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.

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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 °C in 5% CO₂. *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 °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 µg erythromycin ml⁻¹ as described by Blehert *et al.* (2003) and Rickard *et al.* (2006). All stocks were maintained at –80 °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 °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 µl stocks at 1 mM were stored at –80 °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 °C and then diluted 1:500 in AB broth. *V. harveyi* BB152 cultures were grown separately for 14 h at 30 °C and the cell-free supernatants were collected by filter-sterilization using 0.22 µm filter units (VWR). The solutions were then stored at –80 °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 AI-2 activity during growth in batch cultures. The ability of the two species of wild-type and *luxS* mutant streptococci to generate AI-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 AI-2 activity. To study the ability of the streptococci to remove AI-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 csAI-2. In addition, solutions containing only PBS (pH 7.2) and PBS supplemented with 100 µM csAI-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 AI-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 against 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 14 000 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 csAI-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

AI-2 production and removal of activity from solution

To compare relative amounts of AI-2 produced by the two *Streptococcus* species, AI-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

Fig. 1. Growth kinetics and concurrent amount of AI-2 (expressed as fold induction of bioluminescence over unused broth) during batch-culture growth of wild-type and *luxS* mutants of *S. gordonii* DL1 and *S. oralis* 34. (a) Solid lines show the growth kinetics of *S. gordonii* DL1 in Schaedler broth (◆) or Schaedler broth plus 1 mM boric acid (■) and growth kinetics of *S. gordonii* DL1 *luxS* in Schaedler broth (◇) or Schaedler broth plus 1 mM boric acid (□). Dotted lines show AI-2 activity in cell-free supernatant from cultures of *S. gordonii* DL1 in Schaedler broth (◆) or Schaedler broth plus 1 mM boric acid (■) and AI-2 activity in cell-free supernatant from cultures of *S. gordonii* DL1 *luxS* during growth in Schaedler broth (◇) or Schaedler broth plus 1 mM boric acid (□). (b) Solid lines show the growth kinetics of *S. oralis* 34 in Schaedler broth (●) or Schaedler broth plus 1 mM boric acid (▲) and growth kinetics of *S. oralis* 34 *luxS* in Schaedler broth (○) or Schaedler broth plus 1 mM boric acid (△). Dotted lines show AI-2 activity in cell-free supernatant from cultures of *S. oralis* 34 during growth in Schaedler broth (●) or Schaedler broth plus 1 mM boric acid (▲) and AI-2 activity in cell-free supernatant from cultures of *S. oralis* 34 *luxS* during growth in Schaedler broth (○) or Schaedler broth plus 1 mM boric acid (△).

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 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. harvey* 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

Fig. 2. Removal of AI-2 activity by exponential-phase *S. gordonii* DL1 *luxS* cells and *S. oralis* 34 *luxS* cells. Removal was characterized by a percentage decrease in bioluminescence in the *V. harvey* BB170 assay. (a) *S. gordonii* DL1 *luxS* cells suspended in PBS supplemented with 100 μ M csAI-2 (+), cell-free PBS supplemented with 100 μ M csAI-2 (x) and *S. gordonii* DL1 *luxS* in PBS (-). (b) *S. oralis* 34 *luxS* cells suspended in PBS supplemented with 100 μ M csAI-2 (boxed x), cell-free PBS supplemented with 100 μ M csAI-2 (encircled +) and *S. oralis* 34 *luxS* in PBS (-).

smaller biovolume ($P < 0.001$) and were significantly thinner ($P < 0.001$, expressed in COMSTAT as the average thickness) as compared with the isogenic *luxS* mutant (Table 1). Thus, although *x*- and *y*-views of *S. gordonii* DL1 *luxS* biofilms visually appeared to possess less biovolume at 22 h than the wild-type (Fig. 3b versus 3a), the biovolume was actually two times greater. COMSTAT analyses showed no significant difference in the roughness of biofilms between wild-type and mutant. Conversely, neither *S. oralis* 34 nor *S. oralis* 34 *luxS* (Fig. 3c, d) formed substantial biofilms over a 22 h time period. Neither displayed significantly different biofilm biovolume, average thickness or roughness (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).

