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Expression of Functional *Porphyromonas gingivalis* Fimbrillin Polypeptide Domains on the Surface of *Streptococcus gordonii*

ASHU SHARMA,[1]* HIDEKI NAGATA,[1] NOBUSHIRO HAMADA,[1] HAKIMUDDIN T. SOJAR,[1] DENNIS E. HRUBY,[2] HOWARD K. KURAMITSU,[1] AND ROBERT J. GENCO[1,3]

Department of Oral Biology, School of Dental Medicine,[1] and Department of Microbiology, School of Medicine and Biomedical Sciences,[3] State University of New York at Buffalo, Buffalo, New York 14214, and Department of Microbiology, Oregon State University, Corvallis, Oregon 97331[2]

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Genetically engineering bacteria to express surface proteins which can antagonize the colonization of other microorganisms is a promising strategy for altering bacterial environments. The fimbriae of *Porphyromonas gingivalis* play an important role in the pathogenesis of periodontal diseases. A structural subunit of the *P. gingivalis* fimbriae, fimbrillin, has been shown to be an important virulence factor, which likely promotes adherence of the bacterium to saliva-coated oral surfaces and induces host responses. Immunization of gnotobiotic rats with synthetic peptides based on the predicted amino acid sequence of fimbrillin has also been shown to elicit a specific immune response and protection against *P. gingivalis*-associated periodontal destruction. In this study we engineered the human oral commensal organism *Streptococcus gordonii* to surface express subdomains of the fimbrillin polypeptide fused to the anchor region of streptococcal M6 protein. The resulting recombinant *S. gordonii* strains expressing *P. gingivalis* fimbrillin bound saliva-coated hydroxyapatite in a concentration-dependent manner and inhibited binding of *P. gingivalis* to saliva-coated hydroxyapatite. Moreover, the recombinant *S. gordonii* strains were capable of eliciting a *P. gingivalis* fimbrillin-specific immune response in rabbits. These results show that functional and immunologically reactive *P. gingivalis* fimbrillin polypeptides can be expressed on the surface of *S. gordonii*. The recombinant fimbrillin-expressing *S. gordonii* strains may provide an effective vaccine or a vehicle for replacement therapy against *P. gingivalis*. These experiments demonstrated the feasibility of expressing biologically active agents (antigens or adhesin molecules) by genetically engineered streptococci. Such genetically engineered organisms can be utilized to modulate the microenvironment of the oral cavity.

Porphyromonas gingivalis is a virulent gram-negative black-pigmented anaerobe associated with periodontal disease. The fimbriae of *P. gingivalis* have been shown to play an important role in the adherence of the bacteria to host surfaces, including epithelial cells (3, 11), fibronectin-collagen complexes (21), and salivary components (2, 17), as well as to other microorganisms (14, 15, 33). Fimbriae or their major subunit protein (fimbrillin) also induces the production of immunoglobulin G and immunoglobulin A antibodies (4) and stimulates the production of proinflammatory cytokines (interleukin-1, tumor necrosis factor alpha, interleukin-6, and interleukin-8) that are responsible for bone resorption and other inflammatory processes (10, 12, 24). Studies performed with gnotobiotic rats have shown that wild-type heavily fimbriate strains of *P. gingivalis* cause alveolar bone loss while an afimbriate mutant strain of *P. gingivalis* does not cause periodontal bone loss (18). Furthermore, the alveolar bone loss in gnotobiotic rats can be blocked by immunization with *P. gingivalis* fimbriae or synthetic peptides derived from the major subunit protein, fimbrillin (8, 22). Recently, a murine T-cell epitope has been identified within N-terminal amino acid residues 103 to 122 of the fimbrillin molecule. Mice immunized with a synthetic peptide corresponding to residues 103 to 122 of fimbrillin were protected against a normally lethal infection of *P. gingivalis* (5).

Since development of a vaccine against *P. gingivalis* could provide protection against the periodontal disease associated with this organism, we have concentrated on developing and testing a recombinant fimbrial vaccine by using a commensal oral organism, *Streptococcus gordonii*, as a carrier of *P. gingivalis* fimbrial domains. The aim of the present study was to express *P. gingivalis* fimbrial peptides in nonpathogenic oral streptococci and to characterize the function of these peptides. The resulting recombinant oral streptococci can be utilized for antigen presentation (fimbrillin) to induce protective host immune responses. Alternatively, the recombinant organisms can be used as direct competitive inhibitors of *P. gingivalis* colonization in the host oral cavity. Toward this end, we expressed the biologically active N- and C-terminal peptide domains of *P. gingivalis* fimbrillin in *S. gordonii*. These domains were selected because of their role in adherence via binding to saliva-coated oral surfaces and fibronectin (16, 17, 31) or their role in triggering host immune responses (23, 24). The recombinant *S. gordonii* strains can be utilized for developing a fimbrial vaccine against periodontal disease. Utilizing a system recently developed with nonpathogenic oral streptococci for surface expression of heterologous proteins (19, 25, 26), we successfully expressed biologically and immunologically active polypeptide domains of *P. gingivalis* fimbrillin in *S. gordonii.*

