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Role of *Porphyromonas gingivalis* Protease Activity in Colonization of Oral Surfaces

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Cysteine proteases, including Arg-gingipain of *Porphyromonas gingivalis*, have been implicated as important virulence factors in periodontal diseases. These enzymes are also involved in the hemagglutinating activity of the organisms. In order to determine the role of proteases in the colonization of the gingival margin, we have compared the attachment properties of *P. gingivalis* 381 with those of its Arg-gingipain-defective mutant, G-102. Interactions with gram-positive bacteria, human oral epithelial cells, extracellular matrix proteins, and type I collagen were evaluated. In all cases, mutant G-102 was deficient in attachment relative to the parental strain. The mutant's defects could be explained, in part, by the weak autoaggregation displayed by the mutant, which appeared to result from altered fimbrial expression. Both Western blot (immunoblot) and Northern (RNA) blot analyses indicated reduced expression of the major 43-kDa fimbrillin subunit in the mutant. These results suggest that Arg-gingipain may play both direct and indirect roles in the colonization of the gingival margin. In addition, fimbriae may play a direct role in interacting with some host surfaces.

The significant role of *Porphyromonas gingivalis* in periodontal diseases has been well documented (37). These organisms express a number of potential virulence factors which have been implicated in periodontal inflammation (14). Among these, proteases may play a major role in *P. gingivalis* virulence by directly degrading host tissue, activating host proenzymes, or neutralizing host immune systems (18). A strong correlation between gingival protease activity and periodontal inflammation has been demonstrated (43). In addition, a mutant of *P. gingivalis* defective in a cysteine proteinase was recently demonstrated to be less virulent in a mouse model system (8). Therefore, a number of different approaches have suggested that these enzymes are important virulence factors for the organisms.

In addition to degrading host proteins, the proteases of *P. gingivalis* have been implicated in the colonization of the oral cavity (11, 12, 41). Although it is not clear how *P. gingivalis* colonizes the human gingival margin, it has been suggested that this might occur by the organisms directly attaching to saliva-coated teeth (3, 20), by the interaction of the gram-negative anaerobe with gram-positive early colonizers of tooth surfaces (38), or by the binding of the microorganisms to epithelial cells (6) as well as to the extracellular matrix present in the gingival margin (19). In this regard, recent evidence from several laboratories has indicated that a major adhesion of *P. gingivalis* is encoded by a protease gene (1, 32, 45). Therefore, it would be of interest to determine the precise role of individual proteases in the interaction of *P. gingivalis* with host tissue.

Like most organisms, *P. gingivalis* apparently expresses a variety of distinct proteases (32). Recently, several genes coding for protease activity have been isolated and sequenced (31). However, several of these genes contain long regions of homologous sequences (31), and it is not clear whether these represent distinct protease genes. In addition, several *P. gingivalis* genes coding for hemagglutinating activity have been reported to have homology with these protease genes (33). In order to characterize the role of specific proteases in virulence, we have recently constructed a mutant of *P. gingivalis* 381, G-102, which is defective in the RGP-1 cysteine protease (45). This mutant exhibited defects in both protease and hemagglutinating activities, as did protease mutants of strain ATCC 33277 (26). In the present study, we have examined the effects of the protease mutation in G-102 on its ability to colonize various host oral tissues. These results further confirm earlier suggestions that the proteases of *P. gingivalis* are also involved in such interactions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. gingivalis* 381 and mutant G-102, constructed as recently described (45), were maintained anaerobically on blood agar plates containing tryptic soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 1.5% agar, 10% sheep blood, hemin (5 μg/ml), and menadione (1 μg/ml). Mutant G-102 was cultured in tryptic soy broth medium containing erythromycin (10 μg/ml). The fimbrillin-deficient mutant DPG3 (22) was provided by A. Sharma (State University of New York at Buffalo, Buffalo).

Attachment of *P. gingivalis* to oral epithelial cells. Twenty-four-well tissue culture plates were seeded with 5×10^5 human oral epithelial KB cells per ml per well in Dulbecco's modified Eagle medium without antibiotics, and the cultures were incubated overnight. The cells settled overnight and formed confluent monolayers. Bacteria were grown from single colonies for 48 h to late log or early stationary phase. Cultures were harvested, divided into aliquots, washed twice in phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS. Aliquots were added to Dulbecco's modified Eagle medium without serum and antibiotics to give approximately 10^7 cells per ml. Before the assay, the monolayers were washed three times with PBS. One milliliter of bacterial sample was added per monolayer, and each sample was assayed in triplicate. An aliquot of the input bacterial suspension was removed, diluted, and plated for viable counting on tryptic soy broth blood agar plates. Infected monolayers were incubated for 2 h at 37°C in a tissue culture incubator. Monolayers were then washed three times in PBS to remove unattached bacteria, the KB cells were lysed with distilled water, and samples were removed quickly for dilution and viable counting. The level of attachment was expressed as the percentage of bacteria added to the monolayers which attached to the KB epithelial cells.

