



ORIGINAL ARTICLE

Effect of deletion of the *rgpA* gene on selected virulence of *Porphyromonas gingivalis*



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Received 16 November 2015; Final revision received 13 January 2016
Available online 18 April 2016

KEYWORDS

Porphyromonas gingivalis;
rgpA;
mutant;
outer membrane vesicles;
biofilm formation

Abstract *Background/purpose:* The most potent virulence factors of the periodontal pathogen *Porphyromonas gingivalis* are gingipains, three cysteine proteases (RgpA, RgpB, and Kgp) that bind and cleave a wide range of host proteins. Considerable proof indicates that RgpA contributes to the entire virulence of the organism and increases the risk of periodontal disease by disrupting the host immune defense and destroying the host tissue. However, the functional significance of this proteinase is incompletely understood. It is important to analyze the effect of arginine-specific gingipain A gene (*rgpA*) on selected virulence and physiological properties of *P. gingivalis*.

Materials and methods: Electroporation and homologous recombination were used to construct an *rgpA* mutant of *P. gingivalis* ATCC33277. The mutant was verified by polymerase chain reaction and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Cell structures of the mutant were examined by transmission electron microscopy and homotypic biofilm formation was examined by confocal laser scanning microscopy.

Results: Gene analysis revealed that the *rgpA* gene was deleted and replaced by a drug resistance gene marker. The defect of the gene resulted in a complete loss of RgpA proteinase, a reduction of outer membrane vesicles and hemagglutination, and an increase in homotypic biofilm formation.

Conclusion: Our data indicate that an *rgpA* gene deficient strain of *P. gingivalis* is successfully isolated. RgpA may have a variety of physiological and pathological roles in *P. gingivalis*.

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Introduction

Periodontal disease is a chronic inflammatory disease involving bacteria pathogens interacting with host cells, leading to the destruction of the teeth supporting structures. The inflammation has been considered to be a result of an increasing amount of bacteria pathogens in the dental plaque. The major causative agent, *Porphyromonas gingivalis*, is a Gram-negative, nonmotile, asaccharolytic anaerobe.^{1,2} Accumulating data suggest an association between this periodontitis organism infection and systemic inflammatory diseases such as diabetes mellitus, cardiovascular disease.^{3,4} *P. gingivalis* possesses a variety of virulence factors including fimbriae, lipopolysaccharide, outer membrane vesicles (OMVs), and gingipains. The gingipains are *trypsin-like* cysteine proteases that contribute to 85% of the overall proteolytic activity and play the most significant role in its virulence.⁵

Gingipains are located in the culture supernatant, in the membrane surface, and within the cytoplasm. There are two kind of gingipains based on substrate specificity. Arginine-specific gingipains (Rgp) cleave peptide bonds specifically at Arg residues and lysine-specific gingipain (Kgp) cleaves peptide bonds specifically at Lys residues.⁶ There are two Arg-gingipains (RgpA and RgpB), encoded by two genes, *rgpA* and *rgpB*, respectively. Lys-gingipain is encoded by a single *kgp* gene.⁷ Proteinases RgpA and Kgp both consist of an N-terminal propeptide region, a proteolytic domain, and C-terminal HA domains. Their HA domains are highly homologous in sequence. However, RgpB lacks the large C-terminal HA domain.⁸ Depending on complex post-translational processing of the initial translation product, RgpA occurs in at least three isoforms, the initial 95 kDa polyprotein with the 50 kDa catalytic domain non-covalently associated with HA domains, the 50 kDa soluble monomeric form of RgpA secreted into extracellular environment, and 70–90 kDa glycosylation modified RgpA associated with vesicles and bacterial membranes.^{9,10}

Gingipains are critical to physiology of *P. gingivalis*. This primarily manifest in two aspects: (1) gingipains are important for generating peptides or amino acids as energy and carbon source for the organism; and (2) *rgpA* gene is involved in processing mechanisms for bacteria surface proteins, such as fimbriin and Kgp. In Rgp-deficient mutants, fimbriin remains in the precursor form and Kgp is abnormally processed.¹¹ In addition to these physiological roles, gingipains are also used by *P. gingivalis* to manipulate and evade the host immune response via complex mechanisms.^{12,13} It has been reported that gingipains are involved in host invasion, tissue destruction, agglutination and hemolysis of erythrocytes, and disruption and manipulation of the inflammatory response.^{14,15} Proteinase RgpA especially has been shown to be a potent virulence factor in terms of proteolytic destruction of host connective-tissue proteins and disruption of host defense mechanisms.¹⁶ Rgp is implicated in immunological dysregulation and chronic inflammation by inactivating cytokines and their receptors, such as T-cell growth factor interleukin-2, anti-inflammatory cytokines interleukin-4 and interleukin-5,¹⁷ pro-inflammatory cytokines interleukin-12 and tumor necrosis factor- α ,¹⁸ by cleaving T cell co-receptor molecules CD4,

