

## *Porphyromonas gingivalis* Minor Fimbriae Are Required for Cell-Cell Interactions

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**Two distinctive types of fimbriae have been identified in *Porphyromonas gingivalis*. In this report, we demonstrate that minor fimbriae are involved in *P. gingivalis* autoaggregation and colonization. A mutant with a deficiency in minor fimbriae can bind to a saliva-coated surface but does not form microcolonies as the wild-type strain does.**

*Porphyromonas gingivalis* is a gram-negative bacterium closely associated with severe adult periodontitis (16). It is well known that the bacterium can adhere to different surfaces in the oral cavity such as teeth, oral mucosa, and other oral bacteria (8). This adherence ability is important for bacterial colonization and is driven predominantly by peritrichous fimbriae. Two distinct fimbriae are present on the surface of *P. gingivalis* cells (1). Major fimbriae are long, peritrichous, filamentous components. The subunit of the major fimbriae is a 41-kDa protein (FimA, fimbrillin) and is encoded by the *fimA* gene (2). A thin, short secondary fimbrial structure, termed minor fimbriae or short fimbriae (15), is composed of a 67-kDa protein encoded by the *mfa1* gene (4). Both major and minor fimbriae appear to contribute to the pathogenicity of *P. gingivalis* (1). The primary role of FimA is to promote bacterial attachment to oral surfaces. A deficiency in the *fimA* gene leads to a diminished capacity to adhere to human gingival fibroblasts and epithelial cells (5) and attenuation of periodontal bone loss in a gnotobiotic-rat model (10). Major fimbriae are also required for *P. gingivalis* intracellular invasion. *P. gingivalis* fimbria-deficient mutants have a reduced ability to invade human gingival epithelial and KB cells (11, 18, 19). Furthermore, major fimbriae interrupt the host immune system by inducing human peripheral macrophages and neutrophils to overproduce several proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (12, 13). Although the role of minor fimbriae in *P. gingivalis* virulence is less well understood, Hiramane et al. (6) have shown that the 67-kDa fimbrial protein induces IL-1 $\alpha$ , IL-1 $\beta$ , and tumor necrosis factor alpha expression in mouse peritoneal macrophages, suggesting their possible involvement in the inflammatory response during the development of periodontal disease. Recent research has shown that minor fimbriae are necessary for the development of *P. gingivalis* biofilms on streptococcal substrates (7). Coadhesion of *P. gingivalis*-*Streptococcus gordonii* requires specific recognition between the 67-kDa and SspB proteins.

In this study, we demonstrate that minor fimbriae are required for *P. gingivalis* autoaggregation by examining a group of *P. gingivalis* fimbrial mutants. Our results show that only

strains possessing the two distinct fimbriae are able to develop into mature monospecies biofilms. We provide evidence that while major fimbriae are responsible for *P. gingivalis* attachment and initiation of colonization, minor fimbriae are involved in the formation of microcolonies and maturation of *P. gingivalis* biofilms.

**Minor fimbriae (Mfa1) are essential for *P. gingivalis* cell-cell aggregation.** To test the hypothesis that minor fimbriae function distinctively from major fimbriae, a group of fimbrial mutants, including a  $\Delta$ *fimA* mutant, a  $\Delta$ *mfa1* mutant, and a  $\Delta$ *fimA*  $\Delta$ *mfa1* double mutant, were constructed and examined for the ability to autoaggregate in Trypticase soy broth (TSB) supplemented with yeast extract (1 mg/ml), hemin (5  $\mu$ g/ml), and menadione (1  $\mu$ g/ml). *P. gingivalis* ATCC 33277 was used as the parental strain for mutant construction. To construct the  $\Delta$ *fimA* mutant, a 2.5-kb NheI DNA fragment containing the tetracycline gene *tetA(Q)* (9) was inserted into the *fimA* gene cloned into plasmid pFIM. For construction of the  $\Delta$ *mfa1* mutant, a 2.1-kb *ermF-ermAM* cassette (3) was inserted into the *mfa1* gene cloned into plasmid pMFA. The resulting plasmids were linearized with XhoI and introduced into *P. gingi-*

