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Haemophilus ducreyi Is Resistant to Human Antimicrobial Peptides[▽]

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We examined the susceptibility of *Haemophilus ducreyi* to antimicrobial peptides likely to be encountered in vivo during human infection. *H. ducreyi* was significantly more resistant than *Escherichia coli* to the bactericidal effects of all peptides tested. Class I and II *H. ducreyi* strains exhibited similar levels of resistance to antimicrobial peptides.

Haemophilus ducreyi, an extracellular pathogen of human skin, encounters several cell types during human infection, including neutrophils, macrophages, and keratinocytes (3–5) that secrete cationic antimicrobial peptides (APs) (recently reviewed in reference 10). APs secreted by neutrophils include the α-defensins human neutrophil peptides 1 to 4 (HNP-1 to -4) and cathelicidin LL-37 (1, 7). Macrophages secrete human β-defensin 1 (HBD-1) and HBD-2 and LL-37, and keratinocytes secrete HBD-1 to -4 and LL-37 (1, 9, 21). Vaginal epithelial cells also secrete the α-defensin human defensin 5 (HD-5) (14, 15). We previously demonstrated that HNP-1 to -3 are present within natural chancroidal ulcers (5). Because *H. ducreyi* multiplies in an environment with APs, we hypothesized that *H. ducreyi* resists the bactericidal effects of human APs encountered in vivo.

Bactericidal assay. *H. ducreyi* 35000HP and *Escherichia coli* ML35 and their growth conditions have been described previously (2, 8, 12). Protegrin 1 (PG-1) was provided by Robert I. Lehrer. Other APs were purchased from PeproTech (HNP-1, HBD-2, and HBD-3) (Rocky Hill, NJ), Sigma Aldrich (HNP-2) (St. Louis, MO), Peptides International (HNP-3 and HD-5) (Louisville, KY), AnaSpec (HBD-4) (San Jose, CA), and Phoenix Pharmaceuticals (LL-37) (Belmont, CA).

Mid-logarithmic-phase bacteria were suspended in 10 mM sodium phosphate buffer (pH 7.4) with 1% brain-heart infusion broth. Bacteria were mixed with the indicated peptide concentrations in a 96-well polypropylene plate (Costar 3790) and incubated for 1 h at 33°C (*H. ducreyi*) or 37°C (*E. coli*), and the remaining bacteria were quantified by plate count. Survival in the presence of APs was calculated as a percentage of the rate of survival in control wells without APs. Results were subjected to a mixed-model statistical analysis, and the Sidak adjustment was used to control for multiple comparisons (17). *P* values of <0.05 were considered statistically significant.

Differential susceptibilities of *H. ducreyi* and *E. coli* to APs. *H. ducreyi* is susceptible to killing by PG-1, a porcine AP with no human homolog (8). In our assay, both *H. ducreyi* and *E. coli* exhibited <1% survival at a PG-1 concentration of 0.2 μg/ml (data not shown). Thus, our assay detected AP-mediated bactericidal activity.

In assays with α-defensins, *E. coli* was sensitive to HNP-1 to -3, demonstrating 10 to 30% survival at 20 μg/ml (Fig. 1A to C). HD-5 was more potent against *E. coli*, with <1% survival at 20 μg/ml and 16% survival at 2 μg/ml (Fig. 1D). In contrast, *H. ducreyi* exhibited >88% survival at all concentrations of HNP-1 to -3 and HD-5 (Fig. 1) and was significantly more resistant than *E. coli* to α-defensin-mediated killing.

In β-defensin assays, <7% of *E. coli* survived a peptide concentration of 20 μg/ml that 26 to 66% of *H. ducreyi* survived (Fig. 2). At a concentration of 2 μg/ml, only 25 to 30% of *E. coli* but >84% of *H. ducreyi* survived exposure to HBD-3 and HBD-4 (Fig. 2B and C). At a peptide concentration of 20 μg/ml, which represents 5 μM of α-defensin or 2 to 4 μM of β-defensin, the β-defensins exhibited greater bactericidal activity than the α-defensins against *H. ducreyi*. Nevertheless, *H. ducreyi* was significantly more resistant than *E. coli* to killing by HBD-2 to -4 (Fig. 2).

LL-37 exhibited qualitatively greater activity than the de-

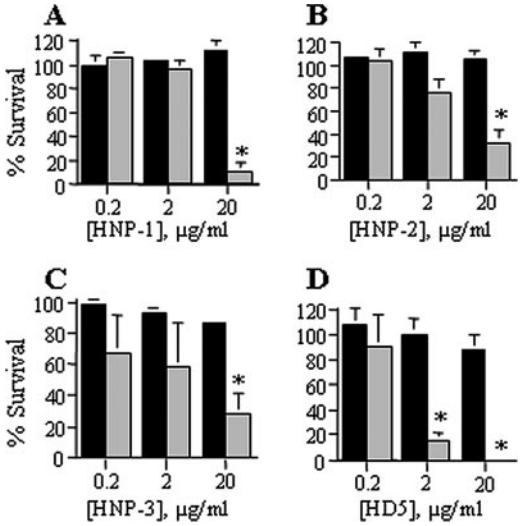

FIG. 1. *H. ducreyi* is significantly more resistant than *E. coli* to the bactericidal effects of α-defensins. Percent survival of bacteria exposed to α-defensins HNP-1 (A), HNP-2 (B), HNP-3 (C), and HD-5 (D). *H. ducreyi* 35000HP, black bars; *E. coli* ML35, gray bars. Data represent the means ± standard errors of the results of three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP, with $P < 0.0001$ (HNP-1 and HD-5), $P = 0.0017$ (HNP-2), and $P = 0.033$ (HNP-3).

