Oral Microbiology Immunology 2009: 24: 260–263
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ORAL MICROBIOLOGY AND IMMUNOLOGY

Short communication

Homotypic biofilm structure of *Porphyromonas gingivalis* is affected by FimA type variations

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Kuboniwa M, Amano A, Inaba H, Hashino E, Shizukuishi S. Homotypic biofilm structure of Porphyromonas gingivalis *is affected by FimA type variations.*
Oral Microbiol Immunol 2009: 24: 260–263. © 2009 John Wiley & Sons A/S.

Introduction: *Porphyromonas gingivalis* is a periodontal pathogen whose long fimbriae (FimA) are classified into six genotypes (types I–V and Ib) based on the diversity of the *fimA* genes. FimA variations were previously shown to be related to the onset and development of adult periodontitis in a general population, while FimA were recently found to be critical mediators of initial biofilm formation. However, it is unclear if FimA variations have effects on biofilm features. Here, we compare the characteristic structures of homotypic biofilms developed by *P. gingivalis* strains with different FimA types.
Methods: Biofilms were formed on saliva-coated glass bottom wells in phosphate-buffered saline and their structures were analysed using confocal laser scanning microscopy. Furthermore, the biovolumes of the biofilms were quantified with a three-dimensional fluorophotometric method.
Results: Biofilm structures formed by the six representative FimA-type strains apparently differed. Type I and Ib *P. gingivalis* formed biofilms with a dense basal monolayer and dispersed microcolonies, whereas those formed by types II, III and IV strains had markedly luxuriant biofilms filled with widely clumped and tall colonies, and their biovolumes were significantly greater than those of types I and Ib. These characteristic features were confirmed to be closely related to FimA type in assays that utilized *fimA*-substituted mutants from type I to II and those from type II to I.
Conclusion: Our results suggest that FimA variations have effects on the structures of biofilms formed by *P. gingivalis*, which may be an important factor in the pathogenesis of periodontitis.

Key words: biofilm; FimA; fimbriae; genotype; *Porphyromonas gingivalis*

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Accepted for publication January 12, 2009

Porphyromonas gingivalis, a gram-negative black-pigmented anaerobe, is a pathogen associated with several forms of severe periodontal disease (22). This opportunistic pathogen can form biofilms along gingival margins, and has shown remarkable resistance to both host immune responses and chemotherapies (11). Two distinct fimbria types, long fimbriae (FimA) and short fimbriae (Mfa1), are present on the surface of *P. gingivalis* cells (4), with the former reported to play an important role in bacterial interactions with various host components (1). FimA has also been suggested to be a critical mediator of biofilm formation, especially in the initial attachment and organization of biofilms during the early phase of biofilm formation (5), because a deficiency in the *fimA* gene leads to a diminished capacity of *P. gingivalis* to form homotypic and heterotypic biofilms (12, 23). FimA are classified into six genotypes (types I–V and Ib) based on the diversity of the *fimA* genes encoding the FimA proteins (1). We also reported that a majority of periodontitis patients harbored *P. gingivalis* with type II FimA, followed by type IV (4), while we found that type II clones adhered to and invaded human epithelial cells *in vitro* more efficiently than other types (8, 15). FimA variation is therefore thought to be an important determinant of the virulence of *P. gingivalis* (2). However, the influence of FimA variations on biofilm development by *P. gingivalis* is unknown. In the present study, we examined the effects of FimA variations on homotypic biofilm formation by

P. gingivalis using representative strains of each FimA type as well as mutants in which the *fimA* gene was substituted with a different genotype.

