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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 September 2013 (06.09.2013)

WIPO | PCT

* WO, 2013/130351, A1 *

(10) International Publication Number
WO 2013/130351 A1

(51) International Patent Classification:
C07K 14/315(2006.01)

(21) International Application Number:
PCT/US2013/027340

(22) International Filing Date:
22 February 2013 (22.02.2013)

(25) Filing Language: English

(26) Publication Language English

(30) Priority Data:
61/603,693 27 February 2012 (27.02.2012) US
61/603,661 27 February 2012 (27.02.2012) US

(71) Applicants: ORAGENICS, INC. [US/US]; 13700 Progress Boulevard, Alachua, FL 32615 (US). THE TEXAS A&M UNIVERSITY SYSTEM [US/US]; Office of Technology Commercialization, 800 Raymond Stotzer Parkway, Suite 2020, College Station, Texas 77845 (US).

(72) Inventors: HILLMAN, Jeffrey D. ; 6424 SW 26th Place, Gainesville, FL 32608 (US). SMITH, James Leif; 606 Coachlight Court, College Station, TX 77845 (US). WILSON-STANFORD, Shawanda R; 1109 Southwest Parkway, Apartment 1811, College Station, TX 77840 (US).

(74) Agent: HILLMAN, Lisa, M.W. ; McDonnell Boehnen Hulbert & Berghoff LLP, 300 South Wacker Drive, Suite 3100, Chicago, IL 60606 (US).

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

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

Published

— *with international search report (Art. 21(3))*

WO 2013/130351 A1 * WO, 2013/130351, A1 *

(54) Title: REPLACEMENT THERAPY FOR DENTAL CARIES

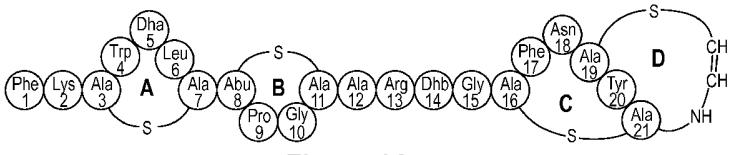


Figure 1A
Wild-type (native) MU1140

(57) Abstract: The invention provides recombinant Streptococcus mutans strains that can be used to improve oral health. An embodiment of the invention provides a method of reducing the incidence or severity of dental caries in a dental caries-susceptible host comprising administering orally to the host an isolated recombinant S. mutans strain of the invention in an amount effective for replacement of dental caries-causing S. mutans host strains in the oral cavity of the host. The isolated recombinant S. mutans strain 10 can be contained in a mouthwash, toothpaste, chewing gum, floss, chewable tablet, food, or beverage.

TITLE: Replacement Therapy for Dental Caries

PRIORITY

This application claims the benefit of U.S. provisional application 61/603,661, filed February 27, 2012, and U.S. provisional application 61/603,693, filed February 27, 2012, both of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Dental caries are one of the most prevalent chronic infectious diseases in the world. Over half of U.S. children age 5 – 9 have at least one cavity or filling; by age 17, nearly 80% of our young people have had a cavity. U.S. Department of Health and Human Services. *Oral Health in America: A Report of the Surgeon General--Executive Summary*. Rockville, MD: US Department of Health and Human Services, National Institute of Dental and Craniofacial Research, National Institutes of Health, 2000.

Annual expenditures on the treatment of dental caries in the U.S. are estimated to be $40 billion a year according to the Dental, Oral and Craniofacial Data Resource Center. Tooth decay is characterized by the demineralization of enamel and dentin, eventually resulting in the destruction of the teeth. Dietary sugar is often misperceived as the cause of tooth decay; however, the immediate cause of tooth decay is lactic acid produced by microorganisms that metabolize sugar on the surface of the teeth. Studies suggest that of the approximately 700 oral microorganisms, *Streptococcus mutans*, a bacterium found in virtually all humans, is the principal causative agent in the development of tooth decay. Residing within dental plaque on the surface of teeth, *S. mutans* derives energy from carbohydrate metabolism as it converts dietary sugar to lactic acid which, in turn, promotes demineralization in enamel and dentin, eventually resulting in a cavity. The rate at which mineral is lost depends on several factors, including the number of *S. mutans* cells that are present and the frequency and amount of sugar that is consumed.

Therapeutic regimens that take advantage of bacterial interference to replace a pathogenic bacterial strain such as *S. mutans* with a non-pathogenic, effector strain are known as replacement therapies. Successful replacement therapy requires an effector strain that: 1) is non-pathogenic, 2) alters the microenvironment to prevent colonization or outgrowth of a pathogenic organism, and 3) persistently colonizes the host at risk to prevent reinfection by the target pathogenic organism,

and aggressively displaces the pathogenic organism from the tissues at risk in the case where the pathogen is part of the host's indigenous flora.

Application of the principles of replacement therapy requires the isolation of a non-cariogenic effector strain of *S. mutans*, *e.g.,* an *S. mutans* strain deficient in lactic acid synthesis that can outcompete native *S. mutans* in the oral cavity of the host. There is a need in the art for stable, lactic acid-deficient, non-cariogenic strains of *S. mutans* that can persistently colonize and aggressively outcompete native *S. mutans* in the oral cavities of the hosts, and that are suitable for use in a replacement therapy in the prevention and/or treatment of dental caries.

The ability of an effector strain to preemptively colonize the human oral cavity and aggressively displace indigenous wild-type strains was initially thought to be a complex phenomenon dependent on a large number of phenotypic properties. However, it was discovered that a single phenotypic property could provide the necessary selective advantage. A naturally occurring strain of *S. mutans* was isolated from a human subject that produces a lantibiotic called MU1140, which is capable of killing virtually all other strains of mutans streptococci against which it was tested. *See e.g.,* Hillman *et al.,* Infect. Immun. 44:141 (1984). Mutants were isolated that produced no detectable MU1140 or that produced approximately three-fold elevated amounts. The mutants were used in a rat model to correlate lantibiotic production to colonization potential. It was found that the ability of these strains to preemptively colonize the host and aggressively displace indigenous strains of *S. mutans* increased significantly as the amount of MU1140 produced increased.

The same relationship between MU1140 production and colonization potential was observed in human subjects, where repeated exposures to the wild-type parent strain were required to achieve persistent colonization (Hillman *et al.* J. Dent. Res. 66:1092 (1985)), whereas a single exposure to the strain producing three-fold elevated amounts of MU1140 was sufficient (Hillman *et al.* J. Dent. Res. 66:1092 (1987)). The latter strain required over a year to completely replace indigenous strains of *S. mutans* in the mouths of the human subjects. During this period, it is presumed that their susceptibility to dental caries persisted until the levels of indigenous *S. mutans* decreased below a threshold level.

In order to further increase the colonization potential of an effector strain for replacement therapy of dental caries, it is desirable to obtain one or more strains of

S. mutans that produce elevated amounts of MU1140 or produce variants of this molecule with increased specific activity. Such strains would reduce the period required for the effector strain to eliminate indigenous, lactic acid-producing strains and thereby achieve full effectiveness. Such strains are also more likely to overcome any inherent resistance to colonization, which, while not currently known, may exist in certain individuals in the population being treated. *See, e.g.,* Hillman, *Antonie van Leeuwenhoek* 82: 361–366, 2002.

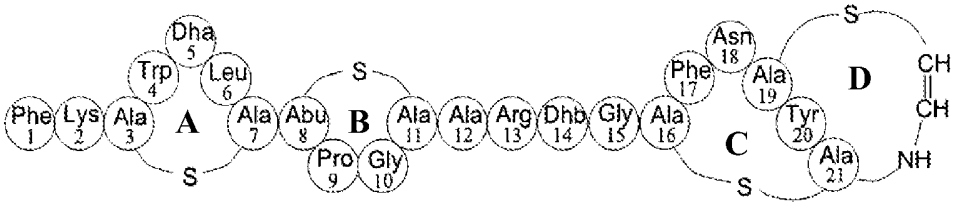
SUMMARY OF THE INVENTION

In one embodiment the invention provides an isolated recombinant *Streptococcus mutans* strain comprising:

(a) a mutation in a polynucleotide involved in lactic acid synthesis such that expression of lactic acid is diminished by about 80% or more as compared to a wild-type *S. mutans* strain;

(b) a recombinant alcohol dehydrogenase polynucleotide;

(c) a recombinant polynucleotide encoding a lantibiotic comprising Formula I:



(SEQ ID NO:1), wherein the following mutations are present: a Phe1Ile mutation or a Phe1Gly mutation; a Trp4Ala mutation; a Dha5Ala mutation; an Arg13Asp mutation; or combinations of two or more of these mutations. The strain can further comprise a Trp4insAla mutation or a ΔTrp4 mutation. The following amino acid substitutions can also be present: Abu8Ala, or Dhb14Ala, or both Abu8Ala and Dhb14Ala. The strain can further comprise a mutation in a polynucleotide involved in ComE, ComC, or both ComE and ComC synthesis such that expression of ComE, ComC, or both ComE and ComC is diminished by about 80% or more as compared to a wild-type *S. mutans* strain. The strain can further comprising a mutation in a polynucleotide involved in D-amino acid synthesis such that expression of the D-amino acid is diminished by about 80% or more as compared to a wild-type *Streptococcus mutans*

strain. The polynucleotide involved in D-amino acid synthesis can be *dal* or a promoter for *dal*. The recombinant alcohol dehydrogenase polynucleotide can be a *Zymomonas mobilis* alcohol dehydrogenase polynucleotide or a *Streptococcus mutans* alcohol dehydrogenase polynucleotide.

Another embodiment of the invention provides a method of reducing the incidence or severity of dental caries in a dental caries-susceptible host comprising administering orally to the host an isolated recombinant *S. mutans* strain of the invention in an amount effective for replacement of dental caries-causing *S. mutans* host strains in the oral cavity of the host. The isolated recombinant *S. mutans* strain can be contained in a mouthwash, toothpaste, chewing gum, floss, chewable tablet, food, or beverage.

Still another embodiment of the invention provides a pharmaceutical composition for reducing the incidence or severity of dental caries comprising an isolated recombinant *S. mutans* strain of the invention and a pharmaceutically acceptable carrier.

Therefore, the invention provides strains of *S. mutans* that are stable, lactic acid-deficient, and non-cariogenic that can aggressively outcompete native *S. mutans* due to, *inter alia,* the expression of a variant MU1140 lantibiotic that has improved biological activity as compared to a wild-type MU1140 lantibiotic.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the wild-type MU1140 structure (SEQ ID NO:1). Figure 1B shows mutation sites of MU1140 (SEQ ID NO:2).

Figure 2 shows the sequence of chromosomal DNA highlighting mutations of variant MU1140 *lanA* polynucleotide sequences with the wild type MU1140 *lanA* polynucleotide sequence.

Figure 3 shows the primers used for mutagenesis of *lanA*, the MU1140 structural gene.

Figure 4A-B shows the results of the zone of inhibition plate assays.

Figure 5 shows the means and standard deviations for the bioactivity of strains producing variants of MU1140 compared to wild-type MU1140.

Figure 6 shows the biological activity of strains producing variants of MU1140 (Phe1Ile and Phe1Gly) compared to wild-type MU1140.

DETAILED DESCRIPTION OF THE INVENTION

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

Streptococcus mutans can be recombinantly manipulated to produce no lactic acid or substantially reduced amounts of lactic acid. Hillman *et al.* J. Appl. Microbiol. 102:1209 (2007). Viable, lactic acid-deficient *S. mutans* strains can be generated by transforming the strains with nucleic acid encoding a recombinant alcohol dehydrogenase (ADH) such that a recombinant alcohol dehydrogenase is expressed, and introducing a mutation in the lactic acid synthesis pathway to render the recombinant ADH-producing strain lactic acid deficient. The recombinant ADH prevents accumulation of metabolites in the bacterium, thus circumventing any lethality of the lactic acid deficiency. Furthermore, *S. mutans* strains can be recombinantly engineered to express a variant MU1140 lantibiotic that has greater biological activity than wild-type MU1140 lantibiotics. These strains can outcompete and replace dental caries-causing wild-type, native *S. mutans* strains in the oral cavity of hosts.

Parent *Streptococcus mutans* Strains

Any *S. mutans* strains can be used to construct the recombinant *S. mutans* strains of the invention. Recombinant *S. mutans* strains of the invention have a selective advantage over wild-type *S. mutans* strains that normally colonize the oral cavity. The selective advantage can be conferred by any of a variety of characteristics (*e.g.,* production of an antibacterial compound, reduced or advantageous relative metabolic needs, greater relative growth rate, production of scavengers for metabolites) that promote oral cavity colonization by the strain and replacement of the resident strain colonizing the oral cavity. In one embodiment of the invention, colonization by the recombinant *S. mutans* strains of the invention will not substantially disrupt colonization by other, non-*S. mutans* strains (*e.g.,* normal bacterial flora not associated with cariogenesis). For example, infection with a recombinant strain of *S. mutans* that produces a variant MU1140 lantibiotic with enhanced lantibiotic activity can result in replacement of the resident, cariogenic *S. mutans* strains without effect upon other resident microbial species of the oral cavity.

Recombinant *Streptococcus mutans* Strains

A recombinant *S. mutans* strain is a non-naturally occurring strain of *S. mutans* that has been generated using any of a variety of recombinant nucleic acid

techniques (*i.e.,* techniques involving the manipulation of DNA or RNA). In general, a recombinant *S. mutans* strain of the invention has a deficiency in lactic acid production; expresses a recombinant alcohol dehydrogenase (ADH) polypeptide; and expresses recombinant polypeptides sufficient to produce a variant MU1140 lantibiotic that has greater biological activity than wild-type MU1140. Recombinant strains of *S. mutans* can optionally be deficient in ComE, ComC, or both ComE and ComC expression and/or can optionally be auxotrophic for an organic substance not normally present in the oral cavity or diet of a particular host (*e.g.*, a D-amino acid).

Variant MU1140

MU1140 has an overall horseshoe-like shape kinked at the "hinge region" between rings B and C. Smith *et al.* (2003) Biochem. 42:10372-10384. This shape is the result of a turn-like motif in the hinge region that folds the amino-terminal AB rings (the lipid II binding domain) towards the carboxy-terminal overlapped rings CD. The flexibility of the hinge region is believed to be important in promoting lateral assembly of MU1140, enabling it to abduct and sequester lipid II. The Ψ angle of Trp4 and Φ angle of Dha5 in ring A help contribute to its flexibility. Also it was determined that the Ψ bond of $_S$Ala7 (a residue that is not confined by the thioether ring) rotates 360º allowing ring A to spin freely with respect to ring B. This flexibility is thought to be important in orienting rings A and B during lipid II binding. The hinge region also contains a potentially enzymatically susceptible arginine at residue 13. Mutations in the structural gene (*lanA*) for MU1140 were generated to determine the effect of the following amino acid alterations: Phe1Ile, Phe1Gly, Trp4Ala, Trp4insAla, ΔTrp4, Dha5Ala, Ala$_S$7insAla, and Arg13Asp. Figure 1B.

It was found that the variants of MU1140 possessing a deletion of Trp4 or insertion of Ala after Trp4 showed bioactivity activity approximately equivalent to the wild-type in a deferred antagonism assay using *Micrococcus luteus* strain ATCC 272 as the target strain. Wilson-Sanford *et al.,* (2009) Appl. Environ. Microbiol. 75:1381. In this assay, activity is determined by calculating the area of the zone of inhibition. These results indicate that shortening or lengthening ring A had no beneficial or deleterious effect on MU1140 activity, indicating an unexpected permissiveness in the structure of ring A. As shown in Figure 5, the Trp4Ala substitution resulted in a statistically significant ($p<.05$) increase in bioactivity when compared to the wild-type. Since both amino acids are uncharged and hydrophobic, it can be speculated that

the difference in bioactivity was due to the size difference between the two amino acids. Replacement of Dha5 with Ala also resulted in a statistically significant (p<.05) increase in bioactivity. Insertion of alanine after $_S$Ala at position 7 resulted in a significant (p<.05) reduction of bioactivity. While not wishing to be bound to any particular theory, since it has been determined that $_S$Ala7 freely rotates 360° allowing ring A to spin freely with respect to ring B, it could be concluded that the Ala$_S$7insAla mutation changed the orientation of the rings during lipid II binding, possibly affecting the affinity of the molecule for its substrate, lipid II. The Arg13Asp substitution showed a very significant (p<.05) increase in bioactivity when compared to the wild-type. While not wishing to be bound to any particular theory, the observed effect may be the result of increased solubility. As shown in Figure 6, both the Phe1Ile and the Phe1Gly substitutions resulted in statistically significant (p<.05) increases in bioactivity when compared to the wild-type. It is noteworthy that substitution of Arg (AGA/AGG/CGT/CGC/CGA/CGG) with Asp (GAT/GAC) or the substitution of Ala (GCT/GCT/GCA/GCG) for Trp (TGG) or the substitution of Ala (GCT/GCT/GCA/GCG) for Ser (AGT/AGC) or the substitution of Ile (ATT/ATG) or Gly (GGT/GGC/CCA/GGG) for Phe (TTT/TTC) are all very unlikely to occur in nature since they involve multiple point mutations, which may include one or more transversions in the affected codon. While not wishing to be bound to any particular theory, the basis for the increase may be due to increased binding affinity to the lipid II target or to improved efficiency in cleavage of the leader sequence. An effector strain producing a variant MU1140 possessing one or more of these site-directed changes (Phe1Ile, Phe1Gly, Trp4Ala, Dha5Ala, and Arg13Asp) has the potential to be superior to an effector strain producing wild type MU1140 by improving its ability to colonize the oral cavity and aggressively displace disease-causing, indigenous strains of *S. mutans*.

Variants of the lantibiotic MU1140 of the invention are polypeptides comprising post-translational modifications. Post-translational modifications are chemical modifications of a polypeptide after it has been translated. A polypeptide is a polymer of two or more amino acids covalently linked by amide bonds. A purified polypeptide is a polypeptide preparation that is substantially free of cellular material, other types of polypeptides, chemical precursors, chemicals used in synthesis of the polypeptide, or combinations thereof. A polypeptide preparation that is substantially

free of cellular material, culture medium, chemical precursors, chemicals used in synthesis of the polypeptide, etc., has less than about 30%, 20%, 10%, 5%, 1% or more of other polypeptides, culture medium, chemical precursors, and/or other chemicals used in synthesis. Therefore, a purified polypeptide is about 70%, 80%, 90%, 95%, 99% or more pure. A purified polypeptide does not include unpurified or semi-purified cell extracts or mixtures of polypeptides that are less than 70% pure.

Wild-type MU1140 is shown in Figure 1A. MU1140 has four rings labeled A, B, C, and D. Two of these rings are formed by lanthionine (Ala-S-Ala) residues, including one in Ring A (Ala_3-S-Ala_7) and one in Ring C (Ala_{16}-S-Ala_{21}); there is a methyl-lanthionine residue (Abu-S-Ala) that forms Ring B comprised of the α-aminobutyrate residue in position 8 and the Ala in position 11 (Abu_8-S-Ala_{11}); and the fourth ring, D, is comprised of the Ala in position 19 linked to an aminovinyl group by a thioether linkage (Ala_{19}–S—CH=CH—NH—).

One embodiment of the invention provides one or more of the following variants of the lantibiotic mutacin, MU1140, shown in Figure 1B (SEQ ID NO:2). That is, the invention includes variants of the wild-type lantibiotic MU1140 (SEQ ID NO:1) with one or more of the following mutations:

1. Phe1Ile or Phe1Gly; that is the phenylalanine at position 1 is changed to isoleucine or glycine.
2. Trp4Ala; that is, the tryptophan at position 4 is changed to alanine.
3. Dha5Ala; that is, the 2,3-didehydroalanine at position 5 is changed to alanine;
4. Arg13Asp; that is, the arginine at position 13 is changed to aspartate.

In one embodiment of the invention a variant of the lantibiotic MU1140 comprises a Phe1Ile or Phe1Gly amino acid substitution; a Trp4Ala amino acid substitution; a Dha5Ala amino acid substitution; an Arg13Asp amino acid substitution; or combinations thereof. An MU1140 variant of the invention can also comprise, e.g., a Trp4insAla in which an alanine is inserted after the fourth tryptophan residue; or a ΔTrp4 in which there is a deletion of the tryptophan at position 4; or both of these changes in the primary amino acid sequence.

Biologically active equivalents of MU1140 lantibiotic polypeptides can have one or more conservative amino acid variations or other minor modifications and retain biological activity. A biologically active equivalent has substantially equivalent

function when compared to the corresponding lantibiotic MU1140. In one embodiment of the invention a lantibiotic mutacin has about 1, 2, 3, 4, or 5 or less conservative amino acid substitutions. A conservative substitution is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and general nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, dha, abu, dhb, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, gly, dha, abu, dhb, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. Biologically active equivalent lantibiotic mutacins or other lantibiotic polypeptides can generally be identified by modifying one of the variant lantibiotic mutacin sequences of the invention, and evaluating the properties of the modified lantibiotic mutacin to determine if it is a biological equivalent. A lantibiotic is a biological equivalent if it reacts substantially the same as a lantibiotic mutacin of the invention in an assay such as a zone of inhibition assay, *e.g.* has 90-110% of the activity of the original lantibiotic mutacin.

Recombinant *S. mutans* strains of the invention comprise a polynucleotide that expresses a functional variant MU1140. Biological activity of a variant MU1140 can be assayed using, *e.g.,* zone of inhibition assays (see Example 2). Recombinant *S. mutans* strains produce enough variant MU1140 to outcompete and substantially eliminate wild-type, cariogenic *S. mutans* from the oral cavity of a host (*e.g.*, reduce the number of wild-type *S. mutans* by about 5, 10, 25, 50, 75, 90, 95, 99, or 100% (or any range between about 5 % and about 100%)).

A lantibiotic of the invention can be covalently or non-covalently linked to an amino acid sequence to which the lantibiotic is not normally associated with in nature, *i.e.,* a heterologous amino acid sequence. A heterologous amino acid sequence can be from a non-*Streptococcus mutans* organism, a synthetic sequence, or an *S. mutans* sequence not usually located at the carboxy or amino terminus of a lantibiotic of the invention. Additionally, a lantibiotic of the invention can be covalently or non-covalently linked to compounds or molecules other than amino acids such as indicator reagents. A lantibiotic of the invention can be covalently or non-covalently linked to an amino acid spacer, an amino acid linker, a signal sequence, a stop transfer sequence, TMR stop transfer sequence, a transmembrane

domain, a protein purification ligand, or a combination thereof. A polypeptide can also be linked to a moiety (i.e., a functional group that can be a polypeptide or other compound) that facilitates purification (e.g., affinity tags such as a six-histidine tag, trpE, glutathione-S-transferase, maltose binding protein, staphylococcal Protein A or com), or a moiety that facilitates polypeptide stability (e.g., polyethylene glycol; amino terminus protecting groups such as acetyl, propyl, succinyl, benzyl, benzyloxycarbonyl or t-butyloxycarbonyl; carboxyl terminus protecting groups such as amide, methylamide, and ethylamide). In one embodiment of the invention a protein purification ligand can be one or more amino acid residues at, for example, the amino terminus or carboxy terminus of a polypeptide of the invention. An amino acid spacer is a sequence of amino acids that are not associated with a polypeptide of the invention in nature. An amino acid spacer can comprise about 1, 5, 10, 20, 100, or 1,000 amino acids.

If desired, a lantibiotic of the invention can be part of a fusion protein, which can contain heterologous amino acid sequences. Heterologous amino acid sequences can be present at the C or N terminus of a lantibiotic of the invention to form a fusion protein. More than one lantibiotic of the invention can be present in a fusion protein. Fragments of lantibiotics of the invention can be present in a fusion protein of the invention. A fusion protein of the invention can comprise one or more lantibiotic of the invention, fragments thereof, or combinations thereof.

In one embodiment of the invention, a recombinant *S. mutans* strain of the invention is ATCC 55676 (deposited under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty)), which has been genetically engineered to express a variant MU1140 as described herein.

