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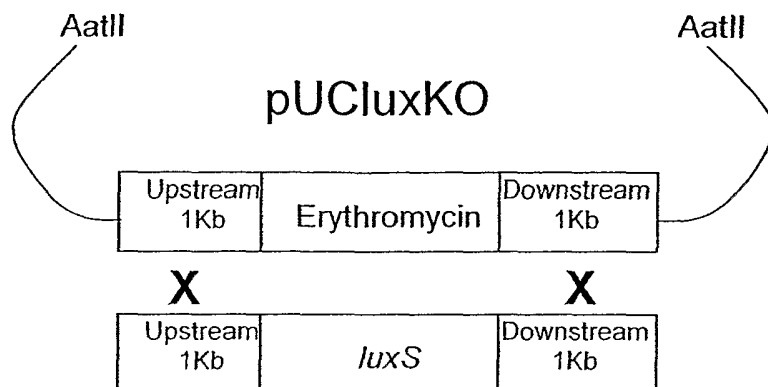
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(54) Title: QUORUM SENSING AND BIOFILM FORMATION



(57) Abstract: The present invention is based on the discovery that interspecies quorum sensing is related to biofilm formation. The present invention provides methods useful for treating or preventing biofilm formation and microbial infections associated with biofilm formation by using an agent that either increases LuxS-dependent pathway or interspecies quorum sensing signal. The present invention also provides compositions containing an anti-microbial agent and an agent that either increases LuxS-dependent pathway or interspecies quorum sensing signal.

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QUORUM SENSING AND BIOFILM FORMATION

FIELD OF THE INVENTION

5 This invention relates generally to the field of biofilm formation, especially bacterial biofilm formation associated with interspecies quorum sensing.

BACKGROUND OF THE INVENTION

10 A bacterial biofilm is a community of bacteria (either single or multiple bacterial species) that adhere to a solid surface (Davey, M.E., et al., *Microbiol Mol Biol Rev*, 64:847-67 (2000)). In recent years, biofilms have received much attention due to their impact on industry and medicine. Biofilms are responsible for a plethora of problems ranging from biofouling of pipelines to facilitating tissue damage in Cystic Fibrosis patients (Barbeau, J., et al., *Can J Microbiol*, 44:1019-28 (1998)); Stickler, D., *Curr Opin Microbiol*, 2:270-5 (1999)); and Wen, Z.T., et al., *Appl Environ Microbiol*, 68:1196-203 (2002)).

 Studies have clearly shown that a bacterial biofilm is not a result of random accretions of bacterial cells; rather, it is the net result of a community of bacteria cooperating to form well-differentiated structures (Costerton, J.W., et al., *Annu Rev Microbiol*, 64:847-67 (2000)). The production of biofilm is dependent on the progression through several steps, from initial attachment to full maturity as a stable ecologic system (Pratt, L., et al., *Curr Opin Microbiol*, 2:598-603 (1999)). Given the tremendous metabolic and physiological changes that are required for the switch from planktonic to biofilm growth, it would seem reasonable that there exist some gene regulators responsible for facilitating this process. Indeed various gene regulation systems have been found to be involved in bacterial biofilm formation (Davies, D.G., et al., *Science*, 280:295-8 (1998)).

30 Quorum sensing is a mechanism for bacteria to change gene expression at very specific cell densities. To date, there are two types of recognized quorum sensing systems in bacteria. The first, known as intraspecies quorum systems, are species

specific. In Gram-negative bacteria, intraspecies quorum signals are composed of an acyl-homoserine lactone backbone with species specific substitutions, while Gram-positive bacteria use various peptides as their signals (Dunny G.M., et al., *Annu Rev Microbiol*, 51:527-64 (1997); Fuqua, C., et al., *Curr Opin Microbiol*, 1:183-9 (1998); and Parsek, M.R., et al., *Proc Natl Acad Sci U S A*, 97:8789-93 (2000)).

Recently, a second quorum sensing system was characterized in *Vibrio harveyi*. This system is referred to as the interspecies quorum system and is believed to operate as a universal quorum system for many bacteria possessing the characteristic *luxS* gene (Bassler, B.L., *Curr Opin Microbiol*, 2:582-7 (1999); Schauder, S., et al., *Mol Microbiol*, 41:463-76 (2001); and Surette, M.G., et al., *Proc Natl Acad Sci USA*, 96:1639-44 (1999)). The *luxS* gene is highly conserved among many species of Gram-negative and Gram-positive bacteria and is thought to be responsible for synthesizing a universally recognized quorum signal referred to as autoinducer-2 (AI-2) (Surette, M.G., et al., *Proc Natl Acad Sci USA*, 96:1639-44 (1999)). The chemical structure of the actual signal is still under investigation, however, crystallographic studies of the AI-2 receptor in *Vibrio harveyi* seem to suggest that AI-2 is a furanosyl borate diester formed from the metabolite 4,5-dihydroxy-2,3-pentadione (Chen, X., et al., *Nature*, 415:545-9 (2002); Ruzheinikov, S.N., et al., *J Mol Biol*, 313:111-22 (2001); and Schauder, S., et al., *Mol Microbiol*, 41:463-76 (2001)).

One feature regarding quorum sensing that has been extensively studied, is the link between intraspecies quorum sensing and biofilm related gene expression. There are several well-characterized examples for the involvement of intraspecies quorum sensing and biofilm formation. For example, *lasI* of *Pseudomonas aeruginosa* directs the synthesis of an acyl-homoserine lactone signal molecule used for *P. aeruginosa* intraspecies quorum signaling (Davies, D.G., et al. *Science*, 280:295-8 (1998)). Mutants in this gene were unable to produce biofilms that progressed beyond the very early stages of biofilm development (Davies, D.G., et al. *Science*, 280:295-8 (1998)). However, exogenous addition of the appropriate signal complemented the defect (Davies, D.G., et al. *Science*, 280:295-8 (1998)).

A similar result was also obtained due to inactivation of the *cep* intraspecies quorum sensing system of *Burkholderia cepacia* (Huber, B., et al., *Microbiology*, 147:2517-28 (2001)). Furthermore, a transposon mutagenesis study of the oral pathogen *Streptococcus gordonii* had detected a severe biofilm deficiency due to disruption of the
5 two component system required for its intraspecies quorum sensing system (Loo, C.Y., et al., *J Bacteriol*, 182:1374-82 (2000)).

In *Staphylococcus aureus*, intraspecies quorum signaling has been implicated as a negative regulator of biofilm formation (Vuong, C., et al., *J Infect Dis*, 182:1688-93 (2000)). LuxS-dependent AI-2 signals have also been detected in a variety of bacterial
10 species and found to be involved in various cellular processes in a cell density dependent manner (Day, W.A., Jr., et al., *Infect Immun*, 69:15-23 (2001); Forsyth, M.H., et al., *Infect Immun*, 68:3193-9 (2000); Frias, J., et al., *Infect Immun*, 69:3431-4 (2001); Joyce, E.A., et al., *J Bacteriol*, 182:3638-43 (2000); Lyon, W.R., et al., *Mol Microbiol*, 42:145-57 (2001); and Sperandio, V., et al., *Proc Natl Acad Sci USA*, 96:15196-201 (1999)).

15 *S. mutans* is a major cariogenic bacterium that normally inhabits a complex, multispecies, biofilm on the tooth surface (dental plaque) (Tanzler, J.M., et al., *J Dent Educ*, 65:1028-37 (2001)). The bacteria produce large amounts of exopolysacchrides, especially in the presence of sucrose, that enable them to adhere to the tooth.