The following *P. gingivalis* strains were used in this study: ATCC33277 [type I *fimA*; *fimA* (I)], HG1691 [*fimA* (Ib)], OMZ314 [*fimA* (II)], 6/26 [*fimA* (III)], HG564 [*fimA* (IV)], and HNA99 [*fimA* (V)], which have been described previously (15). Mutants in which *fimA* was substituted with a different genotype were also employed (8), i.e. 33277 (II) [*fimA*(I) of ATCC33277 was substituted with *fimA*(II) of OMZ314] and OMZ314 (I) [*fimA*(II) of OMZ314 was substituted with *fimA*(I) of ATCC33277]. The *fimA* substitution was carried out with a 7.7 kb DNA fragment containing the flanking regions and a promoter of the *fimA* genes for an efficient homologous recombination in our previous study (8). The regions downstream (2.8 kb) and upstream (3.8 kb) of *fimA* were found to carry 4 and 2 open reading frames (ORFs), respectively. However, these ORFs shared very high DNA sequence homologies between types I and II, which suggested that the proteins encoded by the open reading frames in the *fimA*-flanking region showed negligible influence on our previous results (8). The FimA-deficient mutant MPG1 from strain ATCC33277 (21) was kindly given by Professor N. Hamada (Kanagawa Dental College). Homotypic biofilm formation was assayed as described previously (5). Briefly, *P. gingivalis* cells were grown anaerobically and suspended in prereduced phosphate-buffered saline (PBS, pH 7.4). Bacterial cells (5×10^7) were incubated anaerobically at 37°C in individual wells of 96-well plates that were coated with stimulated saliva collected from at least six healthy individuals, as described previously (10). For microstructural analysis of the biofilms, bacterial cells were stained with 5-(and-6)-carboxyfluorescein and succinimidyl ester (Molecular Probes, Eugene, OR), then washed three times, after which 1×10^8 cells were anaerobically incubated in saliva-coated wells of a chambered coverglass system (Culture Well™; Grace Bio Labs, Bend, OR) in PBS for 24 h at 37°C in the dark on a rotator. The resulting biofilms were examined with a confocal laser scanning microscope (Radiance 2100; Bio-Rad Japan, Tokyo, Japan) with reflected laser light at 488 nm. The images were analysed using the IMAGE J 1.34s (National Institutes of Health, Bethesda, MD) and IMARIS 5.0.1 (Bitplane AG, Zurich, Switzerland) software packages. *Z* stacks of the *x–y* sections in the confocal laser scanning microscope images were converted to composite images with the 'ISO SURFACE' function of the 'SURPASS' option provided by IMARIS, then a biovolume per field was calculated. All data are shown as the mean ± standard error. Multiple comparisons were performed using one-way analysis of variance and Sheffe's test with SPSS 16.0J software (SPSS Japan Inc., Tokyo, Japan).

As shown in Fig. 1, the microstructures of biofilms formed by the different FimA strains were apparently distinct. The type I strain formed biofilms with a dense basal monolayer and dispersed microcolonies, whereas those formed by the type Ib strain as well as the FimA-deficient mutant MPG1 were patchy with smaller dispersed microcolonies. In contrast, the type II strain formed markedly thick biofilms filled with variously-sized microcolonies containing widely clumped and tall colonies. In addition, biofilms formed by the type III strain were also very thick and dense, and contained widely clumped and tall colonies, whereas the type IV strain developed uniformly clustered and chan-

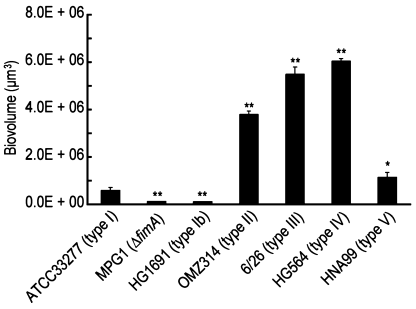

Fig. 2. Biovolumes of homotypic biofilms formed by *Porphyromonas gingivalis* strains with different FimA types. The *z*-stacks of the *x–y* sections in the confocal laser scanning microscope images were converted to the composite 'ISO SURFACE' images as described in FIG. 1, then a biovolume per field was calculated. Data are shown as the mean ± SE; $*P < 0.05$ and $**P < 0.01$ in comparison to strain ATCC33277 (type I *fimA*).

