Drug-delivering bacteria

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Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 September 2023 (21.09.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/175124 A1

(51) International Patent Classification:
A61K 35/74 (2015.01) *C12N 9/02* (2006.01)
A61K 35/741 (2015.01) *C12N 9/06* (2006.01)
A61P 25/16 (2006.01) *C12N 9/10* (2006.01)
C12N 1/20 (2006.01) *C12N 9/12* (2006.01)

(21) International Application Number:
PCT/EP2023/056845

(22) International Filing Date:
17 March 2023 (17.03.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
22163023.9 18 March 2022 (18.03.2022) EP
22212368.9 08 December 2022 (08.12.2022) EP

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV,

(54) Title: DRUG-DELIVERING BACTERIA

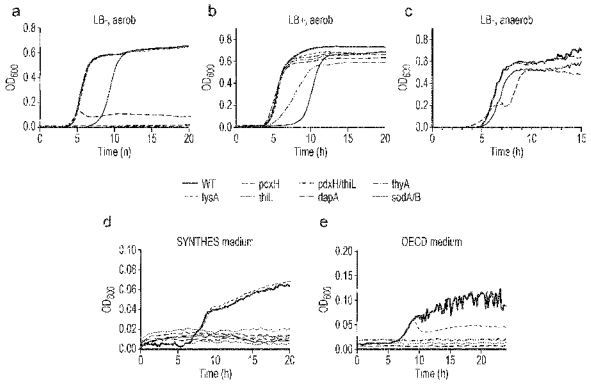

FIG. 1

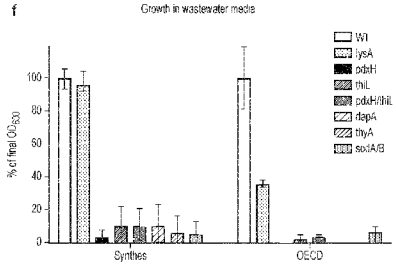

FIG. 1 (Continued)

(57) Abstract: The invention relates to a bacterium deficient in at least two functional genes in the redox pathway; or at least one functional gene in the vitamin B1 synthetic pathway and at least one functional gene in the vitamin B6 pathway. The invention also relates to the bacterium comprising an expression cassette for producing a therapeutic molecule. The invention also relates to a kit with the bacterium and a plasmid; as well as a pharmaceutical formulation comprising the bacterium. The invention also relates to use of the bacterium as a medicament, for example for treating Parkinson's disease.

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

Published:

— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

Drug-delivering bacteria

Field of the Invention

The present invention relates to a bacterium deficient in at least two functional genes in the redox pathway or at least one functional gene in the vitamin B1 synthetic pathway and at least one functional gene in the vitamin B6 pathway. The invention also relates to the bacterium comprising an expression cassette for producing a therapeutic molecule. The invention also relates to a kit with the bacterium and a plasmid; as well as a pharmaceutical formulation comprising the bacterium. The invention also relates to use of the bacterium as a medicament, for example for treating Parkinson's disease.

Background

Genetically engineered bacteria can be used to deliver therapeutic molecules in the mammalian gut. However, their ability to survive and reproduce inside and outside a patient must be controlled to ensure safety and minimize release into the environment.

Summary of the Invention

The inventors have developed novel therapeutic bacteria to deliver therapeutic molecules (medicaments).

These bacteria survive in the mammalian gut to allow the consistent delivery of the therapeutic molecules (medicaments) to the patient. However, when released from the gut, the bacteria do not survive preventing release of recombinant bacteria into the environment.

In a first aspect of the invention there is provided a bacterium deficient in:
a) at least two functional genes in the redox detoxification pathway; or
b) at least one functional gene in the vitamin B1 synthetic pathway and at least one functional gene in the vitamin B6 synthetic pathway.

In a further aspect of the invention, there is provided a bacterium comprising an expression cassette for producing a therapeutic molecule (or medicament), wherein the bacterium is deficient in:

a) at least two functional genes in the redox detoxification pathway; or
b) at least one functional gene in the vitamin B1 synthetic pathway and at least one functional gene in the vitamin B6 synthetic pathway.

In a further aspect of the invention there is provided a kit comprising the bacterium according to the first aspect and a plasmid.

In a further aspect of the invention there is provided a pharmaceutical formulation comprising the bacterium of the first aspect.

In a further aspect of the invention there is provided a bacterium of the first aspect or a pharmaceutical formulation thereof, for use as a medicament, for example, for use in a method of treating Parkinson's disease.

Detailed description

General terms

The bacterium

The bacteria may be a therapeutic bacteria. By therapeutic is meant these cells are non-pathogenic. The bacteria may be commensal. That is, the bacterium may be non-pathogenic and commensal.

The bacteria may be capable of colonizing the gut. The bacteria may be a gut microbe (a bacteria belonging to the human gut microbiome or microflora). Bacteria capable of colonizing the gut may be tested using in vitro artificial models (e.g. SHIME, Simulator of Human Intestinal Microbial Ecosystem®).

By non-pathogenic is meant bacteria that are not capable of causing disease or harmful responses in a host. The non-pathogenic bacteria may be from the genus *Bacteroides* or *Escherichia*. The non-pathogenic bacteria may be a probiotic bacteria. By probiotic is meant live, non-pathogenic microorganisms which can confer health benefits to a host organism, e.g. a human.

Alternatively, the non-pathogenic bacteria may be a synthetic bacteria.

To test if a bacteria is non-pathogenic, the test for haemolytic activity may be used. This test uses blood agar plates and tests for the ability of the bacteria to lyse red blood cells in the vicinity of the bacterial colony. Other types of testing are also available such as sequencing and screening the genome of the bacteria for known pathogenicity factors (de Nies et *al* (2021), Microbiome 9: article number 49).

Suitable bacteria include Escherichia coli, for example E. coli Nissle. Other examples of suitable therapeutic cells include lactic acid bacteria for example Lactobacillus and/or Lactococcus. Other examples of therapeutic cells include Akkermansia, for example Akkermansia muciniphila, Bifidobacterium, Bacteroides, Salmonella or Listeria.

Further examples include Clostridium or Anaerobutyricum soehngenii, for example where the deficiency is in the vitamin B1 or vitamin B6 pathways.

Deficient/non-functional

By the term deficient is meant that the genes specified are rendered inoperable or are not present in the genome to begin with in terms of a synthetic bacteria. The genes or gene functions are deleted. The bacteria does not contain the functional gene. As a result, the enzyme encoded by the gene is not produced (conversely, functional means that the enzyme encoded by the gene is produced). This may be as a result of complete deletion of the coding sequences of the genes or partial deletion.

Alternatively, removing the functional gene may be accomplished by mutation of the coding sequence, for example by way of insertion or substitution of other sequences. For example, one or more mutations (such as frameshift mutations) may be generated such that any resulting RNA transcript codes for a non-functional or truncated protein. In an alternative, an insertion may be made into the chromosomal sequence to disrupt the amino acid code.

For example, the gene may be completely deleted or partially deleted, for example, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the coding sequence may be deleted. This percentage is with respect to the entire sequence.

Non-functionality may be tested by:

- PCR, sequencing the locus; and/or
- Growing the resulting strain on selective media. To test *pdx*H deficiency, the bacteria would be grown on +/- PLP (Vitamin B6); to test *thi*L deficiency, the bacteria would be grown on +/- TPP (Vitamin B1); to test *sod*A/B deficiency, the bacteria would be grown on minimal media +/- oxygen. The bacteria if deficient will not grow without the supplemented media (or cannot grow in oxygen in the case of *sod*A/B).

The resulting cells lacking the Vitamin B1 and Vitamin B6 genes are also known as auxotrophs meaning they are mutant organisms that require a particular additional nutrient which the normal unmutated strain does not.

Expression cassette

The expression cassette includes the machinery needed to produce the therapeutic molecule (medicament). This may include a promoter and the gene sequence of the therapeutic molecule (medicament). Alternatively this may include a promoter and a gene encoding an enzyme which when expressed produces the therapeutic molecule. The bacterium is genetically engineered to include the expression cassette. The bacterium is a genetically engineered bacterium.

The expression cassette may be cloned into one of the native plasmids of a therapeutic bacteria. Alternatively, the expression cassette may be cloned into a plasmid which is not native to the bacteria. The plasmid is then transformed into the bacteria.

Alternatively, the expression cassette may be integrated into the genome of the bacteria (the bacterial chromosome). This can be done using the CRISPR technique. Alternatively this can be done by various other methods including clonetegration (Shearwin *et al* (2013), ACS Synthetic Biology, Vol 2, pp537-541).

Promoters

A promoter is a nucleotide sequence capable of controlling the expression of a gene. The promoter may be a σ^{70} promoter or a modified version of such a promoter where the nucleotide composition has been optimized for *in vivo* expression levels.

The promoters claimed have been tested for predictability and robustness in the mammalian GI tract. They have been selected from a large library of promoters, causing the most stable gene expression under any conditions (e.g. +/- oxygen, in exponential or stationary growth phase, in the upper and lower part of the GI tract, in the lumen vs. in the mucus layer), which are important for making robust therapeutic bacteria.

The promoter may have at least 70, 75, 80, 85, 90, 95 or 100% sequence identity to SEQ ID NO. 74 or 75. The promoter may comprise or consist of SEQ ID NO.s 74 or 75.

For example, the promoter for the tyrosine hydroxylase may have a consensus sequence as follows:

DSNYKNRYDMDHBRNDHYBVHNHNBNDDDDNHKDNN (SEQ ID NO.74)

Where the sequence is in accordance with the IUPAC code below.

IUPAC nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T (or U)	Thymine (or Uracil)
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C
B	C or G or T
D	A or G or T
H	A or C or T
V	A or C or G
N	any base
. or -	gap

For example, the promoter may be SEQ ID NO. 59 or 60 or a sequence comprising 90, 95 or 98% sequence identity with either SEQ ID NO. 59 or 60.

The promoter for any or all of FolE, FolM and/or FolX may be an Anderson promoter (http://parts.igem.org/Promoters/Catalog/Anderson). The promoter for any or all of *Fol*E, FolM and/or FolX may have a consensus sequence as follows (again with reference to the IUPAC code above):

YTKAYRGCTAGCTCAGYCCTWGGKAYWRTGCTAGC (SEQ ID NO. 75).

The promoter may be SEQ ID NO. 55-73 or a sequence comprising 70, 75, 80, 85, 90, 95 or 98% sequence identity with either SEQ ID NO. 55-73.

The promoter may consist of consensus sequence SEQ ID NO. 74 or 75.

Functional variants with different degrees of sequence identity can be checked for retention of activity by comparing expression of a suitable reporter under the control of the variant promoter and compare this activity with the reporter under the control of the non-variant promoter. It is generally preferred that a promoter with less that 100% sequence identity retains at least 25, 50, 75, 80, 85, 90, 95 or 100% activity of the reference promoter.

In addition to sequence identity, the promoters may be shortened at 1 or both ends of the sequence. This shortening may be by 1 or 2 nucleotides at 1 or both ends. These shortened variants can be checked for retention of activity as explained above.

Kit

By kit (or kit of parts) is meant two or more components packaged together.
The bacterium in the kit are as defined herein. The plasmid may comprise any of the promoters described herein.

Therapeutic molecule

The therapeutic molecule may be any of benefit to a patient. That is, the therapeutic molecule is a medicament. By producing is meant the therapeutic molecule or medicament may be a protein encoded in the expression cassette; or the expression cassette may encode an enzyme which enables production of the therapeutic molecule. The enzyme may also be referred to as a medicament, for example, tyrosine hydroxylase as described herein. The medicament may be a heterologous medicament, for example a heterologous protein. By heterologous is meant not from a bacterial species. The medicament may be a mammalian protein. The bacterium may comprise an expression cassette for expression of a gene which encodes a medicament, wherein the bacterium is deficient in:
a) at least two functional genes in the redox detoxification pathway; or
b) at least one functional gene in the vitamin B1 synthetic pathway and at least one functional gene in the vitamin B6 synthetic pathway. The gene may be a heterologous gene. The medicament may be tyrosine hydroxylase.

Examples of therapeutic molecules (medicaments) include L-DOPA, serotonin, dopamine, nor-adrenaline, adrenaline and other tryptophan or tyrosine derivatives.

L-DOPA is a prodrug of dopamine that is administered to patients with Parkinson's due to its ability to cross the blood-brain barrier.

Dopamine which is produced by decarboxylation of L-DOPA, modulates blood pressure, and also has a role in immune modulation, adipose tissue metabolism, nutrient absorption, and modulation of gut-brain axis functions. For production of dopamine, the expression cassette may comprise tyrosine hydroxylase as described above and a L-DOPA decarboxylase.

By derivatives is meant molecules resulting from the biosynthetic pathways of tryptophan or tyrosine. Examples of tryptophan or tyrosine derivatives that have therapeutic potential include: tryptamine (TRM) and/or phenethylamine (PEA). Others include: adrenaline, 5-HTP (5-hydroxytryptophan) and 5-HT (5-hyroxytryptamine).

Genetically engineered

By genetically engineered is meant the bacteria has been modified from its native state. That is, the bacteria is a recombinant bacteria.

<u>The biosynthetic pathways</u>

Vitamin B6 pathway

The vitamin B6 synthetic pathway is shown in Figure 6. The bacteria may be deficient in any gene from this pathway as shown. For example, the non-functional gene may be *epd*, *pdx*B, *ser*C, *pdx*A, *dxs*, *pdx*J or *pdx*H. Preferably the non-functional gene is *pdx*H.

In cases where B6 vitamin can be synthesised through the DXP-independent pathway, the non-functional gene may be Pyridoxal 5'-phosphate synthase subunit *pdx*S or *pdx*T. Therefore, where in the claims it is stated “vitamin B6 synthetic pathway”, this may include the DXP-independent pathway. Therefore, the non-functional gene may be epd, pdxB, serC, pdxA, dxs, pdxJ, pdxH, pdxS or pdxT.

That is, disclosed is a bacterium wherein the bacterium is a therapeutic bacterium (or a bacterium comprising an expression cassette for producing a therapeutic molecule) deficient in:

a) at least two functional genes in the redox detoxification pathway, wherein the redox detoxification pathway comprises *sod*A, *sod*B and *sod*C; or

b) at least one functional gene in the vitamin B1 synthetic pathway, wherein the vitamin B1 synthetic pathway comprises *thi*E or *thi*L; and at least one functional gene

in the vitamin B6 synthetic pathway (wherein the vitamin B6 synthetic pathway comprises *epd*, *pdx*B, *ser*C, *pdx*A, *dxs*, *pdx*J or *pdx*H, *optionally epd*, *pdx*B, *ser*C, *pdx*A, *dxs, pdx*J, *pdx*H, *pdx*S or *pdx*T.

That is, the bacteria may be deficient in:

a) at least two functional genes from the list comprising *sod*A, *sod*B and *sod*C; or

b) at least one functional gene from the list comprising *thi*E or *thi*L; and at least one functional gene from the list comprising: *epd*, *pdx*B, *ser*C, *pdx*A, *dxs*, *pdx*J or *pdx*H, *optionally epd*, *pdx*B, *ser*C, *pdx*A, *dxs, pdx*J, *pdx*H, *pdx*S or *pdx*T

The biocontained strain requires there to be two non-functional genes, the non-functional genes from two different metabolic pathways. Therefore, one of the above vitamin B6 defunct genes must be combined with a non-functional gene from another pathway. For example, the vitamin B1 synthetic pathway.

The resulting bacterium are therefore auxotrophs for vitamin B6. Auxotrophs are conditional lethal mutants in which survival of the bacterium is dependent on the availability of a particular nutrient in its environment.

Vitamin B1 pathway

The vitamin B1 synthetic pathway is shown in Figure 7. The bacteria may be deficient in any gene from this pathway. For example, the non-functional gene may be *thi*E or *thi*L. Preferably the non-functional gene is *thi*L.

The resulting bacterium are therefore auxotrophs for vitamin B1.

Preferably *thi*L and *pdx*H (the latter from the B6 pathway) are both non-functional.

Redox detoxification pathway

The superoxide radicals degradation pathway is shown in Figure 8. This catalyzes the degradation of harmful superoxide into oxygen in the bacteria. The bacteria may be deficient in any gene from this pathway. For example, the non-functional gene may be *sod*A or *sod*B. Preferably both *sod*A and *sod*B are non-functional. *Sod*C may still remain functional. *Sod*A and *sod*B may be the only deficient genes in this pathway.

Other combinations of inoperable genes

The *sod*A and *sod*B genes may be knocked out/otherwise rendered inoperable in combination with *pdx*H/*thi*L. For example *sodA*, *sod*B and *pdx*H may be non-functional. Or sodA, sodB and thiL may be non-functional. Or, *sod*A, *sod*B, *pdx*H and *thi*L may be non-functional.

Further genes may also be rendered non-functional in addition to any of the combinations. For example, other genes which may be rendered inoperable and combined with any of the above double knockouts include any one or more of the following: *hem*F, *hem*N, *chu*A, *ahp*C and/or *suf*A. For example, these may be combined with the *sod*A/*sod*B knockout.

*Sod*A and/or *sodB* may also be rendered non-functional in combination with at least one non-functional gene from the vitamin B1 synthetic pathway and/or vitamin B6 pathway.

Sequence identity

A sequence listing is provided for the genes described above in the vitamin B1 an B6 pathways, and the redox detoxification pathway. The genes provided are from *E. coli Nissle*.

Other therapeutic bacteria may be used. These bacteria may have variations in the DNA sequences for the genes described above.

Therefore, the genes which are mutated; or partially or fully deleted or otherwise rendered inoperable may have at least 70% sequence identity to the SEQ ID NO.s provided. For example, at least 70, 75, 80, 85, 90, 95 or 100% sequence identity.

This includes the genes from the DXP-independent pathway: pdxS or pdxT

Sequence identity may be calculated using any suitable software such as BLAST (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410.)

Pharmaceutical formulation

A pharmaceutical formulation includes excipients to preserve the activity or to deliver the cell to the gut. Preferably the formulation is an oral formulation.

The microbial cell (i.e. bacterium) may be formulated to preserve its activity and/or for delivery to the gut via an oral tablet or capsule or the like.

For example, the microbial cell may be lyophilized and include a lyoprotectant. The formulation may alternatively or additionally include any other excipient required to preserve the activity of the cell.

The formulation may be in an oral dosage form with a coating which allows delivery to the gut, for example an enteric coating.

For use as a medicament

The bacteria can be used to express a therapeutic molecule (medicament), or one or more enzymes which catalyzes one or more reactions to produce a therapeutic molecule.

In either case, the therapeutic molecule can be produced in an amount effective to treat the disease or ameliorate the symptoms of the disease.
As an example only, and to demonstrate the efficacy of the bacteria, the production of L-DOPA is explained below and results showing production of L-DOPA in the bacterial cell lines are provided in the examples.

