J Periodont Res 2007; 42; 114–118

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00922.x

Dense fimbrial meshwork enhances *Porphyromonas gingivalis* adhesiveness: a scanning electron microscopic study

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Hongo H, Takano H, Morita M. Dense fimbrial meshwork enhances Porphyromonas gingivalis *adhesiveness: a scanning electron microscopic study. J Periodont Res 2007; 42: 114–118. © Blackwell Munksgaard 2006*

Background and Objective: The aim of this study was to determine how the fimbriae of *Porphyromonas gingivalis* function in plaque formation.

Material and Methods: We used scanning electron microscopy to examine aggregates and hemaggregates of fimbria-rich ATCC33277 (parent) and fimbria-poor OZ6301C (*pgmA*-knockout, mutant) strains of *P. gingivalis*. We also assessed the hemagglutination activity of the two strains as an indicator of *P. gingivalis* adhesiveness.

Results: Aggregates of *P. gingivalis* were composed of bacterial chains and clusters. Rich fimbriae projecting from cells of the parent strain tended to bunch and form a dense meshwork among bacterial cells. In contrast, cells of the mutant strain projected fewer fimbriae and the meshwork was looser. Hemaggregates including cells of the parent strain contained a detached, dense fimbrial meshwork that adhered to erythrocytes. Hemaggregates comprising cells of the mutant strain included bacterial chains and clusters that adhered to erythrocytes by shorter fimbriae than those of the parent strain. The hemagglutination titer of the parent strain was 10-fold higher than that of the mutant strain, although the number of fimbriae per cell of the parent strain was only double that of the mutant strain.

Conclusion: The results indicate that *P. gingivalis* adhesiveness is prominently enhanced by the dense fimbrial meshwork. Thus, the virulence of *P. gingivalis* is increased by the presence of rich fimbriae.

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Key words: fimbriae; meshwork; *Porphyromonas gingivalis*; scanning electron microscopy

Accepted for publication May 26, 2006

Porphyromonas gingivalis is a gram-negative anaerobic rod that is usually detected in the periodontal pockets of patients with severe periodontitis (1). Lipopolysaccharide, released from the cell membranes of dead *P. gingivalis* in periodontal pockets, activates osteoclasts to resorb alveolar bone (2,3).

P. gingivalis induces alveolar bone resorption by adhering to the subgingival mucosa where it forms aggregates with gram-positive aerobic bacteria (4). Such aggregates stimulate neutrophils and lymphocytes to induce inflammation in the subgingival mucosa, allowing *P. gingivalis* to invade periodontal pockets further (5). Thereafter, *P. gingivalis* adheres to the cementum adjacent to alveolar bone, where osteoclasts stimulated by lipopolysaccharide released from dead *P. gingivalis* cause alveolar bone resorption (6).

Fimbriae are thought to not only mediate initial *P. gingivalis* adherence to the oral mucosa, but also to contribute to its colonization in subgingival pockets (7). Watanabe *et al.* (8) identified a close correlation between the number of fimbriae and the adhesiveness of *P. gingivalis* to

human oral mucosal epithelial cells and fibrocytes *in vitro*. Du *et al*. (9) proved biochemically that fimbriae and hemagglutinating adhesin mediate the adhesion of *P. gingivalis* to epithelial cells. Thus, physiological and biochemical findings both show that fimbrial adhesiveness and fimbria are involved in hemagglutination. However, morphological images, obtained by scanning electron microscopy (SEM), showing how *P. gingivalis* works in plaques and hemaggregates have not been published.

To visualize the function of *P. gingivalis* fimbria using SEM images, we examined cell aggregates and hemaggregates comprising *P. gingivalis* of parent and mutant strains that are fimbria-rich and fimbria-poor, respectively (10). The hemagglutination titers of both strains were also assessed to determine the relationship between fimbrial density and bacterial adhesiveness.

