

RESEARCH ARTICLE OPEN ACCESS

Phenotypic Characterization of *Porphyromonas gulae* Fimbria Mutants

Hiroaki Inaba^{1,2}  | Sho Yoshida² | Naoya Ohara^{3,4,5}  | Ryota Nomura⁶ | Kazuhiko Nakano⁷ | Michiyo Matsumoto-Nakano²

¹Department of Dental Hygiene, Kyoto Koka College, Kyoto, Japan | ²Department of Pediatric Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan | ³Department of Oral Microbiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan | ⁴Advanced Research Center for Oral and Craniofacial Sciences, Dental School, Okayama University, Okayama, Japan | ⁵Research Center for Intestinal Health Science, Okayama University, Okayama, Japan | ⁶Department of Pediatric Dentistry, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan | ⁷Department of Pediatric Dentistry, Graduate School of Dentistry, The University of Osaka, Osaka, Japan

Correspondence: Hiroaki Inaba (h-inaba@mail.koka.ac.jp)

Received: 17 December 2025 | **Revised:** 8 May 2026 | **Accepted:** 22 May 2026

Academic Editor: Suraiya Saleem

Keywords: biofilm | cell invasion | cell migration | fimbriae | periodontitis | *Porphyromonas gulae* | silkworm larvae

ABSTRACT

Porphyromonas gulae has been established as a pathogen in animal periodontal disease and expresses several virulence factors, including fimbriae, lipopolysaccharide, and proteases. *P. gulae* fimbriae are classified into three genotypes (A, B, and C) based on the fimbriillin (*fimA*) gene encoding a fimbrial subunit protein. We previously reported that *P. gulae* organisms with type C fimbriae were clearly more virulent as compared to those with other fimbria types, though the basic function of this is not fully understood. Here, we investigated the function of fimbriae using *fimA*-deficient mutants. In the present study, bacterial fimbriae were related to biofilm tenacity, whereas biofilm formation was not. Also, *P. gulae* fimbriae are involved in bacterial adhesion and entry into gingival epithelial cells. The role of *P. gulae* fimbriae in bacterial pathogenicity was then determined using *fimA*-deficient mutants with a silkworm infection model. Additionally, *P. gulae* infection can induce inflammatory responses in gingival epithelial cells and also inhibit cell migration and proliferation. In contrast, a *P. gulae* *fimA*-deficient mutant did not cause several of those cellular responses. Together, the results show that fimbriae contribute to the pathogenicity of *P. gulae* by affecting bacterial functions and host cell interactions.

1 | Introduction

Fimbriae, nonflagellar hair-like proteinaceous protrusions on the surface of bacteria, are filamentous structures composed of various monomers [1, 2]. These filamentous structures have been characterized as proteins in both Gram-positive and Gram-negative bacteria [3, 4]. Cell-surface organelles can be essentially divided into assembled and type V secretion systems [1, 2, 5]. Fimbriae are largely involved in horizontal gene transfer of pathogenicity islands, including several virulence factors [3, 6]. Moreover, these structures possess various features related to bacterial cell biology,

including biofilm formation, cell aggregation, twitching motility, and phage transduction [3, 4]. In host cell biology experiments, fimbriae have been shown to contribute to the efficiency of colonization and possess important roles for adhesion to eukaryotic cells in the pathogenic process [3, 4]. Additionally, fimbria variations have relationships with bacteria-mediated diseases, such as diarrhea, meningitis, pneumonia, sepsis, sexually transmitted diseases, urinary tract infections, dental caries, and periodontitis [4]. These findings indicate the possibility that fimbriae have a direct role in microbial pathogenicity and are critical virulence factors possessed by bacterial pathogens [1].

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Copyright © 2026 Hiroaki Inaba et al. *Cellular Microbiology* published by John Wiley & Sons Ltd.

Porphyromonas gulae organisms exhibit several characteristics, such as black-pigmented colonies, asaccharolytic, obligate anaerobic growth, Gram-negative coccobacillus, and nonmotile and non-spore-forming cells [7]. The presence of *P. gulae* in canines and cats is known to be an indicator of periodontitis [8]. Moreover, *P. gulae* have been detected in human gingival tissues obtained from healthy subjects, as well as diseased sites of patients with bleeding and bone loss [8, 9]. *P. gulae* expresses several virulence factors, including lipopolysaccharide (LPS) and protease, as well as fimbriae encoded by the chromosomal gene *fimA* [8, 10, 11]. The *fimA* gene has three types (types A, B, and C) based on nucleotide sequence differences, with *fimA* genotype C prevalent in isolates from periodontitis lesions [8]. In addition, *P. gulae* organisms with type C fimbriae have an ability to induce abscess formation in a mouse model and also inhibit human gingival epithelial cell migration, while those with type A or B do not [8]. Recent studies have reported that *P. gulae* has an ability to invade human gingival epithelial cells [10] as well as accelerate silkworm larvae mortality in a *fimA* genotype-dependent manner [11]. Nevertheless, the role of *P. gulae* fimbriae has yet to be fully identified or characterized.

The present study was conducted to delineate the role of *P. gulae* fimbriae in bacterial and host cell biology. Results obtained with *fimA*-deficient mutants indicate that, in addition to its effects on the survival of silkworm larvae, *P. gulae* fimbriae are required for adhesion and invasion efficiency, cell motility inhibition, and inflammatory responses in human gingival epithelial cells. Furthermore, *P. gulae* biofilm strength was found to be affected by the loss of fimbriae.

2 | Materials and Methods

2.1 | Bacteria and Cell Cultures

P. gulae strain D049 was grown in anaerobic jars at 37°C in trypticase soy broth supplemented with yeast extract (1 mg/mL), menadione (1 µg/mL), and hemin (5 µg/mL) and on blood agar plates supplemented with 5% (vol/vol) sheep blood, hemin (5 mg/L), and menadione (1 mg/L), as described previously [10]. *Escherichia coli* DH5a was used as a recipient for all plasmids and was grown in Luria-Bertani (LB) broth and LB agar with appropriate antibiotics, as follows: ampicillin (100 mg/mL), erythromycin (10 mg/mL), and tetracycline (1 mg/mL). Ca9-22 cells that originated from human gingival epithelia were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and cultured in RPMI1640 medium (FUJIFILM Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂.

2.2 | Construction of *P. gulae* Fimbriate Mutant and Complementary Strains

P. gulae *fimA* deletion mutant (Δ *fimA*) and complementary mutant (*cfimA*) strains were constructed as follows (Supporting Information 1: Figures S1 and S2 and Supporting Information 2: Tables S1 and S2). Polymerase chain reaction amplification of the *fimA* region from *P. gulae* D049 chromosomal DNA was performed using *FimA_F* and *FimA_R* primers. The amplified fragment was then subcloned into a pANT Vector (TA-Enhancer

Cloning Kit, Nippon Gene, Tokyo, Japan) to obtain pANT-*fimA*, and its sequence was verified. Then, the erythromycin resistance methylase F (*ermF*) region was amplified from pKD355 using *ErmF_F* and *ErmF_R* primers [12], after which the PCR fragment of the *ermF* region was digested with *NcoI* and inserted into the *NcoI* site of *fimA* in pANT-*fimA*. The resulting plasmid, which contained the *ermF* open reading frame (ORF) in the same direction as the *fimA* ORF, was selected. Inverse PCR using primers *ErmF_inv* and *FimA_inv* was then performed to move the sequence from the start codon of *fimA* to that of *ermF*, thereby placing *ermF* under control of the *fimA* promoter, with the resulting plasmid designated as pANT- Δ *FimA*. The pANT- Δ *FimA* plasmid was linearized by *SmaI* digestion and introduced into *P. gulae* D049 cells by electroporation, resulting in a Δ *fimA* mutant. PCR analysis was performed to verify correct gene replacement occurring via double crossover recombination events (data not shown).

