Autoinducer-2 influences interactions amongst pioneer colonizing streptococci in oral biofilms

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Streptococcus gordonii and Streptococcus oralis are among the first bacterial species to colonize clean tooth surfaces. Both produce autoinducer-2 (AI-2): a family of inter-convertible cell–cell signal molecules synthesized by the LuxS enzyme. The overall aim of this work was to determine whether AI-2 alters interspecies interactions between S. gordonii DL1 and S. oralis 34 within dual-species biofilms in flowing human saliva. Based upon AI-2 bioluminescence assays, S. gordonii DL1 produced more AI-2 activity than S. oralis 34 in batch culture, and both were able to remove AI-2 activity from solution. In single-species, saliva-fed flowcell systems, S. oralis 34 formed scant biofilms that were similar to the luxS mutant. Conversely, S. gordonii DL1 formed confluent biofilms while the luxS mutant formed architecturally distinct biofilms that possessed twofold greater biovolume than the wild-type. Supplementing saliva with 0.1–10 nM chemically synthesized AI-2 (csAI-2) restored the S. gordonii DL1 luxS biofilm phenotype to that which was similar to the wild-type; above or below this concentration range, biofilms were architecturally similar to that formed by the luxS mutant. In dual-species biofilms, S. gordonii DL1 was always more abundant than S. oralis 34. Compared with dual-species, wild-type biofilms, the biovolume occupied by S. oralis 34 was reduced by greater than sevenfold when neither species produced AI-2. The addition of 1 nM csAI-2 to the dual-species luxS–luxS mutant biofilms re-established the biofilm phenotype to resemble that of the wild-type pair. Thus, this work demonstrates that AI-2 can alter the biofilm structure and composition of pioneering oral streptococcal biofilms. This may influence the subsequent succession of other species into oral biofilms and the ecology of dental plaque.

INTRODUCTION

Human oral bacteria are highly interactive organisms that exist within multi-species dental-plaque biofilms (Marsh, 2004). In such biofilms, evidence indicates that inter-species communication alters the ability of bacteria to survive and create a niche (Hojo et al., 2009; Jayaraman & Wood, 2008; Marsh, 2004). For inter-species communication to occur effectively within dental-plaque biofilms, it is proposed that cell–cell association and cell–cell signalling are required (Kolenbrander et al., 2006; Rickard et al., 2008a). Inter-species association can be through non-specific aggregation (Busscher et al., 1992; Busscher & Van der Mei, 2000) or coaggregation: the specific recognition and subsequent adhesion of different species of bacteria (Kolenbrander, 1988; Rickard et al., 2003). Cell–cell association decreases the distance between cells and may enhance cell–cell signalling (Egland et al., 2004; Kolenbrander et al., 2010; Redfield, 2002). One such bacterial cell–cell signalling mechanism, which is the focus of this work, relies upon the production and detection of extracellular molecules collectively called autoinducer-2 (AI-2). AI-2 is an umbrella term for a family of inter-convertible molecules that form from the spontaneous rearrangement of 4,5-dihydroxy-2,3-pentanedione (DPD) (Duerre et al., 1971; Miller et al., 2004; Semmelhack et al., 2005). DPD is generated as a by-product of LuxS activity – a bacterial intracellular enzyme that also converts S-ribosylhomocysteine to homocysteine. Environmental conditions, such as boron availability, shift the equilibrium towards one form or another (Semmelhack et al., 2005). Different species recognize different forms (Chen et al., 2002; Coulthurst Abbreviations: AI-2, autoinducer-2; csAI-2, chemically synthesized AI-2; DPD, 4,5-dihydroxy-2,3-pentanedione.
et al., 2002; Miller et al., 2004) and coaggregation likely allows (by virtue of juxtaposition) even low concentrations of AI-2 to be detected and communication to occur (Rickard et al., 2008a; Shao & Demuth, 2010).

Streptococci account for >50% of the culturable bacteria in healthy dental plaque (Aas et al., 2005; Diaz et al., 2006; Listgarten, 1999). Most, if not all, oral streptococci are able to coaggregate with at least one other streptococcal species (Kolenbrander et al., 1990) and with other indigenous oral species (Kolenbrander, 1993). Many oral bacteria, including streptococci, also produce AI-2 (Frias et al., 2001). Such physical and chemical interactions are important when considering the development of dental-plaque biofilms because both Streptococcus gordonii and Streptococcus oralis are indigenous commensal species that are among the first to colonize clean tooth surfaces (Aas et al., 2005; Diaz et al., 2006; Nyvad & Kilian, 1987; Socransky et al., 1977). S. gordonii and S. oralis belong to a genus that has been referred to as containing ‘pioneering colonizers’ (Kolenbrander et al., 1990) and these species are consistently isolated from dental-plaque biofilm communities, albeit at different relative amounts (Nobbs et al., 2009; Paster et al., 2006; Whittaker et al., 1996). The two species coaggregate with each other and numerous other oral species through complex adhesin–receptor mechanisms (Kolenbrander et al., 2002; Nobbs et al., 2009) and both have also recently been demonstrated to produce AI-2 (Blehert et al., 2003; McNab et al., 2003; Rickard et al., 2006). Such pioneer colonizers may promote the sequential integration of other oral species, including oral pathogens, into the developing dental-plaque communities (Jakubovics & Kolenbrander, 2010; Whitmore & Lamont, 2011).