Production of L-DOPA and treatment of Parkinson's disease

The expression cassette when the therapeutic molecule is L-DOPA may encode a eukaryotic tyrosine hydroxylase (SEQ ID NO. 36, 38, 40 or 42). Additionally, the expression cassette (or an additional expression cassette) may include further genes which express co-factors. For example, the expression cassette may also include FolX, FolE, FolM and/or phhB. Preferably the expression cassette contains the FolE and FolM genes.

Tyrosine hydroxylase

The eukaryotic tyrosine hydroxylase (TyrOH) is a member of the biopterin-dependent aromatic amino acid hydroxylase family of non-heme, iron(II)-dependent enzymes. TyrOH catalyzes the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA) as shown in Figure 9.

The tyrosine hydroxylase of the invention may belong to EC 1.14.16.2. The enzyme may be an animal enzyme.

The sequence of the full length rat tyrosine hydroxylase is as follows:

MPTPSAPSPQPKGFRRAVSEQDAKQAEAVTSPRFIGRRQSLIEDARKEREAAAAAAAAAVA
SSEPGNPLEAVVFEERDGNAVLNLLFSLRGTKPSSLSRAVKVFETFEAKIHHLETRPAQRPL
AGSPHLEYFVRFEVPSGDLAALLSSVRRVSDDVRSAREDKVPWFPRKVSELDKCHHLVTK
FDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYKHGEPIPHVEYTAEEIATWKEVYVTLKGLYA
THACREHLEGFQLLERYCGYREDSIPQLEDVSRFLKERTGFQLRPVAGLLSARDFLASLAF
RVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASDEEIEKL
STVYWFTVEFGLCKQNGELKAYGAGLLSSYGELLHSLSEEPEVRAFDPDTAAVQPYQDQT
YQPVYFVSESFNDAKDKLRNYASRIQRPFSVKFDPYTLAIDVLDSPHTIQRSLEGVQDELHT
LAHALSAIS

The above sequence is SEQ ID NO. 36. The tyrosine hydroxylase may have at least 70, 75, 80, 85, 90, 95, 97 or 100% sequence identity with SEQ ID NO. 36.

The enzyme may be truncated to the core secondary structure elements to provide function, for example by removing 1 to 20 (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20) amino acids from the N and/or C termini of the construct.

Mutant Tyrosine Hydroxylase

The tyrosine hydroxylase may be a mutant, i.e. the enzyme differs from the full length wild type enzyme sequence.

The wild type full length rat enzyme comprises:

- A regulatory domain (amino acids 1-154)
  MPTPSAPSPQPKGFRRAVSEQDAKQAEAVTSPRFIGRRQSLIEDARKEREAAAAAA
  AAAVASSEPGNPLEAVVFEERDGNAVLNLLFSLRGTKPSSLSRAVKVFETFEAKIHH
  LETRPAQRPLAGSPHLEYFVRFEVPSGDLAALLSSVRRVSD (SEQ ID NO. 77)
- A catalytic domain (amino acids 155-456)
  DVRSAREDKVPWFPRKVSELDKCHHLVTKFDPDLDLDHPGFSDQVYRQRRKLIAEI
  AFQYKHGEPIPHVEYTAEEIATWKEVYVTLKGLYATHACREHLEGFQLLERYCGYR
  EDSIPQLEDVSRFLKERTGFQLRPVAGLLSARDFLASLAFRVFQCTQYIRHASSPMH
  SPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASDEEIEKLSTVYWFTVEFGLC
  KQNGELKAYGAGLLSSYGELLHSLSEEPEVRAFDPDTAAVQPYQDQTYQPVYFVS
  ESFNDAKDKLRNYASRIQRPF (SEQ ID NO. 78)
- A tetramer domain (amino acids 457-498)
  SVKFDPYTLAIDVLDSPHTIQRSLEGVQDELHTLAHALSAIS (SEQ ID NO. 79)

The mutant may not comprise the regulatory domain. The entire regulatory domain may be deleted or only part of the regulatory domain may be deleted.

Truncation may be at any point in the regulatory domain to reduce the complexity of the protein for expression in a microbial cell and/or to decrease negative feedback by dopamine for the dopamine-producing microbial cell. The skilled person would be aware of suitable points to truncate the regulatory domain whilst maintaining the activity of the enzyme guided by the crystal structure (Goodwill, K., Sabatier, C., Marks, C. *et al.* Crystal structure of tyrosine hydroxylase at 2.3 Å and its implications for inherited neurodegenerative diseases. *Nat Struct Mol Biol* **4,** 578–585 (1997).

The tyrosine hydroxylase may comprise the catalytic domain (and not the regulatory domain or tetramer domain); or the catalytic domain and the tetramer domain (and not the regulatory domain). These domains may comprise the above amino acids sequences or have at least 70, 75, 80, 85, 90, 95, 99 or 100% sequence identity with the above amino acid sequences, and optionally be further truncated to the core secondary structure elements to provide function, for example by removing 1-20 (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20) amino acids from the N and/or C termini of the constructs.

For example, the truncated enzyme may comprise the catalytic and tetramer domains, amino acids:
SAREDKVPWFPRKVSELDKCHHLVTKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYKHGE
PIPHVEYTAEEIATWKEVYVTLKGLYATHACREHLEGFQLLERYCGYREDSIPQLEDVSRFL
KERTGFQLRPVAGLLSARDFLASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLA
DRTFAQFSQDIGLASLGASDEEIEKLSTVYWFTVEFGLCKQNGELKAYGAGLLSSYGELLHS
LSEEPEVRAFDPDTAAVQPYQDQTYQPVYFVSESFNDAKDKLRNYASRIQRPFSVKFDPYT
LAIDVLDSPHTIQRSLEGVQDELHTLAHALSAIS (amino acids 158-498 of SEQ ID NO. 36; SEQ ID NO. 80). Optionally the truncated enzyme may be SEQ ID NO. 38.

Alternatively, the truncated enzyme may comprise the catalytic domain only:
SAREDKVPWFPRKVSELDKCHHLVTKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYKHGE
PIPHVEYTAEEIATWKEVYVTLKGLYATHACREHLEGFQLLERYCGYREDSIPQLEDVSRFL
KERTGFQLRPVAGLLSARDFLASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLA
DRTFAQFSQDIGLASLGASDEEIEKLSTVYWFTVEFGLCKQNGELKAYGAGLLSSYGELLHS
LSEEPEVRAFDPDTAAVQPYQDQTYQPVYFVSESFNDAKDKLRNYASRIQRPF (amino

acids 158-456 of SEQ ID NO. 36; SEQ ID NO. 81). Optionally the truncated enzyme may be amino acids 1-301 of SEQ ID NO. 38.

The tyrosine hydroxylase may be any sequence having at least 70, 75, 80, 85, 90 or 95% sequence identity to the above truncated forms. The enzyme may additionally be truncated to the core secondary structure elements to provide function, for example by removing 1 to 20 (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20) amino acids from the N and/or C termini of the construct.

The tyrosine hydroxylase may alternatively or additionally comprise a mutation in the catalytic domain. The mutation may be in amino acids 177-198 of SEQ ID NO. 36. These amino acids form a loop as shown by the crystal structure of the enzyme. The amino acid mutated in this loop may be at position 196. The mutant may be Ser 196Glu or Ser196Leu. These are shown below in the rat full length enzyme, and truncated enzyme. The mutation in the truncated form corresponds to position 41, optionally to Glu/Leu (Ser 40 without the start codon).

Full length mutant (loop 177-198 is underlined; mutation 196 is in brackets)

MPTPSAPSPQPKGFRRAVSEQDAKQAEAVTSPRFIGRRQSLIEDARKEREAAAAAAAAAVA
SSEPGNPLEAVVFEERDGNAVLNLLFSLRGTKPSSLSRAVKVFETFEAKIHHLETRPAQRPL
AGSPHLEYFVRFEVPSGDLAALLSSVRRVSDDVRSAREDKVPWFPRKVSELDKCHHLVTK
FDPDLDLDHPGF[**E/L**]DQVYRQRRKLIAEIAFQYKHGEPIPHVEYTAEEIATWKEVYVTLKGL
YATHACREHLEGFQLLERYCGYREDSIPQLEDVSRFLKERTGFQLRPVAGLLSARDFLASL
AFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASDEEIE
KLSTVYWFTVEFGLCKQNGELKAYGAGLLSSYGELLHSLSEEPEVRAFDPDTAAVQPYQD
QTYQPVYFVSESFNDAKDKLRNYASRIQRPFSVKFDPYTLAIDVLDSPHTIQRSLEGVQDEL
HTLAHALSAIS (SEQ ID NO. 82 and 83)

Truncated mutant without the regulatory domain (loop 22-43 is underlined; mutation 41 is in brackets)

MKSAREDKVPWFPRKVSELDKCHHLVTKFDPDLDLDHPGF[**E/L**]DQVYRQRRKLIAEIAFQY
KHGEPIPHVEYTAEEIATWKEVYVTLKGLYATHACREHLEGFQLLERYCGYREDSIPQLEDV
SRFLKERTGFQLRPVAGLLSARDFLASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHV
PMLADRTFAQFSQDIGLASLGASDEEIEKLSTVYWFTVEFGLCKQNGELKAYGAGLLSSYG
ELLHSLSEEPEVRAFDPDTAAVQPYQDQTYQPVYFVSESFNDAKDKLRNYASRIQRPFSVK
FDPYTLAIDVLDSPHTIQRSLEGVQDELHTLAHALSAIS (SEQ ID NO.s 40 and 42)

This mutation at position 196 in the full length or 41 in the truncated form may also be applied to any of the truncated mutants above, for example the truncated form comprising only the catalytic domain.

Therefore, the tyrosine hydroxylase may comprise any of the truncated forms above and additionally comprise a mutation in the loop: CHHLVTKFDPDLDLDHPGFSDQ (SEQ ID NO. 76), optionally at the underlined serine position.

For example, the mutant may be SEQ ID NO. 40 or 42, or a mutant with at least 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO. 40 or 42.

The tyrosine hydroxylase may have at least 70, 75, 80, 85, 90, 95 or 100% sequence identity with any of the above mutant forms. Additionally, the mutant may be further truncated to the core secondary structure elements to provide function, for example by removing 1 to 20 amino acids (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20) from the N and/or C termini of the constructs.

The inventors have surprisingly found that the above mutants (with the mutation at position 196 in the full length sequence and position 41 in the truncated sequence without the regulatory domain) produced less L-DOPA, for example 5, 10, 15 or 20% less L-DOPA compared to the wild-type. This may for example allow modulation of the L-DOPA released.

GTP cyclohydrolase I (folE)

In humans, the production of L-DOPA requires synthesis and regeneration of the co-factor tetrahydrobiopterin. Bacteria and yeast do not produce this co-factor. Therefore, the native cofactor tetrahydromonapterin pathway is exploited instead. GTP cyclohydolase I is an enzyme in this synthesis pathway.

The GTP cyclohydrolase I may belong to E.C. 3.5.4.16.

The GTP cyclohydrolase I may have at least 70, 75, 80, 85, 90, 95 or 100% sequence identity with SEQ ID NO. 44. The enzyme may additionally be truncated to the core secondary structure elements to provide function, for example by removing 1 to 20 (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20) amino acids from the N and/or C termini of the construct.

The mutation may increase hydroxylation of the tyrosine hydroxylase by at least 120% as compared to the native or wild-type unmutated enzyme (under the same conditions).

The mutation may be at any one of the following positions in SEQ ID NO. 44:

D97-E112, K121-D130, N170-H180, S193-L200 and S207-N222. For example, D97, M99, T101, V102, A125, K129, N170, V179, T196, T198 (excluding T198P), S199, L200, S207, H212, E213, F214, L215 and H221.

The mutation may be selected from: D97V, D97L, D97A, D97T, M99C, M99T, M99V, M99L, M99I, T101I, T101V, T101L, V102M, N170K, N170D, N170L, V179A, V179M, T196I, T196V, T196L, T198I, T198V, T198S, T198L, S199Y, S199F, L200P, L200C, L200S, L200A, S207R, S207K, S207M, H212R, H212K, E213K, E213R, F214A, F214G, F214S, L215P, L215Q, L215N, L215D, L215T, L215S, L215G, L215A, L215C, L215F, L215M, H221R and H221K.

The mutant may also comprise any combination of these mutations.

For example, the GTP cyclohydrolase I mutant may have at least 70% sequence identity with SEQ ID NO. 44, and comprise any one or more of the above mutations.

The GTP mutant may be the endogenous, native GTP cyclohydrolase which is mutated i.e. not an additional recombinant copy.

Additional enzymes which aid Tyrosine Hydroxylation activity

In addition or as an alternative to the FolE mutation to increase co-factor production which in turn increases tyrosine hydroxylation, other enzymes in the pathway of Figure 11 may be overexpressed or enzymes involved in the regeneration of the co-factor (such as 4a-hydroxytetrahydrobiopterin dehydratase encoded by the phhB gene).

For example, the microbial cell may over-express (compared to the wild-type under the same conditions) any nucleic acid encoding:

- 4a-hydroxytetrahydrobiopterin dehydratase (SEQ ID NO. 52): phhB (SEQ ID NO. 51); and/or
- dihydroneopterin triphosphate 2'-epimerase (SEQ ID NO. 54): FolX (SEQ ID NO. 53); and/or
- dihydromonapterin reductase (SEQ ID NO. 48): FolM (SEQ ID NO. 47)

The nucleic acid may also be any encoding enzymes with these activities and having at least 70, 75, 80, 85, 90, 95 or 100% sequence identity with the above SEQ ID NO.s. The enzymes may additionally be truncated to the core secondary structure elements to provide function, for example by removing 1 to 20 (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20) amino acids from the N and/or C termini of the constructs.

The microbial cell may have increased activity of FolE and/or FolM. Therefore, the microbial cell comprises a recombinant nucleic acid encoding a eukaryotic tyrosine hydroxylase (for example, a tyrosine hydrolase with at least 70% sequence identity to SEQ ID NO. 38) and upregulated FolE and/or FolM. This may be by additional recombinant FolE and/or FolM being added to the cell. The FolE enzyme may be mutated as described above.

Alternatively, the microbial cell comprises a recombinant nucleic acid encoding a eukaryotic tyrosine hydroxylase (for example, a tyrosine hydrolase with at least 70% sequence identity to SEQ ID NO. 38) and utilizes the endogenous FolE and FolM cofactors. The FolE enzyme may be mutated as described above.

Expression of the tyrosine hydroxylase (or example, a tyrosine hydrolase with at least 70% sequence identity to SEQ ID NO. 4) may be under a promoter comprising or consisting of consensus SEQ ID NO. 74. Expression of one or more of the co-factors (for example, FolE and/or FolM) may be under the control of a promoter comprising or consisting of SEQ ID NO. 75. The enzymes (and optionally the promoters described above) are preferably integrated into the genome of the cell.

Upregulating expression may be via a recombinant nucleic acid, for example an additional copy of the gene on a plasmid or integrated into the genome, or alternatively via upregulating the endogenous sequence.

Sequence identity is calculated as per above.

Sequence listing

All sequences are from *E. coli* Nissle unless specified.

Redox detoxification pathway:

DNA SEQ ID NO	Amino acid SEQ ID NO.	Gene
1	4	*16oda*
2	5	*sod*B
3	6	*sod*C

Vitamin B1 synthetic pathway:

DNA SEQ ID NO	Amino acid SEQ ID NO.	Gene

7	9	*thi*L
8	10	*thi*E

Vitamin B6 synthetic pathway:

DNA SEQ ID NO	**Amino acid SEQ ID NO.**	**Gene**
11	12	*pdx*H
13	14	*epd*
15	16	*pdx*B
17	18	*ser*C
19	20	*pdx*A
21	22	*dxs*
23	24	*pdx*J

Other genes which may be combined with the 17oda/sodB knockout:

DNA SEQ ID NO	**Amino acid SEQ ID NO.**	**Gene**
25	26	*hem*F
27	28	*hem*N
29	30	*chu*A
31	32	*ahp*C
33	34	*suf*A

Promoter:	
1) trc promoter	1) SEQ ID NO. 55
2) trc promoter with lac operator	2) SEQ ID NO. 56
3) trc promoter without lac operator (without lacI binding site)	3) SEQ ID NO. 57
4) Bba_J23100	4) SEQ ID NO. 58
5) J23102	5) SEQ ID NO. 59
6) MSKL7	6) SEQ ID NO. 60
7) MSKL8	7) SEQ ID NO. 61
8) J23101	8) SEQ ID NO. 62
9) J23105	9) SEQ ID NO. 63
10) J23106	10) SEQ ID NO. 64
11) J23107	11) SEQ ID NO. 65
12) J23108	12) SEQ ID NO. 66
13) J23110	13) SEQ ID NO. 67
14) J23111	14) SEQ ID NO. 68
15) J23114	15) SEQ ID NO. 69
16) J23115	16) SEQ ID NO. 70
17) J23116	17) SEQ ID NO. 71
18) J23117	18) SEQ ID NO. 72
19) J23118	19) SEQ ID NO. 73
20) MSKL consensus	20) SEQ ID NO. 74

21) Anderson consensus	21) SEQ ID NO. 75

For producing L-DOPA:

Name	**DNA SEQ ID NO.**	**Amino acid SEQ ID NO.**
Rat Tyrosine Hydroxylase	SEQ ID NO. 35	SEQ ID NO. 36
Truncated Tyrosine Hydroxylase	SEQ ID NO. 37	SEQ ID NO. 38
Truncated and mutated Tyrosine Hydroxylase Ser 41 to Glu Ser 41 to Leu	1) SEQ ID NO. 39 2) SEQ ID NO. 41	1) SEQ ID NO. 40 2) SEQ ID NO. 42
GTP cyclohydrolase (FolE)	SEQ ID NO. 43	SEQ ID NO. 44
FolE codon optimized	SEQ ID NO. 45	SEQ ID NO. 46
FolM	SEQ ID NO. 47	SEQ ID NO. 48
FolM codon optimized	SEQ ID NO. 49	SEQ ID NO. 50
phhB	SEQ ID NO. 51	SEQ ID NO. 52
folX	SEQ ID NO. 53	SEQ ID NO. 54
Loop of Rat tyrosine hydroxylase		SEQ ID NO. 76
Rat Tyrosine hydroxylase regulatory domain		SEQ ID NO. 77
Rat Tyrosine hydroxylase catalytic domain		SEQ ID NO. 78
Rat Tyrosine hydroxylase Tetramer domain		SEQ ID NO. 79
Rat Tyrosine hydroxylase truncated enzyme		SEQ ID NO. 80

Rat Tyrosine hydroxylase Truncated enzyme		SEQ ID NO. 81
Rat Tyrosine hydroxylase Full length mutant (E)		SEQ ID NO. 82
Rat Tyrosine hydroxylase Full length mutant (L)		SEQ ID NO. 83

Throughout the specification, unless the context demands otherwise, the terms 'comprise' or 'include', or variations such as 'comprises' or 'comprising', 'includes' or 'including' will be understood to imply the method or kit includes a stated integer or group of integers, but not the exclusion of any other integer or group of integers.

