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Secretion of *Porphyromonas gingivalis* Fimbrillin Polypeptides by Recombinant *Streptococcus gordonii*

Ashu Sharma,*,[1] Hakimuddin T. Sojar,* Dennis E. Hruby,†
Howard K. Kuramitsu,* and Robert J. Genco*,‡

**Department of Oral Biology, School of Dental Medicine, and ‡Department of Microbiology, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York 14214; and †Department of Microbiology, Oregon State University, Corvallis, OR 97331*

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The fimbriae of *Porphyromonas gingivalis* plays an important role in the pathogenesis of periodontal disease. A structural subunit of the *P. gingivalis* fimbriae, fimbrillin, has been shown to promote adherence of the bacteria to host surfaces and also induce an immune response. Biologically active domains of fimbrillin responsible for adherence or eliciting immune responses have been determined. In a previous study, we engineered the human oral commensal organism *Streptococcus gordonii* to express such biologically active domains on the surface of the bacteria as a vaccine delivery system. In this study we report an alternative approach of secreting fimbrillin polypeptide domains into the medium by modification of the surface-expression system described earlier. Such recombinant *S. gordonii,* in addition to being a source for antigen presentation to trigger a protective immune response, may have the added advantage of directly blocking the fimbriae-mediated adherence of *P. gingivalis* to the oral cavity following implantation. This approach can also be utilized for secreting other biologically important therapeutic molecules on mucosal surfaces for modulating local microenvironments. © 1997 Academic Press

Porphyromonas gingivalis, a black pigmented Gram-negative oral anaerobe, is one of the causative agents of adult periodontitis. Fimbriae play an important role in the pathogenesis of periodontal disease. Fimbrillin is a 43 kDa major subunit protein of fimbriae involved in the fimbriae-mediated adherence of bacteria to host cells and saliva-coated oral surfaces (1-3), and in triggering the tissue destructive host immune responses (4-7). Inactivation of the *fimA* gene encoding fimbrillin also results in a decreased ability of *P. gingivalis* to bind to salivary components and to both bind and invade epithelial cells (8). Moreover, in an animal model, an afimbriated mutant of *P. gingivalis* had a decreased ability to induce periodontal bone resorption compared to the parent strain (9). Strategies proposed for controlling periodontal disease have, therefore, included the development of fimbriae-based vaccines. Studies have shown that vaccination of animals with fimbrial protein, fimbrillin, or synthetic peptides of fimbrillin are able to protect against *P. gingivalis*-associated periodontal tissue destruction (10-12).

Model systems have been described in non-pathogenic oral streptococci for secretion or surface expression of heterologous proteins (13-17). Utilizing the strategy of Pozzi and co-workers (15) for surface expression, we recently generated recombinant strains of *Streptococcus gordonii* that express N- and C- terminal biologically active peptide domains of fimbrillin on the bacterial surface (18). The recombinant fimbrillin-expressing *S. gordonii* also elicited an anti-fimbrillin serum response in rabbits following immunization. Although such fimbrillin-expressing recombinant *S. gordonii* were developed as potential candidates for vectored vaccines against periodontal disease, the present study describes an *in vivo* approach for fimbrillin secretion into the growth medium via modification of the above mentioned surface expression. Such fimbrillin-secreting *S. gordonii,* in addition to being a delivery system for antigen presentation to trigger an antibody response, may have the added advantage of directly blocking fimbriae-mediated colonization of *P. gingivalis*.

MATERIALS AND METHODS

Plasmids, bacterial strains and growth conditions. Escherichia coli DH5α was grown in Luria-Bertani (LB) broth and served as a

[1] To whom correspondence should be addressed: Department of Oral Biology, 208 Foster Hall, 3435 Main Street, State University of New York at Buffalo, Buffalo, NY 14214-3092. Fax: (716) 829-3942. E-mail: sharmaa@acsu.buffalo.edu.

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host for plasmid cultivation. *S. gordonii* recombinants were grown in Todd-Hewitt broth containing 0.2% yeast extract (THY). Plasmids pSGFimN and pSGFimC have been described previously (18).

