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# Expression of Saliva-Binding Epitopes of the *Porphyromonas gingivalis* FimA Protein on the Surface of *Streptococcus gordonii*

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***Porphyromonas gingivalis*, a gram-negative oral anaerobic bacterium, has been implicated in the onset and development of periodontitis. The *P. gingivalis* fimbriae which mediate bacterial adherence to host oral sites and induce host inflammatory responses have been suggested as a potential antigen candidate. for vaccine development. This study was undertaken to generate *Streptococcus gordonii* vectors expressing the major subunit protein (FimA) of *P. gingivalis* fimbriae for testing as a potential live vaccine against periodontitis. We report here the expression of the C-terminal saliva-binding epitopes of *P. gingivalis* FimA on the surface of *S. gordonii* and demonstrate that domains containing free cysteine residues are poorly expressed on the surface of *S. gordonii*.** 

The nonpathogenic oral commensal, *S. gordonii*, has been genetically modified to express heterologous antigens for vaccine development (1, 2). One of the primary aims of our laboratory is to develop a vaccine against *P. gingivalis* associated periodontal disease (periodontitis). A major subunit protein of *P. gingivalis* fimbriae (FimA, fimbrillin) mediates bacterial adherence to the host oral sites via binding to saliva coated oral surfaces (3, 4), facilitates attachment to epithelial cells (5), and directs interaction with other resident bacteria (6) and induces tissue destructive host immune responses (7–10). Immunization with purified *P. gingivalis* fimbriae, fimbrillin or a synthetic peptide corresponding to a subdomain of the FimA protein can induce protection against *P. gingivalis* associated alveolar bone loss in rats (11–13). The peptide domains of FimA involved in bacterial adherence and induction of host immune response have been mapped (14). Since the majority of the FimA domains involved in bacterial adherence to saliva coated oral surfaces and in the induction of protective host immune responses are localized in the C-terminal region from residues 226 to 337 of FimA, this region is an attractive antigen for use as a vaccine (15, 16). Recently, we reported the expression of portions of the N- (residue 55-145) and C- (residues 233-322) terminal regions of *P. gingivalis* FimA on the surface of *S. gordonii* using a strategy developed by Pozzi et al. where the FimA protein was targeted to the *S. gordonii* cell surface as a fusion protein with the C-terminal 'anchor' domain of the M6 molecule. In order for this strategy to be successful in developing a vaccine against *P. gingivalis*, optimal expression of desired FimA epitopes is required on the surface of *S. gordonii*.

In this sudy, we report the expression of the saliva-binding (PRP-1 and Statherin) domains and immunologicaly protective epitope (226–246) of FimA localized in the C-terminal region. We further show that the presence of cysteine residues in the C-terminal portion of FimA retards its surface-expression in *S. gordonii*.

## MATERIALS AND METHODS

*Bacterial strains, plasmids, media and growth conditions.* *Escherichia coli* strain DH5$\alpha$ (Gibco-BRL, Gaithersburg, MD) was grown in Luria-Bertani broth or agar (1.5%). *S. gordonii* GP251 (17) was grown in Todd-Hewitt broth containing 0.2% yeast extract (THY broth) with or without 1.5% agar. For radiolabeling, the cells were grown in THY medium containing 5 $\mu$Ci of [$^3$H]-thymidine (Dupont NEN Research Products, Boston, MA) per ml at 37°C anaerobically (85% $N_2$, 10% $H_2$, 5% $CO_2$). Where appropriate, antibiotics chloramphenicol (Cm) or erythromycin (Em) were added at a concentration of 5 $\mu$g/ml each. Plasmid pUC13Bg12.1 (18) was used as template for

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PCR amplification of the *P. gingivalis fimA* gene. Insertion vector pSMB55 (17), a 5.73Kb *E. coli* plasmid that does not replicate in *S. gordonii* was maintained in *E. coli* strain DH5$\alpha$.

