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Streptococcus gordonii Hsa Environmentally Constrains Competitive Binding by *Streptococcus sanguinis* to Saliva-Coated Hydroxyapatite▽

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Competition between pioneer colonizing bacteria may determine polymicrobial succession during dental plaque development, but the ecological constraints are poorly understood. For example, more *Streptococcus sanguinis* than *Streptococcus gordonii* organisms are consistently isolated from the same intraoral sites, yet *S. gordonii* fails to be excluded and survives as a species over time. To explain this observation, we hypothesized that *S. gordonii* could compete with *S. sanguinis* to adhere to saliva-coated hydroxyapatite (sHA), an in vitro model of the tooth surface. Both species bound similarly to sHA, yet 10- to 50-fold excess *S. gordonii* DL1 reduced binding of *S. sanguinis* SK36 by 85 to >95%. *S. sanguinis*, by contrast, did not significantly compete with *S. gordonii* to adhere. *S. gordonii* competed with *S. sanguinis* more effectively than other species of oral streptococci and depended upon the salivary film on HA. Next, putative *S. gordonii* adhesins were analyzed for contributions to interspecies competitive binding. Like wild-type *S. gordonii*, isogenic mutants with mutations in antigen I/II polypeptides (*sspAB*), amylase-binding proteins (*abpAB*), and Csh adhesins (*cshAB*) competed effectively against *S. sanguinis*. By contrast, an *hsa*-deficient mutant of *S. gordonii* showed significantly reduced binding and competitive capabilities, while these properties were restored in an *hsa*-complemented strain. Thus, Hsa confers a selective advantage to *S. gordonii* over *S. sanguinis* in competitive binding to sHA. Hsa expression may, therefore, serve as an environmental constraint against *S. sanguinis*, enabling *S. gordonii* to persist within the oral cavity, despite the greater natural prevalence of *S. sanguinis* in plaque and saliva.

Within the oral cavity there are hundreds of species with the potential to colonize the tooth surface. Thus, microbes that have a competitive binding advantage are likely to be successful. Competition occurs both between and within species and is strongest for those microbes that target a similar pool of receptors. Furthermore, the outcome of competitive events for one set of bacteria can be expected to influence that of another. This is particularly pertinent for pioneer colonizers, since initial adhesion to the tooth surface by oral microbes is essential for the development and composition of dental plaque. Understanding the factors that influence initial colonization could, therefore, contribute to our understanding of the ecological constraints that govern this polymicrobial community and may suggest novel preventive and control measures for dental plaque.

The pioneer colonizers *Streptococcus sanguinis* and *Streptococcus gordonii* are readily able to attach to the tooth surface (37). The promiscuous adhesive capabilities of these bacteria are facilitated, in part, by specific cell surface adhesins (16, 43, 59). *S. sanguinis* and *S. gordonii* have a high level of 16S rRNA sequence homology and, until recently, were classified as the same species (19). In addition, these two streptococcal species are isolated from the same intraoral sites. Since these streptococci express similar surface proteins, it would be predicted that these two species compete for binding to the same array of available host receptors.

That oral streptococci will compete to adhere to saliva-coated teeth has been suggested by in vitro studies (1, 23), but limited classification of the species and limited characterization of streptococcal surface adhesins meant definitive conclusions were not possible. While interspecies competition was sometimes observed, no explanation of the underlying mechanisms could be provided. Many streptococcal adhesins have now been characterized (16). This study, therefore, aimed to revisit competitive interspecies binding between *S. sanguinis* and *S. gordonii* and establish a molecular basis. Adhesion of a radiolabeled reference species to saliva-coated hydroxyapatite (sHA), an in vitro model of the tooth surface in the mouth, was measured in the presence of an unlabeled competing species to test the hypothesis that *S. gordonii* and *S. sanguinis* compete for binding to salivary receptors. *S. gordonii* was shown to be a strong competitor of *S. sanguinis* adhesion, while *S. sanguinis* was not competitive with *S. gordonii*. A panel of isogenic mutants abrogated in the expression of surface-expressed proteins associated with streptococcal adhesion and colonization, including SspAB, CshAB, ScaA, AbpAB, and Hsa, was screened. From these and subsequent complementation studies, the sialic acid-binding protein Hsa was shown to confer the competitive binding advantages to *S. gordonii*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Streptococci (listed in Table 1) were routinely grown in chemically defined synthetic medium (FMC) (56, 57) at 37°C in 5% CO_2. *Escherichia coli* DH5α cells were grown aerobically at 37°C in Luria-Bertani (LB) medium. When required, antibiotics were added to the medium at the following concentrations: erythromycin, 1 μg ml^{-1} (*S. gordonii*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
S. gordonii strains		
DL1 (Challis)	Wild type	33
V288	Wild type	G. Dunny, University of Minnesota
Blackburn	Wild type	J. Rudney, University of Minnesota
OB277[a]	*cshA3*::*cat cshB3*::*ermAM*	30
OB390[a]	*sspAB*::*ermAM cshA*::*aphA3 cshB*::*cat*	H. Jenkinson, University of Bristol
OB470[a]	*scaA*::*tet*	14
UB1360[a]	Δ(*sspA sspB*)::*aad9*	11
srtA mutant[a]	Δ(*srtA*)::*ermAM*	This study
srtA complemented[a]	Δ(*srtA*)::*ermAM*(pDL276 $srtA^+$)	This study
hsa mutant[a]	Δ(*hsa*)::*ermAM*	This study
hsa complemented[a]	Δ(*hsa*)::*ermAM*(pAS8741 hsa^+)	This study
abpAB mutant[a]	Δ(*abpA*)::*ermAM* Δ(*abpB*)::*aphA3*	This study
S. sanguinis strains		
SK36	Wild type	18
133-79	Wild type	12
S. cristatus CC5A	Wild type	J. Rudney, University of Minnesota
S. mitis SK306	Wild type	J. Rudney, University of Minnesota
S. oralis SK100	Wild type	J. Rudney, University of Minnesota
S. parasanguinis 15912	Wild type	J. Rudney, University of Minnesota
Plasmids		
pGEM-T Easy	3.0 kb; Ap^r; ColE1*ori*	Promega
pGEM-*abpA*::*ermAM*	pGEM-T Easy derived; containing *ermAM* cassette within flanking sequences of *abpA* gene	This study
pGEM-*abpB*::*aphA3*	pGEM-T Easy derived; containing *aphA3* cassette within flanking sequences of *abpB* gene	This study
pVA891	5.4 kb; Em^r, Cm^r; pACYC*ori*; *E. coli*-streptococcal shuttle vector	27
pSF151	3.5 kb; Km^r; ColE1*ori*; *E. coli*-streptococcal-integration shuttle vector	53
pDL276	6.9 kb; Km^r, ColE1*ori*, *E. coli*-streptococcal-integration shuttle vector	53
pAS8741	pAS40S carrying *hsa* within a 7.4-kb HindIII-SphI cloned fragment of *S. gordonii* DL1 genomic DNA; Sp^r	48

