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Insertional Inactivation of an Intrageneric Coaggregation-Relevant Adhesin Locus from *Streptococcus gordonii* DL1 (Challis)

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Transposon Tn*916* was used to insertionally inactivate a coaggregation-relevant locus of *Streptococcus gordonii* DL1 (Challis). One mutant (F11) was isolated that lost the ability to coaggregate with the streptococcal partners of DL1 but retained the ability to coaggregate with partners belonging to other genera. A probe specific for the region flanking the Tn*916* insertion was used to isolate a locus-specific fragment from a chromosomal λ library. Southern analysis of the resulting phagemids revealed that a 0.5-kb *Eco*RI fragment hybridized with the F11 probe. Cloning of the 0.5-kb *Eco*RI fragment into the *E. coli*-streptococcal insertion vector pΩ yielded pCW4, which was used to insertionally inactivate the putative coaggregation-relevant gene in DL1. Insertion mutants showed altered coaggregation with streptococci but retained wild-type coaggregation properties with other genera of bacteria. Comparison of immunoblots of cell surface proteins showed a 100-kDa protein in DL1 which was not detected in the Tn*916* and pCW4 insertion mutants. These results indicate that the 0.5-kb *Eco*RI fragment is part of an adhesin-relevant locus that is involved in the production of a 100-kDa protein at the cell surface.

Results from previous work have identified several streptococcal surface proteins involved in coaggregation with other oral bacteria. Coaggregation between *Streptococcus gordonii* PK488 and *Actinomyces naeslundii* PK606 involves a 34.8-kDa lipoprotein designated ScaA (2). A 76-kDa lipoprotein, HppA (originally SarA), from *S. gordonii* DL1 is a hexapeptide and heptapeptide transporter (21) and was previously shown to be involved in serum-induced cell aggregation and adherence to some species of *Actinomyces* (22). A 290-kDa protein (CshA) is associated with hydrophobicity and intergeneric coaggregation properties with actinomyces partners (40). Several proteins that belong to a family of polypeptides (antigen I/II-like polypeptides) that are present in several oral streptococcal species, notably the mutans group streptococci, have been identified (20, 32). *S. gordonii* DL1 and M5 each express two highly similar but nonidentical antigen I/II polypeptides, SspA and SspB (formerly Ssp-5), that are the products of tandemly arranged chromosomal genes (11). Insertional inactivation of the 210-kDa protein SspA in *S. gordonii* DL1 affects aggregation and adherence to actinomyces (23). The 162-kDa sialic acid-binding lectin SspB from *S. gordonii* M5 binds the human salivary components agglutinin and proline-rich proteins (32). Processing of a 170-kDa antigen I/II-like protein of *S. gordonii* G9B into smaller fragments of 62 or 60 and 45 kDa was proposed for binding to *Porphyromonas gingivalis* (33).

Streptococci are the major early colonizers of the tooth surface (43). They are unusual among the oral bacteria in that they participate in intrageneric coaggregation, a property also shared to a much lesser extent by some oral actinomyces (25, 28) and fusobacteria (30). These interactions among the streptococci are highly specific in that only certain paired strains are coaggregation partners, and all of the coaggregations are inhibited by galactosides (25). In an extensive survey of the adherence properties of 71 oral streptococci, 36 were observed to coaggregate with other streptococci (18). Intrageneric coaggregation may be advantageous because a freshly cleaned tooth surface presents receptor sites within the nascent acquired pellicle that are quickly filled with streptococci that grow and multiply to form a biofilm (34, 35, 48). Successful colonization by other streptococci may be effected by their ability to adhere to streptococci already attached to the acquired pellicle, in addition to other early colonizers, for example, actinomyces and haemophili (29, 35).

The aim of this study was to isolate mutants of *S. gordonii* DL1 that were unable to exhibit intrageneric coaggregation (Cog^-) but maintained coaggregation with members of other genera. With these mutants the adhesins that mediate intrageneric coaggregation among oral streptococci could then be identified. *S. gordonii* DL1 was chosen as the model to study intrageneric coaggregation. It has on its surface the heat- and protease-sensitive adhesin that recognizes galactoside-containing receptors on the surface of *Streptococcus oralis* 34, *S. oralis* C104, and *Streptococcus* SM PK509 (25, 28). A chromosomal library of *S. gordonii* PK488 was also used in this study because it has the same streptococcal partners as *S. gordonii* DL1 (28), and Western blot (immunoblot) analysis showed that cell surface extracts had a protein profile similar to that of *S. gordonii* DL1 when probed with anti-DL1 serum preabsorbed with a spontaneous Cog^- mutant of strain DL1 (8). This same serum blocked coaggregation of both *S. gordonii* DL1 and PK488 with their streptococcal partners but failed to block their coaggregations with other genera (8). This antiserum detected a 100-kDa surface protein on *S. gordonii* DL1. Spontaneously occurring Cog^- mutants of *S. gordonii* DL1 lack this protein (8). No other protein has been shown to be involved in intrageneric coaggregation with either *S. gordonii* DL1 or any other streptococcal strain. We report here the inactivation of a locus that affects the expression of this 100-kDa protein.

