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Differential expression and adherence of *Porphyromonas gingivalis* FimA genotypes

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SUMMARY

***Porphyromonas gingivalis* is a primary pathogen involved in the initiation and progression of adult chronic periodontitis. Its colonization on oral surfaces is a necessary first step leading to infection. FimA, a subunit protein of major (long) fimbriae, is a well-known virulence factor. Based on its nucleotide sequence, FimA is classified into several genotypes. We compared here the transcriptional levels of the *fimA* gene in several *P. gingivalis* strains using real-time polymerase chain reaction analysis, fimbrial display on the *P. gingivalis* surface using transmission electronic microscopy, and the adherence competencies of *P. gingivalis* strains carrying different types of FimAs towards saliva and *Streptococcus gordonii* surfaces using mutagenesis analysis. We demonstrated differential expression of each *fimA* gene in these *P. gingivalis* strains. A correlation of the transcription level of *fimA* and binding activity of *P. gingivalis* was revealed. We show that *P. gingivalis* strains with genotype I and II of FimA are efficient in interaction with saliva or *S. gordonii*. This work highlights the important role of FimA type I and II in *P. gingivalis* attachment to oral surfaces.**

INTRODUCTION

Porphyromonas gingivalis has been shown to have a strong association with several forms of periodontitis (Socransky & Haffajee, 2005). The *P. gingivalis* strains were initially classified into four groups based on the nucleotide sequences of the *fimA* gene (type I and Ib to IV; Fujiwara *et al.*, 1993). Nakagawa *et al.* (2002) later identified a distinctive genotype of *fimA* (type V) from clinical dental plaque samples retrieved from patients with periodontitis. Several clinical studies have been conducted to determine the distribution of *P. gingivalis* strains with different *fimA* genotypes and to establish an association between a specific *fimA* genotype and the occurrence of periodontitis or the severity of the disease. Although the majority of the studies from different countries suggested that *P. gingivalis* strains with type II and IV FimA are periodontitis-associated strains (Amano *et al.*, 1999; van der Ploeg *et al.*, 2004; Miura *et al.*, 2005; Zhao *et al.*, 2007; Nakano *et al.*, 2008; Teixeira *et al.*, 2009), a few studies using the same polymerase chain reaction (PCR) analysis reported that there was no difference in the association of the *fimA* genotypes with the incidence of periodontitis or the severity of the disease (Beikler *et al.*, 2003; Perez-Chaparro *et al.*, 2009). Interestingly, Fujise *et al.* (2005) found that FimA type I positive sites showed little improvement with periodontal treatment, therefore, this group suggested that the presence of *P. gingivalis* FimA type I may be useful as a predictor of poor treatment outcomes. Therefore, the earlier data are not conclusive with respect to association

between particular FimA genotypes and strains of *P. gingivalis*.

The comparative functions of *P. gingivalis* FimA genotypes were investigated in independent laboratories. It appeared that all *P. gingivalis* strains with different *fimA* genotypes adhered to epithelial (KB, a cell line derived from a human carcinoma of the nasopharynx) cells, but with differential efficiencies (Umeda *et al.*, 2006). Kato *et al.* (2007) demonstrated that type II fimbriae showed greater adhesive abilities for $\alpha_5\beta_1$-integrin of epithelial cells than type I fimbriae. A recent study, using confocal microscopy, reported variations in the structures of biofilms formed by *P. gingivalis* strains with different genotypes for FimA (Kuboniwa *et al.*, 2009). Differential host innate immune responses were also observed when mice were infected with either *P. gingivalis* 33277 (FimA I) or OMZ314 (FimA II; Kato *et al.*, 2007; Wang *et al.*, 2009). FimA type II was able to elicit a significantly higher serum interleukin-1β and interleukin-6 level compared with FimA type I. The reciprocal swap mutants' behaviors were consistent with their heterogeneous fimbrial types, suggesting that FimA genotypes have a variable ability to provoke a host immune response. A comprehensive study by Wang *et al.* (2009) demonstrated different effects of FimA type on the bacterially induced bone loss and pro-inflammatory activities as well as intracellular entry. *Porphyromonas gingivalis* 33277 induces significantly more bone loss than OMZ314, but less pro-inflammatory activities compared with OMZ314. Moreover, *P. gingivalis* OMZ314 showed greater invasive ability into macrophages than type I strain 33277. These studies established differential function of FimA types, however, molecular mechanisms of different functional efficiencies have not been revealed. We hypothesize that differential expression of *fimA* in *P. gingivalis* strains carrying different genotypic FimA contributes, at least in part, to their function efficiency. To test this hypothesis, we examined the transcription levels of *fimA* in *P. gingivalis* strains with four different genotypes of FimA (type I, II, III and IV). Differential expression of *fimA* was found in these four strains. In parallel, adherence ability of these strains also varied. The results indicate that the elevated *fimA* expression and adherence ability in *P. gingivalis* strains with particular FimAs may be associated with their persistence and prevalence in the sites of periodontitis.

