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Insertional Inactivation of the Gene Encoding a 76-Kilodalton Cell Surface Polypeptide in *Streptococcus gordonii* Challis Has a Pleiotropic Effect on Cell Surface Composition and Properties

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A library of *Streptococcus gordonii* DL1-Challis DNA was constructed in λgt11. Phage plaques were screened for production of antigens that reacted with antiserum to *S. gordonii* cell surface proteins. A recombinant phage denoted λgt11-cp2 was isolated that carried 1.85 kb of *S. gordonii* DNA and that expressed an antigen with a molecular mass of 29 kDa in *Escherichia coli*. Antibodies that reacted with the expression product were affinity purified and were shown to react with a single polypeptide antigen with a molecular mass of 76 kDa in *S. gordonii* DL1-Challis. A segment (0.85 kb) of the cloned DNA within the transcription unit was ligated into a nonreplicative plasmid carrying an erythromycin resistance determinant and transformed into *S. gordonii* DL1-Challis. The plasmid integrated onto the chromosome, and expression of the 76-kDa polypeptide antigen was abolished. The gene inactivation had no obvious effect on bacterial growth or on a number of phenotypic properties, including hydrophobicity and adherence. However, it abolished serum-induced cell aggregation, mutant cells had reduced aggregation titers in saliva and in colostrum immunoglobulin A, and it also reduced coaggregation with some *Actinomyces* species. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of cell envelope proteins from wild-type and mutant strains showed that as well as lacking the surface-exposed 76-kDa polypeptide, mutant cell envelopes were deficient in several other polypeptides, including those that bound to immunoglobulin A. Expression of the gene encoding the 76-kDa polypeptide in *S. gordonii* appeared to be critical for functional conformation of the cell surface.

Oral streptococci, in particular, strains of *Streptococcus gordonii* and *Streptococcus sanguis*, are the earliest colonizers of clean tooth surfaces and are among the predominant bacteria in human coronal plaque and gingival crevices (17). *S. gordonii* and *S. sanguis* strains are successful at colonizing a variety of sites in the oral cavity, essentially because cells have a high affinity for saliva pellicle (7) and they adhere to a number of different oral bacteria (28). In addition, *S. gordonii* and *S. sanguis* frequently colonize other body sites and are etiologic agents in bacterial endocarditis (51). Adherence of the organisms at sites of endocardial damage is believed to be mediated by plasma components, including fibronectin (36, 51) and platelets (19).

Adherence and aggregation reactions are determinants of growth and survival of oral streptococci in the human body. Adherence of *S. gordonii* and *S. sanguis* to the salivary pellicle involves electrostatic and hydrophobic forces (10) and, probably, lectin-carbohydrate interactions (33, 44, 45). Aggregations of *S. gordonii* and *S. sanguis* cells occur in saliva (12, 35, 50), crevicular fluid (5), and serum (43). Evidence suggests that both adherence to the pellicle and cell aggregation reactions are determined at least in part by proteins present on the streptococcal cell surface. Surface components of *S. gordonii* and *S. sanguis* cells that have been shown to be involved in interactions with salivary components include adherence-blocking proteins (34), a sialic acid-interacting lectin (45), a surface antigen, SSP-5, with a high M_r (9), and a salivary adhesin complex (31). The surface SSP-5 antigen and the salivary adhesin complex are implicated as receptors for salivary agglutinins in saliva-induced cell aggregation. A fibrillar glycoprotein (42) and a fimbrial protein (13) are suggested as being involved in the attachment of cells to the pellicle. Coaggregations of *S. gordonii* and *S. sanguis* with other oral bacteria also involve lectin-carbohydrate interactions. Oligosaccharide sequences that are present on the surfaces of two strains of *S. sanguis* and that act as receptors for *Actinomyces viscosus* fimbrial lectins have recently been identified (4, 40). Thus, adherence and aggregation reactions involve numbers of different cell surface components.

Thirty or more polypeptides are exposed on the cell surface of *S. gordonii* (1, 22), and we attempted to define functions for these polypeptides in physiology and virulence. To try to identify surface proteins involved in adherence and aggregation, mutants of *S. gordonii* and *S. sanguis* deficient in these properties or altered in surface hydrophobicity have been isolated and characterized. Analyses of such mutants have implicated certain proteins in saliva-mediated adherence (41) and in conferring hydrophobicity (25, 41). Generally, though, mutants have shown complex structural and biochemical differences from the parent strains (14, 16).

To extend the genetic analysis of the cell surface of *S. gordonii*, we constructed mutants with defined mutations in genes that encode cell envelope polypeptides. In this study, insertion duplication mutagenesis was used to inactivate a surface protein gene in *S. gordonii*. The insertion had a pleiotropic effect on cell envelope polypeptide composition, and a variety of cell aggregation reactions were affected.

MATERIALS AND METHODS

Bacteria. *S. gordonii* DL1-Challis (previously, *S. sanguis*, [27]) was used throughout this study. The *Actinomyces* strains used in coaggregation tests were *A. viscosus* BE24,

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T14V, and WVU627 and *A. naeslundii* EF1006, NCTC 12104, TF11, and W1544. *Escherichia coli* Y1089 [Δ*lacU169* Δ*lon araD139 strA hflA150* (*chr*::Tn*10*) (pMC9)] and *E. coli* Y1090 [Δ*lacU169* Δ*lon araD139 strA supF*(*trpC22*::Tn*10*) (pMC9)] were both host strains for λgt11. *E. coli* JM105 [Δ(*lac pro*) *thi strA endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q}ZΔM15] (54) and DH5α [*recA* F$^-$ *endA1 gyrA96 thi-1 hsdR17* (r_k^- m_k^+) *sup44 relA1*] were both used as host strains for plasmids.

Media. Streptococci and *Actinomyces* species were grown on BHYN agar (22) at 37°C in a GasPak System (BBL Microbiology Systems, Cockeysville, Md.) and routinely in BHY liquid medium (22) at 37°C without shaking in screw-cap tubes or bottles. For cell adherence studies and preparation of culture protein fractions, streptococci were grown at 37°C in TYT-glucose medium containing 5 g of tryptone per liter, 5 g of yeast extract per liter, 4 g of K_2HPO_4 per liter, 0.5 ml of Tween 80 per liter, and 5 g of glucose per liter (pH 7.5). *E. coli* strains were grown on LB medium (39) at 37°C. Ampicillin or erythromycin was incorporated at 50 μg/ml into medium for growth of *E. coli*, when required. Erythromycin was used at 1 μg/ml into medium for growth of *S. gordonii*.

