

# Transcriptome analysis of LuxS-deficient *Streptococcus mutans* grown in biofilms

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## SUMMARY

We previously reported that LuxS in *Streptococcus mutans* is involved in stress tolerance and biofilm formation. In this study, flowcells and confocal laser scanning microscopy were used to further examine the effects of LuxS-deficiency on biofilm formation. Similar to the wild-type strain (UA159), a strain deficient in LuxS (TW26D) bound efficiently to the flowcells and formed microcolonies 4 h after inoculation. Unlike UA159, which accumulated and formed compact, evenly distributed biofilms after 28 h, TW26D showed only loose, sporadic, thin biofilms. DNA microarray analysis revealed alterations in transcription of more than 60 genes in TW26D biofilms by at least 1.5-fold ( $P < 0.001$ ). Among the upregulated genes were those for sugar-specific enzymes II of the phosphotransferase (PTS) system and the *atp* operon, which codes for the proton-pumping F-ATPase. Of the downregulated genes, several encode proteins with putative functions in DNA repair. Mutation of selected genes caused severe defects in the ability of the mutants to tolerate low pH and oxidative stress. These results provide additional proof that LuxS-deficiency causes global alterations in the expression of genes central to biofilm formation and virulence of *S. mutans*,

including those involved in energy metabolism, DNA repair and stress tolerance.

## INTRODUCTION

*Streptococcus mutans*, the primary etiological agent of human dental caries, exists almost exclusively in biofilms on the tooth surface in a high-density, high-diversity ecosystem known as dental plaque (Burne, 1998). Estimates indicate that nearly 750 different bacterial species can reside in dental plaque (Jenkinson & Lamont, 2005). Although relatively stable once established, the composition and spatial organization of these multi-species communities can be profoundly influenced by the source and availability of nutrients and pH (Burne, 1998; Quivey *et al.*, 2000). Intra- and inter-species communication is also believed to play a crucial role in development of plaque, and ultimately in development of diseases, such as dental caries and periodontitis (Kolenbrander *et al.*, 2002; Jenkinson & Lamont, 2005; Kuramitsu *et al.*, 2007).

One mode of microbial cell–cell communication, commonly referred to as ‘quorum sensing’, involves production, secretion and responses to small signal molecules known as autoinducers (AI). Quorum

sensing is widely used by both gram-positive and gram-negative bacteria and is believed to provide quorum-sensing populations with advantages in accessing complex nutrients, coping with fluctuating environmental conditions and enhancing defenses against other microorganisms or eukaryotic host defense mechanisms (Waters & Bassler, 2005; Ng & Bassler, 2009). Recently, a large number of eubacteria have been found to produce AI-2, which are furanones produced as by-products of the catalytic reaction of the LuxS enzyme. The AI-2 molecules have been shown to serve as signals that confer information about cell numbers, metabolic activity and other properties of cells in a population (Surette & Bassler, 1998; Chen *et al.*, 2002; Ohtani *et al.*, 2002; McNab *et al.*, 2003; Wen & Burne, 2004; Merritt *et al.*, 2005; Vendeville *et al.*, 2005; Rickard *et al.*, 2006). Data continue to accumulate that implicate LuxS (AI-2)-mediated signaling in the global regulation of physiological functions and virulence (reviewed by Henke & Bassler, 2004).

Evidence is also accumulating that inter-species LuxS-mediated signaling plays an important role in the development and spatial organization of oral biofilms, and so can impact the pathogenic potential of dental plaque (Fong *et al.*, 2001; McNab *et al.*, 2003; Yoshida *et al.*, 2005; Rickard *et al.*, 2006; Z.T. Wen and R.A. Burne, unpublished data). LuxS appears to be essential for the development of *Porphyromonas gingivalis*–*Streptococcus gordonii* mixed-species biofilms (McNab *et al.*, 2003). Formation of mutualistic biofilms of *Actinomyces naeslundii* and *Streptococcus oralis* was also shown to be dependent on AI-2 of *S. oralis* (Rickard *et al.*, 2006). These effects on biofilm development appear to be related to changes in gene expression stimulated by AI-2. For example, AI-2 produced by *Aggregatibacter actinomycetemcomitans* is able to complement a *P. gingivalis luxS* mutant to restore normal expression of the *uvrB* and *hasF* genes in the latter organism (Fong *et al.*, 2001).

*S. mutans* possesses a functional homologue of LuxS. Recent studies revealed that LuxS in *S. mutans* affects acid- and oxidative-stress tolerance, bacteriocin production and biofilm formation (Merritt *et al.*, 2003, 2005; Wen & Burne, 2004; Yoshida *et al.*, 2005), traits that are thought to be crucial to the establishment, persistence and cariogenicity of this human pathogen. Recent studies using proteomics and DNA microarray analysis have provided evidence

that LuxS-deficiency in *S. mutans* caused global alterations in the expression of genes (Sztajer *et al.*, 2008; Z.T. Wen and R.A. Burne, unpublished data). In this study, flowcells and confocal laser scanning microscopy (CLSM) were used to further characterize the impact of LuxS-deficiency on the development and architecture of biofilms by *S. mutans*. DNA microarrays were also used to identify the genes involved in LuxS-regulated biofilm formation.

## MATERIALS AND METHODS

### Bacterial strains, growth conditions and construction of isogenic mutants

The *S. mutans* UA159 and its derivatives were maintained on brain–heart infusion (BHI; Difco Laboratories, Detroit, MI). When needed, erythromycin (10 µg ml<sup>-1</sup>) and kanamycin (1 mg ml<sup>-1</sup>) were added to the growth medium. All solid media were prepared similarly, but agar (Difco Laboratories) was added at a concentration of 1.5% (weight/volume). For biofilm formation, *S. mutans* strains were grown in a semi-defined biofilm medium (Loo *et al.*, 2000) with glucose (20 mM, final concentration) (BMG), sucrose (10 mM) (BMS), or glucose (18 mM) plus sucrose (2 mM) (BMGS). All strains deficient for the selected genes were generated using polymerase chain reaction (PCR)–Ligation–Mutation strategy as described previously (Lau *et al.*, 2002; Wen & Burne, 2004), and the deficiency was further verified by PCR and DNA sequence analysis. Unless otherwise stated specifically, all cultures were grown aerobically at 37°C in a 5% CO<sub>2</sub> atmosphere under static conditions.

### Biofilm formation, confocal laser scanning microscopy and image analysis

Biofilm assays on 96-well plates (Corning Inc., Corning, NY) were carried out by following the procedures described previously (Wen & Burne, 2002). Briefly, *S. mutans* strains were grown on BMG and BMS at 37°C in a 5% CO<sub>2</sub> atmosphere under static conditions. After 24 h, biofilms developed on the polystyrene surface were stained using 0.1% crystal violet and measured quantitatively using a spectrophotometer. For confocal analysis, *S. mutans* strains were grown in BMGS in flowcells (4 × 40 × 1 mm, width × length × depth) (Stovall Life Science, Inc.,

Greensboro, NC) maintained in a 37°C warm room. Biofilm development on the glass coverslips was monitored at 4 and 28 h after the initial inoculation by CLSM analysis (Wen *et al.*, 2006). Scans were taken in eight bits at a resolution of 512 × 512 pixels, and for each sample at least five regions were randomly selected for optical dissections. Post acquisition, image stacks were further analyzed by using MCID ELITE 6.0 (Imaging Research, Inc., ON, Canada) to generate xyz, xz and yz reconstructions of biofilms. Quantitative analysis was carried out using image processing software COMSTAT (Heydorn *et al.*, 2000; Klein *et al.*, 2009). Biovolume and area occupied by bacteria in each layer were used to determine the differences between the wild-type and the mutant biofilms. The biovolume is defined as the volume ( $\mu\text{m}^3$ ) of the biomass per  $\mu\text{m}^2$  of substratum area.

### RNA extraction

RNA extractions were carried out by using a hot phenol method, as previously described (Wen & Burne, 2004; Wen *et al.*, 2006). For RNA of planktonic cells, both *S. mutans* wild-type UA159 and the LuxS-deficient mutant, TW26D, a LuxS-deficient mutant that has the whole coding sequence deleted and replaced by an erythromycin-resistance marker (Wen & Burne, 2002), were grown in 50 ml BHI and harvested at an optical density at 600 nm ( $\text{OD}_{600}$ )  $\cong$  0.45 by centrifugation at 3800 *g* at 4°C for 5 min. The pellets were then quickly resuspended and treated with Qiagen RNeasy Protect (Qiagen, Inc., Valencia, CA) using the procedures recommended by the supplier. The treated cells were collected by centrifugation and stored at -80°C until RNA was extracted. For RNA from sessile populations, BMGS was used to grow bacterial cells on sterile glass slides that were deposited in 50-ml Falcon tubes. Adherent populations on the slides were transferred daily to fresh BMGS. After 3 days the biofilms were scratched off the slides with a sterile spatula and immediately treated with the RNeasy Protect reagent as described above. The treated bacterial cells were homogenized in the presence of glass beads using a BeadBeater (Biospect Products, Bartlesville, OK), and RNA was extracted with hot phenol (Wen & Burne, 2004; Wen *et al.*, 2006), precipitated with isopropanol at -80°C overnight, and washed with 75% ethanol. To remove residual DNA, RNA samples were treated with

RNase-free DNase I (Ambion, Inc., Austin, TX) and total RNA was retrieved with the Qiagen RNeasy purification kit. For reference RNA, 3 l of *S. mutans* UA159 were grown in BHI to  $\text{OD}_{600} \cong$  0.45, and total RNA was extracted using the same procedures described above. The purified RNA was then divided into aliquots and stored at -80°C until use.