The bacteria also have the ability to produce large amounts of acids from
20 fermentable sugars in the diet. Acid accumulation can eventually dissolve the hard, crystalline structure of the tooth resulting in a carious lesion (Quivey, R.G., et al., *Crit Rev Oral Biol Med*, 12:301-14 (2001)). Previous studies have established some sophisticated interactions among the oral streptococci as well as with other oral bacteria within the same dental plaque (Kolenbrander, P.E., *Enzymol*, 253:385-97 (1995);
25 Kolenbrander, P.E., *Annu Rev Microbiol*, 54:413-37 (2000); and Kolenbrander, P.E., et al., *Infect Immun*, 63:4584-8 (1995)).

There is a need in the art to develop methods and compositions useful for regulating biofilm formation, especially preventing or treating biofilm formation in association with microbial infections.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that interspecies quorum sensing, e.g., LuxS-dependent signal pathway is related to biofilm formation. Accordingly the present invention provides methods for preventing or inhibiting biofilm formation and
5 methods for treating or preventing disease and other conditions associated with biofilm formation. The present invention also provides compositions that are useful for treating microbial infections and infestations associated with biofilm formation.

In one embodiment, the present invention provides a method of preventing or treating the formation of a biofilm on a surface. The method comprises contacting the
10 surface with an agent that increases LuxS-dependent signal pathway in a bacterium or increases an interspecies quorum sensing signal.

In another embodiment, the present invention provides a method of treating or preventing a disease condition associated with a biofilm. The method comprises administering to a subject in need of such treatment an agent, wherein the agent increases
15 LuxS-dependent signal pathway in a bacterium or increases an interspecies quorum sensing signal.

In yet another embodiment, the present invention provides a composition comprising an anti-microbial agent and an agent that increases LuxS-dependent signal pathway in a bacterium or increases an interspecies quorum sensing signal.

20 In still another embodiment, the present invention provides a method of treating a disease condition associated with bacterial biofilm formation comprising administering to a subject in need of such treatment the composition of the present invention.

In another embodiment, the present invention provides a method of treating a microbial infection associated with biofilm formation. The method comprises
25 administering to a population of bacteria an anti-microbial agent and an agent that increases LuxS-dependent signal pathway in a bacterium or increases interspecies quorum sensing signal.

In yet another embodiment, the present invention provides a method of treating bacterial biofilm formation on a surface. The method comprises contacting the surface
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with an anti-microbial agent and an agent that increases LuxS-dependent signal pathway in a bacterium or increases interspecies quorum sensing signal.

In another embodiment, the present invention provides a method of preventing or inhibiting bacteria biofilm formation or sensitizing bacteria for an anti-bacteria treatment.

- 5 The method comprises administering an agent to a population of bacteria, wherein the agent increases LuxS-dependent signal pathway in a bacterium or increases interspecies quorum sensing signal.

SUMMARY OF THE FIGURES

- 10 Figure 1 shows an alignment of the *S. mutans* LuxS protein (SEQ ID NO. 1) and several other representative LuxS proteins. Residues that coordinate a Zn^{2+} ion and comprise the catalytic center of LuxS (H, H, & C) are printed in bold. 1, *V. harveyi* (SEQ ID NO. 2); 2, *S. mutans*; 3, *E. coli* (SEQ ID NO.3); 4, *S. pyogenes* (SEQ ID NO. 4); 5, *B. subtilis* (SEQ ID NO.5).

- 15 Figure 2 shows *S. mutans luxS* complements a frameshift mutation in *E. coli* DH5 α . *E. coli* DH5 α was transformed with an *E. coli-S. mutans* shuttle vector containing an intact copy of the *S. mutans luxS* gene (pLuxSm). This strain was examined for AI-2 production using the luminescence based AI-2 reporter assay. *V. harveyi* strain BB170 (sensor 1⁻, sensor 2⁺) served as a positive control, while *E. coli* DH5 α served as a negative control. Luminescence is expressed as fold induction relative to the background values.
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- Figure 3 shows AI-2 induction in the presence or absence of sugar. *S. mutans* was grown and assayed as described in Materials and Methods. Cells were grown overnight and resuspended to an OD₆₀₀ of .4 in reporter assay (AB) media and incubated with aeration for 3 hours at 37°C. One sample was incubated in AB alone, while the other had sucrose added to a final concentration of 1%. The presence of sucrose in the media caused a potent reduction in luminescence to below background values.
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- Figures 4A and 4B show *luxS* knockout in *S. mutans*. Figure 4A illustrates the knockout procedure. Plasmids containing either cloned fragments of *S. mutans* DNA as well as the erythromycin cassette were cut using the indicated restriction sites and then
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ligated into a linearized pUC19 backbone. The resulting construct was linearized with the unique AatII site and transformed into *S. mutans* for double crossover. Figure 4B shows that AI-2 production assay confirms that activity was lost in the mutant.

Figure 5 shows the results of biofilms tested for their ability to resist detergent treatment with SDS. Three samples of wildtype and mutant biofilms grown on glass coverslips were shaken at 150 rpm for one hour. The OD₆₀₀ of the resulting supernatants were compared.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates in general to the interspecies quorum sensing, LuxS-dependent signal pathway and their association with biofilm formation. It is the discovery of the present invention that increasing interspecies quorum sensing signal or LuxS-dependent signal pathway decreases biofilm formation and increases biofilm's sensitivity to anti-microbial treatment. The present invention provides methods and compositions useful for preventing or treating biofilm formation or microbial infections associated with biofilm formation.

One feature of the present invention provides a method for preventing or treating biofilm formation. According to the present invention, the formation of a biofilm on a surface can be prevented or treated by contacting the surface, *e.g.*, a biological surface or an inanimate or dynamic solid industrial surface such as a heat exchanger or "clean room" surfaces with an agent or administering an agent to a subject, *e.g.*, a human or an animal in need of such treatment, wherein the agent either increases LuxS-dependent signal pathway in a bacterium or increases an interspecies quorum sensing signal. Alternatively, such agent can be administered to a population of bacteria, *e.g.*, more than one bacterium to prevent or decrease the biofilm formation of the bacteria.

According to another feature of the present invention, bacteria, biofilms, or surfaces containing thereof can be sensitized for anti-microbial, *e.g.*, anti-bacteria treatment using the agents of the present invention. For example, the agents of the present invention can be administered to a population of bacteria or bacteria within a biofilm so that these bacteria are primed or sensitized to be less resistant or more

susceptible to anti-microbial treatments, *e.g.*, antibiotics, target specific therapeutic agents, detergents, and biocides.

Bacteria or biofilms can be sensitized either prior to or simultaneously with any anti-microbial treatment. For example, the agents of the present invention can be used prior to or in combination with an anti-microbial agent to treat a surface, *e.g.*, industrial or biological surface or subject, *e.g.*, human for biofilm formation or microbial infections such as bacterial infection associated with biofilm formation. The subject in need of such treatment can be any suitable subject, *e.g.*, a human or an animal including a domestic animal such as a horse, dog, or cat.

The agent of the present invention can be any agent known or later discovered that is suitable for the present invention. For example, such agent can be any entity capable of activating the LuxS gene, any expression product of the LuxS gene that is involved in the interspecies quorum sensing, any down stream component in the LuxS-dependent signal pathway including any interspecies quorum sensing signal itself, or any agent capable of increasing the function or activity of an interspecies quorum sensing signal.

