Short Fimbriae of Porphyromonas gingivalis and Their Role in Coadhesion with Streptococcus gordonii

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Porphyromonas gingivalis, one of the causative agents of adult periodontitis, attaches and forms biofilms on substrata of Streptococcus gordonii. Coadhesion and biofilm development between these organisms requires the interaction of the short fimbriae of P. gingivalis with the SspB streptococcal surface polypeptide. In this study we investigated the structure and binding activities of the short fimbriae of P. gingivalis. Electron microscopy showed that isolated short fimbriae have an average length of 103 nm and exhibit a helical structure with a pitch of ca. 27 nm. Mfa1, the major protein subunit of the short fimbriae, bound to SspB protein, and this reaction was inhibited by purified recombinant Mfa1 and monospecific anti-Mfa1 serum in a dose-dependent manner. Complementation of a polar Mfa1 mutant with the mfa1 gene restored the coadhesion phenotype of P. gingivalis. Hence, the Mfa1 structural fimbrial subunit does not require accessory proteins for binding to SspB. Furthermore, the interaction of Mfa1 with SspB is necessary for optimal coadhesion between P. gingivalis and S. gordonii.

Porphyromonas gingivalis, a gram-negative anaerobe, is recognized as one of the primary pathogens in severe manifestations of adult periodontitis (33). P. gingivalis colonizes the dental plaque biofilm that accumulates on the supragingival and subgingival tooth surfaces (37, 43). Dental plaque is complex and dynamic, and the initial colonizers comprise predominantly gram-positive commensals such as Streptococcus gordonii and related streptococci, as well as Actinomyces species (14, 21, 30). Later biofilm inhabitants such as P. gingivalis are capable of binding to the antecedent organisms, and these attachment mechanisms are thought to drive the temporal and spatial development of pathogenic plaque (14, 17, 30, 32, 35). In addition, the coadhesion of P. gingivalis with primary colonizing bacteria such as streptococci may be important in the invasion of dentinal tubules by P. gingivalis (19).

P. gingivalis adherence to S. gordonii is multimodal and involves at least two distinct sets of adhesins and receptors. The long (major) fimbriae of P. gingivalis are predominantly comprised of the FimA protein and interact with glyceralddehyde 3-phosphate dehydrogenase on the streptococcal surface (20). Subsequent accretion of P. gingivalis into a mixed species biofilm requires an additional interaction between the short (minor) fimbriae and the Ssp major surface proteins on the streptococcal surface (16). The short fimbriae of P. gingivalis have been described independently by two groups (8, 27) as 0.1 to 0.5 µm in length and antigenically and genetically distinct from the long fimbriae (FimA) that can extend up to 3 µm (8, 27, 47). The short fimbriae are comprised predominantly of the Mfa1 protein. The possible contribution of minor fimbrial components to structure and function has not been investigated, although the mfa1 gene was shown to be cotranscribed with the downstream gene PG0179 (4).

The Ssp proteins are members of the antigen I/II family of streptococcal surface proteins that are highly conserved across all the human oral streptococcal species (2, 13). However, despite the high degree of structural similarity, P. gingivalis can discriminate between antigen I/II proteins from different species. In particular, P. gingivalis adheres to SspA and SspB proteins of S. gordonii but not to the antigen I/II homologue of Streptococcus mutans, SpaP. This species-specific interaction is determined by a discrete domain designated BAR (SspB BAR domain).

In this study, we have examined the morphology of the short fimbriae and defined the role of the Mfa1 protein as a receptor for SspB BAR. Purified recombinant Mfa1 (rMfa) and antibodies to this protein competitively inhibited P. gingivalis-S. gordonii binding. Furthermore, rMfa bound in a dose-dependent manner to the SspB BAR peptide. Complementation of a P. gingivalis Mfa1-deficient mutant with full-length mfa1 in trans restored binding activity of the P. gingivalis strain for S. gordonii. Thus, the Mfa1 major structural subunit protein of the short fimbriae of P. gingivalis is responsible for binding to the S. gordonii SspB BAR domain.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids are listed in Table 1. *P. gingivalis* 33277 and derivatives were grown anaerobically at 37°C in Trypticase soy broth supplemented, per liter, with 1 g of yeast extract, 5 mg of hemin, and 1 mg of menadione. When necessary, gentamicin, erythromycin, or tetracycline was added to the medium at a final concentration of 200, 10, or 5 μg/ml, respectively. Solid medium was prepared by supplementation with 5% sheep blood and 1.5% agar.

