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Review Article

Porphyromonas gingivalis FimA and Mfa1 fimbriae: Current insights on localization, function, biogenesis, and genotype

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ABSTRACT

In general, the periodontal pathogen *Porphyromonas gingivalis* expresses distinct FimA and Mfa1 fimbriae. Each of these consists of five FimA–E and five Mfa1–5 proteins encoded by the *fim* and *mfa* gene clusters, respectively. The main shaft portion comprises FimA and Mfa1, whereas FimB and Mfa2 are localized on the basal portion and function as anchors and elongation terminators. FimC–E and Mfa3–5 participate in the assembly of an accessory protein complex on the tips of each fimbria. Hence, they serve as ligands for the receptors on host cells and other oral bacterial species. The crystal structures of FimA and Mfa1 fimbrial proteins were recently elucidated and new insights into the localization, function, and biogenesis of these proteins have been reported. Several studies indicated a correlation between *P. gingivalis* pathogenicity and the *fimA* genotype but not the *mfa1* genotype. We recently revealed polymorphisms of all genes in the *fim* and *mfa* gene clusters. Intriguingly, *mfa5* occurred in numerous different forms and underwent duplication. Detailed structural and functional knowledge of the fimbrial proteins in the context of the entire filament could facilitate the development of innovative therapeutic strategies for structure-based drug design.

1. Introduction

The gram-negative anaerobe *Porphyromonas gingivalis* is associated with the development of periodontal disease [1] which is a polymicrobial infection. Though *P. gingivalis* only presents in small numbers, it may nonetheless be a keystone pathogen subverting host innate immunity and causing dysbiosis and chronic inflammation [2–4]. *P. gingivalis* may also contribute to systemic disorders such as premature birth and the development of atherosclerosis, rheumatoid arthritis, and Alzheimer's disease [5–8].

P. gingivalis virulence has been attributed to various structures and molecules on its cell surface, including fimbriae, lipopolysaccharides, capsules, gingipains (Arg- and Lys-specific proteases), and hemagglutinins [9]. *P. gingivalis* fimbriae (pili) are proteinaceous, filamentous appendages that protrude from the bacterial cell surface. They play crucial roles in biofilm formation, auto-aggregation, co-aggregation with oral bacteria, adhesion to host molecules, and host cell invasion [10–13]. *P. gingivalis* usually expresses long or major FimA fimbriae and short or minor Mfa1 fimbriae [10,11] (Fig. 1). Each fimbria is composed mainly of FimA and Mfa1 protein polymers encoded by *fimA* and *mfa1* in the *fim* and *mfa* gene clusters, respectively (Fig. 2). Mature fimbriae also contain the minor proteins FimB–E and Mfa2–5 encoded by downstream *fimA* and *mfa1*, respectively [14–19] (Fig. 2). Genome-sequenced *P. gingivalis* strains revealed that the aforementioned gene clusters are remote from each other on the bacterial chromosome [20–22].

The primary amino acid sequences of FimA and Mfa1 share no significant homology with any known types (I-IV) of the bacterial fimbrial proteins. Therefore, *P. gingivalis* possesses a unique class of fimbria subunits [9–11]. Recent structural analyses and mechanistic studies of the fimbrial proteins of *P. gingivalis* [23–25] indicated that *P. gingivalis* fimbriae were formed by a proteinase-mediated donor-strand exchange mechanism, which were classified as a novel type of fimbriae and designated as Type V fimbriae [23,26–28]. Structurally homologous fimbrial proteins are detected only in the *Bacteroidia* class [23], which are integral parts of the human microbiome and are associated with human health and disease. FimA–E and Mfa1–4 are all Type V fimbriae. However, Mfa5 contains a von Willebrand factor type A domain (vWF) and a C-terminal domain (CTD) [14,29]. The latter is translocated to the

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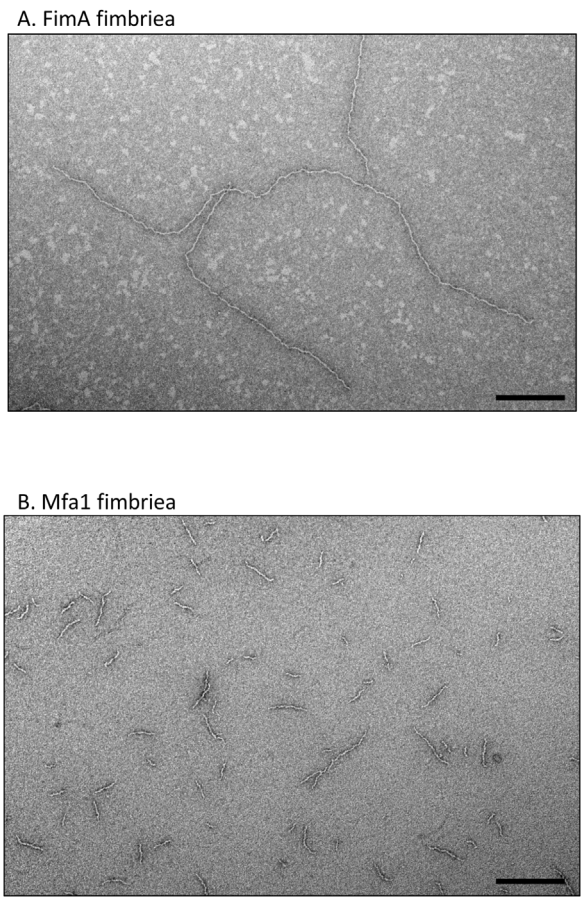

Fig. 1. Structure of the purified fimbriae of *P. gingivalis* strain ATCC 33277. Electron micrograph of negatively stained purified FimA (A) and Mfa1 fimbriae (B). Fimbriae purified according to standard protocols [39,133]. Scale bar = 200 nm.

cell surface by the Type IX Secretion System (T9SS) [30–32] and may, therefore, be assembled by a mechanism differing from those for FimA–E and Mfa1–4 [33].

