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Inactivation of the Gene Encoding Surface Protein SspA in *Streptococcus gordonii* DL1 Affects Cell Interactions with Human Salivary Agglutinin and Oral Actinomyces

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Cell surface protein SSP-5 in the oral bacterium *Streptococcus gordonii* M5 binds human salivary agglutinin in a Ca^{2+}-dependent reaction (D. R. Demuth, E. E. Golub, and D. Malamud, J. Biol. Chem. 265:7120–7126, 1990). The region of the gene encoding an N-terminal segment of a related polypeptide (SspA) in *S. gordonii* DL1 (Challis) was isolated following polymerase chain reaction amplification of genomic DNA. The *sspA* gene in *S. gordonii* DL1 was insertionally inactivated by homologous recombination of the erythromycin resistance (Em^r) determinant *ermAM* onto the streptococcal chromosome. The SspA polypeptide (apparent molecular mass, 210 kDa) was detected on Western blots (immunoblots) of spheroplast extracts and extracellular culture medium proteins from wild-type strain DL1 but was absent from Em^r mutants. One $SspA^-$ mutant (designated OB220) was not altered in rate or extent of aggregation by whole saliva or parotid saliva but showed reduced aggregation in the presence of purified salivary agglutinin. Mutant bacteria were unaffected in their ability to adhere to hydroxylapatite beads coated with whole or parotid saliva and were unaltered in cell surface hydrophobicity. However, the $SspA^-$ strain OB220 was deficient in binding salivary agglutinin and in binding to six strains of *Actinomyces naeslundii*. Therefore, expression of SspA polypeptide in *S. gordonii* is associated with both agglutinin-dependent and agglutinin-independent aggregation and adherence reactions of streptococcal cells.

Streptococci can constitute up to 70% of the cultivable bacteria found in human dental plaque (46), the accumulation of which is associated with development of dental caries and periodontal disease. *Streptococcus gordonii* is a major component of supragingival plaque and is also found on the oropharyngeal mucosa (16, 39). The bacteria adhere to salivary components in pellicle (14, 17), they coaggregate with a multitude of other oral bacterial species (1, 23, 27, 47), and they produce extracellular glucans from sucrose (7, 51). These properties are believed to assist adherence and accumulation of the streptococci in the oral cavity.

S. gordonii produces more than 20 polypeptides that are exposed at the cell surface (2, 21). There is evidence that at least several of these polypeptides are involved in streptococcal cell adherence processes. In *S. gordonii* DL1 (Challis), a 76-kDa polypeptide appears to be involved in cell aggregation reactions (23) and a 290-kDa protein is associated with hydrophobicity and coaggregation properties (37); in strain Channon, a 150-kDa polypeptide may influence saliva-mediated cell aggregation (36). A 38-kDa polypeptide (ScaA) is strongly implicated in coaggregation of *S. gordonii* PK488 with *Actinomyces naeslundii* PK606 (1). Thus, it is likely that aggregation and adherence reactions of *S. gordonii* involve multiple adhesins (19).

The best-characterized adhesin of *S. gordonii* is the SSP-5 polypeptide (10, 11). This polypeptide is antigenically related to P1 (SpaP) and PAc polypeptides in *S. mutans* (5, 10) and to SpaA and PAg in *S. sobrinus* (10). Analysis of the predicted amino acid sequences of SSP-5, SpaP, PAc, and SpaA polypeptides revealed extensive regions of sequence homology (11, 12, 30, 40, 48) and common structural features (30, 34). These polypeptides are all in the predicted molecular mass range 160 to 175 kDa; they have been referred to as the antigen I/II group of polypeptides (34) and appear to be represented in many oral streptococcal species (18, 34, 52, 53).

In *S. mutans*, PAc or P1 (SpaP) polypeptide binds human salivary agglutinin (5). Expression of the polypeptide on the cell surface is necessary for agglutinin-mediated aggregation of cells and for adherence of bacteria to experimental salivary pellicle (26, 31). In *S. gordonii* M5, purified SSP-5 protein (apparent molecular mass, 205 kDa, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) has been shown to bind human salivary agglutinin (11, 12) in a Ca^{2+}-dependent reaction (13) that is sensitive to inhibition by sialic acid (NeuNAc) (11, 12). When the cloned *ssp-5* gene was transformed into *Enterococcus faecalis*, the SSP-5 polypeptide was expressed on the cell surface and the enterococci acquired the property of agglutinin-mediated aggregation (9). These results have provided compelling evidence that SSP-5 polypeptide in *S. gordonii* M5 is a salivary agglutinin receptor.

It is now evident that multiple adhesin-receptor interactions occur between streptococci and other components in the oral environment. However, the relative contributions of P1-like polypeptides to the process of oral cavity colonization by various streptococci are still not clearly defined. *S. gordonii* DL1 (Challis) was found to be similar to strain M5 in that it produced an extracellular polypeptide of approximately 210-kDa molecular mass that reacted with antibodies raised to P1 (SpaP) protein from *S. mutans*. To determine more precisely the role of this P1-like polypeptide (designated SspA) in streptococcal aggregation and adherence reactions, we have constructed isogenic mutants of *S. gordonii* DL1 that are deficient in the production of SspA and characterized their phenotypic properties associated with oral cavity colonization.

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MATERIALS AND METHODS

Bacteria and growth conditions. *S. gordonii* DL1 (Challis) was used throughout, and stocks were maintained at −80°C in BHY medium (37 g of brain heart infusion broth [Difco Laboratories, Detroit, Mich.] and 5 g of yeast extract [Difco] per liter) containing 15% (vol/vol) glycerol. Bacteria were cultured on TSBY agar (30 g of Trypticase soy broth [BBL Microbiology Systems, Cockeysville, Md.], 5 g of yeast extract, and 15 g of agar per liter) at 37°C in a GasPak system (BBL), in BHY medium, or in TY medium (5 g of tryptone [Difco], 5 g of yeast extract, and 4 g of K_2HPO_4 per liter; pH 7.5) containing glucose (8 g/liter). Liquid cultures were grown at 37°C in screw-cap bottles or tubes without shaking. Erythromycin (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.) was included when appropriate. Strains of *A. naeslundii*, formerly *A. viscosus* (24), were cultured under conditions identical to those used for the streptococci.

Escherichia coli DH5α and JM83 were grown aerobically at 37°C in LB medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter; pH 7.5) or on LB medium supplemented with 15 g of agar per liter (44). Ampicillin (50 μg/ml; Sigma) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml; Sigma) were included for strain JM83, and ampicillin and/or erythromycin (50 μg/ml) was included for strain DH5α, when required.