Each document, reference, patent application or patent cited in this text is expressly incorporated herein in their entirety by reference, which means it should be read and considered by the reader as part of this text. That the document, reference, patent application or patent cited in the text is not repeated in this text is merely for reasons of conciseness. Reference to cited material or information contained in the text should not be understood as a concession that the material or information was part of the common general knowledge or was known in any country.

Description of the Figures

Figure 1 shows Growth of auxotroph mutants *in vitro*.
Bacterial growth in LB medium: a) without (-) or b) with (+) supplementation of the missing nutrients, under aerobic conditions; c) Growth in LB medium without supplementation under anaerobic conditions; d) Growth in SYNTHES synthetic wastewater medium, composed to accurately reflect the average abundance of micro- and macronutrients in wastewater; e) Growth in OECD medium, a less complex synthetic medium recommended by the OECD to simulate wastewater conditions (OECD, 2001); f) Summary of the final biomass yield achieved in d) and e), compared to the wild-type. All data are shown as mean +/- standard deviation from at least 3 biological replicates.

Figure 2 shows Survival of auxotrophic strains in simulated sewage conditions. Strains were grown in lab media and washed to remove media supplements, then they were mixed at different ratios with mouse stool diluted in SYNTHES medium. In graphs a) and b), different coloured lined indicate different dilutions of faeces in SYNTHES. Samples were taken out and plated on LB + supplements containing antibiotics selective for the engineered E. coli Nissle, to enumerate surviving cells, at the indicated time points. Graphs c) and d) show results from a separate experiment. C) Survival after 8 weeks in SYNTHES media, at different dilution factors. D) Calculated maximum doublings (replication) of each strain, over the 8 week time period, at different dilutions. The difference in response to the dilution factor demonstrates that auxotroph strains depend on nutrients provided by the faeces (and residing microbiota), whereas the wild-type actually performs better at higher dilutions, due to less competition for the available nutrients from the other bacteria.

Figure 3 shows Behaviour of auxotroph strains in mouse model. Mice were treated with streptomycin in the drinking water and gavaged with 10^8 CFU of bacteria once, then CFU counts were measured in the faeces daily. For cumulative CFU counts, the daily counts were multiplies with an estimated average of 0.5g of faeces produced per animal daily, in order to calculate the total number of bacteria excreted by the mice throughout the 8 days. This number was divided by the 10^8 CFUs used as input, in order to show how much the bacteria replicated in vivo. n = 8 mice per group. The limit of detection in a) to g) was 5000 CFU/g and is shown as a dotted line.

Figure 4 shows Abundance of auxotroph strains in mouse GI tract 8 days after single gavage. Data was collected on the day of dissection. Same mice were used as in Figure 3.

Figure 5 shows Serotonin production in auxotrophic strains. WT and knockout strains were grown in lab media supplemented with the missing nutrients in order to simulate optimal production conditions. Serotonin (5-HT) production is shown on the left axis, production of tryptamine (TRM) and phenethylamine (PEA) by-products is shown on the right y-axis. N = 3 biological replicates per group, mean +/- standard deviation is shown.

Figure 6 shows the PLP (Vitamin B6) biosynthetic pathway

Figure 7 shows the TPP (Vitamin B1) biosynthetic pathway

Figure 8 shows the Superoxide radicals degradation pathway

Figures 9-11 show the L-DOPA pathway. Figure 9 provides an overview of the pathway.

Figures 10 and 11 respectively show in more detail the co-factor needed to synthesize L-DOPA and the making of this co-factor.

Figure 12 shows the Production of tryptamine in the auxotrophic strains. WT and knockout strains were grown in minimal media supplemented with the missing metabolites. Tryptamine production was measured by HPLC and is shown on the y-axis, strains are represented on the x-axis. N = 3 biological replicates per group, mean +/- standard deviation is shown.

Figure 13 shows the Production of L-DOPA. WT and the double knockout strain Δ*pdx*H/*thi*L were grown in a plate-reader in media adjusted to different pH from 3,5-7 with 5% oxygen. L-DOPA concentration measured by HPLC is shown on the y-axis. N= 3 biological replicates per group, mean +/- standard deviation is shown.

Figure 14 shows the Colonization of auxotrophic E.coli Nissle strains in an NMRI mouse model without antibiotic treatment. Figure 14a represents the study layout: 8 groups of conventional NMRI mice (6-weeks old, female, n=6) were acclimatised for 11 days and orally gavaged 3 times in 24-hour intervals, on day 0, 1, and 2, with 10^{11} CFU per animal. CFU counts were measured in the faeces at indicated time points and in gut content at study endpoint day 11. Figure 14b shows colonization in the faeces over the first 24 hours after the first gavage of 10^{11} CFU per animal. Faecal samples were collected and plated, y-axis shows the counted CFU/g faeces normalized to the sample weight, limit of detection is 75 CFU/g, x-axis shows the measured timepoints. N = 5-6 biological replicates per group, mean +/- standard deviation is shown.

Figure 15 shows Colonization over the first 24 hours in faeces after first gavage of 10^{11} CFU per animal for the individual strains compared to the WT. The y-axis gives the abundance of the bacterial strain in CFU/g faeces normalized to the sample weight, limit of detection is 75 CFU/g, x-axis shows the measured timepoints. N = 5-6 biological replicates per group, mean +/- standard deviation is shown.

Figure 16 shows Abundance of auxotrophic strains in faeces over the study duration of 11 days. The strains were gavaged on day 0, 1, and 2 with 10^{11} CFU per animal. The

colonization is measured as described in example 14b). N = 5-6 biological replicates per group, mean +/- standard deviation is shown.

Figure 17 shows Colonization of the individual auxotrophic strains in faeces over 11 days compared to the WT strain as described for figure 15. N = 5-6 biological replicates per group, mean +/- standard deviation is shown.

Figure 18 shows the abundance of auxotrophic strains in faeces and in the mouse GI tract at study endpoint 11 days after first gavage, 9 days after last gavage. The gut was divided in small intestine, cecum, and colon. All samples were weighted, plated and colonies counted after 24 hours incubation at 37°C. Counts were normalized to the sample weight. The abundance of the strains is represented as CFU/g on the y-axis. Limit of detection is 75 CFU/g. N = 5-6 biological replicates per group, mean +/- standard deviation is shown.

Examples

Aspects of the present invention will now be illustrated by way of example only and with reference to the following experimentation.

Example 1: Making of the auxotrophic cell line

Knockouts of essential genes were performed using CRISPR/Cas9 as described previously (Hao Luo et al., ACS Synthetic Biology 2020 9 (3), 494-499 DOI: 10.1021/acssynbio.9b00488). Briefly, a two- plasmid system consisting of an inducible cas9/λ-Red expression plasmid and a guide RNA (gRNA) plasmid were used to introduce double-strand breaks at the desired genomic loci. gRNAs were designed using CRISPy-web after uploading the EcN genome sequence (GenBank: CP007799.1). Templates for homologous recombination at the cut site were generated as follows: loci were amplified using oligos binding 500 bp up and downstream of the gene of interest and then fused using overlap-extension PCR to generate dsDNA products of approximately 1 kb. The resulting dsDNA fragments were purified and co-transformed with the gRNA plasmid to generate markerless gene knockouts in EcN. CRISPR/Cas9 and gRNA expression plasmids were cured from the strains as described previously and the sensitivity to used antibiotic markers was confirmed by plating on solid media +/- antibiotics. Gene knockouts were confirmed by colony PCRs, Sanger sequencing of the genomic locus as well as phenotypic testing for the desired auxotrophy (growth +/- supplementation of the missing nutrient, or growth +/- oxygen).

Example 2: *In vitro* testing of the double knock-outs compared with existing biocontainment cell lines shows the double knock-outs perform better in terms of lack of survival outside of the gut

Existing biocontainment cell lines include *dap*A mutants. These cells lack a functional *dap*A gene. The *dap*A gene, encoding 4-hydroxy-tetrahydropicolinate synthase, is involved in synthesizing lysine and diaminopimelic acid (required for cell wall biosynthesis) and therefore makes the bacteria dependent on exogenous diaminopimelate (DAP) for cell wall biosynthesis and growth. Further lysine auxotrophs include the *lys*A mutant. The LysA protein catalyzes the last step in the lysine biosynthetic pathway forming lysine. The protein expressed from the *dap*A gene catalyses one of the initial steps in the lysine and diaminopimelic acid biosynthetic pathway.

A further known biocontainment cell line is the *thy*A mutant. These cells lack a functional *thy*A gene. The *thy*A gene is involved in synthesis of thymine, a building block of DNA. Therefore *thy*A, like *lys*A and *dap*A, is another essential gene.

Materials and methods

Bacterial growth curves. Three single colonies were picked from each strain, grown in 300 µl LB+ medium (composition see below) in 96 deep-well plates and shaken at 250 rpm at 37°C for 16 hours. The cells were pelleted at 4000 x g for 10 minutes, resuspended in PBS, this washing step was repeated 2 more times. Cells were then again resuspended in PBS and the main culture was inoculated by diluting this preculture 1:100 into 200 µl of the different media detailed below. Cells growth was followed at 37°C with 700 rpm orbital shaking using the BioTek™ ELx800 plate reader by measuring the optical density at 630nm every 10 minutes for 24 h. For anaerobic growth, a plate reader placed in an anaerobic chamber was used.

LB- medium contained 5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl. LB+ medium additionally contained 1 µM TPP, 12 mg/L PLP, 100 mg/L diaminopimelic acid (DAP), 2 mM thymine. SYNTHES medium was prepared according to Aiyuk S, Verstraete W (2004) Sedimentological evolution in an UASB treating SYNTHES, a new representative synthetic sewage, at low loading rates. Bioresour Technol 93(3):269–278. doi:10.1016/ j.biotech.2003.11.006. The medium was prepared using tap water, pH was adjusted to 7.0 and the medium was autoclaved before use. OECD synthetic wastewater medium was prepared according to OECD (2001), Test No. 303: Simulation Test - Aerobic Sewage

Treatment -- A: Activated Sludge Units; B: Biofilms, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris.

Results

The results are shown in Figure 1.

In order to determine biosafety of the developed auxotrophies, the stringency of their growth defect was tested under several conditions. Lysogeny broth (LB) medium is the standard, nutritionally rich growth medium used in the lab for growing *E. coli*, and is used as a control here. It supports the growth of some, but not all, auxotroph strains tested here (Fig 1a), and can support growth to near-WT levels of all strains when the missing nutrients are added (Fig 1b). By measuring *in vitro* growth curves, the three main types of growth defects can be quantified: i) an increase in lag time (e.g. *sod*A/B in Fig 1a), ii) a decrease in the maximum specific growth rate (e.g. *dap*A in Fig 1b), and iii) a decrease in the final amount of cells produced, measured as OD600 (e.g. *pdx*H in Fig 1a). The longer lag phase of the *sod*A/B mutant under aerobic conditions nearly disappears under anaerobic conditions (Fig 1c). Also, the partial growth that is observed in the *pdx*H mutant is completely abolished when combined with knockout of the *thi*L gene.

The two novel knockout combinations Δ*pdx*H/*thi*L and Δ*sod*A/B show no growth under wastewater conditions, which was also confirmed by plating cells onto solid SYNTHES media and incubating for 3 days at 37C, after which no visible growth was observed for the knockouts, while the wild-type had formed full colonies (data not shown).

This data therefore supports that targeting double knockouts in the B1/B6 or redox detoxification pathways produces bacteria which are less likely to grow outside the patient, in wastewater. The novel knockouts are more effective in terms of deficient growth, than the existing cell lines which target the *dap*A, *lys*A and *thy*A genes.

Example 3: Further *in vitro* testing in simulated sewage systems supports lack of survival of double knock-outs outside of the gut

To test the stringency of the new auxotrophies under complex and variable conditions possibly encountered upon exit from the patient, we tested their ability to compete against other microorganisms, and survive in a complex and nutritionally rich simulated sewage

composition. While it is difficult to simulate 'realistic' conditions encountered in different wastewater treatment systems, we aimed to test growth and survival over several orders of magnitude of possible dilution factors of faecal matter in wastewater.

Materials and methods

Strains were grown in LB+ medium and then washed as described in Example 2. Fresh mouse faecal samples were dissolved in PBS (30 mg faecal matter in 1 mL of PBS) to obtain representative 'sewage samples' including live faecal microbiota. Sewage samples were 10-fold serially diluted with SYNTHES medium to simulate different dilutions in wastewater. Washed cells of auxotroph strains were added to the wastewater samples at a density of 10^8 CFU/mL and incubated for 25 days at room temperature in 96-deep-well plates with a loosely fitted lid to limit evaporation but allow some gas transfer. At the indicated time points, samples were taken, diluted in SYNTHES medium and plated on LB+ medium to count viable colonies (CFUs). Total doublings of the auxotroph strains were calculated from the known initial and the maximum cell density reached during the experiment.

Results

The results are shown in Figure 2.

Two independent experiments following the strains' colony-forming units (CFU) over time in a mixture of human stool in SYNTHES media (Fig 2a+b) or murine faeces in SYNTHES media (Fig 2c) demonstrated that both auxotrophs were less able to compete with the remaining faecal microbiota for growth-limiting nutrients and hence disappeared from the mixture faster than the WT. Particularly the Δ*sod*A/B mutant decreased in abundance much faster than the WT, and was mostly undetectable at 25 days in the first experiment (Fig 2b), and completely undetectable at 8 weeks in the second experiment (Fig 2c).

For the Δ*pdx*H/*thi*L mutant, a clear concentration-dependence was observed: the higher the dilution factor, the lower the chance of growth or survival of this auxotroph (Fig 2a+c). This is explained by the need to uptake 2 separate vitamins from the environment at sufficient concentrations to support growth, the probability of which drops exponentially with higher dilution factors. Moreover, it demonstrates that this strain is outcompeted by non-auxotroph, faster-growing bacteria at low nutrient conditions likely found in the environment. Interestingly, while growth was not completely inhibited with our auxotroph strains in these experimental conditions, this was also the case for the Δ*dap*A auxotroph strain, one of two commonly used AMT biocontainment strategies that have already been tested in human

clinical trials (Isabella et al., 2018 and Kurtz et al., 2019). Even though the missing nutrient in this case is a bacterial cell-wall component, growth of this strain was clearly detectable in 3 of the 4 dilutions tested (Fig 2d), as was the case for all auxotrophies tested here, indicating that the chosen conditions were likely very permissive of growth.

Example 4:In vivo testing shows double knock-outs are able to survive in mammalian gut and supply therapeutic molecule at a constant concentration

In order to produce and deliver small molecule therapeutics effectively, AMTs need to be able to survive and remain metabolically active for a certain amount of time inside the patient.

We have tested our novel biocontained strains and compared them to existing Auxotrophies in a streptomycin-treated mouse model to assess these 2 factors. Treatment with streptomycin prior to AMT dosing frees up a niche for E. coli strains in the gut microbiota, enabling high and consistent colonization levels. This was done to assess the maximum survival or growth capacity of each auxotrophy under optimal conditions, and reduce variability based on animal microbiome differences.

Materials and methods

For the in vivo gut colonization experiment, eight groups of conventional NMRI mice (n= 6-weeks old, female) were treated with Streptomycin (5 mg/ml, in drinking water) 4 days prior to inoculation with E. coli Nissle strains and through the experimental period. Mice were orally gavaged once (day 0, 10:00am) with auxotroph EcN strains containing the pMUT1-based plasmid pAuxo which confers kanamycin resistance. For this, bacteria were grown in 2YT media + supplements (see Example 2) for 16h, washed and resuspended in PBS to 10^9 CFU/ml, of which 100 µL were gavaged (10^8 CFU). Faecal samples were collected every 24h for 7 days and EcN cells were counted by plating on selective LB+ media containing 50 mg/L kanamycin. Mice were fasted from 6:00 am on the day of euthanization. At the day of dissection, gut contents (2 sections: small intestine comprising duodenum, jejunum and ileum; and large intestine comprising cecum and colon) were collected for quantification of bacteria. All samples were weighed, colonies were counted after 24 h at 37 °C, and counts normalized by weight of faeces or gut content.

Results

The results are shown in Figures 3 and 4.

After a single oral dose of 108 cells to mice, CFU output was followed in faeces over 8 days (Fig 3).

For the novel double knockout strains Δ*pdx*H/*thi*L (Fig 3b) and Δ*sod*A/B (Fig 3c), no fitness defect was detected *in vivo* compared to the WT.

However, there was a significant reduction in CFU output in faeces over the 8 days in strains Δ*lys*A, Δ*thi*L, Δ*dap*A and particularly Δ*thy*A (Figure 3h). This *in vivo* growth defect was also visible when counting CFUs in the small and large intestine at dissection on day 8, with the Δ*thy*A strain displaying the biggest drop in median CFUs/g remaining in the large intestine, and the largest variability within the group (Fig 4).

Taken together, these data demonstrate that the novel biocontained strains remain viable to a similar degree as the WT, enabling their use as chassis strains for delivery of small molecules that rely on active metabolism in the GI tract.
This feature is not observed in the Δ*dap*A and even less so in the Δ*thy*A strains, which get cleared from the GI tract faster than the WT, and show much larger intra-animal variability within the cohort, making their behaviour in vivo less predictable in a potential patient population (Fig 3 and 4).

Example 5: Testing small molecule production in novel strains shows consistent release of therapeutic molecule

A requirement for effectiveness of small molecule-delivering AMTs is their ability to produce the desired compounds *in vivo*, despite the essential gene knockout(s). While it is challenging to simulate GI tract conditions *in vitro*, testing production under lab conditions enables strain comparison under more controlled conditions and known nutrient availability. Therefore, as a model compound for tyrosine and tryptophan derivate pathways we tested production of serotonin (5-hydroxytryptamine, 5-HT), an important hormone and neurotransmitter produced in the mammalian gut and nervous system, in our engineered and biocontained AMTs, as a representative molecule that requires active amino acid and cofactor metabolism, as well as oxygen usage, when produced in bacteria.

