INFECTION AND IMMUNITY, Dec. 1995, p. 4890–4893 Vol. 63, No. 12
0019-9567/95/$04.00+0
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Identification of a 100-Kilodalton Putative Coaggregation-Mediating Adhesin of *Streptococcus gordonii* DL1 (Challis)

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Received 20 June 1995/Returned for modification 9 August 1995/Accepted 4 October 1995

***Streptococcus gordonii* DL1 (Challis) bears coaggregation-relevant surface proteins which mediate lactose-inhibitable coaggregations with other streptococci. Six spontaneously occurring coaggregation-defective (Cog$^-$) mutants of wild-type strain *S. gordonii* DL1 unable to coaggregate with wild-type streptococcal partners were characterized. Antiserum raised against wild-type cells and absorbed with Cog$^-$ cells specifically blocked lactose-inhibitable coaggregations between *S. gordonii* DL1 and its streptococcal partner strains; it did not block lactose-noninhibitable coaggregations with actinomyces partners. Surface proteins were released from the cells by mild sonication treatment and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A 100-kDa surface protein from *S. gordonii* DL1 was identified by immunoblot analysis with the mutant-absorbed antiserum. Each of the six Cog$^-$ mutants lacked the 100-kDa protein. Several other oral viridans streptococci that exhibit intrageneric lactose-inhibitable coaggregations expressed an immunoreactive protein with about the same size as the 100-kDa putative adhesin. It is proposed that the 100-kDa protein is the adhesin which mediates coaggregation between *S. gordonii* DL1 and its streptococcal partners. The role of this putative adhesin in accretion of streptococci in early colonization of the tooth surface is discussed.**

A freshly cleaned tooth surface is repopulated with higher numbers of viridans streptococci than any other human oral bacteria (17). Subsequent accretion onto the initial streptococcal layer involves additional streptococci as well as actinomyces, haemophili, and veillonellae (17, 19). The ability of streptococci to participate in cell-to-cell interactions with partners within their genus is unusual among oral bacteria; most coaggregations involve partners from different genera (8).

The molecules that mediate cell-to-cell recognition between partner surfaces are usually a complementary set consisting of an adhesin on one cell type and its receptor on the other cell type (11). Many coaggregations are inhibited by lactose, indicating a likely lectin-carbohydrate complementary set of molecules on the respective partners (9). The lectin activity of PlaA (13) from *Prevotella loescheii* PK1295 is found in a 75-kDa adhesive protein (12) that is expressed on the cell surface at a maximum of 400 molecules per cell. It mediates hemagglutination of neuraminidase-treated human erythrocytes (21) and lactose-inhibitable coaggregations with oral streptococci (9).

Streptococcus gordonii DL1 (Challis) was chosen as the model to study intrageneric coaggregation. It has the heat- and protease-sensitive adhesin on the surface which recognizes galactoside-containing receptors on the surface of *Streptococcus oralis* 34, *S. oralis* C104, and *Streptococcus* SM PK509 (2, 8). In addition, *S. gordonii* DL1 is naturally competent for DNA transformation (10, 18). Multiple adhesins mediating lactose-noninhibitable intergeneric coaggregation between *S. gordonii* Challis with actinomyces have been identified (5, 15, 16). These adhesins can be separated into two families. The CshA/B family is composed of large proteins with sizes of about 260 to 290 kDa, and the SspA polypeptide, 210 kDa, is similar to the antigen I/II family of mutans streptococci (4).

To identify adhesin-mediating lactose-inhibitable intrageneric coaggregation, we selected spontaneously occurring mutants of *S. gordonii* DL1 (Challis) which did not coaggregate with the streptococcal partners of DL1 (1). In this study, we show that these mutants are lacking a 100-kDa surface protein and propose that it is a mediator of lactose-inhibitable coaggregations between *S. gordonii* DL1 and its streptococcal partners.

MATERIALS AND METHODS

Cultivation of bacteria. All bacterial strains used in this study are listed in Table 1. Streptococci and actinomyces were cultured in CAMG medium (14) at 37°C under anaerobic conditions with the GasPak system (BBL Microbiology Systems, Cockeysville, Md.). *S. gordonii* PK2975 was resistant to the antibiotics rifamycin (25 μg/ml), spectinomycin (500 μg/ml), and streptomycin (100 μg/ml) (all from Sigma Chemical Co., St. Louis, Mo.) and was selected by plating the wild-type strain DL1 onto each antibiotic, sequentially, as described previously (1).

