1 *Porphyromonas gulae* proteases influence not only bacterial growth,
2 coaggregation, and hemagglutination but also the maintenance of human protein
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Abstract

Porphyromonas gulae, previously known as the animal biotype of the human periodontal pathogen *P. gingivalis*, is Gram-negative, anaerobic, rod-shaped, asaccharolytic, black-pigmented, non-motile, non-spore-forming, and non-motile. *P. gulae* organisms have been isolated from the gingival sulcus of various animal species, including bear, brushtail, possum, dog, cat, coyote, kangaroo, monkey, ovine, wallaby, and wolf. Interestingly, it has also detected in human gingival tissues from healthy and diseased sites. Although recent studies have reported that *P. gulae* possesses a wide variety of virulence factors, such as including fimbriae, LPS, and proteases, the proteases have yet to be well clarified in details. The present study aimed to clarify the characters of the proteases from varieties of *P. gulae* strains available at this time. Based on biochemical and functional characters similar to the factors from *P. gingivalis*, enzyme roles, hemagglutination, and degradation of host proteins were clarified in this study.

***P. gulae* strains possess trypsin protease-like activity:** *P. gulae* exhibits several virulence characteristics similar to those of the human periodontal pathogen *P. gingivalis*. However, the proteolytic enzyme activities of *P. gulae* strains have not been fully elucidated. All of the examined *P. gulae* strains as well as the *P. gingivalis* ATCC 33277 strain consistently produced alkaline phosphatase and showed trypsin activity, while no other enzyme activities were detected in any of the strains tested. Moreover, protease activity was found in both cell extracts and supernatants, with negligible differences among the examined strains. Protease inhibitors, including antipain (cysteine protease inhibitor), phenylmethylsulfonyl fluoride (PMSF; serine proteinase inhibitor), tosyllysine chloromethyl ketone (TLCK; serine endopeptidase specific inhibitor) and leupeptin (serine protease inhibitor as bacterial metabolite, a cathepsin B inhibitor, a calpain inhibitor and a trypsin inhibitor), diminished *P. gulae* proteolytic activity up to 50%.

Hemagglutination activity: *P. gulae* and *P. gingivalis* reportedly possess protease-related and hemagglutinin genes. However, the hemagglutination ability of *P. gulae* has yet to be investigated. The present findings showed distinct hemagglutination activity in *P. gulae* ATCC 51700, and found that protease inhibitors, such as antipain, PMSF, TLCK, and leupeptin, failed to cause agglutination of mouse erythrocytes. These results suggest that *P. gulae* proteases may contribute to the hemagglutination.

***P. gulae* growth:** Previous reports noted that *P. gingivalis* growth mediated by gingipains was reported to increase in chemically defined medium (CDM). After inoculation in CDM for the present assays, *P. gulae* ATCC 51700 was found to be in clearly in the stationary phase from 144 h. Furthermore, antipain, PMSF, TLCK, and leupeptin inhibited the growth of *P. gulae* ATCC 51700, suggesting that *P. gulae* proteases may be essential for bacterial growth.

Coaggregation reaction of *P. gulae* with *A. viscosus*: Coaggregation of *A. viscosus* with *P. gingivalis* has been previously reported. *P. gulae* ATCC 51700 was found to coaggregate with *A. viscosus* ATCC 15987, while inhibition of *P. gulae* proteases using protease inhibitors significantly abrogated that activity of the bacterium. These data suggest that coaggregation

reactions between *P. gulae* ATCC 51700 and *A. viscosus* ATCC 15987 are regulated via the activity of *P. gulae* proteases.

Morphological changes and inhibition of proliferation of human cells by *P. gulae*: *P. gulae* ATCC 51700 caused rounding and detachment of human gingival carcinoma Ca9-22. The nature of morphological changes is reportedly linked to the proliferation rate of host cells infected with microorganisms. *P. gulae* ATCC 51700 inhibited proliferation of Ca9-22 cells in both multiplicity of infection (MOI) at 500. Pretreatments with antipain, PMSF, TLCK, and leupeptin prevented inhibition of Ca9-22 proliferation by *P. gulae* ATCC 51700, suggesting that *P. gulae* proteases cause morphological changes in Ca9-22 cells, leading to inhibition of their proliferation.