The *fimA* genotype was determined either by PCR using genotype-specific primers or from the presence or absence of restriction enzyme cleavage. The *fimA* genotypes I, II, III, IV, and V have been classified and are associated with *P. gingivalis* virulence [11,34,35]. By contrast, *mfa1* may have at least two genotypes including a 70-kDa protein-coding *mfa1* (type $mfa1^{70}$) and a 53-kDa protein-coding *mfa1* (type $mfa1^{53}$) [36]. We phylogenetically analyzed the *mfa1* genotypes of 74 *P. gingivalis* strains using ClustalΩ and further divided $mfa1^{70}$ into the subtypes $mfa1^{70A}$ and $mfa1^{70B}$ [37]. However, the relationship between the *mfa1* genotype and *P. gingivalis* pathogenicity has not been established [38].

In the present review, we summarize the most current findings on the localization, biogenesis, functions, and genotypes of the fimbrial proteins encoded in the *fim* and *mfa* gene clusters in *P. gingivalis*, especially from biochemical, crystallographic, and molecular genetic perspectives (Table 1). Detailed structural and functional knowledge of the fimbrial proteins in the context of the entire filament would be a major asset for the development of new therapeutic strategies for the periodontal disease.

2. FimA and Mfa1 fimbriae structure and encoding genes

The long or major FimA fimbriae of *P. gingivalis* 381 were initially characterized by Yoshimura et al. The authors used an efficient washing method exploiting the fact that the fimbriae are only loosely attached to the bacterial surface [39]. The *fimA* gene encoding the 43-kDa FimA protein was subsequently cloned [40]. FimA were termed 'long fimbriae' because the *P. gingivalis* ATCC 33277 and 381 type strains produce long curly filaments approximately 5 nm in diameter. However, the fimbriae characteristic of these strains are atypical, and their length is attributed to a lack of FimB [19].

Mfa1 was originally described as a 75-kDa protein that co-purified with FimA fimbriae [39]. The 75-kDa protein was subsequently purified and its molecular mass was then reported as approximately 2000 kDa. It was assumed to be a globular outer membrane protein complex [41]. Two independent groups then proposed the presence of other fimbriae shorter than the 33277/381 FimA fimbriae; these were termed 'minor' and 'PgII' fimbriae and were found to have subunit molecular masses of 67 kDa and 72 kDa, respectively [42,43]. However, their *N*-terminal amino acid sequences were identical, and their primary amino acid sequences were highly similar. Thus, the originally identified 75-kDa protein, the 67-kDa protein (Mfa1), and the 72-kDa protein (PgII) are, in fact, the same polypeptide [44–46]. Park et al. used a *fimA*-deficient mutant of ATCC 33277 to purify fimbriae composed of Mfa1 proteins [47]. The Mfa1 fimbrial structure comprised short uniform filaments approximately 6.5 nm wide and 103 nm long.

3. Polymerization of major FimA and Mfa1 subunits

The crystal structures of FimA [23] and Mfa1 [25] were recently elucidated. They revealed a high degree of overall structural homology between subunits. Each one possesses a long, hydrophobic groove that is partially occupied by the *N*-terminal proregion. It was predicted that both fimbriae polymerize via a proteinase-mediated donor-strand exchange mechanism. They were classified as novel fimbriae and designated as Type V [23,27]. The FimA and Mfa1 subunits are synthesized as preproproteins. Each nascent fimbrial subunit contains the *N*-terminal signal sequence so that it can be translocated to the periplasm via the Sec general secretory pathway. Signal peptidase II removes the signal sequence and the newly generated *N*-terminus is lipidated [48,49]. The fimbrillins are then shuttled to the outer membrane where they serve as lipoprotein precursors. The next proteolytic step is mediated by Arg-specific gingipain cleaving at Arg^{46} and Arg^{49} in the amino acid sequences of FimA and Mfa1, respectively [50,51] and yielding the mature forms. Gingipain protease cleavage may release the proregions, free the subunits from the outer membrane, and open the groove for subsequent occupation by a donor β-strand from a neighboring subunit. This mechanism establishes stable subunit-subunit interactions and polymerizes the subunits into fimbrial fibers. Tandem interactions between the termini of the nascent subunits yield the polymeric backbone of the fimbrial structure. In FimA protein polymerization, the *C*-terminal donor strands of each incoming monomer bind the hydrophobic grooves of the preceding monomer [23]. FimA polymerization proceeds independently of the FimB–E proteins [49,52].

Structural investigations have not definitively identified the donor strands associated with the Mfa1 protein [25]. However, the *C*-terminal region might be essential for the extension of the preceding monomer and Mfa1 dimer formation [23,25]. Substitution of the hydrophobic amino acids with charged residues in either the *N*- or *C*-terminal regions yielded Mfa1 proteins that failed to polymerize [53]. Mfa1 fimbrial polymerization might depend on the hydrophobicity of the *N*- and *C*-terminals. Hence, the assembly mechanism might consist of the formation of a hydrophobic binding interface between the Mfa1 subunits at the terminal regions. Alaei et al. reported that the *C*-terminal peptides of Mfa1 and FimA inhibit Mfa1 and FimA protein polymerization, respectively [54]. Nevertheless, the mechanism of the Mfa1 protein subunit-

