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ORIGINAL ARTICLE

S-Aryl-L-cysteine sulphoxides and related organosulphur compounds alter oral biofilm development and AI-2-based cell–cell communication

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Abstract

Aims: To design and synthesize a library of structurally related, small molecules related to homologues of compounds produced by the plant *Petiveria alliacea* and determine their ability to interfere with AI-2 cell–cell communication and biofilm formation by oral bacteria. Many human diseases are associated with persistent bacterial biofilms. Oral biofilms (dental plaque) are problematic as they are often associated with tooth decay, periodontal disease and systemic disorders such as heart disease and diabetes.

Methods and Results: Using a microplate-based approach, a bio-inspired small molecule library was screened for anti-biofilm activity against the oral species *Streptococcus mutans* UA159, *Streptococcus sanguis* 10556 and *Actinomyces oris* MG1. To complement the static screen, a flow-based BioFlux microfluidic system screen was also performed under conditions representative of the human oral cavity. Several compounds were found to display biofilm inhibitory activity in all three of the oral bacteria tested. These compounds were also shown to inhibit bioluminescence by *Vibrio harveyi* and were thus inferred to be quorum sensing (QS) inhibitors.

Conclusion: Due to the structural similarity of these compounds to each other, and to key molecules in AI-2 biosynthetic pathways, we propose that these molecules potentially reduce biofilm formation via antagonism of QS or QS-related pathways.

Significance and Impact of the Study: This study highlights the potential for a non-antimicrobial-based strategy, focused on AI-2 cell–cell signalling, to control the development of dental plaque. Considering that many bacterial species use AI-2 cell–cell signalling, as well as the increased concern of the use of antimicrobials in healthcare products, such an anti-biofilm approach could also be used to control biofilms in environments beyond the human oral cavity.

Introduction

Oral biofilms, the most visually conspicuous being dental plaque, are surface-attached bacterial communities encased within an intricate network of extracellular polymeric substances produced by the constituent bacteria (Kolenbrander *et al.* 2006). The biofilm mode of growth confers distinct advantages over planktonic lifestyles. In the case of the oral cavity, bacterial cell encapsulation within biofilms first and foremost reduces the likelihood of their translocation to inhospitable environments such as the stomach (Kolenbrander and London 1993; Marsh *et al.* 2011). Bacteria within oral biofilms also profit from the local exchange of nutrient by-products from juxtaposed biofilm species (Marsh 2005). Most importantly, the biofilm presents a formidable barricade that not only

protects biofilm bacteria from host immune defences and adverse changes in the environment, but also presents an impenetrable barrier to antibiotics to which the bacteria might otherwise be susceptible (Costerton 1999; Stewart and Costerton 2001; Gilbert *et al.* 2002; Stewart 2002; Lewis 2007). Collectively, these factors make oral biofilms recalcitrant to physical and chemical treatment strategies (Hall-Stoodley *et al.* 2004; Marsh 2005).

From a human health perspective, biofilms present in the oral cavity of individuals impose significant potential for not only the development of oral infections, leading to dental caries and periodontal disease, but may also be linked to cardiovascular disease, obesity, complications during pregnancy, diabetes and respiratory diseases (Scannapieco 1999; Scannapieco *et al.* 2003; Costerton and Keller 2007; Paju and Scannapieco 2007; Selwitz *et al.* 2007; Zeigler *et al.* 2012). Oral biofilms can also serve as a nucleus for the horizontal transfer of antimicrobial resistance genes between bacteria (Roberts and Mullany 2010).

Multiple pathways regulate formation of biofilms, but one key target that has been demonstrated to inhibit or retard biofilm development is quorum sensing (QS) (Kolenbrander *et al.* 2002, 2010; Hojo *et al.* 2009; Shao and Demuth 2010). QS is a population-dependent communication system that allows micro-organisms to coordinate group behaviour in a manner that enables them to act as a multi-cellular single entity. QS uses the accumulation of molecules often referred to as autoinducers to regulate gene expression. Many of these are virulence-related (Parsek and Greenberg 2000; Winans and Bassler 2002; March and Bentley 2004; Vendeville *et al.* 2005). Autoinducer-2 (AI-2) is an umbrella term commonly used to describe a family of inter-convertible molecules in equilibrium formed by spontaneous cyclization of the unstable molecule 4,5-dihydroxy-2,3-pentanedione (DPD) (Semmelhack *et al.* 2005). AI-2 has been proposed to be an inter-species signal, and numerous species have been shown to engage in AI-2-mediated communication (Federle and Bassler 2003; Xavier and Bassler 2003). AI-2 is a by-product of the activated methyl cycle and is generated by the cleavage of *S*-ribosylhomocysteine into homocysteine by the enzyme LuxS (Schauder *et al.* 2001). LuxS/AI-2-based QS has been demonstrated to regulate biofilm formation in an assortment of oral bacteria. *Streptococcus mutans luxS* mutants were shown to be deficient in biofilm formation and displayed downregulation in many biofilm-related genes (Merritt *et al.* 2003; Sztajer *et al.* 2008). Biofilm formation by an *Strep. mutans luxS* mutant has also been shown to be restored by complementation when grown in the presence of certain *Streptococcus*, *Porphyromonas* and *Aggregatibacter* species (Yoshida *et al.* 2005). Furthermore, *Streptococcus oralis* 34 and *Actinomyces naeslundii* T14V were shown to have a mutualistically dependent biofilm-forming relationship that was dependent upon AI-2 in a concentration-dependent manner (Rickard *et al.* 2006). Several other studies link LuxS/AI-2-based signalling with oral biofilm formation and the expression of other virulence factors which ultimately induce acid tolerance, oxidative stress and bacteriocin production states (Blehert *et al.* 2003; Stroeher *et al.* 2003; Wen and Burne 2004; Merritt *et al.* 2005; Ahmed *et al.* 2009; Shemesh *et al.* 2010). Thus, interfering with AI-2-based cell–cell communication may interrupt biofilm formation, which may serve as a viable strategy for improving oral and systemic health. In particular, manipulating the expression of virulence factors while minimizing cytotoxic effects in bacteria may be an attractive approach to disease treatment, as it lowers the pressure of natural selection and therefore may reduce the likelihood of the development of microbial resistance (Rasmussen and Givskov 2006b; Brackman *et al.* 2009; Njoroge and Sperandio 2009; Kalia 2013).

Plants are perpetually exposed to microbes and prone to bacterial invasion. Therefore, it is likely that many flora have developed mechanisms, such as QS inhibition, to defend against harmful pathogens (Rasmussen and Givskov 2006a). Thus, plant metabolites may serve as a significant untapped resource for compounds that mitigate the incursion of infection through inhibition of QS in pathogenic bacteria (Koh *et al.* 2013). In this regard, a number of studies have shown that plant-derived organosulphur natural products such as iberin and ajoene, isolated from horseradish and garlic, respectively, inhibit QS and virulence in *Pseudomonas aeruginosa* (Jakobsen *et al.* 2012a,b). Another interesting class of small molecule natural products is the halogenated furanones initially isolated from the marine algae *Delisea pulchra*, which were demonstrated to inhibit acyl-homoserine lactone (AHL)-mediated communication in several marine bacteria (Givskov *et al.* 1996). Synthetic derivatives of these compounds have since been shown to inhibit AHL-mediated QS and virulence in *Ps. aeruginosa*, AI-2-mediated QS in *Vibrio harveyi* and biofilm formation by several orally associated bacteria (Hentzer *et al.* 2002; Wu *et al.* 2004; Lönn-Stensrud *et al.* 2007; He *et al.* 2012). Previously, we reported that derivatives of metabolites from the flowering plant *Petiveria alliacea*, namely substituted cysteine sulphoxides and diphenyl disulphides, can reduce *Ps. aeruginosa* biofilm formation and QS, as well as bacterial infection in a *Drosophila* model (Cady *et al.* 2012).

The aim of this study was to expand upon the library of *S*-substituted cysteine sulphoxide-based derivatives and to explore the biofilm inhibitory activity of these compounds against bacteria that are relevant to the human oral microbiome, namely *Strep. mutans* UA159, *Streptococcus*

sanguis 10556 and *Actinomyces oris* MG1. *Streptococcus mutans* is most notorious as an etiological agent of dental caries (Ajdić *et al.* 2002). *Streptococcus sanguis* is considered a pioneer colonizer and is known as one of the earliest bacteria to recolonize the tooth surface, postremoval (Ge *et al.* 2008). *Actinomyces oris* is also an early colonizer that attaches to the tooth surface, serving as a foundation for subsequent attachment of other species (Kolenbrander *et al.* 2006; Dige *et al.* 2009). To investigate potential interference of inter-species communication, compounds were also tested in a luminescence-based AI-2 QS reporter, *V. harveyi* BB170. Of the 46 compounds screened, six compounds inhibited biofilm formation in all three indigenous oral bacteria, while five of these six also inhibited luminescence by the AI-2 QS reporter strain *V. harveyi* BB170.

