

Involvement of minor components associated with the FimA fimbriae of *Porphyromonas gingivalis* in adhesive functions

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The FimA fimbriae of *Porphyromonas gingivalis*, the causative agent of periodontitis, have been implicated in various aspects of pathogenicity, such as colonization, adhesion and aggregation. In this study, the four open reading frames (ORF1, ORF2, ORF3 and ORF4) downstream of the fimbriin gene (*fimA*) in strain ATCC 33277 were examined. ORF2, ORF3 and ORF4 were demonstrated to encode minor components of the fimbriae and were therefore renamed *fimC*, *fimD* and *fimE*, respectively. Immunoblotting analyses revealed that inactivation of either *fimC* or *fimD* by an *ermF-ermAM* insertion, but not inactivation of ORF1, was accompanied by concomitant loss of the products from the downstream genes, raising the possibility that *fimC*, *fimD* and *fimE* constitute a transcription unit. The *fimE* mutant produced FimC and FimD, but fimbriae purified from it contained neither protein, suggesting that FimE is required for the assembly of FimC and FimD onto the fimbriin (FimA) fibre. The *fimC*, *fimD* and *fimE* mutants lost autoaggregation abilities. Fimbriae purified from these three mutants showed attenuated binding activities to glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* and to two extracellular matrix proteins, fibronectin and type I collagen. These results suggest that FimE, as well as FimC and FimD, play critical roles in the adhesive activities of the mature FimA fimbriae in *P. gingivalis*.

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INTRODUCTION

Porphyromonas gingivalis is a Gram-negative, black-pigmented obligate anaerobe that has been implicated in adult periodontitis (Lamont & Jenkinson, 1998), which is a major cause of tooth loss in the adult population. This bacterium settles in the gingival crevice by forming a mixed-species consortium with a number of other Gram-negative and -positive bacteria. *P. gingivalis* can also invade and persist within gingival epithelial cells (Andrian *et al.*, 2006). Consistent with these modes of colonization, the adhesion of the bacterium to host tissues and other oral bacteria via its fimbriae has been implicated in its pathogenicity.

P. gingivalis has at least two distinct types of fimbriae (Lamont & Jenkinson, 2000) composed of distinct fimbriins, FimA (the *fimA* gene product) and Mfa1 (the *mfa1* gene product), with apparent molecular masses of about 38 and 75 kDa, respectively. Both types of fimbriae appear to be abundant in some strains and represent evolutionarily unique groups, since no homologues of FimA or Mfa1 have been found in any other bacteria. The FimA fimbriae are approximately 0.3–1.6 µm in length and ~5 nm in diameter (Yoshimura *et al.*, 1984), whereas the Mfa1 (75 kDa) fimbriae are slightly thicker (3.5–6.5 nm in width) and considerably shorter (80–120 nm in length) than the FimA fimbriae (Park *et al.*, 2005).

FimA fimbriae are known to bind to a number of eukaryotic proteins such as fibronectin, collagen, laminin (Hamada *et al.*, 1998), saliva-derived proline-rich protein (Amano *et al.*, 1999) and statherin (Amano *et al.*, 1996), as well as to a prokaryotic protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *Streptococcus oralis*

Abbreviations: CBB, Coomassie brilliant blue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rGAPDH, recombinant glyceraldehyde-3-phosphate dehydrogenase; Sup, supernatant; WC, whole cultures.

The GenBank/EMBL/DDBJ accession no. for the *fimA*, ORF1, *fimC*, *fimD*, and *fimE* sequence of *P. gingivalis* is D42067.

(Maeda *et al.*, 2004b). Interactions between recombinant FimA (rFimA) and some of these proteins were demonstrated with the biomolecular interaction analysis system (BIAcore) (Kontani *et al.*, 1996; Maeda *et al.*, 2004a).

The *fimA* genes from various *P. gingivalis* strains have been classified into six types (I, Ib, II, III, IV and V) (Amano *et al.*, 2004), and each strain carries a single *fimA* gene of one of these types (Dickinson *et al.*, 1988; Takahashi *et al.*, 1992). It has been reported that a large majority of periodontitis patients carry organisms with either type II or IV *fimA*, while type I is the most prevalent *fimA* genotype among *P. gingivalis*-positive healthy adults, although no morphological differences in the fimbriae were reported (Amano *et al.*, 2000). Fimbriae of type I strains (381 and ATCC 33277) have been extensively studied since the 1980s, although the complete genome sequence of this type has not been reported. The genome sequence of the poorly fimbriated strain W83 (type IV) has been completed (Nelson *et al.*, 2003).

In many bacteria, the gene encoding the major fimbriin typically forms a cluster with several additional genes encoding regulatory factors, fimbrial minor components or transport machineries (Soto & Hultgren, 1999). *P. gingivalis* also has a set of fimbriae-related genes flanking the *fimA* gene. Nishikawa *et al.* (2004) identified *fimX*, a gene upstream of *fimA*, as a target of the FimS/FimR two-component regulatory system which controls the expression of *fimA*. Watanabe *et al.* (1996) sequenced the region (~5 kb) downstream of *fimA* and identified four open reading frames (ORF1, ORF2, ORF3 and ORF4). The ORF2 and ORF3 products were reported to be minor components in fimbriae purified from strain 381 and inferred to play critical roles in fimbrial functions (Yoshimura *et al.*, 1993). Takahashi *et al.* (2001) expressed the *fimA* gene of strain 381 in various *P. gingivalis* strains and found that the transformants showed reduced ability to bind some bacteria and mammalian epithelial cells, as well as reduced autoaggregation activity. These results imply that some components that are present on the native fimbriae but absent on the recombinant fimbriae are, at least partly, involved in the adhesion function of FimA fimbriae. Recently, Hajishengallis *et al.* (2006) reported that the wild-type fimbriae can utilize a Toll-like receptor (TLR), TLR1 or TLR6, for cooperative TLR2-dependent activation of transfected cell lines, but the mutant fimbriae lacking the minor components displayed a preference for TLR1 and a stronger inflammatory response than the wild-type fimbriae, suggesting that fimbriae with or without the minor components are discriminated by the innate immune system.