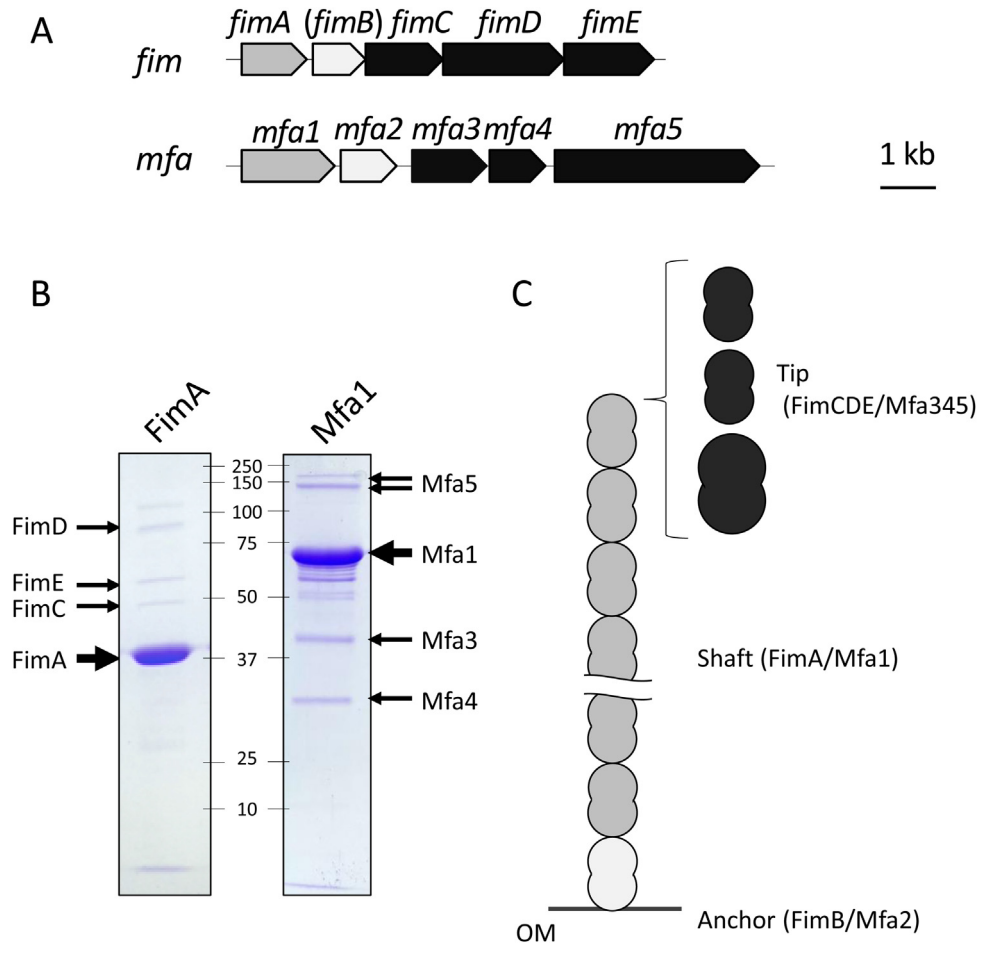

Fig. 2. Fimbrial proteins of FimA and Mfa1 fimbriae of *P. gingivalis*. (A) Gene map of *fim* and *mfa* clusters encoding fimbrial proteins on chromosome. Each cluster contains five genes with same transcriptional direction. *fimB* gene initially reported as *orf1* but nonsense mutation was discovered in *orf1* and restored gene was designated *fimB*. Sequences derived from NCBI accession NC_010729 (*P. gingivalis* ATCC 33277, complete genome) [20]. (B) SDS-PAGE and Coomassie brilliant blue (CBB) staining of purified fimbriae from ATCC 33277 strain. FimA/Mfa1 bands indicated with bold arrows. Fim C, FimD, FimE, Mfa3, Mfa4, and Mfa5 indicated with thin arrows as accessory proteins. (C) Scheme of possible structural model of *P. gingivalis* fimbriae. FimA and Mfa1 fimbriae composed of FimA/Mfa1 polymer associated with accessory proteins of FimCDE/Mfa345 at tip. Outer membrane proteins of FimB/Mfa2 associated with FimA/Mfa1 filaments at base. Sequence orders of tip proteins unknown (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

subunit interactions and the identity of accessory factors, such as chaperones, required for fimbria promotion and expression remain unknown.

4. FimB and Mfa2 function and localization

We purified Mfa1 fimbriae from *P. gingivalis* cSMF1 derived by complementation of an *mfa1*-deficient strain with the *mfa1* gene introduced *in trans* [47] and discovered that this strain had an aberrant phenotype. Mfa1 fimbriae were generated in the form of long filaments loosely associated with cSMF1. A similar structure was observed for the FimA fimbriae of ATCC 33277. The foregoing observations and an earlier discovery that *mfa1* and its adjacent downstream gene *mfa2* are co-transcribed [55] led to the hypothesis that cSMF1 is defective in *mfa2* expression, which results in the aberrant Mfa1 fimbrial phenotype. To test this theory, we constructed the *mfa2*-deletion mutant and complemented it with the wild type *mfa2* allele *in trans*. The *mfa2* mutant presented with aberrant long fimbriae resembling those of cSMF1 whereas the *mfa2*-complemented strain displayed the parental phenotype. Mfa2 was absent in the Mfa1 fimbriae but present in the outer cell membrane. Moreover, Mfa1 and Mfa2 co-precipitated in whole bacteria. Hence, we concluded that this protein anchors to the cell surface and determines the length of the Mfa1 fimbriae [15].

We then analyzed the *fim* cluster because we hypothesized that this DNA sequence may contain mutations in certain strains. We identified an open reading frame (ORF) sequence (357 bp) designated '*orf*1' and located immediately downstream of *fimA* in the ATCC 33277/381 strains [20,56]. However, a longer corresponding ORF (912 bp) occurred at the same locus in other genome-sequenced *P. gingivalis* strains. A comparison of these sequences revealed a possible point mutation in which the 355th bp of the ORF contains an adenine in W83 and OMZ 314 but a thymine in 33277 and 381. At the same locus, the former had a lysine codon while the latter had a stop codon. We named this ORF '*fimB*' and predicted that this defect could alter the FimA fimbriae. Restoration of *fimB* by site-directed mutagenesis resulted in relatively shorter fimbriae (approximately 150 nm) [19]. When FimA was overexpressed, its fimbriae were elongated to $\leq$ 600 nm. By contrast, the FimB-overexpressing strain produced short fimbriae. We concluded that FimB is a terminator and controls the length of FimA fimbriae. In the 33277/381 strains, FimA fimbriae are readily released from the cell surfaces but FimB restoration drastically inhibits their release. Therefore, FimB is an anchor for FimA fimbriae. Taken together, the foregoing results suggest that the 33277/381 strains harbor a mutation in *fimB* encoding FimA fimbriae that are abnormal in terms of

Table 1
Localization, function, biogenesis, and genotype of *P. gingivalis* fimbrial proteins.