Materials and methods

For metabolite production, strains were transformed with plasmid pMUT27 which confers serotonin production capability. Cells were grown in a modified M9+ medium containing 1x M9

salts (M6030, Sigma Aldrich), 0.2% (w/v) glucose, 0.1% (w/v) casamino acids (Cat.No. C2000, Teknova), 1 mM $MgSO_4$, 50 µM $FeCl_3$, 0.2% (v/v) 2YT medium, and 50 mg/L of L-tryptophan. All auxotrophy supplements were added at the concentrations described in Example 2. Kanamycin was added at a final concentration of 50 mg/L. Three single colonies were picked from each strain, grown in 300 µl medium in 96 deep-well plates and shaken at 250 rpm at 37°C for 16 hours. The main culture was inoculated by diluting this preculture 1:100 into fresh medium, and cells were grown for 24 h under the same conditions. Afterwards, the culture supernatant was separated from cells using a 0.2 µm pore size filter, and frozen at -20°C until analysis by LC-MS. All data shown are mean +/- SD from at least 3 biological replicates.

Quantification of metabolites by LC-MS. Detection of serotonin, tryptamine, tryptophan and 5-HTP were conducted by liquid chromatography mass spectrometry (LC-MS) measurements on a Dionex UltiMate 3000 UHPLC (Fisher Scientific, San Jose, CA) connected to an Orbitrap Fusion Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA). The system used an Agilent Zorbax Eclipse Plus C18 2.1 x 100 mm, 1.8 µm column kept at 35°C. Absolute concentrations were determined by comparison to analytical standard curves in spent growth medium.

Results

The results are shown in Figure 5.

In bacterial lab media supplemented with the required nutrients, all tested strains were still able to produce serotonin (Fig 5). Differences among the strains were observed, such as slightly lower production in the Δ*sod*A/B mutant, which can be attributed to its growth defect in the fully aerobic growth conditions used here, which would not apply in the GI tract.

Further, it could also be seen that the selection of gene knockouts can impact metabolite production, such as increased production of the main product (5-HT) compared to wildtype and the complete loss of the by-product tryptamine (TRM), and reduction of phenethylamine (PEA) formation in the Δ*pdx*H and Δ*pdx*H/*thi*L strains. This is likely due to the fact that the heterologous enzyme tryptophan decarboxylase (TDC) – responsible for tryptamine and PEA, but also serotonin production – requires pyridoxal phosphate for activity, shifting the balance favourably towards serotonin in these strains (Fig 5).

These data demonstrate that our novel strains maintain the ability to produce a complex mammalian metabolite in vitro, this result is expected to be transferrable to other therapeutic small molecules and proteins which would need to be secreted actively *in vivo*. The *sod*A/B

double knockout confers strong oxygen sensitivity (data in synthetic wastewater media shows a much faster decrease in abundance compared to WT and was almost undetectable at 25 days after addition to the media) but limited or no growth defect in the low oxygen environment of the gut.

Further advantageous features of the novel mutants here include the following:

Both DAP and Thymidine are needed in relatively high concentrations to supplement the KO, these need to be added to the fermenter when growing the biomass, potentially adding significant cost (DAP: 200 USD / 5g, need 100 mg/L, Thymidine 80 USD / 5g, need 500 mg/L; PLP: 120 USD / 5g, need 12 mg/L; TPP: 40 USD / 5g, need 0.5 mg/L). Adding DAP+THY would add about 12 USD/L broth, PLP+TPP would add 0,3 USD/L. Using *sodA*/B KO, no complementation of the broth is required if grown micro- or anaerobic.

Finally, the better survival *in vivo* of the novel mutants will impact dosing of the therapeutic bacteria in that it may be possible to lower doses or frequencies to achieve the same therapeutic effect.

Example 6: ΔpdxH and ΔpdxH/thiL knockout strains show improved production of tryptamine

Example 5 shows consistent in vitro production of serotonin from the biocontained strains and increased serotonin production in the Δ*pdx*H and Δ*pdx*H/*thi*L knockout strains. In this example we support this data showing production of a second tryptophan derivative: tryptamine.

Materials and methods

For tryptamine metabolite production, strains were transformed with plasmid pMUT28 which confers tryptamine production capability as product. Cells were grown in a modified M9+ medium as described in example 5 with kanamycin added at a final concentration of 50 mg/L. All auxotrophy supplements were added at the concentrations described in example 2. Three single colonies were picked from each strain and grown as in example 5, that includes the pre-cultures and production cultures. The production cultures supernatant were separated and the samples analysed by HPLC with an Agilent Zorbax Eclipse Plus C18 3.0 x 100mm, 3,5 µm column kept at 30°C. The flow rate was 1.0 mL/min with 0.05% Acetic acid (A) and acetonitrile (B) as mobile phase. The gradient started at 5% B and followed a gradient to 70%

over 8 min, and stayed at 70% B for 1 min. All data shown are mean +/- SD with the 3 biological replicates.

Results:

The results are shown in Figure 12.

In the bacterial lab media supplemented with the required nutrients all tested strains were able to produce tryptamine (Fig 12). Differences among the strains were observed, similar to results in example 5, such as increased production with Δ*pdx*H/*thi*L and slightly lower production in the Δ*sod*A/B mutant, which can be attributed to its growth defect in the fully aerobic growth conditions, which would not apply in the GI tract.

Example 7: Δ*pdx*H/*thi*L knockout strains show improved production of L-DOPA

Example 5 and 6 show consistent and increased production from strains producing tryptophan derivatives (5-HT and tryptamine as main product respectively) in combination with the Δ*pdx*H and Δ*pdx*H/*thi*L knockout. In a third example another aromatic amino acid production pathway was tested giving the Δ*pdx*H/*thi*L double knock strain the capability for production of the L-tyrosine derivative L-DOPA.

While it is challenging to simulate GI tract conditions *in vitro*, single parameters like pH and oxygen can be adapted to analyse their impact on production of the therapeutic molecule. Therefore, the L-DOPA production was tested in a pH gradient ranging from 3,5 - 7 and limited oxygen.

Materials and methods

For small metabolite production, strains were transformed with plasmid pMUT-HM181_5.6 which confers L-DOPA production capability. Cells were grown in a modified M9+ medium containing 1x M9 salts (M6030, Sigma Aldrich), 0.2% (w/v) glucose, 1 mM $MgSO_4$, 50 µM $FeCl_3$, 0.2% (v/v) 2YT medium. All auxotrophy supplements were added at the concentrations described in example 2. Kanamycin was added at a final concentration of 50 mg/L. The media was adjusted to pH 3,5 to 7 in different tubes with 0,5 intervals. Three single colonies were picked from each strain and grown as pre-culture as in example 5. The main culture was inoculated by diluting the preculture 1:100 into fresh medium of total volume of 200µl. The cells were grown for 24 hours in a Synergi H1 plate reader, where the oxygen level was set to 5% using a BioTek™ CO_2 and O_2 gas controller. Afterwards, the culture was spun down and the supernatant was separated. The samples were subsequently run on a HPLC. The system used an Cortecs UPLC T3 2.1 x 150mm, 1.6 µm column kept at 30°C. The flow rate was 0,3

mL/min with 10mM ammonium formate (A) and acetonitrile (B) as mobile phase. The gradient started at 0% B and followed a gradient to 70% B from 3,7 min to 4 min, and stayed at 70%B for 1 min. All data shown are mean +/- SD with the 3 biological replicates.

Results:

The results are shown in Figure 13.

The data shows production of L-DOPA from both the WT and Δ*pdx*H/*thi*L knockout strain. A decrease in L-DOPA levels occur with decreasing pH condition. Similar to example 5 and 6 the combination with *pdx*H/*thi*L knockout increased production compared to the wildtype strain, here in the range of pH 6,5-5.

The observed pH range with increased production of the Δ*pdx*H/*thi*L double knockout is highly relevant for production of the AMT under human gut conditions, where the pH gradually increases in the small intestine from pH 6 to about pH 7.4, drops to 5.7 in the caecum and again gradually increases reaching pH 6.7 in the rectum.

In total examples 5,6 and 7 point towards a beneficial effect of combining Δ*pdx*H or Δ*pdx*H/*thi*L knock out with aromatic amino acid production pathways for increased production of aromatic amino acid derivatives.

Example 8: The double knockouts Δ*pdx*H/*thi*L and Δ*sod*A/B show survival in mammalian gut up to 11 days without antibiotics, and better survival than single knockouts

To produce and deliver therapeutic molecules the AMTs must survive and remain in the gut of the patient over a certain period of time. However antibiotic treatment before the first dosing to free up a niche for the E.coli strains in the gut microbiota is not possible in a clinical setting. Also, it is desirable with regard to biosafety that the colonization of the strains should decrease and wash out after treatment with the AMT is stopped.

We here compare the auxotrophic strains with the wild type (WT) and already existing clinically approved auxotrophic strain (Δ*dap*A), in NMRI WT mice. Furthermore a combination of the Δ*sod*A/B and Δ*dap*A was tested. In contrast to example 4 no prior treatment with antibiotics was done to the animals to challenge the auxotrophic strains against the inherent microbiome and allow a more direct comparison to a clinical setting.

Materials and methods

The study lay out is shown in figure 14a). For the in vivo gut colonization experiment without antibiotics, eight groups of conventional NMRI mice (6-weeks old, female, n=6) were acclimatised for 11 days prior the first gavage, with a baseline measurement of the CFU in faeces taken 4 days prior. The mice were orally gavage 3 times with a 24-hour interval, on day 0, 1, and 2, with the auxotrophic EcN strains containing the pMUT1-empty plasmid which confers kanamycin resistance. For this, bacteria were grown in 2YT media + supplements (see Example 2) for 16h, washed and resuspended in PBS to 10^{12} CFU/ml, of which 100 µL were gavaged (10^{11} CFU). Faecal samples were collected for quantification of bacteria 2 times after the first gavage (5- and 8-hours) then in the morning on day 1-5, and day 7, 9 and 11. All samples were dissolved in PBS, 10 fold dilution rows (10^{-1} to 10^{-11}) in PBS were generated and plated in duplicates on selective LB media with supplements containing 50mg/L kanamycin. At dissection (day 11) the gut content was collected and processed similar to the faecal samples. The gut was divided in small intestine comprising duodenum, jejunum and ileum; and cecum and colon. All samples were weighed, colonies were counted after 24 hours incubation at 37 °C, and the count were normalized to volume of PBS (1mL) and weight of the faeces or gut content.

Results

The results are shown in Figures 14-18.

After a single oral dose of 10^{11} cells per animal, colonization of the strains was followed in faeces for 24 hours (Fig 14b and 15). All novel knockout strains, including double knockout Δ*pdx*H/*thi*L and Δ*sod*A/B, showed similar abundance compared to the WT and improved abundance over the auxotrophic knockout Δ*dap*A.

After three oral doses of 10^{11} cells each per animal, abundance of the strains was measured in faeces for 11 days (Fig 16,17), followed by dissection of the animals and analysis in gut content (Fig 18). While the WT strain colonization stayed constant around 10^6 CFU/g throughout the duration of the experiment, the Δ*pdx*H and Δ*thi*L single mutants were no longer detectable at day 7 and 11 respectively in the faeces. In contrast the Δ*dap*A single knockout strain as well as the combination of *dap*A and *sod*A/B knockout were already washed out on day 4, 48 hours after the last gavage, indicating the substantial fitness cost of the Δ*dap*A deletion in the mouse gut. Both double mutant strains Δ*pdx*H/*thi*L and Δ*sod*A/B showed increased duration of colonization over single mutants and were still detectable by the end of the experiment at ca.10^3 CFU/g.

These data demonstrate the novel biocontained double knockout strains ΔpdxH/*thiL and* Δ*sodA/B can* survive for multiple days in the mammalian gut, enabling their use as chassis strains for delivery of small molecules that rely on active metabolism in the GI tract.

5

Claims

1. A bacterium, wherein the bacterium is a therapeutic bacterium and is deficient in:
   a) at least two functional genes in the redox detoxification pathway; or
   b) at least one functional gene in the vitamin B1 synthetic pathway and at least one functional gene in the vitamin B6 synthetic pathway.

2. A bacterium comprising an expression cassette for producing a therapeutic molecule, wherein the bacterium is deficient in:
   a) at least two functional genes in the redox detoxification pathway; or
   b) at least one functional gene in the vitamin B1 synthetic pathway and at least one functional gene in the vitamin B6 synthetic pathway.

3. The bacterium of claim 2, wherein:
   i) the expression cassette is:
      a) comprised in a plasmid; or
      b) integrated into the genome of the bacterium; and/or
   ii) the therapeutic molecule is a medicament.

4. The bacterium of claim 1 wherein the bacterium comprises a promoter, the promoter comprising a sequence with at least 70% sequence identity to SEQ ID NO.s 74 or 75; or the bacterium of claims 2-3 wherein the expression cassette comprises a promoter, the promoter comprising a sequence with at least 70% sequence identity to SEQ ID NO.s 74 or 75.

5. The bacterium of claims 1-4, wherein:
   i)
   a) the redox detoxification pathway genes comprise sodA, sodB and sodC; and/or
   b) the vitamin B1 synthetic pathway genes comprise *thi*E and *thi*L; and/or
   c) the vitamin B6 synthetic pathway genes comprise *epd*, *pdx*B, *ser*C, *pdx*A, *dxs*, *pdx*J and *pdx*H, *optionally epd*, *pdx*B, *ser*C, *pdx*A, *dxs, pdx*J, *pdx*H, *pdx*S and *pdx*T; or
   ii) wherein the bacterium:
   a) does not have a functional sodA and/or sodB gene; and/or
   b) does not have functional pdxH and/or thiL gene.

6. The bacterium of claim 5ii), wherein:

a) the sodA gene has 70% sequence identity to SEQ ID NO. 1;
b) the sodB gene has 70% sequence identity to SEQ ID NO.2;
c) the pdxH gene has 70% sequence identity to SEQ ID NO. 11;
d) the thiL gene has 70% sequence identity to SEQ ID NO. 7.

7. The bacterium of any of claims 1 to 6, wherein the bacterium is any of the following: *Escherichia coli*, optionally *E. coli* Nissle; lactic acid bacterium, optionally *Lactobacillus* or *Lactococcus*; *Akkermansia*, optionally *Akkermansia muciniphila*; *Bifidobacterium*, *Bacteroides*, *Salmonella* or *Listeria*.

8. The bacterium of any of claims 1 to 7, wherein the genes are partially or fully deleted.

9. A kit comprising the bacterium of claim 1 and a plasmid.

10. The kit of claim 9, wherein the plasmid comprises a promoter, optionally wherein the promoter comprises a sequence with at least 70% sequence identity to SEQ ID NO.s 74 or 75.

11. The bacterium of claim 2, wherein the therapeutic molecule, preferably a medicament, is: a) L-DOPA; b) dopamine; c) serotonin; d) a tryptophan derivative; or e) a tyrosine derivative.

12. The bacterium of claim 11a), wherein the expression cassette comprises:

a) a gene encoding a eukaryotic tyrosine hydroxylase, optionally wherein the eukaryotic tyrosine hydroxylase has at least 70% sequence identity to SEQ ID NO.s 36, 38, 40 or 42;

and optionally any one or both of the following:

b) a gene encoding a mutant GTP cyclohydrolase I, the mutant GTP cyclohydrolase I having at least 70% sequence identity to SEQ ID NO. 44, and comprising one or more mutations wherein the mutant provides for an increased hydroxylation activity of the tyrosine hydroxylase; and/or

c) a gene encoding a dihydromonapterin reductase (FolM), optionally wherein the FolM has at least 70% sequence identity to SEQ ID NO. 48.

13. A pharmaceutical formulation comprising any of the bacterium of claims 1 to 8 or 11 to 12.

14. The bacterium of claims 1 to 8 or 11 to 12, or the pharmaceutical formulation of claim 13, for use as a medicament.

15. The bacterium of claim 11a) or claim 12, or a pharmaceutical formulation thereof, for use in a method of treating Parkinson's disease.

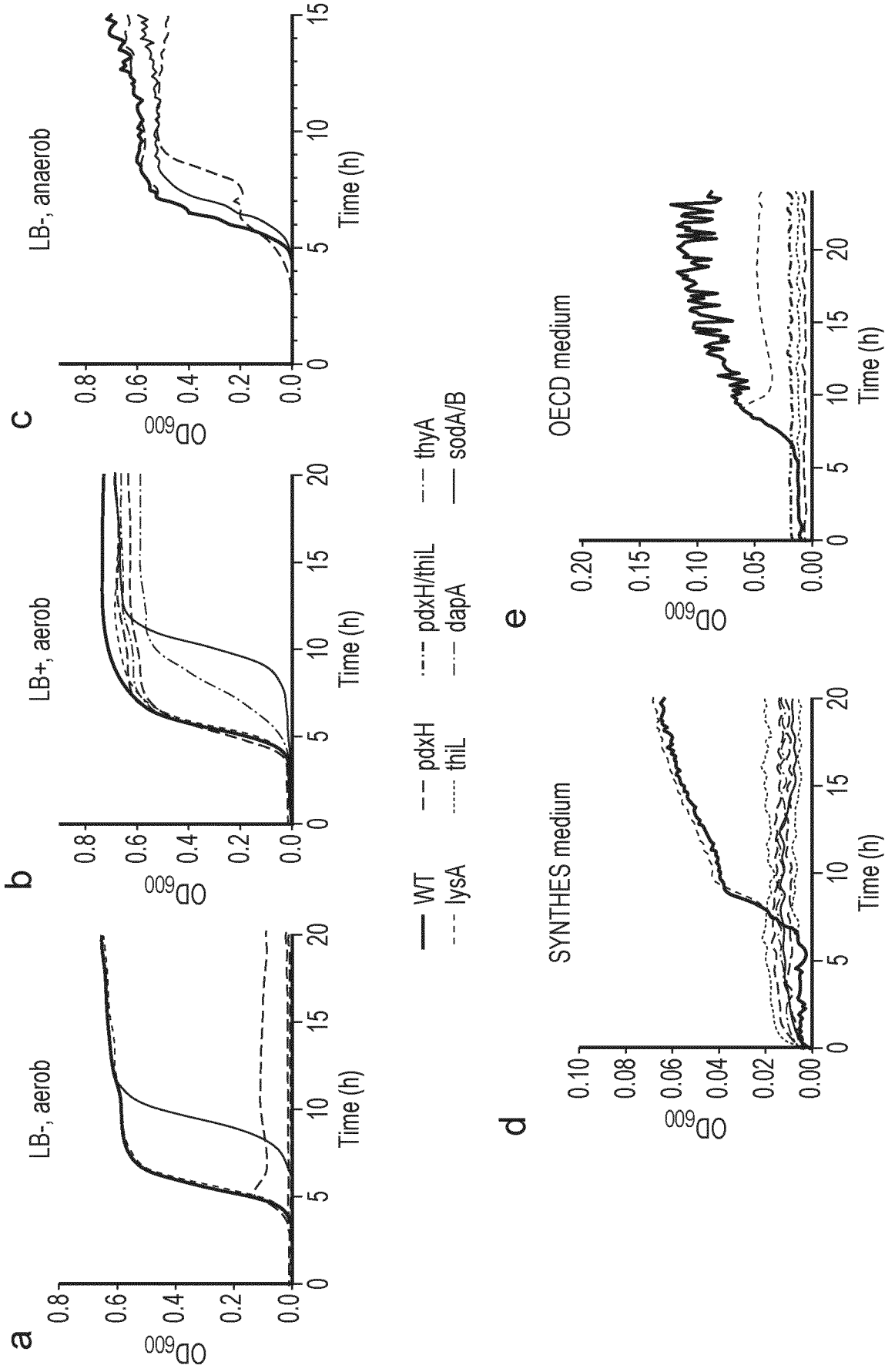
a
LB-, aerob
b
LB+, aerob
c
LB-, anaerob
d
SYNTHES medium
e
OECD medium
OD600
Time (h)
WT
lysA
pdxH
thiL
pdxH/thiL
dapA
thyA
sodA/B

