

PHOSPHOETHANOLAMINE TRANSFERASES IN *HAEMOPHILUS DUCREYI*
MODIFY LIPID A AND CONTRIBUTE TO HUMAN DEFENSIN RESISTANCE

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Michael Patrick Trombley

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Haemophilus ducreyi resists the cytotoxic effects of human antimicrobial peptides (APs), including α -defensins, β -defensins, and the cathelicidin LL-37. Resistance to LL-37, mediated by the sensitive to antimicrobial peptide (Sap) transporter, is required for *H. ducreyi* virulence in humans. Cationic APs are attracted to the negatively charged bacterial cell surface. In other gram-negative bacteria, modification of lipopolysaccharide or lipooligosaccharide (LOS) by the addition of positively charged moieties, such as phosphoethanolamine (PEA), confers AP resistance by means of electrostatic repulsion. *H. ducreyi* LOS has PEA modifications at two sites, and we identified three genes (*lptA*, *ptdA*, and *ptdB*) in *H. ducreyi* with homology to a family of bacterial PEA transferases. We generated non-polar, unmarked mutants with deletions in one, two, or all three putative PEA transferase genes. Mutants with deletions in two PEA transferase genes were significantly more susceptible to β -defensins, and the triple mutant was significantly more susceptible to both α - and β -defensins, but not LL-37; complementation of all three genes restored parental levels of AP resistance. Deletion of all three PEA transferase genes also resulted in a significant increase in the negativity of the mutant cell surface, suggesting these three genes contribute to the addition of positively charged moieties on the cell surface. Mass spectrometric analysis revealed that LptA was required for PEA modification of lipid A; PtdtA and PtdB did not affect PEA

modification of LOS. In human inoculation experiments, the triple mutant was as virulent as its parent strain. While this is the first identified mechanism of resistance to α -defensins in *H. ducreyi*, our in vivo data suggest that resistance to cathelicidin may be more important than defensin resistance to *H. ducreyi* pathogenesis.

Margaret E. Bauer, Ph.D., Chair

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
AP	Antimicrobial Peptide
CCCP	Carbonyl Cyanide m-Chlorophenyl Hydrazone
CFU.....	Colony Forming Unit
CI.....	Confidence Interval
CpxA.....	Two Component Signal Transduction System Kinase, Autokinase, Phosphatase
CpxR	Two Component Signal Transduction System Transcriptional Activator, Repressor
CpxRA	Two Component Signal Transduction System
DltA.....	<i>ducreyi</i> lectin protein A
DppA.....	Dipeptide Protein A
DsrA	<i>ducreyi</i> Serum Resistance Protein A
EDD	Estimated Delivered Dose
FgbA	Fibrinogen Binder Protein A
FLP.....	Flippase
Flp1-3.....	Fimbria-like Protein 1-3
FRT	Flippase Recognition Target
GUD.....	Genital Ulcer Disease
HBD-1-4	Human β -defensin 1-4
HbpA.....	Heme-Binding Protein A
HD-5	Human α -defensin 5
Hfq	Host RNA-binding Protein
HgbA.....	Hemoglobin-Binding Protein A

Hlp.....	<i>H. ducreyi</i> Lipoprotein
HNP-1-4.....	Human Neutrophil Peptide 1-4
LL-37.....	Human Cathelicidin
LOS.....	Lipooligosaccharide
LPS.....	Lipopolysaccharide
LptA.....	Lipid A PEA Transferase
LspA1/A2/B.....	Large Supernatant Protein A1/A2/B
MALDI-MS.....	Matrix-Associated Laser Desorption Ionization Mass Spectrometry
MOMP.....	Major Outer Membrane Protein
MTR.....	Multiple Transferable Resistance Efflux Pump
NcaA.....	Necessary for Collagen Adhesion Protein A
OMP.....	Outer Membrane Protein
OMPA2.....	Outer Membrane Protein A2
OPG.....	Osmoregulated Periplasmic Glucans
OppA.....	Oligopeptide Protein A
PAL.....	Peptidoglycan-Associated Lipoprotein
PEA.....	Phosphoethanolamine
PMF.....	Proton Motive Force
PMN.....	Polymorphonuclear Leukocyte
PtdA/B.....	PEA Transferase of <i>H. ducreyi</i> A/B
RND.....	Resistance-Nodulation-Division
RpoE.....	Extracytoplasmic Function Sigma Factor
Sap.....	Sensitive to Antimicrobial Peptides Transporter

SpecR Spectinomycin Resistance Cassette
Tad Tight Adhesion Protein

CHAPTER ONE: INTRODUCTION

***Haemophilus ducreyi* Basic Information and Phylogeny**

Haemophilus ducreyi is a gram-negative coccobacillus that causes the sexually transmitted disease chancroid (1, 2). *H. ducreyi* is a facultative anaerobe and a member of the *Pasteurellaceae* family (3, 4), and has no known non-human reservoirs in nature, making it an obligate human pathogen (3). *H. ducreyi* is classified as a *Haemophilus* species primarily due to its growth requirements and specific need for heme. More recently, rRNA analysis has revealed *H. ducreyi* is more related to the *Actinobacillus* cluster rather than the *Haemophilus* cluster of the *Pasteurellaceae* family (5). The genome of *H. ducreyi* is 1.7MB in size with 1830 predicted open reading frames.

Clinical Manifestations

Infection occurs primarily in the mucosal epithelium, the stratified squamous epithelium, and occasionally the local lymph nodes (6, 7). The disease is characterized by a painful ulcer at the site of infection (8). *H. ducreyi* infections are endemic among sex workers of resource poor regions in Asia, Africa, and South America (9, 10); the prevalence of *H. ducreyi* infections, while estimated at 6-7 million in 1995 (11) and considered steadily on the decline, is nearly impossible to obtain. A combination of difficulty with culturing the bacteria, a lack of reliable diagnostic testing, and syndromic management of genital ulcer diseases (GUD) with antibiotics that treat both *H. ducreyi* and syphilis have led to vastly underreported numbers (8, 12-14). While considered classically a sexually transmitted disease, recent studies in the South Pacific have identified a non-sexual transmission of *H. ducreyi* that causes cutaneous ulcers in the limbs of patients (15-18).

During the course of an infection, *H. ducreyi* enters the epithelial layer through micro-abrasions in skin, often a result of sexual intercourse (19). As few as one colony forming unit (CFU) is needed to be infectious (20, 21). The disease initially forms small papules within a week of acquisition, although papules can form as early as one day after infection. Papules then develop into pustules after several days, and eventually progress to painful ulcers at the site of infection (22, 23). The ulcers, which can persist for several weeks to several months, ultimately lead to erosion and autoamputation of the infected area if left untreated (3). Currently, the Centers for Disease Control and Prevention recommend treatment with a single dose of azithromycin or ceftriaxone or multiple doses of ciprofloxacin or erythromycin (24).

H. ducreyi is a concern for public health in underdeveloped countries because chancroid has been shown to facilitate both the acquisition and transmission of HIV (8). The presence of GUD, such as chancroid, can increase acquisition of HIV by more than 20-fold (25, 26). The likely mechanism behind this increase is a combination of open ulcers in skin resulting in lack of epithelial barriers and the infiltration during infection of T-cells and macrophages, which contain the HIV-target CD-4 receptor and CCR5 and CXCR4 co-receptors (27). Upon entering the ulcer, the virus is exposed to T-cells and macrophages that are responding to the *H. ducreyi* infection as well as to dendritic cells that may present HIV to additional T-cells (28); in essence, the host's immune response to the *H. ducreyi* infection actually presents HIV with its target cells.

Human Model of *H. ducreyi* Infection

Much has been learned about *H. ducreyi* pathogenesis from the human inoculation experiments developed in the 1990s. While animal models do exist as a model for *H. ducreyi* infection, none are an accurate representative of the human immune response to a *H. ducreyi* infection. Although a human infection can begin with as few as one CFU, the animal models require much larger inocula, indicating that *H. ducreyi* is not as fit in these models as in human infection; mice and macaques require 10^7 CFU and swine and rabbits require 10^4 - 10^5 CFU (1). The swine and rabbit are the more commonly used models, as macaque monkeys are expensive and only the males develop ulcers, and *H. ducreyi* does not survive in mice, with lesions being due to LPS rather than bacterial growth. The swine and rabbit models do, however, illicit an antibody response and spontaneously clear the infection. Antibodies in the swine model are protective, whereas antibodies created during natural human infections are not protective. The rabbit model is also temperature dependent; *H. ducreyi* will only grow if the rabbits are living at 15-17 degrees Celsius (29-31). The animal models of *H. ducreyi* infection do have the advantage of being able to let the infection proceed to the ulcerative stage whereas the human model of *H. ducreyi* infection is halted before ulcers develop. However, since *H. ducreyi* naturally only infects humans, the human is considered the most relevant model for invasion and infection. Since the *H. ducreyi* infection remains local, and the strains used are easily treated with appropriate antibiotics, there is minimal risk to human volunteers.

The development of the human model of *H. ducreyi* infection over the past twenty years has provided the field with a method of examining virulence in the bacteria's

natural host. In the model, healthy volunteers are infected with *H. ducreyi* in the upper arm via an allergen testing device with an estimated delivered dose (EDD) of 90 CFU of 35000HP, a human-passaged variant of a Class I clinical isolate 35000 (21). There are three inoculation sites on one arm, and the subject may also be inoculated in the opposite arm with an EDD of 45-180 CFU of an *H. ducreyi* mutant strain, again at three sites. The infection is followed for 14 days and either spontaneously resolves or is stopped before ulcer formation or development of pain and discomfort of the volunteer. Patients are given one oral dose of ciprofloxacin to treat the infection, and biopsies are taken at the conclusion of the trial (20, 21, 32). While the human inoculation experiment can only provide information regarding the first two weeks of infection, disease progression of wild-type *H. ducreyi* can be compared with that of a mutant strain to examine the necessity of specific genes for virulence.

Upon *H. ducreyi* entry into the host epithelium, there is a vigorous polymorphonuclear leukocyte (PMN) response, which forms the initial epidermal abscess. Macrophages also enter and form a collar at the base of the infection (23). *H. ducreyi* remains extracellular throughout the infection, and it co-localizes with phagocytic immune cells, collagen, and fibrin (33). After 24 hours of infection, additional macrophages, myeloid dendritic cells, NKT cells, and CD4+ and CD8+ T-cells are recruited to the papule site. Since there is a small inoculum and shortened duration of the human trial, there is a limited B-cell response and no serum antibodies for *H. ducreyi* are produced in the human model of infection. In natural infections, however, there is a serum antibody response, although these antibodies are not bactericidal and do not provide immunity (34-36). Due to treatment during early stages of the infection and time

limitations of the human model, little is known about how or why clearance can naturally occur.

***H. ducreyi* Virulence Mechanisms**

During infection, the first line of host immune defense *H. ducreyi* encounters is phagocytic cells. Two large supernatant proteins, LspA1 and LspA2, are expressed as soluble proteins and inhibit phagocytosis (37). A third protein, LspB, is involved in the secretion of these proteins across the cell membrane (38). The specific mechanism of action of LspA1 and LspA2 involves blocking the Fc γ -receptor mediated uptake in phagocytic cells. They do so by inhibiting the phosphorylation of Src family tyrosine kinases, which initiate the signal for phagocytosis (39). The result is phagocytic cells that are unable to ingest and remove the *H. ducreyi* bacteria. Studies done in the human model of *H. ducreyi* infection reveal that LspA1 and LspA2 are required for virulence (40).

H. ducreyi has also been shown to resist complement-mediated killing by normal human serum; attenuated, serum-sensitive strains of *H. ducreyi* are killed by the classical complement pathway (41). Briefly, in this pathway, a complement-reactive protein binds to the antibodies IgG or IgM, which have bound to pathogens. This forms the C-1 complex (42), which cleaves C4 and C2 into multiple parts. The C-1 complex binds with C4b and C2b to form the C3-convertase, which cleaves C3 and binds with C3b. The compound consisting of IgM, C-1, C4b, C2b, and C3b, recruits C5, C6, C7, C8, and C9, which will form the membrane attack complex. This complex creates large pores in the membrane of the pathogen, resulting in cellular death (43).

There are two outer membrane proteins, the *ducreyi* serum resistance protein (DsrA) and the *ducreyi* lectin protein (DltA), found on the *H. ducreyi* cell surface that contribute to serum resistance (44, 45). DsrA is a protein that forms large trimers on the bacterial outer membrane, which prevents the binding of IgM onto the *H. ducreyi* cell surface (16). The DsrA complex inhibits the complement cascade that creates the membrane attack complex. *H. ducreyi* strains lacking the DsrA protein are serum sensitive and fully attenuated in the human model of *H. ducreyi* infection (44, 46). Recent studies have also found that DsrA is involved in fibrinogen, fibronectin, and vitronectin binding, suggesting DsrA plays a large role in *H. ducreyi* adhesion during infection (47, 48). DltA, a fibronectin and carbohydrate-binding surface protein, is also involved in serum resistance. The exact mechanism of action is not yet known, but the *dltA* mutant is moderately sensitive to serum and are partially attenuated in the human model (45, 49).

There have been several additional virulence mechanisms found in *H. ducreyi*. While they may not specifically involve evading the immune system, they have been identified by the human model of *H. ducreyi* infection as necessary for virulence. Iron acquisition is vital to bacterial survival as most host environments are typically iron deficient. The hemoglobin binding protein HgbA found in *H. ducreyi* shares homology with TonB-dependent outer membrane receptor proteins found in other gram-negative bacteria. HgbA binds hemoglobin and is important for iron acquisition in *H. ducreyi* (50, 51). HgbA is required for virulence in the human model of infection (48, 52, 53). The *H. ducreyi* peptidoglycan-associated lipoprotein (PAL), which is homologous to proteins in numerous gram-negative bacteria, is important for linking the outer membrane with the

peptidoglycan, providing stability to the cell envelope (54-56). Studies in the human model have revealed that PAL is necessary for *H. ducreyi* virulence in humans (57). The fibrinogen binder protein FgbA, shown to bind fibrin, is also necessary for virulence. A deletion in *fgbA* resulted in partial attenuation (58); although the mechanism is unknown, it is predicted that this protein could use fibrin deposits to shield the bacterial cell surface from host immune responses as seen in other bacteria (59, 60).

Several virulence genes found in *H. ducreyi* reveal the necessity for adherence and microcolony formation. The necessary for collagen adhesion protein (NcaA) is an outer membrane protein that has been shown to be crucial for *H. ducreyi* binding to type I collagen, an important step in bacterial adherence and invasion. Mutants lacking NcaA are fully attenuated in the human model (61). The fimbria like proteins (Flp1-3) in *H. ducreyi* shares homology with the tight adherence, or Tad, proteins found in many gram-positive and gram-negative bacteria. These Tad proteins are essential for adherence, pathogenesis, and biofilm formation (62). In *H. ducreyi*, loss of Flp1 or Flp2 inhibits bacterial attachment to the foreskin and microcolony formation (63, 64). Mutants with deletions in *flp1-3* and *tadA* were fully attenuated in the human model of *H. ducreyi* infection (63, 65). *H. ducreyi* also has a homolog of the *Vibrio harveyi* LuxS quorum sensing protein. Although *H. ducreyi* does not have a known mechanism of quorum sensing, LuxS could contribute to the formation of microcolonies; a mutant lacking the LuxS protein was partially attenuated in the human model (66).

Clinical isolates of *H. ducreyi* can be separated into two classes. While clinically there exists little variation within each class, being 94% invariant (67), the differences between Class I and Class II strains of *H. ducreyi* are variations in the sequence and

expression of multiple key virulence factors, outer membrane proteins, and lipooligosaccharide (LOS) (67). The outer membrane proteins DsrA and DltA are truncated in the Class II isolates. Although Class II strains are still serum resistant, the Class II DltA protein is four amino acids smaller than its Class I homolog, and the Class II DsrA protein is smaller, shares between 47.8 – 56.0% homology with the Class I DsrA, and is not recognized by class I DsrA antibodies (68). The Class II NcaA protein has a different N-terminus when compared to the Class I NcaA protein. The Hlp is larger in Class II strains than Class I, and Class II strains also contain variant outer membrane proteins (OMPA2 and MOMP) as well as a truncated LOS (68, 69). There are amino acid substitutions and insertions in FgbA when comparing Class I and Class II strains, although both proteins are still functional (58). Both the Sap transporter and MTR efflux pump, transporters involved in AP resistance, are functionally conserved amongst Class I and II strains, although there are some variations throughout the Class I strains (70, 71).

It is unclear the extent of effect that these variations have on the different classes; Class II strains grew slower and formed smaller colonies than Class I strains in vitro, although both strains are still fully virulent in vivo. Recent studies have revealed that recombination is evident amongst Class I strains, but not between Class I and Class II strains. This suggests that these two classes may form separate, distinct species (67).

Regulation of Virulence

An important aspect of virulence genes is their regulation; bacteria are not constitutively expressing every gene in their genome; they regulate genes based on environmental stimuli to enact proper responses. *H. ducreyi* has one known two-component signal transduction regulator system, which is homologous to the two-

component signal transduction system CpxRA found in *Escherichia coli*. CpxRA is comprised of two parts: CpxA, a membrane-bound sensor kinase, and CpxR, a cytoplasmic response regulator. CpxA functions as a kinase, an autokinase, and a phosphatase, whereas CpxR acts as a transcriptional activator or repressor (72-74). In the absence of stress, CpxA will function primarily as a phosphatase to tightly regulate the amount of phosphate on CpxR, which can be phosphorylated by small cytoplasmic molecules such as acetyl phosphate (74). In *E. coli*, the presence of cellular envelope stress activates CpxA, which autophosphorylates itself and then phosphorylates CpxR. CpxR then continues on to regulate the transcription of various virulence genes (75, 76).

In *H. ducreyi*, the CpxRA system has been shown to regulate 165 genes, and it specifically plays an important role in regulating the expression of *lspB*, *lspA2*, *dsrA*, *ompA2*, and *flp1* (77). Deletion of *cpxR* results in an upregulation of *lspB* and *lspA2*, and the *cpxR* mutant is still fully virulent in the human model of *H. ducreyi* infection (66, 78). However, deletion of *cpxA*, which acts as a CpxR phosphatase in the absence of stimuli, results in activation of the CpxRA system. In this mutant, the virulence genes *lspB*, *lspA2*, *dsrA*, and *flp1* are all down regulated (77). Decreased expression of these genes results in a loss of virulence, as seen in the human model (79).

While it is still not entirely clear what stress activates the CpxRA system in *H. ducreyi*, studies have shown that possible membrane stress may play a role in CpxRA activation. In the presence of fetal calf serum, the CpxRA transcripts decrease, which in turn increases the expression of virulence genes necessary for serum survival, such as *dsrA* (66, 77). In the *mtrC* mutant, a strain with a deletion of the periplasmic binding protein in a tripartite efflux pump, CpxRA is activated (71). It is possible that deletion of

function in this efflux pump resulted in accumulation of molecules in the periplasm, which in turn caused membrane stress that then activated CpxA. Current studies are underway to better understand the stressor and mechanism of activation of the CpxRA system.

Recent studies have defined two new regulators of virulence in *H. ducreyi*. A host RNA-binding protein, Hfq, has been found to influence the expression of roughly 16% of *H. ducreyi* genes. Hfq protein binds to mRNA and regulates interactions with small RNAs, likely controlling the degradation and thus expression of the mRNA (80). Hfq is found to primarily regulate expression of virulence genes, such as *flp1*, *lspB*, *lspA2*, and *dsrA*, during stationary phase. A mutant with a deletion of *hfq* was found to be fully attenuated in the human model of *H. ducreyi* infection (81). Additionally, *H. ducreyi* has a homolog of the *E. coli* extracytoplasmic function sigma factor RpoE, which regulates envelope maintenance and repair. In *H. ducreyi*, RpoE regulates the expression of 180 genes that play a role in maintaining and regulating the cellular envelope. While the role of RpoE is distinct from that of CpxRA, both are involved in the upkeep and response of the *H. ducreyi* envelope (82).

Antimicrobial Peptides

During infection, *H. ducreyi* faces the robust immune response of host-secreted antimicrobial peptides (AP). APs are typically small, cationic peptides ranging from 12-100 amino acids in length that target microorganisms. They are practically universally present, isolated from single-celled organisms to complex members of the plant and animal kingdoms (83, 84). There are many different classes of APs that affect many different types of microorganisms; while there are APs that specifically target DNA/RNA/protein/macro-molecule synthesis (85, 86), the majority are known to target the cell membrane and lyse the bacterial cell (87). APs, such as cathelicidin, are primarily stored in neutrophil granules at high concentrations (10 mg/mL); when the APs are released during an infection, the concentration becomes diluted (88-90). However, APs have shown functional activity at concentrations below 10 $\mu\text{g/mL}$ (91). Other APs, such as the defensins, can be stored in circulating immune cells such as neutrophils, macrophages, NK cells, and Paneth cells, as well as expressed in tissue-specific cells such as in the skin or vaginal epithelial layers (92); they can be constitutively expressed or NF- κ B dependent, expressed only during times of inflammation or infection (93-96). *H. ducreyi* encounters defensins and cathelicidin during the course of its infection (22, 23, 33).

Mammalian defensins are divided into three classes; α -defensins, β -defensins, and θ -defensins. They are characterized by six cysteine residues that form three distinct disulfide bonds. α -defensins form disulfide bonds between cysteine residues 1-6, 2-4, and 3-5 to create a triple-stranded β -sheet structure (97). β -defensins form disulfide bonds between cysteine residues 1-5, 2-4, and 3-6 to also create a triple-stranded β -sheet

structure (98). To date, there are six known human α -defensins, and over 30 known human β -defensins. θ -defensins, recently discovered in the rhesus macaque monkey and not found in humans, form a circular peptide. Although its circular secondary structure is not numbered because there is no N or C terminus, it forms disulfide bonds between what would be considered the 1-2, 3-6, and 4-5 cysteine residues in the primary structure (99).

The defensins can be secreted by immune cells or stored in leukocyte granules (88, 89, 100), and they can be expressed either constitutively or induced during infection (101-106). While the exact targets are still debated, the antibacterial activity of defensins is thought to come primarily from targeting the cell membrane and causing permeability in the cell membrane, which eventually will eventually lead to the formation of pores (107-112). These pores then allow potassium to leave the cell, disrupting the osmotic balance as well as destroying the electrochemical gradient, leading to cell death. The permeabilization of the cell membrane is also linked to inhibition of RNA, DNA, and protein synthesis (108); while it is unclear whether the inhibition of these processes or if the formation of pores is the ultimate lethal step, the end result is cell death.

The cathelicidins are another class of APs; while there are many members of this class, the only human cathelicidin is LL-37. This AP, which lacks disulfide bonds, is 37 amino acids long and forms an α -helix in solution (113, 114). LL-37 is produced in a variety of cells including leukocytes, neutrophils, and epithelial cells, and is often found in bone marrow, saliva, sweat, and breast milk (113-116). The LL-37 mechanism of action against *E. coli* has been studied in real time using fluorescence. First, LL-37 crosses the outer membrane in a non-lethal fashion; this step is common amongst APs, and involves the AP attaching to and inserting itself into the outer cell membrane. This

leads to a disturbance of the membrane that induces self-promoting uptake, a non-lethal permeabilization of the outer membrane (117). As LL-37 crosses the periplasm, *E. coli* growth is halted, presumably a result of LL-37 interfering with cell wall synthesis. The peptide then congregates on the cytoplasmic membrane, disrupting and eventually causing the formation of pores and cell death. The entire process from initial attachment to the outer membrane to cell death occurs in roughly 30 minutes (118).

Many mechanisms exist in both gram-positive and gram-negative bacteria that confer resistance to APs. Bacteria can modify their cell surface structure or charge to reduce the ability of APs to bind or penetrate the cell surface; for example, *Vibrio cholerae* will add the positively charged amino-arabinose Ara4N to repel cationic APs (119). Some bacteria such as *Bacillus anthracis* can form spores with a thick spore coat and cortex to prevent AP access to the cell surface (120), and other bacteria such as *Pseudomonas aeruginosa* congregate together and form biofilms, which also provide protection from APs (121). Some bacteria are able to express molecules that either down regulate the expression of APs or bind to extracellular APs to inhibit their capacity to attach to the cell surface; *Shigella flexneri* can down-regulate the expression of LL-37 and the β -defensin HBD-1, and the *Streptococcus pyogenes* protein M1 binds to LL-37 to inactivate it (122, 123). Others are able to produce peptidases and proteases, both as outer membrane proteins or cytoplasmic proteins, which degrade APs before they can reach their intracellular targets; for example, *Staphylococcus aureus* has two proteinases, metalloproteinase and glutamylendopeptidase, that digest and degrade LL-37 before it induces lethality (124). Efflux pumps or channels are present in bacteria to provide resistance to APs; *Klebsiella pneumoniae* has the efflux pump AcrAB that pumps out

polymyxin B out of the cell before lethal damage can be done (125). Lastly, bacteria can also modify the intracellular targets or enzymatic pathways to inhibit AP functionality; this is commonly seen in bacteria such as *E. coli* that resist the AP microcin B17 by the DNA gyrase protein GyrB (126). Taken together, bacteria have evolved and developed numerous mechanisms to resist the lethal modes of action of APs (127, 128).

