INFECTION AND IMMUNITY, June 2005, p. 3351–3357 Vol. 73, No. 6
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.6.3351–3357.2005

Inactivation of *Streptococcus gordonii* SspAB Alters Expression of Multiple Adhesin Genes

Yongshu Zhang,[1] Yu Lei,[1] Angela Nobbs,[1] Ali Khammanivong,[1] and Mark C. Herzberg[1,2]*

Department of Oral Sciences, School of Dentistry,[1] and Mucosal and Vaccine Research Center,[2] University of Minnesota, Minneapolis, Minnesota 55455

Received 5 October 2004/Returned for modification 15 November 2004/Accepted 14 January 2005

SspA and SspB (antigen I/II family proteins) can bind *Streptococcus gordonii* to other oral bacteria and also to salivary agglutinin glycoprotein, a constituent of the salivary film or pellicle that coats the tooth. To learn if SspA and SspB are essential for adhesion and initial biofilm formation on teeth, *S. gordonii* DL1 was incubated with saliva-coated hydroxyapatite (sHA) for 2 h in Todd-Hewitt broth with 20% saliva to develop initial biofilms. Sessile cells attached to sHA, surrounding planktonic cells, and free-growing cells were recovered separately. Free-growing cells expressed more *sspA*-specific mRNA and *sspB*-specific mRNA than sessile cells. Free-growing cells expressed the same levels of *sspA* and *sspB* as planktonic cells. Surprisingly, an SspA$^-$ SspB$^-$ mutant strain showed 2.2-fold greater biofilm formation on sHA than wild-type *S. gordonii* DL1. To explain this observation, we tested the hypothesis that inactivation of *sspA* and *sspB* genes altered the expression of other adhesin genes during initial biofilm formation in vitro. When compared to wild-type cells, expression of *scaA* and *abpB* was significantly up-regulated in the SspA$^-$ SspB$^-$ strain in sessile, planktonic, and free-growing cells. Consistent with this finding, ScaA antigen was also overexpressed in planktonic and free-growing SspA$^-$ SspB$^-$ cells compared to the wild type. SspA/B adhesins, therefore, were strongly suggested to be involved in the regulation of multiple adhesin genes.

Formation of dental plaque is initiated by adhesion of pioneer colonizers to the saliva-coated film or pellicle that coats the tooth. The salivary pellicle expresses specific receptor-like molecules that adsorb from salivary fluid onto the hydroxyapatite (HA)-rich enamel. Adhesion is facilitated when pellicle receptors bind bacterial cell surface adhesins (3). Among these adhesins, antigen I/II proteins are the best characterized and expressed by all oral streptococci, including the early colonizer *Streptococcus gordonii* (5, 14) (B. Grove et al., unpublished data). In *S. gordonii*, SspA and SspB represent the antigen I/II family. Encoded by tandem genes with high homology, SspA and SspB expressed on the cell wall bind to a high-molecular-weight salivary agglutinin glycoprotein (SAG), which is found in the pellicle (4, 5). Mutations in *sspA* or *sspB* cause reduced adhesion of *S. gordonii* to SAG-coated surfaces (5, 14), but it was unclear how adhesion to heterogeneous salivary pellicle with alternative possible pellicle receptors (19, 39) would be affected.

Expression of *sspA* and *sspB* is regulated by environmental signals, including temperature, pH, osmolarity, and saliva (6, 8). SspB may also be transcriptionally regulated by SspA (7). Little is known, however, about the expression of *sspA* and *sspB* by cells associated with early biofilms that form on saliva-coated surfaces. We sought to define the roles of SspA and SspB in the initiation of oral biofilms. To detect the expression of *sspA* and *sspB* in biofilms formed on saliva-coated HA (sHA), a novel oral biofilm model developed in our laboratory (41) was used. With this model, we also analyzed the effect of inactivation of SspA and SspB on the development of initial *S. gordonii* biofilms on sHA.

* Corresponding author. Mailing address: Department of Oral Sciences and Mucosal and Vaccine Research Center, University of Minnesota, 17-164 Moos Tower, 515 Delaware St., SE Minneapolis, MN 55455. Phone: (612) 625-8404. Fax: (612) 626-2651. E-mail: mcherzb@umn.edu.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. gordonii* DL1 (28) was grown in Todd-Hewitt broth (THB) at 37°C in 5% CO_2. *S. gordonii* DL1 OB220 (*sspA*::*ermAM*) (5) and OB219 (*sspA'sspB'*::*ermAM*) (5) were grown in THB supplemented with erythromycin at 5 μg ml^{-1}. *S. gordonii* DL1 SspB$^-$ was grown in THB supplemented with spectinomycin at 500 μg ml^{-1}. Howard F. Jenkinson, University of Bristol, Bristol, United Kingdom, graciously provided the strains.

Preparation of sHA. Human whole saliva was collected from several donors and pooled as described previously (12) using an informed-consent protocol that was reviewed and approved by the University of Minnesota Institutional Review Board. To prepare the sHA used for the biofilm assay as described previously (12), 100 mg ceramic HA (ICN, Aurora, Ohio) was equilibrated for 1 h with 1 mM KH_2PO_4-K_2HPO_4 buffer (pH 6.8) with 50 mM KCl, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ (modified Gibbons' buffer) at ambient temperature. Equilibrated HA was then incubated with 3 ml of sterile whole saliva for 1 h at 37°C in 5% CO_2 and washed three times with ice-cold modified Gibbons' buffer. To prepare sHA for the adhesion assay in buffer, 20 mg of HA was equilibrated for 1 h with modified Gibbons' buffer at ambient temperature. Immediately before use, equilibrated HA was incubated with 1 ml of sterile whole saliva for 1 h at ambient temperature and then washed three times with modified Gibbons' buffer.

sHA adhesion assay. The sHA adhesion assay used was a modification of methods used by Liljemark et al. (21) and Tellefson and Germaine (36). *S. gordonii* DL1 and an SspA$^-$ SspB$^-$ mutant strain were cultured overnight with [^{3}H]thymidine (10 μCi ml^{-1}) in chemically defined synthetic medium (37), centrifuged, and adjusted with modified Gibbons' buffer to give a 2.5 $\times 10^{10}$-cells/ml suspension. The cells (1 ml, ranging from 10^7 to 10^{10}) were then incubated with 20 mg of sHA for 1 h at ambient temperature with continuous inversion on a roto-torque. Under these conditions, there is no detectable synthesis of new streptococcal proteins (M. C. Herzberg and H. F. Jenkinson, unpublished observations). The unattached cells were removed by aspiration. The sHA with attached bacteria was washed three times to remove additional unattached cells. The radioactivity associated with sHA was monitored by liquid scintillation counting. Bacterial suspensions of known concentration were used to calculate the number of radioactive counts per minute per input cell. From this, numbers of adherent or sHA-associated bacteria were determined.