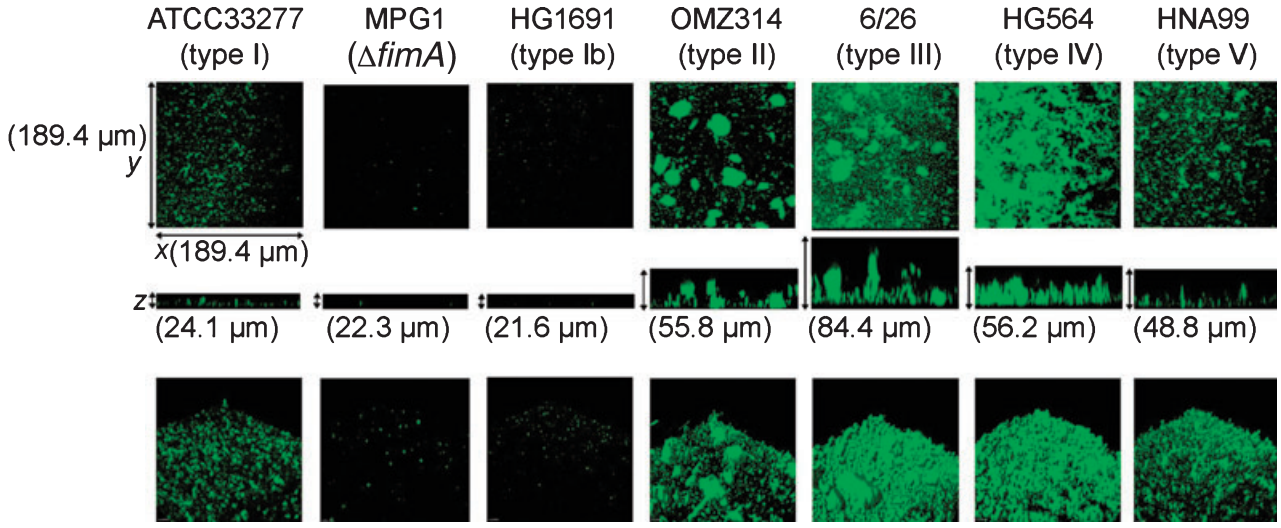

Fig. 1. Homotypic biofilm formation by *Porphyromonas gingivalis* strains with different FimA types and a FimA-deficient mutant. *P. gingivalis* strains were stained with fluorescein (green) and incubated in phosphate-buffered saline for 24 h. After washing, biofilms developed on coverglasses were observed with a confocal laser scanning microscope with a 40 × objective. Optical sections were obtained along the *z*-axis at 0.7-μm intervals and images were reconstructed with imaging software, as described in the text. Upper panels indicate *z*-stacks of the *x–y* sections. Middle panels are representative *x–z* sections. Lower panels show three-dimensional images constructed with the 'ISO SURFACE' function of the 'SURPASS' option using IMARIS software. The 'ISO SURFACE' function was used to present the outline of the biofilm.

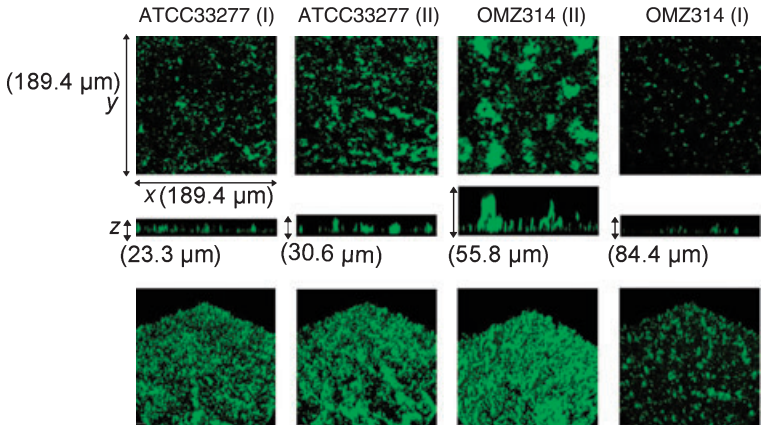

Fig. 3. Homotypic biofilm formation by *fimA*-substituted mutants of *Porphyromonas gingivalis*. ATCC33277 (I) and OMZ314 (II) are wild-type strains. ATCC33277 (II): type I *fimA* of ATCC33277 was substituted with type II *fimA* of OMZ314. OMZ (I): type II *fimA* of OMZ314 was substituted with type II *fimA* of ATCC33277. All strains were stained with fluorescein (green) and incubated in phosphate-buffered saline for 24 h. After washing, biofilms developed on coverglasses were observed as described in Fig. 1.

nel-like biofilms that consisted of tall and large microcolonies. The type V strain also formed thick biofilms with tall and large microcolonies, though with a lower level of density compared with those formed by types III and IV. The biovolumes also varied among the FimA types (Fig. 2). Strains with types II, III, and IV developed very large biofilms with greater biovolumes compared with those developed by types I and Ib, while the type V strains formed biofilms with moderately enlarged biovolumes. These results suggest that FimA variations are closely related to the characteristic features of monotypic biofilms formed by *P. gingivalis.*

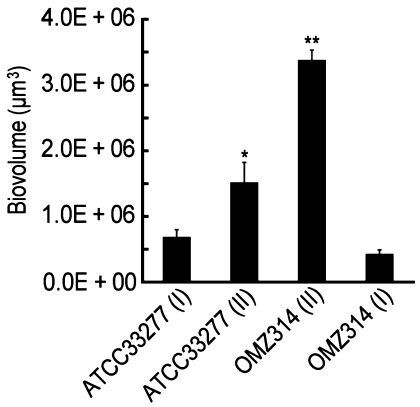

Fig. 4. Biovolumes of homotypic biofilms formed by *fimA*-substituted mutants of *Porphyromonas gingivalis*. The *z*-stacks of the *x*–*y* sections in the confocal laser scanning microscope images were converted to the composite 'ISO SURFACE' images as described in FIG. 1, then a biovolume per field was calculated. Data are shown as the mean ± SE; $*P < 0.05$ and $**P < 0.01$ in comparison to strain ATCC33277 (I).