DNA manipulations. Plasmid DNA was isolated from *E. coli* by a rapid boiling method (44) and from *S. gordonii* in the presence of high salt (35). Plasmids were purified with PlasmidQuik push columns (Stratagene, La Jolla, Calif.). Chromosomal DNA was purified from *S. gordonii* as described previously (22) except that 0.1% (vol/vol) diethylpyrocarbonate (BDH, Poole, England) was included in the cell lysis buffers. Plasmid pUC19 (54) was used for routine cloning and DNA sequencing. Restriction and modifying enzymes (from New England BioLabs, Inc., Beverly, Mass.) were used under the conditions recommended by the manufacturer. To prepare a fragment of streptococcal plasmid pVA736 (35) carrying the erythromycin resistance (Em^r) determinant *ermAM*, plasmid DNA was digested with a combination of *Hin*dIII and *Cla*I. The DNA was then incubated with Klenow enzyme and deoxynucleoside triphosphates to fill the overhanging ends and separated by agarose gel electrophoresis, and the 1.8-kb blunt-ended fragment carrying *ermAM* was purified by electroelution from the gel.

DNA was transferred from agarose gels to Hybond N+ membrane (Amersham Corp., Arlington Heights, Ill.) in alkali by vacuum blotting as described previously (37). Probe DNA (20 ng) was radiolabeled with α-^{35}S-dATP (specific activity, >600 mCi/mmol; Amersham Corp.), using Multiprime (Amersham Corp.) as directed by the manufacturer. Hybridizations were carried out at 65°C for 16 h in 5× Denhardt's solution (44)–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% (wt/vol) SDS. Membranes were washed at 60°C in 0.1× SSC containing 0.5% (wt/vol) SDS, conditions which it was calculated allowed approximately 10% base pair mismatch in hybrids. DNA sequencing was performed with Sequenase (United States Biochemical Corp., Cleveland, Ohio), M13/pUC sequencing primer (−40) 17 mer (New England BioLabs, Inc.), and a set of custom-made primers (from DNA Express, Colorado State University, Fort Collins) that resulted in overlapping sequence data being obtained for both strands of DNA. Autoradiographs were obtained with Hyperfilm β Max (Amersham Corp.).

PCR amplification. Synthetic oligonucleotides (from DNA Express, Colorado State University) were derived from the nucleotide sequence of the *ssp-5* gene (11) and used as primers for polymerase chain reaction (PCR). The coding-strand primer (denoted PCR1) 5′AGTTACAGAGACAACTAGTAC3′ incorporated the sequence recognized by *Spe*I (underlined), and the complementary strand primer (PCR2) 5′CGCTTCTGCGAGTGCTGCAGT3′ incorporated a *Pst*I recognition site (underlined). PCR reactions (0.1 ml) contained *S. gordonii* DL1 DNA (5 ng) and 2 U of *Taq*I polymerase (Amersham Corp.) and were carried out in a thermal reactor (Hybaid Ltd., Teddington, United Kingdom) as follows: 94°C for 2 min, 50°C for 40 s, 72°C for 50 s (1 cycle); 94°C for 25 s, 50°C for 40 s, 72°C for 50 s (30 cycles); and 72°C for 4 min (last cycle). The reaction product was analyzed by agarose gel electrophoresis and purified by phenol extraction and precipitation with ethanol. A portion (0.2 μg) of the DNA product was then digested with a combination of *Spe*I and *Pst*I and was ligated with the compatible ends of pUC19 DNA (0.1 μg) produced by digestion with a combination of *Xba*I and *Pst*I.

DNA-mediated transformation. *E. coli* was made competent for transformation with plasmid DNA by $CaCl_2$ treatment (8). Plasmid DNAs were extracted from transformant colonies by a rapid boiling technique and were sized by agarose gel electrophoresis. Competent cells of *S. gordonii* DL1 were prepared and transformed as described previously (22), and transformants were selected on TSBY agar containing erythromycin (1 μg/ml).

Extraction of bacterial proteins. Late-exponential-phase cells in TY medium containing glucose (10 ml) were harvested by centrifugation (6,000 × *g* at 4°C for 10 min) and washed once with distilled water containing 1 mM EDTA (pH 7.5). Bacteria were suspended in 0.1 ml of spheroplasting buffer (0.02 M Tris hydrochloride [pH 6.8] containing 0.01 M $MgCl_2$, 5 mM phenylmethylsulfonyl fluoride, and 26% [wt/vol] raffinose), mutanolysin (Sigma) was added to a final concentration of 500 U/ml, and the suspension was incubated at 37°C for 30 min (20). To extract proteins, SDS was added to a final concentration 1% (wt/vol), and the suspension was vortexed and then heated at 80°C for 10 min. Suspensions were then centrifuged (10,000 × *g* at 4°C for 10 min), and the supernatants were removed and prepared for electrophoresis by adding 0.1 volume of loading dye (5 mg of bromophenol blue per ml in 70% [vol/vol] glycerol). Culture medium proteins were precipitated from cell-free culture fluid with acetone (80% [vol/vol] final concentration) as described previously (36). Proteins were collected by centrifugation (10,000 × *g* at 4°C for 20 min), dissolved in 0.125 M Tris hydrochloride (pH 6.8) containing 1% (wt/vol) SDS by heating at 70°C for 10 min, and prepared for electrophoresis as described above.

SDS-PAGE and Western blotting (immunoblotting). Proteins were separated by SDS-PAGE (28) and stained with Coomassie blue R250 (BDH) or with silver nitrate (38). Molecular masses of polypeptides were calculated by reference of their mobilities to those of molecular mass marker proteins (215- to 15.3-kDa range, prestained, from Life Technologies, Inc., Gaithersburg, Md.). Proteins were transferred to nitrocellulose by electroblotting in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol at 20 V/cm for 2 h. For immunodetection, blots were incubated with antiserum diluted 1:200, and antibody binding was revealed by using peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulin G (Dako Corp., Carpinteria, Calif.) as described previously (23).

Collection of saliva. Unstimulated whole saliva from five human volunteers was collected on ice, pooled, and clarified by centrifugation (10,000 × *g* at 4°C for 15 min). Some saliva samples were heat treated at 80°C for 10 min and reclarified by centrifugation. Each of the five volunteers had a different combination of blood group and secretor status (presence or absence of blood group substances in saliva): B $Rh(D)^+/Sec^+$, A $Rh(D)^+/Sec^-$, A $Rh(D)^+/Sec^+$, O $Rh(D)^+/Sec^-$, AB $Rh(D)^+/Sec^+$. Stimulated parotid saliva was collected from two of the volunteers with a modified Carlson-Crittenden device (45).