Materials and methods

Strains, standard culture conditions and reagents

The oral strains *Strep. mutans* UA159, *Strep. sanguis* 10556 and *Act. oris* MG1 were used in this study (*Strep. mutans* UA159 was kindly donated by Prof. Tom Wen, Microbiology, Immunology and Parasitology, Louisiana State University, and *Act. oris* MG1 was kindly donated by Dr. Paul Kolenbrander, NIDCR, NIH, Bethesda). All were grown on brain–heart infusion agar and brain–heart infusion broth (BHI; Difco/Becton Dickinson, Franklin Lakes, NJ) at 37°C under 5% CO_2 atmosphere. All reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).

Tested compounds

S-(2-Aminoethyl)-L-cysteine hydrochloride (**1**), taurine (**2**), L-cysteic acid (**3**), glycinamide hydrochloride (**4**), (±)-carnitine hydrochloride (**5**), *S*-carboxylmethyl-L-cysteine (**6**), diphenyl disulphide (**12**), dibenzyl sulphide (**13**), dibenzyl disulphide (**14**), sulfolane (**18**), *S*-methyl-L-cysteine (**28**), L-carnosine (**29**), djenkolic acid (**35**), *S*-ethyl-L-cysteine (**36**), *S*-(4-tolyl)-L-cysteine (**37**), *S*-(2-thienyl)-L-cysteine (**38**), *S*-(2-thiazoyl)-L-cysteine (**39**), *S*-propyl-L-cysteine (**40**) and *S*-phenyl-L-cysteine (**41**) were purchased from Sigma-Aldrich and were used without further purification. Cysteine sulphoxides **7**, **8**, **9**, **10**, **11**, **21**, **22**, **23**, **24**, **25**, **32**, **34**, **42**, **43** and **44** are known compounds and were synthesized by hydrogen peroxide-mediated oxidation of the corresponding cysteines and fully characterized as we have described previously (Kubec *et al.* 2002; Musah *et al.* 2009; He *et al.* 2011; Cady *et al.* 2012). *S*-Substituted **19**, **20**, **26**, **27**, **30** and **45** were synthesized by treatment of cysteine with the corresponding alkyl bromide (obtained from Sigma-Aldrich) in the presence of aqueous NaOH and fully characterized as described previously (Musah *et al.* 2009; He *et al.* 2011; Cady *et al.* 2012). Disulphides **15** and **16** were synthesized by sodium periodate-mediated oxidation of the corresponding sulphides and fully characterized as described previously (Cady *et al.* 2012). Compound **33** was synthesized as described by Johnson and Ambler (1914). Compounds **46**, **17** and **31** were prepared by H_2O_2-promoted oxidation of the corresponding *S*-substituted-L-cysteines as described by us previously (Cady *et al.* 2012). Stock solutions of compounds (100 mmol l^{-1}) were dissolved in DMSO (**7**, **12**–**17**, **37**), 300 mmol l^{-1} NaOH (**1**, **6**, **8**–**11**, **19**–**28**, **32**, **34**–**36**, **38**–**46**) or H_2O (**2**–**5**, **18**, **29**–**33**) depending on solubility.

Static biofilm inhibition assay

A microplate-based assay, modified from work by Gutierrez and colleagues and O'Toole and colleagues, was used to screen compounds for biofilm inhibition (O'Toole *et al.* 1999; Gutierrez *et al.* 2009). The growth media for biofilm formation was 25% (v/v) BHI supplemented with 2% (w/v) sucrose (BHIS). For the initial screen, stock solutions were used to bring the final compound concentration in the growth media to 1 mmol l^{-1}. Briefly, *Strep. mutans* UA159 BHI broth cultures were grown for 18 h at 37°C and 5% CO_2 without agitation. The 18-h culture was diluted 1 : 100 in BHIS with compound. Volume equivalents of DMSO, 300 mmol l^{-1} NaOH and dH_2O were used as positive controls to compounds diluted in each accordingly. An aliquot (100 μl) of each cell/compound mixture was added to three separate wells of a glass-bottom 96-well plate (Thermo Fisher Scientific, Rochester, NY). Total biofilm growth time was 48 h, at 37°C, 5% CO_2. After the first 24 h, spent media/planktonic solution was removed and replaced with fresh media and compound. To quantify biofilm formation after 48 h, planktonic solution was removed and biofilms were washed twice with 100 μl dH_2O. Biofilms were stained with 50 μl of SYTO 9 (Invitrogen, Carlsbad, CA) (5 μmol l^{-1}) for 15 min at room temperature. The fluorescent dye was then removed, and the biofilm was washed twice with 100 μl of dH_2O. The fluorescence intensity (485 nm excitation/520 nm emission) was determined from 12 spots in each well. Confocal laser scanning microscopy was performed using a Leica TCS SP5 II microscope using a 20× oil-immersion lens with 488 nm excitation and 510–540 nm emission detection range. LEICA APPLICATION SUITE v2.2.2 software was used for image acquisition. Subsequent manipulation was performed using IMARIS (Bitplane, Zurich, Switzerland) software.

Autoinducer-2 assay

Quorum sensing inhibition studies were performed using a modified protocol described by Vilchez *et al.* (2007). The strains used for this assay were *V. harveyi* BB170 (Km^r; *luxN*::Tn5; AI-1 positive; AI-2 positive) and *V. harveyi* BB152 (Km^r; *luxLM*::Tn5; AI-1 negative; AI-2 positive). Briefly, *V. harveyi* strains were grown in autoinducer bioassay (AB) medium described by Greenberg *et al.* (1979) with agitation (160 rev min^{-1}) at 30°C under aerobic conditions. Cells were pelleted and then resuspended in AB medium to an $OD_{600} = 0{\cdot}7$. Cells were then incubated at 30°C, 160 rev min^{-1}, for 1·5 h. This was diluted 1 : 5000 in AB medium to produce the working solution (BB170), 180 μl of which was pipetted into each well of a 96-well plate. Twenty microlitres of each test sample was added to each well, with three replicates of each sample. The test sample consisted of compound dissolved in its appropriate solvent and artificial AI-2 (Omm Scientific, Dallas, TX) in water. The final concentrations in each well were 1 mmol l^{-1} and 40 μmol l^{-1} of compound and AI-2, respectively. Positive controls were BB170 supplemented with 40 μmol l^{-1} AI-2 and appropriate solvent by volume, as well as BB152 for reference to wild-type-like luminescence. The negative control was BB170 with no AI-2 and no compound, but a volume equivalent of compound solvent added. Growth (absorbance at 600 nm) and luminescence were monitored every half an hour for 7 h at 30°C in a Tecan M200 microplate reader. Luminescence values were first normalized to cell growth and then used to generate a percentage change from the positive control.

MTT assay

To determine whether the inhibition of QS and biofilm formation was due to growth inhibition or cytotoxic effects, the MTT assay (Roche Applied Science, Mannheim, Germany) was used to estimate cell metabolic activity. The colorimetric assay is based on the breakdown of the yellow tetrazolium salt MTT to purple formazan crystals by the reductase activity of coenzymes NADH and NADPH. Oral bacteria were grown in 100 μl cultures on a 96-well plate (inoculated by a 1 : 100 dilution in BHI from an 18 h culture). Compounds effective at biofilm/QS inhibition were added to the media before inoculation, at a final concentration of 1 mmol l^{-1}. Cells were incubated at 37°C, 5% CO_2. After 24 h incubation, 10 μl of MTT labelling reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 5 mg ml^{-1} in phosphate-buffered saline) was added to each well. Four hours after the addition of labelling reagent, 100 μl of solubilization solution (10% SDS in 0·01 mol l^{-1} HCl) was added to each well. Plates were then incubated for another 24 h, and the absorbance was determined at 560 nm (peak absorbance of breakdown product) and 700 nm (reference wavelength that was subtracted out as background). Controls were biofilms grown without test compound and media without inoculation, representing normal biofilm growth and absence of metabolic activity, respectively.

Saliva collection for microfluidic assay

The protocol for the collection of filter-sterilized cell-free saliva (CFS) preparation was the same as described by Nance *et al.* (2013). Of particular note, stimulated saliva was collected and stored in 30-ml stocks at −80°C. When required, aliquots were thawed at room temperature and were modified for compatibility with single-species biofilm experiments as needed (described below).