### Complementary DNA synthesis, labeling and hybridization

Microarray slides were obtained from the Pathogen Functional Genomics Resource Center (PFGRC) at the J. Craig Venter Institute (<http://pfgrc.jcvi.org/>). The slides have 70-mer oligonucleotides representing 1960 open reading frames of *S. mutans* UA159 printed on the surface of the microarray in four different grids. Array analysis was carried out using the procedures recommended by the PFGRC with minor modifications (Abranches *et al.*, 2006; Wen *et al.*, 2006). Briefly, for complementary DNA (cDNA) synthesis, RNA (10  $\mu\text{g}$ ) was denatured in the presence of 3  $\mu\text{g}$  random hexamers at 70°C for 10 min. After briefly chilling on ice, 9  $\mu\text{l}$  5 $\times$  first-strand buffer, 3  $\mu\text{l}$  0.1 M dithiothreitol, 2  $\mu\text{l}$  12.5 mM dNTP/aa-UTP with 1.5 : 1 ratio of aa-dUTP to dTTP, and 3  $\mu\text{l}$  Superscript III reverse transcriptase were added. Reverse transcription reactions were carried out at 42°C overnight. Following completion of cDNA synthesis, 15  $\mu\text{l}$  of 1 M NaOH was added to hydrolyze the RNA. The aminoallyl-labeled cDNA samples were then purified by using the QIAquick PCR Purification kit (Qiagen, Inc.) and the samples were dried in a speed vacuum. The samples were then resuspended in 5  $\mu\text{l}$  0.1 M  $\text{Na}_2\text{CO}_3$ , pH 9.3, mixed with 5  $\mu\text{l}$  of appropriately resuspended Cy dye (Amersham Biosciences, Piscataway, NJ), and Cy dye coupling was carried out in the dark for 2 h. For all conditions, the reference samples were coupled with Cy5 and both wild-type and the LuxS-deficient mutant samples were coupled with Cy3. After 2 h, the Cy dye-coupled samples were passed through QIAquick PCR purification columns to remove uncoupled dyes and eluted in 100  $\mu\text{l}$  elution buffer. The Cy3-labeled samples were mixed with a Cy5-labeled reference and the mixtures were dried in a speed vacuum. For hybridization, the Cy dye-coupled samples were resuspended in 55  $\mu\text{l}$  hybridization buffer, and hybridization of microarray slides was carried out at 42°C overnight using

a MAUI Hybridization System following the manufacturer's instructions (BioMicro Systems, Salt Lake City, UT). Following the completion of hybridization, slides were washed and scanned at 530 nm and 650 nm by using an Axon GenePix 4000B scanner (Axon Instruments, Union City, CA). At least four separate array slides were performed on RNA isolated from four separate experiments.

### Microarray data analysis

Array data were normalized with the TIGR MICROARRAY DATA ANALYSIS SYSTEM (<http://www.tigr.org/software>) and BRB ARRAY TOOLS 3.01 (developed by Dr Richard Simon and Amy Peng Lam, National Cancer Institute, Bethesda, MD, <http://linus.nci.nih.gov/BRB-ArrayTools.html>) as described elsewhere (Abranches *et al.*, 2006; Wen *et al.*, 2006). Genes that were differentially expressed at statistical significance levels of  $P < 0.001$  and  $P < 0.01$  were then identified using the GROUP COMPARISON program, which was designed to identify the genes that are differentially expressed among groups of specimens collected from different types of tissues or under different experimental conditions, and cutoff was made: genes with a minimum ratio of 1.5 of the transcription level in the LuxS-deficient mutant relative to the wild-type strain were considered upregulated, and those with a ratio of 1.5 or above of the transcription level in the wild-type relative to the LuxS-deficient mutant were considered downregulated.

### Real-time PCR

Gene-specific primers for real-time PCR were designed using BEACON DESIGNER 3.0 (PREMIER Bio-soft International, Palo Alto, CA) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). Real-time PCR were carried out with a Bio-Rad iCycler using procedures detailed elsewhere (Ahn *et al.*, 2006; Wen *et al.*, 2006), and the same RNA preparations that were isolated for DNA array analysis were used for these experiments.

### Acid and hydrogen peroxide killing assays

The abilities of bacterial cells to withstand acid or hydrogen peroxide challenges were determined by using procedures described elsewhere (Wen &

Burne, 2004; Wen *et al.*, 2006). Both planktonic cultures and 3-day biofilms were used for these experiments. For planktonic cultures, bacterial cells were grown on BHI until mid-exponential phase ( $OD_{600} \cong 0.45$ ). Bacterial biofilms were grown on glass slides as described above, and brief sonication (30 s, twice at 10% energy level, with 2 min on ice between treatments) was used to de-chain and disperse biofilms using a Branson Digital Sonifier (model S-250D, Branson Ultrasonics Co, Danbury, CT). For acid killing, bacterial cells were washed once with 0.1 M glycine buffer, pH 7.0, and then incubated in 0.1 M glycine buffer, pH 2.8 for 45 min. For hydrogen peroxide challenge, bacterial cells were prepared similarly, and then incubated in 0.1 M glycine buffer, pH 7.0 containing 0.2% (weight/volume, or 58.8 mM, final concentration) hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) for 90 min. Survival rate was determined by plating serial dilutions in triplicate.

## RESULTS

### LuxS-deficiency impairs biofilm development and alters biofilm architecture

Previously, we examined the impact of LuxS-deficiency on biofilm formation when *S. mutans* strains were grown under static conditions. The results showed that the LuxS-deficient mutants were less efficient at forming biofilms on either polystyrene or hydroxylapatite surfaces than the wild-type strain, and the defects appeared to be related to lack of biofilm development rather than to an impaired ability to adhere to the substratum (Wen & Burne, 2002, 2004). In this study, we used flowcells to support biofilm growth and analyzed the capacity of the LuxS-deficient strain to form biofilms on glass coverslips with constant medium flow as a shear force. Flowcells have proven to be an effective tool to study cell accumulation and biofilm structures under conditions that more closely mimic conditions on tooth surfaces, where saliva flow and mechanical shear force could have a significant impact on bacterial adherence, cell-cell interactions and biofilm maturation (Christensen *et al.*, 1999; Eglund *et al.*, 2004; Rickard *et al.*, 2006; Wen *et al.*, 2006). Moreover, flow across biofilms should affect the accumulation of autoinducer signal. To allow visualization under CLSM, green fluorescence protein (GFP) was used to tag the

**Table 1** Bacterial strains, plasmids and primers used in this study

Strains/plasmid	Relevant characteristics		References
<i>S. mutans</i> UA159	Wild-type		(Ajdic <i>et al.</i> , 2002)
<i>S. mutans</i> TW26D	$\Delta luxS$ , Kan <sup>r</sup>		This study
<i>S. mutans</i> TW297.1	$\Delta smu.44$ , Kan <sup>r</sup>		This study
<i>S. mutans</i> TW238	$\Delta smu.423$ , Kan <sup>r</sup>		This study
<i>S. mutans</i> TW294.1	$\Delta smu.46$ , Kan <sup>r</sup>		This study
<i>S. mutans</i> TW310	$\Delta smu.299$ , Kan <sup>r</sup>		This study
pDL278	Shuttle vector, Sp <sup>r</sup>		(LeBanc & Lee, 1991)
pDL278: <i>luxS</i>	Shuttle carrying <i>luxS</i> , Sp <sup>r</sup>		This study
<i>S. mutans</i> TW26DC	TW26D/pDL278: <i>luxS</i> , Erm <sup>r</sup> and Sp <sup>r</sup>		This study
pAYBG754S	Shuttle vector with a <i>gfp</i> reporter, Kan <sup>r</sup>		(Yoshida & Kuramitsu, 2002)
Primers for mutagenesis	Sequence (5'–3')	Sequence (5'–3')	Flanking region amplified
luxS 5F	55-aaatctgtcattgctgatggac	53-ataccggagctcctactgagtagtc	5' of <i>luxS</i> for mutagenesis
luxS 3F	35-agtccaagcttcaacagtaacttc	33-tgctagacggtgcatagcttgagc	3' of <i>luxS</i> for mutagenesis
smu.44 5F	55-tgttgaaatccaccatataattacgtatc	53-tgcacttctgaattctagtagc	5' of <i>smu.44</i> for mutagenesis
smu.44 3F	35-ttcataacaggagaattcttagatc	33-tcctctcactaaagtgtgtac	3' of <i>smu.44</i> for mutagenesis
smu.46 5F	55-agatagcttgttaacaaacatc	53-agttgccagaattcaatagcatc	5' of <i>smu.46</i> for mutagenesis
smu.46 3F	35-tagaaccaccagaattctattaatcaac	33-acgaccaccagaaccaacaac	3' of <i>smu.46</i> for mutagenesis
smu.423 5F	55-tcactattactgatgtccaaatg	53-tgttcaaatgcatgctgattcat	5' of <i>smu.423</i> for mutagenesis
smu.423 3F	35-tgtagggtgcatgcactttttgtg	33-ttctcactcattttgatg	3' of <i>smu.423</i> for mutagenesis
smu.299 5F	55-atgattcatctcgatcaagcc	53-tacatgtgaattcgttcagcatc	5' of <i>smu.299</i> for mutagenesis
smu.299 3F	35-tagtaaatagctggaattcaggtgc	33-tgaaaccagatcagctgttgac	3' of <i>smu.299</i> for mutagenesis
Primers for qPCR	Forward (5'–3')	Reverse (5'–3')	Application
16S-1 rRNA	cacaccgcccgtcacacc	cagccgcacctccgatac	16S rRNA fragment, 160 bp
smu.1128	aagggtgggttcggacttgg	gcaggcgagcttcaaacattac	<i>ciaH</i> fragment, 115 bp
smu.1528	cggatgctgttgccttactg	ggctgataaccaacggctgatg	<i>atpD</i> fragment, 164 bp
smu.1877	ggctcctcacttggcata	ctgccattgtaagtcatccc	mannose-PST, EIID <sup>Man</sup> , 79 bp
smu.1961	ggaagccctttgacaacagc	gcattcatcaatctggtctat	<i>levD</i> fragment, 94 bp
smu.299	acgatggagctaattggctatgc	agcgtgaagcggcaaaacttg	smu.299 fragment, 140 bp
smu.423	gggtggtgatgattagatgtgc	ccagaccagcctcctaaagc	smu.423 fragment, 137 bp
smu.43	agcaaccagttatcttagg	gtataatagaaatcccgaatagg	smu.43 fragment, 200 bp
smu.44	tggcagactggaaatataagc	ggcaaacctcactcattgacaac	smu.44 fragment, 148 bp
smu.45	gttacaggataccacggctgaag	accttgagttgccatagttcgtag	Smu.45 fragment, 96 bp
smu.46	cacatagtgatgatgtccaaattg	gcatcagactctttaaacttg	smu.46 fragment, 199 bp
smu.47	ctgccacatagacgagaa	gttacaattccaccacaa	smu.47 fragment, 124 bp
smu.610	ttccgatgaaacgaccactac	tcagttccttactcgcactcc	<i>spaP</i> fragment, 115 bp
smu.78	gggacttgggaagtacgagaag	aaacaagagctgctgcaccg	<i>fruA</i> fragment, 148 bp
smu.872	gcggttatgttacgggtacg	aaagcagtggtgcacaaaag	<i>frul</i> fragment, 116 bp
smu.985	cgccgtttatgacaggaggtatc	ggcattggataagcaggcatagc	<i>blgA</i> fragment, 178 bp
smu.987	acgactgcttctcaaacgaatg	ctgctgttcactgttgatgg	<i>wapA</i> fragment, 112 bp