Binding of *P. gingivalis* to type I collagen, laminin, and fibronectin. Acid-soluble rat tail type I collagen, fibronectin, and laminin were purchased from Sigma Chemical Co. (St. Louis, Mo.). The interaction of *P. gingivalis* with type I collagen was detected with either of two assays. For the binding of soluble type I collagen to *P. gingivalis* cells, an enzyme-linked immunosorbent assay was utilized. Whole cells of *P. gingivalis* 381 and G-102 were suspended in 0.2 M sodium carbonate buffer (pH 9.6) and were adsorbed onto 96-well microtiter plates (16 h, 4°C) as solid-phase antigens (10^7 cells per well). The wells were

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washed with PBS-Tween (PBS containing 0.5% Tween 20; 1 h, 37°C) three times and blocked with 1% bovine serum albumin (BSA) containing PBS-Tween (1 h, room temperature). Type I collagen was added in the range of 10 ng to 5 μg (2 h, 37°C). The wells were then washed with PBS-Tween three times, anticollagen antiserum (1/2,000 dilution; Sigma) was added, and the plates were incubated at room temperature for 1 h. After the plates were washed with PBS-Tween six times, goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (1/5,000; Bethesda Research Laboratories, Gaithersburg, Md.) was added for 1 h, and the plates were again washed with PBS-Tween six times. Then, 200 μl of the substrate (0.05% *o*-phenylenediamine dihydrochloride [Bethesda Research Laboratories]–0.01% H_2O_2 in 0.1 M citrate buffer [pH 4.5]) was added. The binding activities were measured by spectrophotometry at A_{450} in duplicate.

The binding activities of *P. gingivalis* to immobilized laminin, type I collagen, and fibronectin were detected as previously described (9). Briefly, the matrix proteins were diluted in bicarbonate buffer (50 mM Na_2CO_3-$NaHCO_3$ [pH 9.5]) to 50 μg/ml and were applied to microtiter plates (Immulon 2; Dynatech Laboratory Inc., McLean, Va.) at 5 μg of protein per well (16 h, 4°C). The wells were washed three times with PBS and incubated with PBS-Tween (1 h, 37°C). *P. gingivalis* cells were grown to early stationary phase, and the harvested cells (10 ml) were washed with cold PBS and adjusted to an A_{550} of 2.0 in PBS-Tween. The cells were added to the coated microtiter wells and incubated for 3 h at 37°C. In some experiments, the densities of the bacterial suspensions were varied. The bacterial suspensions were then carefully aspirated, and the wells were washed with PBS-Tween. The plates were stained with crystal violet (Difco) and washed with PBS-Tween four times. The plates were next dried, and numbers of adherent bacterial cells were measured photometrically (570 nm). The binding experiments were also carried out in the presence of BSA (50 μg/ml) as a negative control. All assays were carried out in duplicate, and the standard deviations were determined.

Autoaggregation of *P. gingivalis*. *P. gingivalis* 381 and mutant G-102 were cultured to early stationary phase. The cells were then washed with PBS twice, vortexed, and suspended with PBS to an A_{550} of 1.0. Each cell suspension in a total volume of 2 ml was monitored for autoaggregation by measurement with a spectrophotometer of the decrease in A_{550} over time at 37°C (25).

Attachment of *P. gingivalis* to gram-positive bacteria. This assay was similar to that outlined by Stinson et al. (42). *P. gingivalis* cells were linked to CNBr-activated Sepharose beads (Pharmacia, Uppsala, Sweden) following the supplier's instructions. Briefly, *P. gingivalis* cells were grown to the stationary phase, washed with PBS buffer, and then resuspended in 0.1 M Na_2CO_3–0.5 M NaCl (pH 7.2) to an optical density of 2 at 600 nm. CNBr-activated Sepharose beads (0.2 g) preactivated with 1 mM HCl were added to the *P. gingivalis* suspension (2×10^9 cells), and the suspension mixture was incubated for 1 h at 22°C. The suspension mixture was blocked with 0.1 M Tris-HCl (pH 7.8) buffer and then washed with 0.1 M sodium carbonate buffer. The mixture was washed with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl and then washed with 0.1 M Tris-HCl buffer (pH 7.8) containing 0.5 M NaCl for three cycles. The pellets (Porphybeads) were then resuspended in PBS buffer. *Streptococcus gordonii* Challis was radiolabeled following growth in Todd-Hewitt broth containing [*methyl*-^{3}H]thymidine (New England Nuclear, Boston, Mass.) at 2.5 μCi/ml. The cultured cells were washed twice with PBS buffer and suspended to an optical density of 1.0 (A_{600}). For the adherence assays, 0.5 ml of radiolabeled *S. gordonii* suspension (1.0×10^8 cells) was combined with 0.5 ml of Porphybead (1.0×10^8 *P. gingivalis* cells) suspension and mixed at 22°C for 1 h. Duplicate samples were collected by filtration on polycarbonate membranes with 8-μm pores (Nuclepore Corp.) and counted by scintillation spectrometry. The results were normalized relative to the *P. gingivalis* cells attached to the beads following determination of cellular protein content with Coomassie Plus protein assay reagent (Pierce, Rockford, Ill.).

