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Mutation of *luxS* Affects Biofilm Formation in *Streptococcus mutans*

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Quorum sensing is a bacterial mechanism for regulating gene expression in response to changes in population density. Many bacteria are capable of acyl-homoserine lactone-based or peptide-based intraspecies quorum sensing and *luxS*-dependent interspecies quorum sensing. While there is good evidence about the involvement of intraspecies quorum sensing in bacterial biofilm, little is known about the role of *luxS* in biofilm formation. In this study, we report for the first time that *luxS*-dependent quorum sensing is involved in biofilm formation of *Streptococcus mutans*. *S. mutans* is a major cariogenic bacterium in the multispecies bacterial biofilm commonly known as dental plaque. An ortholog of *luxS* for *S. mutans* was identified using the data available in the *S. mutans* genome project (http://www.genome.ou.edu/smutans.html). Using an assay developed for the detection of the LuxS-associated quorum sensing signal autoinducer 2 (AI-2), it was demonstrated that this ortholog was able to complement the *luxS* negative phenotype of *Escherichia coli* DH5α. It was also shown that AI-2 is indeed produced by *S. mutans*. AI-2 production is maximal during mid- to late-log growth in batch culture. Mutant strains devoid of the *luxS* gene were constructed and found to be defective in producing the AI-2 signal. There are also marked phenotypic differences between the wild type and the *luxS* mutants. Microscopic analysis of in vitro-grown biofilm structure revealed that the *luxS* mutant biofilms adopted a much more granular appearance, rather than the relatively smooth, confluent layer normally seen in the wild type. These results suggest that LuxS-dependent signal may play an important role in biofilm formation of *S. mutans*.

A bacterial biofilm is a community of bacteria (either single or multiple bacterial species) that adhere to a solid surface (8). In recent years, biofilms have received much attention due to their impact on industry and medicine. Biofilms are responsible for a plethora of problems ranging from biofouling of pipelines to facilitation of tissue damage in cystic fibrosis patients (1, 37, 42).

Studies have clearly shown that a bacterial biofilm is not a result of random accretions of bacterial cells; rather, it is the net result of a community of bacteria cooperating to form well-differentiated structures (7). The production of biofilm is dependent on the progression through several steps, from initial attachment to full maturation into a stable community (29). Given the tremendous metabolic and physiological changes that are required for the switch from planktonic to biofilm growth, it would seem reasonable that there exist some gene regulators responsible for facilitating this process. Indeed, various gene regulation systems, including quorum sensing systems, have been found to be involved in bacterial biofilm formation (9).

Quorum sensing is a mechanism for bacteria to change gene expression at very specific cell densities. To date, there are two types of recognized quorum sensing systems in bacteria. The first, known as intraspecies quorum systems, are species specific. In gram-negative bacteria, intraspecies quorum signals are composed of an acyl-homoserine lactone backbone with species-specific substitutions, while gram-positive bacteria use various peptides as their signals (11, 15, 28). Recently, a second quorum sensing system was characterized for *Vibrio harveyi*. This system has been referred to as the interspecies quorum system and may operate as a universal quorum system for many bacteria possessing the characteristic *luxS* gene (2, 34, 39). The *luxS* gene is highly conserved among many species of gram-negative and gram-positive bacteria and is thought to be responsible for synthesizing a universally recognized cell signal referred to as autoinducer-2 (AI-2) (39). The chemical structure of the actual signal is still under investigation; however, crystallographic studies of the AI-2 receptor in *V. harveyi* seem to suggest that AI-2 is a furanosyl borate diester formed from the metabolite 4,5-dihydroxy-2,3-pentadione (5, 33, 34). The ecological role of *luxS* in bacteria is still poorly characterized, but one logical possibility is that it functions to allow bacteria to optimize gene expression in response to the density of all *luxS*-containing species occupying the same niche.

One feature regarding quorum sensing that has been extensively studied is the link between quorum sensing and biofilm-related gene expression. There are several well-characterized examples for the involvement of intraspecies quorum sensing and biofilm formation. For example, *lasI* of *Pseudomonas aeruginosa* directs the synthesis of an acyl-homoserine lactone signal molecule used for *P. aeruginosa* intraspecies quorum signaling (9). Mutants in this gene were unable to produce biofilms that progressed beyond the very early stages of biofilm development (9). However, exogenous addition of the appropriate signal complemented the defect (9). A similar result was also obtained due to inactivation of the *cep* intraspecies quorum sensing system of *Burkholderia cepacia* (20). Furthermore, a transposon mutagenesis study of the oral pathogen *Streptococcus gordonii* had detected a severe biofilm deficiency due to disruption of the two-component system required for its in-

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Reference(s)
S. mutans 25175	WT, Em^s	ATCC
S. mutans GS-5	WT, Em^s	16
JM03	Δ*luxS* Em^r in 25175 background	This work
JM01	Δ*luxS* Em^r in GS-5 background	This work
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Δ*luxS*	17, 39
E. coli XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZDM15* Tn*10*(Tet^r)]	3

traspecies quorum sensing system (25). In *Staphylococcus aureus*, intraspecies quorum signaling has been implicated as a negative regulator of biofilm formation (41). LuxS-dependent AI-2 signals have also been detected in a variety of bacterial species and have been found to be involved in various cellular processes in a cell density-dependent manner (10, 13, 14, 21, 26, 36). However, to date, no connection between LuxS-dependent AI-2 signals and biofilm formation has been reported. Here we report the first case of mutations of *luxS* affecting biofilm formation of the oral pathogen *Streptococcus mutans*.