CD8,¹⁹ and by degrading complement molecules and immunoglobulins.²⁰ Whereas the structures of gingipains are well defined in previous studies, the functional significance of *rgpA* is not completely understood. Unravelling the function of the *P. gingivalis* *rgpA* gene will contribute to the understanding of the mechanisms applied by this pathogen to evade host immune responses and cause disease. In this study, we provide a new method to isolate an *rgpA*-deficient mutant of *P. gingivalis*. Different from the conventional approach using recombinant plasmids, our method for gene deletion is convenient and efficient. We further characterize micromorphology of the *rgpA* deficient strain, and examine function of this gene in hemagglutination and homotypic biofilm formation.

Materials and methods

Bacterial strains and plasmids

P. gingivalis ATCC33277 and *Escherichia coli* DH5 α were used. Suicide plasmid pPHU281 (provided by Professor Li, Medical School of Nanjing University, Nanjing, China) and pGEM-T-Easy Vector (Promega, Madison, WI, USA) was used to construct the suicide plasmid, which was prepared for *P. gingivalis* gene manipulation. Gene *rgpA* (PGN_1970, GeneBank: AP009380.1) and *ermF-ermAM* cassette (GeneBank: AF219231.1) were respectively derived from *P. gingivalis* genomic DNA and plasmid pVA2198.²¹

Culture conditions

P. gingivalis cells were grown anaerobically (10% CO₂, 10% H₂, and 80% N₂) in trypticase soy broth (TSB) medium (containing, per liter, 30 g of Tryptone Soya Broth, 1 g of yeast extract, 50 mg of hemin, and 1 mg of menadione). For blood agar plate, defibrinated sheep blood was added to Columbia blood agar plate (OXOID, Basingstoke, Hampshire, UK) at 5%. Luria–Bertani medium (containing, per liter, 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride) was used for *E. coli* cell growth. For mutant screening and genetically stability of the antibiotic-resistance strain, erythromycin (300 μ g/mL for *E. coli* and 10 μ g/mL for *P. gingivalis*) was added to the media. Bacterial growth was monitored by measuring the optical density at 660 nm (OD₆₆₀).

Construction of plasmids and donor DNA fragment

Recombinant plasmid pPHU281_up_erm_down was constructed as follows. For construction of the *rgpA*-deletion cassette, the upstream (1.1 kb) and downstream (1 kb) of *rgpA* were amplified by polymerase chain reaction (PCR) from *P. gingivalis* ATCC33277 chromosomal DNA with corresponding primer pairs (upF and upR for upstream, downF and downR for downstream). Primers used in this study are listed in Table 1. The resistance gene, a 2.2 kb *ermF-ermAM* cassette was amplified with pVA2198 as PCR template. According to the method described by Heckman and Pease,²² the *rgpA*-upstream, *ermF-ermAM* and *rgpA*-downstream fragments were spliced by overlap extension to

generate a full-length product, named up_erm_down (4.3 kb). The up_erm_down fragment then was cloned into vector pGEM-T Easy, resulting in plasmid pGT_up_erm_down. pGT_up_erm_down was transformed into *E. coli* DH5 α . The positive clones were screened and propagated, and the recombinant plasmid was prepared, purified, and digested with EcoRI restriction enzyme to dissociate the fragment containing up_erm_down, which was then ligated with EcoRI linearized plasmid pPHU281 to yield a new suicide plasmid (pPHU281_up_erm_down). For efficient electroporation of *P. gingivalis*, a truncated DNA fragment from up_erm_down (named up_erm_down', 3.7 kb) was amplified with pGT_up_erm_down or pPHU281_up_erm_down as PCR templates. Fragment up_erm_down' is also composed of three parts—the 0.8 kb upstream, the 2.2 kb *ermF-ermAM* cassette and the 0.7 kb downstream in a sequential order. DNA fragment up_erm_down' and plasmid pPHU281_up_erm_down were both used as donors for electroporation of *P. gingivalis*.

