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Short communication

# Further evidence that major outer membrane proteins homologous to OmpA in *Porphyromonas gingivalis* stabilize bacterial cells

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*Iwami J, Murakami Y, Nagano K, Nakamura H, Yoshimura F. Further evidence that major outer membrane proteins homologous to OmpA in* Porphyromonas gingivalis *stabilize bacterial cells.*
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**Introduction:** *Porphyromonas gingivalis* is one of the most important bacteria in the progression of chronic periodontal disease. We hypothesized that the major outer membrane proteins Pgm6/7, which are homologous to the OmpA protein in *Escherichia coli*, might contribute to the stabilization of the cell surface. In this study, the effects of Pgm6/7 on the cell surface were examined morphologically.
**Methods:** Deletion mutants of Pgm6/7 (Δ*694*, Δ*695* and Δ*695–694*) were constructed using the polymerase chain reaction-based overlap extension method. Wild-type ATCC 33277 and Pgm6/7 mutants were grown under anaerobic conditions. Whole cells and thin sections of fixed cells were stained and examined by transmission electron microscopy.
**Results:** Compared with the wild-type, numerous vesicles released from cells were observed in each deletion mutant. The outer membrane appeared wavy and irregular. Increased numbers of vesicles were confirmed after their preparation from the culture supernatant. Total gingipain activity in vesicles was increased five- to 10-fold in the deletion mutants.
**Conclusion:** This report provides further evidence that Pgm6/7 proteins in *P. gingivalis* play an important role in the maintenance of bacterial outer membrane integrity.

Key words: bacterial surface; gingipains; OmpA-like proteins; *Porphyromonas gingivalis*; vesicle

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*Porphyromonas gingivalis* is one of the most important bacteria in the initiation and progression of periodontal disease (16, 17). The bacterial membrane acts as a dynamic interface between the bacterium and hostile surroundings so we analyzed the profile of the major outer membrane proteins in *P. gingivalis* (20). These comprise RagA (Pgm1), RagB (Pgm4), 75-kDa protein (Pgm2, which is identical to the short minor fimbriae (MFa1) (7), Lys- and Arg-gingipains (Pgm3 and Pgm5, respectively), and Pgm6/7. A previous report showed that the major outer membrane proteins Pgm6/7 (Pg0695/Pg0694) of *P. gingivalis*, which share homology with the *Escherichia coli* OmpA protein, exist as heterotrimers (22). Since loss of Pgm6/7 affected bacterial growth and membrane permeability, we considered that Pgm6/7 might contribute to the maintenance of the structure of the outer membrane (22). In this study, the effects of Pgm6/7 deficiency on the surface structure were examined morphologically using mutants by deleting the open reading frames that code for Pgm6/7.

Deletion mutants, constructed previously (22), were used in this study. In brief, we used three mutants (Δ*694*, Δ*695*, and Δ*695–694*) derived from *P. gingivalis* wild-type strain ATCC 33277 using the polymerase chain reaction-based overlap extension method, in which the open reading frames *pg0694* and/or *pg0695* were deleted and replaced by *cat*, the gene encoding chloramphenicol acetyl transferase. These mutants form black-pigmented colonies on blood agar plates. The parent and the mutants were cultured in trypticase soy broth (Becton Dickinson,

Sparks, MD) supplemented with yeast extract, hemin, menadione and dithiothreitol (sTSB medium) at 37°C under anaerobic conditions (22).

Bacterial cells, grown for 48 h without washing, were negatively stained on carbon-coated grids with 2% [weight/volume (w/v)] uranyl acetate to examine the production of membrane vesicles (21, 23). To examine the ultrastructure of the cell surface, thin sections were prepared of the cells (10). In brief, washed *P. gingivalis* cells were fixed with 4% (w/v) paraformaldehyde and 5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer at 4°C for 2 h. Samples were post-fixed with 2% (w/v) osmium tetroxide for 90 min and then with 0.5% (w/v) uranyl acetate for 20 min. The fixed cells were dehydrated in ethanol and embedded in L-R white resin (London Resin Company Ltd., Reading, UK). Ultrathin sections were stained with uranyl acetate and lead citrate. Stained sections were observed and photographed using a JEM-1210 electron microscope (JEOL Ltd., Tokyo, Japan).

