



(51) International Patent Classification:

A61L 27/54 (2006.01) *A01N 63/00* (2006.01)
A61L 27/28 (2006.01) *A01P 1/00* (2006.01)
A61L 27/34 (2006.01)

(21) International Application Number:

PCT/US2010/048422

(22) International Filing Date:

10 September 2010 (10.09.2010)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/241,342 10 September 2009 (10.09.2009) US

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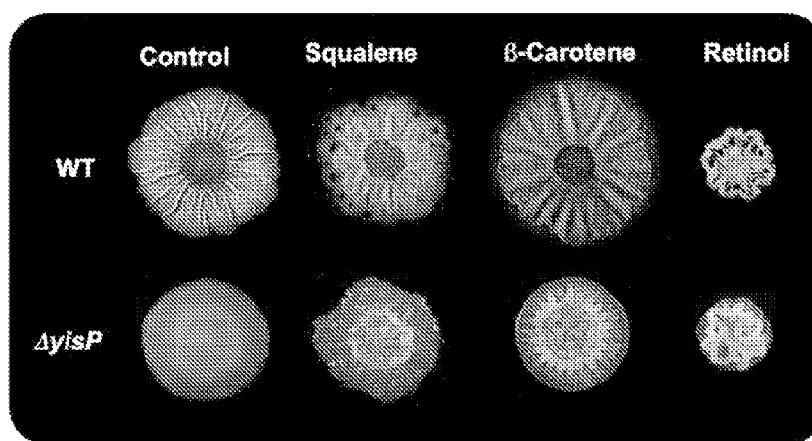
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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, 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, PE, PG, PH, PL, PT, RO, RS, RU, 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, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, 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, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: INHIBITORS OF BIOFILM FORMATION

**FIGURE 7**

(57) Abstract: Disclosed herein is a composition for inhibiting bacterial biofilm formation comprising carrier and an effective amount of an inhibitor of squalene/phytoene synthesis. Inhibitors may inhibit, for example, HMG-CoA Reductase, squalene synthase, 1-deoxy-D-xylulose 5-phosphate synthase. Examples of such inhibitors are a phosphonosulfonate (e.g., BPH-652, BPH-689, BPH-700), a statin (e.g., mevastatin, lovastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin), zaragozic acid, clomazone, and lapauqu Coast acetate or a functional derivative thereof. Also disclosed are substrates comprising the inhibitor of squalene/phytoene synthesis, and methods of inhibiting bacterial biofilm formation.



Published:

— *without international search report and to be republished
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INHIBITORS OF BIOFILM FORMATION

RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional patent application serial number 61/241,342, filed September 10, 2009, the contents of which are herein incorporated by reference in their entirety.

GOVERNMENTAL SUPPORT

[0002] This invention was made with Government support under GM58213 awarded by the National Institutes of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates to the field of microbiology and the inhibition of biofilm formation of microbes.

BACKGROUND OF THE INVENTION

[0004] Despite sterilization and aseptic procedures, bacterial infection remains a major impediment to the utility of medical implants including catheters artificial prosthetics and subcutaneous sensors. Indwelling devices are responsible for over half of all nonsocomial infections, with an estimate of 1 million cases per year in the U.S. alone. Device-associated infections are the results of bacterial adhesion and subsequent biofilm formation at the implantation site. Conventional antibiotic therapies remain ineffective against biofilms. The lack of suitable treatment often leaves extraction of the contaminated device as the only viable option for eliminating the biofilm.

[0005] At the cellular level, implant-associated infections are the result of bacterial adhesion to a biomaterial surface. For a successful implant, tissue integration occurs prior to appreciable bacterial adhesion, thereby preventing colonization at the implant. Host defenses are often not capable of preventing further colonization if bacterial adhesion occurs before tissue integration. A 6 hour post-implantation “decisive period” has been identified during which prevention of bacterial adhesion is critical to long-term success of an implant (Poelstra et al., *J. Biomed. Mater. Res.*, 60: 206 (2002)). Over this period an implant is particularly susceptible to surface colonization. Biofilms are remarkably resistant to both the immune response and systemic antibiotic therapies, and thus their development is the primary cause of

implant-associated infection. The formation of a pathogenic biofilm ensues from the initial adhesion of bacteria to an implant surface. Inhibiting bacterial adhesion is regarded as the most critical step to preventing implant associated infection. The most common pathogens that cause implant infections are Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis*. Other bacteria implicated in implant-associated infections are Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus* group bacteria (e.g., *P. mirabilis* and *P. vulgaris*). With the protective polysaccharide coating and sequestered nutrients, bacteria in biofilms exhibit extreme resistance to antibiotics. In some cases, killing bacteria in a biofilm requires roughly 1000 times the antibiotic dose necessary to achieve the same results in a suspension of cells (A.W. Smith, *Adv. Drug Delivery Rev.*, 57: 1539 (2005)).

[0006] In addition to infection of living organisms, biofilm producing bacteria cause biofouling of surfaces exposed to aquatic environments. For example, surfaces of ships such as the hull, offshore marine structures such as oil rigs, sea water conduit systems for seaside plants, buoys, heat-exchangers, cooling towers, de-salination equipment, filtration membranes, docks, and the like may all experience some degree of fouling when continually exposed to water. In the case of ships, fouling can inhibit vessel performance and capabilities. For example, fouling may substantially increase fuel consumption and may necessitate extensive and more frequent maintenance, all of which raise the overall costs of operation. Biofouling can have a direct adverse economic impact when it occurs in industrial process waters, for example in cooling waters, metal working fluids, or other recirculating water systems such as those used in papermaking or textile manufacture. If not controlled, biological fouling of industrial process waters can interfere with process operations, lowering process efficiency, wasting energy, plugging the water-handling system, and even degrade product quality.

SUMMARY OF THE INVENTION

[0007] One aspect of the invention relates to a composition for inhibiting bacterial biofilm formation comprising a carrier and an effective amount of an inhibitor of squalene/phytoene synthesis. In one embodiment, the inhibitor of squalene/phytoene synthesis inhibits HMG-CoA Reductase. In one embodiment, the inhibitor of squalene/phytoene synthesis inhibits squalene synthase. In one embodiment, the inhibitor of squalene/phytoene synthesis inhibits 1-deoxy-D-xylulose 5-phosphate synthase. In the various embodiments described herein, the inhibitor of squalene/phytoene synthesis may be selected from the group consisting of a phosphonosulfonate, a statin, zaragozic acid,

clomazone, and lapaquistat acetate or a functional derivative thereof. In the various embodiments described herein, the inhibitor of squalene/phytoene synthesis is a phosphonosulfonate or a functional derivative thereof. In one embodiment, the phosphonosulfonate is selected from the group consisting of BPH-652, BPH-689, BPH-700. In one embodiment, the inhibitor of squalene/phytoene synthesis described in the various embodiments above is a statin or a functional derivative thereof. In one embodiment, the statin is selected from the group consisting of mevastatin, lovastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. In the various embodiments described herein the inhibitor of squalene/phytoene synthesis may be zaragozic acid or a functional derivative thereof. In the various embodiments described herein, the inhibitor of squalene/phytoene synthesis may be clomazone or a functional derivative thereof. In the various embodiments described herein, the inhibitor of squalene/phytoene synthesis may be lapaquistat acetate or a functional derivative thereof. In the various embodiments described above, the carrier may be a liquid, a solid, semi-solid, slurry or paste. In the various embodiments described above, the carrier may be a coating agent.

[0008] Another aspect of the present invention relates to a solid or semi-solid substrate comprising an inhibitor of squalene/phytoene synthesis. In one embodiment, the inhibitor of squalene/phytoene synthesis is deposited or absorbed to a surface of the substrate with a composition described above. In one embodiment, the substrate described may be formulated to contain the inhibitor of squalene/phytoene synthesis throughout its entire composition. In one embodiment, one or more of the substrates described herein further comprises additional inhibitors of biofilm or antibacterial agents incorporated therein. In one embodiment, one or more of the substrates described herein is formed as a device, or part thereof, for implantation into a living subject.

[0009] Another aspect of the present invention relates to a method for inhibiting bacterial biofilm formation comprising contacting biofilm producing bacteria with an effective amount of an inhibitor of squalene/phytoene synthesis. In one embodiment, the inhibitor inhibits HMG-CoA Reductase. In one embodiment, the inhibitor is a statin or a functional derivative thereof. In one embodiment of the various methods described herein, the inhibitor inhibits squalene synthase. In one embodiment of the various methods described herein, contacting occurs *in vivo*.

In one embodiment, the contacting occurs in a mammal. In one embodiment of the various methods described herein, the contacting occurs *in vitro*. In one embodiment of the various

methods described herein, contacting occurs in a non-living medium. In one embodiment, the inhibitor is formulated as an antiseptic. In one embodiment of the various methods described herein, the inhibitor is a phosphonosulfonate. In one embodiment, inhibitor inhibits 1-deoxy-D-xylulose 5-phosphate synthase. In one embodiment, the inhibitor is clomazone or a functional derivative thereof. In one embodiment of the various methods described herein, the inhibitor is zaragozic acid, a statin, or lapaquistat acetate or a functional derivative thereof. In one embodiment of the various methods described herein, the statin is selected from the group consisting of mevastatin, lovastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. In one embodiment of the various methods described herein, the phosphonosulfonate is selected from the group consisting of BPH-652, BPH-689, BPH-700. In one embodiment of the various methods described herein, contacting occurs in the presence of an additional agent that impacts the growth and/or attachment and/or virulence of a biofilm forming organism.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figures 1A to 1B contain data that indicate that *yisP* affects the pathway to biofilm formation. Figure 1A is a schematic representation of the signaling pathway leading to biofilm formation in *B. subtilis*. The pathway is triggered by activation of the master regulator Spo0A, via phosphorylation by KinC. Dashed lines represent indirect activation. Figure 1B is a schematic representation of putative metabolic pathway to the formation of distinct polysoprenoids in *B. subtilis*. Enzymes discussed in the text are written in red, next to the reaction they catalyze. Dashed lines represent unknown steps.

[0011] Figure 2 contains data that show that YisP has squalene synthase activity and is involved in the production of a carotenoid. Figure 2 is a bar graph that represents data from experiments showing the enzymatic activity of purified YisP from *B. subtilis* under different conditions. FPP at 3.7 μ M was used as substrate under the optimal conditions specified in Figure 5. Control reaction was performed with no enzyme added.

[0012] Figure 3 is a schematic representation of the two distinct biochemical pathways to produce squalene in *B. subtilis* and *S. aureus*, and where inhibition of biofilm formation by sterol-lowering drugs occurs within these pathways. Zaragozic acid acts as a competitive inhibitor in both routes, since it acts downstream of the formation of FPP. Statins such as mevastatin and lovastatin inhibit the enzyme HMG-CoA reductase and

thus, the route to produce squalene in *S. aureus*. Clomazone inhibits the enzyme 1-deoxy-D-xylulose 5-phosphate synthase and thus, the route to produce squalene in *B. subtilis*.

[0013] Figure 4A shows that *yisP* affects the pathway to biofilm formation upstream of Spo0A. Figure 4A is a schematic representation of the regulatory pathway activated by Spo0A to induce biofilm formation. The active phosphorylated form of Spo0A (Spo0A~P) inhibits the expression of two regulatory repressors, AbrB and SinR, which in turn inhibit the expression of the structural genes downstream of Spo0A. Dashed lines indicate the regulation is not direct.

[0014] Figures 5A to 5B contain data that show enzymatic characterization of YisP as a squalene synthase. Figure 5A is a graph of enzymatic activity of purified YisP using farnesyl pyrophosphate (FPP) as a substrate. The $V_{\max}$ and K_m [FPP] of the equation are presented as m1 and m2, respectively. Figure 5B contains numeric data for biochemical characterization of YisP, for example, optimal conditions and different affinities for several substrates (represented as their respective K_m values). The typical competitive inhibitor of squalene synthases, zaragozic acid, inhibits the activity of the hypothetical squalene synthase YisP (IC_{50} represented). YisP acts as a magnesium-dependent squalene synthase, which preferentially uses FPP as a substrate. GPP is geranyl pyrophosphate and GGPP is geranylgeranyl pyrophosphate.

[0015] Figures 6A to 6B show graphical representation of chemical analyses of extracts from $\Delta yisP$ mutant. Figure 6A is a comparison of the molecular traces of the wild-type strain and the $\Delta yisP$ mutant analyzing the extract from the pellet of cultures using LC-MS. The wild type trace has been offset to overlay the two traces when observed at 280 nm. The major differences are two peaks present in the wild type whose mass were determined to be 738 Da and 600 Da. Figure 6B is a UV-VIS spectrum of the major different peak eluting at 16.9 min on the wild type trace. This spectrum does not match any previously identified molecule and no clear identification could be made.

[0016] Figure 7 shows that exogenous polysoprenoids restore biofilm formation to a *yisP* mutant. Biofilm formation requires the production of an extracellular matrix, which is correlated with the amount of wrinkles observed in the colony (Branda et al. 2001 PNAS 98:11621-11626; Branda et al. 2006 Mol Microbiol 59:1229-1238). Wild type and the $\Delta yisP$ mutant represent positive and negative controls, respectively. Treatment with diverse polyisoprenoids (squalene, β -carotene, and retinol) partially restored the formation of wrinkles in the $\Delta yisP$ mutant.

[0017] Figure 8 is a graphical representation of quantitative analysis of biofilm formation in *S. aureus* in the presence of different concentrations of statins. The crystal violet associated with biofilms was dissolved in ethanol, measured at OD = 595 and used as a read out to quantitatively assay biofilm formation in the presence of different statins. Positive (WT) and negative ($\Delta sarA$) controls are shown along with the samples treated with the different inhibitors. Crystal violet measurements are in arbitrary units.

[0018] Figure 9 is a graphical representation of data obtained from quantitative analysis of the activation of the route of biofilm formation in presence of zaragozic acid. Matrix production was monitored using the reporter P_{yqxM} -*yfp*, a transcriptional fusion of the operon *yqxM-sipW-tasA* responsible for the expression of the matrix-associated protein TasA. Signal was measured in cultures treated and non-treated with zaragozic acid using flow cytometry. At 24 h of growth in the solid medium MSgg, most of cells show maximum expression of the *yqxM* operon (profile with peak at the right). When zaragozic acid was added, the expression of *yqxM* operon dramatically dropped in most cells (profile with peak in between). Control is represented by a non-labeled strain (profile with peak to the left). No defect in growth was detected.

[0019] Table 1: Proteins present in the DRM of *B. subtilis* and *S. Aureus*. Proteins associated to the DRM fraction of *B. subtilis* and *S. aureus* were sequenced by mass spectrometry are listed. KinC has an asterisk to notice that it was detected in DRM fractions using western blot.

[0020] Table 2: Strain list

[0021] Table 3: Primer and mutant list

[0022] Table 4: Bacteria which synthesize squalene from GA3P

[0023] Table 5: Bacteria which synthesize squalene from HMG-CoA

[0024] Table 6: Ratio of FloT-YFP puncta present in the cytoplasmic membrane under different conditions. Counting of the membrane rafts was done by visualization of the number of domains in the membrane where the translational fusion *floT-yfp* localizes. Number of cells analyzed was 200 (N = 200). Standard deviation is shown.

[0025] Table 7: Differential Gene expression of the $\Delta yisP$ mutant compared to the wild type.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0026] An “effective amount”, as the term is used herein, refers to an amount that inhibits biofilm formation in the intended context. Such context may be in the presence or absence of other agents that inhibit the bacterial growth or biofilm formation/function.

[0027] “Inhibit”, as the term is used herein, refers to either partial or complete inhibition of biofilm formation, and is expected to be a reproducibly detectable, statistically significant amount of inhibition, as determined by means known in the art.

[0028] An “indwelling device” is a device that is invasive, placed in or planted within the body, and is associated with a risk of infection.

[0029] A “carrier”, as the term is used herein, is an agent or combination of agents formulated to facilitate functional delivery of the inhibitor of squalene/phytoene synthesis to the desired location (e.g., to an external surface, or for *in vivo* administration). The form the carrier takes will depend upon and/or dictate the intended use of the composition. For example, the carrier may be in liquid form as a solution, dispersion, emulsion, suspension, paste, powder, solid or semi-solid, to result in a composition of similar form.

[0030] “Coating agents” are formulations whereby when applied to a surface, a layer or residue of an effective amount of the inhibitor is left deposited on that surface, to thereby inhibit biofilm formation on the surface. Examples of coating agents include, without limitation, paints, stains, sealants, waxes, and cleaning products such as disinfectants.

[0031] A “substrate surface”, as the term is used herein, refers to the specific surface on which the inhibitor is to be delivered (e.g., via a coating agent). The surface is either external or internal, and is exposed to fluid which may contain biofilm forming bacteria.

As the term is used herein, “contacting” refers to the accomplishment of physical contact of an inhibitor to a bacteria, to an extent which promotes inhibition of biofilm formation by the inhibitor of squalene/phytoene synthesis contained within the agent.

[0032] The term “multicellular organism” is used to refer to an organisms which may be subject to the attack of a biofilm producing organism. The multicellular organisms can be an animal, e.g. mammal. Mammals include rodents (e.g., mice, rats, rabbits, guinea pigs) livestock and pets (e.g., goats, sheep, horses, pigs, cattle, cats, dogs) and primates (humans, chimpanzees, gorillas, etc.). The terms subject and patient are also used to refer to such an animal.

[0033] Aspects of the present invention relate to the finding that biofilm formation in bacteria is dependent upon squalene/phytoene synthesis. Inhibition of this synthesis, e.g. by inhibiting one or more components of the synthetic pathway, inhibits the ability of the bacteria to produce biofilm. Such inhibition has several advantageous applications since the

production of biofilm causes a wide variety of detrimental consequences, from increasing resistance of pathogens to host immune defenses, to aggregation of biofouling organisms to non-living surfaces to cause degradation.

[0034] One aspect of the present invention relates to a composition for inhibiting biofilm formation. The composition comprises an effective amount of an inhibitor of squalene/phytoene synthesis and a carrier. The carrier is an agent for delivery of the inhibitor to the location where one desires to inhibit biofilm producing bacteria. There are two known pathways of squalene synthesis in bacteria, illustrated in Figure 3. Strains such as *B. subtilis* synthesize squalene from GA3P, whereas strains such as *S. aureus* synthesize squalene from HMG-CoA. Both pathways converge downstream to produce IPP. The GA3P pathway is characterized in Takahashi et al., (*PNAS USA* 95: 9879-9884 (1998)). Bacteria that synthesize squalene from GA3P can be inhibited by specific inhibition of this upstream pathway (e.g., inhibition of 1-deoxy-D-xylulose 5-phosphate synthase). Bacteria which are known to synthesize squalene from GA3P are listed in Table 4. The HMG-CoA pathway is characterized in Imogen Wilding et al., (*Journal of Bacteriology*, 182: 4319-327 (2000)). Bacteria that synthesize squalene from HMG-CoA can be inhibited by specific inhibition of this upstream pathway (e.g., inhibition of HMG-CoA Reductase). Bacteria which are known to synthesize squalene from HMG-CoA are listed in Table 5. Both sets of strains of bacterial can be inhibited by utilization of inhibitors which act after the pathway convergence (e.g., inhibition of squalene synthase). Inhibition of the upstream pathway is lethal to the bacteria due, perhaps due to the inhibition of synthesis of other essential factors, however, inhibition of the shared downstream portion of the pathway has surprisingly been shown to be non-lethal. As such, the inhibition of biofilm formation by inhibition of the downstream pathway should promote little to no resistance in the target bacteria.

[0035] In one embodiment, the inhibitor of squalene/phytoene synthesis inhibits HMG-CoA reductase. HMG-CoA reductase is inhibited, for example, by statins. Statins are thought to competitively inhibit the bacterial HMG-CoA similarly to their inhibition of human HMG-CoA reductase. A number of statin molecules are known in the art, such as mevastatin, lovastatin (U.S. Patent No. 4,231,938), atorvastatin (U.S. Patent No. 4,681,893; U.S. Patent No. 5,273,995), cerivastatin (U.S. Patent No. 5,006,530; U.S. Patent No. 5,177,080), fluvastatin (U.S. Patent No. 4,739,073), pitavastatin (U.S. Patent No.s 5,011,930; 5,856,336; 5,872,130), pravastatin (U.S. Patent No. 4,346,227), rosuvastatin (U.S. Patent No. 5,360,440), and simvastatin (U.S. Patent No. 4,444,784). Methods of preparation of the statins are disclosed in U.S. Patent No. 6,777,552. The actual form of the statin used in the

compositions and methods described herein will depend upon the intended use. For example, lovastatin and simvastatin are typically administered to a subject in lactone form, and are converted to the active hydroxy acid in the liver. It may be preferable to instead use the hydroxy acid (prodrug) form itself, especially in situations where such processing is not expected to significantly occur. The salts of the specific statin may also be used, e.g. the sodium salt of pravastatin, or the calcium salt of atorvastatin, rosuvastatin, or pitavastatin. It may be useful to include in the compositions and methods described herein, agents which help stabilize and/or solubilize the statin molecule (e.g., a basifying agent, such as magnesium oxide). Such agents are described, for example, in U.S. Patent No. 5, 180,589). In one embodiment, the composition of the present invention specifically excludes inclusion of one or more statins (e.g., the specific statins disclosed herein). Other known inhibitors of HMG-CoA reductase can also be used in the compositions and methods described herein.