During the course of isolation of fimbria-defective mutants by transposon mutagenesis and purification of fimbriae from ATCC 33277 and its mutant derivatives, we found that ORF4 was longer than previously reported and its size was closely comparable to that of a previously unidentified minor component (60 kDa) of fimbriae purified from

strain 381. Here we report the characterization of the genes downstream of *fimA* and their products, by sequencing the region of two genotype I strains and by constructing isogenic mutants of ATCC 33277 with insertion of an erythromycin-resistance cassette in each gene. The products of three genes, ORF2, ORF3 and ORF4, were found to be minor components of wild-type fimbriae and therefore the genes were renamed *fimC*, *fimD* and *fimE*, respectively (the new nomenclature will be used hereafter). Their products were also shown to play critical roles in autoaggregation and binding to several bacterial and eukaryotic proteins.

METHODS

Bacterial strains and growth conditions. All *P. gingivalis* and *Escherichia coli* strains used in this study are listed in Table 1. The *P. gingivalis* strain ATCC 33277 and its derivatives were grown on blood agar plates (Brucella HK agar base, Kyokuto) supplemented with 5% (w/v) laked rabbit blood, 2.5 µg haemin ml⁻¹, 5.0 µg menadione ml⁻¹ and 0.01% (w/v) DTT at 37 °C under anaerobic conditions. Cells were then cultivated in sTSB liquid medium [trypticase soy broth supplemented with 0.25% (w/v) yeast extract, 2.5 µg haemin ml⁻¹, 5.0 µg menadione ml⁻¹ and 0.01% (w/v) DTT] at 37 °C for 48–60 h under anaerobic conditions. For secondary cultivation, the primary culture was inoculated into sTSB medium at a 1 : 100 dilution and incubated at 37 °C for an additional 24 h. The cells were then harvested and used for subsequent analyses. When necessary, gentamicin (Gm; 200 µg ml⁻¹) or erythromycin (Em; 20 µg ml⁻¹) was added to each medium. *E. coli* strains were grown in LB medium supplemented, when necessary, with kanamycin (50 µg ml⁻¹) or erythromycin (200 µg ml⁻¹).

Cloning and sequencing of ORF1, *fimC* (formerly ORF2), *fimD* (formerly ORF3) and *fimE* (formerly ORF4) in *P. gingivalis* strains 381 and ATCC 33277. Strain SN47-631, isolated as a transposon-generated mutant defective in fimbriation, had an insertion in *fimE* (formerly ORF4), raising the possibility that the gene is involved in biogenesis and function of the FimA fimbriae. We therefore screened a plasmid library of chromosomal DNA from strain 381 (Hasegawa *et al.*, 2003) for *fimE*-positive clones by using primers NSN220 and NSN164 (see Table 2 for primer sequences). Sequencing was carried out using the ABI PRISM Version 1.1 kit and ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems). The revised nucleotide sequence data were deposited in DDBJ (accession no. D42067). Based on the sequence information of the *fimE*-encompassing region from strain 381, a primer set (NSN172 and 227) was designed to amplify the corresponding region from the chromosomal DNA of ATCC 33277. The 2.0 kb fragment was amplified by PCR and cloned into the plasmid vector pCR-BluntI-TOPO (from the Zero Blunt TOPO PCR cloning kit, Invitrogen). Also, the 1.0, 1.4 and 2.1 kb fragments spanning from ORF1 to *fimC* (formerly ORF2), from *fimC* to *fimD* (formerly ORF3) and from *fimD* to *fimE*, respectively, were amplified and cloned into the TOPO vector. The entire DNA sequence from ORF1 to *fimE* of ATCC 33277 was identical to that of strain 381. To construct a plasmid expressing recombinant FimE protein (rFimE), the *fimE* region encoding the putative mature product (nucleotides 112 to 1650) was amplified by PCR using NSN242 and NSN243 as primers and the chromosomal DNA of ATCC 33277 as the template. The resulting fragment was cloned into the expression vector pET200/D-TOPO (Champion pET directional TOPO expression kit, Invitrogen), yielding plasmid pSN140 encoding rFimE His-tagged at its N-terminus. The plasmid

Table 1. Strains used in this study

Strain	Relevant characteristics*	Source or reference
<i>Porphyromonas gingivalis</i>		
381	Heavily fimbriated strain	Yoshimura <i>et al.</i> (1984)
ATCC 33277	Type strain from ATCC, heavily fimbriated	Laboratory stock
SN47-631	Mutant carrying a transposon insertion in <i>fimE</i> , Em ^r	This study
KDP98	<i>fimA</i> insertion mutant of ATCC 33277, Em ^r	Watanabe-Kato <i>et al.</i> (1998)
KO1	ORF1 deletion mutant of ATCC 33277, Em ^r	This study
OZ5001C	ORF2 (<i>fimC</i>) insertion mutant of ATCC 33277, Em ^r	Hongo <i>et al.</i> (1999)
KO3	ORF3 (<i>fimD</i>) insertion mutant of ATCC 33277, Em ^r	This study
KO4	ORF4 (<i>fimE</i>) insertion mutant of ATCC 33277, Em ^r	This study
<i>Escherichia coli</i>		
TOP 10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
BL21 star (DE3)	F ⁻ <i>ompT hsdS_B</i> (<i>r_B⁻ m_B⁻</i>) <i>gal dcm rne131</i> (DE3)	Invitrogen
DH5α	F ⁻ φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i> <i>hsdR17</i> (<i>r_K⁻ m_K⁺</i>) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	Takara Bio

*Em^r, resistance to erythromycin.

was introduced into *E. coli* strain BL21 star (DE3) for overproduction and purification of rFimE.

Protein purification and preparation of antibody. Purification of FimA fimbriae from *P. gingivalis* was performed by a method described previously (Yoshimura *et al.*, 1984) with slight modifications. Purification of rFimE or recombinant glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) (Maeda *et al.*, 2004a) was carried out with a Ni-NTA purification system (Invitrogen), following the manufacturer's instructions. Protein concentrations were measured by using the Micro BCA protein assay kit (Pierce Biotechnology). Polyclonal antibody against rFimE was raised in rabbits as described previously (Yoshimura *et al.*, 1984).