TABLE 1. Oligonucleotide primers used in this study

Gene	Primer sequence
sspA	5′-TCCTGACAAACCTGAGACACC-3′
	5′-TTTAACTTTCAGAGCTTAGTTGCTTTC-3′
sspB	5′-TCCTGACAAACCTGAGACACC-3′
	5′-CATCAAAGATGAAACAAGTCTAAGC-3′
scaA	5′-CACCGAAGAAGAAGGCACTC-3′
	5′-TGTCTCCATCTTCGCCTTTT-3′
abpB	5′-CAAAAACTCCGGAAAAACCA-3′
	5′-GGAGCTTGACTCGGTTCTTG-3′
hsa	5′-CAGAGCTGCAAATCCAAACA-3′
	5′-GCCGAGATACTTGCGCTTAC-3′
cshB	5′-CGTTGTTCAGCAAGGATCAA-3′
	5′-GCCGTTCTGTTGTCCAGTAG-3′
abpA	5′-TGATGCAGTTGAAGGTGGAA-3′
	5′-TAGCTGCACCAACACGTTTC-3′
cshA	5′-CAGACGATGCAACCCCTATT-3′
	5′-TAACGGTCAAGGTCACCACA-3′
16S rRNA	5′-AAGCAACGCGAAGAACCTTA-3′
	5′-GTCTCGCTAGAGTGCCCAAC-3′

Initial sHA biofilm assay. *S. gordonii* DL1 (3 ml) cells were harvested from an overnight culture by centrifugation, resuspended in an equal volume of fresh THB with 20% whole saliva, mixed with 100 mg of fresh sHA, and incubated for 2 h with continuous inversion on a roto-torque at 37°C in 5% CO_2. The planktonic cells and sessile cells attached on sHA were recovered separately as described previously (41). In parallel, cells (3 ml) were cultured in suspension without sHA in THB with 20% saliva under the same incubation conditions. These cells grown in the absence of an adhesion surface were termed free-growing cells. Planktonic cells and free-growing cells were harvested by centrifugation.

RNA extraction. Collected sessile, planktonic, and free-growing cells were resuspended in a lysing reagent (1 ml) containing 150 μl of 2 M sodium phosphate (pH 6.8), 72 μl of H_2O, and 778 μl of RLT (RNeasy Mini Kit; QIAGEN). The planktonic cells and free-growing cells were then mixed with 100 mg HA to correct for nonspecific adsorption of RNA from the sessile cell preparation. In their respective mixtures, sessile, planktonic, and free-growing cells were transferred to FastPrep Blue tubes and processed in a FastPrep FP120 machine (Bio 101) at a speed rating of 6 for 2 min. Disrupted cells were incubated for 5 min at 60°C to permit RNA to desorb from HA and then maintained for an additional 5 min at room temperature. After centrifugation (12,000 × *g*, 10 min), the supernatant was collected and RNA was prepared using an RNeasy Mini Kit (QIAGEN). The integrity of the RNA was checked by electrophoresis in 1.0% (wt/vol) nondenaturing agarose gels. The RNA obtained was then treated with DNase I (Promega, Madison, Wis.) for 2 h at 37°C. The concentration of RNA was determined by measuring the A_{260} in a spectrophotometer.

Reverse transcription. RNA (10 μg) was reverse transcribed into cDNA with random hexamer primers as described previously (41).

Oligonucleotide primers. The sequences of the primers used in this study are listed in Table 1 and were developed by Primer3 (version 0.2) (31). The source code is available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

Real-time PCR. Real-time quantitative PCR amplification, detection, and analysis were performed by the Mx3000 Real-Time PCR system (Stratagene) with Brilliant SYBR Green QPCR Master Mix (Stratagene). Real-time PCR was performed in 50-μl reaction mixtures (1× Brilliant SYBR Green QPCR Master Mix, 1 μM each forward primer and reverse primer, and 3 μl of template). The PCR conditions were (i) 95°C for 10 min and (ii) 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. All the amplified products were then subjected to dissociation curve analysis as follows: 95°C for 1 min, 55°C for 30 s, and 40 successive 30-s plateaus in which the temperature was increased by 1.0°C for each plateau. Purity of the products of PCR amplification was confirmed by electrophoresis in a 1.8% agarose gel.