Preparation of salivary agglutinin protein. Agglutinin was isolated from clarified whole saliva by affinity adsorption of the agglutinin to *S. gordonii* DL1 cells followed by desorption in the presence of EDTA (15, 31). Bacteria were grown in BHY medium (1 liter), harvested by centrifugation (6,000 × *g* at 4°C for 10 min), and washed twice with PBSC (10 mM Na_2HPO_4-KH_2PO_4, pH 7.2, containing 0.1 M NaCl, 0.05 M KCl, and 1 mM $CaCl_2$). Pooled saliva was clarified and diluted with an equal volume of PBSC, and the bacterial cells were suspended in the diluted saliva at a density of approximately 5×10^{10} cells per ml (optical density at 600 nm [OD_{600}] = 30). The cells and saliva were mixed by rotation at 37°C for 30 min and centrifuged at 2,000 × *g* for 15 min, and the bacterial pellet was washed once with PBSC. The cells were suspended in PBS (PBSC minus $CaCl_2$) containing 1 mM EDTA and centrifuged at 5,000 × *g* for 10 min, and the supernatant containing agglutinin was filtered through a 0.45-μm-pore-size nitrocellulose membrane and dialyzed against 10-fold-diluted PBSC at 4°C. Agglutinin preparations were then concentrated by freeze-drying, and their protein contents were determined by a dye-binding microassay (Bio-Rad Laboratories, Richmond, Calif.). The purity of agglutinin preparations was assessed by SDS-PAGE through 8% (wt/vol) acrylamide. Staining of gels with silver nitrate revealed that agglutinin preparations contained a major diffuse band of >300-kDa molecular mass together with several minor bands in the range of 25 to 70 kDa which probably included subunits of secretory immunoglobulin A (see reference 15).

Aggregation assays. Streptococcal cells were grown in TY medium containing glucose, harvested in early stationary phase by centrifugation (6,000 × *g* at 4°C for 10 min), washed twice by suspension in PBSC buffer, and suspended in PBSC buffer at OD_{600} = 5.0. To measure aggregation, cells were diluted to a final OD_{675} of 0.7 in a plastic disposable cuvette containing PBSC, saliva (usually 0.2 ml), or agglutinin (0.5 to 0.8 μg) in a final volume of 1 ml. The contents were mixed, the cuvette was incubated at 37°C, and the OD_{675} of the suspension was measured at intervals. All assays were run in triplicate, and controls containing bacteria in buffer only were included.

Adherence to S-HA beads. Bacterial cells were radioactively labeled by growing them to an OD_{600} of 1.4 in TY medium containing glucose and supplemented with [*methyl*-^{3}H]thymidine (8 μCi/ml; 70 to 85 Ci/mmol). Cells were harvested by centrifugation, washed three times by alternate centrifugation and suspension in KCl buffer (2 mM NaH_2PO_4-K_2HPO_4, pH 6.2, containing 5 mM KCl and 1 mM $CaCl_2$), and suspended in KCl buffer at OD_{600} = 3.0 (equivalent to about 4×10^9 streptococcal cells per ml). Specific radioactivities were between 7×10^{-4} and 2×10^{-3} cpm per cell. Attachment of radioactively labeled bacteria to 10-mg portions of hydroxylapatite beads that had been pretreated with saliva (S-HA beads) or KCl buffer in a final volume of 1.2 ml was measured as described in detail elsewhere (14). Tubes containing bacteria and beads were incubated for 90 min at 20°C, the beads were allowed to settle, and the unbound bacteria were aspirated. The beads were washed three times with KCl buffer, transferred to scintillation vials containing 5 ml of OptiPhase HiSafe 3 fluid (LKB Scintillation Products, Bromma, Sweden), and counted for radioactivity.

To measure the adherence of bacteria to S-HA beads over time, a series of tubes each containing 10 mg of beads (treated with unheated whole saliva) and 2×10^7 radioactive streptococcal cells (final volume, 1.2 ml) was set up, and the tubes were incubated with end-over-end mixing at 20°C for 140 min. At time zero and at intervals, duplicate tubes were removed and the numbers of cells attached to the beads were determined as described above.

Hydrophobicity and glucosyltransferase production. Hydrophobicity was determined by a modified hexadecane partition assay (22). Glucosyltransferase activity in cell-free culture fluid was estimated by activity staining after SDS-PAGE (7).

Adherence of streptococci to immobilized salivary agglutinin. Agglutinin (50 ng) in coating buffer (0.02 M Na_2CO_3-$NaHCO_3$, pH 9.5) was added to flat-bottom wells (0.05 ml per well) of 96-well microtiter plates (Maxisorp; A/S Nunc, Kamstrup, Denmark) which were then kept at 4°C for 16 h. The contents of the wells were discarded, and remaining protein-binding sites were blocked by the addition of TNMC buffer (1 mM Tris hydrochloride, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$; pH 8.0) containing 0.1% (wt/vol) bovine albumin to each well (0.2 ml) and incubation of the plates at 4°C for a further 16 h. The contents of the wells were discarded, and the wells were rinsed twice with TNMC buffer containing 0.05% (vol/vol) Tween 20. Portions (0.05 ml) of streptococcal cell suspension in TNMC buffer (containing 2×10^7 bacteria, radioactively labeled with ^{3}H as described above) were added to each well, and the plates were incubated at 37°C for 2 h on a rotary shaker at 200 rpm. The contents of the wells were discarded, and the wells were washed four times with TNMC buffer containing 0.05% (vol/vol) Tween 20. To remove bound cells from the plates, SDS (1%, wt/vol) in 0.4 N NaOH was added to each well (0.1 ml), and the plates were incubated for 2 to 16 h at room temperature. The contents of the wells were transferred to scintillation vials, mixed with scintillation fluid, and counted for radioactivity as before. Assays were performed in quadruplicate, and control wells (no agglutinin) were included with all assays. For inhibition tests, NeuNAc, *N*-acetyl-β-D-galactosamine (GalNAc), or other sugars were dissolved at a concentration of 0.1 M in TNMC buffer, and the solutions were adjusted to pH 8.0 when necessary. Sugar solution (0.025 ml) was then mixed with streptococcal cell suspension (0.025 ml containing 2×10^7 cells) in microtiter plate wells containing immobilized agglutinin, and binding was assayed as described above.

