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(54) Title: COMPOSITIONS COMPRISING RECOMBINANT PROBIOTIC BACTERIA AND METHODS OF USE THEREOF

(57) Abstract: The invention features probiotic bacteria expressing *Clostridium difficile* SlpA, or fragment thereof, and its use for the treatment or prevention of *Clostridium difficile* infection and gut colonization.

COMPOSITIONS COMPRISING RECOMBINANT PROBIOTIC BACTERIA AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to and the benefit of U.S. Provisional Application No. 62/107,224, filed January 23, 2015, which is incorporated herein by reference in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant Nos. BX1I01BX001183 awarded by the U.S. Department of Veterans Affairs and ARZT-570410-A-02–139 awarded by the U.S. Department of Agriculture. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Clostridium difficile infection (CDI) is a leading cause of antibiotic-associated diarrhea in humans and animals, including agriculturally relevant animals such as calves, foals, and piglets. In the U.S., 400,000 human cases are diagnosed annually, and its treatment and prevention imposes over $3 billion in healthcare-associated costs.

CDI is precipitated when commensal flora are suppressed following antibiotic treatment. The use of antibiotics can suppress the protective normal microbiota causing susceptibility to infection. Exposure to *C. difficile* spores results in colonization of the host gastrointestinal tract. Current treatments include use of antibiotics, which have the potential to alter the bacterial composition of the gut microbiome. Vaccines are being developed for preventing *C. difficile* disease, but do not protect against *C. difficile* colonization.

At present, effective treatments and preventatives for *Clostridium difficile* infection and colonization are lacking. New methods of treatment are urgently required.

SUMMARY OF THE INVENTION

As described below, the present invention features probiotic bacteria (e.g., *Lactoccocus lactis* and *Lactobacillus acidophilus*) expressing the *Clostridium difficile* surface protein SlpA, or fragment or chimera thereof, and its use in treating or preventing *Clostridium difficile* infection and colonization.

In one aspect, the invention provides an isolated polypeptide having a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

In another aspect, the invention provides an isolated nucleic acid molecule encoding a polypeptide having a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

In still another aspect, the invention provides a vector having a nucleic acid sequence encoding a polypeptide having a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

In yet another aspect, the invention provides an isolated cell (e.g., bacterial cell) containing a nucleic acid molecule encoding a polypeptide having a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain or a vector having a nucleic acid sequence encoding a polypeptide having a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

In one aspect, the invention provides a method of treating or preventing *Clostridium difficile* infection in a subject, the method involving administering one or more Lactococcus or Lactobacillus bacterial strains expressing a chimeric SlpA polypeptide to the gut of the subject, where the chimeric SlpA polpypeptide has a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain, thereby treating or preventing *Clostridium difficile* infection in the subject.

In another aspect, the invention provides a method of colonizing the gut of a subject with one or more Lactococcus or Lactobacillus bacterial strains, the method comprising administering one or more Lactococcus or Lactobacillus bacterial strains expressing a chimeric SlpA polypeptide to the gut of the subject, where the chimeric SlpA polpypeptide comprises a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain, thereby colonizing the gut of a subject with Lactococcus or Lactobacillus.

In another aspect, the invention provides a composition (e.g., a therapeutic or pharmaceutical composition) containing an effective amount of one or more Lactoccocus or Lactobacillus bacterial strains expressing a chimeric SlpA polypeptide, where the chimeric SlpA polpypeptide has a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

In still another aspect, the invention provides a kit for treating or preventing *Clostridium difficile* infection in a subject, the kit comprising one or more Lactoccocus or Lactobacillus bacterial strains expressing a chimeric SlpA polypeptide, where the chimeric SlpA polpypeptide has a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain and optionally directions for the use of the kit (e.g., in the methods of any aspect of the invention).

In yet another aspect, the invention provides kit for colonizing the gut of a subject with Lactoccocus or Lactobacillus, the kit containing one or more Lactoccocus or Lactobacillus bacterial strains expressing a chimeric SlpA polypeptide, where the chimeric SlpA polpypeptide has a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain and optionally directions for the use of the kit (e.g., in the methods of any aspect of the invention).

In various embodiments of any of the aspects delineated herein, the SlpA variable domain has the amino acid sequence:

AAPVFAATTGTQGYTVVKNDWKKAVKQLQDGLKDNSIGKITVSFNDGVVG
EVAPKSANKKADRDAAAEKLYNLVNTQLDKLGDGDYVDFSVDYNLENKII
TNQADAEAIVTKLNSLNEKTLIDIATKDTFGMVSKTQDSEGKNVAATKAL
KVKDVATFGLKSGGSEDTGYVVEMKAGAVEDKYGKVGDSTAGIAINLPST
GLEYAGKGTTIDFNKTLKVDVTGGSTPSAVAVSGFVTKDDTDLA

In various embodiments of any of the aspects delineated herein, the SlpA cell wall binding domain is a *Lactobacillus acidophilus* or *Lactobacillus casei* SlpA cell wall binding domain. In certain embodiments, the SlpA cell wall binding domain has the amino acid sequence:

SNTNGKSATLPVVVTVPNVAEPTVASVSKRIMHNAYYYDKDAKRVGTDSV
KRYNSVSVLPNTTTINGKTYYQVVENGKAVDKYINAANIDGTKRTLKHNA
YVYASSKKRANKVVLKKGEVVTTYGASYTFKNGQKYYKIGDNTDKTYVKV
ANFR

In various embodiments of any of the aspects delineated herein, the bacterial secretion signal is a Lactococcus, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei* secretion signal. In certain embodiments, the bacterial secretion signal has the amino acid sequence:

MKKNLRIVSAAAAALLAVAPVAASAVSTVSA

In particular embodiments, the isolated polypeptide has the amino acid sequence:

MKKNLRIVSAAAAALLAVAPVAASAVSTVSAAAPVFAATTGTQGYTVVKN 50
DWKKAVKQLQDGLKDNSIGKITVSFNDGVVGEVAPKSANKKADRDAAAEK 100
LYNLVNTQLDKLGDGDYVDFSVDYNLENKIITNQADAEAIVTKLNSLNEK 150
TLIDIATKDTFGMVSKTQDSEGKNVAATKALKVKDVATFGLKSGGSEDTG 200
YVVEMKAGAVEDKYGKVGDSTAGIAINLPSTGLEYAGKGTTIDFNKTLKV 250
DVTGGSTPSAVAVSGFVTKDDTDLASNTNGKSATLPVVVTVPNVAEPTVA 300
SVSKRIMHNAYYYDKDAKRVGTDSVKRYNSVSVLPNTTTINGKTYYQVVE 350
NGKAVDKYINAANIDGTKRTLKHNAYVYASSKKRANKVVLKKGEVVTTYG 400
ASYTFKNGQKYYKIGDNTDKTYVKVANFR*

In various embodiments of any of the aspects delineated herein, the isolated nucleic acid molecule contains a sequence optimized for expression in Lactococcus, *Lactoccocus lactis*, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei*. In specific embodiments, the isolated nucleic acid has the nucleic acid sequence:

GGATCCATGAAGAAAAATTTAAGAATCGTTAGCGCTGCTGCTGCTGCTTTACTTG
CTGTTGCTCCAGTTGCTGCTTCTGCTGTATCTACTGTTAGCGCTGCTGCACCTGTA
TTTGCTGCAACCACTGGTACACAAGGCTATACGGTGGTTAAGAATGATTGGAAA
AAGGCTGTCAAACAATTACAAGATGGACTTAAAGATAATAGTATTGGTAAGATT
ACGGTCAGTTTCAATGATGGTGTGGTAGGAGAAGTAGCACCTAAATCAGCGAAT
AAGAAAGCAGATCGAGATGCAGCCGCAGAAAAGTTGTATAATCTTGTAAATACA
CAATTAGACAAATTAGGCGATGGCGATTATGTAGATTTTTCTGTTGATTACAATC
TAGAGAATAAGATTATCACCAATCAAGCCGATGCCGAAGCTATTGTTACTAAATT
GAATTCGTTAAATGAAAAGACGCTAATTGATATTGCAACTAAAGATACGTTTGGA
ATGGTGTCTAAAACGCAGGATTCTGAAGGAAAGAATGTTGCGGCAACAAAAGCG
TTAAAAGTAAAAGATGTGGCAACTTTTGGCTTAAAGAGTGGAGGTAGTGAAGAT
ACCGGATATGTTGTCGAAATGAAAGCGGGTGCTGTTGAAGATAAGTATGGTAAA
GTAGGTGATTCTACAGCTGGTATTGCAATCAATCTTCCATCAACAGGTTTAGAAT
ATGCAGGCAAAGGAACAACTATTGATTTCAACAAAACCCTTAAAGTTGATGTAA
CTGGTGGTAGTACACCGAGTGCAGTTGCCGTAAGTGGGTTTGTGACTAAAGATGA
TACAGATTTAGCATCAAATACTAATGGTAAGTCAGCTACTTTGCCAGTAGTTGTT
ACTGTTCCTAATGTTGCTGAGCCAACTGTAGCCAGCGTAAGCAAGAGAATTATGC
ACAACGCATACTACTACGACAAGGACGCTAAGCGTGTTGGTACTGACAGCGTTA
AGCGTTACAACTCAGTAAGCGTATTGCCAAACACTACTACTATCAACGGTAAGAC
TTACTACCAAGTAGTTGAAAACGGTAAGGCTGTTGACAAGTACATCAACGCTGC

AAACATCGATGGTACTAAGCGTACTTTGAAGCACAACGCTTACGTTTACGCATCA
TCAAAGAAGCGTGCTAACAAGGTTGTATTGAAGAAGGGTGAAGTTGTAACTACT
TACGGTGCTTCATACACATTCAAGAACGGCCAAAAGTACTACAAGATCGGTGAC
AACACTGACAAGACTTACGTTAAGGTTGCAAACTTTAGATAATAAAGATCTTCGA
ATTCCCGCGGCCGC

In various embodiments of any of the aspects delineated herein, the vector is a Lactoccocus, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei* expression vector. In certain embodiments, the vector has a Lactococcus or Lactobacillus origin of replication. In specific embodiments, the vector is pMGM10, pMGM11, pTRK848, or pTRK882.

In various embodiments of any of the aspects delineated herein, the vector comprises a first sequence identical to a sequence of a first fragment in a Lactobacillus genome, wherein the first fragment is located at the 5' or 3' terminus of *thyA* gene, or within the *thyA* gene of Lactobacillus. In various embodiments of any of the aspects delineated herein, the vector comprises a second sequence identical to a sequence of a second fragment in a Lactobacillus genome, wherein the second fragment is located at the 5' or 3' terminus of a *thyA* gene. In various embodiments of any of the aspects delineated herein, the vector has a sequence identical to a sequence of a fragment in *Lactobacillus acidophilus*, or *Lactobacillus casei* genome.

In various embodiments of any of the aspects delineated herein, the cell is a Lactoccocus, *Lactoccocus lactis*, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei* cell. In various embodiments of any of the aspects delineated herein, the nucleic acid sequence encoding the chimeric SlpA peptide is integrated into the chromosome of the isolated cell.

In various embodiments of any of the aspects delineated herein, the subject is a human or animal.

In various embodiments of any of the aspects delineated herein, the subject has undergone or is undergoing treatment with one or more antibiotics (e.g., a cephalosporin, metronidazole, fluoroquinolone, such as moxifloxacin or vancomycin and fidaxomycin and the like). In various embodiments of any of the aspects delineated herein, the administration of the Lactoccocus or Lactobacillus expressing a chimeric SlpA polypeptide is by oral administration.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

By "Surface-Layer Protein A (SlpA)" is meant a polypeptide or fragment thereof having at least about 85% or greater amino acid identity to the amino acid sequence provided at NCBI Accession No. CAJ69681 or WP_011254065 and having bacterial adherence activity. Exemplary SlpA amino acid sequences are provided below:

Clostridium difficile SlpA (full length sequence)

```
   1 mnkkniaiam sgltvlasaa pvfaattgtq gytvvkndwk kavkqlqdgl kdnsigkitv
  61 sfndgvvgev apksankkad rdaaaeklyn lvntqldklg dgdyvdfsvd ynlenkiitn
 121 qadaeaivtk lnslnektli diatkdtfgm vsktqdsegk nvaatkalkv kdvatfglks
 181 ggsedtgyvv emkagavedk ygkvgdstag iainlpstgl eyagkgttid fnktlkvdvt
 241 ggstpsavav sgfvtkddtd laksgtinvr vinakeesid idassytsae nlakryvfdp
 301 deiseaykai valqndgies nlvqlvngky qvifypegkr letksandti asqdtpakvv
 361 ikanklkdlk dyvddlktyn ntysnvvtva gedrietaie lsskyynsdd knaitdkavn
 421 divlvgstsi vdglvaspla sektaplllt skdkldssvk seikrvmnlk sdtgintskk
 481 vylaggvnsi skdvenelkn mglkvtrlsg edryetslai adeigldndk afvvggtgla
 541 damsiapvas qlkdgdatpi vvvdgkakei sddaksflgt sdvdiiggkn svskeieesi
 601 dsatgktpdr isgddrqatn aevlkeddyf tdgevvnyfv akdgstkedq lvdalaaapi
 661 agrfkespap iilatdtlss dqnvavskav pkdggtnlvq vgkgiassvi nkmkdlldm
```

Lactobacillus acidophilus

```
  1 mkknlrivsa aaaallavap vaasavstvs aattinasss aintntnaky dvdvtpsvsa
 61 vaantanntp aiagnltgti sasyngktyt anlkadtena titaagstta vkpaelaagv
121 aytvtvndvs fnfgsenagk tvtlgsansn vkftgtnsdn qtetnvstlk vkldqngvas
181 ltnvsianvy ainttdnsnv nfydvtsgat vtngavsvna dnqgqvnvan vvaainskyf
241 aaqyadkkln trtantedai kaalkdqkid vnsvgyfkap htftvnvkat sntngksatl
301 pvvvtvpnva eptvasvskr imhnayyydk dakrvgtdsv krynsvsvlp ntttingkty
361 yqvvengkav dkyinaanid gtkrtlkhna yvyasskkra nkvvlkkgev vttygasytf
421 kngqkyykig dntdktyvkv anfr
```

By "SlpA nucleic acid molecule" is meant a polynucleotide encoding SlpA.

By "SlpA variable domain" is meant a polypeptide having 85% identity to the following sequence:

```
AAPVFAATTGTQGYTVVKNDWKKAVKQLQDGLKDNSIGKITVSFNDGVVG
EVAPKSANKKADRDAAAEKLYNLVNTQLDKLGDGDYVDFSVDYNLENKII
TNQADAEAIVTKLNSLNEKTLIDIATKDTFGMVSKTQDSEGKNVAATKAL
KVKDVATFGLKSGGSEDTGYVVEMKAGAVEDKYGKVGDSTAGIAINLPST
GLEYAGKGTTIDFNKTLKVDVTGGSTPSAVAVSGFVTKDDTDLA
```

In one embodiment, the SlpA variable domain is from *C. difficile* SlpA.

