JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 801–807 Vol. 178, No. 3
0021-9193/96/$04.00+0

Role of the C Terminus in Antigen P1 Surface Localization in *Streptococcus mutans* and Two Related Cocci

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Received 10 August 1995/Accepted 22 November 1995

The C terminus of the major surface protein P1 from *Streptococcus mutans* is composed of a hydrophilic domain, an LPNTGV motif, a hydrophobic domain, and a charged tail. These features are shared by surface proteins from many gram-positive coccal bacteria. To investigate the role of the C-terminal domains in antigen P1 surface localization, full-length and truncated P1 gene constructs, which were expressed on the shuttle vector pDL276, were transformed into the P1-negative mutant *S. mutans* SM3352, *Streptococcus gordonii* DL-1, and *Enterococcus faecalis* UV202. Transformants were tested for expression of P1 by enzyme-linked immunosorbent assaying and Western blotting. The results showed that full-length P1 was expressed by transformants of all three bacteria and was localized on the cell surface. A fusion protein composed of the *Staphylococcus aureus* fibronectin binding protein C terminus and the P1 protein N terminus was found to surface localize in *S. mutans*. Deletion of the entire C-terminal domains resulted in P1 being expressed in the culture supernatant. A P1 truncation, which carried only the hydrophilic domain at its C terminus, was found partially associated with the cell surface. This truncated P1 was readily removed from the isolated cell wall by hot sodium dodecyl sulfate-mercaptoethanol extraction. In contrast, the full-length P1 remained associated with the isolated cell wall after similar treatment, suggesting covalent linkages between the full-length P1 and the cell wall. The results described above showed that antigen P1 was anchored to the cell wall by its C-terminal domains probably via covalent linkages with the cell wall. The results also support a universal mechanism involving the C-terminal domains for protein surface localization among this group of gram-positive bacteria.

There are more than 30 surface proteins from 12 different gram-positive bacteria exhibiting a common structural organization within the carboxy (C) terminus (36, 37). This C terminus includes a hydrophilic (wall-spanning) domain, a consensus LPXTGX motif, a hydrophobic (membrane-spanning) domain, and a charged tail (11, 12, 37). The contribution of the C terminus in surface expression of proteins has been studied by various workers. Hanski et al. (13) demonstrated the surface expression of the *Streptococcus pyogenes* fibronectin binding protein in group A streptococcus and *Enterococcus faecalis*. Rathsam et al. (32) expressed the *Streptococcus salivarius* fructosyltransferase on the surfaces of *Streptococcus gordonii* cells. In addition to these studies, others have demonstrated the ability to express chimeric proteins on the surface of heterologous gram-positive organisms (14, 30). Chimeric fusion proteins which utilize the C terminus of the *S. pyogenes* M protein (30) and the *Staphylococcus aureus* protein A (14) have been used to localize protein antigens on the surfaces of *S. gordonii* and *Staphylococcus xylosus* cells.

The roles of several of the C-terminal domains in protein surface localization have been analyzed for the *S. aureus* protein A (27, 35–37). These studies have demonstrated an important role for the charged tail and the LPXTGX consensus sequence in the anchoring of protein A to the cell surface of *S. aureus*. Schneewind et al. (35) suggested that the anchoring of *S. aureus* protein A requires the cleavage of the Thr-Gly amide bond within the LPXTGX and the subsequent linkage of the protein to the cell wall through an amide bond formed between the C-terminal carboxyl of Thr and the amino group of the pentaglycine cross bridge.

The major surface protein P1 (ca. 185 kDa) of *Streptococcus mutans*, an etiological agent for human dental caries (23), also displays the above-mentioned features at the C terminus (4, 15). Protein P1 has been suggested to behave as an adhesin by promoting the attachment of the organism to a salivary glycoprotein, a constituent of salivary pellicle coating the tooth surface (3, 22). Protein P1 was noted to be associated with the cell wall fraction (33), and immunoelectron microscopic studies revealed P1 as fibrillar structures on the cell surface (1, 22). The present study provides data to show that the C-terminal domains of P1 are required for the protein's surface expression in *S. mutans*, *S. gordonii*, and *E. faecalis*. The results support the idea of a universal mechanism for protein surface localization in gram-positive coccal bacteria (12, 36, 37). The present study also addresses the possible role of the hydrophilic wall-spanning domain in anchoring of the *S. mutans* P1 protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. The streptococci and *E. faecalis* were grown aerobically in Todd-Hewitt broth at 37°C without agitation. *Escherichia coli* cells were cultivated in LB media (1% tryptone, 0.5% yeast extract, 1% NaCl [wt/vol]) at 37°C with agitation. Antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.) and were used at the following concentrations: ampicillin at 50 μg/ml for *E. coli*; kanamycin at 50 μg/ml for *E. coli*, at 500 μg/ml for *S. mutans* and *E. faecalis*, and at 250 μg/ml for *S. gordonii*; and tetracycline at 20 μg/ml for *S. mutans* and at 15 μg/ml for *E. coli* XL-1 Blue.

The full-length *spaP* gene was cloned previously from *S. mutans* NG5 (15, 21). The *spaP* gene, which is carried on the pUC18 plasmid, was designated pSMI/II (15). Plasmid pSMI/II-3 was generated by cloning the *spaP* gene from pSMI/II into the *E. coli*-streptococcus shuttle vector pDL276 (Fig. 1A) (20). The truncated *spaP* genes were generated as described below. The *S. aureus* fibronectin-binding protein (FnBP) gene, *fnbA*, was provided by M. J. McGavin, University of Manitoba (25).