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *Escherichia coli* DH5α (Gibco-BRL, Gaithersburg, Md.) was grown in Luria-Bertani broth or on Luria-Bertani medium containing 1.5% agar. *P. gingivalis* 381 (which originated from the Forsyth Dental Center, Boston, Mass.) was obtained from the culture

* Corresponding author. Mailing address: Department of Oral Biology, 208 Foster Hall, 3435 Main St., State University of New York at Buffalo, Buffalo, NY 14214. Phone: (716) 829-3518. Fax: (716) 829-3942. Electronic mail address: orbashu@ubvms.cc.buffalo.edu.

collection in our laboratory. *P. gingivalis* 381 was grown in half-strength brain heart infusion (18 mg/ml; Difco) supplemented with 5 mg of yeast extract per ml, 5 μg of hemin per ml, and 0.2 μg of menadione per ml and buffered at pH 7.4. *S. gordonii* GP251 (25) was grown in Todd-Hewitt broth containing 0.2% yeast extract (THY) with or without 1.5% agar. For radiolabelling, the cells were grown in THY containing 5 μCi of [^{3}H]thymidine (Dupont NEN Research Products, Boston, Mass.) per ml at 37°C for 2 days anaerobically (85% N_2, 10% H_2, 5% CO_2). When appropriate, the antibiotics chloramphenicol and erythromycin were used at a concentration of 5 μg/ml. Plasmid pUC13Bg12.1 (6) was used as a template for PCR amplification of the *P. gingivalis fimA* gene. Insertion vector pSMB55 (25), a 5.73-kb *E. coli* plasmid that does not replicate in *S. gordonii*, was maintained in *E. coli* DH5α.

***P. gingivalis* fimbrillin-encoding DNA.** DNA fragments encoding the N-terminal portion (amino acid residues 55 to 145) and the C-terminal portion (residues 233 to 322) of the *P. gingivalis* fimbrillin gene (*fimA*) were amplified by PCR by utilizing pUC13Bg12.1 as a template (6). Amplimer set 1 (forward primer 5′C CGGGGTACCGGTGCAATGGAACTGGTTGGC3′ and reverse primer 5′G CGCGAATTCTGTGTAAATGTTATCGTA3′) and amplimer set 2 (forward primer 5′CCGGGGTACCGTTTATGGCAAACTTCAGAAAAACGG3′ and reverse primer 5′GCGCGAATTCCTGTACATTCAAGTGAGCAG3′) were utilized to amplify the *fimA* sequences encoding the N- and C-terminal regions of the fimbrillin, respectively. The forward primers included a four-base "clamp" sequence and a *Kpn*I restriction enzyme site at the 5′ end, whereas the reverse primers included the four-base clamp sequence and a restriction site for *Hin*dIII to facilitate digestion and cloning into the integration vector.

Construction of recombinant *S. gordonii*. Insertion vector pSMB55 (19) was used to obtain translational gene fusions of the *fimA* gene with the M6 gene (*emm6.1*). The *fimA* gene was inserted into the *Kpn*I-*Hin*dIII site of *emm6.1*, and the resulting chimeric plasmid was used to transform *S. gordonii* GP251 as described previously (26). The transformants were selected by plating preparations onto THY agar plates containing 5 μg of erythromycin per ml. Following anaerobic incubation at 37°C for 48 to 72 h, colonies were analyzed for M6-fimbrillin fusion protein expression by performing an immunoblotting and immunofluorescence analysis. Briefly, cultures (10 ml) of *S. gordonii* transformants were grown to the late log phase in THY. Cells were harvested by centrifugation, resuspended in 0.1 ml of protoplasting buffer (100 mM Tris-Cl [pH 8.0], 30% sucrose, 50 mM $MgCl_2$, 5 mM dithiothreitol, 200 μg of lysozyme per ml, 1 mM phenylmethylsulfonyl fluoride), and kept on ice for 1 h. The protoplasts were centrifuged for 3 min at 16,000 × *g* and resuspended in 100 ml of 50 mM Tris-Cl (pH 8.0). Thorough lysis was achieved by five cycles of quick freezing and thawing of the suspension. Unlysed cells and gross debris were eliminated by low-speed centrifugation (1,000 × *g*) for 15 min, whereas the supernatant containing membranes and cytoplasm were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by an immunoblotting analysis (28).

