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(54) **MICROORGANISMS AND METHODS FOR PRODUCING REUTERIN**

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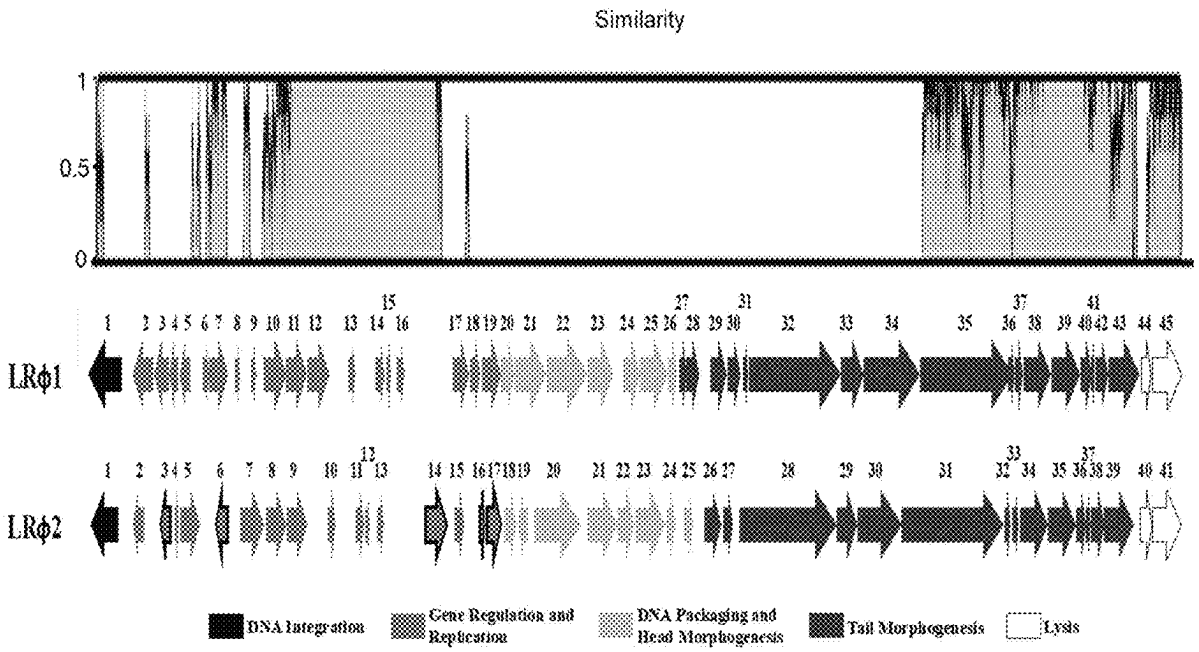
(57) **ABSTRACT**

Microorganisms and methods for producing reuterin. The microorganisms express a phage protein from *Limosilactobacillus reuteri*, Limosilactobacillus mucosae, or *Limosilactobacillus oris*, or a homolog thereof, that enhances the production of reuterin. The methods can include culturing the microorganisms to thereby produce the reuterin.

Specification includes a Sequence Listing.



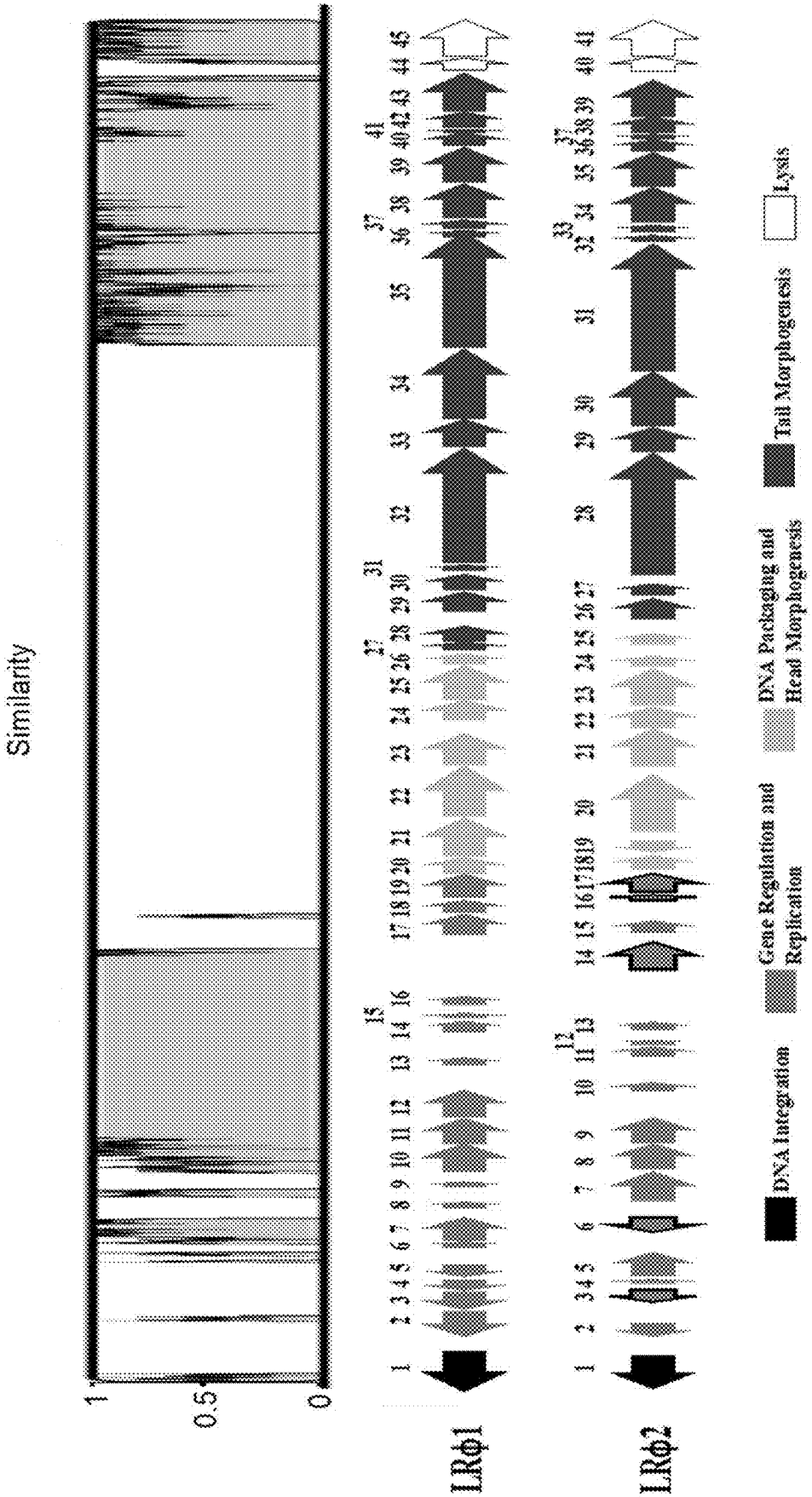

Similarity
1
0.5
0
LRϕ1
LRϕ2
DNA Integration
Gene Regulation and Replication
DNA Packaging and Head Morphogenesis
Tail Morphogenesis
Lysis

FIG. 1

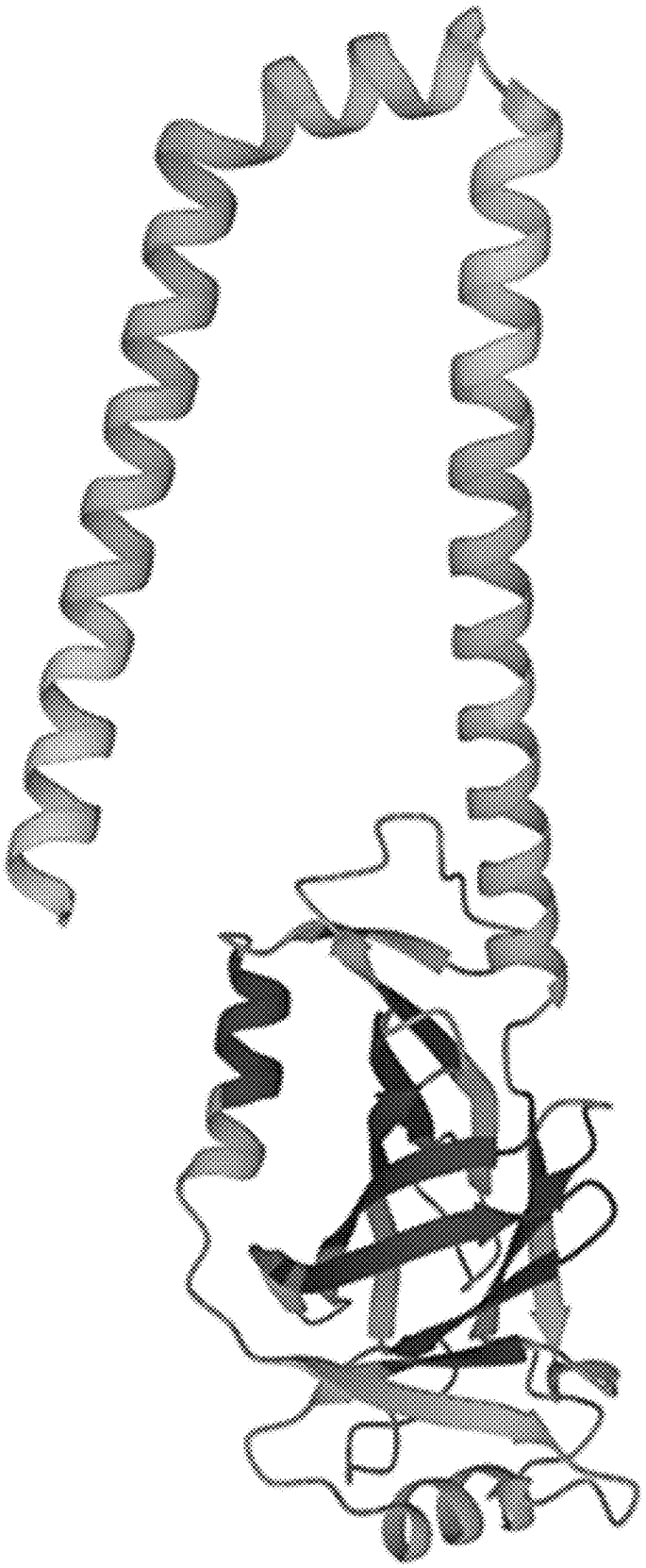


FIG. 2

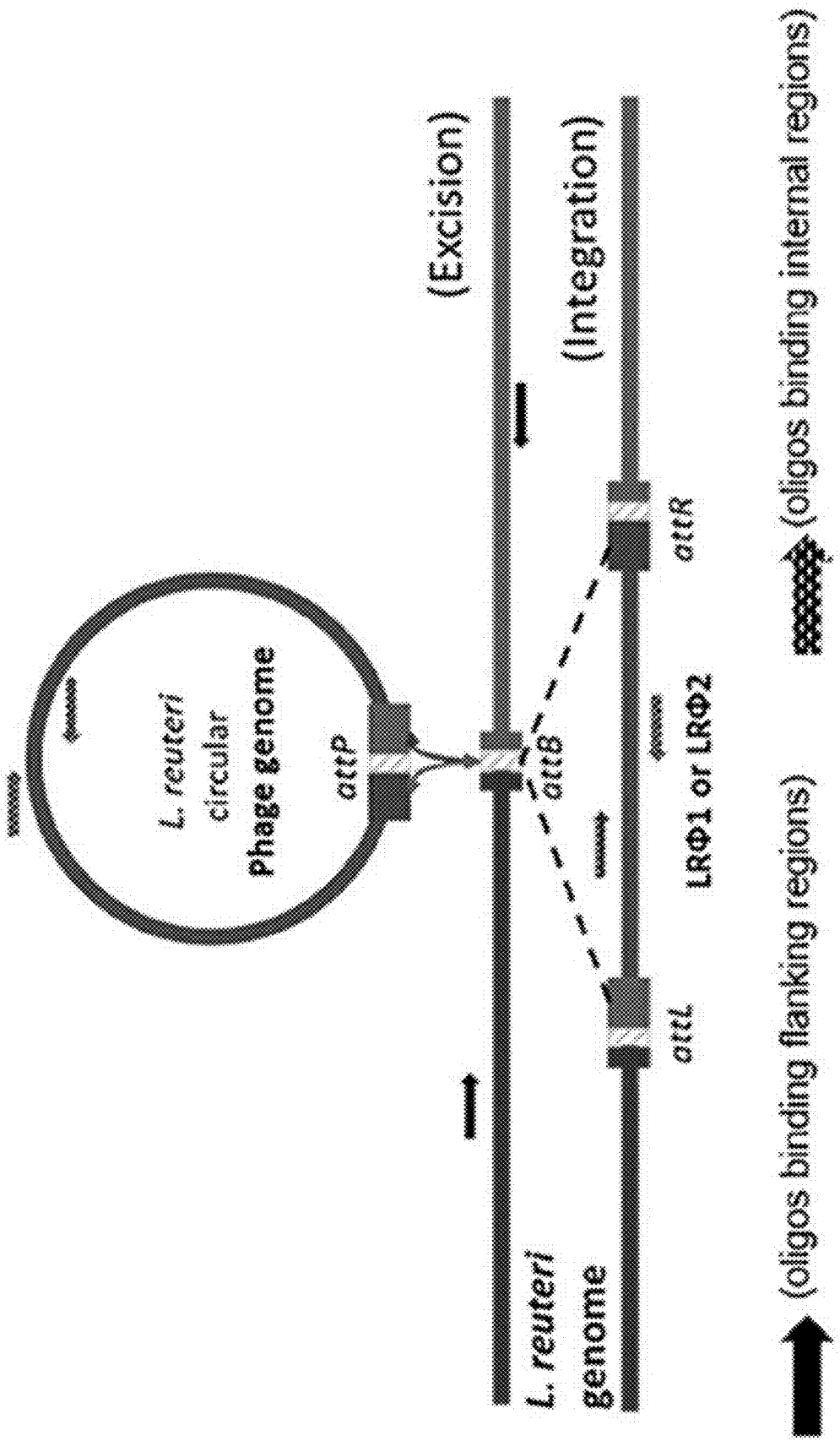

(Excision)
(Integration)
L. reuteri
circular
Phage genome
attP
attB
attR
attL
LRΦ1 or LRΦ2
L. reuteri
genome
(oligos binding flanking regions)
(oligos binding internal regions)

