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

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Treponema denticola, a periodontal pathogen, is relatively resistant to human beta-defensins, which are small cationic antimicrobial peptides produced by a number of cells, including the gingival epithelium. Using two independent methods, we previously demonstrated that *T. denticola* proteases are not responsible for decreased vulnerability to defensins. 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 (h β D-2 and h β D-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 h β D-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 h β D-2 or -3. In contrast, a proton motive force inhibitor, carbonyl cyanide 3-chlorophenylhydrazone, increased the susceptibility of *T. denticola* to killing by h β D-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.

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 several antimicrobial peptides, including the epithelium-derived β -defensins, which have microbicidal activity against *Porphyromonas gingivalis*, actinomycetes, streptococci, and *Candida* species in vitro (34, 39, 47). However, we have previously determined that *Treponema denticola*, a major component of the "red complex" microflora associated with periodontitis (70), is not sensitive to β -defensins (9, 70). Other suspected periodontal pathogens, including *P. gingivalis* and *Actinobacillus actinomycetemcomitans*, exhibit some strain-dependent resistance to β -defensins (34, 48). β -Defensins are found in saliva and gingival crevicular fluid, are expressed by the oral epithelium, tongue, and salivary glands (7, 17, 18, 22, 23, 40, 57), and are upregulated in response to inflammatory stimuli (23, 28, 36). Yet *T. denticola* thrives in this seemingly hostile environment. Because successful treatment of periodontal disease is dependent on a decrease in the number of periodontal pathogens, including oral treponemes (2, 19, 24, 37, 46, 60, 69), understanding how *T. denticola* is able to avoid killing by these peptides may provide insight into the development of effective therapies.

MATERIALS AND METHODS

Bacterial strains and culture. *T. denticola* strains 35404, 33520, 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 μ g/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 $\times g$ for 10 min at 20°C. The bacteria were washed once and resuspended in 10 mM sodium phosphate buffer (pH 7.2) containing 1% Trypticase soy broth (TSB). A total of 1 $\times 10^5$ motile treponemes in 1 ml were added to triplicate tubes and incubated with 10 μ g/ml of human β -defensin 2 (h β D-2) or h β D-3 (Peptides International, Lexington, KY) or 80 μ g/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 μ M), reserpine (10 μ g/ml), verapamil (20 μ g/ml), or sodium orthovanadate (50 μ M), 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

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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 μ M 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 *dlt* 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 $10,000 \times g$ for 10 min. The pellets were washed once with 10 mM sodium phosphate buffer containing 1% TSB and resuspended to a concentration of 1×10^8 treponemes/ml. Then 100 μ l/well was added to Perkin-Elmer Opti96 black plates, and this was followed by addition of 10 μ l/well of 350 μ M CCCP, 100 μ g/ml reserpine, 200 μ g/ml verapamil, 500 μ M sodium orthovanadate, or an appropriate solvent. Acriflavine is a fluorescent dye; as it binds DNA, its fluorescence is quenched (13). We added 10 μ l of a 10.25- μ g/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 505 (bandwidth, 20). The percent quenching of acriflavine fluorescence compared with the control was calculated as follows: $100 - ([\text{average relative fluorescence units of wells with efflux inhibitor}/\text{average relative fluorescence units of wells with solvent only}] \times 100)$. For example, the data indicated that there was 43% more acriflavine quenching in the presence of CCCP than in the presence of the control, which was calculated as follows: $100 - ([4,515 \text{ relative fluorescence units for } T. denticola \text{ with CCCP}/8,037 \text{ relative fluorescence units for } T. denticola \text{ with DMSO}] \times 100)$.

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 *dlt* at a concentration of 1×10^8 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 μ g/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 $14,000 \times g$ 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 μ l 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 μ l 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 μ l 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 μ l streptavidin-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 $14,000 \times g$ for 30 min and then resuspended in 50 μ l PBS and transferred to an enzyme-linked immunosorbent assay plate (Maxisorp; Nunc, Rochester, NY). Beta-defensins were detected by addition of 100 μ l of 3,3',5',5'-tetramethylbenzidine substrate. The reaction was terminated by addition of 100 μ l 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 μ m; average width of *T. denticola* cell, 0.18 μ m; wavelength, 0.9 μ m; and amplitude, 0.15 μ m (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 $\times$ amplitude, which resulted in a total length of 1.83 μ m. The total length of a "stretched-out" *T. denticola* cell is 12.83 μ m. The bacterium was

FIG. 1. Effect of host proteins present in the growth medium on *T. denticola* susceptibility to β -defensins. A total of 1×10^5 *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 *dlt* were incubated in triplicate with 10 μ g/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.