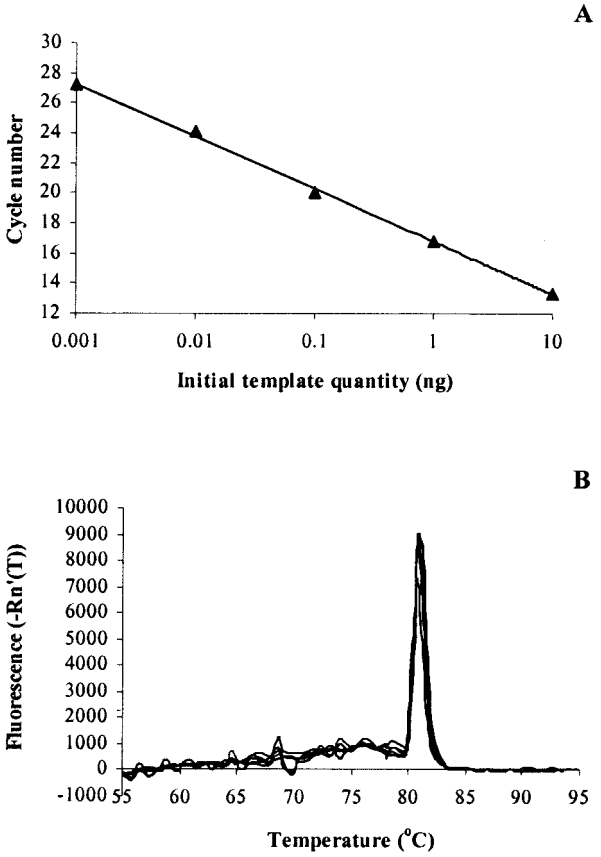

FIG. 1. Real-time PCR of *S. gordonii* DL1 *abpB* gene. (A) Standard curve. The standard curve was a plot of the initial template quantity (*x* axis) versus the threshold cycle (slope = −3.501, *x* intercept = 16.79, R^2 = 0.998, amplification efficiency = 93.0%). (B) Dissociation curve analysis of *abpB* real-time PCR products from reactions to prepare the standard curve as shown in panel A. The plot was based on the first derivative of the fluorescence reading multiplied by −1 [−Rn′(T)]. The specific PCR products melted at 81 ± 1°C.

To estimate the quantity of initial template in a sample, serial real-time PCR was performed with templates of purified *S. gordonii* DL1 genomic DNA (10 ng to 0.001 ng). For each gene, a standard curve was developed as the log of the quantity of initial template plotted against the threshold cycle values for the standard wells. In this way, differences in primer efficiency for each gene could be accommodated. The set of primers used in the real-time PCR for any specific gene was selected for an amplification efficiency ranging from 90 to 100% under the conditions described above (for example, see the *abpB* gene, Fig. 1). From the standard curve, *n*-fold differences could be determined in the level of initial template quantities of a specific gene in one sample relative to another. As reported previously (40, 41), 16S rRNA was selected as an internal standard to normalize for the amount of RNA in each sample.

Estimating the biomass of sessile and planktonic cells. The sHA biofilm assay was performed as described above, and sessile and planktonic cells were each recovered and suspended in 1 ml of 0.3 M sodium phosphate (pH 6.8). To ensure equivalent yields of nucleic acids, the planktonic cells were also mixed with 100 mg HA. In their respective mixtures, sessile and planktonic cells were transferred to FastPrep Blue tubes, processed in a FastPrep FP120 machine (Bio 101) at a speed rating of 6 for 2 min, and centrifuged (12,000 × *g*) and the supernatants were collected. From each supernatant, the biomass of sessile or planktonic cells was estimated as the 16S rRNA gene content of genomic DNA as determined by real-time PCR. The diluted supernatant (1:100) solution (3 μl) was used as the template for a real-time PCR as described above. The sHA biofilm-forming ability of each strain was calculated as the ratio of sessile biomass to total biomass of sessile and planktonic cells.

TABLE 2. *sspA*- and *sspB*-specific mRNA *n*-fold difference in planktonic, sessile, and free-growing *S. gordonii* DL1 cells

Gene	Planktonic cells relative to sessile cells	Free-growing cells relative to sessile cells	Planktonic cells relative to free-growing cells
sspA	1.5	2.0[a]	0.8
sspB	1.8	1.9	1.0

[a] $P < 0.05$.

Preparation of cell lysates for protein antigen analysis. Free-growing and planktonic cells (12 ml) from the sHA biofilm assay were collected as described above, washed, and resuspended in 6 ml of ice-cold phosphate-buffered saline. Collected cells were sonicated on ice for 5 min at a 50-W output and centrifuged at 20,000 × *g* for 15 min. The soluble supernatant was concentrated by ultrafiltration using an Amicon Ultra-15 with an Ultracel PL-10 membrane (Millipore, Bedford, MA), and protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

SDS-PAGE and Western blotting. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17). Gels were stained with Coomassie brilliant blue (Bio-Rad, Hercules, CA) and destained in a methanol-acetic solution (40% methanol, 10% acetic acid). Prestained molecular mass standards (ranging from 207 kDa to 7.7 kDa) were purchased from Bio-Rad. Western blotting was performed by electrotransfer of proteins to nitrocellulose membranes with a trans-blot SD semidry electrophoretic transfer cell (Bio-Rad, Hercules, CA). The membrane was then incubated with rabbit anti-FimA serum (38) and visualized with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Bio-Rad, Hercules, CA) and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-Nitro Blue Tetrazolium (Sigma, St. Louis, MO).

Slot blotting. Total proteins (1 μg protein as estimated by the BCA assay) were slot blotted onto nitrocellulose membrane by a Hybri-slot manifold (Bethesda Research Laboratories, Bethesda, MD) following the instructions of the manufacturer. The membrane was then incubated with rabbit antiserum against paraformaldehyde-fixed cells of *S. gordonii* DL1 (Pacific Immunology Corp., Ramona, CA) and visualized as described for Western blotting.

Statistics. Gene expression and biofilm formation by *S. gordonii* strains were compared for differences using an unpaired two-tailed *t* test.

RESULTS

Sessile cells and planktonic *S. gordonii* cells associated with sHA biofilms differentially express multiple adhesin genes. *S. gordonii* initial biofilms were developed in vitro by incubation in THB medium with 20% saliva for 2 h at 37°C in the presence of sHA. Sessile and planktonic cells were recovered and compared for expression of *sspA* and *sspB* with free-growing cells grown in separate suspension cultures. Free-growing cells expressed more *sspA*-specific mRNA and *sspB*-specific mRNA than sessile cells (Table 2). Free-growing cells expressed the same levels of *sspA* and *sspB* as planktonic cells.