FIG. 3

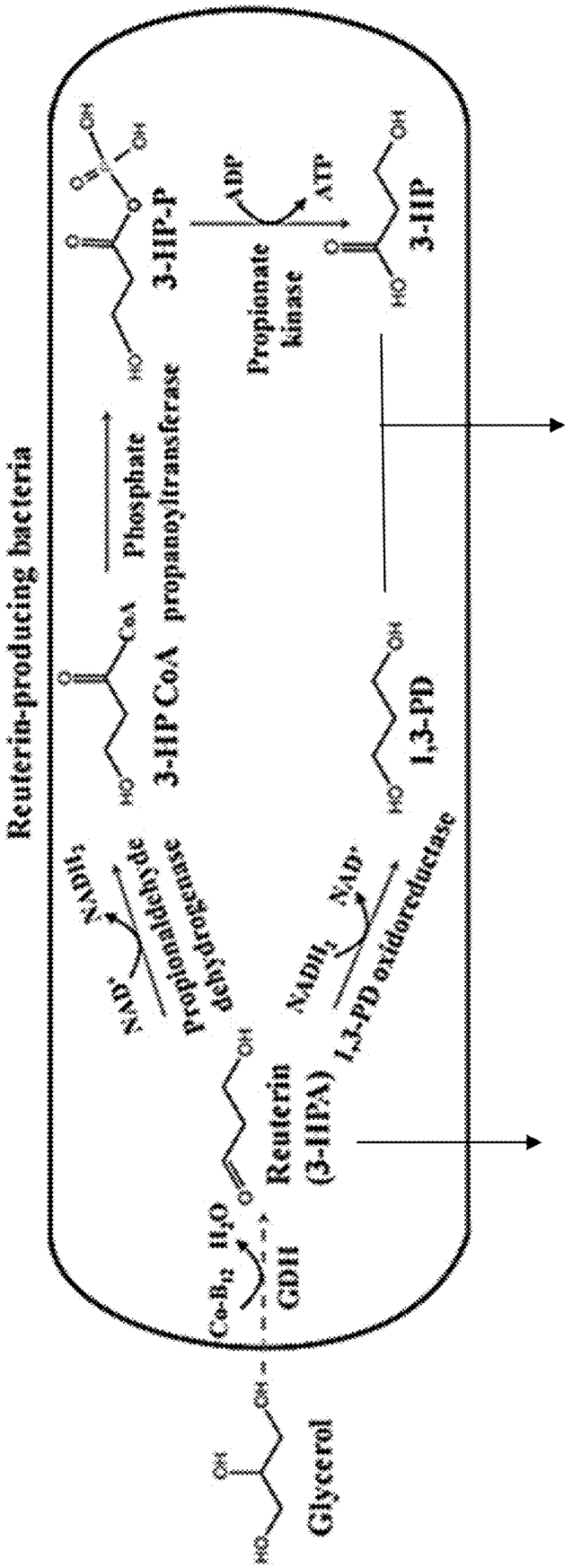

Reuterin-producing bacteria
Glycerol
GDH
Reuterin (3-HPA)
1,3-PD oxidoreductase
1,3-PD
Propionaldehyde dehydrogenase
3-HP CoA
Phosphate propanoyltransferase
3-HP-P
Propionate kinase
3-HP

FIG. 4

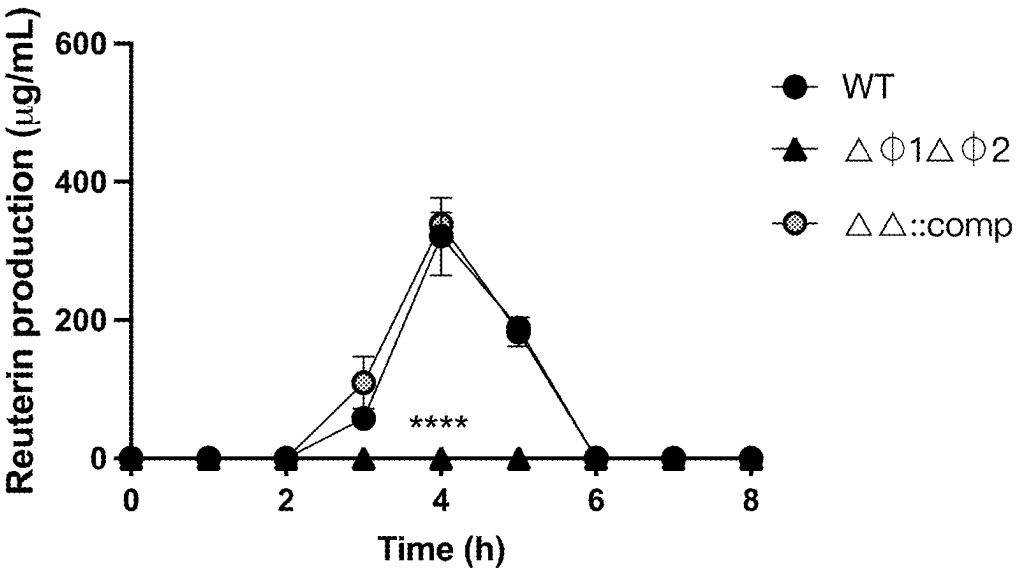

Reuterin production (μg/mL)
600
400
200
0
0
2
4
6
8
Time (h)
****
WT
△Φ1△Φ2
△△::comp

FIG. 5A

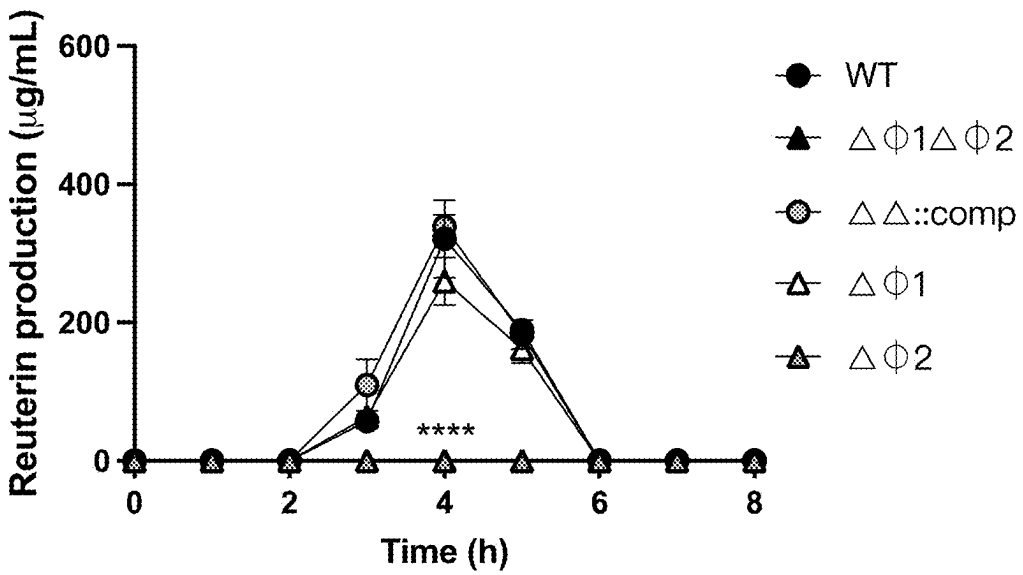

Reuterin production (μg/mL)
600
400
200
0
0
2
4
6
8
Time (h)
****
WT
△Φ1△Φ2
△△::comp
△Φ1
△Φ2

FIG. 5B

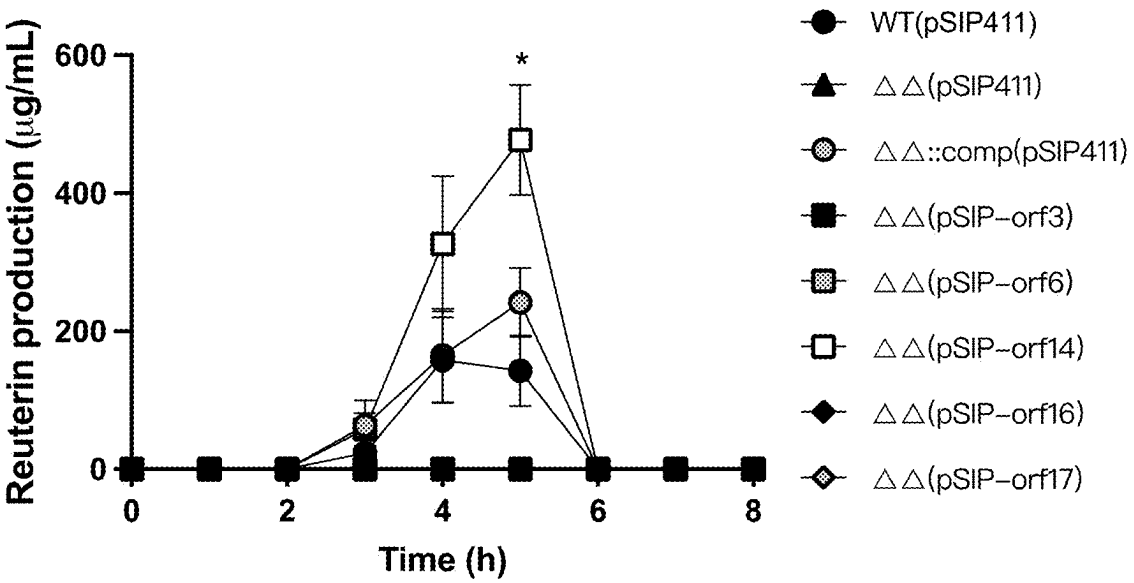

Reuterin production (μg/mL)
600
400
200
0
0
2
4
6
8
Time (h)
*
WT(pSIP411)
△△(pSIP411)
△△::comp(pSIP411)
△△(pSIP-orf3)
△△(pSIP-orf6)
△△(pSIP-orf14)
△△(pSIP-orf16)
△△(pSIP-orf17)

FIG. 5C

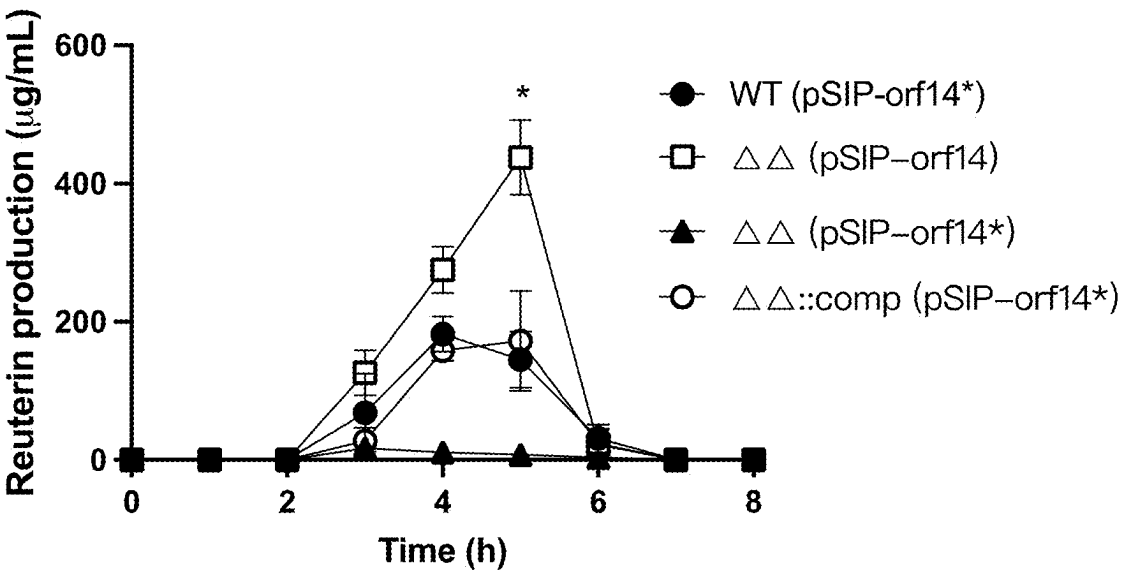

Reuterin production (μg/mL)
600
400
200
0
0
2
4
6
8
Time (h)
*
WT (pSIP-orf14*)
△△ (pSIP-orf14)
△△ (pSIP-orf14*)
△△::comp (pSIP-orf14*)

FIG. 5D

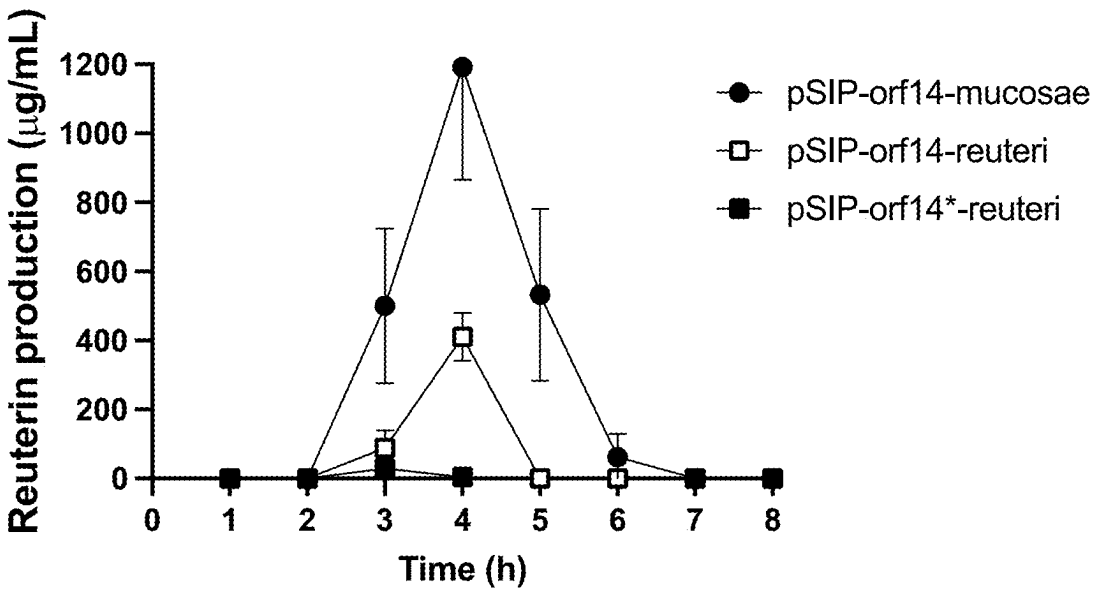

Reuterin production (μg/mL)
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
Time (h)
pSIP-orf14-mucosae
pSIP-orf14-reuteri
pSIP-orf14*-reuteri

FIG. 5E

MICROORGANISMS AND METHODS FOR PRODUCING REUTERIN

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0001] This invention was made with government support under AT011202 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted in XML format and is hereby incorporated by reference in its entirety. The XML copy, created on May 28, 2024, is named USPTO--240528--09824525-P230254US02--APP--SEQ_LIST.xml and is 34,709 bytes in size.

