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Mini review

Surface components of *Porphyromonas gingivalis*

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Background and Objective: Research on *Porphyromonas gingivalis*, a periodontopathogen, has provided a tremendous amount of information over the last 20 years, which may exceed in part than that on other closely related members in terms of phylogenetic as well as proteomic criteria, including *Bacteroides fragilis* and *B. thetaiotaomicron* as major anaerobic, opportunistic pathogens in the medical field. In this minireview, we focused on recent research findings concerning surface components such as outer membrane proteins and fimbriae, of *P. gingivalis*.

Material and Methods: Elucidation of the surface components in *P. gingivalis* was especially difficult because outer membrane proteins are tightly bound to lipopolysaccharide and they are resistant to dissociation and separation from each other, even during sodium dodecyl sulfate–polyacrylamide gel electrophoresis, unless samples are appropriately heated. In addition, *P. gingivalis* is asaccharolytic and therefore a potent proteolytic bacterium, another factor causing difficulty in research. The study of the surface components was carefully carried out considering these unique features in *P. gingivalis* when compared with other gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa*.

Results: Separation of outer membrane proteins, and characterization of OmpA-like proteins and RagAB as major proteins, is described herein. Our recent findings on FimA and Mfa1 fimbriae, two unique appendages in this organism, and on their regulation of expression are also described briefly.

Conclusion: Surface components of *P. gingivalis* somehow have contact with host tissues and cells because of the outermost cell elements. Therefore, such bacterial components are potentially important in the occurrence of periodontal diseases.

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Porphyromonas gingivalis, a gram-negative anaerobe, is a major etiological agent in the initiation and progression of chronic periodontal disease (1,2) and has also been implicated as a contributory factor in the development of systemic diseases such as atherosclerosis (3). The virulence of *P. gingivalis* has been attributed to a variety of potential factors associated with its cell surface, including fimbriae, lipopolysaccharides, capsules, proteases, hemag glutinins and major outer membrane proteins (1,2).

The bacterial cell surface serves as a dynamic interface between the bacterium and the external environment, and facilitates growth, nutrient acquisition, colonization, biofilm formation and evasion of host defense. Therefore, understanding the structure and function of surface components is essential for elucidating the microbial virulence that causes infection and the development of disease.

In this review, we summarize current knowledge on the surface components of *P. gingivalis*, based mainly on recent progress in our work. We focus on the findings for major outer membrane proteins, and on two types of fimbriae (FimA and Mfa1) and the regulation of their expression, with particular

emphasis on the biochemical and molecular genetic aspects.

Outer membrane proteins

Separation of outer membrane

The cell envelope of the gram-negative bacterium consists of two distinct membranes: the inner (cytoplasmic) membrane; and the outer membrane (Fig. 1). The inner membrane is a phopholipid bilayer, whereas the outer membrane is an asymmetric bilayer with an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide. About 50% of the outer membrane mass consists of protein; however, this membrane is dominated by a few major proteins (4–6). The most abundant outer membrane proteins consistently identified are porins and OmpA-like proteins (4,7). It has been estimated that up to 2–3% of the gram-negative bacterial genome encodes outer membrane proteins; to be exact, the β-barrel membrane proteins (8). As the outer membrane is the outermost structure on the surface of most gram-negative bacteria, it serves as a selective permeation barrier that allows the passage of hydrophilic substrates of < 600 Da in *Escherichia coli* and *Salmonella* spp. and exhibits interaction with other bacteria, host cells and their environment (9). Therefore, outer membrane proteins embedded in the outer membrane play important roles in growth, colonization, biofilm formation, establishing infection and the development of disease. However, until recently, major outer membrane proteins in *P. gingivalis* had not been analyzed systematically. To analyze outer membrane proteins, a method for separation of the outer membrane should be established for each organism.

Several attempts have been made to characterize the outer membrane proteins of *P. gingivalis* (10–17). However, most of these studies used differential solubilization with detergents to remove putative inner membrane proteins. This method is known to work well for *E. coli* (18), but it could not be guaranteed that differential solubilization would work as well in *P. gingivalis* as in the other organisms tested. Some studies used *P. gingivalis* outer membranes that were removed by shearing through a hypodermic needle, and they detected several protein bands by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10–13,16). Although this shearing method appears to provide pure outer membrane preparations, it seems to be an inefficient and less productive method of providing outer membrane components for detailed analyses because of limited recovery and/or the possibility of inner membrane contamination as a result of partial cell lysis. Sucrose-density gradient centrifugation after the disruption of bacterial cells using a French pressure cell still remains the most reliable method for isolation of the outer membranes of *E. coli* and *Salmonella* spp., and perhaps for other (as yet unverified) organisms (5,18).

Murakami *et al.* (19) successfully purified the outer membrane from *P. gingivalis* ATCC 33277 by a physical method using sucrose-density gradient centrifugation of a crude whole membrane isolated by French pressure cell disruption. The protein profile of the outer membrane prepared in this manner was confirmed to be little different from that of the fraction obtained by differential solubilization with Triton X-100 in the presence of $MgCl_2$. Therefore, the differential extraction method became a validated separation method for the *P. gingivalis* outer membrane (19).

Identification and characterization of major outer membrane proteins

The N-terminal and internal amino acid sequences of major outer membrane proteins in *P. gingivalis* ATCC 33277 were analyzed (19,20), and the proteins were identified in view of the recent availability of the genomic sequence of *P. gingivalis* (21). These comprised RagA, RagB, Arg-gingipain, Lys-gingipain and OmpA-like proteins (Pgm6/7) (22–26). Adhesin domains of the gingipains Hgp44, Hgp27 and Hgp15 were also identified (Fig. 2).