When considering S. gordonii DL1 and S. oralis 34 as primary co-colonizing oral bacteria, Palmer et al. (2001) showed that S. gordonii DL1 formed dense single-species biofilms in saliva while S. oralis 34 formed sparse biofilms. When co-inoculated, S. gordonii DL1 and S. oralis 34 grew to form dual-species biofilms but the biomass of S. gordonii DL1 predominated (Palmer et al., 2001). S. oralis 34 only formed thick biofilms when in coaggregated mutualistic biofilms with Actinomyces oris T14V (Palmer et al., 2001). No studies of cell–cell signalling-mediated biofilm interactions were performed, however. Recent initial studies by Blehert et al. (2003) have indicated that inactivation of the luxS gene in S. gordonii DL1 subtly altered very early biofilm development. However, it was not clear whether AI-2 and/or metabolic defects due to the inactivation of the luxS gene were responsible for the altered architecture because chemically synthesized AI-2 (csAI-2) was not available at that time. In separate studies, Rickard et al. (2006) used csAI-2 to show that AI-2 influenced the degree of mutualism between S. oralis 34 and A. oris T14V. The optimal concentration of AI-2 for mutualism to occur was between 0.08 and 8 nM, above and below which mutualism was retarded. Collectively these research findings indicate that intra-generic (inter-species) interactions between pioneer-colonizing streptococci may be facilitated by the production and detection of low (pM to nM) concentrations of AI-2.

The overall aim of this work was to determine whether AI-2 influences the development of S. gordonii DL1 and S. oralis 34 single- and dual-species biofilms when human saliva was used as the sole nutrient source. In order to achieve this and help in the interpretation of our findings from dual-species studies, we studied the properties of each wild-type and luxS mutant species, including their relative ability to grow in batch cultures, produce AI-2 and remove AI-2 from their surroundings. Confocal laser scanning microscopy and computational image analysis software were used to quantify the biofilm-forming ability of streptococcal wild-type and isogenic luxS mutants in single- and dual-species biofilms. We demonstrate an AI-2 concentration-dependent role in intra-species signalling between S. gordonii DL1 cells in single-species biofilms as well as a role in influencing inter-species interactions between S. gordonii DL1 and S. oralis 34 in dual-species biofilms. We also show that AI-2 is able to influence the biofilm thickness, biovolume and architecture in these cases.

**METHODS**

**Strains and batch cultures.** Both Streptococcus species and isogenic luxS mutant strains were provided by Paul Kolenbrander (NIDCR, NIH, Bethesda, MD, USA). S. gordonii DL1, S. oralis 34 and their isogenic luxS mutants were grown in Schaedler broth (Difco) that contained 0.01 g haemin l\(^{-1}\). When required 1 mM boric acid was added. Growth was under static conditions at 37\(^\circ\)C in 5% CO\(_2\). Vibrio harveyi BB170 and BB152 (also designated ATCC BAA-1117 and ATCC BAA-1119, respectively), the strains required to measure AI-2 activity, were grown in AB medium (Bassler et al., 1994) at 30\(^\circ\)C with shaking at 200 r.p.m. For general batch cultures and stock preparations, S. gordonii DL1 luxS and S. oralis 34 luxS were grown in broth supplemented with 10 \(\mu\)g erythromycin ml\(^{-1}\) as described by Blehert et al. (2003) and Rickard et al. (2006). All stocks were maintained at -80\(^\circ\)C in 25% (v/v) glycerol.

**Chemical synthesis of AI-2.** DPD, the AI-2 precursor, was chemically synthesized in the Campagna lab using the approach of Semmelhack et al. (2005). Stocks of protected-DPD were stored at -80\(^\circ\)C until required and deprotected as described previously by Semmelhack et al. (2005). Once deprotected, DPD spontaneously forms multiple inter-convertible forms. Collectively this is called csAI-2 and 100 \(\mu\)l stocks at 1 mM were stored at -80\(^\circ\)C for no more than 2 months.

**AI-2 bioluminescence induction assay.** An approach described by Rickard et al. (2010) that is based upon the method of Bassler et al. (1997) was used to test AI-2 activity within solutions. Briefly, V. harveyi BB170 cultures were grown for 14 h at 30 \(^\circ\)C and then diluted 1:500 in AB broth. V. harveyi BB152 cultures were grown separately for 14 h at 30 \(^\circ\)C and the cell-free supernatants were collected by filter-sterilization using 0.22 \(\mu\)m filter units (VWR). The solutions were then stored at -80\(^\circ\)C until required. A Victor 3 multi-label counter (Perkin Elmer) set to luminescence mode was used to detect AI-2 activity. Fold induction values, which are an indicator of AI-2 activity within a test sample, were determined using the approach of Blehert et al. (2003).
Evaluation of Al-2 activity during growth in batch cultures. The ability of the two species of wild-type and luxS mutant streptococci to generate Al-2 activity in Schaedler broth (with or without 1 mM boric acid) was determined by taking samples during batch culture growth at 37 °C in 5% CO2. Samples were filter-sterilized in 0.22 µm filter units (VWR) and immediately stored at −80 °C until required for the bioluminescence induction assay.