BioFlux-based microfluidic flowing-saliva assay

Protocols for the BioFlux system were similar to those used in Nance *et al.* (2013) with minor alterations. The media used for experiments consisted of 25% pooled human filter-sterilized CFS that was supplemented with 25% BHI and 2% sucrose. The experimental CFS also contained 1 mmol l^{-1} of compound **7**, and the control CFS contained a volume equivalent of DMSO (due to compound **7** having initially been dissolved in DMSO). A mid-exponential phase batch-culture inoculum of *Strep. mutans* UA159, grown in BHI broth at 37°C with 5% CO_2 to an OD of 0·4 at 600 nm, was prepared and split into two cultures. Both cultures were pelleted via centrifugation and washed in either the compound-containing media or the control media. In the BioFlux system, CFS was flowed through each channel and incubated at room temperature for 20 min to create an ideal surface for bacterial attachment. The UA159 inoculums that had been washed in either compound-containing or control media (and resuspended to an OD of 0·4 at 600 nm) were then added to each outlet well in the amount of 100 μl. The inoculum was flowed through each channel and allowed to incubate at 37°C for 40 min. After incubation, the remaining inoculum was removed from the outlet wells and discarded. Compound-containing media or control media was added to a final volume of 1 ml to each inlet well, depending on which respective media the inoculum in each channel was previously washed in. After running the media through each channel for 20 h at 37°C, each channel was then stained using live/dead stain (3 μl of both SYTO-9 and propidium iodide per 1 ml of PBS) for 45 min. Biofilms were then imaged directly from the BioFlux plate using a Leica SPE confocal laser scanning microscope equipped with a 40 × 1·25 NA HCX PL

APO infinity-corrected oil objective. Confocal images were rendered in 3D using IMARIS (Bitplane) software. These images were analysed for viability using IMAGEJ (Schneider *et al.* 2012). They were also analysed for biovolume, average thickness and roughness coefficient using COMSTAT 2 software (Heydorn *et al.* 2000).

Quantitative reverse-transcription PCR

Overnight cultures (18 h) of *Strep. mutans* UA159 were obtained from inoculating single colonies into 5 ml BHI media containing either 1 mmol l^{-1} compound **7** or DMSO (solvent control). Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) with the RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's protocol with some modification to the cell lysis steps. Briefly, cells were mixed with 10 ml of the RNAprotect reagent and harvested by centrifugation for 10 min at 5000 ***g***. Each cell pellet was resuspended and lysed in 200 μl TE buffer (Ambion®, Life Technologies, Carlsbad, CA) containing 30 mg ml^{-1} lysozyme (EMD Millipore, Billerica, MA) and 30 mAU Proteinase K (Qiagen). Lysis solutions were left shaking for 10 min at room temperature and then further lysed using a Branson Sonifier Model 450 at 70% power, in pulse mode (10 s on, 5 s off) for a total of 5 min sonication time. After this step, the manufacturer's protocol was followed strictly (Qiagen). Total RNA samples were further treated with DNase I (New England BioLabs, Ipswich, MA) to degrade any residual DNA. Real-time RT-PCR was performed using the one-step QuantiFast SYBR Green RT-PCR kit (Qiagen). The primers shown in Table 3 were used at 250 nM. Cycle conditions were as follows: 30 min at 50°C; 15 min at 95°C; 40 cycles of 15 s at 94°C, 30 s at 50°C and 30 s at 72°C. Gene expression was determined as a function of threshold cycle (Ct) value and was normalized internally using DNA gyrase α-subunit (SMU.1114).

Statistical analysis

Statistical significance was expressed as significant ($P < 0{\cdot}05$), highly significant ($P < 0{\cdot}01$) and extremely significant ($P < 0{\cdot}001$). Statistical analyses included *t*-tests, ANOVAs and Dunnett's multiple comparison tests, as noted.

Results

Biofilm formation by orally associated bacteria is inhibited by several organosulphur compounds

The effects of organosulphur compounds on biofilm formation by the oral strains were investigated through a static biofilm microplate-based screening approach. The initial concentration for the screen was 1 mmol l^{-1}, and biofilm formation in all three organisms was affected to varying degrees by several compounds in the library (Fig. 1). *Actinomyces oris* biofilm formation was significantly inhibited by multiple compounds, particularly **3**, **7**, **12, 14, 17**, **37**, **38** and **46** (≥30% mean reduction). Additionally, for *Act. oris*, several compounds appeared to improve biofilm development, most notably compound **15** (≥30% mean increase). Similar responses were seen for *Strep. mutans*, where compounds **3**, **7**, **9**, **12, 17**, **37**, **38** and **46** most effectively inhibited biofilm formation and compounds **15** and **18** markedly increased biofilm formation (Fig. 1; ≥30% mean reduction/increase, respectively). *Streptococcus sanguis* biofilm formation was significantly reduced by compounds **7**, **12**, **13**, **17**, **26**, **34**, **35**, **36**, **37** and **46** (≥30% mean reduction), while it was enhanced by a number of compounds, most notably **15**, **25**, **27** and **31** (Fig. 1). Compounds that inhibited biofilm formation in all three organisms by at least 25% at 1 mmol l^{-1} included **3**, **7**, **12**, **17**, **37** and **46**. These were selected for further dose–response assays. Compounds that increased biofilm formation are the subject of ongoing investigations.

Biofilm formation as a function of compound concentration revealed dose–response effects

Biofilms were grown in the presence of each of the compounds that were previously shown to significantly inhibit biofilm formation in all three test strains (i.e. compounds **3**, **7**, **12**, **17**, **37** and **46**), in order to determine concentration-dependent effects. An inhibitor-response curve was fit to the observed dose–response data, enabling calculation of the 50% inhibitory concentration (IC_{50}) for each compound (Table 1). The associated dose–response curves are shown in Fig. S1. The data show that the IC_{50} values are similar in magnitude, ranging from high micromolar to low millimolar concentrations, with compound **7** being the most consistent in inhibiting all three bacterial species. Our observations of reduced biofilm formation in the presence of these compounds was corroborated by the results of confocal scanning laser microscopy (CLSM), which was used to visualize the morphologies of the biofilms grown in the presence of inhibitory compounds (Fig. 2). CLSM 3D computational renderings showed that biofilm surface coverage was decreased in bacteria grown in the presence of compounds **3**, **7**, **12, 17**, **37** and **46**, with compound **7** being the most effective across all three species (Fig. 2). In many of these experiments, microcolonies that never developed into mature, confluent biofilms were seen. This may suggest that these compounds do not affect initial bacteria–surface attachment events, but instead affect the progression of biofilm maturation.

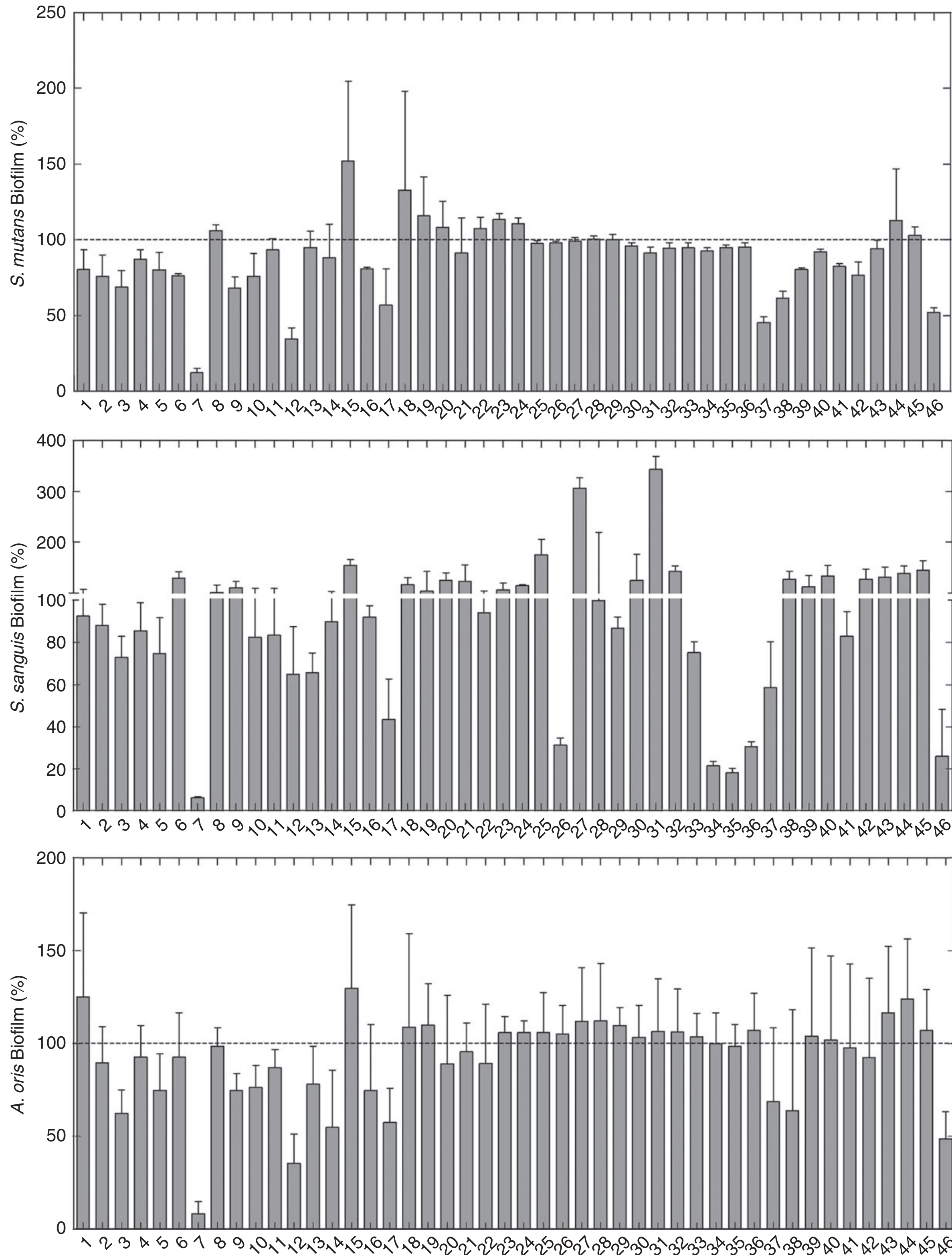


Figure 1 Biofilm formation by oral bacteria in the presence of AI-2 quorum sensing (QS) effectors. The data are scaled with the 100% mark representing biofilm in the absence of added compound. Values below 100% indicate biofilm inhibition, and values above indicate increased biofilm formation. Values are represented as percentages in comparison with the control (not shown) in which tested compounds were absent.