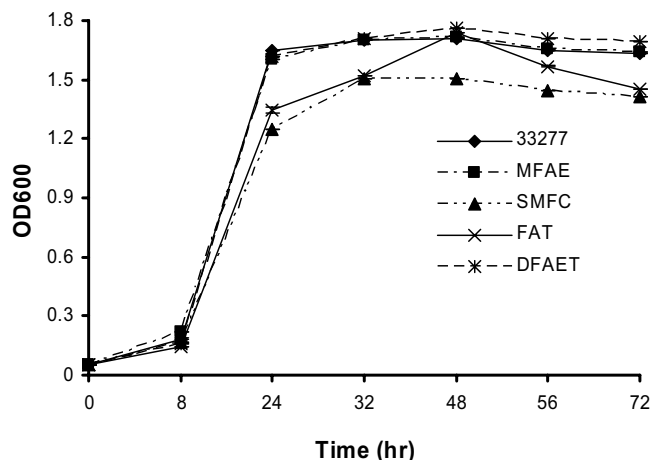
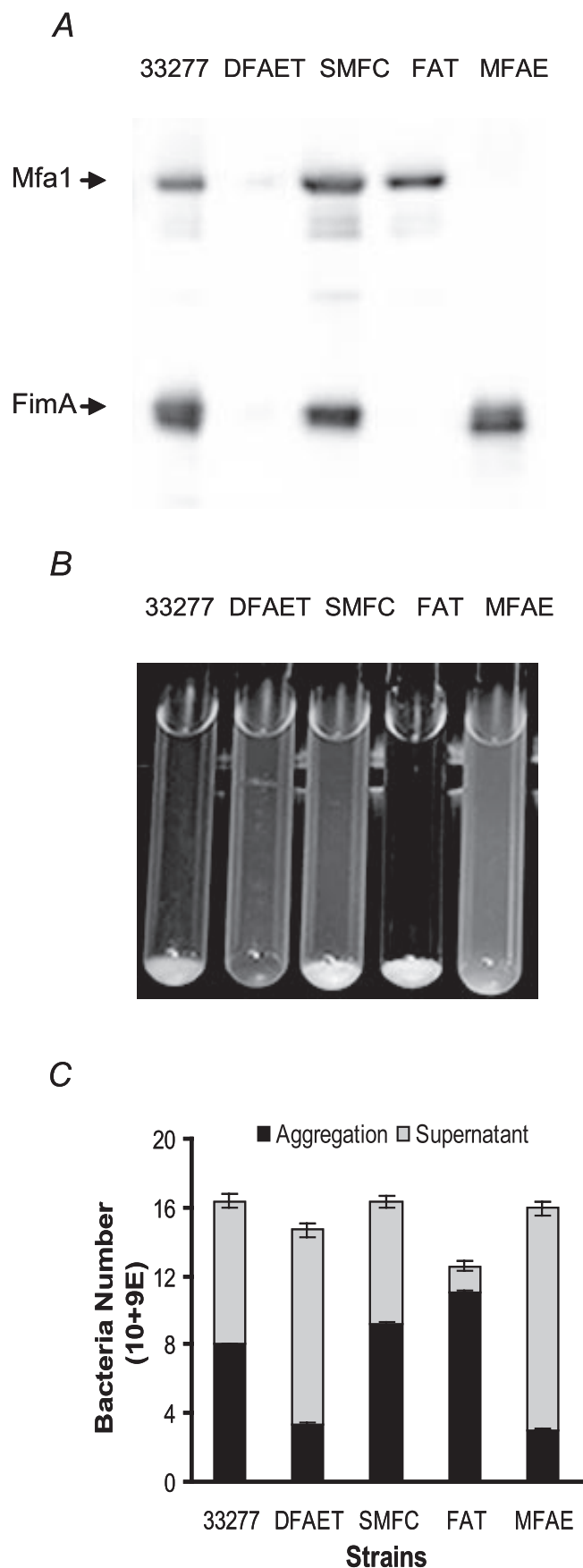


FIG. 1. Comparison of the growth curves of *P. gingivalis* strains. Cells were grown in TSB medium. Shown in the curves are means of four samples, with error bars representing the standard error of the mean. One-milliliter aliquots were taken, and the OD<sub>600</sub> was measured over a period of 72 h.