Kan<sup>r</sup>, Sp<sup>r</sup> and Erm<sup>r</sup>, for kanamycin, spectinomycin and erythromycin resistance, respectively. Sequences underlined are restriction sites engineered for cloning.

*S. mutans* strains. Both strains UA159 and TW26D were transformed with plasmid pAYBG754S, which carries a promoterless *gfp* gene fused with the *S. mutans* glucosyltransferase B gene promoter (*PgtfB*) (Yoshida & Kuramitsu, 2002). The *PgtfB::gfp* system has been shown to be stable and to be useful for biofilm studies in *S. mutans* (Yoshida & Kuramitsu, 2002; Wen *et al.*, 2006). Also, we have previously

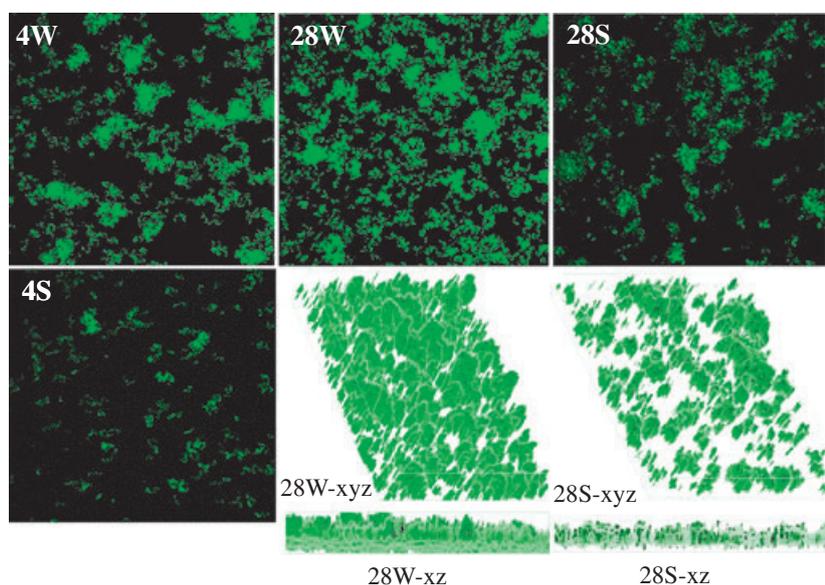
shown by reporter gene fusions that LuxS-deficiency does not have a significant impact on the expression of *gtfB* (Wen & Burne, 2004). No major differences were observed in the expression of GFP between the wild-type and the LuxS-deficient mutant TW26D (Z.T. Wen and R.A. Burne, unpublished data). Possession of GFP as a reporter has no major impact on bacterial growth, adherence or biofilm formation.

When grown in flowcells with only glucose as the supplemental carbohydrate source, *S. mutans* UA159 was able to bind to the coverslips in the flowcell chambers, but the number and size of microcolonies were limited and no significant biofilm accumulation was observed (data not shown). In contrast, adherent forms of *S. mutans* TW26D were not identified in flowcells when glucose was the only supplemental carbohydrate source (data not shown). To promote biofilm formation by *S. mutans* strains in the flowcell system, sucrose was included in the growth medium at a level of 2 mM (Tsumori & Kuramitsu, 1997; Hazlett *et al.*, 1999; Ooshima *et al.*, 2001). The presence of sucrose allows for the formation of extracellular glucans that serve as a scaffolding for adherence and biofilm formation. When sucrose was included in the growth medium (BMGS), both UA159 and TW26D were able to bind to the glass surface and form microcolonies within 4 h after inoculation. However, compared with the more evenly distributed microcolonies formed by the wild-type strain, the microcolonies of the LuxS-deficient mutant were sparse and more variable in size (Fig. 1). As the experiments went on, the wild-type strain continued to grow and accumulate, generating an average biomass of  $1.86 (\pm 0.39) \mu\text{m}^3$  per  $\mu\text{m}^2$  of substratum

after 28 h. The LuxS-deficient mutant, however, had only limited accumulation of biofilms with an average biomass of  $1.07 (\pm 0.19) \mu\text{m}^3$  per  $\mu\text{m}^2$  ( $P = 0.00893$ ) after 28 h. Similar results were also obtained with biofilms grown on glass slides (see Fig. S1). Evidently, growth under static conditions in an aerobic environment with 5%  $\text{CO}_2$  facilitated *S. mutans* biofilm formation dramatically, but the impact of LuxS-deficiency on the ability of *S. mutans* to form biofilms was also consistently demonstrated. The wild-type developed an average of  $35.62 \mu\text{m}^3$  of biomass per  $\mu\text{m}^2$  of substratum surface after 24 h and  $64.32 \mu\text{m}^3$  after 72 h. In contrast, the LuxS-deficient mutant, TW26D accumulated only  $7.67 \mu\text{m}^3$  of biomass ( $P < 0.05$ ) per  $\mu\text{m}^2$  of substratum after 24 h and  $29.48 \mu\text{m}^3$  ( $P < 0.042$ ) after 72 h. Consistent with our previous findings, the biofilms formed by the wild-type were tight, more evenly distributed, while biofilms of the LuxS-deficient mutant were loose, sporadic and had large gaps between cell clusters.

#### The LuxS-deficient mutant has an altered transcriptional profile

DNA microarrays were used to analyze the transcriptional profile of the LuxS-deficient mutants. When



**Figure 1** Confocal laser scanning microscopy analysis of *Streptococcus mutans* biofilms. The *S. mutans* UA159 and TW26D strains that carry a *PgtfB::gfp* reporter system were grown in flowcells in semi-defined biofilm medium with 18 mM glucose and 2 mM sucrose as the carbohydrate sources. Images 4S, 4W, 28S, 28W are representatives of compressed 4-h and 28-h biofilms of the LuxS-deficient mutant TW26D (S) and wild-type UA159 (W), respectively. The xyz and xz images are reconstructed three-dimensional structures. Dimensions of the regions displayed are  $512 \times 512 \mu\text{m}$ .

grown in BHI to mid-exponential phase, 11 genes were found to be altered by at least 1.5-fold ( $P < 0.001$ ) in the LuxS-deficient mutant, with 10 downregulated and one upregulated (Table 2). At  $P < 0.01$ , 33 additional genes were identified as upregulated or downregulated as a result of LuxS-deficiency (see Table S1). Among the altered genes, several are known to be co-transcribed, including a mannose-specific PTS operon (*smu.1877-79*) (Abranches *et al.*, 2003) and the inducible fructose-specific PTS operon (*smu.870-72*) (Wen *et al.*, 2001). As expected, members of these operons were differentially expressed in the same manner in the LuxS-deficient mutant TW26D. To further validate the results of DNA array analysis, we also examined a subset of genes using real-time PCR with gene-specific primers (Table 1) and the same total RNA preparations used for DNA array analysis were used for these assays. As shown in Table 3, the results of real-time PCR were in strong agreement with those of DNA microarray analysis with respect to the trends of expression of the selected genes, with the exception of *spaP* and *fruA*. Contrary to the microarray data (see Table S1), but consistent with the real-time PCR results, *fruA* expression was previously found to be downregulated in response to LuxS-deficiency by more than 50% when analyzed by reporter gene fusions (Wen & Burne, 2004). Different from the microarray data, real-time PCR showed that the expression of *spaP*, which encodes the multi-functional adhesin of *S. mutans*, in TW26D was decreased by more than three-fold (Table 3).