[0036] In one embodiment, the inhibitor of squalene/phytoene synthesis inhibits 1-deoxy-D-xylulose 5-phosphate synthase. 1-deoxy-D-xylulose 5-phosphate synthase is inhibited, for example, by clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone, described in U.S. Patent No. 4,405,357). Clomazone is a potent herbicide. Other known inhibitors of 1-deoxy-D-xylulose 5-phosphate synthase can also be used in the compositions and methods described herein.

[0037] In one embodiment, the inhibitor of squalene/phytoene synthesis inhibits squalene synthase. Squalene synthase is inhibited, for example, by zaragozic acid. Zaragozic acids are a family of natural products produced by fungi. This family of natural products possesses a unique 4,8-dioxabicyclo[3.2.1]octane core. Zaragozic acids are potent inhibitors of *S. cerevisiae*, fungal and mammalian squalene synthase and therefore inhibitors of sterol synthesis (Bergstrom et al., *Annu. Rev. Microbiol.* **49**: 607–39 (1995)). Squalene synthase is the first committed enzyme in sterol synthesis, catalyzing the reductive condensation of farnesyl pyrophosphate to form squalene (Do et al., *Clin. Genet.* **75** (1): 19–29 (2009)). As a squalene synthase inhibitor, zaragozic acid produces lower plasma cholesterol levels in primates (Bergstrom et al., *Annu. Rev. Microbiol.* **49**: 607–39 (1995)). Treatment of rats with zaragozic acid A caused an increase in hepatic low density lipoprotein (LDL) receptor mRNA levels (Ness et al., *Arch. Biochem. Biophys.* **311** (2): 277–85 (1994)). Zaragozic acids also inhibit Ras farnesyl-protein transferase (Dufresne et al., *J. Nat. Prod.* **56** (11): 1923–9 (1993)).

[0038] Squalene synthase is also inhibited by lapaquistat acetate, the active ingredient in TAK 475, is a known squalene synthase inhibitor (Nishimoto et al., *British Journal of*

Pharmacology 139: 911-918 (2003)). Its chemical name is 1-[(3*R*,5*S*)-1-(3-acetoxy-2,2-dimethylpropyl)-7-chloro-5-(2,3-dimethoxyphenyl)-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetyl]piperidine-4-acetic acid (Nishimoto et al., *Br J Pharmacol* 139(5):911-8 (2003)).

[0039] Phosphonosulfonates, also referred to in the art as α -phosphonosulfonates, are also inhibitors of squalene synthase. Phosphonosulfonates and their synthesis are known in the art (U.S. Patent No. 5,712,396, U.S. Patent No. 5,618,964, U.S. Patent No. 5,567,841, U.S. Patent No. 5,332,728). Without limitation, the phosphonosulfonates BPH-652, BPH-698, and BPH-700, BMS-187745 and BMS-188494, can be used in the present invention.

[0040] In addition, other molecules known to inhibit squalene synthase include, without limitation, bisphosphonates (e.g. those disclosed in U.S. Patent No. 5,157,027, U.S. Patent No. 4,871,721), and phosphinylformic acid (U.S. Patent No. 5,025,003). RPR 107393 {3-hydroxy-3-[4-(quinolin-6-yl)phenyl]-1-azabicyclo[2-2-2]octane dihydrochloride} and its R and S enantiomers is another squalene synthase inhibitor (Amin et al., *J Pharmacol Exp Ther* 281: 746-752 (1997)). ER-27856 (4-[N-[(2*E*)-3-(2-Methoxyphenyl)-2-butenyl]-N-methylamino]-1,1-butyldienebisphosphonic acid tris (pivaloyloxymethyl) ester) is another squalene synthase inhibitor (Hiyoshi et al., *J Lipid Res* 41: 1136-44 (2003); Hiyoshi et al., *J Lipid Res* 44: 128-35(2003)). Quinuclidine derivatives comprising pyrrolidine derivatives are also known squalene synthase inhibitors (U.S. Patent Publication 2004/0073045), as are N-aryl-substituted cyclic amine derivatives such as those disclosed in U.S. Patent Publication 2004/0072830 and U.S. Patent No. 7,112,593. BMS-187745 (chemical name (S)-(-)-4-(3-Phenoxyphenyl)-1-phosphonobutanesulfonic acid), and its prodrug ester, BMS-188494, are also inhibitors of squalene synthase (U.S. Patent No. 5,712,396; Charlton-Menys et al., *Drugs* 67: 11-16 (2007); Flint et al., *Toxicol Appl Pharmacol* 145: 91-98 (1997); Sharma et al., *J Clin Pharmacol* 38: 1116-1121 (1998)). EP2300 compounds, such as EP2306 and EP2302, two novel 2-biphenylmorpholine derivatives, also inhibit squalene synthase (Tavridou et al., *Eur J Pharmacol* 535: (2006)). Other known squalene synthase inhibitors can also be used in the compositions and methods described herein.

[0041] It may be useful to utilize multiple inhibitors of squalene/phytoene synthesis (e.g. of one or both pathways, or multiple inhibitors which act after pathway convergence) in a composition, substrate, or method described herein. It may further be useful to also include inhibitors which act on one or both upstream pathways (e.g., selected from the inhibitors disclosed herein) in combination with one or more inhibitors which act downstream after pathway convergence (e.g., selected from the inhibitors disclosed herein). The compositions

described herein can be used to prevent biofilm formation, and/or can be applied to existing bacteria in a biofilm to help degrade the biofilm.

[0042] In addition to the inhibitors described herein, the skilled practitioner will recognize that functional derivatives of the inhibitors may be generated which retain their inhibitory properties. As such, the use of such functional derivatives in the methods and compositions described herein is another aspect of the invention. A functional derivative can be the molecule itself with an additional component, e.g. derivatized to enhance half-life, delivery, solubility, etc. Alternatively, a functional derivative can be a portion of the molecule which retains the desired biological activity (biofilm inhibition).

[0043] The carrier facilitates functional delivery of the inhibitor of squalene/phytoene synthesis to the desired location where biofilms may form (e.g., to an external surface, or for *in vivo* administration).

[0044] In one embodiment, the carrier is a coating agent. Coating agents are formulations whereby when applied to a surface, a layer or residue of an effective amount of the inhibitor is left deposited on that surface, to thereby inhibit biofilm formation on the surface. Examples of coating agents include, without limitation, paints, stains, sealants, waxes, and cleaning products such as disinfectants. In one embodiment, the coating agent carrier is a polymer coating.

[0045] The coating agent will be formulated for the specific surface on which the inhibitor is to be delivered (a substrate surface). In one embodiment, the coating agent is formulated to adhere to or be absorbed by silicone. In another embodiment, the coating agent is formulated to adhere to or be absorbed by a solid polymer (e.g. to a polymeric substrate such as polyvinyl chloride). In another embodiment, the coating agent is formulated to adhere to a metal or a metallic surface (e.g., to inhibit fouling by biofilm producing bacteria, such as degradation of components as in rust, or to inhibit clogging). Metallic and other such surfaces exposed to liquids in which the organisms exist include, without limitation, maritime vehicles and equipment, equipment used in water purification, transport and storage, oil and gas pipelines, cooling towers, heat exchangers, warm water systems, filter systems, water treatment membranes. In one embodiment, the carrier is an "active coating" such as those developed for deposition and delivery of antibacterial agents to a surface.

[0046] It may be useful to further include other anti-fouling or biocidal or antibacterial agents in the compositions described herein. In one embodiment, the carrier itself is a coating agent with antibacterial properties, e.g., a passive coating or as an active coating, each of which themselves, may have other antibacterial properties.

[0047] A number of synthetic surface and coatings that resist bacterial colonization are known in the art and can be formulated to contain (act as carriers of) the inhibitors of squalene/phytoene synthesis described herein. "Passive coatings" reduce bacterial adhesion by altering the physiochemical properties of the substrate so that conditioning films do not form and/or bacteria-substrate interactions are not favorable. Examples of passive coatings are poly(ethylene glycol) (Kingshott et al., *Langmuir*, 2003, **19**, 6912), poly(ethylene oxide) brushes (Kaper et al., *J. Biomater. Sci., Polym. Ed.*, 2003, **14**, 313), hydrophilic polyurethanes (Nagel et al., *J. Biomater. Sci., Polym. Ed.*, 1996, **7**, 769). Active coatings are designed to release high initial fluxes of agents (e.g., antibacterial) during the critical short term post-implantation period (hours) to inhibit the initial adhesion of bacteria. Coatings which actively release agents (e.g., antibiotics) over a longer time period (weeks to months) have also been developed. Such coatings can be adapted for deposition and release of the inhibitors disclosed herein.

[0048] One example is controlled delivery from polymer coatings (e.g., polyurethane, silicone, rubber, polyhydroxyalkanoates etc.) (Schierholz et al., *Biomaterials*, 1997, **18**, 839; Rossi et al., *Antimicrob. Chemother.*, 2004, **54**, 1013). In addition, an additional thin polymer layer can be applied on top of the agent-loaded polymer (e.g., via radio-frequency glow discharge plasma deposition (Kwok et al., *J. Controlled Release*, 1999, **62**, 301). Another example is agent release from biodegradable polymers (e.g., poly(propylenefumarate/methylmethacrylate, collagen, cancellous bone grafts, polyanhydrides, polyorthoesters and polylactide-co-glycolide) (Price, et al., *J. Biomed. Mater. Res.*, 1996, **30**, 281). Another example is agent release from hydroxyapatite coatings (e.g., carbonated hydroxyapatite) (Stigter et al., *ControlledRelease*, 2004, **99**, 127). Another example is anti-infective silver release coatings such as those used for orthopedic fixations pins and silver-coated catheters (Bologna et al., *Urology*, 1999, **54**, 982; Masse et al., *J. Biomed. Mater. Res.*, 2000, **53**, 600; Kumar et al., *Biomaterials*, 2005, **26**, 2081; Kumar et al., *J. Biomed. Mater. Res.*, 2005, **75**, 311; Furno et al., *J. Antimicrob. Chemother.*, 2004, **54**, 1019; Dowling et al., *Surf. Coat. Technol.*, 2003, **163-164**, 637). Another example is controlled release of antibodies to prevent bacterial adhesion (Grainger et al., *Expert Opin. Biol. Ther.*, 2004, **4**, 1029; Poelstra et al., *J. Biomed. Mater. Res.*, 2000, **51**, 224; Rojas et al., *J. Controlled Release*, 2000, **63**, 175; Rediske et al., *Biomaterials*, 2002, **23**, 4565; Poelstra et al., *Tissue Eng.*, 2000, **6**, 401). Another example is nitric oxide release coatings (Marxer et al., *Chem. Mater.*, 2003, **15**, 401; Schoenfisch et al., *Anal. Chem.*, 2000, **72**, 1119; Zhang et al., *Biomaterials*, 2002, **23**, 1485; Frost et al., *Biomaterials*, 2005, **26**, 1685; Nablo et al.,

Biomacromolecules, 2004, **5**, 2034). Another example is the use of lipid-like carriers (e.g., poly-D,L-lactic acid, tocopherol acetate, Softisan 649, Dynasan 118) (Gollwitzer et al., *Journal of Antimicrobial Chemotherapy* 51: 585-591 (2003)). For example, a new anti-infective coating of medical implants to prevent biofilm formation on polytetrafluoroethylene grafts was also recently reported (Matl et al., *Antimicrobial Agents and Chemotherapy* 52: 1957-1963 (2008)) where antibiotics are incorporated into lipid-like carriers (poly-D,L-lactic acid, tocopherol acetate, Softisan 649, Dynasan 118). Another example is antibiotic calcium phosphate coatings (U.S. Patent No. 6,596,338). Another example is coatings which contain enzymes to degrade biofilm structures and/or enzymes with bactericidal effects (U.S. Patent No. 6,830,745). Such coatings can be adapted for delivery of the squalene/phytoene synthesis inhibitors described herein.

[0049] Examples of antibiotics to incorporate into the composition of the present invention include, without limitation, vancomycin, tobramycin, cefamandol, cephalothin, carbenicillin, amoxicillin, ciprofloxacin, and gentamicin.

[0050] In another embodiment, the coating agent is formulated to adhere to a biomaterial surface, such as teeth, bone, skin, etc. In another embodiment, the coating agent is formulated to adhere to or be absorbed by a fabric, cloth or membrane, such as a bandage or other wound dressing. Another example of a membrane is a water treatment membrane. In another embodiment, the carrier is formulated for inclusion into a product for application to a body surface, such as personal care product, to thereby inhibit biofilm formation on the body surface.

[0051] In another embodiment, the carrier is formulated to adhere to a device that is to contact a living medium (the medium around or within a multicellular organism), to thereby inhibit biofilm formation on the device. For example, to be delivered, contacted into, or otherwise implanted, into a living multicellular organism. Such devices are sometimes referred to in the art as indwelling devices. Examples of such devices include, without limitation, catheters, surgical implants, prosthetic devices, surgery tools, endoscopes, contact lenses, etc.

[0052] A composition of the present invention may be prepared in solid form. For example, the carrier and inhibitor(s) may be formulated together as a powder or tablet using means known in the art. The tablets may contain a variety of excipient known in the tableting art such as dyes or other coloring agents, and perfumes or fragrances. Other components known in the art such as fillers, binders, glidants, lubricants, or antiadherents may also be

included. These latter components may be included to improve tablet properties and/or the tableting process.

[0053] The composition may optionally be prepared as a concentrate for dilution prior to its intended use (e.g., application to a substrate surface).

Application of the Composition to the Substrate Surface

[0054] Compositions or formulations that contain an effective amount of an inhibitor of squalene/phytoene synthesis, described herein, can be applied to a substrate surface as an antibiofilm coating. A surface may be treated by applying a suitable amount of a coating that comprises one or more squalene/phytoene synthesis inhibitors described herein. In one embodiment, the coating composition is applied in an amount which is effective to suppress the settlement and/or growth of biofilm forming bacteria and/or enable their facile release by the application of an external shear stress. As known to those of skill in the art, depending on the particular type of surface or surface environment, the mode of applying the coating may vary. In some instances, the composition may be applied to a surface using a brush or mechanical sprayer. In other instances the surface may be dipped, submerged, or infused with the coating.

[0055] Another aspect of the present invention relates to the product which is generated by application of the compositions described herein to a substrate surface. As such, the present invention encompasses such substrates described herein which have an effective amount of the inhibitor of squalene/phytoene synthesis deposited on or absorbed to their surface, following application of the composition described herein. In one embodiment, the invention does not include live or living substrates, especially human. This includes, without limitation, metal substrates, silicone and other polymeric substrates. This also includes substrates designed for specific products which are particularly susceptible to biofilm formation, e.g, products designed for contact and/or implantation into the body of a multicellular organisms, including without limitation, catheters, medical implants, surgery tools, endoscopes, contact lenses, wound dressings. It further includes substrates designed for products such as components of cooling towers, heat exchanger or warm water systems, pipelines (e.g., oil, gas, water).

[0056] To be effective, the inhibitor described herein will be available to at least some of the bacteria such that it may inhibit at least some of the bacteria's squalene/phytoene synthesis. This can occur, for example, through slow release of the inhibitor from the formulation, composition, or substrate described herein into the surrounding environment.

Biofouling

[0057] In one aspect, the invention relates to a composition which comprises a carrier and an effective amount of an inhibitor of squalene/phytoene synthesis as described herein, which is formulated for application to a substrate surface (e.g., non-living) to inhibit biofouling of the surface. Two parallel lines of coatings research and development aimed at reducing fouling have predominated: biocide containing coatings and low surface energy, "non-stick," fouling release coatings. Such coatings may optionally contain other additional components to prevent microbial growth and/or biofilm production.

[0058] Such coatings may be formulated to contain resins (e.g., aldehyde resins), plasticizers, film consumption regulators, solvents.

[0059] Resins such as aldehyde resins are easily prepared by the alkaline autocondensation of acetaldehyde, propionaldehyde, butyraldehydes or mixtures of these aldehydes. Aldehyde resins useful as vehicle in the present invention are prepared by alkaline condensation of starting compounds of the general formula $Ra-CH(OH)-Rb-CH_2O$, where Ra and Rb are non-aromatic organic residues thereby conducting the condensation with elimination of water and other volatile substances in such a way that the final product of condensation preferably contains about 4 to 6 carbon atoms per oxygen atom present in the resin molecule.

[0060] Phthalate plasticizers such as dioctyl phthalate, dimethyl phthalate or dicyclohexyl phthalate; aliphatic dicarboxylate plasticizers such as diisobutyl adipate or butyl sebacate; glycol ester plasticizers such as diethylene glycol dibenzoate or pentaerythritol alkanedioate; phosphate plasticizers such as tricresyl phosphate or trichloroethyl phosphate; epoxy plasticizers such as epoxidized soybean oil or epoxidized octyl stearate; and other plasticizers such as trioctyl trimellitate or triacetin.

[0061] Film consumption regulators are used to retard the rate of dissolution of the surface coating of the present invention. These include, without limitation, chlorinated paraffin, oil, wax, vaseline and liquid paraffin, polyvinyl ether, polypropylene sebacate, partially hydrogenated terphenyl, polyvinyl acetate, polyalkyl (meth)acrylate, alkyd resin, polyester resin, polyvinyl chloride, silicone, epoxy resin, polyurethane resin, urea resin and other hydrophobic polymers having satisfactory compatibility and a low glass transition temperature which retard the rate of dissolution of the paint are useful in the present invention. Other additives which promote film consumption such as monobasic cyclic

organic acids such as rosin, monobutyl phthalate or mono-octyl succinate; oleic acid and castor oil acid, may also be used.

[0062] Solvents include, without limitation, hydrocarbons such as xylene, toluene, ethylbenzene, cyclopentane, octane, heptane, cyclohexane or white spirit; ethers such as dioxane, tetrahydrofuran, ethylene glycol monomethyl ether, ethylene glycol monoethyl ether, ethylene glycol dimethyl ether, ethylene glycol monobutyl ether, ethylene glycol dibutyl ether, diethylene glycol monomethyl ether or diethylene glycol monoethyl ether; esters such as butyl acetate, propyl acetate, benzyl acetate, ethylene glycol monomethyl ether acetate or ethylene glycol monoethyl ether acetate; ketones such as methyl isobutyl ketone or ethyl isobutyl ketone; and alcohols such as n-butanol or propyl alcohol.

Substrates Formulated to Contain the Inhibitor

[0063] Another aspect of the present invention relates to a substrate that is formulated to contain the inhibitor of squalene/phytoene synthesis within/throughout its entire composition to thereby inhibit biofilm formation. Such substrates have the inhibitor(s) added during their generation. Such substrates may be created to take any useful form, e.g., solid, semi-solid, or gels. Without limitation, examples are polymers (e.g., polyethylene, silicone, polyvinyl chloride, polypropylene, polystyrene, polytetrafluoroethylene, polyurethane, polyamide, polyacrylamide, resins). In one embodiment, the substrate is a formed solid, which has been formed into the desired shape and becomes solid or semi-solid upon curing/hardening during the process of manufacture. In one embodiment, the substrate is ductile or plastic, or malleable (e.g., a fabric, a moveable pliable solid, a gel such as a hydrogel)). Substrates which resist or prevent biofilm formation are useful in the production of products which are resistant to biofilm formation, such as surgical implants, artificial heart valves, catheters, membrane filtration devices, materials for wound treatment (e.g., chronic wounds). Such substrates may further comprise additional inhibitors of biofilm formation or antibacterial agents incorporated therein, as described herein and in the art. In one embodiment, the substrate of the present invention specifically excludes inclusion of one or more statins (e.g., the specific statins disclosed herein).

Methods of Inhibiting Biofilm Formation

[0064] Another aspect of the present invention relates to a method for inhibiting bacterial biofilm formation comprising contacting a biofilm producing bacteria with an effective amount of an inhibitor of squalene/phytoene synthesis, as described herein. Such methods

utilize one or more of the inhibitors of squalene/phytoene synthesis described herein. In one embodiment, the inhibitor(s) is in the form of a composition and/or substrate for inhibition of biofilm formation, discussed herein. Such methods may further include the contacting of the biofilm producing bacterial with one or more agents that affects the growth and/or attachment and/or virulence of a biofilm producing bacterial, such as biocidal agents, bacteriostatic agents, antibacterial agents (e.g., antibiotics) and/or other biofilm inhibiting agents (e.g., in addition to the squalene/phytoene synthesis inhibitors disclosed herein) known in the art.