METHODS

Bacterial strains and growth conditions

Porphyromonas gingivalis strains, 33277 (type I), BH18/10, OMZ314, OMZ409 (type II), 49417 (type III), HG564 (type IV) and W83, were grown in Trypticase soy broth (TSB) or on TSB blood agar plates supplemented with yeast extract (1 mg ml^{-1}), hemin (5 μg ml^{-1}) and menadione (1 μg ml^{-1}), at 37°C in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2). *Streptococcus gordonii* DL1 was grown in trypticase peptone broth supplemented with 0.5% glucose at 37°C under aerobic conditions.

RNA isolation and quantitative PCR

The *P. gingivalis* strains were grown anaerobically and harvested by centrifugation at 8000 ***g*** and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). The RNA in the supernatant was then purified using an RNeasy mini spin column (Qiagen, Valencia, CA). Real-time reverse transcription (RT) -PCR analysis was performed by using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) on the iCycler MyiQ™ Real-Time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions. Primers were designed using Primer3 software, and are listed in Table 1. The expression levels of the *fimA* genes for the test sample were determined by using the comparative cycle threshold (ΔC_T) method. By loading the same amount of total RNA for comparable samples, the ΔC_T represents the difference of gene expression between the samples.

Construction of the *fimA* mutants

An insertional *fimA* mutant was generated by using ligation-independent cloning of PCR-mediated mutagenesis (LIC-PCR). A 2.1-kb *ermF-ermAM* cassette was introduced into the *fimA* gene by three steps of PCR to yield a *fimA-erm-fimA* DNA fragment as previously described (Wu *et al.*, 2008). The fragment was then introduced into *P. gingivalis* strains by electroporation. The *fimA*-deficient mutant was generated via a double crossover event that replaced *fimA* with a *fimA-erm-fimA* DNA fragment in the chromosomes

Table 1 Oligonucleotide primers used in this study

Primer name	Primer sequences (5′–3′)	Applications
fimA160F	cagcaggaagccatcaaatc	For RT-PCR of *fimA* (type I, II, III)
fimA160R	cagtcagttcagttgtcaat	
HG564-fimA151F	ctaaaatcgcagcccttgtc	For RT-PCR of *fimA* (type IV)
HG564-fimA151R	gacgcctccaattcgtatgt	
fimA1R-erm	gatgttgcaaataccgatgagcaaccttagtcgcattttcgg	For creating the *fimA* mutant of 33277 and OMZ409
fimA2F-erm	cctctagagtcgacctgcagaatggaactggttggcaaga	
49417-fimA(1)R-erm	gatgttgcaaataccgatgagcaaccttagtcgcattttcgg	For creating the *fimA* mutant of 49417
49417-fimA(2)F-erm	cctctagagtcgacctgcagaatggaattggctggcaaga	
HG564-fimA(1)R-erm	gatgttgcaaataccgatgagccccttcttgaatttgacctg	For creating the *fimA* mutant of HG564
HG564-fimA(2)F-erm	cctctagagtcgacctgcaggccttgacgacttctttgac	
16S rRNAF	tgtagatgactgatggtgaaa	For RT-PCR of *Porphyromonas gingivalis*
16S rRNAR	actgttagcaactaccgatgt	

