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Van Pijkeren et al.

(10) **Patent No.: US 10,376,563 B1**
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(54) **METHODS FOR SYSTEMICALLY DELIVERING POLYPEPTIDES AND MICROORGANISMS THEREFOR**

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(73) Assignee: **WISCONSIN ALUMNI RESEARCH FOUNDATION**, Madison, WI (US)

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(22) Filed: **Feb. 13, 2017**

Related U.S. Application Data

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(51) **Int. Cl.**

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A61K 35/74	(2015.01)
A61K 38/00	(2006.01)
A61K 39/09	(2006.01)
A61K 38/20	(2006.01)
A61K 35/747	(2015.01)
A61K 38/02	(2006.01)
A61K 9/00	(2006.01)
C07K 14/54	(2006.01)
A61K 35/00	(2006.01)

(52) **U.S. Cl.**
CPC ***A61K 38/20*** (2013.01); ***A61K 9/0053*** (2013.01); ***A61K 35/747*** (2013.01); ***A61K 38/02*** (2013.01); ***C07K 14/54*** (2013.01); *A61K 2035/11* (2013.01)

(58) **Field of Classification Search**
CPC C12N 15/74; C12N 15/746; A61K 35/74; A61K 38/00; A61K 39/09
See application file for complete search history.

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(57) **ABSTRACT**

Methods and microorganisms for systemically introducing a polypeptide in the bloodstream of a subject. The methods of the invention include administering into the gastrointestinal tract of a subject a bacterium configured to express and produce and release the polypeptide. The bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream of the subject, preferably in a detectable amount. The microorganisms of the invention include lactic acid bacteria, such as *Lactobacillus reuteri*, that comprise a recombinant gene configured to express a polypeptide to be systemically introduced.

11 Claims, 12 Drawing Sheets

Specification includes a Sequence Listing.

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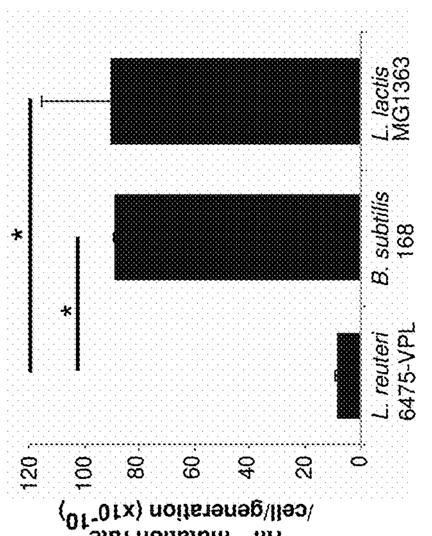

RifR mutation rate /cell/generation (x10-10)
120
100
80
60
40
20
0
*
*
L. reuteri 6475-VPL
B. subtilis 168
L. lactis MG1363

FIG. 1A

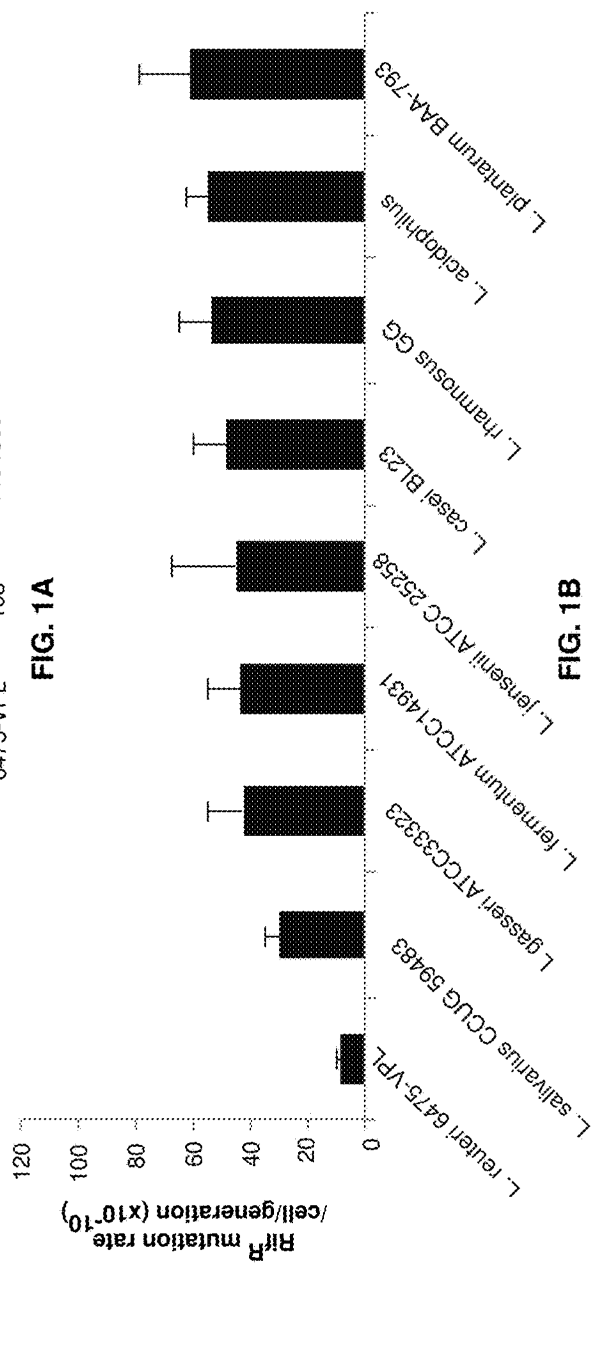

RifR mutation rate /cell/generation (x10-10)
120
100
80
60
40
20
0
L. reuteri 6475-VPL
L. salivarius CCUG 59483
L. gasseri ATCC33323
L. fermentum ATCC14931
L. jensenii ATCC 25258
L. casei BL23
L. rhamnosus GG
L. acidophilus
L. plantarum BAA-793

FIG. 1B

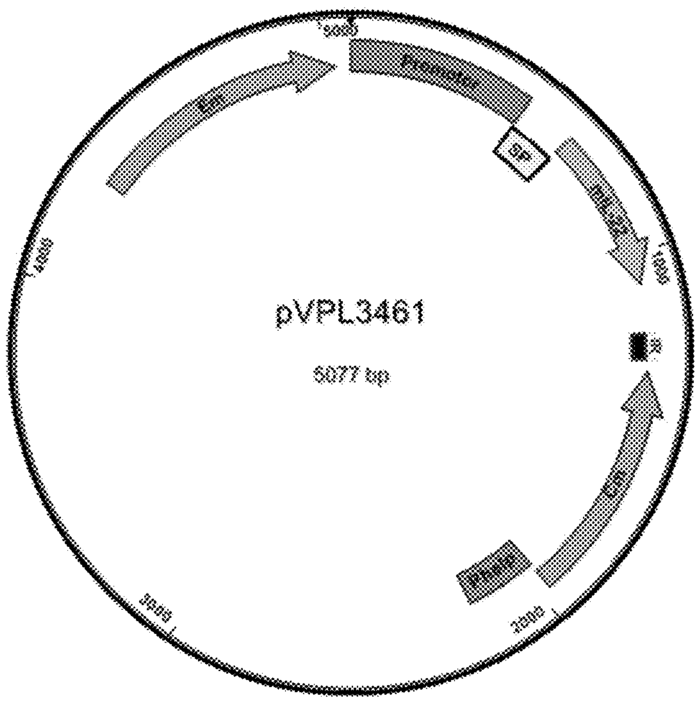


FIG. 2

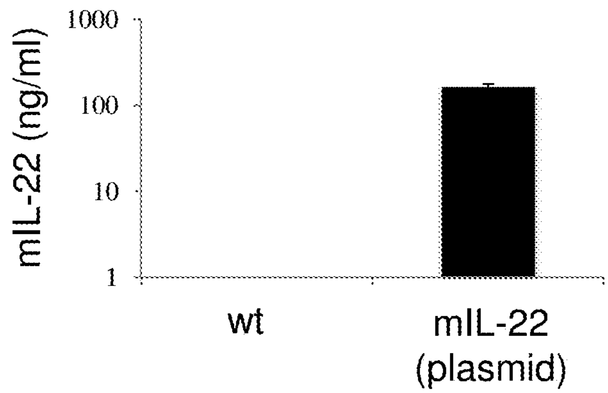

1000
100
10
1
mIL-22 (ng/ml)
wt
mIL-22
(plasmid)

FIG. 3A

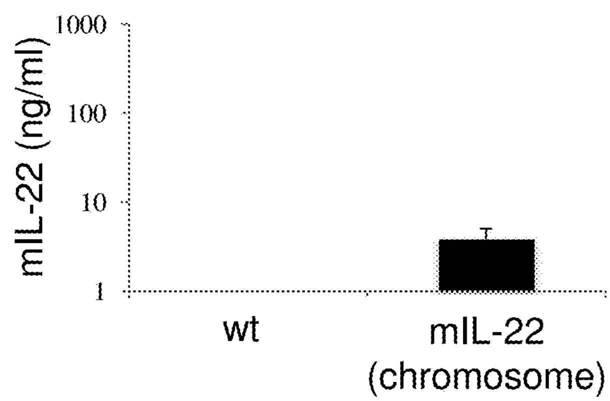

1000
100
10
1
mIL-22 (ng/ml)
wt
mIL-22
(chromosome)

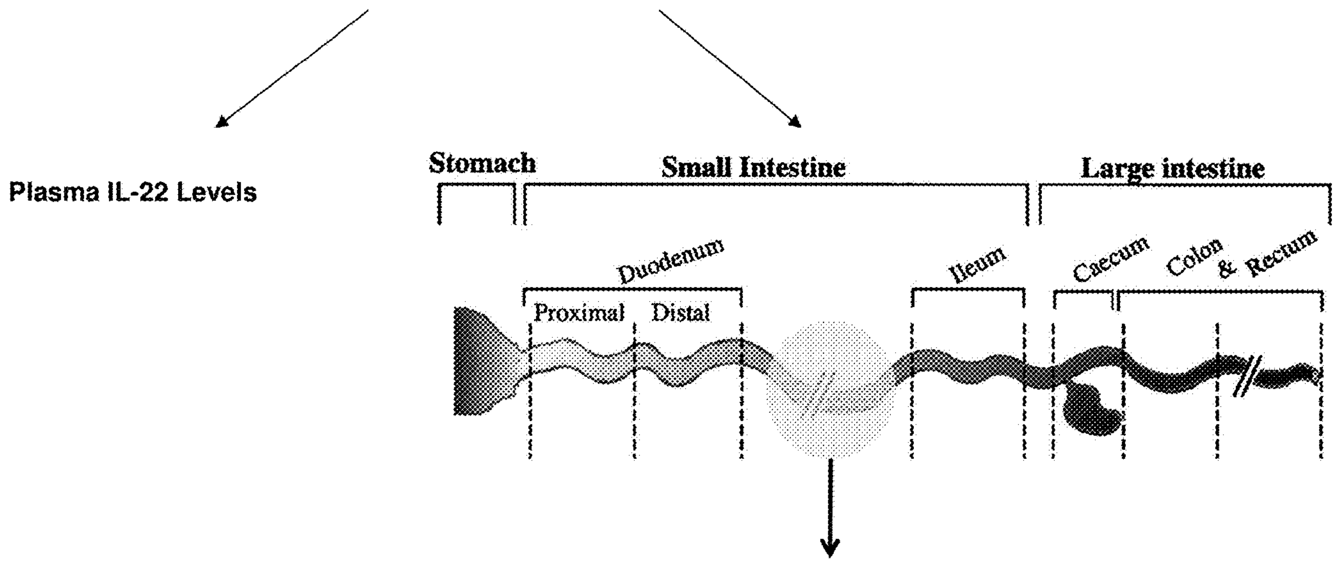
FIG. 3B

In-vivo* assessment of (recombinant) *L. reuteri

Group #1: Sham gavage

#2: LR wild-type (10^9/day for 7 days)

#3: LR(mIL-22) (10^9/day for 7 days)



qRT-PCR analysis on cDNA (REG3G, REG3B)

FIG. 4

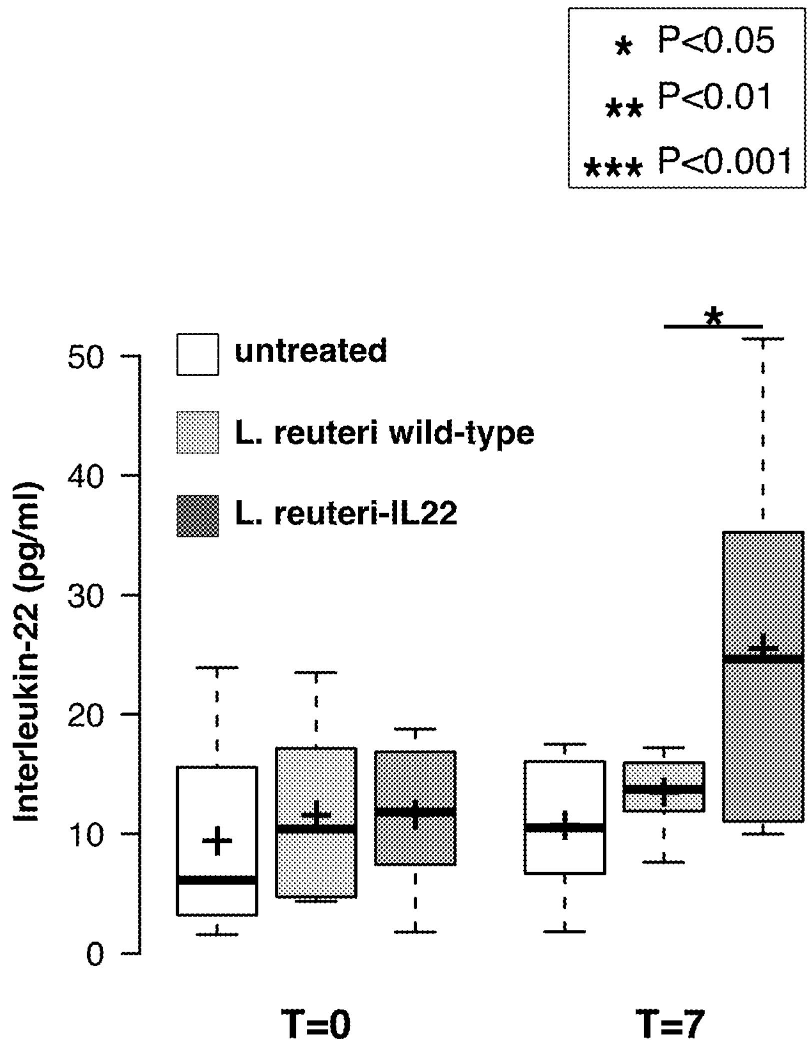

* P<0.05
** P<0.01
*** P<0.001
*
untreated
L. reuteri wild-type
L. reuteri-IL22
Interleukin-22 (pg/ml)
50
40
30
20
10
0
T=0
T=7