Type II *P. gingivalis* is significantly more prevalent in the more severe forms of periodontitis, whereas type I organisms are the most widely distributed type in periodontally healthy subjects (3, 4). In addition, type II strains were shown to be the most virulent and type I clones were found to be avirulent in a mouse abscess model (16). In the present study, biofilms formed by type I and II strains had features that were apparently different from each other. We conducted additional examinations to determine whether these differences were related to FimA type using mutants in which *fimA* was substituted. The substitution of type I *fimA* with type II in strain 33277 altered the structures to thick biofilms comprising colonies that were more clumped and taller (Fig. 3). Furthermore, substitution of type II *fimA* with type I apparently changed the features from thick, tall, and clumped structures, to patchy biofilms with relatively small and sparse microcolonies. The biovolumes of the biofilms were also altered by the substitution of *fimA* genes, as the biovolume was decreased by substitution with type I FimA and increased by substitution with type II FimA (Fig. 4). These results indicate that the characteristic features of biofilms are closely related to FimA type.

Biofilm development proceeds through a series of steps involving the attachment of cells to a surface and growth on that surface, followed by detachment and dissemination to a new site to start the cycle again (14, 18). Defined genetic profiles are considered important for the distinct phases of biofilm development. In this complicated process, FimA has been reported to be required for the initial attachment and organization of homotypic biofilms, which is a crucial event in the early phase of biofilm formation (12). Indeed, we found that all types of FimA were initial positive mediators of biofilm formation. However, FimA type variations had effects on biofilm structure. This was confirmed by the assays using *fimA*-substituted mutants. Type II FimA formed much more luxuriant biofilms filled with widely clumped and tall colonies compared with type I FimA. As a strict anaerobe, *P. gingivalis* is likely to favor an existence deep within a thick biofilm. Alternatively, a luxuriant biofilm may be important to maintain the integrity of channels that allow nutrient penetration. Therefore, biofilms formed by FimA types II, III, and IV may be advantageous for the pathogen. The present findings are the first known report to show the influence of FimA variation on the structure of biofilms formed by *P. gingivalis*. Further study is necessary to determine if the distinct microstructures of biofilms formed by the different FimA strains are related to the periodontal pathogenicity of the bacterium.

Regulation of biofilm development is thought to involve mechanisms that both stimulate and increase the biovolume and limit or stabilize accumulation according to environmental constraints (19, 20). In this study, type I and Ib strains tended to suppress microcolony expansion, whereas other types likely promoted transverse enlargement and longitudinal extension. Although it is unknown whether FimA proteins can regulate dispersion and detachment of microcolonies in biofilms, there seemed to be functional differences with regard to the regulation of biofilm formation among the FimA variants. Autoaggregation driven by non-specific hydrophobic mechanisms is thought to contribute to heterotypic and homotypic biofilm formation (9). However, in our previous study (8) we found that strain 33277 (type I) had significantly higher levels of autoaggregation and hydrophobicity than OMZ314 (type II). Furthermore, the autoaggregation ability of strain 33277 was lost following substitution with type II *fimA* but both the wild-type and substituted mutant of OMZ314 showed no autoaggregation ability, and bacterial hydrophobicity was also negligibly changed by the *fimA* substitution (8). These findings suggest that the variations of biofilm structures caused by different

FimA types are not strongly related to autoaggregation or hydrophobicity. However, the substitution of type II with type I induced more apparent structural alteration, compared with the substitution of type I with type II, by which autoaggregation was diminished. This finding suggests that autoaggregation is partially related to biofilm formation. Additional study is necessary to clarify the factors involved in the impact of FimA type-dependent biofilm structures on biofilm formation by *P. gingivalis*.

In addition to FimA, it has been suggested that other components of *P. gingivalis* mediate and regulate homotypic biofilm formation, such as a putative glycosyltransferase (PG0106) that was shown to be involved in capsular polysaccharide synthesis of *P. gingivalis* (7), UDP-galactose 4-epimerase (GalE), which plays a pivotal role in the modification of lipopolysacchairde O antigen and biofilm formation (17), internalin J protein (5), and UpsA (6), a universal stress protein reported to be required for optimal biofilm accumulation. Autoinducer-2, which regulates proteinase and hemagglutinin activities, hemin and iron acquisition pathways, and stress gene expression, is also considered to be involved in homotypic biofilm formation by *P. gingivalis* (14). Very recently, a low molecular weight tyrosine phosphatase, Ltp1, was reported to affect cascades that constrain homotypic biofilm development (13). The proteins encoded by the open reading frames in the *fimA*-flanking region might also be involved in biofilm formation. It is possible that these molecules also have effects with regard to biofilm structure alterations, in addition to FimA variation. Although varied biofilm structures are thought to have a great effect on the periodontal pathogenicity of *P. gingivalis*, it is necessary to conduct additional examinations of the relationships between bacterial virulence and biofilm so as to elucidate the development of periodontitis caused by bacterial biofilms, a common cause of persistent infections.

Acknowledgment

This research was supported by grants-in-aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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