

Recombinant *Porphyromonas gingivalis* FimA preproprotein expressed in *Escherichia coli* is lipidated and a mature/processed recombinant FimA protein forms a short filament *in vitro*.

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Abstract: The Gram-negative anaerobic bacterium *Porphyromonas gingivalis* is an etiologically important pathogen for chronic periodontal diseases in adults. Our previous study suggested that the major structural components of both Fim and Mfa fimbriae in this organism are secreted through their lipidated precursors. In this study, we constructed *Escherichia coli* strains expressing various *fimA* genes with or without the 5'-terminal DNA region encoding the signal peptide and determined whether lipidation of recombinant FimA proteins occurred in *E. coli*. A recombinant protein from the *fimA* gene with the 5'-terminal DNA region encoding the signal peptide was lipidated, but a recombinant protein from the *fimA* gene without the signal peptide-encoding region was not, as revealed by [³H] palmitic acid labeling experiments. The recombinant protein from the *fimA* gene with the signal peptide-encoding region induced a TLR2-dependent signaling response, whereas a recombinant protein from the *fimA* gene with the signal peptide-encoding region that had a base substitution causing an amino acid substitution (C¹⁹A) did not. Electron microscopic analysis revealed that recombinant FimA(A⁴⁷-W³⁸³) protein was auto-polymerized to form filamentous structures of about 80 nm in length *in vitro*. The results suggest that FimA protein, a major subunit of Fim fimbriae, is transported to the outer membrane by the lipoprotein sorting system, and a mature/processed FimA protein on the outer membrane is

auto-polymerized to form Fim fimbriae.

Key words: *Porphyromonas gingivalis*, Fimbriae, Recombinant FimA protein,
lipoprotein, TLR2

Introduction

Porphyromonas gingivalis is an oral anaerobic pathogen associated with chronic periodontitis in adults, an infection of mucosal tissues surrounding the dentition that causes destruction of alveolar bone and tooth loss (Ezzo et al. 2003). Recent studies have shown that chronic periodontitis is linked to increased risk of coronary vascular disease and have also revealed that *P. gingivalis* is one of the pathogens that enters the bloodstream after crossing the oral mucosal barrier (Beck et al. 2000). This is corroborated by several lines of evidence, most notably the presence of *P. gingivalis* and other species' DNA in atheromatous plaque from carotid endarterectomy specimens (Haraszthy et al. 2000). A number of virulence factors including the capsule, adhesins and extracellular hydrolytic enzymes have been implicated in the pathogenicity of *P. gingivalis*. Among these, *P. gingivalis* fimbriae are important cell structures involved in mucosal pathogenesis and periodontitis by facilitating colonization and invasion of mucosal cells and induction of inflammatory responses (Lamont et al. 1998).

Our previous study suggested that precursor forms of *P. gingivalis* major structural components of both Fim and Mfa fimbriae were lipidated (Shoji et al. 2004). Biogenesis of prokaryotic lipoproteins, especially those in *Escherichia coli*, has been characterized in detail (Tokuda et al. 2004; Tokuda 2009). In general, lipoproteins in prokaryotes

have a signal sequence at their N-termini and are translocated across the inner membrane by a Sec-dependent machinery. Lipid modification of a cysteine residue and proteolytic removal of a signal peptide to yield a mature/processed lipoprotein take place in the inner membrane, and then localization of the mature/processed lipoprotein to either the inner or outer membrane follows. We previously reported that *P. gingivalis* fimbriae might be generated by a novel transport and assembly system using the lipoprotein sorting system and extracellular proteolytic processing/polymerization (Shoji et al. 2004). However, the exact mechanism of biogenesis of *P. gingivalis* fimbriae remains to be determined.

To assess roles in the virulence of *P. gingivalis*, various genes have been cloned and expressed mainly in *E. coli* (Fujimura et al. 2006; Naito et al. 2006). However, expression of these genes cloned in foreign species is not necessarily qualitatively or quantitatively equivalent to that in the original species. In fact, FimA protein is not located on the cell surface of a recombinant *E. coli* strain expressing *P. gingivalis* *fimA* gene, although the recombinant *E. coli* strain produces the *fimA* gene product as revealed by immune-detection with anti-FimA antibody (Yoshimura et al. 1993). The *fimA* gene product in the recombinant *E. coli* strain has not been further analyzed and it has not been elucidated why the recombinant *E. coli* strain cannot generate FimA

fimbriae on the cell surface. In addition, the mechanisms of assembly and polymerization of FimA protein into a fimbrial structure have not been determined.

Here, we report that a recombinant protein from the *fimA* gene with the signal peptide-encoding region is lipidated in *E. coli* and that recombinant FimA (A⁴⁷-W³⁸³) protein has the ability to assemble and form a short filament *in vitro*.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 1.

Media and conditions for bacterial growth

P. gingivalis strains were grown under anaerobic conditions (80% N₂, 10% CO₂, 10% H₂) in enriched brain-heart infusion (BHI) broth (Becton Dickinson) or on enriched Trypto-soya (TS) agar plates (Nissui) supplemented with 5 µg/ml hemin (Sigma) and 0.5 µg/ml menadione (Sigma). Luria-Bertani (LB) broth and LB agar plates were used for growth of *E. coli* strains. Antibiotics were used at the following concentrations: ampicillin (Ap; 100 µg/ml for *E. coli*) and erythromycin (Em; 10 µg/ml for *P. gingivalis*).