Recombinant proteins and antisera. Recombinant Mfa1 protein was produced by PCR amplification of the mfa1 coding sequences on the *P. gingivalis* 33277 chromosomal DNA using primers designed from the TIGR genome sequence. The amplification product was cloned into the pET-30 expression vector (Novagen) with the resulting plasmid encoding the full-length Mfa1 protein. After conjugation, erythromycin- and tetracycline-resistant transconjugants were selected, and plasmid identity was confirmed by restriction analysis.

Immunoblotting of *P. gingivalis*. Expression of Mfa1 was investigated by immunoblotting. Lysates of 5 × 10^9 *P. gingivalis* cells were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Antisera to rMfa or to *P. gingivalis* 33277 whole cells (45) (1:10,000) were used as probes with peroxidase-conjugated secondary antibody (1:3,000). Antigen-antibody binding was developed with 0.05% diaminobenzidine tetrahydrochloride.

ELISA. The level of cell surface Mfa1 protein was determined by enzyme-linked immunosorbent assay (ELISA) after adsorption of *P. gingivalis* strains onto Maxisorp plates (Nunc). Briefly, *P. gingivalis* cells were harvested, washed with PBS, and fixed with 0.5% formalin in PBS at 4°C overnight. After three washes with PBS, 10^6 cells were added to each well for 2 h at 4°C. After being washed to remove unbound bacteria, *P. gingivalis* cells were reacted with rMfa antibodies (1:10,000) followed by peroxidase-conjugated secondary antibody (1:3,000), each for 1 h at 37°C. Antibody-antibody binding was determined by a colorimetric reaction using the 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma Aldrich). Controls included omission of rMfa to fetal bovine serum was subtracted from values obtained with BAR.

Protein binding assay. *P. gingivalis* whole cells were used as control to compare the cell numbers fixed in each well.

Interbacterial binding assay. Adherence of *P. gingivalis* to *S. gordonii* was determined with a nontoxic bilayer assay as described previously (5, 18). Briefly, *S. gordonii* cells were suspended in PBS, and 10^9 bacteria were deposited on nitrocellulose paper in a dot blot apparatus. After adsorption of the streptococcal layers, the plate was washed three times with PBS, blocked for 2 h at room temperature with 1% bovine serum albumin, and incubated for 1 h at room temperature with [3H]thyminidine-labeled *P. gingivalis* (5 × 10^-4 mean cpm/cell) suspended in PBS-T. After being washed with PBS-T to remove unbound organisms, the experimental areas of the filter were excised, and bound *P. gingivalis* was quantitated by scintillation spectroscopy. For antibody inhibition experiments, radiolabeled *P. gingivalis* cells were incubated with antisera to *P. gingivalis* 33277 whole cells was used as control to compare the cell numbers fixed in each well.

### TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><em>S. gordonii</em> DL-1</td>
<td></td>
<td>Laboratory stock; 28</td>
</tr>
<tr>
<td><em>P. gingivalis</em> 33277</td>
<td>Type strain from ATCC</td>
<td></td>
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<tr>
<td>SMF1</td>
<td>Derivative of 33277 with an insertional inactivation of the mfa1 gene; Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>16</td>
</tr>
<tr>
<td>cSMF1</td>
<td>Derivative of SMF1 containing pT-MFA, complemented strain; Em&lt;sup&gt;r&lt;/sup&gt;</td>
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</tr>
<tr>
<td>KDP98</td>
<td>Derivative of 33277 with an insertional inactivation of the fimA gene; Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>42</td>
</tr>
<tr>
<td>E. coli</td>
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<td>DH5α</td>
<td>F 8089lacZΔ(lacZYA-argF)U169 endA1 suppl.E4 recA1 relA1</td>
<td>BRL</td>
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<td>BL21(DE3)pLysS</td>
<td>Host for pET30 vector</td>
<td>Novagen</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pET30</td>
<td>pET expression vector</td>
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<tr>
<td>pET-MFA</td>
<td>pET-30 with the mfa1 open reading frame</td>
<td></td>
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<tr>
<td>pT-COW</td>
<td>Shuttle vector plasmid; Am&lt;sup&gt;r&lt;/sup&gt; Te&lt;sup&gt;c&lt;/sup&gt; in <em>E. coli</em>; Te&lt;sup&gt;c&lt;/sup&gt; in <em>P. gingivalis</em> Mob&lt;sup&gt;r&lt;/sup&gt; Rep&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N. Shoemaker</td>
</tr>
<tr>
<td>pT-MFA</td>
<td>pT-COW containing a 2.5-kb fragment containing the upstream and coding region of the mfa1 gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Resistance to erythromycin (Em<sup>r</sup>), tetracycline (Te<sup>c</sup>), and ampicillin (Am<sup>r</sup>) is indicated. Mob<sup>r</sup>, mobilizable; Rep<sup>c</sup>, ability to replicate in *P. gingivalis*.
Native Mfa1 protein (75 kDa) was purified essentially as described previously (48). Because Mfa1 is not easily sheared off from the cell surface, purification was carried out from fractions of broken cells of a FimA-deficient mutant, KDP98, using gel filtration in the presence of SDS. To avoid breaking fimbriae, bacterial cells were disrupted in a French pressure cell by three passes at 100 MPa in the presence of 100 mM Tris-HCl, pH 7.5, 0.01% SDS, and 0.01% NaN₃ was diluted and negatively stained with 2% phosphotungstic acid (pH 7.4) and observed under a JEM 1010 transmission electron microscope (JEOL). Micrographs were digitized with a resolution of 2 pixels/nm, and electrically expanded images were used to measure the physical dimensions of 100 and 50 different fibers for length and pitch, respectively. The width of thicker and thinner portions was determined by measurement of 20 sites for each region.

Electron microscopy. Purified Mfa1 at 1.8 mg of protein per ml in 10 mM Tris-HCl, pH 7.5, 0.01% SDS, and 0.01% NaN₃ was diluted and negatively stained with 2% phosphotungstic acid (pH 7.4) and observed under a JEM 1010 transmission electron microscope (JEOL). Micrographs were digitized with a resolution of 2 pixels/nm, and electrically expanded images were used to measure the physical dimensions of 100 and 50 different fibers for length and pitch, respectively. The width of thicker and thinner portions was determined by measurement of 20 sites for each region.

RESULTS

Physical characteristics of the short fimbriae. In order to examine the structure of the short fimbriae without contamination by long fimbriae, short fimbriae were purified from the FimA mutant strain KDP98. In addition, sonication was avoided during the isolation process to minimize shearing of the fimbrial structures. The fimbrial preparation was analyzed by electron microscopy, and a micrograph is shown in Fig. 1. The length of the purified short fimbriae, as determined from measurements of 100 fibers, ranged from 60 to 200 nm, consistent with previous observations (8). The majority of the fibers were 80 to 120 nm long, with an average of 103 nm. The short fimbriae displayed a helical structure, with a pitch of ca. 27 nm, and presented a thicker portion ca. 6.5 nm wide along with a thinner portion ca. 3.5 nm wide.