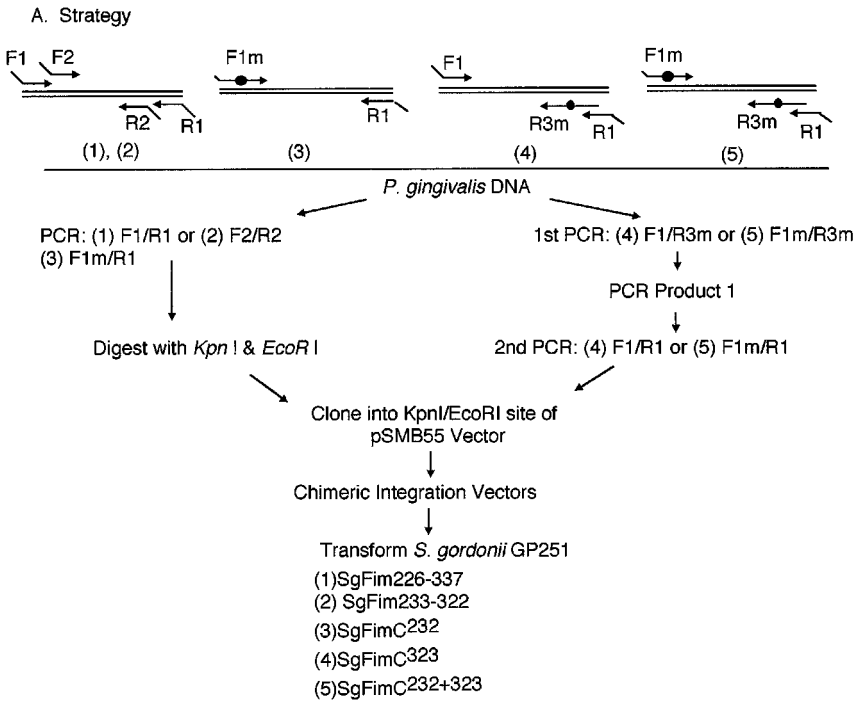
*Construction of recombinant S. gordonii.* DNA fragments encoding C-terminal portions (residues 226 to 337) of the *P. gingivalis* fimbrillin gene (*fimA*) were amplified by PCR utilizing pUC13Bg12.1 as a template (18). Forward primers included the four-base 'clamp' sequence and a *Kpn* I restriction enzyme site at the 5′-end whereas the reverse primers included a four base 'clamp' and a restriction site for *EcoR* I to facilitate digestion and cloning into the integration vector. Insertion vector pSMB55 (1) was used to obtain translational gene fusions of the *fimA* gene with the M6 gene (*emm*6.1). Sequence of the primers used for amplifying wild type FimA encoding DNA or its mutants is shown in Fig. 1. The *fimA* gene was inserted into the *Kpn*I-*EcoR* I site of *emm*6.1 resident on pSMB55 and the resulting chimeric plasmid was used to transform *S. gordonii* GP251 as described previously (19). The transformants were selected by plating on THY-agar plates containing 5 $\mu$g/ml erythromycin. Following anaerobic incubation at 37°C for 48-72 hours, colonies were analyzed for M6-FimA fusion protein expression by immunoblotting. Briefly, cultures (10 ml) of *S. gordonii* transformants were grown in THY broth to an optical density at 600 nm of 1.0. Cells were harvested by centrifugation and 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 $\mu$g/ml lysozyme, 1 mM PMSF) and kept on ice for 1 h. The protoplasts were centrifuged for 3 min at 16,000 x *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 discarded by low speed centrifugation (1,000 x g) for 15 min, whereas the supernatant containing membranes and cytoplasm were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis (19).

*Cell-surface ELISA.* Cell-surface expression of fimbrilin was quantified by a previously described whole cell ELISA technique (19). Briefly, *S. gordonii* cells were grown as above in 15 ml THY broth culture to an absorbance of 1.0 at 600 nm. Cells were harvested by centrifugation at 2,000 x g at 4°C and washed once with 25 ml of cold phophate-buffered saline containing 0.02% sodium azide (PBS-azide). The cells were resuspended in cold PBS-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 $\mu$l of diluted cells were added to each ELISA plate well (Dynatech Immulon II plate, Dynatech Laboratories). Plates were placed at room temperature for 2h to allow coating of wells with *S. gordonii* cells and stored overnight at 4°C. The wells were emptied by inverting the plates and then washed three times with phosphate-buffered saline containing 0.05% Brij 35 (PBS-Brij). The unoccupied sites were blocked by 2% bovine serum albubin in PBS (100 $\mu$l/well, PBS-BSA) for 1 h at 37°C. After blocking, the wells were emptied and incubated for 1h at room temperature with serially diluted primary antibody in PBS-BSA (50 $\mu$l/well). Following incubation, the wells were washed three times with PBS-Brij and were incubated for 2 h at room temperature with alkaline phosphatase labeled goat anti-rabbit antibody (50 $\mu$l/well of 1:1000 dilution in PBS-BSA; Bio-Rad Laboratories, Hercules, CA). After washing the wells three times with PBS-Brij, color was developed using p-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine/1 mM $MgCl_2$, pH 10.0. The plates were read at 405 nm using a Bio-Rad microplate reader.