Production of a mutant MU1140 lantibiotic with enhanced biological activity as compared to a wild-type MU1140 lantibiotic can therefore provide an *S. mutans* with a selective advantage over non-MU1140-producing *S. mutans* strains present in the oral cavity of a host. The variant MU1140, when expressed by a recombinant *S. mutans* strain of the invention, eliminates the resident, MU1140-susceptible *S. mutans* strains, thus interfering with colonization of MU1140-susceptible strains and promoting recombinant *S. mutans* colonization of the oral cavity. Since the wild-type,

native *S. mutans* is displaced from the oral cavity, the incidence and/or severity of dental caries is reduced.

In one embodiment of the invention the effector strain can additionally express lanB, lanC, lanE, lanF, lanG, lanK, lanM, lanP, lanR, lanT or combinations of two or more of these *S. mutans* polypeptides.

Lactic Acid Expression Deficiency

"Lactic acid deficient" or "deficiency in lactic acid production" means that a recombinant *S. mutans* strain produces substantially decreased amounts of lactic acid relative to wild-type *S. mutans*. Substantially decreased amounts of lactic acid are about 40, 50, 60, 70, 80, 90, 95, or 100% (or any range between about 40% and about 100%) less lactic acid than is produced by a wild-type *S. mutans* strain (e.g. *S. mutans* strain UA159 (ATCC 700610)) or other species belonging to the mutans streptococcus group including *Streptococcus sobrinus* (*e.g. S. sobrinus* strain SL1 (ATCC 33478)), *Streptococcus rattus* (*e.g., S. rattus* strain FA1 (ATCC 19645)), *Streptococcus cricetus* (*S. crecitus* strain HS6 (ATCC 19642)), and *Streptococcus ferus* (*S. ferus* strain 8S1)). In one embodiment of the invention, a lactic acid-deficient *S. mutans* effector strain produces no detectable lactic acid. Lactic acid expression can be detected as described in, e.g., Hillman *et al.,* Infect. Immun. 62:60 (1994); Hillman *et al.,* Infect. Immun. 64:4319 (1996); Hillman *et al.,* 1990, Infect. Immun., 58:1290-1295.

Recombinant *S. mutans* strains of the invention can be lactic acid deficient as a result of a non-functional, inactivated, partially functional, or partially inactivated regulatory region, translational signal, transcriptional signal, or structural sequence in the lactic acid synthesis pathway. Regulatory regions, translational signals, and transcriptional signals include, *e.g.,* promoters, enhancers, ribosome binding sites, CAAT box, CCAAT box, Pribnow box, TATA box, etc. Nonfunctional or inactivated means that the known wild-type function or activity of the polynucleotide, gene, polypeptide or a protein has been eliminated or highly diminished by about 80, 90, 95,or 100% (or any range between about 80% and about 100%) as compared to a wild-type polynucleotide, gene, polypeptide or protein. Partially functional or partially inactivated means that the known wild-type function or activity of the polynucleotide, gene, polypeptide or a protein has been partially diminished by about 20, 30, 40, 50,

60, 70, 79% (or any range between about 20% and about 79%) as compared to a wild-type polynucleotide, gene, polypeptide or protein.

Inactivation or partial inactivation, which renders the polynucleotide, gene, polypeptide, or protein non-functional or partially functional, can be accomplished by methods such as incorporating mutations (*e.g.*, point mutations, frame shift mutations, substitutions, deletions (part of or an entire signal, region or structural polynucleotide), interruptions, and/or insertions) in polynucleotides involved in the lactic acid synthetic pathway. A mutation in a polynucleotide involved in lactic acid synthesis can affect expression of lactic acid such that the expressed amount of lactic acid is diminished by about 20, 30, 40, 50, 60, 70, 80, 90, 95% or more as compared to a wild-type *S. mutans* strain.

For example, inactivation or partial inactivation of lactic acid expression can be effected by inactivating or partially inactivating, *e.g.,* the lactate dehydrogenase (*ldh*) gene by deleting part of or the entire *ldh* structural polynucleotide or part of or the entire *ldh* promoter. Also, inactivation or partial inactivation of lactic acid expression can be effected by inactivating or partially inactivating genes encoding enzymes involved in carbohydrate transport, e.g., the phosphoenolpyruvate phosphotransferase system (*pts*) gene(s), by deleting part of or the entire *pts* structural polynucleotide or part of or the entire *pts* promoter. *See e.g.,* Cvitkovitch *et al.,* J. Bacteriol. 177:5704 (1995). Inactivation or partial inactivation of lactic acid expression can be effected by inactivating or partially inactivating genes encoding enzymes involved in intracellular and extracellular polysaccharide storage, e.g., the glycogen synthase (g*lgA*) gene (*see e.g.*, Spatafora *et al.,* Infect. Immun. 63:2556 (1995)) and the fructosyltransferase (*ftf*) gene (*see e.g.,* Schroeder *et al*., Infect. Immun. 57:3560 (1989)), by deleting part of or the entire *glgA* or *ftf* structural polynucleotide or part of or the entire *glgA* or *ftf* promoter.

One or more defects in the lactic acid synthesis pathway can be introduced by mutagenesis (*i.e.,* exposure of *S. mutans* to a mutagen), selection of spontaneous mutants, or genetic manipulation using recombinant techniques. These techniques are well known in the art (see, *e.g.,* Sambrook *et al.,* 1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). In one embodiment of the invention, the lactic acid synthesis pathway defect is introduced using recombinant techniques, *e.g.,* introduction of a defective

ldh structural gene into the bacterium and subsequent site-specific recombination to replace the wild-type *ldh* with the defective *ldh*. The *S. mutans ldh* gene has been cloned, its nucleotide sequence determined (GenBank accession number M72545), and the recombinant *ldh* gene expressed in *Escherichia coli* (Hillman *et al.,* 1990, Infect. Immun., 58:1290-1295; Duncan *et al.,* 1991, Infect. Immun., 59:3930-3934). Hillman *et al.* deleted essentially the entire open reading frame of *ldh* from a *S. mutans* strain (J. Appl. Microbiol. 102:1209 (2007)).

Alcohol Dehydrogenase Production

Because defects in lactic acid synthesis are lethal for *S. mutans*, the defect in the recombinant, lactic acid-deficient *S. mutans* strains must be complemented by the production of a recombinant alcohol dehydrogenase (ADH). *See e.g.,* Hillman *et al.,* Infect. Immun. 64:4319 (1996). Production of the recombinant ADH prevents accumulation of metabolites, *e.g.,* pyruvate, that otherwise causes the death of lactic acid-deficient *S. mutans*.

An *S. mutans* strain can be genetically engineered to express a recombinant alcohol dehydrogenase for example, alcohol dehydrogenase B, alcohol dehydrogenase II, or iron-containing alcohol dehydrogenase from *Zymomonas mobilis* (*see e.g.*, GenBank Accession No. M15394; Conway *et al*., 1987, J. Bacteriol., 169:2591-2597), alcohol dehydrogenase from *Streptococcus rattus*, iron-containing alcohol dehydrogenase from *Commensalibacter intestini*, iron-containing alcohol dehydrogenase from *Azotobacter vinelandii*, iron-containing alcohol dehydrogenase from *Enterobacteriaceae bacterium*, alcohol dehydrogenase from *Pseudomonas fluorescens*, iron-containing alcohol dehydrogenase from *Dickeya zeae*, alcohol dehydrogenase from *Proteus mirabilis*, iron-containing alcohol dehydrogenase from *Rhodoferax ferriredcuens*, iron-containing alcohol dehydrogenase from *Rhodospirillum rubrum*, alcohol dehydrogenase from *Pseudomonas brassicacearum*, alcohol dehydrogenase II from *Pseudomonas syringae*, alcohol dehydrogenase from *Dickeya dadantii*, alcohol dehydrogenase from *Citrobacter rodenitium*, iron-containing alcohol dehydrogenase from *Shewanella putrefaciens*, alcohol dehydrogenase from *Vibrio nigripulchritudo*, alcohol dehydrogenase from *Enterobacter aerogenes*, alcohol dehydrogenase from *Pseudomonas savastanoi*, alcohol dehydrogenase from *Salmonella enterica*, iron-containing alcohol dehydrogenase from *Photobacterium leiognathi*, alcohol

dehydrogenase from *Photobacterium damselae*, alcohol dehydrogenase from *Xenorhabdus nematophila*, alcohol dehydrogenase from *Xenorhabdus bovienii*, alcohol dehydrogenase II from Pseudomonas entomophila, alcohol dehydrogenase II from *Shewanella vilacea*, alcohol dehydrogenase from *Vibrio sinaloensis*, alcohol dehydrogenase from *Shewanella pealeana*, alcohol dehydrogenase from *Vibrio angustum*, alcohol dehydrogenase from *Edwardsiella tarda*, alcohol dehydrogenase from *Salmonella bongori*, iron-containing alcohol dehydrogenase from *Enterobacter asburiae*, alcohol dehydrogenase from *Escherichia coli*, alcohol dehydrogenase 4 from *Vibrio parahaemolyticus*, alcohol dehydrogenase from *Vibrio splendidus*. In one embodiment of the invention, a polynucleotide encoding a bacterial alcohol dehydrogenase or iron-containing alcohol dehydrogenase has at least about 60, 65, 75, 80, 90, 95, 98, 99, or 100% (or any range between about 65% and 100%) homology to *Zymomonas mobilis* alcohol dehydrogenase B.

Additionally, an ADH-encoding polynucleotide can be derived from *S. mutans*, so that introduction of the ADH-encoding polynucleotide, in combination with the native *S. mutans adh* gene, provides for multiples copies of ADH-encoding polynucleotides in the *S. mutans* genome. Alternatively, the recombinant ADH polynucleotide can be generated by introducing a mutation in the regulatory mechanism of the *S. mutans adh* gene to upregulate the production of ADH (*e.g.*, a mutation in the *adh* promoter to provide increased transcription of the *adh* gene).

An *adh* polynucleotide can be introduced into a *S. mutans* strain of the invention using well-known recombinant techniques, for example, transforming the *S. mutans* strain with polynucleotides encoding an ADH polypeptide. Transforming or transformation means that a *S. mutans* has a non-native nucleic acid sequence integrated into its genome or as a plasmid that is maintained through multiple generations. The *adh* polynucleotide expresses a functional ADH polypeptide such that an *S. mutans* strain of the invention is viable despite the inactivation of lactic acid expression.

Methods for identification, cloning, stable transformation, and expression of polynucleotides encoding, *e.g.,* ADH are routine and well known in the art (see, for example, Sambrook *et al.,* 1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). For example, isolation of polynucleotides encoding ADH can be performed by PCR amplification of

the molecule from genomic DNA or from a preexisting clone of the gene. Expression of recombinant ADH can be accomplished by operably linking the *adh* structural polynucleotide to a promoter that facilitates expression in *S. mutans* (e.g., *spaP* or the native *ldh* promoter).

Production of a functional ADH can be assayed by, for example, using conventional ADH activity assays (*e.g.,* assays for NAD-dependent oxidation of ethanol) that are well known in the art (Neal *et al.*, 1986, Eur. J. Biochem., 154:119-124). Hillman *et al.* constructed a strain of *S. mutans* that expressed a functional, recombinant ADH. *See e.g.,* Hillman *et al.,* Infect. Immun. 68:543 (2000).

Auxotrophy

Recombinant *S. mutans* strains of the invention can optionally be genetically engineered to be auxotrophic for an organic substance not normally present in the oral cavity or diet of a host so that the oral cavity colonization by the recombinant *S. mutans* strains can be controlled. That is, the recombinant *S. mutans* strains can optionally be genetically engineered so that they are unable to synthesize a particular organic compound required for growth. For example, the strains of the invention can be auxotrophs for a D-amino acid, such as a D-alanine. Colonization of the auxotrophic strains can then be controlled by regulating the amount of the organic substance in the oral cavity. For example, colonization can be promoted by providing the organic compound periodically to the oral cavity and colonization can be terminated by withholding administration of the organic substance to the oral cavity.

For example, D-alanine is not normally produced or present in the oral cavity or diet of mammals above trace amounts. Therefore, if a recombinant *S. mutans* of the invention was auxotrophic for D-alanine, then D-alanine would need to be periodically delivered to the oral cavity of the mammal to maintain the colonization of a recombinant *S. mutans* of the invention in the oral cavity. In the absence of delivery of D-alanine to the oral cavity, the recombinant *S. mutans* strains of the invention will eventually die out.

In one embodiment of the invention, a recombinant *S. mutans* is alanine racemase deficient. Alanine racemase is required for D-alanine metabolism. "Alanine racemase deficient" or "deficiency in alanine racemase production" means that a recombinant *S. mutans* strain produces substantially decreased amounts of

alanine racemase relative to wild-type *S. mutans*. Substantially decreased amounts of alanine racemase are about 40, 50, 60, 70, 80, 90, 95, or 100% (or any range between about 40% and about 100%) less alanine racemase than is produced by a wild-type *S. mutans* strain. In one embodiment of the invention, an alanine racemase deficient recombinant *S. mutans* strain produces no detectable alanine racemase. Alanine racemase can be assayed as described in, *e.g.*, Wantanabe *et al.,* J. Biochem. 126:781 (1999).

Inactivation or partial inactivation, which renders the polynucleotide, gene, polypeptide, or protein non-functional or partially functional, can be accomplished by methods such as incorporating mutations (*e.g.*, point mutations, frame shift mutations, substitutions, deletions (part of or an entire signal, region or structural polynucleotide), interruptions, and/or insertions in genes involved in the alanine racemase synthesis. A mutation in a polynucleotide involved in alanine racemase synthesis can effect expression of alanine racemase such that the expressed amount of alanine racemase is diminished by about 20, 30, 40, 50, 60, 70, 80, 90, 95% or more as compared to a wild-type *S. mutans* strain.

For example, inactivation or partial inactivation of alanine racemase expression can be effected by inactivating or partially inactivating, *e.g.*, the *dal* gene by deleting part or all of the *dal* structural polynucleotide or part or the entire *dal* promoter.

Bacterial auxotrophs can be generated using a variety of techniques well known in the art, such as chemical mutagenesis, selection of spontaneous mutants, and/or recombinant techniques (*e.g.,* transposon mutagenesis, replacement by recombination with a defective or non-functional gene). For example, D-alanine auxotrophic *S. mutans* strains can be generated by introduction of a defect in the gene encoding alanine racemase (*dal*), the enzyme that converts L-alanine to D-alanine. Such strains have been generated. *See, e.g.,* Hillman *et al.*, J. Appl. Microbiol. 102: 1209-1219 (2007).

ComE Deficiency

Optionally, a recombinant *S. mutans* strain of the invention can comprise an inactivated or non-functional *comE* gene. A strain with an inactivated or non-functional *comE* gene would be less prone to transformation because ComE is

important in the uptake of environmental DNA. Furthermore, *comE* cannot be complemented.

"ComE deficient" or "deficiency in ComE production" means that a recombinant *S. mutans* strain produces substantially decreased amounts of ComE protein relative to wild-type *S. mutans*. Substantially decreased amounts of ComE are about 40, 50, 60, 70, 80, 90, 95, or 100% (or any range between about 40% and about 100%) less ComE protein than is produced by a wild-type *S. mutans* strain. In one embodiment of the invention, a ComE deficient recombinant *S. mutans* strain produces no detectable ComE protein. ComE expression can be assayed as described in, e.g., Chen & Gotschlich, J. Bact. 183:3160 (2001).

Recombinant *S. mutans* strains of the invention can be ComE deficient as a result of a non-functional, inactivated, partially functional, or partially inactivated regulatory region, translational signal, transcriptional signal, or structural sequence in ComE synthesis.

Inactivation or partial inactivation, which renders the polynucleotide, gene, polypeptide, or protein non-functional or partially functional includes methods such as incorporating mutations (*e.g.*, point mutations, frame shift mutations, substitutions, deletions (part of or an entire signal, region or structural polynucleotide), interruptions, and/or insertions) in polynucleotides involved in ComE synthesis. A mutation in a polynucleotide involved in ComE synthesis can effect expression of ComE such that the expressed amount of ComE is diminished by about 20, 30, 40, 50, 60, 70, 80, 90, 95% or more as compared to a wild-type *S. mutans* strain.

For example, inactivation or partial inactivation of ComE expression can be effected by inactivating or partially inactivating, *e.g.,* the *comE* gene by deleting part of or the entire *comE* structural gene or part of or the entire *comE* promoter. Other genes involved in DNA uptake such as *comA*, *comB, comC*, and *comD*, can also or alternatively be inactivated or partially inactivated.

The defect in ComE synthesis can be introduced by mutagenesis (*i.e.*, exposure of the bacterium to a mutagen), selection of spontaneous mutants, or genetic manipulation using recombinant techniques. A *S. mutans* strain with a mutated *comE* gene has been constructed. *See, e.g.,* Hillman *et al.*, J. Appl. Microbiol. 102: 1209-1219 (2007).

Polynucleotides

Polynucleotides of the invention contain less than an entire microbial genome and can be single- or double-stranded nucleic acids. A polynucleotide can be RNA, DNA, cDNA, genomic DNA, chemically synthesized RNA or DNA or combinations thereof. The polynucleotides can be purified free of other components, such as proteins, lipids and other polynucleotides. For example, the polynucleotide can be 50%, 75%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% purified. A nucleic acid molecule existing among hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest are not to be considered an isolated polynucleotide.

The polynucleotides of the invention encode the polypeptides of the invention described above (*e.g.,* MU1140 polypeptides, ADH polypeptides, ComE polypeptides, D-amino acid synthesis polypeptides, and lactic acid synthesis polypeptides). In one embodiment of the invention the polynucleotides encode a variant mutacin 1140 polypeptides shown in SEQ ID NOs:20-27, combinations thereof, or fragments thereof. In one embodiment of the invention the effector strain can additionally express *lanB, lanC, lanE, lanF, lanG, lanK, lanM, lanP, lanR, lanT* or combinations of two or more of these *S. mutans* polynucleotides.

Polynucleotides of the invention can consist of less than about 600, 500, 400, 300, 200, 100, 66, 60, 50, 45, 30, 15 (or any range between about 600 and 15) contiguous polynucleotides. The purified polynucleotides can comprise additional heterologous nucleotides and/or additional homologous polynucleotides. Polynucleotides of the invention can comprise other nucleotide sequences, such as sequences coding for linkers, signal sequences, TMR stop transfer sequences, transmembrane domains, or ligands useful in protein purification such as glutathione-S-transferase, histidine tag, and *Staphylococcal* protein A. One embodiment of the invention provides a purified polynucleotide comprising at least about 6, 10, 15, 20, 25, 30, 40, 45, 50, 60, 66, or more contiguous nucleotides of encoding SEQ ID NOs:20-27.

Polynucleotides of the invention can be isolated. An isolated polynucleotide is a naturally-occurring polynucleotide that is not immediately contiguous with one or both of the 5' and 3' flanking genomic sequences that it is naturally associated with. An isolated polynucleotide can be, for example, a recombinant DNA molecule of any

length. Isolated polynucleotides also include non-naturally occurring nucleic acid molecules. Polynucleotides of the invention can encode full-length polypeptides, polypeptide fragments, and variant or fusion polypeptides.

Degenerate nucleotide sequences encoding polypeptides of the invention, as well as homologous nucleotide sequences that are at least about 80, or about 90, 95, 96, 97, 98, or 99% identical to the polynucleotide sequences of the invention and the complements thereof are also polynucleotides of the invention. Degenerate nucleotide sequences are polynucleotides that encode a polypeptide of the invention or fragments thereof, but differ in nucleic acid sequence from the given polynucleotide sequence due to the degeneracy of the genetic code.

Percent sequence identity has an art recognized meaning and there are a number of methods to measure identity between two polypeptide or polynucleotide sequences. *See, e.g.,* Lesk, Ed., *Computational Molecular Biology*, Oxford University Press, New York, (1988); Smith, Ed., *Biocomputing: Informatics And Genome Projects*, Academic Press, New York, (1993); Griffin & Griffin, Eds., *Computer Analysis Of Sequence Data, Part I*, Humana Press, New Jersey, (1994); von Heinje, *Sequence Analysis In Molecular Biology*, Academic Press, (1987); and Gribskov & Devereux, Eds., *Sequence Analysis Primer*, M Stockton Press, New York, (1991). Methods for aligning polynucleotides or polypeptides are codified in computer programs, including the GCG program package (Devereux *et al*. (1984) *Nuc. Acids Res.* 12:387), BLASTP, BLASTN, FASTA (Atschul *et al*. (1990) *J. Molec. Biol.* 215:403), and Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) which uses the local homology algorithm of Smith and Waterman ((1981) *Adv. App. Math.,* 2:482-489). For example, the computer program ALIGN which employs the FASTA algorithm can be used, with an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2.

When using any of the sequence alignment programs to determine whether a particular sequence is, for instance, about 95% identical to a reference sequence, the parameters are set such that the percentage of identity is calculated over the full length of the reference polynucleotide and that gaps in identity of up to 5% of the total number of nucleotides in the reference polynucleotide are allowed.

Polynucleotides of the invention can be isolated from nucleic acid sequences present in, for example, a bacterial sample. Polynucleotides can also be synthesized in the laboratory, for example, using an automatic synthesizer. An amplification method such as PCR can be used to amplify polynucleotides from either genomic DNA or cDNA encoding the polypeptides.

Polynucleotides of the invention can comprise coding sequences for naturally occurring polypeptides or can encode altered sequences that do not occur in nature. If desired, polynucleotides can be cloned into an expression vector comprising expression control elements, including for example, origins of replication, promoters, enhancers, or other regulatory elements that drive expression of the polynucleotides of the invention in host cells. An expression vector can be, for example, a plasmid. Minichromosomes such as MC and MC1, bacteriophages, phagemids, yeast artificial chromosomes, bacterial artificial chromosomes, virus particles, virus-like particles, cosmids (plasmids into which phage lambda *cos* sites have been inserted) and replicons (genetic elements that are capable of replication under their own control in a cell) can also be used.

Methods for preparing polynucleotides operably linked to an expression control sequence and expressing them in a host cell are well-known in the art. *See, e.g.,* U.S. Patent No. 4,366,246. A polynucleotide of the invention is operably linked when it is positioned adjacent to or close to one or more expression control elements, which direct transcription and/or translation of the polynucleotide.

Compositions Comprising Recombinant *S. mutans* of the Invention

Recombinant *S. mutans* strains of the invention can be characterized by: 1) a lactic acid deficiency, and 2) production of a recombinant ADH, 3) variant MU1140 production, 4) optionally, an auxotrophy for a specific organic substance (e.g., a D-amino acid such as D-alanine), 5) optionally, a deficiency in ComE expression, or combinations thereof.

Compositions of the invention can comprise one or more strains of recombinant *S. mutans* strains as described herein and a pharmaceutically acceptable or nutritionally acceptable carrier. The carrier is physiologically compatible with the area of the subject to which it is administered. Carriers can be comprised of solid-based, dry materials for formulation into tablet, capsule, lozenge, or powdered form. A carrier can also be comprised of liquid or gel-based materials

for formulations into liquid, gel, and chewing gum forms. The composition of the carrier can be varied so long as it does not interfere significantly with the therapeutic activity of the bacterial strains of the invention. .

A composition can be formulated to be suitable for oral administration in a variety of ways, for example in a solid, semi-solid, liquid (including, e.g., a viscous liquid, a paste, a gel, or a solution), a dried mass, a dentifrice, a mouth wash, an oral rinse, a liquid suspension, a beverage, a topical agent, a powdered food supplement, a paste, a gel, a solid food, an oral rinse, a packaged food, a wafer, lozenge, chewing gum and the like. Other formulations will be readily apparent to one skilled in the art. A composition of the invention can include a nutrient supplement component and can include any of a variety of nutritional agents, as are well known, including vitamins, minerals, essential and non-essential amino acids, carbohydrates, lipids, foodstuffs, dietary supplements, and the like.

Compositions of the invention can also include natural or synthetic flavorings and food-quality coloring agents, all of which are compatible with maintaining viability of the bacterial strains of the invention.

A composition of the invention can include one or more gelling agents that can act as an adhesive agent to adhere the composition to the teeth or mouth. The concentration of the gelling agent may be greater than about 2, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60, 70, 80 or less than about 80, 70, 60, 50, 40, 30, or 20 percent by weight of the composition.

