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Purification and characterization of a novel secondary fimbrial protein from *Porphyromonas gulae*

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***Background*:** *Porphyromonas gulae* are black-pigmented anaerobic bacteria isolated from the gingival sulcus of various animal hosts and are distinct from *Porphyromonas gingivalis* originating in humans. We previously reported the antigenic similarities of 41-kDa fimbriae between *P. gulae* ATCC 51700 and *P. gingivalis* ATCC 33277. In this study, to clarify the presence of another type of fimbriae of *P. gulae*, we have purified and characterized the secondary fimbrial protein from *P. gulae* ATCC 51700.

***Methods*:** The secondary fimbrial protein was purified from *P. gulae* ATCC 51700 using an immunoaffinity column coupling with antibodies against the 41-kDa fimbrial protein. The expression of fimbriae on the cell surface of *P. gulae* ATCC 51700 was investigated by transmission electron microscopy. The *N*-terminal amino acid sequence was determined by an amino acid sequencer system.

***Results*:** The molecular mass of this protein was approximately 53-kDa, as estimated by SDS-PAGE. The polyclonal antibodies against the 53-kDa protein did not react with the 41-kDa fimbrial protein of *P. gulae* ATCC 51700. Immunogold electron microscopy revealed that anti-53-kDa fimbrial serum bound to fimbria on the cell surface of *P. gulae* ATCC 51700. The amino acid sequence of the *N*-terminal 15 residues of the 53-kDa fimbrial protein showed only 1 of 15 residues identical to the 41-kDa fimbrial protein.

***Conclusion*:** The 53-kDa fimbriae are different in molecular weight and antigenicity from the 41-kDa fimbrial protein of *P. gulae* ATCC 51700. These results clearly suggest that the 41-kDa and the 53-kDa fimbriae are distinct types of fimbriae expressed simultaneously by this organism.

Keywords: Porphyromonas gulae; Porphyromonas gingivalis; *periodontal disease*; *purification*; *fimbriae*; *53-kDa protein*; *oral microbiology*

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Periodontal disease is a significant oral problem in dogs, characterized by halitosis, gingival inflammation, increased periodontal pocket depth, and alveolar bone loss, resulting in loosening and eventual loss of teeth. In humans, much progress has been made in understanding the disease etiology and interaction between the host and periodontal pathogens. Human periodontitis has been associated with subgingival plaque containing elevated levels of specific Gram-negative anaerobic bacteria, including *Porphyromonas gingivalis*. It possesses virulence factors that include collagenase, lipopolysaccharides, a trypsin-like protease, and fimbriae (1). Some reports have shown that *P. gingivalis* can adhere to other bacteria, erythrocytes, and epithelial cells (2–4). Fimbriae in particular play an important role in facilitating the initial interaction between the bacteria and the host (5–7). Moreover, *P. gingivalis* strains possessed two types of fimbriae on the cell surface (6, 8). We have previously reported that fimbrial protein of *Porphyromonas gulae* ATCC 51700 had the same size and antigenicity as 41-kDa fimbriae of *P. gingivalis* ATCC 33277 (9). There is little information available regarding periodontal disease in companion animals. A black-pigmented anaerobic bacteria (BPAB) have been isolated from the periodontal pockets of dogs, cats, and several wild animals (10–15). In several BPAB, *Porphyromonas spp.* is the predominant species (10, 14). Distinct differences have been noted between human and canine *Porphyromonas* spp. (11, 12, 14, 15). *P. gingivalis* isolates from humans are catalase-negative, whereas *P. gingivalis*-like organism isolates from canine periodontal pockets are catalase-positive. These catalase-positive