Antisera. The rabbit polyclonal antiserum to the *S. mutans* antigen P1 was

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant marker(s)	Bacterial host(s)	Source or reference
Bacteria			
E. coli XL1-Blue	Rec$^-$ Tet^r		Stratagene
S. mutans NG8	Wild type		A. S. Bleiweis (1)
S. mutans SM3352	Tet^r *spaP*		A. S. Bleiweis (7)
S. gordonii DL-1			D. LeBlanc (18)
E. faecalis UV202	Rec$^-$		D. LeBlanc (41)
Plasmids[a]			
pBluescript	2.9 kb, Amp^r	*E. coli*	Stratagene
pDL276 (pUC19 and pVA380-1)	6.9 kb, Kan^r	*E. coli*, streptococci	D. LeBlanc (9)
pSMI/II (pUC18)	8.9 kb, Amp^r	*E. coli*	A. S. Bleiweis (15)
pSMI/II-3 (pDL276)	14.0 kb, Kan^r	*E. coli*, streptococci	S. F. Lee (20)
pSMΔ1490 (pDL276)	13.5 kb, Kan^r	*E. coli*, streptococci	This study
pSMΔ706 (pDL276)	11.1 kb, Kan^r	*E. coli*, streptococci	This study
pP1/FnBP (pDL276)	12.6 kb, Kan^r	*E. coli*, streptococci	This study
pSMΔ1508 (pDL276)	13.5 kb, Kan^r	*E. coli*, streptococci	This study
pSMΔ1464 (pDL276)	13.4 kb, Kan^r	*E. coli*, streptococci	This study

[a] Vectors used to construct the plasmids are indicated in parentheses.

generated as described previously (19). A polyclonal antibody against the *S. aureus* FnBP was produced in New Zealand White rabbits. Briefly, the FnBP (ca. 200 kDa) from the culture supernatant of an overnight culture of *S. aureus* ATCC6538 was concentrated by ammonium sulfate precipitation and was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The FnBP was isolated from the gels by excising unstained areas of the gels as described previously (19). The gel slices were minced and used to immunize two rabbits (19).

DNA isolation and analysis. Plasmid DNA was isolated from *E. coli* by the procedure described by Birnboim and Doly (2) and was purified by CsCl density gradient centrifugation (34). Restriction endonucleases and DNA-modifying enzymes were used according to the instructions of the manufacturer. All of the enzymes were obtained from GIBCO/BRL Life Technologies, Inc. (Burlington, Ontario, Canada) or New England Biolabs (Mississauga, Ontario, Canada) or as otherwise noted. The DNA restriction fragments were separated on horizontal 0.8% (wt/vol) agarose slab gels in Tris-acetate buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.6]) at 15 V/cm of gel.

Recombinant DNA methodology. The truncated *spaP* genes (pSMΔ1490 and pSMΔ706) were generated by restriction of pSMI/II-3 with *Bam*HI (for pSMΔ1490) or *Msc*I and *Sma*I (for pSMΔ706). The resulting DNA fragments were separated by agarose gel electrophoresis. The DNA fragments, whose sizes were 13.5 and 11.1 kb, representing the truncated plasmids pSMΔ1490 and pSMΔ706, respectively, were purified from the agarose gel with a Gene-Clean kit (Bio 101, La Jolla, Calif.) according to the instructions of the manufacturer. The plasmids were recircularized by ligation with T4 DNA ligase at either 4 or 23°C for 18 h. Competent *E. coli* XL-1 Blue cells were prepared by the method described by Kushner (16). Competent cells were transformed with ligated DNA as described by Sambrook et al. (34). *E. coli* cells harboring the plasmid were selected on LB agar plates containing appropriate antibiotics. The deletion plasmid was verified by restriction endonuclease digestion and agarose gel electrophoresis.

Transformation of gram-positive organisms. *S. mutans*, *S. gordonii*, and *E. faecalis* were transformed by the method described by Perry and Kuramitsu (29) as modified by Murchison et al. (26). Cells were cultured in Todd-Hewitt broth supplemented with 5% horse serum (THBS) for 2 consecutive days by inoculation of 5 ml of THBS with 100 μl of cells. For transformation, the culture was diluted 1/40 in THBS, and the dilution was incubated at 37°C without aeration. The optical density at 600 nm was monitored until it reached 0.100 to 0.150. A 1-ml aliquot of cells was removed to a prewarmed sterile glass test tube in a 37°C water bath, and ca. 25 μg of plasmid DNA in 50 μl of 15 mM sodium citrate and 150 mM NaCl were added. The cells were incubated for 30 min at 37°C and were then diluted by the addition of 1 ml of prewarmed THBS. The cultures were incubated for an additional 1 h and were then plated directly on Todd-Hewitt agar containing the appropriate antibiotics. Plates were incubated at 37°C in an anaerobic chamber containing 80% N_2, 10% CO_2, and 10% H_2. Transformants were obtained after 48 h of incubation.

