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# Genetic and antigenic analyses of *Porphyromonas gingivalis* FimA fimbriae

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## SUMMARY

**The periodontal pathogen *Porphyromonas gingivalis* generally expresses two distinct fimbriae, FimA and Mfa1, which play a role in biofilm formation. The *fimA* gene that encodes FimA fimbrilin is polymorphic, and polymerase chain reaction analysis has identified six genotypes called types I–V and Ib. We found recently that fimbriae exhibit antigenic heterogeneity among the genotypes. In the present study, we analysed the *fimA* DNA sequences of 84 strains of *P. gingivalis* and characterized the antigenicity of FimA fimbriae. Strains analysed here comprised 10, 16, 29, 13, 10 and 6 strains of types I, Ib, II, III, IV and V, respectively. DNA sequencing revealed that type Ib does not represent a single cluster and that type II sequences are remarkably diverse. In contrast, the *fimA* sequences of the other types were relatively homogeneous. Antigenicity was investigated using antisera elicited by pure FimA fimbriae of types I–V. Antigenicity correlated generally with the respective genotype. Type Ib strains were recognized by type I antisera. However, some strains showed cross-reactivity, especially, many type II strains reacted with type III antisera. The levels of fimbrial expression were highly variable, and expression was positively correlated with ability of biofilm formation on a saliva-coated plate. Further, two strains without FimA and Mfa1 fimbriae expressed fimbrial structures, suggesting that the strains produce other types of fimbriae.**

## INTRODUCTION

*Porphyromonas gingivalis*, a gram-negative anaerobic bacterium, is closely associated with the initiation and progression of periodontal disease (Lamont & Jenkinson, 1998; Darveau, 2010). Recently, some researchers have designated *P. gingivalis* as a keystone pathogen, meaning that it plays a central role in the development of this disease (Hajishengallis *et al.*, 2011; Darveau *et al.*, 2012). This pathogen causes chronic inflammation in the periodontal tissues by colonizing the gingival sulcus through biofilm formation in cooperation with multiple bacterial species.

*Porphyromonas gingivalis* generally expresses two distinct types of fimbriae called FimA and Mfa1, which are filamentous structures expressed on the bacterial surface (Yoshimura *et al.*, 2009). Each is required for the formation of biofilms. FimA and Mfa1 fimbriae are largely composed of polymers of FimA and Mfa1 proteins, respectively. Several accessory components are associated as minor subunits of both fimbriae. It was thought that FimA fimbriae are long (several micrometres), based on studies of the commonly used strains ATCC 33277 (33277) and 381. However, we showed that these strains have the same single and nonsense mutations in *fimB*, and these mutations caused the elongation of the FimA fimbriae (Nagano *et al.*, 2010). Further, *P. gingivalis* strains do not always exhibit long FimA fimbriae (Kato *et al.*, 2007; Zheng *et al.*, 2011). Hence, the average length of FimA fimbriae is not defined. In

contrast, Mfa1 fimbriae are approximately 100 nm (Park *et al.*, 2005; Hasegawa *et al.*, 2009), although we observed considerable variation in length (unpublished data).

The *fimA* gene encoding FimA exists as a single copy in the chromosome of *P. gingivalis* (Dickinson *et al.*, 1988). A polymerase chain reaction (PCR) -based genotyping method has defined six genotypes called types I–V and Ib (Amano *et al.*, 1999; Nakagawa *et al.*, 2000, 2002; Kuboniwa *et al.*, 2010). We hereafter use the term 'genotype' for this PCR-based classification system. Epidemiologic research showed that strains possessing *fimA* of types II and IV were prevalent in patients with periodontitis, while the type I strain was predominantly detected in healthy individuals (Missailidis *et al.*, 2004; Miura *et al.*, 2005; Kuboniwa *et al.*, 2010; Enersen, 2011). However, an *in vitro* study showed that type II and IV strains did not always show high autoaggregation and adhesion activities to mammalian cells that are associated with the formation of biofilms (Umeda *et al.*, 2006; Inaba *et al.*, 2008; Gao *et al.*, 2012). Hence, the relationship between genotype and pathogenicity is unknown.

Using five polyclonal antisera raised by immunizing mice with pure FimA fimbriae of types I–V (except for type Ib), we recently determined that the FimA fimbriae among the genotypes are antigenically heterogeneous (Nagano *et al.*, 2012a,b). The antisera mostly showed genotype-specific reactivity. Although there are identical or similar amino acid sequences among the genotypes, cross-reactivity was quite low. Additionally, the antisera largely reacted to FimA polymers but only marginally to the monomers. We concluded that based on these results, the specific antibodies recognized preferentially a conformation or a discontinuous epitope of FimA polymers. These findings suggest that the different genotypes account for the variations in structures of the fimbriae, although different bioactivity has not been elucidated.

The goal of the present study was to further characterize FimA fimbriae by analysing 84 strains of *P. gingivalis* collected primarily before the '*fimA* genotype era', (DNA sequencing of *fimA* and characterization of the antigenicity of FimA fimbriae). We revisit here also whether *P. gingivalis* expresses another type of fimbriae.

## METHODS

### Strains and culture conditions of *P. gingivalis*

We used 84 strains of *P. gingivalis* from our laboratory stock (Fig. 1 and Table 1). They were cultivated on Brucella HK Agar (Kyokuto Pharmaceutical Industrial Co., Ltd, Tokyo, Japan) supplemented with 5% laked rabbit blood and in Modified GAM broth (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) at 37°C under anaerobic conditions. The *P. gingivalis* strains 33277, TDC60, 6/26, HG564 and HNA99 were used as prototype strains expressing FimA fimbriae of genotypes I, II, III, IV and V, respectively. We also used two mutants derived from 33277; one is deficient in both FimA and Mfa1 expression caused by deletion of the whole *fim* gene cluster and *mfa1* (Nagano *et al.*, 2012b), and *fimA* is deleted from the other, and therefore only expresses Mfa1 fimbriae (Hasegawa *et al.*, 2009).