The gene resistant to erythromycin (Emr) is underlined.
RT-PCR, reverse transcription polymerase chain reaction.

of the wild-type strains. The mutants were selected on TSB plates containing erythromycin (5 μg ml^{-1}). The insertional mutation was confirmed by PCR analysis, and the mutants were designated *P. gingivalis* 33277_Δ*fimA* mutant (with 59-bp deletion), OMZ409_Δ*fimA* mutant (with 59-bp deletion), 49417_Δ*fimA* mutant (with 59-bp deletion), HG564_Δ*fimA* mutant (with 135-bp deletion).

Adherence assay

Attachment of *P. gingivalis* to saliva-coated surfaces was quantified by the method of O'Toole & Kolter (1998). Saliva collection was approved by the IRB of Meharry Medical College. Human whole saliva was collected from a periodontally healthy female donor. After centrifugation and filter sterilization (pore size 0.22 μm) to remove cell debris, saliva was incubated in a six-well polystyrene plate for 1 h. The plate was then washed with phosphate-buffered saline (PBS; 100 mm NaH_2PO_4, 150 mm NaCl). Bacterial strains were grown in TSB to mid log phase (optical density at 595 nm 1.0) and collected by centrifugation. The bacterial cells were resuspended in PBS. The *P. gingivalis* cells (6×10^8) were added to each well of a six-well polystyrene plate (Corning Incorporated, Corning, NY) that was pre-coated with human whole saliva, and were incubated at 37°C for 2 h.

Heterotypic biofilms of *P. gingivalis*–streptococci were also generated on a polystyrene six-well plate. The *S. gordonii* DL1 (6×10^8) were first incubated in saliva-coated wells at 37°C aerobically for 4 h, and unbound cells were removed by washing with PBS–Tween-20 (0.01%, volume/volume) twice. *Porphyromonas gingivalis* cells (6×10^8) in PBS–Tween-20 were added to the wells covered with streptococcal biofilms and incubated at 37°C anaerobically for 2 h.

The number of *P. gingivalis* cells bound on *S. gordonii* biofilms and saliva-coated surfaces was determined using quantitative PCR (Wu *et al.*, 2008). The *P. gingivalis* cells were lysed with lysis solution (Solution A, Invitrogen). DNA was extracted using an Easy-DNA kit (Invitrogen) and eluted in 250 μl TE buffer (10 mm Tris–HCl, pH 7.5. 1 mM EDTA). The cells in biofilms were enumerated using a QuantiTect SYBR Green PCR Kit (Qiagen) with *P. gingivalis* species-specific 16S ribosomal RNA gene primers. Standards used to determine *P. gingivalis* cell numbers were prepared using genomic DNAs from wild-type strain 33277. A fresh culture of *P. gingivalis* 33277, grown in TSB, was serially diluted in PBS and plated onto TSB plates to obtain colony-forming units per milliliter at each dilution. DNA was also isolated from the dilutions and a quantitative PCR assay was run, as described above, to determine cell numbers. Three trials were performed on three separate cultures.

Transmission electron microscopy of *P. gingivalis*

Porphyromonas gingivalis cells were grown on TSB blood plates for 48 h. Bacterial cells were collected and resuspended in PBS. Twenty microliters of bac-

terial suspension was applied to a Formvar-coated copper grid (200-mesh, Electron Microscopy Sciences, Hatfield, PA) and air dried. The bacterial cells were then negatively stained with 0.5% ammonium molybdate for 30 s and observed with a transmission electron microscope (Philips CM-12, Portland, OR) operated at 80 kV.