***H. ducreyi* and Antimicrobial Peptides**

During infection, *H. ducreyi* encounters host immune cells and resident keratinocytes that express APs (22, 23, 33). PMNs express the α -defensins human neutrophil-1 (HNP-1), HNP-2, HNP-3, and HNP-4, the β -defensin human beta defensin 4 (HBD-4), and the human cathelicidin LL-37 in response to infection (91, 129).

Macrophages express the β -defensins HBD-1 and HBD-2, and the cathelicidin LL-37 in response to inflammation (92, 130). The resident keratinocytes constitutively express the β -defensin HBD-1 and express HBD-2, HBD-3, and HBD-4, and the cathelicidin LL-37 in response to inflammation (131-133). Vaginal epithelial cells constitutively express the α -defensin human defensin 5 (HD-5) (134).

H. ducreyi has been shown to resist the lethal activity of several classes of human APs relevant to infection, including α -defensins, β -defensins, and the human cathelicidin LL-37 (135). In these studies, *H. ducreyi* was significantly more resistant than *E. coli* K-12 to the α -defensins HNP-1, HNP-2, HNP-3, and HD-5, the β -defensins HBD-2, HBD-3, and HBD-4, and the cathelicidin LL-37. The α -defensins used in these studies had virtually no effect on *H. ducreyi* survival at any experimental concentration. This resistance was conserved among both classes of *H. ducreyi*, and involves the ensitive to

antimicrobial peptides (Sap) transporter and the multiple transferable resistance (MTR) efflux pump.

The Sap transporter is an influx pump and belongs to the family of oligopeptide (Opp)–dipeptide (Dpp) peptide and metal ion–uptake ATP binding cassette (ABC) transporters (136-138). Structurally, the Sap transporter has five main components: SapA, SapB, SapC, SapD, and SapF (Figure 1A). SapB and SapC are permease proteins found in the inner membrane and form a pore. SapD and SapF are ATPase subunits that use the energy from converting ATP to ADP to power the influx pump. SapA is a periplasmic binding protein that binds to specific peptides and shuttles them through SapBC by the energy provided from SapDF (136, 138). In *Haemophilus influenzae*, the Sap transporter has been shown to play a role in cellular homeostasis by conferring uptake of heme, and SapD specifically is involved in potassium uptake (138, 139). The Sap transporter also confers resistance to both LL-37 and the β -defensin HBD-3 by directly binding to the APs and shuttling them into the cytoplasm before they can attach to the inner membrane, the lethal target for APs (138, 140, 141). Once inside the cytoplasm, the APs are degraded by cytoplasmic peptidases (137). In addition to *H. influenzae*, the Sap transporter has been found to confer resistance to APs in *Salmonella enterica*, *Erwinia chrysanthemi*, and *Proteus mirabilis* (136, 142, 143).

In *H. ducreyi*, the Sap transporter confers resistance to the human cathelicidin LL-37, but not α - or β -defensins (70, 144). A mutant strain with a deletion of the periplasmic binding protein encoding gene *sapA* was moderately more sensitive than 35000HP to LL-37; when observed in the human model of *H. ducreyi* infection, the *sapA* mutant was partially attenuated (70). When a mutant with deletions of the permease encoding genes

sapB and *sapC* was generated, it was significantly more sensitive than both 35000HP and the *sapA* mutant to LL-37. The *sapBC* mutant was rendered completely attenuated in the human model of *H. ducreyi* infection (144). The results from both the SapA and SapBC studies confirm not only that the Sap transporter is necessary for *H. ducreyi* virulence, but that SapBCDF still has some functionality without the binding protein SapA.

A second mechanism of AP resistance found in *H. ducreyi* is the MTR efflux pump (71). This efflux pump is a member of the resistance-nodulation-division (RND) family of drug efflux pumps, which not only provide resistance to a variety of antibiotics but also export various other toxic substrates such as detergents and APs. These tripartite efflux pumps are powered by proton motive force (PMF), a force generated by the potential energy stored in proton and voltage gradients across a membrane (145-149). In *Neisseria gonorrhoeae*, the MTR efflux pump, a member of the RND family, has three components: MtrC, MtrD, and MtrE (Figure 1B). MtrD is an inner membrane transporter protein involved in proton translocation, and MtrE is an outer membrane channel protein. MtrC is a periplasmic fusion protein that connects MtrD to MtrE. Studies have shown that the MTR efflux pump had three MtrD and three MtrE subunits with six MtrC fusion proteins linking MtrD to MtrE (150). Substrates are thought to be effluxed from both the cytoplasm and periplasm by MTR, and in *N. gonorrhoeae*, resistance to LL-37 and protegrin-1, a β -sheet porcine AP that targets the cell membrane (151), is mediated by MTR (145).

In *H. ducreyi*, exposure to carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a compound that disrupts the proton gradient and PMF (152), revealed there was a PMF-dependent mechanism of resistance to both β -defensins and LL-37 (71). *H. ducreyi* has a

homolog of the *N. gonorrhoeae* MTR efflux pump, and a mutant generated with a deletion in *mtrC* had an increase in sensitivity to β -defensins and LL-37 when compared to 35000HP (71). Deletion of *mtrC* also activated CpxRA, suggesting the loss of MtrC may cause periplasmic stress. While CpxRA activation does increase susceptibility to LL-37, studies revealed the presence of a PMF-dependent mechanism of AP resistance still present in the *cpxA* mutant. This showed that the increase in sensitivity to LL-37 and β -defensins was a result of the loss of the MTR efflux pump and not the activation of CpxRA (71), thus showing that the MTR efflux pump confers resistance to both β -defensins and LL-37 in *H. ducreyi*.

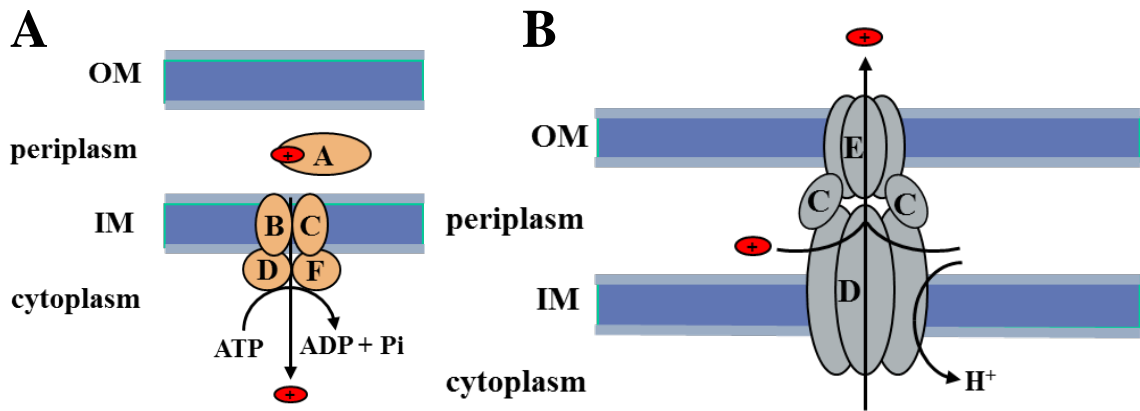


Figure 1. Structure of the Sap Transporter and MTR Efflux Pump. (A) The Sap transporter has five main components: SapA, SapB, SapC, SapD, and SapF. SapA is a periplasmic binding protein; SapB and SapC are permease proteins found in the inner membrane that form a pore; SapD and SapF are ATPase subunits that use the energy from converting ATP to ADP to power the influx pump. OM and IM shown are the outer and inner membrane of the bacteria. (B) The MTR efflux pump has three components: MtrC, MtrD, and MtrE. MtrC is a periplasmic fusion protein; MtrD is an inner membrane transporter protein involved in proton translocation; MtrE is an outer membrane channel protein. MtrD and MtrE have three subunits with six MtrC fusion proteins linking MtrD to MtrE. OM and IM shown are the outer and inner membrane of the bacteria.

Electrostatic Repulsion and Phosphoethanolamine

The Sap transporter and MTR efflux pump confer resistance to LL-37 and β -defensins, but there is still no defined mechanism of resistance to α -defensins in *H. ducreyi*. One focus of ongoing research is to search for new mechanisms in *H. ducreyi* that confer this AP resistance. One mechanism of AP resistance in bacteria is to modify the cell surface with positively charged moieties. These moieties decrease the overall negativity of the cell surface, which can create an electrostatic repulsion of the positively charged APs (153). In gram-positive bacteria, teichoic acids found in the cell wall are modified with alanine groups; alanylation adds positive charges to the bacterial cell surface and contributes to resistance APs (154, 155). Gram-negative bacteria have been shown to modify their lipopolysaccharide (LPS) or LOS with positively charged aminoarabinose or phosphoethanolamine (PEA) (156-163). There is no evidence of the presence of an aminoarabinose transferase in *H. ducreyi* or that *H. ducreyi* modifies its LOS with aminoarabinose. However, *H. ducreyi* LOS contain one PEA modification on the lipid A and a second PEA modification on the KDO of its core oligosaccharide (Figure 2) (164, 165).

Modification of LPS or LOS with PEA has been shown to be advantageous for pathogenesis in several gram-negative bacteria. PEA modification of the cell surface confers resistance to a wide range of APs, including protegrin-1, LL-37, β -defensins, and the antibiotic polymyxin B while also providing protection from human serum (153, 157, 158, 161-163, 166-170). All characterized PEA transferases are members of the YhjW/YjdB/YijP/YbiP superfamily of enzymatic inner membrane proteins, and furthermore, all characterized lipid A PEA transferases in gram-negative pathogens are

members specifically of the Yjdb family of enzymatic proteins (159, 166). Additionally, there are many putative PEA transferases that are members of this superfamily.

In the pathogenic *Neisseria*, three PEA transferases have been identified, including LptA, Lpt-3 and Lpt-6, which PEA-modify lipid A or the Heptose II core sugar at the third or sixth position, respectively (159, 171-173). These PEA modifications contribute to *Neisseria* resistance to polymyxin B, a positively charged antibiotic that targets the cell membrane and often is used as a model cationic AP (174), protegrin-1, human and murine cathelicidin, and human serum (162, 163, 166, 167). Lipid A PEA modification in *Neisseria* has an important role in adhesion to endothelial and epithelial cells, both required for initial infection. This modification also has been shown to reduce the pro-inflammatory cytokines and chemokines secreted by vaginal epithelial cells in female mice (167, 175). Additionally, both the murine female genital tract and the human male urethra models of *N. gonorrhoeae* infection have shown that PEA modification of lipid A contributes to survival in vivo (167, 176). Structural representations of the *Neisseria* LOS can be found in the Appendix Figure 8.

PEA modifications have been shown to contribute to AP resistance in additional gram-negative bacteria. In *Salmonella enterica*, the PEA transferase CptA modifies the Heptose II core sugar, and lipid A is PEA-modified by EptA (157, 158). These PEA modifications of the LPS confer resistance to polymyxin B (153, 157, 158). Additionally, competitive infection experiments in mouse models of *S. enterica* infection showed a moderate decrease in survival of PEA transferase mutants when compared to wild-type strains (157). In *E. coli*, the PEA transferase EptB modifies KDO, and lipid A is PEA-modified by PmrC (161, 168). These PEA modifications of the *E. coli* LPS not

only confer resistance to polymyxin B, but they also play an important role in protecting the cell from environmental stresses, such as the toxic effects from high calcium exposure (161, 168). Structural representations of the Salmonella and *E. coli* LPS can be found in the Appendix Figures 9 and 10.

In addition to LPS and LOS, PEA transferases can modify other bacterial cell surface structures. Recently, studies have shown that the *Campylobacter jejuni* PEA transferase EptC modifies lipid A, flagella, and surface glycans with PEA, contributing to resistance to human and avian β -defensins and polymyxin B (169, 170, 177). Deletion of these PEA modifications in *C. jejuni* also result in a decrease in motility of the organism (169, 178). In contrast to *Neisseria*, in *C. jejuni*, one PEA transferase is responsible for multiple PEA modifications, indicating that each specific PEA modification is not necessarily controlled by an independent or specific PEA transferase. *N. gonorrhoeae* has been shown to modify its Type IV pili with PEA, although the source of this modification and its advantage remains unclear (179). In addition to cell surface structures, the recently described PEA transferase OpgE (also known as YbiP) modifies osmoregulated periplasmic glucans (OPGs) in *E. coli* (180-182). These OPGs, which are important components of some gram-negative bacterial envelopes, play crucial roles in cellular chemotaxis and motility (181, 183, 184). There is no evidence that *H. ducreyi* contains any OPGs.

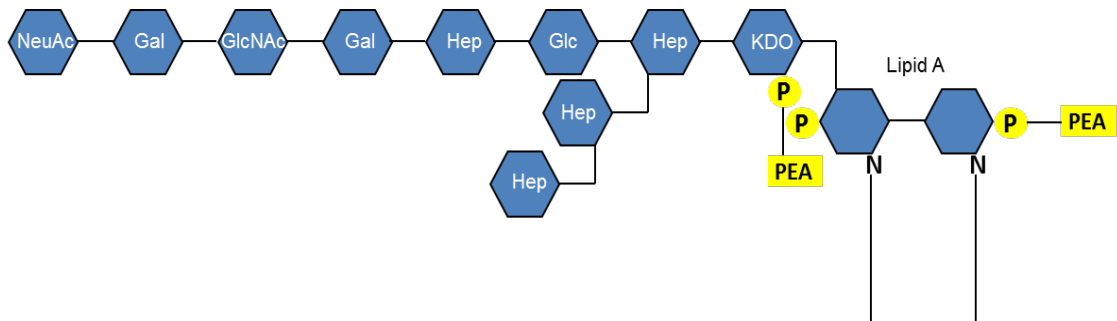
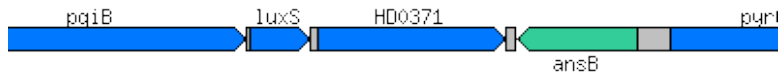


Figure 2. *H. ducreyi* LOS. Depiction of *H. ducreyi* LOS, including known PEA modifications found on the lipid A and on the KDO core sugar. Starting from the right, KDO is 3-Deoxy-D-manno-oct-2-ulosonic acid, Hep is Heptose, Glc is Glucose, Gal is Galactose, GlcNAc is N-Acetylglucosamine, and NeuAc is N-acetylneuraminate. Sugars with a highlighted P are phosphorylated, and PEA indicates a PEA modification at that site.

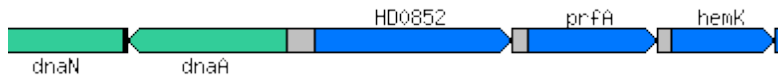
Hypothesis

The *H. ducreyi* genome encodes three genes that have strong homology to the YhjW/YjdB/YijP/YbiP family of PEA transferases (Figure 3). We hypothesized that these genes, lipid A PEA transferase *lptA* (HD0852), PEA transferase of *H. ducreyi* *ptdA* (HD0371) and *ptdB* (HD1598) contribute to AP resistance and virulence of *H. ducreyi*. In this thesis, we generated deletion mutants lacking one, two, or three putative PEA transferase genes in *H. ducreyi*. Using these mutants, we examined the role that these putative PEA transferase genes play in *H. ducreyi* resistance to APs. We also analyzed the contributions of these gene products to cell surface charge and LOS structure. Lastly, we utilized the human model of *H. ducreyi* infection to determine whether these PEA transferase genes are required for virulence in vivo.

A.



B.



C.

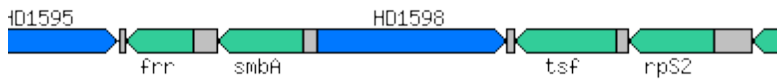


Figure 3. Genomic Map of *ptdA*, *lptA*, and *ptdB*. The location within the genome of each putative PEA transferase is shown above. (A) *HD0371* is the putative PEA transferase *ptdA*, (B) *HD0852* is the putative PEA transferase *lptA*, and (C) *HD1598* is the putative PEA transferase *ptdB*.

CHAPTER TWO: MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids are listed in Table 1. Unless otherwise mentioned, *H. ducreyi* strains were grown at 33°C with 5% CO₂ on chocolate agar plates supplemented with 1% IsoVitalex. If strains contain plasmid vectors or antibiotic resistance cassettes, appropriate antibiotics were added to the agar, including spectinomycin (200 µg/ml), kanamycin (20 µg/ml) or streptomycin (100 µg/ml) (70). Liquid cultures of *H. ducreyi* were grown in Columbia broth supplemented with hemin (50 µg/ml) (Aldrich Chemical Co., Milwaukee, WI), 5% heat inactivated fetal bovine serum (HyClone, Logan, UT), and 1% IsoVitalex and supplemented with half the concentration of appropriate antibiotics used in agar medium. *E. coli* strains were grown at 37°C in Luria-Bertani broth with appropriate antibiotics, which include spectinomycin (50 µg/ml), ampicillin (50 µg/ml), kanamycin (50 µg/ml), or streptomycin (100 µg/ml), with the exception of strain DY380, which was grown in L-Broth at 32°C or 42°C as indicated (79, 185).

Deletion of *ptdA*, *lptA*, and *ptdB* in *H. ducreyi*

We used the recombineering technique, as previously described (71), to generate unmarked, non-polar mutants with deletions in *lptA*, *ptdA*, or *ptdB*, as well as mutants with deletions in two or three of these genes (Table 1). Briefly, as shown in Figure 4, this method replaces the target gene with a spectinomycin resistance (SpecR) cassette flanked by flippase (FLP) recognition target (FRT) sites. Using FLP recombinase, the SpecR cassette is removed, leaving the target gene start ATG, 81 bp of an FLP scar peptide, and the final 21 bp of the target gene, including the stop codon (79). Briefly, PCR products

of 5.5 kb containing *pta*, 3.5 kb containing *lptA*, or 5.0 kb containing *ptaB* were generated with HD0371for3 and HD0371rev3, HD0852for1 and HD0852rev1, or HD1598for3 and HD1598rev3, respectively (Table 2). These products were cloned into TOP10 *E. coli* cells (Invitrogen), which were then transformed with a 2.2 kb PCR fragment containing a SpecR cassette flanked by FRT sites and 50 bp homologous 5' and 3' of the target gene, which was amplified by H1P1HD0371 and H2P2HD0371, H1P1HD0852 and H2P2HD0852, or H1P1HD1598 and H2P2HD1598 (Table 2). After recombination, each fragment was ligated into pRSM2072 to generate the mutagenic plasmid pMEB252 (from *pta*), pMEB256 (from *lptA*), or pMEB251 (from *ptaB*).

Each mutagenic plasmid was individually transformed into *H. ducreyi* as previously described (71), and mutant colonies were selected and transformed with pRSM2975, a temperature sensitive plasmid containing FLP recombinase. Incubation with anhydrotetracycline induced the FLP recombinase, and after selection for loss of SpecR and loss of pRSM2975, the resulting recombined strain is an unmarked, non-polar deletion mutant with the FLP scar in place of the target gene. This process was repeated with each mutagenic plasmid to create a strain collection lacking any combination of one, two, or three of the putative PEA transferase genes *pta*, *lptA*, and *ptaB* (Table 1). In all mutants, PCR and sequencing across the deleted region confirmed loss of target gene(s). For the mutant lacking all three genes, named 35000HP Δ PEAT, whole genome sequencing revealed identical DNA between the mutant and parent strain 35000HP, with the exception of loss of the three target genes. Growth curves comparing 35000HP Δ PEAT to 35000HP were established by measuring the absorbance reading of a broth culture at OD₆₆₀ over a 24 hour period.

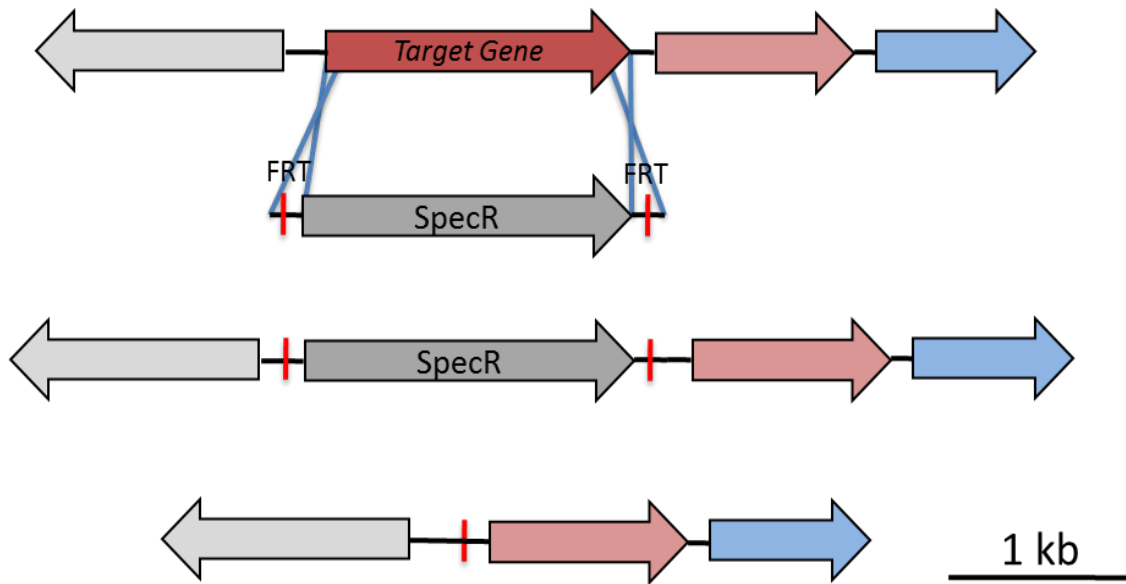


Figure 4. Recombineering of Target Gene. The recombineering technique was used to generate unmarked, non-polar mutants with deletions in *lptA*, *ptdA*, or *ptdB*, as well as mutants with deletions in two or three of these genes. The target gene was replaced via homologous recombination of upstream (H1) and downstream (H2) sites with a spectinomycin resistance (SpecR) cassette flanked by flippase (FLP) recognition target (FRT) sites. Using FLP recombinase, the FRT sites recombined and the SpecR cassette was excised, leaving the target gene start ATG, 81 bp of an FLP scar peptide, and the final 21 bp of the target gene, including the stop codon.

