Mechanisms of Decreased Susceptibility to β-Defensins by *Treponema denticola*

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Periodontal disease is remarkably widespread, afflicting 50% of adults in the United States (38, 62), and severe disease affects 6 million Americans. There is interest in using synthetic antimicrobial peptides as an adjunct to traditional therapies for periodontal disease (26, 45, 73). Humans naturally produce antimicrobial peptides as an adjunct to traditional therapies for periodontal disease. In this study, we confirmed that the major outer membrane protease, dentilisin, is not responsible for *T. denticola* insensitivity to defensins and examined several other possible mechanisms, including reduced binding to the bacterial surface and efflux pump activity. It has been suggested that some bacteria mask their surfaces with serum proteins. *T. denticola* grown in a serum-free medium did not exhibit increased susceptibility to human beta-defensin 2 and 3 (hBD-2 and hBD-3, respectively), suggesting that cloaking of the outer surface with host proteins is not involved in defensin resistance. Nonetheless, we demonstrated that *T. denticola* binds significantly less hBD-2 and -3 than susceptible organisms bind, suggesting that the unusual outer membrane composition of *T. denticola* may discourage cationic peptide binding. Efflux pumps have been shown to mediate resistance to antibiotics and cationic peptides in other bacteria, and their role in *T. denticola*'s relative resistance to β-defensins was investigated. Three inhibitors of bacterial ATP-binding cassette (ABC) efflux pumps had no effect on *T. denticola*'s susceptibility to hBD-2 or -3. In contrast, a proton motive force inhibitor, carbonyl cyanide 3-chlorophenylhydrazone, increased the susceptibility of *T. denticola* to killing by hBD-3, demonstrating a potential role for efflux pumps (other than ABC pumps) in resistance to this peptide. Our data suggest that the combination of decreased defensin binding and efflux of any peptide which enters the cytoplasm may explain *T. denticola*'s relative resistance to human beta-defensins.

**MATERIALS AND METHODS**

**Bacterial strains and culture.** *T. denticola* strains 35404, 35520, and 33521 were a gift from Pamela Braham (University of Washington, Seattle). Strain K1 (dentilisin mutant) and its ATCC 35405 parent were a gift from Kazuyuki Ishihara (Tokyo Dental College, Chiba, Japan) (31). Dentilisin activity was detected by *T. denticola*-induced cleavage of a chromogenic target of chymotrypsin-like activity, succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPNA) (Sigma Chemicals, St. Louis, MO) (54). *T. denticola* was maintained in GM-1 medium (6) or a derivative of OMIZ-W, P4 (75), in an anaerobic jar at 37°C. OMIZ-P4 (ATCC medium 2131) was prepared without sugars, 1,4-dihydroxy-2-naphthoic acid, cholesterol, yeast extract, neopeptone, or human serum. K1 cultures (in GM-1 medium) were supplemented with 40 μg/ml erythromycin. Escherichia coli strain ML35 was obtained from the American Type Culture Collection, Rockville, MD, and was maintained in Luria-Bertani medium at 37°C. The *Staphylococcus aureus* 113 dlt mutant was a gift from Amanda Jones (University of Washington) and was maintained in Todd-Hewitt broth at 37°C.

**Chemicals and reagents.** All chemicals and reagents were purchased from Sigma Chemicals, unless indicated otherwise. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was resuspended in dimethyl sulfoxide (DMSO) at a concentration of 0.1 mM; verapamil hydrochloride was resuspended at a concentration of 10 mg/ml in distilled H2O; acriflavine was resuspended at a concentration of 1 mg/ml in 100% ethanol; reserpine was resuspended at a concentration of 10 mg/ml in 100% ethanol; and sodium orthovanadate was resuspended at a concentration of 0.1 M in distilled H2O. All chemicals were prepared immediately before use.