FIG. 1

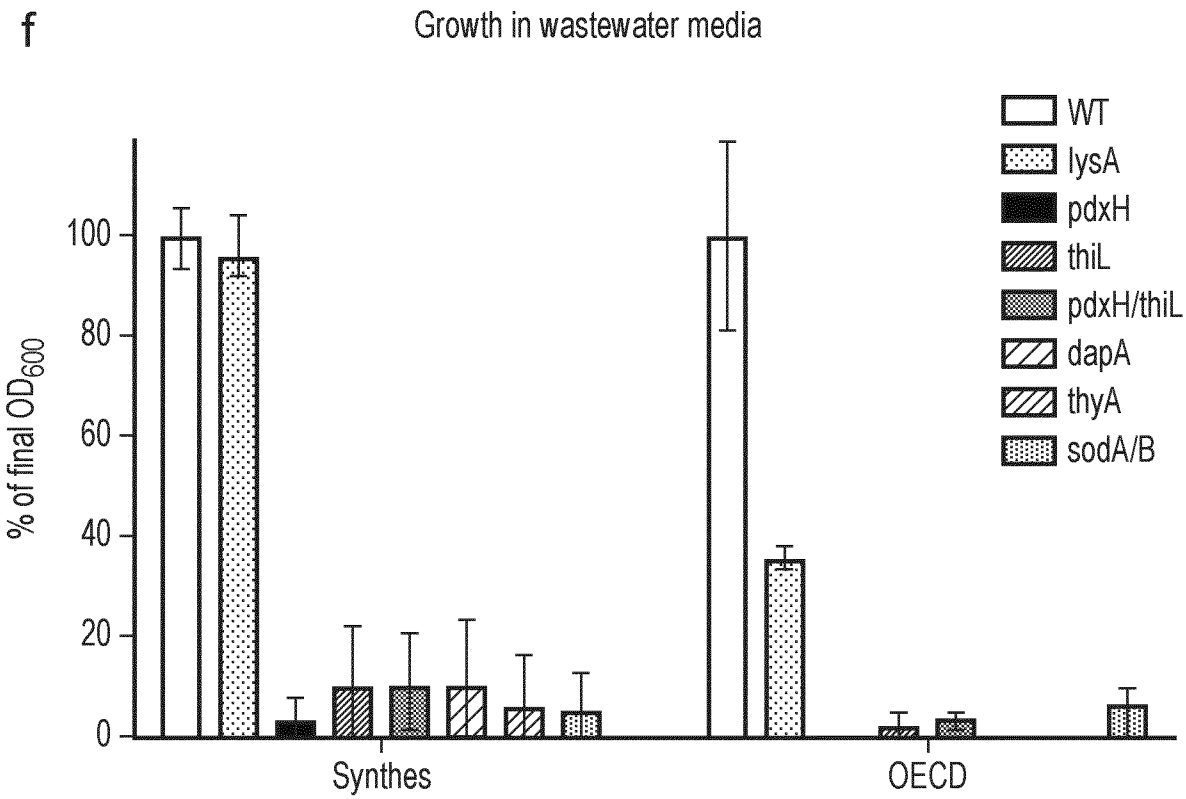

FIG. 1 (Continued)

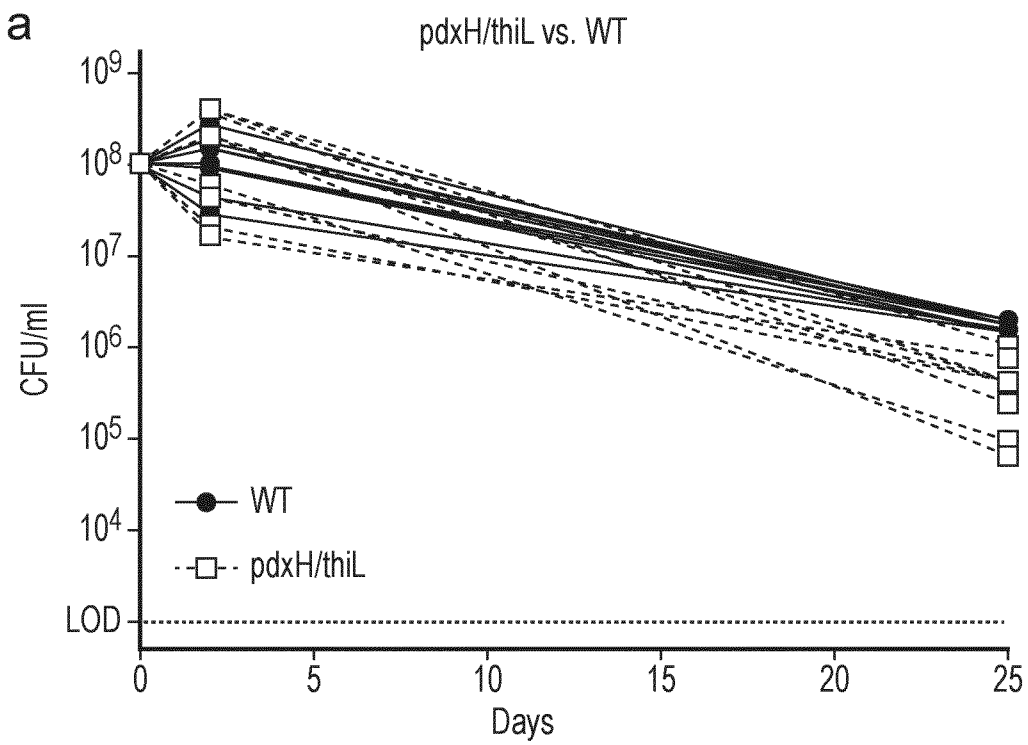

FIG. 2

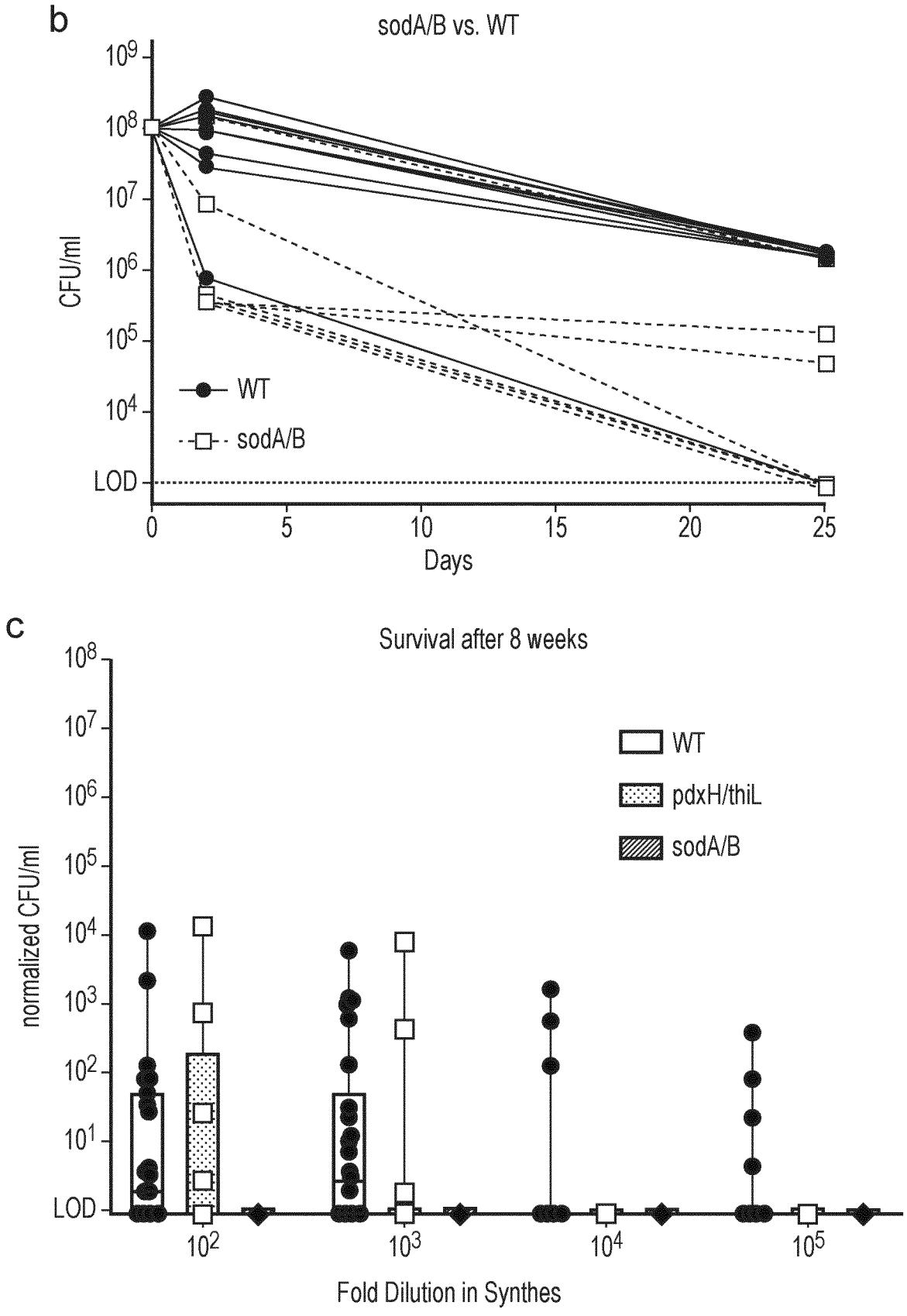

FIG. 2 (Continued)

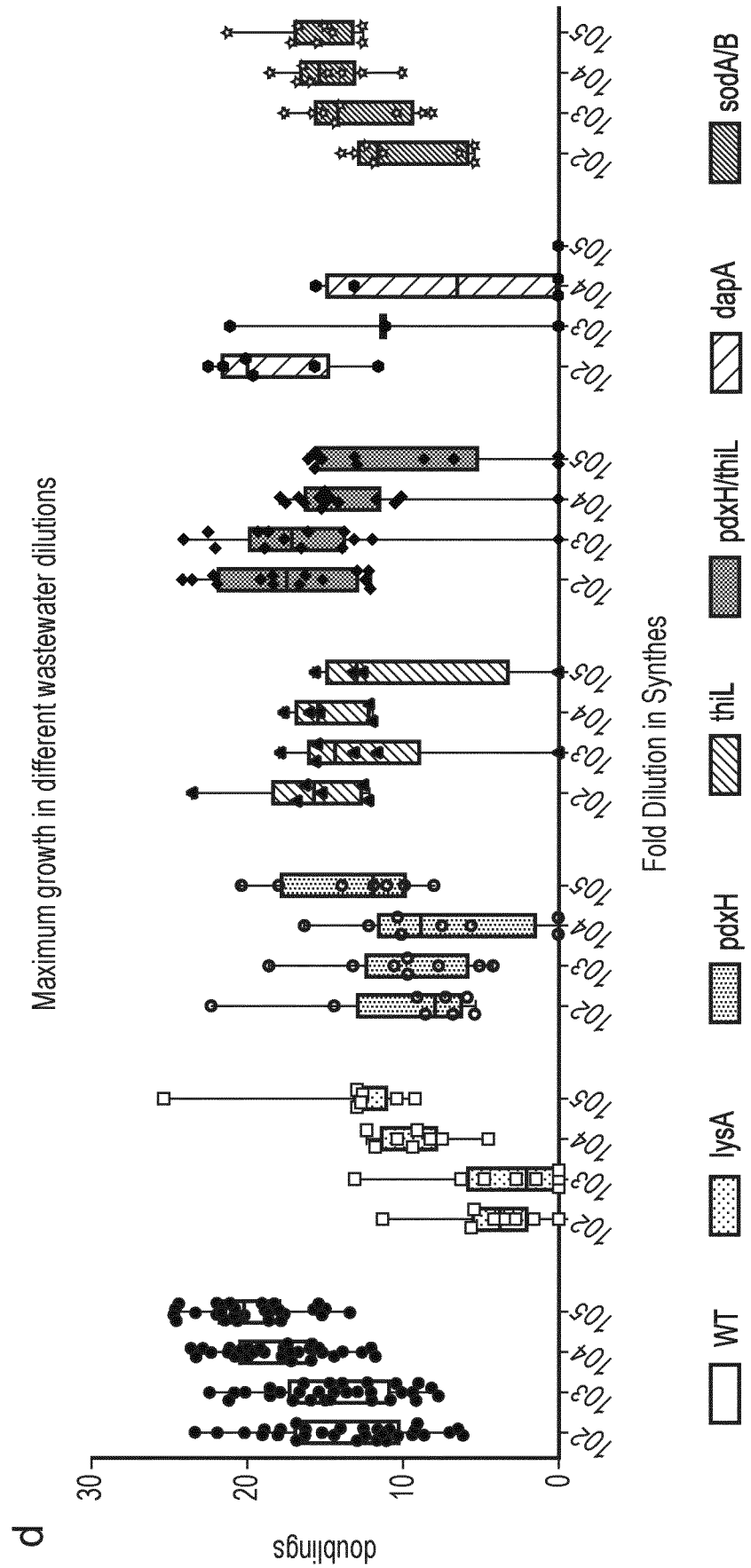

FIG. 2 (Continued)