Veith *et al.* (27) undertook a proteomic study of the outer membrane of *P. gingivalis* W50 prepared by the Sarkosyl solubilization method using two-dimensional gel electrophoresis and peptide mass fingerprinting. Thirty-nine major proteins derived from 31 distinct genes were identified. These included RagA, RagB, Pgm6/7 (equivalent to PG32/33 or Omp40/41; all are OmpA-like proteins), the gingipains and HagA, and their processed adhesin domains.

It is interesting that both Arg-gingipain and Lys-gingipain were shown to exist as major protein bands in the outer membrane (19) although they were initially purified from the culture supernatant (25,26) and had been inferred to be membrane proteins.

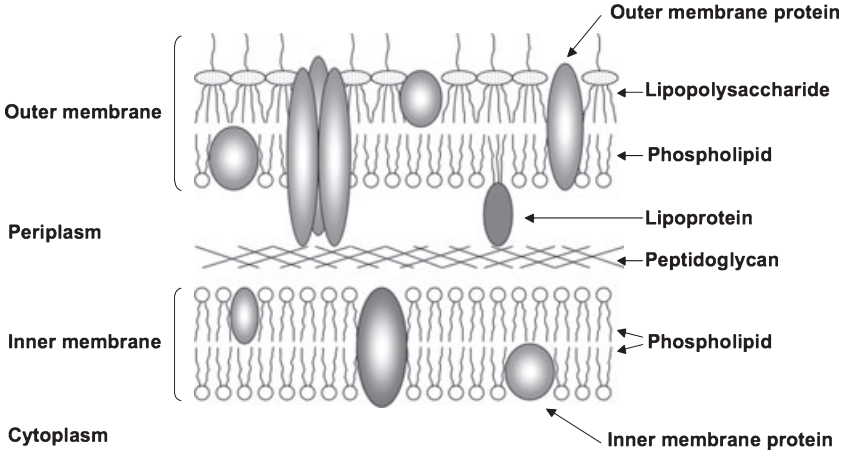

Fig. 1. Schematic illustration of the surface structure of gram-negative bacteria. The asymmetric outer membrane contains lipopolysaccharides, phospholipids, lipoproteins and outer membrane proteins.

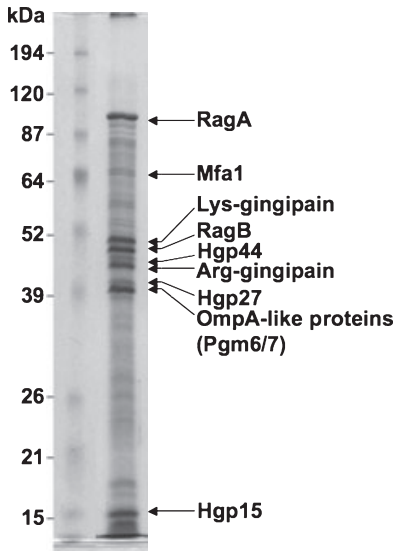

Fig. 2. Typical profile of outer membrane proteins from *Porphyromonas gingivalis* ATCC 33277. The outer membrane fraction was obtained from bacterial cells disrupted using a French pressure cell followed by differential detergent solubilization with Triton X-100 and $MgCl_2$. The fraction was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. The gel was stained with Coomassie Brilliant blue.

These proteinases, as 'landmarks' of *P. gingivalis*, an asaccharolytic bacterium, are closely associated with the pathogenesis of periodontal diseases, and are presumably important in obtaining nutrients from the environment and in the processing and maturation of various cell-surface proteins (25,26). A model of the gingipain proteinase–adhesin complex located at the surface of the outer membrane has been proposed (28). Recent studies have shown some new experimental evidence for a gingipain complex. Takii *et al*. (29) suggested that a gingipain complex containing catalytic and adhesin domains existed in a cell-associated form and was modified by lipopolysaccharide. Subsequently, Pathirana *et al*. (30) characterized the proteinase–adhesin complex. A 75-kDa protein (31), now identified as a major subunit of Mfa1 fimbriae (32,33), was also found in the outer membrane preparations, although a corresponding protein (41–43 kDa) of FimA fimbriae (34) was absent.

Imai *et al*. (35) examined the distribution of major outer membrane proteins among various *P. gingivalis* strains. RagA and OmpA-like proteins were clearly identified in all strains tested. The bands near 50 kDa that were expected to be RagB, Arg-gingipain or Lys-gingipain showed variations in mobility among strains. These proteins were confirmed by Western blot analysis. When the profiles of the major outer membrane proteins in *P. gingivalis* ATCC 33277 and W83 were compared, the mobilities of most proteins were similar. However, the positions of the RagB and Lys-gingipain bands were inverted between the two strains.

Murakami *et al*. (20,36) examined the effects of various culture environments on major outer membrane proteins. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the major outer membrane protein patterns showed little difference over the culturable range of osmolarity and pH. With elevated temperature or prolonged culture, the intensities of the gingipain bands decreased; however, expression of RagA, RagB and the OmpA-like proteins was relatively stable. Culture media type and culture conditions affected major outer membrane protein patterns to some extent. Masuda *et al*. (37) reported the effects of growth conditions on major outer membrane proteins using continuous culture in a chemostat. Gingipains decreased in amount and activity at higher growth rates, and a considerable reduction in gingipain activity under aeration was observed. However, the expression of RagA, RagB and the OmpA-like proteins was essentially unchanged under all conditions tested.

The characterization and functions of the most intriguing and abundant proteins, namely RagA, RagB and OmpA-like proteins, are described in the following sections.

OmpA-like proteins

The OmpA outer membrane protein is one of the most abundant proteins in the outer membrane of *E. coli* and other enterobacteria. This protein can function in adhesion and invasion, participate in biofilm formation, act as both an immune target and as an evasin, and serve as a receptor for several bacteriophages (38). The OmpA protein also produces a nonspecific diffusion channel that allows the slow penetration of various solutes (39). Most OmpA protein exists in a monomeric form (9).