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TABLE 1. List of strains used

Strain	Relevant property or properties	Reference or source
Streptococcus oralis		
34	Reference strain for coaggregation group 3	28
C104	Tc^r; reference strain for coaggregation group 3	28
Streptococcus SM PK509	Reference strain for coaggregation group 5	28
Streptococcus gordonii		
DL1 (Challis)	Reference strain for coaggregation group 1	28
PK488	Reference strain for coaggregation group 6	28
F11	DL1 Cog$^-$ Tn*916* insertion mutant selected with *S. oralis* 34; Tc^r	This study
PK3221	DL1 pCW4 insertion mutant Cog$^-$ with streptococcal partners; Erm^r	This study
PK3222	DL1 pCW4 insertion mutant Cog$^-$ with streptococcal partners; Erm^r	This study
PK3223	DL1 pCW4 insertion mutant Cog$^-$ with streptococcal partners; Erm^r	This study
PK3224	DL1 pCW4 insertion mutant Cog$^-$ with streptococcal partners; Erm^r	This study
PK3225	DL1 pCW4 insertion mutant Cog$^-$ with streptococcal partners; Erm^r	This study
Escherichia coli		
XL1-Blue	*recA* (*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* {F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*})	Stratagene
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+)*supE44*λ- *thi-1 gyrA96 relA1*	Gibco-BRL
PK3320	DH5α containing plasmid pDC1	This study
PK3332	DH5α containing plasmid pCW1	This study
PK3333	DH5α containing plasmid pCW2	This study
PK3334	DH5α containing plasmid pCW3	This study
PK3335	DH5α containing plasmid pCW4	This study

MATERIALS AND METHODS

Strains and culture conditions. All streptococcal strains used for coaggregation in this study, as well as the *Escherichia coli* strains used to clone coaggregation-relevant genes, are listed in Table 1. Additional strains such as *A. naeslundii* T14V, PK947, PK606, and PK984 and *S. gordonii* PK2975 and PK1897 have been identified before (9, 28). All stocks were maintained in cultivating media containing 30% glycerol at −80°C.

Streptococci were cultured in brain heart infusion supplemented with 0.3% yeast extract (Difco Laboratories, Detroit, Mich.). Streptococcal cultures were grown at 37°C under anaerobic conditions with the GasPak system (BBL Microbiology Systems, Cockeysville, Md.). Erythromycin (10 μg/ml) and tetracycline (10 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) were included when appropriate. *S. gordonii* F11 containing Tn*916* was cultured in a medium consisting of tryptone, yeast extract, Tween 80, and glucose buffered to pH 7.5 with K_2HPO_4 (39) in the presence of tetracycline (CAMG/Tc).

E. coli XL1-Blue (Stratagene Cloning Systems, La Jolla, Calif.) and *E. coli* DH5α (Gibco-BRL, Gaithersburg, Md.) were cultured aerobically at 37°C in Luria-Bertani broth or on Luria-Bertani agar (Gibco-BRL). Ampicillin (100 μg/ml; Sigma) for strain XL1-Blue and erythromycin (300 μg/ml; Sigma) or ampicillin (100 μg/ml; Sigma) for strain DH5α were included when appropriate.

All plasmids used in this study are listed in Table 2.

Coaggregation assay. A visual assay was used to measure the ability of transformants to coaggregate by the method described previously (26). Suspensions with a final cell density of about 10^9 cells per ml (260 Klett units, as determined with a 660-nm [red] filter in a Klett-Summerson colorimeter [Klett Manufacturing Co., Inc., New York, N.Y.]) were prepared for each cell type. Equal volumes (0.1 ml) of each cell type were added to a glass test tube (10 by 75 mm) and vortex mixed for 5 s. Coaggregation scoring was done by viewing the tube above an indirect light source (illuminated magnifier no. 39-101; Stocker and Yale, Inc., Beverly, Mass.) while placing the tube on a glass surface attached to the light source and rocking the tube gently to maximize the interaction between two cell types. The change from an evenly turbid suspension before mixing to the formation of visible aggregates consisting of both cell types (coaggregates) either occurred immediately during vortex mixing or developed within a few seconds during the gentle rocking procedure. A coaggregation score ranging from 0 (no change in turbidity and no visible coaggregates) to +4 (maximum coaggregation, with large coaggregates settling immediately, leaving a water-clear supernatant) was given for each pair. A score of +3 indicated the formation of large settling coaggregates but with a slightly turbid supernatant, and a +2 score was given when definite coaggregates were visible but did not settle immediately. The weakest score (+1) represented finely dispersed coaggregates in a turbid background. The reversal of coaggregation was determined by adding *N*-acetyl-D-galactosamine to a final concentration of 15 mM.