### DNA sequencing, genotyping and alignment of *fimA* nucleotide sequences

Genomic DNA purified using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used as a template for PCR. Primers were designed from common sequences among the prototype strains (5′-CTATATGCAAGACAATCTCTAAATG-3′ and 5′-TTCCGATATAGACAAACTATGAAAG-3′), and the sequences were derived from the flanking region of *fimA*. DNA cycle sequencing was performed with the purified PCR product as a template using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The products of the DNA cycle-sequencing reaction were purified and analysed using a 3130 Genetic Analyzer (Applied Biosystems). DNA sequences determined in this study were deposited in the DDBJ (http://www.ddbj.nig.ac.jp/sub-e/html, and each identification number (accession number) is shown in Table 1. The *fimA* genes were genotyped *in silico* by examining whether there are genotype-specific primer sequences and restriction enzyme recognition sites (Amano *et al.*, 1999; Nakagawa *et al.*, 2000, 2002). CLUSTALW (http://www.genome.jp/tools/clustalw/) was used to perform a multiple sequence alignment and to generate a phylogenetic tree. Although using primer sets

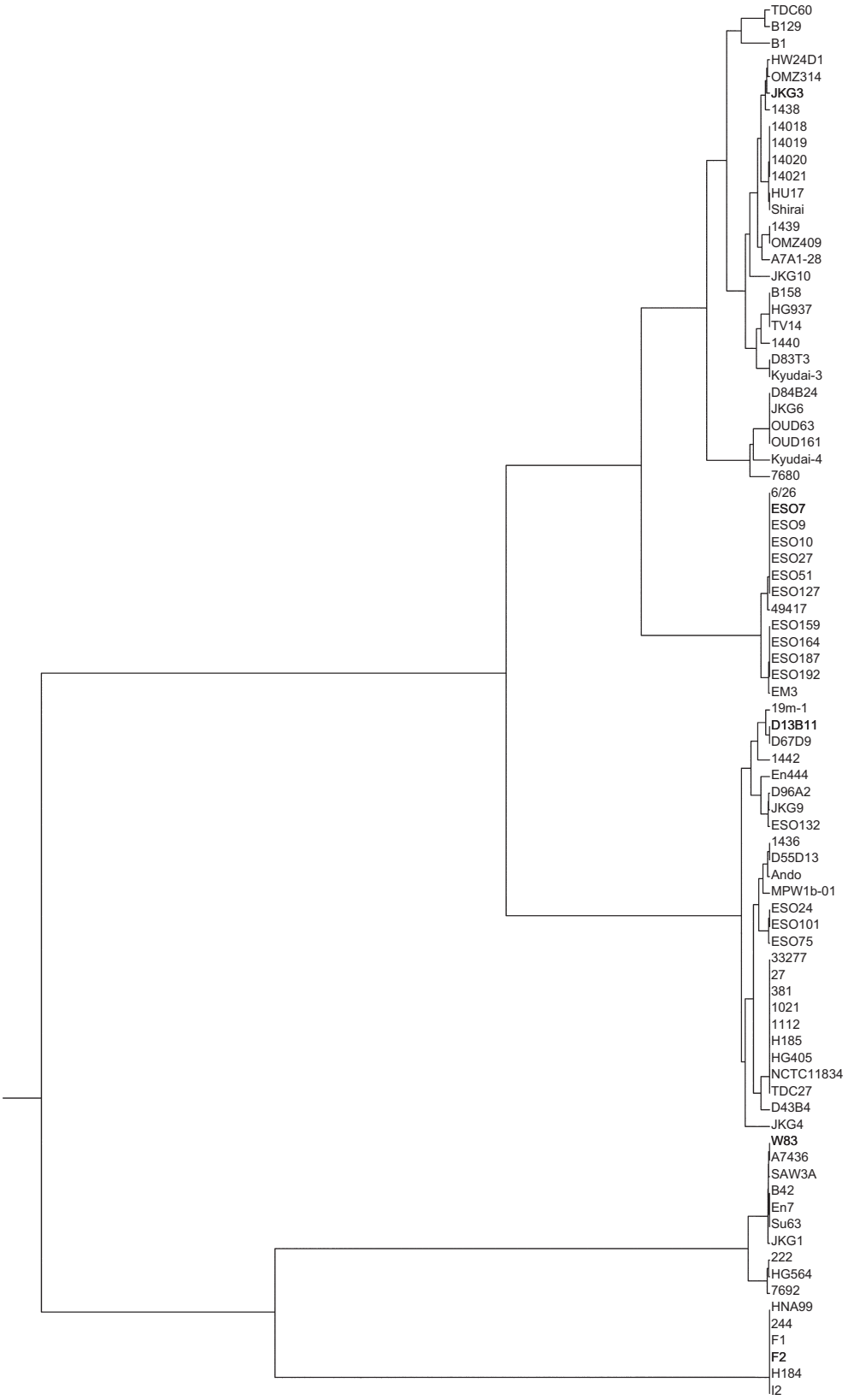


**Figure 1** Phylogenetic tree of *fimA* genes. Whole open reading frames of *fimA* gene were aligned using CLUSTALW.

**Table 1** Characteristics of FimA fimbriae, biofilm formation and fimbrial expression of *Porphyromonas gingivalis* strains