FIG. 5

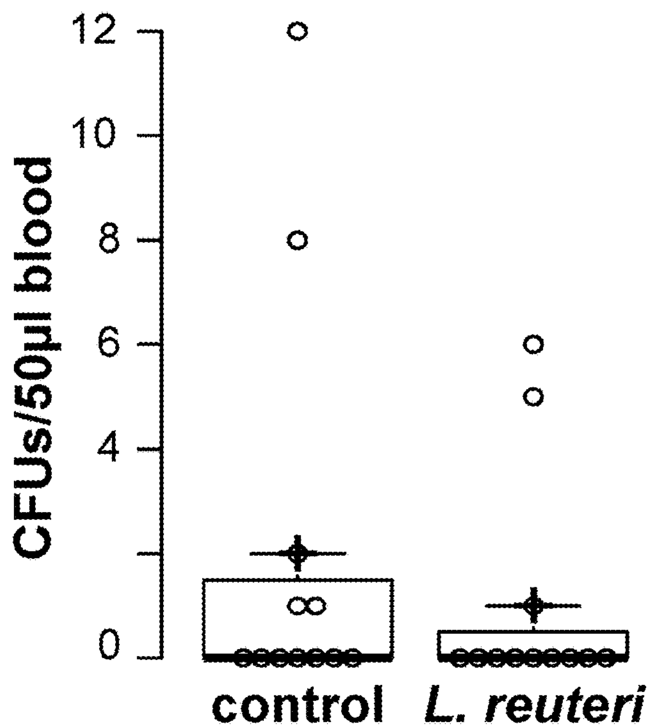


FIG. 6

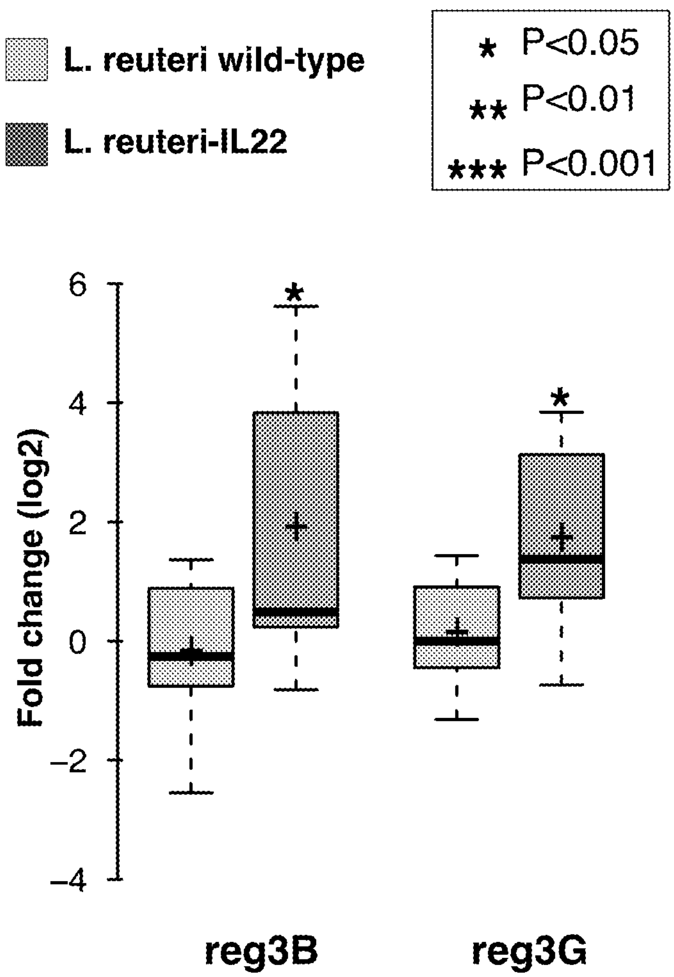

L. reuteri wild-type
L. reuteri-IL22
* P<0.05
** P<0.01
*** P<0.001
Fold change (log2)
6
4
2
0
−2
−4
*
*
reg3B
reg3G

FIG. 7

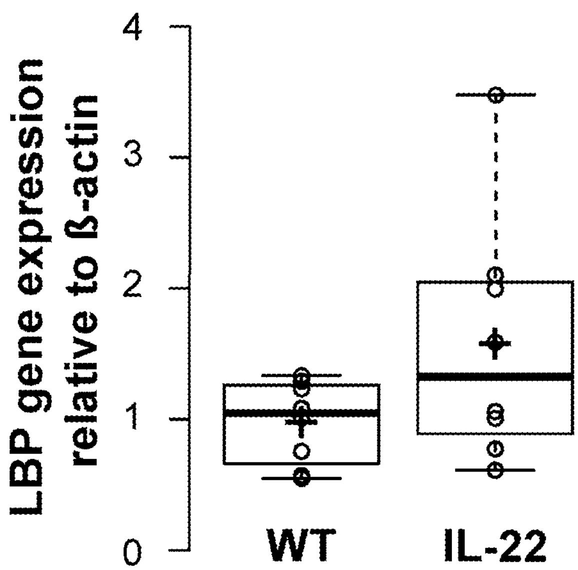


FIG. 8

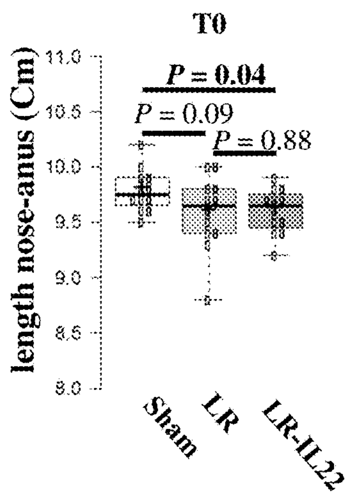


FIG. 9A

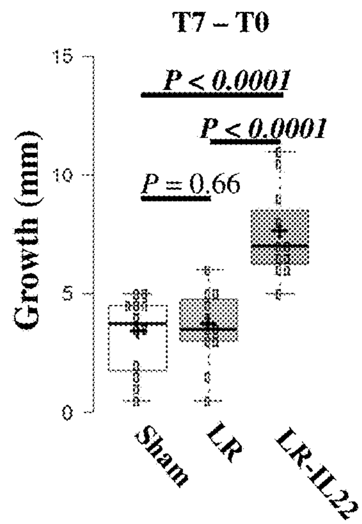


FIG. 9B

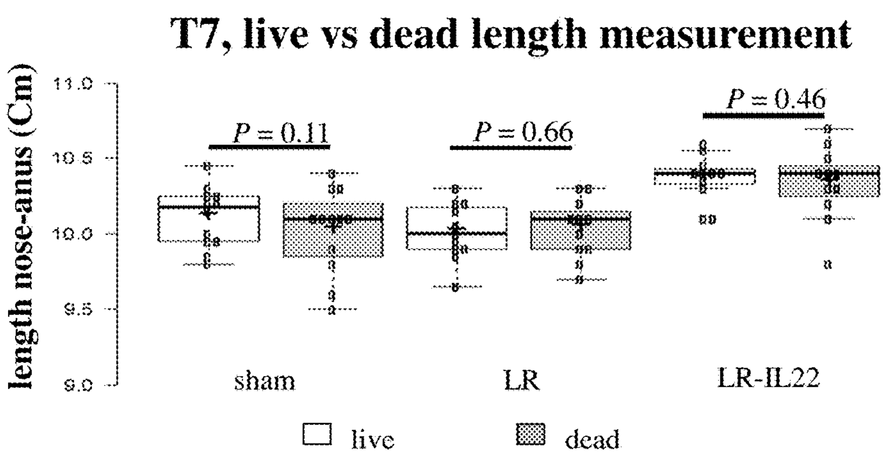


FIG. 9C

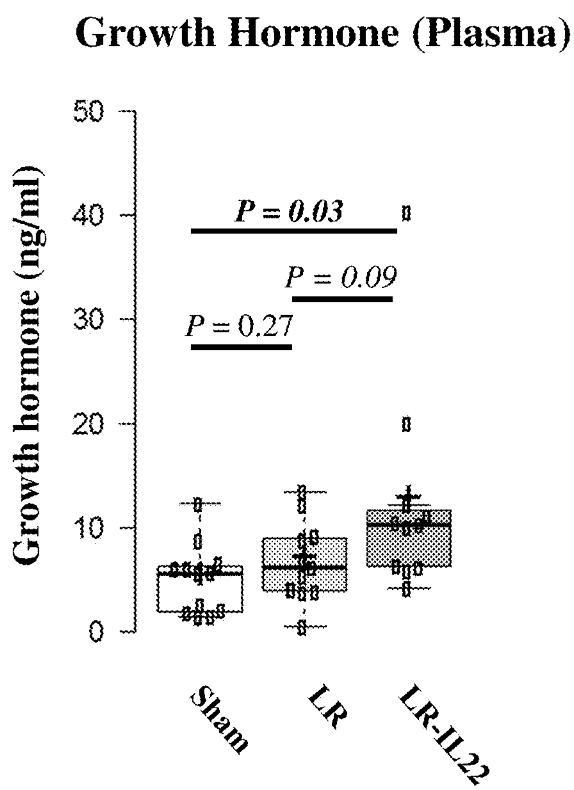

Growth Hormone (Plasma)
Growth hormone (ng/ml)
50
40
30
20
10
0
P = 0.03
P = 0.09
P = 0.27
Sham
LR
LR-IL22

FIG. 10

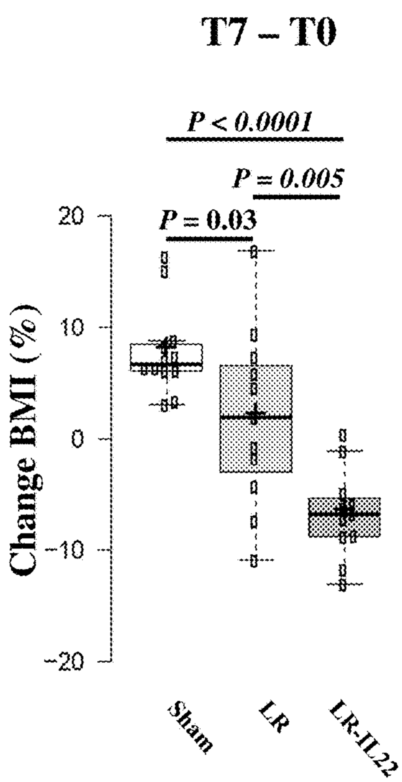


FIG. 11A

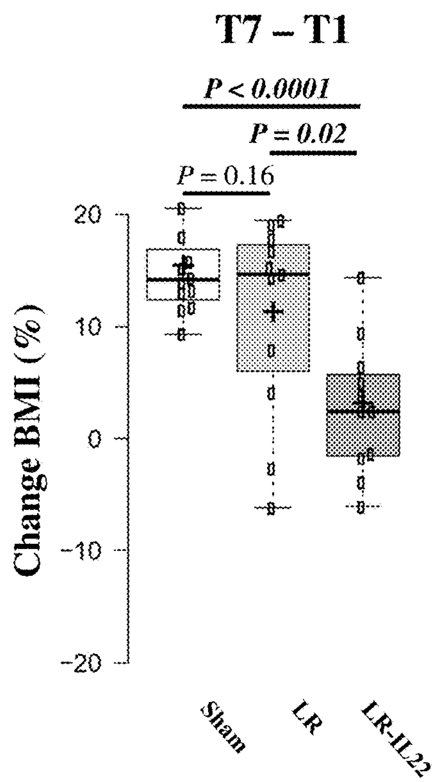


FIG. 11B

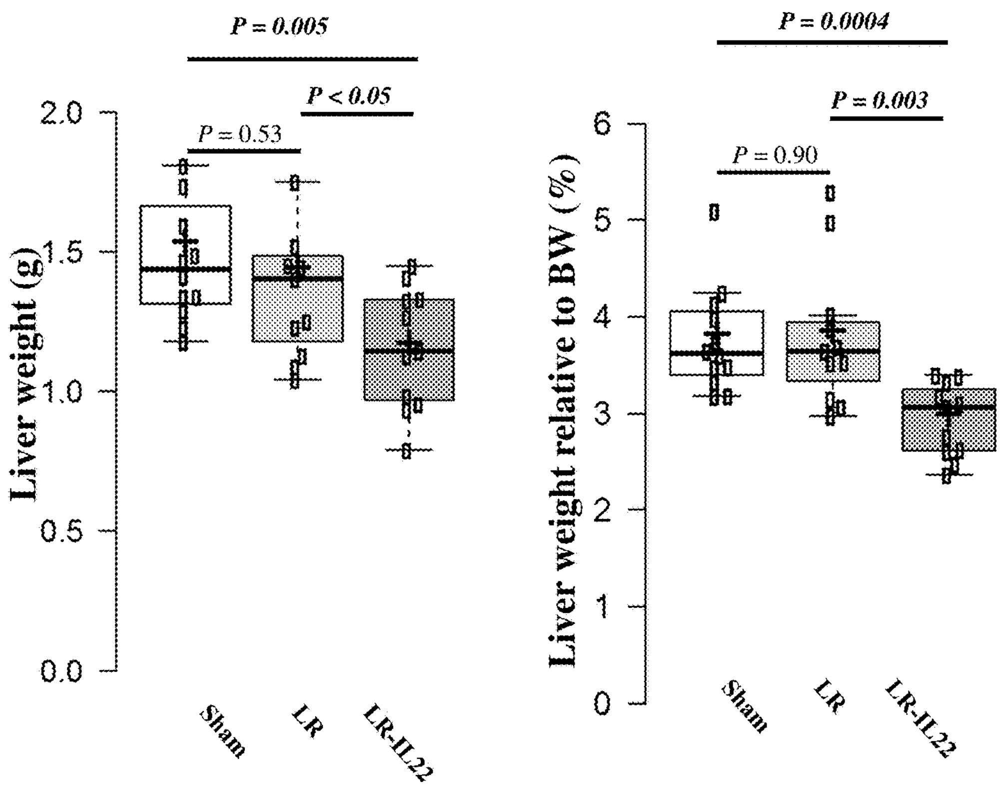


FIG. 12A

FIG. 12B

METHODS FOR SYSTEMICALLY DELIVERING POLYPEPTIDES AND MICROORGANISMS THEREFOR

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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FIELD OF THE INVENTION

The invention is directed to systemically delivering polypeptides to the bloodstream of a subject, such as through administering into the gastrointestinal tract of the subject a microorganism that produces and releases the polypeptides. The invention is also directed to microorganisms suitable for this purpose.