Electroporation of *P. gingivalis*

Electroporation was performed following the instructions of *Current Protocols in Microbiology*.²³ Briefly, *P. gingivalis* cells from blood agar plate were incubated in 10 mL TSB medium at 37°C anaerobically overnight without agitation. The overnight culture was inoculated to 100 mL TSB medium, then incubated an additional 4 hours to obtain a final OD₆₆₀ of 0.55–0.65. Afterwards, *P. gingivalis* cells were harvested by centrifugation, washed with the electroporation buffer (EP buffer, 10% glycerol, 1mM MgCl₂, filter sterilized), and resuspended in 10 mL of the same solution. Ten microliters of pPHU281_up_erm_down plasmid DNA solution or purified up_erm_down' were mixed with 100 μ L of the prepared cell suspension. The whole volume of the DNA-containing cell suspension was poured into a sterile cuvette for electroporation (Pulse cuvette with 0.2 cm electrode gap; Bio-Rad, Hercules, CA, USA). Electroporation was performed under proper experimental conditions (voltage, 2.0 kV; time constant, 5 milliseconds) with an electroporation apparatus (Gene Pulser; Bio-Rad). This procedure was carried out at 4°C. After electroporation, the cell suspension was immediately mixed with 1 mL prewarmed TSB, and incubated anaerobically at 37°C for 16 hours. Cells then were spread on Columbia blood agar plate containing 10 μ g/mL erythromycin and incubated for

7–10 days. The *rgpA* gene was replaced by *ermF-ermAM* cassette via a double crossover event during bacteria chromosome replication. The mutation was confirmed by PCR analysis.

Preparation of RgpA proteinase and polyacrylamide gel electrophoresis

According to previously published protocols,²⁴ RgpA extracts were prepared. Briefly, *P. gingivalis* ATCC33277 was incubated in TSB medium at 37°C under anaerobic conditions. When OD₆₆₀ of *P. gingivalis* reached 1.0, bacteria were centrifuged at 12,000g for 45 minutes at 4°C. Culture supernatant was filtered through a 0.45-mm-pore filter (Millipore, Darmstadt, Germany) to remove residual bacteria and then mixed with precooled acetone in a 40:60 volume ratio. After constant stirring over 15 minutes at –20°C, the solution was centrifuged at 12,000g for 30 minutes at 4°C. Pellet was resuspended in the prepared buffer (150mM NaCl, 20mM Bis–Tris and 5mM CaCl₂). The resuspended pellet was dialyzed overnight at 4°C in a 14,000-molecular-weight cut-off dialysis tubing versus 500 mL of the same buffer containing Aldrithiol-4 (Sigma–Aldrich, St. Louis, MO, USA). During the dialysis, there were three more changes of buffer without Aldrithiol-4. After that, preparation was centrifuged at 34,000g for 1 hour at 4°C and the resulting supernatant was concentrated in an ultracentrifugal filter (Millipore) with a 10,000-molecular-weight cut-off membrane at 4°C. The concentrated gingipain extract was clarified by centrifugation at 192,000g for 1 hour at 4°C. The samples (30 μ L of extract) were then added to a solubilizing buffer and kept at 100°C for 10 minutes. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in a 1.0-mm-thick slab gel (10%). Proteins on gels were stained with Coomassie Brilliant Blue G-250.

Hemagglutination

Overnight cultures of *P. gingivalis* in TSB medium were centrifuged for 15 minutes (4°C, 10,000g), washed with phosphate-buffered saline (PBS), and suspended in PBS. The bacterial suspensions (OD₆₆₀ = 0.4) were then diluted in a two-fold series with PBS. A 100 μ L aliquot of each suspension was mixed with an equal volume of sheep erythrocyte suspension (2.5% in PBS) and incubated in a

Table 1 Primers and sequences designed for *rgpA* mutant construction and genetic analysis.

Primer	Primer sequence (5'–3')
upF (EcoRI)	ccgaattcGGATACGGGTTTCGTCTATGTAGCGGCGGAAAAATGC
upR	CGGAAGCTATCGGGGTACCGTTTTTCATTTTGTATG
ermF	CATCAAAATGAAAAACGGTACCCCGATAGCTTCCG
ermR	CTCCGAGTCCAAGACAGAAGTGCAGGTCGACTCTAG
downF	CTAGAGTCGACCTGCAGTTCTGTCTTGGACTCGGAG
downR (BamHI)	aaggatccTATCTATCTCATGCCGGAGGCGGAAGGTGGTAACTC
upF'	GTGTGGCGCATCAGATATATTTTCATC
downR'	GGACAAGCCTATGGGTCGAGGACATAAG
RgpA F	TCCAATCAGCAATCGGTGAC
RgpA R	CGAGCATCTTCTACCATCC

round-bottom microtiter plate at room temperature for 3 hours. The hemagglutinating activity was assessed visually.