Degradation of human proteins by *P. gulae*: Previous studies have reported that focal contact and adherence junction components, including E-cadherin, β-catenin, focal adhesion kinase (FAK), and paxillin, were associated with epithelial morphology. Following *P. gulae* ATCC 51700 infection at an MOI of 500, E-cadherin, β-catenin, FAK and paxillin also shown to be cleaved at 6 hours. To further evaluate the role of *P. gulae* proteases, *P. gulae* ATCC 51700 was preincubated with several protease inhibitors prior to bacterial infection. Cleavage of focal contact and adherence proteins by *P. gulae* ATCC 51700 was diminished by antipain, PMSF, TLCK, and leupeptin. Furthermore, degradation of recombinant human proteins, γ-globulin and fibrinogen, was observed within 6 hours. Antipain, TLCK, and leupeptin were prevented the cleavage of γ-globulin and fibrinogen, while PMSF did not.

Conclusion: *P. gulae* proteases would be a crucial virulence factors factor for bacterial colonization, such as hemagglutination and coaggregation, and bacterial growth, as well as host defense and cell contact and adherence destruction.

1. Introduction

Porphyromonas gulae, a Gram-negative asaccharolytic, anaerobic, non-motile, non-spore-forming organism, has been described as an animal biotype of a human pathogen that is responsible for human periodontitis [1]. This bacterium has been isolated from the gingival sulcus of several different species, such as cat, dog, monkey, ovine, and marsupial, both with and without a relationship to periodontitis [1-4]. Previously reported that *P. gulae* strains have been shown to possess various virulence factors, including lipopolysaccharide, β-lactamase, alkaline phosphatase, phospholipase, toxins, and capsules, for manipulation of host cells [5-7], as those enable adhesion and invasion of host cells, acquisition of nutrients, and formation of biofilm, and also cause damage to host tissue and regulate coordination with other virulence factors [6]. Based on their functions, the virulence factors can be grouped into those that are requisite fashion or contributory fashion. Some requisite virulence factors, including toxin and polysaccharide, confer pathogenicity and ability to cause disease. In contrast, contributory virulence factors, such as proteases and phospholipases of *Candida albicans*, modify the magnitude of infection and contribute pathogenesis [5].

Proteases comprise a large group of enzymes that hydrolyze peptide bonds. Catalytic quite proteolytic enzymes have been classified as aspartyl protease, cysteine protease, serine protease, metalloprotease, glutamic acid-specific protease, and threonine peptidase, and asparagine peptide lyase [8, 9]. Recent evidence suggested that bacterial proteases are involved in acquiring nutrients for growth, proliferation through host tissue degradation, and evasion of host immune defenses [8], while other studies have shown that bacterial proteases cause the onset of infectious diseases, including cholera, salmonellosis, Legionnaires' disease, bronchiectasis, cystic fibrosis, botulism, tetanus, and anthrax [8, 10]. Serine and cysteine proteases are considered to be crucial pathogenic factors for periodontal pathogens, including *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, and *Fusobacterium nucleatum* [9, 11]. Additionally, several *Porphyromonas* species isolated from animals reportedly have been possess arginine- and lysine-specific proteases [12]. Bacterial proteases, such as serine and cysteine protease, are involved in degradation of periodontal tissues and

inactivation of host defense mechanisms, making the host susceptible to pathological onset of periodontal disease [13), 14)].

The functions of *P. gulae* proteases in regard to bacterial and host cell biology are not fully understood. Here, I report findings showing that *P. gulae* proteases play a vital role in hemagglutination, coaggregation activity with *Actinomyces viscosus*, and bacterial growth, as well as degradation of focal adhesion kinase (FAK), E-cadherin, and paxillin in human gingival epithelial cells, leading to cell proliferation inhibition and morphological changes. Additionally, proteolysis by *P. gulae* proteases were found to contribute to degradation of γ-globulin and fibrinogen.