**Defensin killing assay.** Log-phase cultures of *T. denticola*, *E. coli*, or *S. aureus* 113 dlt were centrifuged at 10,000 × g for 10 min at 20°C. The bacteria were washed once and resuspended in 10 mM sodium phosphate buffer (pH 7.2) containing 1% Trypsicase soy broth (TSB). A total of 1 × 10^9 motile treponemes in 1 ml were added to triplicate tubes and incubated with 10 μg/ml of human β-defensin 2 (hBD-2) or hBD-3 (Peptides International, Lexington, KY) or 80 μg/ml erythromycin (positive control for killing) at 37°C anaerobically for 0.5 to 4 h. In some experiments, efflux pump inhibitors, such as CCCP (final concentration, 35 μM), reserpine (10 μg/ml), verapamil (20 μg/ml), or sodium orthovanadate (50 μM), or equivalent amounts of their solvents were included in the killing assay mixture 10 min before addition of the defensin peptide. Recently, Dorschner et al. (21) indicated that the inhibitory effects of salt on
β-defensin activity could be overcome by cultivating bacteria in mammalian ionic conditions; therefore, we also tested the sensitivity to hBD-2 and -3 of *T. denticola* grown in medium adapted from minimal essential medium containing 27 µM sodium bicarbonate as defined by Dorschner et al. but with additives that permit *T. denticola* growth. No difference in *T. denticola* sensitivity to hBD-2 and -3 was observed in this medium (data not shown).

*T. denticola* viability was determined by determining the number of CFU. After incubation with human β-defensin, bacterial suspensions were diluted 1:30 in 10 mM sodium phosphate buffer containing 1% TSB (pH 7.2) and then added to 25 ml semisolid GM-1 medium (with 0.5% Noble agar and 0.5% gelatin) in 25-cm² tissue culture flasks and allowed to solidify at room temperature. Five milliliters of TSB containing 1% Noble agar was overlaid as a sealant. The flasks were incubated anaerobically at 37°C for 7 to 10 days, and the CFU were counted. As controls for human β-defensin activity, *E. coli* ML35 and the *S. aureus* 113 dl* B* mutant were incubated in the same manner, and viability was determined by plate counting on Luria-Bertani medium and Todd-Hewitt medium, respectively. Student's *t* test assuming unequal variances was used to determine significance; a *P* value of ≤0.05 was considered significant.

**Acriflavine uptake.** *T. denticola* mid- to late-log-phase cultures were collected by centrifugation at 10,000 × g for 10 min. The pellets were washed once with 10 mM sodium phosphate buffer containing 1% TSB and resuspended to a concentration of 1 × 10⁶ treponemes/ml. Then 100 µl well was added to Perkin-Elmer Opti96 black plates, and this was followed by addition of 10 µl of 350 µM CFPCC, 100 µg/ml reserpin, 200 µg/ml verapamil, 50 µM sodium orthovanadate, or an appropriate solvent. Acriflavine is a fluorescent dye; as it binds DNA, its fluorescence is quenched (13). We added 10 µl of a 10.25-µg/ml acriflavine solution to appropriate wells, and the fluorescence was measured immediately and at 2- to 3-min intervals at 37°C using a Perkin-Elmer Fusion instrument with an excitation wavelength of 440 (bandwidth, 35) and an emission wavelength of 505s (bandwidth, 20). The percent quenching of acriflavine fluorescence compared with the control was calculated as follows: 100 – (average relative fluorescence units of wells with efflux inhibitor/average relative fluorescence units of wells with solvent only) × 100. For example, the data indicated that there was 43% more acriflavine quenching in the presence of CFPCC than in the presence of the control, which was calculated as follows: 100 – (0.315 relative fluorescence units for *T. denticola* with CFPCC/0.037 relative fluorescence units for *T. denticola* with DMSO). (× 100).