Transmission electron microscopy

P. gingivalis cells were grown up to early stationary phase in TSB. The cell pellets were washed three times with PBS and fixed with 2.5% glutaraldehyde at 4°C for 2 hours. Then, cell pellets were fixed with 2% osmium tetroxide at 4°C for 90 minutes. The fixed cells were dehydrated in an ethanol series, embedded in 3% agar. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

Preparation of homotypic biofilms

The bacteria were grown in 12-well plates with a glass slip (round, 15 mm in diameter). The concentration of bacteria was adjusted by spectrophotometry after incubation for 24 hours and then standardized by dilution with PBS to 1×10^8 colony forming units/mL each. Every well was filled with 1.5 mL of fresh TSB medium, 100 μ L of bacterial suspension and a glass slip. After incubation of 12 hours, the glass slip with the biofilm was harvested, gently washed with sterile PBS to remove free-floating bacteria, and fixed with 2.5% glutaraldehyde.

Confocal laser scanning microscope

The biofilms on the glass slips were incubated with FITC-ConA (Sigma) at 4°C in the dark for 2 hours. After that, the reagent was removed with the administration of 2 mL of sterile PBS three times. The biofilms were imaged with a confocal laser scanning microscope (Fluoview FV10i; Olympus, Tokyo, Japan) at a magnification of 300 \times .

Results

The coding sequence of *rgpA* was deleted and replaced by resistance gene *ermF-ermAM* by homologous recombination. The overall design for construction of the mutant is shown in

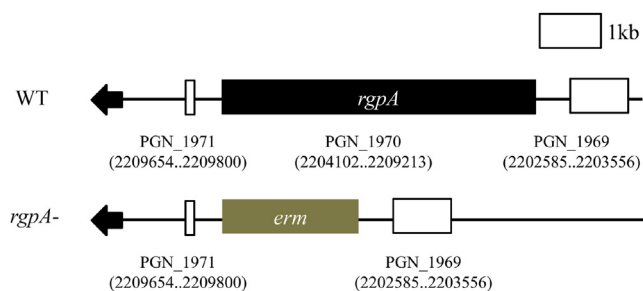


Figure 1 A sketch gene map of *Porphyromonas gingivalis* ATCC33277 (WT) and the *rgpA* mutant strain (*rgpA*-). In the genome of mutant strain, *rgpA* was replaced by *ermF-ermAM* cassette (*erm*). PGN_1969 is the upstream gene and PGN_1971 the downstream gene adjacent to *rgpA*.

Figure 1. The 2.2 kb *ermF-ermAM* cassette, the flanking DNA sequences upstream (1.1 kb) and downstream (1 kb) of *rgpA* were respectively amplified with corresponding primer pairs (Figure 2A). Using overlap-extension PCR, we ligated the above three DNA fragments in a sequential order and got a 4.3 kb fragment, named up_erm_down (Figure 2B). It was cloned into vector pGEM-T Easy and suicide vector pPHU281 (6.9 kb). Recombinant plasmid pPHU281_up_erm_down was digested with EcoRI (one EcoRI restriction site is located at 5' region of primer upF, the other is located at the vector next to primer downR), resulting in two fragments, the 6.9 kb vector and the 4.3 kb up_erm_down. Agarose gel electrophoresis of digested DNA displayed the predicted bands (Figure 2C).

DNA fragment up_erm_down' is a 3.7 kb PCR product amplified with primers upF' and downR' using pGT_u-p_erm_down or pPHU281_up_erm_down as template, as mentioned above. It was introduced to *P. gingivalis* cells by electroporation. The transformed *P. gingivalis* cells were spread on Columbia blood agar plate containing 10 μ g/mL erythromycin, and cultured for 7 days at 37°C. To verify the genotypes of the putative mutants, genomic DNA was extracted and amplified with primers pairs RgpA F/RgpA R and upF'/ermR. Primers RgpA F and RgpA R are located on the coding sequence of *rgpA*. Primers upF' is the upstream primer to amplify up_erm_down' and ermR is the downstream primer to amplify the *ermF-ermAM* cassette. As shown, the mutants had *rgpA* gene deleted (Figure 2D) and *rgpA* was replaced by *ermF-ermAM* cassette (Figure 2E). The wild type *P. gingivalis* ATCC33277 and plasmid pPHU281_up_erm_down was used as negative control and positive control respectively. By DNA sequencing, we confirmed that the insert had no unintentional base changes. These results indicated that *rgpA*-deficient *P. gingivalis* was successfully constructed.