Isolation of Tn*916* insertion coaggregation-defective mutant strain F11. The procedure followed for the transformation of *S. gordonii* DL1 was essentially that described by Behnke (4). Approximately 1.3×10^8 CFU of competent cells was mixed with 100 ng of plasmid pAM120 in a volume of 115 μl and incubated at 37°C for 3 h under anaerobic conditions. The transformation mix was plated on

TABLE 2. List of plasmids used

Plasmid	Relevant properties[a]	Reference and/or source
pAM120	Tc^r Hem^{++} Tn*916* inserted into the *Eco*RI D fragment of pAD1	D. B. Clewell; 16
pDC1	pUC19 with a 6.7-kb *Hin*dIII fragment containing the left end of Tn*916* and *S. gordonii* DL1 flanking DNA	This study
pCW1	pBluescript containing a 6.5-kb *Eco*RI fragment; isolated from the λZapII genomic library of *S. gordonii* PK488	This study
pCW2	pBluescript containing a 5.5-kb *Eco*RI fragment; isolated from the λZapII genomic library of *S. gordonii* PK488	This study
pCW3	pBluescript containing a 5.5-kb *Eco*RI fragment; isolated from the λZapII genomic library of *S. gordonii* PK488	This study
pCW4	pΩ containing a 0.5-kb *Eco*RI fragment isolated from pCW1	This study
pΩ	pBluescript with *ermAM* cloned into the Amp^r determinant	37
pΩ12	pΩ containing a 2-kb *Sau*3AI random genomic fragment from *S. gordonii* Challis	R. D. Lunsford
pΩ13	pΩ containing a 1-kb *Sau*3AI random genomic fragment from *S. gordonii* Challis	R. D. Lunsford

[a] Hem^{++}, hyperhemolytic.

CAMG/Tc agar and incubated for 36 h at 37°C under anaerobic conditions. Tetracycline-resistant transformants were screened for their inability to coaggregate with *S. oralis* 34 by the microtiter plate assay described previously (24). Putative Cog^- mutants were repurified from the microtiter wells, and isolated colonies were retested for their coaggregation with *S. oralis* 34 by the microtiter plate assay. Cog^- mutants were stored in CAMG broth at −40°C. One mutant, designated F11, was found to be unable to coaggregate with any of the streptococcal partners, but it retained coaggregation with actinomyces partners.

Antiserum production. The preparation of the polyclonal antiserum raised against cells of *S. gordonii* DL1 has been described previously (9).

DNA preparation. Plasmid DNA was isolated from *E. coli* with the Magic Minipreps DNA Purification System (Promega Corp., Madison, Wis.). The preparation of streptococcal DNA was prepared as described by Jacob et al. (19). Briefly, cells were grown in brain heart infusion plus D,L-threonine and lysed (5, 6). Protein was removed from the lysate by a sequence of phenol-chloroform and chloroform extractions as described previously (45). Nucleic acids were ethanol precipitated and dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA [pH 7.5]). The suspension was then treated with RNase I (Boehringer Mannheim, Indianapolis, Ind.) followed by proteinase K (Sigma) and subjected to a further phenol-chloroform and chloroform extraction. The DNA was purified by ethanol precipitation and dissolved in TE buffer.

Isolation of a 114-bp junction fragment from the F11 insertion mutant. Tn*916* contains a single *Hin*dIII site 6.5 kb from the left end (16). Genomic DNA from F11 was digested to completion with *Hin*dIII and fractionated by centrifugation in a 10 to 30% sucrose linear gradient (3). Fractions were ethanol precipitated, washed with 70% ethanol, and resuspended in TE buffer. A sample from each fraction was electrophoresed through a 0.5% agarose gel, and Southern blot analysis using a Tn*916* probe identified fractions containing hybridizing fragments of 6 to 8 kb. Sucrose gradient fractions containing these hybridizing fragments were pooled and ligated to dephosphorylated pUC19 linearized with *Hin*dIII. Ligation was carried out at 16°C for 16 h. The resulting plasmid, pDC1, was maintained in *E. coli* DH5α in the presence of ampicillin. Plasmid pDC1 was digested with *Hin*dIII, electrophoresed through a 0.5% agarose gel, transferred onto a nylon membrane, and checked by Southern analysis for inserts that hybridized with biotinylated Tn*916* by using the BluGenes nonradioactive nucleic acid detection system (Gibco-BRL).

DNA Sequencing. Dideoxy sequencing (47) was performed with Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) and ^{35}S-dATPαS (Dupont, NEN Research Products, Boston, Mass.). Primers were synthesized with a PCR-MATE model 391 DNA synthesizer (Applied Biosystems). All sequencing gels were 6% Sequagel (National Diagnostics, Atlanta, Ga.). Autoradiographs were obtained with Kodak Scientific Imaging Biomax MR Film. Sequence analyses, translations, manipulations, and comparisons were conducted with the Genetics Computer Group sequence analysis software package (17). The computer program BLAST was used to search for amino acid sequences that were homologous to any of the deduced proteins (1).