[a] Derived from DL1 (Challis).

OB390) or 5 μg ml^{-1} (*S. gordonii* OB277, *abpAB hsa srtA* mutants); kanamycin, 50 μg ml^{-1} (*E. coli*), 200 μg ml^{-1} (*S. gordonii* OB390), or 250 μg ml^{-1} (*S. gordonii abpAB* mutant); tetracycline, 10 μg ml^{-1} (*S. gordonii*); spectinomycin, 100 μg ml^{-1} (*S. gordonii*); ampicillin, 100 μg ml^{-1} (*E. coli*); and chloramphenicol, 25 μg ml^{-1} (*E. coli*).

sHA adhesion assay. The sHA adhesion assay employed was originally described by Liljemark et al. (25) and Tellefson and Germaine (55) and modified by Gong and Herzberg (6). In brief, human whole saliva was collected from five adult volunteers (as approved by the Committee on the Use of Human Subjects in Research, University of Minnesota) into a chilled tube on ice, pooled, and clarified by centrifugation (2,000 × *g*, 20 min, 4°C). Whole salivary supernatant (1 ml) was then incubated at ambient temperature for 1 h with 20 mg ceramic HA (ICN Biomedicals, Inc.) that had been equilibrated with modified Gibbons' buffer (1 mM KH_2PO_4-K_2HPO_4 buffer [pH 6.8] with 50 mM KCl, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$). The sHA was then washed three times with Gibbons' buffer and transferred to a fresh tube. Streptococci were grown in FMC for 16 to 20 h in the presence of 10 μCi ml^{-1} [*methyl*-^{3}H]thymidine to a specific activity of between 2×10^{-4} and 1.2×10^{-5} cpm $cell^{-1}$, centrifuged, and adjusted with Gibbons' buffer to give a suspension of 2.5×10^{10} cells ml^{-1}. Cells (ranging from 10^7 to 10^{10}) were incubated with 20 mg of sHA (1-ml final volume) for 1 h at ambient temperature with continuous inversion on a rototorque. Unattached cells were aspirated, and cells loosely associated with the sHA were removed by washing a further three times. The radioactivity associated with sHA was monitored by liquid scintillation counting. Bacterial suspensions of known concentration were used to calculate the number of cells per radioactive count per minute. From this, numbers of adherent or sHA-associated bacteria were determined. At the lowest input of 10^7 cells, radioactivity values ranged from 50 to 100 cpm. Background values were ≤15 cpm.

sHA interspecies competition assay. Competing species were grown in the absence of [*methyl*-^{3}H]thymidine, harvested, and adjusted to a 2.5×10^{10} cells ml^{-1} suspension in Gibbons' buffer. Noncompeting, radiolabeled cells (5×10^8) were prepared as described above for the sHA adhesion assay and then incubated with 20 mg sHA using continuous inversion for 1 h at ambient temperature in the presence of a 10- or 50-fold excess of unlabeled, competing cells (1-ml final volume). The sHA was then washed three times in Gibbons' buffer, and numbers of labeled cells bound to the sHA were determined by liquid scintillation counting, as described above for the sHA adhesion assay.

Genetic manipulations. Standard recombinant DNA techniques were employed, as described by Sambrook et al. (41). Plasmids (listed in Table 1) were purified from *E. coli* cells by using the QIAquick Spin Miniprep Purification kit (QIAGEN). Oligonucleotides (listed in Table 2) were synthesized by Integrated DNA Technologies, Inc. Chromosomal DNA was prepared from mutanolysin-treated streptococcal cells by using the QIAGEN 100/G Genomic Tip system. PCR products were purified using the High Pure PCR Product Purification kit (Roche). DNA restriction and modification enzymes were used under the conditions specified by the manufacturer (Promega).