Materials and Methods

Bacterial strains and culture conditions

P. gulae ATCC 51700 and *P. gingivalis* ATCC 33277 were obtained from the American Type Culture Collection. *P. gulae* D040, D044, D049, D066, and D077, clinical isolates obtained from the periodontal pockets of dogs, were kindly provided by Prof. Fumitoshi Asai and Dr. Yukio Kato of Azabu University, and *A. viscosus* ATCC 15987 was kindly provided by Prof. Naoya Ohara of Okayama University. Bacteria were grown anaerobically in Trypticase soya broth supplemented with 1 mg/ml yeast extract (Becton, Dickinson and Company, Sparks, MD, USA), 1 μg/ml menadione (Sigma-Aldrich, St. Louis, MO, USA), and 5 μg/ml hemin (Sigma-Aldrich) at 37°C, as previously described [15]. *P. gulae* and *P. gingivalis* were grown in anaerobic jars (AnaeroPack; Mitsubishi Gas Chemical Co., Tokyo), and maintained at 37°C on blood agar plates, while *A. viscosus* was maintained in Trypticase soy agar.

Human cells, strain and culture conditions

Ca9-22 cells (derived from human gingival carcinoma) were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan). Ca9-22 is an established transformed human gingival cell line that has been used previous studies as a culture model of oral epithelial cells [17), 18)]. Ca9-22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

API-ZYM

An API-ZYM test was applied to *P. gingivalis* ATCC 33277 and *P. gulae* ATCC 51700, D040, D044, D049, D066, D077 cells, and their supernatants. *P. gulae* and *P. gingivalis* strains with a pre-culture density of OD_{600} nm =1.0 were grown overnight, then subsequently cultured for 24h in fresh medium. Cultures were collected, washed, and resuspended in PBS, and adjusted to OD_{600} nm using a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). Culture supernatants were obtained by centrifugation at 15,000 rpm for 10 min at 4°C, then filtered through 0.22 μm polyethersulfone filters (Sartorius minisart, Gottingen, Germany).

Protein contents in the supernatants were measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Cell suspensions and supernatants were added to each of the cupules in an API-ZYM strip (API bioMèrieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Briefly, a bacterial sample ($5x10^7$ cells) or supernatant (protein content 10 μg) was added to each of the cupules. Each panel containing 20 cupules was incubated within its moist chamber, to which distilled water had been added, at 37°C for 4 h. After incubation, 1 drop of reagent A and 1 drop of reagent B reagent were added to each cupule. Color was allowed to develop for 5 min, after which the cupules were exposed to a high intensity light source for 10 s. Evaluation of the activity was done according to the intensity of coloration using a 5-grade scale, with 20 enzyme activities determined. The nearness and degree of enzymatic activity were based on a comparison with the color intensity chart provided by the manufacturer.

Reagents

Antipain, a cysteine and serine protease inhibitor, N^{α}-p-tosyl-$_L$-Lysine chloromethyl ketone (TLCK), a serine protease inhibitor, and phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, were purchased from Sigma-Aldrich, while leupeptin, a cysteine and serine protease inhibitor, was obtained from Peptide Institute (Osaka, Japan). All inhibitors were dissolved in 0.1% dimethyl sulfoxide (DMSO) and stored at -20°C. Samples were preincubated with different concentrations (100 μM) of the inhibitors at 37°C for 2 hours before addition of the substrates.

Protease activity assay

The protease activities of *P. gulae* ATCC 51700 cells and supernatants were determined using a Pierce Protease assay kit (Thermo Scientific), according to the manufacturer's instructions. Briefly, samples ($5x10^7$ cells or 10 μg culture supernatants) were mixed with 100 μl of casein solution. A blank was prepared containing buffer and protease sample without the succinylated casein solution. After incubation for 20 min at room temperature (RT), 50 μl of trinitrobenzenesulfonic acid (TNBSA) was added to each well and incubated for a further 20

min at RT. A standard curve was constructed based on serial dilutions of a 0.5 mg/ml trypsin stock solution. Protease activity was measured on SH-1000 Lab microplate reader (Corona Electric, Ibaraki, Japan) at 450 nm.

Microtiter plate assay of hemagglutination

Hemagglutination assays were performed as previously described [18], with some modifications. *P. gulae* ATCC 51700 cells with a pre-culture density of OD_{600} nm = 1.0 were grown overnight, then cultured for an additional 24 h in fresh medium. Cultures were collected, washed, and resuspended in PBS, and adjusted to OD_{600} nm using a SmartSpec Plus spectrophotometer (Bio-Rad). The bacterial suspensions (5×10^7 cells) were then diluted as a two-fold series with PBS (1:1-11256). Each diluted suspension (100 µl) was mixed with an equal volume of mouse erythrocytes (2.5% in PBS) in a round-bottom 96-well polystyrene microtiter plate and then incubated at RT for 3 h. The hemagglutination titer was assessed visually and the last dilution exhibiting full agglutination of erythrocytes was recorded.