	Component	Localization	Function	Biogenesis	Genotype
FimA fimbriae	FimA	Outside (main shaft portion)	Binding to salivary molecules, such as proline-rich proteins and statherins, cell surface proteins, such as integrins, ICAM-I, keratin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and extracellular matrix proteins, such as collagen type I, fibronectin, laminin, elastin, thrombospondin, and vitronectin. Interaction with streptococci, actinomyces, and *Treponema* spp. Inflammatory response via Toll-like receptors (TLRs) and TLRs co-clusters.	Signal peptidase- and gingipain-dependent	5 (I–V)
	FimB	Outer membrane (filament base)	Anchor and fimbrial elongation terminator.	Signal peptidase-dependent	1
	FimC FimD FimE	Tip (distal end of filament)	Tip complex assembly and optimal surface expression of fimbriae. Binding to complement receptor 3 (CR-3) and GAPDH.	Signal peptidase- and gingipain-dependent	2 (A and B)
Mfa1 fimbriae	Mfa1	Outside (main shaft portion)	Binding to SspB of *Streptococcus gordonii* and dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) of dendritic cells. Inflammatory response via TLR2 and TLR4.	Signal peptidase-and gingipain-dependent	2 (*mfa1*^{70} and *mfa1*^{53})
	Mfa2	Outer membrane (filament base)	Anchor and fimbrial elongation terminator.	Signal peptidase-dependent	2 (*mfa1*^{70} and *mfa1*^{53})
	Mfa3 Mfa4 Mfa5	Tip (distal end of filament)	Tip complex assembly and optimal surface expression of fimbriae.	Signal peptidase-and gingipain-dependent Type IX secretion system-dependent	2 (*mfa1*^{70} and *mfa1*^{53}) 2 (*mfa1*^{70} and *mfa1*^{53}) 5 (A–E)

length and cell anchoring ability. Because these are the two strains that have often been used in the past, we need to review previous reports related to this study and carefully choose the appropriate strains to characterize FimA fimbriae in *P. gingivalis* [52].

The crystal structures of the anchor proteins of FimB [23] and Mfa2 [25] were recently elucidated. Prior structural and functional studies on Mfa2 revealed that its *N*-terminus is not processed by gingipains [17,23], its *N*-terminal lipid modification is retained, and the fimbriae are anchored to the cell surface [23]. Furthermore, *C*-terminal-truncated Mfa2 expressed longer Mfa1 fimbriae than those of the wild type and did not interact with Mfa1 protein. Therefore, *C* termini are critical for the incorporation of these proteins into the fimbriae. Along with polymerizing the major fimbrillin subunits, the anchor proteins may be included in Type V fimbriae despite that fact that they lack the *N*-terminal Arg-gingipain cleavage site and remain attached to the cell surface as lipoproteins [23,26–28].

5. Accessory protein localization and roles in fimbria biogenesis

The intensities of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) bands corresponding to the individual fimbrial proteins suggest that the Mfa3–5 and FimC–E accessory proteins occur in a single purified fimbria in an approximate 1:1:1 ratio [19,25] (Fig. 2B). We constructed deficient mutant strains of these genes to localize Mfa3–5 and established their roles. The absence of even one accessory protein resulted in the loss of all three of them and a reduction in fimbria expression [14,16,17]. Hence, Mfa3–5 proteins might participate in the assembly of the tip complex. Furthermore, the latter must be incorporated into the shaft and is required for optimal Mfa1 fimbria expression on the cell surface. We also performed immunoelectron microscopy with anti-Mfa1 and anti-Mfa3 antisera to localize Mfa3 in the fimbrial filaments. We observed that Mfa3 localizes to the tips or distal ends of the Mfa1 protein filaments [16]. Two Mfa3 size variants (40 kDa and 43 kDa) were detected in whole-cell lysate. The 43-kDa precursor and the mature 40-kDa form were found mainly in the inner and outer membranes, respectively. This finding suggests that at least two steps are involved in incorporating Mfa3 into the fimbrial fiber. Lee et al. showed that mature Mfa3 protein bound Mfa1, Mfa4, and Mfa5 in a concentration-dependent manner. Thus, Mfa3 may be a bridging molecule interlinking Mfa1, Mfa4, and Mfa5 [53]. Moreover, Mfa1 bound neither Mfa4 nor Mfa5 [53].

The crystal structures of the Mfa3 [25] and Mfa4 [23,24] tip proteins were recently elucidated. Structural analyses of the fimbrial proteins revealed that whereas Mfa1 and Mfa2 contain additional *C*-terminal β strands that function as polymerization linkers, neither Mfa3 nor Mfa4 has these structures. Hence, Mfa3 and Mfa4 might be localized to the tip portion [23]. However, the mechanism by which these proteins bind Mfa1 remains unknown.

Unlike Type V fimbrial proteins, such as FimC–E and Mfa3–4, Mfa5 may only have the CTD and vWF domains. Amino acid sequence analyses revealed that these are widely distributed among the archaea, bacteria, and eukaryotes and participate in diverse functions, such as protein-protein interactions and cell adhesion [57]. We recently induced in-frame mutations in the putative CTD and vWF domains to establish their roles in Mfa5. The phenotype of the mutant with an in-frame deletion in CTD was similar to that of the *mfa5* disruption mutant expressing fimbriae at low levels and lacking accessory proteins [14]. The mutant strain of *porU* encoding the *C*-terminal signal peptidase in T9SS [58] and the mutant with an in-frame deletion in CTD were characterized by intracellular Mfa5 accumulation. Hence, Mfa5 function might require T9SS-mediated, CTD-dependent translocation across the outer membrane and subsequent incorporation into fimbrial fibers.

All accessory proteins except Mfa5 were integrated into the Mfa1 fibers in the mutant with the in-frame deletion in vWF. Thus, Mfa5 may associate with other accessory proteins via the vWF domain. However, a fatal mutation might occur in the overall structure of defective Mfa5 and it could be impossible to determine the function of the vWF domain alone. Heidler et al. recently demonstrated via X-ray crystallography that Mfa5 contains a single vWF domain, two Ig-like domains in its *N*-terminal half, and the ARM2 loop structure. The crystal structures of the three *N*-terminal domains of Mfa5 do not resemble those of other fimbrillins [29]. The authors also suggested that Mfa5 structurally resembles the gram-positive adhesins RrgA and GBS104 with a vWF and several IgG-like domains. The latter may form a stalk that projects the vWF domain towards the ligands on host cells. The authors showed that a clear Mfa5 tip location was detected by electron microscopy using the anti-Mfa5 antibody serum, in combination with a gold-conjugated secondary antibody and pure native fimbriae for negative staining [29]. These observations based on crystallography supported our findings on tip localization of accessory proteins and a role as a ligand to receptors on host cells and other oral bacteria exerts a unique function in *P. gingivalis*.