Expression of FimA protein from *P. gingivalis*. *(i) Immunoblot of P. gingivalis* with anti-FimA serum. Whole cells from *P. gingivalis* 381 and mutant G-102 were utilized as samples. Washed cells were extracted following sonication (10 cycles of 1 min each at 4°C in a Branson Ultrasonics [Danbury, Conn.] sonicator, model 450). Each extract was loaded onto sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gels, and Western immunoblot analysis was carried out as described previously (44). Anti-FimA antiserum was used at a 1:1,000 dilution and was kindly provided by A. Sharma.

(ii) Expression of *fimA* mRNA from *P. gingivalis*. Total RNA was isolated from *P. gingivalis* cells grown to mid-log phase as recently described (5). Equal amounts of RNA (10 μg) were loaded onto 1.0% agarose–2.2 M formaldehyde gels, electrophoresed in MOPS (morpholinepropanesulfonic acid) buffer, and transferred onto a Hybond N membrane (Amersham, Arlington Heights, Ill.) following capillary transfer. The *Bam*HI-*Nco*I-digested fragment of pEfim (36) containing the intact *fimA* gene was used as the probe. Detection of mRNA was carried out by using the enhanced chemiluminescence detection system (Amersham) as directed by the supplier.

Electron microscopy. The fimbriae of strain 381 and mutant G-102 were examined with a transmission microscope. Bacterial cells were collected by centrifugation ($8,500 \times g$ for 10 min), washed, and resuspended (5×10^8 cells per ml) in PBS. Ten microliters of cell suspension was applied to a copper grid coated with thin Formvar film and air dried. The cells were then negatively stained for 1 min with 2% uranyl acetate in sterile water, air dried, examined, and photographed with a Hitachi H-600 electron microscope operating at 75 kV. The cells photographed were representative of at least 80% of the cells examined.

TABLE 1. Binding of *P. gingivalis* to epithelial cells

Strain	% Attachment (mean ± SD)	*n*[a]
381	24.4 ± 9.0	5
G-102	11.6 ± 5.7[b]	5
DPG3	0.5 ± 0.3	4

[a] Number of independent experiments with triplicate samples.
[b] Significantly different from value for 381 by the Student *t* test with a *P* value of <0.0276.

RESULTS

Attachment of *P. gingivalis* to epithelial cells. Since recent results have suggested that the adhesion domain of the Arg-gingipain protease may mediate attachment of *P. gingivalis* to eucaryotic cells (1, 26, 32, 45), it was of interest to compare the abilities of the protease mutant G-102 and its parental strain, 381, to bind to oral epithelial cells (Table 1). The results demonstrated that mutant G-102 cells displayed approximately half of the binding capacity of the parental cells. Since, as described below, mutant G-102 was also demonstrated to be altered in fimbrillin expression, it was also of interest to examine a mutant lacking fimbrillin, DPG3, in these assays. DPG3 displayed negligible binding to the epithelial cells.

Attachment of *P. gingivalis* to gram-positive bacteria. The ability of *P. gingivalis* to interact with gram-positive early colonizing bacteria of the gingival margin may be an important factor for virulence (38), and proteases have been implicated in such interactions (41). Therefore, it was of interest to compare the relative abilities of mutant G-102 and its parental strain to interact with the gram-positive early colonizers *S. gordonii* and *Actinomyces naeslundii*. Quantitations of bacterial interactions have been made previously utilizing assays that were based upon the formation of interbacterial aggregates which could be removed from suspensions following low-speed centrifugation (16). In such assays, mutant G-102 displayed poor interaction with gram-positive bacteria compared with its parental strain (data not shown). However, the autoaggregation of strain 381 complicated the interpretation of the resulting data. Therefore, an assay obviating *P. gingivalis* autoaggregation as a confounding factor was utilized. The assay involved the covalent attachment of the *P. gingivalis* strains to Sepharose beads and then the quantitation of labeled *S. gordonii* binding to the Porphybeads. These results indicated that mutant G-102 displayed a reduced capacity to interact with *S. gordonii* relative to the parental strain (Table 2). The *fimA* mutant, DPG3, exhibited even weaker binding to the gram-positive bacterium.