Bacterial cells used for coaggregation assays were pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C, washed three times in coaggregation buffer (1 mM Tris [pH 8.0], 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 150 mM NaCl, 0.02% NaN_3), and stored in coaggregation buffer at 4°C. The visual assay for coaggregation has been described in detail elsewhere (7, 9).

Isolation of spontaneously occurring coaggregation-defective (Cog$^-$) *S. gordonii* DL1 mutants was described by Clemans and Kolenbrander (1). To verify genetic lineage with the wild-type strain DL1, the antibiotic-resistant strain PK2975 was used. After 6 cycles of mixing strain PK2975 with its antibiotic-sensitive partner *S. oralis* C104, pelleting the resultant coaggregates, and adding more partner cells to the supernatant fluid, the final supernatant fluid was serially diluted in CAMG broth and plated onto CAMG agar containing 25 μg of rifamycin per ml of medium. Colonies were screened for the inability to coaggregate by the method of Kolenbrander (6). Genetic lineage was confirmed by plating cells onto agar containing all three antibiotics, rifamycin, spectinomycin, and streptomycin.

Generation of anti-DL1 polyclonal serum. Whole cells of *S. gordonii* DL1 were washed four times in phosphate-buffered saline (PBS; 8.9 mM Na_2HPO_4, 0.83 mM NaH_2PO_4, 1.55 mM KH_2PO_4 [pH 7.2], 0.15 M NaCl) and resuspended in PBS to a final cell density of approximately 10^9 cells per ml (260 Klett units, determined with a 660-nm [red] filter in a Klett-Summerson colorimeter; Klett Manufacturing Co., Inc., New York, N.Y.). A volume of 0.5 ml of whole-cell suspension was injected into the marginal ear vein of a female New Zealand White rabbit twice weekly for 4 weeks; the rabbit was bled from the central ear artery 7 days after the final injection. Subsequent bleedings were done 7 days after a booster injection of the whole-cell suspension. Serum was stored at −40°C. The titer of each antiserum was determined by testing for the agglutination of intact, wild-type *S. gordonii* DL1 cells by serial dilution of the antisera

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TABLE 1. Strains used in this study

Strain	Relevant characteristic(s)	Reference or source
S. gordonii		
DL1 (Challis)	Reference strain for streptococcal coaggregation group 1	7
PK488	Reference strain for streptococcal coaggregation group 6	7
ATCC 10558	Type strain for *S. gordonii*	7
S. oralis		
34	Reference strain for streptococcal coaggregation group 3	7
H1[a]	Reference strain for streptococcal coaggregation group 2	7
J22[a]	Reference strain for streptococcal coaggregation group 4	7
ATCC 10557	Type strain for *S. oralis*	7
C104[a]	Reference strain for streptococcal coaggregation group 3	7
S. sanguis		
12	Coaggregation pattern similar to *S. gordonii* PK488	B. C. McBride
ATCC 10556	Type strain for *S. sanguis*	7
S. sobrinus 6715-10	Noncoaggregating control strain	14
Streptococcus SM PK509	Reference strain for streptococcal coaggregation group 5	7
Enterococcus faecalis GF590[a]	Noncoaggregating control strain	D. B. Clewell
S. gordonii		
PK2975[b]	DL1 Rf^r Sm^r Sp^r	This study
PK1897[b]	PK2975 (Cog^-, selected with *S. oralis* C104)	This study
PK3003[b]	PK2975 (Cog^-, selected with *S. oralis* C104)	This study
PK3017[b]	PK2975 (Cog^-, selected with *S. oralis* C104)	This study
PK3020[b]	PK2975 (Cog^-, selected with *S. oralis* C104)	This study
PK3037[b]	PK2975 (Cog^-, selected with *S. oralis* C104)	This study
PK3050[b]	PK2975 (Cog^-, selected with *S. oralis* C104)	This study
A. naeslundii PK606	Reference strain for actinomyces coaggregation group D	7

[a] Resistant to tetracycline (10 μg/ml).
[b] Resistant to rifamycin (25 μg/ml), streptomycin (100 μg/ml), and spectinomycin (500 μg/ml).

in modified coaggregation buffer containing 100 mM Tris (pH 8.0) and 0.2% bovine serum albumin (Sigma). A 25-μl aliquot of serum was mixed in a 96-well, round-bottom microtiter plate (MicroTest III Flexible Assay Plate, Falcon 3911; Becton Dickinson Labware, Oxnard, Calif.) with 25 μl of a suspension of *S. gordonii* DL1 cells adjusted to approximately 8×10^8 cells per ml of coaggregation buffer (160 Klett units, determined with a 660-nm [red] filter in a Klett-Summerson colorimeter). The plate was mixed for 1 min on a Titertek microplate shaker (Flow Laboratories, Inc., McLean, Va.) and incubated overnight at room temperature.

