THE *HAEMOPHILUS DUCREYI* SAP TRANSPORTER CONTRIBUTES TO ANTIMICROBIAL PEPTIDE RESISTANCE

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ABSTRACT

Kristy Lee Beavers Mount

THE *HAEMOPHILUS DUCREYI* SAP TRANSPORTER CONTRIBUTES TO ANTIMICROBIAL PEPTIDE RESISTANCE

Haemophilus ducreyi is the causative agent of the genital ulcer disease chancroid, which has been shown to facilitate the transmission of HIV. *H. ducreyi* is likely exposed to multiple sources of antimicrobial peptides *in vivo*. APs are small, cationic molecules with both bactericidal and immunomodulatory functions. Because *H. ducreyi* is able to establish and maintain an infection in an environment rich with antimicrobial peptides, we hypothesized that the bacterium was resistant to the bactericidal effects of these peptides. Using a 96-well AP bactericidal assay, we examined *H. ducreyi* susceptibility to eight human APs likely to be encountered at the site of infection, including the α-defensins human neutrophil peptide-1, human neutrophil peptide-2, human neutrophil peptide-3, and human defensin 5, the β-defensins human β defensin-2, human beta defensin-3, and human beta defensin-4, and the human cathelicidin, LL-37. *H. ducreyi* survival was compared to the survival of *Escherichia coli* ML35, a strain known to be susceptible to several antimicrobial peptides. *H. ducreyi* was significantly more resistant than *E. coli* ML35 to the bactericidal effects of all peptides tested. Furthermore, we found that representative class I and class II strains of *H. ducreyi* were each resistant to APs of each functional category, indicating that resistance to antimicrobial peptides could

represent a conserved method of pathogenesis for *H. ducreyi* as a species. The *H. ducreyi* genome contains a homolog for the Sap influx transporter. To study the role of the *H. ducreyi* Sap transporter in AP resistance, we generated an isogenic *sapA* mutant and used the 96-well AP bactericidal assay to compare the AP susceptibility profiles of wild-type *H. ducreyi,* the *sapA* mutant and the *sapA* trans-complement to α-defensins, β-defensins, and LL-37. We observed a 25% decrease in the survival of the *sapA* mutant when it was exposed to LL-37. These findings suggest that the *H. ducreyi* Sap transporter plays a role in *H. ducreyi* resistance to LL-37, but it is likely that other AP resistance mechanisms co-exist within the bacterium.

Margaret E. Bauer, PhD

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LIST OF ABBREVIATIONS

AP……..……..Antimicrobial Peptide
BLAST…….Basic Local Alignment Search Tool
CCR5…….Chemokine (C-C motif) Receptor 5
CCR6…….Chemokine (C-C motif) Receptor 6
CD4……Cluster of Differentiation 4
CD27……....Cluster of Differentiation 27
CD45……Cluster of Differentiation 45
CD8……...….Cluster of Differentiation 8
CDC……..Centers for Disease Control
CDT……..Cytolethal Distending Toxin
CFU……..…….……Colony Forming Units
CGE……Crude Granulocyte Extract
CpxA……...…….Two component sensor protein
CpxR……....Two component regulator protein
Cu,Zn SOD……....Copper, Zinc Superoxide Dismutase
CXCR4……...……..CXC Chemokine Receptor 4
Dig……. Digoxigenin
DltA…….*H. ducreyi* lectin A
DNA……...……Deoxyribonucleic acid
Dpp……..…Dipeptide Transporter
DsrA……..….*H. ducreyi* serum resistance A
DTH…… Delayed-Type Hypersensitivity Response
ECA……...…..Enterobacterial Common Antigen
ECM……..….Extracellular Matrix
EDD…….Estimated Delivered Dose
EIA……...Enzyme Immunoassays
ECM……Extracellular Matrix Proteins
ELISA……..Enzyme Linked Immunosorbant Assay
FgbA……..……Fibrinogen Binder A

FhaB……..……..……..Filamentous Hemagglutinin

Flp……..Fimbria-like protein

FtpA…….……Fine Tangled Pilus A

FPRL1…….….Low affinity receptor to N-formyl-methionyl peptides

G2…….Growth Phase 2

gmhA……. Phosphoheptose isomerase

GUD…..……..Genital Ulcer Disease

HBD-1…….…...….Human Beta Defensin-1

HBD-2…….…….....Human Beta Defensin-2

HBD-3…….Human Beta Defensin-3

HBD-4…….…...…….Human Beta Defensin-4

HD-5……..……..Human Defensin-5

HD-6……...….Human Defensin-6

HFF……..…Human Foreskin Fibroblasts

HgbA……..Hemoglobin Receptor A

hhdAB……...Gene cluster encoding the *H. ducreyi* hemolysin

HIV-1…….…...Human Immunodeficiency Virus Type 1

HIV……...……Human Immunodeficiency Virus Type 1

HL-60 ……..Human Promyelocytic Leukemia Cell Line

HLA-DR…….….Major Histocompatability Complex (MHC II) marker

Hlp……....*H. ducreyi* Lipoprotein

HNP-1…….…..…Human Neutrophil Peptide-1

HNP-2……..……Human Neutrophil Peptide-2

HNP-3……..……Human Neutrophil Peptide-3

IgG……..……..Immunoglobulin G

IgM……...……..Immunoglobulin M

IL-2……Interleukin-2

IL-4……Interleukin-4

IL-5……Interleukin-5

IL-6……Interleukin-6

IL-8……Interleukin-8

INF-γ…….……Interferon gamma

J774A.1 ……Murine Macrophage Cell Line

LbgB……..… Lipooligosaccharide D-glycero-D-manno-heptosyltransferase

LgtF…….……..… UDP-glucose-lipooligosaccharide beta 1-4 glucosyltransferase

LL-37…….Human cathelicidin

LOS……...Lipooligosaccharide

LosB……..LbgB

LPS…….Lipopolysaccharide

LspA1……Large Supernatant Protein 1

LspA2……Large Supernatant Protein 2

LTA…….....Lipotechoic acid

M-PCR……..……Multiplex Polymerase Chain Reaction

MAb……...Molecular Antibody

MDR……....Multidrug Resistance

MEC……Minimum Effective Concentration

MOMP…….Major Outer Membrane Protein

MprF……...lysylphosphatidylglycerol synthetase

mRNA…….Messenger RNA

Mtr……..…..Multiple Transferable Resistance

NcaA…….…….Necessary for Collagen Adhesion Protein A

NP-1…….Rabbit Neutrophil Peptide 1

NTHI…….….Nontypeable *Haemophilus influenzae*

OD…….Optical Density

OM…….Otitis Media

OMP…...……Outer Membrane Protein

OmpA……..……Major Outer Membrane Protein Homolog

Opp……Oligopeptide Transporter

ORF…….……Open Reading Frame

^{32}P……...Radioactive Phosphorus

PAL……..Peptidoglycan Associated Lipoprotein

PBMC…….Peripheral Blood Mononuclear Cells

PCR……..…….Polymerase Chain Reaction

PG-1…….Protegrin-1

PgtE……...……..…..Outer membrane Protease E

PhoP……..……DNA binding transcriptional regulator

PhoQ……..…….Sensor protein

PIA…….….Polysaccharide Intercellular Adhesin

PMN……Polymorphonuclear Leukocytes

Pmr…… Phosphoethanolamine transferase

QacA……...Multidrug efflux protein

qRT-PCR……..Quantitative Reverse Transcriptase Polymerase Chain Reaction

r-cBD-1……...…….Recombinant-Chinchilla Beta Defensin 1

RDA…….……Radial Diffusion Assay

RfaF…… LPS heptosyltransferase II

RfaK……...Hexose Transferase

RNA……...Ribonucleic Acid

rRNA……...……Ribosomal RNA

RT…….……..Reverse Transcriptase

SAP……..…….Sensitive to Antimicrobial Peptides

SCOTS……..….Selective Capture Of Transcribed Sequences

SOD…….…..Superoxide Dismutase

SpoOK…….….*B. subtilis* Response Regulator

STD……..……..Sexually Transmitted Disease

STE6…….. *Saccharomyces cerevisiae* ABC transporter

tad…….Tight Adhesion

TdhA…….……...Heme Receptor

TdX……..…….Uncharacterized Hypothetical Protein

TLR…….….Toll Like Receptor

TNF-α……Tumor Necrosis Factor Alpha

TonB……..……...Energy Transducing Protein B

TrkG……..…Potassium transporter subunit

TrkH……..……Potassium transporter subunit

U-937…….. Human Leukemic Monocyte Lymphoma Cell Line

UNAIDS……United Nations Acquired Immunodeficiency Syndrome Foundation

WecA……..Undecaprenyl-phosphate alpha-N-acetylglucosaminyltransferase

X-gal…….....5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside

LITERATURE REVIEW
SECTION I: *HAEMOPHILUS DUCREYI*

History of Chancroid

Haemophilus ducreyi is a Gram negative bacterium that causes chancroid, a genital ulcer disease (GUD) that is endemic in areas of Africa, Southeast Asia, and Latin America (219). Leon Bassereau was the first physician credited with making a clinical distinction between the chancroid ulcer and the chancre of syphilis (19, 135). In 1889, Augusto Ducrey was able to isolate the causative agent of chancroid through a series of repetitive forearm autoinoculation experiments and the organism was subsequently named in his honor (58, 135). In 1900, Bezancon et al. used culture-purified bacteria to inoculate the forearms of volunteers who later developed soft chancre at the site of inoculation (32, 135). This experiment fulfilled Koch's postulates and *H. ducreyi* was formally defined as the causative agent of chancroid when the bacteria was re-isolated from the lesions (32, 135).

Taxonomy and Genetics of *H. ducreyi*

H. ducreyi is limited in its ability to aquire heme and is only capable of taking up an exogenous porphyrin ring containing source of heme to meet its basic requirements for growth (89, 159). In addition, it has been shown to reduce nitrate and to produce alkaline phosphatase (159). These biochemical characteristics, among others, led researchers to originally classify the organism as a *Haemophilus* species (114). However, after a series of comparative genetic analyses, it was found that the bacterium did not closely resemble a true *Haemophilus* species (55, 56, 159). The bacterium was subsequently re-classified as an *Actinobacillus* (55, 56, 159, 219). A comparative analysis of the competence loci, DNA signal sequences, and 16S RNA of the members of the *Pasteurellaceae* family indicated that *Mannheimia haemolytica, Actinobacillus pleuropneumoniae,* and *H. ducreyi* form a separate genetic lineage apart from the other members of this family (80).

The *H. ducreyi* genome is composed of a single circular 1.7 Mb chromosome and the organism can maintain plasmids (159, 162, 219). An annotated sequence of *H. ducreyi* 35000HP was published in 2003 at www.ncbi.nih.gov. Nineteen *H. ducreyi* clinical isolates were analyzed, and based on observed differences in lipooligosaccharide (LOS) migration patterns and outer membrane protein (OMP) profiles, the strains were grouped into two distinct classes (23, 193, 247). The specific differences between these classes will be described in detail later in this manuscript.

Clinical Features of Chancroid

H. ducreyi likely gains access to the skin through small abrasions caused by the friction of sexual intercourse (135, 159, 219). Small red papules form within a day of entry, but are generally not noticed by patients (135, 159, 219). These papules develop into pustules within two to three days and can go on to ulcerate within seven to fourteen days (135, 159, 219). The painful chancroid ulcers are typically soft, with ragged edges (135, 159, 219). The ulcer base is often covered by a necrotic, purulent exudate that bleeds easily (135, 159, 219). In many cases, the infection progresses to include regional lymphadenopathy and bubo formation (135, 159, 219). Lesions are typically localized to mucosal surfaces or to the stratified squamous epithelium (135, 219). Patients in economically disadvantaged areas, where chancroid is endemic, often seek medical attention only after persistent ulceration (90, 159, 219). *H. ducreyi* grows best between 33°C and 35°C and it is likely that the temperature sensitivity of the organism prevents *H. ducreyi* from spreading throughout the body (135, 227). However, extra-genital lesions can occur due to autoinoculation and in rare cases, *H. ducreyi* can cause infection in the extremities and throat (36, 135, 237).

Diagnosis and Treatment of Chancroid

H. ducreyi is a gram-negative bacillus that auto-aggregates (135, 227). When grown on agar, the organism clumps and colonies can be pushed across the agar surface intact (135, 227). Microscopically, *H. ducreyi* assumes characteristic chaining patterns such as

"railroad tracks," "schools of fish," and "fingerprints" (135, 159). The "railroad track" conformation is often observed after growth in liquid broth, while the "schools of fish" and "fingerprint" conformations are often observed after growth on agar or in specimens isolated from tissue (159). These morphological features, while distinctive, do not allow for the accurate diagnosis of chancroid (135). Nonetheless, culture for *H. ducreyi* remains the main diagnostic method available to most clinical laboratories as it is significantly more cost effective than alternative methods (135). A combination of gonococcal (GC) broth and Mueller-Hinton media are optimal for *H. ducreyi* isolation (135, 227). IsoVitaleX, hemoglobin, and fetal bovine serum or starch can be successfully used for supplementation of growth in culture (135, 159, 227). Vancomycin (3 μg/ml) is often added to clinical cultures to suppress contamination by Gram-positive commensal bacteria (112, 135). Most *H. ducreyi* strains grow well at 33°C in a humid atmosphere containing 5% CO_2 (135, 227). However, due to the fastidious nature of the organism, culture based diagnostic tests are not very sensitive (135). Furthermore, significant difficulties have been encountered in culturing class II *H. ducreyi* strains, and consequently, it is likely that these strains are underrepresented among clinical isolates (160, 247).

While significantly more costly, PCR-based techniques have improved the accuracy of clinical diagnosis (31, 135, 228). The multiplex PCR (M-PCR) system was designed to amplify the DNA of multiple STD pathogens simultaneously (31, 135, 176) and was later modified to include quantitative real time PCR methodology for higher diagnostic accuracy (228). This technology provides clinicians with the capability to distinguish between infections caused by *H. ducreyi, Treponema pallidum,* and herpes simplex virus and is currently regarded as the best diagnostic option available (228). However, due to the prohibitive cost of refrigeration and equipment, and because the test was never made commercially available, it is unfeasible to conduct such an analysis in the poor communities where chancroid is endemic (135). Several serology based techniques have been developed as a means for diagnosing chancroid (135). Unfortunately, each of these strategies has met with limited success, likely due to the lack of an early antibody response to *H. ducreyi* infection (135, 180, 219).

Several antibiotic classes have been shown to exhibit efficacy against *H. ducreyi,* including the macrolides, aminoglycosides, quinolones, and cephalosporins (28, 159, 222). The current recommendation for chancroid treatment is one gram of azithromycin, taken one time, and delivered orally or 500mg ciprofloxacin twice daily for three days or a single 250 mg dose of ceftriaxone delivered intramuscularly (15, 222). The ease of a single dose drug treatment regiment increases patient compliance and could help to prevent the spread of antibiotic resistance strains (104, 135). *H. ducreyi* has the ability to maintain plasmid DNA, and as such, isolates have been reported to encode plasmid-mediated resistance to ampicillin, chloramphenicol, tetracycline, aminoglycosides, sulfonamides and trimethoprim (53, 104, 159). Chromosomal mutations resulting in trimethoprim resistance have also been reported (159). As with all newly emerging antibiotic resistance scenarios, the increased prevalence of *H. ducreyi* strains containing antibiotic resistance genes is of great concern due to a dwindling supply of usable antibiotics and a shortage of new drugs in the developmental pipeline (135, 159).

Epidemiology of Chancroid

As described by Steen et al., chancroid is a disease whose cause can be partially related to economic prosperity and opportunity (222). At one time, chancroid was prevalent is most parts of the world (222). As nations became industrialized, a greater proportion of the population moved into to urban areas (222). However, because economic opportunities were initially more abundant for men than women, some women were forced into temporarily trading sex for goods (222). Prostitution plays a significant role in *H. ducreyi* transmission (159, 219, 222) and is important to the basic epidemiology of the organism (159, 222). As economic opportunities for women increased, the prevalence of chancroid in a given geographic area was reduced (222). These changes in socio-economic conditions, coupled with the advent of antibiotic therapy, led to the near elimination of chancroid from the western world (222). However, chancroid remains endemic in many of the poorest regions of the world, as these regions are now undergoing their own process of industrialization similar to what was previously seen in the Western world (222). Because *H. ducreyi* is a strict human pathogen with no animal

reservoir, and because the economic health of a region is so closely coupled with the transmission of the disease, chancroid is a viable candidate for global eradication (222).

In experimental infection, men developed pustules at rates two fold higher than women (33, 219). Although the exact cause of this disparity has not been consclusively determined, these finding correspond with observations of natural infection, where chancroid infections rates are approximately three times higher in men than in women in endemic regions, and can be as much as 25 times higher in localized outbreaks (90, 135, 219). The transmission rate of chancroid is high (192, 219), a fact that illustrates the importance of using a proper barrier method of protection during sexual intercourse. Male circumcision has been shown to be a highly effective method of reducing the transmission rate of many sexually transmitted diseases, including chancroid (158, 219).

H. ducreyi and HIV

In recent years, *H. ducreyi* has become an organism of considerable interest to researchers because it can co-facilitate the transmission of the human immunodeficiency virus type 1 (HIV) (69, 109, 157, 245). Patients with active GUD have a much greater chance of contracting and spreading HIV (201, 219), and thus contribute to the to the HIV epidemic (201, 219). However, while GUD in general can contribute to HIV acquisition, *H. ducreyi* specifically increases the transmission rate of HIV in several ways (157, 201, 219). At the most basic level, the chancroidal ulcer provides a gateway for the virus to enter the skin (157, 201, 219). Upon entry, the virus encounters CD4+ T-cells and macrophages within the ulcer. In response to infection with *H. ducreyi,* these cells express increased levels of the HIV co-receptors CCR5 and CXCR4 (101, 219). In addition, *H. ducreyi* promotes viral shedding from the chancroidal ulcer and increases the viral load in the blood and semen, making transmission to another person all the more likely (157, 219, 245). Fortunately, the increased HIV viral load associated with chancroid co-infection is associated only with the ulcerative stage of disease, indicating that early treatment of chancroid could help to reduce HIV transmission (107). However, experiments examining the exact molecular mechanisms underlying the interactions of

HIV and *H. ducreyi* should be designed with caution, as unusual and severe clinical pathology has been described in immunocompromised HIV patients infected with *H. ducreyi* (36).

Because of the difficulty associated with developing a HIV vaccine (8), there is considerable interest in attempting to reduce HIV transmission by generating a vaccine against *H. ducreyi* (49). In order for this strategy to be successful, an antibody response must be mounted against the *H. ducreyi* vaccine that results in the production of immunological memory (49, 180). Several *H. ducreyi* proteins have been evaluated with the goal of identifying factors that are immunogenic (5, 16, 60, 62, 121, 126).

Models of Chancroid Infection

In order to study the contribution of individual genes on pathogenesis, it was necessary to develop *in vivo* chancroid models. One of the first attempts to develop an animal model of chancroid resulted in the injection of *H. ducreyi* under the skin of mice (44, 135, 235). While ulceration occurred in this model, it was later demonstrated that the lesions were not caused by live bacteria, but instead, as the result of the inflammatory response stimulated by *H. ducreyi* LOS (44, 135).

Initial experiments with rabbits were unsuccessful because the bacteria failed to replicate at normal skin temperature (44, 135). However, when the rabbits where housed at temperatures of 15 to 17°C, the bacteria survived and produced dermal lesions that persisted for up to two weeks before resolving (135, 198). In comparison, chancroidal lesions in humans persist for up to three months (134, 219), indicating that the kinetics of disease progression vary between humans and rabbits. In addition, a large inoculum was required to initiate infection (198). $1x10^5$ CFU were used to inoculate the rabbits (198, 219), while in contrast, only 1-100 CFU are needed to initiate infection in humans (10, 218). The difference in the size of the inoculum necessary to initiate infection demonstrates that the pathogenic efficiency of the bacterium is reduced in rabbits compared to humans (219).

In the macaque model, $1x10^7$ - $2x10^8$ bacteria were injected into the genital epithelium of primates (135, 136, 219, 233). The male primates developed lesions within one to two days of infection and ulcers reminiscent of human chancroid developed within two weeks (135, 136, 219, 233). However, female macaques failed to develop chancroid ulcers (135, 136, 219, 233). This contrasts human disease which manifests in both males and females, albeit at lower rates in females.

Finally, in the swine model, bacteria were inoculated onto the ears of pigs, using an allergy testing device (100). Papules were apparent within two days of inoculation and pustules formed by day seven (100). Ulcerations were visible by 14 days of infection (100). However, while the histopathology of the swine model mimicked that of human infections, a larger inoculum was required to initiate infection in pigs compared to humans and the bacteria did not replicate (100, 219). These observed differences once again illustrate that *H. ducreyi* is a much less efficient pathogen in animals than in humans (219).

In addition to the problems described above, several inconsistencies have been noted between the human and animal immune response to *H. ducreyi.* Specifically, the human antibody response is delayed compared to that of rabbits, macaques and swine (9, 100, 198, 219, 233). In addition, rabbits and swine, unlike humans, develop productive immunity after exposure to *H. ducreyi* (93, 180). Finally, a mutation that was shown to have a demonstrable effect on virulence in the swine model of chancroid (superoxide dismutase C) was later shown to play no role in the virulence of *H. ducreyi* in humans (34). Together, these findings highlight the fundamentally different immune response of humans and animals to *H. ducreyi.* As a strict human pathogen, *H. ducreyi* has evolved the ability to survive in the specific environment of the human skin (222). Because the bacterium does not naturally cause disease in animals, any pathology that manifests from the animal models cannot be considered to be completely analogous to human infection. As with all artificial disease systems, the findings derived from these models should be evaluated in context of the limitations of each model.

From a historical perspective, the animal models of chancroid have provided us with the preliminary tools to better understand the biology of chancroid. However, to date, the human model of experimental infection is the best method available for evaluating the contribution of individual genes in *H. ducreyi* pathogenesis (10, 219). Volunteers are inoculated on each upper arm with approximately 100 live bacteria via puncture wounds made by an allergy-testing device (10, 219). Isogenic mutant and parent bacterial strains are inoculated on opposite arms in order to prevent cross-contamination (10, 219). The patients are then monitored until a painful pustule develops, until resolution of the disease, or for a maximum of 14 days after inoculation, which ever comes first (10, 219). Papules form at the same rate in males in females (10, 219). However, the pustule formation rate is greater in males, and thus mimics natural infection (33, 219). Mutants who fail to produce pustules at doses ten times higher than the dose at which pustule formation is observed in the wild type are classified as fully attenuated. Mutants who fail to produce pustules or produce pustules at a lower rate than the wild type, at either the same dose or at doses two to three times higher than the wild type, are classified as partially attenuated (10, 17, 219).

The human challenge model (HCM) of chancroid has several clear advantages over the animal models that have been developed. Most importantly, the bacterium is studied in its natural host, with an intact immune response (10, 219). Second, the model evaluates *H. ducreyi* at the natural site of infection, human skin (10, 219). Third, the clinical features of experimental papule and pustule formation are identical to those observed clinically (10, 219). Finally, the histopathology of the experimental lesions closely resembles natural ulcers indicating that the model mimics chancroid clinical pathology (22, 219).

While the HCM may be the best model available for the study of *H. ducreyi,* there remain limitations to be considered. First, in order to ensure the safety of the volunteers, experimental infection can only be sustained through the pustular stage, or for a maximum of 14 days (10, 219). This limitation eliminates the direct study of the ulcerative stage of chancroid (219). However, because the histopathology of, and

localization of bacteria within experimental pustules so closely resembles that of natural ulcers, the applicability of the model could extend beyond the 14-day time period and findings could be extrapolated to apply to the ulcerative stage (22, 219). Second, the use of an allergy testing device for the inoculation of *H. ducreyi* into the skin could disguise the effect of any virulence determinants involved with the initial entry and establishment of infection in the skin (219). However, while these limitations are important and must be considered in the analysis of any data deriving from the model, the HCM remains the most relevant model of *H. ducreyi* infection available (219).

Host Pathogen Interactions

Small abrasions in the epidermis, likely produced during intercourse, are thought to provide *H. ducreyi* with a means of entering the body (25, 159, 219). Upon entry into the epidermis and dermis, the bacteria begin to replicate (25, 219). As part of the healing process, collagen and fibrin are deposited at the wounding site (25, 219). *H. ducreyi* associates with these molecules which form a scaffolding that ultimately supports pustule formation (25, 219). The increased levels of hypertrophic scarring observed in HCM volunteers may be due to the excessive collagen deposition that occurs in some subjects during pustule formation (102).

Chancroidal pustules contain several cell types. PMNs, Macrophages, T-cells, myeloid dendritic cells (DC) and a small number of B-cells accumulate at the pustule site (25, 101, 219). PMNs comprise the majority of the abscess, which is surrounded at its base by a "macrophage collar" (25, 219). Beneath the macrophage collar, macrophages and T-cells accumulate (25, 102, 219). *H. ducreyi* associates with both PMNs and macrophages, but resists phagocytic uptake by both cell types (7, 25, 219). However, *H. ducreyi* is taken up by myeloid DCs, which are in turn partially activated, resulting in stimulation of the immune response (18, 102). The majority of T-cells recruited to the site of infection are CD4+ memory cells, suggesting a "delayed type hypersensitivity response", even in the absence of prior infection (180, 219). These T-cells are activated by *H. ducreyi* specific antigens to produce the cytokines IFN-gamma and IL-10, resulting

in a regulatory T cell response (78). The immunosuppressive activity of IL-10 is thought to interfere with the activity of the phagocytes and thus inhibits the efficient clearance of *H. ducreyi* (78, 102, 219). The T_H1 T-cell response is in turn supported by a DC type 1 response (102).