On negatively stained bacteria, numerous membrane-vesicle-like structures were observed outside the cells in all of the deletion mutants (Fig. 1). Vesicles were occasionally found projecting from the cell surface. These phenomena were most typical from the late-logarithmic phase (24 h) to the early-stationary phase (48 h). The shape and size of the mutant cells were not much different from those of the parent cells. Moreover, the amount of fimbriae surrounding the bacterial cells was observed to be similar among the strains. Thin-section microscopy revealed that the outer membrane in the mutants appeared wavy and uneven and lost its continuity (Fig. 2). The Pgm6/7 proteins share a high degree of similarity to *E. coli* OmpA in the C-terminal region, through which they presumably associate with peptidoglycan (4). These results therefore provided evidence that Pgm6/7 would be responsible for maintaining the rigidity and integrity of the outer membrane.

To further verify that the mutants produced more vesicles than the wild-type cells, membrane vesicles were prepared from each strain after growth in sTSB medium at 37°C for 48 h according to the method of Grenier and Mayrand (6). Vesicle numbers in the mutants, as determined by protein assay, were three- to five-fold higher than in the wild-type cells (Table 1). Major proteins in purified vesicles were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie brilliant blue. They were then identified by N-terminal or internal amino acid sequence analysis followed by a search of the protein databases using BLAST analysis (National Center for Biotechnology Information) (20, 21). Vesicles from the mutants exhibited increased intensities of major protein bands such as Lys-gingipain (Kgp), Arg-gingipain (Rgp), HGP44 and HGP27, which are products of the gingipain genes, and a slight increase in the major fimbrial component FimA (Fig. 3A). To compare components in vesicles and the outer membrane, the envelope fraction from each strain was prepared as previously described (20). The protein patterns of the vesicles were very similar to that of the culture supernatant, as previously reported (21). It has been reported that the membrane vesicle is representative of the outer membrane (5, 6, 14, 15, 24); however, only trace amounts of major outer membrane proteins from *P. gingivalis* (such as RagA, RagB and Mfa1) were found. In the case of the wild-type cells, only a faint Pgm6/7 band was observed in the vesicles, indicating that *P. gingivalis* vesicles carry part of the major outer membrane proteins. The distributions of these outer membrane proteins were confirmed by Western blotting using specific antibodies (data not shown). Lipopolysaccharide (LPS) was detected in the prepared fractions with Pro-Q emerald 300 reagent (32) according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA). LPS profiles in the envelope fraction after proteinase K digestion (1) were almost identical in the parent and the mutants; however, bands of LPS in the vesicle fractions from the mutants were more intense than in the parent (Fig. 3B).

Since the gingipains (Rgp and Kgp) increased in vesicles from mutants, as judged from Coomassie brilliant blue stained SDS–PAGE gels, the activity of gingipains was determined by measuring the hydrolysis of the synthetic chromogenic substrates *N-p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide (Sigma-Aldrich Co., St Louis, MO) and *N*α-benzoyl-L-Arg-*p*-nitroanilide (Sigma-Aldrich Co.), respectively (8).