Increased sHA biofilm formation by inactivation of SspA, SspB, or SspAB in *S. gordonii*. To test whether SspA and SspB contribute to initial biofilm development on sHA, *S. gordonii* DL1, SspA$^-$, SspB$^-$, and SspA$^-$ SspB$^-$ cells were compared (Fig. 2). *S. gordonii* SspA$^-$ showed 1.4-fold, SspB$^-$ showed 1.6-fold, and SspA$^-$ SspB$^-$ showed 2.2-fold greater biofilm-forming ability on sHA than wild-type cells based upon the cellular content of genomic DNA as marked by the 16S rRNA gene.

In otherwise identical experiments, total RNA was quantified from sessile initial biofilms formed by SspA$^-$ SspB$^-$ and wild-type cells. Total RNA extracted from SspA$^-$ SspB$^-$ sessile cells was significantly higher than the total RNA from the wild-type (Fig. 3). Total RNA extracted from SspA$^-$ SspB$^-$ planktonic cells, however, was significantly lower than

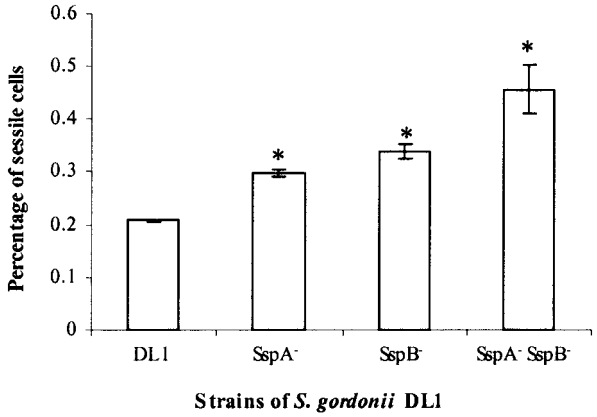

FIG. 2. Initial biofilm formation on sHA by *S. gordonii* DL1 and SspA$^-$, SspB$^-$, and SspA$^-$ SspB$^-$ inactivation mutants. Wild-type and mutant strains were incubated with fresh sHA in THB containing 20% saliva with continuous rotation for 2 h at 37°C. Sessile cells attached to sHA and planktonic cells were collected separately and disrupted. Biomass was measured by real-time PCR as described in Materials and Methods. Data shown are the mean ± standard error ($n = 3$). *, $P < 0.05$.

the total RNA from the wild type. In contrast, similar amounts of total RNA were extracted from free-growing SspA$^-$ SspB$^-$ mutant and wild-type cells. Based on total RNA content, the SspA$^-$ SspB$^-$ cells formed initial biofilms of 1.84-fold greater mass than the wild type, consistent with the data obtained by quantifying the genomic DNA.

Differential expression of adhesin genes by *S. gordonii* DL1 and *S. gordonii* OB219 (SspA$^-$ SspB$^-$). Since SspAB inactivation resulted in a significant increase in biofilm formation, we hypothesized that inactivation of SspAB altered the expression of other adhesin genes. *S. gordonii* DL1 and OB219 (SspA$^-$

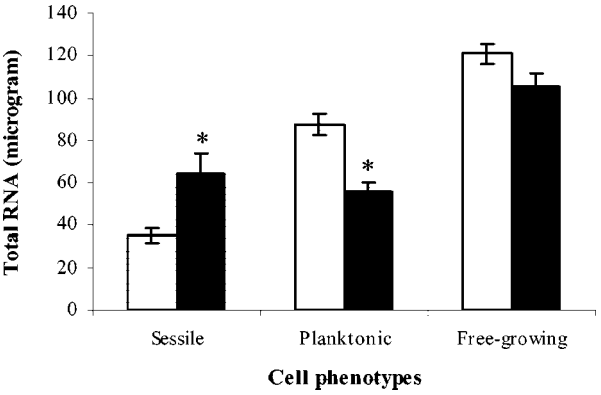

FIG. 3. Total RNA extracted from sessile, planktonic, and free-growing cells of *S. gordonii* DL1 and the SspA$^-$ SspB$^-$ mutant. Wild-type and mutant strains were incubated with fresh sHA in THB containing 20% saliva with continuous rotation for 2 h at 37°C. In parallel, cells were cultured in suspension without sHA in THB with 20% saliva under the same incubation conditions. These cells grown in the absence of an adhesion surface were termed free-growing cells. Sessile cells attached to sHA, planktonic cells, and free-growing cells were collected separately and disrupted. Total RNA was extracted, purified, and quantified as described in Materials and Methods. Symbols: □, *S. gordonii* DL1 wild-type strain; ■, SspA$^-$ SspB$^-$ mutant strain. Data shown are the mean ± standard error ($n \geq 4$). *, $P < 0.05$.

TABLE 3. Adhesin gene-specific mRNA *n*-fold differences between the *sspA sspB* mutant relative to the *S. gordonii* DL1 wild type recovered as initial planktonic and sessile biofilm cells and free-growing cells

Adhesin gene	Planktonic cells	Sessile cells	Free-growing cells
scaA	2.8[a]	2.2[a]	1.9[a]
abpB	2.3[a]	1.9[a]	2.2[a]
hsa	0.7	0.8	0.6
cshB	0.6	0.9	0.5
abpA	1.2	1.1	1.1
cshA	1.2	1.1	1.1

[a] $P < 0.05$.

$SspB^-$) sHA sessile and planktonic biofilm cells and free-growing cells were then compared for expression of other adhesin genes involved in the adhesion to saliva-coated surfaces and coaggregation with other oral bacteria. Compared to wild-type cells, the $SspA^-$ $SspB^-$ strain expressed significantly more *scaA* and *abpB* in sessile, planktonic, and free-growing cells (Table 3). Sessile, planktonic, and free-growing mutant and wild-type cells expressed similar levels of *hsa*, *cshB*, *abpA*, and *cshA*.

sHA adhesion by $SspA^-$ $SspB^-$ strain. We sought to determine if the increase in biofilm formation by $SspA^-$ $SspB^-$ cells was attributable to the adhesion of a greater number of cells to sHA. Adhesion of washed cells to sHA in modified Gibbons' buffer was compared at 1 h at ambient temperature. $SspA^-$ $SspB^-$ and wild-type cells adhered similarly to sHA (Fig. 4).