Bacterial growth assay

Assays of *P. gulae* growth were performed in chemically defined medium (CDM) using methods, as previously described [19], with some modifications. The medium contained 10 mM NaH_2PO_4, 10 mM KCl, 2 mM citric acid, 5 µM boric acid, 20 µM $CaCl_2$, 1.25 mM $MgCl_2$, 3% pancreatic hydrolysate of casein (tryptone), 50 mM α-ketoglutarate, 7.5 µM hemin, and 3 µM menadione. *P. gulae* ATCC 51700 cells were grown overnight at a pre-culture density of OD_{600} nm = 1.0, then the bacterial suspension was diluted 1:5 with fresh CDM and incubation was continued anaerobically at 37ºC, with bacterial growth monitored at 600 nm every 24 h for up to 192 h using a Novaspec III spectrophotometer (Biochrom, Holliston, MA, USA).

Coaggregation assay

Coaggregation of *P. gulae* ATCC 51700 and *A. viscosus* ATCC 15987 was examined as previously described [20]. *P. gulae* and *P. gingivalis* strains were grown overnight at a pre-culture density of OD_{600} nm =1.0, then cultured for an additional 24 h in fresh medium. Cultures were collected and resuspended in coaggregation buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$,

0.1 mM $MgCl_2$, 0.02% NaN_3, 0.15 M NaCl). Equal volumes (500 μl) of both bacterial suspensions were mixed and then incubated at RT for 48 h. Coaggregation was measured at 550 nm using a Novaspec Plus spectrophotometer (Amersham Biosciences, Little Chalfont, UK).

Determination of cell viability

To assess the cytotoxicity of the *P. gulae* proteases and protease inhibitors, the viability of Ca9-22 cells was determined with a cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Ca9-22 cells ($4x10^4$ cells) were seeded into 96 well culture plates and grown overnight. Next, the cells were cultured with DMEM containing protease inhibitors (100 μM) and stimulated with viable *P. gulae* ATCC 51700 cells at a multiplicity of infection (MOI) of 100, 200, and 500. After incubation for 6 h, cell viability was determined and absorbance at 490 nm was recorded with an SH-1000 Lab microplate reader (Corona Electric). The cytotoxicity of *P. gulae* was calculated as the relative decrease in luminescence in comparison to the control.

Western blotting

Ca9-22 cells (1 x 10^6 cells) were seeded into six well culture plates and grown overnight, then incubated with *P. gulae* at 500 MOI for 0, 30, 60, 120, and 360 min. Ca9-22 cells stimulated with *P. gulae* ATCC 51700 cells were solubilized in cell lysis/extraction reagent (Sigma-Aldrich) containing a protease inhibitor cocktail (Thermo Scientific). The soluble fraction was collected by centrifugation at 15,000 rpm for 5 min at 4°C. Immunoblotting was performed as described previously [15]. Briefly, the cell extract (10 μg of protein) was denatured in sodium dodecyl sulfate (SDS) sample buffer and loaded onto an 8% polyacrylamide gel for electrophoresis (SDS-PAGE) (150 V 80 mA) for 90 min. Electrophoretic transfer was carried out at 100 constant current overnight on ice. After transfer, the membranes were blocked with 5% BSA in Tris-buffer saline containing 0.1% Tween 20 at RT for 1 h. Blots were probed with primary antibodies purchased from Cell Signaling Technology, including β-catenin (1:1000), E-cadherin (1:1000), FAK (1:1000), and paxillin (1:1000), overnight at 4°C in a shaker. After reacting with

primary antibodies, the membranes were washed three times with TBS-T at RT for 10 min, then stripped and probed with anti-b-actin antibody (Cell Signaling Technology) as a loading control. Proteins were detected using ECL substrate (GE Healthcare, Amersham, UK)