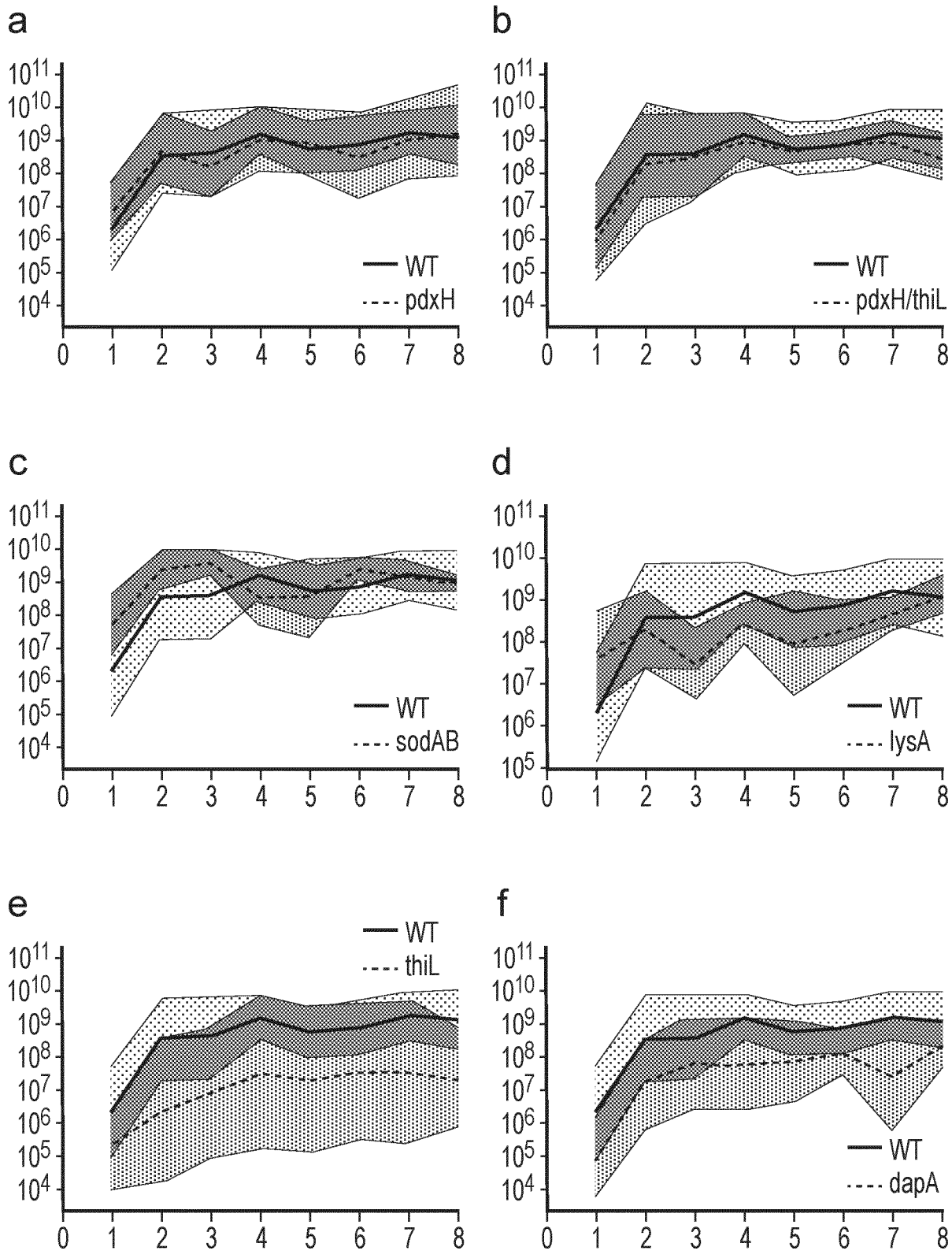

FIG. 3

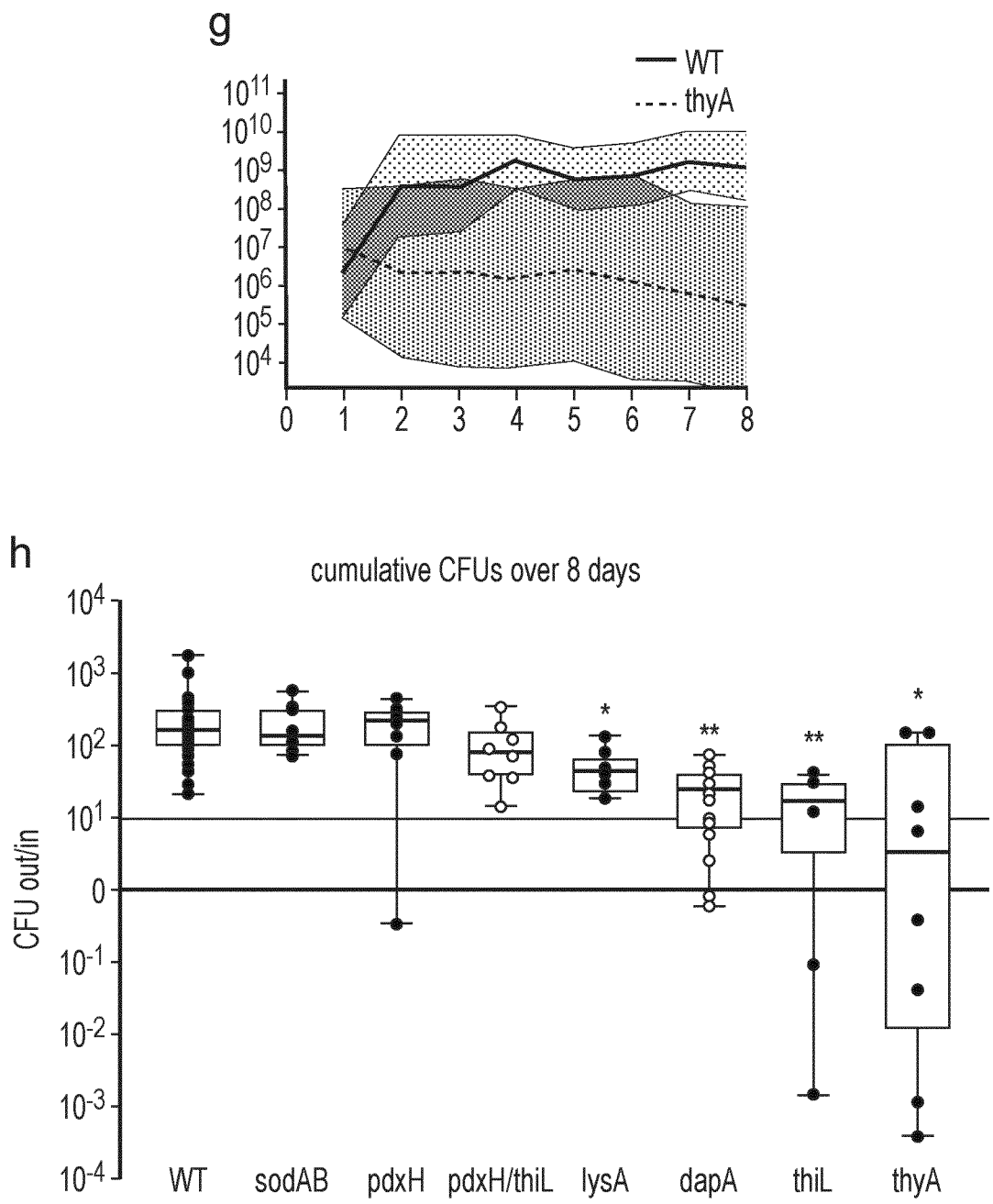

FIG. 3 (Continued)

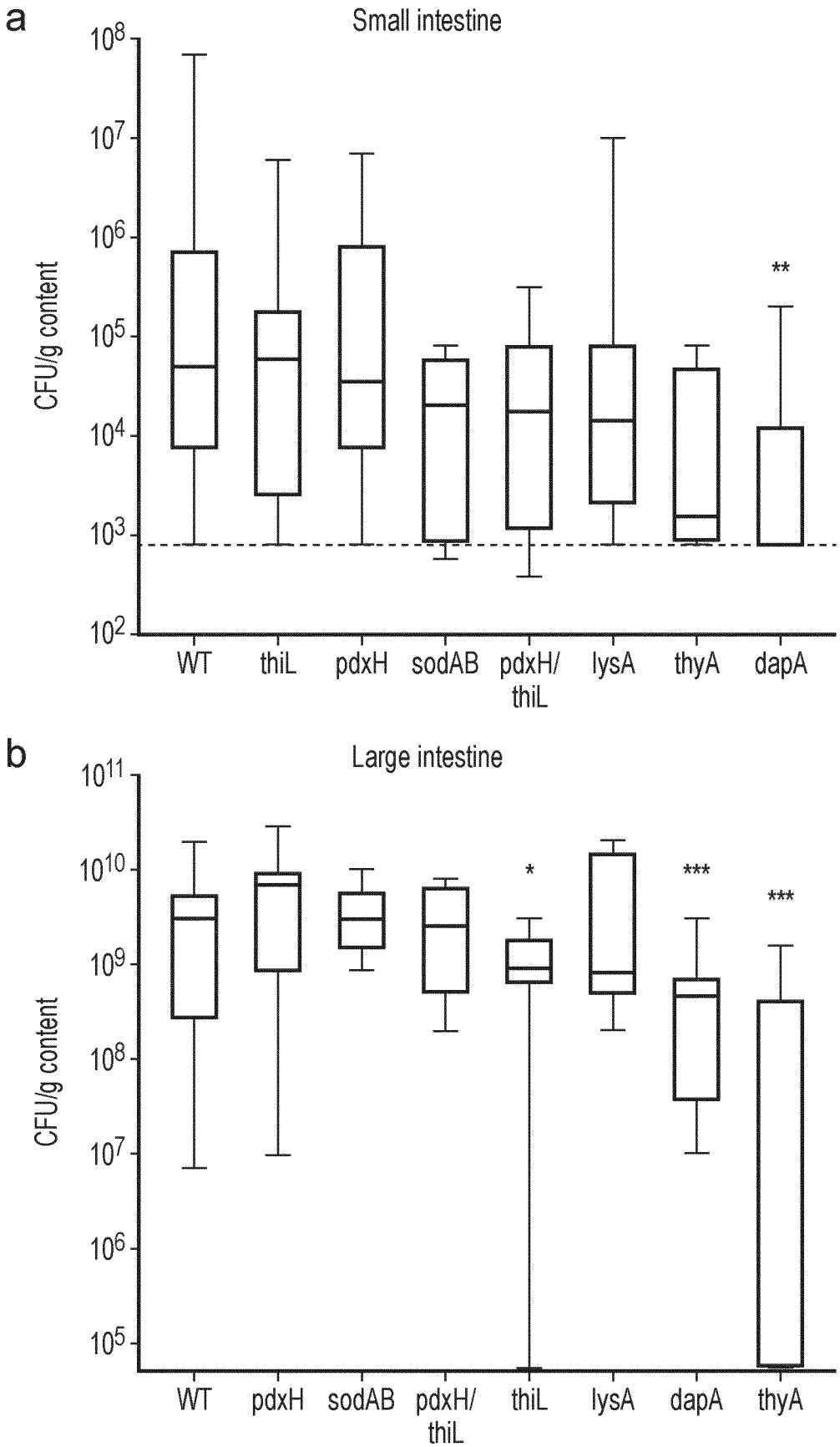
a
Small intestine
CFU/g content
10^8
10^7
10^6
10^5
10^4
10^3
10^2
**
WT
thiL
pdxH
sodAB
pdxH/ thiL
lysA
thyA
dapA
b
Large intestine
CFU/g content
10^{11}
10^{10}
10^9
10^8
10^7
10^6
10^5
*
***
***
WT
pdxH
sodAB
pdxH/ thiL
thiL
lysA
dapA
thyA

FIG. 4

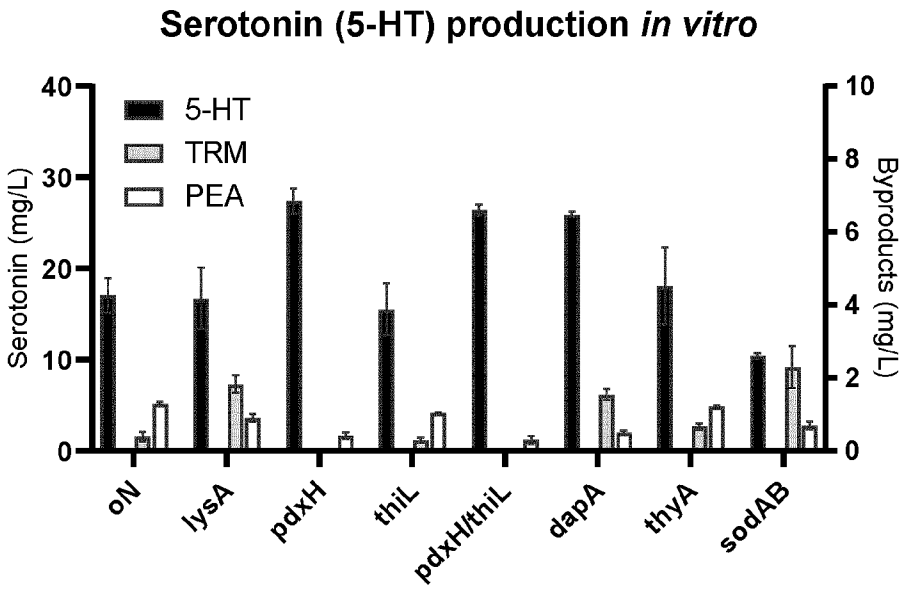
Serotonin (5-HT) production *in vitro*
5-HT
TRM
PEA
Serotonin (mg/L)
Byproducts (mg/L)
0
10
20
30
40
0
2
4
6
8
10
oN
lysA
pdxH
thiL
pdxH/thiL
dapA
thyA
sodAB

Figure 5

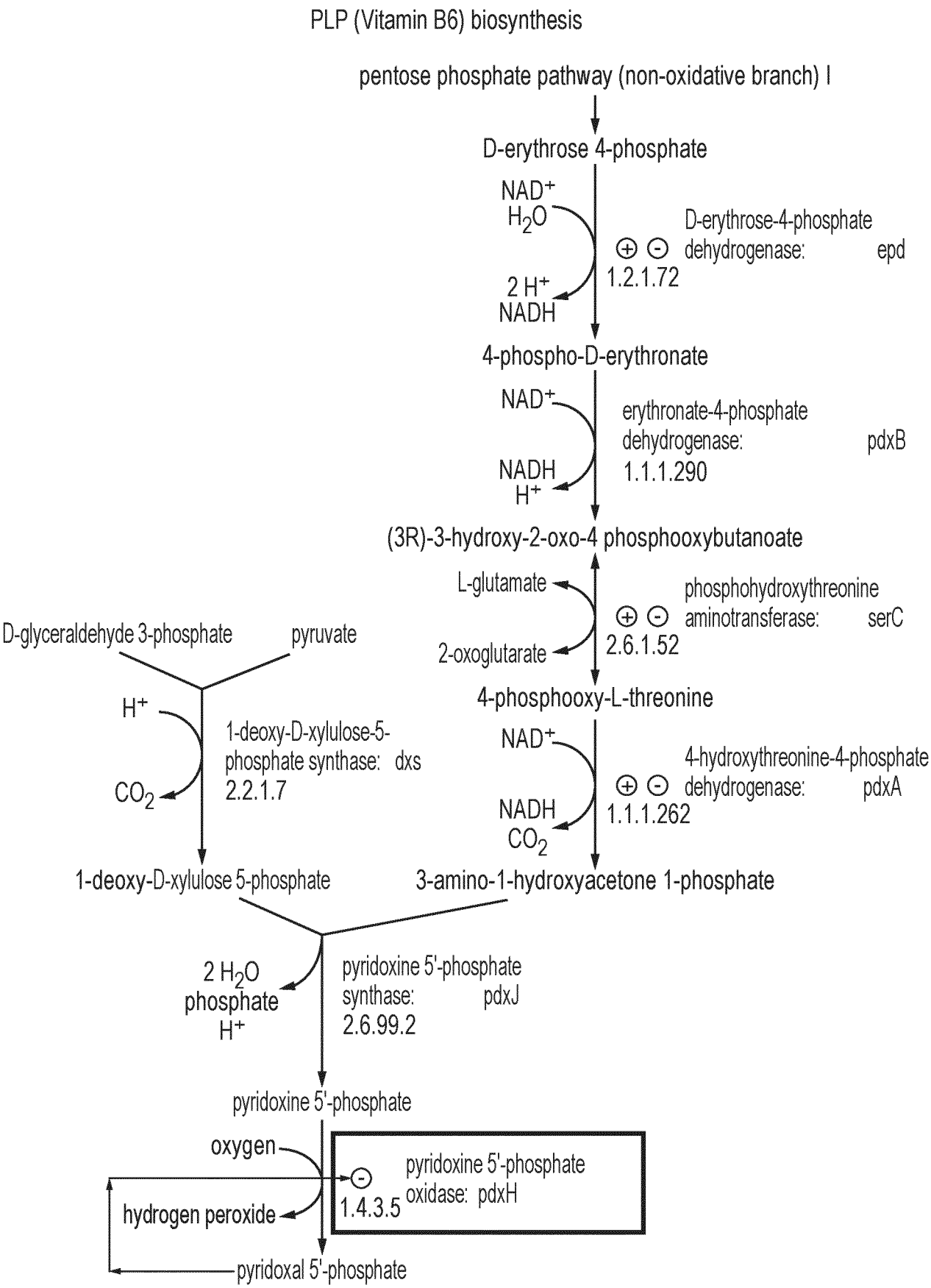
PLP (Vitamin B6) biosynthesis
pentose phosphate pathway (non-oxidative branch) I
D-erythrose 4-phosphate
NAD+
H2O
2 H+
NADH
D-erythrose-4-phosphate dehydrogenase: epd
1.2.1.72
4-phospho-D-erythronate
NAD+
NADH
H+
erythronate-4-phosphate dehydrogenase: pdxB
1.1.1.290
(3R)-3-hydroxy-2-oxo-4 phosphooxybutanoate
L-glutamate
2-oxoglutarate
phosphohydroxythreonine aminotransferase: serC
2.6.1.52
4-phosphooxy-L-threonine
NAD+
NADH
CO2
4-hydroxythreonine-4-phosphate dehydrogenase: pdxA
1.1.1.262
3-amino-1-hydroxyacetone 1-phosphate
D-glyceraldehyde 3-phosphate
pyruvate
H+
CO2
1-deoxy-D-xylulose-5-phosphate synthase: dxs
2.2.1.7
1-deoxy-D-xylulose 5-phosphate
2 H2O
phosphate
H+
pyridoxine 5'-phosphate synthase: pdxJ
2.6.99.2
pyridoxine 5'-phosphate
oxygen
hydrogen peroxide
pyridoxine 5'-phosphate oxidase: pdxH
1.4.3.5
pyridoxal 5'-phosphate

FIG. 6

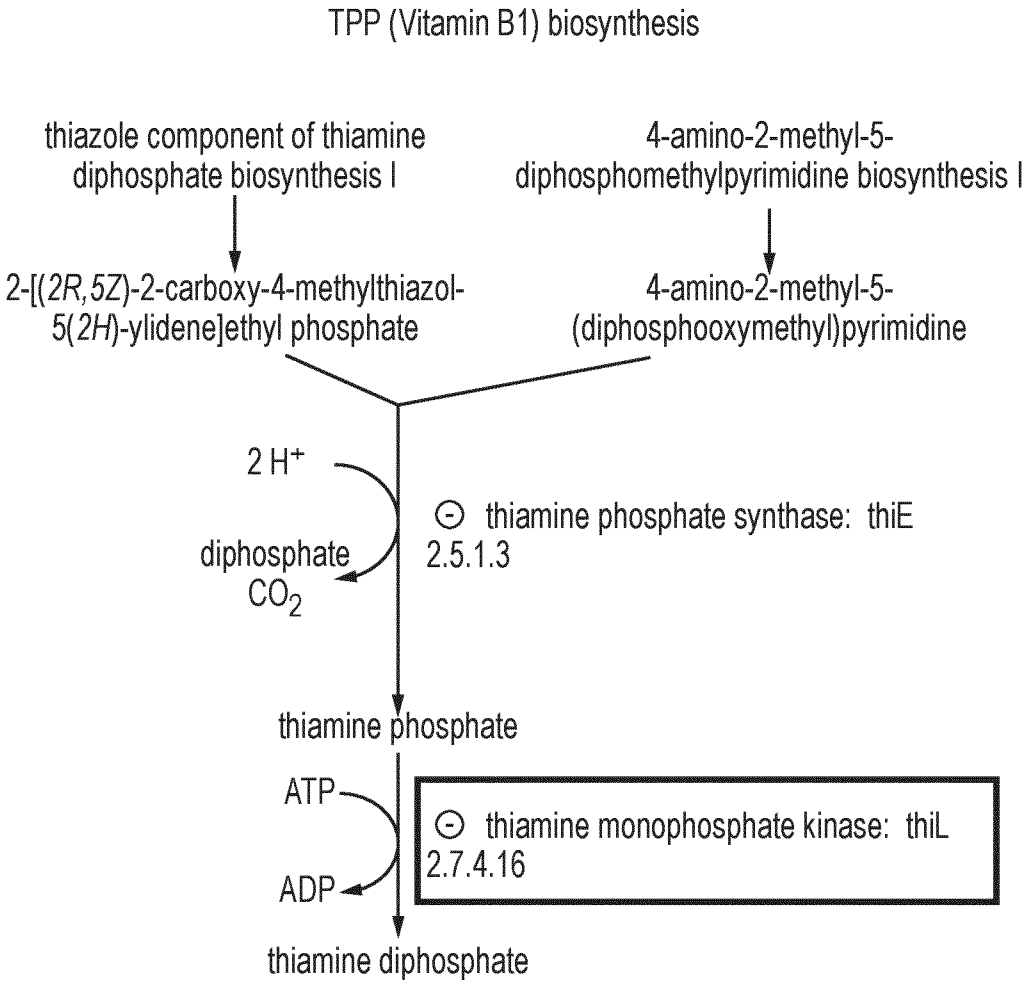
TPP (Vitamin B1) biosynthesis
thiazole component of thiamine diphosphate biosynthesis I
4-amino-2-methyl-5-diphosphomethylpyrimidine biosynthesis I
2-[(2R,5Z)-2-carboxy-4-methylthiazol-5(2H)-ylidene]ethyl phosphate
4-amino-2-methyl-5-(diphosphooxymethyl)pyrimidine
2 H+
diphosphate
CO2
thiamine phosphate synthase: thiE
2.5.1.3
thiamine phosphate
ATP
ADP
thiamine monophosphate kinase: thiL
2.7.4.16
thiamine diphosphate