Preparation of cell fractions. Streptococcal cells were grown in TYT-glucose medium to the late exponential phase (A_{600} = 2.0, about 4 × 10^9 cells per ml), harvested by centrifugation (6,000 × *g*, 6°C, 10 min), and washed by two cycles of centrifugation and suspension of the pellet in 10 mM Tris–0.15 M NaCl that was adjusted to pH 7.5 with HCl. Cells were broken with glass beads in a Braun homogenizer (22), and after three low-speed centrifugations (each at 5,000 × *g*, 4°C, 10 min) to remove unbroken cells and debris, the supernatant was fractionated by centrifugation at 120,000 × *g* (4°C, 45 min) into cytoplasmic (supernatant) and envelope (pellet) fractions. Proteins were solubilized from these fractions with sodium dodecyl sulfate (SDS) extraction buffer (23) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE; see below). Proteins from cell-free culture supernatants were precipitated with 50% (wt/vol) $(NH_4)_2SO_4$, collected by centrifugation, solubilized, and prepared for electrophoresis as described previously (3).

Preparation of whole-cell lysates. Cells from late-exponential-phase cultures of *S. gordonii* (1.3 ml) were collected by centrifugation (10,000 × *g* for 2 min), washed, lysed with lysozyme and SDS (24), and subjected to SDS-PAGE. The amounts of protein in samples were measured by the Folin method (37), with bovine serum albumin used as a standard.

SDS-PAGE. Proteins were separated by SDS-PAGE (29) through 10 or 11.5% (wt/vol) acrylamide gels with 5% (wt/vol) stacking gels and were stained with 0.2% (wt/vol) Coomassie brilliant blue R or PAGE blue (BDH Ltd., Poole, United Kingdom) in methanol-acetic acid-water (40:10:50). Proteins were transferred from acrylamide gels to nitrocellulose by electroblotting (53) in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol (pH 8.3) at 17 V/cm for 2 h. Molecular masses of polypeptide bands were estimated from their distances of migration by reference to a plot relating migration distances for marker proteins to their log molecular masses. The markers were α_2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), bovine albumin (66 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), egg albumin (45 kDa), lactate dehydrogenase (36.5 kDa), triosephosphate isomerase (26.6 kDa), trypsinogen (24 kDa), and lysozyme (14.3 kDa).

^{125}I labeling of cells. Bacteria were surface labeled with ^{125}I by using a lactoperoxidase-catalyzed reaction as described previously (22). Cells were broken with glass beads, and portions of cell lysate were solubilized and subjected to SDS-PAGE. Gels were dried onto Whatman 3MM paper and autoradiographed with Kodak X-Omat film.

Detection of glucosyltransferase and protease activities. Proteins from cell-free culture supernatants were subjected to SDS-PAGE, and glucosyltransferase activities were detected in situ (3). The protease activity associated with cell envelope fractions and in cell-free culture supernatants was detected after SDS-PAGE of samples in gels containing 0.2% (wt/vol) gelatin (20).

Preparation of *S. gordonii* surface antigens. Cells of *S. gordonii* DL1-Challis were extracted with 1% (wt/vol) sodium lauroyl sarcosinate to remove surface proteins (22), and the extract was subjected to ion-exchange high-performance liquid chromatography (24). Antisera were raised in rabbits (22) to a protein fraction containing about eight cell surface polypeptides.

Immunodetection. Plaque lifts and Western immunoblots were incubated with antisera diluted 1:500 or 1:1,000, and antibody binding was detected with ^{125}I-labeled protein A (22). Some Western blots were developed by using horseradish peroxidase (HRP)-linked second antibody as follows. After incubation with primary antibodies, the nitrocellulose was washed once with TBS (50 mM Tris hydrochloride, 0.15 M NaCl [pH 8.0]), then with TBS–0.1% bovine albumin–0.1% Nonidet P-40, and finally with TBS–0.1% bovine albumin (each for 10 min). The blot was incubated at 20°C for 2 h with HRP-conjugated goat anti-rabbit immunoglobulin G (IgG; ICN ImmunoBiologicals, Lisle, Ill.) diluted 1:1,000 with TBS–0.1% bovine albumin, then washed as described above followed by an additional final wash with TBS alone, and developed with 3.5 mM 4-chloro-1-naphthol in TBS containing 0.03% (vol/vol) H_2O_2. Reactions were terminated usually after 30 min of incubation in the dark by rinsing the blot several times with distilled water.

Reaction of Western blots with IgA. Nitrocellulose blots were blocked with 3% bovine albumin in TCB (10 mM Tris hydrochloride [pH 7.2] containing 5 mM $CaCl_2$). They were then incubated for 2 h at 22°C in TCB–0.1% bovine albumin–0.1% Tween 20 containing ^{125}I-labeled IgA (final concentration, 0.6 μg/ml; specific activity, 5 μCi/μg). The blots were washed five times (50 ml, 10 min each time) in TCB–0.1% Tween 20, dried, and autoradiographed. In some experiments blots were blocked as described above and incubated for 2 h with nonradioactive IgA (10 μg/ml) in TCB–0.1% bovine albumin. Blots were washed with TCB–0.1% Tween 20 and then incubated with HRP-conjugated rabbit anti-human IgA α chains (Dakopatts A/S, Glostrup, Denmark) diluted 1:1,000 in TBS–0.1% bovine albumin for 2 h at 22°C. Blots were washed and developed with color reagent as described above.

Reaction of blots with serum agglutinin. Human serum (5 mg of protein) was subjected to preparative SDS-PAGE, the region of the gel (90 to 120 kDa) containing serum agglutinin (43) was excised, and the proteins were electroeluted (3). The final preparation contained 0.3 mg of protein per ml and was active in agglutinating cells of *S. gordonii* DL1-Challis. Binding of ^{125}I-labeled agglutinin (0.1 μg/ml; specific activity, 10 μCi/μg) to Western blots of bacterial cell proteins was performed as described above for IgA.