Host protein cleavage assay

Human recombinant γ-globulin and fibrinogen were purchased from Sigma-Aldrich and recombinant human fibronectin from Fujifilm Wako. *P. gulae* cells were cultured overnight at a pre-culture density of OD_{600} nm = 1.0, then grown for 24 h in fresh medium. The cultures were collected, washed, and resuspended in PBS, then adjusted to OD_{600} nm using a SmartSpec Plus spectrophotometer (Bio-Rad). Recombinant proteins (5 µg) were incubated with the bacterial suspensions (5 x 10^7 cells) at 37°C for 6 hours in the presence or absence of the protease inhibitors (100 µM). Following incubation, *P. gulae* cells were removed by centrifugation at 15,000 rpm and 4°C, and the supernatants were collected. Protein samples were then applied to SDS-PAGE in 8% and 10% polyacrylamide gels, and electroblotted onto a PVDF membrane using a wet blotting system (Bio-Rad, Hercules, CA, USA). Electroblotting was carried out at a 100 mA constant current overnight on ice. PVDF membranes were placed in Coomassie stain (0.5% Coomassie brilliant blue R-250, 50% methanol, 10% acetic acid) for 10 min and then rapidly destained with 90% methanol with several changes, then washed with and stored in redistilled water at 4°C overnight.

Statistical analyses

All values are expressed as the mean ± standard deviation. Statistical analyses were performed using an unpaired Student's *t* test. *P* values <0.05 were considered to indicate significance.

Results

P. gulae strains possess alkaline phosphatase and trypsin protease-like activity

Animal-derived *Porphyromonas* species have been shown to possess arginine- and lysine-specific proteases [12), 21), 22)], while the functions of *P. gulae* proteases have yet to be characterized. I first examined characteristic features of the present *P. gulae* strains, and identified alkaline phosphatase and trypsin activities in each, as well as in *P. gingivalis* (Table 1). Also, trypsin activity in living cells and supernatants were determined. As shown in Figure 1, bacterial protease activity was not significantly different among the *P. gulae* strains or their supernatants. Previous studies reported that bacterial proteolytic enzymes have been blocked by protease inhibitors, including antipain, PMSF, TLCK, and leupeptin [23), 24)]. Thus, I next examined the effects of serine and cysteine protease inhibitors on *P. gulae* protease activities. All serine and cysteine protease inhibitors used in this study showed significant inhibitory effects toward the proteolytic activities of *P. gulae* (Figure 2). These findings suggested that serine and cysteine proteases in all *P. gulae* strains possess such activities.

Determination of hemagglutination activity

Hemagglutination is a known attribute of some bacterial species [25)]. However, protease-related and hemagglutinin genes are found only in *P. gingivalis* and not in any other *Porphyromonas* species [26)], with hemagglutination of *P. gulae* remaining to be clarified. In the present study, *P. gulae* ATCC 51700 showed hemagglutination activity (Figure 3A). Hemagglutinin activity has been reported to be related to the hemagglutinin-adhesin domains of protease-related genes [13)] and also that *P. gingivalis* gingipain protease-specific inhibitors inhibit that activity [20)]. Thus, I examined the effects of serine and cysteine protease inhibitors on *P. gulae* hemagglutinating activity, though none of the protease inhibitors showed inhibition (Figure 3B). These results indicate that *P. gulae* proteases contribute to hemagglutination.

P. gulae growth in chemically defined medium

Bacterial growth has been reported to require proteases, such as *P. gingivalis* gingipains, *Burkholderia cenocepacia* HtrA, Enteroaggregative *Escherichia coli* Pic, and *F. nucleatum* fusolisin shown to be essential for that [14), 27), 28)]. In addition, the role of bacterial proteases in

regard to *P. gingivalis* growth has been clarified using CDM [19]. Therefore, I examined the role of bacterial proteases related to *P. gulae* growth. Between 24 and 144 hours, *P. gulae* cells were in the exponential phase and clearly in the stationary phase by 144 hours following inoculation in CDM (Figure 4A). Subsequently, the growth of *P. gulae* ATCC 51700 was significantly inhibited by the protease inhibitors, such as antipain, PMSF, TLCK, and leupeptin (Figure 4B). These results showed that *P. gulae* proteases contribute to bacterial growth.

Assay of *P. gulae* and *A. viscosus* coaggregation

Coaggregation reportedly promotes interactions between different bacterial species [29]. Furthermore, bacterial proteases are known to contribute to coaggregation of oral bacteria, including *P. gingivalis*, *T. denticola*, and *Streptococcus gordonii* [20), 29), 30)]. Previous reports have also noted that *A. viscosus* strains coaggregate with streptococci, *Eikenella corrodens*, and *P. gingivalis* [20), 31), 32)]. Thus, the effect of bacterial proteases on coaggregation between *P. gulae* and *A. viscosus* was examined. *P. gulae* ATCC 51700 showed coaggregation with *A. viscosus* ATCC 15987 in a time-dependent manner (Figure 5A), while antipain, PMSF, TLCK, and leupeptin had significant inhibitory effects on that coaggregation (Figure 5B). These findings suggest that bacterial proteases mediate *P. gulae* coaggregation activity.