The OmpA-like proteins in *P. gingivalis*, designated Pgm6/7 and PG32/33 or Omp40/41, respectively, with different strains used by two research groups, are encoded by open-reading frames *pg0694* and *pg0695*, which form an operon (40,41). They have a high degree of similarity to *E. coli* OmpA in the C-terminal peptidoglycan-associated region and are predicted to form eight-stranded β-barrels in the N-terminal region (41). PG32/33 were assigned to the immunoreactive 43-kDa and 42-kDa antigens, respectively, of *P. gingivalis* W83 (21,42), and Omp40/41 were assigned, by the same research group, to protein spots at 40 kDa and 41 kDa of *P. gingivalis* W50 (40). Omp40/41 share 47% sequence identity, as do Pgm6/7, and present as a horizontal train of spots on two-dimensional polyacrylamide gel electrophoresis, implying differential post-translational modification; however, their heterogeneity is related to conformational equilibrium (40). In addition, they show heat-modifiability during sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as reported for *E. coli* OmpA (4,5,40). The N-termini of these proteins (Pgm6/7 and Omp40/41) were blocked and identified as pyroglutamate (19,40).

Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with and without 2-mercaptoethanol, Pgm6/7 appear as a single band with apparent molecular masses of 40 and 120 kDa, respectively (19). This suggests that Pgm6/7 form heterotrimers in the outer membrane. To verify the predicted trimeric structure, Nagano *et al*. (41) constructed three mutants by deleting the open-reading frames *pg0695* and/or *pg0694*, which encode Pgm6 and Pgm7, respectively. The double mutant produced no Pgm6/7. The single-deletion mutants contained less Pgm7 and Pgm6 and formed

homotrimers that migrated at 115 and 130 kDa, respectively, under nonreducing conditions. Two-dimensional diagonal electrophoresis and chemical cross-linking experiments have clearly shown that Pgm6/7 mainly form stable heterotrimers via intermolecular disulfide bonds (41). However, it is claimed by Reynolds' group that Omp40/41 form heterodimers thorough disulfide bonds (40) as a result of evidence that they migrate as 34-kDa and 35-kDa proteins, and as a 70-kDa protein, under reducing and nonreducing conditions, respectively. Pgm6/7 (PG32/33) contain two cysteine residues (which are essential for forming intermolecular disulfide bonds) in the mature molecules (40,41).

Loss of Pgm6/7 causes bacterial growth retardation in synthetic media and increases outer membrane permeability, indicating that the mutants have a somewhat defective, weak and leaky outer membrane (41). Similarly, Ross *et al*. (42) reported that PG32/33 play an important role in bacterial growth. Iwami *et al*. (43) have shown that Pgm6/7 deletion mutants have increased vesicle formation and lose their membrane integrity to some extent. Thus, Pgm6/7 in *P. gingivalis* are responsible for the maintenance and stability of the outer membrane, and are key elements that modulate vesicle formation.

The *P. gingivalis* W83 genome has at least three homologs of *E. coli* OmpA, but no homologs of the classical, trimeric, highly efficient *E. coli* porins OmpF and OmpC (9,21). Moreover, *E. coli* OmpA produces nonspecific diffusion channels that allow the slow penetration of various solutes as inefficient porins (39). Therefore, Pgm6/7 have been strongly inferred to be candidates for the major porin in *P. gingivalis*. However, in a swelling assay with proteoliposomes containing bacterial envelopes (44), no obvious difference in the swelling rates of the liposomes between the wild type and the mutants was detected (41). Thus, it appears that Pgm6/7 are unlikely to function as a major porin.

Possible application of OmpA-like proteins in *P. gingivalis* as vaccine candidates was reported. Truncated versions of recombinant PG32/33 showed high levels of protection in *P. gingivalis* murine lesion models. Therefore, they may be potential vaccine candidates (42,45).

RagA and RagB proteins

RagA and RagB were originally identified as immunodominant surface antigens recognized by sera from periodontal patients (15). Sera from patients with periapical lesions also react with RagB antigen (35). It has been reported that *ragB*, which is located adjacent to and downstream of *ragA*, is co-transcribed with *ragA* as a polycistronic message, and that expression of *ragAB* is influenced by temperature (22,24). The *ragAB* operon is proposed to have arisen by horizontal gene transfer, based on its reduced G + C content, from a foreign source such as a component of the periodontal microflora (23).

RagA has homology to TonB-dependent outer membrane receptors, which are involved in the recognition and active transport of specific external ligands by a wide range of gram-negative species (46,47). TonB spans the periplasmic space and physically interacts with outer membrane receptors in a highly conserved region called the TonB box. TonB is associated with ExbB and ExbD, and this system energizes outer membrane transport by the proton motive force of the inner membrane. *P. gingivalis* possesses a gene encoding a putative TonB protein, as well as genes encoding putative ExbB and ExbD proteins (21,48). From its primary amino acid sequence RagB is predicted to be a lipoprotein and it appears to be more diverse than RagA among species (22,23,35). Based on circumstantial evidence, RagA and RagB might form a functionally linked complex on the outer surface of *P. gingivalis*, involved in a TonB-dependent, active process (22). Recently, chemical cross-linking and co-immunoprecipitation experiments revealed a physical association of RagA and RagB molecules (49). In addition, cell-surface labeling showed that both RagA and RagB were exposed on the cell surface (49).