Fig. 3. Representative confocal micrographs from an experiment showing the spatiotemporal development of single-species biofilms of (a) *S. gordonii* DL1, (b) *S. gordonii* DL1 *luxS*, (c) *S. oralis* 34 and (d) *S. oralis* 34 *luxS*. Biofilms were labelled with Alex Fluor 488 anti-*S. gordonii* DL1 antibody (red) and Alexa Fluor 555 anti-*S. oralis* 34 WGA lectin (yellow). Images were collected at 2, 6 and 22 h after inoculation of flowcells. Bars, 50 μ m. Dimensions of the regions shown are 387 $\times$ 387 μ m (*x-y* perspective). Side-views of each biofilm (*x-z* perspective) are under each corresponding 22 h *x-y* biofilm.

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,

Table 1. Characteristics of 22 h single-species biofilms of *S. oralis* 34, *S. gordonii* DL1 and isogenic *luxS* mutants grown in flowing non-supplemented 25 % saliva or with 25 % saliva supplemented with different concentrations of csAI-2

Values in bold are means collected 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 are indicated for each biofilm parameter tested.

	<i>S. oralis</i> 34	<i>S. oralis</i> 34 <i>luxS</i>	<i>S. gordonii</i> DL1	<i>S. gordonii</i> DL1 <i>luxS</i>	<i>S. gordonii</i> DL1 <i>luxS</i> plus csAI-2 (nM)				
					0.01	0.1	1	10	100
Biovolume ($\mu\text{m}^3 \mu\text{m}^{-2}$)	0.18 (0.15)	0.12 (0.10)	1.71 (0.56)	3.69 (0.53)**	3.23 (1.02)**	1.38 (0.36)	1.47 (0.50)	1.56 (0.45)	3.53 (0.46)**
Average thickness (μm)	0.52 (0.26)	0.37 (0.24)	4.08 (1.15)	9.18 (4.55)**	7.73 (2.61)*	4.12 (2.36)	3.53 (2.47)	3.04 (2.30)	8.12 (1.37)*
Roughness coefficient	1.90 (0.10)	1.93 (0.07)	0.49 (0.21)	0.41 (0.14)	0.69 (0.36)	0.74 (0.26)	0.53 (0.30)	0.44 (0.25)	0.64 (0.25)

* $P < 0.05$; ** $P < 0.001$.

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* biovolumes 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.

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

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 μ m. Dimensions of the regions shown are 387 $\times$ 387 μ m (*x-y* perspective).

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 μ m.

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

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 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.

	<i>S. gordonii</i> DL1 + <i>S. oralis</i> 34		<i>S. gordonii</i> DL1 + <i>S. oralis</i> 34 <i>luxS</i>		<i>S. gordonii</i> DL1 <i>luxS</i> + <i>S. oralis</i> 34		<i>S. gordonii</i> DL1 <i>luxS</i> + <i>S. oralis</i> 34 <i>luxS</i>		<i>S. gordonii</i> DL1 <i>luxS</i> + <i>S. oralis</i> 34 <i>luxS</i> plus csAI-2 (nM)		
								0.01	1	100	
Biovolume (μm ³ μm ⁻²)	1.51 (0.31) 0.22 (0.08)	7:1	1.59 (0.57) 0.16 (0.07)	10:1	2.96 (0.88)** 0.19 (0.06)	16:1	2.44 (0.69)* 0.03 (0.01)**	1.87 (0.58) 0.04 (0.03)**	1.82 (0.58) 0.25 (0.11)	1.80 (0.60) 0.07 (0.04)**	
Biovolume ratio‡							81:1	47:1	7:1	26:1	
Average thickness (μm)	8.27 (2.74) 1.99 (1.16)		9.14 (5.30) 1.09 (0.73)		16.50 (7.19)* 2.28 (1.46)		9.63 (4.57) 0.21 (0.13)**	6.40 (3.55) 0.20 (0.24)**	7.60 (7.72) 1.29 (1.84)	7.58 (5.52) 0.26 (0.19)*	
Roughness coefficient	0.91 (0.30) 1.73 (0.23)		0.86 (0.36) 1.86 (0.09)		0.55 (0.24) 1.85 (0.06)		0.59 (0.27) 1.96 (0.03)*	0.81 (0.32) 1.90 (0.13)*	0.60 (0.35) 1.80 (0.15)	0.70 (0.25) 1.83 (0.17)	

* $P<0.05$; ** $P<0.001$.