6. Functions of FimA and Mfa1 fimbriae

The fimbriae of *P. gingivalis* play crucial roles in bacterial adhesion to a wide variety of molecules and oral substrates [10,11,59], including salivary proline-rich proteins [60–66] and statherins [60,62,65–67], various substances on host cells, such as integrins [68–71], ICAM-I [72], keratin [73,74], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [73], extracellular matrix proteins, such as collagen type I [75–77], fibronectin [75–82], laminin [75], elastin [75], thrombospondin [75], and vitronectin [75]. FimA fimbriae also bind to oral bacteria, such as streptococcal GAPDH [83–87], actinomyces [88,89], and *Treponema* spp. [90–92] (Table 1). FimA fimbriae may be responsible for the adhesive properties of *P. gingivalis* and enable it to bind host cells. FimA fimbriae is recognized by immune molecules. Immune responses were evoked through interactions between FimA fimbriae and Toll-like receptors (TLRs), such as TLR2 [93–103] as well as TLR co-clusters, including complement receptor 3 (CR-3) [93,94,104–107], CD14 [95,106], and CXC chemokine receptor 4 (CXCR-4) [108,109].

Mfa1 fimbriae participate in auto-aggregation and regulate homotypic biofilm development in *P. gingivalis* [110,111]. Mfa1 fimbriae bound the SspA/B surface protein of *Streptococcus gordonii* and the DC-SIGN (dendritic, cell-specific, ICAM-3-grabbing nonintegrin) receptor of human dendritic cells [55,112–114]. Interactions between Mfa1 and SspB have been characterized. Daep et al. identified two discrete SspB motifs consisting of the amino acids NITVK and VQDLL (designated BAR). They showed that these motifs are essential for interactions with Mfa1 [115–117]. A synthetic peptide containing NITVK and VQDLL strongly inhibited *P. gingivalis* adhesion to *S. gordonii* and significantly reduced *P. gingivalis* virulence in a murine periodontitis model [115,118]. Purified recombinant Mfa1 lacking tip proteins strongly inhibited *P. gingivalis* adhesion [118]. Therefore, Mfa3, Mfa4, and Mfa5 do not play major roles in SspB interactions. Interactions between Mfa1 and DC-SIGN facilitate *P. gingivalis* penetration into dendritic cells and their subsequent persistence there by evading autophagy and intracellular killing. In this manner, dendritic cell maturation, Th2 effector response stimulation, and proinflammatory cytokine induction are all inhibited [112–114,119,120]. Hamada et al. reported that purified Mfa1 fimbriae upregulated proinflammatory cytokines, such as IL-1α, IL-β, IL-6, and TNFα, via the TLR2 and CD14 pathways in human monocytes and murine peritoneal macrophages [45,121]. A recent study indicated that recognition by TLR2 and TLR4 was involved in the immunomodulatory actions of purified Mfa1 fimbriae and contributes to the overall action of gingival fibroblasts [122].

Several studies evaluating the roles of FimB–E and Mfa2–5 in fimbrial functions are summarized in Table 1. The tip proteins of the Type I and Pap fimbriae of *Escherichia coli* were identified as adhesion molecules directly recognizing their targets [123,124]. Accessory proteins, including FimC, FimD, and FimE, may be responsible for adhesion to CR-3 and GAPDH [18,105]. We reported that *mfa3*- or *mfa4*-deficient mutants lacking all fimbrial accessory proteins strongly auto-aggregate and form biofilms. Hence, accessory proteins may regulate both of these processes [15–17]. Compared with the parental strain, the mutants presented with diminished expression on their fimbrial surfaces [14,17]. Therefore, the latter phenotype may not be a direct consequence of accessory protein loss. Our research group recently compared immunomodulation induction by Mfa1 fimbriae purified from the wild type and the *mfa5*-deficient mutant lacking the Mfa3–5 tip structure [122]. The *mfa5* mutant presented with relatively upregulated immunomodulatory genes. However, the receptors of the Mfa1 and FimA fimbrial tip proteins, and the positions of the accessory proteins on the fimbrial filament remain to be identified.

The PGN_1808 protein was recently identified in *P. gingivalis* ATCC 33277 by *in silico* structural homology search [23]. While PGN_1808 polymerizes in vivo to form filamentous structures [125], its precise function remains to be clarified.

7. Genetic diversity in *fim* and *mfa* clusters

Heterogeneous virulence of *P. gingivalis* has been attributed to a high degree of genetic clonal diversity. Several international studies reported that *fimA* genotypes II and IV were detected predominantly in patients with severe periodontitis whereas *fimA* genotype I was prevalent mainly in healthy patients and mild periodontitis cases [126–129]. However, *fimA* genotype I has also been observed at high frequency in patients with severe periodontitis [130]. A previous study reported no association between the *fimA* genotype and *P. gingivalis* pathogenicity [130]. The results of the foregoing studies suggest that *P. gingivalis* pathogenicity cannot be defined solely on the basis of the *fimA* genotype.

We recently used published genomic information for over 70 *P. gingivalis* strains to analyze the polymorphisms of all genes in the *fim*, *mfa*, and *rag* gene clusters (*fimA*–*fimE*, *mfa1*–*mfa5*, and *ragA* and *ragB*) (Table 2) [37]. Here, we review these genetic polymorphisms.

We classified full length *fimA* [131] and confirmed it in a recent report [37]. The *fimA* gene was categorized into five genotypes (I–V) consistent with PCR genotyping-based classification. By contrast, *fimB* was highly conserved in *P. gingivalis*. We also consistently identified two genotypes (A and B) in each of *fimC*, *fimD*, and *fimE*. Most (82.4%) *fimC*, *fimD*, and *fimE* were classified as genotype A whereas genotype B was frequently detected in *fimA*-IV (6/9 strains) and *fimA*-V (2/2 strains). Thus, there are relationships among the *fimA*, *fimC*, *fimD*, and *fimE* genotypes. Given the high pathogenicity of the *fimA*-genotype IV [126–128], we are interested in the association between genotype B in *fimC* to *fimE,* and bacterial pathogenicity.

The full length of *mfa1* was genotyped and it was divided primarily into $mfa1^{53}$ and $mfa1^{70}$. This approach was consistent with PCR classification [36]. Ando and ATCC 33277 are representative strains of $mfa1^{53}$ and $mfa1^{70}$, respectively, and their overall *mfa1* homology was as low as 52.6%. However, they were nearly identical up to the first 100 bp. We also discovered that $mfa1^{70}$ could be further divided into $mfa1^{70A}$ and $mfa1^{70B}$ subtypes (Fig. 3).