FIELD OF THE INVENTION

[0003] The invention is directed to microorganisms and methods for producing reuterin, specifically, by expressing a phage protein in microorganisms.

BACKGROUND

[0004] Reuterin is an important industrial chemical that is produced by a number of microorganisms during fermentation of glycerol. Reuterin has broad-spectrum antimicrobial properties (Vollenweider et al. 2004) and contributes to the probiosis of a number of probiotics such as *Limosilactobacillus reuteri* by inhibiting gut invaders (Casas et al. 2000). In addition, reuterin is widely used as a food preservative (Schaefer et al. 2010) and an auxiliary therapeutic agent (De Weirdt et al. 2012) and is also an important precursor for several industrial chemicals (Stevens et al. 2011). Previous research has optimized the fermentation conditions of microbes to increase the productivity (Mishra et al. 2018, Begunova et al. 2020). In addition, genes in the propanediol utilization (pdu) operon have been overexpressed to convert glycerol to reuterin more efficiently (Dishisha et al. 2014). However, strategies to further enhance the production of reuterin are needed.

SUMMARY OF THE INVENTION

[0005] One aspect of the invention is directed to recombinant microorganisms.

[0006] In some versions, the microorganisms comprise one or more modifications with respect to a corresponding microorganism not comprising the one or more modifications. In some versions, the one or more modifications comprise a recombinant orf14 gene encoding an Orf14 protein comprising an amino acid sequence of Orf14 of *Limosilactobacillus reuteri* (SEQ ID NO:2), Orf14 of Limosilactobacillus mucosae (SEQ ID NO:4), or Orf14 of *Limosilactobacillus oris* (SEQ ID NO: 6), or an amino acid sequence of a homolog of any of the foregoing.

[0007] In some versions, the Orf14 protein comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

[0008] In some versions, the Orf14 protein comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

[0009] In some versions, the recombinant orf14 gene comprises a promoter and a coding sequence, wherein the promoter is heterologous to the coding sequence.

[0010] In some versions, the recombinant microorganism is a bacterium.

[0011] In some versions, the recombinant microorganism is commensal to human gastrointestinal tract.

[0012] In some versions, the recombinant microorganism comprises a glycerol dehydratase. In some versions, the recombinant microorganism comprises a propanediol utilization gene set.

[0013] In some versions, the recombinant microorganism and the corresponding microorganism produce reuterin.

[0014] In some versions, the recombinant microorganism exhibits enhanced production of reuterin with respect to the corresponding microorganism.

[0015] In some versions, the recombinant microorganism is from a genus selected from the group consisting of *Anaerobutyricum, Bacillus*, Blautia *Citrobacter, Clostridium, Enterobacter, Enterococcus, Flavonifractor, Klebsiella, Lactobacillus, Limosilactobacillus, Listeria, Pediococcus, Salmonella, Streptococcus, Terrisporobacter*, and *Yersina*. In some versions, the recombinant microorganism is *L. reuteri.*

[0016] In some versions, the recombinant microorganism comprises an inactivation of at least one biologically active prophage present in the corresponding microorganism.

[0017] In some versions, the recombinant microorganism is *L. reuteri* and comprises an inactivation of at least one of Φ1 and Φ2.

[0018] In some versions, the recombinant microorganism comprises an inactivation of each biologically active prophage present in the corresponding microorganism.

[0019] In some versions, the recombinant microorganism is *L. reuteri* and comprises an inactivation of each of Φ1 and Φ2.

[0020] Another aspect of the invention is directed to methods of producing reuterin.

[0021] In some versions, the methods comprise culturing a recombinant microorganism of the invention thereby produce reuterin.

[0022] In some versions, the culturing is performed in vivo. In some versions, the culturing is performed in a gastrointestinal tract. Some versions further comprise introducing the recombinant microorganism to a (the) gastrointestinal tract. Some versions further comprise orally administering the recombinant microorganism.

[0023] In some versions, culturing is performed in vitro.

[0024] The objects and advantages of the invention will appear more fully from the following detailed description of the preferred embodiment of the invention made in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] FIG. **1**. Schematic representation of two prophage genomes: *Limosilactobacillus reuteri* 1 (LRΦ1) and Φ2 (LRΦ2) (Oh et al. 2019). Genes are coded based on their predicted functions.

[0026] FIG. **2**. Predicted protein structure of the *Limosilactobacillus reuteri* Orf14.

[0027] FIG. **3**. Schema of prophage deletion, showing location of oligonucleotides on the prophage and bacterial chromosome to screen for integrated or deleted prophages (Oh et al. 2019).

[0028] FIG. **4**. Schema of the pdu pathway in reuterin-producing microorganisms, showing the synthesis and excretion (production) of reuterin (3-HPA) through the action of glycerol dehydratase (GDH, PduCDE). Other enzymes in the pdu pathway include: propionaldehyde dehydrogenase (PduP, also referred to as CoA-acylating propionaldehyde dehydrogenase), which catalyzes the generation of 3-hydroxypropionyl-CoA (3-HP CoA) from reuterin; phosphate propanoyltransferase (PduL, also known as phosphotransacylase), which catalyzes the generation of 3-hydroxypropionyl phosphate (3-HP-P) from 3-HP CoA; propionate kinase (PduW), which catalyzes the generation of 3-hydroxypropionic acid (3-HP) from 3-HP-P, and 1,3-propanediol oxidoreductase (PduQ, 1,3-PD oxidoreductase), which catalyzes the generation of 1,3-propanediol (1,3-PD) from reuterin. See Chen et al. 2017, Sun et al. 2022, and Liang et al. 2021.

[0029] FIGS. **5**A-**5**E. Reuterin production of *L. reuteri* ATCC PTA-6475 and its variants during the growth. Overnight cells were inoculated to reach an initial OD 0.1 in mMRS supplemented with 100 mM glucose and 40 mM glycerol. Supernatant was collected every hour during the growth at 37° C. and the reuterin concentrations were determined. The number of cells of each strain at each time point was normalized to an OD of 3. Data are shown as means±standard deviations for three independent experiments. Statistical significance was determined by unpaired t-test. * $P<0.05$; **** $P<0.0001$. Data are shown as means±standard deviations for three independent experiments. In FIGS. **5**C and **5**D, "orf14" refers to orf14 from *L. reuteri*. In FIG. **5**E, the origins of the orf14 genes (*L. mucosae* or *L. reuteri*) are indicated.

DETAILED DESCRIPTION OF THE INVENTION

[0030] One aspect of the invention is directed to recombinant microorganisms. "Recombinant microorganism" refers to a microorganism that comprises a recombinant nucleic acid, a recombinant gene, or a recombinant polypeptide. A recombinant nucleic acid or polypeptide is one comprising a sequence that is not naturally occurring. A recombinant gene is a gene that comprises a recombinant nucleic acid sequence, is present within a microorganism in which it does not naturally occur, and/or is present at a locus (e.g., genetic locus or on an extrachromosomal plasmid) in which it does not naturally occur.

[0031] The recombinant microorganisms of the invention can comprise one or more modifications with respect to a corresponding microorganism not comprising the one or more modifications. "Corresponding microorganism" refers to a microorganism of the same species having the same or substantially same genetic and proteomic composition as a recombinant microorganism of the invention, with the exception of genetic and proteomic differences resulting from the modifications described herein for the recombinant microorganisms of the invention. The corresponding microorganism is typically a microorganism from which the recombinant microorganisms of the invention are derived and in which the modifications are introduced. In some versions, the corresponding microorganism is the native version of the recombinant microorganism of the invention, i.e., the unmodified microorganism as found in nature. The terms "microorganism" and "microbe" are used interchangeably herein.

[0032] The recombinant microorganisms of the invention preferably comprise a recombinant orf14 gene encoding an Orf14 protein comprising an amino acid sequence of Orf14 of *Limosilactobacillus reuteri* (SEQ ID NO:2), Orf14 of Limosilactobacillus mucosae (SEQ ID NO: 4), or Orf14 of *Limosilactobacillus oris* (SEQ ID NO:6), or an amino acid sequence of a homolog of any of the foregoing. As used herein, the designation "orf14" or "orf14 gene" refers to any gene encoding an Orf14 protein of the invention.

[0033] The *Limosilactobacillus reuteri* Orf14 protein is a protein encoded by 2 of *Limosilactobacillus reuteri* (Oh et al. 2019) (FIGS. **1** and **2**). The amino acid sequence of the *Limosilactobacillus reuteri* Orf14 protein is SEQ ID NO:2:

(SEQ ID NO: 2)

VIYMNKIKRLCKKYQQVIYTLFAVLVIISVVYFALHGFDSFVKWATTDVG

SVADWAGSIGTILAFVAVIWQQGRQENITRAVHIEESRPRFSLTYTPKPV

LKTRVLFWGNGRTAVNINEILGSRSAENYRFISIENISNNVVYDYSIILK

YHSNDNARVRKDYWSSHGLFPRRSVAIVPKFMGTDKDKIGNYIYDELLVK

FTTPANEVGFFISKNINNAQTDSSFGKSQYYFVRGSHVKRVTAINTDKMI

DVDSSICRKLDKEFDEIIGITSLGEINENGKLS

The coding sequence of the *Limosilactobacillus reuteri* Orf14 protein is SEQ ID NO:1:

(SEQ ID NO: 1)

GTGATATATATGAATAAAATAAAAAGGTTATGTAAAAAATACCAGCAAGT

AATTTATACACTATTTGCTGTCCTAGTTATTATAAGTGTTGTTTATTTTG

CTTTGCATGGGTTTGATTCATTTGTAAAGTGGGCAACAACTGATGTGGGG

TCTGTCGCTGATTGGGCTGGAAGTATTGGAACAATTCTTGCTTTTGTTGC

TGTTATATGGCAACAAGGGAGACAAGAAAACATTACAAGAGCTGTTCATA

TCGAAGAATCAAGGCCTAGATTTTCGCTTACATATACACCTAAGCCGGTG

TTGAAAACAAGAGTGTTGTTTTGGGGCAATGGTCGAACGGCAGTTAATAT

AAATGAAATTCTTGGAAGTAGGTCAGCTGAGAATTACAGATTTATTAGTA

TCGAAAATATTTCTAATAATGTAGTTTATGATTATTCCATAATATTAAAG

TATCACTCTAATGATAATGCAAGAGTTAGAAAAGATTATTGGTCAAGTCA

TGGATTATTTCCAAGAAGAAGTGTTGCGATTGTTCCTAAGTTTATGGGCA

CTGATAAAGATAAAATAGGAAATTACATATATGATGAATTACTTGTTAAA

TTTACAACTCCGGCTAATGAAGTAGGATTTTTTATATCGAAGAACATAAA

TAACGCTCAAACAGATTCTAGTTTTGGCAAAAGTCAGTATTATTTTGTAC

GAGGAAGCCACGTTAAGCGTGTAACGGCTATTAACACAGATAAAATGATT

GATGTTGATAGTTCCATTTGTAGGAAATTAGATAAAGAGTTTGACGAAAT

AATTGGTATAACAAGTTTAGGAGAAATAAATGAGAATGGAAAACTTTCTT

AA

[0034] The Limosilactobacillus mucosae and *Limosilactobacillus oris* Orf14 proteins are natural homologs of the *Limosilactobacillus reuteri* Orf14 protein. The amino acid sequence of the Limosilactobacillus mucosae Orf14 protein is SEQ ID NO:4:

(SEQ ID NO: 4)

MKRVKELCKKYQQVIYTLLAVVAIVGVVYFALHGFNSFIKWATTDVGSVA

DWAGSIGTILAFIAVIWQQGRQENITRAVHVEESRPRFSVTYTPKPQAKT

KMLFWGNNRTLSQINTMLNNRNGENYRFINIENISNNVVYDYSIILKYHF

YDNSLVRKDYWSSHGLFPRRSIIIVPKFLGTGKDKIGNYIYDELLIKFTT

PANEIGFFKMTNVNGNRTDSSFGDSQYYFVRGSHIKRVTAINTDEMIKAD

SPLCRKLDREFSETTGGTNFGAIGEDGKAY

The coding sequence of the Limosilactobacillus mucosae Orf14 protein is SEQ ID NO:3:

(SEQ ID NO: 3)

ATGAAAAGGGTAAAAGAATTATGTAAAAAATATCAGCAAGTAATTTACAC

TTTATTAGCTGTTGTCGCTATTGTAGGTGTTGTTTATTTTGCTTTGCATG

GATTTAATTCATTTATAAAATGGGCAACAACTGATGTAGGCTCTGTTGCT

GATTGGGCTGGGAGTATTGGAACTATCCTTGCTTTTATTGCTGTTATTTG

GCAACAAGGAAGACAAGAAAATATCACACGTGCTGTTCATGTTGAAGAAT

CAAGGCCTAGATTTTCTGTCACGTATACTCCCAAACCTCAAGCAAAAACA

AAAATGTTGTTTTGGGGCAACAATCGAACTTTAAGCCAAATAAATACCAT

GTTAAATAATAGAAATGGTGAAAACTATAGATTTATAAATATAGAAAATA

TTTCAAATAATGTTGTCTATGATTATTCAATAATATTAAAATACCATTTT

TATGATAATTCCTTAGTACGAAAGGACTATTGGTCAAGTCATGGATTGTT

CCCGAGAAGAAGTATCATTATTGTTCCAAAATTTTTAGGGACTGGCAAAG

ACAAGATAGGGAATTATATTTATGATGAACTACTTATTAAATTTACAACT

CCAGCTAATGAAATTGGCTTTTTCAAAATGACGAATGTTAATGGTAATCG

AACAGATTCTAGCTTTGGCGATAGTCAATATTATTTTGTCCGTGGAAGCC

ATATTAAACGAGTAACAGCAATTAATACTGATGAAATGATCAAAGCTGAT

AGTCCACTTTGTAGAAAATTAGATAGAGAATTTAGTGAAACAACCGGTGG

AACAAATTTTGGGGCAATAGGAGAAGATGGAAAAGCTTACTAA

The amino acid sequence of the *Limosilactobacillus oris* Orf14 protein is (SEQ ID NO:6):