Cog^- mutant absorption of anti-DL1 serum. Cells of Cog^- mutant PK1897 were suspended to a concentration of 0.5 g (wet weight) per ml of coaggregation buffer. A volume of 1 ml was pelleted by centrifugation ($10,000 \times g$ for 10 min at 4°C), and the pellet was mixed with 5 ml of rabbit whole anti-DL1 serum with constant rotation for 12 h at 4°C. The procedure was repeated several times by pelleting the spent cells and transferring the serum to another 0.5-g cell pellet to yield PK1897-absorbed antiserum. For 3 ml of antiserum, this usually required about 10 to 15 absorptions with 0.5 g of Cog^- mutant cells in each absorption.

Blocking of coaggregation with Cog^- mutant-absorbed anti-DL1 serum. Bacterial cell suspensions were adjusted to approximately 8×10^8 cells per ml of coaggregation buffer. An aliquot of 50 μl of streptococcal cells was mixed in a siliconized glass tube with 10 μl of diluted antiserum and incubated for 30 min at room temperature. The suspension was examined for agglutination before the addition of 50 μl of the partner cells, after which the mixture was scored for coaggregation. The absorbed serum was diluted in modified coaggregation buffer. Controls included the addition of either modified coaggregation buffer or undiluted preimmune serum to the streptococcal cells.

Streptococcal surface preparations. Cells of *S. gordonii* DL1 (wild type), parental strain PK2975, and the spontaneous Cog^- mutants PK1897, PK3003, PK3017, PK3020, PK3037, and PK3050 were washed three times with distilled water by centrifugation ($10,000 \times g$ for 10 min at 4°C) and resuspended to a concentration of 0.5 g (wet weight) of cells per ml of distilled water up to a maximum of 1 ml. The cell suspension was transferred to a 50-ml-capacity conical tube and sonicated on ice for 1 min at maximum power (50 W) with a micro ultrasonic cell disrupter (Kontes, Vineland, N.J.). The sonicated suspension was centrifuged at $20,000 \times g$ for 15 min at 4°C, and the supernatant fluid was stored at −70°C until used. Total protein was determined with the Bio-Rad (Richmond, Calif.) protein assay.

Immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with precast 4 to 12% or 6% Tris-glycine gels (Novex, San Diego, Calif.). Proteins separated on gels were either Western blotted (immunoblotted) or stained by using the Pro-Blue system (Integrated Separation Systems, Natick, Mass.). Western blot transfers to nitrocellulose paper (Schleicher & Schuell, Keene, N.H.) were performed in the Mini-PROTEAN II transfer chamber (Bio-Rad) according to the procedure of Towbin et al. (20). Following the transfer, the filters were treated with a 1,000-fold dilution of PK1897 mutant-absorbed anti-DL1 rabbit serum. Immune complexes were visualized with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Bio-Rad) and a dye indicator system supplied by Promega Corporation (Madison, Wis.). Prestained markers (Bio-Rad) were used to calibrate molecular weights in gels and immunoblots.

RESULTS

Coaggregation-blocking properties of Cog^- mutant-absorbed antiserum. The agglutination titer of anti-*S. gordonii* DL1 serum for parent strain PK2975 and the Cog^- mutant PK1897 were 1:51,200 and 1:25,600, respectively. Preimmune serum did not agglutinate either strain. *S. gordonii* PK1897 mutant-absorbed serum exhibited an agglutination titer of 1:64 with strain PK2975 and did not agglutinate strain PK1897.

S. gordonii PK1897-absorbed antiserum at a 1:55 dilution completely blocked the lactose-inhibitable coaggregations of parent strain PK2975 with *S. oralis* 34, *S. oralis* C104, and *Streptococcus* SM PK509 (Table 2). No agglutination of the PK2975 cells in the coaggregation-blocking assay was observed at an antiserum dilution of 1:55. This was due to the shorter incubation time of the PK2975 cells with absorbed antiserum in the coaggregation-blocking assay versus the agglutination assay (30 min versus overnight, respectively). Complete blocking ability remained for the lactose-inhibitable coaggregations with two of the streptococcal strains at a 1:110 dilution of antiserum. Partial coaggregation blocking was seen at this dilution with *S. oralis* C104. Even at a 1:550 dilution, partial blocking was seen with the streptococcal partners. The coaggregation between *S. gordonii* PK2975 and *Actinomyces naeslundii* PK606, which is not lactose-inhibitable (7), was not affected by the absorbed antiserum. Preimmune serum had no effect on any of the coaggregations.