The genes *mfa2*, *mfa3*, and *mfa4* were also classified into two genotypes consistent with the *mfa1* genotypes. Nevertheless, $mfa1^{70}$ derived from *mfa2, mfa3,* and *mfa4* could not be

Table 2
Genotyping of *fim*, *mfa,* and *rag* gene clusters.

	fimA	*fimB*	*fimC*	*fimD*	*fimE*	*mfa1*	*mfa2*	*mfa3*	*mfa4*	*mfa5-1*	*mfa5-2*	*ragA*	*ragB*
84_3	I	-	A	A	A	53	53	53	53	A1		1	1
7BTORR	II	-	A	A	A	53	53	53	53	A1		2	2
WW2866	□	-	A	A	A	53	53	53	53	A1		3	3
A7A1-28	□	-	A	A	A	53	53	53	53	A1 (M)		3	3
KCOM 3131	□	-	A	A	A	53	53	53	53	A2	E	1	1
KCOM 2801	I	-	A	A	A	53	53	53	53	A2	E	1	1
WW2931	V	-	B	B	B	53	53	53	53	A2	E	1	1
WW2903	II	-	A	A	A	53	53	53	53	A2	E	2	2
AJW4	□	-	A	A	A	53	53	53	53	A2	E	3	3
KCOM 2796	II	-	A	A	A	53	53	53	53	A2	E	3	3
SJD2	I	-	B	B	B	53	53	53	53	A2	E	3	3
KCOM 2798	II	-	A	A	A	53	53	53	53	A2	E (M)	1	1
AFR5B1	□	-	A	A	A	53	53	53	53	Ns	E	2	2
11A	II	-	A	A	A	53	53	53	53	Ns	Ns	2	2
Ando	□	-	A	A	A	53	53	53	53	Ns		1	1
JCVI SC001	II	-	A	A	A	53	53	53	53	Ns		3	3
SJD4	□	-	A	A	A	53	53	53	53	X	X	1	1
ATCC 49417	III	-	A	A	A	53	53	53	53	X	X	1	1
SJD5	I	-	B	B	B	53	53	53	53	X	X	3	3
15_9	IV	-	B	B	Ns	53	53	53	53				
381OKJP	V	-	B	B	B	53	53	53	53				
WW3039	II	-	B	B	B	53	X	53	53	A1		3	3
F0568	□	-	A	A	A	70A	70	70	70	A1		2	2
W4087	□	-	A	A	A	70A	70	70	70	A1		2	2
WW2885	I	-	A	A	A	70A	70	70	70	A1		2	2
F0185	II	-	A	A	A	70A	70	70	70	A1		2	2
381	□	-	A	A	A	70A	70	70	70	A1		4	4
ATCC 33277	I	(M)	A	A	A	70A	70	70	70	A1		4	4
UBA8864	I	(M)	A	A	A	70A	70	70	70	A1		4	4
KCOM 2802	□	-	A	A	A	70A	70	70	70	A2	E	1	1
A7436	IV	-	X	B	B	70A	70	70	70	A2	E	1	1
WW2842	I	-	A	A	A	70A	70	70	70	A2	E	2	2
D83T3	II	-	A	A	A	70A	70	70	70	A2	E	2	2
WW3102	II	-	A	A	A	70A	70	70	70	A2	E	2	2
WW5019	II	-	A	A	A	70A	70	70	70	A2	E	2	2
WW2096	III	-	A	A	A	70A	70	70	70	A2	E	2	2
CP3	IV	-	A	A	A	70A	70	70	70	A2	E	2	2
KCOM 2805	□	-	A	A	A	70A	70	70	70	A2	E	3	3
KCOM 2804	II	-	A	A	A	70A	70	70	70	A2	E	3	3
KCOM 2799	III	-	B	B	B	70A	70	70	70	A2	E	3	3
HG66	I	-	A	A	A	70A	70	70	70	A2	E	4	4
KCOM 2797	II	-	A	A	A	70A	70	70	70	A2	E	4	4
H3	I	-	A	A	A	70A	70	70	70	A2	X	3	3
SU60	IV	-	B	B	B	70A	70	70	70	B (M)	Ns	3	3
WW5127	□	-	B	B	B	70A	70	70	70	C		1	1
3A1	□	-	A	A	A	70A	70	70	70	C		4	4
KCOM 2800	□	-	A	A	A	70A	70	70	70	C		4	4
KCOM 2803	II	-	A	A	A	70A	70	70	70	C		4	4
F0566	I	-	A	A	A	70A	70	70	70	D		3	3
TDC 60	□	-	A	A	A	70A	70	70	70	D		4	4
KCOM 3001	II	-	A	A	A	70A	70	70	70	D		4	4
3_3	I	-	A	A	A	70A	70	70	70	Ns	E	4	4
F0569	II	-	A	A	A	70A	70	70	70	Ns	Ns	4	4
F0570	□	-	A	A	A	70A	70	70	70	X	X	2	2
EM3	III	-	A	A	A	70A	70	70	70	X	X	3	3
SJD11	□	-	A	A	A	70A	70	70	70	X	X	4	4
SJD12	I	-	A	A	A	70A	70	70	70	X	X	4	4
WW2881	I	-	A	A	A	70A	70	70	70	X		3	3
13_1	II	-	A	A	A	70A	70	70	70				
WW2952	□	-	A	A	A	70A	70	70	X	A2	E	2	2
TV14	□	-	A	A	A	70A (M)	70	70	70	D		2	2
MP4-504	□	-	A	A	A	70B	70	70	70	A2	E	2	2
Kyudai-3	□	-	A	A	A	70B	70	70	70	A2	E	2	2
1436	□	-	A	A	A	70B	70	70	70	A2		2	2
JKG9	□	-	A	A	A	70B	70	70	70	A2		2	2
1439	□	-	A	A	A	70B	70	70	70	A2		2	2
B42	□	-	A	A	A	70B	70	70	70	A2		2	2
W83	□	-	B	B	B	X	70	70	70	A2	E	1	1
W50	IV	-	B	B	B	X	70	70	70	A2	X	1	1
JKG10	□	-	A	A	A	X	70	70	70	A2	X	4	4
B158	□	-	A	A	A	X	70	70	70	A2		2	2
Kyudai-4	II	-	A	A	A	X	70	70	70	A2		3	3
222	□	-	B	B	B	φ	φ	φ	φ	X	X	1	1
WW3040	□	-	A	A	A	φ	φ	φ	φ	φ		2	2

