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Resistance of *Porphyromonas gingivalis* ATCC 33277 to Direct Killing by Antimicrobial Peptides Is Protease Independent▽

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Antimicrobial peptides are short, positively charged, amphipathic peptides that possess a wide spectrum of antimicrobial activity and have an important role in the host's innate immunity. Lack of, or dysfunctions in, antimicrobial peptides have been correlated with infectious diseases, including periodontitis. *Porphyromonas gingivalis*, a gram-negative anaerobe and a major pathogen associated with periodontal diseases, is resistant to antimicrobial peptides of human and nonhuman origin, a feature that likely contributes to its virulence. Expressing a robust proteolytic activity, *P. gingivalis* hydrolyzes antimicrobial peptides. In this study, *P. gingivalis* inactivated three antimicrobial peptides, while a D-enantiomer was resistant to degradation. *P. gingivalis* was resistant to the protease-resistant D-enantiomer peptide, and importantly, a protease-deficient *P. gingivalis* mutant was also resistant to the antimicrobial peptide. Finally, the binding of a fluorescently labeled antimicrobial peptide to protease-deficient *P. gingivalis* was much weaker than the binding of susceptible *Escherichia coli*. Our results suggest that the resistance of *P. gingivalis* ATCC 33277 to direct killing by antimicrobial peptides is protease independent and results (at least partially) from the low affinity of antimicrobial peptides to *P. gingivalis*.

Antimicrobial peptides are components of the innate immunity that mediate a broad range of antimicrobial activity. More than 800 cationic peptides have been described for insects, vertebrates, and humans (www.bbcm.units.it/~tossi/pag1.htm). Though highly diverse, antimicrobial peptides share the features of a net positive charge and the ability to adopt an amphipathic structure in solution. The peptides are believed to kill bacteria through a multiple hit mechanism, whose targets can include the outer and inner membranes as well as cytoplasmic components (16). Thus, antimicrobial peptides seem to escape many of the bacterial drug resistance mechanisms and often show a synergistic effect with conventional antibiotics (44).

In mammals, antimicrobial peptides were also found to function as immunomodulators of the innate immune system that alter gene expression in host cells, induce or modulate chemokine and cytokine production, and elicit or inhibit a proinflammatory response (3, 38, 43). Since the recognition of their immunoregulatory functions, antimicrobial peptides are often referred to as host defense peptides.

In the human oral cavity, several kinds of antimicrobial peptides are found. These include α- and β-defensins, histatins, and LL-37 (6, 20, 34). Periodontal disease is a common bacterium-induced inflammatory disease (36) in which the tooth-supporting tissues are attacked. Deficiency of LL-37 in neutrophils and in saliva has been correlated with the occurrence of severe periodontal disease (34).

Porphyromonas gingivalis, the oral pathogen most associated with chronic periodontal disease (40), is resistant to many antimicrobial peptides (1, 15, 27). *P. gingivalis* is known for its robust proteolytic activity, in particular, the expression of extracellular Arg-gingipain and Lys-gingipain cysteine proteases that cleave at the C termini of arginine and lysine, respectively. Arginine and lysine, both being positively charged amino acids, are highly represented in cationic antimicrobial peptides. Therefore, it was rational to hypothesize that gingipains are important for the resistance of *P. gingivalis* to antimicrobial peptides. Our results, however, suggest that although *P. gingivalis* readily digests antimicrobial peptides, its ability to escape killing by antimicrobial peptides is independent of its proteolytic capacity.

Peptide synthesis is relatively straightforward; therefore, antimicrobial peptides present an appealing approach for controlling microbial pathogens. Better understanding of the mechanisms of bacterial resistance to antimicrobial peptides provides insight for the development of effective antibacterial therapies.

MATERIALS AND METHODS

Peptides. The three antimicrobial peptides used in this study have been described by us before (1). Dhvar4 (KRLFKKLLFSLRKY-NH2; molecular weight [MW], 1,839.3) is a derivative of human histatin 5, a member of a family of antifungal and antibacterial histidine-rich proteins secreted from salivary glands (13, 17–19). K_4-S4(1-15)a (ALWKTLLKKVLKAAA-NH_2; MW, 1,653.1) is a

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short derivative of dermaseptin S4, a member of the dermaseptin family of antimicrobial peptides, isolated from the skin of tree frogs (11, 23). Human LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES; MW, 4,493.3) is the only member of the cathelicidin family in humans and is cleaved and released from the C terminus of the cathelicidin hCAP18 propeptide secreted from granules of neutrophils (41) and from epithelial cells (2).