Insertion mutagenesis. All mutants used in this study were derivatives of ATCC 33277 resulting from an erythromycin resistance

gene (Em^r) cassette (*ermF-ermAM*) replacing or being inserted into the gene of interest (Fletcher *et al.*, 1995). The ORF1 mutant (KO1) was constructed by the primer extension method as described previously (Nagano *et al.*, 2005). In brief, the entire coding region of ORF1 (Fig. 1, indicated by a double-headed arrow) in ATCC 33277 was replaced with *ermF-ermAM* by PCR using primers NSN201, 206, 229, 235 and 236 (Table 2). Gene replacement in the resulting strain was confirmed by PCR. The *fimC* mutant (OZ5001C), which has an Em^r cassette insertion at the *Sna*BI site (745 bp) in the *fimC* coding region, was described previously (Hongo *et al.*, 1999). The *fimD* mutant (KO3), which carries an insertion of the whole plasmid sequence including the Em^r cassette between the *Hinc*II and *Bam*HI sites in the *fimD* coding region, was constructed as follows. The *Hinc*II-*Bam*HI fragment (335 bp) of *fimD* was cloned into the suicide vector pVAL7 (Smith & Salyers, 1989). The resulting plasmid, pVAL7-80, was introduced into ATCC 33277 via conjugation and Gm^r Em^r transconjugants were selected. The *fimE* mutant (KO4), which carries an insertion of the Em^r cassette at the *Eco*8II site (384 bp) in the *fimE* coding region, was made by a similar method to that used for the construction of the *gppX* mutant of *P. gingivalis* (Hasegawa *et al.*, 2003).

Table 2. Primers used in this study

Name	Sequence (5'–3')*
NSN164	CTTCCCGGAGCTTGGCTTTC
NSN172	ATGACGGGCAGGTATTATTGG
NSN201	GTAGTCCCGAAGGATGATGG
NSN206	CTGCAGGTCGACTCTAGAG
NSN220	CAAGCTCTACGACGCCAGC
NSN227	CCTGAAAGCGTGGCGCGAG
NSN229	CACCTCGTAAATGCACACGC
NSN235	<u>GCTATCGGGGTACCAACAATGATCTTTGATTG</u>
NSN236	<u>CTAGAGTCGACCTGCAGTTCAGGCTTTCCTCTAC-</u> CG
NSN242	CACCGTAAGCGGTAGCATTGTTC
NSN243	CTAATTCGTACGTGAATCG

*Underlining indicates the overlapping regions of the 5' or 3' end of *ermF-ermAM*.

Sample preparation for detection of fimbriin and fimbrial minor components.

FimA and the products of the genes downstream of *fimA* expressed from *P. gingivalis* cells were detected after precipitation with TCA as described previously (Hongo *et al.*, 1999) except that 2 M Tris was used to neutralize samples instead of washing with diethyl ether. Almost all the bacterial components, including minor, protease-sensitive proteins, could be quantitatively recovered by the TCA precipitation method because strong intrinsic proteolytic activities possessed by this organism were instantaneously inactivated (Hongo *et al.*, 1999). For the preparation of samples from 'whole cultures' (WC), 100 μl 100% (w/v) TCA (Nacalai Tesque) was added directly to 900 μl of each cell culture. Each culture was also fractionated by centrifugation (14 000 g, 3 min). Cells collected from 900 μl of each culture were resuspended in 1 ml 10% (w/v) TCA ('Cell'), whereas 100 μl 100% (w/v) TCA was added to the culture supernatant ('Sup'). In all cases, samples were then incubated on ice for 15 min and subjected to centrifugation (20 000 g, 20 min). After the supernatant was removed, 10 μl 2 M Tris was added to the pellet,

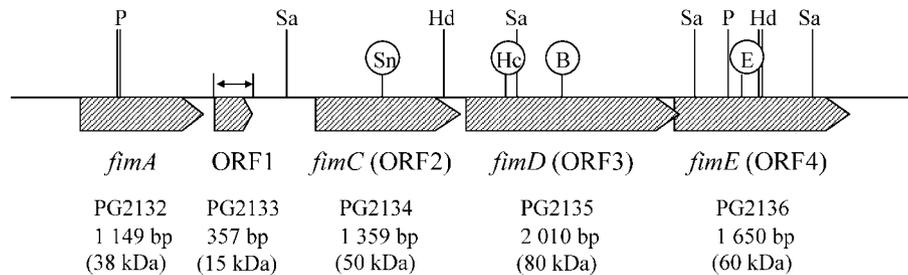


Fig. 1. Schematic diagram of the *fim* gene cluster in *P. gingivalis* strain ATCC 33277. The *fimA* and other coding regions are represented by hatched bars with pointed ends indicating their 3' ends. The genes downstream of *fimA* have been referred to as ORF1, ORF2, ORF3 and ORF4 in the literature (Watanabe *et al.*, 1996); the latter three were renamed *fimC*, *fimD* and *fimE*, respectively, in this study since they were shown to encode minor components of the FimA fimbriae. The PG numbers (from PG2132 to PG2136) below the bars indicate the orthologous ORFs in *P. gingivalis* W83, which were annotated by TIGR. The length of the genes (bp) and the deduced molecular masses of the products are also shown below the bars. Major restriction sites are indicated, with those sites used to insert the *Emf* cassette to construct the *fimC*, *fimD* and *fimE* mutants circled (B, *Bam*HI; E, *Eco*81I; Hc, *Hinc*II; Hd, *Hind*III; P, *Pst*I; Sa, *Sac*I; Sn, *Sna*BI). The double-headed arrow indicates the region exchanged with the *Emf* cassette to construct the ORF1 mutant (see Methods for details).