‡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.

form single-species biofilms. In support of this suggestion, *S. oralis* 34 *luxS* was shown to remove AI-2 activity from suspensions containing csAI-2 (Fig. 2b), and previous studies have shown that the availability of AI-2 influences mutualism between *S. oralis* 34 and *A. oris* T14V (Rickard *et al.*, 2006). Conversely, inactivation of *luxS* in *S. gordonii* DL1 resulted in substantial alteration of biofilm structure (Figs 3 and 4). Blehert *et al.* (2003) have previously reported architectural differences between this wild-type and *luxS* mutant *S. gordonii* DL1 biofilms, albeit subtle in their study, after 4 h development. Importantly and counter to our findings, they did not notice a substantial change in biofilm biovolume of the *luxS* mutant over the wild-type *S. gordonii* DL1 whereas we observed a twofold increase (Table 1). This inconsistency with our findings may be due to the very brief period for which Blehert and coworkers grew *S. gordonii* biofilms (4 h versus 22 h in our system) and/or our use of more advanced and sensitive microscopic and analysis technologies. Regardless, a difference in structure (and in our case, other biofilm parameters, including biovolumes and thickness) could have been due to metabolic pathway changes caused by disruption of the *luxS* gene and not by AI-2-based communication. However, our findings demonstrate that the addition of csAI-2 at a concentration range of 0.1–10 nM to saliva restored the architecture of *S. gordonii* DL1 *luxS* to that of the wild-type, and this strongly supports the potential for a concentration-dependent AI-2-based communication system. Such a concentration dependency on AI-2-mediated communication has not been previously documented, to our knowledge, for *S. gordonii* DL1. As hypothesized by Kolenbrander *et al.* (2010), monitoring AI-2 concentration may allow *S. gordonii* DL1 cells to detect the presence of other species or possibly to differentiate between planktonic and biofilm lifestyles.

In dual-species biofilms, cells of *S. gordonii* DL1 numerically dominated over *S. oralis* 34 regardless of the availability of AI-2 (Fig. 5 and Table 2). However, the abundance of *S. gordonii* DL1 was influenced by whether it was able to produce AI-2 and not by whether AI-2 was produced by *S. oralis* 34 (Table 2). *S. oralis* 34 maintained a significant niche (exhibited as biovolume) as long as AI-2 was produced by itself or *S. gordonii* DL1. The most appreciable change in biovolume ratio was only observed for dual-species biofilms of *S. gordonii* DL1 *luxS*–*S. oralis* 34 *luxS* that were fed saliva that was not supplemented with csAI-2 or with saliva that was supplemented with 0.01 or 100 nM csAI-2 (Table 2). This was also linked to changes in the mosaic-like patterning of *S. oralis* cells in the dual-species biofilm (Fig. 5). Such an interaction/pattern is strikingly different from the pM–nM AI-2-dependent, inter-digitated mutualistic biofilms that form between *S. oralis* 34 and *A. oris* T14V (Palmer *et al.*, 2001; Rickard *et al.*, 2006, 2008b). This phenomenon may relate to juxtaposition (via coaggregation), the differences in amount of AI-2 produced by *S. oralis* 34 and *S. gordonii* DL1 (demonstrated in Fig. 1), and/or the amount of AI-2

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 (Dù & Kolenbrander, 2000), to coaggregate (Hojo *et al.*, 2009; Kolenbrander *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 & Kolenbrander, 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.

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