We synthesized the peptides by the solid-phase method (12), applying Fmoc (9-fluorenylmethyloxycarbonyl) active ester chemistry on an Applied Biosystems model 433A peptide synthesizer (Foster City, CA) as described before (25). The crude peptides were purified to more than 95% chromatographic homogeneity by reversed-phase high-performance liquid chromatography (Alliance-Waters, Milford, MA). The purified peptides were subjected to amino acid analysis and electrospray mass spectrometry (Micromass ZQ; Waters, Milford, MA) to confirm their composition and were stored as lyophilized powder at −20°C.

Bacterial strains and growth conditions. *Streptococcus mutans* ATCC 27351 was cultured in brain heart infusion broth (BHI) (Difco, MD) at 37°C in an atmosphere enriched with 5% CO_2. *P. gingivalis* ATCC 33277 was grown in Wilkin's broth (Oxoid, United Kingdom). *P. gingivalis* KDP136 (*P. gingivalis* ATCC 33277 mutant inactivated in *rgpA*, *rgpB*, and *kgp*), generously supplied by Koji Nakayama (39), was grown in enriched BHI broth (containing, per liter, 37 g of BHI [Difco], 5 g of yeast extract [Difco], 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K_1) supplemented with chloramphenicol, 20 μg/ml; erythromycin, 10 μg/ml; and tetracycline, 0.7 μg/ml. *P. gingivalis* strains were grown in jars containing an anaerobic atmosphere generation system (Oxoid, United Kingdom). *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* ATCC 29523 (26) was cultured in 0.5% yeast extract, 1.5% Bacto tryptone, 0.75% D-glucose, 0.25% NaCl, 0.075% L-cysteine, 0.05% sodium thioglycolate, and 4% $NaHCO_3$ at 37°C in 5% CO_2. *Escherichia coli* ATCC 25922 was grown in BHI under aerobic conditions.

Effect of preincubation with bacterial culture supernatant on peptide antimicrobial activity. Cultures of *P. gingivalis* or *A. actinomycetemcomitans* were grown to stationary phase and centrifuged for 10 min at 10,000 × *g* in a microcentrifuge (Eppendorf, Germany). Supernatants were collected and filter sterilized (0.2 μm; Whatman Schleicher & Schuell, Germany). Peptides K_4-S4(1-15)a (100 μg/ml final concentration), Dhvar4a (800 μg/ml), LL-37 (2,000 μg/ml), and D-K_4-S4(1-15)a (100 μg/ml) were incubated with 20% (final) *P. gingivalis* or *A. actinomycetemcomitans* filtrates for the indicated time.

Overnight cultures of bacteria were diluted 1:10 (for *S. mutans*) or 1:5,000 (for *E. coli*) in culture medium, sonicated for 1 min in a sonication bath (Transsonic T 640; ELMA, Germany), and distributed in aliquots of 190 μl into 96-well plates (Nunc, Denmark). Ten microliters of supernatant-treated peptides was added [K_4-S4(1-15)a and Dhvar4a were added to *S. mutans* cultures, and LL-37 was added to an *E. coli* culture]. Plates were incubated at the appropriate growth conditions. Bacterial growth was determined by measurements of the optical density at 650 nm (OD_{650}) using a ThermoMax microplate spectrophotometer (Molecular Devices). Percent inhibition of bacterial growth by the supernatant-treated peptides was calculated and compared to that for cells where fresh medium was added instead of peptides (no peptide, 0% growth inhibition) and controls where peptides were preincubated with fresh medium instead of culture supernatant (nontreated peptide, 100% growth inhibition). Data are means and standard deviations of three independent experiments performed in triplicate.

The MIC in planktonic bacteria was determined using a microdilution assay as described previously (1).

Fluorescent labeling of peptides. Peptides (1.5 mg) were labeled using the Alexa Fluor 546 protein labeling kit (Molecular Probes, Oregon) and purified using Bio-Gel P-2 (Bio-Rad, Hercules, CA). The first 0.5-ml elution fraction collected (containing the majority of the labeled peptide) was used. Quantification of the labeled LL-37 was measured with a NanoDrop ND-1000 UV-Vis spectrophotometer at 226 nm (NanoDrop Technologies, Wilmington, DE). A calibration curve was made using known amounts of LL-37 at 226 nm to enable estimation of the concentration of the purified, fluorescently labeled LL-37.