By "SlpA cell wall binding domain" is meant a polypeptide having 85% identity to the following sequence:

```
agedrietaielsskyynsddknaitdkavndivlvgstsivdglvasplasektaplll
tskdkldssvkseikrvmnlksdtgintskkvylaggvnsiskdvenelknmglkvtrls
gedryetslaiadeigldndkafvvggtgladamsiapvasqlkdgdatpivvvdgkake
isddaksflgtsdvdiiggknsvskeieesidsatgktpdrisgddrqatnaevlkeddy
ftdgevvnyfvakdgstkedqlvdalaaapiagrfkespapiilatdtlssdqnvavska
vpkdggtnlvqvgkgiassvink
```

In one embodiment, the SlpA cell wall binding domain is from Lactobacillus (e.g., *Lactobacillus acidophilus*).

By "chimeric SlpA" is meant a polypeptide having two or more SlpA sequences from two or more bacterial strains. In one embodiment, a chimeric SlpA has an SlpA variable domain from *C. difficile* SlpA and an SlpA cell wall binding from *Lactobacillus acidophilus*. In another embodiment, a chimeric SlpA has an SlpA signal sequence from *Lactobacillus*

acidophilus, an SlpA variable domain from *C. difficile* SlpA and an SlpA cell wall binding from *Lactobacillus acidophilus*. An exemplary chimeric SlpA sequence is provided below:

```
MKKNLRIVSAAAAALLAVAPVAASAVSTVSAAAPVFAATTGTQGYTVVKN  50
DWKKAVKQLQDGLKDNSIGKITVSFNDGVVGEVAPKSANKKADRDAAAEK 100
LYNLVNTQLDKLGDGDYVDFSVDYNLENKIITNQADAEAIVTKLNSLNEK 150
TLIDIATKDTFGMVSKTQDSEGKNVAATKALKVKDVATFGLKSGGSEDTG 200
YVVEMKAGAVEDKYGKVGDSTAGIAINLPSTGLEYAGKGTTIDFNKTLKV 250
DVTGGSTPSAVAVSGFVTKDDTDLASNTNGKSATLPVVVTVPNVAEPTVA 300
SVSKRIMHNAYYYDKDAKRVGTDSVKRYNSVSVLPNTTTINGKTYYQVVE 350
NGKAVDKYINAANIDGTKRTLKHNAYVYASSKKRANKVVLKKGEVVTTYG 400
ASYTFKNGQKYYKIGDNTDKTYVKVANFR*
```

A codon-optimized nucleic acid sequence of the above chimeric SlpA sequence for expression in Lactobacillus is provided below at Figure 1C.

By "undesirable gut microbiome" is meant a community of microbes comprising a pathogen or having a biological activity associated with a pathogenic process. In one embodiment, an undesirable gut microbiome comprises an increased number or percentage of *Clostridium difficile* relative to the number or percentage of *C. difficile* present in the gut of a healthy control subject.

By "normal gut flora" is meant a population of microbes that is substantially similar to the population of microbes present in the gut of a healthy control subject.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean " includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By "effective amount" is meant the amount of a composition of the invention (e.g., comprising a probiotic bacteria expressing *Clostridium difficile* SlpA, or fragment thereof) required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of probiotic bacteria of the invention varies depending upon the manner of

administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

By “expression vector” is meant a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an *in vitro* expression system. Expression vectors include all those known in the art, such as plasmids, cosmids, and viruses that incorporate the recombinant polynucleotide.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference polypeptide or nucleic acid molecule. A fragment may contain 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids or nucleotides.

The terms "isolated," "purified," or "biologically pure" refers to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. Thus, a purified isolated bacterium that is part of a culture or inoculum is at least about 90%, 95% or 100% free of bacteria, fungi, viruses, or other undefined microbes.

By “marker” is meant any analyte associated with a disease or disorder.

The term “operably linked” refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

The term “promoter” as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term “promoter/regulatory sequence” means a nucleic acid sequence which is required for expression of a gene product operably linked to the

promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

"Pharmaceutically acceptable" refers to those properties and/or substances that are acceptable to the patient from a pharmacological/toxicological point of view and to the manufacturing pharmaceutical chemist from a physical/chemical point of view regarding composition, formulation, stability, patient acceptance and bioavailability. "Pharmaceutically acceptable carrier" refers to a medium that does not interfere with the effectiveness of the biological activity of the active ingredient(s) and is not toxic to the host to which it is administered.

As used herein, the term "pharmaceutical composition" or "pharmaceuticaly acceptable composition" refers to a mixture of at least one compound or molecule useful within the invention with a pharmaceutically acceptable carrier. The pharmaceutical composition facilitates administration of the compound or molecule to a patient. Multiple techniques of administering a compound or molecule exist in the art including, but not limited to, intravenous, oral, aerosol, parenteral, ophthalmic, pulmonary and topical administration.

As used herein, the term "pharmaceutically acceptable carrier" means a pharmaceutically acceptable material, composition or carrier, such as a liquid or solid filler, stabilizer, dispersing agent, suspending agent, diluent, excipient, thickening agent, solvent or encapsulating material, involved in carrying or transporting a compound or molecule useful within the invention within or to the patient such that it may perform its intended function. Typically, such constructs are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation, including the compound useful within the invention, and not injurious to the patient. Some examples of materials that

may serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; surface active agents; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations. As used herein, "pharmaceutically acceptable carrier" also includes any and all coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like that are compatible with the activity of the compound useful within the invention, and are physiologically acceptable to the patient. Supplementary active compounds may also be incorporated into the compositions. The "pharmaceutically acceptable carrier" may further include a pharmaceutically acceptable salt of the compound or molecule useful within the invention. Other additional ingredients that may be included in the pharmaceutical compositions used in the practice of the invention are known in the art and described, for example in Remington's Pharmaceutical Sciences (Genaro, Ed., Mack Publishing Co., 1985, Easton, PA), which is incorporated herein by reference.

By "reference" is meant a standard or control condition.

A "reference sequence" is a defined sequence used as a basis for sequence comparison.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (*e.g.*, a gene described herein), or portions thereof, under various conditions of stringency. (See, *e.g.*, Wahl, G. M.

and S. L. Berger (1987) Methods Enzymol. 152:399; Kimmel, A. R. (1987) Methods Enzymol. 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, *e.g.*, formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, *e.g.*, sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred: embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art.

Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95%, 96%, 97%, 98%, or even 99% or more identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard

deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E depict the generation of plasmid constructs and the biological sequences used. Figure 1A depicts the construction of plasmid pMGM10. Figure 1B depicts the construction of plasmid pMGM11. Figure 1C depicts the codon-optimized slpA chimera nucleic acid sequence that was cloned into the plasmids pMGM10 and pMGM11. Figure 1D depicts the amino acid sequence of the SlpA chimera polypeptide. Figure 1E depicts the promoter sequences used in constructing the plasmids: fructooligosaccharides (Fos) promoter in plasmid pTRK848 / pMGM11 and phosphoglycerate mutase (pgm) promoter pTRK882 / pMGM10. Figure 2 are immunofluorescence images showing the surface expression of *C. difficile* SlpA in *Lactobacillus casei* compared to pre-immune serum and vector only controls.

Figure 3 is a graph showing mean time to death in Syrian Golden hamsters challenged with virulent *Clostridium difficile* and treated with recombinant Lactobacillus expressing a chimeric SlpA (CHI) or Lactobacillus carrying an empty vector (EV) or undergoing antibiotic treatment (Antibiotics), compared to animals that were unchallenged (Unchallenged).

Figure 4 provides a schematic diagram of a plasmid encoding SlpA chimeric protein. The internal fragment of a *thyA* directs the chimera integration into the genome and concomitantly insertionally inactivates the *thyA*, which encodes an essential enzyme thymidylate synthase (ThyA). *Lactobacillus casei* and *Lactobacillus acidophilus* specific constructs were made. A gene *catP* encodes an enzyme Chloramphenicol acetyltransferase, which is an effector of chloramphenicol resistance in bacteria. A region including an *oriR* gene and a $repA^{ts}$ gene constitutes a temperature sensitive broad host replicon based on a

pWVO1 plasmid. YtvA is an anaerobic fluorescent protein used to confirm transformation of Lactobacillus sp and monitor colonization.

Figure 5 provides a schematic diagram of a plasmid encoding SlpA chimeric protein. A 5' seq and a 3' seq are two fragments located at 5' and 3' terminus of a *thyA* gene, respectively, and direct the double homologous recombination to replace the *thyA* gene in the bacterial chromosome with a Slp A chimeric protein encoding sequence and a YtvA fluorescent reporter encoding sequence. *Lactobacillus casei* and *Lactobacillus acidophilus* specific constructs were made. The *thyA* gene encodes an essential enzyme thymidylate synthase (ThyA). The YtvA is an anaerobic fluorescent protein used to confirm transformation of Lactobacillus sp and monitor colonization. A gene *catP* encodes an enzyme Chloramphenicol acetyltransferase, which is an effector of chloramphenicol resistance in bacteria. A region including an *oriR* and a $repA^{ts}$ genes constitutes a temperature sensitive broad host replicon based on pWVO1 plasmid.

Figure 6 provides a partial sequence of a plasmid used for a *thyA* directed integration of a SlpA chimeric protein encoding sequence into a bacterial genome and concomitantly insertionally inactivates *thyA* in *Lactobacillus casei.*

Figure 7 provides a partial sequence of a plasmid used for double homologous recombination-based system for replacing a *thyA* gene in a bacterial chromosome with a Slp A chimeric protein encoding sequence and a YtvA fluorescent reporter encoding sequence in *Lactobacillus casei.*

Figure 8 provides a partial sequence of a plasmid used for a *thyA* directed integration of a SlpA chimeric protein encoding sequence into a bacterial genome and concomitantly insertionally inactivates *thyA* in *Lactobacillus acidophilus.*

Figure 9 provides a partial sequence of a plasmid used for double homologous recombination-based system for replacing a *thyA* gene in a bacterial chromosome with a Slp A chimeric protein encoding sequence and a YtvA fluorescent reporter encoding sequence in *Lactobacillus acidophilus.*

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a probiotic bacteria (e.g., Lactobacillus, Lactococcus) expressing the *Clostridium difficile* surface protein SlpA, or a fragment or chimeric polypeptide thereof, and its use for colonizing the gut or digestive tract of a subject. The invention also provides a method of treating or preventing *Clostridium difficile* infection and

colonization. The invention features use of the probiotic bacteria of the invention for the replacement of a gut microbiome associated with disease.

The invention is based, at least in part, on the discovery that expression of *Clostridium difficile* SlpA (e.g., chimeric SlpA), or fragment thereof, in Lactobacillus or Lactococcus is effective for colonizing the gut with the recombinant bacteria. It was also found that recombinant Lactobacillus protected the gut from virulent *Clostridium difficile* challenge. These findings indicate that administration of gut recombinantly expressing *Clostridium difficile* SlpA, or fragment thereof can be used to treat or prevent *Clostridium difficile* infection and colonization.

Clostridium difficile

Clostridium difficile is a gram-positive, anaerobic, spore-forming bacterium, and causes the antibiotic-associated diarrheal disease, *C. difficile* infection (CDI). It is also a leading cause of bacterial healthcare-associated infections in hospitals in the United States. Like many enteric pathogens, *Clostridium difficile* must associate with the intestinal mucosa to begin the process of host colonization.

Multiple *C. difficile* adhesins have been described, including the flagellin FliC, the flagellar cap protein FliD, fibronectin-binding proteins, a heat-shock protein, GroEL, the surface associated, heat-shock-induced adhesin, Cwp66, and the surface layer protein, SlpA. SlpA contains two biologically distinct entities, the high-molecular weight (HMW) and the low molecular weight (LMW) subunits, which are derived via Cwp84-mediated cleavage of a single precursor protein, and assemble on the bacterial surface into a paracrystalline lattice. The two subunits associate with high affinity through the N-terminus of the HMW protein and the C-terminus of the LMW protein.

Cwp66 and SlpA are encoded by two genes in a 17-gene cluster that encodes many surface-associated proteins. Such S-layer proteins (SLPs) provide structural integrity to the cells, act as molecular sieves, bind to host tissues and extracellular matrix proteins, and contribute to host cell adhesion and immune evasion.

Surface-Layer Protein A (SlpA)

Many gram-positive bacteria including *C. difficile* possess a surface-layer that covers the peptidoglycan-rich cell wall. This "S-layer" consists of many proteins that form a paracrystalline lattice around the bacterial cell. The most abundant S-layer protein in *C.*

difficile is SlpA, a major contributor of adhesion to, and colonization of, intestinal epithelial cells. (Merrigan et al. PLoS ONE 8(11): e78404). Individual subunits of the protein (varying in sequence between strains) mediated host-cell attachment to different extents. Pre-treatment of host cells with crude or purified SlpA subunits, or incubation of vegetative bacteria with anti-SlpA antisera significantly reduce *C. difficile* attachment. SlpA-mediated adherence-interference correlates with the attachment efficiency of the strain from which the protein was derived, with maximal blockage observed when SlpA is derived from highly adherent strains. In addition, SlpA-containing preparations from a non-toxigenic strain effectively blocked adherence of a phylogenetically distant, epidemic-associated strain, and vice-versa. Taken together, these results suggest that SlpA plays a major role in *C. difficile* infection, and that it may represent an attractive target for interventions aimed at abrogating gut colonization by this pathogen.

Therapeutic Compositions

The invention features probiotic bacteria expressing *Clostridium difficile* SlpA or fragment thereof (e.g., chimeric SlpA). In particular, *Clostridium difficile* SlpA, or fragment thereof, is expressed in Lactoccocus (e.g., *Lactoccocus lactis*) or Lactobacillus cells (e.g., *Lactobacillus acidophilus* or *Lactobacillus casei*). In additional embodiments, one or more strains of probiotic bacteria expressing a chimeric SlpA polypeptide are administered or formulated as a therapeutic composition. In certain embodiments, the SlpA expressed is a chimeric SlpA comprising a *C. difficile* SlpA variable domain and Lactobacillus (e.g., *Lactobacillus acidophilus* or *Lactobacillus casei*) SlpA cell wall binding domain. The SlpA additionally includes a bacterial secretion signal that is appropriate for surface expression in its host cell (e.g., Lactoccocus, Lactobacillus, *Lactobacillus acidophilus* or *Lactobacillus casei*). In various embodiments, the invention also includes nucleic acid molecules and vectors encoding a chimeric SlpA polypeptide. Vectors encoding the chimeric SlpA polypeptide can be used to direct or regulate the expression of the chimeric SlpA by the cell (see e.g., Duong et al., Microbial Biotechnoloy 2010, 4(3): 357-367 which is herein incorporated in its entirety by reference). Such vectors contain one or more origin of replication sequences that can be used by a bacterial cell, promoter sequences for expression in a bacterial cell (e.g., constitutive or inducible), genetic markers for selection (e.g., antibiotic resistance); origin of transfer sequences for bacterial conjugation (e.g., traJ), and may also be codon optimized for protein expression.