Downregulation of *spaP* in response to LuxS-deficiency was also recently reported by Merritt *et al.* (2003).

Recently, Sztajer *et al.* (2008) published a similar study on transcriptional profiling of the LuxS-deficient *S. mutans* when grown planktonically. In the study, Sztajer *et al.* used a slightly modified BM medium with 0.5% sucrose (BMS) as the supplemental carbohydrate sources to prepare the cultures for array analysis and the results revealed that the expression of 585 genes was affected in response to LuxS-deficiency. By cross-referencing the two transcriptional profiles, 16 of the 44 genes identified in the LuxS-deficient mutants in this study were also found to be differentially expressed in the Sztajer study (see Table S1). The level of LuxS expression in *S. mutans* has been shown to be growth-phase-dependent (Wen & Burne, 2004; Sztajer *et al.*, 2008), indicative of an influence of environmental conditions on the production of LuxS. The fact that the cultures used for the microarray analyses were grown in two different media, BHI and BMS, could account for the lack of congruence of the two transcriptional profiles.

### LuxS-deficiency affects a different group of genes in biofilms

To further characterize the impact of LuxS-deficiency on *S. mutans* biofilm formation, RNA was extracted from 3-day biofilms of *S. mutans* UA159 and TW26D grown on glass slides. DNA microarray analysis revealed that as compared with UA159,

**Table 2** Upregulated and downregulated genes identified in planktonic cultures ( $P < 0.001$ )

Locus	Description and putative function <sup>1</sup>	Fold-difference <sup>2</sup>	Parametric <i>P</i> value
SMU.1054	Putative glutamine amidotransferase	2.05	4.10E-04
SMU.1347c	Conserved hypothetical protein, possible permease	-3.4	1.40E-05
SMU.1363c	Putative transposase	-7.15	7.30E-05
SMU.1735c	Acetyl-CoA carboxylase $\beta$ subunit	-1.67	6.00E-04
SMU.1739c	3-oxoacyl-(acyl-carrier-protein) synthase	-2.03	7.90E-05
SMU.1740c	3-oxoacyl-acyl-carrier-protein reductase/3-ketoacyl-acyl carrier protein	-2	2.00E-04
SMU.1741c	Malonyl-CoA (acyl-carrier-protein) transacylase	-1.94	6.60E-05
SMU.1877	PTS system, mannose-specific component IIAB	-3.63	2.00E-07
SMU.1878	PTS system, mannose-specific component IIC	-3.1	3.90E-05
<i>SMU.1879</i>	<i>PTS system, mannose-specific component IID</i>	<i>-4.77</i>	<i>1.00E-07</i>
SMU.872	Fructose-specific enzyme IIABC component	-7.67	2.70E-06

<sup>1</sup>Description and putative function of the identified genes are based upon the published *Streptococcus mutans* database.

<sup>2</sup>Defined as levels of expression in the LuxS-deficient mutant relative to those of the wild-type, with minus signs representing downregulation. Genes in italics represent those identified in both planktonic cultures and 3-day biofilm populations.

27 genes were downregulated and 35 were upregulated in TW26D ( $P < 0.001$ , Tables 3–5). At a  $P < 0.01$ , 62 additional genes were found to be altered in TW26D, with 25 downregulated and 37 upregulated (see Table S2). Similar to planktonic cultures, a large number of affected genes were found to encode hypothetical proteins or conserved hypothetical proteins with no known functions. Multiple genes for sugar-specific enzyme II permeases of the PTS system and sugar metabolism were also differentially expressed in biofilm populations as a result of LuxS-deficiency, including a fructose/mannose-specific enzyme II (LevDEFGX) (Smu.1956/61c) (Zeng *et al.*, 2006), a mannose-specific enzyme IID (Smu.1879) (Abranches *et al.*, 2003) and members of the multi-sugar binding system (Smu.878/83) (Cote *et al.*, 2000) (Tables 2–5). Different from planktonic cultures, however, the *lev-DEFGX* operon and the mannose-specific enzyme IID were shown to be upregulated and no major differences were found in expression of the inducible fructose-PTS operon (*smu.870/2*). Unlike in planktonic cultures, there were no significant changes in expression of either multifunctional adhesin SpaP or surface-associated protein WapA in biofilms when

the wild-type and the LuxS-deficient mutant were compared.

Cross-referencing of the transcriptional profiles, sessile vs. planktonic (including data presented here and those recently published by the Sztajer group), revealed that 42 of the 124 genes identified in the LuxS-deficient mutant biofilms were also found to be altered in planktonic cultures of the deficient mutants (see Table S2) (Sztajer *et al.*, 2008). Of the 42 consistently affected genes, four were shown to be reverted to the wild-type level of expression when synthetic AI-2 was included in the growth medium (see Table S2) (Sztajer *et al.*, 2008). It is noted that in the recent study, Sztajer *et al.* carried out the transcriptional profiling using cultures grown on BMS, a condition similar to what we used [BM with glucose (18 mM) and sucrose (2 mM)] to grow biofilms. Therefore, it can be assumed that most, if not all, of these unmatched genes are likely biofilm-associated. A closer examination of these genes revealed that more than half of these unmatched genes encode hypothetical or conserved hypothetical proteins. The exact role of their products in *S. mutans* adherence and biofilm formation as well as their dependence on AI-2 signaling remains to be investigated. These results

**Table 3** RealTime-PCR analysis of selected genes<sup>1</sup>

Locus	Description and putative function	UA159	TW26D	Fold-difference	Parametric <i>P</i> -value
Planktonic					
smu.1128	Sensor/histidine kinase, CiaH	8.17E5(1.13E4)	2.62E5(1.93E4)	-3.12	0.01538
smu.610	Surface antigen SpaP	1.39E6(1.06E5)	3.98E5(2.89E4)	-3.49	0.00172
smu.987	Surface protein WapA	4.52E6(1.54E6)	5.91E6(2.1E6)	1.32	0.02389
smu.872	Fructose-PTS, EIIBC <sup>Fru</sup>	5.39E7(3.0E7)	1.69E6(1.9E6)	-31.8	6.74E-07
smu.78	Exo- $\beta$ -fructosidase, FruA	4.63E5(1.79E4)	1.98E5(7.66E3)	-2.34	6.45E-04
smu.985	$\beta$ -glucosidase, BglA	9.88E4(3.1E3)	8.7E4(2.5E4)	-1.14	0.02897
smu.364	Putative glutamine synthetase	3.59E7(2.03E6)	2.53E7(3.5E6)	-1.43	0.04326
smu.1877	Mannose-PTS, EIID <sup>Man</sup>	4.72E6(8.7E5)	2.00E6(2.6E5)	-2.36	0.00869
Biofilms					
smu.44	DNA mismatch repair protein	6.22E5(4.9E4)	1.13E5(4.8E4)	-5.5	7.85E-04
smu.45	DNA mismatch repair protein	2.44E5(1.3E4)	1.32E4(1.9E3)	-18.48	5.60E-04
smu.46	LuxR-type regulator	4.95E5(4.2E4)	7.21E3(1.3E3)	-68.65	3.43E-04
smu.47	NHN-type endonuclease	1.49E7(6.8E6)	1.93E5(2.7E4)	-77.2	5.11E-04
smu.299	Bacteriocin precursor	8.84E6(7.1E5)	5.84E6(1.3E6)	-1.52	2.59E-04
smu.423	Mutacin-like protein	1.60E5(1.1E5)	8.77E5(1.1E5)	5.48	2.38E-04
smu.985	$\beta$ -glucosidase BglA	2.27E5(9.2E4)	4.2E5(4.4E4)	1.85	4.51E-03
smu.1528	F-ATPase, $\beta$ -subunit	7.5E6(3.8E5)	1.37E7(7.5E6)	1.82	6.74E-03
smu.1877	Mannose-PTS, EIID <sup>Man</sup>	3.55E6(3.38E5)	1.02E6(4.0E5)	-3.48	2.67E-04
smu.1961	Fructose/mannose-PTS, LevD	1.41E6(1.01E6)	3.19E6(1.9E6)	2.26	5.54E-03
16S-1 rRNA	16S rRNA	2.16E7(3.5E6)	2.19E7(5.1E6)	0.99	0.45

<sup>1</sup>Results are expressed as copy numbers in 1  $\mu$ g of total RNA. The data represent mean ( $\pm$  standard deviation) of no less than four independent experiments. See Table 2 for more information.