| Strain | Accession number | PCR-based genotype | Antibody titre ($OD_{490}$) Antisera elicited by FimA fimbriae of genotype I | II | III | IV | V | Western blot of FimA | Biofilm (OD unit) | Fimbrial structure (TEM) |
|---|---|---|---|---|---|---|---|---|---|---|
| TDC60 | NC_015571 | II | 0.043 | 0.559 | 0.098 | 0.001 | −0.010 | + | 0.000 | + |
| B129 | AB795754 | II | 0.000 | 0.856 | 0.118 | 0.001 | −0.010 | + | 2.930 | + |
| B1 | AB795753 | II | −0.002 | 0.081 | −0.002 | 0.002 | −0.009 | – | −0.013 | + |
| HW24D1 | D17797 | II | 0.002 | 0.606 | 0.186 | 0.002 | −0.006 | | 0.845 | |
| OMZ314 | AB795765 | II | −0.002 | 0.935 | 0.483 | −0.004 | 0.015 | | 0.022 | + |
| JKG3 | AB795760 | II | 0.026 | 0.032 | 0.006 | 0.040 | 0.010 | – | −0.013 | + |
| 1438 | AB795744 | II | 0.005 | 0.179 | 0.054 | 0.010 | −0.001 | | 0.044 | |
| 14018 | AB795747 | II | 0.005 | 0.360 | 0.225 | 0.003 | −0.007 | | 0.096 | + |
| 14019 | AB795748 | II | 0.005 | 0.288 | 0.179 | 0.002 | −0.001 | | 0.144 | |
| 14020 | AB795749 | II | −0.003 | 0.252 | 0.156 | 0.002 | 0.015 | | 0.063 | |
| 14021 | AB795750 | II | 0.002 | 0.222 | 0.136 | 0.003 | 0.002 | | 0.053 | |
| HU17 | AB795759 | II | −0.002 | 0.689 | 0.461 | 0.001 | −0.006 | | 0.489 | |
| Shirai | AB795769 | II | −0.002 | 0.499 | 0.310 | 0.004 | 0.016 | | 0.816 | + |
| 1439 | AB795745 | II | 0.003 | 0.645 | 0.288 | 0.003 | −0.002 | | 0.184 | |
| OMZ409 | AB795766 | II | −0.001 | 0.083 | 0.018 | 0.004 | −0.005 | + | 0.124 | + |
| A7A1-28 | AB795752 | II | 0.006 | 0.405 | 0.198 | 0.002 | −0.012 | | 0.134 | |
| JKG10 | AB795762 | II | −0.002 | 0.662 | 0.274 | 0.003 | 0.000 | | 0.127 | |
| B158 | AB795755 | II | −0.001 | 0.031 | 0.003 | 0.002 | 0.014 | – | −0.026 | – |
| HG934 | AB795758 | II | −0.002 | 0.072 | 0.010 | 0.002 | 0.004 | | −0.009 | |
| TV14 | AB795770 | II | −0.005 | 0.018 | 0.004 | 0.009 | 0.010 | – | −0.023 | + |
| 1440 | AB795746 | II | 0.005 | 0.362 | 0.095 | 0.008 | −0.005 | | 0.272 | |
| D83T3 | AB795756 | II | −0.003 | 0.106 | −0.007 | 0.002 | −0.012 | – | 0.087 | + |
| Kyudai-3 | AB795763 | II | −0.001 | 0.008 | −0.006 | 0.000 | −0.011 | – | 0.015 | + |
| D84B24 | AB795757 | II | 0.096 | 0.425 | 0.199 | 0.015 | 0.016 | | 0.008 | |
| JKG6 | AB795761 | II | 0.070 | 0.272 | 0.130 | 0.038 | 0.035 | | 0.406 | |
| OUD63 | AB795767 | II | 0.082 | 0.613 | 0.239 | 0.007 | 0.005 | | 0.787 | |
| OUD161 | AB795768 | II | 0.046 | 0.495 | 0.189 | 0.003 | 0.009 | | 0.132 | |
| Kyudai-4 | AB795764 | II | −0.001 | 0.058 | −0.003 | 0.004 | 0.053 | – | 0.276 | + |
| 7680 | AB795751 | II | 0.004 | 0.094 | 0.028 | 0.000 | 0.001 | | 0.313 | |
| 6/26 | AB795771 | III | 0.003 | 0.137 | 1.170 | 0.007 | −0.005 | | 0.163 | + |
| ESO7 | AB795773 | III | −0.003 | 0.018 | 0.452 | 0.003 | −0.011 | | 0.018 | |
| ESO9 | AB795774 | III | 0.001 | 0.052 | 0.545 | 0.010 | −0.003 | | −0.015 | |
| ESO10 | AB795775 | III | −0.003 | 0.013 | 0.337 | 0.004 | −0.010 | | −0.048 | |
| ESO27 | AB795776 | III | −0.002 | 0.018 | 0.314 | 0.003 | −0.012 | | −0.045 | + |
| ESO51 | AB795777 | III | −0.002 | 0.068 | 0.875 | 0.001 | −0.010 | | 0.211 | |
| ESO127 | AB795778 | III | 0.001 | 0.061 | 0.804 | 0.008 | −0.008 | | 0.069 | |
| ATCC 49417 | D17800 | III | −0.002 | 0.241 | 1.595 | 0.006 | −0.004 | | 0.065 | + |
| ESO159 | AB795779 | III | −0.004 | 0.036 | 0.742 | 0.004 | −0.011 | | 0.050 | |
| ESO164 | AB795780 | III | −0.010 | 0.008 | 0.338 | −0.006 | −0.017 | | 0.018 | |
| ESO187 | AB795781 | III | −0.003 | 0.016 | 0.395 | 0.003 | −0.012 | | 0.095 | |
| ESO192 | AB795782 | III | −0.002 | 0.016 | 0.439 | 0.002 | −0.012 | | 0.033 | |
| EM3 | AB795772 | III | 0.001 | 0.043 | 0.713 | 0.004 | 0.004 | | 0.044 | |
| 19 m-1 | AB795731 | Ib | 0.119 | −0.002 | −0.004 | 0.001 | 0.001 | | −0.033 | |
| D13B11 | AB795733 | Ib | 0.140 | 0.000 | −0.002 | 0.003 | 0.010 | | −0.015 | |
| D67D9 | AB795735 | Ib | 0.170 | −0.001 | −0.004 | 0.001 | 0.006 | | −0.023 | |
| 1442 | AB795730 | Ib | 0.763 | 0.009 | 0.001 | 0.014 | −0.009 | | −0.028 | |
| En444 | AB795737 | Ib | 1.146 | 0.023 | −0.002 | 0.013 | −0.003 | | 0.442 | |
| D96A2 | AB795736 | Ib | 0.998 | 0.077 | 0.023 | 0.029 | 0.033 | | 0.250 | |
| JKG9 | AB795743 | Ib | 1.017 | 0.059 | 0.012 | 0.006 | 0.047 | | 0.108 | |