Suitable gelling agents and adhesion agents useful in the present invention include, for example, silicone, polyethylene oxide, polyvinyl alcohol, polyalkyl vinyl ether-maleic acid copolymer (PVM/MA copolymer) such as, Gantrez AN 119, AN 139, and S-97, polyvinyl alcohol, polyacrylic acid, Poloxamer 407 (Pluronic), polyvinyl pyrrolidone-vinyl acetate copolymer (PVP/VA copolymer), such as Luviskol VA, and Plasdone S PVP/VA, polyvinyl pyrrolidone (PVP, e.g., K-15 to K-120), Polyquaterium-11 (Gafquat 755N), Polyquaterium-39 (Merquat plus 3330), carbomer or carboxypolymethylene (Carbopol), hydroxypropyl methylcellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, corn starch, carboxymethyl cellulose, gelatin and alginate salt such as sodium alginate, natural gums such as gum karaya, xanthan gum, Guar gum, gum arabic, gum tragacanth, and mixtures thereof.

A humectant or plasticizer can be present in compositions of the invention. Humectants or plasticizers include, for example, glycerin, glycerol, sorbitol, polyethylene glycol, propylene glycol, and other edible polyhydric alcohols. The humectants or plasticizers can be present between at about 1% to about 99%, about 10% to about 95%, or at between about 50% and about 80% (or any range between 1% and 99%) by weight of a composition.

Bacteria of the invention can be prepared in, for example, a fermenter. The bacteria can be harvested from the fermenter and can be, for example, concentrated. Bacteria of the invention can be prepared for use by, for example, dehydration, air drying, lyophilizing, freezing, and spray-drying. Bacteria can also be prepared for use by microencapsulation (*see e.g.*, U.S. Pat. No. 6,251,478) or by coating with a protective substance such as, for example, lipid material such as triacylglycerols, waxes, organic esters, soybean oil, cottonseed oil, palm kernel oil, and esters of long-chain fatty acids and alcohols. In one embodiment of the invention the coated or encapsulated bacteria of the invention are released in the oral cavity of the host.

Methods of Treatment and Prevention of Cavities

The recombinant *S. mutans* of the invention can be present in a composition of the invention in a therapeutically effective amount. Therapeutically effective means effective to prevent or reduce the number or incidence (*e.g.*, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% fewer cavities than controls that did not receive the composition) and/or reduce the severity (*e.g.*, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% less severe cavities than controls that did not receive the composition) of cavities.

A therapeutically effective amount or dosage is an amount or dosage of a composition of the invention at high enough levels to prevent caries and/or reduce caries number and/or caries severity, but low enough to avoid serious side effects (at a reasonable benefit/risk ratio), within the scope of sound medical/dental judgment. The therapeutically effective amount or dosage of a composition of the invention may vary with the particular condition being treated, the age and physical condition of the patient being treated, the severity of the condition, the duration of treatment, the nature of concurrent therapy, the specific form of the source employed, and the particular vehicle from which the composition is applied.

The compositions of the invention can be applied in a therapeutically effective amount to the oral cavity of a host for the treatment and/or prevention of cavities. A composition of the invention may be swallowed or may be rinsed around the oral cavity and then spit out, such that it is not substantially delivered to the gastrointestinal tract. That is, less than about 10, 5, 4, 3, 2, or 1, 0.5, or 0.1% (or any range or value between about 10 and 0.1%) of the delivered bacteria are delivered to the gastrointestinal tract. Treatment means inducing a reduction in the amount or intensity (or combination thereof) of cavities.

Prevention means that substantially no dental caries occur after exposure of the host to one or more recombinant *S. mutans* strains of the invention either permanently (as long as the bacteria of the invention remain in sufficient numbers in the subject's oral cavity), or temporarily (e.g., for about 1, 2, 3, 4, 5, 6 or more months). The bacterial strains of the invention can form at least a part of the transient or indigenous flora of the oral cavity and exhibit beneficial prophylactic and/or therapeutic effects.

Treatment means reducing the amount of wild-type *S. mutans* in the oral cavity of a host such that remineralization of small carious lesions can occur and that further damage to larger carious lesions is stopped or slowed. The amount of wild-type *S. mutans* in the oral cavity can be reduced by about 20, 30, 40, 50, 60, 70, 80, 90, or 100% (or any range between about 10 and 100%).

In one embodiment of the invention prevention means prevention in a population of subjects. That is, given a population of subjects, the treatment can prevent dental caries in about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90% or more of the subjects as compared to a control population that did not receive the treatment.

In one embodiment of the invention a composition can comprise one or more isolated recombinant strains of the invention along with one or more isolated *Streptococcus oralis* strains and/or one or more isolated *Streptococcus uberis* strains.

Streptococcus oralis (previously known as *Streptococcus sanguis* Type II) and *S. uberis* are important components in maintaining the normal, healthy balance of microorganisms that compose the periodontal flora. *See*, Socransky *et al.*, *Oral Microbiol. Immunol.* 3:1-7 (1988); Hillman and Shivers, *Arch. Oral. Biol.*, 33:395-401 (1988); Hillman, *et al.*, *Arch. Oral. Biol.*, 30:791-795 (1985). *S. oralis* produces

hydrogen peroxide, which can inhibit periodontal pathogens such as *Actinobacillus actinomycetemcomitans* (Aa), *Bacteroides forsythus,* and *P. intermedia.* Therefore, *S. oralis* and *S. uberis* can be useful in the maintenance of oral health. Compositions of the invention can comprise one or more isolated strains of *S. oralis*, for example, ATCC 35037, ATCC 55229, ATCC 700233, ATCC 700234 and ATCC 9811. Other strains of *S. oralis* include KJ3 and KJ3sm. KJ3sm is a naturally occurring genetic variant of KJ3 that is resistant to streptomycin. The streptomycin resistance is advantageous because it provides a marker for easy isolation of the bacteria. Additionally, streptomycin resistant strains are slightly attenuated and do not survive as long in an oral cavity as wild-type strains. This property is useful where the goal is to non-persistently colonize the oral cavity of an animal with the bacteria.

S. uberis in plaque has been found to correlate with periodontal health, in particular by interfering with the colonization by periodontal pathogens such as *Porphyromonas gingivalis, Campylocbacter recta,* and *Eikenella corrodens.* Compositions of the invention can comprise one or more isolated strains of *S. uberis*, for example, ATCC 13386, ATCC 13387, ATCC 19435, ATCC 27958, ATCC 35648, ATCC 700407, ATCC 9927, strain KJ2 or strain KJ2sm. KJ2sm is a naturally occurring genetic variant of KJ2. That is streptomycin resistant and provides the same advantages as for streptomycin-resistant strains of *S. oralis*. One or more isolated strains of *S. oralis* or one or more isolated strains of *S. uberis*, or both, can be used in compositions and methods of the invention. Additional oral care benefits of these compositions of the invention include, for example, the treatment and/or prevention of periodontitis, oral bacterial infections and diseases, oral wounds, *Candida* or fungal overgrowth, halitosis, or xerostomia-induced dental caries and associated periodontal diseases, the promotion of wound healing, teeth whitening or a combination thereof to a subject.

One embodiment of the invention provides a method for treating dental caries comprising administering a composition comprising one or more recombinant *S. mutans* strains of the invention to the oral cavity of a subject in need thereof. That is, the subject has one or more dental caries.

One embodiment of the invention provides for the prevention of dental caries in normal, healthy subjects. Another embodiment of the invention provides for

treatment and/or prevention of dental caries in subjects having an increased susceptibility to dental caries as compared to normal, healthy subjects. In both embodiments, the method consists of administering a composition comprising one or more recombinant *S. mutans* strains to the oral cavity of a subject.

Subjects have an increased susceptibility to dental caries when they are more likely than a normal, healthy host to develop dental caries. Such hosts may have, for example, decreased saliva production (*e.g.*, patients undergoing radiation therapy on the head or neck, patients having Sjögren's syndrome, diabetes mellitus, gastro-esophageal reflux disease, diabetes insipidus, or sarcoidosis, patients taking antihistamines and antidepressants or other medications that cause “dry mouth”), smokers, smokeless tobacco users, patients having a genetic predisposition (Shuler, J. Dent. Ed. 65:1038 (2001)), or are infants (0 to 2 years old or 6 months to 2 years old), children (3 years to 18 years old), or elderly (older than 65).

The invention also provides a method of reducing the amount in a subject of bacteria that can cause dental caries. The method comprises administering a composition comprising one or more recombinant *S. mutans* strains of the invention to the oral cavity of a subject having one or more strains or species of bacteria that can cause dental caries. The compositions can be administered just once or on a regular basis. The number of the one or more strains or species of bacteria that can cause dental caries in the subject is reduced. The reduction can be about a 5, 10, 25, 50, 75, 90, 95, 99, or 100% (or any range between about 5% and about 100%) reduction in numbers.

Optionally, prior to the administration of the composition of the invention, one or more bacteria that can cause dental caries can be detected and/or quantitated using any detection/quantitation method known in the art. Those of skill in the art are aware of methods of detection of bacteria that cause dental caries. Optionally, prior to the administration of the composition of the invention, one or more dental caries can be diagnosed in the subject using any methodology known in the art.

Another embodiment of the invention provides a method of preventing dental caries in a subject. The method comprises obtaining data regarding a therapeutically effective dosage range for prevention of dental caries in a particular type of subject and determining the effective dosage range of recombinant *S. mutans* for the particular type of subject. A particular type of subject can be, for

example, a subject with decreased saliva production (*e.g.*, patients undergoing radiation therapy on the head or neck, patients having Sjögren's syndrome, diabetes mellitus, gastro-esophageal reflux disease, diabetes insipidus, or sarcoidosis, patients taking antihistamines and antidepressants or other medications that cause "dry mouth"), smokers, smokeless tobacco users, patients having a genetic predisposition, or are infants (0 to 2 years old or 6 months to 2 years old), children (3 years to 18 years old), or elderly (older than 65). The determined therapeutically effective dosage range for the particular type of subject of one or more recombinant *S. mutans* strains of the invention are administered to the oral cavity of the particular type of subject.

Compositions can be administered to the oral cavity of a host or subject such as an animal, including a mammal, for example, a human, a non-human primate, a dog, a cat, a horse, a bovine, a goat, or a rabbit.

The compositions of the invention can be orally administered in for example, food, water, a dentifrice, a gel, a paste, an emulsion, aerosol spray, chewing gum, lozenge, tablet, capsule, or a liquid suspension. The bacteria can either be already formulated into food, water, gel or other carrier or can be a composition (e.g., powder, tablet or capsule) that is added to the carrier (e.g., food, water, dentifrice, gel, paste, emulsion, aerosol spray, or liquid suspension) by the user prior to consumption.

One embodiment of the invention provides a method of non-persistently colonizing an oral cavity of a subject with therapeutically-effective bacteria comprising administering to the oral cavity of a subject a composition of the invention. In one embodiment of the invention the administered bacterial strains do not permanently colonize the oral cavity, rather the strains are present in the oral cavity for about 1 day, about 1 week, about 2 weeks, about 3 weeks, about 1 month, about 3 months or about 12 months after administration of the bacteria.

In another embodiment of the invention, recombinant strains of *S. mutans* persistently colonize an oral cavity of a host for a long term period, e.g., 2 weeks, 1 month, 3 months, 6 months, 1 year, 5 years, or more or for the life of the host.

Compositions of the invention can be administered at a dose of about $1x10^3$, $1x10^5$, $1x10^7$, $1x10^8$, $1x10^9$, or $1x10^{11}$ CFU (or any range or value between about $1x10^3$ and about $1x10^{11}$) of viable bacteria. A dose of a composition of the invention

can be administered at four times a day, three times a day, twice a day, once a day, every other day, two times a week, weekly, biweekly, monthly, or yearly. One, two, or more doses of a composition of the invention can be administered per day for about 1 day, about 1 week, about 2 weeks, about 1 month, about 2 months, about 3 months, about a year or more. In one embodiment of the invention, a composition of the invention is administered one time and is effective for a long term period.

A composition of the invention can comprise bacterial strains at a concentration between about 0.01% and about 50%, or about 0.1% to about 25%, or about 1.0% to about 10% or any ranges or values in between 0.01% and 50% by weight of the composition.

A kit of the invention can contain a single dose, a one week, one month, two month, three month, four month, five month, six month, or 12 month supply of a composition of the invention. A composition of the invention can be packaged and, in turn, a plurality of the packaged compositions can be provided in a storage container or outer package or carton. Where the one or more strains of *S. mutans* are auxotrophic, the kit can include a bacterial auxotroph-maintaining amount of an organic substance, e.g., a composition comprising a D-amino acid such as D-alanine.

Where a composition of the invention comprises one or more strains of *S. mutans* that are auxotrophic for an organic substance, a bacterial auxotroph-maintaining amount of an organic substance can be administered to hosts to maintain the recombinant *S. mutans* in the oral cavity. A "bacterial auxotroph-maintaining amount" is an amount of an organic substance sufficient to maintain viability of the recombinant *S. mutans* auxotroph in the oral cavity. For example, where the recombinant *S. mutans* is auxotrophic for D-alanine, a D-alanine bacterial auxotroph-maintaining amount is an amount of D-alanine sufficient for survival of the D-alanine auxotrophic strain in the host's oral cavity. In general, a single dose of a D-alanine bacterial auxotroph-maintaining amount of D-alanine contains about 1, 5, 10, 20, 25, 50, 75 or 100 mg (or any range between about 1 and about 100 mg). The concentration of D-alanine in a composition in the form of a solution is about 0.01, 1, 10, 25, 50, 75, 100, or 167 mg/ml (the latter being a saturated solution of D-alanine in water at 25ºC) (or any range between about 0.01 and about 167 mg/ml). The

concentrations of D-alanine in a composition can vary according to the carrier used and the saturation point of D-alanine in that specific carrier.

The organic substance, *e.g.*, D-alanine, required for maintenance of the auxotrophic, recombinant *S. mutans* in the oral cavity can be formulated as a mouthwash, chewing gum, dental floss, toothpaste, chewable tablet, food, beverage or any other formulation suitable for oral administration to the host's oral cavity. In addition to the organic substance (*e.g.*, D-alanine), the composition can additionally contain flavoring agents, coloring agents, fragrances, or other compounds that increase the palatability of the composition and/or enhance patient compliance without compromising the effectiveness of the organic substance contained in the composition.

All patents, patent applications, and other scientific or technical writings referred to anywhere herein are incorporated by reference herein in their entirety. The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms, while retaining their ordinary meanings. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

The following are provided for exemplification purposes only and are not intended to limit the scope of the invention described in broad terms above.

EXAMPLES

Example 1: Mutagenesis of MU1140

The *Streptococcus mutans* genome database and *lan* gene cluster, GenBank/EMBL accession number (AF051560), was used to design primers for the mutagenesis and sequencing work. The open reading frame (ORF) of the native MU1140 structural gene (*lanA*) plus 500 base pairs (bp) of 5' and 3' flanking DNA was cloned into the pVA891 plasmid to create p190. The cloned insert in p190 was derived by PCR amplification of chromosomal DNA of *S. mutans* strain JH1140 (ATCC 55676) using the primer sequences of SRWlanA_1 and SRWlanA_2 (see Figure 3). Reagents and media were purchased from Fisher Scientific, and enzymes were purchased from New England BioLabs (Ipswich, MA).

Polymerase Chain Reaction (PCR)

Mutations (see Figure 1B) were introduced into the propeptide region of *lanA,* the structural gene for MU1140, to create the variants of MU1140. *See* Figure 2. The p190 plasmid (J.D. Hillman, unpublished) was used as a template and the site specific mutations were introduced using two-step PCR. In the first step, the upstream and downstream outside primers (SRWlanA_1 and SRWlanA_2) were paired with appropriate inside primers (e.g., SRWlanA_1/Trp4Ala_2 and SRWlanA_2/Trp4Ala_1) (Figure 3), one of which was synthesized to contain an altered base sequence relative to the wild type sequence. The result of this step was the production of two fragments, one that included 5' flanking DNA and a portion of *lanA*, including the site directed base alterations. The second fragment contained the remainder of *lanA* plus 3' flanking DNA. Primers used to produce the MU1140 variants are found in Figure 3. The two fragments were then mixed in equal amounts and subjected to a second round of PCR using the two outside primers, SRWlanA_1 and SRWlanA_2, to yield the final amplicon.

PCR reactions were performed using Taq polymerase in a final volume of 50 µL containing 0.4 µmol of each primer, 50 ng of template DNA, 0.016 mMdNTP, and 1 unit of DNA polymerase in 1X polymerase buffer. Amplification conditions for each fragment were as follows: preheat at 95°C for 1 min, followed by 27 cycles incubation with denaturation (95°C) for 30 sec, annealing (56°C) for 30 sec and extension (72°C) for 2 min followed by a final extension (72°C) for 10 min. Both fragments were combined 50:50 and amplified using the two outside primers

SRWlanA_1 and SRWlanA_2 under the same amplification conditions as mentioned above.

The final PCR product was ligated into a TOPO-TA vector (Invitrogen, Carlsbad, CA) following kit directions and transformed into DH5α-T1®cells (Invitrogen) using standard methods and spread on LB plates containing 50 µg/mL of ampicillin and 40 µL of X-gal (40mg/mL). Blue-white screening was utilized to identify colonies containing an insert. Plasmid DNA from each colony was purified using a PureYield Plasmid Miniprep System (Promega, Madison, WI) according to the manufacturer's instructions. Purified plasmid was subjected to restriction digest using *EcoRI* and examined by agarose gel electrophoresis to identify those that have a cloned insert of proper size (~1100 bp). Plasmids containing the proper sized insert were sequenced using M13 Forward (-20) primer, 5´-GTAAAACGACGGCCAG-3´ (SEQ ID NO:28), to confirm the proper insertion, deletion, or replacement of nucleotide bases.

Recombination

Restriction enzyme digestion was performed on purified plasmid from colonies harboring a confirmed mutation. The insert were separated from the TOPO plasmid by electrophoresis, excised from the gel, and purified using a Qiagen Gel Extraction Kit (Qiagen, Valencia, CA). The purified insert was then ligated into the *S. mutans* suicide vector, pVA891, in a 3:1 insert:vector ratio using T4 DNA ligase at 16°C overnight. The resultant plasmid was then transformed into DH5α cells using standard methods and spread on LB plates containing 300 µg/mL of erythromycin. Colonies which arose following incubation were analyzed to verify proper insert size and sequence as described above.

Purified pVA891 DNA containing confirmed inserts was transformed into *S. mutans* strain JH1140 (ATCC 55676) as follows: *S. mutans* was grown overnight then diluted 1:15 in fresh THyex broth (30 g/L THB, 3 g/L yeast extract), 200µL of diluted cells were added to a 96 well plate and incubated at 37°C for 2 hours. Two microliters of competence stimulating peptide (CSP, 0.1µg/mL; *see e.g.*, Li *et al.,* J. Bacteriol. 183:897 (2001)) was added, and plates were incubated for an additional 6 hours. See Li *et al.*, (2002) J. Bacteriol. 184:2699. Fifty microliters of cells were then plated onto pre-warmed THyex agar plates (30 g/L THB, 3g/L yeast extract, and 15g/L of nutrient agar) containing 300 µg/mL of erythromycin and incubated at 37°C

for 48 hours. Genomic DNA was extracted from clones that arose utilizing a standard chloroform/phenol extraction method and the DNA was used as template for PCR that used SRWlanA_1 and SRWlanA_2 to identify heterodiploid clones presumed to have one wild type and one mutated copy of the *lanA* gene separated by vector DNA, as previously described by Hillman *et al.,* (2000) Infect. Immun. 68:543-549.

Confirming Genetic Identity of Mutant Constructs

Clones containing the desired *lanA* mutations were obtained by spontaneous resolution of the heterodiploid state as follows: several confirmed heterodiploids were grown overnight in 20 mL THyex broth that did not contain erythromycin. The cultures were subcultured (1:20 dilution into fresh media) and again grown overnight to saturation. The cultures were then diluted 100,000 fold and spread onto large THyex agar plates and incubated at 37°C for 48 hours. Resultant colonies were replica patched onto medium with and without erythromycin to identify spontaneous recombinants in which elimination of the pVA891 plasmid (expressing the erythromycin resistance gene) and either the wild-type or mutated *lanA* gene had occurred. Erythromycin sensitive colonies that were identified from the replica plating technique were re-tested on medium with and without erythromycin. The *lanA* region of erythromycin sensitive clones was amplified by PCR as described above. The amplicons generated were sequenced to identify clones possessing only the modified *lanA* genes. BLAST sequence analysis was used to compare the wild-type sequence of *lan*A to the *lanA* of suspected mutants (Figure 2). The mutants generated were: Trp4Ala, Trp4insAla, ΔTrp4, Dha5Ala, Ala_s7insAla, and Arg13Asp.

Example 2: Bioactivity of Mutants

The parent *S. mutans* strain, JH1140 (ATCC 55676), and the mutants were grown to an OD_{600} of 0.8 and diluted to an OD_{600} of 0.2. Samples (2 µL) of the cultures were spotted in triplicate on a pre-warmed THyex agar plate (150 X 15mm) and allowed to air dry. This assay was performed in this manner to help ensure that each sample had the same colony size for comparing zones of inhibition. The plate was incubated for 24 hours at 37ºC, and then placed in an oven at 55°C for thirty minutes to kill the bacteria before the *M. luteus* ATCC 272 indicator strain was overlayed in molten top agar. Heat killing the bacteria prevented any further antimicrobial compound production. *M. luteus* ATCC 272 was grown to an OD_{600nm} between 0.4 and 0.8 and diluted to an OD_{600nm} of 0.2. Then, 400 µl of these cells

was added to 10 ml of molten top agar (42°C) (30g/L Todd Hewitt Broth and 7.5g/L Nutrient agar). All 10 mL of top agar containing the standardized suspension was added to each plate containing approximately 50 mL of THyex agar. The plates were allowed to solidify before being inverted and incubated overnight at 37°C. Each inhibitory zone radius was measured in mm from one edge of the colony to the farthest portion of the zone. The area of the inhibitory zone was calculated for each zone and compared to the average zone area of the wild-type (n=10).

Figure 4 illustrates the bioactivity of strains producing variants of MU1140 compared to wild-type MU1140. The results are summarized in Figure 5, which shows that the strains producing Trp4insAla and ΔTrp4 had zones that were not significantly different (Student's *t* test, $p>.05$) than the wild-type. The strain producing Arg13Asp had the largest inhibitory zone area amounting to a 2.57-fold increase relative to wild-type ($p<.001$). The strains producing Trp4Ala and Dha5Ala produced significant ($p<.001$) 2.12-fold and 1.87-fold increases, respectively, relative to the wild-type. The strain producing Ala_S7insAla had the smallest zone area, which amounted to a significant ($p<.001$) 2-fold reduction in zone area when compared to the wild-type. Figure 6 shows the biological activity of strains producing other variants of MU1140 (Phe1Ile and Phe1Gly) compared to wild-type MU1140. The strains producing Phe1Ile and Phe1Gly demonstrated significant ($p<.001$) 1.82-fold and 1.57-fold increases, respectively, relative to the wild-type.

There has been a number of studies that used site directed mutagenesis of the structural gene for nisin and certain other lantibiotics (reviewed by Chatterjee *et al.* (2005) Chem. Rev. 105:633) to analyze the importance of particular amino acids in the activity of these molecules. Rarely have these mutations resulted in increased bioactivity.

The most interesting result was obtained for the Arg13Asp mutant. This mutation resulted in an unexpected, highly significant increase in bioactivity when compared to the wild-type. Here there was replacement of a positively charged residue with a negatively charged residue in the hinge region. This finding is contrary to the conventional belief that negative charges for lantibiotics should reduce bioactivity since positive charges are thought to aid in the interaction of the antibiotic with negatively charged lipids present in the target cell membrane. This mutation also removed a trypsin cleavable site from the compound, thereby making it more

stable to enzymatic hydrolysis. Furthermore, the Trp4Ala, Dha5Ala, and Arg13Asp are transversion mutations that would likely not naturally occur.