Alternatively, nucleic acid sequences encoding chimeric SlpA may be integrated into the Lactobacillus or Lactococcus genome. In certain embodiments, nucleic acid sequences encoding the chimeric SlpA can be integrated into the Lactobacillus or Lactococcus genome through recombination between vectors comprising the nucleic acid sequence encoding the chimeric SlpA poly peptide and bacterial chromosome. Such techniques are well known in the art (see e.g., Leenhouts, et al., Appl. Environ. Microbiol. 1989, 55(2): 394-400, and Gaspar, et al., Appl. Environ. Microbiol. 2004, 70(3): 1466-74 which are herein incorporated in their entirety by reference).

Probiotic strains may also be engineered with auxotrophic selection, for example requiring thiamine or thymine supplementation for survival.

Methods of the Invention

The present invention provides methods of treating diseases or symptoms thereof associated with the presence of one or more undesirable bacteria in the gut of a subject. Accordingly, the invention provides compositions and methods for treating a subject having or at risk of developing a disease associated with undesirable changes in the gut microbiome, the method involving administering a therapeutically effective amount of a composition comprising a probiotic bacteria of the invention to a subject (e.g., a mammal, such as a human). In particular, the compositions and methods of the invention are effective for treating or preventing *Clostridium difficile* infection, colonization, or diseases and symptoms thereof (e.g., diarrhea). Without being bound to theory, Lactobacillus or Lactococcus expressing *Clostridium difficile* SlpA or fragment thereof (e.g., chimeric SlpA) colonize the gut and compete with *Clostridium difficile* for binding and colonization. Accordingly, the method includes the step of administering to a mammal a therapeutic amount of an amount of a composition comprising one or more probiotic bacteria strains of the invention sufficient to treat the disease or disorder or symptom thereof, under conditions such that the disease or disorder is treated.

Identifying a subject in need of treatment for a disease associated with the gut microbiome can be in the judgment of a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method). In certain embodiments, the subject has undergone or is undergoing treatment with antibiotics. As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not

precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated. As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

The therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of a composition comprising the probiotic bacteria of the invention to a subject (e.g., human) in need thereof. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a disease, disorder, or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider. The compositions herein may be also used in the treatment of any other disorders in which a microbial imbalance in the digestive tract may be implicated.

Methods of Delivery

Compositions comprising the probiotic bacteria of the invention may be administered orally, rectally, or enterally. Preferably compositions administered to a subject in tablet form, by feeding tube, by enema, or by colonoscopy. Preferably, the probiotic bacteria of the invention are diluted in a suitable excipient (e.g., saline solution). An effective dose may include 10^6-10^9 colony forming units of bacteria per day).

Expression of Recombinant Polypeptides

In order to express the fusion protein of the invention, DNA molecules obtained by any of the methods described herein or those that are known in the art, can be inserted into appropriate expression vectors by techniques well known in the art. For example, a double stranded DNA can be cloned into a suitable vector by restriction enzyme linking involving the use of synthetic DNA linkers or by blunt-ended ligation. DNA ligases are usually used to ligate the DNA molecules and undesirable joining can be avoided by treatment with alkaline phosphatase.

Therefore, the invention includes vectors (e.g., recombinant plasmids) that include nucleic acid molecules (e.g., genes or recombinant nucleic acid molecules encoding genes) as described herein. The term "recombinant vector" includes a vector (e.g., plasmid, phage,

phasmid, virus, cosmid, fosmid, or other purified nucleic acid vector) that has been altered, modified or engineered such that it contains greater, fewer or different nucleic acid sequences than those included in the native or natural nucleic acid molecule from which the recombinant vector was derived. For example, a recombinant vector may include a nucleotide sequence encoding an SlpA chimeric polypeptide operatively linked to regulatory sequences, e.g., promoter sequences, terminator sequences, and the like, as defined herein. Recombinant vectors which allow for expression of the genes or nucleic acids included in them are referred to as "expression vectors."

In some of the molecules of the invention described herein, one or more DNA molecules having a nucleotide sequence encoding one or more polypeptides of the invention are operatively linked to one or more regulatory sequences, which are capable of integrating the desired DNA molecule into a prokaryotic host cell. Cells which have been stably transformed by the introduced DNA can be selected, for example, by introducing one or more markers which allow for selection of host cells which contain the expression vector. A selectable marker gene can either be linked directly to a nucleic acid sequence to be expressed, or be introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins described herein. It would be apparent to one of ordinary skill in the art which additional elements to use.

Factors of importance in selecting a particular plasmid or viral vector include, but are not limited to, the ease with which recipient cells that contain the vector are recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Once the vector(s) is constructed to include a DNA sequence for expression, it may be introduced into an appropriate host cell by one or more of a variety of suitable methods that are known in the art, including but not limited to, for example, transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

After the introduction of one or more vector(s), host cells are usually grown in a selective medium, which selects for the growth of vector-containing cells. Expression of recombinant proteins can be detected by immunoassays including Western blot analysis, immunoblot, and immunofluorescence. Purification of recombinant proteins can be carried out by any of the methods known in the art or described herein, for example, any

conventional procedures involving extraction, precipitation, chromatography and electrophoresis. A further purification procedure that may be used for purifying proteins is affinity chromatography using monoclonal antibodies which bind a target protein. Generally, crude preparations containing a recombinant protein are passed through a column on which a suitable monoclonal antibody is immobilized. The protein usually binds to the column via the specific antibody while the impurities pass through. After washing the column, the protein is eluted from the gel by changing pH or ionic strength, for example.

Kits

The invention provides kits for colonizing probiotic bacteria of the invention in the gut of a host. The invention also provides kits for the treatment or prevention of *Clostridium difficile* infection or colonization. In particular embodiments, the kit comprises a sterile container which contains a therapeutic or prophylactic composition comprising the probiotic bacteria of the invention; such containers can be boxes, ampoules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

The kit preferably contains instructions that generally include information about the use of the composition for the expansion of the microbial consortia in the gut of the subject. The kit further contains precautions; warnings; indications; counter-indications; overdosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in

Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1: Probiotic bacterial strains expressing chimeric SlpA are effective at gut colonization and protecting against C. difficile challenge.

Developing novel interventions that avoid the use of antibiotics is important in the treatment of *C. difficile* infection (CDI). Many gram-positive bacteria including *C. difficile* possess a surface-layer that covers the peptidoglycan-rich cell wall. This "S-layer" consists of many proteins that form a paracrystalline lattice around the bacterial cell. Surface layer protein A (SlpA), an adhesin and a major component of the cell surface layer (or S-layer) of *C. difficile*, facilitates gut colonization. Novel probiotic organisms were designed and engineered to express *C. difficile* SlpA on their cell surface (Figures 1A-1E). Without being bound to a particular theory, the engineered probiotic colonizes gut niches specifically occupied by virulent infecting *C. difficile* strains, thus preventing disease.

A chimeric SlpA polypeptide was created that included *C. difficile* SlpA sequences for bacterial adherence. The chimeric SlpA polypeptide was designed with a *L. acidophilus* cell-wall binding domain and secretion signal sequence and the host-cell binding domain of *C. difficile*. Plasmid vectors were constructed to express the *slpA* chimera either constitutively or in response to a fructose oligosaccharide inducer. Plasmid vectors encoding SlpA chimeras were designed and introduced into probiotic bacterial strains *Lactoccocus lactis* and *Lactobacillus acidophilus* via three methods, electroporation, bacterial conjugation (pMTL82151 with pTRK848 comprising traJ; pMTL82151 with pTRK882 comprising traJ), and protoplasting. Transformants were assessed for stable carriage of introduced plasmids, and tested for SlpA surface expression. Western blot analysis and cell surface

immunofluorescence showed that the chimeric SlpA was expressed by the probiotic bacterial cells (Figure 2).

To test the ability of the recombinant bacteria expressing the chimeric SlpA to occlude *C. difficile* competitively, a study was performed in Syrian Golden hamsters (Figure 3). Animals were treated with recombinant Lactobacillus expressing a chimeric SlpA or Lactobacillus carrying an empty vector. Another group of animals underwent treatment with antibiotics. animals that were administered Lactobacillus expressing chimeric SlpA showed extended survival and overall survival at the conclusion of the experiment. By comparison, none of the animals that were treated with antibiotics or administered Lactobacillus with empty vector survived to the end of the experiment. All animals that were not challenged with virulent *C. difficile* survived to the end of the experiment. These results show that Lactobacillus expressing the chimeric SlpA colonized the gut and was able to protect against virulent *C. difficile* challenge. Thus, this indicates that administration of probiotic bacteria expressing chimeric SlpA has the potential to be an effective treatment or preventative for *C. difficile* infection and/or colonization.

Example 2: A chimeric *slpA* polynucleotide can be integrated into the Lactobacillus genome.

Probiotic organisms can include biocontainment features for eventual clinical use and, concomitantly, obviating the requirement of antibiotics for *in vivo* plasmid maintenance and stable SlpA expression. The SlpA expression may also be controlled. The biological containment can be achieved via a two-step mechanism, where both SlpA expression and *Lactobacillus* sp. survival can be controlled. Novel vectors were designed to allow probiotic organisms to express chimeric SlpA stably and under control (Figs. 4 and 5). In one embodiment, the nucleic acid sequence encoding a chimeric SlpA peptide can be integrated into a bacterial chromosome to allow stability of SlpA expression in the absence of selection pressure. The chimeric SlpA expression can be expressed under the control of a fructo-oligosaccharide (FOS) promoter. FOSs are well-tolerated, safe and widely-used supplements in humans and agriculturally-relevant animals.

Probiotic organisms expressing the chimeric SlpA protein, such as the lactic acid bacterium, including both *L. casei* and *L. acidophilus*, can be genetically modified such that complete lethality of the probiotic organisms occurs in the absence of thymine supplementation. This "thymineless death" is predicated on the absolute requirement of

deoxy-thymidine triphosphate (dTTP) for DNA synthesis in all living organisms. Of the two pathways for dTTP synthesis in most bacteria, the *de novo* pathway involves conversion of dUMP to dTMP by the essential enzyme thymidylate synthase (ThyA). The less-used "salvage" pathway involves the conversion of supplemented thymidine into dTMP by thymidine kinase. dTMP is then converted to dTTP. Disruption or mutation of *thyA* in bacteria results in immediate auxotrophy and, in vitro, can be tolerated only by addition of exogenous thymidine that is utilized by the salvage pathway. Withdrawal of thymidine results in rapid, total cell death. Free thymidine is not abundant or bio-available *in vivo* (in the gut), and is unable to support growth of *thyA* auxotrophs. Therefore, the probiotic organisms with *thyA* gene disrupted will necessarily be lost from the gut unless continually administered.

The nucleic acid sequence encoding the chimeric SlpA can be integrated into the *Lactobacillus* sp. through a single homologous recombination at a single site (Fig. 4) or a double homologous recombination (Fig. 5). For the single homologous recombination, one vector is constructed for each species. Thus, a vector is constructed for specific use in *L. casei*, such that the vector includes a nucleic acid sequence that is identical to a nucleic acid sequence of a fragment of the *thyA* gene in *L. casei*. Another vector is constructed for specific use in *L. acidophilus*, such that the vector includes a nucleic acid sequence that is identical to a nucleic acid sequence of a fragment of the *thyA* gene in *L. acidophilus*. The sequence of the vector for the specific use in *L. casei* for single recombination is shown in Fig. 6 and the sequence of the vector for the specific use in *L. acidophilus* for single recombination is shown in Fig. 8.

For the double homologous recombination, one vector is constructed for each species. Thus, a vector is constructed for specific use in *L. casei*, such that the vector includes a first nucleic acid sequence that is identical to a nucleic acid sequence of a fragment located at the 5' of the *thyA* gene in *L. casei* and a second nucleic acid sequence that is identical to a nucleic acid sequence of a fragment located at the 3' of the *thyA* gene in *L. casei* . Another vector is constructed for specific use in *L. acidophilus*, such that the vector includes a first nucleic acid sequence that is identical to a nucleic acid sequence of a first fragment located at the 5' of the *thyA* gene in *L. acidophilus* and a second nucleic acid sequence that is identical to a nucleic acid sequence of a second fragment located at the 3' of the *thyA* gene in *L. acidophilus*. The sequence of the vector for the specific use in *L. casei* for double recombination is shown in

Fig. 7 and the sequence of the vector for the specific use in *L. acidophilus* for double homologous recombination is shown in Fig. 9.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. An isolated polypeptide comprising a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

2. The isolated polypeptide of claim 1, wherein the SlpA variable domain has the amino acid sequence:

AAPVFAATTGTQGYTVVKNDWKKAVKQLQDGLKDNSIGKITVSFNDGVVG
EVAPKSANKKADRDAAAEKLYNLVNTQLDKLGDGDYVDFSVDYNLENKII
TNQADAEAIVTKLNSLNEKTLIDIATKDTFGMVSKTQDSEGKNVAATKAL
KVKDVATFGLKSGGSEDTGYVVEMKAGAVEDKYGKVGDSTAGIAINLPST
GLEYAGKGTTIDFNKTLKVDVTGGSTPSAVAVSGFVTKDDTDLA

3. The isolated polypeptide of claim 1 or 2, wherein the SlpA cell wall binding domain is a *Lactobacillus acidophilus* or *Lactobacillus casei* SlpA cell wall binding domain.

4. The isolated polypeptide of any one of claims 1-3, wherein the SlpA cell wall binding domain has the amino acid sequence:

SNTNGKSATLPVVVTVPNVAEPTVASVSKRIMHNAYYYDKDAKRVGTDSV
KRYNSVSVLPNTTTINGKTYYQVVENGKAVDKYINAANIDGTKRTLKHNA
YVYASSKKRANKVVLKKGEVVTTYGASYTFKNGQKYYKIGDNTDKTYVKV
ANFR

5. The isolated polypeptide of any one of claims 1-4, wherein the bacterial secretion signal is a Lactococcus, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei* secretion signal.

6. The isolated polypeptide of any one of claims 1-5, wherein the bacterial secretion signal has the amino acid sequence:

MKKNLRIVSAAAAALLAVAPVAASAVSTVSA

7. The isolated polypeptide of any one of claims 1-6, having the amino acid sequence:

MKKNLRIVSAAAAALLAVAPVAASAVSTVSAAAPVFAATTGTQGYTVVKN 50
DWKKAVKQLQDGLKDNSIGKITVSFNDGVVGEVAPKSANKKADRDAAAEK 100
LYNLVNTQLDKLGDGDYVDFSVDYNLENKIITNQADAEAIVTKLNSLNEK 150
TLIDIATKDTFGMVSKTQDSEGKNVAATKALKVKDVATFGLKSGGSEDTG 200
YVVEMKAGAVEDKYGKVGDSTAGIAINLPSTGLEYAGKGTTIDFNKTLKV 250
DVTGGSTPSAVAVSGFVTKDDTDLASNTNGKSATLPVVVTVPNVAEPTVA 300
SVSKRIMHNAYYYDKDAKRVGTDSVKRYNSVSVLPNTTTINGKTYYQVVE 350
NGKAVDKYINAANIDGTKRTLKHNAYVYASSKKRANKVVLKKGEVVTTYG 400
ASYTFKNGQKYYKIGDNTDKTYVKVANFR*

8. An isolated nucleic acid molecule encoding a polypeptide of any one of claims 1-7.

9. The isolated nucleic acid molecule of claim 8, comprising a sequence optimized for expression in Lactococcus, *Lactoccocus lactis*, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei*.