(continued)

Table 1. (Continued)

| Strain | Accession number | PCR-based genotype | Antibody titre ($OD_{490}$) Antisera elicited by FimA fimbriae of genotype I | II | III | IV | V | Western blot of FimA | Biofilm (OD unit) | Fimbrial structure (TEM) |
|---|---|---|---|---|---|---|---|---|---|---|
| ESO132 | AB795741 | Ib | 0.499 | 0.013 | −0.002 | 0.002 | −0.009 | | 0.264 | |
| 1436 | AB795729 | Ib | 0.034 | −0.002 | −0.005 | 0.000 | −0.007 | − | −0.034 | + |
| D55D13 | AB795734 | Ib | 1.622 | 0.098 | 0.028 | 0.005 | −0.009 | | 0.126 | |
| Ando | AB795732 | Ib | 0.019 | 0.002 | 0.000 | 0.013 | −0.003 | − | −0.036 | + |
| MPW1b-01 | AB795728 | Ib | 1.306 | 0.058 | 0.008 | 0.002 | −0.011 | | 0.323 | |
| ESO24 | AB795738 | Ib | 1.171 | 0.098 | 0.024 | 0.003 | 0.027 | | −0.014 | |
| ESO101 | AB795740 | Ib | 1.038 | 0.065 | 0.008 | −0.001 | −0.007 | | −0.008 | + |
| ESO75 | AB795739 | Ib | 0.608 | 0.057 | 0.011 | 0.022 | 0.019 | | −0.037 | + |
| 33277 | NC_010729 | I | 0.426 | 0.002 | 0.002 | 0.006 | −0.005 | + | 0.527 | + |
| 27 | AB795720 | I | 1.140 | 0.035 | 0.006 | 0.012 | 0.014 | | 2.261 | |
| 381 | D17794 | I | 0.778 | 0.011 | −0.001 | 0.003 | −0.002 | | 0.272 | |
| 1021 | AB795721 | I | 1.649 | 0.100 | 0.053 | 0.031 | 0.024 | | 3.633 | |
| 1112 | AB795722 | I | 1.564 | 0.067 | 0.047 | 0.011 | 0.019 | | 5.343 | |
| H185 | AB795726 | I | 0.458 | 0.005 | −0.005 | 0.003 | −0.007 | | 0.137 | |
| HG405 | AB795727 | I | 0.395 | 0.011 | 0.000 | 0.006 | 0.022 | | 0.830 | |
| NCTC 11834 | AB795725 | I | 0.894 | 0.016 | 0.002 | 0.008 | −0.002 | | 1.131 | |
| TDC27 | AB795724 | I | 0.862 | 0.015 | −0.003 | 0.005 | 0.001 | | 0.314 | |
| D43B4 | AB795723 | I | 1.186 | 0.006 | −0.003 | 0.002 | 0.006 | | 0.186 | |
| JKG4 | AB795742 | Ib | 0.848 | 0.001 | 0.004 | 0.015 | 0.004 | | 0.006 | |
| W83 | AE015924 | IV | −0.003 | −0.001 | −0.004 | 0.040 | −0.013 | − | −0.040 | − |
| A7436 | AB795785 | IV | −0.001 | −0.006 | −0.008 | 0.626 | −0.004 | | −0.012 | |
| SAW3A | AB795791 | IV | −0.002 | −0.001 | −0.003 | 0.002 | −0.011 | − | −0.043 | + |
| B42 | AB795786 | IV | −0.006 | −0.002 | −0.002 | 1.157 | −0.004 | | −0.034 | |
| En7 | AB795787 | IV | 0.000 | −0.003 | −0.004 | 1.049 | 0.048 | | 0.185 | |
| Su63 | AB795790 | IV | −0.002 | −0.001 | −0.003 | 1.018 | −0.007 | | 0.068 | + |
| JKG1 | AB795789 | IV | −0.003 | −0.002 | −0.004 | 0.983 | 0.012 | | −0.037 | |
| 222 | AB795783 | IV | 0.002 | −0.001 | −0.002 | 0.014 | −0.011 | − | −0.038 | − |
| HG564 | AB795788 | IV | −0.003 | −0.002 | −0.003 | 0.856 | −0.005 | + | 0.234 | + |
| 7692 | AB795784 | IV | −0.002 | −0.001 | −0.002 | 0.017 | −0.012 | − | −0.026 | + |
| HNA99 | AB795792 | V | 0.001 | −0.005 | −0.002 | −0.001 | 0.534 | + | 0.047 | + |
| 244 | AB795793 | V | −0.002 | −0.003 | −0.002 | 0.001 | 0.037 | − | −0.027 | − |
| F1 | AB795794 | V | −0.002 | −0.001 | −0.002 | 0.004 | 0.336 | | 0.071 | |
| F2 | AB795795 | V | 0.001 | 0.000 | −0.004 | 0.004 | 0.576 | | 0.182 | |
| H184 | AB795796 | V | −0.002 | −0.003 | −0.003 | 0.003 | 0.943 | | 0.189 | |
| I2 | AB795797 | V | 0.015 | −0.001 | 0.000 | 0.010 | 1.440 | | 0.134 | + |