Southern blotting. Digested plasmid and chromosomal DNAs were electrophoresed through 0.7% agarose gels, denatured, and transferred by capillary action onto nylon membranes (Gibco-BRL) according to standard procedures (49). Following transfer onto a nylon membrane, the DNA was cross-linked in a model 1800 Stratalinker (Stratagene). Radioactively labeled probes were generated by either end labeling of a 60-base oligonucleotide derived from the 114-bp junction fragment of F11 or random priming of the 922-bp *ermAM* fragment of pΩ (37). Hybridizations were performed at 42°C with Quick-Hyb hybridization solution (Stratagene) according to the manufacturer's instructions. Following hybridization, the nylon membrane was washed as described by Stratagene. The last two washes, 15 min twice in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS), were carried out at 50°C (*ermAM*) and 56°C (60-base oligonucleotide). Following the washes, the nylon sheets were enclosed in clear plastic wrap and exposed to Kodak X-Omat AR film at −70°C prior to development. For probing of the same blot with a second probe, the first probe was stripped from the nylon membrane by the method described previously (45). Before being reprobed, the blot was shown by autoradiography not to have any residual label.

Construction of an *S. gordonii* PK488 Lambda ZAP II library. The *S. gordonii* PK488 library was constructed as described previously (2). Briefly, a partial digest of PK488 DNA was obtained with *Eco*RI. Fragments of 5 to 10 kb were ligated into the Lambda ZAP II vector system, and *E. coli* XL1-blue was used as the host (Stratagene). This library was screened with the 60-base F11-specific probe according to the protocol described by Stratagene. Several plaques that hybridized with the probe were cored from agar plates, and the phage was allowed to diffuse overnight into SM buffer (45) at 4°C. Plaques were purified and subjected to in vivo excision to obtain plasmid (phagemid) derivatives. DNA ligation, packaging, and the in vivo excision of the recombinant plasmid were done according to the Stratagene Lambda ZAP II instruction manual. Plasmids were maintained in *E. coli* DH5α in the presence of ampicillin (100 μg/ml). One of these recombinant plasmids was designated pCW1.

Preparation and analysis of pCW4. One hundred micrograms of pCW1 DNA was digested with *Eco*RI (Boehringer Mannheim). Fragments were separated by electrophoresis on a 0.7% agarose gel, and a 0.5-kb fragment was excised (45). The DNA was purified by using the Geneclean II kit (Bio 101, Inc., La Jolla, Calif.). The *E. coli*-streptococcal insertion vector pΩ (37) was linearized by digestion with *Eco*RI and dephosphorylated with alkaline phosphatase for 30 min at 37°C. This digest was purified with Geneclean II (Bio 101, Inc.) and ligated to the 0.5-kb fragment at 4°C for 15 h. The resultant plasmid, pCW4, was then used to transform *E. coli* DH5α (38). Transformed cells were plated on Luria-Bertani agar containing erythromycin (300 μg/ml). Individual colonies were subcultured, and plasmid preparations were made. Plasmid pCW4 was digested with *Eco*RI (Boehringer Mannheim), and the resulting fragments were separated by electrophoresis on a 0.7% agarose gel. The corresponding Southern blot was probed first with the F11 60-mer to ensure subcloning of the 0.5-kb fragment and then with *ermAM* to show hybridization with the pΩ vector.

***S. gordonii* Challis pΩ insertion library controls.** Plasmids pΩ12 and pΩ13 were isolated from an *S. gordonii* Challis pΩ insertion library that consisted of *Sau*3AI partial digests of *S. gordonii* DNA ligated into the *Bam*HI site of pΩ. The plasmids were maintained in *E. coli* DH5α in the presence of erythromycin (300 μg/ml).

Transformation of *S. gordonii* DL1. Plasmid pCW4 and control plasmids pΩ12 and pΩ13 were used to insertionally mutate *S. gordonii* DL1. An overnight culture of DL1 in Todd-Hewitt broth, pH 7.8 (Difco), supplemented with 5% (vol/vol) heat-inactivated horse serum (THBS medium) was diluted 1:20 into fresh THBS and incubated for 100 min to attain high-level competence. In all transformation assays the final DNA concentration was 5 μg/ml. Twenty-five microliters of the freshly prepared competent cells was added to 450 μl of THBS medium containing 25 μl of the plasmid DNA. The transformation mix was incubated for 2 h at 37°C, and then dilutions were plated on brain heart infusion agar plates supplemented with 0.2% glucose containing erythromycin (10 μg/ml). The plates were incubated for 40 h at 37°C under anaerobic conditions, and erythromycin-resistant transformants were purified. DNA was isolated from *S. gordonii* DL1 and each of the insertional mutants and was digested to completion with *Eco*RI or *Sca*I. Southern blot analysis was carried out with the F11 60-mer and *ermAM* probes described above.