Compounds that inhibit biofilm formation by multiple species also inhibit AI-2-based QS in *Vibrio harveyi* BB170

To investigate the possible connection between QS and biofilm formation in the three strains of bacteria, and to determine whether our panel of lead compounds inhibited biofilm formation by curtailing AI-2-mediated QS, we utilized the *V. harveyi* reporter strain BB170. The effects of our lead biofilm inhibitory compounds on AI-2 QS-mediated communication were investigated by exposing *V. harveyi* to the compounds in the presence of exogenous AI-2 and monitoring *V. harveyi* bioluminescence. Figure 3 illustrates the bioluminescence of *V. harveyi* BB170 grown in the presence of compounds that inhibited biofilm formation by the three oral species, that is **3**, **7**, **12**, **17**, **37** and **46**, after 7 h. The data are normalized to cell growth (as determined by OD_{600}) and scaled so that the negative control (no compound) is equivalent to 100%. The results observed from screening the entire library are shown in Fig S2. Compounds **3**, **7**, **12**, **17** and **46** significantly decreased the QS response in *V. harveyi* BB170 at 1 mmol l^{-1} concentration. Conversely, compound **37** did not, suggesting that it was not an AI-2 inhibitor.

Dose-dependent QSI was also investigated using *V. harveyi* BB170 in the presence of compounds **3**, **7**, **12**, **17** and **46**. An inhibitor-response curve was fit to the data, which is displayed in Fig. S3. The calculated IC_{50} for each compound is shown in Table 2. Compound **46** had the lowest IC_{50} value (0·068 mmol l^{-1}), suggesting it to be the most potent QS inhibitor in this study.

Compounds exhibit differential effects on orally associated bacterial metabolic activity

The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine whether the inhibition of biofilm formation was a result of bactericidal or bacteriostatic effects. In this assay, metabolic activity, and more specifically, the reductive activity of the coenzymes NADH and NADPH, can be related to cell viability. Bacterial cultures with compound present were inoculated into a 96-well plate and incubated for 24 h. MTT reagent was added directly to cultures that included both biofilm and planktonic cells (as controls). *Streptococcus mutans*, *Strep. sanguis* and *Act. oris* displayed differential metabolic activity when grown in the presence of organosulphur biofilm inhibitors (Fig. 4). The metabolic activity of the control (without the addition of compound) was set at 100%, and the other samples were scaled accordingly. Values below 100%, such as was observed for compound **3** when in the presence of all three organisms, represent a decrease in metabolic activity, whereas those above 100% (as observed in the case of compound **46** in all three organisms), are indicative of increased activity relative to the control. The results indicated that, in general, *Strep. mutans* had a greater tolerance for these compounds relative to the other organisms, as its metabolic activity was increased or unaffected by four of the five biofilm/QS inhibitors. *Actinomyces oris* was the most susceptible, as

Table 1 IC_{50} values for compounds that displayed biofilm inhibitory activity

	IC_{50} (mmol l^{-1} ± SE)		
Compound	*Streptococcus mutans*	*Streptococcus sanguis*	*Actinomyces oris*
3	1·37 (±0·05)	1·34 (±0·13)	0·88 (±0·11)
7	0·96 (±0·02)	0·38 (±0·02)	0·61 (±0·06)
12	0·68 (±0·11)	1·56 (±0·19)	1·61 (±0·25)
17	1·44 (±0·20)	0·47 (±0·08)	0·28 (±0·06)
37	0·99 (±0·08)	2·09 (±0·13)	0·92 (±0·15)
46	0·93 (±0·10)	0·70 (±0·03)	0·98 (±0·07)

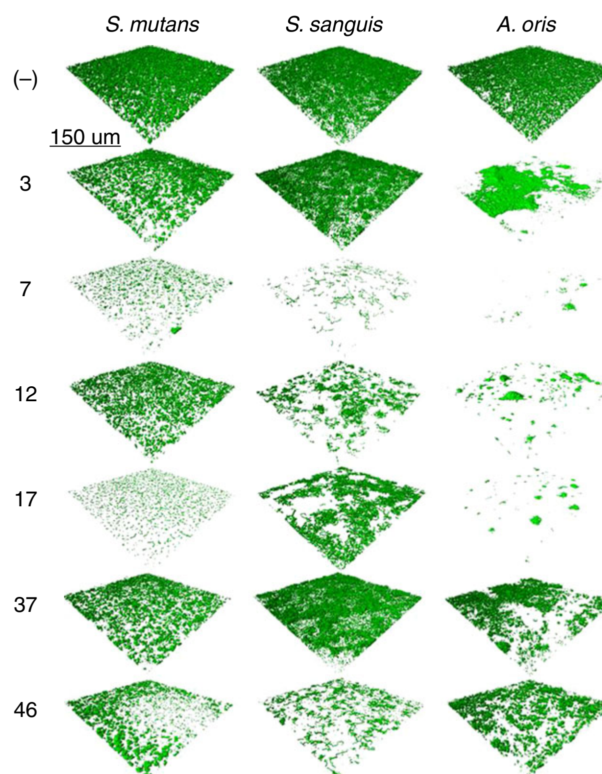


Figure 2 Representative three-dimensional reconstructions of confocal laser scanning microscopy images. The left column shows *Streptococcus mutans*, the middle column *Streptococcus sanguis*, and the right column *Actinomyces oris*. The top row represents the control in which no inhibitor compounds were present, and the subsequent rows show the inhibitor compound, which was presented in the growth media at a concentration of 1 mmol l^{-1}.

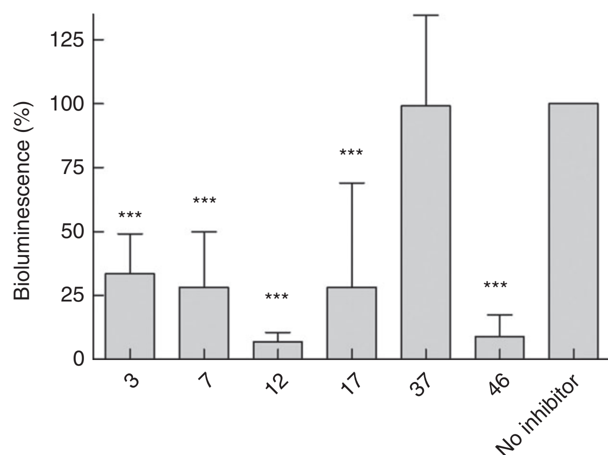


Figure 3 Bioluminescence of *Vibrio harveyi* BB170 with organosulphur biofilm inhibitors at 1 mmol l^{-1} concentration after 7 h of incubation. Bioluminescence of the control (without compound added) was set at 100%, and the response for the tested compounds was normalized accordingly. ANOVA ($P < 0{\cdot}0001$) was performed followed by Dunnett's multiple comparison test, with asterisks indicating significant (***$P < 0{\cdot}001$) reduction in bioluminescence.