**Table 4** Downregulated genes in 3-day biofilms ( $P < 0.001$ )<sup>1</sup>

Locus	Description and putative function	Fold-difference	Parametric <i>P</i> value
SMU.09	Conserved hypothetical protein	-2.67	1.6E-5
SMU.12	Conserved hypothetical protein	-2.80	3.9E-5
SMU.528c	Conserved hypothetical protein	-1.52	3.1E-4
SMU.730	Conserved hypothetical protein	-1.71	8.2E-4
SMU.1574c	Conserved hypothetical protein	-1.99	5.2E-5
SMU.2129c	Conserved hypothetical protein	-1.89	2.7E-4
SMU.735	Hypothetical protein	-1.57	6.0E-4
SMU.1895c	Hypothetical protein	-1.73	2.3E-4
SMU.1896c	Hypothetical protein	-1.91	3.7E-5
SMU.43	Site-specific DNA-methyltransferase restriction-modification protein	-4.62	1.9E-5
SMU.44	Conserved hypothetical protein (possible DNA mismatch repair)	-6.67	1.9E-5
SMU.46	Hypothetic protein (putative response regulator)	-6.57	4.3E-4
SMU.47	Hypothetic protein (putative HNH-type endonuclease)	-4.41	1.0E-3
SMU.127	Putative acetoin dehydrogenase (TPP-dependent), E1 component $\alpha$	-2.24	2.3E-6
SMU.128	Putative acetoin dehydrogenase (TPP-dependent), E1 component $\beta$	-2.79	2.9E-6
SMU.129	Putative dihydrolipoamide acetyltransferase	-2.99	3.8E-6
SMU.130	Putative dihydrolipoamide dehydrogenase	-2.79	4.6E-6
SMU.131	Putative lipoate-protein ligase	-3.33	3.0E-7
SMU.132	Putative hippurate amidohydrolase	-3.01	2.0E-7
SMU.144c	Putative transcriptional regulator	-1.51	2.6E-4
SMU.299c	Putative bacteriocin peptide precursor	-1.62	9.8E-4
SMU.356	Purine operon repressor	-2.18	6.4E-4
SMU.732	Possible inner membrane protein	-1.57	5.4E-4
SMU.930c	Putative transcriptional regulator	-2.59	3.5E-4
SMU.953c	Putative transcriptional regulator/aminotransferase	-1.55	2.7E-4
SMU.1027	Putative transcription regulator	-2.05	3.4E-4
SMU.1452	$\alpha$ -acetolactate synthase	-1.56	3.8E-4

<sup>1</sup>See Table 2 for more information.

also suggest that LuxS-mediated signaling may affect a different set of genes in biofilms.

#### Mutation of genes encoding mutacin-like protein(s) had no impact on biofilm formation

Notably, several genes identified as differentially expressed in the LuxS-deficient mutant biofilms were found to encode a mutacin-like protein (*smu.423*), a bacteriocin precursor (*smu.299*) (Tables 3 and 4 and see Table S2) and a bacteriocin transport accessory protein (data not shown). Mutacins (bacteriocins produced by mutans streptococci) are known for their capabilities to kill or inhibit the growth of other closely related bacterial species in the oral flora (Chikindas *et al.*, 1997), therefore are thought to play an important role in *S. mutans* establishment and persistence in the plaque and ultimately, the development of dental caries. To evaluate the role of these altered genes in *S. mutans* biofilm formation, isogenic mutants,

TW310 and TW238 deficient of *smu.299* and *smu.423*, respectively, were generated with a non-polar kanamycin-resistance element replacing the respective coding sequence. The deficiency of *smu.299* and *smu.423* was confirmed by DNA sequencing, and the non-polar effect of *smu.299* and *smu.423* mutation on the flanking genes was verified by real-time PCR using gene-specific primers (data not shown). The deficient mutants were grown on 96-well plates with either glucose or sucrose as the supplemental carbohydrate source. No major differences in the amount of biofilms were detected between the wild-type and the *smu.299*-deficient or *smu.423*-deficient mutants after 24 or 48 h of incubation under the conditions studied. Although the actual function of the selected genes in *S. mutans* biofilm formation remains unclear, especially when grown in a mixed-species consortium, alteration of expression of genes involving bacteriocin production and processing in response to LuxS-deficiency is consistent with the

**Table 5** Upregulated genes in 3-day biofilms ( $P < 0.001$ )<sup>1</sup>

Locus	Description and putative function	Fold-difference	Parametric $P$ value
SMU.86	Conserved hypothetical protein	1.92	7.9E-4
SMU.475	Conserved hypothetical protein	1.79	7.9E-5
SMU.600c	Conserved hypothetical protein	1.73	9.1E-5
SMU.1404c	Conserved hypothetical protein	1.77	9.3E-4
SMU.68	Hypothetical protein	2.08	9.7E-4
SMU.503c	Hypothetical protein	1.67	3.8E-4
SMU.618	Hypothetical protein	1.61	7.2E-4
SMU.984	Hypothetical protein	1.68	4.7E-4
SMU.1587c	Hypothetical protein	1.96	1.9E-5
SMU.67	Putative acyltransferase	1.78	2.6E-4
SMU.84	Putative tRNA pseudouridine synthase A	1.68	1.7E-4
SMU.101	Putative sorbose PTS system, IIC component	1.77	1.0E-4
SMU.103	Putative sorbose PTS system, IIA component	1.86	7.4E-4
SMU.148	Putative alcohol-acetaldehyde dehydrogenase	2.96	2.0E-7
SMU.423	Mutacin-like protein	19.41	2.0E-3
SMU.558	Isoleucine-tRNA synthetase	2.11	8.9E-5
SMU.878	Multiple sugar-binding ABC transporter, sugar-binding protein precursor	1.94	3.3E-4
SMU.881	Sucrose phosphorylase, GtfA	2.06	6.3E-4
SMU.882	Multiple sugar-binding ABC transporter, ATP-binding protein, MsmK	2.04	5.8E-4
SMU.883	Dextran glucosidase DexB	2.04	1.6E-4
SMU.985	$\beta$ -glucosidase BglA	1.73	6.1E-4
SMU.1077	Putative phosphoglucomutase	1.59	1.2E-4
SMU.1510	Putative phenylalanyl-tRNA synthetase ( $\beta$ subunit)	1.84	5.1E-4
SMU.1511c	Putative acetyltransferase	1.71	5.3E-4
SMU.1531	F1Fo membrane-bound proton-translocating ATPase, $\delta$ subunit	1.74	2.8E-4
SMU.1532	F1Fo membrane-bound proton-translocating ATPase, b subunit	1.67	5.2E-4
SMU.1956c	PTS system, fructose/mannose-specific, LevX	4.72	3.9E-4
SMU.1957c	PTS system, fructose-specific IID component, LevG	4.57	2.7E-4
SMU.1958c	PTS system, fructose-specific IIC component, LevF	4.15	2.9E-4
SMU.1960c	PTS system, fructose-specific IIB component, LevE	3.27	9.1E-4
SMU.1879	PTS system, mannose-specific component IID,	2.02	1.9E-4
SMU.1568	Maltose/maltodextrin ABC transporter, sugar-binding protein MalX	1.68	1.6E-5
SMU.1569	Maltose/maltodextrin ABC transporter, permease protein MalF	1.62	7.8E-5
SMU.1570	Maltose/maltodextrin ABC transporter, MalG permease	1.65	1.3E-5
SMU.1586c	Putative threonyl-tRNA synthetase	2.09	3.8E-4

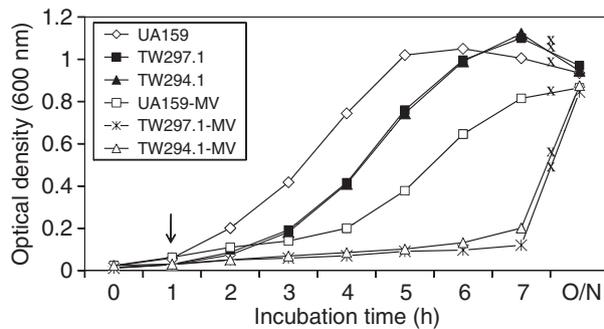
<sup>1</sup>See Table 2 for more information.

role of LuxS-mediated signaling in intra- and inter-species interactions.

#### **Smu.44 and Smu.46 are part of the LuxS-regulated stress response pathway**

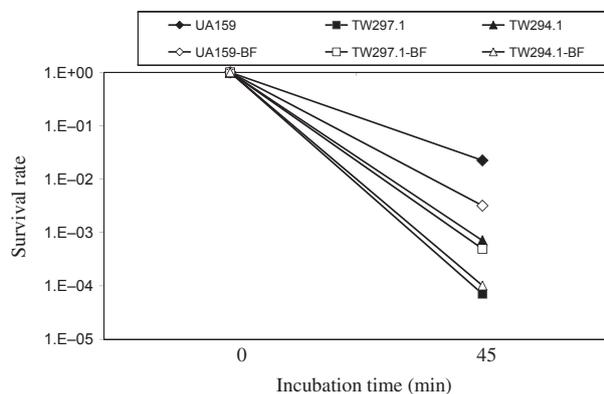
It was shown previously by Northern blot analysis that certain genes known to be involved in DNA repair were altered in response to LuxS-deficiency, including downregulation of *recA*, which could account perhaps in part for the weakened tolerance to acid killing (Wen & Burne, 2004). Of the genes identified as downregulated in the sessile populations, at least eight were also found to encode pro-

teins with putative functions in nucleic acid metabolism and DNA repair, including a putative DNA mismatch repair protein (Smu.44) (Tables 3–5 and see Table S2). To evaluate the role of *smu.44* in *S. mutans* biofilm formation, the coding sequence was deleted and replaced with a non-polar kanamycin-resistance element (Zeng *et al.*, 2006). Deficiency of *smu.44* did not show any major differences in growth rate when BHI or BMG was the growth medium (Fig. 2). When subjected to acid killing by incubating at pH 2.8 in 0.1 M glycine buffer for 45 min (Wen & Burne, 2004), the survival rate of the deficient mutant, TW297.1 was more than 2-logs lower than the wild-type UA159 (Fig. 3), suggesting that



**Figure 2** Growth study. *Streptococcus mutans* strains were grown in brain–heart infusion broth with or without the addition of methyl viologen (MV, 18 mM, final concentration). Data presented here are representatives of more than four independent experiments. Arrow indicates the time when methyl viologen was added to the growth medium, and ‘x’ on growth curves illustrates disruption of measurement of the cultural optical density at 600 nm.