In one embodiment, an agent that increases LuxS-dependent signal pathway is LuxS protein which includes full-length LuxS protein and one or more partial LuxS proteins having substantially the same function of the full-length LuxS protein in synthesizing interspecies quorum sensing signal, *e.g.*, autoinducer-2 in a bacterium. In another embodiment, an agent that increases an interspecies quorum sensing signal is autoinducer-2 which includes autoinducer-2 and an analog or derivative thereof functioning as an interspecies quorum sensing signal or having the same or similar function of autoinducer-2 in interspecies quorum sensing. For example, WO 01/85664 discloses various autoinducer-2 analogs which could be suitable for the present invention.

The methods provided by the present invention can be used to prevent or treat any biofilm. Biofilms in general are coatings formed via bacteria adhering to a surface, *e.g.*, solid surface. Usually a population of bacteria interact or signal with each other directly or indirectly to form a highly hydrated matrix of exopolymers, typically polysaccharide, and other biopolymers on a surface. The formation of biofilm could take several steps and usually includes initial attachment and full maturity into a stable community.

According to the present invention, biofilms prevented or treated by the present invention can contain single species or multiple species bacteria. In one embodiment, the biofilms are associated with microbial infection or a disease condition including, without limitation, dental caries, periodontal disease, prostatitis, osteomyelitis, septic arthritis,
5 and cystic fibrosis. In another embodiment, the biofilms contain Gram positive bacteria including without limitation *Streptococcus*, *e.g.* *S. mutans*. In yet another embodiment, the extracellular matrix or exopolymers of the biofilms is regulated by or responsive to LuxS-dependent interspecies quorum sensing.

In still another embodiment, the biofilms are associated with a surface, *e.g.*, a
10 solid surface. Such surface can be the surface of any industrial structure, *e.g.*, pipeline or the surface of any structure in animals or humans. For example, such surface can be any epithelial surface, mucosal surface, or any host surface associated with bacterial infection, *e.g.*, persistent and chronic bacterial infections. The surface can also include any surface of a bio-device in animals or humans, including without limitation, bio-
15 implants such as bone prostheses, heart valves, and pacemakers.

In addition to surfaces associated with biofilm formation in a biological environment, the surfaces treated by the present invention can also be any surface associated with industrial biofilm formation. For example, the surfaces being treated can be any surface associated with biofouling of pipelines, heat exchangers, air filtering
20 devices, or contamination of computer chips or water-lines in surgical units like those associated with dental hand-pieces.

According to yet another feature of the present invention, it provides compositions useful for treating biofilm formation and infections associated with biofilm formation. The composition of the present invention contains an anti-microbial entity
25 and an agent of the present invention. The anti-microbial entity can be any that is suitable for the present invention, *e.g.*, detergents, biocides, antibiotics or target specific therapeutic entities. The antibiotics include, without limitation, penicillin, quinoline, vancomycin, sulfonamide, ampicillin, ciprofloxacin, and sulfisoxazole. The target specific therapeutic entities can include a targeting moiety coupled to an anti-microbial

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peptide moiety. U.S. Application Serial No. 10/077,624 discloses various target specific anti-microbial entities and is incorporated herein by reference.

5 The composition or agent of the present invention can also include one or more other non-active ingredients, *e.g.*, ingredients that do not interfere with the function of the active ingredients. For example, the composition or agent of the present invention can include a suitable carrier or be combined with other therapeutic agents.

10 A suitable carrier can be a powder, encapsulated solid, or an aqueous carrier including any safe and effective materials for use in the compositions of the present invention. In one embodiment, an aqueous carrier is used for the compositions of the present invention in oral formations and includes, without limitation, thickening materials, humectants, water, buffering agents, abrasive polishing materials, surfactants, titanium dioxide, flavor system, sweetening agents, coloring agents, and mixtures thereof.

15 A suitable carrier can also be a pharmaceutically acceptable carrier that is well known to those in the art. Such carriers include, without limitation, large, slowly metabolized macromolecules, *e.g.*, proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles.

20 Pharmaceutically acceptable salts can also be used in the composition, for example, mineral salts such as sodium or stannous fluorides, or sulfates, as well as the salts of organic acids such as acetates, propionates, carbonates, malonates, or benzoates. The composition can also contain liquids, *e.g.*, water, saline, glycerol, and ethanol, as well as substances, *e.g.*, wetting agents, emulsifying agents, or pH buffering agents.

25 According to another feature of the present invention, the compositions or agents of the present invention can be used to treat or prevent disease conditions associated with biofilm formation. For example, an effective amount of the composition or agent of the present invention can be administered to a subject, *e.g.*, a human or an animal to treat or prevent disease conditions associated with biofilm formation. Alternatively, the agent of the present invention and an anti-microbial agent can be administered as separate compositions, either sequential or simultaneously to a subject to treat or prevent disease conditions associated with biofilm formation. Various disease conditions are associated

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with biofilm formation including, without limitation, dental caries, periodontal disease, prostatitis, osteomyelitis, septic arthritis, cystic fibrosis, and heart valve vegetations. In one embodiment, the disease conditions are on an epithelial surface or a mucosal surface, *e.g.*, mouth, vagina, gastrointestinal tract, and esophageal tract.

5 In generally, an effective amount of the agent or composition of the present invention to be administered to a subject can be determined on a case-by-case basis. Factors to be considered usually include the total surface area to be treated, age, body weight, stage of the condition, other disease conditions, duration of the treatment, and the response to the initial treatment.

10 Typically, the agents or compositions used in the present invention are prepared as a topical or an injectable, either as a liquid solution or suspension. However, solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The composition can also be formulated into an enteric-coated tablet or gel capsule according to known methods in the art. In one embodiment, the composition of the
15 present invention can be formulated onto the surfaces of industrial or biological structures or as coatings thereto, *e.g.*, coatings to prosthetic heart valves, prosthetic hearts, vascular stents, or prosthetic joints.

 The agents or compositions of the present invention may be administered in any way which is industrially or medically acceptable which may depend on the condition or
20 injury being treated. Possible administration routes include injections, by parenteral routes such as intravascular, intravenous, intraepidural or others, as well as oral, nasal, ophthalmic, rectal, vaginal, topical, or pulmonary, *e.g.*, by inhalation. The compositions may also be directly applied to industrial or tissue surfaces. Sustained release, pH dependent release, or other specific chemical or environmental condition mediated
25 release administration is also specifically included in the invention, by such means as depot injections or erodible implants.

EXAMPLES

 The following examples are intended to illustrate but not to limit the invention in
30 any manner, shape, or form, either explicitly or implicitly. While they are typical of

those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

In this study, we demonstrate that *luxS* dependent quorum sensing is involved in biofilm formation of *Streptococcus mutans*. *S. mutans* is a major cariogenic bacterium in the multi-species bacterial biofilm commonly known as dental plaque. An ortholog of *luxS* in *S. mutans* was identified using the data available in the *S. mutans* genome project (<http://www.genome.ou.edu/smutans.html>). Using an assay developed for the detection of the LuxS-associated quorum sensing signal Autoinducer 2 (AI-2), it was demonstrated that this ortholog was able to complement the *luxS* negative phenotype of *E. coli* DH5 α . It was also shown that AI-2 is indeed produced by *S. mutans*. AI-2 production is maximal during mid to late-log growth in batch culture.