Table 2 IC_{50} of AI-2-dependent luminescence by *Vibrio harveyi* BB170. Compound **37** was not considered to be a quorum sensing inhibitor, as an IC_{50} value could not be determined

Compound	*V. harveyi* BB170 IC_{50} (mmol l^{-1})
3	0·727 (±0·050)
7	0·227 (±0·021)
12	0·525 (±0·016)
17	0·412 (±0·037)
46	0·068 (±0·008)

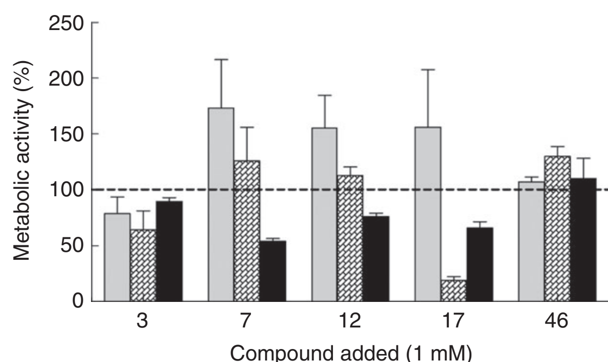


Figure 4 Bacterial metabolic activity as determined by the MTT assay. Metabolic activity was determined for each species and compared to the MTT assay response for bacteria grown in the absence of inhibitory compounds. (□) *Streptococcus mutans*; (▨) *Streptococcus sanguis* and (■) *Actinomyces oris.*

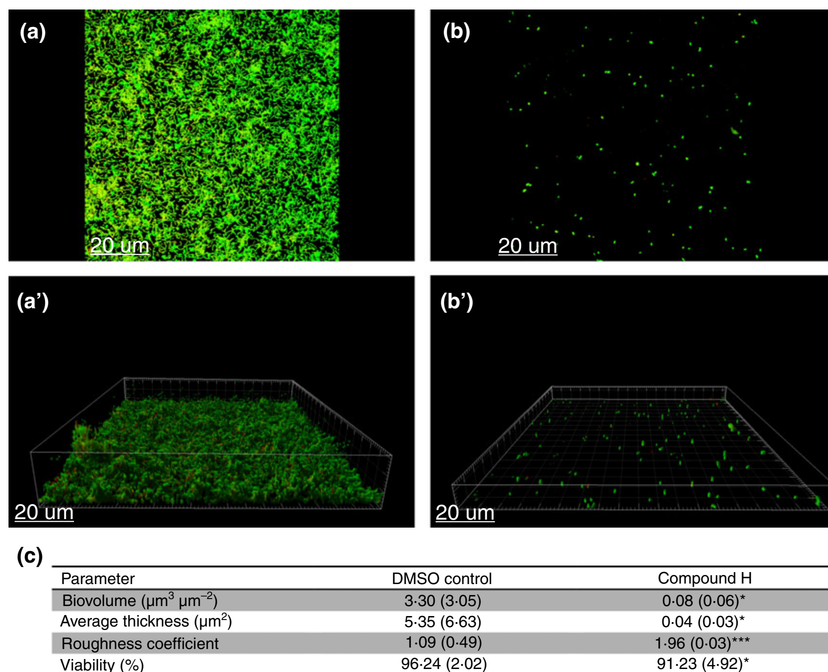
its metabolic activity was negatively affected by four of the five compounds. *Streptococcus sanguis* showed sensitivity similar to that of *Strep. mutans*, with the most notable difference being the case of compound **17**, for which the metabolic activity of *Strep. mutans* was increased and *Strep. sanguis*' metabolic activity was greatly reduced (by *c.* 80%). In general, the biofilm inhibitory activity was greater in magnitude than the reduction in metabolic activity and likely reflects not only changes in growth rate but also changes in gross cell phenotype (e.g. one that does not support biofilm retention). Table S1 compares biofilm formation, metabolic activity and QS effects of the compounds at 1 mmol l^{-1}, with the control (no inhibitor compound present) represented by a relative value of 1.

Compound 7 inhibits *Streptococcus mutans* biofilm formation in a saliva-fed BioFlux flowcell device

The BioFlux system allows for exposure of bacteria to environmentally germane conditions with flowing saliva. A customized 25% saliva-based media supplemented with BHI and sucrose was used for this experiment. Compound **7** (dissolved in DMSO) was added to this media to a final concentration of 1 mmol l^{-1} and was present during the initial inoculation and overnight growth of the biofilms. Control biofilms were grown in the same saliva-based media containing DMSO, but not compound **7**. After 20 h, visualization by CLSM showed that there was significant inhibition of biofilm development in the compound-containing media, as compared to the control (Fig. 5a,b). Specifically, the development of *Step. mutans* biofilms in saliva containing compound **7** resulted in a significant decrease in biofilm biovolume and thickness ($P < 0{\cdot}05$) and a significant increase in roughness ($P < 0{\cdot}001$) (Fig. 5c). The difference in roughness is a reflection of the biofilm architecture changing from a heterogeneous confluent covering (Fig. 5a,a') to sparse archipelago-like arrangements of biofilm microcolonies. Viability analyses indicated a meagre but statistically significant decrease in viability when biofilms were developed in the presence of compound **7** (96·24% *vs* 91·92% viability; Fig. 5c). From a biological perspective, this likely reflects the retention of dead/inactive nonresponsive *Strep. mutans* cells in a biofilm under flowing saliva conditions, and not direct antimicrobial effects of compound **7**.

Transcriptional analysis reveals that compound 7 affects expression levels of biofilm-related genes in *Streptococcus mutans* UA159

We investigated the effects of inhibitory compounds on the expression of several genes associated with biofilm development in *Strep. mutans* (Table 3). *Streptococcus*



Parameter	DMSO control	Compound H
Biovolume ($\mu m^3\ \mu m^{-2}$)	3·30 (3·05)	0·08 (0·06)*
Average thickness (μm^2)	5·35 (6·63)	0·04 (0·03)*
Roughness coefficient	1·09 (0·49)	1·96 (0·03)***
Viability (%)	96·24 (2·02)	91·23 (4·92)*

Figure 5 Effect of compound 7 on biofilm development in flowing saliva, as determined by live/dead staining after 20 h growth and examination under a confocal scanning laser microscopy (CLSM). (a and a') A representative two-dimensional and three-dimensional image of an *Streptococcus mutans* biofilm that had not been exposed to compound 7 (i.e. 25% modified saliva medium plus DMSO). (b and b') A representative two-dimensional and three-dimensional image of an *Strep. mutans* biofilm that had been developed in compound 7 (i.e. 25% modified saliva medium plus DMSO and compound 7). Bars = 20 μm. (c) Computational analyses of biofilms grown in either condition (three 3-D renderings per condition, each from three channels analysed). Student's *t*-test: *$P < 0·05$; ***$P < 0·001$.

mutans UA159 cultures were grown in BHI for 18 h in the presence of either compound **7** (treated) or DMSO (control). RNA was isolated and used for real-time reverse-transcription PCR (RT-PCR) to quantify transcript levels of several biofilm-related genes (Fig. 6). Of these genes, *gtfB* (SMU_1004), which encodes for glucosyltransferase B (which catalyzes conversion of sucrose into insoluble glucans), was shown to be significantly upregulated when *Strep. mutans* was exposed to compound **7**. Conversely, expression of both *gbpB* (SMU_22) and *brpA* (SMU_410), which encode for glucan-binding protein B and biofilm-regulatory protein A, respectively, was significantly reduced in the presence of compound **7**. Expression of *luxS* (SMU_474) also appeared to be downregulated although its reduced expression was not shown to be statistically significant at $P < 0·05$.

Discussion

The data presented in this study indicate that derivatives of natural product metabolites of the plant *P. alliacea* have the ability to inhibit biofilm formation by common oral species. In addition, our data suggest that QS interference/inhibition and the retardation of biofilm formation in oral biofilms may be interconnected and a viable option for future control strategies. Considering the current public health burden caused by oral diseases such as caries and periodontal disease, as well as the unabated development and spread of antimicrobial resistance, the possibility of such a QS-based anti-biofilm strategy is alluring.

The effect of biomimetic organosulphur compounds on oral biofilm formation was investigated using a library of 46 compounds. Six of these (**3**, **7**, **12**, **17**, **37** and **46**) were identified to have 'broad' biofilm inhibitory activity in the high micromolar to low millimolar concentration range. Compounds **3**, **7**, **12**, **17** and **46** were also shown to have a significant inhibitory effect on AI-2-based QS, tested with a *V. harveyi* BB170 QS reporter. Compounds **7**, **17** and **46** are all *S*-substituted cysteine sulphoxides, whereas **3** is a sulphonic acid derivative of cysteine and an intermediate in cysteine biosynthesis, and **12** is the

Table 3 Primers used for quantitative reverse-transcription PCR. Primers for *gtfB*, *brpA*, *gbpB* and *luxS* were reported in Wen *et al.* (2010). Primers for the reference gene, *gyrA*, were those reported in Sztajer *et al.* (2008)

Locus tag	Gene	Protein	Amplicon Length	Forward primer (5'–3')	Reverse primer (5'–3')
SMU.1004	*gtfB*	GtfB, glucan production	98 bp	AGCAATGCAGCCAATCTACAAAT	ACGAACTTTGCCGTTATTGTCA
SMU.410	*brpA*	Transcriptional regulator	148 bp	CGTGAGGTCATCAGCAAGGTC	CGCTGTACCCCAAAAGTTTAGG
SMU.22	*gbpB*	Glucan-binding protein	108 bp	CGTGTTTCGGCTATTCGTGAAG	TGCTGCTTGATTTTCTTGTTGC
SMU.474	*luxS*	*S*-ribosylhomocysteinase	93 bp	ACTGTTCCCCTTTTGGCTGTC	AACTTGCTTTGATGACTGTGGC
SMU.1114	*gyrA*	DNA gyrase α-subunit	163 bp	TTGGTGAAGTCCTGTCACTCG	TCATCATCATTGACGCCTGT

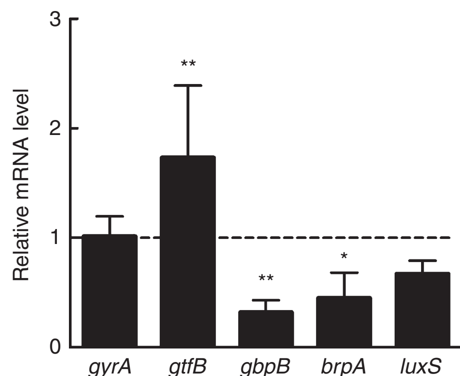


Figure 6 Relative transcript levels of biofilm-related genes in *Streptococcus mutans* in the presence of compound **7** at 1 mM. ANOVA ($P < 0.0001$) was performed followed by Dunnett's multiple comparison test, with asterisks indicating significant ($*P < 0.05$; $**P < 0.01$) difference in the mRNA level. The control transcript level for each gene is equal to 1. DNA gyrase α-subunit (SMU.1114) was used as a reference gene.

disulphide derivative of **7**. Compound **37** did not affect AI-2 QS, which is interesting given its structural similarity to compound **17** (**17** is the sulphoxide derivative of **37**). This observation implicates the involvement of the sulphoxide moiety in the observed QS interference, a heretofore unreported result.

