Characterization of FimA in Porphyromonas gingivalis genotype IV

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Abstract
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 Porphyromonas gingivalis-positive healthy adults. Here we report characterization of recombinant fimbrial protein (rFimA) produced in Escherichia coli from genotype IV-fimA. In SDS-PAGE and immunoblot analysis after partial dissociation, type IV-rFimA showed a ladder-like pattern representing oligomeric/polymeric forms of native fimbrial structure. Unlike anti-type I-native fimbriae which can only recognize conformational epitopes of the respective proteins, both anti-type IV-native fimbriae and anti-type IV-rFimA antibodies recognized conformational as well as linear epitopes in type IV-fimbriae. These results suggest that the type IV-rFimA proteins retain the native fimbrial antigenicity and the antigenicity of type IV-fimbriae is different from that of type I-fimbriae.

Porphyromonas gingivalis fimbriae are critical for the promotion of bacterial infection (Nakagawa et al., 2002). The fimA gene encoding FimA, a subunit of fimbriae, has been classified into six genotypes (I–V and Ib) based on their nucleotide sequences (Nakagawa et al., 2002). It has been reported that there is a significant prevalence of P. gingivalis harboring genotype II and IV fimA in periodontitis patients (Amano et al., 1999, 2000; Nakano et al., 2004).

In a previous study (Lee et al., 1991), anti-native FimA of serotype I strain 2561 was observed to react strongly with FimA from strains of serotype I and weakly with II and III, but not with serotype IV and untypeable strains. The FimA serotype II strains used in the study are now known to belong to fimA genotype II and the serotype IV and untypeable strains belong to genotype IV. The result suggests that serotype II FimA is antigenically and serologically related to serotype I FimA, but serotype IV is distinct from other serotypes. As FimA from these serotype IV and untypeable strains belonging to fimA genotype IV showed different responses to anti-type I FimA in immunoblot analysis, characterization of the type IV-FimA needs to be defined. However, antigenic and serological characterization of type IV-P. gingivalis FimA has not been conducted. This may be due partly to difficulties in purifying type IV-fimbriae from sparsely fimbriated strains such as W83 and W50 (Lee et al., 1991; Sojar et al., 1997). To circumvent the problems of purification, recombinant DNA techniques have been used widely. In the case of type I-rFimA produced in Escherichia coli, the rFimA protein (mature form without signal peptide) cannot be fully assembled into a native fimbrial structure (Shoji et al., 2010). To the best of our knowledge, there is no report on the generation of type IV-rFimA proteins and characterization of their antigenic properties in comparison with native fimbriae. Here we report production and characterization of rFimA from genotype IV-fimA gene.

Prior to generation of type IV-rFimA, fimA genes of P. gingivalis 9-14K-1 and AJW5 were sequenced and their nucleotide sequences were compared with those published previously for type IV-fimA genes of strains W83, W50 and HG564. To amplify the region between 78-nucleotide upstream from the start codon and stop codon of genotype IV-fimA genes of P. gingivalis 9-14K-1 and AJW5, we designed a set of primers based on the chromosomal
DNA sequence of *P. gingivalis* W83 (GenBank accession no. NC_002950): F, GCGGATCCGGTATAGCAAGACAT; R, TTACCAAGTGAAGGGCTGATTAA. The nucleotide sequences of the amplified fragments were determined by direct sequencing of the PCR products, as described previously (PaesHuys et al., 2006). The *fimA* sequences of AJW5 and 9-14K-1 have been deposited in NCBI under accession nos HQ_142987 and HQ_142988, respectively. We compared the nucleotide sequences encoding the mature FimA proteins and the deduced amino acid sequences between different genotype IV-*P. gingivalis* strains (W50, W83, HG564, AJW5 and 9-14K-1). The levels of nucleotide sequence homology among the strains were 98.3–99.9%. The *fimA* sequence of W50 (DDBJ under accession no. D38373) was almost identical to that of W83 except for four nucleotides. The *fimA* sequence of AJW5 was found to be identical to that of HG564 (DDBJ under accession no. D17802) except for a single nucleotide and the deduced amino acid sequences were exactly the same. The sequence similarity between W83/W50 and AJW5/HG564 was about 98%. The *fimA* sequence of 9-14K-1 showed 98.8–99.0% and 99.1–99.2% sequence similarity with the sequences of W83/W50 and AJW5/HG564, respectively. Thus, W83 and AJW5 were chosen as representatives of *fimA* type IV-*P. gingivalis* and rFimA proteins were generated from the *fimA* genes of the strains. Strain 9-14K-1, intermediate between these two strains, was chosen for isolation of native fimbriae.