In experimental infection, volunteers who undergo repeated challenge with *H. ducreyi* can be grouped according to the result of their infection (102, 215). Patients who repeatedly resolve infection after challenge are labeled "resolvers" (RR) while patients who repeatedly form pustules after challenge are labeled "pustule formers" (PP) (102, 215). Pustule formation is dependent on both the host immune response and gender (33, 215). Consequently, great interest has been generated concerning the differences in the immune response of PP vs. RR volunteers. Microarray analysis of myeloid DC indicates that each group generates a distinct immune response after exposure to *H. ducreyi* that is independent of phagocytic capability (102, 215). The characteristics of this "differential response" ultimately contribute to the PP and RR phenotypes (102). Individuals who repeatedly form pustules after challenge with *H. ducreyi* generate a mixed "hyper inflammatory" and regulatory DC transcription profile (102).

Virulence Determinants of *H. ducreyi*

The animal models of chancroid infection have been successfully used for the preliminary evaluation of the role individual genes play in *H. ducreyi* pathogenesis. Contribution to virulence, however, is ultimately determined by attenuation of an isogenic mutant in the human model of experimental infection. A list of all isogenic mutants that have been shown to contribute to *H. ducreyi* virulence in the HCM can be found in Table 1.

Table 1. *H. ducreyi* virulence factors as defined by the human challenge model

Protein	Outcome	Reference
DltA	Partial attenuation for pustule formation	(105)
WecA	Partial attenuation for pustule formation	(17)
FgbA	Partial attenuation for pustule formation	(23)
HgbA	Full attenuation for pustule formation	(11)
PAL	Full attenuation for pustule formation	(71)
DsrA	Full attenuation for pustule formation	(35)
TadA	Full attenuation for pustule formation	(217)
NcaA	Full attenuation for pustule formation	(72)
LspA1 and 2	Full attenuation for pustule formation	(106)

The hemoglobin receptor HgbA is one such virulence determinant (11). HgbA is an outer membrane protein (OMP) whose role in heme transport in dependent on TonB (5, 61, 63). The HgbA protein binds hemoglobin, the only source of heme or iron that can be utilized by *H. ducreyi in vivo* (126). Without a functional HgbA, it is likely that the mutant starves for heme and dies (135, 219). Two other TonB dependent receptors have been characterized in *H. ducreyi:* the heme receptor, TdhA, and an hypothetical protein, TdX (126). A *tdX/tdhA* double mutant retained virulence in the HCM (126), a finding that indicates that HgbA alone is sufficient to provide *H. ducreyi* with the heme necessary for survival (126). Because HgbA is also highly conserved within the species (247), researchers have speculated that it could serve as a potential *H. ducreyi* vaccine candidate (11). Preliminary work was done analyzing the efficacy of an HgbA subunit vaccine in the swine model and the study yielded encouraging results concerning the ability of this protein to elicit a memory response in swine and to protect against re-infection (5).

The peptidoglycan-associated lipoprotein (PAL) has also been determined to be an *H. ducreyi* virulence factor (71). PAL is a outer membrane lipopoprotein that is thought to play a role in membrane stabilization by linking the cytosolic membrane to the outer wall (71, 216). In the HCM, the pustule formation rate was significantly lower at sites inoculated with the PAL mutant as compared to the parent (71). The PAL mutant formed smaller colonies than the wild type and was extremely sensitive to antibiotics (71), suggesting that the loss of PAL yields an unstable outer membrane (219). In addition,

because of the pro-inflammatory nature of lipoproteins, the loss of PAL may have resulted in the stimulation of a weakened inflammatory response to the mutant and consequently less clinical pathology (71, 219).

Another virulence determinant defined by the HCM was the ducreyi serum resistance A (DsrA) protein (35). This OMP contributes to the serum resistance of *H. ducreyi* by blocking the binding of IgM and the subsequent classical complement pathway activation that would normally follow (2, 62). In addition, the protein acts as an adhesin by binding fibronectin and vitronectin (124, 125). In the HCM, volunteers inoculated with the DsrA mutant, FX517, developed significantly smaller papules compared to parent inoculated sites and failed to develop pustules, indicating that serum resistance is an important phenotype for *H. ducreyi* pathogenesis (35).

The OMP ducreyi lectin A (DltA) also contributes to the *H. ducreyi* serum resistance phenotype (127), although it plays a more minor role than does DsrA (105). DltA mediates binding to lactose-related carbohydrates (127). Isogenic *dltA* mutants were generated in both 35000HP and the *dsrA* mutant background (127). Each mutant exhibited increased susceptibility to serum than did the parent and the expression of recombinant DltA conferred serum resistance to *H. influenzae* strain Rd (127). In the HCM, the DltA mutant was found to be partially attenuated for pustule formation (105).

The *H. ducreyi* genome contains an operon that controls the production of fimbriae and has high levels of homology to the well described tight adhesion (tad) operon of *A. actinomycetemcomitans* (166, 217). Three individual mutations were generated in this operon in the 3500HP background (166). Each mutation reduced the bacterium's ability to form microcolonies and to attach to human foreskin fibroblasts (166). Consequently, the mutations were tested in the temperature dependent rabbit model of chancroid (166). The polar *tadA* mutant exhibited a small but significant decrease in virulence in the model, while the *rcpA* and *flp-1 flp-2* mutants each had no effect on pustule formation, indicating that microcolony formation played at best, a minimal role in virulence (166). However, when the *tadA* mutant was evaluated in the HCM, it was fully attenuated (217).

These data suggest that *tadA,* which has homology to the NTPases of type IV section systems in other bacteria, or one of its downstream ORFs, is required for virulence by *H. ducreyi* in humans. (217) Further, this study highlights the importance of studying *H. ducreyi,* a strict human pathogen, in humans.

H. ducreyi binds the extracellular matrix (ECM) proteins fibronectin, laminin, and type I and III collagen *in vitro* (26), associates with fibrin and collagen within experimental papules and pustules (25) and associates with fibrin at the ulcerative stage of disease (22). Consequently, the adherence of *H. ducreyi* to ECM proteins correlates with infection (72, 219). The necessary for collagen adhesion protein A (NcaA) is an *H. ducreyi* OMP that binds collagen *in vitro* and has homology to DsrA (72). When expressed *in trans*, the protein confers to *E. coli* the ability to bind to type I collagen (72). In the swine model, the NcaA mutant exhibited significantly reduced survival compared to the parent. In the HCM, the NcaA mutant was fully attenuated, demonstrating the importance of collagen adherence to *H. ducreyi* virulence (72).

H. ducreyi encodes two large ORFs aptly named large supernatant protein 1 (LspA1) and large supernatant protein 2 (LspA2). These secreted proteins are highly homologous to one another (244). Four *H. ducreyi* strains that were avirulent in animal models were found to have natural mutations that resulted in the loss of either LspA1 or LspA2 production, suggesting that the proteins could be involved in *H. ducreyi* virulence (244). Three isogenic mutants were constructed lacking either one or both of the *lspA1* and *lspA2* ORFs (243). Western blot analysis indicated that the parent contained abundant LspA1 and small amounts of LspA2 (243). The *lspA2* mutant contained comparable amounts of LspA1 to the wild-type, and lacked LspA2 (243). In contrast, the *lspA1* mutant contained significantly up-regulated amounts of LspA2 (243). The *lspA1 lspA2* double mutant was attenuated for virulence in the temperature-dependent rabbit model, indicating that the proteins could play a role in *H. ducreyi* virulence (243).

Wild type 35000HP was shown to inhibit the phagocytosis of IgG opsonized microspheres by differentiated HL-60 (human promyelocytic leukemia cell line) and U-

937 (Human leukemic monocyte lymphoma cell line) cells and by J774A.1 (murine macrophage cell line) cells *in vitro* (238). In contrast, phagocytosis proceeded in the presence of the *lspA1/lspA2* double mutant (238). Likewise, 35000HP resisted phagocytosis itself, while the double mutant was readily taken up by HL-60 and J774A.1 cells (238). Proteins ranging in size from 50 to 60 kDa were harvested from macrophage cell lines exposed to *H. ducreyi.* Using phosphor-specific antibodies and western analysis, these antibodies were shown to exhibit reduced levels of phosphorylation (156). Lyn and Hck, two Src family tyrosine kinases that fall within this size rage and that play an important role in phagocytic signaling, were later shown to have reduced enzymatic activity after exposure to *H. ducreyi* (156). The *lspA1/lspA2* double mutant was subsequently shown to be fully attenuated for virulence in the HCM (106). Taken together, the cumulative data concerning LspA1 and LspA2 indicates that these proteins contribute to *H. ducreyi* virulence by allowing the bacteria to resist phagocytic uptake through the suppression of Src family protein tyrosine kinase activity (106, 156, 238).

Many Gram-negative entero-bacterial species produce a complex cell wall bound carbohydrate named enterobacterial common antigen (ECA) (17, 64). The enzymatic pathway controlling ECA production also contributes to the generation of lipopolysaccharide (LPS) O-antigen and to capsules (17, 129). While *H. ducreyi* does not produce either of these structures, it does encode homologs of the ECA biosynthesis pathway, suggesting that *H. ducreyi* produces ECA (17). The first enzyme in the ECA synthesis pathway, undecaprenyl-phosphate alpha- acetylglucos-aminyltransferase (WecA), was disrupted through the insertion of a polar antibiotic resistance cassette, creating an isogenic 35000HP*wecA* mutant (17, 64, 129). As the presence of ECA has not yet been confirmed in *H. ducreyi,* it is still too early to make a definitive conclusion concerning the exact function of wecA in *H. ducreyi* virulence (17) However, when the mutant was analyzed in the HCM it was found to be partially attenuated, indicating that the *wecA* gene, or a downstream ORF, plays a role in *H. ducreyi* virulence (17).

A gene expression study which compared the expression levels of *H. ducreyi* genes in broth and tissue was used to identify possible genes involved in virulence (24). This

study identified HD0192, a lipoprotein, as a potential virulence factor (24). The recombinant lipoprotein was shown to bind fibrinogen in a ligand blot, and was subsequently renamed fibrinogen binder A (FgbA) (23). An isogenic *fgbA* mutant was constructed in the 35000HP background through the insertion of a non-polar antibiotic resistance cassette (23). In the HCM, the *fgbA* mutant was shown to be partially attenuated, suggesting that *fgbA* contributes to *H. ducreyi* virulence (23).

It is also important to note that not all isogenic *H. ducreyi* mutants tested in the HCM have been shown to contribute to virulence. For example, the *H. ducreyi* LOS molecule has been shown to mediate attachment of the bacterium to keratinocytes *in vitro* (79). However, two separate *H. ducreyi* mutants, each harboring a mutation in the enzymatic pathway controlling LOS synthesis, (*lgbA* and *lgbF)* and consequently producing a truncated LOS molecule, retained their virulence in the HCM (261, 263). This finding illustrates that an *in vitro* phenotype does not always translate to *in vivo* significance. Similarly, the *H. ducreyi* genome encodes the production of a 125 kDa β-hemolysin (181, 232) which has been shown to have cytotoxic activity against human foreskin fibroblasts, human foreskin epithelial cells, human macrophage like cells (U937) and human T-cell like cells (Jurkat) *in vitro* (14, 252). However, the *H. ducreyi* hemolysin did not affect the rate of pustule formation in the HCM, indicating that it is not important for the virulence of the organism (179). In a third example, *H. ducreyi* produces a cytolethal distending toxin (CDT) that induces cell elongation and apoptosis in several cell lines (50, 51, 77) and apoptosis in primary DC, B-cells, T-cells and monocytes (17, 50, 251) by activating the p53/p21 and cyclin B/CDK1 dependent DNA damage response pathways, consequently blocking cell division in the G1 and G2 phases of cell growth (29, 51, 52, 77). The *in vitro* data collected to date suggests that the *H. ducreyi* CDT is important for the virulence of the organism and could provide an explanation for the persistence of untreated chancroid lesions (77, 229). However, the *in vivo* data concerning CDT does not support this conclusion as multiple *H. ducreyi* CDT mutants have been shown to retain virulence in both the temperature dependent rabbit model of chancroid and in the HCM (224). Taken together, the examples described above illustrate the immense value of the HCM as a tool that allows us to interpret *in vitro*

findings in a relevant *in vivo* setting and to classify *H. ducreyi* virulence factors in relation to Koch's molecular postulates (66).

Table 2. *H. ducreyi* structures that did not contribute to virulence in the HCM

Structure	References
Lipooligosaccharide	(20, 21, 26, 44, 79, 223, 261, 263)
Fine tangled pilus	(12, 42)
β-hemolysin	(14, 179, 182)
Superoxide dismutase	(34, 164, 204, 205)
Cytolethal distending toxin	(138, 224, 262)
Major outer membrane proteins	(99, 231)

Two Classes of *H. ducreyi*

As a species, *H. ducreyi* exhibits a high degree of genetic homology (151). However, based on differences in the binding specificity of antibodies to OMPs, the rate of migration of LOS, and growth characteristics, two classes of *H. ducreyi* clinical isolates have been defined (247). White et al. compared the molecular size of the DsrA protein in a variety of *H. ducreyi* clinical isolates (247). In this study, they determined that five of the strains pro duced an alternative version of the protein that was markedly different from that of 35000HP (247). These five strains also expressed variant forms of NcaA, DltA, Hlp, MOMP, and OmpA2, that like DsrA, retained similarity to each other, but were distinct from 35000HP (247). In addition, these strains synthesized a faster-migrating LOS due to genetic differences in the *lbgAB* containing operon (194, 247). The OMP profiles and LOS migration patterns from the remaining *H. ducreyi* isolates were identical to that of 35000HP (247). As such, the 35000HP containing group was classified as Class I and the strains expressing the variant proteins were classified as class II (247). In light of this finding, analyses of potential virulence factors in *H. ducreyi* now generally include an evaluation of the conservation of genes in representative strains encompassing both classes of *H. ducreyi,* and the list of variant protein forms characterized to date has been expanded to include FgbA (23). In addition, a large scale proteomics study was conducted comparing the overall proteome profiles of five *H. ducreyi* strains (193). After an SDS page analysis of the migration patterns of 149

individual proteins, the authors concluded that the overall protein migration profiles of the *H. ducreyi* strains could be divided into two distinct groups, which corresponded to the groups proposed by White et al. (193, 247). Further, they commented that the high level of proteomic diversity between these groups warranted a full genomic sequencing of a representative member of the second class of *H. ducreyi* (193).

LITERATURE REVIEW
SECTION II: ANTIMICROBIAL PEPTIDES

Human Antimicrobial Peptides

Antimicrobial peptides (APs) form part of the nonspecific innate immune system (41, 108). These molecules evolved early in history and are found in both vertebrates and invertebrates (108). APs exhibit cidal activity against many different organisms, including bacteria, enveloped viruses, fungi and parasites (108). Most APs are small cationic secreted molecules and many have both bactericidal and immunomodulatory properties (39, 41, 108, 255).

Cathelicidins are produced by many different mammals (132, 265). The sole human cathelicidin, LL-37, is an α-helical AP that bears a net positive charge (41). Cathelicidins are composed of three distinct regions: a signal sequence, a pro-domain, and an antimicrobial domain (41, 265). Proteolytic cleavage of the antimicrobial domain by proteinase-3 in the secretory granule prior to exocytosis confers activity to the peptide (41, 213). The 37 amino acid long peptide is named for both its size and the L-L amino acid sequence that leads the C-terminally located active peptide which remains after cleavage (213).

Humans produce a second class of APs, referred to as the defensins (74). Defensins are positively charged β-sheet peptides, the secondary structure of which, is dependent on the presence of six cysteine residues that form disulfide bridges within the molecule (74). There are two classes of human defensins, the α- and β-defensins, and the pattern of cysteine bonding is different for each class (74). The α-defensin disulfide bridges are arranged in a "1-6, 2-4, 3-5" pattern (41), while the β-defensin disulfide bridges are spaced in a "1-5, 2-4, 3-6" pattern (41). Humans produce only six α-defensins. In contrast, computational algorithms have predicted the presence of over 20 potential β-defensin genes within the human genome (39, 208). Much like the cathelicidins, defensins are initially synthesized as immature pre-peptides which are then

proteolytically cleaved, leaving behind the C-terminal portion of the peptide, which is active (74).

Antimicrobial Peptides in the Chancroid Environment

H. ducreyi likely encounters many cellular sources of APs during infection (Table 3). Keratinocytes constitutively express the human β-defensin (HBD)–1 (268) and the squamous epithelium of the vagina constitutively expresses the α-defensin human defensin (HD)-5 (199). Wounding of the skin stimulates the production of TNF-α and IL-1, and these cytokines in turn stimulate the release of HBD-2, HBD-3, HBD-4 and LL-37 from keratinocytes (57, 115, 140, 212). In response to bacterial stimulation, PMNs and macrophages are rapidly recruited to the site of infection (25, 101, 180). PMNs secrete the α-defensins HNP-1, HNP-2, HNP-3, and HNP-4 from their azurophilic granules (67), and HBD-4 and LL-37 from their secondary granules (76, 236). Inflammatory stimulators also induce the expression and secretion of HBD-1, HBD-2, and LL-37 from macrophages (59, 141). Inflammatory bacterial products further stimulate cytokine production from both monocytes and lymphocytes via the toll-like-receptor signaling pathway (43). Cellular extracts from both monocytes and, to a lesser degree, lymphocytes, have been shown to increase the production of HBD-1, HBD-2 and HBD-3 from keratinocytes (212).

Several studies have recently established that *H. ducreyi* co-localizes with APs at the site of infection. Bauer et al. visualized PMNs in natural chancroidal ulcers by staining for the α-defensins HNP-1-3 (22). In this study, the authors demonstrated that these peptides were present within PMNs at the ulcerative stage of disease (22). The same lab more recently stained tissue sections from the HCM to demonstrate that HNP-1-3 were also expressed by PMNs at the papular and pustular stages of disease (M.E. Bauer, C.A. Townsend, unpublished data). While still unproven, it is likely that *H. ducreyi* stimulates the degranulation of PMNs. Consequently, these studies establish the relevance of investigating *H. ducreyi* resistance to the APs.

Table 3. Antimicrobial peptide secreting cell types in chancroid

Cell type or tissue	AP Secreted	Class of AP	Reference
Vaginal Mucosal epithelium	HD-5	α-defensin	(199)
Keratinocytes	LL-37	Cathelicidin	(115, 170, 212)
	HBD-1	β-defensin	(95, 268)
	HBD-2	β-defensin	(140, 212)
	HBD-3	β-defensin	(140, 212)
	HBD-4	β-defensin	(240, 254)
Macrophage	LL-37	Cathelicidin	(141)
	HBD-1	β-defensin	(59)
	HBD-2	β-defensin	(59)
PMN	LL-37	Cathelicidin	(83, 213, 236)
	HNP-1	α-defensin	(67, 75)
	HNP-2	α-defensin	(67, 75)
	HNP-3	α-defensin	(67, 75)
	HNP-4	α-defensin	(67, 75)
	HBD-4	β-defensin	(83, 213, 236)

Evidence of Antimicrobial Peptide Activity *In vivo*

Alterations in AP production are known to be a contributing factor to many forms of human disease. Patients with the skin disorder atopic dermatitis express low levels of LL-37, HBD-2, and HBD-3. These patients consequently contract infections of the skin at a rate much higher than would otherwise be expected (115, 171, 175). Conversely, patients with psoriasis express increased levels of the same APs, yet even with a damaged stratum corneum, they develop few skin infections (115, 171, 175). In another example, patients with cystic fibrosis express a dysfunctional Cl^- pump in the apical membrane of the lung epithelium (211, 267). Studies have indicated that the mucus of these patients may have a much higher salt concentration than what is considered to be normal (211, 267). Although still controversial, researchers have hypothesized that the high concentration of salt in the mucus interferes with the bactericidal activity of APs in the lung, and that as a result, cystic fibrosis patients are unusually susceptible to lung infections (149, 211, 250, 267). Another important clinical example of AP activity in the

body can be found in the study of the innate immune system of newborns (39, 133). Infants do not develop a functional adaptive immune system until several months after birth (39, 133). In order to help fight infection, newborns express elevated levels of β-defensins and LL-37, both in utero and following delivery (39, 133). These APs serve to protect the newborn from bacteria and fungi encountered as the infant traverses the birth canal (39, 133). The list of AP associated diseases described here is in no way exhaustive. Instead, it is intended to highlight interesting examples of the *in vivo* relevance of APs. Because APs represent an important part of the innate immune system, the list of disease states linked to AP disregulation continues to rapidly expand alongside our knowledge of the role that APs play in maintaining homeostasis within the body.

Animal studies have also been used to establish the *in vivo* relevance of APs. Mice that were genetically altered to lack CRAMP, a LL-37 homolog, were shown to be more susceptible to bacterial challenge than were wild type mice (39, 170). The mice lacking CRAMP expression exhibited a normal recruitment of PMNs to the site of infection, suggesting that the lack of CRAMP in the PMNs negatively affected the mice's ability to fight infection (39, 170). In experiments using the opposite approach, wild type mice that were infected with cathelicidin-resistant bacteria developed chronic lesions, while cathelicidin-sensitive bacteria were efficiently cleared (39, 170). Similarly, mouse studies have highlighted the contribution of defensins to host defense. Mice that were engineered to lack all mature α-defensins were impaired in their ability to resolve infection and were more likely to die after bacterial challenge (39, 249). Further, a knock-in of human HD-5 into mouse paneth cells markedly increased the effectiveness of the mouse immune response after exposure to bacteria (39, 202).

As was previously detailed, the expression and secretion of many APs are induced by inflammatory mediators (38, 57, 115, 140, 212). In healthy skin, most APs are either undetectable or found at very low levels (38). However, in the presence of infection, the level of AP production by keratinocytes and resident macrophages increases substantially, and both LL-37 and the β-defensins can be found at concentrations in the μg/ml range (38). α-defensins in the skin, produced by PMNs, can be found at

concentration as high as 10mg/ml immediately after degranulation (39). However, their concentration is difficult to measure, as it quickly dilutes as the time after release increases (38). The *in vivo* concentration of a peptide is vital to its activity (39). As a rule, APs generally disrupt membranes at high concentrations and modulate immune responses at lower concentrations (38, 39, 41). It is probable that at low concentrations an AP may not have direct bactericidal activity against an organism, but could still play an important role in the immune response to pathogens.

Antimicrobial Peptide Bactericidal Activity

In order to be effective, APs must associate with the cytoplasmic membrane (108). The interaction of APs with the membrane is dependent on both the amphipathic nature of the APs and on their positive charge (91, 108). In order to gain access to the cytoplasmic membrane of Gram-negative organisms such as *H. ducreyi*, APs must first cross the outer membrane through a process known as "self-promoted uptake" (91, 108, 207). Cationic APs transiently bind to the anionic membrane through electrostatic attraction (108). Consequently, the high concentration of negatively charged lipids on the bacterial membrane is important to the selectivity of APs for bacterial cells over eukaryotic cells (91, 108). Once in the periplasm, the amphipathic and hydrophobic nature of the APs allows them to pass into the inner membrane of the bacterium (54, 108). APs kill bacteria through two main mechanisms (108). They can either directly lyse the prokaryotic inner membrane, an event that is lethal to gram-negative bacteria (131), or they can cross the intact membrane and disrupt the synthesis of anionic intracellular targets such as DNA, RNA, and enzymes through a currently undefined mechanism (91, 108, 253). The α-defensin HNP-1 has been shown to interfere with the production of both nucleic acid and protein in *E. coli* (108, 131).

Because the majority of experiments examining the direct interaction of APs with lipid bilayers have been conducted using synthetic or liposomal model membrane systems it has been difficult to accurately characterize the exact series of molecular events comprising this interaction (91, 113, 253). Regardless of these limitations, several

models have been proposed to describe the relationship of APs with anionic membranes (108). In each of the four following models, APs first interact with the anionic lipid head groups of the membrane before assuming a position parallel to the membrane (108). In the "toroidal pore" model, APs insert themselves perpendicularly into the membrane and the individual lipids within the membrane curve inward with the APs to stabilize a temporary pore (87, 108). LL-37 interacts with the membrane through the formation of a toroidal pore (108, 248). In the "aggregate" model, large clusters of peptides span the membrane and intermix with lipids in a random pattern that leads to disruption of the membrane (108, 150, 253). In the "barrel-stave" model, peptides span the membrane as "staves" in a "barrel"-shaped pore of consistent size (108, 214). Finally, in the "carpet" model, large aggregates of peptide line the lipid bi-layer at concentrations high enough to carpet the membrane (108, 195). At these concentrations, the APs produce large micelles which significantly disturb the stability of the membrane through "detergent-like" activity (108, 248). Due to their amphipathic character, many APs have been shown to behave in this manner at very high concentrations, but the relevance of this model to *in vivo* AP activity is debatable (37, 38, 108).

It is important to remember that each of these models has validity under different circumstances. The examination of a broad range of peptides encompassing a variety of sizes and structures has indicated that each peptide produces a unique signature of membrane interaction (108). No one model is adequate to explain how all APs interact with a particular pathogen. Individual peptides may behave differently at high concentrations that they do at low concentrations (38, 39, 108). It is also likely that individual APs may use more than one mechanism to kill bacteria at a time (108, 184, 196). For example, the β-defensin, HBD-3, has been shown to negatively affect cell growth both by depolarizing the bacterial membrane and by interfering with the functionality of bacterial autolysins (188). Further, while most cationic APs have been characterized as membrane permeabilizing, almost any peptide that is both cationic and amphipathic will cause membrane disruption of synthetic membranes at high concentrations (108). As such, it is likely that the role of membrane disruption in AP research has been overstated, and that in reality, APs exert their bactericidal activity by a

more tempered combination of membrane perturbation and the disruption of intracellular processes (38, 39, 108, 253).