The mutations to MU1140 described herein are therefore unexpected and unpredictable in view of the prior art and result in variant MU1140 molecules that have vastly improved biological and structural characteristics as compared to wild-type MU1140. Mutations that increase activity are important from the standpoint of improving the colonization potential of an *S. mutans* effector strain. The ability of *S. mutans* strains to colonize the oral cavity of rodents and humans has been previously shown to correlate with the amount and/or activity of MU1140 produced. In addition, the ability of *S. mutans* strains to aggressively displace indigenous strains of *S. mutans* in the oral cavity of rodents and humans has been previously shown to correlate with the amount and/or activity of MU1140 produced. *See e.g.,* Hillman *et al.,* Infect. Immun. 44:141 (1984); Hillman *et al.,* J. Dent. Res. 66:1092 (1987).Therefore, an *S. mutans* effector strain of the invention that expresses a variant MU1140 as described herein will have unexpected and improved characteristics as compared to effector *S. mutans* strains that do not express a variant MU1140 of the invention. That is, *S. mutans* effector strains expressing a variant MU1140 will have improved ability to colonize and aggressively outcompete and replace native *S. mutans* in the oral cavities of the hosts relative to *S. mutans* effector strains that do not express a variant MU1140 as described herein.

Example 3 Minimum Inhibitory Concentration

Wild-type mutacin 1140, mutacin 1140 with a F1I mutation, mutacin 1140 with a W4A mutation, and mutacin 1140 with a R13D mutation was purified to about 90% purity (measured via HPLC). The minimum inhibitory concentration (MIC) of MU1140 and variants of MU1140 was determined against several bacteria. The MIC is the lowest concentration of MU1140 that will inhibit the visible growth of a microorganism after 24 hour incubation. A lower MIC is an indication of greater inhibitory activity. Preparation of the antimicrobial agent and bacterial inoculum for minimum inhibitory concentrations (MICs) was performed by following the method described in Clinical Laboratory Standard Institute (CLSI) M07-8A with some minor modifications. *Streptococcus mutans* UA159 was tested overnight in a shaking incubator to maintain uniform dispersion of the bacteria. *Clostridium difficile* UK1

was tested in an anaerobic chamber at 37°C. The medium used was THyex. The results are shown in Table 1.

Table 1

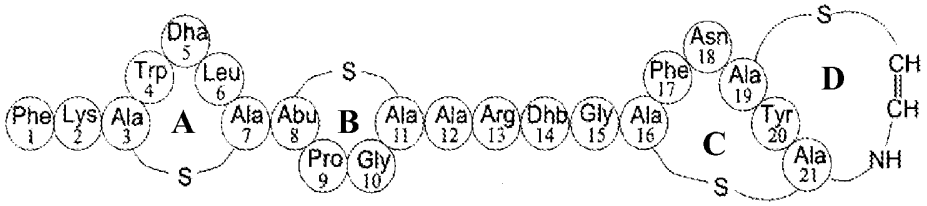
MU1140 Variant	***Streptococcus mutans* UA159**	***Streptococcus pneumonia* FA1**	***Staphylococcus aureus* FA1**	***Micrococcus luteus* ATCC10240**	***Clostridium difficile UK1***
Mu1140 Wild-type	2	0.5	16	0.0625	16
Mu1140 F1I	2	0.25	8	0.0156	8
Mu1140W4A	2	0.125	16	0.0312	8
Mu1140R13D	2	4	>16	0.125	16

While the MIC is not necessarily lower for each organism for each mutant, each mutant may still have advantages over the wild-type MU1140 because it may, for example, be easier to produce, easier to transport, have better shelf stability, have better serum stability, or have better proteolytic stability, among other advantageous properties.

Claims

What is claimed is:

1. An isolated recombinant *Streptococcus mutans* strain comprising:
(a) a mutation in a polynucleotide involved in lactic acid synthesis such that expression of lactic acid is diminished by about 80% or more as compared to a wild-type *S. mutans* strain;
(b) a recombinant alcohol dehydrogenase polynucleotide;
(c) a recombinant polynucleotide encoding a lantibiotic comprising Formula I:



(SEQ ID NO:1), wherein the following mutations are present: a Phe1Ile mutation or a Phe1Gly mutation; a Trp4Ala mutation; a Dha5Ala mutation; an Arg13Asp mutation; or combinations of two or more of these mutations.

2. The isolated recombinant *Streptococcus mutans* strain of claim 1, wherein the mutacin comprising Formula I further comprises a Trp4insAla mutation or a ΔTrp4 mutation.

3. The isolated recombinant *Streptococcus mutans* strain of claim 1, wherein the following amino acid substitutions are present: Abu8Ala, or Dhb14Ala, or both Abu8Ala and Dhb14Ala in the lantibiotic comprising Formula I.

4. The isolated recombinant *Streptococcus mutans* strain of claim 1, further comprising a mutation in a polynucleotide involved in ComE, ComC or both ComE and Com C synthesis such that expression of ComE, ComC, or both ComE and ComC is diminished by about 80% or more as compared to a wild-type *S. mutans* strain.

5. The isolated recombinant *Streptococcus mutans* strain of claim 1, further comprising a mutation in a polynucleotide involved in D-amino acid synthesis such that expression of the D-amino acid is diminished by about 80% or more as compared to a wild-type *Streptococcus mutans* strain.

6. The isolated recombinant *Streptococcus mutans* strain of claim 5, wherein the polynucleotide is *dal* or a promoter for *dal*.

7. The isolated recombinant *Streptococcus mutans* strain of claim 1, wherein the recombinant alcohol dehydrogenase polynucleotide is a *Zymomonas mobilis* alcohol dehydrogenase polynucleotide or a *Streptococcus mutans* alcohol dehydrogenase polynucleotide.

8. A method of reducing the incidence or severity of dental caries in a dental caries-susceptible host comprising administering orally to the host the isolated recombinant *Streptococcus mutans* strain of claim 1 in an amount effective for replacement of dental caries-causing *Streptococcus mutans* host strains in the oral cavity of the host.

9. The method of claim 8, wherein the isolated recombinant *Streptococcus mutans* strain is contained in a mouthwash, toothpaste, chewing gum, floss, chewable tablet, food, or beverage.

10. A pharmaceutical composition for reducing the incidence or severity of dental caries comprising the isolated recombinant *Streptococcus mutans* strain of claim 1 and a pharmaceutically acceptable carrier.

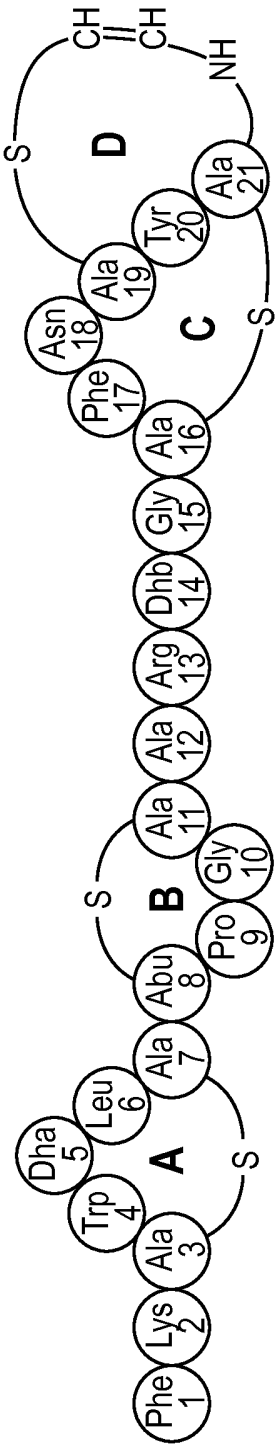


Figure 1A

Wild-type (native) MU1140

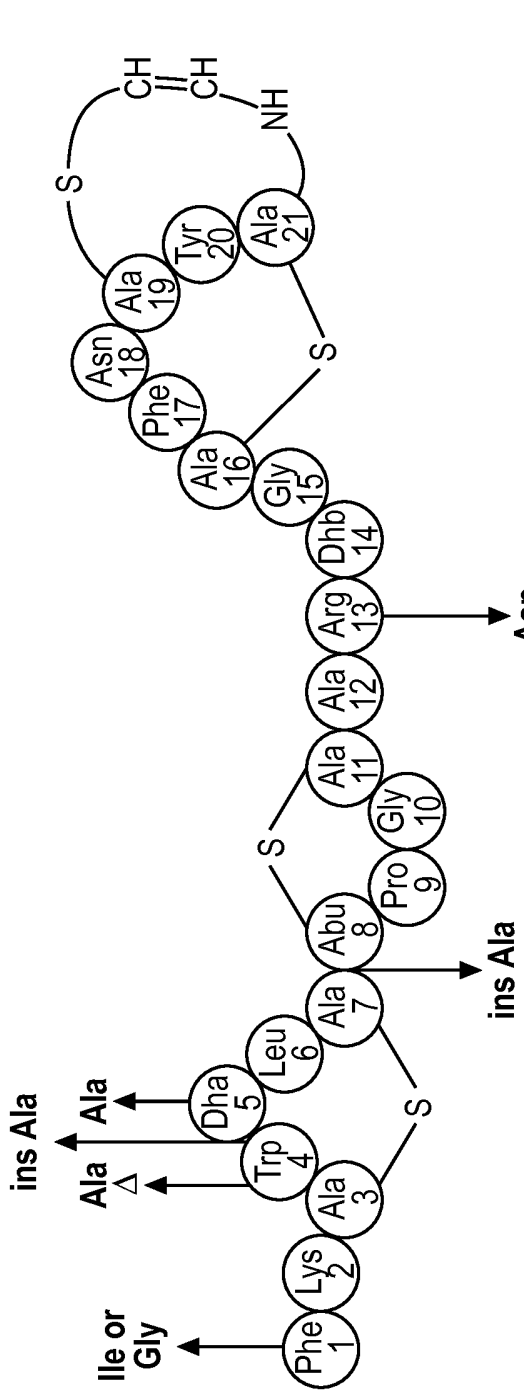


Figure 1B

Schematic of Variations to MU1140

Abbreviations and symbols: ins=insertion and Δ= deletion.

Alignment of mutants to Wild-type Sequence

```
Wild-type     TTCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Phe1Gly       GGCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Phe1Ile       ATCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Trp4Ala       TTCAAAAGTGCA---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Trp4insAla    TTCAAAAGTTGGGCAAGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
ΔTrp4         TTCAAAAGT------AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Ser5Ala       TTCAAAAGTTGG---GCACTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Cys7insAla    TTCAAAAGTTGG---AGCCTTTGTGCAACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Arg13Asp      TTCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAGACACAGGTAGTTTCAATAGTTACTGTTGC
```

Wild-type	SEQ ID NO:19
Phe1Gly	SEQ ID NO:20
Phe1Ile	SEQ ID NO:21
Trp4Ala	SEQ ID NO:22
Trp4insAla	SEQ ID NO:23
ΔTrp4	SEQ ID NO:24
Ser5Ala	SEQ ID NO:25
Cys7insAla	SEQ ID NO:26
Arg13Asp	SEQ ID NO:27

Figure 2

Figure 3: Primers Used for Mutagenesis of MU1140

Oligonucleotide	Sequence (5' – 3')
SRWlanA_1	AGAATTCAGGATGCTATCGCTGCTTTTTTTGTG (SEQ ID NO:1)
SRWlanA_2	AGAATTCAGGAAAGTTGCCATATGGTTTTGTG (SEQ ID NO:2)
Phe1Gly_1	GATCCAGATACTCGT**GGC**AAAAGTTGGAGCCTTTGTACG (SEQ ID NO:15)
Phe1Gly_2	CAACTTTTGCCACGAGTATCTGGATCGTCGTTGC (SEQ ID NO:16)
Phe1Ile_1	GATCCAGATACTCGT**ATC**AAAAGTTGGAGCCTTTGTACG (SEQ ID NO:17)
Phe1Ile_2	CAACTTTTGATACGAGTATCTGGATCGTCGTTGC (SEQ ID NO:18)
Trp4Ala_1	**GCA**AGCCTTTGTACGCCTGGTTG (SEQ ID NO:3)
Trp4Ala_2	ACAAAGGCTTGCACTTTTGAAACG (SEQ ID NO:4)
Trp4insAla_1	**GCA**AGCCTTTGTACGCCTGGTTG (SEQ ID NO:5)
Trp4insAla_2	CAAAGGCTTGCCCAACTTTTGAAACG (SEQ ID NO:6)
ΔTrp4_1	---AGCCTTTGTACGCCTGGTTG (SEQ ID NO:7)
ΔTrp4_2	CGTACAAAGGCTACTTTTGAAACG (SEQ ID NO:8)
Dha5Ala_1	**GCA**CTTTGTACGCCTGGTTGTGC (SEQ ID NO:9)
Dha5Ala_2	GGCGTACAAAGTGCCCAACTTTTGAA (SEQ ID NO:10)
Alas7insAla_1	**GCA**ACGCCTGGTTGTGCAAGGAC (SEQ ID NO:11)
Alas7insAla_2	ACCAGGCGTTGCACAAAGGCTCC (SEQ ID NO:12)
Arg13Asp_1	**GAC**ACAGGTAGTTTCAATAGTTAC (SEQ ID NO:13)
Arg13Asp_2	GAAACTACCTGTGTCTGCACAACCAG (SEQ ID NO:14)
Outside primers are SRWlanA_1 and SRWlanA_2 and are homologous to the 5' and 3' flanking DNA. Underlined section represents the engineered EcoRI site. Mutations are either bolded or dashes. Numbering designates forward (1) and reverse (2) for primers.	

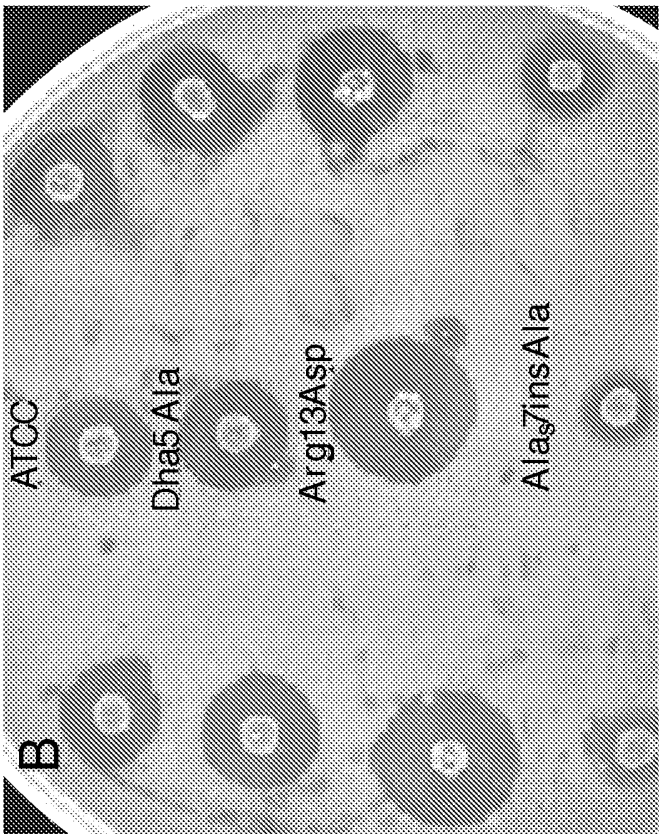


Figure 4B

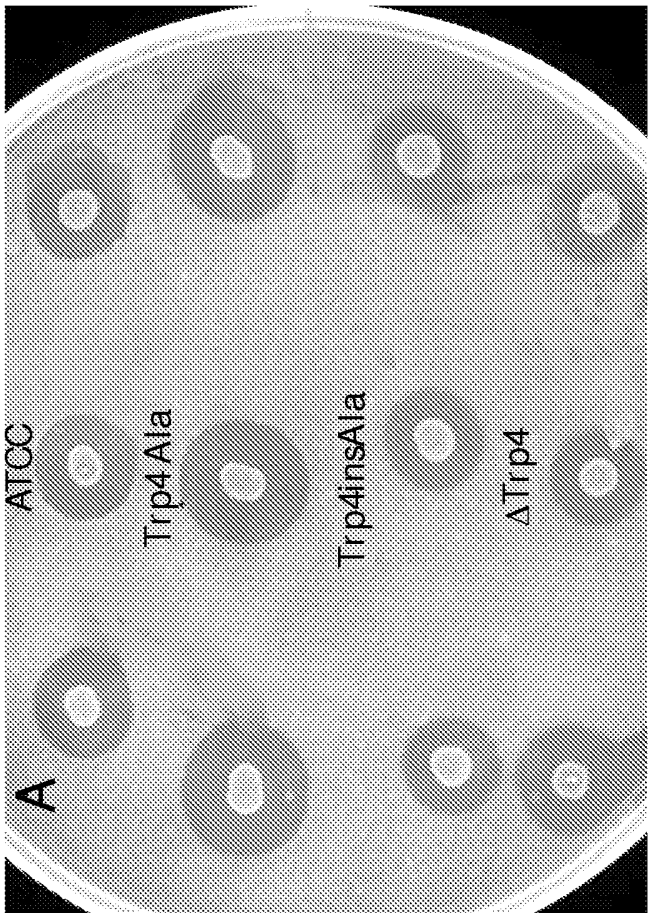


Figure 4A

Figure 4A-B: Zone of Inhibition Plate Assays

Bioactivity of Strains Producing Variants of MU1140 Compared to Wild-Type MU1140

Variant Produced	Mean Area* (mm^2)	Standard Error of the Mean (SEM)	Ratio of Variant to Wild-Type Activities	Statistical Significance (*p* value)#
MU1140 (wild-type)	204.44	8.90	-	-
Phe1Gly	321.85	46.52	1.57	<.001
Phe1Ile	372.78	75.90	1.82	<.001
Trp4Ala	434.80	46.10	2.12	<.001
Trp4insAla	212.37	24.70	1.04	>.05
∇Trp4	217.56	35.37	1.06	>.05
Dha5Ala	382.25	31.40	1.87	<.001
Ala_s7insAla	109.41	9.74	0.54	<.001
Arg13Asp	526.06	55.09	2.57	<.001

* Based on 10 independent samples.
# Student's t Test

Figure 5

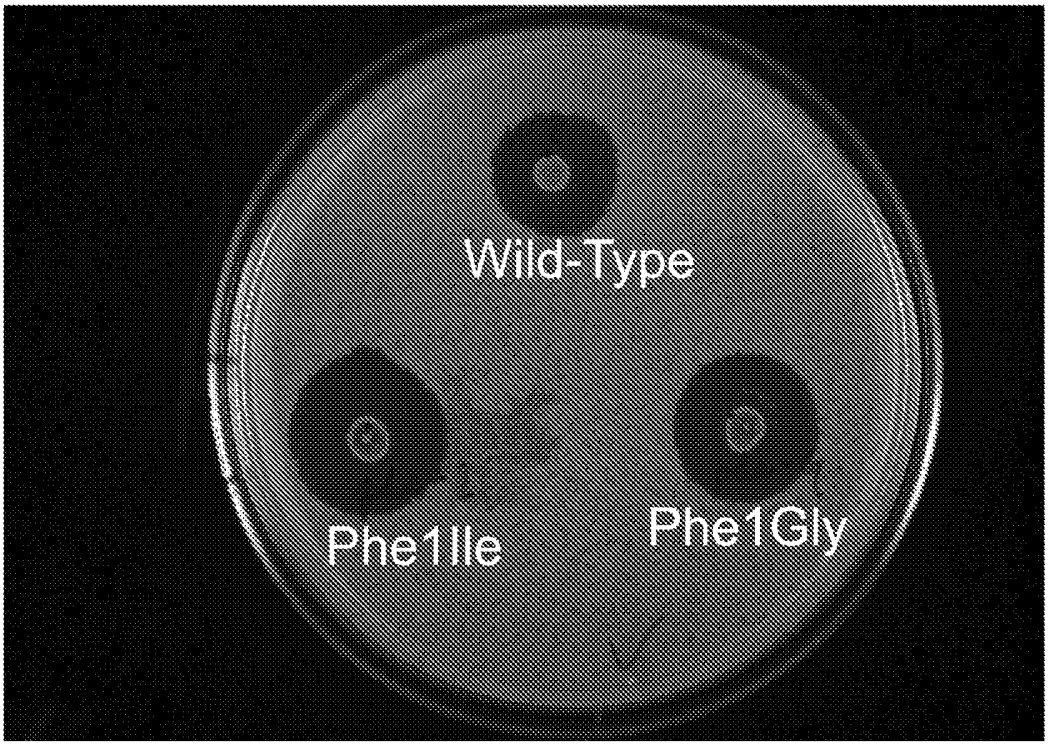


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/027340

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/315 (2013.01)
USPC - 424/49
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 38/00, 38/12, 38/16; A61P 31/04; A61Q 11/00; C07C 323/59; C07K 1/02, 1/06, 1/107, 5/12, 14/00, 14/195, 14/315 (2013.01)
USPC - 424/49, 50, 94.4; 435/183, 220, 252.3, 252.35, 253.4; 514/2.3, 2.4, 2.6, 12.2, 21.4; 530/324, 326, 350; 536/23.1, 23.7

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

CPC - A61K 8/64; A61Q 11/00, 17/005; C07K 1/02, 1/04, 1/062, 1/063, 1/107, 1/1075, 14/32, 14/315 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Patbase, Google Patent, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009/0215985 A1 (KIRICHENKO et al) 27 August 2009 (27.08.2009) entire document	1-10
A	WO 1998/56411 A1 (HILLMAN) 09 June 1998 (09.06.1998) entire document	1-10
A	WO 2008/151434 A1 (HANCOCK et al) 18 December 2008 (18.12.2008) entire document	1-10

☐ 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
14 May 2013	31 MAY 2013

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 104321338 A
(43)申请公布日 2015.01.28

(21)申请号 201380011223.1
(22)申请日 2013.02.22
(30)优先权数据
61/603,693 2012.02.27 US
61/603661 2012.02.27 US
(85)PCT国际申请进入国家阶段日
2014.08.27
(86)PCT国际申请的申请数据
PCT/US2013/027340 2013.02.22
(87)PCT国际申请的公布数据
WO2013/130351 EN 2013.09.06
(83)生物保藏信息
ATCC 55676 1995.06.02
(71)申请人 奥洁克公司
地址 美国佛罗里达州
申请人 得克萨斯农业及机械体系综合大学
(72)发明人 J.D. 希尔曼 J.L. 史密斯
S.R. 威尔逊-斯坦福德
(74)专利代理机构 中国专利代理(香港)有限公司 72001
代理人 黄登高 孟慧岚
(51)Int.Cl.
C07K 14/315(2006.01)

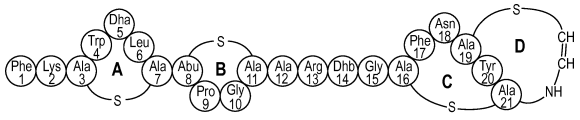
权利要求书1页 说明书19页 附图6页

(54)发明名称

用于龋齿的替代疗法

(57)摘要

本发明提供可用于改善口腔健康的重组变形链球菌菌株。本发明的一个实施方案提供在易患龋齿的宿主中降低龋齿的发生率或严重性的方法,该方法包括口服给予宿主有效量的本发明分离的重组变形链球菌菌株,用于替代宿主口腔中的引起龋齿的变形链球菌宿主菌株。分离的重组变形链球菌菌株10可被包含在漱口剂、牙膏、口香糖、牙线、咀嚼片、食品或饮料中。



野生型(天然) **MU1140**

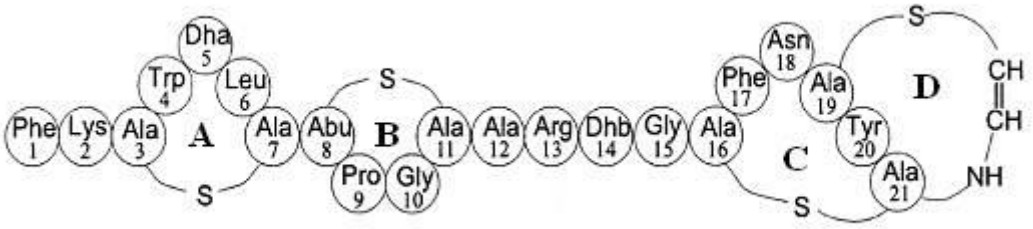
CN 104321338 A

1. 一种分离的重组变形链球菌（*Streptococcus mutans*）菌株，其包含：

(a) 涉及乳酸合成的多核苷酸的突变，以使乳酸的表达在与野生型变形链球菌菌株比较时减少约 80% 或更多；

(b) 重组醇脱氢酶多核苷酸；

(c) 编码包含式 I 的羊毛硫抗生素的重组多核苷酸：



(SEQ ID NO:1)，其中存在以下突变：Phe1Ile 突变或 Phe1Gly 突变；Trp4Ala 突变；Dha5Ala 突变；Arg13Asp 突变；或这些突变的两种或多种的组合。