Construction of a *P. gingivalis mfa1* mutant

P. gingivalis ATCC 33277 genome sequence data were obtained from GenBank (accession No. AP009380). A *P. gingivalis mfa1* mutant was constructed as follows. A DNA fragment corresponding to a region (0.77 kb) within the 5'-terminal portion of the *mfa1* gene (Lamont et al. 2002) was generated by PCR using *P. gingivalis* ATCC 33277

chromosomal DNA as a template with a forward primer, 5'-GGAATTCCCATTGCTCTCATTGG, containing an *Eco*RI site (underlined) and a reverse primer, 5'-GGAATTCCTTCATAGCTCTGGGC, containing an *Eco*RI site (underlined). The resulting fragment was cloned into the pGEM-T Easy vector (Promega) to yield pKD758. A DNA fragment corresponding to a region (0.77 kb) within the 3'-terminal portion of the *mfa1* gene was generated by PCR using *P. gingivalis* ATCC 33277 chromosomal DNA as a template with a forward primer, 5'-GGGATCCCATTAAAGCTACTACT, containing a *Bam*HI site (underlined) and a reverse primer, 5'-GGGATCCGGCACCAAAGGATTC, containing a *Bam*HI site (underlined). The resulting fragment was cloned into the pGEM-T Easy vector to yield pKD759. The *Eco*RI-*Eco*RI region of pKD758 containing the 0.77-kb fragment was inserted into the *Eco*RI site of pKD399, which contains an *ermF-ermAM* Em-resistance cassette (Shoji et al. 2004), resulting in pKD760. The *Bam*HI-*Bam*HI region of pKD759 containing the 0.77-kb fragment was inserted into the *Bam*HI site of pKD760, resulting in pKD761. Proper orientation of the *mfa1* gene in pKD761 was confirmed by DNA sequence analysis. The pKD761 plasmid DNA was linearized by *Bgl*III digestion and introduced into cells of *P. gingivalis* ATCC 33277 by electroporation. The cells were spread on TS agar containing 10 µg/ml Em and incubated under anaerobic conditions

for 7 days. Proper replacement of the Em-resistant transformant (KDP154) was verified by Southern and Western blot analyses.

Plasmid construction for various recombinant FimA proteins

To create various recombinant FimA (rFimA) proteins in *E. coli*, pUC13Bg12.1 (Dickinson et al. 1988) or pKD707 (Shoji et al. 2004) was used as a template for amplifying the *fimA* or *fimA* (C¹⁹A) gene. The 1.1-kb *fimA* gene with the 5'-terminal DNA region encoding the signal peptide (referred to as preproprotein-type shown in Fig. 1) and the *fimA* gene with the signal peptide-encoding region that had a base substitution causing an amino acid substitution (C¹⁹A) (referred to as preproprotein (C¹⁹A)-type shown in Fig. 1) were generated by PCR using pUC13Bg12.1 and pKD707 as templates with a forward primer, 5'-CCATATGAAAAAAAAACAAAGTTTTTCTTG, containing an *NdeI* site (underlined) and a reverse primer, 5'-CCTCGAGCCAAGTAGCATTCTGACC, containing an *XhoI* site (underlined). The resulting fragment was directly digested by *NdeI* and *XhoI*, and it was ligated into the *NdeI-XhoI* region of pET22b(+) vector plasmid (Novagen, Madison, Wis.) to yield pKD762 or pKD763, respectively. The 1.0-kb *fimA* gene without a signal-peptide region (referred to as processed A-type shown in Fig. 1) was generated by PCR using

pUC13Bg12.1 as a template with a forward primer, 5'-CCATATGGCTTTTGGAGTTGGCGAT, containing an *NdeI* site (underlined) and a reverse primer, 5'-CCTCGAGCCAAGTAGCATTCTGACC, containing an *XhoI* site (underlined). The resulting fragment was directly digested by *NdeI* and *XhoI*, and it was ligated into the *NdeI-XhoI* region of pET22b(+) vector plasmid to yield pKD764. To create another *fimA* gene without a signal-peptide region (referred to as processed B-type shown in Fig. 1) as a fusion protein with glutathione S-transferase (GST), a 1.0-kb *fimA* gene (encoding for A⁴⁷-W³⁸³) was generated by PCR using pUC13Bg12.1 as a template with a forward primer, 5'-GGAATTCGCTTTTGGAGTTGGCGAT, containing an *EcoRI* site (underlined) and a reverse primer, 5'-GGCGGCCGCTTACCAAGTAGCATTCTG, containing an *NotI* site (underlined). The resulting fragment was directly digested by *EcoRI* and *NotI*, and it was ligated into the *EcoRI-NotI* region of pGEX-6P3 vector (Amersham Pharmacia Biotech) to yield pKD765. The construct (referred to as rFimA signal fused with β -lactamase shown in Fig. 1) encoding a *fimA'*-*bla* fusion gene that contains the signal peptide-encoding sequence of the *fimA* gene (encoding for M¹-N⁴⁵) following mature β -lactamase was generated as follows. A 0.78-kb DNA fragment encoding mature β -lactamase starting at position +4 after the signal sequence cleavage site was amplified using pUC18 as a

template with a forward primer, 5'-TTTCGAATACGCTGGTGAAAGTAAAAGATG, containing a *Bst*BI site (underlined) and a reverse primer, 5'-CCTCGAGTTACCAATGCTTAATCAGTGAGG, containing an *Xho*I site (underlined). The resulting fragment was cloned into the pGEM-T Easy vector to yield pKD766. The *Bst*BI-*Xho*I fragment of pKD766 was eluted and replaced within the *Bst*BI-*Xho*I region of pKD762 to yield pKD767.