Attachment of *P. gingivalis* to matrix proteins. The attachment of periodontopathic bacteria to the components of the

TABLE 2. Adherence of ^{3}H-labeled *S. gordonii* to *P. gingivalis*-coated Sepharose beads

Strain	Adherence index[a]	% of control[b]
381	3,556.0 ± 344.5	100
G-102	1,730.0 ± 278.5	48.6
DPG3	1,150.0 ± 80.5	32.4

[a] Expressed as counts per minute per milligram of *P. gingivalis* protein. Values are quotients minus background following incubation with equal amounts of *P. gingivalis*-coated Sepharose beads (average ± standard deviation).
[b] Binding to strain 381 was set at 100%.

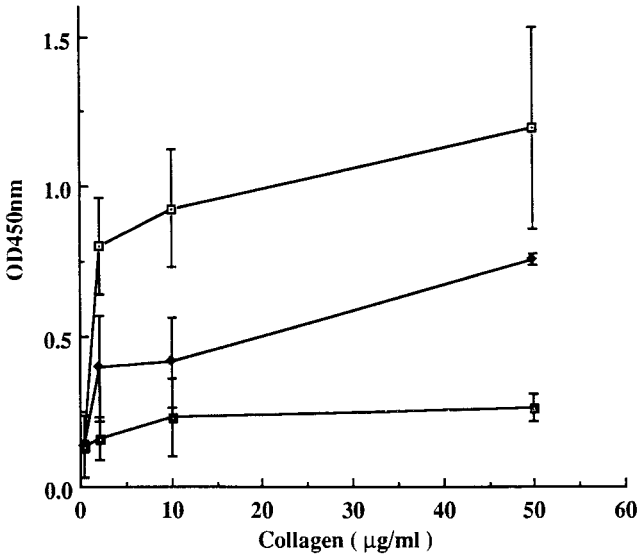

FIG. 1. Binding of soluble type I collagen to *P. gingivalis*. Microtiter plates were precoated with *P. gingivalis* 381 (□) and G-102 (◆) (10^7 cells per well), and the bacteria were incubated with soluble type I collagen at the indicated concentrations. Binding activities were detected with anticollagen serum. *E. coli* JM109 (■) was utilized as a negative control. Values are means ± standard deviations. OD 450 nm, optical density at 450 nm.

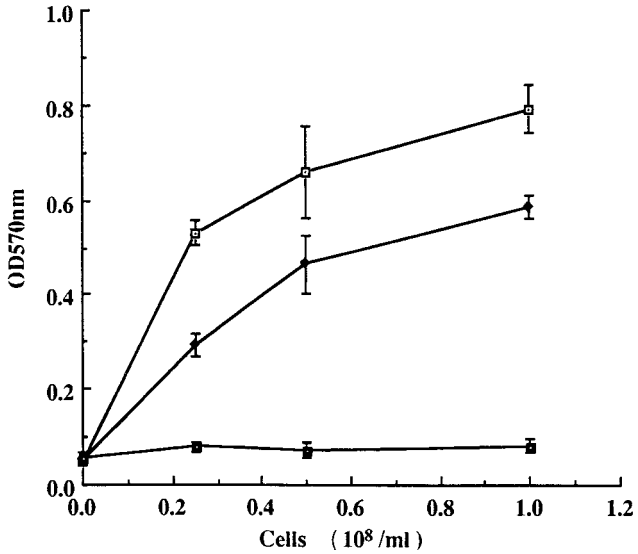

FIG. 2. Binding of *P. gingivalis* to immobilized laminin. *P. gingivalis* 381 (□) and G-102 (◆) were incubated with precoated laminin (50 μg/ml) on microtiter plates. Binding activities were quantitated following spectrophotometric quantitation of cells stained with crystal violet. *E. coli* JM109 (■) was used as a negative control. Values are means ± standard deviations. OD 570 nm, optical density at 570 nm.

extracellular matrix and to type I collagen, a major component of the supporting structure of teeth, may also play a role in *P. gingivalis* colonization of the gingival margin. A comparison of the attachment of strain 381 and that of its protease mutant G-102 to soluble type I collagen indicated that the mutant was defective in interacting with this protein (Fig. 1). As a negative control, *Escherichia coli* was not able to significantly bind collagen. Interactions of both strains with immobilized type I collagen also indicated that mutant G-102 was defective in binding to the substrate (data not shown).