Differential production of ScaA protein by *S. gordonii* DL1 and *S. gordonii* OB219 ($SspA^-$ $SspB^-$). To show that increased expression of the *scaA* gene as shown by real-time reverse transcription-PCR and the gain-of-function results in the initial biofilm assay are associated with higher levels of ScaA protein antigen, sonic cell lysates of planktonic and free-growing cells of *S. gordonii* DL1 and the $SspA^-$ $SspB^-$ strain were prepared. Total protein was estimated by the BCA assay and identical amounts were loaded into each lane for separation by SDS-PAGE (Fig. 5A), followed by Western blotting with anti-FimA serum (Fig. 5B). ScaA protein from *S. gordonii* reacts with anti-FimA serum (38); FimA is the conserved homologue of ScaA in *Streptococcus parasanguinis* (16). Using the anti-FimA probe, free-growing and planktonic $SspA^-$ $SspB^-$ cells appeared to produce more ScaA antigen than wild-type *S. gordonii* DL1. Since the levels of expression of some wild-type and mutant strain proteins appeared to differ in SDS-PAGE, we verified independently that each of the preparations contained the same amount of total antigen per μg of BCA protein. The cell lysates were slot blotted onto nitrocellulose and reacted with rabbit antiserum against whole cells of *S. gordonii* DL1 (Fig. 5C). Each slot corresponding to lanes 2 to 5 appeared to contain the same amount of antigen.

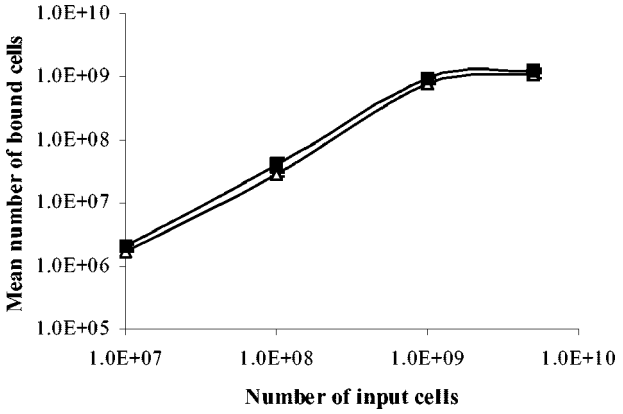

FIG. 4. Adhesion of *S. gordonii* DL1 and $SspA^-$ $SspB^-$ mutant strains to sHA. Both strains were cultured with [^{3}H]thymidine overnight in FMC (37) and washed. Cells (1 ml, containing 10^7 to 10^{10} cells) were incubated with sHA for 1 h at room temperature with continuous rotation. The radioactivity associated with sHA was monitored by liquid scintillation counting. Bacterial suspensions of known concentration were used to calculate the number of radioactive counts per minute per input cell. From this, numbers of adherent or sHA-associated bacteria were determined. Symbols: △, *S. gordonii* DL1 wild-type strain; ■, $SspA^-$ $SspB^-$ mutant strain. Values are given as the mean number of cells bound ± the standard deviation, based on two independent experiments performed in duplicate.

DISCUSSION

In *S. gordonii*, adhesin genes have been suggested to be critical to the ability of these organisms to adhere and colonize biological substrates such as the teeth. The diverse spectrum and abundance of adhesins serve to bind cells to salivary pellicle, soft tissues, and other bacteria with remarkable specificity. In *S. gordonii*, the spectrum of adhesins includes SspA, SspB, AbpA, AbpB, CshA, CshB, and sialic acid-binding protein (Hsa).

A novel oral biofilm model (41) was utilized to test whether expression of *sspA* and *sspB* were regulated during the initiation of biofilms on sHA. Using this model, we now show that sessile and free-growing cells differentially expressed *sspA* and *sspB*, suggesting that production of SspA and SspB may be modulated during the initiation of biofilms on sHA.

In a previous study, we identified a novel two-component system, BfrAB, which is required for biofilm formation by *S. gordonii* (41). Inactivation of BfrAB reduces adhesion to sHA. Furthermore, the *bfrAB* gene is differentially expressed by sessile cells and planktonic cells from sHA biofilms (41), suggesting that BfrAB may be involved in the initiation of biofilms on sHA by *S. gordonii*. It not yet known whether BfrAB is involved in the regulation of *sspA* and *sspB* in initial biofilms. Differential expression of functionally varied genes between sessile and planktonic cells in sHA biofilms, however, suggests that the initiation of oral biofilms is a tightly regulated process.

To test whether inactivation of SspAB affects the initiation of sHA biofilm formation by *S. gordonii*, the $SspA^-$ strain and the $SspB^-$ strain were compared with wild-type *S. gordonii* DL1 in a 2-h sHA biofilm assay. The biomass formed by the strains was estimated by comparing the amounts of 16S rRNA gene. Quantitative real-time PCR was used since it has been shown to be a rapid, reliable, and sensitive method to compare the relative amounts of cells (22). Contrary to our expectations, both the $SspA^-$ and $SspB^-$ strains showed greater biofilm formation on sHA than the wild-type strain. Dual inactivation of SspA and SspB ($SspA^-$ $SspB^-$ strain) further increased the biofilm-forming ability on sHA.