Amino acid sequence analysis of SspB ligand. Previous study (4) of the P. gingivalis ligand for SspB showed that SspB coprecipitated a protein of ca. 100 kDa from a P. gingivalis surface extract. Amino acid sequencing of the 100-kDa protein identified it as Mfa1. In addition, disruption of the mfa1 gene resulted in loss of coadhesion and biofilm formation with S. gordonii. However, as disruption of mfa1 has a polar effect on the downstream cotranscribed gene PG0179, the contribution of Mfa1 to binding and biofilm development could not be determined. Furthermore, the predicted size of Mfa1 (61 kDa) combined with that of the cotranscribed PG0179 protein (37 kDa) would produce a composite protein of approximately 100 kDa. To clarify the identity of the 100-kDa protein recovered in the coprecipitation experiments and to determine the roles of the Mfa1 and PG0179 proteins in coadhesion and biofilm development, a series of experimental approaches was undertaken. We first repeated the pull-down assay described by Chung et al. (4) and coprecipitated P. gingivalis outer membrane proteins with SspB and anti-SspB antibodies. The resulting band was confirmed as containing Mfa1 by immunoblotting with Mfa antibodies (Fig. 2). This SspB-binding component of P. gingivalis was then subjected to more complete amino acid sequence analysis by tandem mass spectrometry of tryptic fragments. Sequences were obtained from peptides that covered more than 50% of Mfa1, including a peptide with a C terminus. No sequence was obtained that was derived from PG0179 or any other P. gingivalis protein. Thus, the SspB ligand would appear to be Mfa1 alone and is not a translational fusion or other composite protein. We conclude that the discrepancy in the sizes of Mfa1 observed by different methodologies results from the extended period of boiling required for complete denaturation of Mfa1 (Fig. 2, compare lanes 3 and 4). Mfa1 has a predicted molecular mass of 61 kDa; however, reported sizes based on SDS-PAGE do vary considerably, from 67 to 75 kDa.
Characteristics and binding properties of the complemented strain, *P. gingivalis* cSMF1. To confirm that Mfa1 mediates *P. gingivalis*-*S. gordonii* coadhesion without the involvement of accessory proteins, an Mfa1-deficient mutant of *P. gingivalis* (SMF1) was complemented in *trans* with the intact *mfa1* gene carried on plasmid pT-COW under the control of the endogenous *mfa1* promoter. Expression of Mfa1 in the complemented strain cSMF1 was confirmed by immunoblot analysis. Figure 4A shows that cSMF1 expresses the Mfa1 protein and associated degradation products. Scanning densitometry and image analysis with Kodak 1D software revealed that the major anti-Mfa reactive band was 58% more intense in cSMF1 compared to the parental strain. Thus, expression of Mfa1 in the complemented strain is higher than in the parental strain. The most likely explanation for the increase in expression is that *mfa* is present on a multicopy plasmid in the recombinant strain. Localization of Mfa1 to the cell surface was determined by ELISA after fixation of *P. gingivalis* strains on 96-well plates. As shown in Fig. 4B, cSMF1 cells showed significantly higher (P < 0.001; t test) reactivity with anti-Mfa serum compared to preimmune serum. The ELISA data also confirmed greater (P < 0.001; t test) expression of Mfa1 in the complemented strain compared to the parent. In contrast, parent, mutant, and complemented mutant strains showed equivalent reactivity with whole *P. gingivalis* antiserum, indicating that equivalent numbers of bacteria were deposited and retained on the plates. Similar ELISA data were obtained with *P. gingivalis* cells cultured to mid-log and early stationary phases of growth (not shown). In an interbacterial binding assay, levels of cSMF1 binding to *S. gordonii* were comparable to those of the parental strain (Fig. 4C). The presence of higher levels of Mfa1 on the cSMF1 strain, thus, did not result in greater binding activity, possibly as a consequence of steric constraints or suboptimal presentation of recombinant protein. The Mfa1-deficient strain showed only low levels of accumulation on *S. gordonii*, consistent with previous reports (16). Thus, expression of *mfa1* in a Mfa1-deficient background restores the ability of *P. gingivalis* to bind to *S. gordonii*. Furthermore, as the complemented strain is deficient in Pg0179 (due to the polar effect of the mutation of the chromosomal *mfa1*), these data provide corroborating evidence that the short fimbrial subunit itself is responsible for binding and does not require the presence of protein PG0179.

**Binding of rMfa to BAR peptide.** SspB, the streptococcal ligand of Mfa1, possesses a discrete region, designated BAR, that spans amino acid residues 1167 to 1250 and is sufficient to promote adherence of *P. gingivalis* cells, without interacting with FimA (5, 16). To determine if rMfa recognized and bound to BAR, we examined the interaction of purified rMfa with...
an immobilized synthetic peptide representing the BAR sequence. As shown in Fig. 5, rMfa was able to bind to the synthetic BAR peptide in a dose-dependent fashion. Binding exhibited second-order kinetics, with half-maximal binding occurring at an input BAR concentration of approximately 2.5 μg/ml. This result is similar to the kinetics of binding of intact \textit{P. gingivalis} cells to BAR (half-maximal binding at 2.0 μg/ml) previously reported (5). Thus, rMfa is capable of interacting with the coadhesion epitope of SspB, consistent with its role as the binding partner for SspB in \textit{P. gingivalis}-\textit{S. gordonii} coadhesion.