TABLE 2. Primers used in this study

Primer	Sequence[a]	Source or reference
abpA.F1	TGATACTGCAGGAACCATC	This study
abpA.F2	CCATCGATGGTCGTAACTCGGAGTTACTACC	This study
abpA.R1	CCATCGATGGAATATCACTGGGCCATTG	This study
abpA.R2	CATGACCAAAGAGCCAAG	This study
abpB.F1	ACCAAGTCCAGAGGAAGC	This study
abpB.R1	CCATCGATGGTCTGTTGAGAAAGCCAGTC	This study
abpB.F2	CCATCGATGGTTTGAAAGTCCTAGACTCCC	This study
abpB.R2	CGGTGATTCTACCAGTTTC	This study
ermAM.F	CCATCGATGGCCATATCATAAAAATCGAAACAGC	This study
ermAM.R	CCATCGATGGTAGGGACCTCTTTAGCTCC	This study
aphA3.F	CCATCGATGGGGCTCCGTCGATACTATG	This study
aphA3.R	CCATCGATGGCCGATACAAATTCCTCGTAG	This study
srtA.F1	CGGGGTACCCATGGCCTGTAGCTCAATC	This study
srtA.R1	GGATCGATGGAAGGAAGCATAAGTTTAATGC	This study
srtA.F2	CCATCGATCCTTCTCGTCTTGCAACTC	This study
srtA.R2	CGCGAGCTCACCTAAGAGACGGTGACCAG	This study
hsaL3BamHI	CGCGGATCCGGATAAGATTTATCAAGACGATCACA	This study
hsaR3SacI	CGCGAGCTCTGTGGCAGACGATGGACTTA	This study
hsaL5KpnI	CGGGGTACCCAAACATAGTTTAAATGCTTTGGA	This study
hsaR5EcoRI	CCGGAATTCCCCCTCTACTTAATTTAATATCCCAAAA	This study
RealsrtA.F	ATGGAAAGCACAGCAACTTC	This study
RealsrtA.R	CTGTAATCCCGAAAACATGG	This study
hsaL1	CAGAGCTGCAAATCCAAACA	65
hsaR1	GCCGAGATACTTGCGCTTAC	65

[a] Underlined letters indicate restriction enzyme sites.

The *abpA* and *abpB* genes of *S. gordonii* DL1 were inactivated by allelic exchange with the erythromycin resistance determinant *ermAM* (amplified from pVA891) or the kanamycin resistance determinant *aphA3* (amplified from pSF151), respectively. PCR amplification with primers abpA.F1/abpA.R1 and abpA.F2/abpA.R2 of *S. gordonii* DL1 DNA template generated two fragments comprising the flanking sequences of the *abpA* gene (468 bp and 512 bp) with a unique ClaI site at their 3′ and 5′ ends, respectively. These were ligated via the ClaI site and cloned into pGEM-T Easy. A DNA fragment (1,023 bp) containing the *ermAM* gene was PCR amplified from plasmid pVA891 by using primers ermAM.F/ermAM.R, which incorporated ClaI restriction sites. The PCR product was digested with ClaI and ligated into the unique site within the combined *abpA* fragments, generating plasmid pGEM-*abpA*::*ermAM*. This process was then repeated to generate plasmid pGEM-*abpB*::*aphA3*, using the primers listed in Table 2. The insert DNA from these two plasmids was PCR amplified using primers abpA.F1/abpA.R2 or primers abpB.F1/abpB.R2, purified, and transformed into *S. gordonii* DL1, thus generating the double mutant. Confirmation of predicted insertions was obtained by PCR amplification and sequencing; loss of function was demonstrated by amylase blot overlay (36).

The *srtA* gene of *S. gordonii* DL1 was inactivated by allelic exchange with the erythromycin resistance determinant *ermAM* as described above, using primers srtA.F1/srtA.R1 and srtA.F2/srtA.R2.

To complement the *srtA* deletion mutant, a DNA fragment (1,484 bp) incorporating the entire *srtA* gene was PCR amplified from *S. gordonii* DL1 chromosomal DNA by using primers srtA.F1/srtA.R2. This was cloned into *E. coli*-streptococcal shuttle vector pDL276, generating plasmid pDL276-*srtA*. This construct was purified and used to transform the *S. gordonii srtA* mutant. Confirmation of predicted insertions was obtained by PCR amplification and sequencing. Complementation was confirmed by detection of the *srtA* RNA transcript by using primers RealsrtA.F/RealsrtA.R. RNA extraction and cDNA synthesis were performed as described below.

The *hsa* gene of *S. gordonii* DL1 was inactivated by allelic exchange with the erythromycin resistance determinant *ermAM*, using primers hsaL3BamH1/hsaR3SacI and hsaL5KpnI/hsaR5EcoR1, as described above.

To complement the *hsa* deletion mutant, the *S. gordonii hsa* mutant was transformed with plasmid pAS8741 (a gift of Y. Takahashi, Nippon Dental University, Tokyo, Japan), which carries *hsa* within a 7.4-kb HindIII-SphI cloned fragment of *S. gordonii* DL1 genomic DNA (48). Complementation was confirmed by detection of the *hsa* RNA transcript with primers hsaL1/hsaR1 (65) and by Western immunoblotting with a goat anti-Hsa antiserum (a gift of B. Bensing, University of California, San Francisco). RNA extraction and cDNA synthesis were performed as described below.

RNA extraction and cDNA synthesis. Bacterial cultures (2 ml) were harvested (3,000 × *g*, 20 min, 4°C) and resuspended in a lysing reagent containing 222 μl H_2O and 778 μl Buffer RLT (RNeasy Mini Kit; QIAGEN). Suspensions were transferred to FastPrep Blue tubes (QBIOgene, Morgan Irvine, CA) and processed in a FastPrep FP120 vibrator (Bio101) at a speed rating of 6 for 2 min. Cell debris was removed by centrifugation (12,000 × *g*, 10 min), the supernatant was collected, and RNA was prepared using an RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. The integrity of the RNA was confirmed by gel electrophoresis, and it was then treated with DNase I (Promega) for 2 h at 37°C. The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. RNA (2 μg) was reverse transcribed into cDNA with random hexamer primers as described previously (64).

Data analysis. Analysis of adhesion data and determination of significance were performed using Student's *t* test. Data are presented based on at least two independent experiments performed in duplicate. Differences were considered significant when a P value of <0.05 was obtained.