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*valis* 33277 by electroporation. Electroporation was carried out by a modification of the procedure of Fletcher et al. (3). *P. gingivalis* 33277 competent cells were obtained by suspending early-log-phase cells in electroporation buffer (10% glycerol, 1.0 mM MgCl<sub>2</sub>). The cells were incubated with linearized plasmid pFIM or pMFA and pulsed with a Bio-Rad (Hercules, CA) Gene Pulser at 2.5 kV. The cells were then immediately added to the TSB and incubated anaerobically for 16 h. The  $\Delta$ *fimA* mutant (FAT) was selected on TSB agar plates containing tetracycline (0.5  $\mu$ g/ml), and the  $\Delta$ *mfa1* mutant (MFAE) was selected on TSB agar plates containing erythromycin (5  $\mu$ g/ml). To construct a  $\Delta$ *fimA*  $\Delta$ *mfa1* double mutant, competent cells of the  $\Delta$ *mfa1* mutant, together with linearized plasmid pFIM, were pulsed with a Bio-Rad (Hercules, CA) Gene Pulser. The  $\Delta$ *fimA*  $\Delta$ *mfa1* double mutant (DFAET) was selected on TSB agar plates containing erythromycin (5  $\mu$ g/ml) and tetracycline (0.5  $\mu$ g/ml). All mutants were confirmed by PCR. The growth rates of these mutants were compared with that of wild-type strain 33277. Dilutions (1:100) of overnight bacterial cultures were inoculated into TSB supplemented with 100  $\mu$ g/ml gentamicin, and the bacteria were grown anaerobically at 37°C. At various times, 1-ml aliquots were taken and the optical density at 600 nm was determined. Wild-type strain 33277 and the fimbrial mutants did not show any significant growth curve differences over a period of 72 h (Fig. 1), suggesting that the growth rate was not affected by the fimbrial mutations.

To determine the production of major and minor fimbriae in these mutants, surface proteins were isolated by sonication and centrifugation from bacteria grown to mid-exponential phase. The soluble proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fimbrial production was detected by Western blotting with a polyclonal anti-FimA or anti-Mfa1 antibody. This analysis revealed that insertional mutations in the *fimA* and/or *mfa1* genes completely abolished fimbrial production (Fig. 2A).

We observed that *P. gingivalis* cells display aggregative behavior, manifested by some cell gathering and sinking to the bottom of the test tubes, when the bacteria were grown in TSB and that the cell aggregation phenotypes of the wild-type strain and the derivative strains were correlated with expression and production of the Mfa1 protein (Fig. 2B). After growth for 24 h in TSB, *P. gingivalis* 33277 showed some degree of aggregation, and approximately 50% of the cells sank to the bottom of the test tube (Fig. 2C). *P. gingivalis*  $\Delta$ *fimA*  $\Delta$ *mfa1* double mutant DFAET, expressing neither *fimA* nor *mfa1*, did not show vis-

FIG. 2. Aggregation of *P. gingivalis* strains in TSB. (A) Immunodetection of fimbrial production with rabbit polyclonal anti-FimA and anti-Mfa1 antibodies. Lane 1, wild-type strain 33277 (*fimA*<sup>+</sup> *mfa1*<sup>+</sup>); lane 2, strain DFAET ( $\Delta$ *fimA*  $\Delta$ *mfa1*); lane 3, strain SMFC (*fimA*<sup>+</sup> *mfa1*<sup>+</sup>); lane 4, strain FAT ( $\Delta$ *fimA* *mfa1*<sup>+</sup>); lane 5, strain MFAE (*fimA*<sup>+</sup>  $\Delta$ *mfa1*). (B) *P. gingivalis* cells were grown in TSB for 24 h. Aggregated cells collected at the bottoms of the test tubes. (C) The number of *P. gingivalis* cells was estimated in a spectrophotometer as described by Soukos et al. (17). OD<sub>600</sub> was determined. Readings of supernatant were taken from 9 ml of top culture, and readings of aggregation were from 1 ml of bottom culture. An OD<sub>600</sub> of 0.1 equals approximately 10<sup>8</sup> *P. gingivalis* cells per ml.