\section*{DISCUSSION}

Colonization of the oral cavity by \textit{P. gingivalis} necessitates adherence to available surfaces such as the preexisting plaque biofilm on tooth surfaces. Consistent with this constraint, \textit{P. gingivalis} can attach to a variety of common oral species including \textit{Fusobacterium nucleatum} and \textit{S. gordonii} (14, 17). Indeed, in vivo studies have shown that introduction of \textit{P. gingivalis} into the mouths of human volunteers results in the organism locating almost exclusively on streptococcal-rich preformed plaque (32). \textit{S. gordonii} is a major component of early plaque on the supragingival tooth surface and can also be found subgingivally (31, 34, 43). \textit{P. gingivalis} cells can adhere to \textit{S. gordonii} in vitro and accumulate into biofilm microcolonies on substrata of \textit{S. gordonii} (16). Initial attachment of \textit{P. gingivalis} to streptococcal cells involves interactions of the long fimbrial protein, FimA (15), with streptococcal glyceraldehyde-3-phosphate dehydrogenase (20). While disruption of the \textit{fimA} gene results in approximately a twofold reduction of coadhesion with \textit{S. gordonii}, a FimA-deficient mutant of \textit{P. gingivalis} retains the capability to form mixed species biofilms with \textit{S. gordonii} in the absence of shear forces (16, 19). In contrast, disruption of the \textit{ssp} genes of \textit{S. gordonii} reduces coadhesion and ablates biofilm formation (16). Thus, coadhesion mediated through the \textit{S. gordonii} Ssp proteins appears to be the predominant adhesive requirement for mixed species biofilm formation. Previous studies have demonstrated that the \textit{S. gordonii} SspB protein binds to the short fimbriae of \textit{P. gingivalis} (4).
However, this report was inconclusive as regards the participation of accessory fimbral components in the binding event. In particular, data from Chung et al. (4) suggested that the product of the PG0179 gene, which is immediately downstream of, and cotranscribed with, the structural subunit gene mfa1, may be associated with the short fimbrae and involved in binding to SspB. However, the results presented in the current study demonstrate that it is the Mfa1 protein that is necessary and sufficient for binding to SspB. This conclusion is based on the following observations: (i) purified recombinant Mfa1 protein can compete with *P. gingivalis* cells for binding sites on *S. gordonii* and impede coadhesion; (ii) monospecific antibodies to rMfa can inhibit *P. gingivalis*-S. gordonii coadhesion; (iii) in contrast, an Mfa1-deficient mutant that is also deficient in expression of the PG0179 gene is deficient in binding to *S. gordonii*, and complementation of this mutant with mfa1 in trans restores binding activity to wild-type levels; (iv) rMfa can bind to the BAR peptide that constitutes the adhesin domain of SspB. Moreover, the finding that coadhesion occurs between the complemented strain cSMF1 and *S. gordonii*, and the ability of rMfa to bind to the BAR domain of SspB, indicates that Mfa1 is the naturally occurring ligand of SspB. As SspB-dependent coadhesion is involved in the initiation of the events that lead to mixed species biofilm formation (16), it is possible that engagement of SspB by Mfa1 may initiate a signal transduction pathway within *P. gingivalis* that culminates in an adaptive response that allows the organism to adopt a “biofilm-ready” phenotype. Participation of additional signaling factors such as AI-2 is then required for biofilm formation to occur (22).

