.g., PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference.

Microorganisms Engineered to Reduce Hyperphenylalaninemia

[079] The genetically engineered microorganisms capable of reducing excess phenylalanine are provided herein. In some embodiments, the genetically engineered microorganisms are bacteria. In some embodiments, the bacteria are non-pathogenic bacteria. In some embodiments, the bacteria are commensal bacteria. In some embodiments, the 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*.

[080] 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).

[081] 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 *Rhodospiridium 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).

[082] 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.

[083] In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise one or more gene(s) encoding PAL, e.g., mutant PAL. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a PAL derived from a prokaryotic species. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a PAL derived from a eukaryotic species. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a PAL derived from a bacterial species, including but not limited to, *Achromobacter xylosoxidans*, *Pseudomonas aeruginosa*, *Photobacterium luminescens*, *Anabaena variabilis*, and *Agrobacterium tumefaciens*.

[084] In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL derived from wild-type *Photobacterium luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL with mutations in one or more amino acid positions selected from 92, 133, 167, 432, 470, 433, 263, 366 and 396 compared to positions in wild type PAL, e.g., *Photobacterium luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL with mutations in one or more amino acid positions selected from S92, H133,

I167, L432, V470, A433, A263, K366, and/or L396 compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133M, H133F, I167K, L432I, V470A, A433S, A263T, K366K (e.g., silent mutation in polynucleotide sequence), and/or L396L (e.g., silent mutation in polynucleotide sequence) compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1.

[085] In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133M, I167K, L432I, and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133F, A433S, and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133F, A263T, K366K (e.g., silent mutation in polynucleotide sequence), L396L (e.g., silent mutation in polynucleotide sequence), and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise mPAL1. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise mPAL2. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise mPAL3.

[086] In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL and exhibit increased stability compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the genetically engineered microorganism, e.g. bacteria, comprising the mutant PAL exhibits about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90% or more than 100% increased stability as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the genetically engineered microorganism, e.g. bacteria, comprising the mutant PAL exhibits about two-, three-, four-, or five-fold increased stability as

compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the genetically engineered microorganism, e.g. bacteria, comprises a mutant PAL and exhibits increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the genetically engineered microorganism, e.g. bacteria, comprising the mutant PAL exhibits about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90% or more than 100% increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the genetically engineered microorganism, e.g. bacteria, comprising the mutant PAL exhibits about two-, three-, four-, or five-fold increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL and exhibits at least a two-fold increase in activity as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL and exhibits at least a three-fold increase in activity as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising the wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL and exhibits at least a four-fold increase in activity as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL and exhibits at least a five-fold increase in activity as compared to a suitable control, e.g. a genetically engineered microorganism, e.g. bacteria, comprising wild type PAL, e.g., *Photorhabdus luminescens* PAL.

[087] In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise additional PME(s), e.g., PAH, LAAD. Exemplary PMEs and

combinations thereof are known in the art, see, e.g., PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL and a wild type PAL.

[088] In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL as described herein and a phenylalanine transporter as described herein, e.g., PheP. In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a mutant PAL as described herein, a LAAD as described herein, and a phenylalanine transporter as described herein, e.g., PheP.

[089] In some embodiments, the genetically engineered microorganisms, e.g., bacteria, comprise a transcriptional regulator, e.g., a non-native transcriptional regulator as described herein.

[090] In these embodiments, the PME, e.g., mutant PAL, phenylalanine transporter, and/or transcriptional regulator present in the genetically engineered microorganism, e.g. bacteria, may be operably linked to one or more promoters. The promoters may be the same or different for each gene or each copy of each gene. In some embodiments, the promoter is a constitutive promoter or an inducible promoter. In some embodiments, the promoter is induced by exogenous environmental conditions. In some embodiments, the promoter is induced by exogenous environmental conditions, such as in the presence of molecules or metabolites specific to the gut of a mammal. In some embodiments, the promoter is induced by low-oxygen, microaerobic, or anaerobic conditions, wherein expression of the gene, is activated under low-oxygen or anaerobic environments, such as the environment of the mammalian gut. In some embodiments, the promoter is an FNR, an ANR, or a DNR promoter. In some embodiments, the oxygen level-dependent promoter is fused to a binding site for a transcriptional activator, e.g., CRP. In some embodiments, the promoter is 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 LAAD expression occurs under aerobic or microaerobic conditions. In one embodiment, PAL expression occurs under anaerobic or low oxygen

conditions and LAAD expression is under the control of the P_{araBAD} promoter. In some embodiments, the one or more PME and/or phenylalanine transporter and/or transcriptional regulator gene is 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 PMEs and/or phenylalanine transporter and/or transcriptional regulator gene is expressed under the control of a promoter that is induced by exposure to tetracycline. In some embodiments, the one or more PMEs and/or phenylalanine transporter gene and/or transcriptional regulator is expressed under the control of a promoter that is induced by exposure to arabinose. In some embodiments the one or more PMEs and/or phenylalanine transporter gene and/or transcriptional regulator is 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 PMEs and/or phenylalanine transporter and/or transcriptional regulator gene is expressed under the control of a promoter that is induced by exposure to rhamnose. In some embodiments, the one or more PMEs and/or phenylalanine transporter and/or transcriptional regulator gene is expressed under the control of a promoter that is induced by exposure to tetracycline. 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 herein.