Streptococcal surface preparations using mild sonication. Streptococci were inoculated into 15 ml of broth and incubated for 17 h, washed three times with distilled water by centrifugation (10,000 × *g* for 10 min at 4°C), and suspended in 0.5 ml of distilled water. The cell suspension was sonicated on ice for 1 min at maximum power (50 W) with a microultrasonic cell disrupter (Kontes, Vineland, N.J.). The sonicated suspension was centrifuged at 20,000 × *g* for 15 min at 4°C, and the supernatant fluid was stored at −80°C until used. Total protein was determined by the Bio-Rad (Richmond, Calif.) protein assay with bovine serum albumin as a standard.

Immunoblot analysis. The proteins from streptococcal surface-sonicate extracts were separated on SDS–6% polyacrylamide gel electrophoresis gels (Novex, San Diego, Calif.) (31). Five micrograms of total protein was loaded per lane and electrophoresed under reducing conditions. The separated proteins were either stained by use of the Pro-Blue system (Integrated Separation Systems, Natick, Mass.) or transferred to nitrocellulose filters by use of a Bio-Rad Mini-Protean II transfer chamber (45). Filters were treated with a 1:1,000 dilution of rabbit anti-DL1 serum preabsorbed with a spontaneously occurring Cog^- mutant, PK1897 (8). Immune complexes were visualized with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G and a dye indicator system supplied by Promega Biotech, Madison, Wis.

Nucleotide sequence accession number. The DNA sequence described in this report has been assigned GenBank accession number U57759.

RESULTS

Sequencing of the *S. gordonii* DL1 junction fragment. Southern blot analysis of *Hin*dIII-digested genomic DNA from the Cog^- Tn*916* insertion mutant F11 revealed two fragments of 6.7 and 14 kb when the DNA was probed with labeled Tn*916*. Plasmid pDC1 contained the 6.7-kb *Hin*dIII fragment from the Cog^- *S. gordonii* DL1 Tn*916* insertion mutant F11, cloned into the *Hin*dIII site of plasmid pUC19. The sequence of the streptococcal DNA flanking the left end of the cloned *Hin*dIII fragment of the Tn*916* insertion in strain F11 was determined by using pDC1. It was 114 bp, and the 60-mer F11-specific oligonucleotide probe was selected from this sequence. The sequence is underlined in Fig. 2.

Isolation and analysis of phagemids isolated from an *S. gordonii* PK488 Lambda ZAP II library. Screening of an *S. gordonii* DL1 genomic library using the F11 60-mer probe gave no positive recombinants. Therefore, because PK488 has coaggregation properties nearly identical to those of DL1 (28), an *S. gordonii* PK488 Lambda ZAP II library (2) was screened. Ten plaques hybridized with the F11 probe and were purified and rescued as phagemids.

*Eco*RI digests of the phagemids revealed two banding patterns, each with a common 0.5-kb fragment combined with

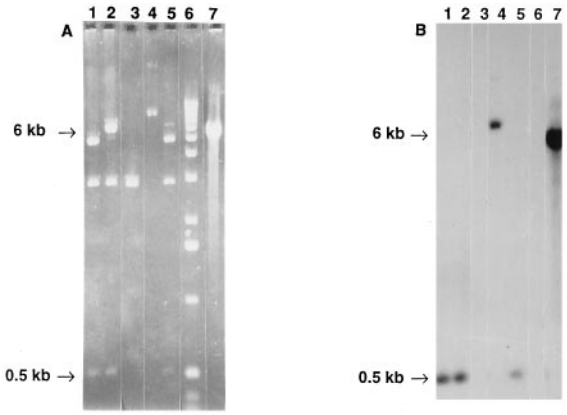

FIG. 1. Southern blot analysis of plasmids isolated from a Lambda ZAP II library of *S. gordonii* PK488. (A) Plasmids pCW3 (lane 1), pCW1 (lane 2), pBluescript (lane 3), and pCW2 (lane 5) were cut with *Eco*RI, electrophoresed through a 0.7% agarose gel, and visualized with ethidium bromide. Also shown are uncut pCW2 (lane 4), a standard 1-kb ladder (lane 6), and the 6.7-kb cloned *Hin*dIII fragment containing the left end of Tn*916* and flanking *S. gordonii* DL1 DNA (lane 7). (B) Corresponding Southern blot of the agarose gel shown in panel A probed with the F11-specific 60-mer. The positions of 6 and 0.5 kb are indicated by the arrows and were determined with the 1-kb ladder.

either a 5-kb fragment (Fig. 1A, lanes 1 and 5 [pCW2 and pCW3, respectively]) or the 6-kb fragment of pCW1 (Fig. 1A, lane 2). Southern analysis of the *Eco*RI digests revealed only the 0.5-kb fragment hybridizing with the F11 probe (Fig. 1B, lanes 1, 2, and 5). A single hybridizing band of greater than 6 kb reacted with the probe with uncut phagemid DNA (Fig. 1B, lane 4).