Determination of removal of Al-2 activity. To study the ability of the streptococci to remove Al-2 activity from solution, exponential-phase S. gordonii DL1 luxS cells and S. oralis 34 luxS cells were washed and suspended in PBS at 180 Klett units (Red filter, Klett Manufacturing Co.) with and without 100 µM csAl-2. In addition, solutions containing only PBS (pH 7.2) and PBS supplemented with 100 µM csAl-2 were prepared. During incubation over 60 min at 37 °C in 5% CO2, samples were collected and filter-sterilized in 0.22 µm filter units (VWR). The samples were immediately stored at −80 °C until being needed for the Al-2 bioluminescence induction assay. Removal was characterized by determining percentage decreases in fold induction values over time.

Preparation and labelling of polyclonal antibodies and wheat-germ agglutinin (WGA). Wheatgerm agglutinin, which binds to S. oralis 34 but not to S. gordonii DL1, was purchased from Sigma-Aldrich. Rabbit serum that contained polyclonal IgG antibodies that react with whole S. gordonii DL1 cells were purchased from Covance. Anti-S. gordonii DL1 polyclonal IgG antibodies were purified in immobilized protein A-Sepharose columns according to the manufacturer's instructions (Invitrogen). These were then labelled with Alexa Fluor 488, and WGA was labelled with Alexa Fluor 555 following the manufacturer's protocols (Invitrogen). To avoid cross-reactivity, labelled anti-S. gordonii DL1 antibodies were mixed with excess S. oralis 34 cells and non-bound antibodies (that only recognize S. gordonii DL1) were concentrated using 30 kDa Microcon centrifugal concentrators (Millipore).

Preparation of human saliva. Saliva was collected from at least six healthy donors who were non-smokers, had not taken antibiotics for at least 3 months prior to donation and had not consumed any food or drinks for at least 2 h before collection. Using a modified method of De Jong & Van der Hoeven (1987), collected saliva was pooled and DTT (Sigma) was added to a final concentration of 2.5 mM. The solution was incubated on ice for 10 min and then centrifuged at 14000 g for 30 min. The supernatant was diluted 1:4 with distilled water to produce 25% saliva and stored at −20 °C. When required the frozen diluted saliva was thawed, passed through a 0.22 µm filter (Vacuum Filtration Systems, VWR) and then used in biofilm experiments.

Continuous flow biofilms. Teflon 2-channel flowcells were constructed in-house using the design of Palmer et al. (2001). The flowcells were connected to a downstream peristaltic pump (Minipuls III, Gilson) and the downstream ports of the flowcell fed into a waste vessel. Flowcells were sterilized with 1% sodium hypochlorite for 24 h and then flushed with sterile water for 2 h to remove residual hypochlorite. Processed saliva replaced the sterile water to condition the flowcell substrata (100 µl min−1 per channel) for 20 min before use.

Prior to flowcell inoculation, S. gordonii DL1, S. oralis 34 and the respective isogenic luxS mutants were cultured separately for 14 h in Schaedler broth and inoculated 1:40 into fresh broth. Cells were grown to mid-exponential phase (4 h) and then diluted to a Klett value of 40 (Red filter, Klett Manufacturing) in Schaedler broth. For inoculation, 250 µl single- or dual-species cell suspension was injected into the flowcells. To inoculate flowcells for dual-species biofilm experiments, cells were mixed together at a 1:1 ratio (both were set to a Klett value of 40 and mixed in equal volumes) prior to co-inoculation. Processed saliva flowed at 100 µl min−1 per channel through the flowcells for up to 22 h. When required, saliva in the flowcell reservoirs was supplemented with csAl-2. All continuous flow experiments were conducted at 37 °C and the flowcells were sampled at 2, 6 and 22 h post-inoculation.

Confocal laser scanning microscopy. To label cells, biofilms were exposed to 250 µl PBS (pH 7.4) containing 10 µg Alexa Fluor 488-conjugated anti-S. gordonii DL1 antibodies ml−1 and/or 10 µg Alexa Fluor 555-conjugated WGA supplemented with 0.1% BSA ml−1 (Rockland). After 20 min the flowcells were flushed with 250 µl PBS and biofilms were examined using a Leica TCS-SP5 confocal microscope (Leica). A ×40, 1.25 NA oil immersion lens was used to visualize labelled S. gordonii DL1 and S. oralis 34 with 488 and 543 nm excitation wavelengths, respectively. Each biofilm was scanned at randomly selected positions and four image stacks per flowcell channel were taken. Optical sectioning was at a thickness of 1 µm.