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Genotype or Description ^a	Source
Strains		
35000HP	Class I Clinical Isolate human-passaged variant	(21)
<i>E. coli</i> TOP10	<i>F</i> - <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>ara</i> Δ <i>I39</i> Δ (<i>ara</i> - <i>leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (<i>StrR</i> ^R) <i>endA1</i> <i>nupG</i> λ -	Invitrogen
<i>E. coli</i> DY380	<i>F</i> - <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) Φ 80 <i>dlacZ</i> <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ <i>I39</i> Δ (<i>ara</i> , <i>leu</i>) 7649 <i>galU</i> <i>galK</i> <i>rspL</i> <i>nupG</i> (λ <i>cI857</i> (<i>cro</i> - <i>bioA</i>) $\langle \rangle$ <i>tet</i>)	(185)
35000HP/pLSSK	35000HP with vector pLSSK; StrepR	(71)
35000HP Δ PEAT	<i>ptdA</i> , <i>lptA</i> , <i>ptdB</i> unmarked deletion mutant of 35000HP	This study
35000HP Δ PEAT/pLSSK	35000HP Δ PEAT with vector pLSSK; StrepR	This study
35000HP Δ PEAT/pPEAT	35000HP Δ PEAT with complement vector pPEAT; StrepR	This study
35000HP Δ ptdA	<i>ptdA</i> unmarked deletion mutant of 35000HP	This study
35000HP Δ lptA	<i>lptA</i> unmarked deletion mutant of 35000HP	This study
35000HP Δ ptdB	<i>ptdB</i> unmarked deletion mutant of 35000HP	This study
35000HP Δ ptdA <i>lptA</i>	<i>ptdA</i> <i>lptA</i> unmarked deletion mutant of 35000HP	This study
35000HP Δ lptA <i>ptdB</i>	<i>lptA</i> <i>ptdB</i> unmarked deletion mutant of 35000HP	This study
35000HP Δ ptdA <i>ptdB</i>	<i>ptdA</i> <i>ptdB</i> unmarked deletion mutant of 35000HP	This study
35000HP <i>SapA</i> /pLSSK	<i>sapA</i> unmarked deletion mutant of 35000HP with vector pLSSK; StrepR	(144)
35000HP <i>sapA</i> / <i>phbpA</i>	35000HP <i>sapA</i> with complement vector <i>phbpA</i> ; StrepR	Unpublished ^b
35000HP <i>sapA</i> / <i>pdppA</i>	35000HP <i>sapA</i> with complement vector <i>pdppA</i> ; StrepR	Unpublished ^b
35000HP <i>sapBC</i> /pLSSK	<i>sapBC</i> unmarked deletion mutant of 35000HP with vector pLSSK; StrepR	(144)
35000HP <i>sapBC</i> / <i>poppA</i>	35000HP <i>sapBC</i> with complement vector <i>poppA</i> ; StrepR	Unpublished ^b
35000HP <i>mtrC</i> /pLSSK	<i>mtrC</i> unmarked deletion mutant of 35000HP with vector pLSSK; StrepR	(71)

35000HP <i>pmtrC/pmtrC</i>	35000HP <i>pmtrC</i> with complement vector <i>pmtrC</i> ; StrepR	(71)
FX517	<i>dsrA:cat</i> insertion mutant of 35000; Cm ^R	(44)
35000HPΔ <i>cpxA</i>	<i>cpxA</i> unmarked deletion mutant of 35000HP	(79)
Plasmids		
pLSSK	<i>H. ducreyi</i> shuttle vector; StrepR	(186)
pPEAT	<i>ptdA lptA ptdB</i> in pLSSK; StrepR	This study
pRSM2072	Suicide vector; AmpR	(187)
pRSM2975	FLP recombinase vector	(79)
pMEB252	<i>ptdA</i> replaced with SpecR cassette in pRSM2072	This study
pMEB256	<i>lptA</i> replaced with SpecR cassette in pRSM2072	This study
pMEB251	<i>ptdB</i> replaced with SpecR cassette in pRSM2072	This study
pCR-XL-TOPO	TA cloning vector; KanR	Invitrogen
pMEB346	<i>ptdA</i> in pCR-XL-TOPO; KanR	This study
pMEB344	<i>lptA</i> in pCR-XL-TOPO; KanR	This study
pMEB348	<i>ptdB</i> in pCR-XL-TOPO; KanR	This study
pMEB355	<i>ptdA</i> in pLSSK; StrepR	This study
pMEB356	<i>lptA ptdB</i> in pCR-XL-TOPO; KanR	This study
<i>pmtrC</i>	<i>mtrC</i> in pLSSK; StrepR	(71)
<i>phbpA</i>	<i>hbpA</i> in pLSSK; StrepR	Unpublished ^b
<i>pdppA</i>	<i>dppA</i> in pLSSK; StrepR	Unpublished ^b
<i>poppA</i>	<i>oppA</i> in pLSSK; StrepR	Unpublished ^b

^a StrepR, resistance to streptomycin; CmR, resistance to chloramphenicol; AmpR,

resistance to ampicillin; KanR, resistance to kanamycin; SpecR, resistance to spectinomycin.

^b Rinker et al., manuscript in preparation

Table 2. Primers used in this study.

Primer	Construct or use	Sequence
HD0371for3	<i>ptdA</i> fragment	ACTAGTGGCTCACCAAGCCATT GGTTACAA
HD0371rev3	<i>ptdA</i> fragment	ACTAGTGCAGGAATTGTACGGT CTGAACG
HD0852for1	<i>LptA</i> fragment	ACTAGTAGGGAAATGATCCGA AGCGAGGA
HD0852rev1	<i>LptA</i> fragment	ACTAGTTCGGTCGTATTAACGT GCTGACCA
HD1598for3	<i>ptdB</i> fragment	ACTAGTTGGCAAATTAACCAC ACGCGGTC
HD1598rev3	<i>ptdB</i> fragment	ACTAGTATGCGCGATATGCTTA ATGCTGGC
H1P1HD0371	pMEB252	AACAATGAGGCTATTTTATTTTC TGCTGACCTTGTTTTATAGATTA TTATGATTCCGGGGATCCGTCG ACC
H2P2HD0371	pMEB252	ATTAACACATAGTTATTAATGC TTTCTAATTAATTGCTGATTGTG GTGTTTGTAGGCTGGAGCTGCT TCG
H1P1HD0852	pMEB256	ATAGCTTGATAGGCATTATTGC TTATGTTTTTATAACAAAGGAAT TTTATGATTCCGGGGATCCGTC GACC
H2P2HD0852	pMEB256	TTTTGCTAAAAAGGCCGCTTAC AAGCGTATTACTCTACTTTATG AGCACATGTAGGCTGGAGCTGC TTCG
H1P1HD1598	pMEB251	ATTAAACAAGGAATAGCGCCCC TATATATTTACTACTAGAATCT ATAATGATTCCGGGGATCCGTC GACC
H2P2HD1598	pMEB251	GCCTGCTTTTTTATTATTAGTAA TCTGTGCTATTCCTGAACGTGC CCATTTGTAGGCTGGAGCTGCT TCG
HD0371compf or1	pMEB346	GCACGTGATGTATTGGCTAAAG GT
HD0371compr ev3	pMEB346	TTTATTTTCTGCTTGCATTAACA CATAGTTATTAAT
HD0852compf or1	pMEB344	GCTTGTAAGGACGTAGCCAAG TG
HD0852compr	pMEB344	TACCGGCTCGGATTTCTAAGAA

ev1		GG
HD1598compf	pMEB348	CCTTGCAATGCCTCACCACTTA
or1		GTT
HD1598compr	pMEB348	TCCGTCTCAATCAGTCGGTGAC
ev1		TA

Complementation of *ptaA*, *lptA*, and *ptdB* in trans

To complement the mutations in 35000HP Δ PEAT, fragments of 2.05 kb, 2.5 kb, and 1.95 kb, containing 164 bp, 340 bp, and 185 bp 5' of the *ptaA*, *lptA*, and *ptdB* ATG start sites respectively, including the predicted promoter regions, were amplified from genomic DNA of 35000HP with primers HD0371compfor1 and HD0371comprev3, HD0852compfor1 and HD0852comprev1, and HD1598compfor1 and HD1598comprev1. These fragments were TA-cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA), resulting in pMEB346 (containing *ptaA*), pMEB344 (containing *lptA*), and pMEB348 (containing *ptdB*).

The 2.05 kb *ptaA* fragment was excised from pMEB346 by digestion with NotI and SpeI and ligated into the shuttle vector pLSSK, resulting in the 5.6 kb pMEB355. The 2.5 kb *lptA* was excised from pMEB344 by digestion with SpeI and XbaI and ligated into SpeI-digested and shrimp alkaline phosphatase treated *ptdB*-containing pMEB348 to form a 8.0 kb pMEB356, with the genes oriented *lptA ptdB*. The 4.5 kb *lptA ptdB* fragment was then excised from pMEB356 by digestion with SpeI and ApaI and ligated into pMEB355. The resulting plasmid (Figure 5), pPEAT, contains a 10.1 kb insert encoding *ptaA*, *lptA*, and *ptdB* with their native promoter regions in vector pLSSK. 35000HP Δ PEAT was transformed with pPEAT to obtain 35000HP Δ PEAT/pPEAT. 35000HP Δ PEAT was transformed with pLSSK to generate 35000HP Δ PEAT/pLSSK.

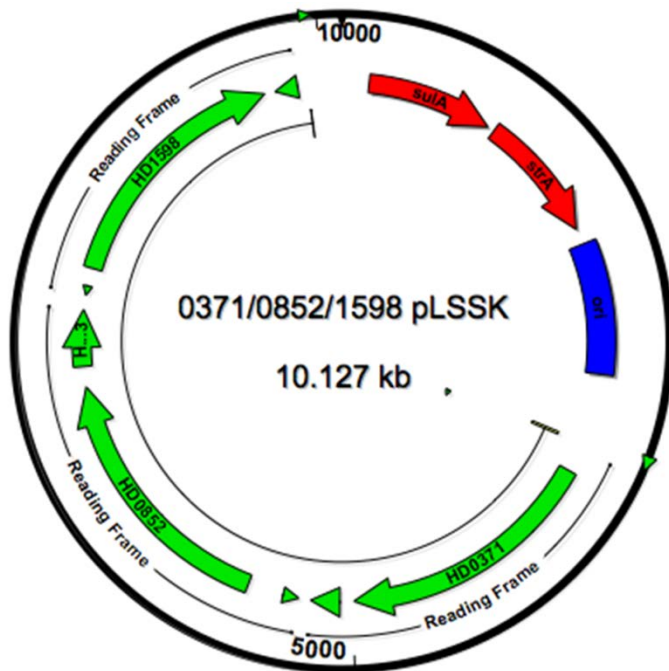


Figure 5. Complementation plasmid pPEAT. The three putative PEA transferase genes were inserted into the plasmid pLSSK in the order shown: *ptdA* (HD0371), *lptA* (HD0852), and then *ptdB* (HD1598). The *ptdA* fragment contains 164 bp 5' of the start codon, the *lptA* fragment contains 340 bp 5' of the start codon, and the *ptdB* fragment contains 185 bp 5' of the start codon. The *ori* indicates the plasmid's origin of replication, *strA* is a gene encoding streptomycin antibiotic resistance, and *sulA* is a gene encoding sulfonamide antibiotic resistance.

qRT-PCR

We analyzed RNA isolated from 35000HP, 35000HP Δ PEAT, and 35000HP Δ PEAT/pPEAT for transcripts of the target genes *lptA*, *ptdA*, and *ptdB*, as well as a control gene *gyrB* (*HD1643*), by quantitative reverse transcriptase PCR (qRT-PCR), as described previously (70). Briefly, RNA was isolated from mid-logarithmic cultures with TRizol Reagent (Invitrogen, Carlsbad, CA), treated twice with DNase I (Ambion, Austin, TX), and purified with an RNeasy spin column (Qiagen, Valencia, CA). After analysis by gel electrophoresis following RT-PCR to ensure lack of DNA contamination, reactions were carried out with the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) and the 7500 Real-Time PCR System (Applied Biosystems, Carlsbad CA). The Pfaffl method, which calculates gene expression from the PCR primer efficiencies and the expression level of a control gene, was used to determine the relative quantification of the target genes compared to the constitutively expressed *gyrB* (*HD1643*) (188), and three biological replicates were used to quantify the RNA.

Isolation of *H. ducreyi* LOS

LOS was extracted from *H. ducreyi* using a microphenol method, as previously described (189) for strains 35000HP/pLSSK, 35000HP Δ PEAT/pLSSK, 35000HP Δ PEAT/pPEAT, 35000HP Δ *lptA*, 35000HP Δ *ptdA*, and 35000HP Δ *ptdB*. Briefly, plate-grown bacteria were harvested, washed with PBS, and then diluted in H₂O. The aqueous phase of a 65°C phenol/water extraction was kept, and the LOS was precipitated overnight at -20°C with cold ethanol. The sample was lyophilized overnight, and samples were either further analyzed by mass spectrometry or run on a tris-glycine gel.

Isolation of *H. ducreyi* outer membrane proteins

Outer membrane protein preparations and analysis were performed as described (190). Briefly, plate-grown *H. ducreyi* were sonicated and subject to 2% sodium lauroyl sarcosinate (Sarkosyl) extraction. The insoluble outer membrane proteins were collected and then resolved by SDS-PAGE and stained with Coomassie blue.

In addition, the OMPs were subject to proteomic post translational modification (PTM) analysis. Samples were suspended in 8M urea, sonicated, and digested overnight in 2 µg trypsin (Promega) after being reduced with 10 mM DTT in 10 mM ammonium bicarbonate and alkylated with 55 mM iodoacetamide in 10 mM ammonium bicarbonate. The peptides were then injected onto a C18 column (NanoAcquity UPLC®) and were eluted with a linear gradient from 3 to 40% acetonitrile (in water with 0.1% formic acid) developed over 240 minutes at room temperature, at a flow rate of 500 nL/min. The effluent was electro-sprayed into a mass spectrometer (Thermo-Fisher Scientific Orbitrap Velos Pro and Waters UPLC system), and the resulting peptide analysis was compared against the Uniprot *H. ducreyi* database using a SequestTM algorithm, searching for PEA modification (S, T, Y; +123.009 Da).

Antimicrobial Peptide Bactericidal Assays

Recombinant α - and β -defensin peptides were purchased from AnaSpec (San Jose, CA: HBD-1), PeproTech Inc. (Rocky Hill, NJ: HNP-1, HBD-2, HBD-3), Peptides International (Louisville, KY: HD-5), and Sigma-Aldrich (St. Louis, MO: HNP-2); synthetic LL-37 was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA). APs were reconstituted as previously described (71).

AP assays were performed as described previously (135). Briefly, approximately 1000 CFU of mid-logarithmic phase *H. ducreyi* were incubated with 0.2, 2.0, and 20 $\mu\text{g}/\text{mL}$ of diluted peptide for one hour. Samples were plated in triplicate on chocolate agar plates supplemented with appropriate antibiotics, and survival at each concentration of peptide after one hour was compared to survival of a control sample receiving diluent but no peptide. We used a Student's t-test with Sidak adjustment for multiple comparisons to determine statistical significance, and comparisons between strains for sensitivity to a given AP were made only when assayed side-by-side.

Serum Bactericidal Assay

35000HP/ pLSSK, 35000HP Δ PEAT/pLSSK, and 35000HP Δ PEAT /pPEAT were assayed for survival in 50% normal human serum, as described previously (44). Briefly, 14 – 16 hour growth from confluent plates was scraped into GC broth and diluted to 1000 CFU/mL. Bacteria were mixed 1:1 with either active or heat-inactivated human serum. Survival was determined by plate count after 45 min incubation. Assays were performed in triplicate, and the percent survival was calculated as the ratio of the (average active serum CFU/plate) / (average heat-inactivated serum CFU/plate) for each strain. The serum-sensitive mutant strain 35000HP*dsrA* was included in the assay as an internal control, and we used a Student's t test with Sidak adjustment for multiple comparisons to determine statistical significance.

Cell Surface Charge Assay

We compared cell surface charges of the *H. ducreyi* strains by adapting protocols used to compare membrane charge changes in red blood cells and yeast using Alcian Blue 8GX, a cationic dye that binds proportionately to negatively charged cell surfaces

(191, 192). *H. ducreyi* strains were grown to mid-logarithmic phase, harvested, washed, and diluted in sterile phosphate buffered saline (PBS). Approximately 1000 CFU of bacteria were incubated for 30 minutes with 100 $\mu\text{g}/\text{mL}$ Alcian Blue 8GX (Sigma-Aldrich, St. Louis, MO) in PBS; control samples were incubated in PBS alone. The bacteria were centrifuged and the supernatant removed, and the pellet was then suspended in 500 μL PBS. Absorbance at O.D.₆₀₇ was measured for both the supernatant and suspended bacteria. Parallel bacterial samples were used to determine dry weight, and the absorbance measurements were normalized to the parent strain. Strains were compared for the amount of dye bound to the bacteria (by both loss of dye in supernatant and gain of dye in bacterial cells), normalized to the dry weight of each bacterial cell sample, using the PBS control samples to account for background absorption levels. A comparative decrease in remaining dye in the supernatant sample, and inversely an increase in dye bound to the bacterial cells, correlates to the sample having a more negative outer membrane charge.

For statistical analysis, mutant-parent pair differences in absorbance readings from both bacteria and supernatants from each strain were computed to account for day-to-day sample variation. Since the absorbance measurements were not normally distributed, non-parametric Wilcoxon signed ranks tests were used to test for significance difference between mutant and parent samples.

MALDI-MS Analysis of *H. ducreyi* LOS

LOS was extracted from *H. ducreyi* as described above. To generate LOS more amendable to mass spectrometric analyses, O-deacylated LOS (O-LOS) samples were prepared by treating 50-100 μg of LOS with 50 μl of anhydrous hydrazine followed by

acetone precipitation as described previously (193). All samples were desalted by drop dialysis using 0.025- μ m pore size nitrocellulose membranes (Millipore, Bedford, MA) and were subsequently lyophilized. Samples were reconstituted in high-performance liquid chromatography (HPLC) grade H₂O; 1 μ l was loaded onto the target, allowed to dry, and then overlaid with either 1 μ l of matrix (50 mg/ml 2,5-dihydroxybenzoic acid (DHB) (Laser Biolabs, Sophia-Antipolis Cedex, France) in 70% acetonitrile) or DHB made as a saturated solution in 70% acetonitrile. Samples were subsequently analyzed using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) on an LTQ linear ion trap mass spectrometer coupled to a vMALDI ion source (MALDI-LIT) (Thermo Fisher, Waltham, MA). The vMALDI source uses a nitrogen laser that operates at 337.1-nm wavelength, 3-ns pulse duration, and 60-Hz repetition rate. Data were collected in the negative ion mode using the automated gain control and the automatic spectrum filter settings. Alternatively, samples were analyzed on the Waters Synapt G2 hybrid mass spectrometer utilizing the MALDI ionization source in the negative ion mode.

Human Model of *H. ducreyi* Infection

For the human model of *H. ducreyi* infection, we used the strains 35000HP and 35000HP/PEAT. Three healthy male and five healthy female volunteers over 21 years of age were recruited for the study. Informed consent was obtained from the subjects for participation and for HIV serology, in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and the Institutional Review Board of Indiana University–Purdue University Indianapolis.

The experimental human challenge protocols were followed as previously described (20, 21, 32, 34, 53, 194). Subjects were observed until they reached the clinical endpoint, defined as either 14 days post-inoculation, resolution of infection at all sites, or development of a pustule that was painful or pruritic or at least 6 mm in diameter. Following the clinical endpoint, biopsies were taken of both a parent site and mutant site. The subjects were then treated with a single dose of oral ciprofloxacin. Individual colonies from the inoculum, surface cultures, and biopsies were analyzed by qRT-PCR to provide confirmation of parent and mutant genotypes. Papule and pustule formation rates for parent and mutant inoculation sites were compared using logistic regression with generalized estimating equations (GEE) to account for the within-subject correlation. Ninety-five percent confidence intervals (95% CI) for papule and pustule formation rates were calculated using GEE-based sandwich standard errors. Day 1 papule size was compared using analysis of variance with a random subject effect.

CHAPTER THREE: RESULTS

Identification of putative PEA transferases in *H. ducreyi*.

Previously, we established that the Sap transporter and MTR efflux pump mediated LL-37 and β -defensin resistance in *H. ducreyi* (70, 71, 144). We next wanted to find what mechanism was responsible for α -defensin resistance. Similar to other bacteria, *H. ducreyi* LOS is modified with the positively-charged PEA, leading to our hypothesis that *H. ducreyi* PEA modifications confer resistance to APs. In gram-negative bacteria, the known and characterized PEA transferases are members of the YhjW/YjdB/YijP/YbiP superfamily of inner membrane proteins (159). Using the Basic Local Alignment Search Tool (BLAST) tool on the National Center for Biotechnology Information website, we searched for *H. ducreyi* homologs of the YhjW/YjdB/YijP/YbiP superfamily; homology searches found three members of this family in the *H. ducreyi* genome (Table 3). *LptA* (HD0852) shared strong homology with characterized lipid A PEA transferase genes in *Neisseria*, *E. coli*, and *S. enterica*; *PtdA* (HD0371) and *ptdB* (HD1598) were homologous to the OPG-modifying PEA transferase OpgE in *E. coli*.

Table 3. Putative *H. ducreyi* PEA transferases.

<i>H. ducreyi</i> Gene ID	<i>H. ducreyi</i> Protein	Homologous Protein (organism)	Expect Value	Site of Action
HD0852	Lipid A PEA transferase LptA	LptA (<i>N. gonorrhoeae</i>)	0	lipid A
		LptA (<i>N. meningitides</i>)	0	lipid A
		EptA/PmrC (<i>E. coli</i>)	3e ⁻¹³⁸	lipid A
		EptA/PmrC (<i>S. enterica</i>)	2e ⁻¹³⁴	lipid A
HD0371	PEA transferase of <i>H. ducreyi</i> PtdA	OpgE/YbiP (<i>E. coli</i>)	1e ⁻⁷⁶	unknown
HD1598	PEA transferase of <i>H. ducreyi</i> PtdB	OpgE/YbiP (<i>E. coli</i>)	1e ⁻⁶²	unknown

Confirmation and initial characterization of the mutants with deletions of the putative PEA transferase genes.

Once we identified the putative PEA transferase genes, we generated mutants with deletions of one, two, or all three genes (Table 2); mutagenesis was confirmed by PCR (Figure 6) and sequencing (data not shown). Additionally, the entire genomes of 35000HP and 35000HP Δ PEAT were sequenced by shotgun sequencing to confirm no additional genomic mutations had occurred during the mutagenesis; the 35000HP and 35000HP Δ PEAT genomes were identical with the exception of the expected loss of *lptA*, *ptdA*, and *ptdB* in the mutant strain. Complementation of 35000HP Δ PEAT with pPEAT was confirmed by qRT-PCR, and the expression level of each PEA transferase gene was within \pm 2-fold of parental expression levels (Figure 7).

In order to determine the effect of the putative PEA transferase deletions on *H. ducreyi* growth, a 24-hour growth curve was measured. There was no significant difference in growth rate between the parent and mutant strains (Figure 8); these data suggests that loss of the putative PEA transferase genes does not affect the bacteria's ability to grow in vitro. We also compared the outer membrane protein (OMP) and LOS profiles of 35000HP and 35000HP Δ PEAT. SDS-PAGE showed identical banding patterns between the parent and mutant strain OMP profiles (Figure 9A). When comparing the LOS profiles, silver-stained gels revealed no noticeable difference between the parent and mutant strains (Figure 9B). Since a single PEA is 123 Da in size, loss of a PEA modification on the OMP or LOS would likely not be observed in SDS or tris-glycine gels; these data suggests that loss of the putative PEA transferase genes does not have a major effect on the OMP and LOS profiles.

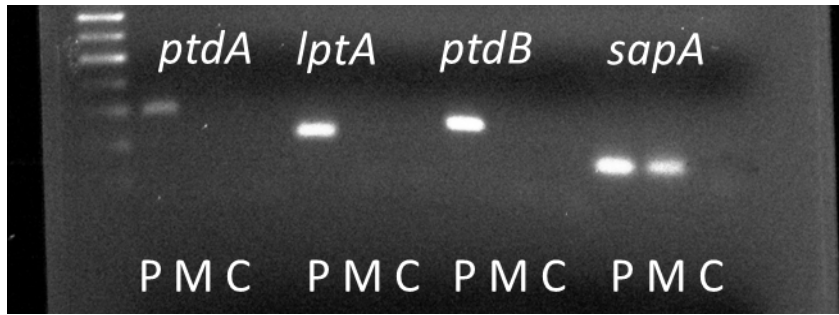


Figure 6. 35000HP Δ PEAT has deletions at *ptdA*, *lptA*, and *ptdB* loci. PCR of (P) 35000HP, (M) 35000HP Δ PEAT, and (C) a no-template control was performed with intragenic primers for *ptdA*, *lptA*, *ptdB*, and *sapA*. Amplicons for each gene are present in 35000HP whereas bands for *ptdA*, *lptA*, and *ptdB* are no longer present in 35000HP Δ PEAT, with *sapA* still present as a positive template control. No bands are present at any no-template control well.