Strains are arranged in the same order as in Fig. 1.
Data represent the mean values in ELISA and biofilm assay.
+, detected; −, not detected; blank, not tested.

for genotyping remains controversial (Moon *et al.*, 2012), we chose ones that have been used widely.

### Enzyme-linked immunosorbent assay

We used antisera to FimA fimbriae that were previously prepared in our laboratory (Nagano *et al.*, 2012b). The antisera were elicited by type I (from 33277), type II (from TDC60), type III (from 6/26), type IV (from HG564) and type V (from HNA99) fimbriae. Sera from unimmunized mice (normal sera) were used as controls. Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (Nagano *et al.*, 2012b). Briefly, whole-cell sonicates of *P. gingivalis* strains (50 μg protein) were coated onto the wells of an ELISA plate. After the wells were blocked, mouse sera diluted 1 : 4000 were added as described above. Anti-mouse

immunoglobulins conjugated to horseradish peroxidase were added and then *o*-phenylenediamine and $H_2O_2$ in citrate buffer (pH 5.0) were added to detect the antigen–antibody complexes. The reaction was terminated and the optical density at 490 nm ($OD_{490}$, reference $OD_{620}$) was measured. The values for the normal sera for each strain and for each antiserum against the FimA- and Mfa1-deficient mutant were subtracted from each value for sera from the immunized mice. As a result, some values are negative.

### Biofilm formation assay

Ability of biofilm formation was examined as previously described (Kuboniwa *et al.*, 2009; Kishi *et al.*, 2012) with slight modifications. Briefly, overnight cultures of *P. gingivalis* strains were adjusted for the turbidity to 0.5 of $OD_{600}$ in a fresh medium. Aliquots (100 μl) were placed on saliva-coated wells of a flat-bottom 96-well polystyrene plate, then incubated in anaerobic conditions for 24 h at 37°C. Unbound bacterial cells were removed by gently washing with phosphate-buffered saline, pH 7.4, then cells were stained with 100 μl of 0.1% (weight/volume) crystal violet for 10 min. After washing with phosphate-buffered saline to remove excess dye, the dye was eluted with 100 μl ethanol. Biofilm volume was evaluated by measuring $OD_{595}$. When $OD_{595}$ values showed over 2.0, they were measured again after appropriate dilution. A blank value (medium alone) was subtracted from each value, and some values were negative.

### Western blot analysis

Western blotting was performed using a standard method. Briefly, whole cell sonicates were denatured by mixing with five-fold concentrated loading buffer (1 M Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate, 50% glycerol, 20% 2-mercaptoethanol and bromophenol blue), boiled for 10 min, and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. We also used antisera to FimA monomer of 33277 (Yoshimura *et al.*, 1985) and Mfa1 monomer of 33277 (Hasegawa *et al.*, 2009). The ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for detection.

### Detection of the *mfa1* gene in chromosomal DNA

Detection of the *mfa1* gene was performed using standard PCR conditions. Because the genomic sequences of 33277 and TDC60 are published (Naito *et al.*, 2008; Watanabe *et al.*, 2011), specific primers were designed using common sequences (5′-GGCTTTGCTTCTTGTAGTAAAGAGG-3′ and 5′-GAATGAACTTTCCAAGGCAAAACTG-3′) that prime the amplification of an internal region of *mfa1*.

### Reverse transcription-PCR

We examined whether *fimA* and *mfa1* were transcribed using reverse transcription (RT-) PCR. Total RNA was isolated using ISOGEN (Nippon Gene Co., Ltd, Tokyo, Japan) and treated with Recombinant DNase I, RNase-free (Takara Bio Inc., Otsu, Japan) to eliminate contaminating genomic DNA. The purified RNA (100 ng) was used to generate cDNA with a PrimeScript RT-PCR Kit (Takara Bio Inc.). The resulting cDNA was used as a template for PCR. The primers used for detecting *fimA* types I, Ib, and II were 5′-AATACGGGTGCAATGGAACTGG-3′ and 5′-CTCTGTGATAGGATTCTCTGGG-3′, and 5′-CTTGTTGGGACTTGCTGCTCTTG-3′ and 5′-CCTTCTTGAATTTGACCTGCATAGACCAT-3′ were used for types IV and V. Primers for *mfa1* were the same as those used to detect the *mfa1* gene. After a standard 25-cycle PCR, the transcriptional activity was determined by detecting the target band using agarose electrophoresis.