Consistent with these results, under otherwise identical conditions, total RNA extracted from the $SspA^-$ $SspB^-$ sessile

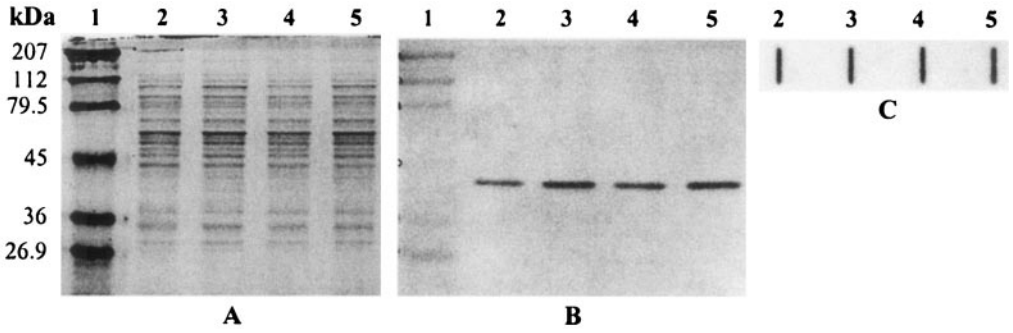

FIG. 5. Expression of FimA-cross-reactive antigens. Planktonic and free-growing cells from *S. gordonii* DL1 and the SspA$^-$ SspB$^-$ mutant were lysed by sonication and centrifuged to clarity, and the proteins in the supernatant were resolved by 10% SDS-PAGE. A replicate gel was stained with Coomassie brilliant blue (A). Resolved proteins were then electrotransferred to nitrocellulose membrane and probed with anti-FimA serum in a Western blot assay (B). Prestained molecular weight standards are shown at the left. Each lane contained 1 μg of protein. Total proteins (1 μg) were also slot blotted onto nitrocellulose membrane and probed with anti-*S. gordonii* DL1 serum (C). Lane 1, standards. Lane 2, free-growing cells of *S. gordonii* DL1. Lane 3, free-growing cells of SspA$^-$ SspB$^-$ mutant strain. Lane 4, planktonic cells of *S. gordonii* DL1. Lane 5, planktonic cells of SspA$^-$ SspB$^-$ mutant strain.

cells was significantly higher than the total RNA from the wild type (Fig. 3). Total RNA extracted from the SspA$^-$ SspB$^-$ planktonic cells, however, was significantly lower than the total RNA from the wild-type cells. On the other hand, similar amounts of total RNA were recovered from parallel free-growing cells of the SspA$^-$ SspB$^-$ mutant and the wild type. These data suggest that the greater sessile biomass of SspA$^-$ SspB$^-$ mutant cells is not caused by the intrinsic differences in growth rates between the SspA$^-$ SspB$^-$ strain and the *S. gordonii* DL1 wild type.

Since multiple adhesins may be involved in the initiation of biofilms, we hypothesized that inactivation of SspAB modulated the profile of adhesin gene expression during initial biofilm formation in vitro by *S. gordonii*. The SspA$^-$ SspB$^-$ and wild-type strains were compared for expression of six adhesin genes of *S. gordonii*, which are known to bind the cell to saliva-coated glass surfaces or other oral bacteria as coaggregation partners (13, 14). These adhesin genes included *scaA*, *hsa*, *abpB*, *abpA*, *cshA*, and *cshB*. ScaA mediates *S. gordonii* coaggregation with *Actinomyces naeslundii* (1, 16). SsaB, the counterpart of ScaA in *S. sanguis*, is involved in the coaggregation with *Actinomyces* and adhesion to sHA (9, 10). The sialic acid-binding protein (Hsa) binds glycoconjugates found in salivary mucins (18, 33), which are major pellicle constituents, and platelets (34). AbpA (20) and AbpB (29) are two amylase-binding proteins in *S. gordonii*. *S. gordonii* AbpA may be the major ligand for binding to amylase in salivary pellicle (29). Inactivation of AbpA reduces the adhesion of *S. gordonii* to amylase-coated HA, biofilm formation in a saliva-coated flow cell system (30), and oral biofilm development in rats (35). AbpB, however, may be required to initiate oral biofilms in starch-eating rats (35). CshA and CshB are involved in the determination of streptococcal cell surface hydrophobicity (24, 26), coaggregation with *Actinomyces* (25), binding to immobilized fibronectin (23), and mucosal colonization of mice (25). The multiplicity of adhesins, each with different structures or alternatively expressed (e.g., CshA and CshB), would likely explain the complex specificity and tropisms shown by the oral streptococci for saliva-coated hard and soft tissue surfaces (11). While CshA levels were up-regulated in *S. sanguis* biofilms formed on glass beads (2), there are no reports known to us to indicate that adhesion of oral streptococci to saliva-coated tooth-like surfaces regulates the expression of adhesin genes.

We now show that inactivation of SspAB significantly up-regulated the expression of *scaA* and *abpB* in sessile, planktonic, and free-growing cells using a real-time reverse transcription-PCR method. Planktonic and free-growing SspA$^-$ SspB$^-$ cells also appeared to express more ScaA antigen than wild-type *S. gordonii* DL1. We attempted to estimate levels of ScaA protein in sessile cells from the wild-type and mutant strains as we reported for RNA (41). Proteins isolated from sessile cells are mixed with salivary proteins associated with sHA. While total protein can be recovered, the proportion that represents bacterial proteins is difficult to quantify. An efficient method to separate sessile cells from the sHA is being developed. Given the consistency that we have shown between *scaA*-specific mRNA and protein antigen levels, and the gain in associated function, the data show that small changes in gene expression may be biologically meaningful during biofilm formation. Pending further study, these findings suggest strongly that other low-magnitude changes in adhesin gene expression during initial biofilm formation may also be biologically important.

As reported previously for the SspA$^-$ strain (15), the washed SspA$^-$ SspB$^-$ strain and wild-type *S. gordonii* DL1 in Gibbons' buffer are now shown to adhere to sHA similarly. Based on the data from this study, SspA$^-$ SspB$^-$ free-growing cells may produce higher levels of adhesins such as ScaA. These adhesins may compensate for the loss of SspAB-mediated adherence to sHA.