Imaging and analysis of confocal microscope stacks. An i5 Intel computer equipped with 4 GB of RAM and a Radeon 5850 graphics card (Advanced Micro Devices) was used for computational analyses. Image stacks were assembled to generate 3D reconstructions using Imaris imaging software (Bitplane AG). Single-species biofilms were rendered using shadow projection to highlight differences in structure. Dual-species biofilms were rendered using maximum image projection in order to ascertain inter-species juxtaposition. Biofilm characteristics, including biovolume, roughness coefficient and average thickness, were determined using COMSTAT software (Heydorn et al., 2000).

Statistical methods. Biofilm growth parameters were evaluated by both the Student’s t-test and Wilcoxon test for comparison of two groups. Analysis of variance (ANOVA) and the Kruskal–Wallis test were used for differences between three or more groups. In the case of significant differences, post hoc analyses were based on Tukey’s multiple comparisons test. Mixed model analyses were performed to confirm the robustness of the results using repeated measures. All pairwise comparisons were adjusted for multiple testing. Differences with P values of <0.05 were considered significant. All statistical analyses were performed with SAS version 9.2 (SAS Institute).

RESULTS

Al-2 production and removal of activity from solution

To compare relative amounts of Al-2 produced by the two Streptococcus species, Al-2 activity was monitored during batch-culture growth for both the wild-type and luxS mutant of S. gordonii DL1 (Fig. 1a) and S. oralis 34 (Fig. 1b). S. gordonii DL1 and S. gordonii DL1 luxS grew similarly in Schaedler broth and Schaedler broth supplemented with 1 mM boric acid and reached stationary phase by 6 h (Fig. 1a). S. oralis 34 and S. oralis 34 luxS grew at comparable rates and reached stationary phase within 8 h (Fig. 1b).

AI-2 activities in cell-free supernatants from batch cultures of S. gordonii DL1, S. oralis 34 and their isogenic luxS mutants were measured by the V. harveyi AI-2 bioluminescence induction assay, which gives an indication of AI-2
concentration (Fig. 1a, b). AI-2 activity was only detected in wild-type S. gordonii DL1 and S. oralis 34 cell-free supernatants after growth in Schaedler broth supplemented with 1 mM boric acid. During stationary phase, S. gordonii DL1 and S. oralis 34 yielded inductions of fivefold and threefold, respectively. AI-2 activity of >twofold was not detected in supernatants from cultures of either luxS mutant after growth in Schaedler broth or Schaedler broth supplemented with 1 mM boric acid.

The ability of S. gordonii DL1 and S. oralis 34 cells to remove AI-2 activity from their surroundings as determined by the V. harveyi BB170 bioluminescence reporter system was investigated by adding 100 μM csAI-2 to cell suspensions of the isogenic luxS mutant of each species (Fig. 2a, b). When csAI-2 was added to PBS (in the absence of cells), AI-2 activity did not change over 60 min (Fig. 2a, b). AI-2 activity was not detected in suspensions containing luxS cells. However, when csAI-2 was added to S. gordonii DL1 luxS or S. oralis 34 luxS cell suspensions, AI-2 activity was rapidly reduced over time. Both S. gordonii DL1 luxS (Fig. 2a) and S. oralis 34 luxS (Fig. 2b) reduced csAI-2 activity by >50% within 20 min of exposure. After 60 min AI-2 activity was reduced by approximately 80% by S. gordonii DL1 luxS while S. oralis 34 luxS reduced the activity >95% (Fig. 2a versus 2b).