Plots illustrating the biofilm inhibitory activities of the five active compounds *vs* QS inhibition for each of the bacteria tested were compared (Fig. 7). Most compounds were more effective at inhibiting QS than they were at inhibiting biofilm formation, which is demonstrated by their location above the diagonal line in the plots (Fig. 7). This may be attributed to the fact that AI-2-mediated bioluminescence in *V. harveyi* is strictly regulated by QS, whereas there are several factors including nutrient availability and stress among other effects that can regulate biofilm formation. The luminescence response to AI-2 in *V. harveyi* BB170 is well documented, but it is less well known how AI-2 regulates biofilm formation, especially in Gram-positive bacteria. Therefore, if these compounds are antagonizing AI-2 QS in Gram-positive bacteria, it is not as straightforward to predict how these bacteria will respond. Compounds **7**, **12** and **46** are among the most effective in inhibiting activity across all three organisms, as indicated by their location within or in close proximity to the smaller box in Fig. 7 (thereby indicating ≥50% reduction in both QS and biofilm formation). In previous work on the effects of organosulphur compounds on *Ps. aeruginosa*, **7** and **12** were both shown to inhibit biofilm formation and **7** was shown to inhibit *las* and *rhl*-based QS, as well as virulence in a *Drosophila* infection model (Cady *et al.* 2012).

In principle, there are several potential mechanisms by which the active compounds described here interfere with QS and biofilm formation. One possibility is through competitive inhibition of AI-2-receptor binding, although this scenario may be viewed as unlikely based on the absence of obvious structural similarity between the active compounds and DPD, or any of the cyclized forms of AI-2. However, the compounds do bear resemblance to various organosulphur small molecules that play key roles in the activated methyl cycle, from which AI-2 is derived. These include cysteine, *S*-ribosyl homocysteine (SRH), *S*-adenosyl homocysteine (SAH), methionine and *S*-adenosyl methionine (SAM). SRH may have the most structural similarity to compounds **7**, **17** and **46**, as both SRH and compounds **7**, **17** and **46** contain sulphur flanked by a ring containing substituent and an amino acid moiety (Fig. 8). Gutierrez *et al.* showed bacterial communication interference in *Vibrio cholerae* using QS inhibitors that block the enzymatic activity that leads to the precursors of the LuxS cleavage substrate (SRH) (Gutierrez *et al.* 2009). It is possible that the compounds presented in this study are functioning similarly and this

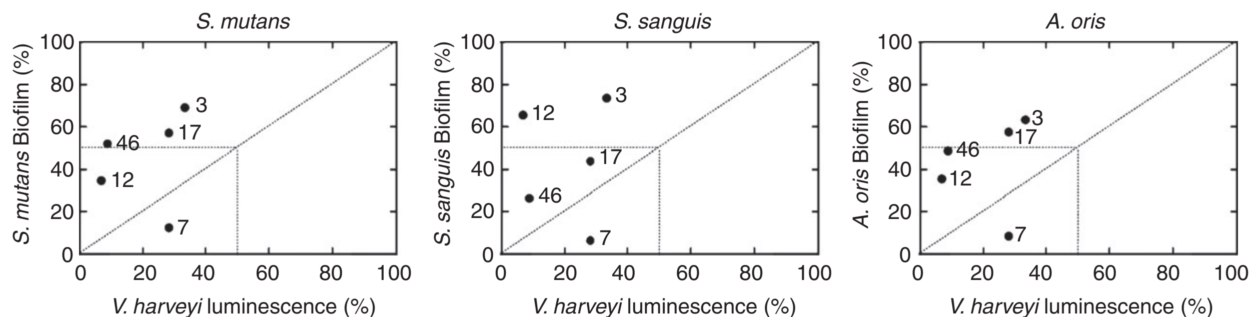


Figure 7 Comparison plots illustrating the relative reduction in bacterial phenotypes when cultured in the presence of the active organosulphur compounds. Plots show the percentage inhibition of biofilm for each organism (*y*-axis) *vs* the percentage reduction in AI-2-mediated bioluminescence (quorum sensing (QS)) in *Vibrio harveyi* (*x*-axis) for compounds with at least 50% reduction in either biofilm formation or bioluminescence. The dotted inset delineates the subset of compounds with 50% reduction in both biofilm formation and luminescence (QS inhibition).

possibility is being investigated. However, it is also entirely possible that QS is not affected in the oral strains and that biofilm inhibition is a result of alternative mechanisms.

While we do not understand the exact mechanism(s) by which cysteine sulphoxide derivatives affect biofilm development and QS among diverse bacteria, there is some structural similarity between compounds **7** and **46** in this study, and a novel signalling molecule, the integrated QS stress signal (IQS), that was recently identified by Lee *et al.* (2013). IQS integrates stress response (specifically to low phosphate conditions) with the *las* QS system in *Ps. aeruginosa*. While *Ps. aeruginosa* does not produce AI-2, Duan *et al.* (2003) have reported that AI-2 activity regulates some of the same virulence factors that are controlled by AHL-based signalling. Compounds **7** and **46** contain aromatic ring systems analogous to that found in IQS, but the importance of this structural feature is as yet unknown. It remains to be seen how commonplace IQS-like integration systems that link stress and population sensing are in other bacteria, and whether compounds such as **7** and **46** are interfering in that signalling. However, such a possibility could provide an explanation for the observed phenotypes of indigenous oral species in this study and those of which we have previously reported with *Ps. aeruginosa* (Cady *et al.* 2012).

Results from our gene expression analyses are similar to those in several other reports (Wen and Burne 2002; Mcnab *et al.* 2003; Merritt *et al.* 2003; Senadheera *et al.* 2005; Yoshida *et al.* 2005; Duque *et al.* 2011). The differential transcript levels of *gtfB* and *gbpB* that we observed in the presence of compound **7** are similar to those reported on *Strep. mutans vicK* knockout mutants (Senadheera *et al.* 2005; Duque *et al.* 2011). VicRK is a two-component signal transduction system, comprised of a membrane-bound sensor kinase (VicK) and an intracellular regulator protein (VicR). Phenotypes of *Strep. mutans* Δ*vicK* include altered biofilm-forming ability when compared to the wild-type strain, as well as drastically reduced transformation efficiency in response to exogenous competence-stimulating peptide, a peptide-based QS signal utilized by multiple streptococci (Senadheera *et al.* 2005). Downregulation of *gbpB* impaired early stages of biofilm formation, which could be partially restored by complementation with exogenous GbpB (Duque *et al.* 2011). It has been suggested that GtfB activity is increased in *Strep. mutans* Δ*luxS* (Merritt *et al.* 2003). This was confirmed on the transcriptional level during *Strep. mutans* log-phase growth (Yoshida *et al.* 2005). The same study also showed that sucrose-dependent biofilm formation was impaired in *Strep. mutans* Δ*luxS*, and the same phenotype was observed in our study. It has also been observed in *Streptoccus gordo-*