Why did the SspA$^-$ SspB$^-$ mutant strain show "normal" sHA adhesion but significantly increased biofilm formation on sHA? In nutritionally rich environments, once bacteria contact attractive surfaces, an adherent biomass begins to accumulate. Sessile, planktonic, and free-growing cells express different phenotypes (27, 32, 41). In the sHA adhesion assay (Fig. 4), bacterial cells were washed, maintained, and assayed in buffer in the absence of carbon and nitrogen sources. Cells appear to be metabolically inactive, without detectable protein synthesis

(Herzberg and Jenkinson, unpublished). Under these conditions, adhering cells cannot reflect the sessile colonial phenotype that is acquired after adhering to sHA in nutrient-rich medium. The sHA adhesion assay therefore can test only the adhesion ability of free-growing cells. In contrast, bacterial cells were maintained and assayed in nutrient-rich medium (THB) in the sHA biofilm assay. The biofilm assay distinguishes the responses of the SspA$^-$ SspB$^-$ mutant and the wild type during initial adhesion to sHA and in the resulting sessile and planktonic cells. We speculate that inactivation of SspAB results in responses to initial adhesion to sHA that differ from the *S. gordonii* DL1 wild type. These altered responses to adhesion may contribute to the increased initial sHA biofilm formation by the SspA$^-$ SspB$^-$ mutant.

Adhesin genes appear to be tightly regulated. For example, SspA and SspB share a common receptor, a high-molecular-weight SAG found in the salivary pellicle that coats the tooth (4, 5). Inactivation of SspA reduces the expression of *sspB* (7). Hence, these two adhesins show similarities in structure, function, and at least one-directional coregulation. Since *bfrAB* appears to regulate initial biofilm formation on sHA (41), a coordinated signaling system may exist. Considering the new data in this report, we propose that SspAB, ScaA, and AbpB are the members of a closely regulated adhesin network.

It is not clear at this time how inactivation of SspAB alters the pattern of expression of these adhesin genes. SspA protein can bind to the upstream regulatory region of the *sspB* gene (7), and may act as a positive transcriptional regulator involved in the expression of *sspB*. We speculate that SspA and perhaps SspB can regulate the expression of *scaA* and *abpB* as negative transcriptional regulators. It is also possible that the proposed adhesin network includes other adhesin or nonadhesin components, of which some may perform regulator roles. Further analyses of the regulatory mechanisms in *S. gordonii* adhesin networks may provide new insights into the processes of adhesion and initial biofilm formation.

ACKNOWLEDGMENTS

This work was supported by NIH grant R01 DE08590.

The assistance of Joel Rudney and James Hodges with the statistical analyses is appreciated.