Recombinant DNA and genetic techniques. Recombinant DNA manipulations were carried out using standard molecular biology procedures (19). A duplex DNA (*Hind* IIISTOP cassette) was obtained by self-complementary annealing of a 5′-phosphorylated oligonucleotide (5′P-AGCTTGCTTAATTAATTAAGCA-3′OH) synthesized at the Center for Advanced Molecular Biology and Immunoloby (CAMBI) Nucleic Acid Facility at the State University of New York at Buffalo. Briefly, the single-stranded oligonucleotide suspended in 50mM Tris/5 mM $MgCl_2$, pH 7.6, was heated at 95°C for 2 min followed by gradual cooling to room temperature (20°C). The self-annealed duplex DNA thus formed contains the translational stop signal (TAA) in both orientations flanked by an *Hind* III compatible cohesive ends. Integration vectors pSGFimN and pSGFimC containing the translational fusions of N- and C- terminal portions of *P. gingivalis* fimbrillin and the M6 protein, respectively, were digested with *Hind* III, followed by calf intestinal alkaline phosphatase treatment. The 5′ phosphorylated duplex DNA (*Hind* III translational stop cassette) was then ligated into the above *Hind* III/CIAP treated vectors. The ligation reactions were transformed into *E. coli* DH5α and plasmid DNA was obtained by an alkaline lysis procedure and confirmed for insertion of the STOP cassette by restriction enzyme digestion and DNA sequencing. The resulting vectors, pSGFimN-STOP and pSGFimC-STOP), were used to transform *S. gordonii* GP251 as described previously (18). The transformants were selected by plating onto THY agar plates containing 5 μg of erythromycin per ml. Following anaerobic incubations for 48-72 h at 37°C, colonies were analyzed for secretion of M6-fimbrillin fusion products by immunoblot analysis.

Immunoblot analysis of the secretion products. Briefly, one milliliter overnight cultures of *S. gordonii* recombinants in THY were diluted 1:10 in fresh THY medium containing antibiotic. Each of these cultures was grown to an optical density at 600 nm of 0.8 (OD_{600}=0.8). Cells were pelleted by centrifugation and culture medium was filterd through a 0.45 micron filter (Amicon Corporation, Beverly, MA). Ammonium sulfate (80% final concentration) was then added to the medium and the mixture was stirred for 4 h at 4°C. Ammonium sulfate precipitated proteins were obtained by centrifugation at 12,000g for 15 minutes at 4°C. Pellets were dialyzed extensively against 5 mM Tris, pH 8.0, and protein content was estimated by the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by an immunoblot analysis utilizing anti-fimbrillin and anti-M6 antibodies (18).

ELISA estimation of secreted fimbrillin peptides. Inhibition-ELISA was utilized to quantitate fimbrillin secreted into the medium. Briefly, microtiter plate wells were coated with recombinant fimbrillin (rFim) by incubating with purified rFim resuspended in 0.1M $NaHCO_3$ buffer, pH 9.6, for 2 h at 37°C (25 ng/well in 100 μl volume). Unoccupied sites were then blocked with 1% BSA in 20 mM Tris-Cl, pH 7.5/0.15M NaCl (TBS-B; 100 μl/well for 30 min at 37°C). Wells were emptied by inverting the plates and then incubated for 1 h at 37°C with a series of increasing concentrations of purified recombinant fimbrillin (rFim; 2.5 ng to 100 ng) and 1:1000 dilution of affinity purified anti-fimbrillin antibody diluted in TBS-B (100 μl per well). Control wells were incubated with antibody alone without recombinant fimbrillin. Following incubation, wells were washed three times with 20 mM Tris-Cl, pH 7.5/0.15M NaCl containing 0.05% Tween-20 (TBS-T) and then incubaterd for 1 h at 37°C with alkaline phosphatase-labeled goat anti-rabbit antiboby (100 μl of 1:1,000 dilution in TBS-B per well; Bio-Rad Laboratories, Hercules, CA). After wells were washed three times with TBS-T buffer, color was developed using p-nitrophenyl phospahte (1 mg/ml) in 10% diethyl amine-1mM $MgCl_2$, pH 10.0). The plates were read at 405 nm with a Bio-Rad microplate reader. A standard inhibition curve was obtained by plotting percentage inhibition of antibody binding against log concentration of recombinant fimbrillin added per well. Percentage inhibition was calculated as:

$$\left(\frac{OD_{405}\text{ control well} - OD_{405}\text{ rFim well}}{OD_{405}\text{ control well}}\right) \times 100.$$

To estimate fimbrillin released into the medium, ammonium sulphate precipitated and TBS dilayzed samples were utilized in the above assay instead of rFim, and inhibition of antibody binding was calculated. The standard curve was then utilized to estimate the amount of fimbrillin in the *S. gordonii* culture medium.