Morphology and proliferation of Ca9-22 cells inhibited by *P. gulae*

A prior study demonstrated that bacterial proteases have various effects on human cells, including rounding, detachment, and inhibition of proliferation [33]. First, the response of gingival epithelial cells (referred to here as Ca9-22 cells) stimulated with *P. gulae* was examined by microscopy, which revealed morphological changes of Ca9-22 (Figure 6A), which prompted me to examine the proliferation of stimulated Ca9-22 cells. *P. gulae* inhibited cell proliferation in MOI-dependent manner (Figure 6B). Additionally, cellular proliferation damage of Ca9-22 cells induced by *P. gulae* was prevented by antipain, PMSF, TLCK, and leupeptin (Figure 6C). These results indicate that damage caused by proliferation and morphological changes are induced by *P. gulae* proteases.

Degradation of human proteins by *P. gulae*

Focal contact and adherence junction components, including E-cadherin, β-catenin, focal

adhesion kinase (FAK), and paxillin, are required for epithelial tissue architecture integrity [34)-36)]. Several bacterial proteases, such as enteroaggregative *Escherichia coli* Pet, group *A Streptococci* SpeB, *P. gingivalis* gingipains, and *Campylobacter jejuni* HtrA, have been shown to have proteolytic activity, leading to distraction of focal contact and adherence junction components [37)-40)]. I examined the effects of *P. gulae* proteases on protein degradation. Following *P. gulae* stimulation, degradation of E-cadherin, β-catenin, FAK, and paxillin was observed (Figure 7A). Subsequently, antipain, PMSF, TLCK, and leupeptin prevented protein degradation mediated by *P. gulae* stimulation (Figure 7B). Previous studies have utilized bacterial proteases, such as those of *P. gingivalis* and *T. forsythia*, for degradation of human proteins, including γ-globulin, and fibrinogen [20), 41)]. In the present experiments, following *P. gulae* stimulation, γ-globulin became gradually degraded in a time-dependent manner, while fibrinogen showed nearly complete digestion (Figure 8A). Additionally, the protease inhibitors antipain, PMSF, TLCK, and leupeptin restricted degradation of human proteins mediated by *P. gulae* proteases (Figure 8B). These findings suggested that *P. gulae* proteases contribute to proteolytic degradation, leading to periodontal destruction.

Discussion

A recent examination of *P. gulae* proteases noted arginyl- and lysyl-specific proteolytic activities [12]. The present results show that *P. gulae* mainly produces serine proteases with trypsin-like activities. Although the function of *P. gulae* proteases have yet to be fully revealed, based on the present biochemical and functional observations, including hemagglutination, coaggregation, and degradation of host proteins, some conclusions can be presented.

P. gulae possess several virulence factors that are similar to those of *P. gingivalis* [12, 42, 43]. All of the present *P. gulae* strains as well as *P. gingivalis* ATCC 33277 produced alkaline phosphatase and showed trypsin activity (Figure 1, Figure 2 and Table 1). These findings indicate the possibility that *P. gulae* proteases are similar to those of *P. gingivalis*. A study to determine whether *P. gulae* proteases perform the same functions as *P. gingivalis* proteases will be performed in the future.

Hemagglutination, which results from aggregation of erythrocytes induced by bacterial proteases adhering to two or more erythrocytes, contributes to bacterial pathogenicity [44]. In other reports, the hemagglutinating activities of *P. gingivalis* and *F. nucleatum* have been considered be linked to bacterial pathogenicity [45, 46]. In the present study, *P. gulae* cells were shown to possess hemagglutinating activity (Figure 3), suggesting that the activity of proteases contributes to the pathogenicity of this organism.

Other studies have also noted that *P. gingivalis* gingipains facilitate bacterial growth in nutrient poor conditions [47-50]. The present findings as well as suggest a role for *P. gulae* proteases, based on results showing that the serine and cysteine protease inhibitors examined here interfere with bacterial growth (Figure 4). Thus, *P. gulae* proteases may be essential for growth of the bacterium.