[091] In some embodiments, the PME, e.g., mutant PAL, phenylalanine transporter, and/or transcriptional regulator expression is controlled by a temperature sensitive mechanism, e.g., the mutant cI857 repressor, the pL and/or pR phage λ promoters. In some embodiments, 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, e.g. 37-42 °C, the functional cI857 dimer is destabilized, binding to the oL or oR DNA sequences is abrogated, and mRNA transcription is initiated. Inducible expression from the ParaBad can be controlled or further fine-tuned through the optimization of the ribosome binding site (RBS), as described herein.

[092] In one embodiment, expression of one or more PME(s), and/or Phe transporter(s), e.g., PheP, and/or transcriptional regulator(s), in the genetically engineered microorganisms, e.g., bacteria, is driven by one or more thermoregulated promoter(s). In one embodiment, expression of PAL is driven by a thermoregulated promoter. In one embodiment, expression of PheP is driven by a thermoregulated promoter. In one

embodiment, expression of LAAD is driven by a thermoregulated promoter.

[093] In some embodiments, more than one PME gene is expressed in the genetically engineered microorganisms, e.g., bacteria, and each gene is expressed under the control of the same promoter, such as any of the promoters discussed herein. In some embodiments, more than one PME gene is expressed in the genetically engineered microorganisms, e.g., bacteria, and each gene is expressed under the control of the same promoter, such as any of the promoters discussed herein. In some embodiments, the PME gene(s) and/or phenylalanine transporter and/or transcriptional regulator gene in the genetically engineered microorganisms, e.g., bacteria is expressed under the control of different promoters, such as any of the promoters discussed herein. In some embodiments, the PME gene(s) and/or phenylalanine transporter and/or transcriptional regulator gene in the genetically engineered microorganisms, e.g., bacteria is expressed under the control of the same promoter, such as any of the promoters discussed herein.

[094] In another embodiment, one or more inducible promoter(s), e.g., thermoregulated, arabinose-inducible, tet-inducible, and IPTG-inducible promoters, in the genetically engineered microorganisms, e.g., bacteria drive the expression of one or more bicistronic message(s). Bicistronic messages 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). In one embodiment, one or more inducible promoter(s) drive the expression of tri-cistronic messages. Tri-cistronic messages 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). In one embodiment, one or more inducible promoter(s) drive the expression of multi-cistronic messages. Multi-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).

[095] The one or more PMEs, phenylalanine transporter and transcriptional regulator gene(s) may be present on a plasmid or chromosome in the genetically engineered microorganism, e.g. bacteria. In some embodiments, expression from the chromosome may be useful for increasing stability of expression of the PME and/or phenylalanine transporter and/or transcriptional regulator. In some embodiments, the one or more PME and/or phenylalanine transporter and/or transcriptional regulator gene(s) is integrated into the chromosome of the microorganism at one or more integration sites in the genetically engineered microorganism. In some embodiments, the one or more PME

and/or phenylalanine transporter and/or transcriptional regulator gene(s) is expressed on a plasmid. In some embodiments, the plasmid is a low copy plasmid. In other embodiments, the plasmid is a high copy plasmid.

[096] In some embodiments, the genetically engineered microorganism, e.g., bacteria, may also comprise one or more gene sequences relating to biosafety and/or biocontainment, e.g., a kill-switch, gene guard system, essential gene for cell growth and/or survival, *thyA*, *dapA*, and/or auxotrophy. The expression of these gene sequence(s) may be regulated using the promoters or promoter systems described herein. The 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 microorganism. Examples and combinations are known in the art, see, e.g., PCT/US2016/032562 and PCT/US2016/062369, U.S. Provisional Application No. 62/184,811, PCT/US2016/062369, the contents of which are hereby incorporated by reference.

[097] In some embodiments, the genetically engineered microorganisms further comprise a native secretion mechanism or non-native secretion mechanism that is capable of secreting a molecule from the cytoplasm in the extracellular environment. Many microorganisms have evolved sophisticated secretion systems to transport substrates across the 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 membrane of the microorganism. Examples of secretion systems are disclosed in PCT/US2016/062369.