considered to be a cylinder, and the surface area of a cylinder is equal to $2(\pi r^2) + (2\pi r)h$, where the *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 μ m². The surface area of *E. coli* was 4.4 μ m², determined using the formula for the surface area of a cylinder with 0.5 μ m as width and 1 μ m as height, and the surface area of *S. aureus* was 3.14 μ m² (the surface area of a sphere is $4\pi r^2$, and the radius was 0.5 μ m). 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 was grown in a chemically defined medium which lacks serum or other proteins and in serum-containing GM-1 medium. Bacteria were collected by centrifugation, washed as described previously, and incubated in the presence or absence of 10 μ g/ml hBD-2 or -3 for 4 h. The bacteria were diluted, and the numbers of CFU were determined; *E. coli* ML35 and *S. aureus* 113 *dlt* were used as controls for defensin activity. Treponemes grown in the chemically defined medium were no more susceptible to killing by hBD-2 or -3 than treponemes grown in GM-1 medium with serum were (Fig. 1). These data suggest that serum proteins present in the standard medium do not account for the resistance of *T. denticola* to β -defensins.

***T. denticola* major outer membrane protease, dentilisin, is not responsible for the lack of sensitivity to β -defensins.** We previously found that *T. denticola* proteases do not degrade β -de-

FIG. 2. (A) Binding of biotinylated β -defensin 2 to bacteria. A total of 1×10^8 cells/ml of *T. denticola* (Td) strains cultured in GM-1 medium, *E. coli*, or *S. aureus* 113 *dlt* were incubated in triplicate with biotinylated β -defensin 2 (10 μ g/ml) for 1 h at 37°C. Bacteria were collected by centrifugation and washed to remove unbound peptide. Biotinylated β -defensins were detected by using streptavidin-HRP. The surface area of each bacterium was determined as described in Materials and Methods. The data are the means and standard errors of four experiments. Significance was determined by Student's *t* test assuming unequal variances; an asterisk indicates that the *P* value was <0.05 for a comparison with either control organism. (B) Correlation of β -defensin 2 binding to and killing of bacteria. Binding assays (right y axis) were performed as described above for panel A. For killing assays (left y axis), 1×10^5 *T. denticola* 35404, *E. coli* ML35, or *S. aureus* 113 *dlt* cells were incubated in triplicate with 10 μ g/ml h β D-2 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.

defensins as an explanation for the decreased vulnerability to defensins, using two independent methods: by demonstrating that there was not increased killing by h β D-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 h β D-2 during coinoculation (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 μ g/ml h β D-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 *dlt* 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 h β D-2, the percentages of the ATCC 35405 parent, K1 mutant, and *S. aureus* 113 *dlt* killed were $33\% \pm 9\%$, $26\% \pm 10\%$, and $65\% \pm 9\%$, respectively. For h β D-3, the percentages of the ATCC 35405 parent, K1 mutant, and *S. aureus* 113 *dlt* killed were $31\% \pm 9\%$, $27\% \pm 12\%$, and $92\% \pm 3\%$, 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

FIG. 3. (A) Binding of biotinylated β -defensin 3 to bacteria. A total of 1×10^8 cells/ml of *T. denticola* (Td) strains cultured in GM-1, *E. coli*, or *S. aureus* 113 *dlt* were incubated in triplicate with biotinylated β -defensin-3 (10 μ g/ml) for 1 h at 37°C. The bacteria were collected by centrifugation and washed to remove unbound peptide. Biotinylated β -defensins were detected by using streptavidin-HRP. The surface area of each bacterium was determined as described in Materials and Methods. The data are the means and standard errors of four experiments. Significance was determined by Student's *t* test assuming unequal variances; an asterisk indicates that the *P* value was <0.05 for a comparison with either control organism. (B) Correlation of β -defensin 2 binding to and killing of bacteria. Binding assays (right y axis) were performed as described above for panel A. For killing assays (left y axis), 1×10^5 *T. denticola* 35404, *E. coli* ML35, or *S. aureus* 113 *dlt* cells were incubated in triplicate with 10 μ g/ml h β D-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.

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 h β D-2 or -3 was incubated with *T. denticola* 35404,

33520, 33521, the ATCC 35405 parent, the ATCC 35405 K1 dentilis mutant, *E. coli* ML35, or *S. aureus* 113 *dlt*. While all five *T. denticola* strains bound measurable amounts of h β D-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 dentilis mutant, which is also not sensitive to β -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 β -defensin binding of *T. denticola* grown in serum-free medium did not differ from the β -defensin binding of *T. denticola* grown in serum-contain-

FIG. 4. Quenching of a fluorescent dye in the presence of efflux pump inhibitors. A total of 1×10^8 cells/ml of *T. denticola* 35404 were incubated in 12 replicates at 37°C with 10 μ g/ml reserpine, 20 μ g/ml verapamil, 50 μ M sodium orthovanadate (NaoV), or the appropriate solvents. Then 10 μ l/well of a 10.25- μ g/ml acriflavine solution was added, and the fluorescence was determined immediately and at 2- to 3-min intervals. The percent quenching of acriflavine fluorescence compared to the fluorescence of the controls (solvent only) was calculated as follows: $100 - ([\text{average relative fluorescence units of wells with efflux inhibitor}/\text{average relative fluorescence units of wells with solvent only}] \times 100)$. This experiment was conducted four times (12 replicates per condition), and the means and standard errors are shown.