In earlier studies, the *ragAB* locus was found in only 17 of the 132 *P. gingivalis* strains (13%) by Southern blot analysis using a DNA probe for *ragB* amplified from *P. gingivalis* W50 (23,50). The *ragAB* locus was detected mostly in virulent strains, including W50 and W83, but not in the *P. gingivalis* strains ATCC 33277 and 381 (23). However, RagA and RagB proteins were actually detected as major proteins in the outer membrane of *P. gingivalis* ATCC 33277 (19,20). In addition, Imai *et al*. (35) showed that RagA and RagB were widely distributed in various *P. gingivalis* strains. Recently, sequence diversity and antigenic variation at the locus were reported. The locus was classified into four alleles (51). For example, W83 and 33277 presented *rag-1* and *rag-4*, respectively. The *ragAB* locus is flanked by the same genes, indicating that it is in the same genomic location among the strains; however, there was some polymorphism (51).

Nagano *et al*. (49) have demonstrated that both *ragA* and *ragB* are required for stable expression of RagA and RagB, using deletion mutants. When *ragA* was deleted, RagB was expressed at a negligible level, mainly because of a polar effect. Interestingly, however, deletion of *ragB* led to RagA degradation. The deletion mutants had retarded bacterial growth in a nutrient-poor synthetic medium, probably because they might not take up larger peptides. Based on sequence comparison with *Bacteroides thetaiotaomicron* SusCD, RagAB has been suggested to play a role in the uptake of macromolecules such as polysaccharides or glycoproteins (22,24,52). However, further work is required to elucidate the exact function of RagAB. In relation to pathogenicity, the deletion mutants had decreased infective activity in a mouse model (49,53).

Other outer membrane proteins

P. gingivalis requires hemin for growth (1,2,48). In most gram-negative bacteria heme utilization is mediated by specific outer membrane receptors (54). Several TonB-dependent outer membrane proteins were reported as

hemin-uptake receptors. HmuR was demonstrated to be involved in hemin and hemoglobin utilization (55,56). Dashper *et al*. (57) reported that IhtB, formerly designated Pga30 (58), was a TonB-dependent outer membrane hemin-binding protein. Another putative TonB-linked outer membrane receptor, Tlr, previously identified as Tla, has been described (59,60). Experimental data suggest that it has an active role in heme acquisition and utilization. Additionally, the TonB-dependent outer membrane receptor HemR was reported; however, biochemical evidence of heme binding or utilization was not shown (61).

Hemin-binding proteins other than TonB-dependent receptors were also reported. HBP35, previously designated as the 40-kDa outer membrane protein, binds hemin and is related to co-aggregation with *Actinomyces viscosus* and *Streptococcus gordonii* (62–65). Specific antibodies to this antigen protect against *P. gingivalis*-induced bone loss and abscesses (66,67). A 26-kDa outer membrane protein that binds heme has also been described; however, the gene encoding this putative receptor has not yet been identified (68).

Several studies have reported other outer membrane proteins that are not involved in hemin utilization. A 28-kDa outer membrane protein (Omp28) was identified and characterized. This protein, expressed widely in *P. gingivalis* strains, is speculated to act as a surface receptor or for adhesion of *P. gingivalis* (69). A 53-kDa outer membrane protein was reported as the immunodominant *P. gingivalis* antigen recognized by sera from periodontitis patients (70,71), although we did not detect it in strains ATCC 33277 and 381, both of which have FimA and Mfa1 fimbriae, at least as a major outer membrane protein. The B-cell and T-cell epitopes for this antigen were identified (72,73). The role of T cells in the humoral responses against the 53-kDa outer membrane protein was also investigated (74). Recently, Nakao *et al*. (75) characterized an Omp85 homolog in *P. gingivalis* and provided evidence that it was a glycosylated homolog involved in biofilm formation. Omp85 is a highly conserved, outer membrane protein in all gram-negative bacteria (76). Omp85 in *Neisseria meningitidis* and *E. coli* is essential for cell viability and is involved in the assembly of outer membrane proteins (77,78). However, the exact function of the Omp85 homolog in *P. gingivalis* remains to be elucidated.

Fimbriae

Fimbriae or pili are proteinaceous, filamentous appendages that protrude outwards from the bacterial cell surface and play a crucial role in virulence by stimulating bacterial attachment to host cells or tissues (79). Research on *P. gingivalis* fimbriae has a long history going back to the 1980s. However, biochemical and later molecular studies began with the purification of fimbriae from *P. gingivalis* 381 in 1984 (34). At that time, different types of fimbriae were not observed morphologically on the cell surface. Later, however, another minor (short) fimbria in *P. gingivalis* were discovered (32), and the presence of a third fimbrial type has been suggested by a biochemical approach, although its characterization has not yet been carried out and it is somewhat related to the second type (80–82). The first fimbriae are called major, long, or FimA fimbriae, and the second ones are referred to as minor, short, or Mfa1 fimbriae. Only recent advances in studies on FimA and Mfa1 fimbriae (these terms are used hereafter to avoid confusion), particularly morphogenic aspects and regulation, especially of FimA fimbrial expression, will be described in the following sections.

FimA fimbriae

Genetic loci for the three fimbriae are all in the chromosome. The loci for FimA and Mfa1 fimbriae are separate from each other and they are immunologically different. However, the gene arrangements of FimA and Mfa1 fimbriae in the chromosome appear to be in part similar to each other, especially in the region encoding a gene for a major structural protein and its several downstream genes, although the full region essential for their morphogenesis was not determined. The orders of the genes are surprisingly similar to each other, where a major structural gene (*fimA* or *mfa1*), an unknown gene [*fimB* (formerly ORF1) or *mfa2*] presumably encoding an outer membrane protein, and three genes (*fimC* to *fimE* and pg0180 to pg0182 in FimA and Mfa1 fimbriae, respectively) encoding minor component proteins, tightly associated with fimbrial filaments, are arranged as clusters with the same transcriptional direction (Fig. 3).