[0065] The inhibitor may be contained within an agent for delivery of the inhibitor to the desired location for the contacting. The agent can be a composition or formulation, or a substrate described herein. The agent may retain the inhibitor in a form which functions to inhibit biofilm formation upon contact of the bacteria to the agent. Alternatively, the agent may release the inhibitor (e.g., slowly over time) such that it sufficiently contacts the bacteria to thereby inhibit biofilm formation.

[0066] In one embodiment, the contacting occurs *in vivo*, e.g., as described herein for *in vivo* uses of formulations, compositions and substrates of the present invention. *In vivo* contacting includes contacting an external surface of the body of a multicellular organism (e.g., an animal as described herein), as well as contacting internal to the body of a subject. In one embodiment, the *in vivo* contacting occurs in the presence of one or more additional agents that impacts the growth and/or virulence of a pathogenic biofilm forming organism, such as microbiocidal agents, bacteriostatic agents, antibacterial agents (e.g., antibiotics) and/or biofilm inhibiting agents (e.g., in addition to the squalene/phytoene synthesis inhibitors disclosed herein) known in the art. The additional agent may be present in the same formulation, or may be contacted (e.g., administered) separately to the subject. In one embodiment, the method excludes the administration (e.g., intravenous, oral) of statins (e.g., one or more specific statins disclosed herein) with a pharmaceutically acceptable carrier, *in vivo*, to the subject (e.g., human). In one embodiment, the method excludes the administration (e.g., intravenous, oral, topical) of an inhibitor (e.g., of squalene synthase inhibitor such as a phosphonosulfonate disclosed herein) in mice.

Administration

[0067] *In vivo* administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal, and transdermal), oral or parenteral. Parenteral administration includes intravenous, subcutaneous, intraperitoneal or intramuscular injection, pulmonary

administration, e.g. , by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration.

[0068] The route of administration may be intravenous (I.V.), intramuscular (I.M.), subcutaneous (S.C.), intradermal (I.D.), intraperitoneal (I.P.), intrathecal (I.T.), intrapleural, intrauterine, rectal, vaginal, topical, intratumor and the like. The compounds of the invention can be administered parenterally by injection or by gradual infusion over time and can be delivered by peristaltic means. Administration may be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays, for example, or using suppositories. For oral administration, the compounds of the invention are formulated into conventional oral administration forms such as capsules, tablets and tonics.

[0069] For topical administration, the pharmaceutical composition is formulated into ointments, salves, gels, or creams, as is generally known in the art. In one embodiment, the inhibitor is formulated as an antiseptic.

[0070] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered can be determined by the skilled practitioner for each individual.

[0071] For *in vivo* delivery, the carrier may be a pharmaceutically acceptable carrier. Compositions for *in vivo* administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Compositions for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Common additives such as surfactants, emulsifiers, dispersants, and the like may be used as known in the art to increase the solubility of the inhibitor, as well as other components in a composition or system. Such

materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

[0072] In another embodiment the contacting occurs *in vitro*. In one embodiment, the contacting occurs in a non-living medium, such as on a non-living substrate, as described herein, for out of body use. In one embodiment, the inhibitor is formulated as one or more of the compositions described herein (e.g., to inhibit biofouling, etc.).

[0073] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0074] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[0075] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used to described the present invention, in connection with percentages means $\pm 1\%$.

[0076] In one respect, the present invention relates to the herein described compositions, methods, and respective component(s) thereof, as essential to the invention, yet open to the inclusion of unspecified elements, essential or not (“comprising”). In some embodiments, other elements to be included in the description of the composition, method or respective component thereof are limited to those that do not materially affect the basic and novel characteristic(s) of the invention (“consisting essentially of”). This applies equally to steps within a described method as well as compositions and components therein. In other embodiments, the inventions, compositions, methods, and respective components thereof, described herein are intended to be exclusive of any element not deemed an essential element to the component, composition or method (“consisting of”).

[0077] All patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an

admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[0078] The present invention may be as defined in any one of the following numbered paragraphs.

1. A composition for inhibiting bacterial biofilm formation comprising a carrier and an effective amount of an inhibitor of squalene/phytoene synthesis.
2. The composition of paragraph 1 wherein the inhibitor of squalene/phytoene synthesis inhibits HMG-CoA Reductase.
3. The composition of paragraph 1 wherein the inhibitor of squalene/phytoene synthesis inhibits squalene synthase.
4. The composition of paragraph 1 wherein the inhibitor of squalene/phytoene synthesis inhibits 1-deoxy-D-xylulose 5-phosphate synthase.
5. The composition of paragraph 1, wherein the inhibitor of squalene/phytoene synthesis is selected from the group consisting of a phosphonosulfonate, a statin, zaragozic acid, clomazone, and lapaquistat acetate or a functional derivative thereof.
6. The composition of paragraph 1, wherein the inhibitor of squalene/phytoene synthesis is a phosphonosulfonate or a functional derivative thereof.
7. The composition of paragraph 6, wherein the phosphonosulfonate is selected from the group consisting of BPH-652, BPH-689, BPH-700.
8. The composition of paragraph 1, 2, or 5, wherein the inhibitor of squalene/phytoene synthesis is a statin or a functional derivative thereof.
9. The composition of paragraph 8, wherein the statin is selected from the group consisting of mevastatin, lovastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin.
10. The composition of paragraph 1, 3 or 5, wherein the inhibitor of squalene/phytoene synthesis is zaragozic acid or a functional derivative thereof.
11. The composition of paragraph 1, 4 or 5, wherein the inhibitor of squalene/phytoene synthesis is clomazone or a functional derivative thereof.

12. The composition of paragraphs 1-11, wherein the inhibitor of squalene/phytoene synthesis is lapauistat acetate or a functional derivative thereof.
13. The composition of paragraphs 1-12, wherein the carrier is a liquid.
14. The composition of paragraphs 1-12, wherein the carrier is a solid, semi-solid, slurry or paste.
15. The composition of paragraphs 1-12, wherein the carrier is a coating agent.
16. A solid or semi-solid substrate comprising an inhibitor of squalene/phytoene synthesis.
17. The substrate of paragraph 16, wherein the inhibitor of squalene/phytoene synthesis is deposited or absorbed to a surface of the substrate with a composition of any one of paragraphs 1-15.
18. The substrate of paragraph 16 or 17, wherein the substrate is formulated to contain the inhibitor of squalene/phytoene synthesis throughout its entire composition.
19. The substrate of paragraphs 16-18 which further comprises additional inhibitors of biofilm or antibacterial agents incorporated therein.
20. The substrate of paragraph 18 or 19 which is formed as a device, or part thereof, for implantation into a living subject.
21. A method for inhibiting bacterial biofilm formation comprising contacting a biofilm producing bacteria with an effective amount of an inhibitor of squalene/phytoene synthesis.
22. The method of paragraph 21, wherein the inhibitor inhibits HMG-CoA Reductase.
23. The method of paragraph 21 or 22, wherein the inhibitor is a statin or a functional derivative thereof.
24. The method of paragraph 21, wherein the inhibitor inhibits squalene synthase.
25. The method of paragraph 21-24, wherein contacting occurs *in vivo*.
26. The method of paragraph 25, wherein the contacting occurs in a mammal.
27. The method of paragraph 21-24 wherein the contacting occurs *in vitro*.
28. The method of paragraph 21-24, wherein contacting occurs in a non-living medium.
29. The method of paragraph 28, wherein the inhibitor is formulated as an antiseptic.

30. The method of paragraph 24, wherein the inhibitor is a phosphonosulfonate.
31. The method of paragraph 21, wherein the inhibitor inhibits 1-deoxy-D-xylulose 5-phosphate synthase.
32. The method of paragraph 31, wherein the inhibitor is clomazone or a functional derivative thereof.
33. The method of paragraph 21, wherein the inhibitor is zaragozic acid, a statin, or lapauistat acetate or a functional derivative thereof.
34. The method of paragraph 23, wherein the statin is selected from the group consisting of mevastatin, lovastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin.
35. The method of paragraph 30, wherein the phosphonosulfonate is selected from the group consisting of BPH-652, BPH-689, BPH-700.
36. The method of paragraphs 21- 35, wherein contacting occurs in the presence of an additional agent that impacts the growth and/or attachment and/or virulence of a biofilm forming organism.

[0079] The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

Introduction

[0080] A feature common to all living cells is the presence of a lipid membrane that defines the boundary between the inside and the outside of the cell. Proteins that localize to the membrane serve a number of essential functions. In eukaryotic cells, membrane proteins that mediate signal transduction and protein secretion are often localized in membrane microdomains enriched in certain lipids, such as sterols and sphingolipids. These microdomains are commonly referred to as “lipid rafts” or “membrane rafts” (Pike 2006; Lingwood and Simons 2010).

[0081] The function of proteins associated with lipid rafts depends on the integrity of these areas. Alterations in the conformation of lipid rafts lead to defects in cell-cell signaling processes and transduction pathways in which these proteins are involved. Thus, disruptions

of lipid rafts are associated with a large variety of human diseases including Alzheimer's, Parkinson's, cardiovascular and prion diseases (Michel and Bakovic 2007). Because of their profound importance on cell physiology, these membrane domains are interesting targets for the development of new pharmacological approaches to cure and prevent these diseases.

[0082] Up to now, lipid rafts have been identified and characterized in eukaryotic cells. However, many bacterial membrane proteins involved in cell-cell signaling and signal transduction pathways are distributed heterogeneously across the cytoplasmic membrane (Meile et al. 2006). These observations suggest that specialized membrane microdomains are also a feature of bacterial cells.

[0083] Certain proteins of eukaryotic membranes have been consistently described as being present in lipid rafts (Lingwood and Simons 2010). Most prominent amongst these is the protein alternatively referred to as Flotillin-1 or Reggie which appears to be involved in the orchestration of diverse processes related to signal transduction, vesicle trafficking, and cytoskeleton rearrangement (Langhorst et al. 2005). Interestingly, bioinformatic analyses indicate that most bacterial genomes encode proteins whose amino acid sequences show some similarity to Flotillin-1. While these bacterial proteins have not been extensively investigated, two reports demonstrated that the flotillin-like proteins from the spore-forming *Bacillus halodurans* and *Bacillus subtilis* are heterogeneously distributed in the cytoplasmic membrane displaying a punctate pattern along the entire cell (Zhang et al. 2005; Donovan and Bramkamp 2009). While no exact function was ascribed to these bacterial proteins, a *B. subtilis* mutant lacking the flotillin-like protein showed a delay in the onset of sporulation and reduced sporulation efficiency (Donovan and Bramkamp 2009). Attempts to determine the type of lipid that led to the punctate distribution of the *B. subtilis* flotillin-like protein were inconclusive, finding only that its localization was not dependent on lipids containing phosphatidylglycerol or cardiolipin (Donovan and Bramkamp 2009). Cardiolipin was of particular interest because it had been shown to occur in patches in the *B. subtilis* membrane (Kawai et al. 2004; Matsumoto et al. 2006; Mileykovskaya and Dowhan 2009). Thus, the function and lipid association of bacterial flotillin-like proteins remains poorly understood.

[0084] All members of the Flotillin family of proteins are members of a superfamily of proteins that contains "SPFH" or "PHB" domains (named after the proteins Stomatin, Prohibitin, Flotillin and HflK/C) (Tavernarakis et al. 1999; Browman et al. 2007). SPFH domain-containing proteins are found associated with lipid rafts and are thought to function in many ways, such as in raft formation, kinase activity enhancement, and ion channel regulation (Morrow and Parton 2005; Kato et al. 2006; Browman et al. 2007). Aside from

those bacterial proteins with high sequence similarity to Flotillin-1 presented above, bacteria also encode other SPFH proteins. While these proteins are widely distributed in bacteria, their functions remain poorly understood. The few genetic studies carried out on SPFH proteins have not yielded clear phenotypes; however, they appear to be involved in stress responses such as high salt and antibiotic treatment (Butcher and Helmann 2006).

Importantly, most bacterial genomes encode multiple SPFH proteins and thus some of their function may be redundant.

[0085] Here is presented with evidence that bacteria contain lipid rafts that are functionally similar to those found in eukaryotes in that they harbor and organize proteins involved in signal transduction, small molecule translocation and protein secretion. The lipids associated with the bacterial rafts are probably polyisoprenoids synthesized via pathways that involve squalene synthases; inhibitors of this enzyme interfere with the formation of bacterial lipid rafts. In addition, a function for the lipid rafts is demonstrated: a mutant devoid of SPFH proteins is defective in a signal transduction pathway whose sensor kinase is found in the rafts. All of these results are consistent with the idea that the organization of physiological processes into microdomains may be a more widespread feature of membranes than previously appreciated.

RESULTS

A lipid synthesis gene involved in signaling biofilm formation in *B. subtilis*.

[0086] The existence of bacterial lipid rafts was discovered while conducting analyses of the signaling pathway involved in biofilm formation by *B. subtilis*. Recently, it was reported that the antifungal agent nystatin and the *B. subtilis* molecule surfactin can signal this bacterium to induce biofilm formation by activating a signal transduction pathway whereby the membrane histidine kinase KinC phosphorylates the transcription factor Spo0A (Lopez et al. 2009) (Figure 1A). The fact that nystatin had an observable effect on *B. subtilis* was surprising because the drug specifically inhibits fungal growth. This inhibition is accomplished through nystatin's ability to bind to, and displace from the membrane, the fungal sterol ergosterol (Bolard 1986). Importantly, ergosterol is not synthesized by bacteria. Thus, it was hypothesized that in *B. subtilis* there might exist a membrane molecule analogous to ergosterol involved in the signal transduction pathway leading to biofilm formation.

[0087] While *B. subtilis* membranes do not contain sterols, structurally similar molecules termed sporulenes have recently been described in this bacterium (Bosak et al. 2008; Kontnik

et al. 2008) (Figure 1B). Both ergosterol and sporulenes are synthesized from the common precursor isoprenyl pyrophosphate (IPP). However, while ergosterol is derived from squalene, this is not the case for sporulenes. Instead, sporulenes are synthesized through a pathway involving the product of the *sqhC* gene, a putative polyisoprenoid cyclase that remains uncharacterized (Bosak et al. 2008; Kontnik et al. 2008). *B. subtilis* forms floating biofilms (pellicles) when cultures are left standing undisturbed. These pellicles are detectable by visual analysis of the cultures in a biofilm formation assay (described below), also referred to herein as a pellicle formation assay. The cells are held together in the pellicle by an extracellular matrix. This matrix is composed of an exopolysaccharide produced by the products of the *eps* operon and amyloid-like fibers of the protein TasA, whose formation requires the three gene operon *yqxM-sipW-tasA* (Branda et al. 2004; Branda et al. 2006; Romero et al. 2010). Thus, a double mutant lacking both operons ($\Delta eps \Delta yqxM$) cannot make matrix and does not form pellicles. To test if sporulenes might be involved in biofilm formation, biofilm formation was tested in this assay using different strains of *B. subtilis*. The *sqhC* gene was deleted, and positive and negative controls were the wild-type strain (WT) and the matrix-deficient mutant ($\Delta eps \Delta yqxM$), respectively (Branda et al. 2004; Branda et al. 2006). The $\Delta sqhC$ mutant formed pellicles that were indistinguishable from those formed by the wild type, indicating that sporulenes were not involved in the biofilm formation signaling pathway (data not shown).

[0088] Having ruled out *sqhC* involvement in biofilm formation, a search for genes whose products might synthesize molecules related to squalene was initiated (Figure 1B). A bioinformatics analysis of the *B. subtilis* genome was carried out to identify known or putative genes that might be involved in the pathway(s) from IPP to squalene. From the set of genes thus identified, research efforts were focused on the first non-essential gene found in this putative pathway, *yisP*. The *yisP* gene product is predicted to have sequence similarity with phytoene or squalene synthases (Kobayashi et al. 2003). The product of *yisP* was shown to be capable of generating a C30 polyisoprenoid by the biofilm formation assay. Deletion of *yisP* resulted in a complete loss of pellicle forming ability in the assay (data not shown). Pellicle formation was restored in the $\Delta yisP$ mutant by re-introducing a functional copy of the gene into the neutral *amyE* locus of the chromosome (data not shown).

[0089] To determine where *yisP* acted in the signaling pathway of biofilm formation, a series of epistasis experiments was carried out. Biofilm formation requires phosphorylation of Spo0A (See Figure 1A). To know whether the action of *yisP* was before or after the

phosphorylation of Spo0A, a constitutively active allele of *spo0A*, whose product does not require phosphorylation (termed *sad67*), was introduced into the $\Delta yisP$ mutant (Ireton et al. 1993). This strain formed pellicles in the biofilm formation assay, thus indicating that the product of *yisP* acts upstream of Spo0A phosphorylation and most likely is involved in the function of KinC (data not shown). Because of the pleiotropic effects of expressing the *sad67* allele, the formation of pellicles was not as robust as in the wild type. However, the action of *yisP* upstream of matrix gene expression was confirmed by double mutant analyses, where two repressors of matrix synthesis genes were deleted this resulted in the production of robust pellicles. Introducing a deletion of either *abrB* or *sinR* genes into the $\Delta yisP$ mutant restored the ability of the $\Delta yisP$ mutant to make biofilm in the pellicle formation assay. Positive and negative controls used were the wild-type strain and $\Delta yisP$ mutant, respectively. These results lead to investigation of the hypothesis that YisP is important for the function of KinC.

The product of *yisP* displays squalene synthase activity *in vitro*.

[0090] Since *yisP* was a gene of unknown function, the activity of its product was characterized to gain some insights as to how it might be involved upstream of Spo0A phosphorylation. The *yisP* gene was cloned and expressed in *Escherichia coli*. As expected from its predicted amino acid sequence, purified recombinant YisP has the enzymatic features of a phytoene or squalene synthase and preferentially uses farnesyl pyrophosphate as a substrate (Figure 5) (Lee and Poulter 2008). In addition, YisP enzymatic activity was dependent on NADH and was blocked by nanomolar concentrations of the competitive inhibitor of squalene synthases, zaragozic acid (Figure 2) (Bergstrom et al. 1993). While squalene itself is not a colored compound, many molecules derived from squalene are indeed pigments, e.g., carotenoids. In this respect, it was interesting to discover that the *yisP* mutant lost a dark-orange pigment that is apparent in the wild type. The color differential was visually detectable in bacterial cultures that were pelleted by centrifugation (data not shown). As initial characterization of this change in pigmentation, preliminary lipid analyses were carried out on the membranes of wild type and the $\Delta yisP$ mutant. Lipids were extracted from the pellet of the cultures of the wild-type strain and the $\Delta yisP$ mutant, and liquid chromatography/mass spectroscopy analyses of these were carried out. Wild-type extract displayed two adjacent peaks in its profile that were absent in the profile of the $\Delta yisP$ mutant. These two peaks correspond to molecules with a molecular mass of 600 and 738 (Figures 6A

and 6B). Further analysis of their UV spectra suggested that these molecules are two new and previously unidentified molecules.

[0091] Some lipids were absent in the $\Delta yisP$ mutant. Accordingly, squalene and the two squalene-derived molecules β -carotene and retinol were tested for their ability to restore biofilm formation to the $yisP$ mutant. Biofilm formation was partially restored in the presence of squalene, β -carotene, and retinol (Figure 7). This indicates that a polyisoprenoid lipid, likely to be a C₃₀ triterpenoid, was involved in the signal transduction pathway that activates KinC and results in biofilm formation.

[0092] Motility assays performed indicate that deletion of $yisP$ affects the function of the KinC. The chimera KinC-DegS inhibits motility in response to the signal nystatin, as previously in Lopez et al. (2009 PNAS 106:280-285). The presence of nystatin in the medium reduced colony spreading when plated on swarming agar (data not shown). Deletion of the gene $yisP$ compromised the functionality of the chimeric kinase so the presence of nystatin did not inhibit colony spreading (data not shown). Motility assays were performed according to the protocol published by Kearns et al (2003 Mol Microbiol 49: 581-590).

KinC and flotillin co-localize in cytoplasmic membrane puncta.

[0093] The finding that a squalene-derived polyisoprenoid was involved in signaling during biofilm formation led to a hypothesis that such a lipid could function to organize KinC in membrane microdomains analogous to lipid rafts in eukaryotic cells. This concept was supported by the finding that not all membrane kinases were affected by the absence of the hypothetical polyisoprenoid ($\Delta yisP$ mutant). ComP, a membrane histidine kinase involved in competence development, was shown to remain functional in the $\Delta yisP$ mutant (data not shown). In addition, KinC has been reported to be heterogeneously localized in the membrane of *B. subtilis* (Meile et al. 2006). All of these observations indicate that there lipid rafts may exist in *B. subtilis*. Standard methods developed to characterize eukaryotic membranes were used to determine if similar membrane microdomains exist in *B. subtilis* and to test if indeed KinC localizes to these microdomains.