**Binding of β-defensin to bacteria.** Eppendorf tubes were treated with phosphate-buffered saline (PBS) containing 1% Tween 20 for 1 h at room temperature to block nonspecific protein binding and then washed once with PBS containing 0.05% Tween 20. Five hundred microliters of *T. denticola* (grown in either GM-1 medium or serum-free chemically defined medium), *E. coli*, or *S. aureus* 113 dl* B* at a concentration of 1 × 10⁶ cells/ml in 10 mM sodium phosphate buffer was incubated with biotinylated β-defensin 2 or 3 (Global Peptide, Fort Collins, CO) at a concentration of 10 µg/ml for 0 to 4 h at 37°C. The maximum peptide binding was observed within 30 min (data not shown). Bacteria were collected by centrifugation at 14,000 × g for 30 min at room temperature. The supernatants were discarded, and the bacteria were washed once with PBS containing 0.05% Tween 20. To exhaust the endogenous peroxidase activity, the bacteria were resuspended in 50 µl of 3% hydrogen peroxide and incubated for 5 min at room temperature. The bacteria were washed again with PBS containing 0.05% Tween 20, resuspended in 50 µl PBS, transferred to new tubes, and stored at −20°C. *T. denticola* cells remained intact under these conditions, as determined by dark-field microscopy (data not shown). In experiments to detect internalized defensin, bacteria were treated with 125 µl of 0.05% Triton X-100 for 5 min at room temperature prior to the final wash. To differentiate between binding and uptake, experiments were conducted at 4 and 37°C; there were no significant temperature-dependent differences in the amount of peptide bound to any of the bacteria tested (data not shown). After addition of 50 µl strepavidin-horseradish peroxidase (HRP) (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and incubation for 1 h at 37°C, bacteria were washed four times by centrifugation at 14,000 × g for 30 min and then resuspended in 50 µl PBS and transferred to an enzyme-linked immunosorbent assay plate (Maxisorp; Nunc, Rochester, NY). Beta-defensins were detected by addition of 100 µl of 3,3,5,5′-tetramethylbenzidine substrate. The reaction was terminated by addition of 100 µl of 2 N H₂SO₄, and the absorbance at 450 nm was determined. The approximate surface area of *T. denticola* was determined using the following parameters: average length of *T. denticola* cell, 11 µm; average width of *T. denticola* cell, 0.18 µm; wavelength, 0.9 µm; and amplitude, 0.15 µm (12). The length was divided by the wavelength to obtain 12.2 waves per bacterium. The total additional length from the height of waves was calculated as follows: 12.2 waves/bacterium × amplitude, which resulted in a total length of 1.83 µm. The total length of a “stretched-out” *T. denticola* cell is 12.83 µm. The bacterium was considered to be a cylinder, and the surface area of a cylinder is equal to 2πr² + 2πrh, where *r* is the radius and *h* is the height. If the radius of a *T. denticola* cell was one-half the width and the height was the total length of *T. denticola*, the surface area of *T. denticola* was approximately 7.3 µm². The surface area of *E. coli* was 4.4 µm², determined using the formula for the surface area of a cylinder with 0.5 µm as width and 1 µm as height, and the surface area of *S. aureus* was 3.14 µm² (the surface area of a sphere is 4πr², and the radius was 0.5 µm). The correlation between the percentage of bacteria killed in the presence of peptide and the ratio of absorbance to surface area was determined by linear regression.

**RESULTS**

*T. denticola* does not mask its surface with host proteins as a mechanism of defensin resistance. Cloaking of peptide-binding sites on the outer membrane of the bacterium by host proteins is a possible mechanism by which *T. denticola* might resist β-defensin killing. Both *T. denticola* and *Treponema pallidum* have been reported to bind host serum proteins (3, 25). To test whether host proteins present in serum contribute to *T. denticola*'s resistance to β-defensins, *T. denticola* 35404 cells grown in GM-1 serum-containing medium or in chemically defined medium (CDM) lacking serum, *E. coli* ML35, or *S. aureus* 113 dl* B* were incubated in triplicate with 10 µg/ml hBD-2 or -3 for 4 h at 37°C. The bacteria were plated on appropriate media, and the numbers of CFU were determined. The data are the means and standard errors from three or more experiments. The data suggest that serum proteins present in the standard medium do not account for the resistance of *T. denticola* to β-defensins.

![FIG. 1](http://iai.asm.org/dx.doi/10.1128/INFECTIMMUN.00028-17)

**FIG. 1.** Effect of host proteins present in the growth medium on *T. denticola* susceptibility to β-defensins. A total of 1 × 10⁶ *T. denticola* 35404 cells grown in GM-1 serum-containing medium or in chemically defined medium (CDM) lacking serum, *E. coli* ML35, or *S. aureus* 113 dl* B* were incubated in triplicate with 10 µg/ml hBD-2 or -3 for 4 h at 37°C. The bacteria were plated on appropriate media, and the numbers of CFU were determined. The data are the means and standard errors from three or more experiments.
fensins as an explanation for the decreased vulnerability to defensins, using two independent methods: by demonstrating that there was not increased killing by hβD-2 in the presence of multiple protease inhibitors and by demonstrating that the presence of T. denticola (with its full proteolytic capability) did not protect E. coli from killing by hβD-2 during coincubation (9).