[098] In some embodiments, wherein the genetically engineered microorganism is a bacterium, the disclosure provides a bacterium comprising one or more phage genome(s), wherein one or more of the phage genomes are defective. In some embodiments, the disclosure provides a bacterium comprising one or more phage genome(s), wherein one or more of the phage genomes are defective such that lytic phage

is not produced. In some embodiments, the disclosure provides a bacterium comprising one or more phage genome(s), wherein one or more of the phage genomes are defective in that one or more phage genes are not expressed. In some embodiments, the disclosure provides a bacterium comprising one or more phage genome(s), wherein one or more phage genes in the one or more phage genome(s) comprise one or more mutations. In some embodiments, the one or more phage genome(s) are present in the natural state of the probiotic bacterium. In some embodiments, the bacteria encode one or more lysogenic phage(s). In some embodiments, the bacteria encode one or more defective or cryptic phage(s) or satellite phage(s). In some embodiments, the bacteria encode one or more tailiocins or gene transfer agents. In some embodiments, the one or more mutations affect the ability of the phage to undergo the lytic cycle, e.g., reduce the frequency or reduce the number of bacteria in a given population that can undergo the lytic stage. In some embodiments, the one or more mutations prevent the phage from infecting other bacteria. In some embodiments, the one or more mutations alters, e.g., increases or reduces, bacterial fitness.

[099] In some embodiments, one or more of the phage genomes of the genetically engineered bacteria are mutated. Such mutations may include one or more deletion(s) of a part of or the complete sequence of one or more phage genes. Alternatively, the mutations may include one or more insertion(s) of one or more nucleotides into one or more phage genes. In another example, the mutations may include one or more substitution(s) of a part of or the complete sequence of one or more phage genes. In another example, the mutations include one or more inversion(s) of a part of or the complete sequence of one or more phage genes in the phage genome. Additionally, the mutations may include any combination of one or more deletions, insertions, substitutions or inversions. In certain embodiments, the one or more mutations reduce or prevent the production and release of phage particles from the bacterium relative to the same bacterium not having the one or more targeted mutations in the one or more phage genomes. In some embodiments, the bacterium is *Escherichia coli* strain Nissle. In some embodiments, the phage genome which is mutated is E coli Nissle Phage 1 genome, the E coli Nissle Phage 2 genome and/or the E coli Nissle Phage 3 genome. In one embodiment, the mutated phage genome is the E coli Nissle Phage 3 genome. In one embodiment, the mutations are located in or comprise one or more genes selected from ECOLIN_09965, ECOLIN_09970, ECOLIN_09975, ECOLIN_09980, ECOLIN_09985, ECOLIN_09990, ECOLIN_09995,

ECOLIN_10000, ECOLIN_10005, ECOLIN_10010, ECOLIN_10015, ECOLIN_10020, ECOLIN_10025, ECOLIN_10030, ECOLIN_10035, ECOLIN_10040, ECOLIN_10045, ECOLIN_10050, ECOLIN_10055, ECOLIN_10065, ECOLIN_10070, ECOLIN_10075, ECOLIN_10080, ECOLIN_10085, ECOLIN_10090, ECOLIN_10095, ECOLIN_10100, ECOLIN_10105, ECOLIN_10110, ECOLIN_10115, ECOLIN_10120, ECOLIN_10125, ECOLIN_10130, ECOLIN_10135, ECOLIN_10140, ECOLIN_10145, ECOLIN_10150, ECOLIN_10160, ECOLIN_10165, ECOLIN_10170, ECOLIN_10175, ECOLIN_10180, ECOLIN_10185, ECOLIN_10190, ECOLIN_10195, ECOLIN_10200, ECOLIN_10205, ECOLIN_10210, ECOLIN_10220, ECOLIN_10225, ECOLIN_10230, ECOLIN_10235, ECOLIN_10240, ECOLIN_10245, ECOLIN_10250, ECOLIN_10255, ECOLIN_10260, ECOLIN_10265, ECOLIN_10270, ECOLIN_10275, ECOLIN_10280, ECOLIN_10290, ECOLIN_10295, ECOLIN_10300, ECOLIN_10305, ECOLIN_10310, ECOLIN_10315, ECOLIN_10320, ECOLIN_10325, ECOLIN_10330, ECOLIN_10335, ECOLIN_10340, and ECOLIN_10345. In one embodiment, the mutations, e.g., one or more deletions, are located in or comprise one or more genes selected from ECOLIN_10110, ECOLIN_10115, ECOLIN_10120, ECOLIN_10125, ECOLIN_10130, ECOLIN_10135, ECOLIN_10140, ECOLIN_10145, ECOLIN_10150, ECOLIN_10160, ECOLIN_10165, ECOLIN_10170, and ECOLIN_10175. pharmaceutically acceptable composition comprising the bacterium disclosed herein and a pharmaceutically acceptable carrier.

[0100] Modifications of phage genomes are known in the art, see, e.g., PCT/US18/38840, the contents of which are hereby incorporated by reference.