Antimicrobial Peptide Immunoregulatory Activity

In early studies establishing the role of APs in the regulation of the immune system, mice were challenged with *Pseudomonas aeruginosa* in the lung, with or without the simultaneous tracheal instillation of the LL-37 precursor, CAP18 (39, 206). The mice that received APs along with bacteria, exhibited a clinical reduction in inflammation, as measured by a decreased production of TNF-α, IL-6 and nitrous oxide, when compared to the mice that were received only bacteria (39, 206). However, a surprising finding of these studies was that similar numbers of bacteria could be recovered from both groups of mice, suggesting that the APs did not have direct bactericidal activity, but instead upregulated the immune response in a controlled manner (39, 206). We now know that APs function not only through their bactericidal activity, but also have immunoregulatory activity (39, 82, 264).

LL-37 plays a role in establishing, maintaining, and regulating the immune response to pathogens (39). LL-37 acts as a chemoattractant of PMNs, T-cells and monocytes (39, 257, 259), and it induces chemotaxis, toll-like receptor expression and degranulation in mast cells (39, 168, 169, 260, 264). The peptide supports the continuation of the immune response by stimulating phagocytosis in PMNs (165), by suppressing apoptosis of both keratinocytes and PMNs (46, 266), and by acting as an angiogenic factor in endothelial cells (39, 117). In addition, LL-37 acts synergistically with GM-CSF and IL1β to upregulate the expression of NFκB and AKT in human leukocytes (165). Finally, LL-37 regulates inflammation overall by binding to and neutralizing bacterial surface components (39, 122, 163).

The α-defensins HNP-1-3 also regulate the immune response (39). These peptides stimulate the production of IL-8 by monocytes and epithelial cells, and induce epithelial cell proliferation (39, 239). In addition, both HNP-1 and HNP-2 have been shown to

chemoattract T-cells and immature dendritic cells, although HNP-1 is a stronger chemoattractant than HNP-2 (39, 48, 74, 230, 258). Likewise, the β-defensin HBD-2 has been shown to chemoattract immature dendritic cells, memory T cells, and PMNs through a process that is dependent on CCR6 (39, 74, 167, 256, 259). Taken together, these data indicate that the APs likely found at the site of infection could potentially play multiple roles in the immune response to *H. ducreyi.*

Bacterial Resistance to Antimicrobial Peptides

Virtually all bacterial pathogens encounter APs in their natural environment (118). Consequently, bacteria have evolved many distinct mechanisms for surviving in the presence of bactericidal APs (118). AP resistance genes have been found in the genomes of the vast majority of pathogenic bacterial species tested (118, 246) and the impressive number of AP resistance mechanisms cataloged to date provides an elegant example of evolution at work (188). Each species has co-evolved with its respective host to adapt to meet the unique challenges present in that bacterium's specific niche (118, 188).

Some general strategies to evade killing by APs include repelling APs from the bacterial surface, inactivating APs in the extracellular milieu and pumping APs out of the periplasm before they can damage the cytosolic membrane (118, 160, 188, 246). The genome of a single species often encodes multiple AP resistance strategies (118). The redundancy and complementation of these strategies allows the bacterium to achieve a high level of resistance to a diverse variety of APs (65, 118). However, while most AP resistance genes are chromosomally encoded, they are not always highly conserved within a species and there can be a large range of AP susceptibility among the clinical isolates of an individual species, a phenomenon that has been observed with commensal *Staphylococcal* and *Streptococcal* species (111, 118, 155). *H. ducreyi* is exposed to many different APs *in vivo* (22, 27). Consequently, it is reasonable to expect that the organism has evolved multiple mechanisms of AP resistance in order to survive in this hostile environment. It is also possible that the class I and class II strains of *H. ducreyi*

could express different AP susceptibility profiles. The experiments described in this thesis directly address these questions.

One of the most prevalent strategies for AP evasion is the alteration of the bacterial membrane or cell wall in a manner that would eliminate or reduce the net negative charge of the membrane, subsequently allowing the bacterium to more closely mimic the substantially less anionic nature of the eukaryotic membrane and to remain undetected by APs (118, 188). For example, many Gram-positive organisms encode the *dltABCD* operon, which modifies the cell wall through D-alanine incorporation, reducing the charge of teichoic acid (1, 118, 119, 189, 197). Similarly, many Gram-negative bacterial species utilize the Pmr enzyme to modify their cell surface by adding aminoarabinose onto lipid A (65, 85, 118, 172). The genomes of many pathogenic bacteria, both Gram-positive and Gram-negative, contain the *mprF* gene (118). This enzyme alters the phosphatidylglycerol of the cell wall with L-lysine, once again reducing the net charge of the cell wall (118, 174, 190, 221, 246). An alternate implementation of the same basic strategy is found in the production of bacterial capsules and biofilms (118). While these structures inhibit the binding of antibodies at the cell surface, they are an inadequate physical barrier against small molecules (45, 118, 241). However, the extremely positive charge of the extracellular matrix of a capsule or biofilm repels APs from the bacterial surface (45, 118, 241). In each of the examples described above, the net charge of the cell surface is made more positive as a mechanism to dampen the electrostatic attraction of APs to the bacterial surface (118).

A second strategy used by bacteria to resist the bactericidal activity of APs is the degradation of APs in the extracellular milieu, before they have the opportunity to damage or cross the membrane (118). One way in which bacteria accomplish this task is through the production of either secreted or membrane bound enzymes that cleave APs in the extracellular environment (118). A few examples of this strategy include the *S. enterica* protease PgtE (84, 118), the *S. aureus* secreted metalloprotease aureolysin and serine protease V8 (118, 210), and the membrane bound *E. coli* OmpT and OmpP (103, 118, 188, 226). In a similar approach, some bacterial species neutralize extracellular

APs by expressing secreted or membrane bound proteins that have the ability to bind and sequester APs, preventing them from interacting with the membrane (118, 188). The *S. aureus* staphylokinase is representative of this strategy (110).

Some bacteria directly bind and transport APs away from the membrane before they can cause damage (118). The multiple transferable resistance (Mtr)CDE pump has been shown to confer resistance to APs in *Neisseria gonorrhoeae* (118, 209). Likewise, the QacA efflux pump contributes to *S. aureus* AP resistance (118, 120), demonstrating that the pumps can be found in both Gram-positive and Gram-negative organisms (118). While most transporters shuttle APs out of the cell, the senstitive to antimicrobial peptides (Sap) transporters of *Salmonella typhimurium* (183), *Proteus mirabilis* (152), *Erwinia chrysanthemi* (142), and *H. influenzae* (146) have been shown act as an influx pump shuttling APs into the cell, where they are presumably degraded (183). The *H. ducreyi* genome contains a homolog of this pump, which will be analyzed in detail in the following chapter of this literature review.

One previous study has been conducted investigating *H. ducreyi* resistance to APs. In this study, Fortney et al. used a radial diffusion assay (RDA) to demonstrate that *H. ducreyi* was susceptible to PG-1, a porcine protegrin with no human homolog (70). It is possible that the presence of animal specific APs such as PG-1 may be a contributing factor to the limited host specificity of this pathogen (160).

LITERATURE REVIEW
SECTION III: SAP TRANSPORTER

The Sap Transporter

As previously discussed, bacterial pathogens have evolved many mechanisms enabling them to resist the bactericidal effects of APs (118, 187). Because *H. ducreyi* is able to not only establish an infection but also to persist in an environment rich with APs (22, 27), we hypothesized that the bacterium was resistant to the APs it was most likely to encounter *in vivo* (160). In order to identify possible AP resistance mechanisms in *H. ducreyi*, we examined the *H. ducreyi* genome for evidence of mechanisms homologous to those described in other bacteria and found high levels of homology to the sensitive to antimicrobial peptides (Sap) transporter. The Sap transporter has been studied in multiple bacterial species and has been found to confer resistance against a variety of APs (47, 81, 142, 144, 146, 152, 183).

Salmonella typhimurium Sap Transporter

The *sap* operon was first identified as a possible mechanism of AP resistance by Groisman et al. Transposon mutagenesis was performed on an AP resistant *S. typhimurium* strain, in an effort to identify genes required for AP resistance (81). The resultant mutants were screened for their susceptibility to protamine, a cationic peptide derived from salmon sperm (81). Twelve mutants were sensitive to protamine on agar plates and eight of these mutants went on to exhibit sensitivity to protamine in a liquid assay (81). The susceptibility of these mutants to rabbit defensin NP-1, frog magainin 2, pig cecropin P1, insect peptides mastoparan and melittin, and human crude granulocyte extract was examined (81). One mutant was extremely sensitive to all of the peptides tested, but was later shown to harbor a mutation in the transcription factor *phoP,* a part of a two-component regulator involved in the regulation of many bacterial virulence factors (68, 81). The remaining mutants fell into groups based on their AP susceptibility (81). The mutants were referred to as Sap mutants for their sensitivity to antimicrobial peptides

and they all contained mutations that mapped to the same operon (81). At the time of this research, it was not yet known that different APs were produced by different cell types within the body. However, when the mutants were inoculated into mice through the intragastric and intraperitoneal routes, differences in virulence were noted (81). The altered susceptibility pattern of the mutants provided some of the first evidence that pathogens were exposed to different APs at different locations within the body (81).

The same lab later built off of their preliminary study by isolating the genes from three *S. typhimurium* mutants which were required for resistance to the melittin and crude granulocyte extracts, but not for resistance to rabbit defensin NP-1 (Table 4) (183). The region of DNA responsible for AP resistance was shown to contain five ORFs: *sapA, sapB, sapC, sapD* and *sapF*, organized in an operon (183). The complex was composed of five proteins exhibiting sequence identity with other known ATP dependent import and export transporters (97, 183, 200). SapD and SapF were shown to be homologous to other prokaryotic ATP binding cassette (ABC) family transporters involved in the uptake of oligopeptides such as the bacterial oligopeptide permease (Opp) (98) and the *B. subtilis* response regulator (SpoOK) (183, 186). SapA was homologous with other periplasmic solute binding proteins involved in peptide transport such as *E. coli* dipeptide transporter (Dpp) (3) and oligopeptide transporter (Opp) (98, 183). The identification of a conserved signaling sequence on the peptide indicated that it was most likely expressed in the periplasm (183). Based on these findings, a model of Sap transporter activity was proposed (Figure 1) (183). Of the three mutants initially analyzed, one contained a mutation in *sapC* (EG123) and two (EG1209 and EG1216) contained mutations in *sapD* (183). The *sapC* mutant was susceptible to both human crude granulocyte extract and melittin, while the *sapD* mutants were only susceptible to the crude granulocyte extract (183). All three mutants retained their resistance to rabbit defensin NP-1 (183). Additional mutants were generated to characterize the activity of the transporter. A mutation that eliminated the entire *sapABCDF* operon was shown to be as susceptible to promatine as the *sapC* mutant (183). An antibiotic resistance cassette was inserted in the *sapF* gene. This mutant was as susceptible to protamine as the *sapD* mutant, indicating that both SapD and SapF are necessary for the full functionality of the transporter (183).

Finally, a non-polar mutation was generated in *sapA*. This mutant retained a low level of residual AP resistance against protamine when compared to the *sapC* and *sapD* mutants, indicating that the the functionality of the transporter, while greater reduced, continued to function at a low level in the absence of SapA (183).

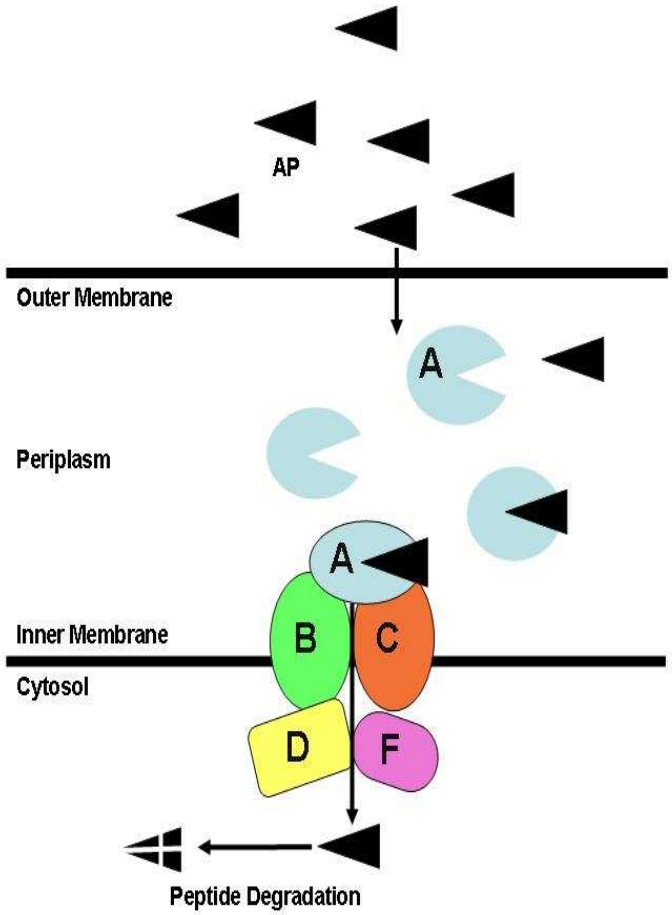


Figure 1. Model of Sap transporter (183). Parra-Lopez et al. proposed a model of the Sap Transporter mechanism of action. SapA was hypothesized to be a periplasmic protein that bound APs and transported them into the SapBCDF membrane complex (183). SapB and SapC were hypothesized to be membrane spanning proteins that form a channel through the membrane (183). SapD and SapF were hypothesized to be ATPases that provided energy for the complex (183). Peptides are thought to be shuttled into the cytosol where they are presumably degraded before they can damage the cytoplasmic membrane or anionic intracellular targets (183).

Proteus mirabilis Sap Transporter

P. mirabilis is a pathogen that primarily causes urinary tract infections, bladder infections and kidney infections (152). *P. mirabilis* encounters many APs *in vivo,* and the high levels of resistance of *P. mirabilis* to these APs could play a role in the virulence of this organism at mucosal surfaces (152). McCoy et al. used transposon mutagenesis to identify genes necessary for resistance to polymixin B (PM), a small cationic and amphipathic antibiotic that causes permeabilization of the bacterial membrane (152). One of the mutants generated contained an insertion in a gene homologous to the *sapD* locus of *S. typhimurium* (152, 183). This mutant was more susceptible than the wild type to α-helical peptide PM, but retained resistance to a β-sheet protegrin analog (Table 4) (152). Based on these results, McCoy et al. argued that the Sap transporter conferred resistance to α-helical peptides, but not to β-sheet peptides, demonstrating that the specificity of the transporter was linked to structure (152). However, a survey with a greater number of APs should be performed in order to substantiate these claims.

Haemophilus influenzae Sap Transporter

Haemophilus influenzae is one of the causative agents of otitis media (OM), or inflammation of the middle ear (147). The β-defensin HBD-2 suppresses the growth of several OM pathogens, including *Streptococcus pneumoniae, Moxaxella catarrhalis,* and nontypeable *H. influenzae* (NTHI) (128, 147). This inhibition of growth is linked to membrane damage, as bacteria exposed to APs were shown to have membrane protrusions and to leak cytoplasmic contents (128, 147). Mason et al. examined the genome of *H. influenzae* for the presence of possible AP resistance mechanisms, including the Sap transporter system (146-148). A putative *sap* operon containing *sapABCDFZ* was revealed (148). The authors demonstrated that the transcription level of *sapA* was 2.8 fold higher in bacteria grown in the middle ear in a chinchilla model of OM vs. bacteria grown *in vitro* (148), and thus generated a non-polar isogenic *H. influenzae sapA* mutant (147). This mutation was shown to render NTHI more sensitive to killing by recombinant chinchilla β-defensin 1 (r-cBD-1), HBD-3, which is homologous to cBD-1, and LL-37 (Table 4) (147). The authors went on to demonstrate the direct association

of NTHI recombinant SapA with r-cBD-1, LL-37, and HBD-3 (146). These studies made three important contributions to our understanding of Sap transporter biology. First, the finding that the NTHI Sap transporter confers resistance against the β-defensins r-cBD-1 and HBD-3 contrasts with the studies conducted with *P. mirabilis* (152) that indicated that the Sap transporter played no role in resistance to β-sheet peptides. Second, the finding that the *H. influenzae* SapA directly associates with r-cBD-1 (146) supports the proposed mechanism of action of the Sap transporter originally hypothesized by Parra-Lopez et al., which postulated that SapA bound APs in the periplasm (183). Finally, the discovery of the *sapZ* gene, which encodes a hypothetical protein with as of yet unknown function, could represent an uncharacterized member of the Sap transporter complex (146, 147).

Erwinia chrysanthemi Sap Transporter

The production of APs is a defense strategy that has been evolutionarily conserved (41, 108). As such, bacterial pathogens of plants are also subjected to the bactericidal activity of APs and have developed AP resistance strategies (142). Lopez-Solanilla et al. investigated the role of the Sap transporter in the bacterial resistance to plant antimicrobial peptides (142). *E. chrysanthemi* is a phytopathogenic bacterium that causes soft rot in a variety of crops (142). The genome of this bacterium encodes a homolog to the *sapABCDF* operon of *S. typhimurium* (142, 183). A polar *sapABCDF E. chrysanthemi* mutant was shown to be more sensitive than was the wild type to wheat α-thionin and to snakin- 1 (Table 4) (142). However, it did not play a role in *E. chrysanthemi* resistance to the defensin Pth-1 (no human homolog) or protamine (142). In addition, the overall virulence of the *sapABCDF* mutant was reduced compared to the wild type (142). This study highlighted two important aspects of Sap transporter biology. First, the Sap transporter confers resistance to APs across kingdoms (47, 142, 144, 146, 152, 183), indicating that it is evolutionarily conserved. Second, the transporter plays a role in AP resistance to only a subset of the APs that the bacterium was likely to encounter during infection, indicating that the Sap transporter binds APs with molecular specificity (142).

Vibrio fischeri Sap Transporter

Based on the data presented thus far, it would be easy to assume that the Sap transporter always confers AP resistance to those organisms in which it is expressed. In 2000, Chen et al. examined the genome of *Vibrio fischeri* and uncovered an ORFs with high levels of homology to the *sapD* and *sapF* ORFs of *E. coli* (47), *S. typhimurium* (183), and *H. influenzae* (146). From this homology, they concluded that "the *sap* operon in the genome enables *V. fischeri* enables [sic] to transport peptides and resist antimicrobial peptides." (47). However, they did not publish empirical data in support of this conclusion (47). In 2002, Lupp et al. published a study contradicting the conclusions of Chen (47). In this study, the authors calculated the MIC of eight different APs against both a polar *sapA* mutant and wild type *V. fischeri* and found the MICs to be identical for the two bacterial strains (144). In contrast, the protamine-susceptible *V. fischeri ompU* (outer membrane protein U) mutant control strain (4) was susceptible to each peptide (144). These data suggest that a functional SapABCDF system in *V. fischeri* does not confer an increased resistance to the APs tested (Table 4) (145).

The lack of an AP susceptible phenotype associated with the *V. fischeri sapA* mutant was unexpected (47, 144). From this study, Lupp et al. concluded that the Sap transporter may not confer resistance to APs in every bacterial system (144). The discrepancy of these studies highlights the need to carefully examine the function of the Sap transporter in each pathogen in which it is expressed. However, it is also important to note two important problems with this study. Six of eight peptides in the panel described in this study were synthetic and *V. fischeri* would not naturally encounter these peptides *in vivo.* Second, the presence of a functional OmpU within *V. fischeri* could mask the effects of the *sap* mutation, if they both play a role in AP resistance. Because the lack of an AP resistance phenotype in the *V. fischeri sapA* mutant was unexpected, Lupp et al. went on to characterize the true function of the Sap transporter in *V. fischeri* (144). The authors observed that when compared to the wild type, the *sapA* mutant displayed a reduced growth grate in rich medium that could not be rescued through the addition of carbon, nitrogen, or phosphorous (144). In addition, they observed a statistically significant reduction in the final population size of the bacterium grown in the host compared to

growth in medium, suggesting that the *V. fischeri sap* operon plays a role in host symbiosis (145). These studies are important to our understanding of Sap transporter biology as a whole because they indicate that a high level of homology between the Sap transporters of various species is not always indicative of a similar function in host pathogenesis, and further, that the Sap transporter may have functions outside the realm of AP resistance.

Homology of *H. ducreyi* Sap to Other Sap Transporters

The *H. ducreyi* genome encodes a *sap* operon consisting of five ORFs (Figure 2): *tyrR (HD1229), sapA (HD1230), sapB (HD1231), sapC (HD1232),* and *sapD (HD1235)* (162). *sapF (HD0863)* is encoded independently (162). The *H. ducreyi tyrR* gene is annotated as a transcriptional regulator of aromatic amino acid metabolism in the published *H. ducreyi* genome available at www.ncbi.nih.gov. The *H. ducreyi* SapABCD and SapF proteins are highly homologous to other SapABCD and SapF proteins reported in the literature (142, 144, 146, 152, 162, 183). The greatest levels of homology were found with *H. influenzae* (146), with percent identities as high as 65% and percent similarities as high as 82% (Table 5). Proteins with high levels of homology to *H. ducreyi* SapA have also been identified in the two organisms most closely related to *H. ducreyi: M. haemolytica* and *A. pleuropneumoniae.* However, while the genome of *A. pleuropneumoniae* also encodes strong homologs of the other Sap transporter components and may in fact express a functional Sap transporter, the closest homologs of SapB, SapC, and SapD in *M. haemolytica,* are annotated as Dpp transporter components. Studies investigating the functionality of the transporter system in these organisms have not been published.

Table 4. Sap transporter mutants and phenotypes

Species	Mutation	Susceptible	Resistant	Source	Structure	Reference
S. typhimurium						
	sapC^{T}	CGE*		Human	α,β	(183)
		Melittin		Insect	β	(183)
			Magainin	Amphibian	β	(183)
			NP-1	Rabbit	β	(183)
			Ceropin	Plant	β	(183)
			Mastoparan	Insect	α	(183)
	sapD^{T}	CGE*		Human	α,β	(183)
			Magainin	Amphibian	β	(183)
			NP-1	Rabbit	β	(183)
			Ceropin	Plant	β	(183)
			Mastoparan	Insect	α	(183)
	sapD^{T}	CGE*		Human	α,β	(183)
			Magainin	Amphibian	β	(183)
			NP-1	Rabbit	β	(183)
			Ceropin	Plant	β	(183)
			Mastoparan	Insect	α	(183)
	sapA^{NP}	Protamine		Fish	β	(183)
	sapF	Protamine		Fish	β	(183)
P. mirabilis	*sapD*	PM-B		Bacteria	α	(152)
			Protegrin Analog	Synthetic	β	(152)
H. influenzae	*sapA*^{NP}		LL-37	Human	α	(147)
			r-cBD-1	Chinchilla	β	(147)
			HBD-3	Human	β	(147)
	sapD		LL-37	Human	α	(146)
			r-cBD-1	Chinchilla	β	(146)
			HBD-3	Human	β	(146)
E. chrysanthemi	*sapABCDF*^{P}	Snakin-1		Plant	β	(142)
		α-thionin		Plant	β	(142)
			Protamine	Fish	β	(142)
			Pth-1 (defensin)	Plant	β	(142)
V. fischeri	*sapA*^{P}		CP11CN	Synthetic	Linear	(145)
			CP26	Synthetic	α	(145)
			CP28	Synthetic	α	(145)
			CP29	Synthetic	α	(145)
			LL-37	Human	α	(145)
			P-CN	Synthetic	α	(145)
			PM-B	Bacteria	α	(145)
			Protamine	Fish	β	(145)

*Crude Granulocyte Extracts, ^{T}Mutant generated by transposon mutagenesis
^{P}Polar mutation, ^{NP}Non-polar mutation

Table 5. Homology between *H. ducreyi* Sap proteins and other Sap proteins

Protein	SapA	SapB	SapC	SapD	SapF
S. typhimurium					
Percent Identity [a]	35%	37%	43%	59%	52%
Percent Similarity [b]	55%	60%	63%	77%	72%
E value[c]	4e-92	4e-54	2e-67	8e-122	3e-81
P. mirabilis					
Percent Identity	35%	36%	43%	59%	51%
Percent Similarity	53%	57%	64%	76%	74%
E value	4e-93	1e-58	5e-69	7e-120	8e-80
H. influenzae					
Percent Identity	44%	46%	52%	65%	59%
Percent Similarity	67%	67%	73%	82%	73%
E value	6e-143	2e-81	4e-90	8e-132	9e-90
E. chrysanthemi					
Percent Identity	35%	31%	44%	58%	51%
Percent Similarity	53%	54%	65%	78%	69%
E value	1e-91	3e-45	6e-61	6e-122	9e-78
V. fischeri					
Percent Identity	33%	38%	42%	56%	50%
Percent Similarity	52%	62%	63%	76%	70%
E value	2e-86	6e-62	8e-61	7e-115	5e-73
M. haemolytica					
Percent Identity	57%	66% (DppB)	64% (DppC)	77%(DppD)	73%
Percent Similarity	71%	82%	80%	89%	86%
E value	0	4e-126	1e-98	4e-167	1e-116
A. pleuropneumoniae					
Percent Identity	71%	79%	81%	88%	78%
Percent Similarity	82%	92%	90%	93%	90%
E value	0	1e-154	9e-132	0	1e-123

[a, b, c] Percent identity and percent similarity and E value as calculated by NCBI BLASTP 2.218+

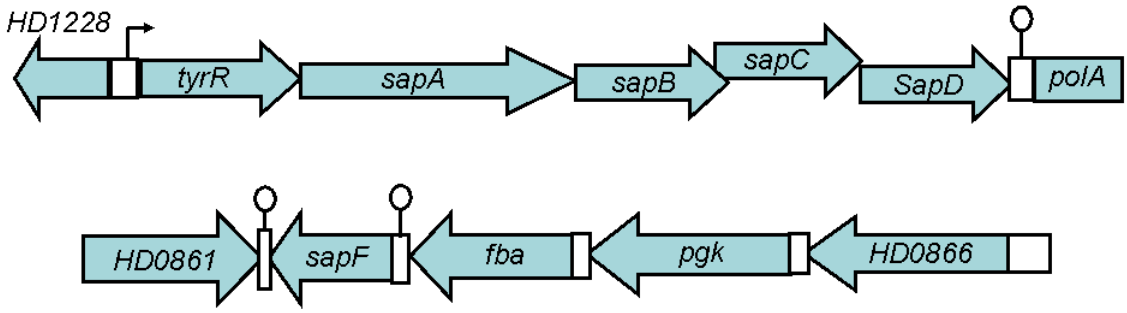


Figure 2. Genetic map of *sap*-containing loci in *H. ducreyi* 35000HP (www.ncbi.nih.gov). Putative operon containing *tyrR* and *sapABCD* located between ORFs HD1228 and HD1236, and unlinked *sapF*-containing locus between ORFs HD0861 and HD0866 and (www.ncbi.nih.gov). ORFs are drawn to scale, arrowheads show direction of transcription, white boxes represent intergenic sequences, arrows represent predicted promoters and ball-and-stick structures indicate predicted stem-loop transcriptional terminators.