2. 权利要求 1 的分离的重组变形链球菌菌株，其中包含式 I 的变异菌素还包含 Trp4insAla 突变或 ΔTrp4 突变。

3. 权利要求 1 的分离的重组变形链球菌菌株，其中在包含式 I 的羊毛硫抗生素中存在以下氨基酸取代：Abu8Ala，或 Dhb14Ala，或 Abu8Ala 和 Dhb14Ala 二者。

4. 权利要求 1 的分离的重组变形链球菌菌株，其还包含在涉及 ComE、ComC 或 ComE 和 Com C 二者合成的多核苷酸中的突变，以使 ComE、ComC，或 ComE 和 ComC 二者的表达在与变形链球菌菌株比较时减少约 80% 或更多。

5. 权利要求 1 的分离的重组变形链球菌菌株，其还包含在涉及 D- 氨基酸合成的多核苷酸中的突变，以使 D- 氨基酸的表达在与野生型变形链球菌菌株比较时减少约 80% 或更多。

6. 权利要求 5 的分离的重组变形链球菌菌株，其中多核苷酸为 *dal* 或用于 *dal* 的启动子。

7. 权利要求 1 的分离的重组变形链球菌菌株，其中重组醇脱氢酶多核苷酸是运动发酵单胞菌（*Zymomonas mobilis*）醇脱氢酶多核苷酸或变形链球菌醇脱氢酶多核苷酸。

8. 一种在易患龋齿的宿主中降低龋齿的发生率或严重性的方法，其包括口服给予宿主有效量的权利要求 1 的分离的重组变形链球菌菌株，用于替代宿主口腔中引起龋齿的变形链球菌宿主菌株。

9. 权利要求 8 的方法，其中分离的重组变形链球菌菌株包含在漱口剂、牙膏、口香糖、牙线、咀嚼片、食品或饮料中。

10. 一种用于降低龋齿的发生率或严重性的药物组合物，其包含权利要求 1 的分离的重组变形链球菌菌株和药学上可接受的载体。

用于龋齿的替代疗法

[0001] 优先权

该申请要求 2012 年 2 月 27 日提交的美国临时申请 61/603,661 和 2012 年 2 月 27 日提交的美国临时申请 61/603,693 的权益，此二者通过引用以其全部内容结合到本文中。

[0002] 发明背景

龋齿是世界上最流行的慢性感染性疾病之一。超过一半的 5-9 岁美国儿童具有至少一个牙洞或牙填料；到 17 岁，几乎 80% 的美国年轻人已有牙洞。美国卫生和公众服务部．美国的口腔健康：一种外科医生的总执行概要 (Oral Health in America: A Report of the Surgeon General--Executive Summary). Rockville, MD：美国卫生和公众服务部，国立牙科和颅面研究，国立卫生研究院，2000。

[0003] 根据牙科，口腔和颅面数据资源中心，在美国有关治疗龋齿的年度支出估算为每年 400 亿美元 ($)。龋齿的特征在于牙釉质和牙本质的脱矿质化，最终导致牙齿的破坏。膳食糖经常被误解为龋齿的原因；然而，龋齿的直接原因是由在牙齿表面上代谢糖的微生物产生的乳酸。研究提示在大约 700 种口腔微生物中，变形链球菌 (*Streptococcus mutans*)，一种几乎在所有人中发现的细菌，是发生龋齿的主要病原体。变形链球菌定居于牙齿表面上的牙菌斑中，当其将膳食糖转化为乳酸时，从碳水化合物代谢获得能量，而乳酸又继而促进牙釉质和牙本质的脱矿质化，最终导致牙洞。矿物质丢失的速率取决于几个因素，包括变形链球菌细胞存在的数目和其消耗糖的频率和量。

[0004] 利用细菌干扰 (bacterial interference)，用非致病的、效应菌株替代致病菌株例如变形链球菌的治疗方案被称为替代疗法。成功的替代疗法需要这样的效应菌株，其：1) 是非致病的，2) 改变微环境以防止病原生物的定居或生长，和 3) 持续地定居于处于风险的宿主以防止由目标病原生物引起的再感染，和在其中病原为宿主的天然菌群的一部分的情况下，从处于风险的组织强有力地置换病原生物。

[0005] 替代疗法原理的应用需要分离变形链球菌的非致龋效应菌株，例如缺乏乳酸合成的变形链球菌菌株，其可比宿主口腔中的天然变形链球菌更具竞争优势 (outcompete)。本领域需要稳定的、乳酸 - 缺乏的、变形链球菌的非致龋株，其可持续地定居于宿主口腔并比其中的天然变形链球菌强有力地更具竞争优势，且其适合用于预防和 / 或治疗龋齿的替代疗法。

[0006] 效应菌株抢先定居人的口腔且强有力地置换本土野生株的能力最初被认为是依赖于大量的表型特性的复杂现象。然而，已发现单一表型特性可提供必要的选择性优势。从人受试者分离出一种天然存在的变形链球菌菌株，其产生称为 MU1140 的羊毛硫抗生素，其在针对其进行的试验中能够杀死几乎所有其它的变形链球菌菌株。参见例如，Hillman 等人，Infect. Immun. 44：141 (1984)。已分离出不产生可检测的 MU1140 或产生大约 3 倍升高量的突变体。将突变体用于大鼠模型以使羊毛硫抗生素产生与定居潜力相关。已发现这些菌株抢先定居于宿主且强有力地置换变形链球菌的本土株的能力随着 MU1140 产生的量增加而显著地增加。

[0007] 在人受试者中观察到 MU1140 产生和定居潜力之间的相同关系，其中需要反复暴

露于野生亲株以获得持久的定居 (Hillman 等人 J. Dent. Res. 66 :1092 (1985)),然而单次暴露于产生 3 倍升高量的 MU1140 的菌株是足够的 (Hillman 等人 J. Dent. Res. 66 : 1092 (1987))。后一菌株完全替代人受试者口腔中的变形链球菌的本土株需要经过一年。在此期间,推测它们对龋齿的易感性持续至本土变形链球菌的水平降至阈值水平之下。

[0008] 为了进一步增加效应菌株的定居潜力用于龋齿的替代疗法,期望获得一或多株变形链球菌,其产生升高量的 MU1140 或产生具有增加的特异性活性的这种分子的变体。这样的菌株将减少效应菌株除去本土的、产乳酸菌株所需的时间并由此获得充分的有效性。这样的菌株也更可能克服任何对定居的固有抗性,虽然目前尚不知道,其可存在于所治疗群体的某些个体中。参见例如,Hillman, *Antonie van Leeuwenhoek* 82 :361 - 366, 2002。

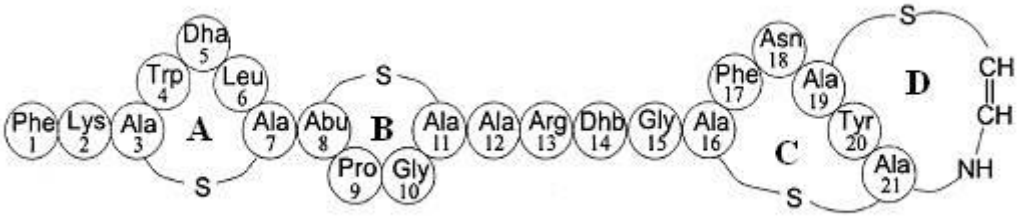
[0009] 发明概述

在一个实施方案中,本发明提供分离的重组变形链球菌菌株,其包含 :

(a) 涉及乳酸合成的多核苷酸的突变,以使乳酸的表达在与野生型变形链球菌菌株比较时减少约 80% 或更多 ;

(b) 重组醇脱氢酶多核苷酸 ;

(c) 编码包含式 I 的羊毛硫抗生素的重组多核苷酸 :



(SEQ ID NO :1),其中存在以下突变 :Phe1Ile 突变或 Phe1Gly 突变 ;Trp4Ala 突变 ; Dha5Ala 突变 ;Arg13Asp 突变 ;或这些突变的两种或多种的组合。所述菌株还可包含 Trp4insAla 突变或 ΔTrp4 突变。以下氨基酸取代也可存在 :Abu8Ala 或 Dhb14Ala,或 Abu8Ala 和 Dhb14Ala 二者。所述菌株还可包含涉及 ComE、ComC,或 ComE 和 ComC 二者合成的多核苷酸的突变,以使 ComE、ComC,或 ComE 和 ComC 二者的表达在与野生型变形链球菌菌株比较时减少约 80% 或更多。所述菌株可进一步包含涉及 D- 氨基酸合成的多核苷酸的突变,以使 D- 氨基酸的表达在与野生型变形链球菌菌株比较时减少约 80% 或更多。涉及 D- 氨基酸合成的多核苷酸可以是 *dal* 或用于 *dal* 的启动子。重组醇脱氢酶多核苷酸可以是运动发酵单胞菌 (*Zymomonas mobilis*) 醇脱氢酶多核苷酸或变形链球菌醇脱氢酶多核苷酸。

[0010] 本发明的另一个实施方案提供一种在易患龋齿的宿主中降低龋齿的发生率或严重性的方法,其包括口服给予宿主本发明的分离的重组变形链球菌菌株,其量有效用于替代在宿主口腔中的引起龋齿的变形链球菌宿主株。分离的重组变形链球菌菌株可包含在漱口剂、牙膏、口香糖、牙线、咀嚼片、食品或饮料中。

[0011] 本发明的另一个实施方案提供一种用于降低龋齿的发生率或严重性的药物组合物,其包含本发明的分离的重组变形链球菌菌株和药学上可接受的载体。

[0012] 因此,本发明提供稳定的、乳酸 - 缺乏的和非致龋的变形链球菌菌株,其可比天然变形链球菌强有力地更具竞争优势,尤其是因为其表达了与野生型 MU1140 羊毛硫抗生素

比较时，生物学活性改善的变体 MU1140 羊毛硫抗生素。

[0013] 附图简述

图 1A 显示野生型 MU1140 结构 (SEQ ID NO :1)。图 1B 显示 MU1140 (SEQ ID NO :2) 的突变位点。

[0014] 图 2 示出了突出显示变体 MU1140 *lanA* 多核苷酸序列突变的染色体 DNA 序列与野生型 MU1140 *lanA* 多核苷酸序列。

[0015] 图 3 显示用于 MU1140 结构基因 *lanA* 诱变的引物。

[0016] 图 4A-B 显示抑菌圈平板测定的结果。

[0017] 图 5 显示产生 MU1140 变体的菌株与产生野生型 MU1140 的菌株比较的生物活性均值和标准差。

[0018] 图 6 显示产生 MU1140 变体 (Phe1Ile 和 Phe1Gly) 的菌株与产生野生型 MU1140 的菌株比较的生物活性。

[0019] 发明详述

如本文所用的，单数形式 ″一个 ″、″一种 ″和 ″所述 ″包括复数对象，除非上下文另外明确指明。

[0020] 变形链球菌可以经重组处理以不产生乳酸或产生显著减少量的乳酸。Hillman 等人 J. Appl. Microbiol. 102 :1209 (2007)。可存活的、乳酸 - 缺乏的变形链球菌菌株可通过用编码重组醇脱氢酶 (ADH) 的核酸转化菌株而生成，以表达重组醇脱氢酶，并在乳酸合成途径中引入突变以使产生重组 ADH 的菌株乳酸缺乏。重组 ADH 防止细菌中代谢物的积累，因此避免乳酸缺乏的任何致死性。此外，可对变形链球菌菌株进行重组工程改造以表达具有比野生型 MU1140 羊毛硫抗生素更高生物学活性的变体 MU1140 羊毛硫抗生素。这些菌株可比宿主口腔中引起龋齿的野生型、天然变形链球菌菌株更具竞争优势并将其替代。

[0021] 变形链球菌亲株

任何变形链球菌菌株可用于构建本发明的重组变形链球菌菌株。本发明的重组变形链球菌菌株相对于通常定居于口腔的野生型变形链球菌菌株具有选择性优势。选择性优势可以通过多种特征的任何一种赋予 (例如，抗菌化合物的产生，减少的或有利的相对代谢需求，更高的相对生长率，代谢物清除剂的产生)，其促进所述菌株的口腔定居并替代定居口腔的定居菌株。在本发明的一个实施方案中，本发明的重组变形链球菌菌株的定居将不会显著破坏其它非变形链球菌菌株 (例如，与龋齿发生无关的正常菌群)。例如，用变形链球菌的重组菌株 (其产生具有提高的羊毛硫抗生素活性的变体 MU1140 羊毛硫抗生素) 感染可导致替代定居的、致龋变形链球菌菌株，而不对口腔的其它定居微生物种类产生影响。

[0022] 重组变形链球菌菌株

重组变形链球菌菌株是非天然存在的变形链球菌菌株，其已使用多种重组核酸技术 (即，涉及 DNA 或 RNA 处理的技术) 的任何一种生成。一般来说，本发明的重组变形链球菌菌株具有乳酸产生的缺乏 ；表达重组醇脱氢酶 (ADH) 多肽 ；和表达足以产生变体 MU1140 羊毛硫抗生素的重组多肽，所述变体 MU1140 羊毛硫抗生素具有比野生型 MU1140 更大的生物学活性。变形链球菌的重组菌株可任选地缺乏 ComE、ComC 或 ComE 和 ComC 二者的表达和 / 或对通常不存在于口腔或特定宿主的饮食 (例如，D- 氨基酸) 中的有机物质而言可任选地为营养缺陷型的。

[0023] 变体 MU1140

MU1140 具有在环 B 和 C 之间的“铰链区”扭结的整体马蹄样形状。Smith 等人 (2003) Biochem. 42 :10372-10384。这种形状是铰链区中的转角样基序 (turn-like motif) 的结果，其将氨基 - 末端 AB 环 (脂质 II 结合域) 朝向羧基 - 末端重叠的环 CD 折叠。认为铰链区的柔性在促进 MU1140 的侧向组装，使其外展并隔离 (abduct and sequester) 脂质 II 方面是重要的。在环 A 中的 Trp4 的 Ψ 角和 Dha5 的 Φ 角有助于促进其柔性。而且，已确定 $_S$Ala7 (一种不受硫醚环限制的残基) 的 Ψ 键旋转 360º，使得环 A 相对于环 B 自由地自旋。这种柔性被认为在脂质 II 结合期间对于环 A 和 B 的取向是重要的。铰链区还在残基 13 上含有潜在的易受酶促影响的精氨酸。产生 MU1140 的结构基因 (*lanA*) 中的突变以确定以下氨基酸改变的作用 :Phe1Ile、Phe1Gly、Trp4Ala、Trp4insAla、ΔTrp4、Dha5Ala、Ala$_s$7insAla 和 Arg13Asp。图 1B。

[0024] 已发现，在延迟拮抗测定中，使用藤黄微球菌 (*Micrococcus luteus*) 菌株 ATCC 272 作为目标菌株，具有 Trp4 缺失或在 Trp4 之后插入 Ala 的 MU1140 变体显示出大致等同于野生型的生物活性。Wilson-Sanford 等人, (2009) Appl. Environ. Microbiol. 75 :1381。在这种测定中，通过计算抑菌圈的面积确定活性。这些结果表明缩短或延长环 A 对 MU1140 活性没有有益或有害作用，表明在环 A 结构中的意想不到的随意性 (permissiveness)。如在图 5 中所示的，当与野生型比较时，Trp4Ala 取代导致生物活性在统计学上显著 (p<.05) 增加。由于两个氨基酸是不带电荷的和疏水的，可以推测生物活性的差异是由于这两个氨基酸之间的大小差异所致。用 Ala 替代 Dha5 也导致生物活性在统计学上显著 (p<.05) 增加。在 7 位的 $_S$Ala 之后插入丙氨酸导致生物活性的显著性 (p<.05) 减少。虽然不希望受限于任何特定理论，但由于已经确定 $_S$Ala7 自由地旋转 360º，使得环 A 相对于环 B 自由地自旋，可以推断 Ala$_s$7insAla 突变改变了环在脂质 II 结合期间的取向，有可能影响分子对其底物脂质 II 的亲和力。当与野生型比较时，Arg13Asp 取代显示出生物活性的非常显著 (p<.05) 的增加。虽然不希望受限于任何特定理论，但观察到的效果可以是增加的溶解性的结果。如在图 6 中所示，当与野生型比较时，Phe1Ile 和 Phe1Gly 二者的取代导致生物活性在统计学上显著 (p<.05) 增加。值得注意的是，用 Asp (GAT/GAC) 取代 Arg (AGA/AGG/CGT/CGC/CGA/CGG) 或用 Ala (GCT/GCT/GCA/GCG) 取代 Trp (TGG) 或用 Ala (GCT/GCT/GCA/GCG) 取代 Ser (AGT/AGC) 或用 Ile (ATT/ATG) 或 Gly (GGT/GGC/CCA/GGG) 取代 Phe (TTT/TTC) 都是极不可能天然存在的，因为它们涉及多点突变，其可包括在受影响的密码子中的一个或多个颠换。虽然不希望受限于任何特定理论，但该增加的基础可能是由于对脂质 II 靶标的结合亲和力增加或由于前导序列的切割效率改善。产生具有一个或多个这些定向位点的改变 (Phe1Ile、Phe1Gly、Trp4Ala、Dha5Ala 和 Arg13Asp) 的变体 MU1140 的效应菌株具有通过改进其定居于口腔和强有力地置换致病的变形链球菌本土株的能力，而优于产生野生型 MU1140 的效应菌株的潜力。

[0025] 本发明的羊毛硫抗生素 MU1140 的变体为包含翻译后修饰的多肽。翻译后修饰是多肽在其被翻译后的化学修饰。多肽是两个或更多个氨基酸经酰胺键共价连接的聚合物。纯化的多肽是基本上没有细胞材料、其它类型的多肽、化学前体、用于合成多肽的化学品或其组合的多肽制剂。基本上没有细胞材料、培养基、化学前体、用于合成多肽的化学品等的多肽制剂，具有少于约 30%、20%、10%、5%、1% 或更少的其它多肽、培养基、化学前体和 / 或用

于合成的其它化学品。因此，纯化的多肽具有约 70%、80%、90%、95%、99% 或更高的纯度。纯化的多肽不包括未纯化的或半 - 纯化的细胞提取物或少于 70% 纯度的多肽的混合物。

[0026] 野生型 MU1140 示于图 1A 中。MU1140 具有标记为 A、B、C 和 D 的 4 个环。这些环中的两个由羊毛硫氨酸 (Ala-S-Ala) 残基形成，包括环 A 中的一个残基 (Ala_3-S-Ala_7) 和环 C 中的一个残基 (Ala_{16}-S-Ala_{21})；存在甲基 - 羊毛硫氨酸残基 (Abu-S-Ala)，其形成包含在 8 位的 α - 氨基丁酸残基和在 11 位的 Ala (Abu_8-S-Ala_{11}) 的环 B；以及第四个环 D，其包含通过硫醚键 (Ala_{19} - S—CH=CH—NH—) 连接于氨基乙烯基的 19 位的 Ala。

[0027] 本发明的一个实施方案提供羊毛硫抗生素变异菌素 MU1140 的一个或多个以下变体，示于图 1B (SEQ ID NO:2) 中。即，本发明包括具有一个或多个以下突变的野生型羊毛硫抗生素 MU1140 (SEQ ID NO:1) 的变体：

1. Phe1Ile 或 Phe1Gly；即，将 1 位的苯丙氨酸改变为异亮氨酸或甘氨酸。

[0028] 2. Trp4Ala；即，将 4 位的色氨酸改变为丙氨酸。

[0029] 3. Dha5Ala；即，将 5 位的 2,3- 二脱氢丙氨酸改变为丙氨酸；

4. Arg13Asp；即，将 13 位的精氨酸改变为天冬氨酸。

[0030] 在本发明的一个实施方案中，羊毛硫抗生素 MU1140 的变体包含 Phe1Ile 或 Phe1Gly 氨基酸取代；Trp4Ala 氨基酸取代；Dha5Ala 氨基酸取代；Arg13Asp 氨基酸取代；或其组合。本发明的 MU1140 变体也可包含，例如 Trp4insAla，其中丙氨酸在第四个色氨酸残基之后插入；或其中 4 位的色氨酸缺失的 ΔTrp4；或在一级氨基酸序列中的这两种改变。

[0031] MU1140 羊毛硫抗生素多肽的生物学活性等价物可具有一个或多个保守的氨基酸变体或其它次要的修饰并保留生物学活性。生物学活性等价物当与相应的羊毛硫抗生素 MU1140 比较时，具有基本上等同的功能。在本发明的一个实施方案中，羊毛硫抗生素变异菌素具有约 1、2、3、4 或 5 或更少的保守氨基酸取代。保守取代是其中氨基酸被另一种具有类似特性的氨基酸取代的取代，使得肽化学领域的技术人员将期望多肽的二级结构和一般特性基本上没有改变。一般来说，以下各组氨基酸代表保守的变化：(1) ala、pro、gly、glu、asp、gln、asn、dha、abu、dhb、ser、thr；(2) cys、ser、tyr、thr；(3) val、ile、leu、met、ala、gly、dha、abu、dhb、phe；(4) lys、arg、his；和 (5) phe、tyr、trp、his。生物学活性等价物羊毛硫抗生素变异菌素或其它羊毛硫抗生素多肽一般可通过如下鉴定：修饰本发明的变体羊毛硫抗生素变异菌素序列之一，并评价修饰的羊毛硫抗生素变异菌素的特性，以确定其是否是生物学等价物。如果羊毛硫抗生素在测定 (例如抑菌圈测定) 中的反应基本上与本发明的羊毛硫抗生素变异菌素相同，例如，具有原始羊毛硫抗生素变异菌素的 90-110% 的活性，它就是生物学等价物。

[0032] 本发明的重组变形链球菌菌株包含表达功能性变体 MU1140 的多核苷酸。变体 MU1140 的生物学活性可使用例如抑菌圈测定 (参见实施例 2) 测定。重组变形链球菌菌株产生足够的变体 MU1140，以比野生型致龋变形链球菌更具竞争优势和基本上从宿主口腔消除野生型致龋变形链球菌 (例如，减少野生变形链球菌的数量约 5、10、25、50、75、90、95、99 或 100% (或在约 5 % 和约 100% 之间的任何范围))。

[0033] 本发明的羊毛硫抗生素可以共价地或非共价地连接于通常羊毛硫抗生素天然不与之关联的氨基酸序列，即异源性氨基酸序列。异源性氨基酸序列可来自非变形链球菌生物体，合成序列，或通常不位于本发明的羊毛硫抗生素的羧基或氨基末端的变形链球菌序

列。此外，本发明的羊毛硫抗生素可以共价地或非共价地连接于非氨基酸的化合物或分子例如指示试剂。本发明的羊毛硫抗生素可以共价地或非共价地连接于氨基酸间隔基、氨基酸连接基、信号序列、终止转移序列、TMR 终止转移序列、跨膜域、蛋白纯化配体或其组合。多肽也可连接于促进纯化（例如，亲和力标签例如 6- 组氨酸标签、trpE、谷胱甘肽 -S- 转移酶、麦芽糖结合蛋白、葡萄球菌蛋白 A 或 com）的部分（即，可以是多肽或其它化合物的官能团），或促进多肽稳定性的部分（例如，聚乙二醇；氨基末端保护基团例如乙酰基、丙酰基、琥珀酰基、苄基、苄氧基羰基或叔丁氧基羰基；羧基末端保护基团例如酰胺、甲酰胺和乙酰胺）。在本发明的一个实施方案中，蛋白纯化配体可以是一个或多个在例如本发明多肽的氨基末端或羧基末端的氨基酸残基。氨基酸间隔基是天然不与本发明的多肽关联的氨基酸序列。氨基酸间隔基可包含约 1、5、10、20、100 或 1,000 个氨基酸。