PCR. CsCl gradient-purified pSMI/II was used as the template for PCR with oligonucleotide primers P1A (5′-*cgg ggt acc* gcc ctt cta tca gta gct tc-3′), P1B (5′-*gcc ggt acc* **tca** agt tga agt ccg cgg tga-3′), and P1C (5′-*cgg ggt acc* **tca** acc aac cgc aat acg ttt cat ttg-3′). The underlined nucleotides represent addition of a *Kpn*I restriction site, and the boldfaced nucleotides represent the addition of a stop codon. The italicized nucleotides represent nontemplate-encoded 5′ additions to the PCR primers required for efficient cleavage of the oligonucleotide by *Kpn*I. The primers P1A/P1B amplify nucleotides 2068 to 4524, and primers

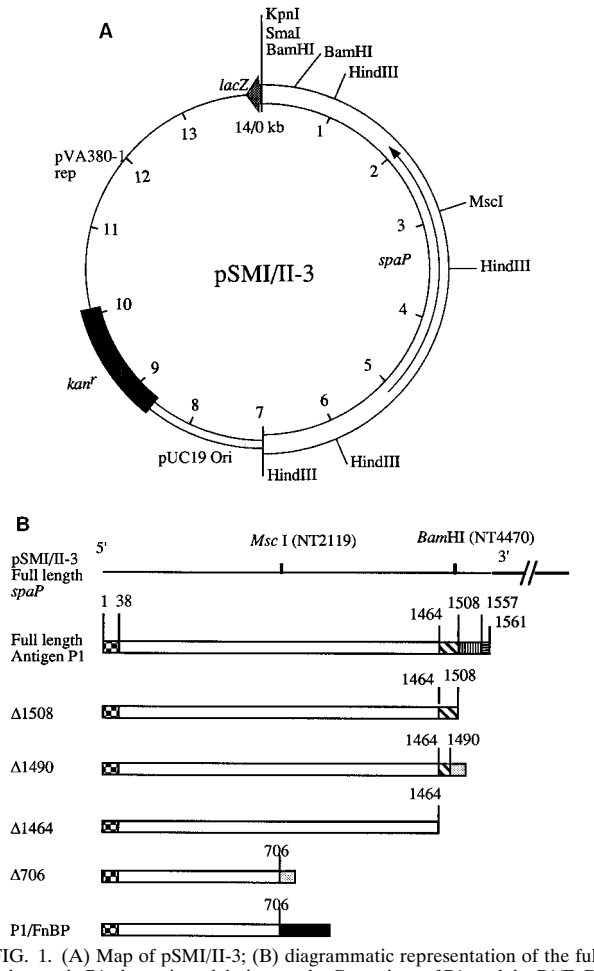

FIG. 1. (A) Map of pSMI/II-3; (B) diagrammatic representation of the full-length protein P1, the various deletions at the C terminus of P1, and the P1/FnBP fusion protein. The nucleotide numbers of the two relevant restriction sites are indicated in parentheses. Other numbers above antigen P1 and truncated P1 are amino acid residue numbers from the start codon. ▬, *spaP* DNA; -/-, pDL276 DNA; ▩, leader sequence; ▨, hydrophilic wall-spanning domain; ▥, hydrophobic domain; ▤, charged tail; ■, the C terminus of *S. aureus* FnBP; and ▧, potential LacZ fragment.

P1A/P1C amplify nucleotides 2068 to 4392 of the *spaP* sequence (GenBank accession number X17390 [15]). Thus, primers P1B and P1C were designed to generate truncation at nucleotide residues 4524 (amino acid residue 1508) and 4392 (amino acid 1464) of the *spaP* gene, respectively.

PCR was carried out by using the *Taq* DNA polymerase. The reaction mixture included 1 ng of template DNA, 2 mM each deoxynucleoside triphosphates, 1.5 mM each primer, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase in 100-μl volumes overlaid with 100 μl of light mineral oil. The amplification was carried out in a DNA minicycler (MJ Research Inc., Watertown, Mass.) and consisted of 30 cycles of denaturation (94°C, 1 min), annealing (53°C, 2 min for primers P1A and P1B; 55°C, 2 min for primers P1A and P1C), and extension (72°C, 2 min). The mineral oil overlay was removed by chloroform extraction. The PCR product was treated with proteinase K (GIBCO/BRL) (6) and was phenol-chloroform extracted. Following digestion with restriction endonuclease *Kpn*I and purification with the Gene-Clean kit, the amplified DNA was ligated into the vector pBluescript (Stratagene). The ligated DNA was transformed into competent *E. coli* XL-1 Blue.

The 2.4- and 2.3-kb DNA fragments from pPCR2.3 and pPCR2.4 carrying the truncated *spaP* sequences were isolated and subcloned with the *Msc*I and the *Kpn*I sites into the shuttle plasmid pSMI/II-3 digested with the same enzymes. Restriction of pSMI/II-3 with *Msc*I and *Kpn*I would cut within and outside the *spaP* sequence (*Kpn*I within the multiple cloning site of the vector). Thus, by ligation of the PCR fragments into these two sites, the 3′ portion of the *spaP* gene was replaced by the truncated sequences. These recombinant plasmids were designated pSMΔ1508 and pSMΔ1464.

The DNA sequences of the entire PCR-generated fragments contained in pSMΔ1508 and pSMΔ1464 were determined with a DNA sequencing kit (Sequenase version 2.0, Amersham Canada Ltd., Oakville, Ontario, Canada). The results confirmed the published sequence of *spaP* (15).

Construction of P1 fusion with *S. aureus* FnBP. The 3′ terminal 632 nucleotides of *fnbA* were isolated by digestion of pBTFN-1 (25) with *Hin*cII and *Sca*I. The *fnbA* fragment was then blunt-end ligated into pSMI/II-3, which had been digested with *Msc*I and *Sma*I (within the multiple cloning site). The correct orientation of the ligated DNA was verified by restriction analysis. The resulting gene fusion was 2.85 kb in length, encoding a protein with a molecular mass of approximately 102,000 Da. This plasmid was designated pP1/FnBP.