Immunoblot analysis of surface extracts of parent strain and six spontaneous Cog^- mutants with Cog^- mutant-absorbed antiserum. Polyclonal rabbit antiserum raised against *S. gordonii* DL1 was absorbed with the cells from the sponta-

TABLE 2. Antibody inhibition of coaggregation between *S. gordonii* PK2975 and partner streptococci and actinomyces

Preincubation supplement to *S. gordonii* PK2975	Coaggregation score[a] with:			
	S. oralis 34	*S. oralis* C104	*Streptococcus* sp. strain SM PK509	*A. naeslundii* PK606
Absorbed serum (dilution ratio)[b]				
1:55	0	0	0	4
1:110	0	1	0	4
1:220	ND	ND	ND	4
1:550	1	1	1	ND
Buffer alone	3	3	3	4
Preimmune serum[c]	3	3	3	4

[a] The method for assigning coaggregation scores has been described by Kolenbrander and Andersen (7). The maximum score is 4; absence of coaggregation was given a score of zero. *S. gordonii* PK2975 cells were preincubated for 30 min at room temperature with PK1897-absorbed anti-DL1 serum. Strain PK1897 is a spontaneously occurring Cog$^-$ mutant of *S. gordonii* PK2975. ND, not done.

[b] PK1897-absorbed anti-DL1 serum at protein concentrations of 0.73 mg/ml (1:55 dilution), 0.36 mg/ml (1:110 dilution), 0.18 mg/ml (1:220 dilution), and 0.07 mg/ml (1:550 dilution) was preincubated with 4×10^7 *S. gordonii* PK2975 cells prior to the addition of the partner bacteria.

[c] Undiluted preimmune serum was preincubated with *S. gordonii* PK2975 (4×10^7 cells) prior to the addition of the partner bacteria. The final antiserum protein concentration was 5.6 mg/ml per reaction mixture.

neous Cog$^-$ mutant PK1897. These absorbed sera were used to probe immunoblots of surface extracts isolated from *S. gordonii* PK2975 (parent strain), PK1897, and five other spontaneous mutants. A Pro-Blue-stained SDS-PAGE gel of surface extracts showed no difference between the parental PK2975 strain and the Cog$^-$ mutants. An immunoblot of an identical gel developed with anti-DL1 polyclonal serum absorbed with the spontaneous mutant PK1897 is shown in Fig. 1. Lane 1 contained the parental *S. gordonii* PK2975 strain, which had a 100-kDa protein that was absent in PK1897 (lane 2) and five other spontaneously occurring Cog$^-$ mutants PK3003, PK3037, PK3017, PK3020, and PK3050 (lanes 3 to 7, respectively). The high-molecular-size protein near the top of the figure varied in intensity in different lanes. Here the protein appeared to be more intense in the lane containing the parent (Fig. 1, lane 1) than in the lanes containing Cog$^-$ mutant samples (Fig. 1, lanes 2 to 7). In other preparations of the parent strain and Cog$^-$ mutants (data not shown), lanes containing Cog$^-$ mutant surface preparations were more heavily stained at this high-molecular-size position than was the parent, suggesting that the variability in the staining intensity of this protein was most likely caused by procedures of sample preparation or due to cell physiology and not to its presence or absence on the cell surface. In contrast, no preparation from any of the Cog$^-$ mutants exhibited a 100-kDa protein.