[0094] Eukaryotic membranes partition into detergent-resistant (DRM) and detergent-sensitive (DSM) fractions. While it is important to emphasize that the DRM fraction is not to be equated with lipid rafts, there is evidence that this fraction includes many of the proteins thought to be present in lipid rafts (Brown 2002). Treatment of eukaryotic cells with Triton X-100 allows for isolation of a DRM fraction and this can be further fractionated by zonal

centrifugation in sucrose gradients. This technique was used to isolate the DRM fraction from *B. subtilis*. The proteins present in the DRM fraction were analyzed by SDS-PAGE in parallel with the proteins present in the DSM fraction (data not shown). Membrane fractionation was performed on wild type (WT), $\Delta yisP$, and WT treated with zaragozic acid (+Z), according to differential sensitivity to detergent solubilization. The membrane fractions sensitive and resistant to detergent solubilization were named DSM and DRM, respectively. Membrane proteins associated with each fraction were visualized in an SDS-PAGE. DRM-associated proteins decreased in $\Delta yisP$ mutant and in the wild-type strain treated with zaragozic acid (+Z). For wild-type *B. subtilis*, the protein profiles from DRM and DSM were dramatically different, suggesting a heterogeneous distribution of lipids and proteins in *B. subtilis* membranes. Importantly, the number and intensity of protein bands in the DRM was greatly decreased in the $\Delta yisP$ mutant or when wild-type cells were treated with zaragozic acid. The DSM fraction of the $\Delta yisP$ mutant still contained significant amounts of protein. However, the protein profile per se displayed some changes probably as a consequence of the pleiotropic effect caused by the mutation in $\Delta yisP$ itself (data not shown).

[0095] In order to detect KinC in either the DSM or DRM fractions, the $\Delta kinC$ mutant was complemented with the translational fusion KinC-YFP (yellow fluorescent protein) and the protein was detected by immunoblotting using monoclonal antibodies against YFP. KinC was present only in the DRM fraction and was not detected in the $\Delta yisP$ mutant or after treatment with zaragozic acid (data not shown). It is possible that in the absence of lipid rafts, KinC is degraded more quickly because it fails to properly localize in the membrane.

[0096] The presence of a sensor kinase in the bacterial DRM fraction suggested a functional similarity with eukaryotes. In eukaryotes, lipid rafts harbor sensor kinases along with protein complexes involved in molecule trafficking and protein secretion (Allen et al. 2007). Thus, the most abundant proteins found in the DRM fraction were identified using mass spectrometry. The majority of the proteins identified were involved in signaling, molecule trafficking, and protein secretion (Table 7). For example, proteins required for uptake of signaling peptides and siderophores, antibiotic export, and protease secretion (Kontinen and Sarvas 1993; Lazazzera et al. 1997; Dorenbos et al. 2002; Ollinger et al. 2006) were detected. As stated above, the proteins identified in the DRM fraction are not necessarily in lipid rafts. However, some of them could be and would thus be expected to co-localize.

[0097] Interestingly, one of the proteins present in the DRM fraction was YuaG, a *B. subtilis* flotillin-like protein, corroborating the results of Donovan and Bramkamp (2009). In eukaryotic cells Flotillin-1 is localized exclusively in lipid rafts and appears to orchestrate diverse processes related to signal transduction, vesicle trafficking, and cytoskeleton rearrangement (Langhorst et al. 2005; Morrow and Parton 2005). YuaG shares 39% amino acid sequence identity (69% similarity) with Flotillin-1. Because of the sequence and localization similarity of YuaG with Flotillin-1, it was renamed to FloT. The membrane distribution of FloT was visualized by constructing a translational fusion with YFP and expression in *B. subtilis*. The resulting strain was used to determine cellular localization of FloT-YFP. If the membrane areas where FloT-YFP localized are indeed analogous to eukaryotic lipid rafts, then KinC, present in the DRM fraction, should co-localize with FloT-YFP. The co-localization of these two proteins was assessed by expressing the fusion proteins KinC-CFP and FloT-YFP simultaneously in a single strain. FloT and KinC was shown to co-localize in a double-labeled strain expressing the translational fusions FloT-YFP and KinC-CFP (data not shown).

[0098] Fluorescence of FloT-YFP was observed distributed in areas along the cytoplasmic membrane (data not shown), consistent with a prior report of heterogeneous distribution of YuaG (Donovan and Bramkamp 2009). This distribution was observed whether the strain producing the FloT-YFP fusion protein contained the wild-type *floT* allele or a deletion allele ($\Delta floT$). (data not shown). On average, approximately six foci of FloT localization per *B. subtilis* cell was detected (Table 6). Distribution of the signal emitted by the translational fusion FloT-YFP was not affected by gene expression or protein levels, as observed in micrographs which showed the distribution pattern of the translational fusion FloT-YFP when expressed under the control of a constitutive artificial promoter in comparison to the distribution pattern when expressed under the control of the natural promoter.

[0099] When cells were treated continuously with zaragozic acid, thus inhibiting the enzymatic activity of YisP, the FloT-YFP translational fusion was rarely detected in the vast majority of the cells. Without being bound by theory, it is thought that inhibition of YisP compromises the integrity of lipid rafts and thus the proteins that are normally present in these membrane regions (such as flotillin) mislocalize and possibly get degraded. To gain a better insight into this loss of visible FloT-YFP, the effect of zaragozic acid treatment on the distribution of FloT-YFP over time was analyzed over several hours. In an analysis of an 8

hour time course following localization of the translational fusion FloT-YFP after the addition of zaragozic acid to the sample, the cells initially (T = 0 h) showed FloT-YFP distributed in numerous discrete foci across the membrane. Two hours after zaragozic acid treatment, these FloT-YFP foci became diffuse throughout the membrane. Diffusion in the localization of FloT-YFP progressed and the level of the signal diminished over the remainder of the time course. Eventually, by eight hours after treatment, little or no FloT-YFP signal could be detected.

[00100] The fact that FloT can localize to discrete foci in membranes containing C₃₀ triterpenoid derived from the enzymatic reaction catalyzed by YisP coupled to complementation of the lack of YisP with several polyisoprenoids, indicates that there is broad latitude with respect to the structure of the lipids that will assemble lipid rafts in *B. subtilis*. In addition to this, these regions can be disrupted by the action of the YisP inhibitor zaragozic acid, which causes FloT to diffuse throughout the membrane, similar to previously reported when lipid rafts are disrupted in eukaryotic cells (Watanabe et al. 2009)

***B. subtilis* SPFH domain-containing proteins enhance KinC activity**

[00101] Since KinC and FloT co-localized, the effect of FloT on KinC function was assessed. When a $\Delta floT$ mutant was tested for KinC activity, the mutant displayed a wild-type phenotype. However, like all flotillin-like proteins, FloT is a member of the superfamily of proteins that contain “SPFH” domains (Tavernarakis et al. 1999; Browman et al. 2007; Hinderhofer et al. 2009). Bioinformatics analysis of the *B. subtilis* genome using the SMART software indicated that this bacterium encodes only one more protein with an SPFH domain, YqfA (Schultz et al. 1998; Letunic et al. 2009). Thus, the effect of eliminating both SPFH domain proteins in *B. subtilis* on the integrity of the lipid rafts and thus, on KinC activity, was determined. A double mutant ($\Delta floT \Delta yqfA$) was constructed and used to characterize the proteins that associated to its DRM fraction by membrane fractionation according to differential sensitivity to detergent solubilization. SDS-PAGE analysis of the proteins associated to the membrane fraction resistant to detergent solubilization DRM in the wild type, the double mutant $\Delta floT \Delta yqfA$ and the $\Delta yisP$ mutant, was performed. Some proteins were missing in the DRM fraction of the $\Delta floT \Delta yqfA$ double mutant as compared to the other strains. Western blot analysis was performed to detect KinC in the DRM fraction of the $\Delta floT \Delta yqfA$ double mutant and $\Delta yisP$ mutant in comparison to the wild type. The Western blot analyses indicated a decrease in the KinC levels in this double mutant (data not

shown). No effects were seen in either of the single mutants, suggesting that these two proteins have redundant function. The KinC activity was tested in the double mutant using an assay that depends on the ability of *B. subtilis* to form pellicles in LB medium in a KinC-dependent manner when surfactin is added (Lopez et al. 2009).

[00102] *B. subtilis* expresses two SPFH domain proteins encoded by FloT and YqfA.

Various deletion mutant strains were used in a biofilm/pellicle formation assay, in the presence and absence of surfactin. The results showed the ability of the $\Delta floT \Delta yqfA$ double mutant to form pellicles in response to the signaling molecule surfactin, when cultured in LB medium (Lopez et al. 2009). The two SPFH-containing proteins in *B. subtilis* FloT and YqfA were deleted. This behavior was seen to be dependent of the histidine kinase KinC, since the *kinC* deficient mutant does not make pellicles when surfactin was added. Pellicles were formed when surfactin was added to the wild-type strain, but not in the *kinC*-deficient background. A weak induction of pellicle formation was observed in the double mutant $\Delta floT \Delta yqfA$ when surfactin was added.

[00103] The $\Delta floT \Delta yqfA$ double mutant phenocopies the $\Delta kinC$ mutant, both being unresponsive to surfactin, as observed in analysis of strains that were deleted in *floT* and *yqfA*. This indicates that indeed in a cell lacking proteins with SPFH domains, KinC activity is compromised. Interestingly, overexpressing KinC in the $\Delta floT \Delta yqfA$ double mutant partially restored pellicle formation in the biofilm pellicle formation assay (data not shown). A possible explanation for this partial restoration of activity would be that FloT might influence KinC activity by increasing local concentrations or promoting multimerization, similar to what has been observed in eukaryotic cells (Browman et al. 2007).

[00104] The dynamic nature of the lipid rafts was indicated by observing the localization pattern of the lipid rafts-associated protein FloT, by following the distribution of the translational fusion FloT-Yfp along the membrane of whole bacteria during a time frame of 1 minute. The distribution at the membrane was seen to change over the course of 1 minute.

[00105] The localization pattern of the SpoIVFB protein homogeneously distributed across the cellular membrane was not affected when treated with zaragozic acid. The membrane-associated metalloprotease SpoIVFB homogeneously distributed across the cellular membrane of *B. subtilis* (Rudner and Losick. 2002 Genes Dev 16:1007-1018), contrasting to the localization in puncta observed in the flotilin-like protein FloT. Moreover, zaragozic acid treatment did not mislocalize the protein SpoIVFB as previously observed in the experiments performed with FloT.

Sequence homologs of Flotillin-1 from other bacteria also show punctate distribution

[00106] Proteins showing sequence similarity to Flotillin-1 are widespread among bacteria. Whether these proteins localize to discrete foci in the membrane in other bacterial species was determined. Fusions of YFP to the Flotillin-1 sequence homologs SA1402 from *Staphylococcus aureus* and YqiK from *E. coli* were constructed. Localization of the translational fusion proteins was determined by detecting the fluorophore YFP in the recipient strains. Distribution of the signal was heterogeneous across the membrane of the bacterium. It was observed that both of the fusion proteins displayed a punctate localization in the membrane. In *S. aureus* the fusion protein localized to a single focus in the bacterial membrane. Consistent with the idea that this microdomain contained a lipid derived from squalene, probably staphyloxanthin or a closely related molecule, localization of the protein was lost after treatment with zaragozic acid (data not shown).

[00107] The finding of a single microdomain in *S. aureus* led to the analysis of the protein content of the DRM fraction in this bacterium in a manner similar to what had been done with *B. subtilis*. Isolation of the membrane fractions of *S. aureus* was performed according to their resistance or sensitivity to detergent. Proteins associated with the detergent-resistant fraction (DRM) and proteins associated with the detergent-sensitive fraction (DSM) were detected by SDS-PAGE. Addition of zaragozic acid to the cultures decreased the proteins detected in the DRM fraction (+Z lane) (data not shown) .

[00108] Proteins associated to the DRM fraction were sequenced and, again, the majority of these proteins function in signal transduction, molecule trafficking, and protein secretion (Table 7). Examples of the proteins present in the DRM fraction are the quorum-sensing regulator involved in virulence, CvfA (Nagata et al. 2008) and the elastin-binding protein EbpS, involved in biofilm formation and tissue colonization (Downer et al. 2002). A Flotillin-1 homolog (SA1402) and a KinC homolog WalK were also identified (Dubrac et al. 2007) as well the protease secretion machinery. This last finding is worthy of note because protease secretion in Gram (+) cocci is known to occur from a single point in the membrane called the ExPortal (Rosch and Caparon 2004). It is presumed that the ExPortal is located in the single membrane raft observed in *S. aureus*. As was observed, treatment with zaragozic acid inhibited protease secretion further supporting the hypothesis that the ExPortal is this single membrane raft (data not shown). A dramatic change in colony color was also observed as a consequence of zaragozic acid treatment (data not shown). This was due to the fact that

the yellow carotenoid staphyloxanthin from *S. aureus* is derived from squalene, thus its synthesis was inhibited by zaragozic acid (Pelz et al. 2005).

Squalene synthesis inhibitors inhibit biofilm formation in *B. subtilis* and *S. aureus*

[00109] Among the proteins found in the DRM fractions of both *B. subtilis* and *S. aureus*, several are involved in the process of biofilm formation. Thus, the effect of treating cultures with small molecule inhibitors that block various points along the pathway leading to squalene synthesis on biofilm formation was assessed.

[00110] *B. subtilis* and *S. aureus* possess different routes to produce squalene (see Figure 3). *S. aureus* has the mevalonate route. This route is also present in humans and can be inhibited by molecules, statins, that act on the key enzyme HMG-CoA reductase (Endo 1981; Wilding et al. 2000). In contrast, *B. subtilis* has the glyceraldehyde-3-phosphate (GA3P) + pyruvate route (Takahashi et al. 1998). This route is also present in plants and can be inhibited by clomazone, a strong inhibitor of 1-deoxy-D-xylulose-5-phosphate synthase (Mueller et al. 2000). Both routes converge into a single pathway with the formation of farnesyl pyrophosphate (FPP). Thus, inhibitors of squalene synthase, such as zaragozic acid, should have an effect on both *B. subtilis* and *S. aureus*. These molecules were tested for their ability to affect biofilm formation in these two bacteria using the biofilm (pellicle) formation assay. Addition of different concentrations of zaragozic acid to the biofilm formation assay of *B. subtilis* and *S. aureus* inhibited biofilm formation. Biofilm formation in *B. subtilis* was observed as a pellicle formed in the surface air-liquid of standing cultures while *S. aureus* forms biofilms attached to the submerged surfaces (at the bottom of the well plate). Crystal violet staining was used in the *S. aureus* assay for better visualization. The effects of a range of drug concentrations were investigated, ranging from 0, 3, 6, 9, and 15 μM zaragozic acid in *B. subtilis*, and from 0, 2, 4, 6, and 10 μM zaragozic acid in *S. aureus*. Pellicle formation was inhibited by as little as 3 and 2 μM , respectively. The assays for each species are different due to the differences in the biofilms they make; *B. subtilis* forms floating pellicles, which can be directly visualized while *S. aureus* forms biofilms attached to submerged solid surfaces that are best visualized when stained.

[00111] The addition of increasing concentrations of clomazone (1, 2, 4, 7, and 10 μM) to cultures of *B. subtilis* inhibited the formation of pellicles in *B. subtilis*. Growth inhibition was also observed. Wild type and the $\Delta\text{eps } \Delta\text{yqxM}$ matrix-deficient mutant were used as positive and negative controls, respectively (Branda et al. 2001 PNAS 98:11621-11626).

[00112] Addition of increasing concentrations (1, 2, 4, 5, and 7.5 μ M) of the statins mevastatin and lovastatin to *S. aureus* inhibited biofilm formation in the assay. Growth inhibition was also observed. Wild type and $\Delta sarA$ strains were used as positive and negative controls, respectively (Beenken et al. 2003 Infect Immun 71:4206-4211). Biofilms were stained with crystal violet for better visualization.

[00113] Small concentrations of clomazone and zaragozic acid potently inhibited pellicle formation in *B. subtilis*; statins and zaragozic acid were potent biofilm inhibitors in *S. aureus*. However, both the statins and clomazone caused cell death due to the essential nature of several of the molecules made along these pathways. In contrast, zaragozic acid did not have a lethal effect. This feature might make zaragozic acid an attractive anti-biofilm agent as one might expect a low incidence of resistance during the use of a non-lethal compound. More importantly, zaragozic acid treatment of *S. aureus* has the added advantage that both biofilm formation and virulence factors would be expected to be decreased simultaneously. Prior reports have indicated that other approaches at reducing biofilm formation in *S. aureus* lead to increased virulence and vice versa (Kong et al. 2006). In fact, aside from the biofilm inhibitory effects of squalene synthase inhibitors reported here, there is a report that squalene synthesis inhibitors inhibit *S. aureus* virulence (Liu et al. 2008).

Discussion

[00114] The finding that bacteria organize several physiological processes in lipid rafts is exciting from several of perspectives. Their conservation across the two most divergent domains of life argues strongly that these membrane microdomains are an ancient feature of cells. The ease of genetic, biochemical and cell biological approaches in bacterial systems means that the functions of lipid rafts may be easier to study using a bacterial model system, which might contribute to clarify some controversial aspects inherent in the study of eukaryotic lipid rafts.

[00115] In relation to the function of lipid rafts in bacteria, the compartmentalization of specific proteins in tightly packed membrane areas might facilitate their activity. For example, the formation of protein complexes required for cell-cell communication (like the Opp signal-uptake machinery) or dimerization of membrane kinases (like KinC or WalK), necessary in most cases to activate the cascades of signaling transduction seems more feasible when these proteins are physically located in restricted membrane areas.

[00116] On a more practical note, it is possible that lipid rafts can be exploited as a new target to control bacterial infections. The fact that disrupting lipid rafts affects several key physiological processes associated with pathogenesis without killing the cell raises the possibility of “anti-raft” compounds as promising anti-infective agents. Remarkably, small molecules that inhibit raft formation simultaneously targeted diverse processes associated with infections in different bacteria, *e.g.* biofilm formation and exoprotease production. Given the enormous variability and complexity of these processes within bacterial species and the difficulty to find a standard treatment against them, the activity of the small molecules that was described herein would be extremely helpful to combat chronic infections by simultaneously targeting many physiological processes associated to the infective process.

[00117] Interestingly, prior reports have indicated that other approaches at reducing biofilm formation lead to increased virulence and vice versa (Kong et al. 2006). As demonstrated herein, the DRM of *S. aureus* harbor the proteins required for biofilm formation, attachment, virulence and signaling. Thus, these processes may all be inhibited by blocking the formation of lipid rafts with the use of these small molecules. Supporting this finding, there is a report describing the use of inhibitors of the squalene synthesis to inhibit *S. aureus* virulence (Liu et al. 2008), in addition to the present observations that squalene synthesis inhibitors also inhibit biofilm formation and exoprotease secretion. Furthermore, it was discovered that the use of zaragozic acid as an anti-raft drug does not affect bacterial growth. It is contemplated that without any selective pressure caused by an agent that kills the cells, the use of zaragozic acid to inhibit bacterial infections might not give rise so rapidly to resistance mechanisms that are observed with many commonly used antibiotics.

[00118] In retrospect, there have been a number of studies describing the efficacy of statins to prevent infections. Patients treated to decrease high cholesterol levels have shown a remarkably reduced incidence of post-operative infection if previously treated with statins as cholesterol-lowering drugs. In other cases, previous treatment with statins strongly reduced the risk of hospitalization for sepsis in patients with chronic kidney disease that were receiving dialysis. Indeed, individuals receiving treatment with statins showed greatly reduced incidence of bacteremias caused by the pathogen *S. aureus* in hospitals (Liappis et al. 2001; Gupta et al. 2007; Falagas et al. 2008; Kopterides and Falagas 2009). It is possible that these patients may have inadvertently been protected against bacterial infections.

Methods of the Examples

[00119] Strains, media and culture conditions. Strains used in this study were *Bacillus subtilis* strain NCIB3610 (Branda et al. 2001) and *Staphylococcus aureus* strains SC-1 and UAMS-1 (Beenken et al. 2003). Additional laboratory strains of *E. coli* DH5 α , *B. subtilis* 168 and *S. aureus* RN4220 were required for cloning purposes. A list of strains used in this study is shown in Table 2.

[00120] Biofilm formation assay for *B. subtilis* 3610 and *S. aureus* SC-1 and UAMS-1.