(SEQ ID NO: 6)

MLNNIKSLYSKFKQFIQGALCIVVLIFGFILIKHITKEWTPFITWFHTDH

GSVADWAGSIGTIAAFLAVFWQVKKQGSIERAIDVERSRPRFSVLFSLTL

PKGTRVLYWNRTDNDANNIISNPEEYRFITIQNISSNVIYDFDVILRYHT

LDNSRSRNDFWSTTGVFPKKTVTFIPKFKGKNNDQQYVYDELLVKFTTPA

NEVGFFRMINVNDVKGDLGLGSGRYYFVKGTHIKGVKAINKDRMIKINSD

ECQRFDSLFEDFNYSTNTIEV

The coding sequence of the *Limosilactobacillus oris* Orf14 protein is (SEQ ID NO:5):

(SEQ ID NO: 5)

ATGTTGAATAATATAAAATCTTTATATTCCAAATTTAAGCAATTTATACA

AGGAGCTTTATGCATAGTTGTACTTATCTTTGGTTTTATTTTAATAAAGC

-continued

ATATAACAAAAGAATGGACACCATTTATTACATGGTTTCATACTGATCAT

GGAAGTGTTGCAGATTGGGCAGGGAGTATTGGAACTATTGCAGCGTTTTT

AGCTGTTTTTTGGCAAGTGAAAAAACAAGGAAGTATTGAAAGAGCTATTG

ATGTTGAAAGAAGCAGGCCTAGATTTTCAGTCTTATTTAGTTTAACTTTA

CCTAAAGGTACAAGAGTACTTTATTGGAATAGGACGGATAATGATGCAAA

TAATATAATTAGTAATCCTGAAGAATACCGATTTATTACGATTCAAAACA

TATCTTCAAATGTAATATATGATTTTGATGTGATTTTGAGATATCATACA

TTAGACAATTCACGTAGTCGTAATGACTTTTGGAGTACGACTGGTGTTTT

TCCAAAGAAAACTGTAACTTTTATACCAAAATTTAAAGGGAAAAATAATG

ACCAACAGTATGTGTATGATGAATTATTAGTAAAATTCACGACTCCAGCT

AATGAAGTTGGTTTCTTTCGGATGATTAATGTAAATGATGTAAAAGGTGA

TTTAGGCTTGGGAAGTGGAAGGTATTATTTTGTAAAGGGTACGCATATTA

AGGGAGTCAAGGCAATTAATAAGGATAGAATGATTAAGATTAATAGTGAT

GAATGCCAAAGATTTGATTCATTATTTGAAGACTTTAATTACTCAACAAA

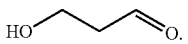
TACTATTGAGGTTTAG

[0035] Homologs of the *L. reuteri, L. mucosae*, and *L. oris* Orf14 proteins can include variants of these proteins comprising an amino acid sequence with at least 80%, 85%, 90%, 95%, 99%, or 100% sequence identity to SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Homologs of the *L. reuteri, L. mucosae*, and *L. oris* Orf14 proteins can also include any native homologs of *L. reuteri, L. mucosae*, and *L. oris* Orf14 proteins found in other organisms, as well as variants of such native homologs comprising an amino acid sequence with at least 80%, 85%, 90%, 95%, 99%, or 100% sequence identity thereto.

[0036] In some versions of the invention, the recombinant gene is configured to be overexpressed in the recombinant microorganism. If a corresponding microorganism endogenously comprises a native orf14 gene, the gene may be modified to exchange or optimize promoters, exchange or optimize enhancers, or exchange or optimize any other genetic element to result in increased expression of the gene. Alternatively, one or more additional copies of the orf14 gene or coding sequence thereof may be introduced to the cell for enhanced expression of the gene product. If a corresponding microorganism does not endogenously comprise a orf14 gene, the gene or coding sequence thereof may be recombinantly introduced for heterologous expression of the gene product. The orf14 gene or coding sequence may be incorporated into the genome of the recombinant microorganism or may be contained on an extra-chromosomal plasmid. The orf14 gene or coding sequence may be introduced to the recombinant microorganism individually or may be included on an operon. Techniques for genetic manipulation are described in further detail below. Accordingly, in some versions of the invention the recombinant orf14 gene comprises a promoter and a coding sequence, wherein the promoter is heterologous to the coding sequence.

[0037] In preferred versions of the invention, the recombinant orf14 gene confers enhanced synthesis, excretion, or

production of reuterin, such that the recombinant microorganism exhibits enhanced synthesis, excretion, or production of reuterin with respect to the corresponding microorganism. "Reuterin" used as herein refers to 3-hydroxypropanal, 3-hydroxypropionaldehyde:



Reuterin is generated in certain microorganisms through the metabolization of glycerol through the action of glycerol dehydratase (GDH, sometimes referred to as PduCDE), typically in the propanediol utilization (pdu) pathway (FIG. **4**, FIG. **2** of Chen et al. 2017 and throughout, Sun et al. 2022, and Liang et al. 2021). "Synthesis" (and grammatical variants thereof such as "synthesize") in this context refers to the generation of reuterin, whether as an intermediate in a metabolic pathway (such as the pdu pathway as described below) or as an end product. "Excretion" (and grammatical variants thereof such as "excrete") refers to the transport of synthesized reuterin from the interior of a cell to the exterior of the cell. "Production" in this context refers to the synthesis and excretion of reuterin. Methods for determining reuterin production levels are provided in the following examples.

[0038] In some versions of the invention, the recombinant microorganism of the invention comprises an inactivation of at least one biologically active prophage present in the corresponding microorganism. In some versions, of the invention, the recombinant microorganism comprises an inactivation of each biologically active lysogenic phage present in the corresponding microorganism. "Prophage" refers to the genetic material of a phage (e.g., bacteriophage) incorporated into the genome of a host organism (such as a recombinant and/or corresponding microorganism of the invention). The term "biologically active" refers to the status of the prophage such that it is able to produce phages if activated. "Inactivation" as used herein with respect to inactivating a biologically active prophage refers to a modification that prevents the ability to produce a phage. The modifications that inactivate a prophage can include genetic mutations to the prophage as it would otherwise exist in the corresponding microorganism or modifications to the non-prophage portions of the genome of the microorganism itself. Such mutations can include substitutions, deletions (whether partial or whole), insertions, etc., of the entire prophage, key genes thereof, or genes or sequences of non-prophage portions of the genome of the microorganism that are responsible for the expression of the prophage genes. The mutations can be introduced by homologous recombination (FIG. **3**) or any other method known in the art.

[0039] An exemplary microorganism of the invention, *L. reuteri*, has two biologically active prophages, Φ1 and Φ2 (Oh ct al. 2019) (FIG. **1**). In some versions of the invention, the recombinant microorganism is *L. reuteri* and comprises an inactivation of at least one of Φ1 and Φ2. In some versions of the invention, the recombinant microorganism is *L. reuteri* and comprises an inactivation of Φ1. In some versions of the invention, the recombinant microorganism is an *L. reuteri* and comprises an inactivation of Φ2. In some versions of the invention, the recombinant microorganism is an *L. reuteri* and comprises an inactivation of Φ1 and Φ2. Sec FIG. **3**, Oh et al. 2019, and the following examples for exemplary methods of inactivating these prophages.

[0040] The recombinant and/or corresponding microorganisms of the invention can comprise bacteria. Bacteria of the invention can include certain commensal or probiotic bacteria, non-commensal bacteria, and other types of bacteria. The bacteria can include non-pathogenic, Gram-positive bacteria capable of anaerobic growth. The bacteria in some cases are viable in the gastrointestinal tract of mammals. The bacteria can be food grade.

[0041] Exemplary recombinant and/or corresponding microorganisms of the invention include commensal microorganisms. The commensal microorganisms may be commensal to humans, such as commensal to the human gastrointestinal tract. The commensal microorganisms may comprise commensal bacteria. Exemplary commensal microorganisms to the human gastrointestinal tract include species of the genus *Achromobacter*, such as *Achromobacter* spp.; species of the genus *Acidaminococcus*, such as *Acidaminococcus fermentans*; species of the genus *Acinetobacter*, such as *Acinetobacter calcoaceticus*; species of the genus *Actinomyces*, such as *Actinomyces viscosus* and *Actinomyces naeslundii*; species of the genus *Aeromonas*; species of the genus *Aggregatibacter*, such as *Aggregatibacter actinomycetemcomitans*; species of the genus *Anaerobiospirillum*; species of the genus, *Anaerobutyricum*, such as *Anaerobutyricum hallii*; species of the genus *Alcaligenes*, such as *Alcaligenes faecalis*; species of the genus *Arachnia*, such as *Arachnia propionica*; species of the genus *Bacillus*; species of the genus *Bacteroides*, such as *Bacteroides gingivalis, Bacteroides fragilis, Bacteroides intermedius, Bacteroides melaninogenicus*, and *Bacteroides pneumosintes*; species of the genus *Bacterionema*, such as *Bacterionema matruchotii*; species of the genus *Blautia*, such as *Blautia obeum*; species of the genus *Corynebacterium*, such as *Corynebacterium matruchotii*; species of the genus *Bifidobacterium*; species of the genus *Buchnera*, such as *Buchnera aphidicola*; species of the genus *Butyriviberio*, such as *Butyriviberio fibrosolvens*; species of the genus *Campylobacter*, such as *Campylobacter coli, Campylobacter sputorum*, and *Campylobacter upsaliensis*; species of the genus *Capnocytophaga*; species of the genus *Clostridium*, such as *Clostridium difficile* and *Clostridium sordellii*; species of the genus *Citrobacter*, such as *Citrobacter freundii*; species of the genus *Corynebacterium*; species of the genus *Cutibacterium*, such as *Cutibacterium acnes*; species of the genus *Eikenella*, such as *Eikenella corrodens*; species of the genus *Enterobacter*, such as *Enterobacter cloacae*; species of the genus *Enterococcus*, such as *Enterococcus faecalis* and *Enterococcus faecium*; species of the genus *Escherichia*, such as *Escherichia coli*; species of the genus *Eubacterium*; species of the genus *Faecalibacterium*; species of the genus *Flavobacterium*; species of the genus *Flavonifractor*, such as *Flavonifractor plautii*; species of the genus *Fusobacterium*, such as *Fusobacterium nucleatum*; species of the genus *Haemophilus*, such as *Haemophilus parainfluenzae* and *Haemophilus* parahphrophilus; species of the genus *Lactobacillus*; species of the genus Leptotrichia, such as Leptotrichia *buccalis*; species of the genus *Methanobrevibacter*, such as *Methanobrevibacter smithii*; species of the genus *Morganella*, such as *Morganella morganii*; species of the genus *Mycobacteria*; species of the genus *Mycoplasma*; species of the genus *Micrococcus*; species of the genus *Mycobacterium*, such as

Mycobacterium chelonae; species of the genus *Neisseria*, such as *Neisseria* sicca; species of the genus Peptococcus; species of the genus *Peptostreptococcus*; species of the genus *Plesiomonas*, such as *Plesiomonas shigelloides*; species of the genus *Porphyromonas*, such as *Porphyromonas gingivalis*; species of the genus *Propionibacterium*; species of the genus *Providencia*; species of the genus *Pseudomonas*, such as *Pseudomonas aeruginosa*; species of the genus *Roseburia*; species of the genus *Rothia*, such as *Rothia dentocariosa*; species of the genus *Ruminococcus*, such as *Ruminococcus bromii*; species of the genus Sarcina; species of the genus *Staphylococcus*, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*; species of the genus *Streptococcus*, such as *Streptococcus anginosus, Streptococcus mutans, Streptococcus oralis, Streptococcus pneumoniae, Streptococcus sobrinus*, and *Streptococcus viridans*; species of the genus *Torulopsis*, such as *Torulopsis glabrata*; species of the genus *Treponema*, such as *Treponema denticola* and *Treponema refringens*; species of the genus *Veillonella*; species of the genus *Vibrio*, such as *Vibrio* sputorum; species of the genus Wolinella, such as Wolinella *succinogenes*; and species of the genus *Yersinia*, such as *Yersinia enterocolitica.*

[0042] Exemplary recombinant and/or corresponding microorganisms of the invention include species of lactic acid bacteria (i.e., species of the order *Lactobacillus*), such as those from the genera *Lactobacillus, Limosilactobacillus, Leuconostoc, Pediococcus, Lactococcus, Streptococcus, Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Fructobacillus, Sporolactobacillus, Tetragenococcus, Vagococcus*, and *Weissella.*

[0043] Exemplary recombinant and/or corresponding microorganisms of the invention include species of the *Lactobacillus* genus. Exemplary species from the *Lactobacillus* genus include *L. acetototerans, L. acidifarinae, L. acidipiscis, L. acidophilus, L. agilis, L. algidus, L. atimentarius, L. amytolyticus, L. amylophilus, L. amylotrophicus, L. amylovorus, L. animatis, L. antri, L. apodemi, L. aviarius, L. bifermentans, L. brevis, L. buchneri, L. camelliae, L. casei, L. catenaformis, L. ceti, L. coleohominis, L. collinoides, L. composti, L. concavus, L. coryniformis, L. crispatus, L. crustorum, L. curvatus, L. delbrueckii* subsp. *delbrueckii, L. delbrueckii* subsp. *butgaricus, L. delbrueckii* subsp. *lactis, L. dextrinicus, L. diolivorans, L. equi, L. equigenerosi, L. farraginis, L. farciminis, L. fermentum, L. fornicalis, L. fructivorans, L. frumenti, L. fuchuensis, L. gallinarum, L. gasseri, L. gastricus, L. ghanensis, L. graminis, L. hammesii, L. hamsteri, L. harbinensis, L. hayakitensis, L. helveticus, L. hitgardii, L. homohiochii, L. iners, L. ingluviei, L. intestinalis, L. jensenii, L. johnsonii, L. katixensis, L. kefiranofaciens, L. kefiri, L. kimchii, L. kitasatonis, L. kunkeei, L. leichmannii, L. lindneri, L. malefermentans, L. mati, L. manihotivorans, L. mindensis, L. mucosae, L. murinus, L. nagelii, L. namurensis, L. nantensis, L. oligofermentans, L. oris, L. panis, L. pantheris, L. parabrevis, L. parabuchneri, L. paracollinoides, L. parafarraginis, L. parakefiri, L. paratimentarius, L. paraplantarum, L. pentosus, L. perolens, L. plantarum, L. pontis, L. psittaci, L. rennini, L. reuteri, L. rhamnosus, L. rimae, L. rogosae, L. rossiae, L. ruminis, L. saerimneri, L. sakei, L. salivarius, L. sanfranciscensis, L. satsumensis, L. secaliphilus, L. sharpeae, L. siliginis, L. spicheri, L. suebicus, L. thailandensis, L. ultunensis, L. vaccinostercus, L. vaginalis, L. versmoldensis, L. vini, L. vitulinus, L. zeae*, and *L. zymae.*

