Determination of efflux pump characteristics in *Haemophilus ducreyi* using efflux pump inhibitors CCCP and PAβN

By

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Allegheny College

Biochemistry Program

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code

and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. Humphreys

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Dr. Deckert

Abstract

Haemophilus ducreyi is the causative agent of the sexually transmitted disease chancroid, which facilitates HIV infection. Although currently treatable, antibiotic resistance is a potential future problem for treating chancroid. One of the potential mechanisms for antibiotic resistance is antibiotic efflux pumps which prevent the antimicrobial drug from affecting the cell by extruding the chemical. A strategy for identifying strains of bacteria that have efflux pumps involves using efflux pump inhibitors and determining antibiotic susceptibility in the presence and absence of such chemicals. This study looked to determine if *H. ducreyi* strains HMC112 and 35000HP have efflux pumps using the efflux pump inhibitors PAβN and CCCP. Disc diffusion assays were performed in the presence and absence of either PAβN or CCCP and the zones of inhibition were measured for the antibiotics ampicillin, erythromycin, tetracycline, and amoxicillin/clavulanic acid. The EPI CCCP did not have a significant impact on the zones of inhibition for any of the antibiotics in either strain. However, PAβN had a significant effect on the zones of inhibition for both HMC112 ($p = 0.0112$) and 35000HP ($p = 0.0328$). From these results, it is possible that *H. ducreyi* does possess efflux pumps that could contribute to antibiotic resistance. Therefore, it would be beneficial to investigate further into *H. ducreyi* efflux pump prevalence on a wider scale.

Introduction

Haemophilus ducreyi is a gram negative bacterium that is the causative agent for the sexually transmitted disease chancroid. *H. ducreyi* infects mucosal surfaces and keratinized squamous epithelium and is generally contracted through small abrasions in the skin acquired

during sexual intercourse (Spinola et al. 2002). Within the first day after infection, small papules form at the infection site followed 2-3 days later by the formation of pustules. Within 5-7 days of initial infection, the pustules rupture and become a pus filled ulcer characterized by yellowish to grey necrotic tissue at the base, jagged edges and extreme pain (Bong et al. 2002). The most common site of infection in women is the entrance to the vagina, but painless internal lesions on the cervix or vaginal wall can also occur. In men, the most common lesion sites are the prepuce, frenulum, and the coronal sulcus (Morse 1989). Generally more men are infected than women, and infection is often associated with exchange of sex for drugs or money (Bong et al. 2002). *H. ducreyi* infection is not common in most industrialized nations but is more common in developing countries with few resources (Morse 1989). One of the main concerns associated with chancroid is that ulcers associated with chancroid facilitate HIV infection (Spinola et. al 2002). Therefore effectively treating chancroid infection is an important component in the attempt to reduce HIV infections in developing nations.

There are currently two different classes of *H. ducreyi*: class I and II. The major differences between the two classes are in the structures of outer membrane components specifically DsrA (*ducreyi* serum resistance A) and lipooligosaccharide (LOS) among others (White et al. 2005). Class II strains express a shortened form of the LOS which does not become sialated (Post et al. 2007). These strains also have an altered form of DsrA that shows that they are only 48% identical at the protein level between class I and class II strains (Post & Gibson 2007).

H. ducreyi is commonly resistant to trimethoprim-sulfamethoxazole and amoxicillin/clavulanic acid (Schmid 1997). Much of *H. ducreyi*'s resistance is due to plasmids containing beta-lactamase enzymes (Rutanarugsa et al. 1990). In addition, some resistance may be related to the region which the strain is located as different strains have different resistance profiles (Rutanarugsa 1990). The most common antibiotics used to treat chancroid are ceftriaxone, erythromycin and azithromycin in one 250mg intramuscular dose, 500mg four times a day for one week, and 1g orally once respectively (Schmid 1997).

Antibiotics have a variety of modes of action on the bacteria that lead to cell death including acting to inhibit the cell wall synthesis (penicillins, cephalosporins and vancomycin), inhibiting protein synthesis (aminoglycosides, tetracyclines, macrolides, and chloramphenicol), inhibiting nucleic acid synthesis (quinolones and fluoroquinolones, rifampin), disruption of the cell membrane (polymyxin B), and antimetabolites (sulfonamides, trimethoprim, Dapsone, and Isoniazid) (Prescott et al. 2009). Antibiotic resistance is a major problem in combating bacterial diseases as resistant strains of pathogenic bacteria are becoming more common. With the introduction of antimicrobial compounds, bacteria have developed new methods of resistance that have been causing problems with treatment using existing antibiotics. One of the methods bacteria have developed to combat antibiotics is altering the target molecules via enzymes in the bacterial cell; altering these enzymes no longer allows antibiotics such as fluoroquinolones to kill the bacterial cell (Nikaido 2009). Another common resistance mechanism is inactivation of the antibiotic by bacterial enzymes which alter the antibiotic itself. This is common with penicillin resistance via the β-lactamase enzyme produced by bacteria. This enzyme

deactivates β- lactam antibiotics by hydrolyzing the β-lactam ring (Babic et al. 2006; Figure 1).

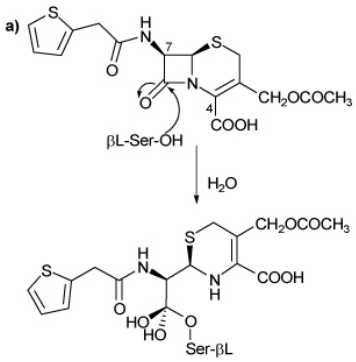


Figure 1: Mechanism of action for β-lactamase enzyme on a β-lactam antibiotic. (Morandi et al. 2008).

Generally, alterations of target molecules are caused by a mutation in the chromosome of the bacterium and usually are non-transferable. Genes that encode for enzymes that inactivate antibiotics are commonly located on transferable plasmids (Nikaido 2009). A third method of resistance is preventing antibiotic access to the targets through efflux. Efflux pumps in particular are major contributors to multidrug resistant phenotypes in many bacteria. Efflux genes can either be located on the chromosome of a bacterium or on a plasmid (Poole 2005). There are five different families of efflux pumps: ATP binding cassette (ABC), small multidrug resistant (SMR), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), and resistance nodulation division (RND) (Poole 2007).

Although each of the efflux pumps is slightly different, they all have a general structure in gram-negative bacteria. Most consist of a three protein complex (Figure 2) consisting of a membrane fusion protein, a periplasmic transitional protein, and an outer membrane protein (Figure 3) (Clark et al. 2009).

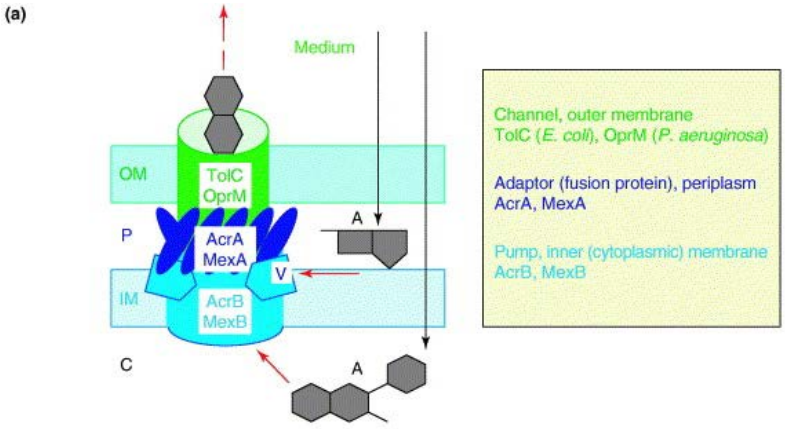


Figure 2: **Cartoon representation of the RND efflux pump system AcrAB-TolC from *E. coli* and MexAB-Oprm from *P. aeruginosa***. Red arrows show the direction of drug efflux. Structures A represent antibiotic molecules. OM signifies the outer membrane; P signifies the periplasmic space; IM signifies the inner membrane; C represents the cytoplasm (Pages et al. 2005).

In the RND type pumps, the membrane fusion protein is where substrates of the pump bind; they are then transferred through the transitional periplasmic protein and extruded from the cell through the outer membrane protein, which generally functions as a channel (Nikaido 2009) (Figure 3).

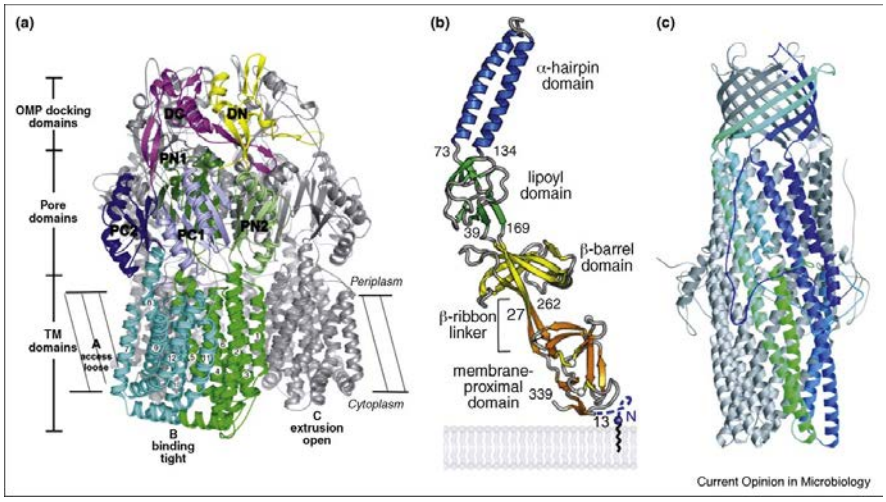


Figure 3: Crystallized Structures of MexA, MexB, and OprM from *P. aeruginosa* (Blair and Piddock 2009).

Recently the crystal structures of each component of the AcrAB-TolC system from *Escherichia coli* was resolved (Blair and Piddock 2009). It is thought that most RND efflux

pumps share a similar structure despite sequence variation between species. The substrate binding sites tend to contain large numbers of phenylalanine residues (Blair and Piddock 2009). It is thought that specific positions in the active site are involved with substrate specificity as point mutations of specific amino acids have altered the binding affinity for different classes of antibiotics like macrolides (Blair and Piddock 2009). RND efflux pumps are fueled by a proton transfer, which in AcrB consists of three amino acids: Asp 407, Asp408, and Lys 940 (Blair and Piddock 2009). Efflux pumps can extrude a variety of different chemical compounds that have different structural characteristics, but all the drugs are generally amphiphilic, hydrophobic, or have either a positive or a neutral charge (Putman et al. 2000). As such, most members of SMR and MFS families and the RND family have a negative portion of the inner membrane protein that is thought to help with the drugs binding to the pump (Putman et al. 2000). Some substrates of the RND pump MexAB-OprM include aminoglycosides, chloramphenicol, cephalosporins, ethidium bromide, macrolides, penicillins, sulfonamides, tetracyclines and trimethoprim among others (Nikaido 2009). It has also been proposed that one mechanism of transport of drugs out of the cell actually occurs in the lipid bilayer of the inner membrane and not from the cytoplasm (Putman et al. 2000). Two specific examples of RND pumps are the MexAB-OprM system in *Pseudomonas aeruginosa* (Figure 3) and the MtrCDE system in *Neisseria meningitidis*. Both of these systems have homologous genes in *H. ducreyi* (Figures B1-B3 & Figures B4-B6). Another system with homology to *H. ducreyi* is the NorM (HmrM) efflux system, a MATE type efflux pump, from *Haemophilus influenzae* (Xu et al. 2003; Figure B7). *H. ducreyi* also has a homologue to the SapABCDF system, a member of the ABC efflux family, from *Salmonella enterica typhimurium* (Parra-Lopez et al 1993; Figures B8-B12).