Statistical analyses

A Student's *t*-test and paired *t*-test were used to determine statistical significance of the differences in *fimA* expression profiles and the adherence competencies of *P. gingivalis* strains with different genotypes of the *fimA* gene. A *P*-value less than 0.05 was considered significant.

RESULTS

Differential expression of different genotype *fimA* genes in *P. gingivalis*

To test the differential expression of *fimA*, seven *P. gingivalis* strains representing *fimA* genotypes I to IV were selected from our laboratory collection and tested for their respective *fimA* expression levels using RT-PCR. The *P. gingivalis* strains were grown in TSB to reach an optical density of 0.6 at 595 nm. The same set of primers was designed for *P. gingivalis* 33277 (type I), BH18/10 (type I), OMZ314 (type II), OMZ409 (type II) and 49417 (type III) corresponding the conserved regions of the *fimA* genes. The *fimA* gene of *P. gingivalis* HG564 and W83 (type IV) was amplified using a different set of primers because of a variation in its nucleotide sequence in the conserved regions. RNA preparations and RT-PCR were conducted in parallel under the same conditions. As shown in Fig. 1, different expression levels of *fimA* were found in these strains, with the significantly higher expression in *P. gingivalis* strains expressing type I and II. To confirm the differential expression of *fimA* and to exclude the possibility that the very low expression level of *fimA* gene in *P. gingivalis* HG564 and W83 was the result of the low efficiency of the primers, the expression level of *fimA* relative to that of 16S ribosomal RNA in each tested *P. gingivalis* strain was examined. The results indicated a similar *fimA* expression order among these four strains (data not shown). Moreover, previous

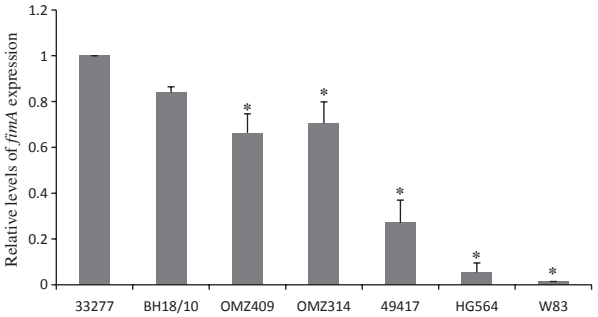

Figure 1 Differential expression of *fimA* gene (pg2132) in *Porphyromonas gingivalis* strains. Strains 33277 (type I), BH18/10 (type I), OMZ314 (type II), OMZ409 (type II), 49417 (type III) and HG564 (type IV) and W83 (type IV) express genotype I, II, III and IV *fimA*, respectively. Total RNAs were extracted from *P. gingivalis* grown in trypticase soy broth. Expression of *fimA* was determined using real time reverse transcription polymerase chain reaction analysis. The transcript level of *fimA* in an individual strain is indicated relative to the expression level of the gene in *P. gingivalis* 33277, set = 1.0. Standard errors are indicated ($n = 3$). Asterisk indicates the statistical significance between *P. gingivalis* 33277 and the tested strains ($P < 0.05$; *t*-test).

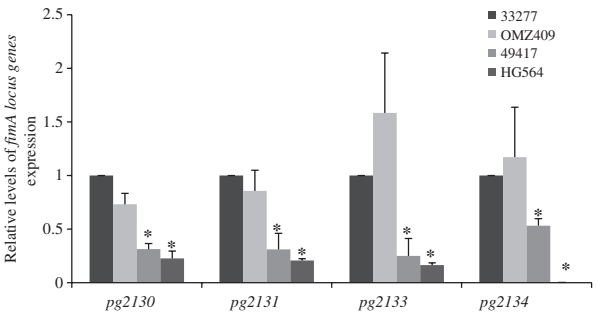

Figure 2 Comparison of expression levels of other *fim* genes in four *Porphyromonas gingivalis* strains. The transcript levels of genes immediately upstream of *fimA*, *pg2130* and *pg2131*, and of genes immediately downstream of *fimA*, *pg2133* and *pg2134*, were determined using quantitative reverse transcription polymerase chain reaction. Expression of these genes in OMZ409, 49417 and HG564 is indicated relative to the expression level of the genes in *P. gingivalis* 33277, set = 1.0. Standard errors are indicated ($n = 3$). Asterisk indicates the statistical significance between *P. gingivalis* 33277 and the tested strains ($P < 0.05$; *t*-test).