Isolation of DNA. Chromosomal DNA from *S. gordonii* was isolated as described previously (23). Large-scale preparations of plasmid DNA from *E. coli* were performed by alkaline lysis (2), and small-scale preparations were made by a rapid boiling technique (21).

Transformation. Transformation of *E. coli* was performed by the method of Dagert and Ehrlich (8). Competent cells of *S. gordonii* DL1-Challis were produced and transformed as described elsewhere (46), and transformants were selected on BHYN agar containing erythromycin.

DNA methodology and hybridization. Restriction and modifying enzymes were used under the conditions recommended by their manufacturers. DNA samples were electrophoresed through 0.7% (wt/vol) agarose gels and stained with ethidium bromide (26). Restriction fragments were purified from agarose gels by electroelution onto DEAE paper and were eluted from the paper with 1 M NaCl. DNA was Southern blotted onto nitrocellulose paper, and blots were hybridized at 68°C for 16 h in 1 M NaCl–0.1 M trisodium citrate–0.1% SDS with 0.5 to 1.0 μg of probe DNA labeled with [α-^{32}P]dATP by nick translation to a specific activity of approximately 10^8 dpm/μg. After hybridization, blots were washed at high stringency (<10% base pair mismatch) at 65°C in 15 mM NaCl–1.5 mM trisodium citrate–0.5% SDS. Autoradiography was carried out at room temperature with Hyperfilm-βmax (Amersham International plc, Little Chalfont, United Kingdom).

Construction of plasmid pNL9740. Plasmid pVA736 (38) was digested with a combination of *Ava*I and *Hin*dIII, and the fragments were separated by agarose gel electrophoresis. A 1.9-kb fragment containing the entire coding region for the erythromycin resistance determinant (38) was isolated and the ends were filled in by using the Klenow enzyme. Plasmid pUC9 (54) was digested with *Nde*I (Bethesda Research Laboratories, Gaithersburg, Md.), the ends were filled in, and the plasmid was ligated with the 1.9-kb fragment carrying the erythromycin resistance determinant. The mixture was transformed into *E. coli* JM105 with simultaneous selection for ampicillin and erythromycin resistances on LB agar plates. Plasmid pNL9740 (4.6 kb) was subsequently purified; it contained the multiple cloning site from pUC9 intact, and accordingly, *E. coli* JM105(pNL9740) formed blue colonies on LB-ampicillin agar containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal).

Cloning in λgt11. Chromosomal DNA from *S. gordonii* DL1-Challis was digested with *Eco*RI, and a portion (approx. 3 μg) was ligated with alkaline phosphatase-treated λgt11 *Eco*RI arms (1 μg; Promega Corp., Madison, Wis.). The ligated DNA was packaged in vitro (Amersham), and the phage titer was determined by plating suitable dilutions in PD buffer (20 mM Tris hydrochloride, 10 mM $MgSO_4 \cdot 7H_2O$, 0.15 M NaCl [pH 7.4]) on *E. coli* Y1090.

Screening of λ plaques. *E. coli* Y1090 cells were grown with shaking at 37°C to the late exponential phase of growth in LB medium containing 0.2% (wt/vol) maltose and ampicillin (50 μg/ml). To samples (0.1 ml) of Y1090 cells was added 0.1 ml of phage suspension appropriately diluted in PD buffer to give approximately 2×10^3 plaques per 90-mm-diameter plate. The phages were incubated with cells at 37°C for 20 min; 4 ml of molten LB top agar containing 10 mM $MgSO_4 \cdot 7H_2O$, isopropyl-β-D-thiogalactopyranoside (IPTG; 0.16 mg/ml), and X-gal (0.1 mg/ml) was added; and the mixtures were poured onto LB agar plates, which were then incubated at 37°C for 16 h. More than 90% of the plaques from the packaging were colorless, indicating insertion of *S. gordonii* DNA into the β-galactosidase (*lacZ*)-coding region of λgt11.

Nitrocellulose filter disks were fitted onto the surfaces of top agars which were previously hardened by incubating the plates at 4°C for 1 h. Plates were incubated at 4°C for an additional 1 h, and then the filters were removed and blocked by incubation at 20°C for 45 min with phosphate-buffered saline (PBS; 50 mM KH_2PO_4, 0.15 M NaCl, adjusted to pH 7.5 with KOH) containing 3% bovine albumin. Filters were then incubated with antiserum diluted 1:1,000 in PBS–1% bovine albumin (10 ml per filter) at 20°C for 3 h, washed twice with PBS–0.1% Tween 20 (20 ml per filter, 10 min each time), and further incubated at 20°C for 2 h with ^{125}I-labeled protein A (2 ng/ml; specific activity, 10 to 20 μCi/μg) in PBS–1% bovine albumin. The filters were washed five times with PBS–0.1% Tween 20 (20 ml per filter), once with PBS, and then dried at 60°C and exposed to Kodak X-Omat film at 20°C for 1 to 5 days as necessary. Areas on the original plates containing positive-reacting plaques with strong signals were identified, the phages were harvested in PD buffer and plated onto Y1090, and plaques were reprobed with antiserum as described above. Once they were purified, recombinant phages were stored lysogenized in the host *E. coli* Y1089 (55).

Preparation of phage and phage DNA. An exponential-phase culture (1 liter) of lysogen was heat shocked at 45°C to induce phage replication (55), and after incubation at 37°C for an additional 2 h, bacteria were sedimented by centrifugation at 4,000 × *g* for 10 min, suspended in PD buffer (15 ml), and incubated at 20°C for 30 min with chloroform (0.3 ml). DNase I (0.02 mg) was added, and the suspension was mixed and allowed to stand for 5 min. The lysate was centrifuged (12,000 × *g*, 4°C, 15 min), the supernatant was removed into a clean tube and was mixed with an equal volume of chloroform, and the phases were separated by centrifugation at 1,600 × *g* for 15 min. The phages were precipitated from the aqueous phase with NaCl and polyethylene glycol 6000 (BDH Ltd.) and purified by centrifuging them through a step gradient of CsCl (26). Phage DNA was prepared as described previously (26) and was purified by centrifugation through a syringe column of Sephadex G50 (39).