RESULTS AND DISCUSSION

In our previous study, N-(residues 55-145) and C-(residues 233-322) terminal domains of fimbrillin were expressed on the surface of *S. gordonii* (SgFimN & SgFimC) by employing a strategy described by Pozzi and co-workers (ref). Briefly, *S. gordonii* were genetically engineered to express fimbrillin domains anchored to the cell surface via the C-terminal hydrophobic and charged 'membrane-anchor domain' of the M6 protein of *S. pyogenes.* This was achieved by constructing integration vectors, pSGfimN and pSGFimC that contain translational fusions of the fimbrillin N- or C-terminal domains to the C-terminal anchor domain of M6 protein. In the present study, we generated recombinant *S. gordonii* that secrete fimbrillin polypeptide domain into the medium. This was achieved by modifying the surface expression system by uncoupling the anchor domain of M6 protein from the N-terminally located fimbrillin domain. A translational stop signal was inserted at the *Hind* III site (3′-end of *fimA;* Fig. 1) of integration vectors pSGFimN and pSGFimC by cloning a *Hind* III-STOP cassette. The fusion protein utilizes the same protein expression machinery (promoter and N-terminal M6 signal peptide portion) as the surface expression system, except that it does not express the membrane anchor domain (Fig. 1). The resulting integration vectors were transformed onto *S. gordonii* GP251 to obtain chromosomal integrants (SgFimN-Sec & SgFimC-Sec). Transformants were analyzed by immunoblotting to detect secreted fusion proteins in the medium (Fig. 2). As expected, a 28 kDa protein band reacted with the anti-fimbrillin antibody. The same 28 kDa band also reacted with monoclonal antibody 3B8 (SIGA Pharmaceuticals, New York, NY) against the M6 protein. This band corresponds to the translational fusion product of the M6 molecule (amino acid residues 1-122), with the 90 amino acid N-terminal polypeptide of fimbrillin (SgFimN-Sec; residues 55-145), or with the C-terminal polypeptide domain of fimbrillin (SgFimC-Sec; residues 233-322). The 28 kDa fusion protein expressed in the medium of Sg-FimN-Sec also reacted with antibody against the N-terminal peptide (residues 69-90 of fimbrillin protein; data not shown) of *P. gingivalis* fimbrillin. Minor bands at lower

molecular weights reacting with both anti-fimbrillin and anti-M6 antibodies likely represent degradation products. A 15 kDa (M6 polypeptide) reacted with monoclonal 3B8 and did not react with anti-fimbrillin antibody in the culture medium of SgCon-Sec (fimbrillin negative control; residues 1-122 of M6). As noted earlier (18), the estimated sizes for the M6 protein or M6-Fim fusion proteins are ~20% larger than the molecular sizes deduced from the DNA sequence due to the anomalous protein migartion on SDS-PAGE. Interestingly, the semi-quantitative immunoblotting results indicated that the yield of secreted N-terminal polypeptide of fimbrillin was lower than the C-terminal polypeptide domain. These results were confirmed by estimating the amount of fimbrillin in the medium by inhibition-ELISA as described in the Materials and Methods section. SgFimN-Sec secreted 28.4±2.8 ng, whereas SgFimC-Sec secreted 112±4.8 ng of fimbrillin polypeptide per milliliter culture medium based on the 41 kDa fimbrillin as the standard at mid-late log phase (OD_{600} of 0.8) conditions. It is interesting to mention