### Transmission electron microscopy

Bacterial cells were negatively stained with 1% ammonium molybdate and observed with a JEM-1210 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

### Statistics

One-way analysis of variance and the Dunnett multiple-comparison test were used to evaluate differences among groups. Significance was defined as a $P$ value $< 0.01$. Pearson correlation coefficients ($r$ values) were used to investigate the association between levels of FimA fimbrial expression and biofilm formation.

## RESULTS AND DISCUSSION

### Multiple alignment of *fimA* DNA sequences

We sequenced the open reading frames of the *fimA* genes of 84 strains. The sequences of nine were previously published. All strains were uniquely classified into one of the genotypes as follows: 10, 16, 29, 13, 10 and 6 strains of types I, Ib, II, III, IV and V, respectively (Table 1). Figure 1 shows a phylogenetic tree generated from the alignments. Type I sequences were almost homogeneous, whereas those of type Ib did not represent a single cluster. Type II sequences were diverse and could be divided into subgroups. When compared with the diversity of type II, types I and Ib are genetically close to each other. Re-classification of the genotypes might be required. Although types III and IV contained two subgroups, they were relatively homogeneous. The type V sequences of six strains were identical. A phylogenetic tree based on the alignments of deduced amino acid sequences was very similar to that generated from the DNA sequences (data not shown).

### Antigenic analysis of FimA fimbriae

The antigenicity of 84 strains was analysed using antisera elicited by pure FimA fimbriae of types I–V. Figure 2 and Table 1 show that all the type I strains reacted with anti-type I antisera (anti-I sera). Type Ib strains were also recognized by anti-I sera, although 1436 and Ando did not react. Types I and Ib did not react with other antisera with the exception of some strains (D96A2, D55D13, ESO24, ESO101, 1021 and 1112), which reacted slightly with anti-II sera. Most type II strains reacted well with anti-II sera. However, many type II strains reacted substantially with anti-III sera, although they all showed higher values with anti-II sera than those of anti-III sera. The strains D84B24, JKG6 and OUD63 cross-reacted slightly with anti-I sera. The type II strains (JKG3, B158, TV14, Kyudai-3 and Kyudai-4) barely reacted with any antisera. All type III strains reacted with anti-III sera, and strains with high titres for the anti-III sera (6/26 and ATCC 49417) also reacted with anti-II sera. Types IV and V strains reacted only with their own antisera. Some strains did not react with any antisera in these types (W83, SAW3A, 222 and 7692 in type IV, and 244 in type V).

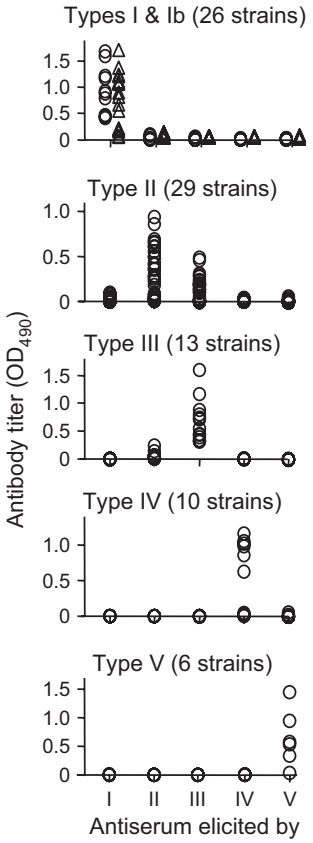


**Figure 2** Seroreactivities of FimA fimbriae. Antisera elicited by pure FimA fimbriae from 33277 (type I), TDC60 (type II), 6/26 (type III), HG564 (type IV) and HNA99 (type V) were used as the primary antibody in enzyme-linked immunosorbent assays. Both values derived from the non-immune sera and sera raised to a FimA-negative mutant were subtracted from all data. Each symbol represents a mean of duplication. Circle and triangle symbols denote types I and Ib, respectively, in top panel.

The antigenicity of FimA fimbriae is not uniform (Suzuki *et al.*, 1988; Lee *et al.*, 1991). Recently, we also found that FimA fimbriae showed differential antigenicity among the genotypes I–V (Nagano *et al.*, 2012b). In the present study, we show heterogeneous antigenicity among fimbriae among 84 strains of *P. gingivalis*. We also show that there are at least five serotypes among FimA fimbriae that are closely associated with the genotype. However, cross-reactivity was detected between some genotypes, in particular, type II strains were recognized by anti-III sera, suggesting that they might express common epitopes. We did not detect a difference between the reactivities of types I and Ib or notice a differential tendency for reactivity with type II, although the sequences

among isolates belonging to this type are highly variable. We plan to conduct computational studies to define the antigenic determinants by analysing unique and common peptide sequences between genotypes.

The antibody titres varied significantly, which could have been caused by differential antigenicity, or differential levels of fimbrial expression, or both. The antibody titres for some strains varied, although their *fimA* and FimA sequences were almost identical, that is, their antigenicities were probably equivalent. Examples include 1436 and D55D13 in type Ib, 222 and HG564 in type IV, and all type V strains. These results indicate that the levels of fimbrial expression vary considerably among strains.

### Biofilm formation assay

We next investigated the ability of biofilm formation on a saliva-coated plate (Table 1 and Fig. 3). In type I, there were strains that showed considerably high values, and the type was statistically significantly higher than the other types. Type II also contained strains that formed massive biofilms and showed a relatively high mean value although this was not significantly different statistically. There were also strains that showed substantial values in the other types.