which was then resuspended in distilled water and SDS loading buffer. The final sample volume was adjusted according to the OD₆₀₀ values measured before harvest.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were carried out as described previously (Lugtenberg *et al.*, 1975; Yoshimura *et al.*, 1989). Proteins were separated with a 12% (w/v) SDS-PAGE gel and visualized by staining with Coomassie brilliant blue (CBB) R-250. For immunoblotting, proteins were transferred onto a nitrocellulose membrane (Hybond ECL nitrocellulose membrane, GE Healthcare Biosciences) and detected by using antibody raised against fimbriin (FimA monomer) (Yoshimura *et al.*, 1984), ORF1, FimC, FimD (Watanabe *et al.*, 1996), or FimE (this study), and peroxidase-conjugated goat anti-rabbit IgG (MP Biomedicals). Signals were visualized with 0.01% (w/v) 4-chloro-1-naphthol in 20 mM Tris/HCl (pH 7.5) containing 0.5 M NaCl supplemented with hydrogen peroxide. To increase sensitivity for the detection of certain proteins, ECL Plus Western blotting detection reagents (GE Healthcare Biosciences) were used for visualization according to the manufacturer's instructions. Before immunoblotting, samples of the same group were analysed by SDS-PAGE, followed by dye-staining. Then, their protein patterns and the intensities of stained protein bands were carefully compared with each other to verify that they had an equivalent amount of proteins for quantitative immunoblotting analysis.

Autoaggregation assay. Cells were grown as described above, harvested by centrifugation at 8000 *g* for 10 min, gently washed twice with 20 mM PBS, pH 6.0, and resuspended in 20 mM PBS. PBS adjusted to pH 6.0 was used throughout the autoaggregation assays. The OD₅₅₀ value of the cell suspension was measured and adjusted by dilution with PBS to 1.0, which corresponds to 1.0×10^8 cells ml⁻¹ (Amano *et al.*, 1997). Aliquots (2 ml each) in test tubes (13 mm diameter) were then shaken at room temperature at a speed of 120 strokes min⁻¹. At 0, 5, 10, 15, 20, 30 and 40 min, the OD₅₅₀ values of the suspensions were measured with a spectrophotometer (mini photo 518R; TAITEC).

Binding assay. Binding of fimbriae to prokaryotic and eukaryotic proteins was examined by ELISA using polystyrene microtitre plates (96-well, Maxisorp; Nalge Nunc) or type I collagen pre-coated plates (Becton Dickinson Labware). Coating of the former with purified

rGAPDH (100 μ l of 10 or 20 μ g ml⁻¹ in 50 mM Na₂CO₃/NaHCO₃ buffer, pH 9.5) and with fibronectin (Sigma-Aldrich; 100 μ l of 10 or 20 μ g ml⁻¹ dissolved in 20 mM PBS, pH 7.2) were carried out at 4 °C overnight and at 37 °C for 2 h, respectively (for fibronectin coating see Nakamura *et al.*, 1999). PBS adjusted to pH 7.2 was used throughout the binding assays. As a negative control, buffer-only wells (100 μ l 50 mM Na₂CO₃/NaHCO₃ buffer or 100 μ l PBS) were prepared and subjected to the same treatments. After washing three times with PBS containing 0.05% (w/v) Tween-20, the wells were incubated with 300 μ l 4% (w/v) casein (Block Ace; Snow Brand) at room temperature for 2 h for blocking, washed again, and incubated with 100 μ l purified fimbriae (2.5 or 5.0 μ g ml⁻¹ in PBS) at room temperature for 2 h and then at 4 °C overnight. After washing, the wells were treated with 100 μ l 4% (w/v) casein or PBS containing anti-fimbriae (polymer) antibody (Yoshimura *et al.*, 1984) (at a 1 : 8000 dilution) and peroxidase-conjugated goat anti-rabbit IgG (at a 1 : 16 000 dilution) (MP Biomedicals). Then the EIA peroxidase substrate, 3,3',5,5'-tetramethylbenzidine (Bio-Rad; 100 μ l), was added to each well. The reactions were stopped by adding 100 μ l 0.5 M H₂SO₄. The binding activities were assessed by measuring the A₄₅₀ values with a microplate reader (model 680; Bio-Rad). All assays were carried out in triplicate and the standard errors were determined. Experiments with pre-coated collagen plates were performed similarly except that coating and blocking procedures were skipped. As a negative control, PBS (100 μ l) without fimbriae protein was applied to several wells which were then subjected to the same procedures as those with fimbriae. For each combination, the mean A₄₅₀ value of the control reactions was subtracted from that of the triplicate assays with fimbriae and the resulting value was defined as net binding.

RESULTS

Genes downstream of *fimA* in type I strains

In this study, we focused on the four ORFs (ORF1, *fimC*, *fimD* and *fimE*) downstream of the *fimA* gene encoding fimbriin in *P. gingivalis* (Fig. 1). The sequences of the four ORFs in strain 381 have already been published (Watanabe *et al.*, 1996). Comparing the *fimA* gene clusters between

strains W83 (Nelson *et al.*, 2003) and 381, we found that ORF1 and *fimE* in 381 are shorter than the corresponding genes (PG2133 and PG2136, respectively) of W83. We sequenced this region in both 381 and ATCC 33277 and found that the size of the *fimE* coding region of 381 (1650 bp) is identical to that of ATCC 33277 and very similar to that of the PG2136 coding region of W83 (1662 bp). The ORF1 sequence of strain 381 was also identical to that of ATCC 33277, but was shorter than that of PG2133 in W83. A schematic diagram of the region downstream of *fimA* in ATCC 33277, used hereafter as the parent strain, is presented in Fig. 1.