Preparation of proteins from induced lysogens. After 2 h at 37°C, a portion (1.5 ml) of the culture of induced lysogen was centrifuged (10,000 × *g*, 3 min), and the cells were suspended in 0.05 ml of SDS extraction buffer (23) and heated at 100°C for 5 min. The viscosity was reduced by brief sonication, and after centrifugation (10,000 × *g*, 5 min) a portion of the supernatant was mixed with loading dye and subjected to gel electrophoresis.

Affinity adsorption of antibodies. Antibodies to the λgt11-cp2 expression product were affinity purified by elution of bound antibodies from nitrocellulose plaque lifts (52). λgt11 and recombinant λgt11-cp2 phages were plated onto *E. coli* Y1090 at a density of about 10^5 plaques per plate, i.e., virtually confluent. Plates were incubated at 42°C for 4 h, and then a nitrocellulose disk was placed on each of the top agars and the plates were incubated at 30°C for an additional 14 h. The filters were removed, rinsed twice with TBS, and blocked with TBS–3% bovine albumin (30 min). *S. gordonii* surface protein antiserum diluted 1:200 in TBS–0.5% bovine albumin (40 ml) was reacted at 20°C for 1 h first with the filter carrying wild-type λgt11 plaques in order to preabsorb antibodies to *E. coli*. This filter was then removed from the dish and replaced by the filter onto which λgt11-cp2 plaques had been adsorbed. After incubation with shaking at 20°C for 6 h, the filter was rinsed with TBS and incubated at 20°C for 8 min in 0.2 M glycine hydrochloride (pH 2.5) containing 0.1% bovine albumin (3.5 ml) to effect the release of bound antibodies. The solution was then neutralized with 1 M K_2HPO_4, diluted to 25 ml with TBS–0.1% bovine albumin,

and used immediately for reaction with Western blots of cell proteins at 4°C for 16 h.

Measurement of adherence properties. Cell surface hydrophobicity was measured by hexadecane partitioning (49) as described previously (23). The numbers of streptococci that adhered to buccal epithelial cells were measured by direct microscopic count (18). Adherence of tritium-labeled cells (25) to saliva-treated hydroxylapatite was measured by the method of Clark et al. (7). Coaggregation reactions of streptococci and *Actinomyces* species were performed in round-bottom wells of microdilution plates. For coaggregation tests, bacterial cells were harvested from cultures in the early stationary phase of growth in TYT-glucose medium, washed twice by centrifugation (10,000 × *g*, 4°C, 10 min) and suspension in 10 mM Tris hydrochloride (pH 7.6) containing 0.15 M NaCl and 5 mM $CaCl_2$ (TBSC buffer), and then suspended in coaggregation buffer (6) containing 0.02% (wt/vol) NaN_3 at an A_{600} of 5.0 (about 1×10^{10} streptococcal cells per ml or 5×10^9 *Actinomyces* cells per ml). Serial twofold dilutions of streptococcal cell suspensions in coaggregation buffer (0.05 ml) were mixed with equal volumes of *Actinomyces* cell suspensions (2.5×10^8 cells), and plates were shaken on an IKA-Vibrax bench top shaker (Janke & Kunkel GMBH & Co., Staufen, Federal Republic of Germany) set at 300 rpm at 20°C for 20 min. Coaggregations were scored as 2+ (completely clumped), 1+ (semi-clumped), or 0 (negative), and coaggregation titers of streptococci are expressed as the reciprocals of the highest dilution of streptococcal cells that caused 1+ coaggregation.

Bacteria-mediated hemagglutination. Human erythrocyte type O from blood drawn in anticoagulant citrate phosphate glucose solution or horse erythrocytes from defibrinated blood were washed three times by centrifugation (400 × *g*, 20°C, 10 min) in TBS (pH 7.4)–0.1% gelatin. Neuraminidase treatment of erythrocytes was performed at 37°C for 1 h with 10% (vol/vol) packed cell suspensions (1 ml) and 1 U of type V neuraminidase (Sigma Chemical Co., St. Louis, Mo.). After treatment, erythrocytes were washed three times by centrifugation in TBS (pH 7.4)–0.1% gelatin. Bacteria were cultured, harvested, and washed as described above for coaggregation measurements but were suspended finally in TBS (pH 7.4)–0.1% gelatin at an A_{600} of 5.0. Visual assays for hemagglutination were performed in round-bottom wells of microdilution plates. Serial dilutions of bacterial cell suspensions in TBS (pH 7.4)–0.1% gelatin (0.05 ml) were mixed with equal volumes of 2% (vol/vol) erythrocytes, and plates were shaken at 20°C for up to 15 min and examined periodically during this time. Hemagglutination activity of bacteria (titer) was expressed as the reciprocal of the highest dilution of bacterial cells that caused complete hemagglutination.

Saliva- and serum-induced bacterial aggregation. Unstimulated whole saliva from several human volunteers was collected and pooled on ice and clarified by centrifugation at 10,000 × *g* (4°C, 20 min). Saliva was used as soon as possible after collection. Some saliva samples were heat treated at 80°C for 30 min. Human serum was obtained from a type O donor, rabbit serum was from nonimmune New Zealand White rabbits, and horse serum was obtained commercially (Sigma). Some serum samples were adjusted to pH 5.0 and treated with neuraminidase (1 U) at 37°C for 2 h (43). Serial dilutions of saliva, serum, and protein fractions were made with TBC in round-bottom wells of microdilution plates (0.05 ml per well). Streptococci were cultured, harvested, and washed as described above for coaggregation tests and were suspended at an A_{600} of 5.0 in TBC containing 0.02% (wt/vol) NaN_3. To measure the aggregation of streptococci in saliva or serum, equal volumes (0.05 ml) of bacterial suspension were added to each well, plates were shaken at 20°C for up to 30 min, and aggregation titers were recorded as the reciprocals of the highest dilutions of saliva or serum that caused complete bacterial aggregation. All assays were run in duplicate or triplicate, and experiments were repeated at least three times with different batches of freshly grown bacterial cells. The effects of EDTA, sialic acid, colominic acid, and various sugars on saliva- or serum-mediated bacterial aggregations were tested by incubating the bacterial cells in serial dilutions of the relevant compound for 15 min before the addition of appropriately diluted saliva or serum.