Cell surface ELISA. Expression of fimbrillin on the cell surface was detected by the whole-cell enzyme-linked immunosorbent assay (ELISA) technique described below. *S. gordonii* cells were grown as described above in a 15-ml THY culture to an A_{590} of 1.0. Cells were harvested by centrifugation at 2,000 × *g* at 4°C and washed once with 25 ml of cold phosphate-buffered saline (PBS) (10 mM phosphate buffer [pH 7.4) containing 0.12 mM NaCl and 2.7 mM KCl) containing 0.02% sodium azide. The cells were resuspended in cold PBS containing 0.02% sodium azide to an optical density at 590 nm of 1.0. The cells were diluted eightfold with 0.1 M sodium bicarbonate buffer, and 100 μl of the diluted cells was used for each ELISA plate well on an Immulon II plate (Dynatech Laboratories). The plates were incubated at room temperature for 2 h to allow coating of the wells with *S. gordonii* cells and were stored overnight at 4°C. The wells were emptied by inverting the plates and then washed three times with PBS containing 0.05% Brij 35. The unoccupied sites were blocked by 2% bovine serum albumin (BSA) in PBS (100 μl per well) for 1 h at 37°C. After blocking, the wells were emptied and incubated for 1 h at room temperature with serially diluted primary antibody in PBS containing 2% BSA (50 μl per well). Following incubation, the wells were washed three times with PBS containing 0.05% Brij 35 and were incubated for 2 h at room temperature with alkaline phosphatase-labeled goat anti-rabbit antibody (50 μl of a 1:1,000 dilution in PBS containing 2% BSA per well; Bio-Rad Laboratories, Hercules, Calif.). After the wells were washed three times with PBS containing 0.05% Brij 35, color was developed by using *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine–1 mM $MgCl_2$ (pH 10.0). The plates were read at 405 nm with a Bio-Rad microplate reader.

Immunofluorescence. Bacteria grown in THY were harvested in the late exponential growth phase and washed once with PBS. Cells were suspended in 2% formaldehyde in PBS, applied to a clean glass slide, and air dried. Each air-dried bacterial spot was washed with distilled water and incubated with 25 μl of M6 or fimbrillin-specific antibodies (1:32 dilution in PBS–0.05% Tween 20–0.5% BSA) at room temperature for 30 min in a humid chamber. After the spots were washed with PBS–0.05% Tween 20, they were flooded with 25 μl of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (1:32 dilution in PBS–0.05% Tween 20–0.5% BSA) and incubated as described above for 30 min. After the slides were washed twice with PBS–0.05% Tween 20 and then with distilled water, they were air dried. The spots were covered with a mounting solution, and the slides were observed with an immunofluorescence microscope.

Binding assay. Binding assays were performed by using the previously described procedure for *P. gingivalis* binding to saliva-coated hydroxyapatite (16). Briefly, 2.5 × 10^7 to 2 × 10^8 ^{3}H-labeled *S. gordonii* cells that had been washed and suspended in 400 μl of buffered KCl (50 mM KCl, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$; pH 6.0) were incubated with 2 mg of whole saliva-coated hydroxyapatite beads for 1 h at room temperature. Following incubation, the beads were washed three times with 1.0 ml of buffered KCl, and the bound radioactivity was quantified by liquid scintillation counting.

***P. gingivalis* binding inhibition assay.** The ability of fimbrillin-expressing recombinant *S. gordonii* to block *P. gingivalis* binding to sHAP beads was determined by an in vitro binding inhibition assay as described previously (16), with the following modifications. A suspension consisting of 150 μl of [^{3}H]thymidine-labeled *P. gingivalis* (1 × 10^8 cells) and 150 μl of *S. gordonii* (1 × 10^8 cells) was incubated with sHAP beads (2 mg) for 1 h at room temperature. The mixture was then layered on 100% Percoll to separate the unbound cells. After the sHAP beads were washed with buffered KCl, the radioactivity of the sHAP bead-bound cells was measured. Inhibition (mean ± standard deviation) of *P. gingivalis* binding was calculated from triplicate readings of each strain.