ELISA. The enzyme-linked immunosorbent assay (ELISA) method used was that described by Voller et al. (40). Cells were grown in Todd-Hewitt broth containing the appropriate antibiotics and were grown at 37°C for 18 h without aeration. The optical density of the culture at 600 nm was determined and was adjusted to 0.2 with phosphate-buffered saline (PBS). The adjusted culture was harvested by centrifugation in a microcentrifuge at 16,000 × *g* for 10 min. The supernatant fluid was recovered, and the cell pellet was washed in PBS and recentrifuged. The washed cells were resuspended in PBS and were serial twofold diluted in PBS, and 100 μl per well was added to polystyrene microtiter plates. The whole cells were fixed to the microtiter plates by 0.25% glutaraldehyde for 1 h at room temperature (8). The glutaraldehyde solution was decanted, and the plates were washed three times in PBS. A 3% (wt/vol) solution of bovine serum albumin (BSA) in PBS was added to each well, and the plates were incubated for 2 h at 37°C. Culture supernatants were also serial twofold diluted in PBS and were dispensed in 100-μl volumes into microtiter plates. The plates were incubated for 1 h at 37°C and were washed three times in PBS-Tween 20 before the addition of the 3% BSA solution. The plates were washed three times with PBS-Tween 20 buffer after blocking. Antibodies were diluted in PBS-Tween 20 and reacted with antigen coated plates for 2 h at 37°C or overnight at 4°C. The antisera used included the rabbit anti-P1 antiserum (dilution, 1/400), the rabbit anti-FnBP antiserum (dilution, 1/200), and the mouse ascitic fluid containing monoclonal anti-P1 antibody 4-10A (dilution, 1/5,000; kindly donated by A. S. Bleiweis, University of Florida [1]). The plates were washed five times in PBS-Tween 20 before being reacted with goat anti-rabbit or goat anti-mouse immunoglobulin G alkaline phosphate conjugates (Sigma) for 1 h at 37°C. The plates were washed five times with PBS-Tween 20 and were developed by the addition of the substrate *p*-nitrophenyl phosphate. The A_{405} was determined by a Bio-Rad microplate reader (model 3550; Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). The results (A_{405}) reported in the graphs for the whole cells and supernatant fluids represented readings from the same dilution for both fractions in side-by-side assays. Thus, the absorbance readings represented proportional distributions of antigen P1 between the two fractions.

Preparation of samples for Western blotting (immunoblotting). One milliliter of overnight (18-h) culture was harvested by centrifugation in a microcentrifuge at 16,000 × *g* for 10 min. The supernatant fluid was recovered for protein precipitation in 10% trichloroacetic acid and was washed twice with ice-cold acetone. The protein pellet was dissolved in 100 μl of the sample buffer described by Laemmli (17). The cell pellet was washed in PBS. The cells were resuspended in a buffer (100 μl) containing 30% (wt/vol) raffinose, 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, and 2 mM phenylmethylsulfonyl fluoride. The cells were treated with 25 kU of mutanolysin and 270 kU of lysozyme per ml at 37°C for 90 min (38). The resulting protoplasts were centrifuged at 16,000 × *g* for 10 min. The supernatant fluid, representing mutanolysin-lysozyme-released proteins from intact cells, was collected. Aliquots (20 μl) of the mutanolysin-lysozyme-released proteins and trichloroacetic acid-precipitated proteins from culture supernatant fluids were separated by SDS-PAGE with 7.5% PAGE gels and the buffer system described by Laemmli (17). Proteins were transferred to nitrocellulose membranes with a Bio-Rad Transblot apparatus and the transfer buffer described by Towbin et al. (39). After transfer, the nitrocellulose was incubated for 1 h in PBS containing 3% BSA to block additional protein-binding sites. Membranes were then incubated in appropriate antisera as described for the ELISA. The blots were developed in 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride dissolved in a solution containing 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM $MgCl_2$ (GIBCO/BRL).

Immunofluorescence. One milliliter of overnight (18-h) culture was harvested by centrifugation. The cell pellet was washed in PBS and resuspended in 1 ml of PBS. A smear of cells was prepared by spreading a 10-μl aliquot onto the surface of a glass slide and allowing it to air dry. The cells were then heat fixed by passing the slide through a flame. A 3% solution of BSA in PBS was applied to the smear, and the slide was incubated for 30 min at room temperature to block additional protein-binding sites. A 20-μl droplet of monoclonal antibody 4-10A diluted 1/500 in PBS was applied to the smear. The glass slide was incubated in a humid box for 30 min at 37°C and then washed several time in a stream of PBS. The second antibody, an anti-mouse Fab-specific fluorescein isothiocyanate-conjugated antibody (Sigma, St. Louis, Mo.), was diluted 1/100 in PBS, and a 20-μl droplet was applied for 30 min at 37°C in a humid dark box. The smear was washed in a stream of PBS and was blotted dry. A drop of emulsion containing 90% glycerol and 10% PBS, supplemented with 0.1% phenylenediamine (Sigma), was applied to prevent rapid quenching of the fluorescence, and a coverslip was placed on top of the emulsion. The cells were viewed with a Zeiss epifluorescence microscope (495-nm excitation, 525-nm emission) and were photographed with Kodak 400 Ectachrome color slide films. Black and white prints were reproduced from color prints.

Preparation of cell walls. *S. mutans* cells were grown in 1-liter aliquots of the *Actinomyces*-defined medium described by Bowden et al. (5), supplemented with 0.2% (wt/vol) tryptone. The inoculated medium was incubated for 20 h in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2). The cells were harvested by centrifugation (10,000 × *g*, 4°C, 20 min) and were washed three times in 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mM pepstatin A. Cells were disrupted in a Mickle tissue disintegrator (Mickle Engineering Co., Gomshall, England) with glass beads until the cells stained red with carbol fuchsia when they were Gram stained (1 to 2 h) (31). Unbroken cells and glass beads were removed by centrifugation at 8,000 × *g* for 15 min. The cell walls were isolated from the supernatant fluids by centrifugation at 27,000 × *g* for 30 min. The pelleted walls were washed three times in phosphate buffer with protease inhibitors and were freeze-dried.