FIG. 7

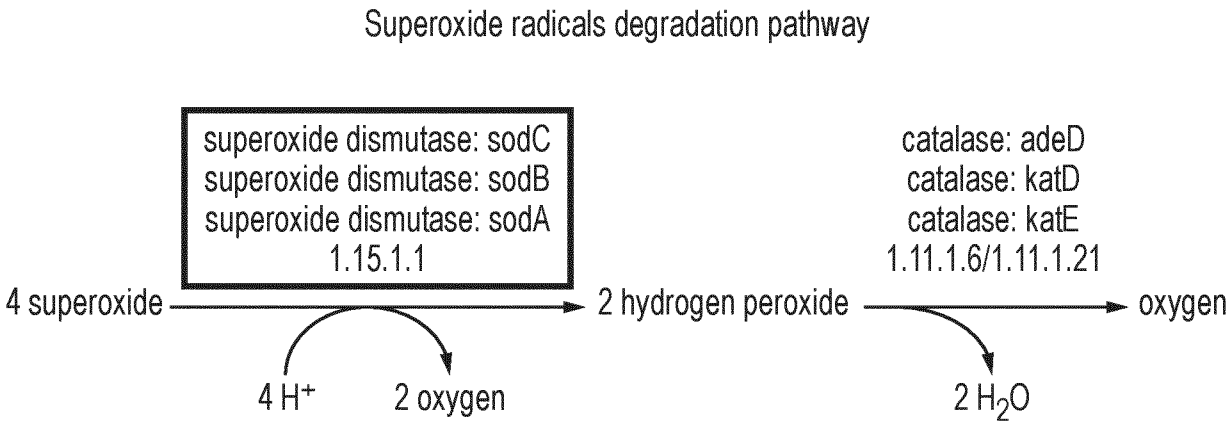
Superoxide radicals degradation pathway
superoxide dismutase: sodC
superoxide dismutase: sodB
superoxide dismutase: sodA
1.15.1.1
catalase: adeD
catalase: katD
catalase: katE
1.11.1.6/1.11.1.21
4 superoxide
2 hydrogen peroxide
oxygen
4 H+
2 oxygen
2 H2O

FIG. 8

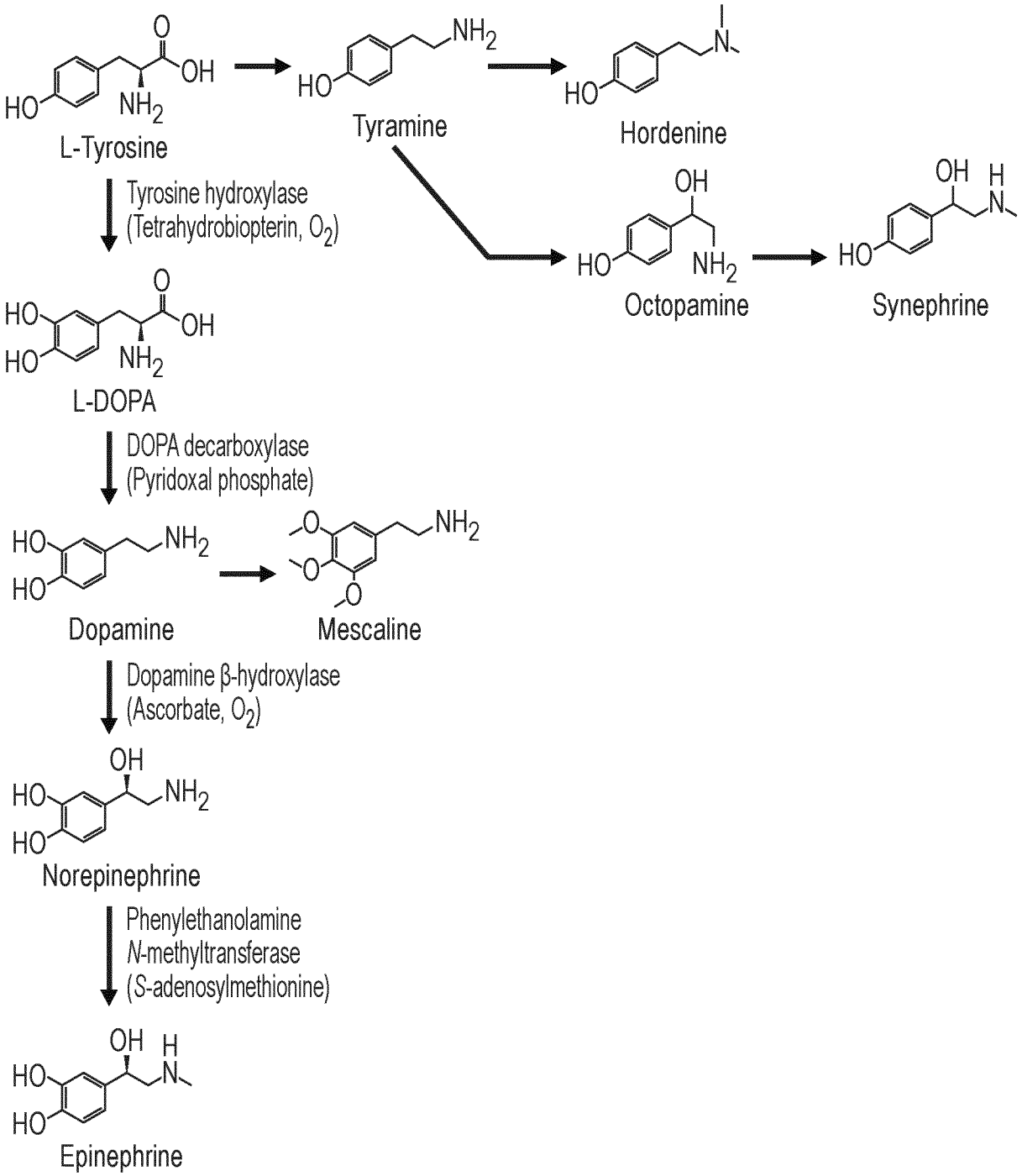
L-Tyrosine
Tyramine
Hordenine
Tyrosine hydroxylase
(Tetrahydrobiopterin, O_2)
Octopamine
Synephrine
L-DOPA
DOPA decarboxylase
(Pyridoxal phosphate)
Dopamine
Mescaline
Dopamine β-hydroxylase
(Ascorbate, O_2)
Norepinephrine
Phenylethanolamine
N-methyltransferase
(*S*-adenosylmethionine)
Epinephrine

FIG. 9

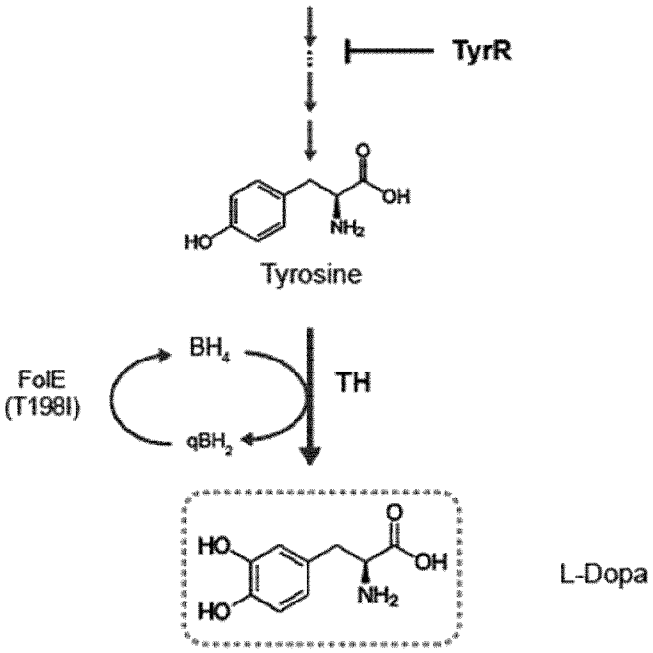
TyrR
OH
O
NH2
HO
Tyrosine
BH4
FolE
(T198I)
TH
qBH2
HO
HO
O
OH
NH2
L-Dopa

Figure 10

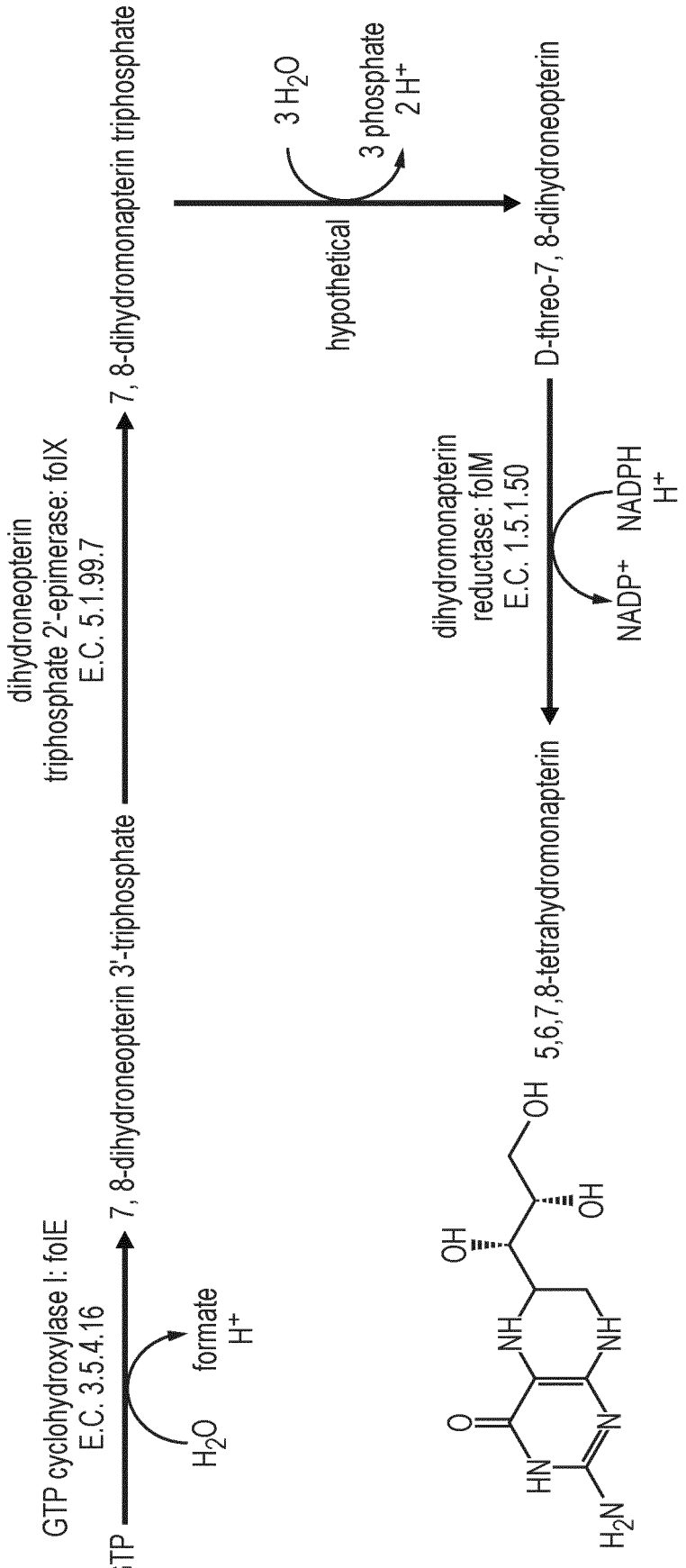
GTP
GTP cyclohydroxylase I: folE
E.C. 3.5.4.16
H_2O
formate
H^+
7, 8-dihydroneopterin 3'-triphosphate
dihydroneopterin
triphosphate 2'-epimerase: folX
E.C. 5.1.99.7
7, 8-dihydromonapterin triphosphate
$3\ H_2O$
hypothetical
3 phosphate
$2\ H^+$
D-threo-7, 8-dihydroneopterin
dihydromonapterin
reductase: folM
E.C. 1.5.1.50
$NADP^+$
NADPH
H^+
5,6,7,8-tetrahydromonapterin
OH
OH
OH
NH
NH
O
HN
N
H_2N

FIG. 11

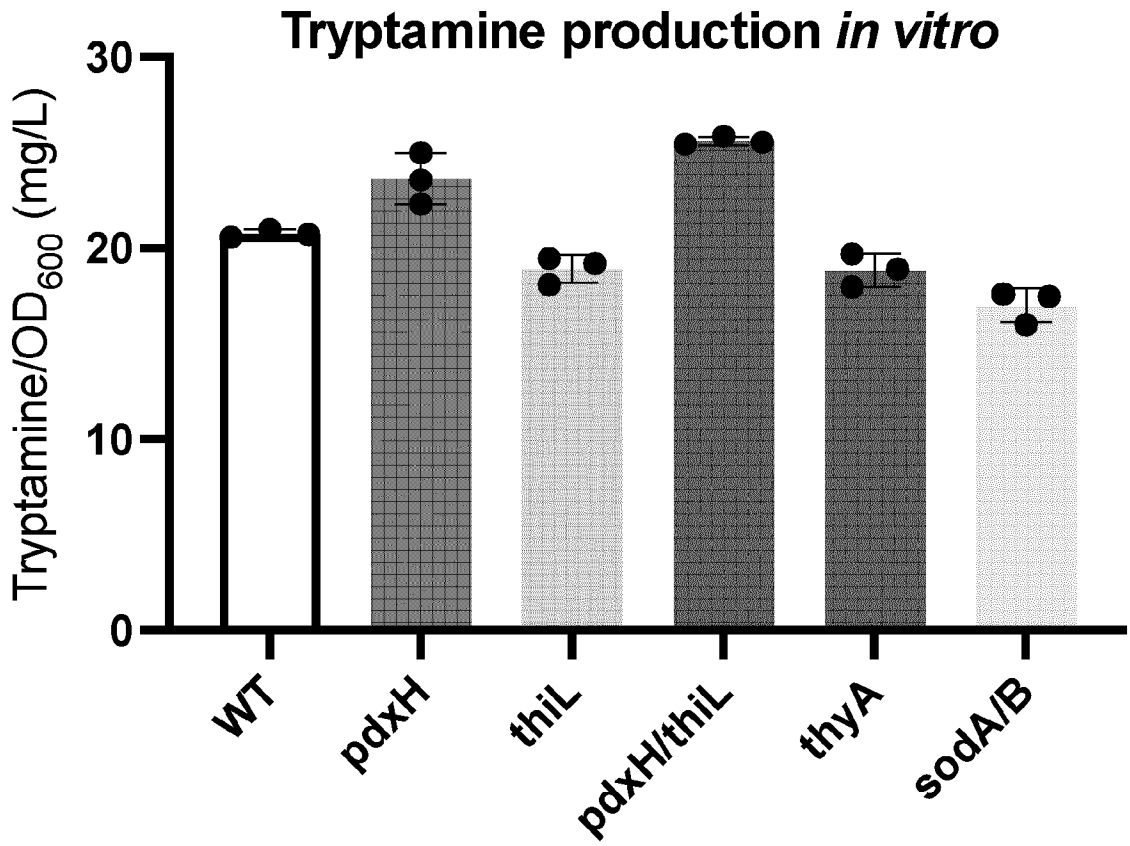
Tryptamine production *in vitro*
Tryptamine/OD600 (mg/L)
30
20
10
0
WT
pdxH
thiL
pdxH/thiL
thyA
sodA/B

FIG. 12

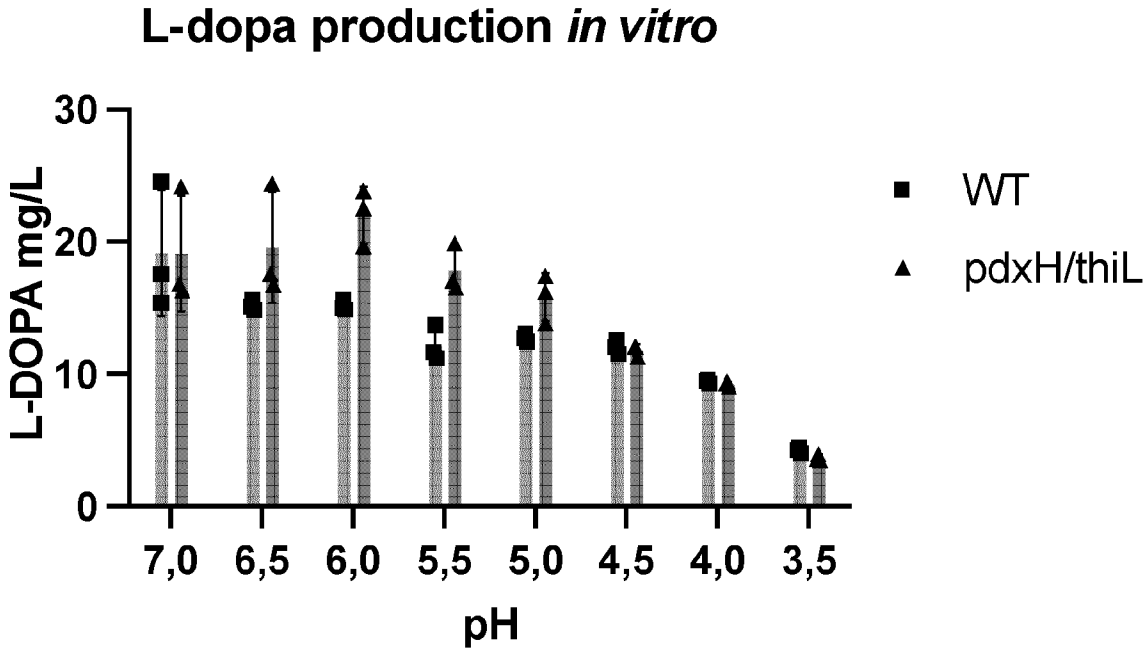
L-dopa production *in vitro*
L-DOPA mg/L
30
20
10
0
7,0
6,5
6,0
5,5
5,0
4,5
4,0
3,5
pH
WT
pdxH/thiL

FIG. 13

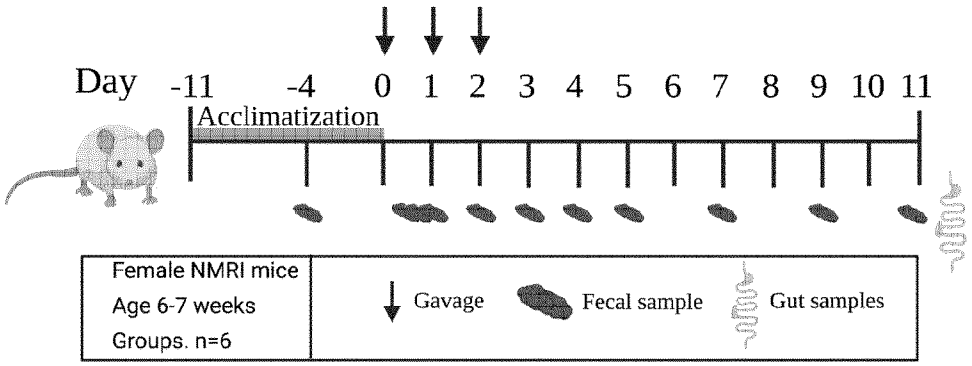

FIG. 14a)