Other Roles of the Sap Transporter

The role of the Sap transporter in AP resistance has been well characterized (142, 144, 146, 152, 183). However, because the transporter is homologous to other transporters involved in peptide uptake (183), it is possible that it also plays a role in nutrient acquisition. Several studies have been performed specifically examining the role of the SapD protein in pathogenesis (96, 146, 152, 185). In *Pasteurella multocida* the *sapD* gene is up-regulated in response to iron-containing compounds (185). The protein was also shown to assist potassium uptake in *E. coli* by acting as an ATPase for the TrkG/TrkH potassium uptake system (96) and it plays a role in potassium uptake in *H. influenzae*, possibly through the same system (146). Loss of SapD resulted in increased susceptibility to APs in *Proteus mirabilis* (152), *H. influenzae* (146), and *S. typhimurium* (81). In addition, the *H. influenzae sapD* mutant had greatly reduced virulence in the

middle ear, was unable to compete with the wild type for survival, and was significantly less fit than the *sapA* mutant (146).

Genetic Regulation of the Sap Transporter

At present there are no hard and fast rules concerning the regulation of the *sap* operon. Multiple systems have been shown to play a role in the regulation of the operon in different bacteria (68, 123, 146). In *E. chrysanthemi,* the expression of the *sap* operon is regulated by the nucleoid-associated protein Fis, which also regulates the expression of many other virulence factors of the bacterium (123). In *Salmonella,* the *sap* operon is regulated by the PhoP-PhoQ two-component system (68, 183). In contrast, *H. influenzae,* which lacks a PhoP-PhoQ regulatory system, has been shown to regulate the *sap* operon through the direct association of the SapA and APs. (146) This association initiates an as of yet undefined signaling event leading to increased *sap* gene expression and the production of Sap proteins required for resistance to APs (146). It is possible that the interaction of SapA with APs in *H. influenzae* could trigger the activation of a two component system other than PhoP-PhoQ (146). This regulatory system could mimic that of *Yersinia,* which resists the action of APs via an efflux pump/potassium antiporter system that is itself regulated by exposure to APs via the RosA/RosB two-component regulator (30).

The *H. ducreyi* Sap Transporter is Expressed during Human Infection

Bauer et al. published a study using the selective capture of transcribed sequences (SCOTS) to pinpoint genes important for *H. ducreyi* virulence (24). In this study, mRNA transcripts were amplified from both broth derived cultures and tissue derived biopsies. The sequences were competitively hybridized in order to selectively identify transcripts that were expressed *in vivo.* Transcripts that were expressed at higher levels in tissue than in broth were hypothesized to be important for *H. ducreyi* virulence. This analysis identified hmologs of *H. influenzae sapA*, *sapB*, and *sapF* genes (24). In order to further confirm the *in vivo* expression of *H. ducreyi sapA*, the same lab later performed nested

RT-PCR on RNA from *H. ducreyi*-infected pustules. The RNA transcripts were amplified in tissue-derived RNA, indicating that *H. ducreyi* expresses the *sap* genes at the pustular stage of disease.

HYPOTHESIS

Based on the previous literature concerning *H. ducreyi,* antimicrobial peptides, and the Sap transporter:

I hypothesized that *H. ducreyi* is resistant to the bactericidal effects of the APs likely to be found at the site of infection (160). The rationale for this hypothesis is derived from two sources. First, the literature indicates that *H. ducreyi* is able to both establish and maintain an infection in an environment known to be rich with APs (22, 25). Second, other bacterial pathogens have been reported to express mechanisms enabling them to resist the bactericidal effects of these peptides (118).

I hypothesized that the putative *H. ducreyi* Sap transporter plays a role in *H. ducreyi* resistance to APs. The rationale for this hypothesis is derived from three sources. First, the *H. ducreyi* genome encodes an operon with high levels of homology to the previously defined *sap* operon of *H. influenzae* (147, 162). Second, the Sap transporter has been reported in the literature to confer resistance to a wide variety of bacterial species including *S. typhimurium, E. chrysanthemi, P. mirabilis,* and *H. influenzae* (81, 142, 146, 147, 152, 183). Third, the *H. ducreyi* Sap transporter was shown to be expressed in chancroidal pustules, indicating that it is expressed *in vivo* (24).

The following studies were undertaken to directly address these hypotheses.

METHODS

All methods described in this section are also detailed, with minor modification, in either Mount et al. (2007) (160) or Mount et al. (2009).

Bacterial Strains and Growth Conditions

H. ducreyi strains (kindly provided by S.M. Spinola) (Table 6) were grown on chocolate agar plates at 33°C with 5% CO_2, for strains 35000HP, HD183, and HD188, or at 30°C with 10% CO_2, for strains CIP542 ATCC, HMC112, and DMC164. 35000HP and CIP542 ATCC cultures were grown with aeration at 33°C in brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) broth containing 0.1% soluble starch (Fisher Scientific, Itasca, Ill.), 1% IsoVitaleX, and 50 µg of hemin (Aldrich Chemical Co., Milwaukee, Wis.) per ml, or in gonococcal broth (15% proteose peptone (BD, Sparks, Maryland), 4% K_2HPO_4, 2% KH_2PO_4, 10% NaCl) supplemented with 10% Fetal Bovine Serum (HyClone, Logan, Utah), 1% IsoVitaleX, and 50 µg of hemin (Aldrich Chemical Co.) per ml (234). *E. coli* ML35 (ATCC# 43827) was grown on Luria Bertani (LB) plates (203). *E. coli* cultures were grown in LB broth at 37°C with aeration. *H. influenzae* 86-028NP, 86-028NPΔ*sapA,* and 86-028NPΔ*sapA*/p*sapA* (kindly provided by K. Mason) were grown on chocolate agar plates at 33°C with 5% CO_2. All *H. influenzae* cultures were grown statically in BHI broth containing 0.2% hemin and 0.2% β-nicotinamide adenine dinucleotide hydrate (Sigma, St. Louis, MO) at 37°C with 5% CO_2.

Sources of Peptides

Recombinant α– and β-defensins were purchased from PeproTech Inc. (Rocky Hill, N.J.), Sigma Aldrich, Peptides International (Louisville, Ken.), and AnaSpec (San Jose, Calif.). Synthetic LL-37 was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, Calif.).

Table 6. Bacterial strains used in this study

Strain	Genotype or Geographic Origin	Source
***E. coli* Strains:**		
HB101	*F*, *thi*-1, *hsd*S20 (r_B, m_B), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (*str*^{r}), *xyl*-5, *mtl*-1	Promega
Top 10	*F- mcrA* Δ*(mrr-hsdRMS-mcrBC)φ80lacZ* Δ*M15* Δ*lacX74recA1ara*Δ*139* Δ*(ara-leu)7697galUgalK rpsL (strR) endA1 nupG*	Invitrogen
***H. ducreyi* Strains:**		
35000HP	Class I; human-passaged (HP) variant of strain *H. ducreyi* 35000HP, Winnipeg, Canada, 1975	(10, 88, 247)
HD183	Class I clinical isolate, Singapore, 1982	(23, 216)
HD188	Class I clinical isolate, Kenya, 1982	(23, 216)
CIP542 ATCC	Class II clinical isolate, Hanoi, Vietnam, 1954	(88, 247)
H MC112	Class II clinical isolate, Origin Not reported, 1984	(247)
DMC164	Class II clinical isolate, Bangladesh Origin and year not reported	(247)
35000HP*sapA*	35000HP*sapA::*kan	This study
35000HP*sapA*/p*sapA*	35000HP*sapA::*kan/p*sapA*	This study

96-well AP Bactericidal Assay

Bacteria were grown to mid-logarithmic phase in gonococcal broth, harvested by centrifugation, washed three times, and suspended in 10 mM sodium phosphate pH 7.4 supplemented with 0.1% BHI (bacterial diluent). Approximately 10^3 cfu of bacteria were mixed with the indicated concentration of peptides in wells of a 96-well polypropylene plate (Costar 3790). All peptide concentrations were tested in duplicate wells. The bacteria and peptides were incubated for one hour at 33°C (*H. ducreyi*) or 37°C (*E. coli*). After incubation, the concentration of bacteria remaining in the wells was determined by

plate count. Results from duplicate wells were averaged, and survival in the presence of APs was calculated as a percentage of survival in control wells without APs. Assay results were subjected to a mixed model analysis in which data were log transformed and modeled with fixed effects for group, concentration, and the group by concentration interaction and included a random effect for sample, so that the correlation of percentages calculated from the same sample were incorporated. The Sidak adjustment was used to control for multiple comparisons and P values < 0.05 were considered to be statistically significant.

Radial Diffusion Assay

H. ducreyi strains were grown for 24 hours on chocolate agar as described above. Approximately 10^7 bacteria scraped from a confluent plate were suspended in a nutrient-poor and salt-free underlay gel containing 0.15% proteose peptone (Difco Laboratories) and 1% agarose (Sigma 6013). Serial half-log dilutions were made for each peptide, and either peptide or peptide diluent was added to 3-mm wells in the underlay. After a three hour incubation period at 33°C (*H. ducreyi*) or 37°C (*E. coli*), a nutrient-rich overlay containing 15% proteose peptone, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.5% NaCl, 0.1% soluble starch (Fisher Scientific) and 1% agar (Difco Laboratories) was added to each plate, and the plates were incubated for another 24 (*E. coli* ML35) or 48 (*H. ducreyi)* hours. The zones of inhibition associated with each well were measured and expressed in units (0.1 mm = 1 U) (70). The diameter (3 mm = 30U) of the well was subtracted from the unit calculation (70). For each experiment, peptides were tested in duplicate.

Polymerase Chain Reaction

Polymerase Chain Reactions (PCR) were conducted using HiFi high fidelity taq polymerase (Invitrogen, Carlsbad, CA) and a PCR Sprint Thermal cycler (Thermo Electron Corporation, Waltham, MA). Reactions were initialized with a 94°C, 2 min melting cycle. Standard reactions used 30 amplification cycles including a 94°C, 30 second melting cycle, a 59°C, 30 second annealing cycle, and a 72°C, 2 minute extension

cycle. Standard reactions concluded with a 72°C, 10 min extension time. Individual reactions necessitated the adjustment of the annealing temperature to account for primer melting temperatures and the adjustment of the extension time based on the size of the anticipated product. All primers were ordered from the Integrated DNA Technologies Company (Coralville, IA). Reactions included a DNA template positive control and a negative control lacking a template. Primers are located in Table 7.

Reverse Transcription PCR

RNA was isolated from mid-log cultures of *H. ducreyi* using the trizol reagent (Invitrogen). The isolated RNA was DNAse treated using a DNA-free kit (Ambion, Austin, TX) and complementary DNA (cDNA) was synthesized using random hexamer primers and an Advantage RT for PCR kit (BD, Palo Alto, CA). Standard PCR was performed on the resultant cDNA. All RT-PCR reactions included a DNA template positive control, a negative control lacking a template, and a negative control that did not receive reverse transcriptase. Primers are located in table 7.

Quantitative RT-PCR

RNA was isolated as described above and quantitated using a Nanodrop UV/Vis spectrophotometer (Thermo Electron Corporation). Primers were designed for *HD1643* (DNA gyrase subunit B), which served as a reference gene, and *H. ducreyi sapD,* the last gene in the *sap* operon. *HD1643* was selected as a control because the expression level of this housekeeping gene was not expected to vary between the wild type and mutant. The cycle threshold was calculated for 10-fold serial dilutions of DNA using each primer set. The linear best fit line was calculated for each primer set and primer efficiencies were calculated as $E = 10^{(1/\text{slope of line})}$. The primer efficiencies were high and reproducible at: 94%, 95%, and 87% for *HD1643* and 94%, 90%, and 99% for *sapD*. The relative expression ratio of the *sapD* and *HD1643* genes, for both 35000HP and 35000HP*sapA,* was calculated as a means to determine if the expression level of *sapD* had changed relative to that of *HD1643*, after the introduction of the kanamycin resistance cassette

into 35000HP*sapA*, thus ensuring that the mutation was non-polar. The relative expression ratio was determined by the Pfaffl method (191): $[E_{target}^{(\Delta Ct\ target\ (control\text{-}treated))}] / [E_{reference}^{(\Delta Ct\ reference\ (control\text{-}treated))}]$. Primers located in table 7.

Southern Blot Analysis

Digoxigenin (Dig) labeled DNA probes specific for the kanamycin resistance cassette, the deleted portion of *sapA,* and 5' end of *tyrR* were synthesized using a PCR Dig Probe Synthesis Kit (Roche, Nutley, NJ) (Primers, Table 7). Genomic DNA was isolated from *H. ducreyi* 35000HP and 35000HP*sapA* using standard methods (203). 5 μg of each DNA preparation was digested with AgeI (New England Biolabs, Ipswich, MA). The digested DNA preparations and 0.5 μl of each probe (positive control) were separated by gel electrophoresis and transferred to a nitrocellulose membrane by capillary transfer (203). The DNA was UV crosslinked and a Dig detection kit (Roche, Nutley, NJ) was used to prepare the membrane for detection. Dig labeled probes were boiled and incubated with the filter at 42°C O/N. The filter was washed extensively and a Dig blocking solution was used to block non-specific binding of the probes before the addition of chemiluminescent substrate. Bands appearing on film were compared to a standardized ladder for sizing.

DNA Sequencing

Genomic DNA was isolated from putative *sapA* mutants using standard methods (203). Primers (Table 7) were designed to bind within the kanamycin resistance cassette and sequence outward into the *sapA* gene and to bind within the flanking genes of the operon and sequence into *sapA*. DNA sequences were obtained by the Indiana University School of Medicine DNA Sequencing Core Facility using a Perkin Elmer / Applied Biosystems 3100 Genetic Analyzer and Big Dye Terminator chemistry v3.1 system. DNA sequences were analyzed by Chromas lite software, version 2.01.

Table 7. Primers Used in PCR, RT-PCR, qRT-PCR, southern, and sequencing

Primer	Sequence
PCR Primers	
SapADelFor1	CGGATAATTCGGTGTTGGCGCATT
SapADelRev1	AAACGCTCGCGGTTAATTGCTTGG
SapAMutFor1	TGCGTACGGCAACTGAGAATAGGT
SapAMutRev1	AACGCCTTGGTTTACGCCAACA
pSap001	TTGAGGCATCGTTGTTACGCCGTT
KanRev2	CCTTTGGAACAGGCAGCTTTCCTT
KanFor2	GCTTTATTGATCTTGGGAGAAGCGGC
pSap004	AATGATTTAATGGATCACGCAA
RT-PCR Primers	
pSap001	TTGAGGCATCGTTGTTACGCCGTT
pSap002	GCGGAATAAGCGGTCGAAATCCAA
pSap003	CTCAGCGTAGCTTGTTTGTC
pSap004	AATGATTTAATGGATCACGCAA
pSap005	TAATGCGATTTCTGCCGGTGTGGT
pSap006	GCCACTTTATCTTGCCGTAACTCAAGCC
pSap007	CAAAGTCCAATGCCAGAATGG
pSap008	GCCCTGTTGGCGTATCAATTTC
pSap009	GGCAGAATTTGAGCCTGTAGTCGT
pSap011	TACGACCGCAATATGGCTCGGATT
SapDFor1	TAAGTGGTGGCAGTGGTTTGGTTG
SapDRev1	AAACGAGGTTGATTAGCCACTGCC
qRT-PCR Primers	
SapDFor2	GCATTGAAATTGATACGCCAACAGGGCGAG
SapDRev2	TCCGCGGTCACAATCCACTCATCTTTCATC
1643For1	TGAAGGGCTTGTTGCGGTGATTTC
1643Rev1	TCCGCTAAACAAGGTCGTGATCGT
Dig Probe Primers	
TyrRSouthernFor	CACAGCTTGATGCGCCCTTATTGA
TyrRSouthernRev	AAGTCGCCTTGTCGCTCACGTAAT
KanSouthernFor	ACCACCTATGATGTGGAACGGGAA
KanSouthernRev	AATGTCATACCACTTGTCCGCCCT
SapAdeleatedFor	CGGATAATTCGGTGTTGGCGCATT
SapAdeletedRev	AAACGCTCGCGGTTAATTGCTTGG
Sequencing Primers	
KanFor2	GCTTTATTGATCTTGGGAGAAGCGGC
KanRev2	CCTTTGGAACAGGCAGCTTTCCTT
pSap001	TTGAGGCATCGTTGTTACGCCGTT
pSap004	AATGATTTAATGGATCACGCAA

Comparative Growth Curves

H. ducreyi 35000HP and *H. ducreyi* 35000HP*sapA* were streaked from freezer stocks onto Chocolate agar plates. A single colony was used to inoculate 20 ml gonococcal broth supplemented with 10% Fetal Bovine Serum (HyClone, Logan, Utah), 1% IsoVitaleX, and 50 µg of hemin (Aldrich Chemical Co.) per ml that was incubated for 16 hours in a 33°C water bath with shaking. 30 ml overnight cultures were diluted to an OD 660nm: 0.065. OD readings were taken every 60 minutes and 1ml was removed every two hours for plate counts. Plates were incubated in a 33°C incubator with 5% CO_2 for 48 hours. Plate counts were performed in triplicate and used to calculate the colony forming units / ml of the culture.

Construction of 35000HP*sapA*

Primers used to generate constructs are listed in Table 8. All plasmids were maintained in *E. coli* TOP10. Plasmids were passed through *E. coli* HB101 before introduction into *H. ducreyi* to increase transformation efficiency into *H. ducreyi* (225). A 517 bp fragment containing the 5' end of *HD1230 (sapA)* was PCR amplified from *H. ducreyi* 35000HP genomic DNA. The DNA fragment was TA cloned into pGEM T-Easy (Promega, Madison, Wis.) to generate pMEB078. A 489 bp fragment containing the 3' end of *HD1230* was PCR amplified from genomic DNA and TA cloned into p-GEM T-Easy (Promega) to generate pMEB080. A non-polar kanamycin resistance cassette from pUC18K2 (153), was inserted downstream of the 5-prime fragment of *sapA* in MEB078 to generate pMEB092. The s*apA'-kan*^{R} fragment of pMEB092 was cloned upstream of the 3-prime region of *sapA* in pMEB080, generating pMEB098. In this construct, 676 bp of *sapA* was replaced with the Kan^R cassette.

The *sapA'-kan*^{R}*-'sapA* fragment was subcloned into pRSM2072, which expresses *lacZ* and acts as a suicide vector in *H. ducreyi* (40). As described above, the vector was passed through *E. coli* HB101 before introductioin into *H. ducreyi* by electroporation (178) In the pRSM2072 system, *lacZ* expression is detectable in by O-nitrophenyl-β-d-galactoside hydrolysis (40). The growth rate of the bacteria grown in the absence of 5-

bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) is unaffected by this reaction (40). However, in the presence of X-gal, colonies appear blue and there is a dramatic reduction in the growth rate due to the expression of the *lacZ* gene encoded on the pRSM2072 backbone (40). The successful completion of a double crossover is phenotypically detectable by the appearance of white healthy colonies (40) Kanamycin resistant transformants were repetitively passed on chocolate agar plates containing X-gal, in order to select for colonies in which the wild type allele had been successfully replaced by the mutagenized allele (40).

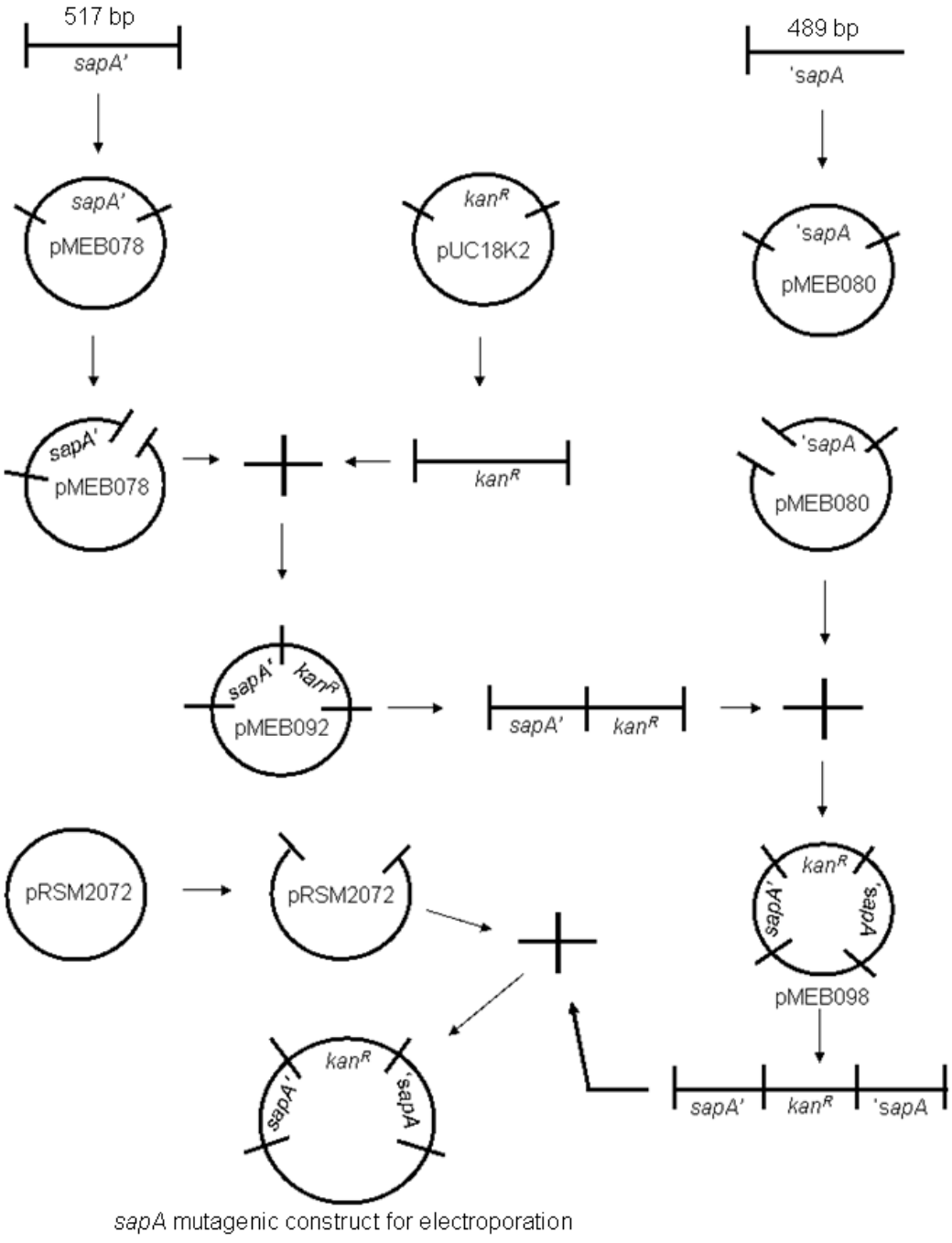
Generation of 35000HP*sapA*/p*sapA*

To complement 35000HP*sapA in trans,* the s*apA* ORF and its putative native promoter were expressed in *H. ducreyi* shuttle vector pLSKS (252) (Figure 4). BPROM software (www.softberry.com) was used to identify the putative promoter located 177 bp upstream of *tyrR* in an untranslated region. The *sapA* ORF was PCR-amplified and TA cloned into pGEM-T-Easy (Promega) to generate pMEB108. The 5' primer used to amplify *sapA* also inserted the nucleotide sequence "CAT" immediately upstream of the *sapA* start codon in order to introduce an NdeI restriction digest site (CATATG) at the 5' end of the *sapA* gene. 237 bp of the putative promoter containing intergenic region located upstream of *tyrR* was PCR-amplified with primers that also introduced an NdeI site at the 3' end of the fragment. The putative promoter region was TA cloned into pSC-A (Stratagene, Cedar Creek, TX) to generate pMEB114. The putative promoter was cut away from pMEB114, digested with NdeI, and then ligated with NdeI-digested pMEB108 to generate pMEB115. In this construct, the *sapA* ORF lies immediately downstream of the untranslated region upstream of *tyrR*. The construct was subcloned into pLSKS to produce pMEB120. pMEB120 was electroporated into *E. coli* HB101 and subsequently introduced into 35000HP*sapA* by electroporation. Transformants were selected for on plates containing both kanamycin and streptomycin. RT-PCR was used to verify that the complemented *sapA* gene was transcribed *in vitro* (data not shown).

