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Susceptibility of Nontypeable *Haemophilus influenzae* to Human β-Defensins Is Influenced by Lipooligosaccharide Acylation

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Nontypeable *Haemophilus influenzae* (NTHI) lipooligosaccharide *htrB* mutants exhibited greater than 45-fold-increased sensitivity to human β-defensin 2 (HBD-2) compared to the wild type. Complementation by *htrB in trans* to acylation competence reversed this increased sensitivity. In contrast, NTHI was more susceptible to HBD-3 and showed no changes in sensitivity as a result of lipooligosaccharide mutations in oligosaccharide and lipid A biosynthesis genes.

β-Defensins are cationic, salt-sensitive, antimicrobial peptides with broad-spectrum activity that contribute to pulmonary mucosal immunity (4, 5, 18, 19). Endotoxin, lipooligosaccharide (LOS), lipopolysaccharide (LPS), and outer membrane proteins are major components of the gram-negative bacterial outer membrane and are the candidate structures to mediate interactions between bacteria and cationic antimicrobial peptides. For example, lipid A composition affects the sensitivity of *Proteus mirabilis* to polymyxin B and protegrins (14). Phosphorylcholine (ChoP) expression on the LOS of nontypeable *Haemophilus influenzae* (NTHI) decreases its sensitivity to the human cathelicidin LL37 (13) and promotes colonization and development of otitis media in a chinchilla model (21). ChoP also contributes to *H. influenzae* airway cell invasion (20) as well as persistence in the airways (24). Furthermore, structural rearrangements of ChoP can affect the sensitivity of *H. influenzae* to C-reactive protein (24). In another example, the Galα1-3Gal moiety on the nonreducing terminus of LOS on *H. influenzae* confers resistance to killing by antibody and complement (23). To date, the susceptibility of NTHI to the β-defensins and the possible role of LOS have not been studied in this common respiratory pathogen. We hypothesized that NTHI is susceptible to β-defensins and that changes in the LOS would alter this sensitivity.

NTHI lipid A acylation mutants have increased susceptibility to HBD-2, and NTHI strains are uniformly sensitive to HBD-3. We used a modified radial diffusion assay to investigate bacterial sensitivity to the β-defensins (11). The broth and agarose plates were changed to brain heart infusion supplemented with 10 μg of both hemin and NAD per ml to optimize the survival and growth of *H. influenzae*. We performed killing assays on strains of NTHI with different LOS modifications (Table 1). The parent *H. influenzae* strain for all the mutants was NTHI 2019. Exceptions to this are the parents of 477.3 and 375.1 which are mutants of the strains 477 and 375, respectively. 477.3 and 375.1 lack CMP-NANA synthase and cannot sialylate their LOS. Recombinant HBD-2 and HBD-3 (Peprotech, Rocky Hill, N.J.) were rigorously analyzed for purity and concentration by mass spectrometry, amino acid composition, and gel electrophoresis. Tobramycin was used as a positive control for bacterial killing. Statistical significance of the sensitivity of the bacteria to the β-defensins was determined using Student's two-tailed *t* test. The results of the antimicrobial assays are shown in Fig. 1. The tobramycin-positive control killed all NTHI strains, with similar MICs between 0.13 and 0.54 μg/ml (±0.01 to 0.14 [standard error of the mean]) Wild-type NTHI and mutants involved in LOS biosynthesis (2019 *pgmB, licD, siaB*, 375.1, and 477.3) were relatively resistant to killing by HBD-2 (Fig. 1A). NTHI 2019 *htrB* mutants (B28 and B29) have a predominantly tetraacyl lipid A compared to the hexaacylated parent strain (10). These *htrB* mutants exhibited a more than 45-fold increase in sensitivity to HBD-2 ($P = 0.008$ and 0.0001, respectively). The two NTHI *htrB* mutants complemented with a pKK plasmid containing *htrB* reverted to a resistant phenotype to HBD-2 ($P = 0.026$ and 0.009, respectively). This complementation experiment demonstrates that a hexaacyl lipid A is required for HBD-2 resistance. Similarly, the double mutant for *htrB* and *pgmB* was significantly more susceptible to HBD-2 ($P = 0.0002$). In contrast, HBD-3 killed all strains of NTHI, regardless of LOS mutation, in low microgram-per-milliliter concentrations (Fig. 1B). It should be pointed out that by the nature of the methods used for this radial diffusion assay, MICs above 79 may indicate a high degree of variability and should be considered as equivalently resistant.

These results are reminiscent of those of Guo et al., who demonstrated that hyperacylation of *Salmonella* LPS increases resistance to cationic antimicrobial peptides (3). Both results indicate that factors controlling the hydrophobicity of the membrane are important determinants of β-defensin sensitivity. Similarly, *Pseudomonas aeruginosa* from cystic fibrosis patients synthesize LPS containing palmitate, aminoarabinose, and a variety of penta- and hexaacylated lipid A structures that are also associated with resistance to cationic antimicrobial peptides (2). In a xenograft model, *htrB* transcription increased

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TABLE 1. NTHI LOS mutants

NTHI strain	Structure with mutation	Reference
2019	Wild type	16
B28	Acylation of lipid A	10
B28+pKK	Complemented acylation	10
B29	Acylation of lipid A	10
B29+pKK	Complemented acylation	10
pgmB	Oligosaccharide chain	20
htrB/*pgmB*	Acylation + oligosaccharide	This study
licD	ChoP	21
siaB	Sialic acid	W. E. Swords, P. Jones, and M. A. Apicella, unpublished data
375	Wild type	6
375.1	Sialic acid	6
477	Wild type	6
477.3	Sialic acid	6

when NTHI was in an airway environment (20a). This increased *htrB* transcription might ensure the hexaacylation of lipid A, leading to decreased β-defensin sensitivity and increased persistence in the airway.