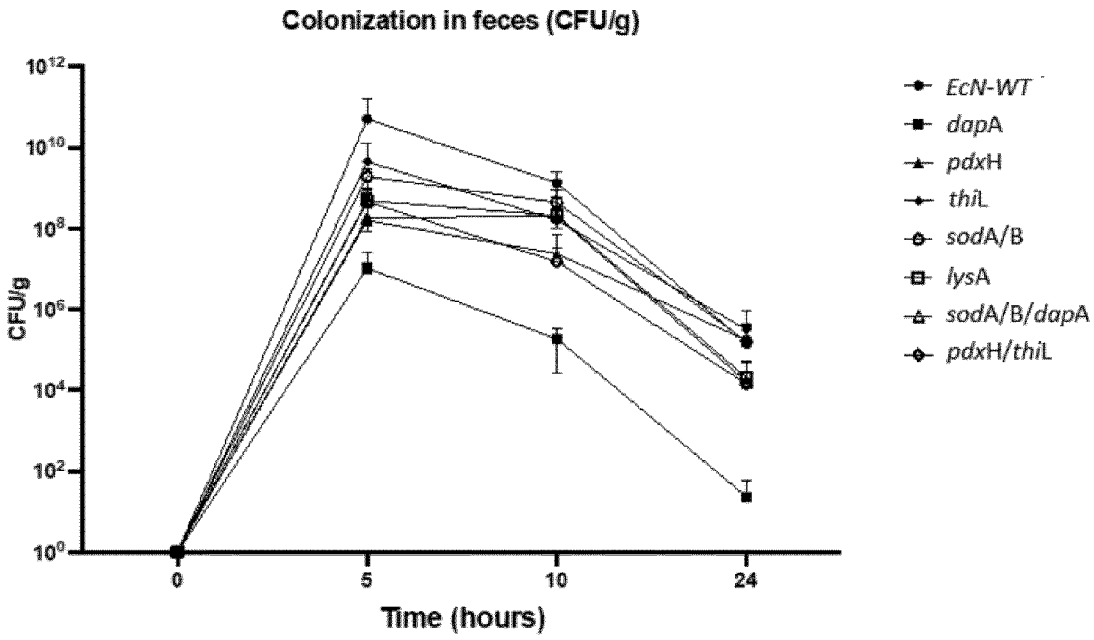

FIG. 14b)

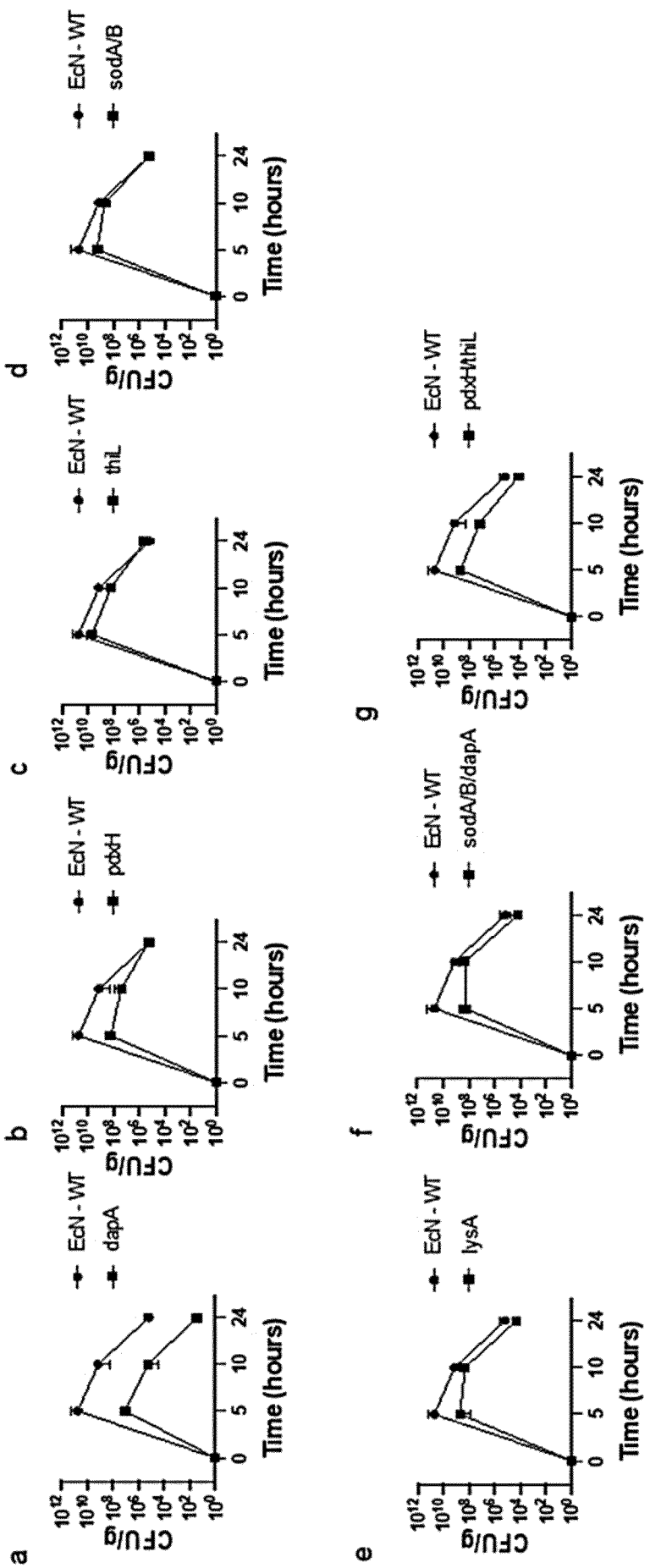
a
b
c
d
e
f
g
CFU/g
Time (hours)
EcN - WT
dapA
pdxH
thiL
sodA/B
lysA
sodA/B/dapA
pdxH/thiL

FIG. 15

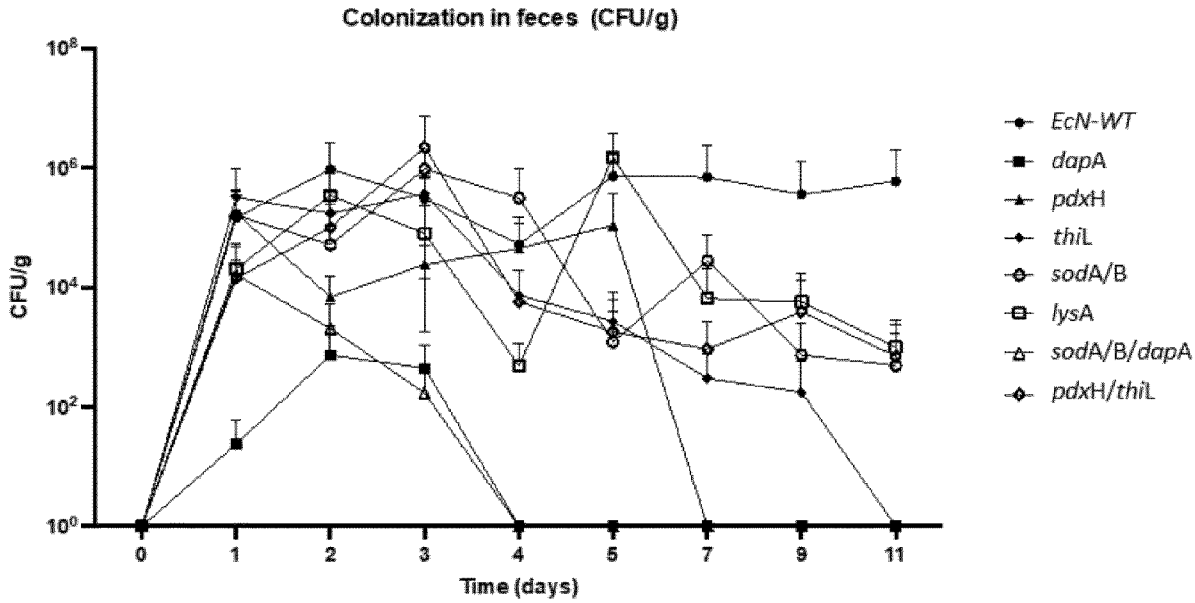
Colonization in feces (CFU/g)
CFU/g
10^8
10^6
10^4
10^2
10^0
0
1
2
3
4
5
7
9
11
Time (days)
EcN-WT
dapA
pdxH
thiL
sodA/B
lysA
sodA/B/dapA
pdxH/thiL

FIG. 16

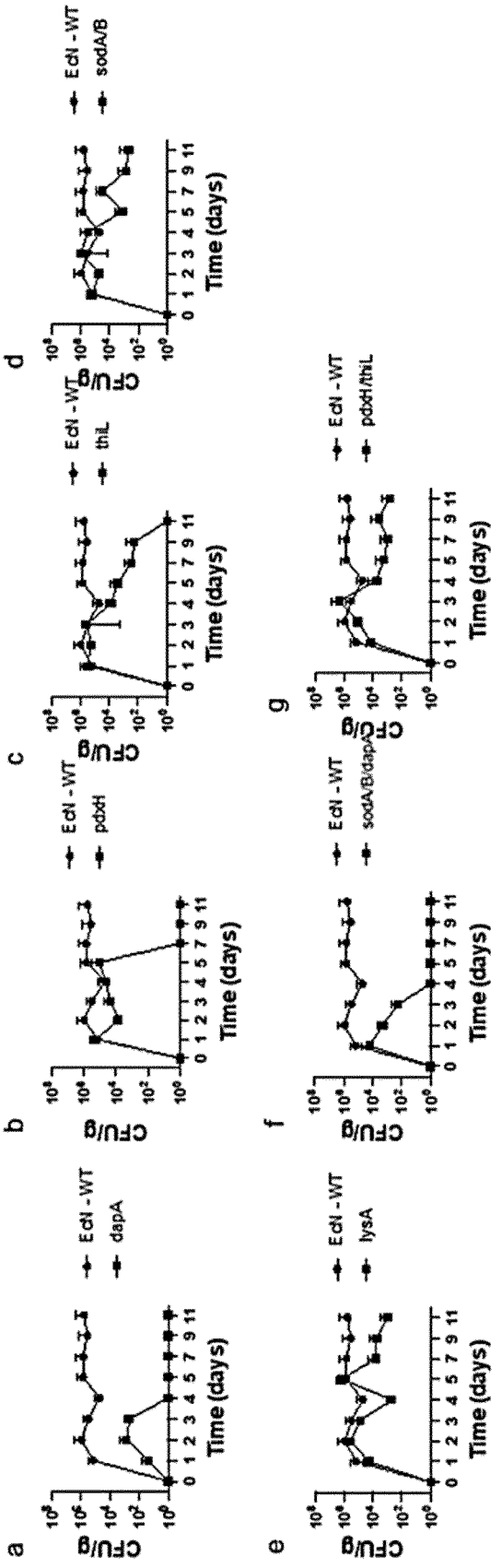
a
b
c
d
e
f
g
CFU/g
Time (days)
EcN - WT
dapA
pdxH
thiL
sodA/B
lysA
sodA/B/dapA
pdxH/thiL

FIG. 17

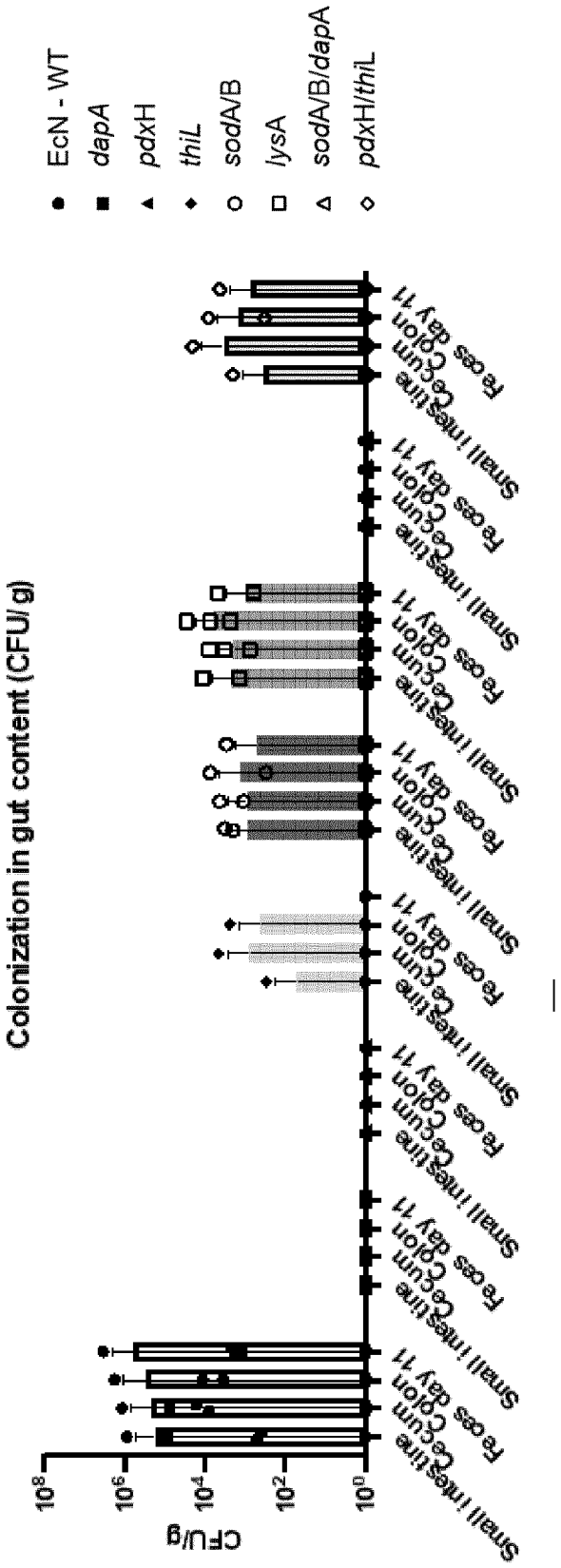
EcN - WT
dapA
pdxH
thiL
sodA/B
lysA
sodA/B/dapA
pdxH/thiL
Colonization in gut content (CFU/ g)
CFU/g
10^8
10^6
10^4
10^2
10^0
Small intestine
Caecum
Colon
Feces
day 11

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/056845

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/74 A61K35/741 A61P25/16 C12N1/20 C12N9/02
C12N9/06 C12N9/10 C12N9/12
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A61K A61P C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATVIG DONALD O. ET AL: "Human Copper-Zinc Superoxide Dismutase Complements Superoxide Dismutase-deficient Escherichia coli Mutants"", THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 262, no. 30, January 1987 (1987-01), pages 14697-14701, XP055949924, the whole document ----- PATEL M ET AL: "Metalloporphyrin class of therapeutic catalytic antioxidants", TRENDS IN PHARMACOLOGICAL SCIENCES, ELSEVIER, HAYWARTH, GB, vol. 20, no. 9, September 1999 (1999-09), pages 359-364, XP004178192, ISSN: 0165-6147, DOI: 10.1016/S0165-6147(99)01336-X the whole document ----- -/--	1-12 1,5-10, 13-15

[x] Further documents are listed in the continuation of Box C.
[x] See patent family annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2023	06/09/2023

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATTHIJSSENS F ET AL: "Prooxidant activity of the superoxide dismutase (SOD)-mimetic EUK-8 in proliferating and growth-arrested Escherichia coli cells", FREE RADICAL BIOLOGY & MEDICINE, ELSEVIER INC, US, vol. 45, no. 5, September 2008 (2008-09), pages 708-715, XP023610800, ISSN: 0891-5849, DOI: 10.1016/J.FREERADBIOMED.2008.05.023 [retrieved on 2008-06-04] pages 708, 709 page 713	1,5-10
X	J. M. BRUNO-BARCENA ET AL: "Role of Antioxidant Enzymes in Bacterial Resistance to Organic Acids", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 76, no. 9, May 2010 (2010-05), pages 2747-2753, XP055329035, US ISSN: 0099-2240, DOI: 10.1128/AEM.02718-09 the whole document	1-3,5-10
A	Caroline B Kurtz ET AL: "An engineered E. coli Nissle improves hyperammonemia and survival in mice and shows dose-dependent exposure in healthy humans", Sci. Transl. Med, 16 January 2019 (2019-01-16), XP055609142, DOI: 10.1126/scitranslmed.aau7975 Retrieved from the Internet: URL:https://stm.sciencemag.org/content/11/475/eaau7975.full.pdf [retrieved on 2019-07-26] the whole document	1-14
A	US 2016/206666 A1 (FALB DEAN [US] ET AL) 21 July 2016 (2016-07-21) the whole document	1-14
A	GROOTE DE M A ET AL: "Periplasmic superoxide dismutase protects Salmonella from products of phagocyte NADPH-oxidase and nitric oxide synthase", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, vol. 94, no. 25, 9 December 1997 (1997-12-09), pages 13997-14001, XP002380881, ISSN: 0027-8424, DOI: 10.1073/PNAS.94.25.13997 the whole document	1-3,5-10

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/056845

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KIM HYUNG JUN ET AL: "The ThiL enzyme is a valid antibacterial target essential for both thiamine biosynthesis and salvage pathways in Pseudomonas aeruginosa", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 295, no. 29, July 2020 (2020-07), pages 10081-10091, XP055964371, US ISSN: 0021-9258, DOI: 10.1074/jbc.RA120.013295 the whole document ----- LAM H-M ET AL: "SUPPRESSION OF INSERTIONS IN THE COMPLEX PDXJ OPERON OF ESCHERICHIA COLI K-12 BY ION AND OTHER MUTATIONS", JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 174, no. 5, March 1992 (1992-03), pages 1554-1567, XP000673192, ISSN: 0021-9193 the whole document -----	1-15 1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/056845

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2023/056845

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15(partially)

A bacterium, wherein the bacterium is a therapeutic bacterium and is deficient in at least two functional genes in the redox detoxification pathway (sodA, sodB and sodC). Related kit, pharmaceutical formulation, and uses as medicament or in treatment.

---

2. claims: 1-15(partially)

A bacterium, wherein the bacterium is a therapeutic bacterium and is deficient in at least one functional gene in the vitamin B1 synthetic pathway (thiE or thiL) and at least one functional gene in the vitamin B6 synthetic pathway (epd, pdxB, serC, pdxA, dxs, pdxJ, pdxH, pdxS and pdxT). Related kit, pharmaceutical composition, and uses in treatment.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/056845

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016206666 A1	21-07-2016	US 2016206666 A1	21-07-2016
		US 2017067065 A1	09-03-2017
		US 2017128499 A1	11-05-2017
		US 2018273956 A1	27-09-2018
		US 2021095297 A1	01-04-2021