REFERENCES

1. **Andersen, R. N., N. Ganeshkumar, and P. E. Kolenbrander.** 1993. Cloning of the *Streptococcus gordonii* PK488 gene, encoding an adhesin which mediates coaggregation with *Actinomyces naeslundii* PK606. Infect. Immun. **61:** 981–987.
2. **Black, C., I. Allan, S. K. Ford, M. Wilson, and R. McNab.** 2004. Biofilm-specific surface properties and protein expression in oral *Streptococcus sanguis*. Arch. Oral Biol. **49:**295–304.
3. **Cassels, F. J., C. V. Hughes, and J. L. Nauss.** 1995. Adhesin receptors of human oral bacteria and modeling of putative adhesin-binding domains. J. Ind. Microbiol. **15:**176–185.
4. **Demuth, D. R., P. Berthold, P. S. Leboy, E. E. Golub, C. A. Davis, and D. Malamud.** 1989. Saliva-mediated aggregation of *Enterococcus faecalis* transformed with a *Streptococcus sanguis* gene encoding the SSP-5 surface antigen. Infect. Immun. **57:**1470–1475.
5. **Demuth, D. R., Y. Duan, W. Brooks, A. R. Holmes, R. McNab, and H. F. Jenkinson.** 1996. Tandem genes encode cell-surface polypeptides SspA and SspB which mediate adhesion of the oral bacterium *Streptococcus gordonii* to human and bacterial receptors. Mol. Microbiol. **20:**403–413.
6. **Du, L. D., and P. E. Kolenbrander.** 2000. Identification of saliva-regulated genes of *Streptococcus gordonii* DL1 by differential display using random arbitrarily primed PCR. Infect. Immun. **68:**4834–4837.
7. **El-Sabaeny, A., D. R. Demuth, and R. J. Lamont.** 2001. Regulation of *Streptococcus gordonii sspB* by the *sspA* gene product. Infect. Immun. **69:** 6520–6522.
8. **El-Sabaeny, A., D. R. Demuth, Y. Park, and R. J. Lamont.** 2000. Environmental conditions modulate the expression of the *sspA* and *sspB* genes in *Streptococcus gordonii*. Microb. Pathog. **29:**101–113.
9. **Ganeshkumar, N., P. M. Hannam, P. E. Kolenbrander, and B. C. McBride.** 1991. Nucleotide sequence of a gene coding for a saliva-binding protein (SsaB) from *Streptococcus sanguis* 12 and possible role of the protein in coaggregation with actinomyces. Infect. Immun. **59:**1093–1099.
10. **Ganeshkumar, N., M. Song, and B. C. McBride.** 1988. Cloning of a *Streptococcus sanguis* adhesin which mediates binding to saliva-coated hydroxyapatite. Infect. Immun. **56:**1150–1157.
11. **Gibbons, R. J.** 1989. Bacterial adhesion to oral tissues: a model for infectious diseases. J. Dent. Res. **68:**750–760.
12. **Gong, K., L. Mailloux, and M. C. Herzberg.** 2000. Salivary film expresses a complex, macromolecular binding site for *Streptococcus sanguis*. J. Biol. Chem. **275:**8970–8974.
13. **Jenkinson, H. F.** 1994. Cell surface protein receptors in oral streptococci. FEMS Microbiol. Lett. **121:**133–140.
14. **Jenkinson, H. F., and D. R. Demuth.** 1997. Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. Mol. Microbiol. **23:** 183–190.
15. **Jenkinson, H. F., S. D. Terry, R. McNab, and G. W. Tannock.** 1993. Inactivation of the gene encoding surface protein SspA in *Streptococcus gordonii* DL1 affects cell interactions with human salivary agglutinin and oral actinomyces. Infect. Immun. **61:**3199–3208.
16. **Kolenbrander, P. E., R. N. Andersen, and N. Ganeshkumar.** 1994. Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene, *scaA*, and ATP-binding cassette. Infect. Immun. **62:**4469–4480.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
18. **Levine, M. J., M. C. Herzberg, M. S. Levine, S. A. Ellison, M. W. Stinson, H. C. Li, and T. van Dyke.** 1978. Specificity of salivary-bacterial interactions: role of terminal sialic acid residues in the interaction of salivary glycoproteins with *Streptococcus sanguis* and *Streptococcus mutans*. Infect. Immun. **19:**107–115.
19. **Li, J., E. J. Helmerhorst, R. F. Troxler, and F. G. Oppenheim.** 2004. Identification of in vivo pellicle constituents by analysis of serum immune responses. J. Dent. Res. **83:**60–64.
20. **Li, L., J. M. Tanzer, and F. A. Scannapieco.** 2002. Identification and analysis of the amylase-binding protein B (AbpB) and gene (*abpB*) from *Streptococcus gordonii*. FEMS Microbiol. Lett. **212:**151–157.
21. **Liljemark, W. F., C. G. Bloomquist, and G. R. Germaine.** 1981. Effect of bacterial aggregation on the adherence of oral streptococci to hydroxyapatite. Infect. Immun. **31:**935–941.
22. **Lyons, S. R., A. L. Griffen, and E. J. Leys.** 2000. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J. Clin. Microbiol. **38:**2362–2365.
23. **McNab, R., A. R. Holmes, J. M. Clarke, G. W. Tannock, and H. F. Jenkinson.** 1996. Cell surface polypeptide CshA mediates binding of *Streptococcus gordonii* to other oral bacteria and to immobilized fibronectin. Infect. Immun. **64:**4204–4210.
24. **McNab, R., and H. F. Jenkinson.** 1992. Gene disruption identifies a 290 kDa cell-surface polypeptide conferring hydrophobicity and coaggregation properties in *Streptococcus gordonii*. Mol. Microbiol. **6:**2939–2949.
25. **McNab, R., H. F. Jenkinson, D. M. Loach, and G. W. Tannock.** 1994. Cell-surface-associated polypeptides CshA and CshB of high molecular mass are colonization determinants in the oral bacterium *Streptococcus gordonii*. Mol. Microbiol. **14:**743–754.
26. **McNab, R., G. W. Tannock, and H. F. Jenkinson.** 1995. Characterization of CshA, a high molecular mass adhesin of *Streptococcus gordonii*. Dev. Biol. Stand. **85:**371–375.
27. **Oosthuizen, M. C., B. Steyn, J. Theron, P. Cosette, D. Lindsay, A. Von Holy, and V. S. Brozel.** 2002. Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. Appl. Environ. Microbiol. **68:**2770–2780.
28. **Pakula, R., and W. Walczak.** 1963. On the nature of competence of transformable streptococci. J. Gen. Microbiol. **31:**125–133.
29. **Rogers, J. D., E. M. Haase, A. E. Brown, C. W. Douglas, J. P. Gwynn, and F. A. Scannapieco.** 1998. Identification and analysis of a gene (*abpA*) encoding a major amylase-binding protein in *Streptococcus gordonii*. Microbiology **144**(Pt. 5)**:**1223–1233.
30. **Rogers, J. D., R. J. Palmer, Jr., P. E. Kolenbrander, and F. A. Scannapieco.** 2001. Role of *Streptococcus gordonii* amylase-binding protein A in adhesion to hydroxyapatite, starch metabolism, and biofilm formation. Infect. Immun. **69:**7046–7056.
31. **Rozen, S., and H. J. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers, p. 365–386. *In* S. Krawitz and S. Misener (ed.), Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, N.J.
32. **Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. **184:**1140–1154.
33. **Takahashi, Y., K. Konishi, J. O. Cisar, and M. Yoshikawa.** 2002. Identifi-

cation and characterization of *hsa*, the gene encoding the sialic acid-binding adhesin of *Streptococcus gordonii* DL1. Infect. Immun. **70:**1209–1218.
34. **Takahashi, Y., A. Yajima, J. O. Cisar, and K. Konishi.** 2004. Functional analysis of the *Streptococcus gordonii* DL1 sialic acid-binding adhesin and its essential role in bacterial binding to platelets. Infect. Immun. **72:**3876–3882.
35. **Tanzer, J. M., L. Grant, A. Thompson, L. Li, J. D. Rogers, E. M. Haase, and F. A. Scannapieco.** 2003. Amylase-binding proteins A (AbpA) and B (AbpB) differentially affect colonization of rats' teeth by *Streptococcus gordonii*. Microbiology **149:**2653–2660.
36. **Tellefson, L. M., and G. R. Germaine.** 1986. Adherence of *Streptococcus sanguis* to hydroxyapatite coated with lysozyme and lysozyme-supplemented saliva. Infect. Immun. **51:**750–759.
37. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11:**649–655.
38. **Viscount, H. B., C. L. Munro, D. Burnette-Curley, D. L. Peterson, and F. L. Macrina.** 1997. Immunization with FimA protects against *Streptococcus parasanguis* endocarditis in rats. Infect. Immun. **65:**994–1002.
39. **Yao, Y., E. A. Berg, C. E. Costello, R. F. Troxler, and F. G. Oppenheim.** 2003. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J. Biol. Chem. **278:**5300–5308.
40. **Yoshida, A., and H. K. Kuramitsu.** 2002. *Streptococcus mutans* biofilm formation: utilization of a *gtfB* promoter-green fluorescent protein (PgtfB::*gfp*) construct to monitor development. Microbiology **148:**3385–3394.
41. **Zhang, Y., Y. Lei, A. Khammanivong, and M. C. Herzberg.** 2004. Identification of a novel two-component system in *Streptococcus gordonii* V288 involved in biofilm formation. Infect. Immun. **72:**3489–3494.

Editor: J. B. Bliska