Table 8. Primers Used in *sapA* Mutagenesis

Primer	Construct	Sequence*
5'SapA Forward	pMEB078	CATATCtctagaGCGGATAGCTTAATTTAT TGCACCAG
5'SapA Reverse	pMEB078	CATATCggtacc*CATATCctcgagGAATCTT TACTGTATATTCATTCGGTGCTG
3'SapA Forward	pMEB080	CATATCtctagaGCGGATAGCTTAATTTATTGC ACCAG
3' SapA Reverse	pMEB080	TAAACGAACTTGCCCGAATGGCTC
SapAcompFor1	pMEB120	GATGTATCATTACTCACTAATATCCCTGCT
SapAcompRev1	pMEB120	CGGTCGAAATCCAACAGAACACAG
SapApromoterFor1	pMEB120	CCTTTAATTTGTTCTAAATACATAATGATCC
SapApromoterRev1	pMEB120	CATATCcatatgTCTTTTCGCCTTAATTTAAGC

*****Underlined regions correspond to restriction digest sites generated during PCR. In pMEB078 a XbaI recognition site was added to the construct by 5'SapA Forward and KpnI and XhoI sites were added to the construct by 5'SapA Reverse. In pMEB080 a KpnI site was added to the construct by 3'SapA Forward. In pMEB120 an NdeI site was added to the construct by SapApromoterRev1.



sapA mutagenic construct for electroporation

Figure 3. Generation of *H. ducreyi sapA* mutant

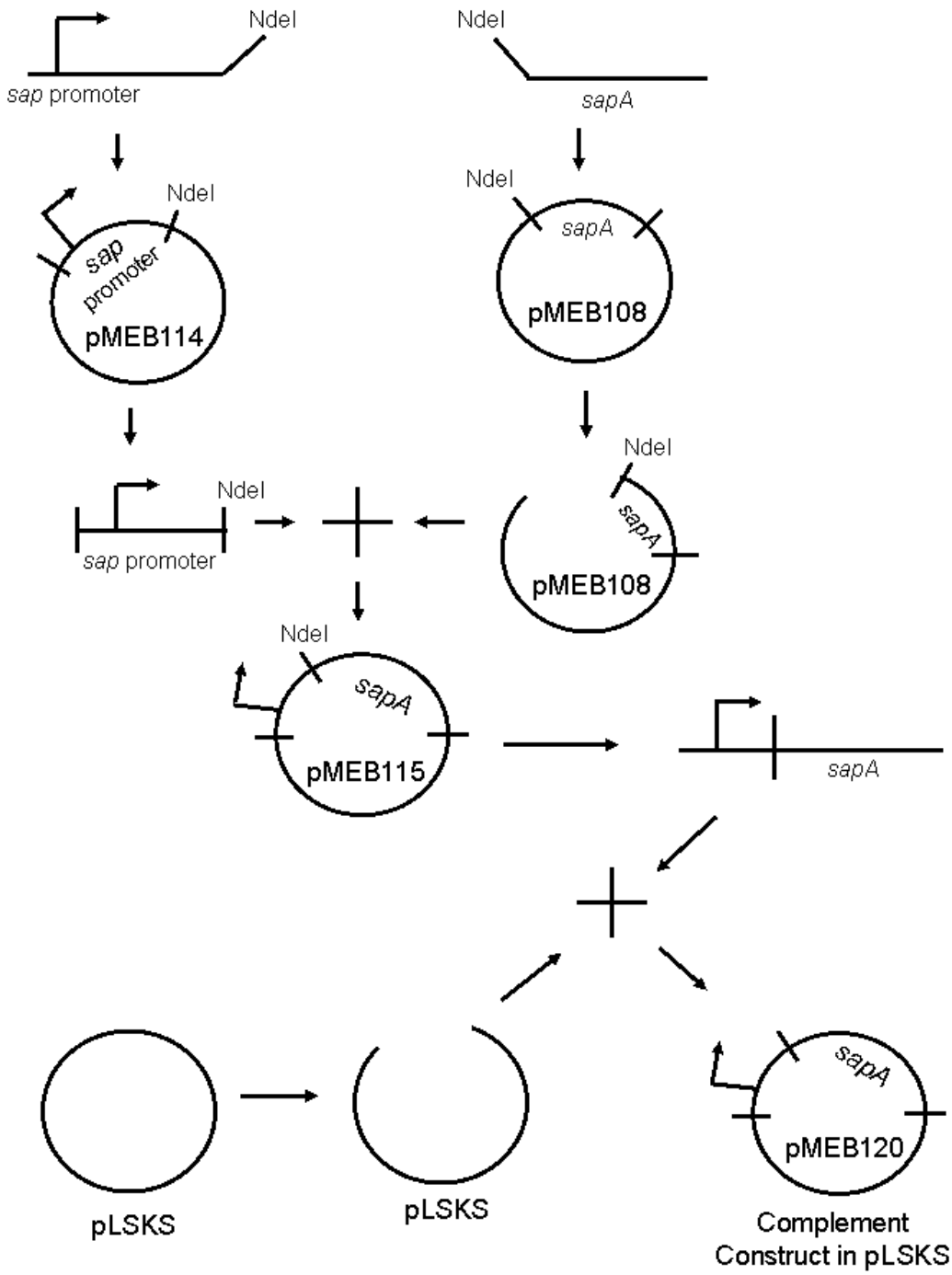


Figure 4. Generation of *H. ducreyi sapA* complement

Table 9. Plasmids used in study

Plasmid	Description	Source or reference
pGEM-T Easy	TA cloning vector, *Kan*^{R}	Promega
pSC-A	TA cloning vector, *Amp*^{R}	Stratagene
pCR XL Topo	TA cloning vector, *Amp*^{R}	Invitrogen
pUC18K2	Source of non-polar, *Kan*^{R}	(153)
pRSM2072	*H. ducreyi* suicide vector	(40)
pMEB078	5' end of *HD1230* in pGEM-T Easy	This study
pMEB080	3' end of *HD1230* in pGEM-T Easy	This study
pMEB092	5' end of *HD1230* + *Kan*^{R} cassette in pGEM-T Easy	This study
pMEB098	5' end of *HD1230* + *Kan*^{R} cassette + 3' end of *HD1230* in pGEM-T Easy	This study
pMEB101	5' end of *HD1230* + *Kan*^{R} cassette + 3' end of *HD1230* in pRSM2072	This study
pMEB114	*sap* operon promoter in pSC-A	This study
pMEB108	*sapA (HD1230)* ORF in pCR XL Topo	This study
pMEB115	*sap* promoter ligated to *sapA* ORF in pCR XL Topo	This study
pMEB120	Putative *sap* operon promoter region + *sapA* in pLSKS	This study
pLSKS	*H. ducreyi* shuttle vector	(252)

RESULTS

SECTION I: *H. DUCREYI* IS RESISTANT TO HUMAN APS

All data and text in this chapter have been previously published, with minor modifications, in Mount et al. (2007) (160).

Validation of 96-well AP Bactericidal Assay

A 96-well AP bactericidal assay was used to measure the bactericidal effects of APs against *H. ducreyi* and *E. coli* ML35, a control strain shown in the literature to be susceptible to many APs (173, 177). We tested the activity of each peptide over a 100-fold range that encompasses most concentrations at which resistance to APs has been demonstrated and included estimated *in vivo* concentrations (130, 199). As was discussed in the introduction, the AP concentration in the skin has been estimated to range from nanograms to milligrams, depending on the AP (39).

Previous work by Fortney et al. demonstrated that *H. ducreyi* is susceptible to killing by PG-1, a porcine AP with no human homolog (70). We therefore used PG-1 to establish the ability of our assay to detect the bactericidal activity of APs. As expected, both *H. ducreyi* 35000HP and *E. coli* ML35 were effectively killed by PG-1 (Table 10), demonstrating that the bactericidal assay can detect killing of *H. ducreyi* by APs.

Table 10. Survival of *E. coli* ML35 and *H. ducreyi* 35000HP exposed to PG-1 (160)

[PG-1]	*E. coli* ML35	*H. ducreyi* 35000HP
0.2 μg/ml	0 ± 0.007[a]	0.62 ± 0.29
2.0 μg/ml	0 ± 0	0 ± 0
20 μg/ml	0 ± 0	0 ± 0

[a] Mean percent survival ± standard error of three independent assays

H. ducreyi Susceptibility to Human α-defensins

H. ducreyi infection leads to a rapid and dense PMN infiltrate (27, 101, 180). α-defensins HNP-1-3 are expressed by PMNs in natural ulcers (22). We therefore tested *H. ducreyi*

susceptibility to α–defensins HNP-1-3. *E. coli* ML35, which has been shown previously to be sensitive to a variety of APs, was used as a control for AP activity (108, 131). *E. coli* ML35 was sensitive to all three HNPs tested, demonstrating only 10-30% survival in the presence of HNPs at 20 μg/ml (Figure 5A-C). In contrast, *H. ducreyi* 35000HP exhibited greater than 90% survival at all concentrations tested (Figure 5A-C). The differences in survival of the two bacteria were statistically significant.

During natural infection of women, *H. ducreyi* likely encounters the α- defensin HD-5, secreted by the squamous epithelium of the vagina (199). We therefore tested *H. ducreyi* susceptibility to HD-5. HD-5 was more potent than HNP-1-3 against *E. coli*, with less than 1% survival at 20 μg/ml and 16% survival at 2 μg/ml (Figure 5D). In contrast, greater than 88% of *H. ducreyi* survived these concentrations of HD-5 (Figure 5D). Taken together, our data demonstrated that *H. ducreyi* 35000HP was significantly more resistant than the control strain to the bactericidal effects of α-defensins (Figure 5).

H. ducreyi Susceptibility to Human β-defensins

Both *H. ducreyi* and the small wounds required to initiate infection act as inflammatory stimulators of keratinocytes at the site of infection, stimulating the expression and secretion of the β-defensins HBD-2, -3, and -4 (139, 212). HBD-2 is also expressed and secreted by macrophages and PMNs that are recruited to the site of infection (59, 242). In addition, HBD-1 is constitutively expressed by both macrophages and keratinocytes (59, 94).

We evaluated the susceptibility of *H. ducreyi* to HBD-2-4 and found that *H. ducreyi* 35000HP was significantly more resistant than *E. coli* ML35 to 20 μg/ml of HBD-2,-3, and -4 and 2 μg/ml of HBD-3 and -4 (Figure 6. Less than 7% of *E. coli* ML35 survived exposure to 20 μg/ml of each β-defensin. In contrast, significantly more *H. ducreyi* (25-66%) survived the same dose of peptide (Figure 6A-C). At the 2 μg/ml concentration of

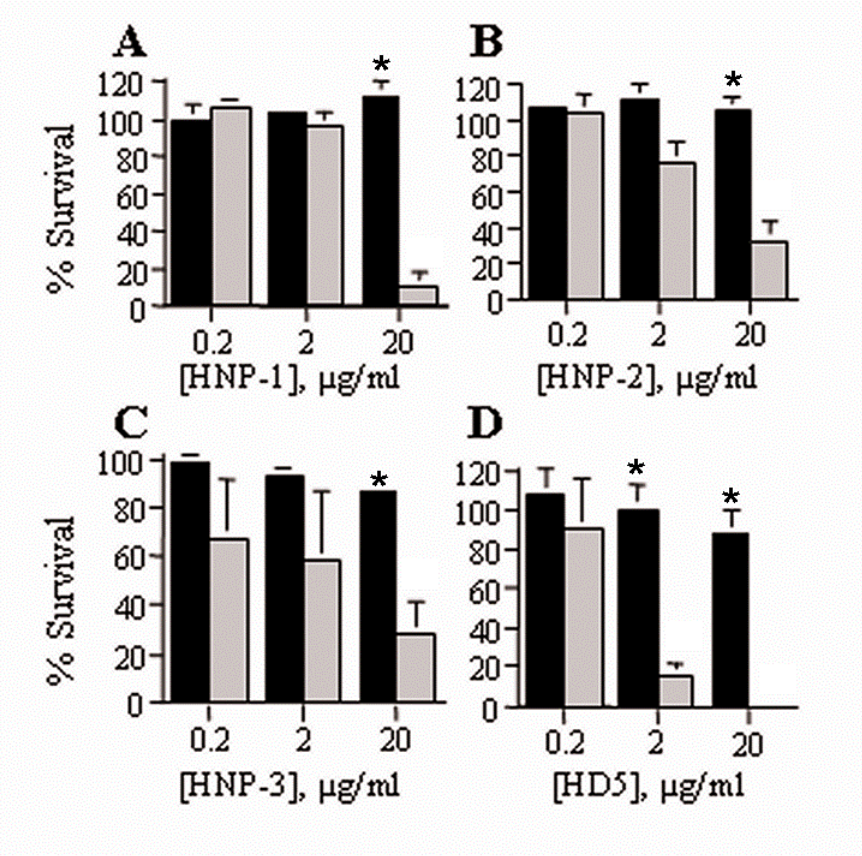


Figure 5. ***H. ducreyi*** **is resistant to α-defensins (160).** Percent survival of bacteria exposed to α-defensins (A) HNP-1, (B) HNP-2, (C) HNP-3, and (D) HD-5. *H. ducreyi* 35000HP is shown in black bars and *E. coli* ML35 is shown in grey bars. Data represent the mean ± standard error for three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP, at P <0.0001 (HNP-1 and HD-5), P =0.0017 (HNP-2), and P =0.0335 (HNP-3).

HBD-3 and HBD-4, the survival of *E. coli* was 25% or 30%, respectively, while at least 84% of *H. ducreyi* 35000HP survived incubation with 2 μg/ml of HBD-3 or HBD-4 (Figure 6B-C). At the highest concentrations tested in our assay, the bactericidal effects of the β-defensins were more potent than the effects of the α-defensins against *H. ducreyi* 35000HP. However, in each case, *H. ducreyi* 35000HP was significantly more resistant than *E. coli* ML35 to the bactericidal effects of HBD-2-4 (Figure 6). We were unable to determine the susceptibility of *H. ducreyi* to HBD-1 because both recombinant and synthetic HBD-1, purchased from three different sources, failed to demonstrate bactericidal activity against *E. coli* ML35 in our assays (data not shown).

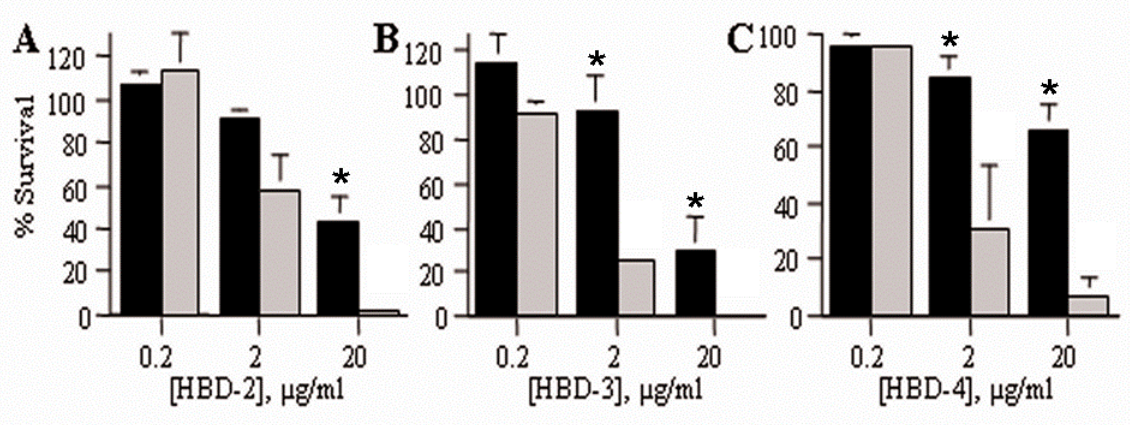


Figure 6. *H. ducreyi* is resistant to β-defensins (160). Percent survival of bacteria exposed to β-defensins (A) HBD-2, (B) HBD-3, and (C) HBD-4. *H. ducreyi* 35000HP is shown in black bars and *E. coli* ML35 is shown in grey bars. Data represent the mean ± standard error for three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP at $P < 0.0001$ (HBD-2, HBD-3, and HBD-4 at 20 μg/ml) and $P = 0.0089$ (HBD-4 at 2 μg/ml).

H. ducreyi Susceptibility to the Human Cathelicidin, LL-37

Only one cathelicidin, LL-37, is produced by humans. This AP is expressed and secreted by macrophages, PMNs, and keratinocytes in response to inflammatory mediators (6, 115, 141). We evaluated the susceptibility of *H. ducreyi* to this peptide. LL-37 had the most potent bactericidal activity against *E. coli* ML35 of all peptides tested. Similarly, *H. ducreyi* 35000HP was much more susceptible to the bactericidal effects of LL-37 than to either the α-defensins or the β-defensins. However, survival of *H. ducreyi* (16%) was significantly greater than survival of *E. coli* (5%) at the 2 μg/ml dose of LL-37 ($P < 0.001$) (Figure 7A).

To define the differences in susceptibility between *E. coli* ML35 and *H. ducreyi* 35000HP to LL-37 in more detail, we exposed the bacteria to 2-fold serial dilutions of LL-37 across a concentration range spanning complete killing of bacteria (4 μg/ml) to at least 90% survival of bacteria (0.125 μg/ml). When the bacteria were exposed to 0.5 μg/ml of LL-37, survival of *H. ducreyi* (43%) was significantly higher than survival of *E. coli* (3%) ($P = 0.0036$) (Figure 7B). Thus, while the overall levels of killing produced by LL-37 appeared qualitatively to be higher than those observed with the other APs tested, our data demonstrate that *H. ducreyi* was significantly more resistant to killing by LL-37, relative to the killing observed when *E. coli* ML35 was exposed to the peptide (Figure 7).

Class I and Class II *H. ducreyi* Strains are Resistant to APs

H. ducreyi as a species shows limited genetic and phenotypic diversity (247). However, based on differences in OMP profiles and LOS migration patterns, two classes of *H. ducreyi* strains have been described (247). We compared the susceptibility of representative members of the two classes of *H. ducreyi* to each class of AP. The class I strain, 35000HP, and the class II strain, CIP542 ATCC, were each exposed to a 100-fold concentration range of α-defensin HNP-1, β-defensin HBD-3, and LL-37. Our data demonstrate that the class I and class II strains of *H. ducreyi* are each resistant to the bactericidal effects of the APs tested (Figure 8). While 35000HP was statistically more resistant than CIP542 ATCC to HBD-3, and CIP542 ATCC was statistically more

resistant than 35000HP to LL-37, both strains exhibited resistance to all of the APs tested (Figure 8). These findings indicate that AP resistance may represent a conserved mechanism of *H. ducreyi* survival.

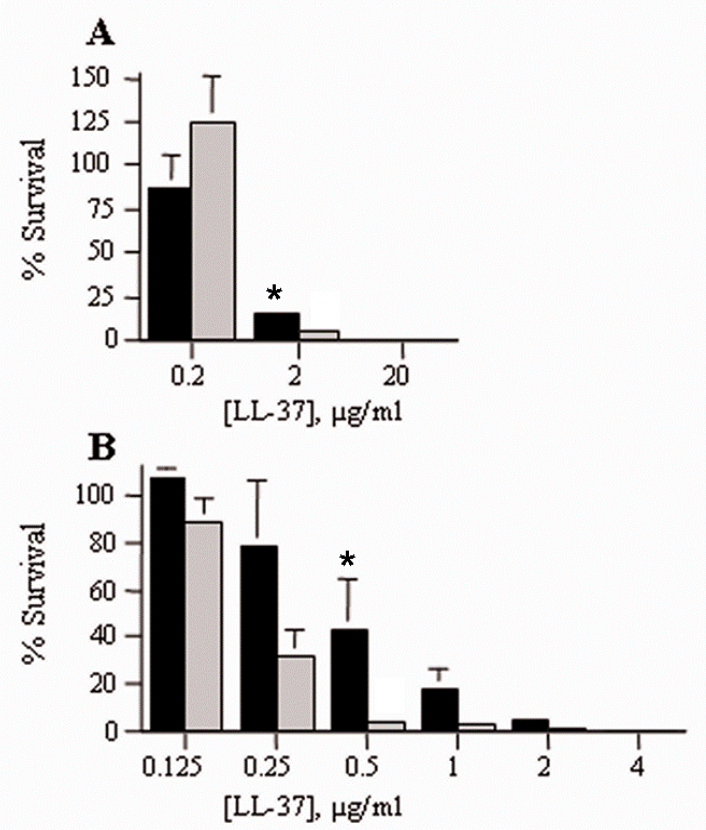


Figure 7. *H. ducreyi* is resistant to the human cathelicidin LL-37 (160). Percent survival of bacteria exposed to (A) 10-fold and (B) 2-fold serial dilutions of the human cathelicidin LL-37. *H. ducreyi* 35000HP is shown in black bars and *E. coli* ML35 is shown in grey bars. Data represent the mean ± standard error for three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP, with $P<0.0001$ (A) and $P=0.0012$ (B).

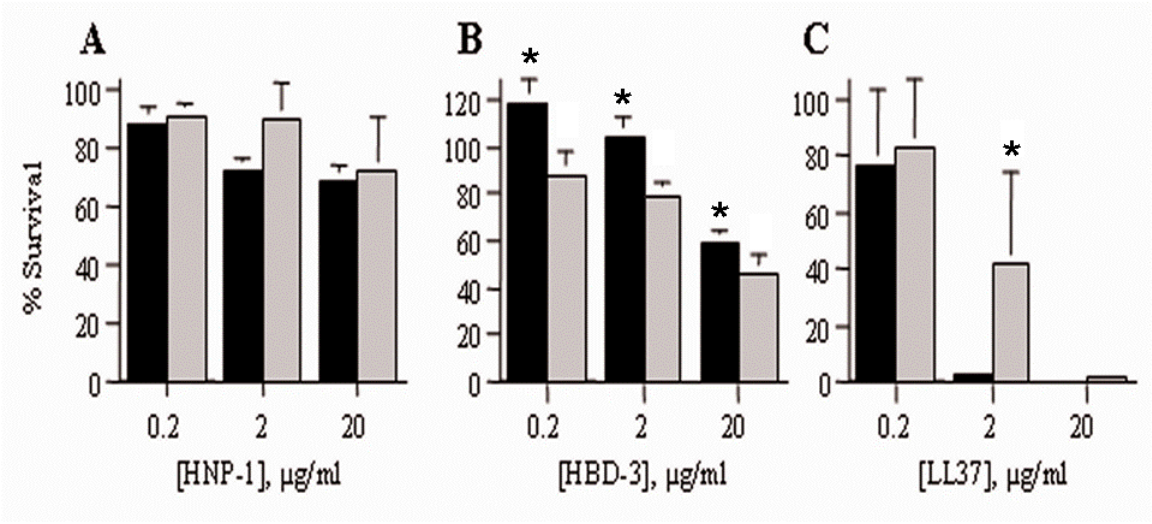


Figure 8. Representative class I and class II *H. ducreyi* strains are resistant to APs (160). Susceptibility of class I 35000HP and Class II CIP542 ATCC to (A) α-defensin HNP-1, (B) β-defensin HBD-3, and (C) cathelicidin LL-37. Class I results are shown in black, and class II results are shown in grey. Data represent the mean ± standard error for three independent assays. Asterisks represent statistically significant differences between strains at the indicated concentration of AP, with $P < 0.02$ for HBD-3 and $P = 0.0036$ for LL-37.

Minimum Inhibitory Concentration of Antimicrobial Peptides

Although *H. ducreyi* was significantly less susceptible than *E. coli* to AP activity (Figure 5-7), some killing of *H. ducreyi* was observed with some β-defensins and LL-37 (Figure 6-7). We therefore assessed the relative activities of these APs against *E. coli* and both class I and class II *H. ducreyi* in a radial diffusion assay (RDA), which provided a more quantitative dose-response curve and allowed for calculation of a minimal effective concentration (MEC) of each peptide against each bacterial strain.

.

As in previous studies, the MEC of each peptide against each bacterial strain was defined as the x-intercept of plots of AP activity over the AP concentration range (70, 220). If fewer than two peptide concentrations exhibited activity against a bacterial strain, no x-intercept could be defined, and the MEC was estimated as > 158 μg/ml, the upper limit of measurable MEC in this assay. The statistical differences among MECs were calculated using Students' *t*-test (Table 11).

All peptides exhibited activity against *E. coli* ML35, with MECs ranging between 2 and 18 μg/ml (Table 11). The MEC of PG-1 against the *H. ducreyi* strains was not significantly different than PG-1 activity against *E. coli* (Figure 9A, $P = 0.1$, two-tailed Students' *t*- test). These results are consistent with a previously published report that *H. ducreyi* is susceptible to killing by PG-1 (70).

In the 96-well bactericidal assay, human cathelicidin LL-37 showed greater activity than other APs against *H. ducreyi* (Figure 7). In the RDA, activity of LL-37 was significantly lower against *H. ducreyi* than against *E. coli* ($P < 0.0001$) (Figure 9B). The MEC of LL-37 was 6.2 μg/ml against *E. coli* ML35 but >158 μg/ml against *H. ducreyi* strains (Table 11). These data confirm that *H. ducreyi* is less susceptible than *E. coli* ML35 to LL-37 mediated activity.

In contrast to porcine protegrin, the human β-defensins demonstrated much less activity against the *H. ducreyi* strains than against *E. coli* ML35 (Figure 9C-E, Table 11). The MECs of human β-defensins against *H. ducreyi* exceeded the upper limits of the assay, with little to no activity even at a peptide concentration of 500 μg/ml (Figure 9C-E). However, the MECs of the β-defensins against *E. coli* were less than 20 μg/ml. The overall activity of each β-defensin, calculated by comparing the slopes of each inhibition curve, was significantly lower against 35000HP than against *E. coli* ML35 ($P < 0.0001$) (Figure 9). These data are consistent with the results of our 96-well AP bactericidal assay (Figure 6), which demonstrated that *H. ducreyi* was significantly more resistant than *E. coli* to killing by human β-defensins.