The 0.5-kb fragment from pCW1 was ligated into pΩ, and the resulting plasmid was designated pCW4. An *Eco*RI digestion of pCW4 confirmed the subcloning of the 0.5-kb fragment that hybridized with the F11 probe. Probing the same Southern blot with *ermAM* revealed hybridization with the uncut pCW4 and with the 2.96-kb pΩ vector (data not shown).

Insertional-duplication mutagenesis of *S. gordonii* DL1 using pCW4. pCW4 was used to transform *S. gordonii* DL1 in order to insertionally inactivate the coaggregation-relevant gene. The cloned chromosomal DNA recognized homologous DNA sequences in the recipient chromosome and targeted insertion of the plasmid (42). Ten transformants were selected and purified. Each transformant was tested for its ability to coaggregate with *S. oralis* 34, *S. oralis* C104, and *Streptococcus* SM PK509 (Table 3). The transformants' abilities to coaggregate with *A. naeslundii* PK984, PK606, PK947, and T14V were also tested (Table 3). Although the results with 5 of the 10 transformants are shown in Table 3, all pCW4 insertion mutants had lost their ability to coaggregate with the three streptococcal partners of the wild type. However, coaggregation with actinomyces remained unaltered. Control transformants of *S. gordonii* Challis with pΩ12 and pΩ13 showed no differences from the wild type in their abilities to coaggregate with any of the wild-type partner strains (data not shown). Identical coaggregation phenotypes were observed for all transformants grown either with or without erythromycin. All the pCW4 insertion mutants were altered specifically in intrageneric coaggregation, but all retained wild-type intergeneric coaggregation reactions.

Sequencing of pCW1. The entire nucleotide sequence of the 0.5-kb streptococcal insert and an adjacent portion of the 6.0-kb fragment of pCW1 was determined by using primers obtained from the sequence of the 114-bp junction fragment described earlier (Fig. 2). A potential open reading frame (ORF) coding for a coaggregation-relevant gene started at nucleotide 277 and stopped at nucleotide 1210 in the 6.0-kb fragment of pCW1 (Fig. 2, ORF 1). A protein of 33.6 kDa is potentially encoded by ORF 1; the 311 amino acids showed 56% identity and 72% similarity to an unknown protein of 34 kDa from *Bacillus subtilis* (41, 44). The first 23 amino acids showed 57% identity and 71% similarity to a hypothetical protein from *Mycoplasma genitalium* (13).

Immunoblot analysis of surface proteins obtained from the insertion mutants. Sonic extracts of cell surface proteins of *S. gordonii* DL1, the Tn*916* insertion mutant strain F11, and the pCW4 insertion mutants were probed with the PK1897 mutant-absorbed anti-DL1 serum. A protein of approximately 100 kDa found in the wild-type DL1 (Fig. 3A, lane 3; Fig. 3B, lane 7) was missing from both strain F11 (Fig. 3A, lane 2) and the pCW4 insertional mutants (Fig. 3B, lanes 2 to 6). The spontaneous mutant previously shown to lack the 100-kDa protein (8) is also shown in Fig. 3 for comparison (lanes 1). The absence of the 100-kDa protein in the mutants suggests that it is involved in intrageneric coaggregation. The 100-kDa protein was present in surface proteins of the *S. gordonii* Cog^+ control insertion mutant transformed with pΩ13 (data not shown).

DISCUSSION

In earlier studies, spontaneously occurring Cog^- mutants of *S. gordonii* DL1 were selected for their inability to coaggregate with their streptococcal partners and were shown to lack a 100-kDa surface protein (8). The present work focused on the

TABLE 3. Coaggregation scores between pCW4 insertion mutants of *S. gordonii* DL1 and streptococcal and actinomyces partners[a]

Partner strain	Coaggregation score with DL1 pCW4 insertion mutant:					Coaggregation score with wild-type DL1
	PK3221	PK3222	PK3223	PK3224	PK3225	
Streptococcus oralis						
C104	−	−	−	−	−	+++[b]
34	−	−	−	−	−	+++[b]
Streptococcus SM PK509	−	−	−	−	−	+++[b]
Actinomyces naeslundii						
PK947	++++	++++	++++	++++	++++	++++
PK606	++++	++++	++++	++++	++++	++++
PK984	++++	++++	++++	++++	++++	++++
T14V	+++	++++	++++	+++	+++	++++

[a] Coaggregation scores are as follows: −, no coaggregation; +++, large clumps formed immediately with some turbidity remaining in the supernatant; and ++++, large clumps formed immediately with a clear supernatant.
[b] Coaggregation was completely inhibited by 15 mM *N*-acetyl-D-galactosamine.