One of the challenges now facing clinicians is how to combat the multidrug efflux pumps of pathogenic bacteria. Recent studies have looked into chemical compounds that will restore the effectiveness of current antibiotics through inhibiting the efflux pumps of resistant bacteria. There are multiple different potential targets for an inhibitor to act on, including altering the regulation of efflux pump expression, blocking the outer membrane channel, developing compounds that will competitively bind to the substrate sites, and eliminating the energy necessary to drive the efflux pump (Pages and Amaral 2008). There are currently several compounds that are functional antibiotic efflux pump inhibitors (EPI) in use in in vitro testing, but only a few are currently in clinical trials because many EPIs have adverse side effects, and only two have received FDA approval (Tohidpour et al. 2009). One EPI used frequently in *in vitro* testing to identify antibiotic efflux pump prevalence in a strain of bacteria is phenylalanyl arginyl β-naphthylamide (PAβN) (Figure 4). PAβN functions by competitively binding to substrate binding sites of efflux pumps, commonly RND pumps, and therefore restores antibiotic concentrations within the cell by being extruded instead of the antibiotic (Blair and Piddock 2009). It is a dipeptide amine from which a number of different chemicals that inhibit efflux pumps have been derived (Barret 2001). The exact mechanism of action on the efflux pumps has not been determined for PAβN (Barret 2001). At high enough concentrations PAβN in itself can affect the membrane integrity (Pages et al. 2005). It is commonly used to assess the overexpression phenotype in multidrug resistant bacteria by using concentrations that do not affect the membrane, but only the efflux pumps. Recent studies performed with *P. aeruginosa* showed that in the presence of PAβN, antibiotics that were previously ineffective showed restored activity (Tohidpour et al. 2009). Multiple strains of *P. aeruginosa* were used in a study where the minimum inhibitory concentrations (MIC) of several antibiotics were determined

through agar dilution in the presence and absence of PAβN (Tohidpour et al. 2009). This type of procedure is a common way to test for efflux pump prevalence in *P. aeruginosa*.

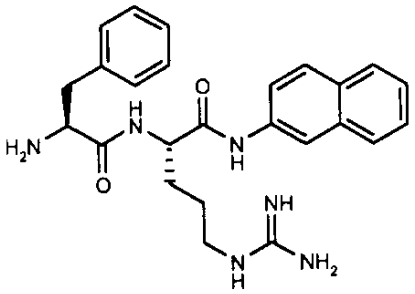


Figure 4: Chemical structure of phenylalanyl-arginine-β-naphthylamide (PAβN) (Barret 2001).

Another type of efflux pump inhibitor is carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Figure 5) which affects the proton gradient required for efflux pumps to function (Pages et al. 2005). The chemical is an ionophore that is able to block the coupling of redox reactions with ATP synthesis and thereby destroying the proton gradient across the membrane (Greksak et al. 1977). Various derivatives of carbonyl cyanide phenylhydrazone have been shown to have an inhibitory effect on growth in yeast and the bacteria *Bacillus cereus* (Greksak et al. 1977). CCCP has been used on *Helicobacter pylori* to restore antibiotic susceptibility in vitro (Zhang et al. 2010). It has also been used to determine efflux characteristics in *S. enterica* serovar Typhimurium and *E .coli* through fluorescence assays (Coldham et al. 2010).

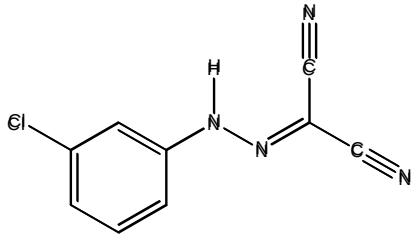


Figure 5: Chemical structure of carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Pages et al. 2005).

The eventual goal of studying efflux pump inhibitors is the hope to use these EPI's in combination with current antibiotics in order to restore their effectiveness. The production of

new antibiotics has been greatly reduced in the past few years, but newly resistant strains of bacteria are still being discovered as bacteria change and adapt to their environment. It would be beneficial to understand which bacteria possess efflux pumps so as to better understand the structure and function of the bacteria. If strains could be easily identified by their efflux pump presence, it may allow physicians to better treat patients with resistant strains of bacterial infection. In addition, the more knowledge of a bacterium's structure and function that is known is beneficial because bacteria can often be used for models of eukaryotic processes as well. Understanding *H. ducreyi* structure and function is especially important because although it is currently very treatable, it facilitates HIV infection. There is some evidence that suggest patients who are HIV positive do not respond as well to treatment as those who are not HIV positive (Schmid 1997). Any information that might lead to faster or more effective treatment could help reduce infection time and reduce the risk of contracting HIV.

Therefore, I determined if *H. ducreyi* has efflux pumps by measuring the zones of inhibition for several antibiotics including Ampicillin (Amp), Tetracycline (Tet), Erythromycin (Ery), and Amoxicillin/Clavulanic acid (AmC) in the presence and in the absence of the efflux pump inhibitor PAβN or CCCP. I expected both PAβN and CCCP to increase the zones of inhibition for all antibiotics tested. I then attempted to determine the MIC of each antibiotic in the presence and absence of the efflux pump inhibitors by microdilution method.

Materials and Methods

Strains and Growth

H. ducreyi strains HMC112 (class II), HD85-023233 (class I), and 35000HP (class I) were maintained on chocolate agar (GC base 3.6% (w/v), hemoglobin 1%(w/v), IsoVitalex 1%(v/v) (B12, L-glutamine, adenine, guanine-HCl, P-aminobenzoic acid, diphosphopyrinine nucleotide oxidized, carboxylase, ferric nitrate, thiamine-HCl, L-cysteine-HCl, L-cystine, dextrose)). HMC112 was grown at 33°C and 10% CO_2; 35000HP was grown at either 33°C and 10% CO_2 or 35°C and 5% CO_2 and HD85-023233 was grown at 35°C and 5% CO_2. Two days prior to an assay a portion of frozen stock of either strain was revived on chocolate agar and incubated overnight. The day before an assay, the bacterial growth was transferred to fresh chocolate agar plates and incubated overnight.

Efflux Pump Inhibitor Preparation

Both CCCP and PAβN are heat sensitive and decompose at high temperatures; they are also extremely toxic. Therefore, both were dissolved in a diluent prior to use in any assay to prevent inhalation and increase ease of handling. The CCCP was dissolved in 100mM DMSO (dimethyl sulfoxide) to a concentration of 50 mg/mL and stored at -20°C. The PAβN was dissolved in 95% ethanol (EtOH) to a final concentration of 25 mg/mL and stored at -20°C.

Agar Preparations

In order to determine the effects of the EPI's on antibiotic susceptibility disc diffusion assays were performed on chocolate agar. Five different types of chocolate agar were used for the assay: plain chocolate agar or chocolate agar supplemented with either 95% EtOH, 100mM DMSO, 20µg/mL CCCP, or 25µg/mL PAβN. Because neither CCCP nor PAβN are water soluble, the EPIs were dissolved in DMSO and EtOH respectively. Therefore, to control for the

solvents, two additional controls, one with DMSO and one with EtOH present in the agar were used. The chocolate agar was made according to protocol except in the case of the supplements. The calculated amount of the supplement was added to the cooled hemoglobin after autoclaving, at the same time the IsoVitalex was added, prior to combining with the hot GC base. For the DMSO control, the chocolate agar plates were $3.125x10^{-4}$% (v/v) DMSO. For the EtOH control, the chocolate agar plates were 0.19% (v/v) EtOH.

Disc Diffusions

A suspension was made containing either HMC112 or 35000HP in sterile saline (0.85%) and adjusted to a McFarland standard 1.0. The McFarland standard is a suspension of barium sulfate which provides a standardized solution turbidity to which solutions can be compared. The McFarland 1.0 standard is equivalent to approximately $3x10^{8}$ colony forming units (CFU)/mL (McFarland Standards). For each assay, seven plates were spread with a lawn of bacteria by swiping the entire plate in three different directions with a sterile swab dipped in the bacterial suspension. One plate in every assay was left as a growth control and had no antibiotic disc. Antibiotic discs were placed onto plates in pairs. Three plates for each assay had tetracycline (30μg) and amoxicillin/clavulanic acid (30μg) and the remaining three plates had erythromycin (15μg) and ampicillin (10μg) discs. The plates were inverted and incubated overnight at the appropriate temperature for either strain (HMC112 or 35000HP). Disc diffusions were performed the same way for each of the five different types of agar: chocolate (growth control), DMSO supplemented (diluent control), EtOH supplemented (diluent control), PAβN supplemented (EPI), and CCCP supplemented (EPI). Each assay was performed in triplicate on three separate

occasions to yield 9 total data points. The zones of inhibition around the antibiotic discs were measured in millimeters after approximately 24 hours of incubation.

MIC Assays

MIC assays were prepared in sterile 96 well plates with strains 35000HP, HMC112 and HD85-023233.A suspension of *H. ducreyi* was made in BHI/starch and adjusted to a McFarland Standard 1.0. In general 100µL BHI(brain-heart infusion)/ starch complete broth was placed into each well of the 96-well plates except the rows for blanks and controls. Then 100µL of ampicillin was added to the first wells of the first six rows and a two-fold serial dilution was performed. Test compound (0.4µL PAβN Figure A2; 0.8µL DMSO Figure A3; 0.4µL EtOH Figure A1) was added to rows 4-6 of the dilution series, then 100µL of inoculated BHI/starch was added to each of the wells including the wells for growth control. For each assay four blanks were set up each having three wells. For the broth only blank, three wells had 150µL BHI/complete and 50µL H_2O to ensure the right optical density. This was an OD control for the solution in the broth only growth control. The ampicillin blank had 150µL BHI/complete and 50µL ampicillin. This was an OD control for the wells with the serial dilution of Ampicillin without test compound (DMSO, EtOH, or PAβN). The test compound blank had 150µL BHI/complete and 50µL H_2O in addition to the calculated amount of test compound necessary (PAβN: 0.4µL; DMSO: 0.8µL, EtOH: 0.4µL). This was the OD control for the growth control containing both broth and test compound. The final blank with both ampicillin and test compound had 100µL BHI/complete, 50µL ampicillin, and either 0.4µL PAβN, 0.4µL EtOH or 0.8µL DMSO. None of the blanks had any bacteria in the wells. This served as the OD control for the wells with the ampicillin serial dilution in the presence of the test compound (EtOH,

DMSO, PAβN). Once completely set up, the 96-well plate was incubated overnight in the appropriate incubator for the strain. At about 24 hours after set up, the plate was read in a plate reader (Thermo Multiskan, EX) with a 595nm filter. For most of the assays, the plates were read at least one more time at about 48 hours after set up.

Data Analysis

For the disc diffusion assays, the mean zone of inhibition was calculated for each antibiotic for each type of agar (chocolate only, DMSO, EtOH, CCCP, PAβN). An ANOVA was run on StatView (Version 5.0.1) to determine whether the means were statistically different from one another. For the MIC assays, the optical density of all the wells of the same composition were averaged (i.e. all three wells with 512μg/mL Amp, broth and bacteria). Then the appropriate blank optical density OD, containing all the background substances in the absence of bacteria, was subtracted from the average of the three wells of identical composition containing bacteria.

Results

In order to determine the susceptibility of *H. ducreyi* to the antibiotics in the presence and absence of the efflux pump inhibitors, disc diffusion assays were performed. However, neither PAβN nor CCCP are soluble in water, therefore, PAβN was dissolved in 95% ethanol, and CCCP was dissolved in 100mM DMSO. Three control trials of the disc diffusion assays were performed in addition to the PAβN and CCCP assays, one with plain chocolate agar, one on agar supplemented with 0.19% EtOH, and one on agar supplemented with 3.125×10^{-4}% DMSO. The concentrations for EtOH and DMSO were the concentrations that were present when the EPI's

were incorporated into the agar. The zones of inhibition were measured for each of the antibiotics: amoxicillin/clavulanic acid, erythromycin, ampicillin, and tetracycline.

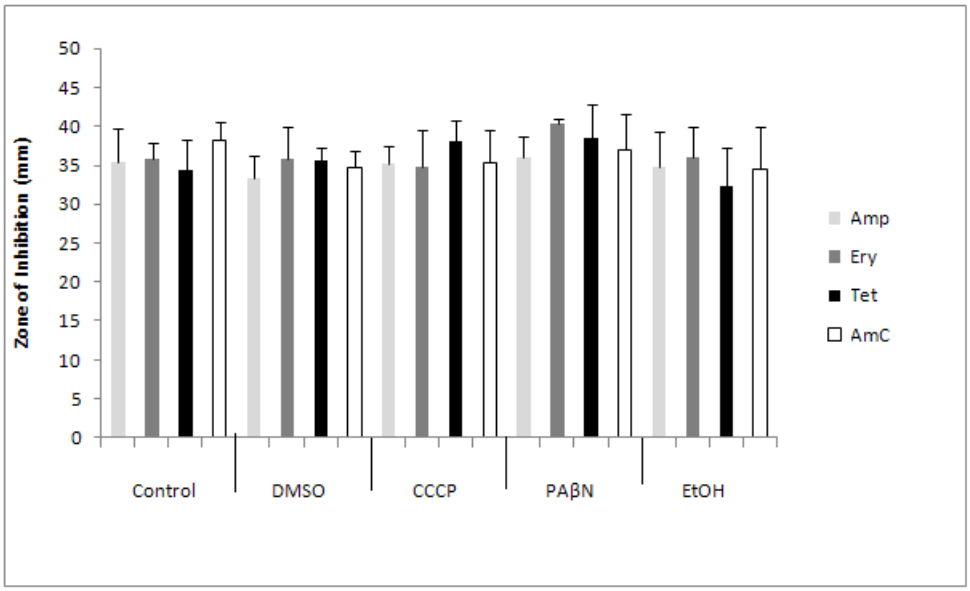


Figure 6: Mean zones of inhibition per antibiotic for each treatment group in *H. ducreyi* strain 35000HP. *H. ducreyi* strain 35000HP was grown on chocolate agar supplemented with the test chemical (none, DMSO, CCCP, PAβN, EtOH) and a disc diffusion assay was performed with the indicated antibiotics. The zones of inhibition for each antibiotic was measured and averaged across all trials (n=9). Error bars indicate standard deviation as calculated by Microsoft Excel.