Strains in boldface indicate that their genomes are assembled in a contig.
Genotyping (I–V; A or B) of each gene is shown in text.
fimB gene could not be classified because of its high homogeneity.
M: a possible point mutation, such as a nonsense mutation, was detected. However, as only one mutation had little influence, genetic analysis was still performed.
X: possible non-point mutation. Although corresponding genes were detected, genetic analyses were not performed.
Ns: sequence detected in gene because of complementation during scaffolding in NGS analysis. Corresponding genes were recognized but genetic analyses were not performed.
φ: corresponding gene not detected.
Light blue box: the genes were detected in the same contig as *mfa1*. i.e., when *mfa1* to *mfa5-1*, *ragA* and *ragB* are light blue, but *mfa5-2* is white, then *mfa1* to *ragB* are successively arranged (but not *mfa5-2*).
All tested strains showed successive arrangement from *fimA* to *fimE* in the same contig.

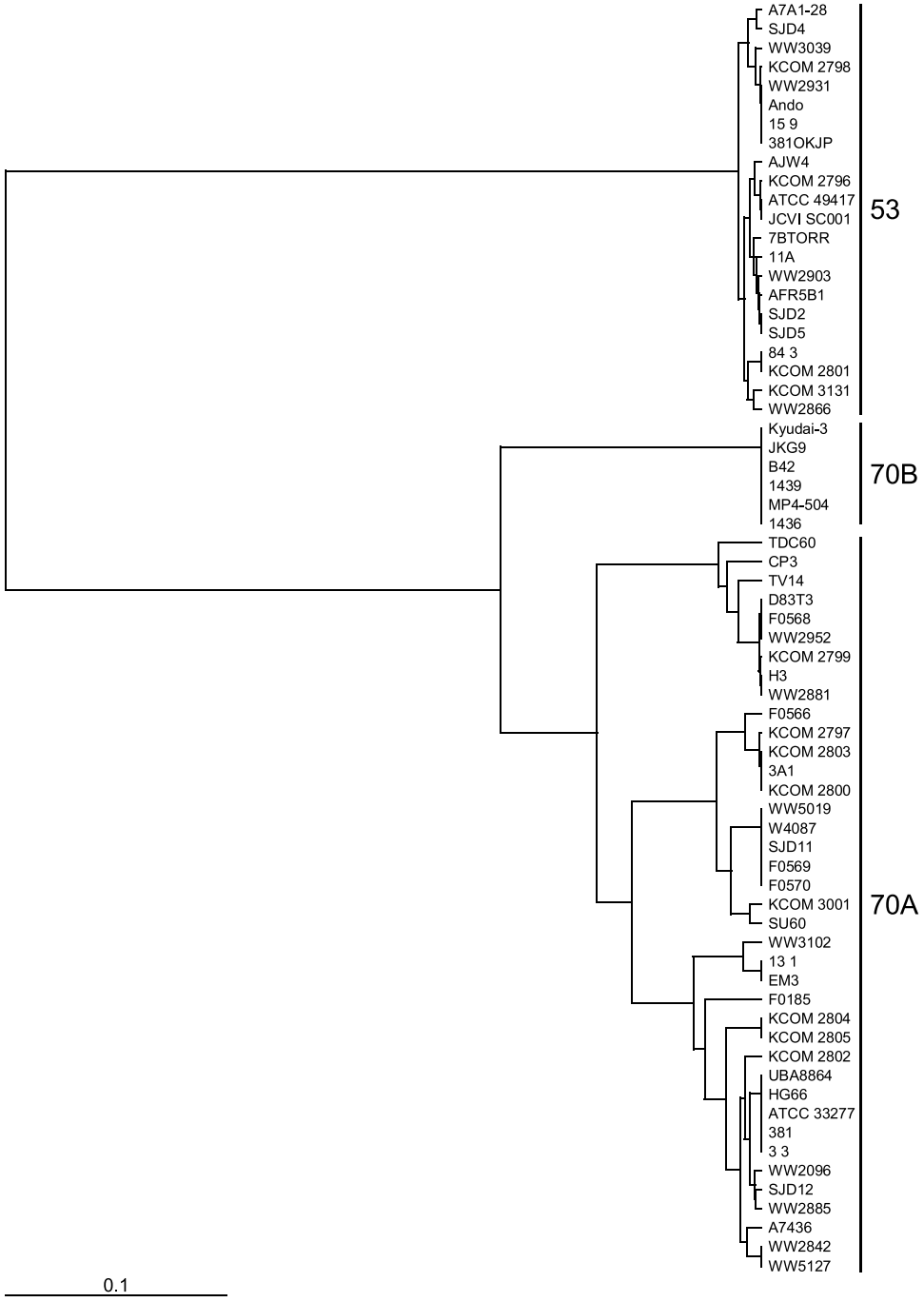

Fig. 3. *mfa1* phylogenetic tree. Phylogenetic tree constructed with TreeView X through multiple sequence alignment analysis with ClustalΩ. *mfa1* primarily classified into *mfa1*^{53} and *mfa1*^{70} genotypes. *mfa1*^{70} also forms *mfa1*^{70A} and *mfa1*^{70B} genotype clusters. Reproduced from Sakae et al. 2021. Permission to reproduce granted by PLoS One [37].

further subdivided. Thus, *mfa1–mfa4* develop during synchronization. However, the phylogenetic distances between *mfa2* genotypes were short, and *mfa3* and *mfa4* had lower diversity than *mfa1*.

Nevertheless, the classification of *mfa5* was completely different from those of the other *mfa* genes. It was classified into the five genotypes A-E, and genotype A was further divided into subtypes A1 and A2 (Fig. 4). They markedly differed in terms of gene length. There were numerous strains tandemly holding two *mfa5* genes. The first (*mfa5-1*) and the second (*mfa5-2*) were exclusively genotypes A2 and E, respectively (Table 2). All genotypes possessed the conserved *C*-terminal half and CTD. For all genotypes, then, T9SS transports Mfa5 to the cell surface. However, *N*-terminals containing the vWF domain, ARM2 loop, and isopeptide bond were only detected in genotypes A1, A2, B, and C. Neither the D nor the E genotype had the *N*-terminal portion. Moreover, genotypes D and E differed in terms of their *N*-terminal amino acid sequences, and a BLAST search failed to annotate the DNA sequences in the *N*-terminal halves of genotypes D and E.