Adherence of streptococci to actinomyces. Strains of *A. naeslundii* were grown at 37°C in TY medium containing glucose to the late exponential phase of growth, and the cells were harvested by centrifugation (8,000 × *g* at 4°C for 10 min) and washed three times with TNMC buffer. Bacteria were suspended at OD_{600} = 0.5 (approximately 10^8 cells per ml) in TNMC buffer, and portions (0.05 ml) were applied to the wells of microtiter plates. The plates were centrifuged (800 × *g* at 20°C for 5 min) to deposit cells onto the bottom surface of the wells and incubated at 4°C for 16 h, and the liquid contents of the wells were then discarded. Additional binding sites were blocked by adding TNMC buffer contain-

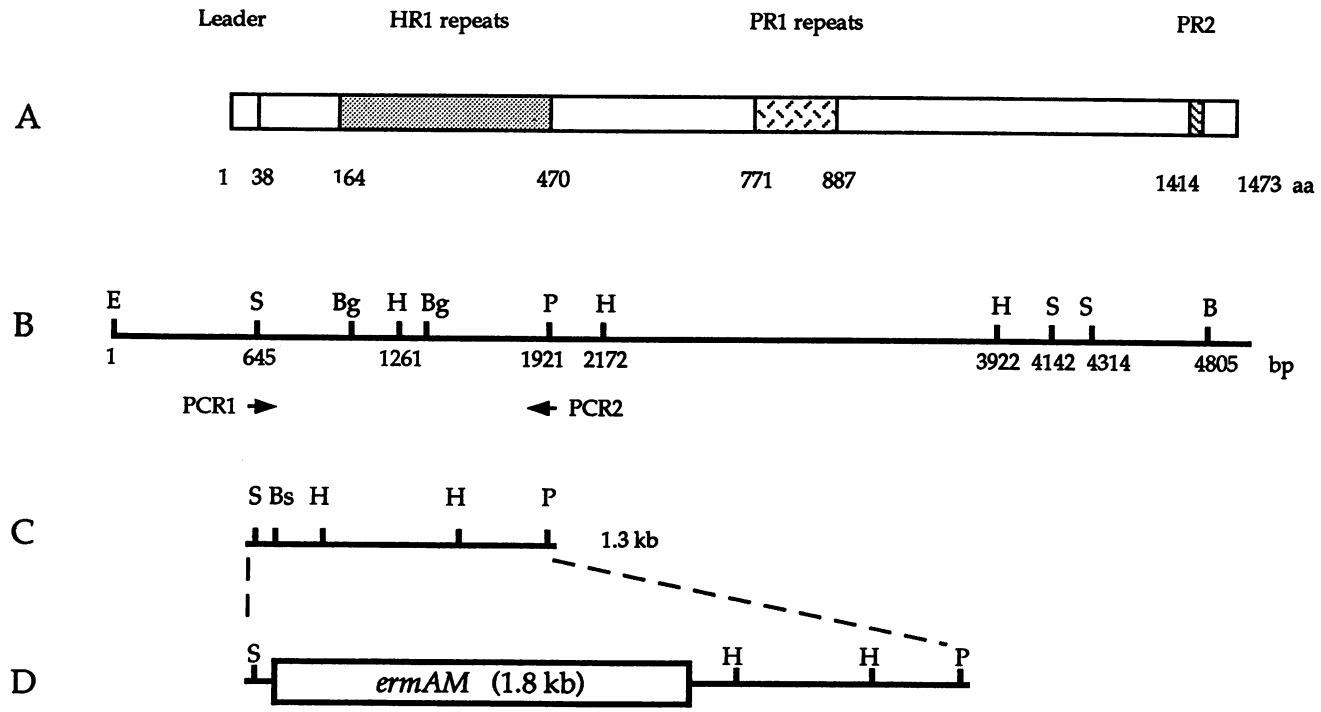

FIG. 1. Schematic representation of the structural features of SSP-5 polypeptide from *S. gordonii* M5 (A) and the region of DNA encoding it (B), drawn from the data in reference 11. Regions of the polypeptide denoted Leader, HR1 repeats, PR1 repeats, and PR2 were designated in reference 11. PCR1 and PCR2 are synthetic oligonucleotide primers that were used to amplify, by PCR, a 1.3-kb DNA fragment from *S. gordonii* DL1 encoding the N-terminal region of a polypeptide homologous to SSP-5. (C) Restriction enzyme sites located within the amplified DNA fragment by electrophoretic sizing of digestion products. Positions of the *Bsr*FI site (106 bp) and *Hin*dIII site at 317 bp were confirmed by the nucleotide sequence. Numbering commences at the 5′ terminus base of the sequence recognized by *Spe*I. (D) The erythromycin resistance determinant *ermAM* was ligated into the unique *Bsr*FI site (Bs). Abbreviations of restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bsr*FI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Spe*I.

ing 0.2% (vol/vol) Tween 20 to each well (0.2 ml) and incubating the plates at 4°C for a further 16 h. Radioactively labeled streptococcal cells (0.05 ml containing 2×10^7 cells) were then added to each well, and numbers bound after incubating with shaking at 37°C for 2 h were determined exactly as described for the agglutinin-binding assay. Inhibition of adherence by sugars was tested as described for the agglutinin-binding assay.

RESULTS

Construction of an SspA-deficient mutant. The complete nucleotide sequence of the gene encoding SSP-5 protein of *S. gordonii* M5 was determined by Demuth et al. (11). The protein was found to be encoded in a single open reading frame of 4,419 bp and contained 1,473 amino acids (Fig. 1). The sequence contained a hydrophobic putative signal peptide (amino acids 1 to 38), a highly charged N-terminal repetitive domain (HR1), a proline-rich repetitive domain (PR1), and a second proline-rich domain (PR2) close to the C terminus of the polypeptide (11). We utilized the sequence obtained for the *ssp-5* gene from strain M5 (11) and synthesized two oligonucleotide primers in order to amplify by PCR a segment of a homologous gene from *S. gordonii* DL1. Oligonucleotide PCR1, which contained the sequence recognized by *Spe*I (at position 645 bp on the map in Fig. 1B), and oligonucleotide PCR2, which contained the sequence recognized by *Pst*I (at position 1921 bp in Fig. 1B), were incubated with *S. gordonii* DL1 DNA for PCR amplification of the DNA bounded by the primers (see Materials and Methods). A single DNA product of approximately 1.3 kb was detected by agarose gel electrophoresis. This fragment was purified, digested with a combination of *Spe*I and *Pst*I, and cloned into plasmid pUC19 to form plasmid pHF1012. Restriction enzyme sites within the cloned fragment of strain DL1 DNA bore no resemblance to the sites within the corresponding fragment of strain M5 DNA: sites for *Hin*dIII within the cloned 1.3-kb fragment are shown in Fig. 1C. A unique *Bsr*FI site at 106 bp (Fig. 1C) was revealed subsequently from DNA sequencing of the fragment.

Sequencing of both strands of the first 450 bp of the insert confirmed that the fragment of DNA inserted in pUC19 encoded a protein similar in sequence to the N-terminal region of SSP-5. The deduced amino acid sequence, commencing translation from the 5′ base of the *Spe*I recognition site and composed of 150 amino acids, was 75% identical to the corresponding deduced amino acid sequence of SSP-5 polypeptide from strain M5 (Fig. 2). The gene encoding the SSP-5-like polypeptide in *S. gordonii* DL1 was designated *sspA*. The SspA sequence also showed overall similarity to SpaP and PAc polypeptides from *S. mutans* serotype c and lesser similarity to SpaA from *S. sobrinus* (Fig. 2).