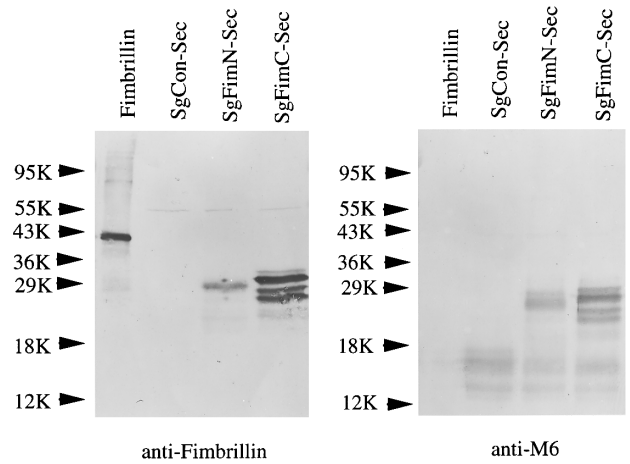

FIG. 2. Immunoblot analysis of the culture medium of recombinant *S. gordonii*. Ammonium sulfate precipitated culture medium was separated on a 15% polyacrylamide SDS-PAGE gel and electroblotted onto nitrocellulose membrane. The membrane was cut into two halves and each half was probed with either affinity purified anti-fimbrillin antibodies or anti-M6 monoclonal antibody. The arrows on the left indicate the position of molecular weight standards.

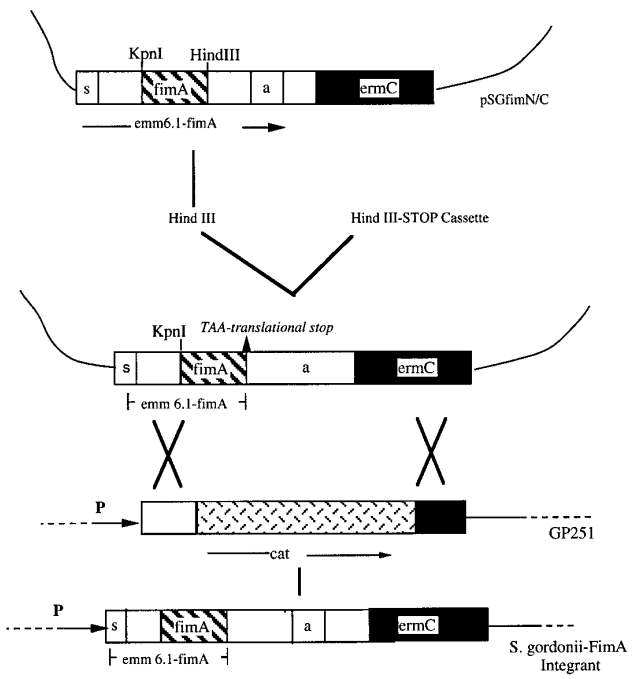

FIG. 1. Strategy utilized in the construction of recombinant *S. gordonii* (SgFimN-sec & SgFimC-sec). In recombinant plasmid PSGFimN/C, the *fimA* gene encoding N- or C-terminal portions of fimbrillin was inserted in frame into the M6 protein gene (*emm* 6.1) such that the translational fusion contains the signal peptide (s) and the C-terminal anchor domain (a) of the M6 protein. *Hind* III-Stop cassette was inserted into the *Hind* III site of pSGFimN/C to delink the anchor domain. Recombinant plasmids were then utilized to transform recipient host, *S. gordoniii* 25i that contains the cat gene downstream from the strong promoter (P); the *cat* (chloramphenicol) gene is flanked by two short fragments, a 145-bp *emm* 6.1 (open box) and a 200-bp *erm* C (erythromycin)gene (solid box), for homologous recombination.

that in contrast to the secretion system described here, the N-terminal fimbrillin domain was found to be better expressed on the surface of *S. gordonii* than the C-terminal domain (18). At present, we do not have an explanation for differences in the ability of *S. gordonii* to secrete N-terminal versus C-terminal portions of fimbrillin. Nevertheless, it is prudent to express a biological domain utilizing both surface expression and a secretion system to select the most efficient *in vivo* delivery system (surface vs secretion system).

Such genetically engineered *S. gordonii* secreting fimbrillin polypeptide may have some advantages for vaccine development. In addition to being a continuous source for antigen presentation to trigger an antibody response, such genetically engineered organisms could also serve as a constant *in vivo* source of fimbrillin peptides in the oral cavity to block fimbriae-mediated *P. gingivalis* colonization. Future studies will focus on this possibility by implanting these organisms into the oral cavity of gnotobiotic rats and testing whether or not they afford protection against subsequent infection by *P. gingivalis*. In conclusion, this study reports the secretion of fimbrillin peptides by modification of the surface expression system. In addition, other biologically important or therapeutic molecules can be engineered to be secreted on mucosal surfaces to modulate the local microenvironment.

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