Gene products of *fimC* and *fimD* (formerly ORF2 and ORF3, respectively), downstream from *fimA*, have been assumed to be two functionally important minor components because their 50-kDa and 80-kDa proteins, respectively, were identified as minor components of fimbriae without evidence of their functions (83). Recently, Nishiyama *et al*. (84) discovered another downstream gene product (60-kDa protein) as the third minor component of the fimbrial filaments and that the three genes, designated *fimC* to *fimE* (formerly ORF4) appear to be cotranscribed as a functional unit, based on analysis of the mutants. As sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of highly purified fimbrial preparations has always shown three faint, but distinct, protein bands migrating at 50, 60 and 80 kDa (83) (F. Yoshimura, unpublished data), and because the 60-kDa protein had not yet been identified, these seem to be reasonable findings suggesting specific roles of the three minor proteins in adhesion as well as immunological reactions such as the host response.

FimA fimbriae are known to bind to a number of eukaryotic proteins (85) as well as a prokaryotic protein, namely glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* (86,87). The binding activities of purified fimbriae from the wild-type and mutant strains (Δ*fimC*, Δ*fimD* and Δ*fimE* mutants) to recombinant glyceraldehyde-3-phosphate dehydrogenase and other proteins were examined (84). The enzyme-linked immunosorbent assay results demonstrated that fimbriae from the wild-type strain bound to

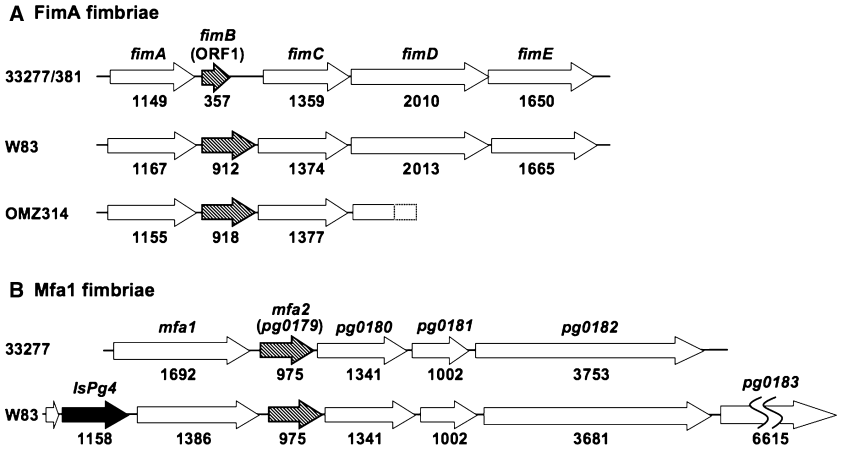

Fig. 3. Schematic diagrams of the downstream genes of *fimA* (A) and *mfa1* (B) in limited strains. (A) The top line shows the FimA gene cluster of *Porphyromonas gingivalis* ATCC 33277 and 381 (*fimA* genotype I). Below are *P. gingivalis* W83 (genotype IV) and OMZ314 (genotype II). Open-reading frames are represented by arrows indicating their 3′ ends. The *fimA* sequences of ATCC 33277, W83 and OMZ314 were obtained from GenBank (D42067) and TIGR GenBank (AB261607), respectively. The downstream sequence of *fimD* in OMZ314 is indicated by a box with a broken line; the sequence further downstream is not yet available. W83 does not produce FimA fimbriae, partly because of a defect in FimSR, a two-component signal transduction system, positively regulating their production. (B) A potential *mfa1* gene cluster in ATCC 33277 and W83. The gene cluster appears to be composed of at least five genes, including *mfa1*, with the same transcriptional direction except that *mfa*1 is split into pg0176 and pg0178 by ISPg4 (pg0177), indicated by the solid arrow, in W83. Therefore, W83 does not produce Mfa1 fimbriae either. The gene *fimB* (and its corresponding genes) and *mfa2* (and its corresponding gene) are highlighted as hatched arrows in FimA and Mfa1 gene clusters, respectively. The number values shown under the genes indicate their lengths in bp.

recombinant glyceraldehyde-3-phosphate dehydrogenase, fibronectin and type I collagen, whereas fimbriae from the three mutants showed significantly weaker binding (only the binding to recombinant glyceraldehyde-3-phosphate dehydrogenase is shown in Fig. 4) and all the mutant fimbriae purified lacked the three minor proteins (FimC, FimD and FimE) simultaneously. Fimbriae from the three mutants are not yet distinguishable from each other morphologically and biochemically, in which they lack all three minor proteins, although FimC and FimD were produced and detected in the whole-cell extracts and culture supernatants of the *fimE* mutant. Fimbriae in the mutants had lost the ability to autoaggregate their mutant cells (84). These results suggest that the minor proteins may play a crucial role in binding and are probably localized in the tips of filaments as an adhesion complex. FimE may play a key role in the formation of this adhesion complex and other components may function as real adhesins. Interestingly, the importance of the minor components in fimbriae has been shown in the innate immune system. The mutant fimbriae without these components displayed a preference for Toll-like receptor 1 and a stronger inflammatory response than the wild-type fimbriae, but the wild-type fimbriae can utilize either Toll-like receptor 1 or Toll-like receptor 6 for co-operative Toll-like receptor 2-dependent activation of transfected cell lines (88). Mutant cells with fimbriae devoid of all accessory proteins (referred to as DAP fimbriae) are dramatically less persistent and virulent than wild-type cells in a mouse periodontitis model. Native fimbriae allow *P. gingivalis* cells to exploit the Toll-like receptor 2/complement receptor 3 pathway for intracellular entry, inhibition of interleukin-12p70 and persistence in macrophages (89). Morphological evidence as to whether the minor components are localized at the fimbrial tip is urgent, and identification of a real adhesin, if it exists in the minor components, remains to be elucidated.