studies indicated that five genes (*pg2130–pg2134*) including *fimA* (*pg2132*) were under the control of a transcriptional activator, FimR, suggesting that these genes compose a *fim* locus (Nishikawa *et al.*, 2004). Transcription profiles of the genes in this cluster were compared among four *P. gingivalis* strains (33277, OMZ409, 49417 and HG564) carrying different FimA genotypes. Consistent with expression level of *fimA*

gene, the mRNA levels of the *pg2130*, *pg2131*, *pg2133* and *pg2134* genes were significantly higher in *P. gingivalis* 33277 (type I) and OMZ409 (type II) compared with those in 49417 (type III) and HG564 (type IV; Fig. 2).

Expression of major (long) fimbriae in *P. gingivalis* strains were further examined using a transmission electron microscopy. The *P. gingivalis* strains with different *fimA* genotypes showed the different morphological properties of fimbriae (Fig. 3). ATCC 33277 (type I *fimA*) expressed longer and curly fimbriae, whereas OMZ409 (type II *fimA*) possessed short and slightly curved sunflower-like fimbriae, which is consistent with the previous descriptions by Kato *et al.* (2007). The fimbriae of 49417 (type III *fimA*) and HG564 (type IV *fimA*) were also short but much sparser than those of 33277 and OMZ409. These observations confirm a differential expression of major fimbriae at both mRNA and protein levels in *P. gingivalis* strains.

Comparative analyses of adherence ability of *P. gingivalis* strains with different genotypes

To investigate the association of *fimA* expression and attachment activity of *P. gingivalis*, the binding abilities of these strains to the saliva-coated surfaces and to an earlier colonizer of dental plaque, *S. gordonii* DL1, were tested. As expected, the binding abilities of *P. gingivalis* strains with type I and II FimA were well above those of strains with type III and IV FimA (Fig. 4). Approximately 10% of *P. gingivalis* 33277 cells (type I) and 5% of OZ409 cells (type II) were able to attach and stay on the saliva-coated and *S. gordonii*-coated surfaces during a 2-h incubation. In contrast, only 0.01% and 0.002% of 49417 (type III) and HG564 (type IV) cells, respectively, bound to saliva-coated surfaces for this period of time (Fig. 4 A). The number of HG564 cells bound to *S. gordonii* surfaces was only 0.002% (Fig. 4B). These overall results are in agreement with the expression levels of