Mutant strains devoid of the *luxS* gene were constructed and found to be defective in producing AI-2 signal. There are also marked phenotypic differences between wildtype and the *luxS* mutants. Microscopic analysis of *in vitro* grown biofilm structure revealed that the *luxS* mutant biofilms adopted a much more granular appearance, rather than the relatively smooth, confluent layer normally seen in wild type. These results demonstrate that LuxS-dependent signal play an important role in biofilm formation of *S. mutans*.

Example 1. Material and Methods

Bacterial strains and culture conditions

All bacterial strains used in this study and their characteristics are listed in Table 1. All *S. mutans* strains were grown in brain heart infusion media (Difco) or on brain heart infusion agar plates. *luxS* deletion mutants were grown using the same media supplemented with 15 μ g/mL erythromycin. All *S. mutans* strains were grown anaerobically at 37°C. *E. coli* cells were grown in Luria-Bertani (LB) medium with aeration at 37°C. *E. coli* cells carrying plasmids were grown in LB medium containing 100 μ g/mL erythromycin. *V. harveyi* BB170 (sensor 1⁻, sensor 2⁺) was kindly provided by B. Bassler (Princeton University) and grown in AB medium (37) overnight at 30°C.

Table 1. Bacterial strains used in this study

Strain	Relevant Characteristics	Reference
<i>S. mutans</i> 25175	WT Em ^s	ATCC
<i>S. mutans</i> GS-5	WT Em ^s	(16)
JM03	$\Delta luxS$ Em ^r in 25175 background	This work
JM01	$\Delta luxS$ Em ^r in GS-5 background	This work
<i>E. coli</i> DH5 α	<i>supE44</i> $\Delta lacUI69$ ($\Phi 80 lacZ \Delta M15$) <i>hsdR17 recA1</i> <i>endA1 gyrA96 thi-1 relA1</i>	(17)
<i>E. coli</i> XL1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1</i> <i>lac</i> [F' <i>proAB lacI^r ZDM15 Tn10(Tet^r)</i>]	(3)

Cloning and analyses of the *S. mutans luxS* gene

- 5 A 990-bp DNA fragment containing the *luxS* gene from *S. mutans* strain 25175 was PCR amplified from genomic DNA using the primers WTlux5 (5'-GATGCTGCACGCTCTGTC-3') (SEQ ID NO. 6) and WTlux3 (5'-GCAGTTAGGGTATCCATCC-3') (SEQ ID NO. 7). Primer sequences were designed using sequence data obtained from the *S. mutans* Genome Sequencing Project, University of Oklahoma (B. A. Roe, R. Y. Tian, H. G. Jia, Y. D. Qian, S. P. Lin, S. Li, S. Kenton, H. Lai, J. D. White, R. E. McLaughlin, M. McShan, D. Ajdic and J. Ferretti. <http://www.genome.ou.edu/smutans.html>). The resulting fragment was cloned into the TOPO TA cloning vector (Invitrogen) and sequenced.

RNA isolation and RT-PCR

- 15 *S. mutans* total RNA was isolated as follows. 25mL cultures were grown overnight as previously described. Cells were centrifuged and resuspended in 1.5mL of TE buffer and then lysed using a Mini-Bead Beater 8 according to manufacturer's instructions. 150 μ L of lysate was then passed through a QiaShredder column (Qiagen) and 100 μ L of the resulting lysate was used for total RNA isolation with the RNeasy mini kit (Qiagen). cDNA buffer was added to RNA samples to obtain a 1X solution and 3 μ L
- 20

of RQ1 RNase free DNase was added to the sample and incubated overnight at 37°C. Reverse Transcription was performed according to manufacturer's protocol using random hexamers.

AI-2 assay

5 The AI-2 luminescence reporter assay was performed essentially as described (38), with the following modifications. To obtain cell free conditioned media, *S. mutans* was grown overnight as described. Stationary phase cells were resuspended in AB media to an OD₆₀₀ of 0.4. Cells were then incubated at 37°C with aeration for 3 hrs. After the incubation period, cells were pelleted by centrifugation, and the resulting supernatant was
10 filtered through a .22 mm pore sized filter (Millipore). The cell free conditioned media was either used immediately or stored at -20°C.

 To determine luminescence, an overnight culture of *V. harveyi* BB170 was diluted 1:1000 into fresh AB medium and 180 µL of cells was added to each 1.5 mL microfuge tube. Conditioned media (20 µL) was then added to the cells at a 10% (vol/vol) final
15 concentration. Positive control samples were obtained by adding cell free conditioned media from an overnight culture of *V. harveyi* BB170 to a final concentration of 10%. In some cases, conditioned media from a wildtype culture of *S. mutans* served as an additional positive control.

 To determine levels of background luminescence, a sample containing 200 µL of
20 1:1000 diluted *V. harveyi* was also included. In some cases, *E. coli* DH5α served as a negative control. All samples were measured hourly until peak induction at the 3hr time point. Measurements were collected using a TD 20/20 luminometer and expressed as arbitrary luminescence units.

Construction and analyses of *S. mutans luxS* mutants

25 2 (1Kb) fragments containing regions of DNA immediately upstream and downstream of the *luxS* translational start and stop codons respectively were amplified from genomic DNA template using the primers 2XupF (5'-GCGGATCCTCAAGCTCTCAAGCGTTCGG-3') (SEQ ID NO. 8) and 2XupR (5'-CGAGATCTATAAGACGGACATAAGGGGC-3') (SEQ ID NO. 9) as well as
30 2XdownF (5'-GCCTCGAGCAGATGATCCTTTTGAGCGTC-3') (SEQ ID NO. 10) and

2XdownR (5'-CGTCTAGACGGATGCAAAGAGAACGAAG-3') (SEQ ID NO. 11).

Primer sequences were designed using sequence data obtained from the *S. mutans*

Genome Sequencing Project, University of Oklahoma

(<http://www.genome.ou.edu/smutans.html>). The resulting fragments were cloned into the

5 TOPO TA cloning vector (Invitrogen).

To obtain the necessary fragments, four separate restriction digests were constructed: the upstream fragment was cut from the vector using BamHI and BglII, the downstream fragment was cut from the vector using XhoI and XbaI, the erythromycin cassette was cut from the plasmid pJT10 (unpublished plasmid by J. P Tsai and W. Shi) using BglII and XhoI, and the pUC19 vector was cut with BamHI and XbaI. The

10 resulting fragments were all gel purified (Qiagen), precipitated, and added to one mass ligation reaction. The resulting construct was then checked by restriction analysis and PCR for the proper configuration of the knockout vector (pUCluxKO).

Next, the plasmid was linearized with the unique cutting enzyme AatII and transformed into *S. mutans*. Transformants were selected for resistance to 15 µg/mL erythromycin. Confirmation of DNA integration was performed by PCR using primers IntluxF (5'-AAGAGTTTGGACCTAAAGGC-3') (SEQ ID NO. 12), IntluxR (5'-CCCACAGGACTCAATAGTTG-3') (SEQ ID NO. 13), UpluxF (5'-CTCGACGAATAGGATCAAAGC-3') (SEQ ID NO. 14), DownluxR (5'-GAGCCATCACACAGCAAAAAC-3') (SEQ ID NO. 15), ermA (5'-AGTGTGTTGATAGTGCAGTATC-3') (SEQ ID NO. 16), and ermM (5'-GAAGCTGTCAGTAGTATACC-3') (SEQ ID NO. 17). All mutants confirmed by PCR were further analyzed for their ability to produce AI-2 using the methods described above.