10. The isolated nucleic acid molecule of claim 8, having the nucleic acid sequence:
GGATCCATGAAGAAAAATTTAAGAATCGTTAGCGCTGCTGCTGCTGCTTTACTTG
CTGTTGCTCCAGTTGCTGCTTCTGCTGTATCTACTGTTAGCGCTGCTGCACCTGTA
TTTGCTGCAACCACTGGTACACAAGGCTATACGGTGGTTAAGAATGATTGGAAA
AAGGCTGTCAAACAATTACAAGATGGACTTAAAGATAATAGTATTGGTAAGATT
ACGGTCAGTTTCAATGATGGTGTGGTAGGAGAAGTAGCACCTAAATCAGCGAAT
AAGAAAGCAGATCGAGATGCAGCCGCAGAAAAGTTGTATAATCTTGTAAATACA
CAATTAGACAAATTAGGCGATGGCGATTATGTAGATTTTTCTGTTGATTACAATC
TAGAGAATAAGATTATCACCAATCAAGCCGATGCCGAAGCTATTGTTACTAAATT
GAATTCGTTAAATGAAAAGACGCTAATTGATATTGCAACTAAAGATACGTTTGGA
ATGGTGTCTAAAACGCAGGATTCTGAAGGAAAGAATGTTGCGGCAACAAAAGCG
TTAAAAGTAAAAGATGTGGCAACTTTTGGCTTAAAGAGTGGAGGTAGTGAAGAT
ACCGGATATGTTGTCGAAATGAAAGCGGGTGCTGTTGAAGATAAGTATGGTAAA
GTAGGTGATTCTACAGCTGGTATTGCAATCAATCTTCCATCAACAGGTTTAGAAT
ATGCAGGCAAAGGAACAACTATTGATTTCAACAAAACCCTTAAAGTTGATGTAA
CTGGTGGTAGTACACCGAGTGCAGTTGCCGTAAGTGGGTTTGTGACTAAAGATGA
TACAGATTTAGCATCAAATACTAATGGTAAGTCAGCTACTTTGCCAGTAGTTGTT
ACTGTTCCTAATGTTGCTGAGCCAACTGTAGCCAGCGTAAGCAAGAGAATTATGC
ACAACGCATACTACTACGACAAGGACGCTAAGCGTGTTGGTACTGACAGCGTTA

AGCGTTACAACTCAGTAAGCGTATTGCCAAACACTACTACTATCAACGGTAAGAC
TTACTACCAAGTAGTTGAAAACGGTAAGGCTGTTGACAAGTACATCAACGCTGC
AAACATCGATGGTACTAAGCGTACTTTGAAGCACAACGCTTACGTTTACGCATCA
TCAAAGAAGCGTGCTAACAAGGTTGTATTGAAGAAGGGTGAAGTTGTAACTACT
TACGGTGCTTCATACACATTCAAGAACGGCCAAAAGTACTACAAGATCGGTGAC
AACACTGACAAGACTTACGTTAAGGTTGCAAACTTTAGATAATAAAGATCTTCGA
ATTCCCGCGGCCGC

11. A vector comprising the nucleic acid molecule of any one of claims 8-10.

12. The vector of claim 11, wherein the vector is a Lactoccocus, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei* expression vector.

13. The vector of claim 11 or 12, comprising a Lactococcus or Lactobacillus origin of replication.

14. The vector of any one of claims 11-13, that is pMGM10, pMGM11, pTRK848, or pTRK882.

15. The vector of any one of claims 11-13, comprising a first sequence identical to a sequence of a first fragment in a Lactobacillus genome, wherein the first fragment is located at the 5' or 3' terminus of a *thyA* gene, or within the *thyA* gene of Lactobacillus.

16. The vector of 15, comprising a second sequence identical to a sequence to a second fragment in Lactobacillus genome, wherein the second fragment is located at the 5' or 3' terminus of a *thyA* gene.

17. The vector of 15, wherein the Lactobacillus is *Lactobacillus casei* or *Lactobacillus acidophilus*.

18. An isolated cell comprising the isolated nucleic acid of any one of claims 8-10.

19. An isolated cell comprising the vector of any one of claims 11-14.

20. The isolated cell of claim 18 or 19, wherein the cell is a Lactoccocus, *Lactoccocus lactis*, Lactobacillus, *Lactobacillus acidophilus*, or *Lactobacillus casei* cell.

21. The isolated cell of claim 18, wherein the isolated nucleic acid molecule is integrated into the chromosome of the isolated cell.

22. A method of treating or preventing *Clostridium difficile* infection in a subject, the method comprising administering Lactoccocus or Lactobacillus expressing a chimeric SlpA polypeptide to the gut of the subject, wherein the chimeric SlpA polpypeptide comprises a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain, thereby treating or preventing *Clostridium difficile* infection in the subject.

23. A method of colonizing the gut of a subject with Lactococcus or Lactobacillus, the method comprising administering Lactoccocus or Lactobacillus expressing a chimeric SlpA polypeptide to the gut of the subject, wherein the chimeric SlpA polpypeptide comprises a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain, thereby colonizing the gut of a subject with Lactococcus or Lactobacillus.

24. The method of any one of claims 22-23, wherein the subject is a human or animal.

25. The method of any of claims 22-24, wherein the subject has undergone or is undergoing treatment with antibiotics.

26. The method of claim 25, wherein the antibiotic is one or more of a cephalosporin, metronidazole, fluoroquinolone, moxifloxacin, vancomycin, and fidaxomycin.

27. The method of any of claims 22-26, wherein the administration of the Lactoccocus or Lactobacillus expressing a chimeric SlpA polypeptide is by oral administration.

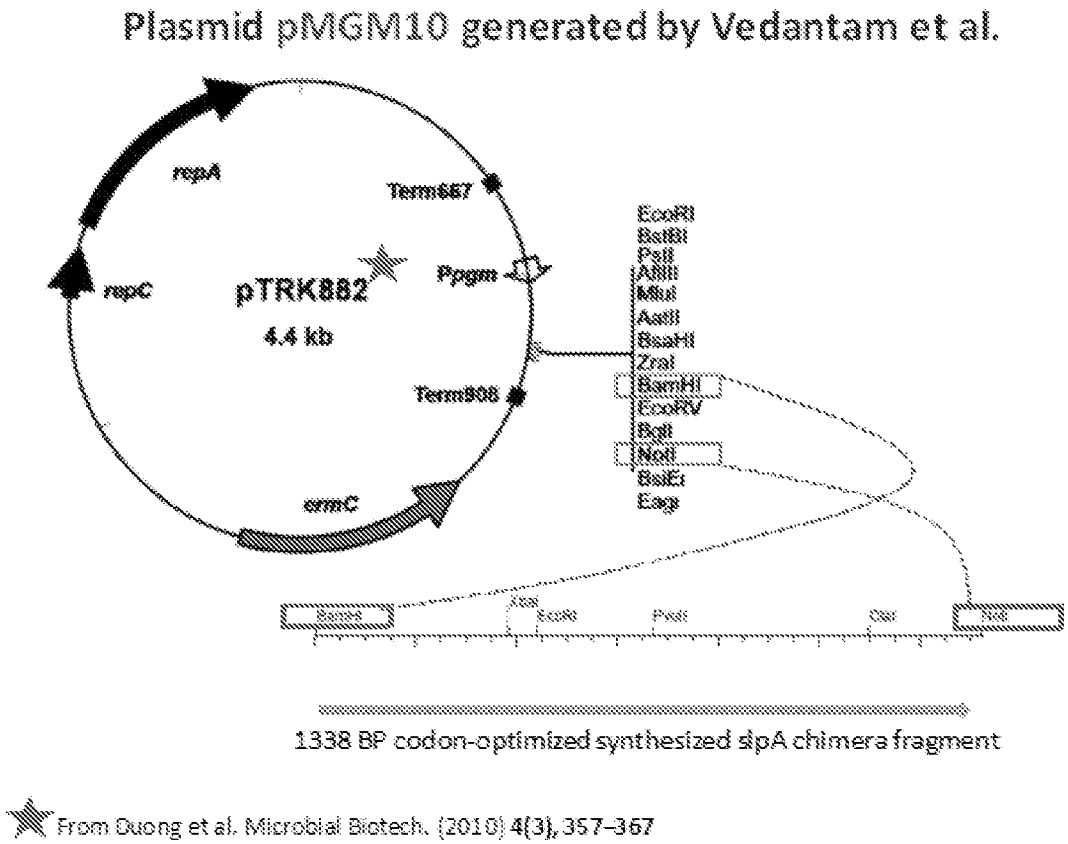
28. A composition comprising an effective amount of Lactoccocus or Lactobacillus expressing a chimeric SlpA polypeptide, wherein the chimeric SlpA polpypeptide comprises

a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

29. A kit for treating or preventing *Clostridium difficile* infection in a subject, the kit comprising Lactoccocus or Lactobacillus expressing a chimeric SlpA polypeptide, wherein the chimeric SlpA polpypeptide comprises a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

30. A kit for colonizing the gut of a subject with Lactoccocus or Lactobacillus, the kit comprising Lactoccocus or Lactobacillus expressing a chimeric SlpA polypeptide, wherein the chimeric SlpA polpypeptide comprises a bacterial secretion signal, *C. difficile* SlpA variable domain, and Lactobacillus SlpA cell wall binding domain.

FIG. 1A



From Duong et al. Microbial Biotech. (2010) 4(3), 357–367

FIG. 1B

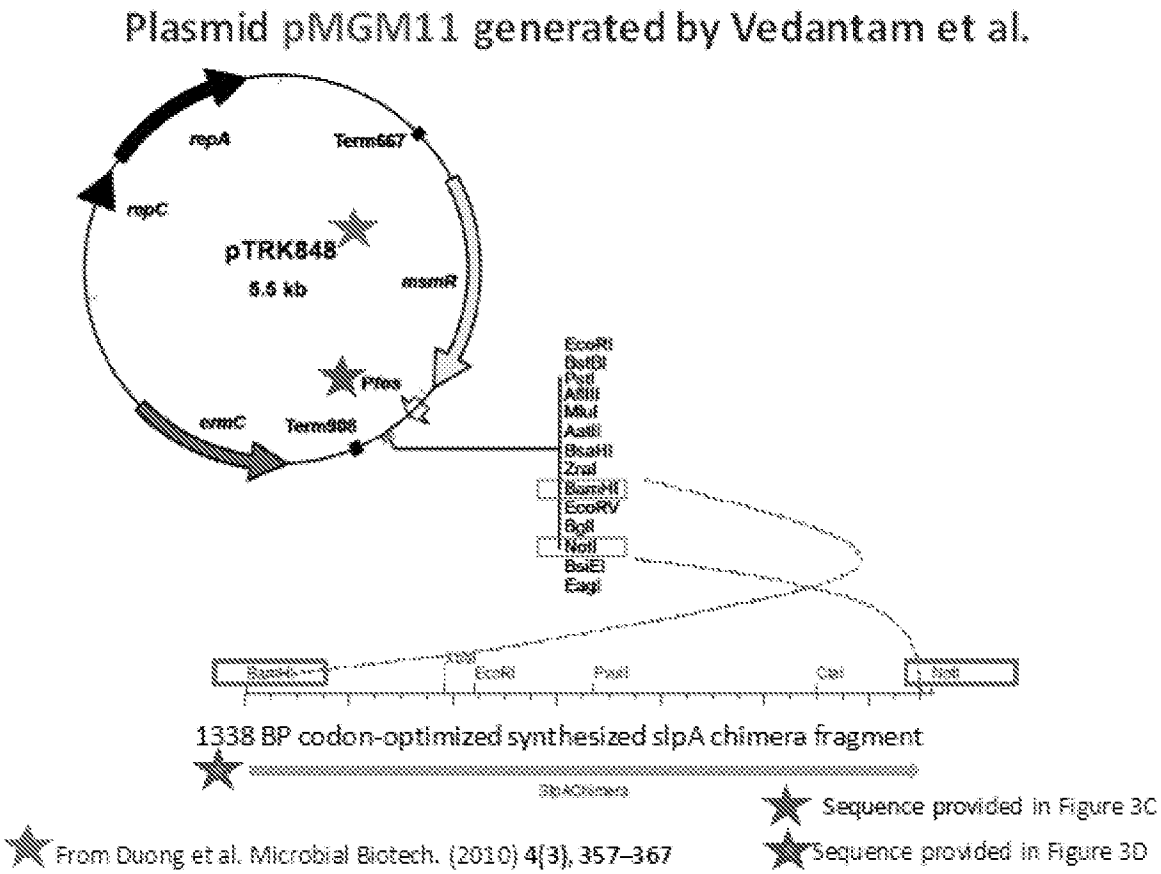

Plasmid pMGM11 generated by Vedantam et al.
repA
Term667
repC
pTRK848
5.5 kb
mcrnR
Ptet
ermC
Term966
EcoRI
BglII
PstI
AflIII
MluI
AseI
SnaBI
ZraI
BamHI
EcoRV
BglI
NotI
BstEII
SapI
BamHI
NotI
EcoRI
PstI
ClaI
SstI
1338 BP codon-optimized synthesized slpA chimera fragment
SlpAChimera
From Duong et al. Microbial Biotech. (2010) 4(3), 357–367
Sequence provided in Figure 3C
Sequence provided in Figure 3D