[0101] In some embodiments, the mutations are located within or encompass one or more genes encoding lytic genes. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more proteases or lysins. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more toxins. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more antibiotic resistance related proteins. In some embodiments, the mutations are located within or encompass one or more genes encoding one or phage translation related proteins. In some embodiments, the one or more mutations are located within or encompass one or more genes encoding structural proteins. Such structural genes include genes encoding polypeptides of the head, tail, collar, or coat. In some embodiments, the one or more mutations are located within or encompass one or more genes encoding polypeptides of the head structure. In some

embodiments, the one or more mutations are located within or encompass one or more genes encoding polypeptides of the tail structure. In some embodiments, the one or more mutations are located within or encompass one or more genes encoding polypeptides of the collar structure. In some embodiments, the one or more mutations are located within or encompass one or more genes encoding tail proteins. In some embodiments, the one or more mutations are located within or encompass one or more genes encoding polypeptides of the coat structure. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more plate proteins. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more proteins require for assembly of the bacteriophage. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more portal proteins. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more polypeptides involved in recombination. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more integrases. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more invertases. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more transposases. In some embodiments, the mutations are located with within or encompass one or more genes encoding one or more polypeptides involved in replication or translation. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more primases. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more tRNA related proteins. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more polypeptides involved in phage insertion. In some embodiments, the mutations are located within or encompass one or more genes encoding an attachment site. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more polypeptides involved in packaging. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more terminases. In some embodiments, the mutations are located within or encompass one or more genes encoding one or more tailiocsins. In some embodiments, the mutations are located within one or more genes associated with lytic growth, horizontal gene transfer, cell lysis, phage structure, phage assembly, phage packaging, recombination, replication, translation, phage insertion, or combinations thereof. In some embodiments, the

mutations are located within or encompass one or more genes encoding one or more host genes. In some embodiments, the mutation is in a gene encoding lipid A biosynthesis (KDO)2-(lauroyl)-lipid IVA acyltransferase, peptidase, zinc ABC transporter substrate-binding protein, zinc ABC transporter ATPase, high-affinity zinc transporter membrane component, ATP-dependent DNA helicase RuvB, ATP-dependent DNA helicase RuvA, Holliday junction resolvase, dihydroneopterin triphosphate pyrophosphatase, aspartyl-tRNA synthetase, hydrolase, DNA polymerase V, MsgA, phage tail protein, tail protein, host specificity protein, peptidase P60, tail protein, tail fiber protein, Minor tail protein U, DNA breaking-rejoining protein, peptidase S14, capsid protein, DNA packaging protein, terminase, lysozyme, holin, DNA adenine methylase, serine protease, antitermination protein, antirepressor, crossover junction endodeoxyribonuclease, adenine methyltransferase, DNA methyltransferase ECOLIN_10240, GntR family transcriptional regulator ECOLIN_10245, cI repressor, Domain of unknown function (DUF4222); DNA recombinase, Multiple Antibiotic Resistance Regulator (MarR), unknown ead like protein in P22, Protein of unknown function (DUF550); 3'-5' exonuclease, excisionase, integrase, tRNA methyltransferase, and combinations thereof.

[0102] In some embodiments, the mutations are located within or encompass genes encoding one or more polypeptides involved in one or more of cell lysis, phage structure, phage assembly, phage packaging recombination, replication or translation, phage insertion, or are host proteins, and combinations thereof.

[0103] In some embodiments, described herein genetically engineered bacteria are engineered *Escherichia coli* strain Nissle 1917. Bioinformatics assessment, as described in PCT/US2018/038840, which is incorporated in herein by reference in its entirety, uncovered three high-confidence, predicted prophage sequences in the *E. coli* Nissle genome, referred herein to as Phage 1, Phage 2, and Phage 3. The longest predicted phage in *E. coli* Nissle (Phage 3) contains a total of 68 proteins, and includes a phage tail, head, portal, terminase, lysin, capsid, and integrase, all of which appear to be intact. Phage 2 contains a total of 69 proteins, and includes phage transposase, lysis, terminase, head, portal, capsid, and tail proteins. Closer inspection of Phage 2 revealed that the *int/xis* gene pair have been disrupted by a mobile genetic element, and that the *cI* repressor has been fragmented into separate DNA-binding and sensing peptides, which would be expected to prevent induction of this phage. The shortest of the intact phages predicted in *E. coli* Nissle, Phage 1, contains a total of 32 proteins, and includes lysis and transposase

functionality. However, the absence of many structural genes within the putative prophage element termed Phage 1 calls into question its potential to release viable phage particles. In some embodiments, the genetically engineered bacteria comprise one or more *E. coli* Nissle bacteriophage, e.g., Phage 1, Phage 2, and Phage 3. In some embodiments, the genetically engineered bacteria comprise Phage 3 of *E. coli* Nissle bacteriophage. PCT/US18/38840, the contents of which are hereby incorporated by reference, provides genes of exemplary phage.

Pharmaceutical Compositions

[0104] Pharmaceutical compositions comprising the genetically engineered microorganisms, e.g., the genetically engineered bacteria comprising mutant PAL, disclosed herein 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 microorganisms, 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 microorganism 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 microorganism that are each engineered to comprise the genetic modifications described herein.