Furthermore, when the *P. gingivalis* strains were compared for their relative abilities to bind to immobilized laminin (Fig. 2) and fibronectin (Fig. 3), it was also observed that mutant G-102 displayed reduced binding capacities. These results suggested that Arg-gingipain protease could play a role, either directly or indirectly, in the attachment of *P. gingivalis* to the extracellular matrix in the gingival margin.

Autoaggregation of *P. gingivalis*. Since mutant G-102 exhibited reduced binding to a variety of different substrates, including matrix proteins, bacterial surfaces, and eucaryotic cells, it appeared unlikely that the adhesin domain of the Arg-gingipain proteases (29) mediated all of these interactions. However, one possible explanation for these multiple defects is that the protease might be involved in the autoaggregation of *P. gingivalis*. Since the binding assays all quantitated the number of bacteria which bound to a substrate, reduced autoaggregation would result in fewer *P. gingivalis* cells accumulating on these surfaces. Therefore, the effect of the *rgp-1* mutation on the autoaggregation of strain 381 was determined (Fig. 4). Parental strain 381 autoaggregated strongly when incubated at 37°C, while mutant G-102 displayed weaker, but significant, self-aggregation. By comparison, the mutant lacking fimbrillin, DPG3, displayed no visible autoaggregation under these conditions. Therefore, these results implicated fimbriae as an important factor in *P. gingivalis* autoaggregation and further suggested the possibility that the *rgp-1* mutation in strain G-102 resulted in decreased expression of fimbriae.

To confirm that the multiple colonization defects displayed by mutant G-102 resulted from the inactivation of the *rgp-1* gene, spontaneous revertants of mutant G-102 were isolated following growth of the mutant in the absence of antibiotic selective pressure. One such revertant resulting from recombination of the two partial copies of the *rgp-1* gene present on the G-102 chromosome (45) was utilized to confirm that revertants displayed wild-type levels of protease, hemagglutinin, and autoaggregation activities (data not shown). These results

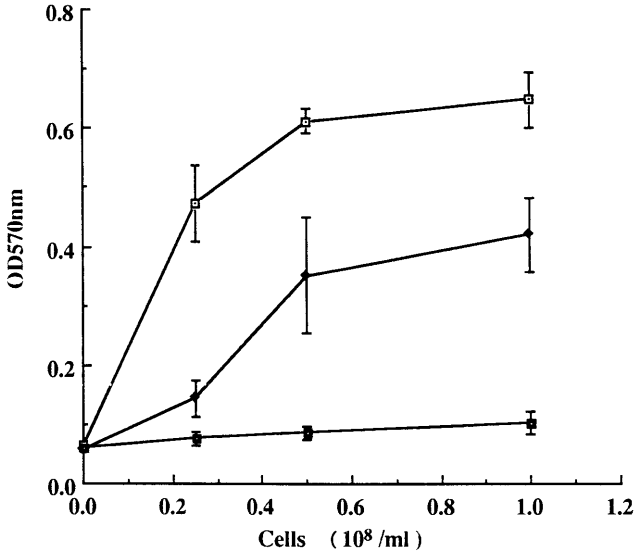

FIG. 3. Binding of *P. gingivalis* to immobilized fibronectin. *P. gingivalis* 381 (□) and G-102 (◆) were incubated with precoated fibronectin (50 μg/ml) on microtiter plates. Binding activities were detected following spectrophotometric quantitation of cells stained with crystal violet. *E. coli* JM109 (■) was used as a negative control. Values are means ± standard deviations. OD 570 nm, optical density at 570 nm.

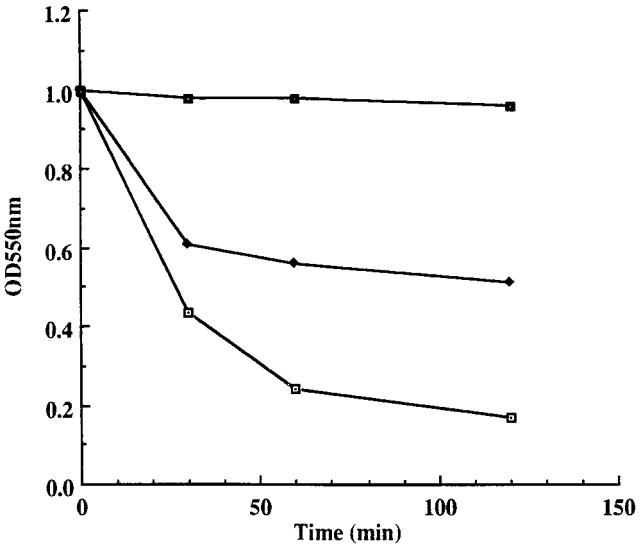

FIG. 4. Autoaggregation of *P. gingivalis* cells. *P. gingivalis* 381 (□), G-102 (◆), and DPG3 (■) were suspended in PBS buffer (optical density at 550 nm [OD_{550}nm], 1.0). Aggregation was determined by measuring the decrease in A_{550} at the indicated intervals.