To insertionally inactivate the *sspA* gene in *S. gordonii* DL1, we proposed to ligate the streptococcal erythromycin resistance determinant (*ermAM*) into the *Bsr*FI site of the 1.3-kb fragment of cloned DNA in pHF1012 and to transform this onto the streptococcal chromosome. However, an additional *Bsr*FI site was present within the ampicillin resistance gene (*bla*) of pHF1012. Accordingly, plasmid pHF1012 was

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SspA     1 TSTANVAVTTTGNPATNLPEAQGEATEAASQSQAQ.GSKDGALPVEVSAD
SSP-5   45 ---ST-E-A---------------MSQV-KE---KA---ES-------SA
SpaP    46 DVDTK-VG-Q-------------SASKQ-E---TK.LERQMVHTI--PKT
PAc     46 DVDTK-VG-Q-------------SASKE-E---TK.LERQMVHTI--PKT
SpaA    50  N--V-GTE-.--------DK-DNPSSQ-ET----A-Q-T--MS-D--TS

SspA    50 DLNKAVTDAKAAGVNLVQGQTSDKGTATTAAENAQKQAEIESDYVKQAEE
SSP-5   95 --D---A---S---KV--DE-K--------TD-----D--K---A-----
SpaP    95 --DQ-AK---S----V--DADVN---VK--E-AV--ET--KE--T----D
PAc     95 --DQ-AK---S----V--DADVN---VK-PE-AV--ET--KE--T----D
SpaA    98 E-DE-AKS-QE---TVS-DA-VN---VE-SD-AN--EP--KD--S---AD

SspA   100 IKKTTEAYKKEVVAHQAETDKINAENKAAEDKYQEDLTAHQAGVEKINTA
SSP-5  145 --T---------A----------------D----K--KS--EE-------
SpaP   145 -----DQ--SD-A--E--VA--K-K-Q-TKEQ-GK-MV--K-E--R--A-
PAc    145 -----DQ--SD-A--E--VA--K-K-Q-TKEQ-EK-MA--K-E--R--A-
SpaA   148 -Q----D--AS-A-N-----R-TQ-NA-KKAQ-EQ--A-NK-E--R-TNE
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FIG. 2. Sequence comparison of SspA from *S. gordonii* DL1 with SSP-5 precursor from *S. gordonii* M5 (11), SpaP precursor from *S. mutans* NG5 (25), PAc precursor from *S. mutans* MT8148 (41), and SpaA precursor from *S. sobrinus* MT3791 (49). Numbers indicate the positions of amino acids within the sequences. The amino acid sequence of SspA was deduced from the nucleotide sequence of the first 450 bp of the PCR product, commencing at the 5′ base of the *Spe*I recognition sequence, as shown in Fig. 1C. Residues in the other proteins identical to those in SspA are indicated with dashes. A period indicates a gap introduced to maximize identity. The line above the sequences commencing at amino acid residue 164 in SSP-5 indicates the start of the first Ala-rich HR1 repeat (see Fig. 1). Identities with SspA were as follows: SSP-5, 78%; SpaP, 53%; PAc, 53%; SpaA, 41%. Sequences of SpaA from *S. sobrinus* 6715 (30), MSL-1 from *S. mutans* KPSK2 (12), and SR protein from *S. mutans* OMZ175 (40) have not been included because alignments of these proteins with SpaA, SpaP, SSP-5, or PAc were made in the original articles.

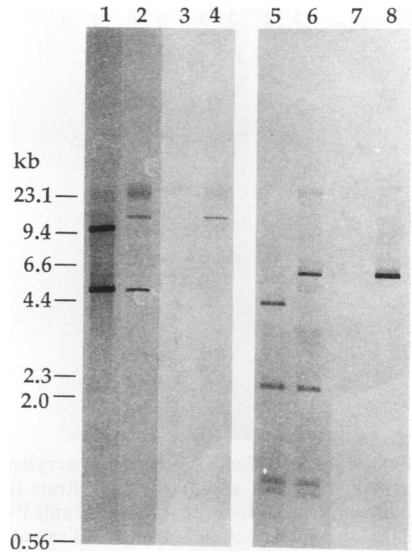

FIG. 3. Insertion of *ermAM* by homologous recombination within the *sspA* gene in mutant strain OB220. Shown are autoradiograms of blots of chromosomal DNAs from *S. gordonii* DL1 (lanes 1 and 3) or OB220 (lanes 2 and 4) digested with *Eco*RI and probed with a radioactively labeled, 1.3-kb *sspA* fragment (lanes 1 and 2) or 1.8-kb *ermAM* fragment (lanes 3 and 4). Lanes 5 through 8 are chromosomal DNAs digested with *Hin*dIII, the probes being identical to those used in lanes 1 to 4. Positions of molecular mass markers (*Hin*dIII digestion products of phage lambda) are indicated on the left. Lanes 5 and 6 each contained an additional hybridizing band of 280 bp (not shown) that was revealed only by more prolonged exposure of the blot to X-ray film.

incubated with *Bsr*FI under conditions promoting partial digestion. Single-stranded ends were filled in with Klenow enzyme, and the plasmid was ligated with the 1.8-kb DNA blunt-ended fragment carrying the *ermAM* erythromycin resistance determinant. The mixture was transformed into *E. coli* DH5α, and transformants were plated onto agar containing ampicillin and erythromycin. This selected for insertion of *ermAM* into pHF1012 but selected against *ermAM* insertion into the *bla* gene. After transformants were screened for plasmid content, a plasmid of anticipated size (5.8 kb) was purified and designated pHF1032. Restriction enzyme mapping of this plasmid confirmed that the *ermAM* fragment was inserted at the *Bsr*FI site within *sspA* (Fig. 1D).