BACKGROUND

Polypeptides, such as enzymes, antibodies, hormones, cytokines, etc., are tremendously useful as therapeutic agents. However, routes for systemically introducing such polypeptides to a subject are limited. Oral administration of the polypeptides is typically not feasible, as the polypeptides are either degraded in the gastrointestinal tract or are blocked from reaching the bloodstream. Direct intravenous administration is therefore the major route by which polypeptides are systemically introduced.

Certain types of genetically engineered bacteria have been used as vehicles for locally delivering polypeptides to various tissues. Engineered *Lactococcus lactis*, for example, has been administered intragastrically for delivering polypeptides such as trefoil factors and interleukin-10 locally to intestinal/mucosal tissues. See Steidler et al. 2000 and Huyghebaert et al. 2005. However, a systemic increase in polypeptides delivered via *Lactococcus lactis* was not found.

Other types of genetically engineered bacteria have been used as vehicles for delivery of polypeptides to tumors in the body. An engineered *Bifidobacterium* strain, for example, has been shown to translocate from the gastrointestinal tract after oral administration and target to, replicate in, and express genes within tumors. See Cronin et al. 2010. This effect, however, depends on the unique ability of the *Bifidobacterium* to translocate from the gastrointestinal tract to extra-intestinal sites in the body. While *Bifidobacterium* may serve as a useful delivery vehicle for some purposes, the systemic distribution of the *Bifidobacterium* is potentially deleterious in certain subject populations such as immunocompromised patients.

Engineered bacteria capable of being administering into the gastrointestinal tract and delivering polypeptides in the bloodstream without systemic levels of the bacteria themselves being increased are needed.

SUMMARY OF THE INVENTION

The invention is directed to methods and microorganisms for systemically introducing a polypeptide in a bloodstream of a subject.

One method comprises administering into the gastrointestinal tract of the subject a bacterium configured to produce and release the polypeptide. The bacterium may comprise a recombinant gene configured to express the polypeptide. The bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream of the subject, preferably in a detectable amount.

In some versions, the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream of the subject without the bacterium being substantially introduced in the bloodstream of the subject.

In some versions, the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream in an amount effective to induce at least one direct systemic effect in the subject. In some versions, the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream in an amount effective to induce at least one direct effect in a non-gastrointestinal tissue in the subject. In some versions, the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream in an amount effective to induce at least one direct effect in a tissue selected from the group consisting of liver, muscles, lungs, kidneys, pancreas, and adipose tissue in the subject.

In some versions, the subject suffers from a condition treatable with systemic introduction of the polypeptide. In some versions, the subject suffers from a condition treatable with systemic introduction of the polypeptide but not treatable with local introduction of the polypeptide to the gastrointestinal tract without systemic introduction of the polypeptide. In either case, the polypeptide is introduced in the bloodstream of the subject in an amount effective to treat the condition, independent of the bacterium getting into the bloodstream.

In some versions, the polypeptide is a therapeutic polypeptide.

In some versions, the polypeptide is selected from the group consisting of a cytokine, a hormone, an antibody, an antimicrobial peptide, and an antigenic peptide.

In some versions, the polypeptide is selected form the group consisting of IL-22, IL-35, insulin, leptin, cathelicidin related antimicrobial peptide, a peptide inhibitor of PCSK9, and an endolysin.

In some versions, the subject suffers from at least one condition selected from the group consisting of insulin resistance, hyperglycemia, lipid dysregulation, hyperlipidemia, and obesity, and wherein the polypeptide is introduced in the bloodstream of the subject in an amount effective to treat the at least one condition.

In some versions, the bacterium comprises a bacterium other than a member of the *Bifidobacterium* genus. In some versions, the bacterium comprises a member of lactic acid bacteria, such as a member of lactic acid bacteria other than a member of the *Lactococcus* genus. In some versions, the bacterium comprises a member of *Lactobacillus*, such as *Lactobacillus reuteri*.

A microorganism of the invention comprises a bacterium comprising a recombinant gene configured to express a polypeptide, wherein the bacterium is configured to produce and release the polypeptide and is capable of introducing the polypeptide in the bloodstream of the subject.

In some versions, the bacterium is capable of introducing the polypeptide in the bloodstream of the subject without the bacterium being substantially introduced in the bloodstream of the subject.

In some versions, the polypeptide is capable of treating a condition in a subject with systemic introduction of the polypeptide in the subject.

In some versions, the polypeptide is selected form the group consisting of IL-22, IL-35, insulin, leptin, cathelicidin related antimicrobial peptide, a peptide inhibitor of PCSK9, and an endolysin.

In some versions, the bacterium comprises a bacterium other than a member of the *Bifidobacterium* genus. In some versions, the bacterium comprises a member of lactic acid bacteria, such as a member of lactic acid bacteria other than a member of the *Lactococcus* genus. In some versions, the bacterium comprises a member of *Lactobacillus*, such as *Lactobacillus reuteri*.

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

FIGS. **1**A and **1**B show mutation rates of various types of bacteria.

FIG. **2** is a plasmid map of the pVPL3461 murine interleukin-22 (mIL-22)-expressing plasmid of the invention, showing an erythromycin resistance gene (Em), an chloramphenicol resistance gene coding sequence (Cm), a phelp promoter (Phelp) (Riedel et al. 2007) for the chloramphenicol resistance gene coding sequence, an mIL-22 coding sequence (mIL-22), a signal peptide for secretion of mIL-22 (SP), a promoter for the signal peptide and the mIL-22 coding sequence (Promoter), and an inverted repeat (IR), which serves as a transcriptional terminator.

FIGS. **3**A and **3**B show secretion of mIL-22 from engineered *L. reuteri* cells. FIG. **3**A shows mIL-22 secretion from *L. reuteri* cells harboring the pVPL3461 plasmid compared to wild-type *L. reuteri* cells as a control. FIG. **3**B shows mIL-22 secretion from *L. reuteri* cells harboring a chromosomal copy of a mIL-22 gene compared to wild-type *L. reuteri* cells as a control.

FIG. **4** shows a schema of methods for assessing delivery of mIL-22 to mice from orally administered *L. reuteri* cells harboring the pVPL3461 plasmid, including detecting plasma mIL-22 levels and determining expression of mIL-22 target genes reg3-beta and reg3-gamma.

FIG. **5** shows plasma IL-22 levels in sham gavaged mice (untreated), mice gavaged with wild-type *L. reuteri* (1×10^9 CFU), and mice gavaged with *L. reuteri* engineered to secrete IL-22 (1×10^9 CFU). Eight-week-old male C57BL/6 mice (n=8/group) were gavaged daily for 7 days. Blood was collected from the animals prior to gavage treatment (T=0) and one hour after gavage at the 7th day of administration (T=7). Center lines show the median values. Box limits indicate the 25th and 75th percentiles as determined by R software. Whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

FIG. **6** shows counts of bacteria detected in blood from the same mice described above for FIG. **5**.

FIG. **7** shows jejunal expression of mIL-22 target genes reg3-beta (reg3B) and reg3-gamma (reg3G) in the same mice described above for FIG. **5**. After 7 days gavage, animals were sacrificed, and part of the small intestine (jejunum) was subjected to total RNA isolation followed by cDNA synthesis and real-time PCR. Fold changes and significance are reported based on comparison to the untreated group, and data is normalized against the housekeeping gene 1-actin. Data were analyzed with the REST software package (Pfaffl et al. 2002). Data are presented in a box-whisker plot (see comments above with respect to FIG. **5** for details).

FIG. **8** shows liver expression of lipopolysaccharide-binding protein (LBP) in the same mice described above for FIG. **5**.

FIGS. **9**A-**9**C show results from length measurements in animals after eight weeks of high-fat diet feeding (T0) and after seven subsequent weeks of treatment (T7) of daily sham gavage of PBS without bacteria (sham), daily gavage of *L. reuteri* VPL1014 (LR), or daily gavage of the IL-22-secreting *L. reuteri* VPL3461 (LR-IL22). FIG. **9**A shows length measurements at T0. FIG. **9**B shows growth at T7. FIG. **9**C shows length measurements of live versus dead animals.

FIG. **10** shows growth hormone levels in the serum of the mice described above for FIGS. **9**A-**9**C at T7.

FIGS. **11**A and **11**B show percentage differences in body mass index (BMI) in the mice described above for FIGS. **9**A-**9**C. FIG. **11**A shows differences in BMI over the course of seven weeks of treatment (T7-T0). FIG. **11**B shows differences in BMI over the course of six weeks of treatment (T7-T1, wherein T1 refers to the time after one week of treatment).

FIGS. **12**A and **12**B show absolute liver weights and liver weights relative to mouse body weights in the mice described above for FIGS. **9**A-**9**C at T7.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides microorganisms such as bacteria that are capable of introducing polypeptides in the bloodstream of a subject. The invention provides microorganisms such as bacteria that are more specifically capable of introducing the polypeptide in the bloodstream of the subject without the microorganism itself being substantially introduced in the bloodstream of the subject. The invention also provides microorganisms such as bacteria that are capable of introducing the polypeptide systemically in the subject without the microorganism itself being substantially introduced systemically in the subject. "Introduce" and its grammatical equivalents refer to delivery to a site in the body. The introducing may result in detectable presence at that site. "Systemically introduce" and its grammatical equivalents refer to delivery to the bloodstream or sites in the body via the bloodstream. The systemic introducing may result in detectable presence in the bloodstream or such sites. The sites in which the polypeptides are systemically introduced include sites or tissues perfused with the bloodstream and which are permeable to polypeptides. The sites in which the polypeptides are systemically introduced include sites or tissues other than those in the gastrointestinal tract. Exemplary sites or tissues include the liver, muscles, lungs, kidneys, pancreas, adipose tissue, or any other site or tissue in the body.

The bacteria of the invention include certain commensal or probiotic bacteria. The bacteria may include non-pathogenic, Gram-positive bacteria capable of anaerobic growth. The bacteria are preferably viable in the gastrointestinal tract of mammals. The bacteria may be food grade.

Exemplary bacteria include species of lactic acid bacteria (i.e., species of the order Lactobacillales). The bacteria may include species of lactic acid bacteria other than species of the *Lactococcus* genus. The bacteria may include species other than species of the *Bifidobacterium* genus Exemplary bacteria more preferably include species of the *Lactobacillus* genus.

Exemplary species from the *Lactobacillus* genus include *L. acetototerans, L. acidifarinae, L. acidipiscis, L. acidophi-*

lus, 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.*

A particularly preferred bacterium is *L. reuteri.*

A bacterium that can bind mucus in the gastrointestinal tract is preferred but not required. We surmise that binding mucus in the gastrointestinal tract may place the bacterium in close proximity to epithelial cells. Release of polypeptides so close to the epithelial cells may help systemic delivery of the polypeptides. A bacterium capable of binding mucus is *L. reuteri*, such as *L. reuteri* VPL1014, discussed below in the examples. The ability to bind mucus can be mediated by the presence of a mucus-binding protein, such as the cell-mucus binding protein CmbA (Jensen et al. 2014).

The bacterium preferably has a low mutation rate. The bacterium preferably has a mutation rate of less than about 100×10^{-10} mutations per cell per generation, less than about 60×10^{-10} mutations per cell per generation, and more preferably less than about 20×10^{-10} mutations per cell per generation.

The bacterium may be engineered to express a polypeptide of interest. The bacterium accordingly may comprise a recombinant gene configured to express the polypeptide of interest. The bacterium may alternatively or additionally comprise a recombinant DNA sequence that results in increased expression of the polypeptide of interest. "Recombinant" used in reference to a gene refers herein to a sequence of nucleic acids that are not naturally occurring in the genome of the bacterium. The non-naturally occurring sequence may include a recombination, substitution, deletion, or addition of one or more bases with respect to the nucleic acid sequence originally present in the natural genome of the bacterium. "Gene" refers to the collection of genetic elements involved in expressing a coding sequence and may include, in addition to the coding sequence, a promoter, a ribosomal binding site, an enhancer, etc. In some versions, increased expression of the polypeptide of interest can result from introducing or modifying (e.g., recombining, substituting, deleting, etc.) genes or other genetic elements responsible for regulating expression of the polypeptide of interest, such as genes for transcription factors or signaling factors.