[0044] Exemplary recombinant and/or corresponding microorganisms of the invention include species of the Limosilactobacillus genus. Exemplary species from the Limosilactobacillus genus include *L. agrestis, L. albertensis, L. alvi, L. antri, L. balticus, L. caviae, L. coleohominis, L. equigenerosi, L. fastidiosus, L. fermentum, L. frumenti, L. gastricus, L. gorilla, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. portuensis, L. reuteri, L. rudii, L. secaliphilus, L. urinaemulieris*, and *L. vaginalis.*

[0045] Exemplary recombinant and/or corresponding microorganisms of the invention include species of *Bifidobacterium*. Exemplary species from the *Bifidobacterium* genus include *B. actinocoloniiforme, B. adolescentis, B. aemilianum, B. aerophilum, B. aesculapii, B. amazonense, B. angulatum, B. animalis, B. anseris, B. apousia, B. apri, B. aquikefiri, B. asteroides, B. avesanii, B. biavatii, B. bifidum, B. bohemicum, B. bombi, B. boum, B. breve, B. callimiconis, B. callitrichidarum, B. callitrichos, B. canis, B. castoris, B. catenulatum, B. catulorum, B. cebidarum, B. choerinum, B. choladohabitans, B. choloepi, B. colobi, B. commune, B. criceti, B. crudilactis, B. cuniculi, B. dentium, B. dolichotidis, B. eriksonii, B. erythrocebi, B. eulemuris, B. faecale, B. felsineum, B. gallicum, B. gallinarum, B. globosum, B. goeldii, B. hapali, B. indicum, B. italicum, B. jacchi, B. lemurum, B. leontopitheci, B. longum, B. magnum, B. margollesii, B. merycicum, B. miconis, B. miconisargentati, B. minimum, B. mongoliense, B. moraviense, B. moukalabense, B. myosotis, B. oedipodis, B. olomucense, B. panos, B. parmae, B. platyrrhinorum, B. pluvialisilvae, B. polysaccharolyticum, B. pongonis, B. porcinum, B. primatium, B. pseudocatenulatum, B. pseudolongum, B. psychraerophilum, B. pullorum, B. ramosum, B. reuteri, B. rousetti, B. ruminale, B. ruminantium, B. saguini, B. saguinibicoloris, B. saimiriisciurei, B. samirii, B. santillanense, B. scaligerum, B. scardovii, B. simiarum, B. simiiventris, B. stellenboschense, B. subtile, B. thermacidophilum, B. thermophilum corrig., B. tibiigranuli, B. tissieri corrig., B. tsurumiense, B. urinalis, B. vansinderenii, B. vespertilionis*, and *B. xylocopae.*

[0046] The recombinant and/or corresponding microorganisms of the invention may comprise microorganisms comprising a glycerol dehydratase (GDH). Glycerol dehydratase belongs to the family of lyases, specifically the hydro-lyases, which cleave carbon-oxygen bonds. The systematic name of this enzyme class is glycerol hydro-lyase (3-hydroxypropanol-forming). Other names in common use include glycerol dehydrase, and glycerol hydro-lyase. Glycerol dehydratase hydrolyzes the chemical reaction (EC 4.2.1.30): glycerol$\leftrightharpoons$3-hydroxypropanal+H_2O. The glycerol dehydratase may be native to the corresponding microorganism or may be engineered into the corresponding and/or recombinant microorganism. The glycerol dehydratase in some microorganisms, including *L. reuteri*, is PduCDE, which is encoded by pduCDE. The pduCDE genes are often included in the propanediol utilization (pdu) gene cluster (FIGS. **4** and **5**), as discussed in further detail below. A number of microorganisms are known to express PduCDE or homologs thereof, including species of *Citrobacter*, such as *Citrobacter freundii*; species of *Yersinia*, such as *Yersinia enterocolitica*; species of *Klebsiella*, such as *Klebsiella oxytoca* and *Klebsiella pneumoniae*, species of *Salmonella*, such as *Salmonella enterica*, species of *Terrisporobacter*, such as *Terrisporobacter glycolicus*; species of *Clotridium*, such as *Clostridium perfringens, Clotridium carboxidiv-*

orans, and *Clotridium pasteuranium*; species of *Lactobacillus*, such as *Lactobacillus reuteri, Lactobacillus brevis*, and *Lactobacillus collinoides*; species of *Streptococcus*, such as *Streptococcus sanguinis*; species of *Enterococcus*, such as *Enterococcus malodoratus*; and species of *Listeria*, such as *Listeria monocytogenes*; among others. Scc, e.g., FIG. **2** of Chen et al. 2017, Chen et al. 2017 at large, and O'Brien et al. 2004.

[0047] The recombinant and/or corresponding microorganisms of the invention may include microorganisms comprising a propanediol utilization gene set. The propanediol utilization (pdu) gene set is a group of genes present in the propanediol utilization (pdu) gene cluster. The pdu gene cluster is a cluster of genes appearing a large number of microorganisms that carry out a metabolic pathway called the propanediol utilization (pdu) pathway (Chen et al. 2017, Dishisha et al. 2014, Chen et al. 2016) (FIG. **4**). The pdu pathway catalyzes dehydration of glycerol to reuterin using glycerol dehydratase (e.g., PduCDE), and further branches to 1,3-propanediol through 1,3-propanediol oxidoreductase (e.g., PduQ), and to 3-hydroxypropionic acid via a series of reactions catalyzed by coenzyme-A acylating propionaldehyde dehydrogenase (e.g., PduP), phosphotransacylasc (e.g., PduL) and propionate kinase (e.g., PduW) (FIG. **4**). The pdu gene cluster in some cases can further encode enzymes used to convert 1,2-propanediol to propionic acid and propanol (Bobik et al. 1997). The pdu gene cluster in some cases can further encode structural proteins making up a microcompartment housing the pathway (Sriramulu et al. 2008). For the purposes herein, the phrase "propanediol utilization (pdu) gene set" refers minimally to a set of genes encoding glycerol dehydratase (e.g., PduCDE), 1,3-propanediol oxidoreductase (e.g., PduQ), coenzyme-A acylating propionaldehyde dehydrogenase (e.g., PduP), phosphotransacylasc (e.g., PduL), and propionate kinase (e.g., PduW). In addition to these, the pdu gene set in some embodiments may further include genes encoding any one or more of PduA, PduB, PduF, PduG, PduH, PduK, PduJ, PduM, PduN, PduO, PduS, PduU, PduV, or any other pdu gene shown in FIG. **2** of Chen et al. 2017 or otherwise described therein, Dishisha et al. 2014, or Chen et al. 2016, or any homologs thereof. A number of microorganisms are known to express pdu gene clusters, including species of *Citrobacter*, such as *Citrobacter freundii*; species of *Yersinia*, such as *Yersinia enterocolitica*; species of *Klebsiella*, such as *Klebsiella oxytoca* and *Klebsiella pneumoniae*, species of *Salmonella*, such as *Salmonella enterica*, species of *Terrisporobacter*, such as *Terrisporobacter glycolicus*; species of *Clotridium*, such as *Clostridium perfringens, Clotridium carboxidivorans*, and *Clotridium pasteuranium*; species of *Lactobacillus*, such as *Lactobacillus reuteri, Lactobacillus brevis*, and *Lactobacillus collinoides*; species of *Streptococcus*, such as *Streptococcus sanguinis*; species of *Enterococcus*, such as *Enterococcus malodoratus*; and species of *Listeria*, such as *Listeria monocytogenes*; among others. Sec, e.g., FIG. **2** of Chen et al. 2017, Chen et al. 2017 at large, and O'Brien et al. 2004.

[0048] In some versions of the invention, recombinant and/or corresponding microorganisms of the invention include microorganisms that synthesize, extracellularly excrete, or produce reuterin. A number of microorganisms are known to produce reuterin natively, including species of *Bacillus, Citrobacter, Clostridium, Enterobacter, Enterococcus, Klebsiella, Lactobacillus, Limosilactobacillus, Listeria, Pediococcus, Salmonella, Streptococcus, Terrisporobacter*, and *Yersina*. See Chen et al. 2017, Nakanishi et al. 2002, Sun et al. 2022, Talarico et al. 1988, El-Ziney 1998, and Vollenweider et al. 2004.

[0049] In some versions of the invention, recombinant and/or corresponding microorganisms of the invention include microorganisms from a genus selected from the group consisting of *Anaerobutyricum, Bacillus, Blautia Citrobacter, Clostridium, Enterobacter, Enterococcus, Flavonifractor, Klebsiella, Lactobacillus, Limosilactobacillus, Listeria, Pediococcus, Salmonella, Streptococcus, Terrisporobacter*, and *Yersina*.

[0050] A recombinant and corresponding microorganism used in the following examples is *L. reuteri* (*Limosilactobacillus reuteri* formerly referred to as *Lactobacillus reuteri*). In addition to *L. reuteri*, other particularly preferred bacteria include *L. plantarum* (e.g., *L. plantarum* BAA-793), *L. rhamnosus* (e.g., *L. rhamnosus* ATCC 53103)), *L. lactis* (e.g., *L. lactis* MG1363), and *L. casei*. For the purposes herein, the "L." in the designation "*L. reuteri*" can refers either to *Limosilactobacillus* or *Lactobacillus*.

[0051] The invention is also directed to methods of synthesizing, excreting, or producing reuterin. The methods can comprise culturing a recombinant microorganism of the invention to thereby synthesize, excrete, or produce reuterin. As used herein, "culturing" refers to the maintenance of the recombinant microorganisms of the invention in conditions suitable for growth.

[0052] In some versions, the culturing is performed in vitro. The in vitro culturing can be performed in liquid culture medium or a solid-phase culture medium (e.g., agar). In some versions, the methods can also comprise isolating the synthesized, excreted, or produced reuterin from the recombinant microorganism. Methods of isolating the synthesized reuterin from the recombinant microorganism can comprise lysing the recombinant microorganism and, optionally, isolating the reuterin from the lysate. Methods of isolating the excreted or produced reuterin from the recombinant microorganism can comprise separating the excreted or produced reuterin from the recombinant microorganism.

[0053] In some versions, the culturing is performed in vivo. In some versions, the culturing is performed in a gastrointestinal tract. In some versions, the culturing is performed in a stomach and/or intestines, such as the small intestines. Some versions of the invention can comprise introducing the recombinant microorganism to the gastrointestinal tract. The recombinant microorganism can be introduced to the gastrointestinal tract by any method known in the art. The recombinant microorganism can be administered orally, rectally, or directly into the gastrointestinal tract via a stoma. The recombinant microorganism is preferably administered directly into or upstream of the small intestines, so that the recombinant microorganism ultimately passes through or into the small intestines. The recombinant microorganism can be swallowed or introduced via a tube. The recombinant microorganism can be combined in a composition with a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other material well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the recombinant microorganism. The precise nature of the carrier or other material may depend on the route of administration. The composition can be liquid, solid, or semi-solid. The composition can comprise a foodstuff or can take the form of a

pharmaceutical composition. Those of relevant skill in the art are well able to prepare suitable compositions.

[0054] Proteins and/or protein sequences are "homologous" when they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Nucleic acid or gene product (amino acid) sequences of any known gene, including the genes or gene products described herein, can be determined by searching any sequence databases known the art using the gene name or accession number a as search term. Common sequence databases include GenBank (www.ncbi.nlm.nih.gov/genbank/), ExPASy (cxpasy.org), KEGG (www.genomc.jp/kegg/), among others. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity (e.g., identity) over 50, 100, 150 or more residues (nucleotides or amino acids) is routinely used to establish homology (e.g., over the full length of the two sequences to be compared). Higher levels of sequence similarity (e.g., identity), e.g., 30%, 35% 40%, 45% 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% or more, can also be used to establish homology. Accordingly, homologs of the genes or gene products described herein include genes or gene products having at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity to the genes or gene products described herein. Methods for determining sequence similarity percentages (e.g., BLASTP and BLASTN using default parameters) are described herein and are generally available. The homologous proteins should demonstrate comparable activities and, if an enzyme, participate in the same or analogous pathways. "Orthologs" are genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same or similar function in the course of evolution. As used herein "orthologs" are included in the term "homologs". For sequence comparison and homology determination, one sequence typically acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence based on the designated program parameters. A typical reference sequence of the invention is a nucleic acid or amino acid sequence corresponding to acsA or other genes or products described herein.

[0055] Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2008)).

[0056] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity for purposes of defining homologs is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=−4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89: 10915).

[0057] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. The above-described techniques are useful in identifying homologous sequences for use in the methods described herein.

[0058] The terms "identical" or "percent identity", in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described above (or other algorithms available to persons of skill) or by visual inspection.

[0059] The phrase "substantially identical" in the context of two nucleic acids or polypeptides refers to two or more sequences or subsequences that have at least about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90, about 95%, about 98%, or about 99% or more nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. Such "substantially identical" sequences are typically considered to be "homologous", without reference to actual ancestry. Preferably, the "substantial identity" exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably, the sequences are substantially identical over at least about 150 residues, at least about 250 residues, or over the full length of the two sequences to be compared.

[0060] Terms used herein pertaining to genetic manipulation are defined as follows.