S. mutans is a major cariogenic bacterium that normally inhabits a complex, multispecies biofilm on the tooth surface (dental plaque) (40). The bacteria produce large amounts of exopolysaccharides, especially in the presence of sucrose, that enable them to efficiently adhere to the tooth. The bacteria also have the ability to produce large amounts of acids from fermentable sugars in the diet. Acid accumulation can eventually dissolve the hard, crystalline structure of the tooth, resulting in a carious lesion (32). Previous studies have established some sophisticated interactions among the oral streptococci as well as with other oral bacteria within the same dental plaque (22–24). For this reason, *S. mutans* and dental plaque comprise an ideal model system for studying the role of interspecies signaling and biofilm formation. In this study, we report that *S. mutans* indeed possesses a functional *luxS* gene that is capable of signaling to *V. harveyi*. Additionally, we show by gene deletion that *luxS* of *S. mutans* is involved in biofilm formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial strains used in this study and their characteristics are listed in Table 1. All *S. mutans* strains were grown in brain heart infusion (BHI) medium (Difco) or on BHI agar plates. *luxS* deletion mutants were grown using the same medium supplemented with 15 μg of erythromycin/ml. All *S. mutans* strains were grown anaerobically (80% N_2, 10% CO_2, 10% H_2) at 37°C. *Escherichia coli* cells were grown in Luria-Bertani medium with aeration at 37°C. *E. coli* cells carrying plasmids were grown in Luria-Bertani medium containing 100 μg of erythromycin/ml. *V. harveyi* BB170 (sensor 1^-, sensor 2^+) was kindly provided by B. Bassler (Princeton University) and grown in AB medium (37) overnight at 30°C.

Cloning and analyses of the *S. mutans luxS* gene. A 990-bp DNA fragment containing the *luxS* gene from *S. mutans* strain 25175 was PCR amplified from genomic DNA using the primers WTlux5 (5′-GATGCTGCACGCTCTGTC-3′) and WTlux3 (5′-GCAGTTAGGGTATCCATCC-3′). Primer sequences were designed using sequence data obtained from the *S. mutans* Genome Sequencing Project, University of Oklahoma (B. A. Roe, R. Y. Tian, H. G. Jia, Y. D. Qian, S. P. Lin, S. Li, S. Kenton, H. Lai, J. D. White, R. E. McLaughlin, M. McShan, D. Ajdic, and J. Ferretti [http://www.genome.ou.edu/smutans.html]). The resulting fragment was cloned into the TOPO TA cloning vector (Invitrogen) and sequenced.

RNA isolation and RT-PCR. *S. mutans* total RNA was isolated as follows. Twenty-five-milliliter cultures were grown overnight as previously described. Cells were centrifuged and resuspended in 1.5 ml of Tris-EDTA buffer and then lysed using a Mini-Bead Beater 8 according to the manufacturer's instructions. One hundred fifty microliters of lysate was then passed through a Qiashredder column (Qiagen), and 100 μl of the resulting lysate was used for total RNA isolation with the RNeasy mini kit (Qiagen). cDNA buffer was added to RNA samples to obtain a 1× solution, and 3 μl of RQ1 RNase-free DNase was added to the sample and incubated overnight at 37°C. Reverse transcription (RT) was performed according to the manufacturer's protocol using random hexamers.

AI-2 assay. The AI-2 luminescence reporter assay was performed essentially as described previously (38), with the following modifications. To obtain cell-free conditioned medium, *S. mutans* was grown overnight as described. Stationary-phase cells were resuspended in AB medium to an optical density at 600 nm (OD_{600}) of 0.4. Cells were then incubated at 37°C with aeration for 3 h. After the incubation period, cells were pelleted by centrifugation, and the resulting supernatant was filtered through a 0.22-mm-pore-size filter (Millipore). The cell-free conditioned medium was either used immediately or stored at −20°C. To determine luminescence, an overnight culture of *V. harveyi* BB170 was diluted 1:1,000 into fresh AB medium, and 180 μl of cells was added to each 1.5-ml microfuge tube. Conditioned medium (20 μl) was then added to the cells at a 10% (vol/vol) final concentration. Positive control samples were obtained by adding cell-free conditioned medium from an overnight culture of *V. harveyi* BB170 to a final concentration of 10%. In some cases, conditioned medium from a wild-type culture of *S. mutans* served as an additional positive control. To determine levels of background luminescence, a sample containing 200 μl of *V. harveyi* (dilution, 1:1,000) was also included. In some cases, *E. coli* DH5α served as a negative control. All samples were measured hourly until peak induction at the 3-h time point. Measurements were collected using a TD 20/20 luminometer and expressed as arbitrary luminescence units.