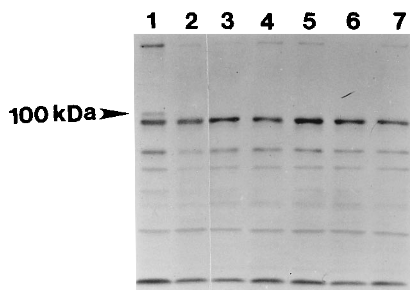

FIG. 1. Immunoblot analysis of parent and Cog$^-$ mutants of *S. gordonii* DL1. Surface extracts of *S. gordonii* PK2975 parent and Cog$^-$ mutant strains were prepared after mild sonication. Five micrograms of total protein was loaded per lane and electrophoresed under reducing conditions through an SDS–4 to 12% PAGE gel. Proteins were transferred from the gel to nitrocellulose paper and subjected to immunoblot analysis using polyclonal rabbit antisera raised against the wild-type strain of *S. gordonii* DL1 and absorbed with the spontaneous Cog$^-$ mutant PK1897. Lanes: 1, strain PK2975, 2, PK1897; 3, PK3003; 4, PK3037; 5, PK3017; 6, PK3020; 7, PK3050. The position of the 100-kDa protein (arrowhead) was determined by using prestained molecular weight markers (data not shown).

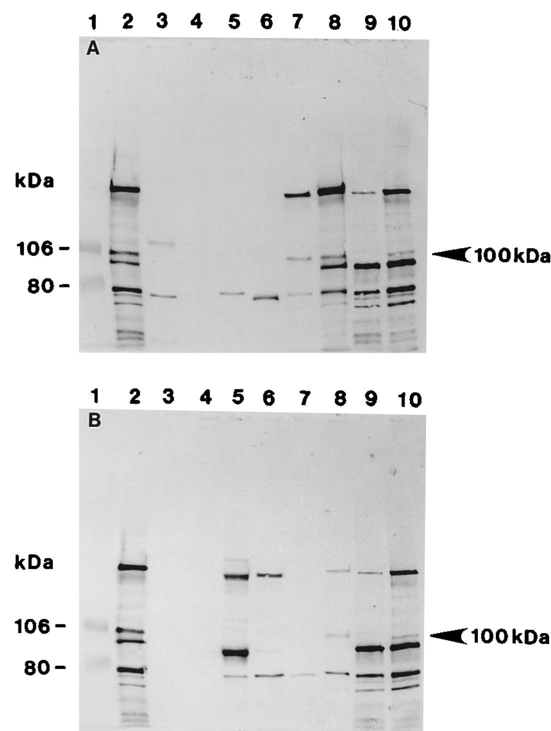

FIG. 2. Immunoblot analysis of proteins released by mild sonication of representative streptococci. Surface extracts of several representative streptococci were prepared after mild sonication and electrophoresed under reducing conditions through an SDS–6% PAGE gel. Proteins were transferred from the gel to nitrocellulose paper and subjected to immunoblot analysis using polyclonal rabbit antisera raised against the parent strain of *S. gordonii* DL1 and absorbed with the spontaneous Cog$^-$ mutant PK1897. (A) Lanes: 2 to 10, *S. gordonii* DL1 (5 μg of protein), *S. oralis* H1 (3.3 μg of protein), *S. oralis* 34 (5.4 μg of protein), *S. oralis* C104 (3.3 μg of protein), *S. oralis* J22 (3.3 μg of protein), *Streptococcus* SM PK509 (5 μg of protein), *S. gordonii* PK488 (4 μg of protein), *S. gordonii* PK1897 (5 μg of protein), and *S. gordonii* PK2975 (5 μg of protein), respectively. (B) lanes 2 to 10, *S. gordonii* DL1 (5 μg of protein), *Enterococcus faecalis* GF590 (5 μg of protein), *Streptococcus sobrinus* 6715-10 (4 μg of protein), *S. sanguis* 12 (4 μg of protein), *S. sanguis* ATCC 10556 (6 μg of protein), *S. oralis* ATCC 10557 (4.5 μg of protein), *S. gordonii* ATCC 10558 (3 μg of protein), *S. gordonii* PK1897 (5 μg of protein), and *S. gordonii* PK2975 (5 μg of protein), respectively. Lanes 1 contained the prestained molecular weight standards phosphorylase *b* (106 kDa) and bovine serum albumin (80 kDa).