[0061] Deletion: The removal of one or more nucleotides from a nucleic acid molecule or one or more amino acids from a protein, the regions on either side being joined together.

[0062] Derived: When used with reference to a nucleic acid or protein, "derived" means that the nucleic acid or polypeptide is isolated from a described source or is at least 70%, 80%, 90%, 95%, 99%, or more identical to a nucleic acid or polypeptide included in the described source.

[0063] Endogenous: An endogenous nucleic acid, gene, gene element (e.g., promoter, enhancer, coding sequence), polypeptide, sequence or any other element in a given cell is one that is naturally occurring in the given cell.

[0064] Exogenous: An exogenous nucleic acid, gene, gene element (e.g., promoter, enhancer, coding sequence), polypeptide, sequence or any other element in a given cell is one that is not naturally occurring in the given cell. The term "heterologous" is used herein interchangeably with "exogenous."

[0065] Expression: The process by which a gene's coded information is converted into the structures and functions of a cell, such as a protein, transfer RNA, or ribosomal RNA. Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated into protein (for example, transfer and ribosomal RNAs).

[0066] Gene: "Gene" refers minimally to a coding sequence and a promoter operationally linked to the coding sequence. A gene may additionally include other elements, such as enhancers and silencers.

[0067] Introduce: When used with reference to genetic material, such as a nucleic acid, and a cell, "introduce" refers to the delivery of the genetic material to the cell in a manner such that the genetic material is capable of being expressed within the cell. Introduction of genetic material includes both transformation and transfection. Transformation encompasses techniques by which a nucleic acid molecule can be introduced into cells such as prokaryotic cells or non-animal eukaryotic cells. Transfection encompasses techniques by which a nucleic acid molecule can be introduced into cells such as animal cells. These techniques include but are not limited to introduction of a nucleic acid via conjugation, electroporation, lipofection, infection, and particle gun acceleration.

[0068] Isolated: An "isolated" biological component (such as a nucleic acid molecule, polypeptide, or cell) has been substantially separated or purified away from other biological components in its original form, such as its native form or the form in which it was originally produced.

[0069] Nucleic acid: Encompasses both RNA and DNA molecules including, without limitation, cDNA, genomic DNA, and mRNA. Nucleic acids also include synthetic nucleic acid molecules, such as those that are chemically synthesized or recombinantly produced. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid molecule can be the sense strand, the antisense strand, or both. In addition, the nucleic acid can be circular or linear.

[0070] Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. An origin of replication is operably linked to a coding sequence if the origin of replication controls the replication or copy number of the nucleic acid in the cell. Operably linked nucleic acids may or may not be contiguous.

[0071] Operon: Configurations of separate genes that are transcribed in tandem as a single messenger RNA are denoted as operons. Thus, a set of in-frame genes in close proximity under the transcriptional regulation of a single promoter constitutes an operon. Operons may be synthetically generated using the methods described herein.

[0072] Overexpress: When a gene is caused to be transcribed at an elevated rate compared to the endogenous or basal transcription rate for that gene. In some examples, overexpression additionally includes an elevated rate of translation of the gene compared to the endogenous translation rate for that gene. Methods of testing for overexpression are well known in the art, for example transcribed RNA levels can be assessed using rtPCR and protein levels can be assessed using SDS page gel analysis.

[0073] Vector or expression vector: An entity comprising a nucleic acid molecule that is capable of introducing the nucleic acid, or being introduced with the nucleic acid, into a cell for expression of the nucleic acid. A vector can include nucleic acid sequences that permit it to replicate in the cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements known in the art. Examples of suitable vectors are found below.

[0074] Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below.

[0075] Exogenous nucleic acids can be introduced stably or transiently into a cell using techniques well known in the art, including electroporation, calcium phosphate precipitation, DEAE-dextran mediated transfection, liposome-mediated transfection, conjugation, transduction, and the like. For stable transformation, a nucleic acid can further include a selectable marker. Suitable selectable markers include antibiotic resistance genes that confer, for example, resistance to

neomycin, tetracycline, chloramphenicol, or kanamycin, genes that complement auxotrophic deficiencies, and the like. (See below for more detail.)

[0076] Various embodiments of the invention use an expression vector that includes a heterologous nucleic acid encoding a protein. Suitable expression vectors include, but are not limited to viral vectors, such as baculovirus vectors or those based on vaccinia virus, polio virus, adenovirus, adeno-associated virus, SV40, herpes simplex virus, and the like; phage vectors, such as bacteriophage vectors; plasmids; phagemids; cosmids; fosmids; bacterial artificial chromosomes; Pl-based artificial chromosomes; yeast plasmids; yeast artificial chromosomes; and any other vectors specific for cells of interest.

[0077] Useful vectors can include one or more selectable marker genes to provide a phenotypic trait for selection of transformed cells. The selectable marker gene encodes a protein necessary for the survival or growth of transformed cells grown in a selective culture medium. Cells not transformed with the vector containing the selectable marker gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli. In alternative embodiments, the selectable marker gene is one that encodes dihydrofolate reductase or confers neomycin resistance (for use in eukaryotic cell culture), or one that confers tetracycline or ampicillin resistance (for use in a prokaryotic cell, such as *E. coli*).

[0078] The coding sequence in the expression vector is operably linked to an appropriate expression control sequence (promoters, enhancers, and the like) to direct synthesis of the encoded gene product. Such promoters can be derived from microbial or viral sources, including CMV and SV40. Depending on the cell/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. can be used in the expression vector (see e.g., Bitter et al. (1987) *Methods in Enzymology,* 153:516-544).

[0079] Suitable promoters for use in prokaryotic cells include but are not limited to: promoters capable of recognizing the T4, T3, Sp6, and T7 polymerases; the P_R and P_L promoters of bacteriophage lambda; the trp, recA, heat shock, and lacZ promoters of *E. coli*; the alpha-amylase and the sigma-specific promoters of *B. subtilis*; the promoters of the bacteriophages of *Bacillus; Streptomyces* promoters; the int promoter of bacteriophage lambda; the bla promoter of the beta-lactamase gene of pBR322; and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol.* 1:277 (1987); Watson et al, Molecular Biology of the Gene, 4th Ed., Benjamin Cummins (1987); and Sambrook et al., In: *Molecular Cloning: A Laboratory Manual,* 3rd ed., Cold Spring Harbor Laboratory Press (2001).

[0080] Non-limiting examples of suitable promoters for use within a eukaryotic cell are typically viral in origin and include the promoter of the mouse metallothionein I gene (Hamer et al. (1982) *J. Mol. Appl. Gen.* 1:273); the TK promoter of Herpes virus (McKnight (1982) *Cell* 31:355); the SV40 early promoter (Benoist et al. (1981) *Nature* (London) 290:304); the Rous sarcoma virus promoter; the cytomegalovirus promoter (Foccking et al. (1980) Gene 45:101); the yeast gal4 gene promoter (Johnston et al. (1982) PNAS (USA) 79:6971; Silver et al. (1984) *PNAS* (USA) 81:5951); and the IgG promoter (Orlandi et al. (1989) PNAS (USA) 86:3833).

[0081] Coding sequences can be operably linked to an inducible promoter. Inducible promoters are those wherein addition of an effector induces expression. Suitable effectors include proteins, metabolites, chemicals, or culture conditions capable of inducing expression. Suitable inducible promoters include but are not limited to the lac promoter (regulated by IPTG or analogs thereof), the lacUV5 promoter (regulated by IPTG or analogs thereof), the tac promoter (regulated by IPTG or analogs thereof), the tre promoter (regulated by IPTG or analogs thereof), the ara-BAD promoter (regulated by L-arabinose), the phoA promoter (regulated by phosphate starvation), the recA promoter (regulated by nalidixic acid), the proU promoter (regulated by osmolarity changes), the cst-/promoter (regulated by glucose starvation), the tetA promoter (regulated by tetracycline), the cadA promoter (regulated by pH), the nar promoter (regulated by anaerobic conditions), the pr. promoter (regulated by thermal shift), the cspA promoter (regulated by thermal shift), the T7 promoter (regulated by thermal shift), the T7-lac promoter (regulated by IPTG), the T3-lac promoter (regulated by IPTG), the T5-lac promoter (regulated by IPTG), the T4 gene 32 promoter (regulated by T4 infection), the nprM-lac promoter (regulated by IPTG), the VHb promoter (regulated by oxygen), the metallothionein promoter (regulated by heavy metals), the MMTV promoter (regulated by steroids such as dexamethasone) and variants thereof.

[0082] Alternatively, a coding sequence can be operably linked to a repressible promoter. Repressible promoters are those wherein addition of an effector represses expression. Examples of repressible promoters include but are not limited to the trp promoter (regulated by tryptophan); tetracycline-repressible promoters, such as those employed in the "TET-OFF"-brand system (Clontech, Mountain View, CA); and variants thereof.

[0083] In some versions, the cell is genetically modified with a heterologous nucleic acid encoding a gene product that is operably linked to a constitutive promoter. Suitable constitutive promoters are known in the art and include constitutive adenovirus major late promoter, a constitutive MPSV promoter, and a constitutive CMV promoter.

[0084] The relative strengths of the promoters described herein are well-known in the art.

[0085] In some versions, the cell is genetically modified with an exogenous nucleic acid encoding a single protein. In other embodiments, a modified cell is one that is genetically modified with exogenous nucleic acids encoding two or more proteins. Where the cell is genetically modified to express two or more proteins, those nucleic acids can each be contained in a single or in separate expression vectors. When the nucleic acids are contained in a single expression vector, the nucleotide sequences may be operably linked to a common control element (e.g., a promoter), that is, the common control element controls expression of all of the coding sequences in the single expression vector.

[0086] When the cell is genetically modified with heterologous nucleic acids encoding two or more proteins, one of the nucleic acids can be operably linked to an inducible promoter, and one or more of the nucleic acids can be

operably linked to a constitutive promoter. Alternatively, all can be operably linked to inducible promoters or all can be operably linked to constitutive promoters.

[0087] Nucleic acids encoding enzymes desired to be expressed in a cell may be codon-optimized for that particular type of cell. Codon optimization can be performed for any nucleic acid by "OPTIMUMGENE"-brand gene design system by GenScript (Piscataway, NJ).

[0088] The introduction of a vector into a bacterial cell may be performed by protoplast transformation (Chang and Cohen (1979) *Molecular General Genetics,* 168:111-115), using competent cells (Young and Spizizen (1961) *Journal of Bacteriology,* 81:823-829; Dubnau and Davidoff-Abelson (1971) *Journal of Molecular Biology,* 56:209-221), electroporation (Shigekawa and Dower (1988) *Biotechniques,* 6:742-751), or conjugation (Kochler and Thorne (1987) *Journal of Bacteriology,* 169:5771-5278). Commercially available vectors for expressing heterologous proteins in bacterial cells include but are not limited to pZERO, pTrc99A, pUC19, pUC18, pKK223-3, pEX1, pCAL, pET, pSPUTK, pTrxFus, pFastBac, pThioHis, pTrcHis, pTrcHis2, and pLEx, in addition to those described in the following Examples.

[0089] Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides therefrom are disclosed by Clontech Laboratories, Inc., Palo Alto, Calif., USA (in the product protocol for the "YEASTMAKER"-brand yeast transformation system kit); Reeves et al. (1992) *FEMS Microbiology Letters* 99:193-198; Manivasakam and Schiestl (1993) *Nucleic Acids Research* 21 (18): 4414-5; and Ganeva et al. (1994) *FEMS Microbiology Letters* 121:159-64. Expression and transformation vectors for transformation into many yeast strains are available. For example, expression vectors have been developed for the following yeasts: *Candida albicans* (Kurtz, et al. (1986) *Mol. Cell. Biol.* 6:142); *Candida maltosa* (Kunze et al. (1985) *J. Basic Microbiol.* 25:141); *Hansenula polymorpha* (Gleeson et al. (1986) *J. Gen. Microbiol.* 132:3459) and Roggenkamp et al. (1986) *Mol. Gen. Genet.* 202:302); *Kluyveromyces fragilis* (Das et al. (1984) *J. Bacteriol.* 158:1165); *Kluyveromyces lactis* (De Louvencourt et al. (1983) *J. Bacteriol.* 154:737) and Van den Berg et al. (1990) Bio/Technology 8:135); *Pichia* quillerimondii (Kunze et al. (1985) *J. Basic Microbiol.* 25:141); *Pichia pastoris* (Cregg et al. (1985) *Mol. Cell. Biol.* 5:3376; U.S. Pat. Nos. 4,837, 148; 4,929,555); *Saccharomyces cerevisiae* (Hinnen et al. (1978) *Proc. Natl. Acad. Sci. USA* 75:1929 and Ito et al. (1983) *J. Bacteriol.* 153:163); *Schizosaccharomyces pombe* (Beach et al. (1981) Nature 300:706); and *Yarrowia lipolytica* (Davidow et al. (1985) *Curr. Genet.* 10:380-471 and Gaillardin et al. (1985) *Curr. Genet.* 10:49). *Limosilactobacillus reuteri, Limosilactobacillus mucosae*, and *Limosilactobacillus oris* are also known as *Lactobacillus reuteri, Lactobacillus mucosae*, and *Lactobacillus oris*, respectively.

[0090] The elements and method steps described herein can be used in any combination whether explicitly described or not.

[0091] All combinations of method steps as used herein can be performed in any order, unless otherwise specified or clearly implied to the contrary by the context in which the referenced combination is made.

[0092] As used herein, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise.

[0093] Numerical ranges as used herein are intended to include every number and subset of numbers contained within that range, whether specifically disclosed or not. Further, these numerical ranges should be construed as providing support for a claim directed to any number or subset of numbers in that range. For example, a disclosure of from 1 to 10 should be construed as supporting a range of from 2 to 8, from 3 to 7, from 5 to 6, from 1 to 9, from 3.6 to 4.6, from 3.5 to 9.9, and so forth.

[0094] All patents, patent publications, and peer-reviewed publications (i.e., "references") cited herein are expressly incorporated by reference to the same extent as if each individual reference were specifically and individually indicated as being incorporated by reference. In case of conflict between the present disclosure and the incorporated references, the present disclosure controls.

[0095] It is understood that the invention is not confined to the particular construction and arrangement of parts herein illustrated and described, but embraces such modified forms thereof as come within the scope of the claims.