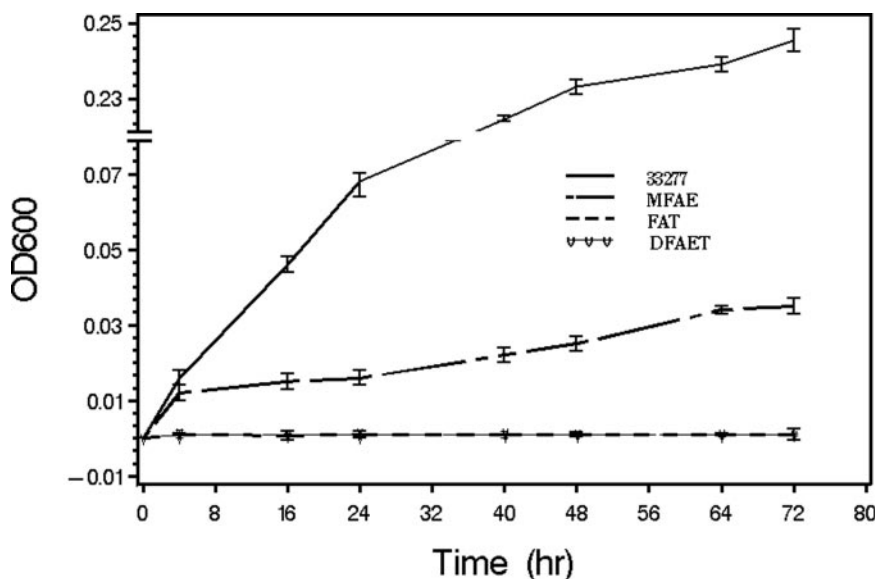


FIG. 3. Quantitation of biofilm formation by *P. gingivalis* on saliva-coated polystyrene wells. *P. gingivalis* biofilms were allowed to form in saliva-coated six-well polystyrene microtiter dishes. The cells bound to the well surfaces were suspended in 1 ml PBS for 4, 16, 24, 40, 48, 64, and 72 h of incubation. The ability of *P. gingivalis* strains to attach and form microcolonies on the surface was scored by measuring cell density. Biofilm formation by wild-type strain 33277, strain DFAET ( $\Delta fimA \Delta mfa1$ ), strain FAT ( $\Delta fimA$ ), and strain MFAE ( $\Delta mfa1$ ) was measured by determining OD<sub>600</sub>. Each data point represents the mean  $\pm$  the standard deviation from at least three independent experiments.

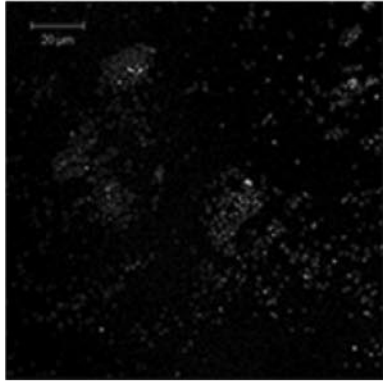
ible aggregation in TSB medium. The *mfa1* mutant MFAE was unable to express *mfa1* and, similar to *P. gingivalis* DFAET, lost its aggregation ability. The highest degree of aggregation was observed in the *fimA* mutant FAT, with 90% aggregation ( $P < 0.01$ ). The evident autoaggregation of FAT may result from low expression of the major fimbriae, which exposes the shorter minor fimbriae. Autoaggregation was restored in a complemented  $\Delta mfa1$  strain (SMFC) carrying plasmid pT-MFA containing a 2.5-kb fragment of the upstream and coding region of the *mfa1* gene (15), suggesting that this phenotype did not result from polar effects. These data demonstrate that the degree of *P. gingivalis* aggregation correlated with the expression levels of *mfa1* and *fimA*. Up-regulation of *mfa1* and down-regulation of *fimA* may promote autoaggregation of *P. gingivalis*.

**Role of major and minor fimbriae in *P. gingivalis* colonization.** The observation that minor fimbriae are associated with cell aggregation led us to hypothesize that minor fimbriae may also play an important role in biofilm formation by *P. gingivalis*. Current models of initial biofilm development in gram-negative organisms include two major steps. In the first step, bacteria attach to the surface in a monolayer. The cells then aggregate into microcolonies. In *Pseudomonas aeruginosa*, flagella are required for formation of the organism monolayer and type IV pili promote the cell-cell interaction that assembles a monolayer into microcolonies (14). We speculate that multiple extracellular proteins may also be involved in *P. gingivalis* biofilm formation. To test this possibility, biofilm formation experiments were performed with six-well polystyrene microtiter dishes (Corning, Inc., Corning, NY) containing TSB medium. The wells were precoated with human whole saliva. The plates were inoculated with individual *P. gingivalis* strains ( $10^8$  cells) and incubated at 37°C in an anaerobic chamber for 4 h. After the nonattached cells were removed, the wells were