Plasmid pHF1032 DNA (0.05 μg) was mixed with competent cells of *S. gordonii*, and transformants were selected on agar containing erythromycin. Several thousand erythromycin-resistant (Em^r) transformants were obtained, and a number were picked at random and purified for further studies. To confirm that *ermAM* had integrated into the chromosome, DNAs were prepared from several transformants and from the wild-type strain DL1 and digested with either *Eco*RI or *Hin*dIII. The DNA fragments were separated by electrophoresis, blotted onto nylon membrane, and hybridized with the ^{35}S-dATP-labeled, 1.3-kb *sspA* gene fragment or 1.8-kb *ermAM* fragment. The results obtained for one transformant (designated strain OB220) are shown in Fig. 3 and were representative of results obtained for other transformants. In the wild-type strain, the *sspA* probe hybridized with two *Eco*RI fragments of 9.6 and 5.3 kb (Fig. 3, lane 1), and in strain OB220 it hybridized with two fragments of 11.4 and 5.3 kb (Fig. 3, lane 2). Probing with *ermAM* showed that the increase in size of the 9.6-kb fragment in OB220 was due to *ermAM* insertion (Fig. 3, lane 4). As expected, *ermAM* did not hybridize with *S. gordonii* DL1 DNA (Fig. 3, lane 3). The *sspA* probe hybridized with five *Hin*dIII fragments of chromosomal DNA in strain DL1 (4.2, 2.1, 0.9, 0.8, and 0.28 kb), and in strain OB220 the 4.2-kb fragment was replaced by a 6.0-kb fragment (Fig. 3, lanes 5 and 6). This new 6.0-kb fragment in strain OB220 was the only fragment detected with the *ermAM* probe (Fig. 3, lane 8). These results were consistent with homologous recombination of sequences flanking *ermAM* having occurred with chromosomal sequences in OB220 and insertion of the resistance determinant at a single site. However, the hybridization of the *sspA* probe with two chromosomal *Eco*RI fragments and five chromosomal *Hin*dIII fragments was unexpected because the probe contained no sites for *Eco*RI and only two sites for *Hin*dIII (see Discussion).

Effect of *ermAM* insertion on SspA polypeptide production. Antiserum raised to purified P1 (SpaP) protein from *S. mutans* serotype c reacts with the P1-like proteins SpaA and SSP-5 (10). In SDS-solubilized extracts of spheroplasts of *S. gordonii* DL1 subjected to SDS-PAGE and Western blotting, the P1 antiserum reacted with a single major band of approximate molecular mass 210 kDa (Fig. 4, lane 5) together with two minor bands of lower molecular mass. No antigenic polypeptides were detected in corresponding extracts of spheroplasts of strain OB220 (Fig. 4, lane 6). Protein profiles of the extracts stained with Coomassie blue were similar, and the 210-kDa polypeptide was not a significant component (Fig. 4, lanes 1 and 2).

The wild-type strain DL1 released into the culture medium a P1 antiserum-reactive polypeptide of approximate molecular mass 205 kDa (Fig. 4, lane 7) which was absent in the culture medium of mutant strain OB220 (Fig. 4, lane 8). Stained protein profiles of culture medium polypeptides of the two strains were similar but not identical (Fig. 4, lanes 3

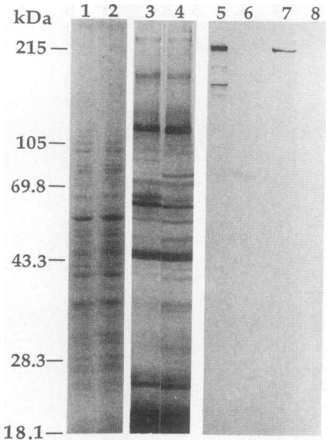

FIG. 4. SDS-PAGE profiles (8% [wt/vol] acrylamide) stained with Coomassie blue (lanes 1 and 2) or silver nitrate (lanes 3 and 4) and corresponding Western blots reacted with anti-P1 (SpaP) antiserum. Lanes 1, 2, 5, and 6 contain polypeptides extracted with SDS from spheroplasts of *S. gordonii* DL1 (lanes 1 and 5) or SspA$^-$ strain OB220 (lanes 2 and 6). Lanes 3, 4, 7, and 8 contain polypeptides released into the culture fluid during growth of wild-type strain DL1 (lanes 3 and 7) or mutant strain OB220 (lanes 4 and 8). Positions of molecular mass markers are indicated on the left.

and 4), and once again, the 205-kDa polypeptide in DL1 could not be clearly distinguished.

Saliva- or salivary agglutinin-mediated aggregation. Cells of the wild-type strain DL1 grown in TY medium containing glucose were aggregated by whole saliva (20% [vol/vol] final concentration) and more slowly by heated whole saliva (Fig. 5A). The rates of aggregation of mutant strain OB220 cells in unheated or heated whole saliva were identical to those of the wild-type cells (Fig. 5B). Neither strain aggregated in buffer alone, but small decreases in OD were observed as the cells settled (Fig. 5A and B). Cells grown in BHY medium were only slowly aggregated by saliva, showing a 30% decrease in OD after 3 h of incubation. No differences were found between the wild-type and mutant strains in their aggregation properties with saliva samples from individuals with different blood groups. Both wild-type and mutant bacteria aggregated at similar rates in 20% (vol/vol) parotid

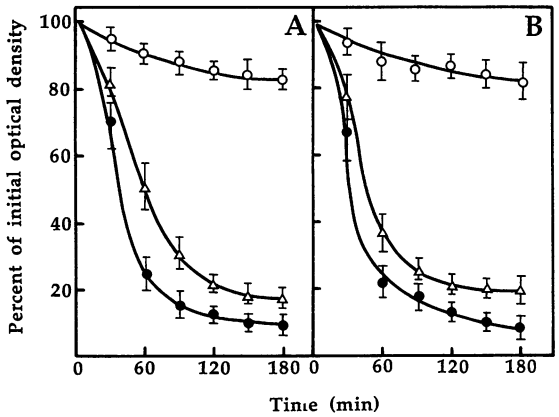

FIG. 5. Aggregation of *S. gordonii* cells of wild-type strain DL1 (A) or SspA$^-$ mutant strain OB220 (B) in PBSC (○) or in 20% (vol/vol) human whole saliva that was unheated (●) or heat treated at 80°C for 10 min (△). Error bars are ± standard deviation (n = 6).

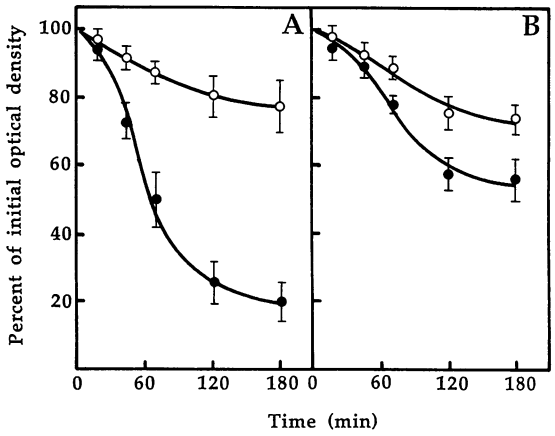

FIG. 6. Aggregation of *S. gordonii* cells of wild-type strain DL1 (A) or SspA$^-$ mutant strain OB220 (B) in PBSC (○) or in PBSC containing 0.8 μg of salivary agglutinin protein per ml (●). Error bars are ± standard deviation (n = 4).

saliva (results not shown). By contrast, cells of strain DL1 were rapidly aggregated in the presence of salivary agglutinin (Fig. 6A), while aggregation of SspA$^-$ mutant cells by agglutinin was considerably reduced (Fig. 6B).