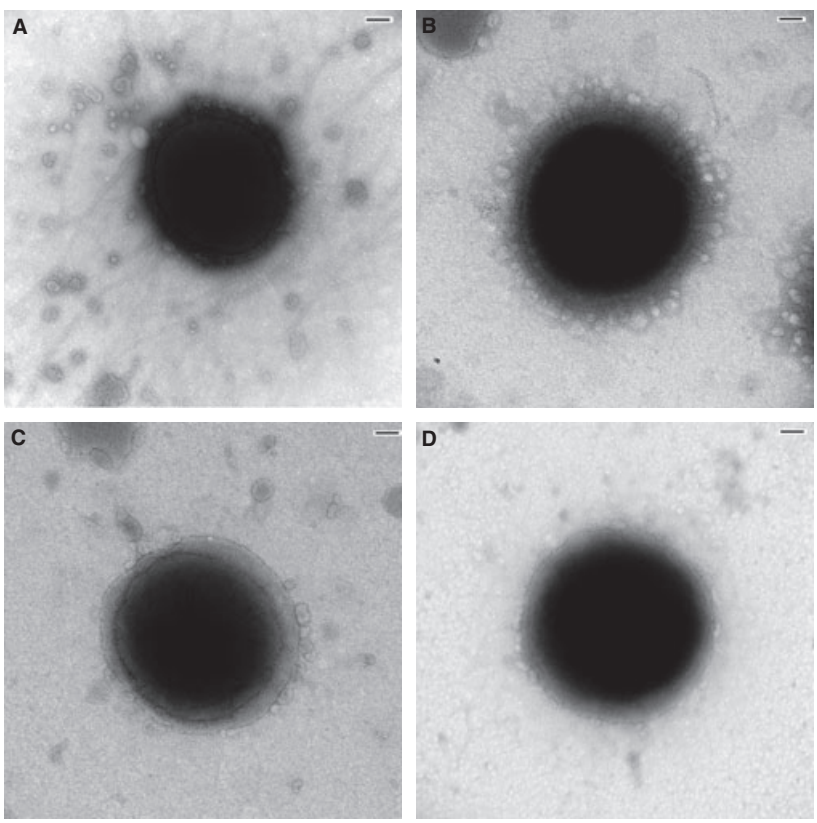

Figure 3 Transmission electron microscopic analysis of *Porphyromonasgingivalis* fimbriae. The *P. gingivalis* strains 33277, type I (A); OMZ409, type II (B); 49417, type III (C); and HG564, type IV (D) were prepared by negatively staining with ammonium molybdate. Fimbrial structures were visualized using transmission electron microscopy. Bars 0.1 μm.

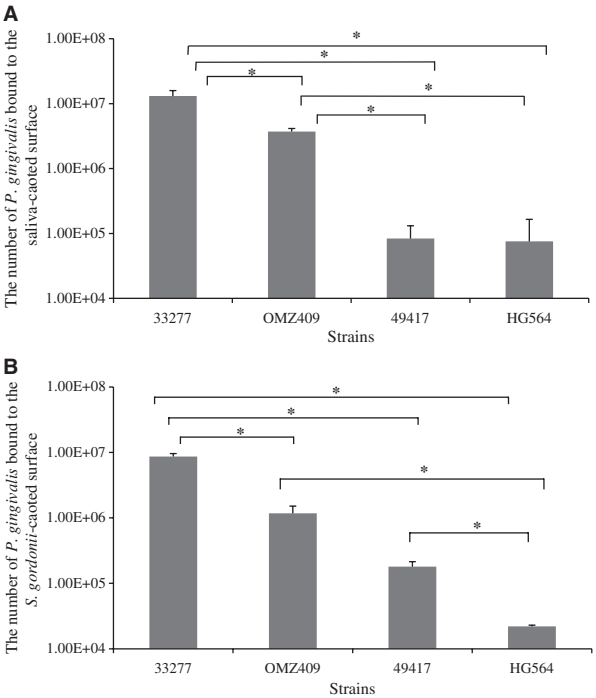

Figure 4 The binding ability of *Porphyromonas gingivalis* strains to a saliva-coated or *Streptococcus gordonii* DL1-covered surface. The *P. gingivalis* cells (6×10^8) were added to polystyrene microtiter wells coated with saliva (A) or *S. gordonii* (B) for 2 h. After washing, the number of *P. gingivalis* cells bound on the bottom of the wells was determined using quantitative polymerase chain reaction. Error bars represent standard deviation ($n = 3$). Comparisons highlighted with an asterisk are significant ($P < 0.05$; Student's *t*-test).