25 Growth of *in vitro* biofilm

Biofilms of *S. mutans* were grown as follows. Individual sterile culture dishes were filled with 2.5 mL of brain heart infusion broth supplemented with 1% (w/v) sucrose. Next, a sterile 18 mm glass microscope cover slip was added to each dish and the culture dish was covered. Each sample was then inoculated with a defined volume of

30 overnight culture. The dishes were incubated anaerobically at 37°C overnight.

Microscopic analyses of *in vitro* grown biofilm

Glass cover slips containing attached biofilm were removed from overnight cultures and rinsed briefly with water. These could be directly viewed using phase contrast and darkfield microscopy. For fluorescently labeled samples, the biofilms were first removed from the culture dish and placed into another dry culture dish. Next, 20 μ L of anti-*S. mutans* mouse monoclonal antibody solution (SWLA1) was added to the attached biofilm in each culture dish and incubated at room temperature for 30 minutes (Shi, W., et al., *Hybridoma*, 17:365-71 (1998)).

After the incubation period, 5 μ L of 2° antibody (FITC conjugated goat anti-mouse) was added to the biofilms and incubated at room temperature for an additional 10 minutes. Finally, the cover slip was briefly rinsed with water to remove excess antibodies and unattached cells and the sample was immediately imaged using fluorescence microscopy.

Treatment of *in vitro* biofilm with SDS and antibiotics

Biofilms were grown overnight using previously described conditions. For treatment with SDS, cover-slips were removed from overnight incubation and rinsed briefly. These were then placed into a fresh culture dish and 2mL of sterile, 1% (w/v) SDS solution was added to each dish. The biofilms were placed on a standard shaker set at 150 rpm for one hour at room temperature. Next, supernatant samples were checked at 40X magnification to confirm that cells were fully individualized and not connected in chains. These samples were measured for their OD₆₀₀ values. For treatment with antibiotics, biofilms were again grown under standard conditions overnight. The following day, the spent media was changed and replaced with BHI supplemented with 1% (w/v) sucrose solution. Ampicillin was added at either 50 μ g/mL or 500 μ g/mL. These biofilms were allowed to grow overnight under anaerobic conditions at 37°C. The following day, biofilms were removed from incubation and sonicated until the cells were fully dispersed. A 10⁶ fold dilution of each sample was plated on non-selective BHI agar plates and incubated anaerobically overnight at 37°C.

Example 2. Identification and isolation of *S. mutans luxS* gene

To determine whether *S. mutans* may possess an interspecies quorum system, it was first necessary to identify a candidate ortholog of *luxS*, the enzyme required for AI-2 production. Using the *luxS* gene from *Vibrio harveyi*, we performed a BLAST search of the University of Oklahoma *Streptococcus mutans* Genome Sequence Database. A candidate open reading frame was identified.

The ORF appears to be an isolated gene (no other convincing ORF's nearby) and encodes a protein of 160 amino acids, which is similar in size to other reported LuxS proteins. It is homologous to the *V. harveyi* LuxS protein with 38% amino acid identity and 57% similarity.

Using this sequence data, primers were designed to amplify a region about 500bp upstream of the translation start site and about 100bp downstream of the translational stop site. This fragment was PCR amplified, cloned into the TOPO TA cloning vector, and sequenced to confirm the identity of the gene. After the fragment was confirmed to be the same ORF identified in the Sequence Database, an NCBI PSI BLAST search of the candidate gene was conducted.

The results yielded strong homologies to numerous LuxS proteins from other Gram positive and Gram negative bacteria. The strongest homologies (identity/similarity) were to other Gram positive species such as *Streptococcus pyogenes* (84% / 92%), *Streptococcus pneumoniae* (83% / 91%), *Lactococcus lactis* (64% / 79%), and *Clostridium perfringens* (45% / 64%), but there were also significant homologies to Gram negative species such as *Neisseria meningitidis* (36% / 58%), *Escherichia coli* (37% / 59%), and *Haemophilus influenzae* (37% / 58%). A multiple alignment of the putative *S. mutans* LuxS protein and those from *V. harveyi*, *E. coli*, *S. pyogenes*, and *B. subtilis* demonstrated a high degree of similarity (Figure 1).

Of greatest interest was the location of several highly conserved amino acids (H, H, & C), which are reported to coordinate a Zn^{+2} ion and form the catalytic center of the protein (19). These amino acids and several others that are reportedly invariant are all conserved in the *S. mutans* LuxS protein (Figure 1).

Example 3. Complementation of an *E. coli luxS* mutant with *S. mutans luxS* gene

After confirming the identity of the candidate ORF, it was necessary to determine whether this gene also had the characteristic AI-2 synthase activity. This was accomplished by complementing an AI-2 production defect in *E. coli* DH5 α . This strain of *E. coli* is known to have a frameshift mutation in its *luxS* gene. Therefore, a plasmid containing the *luxS* gene and some upstream sequence was transformed into DH5 α and AI-2 activity was measured using the reporter assay described by Surette et al (Surette, M.G., et al., *Proc Natl Acad Sci USA*, 95:7046-50 (1998)).

As shown in Figure 2, our assay confirmed that *E. coli* DH5 α was AI-2 negative and also demonstrated that the presence of the *luxS* containing plasmid was sufficient to induce luminescence 89-fold over background.

Example 4. Secretion of an AI-2-like signal by *S. mutans*

To determine whether *S. mutans* had endogenous AI-2 activity, we again employed the AI-2 reporter assay. Initial screens using this assay failed to demonstrate any convincing AI-2 activity. These first experiments were all performed as had been described in previous reports. Cells were grown to various OD₆₀₀ values using standard growth media and the resulting conditioned media was used as a source of AI-2 molecules. However, these experiments consistently yielded background levels of luminescence.

In *E. coli*, it had been demonstrated that the presence of glucose in the growth media caused a strong induction of AI-2 (Surette, M.G., et al., *Proc Natl Acad Sci USA*, 95:7046-50 (1998)). Therefore, we decided to try adding glucose as well as sucrose (*S. mutans* preferred carbon source) to the growth media in an effort to demonstrate AI-2 production. Since *S. mutans* is an acidogenic bacterium, pH readings were taken to ensure that there were no negative effects due to lowered pH. Interestingly, these experiments consistently yielded lower than background levels of luminescence (Figure 3).

In an effort to circumvent this problem, we decided to resuspend overnight cultures in the AI-2 assay media at various OD₆₀₀ ranges. This media only allows for

very limited growth of *S. mutans*, but is able to keep the cells alive long enough for the production of AI-2 signal molecules. After assaying samples produced in this manner, it was immediately possible to measure induced luminescence. Samples assayed at mid to late log growth phase had the strongest induction over background, while the induction
5 tended to drop at stationary phase. Mid to late log samples typically produced about 25-fold induction of the reporter strain (*V. harveyi*) luciferase operon expression over background levels (Figure 3). As a further confirmation, *luxS* was shown to be expressed in *S. mutans* via RT-PCR.