The mutant T. denticola K1 lacks the major outer membrane protease, dentilisin (31). The presence or absence of chymotrypsin-like protease activity was confirmed by cleavage of a chromogenic substrate, SAAPNA; K1 did not cleave SAAPNA, while its parent strain showed the expected activity (data not shown). To test whether the lack of chymotrypsin-like protease activity in this mutant increased the sensitivity to β-defensins, the T. denticola K1 mutant and its parent strain (35405) were incubated in the presence of 10 μg/ml hβD-2 or -3 for 4 h in 10 mM sodium phosphate buffer. The bacteria were then diluted, and the numbers of CFU were determined. S. aureus 113 dtl was used as a control for peptide activity. Experiments were conducted three times with triplicate replicates. There was no difference between the amount of the K1 mutant killed and the amount of its parent strain killed. For hβD-2, the percentages of the ATCC 35405 parent, K1 mutant, and S. aureus 113 dtl killed were 33% ± 9%, 26% ± 10%, and 65% ± 9%, respectively. For hβD-3, the percentages of the ATCC 35405 parent, K1 mutant, and S. aureus 113 dtl killed were 31% ± 9%, 27% ± 12%, and 92% ± 3%, respectively. These results confirm that dentilisin activity is not responsible for T. denticola’s relative insensitivity to β-defensins.

**Binding of β-defensins to T. denticola.** Another mechanism of T. denticola’s resistance to β-defensins may be related to the
overall charge of the bacterium. Defensins are cationic and likely bind negatively charged bacterial structures, such as lipopolysaccharide (LPS), as a preliminary step in bacterial killing. An analysis of the *T. denticola* genome (ATCC 35405) indicated that there is a lack of LPS structural genes (66). The surface of *T. denticola* 33520 is not highly negatively charged (14), and *T. denticola* 33521 outer membrane lipids are dominated by positively charged uronic acid, which may prevent the electrostatic interactions involved in defensin binding (64). Thus, the lack of a negative surface charge may result in reduced binding of defensins to *T. denticola* compared to the binding to other bacteria. To examine this possibility, biotinylated hBD-2 or -3 was incubated with *T. denticola* 35404, 33520, 33521, the ATCC 35405 parent, the ATCC 35405 K1 dentilisin mutant, *E. coli* ML35, or *S. aureus* 113 dlt. While all five *T. denticola* strains bound measurable amounts of hBD-2, they bound significantly less peptide than either *E. coli* or *S. aureus* 113 dlt bound when the values were adjusted for surface area (Fig. 2A). The dentilisin mutant, which is also not sensitive to hBD-defensins, has a disordered outer sheath compared to the parent strain and decreased surface hydrophobicity (31), but these defects did not result in increased defensin binding. The data described above were obtained using *T. denticola* cultured in GM-1 medium. In addition, the hBD-defensin binding of *T. denticola* grown in serum-free medium did not differ from the hBD-defensin binding of *T. denticola* grown in serum-contain-
Similar results were obtained with *H.9252*. These data support the hypothesis that insensitivity to killing by human β-defensins is due, at least in part, to inefficient binding of defensin peptide to the treponeme.

**T. denticola** ABC efflux pumps are not involved in defensin resistance. Efflux of cationic peptides as a resistance mechanism has been observed in *Neisseria* and *Yersinia* species (5, 56, 58, 67, 72). To test whether the numerous ATP-binding cassette (ABC) efflux pumps present in the *T. denticola* genome (66) are involved in defensin resistance, the pump activity was assessed by using three known inhibitors of ABC pumps: verapamil, reserpine, and sodium orthovanadate (35, 59). To ensure that the pump inhibitors were active, a fluorescent dye, acriflavine, was used. As acriflavine is taken up by bacteria, it binds DNA, eliminating the dye’s fluorescence (13). Acriflavine, was used. As acriflavine is taken up by bacteria, it binds DNA, eliminating the dye’s fluorescence (13). Acriflavine was used. As acriflavine is taken up by bacteria, it binds DNA, eliminating the dye’s fluorescence (13).

Proton motive force uncoupler increases *T. denticola* sensitivity to hBD-3. CCCP is a protonophore whose addition to cells results in instantaneous dissipation of the electrochemical gradient of protons (proton motive force) across the cytoplasmic membrane. CCCP has been shown to increase the susceptibility of some bacteria to antimicrobial peptides (49) by inhibiting peptide efflux from the cytoplasm. To assess the activity of CCCP, the acriflavine assay described above was performed. As shown in Fig. 5A, CCCP increased the acriflavine fluorescence quenching in *T. denticola* compared with that in the solvent control. To determine the effect of efflux inhibition on *T. denticola’s* susceptibility to defensins, *T. denticola* 35404 was incubated with CCCP and hBD-2 or -3. Addition of CCCP significantly increased *T. denticola’s* sensitivity to killing
by hβD-3 compared to the sensitivity after incubation with CCCP alone or defense alone, but it had no effect on the sensitivity to hβD-2 (Fig. 5B). These results suggest that efflux has a role in T. denticola’s relative resistance to hβD-3. To confirm that CCCP prevented efflux of hβD-3 from T. denticola, the defensin binding assay was performed in the presence and absence of an inhibitor. T. denticola cells were permeabilized with 0.05% Triton X-100 to allow detection of both internalized and surface peptides. Biotinylated β-defensins were detected by using strepavidin-HRP. The data are the means and standard errors from three experiments. Significance was determined by Student’s t test assuming unequal variances. An asterisk indicates that the P value is <0.05 for a comparison with βD-3 alone and with the nonpermeabilized control containing CCCP plus hβD-3.