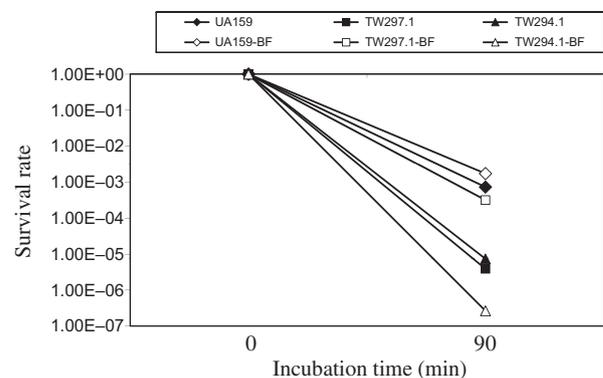
*Smu.44* plays a role in tolerance of low pH. To determine whether sessile populations possessed similar features in acid tolerance, 3-day biofilms were grown on glass slides with glucose (18 mM) and sucrose (2 mM) as the carbohydrate sources and, following brief sonication to disperse the biofilms, these cells were washed and then subjected to acid killing assays as described above. Similar to the planktonic



**Figure 3** Acid-tolerance assays. For acid tolerance, *Streptococcus mutans* strains were grown either in brain–heart infusion (BHI) broth until mid-exponential phase ( $OD_{600} \approx 0.45$ ) or in biofilm medium with glucose (18 mM) plus sucrose (2 mM) (BMGS) on glass slides for 3 days. Brief sonication was used to disperse biofilms. All cultures were washed once with 0.1 M glycine buffer, pH 7.0, and then subjected to acid killing at pH 2.8 for 45 min. The surviving cells were plated on BHI agar plates in triplicate, and expressed as survival rate over time. Data presented here are representatives of more than three independent experiments. Solid symbols represent planktonic cultures and open symbols represent biofilms (BF).

cultures, biofilms of the mutants deficient in *smu.44* also showed a weakened tolerance to acid killing at pH 2.8 for 45 min (Fig. 3). To evaluate the ability of the deficient mutants to tolerate oxidative stress, methyl viologen (paraquat; Sigma, St Louis, MO) was included in the BHI broth (18 mM, final concentration) and its inclusion was found to dramatically inhibit the growth of strain TW297.1, as compared with the parental strain UA159 (Fig. 2). When challenged with hydrogen peroxide, a major endogenous source of oxidative stress in oral bacteria and a product often present in oral care products, the survival rate of the *smu.44*-deficient mutant was also about 2-log lower than strain UA159 (Fig. 4). Similar results were also obtained with 3-day biofilms (Fig. 4). When 96-well plates were used to grow biofilms with glucose as the supplemental carbohydrate source and measured using a spectrophotometer following crystal violet staining (Loo *et al.*, 2000), the deficient mutant, TW297.1 was found to form as much as 62% less biofilm than the parent strain, UA159, although no significant difference was observed when sucrose was used as the carbohydrate source.

The *smu.46* gene is arranged in an apparent operon with *smu.45* and *smu.47* (see Fig. S2), which are predicted to code for another protein with some similarity to the DNA mismatch repair proteins and a



**Figure 4** Hydrogen peroxide killing assays. *Streptococcus mutans* cultures were prepared similarly as those for acid-tolerance assays. Following washes with 0.1 M glycine buffer, pH 7.0, the bacterial cells were incubated in glycine buffer containing 0.2% (volume/volume, 58 mM, final concentration) hydrogen peroxide for 90 min. The survival rates were determined by serial dilutions and plating on brain–heart infusion agar plates. Data presented here are representatives of more than three independent experiments. Solid symbols represent planktonic cultures and open symbols represent biofilms (BF).

putative NHN-type endonuclease (Hsia *et al.*, 2004), respectively. Annotated as a hypothetical protein, the predicted product of the *smu.46* gene shows features of the LuxR-family of regulatory proteins. To analyze the potential role of Smu.46 in *S. mutans*, a deficient mutant was generated using a strategy similar to the one described above. Similar to the *smu.44*-deficient mutants, loss of *smu.46* did not cause any detectable differences in morphology and growth rate under the conditions studied. Addition of methyl viologen to the growth medium also displayed a significant inhibitory effect on growth by the deficient mutant, TW294.1, as compared with strain UA159 (Fig. 2). After incubation in buffer adjusted to pH 2.8 for 45 min or containing 0.2% hydrogen peroxide for 90 min, the survival rates were more than 1-log lower than the wild-type UA159 (Figs 3 and 4), suggesting that deficiency of *smu.46* weakens the ability of the deficient mutants to tolerate acid- and oxidative stress. Similar results were also observed with sessile populations in acid and hydrogen peroxide tolerance (Figs 3 and 4) and in biofilm formation (data not shown). Apparently, the *smu.46*-deficient mutant possesses a phenotype that is similar to the strains lacking *smu.44*. One possible explanation is that *smu.46*-deficiency affects the expression of *smu.44*. To assess this possibility, real-time PCR was used to analyze the expression of *smu.44* in TW294.1, but no major differences in messenger RNA in the wild-type and *smu.46*-deficient mutant were observed, nor was the expression of *smu.45* and *smu.47* altered (data not shown). These results suggest that Smu.46 likely exerts its regulatory function on other gene(s) involving acid and oxidative stress tolerance.

## DISCUSSION

LuxS/AI-mediated signaling in several oral bacteria has been shown to play a critical role in biofilm formation (McNab *et al.*, 2003; Merritt *et al.*, 2003; Wen & Burne, 2004; Yoshida *et al.*, 2005; Rickard *et al.*, 2006). Consistent with our recent findings using scanning electron micrographs of biofilms formed by the LuxS-deficient mutant, TW26D on hydroxylapatite surfaces (Wen & Burne, 2004), the results on flowcells further support that LuxS-deficiency causes major defects in cell–cell interactions and biofilm maturation in *S. mutans*. In addition, the fact that the *luxS* mutant did not adhere well to the glass slides in

the absence of sucrose, and formed fewer microcolonies than the wild-type, suggests that defects in initial adherence may be part of the reason the TW26D strain shows diminished biofilm forming capacity. In the absence of sucrose, SpaP (also called P1), a multi-functional adhesin that interacts with salivary agglutinin, is considered a major factor in *S. mutans* adherence and biofilm initiation on the tooth surface (Bowen *et al.*, 1991; Crowley *et al.*, 1999). Although no significant differences were identified in sessile populations between the wild-type and the LuxS-deficient mutant, real-time PCR analysis with total RNA extracted from planktonic cultures grown on BHI revealed that the expression of *spaP* was downregulated by more than threefold in response to LuxS-deficiency (Table 3), which is consistent with recent findings of Merritt *et al.* (2005). We have also used BIAcore assays to analyze the capacity of the LuxS-deficient mutant to bind to salivary agglutinin immobilized on a Pioneer F1 sensor chip (Oli *et al.*, 2006). Preliminary data showed that, as compared with the wild-type strain, P1-mediated whole cell–ligand interaction of the LuxS-deficient mutant with salivary agglutinin was decreased by 17%, with the resonance signal for the wild-type at  $938.95 \pm 102.45$  vs.  $785.5 \pm 84.5$  for the LuxS-deficient mutant ( $P < 0.05$ ). These results suggest that deficiency of LuxS and/or AI-2-mediated signaling affects the expression of the multifunctional adhesin, which consequently could modulate the ability of the deficient mutant to colonize the tooth surface *in vivo*. However, considering the fact that the flowcells were not coated with saliva, the impact of SpaP deficiency on bacterial adherence and biofilm initiation under the current conditions is probably limited.

It is evident that LuxS-deficiency in *S. mutans* causes substantial alterations in gene expression in both planktonic (Tables 2 and 3 and see Tables S1 and S2) (Sztajer *et al.*, 2008) and sessile populations (Tables 3–5 and see Table S2). Based on the description and putative functions of the affected genes, LuxS-deficiency in *S. mutans* when grown in biofilms also affects nearly every aspect of cellular physiology and virulence, including carbohydrate and central metabolism (37), nucleic acid metabolism and DNA repair (8), amino acid and protein synthesis (6), stress responses (6), fatty acid synthesis (1), two component signal transduction (1), cell envelope (4), membrane transporters (5), transcription regulation (7),

and bacteriocin and other cellular process (5). Of the genes affected by LuxS-deficiency in biofilms, at least 52 were found to code for proteins with a potential role in metabolic processes, which is consistent with findings in planktonic cultures of *S. mutans* (Sztajer *et al.*, 2008) and some other bacteria (DeLisa *et al.*, 2001; McNab *et al.*, 2003; Winzer *et al.*, 2003). Cross-referencing of the transcriptional profiles also provided evidence that of the genes consistently affected by LuxS-deficiency in both planktonic and sessile populations, only a few (*smu.44*, *smu.103*, *smu.132* and *smu.1511*) were shown to respond to synthetic AI-2. However, it awaits further investigation how many of the remaining biofilm-associated genes are AI-2-dependent.