Overnight cultures were diluted 1:100 in biofilm-inducing medium (MSgg for *B. subtilis*, TSB NaCl 3% Glucose 0.5% for *S. aureus* SC-01), serum-coated surfaces were required for UAMS-1 as described in Beenken et al. (2003). Cells were dispensed in polystyrene well plates and incubated for 24 h at 30° C in the case of *B. subtilis* or 37° C in the case of *S. aureus*. Biofilms formed by *S. aureus* were stained with crystal violet for better visualization, according to the protocol described by O'Toole *et al* (O'Toole and Kolter 1998). In *S. aureus*, biofilm formation was quantitatively monitored by dissolving the crystal violet associated with biofilms and measured spectrophotometrically at OD = 595 nm (O'Toole and Kolter 1998). Exoprotease production assay was performed in LB plates supplemented with non-fat powder milk (1%). Plates were incubated at 37° C for two days. MSgg plates were supplemented with polyisoprenoids for the $\Delta yisP$ mutant complementation. Squalene, β -carotene, retinol and staphyloxanthin were diluted into the medium at 100 μ M prior to the addition of cells. Pellicle formation in response to surfactin was assayed in LB medium. Strains were grown in LB shaking medium at 37°C in exponential phase for 20 generations. Cultures were transferred to polystyrene well plates and surfactin was added to a final concentration of 20 μ M. Cultures were incubated not shaking at 30°C for 10 h prior to imaging. Small molecules such as statins, clomazone, zaragozic acid, surfactin were added directly to the pellicle formation assay at specified concentrations.

[00121] Strain construction and reporters. Deletion mutants $\Delta yisP::km$, $\Delta sqhC::km$, $\Delta sqhC::mls$, $\Delta yuaG::km$, $\Delta yqfA::spc$, were generated using long flanking homology PCR (Wach 1996) (using the primers listed in Table 3). For the translational fusion *yuaG-yfp* and for complementation of *yisP*, a wild type copy of *yisP* was cloned in pKM008 or pKM003 vectors and inserted by double recombination into the neutral integration site *amyE* in the

genome of *B. subtilis* strain 168 by inducing natural competence (Hardwood and Cutting 1990). Translational fusions *yqiK-yfp* and *sal402-yfp* were cloned in pKM008 and pMAD (Arnaud et al. 2004) plasmids, respectively and introduced into *E. coli* and *S. aureus* by electroporation. Co-localization of KinC with FloT in *B. subtilis* required overexpression of the translational fusion *kinC-cfp* under the control of the IPTG-inducible promoter $P_{hyperspank}$ in pDR111. The construction $P_{hyperspank}$ -*kinC-cfp* was transferred to the plasmid pDR183 and inserted into the *lacA* neutral integration site by double recombination. Constructions were transferred to the strain NCIB3610 by phage transduction (Yasbin and Young 1974; Novick 1991).

[00122] Image Capture and Analysis. Colonies were photographed using a Zeiss Stemi SV6 Stereoscope connected to a color AxioCam®. Microscopy images were taken on a Nikon Eclipse TE2000-U microscope equipped with an X-cite 120 illumination system, using a Hamamatsu digital camera model ORCA-ER. Fluorescence signal was detected with a Ex436/500 filter. Image processing was done using MetaMorph® Software and Photoshop®. Biofilm formation in well plates was photographed by using a Nikon D100 digital camera.

[00123] Overexpression and purification of YisP. *yisP* was expressed under the control of an IPTG-inducible promoter in the plasmid pET15-B (Novagen) using the restriction sites *BamHI* and *NdeI* of the MCS. The protein expressed has an N-terminal His-Tag sequence for further purification. 500 ml of LB culture of *E. coli* BL21-DE3 Gold® carrying the plasmid was grown in the presence of ampicillin to a density of $OD_{600} = 0.8$ and 0.5 mM IPTG was added to induce expression. After two hours of incubation, cells were pelleted and lysed using CellLytic B (Sigma). Purification of the His-tagged YisP was carried out using a His-select Nickel affinity gel (Sigma).

[00124] Enzymatic activity and characterization of YisP. An enzymatic assay to monitor the enzymatic activity of YisP was performed according to (Debruyne 1983) with specific modifications. Release of inorganic phosphate derived from the condensation of two molecules of FPP was monitored using the ability of rhodamine B to precipitate, once it is chelated with the inorganic phosphate released from the reaction of condensation ($2FPP \rightarrow$ Squalene + $2PP_i$). The precipitate was washed, dissolved in acetone and measured

spectrophotometrically at 555 nm. 2 U of pyrophosphatase were added to the reaction mix to increase the sensitivity of the assay ($2\text{PPi} \rightarrow \text{Pi} + \text{Pi}$). The reaction buffer contained 50 mM Tris-HCl, 20mM MgCl_2 , pH = 7.4 and different substrates (FPP, GPP and GGPP) were used pair wise to optimize the reaction. Purified enzyme was pre-incubated in the reaction buffer for 20 min prior to enzymatic analysis.

[00125] Pigment production and analysis in *B. subtilis*. To boost the production of pigment in *B. subtilis*, Mmsg was mixed with LB (with 1% NaCl) at a ratio of 3:1 and 500 ml of culture was grown in a 1 L flask shaking at 30° C until stationary phase was reached. Next, the culture was allowed to stand in the dark until color appeared (approximately 8 h). Cell pellets was dissolved in ethanol to 1:1 (v/v) and incubated at 45°C during 2h. After centrifugation, pellet was discarded and the ethanol containing the pigments was concentrated by evaporation 10x prior LC-MS analysis.

[00126] Cell membrane fractionation according to detergent resistance. To purify the membrane fraction, cells were treated with lysozyme (*B. subtilis*) or lysostaphin (*S. aureus*) and lysed by sonication. Cell debris was eliminated by normal centrifugation (13,000 RPM for 2 min) and the membrane fraction was precipitated from the supernatant by ultracentrifugation (75,000 RPM for 40 min). Next, the membrane fraction was treated with the CellLytic MEM protein extraction kit (Sigma) to purify proteins associated with detergent resistant fractions. Samples were run on SDS-PAGE and proteins were detected by immunoblot, as described in Lopez et al. (2009), and by coomassie staining. Coomassie stained bands were analyzed by mass spectrometry (Thermo Scientific LTQ Orbitrap XL).

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Table 1: Proteins present in the DRM of *B. subtilis* and *S. Aureus*

Proteins present in the DRM of <i>B. subtilis</i>	
Protein name	Function
KinC ⁺	Sensor histidine kinase involved in biofilm formation.
FliT ⁺	Lipid raft-associated protein
Praa	Post-translocation molecular chaperone involved in protease secretion.
OppA	Oligopeptide ABC transporter (binding protein)
OppB	Oligopeptide ABC transporter (binding protein)
BdbC	Thiol-disulfide oxidoreductase
BdbD	Thiol-disulfide oxidoreductase
FeuA	Iron-uptake system (Iron-binding protein)
FeuB	Iron-uptake system (Integral membrane protein)
FhuD	Ferriochrome transporter ATP-binding protein
FhuB	Ferriochrome transporter permease
PcpC ⁺	Penicillin-binding protein 3.
DacA ⁺	Penicillin-binding protein 5 (D-alanyl-D-alanine carboxypeptidase)
DacC ⁺	Penicillin-binding protein (D-alanyl-D-alanine carboxypeptidase)
AlpA ⁺	ATP synthase (subunit alpha)
AlpB	ATP synthase (subunit a)
yxeB	Unknown. Similar to ABC transporter (binding protein)
yxeA	Unknown. Similar to ABC transporter (ATP-binding protein)
yknZ	Unknown. Similar to ABC transporter (permease)
Proteins present in the DRM of <i>S. aureus</i> .	
Protein name	Function
WalK	Sensor histidine kinase involved in biofilm formation.
FliT (SA1403)	Lipid raft-associated protein
Praa	Post-translocation molecular chaperone involved in protease secretion
KdpB1	Potassium-transporting ATPase B chain 1
KdpC2	Potassium-transporting ATPase C chain 2
KdpA2	Potassium-transporting ATPase A chain 2
Sip1B	Protein secretion
SecA ⁺	Protein translocase subunit
Ebps	Elastin-binding protein
CvxA	Virulence quorum-sensing regulator
Tcaa	Teicoplanin-resistance protein
SA1283 ⁺	Penicillin-binding protein 2
SA168	Unknown. Similar to ABC transporter (Permease)
SA1977	Unknown. Similar to ABC transporter (Permease)
SirB	Siderophore-uptake ABC transporter (Permease)
SA1911 ⁺	ATP synthase (A chain)
SA1909 ⁺	ATP synthase (B chain)
SA1615 ⁺	ATP synthase (Hydrolase)

Table 2: Strain List

Strain	Genotype	Reference
<i>Bacillus subtilis</i>		
DL1	wild type (WT)	(B)
DL7	$\Delta\text{eps::tet}$, $\Delta\text{yqxM::km}$	(D)
DL1064	$\Delta\text{yisP::Km}$	This study
DL1201	$\Delta\text{sqhC::km}$	This study
DL346	$\Delta\text{kinC::mls amyE::kinC-yfp}$	(C)
DL1176	$\Delta\text{yisP::km}$, $\text{amyE::yisP-yfp (spc)}$	This study
DL1175	$\Delta\text{yisP::km}$, amyE::yisP (spc)	This study
DL1157	$\Delta\text{yisP::km}$, amyE::sad67 (spc)	This study
DL1207	$\text{amyE::floT-yfp (spc)}$	This study
DL1295	$\text{amyE::P}_{\text{yaaF}}\text{-floT-yfp (spc)}$	This study
DL1235	$\text{amyE::floT-yfp (spc)}$, $\text{lacA::P}_{\text{hp}}\text{kinC-cfp}$	This study
DL1241	$\Delta\text{floT::km}$, $\Delta\text{yqjA::spc}$	This study
DL1275	$\Delta\text{floT::km}$, $\Delta\text{yqjA::spc}$, $\text{lacA::P}_{\text{hp}}\text{kinC-cfp}$	This study
DL 1247	$\Delta\text{yisP::km}$, $\Delta\text{abrB::tet}$	This study
DL 1142	$\Delta\text{yisP::km}$, $\Delta\text{sinR::spc}$	This study
DL 1281	$\Delta\text{floT::km}$, $\text{amyE::floT-yfp (spc)}$	This study
DL 1315	$\Delta\text{floT::km}$, $\Delta\text{yqjA::spc}$, $\Delta\text{kinC::cm}$, $\text{lacA::kinC-yfp (mls)}$	This study
<i>Staphylococcus aureus</i>		
DL1127	Wild type UAMS-1	(A)
DL1128	Wild type SC-01	(A)
DL1129	ΔsarA	(4)
DL1231	UAMS-1 pMAD <i>sa1402-yfp</i>	This study
<i>Escherichia coli</i>		
DL95	Wild type	(E)
DL1205	pKM008 <i>yqjK-yfp</i>	This study
DL1160	pET15b His ⁶ <i>yisP</i>	This study

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Table 3: Primer List

Purpose	Name	Sequence (5'-3')	
Deletion mutants	yisP1	GAATAGGTTTTAATGTTCTGC	SEQ ID NO: 1
	yisP2	CCTATCACCTCAAATGGTTCGCTGCTGCTTCTTCAAGTCTAGTATG	SEQ ID NO: 2
	yisP3	CGAGCGCCTACGAGGAATTTGTATCGCCCGGCCCTTGATTGAGCCG	SEQ ID NO: 3
	yisP4	AAGCTGCTGAAAATCGGC	SEQ ID NO: 4
	sqhc1	GGCCAAGTCTGCTGTGC	SEQ ID NO: 5
	sqhc2	CCTATCACCTCAAATGGTTCGCTGCACGCTCAACTCCTTCA	SEQ ID NO: 6
	sqhc3	CGAGCGCCTACGAGGAATTTGTATCGCCCGGAGATGTTCAATTGG	SEQ ID NO: 7
	sqhc4	GGGATCACATCCCATTG	SEQ ID NO: 8
	yuaG1	GACATTACCATATATATAAGC	SEQ ID NO: 9
	yuaG2	CCTATCACCTCAAATGGTTCGCTGAAATGGGTTTCATGCGGAG	SEQ ID NO: 10
	yuaG3	CGAGCGCCTACGAGGAATTTGTATCGGAGTAAGGAAAGGGCAGA	SEQ ID NO: 11
	yuaG4	AACTTATAATGCGACTTACAT	SEQ ID NO: 12
	yqjA1	GCTGTATATATAACGATACG	SEQ ID NO: 13
	yqjA2	CGTTACGTTATTAGCGAGCCAGTCGCCACCAGCAGACAACGC	SEQ ID NO: 14
	yqjA3	CAATAAACCCCTTGCCCTCGCTACGCCGCTTATTATTGCAGCG	SEQ ID NO: 15
	yqjA4	GCTGTATATATAACGATACG	SEQ ID NO: 16
Overexpression Of YisP	yis15fwNdeI	ACATAGGAGTGAGCATATGAAAGAGATAAAAGAA	SEQ ID NO: 17
	yis15rvBamHI	TTTTGGATCCTTAGATATCAGACAGAATCTG	SEQ ID NO: 18
Translational fusion KinC-Cfp	kinCcfpsalI	TTTTGTGACGGGATACTTTACATATTTTAC	SEQ ID NO: 19
	cfprvNheI	TTTTGCTAGCTTACTTATAAAGTTCTGTTCA	SEQ ID NO: 20
Translational fusion YuaG-yfp	yuaGfw	GCTAAGCTTACATAAGGAGGAAGTACTATGACAATGCCGATTATA	SEQ ID NO: 21
	yuaGyfpv	GAAAAGTTCTTCTCCTTTACTCATCTCTGATTTTTGGATCGTTT	SEQ ID NO: 22
	PyuaFfw	AAAAGAATTCCGCAGCAGTCAGCTGC	SEQ ID NO: 23
	PyuaFrv	AAAAAAGCTTATCAATGCATTGATGAACGG	SEQ ID NO: 24
Translational fusion Sa1402-yfp	sa1402up	AAAAGTCGACACCATGTTTAGTTTAAGTTTAA	SEQ ID NO: 25
	sa1402rv	AAGTTCTTCTCCTTTACTCATATGTTTCAGGTGACTCATCATCACTTTG	SEQ ID NO: 26
Translational fusion YqjK-yfp	yqjKup	AAAAGTCGACATAATGGATGATATTGTTAATTC	SEQ ID NO: 27
	yqjKrv	AAGTTCTTCTCCTTTACTCATCTTTTCTTCGACGGGAGT	SEQ ID NO: 28
Translational fusion YisP-yfp	yisptwEcorI	AAAAGAATTCAGCGATGATATGGTGTAATAT	SEQ ID NO: 29
	yispyfpuprv	GAAAAGTTCTTCTCCTTTACTCATGATATCAGACAGAATCTGC	SEQ ID NO: 30
Yfp amplification	yfpfw	ATGAGTAAAGGAGAGAAGACTT	SEQ ID NO: 31
	yfpv	CTCAGGATCCTTATTTGTAT	SEQ ID NO: 32
Cfp amplification	cfpfw	ATGGTTTCAAAGGCCGAAGAA	SEQ ID NO: 33
	cfprv	CTCAGGATCCTTAAAGATACT	SEQ ID NO: 34

Table 4. Bacteria Which Synthesize Squalene Via GA3P Pathway

Prokaryotes (927)

Bacteria (863)

Proteobacteria (461)

Gamma/enterobacteria (90)

eco Escherichia coli K-12 MG1655
 ecj Escherichia coli K-12 W3110
 ecd Escherichia coli K-12 DH10B
 ebw Escherichia coli BW2952
 ece Escherichia coli O157 EDL933 (EHEC)
 ecs Escherichia coli O157 Sakai (EHEC)
 ect Escherichia coli O157 EC4115 (EHEC)
 eci Escherichia coli UTI89 (UPEC)
 ecp Escherichia coli 536 (UPEC)
 ecv Escherichia coli APEC O1
 ecw Escherichia coli E24377A
 ecx Escherichia coli HS
 ecm Escherichia coli SECEC
 ecy Escherichia coli SE11
 ecl Escherichia coli ATCC 8739
 ecg Escherichia coli O127
 eck Escherichia coli 55989
 ecq Escherichia coli ED1a
 ecr Escherichia coli IAI1
 ect Escherichia coli IAI39
 ecz Escherichia coli S88
 eum Escherichia coli UMN026
 elf Escherichia coli LF82
 efe Escherichia fergusonii
 sty Salmonella enterica subsp. enterica serovar Typhi CT18
 stt Salmonella enterica subsp. enterica serovar Typhi Ty2
 spt Salmonella enterica subsp. enterica serovar Paratyphi A
 sek Salmonella enterica subsp. enterica serovar Paratyphi A AKU12601
 spq Salmonella enterica subsp. enterica serovar Paratyphi B
 sei Salmonella enterica subsp. enterica serovar Paratyphi C
 sec Salmonella enterica subsp. enterica serovar Choleraesuis
 seh Salmonella enterica subsp. enterica serovar Heidelberg
 see Salmonella enterica subsp. enterica serovar Newport
 sew Salmonella enterica subsp. enterica serovar Schwarzengrund
 sea Salmonella enterica subsp. enterica serovar Agona
 sed Salmonella enterica subsp. enterica serovar Dublin
 seg Salmonella enterica subsp. enterica serovar Gallinarum
 set Salmonella enterica subsp. enterica serovar Enteritidis
 ses Salmonella enterica subsp. arizonae
 stm Salmonella typhimurium LT2
 ype Yersinia pestis CO92
 ypk Yersinia pestis KIM
 ypm Yersinia pestis Mediaevails
 ypa Yersinia pestis Antiqua
 ypn Yersinia pestis Nepal516
 ypp Yersinia pestis Pestoides
 ypg Yersinia pestis Angola

yps *Yersinia pseudotuberculosis* IP32953
 ypi *Yersinia pseudotuberculosis* IP31758
 ypy *Yersinia pseudotuberculosis* YPIII
 ypb *Yersinia pseudotuberculosis* PB1/+
 yen *Yersinia enterocolitica*
 sfl *Shigella flexneri* 301 (serotype 2a)
 sfx *Shigella flexneri* 2457T (serotype 2a)
 sfv *Shigella flexneri* 8401 (serotype 5b)
 ssn *Shigella sonnei*
 sbo *Shigella boydii* Sb227
 sbc *Shigella boydii* CDC 3083-94
 sdy *Shigella dysenteriae*
 eca *Erwinia carotovora*
 eta *Erwinia tasmaniensis*
 plu *Photobacterium luminescens*
 buc *Buchnera aphidicola* APS
 bas *Buchnera aphidicola* Sg
 bap *Buchnera aphidicola* 5A
 bau *Buchnera aphidicola* Tuc7
 wbr *Wigglesworthia glossinidia*
 sgl *Sodalis glossinidius*
 ent *Enterobacter* sp. 638
 esa *Enterobacter sakazakii*
 kpn *Klebsiella pneumoniae*
 kpe *Klebsiella pneumoniae* 342
 kpu *Klebsiella pneumoniae* NTUH-K2044
 cko *Citrobacter koseri* ATCC BAA-895
 spe *Serratia proteamaculans*
 pmr *Proteus mirabilis*
 eic *Edwardsiella ictaluri*
 bfl *Candidatus Blochmannia floridanus*
 bpn *Candidatus Blochmannia pennsylvanicus*
 hde *Candidatus Hamiltonella defensa*
 dda *Dickeya dadantii*
 Gamma/others (136)
 hin *Haemophilus influenzae* Rd KW20 (serotype d)
 hit *Haemophilus influenzae* 86-028NP (nontypeable)
 hip *Haemophilus influenzae* PittEE
 hiq *Haemophilus influenzae* PittGG
 hdu *Haemophilus ducreyi*
 hap *Haemophilus parasuis*
 hso *Haemophilus somnus* 129PT
 hsm *Haemophilus somnus* 2336
 pmu *Pasteurella multocida*
 msu *Mannheimia succiniciproducens*
 apl *Actinobacillus pleuropneumoniae* L20 (serotype 5b)
 apj *Actinobacillus pleuropneumoniae* JL03 (serotype 3)
 apa *Actinobacillus pleuropneumoniae* AP76 (serotype 7)
 asu *Actinobacillus succinogenes*
 xfa *Xylella fastidiosa* 9a5c
 xfl *Xylella fastidiosa* Temecula1
 xfm *Xylella fastidiosa* M12
 xfn *Xylella fastidiosa* M23
 xcc *Xanthomonas campestris* pv. *campestris* ATCC 33913
 xcb *Xanthomonas campestris* pv. *campestris* 8004