Construction and analyses of *S. mutans luxS* mutants. Two 1-kb fragments containing regions of DNA immediately upstream and downstream of the *luxS* translational start and stop codons, respectively, were amplified from a genomic DNA template using the primers 2XupF (5′-GCGGATCCTCAAGCTCTCAAGCGTTCGG-3′) and 2XupR (5′-CGAGATCTATAAGACGGACATAAGGGGC-3′) as well as 2XdownF (5′-GCCTCGAGCAGATGATCCTTTTGAGCGTC-3′) and 2XdownR (5′-CGTCTAGACGGATGCAAAGAGAACGAAG-3′). Primer sequences were designed using sequence data obtained from the *S. mutans* Genome Sequencing Project, University of Oklahoma (http://www.genome.ou.edu/smutans.html). The resulting fragments were cloned into the TOPO TA cloning vector (Invitrogen). To obtain the necessary fragments, four separate restriction digests were constructed: the upstream fragment was cut from the vector using *Bam*HI and *Bgl*II, the downstream fragment was cut from the vector using *Xho*I and *Xba*I, the erythromycin cassette was cut from the plasmid pJT10 (J. P. Tsai and W. Shi, unpublished data) using *Bgl*II and *Xho*I, and the pUC19 vector was cut with *Bam*HI and *Xba*I. The resulting fragments were all gel purified (Qiagen), precipitated, and added to one mass ligation reaction. The resulting construct was then checked by restriction analysis and PCR for the proper configuration of the knockout vector (pUCluxKO). Next, the plasmid was linearized with the unique cutting enzyme *Aat*II and transformed into *S. mutans*. Transformants were selected for resistance to 15-μg/ml erythromycin. Confirmation of DNA integration was performed by PCR using the primers IntluxF (5′-AAGAGTTTGGACCTAAAGGC-3′), IntluxR (5′-CCCACAGGACTCAATAGTTG-3′), UpluxF (5′-CTCGACGAATAGGATCAAAGC-3′), DownluxR (5′-GAGCCATCACACAGCAAAAAC-3′), ermA (5′-AGTGTGTTGATAGTGCAGTATC-3′), and ermM (5′-GAAGCTGTCAGTAGTATACC-3′). All mutants confirmed by PCR were further analyzed for their ability to produce AI-2 using the methods described above.

Growth of in vitro biofilm. Biofilms of *S. mutans* were grown as follows. Individual sterile culture dishes were filled with 2.5 ml of BHI broth supplemented with 1% (wt/vol) sucrose. Next, a sterile 18-mm-diameter glass microscope coverslip was added to each dish, and the culture dish was covered. Each sample was then inoculated with a defined volume of overnight culture. The dishes were incubated anaerobically at 37°C overnight.

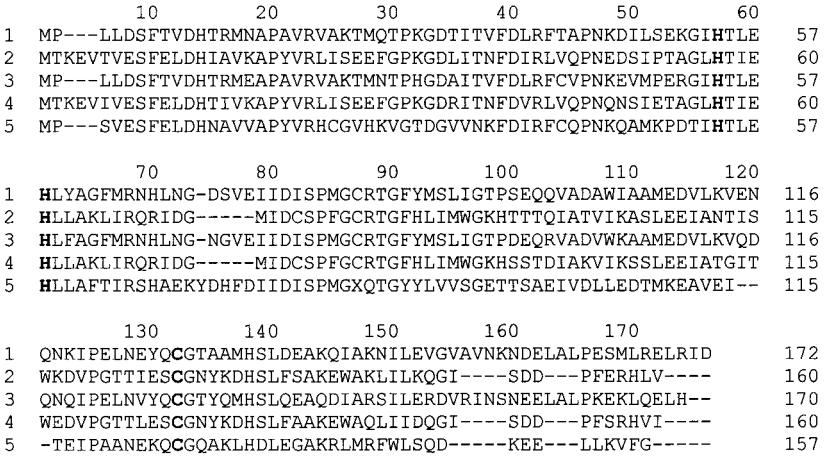

FIG. 1. Alignment of the *S. mutans* LuxS protein and several other representative LuxS proteins. Residues that coordinate a Zn^{2+} ion and comprise the catalytic center of LuxS (H, H, and C) are printed in bold. 1, *V. harveyi*; 2, *S. mutans*; 3, *E. coli*; 4, *S. pyogenes*; 5, *B. subtilis*.

Microscopic analyses of in vitro-grown biofilm. Glass coverslips containing attached biofilm were removed from overnight cultures and rinsed briefly with water. These could be directly viewed using phase-contrast and dark-field microscopy. For fluorescently labeled samples, the biofilms were first removed from the culture dish and placed into another dry culture dish. Next, 20 μl of anti-*S. mutans* mouse monoclonal antibody solution (SWLA1) was added to the attached biofilm in each culture dish and incubated at room temperature for 30 min (35). After the incubation period, 5 μl of fluorescein isothiocyanate-conjugated goat anti-mouse antibody was added to the biofilms and incubated at room temperature for an additional 10 min. Finally, the coverslip was briefly rinsed with water to remove excess antibodies and unattached cells, and the sample was immediately imaged using fluorescence microscopy.