Extraction of cell wall proteins. Twenty milligrams of freeze-dried cell walls was resuspended in 300 μl of 3× concentrated SDS-mercaptoethanol buffer (20 mM Tris [pH 6.8], 0.3% glycerol, 6% SDS, 0.15% β-mercaptoethanol) as described by Laemmli (17), and the suspension was boiled for 20 min. The heated suspension was centrifuged at 10,000 × *g* for 20 min, and the supernatant fluid was recovered, leaving the residual cell walls in the pellet. The residual cell walls were washed three times in distilled water and were resuspended in a final volume of 150 μl. A 10-μl aliquot of residual cell walls and 5-μl aliquots of untreated cell walls and the extracted proteins were analyzed by Western immunoblotting with the monoclonal antibody 4-10A to detect antigen P1.

RESULTS

Expression of *spaP* in gram-positive coccal bacteria. The full-length P1 gene (*spaP*) carried on the shuttle plasmid pSMI/II-3 (Fig. 1) was transformed into *S. mutans* SM3352 (the P1-deficient isogenic mutant of strain NG8 [7]), *S. gordonii* DL-1, and *E. faecalis* UV202. Kanamycin-resistant transformants were tested by ELISA for expression and distribution of the recombinant P1 protein. Initial ELISA results indicated that P1 was predominantly cell associated in the transformants (data not shown). One transformant from each of the three bacteria was selected for further studies. The three transformants *S. mutans* SMI/II-3/SM3352, *S. gordonii* SMI/II-3/DL-1, and *E. faecalis* SMI/II-3/UV202 were treated with mutanolysin-lysozyme, and the released proteins were subjected to Western blot analysis. Figure 2 shows that definite but multiple immunoreactive bands indicative of antigen P1 were present in the samples. The wild-type P1 antigen from *S. mutans* NG8 also appeared as multiple bands when extracted by the same muralytic enzymes (Fig. 2, lane 1). The highest-molecular-weight band was in the range of 185,000, the weight expected for full-size P1 (15). These results indicate that *spaP* was expressed in the P1-deficient *S. mutans*, *S. gordonii*, and *E. faecalis*. The gene product appeared to have the expected size and was surface localized in the transformants.

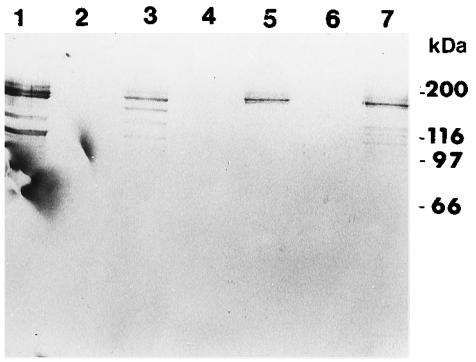

FIG. 2. Detection of antigen P1 in recombinant *S. mutans*, *S. gordonii*, and *E. faecalis* carrying the full-length *spaP* by Western blotting. Samples shown are mutanolysin-lysozyme released materials from whole cells. Antigen P1 was analyzed with the anti-P1 monoclonal antibody 4-10A. Lanes: 1, *S. mutans* NG8; 2, *S. mutans* SM3352; 3, *S. mutans* SMI/II-3/SM3352; 4, *S. gordonii* DL-1; 5, *S. gordonii* SMI/II-3/DL-1; 6, *E. faecalis* UV202; and 7, *E. faecalis* SMI/II-3/UV202.

Immunofluorescence. To further demonstrate that the P1 detected in the transformants was in fact found on the cell surface, immunofluorescence studies were performed. As shown in Fig. 3A, C, and E, slight to intense fluorescence was present on the cell surfaces of transformants *S. mutans* SMI/II-3/SM3352, *S. gordonii* SMI/II-3/DL-1, and *E. faecalis* SMI/II-3/UV202 and absent in the background bacteria (Fig. 3B, D, and F). These results support the interpretation of surface localization of antigen P1 in these transformants.

Expression of the P1/FnBP fusion protein. To provide further evidence in support of a common mechanism for surface localization of gram-positive proteins, a gene fusion was created by ligating the 5′ DNA from *spaP* in frame to the 3′ DNA