To amplify the region encoding mature FimA without the 5′-terminal DNA region encoding the signal peptide, a set of primers was designed based on the *fimA* sequences of W83 and AJW5 and amino-terminal sequences of mature FimA proteins directly isolated from genotype IV-*P. gingivalis* strains (Lee et al., 1991): F, CGGGATCCGGTATAGGGATGCGCCTTGCA; R,CCAAGCTTTTACCAAGTACGACCCTGTAGA. BamHI and HindIII recognition sites were incorporated in the forward and reverse primers, respectively. The PCR products were then digested with BamHI/HindIII and ligated to pSET/B vector (Invitrogen) at the two restriction sites. The successfully transformed *E. coli* strain BL21 (DE3) pLysS was grown at 37 °C for 3 h, the cells were lysed by sonication on ice for 1 min in Z buffer (8 M urea, 100 mM NaCl, 20 mM HEPES, pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride (Amresco), centrifuged, and the supernatant purified on a Ni^{2+}-NTA (Invitrogen) affinity column using an imidazole gradient (100, 250 and 500 mM) in Z buffer. The eluted proteins were further purified using a Detoxi-Gel (Thermo Scientific, Rockford, IL) column. Native fimbrial proteins were purified from *P. gingivalis* strains 9-14K-1 (type IV) and 2561 (type I), as described previously (Lee et al., 1995). Purity of the native fimbrial proteins was confirmed by SDS-PAGE, followed by silver nitrate staining. Rabbit polyclonal antibodies were raised against the purified native fimbriae and rFimA, under the guidance of the Institutional Animal Care and Use Committee (IACUC) at Kyung Hee Medical Center using an approved protocol (KHMC IACUC 10-045). IgG faction was isolated from the antiserum using a Melon™ IgG Gel Spin Purification Kit (Thermo Scientific).

For SDS-PAGE and immunoblot analyses, sonic extracts (crude fimbriae) were obtained from *P. gingivalis* strains representing different genotypes as described previously (Lee et al., 1995). The sonic extracts were treated at 100 °C for 10 min with β-mercaptoethanol (β-ME) or 80 °C for 5 min without β-ME. The samples were subjected to SDS-PAGE followed by immunoblot analyses with IgG antibodies to native fimbriae or rFimA.

Previously, purified type I-fimbriae showed a ladder-like pattern when treated at 80 °C for 5 min under non-reducing conditions, and could be dissociated into its monomeric from of 43 kDa after heating at 100 °C under reducing conditions (Sojar et al., 1991; Lee et al., 1995). In this study, purified type IV-fimbriae were completely dissociated into monomeric forms, showing an apparent molecular mass of 42 kDa when treated at 100 °C for 10 min with reducing agent β-ME (Fig. 1a). Type IV-rFimA proteins fused into a short N-terminal peptide containing six histidine residues in tandem with a predicted molecular mass of 3.6 kDa showed a molecular mass of 46 kDa. A characteristic ladder-like pattern representing oligomeric/polymeric forms of the fimbrial proteins was observed when the rFimA and native fimbriae were treated at 80 °C for 5 min in the absence of β-ME (Fig. 1b).

Anti-type I-fimbriae recognized the oligomeric/polymeric fimbrial proteins when the sonic extracts from *P. gingivalis* strains possessing genotype I-*fimA* were treated at 80 °C in the absence of β-ME, and showed no reaction with the monomeric forms of FimA proteins treated at 100 °C in the presence of β-ME (Fig. 2a). This finding is consistent with previous studies (Yoshimura et al., 1984; Sojar et al., 1991; Lee et al., 1995). The same result was obtained from the immunoblot using purified type I-native fimbriae with anti-type I fimbriae (result not shown). On the other hand, antibodies against type IV-fimbriae recognized not only oligomeric/polymeric forms but also monomeric forms of their respective proteins (Fig. 2b). Antibodies to type IV-rFimA reacted with both monomeric and oligomeric/polymeric forms of type IV-fimbriae (Fig. 2c) in the same way as antibodies to type IV-fimbriae did. It suggests that the antibodies to
type IV-fimbriae as well as type IV-rFimA can recognize both linear and conformational epitopes in type IV-fimbriae, unlike anti-type I-fimbriae, which can only recognize conformational epitopes of the respective proteins. It appears that the type IV-rFimA proteins are self-assembled into native-like structures of oligomers/polymers and thus retain native antigenicity of type IV-fimbriae.

Immunoblot analyses demonstrated that there was no difference in the immunoreactivities of antibodies against 9-14K-1FimA, W83 rFimA and AJW5 rFimA with fimbrial proteins from different strains of genotype IV-fimA (Fig. 2). Therefore, sequence differences formed between the strains of genotype IV-P. gingivalis are not indicative of structural or antigenic variation in fimbrial proteins. In a previous study (Lee et al., 1991), antibodies to type I-fimbriae were found to react with type I- as well as type II- and type III-fimbriae. In the present study, the antibodies to type IV-fimbriae and type IV-rFimA did not
react with fimbrial proteins from \textit{P. gingivalis} strains of other genotypes (data not shown), suggesting distinct antigenic characteristics of type IV-fimbrial proteins.

It is known that \textit{P. gingivalis} fimbriae are highly immunogenic and immunization with fimbriae purified from \textit{P. gingivalis} 381 blocks the progression of experimental periodontal disease caused by the homologous strain (Evans \textit{et al.}, 1992). Although purified native fimbriae may be a useful immunogen for vaccination to block or reduce the severity of periodontitis, type I-rFimA vaccine-induced antibody responses may differ substantially from naturally acquired responses because type I-rFimA proteins do not fully represent the native structure (Shoji \textit{et al.}, 2010). However, our results suggest that rFimA proteins of genotype IV have conformational and/or linear epitopes similar to those expressed on native fimbrial proteins directly isolated from genotype IV- \textit{P. gingivalis} strains. Therefore, type IV-rFimA may be used as a potential vaccine candidate for prevention of genotype IV- \textit{P. gingivalis}-induced periodontitis.

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\section*{Authors’ contribution}
Y.-S.C. and J.-H.M. contributed equally to this work.

\section*{References}