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P. gingivalis-like organisms may well represent isolates of *P. gulae* (15). The most frequently isolated BPAB in dog and cat periodontal pockets are *P. gulae*, *P. salivosa* and *P. denticanis* (11). Each of these isolates was demonstrated to be pathogenic in a mouse model of periodontal disease. In humans, *P. gingivalis* is the BPAB associated with periodontal destruction (16–22). *P. gingivalis* is considered to be one of the most prominent periodontopathogens, possessing several characteristics of an overt pathogenic organism. *P. gingivalis* adheres to salivary components (23), epithelial cells (24–26), erythrocytes (4, 27), fibronectin-collagen complexes (28), and other bacteria (2). This adherence capacity is thought to be mediated by various surface proteins. The fimbriae in particular have been suggested to play an important role in facilitating the initial interaction between bacteria and host (29). Moreover, the fimbriae mediate bacterial cell-to-cell interaction. It has been reported that the fimbriae of *P. gingivalis* mediate the adherence between *P. gingivalis* and *Streptococcus gordonii* (30). In this study, to clarify the presence of another type of fimbriae of *P. gulae*, we have purified and characterized the secondary fimbrial protein from *P. gulae* ATCC 51700. The secondary fimbrial protein of *P. gulae* ATCC 51700 and the 53-kDa fimbrial protein of *P. gingivalis* strain 381 are immunologically cross-reactive. Moreover, the secondary fimbrial protein gene (*mfa1*) of *P. gulae* ATCC 51700 and the 53-kDa fimbrial protein gene of *P. gingivalis* strain 381 are highly homologous. We suggest that the secondary fimbrial protein of *P. gulae* could become an effective vaccine antigen to prevent the initiation and progression of periodontitis in companion animals.

Materials and methods

Strains and cultivation conditions

P. gulae ATCC 51700 and *P. gingivalis* strain 381 and ATCC 33277 were cultivated (5% CO_2, 10% H_2, and 85% N_2) in an anaerobic chamber (ANX-1, HIRASAWA, Japan) at 37°C in pre-reduced brain–heart infusion (BHI) broth (Difco Laboratories, USA) supplemented with yeast extract (0.5%, Difco Laboratories), hemin (5 μg/ml, Wako, Japan), and vitamin K (10 μg/ml, Wako).

Purification of fimbriae from P. gulae ATCC 51700

P. gulae ATCC 51700 was incubated anaerobically for 18 h in BHI broth. The bacterial cell pellet was harvested by centrifugation at 8,000 × g for 30 min and washed twice with 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ and 1.5 M NaCl by repeated pipetting. The suspension was subjected to ultrasonication with a 3-mm microtip at 25-W output on the pulse setting with five cycles of 1 min in an icebox, and then the suspension was recentrifuged at 8,000 × g for 30 min. After centrifugation, ammonium sulfate was added to the supernatant to 40% saturation and the precipitated proteins were collected by centrifugation and suspended in a small volume of 20 mM Tris-HCl buffer. The suspension was then dialyzed against 20 mM Tris-HCl for a day. The crude fimbrial preparation was applied to a DEAE Sepharose CL-6B anion exchange column equilibrated with 20 mM Tris-HCl (pH 8.0). The column was washed with 20 mM Tris-HCl buffer and then eluted with a linear gradient of 0 to 0.3 M NaCl at room temperature. The 41-kDa and the 53-kDa fimbrial proteins were eluted at 0.15 M NaCl. The fraction containing fimbrial protein was dialyzed against 2 mM Tris-HCl for 1 day and then applied to an immunoaffinity column chromatography (Affi-Gel Hz Immunoaffinity Kit; Bio-Rad, USA) binding the polyclonal antibodies (PAbs) against the 41-kDa fimbriae of *P. gingivalis* ATCC 33277. The unbound proteins were eluted at phosphate-buffered saline (PBS) containing 0.5 M NaCl and then 41-kDa fimbrial protein bound with the column was eluted at 0.2 M glycine-HCl (pH 2.5).

SDS-PAGE

Protein extracts were heated at 100°C for 5 min in loading buffer (62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, and 0.1% bromophenol blue). Samples were applied to 12.5% polyacrylamide slab gels with a 4% stacking gel and electrophoresed at 30 mA constant current for 1 h. The proteins were stained with Coomassie brilliant blue R-250. For molecular weight calibration, Precision Plus Protein Unstained Standards (Bio-Rad) were used.