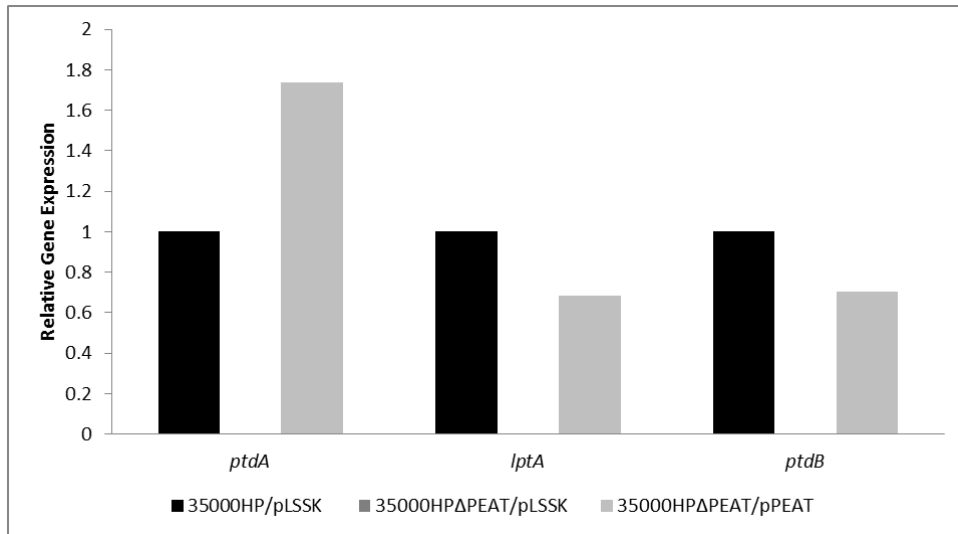


Figure 7. 35000HPΔPEAT/pPEAT restores expression of PEA transferase genes.

qRT-PCR of expression levels of *ptdA*, *lptA*, and *ptdB* in 35000HP/pLSSK (normalized to 1), 35000HPΔPEAT/pLSSK, and 35000HPΔPEAT/pPEAT. 35000HPΔPEAT/pLSSK has no expression of the putative PEA transferase genes, and expression of these genes is restored by pPEAT.

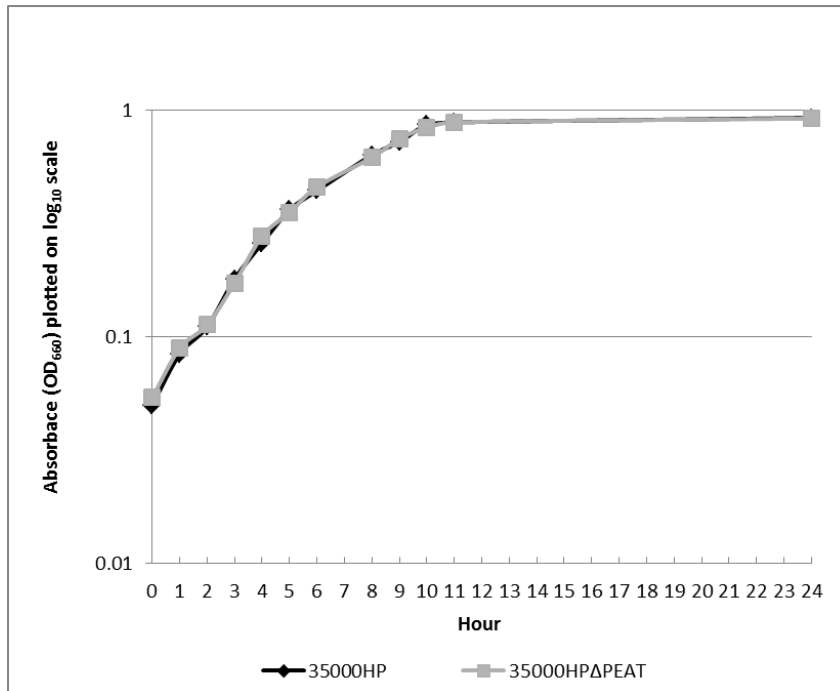


Figure 8. Deletion of PEA transferase genes does not affect growth rate compared to 35000HP. Growth rates of 35000HP and 35000HPΔPEAT were determined by measuring the absorbance at OD₆₆₀ over a 24 hour time period. There is no difference between 35000HP and 35000HPΔPEAT when plotted on a log₁₀ scale.

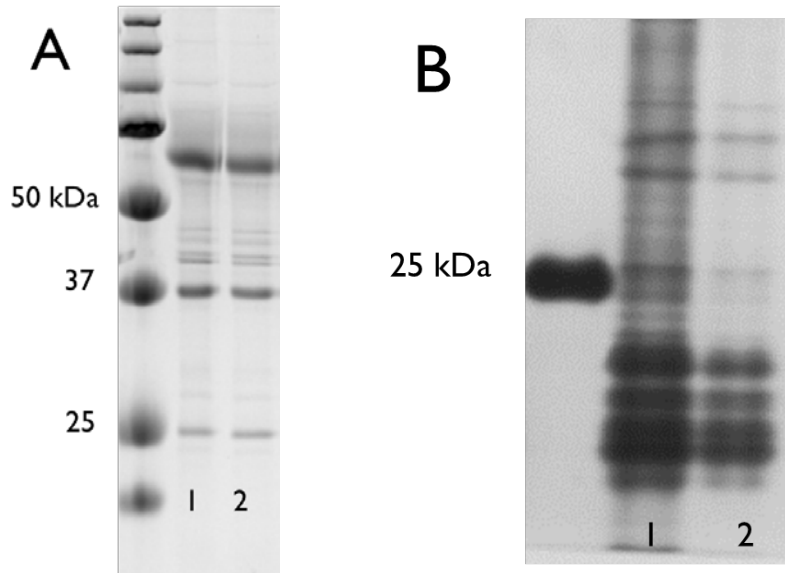


Figure 9. Outer membrane protein profile and lipooligosaccharide profile of 35000HP Δ PEAT remain unchanged from parent. A) Coomassie-stained SDS-PAGE outer membrane protein profile of (1) 35000HP and (2) 35000HP Δ PEAT shows no difference between parent and mutant. The first lane on the left is a protein ladder. B) Silver-stained Tris-glycine LOS profile of 35000HP (1) and 35000HP Δ PEAT (2) shows no difference between parent and mutant. The first lane on the left is a protein ladder.

The *H. ducreyi* PEA transferase genes confer resistance to α - and β -defensins but not to cathelicidin or human serum.

In order to determine the contribution of each putative PEA transferase to AP resistance, we assessed the role they had in resistance to human APs by assaying the parent and mutant strains for sensitivity to the α -defensin HD-5, the β -defensin HBD-3, and the human cathelicidin, LL-37, at various concentrations.

When comparing 35000HP to mutants with single deletions in *lptA*, *ptdA*, or *ptdB*, we found no significant changes in sensitivity to HD-5, HBD-3, or LL-37 (Figures 10-12). This suggests that loss of any one PEA transferase does not have an effect on AP resistance. We next compared 35000HP to mutants with deletions in two putative PEA transferases. We found that all three mutants were more susceptible than 35000HP to HBD-3 (Figures 13B, 14B, 15B). We additionally found statistically significant differences between 35000HP and 35000HP Δ *lptA ptdA* at one concentration of HD-5 and LL-37 (Figure 13A, 13C.). Similarly, we found a statistically significant difference in sensitivity to LL-37 at one concentration between 35000HP and 35000HP Δ *ptdA ptdB* (Figure 15A). It was unclear if this data was biologically significant or a product of chance due to day-to-day variations in the assays, so we next characterized the mutant with deletions in all three putative PEA transferases.

To further determine the role of all three PEA transferase genes in AP resistance, we assayed 35000HP/pLSSK, 35000HP Δ PEAT/pLSSK, and 35000HP Δ PEAT/pPEAT for sensitivity to α -defensins, β -defensins, and LL-37 at various concentrations. We found that 35000HP Δ PEAT/pLSSK was significantly more sensitive to the α -defensin HD-5 at all three concentrations (Figure 16A); complementation with pPEAT restored the parental

resistance phenotype. We next wanted to determine if the putative PEA transferases conferred resistance to additional α -defensins. We found 35000HP Δ PEAT/pLSSK had an increased susceptibility compared to 35000HP/pLSSK when challenged with HNP-1 and HNP-2 (Figures 16B, 16C); complementation with pPEAT restored the parental resistance phenotype. These data indicate that the PEA transferase gene products contribute to α -defensin resistance in *H. ducreyi*.

Next, we found that 35000HP Δ PEAT/pLSSK was significantly more susceptible to the β -defensin HBD-3 at 2 concentrations (Figure 17A); complementation with pPEAT restored the parental resistance phenotype. We next wanted to determine if the putative PEA transferases conferred resistance to additional β -defensins. We found 35000HP Δ PEAT/pLSSK had a greater sensitivity than 35000HP/pLSSK to HBD-2 (Figure 17B); complementation with pPEAT restored the parental resistance phenotype. These data suggest that the PEA transferase gene products contribute to β -defensin resistance in *H. ducreyi*.

While we did see statistically significant decreases in survival when exposed to LL-37 in 35000HP Δ lptA ptdA and 35000HP Δ lptA ptdB, we found no significant difference in sensitivity to LL-37 between 35000HP/pLSSK and 35000HP Δ PEAT/pLSSK at any concentration (Figure 18). The assays with 35000HP Δ PEAT/pLSSK, which displays the greatest sensitivity to APs amongst all the PEA transferase mutants, were also performed in a greater number of replicates. All data taken together suggest that we likely saw a statistical anomaly in the double mutants, and the *H. ducreyi* PEA transferase gene products likely contribute to α - and β -defensin resistance but do not contribute significantly to cathelicidin resistance.

Previous studies have shown that addition of PEA to LOS contributes to serum resistance in *Neisseria gonorrhoeae* (162). In contrast, we found no significant difference in sensitivity between 35000HP and 35000HP Δ PEAT to normal human serum (Figure 19). These data indicate that the putative PEA transferases of *H. ducreyi* do not contribute to resistance to human serum.

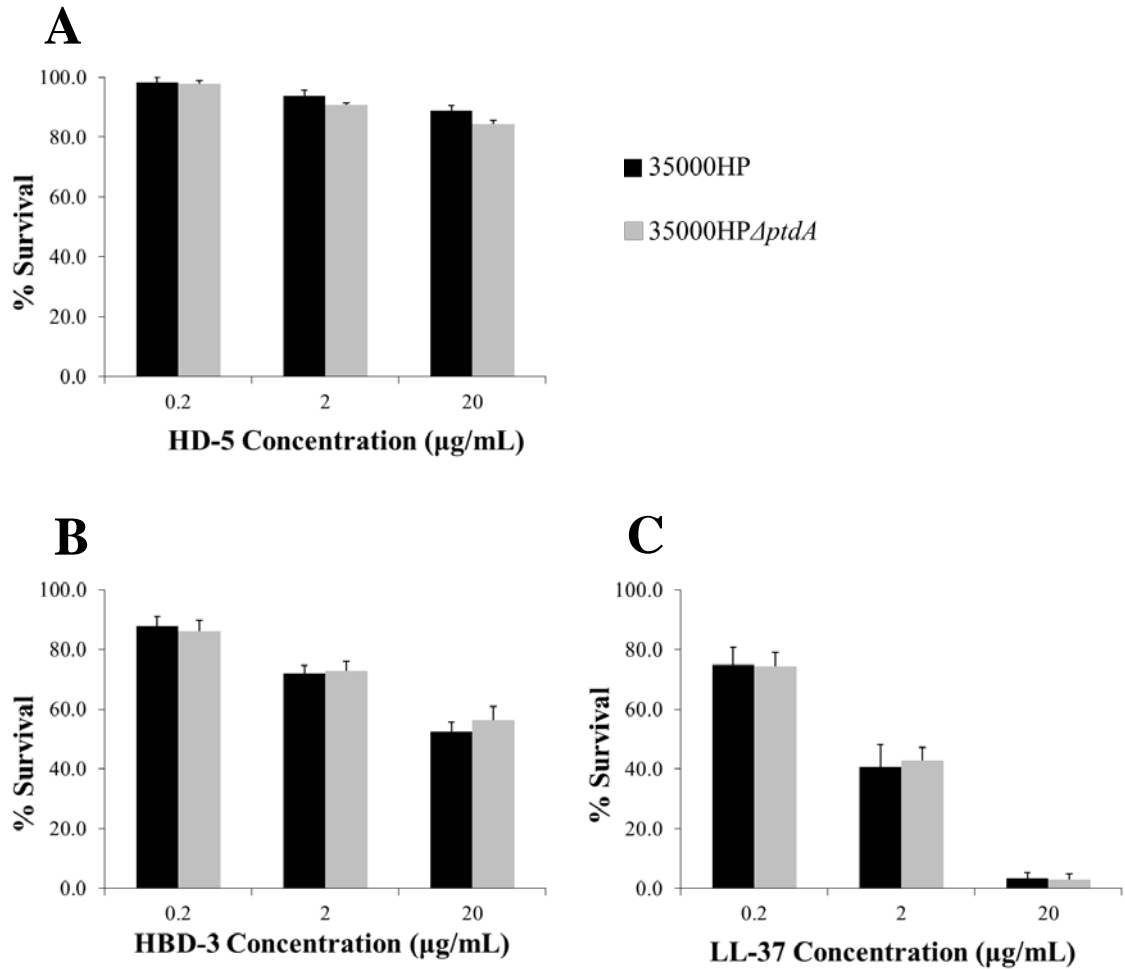


Figure 10. *ptdA* alone does not confer resistance to α - and β -defensins or cathelicidin in *H. ducreyi*. 35000HP and 35000HP Δ *ptdA* were examined for resistance to the (A) α -defensin HD-5 (B) β -defensin HBD-3, and (C) human cathelicidin LL-37. There were no statistically significant differences between 35000HP and 35000HP Δ *ptdA* for HD-5, HBD-3 or LL-37. Data represents average \pm standard error of three independent assays, and statistical significance was determined by Student's t-test.

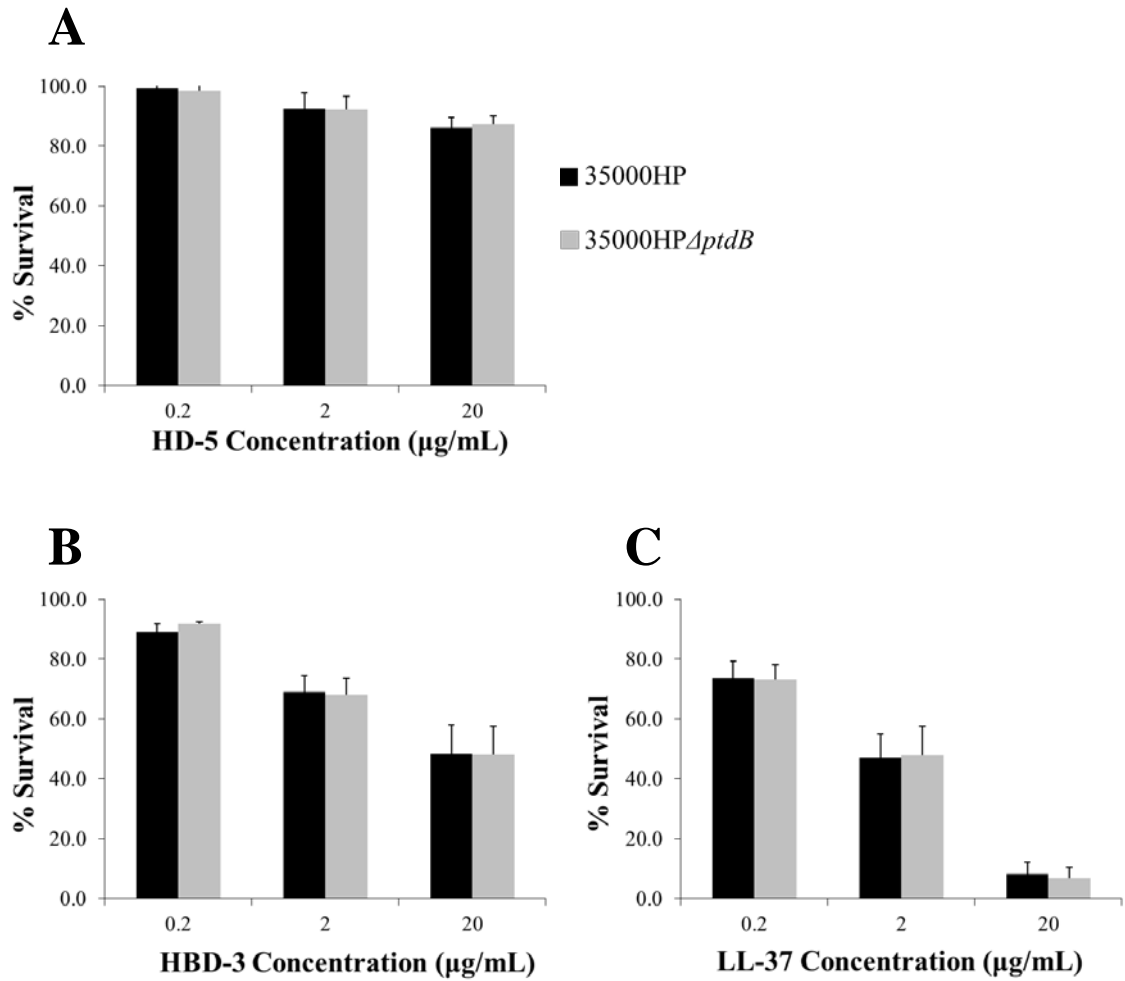


Figure 11. *ptdB* alone does not confer resistance to α - and β -defensins or cathelicidin in *H. ducreyi*. 35000HP and 35000HP Δ *ptdB* were examined for resistance to the (A) α -defensin HD-5 (B) β -defensin HBD-3, and (C) human cathelicidin LL-37. There were no statistically significant differences between 35000HP and 35000HP Δ *ptdB* for HD-5, HBD-3 or LL-37. Data represents average \pm standard error of three independent assays, and statistical significance was determined by Student's t-test.

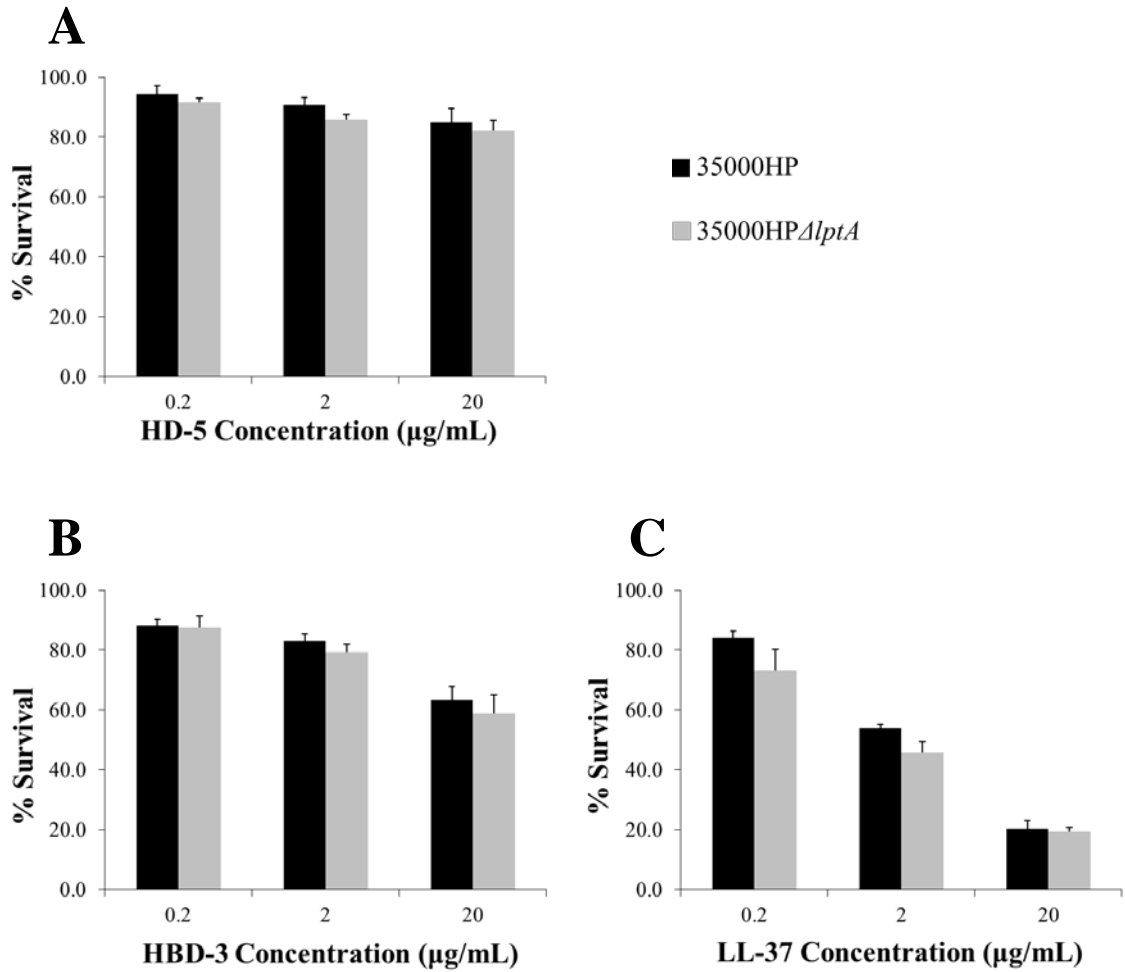


Figure 12. *lptA* alone does not confer resistance to α - and β -defensins or cathelicidin in *H. ducreyi*. 35000HP and 35000HP Δ *lptA* were examined for resistance to the (A) α -defensin HD-5 (B) β -defensin HBD-3, and (C) human cathelicidin LL-37. There were no statistically significant differences between 35000HP and 35000HP Δ *lptA* for HD-5, HBD-3 or LL-37. Data represents average \pm standard error of three independent assays, and statistical significance was determined by Student's t-test.

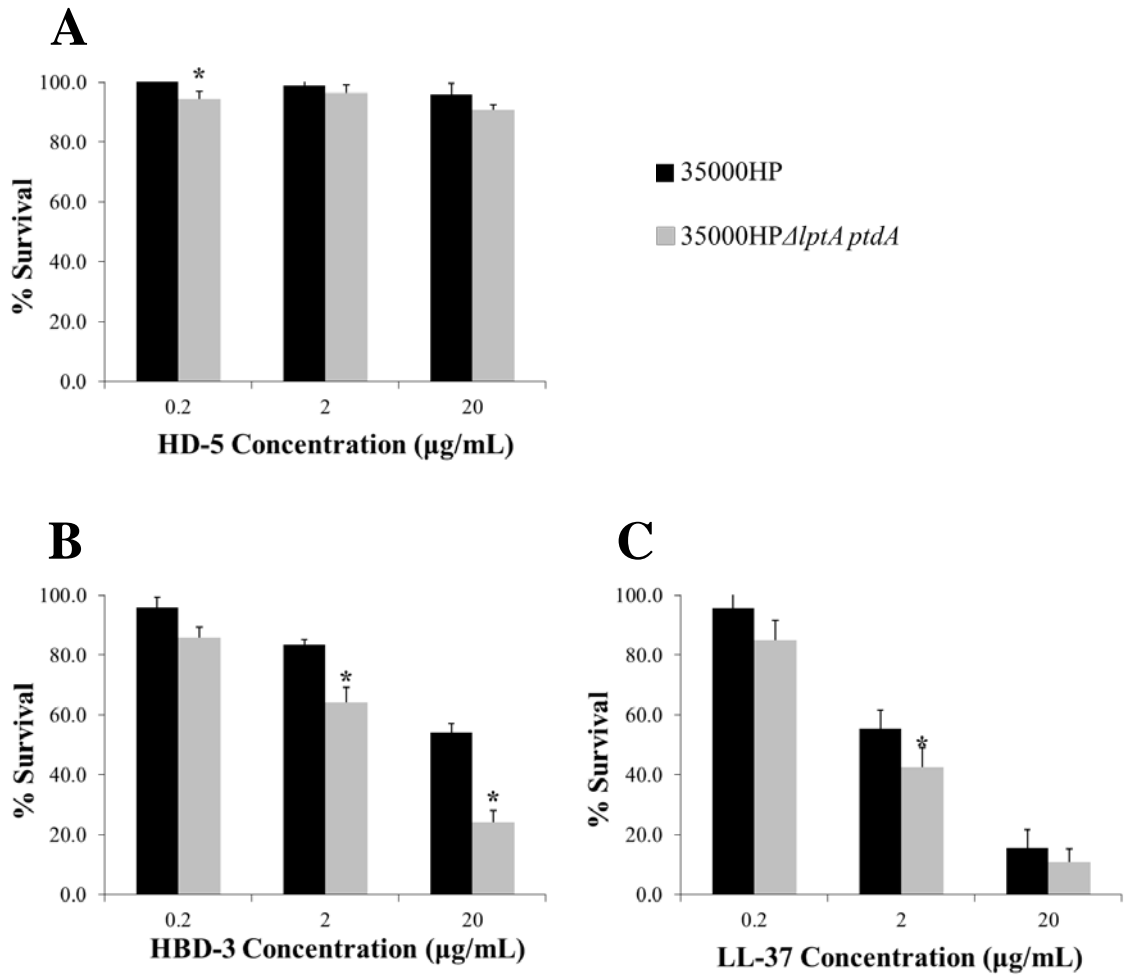


Figure 13. *lptA* and *ptdA* together confer resistance to β -defensins, and possibly to α -defensins and cathelicidin, in *H. ducreyi*. 35000HP and 35000HPΔ*lptA ptdA* were examined for resistance to the (A) α -defensin HD-5 (B) β -defensin HBD-3, and (C) human cathelicidin LL-37. Asterisks indicate statistically significant differences from 35000HP ($P < 0.05$). Data represent average \pm standard error of three to four independent replicates, and statistical significance was determined by Student's t-test.