Examples

INTRODUCTION

[0096] The present examples show the enhancement of reuterin production in active cells by overexpressing the phage protein referred to herein as Orf14 in *Limosilactobacillus reuteri* as an exemplary microorganism.

Methods

Characterization and comparison of Φ1 and Φ2 of *L. reuteri* ATCC PTA-6475

[0097] The identification and comparison of prophages Φ1 and Φ2 in *L. reuteri* ATCC PTA-6475 are described in (Oh et al. 2019). Maps of the phages are shown in FIG. **1**.

Construction of Prophage Deletion Strains (LRΔΦ1, LRΔΦ2, and LRΔΦ1Φ2) and Restoring the Prophages in LRΔΦ1Φ2

[0098] *L. reuteri* strains including a deletion of Φ1 (LRΔΦ1), Φ2 (LRΔΦ2), or both Φ1 and Φ2 (LRΔΦ1Φ2) were generated as described in (Oh et al. 2019). See also FIG. **3**.

Cloning of Individual Φ2 Genes in LRΔ$14$2

[0099] Plasmid-based constructs containing genes unique to Φ2 (orf3, orf6, orf14 (SEQ ID NOS: 1-2), orf16, and orf17) were used to assess the impact of these genes on the reuterin production. By PCR (Phusion Hot Start polymerase II, Thermo Scientific) each gene was amplified. See Table 1 for primer pairs. A backbone was amplified with oligos oVPL399 and oVPL400 from pSIP411. Amplicons were fused to backbone by Ligase cycling reaction (de Kok et al. 2014) to generate pSIP-orf3, pSIP-orf6, pSIP-orf14, pSIP-orf16, and pSIP-orf17. After transferring plasmids into *Escherichia coli* EC1000, the integrity of the constructs was verified by Sanger sequencing. Confirmed plasmid constructs were transformed to LRΔΦ1ΔΦ2 using previously described protocols (Oh et al. 2019) and transformants were confirmed by PCR (Table 1) following by sanger sequencing. The original plasmid pSIP411 was transformed to LR wildtype and LRΔΦ1ΔΦ2, which served as controls.

TABLE 1

Oligonucleotides used in this study.

Aim	Primer	Sequence (5'→3')
pSIP411 backbone	oVPL399	GGCTAAAATCTCCTTGTAATAGTATTTTATAG (SEQ ID NO: 7)
	oVPL400	TAATCTAGACTCGAGGAATTCGGTAC (SEQ ID NO: 8)
orf3	oVPL4581	TTGAACGATGACATTTTACGAGAAAAGA (SEQ ID NO: 9)
	oVPL4582	CTAGTCATCGTTCTTTCGATACTTTTTAGG (SEQ ID NO: 10)
Bridging oligo for orf3	oVPL4583	ATAAAATACTATTACAAGGAGATTTTAGCCTT GAACGATGACATTTTACGAGAAAAGATA (SEQ ID NO: 11)
	oVPL4584	CCTAAAAAGTATCGAAAGAACGATGACTAGT AATCTAGACTCGAGGAATTCGGTACCCCG (SEQ ID NO: 12)
orf6	oVPL4585	ATGCAAGATAAAATTGTTGTTAGCTCTTTT (SEQ ID NO: 13)
	oVPL4586	CTATTTCCATTTTGACCGCCATTGT (SEQ ID NO: 14)
Bridging oligo for orf6	oVPL4587	ATAAAATACTATTACAAGGAGATTTTAGCCAT GCAAGATAAAATTGTTGTTAGCTCTTTT (SEQ ID NO: 15)
	oVPL4588	TTCCGACAATGGCGGTCAAAATGGAAATAGT AATCTAGACTCGAGGAATTCGGTACCCCG (SEQ ID NO: 16)
orf14	oVPL4589	GTGATATATATGAATAAAATAAAAAGGTTAT GTAAAAAATACCA (SEQ ID NO: 17)
	oVPL4590	TTAAGAAAGTTTTCCATTCTCATTTATTTCTCC (SEQ ID NO: 18)
Bridging oligo for orf14	oVPL4591	ATAAAATACTATTACAAGGAGATTTTAGCCGT GATATATATGAATAAAATAAAAAGGTTA (SEQ ID NO: 19)
	oVPL4592	GAAATAAATGAGAATGGAAAACTTTCTTAAT AATCTAGACTCGAGGAATTCGGTACCCCG (SEQ ID NO: 20)
orf16	oVPL4593	ATGGCAGAATTAAGTGAGGCAC (SEQ ID NO: 21)
	oVPL4594	TTAATTGTTATTCAACTTATCATCAATCATCTT ACG (SEQ ID NO: 22)
Bridging oligo for orf16	oVPL4595	ATAAAATACTATTACAAGGAGATTTTAGCCAT GGCAGAATTAAGTGAGGCACGGCGCAAA (SEQ ID NO: 23)
	oVPL4596	ATGATTGATGATAAGTTGAATAACAATTAATA ATCTAGACTCGAGGAATTCGGTACCCCG (SEQ ID NO: 24)
orf17	oVPL4597	ATGCCAAGGTATAGAAGATGTAGACA (SEQ ID NO: 25)
	oVPL4598	TTATTTTTGGTGCATCAATAGAACGATTTT (SEQ ID NO: 26)
Bridging oligo for orf17	oVPL4599	ATAAAATACTATTACAAGGAGATTTTAGCCAT GCCAAGGTATAGAAGATGTAGACAACCT (SEQ ID NO: 27)
	oVPL4600	AAAATCGTTCTATTGATGCACCAAAAATAATA ATCTAGACTCGAGGAATTCGGTACCCCG (SEQ ID NO: 28)
Screening	oVPL659	TGCCCCGTTAGTTGAAGAAG (SEQ ID NO: 29)
	oVPL660	ATTCTGCTCCCGCCCTTATG (SEQ ID NO: 30)
pSIP-orf14*	oVPL4690	TTAAGCAACAAAAGCAAGAATTGTTCCAATA CTTC (SEQ ID NO: 31)
	oVPL4691	TGAGTTATATGGCAACAAGGGAGACAAGAAA ACAT (SEQ ID NO: 32)

TABLE 1-continued

Oligonucleotides used in this study.

Aim	Primer	Sequence (5'→3')
MAMA oligo for orf14*	oVPL4723	GTCTCCCTTGTTGCCATATAACTCATTA (SEQ ID NO: 33)

Construction of pSIP-orf14*

[0100] Two in frame stop codons were incorporated into pSIP-orf14 to generate pSIP-orf14*, which loses the function of Orf14 but has the same transcriptional level as pSIP-orf14. By standard blunt-end ligation using T4 DNA ligase (Thermo Scientific), oligonucleotideS oVPL4690 and oVPL4691 were used to generate linear pSIP-orf14* from the backbone of pSIP-orf14. After ligation, DNA was transformed into *E. coli* EC1000. OligonucleotideS oVPL4723 and oVPL659 were used to confirm the mutation by PCR, followed by Sanger sequencing. Confirmed plasmid pSIP-orf14* was further transformed to LR wildtype, LRΔΦ1ΔΦ2, and LR ΔΦ1ΔΦ2:: comp, respectively.

Construction of pSIP-Orf14-Mucosae and pSIP-Orf14-Oris

[0101] To compare the impact of orf14 homologues on the reuterin production, the sequences of two orf14 homologues originally from *L. mucosae* (SEQ ID NOS: 3-4) and *L. oris* (SEQ ID NOS: 5-6) were codon optimized for expression in *L. reuteri* using the OPTIMIZER web server (Puigbo et al. 2007) (Table 2), and double-strand DNA was synthesized by GeneWiz. Synthesized DNA fragments were cloned to pSIP411 backbone as described above.

Determination of Reuterin Formation

[0102] To characterize the reuterin production during growth, strains were cultured at 37° C. anacrobically in deMan-Rogosa-Sharpe medium (MRS; 288110, BD BioSciences) for 18 h, and then bacteria were sub-cultured to reach an initial OD_{600} of 0.1 in modified MRS (10 g peptone, 10 g beef extract, 5 g yeast extract, 2 g ammonium citrate dibasic, 2 g K2HPO4, 0.1 g MgSO4, 0.05 g MnSO4, and 1.0 g Tween 80 per liter) supplemented with 100 mM glucose. Cultures were incubated anaerobically at 37° C. Strains harboring pSIP411 or its variants were grown in mMRS supplemented with 5 μg/mL erythromycin. Where applicable, induction peptide (10 ng/ml) (MAGNSSNFIHKIKQIFTHR (SEQ ID NO:34)) was added. Samples were collected every hour up to 8 hours. Cell density was monitored using GENESYS 20 visible spectrophotometer (Thermo Scientific) and reuterin production was measured as described in Zhang et al. 2020. Briefly, cell-free supernatants (50 μL) were mixed with 37.5 μL tryptophan (Sigma-Aldrich) in a 96 well plate, followed by an addition of 150 μL 37% HCl (Thermo scientific). After incubating at 37° C. for 20 min, OD_{560} was measured by GloMax plate reader (Promega). Two-fold dilutions of acrolein (06-722-501, ThermoFisher) were used to generate a standard curve to quantify reuterin (Doleyres et al. 2005). Reuterin concentration was normalized to OD_{600} of 3 in FIGS. **5**A-**5**D.

Statistical Analyses

[0103] Data were obtained in 3 biological replicates and are expressed as means±standard deviations. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA). The least significant difference (LSD) was used to test the difference among means using a P value of 0.05.

Results

[0104] To understand the role of prophages in reuterin production, we used the previously developed isogenic mutant *L. reuteri*ΔΦ1ΔΦ2 in which both prophage genomes were deleted. We determined that *L. reuteri*ΔΦ14Φ2 does not produce reuterin, which could be restored by complementation of two prophages (FIG. **5**A). To determine which prophage drives reuterin production, we tested the single prophage deletion strains, and found that deletion of Φ2 but not deletion of Φ1 abolished reuterin production (FIG. **5**B). We therefore hypothesized that Φ2-encoding regulatory genes contribute to reuterin production.

[0105] By comparing the two prophage genomes, we identified five unique genes on Φ2 (orf3, orf6, orf14, orf16, and orf17) (FIG. **1**), which were cloned into the inducible vector pSIP411. Plasmid pSIP411 and its variants were transferred to *L. reuteri*ΔΦ1ΔΦ2. Wildtype and complementary strains with pSIP411 served as controls. Overexpressing Orf14 in *L. reuteri*ΔΦ1ΔΦ2 not only rescued reuterin production in the *L. reuteri*ΔΦ1ΔΦ2 background, but also resulted in a 3-fold increase ($P<0.05$) in reuterin production compared to that in the wildtype strain following 5 h incubation (FIG. **5**C). The control plasmid in which the coding sequence of orf14 was interrupted by two in-frame stop codons did not restore reuterin production (FIG. **5**D).

[0106] Two Orf14 homologues were identified in *Limosilactobacillus* mucosae (SEQ ID NOS: 3 and 4) and *Limosilactobacillus oris* (SEQ ID NOS: 5 and 6), with amino acid identity of 77.34% and 47.74% respectively by Protein BLAST search in NCBI (National Center for Biotechnology Information). Overexpression of Orf14-mucosae yielded 2.9-fold higher reuterin production in *L. reuteri* compared to the overexpression of native Orf14 (FIG. **5**E), indicating the application of Orf14 in related species (homology>77%).

[0107] Taken together, we engineered a hyper reuterin producer *L. reuteri*ΔΦ14Φ2 (pSIP-orf14) by deleting prophages and overexpressing the phage-encoding gene orf14.

CONCLUSIONS

[0108] The high-yield production of reuterin in the engineered strain *L. reuteri* ΔΦ1ΔΦ2 (pSIP-orf14) highlighted its usefulness as a robust strain for industrial applications. This *L. reuteri* mutant produces 3-fold more reuterin compared to the control strain. This strain is advantageous because not only is the reuterin production more robust, but reuterin can be harvested from actively growing cells without "leakage" of bacteriophages. The culture media can be directly used for reuterin extraction. This contrasts conventional methods, wherein resting cells need to be harvested, washed, and resuspended in glycerol solution for bioconversion, thereby shortening the process from a two-stage

process to a one-stage process. The increased reuterin production by *L. reuteri* ΔΦ1ΔΦ2 (pSIP-orf14) is also reflected in the antimicrobial activity against ESKAPE pathogens, which may strengthen the probiosis of *L. reuteri*. Overexpression of orf14 in phage-deleted microbes may also promote the probiotic gut fitness through upregulating the pdu cluster to take advantage of 1,2-propanedial produced by other microbes. The engineering strategies provided herein can be used in biochemistry industry to maximize reuterin production. The resulting strains can be used as advanced probiotic with promoted function and gut fitness.