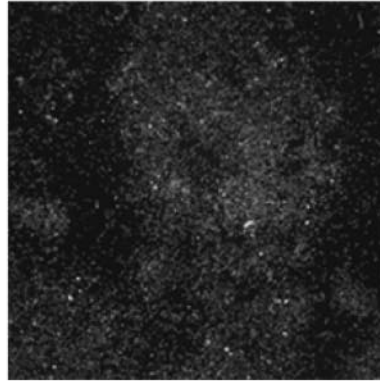
washed three times with phosphate-buffered saline (PBS). The attached bacteria were subsequently grown in TSB anaerobically for 72 h with a culture medium (TSB) change every 24 h. At various time intervals, bound bacteria were suspended in 1 ml of PBS and quantified by determination of the optical density at 600 nm (OD<sub>600</sub>). As shown in Fig. 3, *P. gingivalis*  $\Delta fimA$  mutant FAT and  $\Delta fimA \Delta mfa1$  double mutant DFAET displayed poor adherence to the surface at all time points, which is likely due to its lack of FimA production. Wild-type strain 33277 bound to the saliva-coated wells and formed microcolonies visible to the naked eye after 48 h. The number of attached cells peaked at 72 h. *P. gingivalis*  $\Delta mfa1$  mutant MFAE was able to bind to the surfaces of the wells. There was no significant difference in the degree of bacterial attachment between wild-type strain 33277 and *mfa1*-deficient mutant MFAE during the first 4 h of incubation, suggesting that these two strains have similar adherence abilities. However, the number of attached *P. gingivalis*  $\Delta mfa1$  mutant cells was only slightly increased after initial binding. The *mfa1*-deficient mutant also failed to form microcolonies visible to the naked eye after 48 h of growth. These findings support the aggregation results, suggesting that the minor fimbriae are involved in cell-cell aggregation, an essential step in microcolony formation.

To visualize *P. gingivalis* biofilms, we examined bacterial attachment and aggregation by using inverted confocal microscopy. *P. gingivalis* 33277, the  $\Delta fimA$  mutant FAT, the  $\Delta mfa1$  mutant MFAE, and the  $\Delta fimA \Delta mfa1$  double mutant DFAET were grown in TSB for 24 h, harvested by centrifugation, and resuspended in PBS. The cells were labeled with fluorescein 5-isothiocyanate (FITC; final concentration, 4  $\mu$ g/ml; Sigma). *P. gingivalis*-FITC suspensions were incubated anaerobically at 4°C for 20 min in the dark with gentle shaking. The labeled cells were then washed twice with PBS, and 2 ml of cell sus-

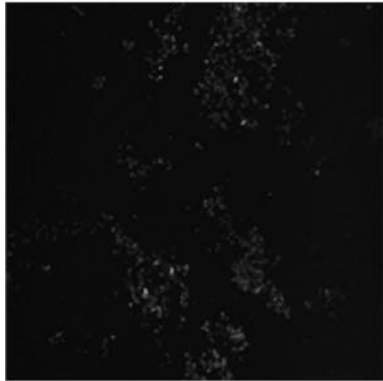
A 33277 (*fimA*<sup>+</sup> *mfa1*<sup>+</sup>)



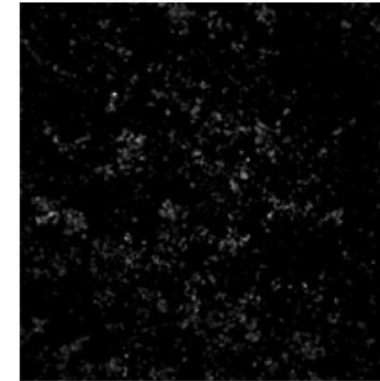
E 33277 (*fimA*<sup>+</sup> *mfa1*<sup>+</sup>)



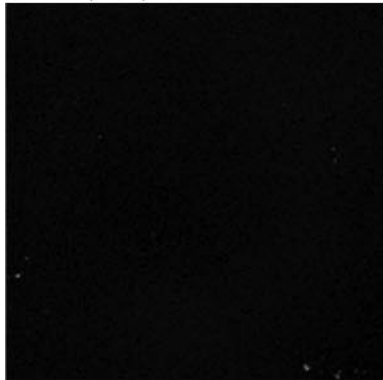
B MFAE (*mfa1*)



F MFAE (*mfa1*)



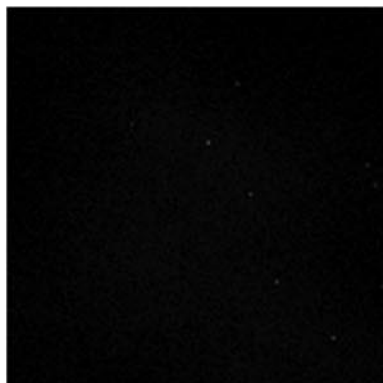
C FAT (*fimA*)