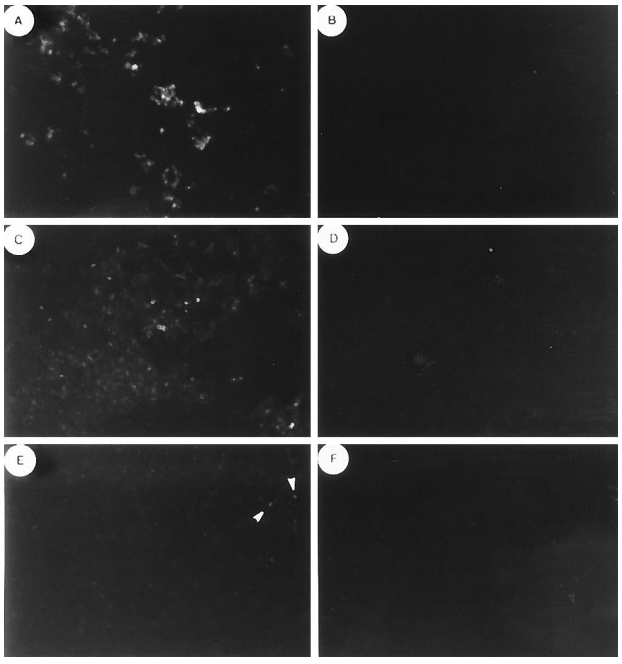

FIG. 3. Detection of antigen P1 in *S. mutans*, *S. gordonii*, and *E. faecalis* transformants carrying the full-length *spaP* by immunofluorescence labelling. (A) *S. mutans* SMI/II-3/SM3352; (B) *S. mutans* SM3352; (C) *S. gordonii* SMI/II-3/DL-1; (D) *S. gordonii* DL-1; (E) *E. faecalis* SMI/II-3/UV202; and (F) *E. faecalis* UV202. Weak fluorescence in *E. faecalis* SMI/II-3/UV202 is indicated by arrowheads.

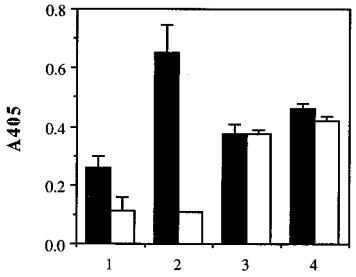

FIG. 4. Expression of the fusion gene *spaP*/*fnbA* in *S. mutans* SM3352 and *S. gordonii* DL-1. The recombinant protein was analyzed with the rabbit polyclonal anti-P1 antiserum in ELISA. A_{405} readings were determined from diluted samples equivalent to culture optical densities at 600 nm of 0.1 and 0.025 for *S. mutans* and *S. gordonii*, respectively. Bars: 1, *S. mutans* SM3352; 2, P1/FnBP/SM3352; 3, *S. gordonii* DL-1; 4, P1/FnBP//DL-1. ■, whole cells; □, culture supernatant fluids. Error bars indicate standard deviations of triplicate samples.

coding for the C terminus of the *S. aureus* FnBP (Fig. 1). The resulting fusion protein would, therefore, express the C-terminal domains from the *S. aureus* FnBP (211 amino acids) and the N terminus of antigen P1 (706 amino acids). The fusion protein would total 917 amino acids, generating a polypeptide of 102 kDa before cleavage of the signal sequence and a polypeptide of 98 kDa after signal sequence cleavage. With the same shuttle vector (pDL276), the construct pP1/FnBP was transformed into both *S. mutans* SM3352 and *S. gordonii* DL-1. Kanamycin-resistant transformants were screened for expression of the fusion protein. ELISA analysis with anti-P1 antisera indicated that the fusion protein was primarily on the cell surface of one of the transformants, *S. mutans* P1/FnBP/SM3352 (Fig. 4, bar 2). In the case of transformant *S. gordonii* P1/FnBP/DL-1, the amount of reactivity detected with the whole cells and culture supernatant fluid was marginally greater than that for the background *S. gordonii* DL-1. Expression of the chimeric protein P1/FnBP in the transformants was also detected by Western immunoblotting with both the rabbit anti-P1 antiserum and rabbit anti-FnBP antiserum (Fig. 5). An immunoreactive band with a molecular mass of approximately 98 kDa was observed with both antisera from samples prepared from the transformants. These results indicate that the chimeric protein was expressed in both *S. mutans* SM3352 and *S. gordonii* DL-1.

Construction of deletion mutants of *spaP*. Computer analysis of the *spaP* amino acid sequence with the PLOT.A/HYD

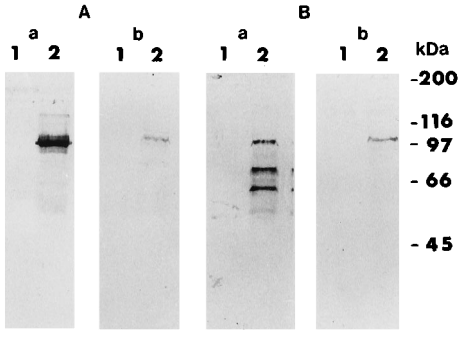

FIG. 5. Detection of P1/FnBP fusion proteins expressed by *S. mutans* (A) and *S. gordonii* (B) transformants by Western blotting. Samples are culture supernatant fluids from late-exponential-phase cultures. Antigen P1/FnBP fusion protein was analyzed with the rabbit polyclonal anti-P1 antibody (blots a) and the rabbit polyclonal anti-FnBP antibody (blots b). Lanes 1, *S. mutans* SM3352 or *S. gordonii* DL-1. Lanes 2, P1/FnBP/SM3352 or P1/FnBP/DL-1.