confirmed that the multiple properties displayed by the mutant resulted from a single gene mutation.

Electron microscopic examination of mutant G-102. In order to examine directly the expression of fimbriae in mutant G-102, electron micrographs of representative mutants and the parental organisms were compared (Fig. 5). Following negative staining, bundles of fimbriae were clearly visible on the surface of parental strain 381. However, no such structures were observed on the surface of the mutant. Instead, low-density long fibrils were associated with this strain. Therefore, direct examination of the cells indicated that mutant G-102 was indeed altered in fimbrial expression. Reduced fimbrial expression was also suggested by the reduced hydrophobicity of mutant G-102 relative to strain 381 (data not shown). It was also observed that the mutant cells displayed larger amounts of vesicular structures (blebbing) than did cells of the parental organism.

Expression of the FimA subunit protein in mutant G-102. One possible explanation for the absence of normal fimbriae on the surface of mutant G-102 would be that the mutant is altered in the expression of the major fimbrillin subunit protein, FimA (46). Therefore, the relative levels of the FimA protein in the mutant and parental strain were compared by Western blotting (Fig. 6). These results indicated that lower levels of FimA were associated with the mutant cells than with cells of the parental organism. In addition, the estimated mass of the FimA protein in the mutant, 43 kDa, indicated that normal processing of the protein in the mutant had occurred (46). In order to determine whether the decreased expression of the FimA protein in the mutant was transcriptionally regulated, Northern (RNA) blot analysis of the *fimA* mRNA was carried out utilizing a *fimA* probe (Fig. 7). These results clearly indicated that lower levels of *fimA* mRNA were expressed in the protease mutant cells than in the parental cells. These results suggested that the reduced expression of the fimbriae on the surface of mutant G-102 resulted from an alteration in the transcription of the *fimA* gene due to the protease defect.

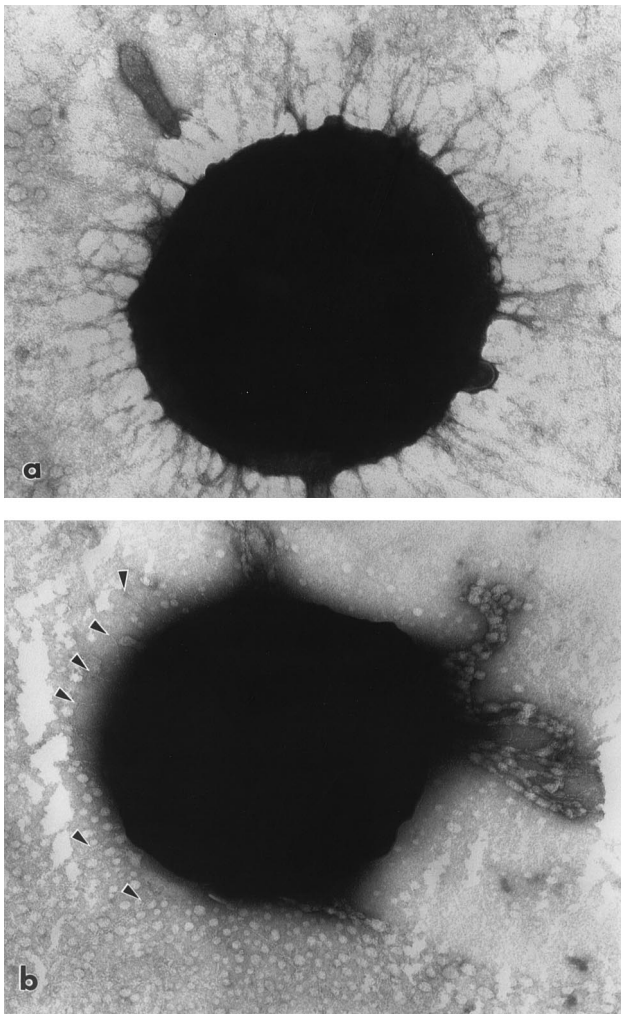

FIG. 5. Electron microscopic analysis of *P. gingivalis*. (a) Parental strain 381 showing intact fimbriae; (b) Arg-gingipain-deficient mutant G-102 showing altered fimbrial expression. Arrowheads, thin filamentous structures. Magnification, ×8,100.