Treatment of in vitro biofilm with sodium dodecyl sulfate (SDS) and antibiotics. Biofilms were grown overnight using previously described conditions. For treatment with SDS, coverslips were removed from overnight incubation and rinsed briefly. These were then placed into a fresh culture dish, and 2 ml of sterile, 1% (wt/vol) SDS solution was added to each dish. The biofilms were placed on a standard shaker set at 150 rpm for 1 h at room temperature. Next, supernatant samples were checked at magnification ×40 to confirm that cells were fully individualized and not connected in chains. These samples were measured for their OD_{600} values. For treatment with antibiotics, biofilms were again grown under standard conditions overnight. The following day, the spent medium was changed and replaced with BHI supplemented with 1% (wt/vol) sucrose solution. Ampicillin was added at a concentration of either 50 or 500 μg/ml. These biofilms were allowed to grow overnight under anaerobic conditions at 37°C. The following day, biofilms were removed from incubation and sonicated until the cells were fully dispersed. A 10^6-fold dilution of each sample was plated on nonselective BHI agar plates and incubated anaerobically overnight at 37°C.

RESULTS

Identification and isolation of the *S. mutans luxS* gene. To determine whether *S. mutans* may possess an interspecies quorum system, it was first necessary to identify a candidate ortholog of *luxS*, the enzyme required for AI-2 production. Using the *luxS* gene from *V. harveyi*, we performed a BLAST search of the University of Oklahoma *S. mutans* genome sequence database. A candidate open reading frame (ORF) was identified. The ORF appears to be an isolated gene (no other convincing ORFs nearby) and encodes a protein of 160 amino acids, which is similar in size to other reported LuxS proteins. It is homologous to the *V. harveyi* LuxS protein, with 38% amino acid identity and 57% similarity. Using this sequence data, primers were designed to amplify a region about 500 bp upstream of the translation start site and about 100 bp downstream of the translational stop site. This fragment was PCR amplified, cloned into the TOPO TA cloning vector, and sequenced to confirm the identity of the gene. After the fragment was confirmed to be the same ORF identified in the sequence database, a National Center for Biotechnology Information PSI BLAST search of the candidate gene was conducted. The results yielded strong homologies to numerous LuxS proteins from other gram-positive and gram-negative bacteria. The strongest homologies (identity/similarity) were to other gram-positive species, such as *Streptococcus pyogenes* (84%/92%), *Streptococcus pneumoniae* (83%/91%), *Lactococcus lactis* (64%/79%), and *Clostridium perfringens* (45%/64%), but there were also significant homologies to gram-negative species, such as *Neisseria meningitidis* (36%/58%), *E. coli* (37%/59%), and *Haemophilus influenzae* (37%/58%). A multiple alignment of the putative *S. mutans* LuxS protein and those from *V. harveyi*, *E. coli*, *S. pyogenes*, and *B. subtilis* demonstrated a high degree of similarity (Fig. 1). Of greatest interest was the location of several highly conserved amino acids (H, H, and C), which are reported to coordinate a Zn^{+2} ion and form the catalytic center of the protein (19). These amino acids and several others that are reportedly invariant are all conserved in the *S. mutans* LuxS protein (Fig. 1).

Complementation of an *E. coli luxS* mutant with *S. mutans luxS* gene. After confirming the identity of the candidate ORF, it was necessary to determine whether this gene also had the characteristic AI-2 synthase activity. This was accomplished by complementing an AI-2 production defect in *E. coli* DH5α. This strain of *E. coli* is known to have a frame-shift mutation in its *luxS* gene (39). Therefore, a plasmid containing the *luxS* gene and some upstream sequence was transformed into DH5α, and AI-2 activity was measured using the reporter assay described by Surette et al. (38). As shown in Fig. 2, our assay confirmed that *E. coli* DH5α was AI-2 negative and also demonstrated that the presence of the *luxS*-containing plasmid was sufficient to induce luminescence 89-fold over background levels.

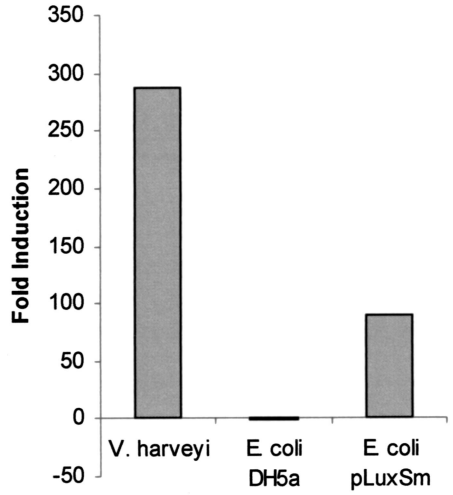

FIG. 2. *S. mutans luxS* complements a frameshift mutation in *E. coli* DH5α. *E. coli* DH5α was transformed with an *E. coli-S. mutans* shuttle vector containing an intact copy of the *S. mutans luxS* gene (pLuxSm). This strain was examined for AI-2 production using the luminescence-based AI-2 reporter assay. *V. harveyi* strain BB170 (sensor 1^-, sensor 2^+) served as a positive control, while *E. coli* DH5α served as a negative control. Luminescence is expressed as fold induction relative to the background values.