For complementation, *fimA* was inserted into the *ermF* ORF locus of the Δ *FimA* genome. To create unique *XhoI* and *SacI* sites in the *ermF* ORF of pANT- Δ *FimA*, inverse PCR was performed using *ErmF_Xh* and *ErmF_Sa* primers, with the resulting plasmid designated as pANT- Δ *FimA* SX. The *fimA* region containing the ORF, promoter, and *fimA* terminator was PCR amplified using *FimA_EX_F* and *FimA_EX_R* primers. The fragment was then digested with *XhoI* and *BamHI* and cloned into the corresponding sites of pBluescript II SK(-) to obtain pBSSK-*FimA*. Next, pTIO-1T was digested with *XbaI* and *SacI*, and the fragment containing the tetQ cassette was inserted into the *XbaI/SacI* sites of pBSSK-*FimA*, resulting in pBSSK-*FimA* tet. Finally, the *XhoI-SacI* fragment from pBSSK-*FimA* tet was inserted into the corresponding sites of pANT- Δ *FimA* SX to obtain pANT- Δ *FimA* SX tet. pANT- Δ *FimA* SX tet was then linearized by *SmaI* digestion and introduced into the strain by electroporation, resulting in a *fimA* complementary mutant.

2.3 | Protease Activity Assay

Bacterial cells were harvested and adjusted to a final density of 5×10^7 cells for whole-cell analysis. Protease activity in culture supernatants was measured using the Pierce Protease Assay Kit (Thermo Scientific, Rockford, IL), with 10 µg of supernatant protein used per reaction. Briefly, each sample was mixed with the supplied casein substrate and reaction buffer and incubated at room temperature (RT) for 20 min. The reaction was then developed by the addition of trinitrobenzene sulfonic acid (TNBS), followed by an additional 20 min incubation at RT. TNBS derivatizes newly generated primary amines, yielding a yellow-orange chromogenic product. Absorbance was measured at 450 nm using an SH-1000 Lab microplate reader (Corona Electric).

2.4 | Biofilm Assay

- i. Visualization. Biofilms formed by *P. gulae* D049 and its mutants on glass plates were analyzed by confocal microscopy, as previously described [13], with some modifications. Briefly, the bacterial strains (1×10^8 cells/well) were labeled with hexidium iodide (15 µg/mL; Molecular

Probes, Eugene, OR, United States) in TSB and cultured anaerobically for 24 h at 37°C in eight-well Lab-TeK II Chamber slides (Nalge Nunc International, Roskilde, Denmark). Visualization of the stained biofilms was performed using an LSM 780 confocal laser scanning microscope with an emission wavelength of 568 nm (Axio Observer Z1, Carl Zeiss, Germany). The images were then digitally reconstructed using ZEN LITE 2012 64-bit version (Black edition; Carl Zeiss) and exported to ImageJ, Version 1.48 (freeware), and biomass density was determined based on pixel intensity.

- ii. Quantification. *P. gultae* D049 and its mutants were inoculated into TSB incubated anaerobically, and 5×10^7 cells/well were seeded into 96-well plates (Nunc, Roskilde, Denmark) for 24 h. Thereafter, the medium was removed, and the wells were washed with phosphate-buffered saline (PBS), stained with 1% crystal violet (CV), and subsequently destained with 95% ethanol. Absorbance was determined at 595 nm using an SH-1000 Lab microplate reader (Corona Electric, Ibaraki, Japan).

2.5 | Sonic Disruption Assay

Biofilm disruption of *P. gultae* D049 and its mutants was performed using an ultrasonic disruptor and then quantified, as previously described [13], with some modifications. *P. gultae* D049 and its mutants (2×10^8 CFU/well) were incubated in six-well plates (Nunc) for 60 h at 37°C, and the resulting biofilms were sonicated for 5 s at Output Level 1 (output power 25 W, oscillating frequency 28 kHz, tip diameter 2.5 mm) using a Handy ultrasonic disruptor (UR-20P, Tomy Seiko, Tokyo, Japan). Following ultrasonic treatment, supernatants containing detached cells were carefully removed by aspiration, and any wells containing adherent biofilms were gently washed with PBS. The biofilms were then stained with 1% CV, and the bound dye was eluted with 95% ethanol. Biofilm biomass was quantified by measuring absorbance at 595 nm using an SH-1000 Lab microplate reader (Corona Electric, Ibaraki, Japan).

2.6 | Protection Assay

P. gultae adhesion and invasion were quantitated using antibiotic protection assay findings as described previously [10], with some modifications. *P. gultae* D049 and its mutants were harvested, washed, and resuspended in RPMI1640 without antibiotics, and then Ca9-22 cells were infected with the bacteria at a multiplicity of infection (MOI) of 100 for 90 min on 24-well plates (Corning Costar, Cambridge, MA, United States) and washed with PBS. For determining total adhesion and invasion levels, cells were lysed with sterile distilled water for 15 min, and then lysate dilutions were plated and cultured anaerobically on blood agar plates. For invasion assays, extracellular bacteria were killed with ampicillin (200 µg/mL) and gentamicin (300 µg/mL) for 60 min. Cells were lysed, and intracellular bacteria enumerated. The resulting lysates were serially diluted (10^0 – 10^{-7}) in PBS, and 20 µL of each dilution was plated onto blood agar plates and incubated anaerobically at 37°C for 4 days, after which colony-forming units (CFUs) were counted. The numbers of adherent and internalized bacteria

were then determined based on CFU counts, and adhesion and invasion rates were calculated as percentages relative to the initial inoculum.

2.7 | Tetrazolium Proliferation Assay

Cells (1×10^4 cells/well) were seeded into 96-well plates (Corning Costar) and incubated at 37°C for 24 h, followed by *P. gultae* D049 and its mutants at an MOI of 100 for an additional 24 h. Infected or control cell proliferation was incubated with a Cell Counting Kit (Dojindo, Kumamoto, Japan) for 2 h at 37°C, and absorbance was measured at 570 nm using an SH-1000 Lab microplate reader (Corona Electric).

2.8 | Cell Migration Scratch Assay

The migration capability of infected and control Ca9-22 cells was assessed using a wound healing scratch assay as described previously [14], with some modifications. Ca9-22 cells were cultured until confluency in six-well culture plates, and then a scratch was made in the confluent monolayer with a plastic tip. The cells were then infected with each bacterial strain at an MOI of 100 for 2 h and subsequently maintained in RPMI 1640 medium containing 10% FBS for an additional 4 h, resulting in a total infection duration of 6 h. Cell migration was analyzed using ImageJ software, as described previously [15]. Throughout the assay, Ca9-22 cell morphology and adherence were monitored by phase contrast microscopy, with no apparent cell detachment or significant cell death observed.

2.9 | Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated using TRIsure (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions and then converted into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was performed with the following primers: for IL- β , 5'-CTTTGAAGCTGATGGCCCTAAA-3' and 5'-AGTGGTGGTCGGAGATTTCGT-3' [16]; for COX2, 5'-CACAGGCTTCCATTGACCAGA-3' and 5'-GTGCTCCAACCTTACCATTGG-3' [16]; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GTCTTCACCACCATGGAGAAG-3' and 5'-GTTGTCTATGGATGACCTTGGC-3' [16]. Negative RT reactions were included in each assay. Expression values for mRNA were quantified by the $\Delta\Delta C_t$ method using GAPDH as the control.

2.10 | ELISA

Cells (1×10^4 cells/well) were seeded into 96-well plates (Corning Costar) and incubated at 37°C for 24 h. Next, cells were infected with *P. gultae* D049 and its mutants at an MOI of 100 for 4 h. Following incubation, cell culture supernatants were collected for IL-1 β quantification, and cell lysates were prepared for COX2 analysis. ELISA kits were used to measure the concentrations of COX2 (R&D Systems, Minneapolis, MN) and IL-1 β

(Proteintech Group Inc., Rosemont, IL, United States), according to the manufacturer's instructions. Absorbance was determined at 450 nm, with correction to 550 nm, using a SH-1000 Lab microplate reader (Corona Electric, Ibaraki, Japan).

2.11 | *P. gulyae* Infection of Silkworm Larvae Model

Bacterial pathogenesis and toxicity in silkworms infected with *P. gulyae* D049 and its mutants were examined as previously described [11]. Briefly, silkworms were grown from fertilized eggs to the four-molt stage. Hatched four-instar larvae were given antibiotic-free food (SilkMate 2S, Nosan Corporation, Kanagawa, Japan) for 1 day, and then bacterial suspensions (5×10^7 CFU in $50 \mu\text{l}$ PBS) were injected into the hemolymph of the silkworms

($n = 10$). Following the injection, the larvae were further incubated at 37°C without feeding, and survival was examined every 12 h for up to 156 h.