The Mfa1 protein was originally identified as a 75-kDa protein that copurified with the long fimbrae. Subsequently, the 75-kDa protein was purified as an immunodominant surface antigen forming a large, stable complex with an apparent molecular mass of about 2,000 kDa that was assumed (in the absence of morphological observation) to be a globular outer membrane or surface protein complex (48). Two independent groups later proposed the existence of short fimbrae, which they called minor and PGII fimbrae, with subunit molecular masses of 67 kDa and 72 kDa, respectively (8, 27). Little is known about the morphology of these fimbrae, however, in part because purification is difficult in the presence of the long fimbrae. Nonetheless, it is now well established that the 75-kDa protein, Mfa1 (67 kDa), and PGII (72 kDa) are the same polypeptides, based on their identical N-terminal amino acid sequences and extensive similarity of primary amino acid sequence deduced from the gene sequences (9, 26, 41). Furthermore, the short fimbrae comprised of Mfa1 are distinct from a third fimbral type consisting of a 53-kDa protein (1). In this study, electron micrographs were taken of the short fimbrae purified from a FimA-deficient strain. The short fimbrae were slightly wider and much shorter (width, ca. 6.5 nm; average length, 103 nm) than the FimA fimbrae (width, ca. 5 nm; length, up to 3 μm) although both appear to exhibit a helical structure. The short fimbrae are also more uniform in length, consistent with the elution pattern of an almost symmetrical peak from gel filtration during purification (48), whereas the long FimA fimbrae elute as a much broader peak under similar conditions (data not shown). The fact that the short fimbrae were difficult to shear off the surface of bacteria without sonication, in contrast to the FimA fimbrae, is also consistent with fimbrae of a short type. Indeed, the short fimbrae were distributed to the soluble as well as the membrane fractions when cells were broken. Furthermore, a residual population remained in the peptidoglycan fraction even after intensive SDS extraction (23; unpublished data), indicating that they may bind or penetrate through the peptidoglycan layer. Such a location could potentially allow the short fimbrae to function as a part of signal transduction pathway following binding of SspB to the exposed surface regions of the molecule; however, more research is required to address this possibility.

Complementation of gene disruptions in *P. gingivalis* has been achieved in only a limited number of studies, mostly with proteolytic enzymes (25, 29) and with the response regulator fimR (24). In the case of surface fimbrial adhesins, Takahashi et al. (36) successfully expressed the long fimbrae on the surface of heterologous *P. gingivalis* stains that produce serologically distinct FimA. In this situation, with both recombinant and native FimA present, the recombinant fimbrae interfered with the adhesive activity of the host strain. In the current study we utilized the shuttle vector plasmid pT-COW (7, 24) for complementation of a *P. gingivalis* strain deficient in short fimbrae. Expressed Mfa protein derived from this plasmid was transported to the surface of *P. gingivalis* and displayed functional activity. As this shuttle vector replicates in *P. gingivalis* 33277, this system may prove to be applicable to the complementation in *trans* of mutations in other *P. gingivalis* genes for surface proteins.

Both the short and long fimbrae of *P. gingivalis* are comprised of a major structural subunit. Studies have suggested that proteins PG2134 and PG2135, encoded by genes downstream of fimA (PG2132), may be minor components of long fimbrae (40, 46). It is not known if the short fimbrae are comprised solely of Mfa1 or also contain minor components. It remains possible that PG0179, which is cotranscribed with Mfa1, could be a short fimbral component or could be necessary for optimal presentation of the short fimbrae; however, PG0179 does not contribute to binding to *S. gordonii*. In addition to their distinct physical properties, and their individual streptococcal receptor specificity, the long and short fimbrae have other contrasting biological properties. The short fimbrae are more active in promoting bone resorption in rats and elicit a secreted cytokine profile from macrophages distinct from that of the long fimbrae (9, 10). Adherence of FimA and Mfa1 mutants to epithelial cells is also dissimilar (38). While FimA has been shown to bind to integrins on the gingival epithelial cell surface (45), the binding receptor for Mfa1 is unknown.

In conclusion, the short fimbrae of *P. gingivalis* are ca. 6.5 nm wide and on average 103 nm long. The short fimbral subunit protein Mfa1 is the naturally occurring binding partner for the SspB streptococcal adhesin, and interaction between SspB and Mfa1 is necessary for the optimal coadhesion between *P. gingivalis* and *S. gordonii*.

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