Bacterial coaggregation is considered to promote interactions among oral pathogens, leading to formation of dental plaque and biofilm [29, 51, 52]. Several different types of bacterial proteases, such as *P. gingivalis* gingipains, *T. denticola* chymotrypsin-like proteases, and *S. gordonii* Challisin, have been shown to contribute to coaggregation of various oral species, resulting in microbial community development and host tissue pathogenesis [20, 29, 30].

Actinomyces species are early colonizers and experiments have shown their important roles as key bacteria during initial biofilm formation on a salivary pellicle-coated enamel surface [53), 54)]. *A. viscosus* has also been found to be related to root canal caries and periodontal disease [55), 56)], as well as biofilm formation at the bottom of human periodontal pockets and progression of periodontitis [56)]. Other reports have indicated that *P. gingivalis* virulence factors, including fimbriae and gingipain, are involved with coaggregation with *A. viscosus* [20), 57)]. In this study, coaggregation of *P. gulae* with *A. viscosus* was found to be dependent on *P. gulae* proteases (Figure 5), suggesting that *P. gulae* proteases are involved in coaggregation of *P. gulae* and *A. viscosus*.

P. gingivalis gingipains are thought to contribute to the pathogenesis and development of periodontitis [49), 50)], and have been shown to have proteolytic activity against focal contact and adherence junction components, such as E-cadherin, paxillin, FAK, and β-catenin [39), 40), 58)-61)], while it has also been suggested that those activities might contribute to the pathogenesis of periodontal disease [60)]. The present study results indicate that *P. gulae* proteolytic enzymes degrade human proteins, including E-cadherin, paxillin, FAK, and β-catenin, resulting in reduced cell contact and gingival epithelial cell proliferation defects (Figure 6 and 7). Additionally, γ-globulin and fibrinogen have been reported be digested by *P. gingivalis* proteases [20)], while degradation of γ-globulin, related to host defense mechanisms, reportedly facilitates and prolongs periodontal disease [62)]. Fibrinogen is abundant in sites affected by periodontal disease, where tissue destruction and spontaneous bleeding can be observed [63)]. In the present experiments, *P. gulae* proteases induced degradation of γ-globulin and fibrinogen (Figure 8). Together, these findings suggest that *P. gulae* may facilitate host proteins proteolysis, leading to periodontal pathogenesis.

In summary, *P. gulae* proteases are important virulence factors for bacterial biology as well as host cell biology, thus raising the possibility that they may be important therapeutic targets for periodontal disease treatment strategies. Additionally, my findings suggest that *P. gulae* may contribute to the pathogenesis of periodontitis in both animals and humans.

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Figure legends

Figure 1. Protease activities of six *P. gulae* strains.

Protease activities were determined using a Pierce Protease assay kit, as described in Materials and Methods. (A) Six *P. gulae* strains and *P. gingivalis* ATCC 33277 were grown overnight to a pre-culture density of OD_{600} nm = 1.0, then cultured for 24 h in fresh medium. The cultures were collected, washed, and resuspended in PBS, and adjusted to OD_{600} nm. Bacterial suspensions (5 x 10^7 cells) were analyzed for their effects on trypsin activity. (B) Culture supernatants were obtained and filtered through a 0.22 μm polyethersulfone filter, then analyzed for effects on trypsin activity. Enzyme activity indicated by the microplate reader (OD_{450} nm) is expressed as arbitrary units. Data are shown as the mean ± SD of three independent experiments and were analyzed with a *t*-test.

Figure 2. Inhibitory effects of protease inhibitors against *P. gulae* ATCC 51700 proteolytic activity.

P. gulae ATCC 51700 cells were exposed to antipain, PMSF, TLCK, and leupeptin (100 μM) for 2 h. Enzyme activity indicated by the microplate reader (OD_{450} nm) with arbitrary units. Data are shown as the mean ± SD of three independent experiments and were analyzed with a *t*-test. **P* <0.01 as compared with control (*P. gulae* ATCC 51700 living cells without protease inhibitors).

Figure 3. Hemagglutination activity of *P. gulae* ATCC 51700.