3 | Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test and are presented as the mean $\pm$ standard deviation (SD). Results presented are representative of at least three biological replicates. Silkworm survival rates were determined using a Kaplan–Meier plot and compared with a log-rank test (StatView software, Version 5.0; SAS Institute Inc., Cary, NC, United States). Statistical significance was considered at $p < 0.01$ or $p < 0.05$.

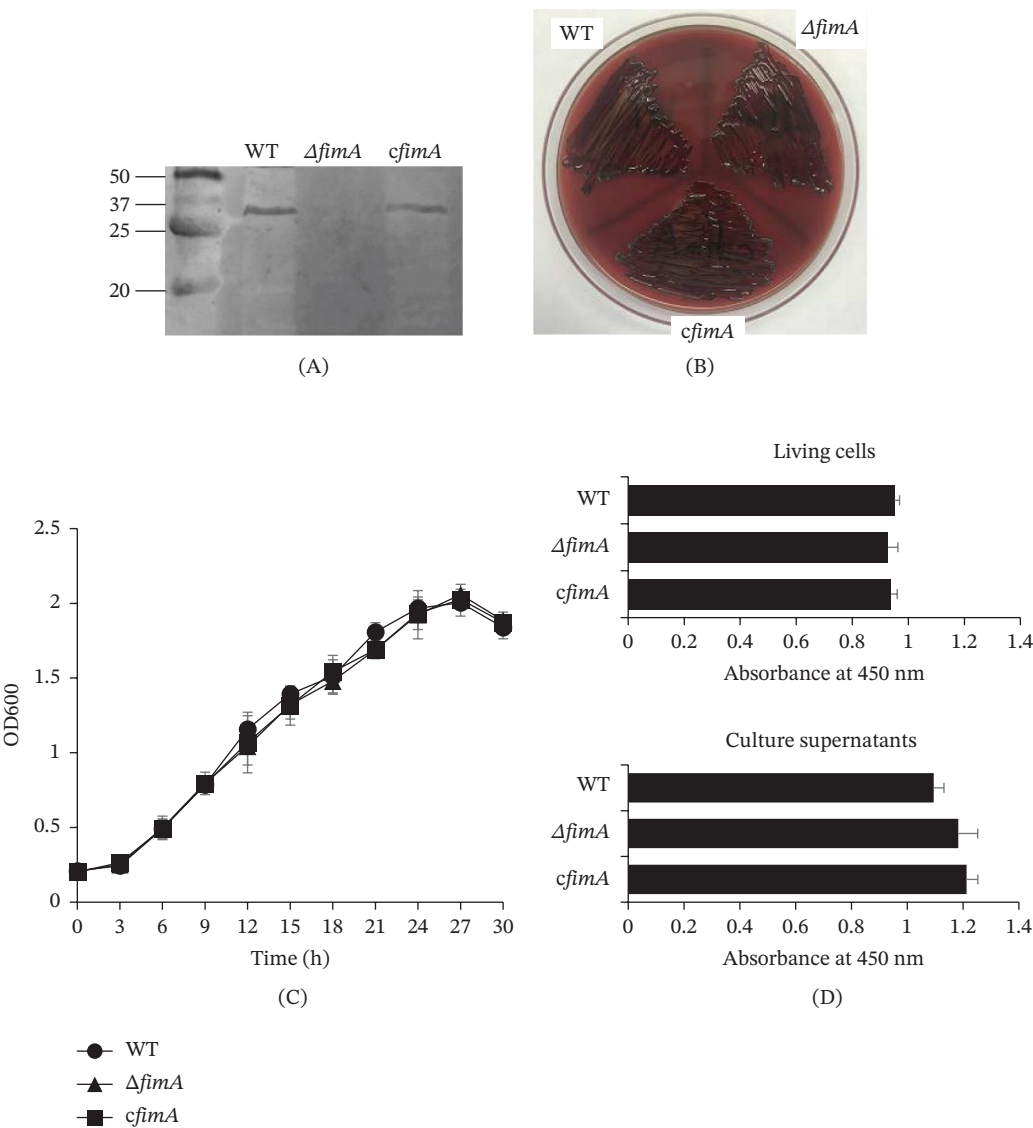


FIGURE 1 | Characterization of *P. gulyae* *finA*-deficient mutant. (A) Western blotting analysis of *P. gulyae* D049 (wild type [WT]), *finA*-deficient mutant ($\Delta finA$), and complement (*cfimA*) strains. (B) Colonial pigmentation. *P. gulyae* D049 WT, $\Delta finA$, and *cfimA* strains were anaerobically grown on blood agar plates at 37°C for 7 days. (C) Anaerobic growth profile of *P. gulyae* *finA*-deficient mutant. Growth was monitored by measuring optical density at 600 nm every 3 h up to 30 h. Data are shown as the mean $\pm$ SD of three independent experiments. (D) Quantification of protease activities of WT, $\Delta finA$, and *cfimA* *P. gulyae* strains (5×10^7 CFU) and supernatants ($10 \mu\text{g}$ of protein contained in supernatant). Enzyme activities were determined with a microplate reader and expressed as arbitrary units. Data are shown as the mean $\pm$ SD of three independent experiments.

4 | Results

4.1 | Construction of *P. gulate fimA*-Deficient Mutant and Complemented Strain

Construction of the *P. gulate* Δ *fimA* strain was performed by inactivating the *fimA* gene with a double cross-over recombination in the *P. gulate* D049 strain (Supporting Information 1: Figure S1). The upstream and downstream regions of the *fimA* gene and the *ermF* DNA cassette from the pTIO-1 vector underwent PCR amplification. These amplified DNA fragments were then cloned into a pANT vector (*fimA*-*ermF* in pANT), and the product was purified and transformed into *P. gulate* D049 following electroporation. The *P. gulate* Δ *fimA* strain was complemented *in trans* with the intact *fimA* gene carried on plasmid pTIO-1Te, after which the plasmid was amplified using PCR and cloned into a pANT vector (*fimA*-*tetQ* in pANT) (Supporting Information 1: Figure S2). Western blot analysis was performed to examine the fimbria expression of each *P. gulate* strain (Figure 1A). No difference in that expression between the *P. gulate* parent strain and

cfimA was observed, whereas the *P. gulate* Δ *fimA* strain was not. There were no differences in colonial pigmentation, bacterial growth, or proteolytic activity observed among the examined strains (Figure 1B–D).

4.2 | Biofilm Formation Associated With *P. gulate fimA*-Deficient Mutant

The long filamentous structures of *Porphyromonas gingivalis*, *E. coli*, *Klebsiella pneumoniae*, and *Burkholderia pseudomallei*, known as pili or fimbriae, have been shown to be important for biofilm formation [17–20]. We analyzed the role of *P. gulate* fimbriae in biofilm formation using isogenic mutants. As shown in Figure 2A,B, *P. gulate* fimbriae did not have effects on biofilm formation or quantity. On the other hand, biofilm formed by the *P. gulate* fimbria-deficient strain showed a significant decrease in tenacity, though biofilm strength was similar between the parental and complemented strains (Figure 2C).