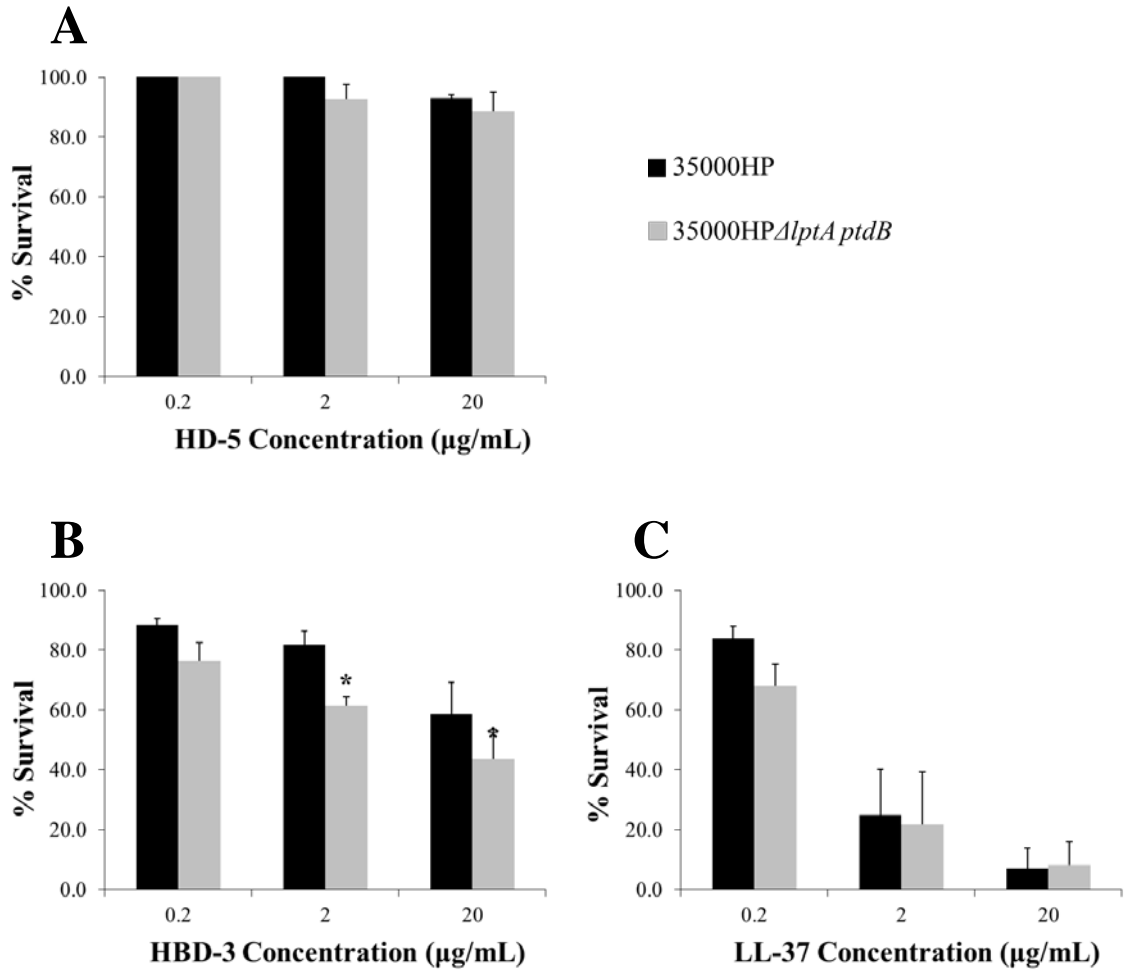


Figure 14. *lptA* and *ptdB* together confer resistance to β -defensins, but not α -defensins or cathelicidin, in *H. ducreyi*. 35000HP and 35000HPΔ*lptA ptdB* were examined for resistance to the (A) α -defensin HD-5 (B) β -defensin HBD-3, and (C) human cathelicidin LL-37. Asterisks indicate statistically significant differences in survival of the mutant versus 35000HP ($P < 0.05$). Data represent average \pm standard error of three independent replicates, and statistical significance was determined by Student's t-test.

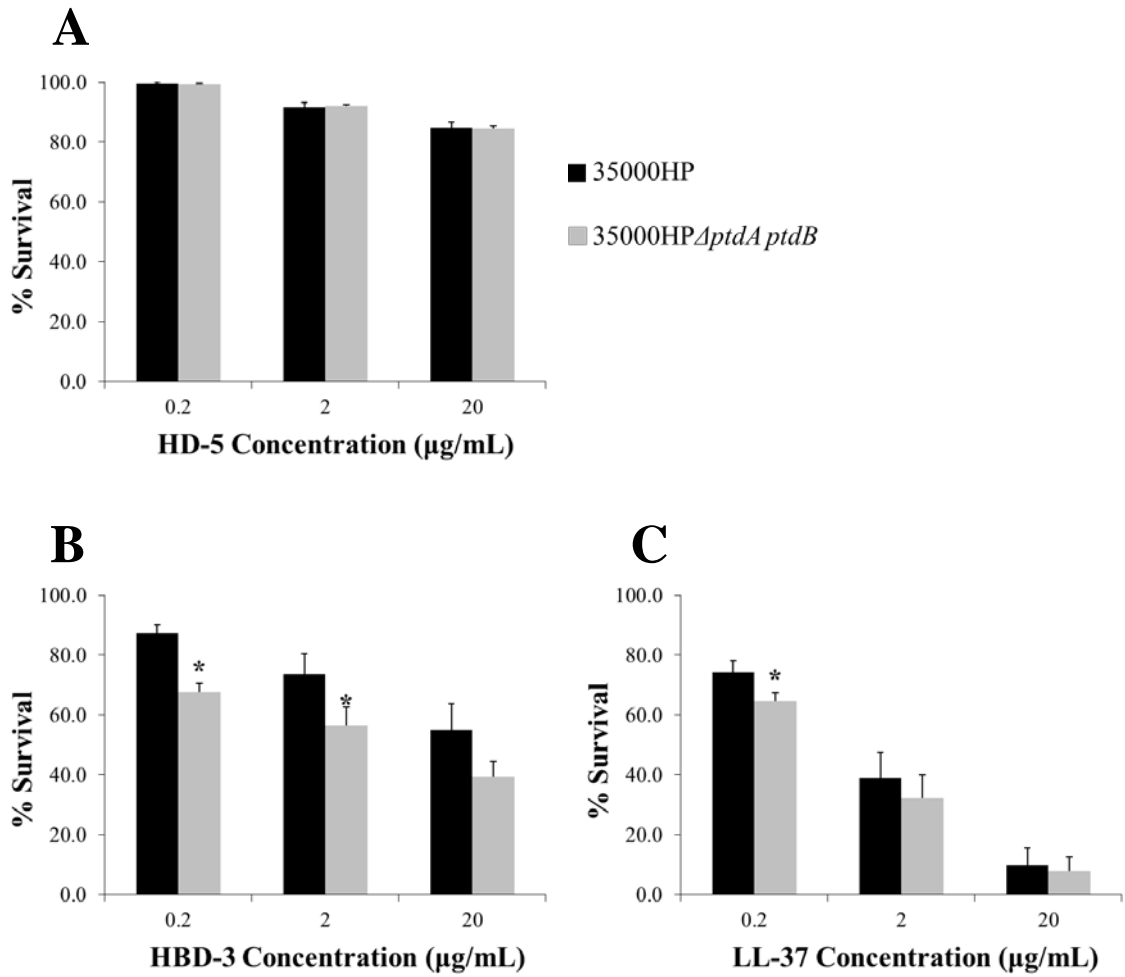


Figure 15. *ptdA* and *ptdB* together confer resistance to β -defensins, possibly cathelicidin, but not α -defensins, in *H. ducreyi*. 35000HP and 35000HPΔ*ptdA ptdB* were examined for resistance to the (A) α -defensin HD-5 (B) β -defensin HBD-3, and (C) human cathelicidin LL-37. Asterisks indicate statistically significant differences from 35000HP ($P < 0.05$). Data represent average \pm standard error of four independent replicates, and statistical significance was determined by Student's t-test.

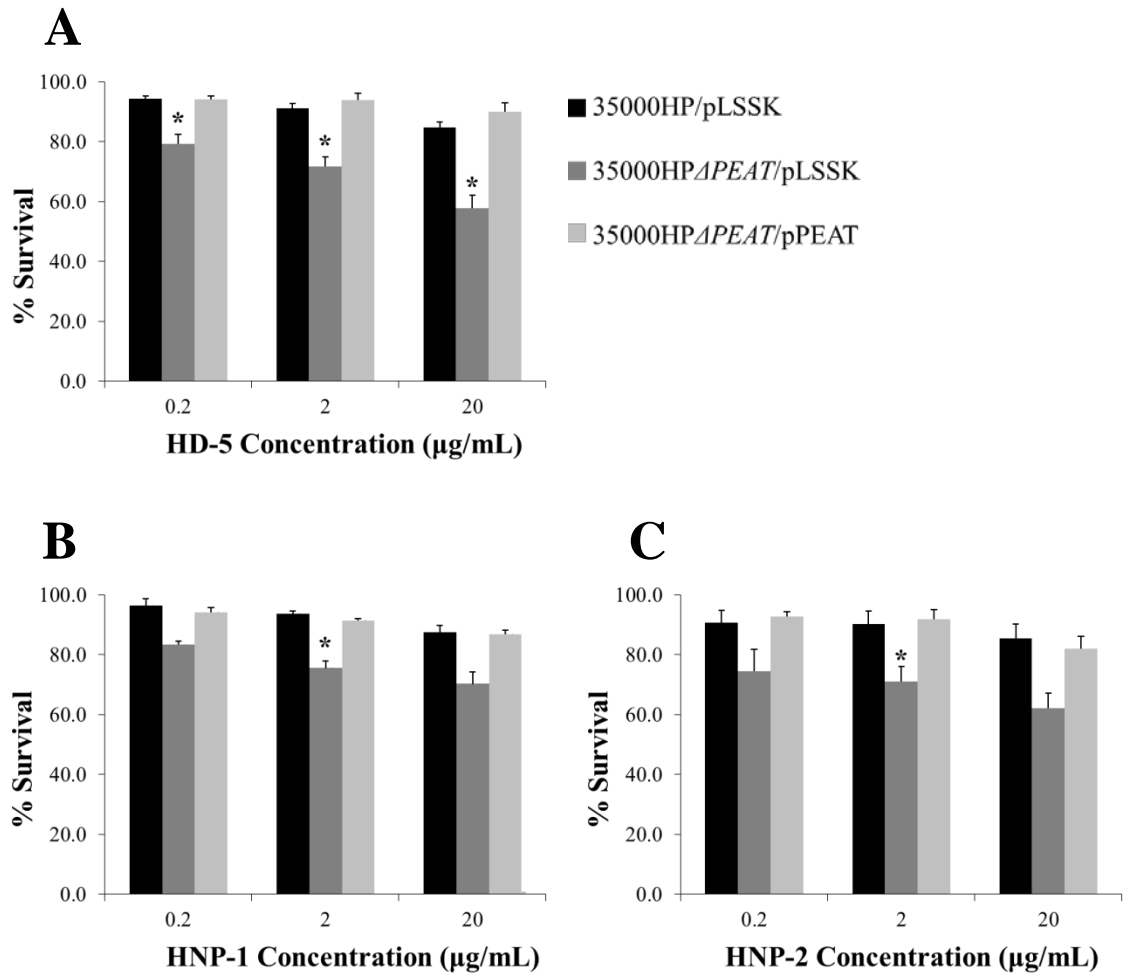


Figure 16. PEA transferases confer resistance to the α -defensins HD-5, HNP-1, and HNP-2 in *H. ducreyi*. 35000HP/pLSSK, 35000HP Δ PEAT/pLSSK and 35000HP Δ PEAT/pPEAT were tested for resistance to the α -defensins (A) HD-5, (B) HNP-1, and (C) HNP-2. Asterisks indicate statistically significant differences from 35000HP ($P < 0.05$). Complementation with pPEAT restored parental levels of susceptibility to defensins. Data represent average \pm standard error of six independent replicates, and statistical significance was determined by Student's t-test.

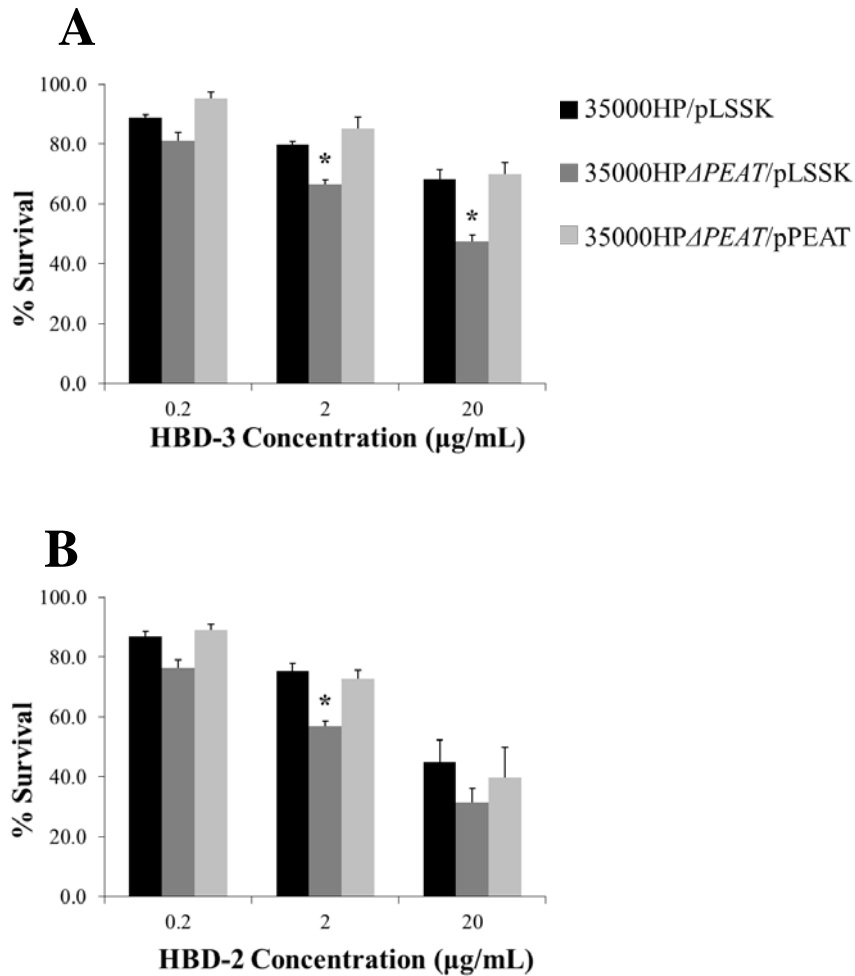


Figure 17. PEA transferases confer resistance to the β -defensins HBD-2 and HBD-3 in *H. ducreyi*. 35000HP/pLSSK, 35000HP Δ PEAT/pLSSK, and 35000HP Δ PEAT/pPEAT were tested for resistance to the β -defensins (A) HBD-2 and (B) HBD-3. Asterisks indicate statistically significant differences from 35000HP ($P < 0.05$). Complementation with pPEAT restored parental levels of susceptibility to defensins. Data represent average \pm standard error of three to four independent replicates, and statistical significance was determined by Student's t-test.

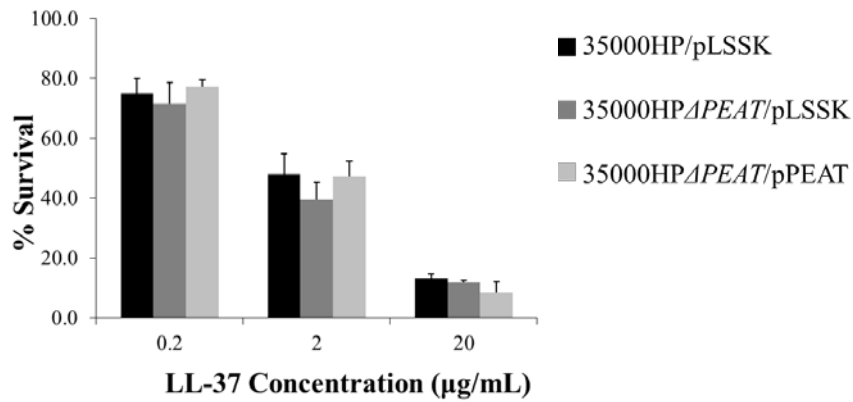


Figure 18. PEA transferases do not confer resistance to cathelicidin in *H. ducreyi*.

35000HP/pLSSK, 35000HP Δ PEAT/pLSSK and 35000HP Δ PEAT/pPEAT were tested for resistance to the human cathelicidin LL-37. There was no statistically significant difference between 35000HP and 35000HP Δ PEAT/pLSSK. Data represent average \pm standard error of six independent replicates, and statistical significance was determined by Student's t-test.

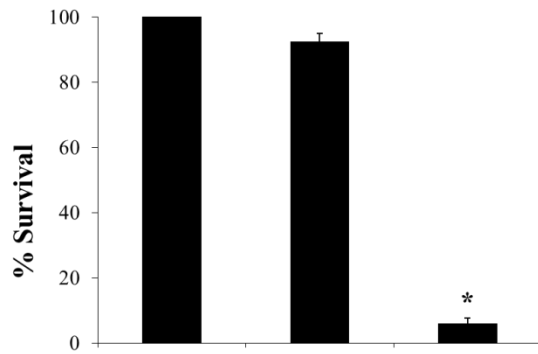


Figure 19. PEA transferases do not confer resistance to human serum in *H. ducreyi*.

35000HP, 35000HPΔPEAT and FX517, a serum-sensitive *dsrA* mutant, were examined for resistance to human serum. There was no significant difference in sensitivity to serum between 35000HP and 35000HPΔPEAT; FX517 was significantly more sensitive to serum than 35000HP, indicated by asterisk ($P < 0.05$). Data represent average \pm standard error of six independent assays, and statistical significance was determined by Student's t-test.

35000HP Δ PEAT had a more negative cell surface charge than 35000HP.

Our initial hypothesis states that these PEA transferases alter the cell surface charge to confer AP resistance by electrostatic repulsion. Once we established that the PEA transferase genes contributed to the cationic defensin resistance, we next examined whether these transferase genes affect the cell surface charge of *H. ducreyi*. To do this, we modified a protocol that uses the cationic dye Alcian Blue 8GX, which proportionately binds to the cell surface based on charge, to compare the relative cell surface charges of 35000HP and 35000HP Δ PEAT. We hypothesized more Alcian Blue would bind to 35000HP Δ PEAT than 35000HP, thus indicating a greater negatively charged cell surface on 35000HP Δ PEAT.

When comparing 35000HP Δ PEAT to 35000HP (Table 4.), we found 17.5% more dye bound to 35000HP Δ PEAT cells than to 35000HP cells ($P < 0.0001$). We also found 16.5% less dye remaining in the supernatant from 35000HP Δ PEAT cells than from 35000HP cells ($P < 0.0001$). Complementation of 35000HP Δ PEAT/pLSSK with pPEAT genes restored the level of dye bound to that of 35000HP/pLSSK (data not shown). These data indicate that the cell surface of 35000HP Δ PEAT was more negatively charged than 35000HP, suggesting that the PEA transferase gene products conferred the addition of positive moieties on the cell surface.

We next compared the relative cell surface charge of 35000HP, the three single mutants, 35000HP Δ *ptdA ptdB*, and 35000HP Δ PEAT (Figure 20). We found no significant difference in cell surface charge between 35000HP and any of the single mutants. However, a statistically significant trend was found indicating that more dye bound to 35000HP Δ *ptdA ptdB* (roughly 9.5% more) and 35000HP Δ PEAT (roughly 21%

more) than 35000HP. These data suggest that loss of one putative PEA transferase does not affect the cell surface charge. However, there does appear to be an additive effect, where loss of two PEA transferase gene products increases the negativity of the cell surface, and loss of all three PEA transferases has the greatest effect of cell surface charge.

Table 4. Cell surface of 35000HP Δ PEAT is more negatively charged than 35000HP.

	Strain	Mean	Std^b	Median	Wilcoxon p-value^c
Bacterial Cells	35000HP	0.280 ^a	0.203	0.217	<0.0001
	35000HP Δ PEAT	0.329	0.237	0.268	
Supernatant	35000HP	0.588	0.602	0.374	<0.0001
	35000HP Δ PEAT	0.491	0.532	0.313	

^a absorbance measurement at O.D.₆₆₀

^b Std, Standard deviation

^c Statistical significance determined by comparing median values with a non-parametric Wilcoxon signed ranks test because the absorbance measurements were not normally distributed; n = 24

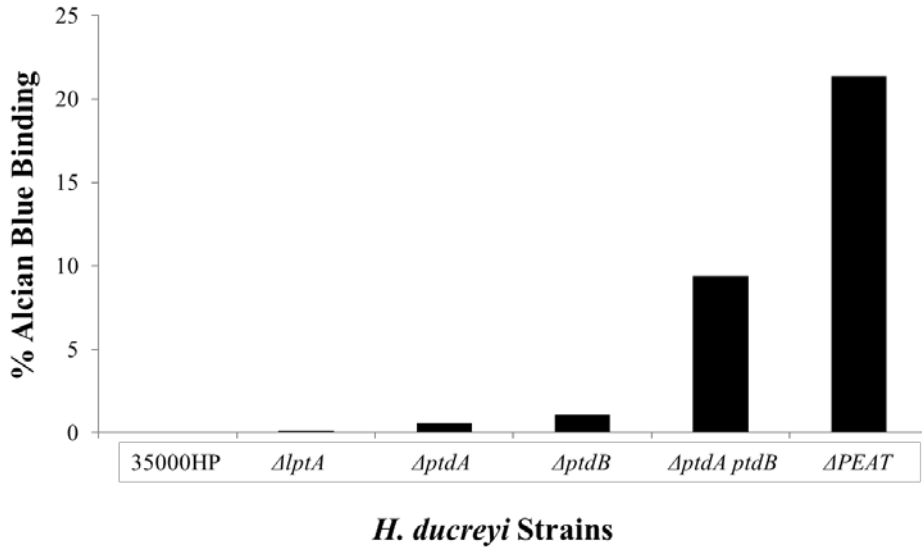


Figure 20. Loss of putative PEA transferases *ptdA* and *ptdB* contributes to increased cell surface negativity. The cell surface charges of 35000HP, 35000HP $\Delta lptA$ ($\Delta lptA$), 35000HP $\Delta ptdA$ ($\Delta ptdA$), 35000HP $\Delta ptdB$ ($\Delta ptdB$), 35000HP $\Delta ptdA ptdB$ ($\Delta ptdA ptdB$), and 35000HP $\Delta PEAT$ ($\Delta PEAT$) were examined. The percentage of Alcian blue dye that bound to the bacteria, which correlates with a negatively charged cell surface, was normalized to 35000HP for each sample. Data indicates the average of five independent assays, and a significant trend was observed (P = 0.036).

***H. ducreyi* LptA modifies the LOS lipid A with phosphoethanolamine.**

After determining that the PEA transferase genes play a role in modifying the *H. ducreyi* cell surface charge, we next hypothesized that the observed change in surface charge correlated with the loss of PEA modification on *H. ducreyi* LOS. We tested this hypothesis by analyzing the LOS structures of each single mutant as well as the parent, the triple mutant, and the complemented triple mutant via mass spectrometry.

LOS samples from each strain were prepared in triplicate and then *O*-deacylated to generate water soluble *O*-LOS that was analyzed by MALDI-MS. Lipid A and oligosaccharide (OS) “prompt fragments” are generated within the instrument during the ionization process. These fragments can provide useful information in determining LOS structures.