Adherence to S-HA beads and to immobilized agglutinin. Approximately 25% of input cells of *S. gordonii* DL1 adhered to S-HA beads, and a similar percentage of input cells of mutant strain OB220 adhered (results not shown). In these assays, beads were washed three times with buffer before they were counted for radioactivity to estimate numbers of cells attached. Washing up to seven times did not reduce significantly the numbers of wild-type or mutant strain cells attached. There were no differences in the kinetics of adsorption of wild-type or mutant cells to S-HA beads. Both strains showed an initial rapid linear rise in binding which was followed by approach to equilibrium, and adherence was essentially maximal by 90 min (data not shown).

The abilities of wild-type and mutant strain cells to bind immobilized agglutinin were measured by a solid-phase assay utilizing microtiter plate wells. Agglutinin-coated hydroxylapatite beads were not used for cell adherence assays because of the small quantities of agglutinin available for coating. Approximately 40% of input cells of strain DL1 bound to immobilized agglutinin compared with 22% of input cells of OB220 (Table 1). Binding of strain DL1 cells to agglutinin was sensitive to inhibition by 50 mM NeuNAc, but binding of the mutant cells was not (Table 1). The presence of 50 mM GalNAc (Table 1), lactose, or fucose had no effect on streptococcal adherence.

TABLE 1. Adherence of *S. gordonii* DL1 or OB220 (SspA$^-$) mutant to immobilized salivary agglutinin

Substrate[a]	Streptococcal cells bound (10^6) ± SD (n = 4)[b]	
	DL1	SspA$^-$
Agglutinin	8.1 ± 0.84	4.5 ± 0.50
+ 50 mM NeuNAc	4.5 ± 0.60	4.8 ± 0.59
+ 50 mM GalNAc	8.6 ± 0.81	5.2 ± 0.41
Control	0.72 ± 0.04	0.91 ± 0.07

[a] 50 ng of salivary agglutinin applied to microtiter plate wells.
[b] Input cell concentration, 2 × 10^7.

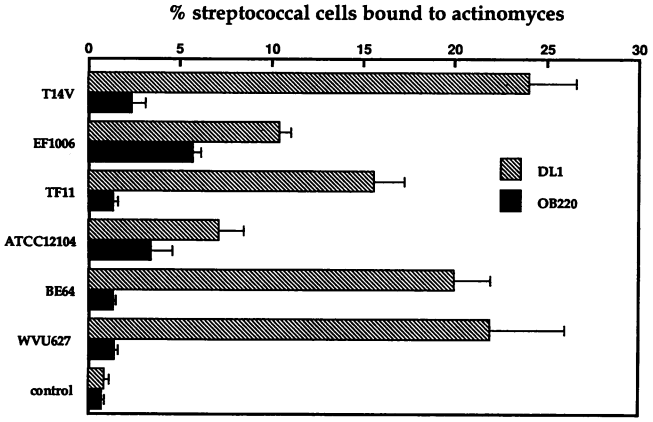

FIG. 7. Adherence of *S. gordonii* DL1 or OB220 to cells of six strains of *A. naeslundii* (T14V, EF1006, TF11, ATCC 12104, BE64, and WVU267) immobilized on the bottom surfaces of microtiter plate wells. Control represents wells to which no actinomyces cells were attached. Adherence values are expressed as the percentage of input streptococcal cells (2×10^7 added to each well) bound. Error bars are standard deviations of the mean ($n = 6$).

Cell surface hydrophobicity and glucosyltransferase production. Cell surface hydrophobicities of wild-type and mutant bacteria were compared by measuring the numbers of cells of each strain adsorbing to hexadecane. For strain DL1, 79% ± 5% of cells adsorbed to the hexadecane phase compared with 81% ± 6% for strain OB220, showing that inactivation of SspA production had no effect on cell surface hydrophobicity. Strains DL1 and OB220 produced identical glucosyltransferase activities and had similar growth rates in TY-glucose medium and identical colony morphologies on TSBY or mitis salivarius (Difco) agar.

Adherence of streptococci to actinomyces. *S. gordonii* DL1 cells adhered to varying extents to cells of six different strains of *A. naeslundii* immobilized in microtiter plate wells. The highest percentages of input streptococcal cells were bound to *A. naeslundii* T14V, WVU267, and BE64 (Fig. 7). Inactivation of *sspA* in strain OB220 resulted in reduced binding to all actinomyces strains tested. In particular, there were >90% reductions in binding to *A. naeslundii* WVU267, BE64, and T14V (Fig. 7). Binding of wild-type strain DL1 cells to actinomyces was not inhibited by 50 mM concentrations of NeuNAc, GalNAc, lactose, or fucose.

DISCUSSION

There is good evidence that the P1-like proteins (antigen I/II) are major salivary adhesins in a number of strains of mutans streptococci (6, 26, 31). Although the role of these polypeptides in the establishment of streptococci in the oral cavity is unclear (3), local passive immunization with monoclonal antibodies to antigen I/II were effective in preventing recolonization of the human mouth by *S. mutans* (33). In *S. mutans* serotype c, the expression of P1 (SpaP) was essential for binding of bacterial cells to experimental salivary pellicle (parotid saliva-coated hydroxylapatite beads) but not for binding of bacteria to beads coated with glucan formed in situ (3). Furthermore, it was shown that P1 protein was directly responsible for binding of *S. mutans* to agglutinin-coated hydroxylapatite beads and that P1 protein in the fluid phase acted as a competitive inhibitor of cell binding (6). On the other hand, the contribution of the P1-like protein SpaA to adherence and aggregation reactions of *S. sobrinus* is not known. In a study of adherence to agglutinin-coated beads of four serotype g strains expressing SpaA on their cell surfaces, only two strains adhered to agglutinin-coated beads (6). The non-adherent strains also failed to be aggregated with salivary agglutinin (6).