Chemicals. Restriction and DNA-modifying enzymes and radiochemicals were purchased from Amersham (unless indicated otherwise). Acrylamides, SDS, Tris, and glycine were purchased from BDH Ltd. Human serum without IgG, human serum without IgA, purified human serum IgG, purified human colostrum IgA, bovine albumin, equine transferrin, and all other special chemicals were obtained from Sigma, unless stated otherwise.

RESULTS

Cloning of cell surface antigen gene from *S. gordonii*. A λgt11 library of *S. gordonii* DL1-Challis was screened for production of antigens that reacted with surface protein antiserum (see Materials and Methods). One phage isolate that formed plaques that gave strong signals with the probe was purified. Restriction enzyme analysis of the DNA isolated from this phage showed an additional single *Eco*RI fragment of 1.85 kb in the *lacZ* cloning site (Fig. 1). Restriction sites for several enzymes within the insert of the recombinant phage (denoted λgt11-cp2) are shown in Fig. 1.

Expression of cloned gene in *E. coli*. To identify the protein product encoded by the cloned fragment, *E. coli* lysogens carrying the wild-type phage λgt11 or recombinant λgt11-cp2 were grown with or without the *lac* inducer IPTG and were induced by heat shock. Cell proteins were solubilized, separated by SDS-PAGE, and electroblotted onto nitrocellulose. Western blots were incubated with surface protein antiserum (diluted 1:500), and antibody binding was detected with ^{125}I-labeled protein A. The recombinant phage λgt11-cp2 expressed a polypeptide with an approximate molecular mass of 29 kDa (Fig. 2, lane 1). Some lower-molecular-mass bands reacted faintly with the antiserum in Fig. 2, lane 1, and may be proteolytic fragments since no cross-reacting proteins were detected in the lysate of an induced lysogen carrying wild-type λgt11 phage (Fig. 2, lane 3). The presence of IPTG had no effect on the immunoblot profile (Fig. 2, lane 2), indicating that production of the antigenic polypeptide was not under control of the *lacZ* (β-galactosidase) promoter within the vector DNA. There was no binding of preimmune rabbit serum (diluted 1:500) to the protein blots.

Reaction of affinity-purified antibodies with *S. gordonii* proteins. Antibodies within the surface protein antiserum that reacted with the 29-kDa expression product were affinity purified by elution from nitrocellulose filter lifts of λgt11-cp2 plaques (see Materials and Methods). These affinity-purified antibodies were used to probe Western blots of proteins from whole-cell lysates of *S. gordonii* DL1-Challis, and they reacted with a single polypeptide band with a molecular mass of 76 kDa (data not shown). This polypeptide was present in the cell envelope fraction obtained from cells that were broken with glass beads (Fig. 3, lane 5), but it was not detected in a cytoplasmic protein fraction (Fig. 3, lane 7).

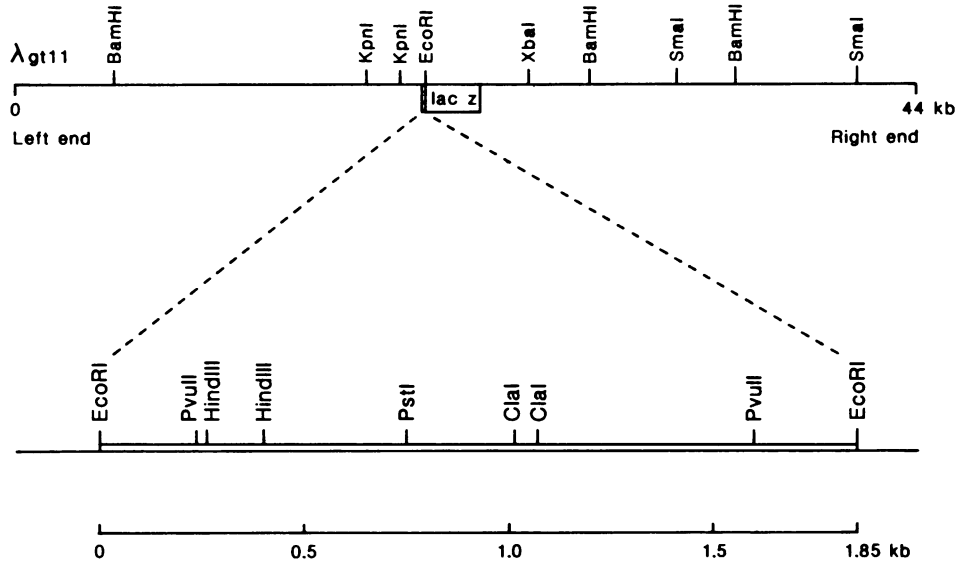

FIG. 1. Restriction endonuclease map of the 1.85-kb fragment of *S. gordonii* DL1-Challis DNA cloned into the *Eco*RI site of λgt11. Only sites for enzymes that recognized hexanucleotide sequences are shown. There were no sites in the streptococcal DNA insert for enzymes *Bam*HI, *Kpn*I, *Sal*I, *Sma*I, *Sst*I, or *Xba*I.

Subcloning and expression of cloned gene. The internal *Pvu*II segment comprising 1.35 kb of the original cloned *Eco*RI fragment (Fig. 1) was ligated into the unique *Sma*I site of plasmid pNL9740 (see Materials and Methods). The presence of the *Pvu*II fragment in pNL9740 was deleterious to the growth of *E. coli* JM105. Transformant colonies containing this plasmid (denoted pNL9743) grew weakly on agar plates, and the streptococcal DNA insert became deleted when cells were grown in liquid medium. Immunoblot analysis of proteins solubilized from colonies carrying pNL9743 showed the presence of several polypeptide bands in the molecular mass range of 21 to 25 kDa that reacted with the *S. gordonii* surface protein antiserum (data not shown).

Plasmid pNL9743 was digested with *Pst*I, which produced two fragments of 5.1 and 0.85 kb. The smaller fragment was equivalent to the internal *Pst*I-*Pvu*II fragment from the original 1.85-kb cloned insert (Fig. 1), but it carried an additional few base pairs of multiple-cloning-site DNA at the *Pvu*II site end. The 0.85-kb *Pst*I fragment was ligated with *Pst*I-digested pNL9740, and the mixture was transformed into *E. coli* JM105 with selection for ampicillin resistance on LB agar plates containing X-gal. Two plasmids denoted pNL9750d1 and pNL9750d3 were purified with the 0.85-kb *Pst*I fragment cloned in opposite orientations. The plasmids were stably maintained in *E. coli* JM105, and on Western immunoblots of transformant cell lysates carrying these plasmids, no antigens were detected that reacted with the surface protein antiserum (data not shown).