Polyclonal antibodies

PAbs to the 53-kDa fimbrial protein were prepared using purified protein described above as immunogen. BALB/c mice (Nihon SLC, Japan) were injected at multiple sites subcutaneously with 50 μg of the appropriately conjugated protein in Freund's incomplete adjuvant (Difco). After 2 weeks, the mice were injected weekly for 4 weeks with the immunogen. Each mouse was bled after the last booster injection and the antibodies were tested against the corresponding antigen by Western blotting. After an adequate antibody titer was obtained, the mice were bled by cardiac puncture and the sera were prepared and stored at −20°C. PAbs against the purified 41-kDa fimbriae protein were raised in New Zealand White Rabbits (Nihon SLC, Japan). The experimental procedures for this study were reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental College.

Western blotting

For immunoblot analysis, the proteins separated by SDS-12.5% PAGE were transferred to a polyvinylidene difluoride membrane (PVDF membrane, Immobilon; Nihon Millipore Kogyo, Japan) at 200 mA for 1 h.

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The membranes were then treated with TBS (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl) containing 1% bovine serum albumin (BSA) to block unoccupied protein-binding sites. They were then incubated with the PAbs specific for the 53-kDa fimbrial protein of *P. gulae* ATCC 51700 at 37°C for 1 h, washed in TBS-Tween, incubated for 1 h with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase, and then immersed in a 4-chloro-1-naphthol (Tokyo Chemical Industry, Japan) solution to develop the color. The reaction was stopped by immersing the membranes in distilled water and the membranes were then dried.

Immunoelectron microscopy

Bacterial cells from 18-h anaerobic culture were harvested by centrifugation at 10,000 × g for 1 min and resuspended in PBS (pH 7.4). Copper grids (150 mesh) were covered with a thin film of collodion, which was then coated with carbon. The supported films were made hydrophilic by ion bombardment before use. A drop of cell suspension or purified protein was applied to the specimen grid. For Immunogold labeling, a cell suspension of *P. gulae* ATCC 51700 was transferred to a collodion-coated film nickel grid. The cells were incubated with 5 µl mouse PAbs against the 53-kDa fimbriae and 5 µl rabbit PAbs to the 41-kDa fimbrial of *P. gulae* ATCC 51700 (diluted 1:5,000 in PBS containing 1% BSA) at 37°C for 1 h. After five washes with PBS, the cells were incubated with EM Goat anti-mouse IgG: 5-nm gold (BBInternational, UK) and EM Goat anti-rabbit IgG: 10-nm gold (BBInternational, UK) at 37°C for 30 min. Cells were stained with 2% uranyl acetate for 1 min after five washes with PBS. The specimens were examined and photographed with a JEM-200CX electron microscope (Nippon Denshi Co., Japan) operated at 80 kV.

N-*terminal amino acid sequences*

Purified fimbrial protein was electrophoresed on a 12.5% SDS-polyacrylamide gel and then transferred onto a PVDF membrane operated at 200 mA for 1 h. After the membrane was stained with Coomassie brilliant blue R-250, the purified fimbrial protein band was excised and analyzed using a PPSQ-21 amino acid sequencer system (Shimadzu, Japan).

Nucleotide sequence of a mfa1 *gene*

P. gulae ATCC 51700 chromosomal DNA was used as the template for amplification of the *mfa1* gene. Two pairs of PCR primers, PG1F, PG1R and PG2F, PG2R, were designed based on the *mfa1* gene of *P. gingivalis* strain 381 for amplification of the *mfa1* gene with the open reading frame (ORF) and promoter region. PG1F (5'TCCGGATTCTTTTGTTATTTAGTG3') and PG1R (5'ATAAGGCACAGTGGGGACAT3') were used to identify the 5' region of *mfa1* gene. PG2F (5'GGTAGCCCAGTACGAAAAGAA3') and PG2R (5'GAGATACTCCCGAAAAGACAATC3') were used to identify the 3' region of *mfa1* gene. PCR amplification was performed in a total volume of 50 µl using AccuPrime *Pfx* DNA polymerase (Invitrogen, USA). The amplification reaction was performed in an iCycler (Bio-Rad) with the following cycling parameters, both PG1 and PG2: initial denaturation at 94°C for 2 min; 35 cycles consisting of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; and then a final elongation step at 72°C for 7 min. Amplicons were detected by electrophoresis of 10 µl PCR product on a 0.7% agarose gel. Band sizes were confirmed with reference to molecular size markers (Smart Ladder 0.2–10 kbp; NIPPON GENE, Japan). The nucleotide sequence of the gene was determined using a dye-terminator reaction with a model 310 Genetic Analyzer (PE Applied Biosystems, USA).