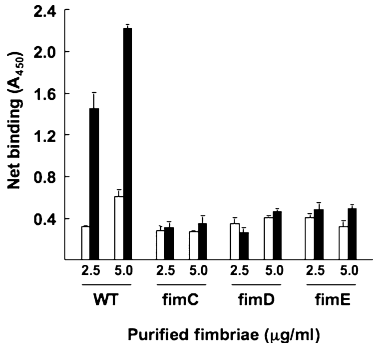

Fig. 4. Specific binding of the wild-type and mutant fimbriae to a prokaryotic protein, recombinant glyceraldehyde-3-phosphate dehydrogenase. The figure is a simplified and modified version of Fig. 5(a) published in a recent report (84). Wells of microtiter plates contained buffer with casein but without specific ligands (white bars), or with 20 mg/mL of recombinant glyceraldehyde-3-phosphate dehydrogenase (black bars).

Mfa1 fimbriae

As described above, Mfa1 fimbriae were found as the second fimbrial type on a *P. gingivalis* mutant deficient in the *fimA* gene (32), suggesting short and/or fewer filaments hidden by long FimA fimbriae in cells. They were initially thought to be a major surface antigen as a large complex, probably because of the short filaments (31). Indeed, some strains, such as ATCC 33277 and 381, appear to produce as many Mfa1 fimbriae as FimA fimbriae, based on a comparison of two major FimA and Mfa1 protein bands with molecular masses of 41–43 kDa and 67–75 kDa (depending on the research group), respectively, in whole-cell lysates, although the first report called them 'minor fimbriae' (32). Neither Mfa1 nor FimA fimbriae are produced in a few strains such as W83, although the precise prevalence of Mfa1 fimbriae among strains has not yet been reported. Little is known about the function of Mfa1 fimbriae, although recent studies have shown that they are involved in co-adhesion with *S. gordonii* (33,90,91), in autoaggregation and in colonization

(92), and, similarly to FimA fimbriae, they can also stimulate potent inflammatory responses (93–96). However, we do not know why some *P. gingivalis* strains produce large amounts of both Mfa1 and FimA fimbriae, or how they share their functions and roles in this organism.

Recently, Mfa1 fimbriae purified from a *fimA* mutant were indeed found to be short filaments of uniform length (80 to 120 nm long with an average of about 100 nm) (33). However, a mutant deficient in *mfa2*, a downstream gene of *mfa1* encoding the major fimbrilin Mfa1, from ATCC 33277 produced long filaments (F. Yoshimura, J. Iwami & Y. Murakami, unpublished data). This finding came from an attempt to isolate Mfa1 fimbriae in an *mfa1* mutant complemented by the introduction of the wild-type *mfa1* gene *in trans* (cSMF1). Like FimA fimbriae, Mfa1 fimbriae can be easily shed from the cell surface of the mutant cells, and the Mfa1 fimbriae that were shed appeared to be much longer than those in the wild type, although the results were somehow ambiguous because of the presence of long and loosely attached FimA fimbriae in the strain. Therefore, later studies on *mfa2* and its product (Mfa2) were carried out with the background of a *fimA*-deficient mutant. As described in a previous report (91), *mfa1* and *mfa2* were confirmed to be co-transcribed as an operon, based on reverse transcription–polymerase chain reaction results (Y. Hasegawa, Y. Murakami & F. Yoshimura, unpublished data). Further downstream genes (pg0180, pg0181 and pg0182) appear to be expressed in a regulated manner closely linked to *mfa1* and *mfa2*. Products of pg0180, pg0181 and pg0182 were identified as three minor components in the Mfa1 fimbriae purified. Mfa2 was not found in Mfa1 fimbriae, but was found in the outer membrane, indicating that this protein may function as an anchor for the Mfa1 fimbriae and also as a regulator of their length (Y. Hasegawa, Y. Murakami & F. Yoshimura, unpublished data).

It may be pertinent to mention here that genetic variants of *mfa1* (i.e. *mfa1* genotypes), such as *fimA*, and their distribution have been reported, although they were designated the *Pg-II fim* gene for Pg-II fimbriae (97).

Functional exploration for *mfa2*, an adjacent, downstream gene of *mfa1*, reminds us of the presence of a similar gene (*fimB*) and genetic structure (*fimA–fimB–fimC* to *fimE*) in FimA fimbriae (see Fig. 3). In a morphological sense, Mfa1 and FimA fimbriae may be difficult to distinguish from each other in strains such as OMZ314 that contain the *fimA* genotype II. Because FimA fimbriae are easily shed only from ATCC 33277 and strain 381 (genetically similar strains and both of the *fimA* genotype I), but not isolated selectively from other strains with different *fimA* genotypes, most strains with the *fimA* genotype I may possibly produce unusual FimA fimbriae that are very long and loosely attached to the cell surface, and tend to be easily shed by a simple, efficient washing method (34) (F. Yoshimura, unpublished data). However, because *fimB* (corresponding to *mfa2*) in *P. gingivalis* ATCC 33277 and 381 appears to be truncated when compared with other strains, including W83 (84) as shown in Fig. 3, they may be mutants of FimA fimbriae, as *E. coli* K12 is known to be an O-antigen-defective mutant. Further integrated research on genes for Mfa1 and FimA fimbriae is necessary. For example, other strains genetically different from ATCC 33277 and 381 with the same *fimA* genotype I should be examined to determine whether they have long FimA fimbriae loosely attached to cells and what their Mfa1 fimbriae look like.