Material and methods

Strains

P. gingivalis ATCC33277 (parent strain) was obtained from the American Type Culture Collection (Rockville, MD, USA), and the OZ6301C (*pgmA*-knockout, mutant) was supplied by Aichi Gakuin University (Aichi, Japan). The *pgmA* gene of ATCC33277 (11) is inactivated in OZ6301C by Fletcher's DNA cassette that is erythromycin resistant (12). Strain OZ6301C expresses about half of the amount of fimbrilin found in ATCC33277 (10).

Method of incubation

The bacteria were suspended in 0.5 ml of Brain–Heart infusion (BHI) broth (Difco, Detroit, MI, USA) diluted to 50% of the standard concentration. One drop of the suspension medium was streaked onto plates containing Brucella agar medium (Kyokutou, Tokyo, Japan) and the plates were incubated under anaerobic conditions at 37°C.

To confirm the effectiveness of the DNA cassette, the *pgmA*-knockout strain was incubated under anaerobic conditions for 3 d at 37°C on agar media containing erythromycin (final concentration, 20 μg/ml). Pieces of colonies scraped from plates using an inoculating loop were mixed with 300 ml of GAM bouillon (Nissui, Tokyo, Japan). Flasks containing parent and mutant strains were incubated for 3.0–3.5 and 4.0–4.5 d, respectively. The mutant strain grew more slowly than the parent strain, and was incubated for 1 d longer than the parent strain to equalize the amount of aggregates of both. We incubated parent and mutant strains 23 and 12 times, respectively, to generate the data shown here.

Preparation of *P. gingivalis* cells for SEM

Flasks containing mutant and parent *P. gingivalis* aggregates in GAM bouillon were gently shaken to form homogeneous cell suspensions of about the same color.

One drop of each suspension was removed using a sterilized chopstick and placed on silane-coated 7-mm^2 glass slides. After 10 min, the slides were gently immersed in Petri dishes containing 0.02 M cacodylate buffer (pH 7.4), 0.15 M NaCl and 1% glutaraldehyde. The samples were fixed for 5 h, dehydrated in a graded series of ethanol, immersed in isoamyl acetate, dried in a critical point dryer, coated with osmium vapor for 1 s using an osmium plasma coater (Nippon Laser, Tokyo, Japan) and observed by SEM (S4500; Hitachi, Tokyo, Japan). We initially selected fields where the bacterial cells were intact at low magnification, and then photographed them at 5×10^4 magnification. Bacterial cells were considered intact or damaged when the fimbriae were rich and long, or sparse and shorter, respectively.

Semiquantitative method for counting fimbriae

We counted the number of fimbriae per cell from 203 and 74 photographs of 10–12 cells of parent and mutant strains, respectively, in which cells did not overlap. The fimbriae were counted near the cell surface, where they were not too bunched, under a magnifying glass (×8) on a negative film placed on a light box. Half of the fimbriae per cell were counted in this manner.

Hemagglutination activity

The hemagglutination titer of *P. gingivalis* was examined using the modified method of Chu *et al*. (13). Cell suspensions in liquid medium, taken from flasks prepared as described above, were diluted from eight- to 350-fold with saline and then mixed with an equal amount of 5% chicken erythrocytes in saline (Nippon Biotest, Tokyo, Japan) on blood test plates. To prevent damage of the fimbriae, the bacteria were not washed before the hemagglutination test, and mixed using a micropipette with a tip that had been cut with scissors. After 1 h of incubation at room temperature, the highest dilution of the bacterial cultures that induced hemagglutination was recorded.

To determine the optical density of the cells before dilution, cultures were thoroughly dispersed, liquid medium was replaced with saline by centrifugation, and the optical density of each cell suspension was measured by spectrophotometry at 600 nm (UV mini 1240; Shimadzu, Kyoto, Japan).