xca *Xanthomonas campestris* pv. *campestris* B100
 xcv *Xanthomonas campestris* pv. *vesicatoria*
 xac *Xanthomonas axonopodis*
 xoo *Xanthomonas oryzae* KACC10331
 xom *Xanthomonas oryzae* MAFF311018
 xop *Xanthomonas oryzae* PXO99A
 smi *Stenotrophomonas maltophilia*
 smt *Stenotrophomonas maltophilia* R551-3
 vch *Vibrio cholerae* O1
 vco *Vibrio cholerae* O395
 vcm *Vibrio cholerae* M66-2
 vcj *Vibrio cholerae* MJ-1236
 vvu *Vibrio vulnificus* CMCP6
 vvy *Vibrio vulnificus* YJ016
 vpa *Vibrio parahaemolyticus*
 vha *Vibrio harveyi*
 vsp *Vibrio splendidus*
 vfi *Vibrio fischeri*
 vfm *Vibrio fischeri* MJ11
 vsa *Aliivibrio salmonicida* LFI1238
 ppr *Photobacterium profundum*
 pae *Pseudomonas aeruginosa* PAO1
 pau *Pseudomonas aeruginosa* UCBPP-PA14
 pap *Pseudomonas aeruginosa* PA7
 pag *Pseudomonas aeruginosa* LESB58
 ppu *Pseudomonas putida* KT2440
 ppf *Pseudomonas putida* F1
 ppg *Pseudomonas putida* GB-1
 ppw *Pseudomonas putida* W619
 pst *Pseudomonas syringae* pv. *tomato* DC3000
 psb *Pseudomonas syringae* pv. *syringae* B728a
 psp *Pseudomonas syringae* pv. *phaseolicola* 1448A
 pfl *Pseudomonas fluorescens* Pf-5
 pfo *Pseudomonas fluorescens* Pf0-1
 pfs *Pseudomonas fluorescens* SBW25
 pen *Pseudomonas entomophila*
 pmy *Pseudomonas mendocina*
 psa *Pseudomonas stutzeri*
 cja *Cellvibrio japonicus*
 avn *Azotobacter vinelandii*
 par *Psychrobacter arcticum*
 pcr *Psychrobacter cryohalolentis*
 prw *Psychrobacter* sp. PRwf-1
 aci *Acinetobacter* sp. ADP1
 acb *Acinetobacter baumannii* ATCC 17978
 abm *Acinetobacter baumannii* SDF
 aby *Acinetobacter baumannii* AYE
 abc *Acinetobacter baumannii* ACICU
 abn *Acinetobacter baumannii* AB0057
 abb *Acinetobacter baumannii* AB307-0294
 son *Shewanella oneidensis*
 sdn *Shewanella denitrificans*
 sfr *Shewanella frigidimarina*
 saz *Shewanella amazonensis*
 sbl *Shewanella baltica* OS155

sbm *Shewanella baltica* OS185
 sbn *Shewanella baltica* OS195
 sbp *Shewanella baltica* OS223
 slo *Shewanella loihica*
 spc *Shewanella putrefaciens*
 sse *Shewanella sediminis*
 spl *Shewanella pealeana*
 she *Shewanella* sp. MR-4
 shm *Shewanella* sp. MR-7
 shn *Shewanella* sp. ANA-3
 shw *Shewanella* sp. W3-18-1
 shl *Shewanella halifaxensis*
 swd *Shewanella woodyi* ATCC 51908
 swp *Shewanella piezotolerans* WP3
 ilo *Idiomarina loihiensis*
 cps *Colwellia psychrerythraea*
 pha *Pseudoalteromonas haloplanktis*
 pat *Pseudoalteromonas atlantica*
 sde *Saccharophagus degradans*
 maq *Marinobacter aquaeolei*
 amc *Alteromonas macleodii*
 pin *Psychromonas ingrahamii*
 mca *Methylococcus capsulatus*
 ftu *Francisella tularensis* subsp. *tularensis* SCHU S4
 ftf *Francisella tularensis* subsp. *tularensis* FSC 198
 ftw *Francisella tularensis* subsp. *tularensis* WY96-3418
 fti *Francisella tularensis* subsp. *holarctica* LVS
 fth *Francisella tularensis* subsp. *holarctica* OSU18
 fta *Francisella tularensis* subsp. *holarctica* FTNF002-00
 ftn *Francisella tularensis* subsp. *novicida* U112
 ftm *Francisella tularensis* subsp. *mediasiatica* FSC147
 fph *Francisella philomiragia*
 noc *Nitrosococcus oceani*
 aeh *Alkalilimnicola ehrlichei*
 hha *Halorhodospira halophila*
 tgr *Thioalkalivibrio* sp. HL-EbGR7
 hch *Hahella chejuensis*
 csa *Chromohalobacter salexigens*
 abo *Alcanivorax borkumensis*
 mmw *Marinomonas* sp. MWYL1
 aha *Aeromonas hydrophila*
 asa *Aeromonas salmonicida*
 tau *Tolumonas auensis*
 afe *Acidithiobacillus ferrooxidans* ATCC 53993
 afr *Acidithiobacillus ferrooxidans* ATCC 23270
 bci *Baumannia cicadellinicola*
 rma *Candidatus Ruthia magnifica*
 vok *Candidatus Vesicomysocius okutanii*
 Beta (70)
 nme *Neisseria meningitidis* MC58 (serogroup B)
 nma *Neisseria meningitidis* Z2491 (serogroup A)
 nmc *Neisseria meningitidis* FAM18 (serogroup C)
 nmh *Neisseria meningitidis* 053442 (serogroup C)
 ngo *Neisseria gonorrhoeae*
 ngk *Neisseria gonorrhoeae* NCCP11945

cvi *Chromobacterium violaceum*
 lhk *Laribacter hongkongensis*
 rso *Ralstonia solanacearum*
 rpi *Ralstonia pickettii* 12J
 rpf *Ralstonia pickettii* 12D
 reu *Ralstonia eutropha* JMP134
 reh *Ralstonia eutropha* H16
 rme *Ralstonia metallidurans*
 cti *Cupriavidus taiwanensis*
 bma *Burkholderia mallei* ATCC 23344
 bmv *Burkholderia mallei* SAVP1
 bml *Burkholderia mallei* NCTC 10229
 bmn *Burkholderia mallei* NCTC 10247
 bxe *Burkholderia xenovorans*
 bvi *Burkholderia vietnamiensis*
 bur *Burkholderia* sp. 383
 bcn *Burkholderia cenocepacia* AU1054
 bch *Burkholderia cenocepacia* HI2424
 bcm *Burkholderia cenocepacia* MC0-3
 bcj *Burkholderia cenocepacia* J2315
 bam *Burkholderia cepacia*
 bac *Burkholderia ambifaria* MC40-6
 bmu *Burkholderia multivorans* ATCC 17616 (JGI)
 bmj *Burkholderia multivorans* ATCC 17616 (Tohoku)
 bps *Burkholderia pseudomallei* K96243
 bpm *Burkholderia pseudomallei* 1710b
 bpl *Burkholderia pseudomallei* 1106a
 bpd *Burkholderia pseudomallei* 668
 bpr *Burkholderia pseudomallei* MSHR346
 bte *Burkholderia thailandensis*
 bph *Burkholderia phymatum*
 bpy *Burkholderia phytofirmans*
 bgl *Burkholderia glumae*
 prn *Polynucleobacter* sp. QLW-P1DMWA-1
 pne *Polynucleobacter necessarius*
 bpe *Bordetella pertussis*
 bpa *Bordetella parapertussis*
 bbr *Bordetella bronchiseptica*
 bpt *Bordetella petrii*
 bav *Bordetella avium*
 rfr *Rhodoferrax ferrireducens*
 pol *Polaromonas* sp. JS666
 pna *Polaromonas naphthalenivorans*
 aav *Acidovorax avenae*
 ajs *Acidovorax* sp. JS42
 vei *Verminephrobacter eiseniae*
 dac *Delftia acidovorans*
 dia *Diaphorobacter* sp. TPSY
 vap *Variovorax paradoxus*
 mpt *Methylobium petroleiphilum*
 har *Herminiimonas arsenicoxydans*
 mms *Minibacterium massiliensis*
 lch *Leptothrix cholodnii*
 neu *Nitrosomonas europaea*
 net *Nitrosomonas eutropha*

nmu *Nitrosospira multiformis*
 eba *Aromatoleum aromaticum* EbN1
 azo *Azoarcus* sp. BH72
 dar *Dechloromonas aromatica*
 tmz *Thauera* sp. MZ1T
 tbd *Thiobacillus denitrificans*
 mfa *Methylobacillus flagellatus*
 Epsilon (24)
 hpy *Helicobacter pylori* 26695
 hpj *Helicobacter pylori* J99
 hpa *Helicobacter pylori* HPAG1
 hps *Helicobacter pylori* Shi470
 hpg *Helicobacter pylori* G27
 hpp *Helicobacter pylori* P12
 hhe *Helicobacter hepaticus*
 hac *Helicobacter acinonychis*
 wsu *Wolinella succinogenes*
 tdn *Thiomicrospira denitrificans*
 cje *Campylobacter jejuni* NCTC11168
 cjr *Campylobacter jejuni* RM1221
 cjj *Campylobacter jejuni* 81-176
 cju *Campylobacter jejuni* 81116
 cjd *Campylobacter jejuni* subsp. *doylei* 269.97
 cff *Campylobacter fetus*
 ccv *Campylobacter curvus*
 cha *Campylobacter hominis* ATCC BAA-381
 cco *Campylobacter concisus* 13826
 cla *Campylobacter lari*
 abu *Arcobacter butzleri*
 nis *Nitratiruptor* sp. SB155-2
 sun *Sulfurovum* sp. NBC37-1
 nam *Nautilia profundicola*
 Delta (29)
 gsu *Geobacter sulfurreducens*
 gme *Geobacter metallireducens*
 gur *Geobacter uraniumreducens*
 glo *Geobacter lovleyi*
 gbm *Geobacter bemidjiensis*
 geo *Geobacter* sp. FRC-32
 pca *Pelobacter carbinolicus*
 ppd *Pelobacter propionicus*
 dvu *Desulfovibrio vulgaris* Hildenborough
 dvl *Desulfovibrio vulgaris* DP4
 dvm *Desulfovibrio vulgaris* Miyazaki F
 dde *Desulfovibrio desulfuricans* G20
 dds *Desulfovibrio desulfuricans* ATCC 27774
 dma *Desulfovibrio magneticus*
 lip *Lawsonia intracellularis*
 dps *Desulfotalea psychrophila*
 dol *Candidatus Desulfococcus oleovorans*
 dal *Desulfatibacillum alkenivorans*
 dat *Desulfobacterium autotrophicum*
 ade *Anaeromyxobacter dehalogenans* 2CP-C
 acp *Anaeromyxobacter dehalogenans* 2CP-1
 afw *Anaeromyxobacter* sp. Fw109-5

ank Anaeromyxobacter sp. K
 sat Syntrophus aciditrophicus
 sfu Syntrophobacter fumaroxidans
 Alpha/rickettsias (28)
 wol Wolbachia wMel
 wbm Wolbachia wBm
 wri Wolbachia sp. wRi
 wpi Wolbachia pipientis
 ama Anaplasma marginale St. Maries
 amf Anaplasma marginale Florida
 aph Anaplasma phagocytophilum
 eru Ehrlichia ruminantium Welgevonden (South Africa)
 erw Ehrlichia ruminantium Welgevonden (France)
 erg Ehrlichia ruminantium Gardel
 ecn Ehrlichia canis
 ech Ehrlichia chaffeensis
 nse Neorickettsia sennetsu
 pub Candidatus Pelagibacter ubique
 Alpha/rhizobacteria (53)
 pla Parvibaculum lavamentivorans
 sme Sinorhizobium meliloti
 smd Sinorhizobium medicae
 atu Agrobacterium tumefaciens C58
 atc Agrobacterium tumefaciens C58 (Cereon)
 ara Agrobacterium radiobacter K84
 avi Agrobacterium vitis S4
 ret Rhizobium etli CFN 42
 rec Rhizobium etli CIAT 652
 rle Rhizobium leguminosarum
 rlt Rhizobium leguminosarum bv. trifolii WSM2304
 rlg Rhizobium leguminosarum bv. trifolii WSM1325
 rhi Rhizobium sp. NGR234
 bja Bradyrhizobium japonicum
 bra Bradyrhizobium sp. ORS278
 bbt Bradyrhizobium sp. BTAi1
 rpa Rhodopseudomonas palustris CGA009
 rpb Rhodopseudomonas palustris HaA2
 rpc Rhodopseudomonas palustris BisB18
 rpd Rhodopseudomonas palustris BisB5
 rpe Rhodopseudomonas palustris BisA53
 rpt Rhodopseudomonas palustris TIE-1
 nwi Nitrobacter winogradskyi
 nha Nitrobacter hamburgensis
 oca Oligotropha carboxidovorans
 xau Xanthobacter autotrophicus
 azc Azorhizobium caulinodans
 mex Methylobacterium extorquens
 mea Methylobacterium extorquens AM1
 mrd Methylobacterium radiotolerans
 met Methylobacterium sp. 4-46
 mpo Methylobacterium populi
 mch Methylobacterium chloromethanicum
 mno Methylobacterium nodulans
 bid Beijerinckia indica
 msl Methylocella silvestris

Alpha/others (30)

ccr *Caulobacter crescentus* CB15
 ccs *Caulobacter crescentus* NA1000
 cak *Caulobacter* sp. K31
 pzu *Phenylobacterium zucineum*
 sil *Ruegeria pomeroyi*
 sit *Silicibacter* sp. TM1040
 rsp *Rhodobacter sphaeroides* 2.4.1
 rsh *Rhodobacter sphaeroides* ATCC 17029
 rsq *Rhodobacter sphaeroides* ATCC 17025
 rsk *Rhodobacter sphaeroides* KD131
 jan *Jannaschia* sp. CCS1
 rde *Roseobacter denitrificans*
 pde *Paracoccus denitrificans*
 dsh *Dinoroseobacter shibae*
 mmr *Maricaulis maris*
 hne *Hyphomonas neptunium*
 zmo *Zymomonas mobilis*
 nar *Novosphingobium aromaticivorans*
 sal *Sphingopyxis alaskensis*
 swi *Sphingomonas wittichii*
 eli *Erythrobacter litoralis*
 gox *Gluconobacter oxydans*
 gbe *Granulobacter bethesdensis*
 acr *Acidiphilium cryptum* JF-5
 gdi *Gluconacetobacter diazotrophicus* PAI 5 (Brazil)
 gdj *Gluconacetobacter diazotrophicus* PAI 5 (JGI)
 rru *Rhodospirillum rubrum*
 rce *Rhodospirillum centenum*
 mag *Magnetospirillum magneticum*

Magnetococcus (1)

mgm *Magnetococcus* sp. MC-1

Acidobacteria (3)

aba *Acidobacteria bacterium*
 aca *Acidobacterium capsulatum*
 sus *Solibacter usitatus*

Firmicutes (173)

Bacillales (61)

bsu *Bacillus subtilis*
 bha *Bacillus halodurans*
 ban *Bacillus anthracis* Ames
 bar *Bacillus anthracis* Ames 0581
 baa *Bacillus anthracis* A2012
 bat *Bacillus anthracis* Sterne
 bah *Bacillus anthracis* CDC 684
 bai *Bacillus anthracis* A0248
 bce *Bacillus cereus* ATCC 14579
 bca *Bacillus cereus* ATCC 10987
 bcz *Bacillus cereus* ZK
 bcy *Bacillus cereus* subsp. cytotoxis NVH 391-98
 bcr *Bacillus cereus* AH187
 bcb *Bacillus cereus* B4264
 bcu *Bacillus cereus* AH820
 bcg *Bacillus cereus* G9842
 bcq *Bacillus cereus* Q1

bcx *Bacillus cereus* 03BB102
 btk *Bacillus thuringiensis* 97-27
 btl *Bacillus thuringiensis* Al Hakam
 bwe *Bacillus weihenstephanensis*
 bli *Bacillus licheniformis* ATCC 14580
 bld *Bacillus licheniformis* DSM13
 bcl *Bacillus clausii*
 bay *Bacillus amyloliquefaciens*
 bpu *Bacillus pumilus*
 gka *Geobacillus kaustophilus*
 gtn *Geobacillus thermodenitrificans*
 gwc *Geobacillus* sp. WCH70
 afl *Anoxybacillus flavithermus*
 lsp *Lysinibacillus sphaericus*
 esi *Exiguobacterium sibiricum*
 eat *Exiguobacterium* sp. AT1b
 mcl *Macrococcus caseolyticus*
 lmo *Listeria monocytogenes* EGD-e
 lmf *Listeria monocytogenes* F2365
 lmh *Listeria monocytogenes* HCC23
 lmc *Listeria monocytogenes* Clip81459
 lin *Listeria innocua*
 lwe *Listeria welshimeri* SLCC5334
 bbe *Brevibacillus brevis*
 Clostridia (46)
 cac *Clostridium acetobutylicum*
 cpe *Clostridium perfringens* 13
 cpf *Clostridium perfringens* ATCC 13124
 cpr *Clostridium perfringens* SM101
 ctc *Clostridium tetani* E88
 cno *Clostridium novyi*
 cth *Clostridium thermocellum*
 cdf *Clostridium difficile*
 cbo *Clostridium botulinum* A ATCC 3502
 cba *Clostridium botulinum* A ATCC 19397
 cbh *Clostridium botulinum* A Hall
 cby *Clostridium botulinum* A2
 cbl *Clostridium botulinum* A3 Loch Maree
 cbk *Clostridium botulinum* B Eklund 17B
 cbb *Clostridium botulinum* B1 Okra
 cbi *Clostridium botulinum* Ba4
 cbt *Clostridium botulinum* E3
 cbf *Clostridium botulinum* F Langeland
 cbe *Clostridium beijerinckii*
 ckl *Clostridium kluyveri* DSM 555
 ckr *Clostridium kluyveri* NBRC 12016
 cpy *Clostridium phytofermentans*
 cce *Clostridium cellulolyticum*
 amt *Alkaliphilus metalliredigens*
 aoe *Alkaliphilus oremlandii*
 sth *Symbiobacterium thermophilum*
 swo *Syntrophomonas wolfei*
 ate *Anaerocellum thermophilum*
 chy *Carboxydotherrmus hydrogenoformans*
 dsy *Desulfitobacterium hafniense* Y51

dhd *Desulfitobacterium hafniense* DCB-2
 drm *Desulfotomaculum reducens*
 pth *Pelotomaculum thermopropionicum*
 dau *Candidatus Desulforudis audaxviator*
 hmo *Heliobacterium modesticaldum*
 eel *Eubacterium eligens*
 ere *Eubacterium rectale*
 tte *Thermoanaerobacter tengcongensis*
 tex *Thermoanaerobacter* sp. X514
 tpd *Thermoanaerobacter pseudethanolicus*
 csc *Caldicellulosiruptor saccharolyticus*
 mta *Moorella thermoacetica*
 cpo *Coprothermobacter proteolyticus*
 nth *Natranaerobius thermophilus*
 hor *Halothermothrix orenii*
 Tenericutes (24)
 Mollicutes (24)
 mpe *Mycoplasma penetrans*
 mga *Mycoplasma gallisepticum*
 Actinobacteria (68)
 mtu *Mycobacterium tuberculosis* H37Rv
 mtc *Mycobacterium tuberculosis* CDC1551
 mra *Mycobacterium tuberculosis* H37Ra
 mtf *Mycobacterium tuberculosis* F11
 mbo *Mycobacterium bovis* AF2122/97
 mbb *Mycobacterium bovis* BCG Pasteur 1173P2
 mbt *Mycobacterium bovis* BCG Tokyo 172
 mle *Mycobacterium leprae* TN
 mlb *Mycobacterium leprae* Br4923
 mpa *Mycobacterium avium* paratuberculosis
 mav *Mycobacterium avium* 104
 msm *Mycobacterium smegmatis*
 mul *Mycobacterium ulcerans*
 mva *Mycobacterium vanbaalenii*
 mgi *Mycobacterium gilvum*
 mab *Mycobacterium abscessus* ATCC 19977T
 mmc *Mycobacterium* sp. MCS
 mkm *Mycobacterium* sp. KMS
 mjl *Mycobacterium* sp. JLS
 mmi *Mycobacterium marinum* M
 cgl *Corynebacterium glutamicum* ATCC 13032 (Kyowa Hakko)
 cgb *Corynebacterium glutamicum* ATCC 13032 (Bielefeld)
 cgt *Corynebacterium glutamicum* R
 cef *Corynebacterium efficiens*
 cdi *Corynebacterium diphtheriae*
 cjk *Corynebacterium jeikeium*
 cur *Corynebacterium urealyticum*
 car *Corynebacterium aurimucosum*
 nfa *Nocardia farcinica*
 rha *Rhodococcus* sp. RHA1
 rer *Rhodococcus erythropolis*
 rop *Rhodococcus opacus*
 sco *Streptomyces coelicolor*
 sma *Streptomyces avermitilis*
 sgr *Streptomyces griseus*

twh *Tropheryma whipplei* Twist
 tws *Tropheryma whipplei* TW08/27
 lxx *Leifsonia xyli xyli* CTCB07
 cmi *Clavibacter michiganensis* subsp. *michiganensis*
 cms *Clavibacter michiganensis* subsp. *sepedonicus*
 art *Arthrobacter* sp. FB24
 aau *Arthrobacter aurescens*
 ach *Arthrobacter chlorophenolicus*
 rsa *Renibacterium salmoninarum*
 krh *Kocuria rhizophila*
 mlu *Micrococcus luteus*
 bcv *Beutenbergia cavernae*
 pac *Propionibacterium acnes*
 nca *Nocardioideis* sp. JS614
 tfu *Thermobifida fusca*
 fra *Frankia* sp. Ccl3
 fre *Frankia* sp. EAN1pec
 fal *Frankia alni*
 ace *Acidothermus cellulolyticus*
 kra *Kineococcus radiotolerans*
 sen *Saccharopolyspora erythraea*
 stp *Salinispora tropica*
 saq *Salinispora arenicola*
 blo *Bifidobacterium longum* NCC2705
 blj *Bifidobacterium longum* DJO10A
 bln *Bifidobacterium longum* subsp. *infantis* ATCC 15697
 bad *Bifidobacterium adolescentis*
 bla *Bifidobacterium animalis* subsp. *lactis* AD011
 blc *Bifidobacterium animalis* subsp. *lactis* BI-04
 blt *Bifidobacterium animalis* subsp. *lactis* DSM 10140
 rxy *Rubrobacter xylanophilus*
Fusobacteria (1)
 fnu *Fusobacterium nucleatum*
Gemmatimonas (1)
 gau *Gemmatimonas aurantiaca*
Planctomyces (1)
 rba *Rhodopirellula baltica*
Verrucomicrobia (3)
 ote *Opitutus terrae*
 min *Methylophilum inferorum*
 amu *Akkermansia muciniphila*
Chlamydia (13)
 ctr *Chlamydia trachomatis* D/UW-3/CX (serovar D)
 cta *Chlamydia trachomatis* A/HAR-13 (serovar A)
 ctb *Chlamydia trachomatis* 434/Bu
 cti *Chlamydia trachomatis* L2b/UCH-1/proctitis
 cmu *Chlamydia muridarum*
 cpn *Chlamydophila pneumoniae* CWL029
 cpa *Chlamydophila pneumoniae* AR39
 cpj *Chlamydophila pneumoniae* J138
 cpt *Chlamydophila pneumoniae* TW183
 cca *Chlamydophila caviae*
 cab *Chlamydophila abortus*
 cfe *Chlamydophila felis*
 pcu *Candidatus Protochlamydia amoebophila*