*Adherence assay.* Adherence assays to determine the relative binding abilities of recombinant *S. gordonii* to saliva were carried out by a modification of a previuosly described assay (20). Microtiter plate wells were coated with human saliva (3) diluted 1:10 in 0.1 M $NaHCO_3$, pH 9.6 bufffer, for 2 h at 37°C (100 $\mu$l/well). Unoccupied sites were blocked with 0.05% Tween-20 in PBS (PBST) and wells were then incubated for 1 h at room temperature with 1 x $10^5$ to 1 x $10^7$ $^3$H-labeled *S. gordonii* cells washed and suspended in 50 $\mu$l PBS/0.02% sodium azide. Following incubation, wells were washed with PBST and bound-cells were dissociated by incubating each well with 100 $\mu$l of 0.5 N NaOH/1% sodium dodecyl sulfate solution. Prior to scintillation counting to measure bound radioactivity. samples were neutralized by adding 100 $\mu$l of 0.5 N HCl. The percentage of cell binding of each of the recombinant at sub-saturating concentration was calculated and Tukey–Kramer multiple comparison tests of significance were performed to determine statistical differences in mean binding.

## RESULTS AND DISCUSSION

The C-terminal region of the *P. gingivalis* FimA protein (residues 226-337) contains domains responsible for bacterial adherenece to saliva-coated oral surfaces and the immunologically active peptide domain (peptide 226-246) that confers protection against *P. gingivalis* associated alveolar bone loss in rats. We reported earlier (19) that full length FimA protein or its polypeptide domains containing free cysteine residues express poorly on the surface of *S. gordonii.* The aim of the present study was therefore to construct recombinant *S. gordonii* expressing the C-terminal region of FimA and to study the contribution of free sulfhydryls on surface expression. The C-terminal region contains cysteines at positions 232 and 333 respectively. To analyze the effect of free sulfhydryls on the surface expression of FimA protein via the gram positive C-terminal anchor domain on *S. gordonii,* wild type and mutant C-terminal (226–337) polypeptdes of *P. gingivalis* fimbrillin with either one (residue 232 or 323) or both (232 and 323) of the cysteines changed to serines were constructed by a PCR-based strategy depicted in Fig. 1. The *fimA* gene fragments were inserted in frame with the M6 gene residing in pSMB55 to obtain translational fusions of the FimA and M6 proteins. The correct in-frame fusion and the nucleotide changes in the cysteine to serine encoding *fimA* gene fragments were confirmed by DNA sequencing. The recombinant plasmids were transformed into recipient *S. gordonii* strain (GP251) and the transformants selected on erythromycin selective plates were characterized by immunoblot analysis of the total cell extracts and cell-surface expression of M6-FimA polypeptides was quantified by cell-surface ELISA using anti-fimbrillin and anti-M6 monoclonal antibodies. The binding ability of the recombinants was determined by an *in vitro* binding assay. Immunoblot analysis of cell extracts with anti-FimA (Fig. 2) and anti-M6 (data not shown) antibodies confirmed that M6-FimA fusion proteins of expected size (45-kDa band) were expressed in total cell extracts of the *S. gordonii*-FimA recombinants. Minor bands reacting with the anti-fimbrillin antibody likely represented the products of the post translational processing and/or the degradation products of the M6-FimA fusion protein. Recombinants SgFim226-337 and




B. Primer sequences. Numbers in parenthesis signify nucleotide positions in *fimA* gene. Nucleotide changes in mutatnt primers are shown in bold. Restriction enzyme sites introduced in primer sequences to facilitate cloning are underlined.