RESULTS

sHA adhesion by *S. gordonii* and *S. sanguinis*. As *S. gordonii* and *S. sanguinis* are pioneer colonizers, their ability to adhere to sHA models adhesion to the tooth. Given the array of surface adhesins that could potentially mediate binding to sHA, well-characterized laboratory strains were chosen to represent both species. Using a range of input bacteria, *S. gordonii* (strain DL1 or V288) and *S. sanguinis* SK36 were shown to adhere directly to sHA with similar isotherms (Fig. 1A). In each case, the binding curve appeared to approach saturation at approximately 10^9 bound cells per 20 mg of sHA. A comparable binding profile was also seen for *S. sanguinis* 133-79 (data not shown). In the absence of saliva, an input of 10^9 cells resulted in ≤5% adhesion for each strain (data not shown), indicating that these streptococcal strains interact primarily with a salivary component(s) in the saliva coating film or pellicle.

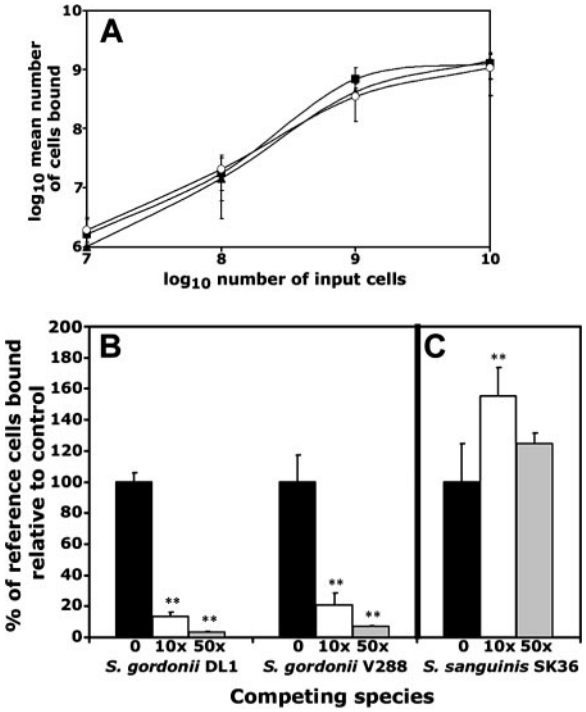

FIG. 1. Adhesion and competition between *S. gordonii* and *S. sanguinis* for sHA. (A) Symbols: ■, *S. gordonii* DL1; ▲, *S. gordonii* V288; ○, *S. sanguinis* SK36. Radioactively labeled bacteria were incubated with sHA for 1 h, and numbers of attached cells were determined as described in Materials and Methods. Data are presented as the mean ± standard deviation. (B and C) Radioactively labeled *S. sanguinis* SK36 cells (B) or *S. gordonii* V288 cells (C) were incubated with sHA in the presence of a 10-fold (□) or 50-fold (▨) excess of unlabeled competing cells (*S. gordonii* strain DL1 or V288 [B] or *S. sanguinis* SK36 [C]). Numbers of attached reference (labeled) cells were then determined as described in Materials and Methods. As a control, reference cells were incubated with sHA in the absence of any competing cells (■), and these adhesion levels were set to 100%. Data are shown as the relative percentage of cells bound ± standard deviation. **, $P < 0.01$ compared to controls in the absence of competition.

Competitive binding between *S. gordonii* and *S. sanguinis*. As *S. gordonii* and *S. sanguinis* bound sHA similarly, we hypothesized that these two species may compete when present together. This hypothesis was tested using an adapted sHA adhesion assay in which one (noncompeting) species is radioactively labeled, while a second (competing) species is unlabeled. Based upon the sHA binding profiles of *S. gordonii* and *S. sanguinis* (Fig. 1A), an input of 5×10^8 noncompeting cells was selected as producing half-maximal binding to sites on sHA. The adhesion of 5×10^8 noncompeting radiolabeled cells was arbitrarily set as 100%. Competition would, therefore, reduce the percentage bound; cooperativity would increase the percentage as maximal binding was approached. Noncompeting radiolabeled cells were then incubated with sHA in the presence of a 10- or 50-fold excess of competing cells, and numbers of bound noncompeting cells were determined by liquid scintillation counting. When present at a 10-fold excess, *S. gordonii* DL1 or V288 reduced binding levels of *S. sanguinis* SK36 by >80%, which increased to >95% at a 50-fold excess (Fig. 1B). A similar reduction in adhesion was also found with *S. sanguinis* 133-79 (data not shown). By contrast, *S. sanguinis* did not significantly impair the ability of *S. gordonii* DL1 to adhere to sHA at any concentration tested (data not shown) and actually promoted adhesion by *S. gordonii* V288 up to 50% (Fig. 1C), suggesting interspecies cooperativity in binding. The ability of *S. gordonii* to resist competition by *S. sanguinis* was dependent upon the presence of saliva and was not seen using HA alone (data not shown). In self-inhibition studies, in which unlabeled and labeled cells of the same strain were combined, the presence of a 10-fold excess of competing cells resulted in 65 to 75% inhibition (data not shown).

Competitive binding by other oral streptococci. To determine if the one-way competition was species specific, the abilities of *S. gordonii* and *S. sanguinis* to resist competition by other oral streptococci were examined. It was first confirmed that each of the species tested bound similarly to sHA. Competition with *S. sanguinis* SK36 was then investigated. Of the six competing strains tested, *S. gordonii* strains DL1 and Black-