Spirochete (18)

tpa *Treponema pallidum* subsp. *pallidum* Nichols
 tpp *Treponema pallidum* subsp. *pallidum* SS14
 tde *Treponema denticola*
 lil *Leptospira interrogans* serovar *lai*
 lic *Leptospira interrogans* serovar *Copenhageni*
 lbj *Leptospira borgpetersenii* JB197
 lbl *Leptospira borgpetersenii* L550
 lbi *Leptospira biflexa* serovar *Patoc* Patoc 1 (Paris)
 lbf *Leptospira biflexa* serovar *Patoc* Patoc 1 (Ames)
 bhy *Brachyspira hyodysenteriae*

Cyanobacteria (36)

syn *Synechocystis* sp. PCC6803
 syw *Synechococcus* sp. WH8102
 syc *Synechococcus elongatus* PCC6301
 syf *Synechococcus elongatus* PCC7942
 syd *Synechococcus* sp. CC9605
 sye *Synechococcus* sp. CC9902
 syg *Synechococcus* sp. CC9311
 syr *Synechococcus* sp. RCC307
 syx *Synechococcus* sp. WH7803
 syp *Synechococcus* sp. PCC7002
 cya *Cyanobacteria* Yellowstone A-Prime
 cyb *Cyanobacteria* Yellowstone B-Prime
 tel *Thermosynechococcus elongatus*
 mar *Microcystis aeruginosa*
 cyt *Cyanothece* sp. ATCC 51142
 cyp *Cyanothece* sp. PCC 8801
 cyc *Cyanothece* sp. PCC 7424
 cyn *Cyanothece* sp. PCC 7425
 gvi *Gloeobacter violaceus*
 ana *Anabaena* sp. PCC7120
 npu *Nostoc punctiforme*
 ava *Anabaena variabilis*
 pma *Prochlorococcus marinus* SS120
 pmm *Prochlorococcus marinus* MED4
 pmt *Prochlorococcus marinus* MIT9313
 pmn *Prochlorococcus marinus* NATL2A
 pmi *Prochlorococcus marinus* MIT9312
 pmb *Prochlorococcus marinus* AS9601
 pmc *Prochlorococcus marinus* MIT 9515
 pmf *Prochlorococcus marinus* MIT 9303
 pmg *Prochlorococcus marinus* MIT 9301
 pmh *Prochlorococcus marinus* MIT 9215
 pmj *Prochlorococcus marinus* MIT 9211
 pme *Prochlorococcus marinus* NATL1A
 ter *Trichodesmium erythraeum*
 amr *Acaryochloris marina*

Bacteroides (15)

bth *Bacteroides thetaiotaomicron*
 bfr *Bacteroides fragilis* YCH46
 bfs *Bacteroides fragilis* NCTC9343
 bvu *Bacteroides vulgatus*
 pgi *Porphyromonas gingivalis* W83
 pgn *Porphyromonas gingivalis* ATCC 33277

pdi *Parabacteroides distasonis*
 aps *Candidatus Azobacteroides pseudotrichonymphae*
 sru *Salinibacter ruber*
 chu *Cytophaga hutchinsonii*
 Green sulfur bacteria (11)
 cte *Chlorobaculum tepidum*
 cpc *Chlorobaculum parvum* NCIB 8327
 cch *Chlorobium chlorochromatii*
 cph *Chlorobium phaeobacteroides* DSM 266
 cpb *Chlorobium phaeobacteroides* BS1
 cli *Chlorobium limicola*
 pvi *Prosthecochloris vibrioformis*
 plt *Pelodictyon luteolum*
 pph *Pelodictyon phaeoclathratiforme*
 cts *Chloroherpeton thalassium*
 paa *Prosthecochloris aestuarii*
 Green nonsulfur bacteria (10)
 det *Dehalococcoides ethenogenes*
 deh *Dehalococcoides* sp. CBDB1
 deb *Dehalococcoides* sp. BAV1
 tro *Thermomicrobium roseum*
 Termite group (2)
 emi *Elusimicrobium minutum*
 rsd Uncultured Termite group 1 bacterium phylotype Rs-D17
 Deinococcus-Thermus (5)
 dra *Deinococcus radiodurans*
 dge *Deinococcus geothermalis*
 ddr *Deinococcus deserti*
 tth *Thermus thermophilus* HB27
 ttj *Thermus thermophilus* HB8
 Hyperthermophilic bacteria (18)
 aae *Aquifex aeolicus*
 hya *Hydrogenobaculum* sp. Y04AAS1
 sul *Sulfurihydrogenibium* sp. Y03AOP1
 saf *Sulfurihydrogenibium azorense*
 pmx *Persephonella marina*
 tma *Thermotoga maritima*
 tpt *Thermotoga petrophila*
 tle *Thermotoga lettingae*
 trq *Thermotoga* sp. RQ2
 tna *Thermotoga neapolitana*
 tme *Thermosipho melanesiensis*
 taf *Thermosipho africanus*
 fno *Fervidobacterium nodosum*
 pmo *Petrotoga mobilis*
 kol *Kosmotoga olearia*
 dth *Dictyoglomus thermophilum*
 dtu *Dictyoglomus turgidum*
 tye *Thermodesulfovibrio yellowstonii*

Table 5. Bacteria Which Synthesize Squalene Via HMG-CoA PATHWAY

Prokaryotes (927)

Bacteria (863)

Proteobacteria (461)

Gamma/others (136)

cbu *Coxiella burnetii* RSA 493
 lpn *Legionella pneumophila* Philadelphia 1
 lpf *Legionella pneumophila* Lens
 lpp *Legionella pneumophila* Paris
 lpc *Legionella pneumophila* Corby
 tcx *Thiomicrospira crunogena*
 dno *Dichelobacter nodosus*

Delta (29)

bba *Bdellovibrio bacteriovorus*
 mxa *Myxococcus xanthus*
 scl *Sorangium cellulosum*

Firmicutes (173)

Bacillales (61)

oih *Oceanobacillus iheyensis*
 sau *Staphylococcus aureus* N315
 sav *Staphylococcus aureus* Mu50
 saw *Staphylococcus aureus* Mu3
 sam *Staphylococcus aureus* MW2
 sar *Staphylococcus aureus* MRSA252
 sas *Staphylococcus aureus* MSSA476
 sac *Staphylococcus aureus* COL
 sab *Staphylococcus aureus* RF122
 saa *Staphylococcus aureus* USA300
 sax *Staphylococcus aureus* USA300 TCH1516
 sao *Staphylococcus aureus* NCTC8325
 saj *Staphylococcus aureus* JH9
 sah *Staphylococcus aureus* JH1
 sae *Staphylococcus aureus* Newman
 sep *Staphylococcus epidermidis* ATCC 12228
 ser *Staphylococcus epidermidis* RP62A
 sha *Staphylococcus haemolyticus*
 ssp *Staphylococcus saprophyticus*
 sca *Staphylococcus carnosus*
 lmo *Listeria monocytogenes* EGD-e
 lmf *Listeria monocytogenes* F2365
 lmh *Listeria monocytogenes* HCC23
 lmc *Listeria monocytogenes* Clip81459
 lin *Listeria innocua*
 lwe *Listeria welshimeri* SLCC5334

Lactobacillales (66)

lla *Lactococcus lactis* subsp. *lactis* IL1403
 llic *Lactococcus lactis* subsp. *cremoris* SK11
 llim *Lactococcus lactis* subsp. *cremoris* MG1363
 spy *Streptococcus pyogenes* SF370 (serotype M1)
 spz *Streptococcus pyogenes* MGAS5005 (serotype M1)
 spm *Streptococcus pyogenes* MGAS8232 (serotype M18)

spg *Streptococcus pyogenes* MGAS315 (serotype M3)
 sps *Streptococcus pyogenes* SSI-1 (serotype M3)
 sph *Streptococcus pyogenes* MGAS10270 (serotype M2)
 spi *Streptococcus pyogenes* MGAS10750 (serotype M4)
 spj *Streptococcus pyogenes* MGAS2096 (serotype M12)
 spk *Streptococcus pyogenes* MGAS9429 (serotype M12)
 spf *Streptococcus pyogenes* Manfredo (serotype M5)
 spa *Streptococcus pyogenes* MGAS10394 (serotype M6)
 spb *Streptococcus pyogenes* MGAS6180 (serotype M28)
 soz *Streptococcus pyogenes* NZ131 (serotype M49)
 spn *Streptococcus pneumoniae* TIGR4
 spr *Streptococcus pneumoniae* R6
 spd *Streptococcus pneumoniae* D39
 spv *Streptococcus pneumoniae* Hungary19A 6
 spw *Streptococcus pneumoniae* CGSP14
 spx *Streptococcus pneumoniae* G54
 sne *Streptococcus pneumoniae* ATCC 700669
 snm *Streptococcus pneumoniae* 70585
 sjj *Streptococcus pneumoniae* JJA
 spp *Streptococcus pneumoniae* P1031
 snt *Streptococcus pneumoniae* Taiwan19F-14
 sag *Streptococcus agalactiae* 2603 (serotype V)
 san *Streptococcus agalactiae* NEM316 (serotype III)
 sak *Streptococcus agalactiae* A909 (serotype Ia)
 smu *Streptococcus mutans*
 stc *Streptococcus thermophilus* CNRZ1066
 stl *Streptococcus thermophilus* LMG18311
 ste *Streptococcus thermophilus* LMD-9
 ssa *Streptococcus sanguinis*
 ssu *Streptococcus suis* 05ZYH33
 ssv *Streptococcus suis* 98HAH33
 sgo *Streptococcus gordonii*
 seq *Streptococcus equi* subsp. zooepidemicus
 sez *Streptococcus equi* subsp. zooepidemicus MGCS10565
 seu *Streptococcus equi* subsp. equi 4047
 sub *Streptococcus uberis*
 lpl *Lactobacillus plantarum*
 ljo *Lactobacillus johnsonii*
 lac *Lactobacillus acidophilus*
 lsa *Lactobacillus sakei*
 lsl *Lactobacillus salivarius*
 ldb *Lactobacillus delbrueckii* ATCC 11842
 lbu *Lactobacillus delbrueckii* ATCC BAA-365
 lbr *Lactobacillus brevis*
 lca *Lactobacillus casei* ATCC 334
 lcb *Lactobacillus casei* BL23
 lga *Lactobacillus gasseri*
 lre *Lactobacillus reuteri* DSM 20016
 lrf *Lactobacillus reuteri* JCM 1112
 lhe *Lactobacillus helveticus*
 lfe *Lactobacillus fermentum*
 ppe *Pediococcus pentosaceus*
 efa *Enterococcus faecalis*
 lme *Leuconostoc mesenteroides*
 lci *Leuconostoc citreum*

Tenericutes (24)
 Mollicutes (24)
 acl *Acholeplasma laidlawii*
 Actinobacteria (68)
 mul *Mycobacterium ulcerans*
 mmi *Mycobacterium marinum* M
 ckp *Corynebacterium kroppenstedtii*
 nfa *Nocardia farcinica*
 Spirochete (18)
 bbu *Borrelia burgdorferi*
 bga *Borrelia garinii*
 baf *Borrelia afzelii*
 bbz *Borrelia burgdorferi* ZS7
 btu *Borrelia turicatae*
 bhr *Borrelia hermsii*
 bdu *Borrelia duttonii*
 bre *Borrelia recurrentis*
 Bacteroides (15)
 gfo *Gramella forsetii*
 fjo *Flavobacterium johnsoniae*
 fps *Flavobacterium psychrophilum*
 Green nonsulfur bacteria (10)
 rrs *Roseiflexus* sp. RS-1
 rca *Roseiflexus castenholzii* DSM13941
 cau *Chloroflexus aurantiacus*
 cag *Chloroflexus aggregans*
 chl *Chloroflexus* sp. Y-400-fl
 hau *Herpetosiphon aurantiacus*
 Archaea (64)
 Euryarchaeota (39)
 hal *Halobacterium* sp. NRC-1
 hsl *Halobacterium salinarum* R1
 hma *Haloarcula marismortui*
 hwa *Haloquadratum walsbyi*
 nph *Natronomonas pharaonis*
 hla *Halorubrum lacusprofundi*
 pto *Picrophilus torridus*
 Crenarchaeota (23)
 sso *Sulfolobus solfataricus*
 sto *Sulfolobus tokodaii*
 sai *Sulfolobus acidocaldarius*
 sis *Sulfolobus islandicus* L.S.2.15
 sia *Sulfolobus islandicus* M.14.25
 sim *Sulfolobus islandicus* M.16.27
 sid *Sulfolobus islandicus* M.16.4
 siy *Sulfolobus islandicus* Y.G.57.14
 sin *Sulfolobus islandicus* Y.N.15.51
 mse *Metallosphaera sedula*

Table 6

Strain	Domains in membrane	Domains in spore
Wild type	6.1 ± 0.5	2.2 ± 0.4
+Zaragozic	1.5 ± 0.5	5.6 ± 1.0
$\Delta yisP$	1.9 ± 0.2	5.2 ± 0.6
$\Delta sqhC$	4.0 ± 0.6	0.2 ± 0.2
$\Delta sqhC$ +Zaragozic	0.5 ± 0.3	0.2 ± 0.2

Table 7: Differential gene expression of the $\Delta yisP$ mutant compared to the wild-type

Genes			Induction ratio ($\Delta yisP$ /wt)
Central metabolism	Master regulatory genes	<i>ccpC</i>	-3
		<i>rocR</i>	6
		<i>glnR</i>	5
		<i>ccpA</i>	-4
		<i>codY</i>	-3
	Structural genes	<i>citZCH</i>	12
		<i>citB</i>	30
		<i>rocABC</i>	4
		<i>rocDEF</i>	8
		<i>rocG</i>	4
		<i>ureABC</i>	-8
		<i>hprK</i>	-6
		<i>relA</i>	-5
Developmental metabolism	Master regulatory genes	<i>abrB</i>	8
		<i>abh</i>	3
		<i>sinIR</i>	-4
		<i>mta</i>	4
		σ^D	5
		σ^X	3
		σ^W	3
	Structural genes	<i>srfAA-AD</i>	3
		<i>pgsBCA</i>	8
		<i>comGA-GG</i>	3
		<i>yqxM-sipW-tasA</i>	-5
		<i>skfA-H</i>	-5
		<i>dltA-E</i>	6
		<i>albA-G</i>	4

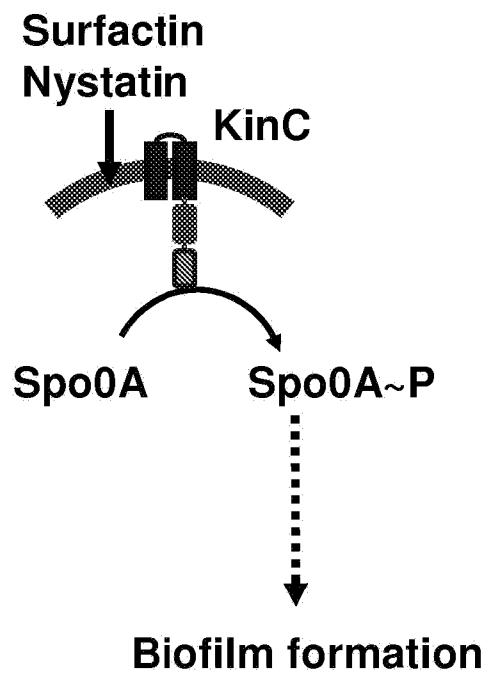
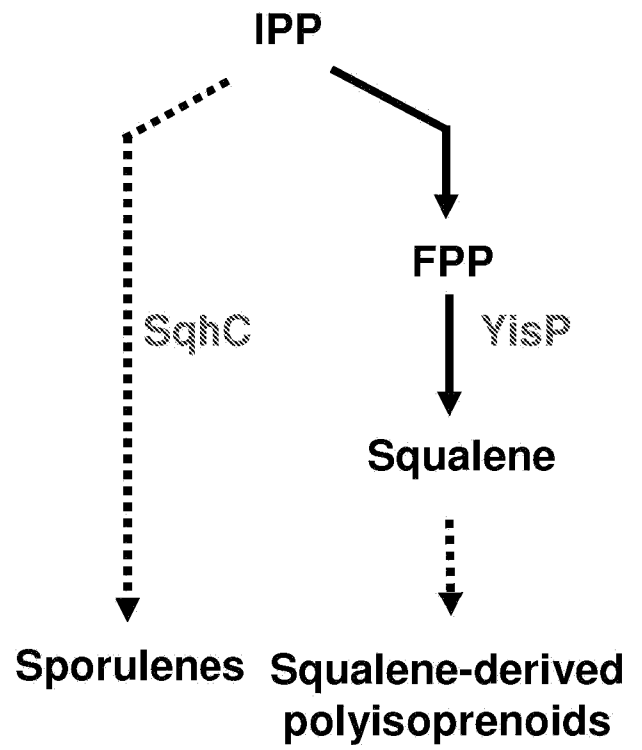
What is claimed:

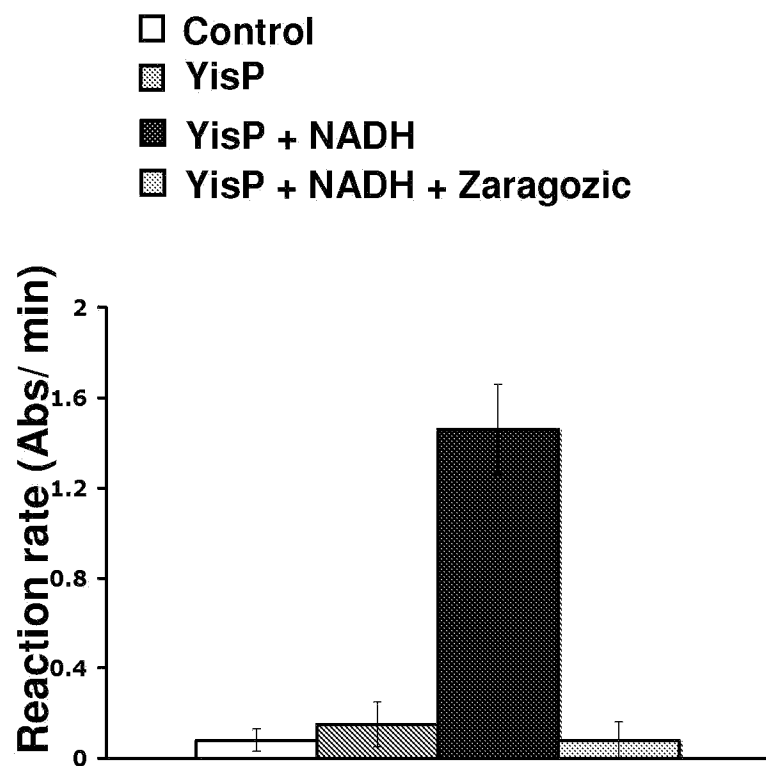
1. A composition for inhibiting bacterial biofilm formation comprising a carrier and an effective amount of an inhibitor of squalene/phytoene synthesis.
2. The composition of claim 1 wherein the inhibitor of squalene/phytoene synthesis inhibits HMG-CoA Reductase.
3. The composition of claim 1 wherein the inhibitor of squalene/phytoene synthesis inhibits squalene synthase.
4. The composition of claim 1 wherein the inhibitor of squalene/phytoene synthesis inhibits 1-deoxy-D-xylulose 5-phosphate synthase.
5. The composition of claim 1, wherein the inhibitor of squalene/phytoene synthesis is selected from the group consisting of a phosphonosulfonate, a statin, zaragozic acid, clomazone, and lapaquistat acetate or a functional derivative thereof.
6. The composition of claim 1, wherein the inhibitor of squalene/phytoene synthesis is a phosphonosulfonate or a functional derivative thereof.
7. The composition of claim 6, wherein the phosphonosulfonate is selected from the group consisting of BPH-652, BPH-689, BPH-700.
8. The composition of claim 1, 2, or 5, wherein the inhibitor of squalene/phytoene synthesis is a statin or a functional derivative thereof.
9. The composition of claim 8, wherein the statin is selected from the group consisting of mevastatin, lovastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin.
10. The composition of claim 1, 3 or 5, wherein the inhibitor of squalene/phytoene synthesis is zaragozic acid or a functional derivative thereof.