Our analysis confirmed that *ragA*/*ragB* located immediately downstream of *mfa5* are classified into four genotypes [132], both genotypes were fully aligned, and there was an association between the *ragA*/*ragB* and *mfa1* genotypes. In *P. gingivalis* strains with the *mfa1*^{53} genotype, *ragA-1–ragA-3* were highly abundant but *ragA-4* was absent. In *P. gingivalis* strains with the *mfa1*^{70} genotype, *ragA-*

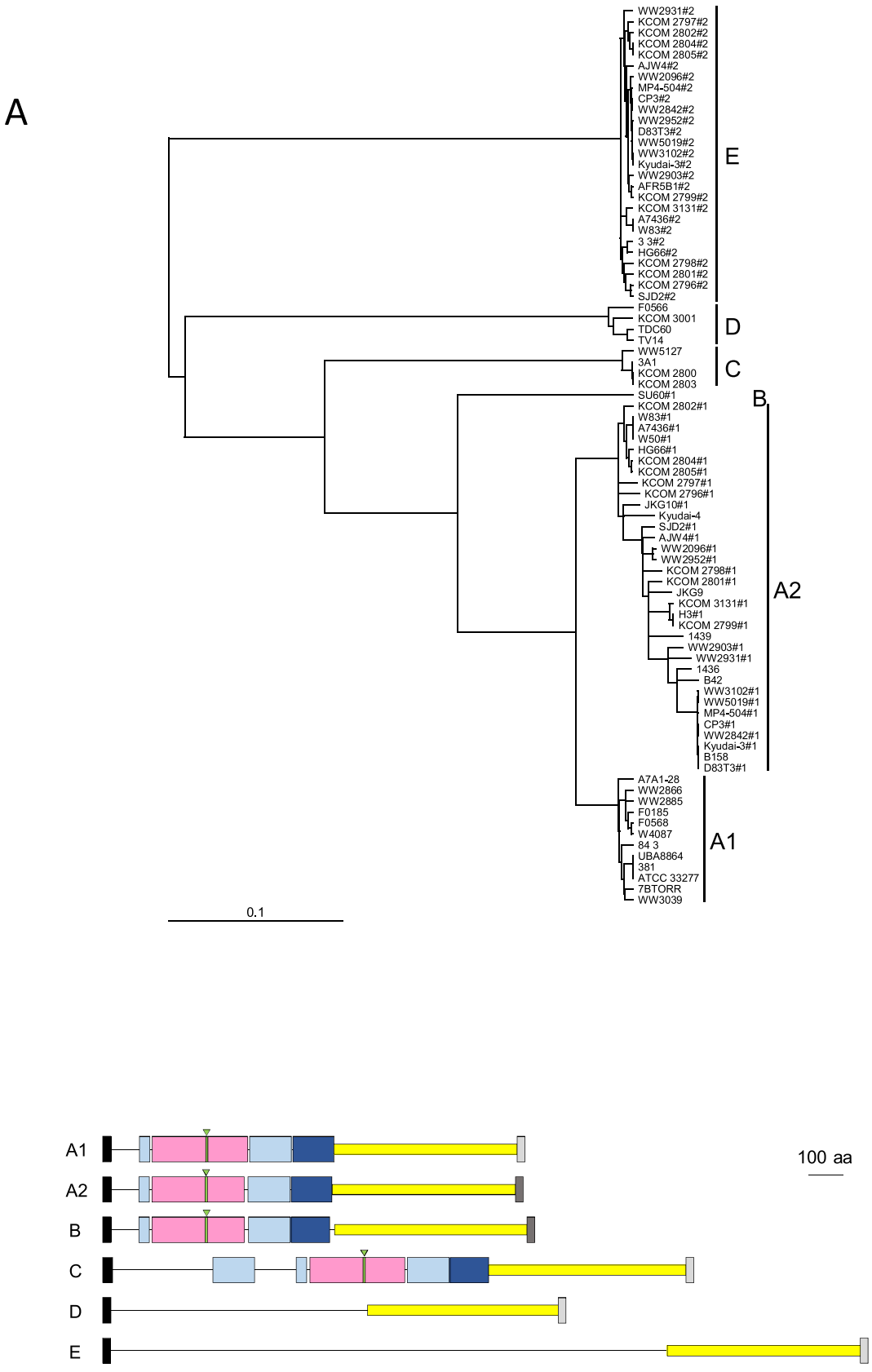

Fig. 4. *mfa5* diversity. Phylogenetic tree of *mfa5* gene (A). Schematic diagram of amino acid sequences among *mfa*5 of representative strains in respective genotype (B). Reproduced from Sakae et al. 2021. Permission to reproduce granted by PLoS One [37].

2–ragA-4 were highly abundant but only 7.9% *ragA-1* was detected. All six *P. gingivalis* strains with genotype *mfa1*^{70B} harbored *ragA-2*. Thus, *ragA-4* was only detected in strains with the *mfa1*^{70A} genotype. However, there was no correlation between the *mfa5* and *ragA*/*ragB* genotypes. The tandem arrangement of the *mfa1* and *ragA*/*ragB* gene clusters explain the relationship between the *mfa1* and *ragA*/*ragB* genotypes. However, their *mfa5* diversities were apparently unrelated, and there was no association between the *mfa1* and *fimA* genotypes or the *mfa5* and *fimA* genotypes.

8. Conclusion

Porphyromonas gingivalis fimbriae participate in biofilm formation, auto-aggregation, co-aggregation to oral bacteria, and bacterial adhesion to host molecules. Among the structural proteins of fimbriae encoding the *fim* and *mfa* clusters, the accessory proteins forming the fimbrial tips could play important roles. They regulate fimbrial formation and mediate fimbrial functions. However, the fimbrial structure and function in the tip complex and the genes involved in virulence remain to be established. Further work is required to resolve fimbria structure and mechanisms. Elaborate understanding of the structures of the fimbrial proteins encoded by the *fim* and *mfa* gene cluster within the entire filament could facilitate the development of innovative therapeutic strategies for structure-based drug design.

Contributions

Y.H. and K.N. prepared figures and wrote the manuscript.

Conflict of interest

The authors declare that there are no potential conflicts of interest.

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