Extracts from culture supernatant of *P. gingivalis* and the *rgpA* mutant were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis to verify the protein expression (Figure 3). A protein band of about 50 kDa was observed in culture supernatant of the wild type, but disappeared in the *rgpA* mutant. Since secretory RgpA is in soluble monomeric form with molecular mass about 50 kDa, it is most likely that the disappearance of the protein band in *rgpA* mutant is attributed to deficiency of *rgpA* gene.

Visually, the mutant strain had normal pigmentation on blood agar plate (Figure 4B). *P. gingivalis* has the ability to agglutinate erythrocytes, which is one of the significant features of this organism. The hemagglutinating activity was attributed to HA domain structure of Rgp–Kgp complexes. Since the mutant in this study lacked the HA domain of *rgpA*, we determined hemagglutinating activity of the mutant cells. The mutant showed lower hemagglutinating activity than the wild type on sheep erythrocytes (Figure 4A).

We examined cell structure of the *rgpA* mutant and the wild type strain by transmission electron microscopy. As shown in Figure 5, the mutant exhibited identical morphology and microstructure with the wild type, except fewer OMVs.

We examined the homotypic biofilm formation of the *rgpA* mutant by confocal laser scanning microscopy. FITC-ConA stained biofilms of the wild type strain and the *rgpA* mutant were imaged by software program FV10-ASW 2.1 Viewer (Fluoview FV10i; Olympus, Tokyo, Japan). As shown in Figure 6, homotypic biofilm formation of the *rgpA* mutant

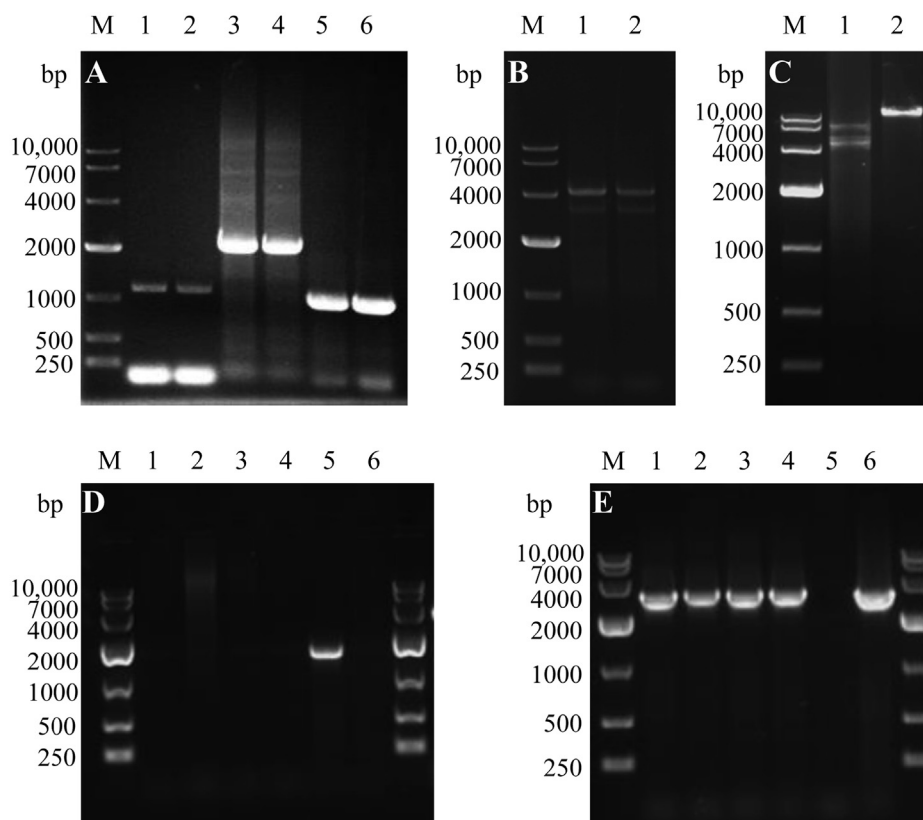


Figure 2 Construction and verification of *rgpA* mutant strain. DNA products were analyzed by 1% agarose gel electrophoresis. (A) Upstream (lanes 1 and 2, ~1.1 kb) and downstream (lanes 3 and 4, ~1 kb) of *rgpA*, *ermF-ermAM* cassette (lanes 3 and 4, ~2.2 kb) were respectively amplified by polymerase chain reaction. (B) Overlap extension products of the above three DNA fragments, named up_erm_down (lanes 1 and 2, ~4.3 kb). (C) Restriction enzyme reaction was performed to identify the recombinant plasmid pPHU281_up_erm_down. pPHU281_up_erm_down was linearized by restriction enzyme *SacI* (lane 2, 11.2 kb), and cleaved into two fragments (lane 1, 2 bands, 6.9 kb and 4.3 kb) by *EcoRI*. (D) The genomic DNA of putative strain was amplified with specific primer pairs RgpA F and RgpA R. Mutant strains (lanes 1–4, no DNA band) were *rgpA*-deficient genotypes. (E) Genomic DNA of the putative strain was amplified with specific primer pairs upF' and ermR. It had been confirmed that *ermF-ermAM* cassette was inserted into the mutant strain (lane 1–4, ~3 kb) genome at the *rgpA* locus. In (D) and (E), *Porphyromonas gingivalis* ATCC33277 (lane 5) and plasmid pPHU281_up_erm_down (lane 6) were used as negative control and positive control, respectively. M = DNA marker, bp.