To define the full contribution of the putative PEA transferases on LOS structure, triplicate *O*-LOS samples from 35000HP/pLSSK, 35000HP Δ PEAT/pLSSK, and 35000HP Δ PEAT/pPEAT were prepared and analyzed by MALDI-MS (Figure 21, Table 5). The *O*-deacylated monophosphorylated lipid A (MPLA) at m/z 951.6 or 951.4 was observed in 35000HP/pLSSK as well as in 35000HP Δ PEAT and its corresponding complemented strain. Since PEA is 123 Da in size, the peak at m/z 1074.5 corresponds to a MPLA with the addition of one PEA; this peak was observed in the parent strain and the complement strain, but was not observed in 35000HP Δ PEAT, indicating loss of PEA modification in the mutant lipid A.

Evaluation of the full *O*-LOS glycoforms showed that all three strains had PEA present on the oligosaccharide. Glycoforms containing one PEA were observed in all three strains at m/z 3123, 2832, 2670, 2467, and 2304 (see Table 5 for full glycoform

compositions). Glycoforms containing two PEA groups were observed in both the parent strain as well as the complemented strain at m/z 3268 and 2977 (see Table 5 for full glycoform compositions). The masses corresponding to *O*-LOS structures with two PEA groups were not observed in 35000HP Δ PEAT. The observation that the lipid A from 35000HP Δ PEAT lacked the addition of PEA, combined with the presence of a PEA on the oligosaccharide of the *O*-LOS from this strain, demonstrates that the lipid A PEA transferase is inactive but that a second PEA transferase, responsible for the addition of PEA onto the oligosaccharide, is still active.

We next analyzed the lipid A fragments from each single mutant strain to provide further information about the *H. ducreyi* *O*-LOS. Representative spectra of the lipid A regions of the *O*-LOS from the single mutant strains and the corresponding parent strain are shown in Figure 22. The peak at m/z 951.5, corresponding to the MPLA, was observed in all three single mutants (35000HP Δ ptdB, 35000HP Δ ptdA, and 35000HP Δ lptA) as well as the parent strain. The MPLA with the addition of one PEA, found at m/z 1074.5, was observed in the parent strain as well as the 35000HP Δ ptdB and 35000HP Δ ptdA strains, but was not observed in the 35000HP Δ lptA strain. These data indicate that LptA alone confers the PEA modification of lipid A. Studies indicated no change in cell surface charge when comparing 35000HP to 35000HP Δ lptA (Figure 20.), suggesting that loss of the lipid A PEA modification does not have a significant effect on cell surface charge, and that it is the modification of something other than LOS that additionally contributes the observed change in cell surface charge.

Table 5. *H. ducreyi* O-LOS glycoforms and corresponding monoisotopic masses.

Glycoform ^a	Hex	HexN Ac	PE A	NeuA c	[M-H] ⁻ calc	[M-H] ⁻ _{obs}		
						35000H P/ pLSSK	35000H P Δ PEAT/ pLSSK	35000HP Δ PEAT/ pPEAT
^b A ₅ a ₁ **	3	1	2	1	3268.0 7	3268.18	^c nd	3267.82
A ₅ a ₁ *	3	1	1	1	3123.0 8	3123.00	3122.73	3122.73
A ₅ a ₁	3	1	0	1	3000.0 7	2999.91	2999.73	2999.55
^b A ₅ **	3	1	2	0	2976.9 8	2977.09	nd	2976.73
A ₅ *	3	1	1	0	2831.9 8	2831.91	2831.73	2831.64
A ₅	3	1	0	0	2708.9 8	2708.82	2708.64	2708.55
A ₄ *	2	1	1	0	2669.9 3	2669.82	2669.64	2669.55
A ₄	2	1	0	0	2546.9 2	2546.73	2546.64	2546.55
A ₃ *	2	0	1	0	2466.8 5	2466.64	2466.64	2466.45
A ₃	2	0	0	0	2343.8 4	2343.64	2343.55	2343.45
A ₂ *	1	0	1	0	2304.8 0	2304.55	2304.55	2304.36

^aAll molecular masses contain four Heptoses, Kdo(P), and O-deacylated lipid A.

Asterisks indicate the number of PEA present in the structure.

^bGlycoforms were observed in their sodiated forms.

^cnd indicates that the particular glycoform was not detected in the sample.

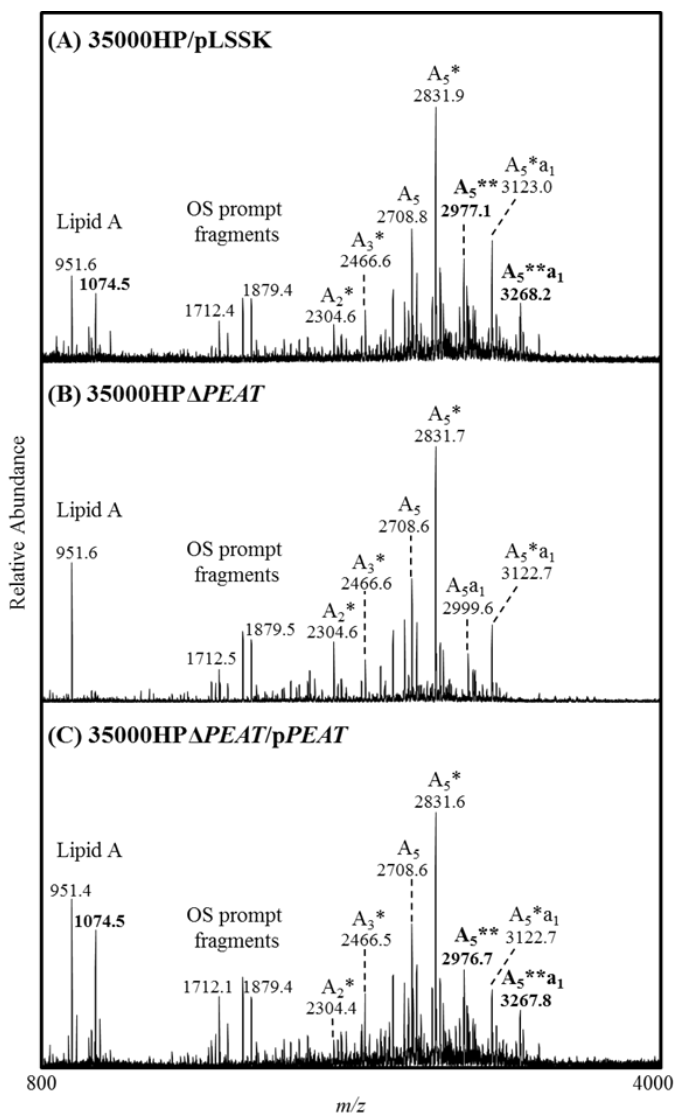


Figure 21. *H. ducreyi* putative PEA transferases contribute to modification of Lipid A with PEA. Negative-ion MALDI-MS spectra of *O*-LOS from (A) 35000HP/pLSSK, (B) 35000HPΔPEAT/pLSSK, and (C) 35000HPΔPEAT/pPEAT. The compositions of the glycoforms are described in Table 5. Masses labeled with two asterisks only observed in the parent strain and the corresponding *PEAT* complemented strain. The asterisk in the glycoform nomenclature designates the number of PEA groups present on the *O*-LOS.

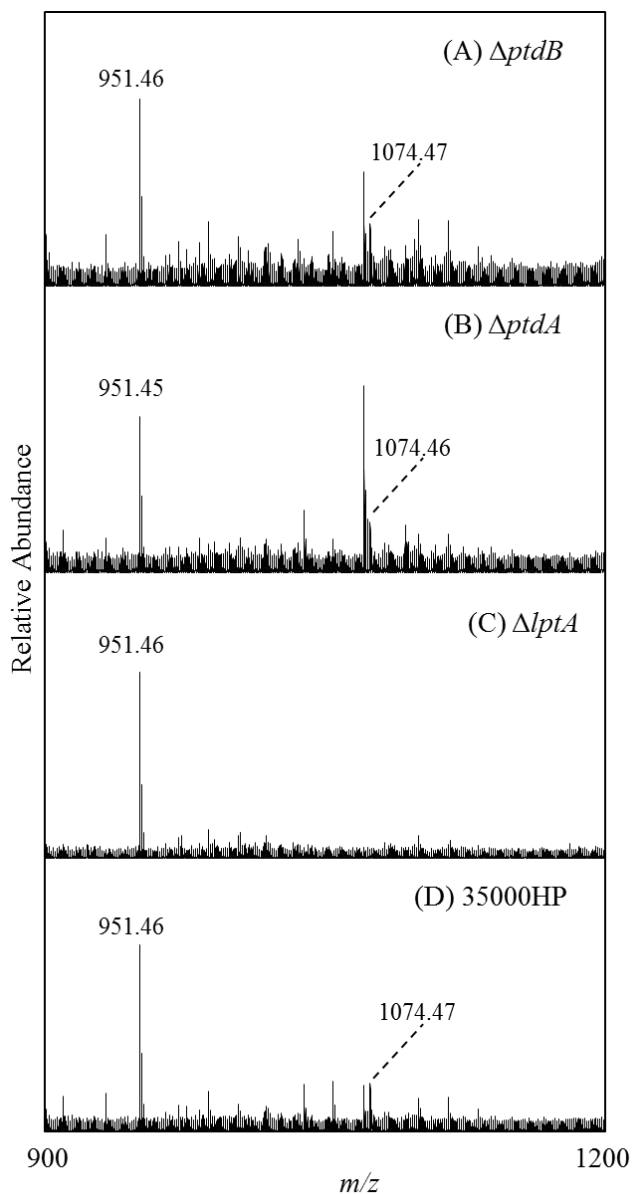


Figure 22. LptA contributes to modification of Lipid A with PEA. Negative-ion MALDI-MS spectra of *O*-LOS from (A) 35000HP Δ *ptdB*, (B) 35000HP Δ *ptdA*, (C) 35000HP Δ *lptA*, and (D) 35000HP. The figure shows zoomed images from representative spectra for each strain. The *O*-deacylated monophosphorylated lipid A (MPLA) was observed at *m/z* 951.46 or 951.45; this structure plus the addition of PEA was observed at *m/z* 1074.5. The MPLA plus PEA was not observed in the 35000HP Δ *lptA* samples.

H. ducreyi ptdA, lptA, and ptdB are not required for survival in vivo.

We next examined the role of the PEA transferase genes in virulence in vivo by using the human model of *H. ducreyi* infection. To do this, we challenged eight healthy adult volunteers with between 58 – 139 CFU of 35000HP and between 40 – 224 CFU of 35000HP Δ PEAT, with the exception of the final three volunteers who were challenged with roughly equal doses of parent and mutant (Table 6).

Of the patient sites inoculated with 35000HP, 23 of 24 (95.8%) developed papules, while 21 of 24 (87.5%) sites inoculated with 35000HP Δ PEAT developed papules. Pustules developed at 5/24 (20.8%) parent-inoculated sites and 5/24 (20.8%) mutant-inoculated sites. The average parent papule size at day 1 was 8.7 mm² whereas the average mutant papule size at day 1 was 5.0 mm².

When comparing 35000HP and 35000HP Δ PEAT, there was no statistical difference between papule formation rate (parent = 95.8% [95% C.I., 88.2 – 99.9%], mutant = 87.5% [95% C.I., 76.3 – 98.7%], $P = 0.103$) or pustule formation rate (parent = 20.8% [95% Confidence Interval (C.I.), 9.7 – 32.0%], mutant = 20.8% [95% C.I., 4.8 – 36.9%], $P = 1.0$). The difference in day 1 papule size (parent 8.7 mm², mutant 5.0 mm²) did approach statistical significance, with a P of 0.051. However, taken together, these data suggest that the gene products of *ptdA*, *lptA*, and *ptdB* do not contribute to *H. ducreyi* virulence during human infection.

Table 6. 35000HP Δ PEAT is fully virulent in vivo.

Response to inoculation of live <i>H. ducreyi</i> strains						
Volunteer^a	Gender^b	Observation period (days)	Isolate^c	Dose (CFU)	No. of initial papules	No. of pustules at endpoint
441	M	7	P	58	3	1
			M	56-224 ^d	2	2
442	M	7	P	58	3	1
			M	56-224 ^d	3	1
444	F	9	P	70	3	
			M	40-159 ^e	3	1
445	F	7	P	70	3	1
			M	40-159 ^e	2	
446	F	7	P	70	3	1
			M	40-159 ^e	3	
447	M	8	P	139	3	
			M	112	3	
451	F	7	P	108	3	
			M	88	3	
453	F	12	P	108	2	1
			M	88	2	1

^a Volunteers 441 and 442 were inoculated in the first iteration. Volunteer 443 withdrew from the study prior to inoculation. Volunteers 444, 445, and 446 were inoculated in the second iteration. Volunteer 449 was excluded from the trial; volunteers 448 and 450 withdrew from the study prior to inoculation. Volunteer 447 was inoculated in the third iteration. Volunteers 451 and 453 were inoculated in the fourth iteration. Volunteer 452 was excluded from the study prior to inoculation.

^b M, Male; F, Female

^c P, 35000HP (parent); M, 35000HP Δ PEAT (mutant)

^d Mutant-inoculated sites received EDD of 56, 112, or 224 CFU.

^e Mutant-inoculated sites received EDD of 40, 80, or 159 CFU.

CHAPTER FOUR: DISCUSSION

The relationship between host and pathogen is like a giant chess game; one of fluidity and constant evolution, with each side trying to outplay the other. The interplay between *H. ducreyi* and its human host is no different. For every component of the immune system used as defense during infection, *H. ducreyi* has developed a mechanism of resistance. Our research primarily focuses on the evasion of host antimicrobial peptides. Previous studies have established that LL-37 resistance is mediated by the Sap transporter and the MTR efflux pump and β -defensin resistance is mediated by the MTR efflux pump as well. Our research was aimed at discovering a mechanism of α -defensin resistance in *H. ducreyi*.

Several bacteria have used modification of their cell surface as a way to avoid the immune system. Surface proteins may be modified to serve as camouflage or to directly interact with immune system components. In gram-negative bacteria, PEA transferases modify a variety of substrates, including carbohydrate components of LPS, other sugars in the gram-negative envelope, and proteinaceous surface structures; the best characterized PEA transferases are those that PEA-modify the LPS of enteric pathogens or the LOS of *N. gonorrhoeae*. PEA modification of LPS or LOS contributes to AP resistance and serum resistance in vitro and to virulence in vivo. Structurally, all known gram-negative PEA transferases are members of the YhjW/YjdB/YijP/YbiP family of inner membrane proteins. In *H. ducreyi*, both lipid A and core components of the LOS are modified with PEA; we identified three *H. ducreyi* genes with strong homology to known PEA transferases. Hypothesizing that these PEA transferases may be involved in

α -defensin resistance, our goal was to characterize the role of these PEA transferases in *H. ducreyi* pathogenesis.

We examined the in vitro effect of PEA modification on resistance to human APs relevant to *H. ducreyi* infection, including α -defensins, β -defensins, and the cathelicidin LL-37. Using mutants with deletions in one, two, and all three putative PEA transferases, we observed that PEA modification did not appear to confer resistance to LL-37 (Figures 10-16). While there were several slight albeit statistically significant differences between the parent and double PEA transferase mutants in LL-37 sensitivity, these differences were not present in the triple mutant. Since we performed a greater number of assays with the triple mutant and consistently observed no difference in LL-37 susceptibility between the parent and triple mutant, the statistical significance found when comparing LL-37 sensitivity between the parent and double mutants was likely not biologically relevant. It is more likely a product of day-to-day variation that produced a statistical anomaly.

PEA transferases have been shown to confer resistance to LL-37 in pathogenic *Neisseria*, but previous studies have already established that both Sap and MTR play major roles in LL-37 resistance in *H. ducreyi* (70, 71, 144). It is still possible that PEA transferases contribute to LL-37 resistance, but the presence of an intact Sap transporter and a functioning MTR efflux pump may be masking any contribution of PEA modification to LL-37 resistance. Inactivating or deleting both the Sap transporter and the MTR efflux pump in the triple PEA transferase mutant could provide more insight to what role PEA modifications have in LL-37 resistance. It is also possible that the presence of PEA-modified KDO in 35000HP Δ PEAT still confers resistance to LL-37; to

fully understand the contribution of PEA modifications to LL-37 resistance, this active PEA transferase would need to be deleted as well.

It was clear, however, that PEA modification plays a role in *H. ducreyi* resistance to both α - and β -defensins. When any one of the PEA transferase genes was deleted, we observed no difference in AP susceptibility between the parent and mutant strains; however, when two PEA transferase genes were deleted, the mutants became more susceptible to β -defensins (Figures 13B, 14B, 15B). It was only when all three PEA transferase genes were deleted that the mutant became more susceptible than the parent strain to the α -defensins as well. Thus, all three putative PEA transferases contribute to defensin resistance (Figures 16A, 17). We likely see a significant increase in susceptibility to HBD-3 with two PEA transferase deletions because HBD-3 has a greater overall net positive charge (+13) than HD-5 (+6). We hypothesized that the addition of the positively charged PEA onto the cell surface conferred AP resistance, so it is not surprising that the AP with the greatest overall net positive charge is most affected by PEA addition. It is worth noting that while the increased sensitivity to the defensins is considered moderate, roughly between a 25-35% decrease in survival when compared to parent, it is consistent throughout the defensins tested (Figures 16, 17). Thus, this is the first known mechanism of α -defensin resistance found in *H. ducreyi*.

When all three putative PEA transferases were deleted, we also observed a significant change in surface charge of the organism (Table 4). Loss of *lptA* alone, which we found is necessary to PEA-modify Lipid A, had no effect on surface charge in our assay. Our inability to detect changes in surface charge in any of the single mutants may reflect a lack of sensitivity of the cationic dye assay, but this data is consistent with what

we observed in AP resistance; loss of one putative PEA transferase does not appear to have a large enough effect on cell surface charge to influence AP resistance. Likewise, the greatest effect on surface charge observed in the *lptA ptdA ptdB* mutant does correlate with the increased sensitivity to defensins. When we examined the cell surface charge of 35000HP Δ *ptdA ptdB*, we saw an intermediate between the single mutants and triple mutant. Deletion of these two putative PEA transferases increases *H. ducreyi* susceptibility to HBD-3, which correlates with the increase in negativity of the cell surface seen in the Alcian blue assay. It is difficult to predict, however, the limitations of our surface charge assay. Ideally, we would examine the relative surface charges of each single, double, and triple mutant compared to parent with techniques sensitive enough to detect minute changes in absorbance and weight. These data suggest that it is the additive effect of PEA modification at multiple sites on the *H. ducreyi* surface that provides sufficient positivity to repel cationic APs.

The results of the AP and cell surface charge assays, taken together, raise the question about gene regulation. *LptA*, *ptdA*, and *ptdB* are not controlled by any known regulons in *H. ducreyi*, nor are they found in an operon, suggesting that, if they were regulated, it is done on an individual gene-by-gene basis. It is possible the lack of AP resistance seen in the *lptA* mutant a result of this gene product alone having a minimal effect on cell surface charge, as seen in the cell surface charge assay, or a result of an up-regulation of the putative PEA transferases *ptdA* and *ptdB*. Expression levels of *lptA*, *ptdA*, and *ptdB* could be measured and compared in 35000HP, each single mutant and each double mutant. We then can determine if these genes are constitutively expressed or if their expression is increased with the loss of one or more PEA transferase. If they are

constitutively expressed, then we can infer that each PEA transferase gene product alone may not affect cell surface charge enough to confer AP resistance. However, if expression of *ptdA* and *ptdB* increases in the *lptA* mutant, then there is likely a compensatory regulation mechanism present. This regulation could then account for why we do not see a change in cell surface charge or AP sensitivity in the *lptA* mutant.

In contrast to observations in *N. gonorrhoeae*, loss of the three PEA transferase genes did not affect susceptibility of *H. ducreyi* to human serum (162). Our results indicate that, in *H. ducreyi*, PEA modification likely does not play a role in evasion of host complement. *H. ducreyi* expresses two surface proteins, DsrA and DltA, which have been previously shown to confer resistance to complement-mediated killing (44, 45). DsrA blocks binding of IgM to the *H. ducreyi* surface and prevents initiation of the complement cascade, whereas the contribution of the structurally related DltA to serum resistance remains unclear (44, 45). Thus, in a similar masking effect that the Sap transporter and MTR efflux pump may have on AP resistance, the activities of DsrA and DltA may be concealing any contribution of surface modification with PEA on serum resistance. DsrA is a large surface protein that likely acts as a shield, preventing IgM from binding to the *H. ducreyi* cell surface; this blocking action would likely take place before IgM would come in contact with the PEA modifications found on the cell surface. It is possible that deleting *dsrA* and *dltA* in the triple PEA transferase mutant may reveal that PEA modification has a slight contribution to serum resistance, but as we've shown, these PEA transferases likely do not appear to confer resistance to human serum.

Our data suggest that all three PEA transferase genes in *H. ducreyi* contribute to lessening the negativity of the cell surface (Table 4). Mass spectrometric analysis

indicated that LptA does this by modifying lipid A with PEA (Figure 21); the functions of *ptdA* and *ptdB* are less clear. Loss of either gene alone had no effect on the modification of lipid A, but both genes contributed to effects on cell surface charge and AP resistance (Figure 19, Table 4). The closest characterized homolog of these genes, *opgE*, encodes a PEA transferase that targets OPGs. OPGs and related periplasmic glucans have been found in disparate gram-negative bacteria, from the *Enterobacteriaceae* to *Alphaproteobacteria* (180). However, no OPG-like molecules have been described among the *Pasteurellaceae*, which includes *H. ducreyi*; further, we were unable to find homologs of any known OPG biosynthesis or modification genes in the genomes of the *Pasteurellaceae*, except for *opgE*. Thus, OpgE homologs in the *Pasteurellaceae*, such as PtdA and PtdB, likely target other molecules; our cell surface charge data suggest that PtdA and PtdB modify surface structures with positively charged moieties. Since *ptdA* and *ptdB* share this homology with the *E. coli* OpgE, we could generate an *E. coli* mutant with a deletion in *opgE* and attempt to complement this strain with a plasmid carrying the *H. ducreyi ptdA* or *ptdB* genes. If PEA modification of Opg is restored, then we can say with more certainty that *ptdA* and *ptdB* are, in fact, PEA transferases. We could then search for additional cell surface proteins in *H. ducreyi* that could potentially be modified with PEA by PtdA and PtdB.

There is precedence for PEA transferases to modify surface proteins. *C. jejuni* modifies its flagellum with PEA, and *N. gonorrhoeae* modifies its type IV pilus with PEA (169, 179). *H. ducreyi* does not have a flagellum, and there is no evidence that the organism expresses a type IV pilus. However, the three *H. ducreyi* genes *pilA* (*HD1123*), *pilB* (*HD1124*), and *pilC* (*HD1125*) share homology to known and characterized type IV

pilus genes found in pathogenic *Neisseria* and *Pseudomonas aeruginosa*. Analysis of these putative genes may help reveal if these homologs have a functional role in *H. ducreyi* or if they are pseudogenes that are vestigial from an ancient ancestor and are no longer expressed. We could also analyze the function of these three putative genes by generating a mutant with deletions in all three genes. We could then examine this mutant for defects in growth and motility as well as what contribution, if any, it has in virulence using the human model of *H. ducreyi* infection. If we do see expression and function of the putative type IV pili in *H. ducreyi*, we can also attempt to isolate the putative type IV pilus and examine its structure for PEA modification. If PEA modification is found, it may be plausible that PtdA or PtdB is responsible for modifying this protein with PEA; mass spectrometry analysis of the 35000HP putative type IV pilus compared to that of the *ptdA*, the *ptdB*, and the *ptdA ptdB* mutants could further reveal if these PEA transferases are responsible for modifying the putative type IV pilus with PEA.

The function of PtdA and PtdB may not be limited to what has previously been described. They potentially could modify any surface protein; our cell surface charge assays examine the overall surface charge, not just that of a specific region or protein. Preliminary proteomic post translational modification (PTM) analysis of all surface proteins found in the *H. ducreyi* genome was inconclusive; one culture of 35000HP revealed PEA modification of the major outer membrane protein (MOMP) and outer membrane protein A2 (OMPA2), while a second culture of 35000HP had no PEA modification of any outer membrane proteins. Similarly, one culture of the 35000HP*PEAT* that underwent PTM analysis showed PEA modification of MOMP and OMPA2 and the second culture revealed no modification (data not shown). While the

preliminary data was inconclusive, the first step would be to have additional PTM analysis of 35000HP to better determine if there are outer membrane proteins modified with PEA. Next, PTM analysis of 35000HP Δ *ptdA* and 35000HP Δ *ptdB* would confirm if these PEA transferases confer modification of any outer membrane proteins.