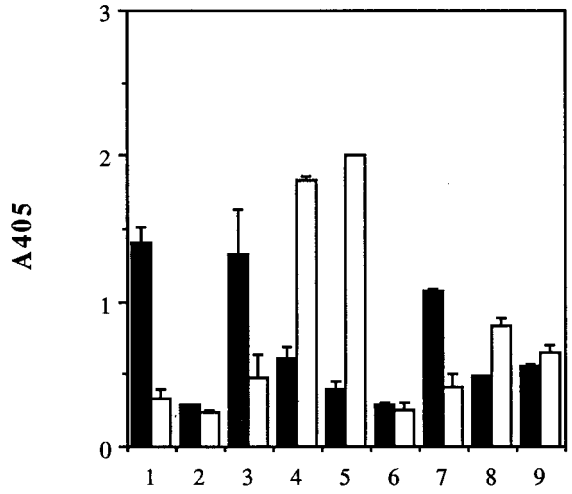

FIG. 6. Expression of full-length *spaP* (pSMI/II-3) and truncated *spaP* (pSMΔ1490 and pSMΔ1508) in *S. mutans* SM3352 and *S. gordonii* DL-1. Antigen P1 was analyzed in both the whole cells (■) and culture supernatant fluids (□) by ELISA with the anti-P1 monoclonal antibody 4-10A. A_{405} readings were determined from diluted samples equivalent to culture optical densities at 600 nm of 0.05 and 0.1 for *S. mutans* and *S. gordonii*, respectively. Bars: 1, *S. mutans* NG8; 2, *S. mutans* SM3352; 3, *S. mutans* SMI/II-3/SM3352; 4, *S. mutans* Δ1490/SM3352; 5, *S. mutans* Δ1508/SM3352; 6, *S. gordonii* DL-1; 7, *S. gordonii* SMI/II-3/DL-1; 8, *S. gordonii* Δ1490/DL-1; and 9, *S. gordonii* Δ1508/DL-1. Error bars indicate standard deviations from triplicate samples.

MacProt program identified a 44-amino-acid stretch (residues 1464 to 1508) as the hydrophilic wall-spanning domain. This hydrophilic stretch of amino acids is found N terminally to the LPNTGV consensus sequence at residues 1528 to 1533. Thus, the C-terminal domains of antigen P1 begin at amino acid residue 1464 to the last amino acid of the protein (Fig. 1B).

To assist in the definition of the C-terminal domains of antigen P1 in protein surface localization, a number of deletion mutants of P1 were generated (Fig. 1). The first deletion mutant was Δ706, which had P1 truncated at amino acid residue 706. This deletion mutant contained none of the C-terminal domains. The second deletion mutant was truncated at residue 1464 (Δ1464), which is N terminal to the wall-spanning domain. Two other deletion mutants with full or partial wall-spanning domains were also created (Δ1508 and Δ1490). Deletion mutant Δ1490 still carried 26 of the 44 amino acid residues of the wall-spanning domain. Δ706 and Δ1490 were generated from the convenient restriction sites *Msc*I and *Bam*HI, respectively, and computer analysis of the DNA sequence suggested that they both carried a 3′ in-frame fusion with the *lacZ* gene from the plasmid vector. Δ1508 and Δ1464 were generated by PCR as described in Materials and Methods.

Expression and localization of *spaP* deletion mutants. *S. mutans* and *S. gordonii* transformants carrying plasmid pSMΔ706 were able to express the truncated *spaP*, and the bulk of the gene product was found in the culture supernatant fluids (data not shown). Similarly, transformants carrying pSMΔ1464 were found to express P1 in the culture supernatant fluids. These results confirm a role for the C terminus in surface localization of antigen P1 in *S. mutans* and *S. gordonii*.

Transformants of *S. mutans* and *S. gordonii* carrying part (Δ1490) or whole (Δ1508) of the hydrophilic wall-spanning domain were analyzed by ELISA (Fig. 6). The results showed that the majority of the gene product was found in the culture supernatant fluids (Fig. 6, bars 4, 5, 8, and 9). A small amount of recombinant P1 may also be present on the cells, as indicated by the higher-than-background readings exhibited by the whole cells. In contrast, in *S. mutans* and *S. gordonii* transformants carrying the intact *spaP*, P1 was mostly cell associated (Fig. 6, bars 3 and 7). Western immunoblotting with the monoclonal anti-P1 antibody indicated that the presence of multiple immunoreactive bands in the culture supernatant fluids and mutanolysin-lysozyme released wall proteins from *S. mutans* Δ1508/SM3352 and *S. gordonii* Δ1508/DL-1. The highest molecular mass band was ca. 185 kDa (data not shown).

E. faecalis UV202 was also transformed with the deletion mutant pSMΔ1490. ELISA data demonstrated similar results to those found with pSMΔ1490 transformed *S. gordonii* (data not shown). The expression of the recombinant P1 antigen in *E. faecalis* UV202 was variable and decreased with continued subculturing.

Analysis of antigen P1 in cell walls. Cell walls from *S. mutans* NG8, *S. mutans* SM3352 harboring pSMI/II-3, and *S. mutans* SM3352 harboring pSMΔ1508 were isolated and analyzed by Western immunoblotting. Antigen P1 could be identified from wall preparations from *S. mutans* NG8 (Fig. 7A, lane 1), SMI/II-3/SM3352 (lane 2), and Δ1508/SM3352 (lane 3). As indicated by the intensity of the reactive protein bands, the relative abundance of the truncated P1 associated with cell walls of Δ1508/SM3352 was much less than that from the wild-type NG8 and SMI/II-3/SM3352. This result is consistent with the ELISA data (Fig. 6), according to which the reactivity of the Δ1508/SM3352 cells was marginally greater than that of the nontransformant SM3352. The multibanding pattern of protein P1 was observed for each of the samples, although fewer bands were seen in the case of the truncated P1.

Cells walls from all three strains were subjected to hot-SDS extraction to remove noncovalent proteins associated with the cell walls. The residual cell walls (Fig. 7B) and the SDS-extracted proteins (Fig. 7C) for each of the samples were analyzed for the presence of antigen P1. The antigen was detected in the SDS-extracted residual cell walls from *S. mutans* NG8 and SMI/II-3/SM3352 (Fig. 7B, lanes 1 and 2, respectively). In contrast, P1 was not detected in the residual cell walls from Δ1508/SM3352 (Fig. 7B, lane 3). Antigen P1 was detected in the SDS-extracted proteins in all three samples (Fig. 7C).