was markedly increased compared to that of wild type strain.

Discussion

Gingipains are the primary virulence factors of *Porphyromonas gingivalis*, the main periodontopathogen. It is expected that inhibition of gingipain activity *in vivo* could prevent or slow down the progression of adult periodontitis.²⁵ Therefore, elucidating pathogenic mechanisms of these proteolytic enzymes has important implications. The three approaches most adopted to investigate gingipains include immunization, analysis of knockout strains, and testing of specific protease inhibitors using murine models of infection or bone loss. To determine the significance of *rgpA* to pathogenicity of *P. gingivalis*, we constructed a *rgpA* mutant by integration of DNA fragment up_erm_down' or plasmid pPHU281_up_erm_down into the bacterium's genome. They both contain the flanking genes of *rgpA* and *ermF-ermAM* cassette and are used as donors for electroporation. Instead of enzymatic ligation, we used overlap

extension PCR to get the target DNA fragment up_erm_down. It is a simple and accurate technique for mutagenesis and gene splicing. The DNA fragment was then cloned into suicide plasmid pPHU281 to get the recombinant plasmid pPHU281_up_erm_down. The truncated DNA fragment up_erm_down' or plasmid pPHU281_up_erm_down entered *P. gingivalis* competent cells by electroporation and integrated into the bacterial genome. Gene *rgpA* (5.1 kb) was replaced by resistant gene *ermF-ermAM* (2.2 kb) through homologous recombination. Based on our work, we find that using DNA fragment as electroporation donor is an efficient approach for gene disruption, or at least for *P. gingivalis*. This is manifested in two aspects. On one hand, DNA fragment is smaller in size than plasmid, which makes it easier to pass through cell membrane and reach to bacterial chromosome. On the other hand, bacterial colonies on antibiotic agar plate are mutant strains with hardly any false positives. This method improves work efficiency and saves the time of mutant screening.

P. gingivalis are black-pigmented colonies resulting from heme acquisition on cell surface. Hemoglobin from erythrocytes is captured by hemoglobin receptor protein on the

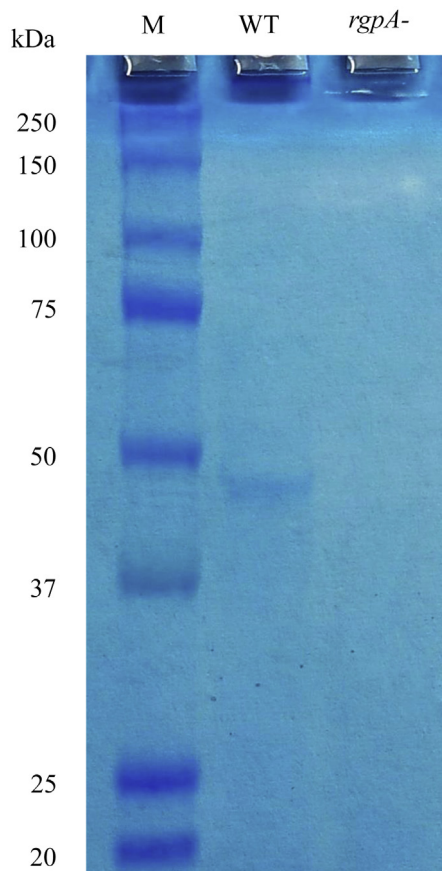


Figure 3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of gingipain extracts of *Porphyromonas gingivalis* ATCC33277 (WT) and the *rgpA* mutant (*rgpA*⁻) with Coomassie brilliant blue staining. M = molecular weight marker, kDa.

cell surfaces, and digested by gingipain to yield heme and peptides.²⁶ Heme and peptides transfer into bacteria to be used as nutrients. It has been postulated that heme acquisition is a defense mechanism of *P. gingivalis* against reactive oxygen species in the oral inflammatory microenvironment and a mechanism for iron storage.²⁷ Gingipains Kgp and RgpA are involved in heme acquisition, binding and