Expression and purification of *P. gingivalis* rFimA proteins in *E. coli*

For expression of the recombinant proteins, pKD762, pKD763, pKD764 or pKD767 was transformed into *E. coli* BL21(DE3) (Novagen, Madison, Wis.) and pKD765 was transformed into *E. coli* Top10 (Invitrogen). The transformants were grown in LB broth containing 100 µg/ml ampicillin at 37°C with shaking until OD₆₀₀ of 0.4~0.6, and protein expression was induced by adding 1 mM isopropyl-β-D-galactopyranoside (IPTG). After 3 h of incubation, the cells were harvested by centrifugation and suspended in native binding buffer as described by the manufacturer/supplier (Novagen) and then disrupted by sonication. Since rFimA His-tagged proteins or GST-rFimA fusion protein were found to be solubilized in the supernatant after removal of cellular debris by centrifugation, proteins in the supernatant were subjected to affinity

chromatography (Ni affinity beads; Invitrogen or Glutathione Sepharose 4B; Amersham Pharmacia Biotech) as recommended by the manufacturer. The GST-rFimA fusion protein was treated with PreScission protease (Amersham Pharmacia Biotech), and the cleavage target product was recovered in the flowthrough fraction. The purity of rFimA proteins was tested by acrylamide gel electrophoresis and Coomassie brilliant blue staining. N-terminal sequence analysis was performed as described previously (Shoji et al. 2004).

Labeling rFimA proteins with [³H]-palmitic acid

Fresh overnight culture of *E. coli* BL21(DE3) containing the plasmid pKD762, pKD763, pKD764 or pKD767 was diluted 1:100 in LB and incubated at 37°C for 3 h. Then final concentrations of 1 mM IPTG and 20 µCi/ml [9,10(n)-³H]-palmitic acid (Amersham Biosciences) were added, followed by 3-h incubation. *E. coli* cells were then harvested, washed three times in ice-cold PBS, and suspended with PBS buffer and solubilized with an equal volume of the 2 x sample buffer. The released proteins were separated by SDS-PAGE, and radiolabeled lipoproteins in the gel were detected by a Bio imaging analyzer BAS-5000 (Fuji, Tokyo, Japan).

Preparation of *P. gingivalis* Fim fimbriae

P. gingivalis Fim fimbriae were isolated and purified using the method of Yoshimura et al. (1984). Briefly, *P. gingivalis* Fim fimbriae were isolated by pipetting the cell suspension from an *mfa1* mutant (KDP154) and then purified by anion exchange chromatography. FimA-containing fractions were pooled and the purity of Fim fimbriae was tested by acrylamide gel electrophoresis and Coomassie brilliant blue staining, which showed a major single band at 43 kDa in a reducing condition.

Elimination of LPS from purified rFimA and native Fim fimbriae-containing fraction

Purified recombinant FimA proteins or native Fim fimbriae were dialyzed against phosphate buffered saline (PBS), and the removal of lipopolysaccharide (LPS) was accomplished by the method of Triton X-114 (Sigma) phase separation (Liu et al. 1997). LPS concentration of rFimA proteins used in this study was less than 10 pg/ μ g protein.

Cell lines

Chinese hamster ovary (CHO) reporter cells were grown as adherent monolayers at 37°C in a 5% saturated CO₂ atmosphere, and they were passaged at least twice in one

week for maintenance of logarithmic growth. The engineering of the CD14-expressing CHO reporter cell line, also known as clone 3E10, has been previously described in detail (Delude et al. 1998). This clonal cell line expresses surface CD25 antigen under the control of a region from the human E-selectin promoter containing the nuclear factor- κ B (NF- κ B) binding site. The cell line 7.19, which was a nonresponder to LPS derived from 3E10/CD14, was also used (Schromm et al. 2001). 3E10 or 7.19 cells were stably transfected with cDNA for human TLR2 or TLR4 as described previously (Kishimoto et al. 2006; Lien et al. 1999) and were grown in the presence of G418 (1 mg/ml) and hygromycin B (400 U/ml)

Flow cytometric analysis

CHO transfectants were plated in 24-well tissue culture dishes at a density of 10^5 cells per well (Lien et al. 2000). After overnight incubation, confluent monolayers of CHO cells were stimulated with preproprotein-type rFimA (1-10 μ g/ml) and preproprotein (C¹⁹A)-type rFimA (1-10 μ g/ml). Following incubation for 18 h, the cells were treated with trypsin/EDTA for 1 min, and the detached cells were assessed by flow cytometry for the presence of surface CD25 as described previously (Yoshimura et al. 1999).

Electron microscopy

Samples were negatively stained with a 0.5% uranyl acetate solution and examined in a JEM 2000EX electron microscope (JEOL) at 100 kV.

Other methods

DNA manipulations and Southern and Western blot analyses were carried out as described previously (Shoji et al. 2004).

Results

Lipidation of the preproprotein-type rFimA protein in *E. coli*

Previously, we showed that C¹⁹ within the signal peptide-encoding region of the *fimA* gene was lipidated in *P. gingivalis* (Shoji et al. 2004). To examine whether the *fimA* gene product in *E. coli* is lipidated or not, preproprotein-type rFimA and preproprotein (C¹⁹A)-type rFimA protein over-expression systems in *E. coli* were constructed (Fig. 1). Incorporation of radioactivity into a protein with an apparent molecular mass of 45 kDa was observed when expression of preproprotein-type rFimA in BL21(DE3) was induced with 1 mM IPTG and incubated with [³H] palmitic acid, but no radiolabel was detected in preproprotein (C¹⁹A)-type rFimA (Figs. 2B,E). In addition, significant incorporation of radioactivity into a protein was also observed when expression of the fusion gene product that contained the signal peptide sequence (M¹-N⁴⁵) of FimA following mature β -lactamase was induced with 1 mM IPTG and incubated with [³H] palmitic acid (Fig. 2B). The above observations suggest that the *fimA* gene product in *E. coli* is a lipoprotein and that lipidation is dependent on C¹⁹ as seen in *P. gingivalis* (Shoji et al. 2004). N-terminal amino acid sequence analysis revealed that the purified preproprotein-type rFimA protein started from ¹MKKTKFFLLG¹⁰, suggesting that the

lipoprotein-specific signal peptidase in *E.coli* cannot cleave the signal peptide region of FimA protein.