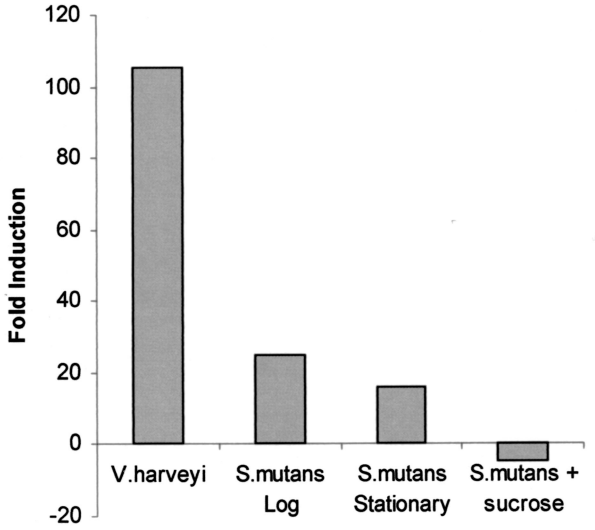

FIG. 3. AI-2 induction in the presence or absence of sugar. *S. mutans* was grown and assayed as described in Materials and Methods. Cells were grown overnight and resuspended to an OD_{600} of 0.4 in reporter assay (AB) medium and incubated with aeration for 3 h at 37°C. One sample was incubated in AB alone, while for the other, sucrose was added to a final concentration of 1%. The presence of sucrose in the medium caused a potent reduction in luminescence to below background values.

Secretion of an AI-2-like signal by *S. mutans*. To determine whether *S. mutans* had endogenous AI-2 activity, we again employed the AI-2 reporter assay. Initial screens using this assay failed to demonstrate any convincing AI-2 activity. These first experiments were all performed as had been described in previous reports (38, 39). Cells were grown to various OD_{600} values using standard growth medium, and the resulting conditioned medium was used as a source of AI-2 molecules. However, these experiments consistently yielded background levels of luminescence. With *E. coli*, it had been demonstrated that the presence of glucose in the growth medium caused a strong induction of AI-2 (38). Therefore, we decided to try adding glucose as well as sucrose (the preferred carbon source of *S. mutans*) to the growth medium in an effort to demonstrate AI-2 production. Since *S. mutans* is an acidogenic bacterium, pH readings were taken to ensure that there were no negative effects due to lowered pH. Interestingly, these experiments consistently yielded lower than background levels of luminescence (Fig. 3). In an effort to circumvent this problem, we decided to resuspend overnight cultures in the AI-2 assay medium at various OD_{600} ranges. This medium only allows for very limited growth of *S. mutans* but is able to keep the cells alive long enough for the production of AI-2 signal molecules. After assaying samples produced in this manner, it was immediately possible to measure induced luminescence. Samples assayed at mid- to late-log growth phase showed the strongest induction over background levels, while the induction tended to drop at stationary phase. Mid- to late-log samples typically produced about 25-fold induction of the reporter strain (*V. harveyi)* luciferase operon expression over background levels (Fig. 3). As a further confirmation, *luxS* was shown to be expressed in *S. mutans* via RT-PCR (data not shown).

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

General phenotypic characterization of *S. mutans luxS* mutants. After confirmation of the deletion of *luxS* and subsequent loss of AI-2 activity, the next step was to quantify any resulting physiological changes in numerous *luxS* mutant isolates. When the mutant colonies were plated, there were no obvious phenotypic differences in colony morphology. In batch culture there was no obvious difference in growth rate, nutrient requirements, or acid production (data not shown).

Altered biofilm structure of the *S. mutans luxS* mutants. Our next investigation of mutant phenotypes was to determine if there were any alterations of biofilm structure as a result of loss of AI-2 production. Both wild-type and mutant cells were able to form biofilms when grown on a solid surface overnight. Upon visual inspection, there was a noticeable difference in biofilm structure from the that of the wild type. Without the aid of magnification, wild-type biofilm generally has a very confluent appearance with no major discernible features. In contrast, the *luxS* mutant biofilm had a very rough texture. Under magnifications of ×20 and ×40, this difference is even