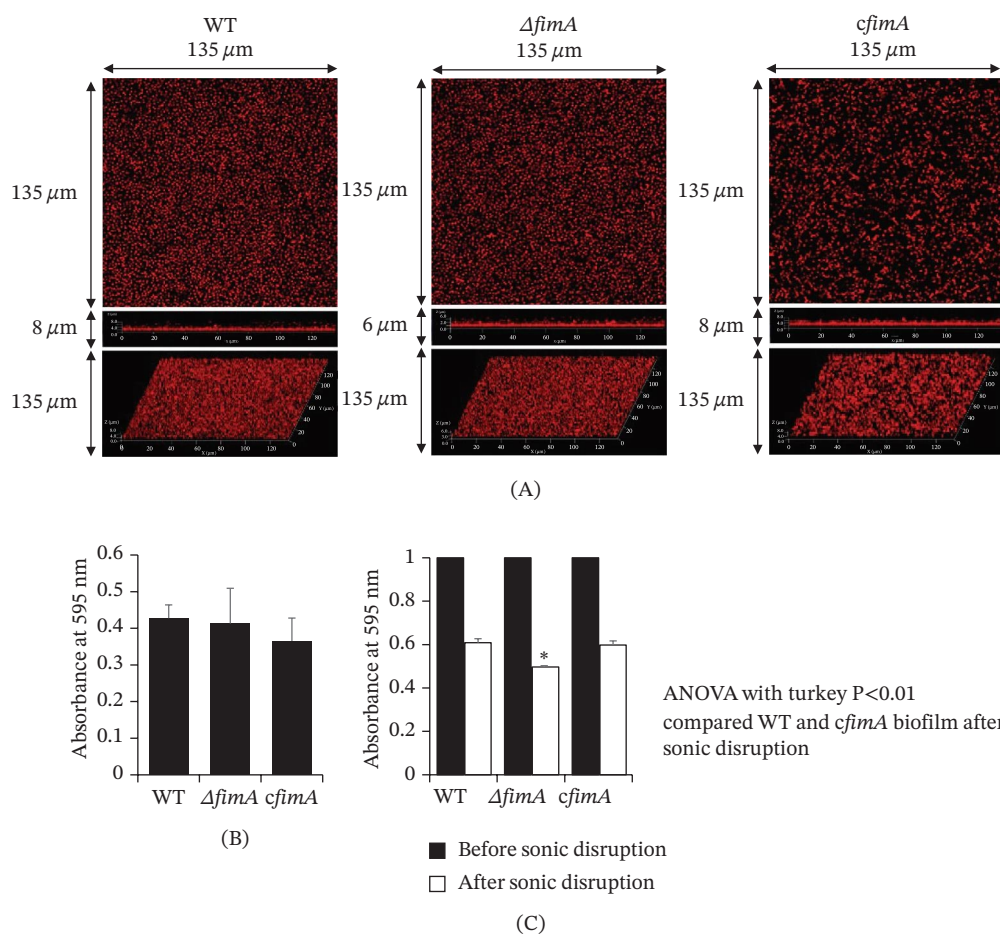


FIGURE 2 | Biofilm formation by *P. gulate* *fimA*-deficient mutant. (A) *P. gulate* strains were stained with hexidium iodide and incubated in TSB for 24 h. Biofilm development was observed with a confocal laser scanning microscope equipped with a 40x objective. Upper panels show horizontal sections (XY), middle panels show vertical sections (XZ), and lower panels show three-dimensional images, each created by ZEN LITE (2012, 64-bit version). Data shown are representative of three independent experiments. (B) Integrated density measurements of *P. gulate* biofilms were performed. (C) *P. gulate* biofilms were formed in six-well plates. Following sonication for 5 s, the remaining biofilm was stained with CV and quantified. Data are shown as the mean $\pm$ SD of three independent experiments. WT (wild type) = D049, Δ *fimA* = *fimA*-deficient mutant, *cfimA* = complement strain. **p* < 0.05 by ANOVA with Tukey's multiple-comparison test compared to *P. gulate* WT and *cfimA* biofilm after sonic disruption.

4.3 | Adhesion and Invasion of *P. gulae* *fimA*-Deficient Mutant

Interactions between fimbriae and human gingival epithelial cells are important for bacterial adhesion and invasion [21]. We found that *P. gulae* fimbria-deficient strain had a 60% reduction in adhesion as compared to the parent and complemented strains (Figure 3A). The invasive ability of the examined *P. gulae* strains is shown in Figure 3B. While the invasion abilities did not differ significantly among the parent and complemented strains, the fimbria-deficient mutant showed a 67% reduction in invasion.

4.4 | *P. gulae* Fimbriae Slow Proliferation and Cell Migration of Ca9-22 Cells

P. gingivalis has been reported to inhibit proliferation and cell migration [22]. Thus, findings showing bacterial adhesion and invasion prompted an investigation of infected Ca9-22 cell proliferation and migration rate. Tetrazolium proliferation assay results showed that the *P. gulae* wild-type and *cfimA* strains inhibited the proliferation of Ca9-22 cells, whereas the $\Delta fimA$ strain did not (Figure 4A). Additionally, scratch assay findings showed that the wild-type and *cfimA* strains significantly inhibited cell migration, in contrast to the $\Delta fimA$ strain (Figure 4B,C).

4.5 | Inflammatory Responses by *P. gulae* Fimbriae in Ca9-22 Cells

A previous study showed that *P. gulae* induces inflammatory cytokines and mediators, including IL-1 β and COX2 [22]. Thus, we investigated whether *P. gulae* fimbriae have a role in the inflammatory responses of Ca9-22 cells to *P. gulae*. At the mRNA and protein levels, IL-1 β and COX2 were increased by *P. gulae*

wild-type and *cfimA* strains (Figure 5), while the $\Delta fimA$ strain did not show a significant increase. These findings indicate a predominant role for *P. gulae* fimbriae in inflammatory responses at both the mRNA and protein levels.

4.6 | Infection of Silkworm Larvae With *P. gulae* *fimA*-Deficient Mutant

Silkworms have been used as an animal model to investigate the pathogenicity of bacteria [23]. To evaluate the pathogenic potential of *P. gulae* fimbriae, we investigated the pathogenic role of *P. gulae* fimbriae using a silkworm-infection model (Figure 6). Following infection with the *P. gulae* wild-type or *cfimA* strain, 40% of the silkworms were found dead within 156h, while all infected with the $\Delta fimA$ strain remained alive.

5 | Discussion

Periodontal disease refers to both gingivitis and periodontitis and is an infectious condition caused by periodontal pathogens [24, 25], which are caused by complex interactions between the formation of biofilm and inflammatory responses. Based on previous findings, it is estimated that inflammatory response increases the risk of tissue damage by nearly 80% [26, 27]. Additionally, in vitro and in vivo studies have found relationships between virulence factors, defined as components of a pathogen that can damage a susceptible host [28], and periodontal pathogenesis factors, such as bacterial cell invasion [10], biofilm formation [27], epithelial cell migration efficacy [8, 14], silkworm larvae survival [13], gingival epithelial cell degradation [29], and inflammation responses [18, 30]. Pathogenic microorganisms have also been shown to possess several virulence factors, including LPS, proteases, fimbriae, capsules, and secretion systems [31, 32]. Among those, fimbriae are considered to be critical factors involved in the mediation of host tissue interactions with periodontal pathogens, such as *P. gingivalis* and *P. gulae*, and also shown to be related to the development of periodontitis in humans and animals [8, 30]. Although the functions of *P. gulae* fimbriae have yet to be fully elucidated, based on the present bacterial and host cell biology findings, including biofilm formation, adhesion/invasion efficiency, cytotoxic effects, inflammatory responses, and silkworm infection model results, some conclusions can be drawn.

Specific fimbriae (type II) confer an ability to enhance bacterial adhesion/invasion efficiency to *P. gingivalis* strains, resulting in severe inflammation and periodontitis development [30]. Another study noted that type II *P. gingivalis* was more frequently found in deeper periodontal pockets of patients with severe periodontitis [33]. *P. gulae* organisms are closely related to *P. gingivalis*, and their type C fimbriae have similarities to those of type II *P. gingivalis*, including the following: higher frequency in periodontal lesions and invasion efficiency [8, 10]. The present results show that the *fimA*-deficient mutant strain inhibited adhesion and invasion, suggesting important roles for *P. gulae* fimbriae (Figure 3). Additionally, *P. gulae* proteases were previously found to be essential for adhesion and invasion of gingival epithelial cells [10], while the

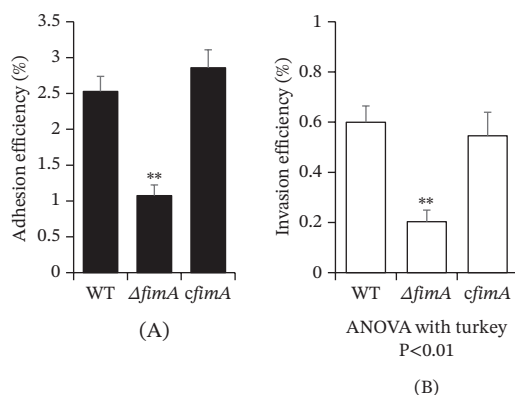


FIGURE 3 | Adhesion/invasion of Ca9-22 cells by *P. gulae* *fimA*-deficient mutant. Invasion of *P. gulae* strains was assessed by antibiotic protection assays. Ca9-22 cells were infected with bacteria at an MOI of 100 for 90 min. The number of adherent and/or intracellular bacteria was determined by counting viable cell lysates and is expressed as a percentage of bacterial cell number. Values are shown as the mean $\pm$ SD of three independent experiments. (A) Adhesion efficiency. (B) Invasion efficiency. WT (wild type)=D049, $\Delta fimA$ =*fimA*-deficient mutant, *cfimA*=complement strain. ** p < 0.01 by ANOVA with Tukey's multiple-comparison test.