Ability of preproprotein-type rFimA to induce a TLR2-dependent signaling response

In an attempt to determine whether preproprotein-type rFimA is a lipoprotein by another approach, we examined whether preproprotein-type rFimA has the ability to induce a TLR2-dependent signaling response by using Chinese hamster ovary cell lines. We used the stably transfected CD14⁺ Chinese hamster ovary (CHO) reporter cell line 3E10/huTLR2, which expresses inducible cell surface CD25 under the control of an NF- κ B-dependent promoter in response to a TLR2 agonist such as lipoprotein and peptidoglycan (Kishimoto et al, 2006; Lien et al, 2000). Cell surface expression of CD25 was evaluated by flow cytometric analysis after staining with fluorescein isothiocyanate-conjugated anti-CD25 mAb, as described previously (Kishimoto et al, 2006). The preproprotein-type rFimA activated CD25 expression, but preproprotein (C¹⁹A)-type rFimA did not (Fig. 3). This result indicates that the preproprotein-type rFimA activated TLR2, strongly suggesting that preproprotein-type rFimA is lipidated at C¹⁹.

Microscopic analysis of the recombinant FimA proteins

As described previously, maturation of FimA protein processed by arginine-specific protease (Arg-gingipain, Rgp) is essential for generation of Fim fimbriae in this organism (Nakayama et al. 1996). We hypothesized that assembly and polymerization of FimA protein occur on the cell surface because Rgp has been shown to be anchored on the cell surface (Shoji et al. 2002). Therefore, we examined whether the recombinant FimA protein assembles and polymerizes by itself *in vitro*. Microscopic observation revealed that processed B-type rFimA protein derived from a glutathione S-transferase (GST)-FimA fusion protein, which was kept at 4°C, formed short filaments (Fig. 4A). On the other hand, preproprotein-type rFimA protein and processed A-type rFimA protein that has His-tags at the C-terminal region were observed as small aggregates (Figs. 4C,D). The length of the short filaments was only about 80 nm, which was over 17-fold less than that of native FimA fimbriae purified from an Mfa1-deficient mutant (KDP154) (Fig. 4B). Twisted parts of the short filaments were very similar to those of native Fim fimbriae in length and width (Table 2). Incubation of a suspension of processed B-type rFimA protein at 37°C accelerated polymerization of the protein (Fig. 5). These results suggest that mature/processed FimA protein itself has the ability to auto-assemble and polymerize, although other factors such as FimC, FimD and FimE

proteins may be necessary for generating native Fim fimbriae

Discussion

The results of a [³H] palmitic acid labeling experiment in this study (Figs. 2B,E) indicate that a preproprotein-type rFimA expressed in *E. coli* is a lipoprotein. Such acylation was not observed in the C¹⁹A mutant protein (Figs. 2B,E). In *E.coli*, mature forms of lipoproteins have three fatty acid molecules at N-terminal cysteine: two as diglycerides on the SH group and one as a free fatty acid on the NH₂-terminal group. However, in case of the preproprotein-type rFimA, N-terminal of the purified protein was M¹, suggesting that the purified protein, which was lipidated, still had the signal peptide region. It has been reported that Lpp lipoprotein of *E. coli* cells treated with globomycin, an inhibitor of lipoprotein-specific signal peptidase, is lipidated and that the N-terminal of the Lpp lipoprotein is methionine that is encoded by the initiation codon of the Lpp gene, indicating that cleavage of a signal peptide by lipoprotein-specific signal peptidase takes place after lipidation at a cysteine residue (Hayashi et al. 1985). These results suggest that *E. coli* lipoprotein-specific signal peptidase cannot cleave the signal peptide of *P. gingivalis* FimA protein. The finding that expression of an exclusively NF-κB-dependent reporter gene was induced upon stimulation with preproprotein-type rFimA in the presence of TLR2 on CHO cells but

not with preproprotein (C¹⁹A)-type rFimA also indicated that the preproprotein-type rFimA is a lipoprotein (Fig. 3).

At present, the detailed mechanism of *P. gingivalis* fimbriation remains unclear. Five open reading frames encoding 63- (PgmA), 15- (Orf1), 50- (FimC), 80- (FimD), and 60-kDa (FimE) polypeptides were reported to exist around the *fimA* gene in strain 381 (Yoshimura et al. 1993; Watanabe et al. 1996). Among these, the FimC, FimD, and FimE proteins have been confirmed to be minor structural components of Fim fimbriae, and it has been shown that they enhance adhesive activities to bind to the extracellular matrix (Nishiyama et al. 2007) and limit cellular activation through TLR2 signaling via CXCR4 (Pierce et al. 2009). Takahashi et al. (1999) showed by transformation experiments that introduction of a fragment containing the *fimA* gene into *P. gingivalis* was sufficient for construction of fimbrial-like structures. Several lines of evidence indicate that final processing of FimA protein occurs on the outer leaflet of the *P. gingivalis* outer membrane by gingipain proteases. Firstly, proprotein-type FimA which has T⁴¹ at the N-terminal is accumulated on the cell surface of a gingipain-null mutant (Shoji et al. 2004). Secondly, treatment of the gingipain-null mutant with vesicle-depleted supernatant containing soluble gingipains elicits generation of native fimbriae on the cell surface (Kato et al. 2007). Therefore, we speculated that the

processed-type rFimA protein can assemble and polymerize by itself *in vitro*. Processed B-type rFimA protein was found to form short filaments, although the length was much shorter than that of native Fim fimbriae (Figs. 4C,D). This result suggested that another factor(s) was necessary for formation of a native fimbrial structure. In this context, it has recently been reported that *fimC* and *fimE* mutants, which still express the FimA protein, are incapable of assembling as long fimbriae (Wang et al. 2007).