The RDAs were performed with both class I and class II strains of *H. ducreyi*. The two strains showed similar levels of susceptibility to all peptides tested, with both strains being relatively susceptible to killing by PG-1 and resistant to killing by human APs (Figure 9, Table 11). These data support the conclusion that resistance to AP-mediated killing is a phenotype common to both known classes of *H. ducreyi* strains.

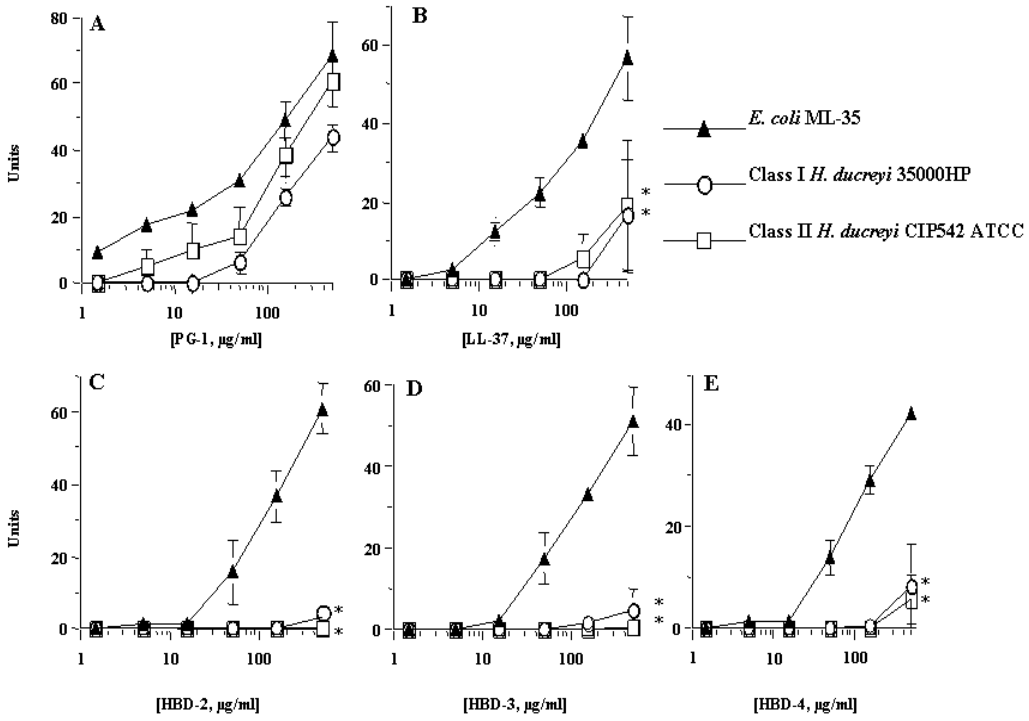


Figure 9. AP-mediated inhibition of bacterial growth (160). Serial dilutions of the indicated peptides were tested for activity against class I *H. ducreyi* 35000HP (open circles), class II *H. ducreyi* CIP542 ATCC (open squares), and *E. coli* ML35 (filled triangles). Data represent the mean ± standard error for three independent assays, each performed in duplicate. The units of inhibition shown on the y-axis represent the diameter of the zone of inhibition surrounding the well, minus the diameter of the well itself. Asterisks represent statistically significant differences in peptide activity compared with *E. coli* ML35 ($P < 0.001$).

Table 11. Minimum effective concentrations of APs (160)

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

[a] MEC in μg/ml, calculated as the *x*-intercept of the best fit line. Data are the mean MEC from three independent assays, each performed in duplicate.
[b] If less than two peptide concentrations exhibited activity, no x-intercept could be defined, and the MEC was reported as >158 μg/ml, the upper limit of measurable MEC in this assay.

AP Resistance is Conserved within the Species

In order to confirm that the AP resistance phenotype was conserved within the species, we used an RDA to calculate the MEC of LL-37 against three class I and three class II strains of *H. ducreyi.* The MEC of LL-37 against each strain exceed the upper limit of measurable MEC in the assay, and was thus reported as >158 μg/ml (Table 12). Because AP resistance is conserved within the species, the phenotype could represent a putative *H. ducreyi* virulence factor.

Table 12. AP resistance is conserved within the species

H. ducreyi strain	Class	MEC LL-37 (μg/ml)
35000HP	I	>158 μg/ml
HD183	I	>158 μg/ml
HD188	I	>158 μg/ml
CIP542 ATCC	II	>158 μg/ml
HMC112	II	>158 μg/ml
DMC64	II	>158 μg/ml

RESULTS

SECTION II: THE *H. DUCREYI* SAP TRANSPORTER PLAYS A ROLE IN AP RESISTANCE

All data and text described in this chapter have been prepared for publication, with only minor modification, in Mount et al. (2009).

Our data indicate that *H. ducreyi* is resistant to the bactericidal effects of the APs likely to be found at the site of infection. This AP resistance phenotype is conserved within the species and could thus represent a putative *H. ducreyi* virulence factor. As such, we examined the *H. ducreyi* genome (available at www.ncbi.nih.gov) for evidence of regions of homology to other known AP resistance mechanisms. We uncovered an operon with high levels of homology to the previously described *sap* operon of *S. typhimurium, P. mirabilus, H. influenzae, E. chrysanthemi,* and *V. fischeri* (Table 5) (142, 144, 147, 152, 183). *sapA, sapB,* and *sapF* transcripts were identified as putative virulence determinants though the use of the SCOTS procedure, which compares the transcription levels of genes in broth derived cultures and tissue derived biopsies (24). We therefore designed a strategy to mutagenize the *sapA* gene *(HD1230)* of *H. ducreyi* as a means to examine the role of the Sap transporter in *H. ducreyi* AP resistance. We choose to mutate *sapA* specifically, as previous studies with *S. typhimurium* and *H. influenzae* have indicated that the loss of *sapA* dramatically decreases the functionality of the transporter as a whole (147, 183). By adopting this mutagenesis strategy we have hypothesized that the loss of of *sapA* in *H. ducreyi* will phenotypically correspond to a loss of all AP resistance functionality of the transporter in the organism.

Construction of a *sapA* Mutant in *H. ducreyi*

We used RT-PCR to map the operon structure of the *H. ducreyi sapABCD* locus in broth culture and found that the four contiguous *sap* genes, along with the upstream *tyrR*, are co-transcribed (Figure 10). We therefore used a non-polar kanamycin resistance cassette (154) in the construction of the 35000HP*sapA* mutant in order to ensure that transcription

of the downstream genes was not disrupted. The s*apA* mutation in 35000HP*sapA* was confirmed by PCR analyses (Figure 11), DNA sequencing, and southern blotting (Figure 12). 35000HP and 35000HP*sapA* demonstrated similar growth rates in broth (Figure 13). Standard RT-PCR (Figure 14) was performed in order to confirm that *sapD,* the most downstream gene of the *sap* operon, was transcribed after the mutagenesis procedure was complete. Quantitative RT-PCR was performed to ensure that the transcription level of *sapD* was unaffected by the mutagenesis procedure. The expression level of *sapD* in 35000HP and 35000HP*sapA* was normalized to the expression level of the housekeeping gene *HD1643* (DNA gyrase B) and the expression level of *sapD* was compared between the two stains. The expression level of *HD1643* was expected to be unaffected by the mutagenesis procedure and thus served as a reference for gene expression in our system. Three assays were performed, yielding an average relative expression ratio of 1.44 (*sapD* to *HD1643*) with a standard error of 0.19. These data indicate that *sapD,* the most downstream gene of the *sapABCD* operon, is equally expressed in 35000HP and 35000HP*sapA,* and that the *sapA* mutation in 35000HP*sapA* is non-polar.

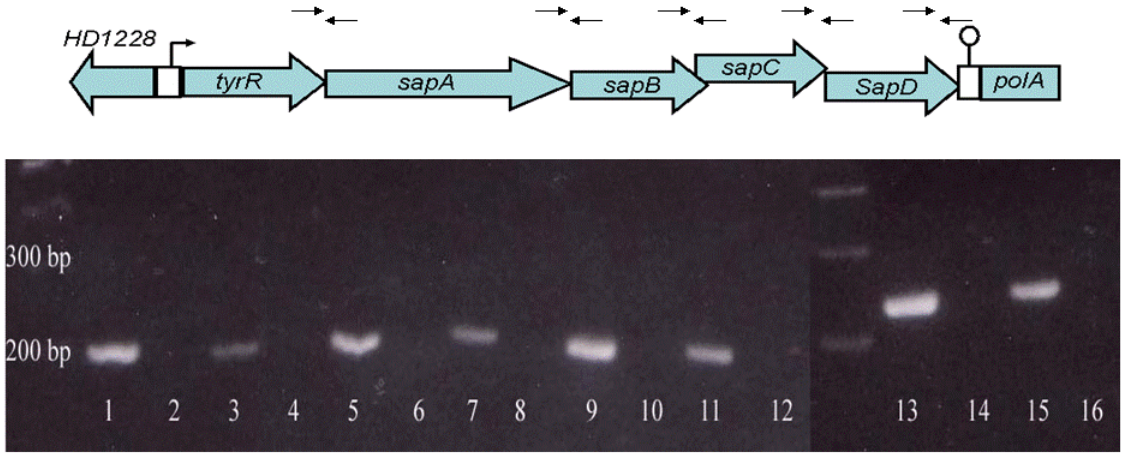


Figure 10. The *sap*-containing locus of *H. ducreyi* is transcribed as an operon. A. Representation of *sap* operon. B. RT-PCR analysis was used to determine if the *sap* containing mRNA encoded a poly-cistronic message. Lanes 1, 5, 9 and 13 contain DNA as a template. Lanes 2, 6, 10 and 14 lack a template. Lanes 3, 7, 11 and 15 contain cDNA. Lanes 4, 8, 12 and 16 contain RNA that was not reverse transcribed. Lanes 1-4 used primers that spanned the *tyrR/sapA* junction. Lanes 5-8 used primers that spanned the *sapA/sapB* junction. Lanes 9-12 used primers that spanned the *sapB/sapC* junction. Lanes 13-16 used primers that spanned the *sapC/sapD* junction.

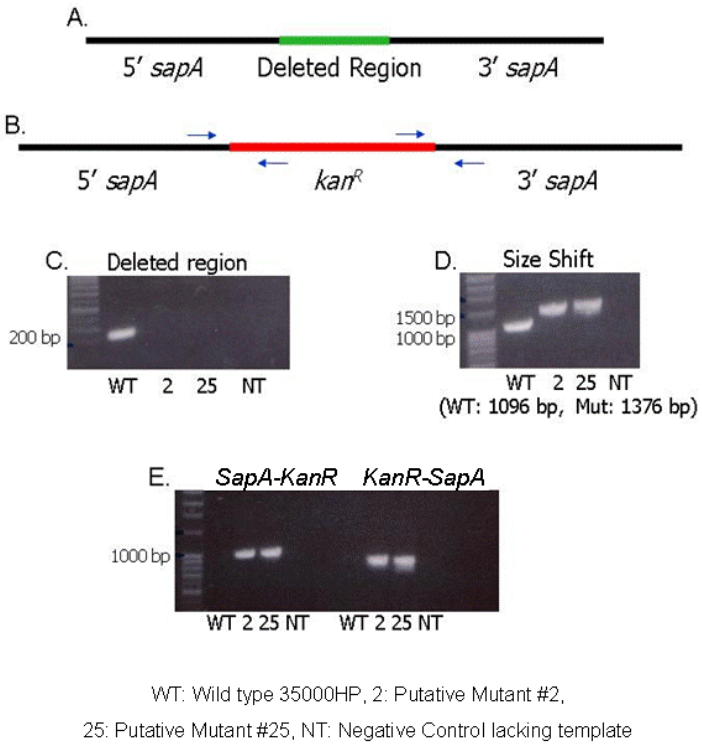


Figure 11. PCR Confirmation of 35000HP*sapA*. PCR was used to characterize two putative 35000HP*sapA* mutants (2 and 25). Prior to analysis, kanamycin resistant transformants were propagated on chocolate agar plates containing X-gal, in order to identify colonies that had undergone allelic exchange. (A) A representation of the WT *H. ducreyi* genome compared to (B) the expected conformation of 35000HP*sapA*. The kanamycin resistance cassette is larger than the DNA sequence deleted from the WT *sapA* gene, resulting in a size shift that can be detected by PCR primers binding outside the kanamycin resistance cassette. (C) Products of PCR reaction amplifying *sapA* deleted region. (D) Products of PCR reaction detecting size shift between WT and *sapA* mutant genomes. (E) Products of PCR reaction using primers that amplify across the junction between the *H. ducreyi sapA* gene and the kanamycin resistance cassette.

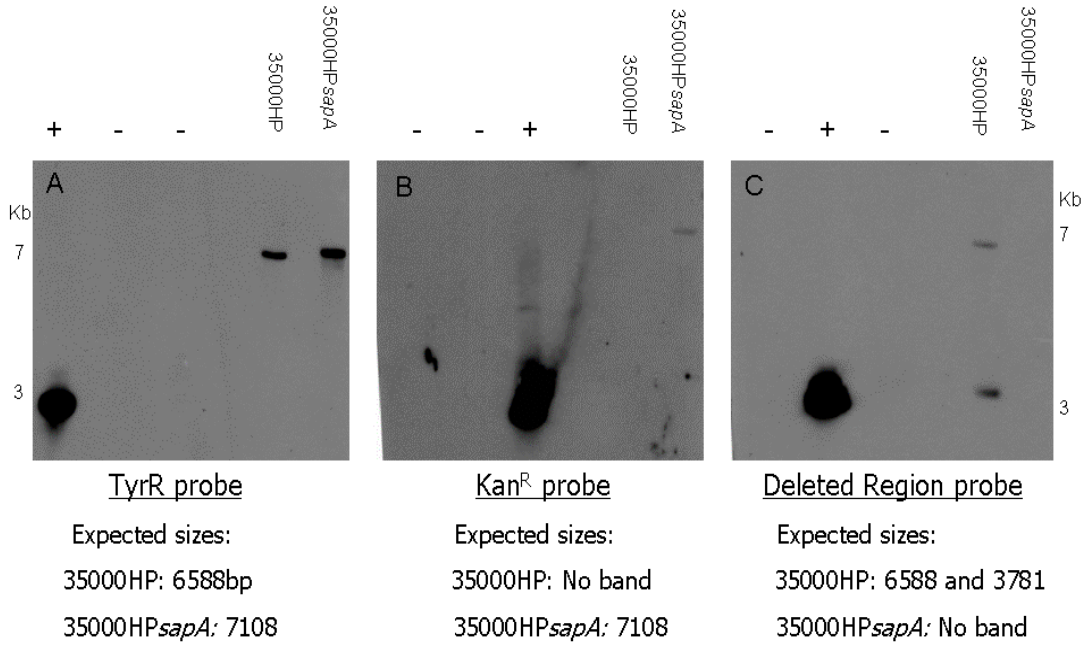


Figure 12. Southern analysis of 35000HP*sapA*. A southern analysis was performed to verify that the kanamycin resistance cassette had inserted into only one location in the 35000HP genome in the creation of 35000HP*sapA*. Genomic DNA was digested, separated by electrophoresis, and transferred to a nitrocellulose membrane. Blots were probed with probes specific for *tryR,* the *kan*^{R} cassette, or the deleted region of 35000HP. As a positive control for each blot, the probe was shown to bind to itself. As a negative control for each blot, the probe was shown to not bind to the other probes used in the study.

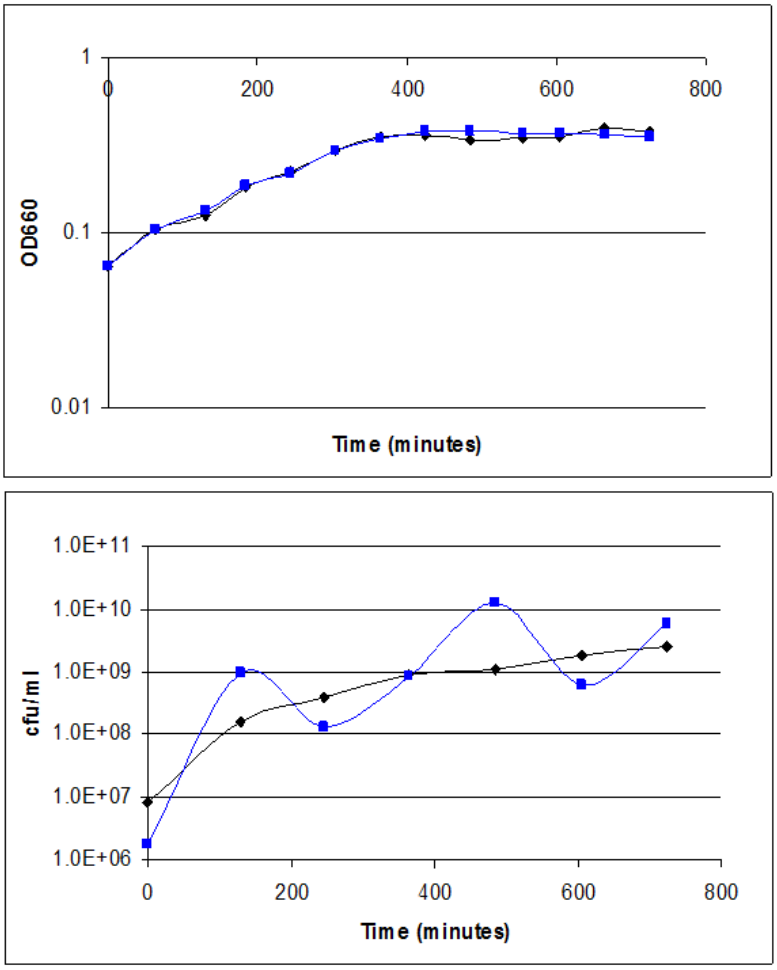


Figure 13. The 35000HP*sapA* mutation did not affect *H. ducreyi* growth *in vitro*. Mid-log cultures of *H. ducreyi* 35000HP (Black diamond) and 35000HP*sapA* (Blue square) were grown in a 33°C water bath. The OD660 nm was measured every 60 minutes. One ml of culture was removed every two hours to perform plate counts. Plate counts were performed in triplicate and results were averaged. Data shown is representative of three independent assays.

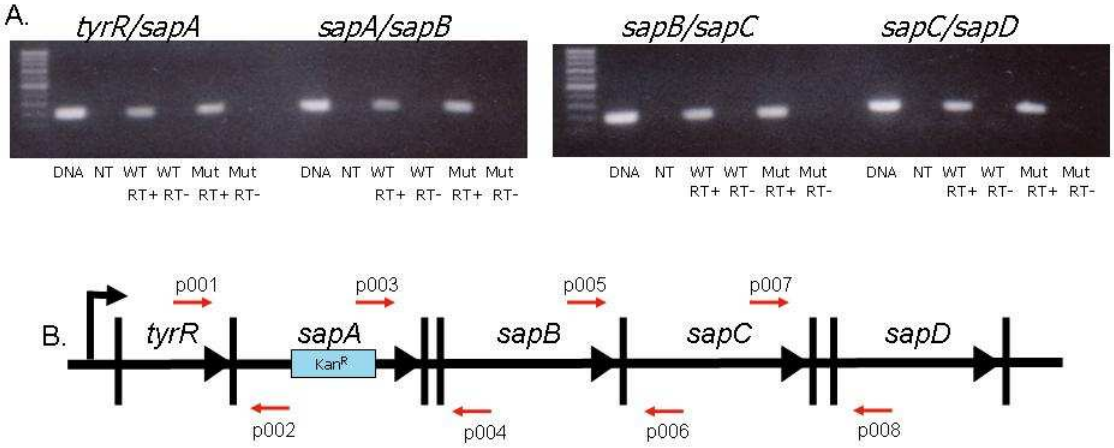


Figure 14. RT-PCR confirmation of putative 35000HP*sapA* mutant. RT-PCR analysis of *sap*-containing locus was used to determine if the 35000HP*sapA* mutation altered the transcription of the *sap* operon encoded poly-cistronic message. NT: Sample lacking template. WT: Wild type *H. ducreyi* 35000HP. Mut: 35000HP*sapA*. RT+: RNA sample received reverse transcriptase. RT-: RNA sample that did not receive reverse transcriptase. p001-p008: primers 001-008.

The *H. ducreyi* Sap transporter Confers Resistance to LL-37

A 96-well AP bactericidal assay comparing the survival of *H. ducreyi* 35000HP, 35000HP*sapA,* and the *sapA* complement, 35000HP*sapA*/p*sapA,* was performed in order to ascertain what role the Sap transporter played in *H. ducreyi* resistance to LL-37. In an assay with 10-fold serial dilutions of LL-37, 35000HP was significantly more resistant than 35000HP*sapA* to LL-37 at all but the lowest concentrations (Figure 15A). An assay with two-fold serial dilutions of LL-37 was performed to more specifically characterize the effect of the *sapA* mutation on *H. ducreyi* resistance to LL-37. Wild-type *H. ducreyi* was found to have significantly higher levels of survival than the *sapA* mutant in the presence of between 0.25 μg/ml and 2 μg/ml LL-37 (Figure 15B). Statistically significant complementation was observed at LL-37 concentrations as high as 2 μg/ml. This finding indicates that the observed differences in LL-37 susceptibility of the parent and mutant are due to the activity of SapA. These data suggest that *H. ducreyi* resistance to LL-37 is due, at least in part, to the activity of the Sap transporter.

The *H. ducreyi* Sap Transporter does not Confer Resistance to β-defensins

H. ducreyi is exposed to multiple cellular sources of β-defensins at the site of infection (74) and our data demonstrate that the bacterium is resistant to the bactericidal effects of HBD-2, HBD-3, and HBD-4 (Figure 6) (160). In this study, we examined the role of the Sap transporter in *H. ducreyi* resistance to these β-defensins. In a 96-well AP bactericidal assay with 10-fold serial dilutions of peptide, there was no discernable difference in AP resistance levels of 35000HP, 35000HP*sapA,* and 35000HP*sapA/*p*sapA* exposed to the β-defensins HBD-2, HBD-3, or HBD-4 (Figure 16). This finding indicates that the *H. ducreyi* Sap transporter is not involved in the bacterium's resistance to the β-defensins tested.

Mason et al. previously reported that the *H. influenzae sapA* mutant is susceptible to HBD-3, a finding that contradicts our findings with *H. ducreyi* (147). The assay was repeated with *H. influenzae* 86-028NP and 86-028NP*sapA* (kindly provided by Kevin Mason). In these assays, the *H. influenzae sapA* mutant was more susceptible than the

wild type to 0.2 μg/ml and 2 μg/ml HBD-3 (data not shown), a finding that correlates with the previously published results (147). Taken together, these data indicate that there could be differences in the ligand specificity of the *H. ducreyi* and *H. influenzae* Sap transporters.

The *H. ducreyi* Sap Transporter Does not Confer Resistance to α-defensins

H. ducreyi is exposed to PMN derived α-defensins at all stages of disease progression (22), and *H. ducreyi* is likely exposed to the α-defensin HD-5 during vaginal and cervical infection (199). We have demonstrated that *H. ducreyi* is resistant to the bactericidal activity of these peptides (Figure 5) (160). However, we found no significant difference in the rate of survival of 35000HP, 35000HP*sapA* and 35000HP*sapA*/p*sapA* after exposure to increasing concentrations of HNP-1, HNP-2, and HD-5 (Figure 17). This finding suggests that the Sap transporter does not confer *H. ducreyi* resistance to the α-defensins surveyed.

The *sapA* Gene is Conserved among Both Class I and Class II *H. ducreyi*

Two phenotypic classes of *H. ducreyi* have been described (247). Because both classes of *H. ducreyi* are resistant to APs (160), Carisa Townsend of the Bauer lab examined whether a panel of clinical isolates would also harbor the *sapA* gene. PCR was performed using *sapA*-specific primers designed from the 35000HP sequence. The PCR primers were internal to the open reading frame of the gene. *SapA* was amplified from genomic DNA of all ten clinical isolates tested, including six class I stains and four class II stains. All amplimers co-migrated with the corresponding amplimer in 35000HP genomic DNA. Thus, the Sap transporter may be important for AP resistance in both classes of *H. ducreyi* and could act as an *H. ducreyi* virulence factor.