The bacterium may be engineered to produce and release the polypeptide. As used herein, "release" used with respect to the bacterium releasing the polypeptide refers to disposing the polypeptide outside the bacterium, i.e., in the extracellular environment of the microorganism. Release may occur through secretion of the polypeptide or through lysis of the bacterium, among other possible mechanisms. Elements for engineering a bacterium to secrete a polypeptide are well known in the art. Typical elements include a signal peptide-encoding sequence placed upstream of—and in-frame with—the coding sequence of the polypeptide to be secreted. The sequences of a large number of signal peptides for bacteria are known in the art. Exemplary signal peptide sequences are available at www.cbs.dtu.dk/services/SignalP/. Elements for inducing a bacterium to lyse include lytic proteins, which can be expressed from a bacterium through recombinant engineering. As used herein, "lytic protein" refers to any protein that causes or aids in the lysis of a microorganism.

Lytic proteins are well known in the art. A number of lytic proteins, for example, are found in bacteriophages and serve to lyse microorganisms during the lytic stages of the bacteriophage's life cycle. These include holins and lysins (Sheehan et al. 1999). During bacteriophage replication, biologically active lysins are present in the cytosol but require expression of a membrane protein, holin, to release the virions from the cell. When holin levels are optimal, the lysin can access the peptidoglycan layer for cleavage which leads to bacterial cell lysis (Wang et al. 2000). So far, five main groups of lysins have been identified that can be distinguished from one and another based on the cleavage specificity of the different bonds within the peptidoglycan (Fischetti 2009). Structurally, lysins can consist of a single catalytic domain, which generally is typical for lysins derived from bacteriophages targeting Gram-negative bacteria (Cheng et al. 1994). Bacteriophages targeting Gram-positive bacteria typically encode lysins that contain multiple domains: a N-terminal catalytic domain and a C-terminal cell-wall binding domain (Nelson et al. 2006, Navarre et al. 1999). A few lysins have been identified that have three domains (Becker et al. 2009).

A number of other lytic proteins are native to the microorganisms themselves (Feliza et al. 2012, Jacobs et al. 1994, Jacobs et al. 1995, López et al. 1997). These lytic proteins may affect cell wall metabolism or introduce nicks in the cell wall. Five protein classes are differentiated by the wall component they attack (Loessner et al. 2005, Loessner et al. 2002).

In some versions of the invention, the microorganism is configured to constitutively express a lysin and to express a holin in a maltose-dependent manner. In some versions, the microorganism is configured to express both a lysin and a holin in a maltose-dependent manner.

Lytic proteins can be expressed in a maltose-dependent manner by operably connecting the coding sequence of the lytic protein to a maltose-sensitive promoter. "Coding sequence" refers to a nucleic acid in a gene that encodes the gene product. "Promoter" refers to any nucleic acid that confers, activates, or enhances expression of an operably connected coding sequence. "Operably connected" generally refers to a connection of two genetic elements in a manner wherein one can operate on or have effects on the other. "Operably connected" used in reference to a promoter and a coding sequence refers to a connection between the promoter and the coding sequence such that the coding sequence is under transcriptional control of the promoter. For example, promoters are generally positioned 5' (upstream) of a coding sequence to be operably connected to the promoter. In the construction of heterologous promoter/coding sequence combinations, it is generally preferred to position the promoter at a distance from the transcription start site that is approximately the same as the distance between that promoter and the coding sequence it controls in its natural setting, i.e. in the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function.

Operably connecting a maltose-inducible promoter to the coding sequence of a lytic protein induces lysis of the microorganism, release of the lytic protein, and release of any other polypeptides made by the microorganism, in a maltose-dependent manner. Such release occurs in the gastrointestinal tract due to natural levels of maltose therein.

An exemplary maltose-inducible promoter is represented by SEQ ID NO: 1, which is a maltose-inducible promoter found in *L. reuteri*. The maltose-inducible promoter represented by SEQ ID NO: 1 or variants thereof are suitable for use in the present invention. Variants of SEQ ID NO:1 include sequences at least about 80% identical, at least about 83% identical, at least about 85% identical, at least about 87% identical, at least about 90% identical, at least about 83% identical, at least about 95% identical, at least about 97% identical, at least about 98% identical, or at least about 99% identical to SEQ ID NO: 1 Other methods of inducing lysis of bacteria in vivo are known.

The bacteria can be engineered using any methods known in the art. General methods are provided in Green et al. 2012. Methods for engineering lactic acid bacteria such as *L. lactis* are provided by van Pijkeren et al. 2012, Oh et al. 2014, and Barrangou et al. 2016.

The recombinant gene may be incorporated into the chromosome of the bacterium or may be included on an extra-chromosomal plasmid. The extra-chromosomal plasmid may replicate at any copy number in the cell and, accordingly, be a single-copy plasmid, a low-copy plasmid, or a high-copy plasmid. The extra-chromosomal plasmid is preferably substantially stable within the bacterium. The rate of loss of the extra-chromosomal plasmid from the bacterium is preferably less than about 10% per generation, less than about 5% per generation, or less than about 1% per generation, wherein percent per generation refers to the percent of the population per generation in which the plasmid is lost.

The bacterium may be engineered to produce and release any polypeptide of interest. The polypeptide may have any of a number of amino acid chain lengths. In some versions, the polypeptide may have an amino acid chain length of from about 2 to about 4000 amino acids, from about 2 to about 3000 amino acids, from about 2 to about 2000 amino acids, from about 2 to about 1500 amino acids, from about 2 to about 1000 amino acids, from about 2 to about 500 amino acids, from about 3 to about 250 amino acids, or from about 3 to about 225 amino acids. The polypeptide may have a net positive charge at neutral pH, a net negative charge at neutral pH, or a net neutral charge at neutral pH. The polypeptide is preferably soluble in water. The polypeptide may form a globular or fibrous structure or may have an intrinsically disordered structure.

The polypeptide may have any of a number of functionalities. The polypeptide, for example, may be enzymatic or non-enzymatic. The polypeptide may be fluorescent or non-fluorescent. Within the physiological context of a mammal, the polypeptide may be a cytokine, a hormone, an antibody, an antimicrobial peptide, and an antigenic peptide, among others.

Exemplary classes of cytokines include interleukins, lymphokines, monokines, interferons (IFNs), colony stimulating factors (CSFs), among others. Specific exemplary cytokines include IL-1 alpha (IL1a), IL-1 beta (IL1b), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, IL-33, IL-35, IL-36, IFN-alpha, IFN-beta IFN-gamma, TNF-alpha, TNF-beta, CNTF (C-NTF), LIF, OSM (oncostatin-M), EPO (erythropoietin), G-CSF (GCSF), GM-CSF (GMCSF), M-CSF (MCSF), SCF, GH (growth hormone), PRL (prolactin), aFGF (FGF-acidic), bFGF (FGF-basic), INT-2, KGF (FGF7). EGF, TGF-alpha, TGF-beta, PDGF, betacellulin (BTC), SCDGF, amphiregulin, and HB-EG, among others.

Exemplary hormones include epinephrine, melatonin, triiodothyronine, thyroxine, amylin (or islet amyloid polypeptide), adiponectin, adrenocorticotropic hormone (or corticotropin), angiotensinogen, angiotensin, antidiuretic hormone (or vasopressin, arginine vasopressin), atrial-natriuretic peptide (or atriopeptin), brain natriuretic peptide, calcitonin, cholecystokinin, corticotropin-releasing hormone, cortistatin, encephalin, endothelin, erythropoietin, follicle-stimulating hormone, galanin, gastric inhibitory polypeptide, gastrin, ghrelin, glucagon, glucagon-like peptide-1, gonadotropin-releasing hormone, growth hormone-releasing hormone, hepcidin, human chorionic gonadotropin, human placental lactogen, growth hormone, inhibin, insulin, insulin-like growth factor (or somatomedin), leptin, lipotropin, luteinizing hormone, melanocyte stimulating hormone, motilin, orexin, oxytocin, pancreatic polypeptide, parathyroid hormone, pituitary adenylate cyclase-activating peptide, prolactin, prolactin releasing hormone, relaxin, renin, secretin, somatostatin, thrombopoietin, thyroid-stimulating hormone (or thyrotropin), thyrotropin-releasing hormone, and vasoactive intestinal peptide, among others.

Other physiologically active peptides include glucagon-like peptide-1 (GLP-1); tachykinin peptides, such as substance P, kassinin, neurokinin A, eledoisin, and neurokinin B; peptide PHI 27 (peptide histidine isoleucine 27); pancreatic polypeptide-related peptides, such as NPY (neuropeptide Y), PYY (peptide YY), and APP (avian pancreatic polypeptide); opioid peptides, such as proopiomelanocortin (POMC) peptides and prodynorphin peptides; AGG01; B-type natriuretic peptide (BNP); lactotripeptides; and peptides that inhibit PCSK9 (Zhang et al. 2014).

Exemplary antibodies include single-chain antibodies, single-domain antibodies (sdAbs), and single-chain variable fragments (scFvs).

Exemplary antimicrobial peptides include cathelicidins, defensins, protegrins, mastoparan, poneratoxin, cecropin, moricin, melittin, magainin, dermaseptin, and others.

In preferred versions, the polypeptide that is systemically introduced is a polypeptide capable of treating a condition in a subject with its systemic introduction. In some versions, The polypeptide that is systemically introduced is a polypeptide capable of treating a condition in a subject with its systemic introduction but is not capable of treating a condition in the subject with its local introduction to the gastrointestinal tract alone. The condition may be any condition described herein.

The inventors have unexpectedly found that administering *L. reuteri* to the gastrointestinal tract is capable of delivering produced polypeptides to the bloodstream without the bacteria themselves being introduced in the bloodstream. Accordingly, an aspect of the invention includes administering an amount of a bacterium of the invention into the gastrointestinal tract of a subject. The bacterium may be administered in any amount effective to introduce the polypeptide in the bloodstream of the subject. Exemplary amounts include from about 1×10^3 to about 1×10^{15}, from about 1×10^5 to about 1×10^{13}, from about 1×10^7 to about 1×10^{11}, or about 1×10^9 colony forming units (CFU). Amounts above and below these ranges may be acceptable.

The bacterium can be administered to the gastrointestinal tract by any method known in the art. The bacterium may be administered orally, rectally, or directly into the gastrointestinal tract via a stoma. The bacterium is preferably administered directly into or upstream of the small intestines, so that the bacterium ultimately passes through or into the small intestines. The bacterium may be swallowed or introduced via a tube.

The bacterium may 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 bacterium. The precise nature of the carrier or other material may depend on the route of administration. The composition may be liquid, solid, or semi-solid. The composition may comprise a foodstuff or may take the form of a pharmaceutical composition. Those of relevant skill in the art are well able to prepare suitable compositions.

The subject to which the bacterium is administered may be an animal, such as a mammal or, more specifically, a human.

In some versions of the invention, the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream in an amount effective to induce at least one systemic effect in the subject, such as at least one effect in a non-gastrointestinal tissue in the subject. As used herein, "systemic effect" refers to an effect that occurs at a site or tissue in the body other than where the polypeptide is initially released from the bacterium. In the present case, the term refers to an effect that occurs at a site or tissue other than the gastro-intestinal tract, such as the liver, muscles, lungs, kidneys, pancreas, adipose tissue, or others. The systemic effect preferably occurs by virtue of the polypeptide being systemically introduced and accessing a site or tissue other than gastrointestinal tissue via bloodstream, such that the effect is a direct effect of the polypeptide and is not a secondary effect of a primary effect of the polypeptide in the gastrointestinal tissue. Such effects are referred to herein as "direct systemic effects." Direct systemic effects of the systemically introduced polypeptide can be distinguished from secondary effects, for example, by comparing effects resulting from administering the polypeptide-producing bacterium into the gastrointestinal tract with effects resulting from systemically administering the polypeptide directly into the bloodstream and effects resulting from locally administering the polypeptide directly into the gastrointestinal tract. Direct systemic effects are those that are mirrored by the systemic administration of the polypeptide into the bloodstream but not the local administration of the polypeptide into the gastrointestinal tract. The presence of direct systemic effects of the polypeptide resulting from administering the polypeptide-producing bacterium into the gastrointestinal tract can be an indicator of the polypeptide entering the bloodstream, whether or not the polypeptide itself is detected in the bloodstream.

In some versions, the bacterium is administered to a subject that suffers from a condition treatable with systemic introduction of the polypeptide. In yet other versions, the bacterium is administered to a subject that suffers from a condition treatable with systemic introduction of the polypeptide but is not treatable with only local introduction of the polypeptide to the gastrointestinal tract. In either case, the polypeptide is introduced in the bloodstream of the subject in an amount effective to treat the condition. As used herein, "treat" used in reference to a condition refers to ameliorating to any extent the condition itself or any symptom associated therewith.

In some versions, the bacterium is administered to a subject to prevent or inhibit the development of any of the conditions or associated symptoms described herein. The subject in such a case may show early signs of the condition or symptom, have a genotype that predisposes the subject to develop the condition or symptom, have a behavior or environmental situation that predisposes the subject to develop the condition or symptom, or otherwise be predisposed to develop the condition or symptom.