Immunoblot analysis of surface extracts of representative oral streptococci with Cog$^-$ mutant-absorbed antiserum. Anti-DL1 serum absorbed with the Cog$^-$ mutant PK1897 was used to probe immunoblots of surface extracts of several representative oral streptococci (Fig. 2). Surface extracts from representatives of the six streptococcal coaggregation groups (8) are shown in panel A (Fig. 2), and other representative streptococci are shown in panel B (Fig. 2). *S. gordonii* DL1 (Fig. 2, lanes 2) and the parent strain PK2975 (Fig. 2, lanes 10) served as positive controls and showed the 100-kDa band (arrowheads). The spontaneous Cog$^-$ mutant PK1897 served as the negative control (Fig. 2, lanes 9) and lacked the 100-kDa

band. The reference streptococcal strains (8) *Streptococcus* SM PK509 (Fig. 2A, lane 7) and *S. gordonii* PK488 (Fig. 2A, lane 8) showed a 100-kDa band. In fact, the surface proteins of strain PK488 (Fig. 2A, lane 8) appeared to immunoreact nearly identically to those from strains DL1 (Fig. 2A, lane 2) and PK2975 (Fig. 2A, lane 10). None of the other reference streptococci (Fig. 2A, lanes 3 to 6) or certain other representative streptococci (Fig. 2B, lanes 3 and 4) had a band that comigrated with the 100-kDa band found in *S. gordonii* DL1. None of these strains express a protease-inactivated adhesin that mediates lactose-inhibitable intrageneric coaggregation (8). However, lanes 5 to 8 (Fig. 2B) have faint bands which migrated above the 100-kDa band, and the streptococci represented in these lanes all participated in lactose-inhibitable intrageneric coaggregation. Although Cog$^-$ mutant-absorbed serum blocked intrageneric coaggregations between DL1 and its partners, this antiserum did not block the intrageneric coaggregations between any of the streptococci in lanes 5 to 8 (Fig. 2B) and their partners.

Thus, most of the human oral streptococci that exhibit lactose-inhibitable intrageneric coaggregations express a protein with a size of about 100-kDa that immunoreacts with antiserum that blocks intrageneric coaggregation between *S. gordonii* DL1 and its streptococcal partners. These results suggest that the 100-kDa surface protein may be the lactose-sensitive adhesin that mediates their coaggregation.

DISCUSSION

Spontaneous Cog$^-$ mutants were used to identify a putative 100-kDa adhesin from *S. gordonii* DL1. This protein is proposed to mediate lactose-sensitive intrageneric coaggregations. It is clearly distinct from the adhesins with sizes of greater than 200-kDa on this strain that mediate intergeneric coaggregation with actinomyces (4, 5, 15, 16). Also, absorbed antiserum that blocked lactose-inhibitable coaggregations had no effect on intergeneric lactose-noninhibitable coaggregations (Table 2).

Immunoblot analysis of reference streptococci from the streptococcal coaggregation groups and other representative streptococci showed that *S. gordonii* PK488 and *Streptococcus* sp. strain SM PK509 contained a 100-kDa protein that comigrated with the 100-kDa protein found in wild-type *S. gordonii* DL1 and parent strain PK2975. Like *S. gordonii* DL1 and PK2975, both PK488 and PK509 coaggregated with *S. oralis* 34 (1, 8). The Cog$^-$ mutant-absorbed serum partially blocked coaggregations between *S. gordonii* PK488 and its streptococcal partners. Mutants of one of these streptococcal partners, *S. oralis* 34, which are unable to coaggregate with DL1 also failed to coaggregate with PK488 (9). Taken together, these data suggest that the 100-kDa protein on PK488 may be functionally similar to the proposed 100-kDa adhesin found on DL1. Immunoblot analysis of four other representative oral streptococci (Fig. 2B, lanes 5 to 8) showed faint bands which migrated more slowly than the 100-kDa band found in DL1 and PK2975. All of these streptococci participate in intrageneric coaggregation. Cog$^-$ mutant-absorbed serum did not block the intrageneric coaggregations between these streptococci and their partners. Therefore, these faint bands appear to be less related to the proposed 100-kDa adhesin found on DL1. Several bands with molecular sizes less than 100 kDa are visible (Fig. 2). Their relationships to the 100-kDa protein are unknown; some may be monomers or smaller fragments of the high-molecular-size molecules.

Initial colonizers like these viridans streptococci express adherence-relevant surface proteins (4). These proteins include the antigen I/II family of the mutans group and the LraI family of lipoprotein receptor antigens (4). Several more from *S. gordonii* DL1 have been identified, such as SarA (3), CshA/B (16), and SspA (5). We have identified a putative 100-kDa adhesin from *S. gordonii* DL1, and it is proposed to mediate lactose-inhibitable intrageneric coaggregations.

ACKNOWLEDGMENTS

We thank C. Bouma, C. Whittaker, J. London, R. D. Lunsford, R. N. Andersen, and N. Ganeshkumar for giving helpful criticism during the writing of the manuscript.

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Editor: V. A. Fischetti