(A) Bacterial suspensions and two-fold series dilutions applied to the wells of a microtiter plate are shown from left to right. Mouse erythrocytes were mixed with each suspension and incubated at RT for 3 h. (B) *P. gulae* ATCC 51700 cells were exposed to antipain, PMSF, TLCK, and leupeptin (100 μM) at 37°C for 2 h. Next, an equal volume of erythrocyte suspension was added and incubation was performed in a round-bottomed microtiter plate at RT for 3 h. Results from three independent experiments are shown.

Figure 4. Growth behavior of *P. gulae* ATCC 51700 in CDM.

(A) *P. gulae* ATCC 51700 overnight cultures (OD_{600} nm = 1.0) were diluted 5-fold with fresh CDM, then growth in the cultures at 37°C was monitored as an increase in OD_{600} nm for the

indicated times. Values shown are representative of three biological replicates. (B) *P. gulae* ATCC 51700 overnight cultures (OD_{600} nm = 1.0) were diluted 5-fold with fresh CDM with/without antipain, PMSF, TLCK, and leupeptin. The endpoint for bacterial growth (OD_{600} nm) was reached after 144 h.

Figure 5. Coaggregation of *P. gulae* ATCC 51700 and *A. viscosus* ATCC 15987.

(A) Time course of *P. gulae* and *A. viscosus* coaggregation. Equal volumes (500 μl) of the two bacterial suspensions were mixed and incubated at RT for the indicated times. Coaggregation was monitored as increase in OD_{550} nm. Data are shown as the mean percentage of coaggregation ± SD of three independent experiments. The endpoint for bacterial growth (OD_{550} nm) was reached after 48 h. (B) *P. gulae* and *A. viscosus* suspensions were treated with/without antipain, PMSF, TLCK, and leupeptin (100 μM). The endpoint for bacterial growth (OD_{600} nm) was reached after 144 h. **P* <0.05 and ***P* <0.01 indicate significant difference (Student's *t* test) as compared to *P. gulae* with DMSO without an inhibitor. All experiments were performed in triplicate and repeated three times.

Figure 6. *P. gulae* ATCC 51700 induces shape change and inhibits proliferation of Ca9-22 cells.

(A) Ca9-22 cells were stimulated with *P. gulae* ATCC 51700 at an MOI 500 for 24 h. Changes in cell morphology were observed by phase-contrast microscopy. Control cells were uninfected. (B) Ca9-22 cell proliferation was measured as 450 nm following stimulation with *P. gulae* ATCC 51700 at the indicated MOI. Data are shown as the mean relative ratio of infected/uninfected ± SD from three independent experiments and were analyzed with a *t*-test. **P*<0.01 as compared with uninfected cells. (C) Ca9-22 cells were stimulated with viable *P. gulae* ATCC 51700 cells at an MOI 500 with/without antipain, PMSF, TLCK, and leupeptin (100 μM). Cell proliferation was detected by measuring absorbance at 450 nm. Relative ratios were calculated relative to infected cells without an inhibitor (t test), with results based on three experiments shown.

Figure 7. Cell adhesion-related protein degradation by *P. gulae* ATCC 51700.

(A) Ca9-22 cells were infected with *P. gulae* ATCC 51700 at an MOI of 500 for 360 min. (B) Ca9-22 cells stimulated with *P. gulae* ATCC 51700 at an MOI of 500 were treated with/without antipain, PMSF, TLCK, and leupeptin (100 μM). The samples (10 μg) were analyzed by polyacrylamide electrophoresis using 8% and 10% SDS-PAGE, then subjected to immunoblotting and probed against β-catenin, E-cadherin, FAK, and paxillin. Results were determined from three different experiments.

Figure 8. Recombinant human proteins degradation by *P. gulae* ATCC 51700.

(A) The recombinant proteins γ-globulin and fibrinogen (5 μg) were stimulated with *P. gulae* ATCC 51700 ($5x10^7$ cells) at 37ºC for the indicated times. (B) Effects of protease inhibitors (100 μM) on recombinant protein degradation mediated by *P. gulae* stimulation. γ-globulin and fibrinogen were stimulated with *P. gulae* ATCC 51700 ($5x10^7$ cells) with/without antipain, PMSF, TLCK, and leupeptin, and those in combination for 360 min, then analyzed by polyacrylamide electrophoresis using 8% SDS-PAGE. Proteins were electroblotted onto PVDF membranes, which were then stained with 0.1% Coomassie Brilliant blue R-250. Each particle protein profile was analyzed a minimum of three times following separation.