Attachment of fluorescently labeled peptide to *P. gingivalis* and to *E. coli*. Overnight bacterial cultures were diluted (1:5 for *P. gingivalis* KDP136 and 1:100 for *E. coli* ATCC 25922) and grown to an OD_{600} of approximately 1 (approximately 8 h for *P. gingivalis* and 3 h for *E. coli*). Cells were sedimented for 3 min at 10,000 × *g* and brought to an OD_{600} of 1 in phosphate-buffered saline (PBS). Labeled peptides (0 [negative control], 1, 4, or 16 μl [estimated concentration 0.8 mg/ml]) were added to 100 μl of bacterial suspension and incubated at room temperature for 10 min. Cells were washed in 0.5 ml PBS, resuspended in 0.1 ml PBS, and transferred to wells of a 96-well microtiter plates (Nunc, Denmark). Fluorescence of bacterium-attached labeled peptide was determined by using a fluorescence microplate reader (excitation, 544 nm; emission, 590 nm; FLUOstar$^+$ Galaxy; BMG Laboratories, Offenburg, Germany). In order to test the integrity of peptide incubated with *P. gingivalis* KDP136, the supernatant of the labeling reaction mixture (containing unbound peptide) was used to react with *E. coli* ATCC 25922 as described above. Experiments were performed with minimal exposure to light. Means and standard deviations of three independent experiments are presented. Two microliters of each adherence reaction mixture was placed on a microscope slide and analyzed using an Axiovert 200, SensiCam Zeiss, PCO microscope (Zeiss, Germany).

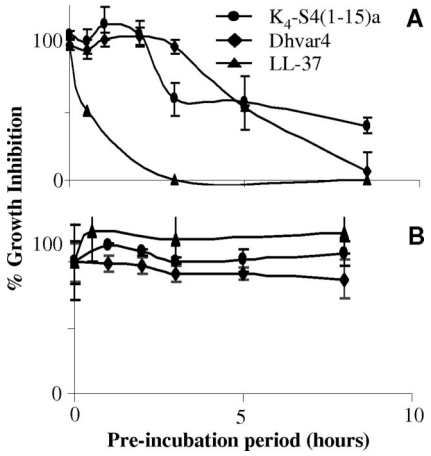

FIG. 1. Effect of *P. gingivalis* ATCC 33277 or *A. actinomycetemcomitans* culture supernatant fluid on peptide antimicrobial activity: Peptides K_4-S4(1-15)a, Dhvar4, and LL-37 were preincubated with supernatants prepared from *P. gingivalis* (A) or *A. actinomycetemcomitans* (B) for the indicated time and added to a growing culture of *S. mutans* [K_4-S4(1-15)a (5 μg/ml final concentration), Dhvar4 (40 μg/ml final concentration)], or *E. coli* (LL-37, [100 μg/ml final concentration]). Percent bacterial growth inhibition by the supernatant-treated peptides was calculated as described in Materials and Methods. Data are means and standard deviations (error bars) of three independent experiments.

RESULTS

Peptide antimicrobial activity diminishes following incubation with culture supernatant prepared from *P. gingivalis*. Previous results by others (15, 21) and ourselves (1) demonstrated that most tested *P. gingivalis* strains showed resistance to antimicrobial peptides. The fact that *P. gingivalis* is a very proteolytic organism suggested that its resistance to antimicrobial peptides might stem from its ability to hydrolyze them. Proteolysis has been suggested before as a resistance mechanism against antimicrobial peptides in yeast (*Candida albicans*) (35) and bacteria (14, 37), including *P. gingivalis* (10).