LuxS in *S. mutans* is shown to be differentially expressed at different growth phases (Wen & Burne, 2004; Sztajer *et al.*, 2008), suggesting that changes in environmental conditions, such as pH, nutrient availability and cell density, and growth mode (planktonic vs. sessile) could have an impact on the transcription of LuxS, and consequently affect the AI-2 activity. Of note, the expression of *dnaK* and *groEL* was found in our earlier study to be upregulated in the LuxS-deficient mutant grown in BHI broth, especially in the late-exponential phase ( $OD_{600} \cong 0.5$ ) (Wen & Burne, 2004). The expression of *recA* was shown to be downregulated in early-exponential phase ( $OD_{600} \cong 0.3$ ), but was upregulated in late exponential phase. However, none of these genes were identified in our DNA microarray profiling. While efforts were made to use similar conditions to carry out the microarray analysis, differences may exist between the conditions when the cultures were harvested for total RNA extraction, contributing at least in part to the discrepancies observed. The differences in growth conditions and growth mode (planktonic vs. sessile) could also be attributed to some of the variations in the results presented here and those published by other groups (Merritt *et al.*, 2005; Sztajer *et al.*, 2008). In a recent study by Eglund *et al.* (2004), it was found that expression of GFP in *S. gordonii* under the direction of amylase gene promoter was regulated in response to a signal produced by *Veillonella atypica*. However, only cells that were in close contact with *V. atypica* turned on the expression, suggesting that the signal concentration, as well as the proximity of bacterial species, are crucial for effective signal transduction in certain condi-

tions. Although it remains unclear how the expression of LuxS is regulated when *S. mutans* is grown in the presence of a surface and in a high-density environment, the concentration and accessibility of AI-2 signal molecules in biofilms, a high cell-density community, may differ from planktonic cultures, attributing to the differences in gene transcription observed in the LuxS-deficient mutants when grown in planktonic vs. biofilm mode. However, the actual mechanism remains to be further investigated.

While the mechanisms are not yet fully understood, the intimate linkage between stress tolerance and biofilm formation is well documented (Li *et al.*, 2002; Lemos *et al.*, 2004, 2005; Wen & Burne, 2004; Abranches *et al.*, 2006; Wen *et al.*, 2006). Compared with the parental strain, one of the features of the LuxS-deficient mutants is the significantly weakened tolerance to low pH and acid killing (Wen & Burne, 2004). Of the genes affected by LuxS-deficiency, several have been shown to play a major role in acid tolerance, including downregulation of *brpA* and *ciaH* in planktonic cultures (Ahn *et al.*, 2006; Wen *et al.*, 2006), contributing at least in part to the increased susceptibility of the deficient mutants to acid stress. Proton extrusion by the membrane-associated F-ATPase is the primary mechanism employed by mutans streptococci to maintain intracellular pH homeostasis (Marquis, 1995). In contrast to planktonic cultures, the expression of the F-ATPase operon was found to be upregulated in TW26D when grown in biofilms (Tables 3 and 4). Interestingly, when analyzed for ATPase activity using an established method (Belli & Marquis, 1991), no significant differences were observed between the LuxS-deficient mutant biofilms and the wild-type biofilms (data not shown) (Wen & Burne, 2004). F-ATPase is a highly complex, membrane-associated protein, and as such, downregulation of genes that are known to be involved in fatty acid and phospholipids synthesis (*smu.1734c/45c*) (Table 2 and see Table S1) and protein secretion and membrane integrity (*ffh*) (Wen & Burne, 2004), which were identified in the planktonic populations but not in biofilms of the LuxS-deficient mutant, will likely have an impact on the conformation of and function in proton extrusion by the F-ATPase enzyme. Therefore, upregulation of F-ATPase expression may be a compensatory effort of the LuxS-deficient mutants to maintain pH homeostasis and combat defects in acid tolerance, especially

when grown in biofilms, as a consequence of a change in either the activity or stability of the ATPase arising from alterations in the membrane or in cellular proteolysis.

Many stresses encountered by oral bacteria, such as acid and oxidative stress, can induce DNA damage (Lemos & Burne, 2008). Recent studies have shown that several enzymes of DNA repair systems, including recombination protein RecA, AP endonuclease Smx and UV repair excinuclease UvrA, are involved in the ability of *S. mutans* to survive challenges imposed by acid and low pH and hydrogen peroxide (Quivey *et al.*, 1995; Hahn *et al.*, 1999; Hanna *et al.*, 2001; Faustoferri *et al.*, 2005). In our previous study by Northern blotting analysis, the expression of *recA*, *smx* and *nth* was found to be altered as a result of LuxS-deficiency, and the most dramatic differences were observed in early-exponential phase ( $OD_{600} \cong 0.3$ ) of the LuxS-deficient mutants (Wen & Burne, 2004). In this study, DNA microarray analysis of the sessile populations revealed downregulation of several genes coding for proteins with putative function in nucleic acid metabolism and DNA repair, including *smu.44* for a putative protein of DNA mismatch repair. Like Smx and UvrA (Hanna *et al.*, 2001; Faustoferri *et al.*, 2005), SMU.44-deficiency was found to affect the ability of the deficient mutants to endure acid and hydrogen peroxide killing. Although the underlying mechanism of SMU.46-mediated regulation awaits further investigation, strains deficient in SMU.46, a putative transcriptional regulator, also displayed a phenotype similar to the SMU.44-deficient mutant, TW297.1. Of note, downregulation of *smu.44*, *smu.46* and *recA* in planktonic cultures were also recently reported by Sztajer *et al.* (2008) in transcriptional profiling of a LuxS-deficient *S. mutans*. In addition, inclusion of synthetic AI-2 to the growth medium was found to be able to restore the transcription of *smu.44* to the level of the wild-type, suggesting its AI-2 dependence. As shown by real-time PCR, however, no major differences were identified in expression of either *smu.44* or *smu.46* between planktonic cultures and sessile populations of the wild-type UA159 (Z.T. Wen *et al.*, data not shown). Neither one of these genes was identified in a recent DNA microarray analysis of genes associated with biofilm thickness by Shemesh *et al.* (2007). These results further suggest that DNA repair is part of the LuxS-regulated stress response circuit and that the weakened stress toler-

ance as a result of downregulation of enzymes like SMU.44 and SMU.46 is part of the underlying factors in biofilm formation by the LuxS-deficient mutants. Effort is currently directed to further characterization of the LuxS-regulon and their roles in biofilm formation by *S. mutans*.

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## REFERENCES

- Abranches, J., Chen, Y.Y. and Burne, R.A. (2003) Characterization of *Streptococcus mutans* strains deficient in EIIAB<sup>Man</sup> of the sugar phosphotransferase system. *Appl Environ Microbiol* **69**: 4760–4769.
- Abranches, J., Candella, M., Wen, T.Z., Baker, H.V. and Burne, R.A. (2006) Different roles of EIIAB<sup>Man</sup> and EII<sup>Glc</sup> in the regulation of energy metabolism, biofilm development and competence in *Streptococcus mutans*. *J Bacteriol* **188**: 3748–3756.
- Ahn, S.J., Wen, Z.T. and Burne, R.A. (2006) Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. *Infect Immun* **74**: 1631–1642.
- Ajdic, D., McShan, W.M., McLaughlin, R.E. *et al.* (2002) Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci USA* **99**: 14434–14439.
- Belli, W.A. and Marquis, R.E. (1991) Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Appl Environ Microbiol* **57**: 1134–1138.
- Bowen, W.H., Schilling, K., Giertsen, E. *et al.* (1991) Role of a cell surface-associated protein in adherence and dental caries. *Infect Immun* **59**: 4604–4609.