Given the large number of surface proteins and the variability seen in the preliminary analysis, it may be more efficient to specifically isolate any outer membrane proteins in *H. ducreyi* in which PEA modification has been shown. The mass spectrometry data of the *H. ducreyi* LOS indicated that even known sites of PEA modification, such as lipid A, exist in both modified and unmodified forms. By isolating specific proteins, it may provide us with less background and a better analysis of each protein. When attempting to isolate a specific protein, we can recombinantly express it on a plasmid the surface protein gene flanked with a B-terminus polyhistidine-tag to allow for pull-down elution in a nickel column isolate large quantities of our target surface protein. Thus, instead of globally searching through hundreds of proteins for a small 123 Da PEA modification, we can have more stringent analysis of one specific site on one specific protein. In a sense, this is similar to examining RNA expression in a microarray versus using qRT-PCR; while microarrays are useful when looking at global expression of genes during specific conditions, they can potentially be unreliable as a sole analysis due to background and a high number of false negatives or positives. By using qRT-PCR, the expression of one specific gene can be determined by comparing it to a constitutively expressed “housekeeping” gene rather than comparing the target gene expression to a large number of genes. Isolation and analysis of several potentially PEA

modified outer membrane proteins in 35000HP, 35000HP Δ *pta*, and 35000HP Δ *ptdB* could reveal the function and modification site of PtdA and PtdB.

The other question that the LOS mass spectrometry analysis raised is what gene product is responsible for modifying *H. ducreyi* KDO with PEA. The PEA modification of KDO was detected by mass spectrometry in all strains examined in this study, including the triple mutant strain, 35000HP Δ *PEAT* (Figure 20). These data indicate that an additional PEA transferase exists in *H. ducreyi* that has yet to be identified.

Homology searches do not reveal additional putative PEA transferases in *H. ducreyi*. Since we are specifically looking for gene products that modify KDO with PEA, we could perform transposon mutagenesis to introduce random mutations throughout the *H. ducreyi* genome. We could initially screen for sensitivity to polymyxin B, whose cationic structure that targets bacterial membranes resembles that of many APs. Once we have found a strain susceptible to polymyxin B, we would then analyze the mutant by mass spectrometry to determine if KDO is modified with PEA. If we found a gene responsible for the PEA addition on KDO, we would then have to undergo a similar process of deletion and characterization as seen with *lptA*, *pta*, and *ptdB*. While mass spectrometry analysis will ultimately determine if this gene confers PEA modification to the KDO core sugars in the *H. ducreyi* LOS, we are still interested in the effect these putative proteins would have on cell surface charge and AP resistance.

We could construct an additional mutant with deletions in all PEA transferase genes, including *lptA*, *pta*, *ptdB*, and any newly discovered putative PEA transferases, and assay for additive contribution to cell surface charge and AP resistance. We would likely see a greater change in cell surface charge and an increase in sensitivity to APs in

the multi-PEA transferase mutant when compared to 35000HP, and we may also see an impact on virulence with this mutant in the *H. ducreyi* model of human infection.

When tested in the human model of *H. ducreyi* infection, 35000HP Δ PEAT was fully virulent. This result differs from previous in vivo studies in animal and human models of *S. enterica* and *N. gonorrhoeae* infection. An *eptA cptA* mutant of *S. enterica* was less fit than its parent strain in mice, although the reduced in vivo survival of the mutant was considered modest (157). In *N. gonorrhoeae*, LptA-mediated modification of lipid A with PEA provided an increased fitness advantage in both female mice and human male volunteers (176). While competition infection assays are more sensitive in determining fitness in vivo, they are not a reliable method that can be used with *H. ducreyi*. Due to the clumping nature of *H. ducreyi*, it is nearly impossible to determine inoculation. This would make it very difficult to inoculate the volunteer with an exact 1:1 ratio of parent to mutant strains as well as to determine the exact number of CFU recovered post infection. Additionally, *N. gonorrhoeae* competition infections use large inoculum (10^5 - 10^6), whereas *H. ducreyi* requires as few as one CFU to be infectious. Using such small inoculation sizes in *H. ducreyi* would have a greater impact on the statistical analysis.

In the *H. ducreyi* human challenge model, spontaneous resolution of all parent-inoculated sites occurs in 24.8% of participants (n=299 subjects); the overall parent pustule formation rate is 53.8% (n=803 sites) (195). In the present study, the pustule formation rate at parent-inoculated sites was only 20.8%. With such a low parent pustule formation rate, it would be difficult to discern a difference in pustule formation rates between parent and mutant strains even if the mutant was partially attenuated. Since

KDO is still modified with PEA, we cannot say for certain that PEA modifications do not contribute to virulence in the humans, but we can conclude that the gene products of *ptdA*, *lptA*, and *ptdB* likely do not contribute significantly to virulence in the human model of *H. ducreyi* infection.

The human model of *H. ducreyi* infection is an invaluable tool, especially since the current swine and rabbit models of invasion and infection have major limitations. Animal models require a much larger inoculum than the human model, and both swine and rabbits clear the infection and produce protective antibodies (29-31), neither of which are representative of the human immune response to a *H. ducreyi* infection. In many animal models of infection, large numbers of genetically identical subjects are challenged. While this may allow for a more discernable difference in virulence by eliminating potential variables, it does not account for the genetic diversity found in a normal population.

It is important, however, to also acknowledge the limitations of the human model. There are strict regulations that limit the number of human volunteers used in each trial; statistics become more powerful with greater numbers, and we would like to have a high number of test subjects to allow for a more stringent statistical analysis. In an ideal setting, subjects would also be more representative of the global population in both ethnicity and age; the current human model of *H. ducreyi* infection has been limited to individuals aged 18-68, with 80% of the subjects being Caucasian, 18% were African American, and only 2% were Asian American. Currently, there is no apparent association with race or sex and papule formation (195). The human model is limited to the first two weeks of infection; it is possible that there are virulence mechanisms that

may not be critical for initial invasion and infection but rather for sustained, long-term infection. Ideally, we would be able to challenge human volunteers throughout the course of the entire *H. ducreyi* infection to better determine the impact of each gene product on virulence.

When we examine both our in vitro and in vivo data, potentially the lack of contribution to virulence in vivo can be attributed to the modest difference in susceptibility to defensins between the parent and mutant strains observed in vitro. A 25-35% increase in sensitivity to defensins may not be significant enough to correlate with attenuation in vivo. But these results of this trial could also raise the question of the overall importance of defensins in the host's response to *H. ducreyi* infection. Previous human trials examining the importance to pathogenesis of the Sap transporter, which confers resistance to LL-37 but not defensins, established a direct correlation between LL-37 resistance in vitro and virulence in vivo: a *sapA* mutant with a 15-20% loss of transporter-mediated LL-37 resistance was partially attenuated in vivo, and a *sapBC* mutant, with no transporter activity and a 35-45% loss in resistance to LL-37, was fully attenuated for virulence (70, 144). These data, together with the current study, suggest that, during *H. ducreyi* infection, LL-37 plays a more significant role than defensins in host defense. A greater impact of LL-37 than defensins on host defense could also account for the greater role observed for *N. gonorrhoeae* LptA in human infection; unlike its *H. ducreyi* homolog, *N. gonorrhoeae* LptA contributes to LL-37 resistance (162).

In summary, we identified three *H. ducreyi* genes that shared a high degree of homology to known PEA transferases found in other gram-negative bacteria. Deletion of all three genes, including *lptA*, *ptdA*, and *ptdB*, resulted in an increase in sensitivity to α -

and β -defensins, a more negatively charged cell surface, and the loss of PEA modification on lipid A. While this is the first known mechanism of α -defensin resistance and the first characterized PEA transferase found in *H. ducreyi*, the *lptA ptdA ptdB* mutant was fully virulent when tested in the human model of *H. ducreyi* infection. Our results suggest that AP resistance is conferred by multiple mechanisms in *H. ducreyi* and that defensins may play a lesser role than LL-37 in host defense against *H. ducreyi*.

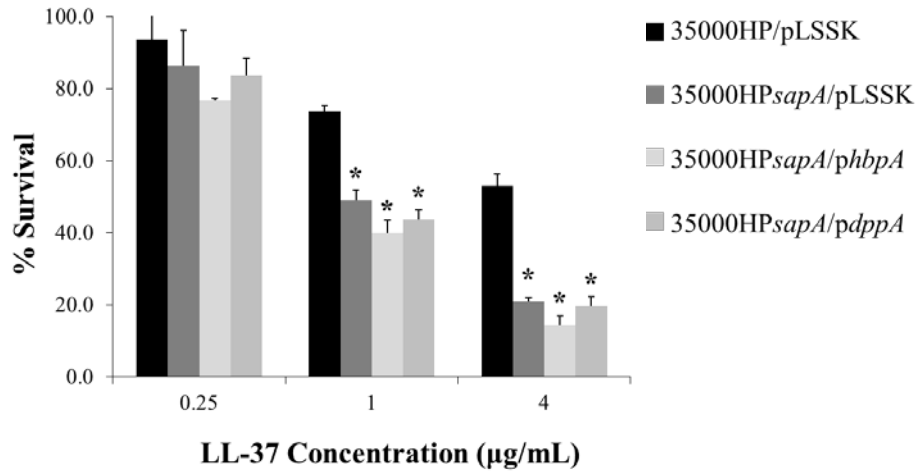
APPENDIX

Part 1: Sap Periplasmic Binding Protein Homologs

Previous studies have shown that the *H. ducreyi* Sap transporter (Figure 1A) confers resistance to the bactericidal effects of LL-37, and that this mechanism of AP resistance is vital for virulence in the human model of *H. ducreyi* infection (70, 144). When observing the in vivo sensitivity to LL-37, we noticed that the *sapA* mutant had a modest resistance phenotype whereas the *sapBC* mutant was highly sensitive to LL-37 compared to 35000HP. Since the *sapA* mutant still had an intact inner membrane channel (SapB and SapC) as well as functioning ATPases (SapD and SapF), these data suggest that the Sap transporter retains partial function in the absence of the periplasmic binding protein SapA; the Sap transporter is only completely disabled when the inner membrane permeases are deleted.

SapA shares homology to the periplasmic binding proteins heme-binding lipoprotein (*hbpA*) and the dipeptide-binding protein (*dppA*). In nontypeable *H. influenzae*, HbpA is necessary to bind and deliver heme into the cytoplasm by means of the DppBC inner membrane channel (139). DppA has been found in other *Haemophilus* species and is predicted to be a periplasmic transport protein. The Sap transporter is also involved in heme acquisition and transportation, suggesting the possibility that HbpA and DppA may also utilize the Sap permeases to transport heme into the cell (139). Since there appears to be an interplay between the Sap transporter and HbpA and DppA, we hypothesized that, in the absence of SapA, HbpA and DppA may be able to transport LL-37 through the Sap permease proteins and restore partial function of the transporter.

We approached this hypothesis by generating plasmids expressing either *hbpA* or *dppA*, with the aim to complement 35000HP*sapA* with *phbpA* or *pdppA*. We then transformed the 35000HP*sapA* with either *phbpA* or *pdppA* and examined the resistance to LL-37. We saw no difference between 35000HP*sapA*/pLSSK and 35000HP*sapA*/*phbpA* or 35000HP*sapA*/*pdppA* (Appendix Figure 1). These data suggest that HbpA and DppA are not able to transport LL-37 through the Sap permease channel. However, it does not rule out the possibility that there may be additional periplasmic binding proteins that may function to transport LL-37 through the Sap permease in the absence of SapA. One likely possibility is a protein homologous to the periplasmic transport protein oligopeptide-binding protein (OppA) found in the *Haemophilus* genus. Subsequent studies done by the Bauer laboratory post-doctoral fellow Sherri Rinker show that loss of OppA increases *H. ducreyi* sensitivity to LL-37, and that OppA is not only able to bind LL-37, but it can also partially complement the *sapA* mutant. These data suggest that OppA contributes to transporting LL-37 through the Sap channel in the absence of SapA.



Appendix Figure 1. *hbpA* and *dppA* cannot confer AP resistance through the Sap transporter. 35000HP/pLSSK, 35000HPsapA, 35000HPsapA/phbpA, and 35000HPΔSapA/pdppA were examined for resistance to the human cathelicidin LL-37. Asterisks indicate statistically significant differences from 35000HP (P < 0.05). Data represents average ± standard error of three independent assays, and statistical significance was determined by Student's t-test.

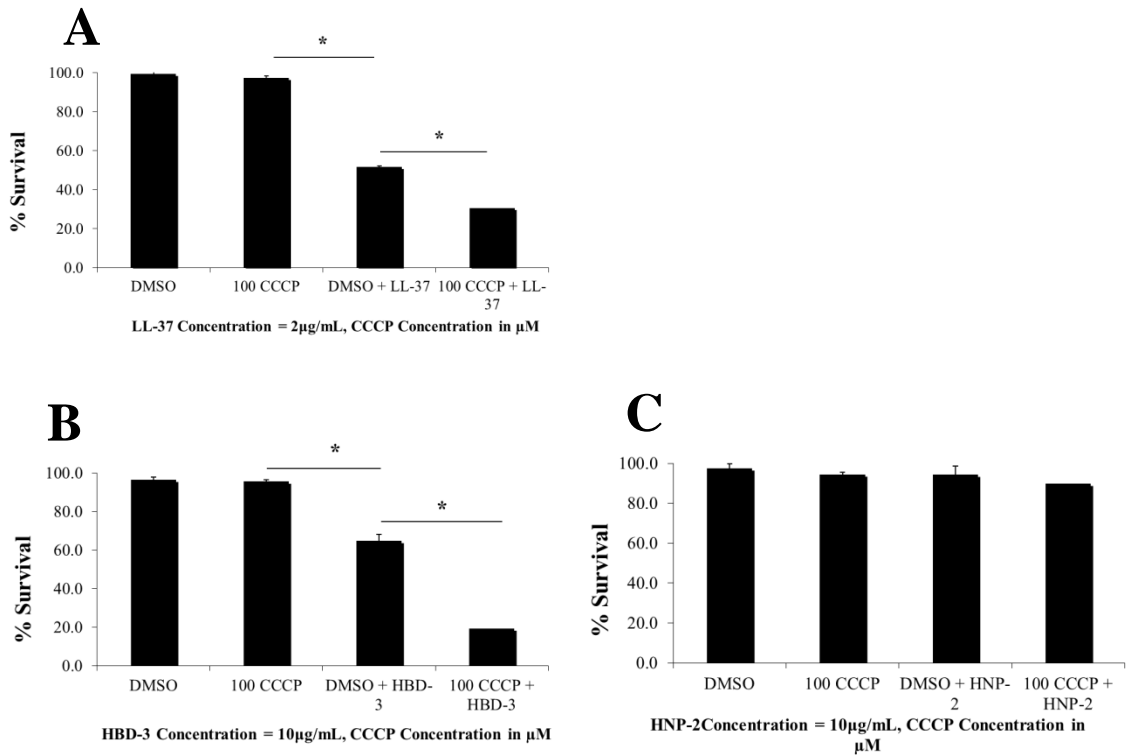
Part 2: MtrC Contributes to AP Resistance

Resistance-nodulation-division (RND) efflux pumps are a common mechanism of AP resistance in gram negative bacteria (145, 146, 148). RND efflux pumps, such as the MTR pump found in *N. gonorrhoeae*, are powered by proton motive force (PMF) (145, 149). To determine if there was a PMF-dependent mechanism of AP resistance in *H. ducreyi*, we exposed 35000HP to carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a proton uncoupler which disrupts the proton gradient and PMF, and then performed our AP assay as previously described in the Methods section above (135, 152). We found that 35000HP was more susceptible to both the β -defensin HBD-3 and LL-37, but not the α -defensin HNP-2, after exposure to CCCP (Appendix Figure 2), indicating the presence of a PMF-dependent mechanism of AP resistance. Homology searches revealed *H. ducreyi* has a homolog of the *N. gonorrhoeae* MTR efflux pump, and we next generated a mutant with a deletion in *mtrC* (work done by post-doctoral fellow Sherri Rinker).

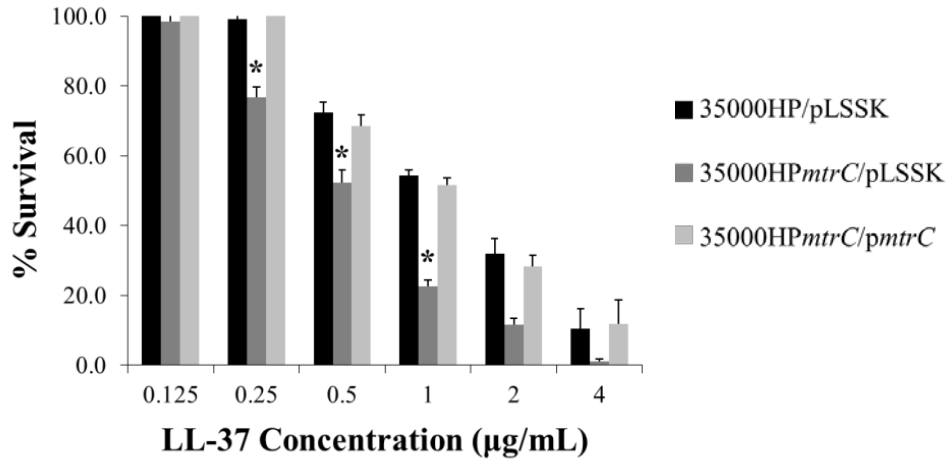
After generating the *mtrC* mutant, we next examined its susceptibility to APs. We found that 35000HP*mtrC*/pLSSK was more sensitive than 35000HP/pLSSK to both LL-37 and multiple β -defensins, but not α -defensins (Appendix Figures 3-5); complementation with *pmtrC* restored the parental resistance phenotype. Further studies revealed that a deletion in *mtrC* activated the CpxRA system (71), which also increases susceptibility to LL-37 (work done by post-doctoral fellow Sherri Rinker). We therefore next wanted to determine if the increased sensitivity to APs in the *mtrC* mutant is a result of CpxRA activation or of the loss of MtrC function. When we compared 35000HP, 35000HP*mtrC*, and 35000HP Δ *cpxA* sensitivity to LL-37 side-by-side, we observed greater cathelicidin susceptibility in 35000HP*mtrC* than in 35000HP Δ *cpxA* (Appendix

Figure 6), suggesting that loss of *mtrC* affects AP resistance in more ways than just activation of CpxRA.

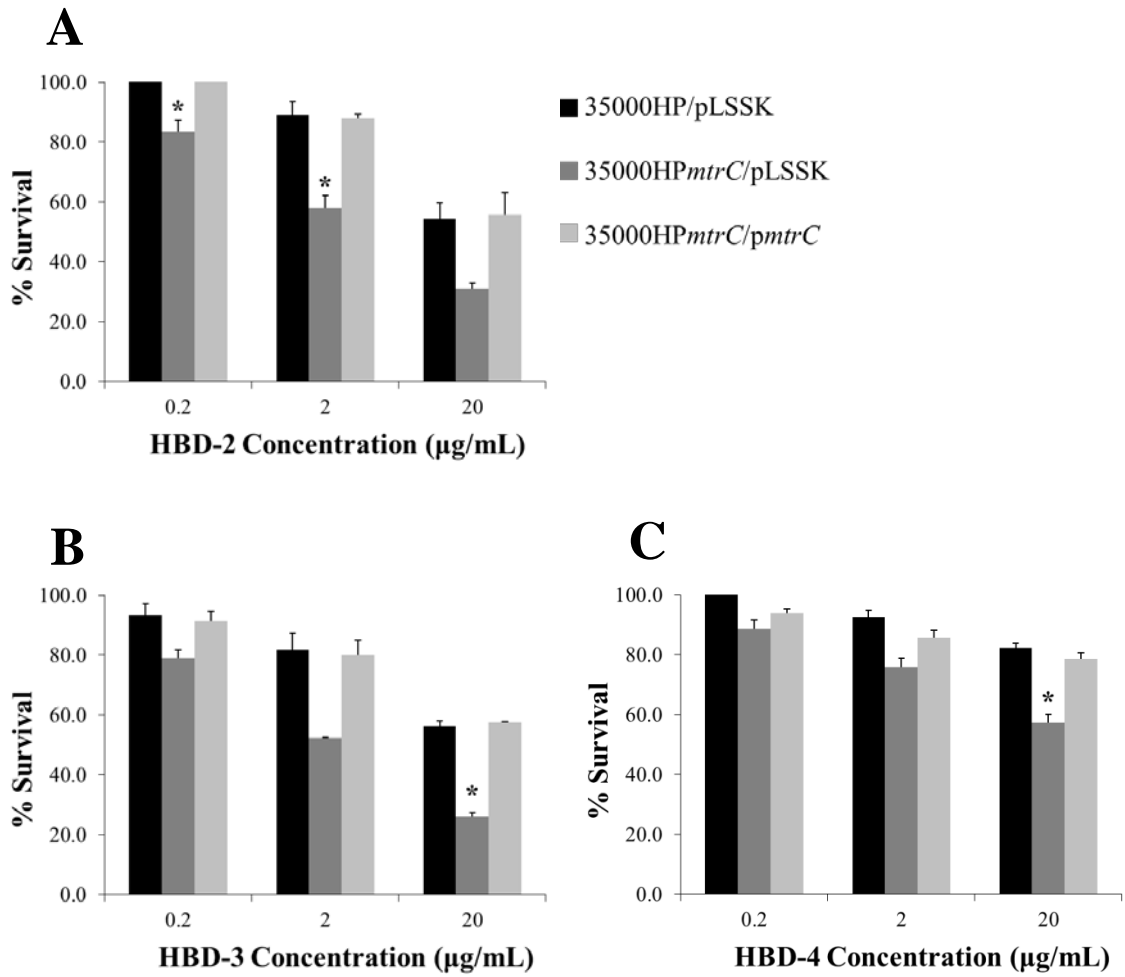
To further determine the independent contributions of CpxRA and MTR to AP resistance, we exposed 35000HP, 35000HP*mtrC*, and 35000HP Δ *cpxA* to CCCP and then examined sensitivity to both LL-37 and the β -defensin HBD-3. We found that the addition of CCCP increased 35000HP and 35000HP*cpxA* susceptibility to both LL-37 and HBD-3 but did not alter 35000HP*mtrC* susceptibility to these peptides (Appendix Figure 7). This demonstrates the presence of a PMF-dependent mechanism of AP resistance, likely MTR, still present in the *cpxA* mutant. If the decreased AP resistance phenotype seen in 35000HP*mtrC* was only because of CpxRA activation, we would not have expected to see any change in AP resistance between the *cpxA* mutant with or without CCCP. These data taken together strongly indicate that MTR and CpxRA work through separate mechanisms to confer resistance to APs.



Appendix Figure 2. *H. ducreyi* has a PMF-dependent mechanism for LL-37 and HBD-3 resistance, but not HNP-2 resistance. 35000HP was pretreated with the PMF inhibitor CCCP (1000µM), or its diluent DMSO, and then assayed for resistance to (A) LL-37 (2 µg/ml), (B) HBD-3 (10 µg/ml), or (C) HNP-2 (10 µg/ml). Asterisks indicate statistical significance between pairs shown with lines ($P < 0.05$). Data represents average \pm standard error of four independent assays, and statistical significance was determined by Student's t-test.