DISCUSSION

Bacterial proteases have been proposed to act as virulence factors by providing essential amino acids to organisms, degrading host immune proteins, exposing host cell cytotopes, and playing a role in adhesion to host tissue (11, 23). Relative to other oral bacteria, *P. gingivalis* has been demonstrated to be highly proteolytic (14). Recent results have indicated that the major proteases produced by these organisms are cysteine proteases (4, 15, 35). Proteases exhibiting both Arg bond (30) and Lys bond (30, 34) specificities have been purified from *P. gingivalis*. The gene coding for the Arg-specific cysteine protease, *rgp-1*, has recently been isolated and sequenced (1, 29). In addition, genes coding for proteases and exhibiting extensive homology with the *rgp-1* gene have been isolated and sequenced (7, 17, 27). However, the relationship between these genes has not yet been clarified and the number of distinct cysteine protease genes in each *P. gingivalis* strain has not yet been determined.

P. gingivalis may initially colonize the gingival margin by attaching directly to saliva-coated teeth (3, 20), by aggregating with gram-positive bacteria which are early colonizers of the

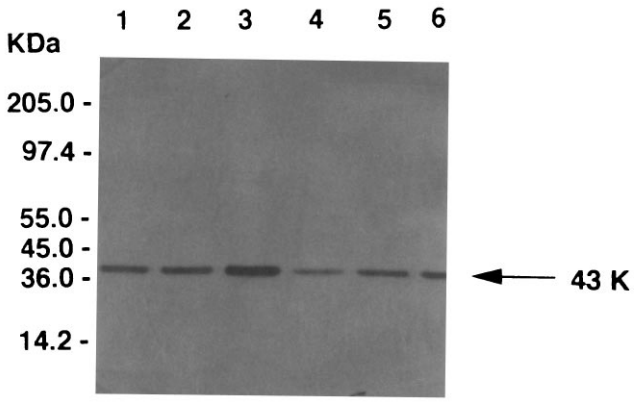

FIG. 6. Western blot analysis of *P. gingivalis* 381 and G-102 with anti-FimA sera. Lanes 1 to 3, 381 whole cells (1.0×10^5, 5.0×10^5, and 1.0×10^6 cells per lane, respectively); lanes 4 to 6, G-102 whole cells (1.0×10^5, 5.0×10^5, and 1.0×10^6 cells per lane, respectively). The FimA protein (43 kDa) is indicated by the arrow. Molecular mass markers are on the left.

gingival margin (38), by binding to type I collagen, which is the primary supporting structure of teeth (11), by interacting with components of the extracellular matrix (19), or by directly binding to epithelial cells which line the gingival margin (6). Inflammation induced by the presence of early-colonizing gram-positive bacteria may expose type I collagen and extracellular matrix proteins for interaction with *P. gingivalis*. However, it is not clear whether direct interaction with the tooth pellicle is important in *P. gingivalis* incorporation into subgingival plaque. Recent results utilizing protease inhibitors have suggested a potential role for the cysteine proteases in *P. gingivalis* attachment to gram-positive bacteria (21, 41), as well as to proteins of the extracellular matrix (19). The present results have demonstrated that a cysteine protease mutant of strain 381 defective in the *rgp-1* gene, G-102, displayed reduced interaction with gram-positive bacteria, immobilized extracellular matrix proteins, type I collagen, and human epithelial cells. Therefore, the utilization of the monospecific mutant has

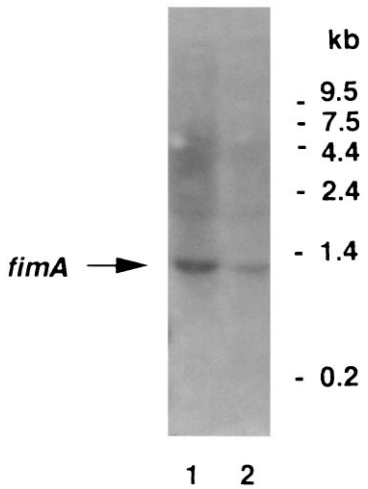

FIG. 7. Northern blot analysis of *fimA* mRNA from *P. gingivalis* 381 and G-102. Equal amounts of RNA (20 μg) were taken from 381 cells (lane 1) and mutant G-102 cells (lane 2). The position of *fimA* mRNA (1.35 kb) is indicated by an arrow. The probe was a 984-bp fragment containing the *fimA* gene. Molecular size markers are on the right.

supported the suggestion that proteases could play a significant role in *P. gingivalis* colonization of the gingival margin.