[0034] 如果需要，本发明的羊毛硫抗生素可以是融合蛋白的一部分，其可含有异源性氨基酸序列。异源性氨基酸序列可存在于本发明的羊毛硫抗生素的 C 或 N 末端以形成融合蛋白。多于一个的本发明的羊毛硫抗生素可存在于融合蛋白中。本发明的羊毛硫抗生素的片段可存在与本发明的融合蛋白中。本发明的融合蛋白可包含一个或多个本发明的羊毛硫抗生素、其片段或其组合。

[0035] 在本发明的一个实施方案中，本发明的重组变形链球菌菌株是 ATCC 55676（根据国际承认用于专利程序目的的微生物保藏的布达佩斯条约条款及其规章（布达佩斯条约）而保藏），其已如本文所述经基因工程改造以表达变体 MU1140。

[0036] 因此，与野生型 MU1140 羊毛硫抗生素比较而具有提高的生物学活性的突变体 MU1140 羊毛硫抗生素的产生可给变形链球菌提供相对于存在于宿主口腔中的不产生 MU1140 的变形链球菌菌株的选择性优势。变体 MU1140，当通过本发明的重组变形链球菌菌株表达时，消除定居的、MU1140- 易感变形链球菌菌株，因此干扰 MU1140- 易感菌株的定居并促进重组变形链球菌定居于口腔。由于野生型天然变形链球菌从口腔中被置换，因而龋齿的发生率和 / 或严重性降低。

[0037] 在本发明的一个实施方案中，效应菌株可另外地表达 lanB、lanC、lanE、lanF、lanG、lanK、lanM、lanP、lanR、lanT 或这些变形链球菌多肽的两个或更多个的组合。

[0038] 乳酸表达缺乏

″乳酸缺乏″或″乳酸产生缺乏″意指相对于野生型变形链球菌，重组变形链球菌菌株产生显著降低量的乳酸。乳酸的显著降低量为比野生型变形链球菌菌株（例如变形链球菌菌株 UA159 (ATCC 700610)）或其它属于变形链球菌群的种类产生的乳酸少约 40、50、60、70、80、90、95 或 100%（或约 40%- 约 100% 之间的任何范围），所述变形链球菌群包括表兄链球菌 (*Streptococcus sobrinus*)（例如表兄链球菌菌株 SL1 (ATCC 33478)）、鼠链球菌 (*Streptococcus rattus*)（例如，鼠链球菌菌株 FA1 (ATCC 19645)）、大鼠链球菌 (*Streptococcus cricetus*)（大鼠链球菌菌株 HS6 (ATCC 19642)）和野生链球菌 (*Streptococcus ferus*)（野生链球菌菌株 8S1））。在本发明的一个实施方案中，乳酸 - 缺乏的变形链球菌效应菌株产生不可检测的乳酸。乳酸表达可如在例如 Hillman 等人，Infect. Immun. 62 :60 (1994)；Hillman 等人，Infect. Immun. 64 :4319 (1996)；Hillman 等人，1990, Infect. Immun., 58 :1290-1295 中所述进行检测。

[0039] 作为在乳酸合成途径中的非功能性的、失活的、部分功能性的，或部分失活的调控

区、翻译信号、转录信号或结构序列的结果，本发明的重组变形链球菌菌株可以是乳酸缺乏的。调控区、翻译信号和转录信号包括，例如启动子、增强子、核糖体结合位点、CAAT 框、CCAAT 框、普里布诺框 (Pribnow box)、TATA 框等。非功能性的或失活的意指已知的多核苷酸、基因、多肽或蛋白的野生型功能或活性已经消除或与野生型多核苷酸、基因、多肽或蛋白比较大大减少，减少约 80、90、95 或 100% （或约 80%- 约 100% 之间的任何范围）。部分功能性的或部分失活的意指已知的多核苷酸、基因、多肽或蛋白的野生型功能或活性与野生型多核苷酸、基因、多肽或蛋白比较已经部分地减少约 20、30、40、50、60、70、79% （或约 20%- 约 79% 之间的任何范围）。

[0040] 失活或部分失活（其使得多核苷酸、基因、多肽或蛋白为非功能性的或部分功能性的）可通过方法例如在涉及乳酸合成途径的多核苷酸中掺入突变（例如，点突变、移码突变、取代、缺失（部分或完整信号、区域或结构多核苷酸）、中断和 / 或插入）来实现。涉及乳酸合成的多核苷酸的突变可影响乳酸的表达，以使乳酸表达的量在与野生型变形链球菌菌株比较时减少约 20、30、40、50、60、70、80、90、95% 或更大。

[0041] 例如，乳酸表达的失活或部分失活可通过缺失部分或完整 *ldh* 结构多核苷酸或部分或完整 *ldh* 启动子使例如乳酸脱氢酶 (*ldh*) 基因失活或部分失活来实现。此外，乳酸表达的失活或部分失活可通过失活或部分失活编码涉及糖转运的酶的基因，例如磷酸烯醇丙酮酸磷酸转移酶系统 (*pts*) 基因，通过缺失部分或完整 *pts* 结构多核苷酸或部分或完整 *pts* 启动子来实现。参见例如，Cvitkovitch 等人，J. Bacteriol. 177 :5704 (1995)。乳酸表达的失活或部分失活可通过失活或部分失活编码涉及细胞内和细胞外多糖储存的酶的基因，例如糖原合成酶 (g*lgA*) 基因（参见例如，Spatafora 等人，Infect. Immun. 63 :2556 (1995)）和果糖基转移酶 (*ftf*) 基因（参见例如，Schroeder 等人，Infect. Immun. 57 : 3560 (1989)），通过缺失部分或完整 *glgA* 或 *ftf* 结构多核苷酸或部分或完整 *glgA* 或 *ftf* 启动子来实现。

[0042] 乳酸合成途径中的一个或多个缺陷可通过诱变（即，使变形链球菌暴露于诱变剂）、自发突变体的选择，或使用重组技术的基因操纵引入。这些技术是本领域熟知的（参见，如，Sambrook 等人，1989, 分子克隆 :实验室手册 (Molecular Cloning :A Laboratory Manual)，第二版，Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.）。在本发明的一个实施方案中，乳酸合成途径缺陷使用重组技术引入，例如，将缺陷的 *ldh* 结构基因引入细菌和随后进行位点 - 特异性重组，以用缺陷的 *ldh* 替代野生型 *ldh*。已克隆变形链球菌 *ldh* 基因并测定其核苷酸序列 (GenBank 检索号 M72545)，并在大肠杆菌 (*Escherichia coli*) 中表达重组 *ldh* 基因 (Hillman 等人，1990, Infect. Immun., 58 : 1290-1295 ;Duncan 等人，1991, Infect. Immun., 59 :3930-3934)。Hillman 等人使变形链球菌菌株的 *ldh* 的完整开放阅读框基本缺失 (J. Appl. Microbiol. 102 :1209 (2007))。

[0043] 醇脱氢酶产生

由于乳酸合成的缺陷对于变形链球菌是致死的，重组的、乳酸 - 缺乏的变形链球菌菌株的缺陷必须通过重组醇脱氢酶 (ADH) 的产生来补充。参见例如，Hillman 等人，Infect. Immun. 64 :4319 (1996)。重组 ADH 的产生防止代谢物例如丙酮酸的积累，否则丙酮酸引起乳酸 - 缺乏的变形链球菌死亡。

[0044] 可对变形链球菌菌株进行基因工程改造以表达重组醇脱氢酶例如，醇脱氢酶

B、醇脱氢酶 II，或来自运动发酵单胞菌的含铁醇脱氢酶（参见例如，GenBank 检索号 M15394 ;Conway 等人， 1987, J. Bacteriol., 169 :2591-2597)，来自鼠链球菌的醇脱氢酶，来自小肠共生菌 (*Commensalibacter intestine*) 的含铁醇脱氢酶，来自棕色固氮菌 (*Azotobacter vinelandii*) 的含铁醇脱氢酶，来自肠杆菌属 (*Enterobacteriaceae*) 细菌的含铁醇脱氢酶、来自荧光假单胞菌 (*Pseudomonas fluorescens*) 的醇脱氢酶，来自水稻基腐细菌 (*Dickeya zeae*) 的含铁醇脱氢酶，来自奇异变形杆菌 (*Proteus mirabilis*) 的醇脱氢酶，来自铁还原红育菌 (*Rhodoferax ferriredcuens*) 的含铁醇脱氢酶，来自深红红螺菌 (*Rhodospirillum rubrum*) 的含铁醇脱氢酶，来自油菜假单胞菌 (*Pseudomonas brassicacearum*) 的醇脱氢酶，来自丁香假单胞菌 (*Pseudomonas syringae*) 的醇脱氢酶 II，来自 *Dickeya dadantii* 的醇脱氢酶，来自鼠柠檬酸杆菌 (*Citrobacter rodenitium*) 的醇脱氢酶，来自腐败希瓦氏菌 (*Shewanella putrefaciens*) 的含铁醇脱氢酶，来自黑美人弧菌 (*Vibrio nigripulchritudo*) 的醇脱氢酶，来自产气肠杆菌 (*Enterobacter aerogenes*) 的醇脱氢酶，来自萨氏假单胞菌 (*Pseudomonas savastanoi*) 的醇脱氢酶，来自肠沙门氏菌 (*Salmonella enterica*) 的醇脱氢酶，来自鳆发光杆菌 (*Photobacterium leiognathi*) 的含铁醇脱氢酶，来自美人鱼发光杆菌 (*Photobacterium damselae*) 的醇脱氢酶，来自嗜线虫致病杆菌 (*Xenorhabdus nematophila*) 的醇脱氢酶，来自伯氏致病杆菌 (*Xenorhabdus bovienii*) 的醇脱氢酶，来自虫媒假单胞菌 (*Pseudomonas entomophila*) 的醇脱氢酶 II，来自藤黄希瓦氏菌 (*Shewanella vilacea*) 的醇脱氢酶 II，来自锡那罗州弧菌 (*Vibrio sinaloensis*) 的醇脱氢酶，来自 *Shewanella pealeana* 的醇脱氢酶，来自短尾蛛弧菌 (*Vibrio angustum*) 的醇脱氢酶，来自迟缓爱德华氏菌 (*Edwardsiella tarda*) 的醇脱氢酶，来自邦戈尔沙门氏菌 (*Salmonella bongori*) 的醇脱氢酶，来自阿氏肠杆菌 (*Enterobacter asburiae*) 的含铁醇脱氢酶，来自大肠杆菌 (*Escherichia coli*) 的醇脱氢酶，来自副溶血性弧菌 (*Vibrio parahaemolyticus*) 的醇脱氢酶 4，来自灿烂弧菌 (*Vibrio splendidus*) 的醇脱氢酶。在本发明的一个实施方案中，编码细菌醇脱氢酶或含铁醇脱氢酶的多核苷酸具有与运动发酵单胞菌醇脱氢酶 B 至少约 60、65、75、80、90、95、98、99 或 100% （或约 65%-100% 之间的任何范围）的同源性。

[0045] 此外，ADH- 编码多核苷酸可来源于变形链球菌，使得 ADH- 编码多核苷酸与天然变形链球菌 *adh* 基因的组合引入提供变形链球菌基因组中的 ADH- 编码多核苷酸的多个拷贝。或者，重组 ADH 多核苷酸可通过将突变引入变形链球菌 a*dh* 基因的调节机制中，以上调 ADH（例如，启动子中的突变以提供增加的 *adh* 基因转录）的产生而生成。

[0046] *adh* 多核苷酸可使用熟知的重组技术引入本发明的变形链球菌菌株中，例如，用编码 ADH 多肽的多核苷酸转化变形链球菌菌株。转化或转化作用意指变形链球菌具有整合到其基因组中的非天然核酸序列或作为通过多次传代而维持的质粒。*adh* 多核苷酸表达功能性 ADH 多肽，以使本发明的变形链球菌菌株尽管乳酸表达失活，但仍为可存活的。

[0047] 用于多核苷酸（例如编码 ADH 的多核苷酸）的鉴定、克隆、稳定转化和表达的方法是本领域常规的和熟知的。参见，例如 Sambrook 等人， 1989, 分子克隆 :实验室手册 (Molecular Cloning :A Laboratory Manual), 第二版, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.)。例如，编码 ADH 的多核苷酸的分离可通过分子自基因组 DNA 或自先前存在的基因克隆的 PCR 扩增来进行。重组 ADH 的表达可通过将 *adh* 结构

多核苷酸与促进在变形链球菌中的表达的启动子（例如，*spaP* 或天然 *ldh* 启动子）有效连接来实现。

[0048] 功能性 ADH 的产生可通过例如使用本领域熟知的常规 ADH 活性测定（例如，对乙醇的 NAD- 依赖性氧化的测定）来测定 (Neal 等人， 1986, Eur. J. Biochem.， 154：119-124)。Hillman 等人构建了表达功能性重组 ADH 的变形链球菌菌株。参见例如，Hillman 等人， Infect. Immun. 68：543 (2000)。

[0049] 营养缺陷型

本发明的重组变形链球菌菌株可任选地经基因工程改造为对通常不存在于口腔或宿主饮食中的有机物质而言是营养缺陷型的，使得可控制重组变形链球菌菌株的口腔定居。即，重组变形链球菌菌株可任选地经基因工程改造，以使它们不能合成生长所需的特定有机化合物。例如，本发明的菌株对于 D- 氨基酸，例如 D- 丙氨酸而言可以是营养缺陷型。营养缺陷型菌株的定居则可通过调节口腔中的有机物质的量来控制。例如，通过周期性地向口腔提供有机化合物可促进定居并通过阻止给予口腔该有机物质而终止定居。

[0050] 例如，哺乳动物口腔或饮食中通常不产生 D- 丙氨酸或不以超过痕量存在。因此，如果本发明的重组变形链球菌对于 D- 丙氨酸是营养缺陷型的，则需要周期性地将 D- 丙氨酸递送至哺乳动物的口腔，以维持本发明的重组变形链球菌在口腔中定居。在没有 D- 丙氨酸递送至至口腔时，本发明的重组变形链球菌菌株将最终消失。

[0051] 在本发明的一个实施方案中，重组变形链球菌是丙氨酸消旋酶缺乏的。丙氨酸消旋酶对于 D- 丙氨酸代谢是必需的。″丙氨酸消旋酶缺乏″或″丙氨酸消旋酶产生的缺乏″意指相对于野生型变形链球菌，重组变形链球菌菌株产生显著降低量的丙氨酸消旋酶。显著降低量的丙氨酸消旋酶是比由野生型变形链球菌菌株产生的丙氨酸消旋酶少约 40、50、60、70、80、90、95 或 100% （或约 40%- 约 100% 之间的任何范围）。在本发明的一个实施方案中，丙氨酸消旋酶缺乏的重组变形链球菌菌株产生不可检测的丙氨酸消旋酶。丙氨酸消旋酶可如在例如，Wantanabe 等人， J. Biochem. 126：781 (1999) 中所述测定。

[0052] 失活或部分失活（其使多核苷酸、基因、多肽或蛋白为非功能性的或部分功能性的）可通过方法例如在涉及丙氨酸消旋酶合成的基因中掺入突变（例如，点突变、移码突变、取代、缺失（部分或完整信号、区域或结构多核苷酸）、间断和 / 或插入而实现。涉及丙氨酸消旋酶合成的多核苷酸的突变可影响丙氨酸消旋酶的表达，以使丙氨酸消旋酶的表达量在与野生型变形链球菌比较时减少约 20、30、40、50、60、70、80、90、95% 或更多。

[0053] 例如，丙氨酸消旋酶表达的失活或部分失活可例如通过缺失部分或所有的 *dal* 结构多核苷酸或部分或完整的 *dal* 启动子使 *dal* 基因失活或部分失活来实现。

[0054] 细菌营养缺陷型可使用多种本领域熟知的技术，例如化学诱变、自发突变体的选择，和 / 或重组体技术（例如转座子诱变、通过用缺陷的或非功能性的基因重组替代）生成。例如，D- 丙氨酸营养缺陷型的变形链球菌菌株可通过将缺陷引入编码丙氨酸消旋酶 (*dal*)（该酶将 L- 丙氨酸转化为 D- 丙氨酸）的基因而生成。已生成这样的菌株。参见，例如 Hillman 等人， J. Appl. Microbiol. 102：1209-1219 (2007)。

[0055] ComE 缺乏

任选地，本发明的重组变形链球菌菌株可包含失活的或非功能性 *comE* 基因。具有失活的或非功能性 *comE* 基因的菌株将不太易于转化，因为 ComE 在环境 DNA 的摄取中是重要的。

此外，*comE* 不能被补充。

[0056] “ComE 缺乏”或"ComE 产生的缺乏”意指相对于野生型变形链球菌，重组变形链球菌菌株产生显著降低量的 ComE 蛋白。ComE 的显著降低量是比由野生型变形链球菌菌株产生的 ComE 蛋白少约 40、50、60、70、80、90、95 或 100% （或约 40%- 约 100% 之间的任何范围）。在本发明的一个实施方案中，缺乏 ComE 的重组变形链球菌菌株产生不可检测的 ComE 蛋白。ComE 表达可如例如在 Chen & Gotschlich, J. Bact. 183 :3160 (2001) 中所述测定。

[0057] 作为 ComE 合成中非功能性的、失活的、部分功能性的或部分失活的调控区、翻译信号、转录信号或结构序列的结果，本发明的重组变形链球菌菌株可以是 ComE 缺乏的。

[0058] 失活或部分失活（其使得多核苷酸、基因、多肽或蛋白为非功能性的或部分功能性的）包括方法例如在涉及 ComE 合成的多核苷酸中掺入突变（例如，点突变、移码突变、取代、缺失（部分或完整信号、区域或结构多核苷酸）、间断和 / 或插入）。涉及 ComE 合成的多核苷酸的突变可影响 ComE 的表达，以使 ComE 的表达量在与野生型变形链球菌菌株比较时减少约 20、30、40、50、60、70、80、90、95% 或更多。

[0059] 例如，ComE 表达的失活或部分失活可例如通过缺失部分或完整 *comE* 结构基因或部分或完整 *comE* 启动子，使 *comE* 基因失活或部分失活而实现。或者，涉及 DNA 摄取的其它基因例如 *comA*、*comB*、*omC* 和 *comD* 也可以是失活的或部分失活的。

[0060] ComE 合成的缺陷可通过诱变（即，使细菌暴露于诱变剂）、自发突变体的选择、或使用重组体技术的基因操纵来引入。已构建具有突变的 *comE* 基因的变形链球菌菌株。参见，例如 Hillman 等人， J. Appl. Microbiol. 102 :1209-1219 (2007)。

[0061] 多核苷酸

本发明的多核苷酸含有少于整个的微生物基因组并且可以是单链或双链核酸。多核苷酸可以是 RNA、DNA、cDNA、基因组 DNA、化学合成的 RNA 或 DNA，或其组合。多核苷酸可以是纯化的，没有其它成分，例如蛋白、脂质和其它多核苷酸。例如，多核苷酸可以是 50%、75%、90%、95%、96%、97%、98%、99% 或 100% 纯化的。在例如 cDNA 或基因组文库，或含有基因组 DNA 限制酶消化片段的凝胶切片中的数百至数百万其它核酸分子中存在的核酸分子不认为是分离的多核苷酸。

[0062] 本发明的多核苷酸编码上述的本发明多肽（例如，MU1140 多肽、ADH 多肽、ComE 多肽、D- 氨基酸合成多肽和乳酸合成多肽）。在本发明的一个实施方案中，多核苷酸编码示于 SEQ ID NOs :20-27 中的变体变异菌素 1140 多肽、其组合或其片段。在本发明的一个实施方案中，效应菌株可另外表达 *lanB*、*lanC*、*lanE*、*lanF*、*lanG*、*lanK*、*lanM*、*lanP*、*lanR*、*lanT* 或这些变形链球菌多核苷酸的两个或更多个的组合。

[0063] 本发明的多核苷酸可由少于约 600、500、400、300、200、100、66、60、50、45、30、15 个（或约 600-15 之间的任何范围）相邻多核苷酸组成。纯化的多核苷酸可包含另外的异源性核苷酸和 / 或另外的同源性多核苷酸。本发明的多核苷酸可包含其它核苷酸序列，例如编码以下的序列 :连接基、信号序列、TMR 终止转移序列、跨膜域，或用于蛋白纯化的配体例如谷胱甘肽 -S- 转移酶、组氨酸标签和葡萄球菌蛋白 A。本发明的一个实施方案提供包含编码 SEQ ID NOs :20-27 的至少约 6、10、15、20、25、30、40、45、50、60、66 或更多个相邻核苷酸的纯化多核苷酸。

[0064] 可分离本发明的多核苷酸。分离的多核苷酸是天然存在的多核苷酸，其与天然关联的 5’和 3’侧翼基因组序列之一或二者并不是紧邻的。分离的多核苷酸可以是，例如任何长度的重组 DNA 分子。分离的多核苷酸也包括非天然存在的核酸分子。本发明的多核苷酸可编码全长多肽、多肽片段和变体或融合多肽。

[0065] 本发明的编码多肽的简并核苷酸序列以及与本发明的多核苷酸序列至少约 80，或约 90、95、96、97、98 或 99% 相同的同源性核苷酸序列及其互补序列也是本发明的多核苷酸。简并核苷酸序列是编码本发明的多肽或其片段的多核苷酸，但由于遗传密码的简并性，核酸序列不同于给定的多核苷酸序列。

[0066] 序列同一性百分比具有本领域公认的含义并且有许多测量两个多肽或多核苷酸序列之间的同一性的方法。参见，例如 Lesk, Ed., *Computational Molecular Biology*, Oxford University Press, New York, (1988) ;Smith, Ed., *Biocomputing :Informatics And Genome Projects*, Academic Press, New York, (1993) ;Griffin & Griffin, Eds., 序列数据的计算机分析 (*Computer Analysis Of Sequence Data*), 部分 I, Humana Press, New Jersey, (1994) ;von Heinje, 分子生物学的序列分析 (*Sequence Analysis In Molecular Biology*), Academic Press, (1987) ;和 Gribskov & Devereux, Eds., 序列分析引物 (*Sequence Analysis Primer*), M Stockton Press, New York, (1991)。用于对比多核苷酸或多肽的方法已被编程为计算机程序，包括 GCG 程序包 (Devereux 等人 (1984) *Nuc. Acids Res.* 12 :387), BLASTP, BLASTN, FASTA (Atschul 等人 (1990) *J. Molec. Biol.* 215 :403)，和 Bestfit 程序 (Wisconsin 序列分析包， 用于 Unix 的版本 8, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711)，其使用 Smith 和 Waterman 的局部同源性算法 ((1981) *Adv. App. Math.*, 2 : 482-489)。例如，可使用采用 FASTA 算法的计算机程序 ALIGN，对于仿射空位搜索 (affine gap search) 使用为 -12 的空位开放罚分 (gap open penalty) 和为 -2 的空位延伸罚分 (gap extension penalty)。

[0067] 当使用任何序列对比程序以确定特定序列，例如，是否与参照序列具有约 95% 同一性时，设定参数以便在参照多核苷酸的全长内计算同一性百分比并允许参照多核苷酸的核苷酸总数的至多 5% 的同一性空位。

[0068] 本发明的多核苷酸可从存在于例如细菌样品中的核酸序列中分离。多核苷酸也可在实验室中，例如，使用自动合成仪合成。扩增方法例如 PCR 可用于从基因组 DNA 或者编码多肽的 cDNA 扩增多核苷酸。

[0069] 本发明的多核苷酸可包含天然存在的多肽的编码序列或可编码非天然存在的改变的序列。如果需要，可将多核苷酸克隆到包含表达控制元件 (包括例如，复制起点、启动子、增强子或在宿主细胞中驱动本发明的多核苷酸表达的其它调控元件) 的表达载体中。表达载体可以是例如质粒。也可使用微染色体 (Minichromosomes) 例如 MC 和 MC1、噬菌体、噬菌粒、酵母人工染色体、细菌人工染色体、病毒颗粒、病毒 - 样颗粒、粘粒 (已插入噬菌粒 λ*cos* 位点的质粒) 和复制子 (能够在其自身控制下在细胞中复制的遗传元件)。

[0070] 制备有效连接于表达控制序列的多核苷酸和在宿主细胞中表达它们的方法为本领域熟知的。参见，例如美国专利号 4,366,246。当本发明的多核苷酸位于邻接或接近于一个或多个指导多核苷酸的转录和 / 或翻译的表达控制元件时，所述多核苷酸是有效连接