Data analysis of nucleotide sequence and amino acid sequence

Data analyses of nucleotide sequences and deduced amino acid sequences were performed with GENETYX-MAC/DB (Software Development, Japan). Multiple alignment analysis was performed with CLUSTAL W in the DNA Data Bank of Japan (DDBJ; Japan). Sequence data of the *mfa1* genes of *P. gingivalis* strain 381 and ATCC33277 were obtained from DDBJ under accession nos. AB524739 and AB016284, respectively.

Results

Purification of fimbrial protein and immunologic reactions

The secondary fimbrial protein of *P. gulae* ATCC 51700 was purified from the fraction using an immunoaffinity column, from which it was eluted in PBS containing 0.5M NaCl. The protein showed a single band of molecular mass 53-kDa in SDS-PAGE. The 41-kDa fimbrial protein was eluted at 0.2M glycine-HCl (pH 2.5) using the same column (Fig. 1A, lanes 1, 2, and 3). On Western blotting analysis, the PAbs against the secondary fimbrial protein from *P. gulae* ATCC 51700 reacted with the crude fimbrial preparation and purified the secondary fimbrial protein but did not react with purified 41-kDa fimbrial protein (Fig. 1B, lanes 1, 2, and 3). Thus, these two types of fimbrial proteins did not show cross-reactivity.

Transmission electron micrograph of immunogold labeling

The expression of fimbriae on the cell surface of *P. gulae* ATCC 51700 was investigated by transmission electron microscopy (Fig. 2A). In immunogold labeling, the fimbriae of *P. gulae* ATCC 51700 were labeled with the PAbs against 53-kDa protein (Fig. 2B). An immunogold

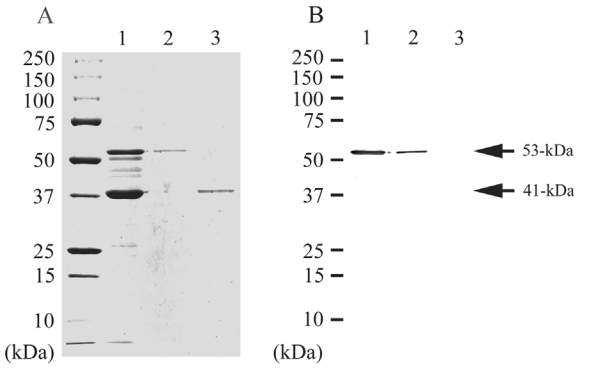

Fig. 1. SDS-PAGE and Western blotting of the purified proteins from *P. gulae* ATCC 51700. Proteins were electrophoresed on a 12.5% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250 (A). Western blotting analysis was performed with the PAbs against the 53-kDa fimbrial protein (B). Lane 1, crude fimbrial preparation from *P. gulae* ATCC 51700; Lane 2, Purified secondary fimbrial protein from *P. gulae* ATCC 51700; Lane 3, Purified 41-kDa fimbrial protein from *P. gulae* ATCC 51700.

double-labeling method was used to visualize the binding of two fimbriae-specific PAbs to *P. gulae* ATCC 51700 cells. The mouse PAbs against the 53-kDa and the rabbit PAbs against the 41-kDa bound to fimbriae on the cell surface, respectively. The 5-nm collodion gold-labeled goat anti-mouse serum bound to the 53-kDa fimbriae. The 10-nm collodion gold bound to the 41-kDa fimbriae.