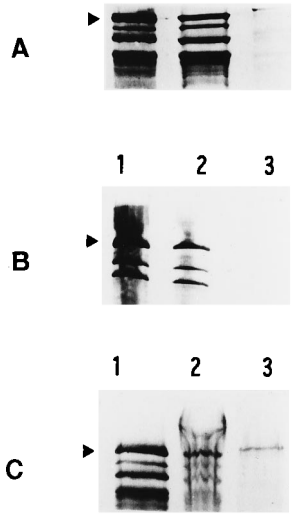

FIG. 7. Western blotting of antigen P1 in cell wall preparations. (A) Untreated cell walls; (B) SDS-mercaptoethanol-extracted residual cell walls; and (C) SDS-mercaptoethanol-solubilized cell wall P1. Lanes: 1, *S. mutans* NG8; 2, *S. mutans* SMI/II-3/SM3352; and 3, *S. mutans* Δ1508/SM3352. Arrowheads indicate the ca. 185-kDa full-length P1.

DISCUSSION

In the present study, the role of the C-terminal domains in surface localization of P1 in *S. mutans* as well as in *S. gordonii* and *E. faecalis* was investigated. The results clearly indicate that the C-terminal domains of P1 were responsible for protein surface localization in these three bacteria. This statement is supported by the findings that the intact P1 was surface localized in these organisms while the C terminus truncated P1 was not. The last (C-terminal) 97 amino acid residues of P1 appeared to be responsible for protein surface localization, as suggested by results with deletion mutant Δ1464. That P1 was able to surface localize in *S. gordonii* and *E. faecalis* and that the *S. aureus* FnBP C-terminal domains were able to surface localize P1 in *S. mutans* strongly indicate that the C-terminal domains from one organism were able to function in another organism. Thus, these results provide strong evidence in support of a common mechanism for surface protein localization and anchoring among this group of gram-positive bacteria (36, 37). In this mechanism, the C-terminal domains of these surface proteins carry the information for surface localization, and the cellular anchoring machinery in one organism apparently was able to recognize and process information encoded by the C-terminal domains from another organism.

In the P1/FnBP fusion protein expression studies, the immunoblotting results clearly showed that the correct fusion was made and that the chimeric protein was expressed by both *S. mutans* and *S. gordonii*. The ELISA data suggest that the chimeric protein was associated with the cell surface of *S. mutans*. However, in *S. gordonii*, the chimeric protein was only marginally detected. The lack of detection of P1/FnBP on the surface of *S. gordonii* is not very clear at this time. Perhaps when the fusion protein was present on the surfaces of *S. gordonii* cells, it was folded in such a way that the antigenic epitopes were inaccessible to the antibody.

The wall-spanning domain appeared to play the role of intercalating P1 to the cell wall. Antigen P1 was observed in the cell walls isolated from the deletion mutant Δ1508 expressed in *S. mutans* SM3352, although the majority of the P1 protein was detected in the culture supernatant. Treatment of Δ1508/SM3352 cell walls with boiling SDS-mercaptoethanol resulted in the removal of the P1 from the cell walls, suggesting that the protein is not covalently associated with the cell wall. The native P1 protein and the expressed recombinant full-length P1 were associated with the cell wall. However, hot SDS-mercaptoethanol treatment could not completely remove the native P1 protein or the full-length recombinant P1 protein from purified cell walls, suggesting a possible covalent link with the cell wall.

It is not clear at this time what the roles of the LPNTGV motif, the hydrophobic domain, and the charged tail of *S. mutans* P1 in the protein surface localization are. In the model given by protein A (35, 37), the LPXTGX motif and charged tail are required for signal recognition and posttranslational modification of the protein to allow covalent linking of protein A to the cell wall cross bridges. Given the similarity between the C termini of P1 and *S. aureus* protein A, it is likely that the two domains in P1 will have similar functions.

P1 protein released from intact cells by muralytic enzymes appeared as multiple bands in Western blots. A ladder of protein bands has also been observed for *S. pyogenes* M protein (10) and *S. aureus* protein A (36, 37) when the proteins were extracted from whole cells. In the case of protein A, the multibanding pattern was thought to be caused by mucopeptide fragments attached to the protein. Pancholi and Fischetti (28) isolated the cell wall-associated portion of the *S. pyogenes* M protein and were unable to detect any amino sugars associated with the wall-associated fragment. Earlier studies by Russell (33) have suggested that *S. mutans* antigen P1 is a glycoprotein, since cell wall carbohydrate has been associated with the extracted protein. However, whether antigen P1 represents a glycoprotein or a protein-carbohydrate complex has remained unresolved (24). It should be pointed out that the multiple banding pattern may also be attributed to proteolytic activities.

In conclusion, the results presented in this paper showed that *S. mutans* P1 protein is anchored to the cell surface by the C terminus, possibly via covalent linkage to the cell wall. The wall-spanning domain of the *S. mutans* P1 antigen contributes to the noncovalent retention of the P1 protein on the cell surface. The absence of the LPXTGX motif, hydrophobic domain, and charged tail resulted in impaired anchoring of P1 to the cell surface. Therefore, the findings would support the model for surface localization and anchoring of gram-positive bacterial proteins previously described (36, 37).

ACKNOWLEDGMENTS

We thank G. H. Bowden for his assistance in the isolation of cell walls.

This study was supported by a grant (MT-11580) to S.F.L. from the Medical Research Council of Canada. S.F.L. is a recipient of a development grant (DG-N023) for salary support from the Medical Research Council. M.K.H.M. is a recipient of the University of Manitoba Graduate Student fellowship.

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