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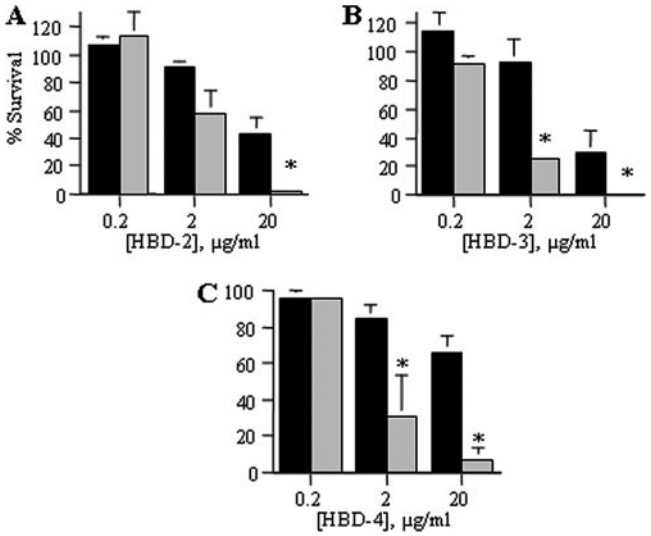

FIG. 2. *H. ducreyi* is significantly more resistant than *E. coli* to the bactericidal effects of β-defensins. Percent survival of bacteria exposed to β-defensins HBD-2 (A), HBD-3 (B), and HBD-4 (C). *H. ducreyi* 35000HP, black bars; *E. coli* ML35, gray bars. Data represent the means ± standard errors of the results of three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP, with $P < 0.0001$ (HBD-2, HBD-3, and HBD-4 at 20 μg/ml) and $P = 0.0089$ (HBD-4 at 2 μg/ml).

fensins against *E. coli* and *H. ducreyi*. Nonetheless, the rate of survival of *H. ducreyi* (16%) was significantly greater than the rate of survival of *E. coli* (5%) at a dose of 2 μg/ml peptide (Fig. 3A). When LL-37 activity was assessed with serial twofold dilutions, the rate of survival of *H. ducreyi* (43%) was significantly higher than the rate of survival of *E. coli* (3%) at a dose of 0.5 μg/ml (Fig. 3B). These data shown that, relative to the level of resistance of *E. coli*, *H. ducreyi* resists LL-37 activity.

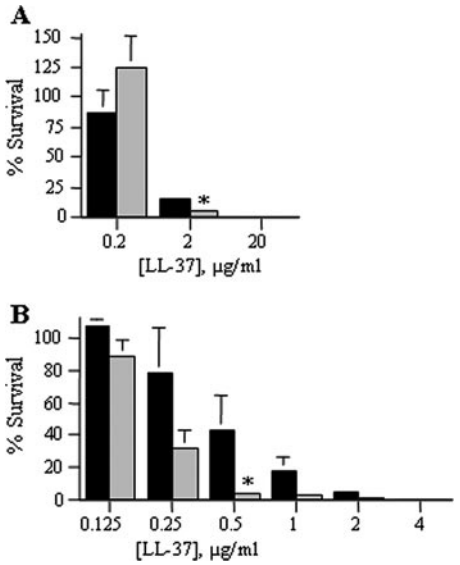

FIG. 3. *H. ducreyi* is significantly more resistant than *E. coli* to the bactericidal effects of the human cathelicidin LL-37. Percent survival of bacteria exposed to 10-fold (A) and 2-fold (B) serial dilutions of LL-37. *H. ducreyi* 35000HP, black bars; *E. coli* ML35, gray bars. Data represent the means ± standard errors of the results of three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP, with $P < 0.0001$ (A) and $P = 0.0012$ (B).