Effect of luxS disruption on single-species streptococcal biofilm development

The spatiotemporal development of single-species biofilms of S. gordonii DL1, S. oralis 34 and their isogenic luxS mutants were determined by confocal laser scanning microscopy and computer-based image analysis. Spatiotemporal single-species biofilm development of S. gordonii DL1 and S. gordonii DL1 luxS was dissimilar. By 6 h the S. gordonii DL1 luxS biofilm cells were often less scattered than the wild-type, and by 22 h the architectural differences were even more pronounced. (Fig. 3a versus 3b). At 22 h, S. gordonii DL1 formed confluent homogeneous biofilms covering most of the surface. Conversely, S. gordonii DL1 luxS formed biofilms that, through visual inspection of confocal stacks in the x- and y-dimensions, appeared to cover less of the surface (Fig. 3). Visualization in the z-dimension and COMSTAT analysis, however, showed that S. gordonii DL1 biofilms occupied a significantly
As compared with the concentration-dependent changes in biofilm architecture rendering yielded an especially noticeable pattern of AI-2 the change in architecture (Fig. 4). Unlike the 2D views, 3D and computationally rendered in 2D and 3D to examine different concentrations of csAI-2. Biofilms were visualizedbiofilms were grown with 25 % saliva supplemented with different concentrations of csAI-2. Biofilms were visualized and computationally rendered in 2D and 3D to examine the change in architecture (Fig. 4). Unlike the 2D views, 3D rendering yielded an especially noticeable pattern of AI-2 concentration-dependent changes in biofilm architecture (Fig. 4). As compared with the S. gordonii DL1 biofilms, the S. gordonii DL1 luxS biofilms were relatively rutted after 22 h (Fig. 4a versus 4b) and possessed cellular masses that projected into the lumen of the flowcells. Chemical complementation with 0.1, 1 or 10 nM csAI-2 restored the S. gordonii DL1 luxS biofilm architecture to that which was akin to the wild-type. Biofilms developed in saliva containing the lowest or highest concentrations of csAI-2 (0.01 or 100 nM) resulted in the development of S. gordonii DL1 luxS biofilms that resembled S. gordonii DL1 luxS biofilms grown in the absence of csAI-2 (Fig. 4c, g versus 4b). COMSTAT analysis confirmed the visual observations from the 2D projections and 3D renderings. S. gordonii DL1 luxS mutant biofilms grown in saliva supplemented with 0.1, 1 and 10 nM csAI-2 possessed a biovolume and average thickness that was not significantly different (P>0.05) to that of the wild-type grown in non-supplemented saliva (Table 1). When csAI-2 was added to S. gordonii DL1 luxS biofilms at the lowest and highest concentrations studied (0.01 or 100 nM), the biofilms possessed biovolumes and average thicknesses that were statistically different from those formed by the wild-type (P<0.05) but similar to the S. gordonii DL1 luxS biofilms in saliva-lacking, exogenously supplied csAI-2 (Table 1).

Effect of exogenous AI-2 on single-species streptococcal biofilm development

To determine whether changes in S. gordonii DL1 biofilm development were due to AI-2 signalling, luxS mutant biofilms were grown with 25 % saliva supplemented with different concentrations of csAI-2. Biofilms were visualized and computationally rendered in 2D and 3D to examine the change in architecture (Fig. 4). Unlike the 2D views, 3D rendering yielded an especially noticeable pattern of AI-2 concentration-dependent changes in biofilm architecture (Fig. 4). As compared with the S. gordonii DL1 biofilms, the S. gordonii DL1 luxS biofilms were relatively rutted after 22 h (Fig. 4a versus 4b) and possessed cellular masses that projected into the lumen of the flowcells. Chemical complementation with 0.1, 1 or 10 nM csAI-2 restored the S. gordonii DL1 luxS biofilm architecture to that which was akin to the wild-type. Biofilms developed in saliva containing the lowest or highest concentrations of csAI-2 (0.01 or 100 nM) resulted in the development of S. gordonii DL1 luxS biofilms that resembled S. gordonii DL1 luxS biofilms grown in the absence of csAI-2 (Fig. 4c, g versus 4b). COMSTAT analysis confirmed the visual observations from the 2D projections and 3D renderings. S. gordonii DL1 luxS mutant biofilms grown in saliva supplemented with 0.1, 1 and 10 nM csAI-2 possessed a biovolume and average thickness that was not significantly different (P>0.05) to that of the wild-type grown in non-supplemented saliva (Table 1). When csAI-2 was added to S. gordonii DL1 luxS biofilms at the lowest and highest concentrations studied (0.01 or 100 nM), the biofilms possessed biovolumes and average thicknesses that were statistically different from those formed by the wild-type (P<0.05) but similar to the S. gordonii DL1 luxS biofilms in saliva-lacking, exogenously supplied csAI-2 (Table 1).

Effect of disruption of luxS on dual-species streptococcal biofilm interactions

To determine whether luxS was required for S. gordonii DL1 and/or S. oralis 34 to maintain a presence within dual-species streptococcal biofilms, the wild-type and luxS mutant strains of both species were grown in saliva-fed biofilms in all four possible dual-species combinations. For all pairings, S. gordonii DL1 was more abundant than S. oralis 34 by 22 h (Fig. 5). Post co-inoculation, coaggregated micro-colonies were conspicuous at 2 and 6 h in most of the cases (Fig. 5), although at 6 h dual luxS mutant pairs showed a decrease in the number of coaggregated micro-colonies (Fig. 5d). After 22 h, when one or both of the two partners produced AI-2 (wild-type), the biofilms developed to give an undulating architecture with island-like topographies and mosaics of S. oralis 34 cell clusters (yellow) surrounded by S. gordonii DL1 cells (red) (Fig. 5a–c).
Dual-species biofilms of *S. gordonii* DL1 *luxS* and *S. oralis* 34 *luxS* were more architecturally homogeneous, containing relatively sparse and small clusters of *S. oralis* 34 *luxS* (Fig. 5d). COMSTAT analysis confirmed that the biovolume occupied by *S. gordonii* DL1 was always greater than that of *S. oralis* 34, regardless of whether one or both were wild-type or *luxS* mutants (Table 2). In dual-species, wild-type-*luxS* mutant biofilms where only one of the species produced AI-2 (Fig. 5b, c), biofilms were visually more similar to dual-species, wild-type biofilms than to dual-species, *luxS* mutant biofilms (Fig. 5b, c versus a or d). However, dual-species biofilms containing *S. gordonii* DL1 *luxS* exhibited significantly increased biovolumes (*P* < 0.05) compared with *S. gordonii* DL1 wild-type, irrespective of whether the partner species was *S. oralis* 34 or *S. oralis* 34 *luxS* (Table 2). When both species were *luxS* mutants, *S. oralis* 34 *luxS* was in much lower quantities compared with all other combinations (81:1, Fig. 5d). Taken together, the biovolume data and ratio calculations (Table 2) indicate that the biofilm properties of *S. oralis* *luxS* can be biologically complemented by *S. gordonii* DL1 in dual-species, wild-type-*luxS* mutant biofilms but *S. gordonii* DL1 *luxS* cannot be biologically complemented by *S. oralis* 34.