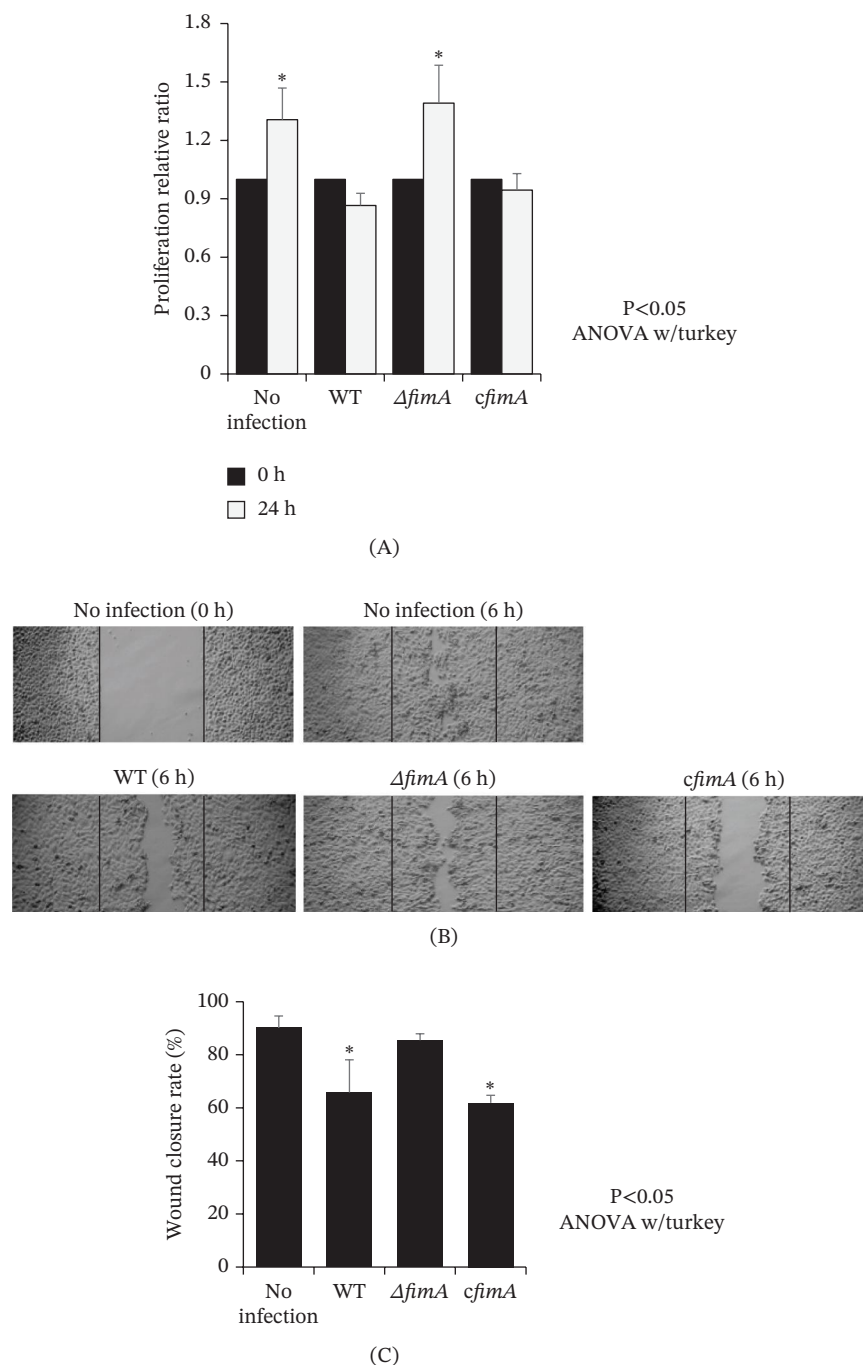


FIGURE 4 | *P. gulyae* fimbriae inhibit cell proliferation and cell migration of Ca9-22 cells. (A) Ca9-22 cell proliferation was determined using tetrazolium following infection with each *P. gulyae* strain at an MOI of 100 for 24 h. Data are expressed as a relative infected/uninfected ratio and presented as the mean $\pm$ SD of three independent experiments. (B) Scratch assay to investigate migration of Ca9-22 cells infected with *P. gulyae* strains at an MOI of 100. (C) Wound area was calculated using ImageJ and expressed as a percentage compared to the area at 0 h. Data are expressed as a relative infected/uninfected ratio and presented as the mean $\pm$ SD of three independent experiments. WT (wild-type) = D049, $\Delta fimA$ = *fimA*-deficient mutant, *cfimA* = complement strain. * $p < 0.05$ by ANOVA with Tukey's multiple comparison test.

present results indicate that bacteria adhesion and invasion are affected by multiple virulence factors, including fimbriae and proteases. These findings also indicate the possibility that *P. gulyae* fimbrial proteins may be processed into their mature form by their proteases.

The oral cavity constitutes a unique environment in which saliva, which contains numerous microorganisms, contributes

to wound healing processes [34], with epithelial cell migration a critical component of wound healing and epithelial barrier re-establishment [35]. In the present study, infection with the *P. gulyae* wild-type strain significantly inhibited gingival epithelial cell migration, whereas the inhibitory effect was absent in cells infected with the fimbria-deficient mutant (Figure 4). Notably, migratory inhibition was restored in the complemented strain, suggesting that suppression of epithelial cell