The data for the zones of inhibition were analyzed with an ANOVA (StatView Version 5.0.1) to determine if the means were statistically different between antibiotics and between test condition (control, EtOH, PAβN, CCCP and DMSO; Figure 6). For 35000HP, none of the antibiotics were statistically different from one another ($p > 0.05$) except for ampicillin and erythromycin ($p=0.0349$). In the treatment condition, the zones of inhibition on the control (chocolate agar) were not statistically different than the zones on diluent control agar (DMSO and EtOH), or the inhibitor CCCP agar ($p>0.05$). However, the zones of inhibition on control agar were statistically different from the zones on inhibitor PAβN agar ($p=0.0328$). In addition,

the zones on PAβN agar were statistically different from the zones on EtOH agar in which it was dissolved ($p<0.0001$). The zones of inhibition for PAβN were larger than the zones for either the chocolate agar control or the EtOH diluent control (Figure 6).

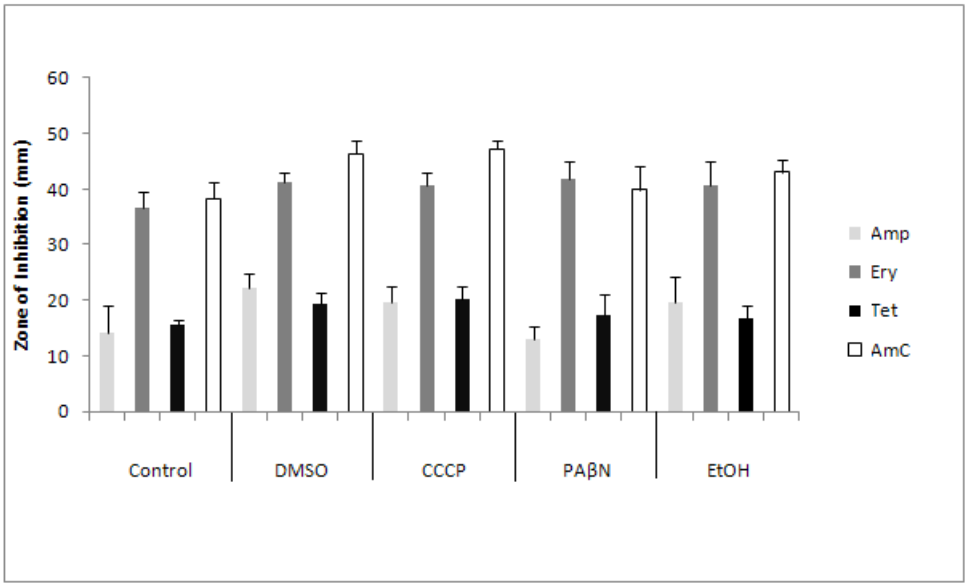


Figure 7: Mean zones of inhibition per antibiotic for each treatment group in *H. ducreyi* strain HMC112. *H. ducreyi* strain HMC112 was grown on chocolate agar supplemented with the test chemical (none, DMSO, CCCP, PAβN, EtOH), and a disc diffusion assay was performed with the indicated antibiotics. The zones of inhibition for each antibiotic were measured and averaged across all trials (n=9). Note: n=8 for amoxicillin/clavulanic acid on DMSO supplemented agar. Error bars show standard deviation as calculated by Microsoft Excel.

The data were analyzed with an ANOVA in the same way as the data for 35000HP. For HMC112, the zones on control (chocolate agar only) were statistically different from the zones on DMSO agar (diluent control) ($p< 0.0001$), EtOH agar (diluent control) ($p < 0.0001$), PAβN agar ($p = 0.0112$), and CCCP agar ($p < 0.0001$). The CCCP treatment did not have zones that were statistically different than the zones for its diluent DMSO ($p = 0.2951$). The zones for PAβN were significantly different from its diluent EtOH ($p = 0.0073$).

In addition to disc diffusion assays, an attempt was made to determine the MIC's of each antibiotic in the presence and absence of each EPI (PAβN, CCCP). In addition, an attempt was made to determine the MIC in the presence and absence of diluent controls (DMSO, EtOH). The raw optical density data obtained from the plate reader was analyzed prior to forming the graphs. In order to determine the amount of bacterial growth in each of the wells, the optical density (OD) of the appropriate blank was subtracted from the wells containing bacteria. For example: for the growth control containing just broth and bacteria, the OD of all the wells containing just broth and bacteria was averaged. Then the OD of the broth blanks was averaged, and this number was subtracted from the broth/bacteria growth control. This is done to take into account the background of the solution so that the bacterial growth can be accurately measured. The first attempt to determine MIC was made using strain 35000HP (Figure A1).

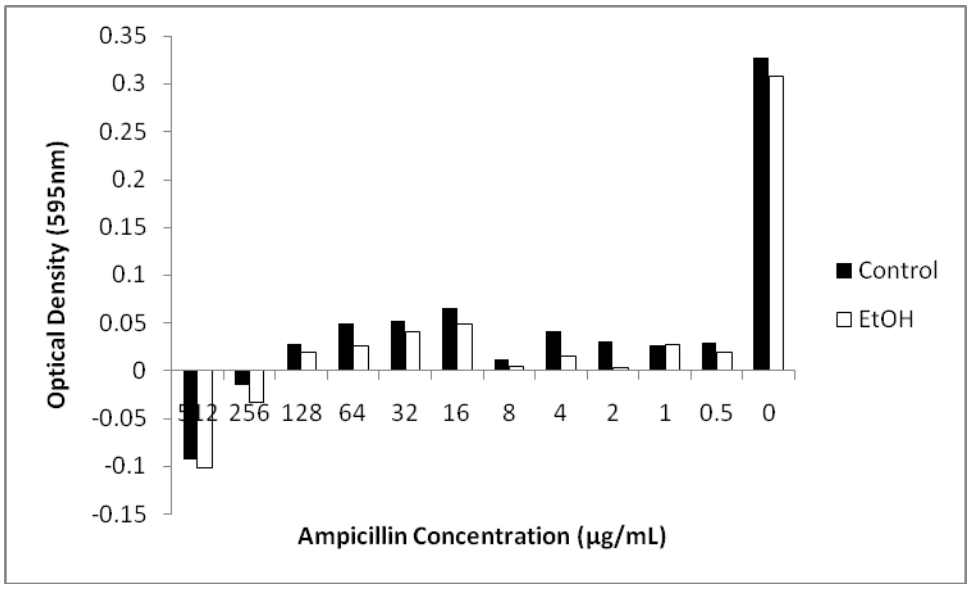


Figure 8: MIC assay for strain 35000HP using ampicillin and testing the broth vs. ethanol measured at 24 hours with 595nm filter.

The growth controls here were successful; however, there was no growth in either of the test conditions (Ampicillin; EtOH + Ampicillin) in the presence of ampicillin at any dilution (Figure 8). The wells containing just bacteria and broth were the growth controls, and these had an optical density that was greater than the optical density of the broth blanks. A second MIC was attempted using 35000HP and instead of EtOH as the test chemical DMSO was used (Figure A4).

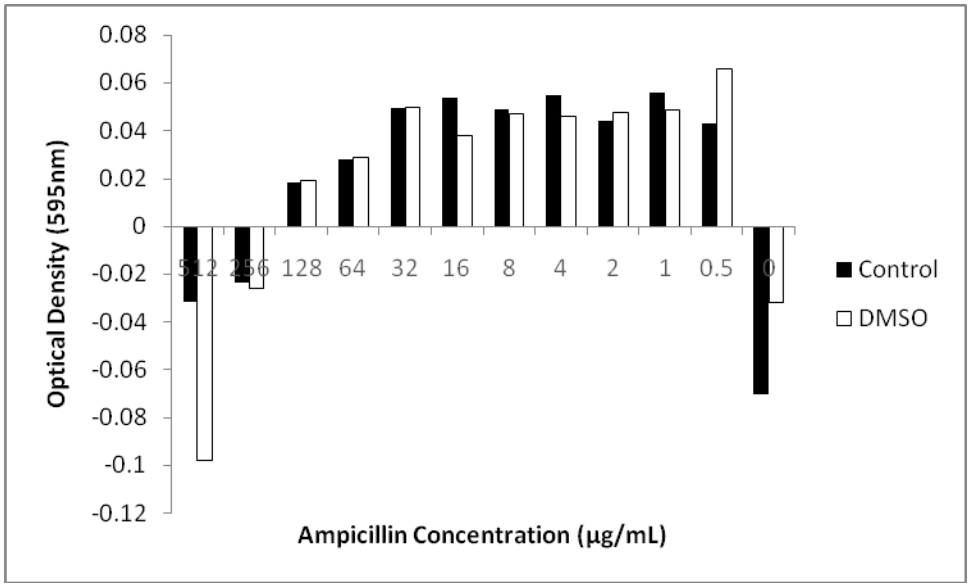


Figure 9: MIC assay for strain 35000HP using ampicillin and testing the broth vs. DMSO measured at 24 hours with 595nm filter.

The MIC with 35000HP comparing a broth control with the DMSO was unsuccessful. None of the growth controls (Control: broth and bacteria; DMSO: broth, DMSO (0.8µL), and bacteria) nor the wells with the ampicillin serial dilutions grew (Figure 9). The next MIC that was attempted was for HMC112 (Figure A2) and was performed across a wider range of ampicillin concentration because in the MIC for 35000HP all the wells containing ampicillin

showed no growth. The wider range of concentrations was to take into account *H. ducreyi*'s sensitivity to ampicillin for 35000HP.

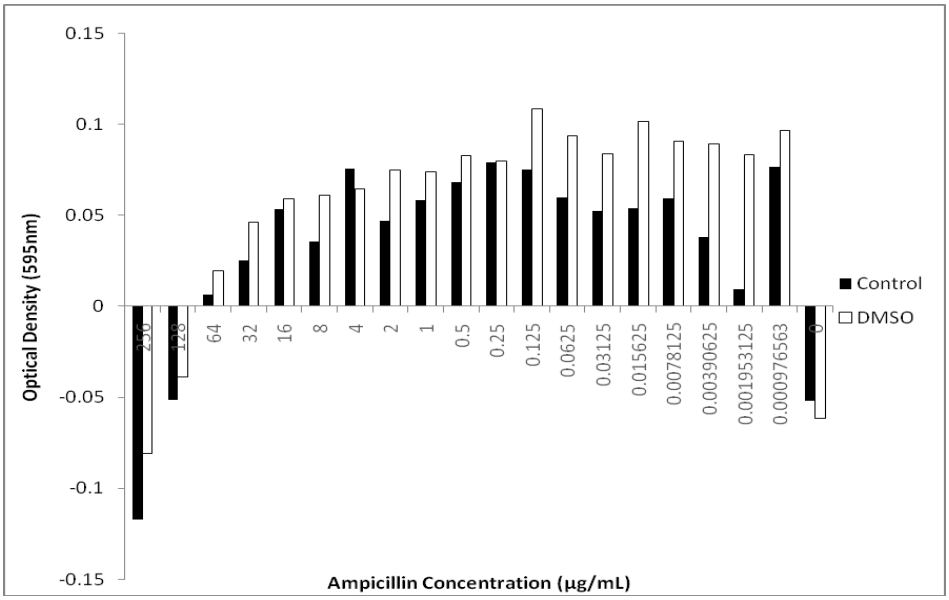


Figure 10: MIC assay for strain HMC112 using ampicillin and testing the control vs. DMSO measured at 47 hours at 595nm filter.