的。

[0071] 包含本发明的重组变形链球菌的组合物

本发明的重组变形链球菌菌株的特征可在于 :1) 乳酸缺乏,和 2) 重组 ADH 的产生,3) 变体 MU1140 产生,4) 任选地,对于特定有机物质 (例如, D- 氨基酸例如 D- 丙氨酸) 为缺陷营养型,5) 任选地,ComE 表达的缺乏,或其组合。

[0072] 本发明的组合物可包含一或多株如本文所述的重组变形链球菌菌株和药学上可接受的或营养学上可接受的载体。载体是与其给予的受试者的区域在生理学上相容的。载体可包含用于配制成片剂、胶囊、锭剂,或粉末形式的固体 - 基的干燥材料。载体也可包含用于配制成液体、凝胶和口香糖形式的液体或凝胶基材料。载体的组成可以是变化的,只要它不显著干扰本发明的细菌菌株的治疗活性。

[0073] 可将组合物配制为适合以各种方式经口给予,例如以固体、半 - 固体、液体 (包括,例如粘性液体、糊剂、凝胶或溶液剂)、干燥团块 (mass)、洁齿剂、漱口剂、口腔清洗剂、液体混悬剂、饮料、局部施用剂、粉末状食品补充剂、糊剂、凝胶、固体食品、口腔清洗剂、包装食品、薄片 (wafer)、锭剂、口香糖等。其它制剂对本领域技术人员而言将是显然的。本发明的组合物可包含营养补充成分并可包含如所熟知的多种营养剂的任何一种,包括维生素、矿物质、必需和非必需氨基酸、碳水化合物、脂质、食品、膳食补充剂等。

[0074] 本发明的组合物也可包括天然或合成的调味剂和食品级着色剂,所有这些均与维持本发明细菌菌株的生存力相容

本发明的组合物可包含一或多种胶凝剂,其可用作粘合剂,以使组合物粘附至牙齿或口腔。胶凝剂的浓度可大于约 2、4、6、8、10、15、20、30、40、50、60、70、80 或少于约 80、70、60、50、40、30 或 20% 重量的组合物。

[0075] 用于本发明的合适胶凝剂和粘合剂包括,例如,硅酮、聚环氧乙烷、聚乙烯醇、聚烷基乙烯基醚 - 马来酸共聚物 (PVM/MA 共聚物) 例如 Gantrez AN 119、AN 139 和 S-97、聚乙烯醇、聚丙烯酸、泊洛沙姆 407 (Pluronic)、聚乙烯吡咯烷酮 - 乙烯乙酸酯共聚物 (PVP/VA 共聚物),例如 Luviskol VA 和 Plasdone S PVP/VA、聚乙烯基吡咯烷酮 (PVP,例如 K-15 至 K-120)、聚季胺 (Polyquaterium)-11 (Gafquat 755N)、聚季胺 -39 (Merquat plus 3330)、卡波姆或聚羧乙烯 (Carbopol)、羟丙基甲基纤维素、羟乙基纤维素、羟丙基纤维素、玉米淀粉、羧甲基纤维素、明胶和藻酸盐例如藻酸钠、天然树胶例如刺梧桐胶、黄原胶、瓜尔胶、阿拉伯胶、黄蓍胶及其混合物。

[0076] 湿润剂或增塑剂可存在于本发明的组合物中。湿润剂或增塑剂包括,例如,甘油、丙三醇、山梨醇、聚乙二醇、聚丙二醇和其它可食用多元醇。湿润剂或增塑剂可以组合物的约 1%- 约 99%、约 10%- 约 95% 或以约 50%- 约 80% 之间 (或 1%-99% 之间的任何范围) 重量存在。

[0077] 本发明的细菌可在例如发酵罐中制备。可从发酵罐中收获细菌并可例如浓缩。本发明的细菌可通过例如脱水、空气干燥、冷冻干燥、冷冻和喷雾干燥制备以便使用。细菌也可通过微囊化 (参见例如,美国专利号 6,251,478) 或通过使用保护性物质例如脂质材料例如三酰基甘油、蜡、有机酯、豆油、棉籽油、棕榈仁油和长链脂肪酸与醇的酯包被来制备以便使用。在本发明的一个实施方案中,本发明的包被或包封的细菌在宿主口腔中释放。

[0078] 牙洞的治疗和预防方法

本发明的重组变形链球菌可以治疗有效量存在于本发明的组合物中。治疗有效的意指有效预防或减少牙洞的数目或发生率（例如，比未接受组合物的对照少 5、10、20、30、40、50、60、70、80、90 或 100% 的牙洞）和 / 或降低严重性（例如，比未接受组合物的对照少 5、10、20、30、40、50、60、70、80、90 或 100% 的严重牙洞）。

[0079] 治疗有效量或剂量是足以预防龋齿和 / 或减少龋齿数目和 / 或龋齿严重性的高水平，但低至足以避免严重副作用（以合理的利益 / 风险比），在合理的医学 / 牙科判断范围内的本发明组合物的量或剂量。本发明组合物的治疗有效量或剂量可随着待治疗的特定病症，待治疗患者的年龄和身体条件，病症的严重性，治疗持续时间，联合治疗的性质，所用来源的具体形式和组合物所用的具体溶媒而变化。

[0080] 本发明的组合物可以治疗有效量施用于宿主的口腔，用于治疗和 / 或预防牙洞。本发明的组合物可被吞咽或可在整个口腔中清洗，然后吐出，以使其基本上不递送至胃肠道。即，少于约 10、5、4、3、2 或 1、0.5 或 0.1% （或约 10-0.1% 之间的任何范围或值）的递送细菌被递送至胃肠道。治疗意指引起牙洞的数量或强度（或其组合）的减少。

[0081] 预防意指在宿主永久地（只要本发明的细菌以足够数目保留在受试者的口腔中）或暂时地（例如，约 1、2、3、4、5、6 或更多个月）暴露于一个或多个本发明的重组变形链球菌菌株后，基本上没有龋齿发生。本发明的细菌菌株可构成口腔的至少部分暂时或本土菌群并呈现有益的预防和 / 或治疗效果。

[0082] 治疗意指减少野生型变形链球菌在宿主口腔的量，以使小龋损害的再矿化可以发生并终止或减缓对大龋损害的进一步损害。口腔中的野生型变形链球菌的量可减少约 20、30、40、50、60、70、80、90 或 100% （或约 10-100% 之间的任何范围）。

[0083] 在本发明的一个实施方案中，预防意指在受试者群体中的预防。即在给定的受试者群体中，与未接受治疗的对照群体比较，治疗可预防约 5、10、20、30、40、50、60、70、80、90% 或更多受试者的龋齿。

[0084] 在本发明的一个实施方案中，组合物可包含一个或多个本发明的分离的重组菌株以及一个或多个分离的口腔链球菌（*Streptococcus oralis*）菌株和 / 或一个或多个分离的乳房链球菌（*Streptococcus uberis*）菌株。

[0085] 口腔链球菌（以前称为血链球菌（*Streptococcus sanguis*） II 型）和乳房链球菌在维持构成牙周菌群的微生物的正常、健康平衡方面是重要组分。参见，Socransky 等人，*Oral Microbiol. Immunol.* 3 :1-7 (1988) ;Hillman 和 Shivers, *Arch. Oral. Biol.*, 33 :395-401 (1988) ;Hillman 等人，*Arch. Oral. Biol.*, 30 :791-795 (1985)。口腔链球菌产生过氧化氢，其可抑制牙周病原体例如伴放线菌放线杆菌（*Actinobacillus actinomycetemcomitans*）(Aa)、福赛斯拟杆菌（*Bacteroides forsythus*）和中间普氏菌（*P. intermedia*）。因此，口腔链球菌和乳房链球菌可用于维持口腔健康。本发明的组合物可包含一个或多个分离的口腔链球菌菌株，例如 ATCC 35037、ATCC 55229、ATCC 700233、ATCC 700234 和 ATCC 9811。其它口腔链球菌菌株包括 KJ3 和 KJ3sm。KJ3sm 是天然存在的抵抗链霉素的 KJ3 遗传变体。链霉素抗性是有利的，因为其提供容易分离细菌的标记物。此外，链霉素抗性株是稍微减毒且不如野生株在口腔中存活那么长。当目标是用细菌非持久地定居于动物的口腔时，这种特性是有用的。

[0086] 已发现牙菌斑中的乳房链球菌与牙周健康有关，特别是通过干扰牙周病原体例如

牙龈卟啉单胞菌 (*Porphyromonas gingivalis*)、直肠弯曲菌 (*Campylocbacter recta*) 和啮蚀艾肯菌 (*Eikenella corrodens*) 的定居。本发明的组合物可包含一个或多个分离的乳房链球菌菌株，例如 ATCC 13386、ATCC 13387、ATCC 19435、ATCC 27958、ATCC 35648、ATCC 700407、ATCC 9927、KJ2 株或 KJ2sm 株。KJ2sm 是 KJ2 天然存在的遗传变体。其是链霉素抵抗的并提供与口腔链球菌的链霉素 - 抗性株相同的优点。一个或多个分离的口腔链球菌菌株或一个或多个分离的乳房链球菌菌株，或者二者可用于本发明的组合物和方法。本发明的这些组合物的另外口腔护理益处包括，例如，治疗和 / 或预防受试者的牙周炎、口腔细菌感染和疾病、口腔创伤、假丝酵母 (*Candida*) 或真菌过度生长、口臭或口腔干燥 - 引起的龋齿和相关的牙周疾病，促进伤口愈合、牙齿美白或其组合。

[0087] 本发明的一个实施方案提供用于治疗龋齿的方法，其包括向有需要的受试者的口腔给予包含一个或多个本发明的重组变形链球菌菌株的组合物。即，受试者具有一个或多个龋齿。

[0088] 本发明的一个实施方案提供对正常、健康的受试者中龋齿的预防。本发明的另一个实施方案提供对患有增加的龋齿易感性的受试者 (与对正常、健康的受试者比较) 中龋齿的治疗和 / 或预防。在这两个实施方案中，该方法由以下组成 :向受试者的口腔给予包含一个或多个重组变形链球菌菌株的组合物。

[0089] 当受试者比正常、健康的宿主更可能发生龋齿时，他们具有增加的对龋齿的易感性。这样的宿主可例如具有降低的唾液产生 (例如，在头或颈部经历放射疗法的患者、患有干燥综合征 (Sjögren's syndrome)、糖尿病、胃 - 食管反流疾病、尿崩症，或类肉瘤病的患者、服用抗组胺剂和抗抑郁剂或引起“口干”的其它药物的患者)、吸烟者、无烟烟草使用者 (smokeless tobacco users)、具有遗传倾向的患者 (Shuler、J. Dent. Ed. 65 :1038 (2001))，或为婴儿 (0-2 岁龄或 6 月至 2 岁龄)、儿童 (3 岁至 18 岁龄) 或老年人 (大于 65 岁)。

[0090] 本发明也提供在受试者中减少可引起龋齿的细菌量的方法。该方法包括向具有可引起龋齿的细菌的一个或多个菌株或种类的受试者的口腔给予包含一个或多个本发明的重组变形链球菌菌株的组合物。可仅给予组合物一次或定期给予。减少了受试者中可引起龋齿的细菌的一个或多个菌株或种类的数目。所述减少可以是数目的约 5、10、25、50、75、90、95、99 或 100% (或约 5%- 约 100% 之间的任何范围) 的减少。

[0091] 任选地，在给予本发明的组合物之前，可使用本领域已知的任何检测 / 定量方法检测可引起龋齿的一或多种细菌和 / 或对其定量。本领域技术人员知道检测可引起龋齿的细菌的方法。任选地，在给予本发明的组合物之前，可使用本领域已知的任何方法，在受试者中诊断一个或多个龋齿。

[0092] 本发明的另一个实施方案提供预防受试者的龋齿的方法。该方法包括获得关于在特定类型的受试者中预防龋齿的治疗有效的剂量范围和确定用于特定类型的受试者的重组变形链球菌的有效剂量范围的数据。特定类型的受试者可以是例如，具有降低唾液产生的受试者 (例如，在头或颈部经历放射疗法的患者、患有干燥综合征、糖尿病、胃 - 食管反流疾病、尿崩症，或类肉瘤病的患者、服用抗组胺剂和抗抑郁剂或引起“口干”的其它药物的患者)、吸烟者、无烟烟草使用者、具有遗传倾向的患者)，或为婴儿 (0-2 岁龄或 6 月至 2 岁龄)、儿童 (3 岁至 18 岁龄) 或老年人 (大于 65 岁)。对于特定类型的受试者，将确定的治

疗有效的剂量范围的一个或多个本发明的重组变形链球菌菌株给予特定类型的受试者的口腔。

[0093] 可将组合物给予宿主或受试者例如动物的口腔，所述动物包括哺乳动物，例如人、非人灵长类动物、狗、猫、马、牛、山羊或兔。

[0094] 本发明的组合物可以例如食品、水、洁齿剂、凝胶、糊剂、乳剂、气溶胶喷雾剂、口香糖、锭剂、片剂、胶囊或液体混悬剂经口给予。细菌可以已经被配制到食品、水、凝胶或其它载体中或者可以是由用户在消费之前加入到载体（例如，食品、水、洁齿剂、凝胶、糊剂、乳剂、气溶胶喷雾剂或液体混悬剂）中的组合物（例如，粉末剂、片剂或胶囊）。

[0095] 本发明的一个实施方案提供用治疗有效的细菌非永久地定居受试者的口腔的方法，其包括给予受试者的口腔本发明的组合物。在本发明的一个实施方案中，给予的细菌菌株并非永久地定居于口腔，而是在给予细菌后，所述菌株在口腔中存在约 1 天、约 1 周、约 2 周、约 3 周、约 1 个月、约 3 个月或约 12 个月。

[0096] 在本发明的另一个实施方案中，变形链球菌的重组菌株永久地定居于宿主的口腔达一段长的时期，例如 2 周、1 个月、3 个月、6 个月、1 年、5 年或更长或宿主终生。

[0097] 本发明的组合物可以约 $1x10^3$、$1x10^5$、$1x10^7$、$1x10^8$、$1x10^9$ 或 $1x10^{11}$ CFU（或约 $1x10^3$- 约 $1x10^{11}$ 之间的任何范围或值）活菌的剂量给予。本发明组合物的剂量可以一天 4 次、一天 3 次、一天 2 次、一天 1 次、每隔一天 1 次、一周两次、每周 1 次、每两周 1 次、每月 1 次或每年 1 次给予。本发明的组合物的 1 个、2 个或更多个剂量可每天给予，持续约 1 天、约 1 周、约 2 周、约 1 个月、约 2 个月、约 3 个月、约一年或更长。在本发明的一个实施方案中，本发明的组合物给予 1 次并在一段长的时期内有效。

[0098] 本发明的组合物可包含组合物重量的约 0.01%- 约 50% 之间的浓度，或约 0.1%- 约 25%，或约 1.0%- 约 10% 或 0.01%-50% 之间的任何范围或值的细菌菌株。

[0099] 本发明的药盒可含有单一剂量、1 周、1 个月、2 个月、3 个月、4 个月、5 个月、6 个月，或 12 个月供应量的本发明的组合物。可包装本发明的组合物，并且多个包装的组合物可继而在贮藏容器或外包装或纸板箱中提供。当一个或多个变形链球菌菌株为营养缺陷型时，药盒可包括细菌营养缺陷型 - 维持量的有机物质，例如包含 D- 氨基酸例如 D- 丙氨酸的组合物。

[0100] 当本发明的组合物包含对有机物质为营养缺陷型的一个或多个变形链球菌菌株时，可将细菌营养缺陷型 - 维持量的有机物质给予宿主，以维持口腔中的重组变形链球菌。″细菌营养缺陷型 - 维持量″是足以维持口腔中的重组变形链球菌营养缺陷型的生存力的有机物质的量。例如，当重组变形链球菌对 D- 丙氨酸为营养缺陷型时，D- 丙氨酸细菌营养缺陷型 - 维持量是足以使 D- 丙氨酸营养缺陷型菌株在宿主口腔中存活的 D- 丙氨酸的量。一般来说，D- 丙氨酸的 D- 丙氨酸细菌营养缺陷型 - 维持量的单一剂量含有约 1、5、10、20、25、50、75 或 100 mg（或约 1- 约 100 mg 之间的任何范围）。在以溶液形式的组合物中，D- 丙氨酸浓度为约 0.01、1、10、25、50、75、100 或 167 mg/ml（后者是在 25℃时 D- 丙氨酸在水中的饱和溶液）（或约 0.01- 约 167 mg/ml 之间的任何范围）。组合物中 D- 丙氨酸浓度可根据所用的载体和 D- 丙氨酸在该特定载体中的饱和点而变化。

[0101] 维持口腔中的营养缺陷型的、重组变形链球菌所需的有机物质，例如 D- 丙氨酸可被配制为漱口剂、口香糖、牙线、牙膏、咀嚼片、食品、饮料或任何其它适合于经口给予宿主

口腔的制剂。除了有机物质（例如，D- 丙氨酸），组合物还可任选含有调味剂、着色剂、香料或其它增加组合物的适口性和 / 或提高患者的依从性而不削弱组合物中包含的有机物质的有效性的化合物。

[0102] 本文中任何地方提及的所有专利、专利申请和其它科学或技术文本，均通过引用以其整体结合到本文中。在本文中说明性阐述的本发明，适当地可在本文中未具体公开的任何一个或多个要素、限制不存在的情况下实施。因此，例如，在本文的各实例中，术语“包括”、“基本上由……组成”和“由……组成”的任何一个，可用另两个术语中的任一个替换，同时保留其通常含义。已采用的术语和表述是用作描述而非限制的术语，并且不意图在使用这类术语和表述时将所示和所述特征的任何等价物或其部分排除在外，但认识到的是，在要求保护的本发明的范围之内的多种修改是可能的。因此，应理解的是，尽管已通过实施方案具体公开本发明，但是本领域技术人员仍可采用本文所公开的构思的可选特征、修改和变动，并且认为所述修改和变动在通过说明书和随附权利要求所界定的本发明的范围之内。

[0103] 此外，当以马库什 (Markush) 群组或其它可选群组的方式阐述本发明的特征或方面时，本领域技术人员将认识到的是，本发明亦因此以马库什群组或其它群组的任何单个成员或成员亚群的形式来阐述。

[0104] 提供以下仅用于举例说明目的，而非意图限制在上文中以广义术语描述的本发明的范围。

实施例

[0105] 实施例 1 :MU1140 的诱变

将变形链球菌基因组数据库和 *lan* 基因簇 (GenBank/EMBL 检索号 (AF051560)) 用于设计诱变和测序工作的引物。将天然 MU1140 结构基因 (*lanA*) 的开放阅读框 (ORF) 加上 5’和 3’侧翼 DNA 的 500 个碱基对 (bp) 克隆到 pVA891 质粒以构建 p190。克隆到 p190 中的插入片段，通过使用引物序列 SRWlanA_1 和 SRWlanA_2 （见图 3) 对变形链球菌菌株 JH1140 (ATCC 55676) 的染色体 DNA 进行 PCR 扩增来得到。试剂和培养基购自 Fisher Scientific，而酶购自 New England BioLabs (Ipswich, MA)。

[0106] 聚合酶链式反应 (PCR)

将突变（参见图 1B) 引入 *lanA* (MU1140 的结构基因）的前肽区，以构建 MU1140 的变体。参见图 2。将 p190 质粒 (J.D. Hillman，未公布）用作模板并使用两步 PCR 引入定点突变。在第一步中，使上游和下游外引物 (SRWlanA_1 和 SRWlanA_2) 与适当的内引物配对（例如 SRWlanA_1/Trp4Ala_2 和 SRWlanA_2/Trp4Ala_1) （图 3)，所述内引物之一合成为相对于野生型序列含有改变的碱基序列。此步骤的结果为产生两个片段，一个片段包含 5’侧翼 DNA 和 *lanA* 的一部分，其包含定点碱基改变。第二个片段包含 *lanA* 的剩余部分加上 3’侧翼 DNA。用于产生 MU1140 变体的引物见图 3。然后等量混合所述两个片段，并使其经历采用两个外引物 SRWlanA_1 和 SRWlanA_2 的第二轮 PCR，得到最终的扩增子。

[0107] 使用 Taq 聚合酶以 50 μL 的终体积进行 PCR 反应，其包含 0.4 μmol 各引物、50 ng 模板 DNA、0.016 mM dNTP 以及含 1 个单位 DNA 聚合酶的 1X 聚合酶缓冲液。用于各片段的扩增条件如下 :95℃预热 1 min，接着 27 个循环的变性 (95℃）孵育 30 秒，退火 (56℃） 30

秒并延伸（72℃） 2 min，接着最终延伸（72℃） 10 min。将两个片段以 50:50 合并，并使用两个外引物 SRWlanA_1 和 SRWlanA_2 在如上所提及的相同扩增条件下进行扩增。

[0108] 按照试剂盒说明书将最终 PCR 产物连接到 TOPO-TA 载体（Invitrogen, Carlsbad, CA），使用标准方法转化到 DH5α-T1® 细胞（Invitrogen），并涂布至含 50 μg/mL 氨苄西林和 40 μL X-gal（40mg/mL）的 LB 平板上。采用蓝白筛选来鉴定含插入片段的菌落。根据制造商说明书使用 PureYield Plasmid Miniprep System（Promega, Madison, WI）纯化来自各菌落的质粒 DNA。使纯化的质粒经历使用 *EcoRI* 的限制酶消化，并通过琼脂糖凝胶电泳检测以鉴定具有适当大小（约 1100 bp）的克隆插入片段的质粒。使用 M13 正向（-20）引物 5′-GTAAAACGACGGCCAG-3′（SEQ ID NO: 28）对含有适当大小插入片段的质粒测序，以确认核苷酸碱基的适当插入、缺失或置换。

[0109] 重组

对来自含有确认突变的菌落的纯化质粒进行限制酶消化。通过电泳将所述插入片段从 TOPO 质粒中分离，从凝胶中切下并使用 Qiagen Gel Extraction 试剂盒（Qiagen, Valencia, CA）纯化。然后使用 T4 DNA 连接酶以 3:1 的插入片段：载体比率将纯化的插入片段于 16℃过夜连接至变形链球菌自杀载体 pVA891。然后使用标准方法将所得质粒转化至 DH5α 细胞并涂布至含 300 μg/mL 红霉素的 LB 平板上。对培养之后出现的菌落进行分析，以验证如上所述的适当的插入片段大小及序列。

[0110] 如下将含有确认插入片段的纯化 pVA891 DNA 转化至变形链球菌菌株 JH1140（ATCC 55676）：使变形链球菌生长过夜并随后以 1:15 稀释到新鲜 THyex 肉汤（30 g/L THB、3 g/L 酵母提取物）中，将 200μL 稀释的细胞加至 96 孔板并于 37℃培养 2 小时。加入 2 微升感受态刺激肽（CSP，0.1μg/mL；参见例如 Li 等，J. Bacteriol. 183:897（2001）），并使平板再培养 6 小时。参见 Li 等，（2002）J. Bacteriol. 184:2699。然后将 50 微升细胞涂布至含 300 μg/mL 红霉素的预热 THyex 琼脂平板（30 g/L THB、3g/L 酵母提取物和 15g/L 营养琼脂）并于 37℃培养 48 小时。利用标准氯仿 / 酚提取法从出现的克隆中提取基因组 DNA，并将所述 DNA 用作使用 SRWlanA_1 和 SRWlanA_2 的 PCR 的模板，以鉴定异双倍体克隆，推测所述异双倍体克隆含有通过载体 DNA 分离的 *lanA* 基因的一个野生型拷贝和一个突变拷贝，如先前由 Hillman 等，（2000）Infect. Immun. 68:543-549 所述。