FIG. 1C

1338 BP codon-optimized synthesized slpA chimera fragment

GGATCCATGAAGAAAAATTTAAGAATCGTTAGCGCTGCTGCTGCTGCTTTACTTGCTGTTGCTCCA
GTTGCTGCTTCTGCTGTATCTACTGTTAGCGCTGCTGCACCTGTATTTGCTGCAACCACTGGTACAC
AAGGCTATACGGTGGTTAAGAATGATTGGAAAAAGGCTGTCAAACAATTACAAGATGGACTTAAA
GATAATAGTATTGGTAAGATTACGGTCAGTTTCAATGATGGTGTGGTAGGAGAAGTAGCACCTAAA
TCAGCGAATAAGAAAGCAGATCGAGATGCAGCCGCAGAAAAGTTGTATAATCTTGTAAATACACAA
TTAGACAAATTAGGCGATGGCGATTATGTAGATTTTTCTGTTGATTACAATCTAGAGAATAAGATTAT
CACCAATCAAGCCGATGCCGAAGCTATTGTTACTAAATTGAATTCGTTAAATGAAAAGACGCTAATT
GATATTGCAACTAAAGATACGTTTGGAATGGTGTCTAAAACGCAGGATTCTGAAGGAAAGAATGTT
GCGGCAACAAAAGCGTTAAAAGTAAAAGATGTGGCAACTTTTGGCTTAAAGAGTGGAGGTAGTG
AAGATACCGGATATGTTGTCGAAATGAAAGCGGGTGCTGTTGAAGATAAGTATGGTAAAGTAGGT
GATTCTACAGCTGGTATTGCAATCAATCTTCCATCAACAGGTTTAGAATATGCAGGCAAAGGAACA
ACTATTGATTTCAACAAAACCCTTAAAGTTGATGTAACTGGTGGTAGTACACCGAGTGCAGTTGCC
GTAAGTGGGTTTGTGACTAAAGATGATACAGATTTAGCATCAAATACTAATGGTAAGTCAGCTACTT
TGCCAGTAGTTGTTACTGTTCCTAATGTTGCTGAGCCAACTGTAGCCAGCGTAAGCAAGAGAATTA
TGCACAACGCATACTACTACGACAAGGACGCTAAGCGTGTTGGTACTGACAGCGTTAAGCGTTACA
ACTCAGTAAGCGTATTGCCAAACACTACTACTATCAACGGTAAGACTTACTACCAAGTAGTTGAAAA
CGGTAAGGCTGTTGACAAGTACATCAACGCTGCAAACATCGATGGTACTAAGCGTACTTTGAAGCA
CAACGCTTACGTTTACGCATCATCAAAGAAGCGTGCTAACAAGGTTGTATTGAAGAAGGGTGAAG
TTGTAACTACTTACGGTGCTTCATACACATTCAAGAACGGCCAAAAGTACTACAAGATCGGTGACA
ACACTGACAAGACTTACGTTAAGGTTGCAAACTTTAGATAATAAAGATCTTCGCGGCCGCATCACT
AGTGAATTCGCGGCCGC

FIG. 1D

TRANSLATED SlpA chimera fragment

```
MKKNLRIVSAAAAALLAVAPVAASAVSTVSAAAPVFAATTGTQGYTVVKN      50
DWKKAVKQLQDGLKDNSIGKITVSFNDGVVGEVAPKSANKKADRDAAAEK     100
LYNLVNTQLDKLGDGDYVDFSVDYNLENKIITNQADAEAIVTKLNSLNEK     150
TLIDIATKDTFGMVSKTQDSEGKNVAATKALKVKDVATFGLKSGGSEDTG     200
YVVEMKAGAVEDKYGKVGDSTAGIAINLPSTGLEYAGKGTTIDFNKTLKV     250
DVTGGSTPSAVAVSGFVTKDDTDLASNTNGKSATLPVVVTVPNVAEPTVA     300
SVSKRIMHNAYYYDKDAKRVGTDSVKRYNSVSVLPNTTTINGKTYYQVVE     350
NGKAVDKYINAANIDGTKRTLKHNAYVYASSKKRANKVVLKKGEVVTTYG     400
ASYTFKNGQKYYKIGDNTDKTYVKVANFR*
```

FIG. 1E

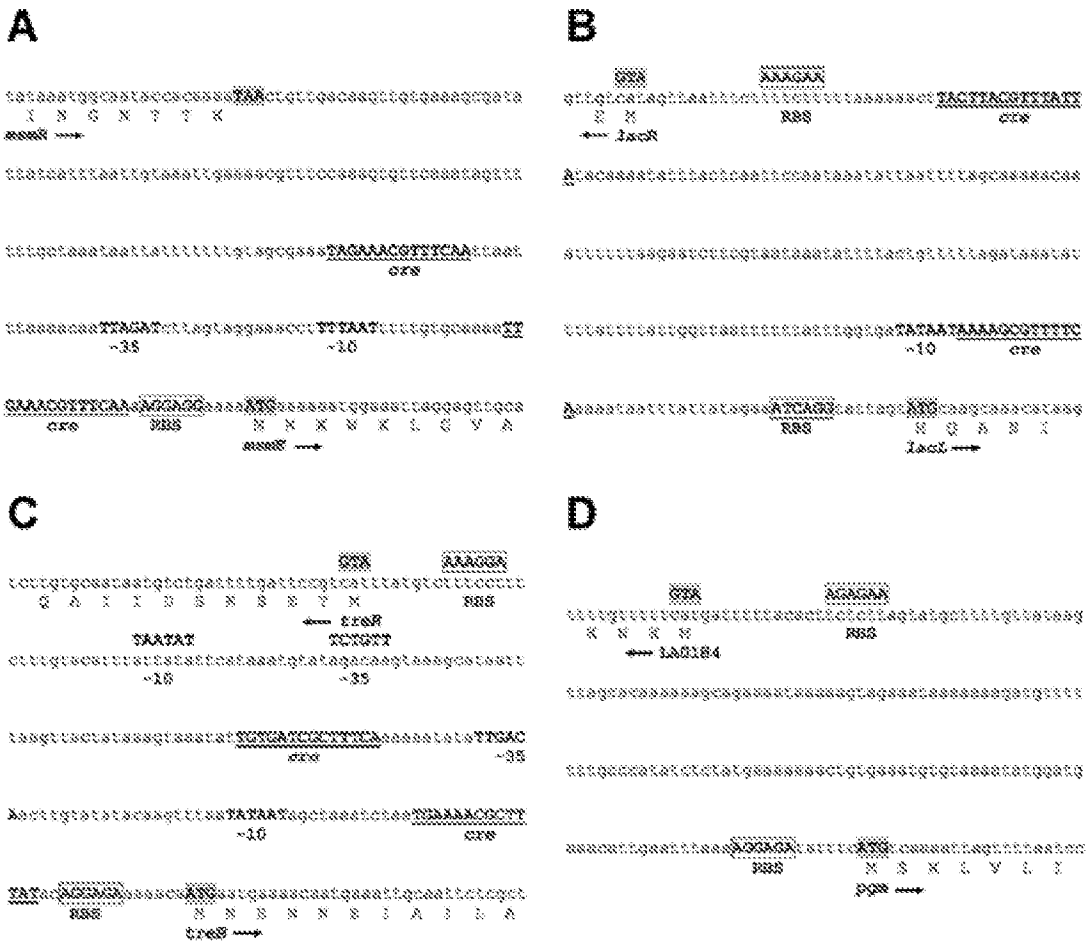


FIG. 2

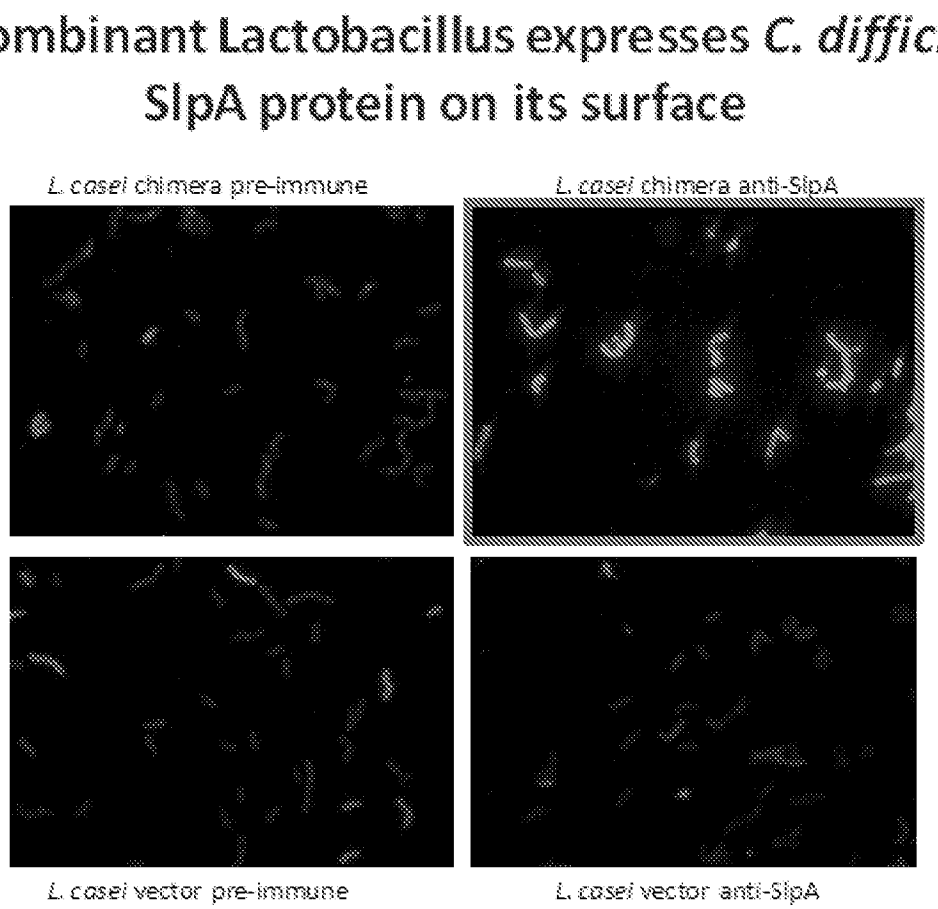

Recombinant Lactobacillus expresses C. difficile SlpA protein on its surface
L. casei chimera pre-immune
L. casei chimera anti-SlpA
L. casei vector pre-immune
L. casei vector anti-SlpA

FIG. 3

- Syrian Golden hamsters are safely and robustly colonized by recombinant Lactobacillus sp.
- Recombinant Lactobacillus protects hamsters from virulent *C. difficile* challenge

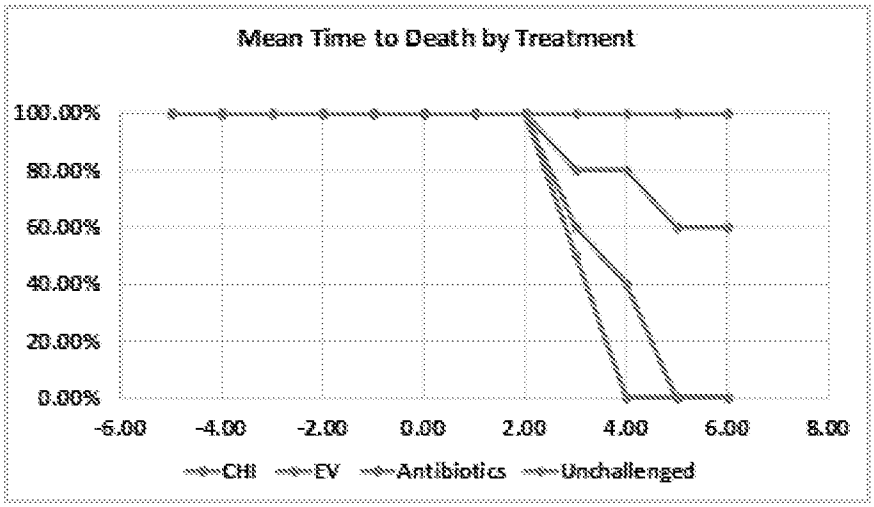


FIG. 4

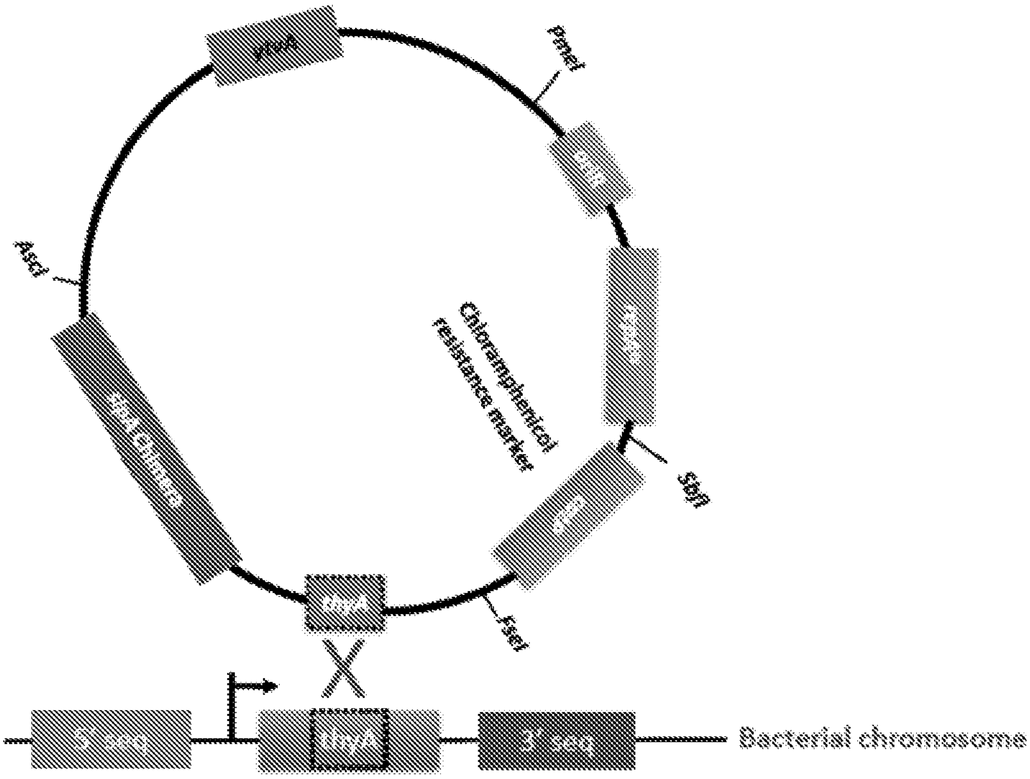

PmeI
AscI
Chloramphenicol resistance marker
SbfI
FseI
5' seq
thyA
3' seq
Bacterial chromosome

FIG. 5

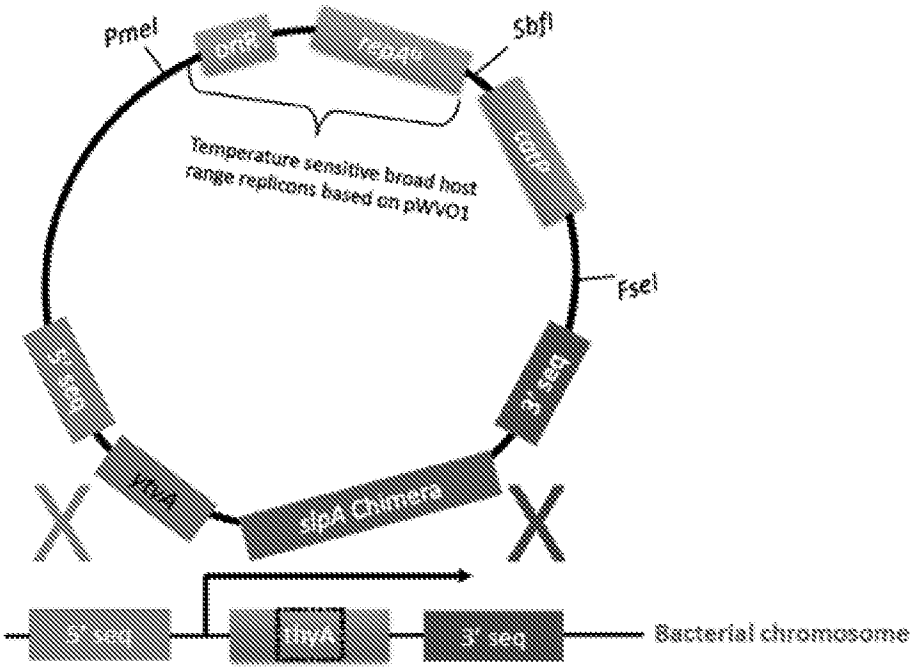

PmeI
SbfI
Temperature sensitive broad host range replicons based on pWV01
FseI
sipA Gamma
Bacterial chromosome