G FAT (*fimA*)



D DFAET (*fimA mfa1*)



H DFAET (*fimA mfa1*)

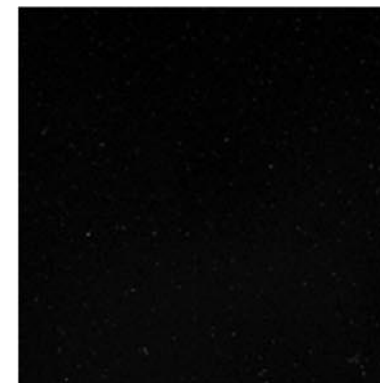


FIG. 4. Biofilm formation by *P. gingivalis* on saliva-coated MatTek glass bottom culture dishes. *P. gingivalis* cells were stained with FITC and incubated in saliva-coated glass bottom culture dishes for 1 (A, B, C, and D) or 24 (E, F, G, and H) h. *P. gingivalis* biofilms were visualized with a confocal laser scanning microscope.

pension ( $10^8$  cells) was inoculated into MatTek glass bottom culture dishes (1 mm by 3.5 mm; MatTek Corp., Ashland, MA). Each dish was pretreated with human whole saliva. After anaerobic incubation for 1 or 24 h, the unbound cells were removed and the dishes were washed three times with PBS and refilled with 1 ml of sterile PBS. The bacteria attached to the glass bottom culture dishes were examined under a 510 inverted confocal laser scanning microscope (Carl Zeiss Micro-Imaging, GmbH, Germany). *P. gingivalis* biofilms were analyzed by using the LSM software, which calculates measurement by the  $x$ - $y$ - $z$  pixel dimensions. As showed in Fig. 4, 1 h after inoculation, wild-type strain 33277 formed monolayers punctuated by a few small microcolonies ( $<1$   $\mu\text{m}$  thick) on the surface of a saliva-coated glass bottom culture dish (Fig. 4A). A similar observation was made with the *mfa1* mutant (Fig. 4B). The results are consistent with the biofilm assays, suggesting that *mfa1* mutation does not affect the ability of *P. gingivalis* to adhere to saliva-coated surfaces. However, the binding ability of the *fimA* mutant FAT and the *fimA mfa1* double mutant DFAET was abolished. Very few cells were detected on the saliva-coated surfaces (Fig. 4C and D), even after 24 h of exposure to saliva-coated surfaces (Fig. 4G and H). Wild-type strain 33277 continued to develop microcolonies, eventually covering 16% of the surface after 24 h of incubation (Fig. 4E). *P. gingivalis* 33277 microcolonies were about 800  $\mu\text{m}^2$  in size and 5  $\mu\text{m}$  thick. The *mfa1* mutant formed a progressively denser monolayer with small dispersed microcolonies after 24 h (Fig. 4F). Statistical analyses of microcolony values were performed with superANOVA (version 1.11; Abicus Software). The MFAE ( $\Delta mfa1$ ) microcolonies on a saliva-coated surface were significantly smaller and thinner (25  $\mu\text{m}^2$  in area and 3.5  $\mu\text{m}$  thick;  $P < 0.0001$ ) and only covered 9% of the surface. These data further demonstrate that the minor fimbriae are required for cell-cell interactions and aggregation. The *mfa1* mutant was capable of binding to the saliva-coated surface but could not efficiently recruit cells to form microcolonies. Our observations suggest that, in the early stage, the developmental pathway of *P. gingivalis* biofilms may also include two steps, from a monolayer to microcolonies. The *P. gingivalis* major fimbriae are required for initial attachment. However, in the later stages of biofilm formation, the minor fimbriae appear to play an important role in microcolony formation by facilitating cell-cell interactions.

**Conclusions and perspective.** *P. gingivalis* is considered a major periodontal pathogen and a secondary colonizer of dental plaque. The abilities of *P. gingivalis* to bind to oral surfaces are essential characteristics of bacterial pathogenicity. Specific binding allows *P. gingivalis* to remain at periodontal sites and avoid elimination by salivary flow through either expectoration or swallowing. In this study, we report that the minor fimbriae may be responsible for *P. gingivalis* autoaggregation and microcolony formation. The minor fimbriae could be one of the key elements that allow the organism to survive shearing forces

and to establish *P. gingivalis* colonization. Inhibition of production of minor fimbriae in *P. gingivalis* may restrain accumulation of the organism, which is required for efficient infection.

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