10 **Example 5. Construction of *S. mutans luxS* mutants**

After confirming that this ORF was a functional *luxS* gene and that it was involved in the production of AI-2 signal molecules, we decided to disrupt the function of this gene by allelic replacement to check for any resulting phenotypic changes. *luxS* was deleted using a double crossover construct as illustrated in Figure 4A and described in
15 Example 1. The double crossover event was confirmed by various PCR reactions. As a further confirmation of the construct, we tested these mutants for the loss of AI-2 activity. As shown in Figure 4B, wildtype *S. mutans* had a luminescence induction of about 25 fold, while the mutant retained essentially background levels of luminescence.

20 **Example 6. General phenotypic characterization of *S. mutans luxS* mutants**

After confirmation of the deletion of *luxS* and subsequent loss of AI-2 activity, the next step was to quantify any resulting physiological changes in the *luxS* mutant. When the mutant colonies were plated, there were no obvious phenotypic differences in colony morphology. In batch culture there was no obvious difference in growth rate, nutrient
25 requirements, or acid production.

Example 7. Altered biofilm structure of the *S. mutans luxS* mutants

Our next investigation of mutant phenotypes was to determine if there were any alterations of biofilm structure as a result of loss of AI-2 production. Both wild-type and
30 mutant cells were able to form a biofilm when grown on a solid surface overnight.

Upon visual inspection, there was a noticeable difference in biofilm structure from the wild-type. Without the aid of magnification, wild-type biofilm generally has a very confluent appearance with no major discernable features. In contrast, the *luxS* mutant biofilm had a very rough texture. Under 20X and 40X magnification, this difference is even more apparent. Wild-type biofilms are very uniform with complete coverage of the attached surface. They also tend to have relatively small aggregates spread fairly evenly throughout the biofilm matrix. *luxS* mutant biofilms are quite different. Their organization seems much more heterogeneous. There were noticeable large gaps in the biofilm matrix and the cell aggregates appeared much larger. Using fluorescence imaging, there is a clear indication that the sizes of mutant aggregates tended to be much larger than those of wild-type.

As biofilms are known to be very resistant to detergents and biocides, we were also interested to determine if this property was influenced by the *luxS* mutation. Overnight biofilms of wildtype and the *luxS* mutant were incubated in a 1% solution of SDS and shaken at 150 rpm for one hour. These supernatants were then checked by microscopy to ensure that cells were not clumped, and subsequently, measured for the OD₆₀₀. It was found that the wildtype supernatants had an OD₆₀₀ value that averaged about 6 fold greater than that of the mutant (figure 5B), which suggested that *luxS* mutant biofilms were more resistant to detergent treatment. We also treated biofilms of wildtype and the *luxS* mutant with the antibiotic ampicillin at 50 µg/mL or 500 µg/mL for 16 hours. We found that very few (<1%), if any, wildtype cells survived treatment with 50 µg/mL ampicillin and that none survived the treatment with 500 µg/mL. In contrast, numerous cells (>10%) within the *luxS* mutant biofilms survived the treatment with 50 µg/mL ampicillin and even with 500 µg/mL ampicillin.

Within the past couple of years, there has been a plethora of data describing various physiological functions from both Gram positive and Gram negative species which are subject to regulation by *luxS* (Chung, W.O., et al., *J Bacteriol*, 183:3903-9 (2001); Day, W.A., Jr., et al., *Infect Immun*, 69:15-23 (2001); Fong, K.P., et al., *Infect Immun*, 69:7625-34 (2001); Lyon, W.R., et al., *Mol Microbiol*, 42:145-57 (2001); and Sperandio, V., et al., *Proc Natl Acad Sci USA*, 96:15196-201 (1999)). Much of the

reported data has supported the hypothesis that *luxS* is somehow involved in regulating virulence factor expression. Furthermore, there has not been a reported scenario in which mutating this gene has lead to severe growth impairment, which suggests that the production of AI-2 is not a requirement for basic metabolic processes.

5 Our current findings are consistent with these same trends. After deletion of the *luxS* gene, there were no noticeable changes in growth patterns or basic nutrient requirements. However, *S. mutans* is a predominantly biofilm dwelling organism and as such depends on its biofilm production for virulence in the oral cavity. The involvement of *luxS* in the proper development of *S. mutans* biofilms also yields some insight into the
10 role of interspecies communication in multispecies biofilm formation. *S. mutans* normally grows amongst hundreds of other competing species of oral bacteria and must therefore employ strategies to survey and respond to other species that compete for control of available ecological niches in the mouth.

 One possible strategy to accomplish this goal would be through the use of *luxS*
15 based interspecies signaling. We believe that as an early colonizer of the tooth surface, AI-2 signal molecules is an important factor in regulating biofilm related gene expression to help modulate energy utilization for growth in an extremely competitive environment.

 Through a search of the *S. mutans* genome database, we were able to find a candidate *luxS* ortholog. Despite having a gene whose encoded protein aligned
20 exceedingly well with other known LuxS proteins, initial attempts to demonstrate the production of AI-2 molecules all seemed to suggest that they were not produced. Indeed, there are reports of other *luxS* containing bacteria that have not been shown to induce the AI-2 reporter assay (Frias, J., et al., *Infect Immun*, 69:3431-4 (2001) and Surette, M.G., et al., *Proc Natl Acad Sci USA*, 96:1639-44 (1999)). It was especially perplexing to find
25 that the presence of glucose and sucrose in the growth media caused the AI-2 reporter assay to yield values below background levels, while glucose was known to cause a potent induction of AI-2 activity in *E. coli*.

 Part of the answer came from the media itself. It seems that detectable AI-2 activity drops in rich media and is potently inhibited in the presence of glucose and to an
30 even greater extent with sucrose. This phenomenon may explain why a recent screen for

AI-2 activity in various oral pathogens failed to demonstrate AI-2 activity in *S. mutans* strain 25175 (Frias, J., et al., *Infect Immun*, 69:3431-4 (2001)).

There also exists the possibility that AI-2 is still produced in rich media and/or in the presence of sugar, but the reporter assay is inhibited by some factor(s) secreted by *S. mutans* when grown under these conditions. Indeed, *S. mutans* is known to produce a battery of various inhibitory molecules such as lantibiotics and non-lantibiotic bacteriocins (Chen, P., et al., *Appl Environ Microbiol*, 65:1356-60 (1999); Qi, F., et al., *Appl Environ Microbiol*, 67:15-21 (2001); and Qi, F., et al., *Appl Environ Microbiol*, 65:3880-7 (1999)). In addition, it is also known that in the presence of sugars, *S. mutans* has a distinct growth advantage over other competing oral bacteria. Therefore, it is possible that sugar stimulates *S. mutans* to produce an inhibitor that affected either luciferase production in the AI-2 assay or perhaps even an inhibitor that acted at level of the AI-2 signaling process. Even though an AI-2 related inhibitory signal in *S. mutans* is currently unknown, its production could give additional explanations for *S. mutans* ability to gain a growth advantage over competitors within its multispecies biofilm.

Our data show that both wild-type and the *luxS* mutant of *S. mutans* are able to form biofilms on solid surfaces. This is consistent with previous reports (Wen, Z.T., et al., *Appl Environ Microbiol*, 68:1196-203 (2002)). Our investigation demonstrates that the *luxS* mutant has several altered biofilm phenotypes: increased size of cell aggregates, altered biofilm structure, and an increased biofilm resistance to detergents and antibiotics. These phenotypes reveal that *luxS* has a regulatory role on one or more genes related to biofilm formation. In *S. mutans*, EPS consists of insoluble glucans, which are primarily synthesized by the activity of the GtfB enzyme. Glucans make the cells more generally adherent in a nonspecific manner, but are also known to interact with glucan binding proteins for specific interactions (Hazlett, K.R., et al., *Infect Immun*, 67:3909-14 (1999) and Mattos-Graner, R.O., et al., *Infect Immun*, 69:6931-41 (2001)). Therefore, the observed phenotypes can be partially due to a greater production of glucan, an increase in specific receptors for glucan or other cell wall components, or possibly a combination of both factors. These questions are currently being addressed using a proteomics approach.