The *fimE* gene encodes a minor component of the FimA fimbriae

The *fimC* and *fimD* products co-purified with FimA fimbriae and therefore appeared to be minor components of the fimbriae (Yoshimura *et al.*, 1993), whereas the ORF1 and *fimE* products were not detected in the same preparation (Watanabe *et al.*, 1996). Here we raised a polyclonal antibody against full-length recombinant FimE, and examined fimbriae purified from ATCC 33277 by immunoblotting with the new antibody. FimE was detected in the purified fimbriae from the wild-type strain (Fig. 2, lane WT in panel Anti-FimE). The apparent molecular mass of the product was approximately 60×10^3 , in good agreement with that deduced from the nucleotide sequence (60639.43 for the precursor form and 56672.91 for the

predicted mature form). FimE, as well as FimC and FimD, could be detected by CBB staining when sufficient amounts (more than 30 μg protein) of the wild-type fimbriae were applied (data not shown). These results demonstrate that FimE is produced in *P. gingivalis* and is associated with FimA fimbriae, and therefore we conclude that FimE, like FimC and FimD, is a minor component of the FimA fimbriae. On the other hand, the ORF1 product was not detected in the fimbriae preparation (data not shown) as reported previously (Watanabe *et al.*, 1996).

Effects of mutations of ORF1, *fimC*, *fimD* and *fimE* on the levels of the minor components and fimbrilin

We constructed and characterized a series of mutants with the Em^r cassette (*ermF-ermAM*) inserted into ORF1, *fimC*, *fimD* or *fimE*. First, whole cultures (WC) of the wild-type and mutant strains, as well as supernatants (Sup) and pellets (Cell) after low-speed centrifugation of the whole cultures, were analysed by immunoblotting with antibodies raised against ORF1, FimC, FimD and FimE after precipitating proteins with TCA to minimize degradation by endogenous proteases of *P. gingivalis*, which is a potent proteolytic organism (Hongo *et al.*, 1999). The ORF1 product was not detected in any sample tested (data not shown). The ORF1 mutant had slightly lower amounts of FimC, FimD and FimE than the wild-type strain (Fig. 3, compare lanes ORF1 and WT in panels FimC/WC through

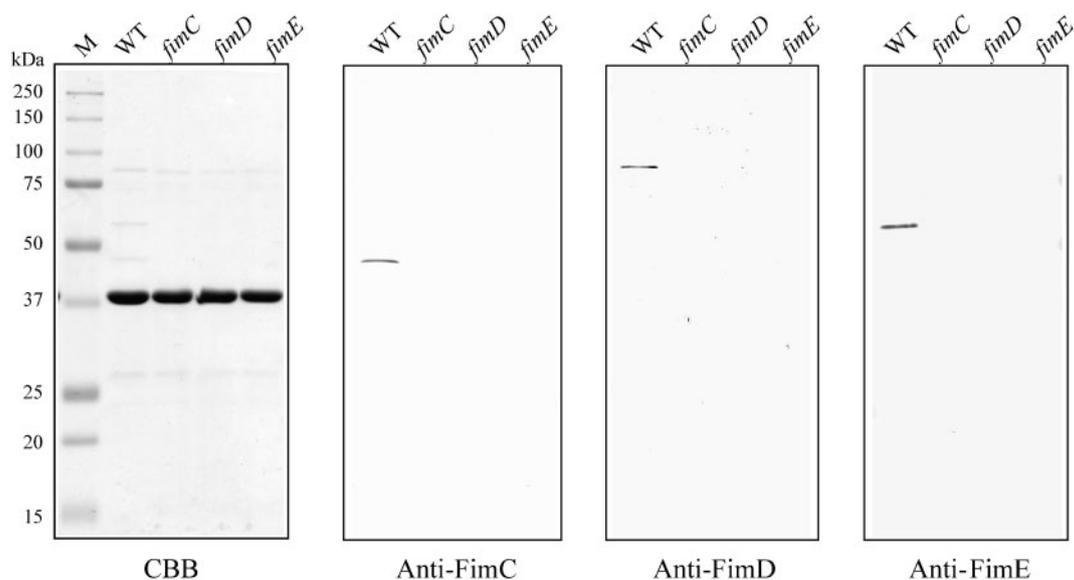


Fig. 2. Detection of fimbrilin (FimA) and minor components in purified fimbriae from the wild-type and mutant *P. gingivalis* strains. Fimbriae (10 μg each) purified from the wild-type strain (ATCC 33277) and its *fimC* and *fimE* derivatives were subjected to SDS-PAGE followed by Coomassie brilliant blue staining (CBB) and immunoblotting with antibodies raised against recombinant FimC (Anti-FimC), FimD (Anti-FimD) and FimE (Anti-FimE). Lanes: M, markers; WT, wild-type strain ATCC 33277; *fimC*, *fimC* mutant OZ5001C; *fimD*, *fimD* mutant KO3*fimE*, *fimE* mutant KO4.