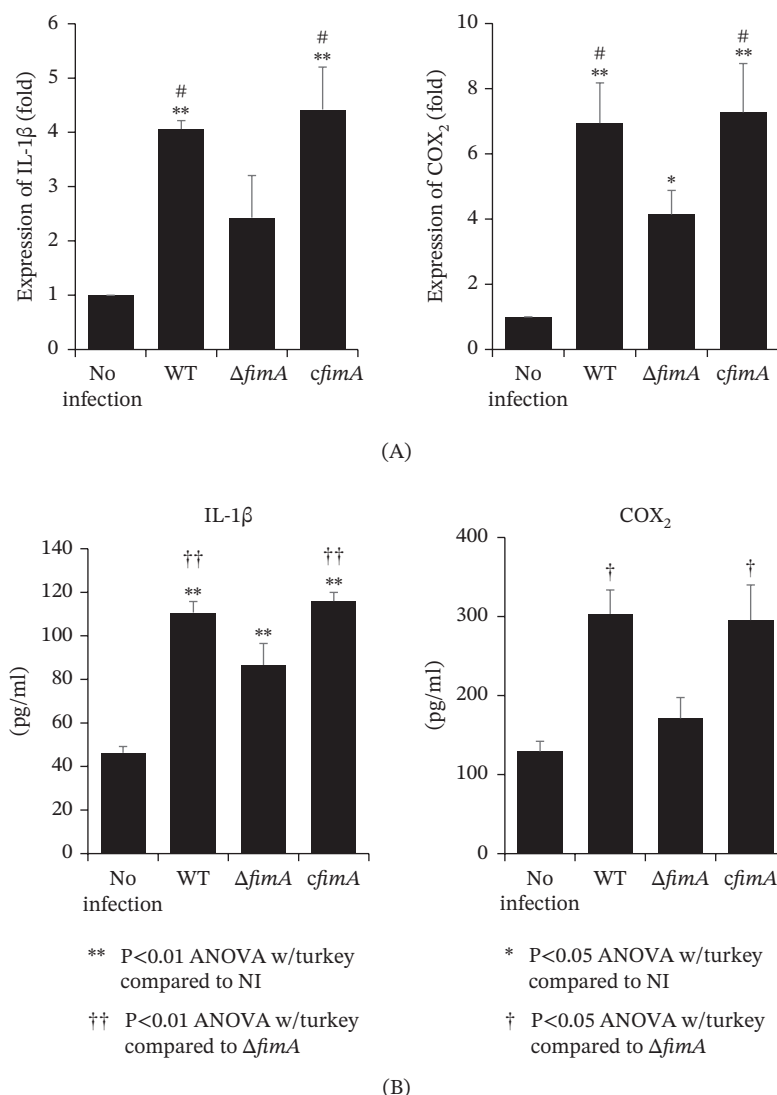


FIGURE 5 | (A, B) Elevated inflammatory response of Ca9-22 cells infected with *P. gulyae* *fimA*-deficient mutant. Ca9-22 cells were stimulated with each *P. gulyae* strain at an MOI of 100 for 4 h, and then protein production (for IL-1 β) and cell lysate (for COX2) related to inflammatory response were examined using an ELISA. Data are shown as the mean $\pm$ SD of three independent experiments. WT (wild type) = D049, $\Delta fimA$ = *fimA*-deficient mutant, *cfimA* = complement strain. * $p < 0.05$ and ** $p < 0.01$, as compared to no infection, and $^{\dagger}p < 0.01$ and $^{\dagger\dagger}p < 0.05$, as compared to $\Delta fimA$, based on findings of ANOVA with Tukey's multiple-comparison test.

migration is a fimbria-dependent and/or strain-specific phenomenon rather than a consequence of nonspecific bacterial alterations. These findings are consistent with previous reports demonstrating that bacterial fimbriae inhibit cell migration [36].

Epithelial cell migration is tightly regulated by dynamic modulation of cell adhesion, cytoskeletal reorganization, and intracellular signaling pathways [37]. The present findings indicate that *P. gulyae* fimbriae interfere with these processes during early host–pathogen interactions, leading to reduced epithelial cell motility without overt cytotoxicity. Such fimbria-dependent suppression of epithelial migration may compromise wound repair at sites of infection, thereby facilitating persistent bacterial colonization and contributing to chronic inflammatory conditions associated with periodontal disease. However, a potential limitation of scratch (wound-healing) assays is that reduced wound closure may indicate not only

impaired cell motility but also cytotoxicity or inhibition of cell proliferation following a bacterial infection. This possibility was carefully considered when interpreting the present results.

In the present study, Ca9-22 cells were infected with each *P. gulyae* strain for 2 h. Because of the relatively short infection period, it is unlikely that extensive cell death was induced during the subsequent wound healing observation period. Consistent with this, serial phase-contrast microscopic imaging revealed no apparent cell detachment or pronounced morphological damage under the infection conditions used, consistent with previous reports [29]. Furthermore, independent proliferation assays of cells infected for 24 h showed no obvious cell detachment or marked cytotoxic changes, and cell proliferation was also not significantly inhibited (Figure 4). These findings suggest that *P. gulyae* exerts minimal cytotoxic effects, even with prolonged exposure.

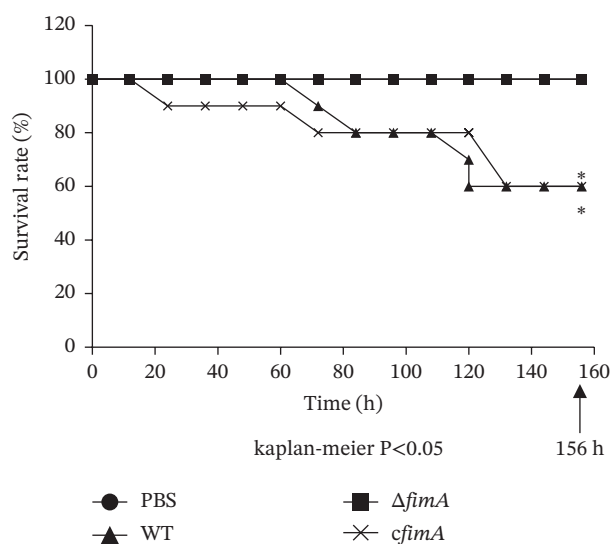


FIGURE 6 | Survival of silkworm larvae infected with *P. gulyae* *fimA*-deficient mutant. Ten silkworms were injected with *P. gulyae* (5×10^7 cells), and larval survival was monitored every 12 h up to 156 h. Data shown are representative of three independent experiments. Survival of silkworm larvae in each group was evaluated using a Kaplan–Meier plot and compared with a log-rank test. * $p < 0.05$.

Despite identical MOI and incubation times across all of the examined strains, inhibition of wound closure was observed only with the wild-type and complemented strains, whereas the fimbria-deficient mutant showed no such effect. Had non-specific cytotoxicity or growth inhibition been responsible for the reduced wound closure, similar effects would have been observed across all strains. Moreover, the protease activity of *P. gulyae*, previously implicated in protein degradation and cell detachment [29], did not differ among the bacterial strains tested (Figure 1D). Taken together, these findings indicate that the observed impairment in wound healing predominantly reflects fimbria-dependent suppression of epithelial cell migration rather than secondary effects of cell death or reduced proliferation.

Various pathogens, such as uropathogenic *E. coli* and *Salmonella enterica* serovar Typhimurium, have been reported to induce inflammatory reactions that are mediated by fimbriae [13, 38]. In the present study, we found that inflammatory responses, IL-1 β secretion, and COX2 expression were induced by the *P. gulyae* wild type and the complemented strain but not the fimbria-deficient mutant (Figure 5). These findings suggest that induction of inflammatory responses in gingival epithelial cells by *P. gulyae* is dependent on the presence of fimbriae. These findings are consistent with a previous report that noted enhancement of IL-1 β and COX2 expression in gingival epithelial cells by *P. gulyae* infection [16]. Importantly, the results obtained with fimbria-deficient and complemented strains in the present study further clarify the contribution of fimbriae to inflammatory mediator induction in gingival epithelial cells. As key bacterial structures involved in early interactions between bacteria and host cells, fimbriae have been suggested to function not only as adhesion factors but also as modulators of host cell responsiveness [39]. Based on our findings, *P. gulyae* fimbriae may influence both the nature

and magnitude of stimuli delivered to gingival epithelial cells, thereby promoting the production of inflammatory mediators.

In the present study, *P. gulyae* organisms formed biofilm regardless of whether the FimA protein was displayed on the bacterial surface as a fimbrial organelle (Figure 2). On the other hand, *P. gulyae* fimbriae were shown to be involved in biofilm durability. However, previous findings indicated that *P. gingivalis* fimbria-deficient mutants were more resistant to sonic disruption as compared to fimbriated strains [40]. The present findings are not consistent with previous reports; thus, it is suggested that biofilm formation and tenacity vary depending on the bacterial species. Additionally, the current study showed that fimbriae contribute to the tenacity of biofilm formed by oral microorganisms. Our findings suggest that the diversity of *P. gulyae* fimbriae has an influence on biofilm tenacity, as well as pathogenicity and development of disease, and biofilm tenacity may contribute to the pathogenesis of periodontitis mediated by *P. gulyae*.

Silkworm larvae have been reported to be suitable as an animal model for screening and identification of various factors, including antimicrobial agents, bacterial pathogenicity, and drug toxicology [38]. The present results suggest *P. gulyae* fimbria pathogenicity, based on findings showing that use of the wild-type strain resulted in a markedly reduced silkworm larvae survival rate, whereas the fimbria-deficient mutant did not show such effects (Figure 6). Previous studies have noted that fimbriae contribute to the pathogenicity of various bacteria, such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Vibrio cholerae*, *Salmonella typhi*, and *E. coli* [1, 13]. Therefore, *P. gulyae* fimbriae may have potential for involvement in the pathogenesis of periodontal disease.