In terms of gene organization and protein similarities between *fim* and *mfa* gene loci, Mfa1 protein is expected to possess the same secretion and polymerization machinery as that of FimA protein. Downstream accessory proteins such as FimC, FimD, FimE, Mfa2, PGN_0289 and PGN_0290 are also expected to use the same secretion pathway as that used by FimA protein, because these proteins possess putative lipoprotein signal peptide sequences. Desvaux et al. (2009) proposed that the secretion systems of type 1 pilus and curli fimbriae be referred to as Type VII and Type VIII secretion systems, respectively, because of the lack of structural and sequence homology. The major structural component protein CsgA of *E. coli* curli fimbriae assembles on the cell surface by nucleation-dependent polymerization (Chapman et al. 2002), which seems to resemble assembly and polymerization of FimA protein on the *P. gingivalis* cell surface. However, *E. coli* CsgA protein does not use a lipoprotein sorting system for

translocation to the cell surface. The secretion system of *P. gingivalis* Fim and Mfa fimbrial components may be classified as a new secretion system.

In conclusion, we have shown that the preproprotein-type rFimA expressed in *E. coli* is a lipoprotein and that the processed B-type rFimA has the ability to assemble and polymerize to form a filamentous structure by itself.

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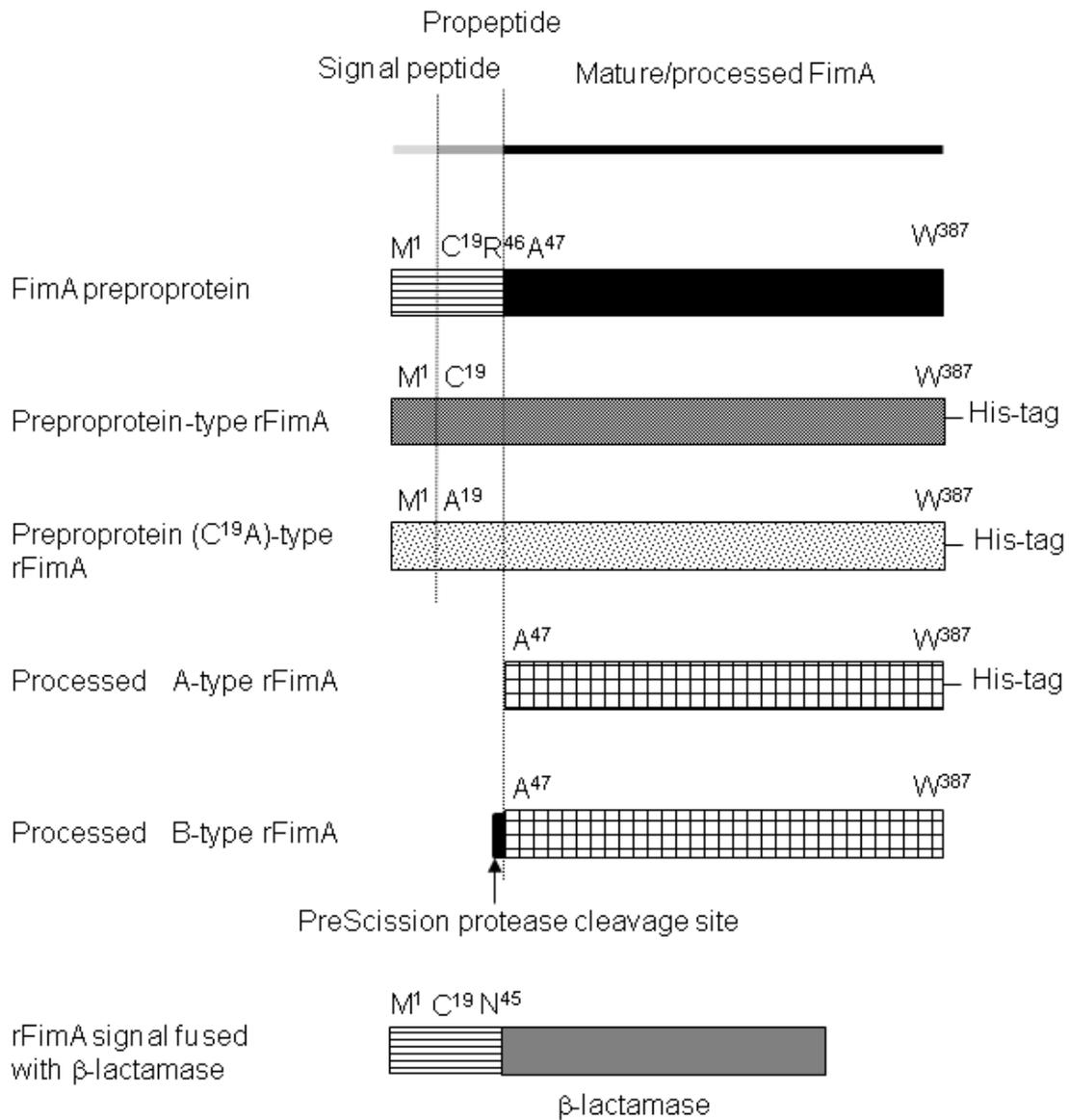


Fig. 1. Diagram of rFimA proteins expressed in *E. coli*. Preproprotein-type, preproprotein (C¹⁹A)-type and processed A-type rFimA proteins were expressed as His-tagged proteins. Processed B-type rFimA protein was initially expressed as a GST-FimA fusion protein.