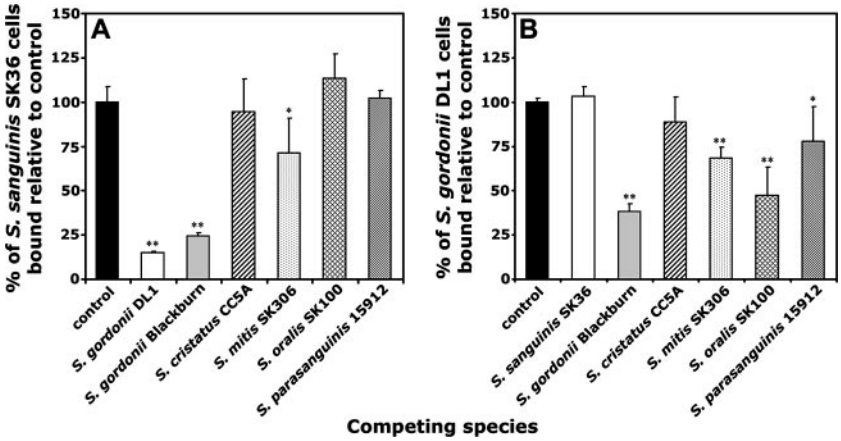

FIG. 2. Adhesion of *S. sanguinis* SK36 or *S. gordonii* DL1 to sHA in the presence of competing oral streptococci. Radioactively labeled *S. sanguinis* SK36 cells (A) or *S. gordonii* DL1 cells (B) were incubated with sHA in the presence of a 10-fold excess of unlabeled competing streptococcal cells. Numbers of attached reference (labeled) cells were then determined as described in Materials and Methods. As a control, reference cells were incubated with sHA in the absence of any competing cells, and these adhesion levels were set to 100%. Data are shown as the relative percentage of cells bound ± standard deviation. *, $P < 0.05$; **, $P < 0.01$ (compared to controls in the absence of competition).

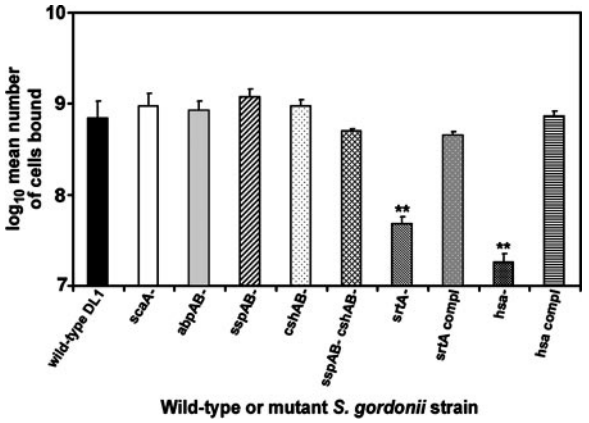

FIG. 3. Adhesion of isogenic mutants of *S. gordonii* DL1 to sHA. Radioactively labeled bacteria (input of 10^9) were incubated with sHA for 1 h, and numbers of attached cells were determined as described in Materials and Methods. Data are presented as the mean ± standard deviation. **, $P < 0.01$ compared to the parent strain.

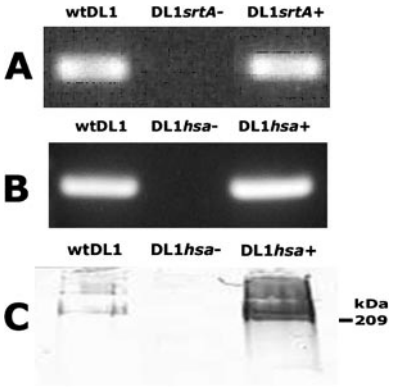

FIG. 4. Confirmation of the genetic manipulations of *S. gordonii* DL1 *srtA* and *hsa* genes. (A) RNA was extracted from wild-type *S. gordonii* (wtDL1) and the *srtA* deletion (DL1*srtA*−) and complemented (DL1*srtA*+) mutants and reverse transcribed into cDNA, and the *srtA* transcript was detected by PCR. (B) RNA was extracted from wild-type *S. gordonii* and the *hsa* deletion (DL1*hsa*−) and complemented (DL1*hsa*+) mutants and reverse transcribed into cDNA, and the *hsa* transcript was detected by PCR. (C) Cell wall protein fractions were collected by mutanolysin digest and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Hsa was detected by Western immunoblotting.

burn reduced adhesion levels of *S. sanguinis* SK36 by 85% and 75%, respectively (Fig. 2A). This level of inhibition was significantly higher than that seen with the other species tested. *S. mitis* SK306 reduced *S. sanguinis* binding by only 29%, while *S. oralis* SK100, *S. cristatus* CC5A, or *S. parasanguinis* 15912 had no significant effects (Fig. 2A). Competition for adhesion to sHA by *S. gordonii* DL1 was then tested. *S. gordonii* Blackburn was found to be the strongest competitor, reducing strain DL1 adhesion by 62%, followed by *S. oralis* SK100, which inhibited by 53% (Fig. 2B). A 20 to 30% reduction in DL1 adhesion levels was found in the presence of excess *S. mitis* SK306 or *S. parasanguinis* 15912 (Fig. 2B). Neither *S. sanguinis* SK36 nor *S. cristatus* CC5A had any significant effects on binding to sHA by *S. gordonii* DL1 (Fig. 2B). Taken collectively, these data suggest that competitive binding by oral streptococci is species dependent. *S. gordonii* was consistently shown to be a strong competitor of *S. sanguinis*. Unlike *S. sanguinis*, other species of streptococci could inhibit binding of *S. gordonii* to sHA.

LPXTG-containing adhesins, notably Hsa, mediate the competitive capabilities of *S. gordonii*. To explain the one-way competitive binding, putative *S. gordonii* surface adhesins were considered for their potential to confer a selective binding advantage over *S. sanguinis* cells. A series of *S. gordonii* isogenic mutants and parent strains (Table 1) were tested in sHA adhesion and competition assays. Potential candidates for the observed competition included mutants with deletions in the following genes: *srtA*, as a general screen for the involvement of LPXTG motif-containing adhesins; *hsa*, encoding an adhesin known to bind sialylated substrates, as are found in the sHA model; and *abpAB*, encoding amylase-binding proteins that are present in *S. gordonii* but not *S. sanguinis*. Mutants lacking proteins SspA and -B (multifunctional polypeptides), CshA and -B (associated with fibronectin binding and hydrophobicity), and ScaA (virulence-associated lipoprotein) were also tested; these are all adhesins previously reported to promote streptococcal adhesion and colonization.