Previously, we reported that *P. gingivalis* is resistant to the antimicrobial peptides K_4-S4(1-15)a, LL-37, and Dhvar4 (1). To test whether *P. gingivalis* can inactivate these three peptides, the growth inhibitions of *S. mutans* [susceptible to K_4-S4(1-15)a and Dhvar4] and *E. coli* (susceptible to LL-37) (1) were tested with or without preincubation of the peptides with protease-containing *P. gingivalis* culture supernatant. As can be seen in Fig. 1A, the antimicrobial activity of all three antimicrobial peptides was diminished following preincubation with the culture supernatant prepared from *P. gingivalis* ATCC 33277 in a time-dependent manner. Culture supernatant prepared from the nonproteolytic periodontal pathogen *A. actinomycetemcomitans* in a manner similar to that described

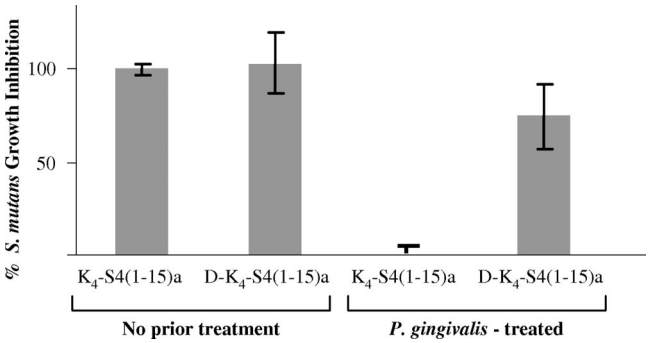

FIG. 2. Effect of *P. gingivalis* supernatant on antibacterial activity of D-K_4-S4(1-15)a, the D-enantiomer of K_4-S4(1-15)a. Peptides K_4-S4(1-15)a and D-K_4-S4(1-15)a were treated (or not) for 8 h with supernatant prepared from *P. gingivalis* and added (5 μg/ml) to a growing culture of *S. mutans*. Percent inhibition was calculated and compared to those for cells grown without antimicrobial peptides (0% inhibition) and cells grown in the presence of untreated K4-S4(1-15)a (100% inhibition). Data are means and standard deviations (error bars) of three independent experiments.

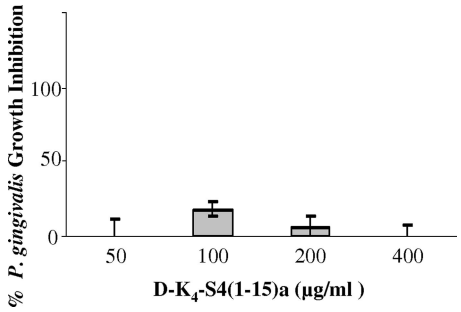

FIG. 3. Effect of D-K_4-S4(1-15)a on *P. gingivalis* growth. Increasing amounts of D-K_4-S4(1-15)a were added to a growing culture of *P. gingivalis*. Percent inhibition was calculated according to that for nontreated cells (0%). Data are means and standard deviations (error bars) of three independent experiments.

above did not reduce the antimicrobial activity of the peptides (Fig. 1B).

D-Enantiomer conformation of K_4-S4(1-15)a renders resistance to inactivation by culture supernatants of *P. gingivalis* ATCC 33277. Peptides synthesized using D-amino acids acquire an increased resistance to proteolysis by bacterial proteases. Unlike the "parent" K_4-S4(1-15)a peptide, the D-enantiomer retained anti-*S. mutans* activity following 8 h of incubation with culture supernatant prepared from *P. gingivalis* (Fig. 2). This observation suggested that the peptide in the D-conformation was resistant to degradation by *P. gingivalis*.

***P. gingivalis* is resistant to the D-enantiomer of K_4-S4(1-15)a.** Although D-K_4-S4(1-15)a remained effective against *S. mutans*, even after an 8-h preincubation with *P. gingivalis* supernatant, *P. gingivalis* ATCC 33277 remained resistant to D-K_4-S4(1-15)a (Fig. 3). Some inhibition of D-K_4-S4(1-15)a could be detected at 100 μg/ml; however, this effect was not observed at higher concentrations. This result suggested that the resistance of *P. gingivalis* ATCC 33277 to K_4-S4(1-15)a is independent of its proteolytic capacity.