A particular aspect of the invention is directed to introducing interleukin-22 (IL-22) in the bloodstream of a subject by administering a bacterium comprising a recombinant interleukin-22 (IL-22) gene. The IL-22 may be introduced in the bloodstream of the subject in an amount effective to induce at least one IL-22-dependent effect in a non-gastrointestinal tissue in the subject. An exemplary IL-22 that can be systemically introduced comprises a sequence of SEQ ID NO:2 or a sequence at least about 90%, 95%, 97%, 99% or more identical thereto.

IL-22 is capable of inducing a number of effects in non-gastrointestinal tissues when circulating systemically through the bloodstream. In respiratory epithelial cells, for example, IL-22 can increase antibacterial defense, elevate mucus production, enhance proliferation, and raise production of granulocyte-attracting chemokines. In synovial fibroblasts, IL-22 can elevate RANKL expression and increase production of monocyte-attracting chemokines. In pancreatic cells, IL-22 can increase protection against damage, inhibit autophagy, and enhance islet proliferation. In hepatocytes, IL-22 can increase acute-phase protein production, increase protection against damage, and elevate liver progenitor cell proliferation. In epidermal keratinocytes, IL-22 can increase antibacterial defense, retard differentiation and cornification, induce production of granulocyte-attracting chemokines, elevate migration and tissue remodeling, and enhance STAT3 and IL-20 expression. See Sabat et al. 2014 and Wang et al. 2014 for additional direct IL-22-dependent effects.

Another particular aspect of the invention is directed to introducing interleukin-22 (IL-22) in the bloodstream of a diabetic subject by administering a bacterium comprising a recombinant interleukin-22 (IL-22) gene. The diabetic subject may suffer from type 1 or type 2 diabetes. IL-22 has been shown to have a number of ameliorative effects in diabetic subjects. In type 2 diabetic subjects, these effects include ameliorating hyperglycemia, insulin resistance, hyperlipidemia, lipid dysregulation in the liver and adipose tissues, endotoxemia, and chronic inflammation. See Wang et al. 2014. As shown in the present examples, IL-22 is also capable of reducing body mass index (BMI).

More generally, subjects in which IL-22 is introduced may include those suffering from of insulin resistance, hyperglycemia, lipid dysregulation, hyperlipidemia, obesity, or other manifestations of metabolic syndrome. The systemic effect of the systemic introduction of IL-22 to such subjects may include a reduction in body mass index (BMI), liver weight, liver triglycerides, glucose intolerance, and insulin resistance, or other effects described elsewhere herein.

Another polypeptide that may be introduced in the bloodstream of a subject with the bacteria of the invention is interleukin-35 (IL-35). Systemic administration of IL-35 treats type-1 diabetes and inhibits or slows the development of type-1 diabetes. See Singh et al. 2015. An exemplary IL-35 that can be systemically introduced is a human recombinant IL-35 comprising a sequence of SEQ ID NO:3 or a sequence at least about 90%, 95%, 97%, 99% or more identical thereto. Bacteria of the invention comprising a recombinant IL-35 gene can be administered to subjects with diabetes, such as type 1 diabetes, to systemically introduce the IL-35 polypeptide in the bloodstream of the subject in an amount effective to treat the diabetes in the subject.

Another polypeptide that may be introduced in the bloodstream of a subject with the bacteria of the invention is insulin. The insulin can be produced in single-chain form. See, e.g., Rajpal et al. 2009. An exemplary insulin that can be systemically introduced includes the insulin A-chain connected to the insulin B-chain by the linker sequence QRGGGGGQR (SEQ ID NO:4). See Rajpal et al. 2009. Single-chain insulin retains all of the physiological effects of traditional two-chain insulin, including stimulation of glucose uptake into adipocytes, and suppression of hepatic gluconeogenesis. Bacteria of the invention comprising a recombinant insulin gene can be administered to subjects with diabetes, insulin resistance, or hyperglycemia to systemically introduce the insulin polypeptide in the bloodstream of the subject in an amount effective to treat the diabetes, or hyperglycemia in the subject.

Another polypeptide that may be introduced in the bloodstream of a subject with the bacteria of the invention is leptin. Leptin is made by adipose tissue and regulates energy balance by acting on receptors in the brain. Congenital leptin deficiency (CLD), or generalized lipodystrophy results in a lack of leptin and can lead to a litany of disorders, including morbid obesity (in the case of CLD), diabetes, and infertility. Systemic leptin replacement therapy mitigates these disorders. An exemplary leptin polypeptide that can be systemically introduced includes a polypeptide comprising a sequence of SEQ ID NO:5 or a sequence at least about 90%, 95%, 97%, 99% or more identical thereto. Bacteria of the invention comprising a recombinant leptin gene can be administered to subjects with congenital leptin deficiency or generalized lipodystrophy to systemically introduce the leptin polypeptide in the bloodstream of the subject in an amount effective to treat the obesity, diabetes, infertility or any other aspect of the congenital leptin deficiency or generalized lipodystrophy.

Another polypeptide that may be introduced in the bloodstream of a subject with the bacteria of the invention is cathelicidin related antimicrobial peptide (CRAMP). CRAMP is a small peptide produced by pancreatic islets in response to gut microbiota-derived short-chain fatty acids. Synonyms for CRAMP include CAMP, CAP18, Cnlp, FALL39, and MCL. The islet-derived CRAMP maintains immune homeostasis. CRAMP production is defective in non-obese diabetic mice, leading to inflammation and activation of diabetogenic T-cells and resulting in type 1 diabetes (Sun et al. 2015). This process can be reversed by direct systemic administration of CRAMP. An exemplary CRAMP polypeptide that can be systemically introduced includes a polypeptide comprising a sequence of SEQ ID NO:6 or a sequence at least about 90%, 95%, 97%, 99% or more identical thereto. Bacteria of the invention comprising a recombinant CRAMP gene can be administered to non-obese diabetic subjects to systemically introduce the CRAMP polypeptide in the bloodstream of the subject in an amount effective to treat the diabetes and/or inflammation in these subjects.

Other polypeptides that may be introduced in the bloodstream of a subject with the bacteria of the invention include peptide inhibitors of PCSK9. PCSK9 (proprotein convertase subtilisin/kexin type 9) is a negative regulator of the hepatic low density lipoprotein receptor. Inhibition of PCSK9 results in LDL cholesterol-lowering effects. A number of peptide inhibitors of PCSK9 are known in the art. See Zhang et al. 2014. Any of these polypeptides or others can be introduced in the bloodstream of a subject with the bacteria of the invention. A particularly preferred peptide inhibitor of PCSK9 is referred to as "Pep2-8," which comprises a sequence of SEQ ID NO:7. Bacteria of the invention comprising a recombinant gene configured to express one or more peptide inhibitors of PCKS9 can be administered to subjects with hypercholesterolemia to systemically introduce the peptide inhibitor of PCSK9 in the bloodstream of the subject in an amount effective to treat the hypercholesterolemia.

Other polypeptides that may be introduced in the bloodstream of a subject with the bacteria of the invention include lysins, such as bacteriophage-derived lysins (endolysins), i.e. enzybiotics. One of the largest concerns in 21st century medicine is the development of microbial antibiotic-resistance. Little progress has been made in the discovery and development of novel antibiotics, and bacteriophage-derived lysins (enzybiotics) constitute promising alternatives to antibiotics. The enzybiotics interfere with peptidoglycan cell wall synthesis, mainly of Gram positive bacteria, but do so in a species specific manner. Exemplary lysins that can be systemically introduced include those described in the references cited herein, all of which are incorporated herein by reference. Bacteria of the invention comprising a recombinant gene configured to express one or more lysins can be administered to subjects with sepsis or infection with pathogens such as *S. aureus* to systemically introduce the lysin in the bloodstream of the subject in an amount effective to treat the sepsis or infection.

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

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.

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

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.

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.

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

Bacterial strains, plasmids, media, and culture.

Bacterial strains and plasmids used in the present examples are listed in Table 1. *Lactobacillus reuteri* VPL1014 and its derivatives were routinely cultured at 37° C. in deMan Rogosa Sharpe (MRS) medium (Difco, BD Biosciences). Where appropriate, erythromycin was added to a final concentration of 5 μg/ml. Competent cells of *L. reuteri* were prepared as described before (Ahrnd et al. 1992). To test IL-22 expression and to prepare bacteria for animal experiments, bacteria were cultured in Lactobacilli Defined Medium-III (LDM-III, Table 2).

Specifically, *L. reuteri* VPL1014 was inoculated in 10 ml MRS broth, and *L. reuteri* VPL3461 was inoculated in 10 ml MRS containing 5 g/ml erythromycin at 37° C. Overnight-cultures of each strain were sub-cultured in MRS at OD_{600}=0.1. At OD600≥1≤1.2 cells were harvested by centrifugation (5,000 rpm for 5 min), and the cell pellets were washed twice with LDM-III. Washed cell pellets, each derived from 10-ml culture, were stored at −80° C. until use. From this step onwards, no antibiotics were supplemented to the culture media. At the day of gavaging, the cell pellets were resuspended in 10 ml freshly prepared pre-warmed LDM-III and incubated at 37° C. until OD_{600}≥3.5≤3.7. Bacteria were concentrated 10-fold, and suspensions were made containing ~10^{10} CFU/ml *L. reuteri* VPL1014 or VPL3461. For animal experiments (see below) 100 al of the suspension was administrated by oral gavage to the corresponding animals.

TABLE 1

Bacterial strains and plasmids used in the present examples.

Strain or plasmid	Characteristics	Source
L. reuteri VPL1014	A derivative of *L. reuteri* ATCC PTA 6475, human breast milk isolate	ATCC PTA 6475, U.S. Pat. 7,344,867 to BioGaia AB (Lerum, SE), and van Pijkeren et al. 2012
L. reuteri VPL3461	L. reuteri VPL1014 harboring pVPL3461	Described herein
pJP028	Em^R, derivative of pNZ8048 containing promoter from *L. reuteri* SD2112, signal peptide from *L. reuteri* JCM1112, and LPXTG (cell wall anchor domain)	Described herein
pVPL3461	Em^R, derivative of pJP028 omitting LPXTG domain and harboring mIL-22 gene	Described herein

TABLE 2

Lactobacilli Defined Medium-III (LDM3) composition.

Basal Medium	Amount per Liter	
Ingredient		
K_2HPO_4.	1.50 g	
KH_2PO_4	1.50 g	
Sodium Acetate	15.00 g	
Sodium Citrate, Dihydrate	0.25 g	
Tryptophan	0.05 g	
Asparagine, Monohydrate	0.23 g	
Vitamin-free Casamino acid	10.00 g	
Cysteine-HCl, Monohydrate	0.22 g	
Tween80 (10% v/v)	10 ml	
Dissolve in 937.5 ml of DI water and autoclave at 121° C. for 15 min. Prepare stock solutions (as below) and add to sterile Basal Medium.		
Vitamin Solution (in 25 ml dH_2O)		
Thiamin HCl	10 mg	0.5 ml
ρ-aminobenzonoic acid	2 mg	
Calcium pantothenic acid	20 mg	
Niacin	50 mg	
Pyridoxin HCl	25 mg	
Biotin Solution (in 50 ml 0.01M HCl)		
Biotin	4 mg	0.5 ml
96% EtOH	40 μL	
Riboflavin Solution (in 50 ml dH_2O)		
Riboflavin	4 mg	5 ml
Folic Acid Solution (in 50 ml 0.001M NaOH)		
Folic acid	10 mg	0.5 ml
Nucleic Acid Solution (in 15 ml 1M HCl)		
Adenine hemisulfate	50 mg	3 ml
Guanine	40.3 mg	
Cytidylic acid	150 mg	
Uracil Solution (in 10 ml 1M NaOH)		
Uracil	200 mg	1 ml
Thymidine Solution (in 12.5 ml dH_2O)		
Thymidine	20 mg	1 ml
Salt Solution (in 10 ml dH_2O)		
$MgSO_4$	0.793 g	1 ml

TABLE 2-continued

Lactobacilli Defined Medium-III (LDM3) composition.		
$MnSO_4$	0.128 g	
$FeSO_4$	0.130 g	
Glucose (40% w/v)		50 ml
Total 1 L (LDM), final pH6.5		

Mutation Rate.

A suitable microorganism for delivering peptides preferably has a low mutation rate. The mutation rate of *L. reuteri* VPL1014 was compared with that of a number of other microorganisms using conventional methods (Rosche et al. 2000, Foster et al. 2006). As shown in FIGS. **1**A and **1**B, *L. reuteri* displayed an exceptionally low mutation rate, particularly compared to the other tested probiotic microorganisms.

Reagents and Enzymes.

Cloning was performed via ligation cycle reaction (LCR; Kok et al. 2014). Enzymes and reagents for LCR were purchased from Fermentas. Polymerase chain reaction (PCR) for cloning purposes was performed with the high-fidelity enzyme Phusion Hot Start Polymerase II (Fermentas). PCR for screening purposes was performed with Taq polymerase (Denville Scientific). To concentrate the LCR reaction prior to electrotransformation into *L. reuteri*, we used Pellet Paint Co-Precipitant (Novagen). Oligonucleotides and synthetic double-stranded DNA fragments were purchased from Integrated DNA Technologies. All oligonucleotides and synthetic DNA fragments used in this study are listed in Table 3.

TABLE 3

Oligonucleotides and synthetic DNA used in the present examples.