- Burne, R.A. (1998) Oral streptococci...products of their environment. *J Dent Res* **77**: 445–452.
- Chen, X., Schauder, S., Potier, N. *et al.* (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545–549.
- Chikindas, M.L., Novak, J., Caufield, P.W., Schilling, K. and Tagg, J.R. (1997) Microbially-produced peptides having potential application to the prevention of dental caries. *Int J Antimicrob Agents* **9**: 95–105.
- Christensen, B.B., Sternberg, C., Andersen, J.B. *et al.* (1999) Molecular tools for study of biofilm physiology. *Methods Enzymol* **310**: 20–42.
- Cote, C.K., Cvitkovitch, D., Bleiweis, A.S. and Honeyman, A.L. (2000) A novel  $\beta$ -glucoside-specific PTS locus from *Streptococcus mutans* that is not inhibited by glucose. *Microbiology* **146**: 1555–1563.
- Crowley, P.J., Brady, L.J., Michalek, S.M. and Bleiweis, A.S. (1999) Virulence of a spaP mutant of *Streptococcus mutans* in a gnotobiotic rat model. *Infect Immun* **67**: 1201–1206.
- DeLisa, M.P., Wu, C.F., Wang, L., Valdes, J.J. and Bentley, W.E. (2001) DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. *J Bacteriol* **183**: 5239–5247.
- Egland, P.G., Palmer, R.J. Jr and Kolenbrander, P.E. (2004) Interspecies communication in *Streptococcus gordonii*–*Veillonella atypica* biofilms: signaling in flow conditions requires juxtaposition. *Proc Natl Acad Sci USA* **101**: 16917–16922.
- Faustoferri, R.C., Hahn, K., Weiss, K. and Quivey, R.G. Jr (2005) Smx nuclease is the major, low-pH-inducible apurinic/aprimidinic endonuclease in *Streptococcus mutans*. *J Bacteriol* **187**: 2705–2714.
- Fong, K.P., Chung, W.O., Lamont, R.J. and Demuth, D.R. (2001) Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. *Infect Immun* **69**: 7625–7634.
- Hahn, K., Faustoferri, R.C. and Quivey, R.G. Jr (1999) Induction of an AP endonuclease activity in *Streptococcus mutans* during growth at low pH. *Mol Microbiol* **31**: 1489–1498.
- Hanna, M.N., Ferguson, R.J., Li, Y.H. and Cvitkovitch, D.G. (2001) *uvrA* is an acid-inducible gene involved in the adaptive response to low pH in *Streptococcus mutans*. *J Bacteriol* **183**: 5964–5973.
- Hazlett, K.R., Mazurkiewicz, J.E. and Banas, J.A. (1999) Inactivation of the *gfpA* gene of *Streptococcus mutans* alters structural and functional aspects of plaque biofilm which are compensated by recombination of the *gtfB* and *gtfC* genes. *Infect Immun* **67**: 3909–3914.
- Henke, J.M. and Bassler, B.L. (2004) Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J Bacteriol* **186**: 3794–3805.
- Heydorn, A., Nielsen, A.T., Hentzer, M. *et al.* (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146**(Pt 10): 2395–2407.
- Hsia, K.C., Chak, K.F., Liang, P.H., Cheng, Y.S., Ku, W.Y. and Yuan, H.S. (2004) DNA binding and degradation by the HNH protein ColE7. *Structure* **12**: 205–214.
- Jenkinson, H.F. and Lamont, R.J. (2005) Oral microbial communities in sickness and in health. *Trends Microbiol* **13**: 589–595.
- Klein, M.I., Duarte, S., Xiao, J., Mitra, S., Foster, T.H. and Koo, H. (2009) Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilm development. *Appl Environ Microbiol* **75**: 837–841.
- Kolenbrander, P.E., Andersen, R.N., Blehert, D.S., Egland, P.G., Foster, J.S. and Palmer, R.J. Jr (2002) Communication among oral bacteria. *Microbiol Mol Biol Rev* **66**: 486–505.
- Kuramitsu, H.K., He, X., Lux, R., Anderson, M.H. and Shi, W. (2007) Interspecies interactions within oral microbial communities. *Microbiol Mol Biol Rev* **71**: 653–670.
- Lau, P.C.Y., Sung, C.K., Lee, J.H., Morrison, D.A. and Cvitkovitch, D.G. (2002) PCR ligation mutagenesis in transformable streptococci: application and efficiency. *J Microbiol Methods* **49**: 193–201.
- LeBanc, D. and Lee, L. (1991) Replication function of pVA380-1. In: Dunny G., Cleary P.P. and McKay L.L., eds. *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci*. Washington, DC: American Society for Microbiology: 235–239.
- Lemos, J.A. and Burne, R.A. (2008) A model of efficiency: stress tolerance by *Streptococcus mutans*. *Microbiology* **154**: 3247–3255.
- Lemos, J.A., Brown, T.A. Jr and Burne, R.A. (2004) Effects of RelA on key virulence properties of planktonic and biofilm populations of *Streptococcus mutans*. *Infect Immun* **72**: 1431–1440.
- Lemos, J.A., Abranches, J. and Burne, R.A. (2005) Responses of cariogenic streptococci to environmental stresses. *Curr Issues Mol Biol* **7**: 95–107.
- Li, Y.H., Lau, P.C., Tang, N., Svensater, G., Ellen, R.P. and Cvitkovitch, D.G. (2002) Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. *J Bacteriol* **184**: 6333–6342.
- Loo, C.Y., Corliss, D.A. and Ganeshkumar, N. (2000) *Streptococcus gordonii* biofilm formation: identification

- of genes that code for biofilm phenotypes. *J Bacteriol* **182**: 1374–1382.
- Marquis, R.E. (1995) Oxygen metabolism, oxidative stress and acid–base physiology of dental plaque biofilms. *J Ind Microbiol* **15**: 198–207.
- McNab, R., Ford, S.K., El-Sabaeny, A., Barbieri, B., Cook, G.S. and Lamont, R.J. (2003) LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J Bacteriol* **185**: 274–284.
- Merritt, J., Qi, F., Goodman, S.D., Anderson, M.H. and Shi, W. (2003) Mutation of luxS affects biofilm formation in *Streptococcus mutans*. *Infect Immun* **71**: 1972–1979.
- Merritt, J., Kreth, J., Shi, W. and Qi, F. (2005) LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory component. *Mol Microbiol* **57**: 960–969.
- Ng, W.L. and Bassler, B.L. (2009) Bacterial quorum-sensing network architectures. *Annu Rev Genet* **43**: 197–222.
- Ohtani, K., Hayashi, H. and Shimizu, T. (2002) The luxS gene is involved in cell–cell signaling for toxin production in *Clostridium perfringens*. *Mol Microbiol* **44**: 171–179.
- Oli, M.W., McArthur, W.P. and Brady, L.J. (2006) A whole cell BIAcore assay to evaluate P1-mediated adherence of *Streptococcus mutans* to human salivary agglutinin and inhibition by specific antibodies. *J Microbiol Methods* **65**: 503–511.
- Ooshima, T., Matsumura, M., Hoshino, T., Kawabata, S., Sobue, S. and Fujiwara, T. (2001) Contributions of three glycosyltransferases to sucrose-dependent adherence of *Streptococcus mutans*. *J Dent Res* **80**: 1672–1677.
- Quivey, R.G. Jr, Faustoferri, R.C., Clancy, K.A. and Marquis, R.E. (1995) Acid adaptation in *Streptococcus mutans* UA159 alleviates sensitization to environmental stress due to RecA deficiency. *FEMS Microbiol Lett* **126**: 257–261.
- Quivey, R.G. Jr, Kuhner, W.L. and Hahn, K. (2000) Adaptation of oral streptococci to low pH. *Adv Microb Physiol* **42**: 239–274.
- Rickard, A.H., Palmer, R.J. Jr, Blehert, D.S. *et al.* (2006) Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. *Mol Microbiol* **60**: 1446–1456.
- Shemesh, M., Tam, A. and Steinberg, D. (2007) Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. *Microbiology* **153**: 1307–1317.
- Surette, M.G. and Bassler, B.L. (1998) Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proc Natl Acad Sci USA* **95**: 7046–7050.
- Sztajer, H., Lemme, A., Vilchez, R. *et al.* (2008) Autoinducer-2-regulated genes in *Streptococcus mutans* UA159 and global metabolic effect of the luxS mutation. *J Bacteriol* **190**: 401–415.
- Tsumori, H. and Kuramitsu, H. (1997) The role of the *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: essential role of the GtfC enzyme. *Oral Microbiol Immunol* **12**: 274–280.
- Vendeville, A., Winzer, K., Heurlier, K., Tang, C.M. and Hardie, K.R. (2005) Making ‘sense’ of metabolism: autoinducer-2, LuxS and pathogenic bacteria. *Nat Rev Microbiol* **3**: 383–396.
- Waters, C.M. and Bassler, B.L. (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**: 319–346.
- Wen, Z.T. and Burne, R.A. (2002) Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. *Appl Environ Microbiol* **68**: 1196–1203.
- Wen, Z.T. and Burne, R.A. (2004) LuxS-mediated signaling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation. *J Bacteriol* **186**: 2682–2691.
- Wen, Z.T., Browngardt, C. and Burne, R.A. (2001) Characterization of two operons that encode components of fructose-specific enzyme II of the sugar:phosphotransferase system of *Streptococcus mutans*. *FEMS Microbiol Lett* **205**: 337–342.
- Wen, Z.T., Baker, H.V. and Burne, R.A. (2006) Influence of BrpA on critical virulence attributes of *Streptococcus mutans*. *J Bacteriol* **188**: 2983–2992.
- Winzer, K., Hardie, K.R. and Williams, P. (2003) LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria. *Adv Appl Microbiol* **53**: 291–396.
- Yoshida, A. and Kuramitsu, H.K. (2002) *Streptococcus mutans* biofilm formation: utilization of a gtfB promoter-green fluorescent protein (Pg<sub>gtfB</sub>::gfp) construct to monitor development. *Microbiology* **148**: 3385–3394.
- Yoshida, A., Ansai, T., Takehara, T. and Kuramitsu, H.K. (2005) LuxS-based signaling affects *Streptococcus mutans* biofilm formation. *Appl Environ Microbiol* **71**: 2372–2380.
- Zeng, L., Wen, Z.T. and Burne, R.A. (2006) A novel signal transduction system and feedback loop regulate fructan hydrolase gene expression in *Streptococcus mutans*. *Mol Microbiol* **62**: 187–200.

**SUPPORTING INFORMATION**

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

**Figure S1.** Representative confocal laser scanning microscopy images of *Streptococcus mutans* biofilms grown on glass slides.

**Figure S2.** Schematic diagram of *smu.44* and *smu.46* and their flanking regions.

**Table S1.** Classification of genes identified in LuxS-deficient *Streptococcus mutans* planktonic cultures ( $P < 0.01$ ).

**Table S2.** Classification of genes identified in LuxS-deficient *Streptococcus mutans* biofilms ( $P < 0.01$ ).

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