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   1  GAATTCAAATGGCGGGAGCTGGGCATCCCTTACAAGCTGGAAGGGGTTAAGCCTCCGACA

  61  GCTGATCGGGTCAAGAATGCCAAGGAGCTCATGCAGACTGAATCTTATACAGAGTATATG

 121  AATCGGGTGCATAATAGCTGATCTGTTTTGAGTATCAATAAAAGGACTGGTGAGTTGACC

 181  AGTCCTTTTTTCAAAAAATATTCTTCTGTTTTTGTGAAATGCTTGTAATTGTGGTAAAAT

                          >start orf 1>
 241  AAATGCAATAAAGTTTATATAAAAAAGAGGTTTATCATGTCTAAAATTTTAGTTTTCGGT
                ↑                    M  S  K  I  L  V  F  G

 301  CACCAAAATCCTGACTCAGATGCTATCGGTTCATCTTACGCATTTGCTTACTTGGCTCGC
      H  Q  N  P  D  S  D  A  I  G  S  S  Y  A  F  A  Y  L  A  R

 361  GAAGCTTATGGCTTGGATACAGAAGCTGTTGCATTGGGAGAGCCTAATGAAGAAACTGCC
      E  A  Y  G  L  D  T  E  A  V  A  L  G  E  P  N  E  E  T  A

 421  TTTGTATTGGATTACTTTGGTGTGGCTGCACCGCGCGTGATTACATCTGCTAAGGCAGAG
      F  V  L  D  Y  F  G  V  A  A  P  R  V  I  T  S  A  K  A  E

 481  GGGGCTGAGCAGGTTATTCTGACAGACCACAATGAATTCCAGCAGTCTGTGGCAGATATC
      G  A  E  Q  V  I  L  T  D  H  N  E  F  Q  Q  S  V  A  D  I

 541  GCTGAAGTTGAAGTTTATGGCGTGGTGGACCACCACCGTGTCGCTAACTTCGAAACGGCT
      A  E  V  E  V  Y  G  V  V  D  H  H  R  V  A  N  F  E  T  A

 601  AACCCCCTATATATGCGCTTGGAGCCAGTTGGCTCTGCTTCATCTATTGTTTACCGTATG
      N  P  L  Y  M  R  L  E  P  V  G  S  A  S  S  I  V  Y  R  M

 661  TTCAAGGAGCATAGCGTAGCAGTTTCAAAAGAAATTGCAGGATTGATGCTGTCTGGTTTG
      F  K  E  H  S  V  A  V  S  K  E  I  A  G  L  M  L  S  G  L

 721  ATTTCTGATACTCTCTTGCTCAAGTCACCAACAACTCATCCGACTGACAAGGCCATTGCA
      I  S  D  T  L  L  L  K  S  P  T  T  H  P  T  D  K  A  I  A

 781  CCAGAATTGGCTGAGCTGGCTGGTGTCAACTTGGAAGAGTACGGTTTGGCTATGCTTAAG
      P  E  L  A  E  L  A  G  V  N  L  E  E  Y  G  L  A  M  L  K

 841  GCAGGTACAAACTTGGCTAGCAAGTCTGCAGAAGAATTGATCGACATCGATGCGAAAACA
      A  G  T  N  L  A  S  K  S  A  E  E  L  I  D  I  D  A  K  T

 901  TTTGAGTTGAACGGAAACAATGTCCGTGTAGCACAGGTCAATACTGTTGATATTGCAGAA
      F  E  L  N  G  N  N  V  R  V  A  Q  V  N  T  V  D  I  A  E

 961  GTTTTGGAGCGTCAAGCAGAAATTGAAGCAGCTATTGAAAAAGCAATTGCTGACAATGGC
      V  L  E  R  Q  A  E  I  E  A  A  I  E  K  A  I  A  D  N  G

1021  TACTCTGACTTCGTCTTGATGATTACAGACATCATCAATTCAAACTCAGAAATTTTGGCT
      Y  S  D  F  V  L  M  I  T  D  I  I  N  S  N  S  E  I  L  A

1081  ATCGGAAGCAACATGGATAAGGTTGAAGCAGCCTTCAACTTTGTTCTTGAAAACAACCAT
      I  G  S  N  M  D  K  V  E  A  A  F  N  F  V  L  E  N  N  H

1141  GCTTTCCTTGCAGGCGCTGTTTCTCGTAAAAAGCAAGTGGTGCCTCAACTGACAGAAAGT
      A  F  L  A  G  A  V  S  R  K  K  Q  V  V  P  Q  L  T  E  S