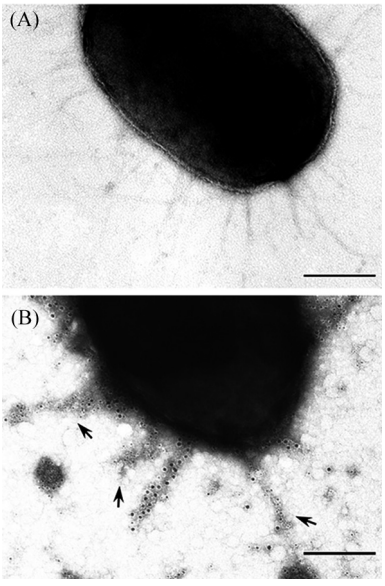

Fig. 2. Transmission electron micrographs of double immunogold labeling. *P. gulae* ATCC 51700 possessed fimbriae on its cell surface (A). The 5-nm collodion gold-labeled goat anti-mouse serum bound to the 53-kDa fimbriae (arrows). The 10-nm collodion gold bound to the 41-kDa fimbriae. Fimbriae of *P. gulae* were specifically labeled with gold particles (B). Bars, 0.2 μm.

Fimbriae of *P. gulae* were specifically labeled with two kinds of gold particles. These results suggested that *P. gulae* ATCC 51700 possesses 53-kDa fimbriae antigenicity distinct from 41-kDa fimbriae. The 53-kDa fimbriae are produced along with 41-kDa fimbriae on the *P. gulae* ATCC 51700.

Immunological reactivities between P. gulae *and* P. gingivalis

Crude fimbrial proteins of *P. gulae* ATCC 51700 and *P. gingivalis* strains 381 and ATCC 33277 were analyzed by SDS-PAGE (Fig. 3A, lanes 1, 2, and 3). On Western blotting analysis, the PAbs against the 53-kDa fimbrial protein of *P. gulae* ATCC 51700 reacted with the 53-kDa band of crude fimbrial protein from *P. gingivalis* strain 381 but did not react with crude fimbrial protein from *P. gingivalis* ATCC 33277 (Fig. 3B, lanes 1, 2, and 3).

Comparison of mfa1 *gene*

The *mfa1* gene was amplified using two pairs of PCR primers, PG1 and PG2, from *P. gulae* ATCC 51700. The sequence of *P. gulae* ATCC 51700 *mfa1* gene is available from DDBJ (accession no. AB510743). Multiple alignment analysis showed that this gene fragment shares considerably high homology with the 53-kDa fimbrial protein of *P. gingivalis* strain 381 (89%). The deduced amino acid sequence encoding the 53-kDa fimbrial protein of *P. gulae* ATCC 51700 showed 92% similarity with the 53-kDa fimbrial protein of *P. gingivalis* strain 381. However, the deduced amino acid sequence encoding the 67-kDa fimbrial protein of *P. gingivalis* ATCC 33277 showed 34% similarity with the 53-kDa

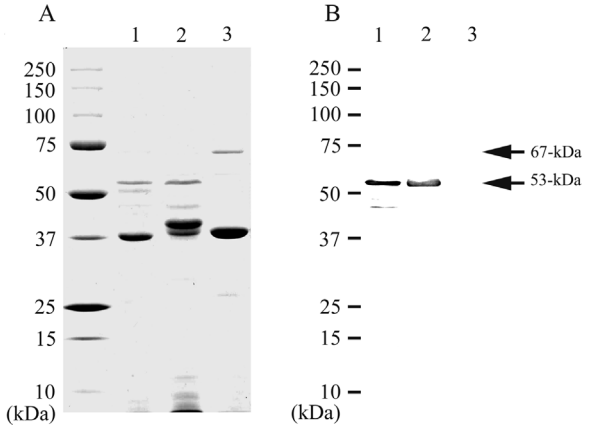

Fig. 3. SDS-PAGE analysis and Western blotting of crude proteins. Proteins were electrophoresed on a 12.5% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250 (A). Western blotting analysis was performed with the PAbs against the secondary fimbrial protein from *P. gulae* ATCC 51700 (B). Lane: 1, crude fimbrial preparation from *P. gulae* ATCC 51700; Lane 2, crude fimbrial preparation from *P. gingivalis* strain 381; Lane 3, crude fimbrial preparation from *P. gingivalis* ATCC 33277.