In summary, we have identified a *luxS* ortholog in *S. mutans* and demonstrated a corresponding AI-2 activity in the AI-2 assay. When *luxS* was inactivated, AI-2 activity was abolished with concomitant changes in biofilm structure and resistance to detergents and antibiotics. Taken together, this study provides evidence for the involvement of
5 LuxS in bacterial biofilm formation. It also demonstrates that AI-2 molecules or its analogs could be used to alter biofilm structure in *S. mutans* and/or make the bacteria within its biofilm more accessible to detergent and/or antibiotic treatments.

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made
10 without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A method of preventing or treating the formation of a biofilm on a surface comprising contacting the surface with an agent that increases LuxS-dependent signal pathway in a bacterium.
5
2. The method of claim 1, wherein the agent increases an interspecies quorum sensing signal.
- 10 3. The method of claim 1, wherein the agent is an interspecies quorum sensing signal.
4. The method of claim 1, wherein the agent is autoinducer-2.
- 15 5. The method of claim 1, wherein the agent is LuxS.
6. The method of claim 1, wherein the agent increases the activity of LuxS in interspecies quorum sensing.
- 20 7. The method of claim 1, wherein the biofilm contains Gram positive bacteria.
8. The method of claim 1, wherein the biofilm contains more than one species of bacteria.
- 25 9. The method of claim 1, wherein the biofilm contains *Streptococcus*.
10. The method of claim 1, wherein the biofilm contains *S. mutans*.
11. The method of claim 1, wherein the extracellular matrix of the biofilm is regulated by LuxS-dependent signal pathway.
30

12. The method of claim 1, wherein the surface is a solid surface.
13. The method of claim 1, wherein the surface is an epithelial or mucosal surface of
5 a mammal.
14. The method of claim 1, wherein the surface is associated with bacterial infection.
15. A method of preventing or inhibiting the formation of a biofilm on a surface
10 comprising contacting the surface with an agent that increases an interspecies
quorum sensing signal.
16. A method of treating or preventing a disease condition associated with a biofilm
comprising administering to a subject in need of such treatment an agent, wherein
15 the agent increases LuxS-dependent signal pathway in a bacterium.
17. A method of treating or preventing a disease condition associated with bacterial
biofilm formation comprising administering an agent to a subject in need of such
treatment, wherein the agent increases an interspecies quorum sensing signal.
20
18. The method of claim 16, wherein the agent increases an interspecies quorum
sensing signal.
19. The method of claim 16, wherein the agent is an interspecies quorum sensing
25 signal.
20. The method of claim 16, wherein the agent is autoinducer-2.
21. The method of claim 16, wherein the agent is LuxS.
30

22. The method of claim 16, wherein the agent increases the activity of LuxS in interspecies quorum sensing.
23. The method of claim 16, wherein the biofilm contains Gram positive bacteria.
- 5 24. The method of claim 16, wherein the biofilm contains more than one species of bacteria.
25. The method of claim 16, wherein the biofilm contains *Streptococcus*.
- 10 26. The method of claim 16, wherein the biofilm contains *S. mutans*.
27. The method of claim 16, wherein the extracellular matrix of the biofilm is regulated by LuxS-dependent signal pathway.
- 15 28. A composition comprising an anti-microbial agent and an agent that increases LuxS-dependent signal pathway in a bacterium.
29. A composition comprising an anti-microbial agent and an agent that increases an interspecies quorum sensing signal.
- 20 30. The composition of claim 28, wherein the agent increases an interspecies quorum sensing signal.
- 25 31. The composition of claim 28, wherein the agent is an interspecies quorum sensing signal.
32. The composition of claim 28, wherein the agent is autoinducer-2.
- 30 33. The composition of claim 28, wherein the agent is LuxS.

34. The composition of claim 28, wherein the agent increases the activity of LuxS in interspecies quorum sensing.

5 35. The composition of claim 28, wherein the anti-microbial agent is an antibiotic.

36. The composition of claim 35, wherein the antibiotic is selected from the group consisting of penicillin, quinoline, vancomycin, and sulfonamide.

10 37. The composition of claim 35, wherein the antibiotic is selected from the group consisting of ampicillin, ciprofloxacin, and sulfisoxazole.

15 38. The composition of claim 28, wherein the anti-microbial agent comprising a targeting moiety and an anti-microbial peptide moiety, wherein the targeting moiety is coupled to the anti-microbial peptide moiety and recognizes a target microbial organism and wherein the composition has an anti-microbial effect on a target microbial organism.

20 39. The composition of claim 28 further comprising a pharmaceutically acceptable carrier.

25 40. A method of treating a disease condition associated with bacterial biofilm formation comprising administering to a subject in need of such treatment the composition of claim 28.

41. A method of treating a disease condition associated with bacterial biofilm formation comprising administering to a subject in need of such treatment the composition of claim 29.

30

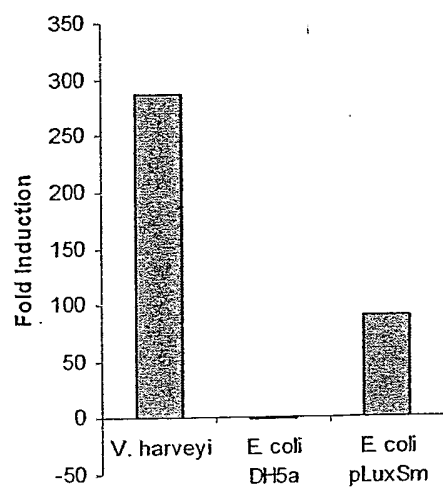
42. The method of claim 40, wherein the disease condition is on an epithelial or a mucosal surface.
43. The method of claim 42, wherein the mucosal surface is selected from the group consisting of mouth, vagina, gastrointestinal tract, and esophageal tract.
44. The method of claim 40, wherein the disease condition is an oral infection.
45. The method of claim 40, wherein the disease condition is caused by *S. mutans*.
46. A method of treating a microbial infection associated with biofilm formation comprising administering to a population of bacteria an anti-microbial agent and an agent that increases LuxS-dependent signal pathway in a bacterium.
47. A method of treating microbial infection associated with biofilm formation comprising administering to a population of bacteria an anti-microbial agent and an agent that increases interspecies quorum sensing signal.
48. A method of treating bacterial biofilm formation on a surface comprising contacting the surface with an anti-microbial agent and an agent that increases LuxS-dependent signal pathway in a bacterium.
49. A method of treating bacterial biofilm formation on a surface comprising contacting the surface with an anti-microbial agent and an agent that increases interspecies quorum sensing signal.
50. A method of preventing or inhibiting bacteria biofilm formation or sensitizing bacteria for an anti-bacteria treatment comprising administering an agent to a population of bacteria, wherein the agent increases LuxS-dependent signal pathway in a bacterium.