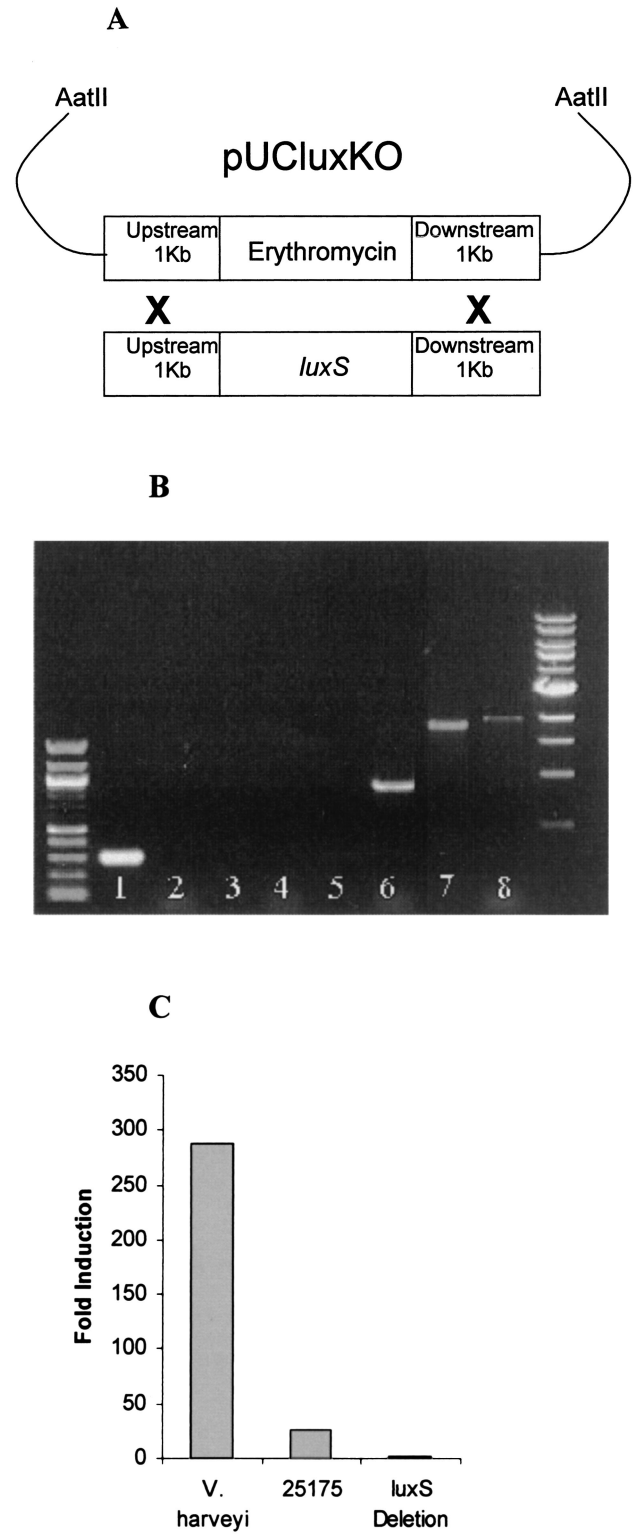

FIG. 4. *luxS* knockout in *S. mutans*. (A) Illustration of the knockout procedure. Plasmids containing cloned fragments of *S. mutans* DNA as well as the erythromycin cassette were cut using the indicated restriction sites and then ligated into a linearized pUC19 backbone. The resulting construct was linearized with the unique *Aat*II site and transformed into *S. mutans* for double crossover. (B) Confirmation of crossover event. In lanes 1 to 4, wild-type DNA was amplified with primers: internal to *luxS*, the erythromycin cassette, upstream external *luxS* plus erythromycin, or downstream external *luxS* plus erythromycin. In lanes 5 to 8, *luxS* mutant DNA was amplified using the same primer combinations. External *luxS* primers bind to sites that were not subject to crossover. (C) AI-2 production was assayed to confirm that activity was lost in the mutant.

more apparent (Fig. 5A). Wild-type biofilms are very uniform, with complete coverage of the attached surface. They also tend to have relatively small aggregates spread fairly evenly throughout the biofilm matrix. *luxS* mutant biofilms are quite different. Their organization seems much more heterogeneous. There were noticeable large gaps in the biofilm matrix, and the cell aggregates appeared much larger. Using fluorescence imaging, there is a clear indication that the sizes of mutant aggregates tended to be much larger than those of the wild type (data not shown).

Since biofilms are known to be very resistant to detergents and biocides, we were also interested to determine if this property was influenced by the *luxS* mutation. Overnight biofilms of the wild type and the *luxS* mutant were incubated in a 1% solution of SDS and shaken at 150 rpm for 1 h. These supernatants were then checked by microscopy to ensure that cells were not clumped, and subsequently, the OD_{600} was measured. It was found that the wild-type supernatants had an OD_{600} that averaged about sixfold higher than that of the mutant (Fig. 5B), which suggested that *luxS* mutant biofilms were more resistant to detergent treatment. We also treated biofilms of the wild type and the *luxS* mutant with the antibiotic ampicillin at a concentration of 50 or 500 μg/ml for 16 h. We found that very few (<1%), if any, wild-type cells survived treatment with 50 μg of ampicillin/ml and that none survived the treatment with 500 μg/ml. In contrast, numerous cells (>10%) within the *luxS* mutant biofilms survived the treatment with 50 μg of ampicillin/ml and even with 500 μg of ampicillin/ml.

DISCUSSION

In this paper we report the identification of the *luxS* gene for *S. mutans*. This gene is recognized as the enzyme primarily responsible for the production of autoinducer-2 (AI-2) interspecies quorum signals for *V. harveyi*. We demonstrated that the *S. mutans* LuxS protein exhibited high degrees of similarity to other LuxS proteins in the National Center for Biotechnology Information database and was able to complement a LuxS defect in *E. coli*. Furthermore, conditioned medium from *S. mutans* was capable of inducing the luciferase operon expression in *V. harveyi*, and deletion of this gene abolished this ability. Finally, loss of AI-2 activity was associated with prominent changes in biofilm structure.