Insertional inactivation of the gene encoding the 76-kDa

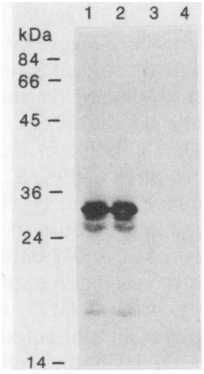

FIG. 2. Antigenic polypeptides expressed from recombinant λgt11-cp2. *E. coli* lysogens carrying either λgt11-cp2 (lanes 1 and 2) or wild-type λgt11 (lanes 3 and 4) were induced by heat shock. Total cell proteins were separated by SDS-PAGE, and Western blots were incubated with *S. gordonii* surface protein antiserum (diluted 1:500) and ^{125}I-labeled protein A. Lanes: 1, λgt11-cp2 lysogen; 2, λgt11-cp2 lysogen with IPTG present in culture medium; 3, λgt11 lysogen; 4, λgt11 lysogen plus IPTG. Migration distances of molecular mass markers are shown for reference. Lanes were each loaded with 50 μg of protein.

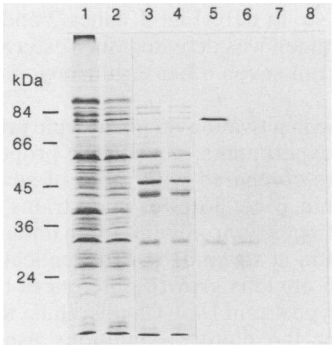

FIG. 3. SDS-PAGE (11.5% acrylamide) patterns of polypeptides (lanes 1 to 4, stained with PAGE blue) and corresponding immunoblots (lanes 5 to 8, reacted with antibodies affinity purified to the λgt11-cp2 expression product) from envelope or cytoplasmic fractions of *S. gordonii* DL1-Challis and mutant strain d1b. Lanes 1 and 5, DL1-Challis cell envelope fraction; lanes 2 and 6, d1b cell envelope fraction; lanes 3 and 7, DL1-Challis cytoplasmic fraction; lanes 4 and 8, d1b cytoplasmic fraction. Lanes 1 and 2 were loaded with about 45 μg of protein; lanes 3 and 4 were loaded with about 30 μg of protein. Migration distances for molecular mass markers are shown.

polypeptide in *S. gordonii*. Nucleotide sequence data on the *Pst*I-*Pvu*II fragment showed an open reading frame throughout its length and thus suggested that this fragment lay wholly within the transcription unit encoding the 29-kDa antigen in *E. coli*. These sequence data also provided an explanation for the absence of antigen production by *E. coli* JM105 transformants carrying pNL9750d1 or pNL9750d3.

So as not to introduce the intact ampicillin resistance gene into *S. gordonii* in the following experiments, plasmid pNL9750d1 was digested with *Cfr*10I, treated with exonuclease III followed by S1 nuclease, and then religated to produce a derivative with a small deletion. The resulting plasmid, pNL9760d1 (5.4 kb), conferred erythromycin resistance but not ampicillin resistance in *E. coli* DH5α. Plasmid pNL9760d1 (which was unable to replicate in *S. gordonii*) was transformed into *S. gordonii* DL1-Challis with selection for erythromycin resistance (1 μg/ml). The plasmid should recombine as its only chromosomal site of homology (within the gene encoding the 76-kDa polypeptide) and disrupt the gene with integration of the entire plasmid (11). From one experiment, $>10^4$ erythromycin-resistant transformants of *S. gordonii* DL1-Challis were obtained per μg of pNL9760d1 DNA.

To confirm plasmid integration, DNAs were extracted from cells of three randomly picked transformants (and from wild-type strain DL1-Challis as a control) and digested with *Eco*RI, and the fragments were separated by agarose gel electrophoresis. The DNA fragments were capillary blotted onto nitrocellulose, and the blot was incubated with nick-translated pNL9750d1, which was used as a probe. The probe reacted with a single *Eco*RI fragment of 1.85 kb in strain DL1-Challis DNA, which was identical in size to the original cloned fragment (Fig. 1). In the mutant strains, the probe reacted with two *Eco*RI fragments of chromosomal DNA with approximate sizes of 6.0 and 1.0 kb (data not shown), providing evidence of plasmid insertion at the expected site.

The SDS-PAGE profiles of proteins in envelope, cytoplasmic, and culture supernatant fractions of strain DL1-Challis (wild type) and one mutant strain d1b are compared in Fig. 3. The 76-kDa antigen, which was found exclusively in the cell envelope fraction of strain DL1-Challis cells (Fig. 3, lanes 1 and 5), was absent from Western blots of cell envelope proteins from strain d1b (Fig. 3, lanes 2 and 6). No 76-kDa polypeptide antigen was detected on Western blots of whole-cell proteins from seven other erythromycin-resistant transformants.

Effect of gene inactivation on phenotypic properties. For all the following experiments, phenotypic properties of mutant strain d1b were compared with those of strain DL1-Challis carrying plasmid pVA736 (38), and strains were grown in media containing erythromycin to maintain selection for plasmid insertion in strain d1b or for replication of pVA736. There were no obvious growth differences between mutant d1b and wild-type strain DL1-Challis; thus, the gene was not essential under the culture conditions used. In addition, there were no differences in the sugars fermented by mutant and wild-type strains (the strains conformed to the patterns described previously [27]), and both strains were identical in their susceptibilities to a number of antibiotics tested (bacitracin, cephalothin, fusidic acid, gentamicin, kanamycin, nitrofurantoin, and sulfamethoxazole). The mutant strain was also identical to strain DL1-Challis in the following properties: glucosyltransferase production (approximate molecular mass, 170 kDa), production of cell-bound and extracellular proteases (approximate molecular masses, 250 to 300 kDa), SDS-PAGE profiles of proteins in cell-free culture fluid, and competence for DNA-mediated transformation.