51. A method of preventing or inhibiting bacteria biofilm formation or sensitizing bacteria for an anti-bacteria treatment comprising administering an agent to a population of bacteria, wherein the agent increases interspecies quorum sensing signal.
- 5

	10	20	30	40	50	60	
1	MP---	LLDSFTVDHTRMNA	PAVRVAKTMQTPK	GDITVFDLRFTAP	NKDILSEKGIHTLE		57
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3	MP---	LLDSFTVDHTRMEAP	AVRVAKTMNTPHGD	AITVFDLRFCVPN	KEVMPERGIHTLE		57
4	MTKEVIVESFELD	HITIVKAPYVRLISE	EEFGPKGDRITNF	DVRLVQPNQNSIET	AGLHTIE		60
5	MP---	SVESFELDHNAVVAP	YVRHCGVHKVGT	DGVVNKFDIRFCQ	PNKQAMKPDTHLE		57
	70	80	90	100	110	120	
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2	HLLAKLIRQRIDG	-----MIDCS	PFGCRTGFHLMWG	KHTTTQIATVIKAS	LEEIANTIS		115
3	HLFAGFMRNHLNG	-NGVEIIDISPMGC	RRTGFYMSLIGTP	DEQRVADVWKAAME	DVLKVQD		116
4	HLLAKLIRQRIDG	-----MIDCS	PFGCRTGFHLMWG	KHSSTDIKVIKSS	LEEIATGIT		115
5	HLLAFTIRSHA	EKYDHF	DIIDISPMGXQT	GYLVVSGETTS	AEIVDLLED	TMKEAVEI--	115
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3	QNQIPELNVYQCG	TYQMHSLQEAQDI	ARSILERDVRINS	NEELALPKEKLQ	ELH--		170
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FIGURE 1

2/6

**FIGURE 2**

3/6

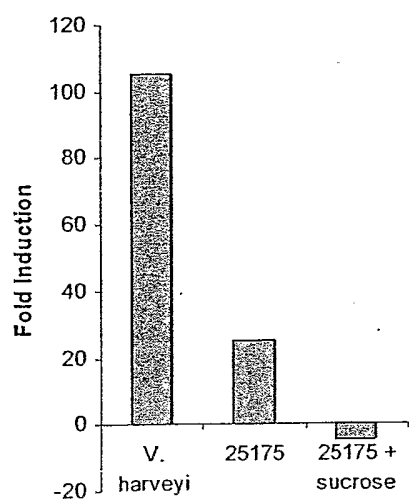


FIGURE 3

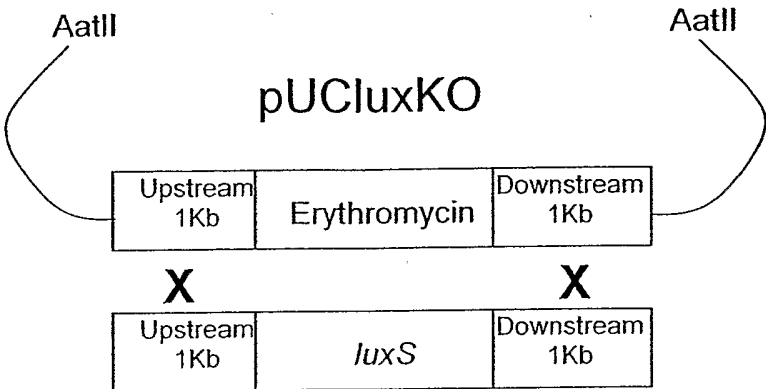


FIGURE 4A

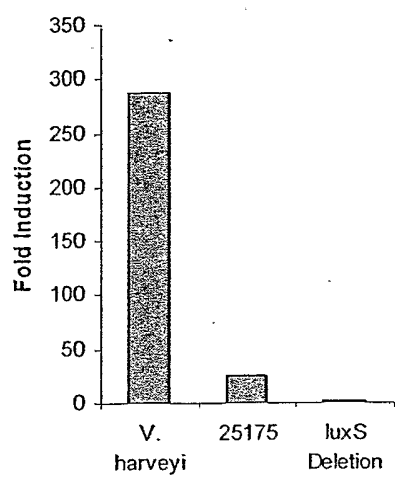


FIGURE 4B

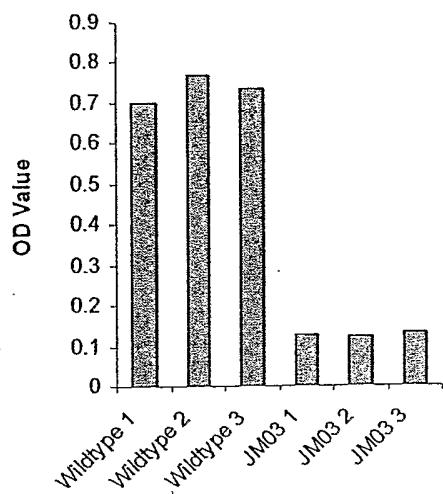


FIGURE 5

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C3 Scientific Corporation

WENYUAN, Shi

ANDERSON, Maxwell H.

Merritt, Justin

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Arg Asn His Leu Asn Gly Asp Ser Val Glu Ile Ile Asp Ile Ser Pro
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Met Gly Cys Arg Thr Gly Phe Tyr Met Ser Leu Ile Gly Thr Pro Ser
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Glu Gln Gln Val Ala Asp Ala Trp Ile Ala Ala Met Glu Asp Val Leu
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Lys Val Glu Asn Gln Asn Lys Ile Pro Glu Leu Asn Glu Tyr Gln Cys
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35 40 45

Pro Glu Arg Gly Ile His Thr Leu Glu His Leu Phe Ala Gly Phe Met

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Arg Asn His Leu Asn Gly Asn Gly Val Glu Ile Ile Asp Ile Ser Pro

65 70 75 80

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35 40 45

Asn Ser Ile Glu Thr Ala Gly Leu His Thr Ile Glu His Leu Leu Ala
50 55 60

Lys Leu Ile Arg Gln Arg Ile Asp Gly Met Ile Asp Cys Ser Pro Phe
65 70 75 80

Gly Cys Arg Thr Gly Phe His Leu Ile Met Trp Gly Lys His Ser Ser
 85 90 95

Thr Asp Ile Ala Lys Val Ile Lys Ser Ser Leu Glu Glu Ile Ala Thr
 100 105 110

Gly Ile Thr Trp Glu Asp Val Pro Gly Thr Thr Leu Glu Ser Cys Gly
 115 120 125

Asn Tyr Lys Asp His Ser Leu Phe Ala Ala Lys Glu Trp Ala Gln Leu
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Ile Ile Asp Gln Gly Ile Ser Asp Asp Pro Phe Ser Arg His Val Ile
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Val Asn Lys Phe Asp Ile Arg Phe Cys Gln Pro Asn Lys Gln Ala Met
35 40 45

Lys Pro Asp Thr Ile His Thr Leu Glu His Leu Leu Ala Phe Thr Ile
50 55 60

Arg Ser His Ala Glu Lys Tyr Asp His Phe Asp Ile Ile Asp Ile Ser
65 70 75 80

Pro Met Gly Xaa Gln Thr Gly Tyr Tyr Leu Val Val Ser Gly Glu Thr
85 90 95

Thr Ser Ala Glu Ile Val Asp Leu Leu Glu Asp Thr Met Lys Glu Ala
100 105 110

Val Glu Ile Thr Glu Ile Pro Ala Ala Asn Glu Lys Gln Cys Gly Gln
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