1201  TTTAATGCATAG
      F  N  A  *
```

FIG. 2. Nucleotide sequence and deduced amino acid sequence of ORF 1 of pCW1. The putative start site and the likely direction of transcription (>) of ORF 1 are indicated. The start site is deduced solely on the basis of the DNA sequence. The deduced amino acid sequence is shown with the one-letter code. The 60-base F11-specific probe is underlined. The point of insertion of Tn*916* (↑) is indicated. The *Eco*RI site at the junction of the 0.5- and 6.0-kb fragments is underlined. The stop site is shown by the asterisk.

isolation of two groups of insertional Cog$^-$ mutants of *S. gordonii* DL1 that exhibited a phenotype like that of the spontaneous Cog$^-$ mutants. As with the spontaneously occurring Cog$^-$ mutants, surface sonic extracts of both types of insertional mutants lacked the 100-kDa protein. This gave further support for the idea that the 100-kDa protein is a putative adhesin mediating intrageneric coaggregation. Such adhesins may be critical to successful colonization of oral surfaces.

A high-molecular-mass band near the top of the immunoblot of surface sonic extracts (Fig. 3) exhibited different staining intensities with different strains. Although a given preparation always exhibited the same staining density, the immunoblots shown in Fig. 3 give examples of different preparations of wild-type DL1 (Fig. 3A, lane 3; Fig. 3B, lane 7) showing the variation in staining intensity of this high-molecular-mass band. The reason for the variation is unknown.

A small number of potentially adhesive proteins on the surfaces of streptococci have been identified and characterized, including proteins thought to be responsible for adherence to either saliva-coated hydroxyapatite (SHA), a model surface for tooth enamel, or other bacteria. For example, our laboratory (2, 26, 27) identified a 34.8-kDa lipoprotein (ScaA) involved in the interaction of *S. gordonii* PK488 with *A. naeslundii* PK606. Homologous proteins have been reported for several oral

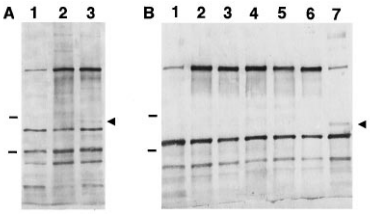

FIG. 3. Immunoblot analysis of sonic surface extracts of spontaneous Cog$^-$ mutant strain PK1897, Tn*916* insertion Cog$^-$ mutant strain F11, and pCW4 insertion Cog$^-$ mutants with wild-type *S. gordonii* DL1. The immunoblots were scanned directly with an Agfa Arcus Plus scanner. The images were labeled with Photoshop, version 3.0, for Macintosh (Adobe Systems, Inc., Mountain View, Calif.). (A) Spontaneous Cog$^-$ mutant strain PK1897 (lane 1), Tn*916* insertion Cog$^-$ mutant strain F11 (lane 2), wild-type DL1 (lane 3) are shown. (B) The PK1897 spontaneous Cog$^-$ mutant (lane 1); pCW4 insertion Cog$^-$ mutants PK3221, PK3222, PK3223, PK3224, and PK3225 (lanes 2 to 6, respectively); and wild-type strain DL1 (lane 7) are shown. The positions of 100 kDa (arrows) were determined by using prestained molecular weight standards (Bio-Rad). The standards of 112 and 84 kDa are indicated by dashes.

streptococci (10, 12, 14, 15), for *Streptococcus pneumoniae* (46), and for an endocarditis isolate of *Enterococcus faecalis* (36) and are thought to be involved in other adherence phenomena, including binding to SHA. *S. gordonii* DL1 binds to SHA (7) and has been shown to have surface proteins which on immunoblots react with anti-ScaA antiserum (26). The relationship of the ScaA homolog of strain DL1 and the ability to bind cells to SHA has not been reported, although this antigen could be important in streptococcal colonization.

Much of the past work with these oral streptococci has focused on intergeneric coaggregation. While these streptococci participate in intergeneric coaggregation (20, 25), the work presented here was carried out in order to more clearly define the role of streptococcal surface proteins in intrageneric coaggregation. The DNA cloned and identified in this work appeared to be distinct from the genes that have been isolated previously. In this work two different groups of Cog$^-$ mutants of *S. gordonii* DL1 that were unable to mediate intrageneric coaggregation were isolated. Tn*916* transposon mutagenesis of *S. gordonii* DL1 yielded strain F11, which was unable to coaggregate with the wild-type streptococcal coaggregation partners but was still able to coaggregate with actinomyces and other coaggregation partners. Likewise, streptococcal transformants containing pCW4 inserted into the chromosome had specifically lost their ability to participate in intrageneric coaggregation but were still able to coaggregate with other wild-type partners.

Both kinds of insertional mutants lacked a 100-kDa surface protein from mild sonic extracts of whole cells, whereas the wild-type *S. gordonii* DL1 and also the control insertion mutant transformed with pΩ13 expressed this protein. The Cog$^-$ mutant-absorbed antiserum recognizing this protein specifically blocked the galactoside-inhibitable intrageneric coaggregation of *S. gordonii* DL1 but not coaggregation reactions between DL1 and other partners (8). We conclude from these data that we have inactivated a locus necessary for the synthesis of a 100-kDa protein that is involved specifically in intrageneric coaggregation. Partial sequencing of pCW1 identified a potential ORF (ORF 1). The potentially encoded protein of approximately 34 kDa showed 56% identity with a 34-kDa hypothetical protein from *B. subtilis*, suggesting that ORF 1 is not the structural gene for a 100-kDa protein cloned in pCW1. The locus may encode a regulatory protein involved in expression of the 100-kDa adhesin on the cell surface, or it may be a locus involved in expressing transport or secretory proteins necessary for proper positioning of a surface adhesin.

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