FIG. 6


Construct 1: Lactobacillus casei single-integration system (DNA sequence)
L casei camp int (3166 bp)

FIG. 6 (CONTINUED)

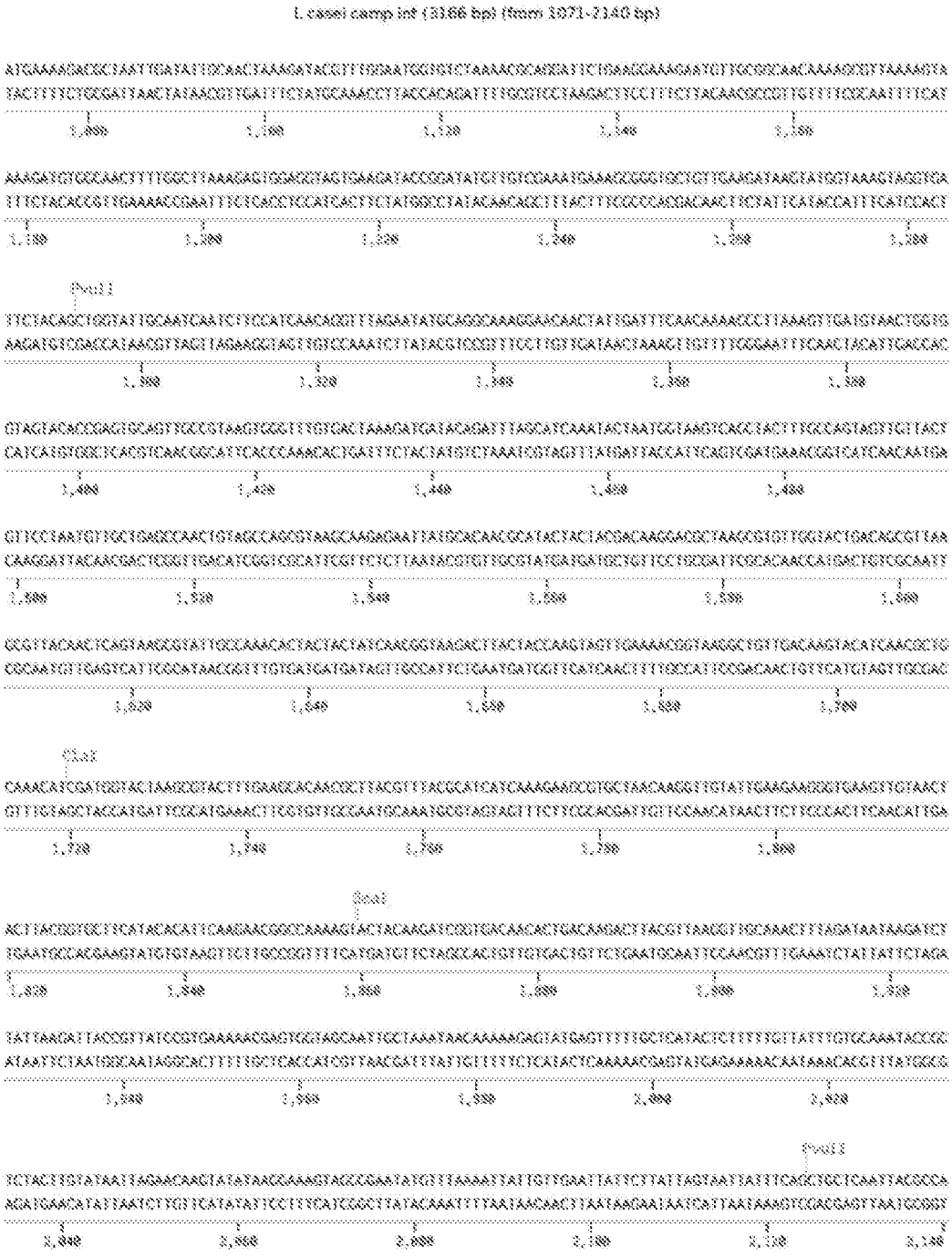


FIG. 6 (CONTINUED)

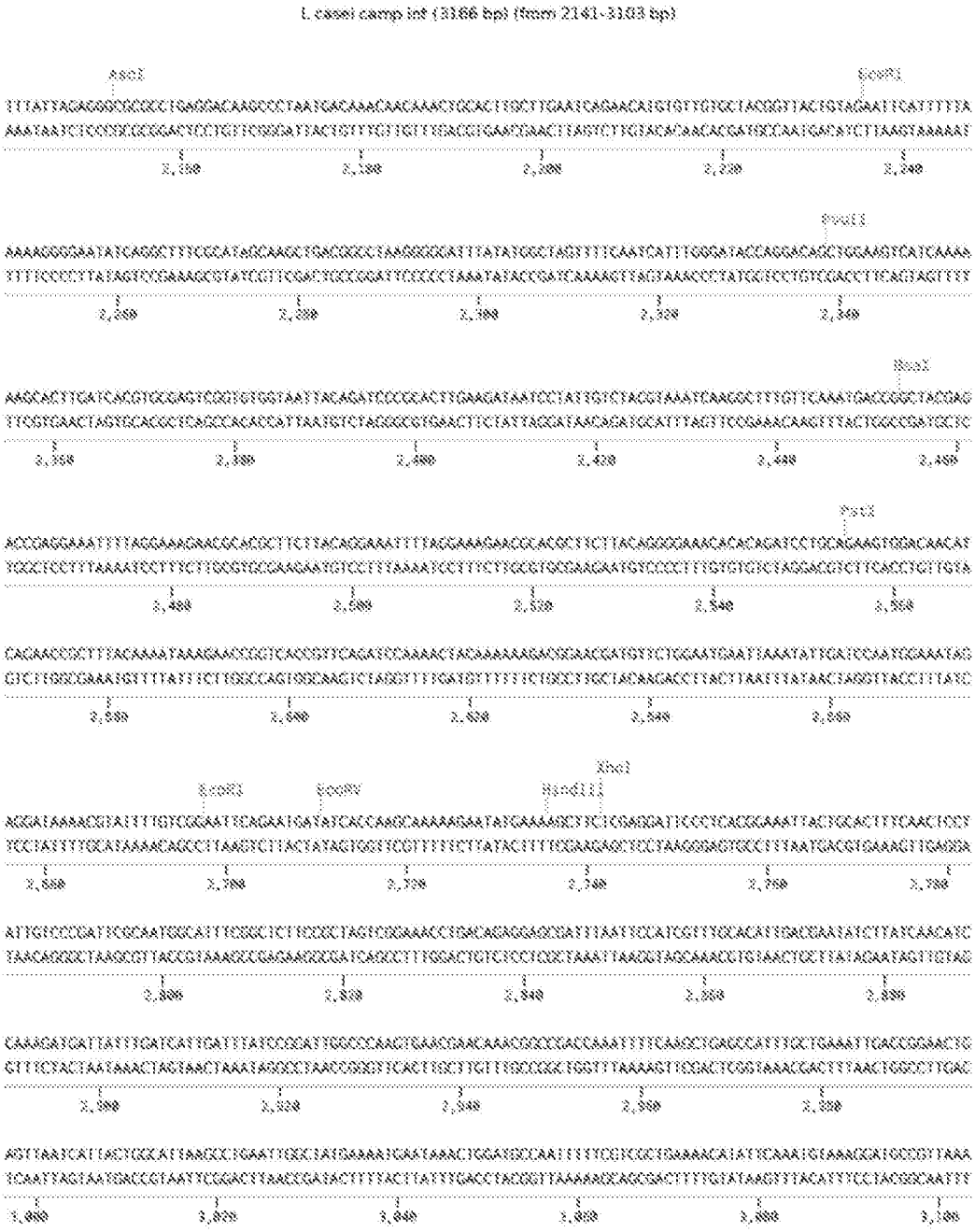


FIG. 6 (CONTINUED)

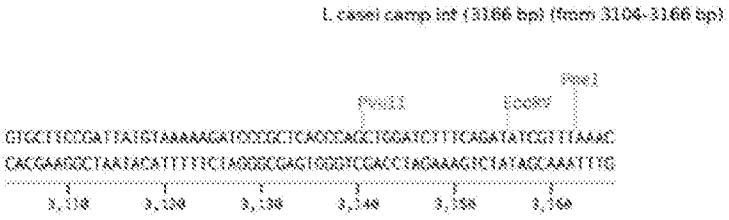


FIG. 7

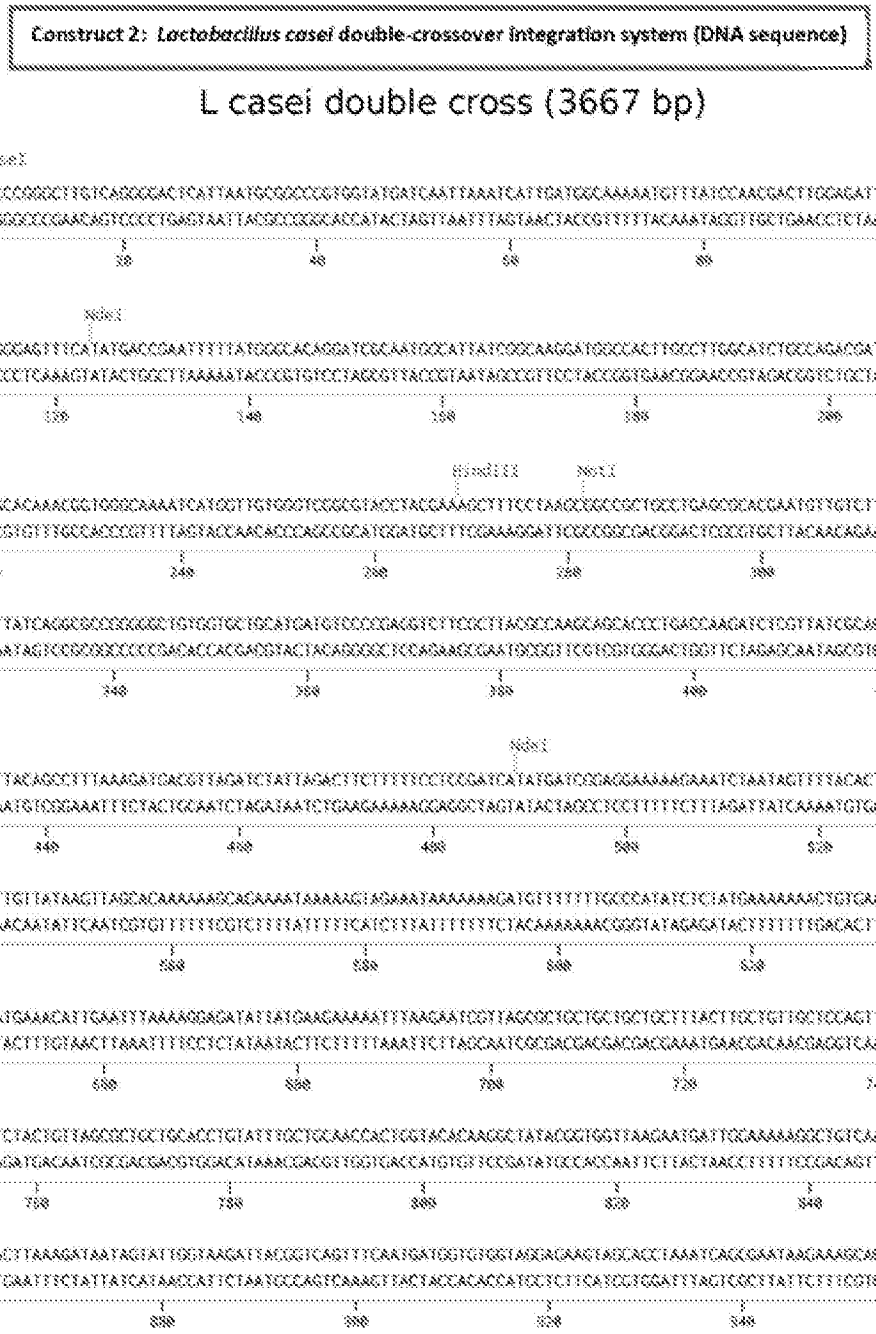

Construct 2: Lactobacillus casei double-crossover integration system (DNA sequence)
L casei double cross (3667 bp)

FIG. 7 (CONTINUED)

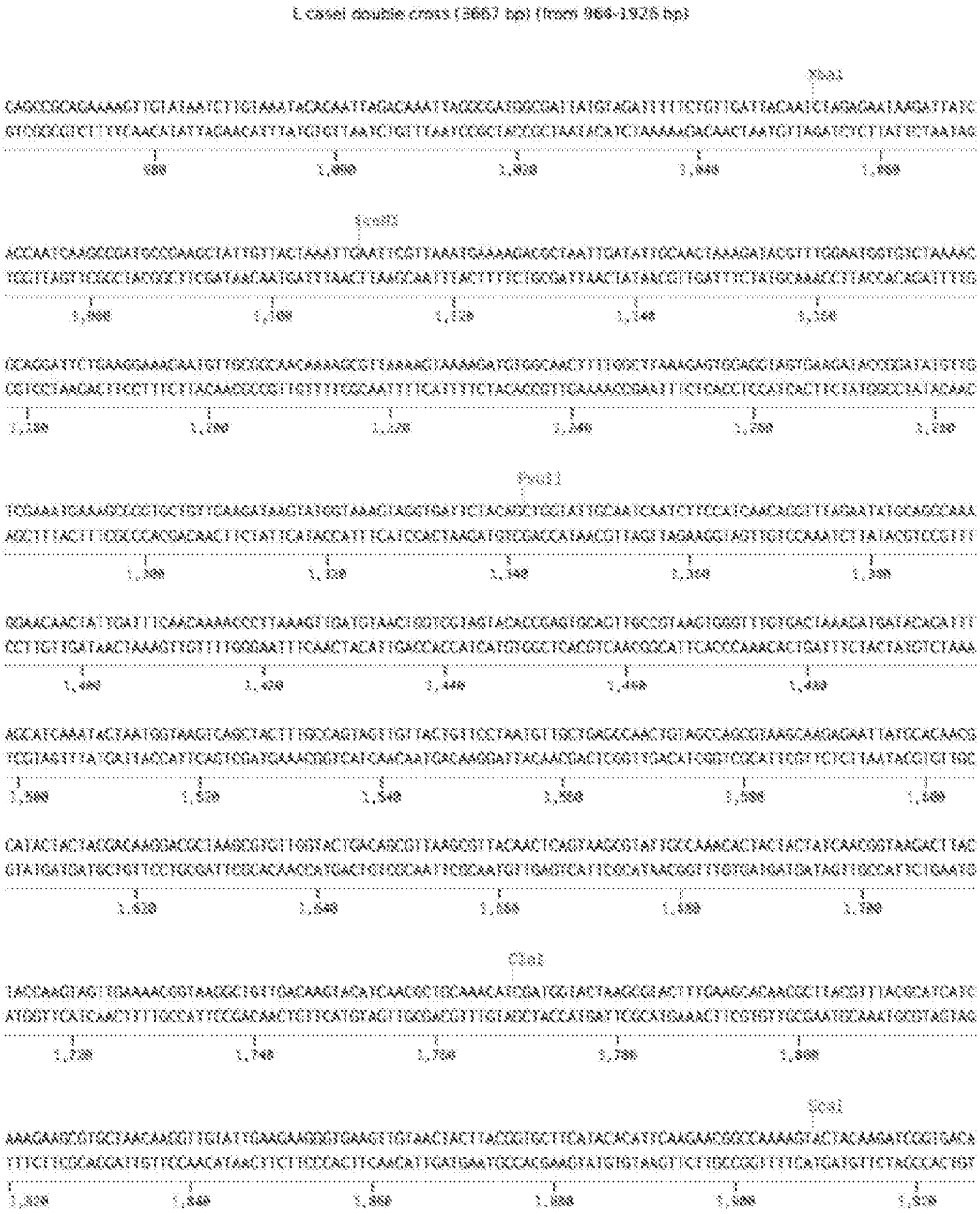


FIG. 7 (CONTINUED)