Regulation of fimbrial expression

Regulation of FimA

Biosynthesis of the subunit proteins of FimA fimbriae is positively regulated by the FimS–FimR two-component system. In prokaryotes the two-component system is a common signal transduction mechanism used to sense and respond to environmental changes (98). The two-component system typically comprises an inner membrane-localized sensory histidine kinase and a cognate cytoplasmic response regulator. Sensing of an environmental signal (ion, molecule, temperature, osmotic pressure, etc.) by the periplasmic sensor domain of a histidine kinase induces autophosphorylation of a conserved histidine residue in the cytoplasmic kinase domain. The phosphoryl group is then transmitted to a conserved aspartate residue in the receiver domain of the cognate response regulator, inducing its conformational change. The activated response regulator typically functions as a transcriptional regulator to modulate the expression level of target genes. Many studies have revealed significant roles of various two-component systems in modulating the expression of virulence-determinant genes of pathogenic bacteria. In *P. gingivalis* the first two-component system identified was FimS–FimR (99), discovered during screening for fimbriae-deficient transposon mutants (100). The histidine kinase FimS, encoded by *fimS*, has a putative periplasmic domain between two membrane-spanning regions in the N-terminal half of the polypeptide, with very poor homology to known histidine kinase sensor domains. The only conserved motif found in the center of the periplasmic domain is the tetratricopeptide repeat, which is identified in a wide variety of proteins as a tandemly repeated degenerate amino acid sequence that forms a scaffold to mediate protein–protein interactions (101). The *fimR*-encoding response regulator, FimR, has a helix-turn-helix motif in the C-terminal effector domain, implying its function as a DNA-binding transcriptional regulator. Disruption of *fimS–fimR* loci causes a drastic reduction of FimA production, and the *fimR* insertion mutant is successfully complemented by the wild-type gene *in trans* using an expression plasmid (102). Northern blot analysis has revealed that this regulation takes place at the transcriptional level. Comparative expression analysis of *fimR* wild-type and mutant strains using microarrays showed that only a small number of genes were consistently up-regulated by FimR. Some of the FimR-responsive

genes with high fold-change values are clustered around the *fimA* locus, indicating that the FimS–FimR two-component system has a dedicated role in the expression of the *fim* gene cluster (103). It is intriguing that the *fimS–fimR* loci are located far from the *fimA* locus (i.e. an interval of about 720 kb on the W83 genome) because two-component system genes are often located adjacent to the target genes and comprise operons.

Despite the distinct phenotypes of *fimS* and *fimR* mutants in terms of *fimA* expression, FimR does not directly activate the transcription of *fimA*. Chromatin immunoprecipitation and gel shift assays (e.g. electrophoretic mobility shift assays) have revealed that the response regulator binds exclusively to the promoter region of a previously overlooked open-reading frame (103) that is located immediately upstream of the *pgmA* locus (17), and consequently becomes the first gene of the *fim* gene cluster. This target gene of FimR, designated *fimX*, encodes a 193 amino acid polypeptide, FimX, with an unknown function. As predicted by signal peptides and lipoprotein motifs, both FimX and PgmA seem to localize in the membrane fraction (K. Nishikawa, unpublished data). The transcription profiles of *fimR* and *fimX* insertion mutants are essentially identical: mutations in *fimR* or *fimX* abrogate transcription of *pgmA*, *fimA* and the rest of the downstream genes that encode minor components of FimA fimbrilin (84). By contrast, transcription of *fimA* is still detectable in the *pgmA* mutant, and its expression of FimA protein still maintained at approximately 50% of the wild-type level. The *fimA* knockout mutant still maintains expression of *fimX* and *pgmA*, but not the downstream genes. These findings substantiate a regulation cascade model comprising at least four steps, as shown in Fig. 5. The first step for activation of FimR starts from FimS sensing an unidentified environmental signal, which causes autophosphorylation of the histidine kinase, and the phosphoryl group is rapidly transmitted to FimR and induces its conformational change. In the second step, the activated form of the response regulator binds to the promoter region of *fimX* and up-regulates the transcription of the *fimX–pgmA* operon. In the third step, the products of the operon, principally FimX, promote transcription of *fimA* and produce the major subunit of FimA fimbriae. In the fourth step, FimA protein acts as a positive regulator for the transcription of downstream genes encoding minor components of the fimbriae.

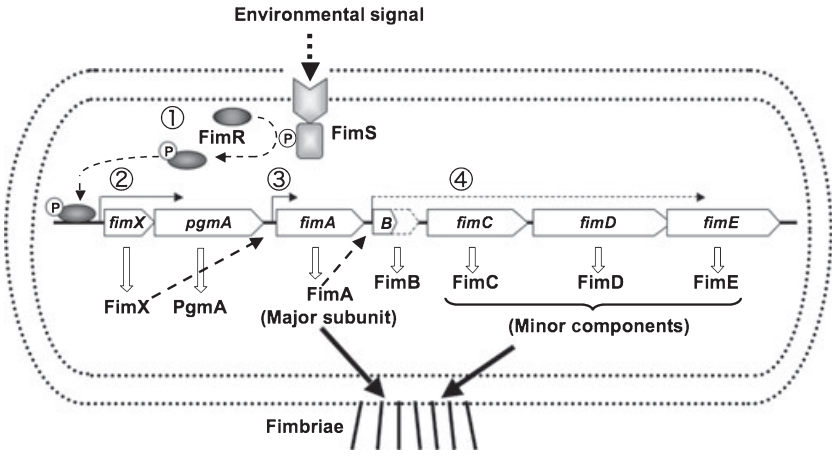

Fig. 5. A regulation cascade model for expression of the *Porphyromonas gingivalis fim* gene cluster. An unknown environmental signal is received by the FimS sensor kinase and transmitted to the cognate FimR response regulator in the cytoplasm ①. The activated FimR up-regulates the transcription of the *fimX–pgmA* operon ②, followed by the expression of *fimA*, encoding a major subunit fimbrilin (FimA), in a FimX-dependent manner ③. Finally, the expressed FimA acts as a positive regulator for the transcription of downstream genes encoding minor structural components ④. The FimA and minor components may be polymerized to unique single-stranded filaments, possibly through a proposed mechanism (113). Experimental details of the regulation cascade have been described previously (103).