F1: GCGGGGTACC(941)ATTCATCCGACAATCCTGTGT(963)
F1m: GCGGGGTACC(941)ATTCATCCGACAATCCTG**A**GTGTTTATGGCAAACTTCAG(979)
R1: GCGCGAATTC(1276)CCAAGTAGCATTCTGACCAACGAGAACCCACTCAGCTACAG**T**(1235)
F2: CGGGGTACC(962)GTTTATGGCAAACTTCAGAAAAACGG((987)
R2: GCGCGAATTC(1231)CTGTACATTCAAGTGAGCAG(1220)
R3m: (1255)GAGAACCCACTCAGCTACAGTGC**T**CTGTACATTCAAGTGAGCAGA(1211)

**FIG. 1.** (A) Strategy utilized in the construction of recombinant *S. gordonii*. (B) Primer sequences.

SgFim233-322 expressed amino acid residues 226 to 337 and 233 to 322 of *P. gingivalis* 381 FimA whereas recombinants SgFimC$^{232}$, SgFimC$^{323}$, and SgFimC$^{232+323}$ have cysteines at positions 232, 323 or positions 232 and 323 changed to serine residues respectively. The results of ELISA with either anti-Fim or anti-M6 showed that the M6-FimA fusion polypeptides expression on the surface of *S. gordonii* decreased significantly when sulfhydryl groups were present (Fig. 3). The yields of M6-FimA fusion proteins on the surface of the recombinants were calculated relative to expression in SgFimC$^{233\text{-}322}$, a recombinant expressing FimA polypeptide devoid of any free sulfhydryls. The results showed that expression of M6-FimA polypeptides on SgFimC$^{232}$ or SgFimC$^{323}$ reduced to 69 and 75% respectively whereas it were reduced by 50% in SgFim226-337 (Fig. 3). On the other hand, when both sulfhydryls were changed to serines (SgFimC$^{232+323}$) there was no significant difference on the surface expression relative to SgFim233-323. The binding abilities of the recombinant *S. gordonii* to saliva was estimated by the *in vitro* assay. As shown in Table 1, saliva binding potentials of SgFimC$^{232+323}$, SgFim226-337 and SgFim233-323 were significantly better compared to poorly expressing recombinants of FimA protein (SgFimC$^{232}$ and SgFimC$^{323}$) or to a non-FimA expressing negative control (GP251). The saliva-binding was

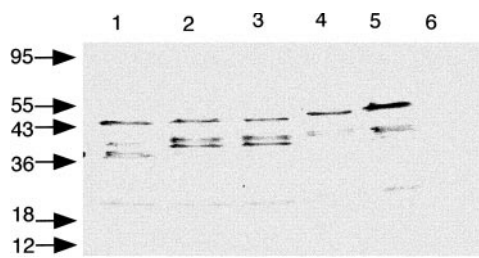



**FIG. 2.** Immunoblot analyis of the cell extracts of recombinant *S. gordonii*. SDS-PAGE separated fractions were electroblotted onto a nitrocellulose membrane and probed with affinity purified anti-fimbrillin antibody. The arrows on the left indicate the positions of molecular weight standards in kilodaltons. Lanes: 1, SgFimC233-322; 2, SgFim226-337; 3, SgFimC$^{232+322}$; 4, SgFimC$^{232}$; 5, SgFimC$^{323}$; 6, GP251 (parent strain).