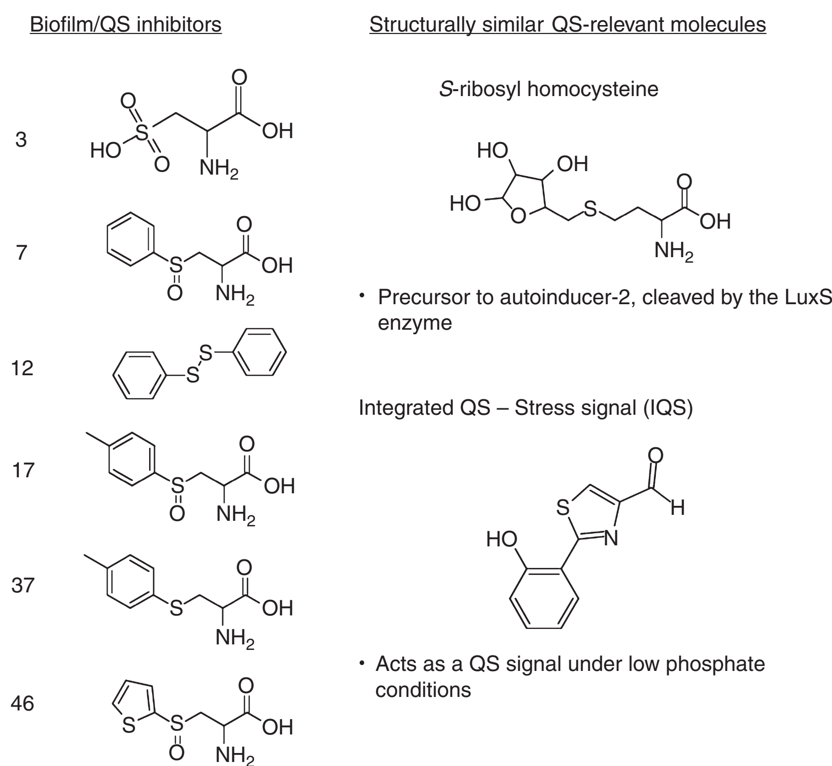


Figure 8 Structures of the compounds that were effective in this study and structurally-similar molecules relevant for quorum sensing (QS). Compound 37 did not inhibit bioluminescence (QS) but did inhibit biofilm formation. Zhao *et al.* showed LuxS inhibition using *S*-ribosyl homocysteine (SRH) analogues (Zhao *et al.* 2003). Lee *et al.* recently identified QS stress signal (IQS), a signalling molecule that integrates stress response with QS.(Lee *et al.* 2013).

nii that LuxS controls glucosyltransferase activity and that utilization of AI-2 affects metabolism of carbohydrates (Mcnab *et al.* 2003). Additionally, it was shown that *Strep. mutans* Δ*brpA* did not alter initial cell attachment after 6 h, but biofilm formation after 24 h was substantially reduced (Wen and Burne 2002). This coincides with our observation that our compounds were ineffective at inhibiting *Strep. mutans* biofilm formation when added after 4 h, along with a significant reduction in *brpA* expression (data not shown). Taken together, all of these observations support our working hypothesis that the organosulphur compounds used in this study may not directly affect initial cell attachment, but rather affect other events related to biofilm development, including cell–cell signalling.

In addition to the development of more potent biofilm and QS inhibitors, global transcriptomic and proteomic analyses of bacteria exposed to the active compounds are being actively pursued. The effectiveness of these compounds in multispecies biofilm systems, as well as against clinical oral biofilm samples to better understand how they may affect the normal oral flora, is also being investigated. Given the concern that the human oral cavity is subject to selection pressures that will result in the development of antimicrobial resistance in the residing oral species (Roberts and Mullany 2010), strategies based upon preventing QS are particularly enticing, especially if shown to be effective against natural oral biofilms that can contain hundreds of oral species.

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Conflict of interest

The authors declare no conflict of interest.

References

Ahmed, N.A., Petersen, F.C. and Scheie, A.A. (2009) AI-2/LuxS is involved in increased biofilm formation by *Streptococcus intermedius* in the presence of antibiotics. *Antimicrob Agents Chemother* **53**, 4258–4263.

Ajdić, D., McShan, W.M., McLaughlin, R.E., Savić, G., Chang, J., Carson, M.B., Primeaux, C., Tian, R. *et al.* (2002) Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci USA* **99**, 14434–14439.

Blehert, D.S., Palmer, R.J., Xavier, J.B., Almeida, J.S. and Kolenbrander, P.E. (2003) Autoinducer 2 production by *Streptococcus gordonii* DL1 and the biofilm phenotype of a *luxS* mutant are influenced by nutritional conditions. *J Bacteriol* **185**, 4851–4860.

Brackman, G., Celen, S., Baruah, K., Bossier, P., Van Calenbergh, S., Nelis, H.J. and Coenye, T. (2009) AI-2 quorum-sensing inhibitors affect the starvation response and reduce virulence in several Vibrio species, most likely by interfering with LuxPQ. *Microbiology* **155**, 4114–4122.

Cady, N.C., McKean, K.A., Behnke, J., Kubec, R., Mosier, A.P., Kasper, S.H., Burz, D.S. and Musah, R.A. (2012) Inhibition of biofilm formation, quorum sensing and infection in *Pseudomonas aeruginosa* by natural products-inspired organosulfur compounds. *PLoS One* **7**, e38492.

Costerton, J.W. (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.

Costerton, J. and Keller, D. (2007) Oral periopathogens and systemic effects. *Gen Dent* **55**, 210–215.

Dige, I., Raarup, M.K., Nyengaard, J.R., Kilian, M. and Nyvad, B. (2009) *Actinomyces naeslundii* in initial dental biofilm formation. *Microbiology* **155**, 2116–2126.

Duan, K., Dammel, C., Stein, J., Rabin, H. and Surette, M.G. (2003) Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol Microbiol* **50**, 1477–1491.

Duque, C., Stipp, R.N., Wang, B., Smith, D.J., Ho, F., Kuramitsu, H.K., Duncan, M.J. and Mattos-Graner, R.O. (2011) Downregulation of GbpB, a component of the VicRK regulon, affects biofilm formation and cell surface characteristics of *Streptococcus mutans*. *Infect Immun* **79**, 786–796.

Federle, M.J. and Bassler, B.L. (2003) Interspecies communication in bacteria. *J Clin Invest* **112**, 1291–1299.

Ge, X., Kitten, T., Chen, Z., Lee, S.P., Munro, C.L. and Xu, P. (2008) Identification of *Streptococcus sanguinis* genes required for biofilm formation and examination of their role in endocarditis virulence. *Infect Immun* **76**, 2551–2559.

Gilbert, P., Allison, D.G. and Mcbain, A.J. (2002) Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *J Appl Microbiol* **92**, 98–110.

Givskov, M., Nys, R.D.E., Manefield, M., Gram, L., Maximilien, R.I.A., Eberl, L.E.O., Molin, S., Steinberg, P.D. *et al.* (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J Bacteriol* **178**, 6618–6622.

Greenberg, E.P., Hastings, J.W. and Ulitzur, S. (1979) Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. *Arch Microbiol* **120**, 87–91.

Gutierrez, J.A., Crowder, T., Rinaldo-Matthis, A., Ho, M.-C., Almo, S.C. and Schramm, V.L. (2009) Transition state analogs of 5′-methylthioadenosine nucleosidase disrupt quorum sensing. *Nat Chem Biol* **5**, 251–257.

Hall-Stoodley, L., Costerton, J.W. and Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**, 95–108.

He, Q., Kubec, R., Jadhav, A.P. and Musah, R.A. (2011) First insights into the mode of action of a "lachrymatory factor synthase"–implications for the mechanism of lachrymator formation in *Petiveria alliacea*, *Allium cepa* and Nectaroscordum species. *Phytochemistry* **72**, 1939–1946.

He, Z., Wang, Q., Hu, Y., Liang, J., Jiang, Y., Ma, R., Tang, Z. and Huang, Z. (2012) Use of the quorum sensing inhibitor furanone C-30 to interfere with biofilm formation by *Streptococcus mutans* and its *luxS* mutant strain. *Int J Antimicrob Agents* **40**, 30–35.

Hentzer, M., Riedel, K., Rasmussen, T.B., Heydorn, A., Andersen, J.B., Parsek, M.R., Rice, S.A., Eberl, L. *et al.* (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* **148**, 87–102.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B.K. and Molin, S. (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146**, 2395–2407.

Hojo, K., Nagaoka, S., Ohshima, T. and Maeda, N. (2009) Bacterial interactions in dental biofilm development. *J Dent Res* **88**, 982–990.

Jakobsen, T.H., Bragason, S.K., Phipps, R.K., Christensen, L.D., van Gennip, M., Alhede, M., Skindersoe, M., Larsen, T.O. *et al.* (2012a) Food as a source for quorum sensing inhibitors: iberin from horseradish revealed as a quorum sensing inhibitor of *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **78**, 2410–2421.

Jakobsen, T.H., van Gennip, M., Phipps, R.K., Shanmugham, M.S., Christensen, L.D., Alhede, M., Skindersoe, M.E., Rasmussen, T.B., *et al.* (2012b) Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. *Antimicrob Agents Chemother* **56**, 2314–2325.

Johnson, T.B. and Ambler, J.A. (1914) The alkylation and hydrolysis of aliphatic sulfonamides. A new synthesis of sarcosine. *Annales* **87**, 372–385.

Kalia, V.C. (2013) Quorum sensing inhibitors: an overview. *Biotechnol Adv* **31**, 224–245.

Koh, C., Sam, C., Yin, W., Tan, L.Y. and Krishnan, T. (2013) Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors* **13**, 6217–6228.

Kolenbrander, P.E. and London, J. (1993) Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* **175**, 3247–3252.

Kolenbrander, P.E., Andersen, R.N., Blehert, D.S., Egland, P.G., Foster, J.S. and Palmer, R.J. Jr (2002) Communication among Oral Bacteria. *Microbiol Mol Biol Rev* **66**, 486–505.

Kolenbrander, P.E., Palmer, R.J., Rickard, A.H., Jakubovics, N.S., Chalmers, N.I. and Diaz, P.I. (2006) Bacterial interactions and successions during plaque development. *Periodontol 2000* **42**, 47–79.