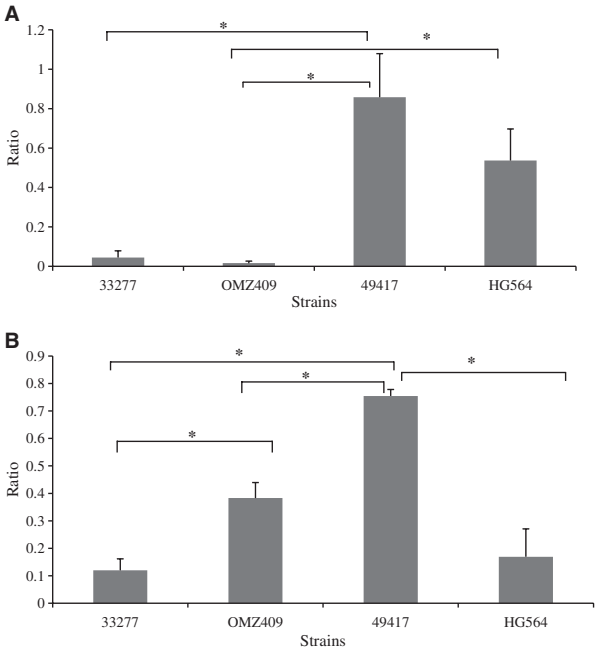

Figure 5 Impact of *fimA* mutations on the binding ability of *Porphyromonas gingivalis* strains. The *P. gingivalis* cells (6×10^8) were introduced onto the saliva-coated (A) or *Streptococcus gordonii* (DL1)-covered (B) polystyrene microtiter dishes and incubated for 2 h. The unbound cells were removed by washing with phosphate-buffered saline. The number of *P. gingivalis* cells left bound on the bottom was determined using quantitative polymerase chain reaction. The ratio of the number of each *fimA* mutant-containing strain bound to the saliva-coated surface or to an *S. gordonii*-covered surface to the number of the parent strain bound is presented. An asterisk indicates the statistical significance of binding activity differences between the mutant and the parent strain ($P < 0.05$; *t*-test).

fimA in different *P. gingivalis* strains and also with previous reports (Hamada *et al.*, 1994; Lin *et al.*, 2006) supporting a critical role of FimA in promoting the attachment of *P. gingivalis* to various surfaces.

To confirm the different abilities of the various genotypes of FimA, four *P. gingivalis* mutants, each with a *fimA* deficiency, were constructed from the four parent strains. The attachment properties of these FimA mutants to saliva-coated and *S. gordonii* surfaces were examined and compared with those of the parent strains. Binding ability to the saliva surface was abolished in the 33277_Δ*fimA* mutant and OMZ409_Δ*fimA* mutant compared with their parent strains (Fig. 5A), whereas 50% of the ability of the HG564_Δ*fimA* mutant remained compared with its parent strain. Interestingly, a significant difference was not observed in the saliva binding ability when comparing the 49417_Δ*fimA* mutant and its parent strain, suggesting that type III FimA is not essential for the binding ability.

All mutant strains did have a significantly lower ability to bind to *S. gordonii* surfaces compared with their parent strains, suggesting that FimA plays a role in these interactions. However, the extent of that reduced ability varied among the mutants (Fig. 5B). The binding ability of the 49417_Δ*fimA* mutant to *S. gordonii* was reduced by only about 25% of that found in the wild-type strain. The reduced binding ability for other three types ranged from 60% to 90%; the 33277_Δ*fimA* mutant lost the greatest fraction of its binding activity compared with the other mutants. It is noteworthy that the OMZ409_Δ*fimA* mutant nearly lost its ability to bind to saliva, but maintained 40% of its binding with respect to *S. gordonii*. Based

on the results, we conclude that type I, II, III and IV FimAs contribute, to different degrees, to the interaction between *P. gingivalis* and *S. gordonii*.

DISCUSSION

FimA has been recognized as one of the major adhesins displayed on the surface of *P. gingivalis* strains. The gene coding FimA has been identified in all *P. gingivalis* strains that were sequenced for this gene (Fujiwara *et al.*, 1993). Although the *fimA* gene is classified into several genotypes based on their sequences of nucleotides, identical sequences were detected in the promoter region of all genotypes of *fimA* (Fujiwara *et al.*, 1993). Kato *et al.* (2007)demonstrated that type II FimA possesses stronger epithelial cell adhesive efficiency than type I FimA. In the present study, we demonstrated differential expression of *fimA* gene in *P. gingivalis* strains with different *fimA* genotypes. Transcription of the *fimA* gene in *P. gingivalis* 33277 (type I) was the highest among these seven strains, followed by strains BH18/10 (type II), OMZ314 (type II) and OMZ409 (type II; Fig. 1). As much as a 20-fold decrease in the transcription of *fimA* was found in HG564 (type IV) in comparison with that of 33277, which is likely to be responsible for the differential ability of these strains to interact with human whole saliva and an earlier colonizer of dental plaque, *S. gordonii*.