ing medium (data not shown). There was a suggestion of a correlation between the amount of defensin binding to a bacterium and the amount of defensin killing (Fig. 2B) ($R^2 = 0.83$). Similar results were obtained with h β D-3 (Fig. 3) ($R^2 = 0.73$). These data support the hypothesis that *T. denticola*'s 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 quenching occurs at a higher rate in the absence of active efflux pumps (13). As shown in Fig. 4, all three ABC pump inhibitors enhanced acriflavine fluorescence quenching in *T. denticola*. *T. denticola* 35404 was incubated with an inhibitor or solvent in the presence or absence of h β D-2 and -3 for 1 to 4 h and plated on semisolid agar. None of the inhibitors increased the *T. denticola* sensitivity to killing by h β D-2 or -3 compared with the killing with the inhibitor or defensin alone, suggesting that ABC efflux pumps are not involved in the *T. denticola* insensitivity to β -defensins (data not shown).

FIG. 5. Dissipation of proton motive force increases *T. denticola* sensitivity to h β D-3. (A) A total of 1×10^8 cells/ml of *T. denticola* 35404 were incubated in triplicate at 37°C with 35 μ M CCCP or DMSO (solvent). Then 10 μ l/well of a 10.25- μ g/ml acriflavine solution was added, and the fluorescence was determined immediately and at 2- to 3-min intervals. The percent quenching of acriflavine compared to the fluorescence of the controls (solvent only) was calculated as follows: $100 - ([\text{average relative fluorescence units of wells with efflux inhibitor}/\text{average relative fluorescence units of wells with solvent only}] \times 100)$. This experiment was conducted at least four times with similar results; the results of a representative experiment are shown. (B) A total of 1×10^5 *T. denticola* 35404 cells were incubated with 35 μ M CCCP for 10 min prior to the addition of 10 μ g/ml h β D-2 or -3 and incubation for 1 h at 37°C. The bacteria were plated on the appropriate medium, and the numbers of CFU were determined. The data are the means and standard errors from six experiments. Significance was determined by Student's *t* test assuming unequal variances. One asterisk, $P < 0.05$ for a comparison with CCCP; two asterisks, $P < 0.05$ for a comparison with defensin alone.

Proton motive force uncoupler increases *T. denticola* sensitivity to h β D-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 h β D-2 or -3. Addition of CCCP significantly increased *T. denticola*'s sensitivity to killing

FIG. 6. Effect of dissipation of proton motive force on total association of β -defensins with *T. denticola*. A total of 1×10^8 cells/ml of *T. denticola* 35404 were incubated with biotinylated β -defensin 2 or 3 (10 μ g/ml) and 50 μ M CCCP or an equivalent amount of solvent (DMSO) for 1 h at 37°C. The bacteria were collected by centrifugation and washed to remove unbound peptide. The amount of surface-associated defensin was determined immediately, or bacteria were permeabilized with 0.05% Triton X-100 to allow detection of both internalized and surface peptides. Biotinylated β -defensins were detected by using streptavidin-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 β D-3 alone and with the nonpermeabilized control containing CCCP plus β D-3.

by h β D-3 compared to the sensitivity after incubation with CCCP alone or defensin alone, but it had no effect on the sensitivity to h β D-2 (Fig. 5B). These results suggest that efflux has a role in *T. denticola*'s relative resistance to h β D-3. To confirm that CCCP prevented efflux of h β D-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 intracellular defensin, as well as surface-bound peptide. As shown in Fig. 6, the total amount of h β D-3 associated with *T. denticola*, but not the total amount of h β D-2, was indeed significantly increased in the presence of CCCP, suggesting that h β D-3 accumulated in the organism due to the inhibition of efflux.

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 h β D-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 h β D-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 h β D-1 (4). We previously showed that proteolytic destruction of h β D-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 streptavidin-HRP detection of biotinylated h β D-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 h β D-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 h β D-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 h β D-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 h β D-3, but it had no effect on *T. denticola*'s sensitivity to h β D-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 h β D-3 appears to be synergistic rather than additive (e.g., when CCCP and h β D-3 killing values were added separately, the result was 24% $\pm$ 10% killing, while when CCCP and h β D-3 were incubated together, the result was 67% $\pm$ 10% killing). To address whether CCCP treatment increases the amount of peptide internalized by *T. denticola*, we measured the association of h β D-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 h β D-3, but not the amount of h β D-2, was significantly increased in the presence of CCCP. While the results are not conclusive, association of

increased amounts of h β D-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 h β D-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 h β D-3 peptide which manages to enter the cytoplasm in order to withstand killing by h β D-3. The relative resistance to h β D-2 may simply be due to decreased binding of this peptide; h β D-3 has a higher positive charge than h β D-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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