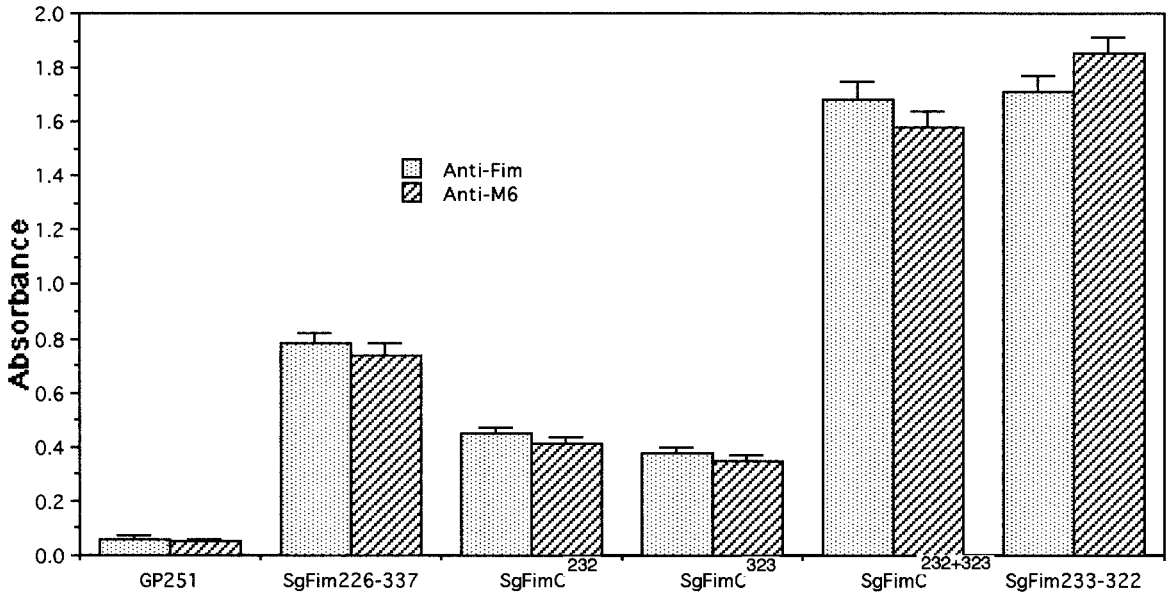



**FIG. 3.** Cell surface ELISA. Cells absorbed on microtiter plate wells were incubated with either affinity purified anti-fimbrillin or monoclonal anti-M6 antibody followed by peroxidase coupled secondary goat anti-rabbit IgG or goat anti-mouse IgG antibody respectively. Absorbance at 490 nm was measured following color development. Experiments were repeated at least three times with simialr results. Each assay was performed in triplicate and the data were expressed as mean ± s.d.

dose dependent and saturable. The *S. gordonii* recombinants did not bind to non-saliva coated plastic wells blocked with TBST alone (data not shown). The saliva binding activity of the steptococcal recombinants was dependent on the yield and the total number of PRP-1 and statherin domains expressed on FimA polypeptides. Additionally, SgFimC$^{232+323}$ bound significantly better to saliva compared to SgFim233-322 or SgFim226-337 ($p \leq 0.001$). The above results showed that the presence of cysteines inhibited the surface expression and the presence of a single sulfhydryl group in the polypeptide chain had more significant effects on expression. We speculate that free sulfhydryls (as in SgFimC$^{232}$ or SgFimC$^{323}$) could form interchain disulfide bonds with components of the translocation machinery involved in cell surface trafficking thereby inhibiting surface localization. On the other hand intrachain disulfide bonds are likely formed within the FimA polypeptde in SgFim226-337. Free sulfhydrayls are therefore unavailable for reacting with components of the translocation machinery. We are not aware of any study that addresses the effects of sulfhydryl groups on the surface localization of proteins in gram postive bacteria that traget via the C-terminal anchor domain. Interestingly, the majority of the gram positive surface-expressed proteins targeted via the C-terminal anchor machinery deposited in the GenBank Data base were found to be devoid of cysteine residues. The detrimental role that sulfhydryl groups may play on surface expression of M6-FimA protein is important, since the development of an *S. gordonii:*FimA vaccine depends on optimal cell surface expression of the FimA polypeptide.

In conclusion, this study demonstrates that polypeptides of FimA that contain free sulfhydryl groups are poorely expressed on the surface of *S. gordonii* when targeted by a C-terminal gram postive anchor domain. Additionally, the recombinant SgFimC$^{232+323}$ with en-

**TABLE 1**

Adherence of *S. gordonii* Recombinant Cells to Saliva-Coated Microtiter Plate Wells

| Strain | Adherence (%) (mean ± s.d.) |
|---|---|
| SgFim233-322 | 3.45 ± 0.16[a] |
| SgFim226-337 | 3.28 ± 0.31[b] |
| SgFimC$^{232+322}$ | 6.33 ± 0.99[c] |
| SgFimC$^{232}$ | 1.19 ± 0.21[ns] |
| SgFimC$^{323}$ | 0.82 ± 0.18[ns] |
| GP251, Parent Strain | 1.12 ± 0.13 |

*Note.* Adherence is expressed as percentage of $^{3}$H-labeled cells at subsaturating concentrations ($10^{7}$ cells/well) that bound to saliva-coated wells. Each assay was done in triplicate and the data were expressed as the mean ± standard deviation.

[a] P value in comparison with GP251, SgFimC$^{232}$ & SgFimC$^{323}$, $p < 0.01$ (significant).

[b] P value in comparison with GP251, SgFimC$^{232}$ & SgFimC$^{323}$, $p < 0.01$ (significant); P value in comparison with SgFim233-322, $p > 0.05$ (not significant).

[c] P value in comparison with GP251, SgFimC$^{232}$ & SgFimC$^{323}$, SgFim233-322 & SgFim226-337, $p < 0.001$ (highly significant).

[ns] P value not significant compared with GP251.

hanced surface expression and saliva-binding capabilities is a likely vaccine candidate. The studies are in progress to test the efficacy of $SgFimC^{232+323}$ to protect against *P. gingivalis* associated alveolar bone loss in rats.

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