Binding of fluorescently labeled peptides to *P. gingivalis*. Fluorescent labeling of the K_4-S4(1-15)a peptide abolished its bacterial binding capacity (data not shown), presumably because of steric interference by the chromophore, which has a significant molecular mass (410 Da) compared to that of the short K_4-S4(1-15)a peptide (1,653 Da). However, fluorescently labeled LL-37 peptide bound *E. coli* ATCC 25922 in a dose-dependent manner (Fig. 4A and C). Fluorescently labeled LL-37 was immediately digested by *P. gingivalis* ATCC 33277 (data not shown). Therefore, a protease-deficient *P. gingivalis* strain (KDP136) (39) was used to determine the binding of LL-37 to *P. gingivalis*. Unlike *E. coli*, which exhibited strong binding to LL-37 (Fig. 4A and C), the protease-deficient *P. gingivalis* KDP136 strain demonstrated weak binding to the fluorescently labeled LL-37 peptide (Fig. 4A). Poor binding to *P. gingivalis* KDP136 (Fig. 4D) did not result from peptide degradation because the unbound peptide that remained in the incubation supernatant after removal of *P. gingivalis* KDP136 by centrifugation bound to *E. coli* with an affinity similar to that of the peptide not incubated with *P. gingivalis* KDP136 (Fig. 4B).

Protease-deficient *P. gingivalis* KDP136 is resistant to LL-37. Though not capable of degrading LL-37 (Fig. 4B), the protease-deficient *P. gingivalis* KDP136 mutant was found to be resistant to the LL-37 antimicrobial peptide (MIC higher than 200 μg/ml [data not shown]), supporting the above results indicating that the resistance of *P. gingivalis* to direct killing by antimicrobial peptides is protease independent.

DISCUSSION

P. gingivalis is a major pathogen involved in periodontal disease that flourishes in the gingival crevice, although it is bathed with antimicrobial peptides (6, 45). Indeed, in a recent study (1), *P. gingivalis* was found to be resistant to the three tested antimicrobial peptides (from human and nonhuman origins). Resistance to antimicrobial peptides is likely to contribute to the virulence of *P. gingivalis*. The high proteolytic capacity of *P. gingivalis* suggested that peptide degradation is the mechanism by which *P. gingivalis* resists host antimicrobial peptides. Indeed, in this study, wild-type *P. gingivalis* inactivated all three antimicrobial peptides in a time-dependent manner. Of the three antimicrobial peptides, the activity of human LL-37 was reduced the most and most rapidly (Fig. 1A). This might result from its increased length (37 amino acids) and its abundance of lysines (6) and arginines (5) that are the cleavage sites for the Arg- and Lys-gingipains of *P. gingivalis*. Dhvar4 (a derivative of the human salivary histatin 5 antimicrobial peptide) is 14 amino acids long and contains four lysines and two arginines and was more susceptible to proteolysis by *P. gingivalis* than was K_4-S4(1-15)a (an analog of the amphibian dermaseptin S4 peptide), which is 15 amino acids long but contains only four lysines (Fig. 1A).

The fact that wild-type *P. gingivalis* ATCC 33277 is resistant to D-K_4-S4(1-15)a (Fig. 3), the protease-resistant D-enantiomer of K_4-S4(1-15)a, and that its protease-deficient mutant progeny *P. gingivalis* KDP136 retains resistance to LL-37, although it is incapable of degrading it, testify that the resistance of *P. gingivalis* ATCC 33277 to direct killing by these antimicrobial peptides is independent of protease activity. Rather, our results suggest that the resistance of *P. gingivalis* ATCC