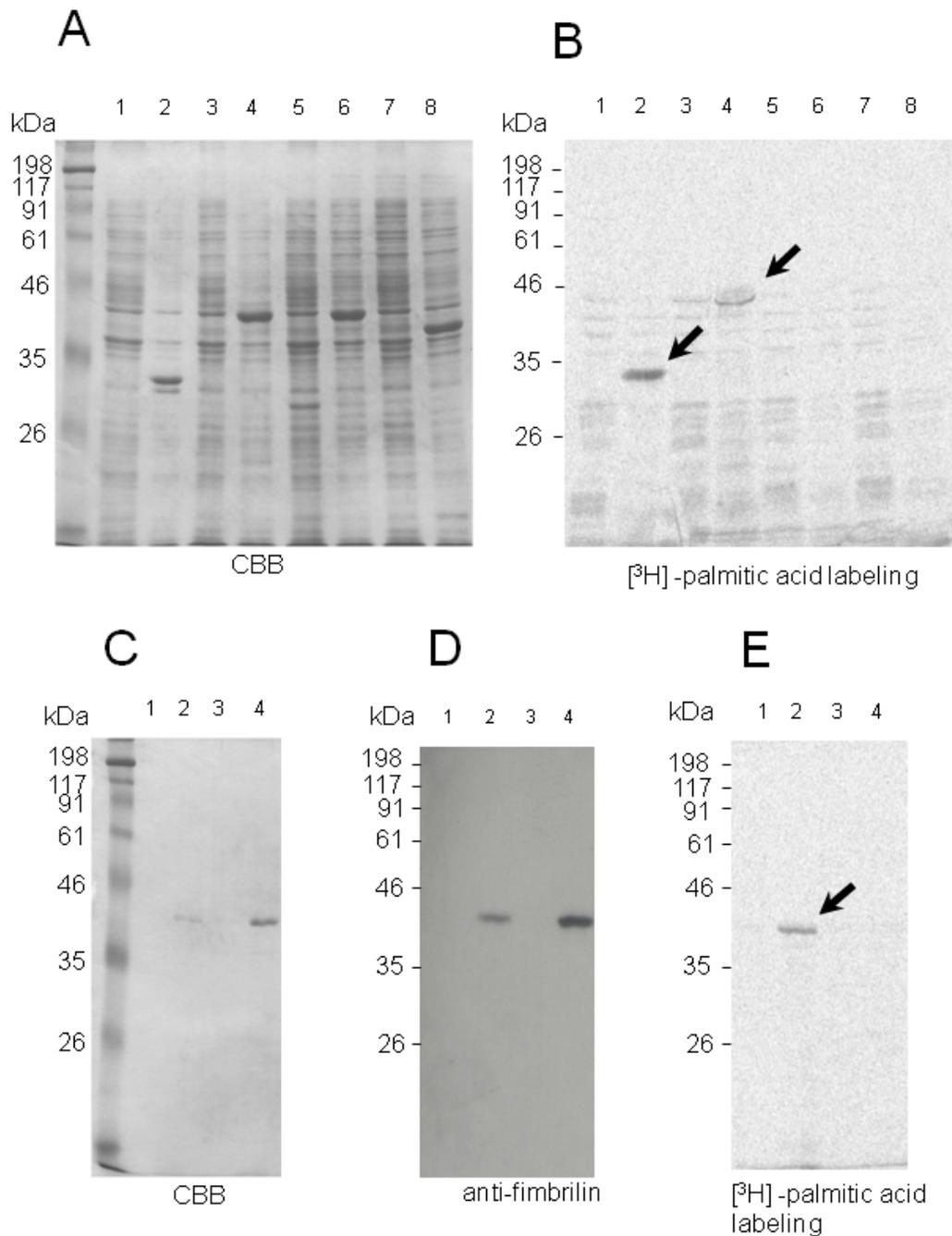


Fig. 2. Lipidation of preprotein-type rFimA. Incorporation of $[^3\text{H}]$ -labeled palmitic acid into rFimA proteins in *E.coli*. (A) The rFimA signal fused with β -lactamase, preprotein-type, preprotein (C^{19}A)-type and processed-type rFimA proteins in *E. coli* were expressed by IPTG induction (Lanes 2, 4, 6 and 8) or not (Lanes 1, 3, 5 and 7)

and separated by SDS-PAGE, and the gel was stained by Coomassie brilliant blue. (B) Lipidated rFimA signal fused with β -lactamase and preproprotein-type rFimA was detected by autoradiography. Lanes 2 and 4 show the presence of the labeled protein (see arrows). (C) Purified preproprotein-type and preproprotein ($C^{19}A$)-type rFimA using Ni-beads after [3H] palmitic acid labeling and IPTG inducton (Lanes 2 and 4) or not (Lanes 1 and 3) were separated by SDS-PAGE, and the gel was stained by Coomassie brilliant blue. (D) Immunoblot analysis of the purified rFimA proteins by anti-FimA antibody. (E) Lipidated preproprotein-type rFimA was detected by autoradiography (see arrow).