Immunization of rabbits. New Zealand white rabbits (3 kg) were immunized subcutaneously with 10^9 live streptococcal cells emulsified in a copolymer adjuvant (TiterMax; Cytrex Corp., Norcross, Ga.). The N- and C-terminal fimbrillin-expressing *S. gordonii* strains (designated Sg-FimN and Sg-FimC, respectively) were pooled and used to immunize two rabbits. Two weeks after the primary immunization, the rabbits were given subcutaneous booster injections of the same streptococcal emulsion. The rabbits were bled 4 weeks after the booster injections, and the sera were pooled. The antibodies in the sera were tested by immunoblotting against *P. gingivalis* fimbrillin polypeptides produced in *E. coli* (2, 28).

RESULTS

Construction and characterization of the *S. gordonii*-fimbrillin recombinants. The genetic system for expression of *P. gingivalis* fimbrillin on the surface of *S. gordonii* is based on the construction and expression of translational fusions with the M6 protein gene (*emm6.1*) developed by Pozzi et al. (26). The N-terminal (amino acid residues 55 to 145) and C-terminal (residues 233 to 322) fimbrillin-encoding DNA fragments obtained by PCR were digested with restriction endonucleases *Kpn*I and *Hin*dIII and were purified from the agarose gels. The resulting 282- and 279-bp DNA fragments were unidirectionally cloned into the *Kpn*I and *Eco*RI sites of integration plasmid pSMB55 to obtain the chimeric plasmids pSGFimN and pSGFimC, respectively. The correct in-frame fusion of the *fimA* gene fragments with the M6 gene was confirmed by DNA sequencing. The linearized plasmids were used to transform *S. gordonii* GP251, and transformants were plated onto THY agar containing erythromycin. Transformants that were resistant to erythromycin and sensitive to chloramphenicol (strategy summarized in Fig. 1) were analyzed to confirm *fimA* gene integration by Southern blotting and PCR analysis (data not shown).

Cell extracts of *S. gordonii*-FimA integrants were then analyzed by SDS-PAGE and immunoblotting. A major 45-kDa protein band reacted with the anti-fimbrillin antibody in both the N- and C-terminal fimbrillin-expressing *S. gordonii* cell extracts (Fig. 2). The same 45-kDa band also reacted with monoclonal antibody 3B8 (SIGA Pharmaceuticals, Inc., New York, N.Y.) against the M6 molecule. This band therefore corresponds to the translational fusion product of the M6 molecule (amino acid residues 1 to 122 and 302 to 441) with the 90-amino-acid N-terminal polypeptide of fimbrillin (residues 55 to 145) or with the 89-amino-acid C-terminal polypeptide of fimbrillin (residues 233 to 322). Minor bands at lower molecular weights also reacted with both anti-M6 and anti-fimbrillin antibodies. These minor bands probably represent the degradation products of the M6-fimbrillin fusion protein. An ~34-kDa band (M6 polypeptide) reacted in *S. gordonii* cells transformed with integration vector pSMB55. The ~34-kDa band therefore corresponds to the expressed M6-polypeptide alone. It should also be noted that as previously observed

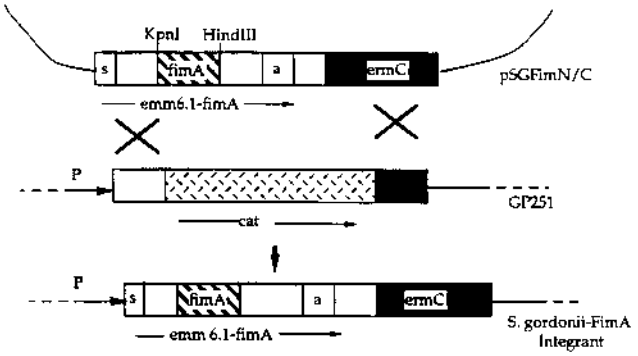

FIG. 1. Integration of the emm6.1-FimA translational fusion into the chromosome of *S. gordonii*. In recombinant plasmid pSGFimN/C, the *fimA* gene encoding N- or C-terminal portions of the fimbrillin is inserted in frame into the M6 protein gene (*emm6.1*). The regions of *emm6.1* encoding the signal peptide (s) and the C-terminal anchor (a) of the M6 protein are indicated. GP251 is a recipient *S. gordonii* strain containing the *cat* gene (broken cross-hatched lines) downstream from a strong promoter (P); the *cat* gene is flanked by two short fragments, a 145-bp *emm6.1* fragment (open box) and a 200-bp *ermC* gene fragment (solid box), for homologous recombination.