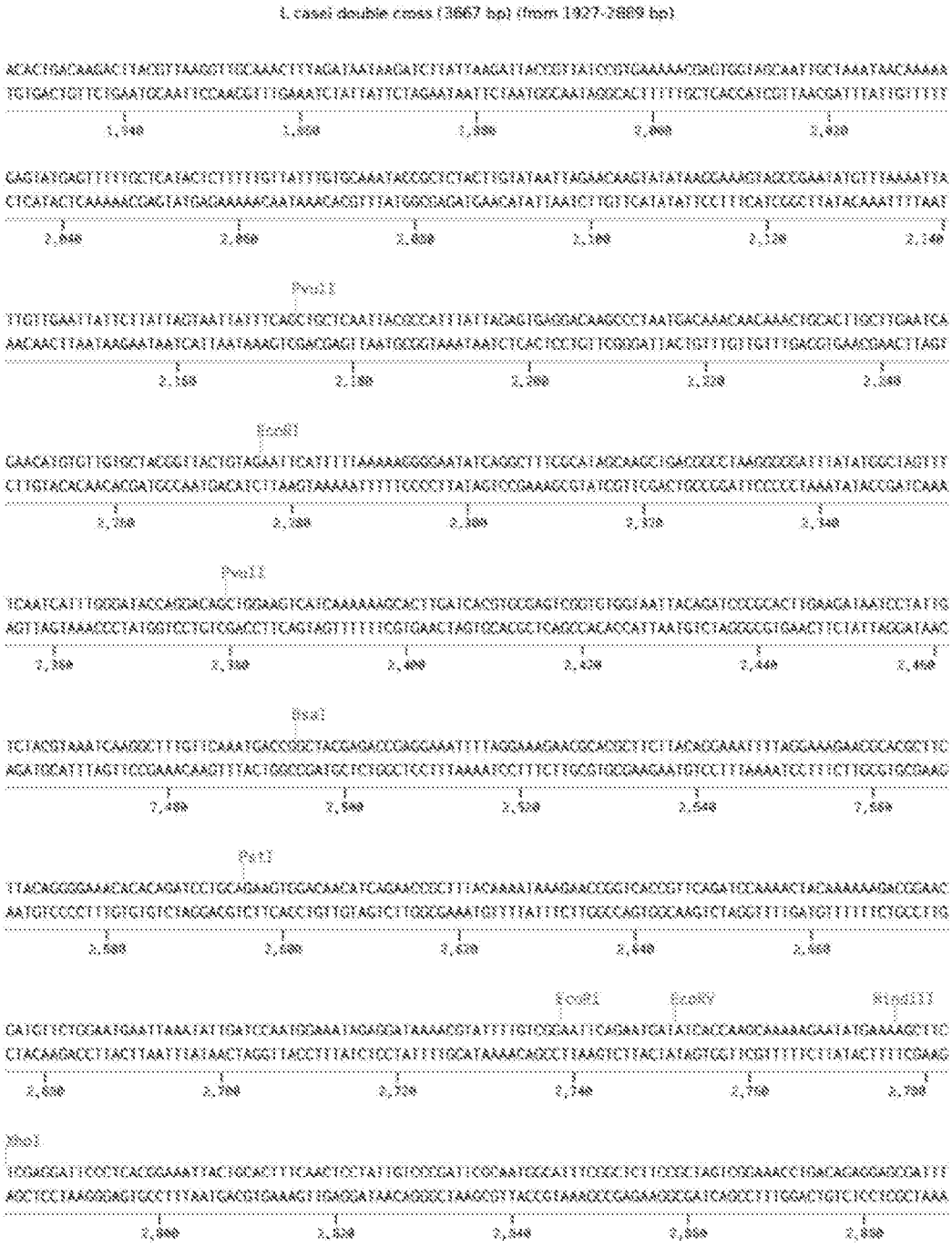


FIG. 7 (CONTINUED)

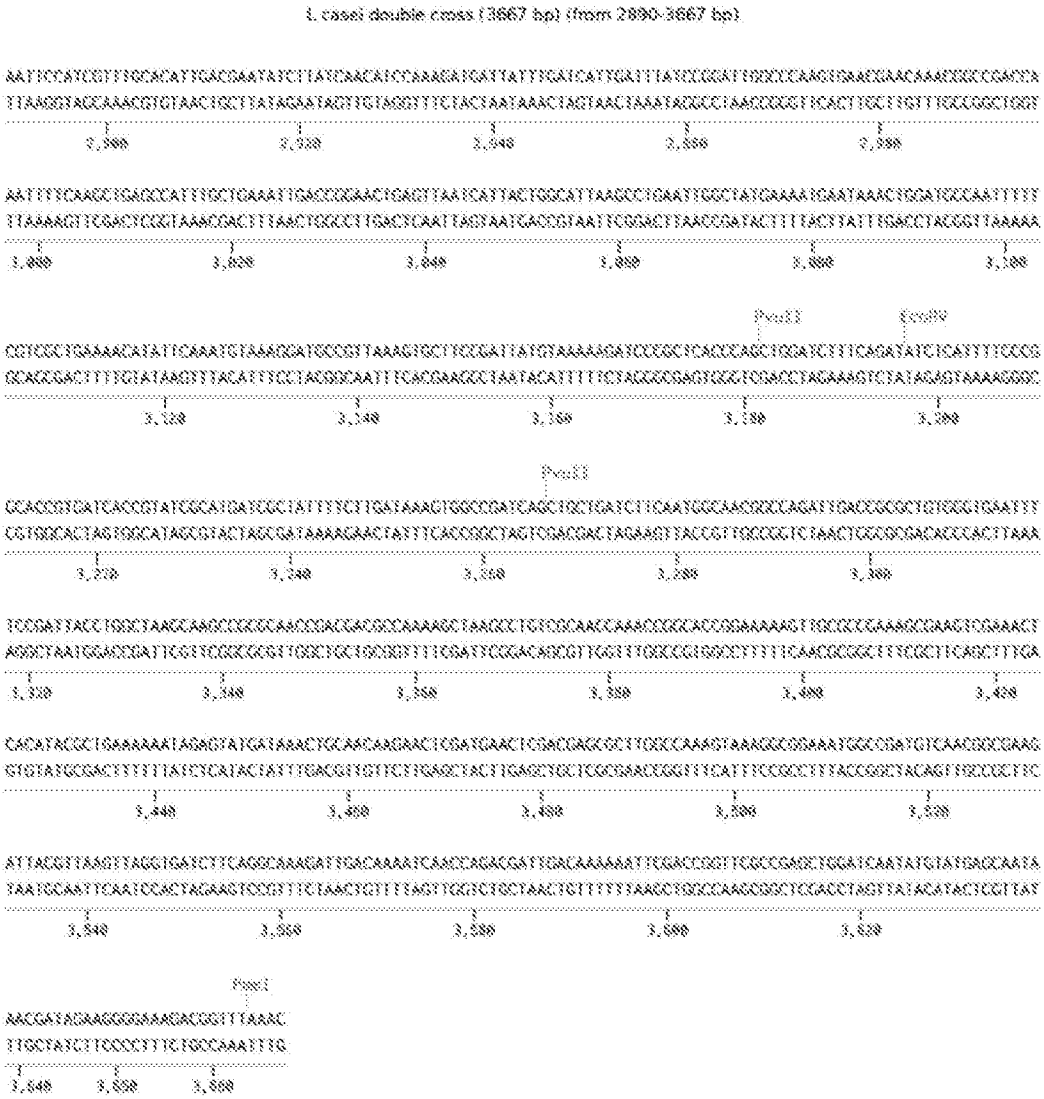


FIG. 8


Construct 3: Lactobacillus acidophilus single-integration system (DNA sequence)
LA slpA Chi (3166 bp)

FIG. 8 (CONTINUED)

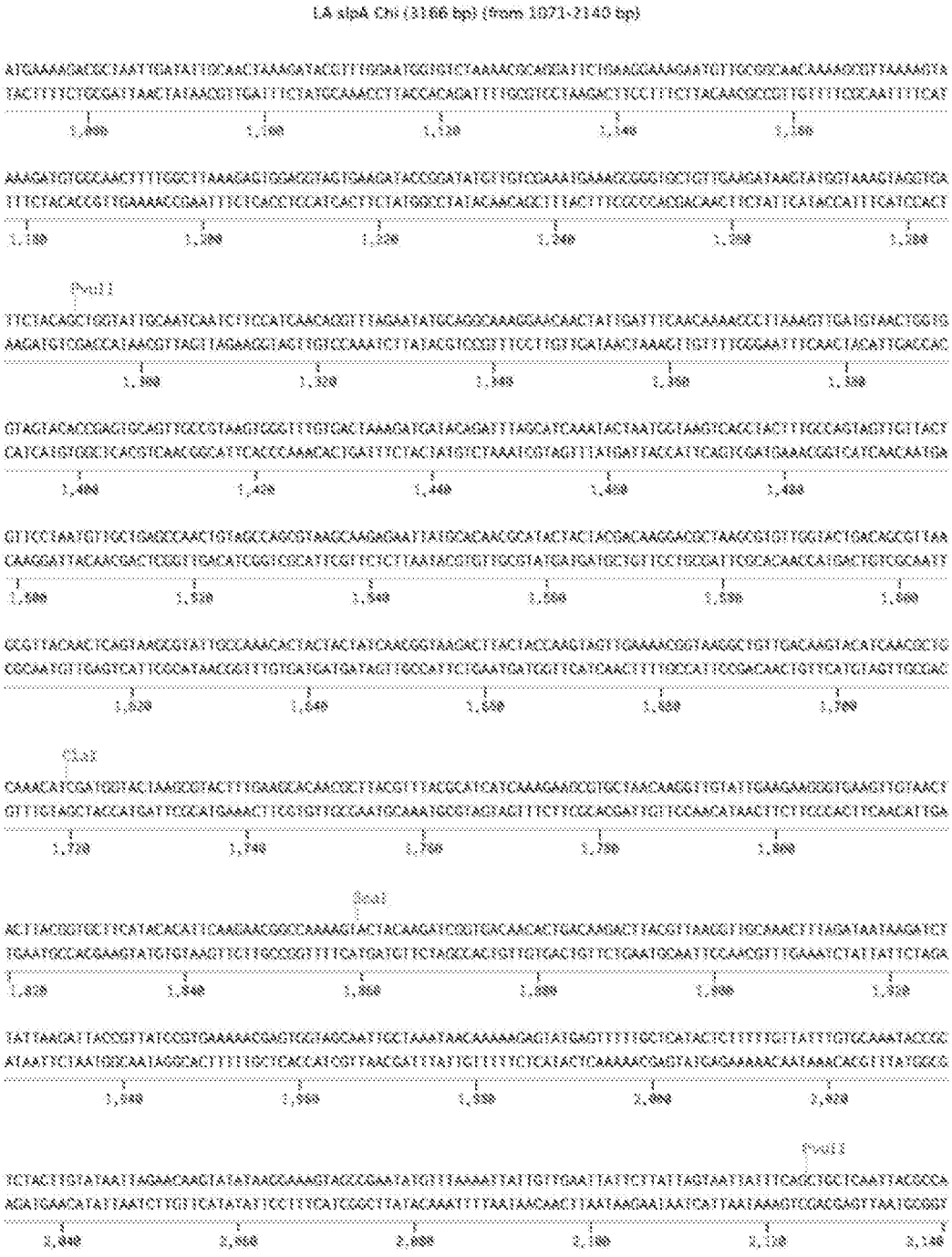


FIG. 8 (CONTINUED)

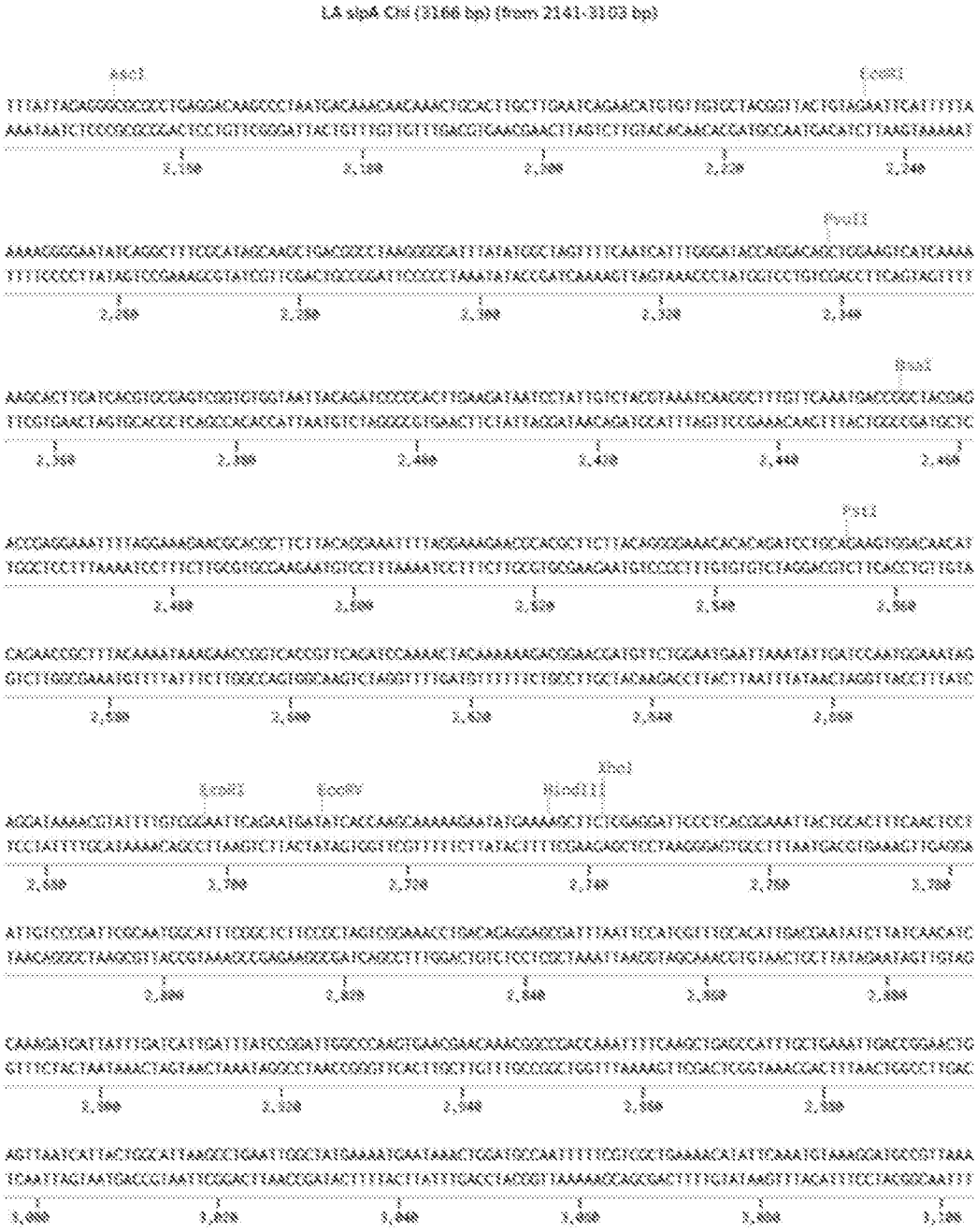


FIG. 8 (CONTINUED)