[0105] The pharmaceutical compositions described herein may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into compositions for pharmaceutical use. Methods of formulating pharmaceutical compositions are known in the art (*see, e.g.*, "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA). In some embodiments, the pharmaceutical compositions are subjected to tableting, lyophilizing, direct compression, conventional mixing, dissolving, granulating, levigating, emulsifying, encapsulating, entrapping, or spray drying to form tablets, granulates, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. Appropriate formulation depends on the route of administration.

[0106] The genetically engineered microorganisms 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). In some embodiments, the genetically engineered microorganism is a bacterium. 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.

[0107] The genetically engineered microorganisms 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 microorganisms 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 microorganisms 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.

[0108] The genetically engineered microorganisms disclosed herein may be administered topically and formulated in the form of an ointment, cream, transdermal

patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form well-known to one of skill in the art. *See, e.g., "Remington's Pharmaceutical Sciences,"* Mack Publishing Co., Easton, PA. In an embodiment, for non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity greater than water are employed. Suitable formulations include, but are not limited to, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, etc., which may be sterilized or mixed with auxiliary agents (*e.g.,* preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, *e.g.,* osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (*e.g.,* a gaseous propellant, such as freon) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms. Examples of such additional ingredients are well known in the art. In one embodiment, the pharmaceutical composition comprising the recombinant microorganism of the invention may be formulated as a hygiene product. For example, the hygiene product may be an antimicrobial formulation, or a fermentation product such as a fermentation broth. Hygiene products may be, for example, shampoos, conditioners, creams, pastes, lotions, and lip balms.

[0109] The genetically engineered microorganisms disclosed herein may be administered orally and formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, etc. Pharmacological compositions for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients include, but are not limited to, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose compositions such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, 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.

[0110] Tablets or capsules can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.,* pregelatinised maize

starch, polyvinylpyrrolidone, hydroxypropyl methylcellulose, carboxymethylcellulose, polyethylene glycol, sucrose, glucose, sorbitol, starch, gum, kaolin, and tragacanth); fillers (*e.g.*, lactose, microcrystalline cellulose, or calcium hydrogen phosphate); lubricants (*e.g.*, calcium, aluminum, zinc, stearic acid, polyethylene glycol, sodium lauryl sulfate, starch, sodium benzoate, L-leucine, magnesium stearate, talc, or silica); disintegrants (*e.g.*, starch, potato starch, sodium starch glycolate, sugars, cellulose derivatives, silica powders); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. A coating shell may be present, and common membranes include, but are not limited to, polylactide, polyglycolic acid, polyanhydride, other biodegradable polymers, alginate-polylysine-alginate (APA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroxymethylacrylate-methyl methacrylate (HEMA-MMA), multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitrile/sodium methallylsulfonate (AN-69), polyethylene glycol/poly 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.

[0111] In some embodiments, the genetically engineered microorganisms are enterically coated for release into the gut or a particular region of the gut, for example, the large intestine. The typical pH profile from the stomach to the colon is about 1-4 (stomach), 5.5-6 (duodenum), 7.3-8.0 (ileum), and 5.5-6.5 (colon). In some diseases, the pH profile may be modified. In some embodiments, the coating is degraded in specific pH environments in order to specify the site of release. In some embodiments, at least two coatings are used. In some embodiments, the outside coating and the inside coating are degraded at different pH levels.

[0112] Liquid preparations for oral administration may take the form of solutions, syrups, suspensions, or a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable agents such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives, or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol, or fractionated

vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring, and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated for slow release, controlled release, or sustained release of the genetically engineered microorganisms described herein.

[0113] In certain embodiments, the genetically engineered microorganisms may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

[0114] In another embodiment, the pharmaceutical composition comprising the recombinant microorganisms 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 microorganism 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 microorganisms 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.

[0115] In some embodiments, the composition is formulated for intrainestinal administration, intrajejunal administration, intraduodenal administration, intraileal administration, gastric shunt administration, or intracolic administration, via nanoparticles, nanocapsules, microcapsules, or microtablets, which are enterically coated or uncoated. The pharmaceutical compositions may also be formulated in rectal compositions such as suppositories or retention enemas, using, *e.g.*, conventional suppository bases such as cocoa butter or other glycerides. The compositions may be suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain suspending, stabilizing and/or dispersing agents.

[0116] The genetically engineered microorganisms described herein may be administered intranasally, formulated in an aerosol form, spray, mist, or in the form of drops, and conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant (*e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas). Pressurized aerosol dosage units may be determined by providing a valve to deliver a metered amount. Capsules and cartridges (*e.g.*, of gelatin) for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0117] The genetically engineered microorganisms may be administered and formulated as depot preparations. Such long acting formulations may be administered by implantation or by injection, including intravenous injection, subcutaneous injection, local injection, direct injection, or infusion. For example, the compositions may be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives (*e.g.*, as a sparingly soluble salt).

[0118] 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.

[0119] 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.