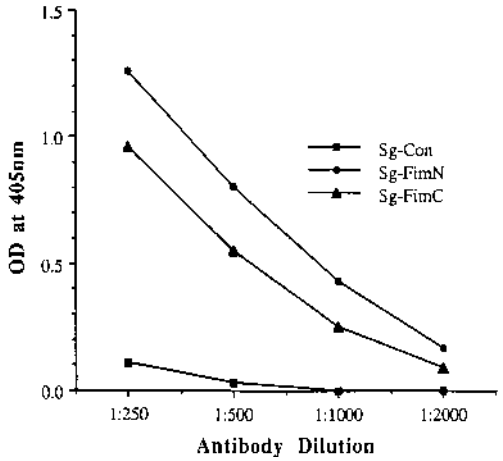

FIG. 3. Cell surface ELISA of expressed fimbrillin peptides. Microtiter plate wells coated with *S. gordonii* cells were incubated with serial dilutions of affinity-purified rabbit anti-fimbrillin antibody and then with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G. Color was developed with *p*-nitrophenyl phosphate and read at 405 nm. OD, optical density.

for M6-E7 fusion proteins produced in *S. gordonii* (26), the molecular sizes estimated for the M6-Fim fusion protein and a portion of the M6 protein are ~20% larger than the molecular sizes deduced from the DNA sequence (i.e., 45 kDa compared with 38 kDa for the M6-Fim fusion and 34.5 kDa compared with 29 kDa for the M6 portion alone), perhaps because of conformational features of the M protein and fimbrillin sequences. A representative N-terminal fimbrillin-expressing *S. gordonii* recombinant was designated Sg-FimN, while a C-terminal fimbrillin-expressing recombinant of *S. gordonii* was designated Sg-FimC. A non-fimbrillin-expressing recombinant obtained by transformation with integration vector pSMB55 alone was also obtained; this recombinant was designated Sg-Con and was used as a negative control.

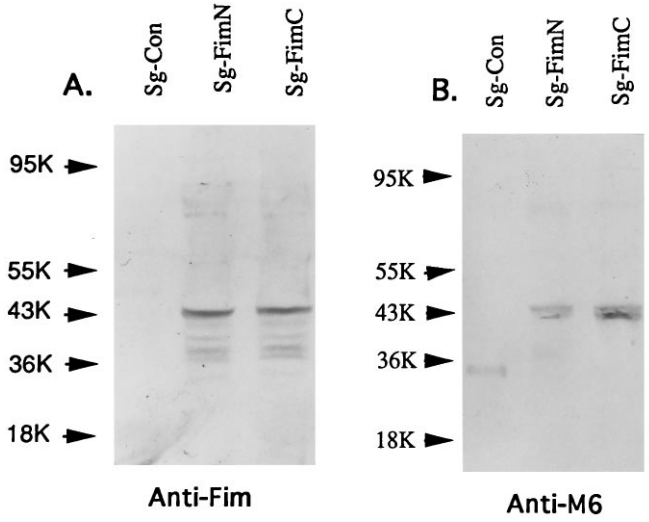

FIG. 2. Immunoblot analysis of cell extracts of recombinant *S. gordonii* strains. Cell extracts of control strain *S. gordonii* GP251 and fimbrillin-expressing derivatives Sg-FimN and Sg-FimC were separated on a 12% polyacrylamide SDS–PAGE gel and electroblotted onto a nitrocellulose membrane. The membrane was cut into two halves, and each half was probed with either affinity-purified anti-fimbrillin antibodies obtained from rabbits immunized with purified recombinant fimbrillin protein produced in *E. coli* (1:1,000 dilution) or anti-M6 monoclonal antibody (1:500 dilution). The arrows on the left indicate the positions of the molecular weight standards.

Surface expression. To verify that a fimbrillin peptide was expressed on the surfaces of Sg-FimN and Sg-FimC, a cell surface ELISA and an immunofluorescence analysis were carried out by utilizing affinity-purified anti-fimbrillin and anti-M6 monoclonal antibodies. The results of the ELISA showed that the anti-fimbrillin antibody reacted with cell surface-expressed fimbrillin at various antibody dilutions (Fig. 3). Sg-Con did not react with anti-fimbrillin antibody. All three genetically modified *S. gordonii* recombinants (Sg-Con, Sg-FimN, and Sg-FimC) reacted with anti-M6 monoclonal antibody (data not shown).

The ELISA results were confirmed by the fact that Sg-FimN and Sg-FimC exhibited positive fluorescence when they reacted with anti-fimbrillin antibody (Fig. 4), whereas no fluorescence was detected with Sg-Con. Anti-M6 antibody on the other hand detected positive fluorescence for each of the three recombinant strains (data not shown).