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fimbrial protein of *P. gulae* ATCC 51700 (Fig. 4). The amino acid sequence of the *N*-terminal 15 residues of the 53-kDa fimbrial protein of *P. gulae* ATCC 51700 (AGDNDYNHVGEYGGI) showed 12 of 15 residues identical to the 53-kDa fimbrial protein of *P. gingivalis* strain 381 (AGDNDYNPIGEYGGV) and 4 of 15 residues identical to the 67-kDa fimbrial protein of *P. gingivalis* ATCC 33277 (AGDGQDQANPDYHYV) but only 1 of 15 residues identical to the 41-kDa fimbrial protein of *P. gulae* ATCC 51700 (AFGVADDEAKVAKLT).

Discussion

We previously reported the existence of 41-kDa fimbriae of *P. gulae* ATCC 51700 and molecular and antigenic similarities of 41-kDa fimbrial protein between *P. gulae* ATCC 51700 and *P. gingivalis* ATCC 33277 (9). In this study, we have succeeded in purifying and characterizing secondary fimbrial protein of *P. gulae* ATCC 51700. The purified secondary fimbrial protein was observed as a single band of 53-kDa by SDS-PAGE analysis, and had antigenicity distinct from that of 41-kDa fimbrial protein (Fig. 1). The *N*-terminal amino acid sequence of 53-kDa and 41-kDa fimbrial proteins were identical at only 1 of 15 positions. In immunogold labeling, the PAbs against 53-kDa protein bound to fimbrial structures on the surface of *P. gulae* ATCC 51700 (Fig. 2). The 5-nm collodion gold-labeled goat anti-mouse serum bound to the 53-kDa fimbriae. The 10-nm collodion gold bound to the 41-kDa fimbriae. Fimbriae of *P. gulae* were specifically labeled with two kinds of gold particles. The immunoelectron-microscopic findings suggested that the PAbs react with unique determinants on major and minor fimbrial proteins. Hamada et al. (9) reported that the fimbriae of *P. gulae* ATCC 51700 was labeled with PAbs against 41-kDa fimbrial protein. Some fimbriae on the cell surface were not labeled with the antibody. Thus, the 53-kDa fimbrial protein was antigenicity distinct from 41-kDa fimbrial protein, demonstrating that two distinctly different fimbriae are expressed by the same *P. gulae* ATCC 51700. The secondary fimbrial protein purified from *P. gingivalis* ATCC 33277 markedly induced IL-1α, IL-β, IL-6, and TNF-α cytokine expression in mouse peritoneal macrophages (31). It has been reported that the 53-kDa protein of *P. gingivalis* strain 381 reacted strongly with the serum of patients with periodontal disease (32, 33). On Western blotting analysis, the 53-kDa fimbrial protein of *P. gulae* ATCC 51700 and that of *P. gingivalis* strain 381 showed immunological cross-reactivity (Fig. 3). Moreover, the amino acid sequence of the 15 *N*-terminal residues of the 53-kDa fimbrial protein of *P. gulae* ATCC 51700 had 12 of 15 residues identical to those of *P. gingivalis* strain 381 (Fig. 4). It is possible that *P. gulae* exists frequently in companion animals and the secondary fimbrial protein of *P. gulae* plays a role as a strong antigen. These results suggest that the 53-kDa fimbrial protein of *P. gulae* ATCC 51700 may play an important role in periodontal pathogenicity and the host immune response. It was reported that *P. gulae* was pathogenic in a mouse model of periodontal disease. Whole-cell bacterin preparation