Kolenbrander, P.E., Palmer, R.J., Periasamy, S. and Jakubovics, N.S. (2010) Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* **8**, 471–480.

Kubec, R., Kim, S. and Musah, R.A. (2002) S-Substituted cysteine derivatives and thiosulfinate formation in *Petiveria alliacea*-part II. *Phytochemistry* **61**, 675–680.

Lee, J., Wu, J., Deng, Y., Wang, J. and Wang, C. (2013) A cell-cell communication signal integrates quorum sensing and stress response. *Nat Chem Biol* **9**, 339–343.

Lewis, K. (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**, 48–56.

Lönn-Stensrud, J., Petersen, F.C., Benneche, T. and Scheie, A.A. (2007) Synthetic bromated furanone inhibits autoinducer-2-mediated communication and biofilm formation in oral *Streptococci*. *Oral Microbiol Immunol* **22**, 340–346.

March, J.C. and Bentley, W.E. (2004) Quorum sensing and bacterial cross-talk in biotechnology. *Curr Opin Biotechnol* **15**, 495–502.

Marsh, P.D. (2005) Dental plaque: biological significance of a biofilm and community life-style. *J Clin Periodontol* **32**, 7–15.

Marsh, P.D., Moter, A. and Devine, D.A. (2011) Dental plaque biofilms: communities, conflict and control. *Periodontol 2000* **55**, 16–35.

Mcnab, R., Ford, S.K., El-Sabaeny, A., Barbieri, B., Cook, G.S. and Lamont, R.J. (2003) LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J Bacteriol* **185**, 274–284.

Merritt, J., Qi, F., Goodman, S.D., Maxwell, H., Shi, W. and Anderson, M.H. (2003) Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. *Infect Immun* **71**, 1972–1979.

Merritt, J., Kreth, J., Shi, W. and Qi, F. (2005) LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory component. *Mol Microbiol* **57**, 960–969.

Musah, R.A., He, Q., Kubec, R. and Jadhav, A. (2009) Studies of a novel cysteine sulfoxide lyase from *Petiveria alliacea*: the first heteromeric alliinase. *Plant Physiol* **151**, 1304–1316.

Nance, W.C., Dowd, S.E., Samarian, D., Chludzinski, J., Delli, J., Battista, J. and Rickard, A.H. (2013) A high-throughput microfluidic dental plaque biofilm system to visualize and quantify the effect of antimicrobials. *J Antimicrob Chemother* **68**, 2550–2560.

Njoroge, J. and Sperandio, V. (2009) Jamming bacterial communication: new approaches for the treatment of infectious diseases. *EMBO Mol Med* **1**, 201–210.

O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B. and Kolter, R. (1999) Genetic approaches to study of biofilms. *Methods Enzymol* **310**, 91–109.

Paju, S. and Scannapieco, F.A. (2007) Oral biofilms, periodontitis, and pulmonary infections. *Oral Dis* **13**, 508–512.

Parsek, M.R. and Greenberg, E.P. (2000) Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc Natl Acad Sci USA* **97**, 8789–8793.

Rasmussen, T.B. and Givskov, M. (2006a) Quorum sensing inhibitors: a bargain of effects. *Microbiology* **152**, 895–904.

Rasmussen, T.B. and Givskov, M. (2006b) Quorum-sensing inhibitors as anti-pathogenic drugs. *Int J Med Microbiol* **296**, 149–161.

Rickard, A.H., Palmer, R.J., Blehert, D.S., Campagna, S.R., Semmelhack, M.F., Egland, P.G., Bassler, B.L. and Kolenbrander, P.E. (2006) Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. *Mol Microbiol* **60**, 1446–1456.

Roberts, A.P. and Mullany, P. (2010) Oral biofilms: a reservoir of transferable, bacterial, antimicrobial resistance. *Expert Rev Anti Infect Ther* **8**, 1441–1450.

Scannapieco, F.A. (1999) Role of oral bacteria in respiratory infection. *J Periodontol* **70**, 793–802.

Scannapieco, F.A., Bush, R.B. and Paju, S. (2003) Associations between periodontal disease and risk for atherosclerosis, cardiovascular disease, and stroke. A systematic review. *Ann Periodontol* **8**, 38–53.

Schauder, S., Shokat, K., Surette, M.G. and Bassler, B.L. (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **41**, 463–476.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W. (2012) NIH Image to ImageJ : 25 years of image analysis. *Nat Methods* **9**, 671–675.

Selwitz, R.H., Ismail, A.I. and Pitts, N.B. (2007) Dental caries. *Lancet* **369**, 51–59.

Semmelhack, M.F., Campagna, S.R., Federle, M.J. and Bassler, B.L. (2005) An expeditious synthesis of DPD and boron binding studies. *Org Lett* **7**, 569–572.

Senadheera, M.D., Guggenheim, B., Grace, A., Huang, Y.C., Choi, J., David, C.I., Treglown, J.S., Goodman, S.D. *et al.* (2005) A VicRK signal transduction system in *Streptococcus mutans* affects *gtfBCD*, *gbpB*, and *ftf* expression, biofilm formation, and genetic competence development. *J Bacteriol* **187**, 4064–4076.

Shao, H. and Demuth, D.R. (2010) Quorum sensing regulation of biofilm growth and gene expression by oral bacteria and periodontal pathogens. *Periodontol 2000* **52**, 53–67.

Shemesh, M., Tam, A., Aharoni, R. and Steinberg, D. (2010) Genetic adaptation of *Streptococcus mutans* during biofilm formation on different types of surfaces. *BMC Microbiol* **10**, 51.

Stewart, P.S. (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* **292**, 107–113.

Stewart, P.S. and Costerton, J.W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135–138.

Stroeher, U.H., Paton, A.W., Ogunniyi, A.D. and Paton, J.C. (2003) Mutation of *luxS* of *Streptococcus pneumoniae* affects virulence in a mouse model. *Infect Immun* **71**, 3206–3212.

Sztajer, H., Lemme, A., Vilchez, R., Schulz, S., Geffers, R., Yip, C.Y.Y., Levesque, C.M., Cvitkovitch, D.G. *et al.* (2008) Autoinducer-2-regulated genes in *Streptococcus mutans* UA159 and global metabolic effect of the *luxS* mutation. *J Bacteriol* **190**, 401–415.

Vendeville, A., Winzer, K., Heurlier, K., Tang, C.M. and Hardie, K.R. (2005) Making "sense" of metabolism: autoinducer-2, LuxS and pathogenic bacteria. *Nat Rev Microbiol* **3**, 383–396.

Vilchez, R., Lemme, A., Thiel, V., Schulz, S., Sztajer, H. and Wagner-Döbler, I. (2007) Analysing traces of autoinducer-2 requires standardization of the *Vibrio harveyi* bioassay. *Anal Bioanal Chem* **387**, 489–496.

Wen, Z.T. and Burne, R.A. (2002) Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. *Appl Environ Microbiol* **68**, 1196–1203.

Wen, Z.T. and Burne, R.A. (2004) LuxS-mediated signaling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation. *J Bacteriol* **186**, 2682–2691.

Wen, Z.T., Yates, D., Ahn, S. and Burne, R.A. (2010) Biofilm formation and virulence expression by *Streptococcus mutans* are altered when grown in dual-species model. *BMC Microbiol* **10**, 111.

Winans, S.C. and Bassler, B.L. (2002) Mob psychology. *J Bacteriol* **184**, 873–883.

Wu, H., Song, Z., Hentzer, M., Andersen, J.B., Molin, S., Givskov, M. and Høiby, N. (2004) Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice. *J Antimicrob Chemother* **53**, 1054–1061.

Xavier, K.B. and Bassler, B.L. (2003) LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol* **6**, 191–197.

Yoshida, A., Ansai, T., Takehara, T. and Kuramitsu, H.K. (2005) LuxS-based signaling affects *Streptococcus mutans* biofilm formation. *Appl Environ Microbiol* **71**, 2372–2380.

Zeigler, C.C., Persson, G.R., Wondimu, B., Marcus, C., Sobko, T. and Modéer, T. (2012) Microbiota in the oral subgingival biofilm is associated with obesity in adolescence. *Obesity (Silver Spring)* **20**, 157–164.

Zhao, G., Wan, W., Mansouri, S., Alfaro, J.F., Bassler, B.L., Cornell, K.A. and Zhou, Z.S. (2003) Chemical synthesis of S-ribosyl-l-homocysteine and activity assay as a LuxS substrate. *Bioorg Med Chem Lett* **13**, 3897–3900.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Dose-dependent biofilm formation of orally associated bacteria in presence of biofilm inhibitor.

Figure S2 *V. harveyi* BB170 quorum sensing reporter dose response to quorum sensing inhibitors.

Figure S3 Luminescence of *V. harveyi* BB170 in the presence of entire compound library at 1 mmol l^{-1}.

Table S1 Summary of results for organosulfur compounds that inhibited both AI-2 based QS and oral bacterial biofilm formation.