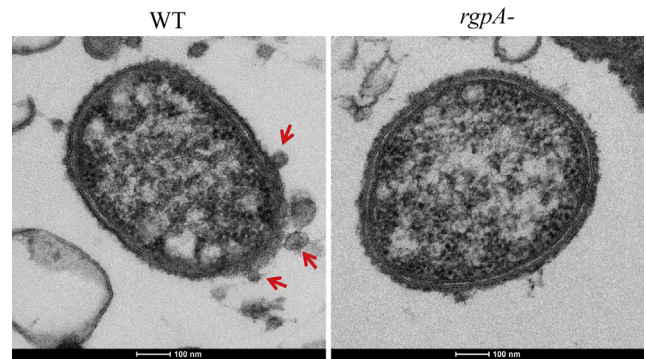


Figure 5 Transmission electron micrographs of *Porphyromonas gingivalis* ATCC33277 (WT) and *rgpA* mutant (*rgpA*⁻). Bubbles on the cell surfaces indicate the presence of outer membrane vesicles (red arrows).

accumulation of *P. gingivalis*, and Kgp is apparently the primary enzyme involved in binding of hemoglobin and bleeding.²⁸ In our study, the *rgpA* mutant strain is black pigmented, with no difference with the wild type. This may suggest that *rgpA* gene plays little role in heme acquisition.

Hemagglutination is a distinctive property of *P. gingivalis*, and differentiates the microorganism from other asaccharolytic black-pigmented anaerobic bacteria such as *Porphyromonas asaccharolytica* and *Porphyromonas endodontalis*. Erythrocytes are the largest reserves of heme and iron. For nutrient acquisition, *P. gingivalis* first efficiently agglutinates erythrocytes and then slowly lyses them to release hemoglobin.²⁹ It has been indicated that hemagglutination of *P. gingivalis* is caused by the *rgpA*⁻, *kgp*⁻, and *hagA*-encoding HA domains.^{13,30} The HA subunits of RgpA consist of four sequence-related adhesion domains, Rgp15, Rgp17, Rgp27, and Rgp44. Extensive studies have been carried out to identify the hemagglutination motifs within the gingipain amino acid sequences. The peptide G907-T931 of RgpA from *P. gingivalis* W83 was recognized as being hemagglutination associated by monoclonal antibody MAb61BG1.³¹ The small peptide PVQNL or PVKNLK within the G907-T931 sequence was verified as a hemagglutination-associated short motif. This short motif is located at the C-terminus end of RgpA proteolytic

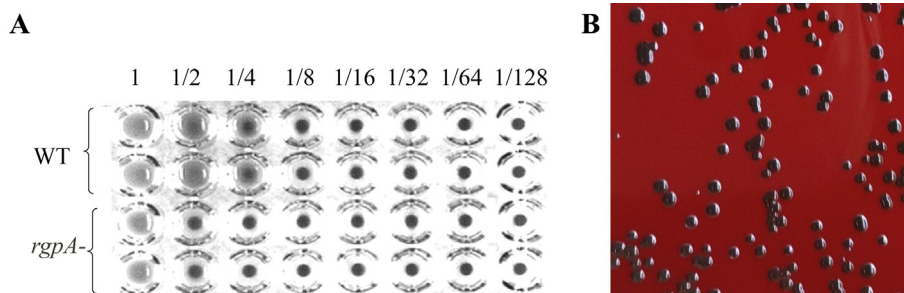


Figure 4 Hemagglutination and pigmentation of the *rgpA* mutant strain. (A) Hemagglutination activity. *Porphyromonas gingivalis* cells were grown in enriched trypticase soy broth, washed with phosphate-buffered saline, and suspended in phosphate-buffered saline at an optical density at 660 nm of 0.4. The suspension and its dilutions in a two-fold series were applied to the wells of a microtiter plate from left to right and mixed with sheep erythrocyte suspension (2.5%). (B) Colonial pigmentation. The mutant cells formed glossy roundish black colonies on blood agar plate after incubation anaerobically for 7 days. WT = wild type; *rgpA*⁻ = *rgpA* mutant strain.