Preparation of SEM samples of *P. gingivalis* hemaggregates

Hemaggregates of *P. gingivalis* were collected from the bottom of blood test plates, placed on silane-coated glass slides using a micropipette and processed, as described above, for SEM.

Results

SEM observation of *P. gingivalis* aggregates of parent and mutant strains cultured in liquid medium

Cells of *P. gingivalis* with fimbriae were distinct (Fig. 1). Abundant cells of the parent strain adhered well to glass slides, whereas mutant cells adhered sparsely (Fig. 2). Aggregates of the parent strain were composed of long and abundant bacterial chains and clusters of 20–80 μm in diameter,

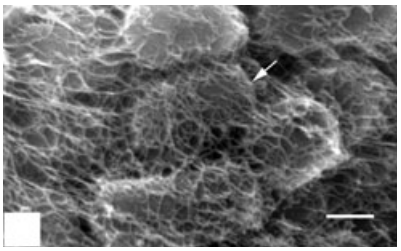

Fig. 1. Scanning electron micrograph of *Porphyromonas gingivalis* in parent strain cluster. Fimbriae bunch and form a dense meshwork containing embedded bacterial cells (arrow). Bar, 0.3 μm.

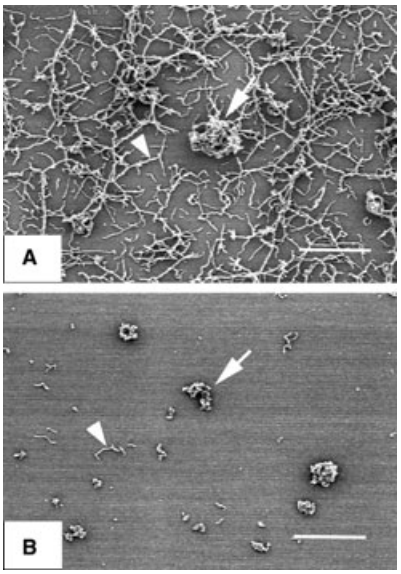

Fig. 2. Scanning electron micrograph of *Porphyromonas gingivalis* cultured in liquid medium. (A) Parent strain. (B) Mutant strain. Bar, 30 μm. More parent *P. gingivalis* strain is attached to slide than mutant. Chains of the parent strain are long and abundant, whereas chains of the mutant are short and sparse. Clusters and chains are indicated by arrows and arrowheads, respectively.

of which the margins were wreathed with chains (Figs 2A and 3A). In contrast, those of the mutant strain were composed of few chains and isolated clusters of 10–15 μm in diameter, but the margins were also wreathed with chains (Figs 2B and 3B). Aggregates of the mutant strain comprised shorter chains and smaller clusters than those of the parent strain.

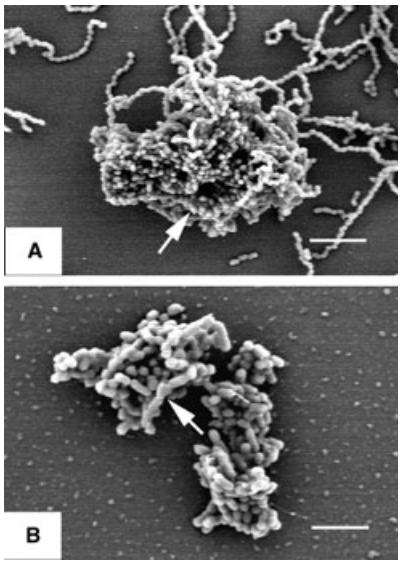

Fig. 3. Enlarged photographs of bacterial cluster shown in Fig. 1. (A) Parent strain. (B) Mutant strain. Bar, 3 μm. Clusters of parent and mutant strains are wreathed with chains (arrow).