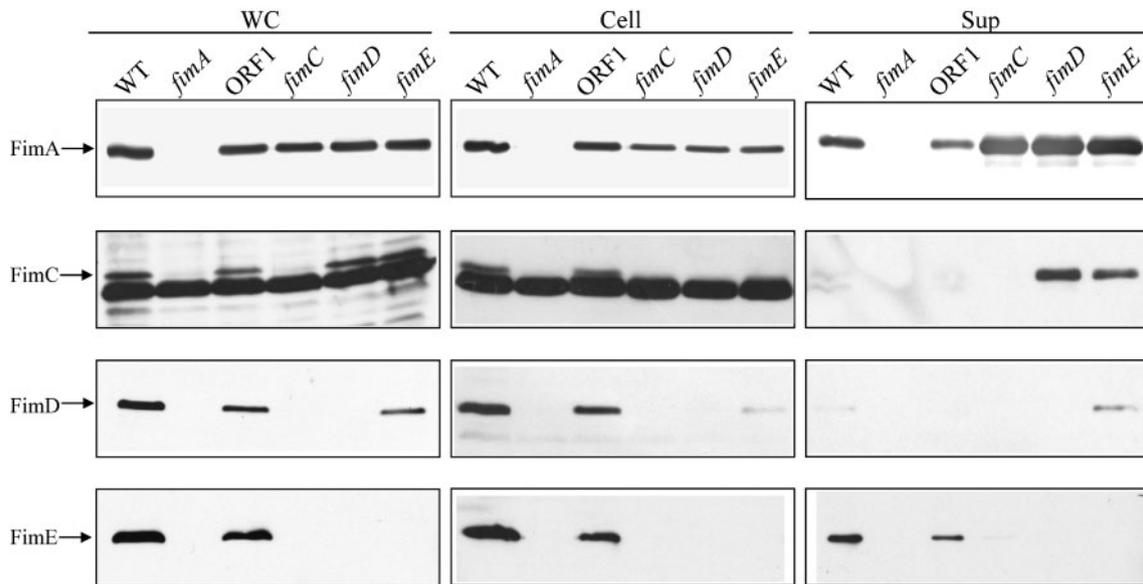


Fig. 3. Detection of fimbrilin (FimA) and minor components in cultures of the wild-type and mutant *P. gingivalis* strains. Proteins from whole cultures (panels labelled WC) of the wild-type and mutant strains, as well as those from their pellets (panels labelled Cell) and supernatants (panels labelled Sup) after low-speed centrifugation (14 000 *g*, 3 min), were precipitated with TCA and subjected to SDS-PAGE followed by immunoblotting with antibodies raised against fimbrilin (FimA monomer) and recombinant minor components (bands indicated with FimA, FimC, FimD and FimE). To detect signals of FimC, FimD and FimE, the ECL Plus system (GE Healthcare Biosciences) was used. Arrows indicate corresponding protein bands. Each lane contained a sample corresponding to 50 μ l (FimA panels labelled WC and Cell), 200 μ l (all panels labelled Sup), or 100 μ l (all other panels) of cell culture with an OD₆₀₀ value of 1.0. Lanes: WT, wild-type strain ATCC 33277; *fimA*, *fimA* mutant KDP98; ORF1, ORF1 mutant KO1; *fimC*, *fimC* mutant OZ5001C; *fimD*, *fimD* mutant KO3; *fimE*, *fimE* mutant KO4.

FimE/WC), but retained almost wild-type ratios of released (Sup) to cell-associated (Cell) amounts of the minor components (Fig. 3, compare lanes ORF1 and WT in panels FimC/Cell through FimE/Sup). In the *fimC* mutant, not only FimC itself but also FimD and FimE were absent (Fig. 3, lanes *fimC* in panels FimC/WC through FimE/WC). Similarly, the *fimD* mutant did not have FimE or FimD itself (Fig. 3, lanes *fimD* in panels FimC/WC through FimE/WC). Thus, the inactivation of the *fimC* or *fimD* gene resulted in concomitant loss of the downstream gene products, probably due to polar effects of the insertions. The inactivation of *fimE* did not have a significant effect on the levels of FimC but had a slight negative effect on the level of FimD (Fig. 3, compare lanes *fimE* and WT in panels FimC/WC and FimD/WC). The *fimD* and *fimE* mutations were also shown to affect cellular localization of the remaining minor components; FimC in the *fimD* mutant and FimC and FimD in the *fimE* mutant appeared to leak out into the culture supernatant (Fig. 3, lane *fimD* in panel FimC/Sup and lanes *fimE* in panels FimC/Sup and FimD/Sup). Consistent with this finding, no minor components were found in fimbriae purified from the *fimC*, *fimD* and *fimE* mutants (Fig. 2, lane *fimC*, *fimD* and *fimE*). These results suggest that FimE is required for the assembly of FimC and FimD onto a fimbrial fibre consisting of

fimbrilin (FimA). The strong band immediately below the FimC band (Fig. 3 FimC panels WC and Cell) is an unrelated protein cross-reacting with anti-FimC antibody.

Next, we examined the cellular amount of FimA in the same TCA-precipitated samples by immunoblotting with anti-FimA antibody (Fig. 3, panels FimA). Quantitative image analyses using the software Image J (<http://rsb.info.nih.gov/ij/>) showed that the FimA signals in whole cultures of the ORF1, *fimC*, *fimD* and *fimE* mutants were approximately 80% of that of wild-type (Fig. 3, panel FimA/WC). Interestingly, immunoblotting with pellets (Fig. 3, panel FimA/Cell) and culture supernatants (Fig. 3, panel FimA/Sup) gave different profiles for the mutants. The FimA signals in the pellets from the *fimC*, *fimD* and *fimE* mutants were approximately 50% of that of wild-type, whereas the signal of the ORF1 mutant was approximately 80%. The FimA signals in the culture supernatants of the *fimC*, *fimD* and *fimE* mutants were about twice that of the wild-type. After ultracentrifugation of the culture supernatants, FimA was detected in the precipitates, suggesting that it was released as filaments rather than monomers (data not shown). Thus, fimbriae of the *fimC*, *fimD* and *fimE* mutants seem more apt to detach from cells than those of the wild-type. Since all of the *fimC*,

fimD and *fimE* mutants lack FimE (Fig. 3, panel FimE/WC), it appears that the loss of FimE destabilizes the fimbrial attachment to the cell surface.

Autoaggregation activities of the ORF1, *fimC*, *fimD* and *fimE* mutants

We then examined whether the minor components are involved in autoaggregation of *P. gingivalis*, which seems to depend on the formation of the FimA fimbriae (Tokuda *et al.*, 1996) and to have some correlation with colonization (Tokuda *et al.*, 1996) and virulence (Eick *et al.*, 2002). Wild-type and mutant cells, gently washed with PBS, were prepared, and each suspension (2 ml) was shaken in a test tube. Formation of aggregates and cleared supernatants of wild-type cells were apparent just by eye, whereas the *fimC*, *fimD* and *fimE* mutants, but not the ORF1 mutant, showed much less aggregation (Fig. 4a). For quantitative analysis, the OD₆₆₀ value of each suspension was monitored