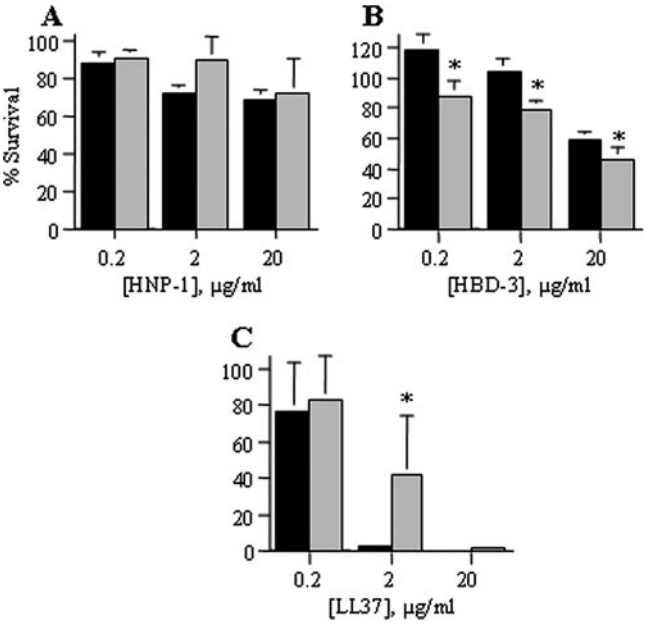

FIG. 4. Representative class I and class II *H. ducreyi* strains are both resistant to APs. Susceptibility of class I 35000HP and class II CIP542 ATCC to α-defensin HNP-1 (A), β-defensin HBD-3 (B), and cathelicidin LL-37 (C). Class I results are shown in black, and class II results are shown in gray. Data represent the means ± standard errors of the results of three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP, with $P < 0.02$ for HBD-3 and $P = 0.0036$ for LL-37.

Both *H. ducreyi* classes are resistant to APs. *H. ducreyi* strains comprise two phenotypic classes (20). We compared the susceptibilities of the class I strain 35000HP and the class II strain CIP542 ATCC (8) to PG-1, HNP-1, HBD-3, and LL-37. As with 35000HP, CIP542 ATCC was susceptible to PG-1, with <2% survival at a dose of 0.2 μg/ml (data not shown). 35000HP was significantly more resistant than CIP542 ATCC to HBD-3 but less resistant than CIP542 ATCC to LL-37 (Fig. 4A and B). Importantly, both the class I and class II strain exhibited resistance to all human APs tested (Fig. 4), indicating that AP resistance may represent a conserved mechanism of *H. ducreyi* survival.

Radial diffusion assays. Bactericidal assays showed low levels of killing of *H. ducreyi* with the β-defensins and LL-37 (Fig. 2 and 3). We thus assessed the activities of these APs in a radial diffusion assay (RDA), which enabled the calculation of a minimum effective concentration (MEC) of each peptide against each strain. The RDA was performed as previously described (8) except that salts were omitted from the agarose underlay to prevent the inactivation of salt-sensitive APs. The units of AP activity were derived from measuring the zones of inhibition surrounding AP-impregnated wells. The MEC of each peptide was defined as the *x* intercept of plots of AP activity over the AP concentration range (Table 1) (8, 18). If less than two concentrations exhibited activity against a strain, no *x* intercept could be defined, and the MEC was estimated as >158 μg/ml, the upper limit of measurable MEC in this assay.

TABLE 1. MECs of APs[a]

Peptide	*E. coli* ML35	*H. ducreyi* 35000HP	*H. ducreyi* CIP542 ATCC
PG-1	2.5	39.0	23.1
HBD-2	11.1	>158	>158
HBD-3	17.4	>158	>158
HBD-4	12.6	>158	>158
LL-37	6.2	>158	>158

[a] MEC in μg/ml, calculated as the *x* intercept of the best-fit line. Data are the mean MECs from three independent assays, each performed in duplicate.

All peptides exhibited activity against *E. coli*, with MECs between 2 and 18 μg/ml (Table 1). The MECs of PG-1 against the *H. ducreyi* strains were not significantly different than the PG-1 activity against *E. coli* (Student's *t* test; $P = 0.1$). With LL-37 and the β-defensins, the dose curves from the RDAs showed significantly less activity against either *H. ducreyi* strain than against *E. coli* ($P < 0.001$; data not shown). The MECs of LL-37 and the β-defensins against *H. ducreyi* exceeded the upper limits of the assay, while the MECs of the human APs against *E. coli* were <20 μg/ml (Table 1). These data confirm that both known classes of *H. ducreyi* are significantly more resistant than *E. coli* to LL-37 and HBD-2 to -4.

Concluding remarks. We have demonstrated that *H. ducreyi* is resistant to human APs likely encountered during infection. Control assays confirmed previous studies demonstrating that the organism is susceptible to PG-1, which *H. ducreyi* does not naturally encounter (8). The differential susceptibility of *H. ducreyi* to human and animal APs may contribute to both its limited host range (humans) and differences in the organism's survival in human and animal models of infection (16, 19), although a much broader panel of APs tested against more *H. ducreyi* strains is needed to address this hypothesis.

Pathogens have evolved various mechanisms to overcome bactericidal APs, including modulating AP production, inactivating APs, pumping APs out of the cell or into the cytoplasm, and repelling APs electrostatically (6, 11, 13). The expression of multiple AP-resistance strategies in pathogens such as *Neisseria gonorrhoeae*, *Salmonella enterica*, and *Staphylococcus aureus* demonstrates the importance to pathogenesis of combating APs. Future work will focus on elucidating the mechanism(s) of AP resistance in *H. ducreyi*.

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