TABLE 1. Coaggregation of *S. gordonii* DL1-Challis and mutant strain d1b with various *Actinomyces* species[a]

Actinomyces strain	Coaggregation titer of *S. gordonii*[b]:	
	DL1-Challis	d1b
A. viscosus BE64	32	16
A. viscosus T14V	32	32
A. viscosus WVU627	32	2
A. naeslundii EF1006	0[c]	0
A. naeslundii NCTC 12104	2	2
A. naeslundii TF11	32	8
A. naeslundii W1544	32	4

[a] Experiments were performed in triplicate and repeated three times; titers of triplicates were identical.

[b] Titer is expressed as the reciprocal of the highest dilution of streptococcal cells showing a 1+ coaggregation score with 2.5×10^8 *Actinomyces* cells.

[c] No coaggregation was observed when 5×10^8 streptococcal cells were mixed with 2.5×10^8 *Actinomyces* cells.

Effect of gene inactivation on adherence and aggregation properties. Since the 76-kDa polypeptide was extracted from *S. gordonii* cells by a method that released surface proteins (22), it was possible that mutants that lacked the polypeptide had altered cell surface properties. Cells of DL1-Challis and mutant d1b had similar surface hydrophobicities and adhered to the same extent to buccal epithelial cells or to saliva-treated hydroxylapatite. However, there were differences in the abilities of the wild-type and disruptant strains to coaggregate with *Actinomyces* species. Mutant strain d1b coaggregated with *A. viscosus* T14V in a manner similar to that of the wild-type strain, but it had reduced coaggregating ability with most of the other *Actinomyces* species, especially with *A. viscosus* WVU267 (Table 1).

Aggregations of mutant and wild-type strains were assayed by using microdilution plates with twofold serial dilutions of saliva, sera, or serum components. The saliva-induced aggregation titer of mutant strain d1b was 8- to 16-fold lower than the corresponding titer of strain DL1-Challis (Table 2). Heating of the saliva at 80°C for 30 min reduced significantly the DL1-Challis titer but not the titer for strain d1b. Saliva-induced aggregation was Ca^{2+} dependent and was abolished by the addition of >5 mM EDTA. Aggregation was also abolished by the addition of 10 mM sialic acid. There were no differences in hemagglutination activities of strain DL1-Challis or strain d1b with fresh human or horse erythrocytes (titers, 32/64). Bacteria did not agglutinate neuraminidase-treated erythrocytes.

Various sera induced aggregation of wild-type DL1-Challis cells (titers, between 512 and 2,048), but cells of mutant strain d1b were usually not aggregated (Table 2). Human serum that had been heated at 60°C, with the ensuing clot removed, was as active as unheated serum in inducing aggregation of strain DL1-Challis cells (Table 2). Serum-induced aggregations were promoted by 5 mM $CaCl_2$ and were inhibited in the presence of >30 mM EDTA. Aggregations were not affected by the addition (to 0.1 M) of various sugars, including D-galactose, D-glucose, D-mannose, L-rhamnose, or *N*-acetyl-D-glucosamine, or by 10 mM sialic acid or colominic acid. Treatment of human serum with neuraminidase reduced the aggregation titer for *S. gordonii* DL1-Challis to 128/256.

Human serum from which IgG was removed mediated cell

TABLE 2. Aggregation titers of *S. gordonii* DL1-Challis and mutant strain d1b with saliva, serum, and protein fractions[a]

Agglutinin	Aggregation titer of *S. gordonii*[b]:	
	DL1-Challis	Strain d1b
Whole saliva	64/128	4/8
Heated saliva (80°C, 30 min)	16	2/4
Human serum	1,024/2,048	0/8
Horse serum (defibrinated)	512	0/4
Rabbit serum (defibrinated)	256/512	0/2
Heated human serum (60°C, 30 min)	2,048	2/8
Human serum minus IgG[c]	1,024	2/8
Human serum minus IgA[d]	512	2/4
Human serum IgG (1 mg/ml)	0[e]	0
Human colostrum IgA (1 mg/ml)	16/32	4

[a] Experiments were performed in duplicate and repeated four times; results of saliva aggregation are for one batch, as titers varied with batches.

[b] Titer is expressed as the reciprocal of the highest dilution of saliva, serum, or protein fraction causing aggregation of 5×10^8 streptococcal cells; duplicate titers were identical and the ranges over experiments are shown.

[c] Protein concentration, 68 mg/ml.

[d] Protein concentration, 54 mg/ml.

[e] No aggregation when 5×10^8 streptococcal cells were incubated with an equal volume (0.05 ml) of serum protein solution.

aggregation of *S. gordonii* DL1-Challis (Table 2). Cells were not aggregated by purified IgG, albumin, or transferrin. Aggregation titers for DL1-Challis were slightly reduced in human serum from which IgA was removed. Cells of strain DL1-Challis were aggregated by IgA from human colostrum, and the IgA-induced aggregation titer for the mutant d1b was reduced (Table 2).

Surface proteins and IgA binding. Cells of strain DL1-Challis and mutant d1b were surface labeled with ^{125}I, and surface-exposed proteins were detected by autoradiography after SDS-PAGE. The 76-kDa polypeptide was 1 of about 30 surface-labeled polypeptides in strain DL1-Challis and was missing from strain d1b (Fig. 4). Results of the experiments also suggested reduced labeling of several other bands in the mutant strain (Fig. 4). A more detailed analysis of SDS-PAGE profiles of cell envelope proteins from wild-type and mutant strains showed that as well as being deficient in the 76-kDa polypeptide, cell envelopes of mutant strain d1b had reduced amounts of the 97-, 39-, 33-, 28-, 26-, 20.5-, and 20-kDa polypeptides (Fig. 5, lane 6). When Western blots of these gels were reacted with ^{125}I-labeled IgA, the 28- and 26-kDa polypeptide bands were the major IgA-binding proteins (Fig. 5, lanes 1 and 3). A total of five polypeptide bands in strain DL1-Challis reacted with IgA (Fig. 5, lanes 1 and 3), and the amounts of all these were reduced in mutant strain d1b (Fig. 5, lanes 2 and 4). The 76-kDa polypeptide did not bind IgA (Fig. 5). Partially purified ^{125}I-labeled serum agglutinin was also used to probe nitrocellulose blots of DL1-Challis and d1b cell envelope proteins. The labeled probe bound to about 35 polypeptide bands in each strain, it did not bind to the 76-kDa band in DL1-Challis, and no clear differences could be seen between agglutinin-binding protein profiles of the wild-type and mutant cells (data not shown).