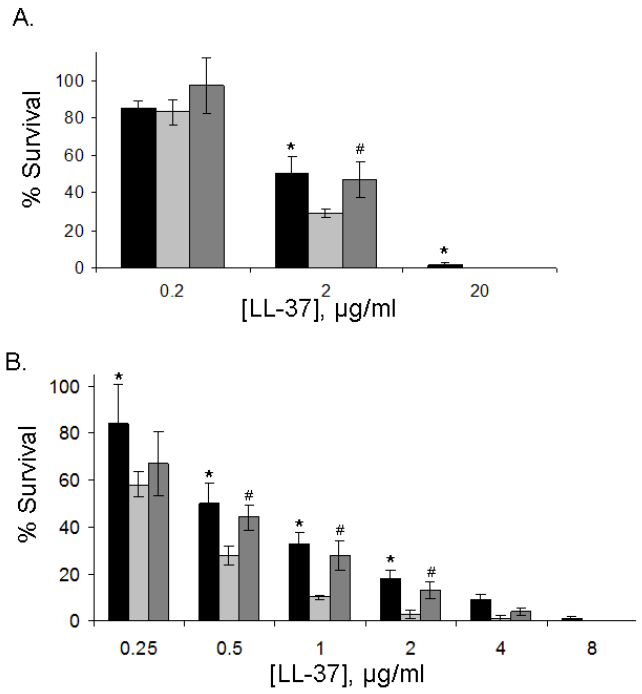


Figure 15. The *H. ducreyi* Sap transporter confers resistance to LL-37. Bactericidal assay comparing the percent survival of *H. ducreyi* 35000HP, 35000HP*sapA*, and 35000HP*sapA*/p*sapA* exposed to LL-37 for 1 hour. Data represent the mean ± standard error for three independent assays. Black bars: 35000HP. Light gray bars: 35000HP*sapA*. Dark gray bars: 35000HP*sapA*/p*sapA*. Asterisks represents that 35000HP has a significantly greater mean percent of control than 35000HP*sapA*. Pound sign represents that 35000HP*sapA*/p*sapA* has a significantly greater mean percent of control than 35000HP*sapA*. Figure 15A: Bactericidal assay with a 10-fold serial dilution of LL-37. In comparison of 35000HP and 35000HP*sapA,* for 2 µg/ml, $P = 0.0032$. For 20 µg/ml, $P=0.0059$. In comparison of 35000HP*sapA*/p*sapA* and 35000HP*sapA,* $P = 0.0009$. Figure 15B: Bactericidal assay with a 2-fold serial dilution of LL-37. In comparison of 35000HP and 35000HP*sapA,* for 0.25 µg/ml, $P = 0.01$. For 0.50 µg/ml, $P < 0.0001$. For 1.0 µg/ml, $P < 0.0001$. For 2.0 µg/ml, $P < 0.0001$. In comparison of 35000HP*sapA*/p*sapA* and 35000HP*sapA,* for 0.5 µg/ml, $P = 0.0009$. For 1 µg/ml, $P < 0.0001$. For 2 µg/ml, $P < 0.0001$.

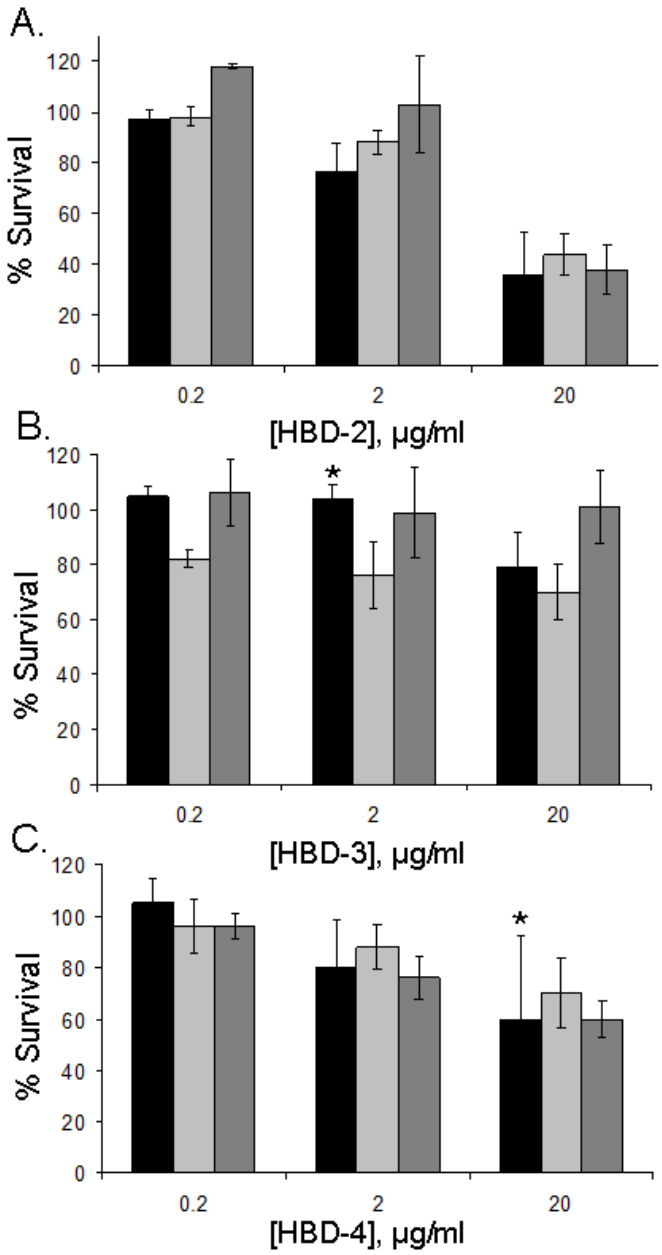


Figure 16. The *H. ducreyi* Sap transporter does not confer resistance against β-defensins. Bactericidal assay comparing the percent survival of *H. ducreyi* 35000HP, 35000HP*sapA*, and 35000HP*sapA*/p*sapA* exposed to HBD-2 (a), HBD-3 (b) or HBD-4 (c) for 1 hour. Data represent the mean ± standard error for three independent assays. Black bars: 35000HP. Light gray bars: 35000HP*sapA*. Dark gray bars: 35000HP*sapA*/p*sapA*. For HBD-3, $P = 0.0157$. For HBD-4, $P = 0.0039$.

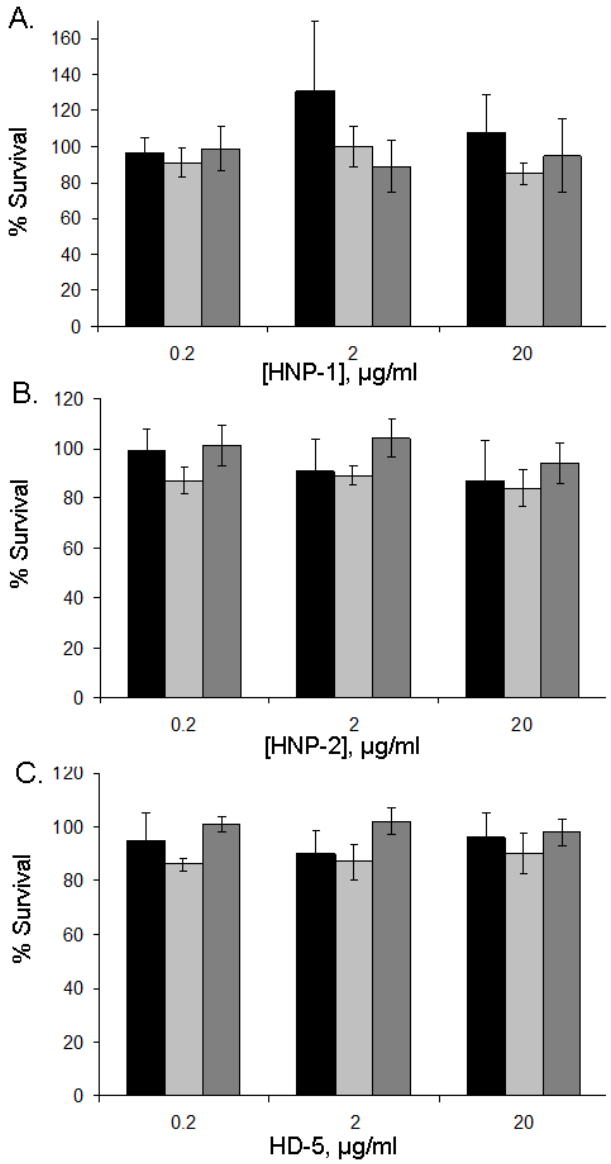


Figure 17. The *H. ducreyi* Sap transporter does not confer resistance against α-defensins. Bactericidal assay comparing the percent survival of *H. ducreyi* 35000HP, 35000HP*sapA*, and 35000HP*sapA*/p*sapA* exposed to HNP-1 (a), HNP-2 (b) or HD-5 (c) for 1 hour. Data represent the mean ± standard error for three independent assays. Black bars: 35000HP. Light gray bars: 35000HP*sapA*. Dark gray bars: 35000HP*sapA*/p*sapA*. No statistical difference was observed between parent and mutant for HNP-1, HNP-2 or HD-5, at any concentration. $P > 0.05$ for all comparisons.

DISCUSSION

Portions of the data analysis detailed in the following section has also been published in Mount et al. (2007) (160) and will be submitted for publication in Mount et al. (2009).

H. ducreyi is a human pathogen (159) which facilitates the transmission of HIV in endemic areas (101, 116, 201). As such, it is important to understand how *H. ducreyi* interacts with the immune system. Because *H. ducreyi* is able to both establish and maintain an infection in an environment rich with APs (22, 25), we hypothesized that the organism would exhibit an AP resistance phenotype. Section I of the Results demonstrates that *H. ducreyi* is resistant to the three major classes of APs found in human skin. We further demonstrate that the AP resistance phenotype is conserved within the species, suggesting that AP resistance could represent a virulence factor for the bacterium. We examined the *H. ducreyi* genome for evidence of homology to other known AP resistance mechanisms and found a strong homolog of the well characterized Sap transporter. Section II of the Results explores the role of this transporter in AP resistance. These data demonstrate that the Sap transporter plays a role in the resistance of *H. ducreyi* to the cathelicidin LL-37. However, because the Sap transporter did not play a role in *H. ducreyi* resistance to many other APs likely to be encountered by the bacterium *in vivo,* we have now hypothesized that additional resistance mechanisms may be present within the bacterium.

In vivo, *H. ducreyi* encounters PMNs, macrophages, and keratinocytes at the site of infection (25, 27). In the presence of bacterial stimulation, PMNs actively secrete the α-defensins HNP-1-4, the β-defensin HBD-4, and the cathelicidin LL-37 (67, 236). Upon stimulation by bacteria, keratinocytes show increased levels of both the expression and the secretion of the β-defensins HBD-2-4 and the cathelicidin LL-37 (115, 139). Inflammatory mediators further induce the expression of HBD-2 and LL-37 from macrophages (59, 139). Finally, because chancroid is a genital ulcer disease, we also investigated the susceptibility of *H. ducreyi* to HD-5, an α-defensin found in the vaginal

mucosa (199). Our results demonstrate that *H. ducreyi* was significantly more resistant than *E. coli* ML35 to the bactericidal effects of all peptides tested, including 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 effectiveness of the APs against *H. ducreyi* varied by the class of the peptide, with α-defensins exhibiting the least bactericidal activity and LL-37 the most. Very little reduction in survival was observed when *H. ducreyi* was exposed to α-defensins HNP-1-3 or HD-5, although bactericidal activity against *E. coli* in the same dose range indicated that the peptides were active (Figure 5). The β-defensins showed a modest dose-dependent effect on *H. ducreyi* that was significantly less than the effect on *E. coli* ML35 (Figure 6). LL-37 exhibited the most potent bactericidal activity against *H. ducreyi* of all of the peptides tested (Figure 9) but nonetheless continued to exhibit significantly higher levels of survival compared to *E. coli* ML35.

While *H. ducreyi* was more resistant than *E. coli* ML35 to all of the APs tested in the 96-well assay, some residual killing was observed in assays with the β-defensins and LL-37. In order to more accurately quantitate *H. ducreyi* AP resistance to these peptides, we performed a RDA that enabled us to calculate the MEC of the peptides against *H. ducreyi* 35000HP and *E. coli* ML35. In this assay, we found that each of the peptides exhibited activity against *E. coli* ML35, with MECs ranging between 2 and 18 μg/ml (Table 11). In contrast, the MECs of the β-defensins and LL-37 against *H. ducreyi* exceeded the upper limits of the assay, with little to no activity, even at a peptide concentration of 500 μg/ml (Fig. 8B-E). These results indicate that while the β-defensins and LL-37 may exhibit more activity against *H. ducreyi* than do the α-defensins, *H. ducreyi* is resistant to their bactericidal effects *in vitro*.

Prior to this work, the only study to investigate *H. ducreyi* susceptibility to APs demonstrated that the organism is susceptible to protegrin, a porcine AP with no human homolog (70). In contrast, the work described in this thesis demonstrates that *H. ducreyi* is resistant to each of the human APs tested in our assay. *H. ducreyi* is a strict human

pathogen (159, 219). As such, one could speculate that the diversity in the spectrum of APs found between humans and animals could be one of the many characteristics that limit the pathogenesis of *H. ducreyi* to humans. For example, the resistance of *H. ducreyi* to human APs, but not to porcine protegrin, could contribute to reported differences in the survival of the organism in the human and swine models of infection. *H. ducreyi* multiplies during human infection, with bacterial recovery typically 10-1000 fold greater than the inoculum (100, 219). In contrast, bacterial recovery from porcine skin is usually less than the inoculum used, suggesting that the bacteria do not multiply in porcine skin as efficiently as they do in human skin (204).

H. ducreyi is resistant to the APs that it naturally encounters *in vivo,* while maintaining susceptibility to the one animal derived AP tested. Based on this observation, one could argue that non-human APs could be a possible source of bactericidal agents to investigate in the development of therapies against *H. ducreyi*. While there is considerable interest in advancing this research, many studies with non-human APs have shown limited success in phase three clinical trials against other types of infections (92). Many of the problems associated with these therapies stem from the fact that non-human APs have bactericidal activity against not only human pathogens, but against our normal flora (92). Disruption of the normal flora can provide pathogens with an unoccupied niche to habitate (92). Consequently, the use of non-human APs as a therapeutic tool against *H. ducreyi* should be investigated with caution.

H. ducreyi clinical isolates can be divided into two phenotypic classes (247). In order to determine whether the AP resistance phenotype was specific to one class of *H. ducreyi*, we compared the percent survival of 35000HP, a class I strain, with that of CIP542 ATCC, a class II strain, in the 96-well bactericidal assay. Representative strains of both classes exhibited resistance to killing by α-defensin HNP-1, β-defensin HBD-3, and cathelicidin LL-37. We further tested this preliminary conclusion by challenging three class I and three class II *H. ducreyi* strains to increasing concentrations of LL-37 in a radial diffusion assay. From this experiment, we were able to calculate the average MEC of the peptide against both class I and class II strains. We found that the MEC of LL-37

against each stain tested exceeded the limit of detection for the assay. Taken together, these data suggest that resistance to APs is a phenotype of both class I and class II *H. ducreyi* strains and that it could represent a conserved role in the virulence of *H. ducreyi* as a species.

Successful bacterial pathogens have evolved a variety of mechanisms for overcoming the bactericidal activity of host APs (118). These mechanisms are often expressed simultaneously, highlighting the importance of AP resistance for successful bacterial pathogenesis (118). Some general strategies to evade killing by APs include inactivating APs in the extracellular milieu, pumping APs out of the cytosol, and repelling APs from the bacterial surface (118, 188). In order to identify putative mechanisms of *H. ducreyi* resistance to APs, we surveyed the *H. ducreyi* genome (www.ncbi.nih.gov) for regions of homology to other known AP resistance factors. From this study, we found that the *H. ducreyi* genome encodes a strong homolog of the Sap transporter, an influx pump thought to shuttle APs into the cytosol where they are presumably degraded before they damage the cytosolic membrane or anionic intracellular targets such as DNA or proteins (183).

The *sap* operon was originally identified as a possible mechanism of AP resistance by Groisman et al. in 1992, and the first *sap* mutants were generated in *S. typhimurium* (81, 183). Since then, s*ap* mutants have been successfully generated in a variety of organisms, each with a distinct AP susceptibility profile (47, 142, 144, 147, 152, 183). In *S. typhimurium,* three *Sap* mutants were characterized, each of which was more susceptible than the wild type bacteria to human crude granulocyte extracts, but retained their resistance to rabbit defensin NP-1 (183). McCoy et al. generated an AP susceptible mutant harboring an insertion within the *sapD* gene of *P. mirabilis* (152). This mutant was susceptible to the α-helical peptide polymixin B while it retained resistance to a β-sheet protegrin analog (152). In a *H. influenzae* Sap mutant, the transporter was shown to confer resistance to the r-cBD-1, HBD-3, and LL-37 (147).

The virulence of the Sap transporter has been conserved among many different bacteria, as evidenced by the activity of the Sap transporter in the plant pathogen *E. chrysanthemi*

(142). Interestingly, while the Sap transporter plays a role in the AP resistance of many different bacteria, it does not play a role in AP resistance in all of the bacteria in which it has been studied. In *V. fischeri,* a polar mutation within the *sapABCDF* operon does not result in susceptibility to any of the eight APs tested, including LL-37 (145). Taken together, these studies indicate that the Sap transporter has evolved distinct functionality within each bacterial species to meet the need of that particular pathogen.

It is important to note that the transporter confers resistance to a different subset of APs in each of the previously described systems, illustrating the need to characterize its function in each pathogen in which it is expressed. Further, the functionality of the transporter can be most accurately assessed by challenging *sap* mutants with APs that are actually found in the unique environmental niche of the organism in question. As demonstrated by the literature, what is true in one system cannot be broadly assumed to be true in other systems (144).

As an influx pump, it is likely that the original function of the Sap transporter was for nutrient acquisition, be it through the uptake of nutrients such as potassium, or by scavenging for peptides which could be broken down for parts (3, 97, 183). The AP resistance functionality of the transporter may have evolved out of its role in basic nutrient acquisition, to meet the specific needs of each bacterial species, under the selective pressure of each unique niche.

In order to study the role of the Sap transporter in *H. ducreyi* resistance to APs, we generated an *H. ducreyi sapA* isogenic mutant in the 35000HP background. The *H. ducreyi* Sap transporter is encoded in an operon containing the *tryR, sapA, sapB, sapC* and *sapD* ORFs, while *sapF* is encoded independently. Traditional cloning methods were used to generate an isogenic non-polar insertion/deletion *sapA* mutant through the insertion of a promoter-less kanamycin resistance cassette into *sapA* (153). The use of a non-polar cassette ensures that the 35000HP*sapA* mutant retains transcription through the operon, downstream of the *sapA* mutation (153).

We used the 96-well bactericidal assay to examine the role of the Sap transporter in *H. ducreyi.* The percent survival of wild type *H. ducreyi* 35000HP, 35000HP*sapA,* and the 35000HP*sapA/pSapA* complement were calculated after exposure to increasing concentrations of APs. An approximately 25% reduction in the percent survival of the 35000HP*sapA* mutant was observed as compared to the wild type, after exposure to LL-37. LL-37 resistance was restored in the complemented mutant. This finding indicates that the Sap transporter plays a role in LL-37 resistance in *H. ducreyi.* By importing LL-37 into the cell, where it is presumably degraded, both the direct bactericidal activity and the immunomodulatory activity of the LL-37 can be circumvented or decreased. Interestingly, there was no discernable difference in the percent survival of 35000HP and 35000HP*sapA* after exposure to the α-defensins HNP-1, HNP-2 and HD-5, or the β-defensins HBD-2, HBD-3 and HBD-4, indicating that the *H. ducreyi* Sap transporter does not play a role in resistance to these APs and that it has a much higher level of molecular specificity than was originally anticipated, or alternatively, that the functionality of the transporter in regards to these peptides is redundant with other AP resistance mechanisms.

Chancroid is a persistent infection and as such, *H. ducreyi* is exposed to multiple AP secreting cell types within the body (25). We observed only a 25% decrease in the survival rate of the 35000HP*sapA* mutant compared to the wild type, after exposure to LL-37. This observation suggests that multiple AP resistance mechanisms exist simultaneously within *H. ducreyi* and that their functionality is redundant. Investigations with *S. enterica* and *S. aureus* have revealed the presence of several redundant resistance mechanisms within the same bacterial species (65, 118, 187). These redundant mechanisms seem to complement one another to achieve high-level resistance to a broad spectrum of APs (65, 118, 187). As discussed in the Future directions section of this thesis, future work on this project will focus on determining the role of these other putative resistance factors both alone and in concert with the Sap transporter. Our data indicate that the Sap transporter confers to *H. ducreyi* resistance to only a subset of the APs to which it is likely exposed. The presence of other AP resistance mechanisms

within the bacterium may mask the role of the transporter in resistance to additional APs and could help to explain this observation.

In generating the 35000HP*sapA* mutant, we chose to knock out *sapA* as a way of eliminating AP resistance activity without affecting other putative functions of the transporter. The SapA protein has been shown to directly bind APs in *H. influenzae* (146). SapA binds APs and shuttles them through a channel formed from SapB, SapC, SapD, and SapF (183). We reasoned that if SapA were required to bind APs in the periplasm for the Sap transporter to function, then loss of this protein would render the entire complex useless, as was shown in *H. influenzae* and *S. typhimurium* (147, 183). However, because we observed that the loss of SapA in *H. ducreyi* resulted in susceptibility to only one class of APs, we cannot rule out the possibility that the SapBCDF complex could continue to function by directly binding and transporting other peptides into the cell for degradation in the absence of SapA, or that another *H. ducreyi* periplasmic peptide binding protein could mimic the AP binding activity of SapA in the Sap transporter. Other periplasmic binding proteins have been described in *H. ducreyi* (137), but their interaction with the Sap membrane complex has not been examined.

The work described in this thesis is the first to examine the role of the Sap transporter in AP resistance using a broad panel of human APs encompassing multiple members of the same peptide class against a specific human pathogen. In previous studies, homologous transporters were characterized using APs derived from multiple species or with synthetic peptides. Our study examines in detail what role the transporter plays in conferring resistance to not only broad classes of APs, but to individual peptides within a class. As such, our work adds an important element of complexity to the field as a whole.

Finally, *sapA* was detected by PCR in all ten *H. ducreyi* strains tested, including both class I and class II *H. ducreyi*. This finding suggests that the transporter is conserved within the species. Conservation of AP resistance mechanisms within a species is not always guaranteed (111, 118, 155). Work with *S. enterica* and *S. aureus* demonstrates that isolates of a species may vary widely in their expression of AP resistance

mechanisms and in their susceptibility to APs (111, 118, 155). Because the expression of the *H. ducreyi* Sap transporter is conserved within the species, it could represent a virulence factor for the organism.

H. ducreyi is an extracellular pathogen (22, 27) that likely encounters multiple antimicrobial peptides *in vivo*. These peptides are secreted by macrophages, PMNs, keratinocytes, and the vaginal epithelium (73). The work detailed in this thesis demonstrates that *H. ducreyi* has evolved at least one mechanism necessary to resist the bactericidal activity of these peptides (160), and further, that the Sap transporter plays an important role in the *H. ducreyi* AP resistance phenotype. As such, the Sap transporter contributes to *H. ducreyi* pathogenesis by allowing the bacterium to thrive in the presence of this ancient innate immune response (108).

FUTURE DIRECTIONS

The data presented in this thesis adds a critical level of detail to the study of bacterial resistance to APs, which was previously lacking from the field. Prior to this study, researchers had not examined, at the same level as cataloged in this work, the interaction of a specific pathogen with the APs unique to the organism's particular niche and host. By observing how a pathogen interacts with not only broad classes of APs, but with individual peptides within a class, we have added an important element of detail to the rapidly expanding field of bacterial AP resistance.

Because APs exert their bactericidal effects by different mechanisms depending on their concentration (108), it will be important to expand on our current work and to define the specific kinetics of this interaction as it relates to *H. ducreyi.* We have reported that *H. ducreyi* is resistant to eight specific APs. One of the limits currently encountered when considering *H. ducreyi* AP resistance, is our lack of understanding of how various peptides interact with the *H. ducreyi* cytosolic membrane. For example, it is entirely possible that the cathelicidin LL-37 interacts with the membrane differently than does the α-defensin HD-5, considering their different structures, yet we have reported that *H. ducreyi* is resistant to both peptides. Likewise, it is possible that a single peptide, for example, LL-37, interacts with the membrane differently at high vs. low concentrations. It would be interesting to catalog the similarities and differences of the *H. ducreyi* AP resistance response after treatment with a larger range of peptide concentrations, encompassing both more dilute and more concentrated concentrations that which were analyzed in this study. By broadening the range of concentrations tested in our assays, we will be better able to accurately define the physiologically relevant concentrations of specific peptides as they interact with *H. ducreyi.* In addition, completion of these more detailed dose response studies with multiple classes of peptides would allow us to propose a basic set of rules concerning the roles that AP structure plays in governing the interactions of APs with *H. ducreyi*.

A considerable effort was made to take into account the future direction of this project when constructing the 35000HP*sapA* mutant. 35000HP*sapA* was generated using one of only two non-polar antibiotic resistant cassettes approved for use in the HCM. Because 35000HP*sapA* was resistant to LL-37 *in vitro,* indicating that the Sap transporter could possibly contribute to *H. ducreyi* virulence, testing of this mutant in the HCM was undertaken. It is interesting to consider the possible contribution that this transporter could make to the virulence of *H. ducreyi in vivo.* Had we seen results suggesting that the transporter played a role in resistance to all of the APs likely to be encounter *in vivo,* then it would have been tempting to hypothesize that 35000HP*sapA* would be fully attenuated in the HCM. However, because we saw that the transporter plays a much more subtle role in *H. ducreyi* AP resistance as a whole, with no change in the survival rate of the WT and mutant when exposed to seven of the eight peptides tested, then it is more reasonable to hypothesize that partial attenuation will be observed. Experiments performed to date indicate that 35000HP*sapA* is in fact partially attenuated in the HCM, supporting this hypothesis (Unpublished data, D. Janowicz and S. Spinola).