[0120] Dosage regimens may be adjusted to provide a therapeutic response. Dosing can depend on several factors, including severity and responsiveness of the disease, route of administration, time course of treatment (days to months to years), and time to amelioration of the disease. For example, a single bolus may be administered at one time, several divided doses may be administered over a predetermined period of time, or the dose may be reduced or increased as indicated by the therapeutic situation. The specification for the dosage is dictated by the unique characteristics of the active compound and the particular therapeutic effect to be achieved. Dosage values may vary with the type and severity of the condition to be alleviated. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgment of the treating clinician. Toxicity and therapeutic efficacy of compounds provided herein can be determined by standard pharmaceutical procedures in cell culture or animal models. For example, LD₅₀, ED₅₀, EC₅₀, and IC₅₀ may be determined, and the dose ratio between toxic and therapeutic effects (LD₅₀/ED₅₀) may be calculated as the therapeutic index. Compositions that exhibit toxic side effects may be used, with careful modifications to minimize potential damage to reduce side effects. Dosing may be estimated initially from cell culture assays and animal models. The data obtained from *in vitro* and *in vivo* assays and animal studies can be used in formulating a

range of dosage for use in humans.

[0121] 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

[0122] 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 microorganism, *e.g.*, 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 microorganism comprising gene sequence encoding one or more PME's, *e.g.*, PAL including mutant PAL, PAH, and/or LAAD.

[0123] In some embodiments, the method of treatment comprises administering a microorganism, *e.g.*, bacterium, comprising a mutant PAL derived from wild-type

Photorhabdus luminescens PAL, e.g., SEQ ID NO: 1. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL with mutations in one or more amino acid positions selected from 92, 133, 167, 432, 470, 433, 263, 366 and 396 compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL with mutations in one or more amino acid positions selected from S92, H133, I167, L432, V470, A433, A263, K366, and/or L396 compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133M, H133F, I167K, L432I, V470A, A433S, A263T, K366K (e.g., silent mutation in polynucleotide sequence), and/or L396L (e.g., silent mutation in polynucleotide sequence) compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1.

[0124] In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133M, I167K, L432I, and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133F, A433S, and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL with mutations in one or more amino acid positions selected from S92G, H133F, A263T, K366K (e.g., silent mutation in polynucleotide sequence), L396L (e.g., silent mutation in polynucleotide sequence), and V470A compared to positions in wild type PAL, e.g., *Photorhabdus luminescens* PAL, e.g., SEQ ID NO: 1.

[0125] In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising mPAL1. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising mPAL2. In some embodiments, the method of treatment comprises administering a microorganism,

e.g., bacterium, comprising mPAL3.

[0126] In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL that exhibits increased stability compared to wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL that exhibits increased activity or ability to metabolize phenylalanine and/or reduce hyperphenylalaninemia compared to a wild type PAL, e.g., *P. luminescens* PAL. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL that exhibits at least a two-fold increase in activity compared to wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL that exhibits at least a three-fold increase in activity compared to the wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL that exhibits at least a four-fold increase in activity compared to the wild type PAL, e.g., *Photorhabdus luminescens* PAL. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL that exhibits at least a five-fold increase in activity compared to the wild type PAL, e.g., *Photorhabdus luminescens* PAL.

[0127] In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, further comprising additional PME(s), e.g., PAH, LAAD, and/or phenylalanine transporter(s). Exemplary PMEs and combinations thereof are known in the art, see, e.g., PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference. In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, comprising a mutant PAL and a wild type PAL.

[0128] In some embodiments, the method of treatment comprises administering a microorganism, e.g., bacterium, further comprising a transcriptional regulator, e.g., a non-native transcriptional regulator as described herein. In these embodiments, the PME, e.g., mutant PAL, phenylalanine transporter, and/or transcriptional regulator may be operably linked to one or more promoters as disclosed herein, e.g., a constitutive promoter, an inducible promoter, a thermoregulated promoter, an oxygen-level dependent promoter, etc.

[0129] In some embodiments, the genetically engineered microorganism, e.g.,

bacteria, may also comprise one or more gene sequences relating to biosafety and/or biocontainment as described herein, e.g., a kill-switch, gene guard system, essential gene for cell growth and/or survival, *thyA*, *dapA*, auxotrophy, etc.

[0130] 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.

[0131] In certain embodiments, the genetically engineered microorganisms 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 microorganisms are delivered simultaneously with dietary protein. In other embodiments, the genetically engineered bacteria are not delivered simultaneously 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 microorganisms 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 microorganisms 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 microorganisms need not be delivered simultaneously with dietary protein, and a phenylalanine gradient is generated,

e.g., from blood to gut, and the genetically engineered microorganisms metabolize phenylalanine and reduce hyperphenylalaninemia.