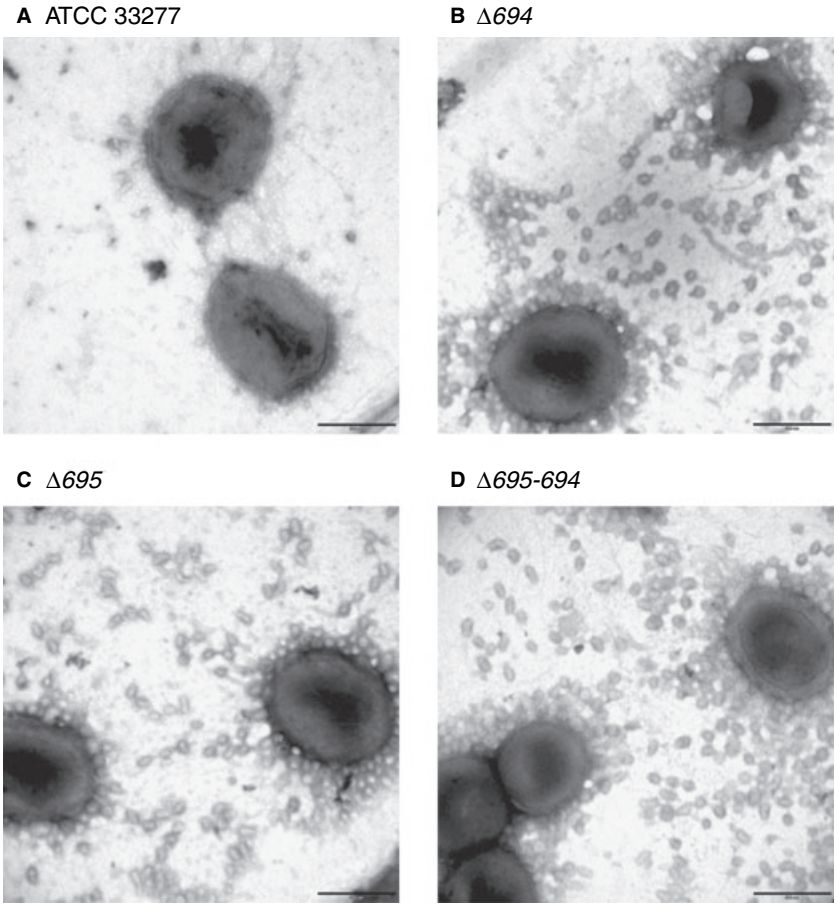


*Fig. 1.* Vesicle formation of the *Porphyromonas gingivalis* parent and Pgm6/7 mutants. The whole cells were negatively stained with 2% uranyl acetate; bars represent 0.5 μm. (A) ATCC 33277, (B) Δ*694*, (C) Δ*695*, (D) Δ*695–694*.

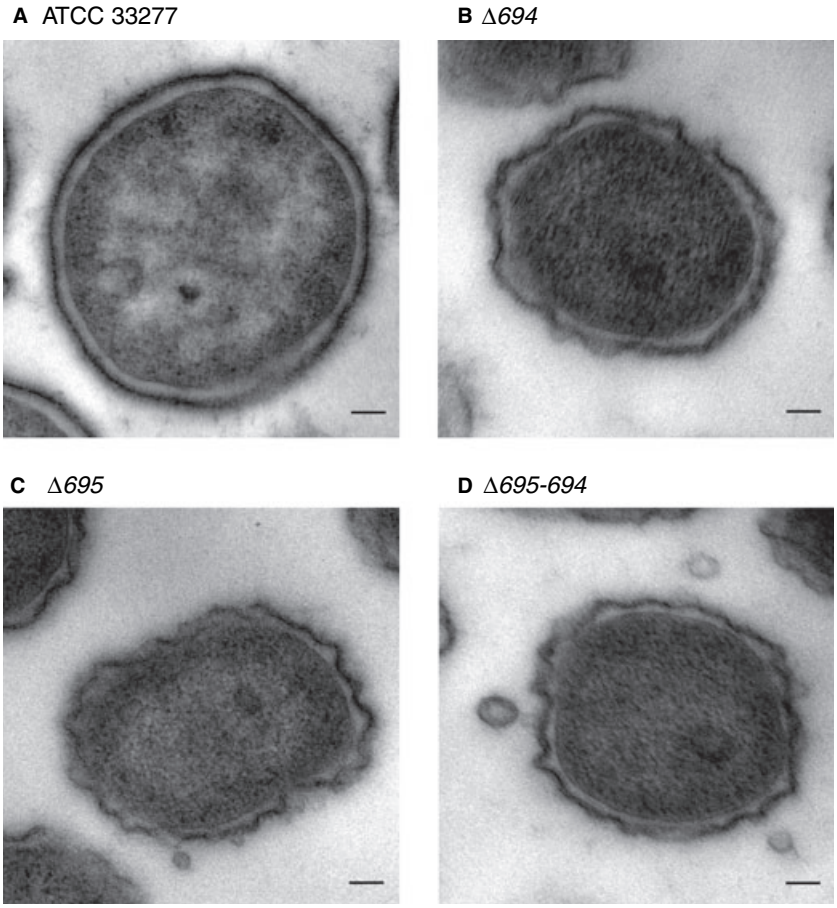


*Fig. 2.* Electron micrographs of ultrathin-sectioned cells in the parent and Pgm6/7 mutants. The cells were stained with uranyl acetate and lead citrate; bars represent 0.1 μm. (A) ATCC 33277, (B) Δ*694*, (C) Δ*695*, (D) Δ*695–694*.

*Table 1.* Gingipain activities in purified membrane vesicles

| Strain | Amount of vesicles[1] (mg) | Gingipain activity[2] ($\Delta OD_{405}$/mg/min) | | Relative total activity (fold) | |
|---|---|---|---|---|---|
| | | Rgp | Kgp | Rgp | Kgp |
| 33277 | 2.39 | 5.70 ± 0.03 | 0.59 ± 0.09 | 1 | 1 |
| Δ*694* | 12.93 | 10.57 ± 0.64*[3] | 1.03 ± 0.06* | 10.0 | 9.4 |
| Δ*695* | 7.89 | 8.98 ± 0.04* | 0.87 ± 0.07 | 5.2 | 4.9 |
| Δ*695–694* | 9.56 | 8.02 ± 0.13* | 0.90 ± 0.10 | 5.6 | 6.1 |

[1]Vesicles were prepared from 100 ml of the culture supernatant after *P. gingivalis* were anaerobically grown in sTSB medium at 37 °C for 48 h.
[2]Values are the means ± standard deviations of triplicate assays.
[3]$P < 0.05$ compared with ATCC 33277 as determined by the Student's *t*-test.