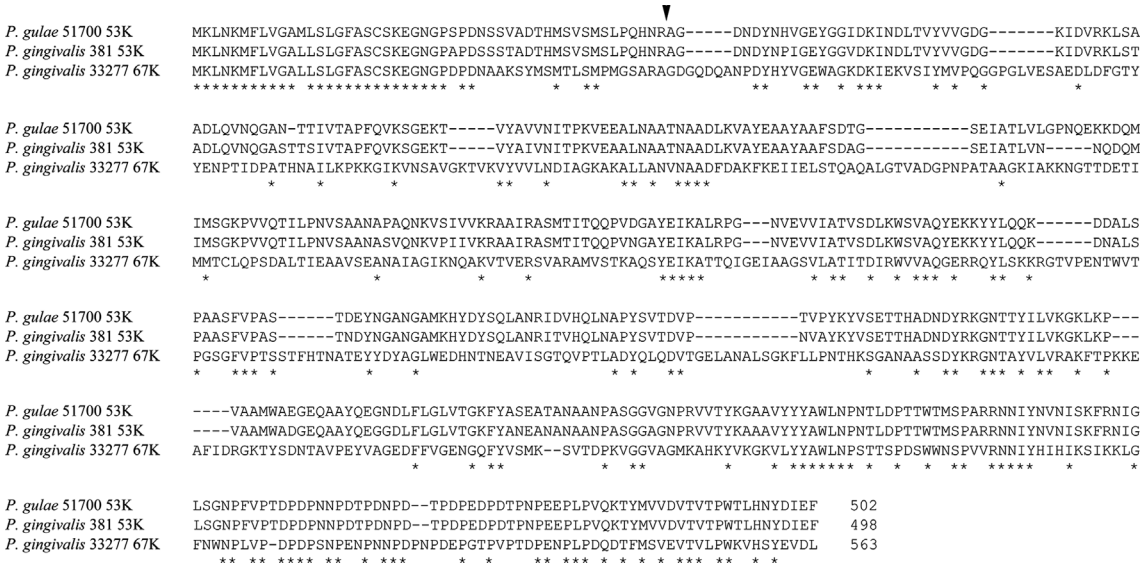

Fig. 4. Comparison of predicted amino acid sequences for Mfa1encoded by the *mfa1* genes of *P. gulae* and *P. gingivalis* strains. Amino acid identities are shown by asterisks. Hyphens are used to indicate the positions of gaps in the multiple alignment. The alignment of the deduced amino acid sequences was performed with the CLUSTALW program of the DNA Data Bank of Japan. The nucleotide sequences had been deposited with DDBJ/EMBL/GenBank under the accession numbers AB510743 for *P. gulae* ATCC 51700, AB524739 for *P. gingivalis* 381 and AB016284 for *P. gingivalis* ATCC33277. The arrow indicates the cleavage site.

of *P. gulae* displayed significantly reduced alveolar bone loss (34). The 53-kDa protein isolated from *P. gingivalis* 381 (8), which has been demonstrated to be the same molecule as a 53-kDa major outer membrane protein (35), has reported as a minor fimbriae and a major immunodominant protein likely to contribute to host–bacteria interaction (36). Those two types of minor fimbrial proteins showed no immunological cross-reactivity. The roles of these distinct molecules and the difference in antigenicity in relation to bacterial virulence are yet unclear. The role of minor fimbriae in virulence is less understood. However, the minor fimbriae are necessary for the development of synergistic biofilms between *P. gingivalis* and *S. gordonii* via a specific interaction with the streptococcal SspB protein (37). Moreover, Lin et al. reported that the minor fimbriae are involved in *P. gingivalis* autoaggregation and colonization. A mutant with a deficiency in minor fimbriae can bind to a saliva-coated surface but does not form microcolonies as the wild-type strain does. The major fimbriae are required for initial attachment and organization of biofilms. The minor fimbriae promoted bacterial autoaggregation, whereas major fimbriae suppressed it (38, 39). Our further studies will be directed toward elucidating the biochemical and immunobiological functions of the secondary fimbriae.

Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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