We then analysed correlation between levels of biofilm formation and FimA fimbrial expression (ELISA values). Figure 4 showed that there were positive correlations in all genotypes. The strongest correlation was observed relative to type I strains. Type V also showed a strong correlation. However, the fimbrial expression was not always a crucial factor for biofilm formation because there were strains that did not form biofilms even though they showed high FimA fimbrial expression. Indeed, *P. gingivalis* biofilm formation is affected by many factors, such as the cell surface hydrophobicity, gingipains, haemagglutinin, capsule and other types of fimbriae (Davey & Duncan, 2006; Amano, 2010; Bostanci & Belibasakis, 2012).

Studies on *P. gingivalis* have analysed the correlation between genetic variability and pathogenicity (Igboin *et al.*, 2009; Kuboniwa *et al.*, 2010; Enersen, 2011; Enersen *et al.*, 2013); types II and IV were strongly associated with the severe periodontitis, and type II actively adhered to and invaded into the epithelial cells. Here we showed that type I exhibited the highest value, on average, for biofilm formation, but there were strains even in other genotypes that showed a high activity of biofilm formation. Importantly, the fimbrial expression levels were correlated with the ability of biofilm formation. Hence, it is necessary to pay attention not only to the genotype but also to the fimbrial expression level.

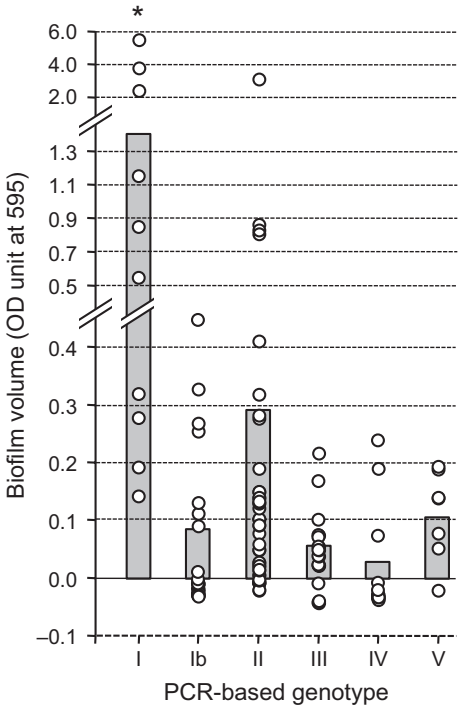


**Figure 3** Biofilm formation assay. *Porphyromonas gingivalis* strains (0.5 of $OD_{600}$) were anaerobically incubated for 24 h on a saliva-coated plate. Biofilm formation was evaluated by measuring eluted crystal violet at $OD_{595}$. Each symbol represents a mean of six sets of measurements, and grey column represents a mean of each genotype. Type I showed higher than the other types with statistical significance (*$P < 0.01$).

### Examination of FimA protein expression of strains with low values in ELISA

Certain strains did not react with any antisera tested here. Further, Western blot analysis did not detect a FimA-specific band (Table 1). Hence, we predicted that they rarely express FimA proteins. To investigate this in more detail, we examined whether transcription occurred in strains without expression of FimA protein using RT-PCR. Many of them (B158 and TV14 in type II, 1436 in type Ib, 222 and 7692 in type IV, and 244 in type V) showed a faint band, indicating that they had a slight transcriptional activity. Although these might express FimA fimbriae/proteins, it was very low and could not be detected by ELISA and

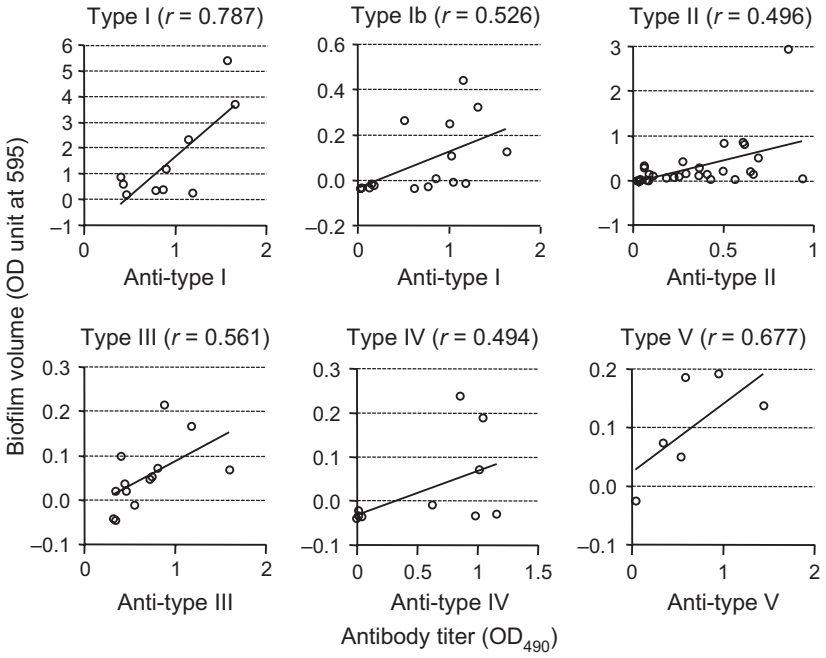


**Figure 4** Correlation between levels of FimA fimbrial expression and biofilm formation. *r* denotes correlation coefficient.

Western blotting. JKG3 (type II), Kyudai-3 (type II), Ando (type Ib) and SAW3A (type IV) did not show any signs of transcription. The afimbrial strain W83 (type IV) has only a trace amount of FimA transcriptional activity because of a deficiency in signal transduction systems (Nishikawa & Duncan, 2010). These strains might also have a similar defect. However, Kyudai-4 showed a similar level of transcription as the prototype strain TDC60 (data not shown). Further study is therefore required to understand why the protein was not detected, although transcriptional activity was high.