[0111] 确认突变构建体的遗传同一性

如下通过自发拆分异双倍体状态来获得含有所需 *lanA* 突变的克隆：使数个确认的异双倍体在不含红霉素的 20 mL THyex 肉汤中生长过夜。将培养物传代培养（1:20 稀释到新鲜培养基中）并再次生长过夜至饱和。然后将培养物稀释 100,000 倍，涂布至大型 THyex 琼脂平板上并于 37℃培养 48 小时。将所得菌落复制接种至含和不含红霉素的培养基上以鉴定其中发生 pVA891 质粒（表达红霉素抗性基因）以及野生型或突变 *lanA* 基因缺失的自发重组。用含和不含红霉素的培养基重新检验由复制平板技术鉴定的红霉素敏感菌落。通过如上所述的 PCR 扩增红霉素敏感克隆的 *lanA* 区。对所产生扩增子测序以鉴定仅具有修饰的 *lanA* 基因的克隆。使用 BLAST 序列分析将 *lanA* 的野生型序列与 *lanA* 的疑似突变体序列进行比较（图 2）。产生的突变体为 Trp4Ala、Trp4insAla、ΔTrp4、Dha5Ala、Ala_S7insAla 和 Arg13Asp。

[0112] 实施例 2：突变体的生物活性

使亲代变形链球菌菌株 JH1140 (ATCC 55676) 及突变体生长至 OD_{600} 0.8 并稀释成 OD_{600} 0.2。将培养物的样品 (2 μL) 一式三份印迹到预热的 THyex 琼脂平板 (150 X 15mm) 上并允许风干。以此方式进行该测定，以帮助确认各样品具有相同的菌落大小以用于比较抑菌圈。将平板于 37℃培养 24 小时，并随后置于 55℃烘箱中达 30 分钟以杀死细菌，然后以熔化的顶层琼脂形式覆盖藤黄微球菌 (*M. luteus*) ATCC 272 指示菌株。热杀灭细菌防止任何进一步的抗微生物化合物产生。使藤黄微球菌 ATCC 272 生长至 OD 600_{nm} 介于 0.4-0.8 之间并稀释成 OD 600_{nm} 0.2。然后将 400 μl 这些细胞加至 10 ml 熔化的顶层琼脂 (42℃) (30g/L Todd Hewitt 肉汤和 7.5g/L 营养琼脂)。向含有约 50 ml THyex 琼脂的各平板中加入全部 10 mL 的含标准化悬浮液的顶层琼脂。使平板凝固，然后倒置并于 37℃培养过夜。从菌落的一个边缘至抑菌圈的最远部分以 mm 测量各抑菌圈半径。对各抑菌圈计算抑菌圈的面积并与野生型 (n=10) 的平均抑菌圈面积进行比较。

[0113] 图 4 阐述了产生 MU1140 变体的菌株与产生野生型 MU1140 的菌株比较的生物活性。结果概述于图 5，其显示产生 Trp4insAla 和 ΔTrp4 的菌株与野生型相比具有的抑菌圈无显著差异 (斯氏 t 检验，$p>.05$)。产生 Arg13Asp 的菌株具有最大的抑菌圈面积，相对于野生型总计有 2.57 倍增加 ($p<.001$)。产生 Trp4Ala 和 Dha5Ala 的菌株，相对于野生型分别产生显著的 ($p<.001$) 2.12 倍和 1.87 倍增加。产生 Ala_S7insAla 的菌株具有最小的抑菌圈面积，与野生型相比，其在抑菌圈面积上总计有显著的 ($p<.001$)2 倍减少。图 6 显示产生其它 MU1140 变体 (Phe1Ile 和 Phe1Gly) 的菌株与野生型 MU1140 进行比较的生物活性。产生 Phe1Ile 和 Phe1Gly 的菌株，相对于野生型分别显示显著的 ($p<.001$) 1.82 倍和 1.57 倍增加。

[0114] 有许多研究使用乳酸链球菌肽 (nisin) 和某些其它羊毛硫抗生素 (由 Chatterjee 等人 (2005) Chem. Rev. 105 :633 综述) 的结构基因的位点定向诱变，以分析特定氨基酸在这些分子的活性中的重要性。这些突变很少导致增加的生物活性。

[0115] 对于 Arg13Asp 突变体，获得最引人关注的结果。当与野生型比较时，这种突变导致生物活性的意想不到的、高度显著性的增加。在此，在铰链区中用荷负电的残基替代荷正电的残基。这种结果违背常规信念，即羊毛硫抗生素的负电荷将减少生物活性，因为正电荷被认为有助于抗生素与存在于靶细胞膜中的带负电荷的脂质的相互作用。这种突变也从化合物除去胰蛋白酶可切割位点，从而使其对酶促水解更稳定。此外 Trp4Ala、Dha5Ala 和 Arg13Asp 为很可能不会天然发生的颠换突变。

[0116] 因此，考虑到现有技术，本文描述的对 MU1140 的突变是意想不到的和不可预测的，并导致与野生型 MU1140 相比已极大改善生物学和结构特征的变体 MU1140 分子。从改进变形链球菌效应菌株的定居潜力的观点而言，增加活性的突变是重要的。先前已表明变形链球菌菌株定居啮齿动物和人的口腔的能力与产生的 MU1140 的量和 / 或活性有关。此外，先前已表明变形链球菌菌株强有力地替代啮齿动物和人口腔中的变形链球菌本土株的能力与产生的 MU1140 的量和 / 或活性有关。参见例如，Hillman 等人、Infect. Immun. 44 : 141 (1984) ; Hillman 等人、J. Dent. Res. 66 :1092 (1987)。因此，与不表达本发明的变体 MU1140 的变形链球菌效应菌株比较，表达如本文所述的变体 MU1140 的本发明变形链球菌效应菌株具有意想不到的和改善的特征。即，相对于不表达如本文所述的变体 MU1140 的变形链球菌效应菌株，表达变体 MU1140 的变形链球菌效应菌株具有改进的定居和比宿主

口腔中天然变形链球菌强有力地更具竞争优势并将其替代的能力。

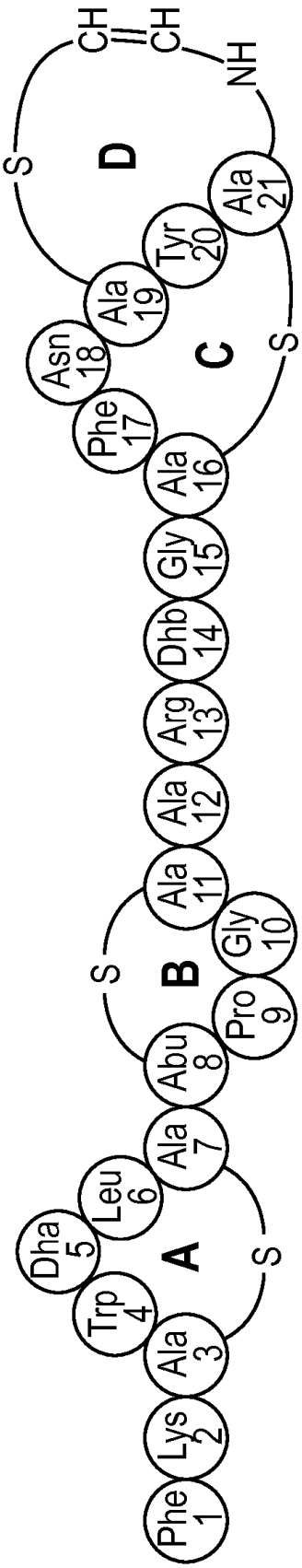
[0117] 实施例 3 :最小抑制浓度

将野生型变异菌素 1140、具有 F1l 突变的变异菌素 1140、具有 W4A 突变的变异菌素 1140 和具有 R13D 突变的变异菌素 1140 纯化至约 90% 纯度（经由 HPLC 测定）。针对数种细菌确定 MU1140 和 MU1140 变体的最小抑制浓度 (MIC)。MIC 为在 24 小时培养之后抑制可见的微生物生长的 MU1140 的最低浓度。越低的 MIC 表明越大的抑制活性。用于最小抑制浓度 (MIC) 的抗微生物剂和细菌接种物的制备，通过遵循具有某些小改动的 Clinical Laboratory Standard Institute (CLSI) M07-8A 中所述的方法进行。在振荡培养箱中过夜测试变形链球菌 UA159 以维持细菌的均匀分布。在厌氧培养室中于 37℃测试艰难梭菌 UK1。使用的培养基为 THyex。结果在表 1 中显示。

[0118] 表 1

MU1140 变体	变形链球菌 UA159	肺炎链球菌 FA1	金黄色葡萄球菌 FA1	藤黄微球菌 ATCC10240	艰难梭菌 UK1
Mu114 野生型	2	0.5	16	0.0625	16
Mu1140 F1I	2	0.25	8	0.0156	8
Mu1140W4A	2	0.125	16	0.0312	8
Mu1140R13D	2	4	>16	0.125	16

虽然对于各突变体而言，各生物的 MIC 未必更低，但各突变体相对于野生型 MU1140 仍具有优势，因为除了其它有利特性之外，其还可例如更易于生产、更易于运输、具有更好的贮存稳定性、具有更好的血清稳定性或者具有更好的蛋白水解稳定性。



野生型(天然) **MU1140**

图 1A

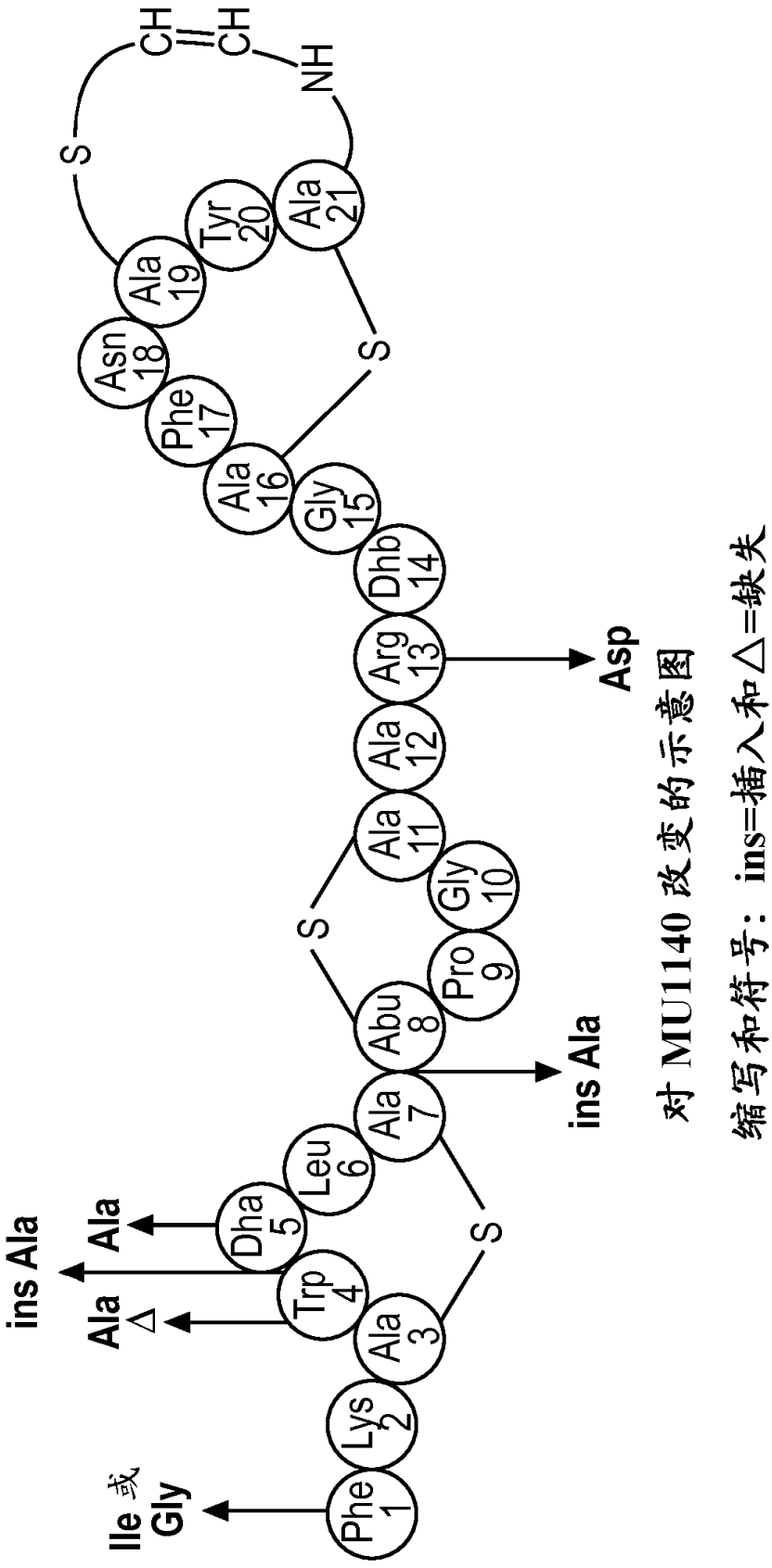

Phe 1
Lys 2
Ala 3
Trp 4
Dha 5
Leu 6
Ala 7
Abu 8
Pro 9
Gly 10
Ala 11
Ala 12
Arg 13
Dhb 14
Gly 15
Ala 16
Phe 17
Asn 18
Ala 19
Tyr 20
Ala 21
S
S
S
S
CH
CH
NH
Ile 或 Gly
ins Ala
Ala
Δ
Ala
ins Ala
Asp
对 MU1140 改变的示意图
缩写和符号：ins=插入和△=缺失

图 1B

突变体与野生型序列的比对

野生型	TTCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Phe1Gly	GGCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Phe1Ile	ATCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Trp4Ala	TTCAAAAGTGCA---AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Trp4insAla	TTCAAAAGTTGGGCAAGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
ΔTrp4	TTCAAAAGT------AGCCTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Ser5Ala	TTCAAAAGTTGG---GCACTTTGT---ACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Cys7insAla	TTCAAAAGTTGG---AGCCTTTGTGCAACGCCTGGTTGTGCAAGGACAGGTAGTTTCAATAGTTACTGTTGC
Arg13Asp	TTCAAAAGTTGG---AGCCTTTGT---ACGCCTGGTTGTGCAGACACAGGTAGTTTCAATAGTTACTGTTGC

野生型	SEQ ID NO:19
Phe1Gly	SEQ ID NO:20
Phe1Ile	SEQ ID NO:21
Trp4Ala	SEQ ID NO:22
Trp4insAla	SEQ ID NO:23
ΔTrp4	SEQ ID NO:24
Ser5Ala	SEQ ID NO:25
Cys7insAla	SEQ ID NO:26
Arg13Asp	SEQ ID NO:27

图 2

用于 MU1140 诱变的引物

寡核苷酸	序列 (5' – 3')
SRWlanA_1	AGAATTCAGGATGCTATCGCTGCTTTTTTTGTG (SEQ ID NO:1)
SRWlanA_2	AGAATTCAGGAAAGTTGCCATATGGTTTTGTG (SEQ ID NO:2)
Phe1Gly_1	GATCCAGATACTCGT**GGC**AAAAGTTGGAGCCTTTGTACG (SEQ ID NO:15)
Phe1Gly_2	CAACTTTTGCCACGAGTATCTGGATCGTCGTTGC (SEQ ID NO:16)
Phe1Ile_1	GATCCAGATACTCGT**ATC**AAAAGTTGGAGCCTTTGTACG (SEQ ID NO:17)
Phe1Ile_2	CAACTTTTGATACGAGTATCTGGATCGTCGTTGC (SEQ ID NO:18)
Trp4Ala_1	**GCA**AGCCTTTGTACGCCTGGTTG (SEQ ID NO:3)
Trp4Ala_2	ACAAAGGCTTGCACTTTTGAAACG (SEQ ID NO:4)
Trp4insAla_1	**GCA**AGCCTTTGTACGCCTGGTTG (SEQ ID NO:5)
Trp4insAla_2	CAAAGGCTTGCCCAACTTTTGAAACG (SEQ ID NO:6)
ΔTrp4_1	---AGCCTTTGTACGCCTGGTTG (SEQ ID NO:7)
ΔTrp4_2	CGTACAAAGGCTACTTTTGAAACG (SEQ ID NO:8)
Dha5Ala_1	**GCA**CTTTGTACGCCTGGTTGTGC (SEQ ID NO:9)
Dha5Ala_2	GGCGTACAAAGTGCCCAACTTTTGAA (SEQ ID NO:10)
Alas7insAla_1	**GCA**ACGCCTGGTTGTGCAAGGAC (SEQ ID NO:11)
Alas7insAla_2	ACCAGGCGTTGCACAAAGGCTCC (SEQ ID NO:12)
Arg13Asp_1	**GAC**ACAGGTAGTTTCAATAGTTAC (SEQ ID NO:13)
Arg13Asp_2	GAAACTACCTGTGTCTGCACAACCAG (SEQ ID NO:14)

外引物是 SRWlanA_1 和 SRWlanA_2)并且与 5'和 3'侧翼 DNA 是同源的。
下划线部分表示工程改造 EcoR1 位点。突变为粗体或加虚线。
编号指明正向引物(1)和反向引物(2)。

图 3

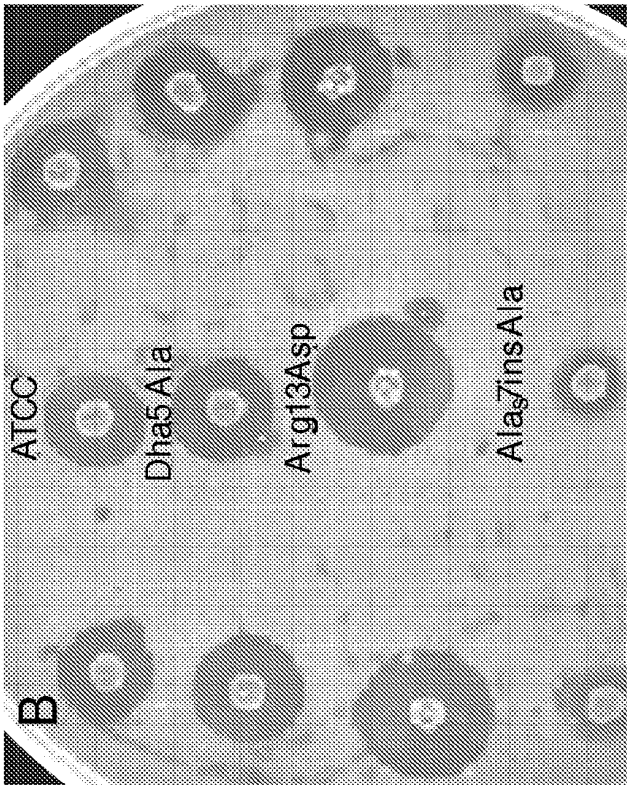


图 4B

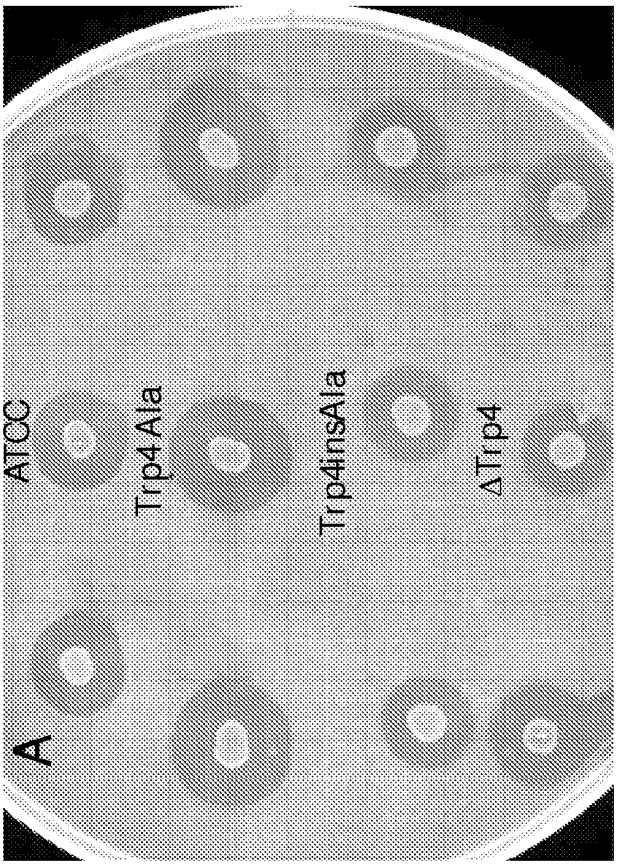


图 4A

图 4A-B: 抑菌圈平板测定

产生 MU1140 变体的菌株与产生野生型 MU1140 的菌株比较的生物活性

产生的变体	平均面积 * (mm^2)	均值的标准误(SEM)	变体对野生型的活性比率	统计学显著性 (p 值) #
MU1140 (野生型)	204.44	8.90	-	-
Phe1Gly	321.85	46.52	1.57	<.001
Phe1Ile	372.78	75.90	1.82	<.001
Trp4Ala	434.80	46.10	2.12	<.001
Trp4insAla	212.37	24.70	1.04	>.05
∇Trp4	217.56	35.37	1.06	>.05
Dha5Ala	382.25	31.40	1.87	<.001
Ala_s7insAla	109.41	9.74	0.54	<.001
Arg13Asp	526.06	55.09	2.57	<.001

* 基于 10 个独立样品。
# 斯氏 t 检验

图 5

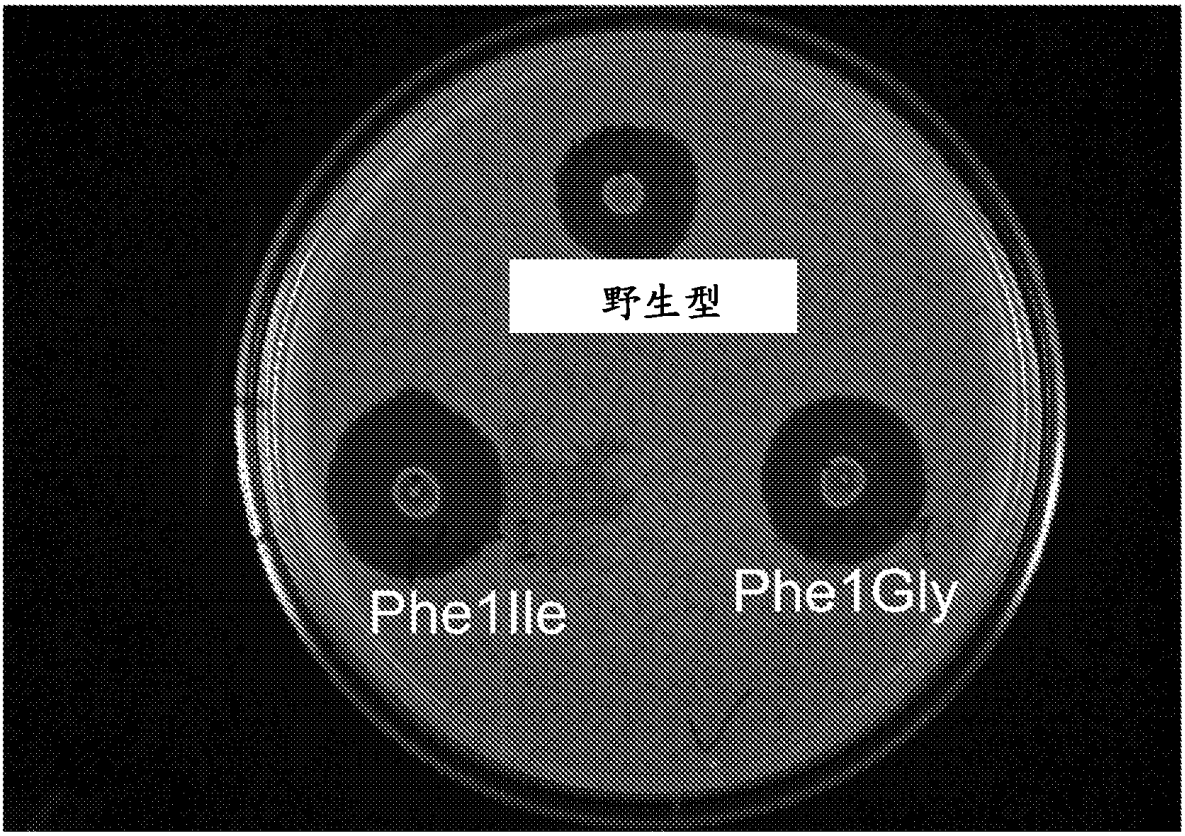


图 6