Within the past couple of years, there has been a plethora of data describing various physiological functions from both gram-positive and gram-negative species which are subject to regulation by *luxS* (6, 10, 12, 26, 36). Much of the reported data have supported the hypothesis that *luxS* is somehow involved in regulating virulence factor expression. Furthermore, there has not been a reported scenario in which mutating this gene has led to severe impairment of growth, which suggests that the production of AI-2 is not a requirement for basic metabolic processes. Our present findings are consistent with these same

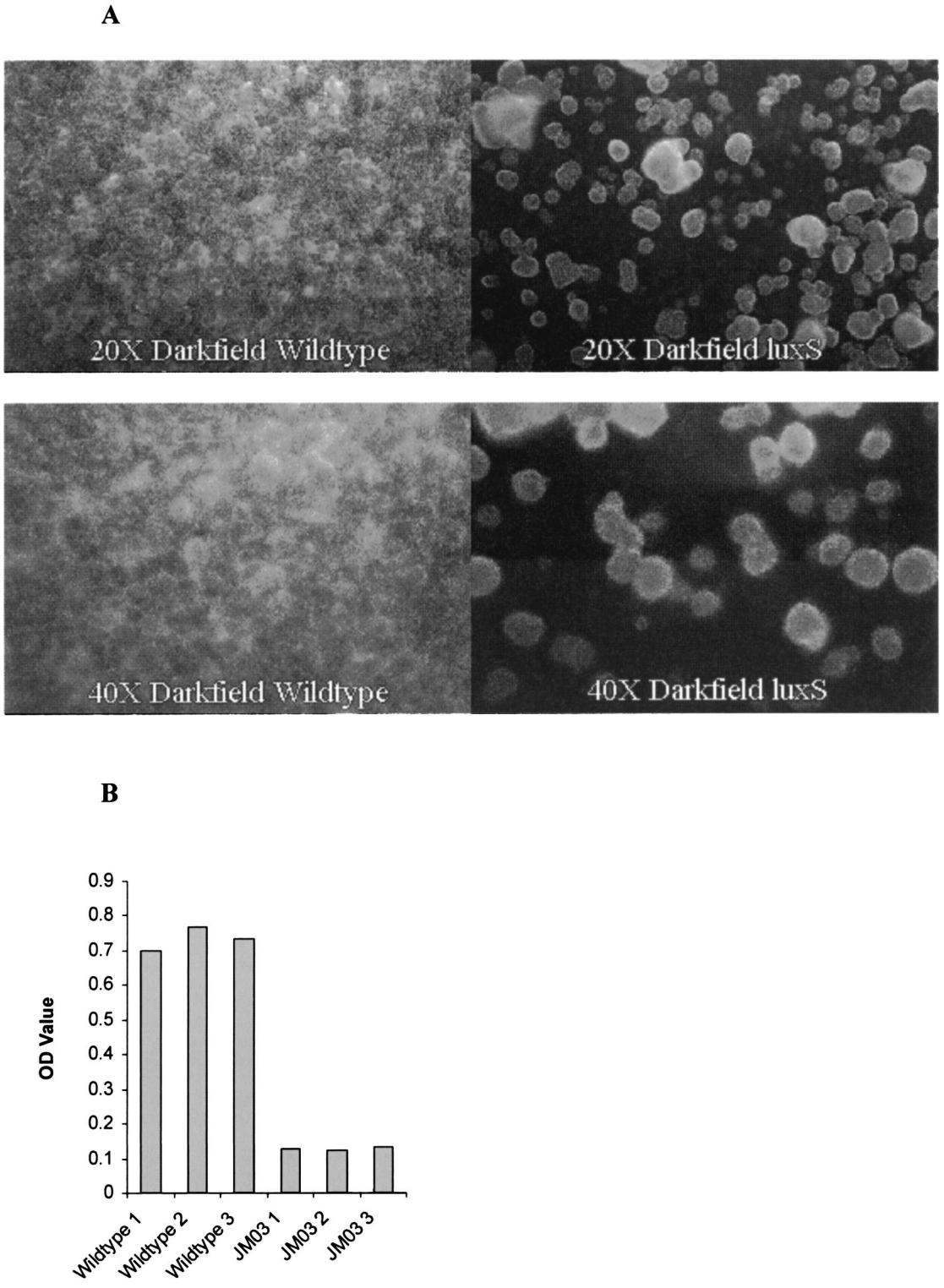

FIG. 5. Mutation of *luxS* causes an alteration in *S. mutans* biofilm. (A) Two images of in vitro biofilms representing both the wild type and the mutant using dark-field microscopy at magnifications ×20 and ×40. Cells were grown on glass coverslips as described in Materials and Methods. Similar results were obtained for more than 100 independent wild-type and *luxS* mutant biofilms examined. (B) Biofilms were tested for their ability to resist detergent treatment with SDS. Three samples of wild-type and mutant biofilms grown on glass coverslips were shaken at 150 rpm for 1 h. The OD_{600}s of the resulting supernatants were compared.

trends. After deletion of the *luxS* gene, there were no noticeable changes in growth patterns or basic nutrient requirements. However, *S. mutans* is a predominantly biofilm-dwelling organism and as such depends on its biofilm production for virulence in the oral cavity. The involvement of *luxS* in the proper development of *S. mutans* biofilms may also yield some insight into the role of interspecies communication in multispecies biofilm formation. *S. mutans* normally grows among hundreds of other competing species of oral bacteria and must therefore employ strategies to survey and respond to other species that compete for control of available ecological niches in the mouth. One possible strategy to accomplish this goal would be the use of *luxS*-based interspecies signaling. As an early colonizer of the tooth surface, AI-2 signal molecules may be an important factor in regulating biofilm-related gene expression to help modulate energy utilization for growth in an extremely competitive environment.