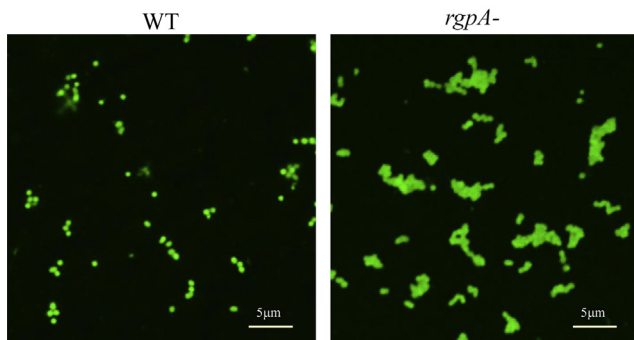


Figure 6 Homotypic biofilm formation of *Porphyromonas gingivalis* ATCC33277 (WT) and *rgpA* mutant (*rgpA*⁻). Bacterial cells were incubated with a glass slip in trypticase soy broth for 12 hours. Homotypic biofilms developed on the glass slip were stained with FITC (green) and observed with a confocal laser scanning microscope. Representative images are shown.

domain, in the Rgp44 and Rgp17 regions.³² Sakai et al³³ reported that a recombinant protein Rgp44 was able to bind to the erythrocyte membrane protein, glycophorin A. Considering that homologous sequences are found at multiple loci in the *P. gingivalis* genome, HA domains are supposed to have a variety of pathogenic activities.

Gingipains are produced as secreted or membrane-associated forms on the cell surface.³⁴ Cell-associated gingipains comprise the majority (80%) of Rgp and Kgp activities. Since RgpA are mostly located on the outer membrane of *P. gingivalis*, inactivation of *rgpA* may modify cell surface properties. Surface microstructures of the *rgpA* mutant and *P. gingivalis* were examined by transmission electron microscopy. The *rgpA* mutant presents decreased OMVs compared with the wild type. OMVs are spherical, microstructural bodies on or around the surface of bacterial cells. They range from 20 nm to 250 nm in diameter and consist of outer membrane and encapsulated periplasmic components. As a vehicle for antigens and active proteases, OMVs of *P. gingivalis* carry a wide range of toxic substances such as lipopolysaccharide, gingipain, hemagglutinin, carboxypeptidase, peptidylarginine deiminase, hemin-binding protein, and fimbrial protein.³⁵ These substances are considered to be released into the crevicular environment, which play potential roles in host immune disorder and tissue destruction during *P. gingivalis* infection. About 17% of Rgp and 7% of Kgp were located in OMVs. The *rgpA* mutant expressed reduced OMVs. This may result in decreased virulence of *P. gingivalis*. The fimbriation of the mutant had no difference with the wild type. It has been reported that there is little or no fimbriation on the cell surface of *rgpA rgpB* double mutant, whereas the number of fimbriae of the *rgpA* or *rgpB* mutant is similar to the parent strain.³⁶ The major component of fimbriae, fibrillin, remained in the precursor form only in the *rgpA rgpB* double knockout strains.¹¹ These indicate that a combination of RgpA and RgpB may affect maturation of fimbriae. However, the interaction of RgpA proteinase and the vesicles, fimbriation needs to be further studied.

Biofilm formation is related to the bacterial cell surface. Recent findings indicate that gingipains are involved in both homotypic (monospecies) biofilm formation by *P. gingivalis*,

but also synergistic biofilm formation with other bacteria such as *Fusobacterium nucleatum*.³⁴ Cranberry constituents, which are inhibitors of *P. gingivalis* proteinases, can prevent *P. gingivalis* biofilm formation.³⁷ An iron-binding glycoprotein, lactoferrin displays biofilm inhibition activity against *P. gingivalis*, and the antibiofilm effect is attributable to its antigingipain activity.³⁸ We first evaluated the roles of *rgpA* in homotypic biofilm formation in this study. When cultured in TSB medium for 12 hours, wild type formed biofilms with dispersed and sparser microcolonies. The mutant developed clustered, increased biofilm. Thus, *rgpA* seems to be a negative regulator of biofilm formation. Consistent with our results, Kuboniwa and colleagues³⁹ found that the biovolumes of homotypic biofilms were significantly increased in *rgp* mutant or *kgp* mutant in the early maturation phase, as compared to the wild type. This negative regulation of *rgpA* may be essential for the initiation and maturity of biofilms. Biofilm development is in dynamic changes that both stimulative and restrictive factors act synergistically. The *rgpA* mutant in our study exhibited reduced outer membrane vesicles. It is presumed that *rgpA* defection and the resulted downstream changes of bacterial cell surface may be related to the increased biofilm development.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

We appreciate the guidance of Professor Huang (Department of Biochemistry and Molecular Biology, Jiangsu University, Zhenjiang, China) for construction of *rgpA*-mutant. This work was supported by the National Natural Science Foundation of China (81271155, 51472115, 81300852), Key Project supported by Medical Science and Technology Development Foundation, Department of Health, Medical School of Nanjing University (ZKX13050).

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