### Effect of exogenous AI-2 on dual-species streptococcal biofilm interactions

The finding that *luxS* disruption in both *Streptococcus* species altered the dual-species biofilm architecture and the relative abundance (expressed as biovolume ratio, Table 2) of each species suggested that either LuxS has a metabolic function(s) relevant to biofilm formation or AI-2 plays a role in the establishment of the biofilms. An approach to determine whether cell–cell signalling mediates dual-species biofilm interactions is to add known concentrations of csAI-2 to the saliva that feeds the biofilms. To saliva, csAI-2 was added to final concentrations of 0.01, 1 and 100 nM. These concentrations represent the highest and lowest concentrations (0.01 and 100 nM) and the anticipated optimal concentration (1 nM) based upon results from the single-species experiments described earlier. Confocal images of 2 and 6 h biofilms showed microcolonies with both species closely juxtaposed (coaggregated) regardless of addition or concentration of csAI-2 (Fig. 5e–g). However, after 22 h of development biofilms that were fed csAI-2 concentrations of 0.01 and 100 nM in saliva were more architecturally akin to each other (Fig. 5e, g) and to the dual-species *luxS* mutant biofilm (Fig. 5d). The biovolume ratios were 47:1, 7:1 and 26:1 for those that were exposed to 0.01, 1 and 100 nM csAI-2,
DISCUSSION

This study demonstrates that AI-2 influences intra-species interactions between S. gordonii DL1 biofilm cells and inter-species interactions between S. oralis 34 and S. gordonii DL1. Both species are pioneer colonizers of dental-plaque biofilms, producers of AI-2 and can reduce AI-2 activity in solutions. In particular, this study demonstrated that different AI-2 concentrations can alter biovolume (and biovolume ratio in dual-species biofilms), average biofilm thickness and the architecture of single- and dual-species biofilms. An interesting finding, when considering that many AI-2-based studies have focused on single species, was that AI-2 influenced the biovolume and average biofilm thickness of S. oralis 34 in dual-species biofilms but not in single-species biofilms.

Both wild-type Streptococcus species generated detectable AI-2 activity in Schaedler broth supplemented with boric acid, while the isogenic luxS mutant of each strain did not produce AI-2 (>twofold, Fig. 1). AI-2 activity was not detected in non-supplemented broth from wild-type species or the luxS species. Schaedler broth is a general purpose liquid medium for the growth of fastidious aerobic and anaerobic bacteria (Schaedler et al., 1965) that has been used in the testing of oral bacteria for the production of AI-2 (Frias et al., 2001). Until now, Schaedler broth has not been supplemented with boric acid and requirement for boric acid to detect AI-2 produced by streptococci in Schaedler broth has not been shown before, to our knowledge. This likely relates to the ability of the bioluminescent reporter system (V. harveyi BB170) to recognize a boronated form of AI-2 (Chen et al., 2002; DeKeersmaecker & Vanderleyden, 2003) and a low concentration of AI-2 being produced by the streptococci (Frias et al., 2001). By supplementing broth with 1 mM boron, the equilibrium of different AI-2 forms is likely shifted towards boronated respectively (Table 2). The large changes in biovolume ratios were solely due to differences in the amount of S. oralis 34 luxS in the biofilms (Table 2). The biovolume ratio for dual-species luxS mutant biofilms that were supplemented with 1 nM csAI-2 was not significantly different from that of the dual-species, wild-type biofilms. Dual-species, luxS mutant biofilms supplemented with either 0.01 or 100 nM csAI-2 yielded S. oralis 34 luxS biofilms that were significantly different from those of wild-type biofilms (P<0.001). Differences in other biofilm parameters, such as average thickness and roughness, were less discernible (Table 2). Although compared with dual-species, wild-type biofilms, the average thickness of S. oralis 34 luxS in dual-species, luxS mutant biofilms was significantly reduced (P<0.05) when saliva was not supplemented with csAI-2, or if the concentration was above or below 1 nM, further emphasizing the sensitivity of S. oralis 34 to AI-2 in dual-species biofilms. Collectively these results suggest a role for AI-2 in mediating changes in dual-species biofilm properties including relative species abundance.
Fig. 4. Representative 2D and 3D confocal micrographs from an experiment showing the effect of csAI-2 on the spatiotemporal development of single-species biofilms of *S. gordonii* DL1 *luxS* biofilms. Images taken after 2, 6 and 22 h of growth in flowing 25% saliva and showing biofilm development of (a) *S. gordonii* DL1, (b) *S. gordonii* DL1 *luxS* and (c–g) *S. gordonii* DL1 *luxS* in 25% saliva supplemented with 0.01, 0.1, 1, 10 and 100 nM csAI-2. Twenty-two hour biofilms are also shown in 3D to exemplify changes in structure. Biofilms were labelled with Alex Fluor 488 anti-*S. gordonii* DL1 antibody. Bars, 50 µm. Dimensions of the regions shown are 387×387 µm (x-y perspective).
forms, thus raising the effective concentration of the form that is detectable by *V. harveyi* BB170 (Semmelhack *et al.*, 2004). Importantly, a direct comparison of fold inductions indicates that *S. gordonii* DL1 produces relatively more bioavailable AI-2 than *S. oralis* 34 (Fig. 1a, b) and this is important when considering the different rates of removal of AI-2 activity and biofilm interactions. It is not known whether oral streptococci recognize a borated or non-borated form of AI-2, although boron is present in the oral cavity at concentrations ≥ 400 nM (Ward, 1993).