The proteases of *P. gingivalis* could play an important role in colonization either directly, by acting as an adhesin, or indirectly, by affecting the expression of an adhesin. The former role has been suggested for the RGP-1 cysteine protease in hemagglutination since an adhesin domain has been identified in the carboxyl-terminal region of the protein (1, 29). This suggestion has been confirmed by the demonstration that *P. gingivalis* protease mutants are also defective in hemagglutination (26, 45). It is likely that the adhesin recognizes a receptor common to eucaryotic cells, and this may explain the inability of mutant G-102 to interact normally with erythrocytes (45) and buccal epithelial cells (Table 1). However, confirmation of this suggestion is required. Previous results have demonstrated that *P. gingivalis* has the ability to attach to human cells, including erythrocytes and buccal epithelial cells (6, 28). This property may enable the organisms to colonize the epithelial lining of the gingival margin. The present results further suggest another factor which may influence *P. gingivalis* attachment to epithelial cells. Since the mutant of strain 381 lacking fimbrillin does not bind efficiently to epithelial cells (Table 1) and the protease mutant is defective in normal fimbrial expression (Fig. 5), the reduced interaction of mutant G-102 with the epithelial cells could result, in part, from this defect. This may be a direct result of the defect, if the fimbriae are directly involved in attachment to epithelial cells, or an indirect one, stemming from decreased aggregation of the *P. gingivalis* cells. The former possibility is further suggested by recent results demonstrating that a *fimA* mutant of *P. gingivalis* 33277 exhibited weak binding to fibroblast and epithelial cells (13).

It seems unlikely that the adhesin domain of the Arg-gingipain protease which recognizes eucaryotic cells is also directly involved in interaction with gram-positive bacteria and with the extracellular matrix proteins, although the present results do not rule out this possibility. However, it is likely that the reduced attachment of mutant G-102 to these diverse surfaces results, in part, from the reduced autoaggregation of the mutant cells. Since the assays employed quantitate the total numbers of bacteria which attach to these surfaces, a reduction in autoaggregation would be reflected in apparent reduced attachment. Since the present results indicate that the fimbriae of *P. gingivalis* play an important role in autoaggregation (Fig. 4), autoaggregation is likely to play an important role in multiple colonization mechanisms in vivo. It is also possible that the fimbriae themselves play a direct role in the interaction of *P. gingivalis* with some of these varied surfaces (10). Therefore, the fimbriae of these organisms may influence attachment to gram-positive bacteria and the extracellular matrix proteins both directly and indirectly.

The present results demonstrated that mutant G-102 was also defective in interacting with *S. gordonii* as previously described (Table 2). However, in a recent study (22) the afimbriate mutant DPG3 appeared to bind normally to *S. gordonii* G9B. It is not clear whether the utilization of different strains of the gram-positive bacterium or the use of distinct assays is responsible for these differences. In addition, since multiple *P. gingivalis* adhesins could be involved in such interactions, the differential expression of fimbriae on the surface of the mutants could be responsible for such differences. Therefore, the precise role of the *P. gingivalis* cell surface receptor(s) in such interactions still needs to be defined.

The present results are another indication of the importance of the environment in influencing the expression of the *P. gingivalis* fimbriae. Recent results have indicated that the elevated temperatures which occur in inflamed tissue can reduce

the expression of these cell surface appendages (2). The present results further suggest that a reduction in the availability of essential amino acids resulting from a protease deficiency can also reduce the expression of the fimbriae. This regulation appears to occur at the level of transcription (Fig. 6), since mutant G-102 appears to transcribe the *fimA* gene at a reduced rate relative to parental strain 381. However, the molecular basis for the regulation of fimbrial expression in *P. gingivalis* remains to be determined.

It is also of interest that mutant G-102 appeared to express extensive surface blebbing which results in increased extracellular vesicle formation (Fig. 5). Such blebbing is observed in cultures of *P. gingivalis* that have been nutritionally stressed following iron limitation (24) or following growth into the stationary phase (39). Since the vesicles are rich in protease activity, this property appears to be a stress response of the organisms to alleviate amino acid deprivation. Such alterations in the potential virulence properties of *P. gingivalis* as a result of environmental changes may be of clinical significance since periodontal infections are cyclical (40). Therefore, reversible alterations in the properties of the periodontopathic bacteria present in subgingival plaque could play a role in such fluctuations. It is clear that studies directed toward determining the molecular basis for such changes will be important in defining the etiology of periodontal diseases.

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