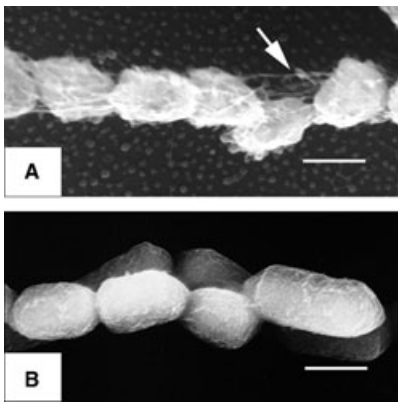

Fig. 4. Enlarged scanning electron micrograph of bacterial chains. (A) Parent strain. (B) Mutant strain. Bar, 0.5 μm. Fimbriae are rich on parent cells. Bacterial cells of parent strain in chains connect to other cells via dense meshworks (arrow) (A). In contrast, fimbriae are sparse in chains of the mutant strain and the fimbrial meshwork is virtually absent (B).

Cells of both strains were short rods of about 0.8 and 0.5 μm in long and short diameters, respectively. Cells of the parent strain had abundant fimbriae that formed dense fimbrial meshworks in bacterial chains and clusters (Figs 1, 4A and 5A). Cells in chains of the parent strain were connected to

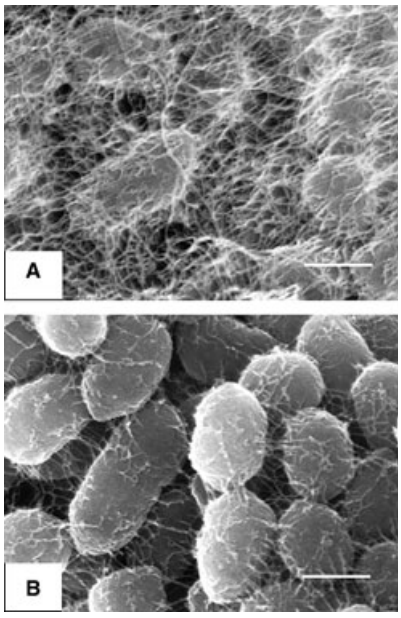

Fig. 5. Enlarged scanning electron micrograph of bacterial clusters. (A) Parent strain. (B) Mutant strain. Bar, 0.5 μm. Bacteria in parent strain clusters are connected by rich, long fimbriae that form dense meshworks (A); those of the mutant strain are connected by fewer, shorter fimbriae that form loose meshworks (B).

other cells by fimbria meshworks (Fig. 4A), whereas chains on the surface of mutant cells contained fewer and shorter fimbriae (Fig. 4B). Clusters of the parent strain contained bunched fimbriae that formed dense, large-scale meshworks with apparently embedded bacteria (Figs 1 and 5A). On the other hand, clusters of the mutant strain contained sparse fimbriae that formed loose meshworks in intercellular spaces (Fig. 5B).

Comparison of the number of fimbriae per cell in aggregates

The numbers of fimbriae per parent and mutant cell were 196.4 ± 23.6 and 93.2 ± 10.6 (means ± standard deviation), respectively. The parent strains contained about twice as many fimbriae as the mutant strain.

SEM observation of hemaggregates of parent and mutant cells

The dense fimbrial meshworks were detached from parent bacterial cells in

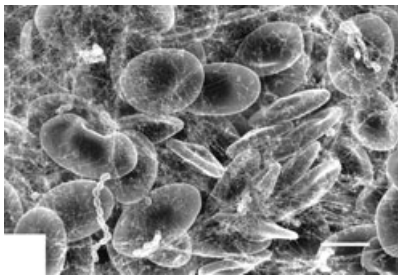

Fig. 6. Scanning electron micrograph of hemaggregate including *Porphyromonas gingivalis* of the parent strain. Erythrocyte aggregation by *P. gingivalis* of the parent strain is mediated by dense fimbrial meshwork including some bacterial cells. Bar, 10 μm.