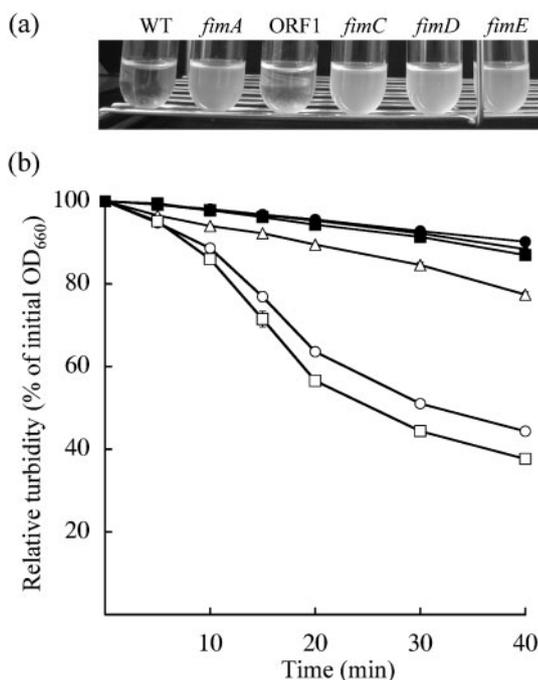


Fig. 4. Autoaggregation activities of the wild-type and mutant *P. gingivalis* strains. (a) Aggregation of cells after incubation for 60 min. Strains are labelled as described in the legend to Fig. 3. (b) Time-course of autoaggregation. The OD₆₆₀ value of each cell suspension was measured at the indicated times. Relative turbidities, defined as the relative OD₆₆₀ values (in percent) normalized to the initial value of each suspension (i.e. at time zero; approximately 0.7), are plotted against incubation time. All assays were performed in triplicate and the means ± SE are shown (standard errors not shown where smaller than symbols). ○, Wild-type strain ATCC 33277; △, *fimA* mutant KDP98; □, ORF1 mutant KO1; ●, *fimC* mutant OZ5001C; ▲, *fimD* mutant KO3; ■, *fimE* mutant KO4.

(Fig. 4b). The OD₆₆₀ values for the wild-type and the ORF1 mutant strains decreased gradually during constant shaking, reaching 44% and 38% of the initial OD₆₆₀ values, respectively, after 40 min, indicative of autoaggregation activity. In contrast, the *fimC*, *fimD* and *fimE* mutants still had relative OD₆₆₀ values of approximately 86–90%, which were actually slightly higher than that of the *fimA* mutant (77%). Continuous shaking could detach fimbriae from cells during the autoaggregation experiment, although diluted cell suspensions were shaken gently. In fact, the *fimC*, *fimD* and *fimE* mutants seemed to release their fimbriae into the medium during growth (Fig. 3, panel FimA/Sup). However, immunoblotting assays detected no significant decrease in the amount of bound fimbriae in any of the mutant strains after 40 min shaking (data not shown). Therefore, we conclude that the attenuated autoaggregation activities of the *fimC*, *fimD* and *fimE* mutants resulted from the lack of FimE or other minor component(s) of the fimbriae rather than the loss of fimbriae themselves.

Binding of fimbriae purified from the wild-type, *fimC*, *fimD* and *fimE* strains to rGAPDH and extracellular matrix proteins

We also examined the binding activities of purified fimbriae to rGAPDH from *Streptococcus oralis* and the two extracellular matrix proteins, fibronectin and type I collagen, which have been reported by several groups (Maeda *et al.*, 2004a; Nakamura *et al.*, 1999). The ELISA results demonstrated that fimbriae from the wild-type bind to these proteins, whereas those from the *fimC*, *fimD* and *fimE* mutants showed significantly lower binding (Fig. 5). These binding experiments were based on the assumption that the wild-type and mutant fimbrial preparations (i.e. fimbriae with and without minor components) have similar reactivity with the anti-fimbriae serum. Indeed, control experiments using wells coated only with fimbriae showed no significant difference in reactivity among the mutant fimbriae. Since all three mutants lack FimE (Fig. 3, panel FimE/WC, lanes *fimC*, *fimD* and *fimE*) and the *fimE* fimbriae lack FimC and FimD (Fig. 2, panels Anti-FimC and Anti-FimD), these results suggest that some of the three minor components are involved in the binding of the FimA fimbriae to these bacterial and eukaryotic proteins. Which minor component(s) serve directly as adhesin(s) remains to be elucidated.

DISCUSSION

In this study, we characterized the four genes (ORF1, *fimC*, *fimD* and *fimE*) downstream of *fimA*, the gene for a major subunit of long fimbriae (Fig. 1) and found that the most distal gene *fimE* encodes a minor component that is closely associated with the intact FimA fimbriae (Fig. 2) as previously reported for the other two genes *fimC* and *fimD* (Yoshimura *et al.*, 1993). Fimbriae isolated from the *fimC*,