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SEQUENCE LISTING

Sequence total quantity: 34

SEQ ID NO: 1 moltype = DNA length = 852
FEATURE Location/Qualifiers
source 1..852
mol_type = genomic DNA
organism = Lactobacillus reuteri

SEQUENCE: 1
gtgatatata tgaataaaat aaaaaggtta tgtaaaaaat accagcaagt aatttataca 60
ctatttgctg tcctagttat tataagtgtt gtttattttg ctttgcatgg gtttgattca 120
tttgtaaagt gggcaacaac tgatgtgggg tctgtcgctg attgggctgg aagtattgga 180
acaattcttg cttttgttgc tgttatatgg caacaaggga gacaagaaaa cattacaaga 240
gctgttcata tcgaagaatc aaggcctaga ttttcgctta catatacacc taagccggtg 300
ttgaaaacaa gagtgttgtt ttggggcaat ggtcgaacgg cagttaatat aaatgaaatt 360
cttggaagta ggtcagctga gaattacaga tttattagta tcgaaaatat ttctaataat 420
gtagtttatg attattccat aatattaaag tatcactcta atgataatgc aagagttaga 480
aaagattatt ggtcaagtca tggattattt ccaagaagaa gtgttgcgat tgttcctaag 540
tttatgggca ctgataaaga taaaatagga aattacatat atgatgaatt acttgttaaa 600
tttacaactc cggctaatga agtaggattt tttatatcga agaacataaa taacgctcaa 660
acagattcta gttttggcaa aagtcagtat tattttgtac gaggaagcca cgttaagcgt 720
gtaacggcta ttaacacaga taaaatgatt gatgttgata gttccatttg taggaaatta 780
gataaagagt ttgacgaaat aattggtata acaagtttag gagaaataaa tgagaatgga 840
aaactttctt aa 852

SEQ ID NO: 2 moltype = AA length = 283
FEATURE Location/Qualifiers
source 1..283
mol_type = protein
organism = Lactobacillus reuteri

SEQUENCE: 2
VIYMNKIKRL CKKYQQVIYT LFAVLVIISV VYFALHGFDS FVKWATTDVG SVADWAGSIG 60
TILAFVAVIW QQGRQENITR AVHIEESRPR FSLTYTPKPV LKTRVLFWGN GRTAVNINEI 120
LGSRSAENYR FISIENISNN VVYDYSIILK YHSNDNARVR KDYWSSHGLF PRRSVAIVPK 180
FMGTDKDKIG NYIYDELLVK FTTPANEVGF FISKNINNAQ TDSSFGKSQY YFVRGSHVKR 240
VTAINTDKMI DVDSSICRKL DKEFDEIIGI TSLGEINENG KLS 283

SEQ ID NO: 3 moltype = DNA length = 843
FEATURE Location/Qualifiers
source 1..843
mol_type = genomic DNA
organism = Lactobacillus mucosae

SEQUENCE: 3
atgaaaaggg taaaagaatt atgtaaaaaa tatcagcaag taatttacac tttattagct 60
gttgtcgcta ttgtaggtgt tgtttatttt gctttgcatg gatttaattc atttataaaa 120
tgggcaacaa ctgatgtagg ctctgttgct gattgggctg ggagtattgg aactatcctt 180
gcttttattg ctgttatttg gcaacaagga agacaagaaa atatcacacg tgctgttcat 240
gttgaagaat caaggcctag attttctgtc acgtatactc ccaaacctca agcaaaaaca 300
aaaatgttgt tttggggcaa caatcgaact ttaagccaaa taaataccat gttaaataat 360
agaaatggtg aaaactatag atttataaat atagaaaata tttcaaataa tgttgtctat 420
gattattcaa taatattaaa ataccatttt tatgataatt ccttagtacg aaaggactat 480
tggtcaagtc atggattgtt cccgagaaga agtatcatta ttgttccaaa atttttaggg 540
actggcaaag acaagatagg gaattatatt tatgatgaac tacttattaa atttacaact 600
ccagctaatg aaattggctt tttcaaaatg acgaatgtta atggtaatcg aacagattct 660
agctttggcg atagtcaata ttattttgtc cgtggaagcc atattaaacg agtaacagca 720
attaatactg atgaaatgat caaagctgat agtccacttt gtagaaaatt agatagagaa 780

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tttagtgaaa caaccggtgg aacaaatttt ggggcaatag gagaagatgg aaaagcttac  840
taa                                                                843

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FEATURE                Location/Qualifiers
source                 1..280
                       mol_type = protein
                       organism = Lactobacillus mucosae
SEQUENCE: 4
MKRVKELCKK YQQVIYTLLA VVAIVGVVYF ALHGFNSFIK WATTDVGSVA DWAGSIGTIL  60
AFIAVIWQQG RQENITRAVH VEESRPRFSV TYTPKPQAKT KMLFWGNNRT LSQINTMLNN  120
RNGENYRFIN IENISNNVVY DYSIILKYHF YDNSLVRKDY WSSHGLFPRR SIIIVPKFLG  180
TGKDKIGNYI YDELLIKFTT PANEIGFFKM TNVNGNRTDS SFGDSQYYFV RGSHIKRVTA  240
INTDEMIKAD SPLCRKLDRE FSETTGGTNF GAIGEDGKAY                        280

SEQ ID NO: 5           moltype = DNA  length = 816
FEATURE                Location/Qualifiers
source                 1..816
                       mol_type = genomic DNA
                       organism = Lactobacillus oris
SEQUENCE: 5
atgttgaata atataaaatc tttatattcc aaatttaagc aatttataca aggagcttta  60
tgcatagttg tacttatctt tggttttatt ttaataaagc atataacaaa agaatggaca  120
ccatttatta catggtttca tactgatcat ggaagtgttg cagattgggc agggagtatt  180
ggaactattg cagcgttttt agctgttttt tggcaagtga aaaaacaagg aagtattgaa  240
agagctattg atgttgaaag aagcaggcct agattttcag tcttatttag tttaacttta  300
cctaaaggta caagagtact ttattggaat aggacggata atgatgcaaa taatataatt  360
agtaatcctg aagaataccg atttattacg attcaaaaca tatcttcaaa tgtaatatat  420
gattttgatg tgattttgag atatcataca ttagacaatt cacgtagtcg taatgacttt  480
tggagtacga ctggtgtttt tccaaagaaa actgtaactt ttataccaaa atttaaaggg  540
aaaaataatg accaacagta tgtgtatgat gaattattag taaaattcac gactccagct  600
aatgaagttg gtttctttcg gatgattaat gtaaatgatg taaaaggtga tttaggcttg  660
ggaagtggaa ggtattattt tgtaaagggt acgcatatta agggagtcaa ggcaattaat  720
aaggatagaa tgattaagat taatagtgat gaatgccaaa gatttgattc attatttgaa  780
gactttaatt actcaacaaa tactattgag gtttag                            816

SEQ ID NO: 6           moltype = AA  length = 271
FEATURE                Location/Qualifiers
source                 1..271
                       mol_type = protein
                       organism = Lactobacillus oris
SEQUENCE: 6
MLNNIKSLYS KFKQFIQGAL CIVVLIFGFI LIKHITKEWT PFITWFHTDH GSVADWAGSI  60
GTIAAFLAVF WQVKKQGSIE RAIDVERSRP RFSVLFSLTL PKGTRVLYWN RTDNDANNII  120
SNPEEYRFIT IQNISSNVIY DFDVILRYHT LDNSRSRNDF WSTTGVFPKK TVTFIPKFKG  180
KNNDQQYVYD ELLVKFTTPA NEVGFFRMIN VNDVKGDLGL GSGRYYFVKG THIKGVKAIN  240
KDRMIKINSD ECQRFDSLFE DFNYSTNTIE V                                 271

SEQ ID NO: 7           moltype = DNA  length = 32
FEATURE                Location/Qualifiers
source                 1..32
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 7
ggctaaaatc tccttgtaat agtattttat ag                                32

SEQ ID NO: 8           moltype = DNA  length = 26
FEATURE                Location/Qualifiers
source                 1..26
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 8
taatctagac tcgaggaatt cggtac                                       26

SEQ ID NO: 9           moltype = DNA  length = 28
FEATURE                Location/Qualifiers
source                 1..28
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 9
ttgaacgatg acattttacg agaaaaga                                     28

SEQ ID NO: 10          moltype = DNA  length = 30
FEATURE                Location/Qualifiers
source                 1..30
                       mol_type = other DNA
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                     organism = synthetic construct
SEQUENCE: 10
ctagtcatcg ttctttcgat acttttagg                                    30

SEQ ID NO: 11          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 11
ataaaatact attacaagga gattttagcc ttgaacgatg acattttacg agaaaagata  60

SEQ ID NO: 12          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 12
cctaaaaagt atcgaaagaa cgatgactag taatctagac tcgaggaatt cggtaccccg  60

SEQ ID NO: 13          moltype = DNA  length = 30
FEATURE                Location/Qualifiers
source                 1..30
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 13
atgcaagata aaattgttgt tagctctttt                                   30

SEQ ID NO: 14          moltype = DNA  length = 25
FEATURE                Location/Qualifiers
source                 1..25
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 14
ctatttccat tttgaccgcc attgt                                        25

SEQ ID NO: 15          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 15
ataaaatact attacaagga gattttagcc atgcaagata aaattgttgt tagctctttt  60

SEQ ID NO: 16          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 16
ttccgacaat ggcggtcaaa atggaaatag taatctagac tcgaggaatt cggtaccccg  60

SEQ ID NO: 17          moltype = DNA  length = 44
FEATURE                Location/Qualifiers
source                 1..44
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 17
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SEQ ID NO: 18          moltype = DNA  length = 33
FEATURE                Location/Qualifiers
source                 1..33
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 18
ttaagaaagt tttccattct catttatttc tcc                               33

SEQ ID NO: 19          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 19
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```

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source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 20
gaaataaatg agaatggaaa actttcttaa taatctagac tcgaggaatt cggtaccccg  60

SEQ ID NO: 21          moltype = DNA  length = 22
FEATURE                Location/Qualifiers
source                 1..22
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 21
atggcagaat taagtgaggc ac                                          22

SEQ ID NO: 22          moltype = DNA  length = 36
FEATURE                Location/Qualifiers
source                 1..36
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 22
ttaattgtta ttcaacttat catcaatcat cttacg                           36

SEQ ID NO: 23          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 23
ataaaatact attacaagga gattttagcc atggcagaat taagtgaggc acggcgcaaa  60

SEQ ID NO: 24          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 24
atgattgatg ataagttgaa taacaattaa taatctagac tcgaggaatt cggtaccccg  60

SEQ ID NO: 25          moltype = DNA  length = 26
FEATURE                Location/Qualifiers
source                 1..26
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 25
atgccaaggt atagaagatg tagaca                                      26

SEQ ID NO: 26          moltype = DNA  length = 30
FEATURE                Location/Qualifiers
source                 1..30
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 26
ttatttttgg tgcatcaata gaacgatttt                                  30

SEQ ID NO: 27          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 27
ataaaatact attacaagga gattttagcc atgccaaggt atagaagatg tagacaacct  60

SEQ ID NO: 28          moltype = DNA  length = 60
FEATURE                Location/Qualifiers
source                 1..60
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                       organism = synthetic construct
SEQUENCE: 28
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SEQ ID NO: 29          moltype = DNA  length = 20
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source                 1..20
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SEQUENCE: 29
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SEQ ID NO: 30       moltype = DNA  length = 20
FEATURE             Location/Qualifiers
source              1..20
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                    organism = synthetic construct
SEQUENCE: 30
attctgctcc cgcccttatg                                          20

SEQ ID NO: 31       moltype = DNA  length = 35
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source              1..35
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                    organism = synthetic construct
SEQUENCE: 31
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FEATURE             Location/Qualifiers
source              1..35
                    mol_type = other DNA
                    organism = synthetic construct
SEQUENCE: 32
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FEATURE             Location/Qualifiers
source              1..28
                    mol_type = other DNA
                    organism = synthetic construct
SEQUENCE: 33
gtctcccttg ttgccatata actcatta                                 28

SEQ ID NO: 34       moltype = AA  length = 19
FEATURE             Location/Qualifiers
source              1..19
                    mol_type = protein
                    organism = synthetic construct
SEQUENCE: 34
MAGNSSNFIH KIKQIFTHR                                           19
```

1. A recombinant microorganism comprising one or more modifications with respect to a corresponding microorganism not comprising the one or more modifications, wherein the one or more modifications comprise a recombinant orf14 gene encoding an Orf14 protein comprising an amino acid sequence of Orf14 of *Limosilactobacillus reuteri* (SEQ ID NO:2), Orf14 of *Limosilactobacillus* mucosae (SEQ ID NO:4), or Orf14 of *Limosilactobacillus oris* (SEQ ID NO: 6), or an amino acid sequence of a homolog of any of the foregoing.

2. The recombinant microorganism of claim **1**, wherein the Orf14 protein comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO:2, SEQ ID NO: 4, or SEQ ID NO:6.

3. The recombinant microorganism of claim **1**, wherein the Orf14 protein comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

4. The recombinant microorganism of claim **1**, wherein the recombinant orf14 gene comprises a promoter and a coding sequence, wherein the promoter is heterologous to the coding sequence.

5. The recombinant microorganism of claim **1**, wherein the recombinant microorganism is a bacterium.

6. The recombinant microorganism of claim **1**, wherein the recombinant microorganism is commensal to human gastrointestinal tract.

7. The recombinant microorganism of claim **1**, wherein the recombinant microorganism comprises a glycerol dehydratase.

8. The recombinant microorganism of claim **1**, wherein the recombinant microorganism comprises a propanediol utilization gene set.

9. The recombinant microorganism of claim **1**, wherein the recombinant microorganism and the corresponding microorganism produce reuterin.

10. The recombinant microorganism of claim **1**, wherein the recombinant microorganism exhibits enhanced production of reuterin with respect to the corresponding microorganism.

11. The recombinant microorganism of claim **1**, wherein the recombinant microorganism is from a genus selected from the group consisting of *Anaerobutyricum, Bacillus, Blautia Citrobacter, Clostridium, Enterobacter, Enterococcus, Flavonifractor, Klebsiella, Lactobacillus, Limosilactobacillus, Listeria, Pediococcus, Salmonella, Streptococcus, Terrisporobacter*, and *Yersina*.

12. The recombinant microorganism of claim **1**, wherein the recombinant microorganism is *L. reuteri*.

13. The recombinant microorganism of claim **1**, wherein the recombinant microorganism comprises an inactivation of at least one biologically active prophage present in the corresponding microorganism.

14. The recombinant microorganism of claim **13**, wherein the recombinant microorganism is *L. reuteri* and comprises an inactivation of at least one of Φ1 and Φ2.

15. The recombinant microorganism of claim **1**, wherein the recombinant microorganism comprises an inactivation of each biologically active prophage present in the corresponding microorganism.

16. The recombinant microorganism of claim **15**, wherein the recombinant microorganism is *L. reuteri* and comprises an inactivation of each of Φ1 and Φ2.

17. A method of producing reuterin, the method comprising culturing the recombinant microorganism of claim **1** to thereby produce reuterin.

18-20. (canceled)

21. The method of claim **17**, further comprising orally administering the recombinant microorganism to introduce the recombinant microorganism to a gastrointestinal tract, wherein the culturing is performed in the gastrointestinal tract.

22. The method of claim **17**, wherein the culturing is performed in vitro.

23. The recombinant microorganism of claim **1**, wherein:

the Orf14 protein comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6;

the recombinant orf14 gene comprises a promoter and a coding sequence, wherein the promoter is heterologous to the coding sequence;

the recombinant microorganism is *L. reuteri* and comprises an inactivation of each of Φ1 and Φ2; and

the recombinant microorganism exhibits enhanced production of reuterin with respect to the corresponding microorganism.

* * * * *