Binding assay. None of the recombinant *S. gordonii* strains (Sg-FimN, Sg-FimC, and Sg-Con) bound significantly to HAP beads saturated with buffered KCl (data not shown). On the other hand, all three recombinants bound to sHAP beads. The binding was concentration dependent and saturable at a concentration of 1×10^8 added cells. At the saturation point binding of Sg-FimN and Sg-FimC was significantly higher than binding of Sg-Con (Fig. 5). When Sg-FimN was compared with Sg-FimC, the former bound better than the later (about 50% more binding). The binding of Sg-Con to sHAP beads was comparable to the reported abilities of *S. gordonii* wild-type strains to bind to sHAP (9).

***P. gingivalis* binding inhibition assay.** The ability of Sg-FimN and Sg-FimC to inhibit the binding of *P. gingivalis* to sHAP beads was examined by performing a binding inhibition assay. The inhibitory activity of each recombinant against *P. gingivalis* binding to sHAP was tested in reaction mixtures containing 2 mg of sHAP beads incubated with 1×10^8 *P. gingivalis* cells and an equal number of *S. gordonii* cells. The numbers of *P. gingivalis* cells used in this experiment were based on the results of previous studies, whereas the *S. gordonii* cell concentrations were based on the results of the binding assay mentioned above. Almost complete saturation was reached with each of

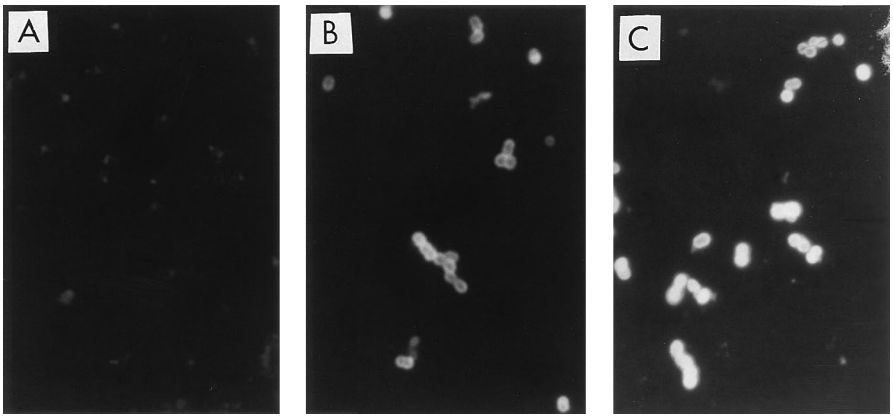

FIG. 4. Immunofluorescence of fimbrillin-expressing recombinants Sg-FimN and Sg-FimC. Cells were treated with affinity-purified rabbit anti-fimbrillin antibodies and with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G. Positive fluorescence was observed with both Sg-FimC (B) and Sg-FimN (C). Sg-Con was used as a negative control (A).

the *S. gordonii* strains at a level of 1×10^8 cells under similar conditions (2 mg of sHAP beads), and therefore this level was used for the inhibition studies (Fig. 6). *P. gingivalis* binding to sHAP beads was inhibited significantly more by Sg-FimN and Sg-FimC than by Sg-Con (75, 55, and 20%, respectively). These results indicated that the N-and C-terminal domains of *P. gingivalis* fimbrillin expressed on the *S. gordonii* surface were responsible for the enhanced inhibition activity of Sg-FimN and Sg-FimC, respectively.

Immunogenicity of the fimbrillin polypeptide expressed on the surface of *S. gordonii*. The immunogenicity of the M6-fimbrillin fusion protein expressed on the surface of *S. gordonii* was examined by subcutaneously immunizing rabbits with 10^8 live Sg-FimN and Sg-FimC cells emulsified in copolymer adjuvant (TiterMax; Cytrex Corp.). Immunoblotting was performed to detect the antibody response to *P. gingivalis* fimbrillin protein. The pooled sera of two rabbits immunized with recombinant fimbrillin-expressing *S. gordonii* strains reacted with the *P. gingivalis* fimbrillin expressed and purified from *E. coli* (Fig. 7). No antibodies to the fimbrillin were seen in the sera of rabbits immunized with Sg-Con. The immunization data indicated that the fimbrillin polypeptide domains are immunocompetent as they appear on the surface of *S. gordonii* and induce antibodies with specific reactivity to the *P. gingivalis* fimbrillin.