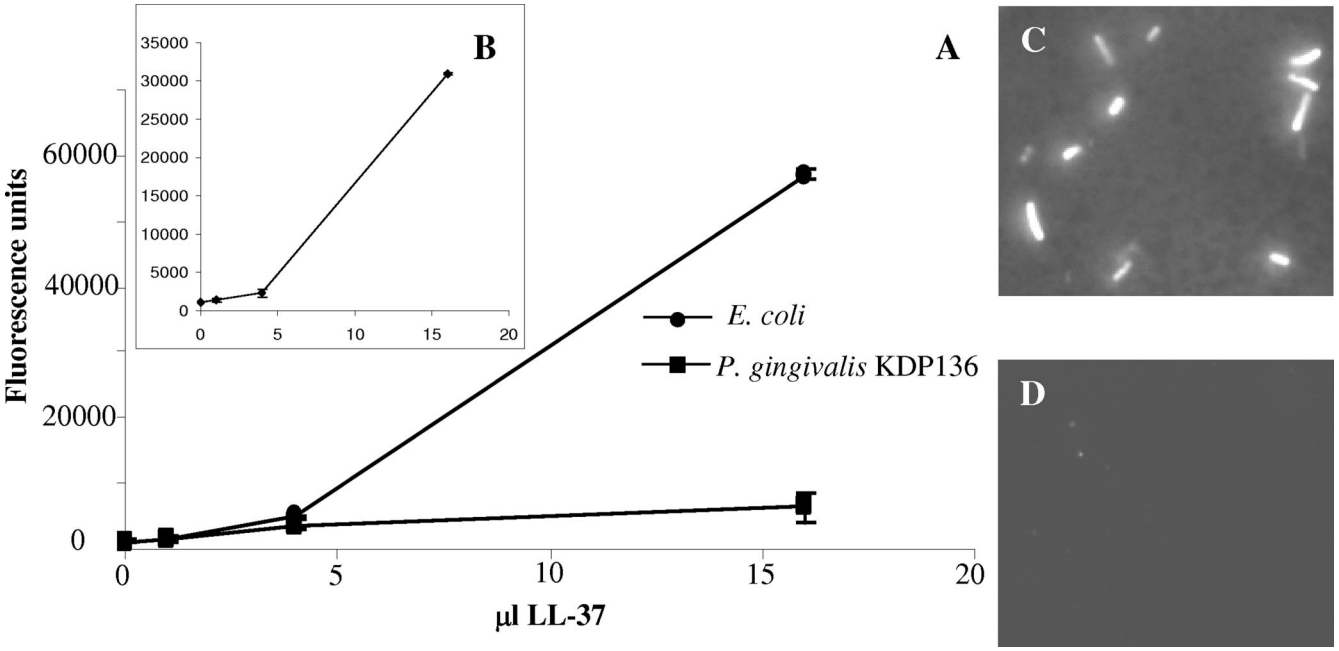

FIG. 4. Binding of Alexa Fluor-labeled LL-37 to *E. coli* ATCC 25922 or *P. gingivalis* KDP136. (A) Total fluorescence of bacteria incubated with Alexa Fluor-labeled LL-37 (concentration is estimated at 0.8 mg/ml [see Materials and Methods]). (B) Total fluorescence of *E. coli* ATCC 25922 incubated with the supernatant of Alexa Fluor-labeled LL-37 that was preincubated with *P. gingivalis* KDP136 (see Results). Images in panels C and D show fluorescence of bacteria incubated with Alexa Fluor-labeled LL-37 recorded using identical instrument settings. Of the 13 *E. coli* cells present in the field of view presented in panel C, 12 were labeled. Out of the 51 *P. gingivalis* KDP136 cells present in the field of view presented in panel D, only two were labeled. Error bars indicate standard deviations.

33277 to antimicrobial peptides derives, at least in part, from its low affinity to these positively charged peptides (Fig. 4). Cationic antimicrobial peptides bind lipopolysaccharides (LPS) of gram-negative bacteria (32), and LPS structure has been shown to affect susceptibility or resistance to antimicrobial peptides (28). In accordance with our observations, it was suggested previously (9, 10) that the unique LPS structure of *P. gingivalis* that induces different host responses than those of most studied LPS (7, 24, 33) might contribute to its resistance to antimicrobial peptides. Alterations in LPS composition might also explain differences in antimicrobial susceptibility profiles observed among several strains of *P. gingivalis* (21). Modifications in LPS (9) or bacterial cell surfaces have previously been demonstrated to contribute to resistance to antimicrobial peptides in several bacterial pathogens, including *Haemophilus influenzae* (42), *Staphylococcus aureus* (8, 29, 30), and *Pseudomonas aeruginosa* (31). *Treponema denticola* is a periodontal pathogen that, in a manner similar to that of *P. gingivalis*, expresses robust proteolytic activity. The resistance of *T. denticola* to β-defensins was also shown to be associated with its unique cell surface structure that lacks LPS and demonstrated reduced defensin binding (4, 5).

In dental plaque, *P. gingivalis* is found in a closely packed interdigitated mixed-species biofilm. Though we demonstrated that the highly active gingipains of *P. gingivalis* are not essential for its resistance to direct killing by antimicrobial peptides, we propose (22) that the *P. gingivalis* proteases, by degrading local antimicrobial peptides, can provide protection to proximal, otherwise peptide-susceptible bacteria, such as *Fusobacterium nucleatum* (1) and *A. actinomycetemcomitans* (34). It also seems reasonable to speculate that in vivo, the degradation of local antimicrobial peptides by *P. gingivalis* disrupts the immunoregulatory functions of the peptides and assists *P. gingivalis* (and proximal plaque community species) in evading an otherwise orchestrated host immune response.

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