There was no growth for either the controls or the test conditions in this assay (Figure 10). The OD's were very miniscule across all concentrations and in some cases, like the two growth controls (Control: bacteria + broth; DMSO: bacteria +broth +DMSO), the OD was negative, indicating that the blank (no bacteria) had a higher OD than did the wells with bacteria. If there is bacterial growth this should not be the case; when there is bacterial growth the wells containing bacteria should have a higher OD than those not containing bacteria.

The third strain investigated for MIC was the ampicillin resistant strain HD85-023233 (Figure A3).

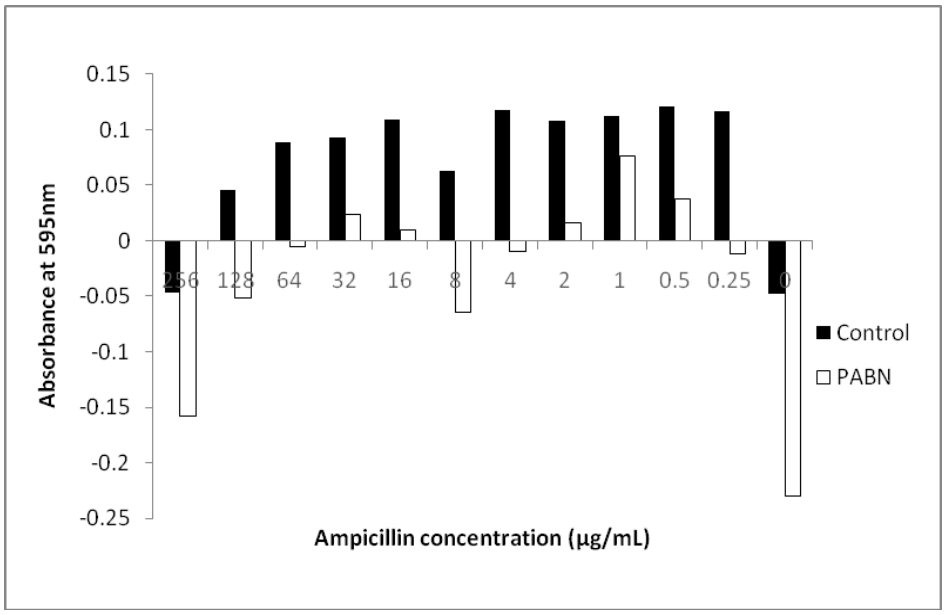


Figure 11: MIC assay for strain HD85-023233 comparing using ampicillin and testing the control vs. PAβN measured at 24 hours at a 595nm filter.

There was no growth for the control or treatment groups for this assay (Figure 11). Similarly to HMC112, the two growth controls (Control: broth + bacteria; PAβN: PAβN + broth + bacteria) had negative ODs indicating that the blanks for each of these conditions had greater ODs than the wells containing bacteria. Without a functioning growth control, the assay is invalid and cannot be analyzed. In addition, all the ampicillin containing wells showed either negative ODs or very small ODs in comparison to the appropriate blanks (no bacteria) indicating no growth. The MIC assays were unsuccessful across all strains tested, mostly because the bacteria were not growing.

Discussion

The purpose of this project was to determine whether *H. ducreyi* has efflux pumps using two different efflux pump inhibitors: PAβN and CCCP. The assays used to determine this were disc diffusion (Kirby-Bauer) and MIC microdilution assays. The disc diffusion assays had different results between the two strains of *H. ducreyi* tested (HMC112 and 35000HP) and showed some interesting results with regards to the controls. The zones of inhibition on the

chocolate agar alone were statistically different from the zones on either of the diluent controls (EtOH, DMSO) for strain HMC112 which was not the case for strain 35000HP. Although the amount of each diluent was small (EtOH: 0.19%; DMSO: 3.125×10^{-4}%), there was still a difference in zone size when there was diluent present in the agar than when the disc diffusion was on control chocolate agar for HMC112 (Figure 7). This was contrary to what was expected. Although DMSO has been shown to inhibit bacterial growth at some concentrations, it is commonly used as a cryoprotectant for eukaryotic and prokaryotic cells (McGann and Walterson, 1987). The concentration typical for DMSO cryoprotection is about 10% with some variation on occasion (Wolf 2010 UMBC). It is possible that the ethanol did not evaporate like expected but rather remained in the agar; it could have inhibited some growth of the bacteria so that when the antibiotic was used, the zones were slightly larger. Ethanol has been shown to have a concentration dependent effect on growth rate in *Vibrio haemolyticus* (Chiang et al. 2006). Solvents like ethanol tend to disrupt the plasma membrane integrity which can lead to cell lysis (Chiang et al. 2006). It is possible that if the ethanol didn't evaporate as expected, the concentration was low enough to alter the membrane permeability of *H. ducreyi* and increase antibiotic susceptibility. In addition, it is possible that HMC112 and 35000HP have different susceptibilities to ethanol as previous studies have shown that the ability to endure organic solvent exposure can vary between strains of bacteria (Sardessai & Bhosle 2002). This could account for the fact that the EtOH diluent control was not statistically different from the chocolate agar only control for strain 35000HP but was statistically different for HMC112.

In the case of DMSO, it is possible that it inhibited the growth of the bacteria. DMSO has been shown to affect antibiotic resistance in *Pseudomonas* and *Mycobacterium tuberculosis* (Jacob and Wood, 1967). It is possible that *H. ducreyi* is particularly susceptible to DMSO

toxicity, and even the minute amounts of the chemical in the agar had an effect on the antibiotic susceptibility. One study determined the MIC of DMSO solutions for a number of different strains of bacteria and fungi and found the lowest MIC to be 4% (Basch and Gadebusch, 1968). This concentration is small, but is still larger than the concentration of DMSO present in the agar (3.125×10^{-4}%). In addition, DMSO has been shown to increase membrane permeability in high concentrations (Pottz et al. 1967). This could have been the reason for the larger zones of inhibition of the antibiotics when HMC112 was grown on DMSO supplemented agar (Figure 7). The same effect was not seen with 35000HP, but it is possible that this particular strain is less susceptible to DMSO toxicity. DMSO could have similar strain dependent effects like those that are seen for EtOH. If a different solvent could be found for the CCCP it is possible that the disc diffusions could be performed with no statistical significance between the controls. Due to the chemicals structure of CCCP (Figure 5), it would be difficult to find a suitable solvent that would not be toxic to the bacteria. This is one of the reasons DMSO was thought to be ideal, because it is generally considered relatively non-toxic at low concentrations (Pottz et al. 1967) and is often used as both a solvent and a cryoprotectant. It would be interesting to compare other class I and II strains of *H. ducreyi* to see if a difference between the control and the diluent controls would be observed. There may be a difference in the way that DMSO and EtOH effect class I and class II strains in general.

The zones of inhibition on CCCP supplemented agar were not statistically different from the zones of inhibition on DMSO supplemented agar for either strain but they were significantly different from the zones of inhibition on the plain chocolate agar control in HMC112 (Figure 7). The fact that the zones on CCCP agar were not statistically different than the zones on the DMSO agar indicates that the difference in zone size was likely due to the DMSO diluent rather

than the CCCP which was dissolved in DMSO prior to incorporation into the agar. CCCP has been shown to effect RND and MATE type efflux pumps (Zhang et al. 2010). Therefore, it was expected to increase the zones of inhibition of all the antibiotics tested on both strains of *H. ducreyi* tested (HMC112, 35000HP). It is possible that the concentration of CCCP used (20μg/mL) was not high enough to have an impact on any efflux pumps that *H. ducreyi* might have. Another possibility is that because both HMC112 and 35000HP are already sensitive to the antibiotics tested (Figure 6 & 7) there was no effect on the zones of inhibition. Previously CCCP was shown to be less effective in decreasing the MICs of antibiotics in already sensitive strains of *Helicobacter pylori* (Zhang et al. 2010). It's possible that if the efflux pumps are present, they are not a major contributor to antibiotic resistance in either strain tested. From this it would be beneficial to investigate whether strains of *H. ducreyi* that do show antibiotic resistance would be affected by CCCP. Using a strain like HD85-023233 which is resistant to ampicillin might give a more accurate assessment of *H. ducreyi*'s efflux pump prevalence when using the inhibitor CCCP.

The PAβN on the other hand was effective in altering the zones of inhibition of each of the antibiotics for both strains. The zones of inhibition on PAβN agar were significantly different from both the chocolate agar only control and the EtOH diluent control for both HMC112 and 35000HP (Figures 6 &7). Each strain must be looked at separately though because for strain 35000HP it is clear that the zones of inhibition were larger on the PAβN supplemented agar when compared with either the chocolate agar only or the EtOH diluent control zones (Figure 6), whereas it is unclear whether the zones of inhibition were larger or smaller when on the PAβN agar for HMC112. For strain HMC112, zones of inhibition on the EtOH diluent control agar was significantly different from zones of inhibition on the chocolate agar only control (Figure 7).

Because of this, the difference observed between the zones of inhibition on PAβN agar and chocolate only or EtOH agar must be carefully assessed and accurate conclusions are difficult to draw. The zones of inhibition for erythromycin and tetracycline were larger when on PAβN agar than on the EtOH agar, but the zones of ampicillin and amoxicillin/clavulanic acid were larger on the EtOH agar than on the PAβN supplemented agar (Figure 7). This makes it difficult to assess what effect the PAβN actually had on the bacteria. All the antibiotics used (Amp, Ery, Tet, AmC) are substrates of RND type efflux pumps (Poole 2007). In addition, both Ery and Tet are substrates of MF family efflux pumps while as of yet Amp and AmC have not been shown to be substrates of these kinds of efflux pumps (Poole 2007). Although PAβN is usually used to assess RND pump prevalence, it is possible that HMC112 also has MF family pumps that were impaired by the EPI and led to the increased zones of inhibition on the PAβN agar.

The effect of PAβN on 35000HP is clearer than for HMC112. The zones of inhibition on the EtOH supplemented agar were not statistically different from the zones on the chocolate agar only, but the zones on PAβN agar were significantly different from both the zones on control agar and the EtOH agar (Figure 6). This indicates that the PAβN increased the zones of inhibition for each of the antibiotics rather than its diluent (EtOH). This indicates that 35000HP probably does have efflux pumps, possibly of the RND type. This EPI has been used previously to demonstrate the presence of RND efflux pumps in *H. pylori* (Hirata et al. 2009), *P. aeruginosa* (Tohidpour et al. 2009), *S. enterica* serovar Typhimurium and *E. coli* (Coldham et al. 2010). Therefore, it is possible that 35000HP also has efflux pumps of the RND type like the previously mentioned bacteria. These observations are consistent with the BLAST analyses showing sequence homology between *H. ducreyi* genes and those for RND pumps from *P. aeruginosa* (Figure B1-B3) and *N. meningitidis* (Figure B4-B6). From the results of this experiment with

strain 35000HP, it appears that disc diffusions in the presence of PAβN show some promise for determining the presence of efflux pumps in *H. ducreyi*.

The other assays attempted in order to determine the efflux characteristics were MIC microdilution assays. The MIC assays performed were unsuccessful. Three different strains of *H. ducreyi* were used to test the effectiveness of EPI to increase antibiotic susceptibility, but neither HMC112 nor HD85-023233 grew in BHI/Starch complete media in the 96- well plates (Figures 10 & 11). The growth controls for the first attempt looking at EtOH and the Control (broth) with 35000HP were successful; however, all the wells containing ampicillin did not grow (Figure 8). The second attempt at obtaining an MIC for 35000HP, this time in the presence and absence of DMSO, was unsuccessful (Figure 9). Like HMC112 and HD85-023233, neither growth controls nor the wells with the serial dilutions of ampicillin grew. In the case of HD85-023233, it is possible that instead of starch it would grow better with serum in the media (Zac Saylor, personal correspondence). In the case of 35000HP, the concentration of ampicillin was probably too high, killing all the bacteria, and should be reduced for assays in the future. HMC112 did not grow in the control wells or the wells containing ampicillin (Figure 10). Both strains HMC112 and 35000HP are susceptible to ampicillin, albeit HMC112 less so than 35000HP, but HD85-023233 is resistant up to 128μg/mL (Zac Saylor, personal correspondence). It was not surprising that the 35000HP growth controls were successful while the bacteria in the presence of ampicillin were killed. It was unexpected for none of the strains 35000HP (second attempt), HMC112 (Figure 10) or HD85-023233 (Figure 11) to grow in either just the broth controls or the wells with ampicillin. A second attempt at determining the MIC for ampicillin in the presence and absence of PAβN for HD85-023233 was similarly unsuccessful to the first attempt with no growth in any of the wells.