Binding of the mutants and binding of wild-type *S. gordonii* to sHA were compared at a range of input bacteria (10^7 to 10^{10} cells per 20 mg of sHA). The maximal binding capacity of the sHA was confirmed to be approximately 10^9 bound cells. Using 10^9 cells as the input dose, strains abrogated in the production of ScaA, AbpAB, SspAB, and CshAB bound to sHA at levels comparable to that of the wild-type strain (Fig. 3). By contrast, *srtA* and *hsa* mutants at this input dose bound sHA 15- to 20-fold less effectively than the parent strain (Fig. 3). To rule out that potential indirect effects of the mutations were responsible for this loss of binding ability, complementation of the *srtA* and *hsa* mutants was performed. Reverse transcription-PCR and Western immunoblotting were first used to confirm restoration of protein expression (Fig. 4), and sHA adhesion assays were then performed. Adhesion levels for *srtA* and *hsa* complementation mutants were restored to 70% and 100% of those of the wild-type strain, respectively (Fig. 3).

Isogenic mutants were then tested in sHA competition assays. The wild-type and isogenic mutants of *S. gordonii* were compared initially for competition with *S. sanguinis* SK36 for binding to sHA. Like the wild-type DL1, mutants with mutations in *scaA*, *abpAB*, *sspAB*, and *cshAB* reduced adhesion of *S. sanguinis* to sHA by 80 to more than 95% when present at 10- and 50-fold excesses, respectively (Table 3). Compared to the wild type, however, the *srtA* and *hsa* mutants used at identical input doses were unable to compete with *S. sanguinis* (Table 3). Indeed, *S. sanguinis* binding may be slightly increased in the presence of 10- and 50-fold excesses of the *srtA* mutant. The *srtA*-complemented mutant, however, regained the ability to compete, inhibiting by 73% and 85%, respectively (Table 3). At a 10-fold excess, the *hsa* mutant reduced *S. sanguinis* binding by only 13%, which increased to 39% at a 50-fold excess. Levels of inhibition similar to those of wild-type *S. gordonii*, however, were shown upon complementation of the *hsa* mutation. When present at 10- and 50-fold excesses, the *hsa*-complemented mutant reduced *S. sanguinis* adhesion by 60% and 94%, respectively (Table 3).

Each mutant was then compared for adhesion in the pres-

TABLE 3. Adhesion of *S. sanguinis* SK36 to sHA in the presence of excess competing species

Competing *S. gordonii* strain	% of *S. sanguinis* cells bound relative to control (mean ± SD) with[a]:	
	10-fold excess competitor	50-fold excess competitor
Control (no competitor)	100.0 ± 11.3	100.0 ± 11.3
DL1 (Challis)	13.3 ± 2.6	3.4 ± 0.4
scaA mutant (OB470)	10.7 ± 1.0	3.7 ± 1.4
abpAB mutant	12.4 ± 1.3	4.9 ± 0.2
sspAB mutant (UB1360)	8.3 ± 1.3	4.4 ± 1.9
cshAB mutant (OB277)	7.5 ± 1.4	2.4 ± 0.2
sspAB cshAB mutant (OB390)	17.4 ± 7.8	4.1 ± 1.8
srtA mutant	116.0 ± 12.1**	126.7 ± 30.8**
srtA-complemented strain	26.8 ± 9.8	15.0 ± 5.9
hsa mutant	86.8 ± 16.7**	61.1 ± 8.5**
hsa-complemented strain	39.8 ± 6.8**	5.5 ± 0.9

[a] Radioactively labeled *S. sanguinis* SK36 cells were incubated with sHA in the presence of a 10- or 50-fold excess of unlabeled competing streptococcal cells. Numbers of attached *S. sanguinis* cells were then determined as described in Materials and Methods. As a control, *S. sanguinis* was incubated with sHA in the absence of any competing cells, and these adhesion levels were set to 100%. **, value is significantly higher ($P < 0.01$) than that for wild-type DL1.

TABLE 4. Adhesion of wild-type *S. gordonii* DL1 and isogenic mutants to sHA in the presence of excess competing *S. sanguinis* cells

S. gordonii strain	% of *S. gordonii* cells bound relative to control (mean ± SD) with[a]:	
	10-fold excess SK36 cells	50-fold excess SK36 cells
Control (no competitor)	100.0	100.0
DL1 (Challis)	111.1 ± 6.3	89.8 ± 4.8
scaA mutant (OB470)	118.8 ± 14.1	90.4 ± 12.0
abpAB mutant	105.9 ± 5.6	77.6 ± 11.2**
sspAB mutant (UB1360)	117.6 ± 13.6	74.6 ± 9.0**
cshAB mutant (OB277)	102.8 ± 8.7	88.3 ± 2.9**
sspAB cshAB mutant (OB390)	121.9 ± 34.4	87.4 ± 1.7**
srtA mutant	102.6 ± 11.5	30.5 ± 7.9**
srtA-complemented strain	105.0 ± 6.6	79.0 ± 15.0
hsa mutant	58.9 ± 8.2**	29.5 ± 5.5**
hsa-complemented strain	93.4 ± 13.9	94.7 ± 9.8

[a] Radioactively labeled *S. gordonii* cells were incubated with sHA in the presence of a 10- or 50-fold excess of unlabeled *S. sanguinis* cells. Numbers of attached *S. gordonii* cells were then determined as described in Materials and Methods. As a control, *S. gordonii* strains were incubated with sHA in the absence of any competing cells, and these adhesion levels were set to 100%. **, value is significantly lower ($P < 0.01$) than that for controls in absence of competition.