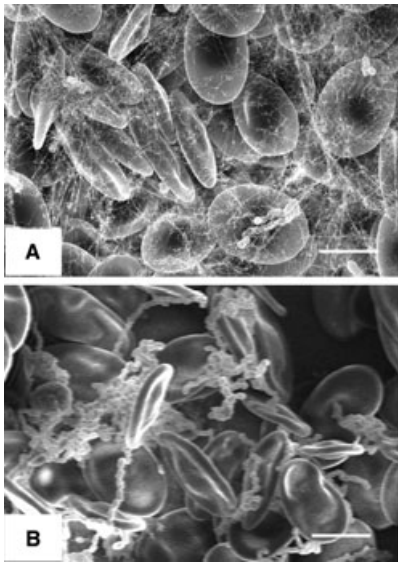

Fig. 7. Scanning electron micrograph of hemaggregates including *Porphyromonas gingivalis*. (A) Parent strain. (B) Mutant strain. Bar, 5 μm. Erythrocyte aggregation by the parent *P. gingivalis* strain is mediated by a dense fimbrial meshwork (A); that of the mutant strain appears to be mediated by bacterial clusters and chains (B). Hemagglutination was induced using a higher concentration of mutant cells than of parent cells.

hemaggregates and involved a few bacterial chains with aggregated erythrocytes (Figs 6, 7A and 8A). Bacterial chains with long fimbriae occasionally mediated hemagglutination (Fig. 9A). On the other hand, mutant bacterial chains and clusters appeared to mediate hemaggregation (Figs 7B and 8B). Mutant cells in

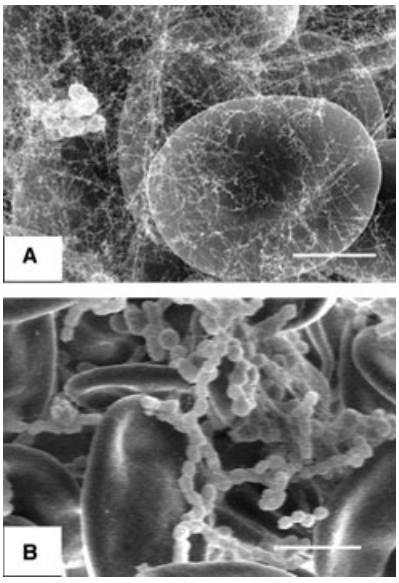

Fig. 8. Enlarged photographs of *Porphyromonas gingivalis* hemaggregate. (A) Parent strain. (B) Mutant strain. Bar, 3 μm. A dense fimbrial meshwork adheres to the surface of erythrocytes in the hemaggregates containing parent strain *P. gingivalis* (A). The meshwork consists of fimbriae detached from cells by using a micropipette to mix bacteria and erythrocytes. Bacterial chains and clusters adhere to the erythrocyte surface in hemaggregates of mutant *P. gingivalis* (B). Hemagglutination was induced using a higher concentration of mutant cells than of parent cells.

chains and clusters had shorter fimbriae than those of the parent strain in hemaggregates (Fig. 9B).

Comparison of hemagglutination titers

The minimal optical density of the parent and mutant strains required to induce hemagglutination, as calculated from the original optical density and the dilution factor, were 0.04 ± 0.02 and 0.4 ± 0.1 (means ± standard deviation) at 600 nm, respectively. Thus, the hemagglutination titer of the parent strain was 10-fold higher than that of the mutant strain.

Discussion

We obtained clear SEM images of *P. gingivalis* with fimbriae. The SEM images revealed that the powerful