Therefore, it might be beneficial to determine the MIC's via the agar dilution method instead of through broth microdilution. All three strains used (35000HP, HMC112, and HD85-023233) grew well on chocolate agar even when supplemented with the efflux pump inhibitors (CCCP and PAβN). The agar dilution method has been used previously on strains of *P. aeruginosa* and was effective for determining the MIC in the presence and absence of the EPI PAβN (Tohidpour et al. 2009). This method might work better for *H. ducreyi* which did not grow successfully in broth. One drawback to this method would be potential interaction between antibiotics and the efflux pump inhibitors in the agar. A different possible method to use would be an E-test in which a strip having a gradient of antibiotic concentrations is placed onto agar instead of the single concentration antibiotic disc (Varela et al. 2008). This would allow for the determination of MIC without using an exorbitant number of plates and would reduce concerns about negative interactions of EPI and antibiotic.

In the future it would be interesting to determine if other strains of *H. ducreyi* have similar susceptibilities to these antibiotics (Amp, Ery, Tet, AmC) in the presence of EPIs. It would be especially interesting to look at strains that already show antibiotic resistance, such as HD85-023233 which is ampicillin resistant. If these strains show increased zones of inhibition, then efflux pumps would probably contribute to their resistance. Class I strains would probably be the best jumping off point from these data as the results with the class I strain 35000HP were much clearer and easier to interpret than those of the class II strain HMC112. It would also be possible to look at a broader range of antibiotics to determine if efflux pumps play a role in reducing susceptibility to other classes of antibiotics. Many antibiotics are substrates of the RND efflux pumps suspected to be present in *H. ducreyi* including aminoglycosides and fluoroquinolones (Poole 2007). It might also be interesting to look at antibiotics that are not

substrates of RND pumps, like streptogramins which are substrates of ABC type pumps (Poole 2007), to see if *H. ducreyi* might have other types of efflux pumps. In addition to more strains and antibiotics, there are a number of other EPIs currently available to use in in vitro testing; one in particular 1-(1-naphthylmethyl)-piperazine (NMP) might be another option instead of CCCP for future testing. This EPI has been used on strains of *Enterobacteriaceae* to determine efflux pump characteristics (Schumacher et al. 2006). It has been shown to inhibit RND type efflux pumps to a slightly lesser extent than PAβN (Schumacher et al. 2006). It would be interesting to see if this inhibitor might show a more significant impact than CCCP in *H. ducreyi*. A completely different approach could also be taken using a fluorescence assay with efflux pump inhibitors to assess the efflux characteristics of *H. ducreyi*. In this type of assay the accumulation of a fluorescent dye that is a substrate for efflux pumps, like Hoecht 33342, is measured in the presence and absence of EPI's (Piddock et al. 2010). If the accumulation increases in the presence of the EPI, it can be determined that the strain likely has efflux pumps that contribute to drug resistance (Piddock et al. 2010). This kind of assay would provide a more specific measure of the efflux characteristics than disc diffusions can provide.

Overall, from the data collected here, it is possible that *H. ducreyi* 35000HP does contain some efflux pumps of the RND type. Because of the conflicting data in HMC112 it is not really possible to say the same for HMC112. In the future it would be beneficial to determine the extent to which efflux pumps are prevalent across strains, in particular in resistant strains like HD85-023233.There are a number of future experiments that are possible for a more in depth understanding of *H. ducreyi*'s efflux pump characteristics including adapting the MIC analysis and incorporating a fluorescence assay. Continuing to assess the efflux pump prevalence in *H. ducreyi* is an important strategy to combat resistance and improve treatment of chancroid. By

understanding the prevalence of efflux pumps in *H. ducreyi* clinicians and researchers may be able to use more effective treatments on these resistant strains. Therefore, investigations into *H. ducreyi*'s efflux pump prevalence should be continued in the future.

Appendix A

35000HP MIC for Ampicillin in the presence and absence of EtOH												
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control
Control	512	256	128	64	32	16	8	4	2	1	0.5	Growth control
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control (EtOH)
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control (EtOH)
EtOH (0.19%)	512	256	128	64	32	16	8	4	2	1	0.5	Growth control (EtOH)
	Broth Blank	Broth Blank	Broth Blank	Broth/ Amp	Broth/ Amp	Broth/ Amp	Broth/ EtOH	Broth/ EtOH	Broth/ EtOH	Broth/ Amp/ EtOH	Broth/ Amp/ EtOH	Broth/ Amp/ EtOH
	Growth Control	Growth Control	Growth Control	Growth Control	Growth Control	Growth Control	Growth Control/ EtOH	Growth Control/ EtOH	Growth Control/ EtOH	Growth Control/ EtOH	Growth Control/ EtOH	Growth Control/ EtOH

Figure A1: Diagram of 96-well plate set up for MIC assay with *H. ducreyi* strain 35000HP. All the wells with a number indicate the concentration (µg/mL) of ampicillin present in the well after serial two-fold dilution. These wells have 100µL of inoculated BHI/starch. The growth controls had 50µL BHI/starch complete in addition to 50µL of DI H_2O and 100µL inoculated BHI/starch. The growth controls with EtOH also had 0.4µL EtOH (95%). The broth blanks have 150µL BHI/starch complete and 50µL DI H_2O. The wells labeled Broth/amp had 150µL BHI/starch complete and 50µL Ampicillin (concentration in well: 512µg/mL). The Broth/EtOH blanks had 150µL BHI/starch complete, 50µL DI H_2O, and 0.4µL EtOH (95%). The Broth/Amp/EtOH blanks had 150µL BHI/starch complete, 50µL ampicillin, and 0.4µL EtOH (95%).

HD85-023233 MIC for Ampicillin in the presence and absence of PAβN												
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control
Control	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control (PAβN 50µg/mL)
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control (PAβN 50µg/mL)
PAβN (50µg/mL)	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control (PAβN 50µg/mL)
	Broth Blank	Broth Blank	Broth Blank	Broth/ Amp	Broth/ Amp	Broth/ Amp	Broth/ PAβN	Broth/ PAβN	Broth/ PAβN	Broth/ Amp/ PAβN	Broth/ Amp/ PAβN	Broth/ Amp/ PAβN

Figure A2: Diagram of 96-well plate set-up for MIC assays with HD85-023233 All the wells with a number indicate the concentration (µg/mL) of ampicillin present in the well after serial two-fold dilution. These wells have 100µL of inoculated BHI/starch. The growth controls had 50µL BHI/starch complete in addition to 50µL of DI H_2O and 100µL inoculated BHI/starch. The growth controls with PAβN also had 0.4µL PAβN (final well concentration of 50µg/mL). The broth blanks have 150µL BHI/starch complete and 50µL DI H_2O. The wells labeled Broth/amp had 150µL BHI/starch complete and 50µL Ampicillin (concentration in well: 256µg/mL). The Broth/ PAβN blanks had 150µL BHI/starch complete, 50µL DI H_2O, and 0.4µL PAβN (final concentration in well of 50µg/mL). The Broth/Amp/ PAβN blanks had 150µL BHI/starch complete, 50µL ampicillin, and 0.4µL PAβN (final concentration of 50µg/mL). This assay was repeated to similar results.

MIC for HMC112 in the presence and absence of DMSO												
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control
	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.001953125	0.000976563	Broth Blank	Broth Blank	Broth Blank	Growth control
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control
Control	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.001953125	0.000976563	Broth/ Amp	Broth/ Amp	Broth/ Amp	Growth Control
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control (DMSO)
	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.001953125	0.000976563	Broth/ DMSO	Broth/ DMSO	Broth/ DMSO	Growth control (DMSO)
	256	128	64	32	16	8	4	2	1	0.5	0.25	Growth control (DMSO)
DMSO (3.125×10^{-4}%)	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.001953125	0.000976563	Broth/ Amp/ DMSO	Broth/ Amp/ DMSO	Broth/ Amp/ DMSO	Growth control (DMSO)

Figure A3: Diagram of 96-well plate set-up for MIC assays with HMC112 All the wells with a number indicate the concentration (μg/mL) of ampicillin present in the well after serial two-fold dilution. These wells have 100μL of inoculated BHI/starch. The growth controls had 50μL BHI/starch complete in addition to 50μL of DI H_2O and 100μL inoculated BHI/starch. The growth controls with DMSO also had 0.8μL DMSO (100mM). The broth blanks have 150μL BHI/starch complete and 50μL DI H_2O. The wells labeled Broth/amp had 150μL BHI/starch complete and 50μL Ampicillin (concentration in well: 256μg/mL). The Broth/ DMSO blanks had 150μL BHI/starch complete, 50μL DI H_2O, and 0.8μL DMSO (100mM). The Broth/Amp/ PAβN blanks had 150μL BHI/starch complete, 50μL ampicillin, and 0.8μL DMSO (100mM).

35000HP MIC for Ampicillin in the presence and absence of DMSO												
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control
Control	512	256	128	64	32	16	8	4	2	1	0.5	Growth control
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control (DMSO)
	512	256	128	64	32	16	8	4	2	1	0.5	Growth control (DMSO)
DMSO (3.125×10^{-4}%)	512	256	128	64	32	16	8	4	2	1	0.5	Growth control (DMSO)
	Broth Blank	Broth Blank	Broth Blank	Broth/ Amp	Broth/ Amp	Broth/ Amp	Broth/ DMSO	Broth/ DMSO	Broth/ DMSO	Broth/ Amp/ DMSO	Broth/ Amp/ DMSO	Broth/ Amp/ DMSO
	Growth Control	Growth Control	Growth Control	Growth Control	Growth Control	Growth Control	Growth Control/ DMSO	Growth Control/ DMSO	Growth Control/ DMSO	Growth Control/ DMSO	Growth Control/ DMSO	Growth Control/ DMSO

Figure A4: Diagram of second 96-well plate set up for MIC assay with *H. ducreyi* strain 35000HP. All the wells with a number indicate the concentration (μg/mL) of ampicillin present in the well after serial two-fold dilution. These wells have 100μL of inoculated BHI/starch. The growth controls had 50μL BHI/starch complete in addition to 50μL of DI H_2O and 100μL inoculated BHI/starch. The growth controls with EtOH also had 0.8μL DMSO (100mM). The broth blanks have 150μL BHI/starch complete and 50μL DI H_2O. The wells labeled Broth/amp had 150μL BHI/starch complete and 50μL Ampicillin (concentration in well: 512μg/mL). The Broth/DMSO blanks had 150μL BHI/starch complete, 50μL DI H_2O, and 0.8μL DMSO (100mM). The Broth/Amp/EtOH blanks had 150μL BHI/starch complete, 50μL ampicillin, and 0.8μL DMSO (100mM).

Appendix B

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MexA Score = 62.8 bits (151),  Expect = 1e-11, Method: Compositional matrix adjust.
 Identities = 53/227 (23%), Positives = 101/227 (44%), Gaps = 16/227 (7%)

Query  80   KEGSDVKAGQQLYQIDPATYEADYQSAQANLASTQEQAQRYKLLVADQAVSKQQYADANA  139
            K G  V  GQ L ++D +  +A   ++++ L + +    RY+ LVA ++ SK +   A A
Sbjct  122  KSGERVTRGQLLIELDSSVEQATLHASESQLPNAKATLSRYRSLVASKSASKAELDKAQA  181

Query  140  AYLQSKAAVEQARINLRYTKVLSPISGRIGRSAVTEGALVTNGQANAMATVQQLDPIYVD  199
             Y Q  A +E  +  ++  ++ +P  G +G   V  G  +T   A  +  V+    + V
Sbjct  182  TYNQLVANIESLKAAIKRRQIYAPFDGIVGIVKVNLGEYIT--AATEIVRVEDSSQMKVS  239

Query  200  VTQPSTALLRLRRELASGQLERAGDNAAKVSLKLEDGSQYPLEGRLEFSEVSVDEGTGSV  259
               P T L     ++  GQ         +V+  ++       E ++   + +V + TG V
Sbjct  240  FNVPQTNL----ADITIGQ---------RVAFSVDAIPNQTFEAKVSAIDSAVTKSTGLV  286