Before examining the effect of AI-2 on biofilm development, it was important to establish whether it is recognized/removed by *S. gordonii* DL1 and *S. oralis* 34 cells. To determine this, csAI-2 was added to single-species cell suspensions of *S. gordonii* DL1 *luxS* and *S. oralis* 34 *luxS* (Fig. 2a, b). The *luxS* mutants were studied because neither produced AI-2. As inferred by the bioluminescence detection system, *S. oralis* 34 *luxS* removed the vast majority of detectable AI-2 activity from the suspension within 60 min, while *S. gordonii* DL1 *luxS* reduced the AI-2 activity by less (approx. 80%, Fig. 2a versus 2b). We hypothesize that the reduction in AI-2 activity is due to an active uptake system such as that which exists in *Salmonella typhimurium* and *Aggregatibacter actinomycetemcomitans*. These species can remove AI-2 from solution through the expression of ABC-type transporters (James *et al.*, 2006; Shao *et al.*, 2007; Taga *et al.*, 2001, 2003). Assuming that the reduction of AI-2 activity by *S. gordonii* DL1 and *S. oralis* 34 is also due to the removal by transporters, the difference in rates of removal along with different rates of production of AI-2 will contribute to the amount of AI-2 that is bioavailable for cell–cell communication. Collectively the data suggest that *S. oralis* 34 produces less bioavailable AI-2 than *S. gordonii* DL1, which may explain why *S. gordonii* DL1 cannot be biologically complemented by *S. oralis* 34 in dual-species biofilms (Fig. 5, Table 2).

The biofilm studies presented here demonstrate that *S. gordonii* DL1 forms more substantial single-species biofilms in saliva than does *S. oralis* 34. Such a phenomenon has been observed before (Palmer *et al.*, 2001; Rickard *et al.*, 2006) and is probably because *S. gordonii*, but not *S. oralis*, adheres strongly to salivary alpha-amylase (Brown *et al.*, 1999; Scannapieco *et al.*, 1994). Also, *S. gordonii* DL1 is able to more efficiently use saliva for growth (Palmer *et al.*, 2001). In single-species biofilms, inactivation of the *luxS* gene in *S. gordonii* DL1 altered biofilm architecture while the inactivation of *luxS* in *S. oralis* 34 did not. An absence of change in architecture does not necessarily mean that *S. oralis* 34 does not respond to AI-2; rather, simply that it does not affect the ability of this species to

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**Fig. 5.** Effect of disruption of *luxS* and addition of csAI-2 on the spatiotemporal development of dual-species biofilms of *S. gordonii* DL1 and *S. oralis* 34. Representative confocal micrographs from an experiment show typical biofilms developed after 2, 6 and 22 h post-inoculation. Development of biofilms: (a) *S. gordonii* DL1 and *S. oralis* 34, (b) *S. gordonii* DL1 and *S. oralis* 34 *luxS*, (c) *S. gordonii* DL1 *luxS* and *S. oralis* 34, (d) *S. gordonii* DL1 *luxS* and *S. oralis* 34 *luxS*, and (e–g) *S. gordonii* DL1 *luxS* and *S. oralis* 34 *luxS* biofilms developed in 25% saliva supplemented with 0.01, 1 and 100 nM csAI-2. Streptococcal communities were labelled with Alex Fluor 488 anti-*S. gordonii* DL1 antibody (red) and Alexa Fluor 555 anti-*S. oralis* 34 WGA lectin (yellow). Side-views of each biofilm (x–z perspective) are under each corresponding 22 h *x–y* biofilm. Bars, 50 μm.
Table 2. Biofilm characteristics of 22 h dual-species biofilms of *S. gordonii* DL1 and *S. oralis* 34 wild-type and luxS mutant combinations grown in flowing 25% saliva as well as the biofilm characteristics of 22 h *S. gordonii* DL1 luxS and *S. oralis* 34 luxS dual-species biofilms developed in flowing 25% saliva supplemented with different concentrations of csAI-2

Upper and lower mean values are from *S. gordonii* DL1 and *S. oralis* 34, respectively. Mean values in bold were derived from 12 confocal laser scanning microscope image stacks from three experiments. Numbers in parentheses are associated SDs of each mean value. Significant differences between *S. oralis* or *S. gordonii* DL1 luxS mutants and their respective wild-type strains in *S. gordonii* DL1–*S. oralis* 34 biofilms are highlighted for each biofilm parameter tested.