6 | Conclusion

Results obtained with a silkworm model demonstrate that *P. gulyae* fimbriae have critical roles in multiple aspects of the pathogenesis of periodontal disease, including bacterial adhesion and invasion, inhibition of epithelial cell migration, induction of inflammatory responses, biofilm tenacity, and fimbrial pathogenicity. Additionally, they indicate that fimbria function in concert with other virulence factors, such as gingipains, to enhance periodontal disease onset and development. Namely, because of their impairment of epithelial wound healing, stimulation of inflammation, and enhancement of biofilm stability, *P. gulyae* fimbriae are critical contributors to the chronicity and severity of periodontal disease. These findings not only expand understanding regarding the pathogenic functions of *P. gulyae* but also highlight fimbriae as potential therapeutic intervention targets for the prevention and management of periodontitis.

Author Contributions

Hiroaki Inaba: conceptualization, funding acquisition, formal analysis, data curation, investigation, resources, validation, methodology, visualization, writing—review and editing, and writing original draft. Sho Yoshida: funding acquisition, formal analysis, data curation, investigation, validation, and visualization. Naoya Ohara: formal analysis,

data curation, investigation, resources, validation, methodology, visualization, writing—review and editing, and writing original draft. Ryota Nomura: conceptualization, resources, methodology, and writing—review and editing. Kazuhiko Nakano: conceptualization, resources, and writing—review and editing. Michiyo Matsumoto-Nakano: conceptualization, funding acquisition, data curation, methodology, writing—review and editing.

Funding

This study was funded by the Ministry of Education, Culture, Sports, Science and Technology of Japan (23K09137 and 23H03115).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

References

1. D. G. Thanassi, J. B. Bliska, and P. J. Christie, "Surface Organelles Assembled by Secretion Systems of Gram-Negative Bacteria: Diversity in Structure and Function," *FEMS Microbiology Reviews* 36, no. 6 (2012): 1046–1082, <https://doi.org/10.1111/j.1574-6976.2012.00342.x>.
2. T. Shanmugasundarasamy, D. Karaiyagowder Govindarajan, and K. Kandaswamy, "A Review on Pilus Assembly Mechanisms in Gram-Positive and Gram-Negative Bacteria," *Cell Surface* 8, no. 8 (2022): 100077, <https://doi.org/10.1016/j.tcsu.2022.100077>.
3. J. L. Telford, M. A. Barocchi, I. Margarit, R. Rappuoli, and G. Grandi, "Pili in Gram-Positive Pathogens," *Nature Reviews Microbiology* 4, no. 7 (2006): 509–519, <https://doi.org/10.1038/nrmicro1443>.
4. T. Proft, and E. N. Baker, "Pili in Gram-Negative and Gram-Positive Bacteria - Structure, Assembly and Their Role in Disease," *Cellular and Molecular Life Sciences* 66, no. 4 (2009): 613–635, <https://doi.org/10.1007/s00018-008-8477-4>.
5. K. A. Kline, K. W. Dodson, M. G. Caparon, and S. J. Hultgren, "A Tale of Two Pili: Assembly and Function of Pili in Bacteria," *Trends in Microbiology* 18, no. 5 (2010): 224–232, <https://doi.org/10.1016/j.tim.2010.03.002>.
6. J. Hacker, G. Blum-Oehler, I. Mühldorfer, and H. Tschäpe, "Pathogenicity Islands of Virulent Bacteria: Structure, Function and Impact on Microbial Evolution," *Molecular Microbiology* 23, no. 6 (1997): 1089–1097, <https://doi.org/10.1046/j.1365-2958.1997.3101672.x>.
7. D. Fournier, C. Mouton, P. Lapierre, T. Kato, K. Okuda, and C. Ménard, "*Porphyromonas gulae* sp. nov., an Anaerobic, Gram-Negative Coccobacillus From the Gingival Sulcus of Various Animal Hosts," *International Journal of Systematic and Evolutionary Microbiology* 51, no. 3 (2001): 1179–1189, <https://doi.org/10.1099/00207713-51-3-1179>.
8. Y. Yamasaki, R. Nomura, K. Nakano, et al., "Distribution and Molecular Characterization of *Porphyromonas gulae* Carrying a New fimA Genotype," *Veterinary Microbiology* 161, no. 1–2 (2012): 196–205, <https://doi.org/10.1016/j.vetmic.2012.07.026>.
9. E. Gaetti-Jardim, M. F. Pereira, E. M. Vieira, C. M. Schweitzer, A. C. Okamoto, and M. J. Ávila-Campos, "Occurrence of Periodontal Pathogens in Ethnic Groups From a Native Brazilian Reservation," *Archives of Oral Biology* 60, no. 6 (2015): 959–965, <https://doi.org/10.1016/j.archoralbio.2015.01.002>.
10. H. Inaba, R. Nomura, Y. Kato, et al., "Adhesion and Invasion of Gingival Epithelial Cells by *Porphyromonas gulae*," *PLoS One* 14, no. 3 (2019): e0213309, <https://doi.org/10.1371/journal.pone.0213309>.
11. S. Yoshida, H. Inaba, R. Nomura, et al., "Efficacy of FimA Antibody and Clindamycin in Silkworm Larvae Stimulated With *Porphyromonas gulae*," *Journal of Oral Microbiology* 13, no. 1 (2021): 1914499, <https://doi.org/10.1080/20002297.2021.1914499>.
12. S. Ono, M. Nakayama, M. Tachibana, et al., "Construction and Characterization of a PGN_0297 Mutant of *Porphyromonas gingivalis*: Evidence of the Contribution of PGN_0297 to Gingipain Activity," *Acta Medica Okayama* 73, no. 4 (2019): 315–323, <https://doi.org/10.18926/AMO/56933>.
13. S. Yoshida, H. Inaba, R. Nomura, K. Nakano, and M. Matsumoto-Nakano, "Role of Fimbriae Variations in *Porphyromonas gulae* Biofilm Formation," *Journal of Oral Biosciences* 66, no. 4 (2024): 28–33, <https://doi.org/10.1016/j.job.2024.08.003>.
14. K. Imamura, E. Kokubu, D. Kita, et al., "Role of Mitogen-Activated Protein Kinase Pathways in Migration of Gingival Epithelial Cells in Response to Stimulation by Cigarette Smoke Condensate and Infection by *Porphyromonas gingivalis*," *Journal of Periodontal Research* 51, no. 5 (2016): 613–621, <https://doi.org/10.1111/jpre.12341>.
15. J. C. Rincon, H. R. Haase, and P. M. Bartold, "Effect of Emdogain on Human Periodontal Fibroblasts in an In Vitro Wound-Healing Model," *Journal of Periodontal Research* 38, no. 3 (2003): 290–295, <https://doi.org/10.1034/j.1600-0765.2003.00610>.
16. H. Inaba, S. Yoshida, and R. Nomura, "*Porphyromonas gulae* Lipopolysaccharide Elicits Inflammatory Responses Through Toll-Like Receptor 2 and 4 in Human Gingival Epithelial Cells," *Cellular Microbiology* 22, no. 12 (2020): e13254, <https://doi.org/10.1111/cmi.13254>.
17. R. J. Lamont, and M. Kuboniwa, "The Polymicrobial Pathogenicity of *Porphyromonas gingivalis*," *Frontiers in Oral Health* 5 (2024): 1404917, <https://doi.org/10.3389/froh.2024.1404917>.
18. J. A. Garnett, V. I. Martínez-Santos, Z. Saldaña, et al., "Structural Insights Into the Biogenesis and Biofilm Formation by the *Escherichia coli* Common Pilus," *Proceedings of the National Academy of Sciences of the United States of America* 109, no. 10 (2012): 3950–3955, <https://doi.org/10.1073/pnas.1106733109>.
19. M. D. Alcántar-Curiel, D. Blackburn, Z. Saldaña, et al., "Multi-Functional Analysis of *Klebsiella pneumoniae* Fimbrial Types in Adherence and Biofilm Formation," *Virulence* 4, no. 2 (2013): 129–138, <https://doi.org/10.4161/viru.22974>.
20. U. Okaro, S. Mou, G. Lenkoue, et al., "A Type IVB Pilin Influences Twitching Motility and In Vitro Adhesion to Epithelial Cells in *Burkholderia pseudomallei*," *Microbiology* 168, no. 3 (2022): 001150, <https://doi.org/10.1099/mic.0.001150>.
21. A. Weinberg, C. M. Belton, Y. Park, and R. J. Lamont, "Role of Fimbriae in *Porphyromonas gingivalis* Invasion of Gingival Epithelial Cells," *Infection and Immunity* 65, no. 1 (1997): 313–316, <https://doi.org/10.1128/iai.65.1.313-316.1997>.
22. E. Hintermann, S. K. Haake, U. Christen, A. Sharabi, and V. Quaranta, "Discrete Proteolysis of Focal Contact and Adherence Junction Components in *Porphyromonas gingivalis*-Infected Oral Keratinocytes: A Strategy for Cell Adhesion and Migration Disabling," *Infection and Immunity* 70, no. 10 (2002): 5846–5856, <https://doi.org/10.1128/IAI.70.10.5846-5856.2002>.
23. Y. Matsumoto, and K. Sekimizu, "Silkworm as an Experimental Animal for Research on Fungal Infections," *Microbiology and Immunology* 63, no. 2 (2019): 41–50, <https://doi.org/10.1111/1348-0421.12668>.
24. B. L. Pihlstrom, B. S. Michalowicz, and N. W. Johnson, "Periodontal Diseases," *Lancet* 366, no. 9499 (2005): 1809–1820, [https://doi.org/10.1016/S0140-6736\(05\)67728-8](https://doi.org/10.1016/S0140-6736(05)67728-8).
25. D. F. Kinane, P. G. Stathopoulou, and P. N. Papapanou, "Periodontal Diseases," *Nature Reviews Disease Primers* 3, no. 1 (2017): 17038, <https://doi.org/10.1038/nrdp.2017.38>.