Query  260  TIRAVFPNPNNELLPGMFVHAQLQEGVKQKAILAPQQGVTRDLKGQA  306
             + AV  +   +L+ GMF    +    +   ++ PQ  VT  + G++
Sbjct  287  AVEAVV-DGRGQLMSGMFARLNIALATEMDQVVVPQIAVTYTMYGES  332
```

Figure B1: Sequence homology of *P. aeruginosa* strain PAO1 outer membrane protein MexA with *H. ducreyi* strain 35000HP whole genome

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Score =  400 bits (1029),  Expect = 6e-113, Method: Compositional matrix adjust.
 Identities = 308/1038 (29%), Positives = 533/1038 (51%), Gaps = 47/1038 (4%)

Query  2     SKFFIDRPIFAWVIALVIMLAGGLSILSLPVNQYPAIAPPAIAVQVSYPGASAETVQDTV  61
             +  FI RP+ A  I++V ++ G  ++  L V +YP +    I V  +Y GA AET+Q  +
Sbjct  3     TDIFIKRPVLAISISIVFIILGLQAMFKLQVREYPEMTNTIITVTTNYAGADAETMQAFI  62

Query  62    VQVIEQQMNGIDNLRYISSESNSDGSMTITVTFEQGTDPDIAQVQVQNKLQLATPLLPQE  121
                +E+ +   DN+ YI S+S    S  IT+  +  T+P IA   + +K+      LP
Sbjct  63    TSKLEESIAQADNIDYIKSDSAPSTS-NITIKMKLNTNPGIAVADITSKVNAVRSQLPSN  121

Query  122   VQRQGIRVTKAVKNFLMVVGVVSTDGSMTKEDLSNYIVSNIQDPLSRTKGVGDFQVFG-S  180
             +    IR +      ++ +   S +  ++   L++YI   ++  +    GV     FG +
Sbjct  122   IDDPSIRSSTGAMEAIVFIRFSSKE--LSAPQLTDYIERVVKPQMFTVNGVSKINYFGGA  179

Query  181   QYSMRIWLDPAKLNSYQLTPGDVSSAIQAQNVQISSGQLGGLPAVKGQQLNATIIGKTRL  240
              + +RIWLDPAK+    L+  +V++A+ A NVQ ++G   G       ++      +T
Sbjct  180   SFGLRIWLDPAKIAGLGLSANEVTAALAANNVQTAAGNSNGYYTEYRNKV------ETTT  233

Query  241   QTAEQFENILLKVNPDGSQVRLKDVADVGLGGQDYSINAQFNGSPASGIAIKLATGANAL  300
             ++ E+  ++++K  P+GS ++L D+A++ L           NG  A  +A++ A+ ANAL
Sbjct  234   KSVEELASLVIKTLPNGSNIKLGDIAEISLEKARDDSRGIVNGREAVVLAVEAASTANAL  293

Query  301   DTAKAIRQTIANLEPFMPQGMKVVYPYDTTPVVSASIHEVVKTLGEAILLVFLVMYLFLQ  360
             D AK I    A ++  +P  ++    YD T  +++SI EVVKT+ EA+++V +V+ LFL
Sbjct  294   DVAKHIYPKFAEIQSNLPASVEAEVIYDKTIAINSSIKEVVKTILEAVIIVLVVIVLFLG  353

Query  361   NFRATLIPTIAVPVVLLGTFGVLAAFGFSINTLTMFGMVLAIGLLVDDAIVVVENVERVM  420
             +FRA +IP + +P+ L+G   VL  FGF+IN LT+ G++LAIGL+VDDAIVV+ENVER +
Sbjct  354   SFRAMIIPIVTIPISLIGVLMVLQGFGFTINLLTLLGLILAIGLVVDDAIVVLENVERHI  413

Query  421   AEEGLSPREAARKSMGQIQGALVGIAMVLSAVFLPMAFFGGSTGVIYRQFSITIVSAMAL  480
              + G +P  AA     +I   ++ + + L AV+ PMA   G TG ++++FS+T+  A+ +
Sbjct  414   KQNGETPFRAAIIGTREIATPVIAMTITLMAVYSPMALMSGITGSLFKEFSLTLAGAVFI  473

Query  481   SVIVALILTPALCATMLKPIEKGDHGEHKGGFFGWFNRMFLSTTHGYERGVASILKHRAP  540
             S + AL L+P + + +LK  EK    E +        +     T  Y + +  ++  R
Sbjct  474   SGVAALTLSPMMTSKILKQSEKPSWLEQR------IEKTLHKVTDIYTKILLVVMASRYA  527

Query  541   YLLIYVVIVAGMIWMFTRIPTAFLPDEDQGVLFAQVQTPPGSSAERTQVVVDSMREYLLE  600
              L+   +I A +  +F  + +   P ED+G++      P   + +  Q V+  + + +
Sbjct  528   ILVFAGLIFASLPMLFNSLSSEVAPAEDRGIVMGMASGPANVNMDYMQDVMFPVAQQI--  585

Query  601   KESSSVSSVFTVTGFNFAGRGQSSGMAFIM--LKPWEERPGGENSVFELAKRAQMHFFSF  658
              +   V++   + GF       SS  +F++  LK W ER   +  V     +
Sbjct  586   NQLPEVNATQIMPGF------PSSNGSFMLVPLKDWSERTRSQKEVLAEVNKITNE---V  636

Query  659   KDAMVFAFAPPSVLELGNATGFDLFLQDQAGVGHEVLLQARNKFLMLAAQNPALQRVRPN  718
              +A V  F  P +    +   F   +       +  L Q  + FL+ A  +     + +
Sbjct  637   TEASVIGFEFPEISTGESGLPFSFVI--TTANDYRDLAQVASDFLLAAQSSGQFFFNQLD  694
```

```
Query  719   GMSDEPQYKLEIDDEKASALGVSLADINSTV-SIAWGSSYVNDFIDRGRVKRVYLQGRPD  777
              D     ++ID EK    GV+ ADI S + S   G++     ID GR   +  Q +
Sbjct  695   LKFDTASMAIKIDREKMGTYGVTAADIRSVLGSFLSGATLARVDID-GRAYDIISQVKRS  753

Query  778   ARMNPDDLSKWYVRNDKGEMVPFNAFATGKWEYGSPKLERYNGVPAMEILGEPAPGLSSG  837
               R++P DL+K+YV+   G  VP + F + + +     L + N + +  I G  A   S G
Sbjct  754   ERLSPQDLAKYYVKLKDGSSVPLSTFVSMELKTQPRTLPKLNQLNSATITGVLAG--SMG  811

Query  838   DAMAAVE-EIVKQLPKGVGYSWTGLSYEERLSGSQAPALYALSLLVVFLCLAALYESWSI  896
              +A+   E +  + LP G  Y +   S + +  G+     + L++++++L LA  +ESW
Sbjct  812   NAVKWAEAKAAEILPAGYYYDFMSESRQYKQEGNALALTFLLAIVMIYLVLAVQFESWRD  871

Query  897   PFSVMLVVPLGVIGALLATSM--------RGLSNDVFFQVGLLTTIGLSAKNAILIVEFA  948
              P  +++ VPL + GAL+  ++         G + +++ QVGL+T +GL  K+ IL+ E A
Sbjct  872   PMVILVSVPLAISGALVVLNILALPMIGKAGATLNIYSQVGLITLVGLITKHGILMCEVA  931

Query  949   KE--LHEQGKGIVEAAIEACRMRLRPIVMTSLAFILGVVPLAISTGAGSGSQHAIGTGVI  1006
              KE  LH  GK   +A   A ++RLRPI+MT+ A I G++PL  +TGAG+  + +IG  ++
Sbjct  932   KEEQLH-HGKNKFDAITYAAQIRLRPILMTTAAMIAGLIPLLYATGAGAVMRFSIGIVIV  990

Query  1007  GGMVTATVLAIFWVPLFY  1024
               G+   T+  +F +P+ Y
Sbjct  991   AGLAIGTLFTLFVLPVIY  1008
```

Figure B2: Sequence homology of *P. aeruginosa* strain PAO1 transporter protein MexB with *H. ducreyi* strain 35000HP whole genome.

```
Score = 92.4 bits (228),  Expect = 2e-20, Method: Compositional matrix adjust.
 Identities = 115/481 (23%), Positives = 213/481 (44%), Gaps = 30/481 (6%)

Query  1    MKRSFLSLAVA-AVVLSGC--SLIPDYQRPEAPVAAAYPQGQAYGQNTGAAAVPAADIGW  57
            MK S L+L +  A  LS C  S+  D    +A  +      Q Y + T    +   D  W
Sbjct  1    MKFSKLALFITLASFLSACTSSMTKDGSLEQAKTSY-----QQYEELTKQFNI---DEEW  52

Query  58   REFFRDPQLQQLIGVALENNRDLRVAALNVEAFRAQYRIQRADLFPRIGVDGSGTRQR-L  116
             + + DPQL +L+  AL NN +L  +A+ V        +  A L P     GS +  + L
Sbjct  53   WKGYNDPQLNRLVSQALANNVNLAKSAILVNKALYNANLVGASLVPTFSGSGSSSASKLL  112

Query  117  PGDLSTTGSPAISSQYGVTLGTT---AWELDLFGRLRSLRDQALEQYLATEQAQRSAQTT  173
              D S   S +++    V    +   ++ LDL+ RL      +  +Y AT +  ++ + +
Sbjct  113  VSDGSLQSSNSVTKASNVVHNASFNLSYTLDLWRRLADSASASEWEYKATIEDLKATRLS  172

Query  174  LVASVATAYLTLKADQAQLQLTKDTLGTYQKSFDLTQRSYDVGVASALDLRQAQTAVEGA  233
            ++ +V  +Y  +   +  + +++ T+  Y++   + +   +VG    L + QA+ A   A
Sbjct  173  IINAVVASYYQIAYLKDVINVSEQTIKNYEQINHILENKMEVGAIDHLAVEQAKQATLNA  232

Query  234  RATLAQY-TRLVAQDQ---NALVLLLGSGIPANLPQGLGLDQTLLTEVPAGLPSDLLQRR  289
            R  L  Y T L   +Q   N L L     +P   P  L ++   L  V   +P   +  R
Sbjct  233  RNGLIGYRTTLKVAEQTLRNLLNLKPNEPLPIEYPAILSVE---LQGVDMNVPISAIANR  289

Query  290  PDILEAEHQLMAANASIGAARAAFFPSISLTANAGTMSRQLSGLFDAGSGSWLFQPSINL  349
            PD+     +L +A  S+ A   ++FP+++L A+  + + +L  + D   G+ +   S+NL
Sbjct  290  PDVAAKLKRLQSAFKSLSATEKSWFPTVTLGASISSSAAKLRNIGDNQLGNSMI--SLNL  347

Query  350  PIFTAGSLRASLDYAKIQKDINVAQYEKAIQTAFQEVAD-GLAARGTF--TEQLQAQRDL  406
            P     +++ ++  ++    +    YE+ I +A  EV +   A +  F     LQ   +
Sbjct  348  PFLDWQTVKNNVKLSEADYRLAKLNYEQVITSALNEVDNYYYAYQQAFDSYSTLQQVYEH  407

Query  407  VKASDEYYQLADKRYRTGVDNYLTLLDAQRSLFTAQQQLITDRLNQLTSEVNLYKALGGG  466
             K    YY+    RY  GV  +   ++A     T+Q  ++  +   L  E  +Y+++ G
Sbjct  408  NKKISFYYK---NRYELGVAEFREWVNAMNVELTSQLAILNQKYTILAKENAVYQSMAGK  464

Query  467  W  467
            +
Sbjct  465  Y  465
```

Figure B3:Sequence homology of *P. aeruginosa* strain PAO1 outer membrane protein OprM with *H. ducreyi* strain 35000HP whole genome.