ence of excess *S. sanguinis*. In the presence of a 10-fold excess of *S. sanguinis* cells, binding by wild-type *S. gordonii* DL1 or *abpAB* or *cshAB* mutants was unaffected. In the presence of *S. sanguinis*, adhesion of *scaA*, *sspAB*, and *sspABcshAB* mutants tended to increase by 15 to 20%; the increase was statistically significant ($P < 0.05$) for the *scaA* mutant (Table 4). Adhesion of wild-type *S. gordonii* and the *scaA* mutant was unaffected by 50-fold excess *S. sanguinis* cells, while binding by *abpAB*, *sspAB*, *cshAB*, or *sspAB cshAB* mutants was reduced by just 10 to 25% (Table 4). Once again, the *srtA* and *hsa* mutants exhibited a different profile. In the presence of 10-fold excess *S. sanguinis* cells, adhesion by the *srtA* mutant was unaffected, but at 50-fold excess, its binding was inhibited by 70% (Table 4). Similar inhibition was seen with the *hsa* mutant, the binding of which was also reduced by 40% at a 10-fold excess of *S. sanguinis* cells (Table 4). Like wild-type cells, the *srtA* and *hsa*-complemented mutants showed no significant reduction in adhesion, even at a 50-fold excess of *S. sanguinis* cells (Table 4).

DISCUSSION

Dental plaque is a dynamic environment in which microbes must overcome constant challenges to survive. With more than 600 species occupying the oral cavity at any one time, bacteria relentlessly compete or cooperate with other microflora for available receptors, while contending with a multitude of host defenses. In addition, bacteria must colonize in continuously changing environmental conditions generated by salivary flow, vacillating temperature and humidity, and dietary intake. The success with which each species can utilize available resources and tolerate adverse conditions determines its ability to thrive and thereby establish its own unique environmental niche. Collectively, these niches then specify the polymicrobial community that constitutes dental plaque.

Employing one or more adhesins, bacteria such as *S. gordonii* and *S. sanguinis* interact with a wide range of potential host receptors and other oral microbes in the vicinity to facilitate colonization of the oral cavity. Salivary pellicle constituents identified as putative receptors for *S. sanguinis* can also be utilized by *S. gordonii* and vice versa. Such receptors include proline-rich proteins (5, 40), glycoproteins (31, 40, 49), salivary agglutinin (3, 9), secretory immunoglobulin A (sIgA) (24, 39), lysozyme (38, 55), and lactoferrin (38). An ability to bind directly to α-amylase has been shown for *S. gordonii* and is lacking for *S. sanguinis* (45), while *S. sanguinis* has been shown to utilize a binding site formed by a complex of α-amylase and the light chain of sIgA (7). Given the genetic similarities between these two species (17), it is perhaps unsurprising that they showed similar isotherms for binding to sHA. Despite this similarity in binding, however, *S. gordonii* and *S. sanguinis* differed significantly in their abilities to compete with one another to adhere.

Remarkably, the numbers of *S. gordonii* cells bound to sHA were unaffected by the presence of excess *S. sanguinis* cells. Conversely, excess *S. gordonii* cells significantly reduced *S. sanguinis* adhesion levels, an example of interspecies antagonism for binding sites. The principle that colonization of the tooth surface by one bacterium could impair attachment by a second species has been fundamental to the models of plaque development proposed over the years. This study provides, however, the first proof of principle of a specific surface-expressed adhesin playing a role in the mechanism of interspecies competition for adhesion to the tooth surface.

Other forms of interspecies antagonism among oral bacteria are often associated with the production of bacteriocins or toxic metabolic by-products (8, 13, 21). *S. gordonii* DL1 (Challis) produces both bacteriocins (58) and hydrogen peroxide (2), which impair bacterial replication. In our experimental conditions, however, cells neither synthesize new proteins (M. C. Herzberg and H. F. Jenkinson, unpublished observations) nor replicate during the short period of incubation with sHA. Furthermore, extensive washing of the cells prior to incubation with sHA would effectively remove any products potentially

released during the labeling phase. Thus, since such extracellular antagonisms are unlikely to be operative, the competition with *S. gordonii* appears to reflect the specific inability of *S. sanguinis* to bind to sHA in the presence of the competitor.

To characterize the basis of its competitive advantage, a series of isogenic *S. gordonii* adhesin mutants were screened. Mutants with mutations in antigen I/II polypeptides (3), Csh adhesins (30), and amylase-binding protein (Abp) (42, 44, 45) were selected, since there is evidence that these enable colonization. In addition, Hamada et al. (9) recently found that synthetic SspB peptides of *S. gordonii* 10558 could inhibit the binding of *S. sanguinis* 10556 to salivary components, as detected using a BIAcore system. Abp expression by *S. gordonii* is also a taxonomic distinction from *S. sanguinis*, which lacks the *abpAB* genes (20). The sialic acid-binding protein Hsa (48) was studied since abundant sialylated proteins in saliva could form potential binding sites in the salivary pellicle (60). ScaA was tested since it forms part of a compensatory adhesin mechanism in *S. gordonii* utilized during sHA biofilm formation (65), while a sortase (SrtA) mutant allowed a general screen for the role of LPXTG-containing adhesins. Despite their putative roles in facilitating colonization of the oral cavity, loss of the antigen I/II polypeptides or the Csh or Abp adhesins had no significant effects on the ability of *S. gordonii* to bind to sHA or to effectively compete with *S. sanguinis* for adhesion. The functions of such adhesins may be compensated for by altered expression of alternative adhesins, a system that has recently been demonstrated during *S. gordonii* biofilm formation in vitro (65). By contrast, mutations in both the *srtA* and *hsa* genes strongly impaired *S. gordonii* binding to sHA. In addition, these mutants were less effective competitors than the parent strain, and excess *S. sanguinis* cells inhibited their adhesion to sHA. This implied that LPXTG-containing adhesins, notably Hsa, are required for maximal binding to sHA by *S. gordonii* and for competition resistance against *S. sanguinis*. This hypothesis was then further confirmed, with restoration of wild-type *S. gordonii* competitive capabilities upon complementation of the *srtA* or *hsa* mutations.