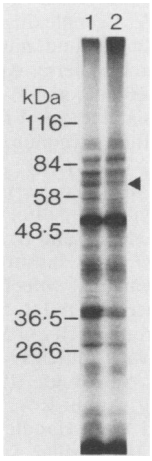

FIG. 4. SDS-PAGE (10% acrylamide) profiles of polypeptides from cells of *S. gordonii* DL1-Challis (lane 1) or d1b (lane 2) surface labeled with ^{125}I. Labeled cells were broken with glass beads, and samples of extract were solubilized and electrophoresed. Each lane contained about 50 μg of protein. The positions of molecular mass markers are indicated, and the arrowhead indicates the position of the 76-kDa band.

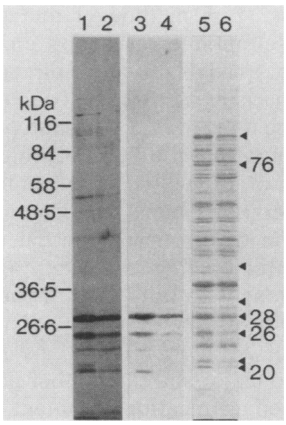

FIG. 5. Reaction of Western blots of cell envelope polypeptides separated by SDS-PAGE (10% acrylamide) from strain DL1-Challis (lanes 1, 3, and 5) or strain d1b (lanes 2, 4, and 6) with IgA. Lanes 1 and 2, autoradiograph of blot reacted with ^{125}I-labeled IgA; lanes 3 and 4, blot reacted with nonradioactive IgA (10 μg/ml) and developed with HRP-conjugated second antibody; lanes 5 and 6, stained gel lanes (Coomassie brilliant blue R) corresponding to blots. Gel lanes were loaded with 65 μg of protein. Positions of molecular mass markers are indicated for reference. Arrowheads on the right indicate polypeptides that were absent or reduced in the cell envelope of strain d1b (see text).

DISCUSSION

S. gordonii and *S. sanguis* cells present an array of polypeptides at their cell surfaces (1, 22, 25). Precisely which of these are involved in determining cell adherence and aggregation properties is not known. Several genes encoding surface polypeptides in *S. gordonii* or *S. sanguis* were cloned and expressed in *E. coli*. Two of these genes encode proteins capable of binding salivary agglutinin and are involved in saliva-induced aggregation (9, 47). An additional two cloned genes encode protein components of fibrils (15) and fimbriae (13), structures that are suggested to be involved in the adherence of cells to saliva-treated hydroxylapatite. The gene cloned and identified in this report is distinct both structurally and functionally from these other genes that were isolated.

Insertion duplication mutagenesis was used to inactivate the gene encoding a 76-kDa surface polypeptide in *S. gordonii* in order to determine the role of this polypeptide in cell

surface functions. This method of mutagenesis was used primarily because suitable restriction enzyme sites within the gene were not available to allow direct insertion in vitro of a selectable marker, such as has been done to inactivate the gene encoding antigen P1 in *Streptococcus mutans* (32). Plasmid insertion within the 76-kDa protein gene caused a pleiotropic effect on the polypeptide composition of the cell envelope and alterations in cell aggregation properties of the cells. Aggregation of oral streptococcal cells in saliva involves cell receptor binding of salivary agglutinins (33, 45) and cell interactions with IgA (35, 50), bacterial aggregating factors (12), and lysozyme (30). Analysis of IgA binding to Western blots of *S. gordonii* proteins revealed that the organism has two major and three other polypeptides which bind IgA. Reduced aggregation of mutant d1b cells in saliva could be explained by the observation that amounts of these IgA-binding proteins were clearly reduced in the cell envelope. This explanation for a reduced saliva aggregation titer in the mutant was supported by two independent observations. First, the mutant cells showed reduced aggregation in purified IgA from colostrum, and second, heating of saliva at 80°C, which inactivated IgA but not salivary glycoprotein agglutinin, reduced the aggregation titer of wild-type DL1-Challis but not that of the mutant strain. The gene inactivation did not affect the sialic acid-reversible cell interaction with salivary agglutinin. Interestingly, the gene inactivation also did not affect adherence of mutant cells to saliva-treated hydroxylapatite. This emphasizes the distinction that has been made (48) between saliva-induced cell aggregation and saliva-mediated cell adherence.

The most pronounced phenotypic effect of the gene inactivation was on serum-mediated aggregation. IgA binding did not seem to be a major factor in this reaction, unlike that in saliva-mediated aggregation, since high-titer aggregation of strain DL1-Challis cells nevertheless occurred in serum from which IgA was removed. The characteristics of the serum-induced aggregation of *S. gordonii* DL1-Challis suggest that it involves a serum glycoprotein agglutinin, as has been described for serum-induced aggregation of *S. sanguis* 12 (43). However, we could not show that blots of cell envelope polypeptides from wild-type and mutant strains differed in their serum agglutinin-binding profiles. Thus, the results do not identify a particular protein receptor for bacterial agglutinin in serum. Indeed, it is likely that aggregation reactions of cells in saliva and serum and with some *Actinomyces* species involve multiple components on the streptococcal cell surface.

The changes in composition of the cell envelope in the mutant strain could be due to the fact that plasmid insertion affects expression of neighboring genes; alternatively, or in addition, the failure to produce and thus incorporate the 76-kDa polypeptide into the cell envelope may affect the assembly of other components. It follows that regulation of the expression of the 76-kDa protein gene in *S. gordonii* might modulate cell surface properties and bacterial virulence. The 76-kDa polypeptide did not itself bind IgA or serum agglutinin on Western blots. However, a role for this polypeptide in binding of aggregating factors present in saliva (12) and serum and associated with the surfaces of *Actinomyces* cells cannot be excluded. Sequence analysis and mutagenesis of the genomic region encompassing the 76-kDa protein gene should reveal further how cell surface structure is determined by expression at this locus.

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