<table>
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<tr>
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<th><em>S. gordonii</em> DL1 + <em>S. oralis</em> 34</th>
<th><em>S. gordonii</em> DL1 + <em>S. oralis</em> 34 luxS</th>
<th><em>S. gordonii</em> DL1 luxS + <em>S. oralis</em> 34</th>
<th><em>S. gordonii</em> DL1 luxS + <em>S. oralis</em> 34 luxS plus csAI-2 (nM)</th>
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<tr>
<td>Biovolume (µm³ µm⁻²)</td>
<td>1.15 (0.21)</td>
<td>1.59 (0.57)</td>
<td>2.96 (0.88)**</td>
<td>2.44 (0.69)*</td>
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<td>0.22 (0.08)</td>
<td>0.16 (0.07)</td>
<td>0.19 (0.06)</td>
<td>0.03 (0.01)**</td>
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<tr>
<td></td>
<td>0.01 1 100</td>
<td>1 10:1 1 6:1 8 1:1 4 7:1 7:1 2 6:1</td>
<td>1.87 (0.58)</td>
<td>1.82 (0.58)</td>
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<td></td>
<td>0.88 (5.30)</td>
<td>2.28 (1.46)</td>
<td>2.40 (3.55)</td>
<td>2.60 (7.72)</td>
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<td>0.85 (0.36)</td>
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*P<0.05; **P<0.001.

Value is derived by dividing the biovolume of *S. gordonii* DL1 by the biovolume of *S. oralis* 34 and is an indication of the relative abundance of these species.
activity removed by either species (demonstrated in Fig. 2). Ultimately, three conclusions can be drawn from our single- and dual-species cell–cell signalling studies. First, AI-2 mediates biofilm interactions between the two species of streptococci. Second, *S. gordonii* DL1 luxS requires AI-2 at a concentration of 0.01–100 nM to present a similar phenotype to the wild-type in dual-species biofilms and *S. oralis* cannot provide such a threshold, bioavailable AI-2 concentration. The threshold range is narrower in single-species biofilms (0.1–10 nM). Finally, there is a requirement for *S. oralis* 34 to be exposed to AI-2: either from itself, from *S. gordonii* DL1 or by the addition of approximately 1 nM csAI-2 to saliva in order for it to optimally maintain a niche in the dual-species biofilms. How the two species use AI-2 to effectively compete to increase their relative abundance in these dual-species biofilms is, at present, unknown. Competitive biofilm interactions are known to occur between streptococci via the production of bacteriocins (*Streptococcus mutans*) and hydrogen peroxide (*Streptococcus sanguis*) (Kreth et al., 2005) but not as a function of AI-2 cell–cell signalling and/or bioavailability.

In conclusion, this work demonstrates that AI-2 can alter single-species (*S. gordonii* DL1) and dual-species (*S. gordonii* DL1–*S. oralis* 34) biofilm development in flowing human saliva. Both *S. oralis* and *S. gordonii* can be isolated from immature dental-plaque biofilms and are among the first to colonize clean tooth surfaces (Aas et al., 2005; Diaz et al., 2006; Jenkinson, 1994). The relative amount of each species varies depending upon age of the dental-plaque biofilm, the physical position on tooth surfaces of the biofilm community and periodontal disease status (Aas et al., 2005; Abiko et al., 2010; Whitmore & Lamont, 2011). However, when considering the ability of these two species to be the first to colonize exposed tooth surfaces, a number of factors are likely to be important for abundance. These include their propensity to attach to saliva-coated tooth surfaces (van der Mei et al., 2008), to differentially express genes when attached to saliva-coated surfaces (Dü & Kolkenbrander, 2000), to coaggregate (Hojo et al., 2009; Kolkenbrander et al., 2006), and as shown here, to coordinate behaviour through AI-2-mediated cell–cell signalling. Our finding is potentially significant to the subsequent development of polymicrobial dental-plaque biofilms as it was recently demonstrated that the periodontal pathogen *Porphyromonas gingivalis* ATCC 33277 did not form dual-species biofilms with *S. oralis* 34 and instead formed dense dual-species biofilms when paired with *S. gordonii* DL1 (Periasamy & Kolkenbrander, 2009). Thus, dental-plaque biofilms that contain significant amounts of *S. oralis* 34 may be less susceptible to the integration of periodontal pathogens than *S. gordonii* DL1.

ACKNOWLEDGEMENTS

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