DISCUSSION

Model systems have been developed recently for expressing heterologous proteins in nonpathogenic oral streptococci, such as *S. gordonii* (13, 26, 29, 30). *Arthrobacter* dextranase (13) and a glucan-binding domain of *Streptococcus mutans* (29, 30) have been successfully expressed in biologically active forms in *S. gordonii*. Pozzi and coworkers have, on the other hand, expressed heterologous proteins on the surface of *S. gordonii*. The genetically engineered streptococci have been shown to elicit host immune responses to the expressed proteins following colonization of animals (19).

The present study was undertaken to prepare recombinant streptococci (*S. gordonii*) capable of surface expressing biologically active polypeptides of *P. gingivalis* fimbrillin by utilizing the strategy developed by Pozzi et al. (26). Briefly, the N-terminal portion of the M6 molecule (leader sequence through amino acid residue 122 to enable translocation), together with the fimbrillin polypeptide, is expressed on the cell surface, whereas the C-terminal region (the attachment motif) of the M6 molecule remains buried in the cell wall. To construct such M6-fimbrillin fusion molecules, PCR-amplified fragments encoding N-terminal (amino acid residues 55 to 145) and C-terminal (residues 233 to 322) regions of the fimbrillin gene were cloned in-frame with the M6 gene present in integration vector pSMB55. The recombinant integration vector was then used to transform *S. gordonii* GP251. The chromosomal integration events (summarized in Fig. 1) were identified following selection of transformants on THY agar plates containing 5 μg of erythromycin per ml. Transformants that were resistant to erythromycin but sensitive to chloramphenicol (Em^+ Cm^-) were tested for the correct *emm6.1-fimA* fusions by Southern blot and Western blot (immunoblot) analyses. Surface expression of fimbrillin polypeptides was confirmed by performing total cell surface ELISA and immunofluorescence analyses with affinity-purified anti-*P. gingivalis* fimbrillin antibody and anti-M6 monoclonal antibody. Both Sg-FimN and Sg-FimC reacted positively with anti-fimbrillin and anti-M6 antibodies in ELISA and immunofluorescence experiments. The semiquantitative results of the ELISA indicated that although the fimbrillin polypeptides were expressed on the surfaces of both

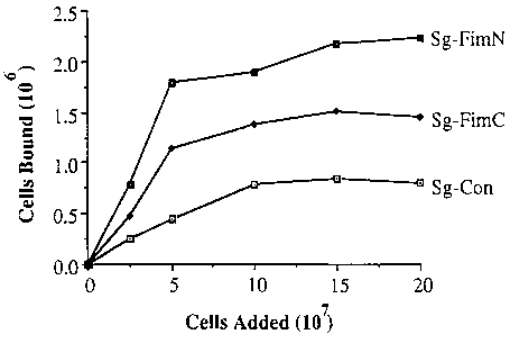

FIG. 5. Binding of ^{3}H-labeled Sg-Con (control), Sg-FimN, and Sg-FimC to saliva-coated HAP beads. A 2-mg portion of HAP beads equilibrated with buffered KCl or clarified saliva was added to a siliconized borosilicate tube and incubated with different numbers of labeled cells (2.5×10^7 to 2×10^8 cells) in a total volume of 400 μl with gentle agitation for 1 h at room temperature. After the preparation was washed to separate unbound cells from bead-bound cells, the radioactivity of the bead-bound cells was quantified. The results shown are the mean values of duplicate samples that were representative of several experiments.

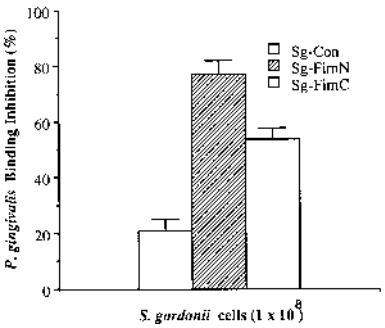

FIG. 6. Inhibitory effect of recombinant *S. gordonii* strains on the binding of *P. gingivalis* to sHAP beads. A 2-mg portion of sHAP beads was incubated with 1×10^8 *P. gingivalis* 2561 cells in the presence of 1×10^8 *S. gordonii* cells in a total volume of 300 μl for 1 h at room temperature. After incubation, the reaction mixture was layered onto 100% Percoll to separate unbound cells from the sHAP bead-bound cells. The sHAP beads were then washed with buffered KCl, and the radioactivity of the washed sHAP-bound cells was quantified. The results shown are means ± standard deviations and represent several experiments.