[0132] The method may comprise preparing a pharmaceutical composition with at least one genetically engineered species, strain, or subtype of microorganism, *e.g.*, bacterium, described herein, and administering the pharmaceutical composition to a subject in a therapeutically effective amount. In some embodiments, the genetically engineered microorganisms of the invention are administered orally, *e.g.*, in a liquid suspension. In some embodiments, the genetically engineered microorganisms of the invention are lyophilized in a gel cap and administered orally. In some embodiments, the genetically engineered microorganisms of the invention are administered via a feeding tube or gastric shunt. In some embodiments, the genetically engineered microorganism of the invention are administered rectally, *e.g.*, by enema. In some embodiments, the genetically engineered microorganisms of the invention are administered topically, intraintestinally, intrajejunally, intraduodenally, intraileally, and/or intracolically.

[0133] 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.

[0134] 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.

[0135] 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.

[0136] In some embodiments, the activity (e.g., phenylalanine degrading activity) of genetically engineered microorganism expressing PAL, e.g., mutant PAL, 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). Phenylalanine 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.

[0137] 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.

[0138] 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 microorganism of the present disclosure, e.g., microorganism 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 hyperphenylalaninemia 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 hyperphenylalaninemia 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.

[0139] 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.

[0140] 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.

[0141] 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.

[0142] 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.

[0143] 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.

[0144] 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-beta-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, eosinophils, and monocytes), and platelets. Other non-limiting 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.

[0145] 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).

[0146] In some embodiments, the genetically engineered microorganisms are administered in combination with one or more recombinantly produced PME enzymes, *e.g.* recombinant PAL, LAAD or PAH. In some embodiments, the recombinant PAL is a mutant PAL. 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 microorganism is delivered as amorphous silica PME particles.

Examples

Example 1. Construction of PAL Plasmids and Transforming Bacteria

[0147] To facilitate inducible production of PAL in *Escherichia coli* Nissle, the *PAL* gene of *Anabaena variabilis* or *Photorhabdus luminescens*, 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 promoters are provided herein.

[0148] 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 OD₆₀₀ 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.

[0149] To facilitate inducible production of mutant, mPAL1, mPAL2 and mPAL3 were cloned into low copy plasmids (pSC101 origin of replication) under control of an anhydrous tetracycline (aTc)-responsive promoter and transferred to Nissle bacteria.

Example 2. Screening Process, Including Identification of mPAL1, mPAL2 and mPAL3

[0150] To generate PAL activity in strains, cultures containing plasmids expressing wild type PAL3, mPAL1, mPAL2, and mPAL3 were first grown overnight. The next morning, overnight cultures were used to back-dilute into fresh media at an OD₆₀₀ = 0.1 and cultures were grown to early log phase. Upon entry into early log phase, aTc was added at a concentration of 200 ng/mL for induction of PAL, and the induction proceeded for 5 hours. At the end of the induction phase, the cultures were centrifuged, the supernatant discarded, and the pellets resuspended in 15% glycerol. The cell material was stored at -80 degrees C until the day of testing in vitro PAL activity (TCA production).

[0151] To test for PAL activity from activated cells, frozen cell aliquots were thawed and resuspended in sodium bicarbonate buffer at 5.0×10^9 CFU/mL. This solution was then mixed with equal parts of simulated gastric fluid (SGF) and incubated for 2 hours at 37°C with shaking. After 2 hours, samples were removed and cells were pelleted by centrifugation. The supernatant was recovered and analyzed for *trans*-cinnamate

(TCA) (see FIGS. 1 and 2).

[0152] Quantification of trans-cinnamic acid (TCA) was performed using a Shimadzu HPLC-PDA system. TCA standards were prepared in assay media with the following concentrations: 0.005, 0.03, 0.1, 0.7, 3.4, 6.7, 16.9, 33.7, 50.6 mM. Bacterial supernatant samples were thawed and centrifuged at 4000 rpm for 5min. In a 96-well plate, 5 μ L of the standards and samples were transferred, followed by the addition of 195 μ L of water. The plate was heat-sealed with a ClearSeal cover and mixed.

[0153] The injection volume used was 20 μ L and the run time was 10 min at a flow rate of 0.35mL/min. Mobile phase A was 0.1% Trifluoroacetic acid in water and mobile phase B was 0.1% Trifluoroacetic acid in acetonitrile. Chromatographic separation was carried out using a Thermo Scientific Hypersil Gold, 100x21mm, 1.9 μ , Part No. 25002-102130, with the following gradient: 5% $\rightarrow$ 35% B from 0 to 2 min, 35% B from 2 to 4 min, 90% B from 4.01 to 4.50 min, 5% B from 4.51 to 6 min, stop at 10 min. Retention time for TCA was 6.05 min, absorbing at 315 nm. (FIG. 1 and FIG. 2).

Example 3. Efficacy of Mutant PAL in a Mouse Model of PKU

[0154] For *in vivo* studies, BTBR-*Pah*^{emu2} mice are obtained from Jackson Laboratory and bred to homozygosity for use as a model of PKU. Bacteria harboring the PAL mutant described herein are grown. Bacteria are resuspended in phosphate buffered saline (PBS) and administered to mice by oral gavage. The bacteria may be induced by ATC for 2 hours prior to administration.