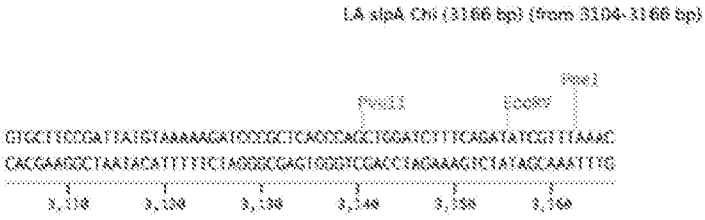


FIG. 9

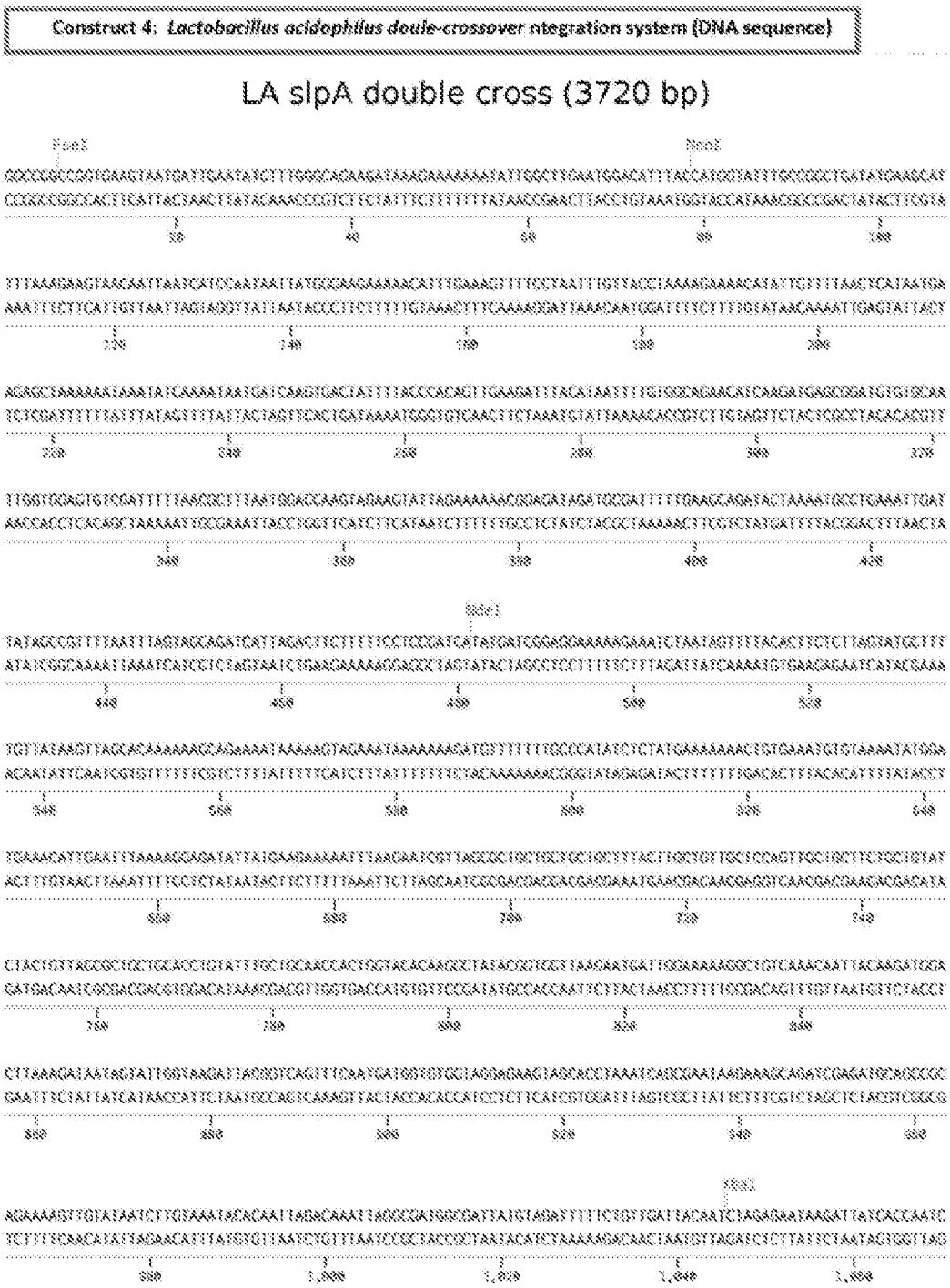

Construct 4: Lactobacillus acidophilus doule-crossover ntegration system (DNA sequence)
LA slpA double cross (3720 bp)
PstI
NcoI
XbaI

FIG. 9 (CONTINUED)

FIG. 9 (CONTINUED)

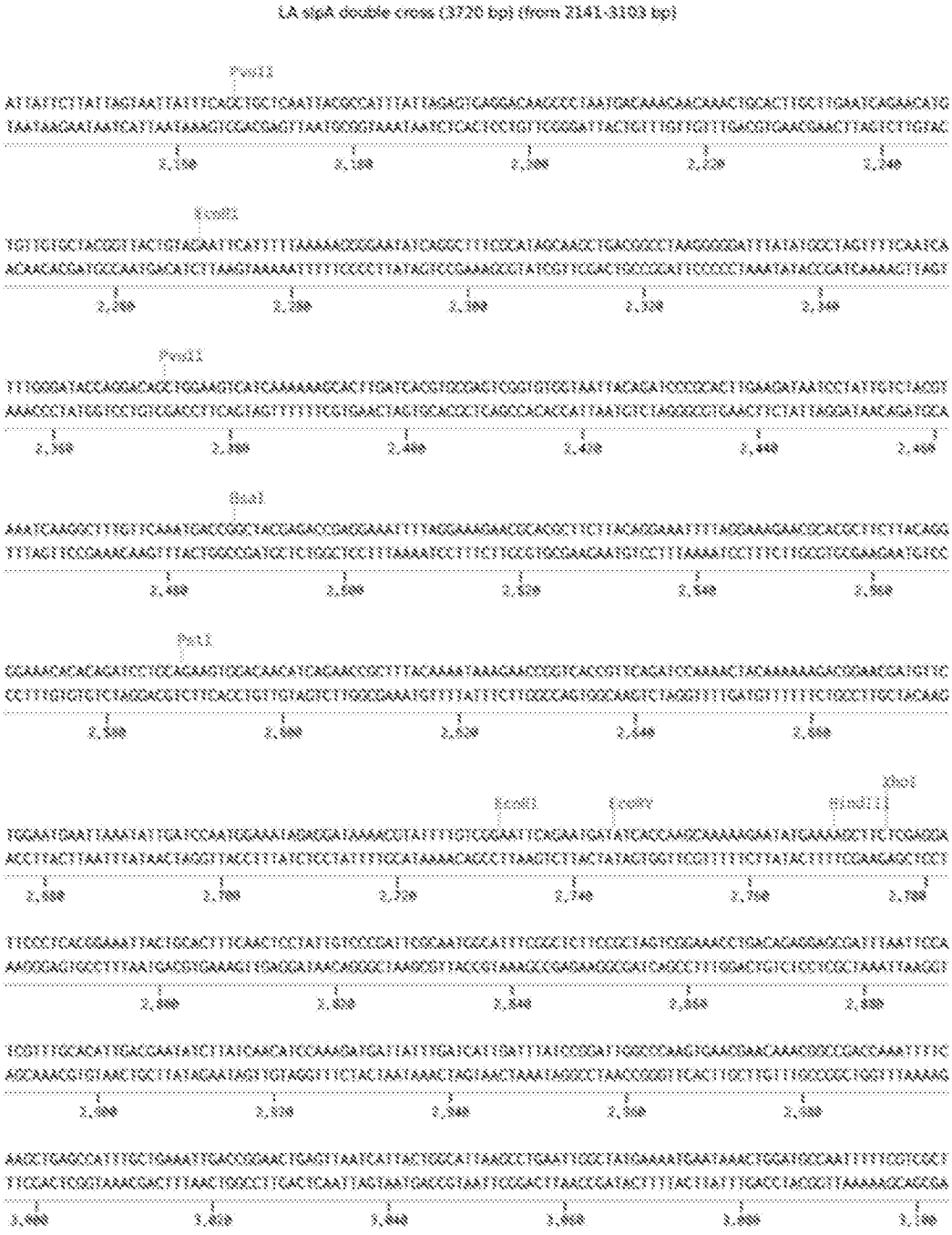
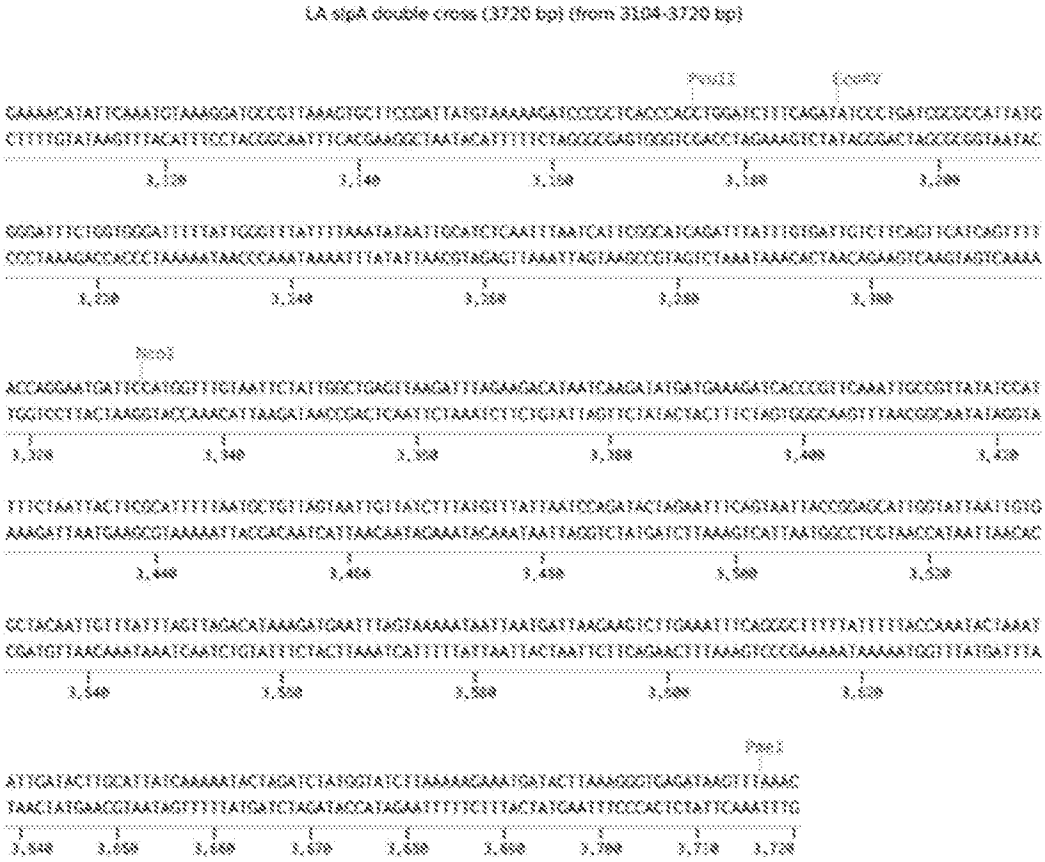


FIG. 9 (CONTINUED)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/14590

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/08, A61K 35/74, A61P 31/04 (2016.01)
CPC - A61K 39/08, A61K35/74, C07K2319/00, C07K14/335, C07K14/33, A61K 38/02, A61K38/16, A61K 38/164
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 39/08, A61K 35/74, A61P 31/04 (2016.01)
CPC - A61K 39/08, A61K35/74, C07K2319/00, C07K14/335, C07K14/33, A61K 38/02, A61K38/16, A61K 38/164

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Keyword search; search terms below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar, Google Patents. chimeric polypeptide, probiotic, slpA, Lactobacillus, Clostridium difficile, C. difficile, enhanced, recombinant, slpA chimer, intestinal adhesion, cell wall binding domain, adherence, blocking, intestinal mucosa, infection, epithel*, pathogen, genetically engineered/modified, chimera, heterologous

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0233408 A1 (POUWELS et al.) 20 October 2005 (20.10.2005) abstract, para [0026], [0028], [0069], [0076], [0101], [0102], [0114], [0123], [0165], [0167], [0168], [0175], [0228]	1-3, 22-24, 28-30
Y	MERRIGAN et al. Surface-Layer Protein A (SlpA) Is a Major Contributor to Host-Cell Adherence of Clostridium difficile. 12 November 2013 (12.11.2013) Volume 8 Issue11:e78404, author manuscript pages 1-12. abstract, page 11 col 1 para 2, col 2 para 2.	1-3, 22-24, 28-30
Y	Q1JU94, UniProtKB Accession No: Q1JU94, S-layer protein, 13 June 2006 [online]. [Retrieved on 3 May 2016]. Retrieved from the Internet <URL: http://www.uniprot.org/uniprot/Q1JU94> Entire document	2, 3/2

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
03 May 2016	19 MAY 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/14590

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

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

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

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

3. ☒ Claims Nos.: 4-21 and 25-27
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.