It is interesting that *P. aeruginosa* mutants defective in intraspecies quorum sensing produced weaker biofilms that were more sensitive to detergents (9), while *S. mutans* mutants defective in interspecies cell signaling generated stronger biofilms that were more resistant to detergents (Fig. 5B). While this difference could simply be attributed to different regulatory mechanisms between species, it is also possible that the defect in intraspecies quorum sensing renders the bacterium unable to detect its fellow species nearby, thus preventing biofilm maturation. In contrast, the defect in interspecies signaling could create an environment in which the mutant perceives there to be fewer other competing bacterial species, and thus, it initiates its biofilm production at an increased capacity.

Through a search of the *S. mutans* genome database, we were able to find a candidate *luxS* ortholog. Despite having a gene whose encoded protein aligned exceedingly well with other known LuxS proteins, initial attempts to demonstrate the production of AI-2 molecules all seemed to suggest that they were not produced. Indeed, there are reports of other *luxS*-containing bacteria that have not been shown to induce the AI-2 reporter assay (14, 39). It was especially perplexing to find that the presence of glucose and sucrose in the growth medium caused the AI-2 reporter assay to yield values below background levels, while glucose was known to cause a potent induction of AI-2 activity with *E. coli*. Part of the answer came from the medium itself. It seems that detectable AI-2 activity drops in rich medium and is potently inhibited in the presence of glucose and to an even greater extent with sucrose. This phenomenon may explain why a recent screen for AI-2 activity in various oral pathogens failed to demonstrate AI-2 activity for *S. mutans* strain 25175 (14). There also exists the possibility that AI-2 is still produced in rich medium and/or in the presence of sugar but the reporter strain is inhibited by some factor(s) secreted by *S. mutans* when grown under these conditions. Indeed, *S. mutans* is known to produce a battery of various inhibitory molecules, such as lantibiotics and nonlantibiotic bacteriocins (4, 30, 31). In addition, it is also known that in the presence of sugars, *S. mutans* has a distinct growth advantage over other competing oral bacteria. Therefore, it is possible that sugar stimulates *S. mutans* to produce some inhibitor that affected either luciferase production in the AI-2 assay or perhaps even an inhibitor that acted at the level of the AI-2 signaling process. Even though an AI-2-related inhibitory signal for *S. mutans* is currently unknown, its production could give additional explanations for *S. mutans*' ability to gain a growth advantage over competitors within its multispecies biofilm.

A recent report by Wen and Burne concluded that *luxS* may not be involved in biofilm formation (42). In contrast, we report here that the *luxS* mutant has several altered biofilm phenotypes: increased size of cell aggregates, altered biofilm structure, and an increased biofilm resistance to detergents and antibiotics. This apparent inconsistency is most likely due to different methods of biofilm growth and evaluation. In the biofilm assay employed by Wen et al., *S. mutans* biofilm was established in a microtiter plate and stained with crystal violet dye to visualize the adherent biofilm. This assay is very useful for selecting mutants defective in biofilm formation but is not likely to identify mutants with an altered mature biofilm structure, such as *luxS*. Our data show that the *luxS* mutant of *S. mutans* is still able to form biofilms on solid surfaces, consistent in this regard with the findings of Wen et al. However, our detailed microscopic analyses indicate a clear difference in mature biofilm structure between the wild type and *luxS* mutants (Fig. 5). Furthermore, components in the growth medium used for *S. mutans* biofilm formation can have a profound impact upon the structure of the biofilm. Sucrose, in particular, seems to be very critical in this regard. Since there is no proven standard for the development of the most medically relevant *S. mutans* biofilm, a diverse array of procedures has been used. For this reason, we also grew our biofilms using an alternate protocol employing biofilm growth medium devoid of sucrose and found that both the wild type and the *luxS* mutant biofilms were structurally distinct from those formed in the presence of sucrose. In this case, there was no noticeable difference between the wild-type and *luxS* mutant biofilms, which is also in agreement with the findings of Wen et al. However, when sucrose was added to biofilm growth medium, there was a profound change in the observed biofilm structure. In addition, the structural differences between wild-type and *luxS* mutant biofilms became very similar to those observed in our previous assays (data not shown).

Our data suggest that *luxS* has a regulatory role for one or more genes related to biofilm formation. Preliminary studies in the laboratory of S. D. Goodman indicate that there is increased glucosyltransferase (GtfB) activity in the *luxS* mutants (unpublished data). For *S. mutans*, sucrose-derived exopolysaccharides consist of insoluble glucans, which are primarily synthesized by the activity of the GtfB enzyme. Glucans make the cells more generally adherent in a nonspecific manner but are also known to interact with glucan binding proteins for specific interactions (18, 27). Therefore, the observed phenotypes can be partially due to a greater production of glucan, an increase in specific receptors for glucan and/or other cell wall components, or possibly a combination of both factors. These questions are currently being addressed using a proteomics approach.

In summary, *S. mutans* exists in a highly competitive multispecies biofilm in the oral cavity. For this reason, we were interested in whether *S. mutans* could possibly participate in some form of interspecies communication and what effect, if any, this would have on gene expression. To this end, we have identified a *luxS* ortholog in *S. mutans* and demonstrated a

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

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