Aliquots of the whole cell culture, the culture supernatant or the vesicle fraction were used to make a reaction mixture containing 50 mM Tris–HCl (pH 8.5) and 10 mM dithiothreitol. The reaction was started by addition of substrate (at a final concentration of 0.2 mM); this was followed by incubation at 37°C for 10 min. The reaction was stopped by adding 0.2 ml of 50% (v/v) acetic acid. The release of *p*-nitroanilide was determined by measuring the optical density at 405 nm ($OD_{405}$). Protease activity was divided by the $OD_{600}$ values of cell density to normalize all values of the whole cell culture and the culture supernatant.

The enzyme activity of the vesicles was more than 1.5-fold higher in the mutants (Table 1). When total activity was calculated, that of the mutants was five- to 10-fold higher than that of the parent cells. Enzyme activity in the culture supernatants containing membrane vesicles was also three- to five-fold higher in the mutants, whereas that in the whole cell culture only changed slightly (Fig. 4).

When cell viability was examined during the stationary phase (48 h), almost the same number of colony-forming units was detected in the parent as in the mutants. Viability was neither reduced nor different among these strains until 72 h. Thus increased release of vesicles in the mutants was not the result of cell death. To examine whether LPS and gingipains were present in the supernatant in soluble form, a vesicle-free supernatant was prepared as described elsewhere (29). Regardless of the strains used, about 10% of the total gingipain activities found in whole cell cultures remained in the vesicle-free supernatant fraction, and only a small amount of LPS was found in the same fraction (data not shown). Several studies have reported that about 20% of total gingipain activity is distributed in the vesicle-free supernatant (25, 30, 31).

It is not clear why the vesicles in the mutants selectively contained increased amounts and activities of gingipains. Recently, it was reported that gingipains form a large complex with adhesions and LPS on the cellular surface (33). We speculate that the gingipain complex may lose its tight association with the outer membrane when either of the Pgm6/7 proteins is lacking and will tend to be ejected in vesicles.

Vesicles and outer membrane vesicles have been known in gram-negative bacteria for a long time. Recent data show that vesicles produced from pathogenic bacteria can transmit virulence factors to host cells (15). Vesicles from *P. gingivalis* and *Actinobacillus actinomycetemcomitans* possess gingipains (25, 29–31) and leukotoxin (13), respectively. *P. gingivalis* vesicles induce the formation of murine macrophage foam cells (26) and are also potent activators and aggregative factors for murine platelets (28). Microspheres coated with *P. gingivalis* vesicles are adhesive and interact with oral bacteria as well as host cells (5, 12, 34), and they even invade the cells and result in cell death (12, 34). Pgm6/7 mutants producing more vesicles than the parent may be considered to be more virulent in particular conditions. However, because nutrient-limited conditions retard the growth of mutants (22), they are unlikely to be more virulent than the parent *in vivo*. When various *P. gingivalis* strains were investigated, all of them expressed a large amount of Pgm6/7 proteins (11). Therefore, we speculate that spontaneous Pgm6/7 mutants may not occur *in vivo*.