**DISCUSSION**

The discovery of naturally occurring antimicrobial peptides has resulted in much interest in the scientific, dental, and medical communities. In this age of antibiotic resistance, the idea of using natural antimicrobial peptides or their synthetic derivatives to combat bacterial and fungal infections is particularly appealing. The use of antimicrobial peptides as dental therapeutics to combat periodontal disease and caries has been posited, and a number of oral microbes have demonstrated sensitivity to these peptides in vitro (34, 41, 43, 44, 47, 48). However, we have demonstrated that T. denticola and several other oral treponemes are not vulnerable to human β-defensins (9, 10). T. denticola is part of the “red complex” of periodontal pathogens associated with severe periodontal disease (70), and good therapeutic outcomes require a decrease in the number of periodontal pathogens, including oral treponemes (2, 19, 24, 37, 46, 60, 69).

A number of potential mechanisms may be used by T. denticola to resist killing by human β-defensins. One possibility is that T. denticola cloaks itself in host serum proteins derived from the culture media or saliva. Both T. denticola and the syphilis spirochete T. pallidum have been reported to bind host proteins to their outer sheaths, perhaps as a mechanism for host mimicry immune evasion or as a physical barrier to specific antibodies (3, 25). In this study we examined the sensitivity of T. denticola grown in a chemically defined medium lacking serum or other host proteins to hβD-2 and -3 and found that the absence of host proteins in the growth medium has no effect on T. denticola’s sensitivity to the peptides. In addition, there was no difference in the binding of hβD-2 or -3 to T. denticola grown in the absence of serum. While binding of host serum proteins to T. denticola is probably important for treponeme-host interactions, masking of the bacterium’s outer surface is not required for resistance to β-defensin killing.

Some bacteria employ proteases to destroy antimicrobial peptides. For example, S. aureus aureolysin degrades the human epithelial antimicrobial peptide LL-37, and strains that produce more of this protease are less susceptible to killing by LL-37 (68). ZapA, a metalloprotease from Proteus mirabilis that indiscriminately degrades a number of host proteins, inactivates LL-37 and hβD-1 (4). We previously showed that proteolytic destruction of hβD-2 is not responsible for T. denticola’s invulnerability to defensins by two independent methods (9). In this study, we tested the sensitivity of a protease mutant, K1, which lacks the major outer membrane protease, dentilisin (32). Dentilisin is a chymotrypsin-like protease which can degrade the small chemokine interleukin-8 (20). There was no difference between the vulnerability of the mutant to defensins and the vulnerability of its parent strain, demonstrating that dentilisin activity plays no role in T. denticola’s sensitivity to defensins. These data corroborate our previous observations that T. denticola’s impressive proteolytic activity is not involved in its avoidance of defensin killing (9).

The available evidence suggests that T. denticola lacks a traditional negatively charged LPS, and the outer membrane of at least one strain, 33521, is dominated by a positively charged uronic acid species (15, 64, 66). It is possible that β-defensins do not bind well to the surface of the organism. Indeed, alteration of the surface charge by decoration of LPS or lipoteichoic acid with positively charged moieties is the most common mechanism employed by bacteria to avoid killing by cationic peptides. For example, lipid A modification plays a role in Haemophilus influenzae resistance to human β-defensins (71), and some S. aureus isolates have teichoic acids decorated with D-alanine- or L-lysine-modified phospholipids; mutants lacking these modifications are vulnerable to β-defensins (14, 51, 52). In this study, we measured the binding of β-defensins to T. denticola by strepavidin-HRP detection of biotinylated hβD-2 and -3 incubated with the bacteria. In order to compare binding of labeled defensin (as measured by absorbance) by T. denticola to binding by the smaller organism S. aureus or E. coli, we normalized the data by accounting for surface area. T. denticola binds significantly less hβD-2 than susceptible E. coli or S. aureus binds when the data are expressed as a ratio of absorbance to surface area. Similar results were obtained with...
labeled hβD-3. These results support the hypothesis that β-defensin peptides interact poorly with T. denticola’s outer surface. Interestingly, T. denticola is resistant to polymyxin B, another cationic peptide that interacts strongly with LPS and other negatively charged bacterial surface structures (1, 27). Several studies have demonstrated that spirochetes which lack LPS are also relatively resistant to antimicrobial peptides, while Leptospira strains, which have a traditional LPS, have sensitivities comparable to those of other gram-negative organisms (8, 16, 61, 65, 74). While the structure and amphipathic nature of antimicrobial peptides are certainly involved, the overall charge has repeatedly been demonstrated to be an important consideration in peptide binding and killing of microorganisms (29, 30, 33, 63, 76). Our data suggest that there is a correlation between the amount of peptide bound to a bacterium and the level of killing.