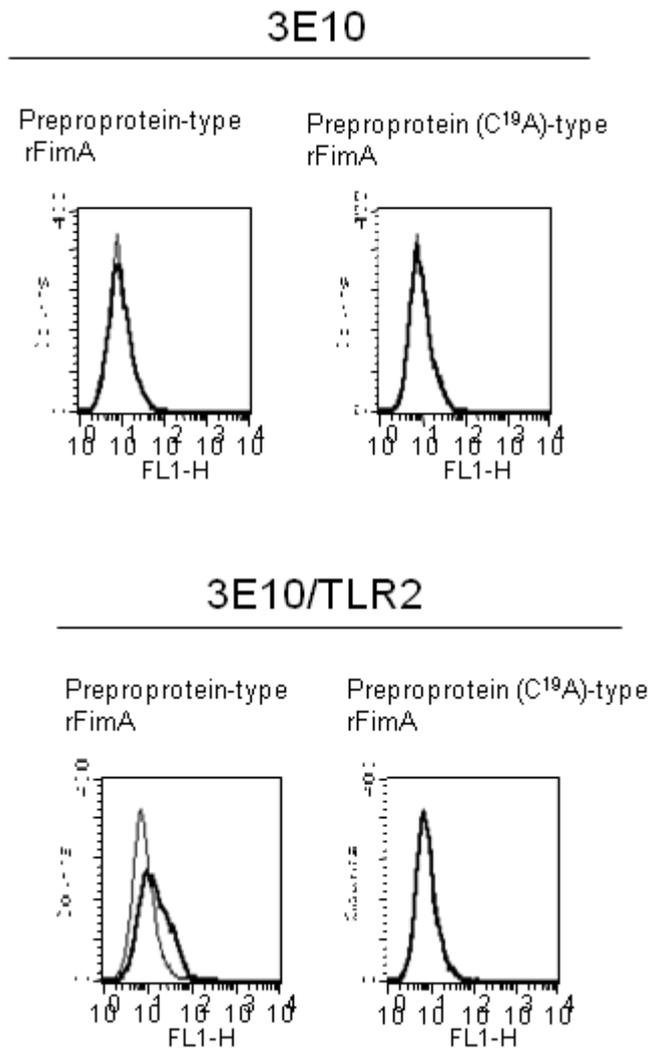


Fig. 3. TLR2-dependent NF- κ B activation by preprotein-type rFimA protein. CHO/CD14 cells or CHO/CD14/TLR2 cells were stimulated with preprotein-type rFimA or preprotein (C¹⁹A)-type rFimA at a concentration of 1 μ g/ml. After 18 h of incubation, the cells were stained with FITC-labeled anti-CD25 mAb and subjected to flow cytometric analysis for NF- κ B-driven CD25 expression. The solid line indicates no presence of a stimulant, and the bold line indicates presence of a stimulant. Tests with higher concentrations of fimbrial proteins (3 and 10 μ g/ml) produced identical results.

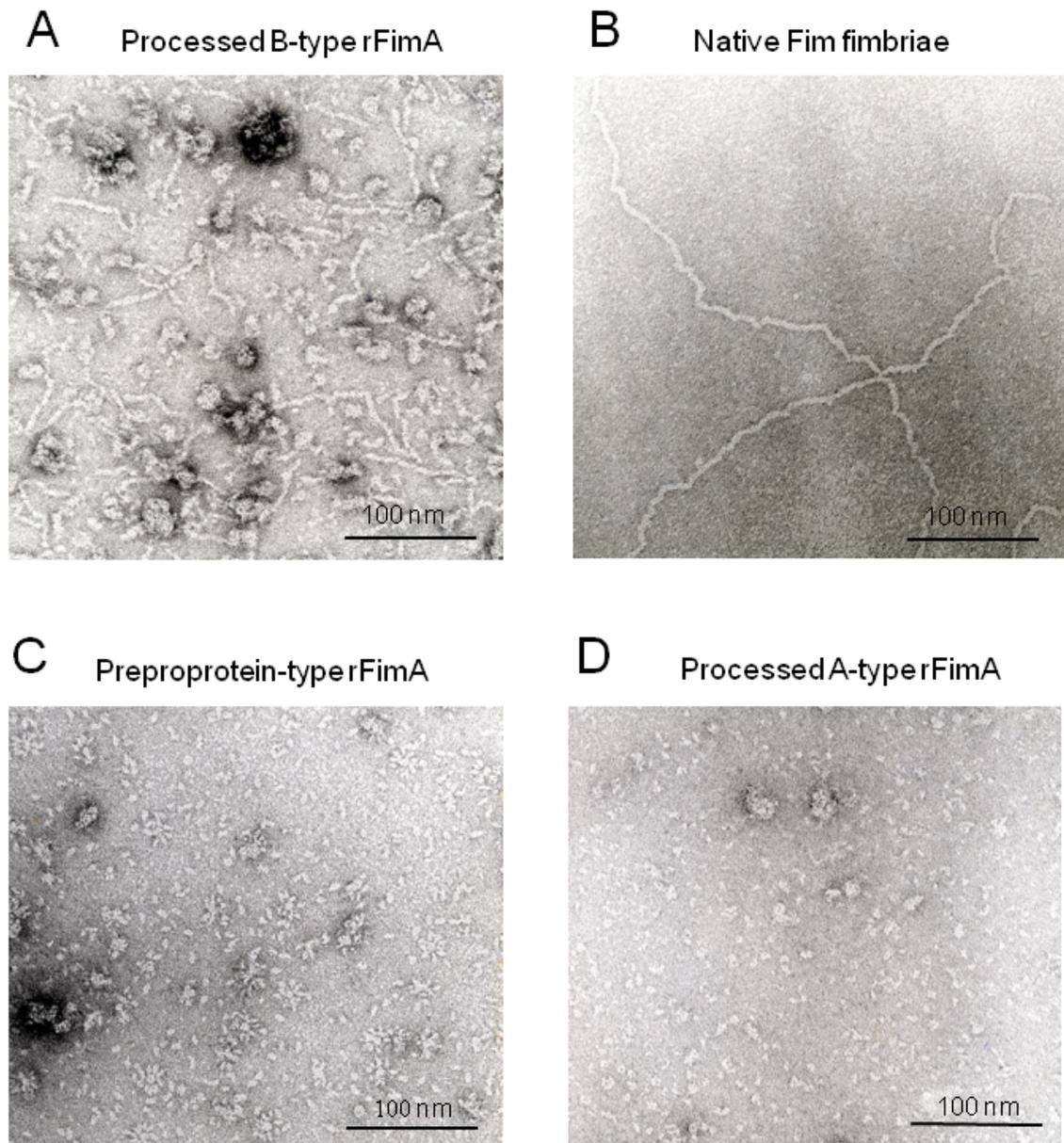


Fig. 4. Electron micrographs of rFimA proteins and native Fim fimbriae. The purified preparations were negatively stained with 2% uranyl acetate. (A) processed B-type rFimA, (B) native Fim fimbriae, (C) preprotein-type rFimA, (D) processed A-type rFimA. Scale bar represents 100 nm.