It is proposed that vesicle formation is the result of cell wall turnover during

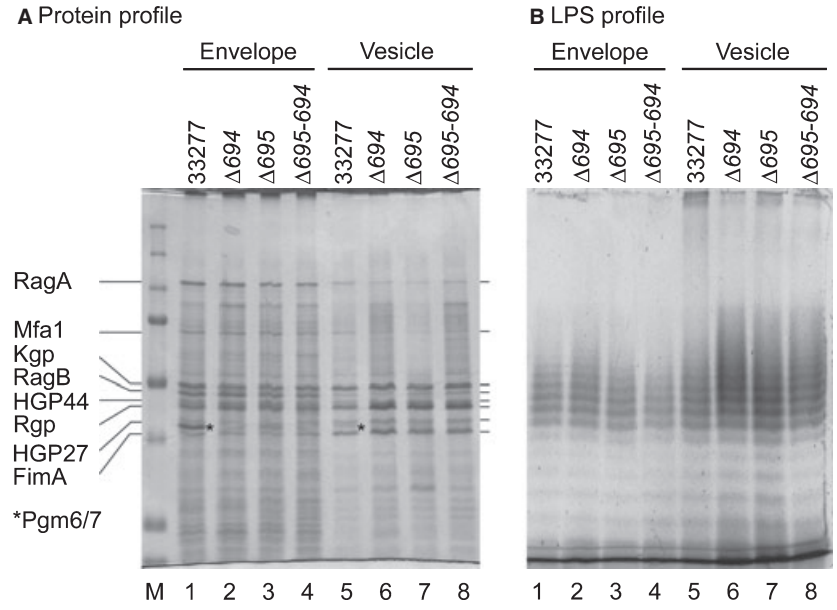


*Fig. 3.* Typical protein and LPS patterns of the envelope and vesicle fractions from the parent and Pgm6/7 mutants. (A) Protein profile: 50 μg protein was applied for the envelope fraction, and 30 μg for the vesicle fraction. The gel was stained with Coomassie brilliant blue and protein bands were identified by N-terminal or internal amino acid sequence analysis. (B) LPS profile: proteinase K-digested proteins from the envelope and vesicle fractions were loaded, the amount of proteins used was the same as above. The gel was stained with Pro-Q emerald 300. Lane M, molecular mass marker. From top to bottom, 250, 150, 100, 75, 50, 37, 25 and 20 kDa. Lanes 1 to 4, envelope fraction; lanes 5 to 8, vesicles. Lanes 1 and 5, ATCC 33277; lanes 2 and 6, Δ*694*; lanes 3 and 7, Δ*695*; lanes 4 and 8, Δ*695–694*.

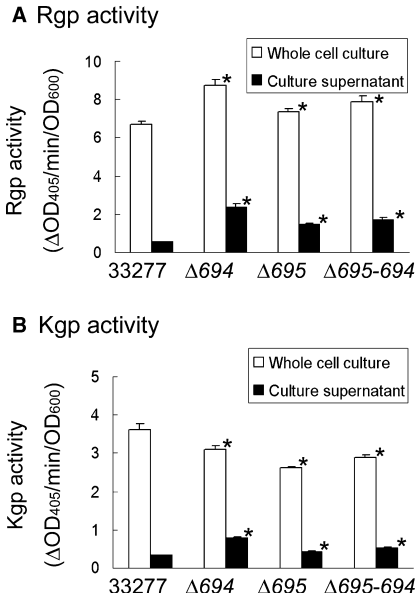


*Fig. 4.* Gingipain activities of the whole cell culture and the culture supernatant from the parent and Pgm6/7 mutants. (A) Rgp activity, (B) Kgp activity. *P. gingivalis* were anaerobically grown in sTSB medium at 37°C for 48 h. All the data were divided by the value of the cell density to normalize proteinase activity per $OD_{600}$ unit. Asterisks indicate $P < 0.05$ compared with ATCC 33277 as determined by Student's *t*-test.

growth (15, 34, 35). Recent molecular models illustrated that vesicle formation would originate in areas that lacked outer membrane–peptidoglycan linkages (15, 18). Several factors affecting vesicle release in gram-negative bacteria have been described. In *E. coli*, the *ompA* mutant itself could not promote vesicle release; however, *ompA, pal* and major lipoprotein *lpp* mutants formed large numbers of vesicles (2). *P. gingivalis* Pgm6/7, homologs of OmpA, may share a function similar to that of Pal because they have a structural similarity to Pal at the C-terminal domain (22). In contrast, deletion of the *yfgL* lipoprotein gene, which is partially involved in the turnover of peptidoglycan, resulted in decreased release of *E. coli* outer membrane vesicles (27), although an autolysin mutant of *P. gingivalis* was reported to produce an elevated number of vesicles (9). In addition, *P. gingivalis* mutants carrying a disruption in the gene encoding Kgp or a glycosyl transferase formed no or very few vesicles (3), although a recent study reported that only a few low-vesiculation mutants and no null mutants were recovered in *E. coli* (19). More work is needed to elucidate the formation and pathogenic roles of vesicles in *P. gingivalis*.

In this study, we have shown that *P. gingivalis* deletion mutants for OmpA-like proteins have increased vesicle formation and lose their membrane integrity to some extent. Thus, Pgm6/7 in *P. gingivalis* was responsible for the maintenance and stability of the outer membrane, and is a factor that modulates vesicle formation.

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