recombinants, the expression was greater in Sg-FimN than in Sg-FimC. During our studies we also found that full-length fimbrillin polypeptide (length, 337 amino acids) or its peptide domains containing free thiol groups were not expressed on the surface of *S. gordonii*. Neither the N-terminal domain nor the C-terminal domain contained any cysteine residues in our expression experiments. The primary and/or secondary structures of the fimbrillin domains (N terminal and C terminal) may be responsible for the differential expression on the surface of *S. gordonii.*

The biological activity of the surface-expressed fimbrillin polypeptides was examined by determining the ability of recombinant strains Sg-FimN and Sg-FimC to bind to sHAP beads. The results showed that the N- and C-terminal domains of *P. gingivalis* fimbrillin expressed on the surface of *S. gordonii* bind better to sHAP beads. The enhanced binding of Sg-FimN and Sg-FimC to sHAP compared with Sg-Con binding can be attributed to the specific interactions of N- or C-terminal fimbrillin domains with the salivary components statherin and proline-rich proteins (1). Binding of Sg-Con to sHAP can be attributed to the inherent ability of *S. gordonii* to bind to salivary proteins, such as proline-rich proteins (9), salivary mucins (20, 32), and α-amylases (27). Although it is thought that the binding between *P. gingivalis* fimbrillin and sHAP is primarily mediated by the C-terminal peptide domains (16, 17), an N-terminal peptide domain (residues 126 to 145) has also been shown recently to mediate binding of fimbrillin to sHAP (1) and fibronectin (31). The results of the present study revealed that the binding of Sg-FimN to sHAP was greater than the binding of Sg-FimC. This may be due to differences in the expression of fimbrillin N- and C-terminal domains on *S. gordonii*, as shown by the semi-quantitative cell surface ELISA. The results suggested that there is a lower level of expression of C-terminal fimbrillin peptides than of N-terminal fimbrillin peptides. In addition, the conformations of the N- and C-terminal peptides expressed on the surface of *S. gordonii* also might have contributed to the degree of binding to sHAP. However, we have no direct evidence to support this hypothesis.

To test the ability of Sg-FimN and Sg-FimC to inhibit *P. gingivalis* binding to sHAP beads, in vitro binding inhibition assays were performed. The results showed that fimbrillin-expressing strains Sg-FimN and Sg-FimC are more effective in blocking *P. gingivalis* binding to sHAP than the fimbrillin-negative control, strain Sg-Con, is. In addition, the immunological activities of Sg-FimN and Sg-FimC were determined in rabbits following immunization. The immunization data indicated that the fimbrillin polypeptide domains are accessible to the immune system for induction of specific antibodies able to recognize the *P. gingivalis* fimbrillin.

Our future studies will involve oral immunization of animals with Sg-FimN and Sg-FimC to determine if fimbrillin-specific mucosal and systemic antibody responses can be obtained. If these studies are successful, the recombinants will be utilized as a candidate fimbrial vaccine against *P. gingivalis* infection in a rat model (7). The recent studies of Medaglini and coworkers (19) demonstrated that colonization with recombinant oral streptococci expressing surface-oriented heterologous antigens is an effective way of inducing mucosal and systemic immune responses. There is also potential to utilize these strains to block the sites for fimbria-mediated colonization of *P. gingivalis* in the oral cavity, thereby replacing the pathogenic *P. gingivalis* cells with nonpathogenic streptococci (replacement therapy).

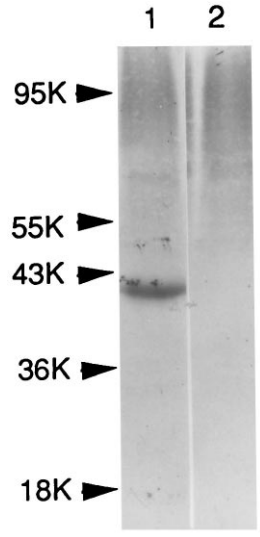

FIG. 7. Immunoblot analysis for the detection of fimbrillin-specific antibodies in the sera of rabbits immunized with recombinant *S. gordonii. P. gingivalis* fimbrillin protein (5 μg) was separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. Membrane strips were cut out, blocked with 2% nonfat milk, and incubated with rabbit antiserum (1:200 dilution) obtained following immunization with recombinant *S. gordonii* (lane 1) or rabbit antiserum (negative control; 1:200 dilution) from rabbits immunized with fimbrillin-negative control strain Sg-Con (lane 2). After washing, the membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G(H+L). The bound antibodies were visualized with the 3,3′,5,5′-tetramethylbenzidine membrane substrate system (Kirkegaard & Perry, Inc., Gaithersburg, Md.). The arrowheads on the left indicate the positions of the molecular weight standards.

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