Although there has been a substantial body of work published detailing the function of the Sap transporter in other organisms, it is important to determine how the Sap transporter functions in *H. ducreyi* specifically. The generation of the non-polar *sapA* mutation in *H. ducreyi* allowed us to simultaneously examine the role of the transporter in AP resistance, while preserving other putative functions of the complex. By knocking out only the periplasmic component of the transporter, the remaining membrane bound portion of the transporter, composed of SapB, SapC, SapD and SapF, remained intact and could theoretically remain active if SapB and SapC directly bound peptide. Although the direct association of SapA with APs has been hypothesized in several organisms, it has only been experimentally demonstrated in one organism, *H. influenzae* (146). The formal possibility remains that APs could be shuttled into the cell by directly binding the SapBCDF complex, even in the absence of SapA. Individual transporter component mutations were generated *S. typhimurium* and *H. influenzae* (147, 183). In each of these studies, *sapC* mutants were susceptible to lower AP concentrations than were *sapA* mutants, indicating that the most effective transporter alterations involve the loss of the

membrane bound components of the transporter and that a low level of activity remained when only SapA was missing. In addition, the *H. ducreyi* genome encodes a DppA homolog, which could conceivably interact with the remaining Sap complex. In the future, non-polar mutations should be generated in each individual gene within the *H. ducreyi sap* operon, allowing us to study the contribution of each component of the transporter in AP resistance.

Finally, an intact SapD protein could play a role in potassium uptake in the cell through a homolog of the *E. coli* Trk system. If *H. ducreyi* SapD plays a role in potassium uptake that is analogous to that of the *H. influenzae* SapD (146), the *S. typhimurium* SapD (183), or the *E. coli* SapD (96), then an *H. ducreyi* strain carrying a mutation in *sapABC* is likely to display a more robust growth phenotype than a strain carrying a *sapABCD* mutation, because nutrient acquisition could be affected by the loss of SapD. The generation of mutations resulting in the loss of function of both individual and combinatory Sap transporter components would allow us to evaluate these hypotheses.

Two of the most interesting questions to arise from this research are: on what basis does the *H. ducreyi* Sap transporter recognize APs, and why does *H. ducreyi* Sap transporter confer resistance to a different subset of AP than the Sap transporters of other organisms? Our data indicate that the *H. ducreyi* Sap transporter confers resistance against the cathelicidin LL-37, but not against either the α- or β-defensins. It is not sufficient to explain the selectivity of the transporter on the basis of structure alone, when we have, as of yet, only tested the role of the transporter with a single α-helical peptide. Further, our knowledge of the Sap transporters of other organisms is insufficient to assign rules for the recognition of APs, as an analysis of the literature to date fails to yield a discernable pattern of structure based AP recognition (47, 81, 142, 144, 146, 147, 152, 160, 183). In the future, the ability of the transporter to bind other α-helical structures should be analyzed. However, as *H. ducreyi* does not naturally encounter α-helical peptides other than LL-37 *in vivo,* relevant research in this area is not feasible with the *H. ducreyi* Sap transporter. It remains possible that the size, the charge, or the inherent flexibility of the peptide could also play a role in its recognition by the Sap transporter. Thus, we should

examine the individual differences in the peptides in greater detail in order to determine what characteristics are important for recognition and transport by the transporter. Finally, protein crystallography could be used to map the SapA/AP binding site and provide more basic information about the protein: protein interaction. As an influx transporter, it is possible that the Sap complex originally evolved for the purpose of nutrient acquisition and that it acquired AP resistance functionality independently in each bacterial species in which it is expressed. This hypothesis would help to explain the differences in the AP resistance functionality profiles of the Sap transporters of the various bacterial species in which the organism has been studied.

Another important element to consider when characterizing the functionality of the Sap transporter is its regulation. The Sap transporter of *S. typhimurium* is regulated by the PhoP-PhoQ two component regulatory system (183). In contrast, *H. influenzae,* lacks a PhoP-PhoQ homolog and regulates the Sap transporter through a system involving the direct interaction of SapA with APs (146). Three preliminary approaches should be considered to begin the characterization of the regulation of the *H. ducreyi* Sap transporter. While a PhoP-PhoQ homolog has not been identified in the *H. ducreyi* genome, genes with high homology to the CpxA-CpxR and the Sigma E regulators have been identified (24). The intergenic region upstream of the *sap* operon contains consensus binding sequence boxes for each regulator (161). In other organisms, these regulators are activated in response to envelope stress and they should therefore be evaluated for their contribution to the regulation of the Sap transporter. In addition, the *H. ducreyi sap* operon contains an ORF coding for a *tyrR* transcriptional regulator that could play a role in the regulation of the transporter. The generation of an isogenic *tyrR* mutant in 35000HP would allow for the analysis of the role of *tyrR* in AP resistance. An examination of the up-regulation of the *sap* genes in response to outside stimuli, including direct exposure to various APs, should also be undertaken (146).

A non-polar *sapA* mutant was generated in *H. influenzae* (146, 147). *H. influenzae* is the nearest relative of *H. ducreyi* in which the Sap transporter has been studied. As such, it is important to note the similarities and differences between these studies and those

presented here. In *H. influenzae,* the Sap transporter was shown to confer resistance to LL-37, r-cβD-1, and HBD-3 (147). The *H. ducreyi* Sap transporter also contributes to resistance to LL-37. However, we did not observe a statistically significant difference in the susceptibility of 35000HP and 35000HP*sapA* to HBD-3. In the future, experiments comparing the basic structural and regulatory differences in these transporters may shed some light on this discrepancy. However, in order to determine if the difference in the specificity of the transporters lies with SapA, two experiments should be performed. First, just as was done in *H. influenzae* (146), ligand blots could be used to characterize the AP binding pattern of *H. ducreyi* recombinant SapA. Second, the *H. ducreyi* SapA protein could be expressed *in trans* in the *H. influenzae sapA* mutant, in order to determine if the expression of the *H. ducreyi* SapA protein changes the specificity of the *H. influenzae* transporter. A cursory examination of the Sap transporter literature could lead researchers to conclude that it functions by the same mechanism in each bacterial species, against every AP. However, the model of Sap transporter activity proposed by Parra-Lopez et al. was based only on research conducted in *S. typhimurium.* Future researchers should appreciate the complexity of this transporter and consider it when designing experiments to characterize its function in each unique bacterial species in which it is shown to be expressed and by testing its function with peptides found at the site of infection of that particular pathogen.

Finally, the question remains as to what other AP resistance mechanisms might be active in *H. ducreyi.* While a transposon mutagenesis procedure coupled with a simple AP susceptibility screen would allow us to identify other *H. ducreyi* genes involved with novel AP resistance mechanisms, the *H. ducreyi* genome also contains homologs of many previously characterized putative bacterial AP resistance factors. The generation of isogenic mutations in any of these putative factors would prove valuable in determining their contribution to AP resistance. In addition, the generation of double isogenic mutants, where multiple putative AP resistance factors are eliminated simultaneously, would allow researchers to evaluate the redundancy of these AP resistance factors in *H. ducreyi.* One *H. ducreyi* putative AP resistance mechanism that is of considerable interest is the Mtr efflux pump which has been characterized in *N. meningitidis* and *N.*

gonorrhoeae (86, 209). The pump functions by binding APs in the cytosol and periplasm and shuttling them out of the cell before they can damage the cytosolic membrane (209). The construction of an isogenic mutation, eliminating one, two, or all of the *H. ducreyi mtr* genes would prove very useful in ascertaining the role of this efflux pump in *H. ducreyi* AP resistance. I have completed preliminary work towards this end, which is detailed in the appendix of this manuscript. The Mtr project will be carried on by Dr. Sherri Rinker of the Bauer lab.

The studies presented in the manuscript both help to clarify the role of the Sap transporter in AP resistance, and illustrate the need for more detailed, niche specific, analysis within the field. AP resistance is an important mechanism for bacterial survival. Clinically, we are faced with an ever dwindling supply of antibiotics to which bacteria have not developed resistance, while simultaneously we can observe that the research and development of new antibiotics is stalling. Research into the use of APs as clinical therapy is already underway and could represent a significant therapeutic tool in the future (92). As such, it is important to understand the ways in which bacteria have evolved resistance to these peptides. The experiments presented in this work help us to take a small step in that direction by allowing us to better characterize the interaction of *H. ducreyi* with APs.

APPENDIX
MTR EFFLUX TRANSPORTER MUTATGENESIS

The multiple transferable resistance (Mtr)CDE pump has been shown to confer resistance to APs in *N. gonorrhoeae* (86, 209). The *H. ducreyi* genome contains a homolog of the Mtr efflux transporter of *N. gonorrhoeae* (209). This transporter is composed of the MtrC, MtrD and MtrE proteins (209). In *H. ducreyi, mtrC* and *mtrD* homologs are co-transcribed in an operon, while a *mtrE* homolog is encoded separately (Figure 18). MtrC is a periplasmic protein that links MtrD and MtrE to form an efflux transporter complex for expulsion of APs from the cell (209).

In order to examine the role of the putative Mtr transporter in *H. ducreyi,* we designed a strategy to create an isogenic *mtrC* mutant in the 35000HP background, using a non-polar chloramphenicol acetyl transferase (CAT) cassette (143) (Figure 19). The *mtrC* ORF *(HD1513)*, along with approximately 1500 bp of upstream and downstream flank, were PCR amplified (Primers Table 13) and the resulting amplicon was TA cloned into pCR XL Topo to generate pMEB052. pMEB052 was digested with PshA1 and AgeI to liberate a 597 bp section of *mtrC.* The remaining portion of the pMEB052 was treated with the large fragment of DNA polymerase B (Invitrogen) to produce blunt ends and was ligated with the CAT cassette of pSL1 (143) to produce pMEB088. Multiple unsuccessful attempts were made to liberate the disrupted *mtrC* gene from pMEB088 and to sub-clone it into pRSM2072. Consequently, a shortened version of the disrupted gene, containing only 500 bp of flank on either side of the CAT cassette was PCR amplified from pMEB088 using primers that added an XhoI restriction site to the 5' end of the construct and a BamHI site to the 3' end of the construct. The resultant amplicon was digested with XhoI and BamHI directly and ligated into pRSM2072 (40). It was then digested with the same enzymes to produce pMEB118. pMEB118 was electroporated into *E. coli* HB101, to produce pMEB119. Many unsuccessful attempts were made to introduce pMEB119 into 35000HP by electroporation. However, the resultant transformants were not more resistant than wild type *H. ducreyi* to chloramphenicol (0.3

μg/ml, 0.5 μg/ml, 10 μg/ml, or 33 μg/ml). We hypothesized that the native promoter of the mutagenic *mtr* operon was insufficient to promote the transcription of the promoter-less non-polar CAT cassette.

To overcome this technical set back, a new strategy for the mutagenesis of *mtrC* was designed (Figure 20). pMEB052 will be digested with PshA1 and AgeI, and treated with the large fragment of DNA polymerase B, as described above. The resultant 6.7Kb fragment will then be ligated with the SmaI/EcoRV digested Ω CAT cassette of pBSL119 (13). The resultant disrupted *mtrC* construct will then be PCR amplified to shorten the flank to facilitate ligation with pRSM2072, as described above. The amplicon and pRSM2072 (40) will each be digested with BamHI and XhoI and ligated together to generate the final *mtrC* mutagenic construct. This construct will be electroporated into *E. coli* HB101, and introduced into 35000HP by electroporation. The ΩCAT cassette contains its own promoter, which should result in an increase in the transcription of the chloramphenicol resistance gene and yield chloramphenicol resistant transformants.

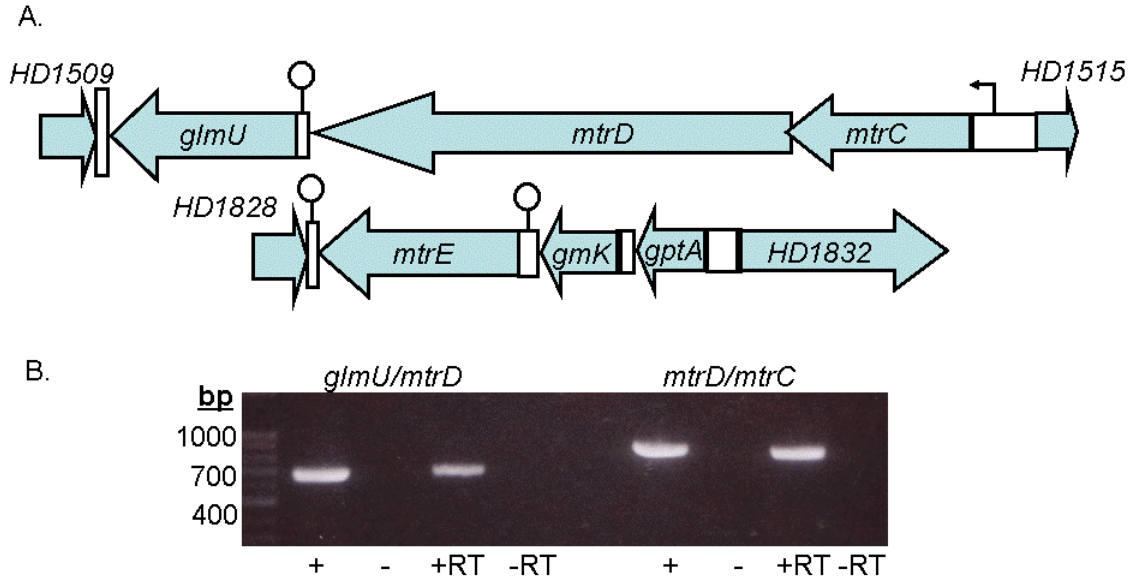


Figure 18. *mtr* efflux pump operon (162). A. Schematic diagram of *H. ducreyi mtrCD* and *mtrE* containing locus (162). B. RT-PCR analysis of *mtr*-containing loci was used to confirm that *mtrC* and *mtrD* were transcribed as an operon.

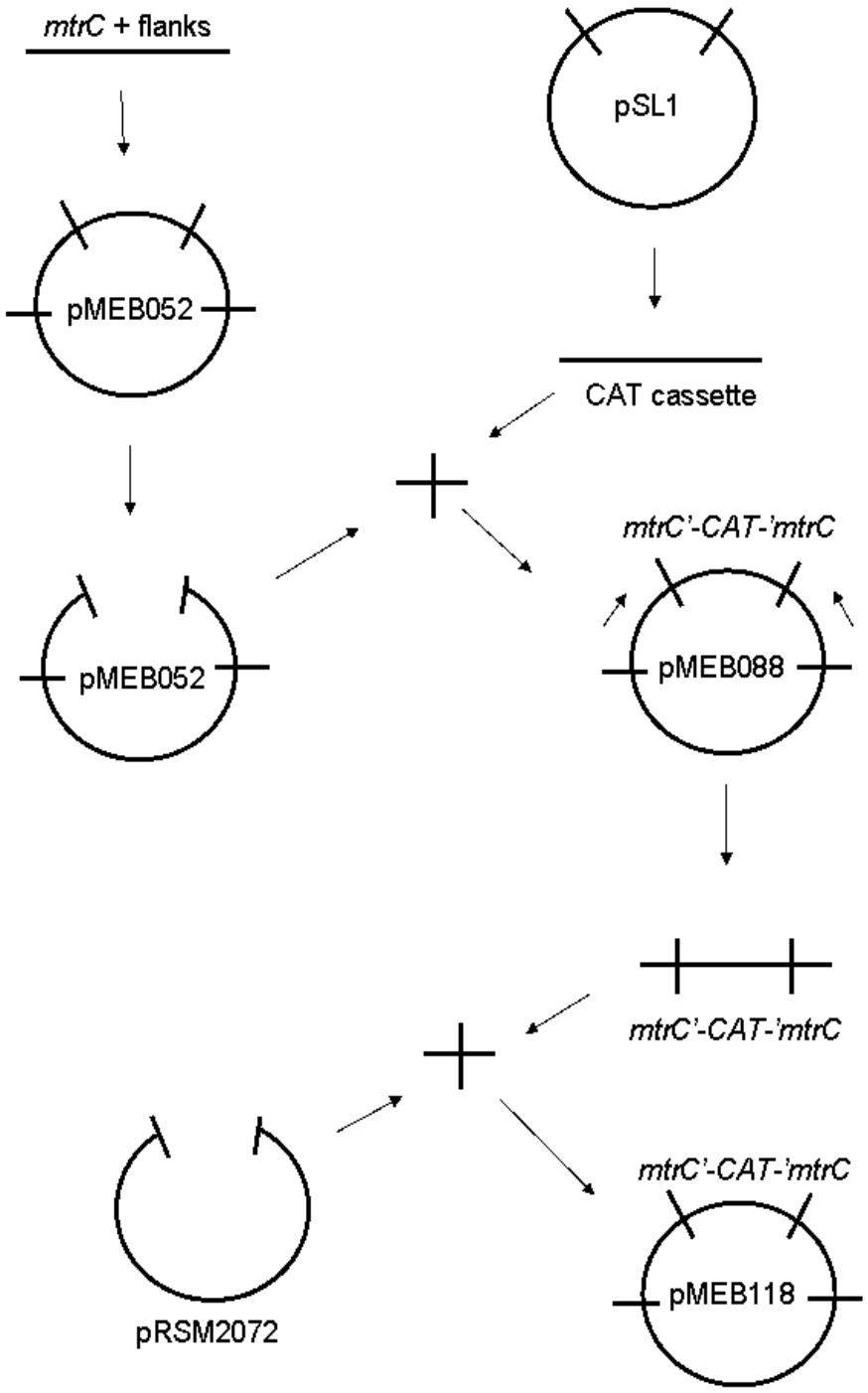


Figure 19. *mtrC* mutatgenic strategy

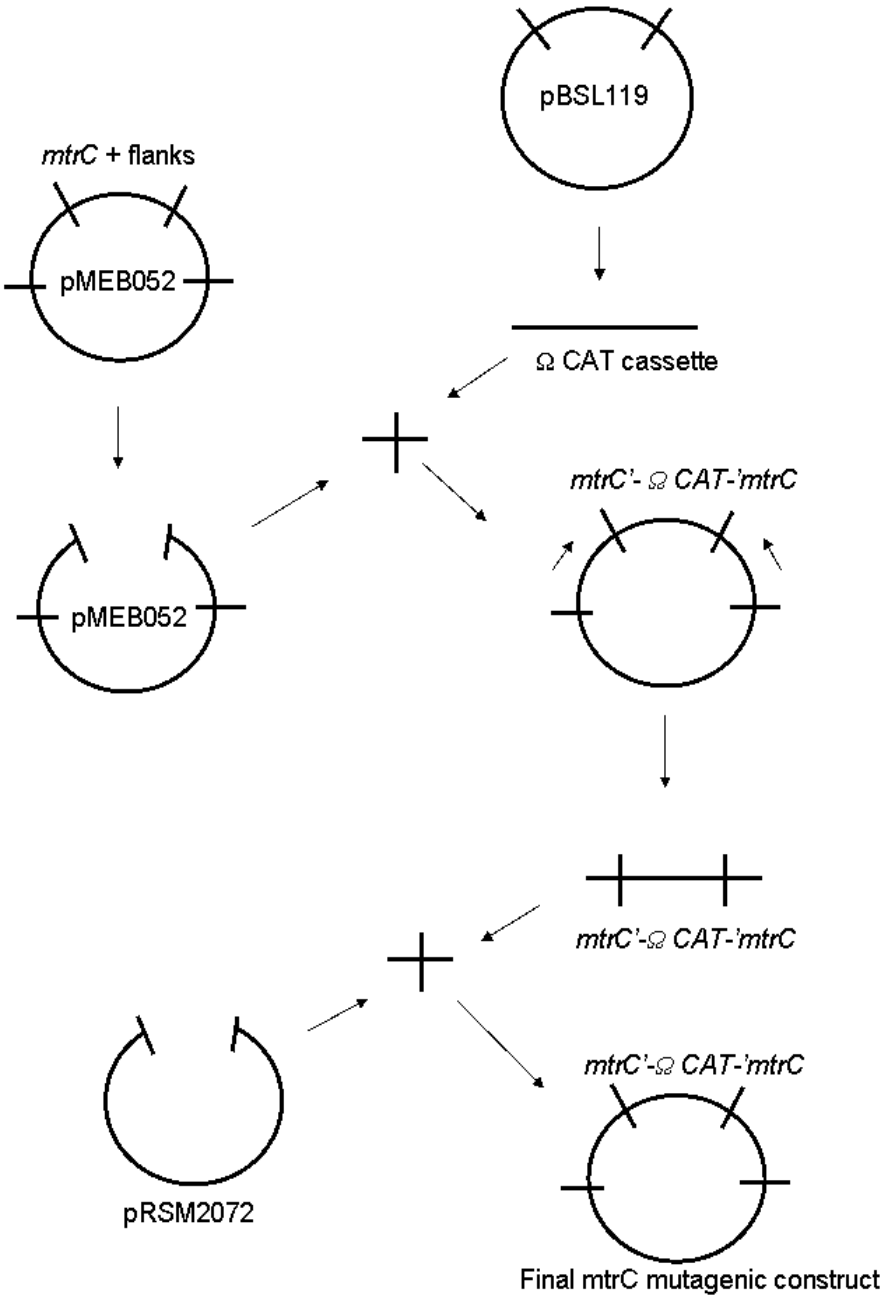


Figure 20. Revised *mtrC* mutagenesis strategy

Table 13. Primers used in *mtrC* mutagenesis and RT-PCR

Primer	Construct	Sequence
MtrC forward 1	pMEB053	CAGTTTGCCCAATCGTGGCGATAA
MtrC Reverse 1	pMEB052	ACCGCTCCAGCAAGTTGGAAGGAA
XhoI Forward I	pMEB118	CATATCctcgagCTATGGCTGGCTTTGAGA
BamHI Reverse I	pMEB118	CATATCggatccACTCATTAAATATGG CCAATCA
pMtr001	*glmU/mtrD*	TTTAATAAGTCACCGCCGTGCCCA
pMtr002	*glmU/mtrD*	GTTGGCTTGATCACCAAACACGGT
pMtr003	*mtrC/mtrD*	AGCGCCAGTGGAAGAACGAATAGA
pMtr004	*mtrC/mtrD*	TAATGTCGGGAATGTTTGCGCGTC

*Underlined regions correspond to restriction digest sites generated during PCR. In MEB118 an XhoI recognition site was added to the construct by XhoI Forward I and a BamHI site was added to the construct by BamHI Reverse I.

Table 14. Plasmids Used in *mtrC* Mutagenesis

Plasmid	Description	Source or reference
Plasmids:		
pCR XL Topo	TA cloning vector, Amp^R	Invitrogen
pMEB052	*mtrC (HD1513)* + flank in pCR XL Topo	This study
pMEB088	Full length *mtrC'*+ Non-polar CAT + *'mtrC* in pCR XL Topo	This study
pMEB118	Shortened *mtrC'*+ Non-polar CAT + *'mtrC* in pRSM2072	This study
pSL1	Vector containing non-polar CAT cassette	(143)
pBSL119	Vector containing Ω CAT cassette	(13)
pRSM2072	*H. ducreyi* suicide vector	(40)

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CURRICULUM VITAE

Kristy Lee Beavers Mount

Education

Indiana University
Indianapolis, Indiana
PhD. Microbiology and Immunology, 2009

Purdue University
West Lafayette, Indiana
B.S. Biology, 2003

Research Experience

Doctoral Research: 2005-2009
PI: Dr. Margaret Bauer, IUSM, Department of Microbiology and Immunology:

Project: I established that *H. ducreyi,* the causative agent of chancroid, is resistant to the bactericidal effects of the antimicrobial peptides most likely to be encountered *in vivo,* including 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. Based on the literature, I hypothesized that the Sap influx transporter contributed to this phenotype. I generated an isogenic non-polar *sapA* mutant in *H. ducreyi* 35000HP and found that the mutant was more susceptible than the wild type to LL-37. These data suggest that the Sap transporter contributes to antimicrobial peptide resistance in *H. ducreyi.*

Proctor and Gamble, Cincinnati, Ohio, Summer Internships: 2001 and 2002
PI: Dr. Heather Rocchetta, 2002

Project: I worked to characterize the bacterial populations found in the axilla and performed antimicrobial testing against these bacterial populations in an effort to develop a natural antimicrobial deodorant.

Undergraduate Research: 2000
PI: Dr. David Franklin, Purdue University, Department of Biology

Project: Comparison of embryonic development of p59, p18, p19, and p21 knockout mice.

Teaching Experience

Indiana University School of Nursing, Microbiology J210: Microbiology
Guest Lecturer: 2005-2008
Lectures: Enteric Bacterial Infection, Pathogenic Mycology, Parasitology

Indiana University School of Nursing, Nursing J210: Microbiology
Teaching Assistant: 2005

Purdue University, Biology 132: Structure, Development and Function
Teaching Intern: 2002 and 2003

Purdue University Biology 122: Ecology
Teaching Intern: 2002

Publications

Mount, K.L.B., C.A. Townsend, M.E. Bauer. (2007) "*Haemophilus ducreyi* is Resistant to Human Antimicrobial Peptides." Antimicrobial Agents and Chemotherapy. V51(9):3391-3

Mount, K.L.B., C.A. Townsend, M.E. Bauer. (2009) "The *Haemophilus ducreyi* Sap Transporter Contributes to Cathelicidin Resistance." Manuscript in Preparation

Abstracts and Presentations

Mount, K.L.B., C.A. Townsend, M.E. Bauer. (2007) "*Haemophilus ducreyi* is Resistant to Human Antimicrobial Peptides." American Society for Microbiology General Meeting, Orlando, Florida, 2006

Mount, K.L.B., C.A. Townsend, M.E. Bauer. (2007) "*Haemophilus ducreyi* is Resistant to Human Antimicrobial Peptides." Midwest Microbial Pathogenesis Meeting, Cincinnati, Ohio, 2006

Mount, K.L.B., C.A. Townsend, M.E. Bauer. (2009) "The *Haemophilus ducreyi* Sap Transporter Contributes to Cathelicidin Resistance." Midwest Microbial Pathogenesis Meeting, Madison, Wisconsin, 2008

Awards

Harold Raidt Teaching Award: 2007
Sigma Xi Research Competition, Second Place: 2006
American Society of Microbiology Travel Award: 2006
Indiana University School of Medicine Travel Award: 2006
Howard Hughes Undergraduate Research Fellowship: 2000