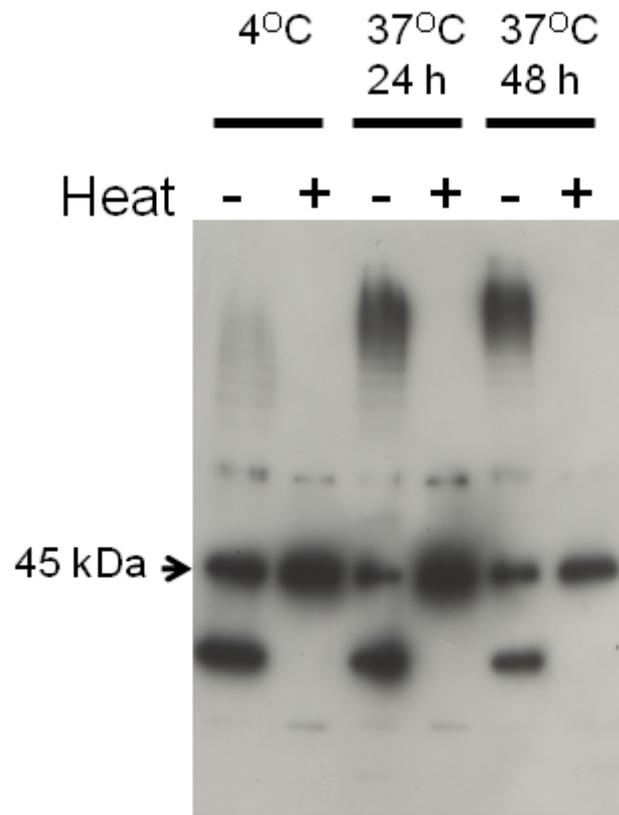


Fig. 5. *In vitro* auto-polymerization of processed B-type rFimA protein. Purified processed B-type rFimA was incubated at 37°C for 24 h or 48 h. Solubilized samples in the presence of β -mercaptoethanol were heat-denatured or not and then separated by SDS-PAGE and detected by anti-FimA antibody.

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
<i>P. gingivalis</i> strains		
33277	Wild type	ATCC
KDP154	<i>mfa1</i> ::Em ^r	This study
<i>E. coli</i> strains		
BL21(DE3)	Host strain for expression vector pKD762, pKD763, pKD764 or pKD767	Novagen
Top10	Host strain for expression vector pKD765	Invitrogen
XL1-Blue	General-purpose host strain for cloning	Stratagene
Plasmids		
pET22b(+)	Ap ^r , expression vector plasmid	Novagen
pGEM-T Easy	Ap ^r , PCR TA cloning vector plasmid	Promega
pGEX-6P3	Ap ^r , expression vector plasmid	Amersham Pharmacia Biotech
pKD399	Ap ^r Em ^r , contains <i>ermF ermAM</i> in pUC18	Shoji et al. 2004
pKD704	Ap ^r Em ^r , contains the 0.77 kb <i>fimA</i> -upstream, <i>ermF ermAM</i> and 0.78 kb <i>fimA</i> -downstream in pBluescript SK-	Shoji et al. 2004
pKD707	Ap ^r FimA(C ¹⁹ A), contains the 2.5 kb <i>fimA</i> region cloned in pBluescript SK-	Shoji et al. 2004
pKD758	Ap ^r , contains the 0.77 kb 5'-terminal portion of the <i>mfa1</i> gene cloned in pGEM-T Easy	This study
pKD759	Ap ^r , contains the 0.77 kb 3'-terminal portion of the <i>mfa1</i> gene cloned in pGEM-T Easy	This study
pKD760	Ap ^r Em ^r , contains the 0.77 kb 5'-terminal portion of the <i>mfa1</i> gene inserted in <i>EcoRI</i> site of pKD399	This study
pKD761	Ap ^r Em ^r , contains the 0.77 kb 3'-terminal portion of the <i>mfa1</i> gene inserted in <i>BamHI</i> site of pKD760	This study
pKD762	Ap ^r , contains preproprotein-type encoding gene cloned within <i>NdeI-XhoI</i> region of pET22b(+)	This study
pKD763	Ap ^r , contains preproprotein (C ¹⁹ A)-type encoding gene cloned within <i>NdeI-XhoI</i> region of pET22b(+)	This study
pKD764	Ap ^r , contains processed A-type encoding gene cloned within <i>NdeI-XhoI</i> region of pET22b(+)	This study
pKD765	Ap ^r , contains processed B-type encoding gene cloned within <i>EcoRI-NotI</i> region of pGEX-6P3	This study
pKD766	Ap ^r , contains the 0.78 kb mature beta-lactamase encoding gene cloned in pGEM-T Easy	This study
pKD767	Ap ^r , contains FimA(M ¹ -N ⁴⁵) fused with mature beta-lactamase encoding gene inserted within <i>NdeI-XhoI</i> region of pET22b(+)	This study
pUC13Bg12.1	Ap ^r FimA ⁺ , contains the 2.5 kb <i>fimA</i> region cloned in pUC13	Yoshimura et al. 1993
pUC18	Ap ^r , cloning vector plasmid	Stratagene

Table 2

Comparison between processed B-type rFimA filaments and native Fim fimbriae.

	Processed B-type rFimA filaments	Purified native Fim fimbriae
Length (nm)	82.00 ± 14.40	>1400
Twisted part length (nm)*	34.17 ± 7.33	35.47 ± 10.10
Width (nm)	5.29 ± 0.62	5.25 ± 0.69

* Fibrous structures of processed B-type rFimA filaments and native Fim fimbriae were composed of repeated twisted parts, and we measured the length of one twisted part.