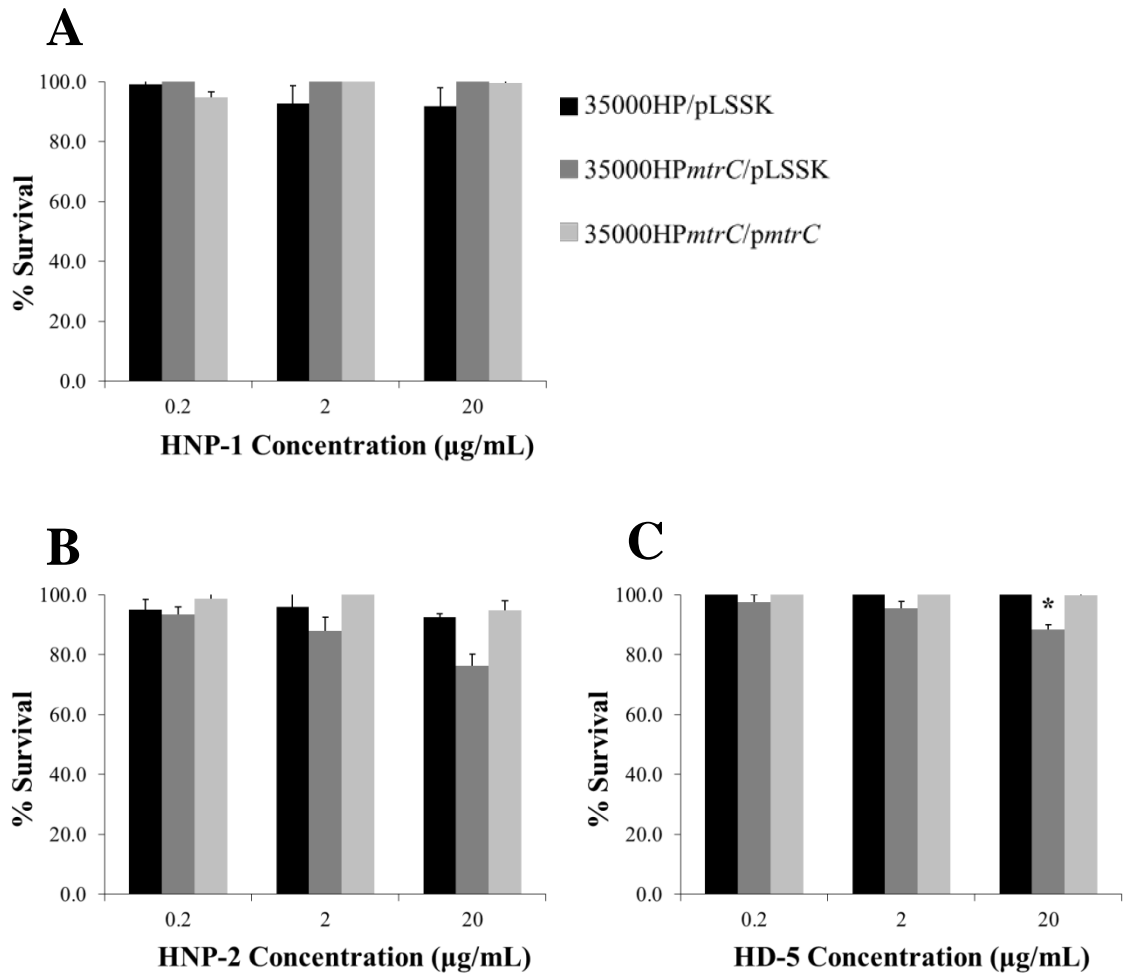


Appendix Figure 3. MTR confers resistance to the human cathelicidin LL-37 in *H. ducreyi*. 35000HP/pLSSK, 35000HPmtrC/pLSSK and 35000HPmtrC/pmtrC were examined for resistance to the cathelicidin LL-37. Asterisks indicate statistical significance from 35000HP ($P < 0.05$). Data represents average \pm standard error of five independent assays, and statistical significance was determined by Student's t-test.



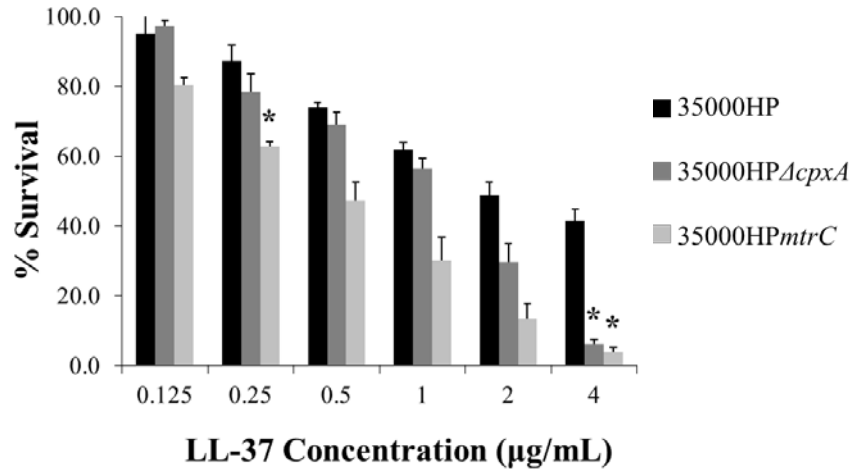
Appendix Figure 4. MTR confers resistance to β -defensins in *H. ducreyi*.

35000HP/pLSSK, 35000HPmtrC/pLSSK and 35000HPmtrC/pmtrC were examined for resistance to the β -defensins (A) HBD-2, (B) HBD-3, and (C) HBD-4. Asterisks indicate statistical significance from 35000HP ($P < 0.05$). Data represents average \pm standard error of two to five independent assays, and statistical significance was determined by Student's t-test.

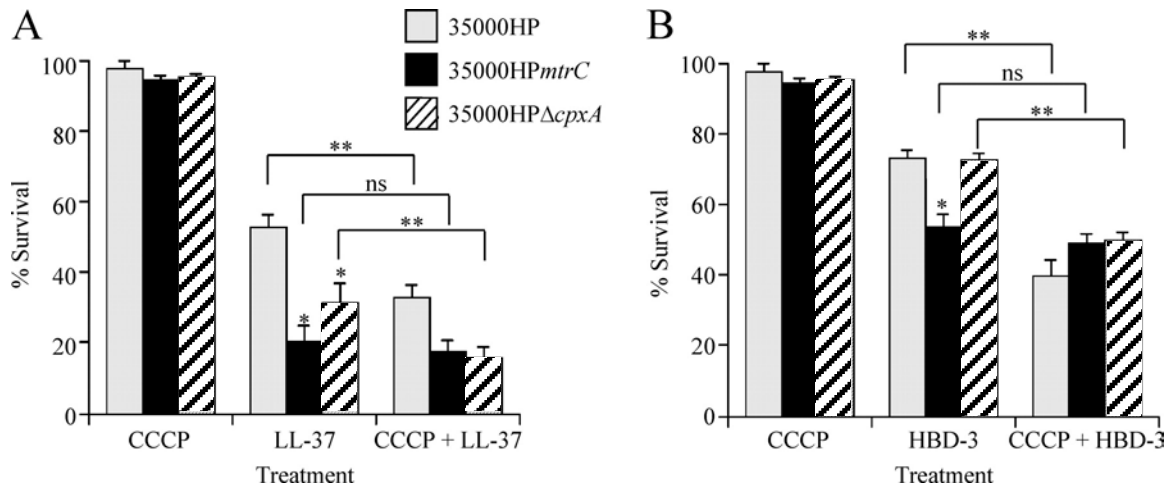


Appendix Figure 5. MTR does not confer resistance to α -defensins in *H. ducreyi*.

35000HP/pLSSK, 35000HP*mtrC*/pLSSK and 35000HP*mtrC*/*pmtrC* were examined for resistance to the α -defensins A) HNP-1, B) HNP-2 and C) HD-5. Asterisks indicate statistical significance from 35000HP ($P < 0.05$). Data represents average \pm standard error of three to five independent assays, and statistical significance was determined by Student's t-test.



Appendix Figure 6. Loss of MTR in *H. ducreyi* has a greater effect on resistance to the human cathelicidin LL-37 than just loss of *cpxA*. 35000HP, 35000HPΔ*cpxA*, and 35000HP*mtrC* were examined for resistance to the cathelicidin LL-37. Asterisks indicate statistical significance from 35000HP ($P < 0.05$). Data represents average \pm standard error of three independent assays, and statistical significance was determined by Student's t-test.



Appendix Figure 7. Proton motive force dependence of *mtrC*- and *cpxA*-mediated AP resistance. Strains 35000HP, 35000HP $mtrC$, and 35000HP $\Delta cpxA$ were pretreated with DMSO alone or CCCP in DMSO and challenged with (A) LL-37 (2 μ g/ml) or (B) HBD-3 (10 μ g/ml). Asterisks indicate statistical significance between pairs shown with a bracket ($P < 0.05$). Data represents average \pm standard error of six independent assays, and statistical significance was determined by Student's t-test.

Part 3: *H. ducreyi* Whole Genome Sequencing

After construction of 35000HP Δ PEAT, we confirmed by whole genome sequencing that the genomes of 35000HP and 35000HP Δ PEAT were identical with the exception of the three putative PEA transferase genes. While there was no difference between the two strains, we did observe differences between 35000HP and the published *H. ducreyi* genome sequence. Although bacteria can develop random mutations, the 35000HP strain used is taken fresh from a freezer stock to avoid extended passaging and increased chance of mutation. While it remains unclear if these observed differences happen to be isolated to one specific colony or perhaps errors in sequencing, there alterations seen in six genes, four intergenic regions, and one t-RNA transcript code, with the changes shown in Appendix Table 1.

The six genes with varying sequences were HD0110 (Mu-like prophage FluMu protein), HD0264 (malate dehydrogenase), HD0885 (glycosyl transferase), HD1455 (nicotinamide phosphoribosyl transferase), and HD1449 and HD1973 (both hypothetical proteins). The difference in HD0110 was a single base pair change that resulted in a proline becoming a glutamine. The differences in HD0264 included four separate insertions that all resulted in stop codons and truncation of the protein. Instead of the normal 324 amino acid protein, the variants contained a protein either 69, 75, or 79 amino acids in length. The differences in HD0885, HD1449, and HD1455 were all single base pair changes that resulted in a valine becoming an alanine, a leucine becoming a proline, and a phenylalanine becoming a cysteine, respectively. Lastly, the differences in HD1973 were insertions that either eliminated the open reading frame of the protein or inserted a glycine-leucine-tyrosine amino acid chain. While it is hard to predict how the

elimination of the open reading frame in the hypothetical protein HD1973 or how the single amino acid changes affect protein structure or function, the truncation found in malate dehydrogenase (HD0264) could have an effect on growth or metabolism. Malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate and functions as a part of the carbohydrate metabolic pathways. It is unclear if there is a significant difference in growth or virulence between 35000HP and the published strain or if this mutation has any effect on cellular metabolism, although this is unlikely given the consistency of 35000HP growth in the human model of *H. ducreyi* infection.

The four intergenic regions with varying sequences were pre-HD0001 (glucose inhibited division protein A), pre-HD0887 (nucleoside transport), pre-HD1968 (50S Ribosomal protein L14), and pre-HD1448 (unknown hypothetical protein). The mutations before HD0001 were both single base pair changes and insertions, all occurring between 287-295 nucleotides before HD0001. The differences before HD0887 and HD1448 were a single base pair change or a single base to double base change respectively, occurring 89 and 68 nucleotides before the start of the genes. The differences found before HD1968 included single base pair changes, base pair deletions, or small insertions, all of which occurred between 90-92 nucleotides before the gene. Lastly, the t-RNA transcript code with a single base pair change was tRNA-Asp-2; however no open reading frame was detected in either the published sequence or 35000HP. It is unclear if any of these alterations have an effect on gene expression or protein function.

If a bacterial strain is passaged numerous times, it would not be uncommon for its genome to change over time; however, 35000HP is not passaged, so these results were

somewhat unexpected. To confirm the differences, the eleven gene or intergenic sequences could be PCR amplified and sequenced; this would likely be more accurate and efficient than sequencing the entire genome again. Should these differences still exist, for example in malate dehydrogenase, additional testing could be done to search for phenotypic differences. Functional assays could determine if growth or metabolism has changed between 35000HP and the published strain, and mutagenesis could determine if the gene products are even necessary for growth and virulence. While it is unclear which sequence is considered more accurate, this does show that the *H. ducreyi* genome remains very stable.

Appendix Table 1. Variations between 35000HP and published *H. ducreyi* genomic sequence.

POS^a	Gene ID	REF^b	ALT^c	Result^d
1	Pre-HD0001 (glucose inhibited division protein A)	A	T	Single bp change
2	Pre-HD0001	C	T	Single bp change
3	Pre-HD0001	C	A	Single bp change
4	Pre-HD0001	G	T	Single bp change
4	Pre-HD0001	GTTTA	GTGGATAACTTTACCG TTTA,GATAACTTTACC GTTTA,GGTGGATAACT TTACCGTTTA	Insertion
5	Pre-HD0001	TT	TTAACTTTACCGTT,TG GATAACTTTACCGTT	Insertion
6	Pre-HD0001	T	TAACTTTACCGTT,TAC CGTT	Insertion
7	Pre-HD0001	TA	TACTTTACCGTTTA	Insertion
8	Pre-HD0001	A	ACCGTTTA	Insertion
81481	HD0109 (hypothetical)	C	A	Silent Mutation
81481	HD0110 (Mu-like prophage)	C	A	P to Q
199946	HD0264 (malate dehydrogenase)	A	ATGCGGGT	Truncation
199947	HD0264	A	AGGGTG	Truncation
199948	HD0264	A	ACGGGTGA	Truncation
199954	HD0264	CG	CTAATTTAG	Truncation
708646	HD0885 (glycosyl transferase)	T	C	V to A
710026	Pre-HD0887 (nucleoside transport)	G	T	Single bp change
118593 8	Pre-HD1448 (hypothetical)	C	CA	Insertion
118606 7	HD1449 (hypothetical)	T	C	L to P
119069 5	HD1455 (nicotinamide)	T	G	F to C

phosphoribosyl transferase)				
164535 3	Pre-HD1968 (50S Ribosomal protein)	GGT	GAAGCAAAAAGT,GAA GT,G	Insertion
164535 5	Pre-HD1968	T	C	Single bp change
164612 0	HD1973 (hypothetical)	CC	CGGATTGTAC,CCGGAT TGTAC	Eliminates ORF, GLY insertion
169894 8	n/a	ATAAC TTT	ATAACTTTACCGTTTAA CTTT	Insertion
169895 0	n/a	AACTT T	AACTTTACTTT	Insertion
169895 1	n/a	ACTTT	ACTTTACCCTTT,ACTTT ACCGTCTTT,ACTTTACC GTTTATCTTT	Insertion

^a Location of mutation on *H. ducreyi* chromosome

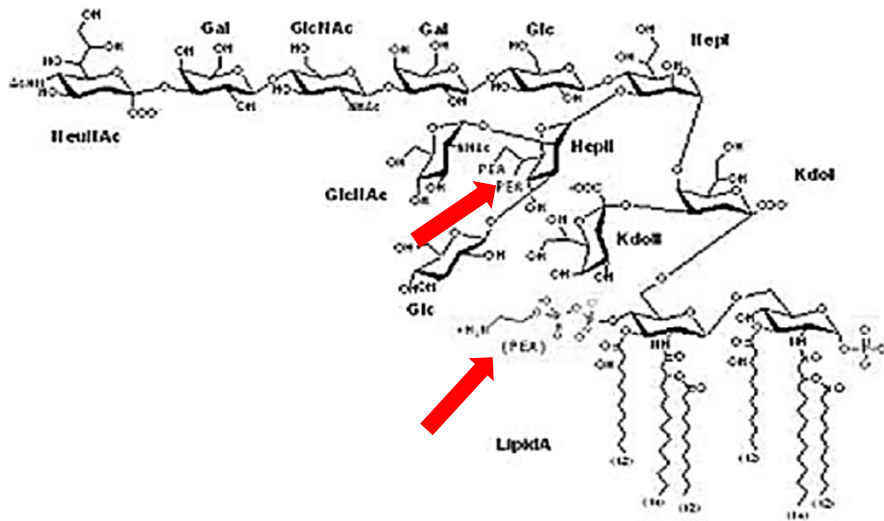
^b Published *H. ducreyi* sequence

^c 35000HP genome sequence

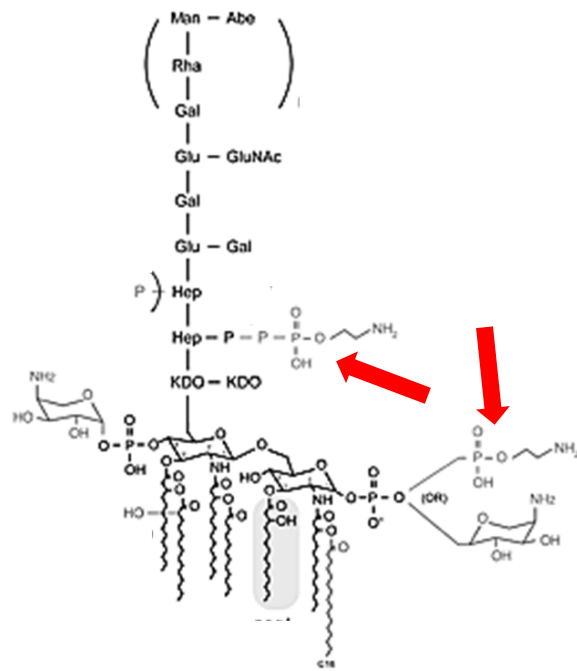
^d Result; expected impact mutation has on gene

Part 4: Bacterial LOS, LPS Structures

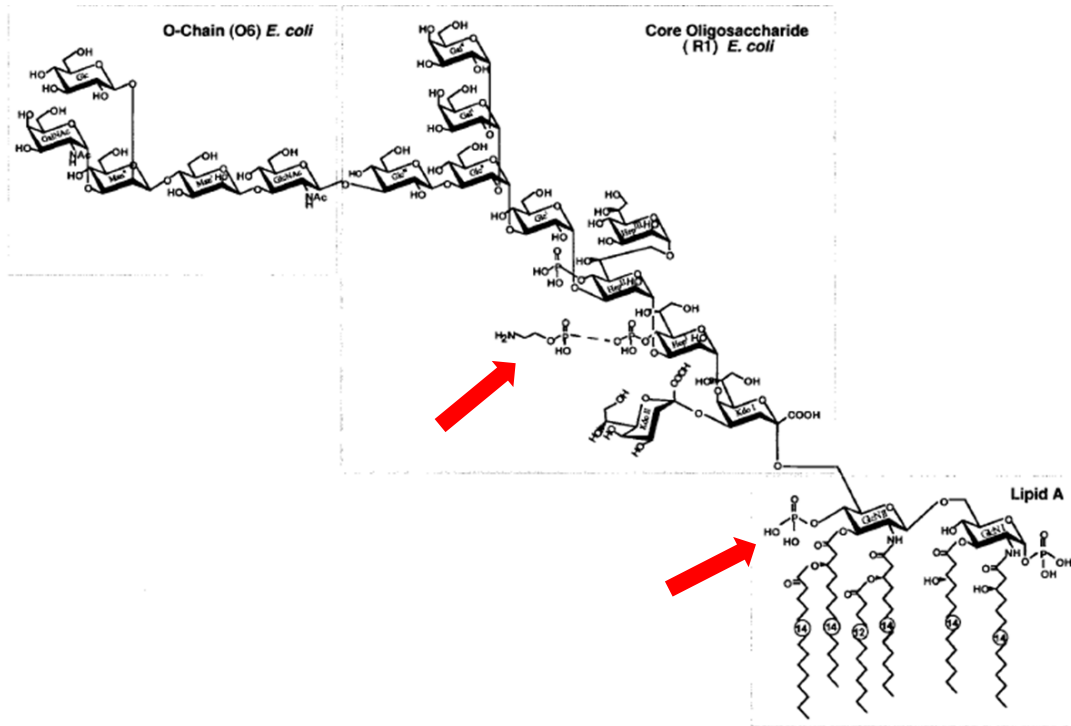
The three *H. ducreyi* genes of interest in this study, *ptdA*, *lptA*, and *ptdB*, share strong homology with PEA transferases found in other bacteria, including *N. gonorrhoeae*, *N. meningitidis*, *S. enterica*, and *E. coli*. Here, we show the structures of the *Neisseria* LOS (Appendix Figure 8), *Salmonella* LPS (Appendix Figure 9), and *E. coli* LPS (Appendix Figure 10).



Appendix Figure 8. *Neisseria* LOS. Depiction of the structure of *Neisseria* LOS (196). PEA modifications are indicated by the red arrows and occur on the Hep II core sugar on carbons 3 and 6 as well as on the lipid A.



Appendix Figure 9. *Salmonella* LPS. Depiction of *Salmonella* LPS (197). PEA modifications are indicated by the red arrows and occur on the Hep II core sugar as well as on the lipid A.



Appendix Figure 10. *E. coli* LPS. Depiction of *E. coli* LOS (198). PEA modifications are indicated by the red arrows and occur on the KDO core sugar as well as on the lipid A (PEA modification on the lipid A is not shown in this figure, but the modification can occur where the arrow indicates).

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CURRICULUM VITAE

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Education

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Doctor of Philosophy in Microbiology and Immunology, Minor in Life Sciences

Butler University, Indianapolis, IN, May 2009
Bachelor of Science in Biology, Minor in Chemistry

Honors, Awards, Fellowships

Indiana University
Indiana University School of Medicine Travel Fellowship Award
IUPUI Graduate School Travel Fellowship Award
American Society for Microbiology Travel Award
Indiana University Immunology and Infectious Disease T32 Training Program Grant, NIH AI060519 (Appointed July 2011, reappointed July 2012 and July 2013)
Phi Kappa Psi District Academic and Service Scholarship

Butler University
Butler University Robert C. Karn Award
Butler University Academic Scholarship
Elise S. Turner Biology Departmental Scholarship
Mercy Health Partners Academic Scholarship
American Legion Leadership Scholarship
Phi Kappa Psi Academic Scholarship

Professional Experience

Instructor of Biological Sciences, Butler University, August 2014 – present
Taught courses for ~160 undergraduate students
Fundamentals of Genetics: 4 sections of lecture and lab
Principals of Microbiology: 2 sections of lab
Biology and Society: 1 section of lecture and lab
Academic advisor for 10 undergraduate Biology students
Teaching Assistant, Indiana University School of Nursing, January 2012 – May 2012
Taught Microbiology Laboratory section for 35 first-year Nursing students

Tutor, Butler University, Biology and Physics Departments, August 2008 – May 2009

Employed as private tutor through the Learning Resource Center for 4 students

Lab Assistant, Butler University Biology Department, August 2008 – May 2009

Employed to assist professors in lab set-up, administer exams, and aid ~100 students total in 4 lab sections

Butler Summer Institute, Butler University, May 2008 – August 2008

Undergraduate independent research summer internship

Publications

Michael P. Trombley, Deborah M. B. Post, Sherri D. Rinker, Lorri M. Reinders, Kate R. Fortney, Beth W. Zwickl, Diane M. Janowicz, Barry P. Katz, Stanley M. Spinola, and Margaret E. Bauer. *Phosphoethanolamine Transferase LptA in Haemophilus ducreyi Modifies Lipid A and Contribute to Human Defensin Resistance in vitro*. PLOS One. In press.

Rinker, S.D., M.P.Trombley, X. Gu, K.R. Fortney, and M.E.Bauer. *Deletion of mtrC in Haemophilus ducreyi increases sensitivity to human antimicrobial peptides and activates the CpxRA regulon*. Infect. Immun. June 2011

Conferences Attended

Michael P. Trombley, Sherri D. Rinker, Deborah M. B. Post, Bradford W. Gibson, Margaret E. Bauer. *Haemophilus ducreyi Resists Human Defensins by Electrostatic Repulsion*. American Society for Microbiology General Conference. May 2014, Boston, MA.

Michael P. Trombley, Sherri D. Rinker, Deborah M. B. Post, Bradford W. Gibson, Margaret E. Bauer. *Haemophilus ducreyi Resists Human Defensins by Electrostatic Repulsion*. Midwest Microbial Pathogenesis Conference. August 2013, Columbus, OH.

SD Rinker, MP Trombley, KR Fortney, BW Zwickl, BP Katz, DM Janowicz, SM Spinola, ME Bauer. *Permease Proteins of the Sap Transporter are Required for Virulence in Haemophilus ducreyi in Humans*. Midwest Microbial Pathogenesis Conference. September 2012, Wisconsin, MI.

SD Rinker, MP Trombley, KR Fortney, BW Zwickl, BP Katz, DM Janowicz, SM Spinola, ME Bauer. *Permease Proteins of the Sap Transporter are Required for Virulence in Haemophilus ducreyi in Humans*. American Society for Microbiology General Conference. June 2012, San Francisco, CA.

Michael P. Trombley. *Innate Immune Resistance in Haemophilus ducreyi*, Invited Guest Speaker. Butler University, April 2012

Sherri D. Rinker, Michael P. Trombley, Xiaoping Gu, Kate R. Fortney, Margaret E. Bauer. *Deletion of MtrC Increases Susceptibility to Human APs and Activated the Cpx Regulon in H. ducreyi*. Midwest Microbial Pathogenesis Conference. September 2010, St. Louis, MO.

Michael P. Trombley. *Auxin Concentrations in Foliar Embryo of Kalanchoe pinnata during Leaf Development and Leaf Detachment via Agrobacterium Transformation and DR5:GUS Analysis*. Undergraduate Research Conference. Butler University, March 2009

Michael P. Trombley, Philip Villani. *Auxin Concentrations in Foliar Embryo of Kalanchoe pinnata during Leaf Development and Leaf Detachment via Agrobacterium Transformation and DR5:GUS Analysis*. Butler Summer Institute Poster Session. Butler University, August 2008