MtrC GENE ID: 1490957 macA | macrolide-specific efflux protein [Haemophilus ducreyi 35000HP]

```
 Score = 52.8 bits (125),  Expect = 2e-08, Method: Compositional matrix adjust.
 Identities = 52/185 (29%), Positives = 92/185 (50%), Gaps = 15/185 (8%)

Query  65   GRLESLRTADVRAQVGGIIQKRLFQEGSYVRAGQPLYQIDSSTYE-------AGLESara  117
            G L+++   ++ AQV G IQ  L  EG YV+ G+ L  ID    +       A LES++A
Sbjct  53   GILQAVDQVNIGAQVSGQIQDILVSEGQYVKKGELLAIIDPQLAQSELKLAKAELESSQA  112

Query  118  qlataqatlakadadlarYKPLVAAEAVSRQEYDAAVTAKRSAEAGVKAAQAAIKSAGIS  177
             +   QA L +   +  R++ L+   A S+QE+D A T    A+A ++ AQ+ ++SA +
Sbjct  113  NMLAKQAILKQYQTNFERHRKLIKQNASSQQEFDEARTRLEVAKADIRIAQSNLESANLR  172

Query  178  LNRSR-------ITAPISGFIGQSKVSEGTLLNAGDAT-VLATIRQTNPMYVNVTQSASE  229
            + R++       I +P+ G +       G  L A      L T+   + M ++   S ++
Sbjct  173  VTRAKTQLAYTEIRSPVDGTVVSIIAQSGQTLAAQQQVPTLMTLANIDTMKISAKISEAD  232

Query  230  VMKLR  234
            +M ++
Sbjct  233  IMHVQ  237
```

Figure B4: Sequence homology from comparison of Neisseria meningitidis strain MC58 efflux protein gene MtrC with *H. ducreyi* strain 35000HP whole genome

MtrD GENE ID: 1491387 HD1512 | acriflavine resistance protein
[Haemophilus ducreyi 35000HP]

```
 Score =  299 bits (766),  Expect = 2e-82, Method: Compositional matrix adjust.
 Identities = 314/1050 (30%), Positives = 526/1050 (51%), Gaps = 68/1050 (6%)

Query  5     FIDRPIFAWVISifiiaagifgiKSLPVSQYPSVAAPTITLRATYPGASAQVMEDSVLSV  64
             FI RP+ A  ISI   I  G+  +  L V +YP +    IT+   Y GA A+ M+  + S
Sbjct  6     FIKRPVLAISISIVFIILGLQAMFKLQVREYPEMTNTIITVTTNYAGADAETMQAFITSK  65

Query  65    IERNMNGVEGLDYMstsadssgsgsvsLTFTPDTDENLAQVEVQNKLSEVLSTLPATVQQ  124
             +E ++   + +DY+ + +  S S    +    +T+  +A  ++ +K++ V S LP+ +
Sbjct  66    LEESIAQADNIDYIKSDSAPSTSNIT-IKMKLNTNPGIAVADITSKVNAVRSQLPSNIDD  124

Query  125   YGVTVSKARSNFLMIVMLSSDVQSTEEMNDYAQRNIVPELQRIEGVGQVRLFG-AQRAMR  183
               +  S      ++ +  SS   S  ++ DY +R + P++  + GV ++  FG A   +R
Sbjct  125   PSIRSSTGAMEAIVFIRFSSKELSAPQLTDYIERVVKPQMFTVNGVSKINYFGGASFGLR  184

Query  184   IWVDPKKLQNYNLSFADVGSALSAQNVQISAGSIGSLPavrgqtvtatvtaqgqLGTAEE  243
             IW+DP K+    LS  +V +AL+A NVQ +AG+        G         +    + EE
Sbjct  185   IWLDPAKIAGLGLSANEVTAALAANNVQTAAGN------SNGYYTEYRNKVETTTKSVEE  238

Query  244   FGNVILRANTDGSNVYLKDVARVGLGMEDYSSSTRLNGVNTTGMAVMLSNSGNAMATAKA  303
               +++++   +GSN+ L D+A + L      S   +NG     +AV  +++ NA+  AK
Sbjct  239   LASLVIKTLPNGSNIKLGDIAEISLEKARDDSRGIVNGREAVVLAVEAASTANALDVAKH  298

Query  304   VKERMATLEKYFPQGMSWKTPYDTSKFVEISIEKVIHTLIEAmvlvfvvmylflQNIRYT  363
             +  + A ++   P  +  +  YD +  +  SI++V+ T++EA+++V VV+ LFL + R
Sbjct  299   IYPKFAEIQSNLPASVEAEVIYDKTIAINSSIKEVVKTILEAVIIVLVVIVLFLGSFRAM  358

Query  364   LIPTIVVPISLLGGFAFISYMGMSINVLTMFAMvlvigivvddaivvvenvERIMAGEGL  423
             +IP + +PISL+G    +   G +IN+LT+  ++L IG+VVDDAIVV+ENVER +   G
Sbjct  359   IIPIVTIPISLIGVLMVLQGFGFTINLLTLLGLILAIGLVVDDAIVVLENVERHIKQNGE  418

Query  424   PPKEATKKAMGQISGAVIGITAVLISVFVPLAMFSGATGNIYKQFALTMassiafsafla  483
              P  A      +I+  VI +T  L++V+ P+A+ SG TG+++K+F+LT+A ++  S   A
Sbjct  419   TPFRAAIIGTREIATPVIAMTITLMAVYSPMALMSGITGSLFKEFSLTLAGAVFISGVAA  478

Query  484   ltltpalcatmlKTIPKGHHEEkkgffgwfnkkfnSWTHGYEGRVAKVLRK-----TFRM  538
             LTL+P + + +LK   K                 SW    E R+ K L K     T  +
Sbjct  479   LTLSPMMTSKILKQSEK----------------PSW---LEQRIEKTLHKVTDIYTKIL  518

Query  539   MVVYIGLAVVGVF----------LFMRLPTSFLPTEDQGFVMVSVQLPAGATQERTNATL  588
             +VV      + VF          LF  L +   P ED+G VM     PA    +     +
Sbjct  519   LVVMASRYAILVFAGLIFASLPMLFNSLSSEVAPAEDRGIVMGMASGPANVNMDYMQDVM  578
```

```
Query  589   AQVTQLAKSIPEIENIITVSGFSFSGSGQNMAMGFAILKDWNERTAPGSDAVAIAGKLTG  648
              V Q    +PE+     + GF  S +G  M +    LKDW+ERT    + +A   K+T
Sbjct  579   FPVAQQINQLPEVNATQIMPGFP-SSNGSFMLVP---LKDWSERTRSQKEVLAEVNKITN  634

Query  649   MMMGTLKDGFGIAVVPPPILELGNGSGLSINLQDRNNTGHTALLAKRNELIQKMRASGLF  708
               +      GF      P I    +G   S  +   N+    A +A    L  +      F
Sbjct  635   EVTEASVIGFEF----PEISTGESGLPFSFVITTANDYRDLAQVASDFLLAAQSSGQFFF  690

Query  709   DPSTVRAGGLEDSPQLKIDINRAAAAAQGISFADIRTalasalsssYVSDFPNQGRLQRV  768
              +   ++     D+  + I I+R      G++ ADIR+ L S LS + ++     GR   +
Sbjct  691   NQLDLKF----DTASMAIKIDREKMGTYGVTAADIRSVLGSFLSGATLARVDIDGRAYDI  746

Query  769   MVQADEDARMQPADILNLTVPNKSGVAVPLSTIATVSWENGTEQSVRFNGYPSMKLSASP  828
              + Q     R+ P D+    V  K G +VPLST  ++  +       + N     +L+++
Sbjct  747   ISQVKRSERLSPQDLAKYYVKLKDGSSVPLSTFVSMELKTQPRTLPKLN-----QLNSAT  801

Query  829   ATGVSTGQAMAAVQ----KMVDELGGGYSLEWGGQSREEAKGGSQTLILYGlavaavflv  884
               TGV  G    AV+    K  + L  GY  ++  +SR+  + G+   + + LA+  ++LV
Sbjct  802   ITGVLAGSMGNAVKWAEAKAAEILPAGYYYDFMSESRQYKQEGNALALTFLLAIVMIYLV  861

Query  885   laalYESWSIPLAVILVIPLGLIGAAAGVTGRNLFEGLLGSVPSFANDIYFQVGFVTVMG  944
              LA  +ESW  P+ +++ +PL + GA   V    L   ++G   +  N IY QVG +T++G
Sbjct  862   LAVQFESWRDPMVILVSVPLAISGAL--VVLNILALPMIGKAGATLN-IYSQVGLITLVG  918

Query  945   LSAKNAILIIEFAKDLQ-AQGKSAVeaaleaarlrFRPIIMTSFAFILGVVPLYIAGGAS  1003
              L  K+ IL+ E AK+ Q   GK+  +A   AA++R RPI+MT+ A I G++PL  A GA
Sbjct  919   LITKHGILMCEVAKEEQLHHGKNKFDAITYAAQIRLRPILMTTAAMIAGLIPLLYATGAG  978

Query  1004  SASQRAIGTTVFWGMLIGTLLSVFLVPLFY  1033
              +  + +IG  +  G+ IGTL ++F++P+ Y
Sbjct  979   AVMRFSIGIVIVAGLAIGTLFTLFVLPVIY  1008
```

Figure B5: Sequence homology from comparison of Neisseria meningitidis strain MC58 efflux protein gene MtrD with *H. ducreyi* strain 35000HP whole genome

MtrE GENE ID: 1491675 HD1829 | outer membrane protein [Haemophilus ducreyi 35000HP]

```
 Score =  106 bits (264),  Expect = 1e-24, Method: Compositional matrix adjust.
 Identities = 104/426 (25%), Positives = 203/426 (48%), Gaps = 20/426 (4%)

Query  50   VDLGWHDYFADPRLQKLIDIALERNTSL-RTAVL-NSEIYRKQY----MIERnnllptla  103
            +D  W   + DP+L +L+  AL  N +L ++A+L N  +Y        ++   +   + +
Sbjct  48   IDEEWWKGYNDPQLNRLVSQALANNVNLAKSAILVNKALYNANLVGASLVPTFSGSGSSS  107

Query  104  ananDSRQGSLSGGN-VSSSYKVGLGAA---SYELDLFGRVRSSSEAALQGYFASTANRD  159
            A+      GSL   N V+ +  V   A+   SY LDL+ R+  S+ A+    Y A+  +
Sbjct  108  ASKLLVSDGSLQSSNSVTKASNVVHNASFNLSYTLDLWRRLADSASASEWEYKATIEDLK  167

Query  160  AAHLSLIATVAKAYFNERYAEEAMSLAQRVLKTREETYKLSELRYKAGVISAVALRQQEA  219
            A  LS+I  V  +Y+   Y ++ ++++++ +K  E+   + E + + G I  +A+ Q +
Sbjct  168  ATRLSIINAVVASYYQIAYLKDVINVSEQTIKNYEQINHILENKMEVGAIDHLAVEQAKQ  227

Query  220  LIESAK---ADYAHAARSREQA-RNALATLINQPIPEDLPAGLPLDKQFFVEKLPAGLSS  275
               +A+     Y    +  EQ  RN L    N+P+P + PA L ++ Q     +P
Sbjct  228  ATLNARNGLIGYRTTLKVAEQTLRNLLNLKPNEPLPIEYPAILSVELQGVDMNVPIS---  284

Query  276  EVLLDRPDIRAAEHALKQananigaaraaFFPSIRLTGTVGTGSAELGGLFKSGTGVWSF  335
              + +RPD+ A    L+ A  ++ A   ++FP++ L  ++ + +A+L  +  +  G  +
Sbjct  285  -AIANRPDVAAKLKRLQSAFKSLSATEKSWFPTVTLGASISSSAAKLRNIGDNQLG--NS  341

Query  336  APSITLPIFTWGTNKANLDVAKLRQQVQIVAYESAVQSAFQDVANALAAREQLDKAYDAL  395
              S+ LP   W T K N+ +++   ++  + YE  + SA  +V N   A +Q   +Y  L
Sbjct  342  MISLNLPFLDWQTVKNNVKLSEADYRLAKLNYEQVITSALNEVDNYYYAYQQAFDSYSTL  401

Query  396  SKQSRASKEALRLVGLRYKHGVSGALDLLDAERSSYAAEGAALSAQLTRAENLADLYKAL  455
```

```
            +    +K+       RY+ GV+   + ++A      ++ A L+ + T       +Y+++
Sbjct  402  QQVYEHNKKISFYYKNRYELGVAEFREWVNAMNVELTSQLAILNQKYTILAKENAVYQSM  461

Query  456  GGGLKR  461
            G  KR
Sbjct  462  AGKYKR  467
```