26. S. G. Grossi, J. J. Zambon, A. W. Ho, et al., "Assessment of Risk for Periodontal Disease. I. Risk Indicators for Attachment Loss," *Journal of Periodontology* 65, no. 3 (1994): 260–267, <https://doi.org/10.1902/jop.1994.65.3.260>.
27. J. Meyle, and I. Chapple, "Molecular Aspects of the Pathogenesis of Periodontitis," *Periodontology* 2000 69, no. 1 (2015): 7–17, <https://doi.org/10.1111/prd.12104>.
28. A. Casadevall, and L. A. Pirofski, "Virulence Factors and Their Mechanisms of Action: The View From a Damage-Response Framework," *Journal of Water and Health* 1 (2009): S2–S18, <https://doi.org/10.2166/wh.2009.036>.
29. A. S. Urmi, H. Inaba, R. Nomura, et al., "Roles of *Porphyromonas gulae* Proteases in Bacterial and Host Cell Biology," *Cellular Microbiology* 23, no. 8 (2021): e13312, <https://doi.org/10.1111/cmi.13312>.
30. M. Enersen, K. Nakano, and A. Amano, "*Porphyromonas gingivalis* Fimbriae," *Journal of Oral Microbiology* 5, no. 1 (2013): 20265, <https://doi.org/10.3402/jom.v5i0.20265>.
31. V. Cepas, and S. M. Soto, "Relationship between Virulence and Resistance among Gram-Negative Bacteria," *Antibiotics* 9, no. 10 (2020): 719, <https://doi.org/10.3390/antibiotics9100719>.
32. J. Zhu, T. Wang, L. Chen, and H. Du, "Virulence Factors in Hypervirulent *Klebsiella pneumoniae*," *Frontiers in Microbiology* 12 (2021): 642484, <https://doi.org/10.3389/fmicb.2021.642484>.
33. A. Amano, I. Nakagawa, K. Kataoka, I. Morisaki, and S. Hamada, "Distribution of *Porphyromonas gingivalis* Strains With *fimA* Genotypes in Periodontitis Patients," *Journal of Clinical Microbiology* 37, no. 5 (1999): 1426–1430, <https://doi.org/10.1128/JCM.37.5.1426-1430.1999>.
34. C. Politis, J. Schoenaers, R. Jacobs, and J. O. Agbaje, "Wound Healing Problems in the Mouth," *Frontiers in Physiology* 7 (2016): 507, <https://doi.org/10.3389/fphys.2016>.
35. I. Aukhil, "Biology of Wound Healing," *Periodontology* 22, no. 1 (2000): 44–50, <https://doi.org/10.1034/j.1600-0757.2000.2220104.x>.
36. I. Nakagawa, H. Inaba, T. Yamamura, et al., "Invasion of Epithelial Cells and Proteolysis of Cellular Focal Adhesion Components by Distinct Types of *Porphyromonas gingivalis* Fimbriae," *Infection and Immunity* 74, no. 7 (2006): 3773–3782, <https://doi.org/10.1128/IAI.01902-05>.
37. S. Seetharaman, and S. Etienne-Manneville, "Cytoskeletal Crosstalk in Cell Migration," *Trends in Cell Biology* 30, no. 9 (2020): 720–735, <https://doi.org/10.1016/j.tcb.2020.06.004>.
38. S. Panthee, A. Paudel, H. Hamamoto, and K. Sekimizu, "Advantages of the Silkworm as an Animal Model for Developing Novel Antimicrobial Agents," *Frontiers in Microbiology* 7, no. 8 (2017): 373, <https://doi.org/10.3389/fmicb.2017.00373>.
39. V. Sharma, I. von Ossowski, and Krishnan, "Exploiting Pilus-Mediated Bacteria-Host Interactions for Health Benefits," *Molecular Aspects of Medicine* 81 (2021): 100998, <https://doi.org/10.1016/j.mam.2021.100998>.
40. M. Kuboniwa, A. Amano, E. Hashino, et al., "Distinct Roles of Long/Short Fimbriae and Gingipains in Homotypic Biofilm Development by *Porphyromonas gingivalis*," *BMC Microbiology* 9, no. 1 (2009): 105, <https://doi.org/10.1186/1471-2180-9-105>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Supporting Information 1.** Figure S1: Construction of *fimA* deletion mutants. PCR amplification of the *fimA* region from *P. gulae* D049 chromosomal DNA was performed using primers *FimA_F* and *FimA_R*. The amplified fragment was sub-cloned into the pANT vector (TA-Enhancer Cloning Kit, Nippon Gene, Toyama, Japan) to generate pANT-*FimA*, and its sequence was verified. The *ermF* region was then amplified from pKD355 [1] using primers *ErmF_F* and *ErmF_R*. Next, the PCR fragment of the *ermF* region was digested with *NcoI* and inserted into the *NcoI* site of *fimA* in pANT-*FimA*, with the resultant plasmid containing the *ermF* ORF in the same direction as the *fimA* ORF, subsequently selected. Inverse PCR was performed using primers *ErmF_inv* and *FimA_inv* to remove the sequence from the start codon of *fimA* to that of *ermF* to drive *ermF* by the *fimA* promoter, resulting in pANT- Δ *FimA*. The pANT- Δ *FimA* plasmid was linearized by *SmaI* digestion and introduced into *P. gulae* D049 cells by electroporation, resulting in a Δ *fimA* mutant. Correct gene replacement that occurred through double crossover recombination events was verified by PCR analysis (data not shown). Figure S2: Construction of *fimA*-complemented strains. To perform *fimA* complementation, *fimA* was inserted into the *ermF* ORF in the genome of Δ *FimA*. To create unique *XhoI* and *SacI* sites within the *ermF* ORF of pANT- Δ *FimA*, inverse PCR was performed using primers *ErmF_Xh* and *ErmF_Sa*, with the resultant plasmid designated pANT- Δ *FimA* SX. The promoter, containing the *fimA* region ORF, and terminator of *fimA* were PCR amplified using *FimA_EX_F* and *FimA_EX_R* primers. The PCR fragment was digested with *XhoI* and *BamHI*, then ligated into the corresponding sites of pBluescript II SK(-) to generate pBSSK-*FimA*. Next, pTIO-1T [2] was digested with *XbaI* and *SacI*, and the fragment containing the *tetQ* cassette was inserted into the *XbaI/SacI* sites of pBSSK-*FimA*, with the resultant plasmid designated as pBSSK-*FimA* tet. An *XhoI-SacI* fragment from pBSSK-*FimA* tet was then inserted into the corresponding sites of pANT- Δ *FimA* SX to obtain Δ *FimA* SXtet. Finally, the pANT- Δ *FimA* SXtet plasmid was linearized by *SmaI* digestion and introduced into Δ *FimA* by electroporation, resulting in a *fimA*-complemented strain. **Supporting Information 2.** Supporting information table.