[0155] At the beginning of the study, mice are given water that was supplemented with 100 micrograms/mL ATC and 5% sucrose. Mice are fasted by removing chow overnight (10 hrs), and blood samples are collected by mandibular bleeding the next morning in order to determine baseline phenylalanine levels. Blood samples are collected in heparinized tubes and spun at 2G for 20 min to produce plasma, which is then removed and stored at -80° C. Mice are given chow again, and are gavaged after 1 hr. with 100 μ L (5×10^9 CFU) of bacteria that had previously been induced for 2 hrs with ATC. Mice are put back on chow for 2 hrs. Plasma samples are prepared as described above. Phenylalanine levels before and after feeding are measured and compared to controls.

[0156] For subcutaneous phenylalanine challenge, beginning at least 3 days prior to the study (i.e., Days -6 to -3), homozygous BTBR-*Pah*^{emu2} mice (approx. 6-12 weeks of age) are maintained on phenylalanine-free chow and water supplemented with 0.5 grams/L phenylalanine. On Day 1, mice are randomized into treatment groups and blood samples

are collected by sub-mandibular skin puncture to determine baseline phenylalanine levels. Mice are also weighed to determine the average weight for each group. Mice are 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 μ L of H₂O (n=30), control bacteria, or the bacteria comprising mutant PAL are administered to mice by oral gavage. Blood samples are collected at 2 hrs and 4 hrs following phenylalanine challenge, and phenylalanine levels in the blood are measured using mass spectrometry.

[0157] Additional assays of PAL activity, e.g., mutant PAL activity, are known in the art. See, e.g., PCT/US2016/032562 and PCT/US2016/062369, the contents of which are hereby incorporated by reference.

Example 4. Kinetic Measurements of PAL Variants

[0158] Michaelis-Menten graphs with rate V (μ M TCA/min) as a function of Phe concentration [Phe] (mM) were generated for wild type PAL3, mPAL1, mPAL2, and mPAL3. Bacteria were inoculated 1:100 from a saturated overnight pre-culture, followed by induction with 200 ng/mL ATC two hours later. After four hours of induction, cells were pelleted, washed in PBS, normalized to OD₆₀₀=50 in PBS, and diluted 2-fold into 50% glycerol for storage at -80°C. Lysate from each strain was prepared via sonication using a Branson Digital Sonifier with microtip. The soluble fraction of the lysate samples was used for the kinetic assay. Total protein in the lysate samples was measured via Bradford Assay, and all samples were normalized to 10 μ g total protein loading per well for the kinetic assay. The lysate samples were incubated in 1X M9 0.5% glucose with Phe concentrations ranging from 40 mM Phe down to 39 μ M with 2-fold dilutions. The kinetic assay was performed in UV-star 96-well microplates (Greiner) with TCA quantified by A290 measurements every minute using a BioTek Synergy H1 microplate reader set to 37°C static incubation. The data points on each graph are rate (V in μ M TCA/min) calculated from the first hour of activity for each Phe concentration tested, where activity remained linear. (FIG. 3).

Example 5. Efficacy of Mutant PAL in a Cynomolgus Monkey Model

[0159] To evaluate the in vivo efficacy of the mutant PAL described herein, genetically engineered E. coli Nissle comprising mPAL2 (SYN7262) was administered via nasal gastric gavage as described in U.S. Patent No. 10,610,546, the contents of which are hereby incorporated by reference in its entirety. Briefly, SYNB1618 and SYN7262 strains

were grown in a bioreactor and PAL expression induced via addition of anaerobiosis/IPTG or ATC/IPTG, respectively. At the end of fermentation, cells were spun down and stored in 15% glycerol at -80C. On the day of dosing, each animal was administered 5.5 g of protein in the form of peptone, and 250 mg of D5-Phe, followed by a 1e11 live cell dose of either SYN1618 or SYN7262. Blood and urine were collected over a six-hour period. Plasma TCA areas under the curve, as well as excretion of urinary hippurate were analyzed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Max triple quadrupole mass spectrometer. (FIG.4).

Example 6. Whole Cell Activity of PKU Strains

[0160] Bacterial strains comprising different copy numbers of wild type PAL3 were prepared as described herein. A portion of cells from each strain were then lysed. The soluble fraction of the lysate samples was used for the activity assay. PAL3 activity of intact bacteria and lysates was measured as described previously. Increased copy number (expression) of PAL3 has little effect on whole-cell rate of TCA production. In contrast, when the same cell material is lysed, increased copy number (expression) corresponds to increased activity. Lysate PAL activity, measured by conversion of d5-Phe to d5-TCA, decreases in the presence of increasing exogenous unlabeled TCA, demonstrating that the enzyme is feedback inhibited by its product. Addition of salicylate, an inducer of efflux pumps in *E. coli*, during induction of PAL led to increased rates of whole-cell PAL activity in vitro. (FIG.5).