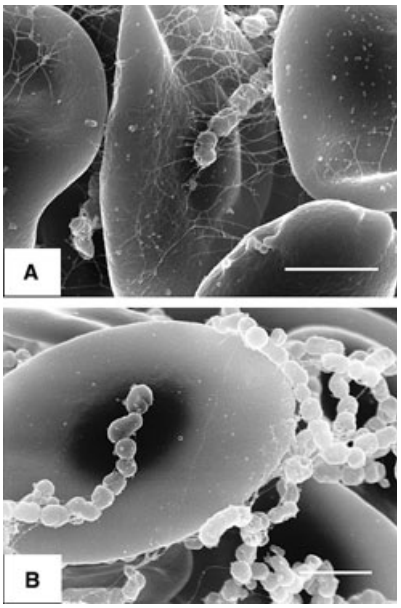

Fig. 9. Enlarged photographs of chains in aggregates of erythrocytes caused by *Porphyromonas gingivalis*. (A) Parent strain. (B) Mutant strain. Bar, 2 μm. Chains of bacterial cells with long fimbriae mediate hemagglutination in hemaggregates caused by the parent strain of *P. gingivalis* (A), and short fimbriae on mutant *P. gingivalis* cells, constituting chains or clusters, also mediate hemagglutination (B). Short fimbriae might be artifacts of mechanical disruption.

adhesiveness and hemagglutination activity depends on dense fimbrial meshworks and that the density of the meshwork depends on the number of fimbriae per cell. This study is the first to show morphological evidence of fimbrial adhesiveness and involvement in bacterial hemagglutination.

We observed fimbriae by SEM because we used a high-power SEM and an osmium plasma coater, incubated the cells in abundant medium and ensured minimal disruption of samples before fixation.

The hemagglutination titer of the parent strain was about 10-fold higher than that of the mutant strain. Optical density is a variable of a hemagglutination titer and reflects the numbers of cells and fimbriae in the sample. As the hemagglutination titer was not proportional to the number of fimbriae per cell, the high hemagglutination titers of the parent strain can be explained by the presence of the dense fimbrial meshwork.

Mutant cells contained fewer and shorter fimbriae in aggregates than the parent strain. We consider that aggregation is difficult because the fimbriae were sparse, and thus easily damaged during sample preparation. The mutant fimbriae in aggregates differed from those, ≈ 0.1 μm in length, described by Park *et al.* (14).

Slots & Gibbons (15) and Okuda *et al.* (16) have shown physiological evidence that fimbriae have adhesive properties and that isolated fimbriae can induce hemagglutination. The former observed fimbrial aggregates in hemaggregates by transmission electron microscopy. Our SEM study revealed a detached dense fimbrial meshwork that mediated hemagglutination. We used a pipette to mix the bacterial cells and erythrocytes on blood test plates. Thus, fimbriae detached from the cells by mechanical disruption formed a dense meshwork that mediated hemagglutination. This finding indicates that the fimbriae themselves are adhesive.

Noiri *et al.* (6) immunohistochemically localized *P. gingivalis* fimbriae in periodontal biopsy tissues obtained from patients with severe periodontitis. They found that *P. gingivalis* fimbriae localize in plaques attached to the cementum. The dense fimbrial meshwork of *P. gingivalis* observed in the present study probably contributes to the attachment of plaques to the cementum, which is the optimal location for alveolar bone resorption.

The SEM images of aggregates of *P. gingivalis* of the parent strain suggest how fimbriae support the process of colonization. Two daughter cells of *P. gingivalis* were connected with fimbriae along the long axis of rods. Thus, the repetition of amitosis causes the formation of long chains supported by fimbrial meshwork.

A bacterial cluster might then be formed by chain aggregation, because the surfaces of clusters were wreathed with such chains. When chains become dense, each cell in a chain connects to other cells in neighboring chains via fimbriae, forming fimbrial meshworks.

Subgingival plaque contains live and dead bacteria (17) and endotoxin released from dead *P. gingivalis* stimulates bone resorption (18). When released deep inside large plaques, endotoxin might diffuse smoothly through fimbrial meshworks.

In conclusion, the presence of rich fimbriae helps *P. gingivalis* to form dense fimbrial meshworks that prominently enhance its adhesiveness. Thus, *P. gingivalis* forms plaques with other bacteria in deep subgingival pockets and becomes a potential threat within the oral cavity.

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