Efflux pumps in bacteria can recognize a variety of amphipathic molecules; certain efflux pumps are involved in the resistance of Neisseria gonorrhoeae and Yersinia enterocolitica, as well as other bacteria, to antimicrobial peptides and other toxic cationic compounds (5, 11, 42, 53, 72). According to the recently published genome sequence, T. denticola possesses an unusually large number of ABC efflux pumps, as well as several homologs of the NorM family of efflux pumps (66). In N. gonorrhoeae and Neisseria meningitidis, a NorM homolog is responsible for the efflux of cationic compounds, including the fluorescent dye acriflavine (56). T. denticola actively effluxes acriflavine from its cytoplasm, and in the presence of efflux inhibitors, T. denticola is unable to remove acriflavine, which accumulates inside the cell. These data indicate that at least some of T. denticola’s efflux pumps are active and involved in removal of a cationic compound. However, three inhibitors of ABC pumps (verapamil, reserpine, and sodium orthovanadate) had no effect on T. denticola’s susceptibility to hβD-2 or -3, suggesting that while ABC pumps may be involved in the efflux of some cationic compounds, they are not involved in β-defensin resistance. Other efflux systems encoded in the T. denticola genome (66), such as MATE transporters, may still be involved in T. denticola’s relative resistance to β-defensins.

Transport of material from the cytoplasm across bacterial membranes requires energy in the form of a proton gradient, and disruptors of the proton motive force are often used to examine the role of efflux in resistance to antibiotics (50, 55). A proton motive force inhibitor, CCCP, significantly increases T. denticola’s susceptibility to killing by hβD-3, but it had no effect on T. denticola’s susceptibility to hβD-2; similar results were observed for incubation times ranging from 0.5 to 4 h (data not shown). The increased killing of T. denticola in the presence of CCCP and hβD-3 appears to be synergistic rather than additive (e.g., when CCCP and hβD-3 killing values were added separately, the result was 24% ± 10% killing, while when CCCP and hβD-3 were incubated together, the result was 67% ± 10% killing). To address whether CCCP treatment increases the amount of peptide internalized by T. denticola, we measured the association of hβD-2 and -3 with T. denticola in the presence of CCCP. When cells were permeabilized to determine the total amount (surface plus internalized) of defensin associated with T. denticola, the amount of hβD-3, but not the amount of hβD-2, was significantly increased in the presence of CCCP. While the results are not conclusive, association of increased amounts of hβD-3 with T. denticola in the presence of a proton motive force inhibitor suggests that efflux has a role in the T. denticola insensitivity to hβD-3. We are continuing to examine the role of efflux in defensin resistance.

Most likely, T. denticola employs more than one strategy to avoid killing by host antimicrobial peptides. Our data support a model in which T. denticola uses a combination of decreased peptide binding plus active efflux of any hβD-3 peptide which manages to enter the cytoplasm in order to withstand killing by hβD-3. The relative resistance to hβD-2 may simply be due to decreased binding of this peptide; hβD-3 has a higher positive charge than hβD-2 (+11 versus +6) and exhibits more efficient binding and killing of T. denticola (compare Fig. 2B and 3B). This intrinsic ability to avoid an important component of the host innate immune system may help explain the persistence of T. denticola in periodontal disease, even in the face of a vigorous inflammatory response. T. denticola associates with the epithelium, in close proximity to the highest concentrations of β-defensins. Other oral treponemes, which are also found at high levels in the periodontal pocket, share T. denticola’s insensitivity to β-defensins (10).

In this setting, treponemes may serve as a protective physical barrier between epithelium-derived antimicrobial peptides and other defensin-sensitive bacteria involved in periodontal disease, thus contributing to the survival of these bacteria in the gingival crevice.

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