### Transmission electron microscopy observation of fimbriae

Next, we determined whether fimbrial structures could be observed on cells of the strains that expressed low levels of FimA (Table 1 and Fig. 5). We did not find fimbrial structures on B158, W83, 222 and 244. However, the others expressed variable numbers of fimbrial structures.

### Detection of Mfa1 and transcription of *mfa1*

Because there was a possibility that strains that either expressed low or undetectable levels of FimA fimbriae expressed Mfa1 fimbriae, we performed Western blotting to detect Mfa1 fimbriae. Expression was detected in JKG3, Kyudai-3, Kyudai-4 and 1436, all of which rarely produce FimA fimbriae. It is therefore reasonable to conclude that fimbrial structures present on the cells of these strains represent Mfa1 fimbriae. However, we do not exclude the possibility that other strains express Mfa1 fimbriae. We used an anti-Mfa1 antiserum elicited by Mfa1 derived from 33277; however, as was the case for FimA fimbriae, the antiserum might not recognize any types of Mfa1 fimbriae because of differences in antigenicities from the Mfa1 of 33277. Indeed, the *mfa1* DNA sequences of 33277 and TDC60 are different, suggesting the antigenic heterogeneity of Mfa1 as well, although the anti-Mfa1 antiserum recognized the Mfa1 of TDC60.

We next examined the *mfa1* gene and its transcription. The *mfa1* gene was not detected in Ando, SAW3A, 222, 7692 or 244. However, this could be due to genetic polymorphism. We examined the transcriptional activity of strains in which *mfa1* was detected. Two strains (B158 and TV14) that were negative in Western blot analysis exhibited transcriptional activity. These might express Mfa1 fimbriae with different antigenicity from Mfa1 fimbriae of 33277.

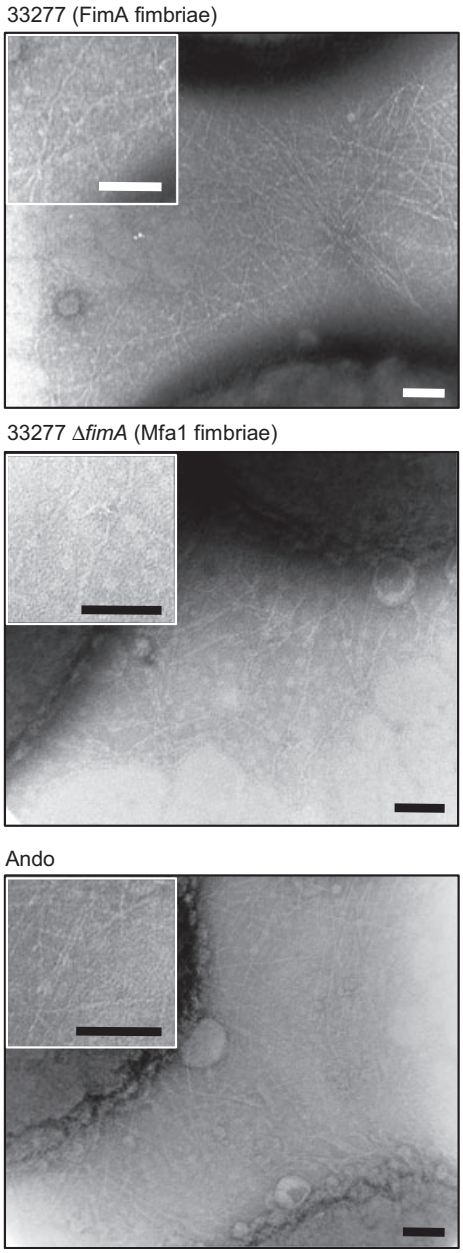


**Figure 5** Transmission electron micrographs of bacterial surface structures. Intact bacteria were negatively stained with 1% ammonium molybdate. Strain 33277 abundantly expressed FimA fimbriae, while a *fimA*-deletion mutant of 33277 only expressed Mfa1 fimbriae. Fimbrial structures were observed on cells of the Ando strain, although this strain did not detectably express *fimA*/FimA or *mfa1*/Mfa1. Bars indicate 50 nm.

### Other types of fimbriae

Finally, Ando and SAW3A did not detectably express *fimA/mfa1* or FimA/Mfa1, although they expressed fimbrial structures. Ando expressed abundant fimbrial structures (Fig. 5), and SAW3A expressed only a few. Filaments formed by FimA and Mfa1 fimbriae were curly (Fig. 5), consistent with previous reports (Yoshimura *et al.*, 1984; Park *et al.*, 2005), while the fimbriae of Ando were almost straight.

It has been reported that a *P. gingivalis* strain expresses another type of fimbriae called 53k fimbriae (Hongyo *et al.*, 1997; Arai *et al.*, 2000; Togashi *et al.*, 2006). The fimbriae of Ando and SAW3A might represent the 53k fimbriae. However, these fimbriae have not been studied in detail; future study of their fimbrial features and functions is planned.

## CONCLUSIONS

Analysis of 84 strains of *P. gingivalis* revealed that FimA fimbriae showed five distinct antigenicities, which were almost consistent with the *fimA* genotype, although the DNA sequences were quite divergent among some genotypes. We also showed that the expression level of the fimbriae varied significantly. Type I showed a high ability of biofilm formation. Moreover, all genotypes showed a positive correlation between levels of FimA fimbrial expression and biofilm formation. We may have discovered strains that express fimbriae other than FimA and Mfa1.

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