In *S. gordonii* M5, the SSP-5 polypeptide has been shown to bind salivary agglutinin in a NeuNAc-sensitive and Ca^{2+}-dependent reaction (11–13). Despite the similarity of SSP-5 to PAc, SpaP, and MSL-1 proteins from *S. mutans* serotype c (about 60% at the amino acid level), there is evidence of functional differences between the *S. gordonii* and *S. mutans* proteins. For example, SSP-5 and MSL-1 both bind salivary agglutinin, but the reaction of MSL-1 is sensitive to inhibition by fucose and not to inhibition by NeuNAc (11, 12). The significance of streptococci binding salivary agglutinin is that in the fluid phase it represents a mechanism for bacterial clearance. When immobilized on oral surfaces, either host or bacterial (29), the agglutinin provides a receptor for bacterial adherence. To more clearly define the role of P1-like polypeptide SspA in adherence and aggregation of *S. gordonii* DL1, we constructed a mutant defective in SspA expression. PCR was used to obtain a 1.3-kb fragment of *S. gordonii* DL1 DNA that was homologous to the sequence of *ssp-5* in strain M5. No conservation of restriction sites was found between the fragment amplified by PCR from strain DL1 and the sequence from strain M5 (the *Spe*I and *Pst*I sites were defined by the primers). This was unexpected in view of the fact that restriction fragment length polymorphism analysis of the N-terminal coding region (including the A region) of *spaP* in eight strains of *S. mutans* serotype c suggested several conserved restriction sites (4). Nucleotide sequencing of the *sspA* fragment revealed 78% homology between the deduced amino acid sequences of SspA and SSP-5. This was somewhat surprising in view of the fact that SR (from *S. mutans* serotype f) and PAc (from *S. mutans* serotype c) are 86% identical (30, 40) and SpaP and PAc (from *S. mutans* serotype c) are 96% identical (30). These observations raised the possibility that SspA may show differences in activity or function compared with SSP-5.

To inactivate the *sspA* gene in strain DL1, *ermAM* was inserted into the N-terminal coding region of the gene. Extraction of the SspA polypeptide intact from strain DL1 cells was facilitated by first digesting the cell wall with mutanolysin. Breakage of the cells with glass beads was not a suitable method for releasing the protein because it resulted in fragmentation of the polypeptide. SDS-PAGE and Western immunoblot analyses of proteins extracted from spheroplasts, and of proteins released into the culture fluid, confirmed that production of SspA by strain OB220 had been abolished. On the basis of the SSP-5 sequence, the insertion of *ermAM* at bp 106 in the *sspA* sequence would result in production of a truncated precursor polypeptide of about 68 amino acids. It seems highly unlikely that this protein product, if indeed stable, would have been at all functional. By contrast, insertional inactivation of *spaP* in *S. mutans* 834 resulted in production of a truncated P1 polypeptide corresponding to the amino-terminal 612 amino acids (5, 6, 31). Cells expressing this polypeptide were not adherent to agglutinin-coated hydroxylapatite beads but showed some degree of aggregation by salivary agglutinin (6).

In mutant strain OB220, it was demonstrated that *ermAM* was incorporated into the genome at a single site. Increases in size of single *Eco*RI and *Hin*dIII fragments corresponding in each case to an insertion of *ermAM* (1.8 kb) were observed. However, probing the blots of *S. gordonii* chro-

mosomal DNAs with the *sspA* gene fragment revealed two hybridizing *Eco*RI fragments and five *Hin*dIII fragments. These observations were inconsistent with the restriction enzyme sites present in the probe fragment which contained no *Eco*RI site and only two *Hin*dIII sites (Fig. 1). Since the stringency of hybridization and washing would have allowed no more than approximately 10% mismatch of base pairs in hybrids, these data suggest the existence of a second chromosomal locus with sequence homology to *sspA*. If the presence of a homolog to *sspA* is invoked, then account must be taken of the fact that insertion of *ermAM* onto the chromosome of strain OB220 abolished production of all antigenic material reacting with P1 antiserum. Therefore, an *sspA* gene homolog would have to be either nontranscribed or normally cotranscribed with *sspA* such that expression of the homolog was also inactivated by *ermAM* insertion into *sspA*. These possibilities are currently under investigation.

Abolishing SspA expression did not affect the ability of *S. gordonii* cells to be aggregated in whole or parotid saliva or to adhere to hydroxylapatitie beads coated with whole or parotid saliva. By comparison, inactivation of *pac* expression in *S. mutans* MT8148 abolished whole-saliva-mediated aggregation of cells and considerably reduced binding of bacteria to S-HA beads (26). Likewise, disruption of *spaP* in *S. mutans* 834 abolished adherence of cells to parotid saliva-coated hydroxylapatite beads (3). Therefore, unlike the P1-like proteins in *S. mutans*, SspA does not appear to play a pivotal role in whole or parotid saliva-mediated aggregation and adherence in *S. gordonii* DL1. Presumably, this is at least partly due to the ability of *S. gordonii* cells to bind a variety of salivary components. Salivary components able to aggregate *S. gordonii* cells include agglutinin (9, 10), low-molecular-mass mucin MG-2 (32), and histidine-rich polypeptides (42). Polypeptides in salivary pellicle that are able to promote *S. gordonii* adherence include MG-2 (32), proline-rich proteins (17), proline-rich glycoprotein (32), and α-amylase (50). Evidence is provided herein that SspA protein in *S. gordonii* DL1 interacts with salivary agglutinin and that binding to agglutinin was inhibited by NeuNAc. However, inactivation of the gene encoding SspA did not abolish completely the adherence of strain OB220 cells to immobilized agglutinin or, completely, their aggregation in the presence of agglutinin. It is suggested, therefore, that *S. gordonii* DL1 may carry another adhesin on the cell surface that binds salivary agglutinin.

Of particular interest was the observation that SspA$^-$ mutant cells were deficient in the ability to adhere to actinomyces. Coaggregation of actinomyces and streptococci can be assessed by mixing the organisms in suspension; however, with the actinomyces cells immobilized, more accurate and reproducible measurements of numbers of streptococcal cells bound were obtained. Many intergeneric coaggregations of oral bacteria involve lectin-carbohydrate interactions between the cells (27). The mechanisms of adherence of *S. gordonii* DL1 to the various *A. naeslundii* strains (Fig. 7) are not yet elucidated; however, the reactions were not inhibitable by fucose, lactose, GalNAc, or NeuNAc. Evidence suggests that coaggregation of *A. naeslundii* T14V (actinomyces coaggregation group A) with *S. gordonii* DL1 (streptococcus coaggregation group 1) in suspension involves a protein on the streptococcus reacting with a heat-stable component on the actinomyces (27). The simplest explanation for the SspA$^-$ mutant cell phenotype is that SspA polypeptide, besides interacting with salivary agglutinin, interacts with a receptor on the cell surfaces of actinomyces. However, the reaction would appear to be independent of the NeuNAc-sensitive binding of streptococci to salivary agglutinin. This suggestion, that SspA might therefore have more than one adhesin function, is not unreasonable considering evidence from other recent studies of P1-like proteins. For example, antigen I/II binds basic proline-rich proteins in saliva (43), and antibodies to P1 were found to reduce the binding of *S. mutans* KPSK2 cells to *S. sanguis* G9B (29). A possible alternative explanation for the results is that inactivation of *sspA* could affect the synthesis and/or assembly of other cell surface components necessary for streptococcal cell binding to actinomyces. Purifying recombinant SspA polypeptides from *E. coli* and testing their ability to inhibit adherence of streptococci to actinomyces will determine whether SspA is involved directly in cell-cell adhesion.

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