Oligo-nucleo-tides	Sequence
oVPL329	attccttggacttcatttactgggtttaac (SEQ ID NO: 8)
oVPL363	taatatgagataatgccgactgtac (SEQ ID NO: 9)
oVPL1219	ttcatggggatgaatgcttctgctaatacattaccagttaa tactcgttg (SEQ ID NO: 10)
oVPL1220	cttggttttctaattttggttcaaagatcaaacacaagcat tacgtaaactc (SEQ ID NO: 11)
oVPL1221	gcttgaaacgttcaattgaaatggca (SEQ ID NO: 12)
oVPL1222	tgtaaaaccaataaggactgaagc (SEQ ID NO: 13)
oVPL1223	ggagttgcttcagtccttattggttttacattcatggggat gaatgcttctgctaataca (SEQ ID NO: 14)
oVPL1224	tgatctttgaaccaaaattagaaaaccaaggcttgaaacgt tcaattgaaatggcaatta (SEQ ID NO: 15)
oVPL1313	actccctgaagaatataccctcc (SEQ ID NO: 16)

TABLE 3-continued

Oligonucleotides and synthetic DNA used in the present examples.

Oligo-nucleo-tides	Sequence
oVPL1314	cgctattgagcacagatacgag (SEQ ID NO: 17)
oVPL1315	atgcttccccgtataaccatca (SEQ ID NO: 18)
oVPL1316	ggccatatctgcatcataccag (SEQ ID NO: 19)
oVPL1321	gatcaccgacaagggcctg (SEQ ID NO: 20)
oVPL1322	ggctatgaaactcgtactgcc (SEQ ID NO: 21)
oVPL1325	ggctgtattcccctccatcg (SEQ ID NO: 22)
oVPL1326	ccagttggtaacaatgccatgt (SEQ ID NO: 23)
gVPL1	ATTCATGGGGATGAATGCTTCTGCTAATACATTACCAGTTA ATACTCGTTGTAAATTAGAAGTTAGTAATTTTCAACAACCA TATATTGTTAATCGTACTTTTATGTTAGCTAAAGAAGCTAG TTTAGCTGATAATAATACTGATGTTCGTTTAATTGGTGAAA AATTATTTCGTGGTGTTAGTGCTAAAGATCAATGTTATTTA ATGAAACAAGTTTTAAATTTTACTTTAGAAGATGTTTTATT ACCACAAAGTGATCGTTTTCAACCATATATGCAAGAAGTTG TTCCATTTTTAACTAAATTAAGTAATCAATTAAGTAGTTGT CATATTAGTGGTGATGATCAAAATATTCAAAAAAATGTTCG TCGTTTAAAAGAAACTGTTAAAAAATTAGGTGAAAGTGGTG AAATTAAAGCTATTGGTGAATTAGATTTATTATTTATGAGT TTACGTAATGCTTGTGTTTGATCTTTGAACCAAAATTAGAA AACCAAGG (SEQ ID NO: 24)

Construction of *L. reuteri* VPL1014 that Secretes mIL-22.

Our aim was to engineer *Lactobacillus reuteri* VPL1014 to secrete the murine cytokine interleukin-22 (mIL-22). We first opted for expression from the multicopy plasmid pJP028 to maximize mIL-22 production. pJP028 is a derivative of pNZ8048 (de Ruyter et al. 1996) in which the nisin-expression cassette was replaced with a secretion cassette. By PCR (oVPL1221-oVPL1222), we amplified the backbone of pJP028, omitting the cell wall anchor domain, to yield a 4.579 kb product. For optimal expression of mIL-22 in our expression host, *L. reuteri*, we first applied in-silico codon optimization of the mIL-22 coding sequence using the online software, OPTIMIZER (genomes.urv.es/OPTIMIZER/, Table 4) followed by synthesis. The resulting synthetic product (gVPL1) was amplified by PCR (oVPL1219 and oVPL1220), followed LCR (Kok et al. 2014) placing the gVPL1 fragment between the start and stop codon located on the pJP028 backbone. The LCR mixture was precipitated and transformed in *L. reuteri* VPL1014. Transformants were screened by PCR (oVPL329-oVPL363) to confirm cloning of mIL-22. One positive clone was colony purified, a 1.584 kb amplicon was generated by colony PCR, and the integrity of the construct was confirmed by DNA sequencing (GeneWiz). The resultant strain was named VPL3461. We hereafter refer to pVPL3461 when it concerns the plasmid that encodes codon-optimized mIL-22 (FIG. **2**).

The nucleotide sequence of pVPL3461 is represented by SEQ ID NO:25. The nucleotide sequence of the IL-22 promoter (*L. reuteri* native promoter) in pVPL3461 is represented by SEQ ID NO: 26. The nucleotide sequence encoding the signal peptide (SP) in pVPL3461 is represented by SEQ ID NO:27. The nucleotide sequence encoding IL-22 in pVPL3461 is represented by SEQ ID NO:28. The nucleotide sequence of the inverted repeat (IR) in pVPL3461 is represented by SEQ ID NO:29. The nucleotide sequence of the chloramphenicol marker (Cm) in pVPL3461 is represented by SEQ ID NO:30. The nucleotide sequence of the Phelp promoter in pVPL3461 is represented by SEQ ID NO:31. The nucleotide sequence of the erythromycin marker (Em) in pVPL3461 is represented by SEQ ID NO:32.

Additionally, a construct from the pVPL3461 plasmid comprising the promoter, signal peptide, and mIL-22 coding sequence was excised from pVPL3461 and incorporated in the *L. reuteri* chromosome using methods known in the art. The resultant strain was named VPL3461chr.

Animal Trial.

Twenty-four 6-week old male B6 mice (C57BL/6J) were purchased from Jackson Labs (Bar Harbor, Me.). Animals were housed at an environment-controlled facility with a 12-hour light and dark cycle. Both diet (standard chow, LabDiet, St Louis, Mo.) and water were freely available to the animals. After transport, animals were allowed to adjust to the new environment for two weeks, after which treatment by gavage started. Three groups of 8 animals per group were treated daily for 7 consecutive days. Treatments were sham gavage where the animals were subjected to insertion of a gavaging needle without administering anything (control), gavage of *L. reuteri* VPL1014 (WT group) and gavage of *L. reuteri* VPL3461 (LR_mIL-22). Bacterial load administrated was ~1×10^9 CFU in a volume of 100 al of the respective bacterial supernatant. See FIG. **4**.

TABLE 4

Codon optimization table for *L. reuteri* F275.*#
Fields: [sequence of nucleotide triplet] [frequency: per thousand] ([number])

UUU 29.5 (16802)	UCU 9.7 (5521)	UAU 24.8 (14106)	UGU 4.4 (2479)
UUC 11.3 (6447)	UCC 4.0 (2249)	UAC 12.4 (7068)	UGC 1.5 (861)
UUA 36.4 (20706)	UCA 15.0 (8537)	UAA 2.3 (1307)	UGA 0.4 (219)
UUG 15.4 (8765)	UCG 4.3 (2458)	UAG 0.7 (374)	UGG 10.8 (6134)
CUU 22.1 (12555)	CCU 9.7 (5489)	CAU 15.3 (8708)	CGU 14.1 (7995)
CUC 6.3 (3597)	CCC 3.4 (1927)	CAC 8.0 (4562)	CGC 5.9 (3360)
CUA 9.4 (5359)	CCA 17.7 (10090)	CAA 35.0 (19877)	CGA 8.6 (4869)
CUG 5.3 (3005)	CCG 6.0 (3428)	CAG 11.7 (6653)	CGG 9.9 (5610)
AUU 50.7 (28857)	ACU 22.3 (12657)	AAU 36.1 (20541)	AGU 15.6 (8850)
AUC 16.7 (9524)	ACC 9.8 (5550)	AAC 15.8 (8968)	AGC 6.7 (3786)
AUA 5.8 (3300)	ACA 16.6 (9464)	AAA 36.6 (20829)	AGA 3.3 (1872)
AUG 26.9 (15321)	ACG 9.2 (5230)	AAG 30.3 (17232)	AGG 1.4 (792)
GUU 35.0 (19884)	GCU 29.0 (16508)	GAU 43.5 (24740)	GGU 24.3 (13830)
GUC 9.2 (5210)	GCC 12.4 (7053)	GAC 15.2 (8617)	GGC 10.9 (6178)
GUA 16.1 (9176)	GCA 25.3 (14409)	GAA 45.6 (25918)	GGA 19.8 (11244)
GUG 7.7 (4354)	GCG 9.8 (5573)	GAG 11.2 (6360)	GGG 10.1 (5771)

*This table was made based on 568,715 codons among 1,900 CDSs on chromosomal DNA of strain F275.
#Coding GC 39.50% 1st letter GC 51.33% 2nd letter GC 35.17% 3rd letter GC 32.00%.

Determine mIL-22 Secretion.

Strains *L. reuteri* VPL1014 and VPL3461 were cultured in LDM-III as described above, and the supernatants were collected after centrifugation (5 min at 3,214×g), followed by filter-sterilization (0.22 am, Millipore). One hundred microliters of filter-sterilized supernatant from *L. reuteri* VPL1014 and VPL3461 was assessed for the presence of mIL-22 by ELISA (R&D systems). Production of mIL-22 could not be detected for *L. reuteri* 6575-VPL (15 pg/ml cut-off limit), while strain VPL3461 secreted mIL-22 at levels of 164.2±13.1 ng/ml (n=3). See FIG. **3**A.

Secretion of mIL-22 from VPL3461chr was similarly tested. VPL3461chr showed detectable mIL-22 secretion. See FIG. **3**B.

Plasmid Stability of pVPL3461 in *L. reuteri* VPL1014.

Prior to assessing biological activity of the *L. reuteri*-produced mIL-22 in mice, we first assessed the stability of pVPL3461. Normally, selection of plasmids is achieved by supplementation of an antibiotic, but we wanted to avoid the supplementation of antibiotics in mice to maintain a fully competent microbiota. VPL3461 was cultured overnight in LDM-III (supplemented with antibiotics). Cells were washed to remove residual antibiotics, followed by subculturing to antibiotic-free LDM-III (OD600=0.1). After 1 passage (20 hr, ~10 generations), we showed that 96% of the cell population was resistant to erythromycin, demonstrating that the rate of loss of pVPL3461 was 0.4% per generation, and would be considered stable enough for in-vivo assessment of biological activity.

Blood collection to assess plasma IL-22 levels.

At T=0 (prior to the start of treatment) and at T=7 (2 hours after the last gavage) of the animal trial, blood was collected (50 al per animal) via retro orbital puncture. Plasma was isolated from whole blood sample by centrifugation at 9,000 rpm for 7 min and the plasma fraction was stored at −80° C. until use. By ELISA (as described above) we determined plasma mIL-22 levels. See FIG. **4**.

Plasma IL-22 levels after 7 days gavage are shown in FIG. **5**. The mice administered *L. reuteri* VPL3461 showed a statistically significant increase in plasma IL-22 levels compared to controls.

We also assessed whether the administered *L. reuteri* VPL1014 and *L. reuteri* VPL3461 could be detected in the bloodstream of the animals after 7 days gavage. From each animal, 50 μl blood was plated on deMan Rogosa Sharp (MRS) medium that is selective for a broad range of lactic acid bacteria, including *L. reuteri*. The results are shown in FIG. **6**. The prolonged daily administration of *L. reuteri* did not increase the total number of lactic acid bacteria in the bloodstream. As determined by colony morphology, the bacteria detected in the bloodstream of animals that were gavaged with *L. reuteri* were not *L. reuteri*. *L. reuteri* yields (after 48 h) on MRS plates small-medium sized colonies that are opaque. From the 3 animals in which we detected bacteria in the bloodstream, all colonies were pigmented, mostly yellow, and some were red-ish. Some colonies were also extremely large. The size combined with the pigmented phenotype made it evident that the recovered bacteria in the bloodstream were not *L. reuteri*.

These results indicate that the systemic increase of IL-22 was not a result of *L. reuteri* VPL3461 itself entering the bloodstream.

cDNA Synthesis.

To assess biological functionality of *L. reuteri* secreted mIL-22, we assess gene expression levels of reg3-beta and reg3-gamma. Both genes are known to be upregulated by IL-22 (Loonen et al. 2013, Sovran et al. 2015). Part of the small intestine (jejunum) of each animal was processed for RNA isolation. First, samples were homogenized (Omni TH, Omni International) followed by RNA isolation and on-column DNaseI treatment (Qiagen), after which an additional DNase treatment was conducted (RQI DNase; Promega, Madison, Wis.). RNA was quantified by Qubit analysis (Invitrogen). One ag RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Richmond, Calif.). See FIG. **4**.

Quantitative Real-Time PCR.

Relative gene expression levels were determined using the CFX96™ real-time PCR (Bio-Rad). Expression of reg3-beta and reg3-gamma was determined relative to that of the housekeeping gene β-actin. The qRT-PCR was performed with the SYBR Green PCR master mix (Bio-Rad). Primers for amplification of: reg3b (oVPL1313-oVPL1314), reg3g (oVPL1315-oVPL1316), and β-actin (oVPL1325-oVPL1326) are listed in Table 3. Gene expression of the reg genes in the jejunum tissues relative to β-actin was determined by the Relative Expression Software Tool (REST), which allows comparison of gene expression between groups of animals (Pfaffl et al. 2001 and 2002).

As shown in FIG. **7**, mice administered IL-22-expressing *L. reuteri* VPL3461 showed an average of 4.7-fold and 3-fold increased expression of reg3-beta and reg3-gamma, respectively in the jejunum compared to mice administered wild-type *L. reuteri*, demonstrating that the secreted IL-22 is biologically active.

We also determined liver expression of the gene encoding the lipopolysaccharide-binding protein (LBP), which is known to be regulated by IL-22. Expression levels were relative to that of the control (1-actin). As shown in FIG. **8**, liver LBP expression was not changed in animals that received wild-type *L. reuteri*. Animals administered the IL-22-expressing *L. reuteri* VPL3461 displayed an increased level of LBP gene expression, varying from 1.5-fold to 3.5-fold. We did not detect a statistical difference in gene expression, but we predict including more animals per group will show a statistical difference.

Metabolic Syndrome Trial.