While possessing some features of membrane-localized transcriptional regulators such as the ToxR/ToxS and ToxR/TcpP systems in *Vibrio cholerae* (104,105), FimX and PgmA could be the components of a chaperon/usher assembly pathway. Genes involved in the biosynthesis of fimbriae belonging to the chaperon/usher assembly class are clustered into operons, which encode at least three different proteins: a major structural subunit; a chaperon; and an usher (106). These operons often contain additional genes encoding structural proteins such as minor fimbrial subunits. Although FimX and PgmA lack homology to any known chaperon/usher protein, the organization of the *fim* gene cluster, as well as the existence of *fimX–pgmA* homologs in major *Bacteroides* species (i.e. *Bacteroides fragilis* and *B. thetaiotaomicron*), implies the existence of a new member of chaperon/usher proteins unique to *Bacteroidetes* (107).

Regulation of Mfa1

So far little is known with regard to the regulation mechanism of Mfa1 biosynthesis, except for the possible involvement of FimS–FimR in the transcription of *mfa1*. In addition to the essential role of this two-component system in the regulation of the *fim* gene cluster, comparative gene expression analyses using W83-based DNA microarrays also detected marginal and unstable (about two-fold or less) down-regulation of genes clustered around the *mfa1* locus (from PG0176 to PG0182 by The Institute for Genomic Research) in a *fimR* mutant (Oral Pathogen Microarray Database [OPMD], http://array.brop.org). However, repetition of the western blot analyses revealed that the production level of Mfa1 protein in the *fimR* mutant was still abundant and comparable to the level in the parent strain (K. Nishikawa and F. Yoshimura, unpublished data), suggesting a minor, if any, contribution of FimS–

FimR to the regulation of Mfa1 biosynthesis. It is noteworthy that such a discrepancy between the levels of transcription and translation in *mfa1* expression is not observed for *fimA* expression. A recent study using real-time polymerase chain reaction also detected down-regulation of *mfa1* in a *fimR* mutant, and the reduction of Mfa1 protein in the mutant was estimated to be 50% (108). In the same study, recombinant FimR was shown to bind to a sequence located more than 400 bp upstream of the putative start codon of *mfa1*. However, the transcriptional start site determined in the study was 390 bp upstream of the site previously reported (109). Because the transcriptional start site was used as a guide to predict the possible promoter region of *mfa1*, it is arguable whether the probe used in electrophoretic mobility shift assays contained a real promoter sequence recognized by FimR *in vivo*. The probe comprised a long AT-rich sequence and, consequently, should have contained numerous potential consensus binding sites for helix-turn-helix motifs of DNA-binding regulators. No explanation has been given for such different results obtained using the same type strain grown under the same culture conditions, indicating the need for further deliberate investigations. Chromatin immunoprecipitation–polymerase chain reaction analysis did not detect enrichment of the FimR–*mfa1* promoter complex from the type strain ATCC 33277 (K. Nishikawa, unpublished data), suggesting that FimS–FimR might have an indirect role in the transcription of *mfa1* as they do in the regulation of *fimA*. There is an analogy between *fimA* and *mfa1* in terms of the organization of genes clustered around them. We cannot rule out the possibility that Mfa1 might be constitutively expressed, or if induced, the expression apparently needs regulatory mechanisms other than that via the FimS–FimR two-component system.

Temperature stress affects expression of FimA and Mfa1

To date, temperature stress seems to be the only robust environmental stimulus that affects the expression levels of FimA and Mfa1 (20,110). A slight elevation of the culture temperature (2–3°C) from the normal growth temperature (37°C) was sufficient to induce drastic reductions in expression of both FimA and Mfa1. This stress response, however, has not yet been linked to regulation via FimS–FimR or the other two-component systems.

Concluding remarks

This article describes some surface components of *P. gingivalis*, especially outer membrane proteins, fimbriae and fimbrial regulation. We should explain that this article was not planned as a comprehensive review on these topics, but was intended to describe recent research findings of our laboratory. The genome of *P. gingivalis* W83 has been sequenced; the genome analysis was published in 2003 (21) and this has had a tremendous impact on current research in this field as well as in other studies of *P. gingivalis*, although the studies described here were mainly carried out using ATCC 33277. Over the last 5 years, characterization of outer membrane proteins of this organism has shown remarkable progress, and this is the main topic discussed herein.

One of the authors has been working on and considering this topic for about 25 years, ever since Drs E. Newbrun and C. I. Hoover kindly sent a *Bacteroides gingivalis* (former name of *P. gingivalis*) strain on an agar plate to Japan by airmail without any notice about its origin, or even its strain number. Later, they let us know it as strain 381 when a report on a protease, a so-called trypsin-like protease, at the beginning of the gingipain study, was co-operatively submitted to *Archives of Oral Biology* (111). Subsequently, we started using ATCC 33277 instead of 381 in the mid-1990s, mainly because the former strain was found to be subtly different from the latter, being a more efficient recipient for transposon mutagenesis (100), although the two strains had been believed to be the same. The authors have mainly used this type-strain in *P. gingivalis* research for the last 10 years, and research on the topics discussed in this minireview was carried out by the authors during this period. Meanwhile, sequencing the genome of the strain began some time ago, and the sequence has been comparatively analyzed with W83 in Japan (112). We hope that this minireview will be useful for people interested in this fascinating organism.

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