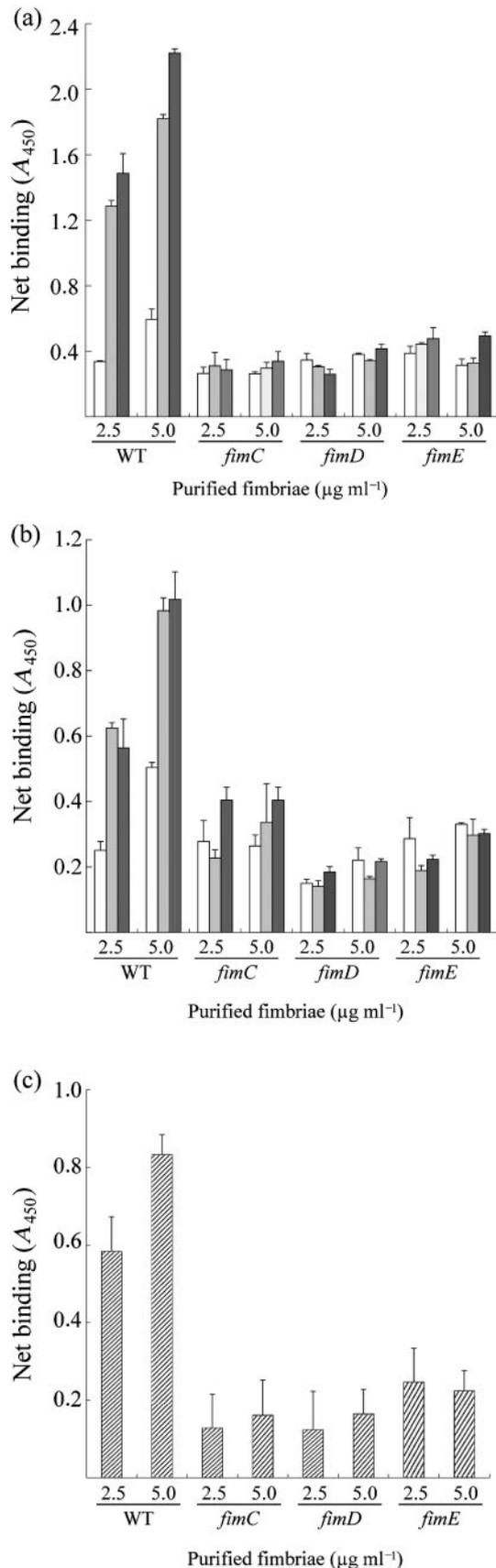


Fig. 5. Specific binding of the wild-type and mutant fimbriae to prokaryotic and eukaryotic proteins. (a, b) Binding of fimbriae to rGAPDH (a) and fibronectin (b). Wells of microtitre plates were treated with buffer containing casein but without specific ligands (white bars), or with 10 $\mu\text{g ml}^{-1}$ (light grey bars) or 20 $\mu\text{g ml}^{-1}$ (dark grey bars) rGAPDH (a) or fibronectin (b) and then with fimbriae (2.5 or 5.0 $\mu\text{g ml}^{-1}$) purified from the wild-type, *fimC*, *fimD* or *fimE* strains (for detailed procedures see Methods). (c) Binding of fimbriae to type I collagen. Wells of microtitre plates pre-coated with type I collagen were treated with purified fimbriae as described above. For each sample, the mean A_{450} value of the control reactions was subtracted from that with fimbriae and the resulting value was defined as net binding. Bars indicate the means \pm SE values of each combination (in triplicate).

fimD and *fimE* mutants had lost all the three minor components (Fig. 2), although FimC and FimD were produced and detected in the *fimE* mutant (Fig. 3). Fimbriae from all of these mutants had lost autoaggregation ability (Fig. 4) and the ability to adhere to the bacterial and eukaryotic ligands, GAPDH, fibronectin and collagen type I (Fig. 5). Since all of these mutants lack FimE, our results suggest that FimE at least is involved in the adhesive activities of the FimA fimbriae. Incorporation of minor components into mature fimbriae and their contribution to the adhesive function are commonly found in various bacterial pili as reported for type I fimbriae and P pili of *E. coli* (Jones *et al.*, 1995; Kuehn *et al.*, 1992).

It is unclear whether FimE is directly involved in adhesion functions. In the *fimE* mutant, FimC and FimD were produced and detected in whole cultures (Fig. 3), but they were not incorporated into fimbriae (Fig. 2) and were released into the culture supernatant (Fig. 3), suggesting that FimE is required for the integration of FimC and FimD into the mature and functional fimbriae. FimE could be required for the assembly of a putative tip complex consisting of FimC, FimD and FimE, one or more of which might serve as adhesin(s). Alternatively, FimE might not be directly responsible for adhesion but could help adhesin(s) FimC and/or FimD incorporate into fimbriae, e.g. by serving as a molecular chaperone. Preliminary analyses of the FimC, FimD and FimE sequences failed to detect a significant level of similarity to any of the known adhesins or other fimbrial proteins. It will be necessary to test whether any of these minor components can bind directly to the bacterial and eukaryotic ligands.

The *fimC* mutant simultaneously lost the ability to produce FimD and FimE, and the *fimD* mutant lacks FimE. A simple interpretation of these polar effects is that the genes from *fimC* through *fimE* may form an operon and that their translation is coupled. Translational coupling is a commonly used strategy to maintain strict stoichiometry of components of supramolecular complexes, including fimbriae (Draper, 1996). Similarly, the *fimA* mutant lacks all of the minor components (Fig. 3). However, this cannot be attributed to a polar effect of the insertion since the

fimA mRNA has been reported to be monocistronic (Amano *et al.*, 1994; Dickinson *et al.*, 1988) and the replacement of ORF1, the gene immediately downstream of *fimA* and immediately upstream of *fimC* (Fig. 1), by the Em^r cassette did not significantly affect the amounts of the minor components (Fig. 3). There might be some regulatory circuit to prevent synthesis of the minor components in the absence of fimbriae. Also, it appears that the level of the *fimD* product is decreased in the *fimE* mutant (Fig. 3, panel FimD/WC). The mechanism of this negative effect of the *fimE* insertion on the upstream gene remains to be elucidated.

Among the four genes downstream of *fimA*, ORF1 appears to play a minor role in fimbriae assembly and function since its inactivation gave a very weak or no phenotype. The ORF1 sequences of the *fimA* genotype I strains 381 and ATCC 33277 were identical (357 bp in length) and shorter than the corresponding gene PG2133 (909 bp in length) of W83, a poorly fimbriated strain. The 15 kDa ORF1 and 30 kDa PG2133 products were not detected in whole cell samples or in purified fimbriae (data not shown). The ORF1 mutant showed a slight decrease in FimA, but the ratio of cell-associated/culture supernatant FimA and the autoaggregation activity were very close to those of wild-type (Fig. 3). Taking these results together, ORF1 is not likely to be involved in fimbrial formation at least in strains ATCC 33277 and 381. However, the ORF1 mutant showed a slight but reproducible increase in autoaggregation (Fig. 4). Therefore, the ORF1 product might play a role in the negative regulation of adhesion, although its contribution appears to be marginal, at least under the conditions tested.

Fimbriae from strains other than those carrying *fimA* of genotype I have not been purified thus far, and their morphology and antigenic variations have not been explored. Our preliminary purification data suggest that fimbriae from several *fimA* genotype Ib (close to genotype I) and II strains have at least three minor components equivalent to those from the genotype I strains 381 and ATCC 33277 but do not contain a component corresponding to the gene product of PG2133 (or ORF1) (S. N. & F. Y., unpublished results). It will be of great interest in terms of molecular function and evolution to compare the sequences and the natures of the minor components of the FimA fimbriae with different *fimA* genotypes, especially those of genotype II, which is predominant among periodontitis patients (Amano *et al.*, 2000).

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