Most of the studies examining the interactions between *P. gingivalis* and the surfaces of the oral cavity involve *P. gingivalis* strains with type I FimA, such as 33277 and 381. We compared here the abilities of *P. gingivalis* strains with different FimA genotypes to bind to saliva-coated and *S. gordonii*-coated surfaces. A correlation between the FimA expression level and binding activity was observed in most strains, but particularly for those expressing type I and type II FimA. Expression of *fimA* in *P. gingivalis* 33277 and OMZ409 was significantly higher than that of the other strains with type III and IV FimA. Accordingly strains with type I and II FimA showed much higher adherence activities, suggesting a critical role of these FimAs in the bacterial adherence to oral surfaces. It is important to note that not all binding activities of *P. gingivalis* strains were affected by its expression of FimA. *Porphyromonas gingivalis* 49417 with type III FimA produced significantly less *fimA* than 33277 and OMZ409, but produced more mRNA of *fimA* than HG564 (Fig. 1). There was, however, no significant difference in the ability of *P. gingivalis* 49417 and HG564 to bind to the saliva-coated surfaces (Fig. 3A). This result implies that type III FimA cannot efficiently interact with other salivary molecules in contrast to the other FimA genotypes. This assumption was confirmed by our mutagenesis analysis. In fact, the *fimA* mutation had no significant effect on the ability of *P. gingivalis* 49417 to interact with a saliva-coated surface, whereas a *fimA* deficiency in *P. gingivalis* 33277, OMZ409 and HG564 resulted in significantly decreased binding of these bacteria to saliva-coated surfaces. These results suggest that binding activity of *P. gingivalis* depends not only on the level of *fimA* gene expression, but is also likely to depend on a particular feature of the structure of the FimA protein.

A multimodal model has been proposed to describe *P. gingivalis* adherence to *S. gordonii* (Park *et al.*, 2005). Both major (long) and minor (short) fimbriae of *P. gingivalis* appear to be involved in recognizing and interacting with different surface proteins of *S. gordonii*. FimA, the subunit protein of major fimbriae, interacts with glyceraldehyde-3-phosphate dehydrogenase, whereas Mfa1, the subunit protein of minor fimbriae, interacts with SspB protein, a member of the streptococcal antigen I/II family (Daep *et al.*, 2008). Based on this model, it is anticipated that a genotype-specific *fimA* deficiency may only partially suppress the interaction between *P. gingivalis* and *S. gordonii*. We indeed found a different net effect of a particular *fimA* deficiency upon *P. gingivalis* and *S. gordonii* interactions, based on the type of FimA involved. *Porphyromonas gingivalis* 33277 lost 90% of its ability to interact with *S. gordonii* because of a mutation of its *fimA* gene (Fig. 5B). In *P. gingivalis* OMZ409, the mutation in the *fimA* gene led to complete suppression of the adherence to the saliva-coated surface but had only a 60% negative effect on its adherence to *S. gordonii*. In *P. gingivalis* 49417, the mutation had much less of an effect on its interaction with *S. gordonii* (only $\sim$20% loss), further demonstrating the differential properties of these strains, including their adherence to saliva and *S. gordonii*. Differences in the regulation of *fimA* expression in *P. gingivalis* with different genotypes of FimA and in the adherent properties of these FimA-expressing bacteria may provide a molecular basis for the development of strategies against *P. gingivalis* colonization in the oral cavity.

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