Given that ChoP expression results in decreased susceptibility to LL37 (13), it was somewhat surprising that there were no significant differences in β-defensin sensitivity with the ChoP mutants or with the *pgmB* mutant which is missing not only the terminal group but also all of the oligosaccharide chains extending from the triheptose structure. We anticipated that the cationic β-defensins might have increased activity against mutants without the positively charged ChoP because of increased electrostatic attraction. This result emphasizes that different classes of cationic antimicrobial peptides may have unique interactions with bacteria.

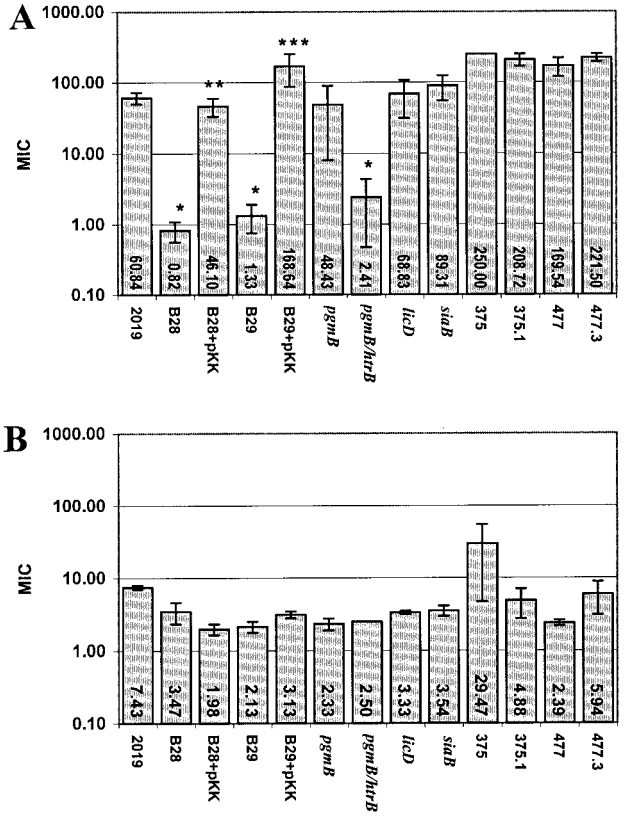

FIG. 1. Sensitivity of NTHI LOS mutants to HBD-2 (A) and HBD-3 (B). All data are the mean MIC (in micrograms per milliliter) as determined by the radial-diffusion assay ($n = 3$ to 7). Symbols: ∗, $P < 0.01$ compared to 2019; ∗∗, $P = 0.026$ compared to uncomplemented *htrB* mutant B28, ∗∗∗, $P = 0.01$ compared to uncomplemented *htrB* mutant B29. Error bars represent the standard error of the mean.

Another surprising result was the striking difference in the antimicrobial activities of the two β-defensins. β-Defensins are cationic peptides of 33 to 47 amino acids with a conserved 6-cysteine motif forming a specific pattern of three disulfide bonds that confers an amphipathic tertiary structure (17). Their initial interactions with negatively charged bacterial membranes are thought to be electrostatic. Defensins permeabilize bacterial membranes by forming pores (25), perhaps with oligomeric structures (7, 17), but their mechanisms of action are incompletely understood. If the human β-defensins have similar mechanisms of action, one might expect to see similar patterns of antimicrobial sensitivity, possibly with different potencies. These studies, however, demonstrate significant differences in the antimicrobial activities of the β-defensins against the NTHI LOS mutants. The greater activity of HBD-3 may reflect its higher net cationic charge density (4, 9), its ability to form oligomers (17), a different mechanism of action, or interaction with different binding sites on bacteria.

These are the first data to show that the human β-defensins exhibit antimicrobial activity against *H. influenzae*. NTHI is a common commensal in the human upper respiratory tract (12) and is also a significant disease-associated pathogen (15). Because β-defensins are widely expressed in the respiratory tract mucosa (4, 9, 18, 19) these results have implications for understanding innate host defense against this common organism.

The virulence of NTHI and its ability to persist in the airway may be influenced by β-defensin sensitivity. NTHI *htrB* mutants have a decreased ability to multiply and cause infection in a chinchilla model of otitis media (1). Recent data also indicate that NTHI *htrB* mutants have a significantly reduced ability to persist in airway epithelia in vitro and that they elicit lesser degrees of cytoskeletal rearrangements and stimulate less host cell signaling in vivo (Swords et al., submitted). One possible explanation for this decreased virulence is that the NTHI *htrB* mutants are more sensitive to inducible defensins such as HBD-2. Since the acylation state of lipid A influences the sensitivity of NTHI to killing by human β-defensins, we speculate that steps in this process may provide targets for future protection and intervention strategies. Deacetylins have been developed that reduce lipid A acylation in *Salmonella enterica* serovar Typhimurium (22) and competitively inhibit lipid A biosynthesis enzymes and have antimicrobial activity against *Escherichia coli* (8). Furthermore, the single-copy genes involved in lipid A biosynthesis are highly conserved, and homologues of these genes are present in most sequenced gram-negative bacteria. Development of pharmacotherapeutic agents that inhibit the acylation of lipid A may increase the sensitivity of NTHI to β-defensins and thereby enable innate immune factors to more readily clear infections by this common pathogen.

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