Hsa is a member of a family of serine-rich repeat polypeptides common among oral streptococci. Hsa or its homologue, GspB, have been found in all strains of *S. gordonii* tested to date. These proteins, originally identified in *S. gordonii* strains DL1 and M99, respectively, are nearly identical, and both have been shown to promote attachment to platelets (48, 49, 51, 63), epithelial cells (15), and the α2-3-linked sialoglycoconjugates of salivary mucins and sIgA (26, 52). An Hsa-like protein, designated SrpA, was recently identified in *S. sanguinis* strains SK36 and 133-79 (34, 47) and *S. cristatus* CC5A (10). SrpA also mediates adhesion to platelets, but expression appears to be strain dependent (34, 47). A serine-rich repeat protein (Fap1) similar to Hsa is expressed by *S. parasanguinis* FW213 (61, 62) and appears to promote adhesion to sHA (46).

The GspB homologues can be split into two groups based on the isoelectric point (pI) of the intervening region between the two sets of serine-rich repeats (51). Hsa and GspB have an intervening region with a basic pI (termed the basic region [BR]), and SrpA and Fap1 have region with an acidic pI (termed the acidic region [AR]). The BR mediates adhesion of both *S. gordonii* strains DL1 and M99 to carbohydrate moieties on platelets, but variations in primary sequence generate, at least in part, differences in sialylated glycoprotein specificities and affinities between these strains (51). By contrast, the AR-containing GspB homologues of *Streptococcus agalactiae* and *S. parasanguinis* bind poorly to platelets (34, 51). SrpA of *S. sanguinis* does mediate adhesion to platelet receptor GPIbα in a sialic acid-dependent manner (34). However, unlike the AR of *S. agalactiae*, the SrpA AR is somewhat similar to that of Hsa, sharing 48% sequence identity (51). Thus, although the carbohydrate structures recognized by SrpA have yet to be determined, it might be predicted that they will prove to be similar to that of *S. gordonii*. Nevertheless, given the variation in primary sequence and the evidence that other domains within the protein and glycosylation levels may influence adhesion (49, 50), it seems likely that differences in specific binding properties could exist between these proteins. In this study, all Hsa-carrying strains were strong competitors against *S. sanguinis*, whereas species with an acidic product of the *srpA* gene competed poorly. We speculate, therefore, that the presence of an Hsa-like adhesin and the structure of the intervening BR specify the competitive capabilities of a strain. *S. gordonii srtA* and *hsa* mutants do, however, partially bind sHA and compete with *S. sanguinis*. Hence, other, non-LPXTG-containing adhesins may also facilitate these interactions, albeit it to a lesser extent. Thus, for some streptococcal species and strains, adhesins other than Hsa may play major roles in sHA adhesion. Nonetheless, for *S. gordonii*, Hsa is essential for efficient binding.

Competitive binding of *S. gordonii* Hsa is strongly suggested to occur through interactions with a sialylated pellicle constituent(s), maintaining adhesion to sHA even in the presence of competing cells. Likely pellicle receptors for Hsa include gp340, mucin MG2, and the sIgA heavy chain (15, 52). By elucidating the salivary receptor(s) in this competition phenomenon, we will determine whether Hsa-mediated attachment of *S. gordonii* directly blocks the same receptor utilized by *S. sanguinis* or prevents access of *S. sanguinis* to its own pellicle receptor(s). For example, both *S. gordonii* and *S. sanguinis* have been shown to bind salivary mucin MG2 (35, 52). Alternatively, salivary pellicle binding sites identified for *S. sanguinis* include a complex of α-amylase and the light chain of sIgA (7). One could speculate, therefore, that *S. gordonii* Hsa may bind to the sialylated residues of sIgA and sterically interfere with attachment of *S. sanguis* to the α-amylase-sIgA complex. Another mechanism that cannot be ruled out is that Hsa facilitates binding of *S. gordonii* to surface components of *S. sanguinis* and in this way physically blocks attachment of *S. sanguinis* to the salivary pellicle. It should be noted, however, that no aggregates of cells could be visualized during these studies, and the one-sided nature of this competition phenomenon makes this unlikely. It is also possible that the range of binding specificities of Hsa allow *S. gordonii* to target a larger number of salivary pellicle receptors in comparison to *S. sanguinis*, enabling *S. gordonii* to attach more effectively. Future work will now aim to elucidate the precise mechanism(s) underlying this competitive binding phenomenon in greater detail.

In vivo, oral streptococci represent approximately 20% of the total bacteria present in saliva (29) and can constitute up to 80% of early plaque (37). Regardless of the detection technique employed, *S. sanguinis* is consistently found in excess

relative to *S. gordonii* in saliva (22, 54), dental plaque (4, 28, 32), and oral soft tissue surfaces (28). Nevertheless, *S. gordonii* seems to defy the evolutionary odds and persists over time in comparatively low numbers within the oral cavity, even when outnumbered by one of its major competitors, *S. sanguinis*. Thus, in facilitating adhesion to the salivary pellicle, *S. gordonii* Hsa also appears to serve as an environmental constraint, limiting the binding of *S. sanguinis* to the same intraoral sites. Consequently, *S. gordonii* is able to establish an environmental niche in the presence of *S. sanguinis*, which allows these two genetically similar species to coexist. *S. gordonii* can, however, be outcompeted by other oral streptococci, namely, *S. oralis*, which can also be isolated from the same oral sites. This may therefore explain why, despite its ability to bind to the salivary pellicle, *S. gordonii* is not present in abundance within the oral cavity but rather consistently maintains a low-level presence. Since the early-colonizer population influences the overall composition of dental plaque, such mechanisms have important implications for our understanding of the microbial ecology of dental plaque and for the development of novel preventive and control regimens.

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