IL-22 has been shown to alleviate metabolic disorders and provide other therapeutic effects in diabetic subjects. See Wang et al. 2014. We tested whether administering IL-22-secreting *L. reuteri* to mice with diet-induced obesity could recapitulate these effects.

Thirty-six 6-week old male B6 mice (C57BL/6J) were purchased from Jackson Labs (Bar Harbor, Me.). Animals were housed at an environmental controlled facility with a 12-hour light and dark cycle. After transport, animals were caged (4 mice per cage) and immediately placed on an ad libitum high-fat diet: 45% kcal fat diet containing 21% milk fat and 2% soybean oil (Cat. No. TD08811, Envigo, Indianapolis, Ind.), for eight weeks. Based on prior work, animals placed on this diet for eight weeks develop signs of metabolic syndrome, including glucose intolerance and insulin sensitivity.

After eight weeks on the high-fat diet, we initiated the treatment of daily gavage for a period of seven weeks. Animals in a first group (12 animals) received a sham gavage of 100 μl PBS without bacteria. Animals in a second group (12 animals) received 100 μl of *L. reuteri* VPL1014 (10^9 CFU). Animals in a third group (12 animals) received 100 al of the IL-22-secreting *L. reuteri* VPL3461 (10^9 CFU).

The length (nose to anus) of the animals was determined after eight weeks high-fat diet feeding (T0), and was subsequently determined every week after for seven weeks (T7). Each animal was measured three times and the values were averaged. After eight weeks high-fat diet feeding but prior to the start of the treatment (T0) we observed that the animals assigned to be receiving treatment were similar in length (P=0.88) but both groups were marginally smaller than the control group (control vs WT, P=0.09; control vs recombinant, P=0.04). See FIG. **9**A. After seven weeks of treatment (T7) we observed that the animals gavaged with the IL-22-secreting *L. reuteri* grew faster than animals gavaged with *L. reuteri* wild-type (P<0.0001) or PBS control (P<0.0001). See FIG. **9**B. The increased growth is purely driven by recombinant IL-22 that is delivered by *L. reuteri* because *L. reuteri* wild-type does not influence growth compared to the PBS control (P=0.66)

Healthy mice have a natural curve in their spine. When mice are obese, the excess weight will press the spine down. When measuring length of the animal from nose to anus, obese mice may be perceived to be longer. To determine if this would have affected our body length data, we measured animals alive and after euthanasia at T7. When anesthetized or dead, any bias derived from differences in curvature will be lost as the animal is completely relaxed. As shown in FIG. **9**C, there is no difference in the body length of the animals when measured alive or dead. This finding conclusively confirmed that recombinant *L. reuteri* secreting IL-22 promotes growth.

Our growth data led us to measure growth hormone levels in the serum. As shown in FIG. **10**, mice treated with IL-22-secreting *L. reuteri* had increased levels of growth hormone compared to the PBS control (P=0.03) at T7. Levels were also higher in the recombinant group compared to the wild-type but this was not statistically significant (P=0.09).

In mice, the body mass index (BMI) can be an indication of metabolic syndrome. We determined the BMI of the mice as follows: (body weight (g)/[nose-anus length (mm)]^2). As shown in FIGS. **11**A and **11**B, *L. reuteri*-derived IL-22 reduces the change in BMI over the course of seven weeks (T7-T0) and six weeks (T7-T1), respectively.

Following seven weeks treatment (T7), animals were killed, tissues were harvested, and liver weights were determined. Liver weights are shown as absolute liver weights (FIG. **12**A) or liver weight relative to mouse body weight (FIG. **12**B). Both metrics showed that IL-22-secreting *L. reuteri* yielded significantly lower liver weights compared to the wild-type *L. reuteri* or PBS control.

These results show that oral administration of recombinant *L. reuteri* engineered to secrete IL-22 systemically delivers IL-22 in a manner that results in systemic physiological effects. In the present case, the systemic physiological effects included an increase in growth, an increase in growth hormone in the plasma, a reduction in BMI, and a reduction in liver weight. The administration reversed many effects associated with metabolic syndrome. We predict that systemic delivery of IL-22 will also reverse many metabolic symptoms of diabetic subjects, including hyperglycemia and insulin resistance, and will improve insulin sensitivity, preserve gut mucosal barrier and endocrine functions, decrease endotoxemia and chronic inflammation, and reverse the dysregulation of lipid metabolism in liver and adipose tissues.

Delivery of Peptides Other than IL-22.

L. reuteri can be used to systemically deliver polypeptides other than IL-22. This can be performed by replacing the mIL-22 reading frame from the pVPL3461 plasmid and replacing it with the reading frame of any polypeptide of interest. The edited plasmid can then be introduced into *L. reuteri* using methods described above, and the *L. reuteri* harboring the edited plasmid can be administered as described above. We predict that the *L. reuteri* so modified will be capable of systemically delivering any polypeptide of interest without the bacterium itself being distributed systemically. We predict that diseases and conditions that are alleviated by systemic administration of such polypeptides will be alleviated with the *L. reuteri*-dependent systemic delivery of the peptides.

Statistical Analysis.

In the present examples, ANOVA (analysis of variance) was used for data analysis, and significance in comparisons between groups was analyzed by t-test. Significant difference was considered when P-value is lower than 0.05.

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    130   135   140

Tyr Trp Ile Arg Tyr Lys Arg Gln Gly Ala Ala Arg Phe His Arg Val
145   150   155   160

Gly Pro Ile Glu Ala Thr Ser Phe Ile Leu Arg Ala Val Arg Pro Arg
                165   170   175

Ala Arg Tyr Tyr Val Gln Val Ala Ala Gln Asp Leu Thr Asp Tyr Gly
            180   185   190

Glu Leu Ser Asp Trp Ser Leu Pro Ala Thr Ala Thr Met Ser Leu Gly
        195   200   205

Lys

<210> SEQ ID NO 4
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide Linker

<400> SEQUENCE: 4

Gln Arg Gly Gly Gly Gly Gly Gln Arg
1   5

<210> SEQ ID NO 5
<211> LENGTH: 146
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr
1   5   10   15

Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser Ser
            20   25   30

Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro Ile
        35   40   45

Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile
    50   55   60

Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln Ile Ser Asn Asp Leu
65   70   75   80

Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys
                85   90   95

His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly Gly
            100   105   110

Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg
        115   120   125

Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro
    130   135   140

```
Gly Cys
145


<210> SEQ ID NO 6
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Phe Ala Leu Leu Gly Asp Phe Phe Arg Lys Ser Lys Glu Lys Ile Gly
1               5                   10                  15

Lys Glu Phe Lys Arg Ile Val Gln Arg Ile Lys Asp Phe Leu Arg Asn
            20                  25                  30

Leu Val Pro Arg Thr Glu Ser
        35


<210> SEQ ID NO 7
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide PCSK9 Inhibitor

<400> SEQUENCE: 7

Thr Val Phe Thr Ser Trp Glu Glu Tyr Leu Asp Trp Val
1               5                   10


<210> SEQ ID NO 8
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL329

<400> SEQUENCE: 8

attccttgga cttcatttac tgggtttaac                                      30


<210> SEQ ID NO 9
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL363

<400> SEQUENCE: 9

taatatgaga taatgccgac tgtac                                           25


<210> SEQ ID NO 10
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1219

<400> SEQUENCE: 10

ttcatgggga tgaatgcttc tgctaataca ttaccagtta atactcgttg                50


<210> SEQ ID NO 11
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1220

<400> SEQUENCE: 11
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cttggttttc taattttggt tcaaagatca aacacaagca ttacgtaaac tc 52

<210> SEQ ID NO 12
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1221

<400> SEQUENCE: 12

gcttgaaacg ttcaattgaa atggca 26

<210> SEQ ID NO 13
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1222

<400> SEQUENCE: 13

tgtaaaacca ataaggactg aagc 24

<210> SEQ ID NO 14
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1223

<400> SEQUENCE: 14

ggagttgctt cagtccttat tggttttaca ttcatgggga tgaatgcttc tgctaataca 60

<210> SEQ ID NO 15
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1224

<400> SEQUENCE: 15

tgatctttga accaaaatta gaaaaccaag gcttgaaacg ttcaattgaa atggcaatta 60

<210> SEQ ID NO 16
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1313

<400> SEQUENCE: 16

actccctgaa gaatataccc tcc 23

<210> SEQ ID NO 17
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1314

<400> SEQUENCE: 17

cgctattgag cacagatacg ag 22

<210> SEQ ID NO 18
<211> LENGTH: 22
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1315

<400> SEQUENCE: 18

atgcttcccc gtataaccat ca 22

<210> SEQ ID NO 19
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1316

<400> SEQUENCE: 19

ggccatatct gcatcatacc ag 22

<210> SEQ ID NO 20
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1321

<400> SEQUENCE: 20

gatcaccgac aagggcctg 19

<210> SEQ ID NO 21
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1322

<400> SEQUENCE: 21

ggctatgaaa ctcgtactgc c 21

<210> SEQ ID NO 22
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1325

<400> SEQUENCE: 22

ggctgtattc ccctccatcg 20

<210> SEQ ID NO 23
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer oVPL1326

<400> SEQUENCE: 23

ccagttggta acaatgccat gt 22

<210> SEQ ID NO 24
<211> LENGTH: 500
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gVPL1 Codon-Optimized IL-22 Coding Sequence

<400> SEQUENCE: 24

attcatgggg atgaatgctt ctgctaatac attaccagtt aatactcgtt gtaaattaga 60

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agttagtaat tttcaacaac catatattgt taatcgtact tttatgttag ctaaagaagc    120

tagtttagct gataataata ctgatgttcg tttaattggt gaaaaattat ttcgtggtgt    180

tagtgctaaa gatcaatgtt atttaatgaa acaagtttta aattttactt tagaagatgt    240

tttattacca caaagtgatc gttttcaacc atatatgcaa gaagttgttc catttttaac    300

taaattaagt aatcaattaa gtagttgtca tattagtggt gatgatcaaa atattcaaaa    360

aaatgttcgt cgtttaaaag aaactgttaa aaaattaggt gaaagtggtg aaattaaagc    420

tattggtgaa ttagatttat tatttatgag tttacgtaat gcttgtgttt gatctttgaa    480

ccaaaattag aaaaccaagg                                                500
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<210> SEQ ID NO 25
<211> LENGTH: 5090
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Vector pVPL3461

<400> SEQUENCE: 25

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aatgattgaa aacaaaattg actttaatga aaaaagaaat ttgatgattt catcggttat     60

tttagtaatt gggattggaa atgcctatct tcaattagga aaatatcaat tctctggatt    120

agctgttgca gctgtcctcg ggataataat gaatttaatc cttccacaaa aagccaaaag    180

tgaaatgtag tttgaatttt taagcagagt aagaaaaggc atccgacctc ttactctgct    240

tttttcgtat gataacaaaa caaaaaatta agaggtcgtg gaaaaaacag attgtaagaa    300

aaagtttatc aagtctaaga aaacgttttc tttacactct tatcaattga ttagtaggta    360

tatataaacc taattttttt tgaattttaa cgaaattaat gatatattaa cattcgtaac    420

ttggggtagt tcgttttatt tgcaacgttc atccaattta caagccaaat tattgttata    480

aggaaagagg tggaatatga atgttatcga agaataatcg aaaggaacaa ttccggaaac    540

aagagccgaa aaagcaacgt tttgcaatta aaaagctcac tgtcggagtt gcttcagtcc    600

ttattggttt tacattcatg ggatgaatg cttctgctaa tacattacca gttaatactc     660

gttgtaaatt agaagttagt aattttcaac aaccatatat tgttaatcgt acttttatgt    720

tagctaaaga agctagttta gctgataata atactgatgt tcgtttaatt ggtgaaaaat    780

tatttcgtgg tgttagtgct aaagatcaat gttatttaat gaaacaagtt ttaaatttta    840

ctttagaaga tgttttatta ccacaaagtg atcgttttca accatatatg caagaagttg    900

ttccattttt aactaaatta agtaatcaat taagtagttg tcatattagt ggtgatgatc    960

aaaatattca aaaaaatgtt cgtcgtttaa aagaaactgt taaaaaatta ggtgaaagtg   1020

gtgaaattaa agctattggt gaattagatt tattatttat gagtttacgt aatgcttgtg   1080

tttgatcttt gaaccaaaat tagaaaacca aggcttctgc taatacattg aaacgttcaa   1140

ttgaaatggc aattaaacaa attacagcac gtgttgcttt gattgatagc caaaaagcag   1200

cagttgataa agcaattact gatattgctg aaaaattgta atttataaat aaaaatcacc   1260

ttttagaggt ggttttttta tttataaatt attcgtttga tttcgctttc gatagaacaa   1320

tcaaagcgag attataaaag ccagtcatta ggcctatctg acaattcctg aatagagttc   1380

ataaacaatc cagcatgata accatcacaa acagaatgat gtacctgtaa agatagcggt   1440

aaatatattg aattaccttt attaatgaat tttcctgctg taataatggg tagaaggtaa   1500

ttactattat tattgatatt taagttaaac ccagtaaatg aagtccaagg aataatagaa   1560
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agagaaaaag cattttcagg tataggtgtt ttgggaaaca atttccccga accattatat   1620
ttctctacat cagaaaggta taaatcataa aactctttga agtcattctt tacaggagtc   1680
caaataccag agaatgtttt agatacacca tcaaaaattg tataaagtgg ctctaactta   1740
tcccaataac ctaactctcc gtcgctattg taaccagttc taaaagctgt atttgagttt   1800
atcacccttg tcactaagaa aataaatgca gggtaaaatt tatatccttc ttgttttatg   1860
tttcggtata aaacactaat atcaatttct gtggttatac taaaagtcgt ttgttggttc   1920
aaataatgat taaatatctc ttttctcttc caattgtcta aatcaatttt attaaagttc   1980
atgggtttca ctctccttct acatttttta acctaataat gccaaatacc gtttgccacc   2040
cctctctttt gataattata atattggcga aattcgcttc taaagatgaa acgcaatatt   2100
atatgcttgc tttataattg tgagcgctca caatttatgt gattatacca gccccctcac   2160
tacatgtcaa gaataaactg ccaaagcata atgtaaggaa gataaatccc ataagggcgg   2220
gagcagaatg tccgagacta atgtaaattt gtccaccaat taaaggaccg ataacgcgag   2280
cttctcgagt gcatattttc ggcaatcttc tcaatgagat gctcttcagc atgttcaatg   2340
atgtcgattt tttattaaaa cgtctcaaaa tcgtttctga gacgttttag cgtttatttc   2400
gtttagttat cggcataatc gttaaaacag gcgttatcgt agcgtaaaag cccttgagcg   2460
tagcgtgctt tgcagcgaag atgttgtctg ttagattatg aaagccgatg actgaatgaa   2520
ataataagcg cagcgtcctt ctatttcggt tggaggaggc tcaagggagt ttgagggaat   2580
gaaattccct catgggtttg attttaaaaa ttgcttgcaa ttttgccgag cggtagcgct   2640
ggaaaatttt tgaaaaaaat ttggaatttg gaaaaaaatg gggggaaagg aagcgaattt   2700
tgcttccgta ctacgacccc ccattaagtg ccgagtgcca atttttgtgc caaaaacgct   2760
ctatcccaac tggctcaagg gtttgagggg tttttcaatc gccaacgaat cgccaacgtt   2820
ttcgccaacg ttttttataa atctatattt aagtagcttt attgttgttt ttatgattac   2880
aaagtgatac actaatttta taaaattatt tgattggagt tttttaaatg gtgatttcag   2940
aatcgaaaaa aagagttatg atttctctga caaaagagca agataaaaaa ttaacagata   3000
tggcgaaaca aaaaggtttt tcaaaatctg cggttgcggc gttagctata gaagaatatg   3060
caagaaagga atcagaacaa aaaaaataag cgaaagctcg cgtttttaga aggatacgag   3120
ttttcgctac ttgtttttga taaggtaata tatcatggct attaaaaata ctaaagctag   3180
aaattttgga tttttattat atcctgactc aattcctaat gattggaaag aaaaattaga   3240
gagtttgggc gtatctatgg ctgtcagtcc tttacacgat atggacgaaa aaaaagataa   3300
agatacatgg aatagtagtg atgttatacg aaatggaaag cactataaaa aaccacacta   3360
tcacgttata tatattgcac gaaatcctgt aacaatagaa agcgttagga acaagattaa   3420
gcgaaaattg gggaatagtt cagttgctca tgttgagata cttgattata tcaaaggttc   3480
atatgaatat ttgactcatg aatcaaagga cgctattgct aagaataaac atatatacga   3540
caaaaaagat attttgaaca ttaatgattt tgatattgac cgctatataa cacttgatga   3600
aagccaaaaa agagaattga agaatttact tttagatata gtggatgact ataatttggt   3660
aaatacaaaa gatttaatgg cttttattcg ccttagggga gcggagtttg gaattttaaa   3720
tacgaatgat gtaaaagata ttgtttcaac aaactctagc gcctttagat tatggtttga   3780
gggcaattat cagtgtggat atagagcaag ttatgcaaag gttcttgatg ctgaaacggg   3840
ggaaataaaa tgacaaacaa agaaaaagag ttatttgctg aaaatgagga attaaaaaaa   3900
gaaattaagg acttaaaaga gcgtattgaa agatacagag aaatggaagt tgaattaagt   3960
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acaacaatag atttattgag aggagggatt attgaataaa taaaagcccc cctgacgaaa 4020

gtcgacggca atagttaccc ttattatcaa gataagaaag aaaaggattt ttcgctacgc 4080

tcaaatcctt taaaaaaaca caaaagacca catttttttaa tgtggtcttt attcttcaac 4140

taaagcaccc attagttcaa caaacgaaaa ttggataaag tgggatattt ttaaaatata 4200

tatttatgtt acagtaatat tgacttttaa aaaaggattg attctaatga agaaagcaga 4260

caagtaagcc tcctaaattc actttagata aaaatttagg aggcatatca aatgaacgag 4320

aaaaatataa aacacagtca aaactttatt acttcaaaac ataatataga taaaataatg 4380

acaaatataa gattaaatga acatgataat atctttgaaa tcggctcagg aaaaggccat 4440

tttacccttg aattagtaaa gaggtgtaat ttcgtaactg ccattgaaat agaccataaa 4500

ttatgcaaaa ctacagaaaa taaacttgtt gatcacgata atttccaagt tttaaacaag 4560

gatatattgc agtttaaatt tcctaaaaac caatcctata aaatatatgg taatatacct 4620

tataacataa gtacggatat aatacgcaaa attgtttttg atagtatagc taatgagatt 4680

tatttaatcg tggaatacgg gtttgctaaa agattattaa atacaaaacg ctcattggca 4740

ttacttttaa tggcagaagt tgatatttct atattaagta tggttccaag agaatatttt 4800

catcctaaac ctaaagtgaa tagctcactt atcagattaa gtagaaaaaa atcaagaata 4860

tcacacaaag ataaacaaaa gtataattat ttcgttatga aatgggttaa caaagaatac 4920

aagaaaatat ttacaaaaaa tcaatttaac aattccttaa aacatgcagg aattgacgat 4980

ttaaacaata ttagctttga acaattctta tctcttttca atagctataa attatttaat 5040

aagtaataat atgagataat gccgactgta ctttttacag tcggttttct 5090

<210> SEQ ID NO 26
<211> LENGTH: 500
<212> TYPE: DNA
<213> ORGANISM: Lactobacillus reuteri

<400> SEQUENCE: 26

aatgattgaa aacaaaattg actttaatga aaaaagaaat ttgatgattt catcggttat 60

tttagtaatt gggattggaa atgcctatct tcaattagga aaatatcaat tctctggatt 120

agctgttgca gctgtcctcg ggataataat gaatttaatc cttccacaaa aagccaaaag 180

tgaaatgtag tttgaatttt taagcagagt aagaaaaggc atccgacctc ttactctgct 240

tttttcgtat gataacaaaa caaaaaaatta agaggtcgtg gaaaaaacag attgtaagaa 300

aaagtttatc aagtctaaga aaacgttttc tttacactct tatcaattga ttagtaggta 360

tatataaacc taattttttt tgaattttaa cgaaattaat gatatattaa cattcgtaac 420

ttggggtagt tcgttttatt tgcaacgttc atccaattta caagccaaat tattgttata 480

aggaaagagg tggaatatga 500

<210> SEQ ID NO 27
<211> LENGTH: 144
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Artificical signal peptide in pVPL3461

<400> SEQUENCE: 27

atgttatcga agaataatcg aaaggaacaa ttccggaaac aagagccgaa aaagcaacgt 60

tttgcaatta aaaagctcac tgtcggagtt gcttcagtcc ttattggttt tacattcatg 120 gggatgaatg cttctgctaa taca 144

<210> SEQ ID NO 28
<211> LENGTH: 438
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 28

ttaccagtta atactcgttg taaattagaa gttagtaatt ttcaacaacc atatattgtt 60

aatcgtactt ttatgttagc taaagaagct agtttagctg ataataatac tgatgttcgt 120

ttaattggtg aaaaattatt tcgtggtgtt agtgctaaag atcaatgtta tttaatgaaa 180

caagttttaa attttacttt agaagatgtt ttattaccac aaagtgatcg ttttcaacca 240

tatatgcaag aagttgttcc atttttaact aaattaagta atcaattaag tagttgtcat 300

attagtggtg atgatcaaaa tattcaaaaa aatgttcgtc gtttaaaaga aactgttaaa 360

aaattaggtg aaagtggtga aattaaagct attggtgaat tagatttatt atttatgagt 420

ttacgtaatg cttgtgtt 438

<210> SEQ ID NO 29
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Inverted repeat in pVPL3461

<400> SEQUENCE: 29

aaaaattgta atttataaat aaaaatcacc ttttagaggt ggttttttta tttataaatt 60

attcgtttga 70

<210> SEQ ID NO 30
<211> LENGTH: 648
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chloramphenicol marker in pVPL3461

<400> SEQUENCE: 30

atgaacttta ataaaattga tttagacaat tggaagagaa aagagatatt taatcattat 60

ttgaaccaac aaacgacttt tagtataacc acagaaattg atattagtgt tttataccga 120

aacataaaac aagaaggata taaattttac cctgcattta ttttcttagt gacaagggtg 180

ataaactcaa atacagcttt tagaactggt tacaatagcg acggagagtt aggttattgg 240

gataagttag agccacttta tacaattttt gatggtgtat ctaaaacatt ctctggtatt 300

tggactcctg taaagaatga cttcaaagag ttttatgatt tatacctttc tgatgtagag 360

aaatataatg gttcggggaa attgtttccc aaaacaccta tacctgaaaa tgcttttttct 420

ctttctatta ttccttggac ttcatttact gggtttaact taaatatcaa taataatagt 480

aattaccttc tacccattat tacagcagga aaattcatta ataaaggtaa ttcaatatat 540

ttaccgctat ctttacaggt acatcattct gtttgtgatg gttatcatgc tggattgttt 600

atgaactcta ttcaggaatt gtcagatagg cctaatgact ggctttta 648

<210> SEQ ID NO 31
<211> LENGTH: 211
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Phelp promoter in pVPL3461

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<400> SEQUENCE: 31

cattatgctt tggcagttta ttcttgacat gtagtgaggg ggctggtata atcacataaa     60

ttgtgagcgc tcacaattat aaagcaagca tataatattg cgtttcatct ttagaagcga    120

atttcgccaa tattataatt atcaaaagag aggggtggca aacggtattt ggcattatta    180

ggttaaaaaa tgtagaagga gagtgaaacc c                                   211


<210> SEQ ID NO 32
<211> LENGTH: 735
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Erythromycin marker in pVPL3461

<400> SEQUENCE: 32

atgaacgaga aaaatataaa acacagtcaa aactttatta cttcaaaaca taatatagat     60

aaaataatga caaatataag attaaatgaa catgataata tctttgaaat cggctcagga    120

aaaggccatt ttacccttga attagtaaag aggtgtaatt tcgtaactgc cattgaaata    180

gaccataaat tatgcaaaac tacagaaaat aaacttgttg atcacgataa tttccaagtt    240

ttaaacaagg atatattgca gtttaaattt cctaaaaacc aatcctataa aatatatggt    300

aatatacctt ataacataag tacggatata atacgcaaaa ttgtttttga tagtatagct    360

aatgagattt atttaatcgt ggaatacggg tttgctaaaa gattattaaa tacaaaacgc    420

tcattggcat tacttttaat ggcagaagtt gatatttcta tattaagtat ggttccaaga    480

gaatattttc atcctaaacc taaagtgaat agctcactta tcagattaag tagaaaaaaa    540

tcaagaatat cacacaaaga taaacaaaag tataattatt tcgttatgaa atgggttaac    600

aaagaataca agaaaatatt tacaaaaaat caatttaaca attccttaaa acatgcagga    660

attgacgatt taaacaatat tagctttgaa caattcttat ctcttttcaa tagctataaa    720

ttatttaata agtaa                                                     735
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We claim:

1. A method of systemically introducing a polypeptide into a bloodstream of a subject, the method comprising administering into the gastrointestinal tract of the subject a bacterium that produces and releases the polypeptide, wherein the bacterium comprises a recombinant gene configured to express the polypeptide, wherein the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream of the subject in a detectable amount without the bacterium being introduced in the bloodstream of the subject in a detectable amount, wherein the bacterium is *Lactobacillus reuteri*.

2. The method of claim **1**, wherein the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream in an amount effective to induce at least one direct systemic effect in the subject.

3. The method of claim **1**, wherein the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream in an amount effective to induce at least one direct effect in a non-gastrointestinal tissue in the subject.

4. The method of claim **1**, wherein the bacterium is administered in an amount effective to introduce the polypeptide in the bloodstream in an amount effective to induce at least one direct effect in a tissue selected from the group consisting of liver, muscles, lungs, kidneys, pancreas, and adipose tissue in the subject.

5. The method of claim **1**, wherein the subject suffers from a condition treatable with systemic introduction of the polypeptide and wherein the polypeptide is introduced in the bloodstream of the subject in an amount effective to treat the condition.

6. The method of claim **1**, wherein the subject suffers from a condition treatable with systemic introduction of the polypeptide but not treatable with local introduction of the polypeptide to the gastrointestinal tract without systemic introduction of the polypeptide, and wherein the polypeptide is introduced in the bloodstream of the subject in an amount effective to treat the condition.

7. The method of claim **1**, wherein the polypeptide is selected from the group consisting of a cytokine, a hormone, an antibody, an antimicrobial peptide, and an antigenic peptide.

8. The method of claim **1**, wherein the polypeptide is selected from the group consisting of interleukin-22 (IL-22), interleukin-35 (IL-35), insulin, leptin, cathelicidin related antimicrobial peptide, a peptide inhibitor of proprotein convertase subtilisin/kexin type 9 (PCSK9), and an endolysin.

9. The method of claim **8**, wherein the subject suffers from at least one condition selected from the group consisting of insulin resistance, hyperglycemia, lipid dysregulation, hyperlipidemia, and obesity, and wherein the polypeptide is

introduced in the bloodstream of the subject in an amount effective to treat the at least one condition.

10. The method of claim **1**, wherein the polypeptide is interleukin-22 (IL-22).

11. The method of claim **1**, wherein the polypeptide is a cytokine.

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