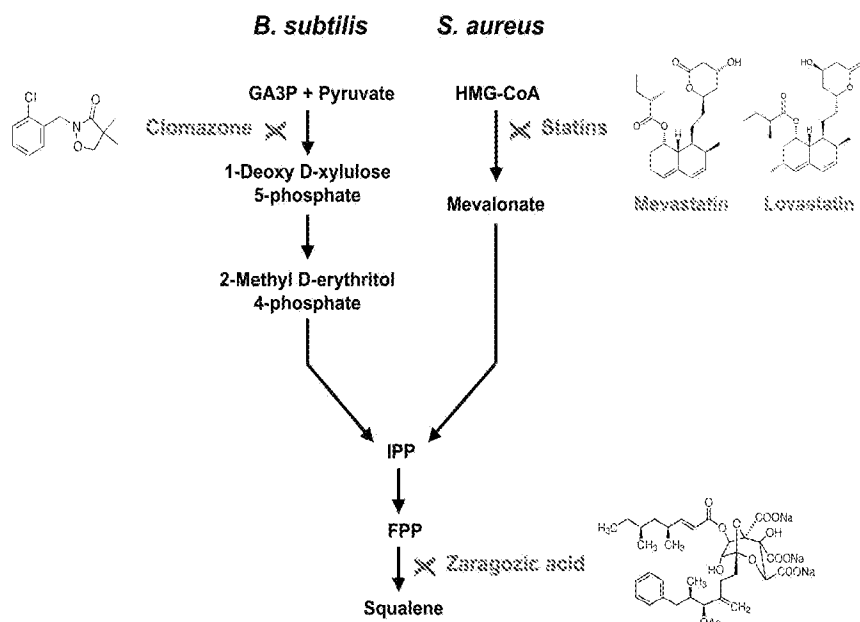
11. The composition of claim 1, 4 or 5, wherein the inhibitor of squalene/phytoene synthesis is clomazone or a functional derivative thereof.
12. The composition of claims 1-11, wherein the inhibitor of squalene/phytoene synthesis is lapauisat acetate or a functional derivative thereof.
13. The composition of claims 1-12, wherein the carrier is a liquid.
14. The composition of claims 1-12, wherein the carrier is a solid, semi-solid, slurry or paste.
15. The composition of claims 1-12, wherein the carrier is a coating agent.
16. A solid or semi-solid substrate comprising an inhibitor of squalene/phytoene synthesis.
17. The substrate of claim 16, wherein the inhibitor of squalene/phytoene synthesis is deposited or absorbed to a surface of the substrate with a composition of any one of claims 1-15.
18. The substrate of claim 16 or 17, wherein the substrate is formulated to contain the inhibitor of squalene/phytoene synthesis throughout its entire composition.
19. The substrate of claims 16-18 which further comprises additional inhibitors of biofilm or antibacterial agents incorporated therein.
20. The substrate of claim 18 or 19 which is formed as a device, or part thereof, for implantation into a living subject.
21. A method for inhibiting bacterial biofilm formation comprising contacting a biofilm producing bacteria with an effective amount of an inhibitor of squalene/phytoene synthesis.
22. The method of claim 21, wherein the inhibitor inhibits HMG-CoA Reductase.

23. The method of claim 21 or 22, wherein the inhibitor is a statin or a functional derivative thereof.
24. The method of claim 21, wherein the inhibitor inhibits squalene synthase.
25. The method of claim 21-24, wherein contacting occurs *in vivo*.
26. The method of claim 25, wherein the contacting occurs in a mammal.
27. The method of claim 21-24 wherein the contacting occurs *in vitro*.
28. The method of claim 21-24, wherein contacting occurs in a non-living medium.
29. The method of claim 28, wherein the inhibitor is formulated as an antiseptic.
30. The method of claim 24, wherein the inhibitor is a phosphonosulfonate.
31. The method of claim 21, wherein the inhibitor inhibits 1-deoxy-D-xylulose 5-phosphate synthase.
32. The method of claim 31, wherein the inhibitor is clomazone or a functional derivative thereof.
33. The method of claim 21, wherein the inhibitor is zaragozic acid, a statin, or lapaquistat acetate or a functional derivative thereof.
34. The method of claim 23, wherein the statin is selected from the group consisting of mevastatin, lovastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin.
35. The method of claim 30, wherein the phosphonosulfonate is selected from the group consisting of BPH-652, BPH-689, BPH-700.

36. The method of claims 21- 35, wherein contacting occurs in the presence of an additional agent that impacts the growth and/or attachment and/or virulence of a biofilm forming organism.

1A**1B****Figure 1**

**FIGURE 2**

**FIGURE 3**

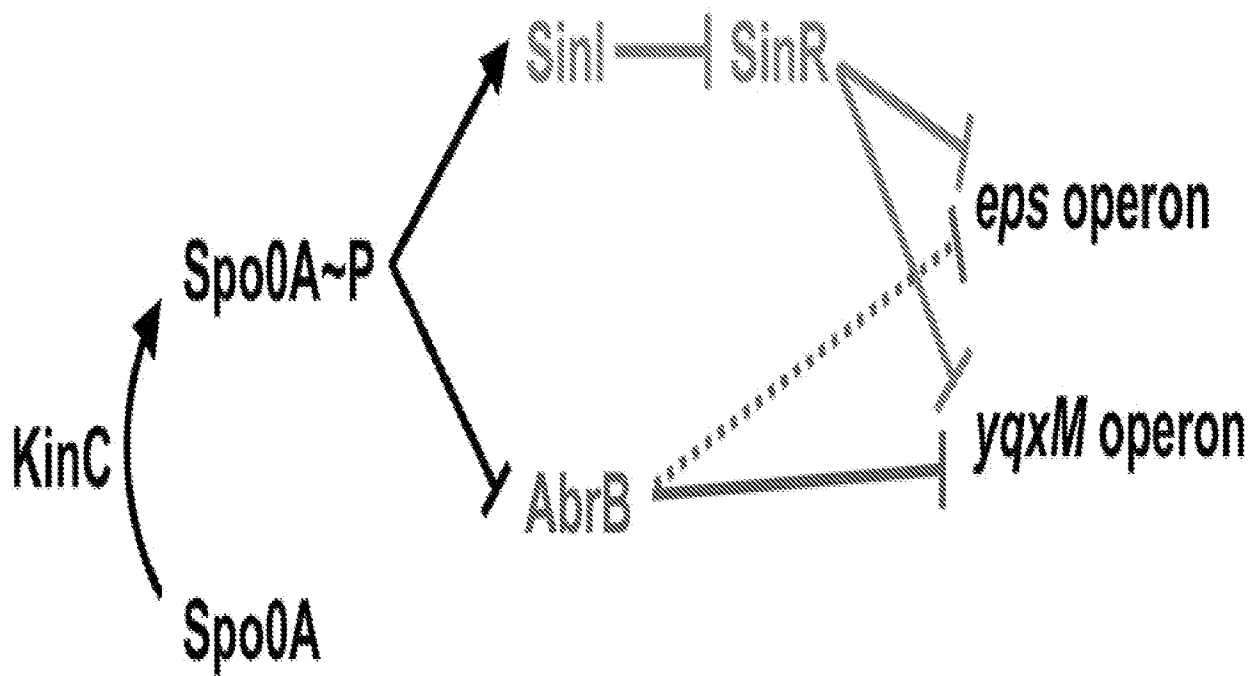
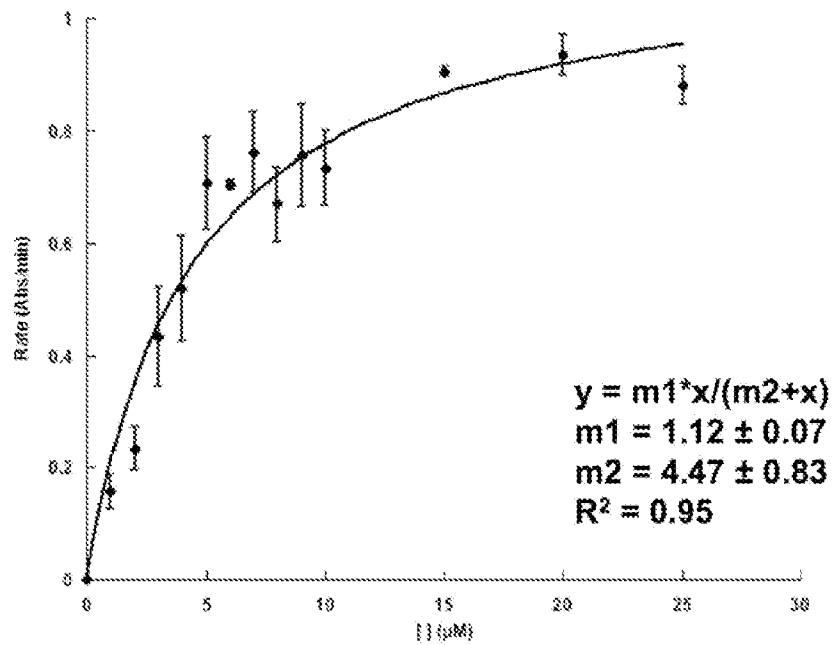


FIGURE 4

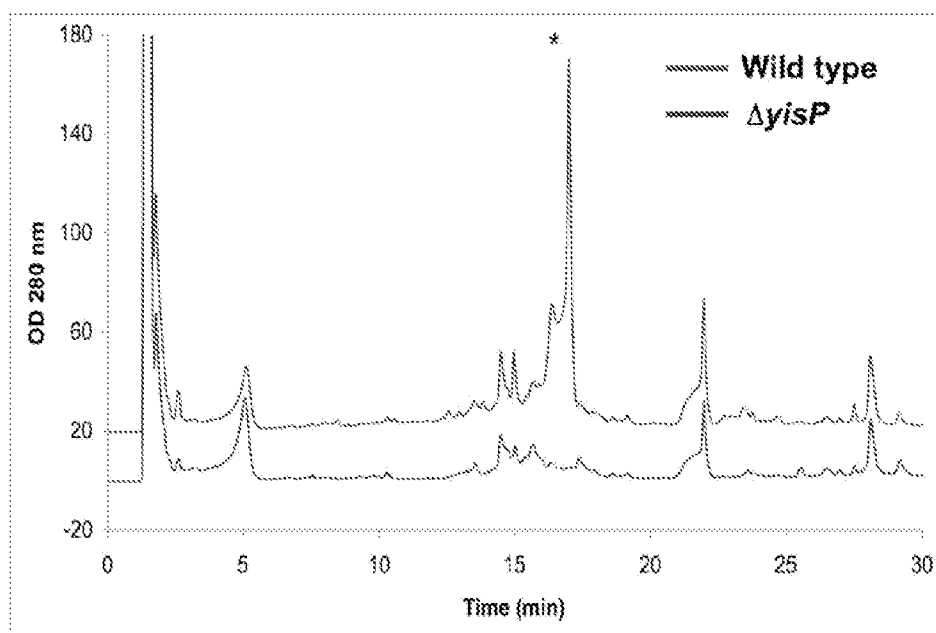
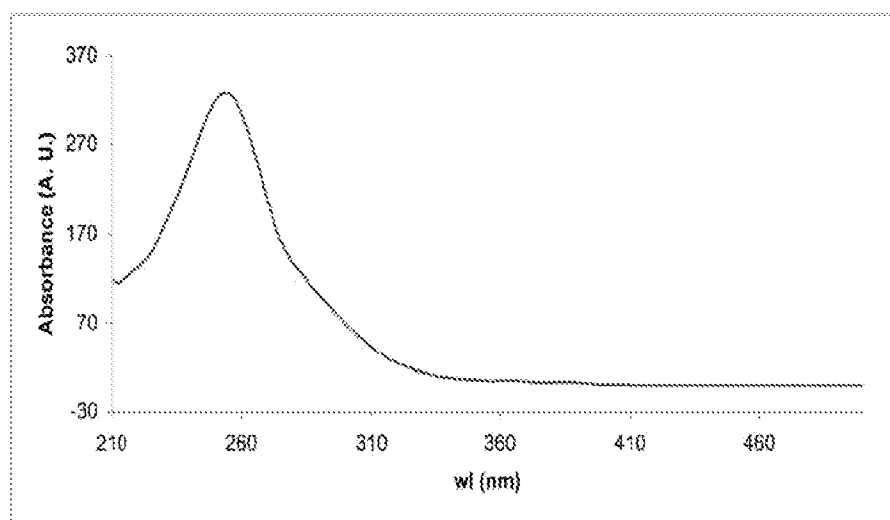
A**B**

pH = 7.4
T ($^{\circ}\text{C}$) = 42
Mg⁺² = 20 mM

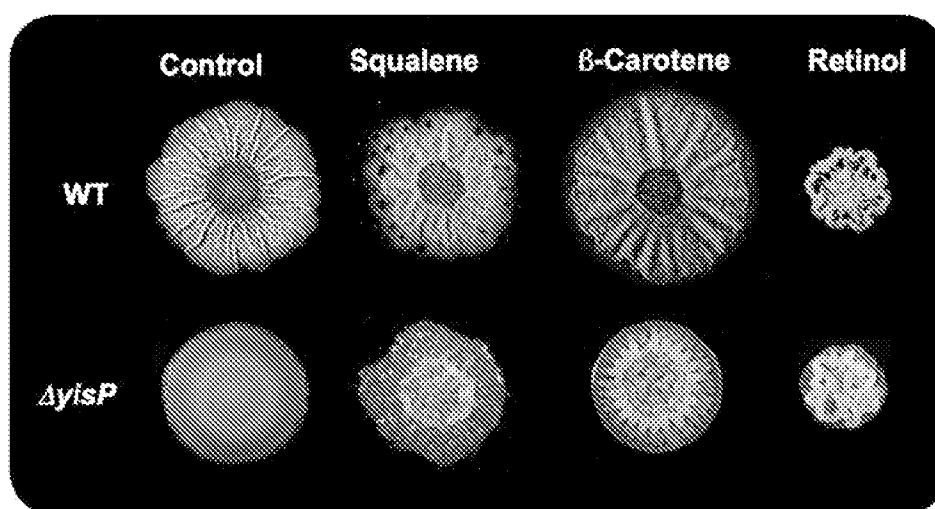
K_m [FPP] = 4.47 μM
K_m [GPP] = 14.9 μM
K_m [GGPP] = >50 μM
K_m [NADH] = 250 μM

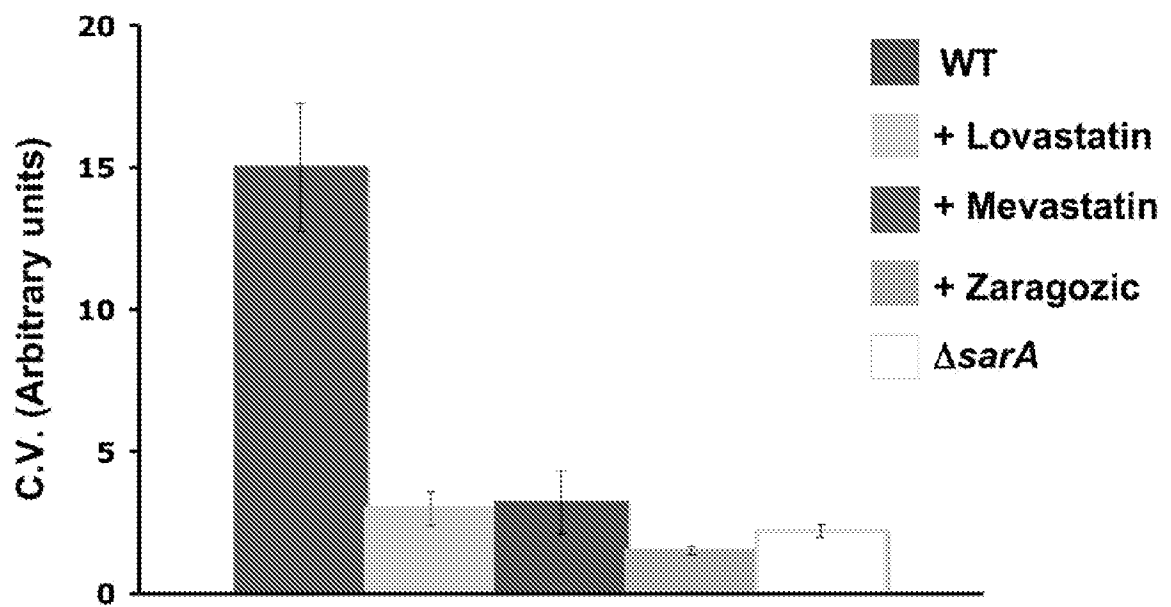
Zaragocic acid IC₅₀ = 322 $\pm$ 102 nM

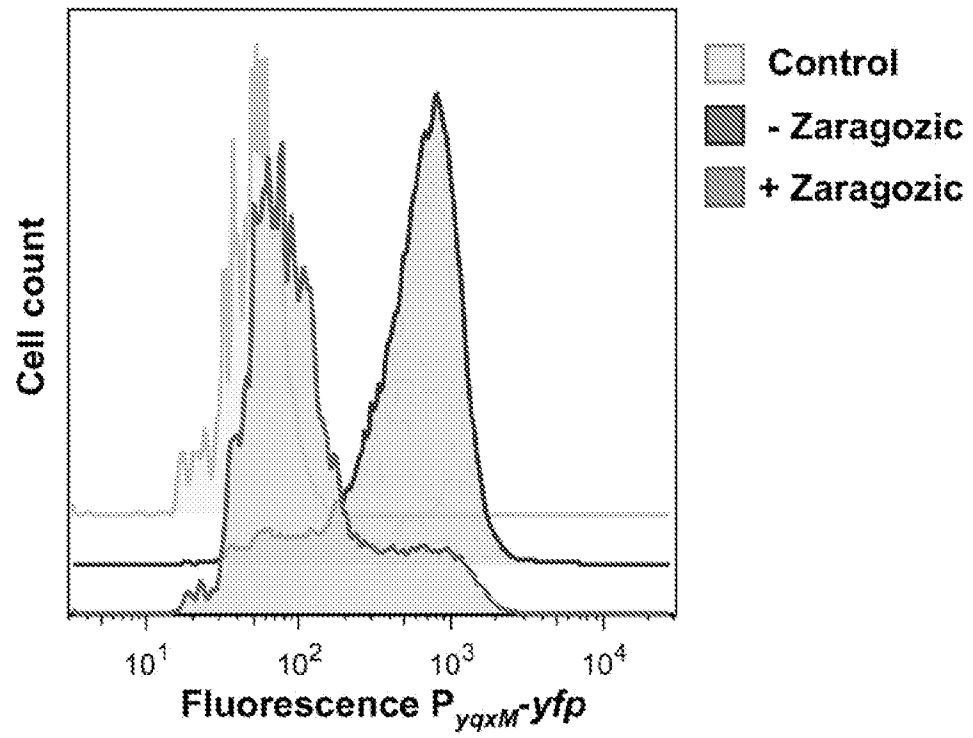
FIGURE 5

A**B****FIGURE 6**

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**FIGURE 7**

**FIGURE 8**

**FIGURE 9**