Figure B6: Sequence homology from comparison of Neisseria meningitidis strain MC58 efflux protein gene MtrE with *H. ducreyi* strain 35000HP whole genome

```
GENE ID: 1491005 norM | multidrug resistance protein NorM
[Haemophilus ducreyi 35000HP]

 Score =  374 bits (959),  Expect = 4e-105, Method: Compositional matrix adjust.
 Identities = 218/439 (50%), Positives = 305/439 (70%), Gaps = 0/439 (0%)

Query  26   AQIAQNSMGLADTIMAGRVSSTDMAAISIGASIWmplmlfgqglllalppTISYLNGSGQ  85
            +Q++ + MGLAD +MAG VS  D++AI++  SI+ PL LF  G+L A+ PT+SYLNG+ Q
Sbjct  25   SQLSASGMGLADIVMAGLVSDDDVSAIAVSNSIYFPLFLFVLGVLNAITPTVSYLNGANQ  84

Query  86   RHRIAHQVRQGIWLVLGVSIPLGLLIYFCEIPLQYMQMESKMSDLARDYLHAMLWGLPAY  145
            R+ IAHQ+RQG WLV    IPL ++       L YM   +  +  ++DYL  M  G+
Sbjct  85   RNLIAHQIRQGFWLVWAFVIPLIVVFLNSHWILDYMNTPAVFAQKSQDYLAIMAIGIVPA  144

Query  146  LMLINFRCLNDGIAKTKPAMVITFLGLLINIPLNYIFIYGKFGIPAFGAVGCGIATAIVN  205
            L+ +N RC+NDG+A  KPAM ITF+GLL NIPLNYIFI+GKFG+P  GAVGCG+ATAIVN
Sbjct  145  LLTVNLRCMNDGLANPKPAMRITFIGLLCNIPLNYIFIFGKFGVPEMGAVGCGVATAIVN  204

Query  206  WAMCLMMIFYSYTNAQERSLKVFSQLIEMPNlktlkkllrlglPIAIAICCEVALFALTS  265
            W+M LMM  Y YTN  ++ + +F++  EMP+ KTL K+ +LGLPI  A   EV LF+ ++
Sbjct  205  WSMFLMMFHYCYTNKPQKDIGLFNKWFEMPSGKTLLKICKLGLPIGFATFTEVMLFSTSA  264

Query  266  LMLSPLGATIVASHQITLNTSSFIFMFPMSIGMAATILVGQALGTGSPQNAKKIGYaall  325
            L LSPLG+ +VASHQ  L TSS +FM P+S G+A TI++G+ LG    + AK I Y AL+
Sbjct  265  LFLSPLGSQVVASHQTALQTSSMLFMVPLSFGIATTIVLGRVLGQKLVEEAKIISYHALI  324

Query  326  lgltvtiitalitiFFRYEIASIFVTDEIVIamaanlllfaalYQFSDTIQMVVGGILRG  385
             G+   +I A++ + F   I   F  D + IA+A++LLLFAA+YQ  D++Q+V  GILRG
Sbjct  325  TGVIFAVIAAILIVLFNNIIPLAFTRDPVSIAIASHLLLFAAVYQIPDSLQVVANGILRG  384

Query  386  YKDTKMILYITLFSYWVIGVPLGYTLGRTDWLIPHIDAKGFWIAFVVSLTFAAFLLSLRM  445
            YK TK +LY+T+F YW IG+P GY L RTDW++  + A GFW+ F VSL+ A+ LL  ++
Sbjct  385  YKHTKPVLYVTVFCYWGIGIPFGYVLARTDWIVQPMAAAGFWLIFCVSLSLASGLLIYQI  444

Query  446  KKMQAMSDNAILQRLEKLK  464
            +K+Q +    ++ +LE++K
Sbjct  445  RKIQKIPAATLIAKLERIK  463
```

Figure B7: Sequence homology from comparison of Haemophilus influenzae strain86-028NP multidrug resistance protein gene NorM with *H. ducreyi* strain 35000HP whole genome

```
SapA GENE ID: 1491141 sapA | peptide transport periplasmic protein SapA
[Haemophilus ducreyi 35000HP] (10 or fewer PubMed links)

 Score =  323 bits (829),  Expect = 5e-90, Method: Compositional matrix adjust.
 Identities = 188/532 (36%), Positives = 293/532 (56%), Gaps = 32/532 (6%)

Query  32   DIRDSGFVYCVSGQVNTFNPQKASSGLIVDTLAAQLYDRLLDVDPYTYRLVPELAESWEV  91
            ++     +YC S    +FNPQKA  G  ++ +  Q+YD+L ++D +T+R++P LAE++ V
Sbjct  33   ELSADSLIYCTSISGLSFNPQKADVGTNMNVVTEQIYDKLFEIDRHTHRVIPSLAETFSV  92

Query  92   LDNGATYRFHLRRDVSFQKTAWFTPTRKLNADDVVFTFQRIF------------------  133
             D+G     +LRR V+F KT WFTPTR  NA+DVVF+  R+
Sbjct  93   SDDGKEITLNLRRQVAFHKTPWFTPTRLFNAEDVVFSLNRMIGNVEELPALDFNEDSKEQ  152

Query  134  ---DRRHPWH-NINGSSFPYFDSLQFADNVKSVRNVDNNTVEFRLTQPDASFLWHLATHY  189
```

```
              ++R+ +H   N + +PYF+S+     +  +   +  TV+  L  PD S L HLA+ Y
Sbjct  153  FQQNQRYAYHFKANLAHYPYFESVALKKKIAKISAPNEYTVKIHLVAPDNSVLAHLASQY  212

Query  190  ASVMSAEYAAQLSRKDRQELLDRQPVGTGPFQLSEYRAGQFIRLQRHDGFWRGKPLMPQV  249
            A ++S EYA  L+  +    LD  PVGTG +QLS+Y   +++RL+ +  +W  K  +  V
Sbjct  213  AVILSKEYALLLNADENLAQLDLLPVGTGVYQLSDYIQNEYVRLKPNPVYWGEKAKINNV  272

Query  250  VVDLGSGGTGRLSKLLTGECDVLAWPAASQltilrddprlrltlrpGMNIAYLAFNTDKP  309
            VVD  S  TGR++K L  ECD++A P  SQ    R      +   PG N+A+LAFN  K
Sbjct  273  VVDFSSNSTGRMAKYLNQECDIVAQPEPSQ---RRVISSYEIVESPGANLAFLAFNMQKE  329

Query  310  PLNNPAVRHALALSINNQRLMQSIYYGTAETAASILPRASWAYDNDAKITEYNPQKSREQ  369
             + + A R  +A +IN +RL+++++YG+AE A ++LP A +A  N A      PQ   +
Sbjct  330  KMQDIAFRRQIAQAINRERLVKALFYGSAEVADNVLPSALFAQKNPAAYPYKAPQPRAKN  389

Query  370  LKALGIENLTLHLWVPTSSQAWNPSPLKTAELIQADMAQvgvkvvi--vpvEGRFQEARL  427
             K   ++ L    WV   S+ +N  PLK AE+I+ D+ ++ + V+I  V      Q A
Sbjct  390  AK---LDRLI--FWVLDESRVYNLHPLKMAEMIRNDLKKINIDVIIRPVSRAKVVQLAAA  444

Query  428  MDMNHDLTLSGWATDSNDPDSFFRPLLSCAAINSQTNFAHWCNPEFDSVLRKALSSQQLA  487
               ++DL L+GW  ++ DP++F  P+LSC   N  TN A+WC+ +FD  L  A ++Q
Sbjct  445  GKADYDLILTGWLANNLDPNAFLSPILSCRTQNKVTNLANWCHQQFDEWLEIAKANQVPY  504

Query  488  SRIEAYEEAQNILEKELPILPLASSLRLQAYRYDIKGLVLSPFGNASFAGVS  539
             R   Y++ Q +LE++LPILPL  + R       IK   + PFG    + ++
Sbjct  505  VRNMIYKQTQALLEEQLPILPLLHAQRSLFVNQKIKNAHIEPFGQVRLSELT  556
```

Figure B8: Sequence homology from comparison of *Salmonella enterica* subsp. Serovar typhimurium transport protein gene SapA with *H. ducreyi* strain 35000HP whole genome

SapB GENE ID: 1491142 sapB | peptide transport system permease protein SapB [Haemophilus ducreyi 35000HP]

```
 Score =  196 bits (497),  Expect = 7e-52, Method: Compositional matrix adjust.
 Identities = 105/277 (38%), Positives = 166/277 (60%), Gaps = 0/277 (0%)

Query  42   NAWVFWFNGLLHWDFGVSSINGQLISEQLKEVFPATMELCILAFGFALMVGIPVGTVAGV  101
             A+  +  GLLH DFG+S  NG  I+ Q+  VFPAT+ LC  A   ++++GIP+G VA
Sbjct  42   QAYFSYVMGLLHGDFGISYSNGDPIANQILNVFPATISLCFAALFVSVIIGIPLGFVAAS  101

Query  102  TRSKWPDRFISALALLGFSIPVfwlallltlffsltlgwlPVSGRFDLLYEVKPVTGFAI  161
             R     + ++ ++    +IPVFWLA++   + +     +   G    +YE+  VTGF +
Sbjct  102  FRYNVVGKLLAIVSAFSLAIPVFWLAIMALYYAASNDWQIAAVGELHPIYEISLVTGFRL  161

Query  162  IDAWISDCPWRDEMVMSAIRHMVLPVLTLSVAPTTEVIRLMRISTIEVYDQNYVKAAATR  221
            +D +++D P++ +M+ S + H+ LP L L++  T EVIR  R     V  QNY+K A TR
Sbjct  162  LDIFLADSPYKLKMMQSVLHHLALPTLILALPATLEVIRFTRQRAEYVMKQNYIKVARTR  221

Query  222  GLSRFTILRRHVLHNALPPVIPRLGLQFSTMLTLAMITEMVFSWPGLGRWLIHAIRQQDY  281
            G S + I  +H+L N LP +IP +    + +   AM+ E +FSW G+G WLI+A+  QDY
Sbjct  222  GWSPYKIWLKHILRNTLPALIPMIARNLTLVFAFAMLVENIFSWGGIGLWLINALAIQDY  281

Query  282  AAISAGVMVIGSLVIVVNVISDILGAMANPLKHKEWY  318
             AISAGV+ IG  V+ V+++  ++  + +P + K+WY
Sbjct  282  NAISAGVVAIGLFVLGVDILVRLVTTLLDPSQKKDWY  318
```

Figure B9: Sequence homology from comparison of *Salmonella enterica* subsp. Serovar typhimurium transport protein gene SapB with *H. ducreyi* strain 35000HP whole genome

SapC GENE ID: 1491143 sapC | peptide transport system permease protein SapC [Haemophilus ducreyi 35000HP]

```
 Score =  207 bits (527),  Expect = 3e-55, Method: Compositional matrix adjust.
 Identities = 124/286 (44%), Positives = 181/286 (64%), Gaps = 5/286 (1%)
```

```
Query  8    SEKRPPGTLRTAWRKFYSDAPAMVglygcaglallcIFGGWIAPYGIDQQFLGYQLLPPS  67
            + R     +  W +   D  A+  LY    L  L   G  IAPY ++ QF+G++LLPPS
Sbjct  8    EQFRQSDYAKQFWLELRQDKVALASLYFFLILLFLTFAGELIAPYQVNTQFVGFELLPPS  67

Query  68   WSRYGEVSFFLGTDDLGRDVLSRLLSGAAPTVGGAFIVTLAATLCGLVLGVVAGATHGLR  127
            W  YG++S F GTDDLGRD+ SR+L+G   TVG A +++ A  + G V+GV+AG +   R
Sbjct  68   WDDYGQISHFFGTDDLGRDIFSRILAGFYYTVGAALLISFAIAIIGGVIGVLAGTS---R  124

Query  128  SAV--LNHildtllsipslllaiiVVAFAGPHLSHAMFAVWLALLPRMVRSVYSMVHDEL  185
             A+  L H+ DT L IP+L++AII+       L +AM A++LA+LP  +  +Y     +L
Sbjct  125  KAISFLGHLFDTFLFIPTLIIAIIIATLMEASLINAMLAIFLAMLPHFIHKIYQATEQQL  184

Query  186  EKEYVIAARLDGATTLNILWFAILPNITAGLVTEITRALSMAILDIAALGFLDLGAQLPS  245
            ++EYVI  RLDG +   ++   +LPN+TA  V E+T    +A+LDI AL F+ LGAQ P
Sbjct  185  KREYVITLRLDGISRWQLIKEVVLPNLTALAVKEMTHICIIAVLDINALSFIGLGAQSPM  244

Query  246  PEWGAMLGDALELIYVAPWTVMLPGAAITLSVLLVNLLGDGIRRAI  291
            PEWG M+ D++ELIY+APWTV+LPG    L +L+++LG+ I R +
Sbjct  245  PEWGVMIKDSIELIYIAPWTVILPGIITILVILIISMLGNSISRVL  290
```

Figure B10: Sequence homology from comparison of *Salmonella enterica* subsp. Serovar typhimurium transport protein gene SapC with *H. ducreyi* strain 35000HP whole genome

SapD GENE ID: 1491144 sapD | peptide transport system ATP binding protein SapD [Haemophilus ducreyi 35000HP]

```
 Score =  407 bits (1045),  Expect = 2e-115, Method: Compositional matrix adjust.
 Identities = 195/327 (60%), Positives = 253/327 (78%), Gaps = 3/327 (0%)

Query  1    MPLLDIRNLTIEFKTSEGWVKAVDRVSMTLSEGEIRGLVGESGSGKSLIAKAICGVAKDN  60
            M LLDIR+L+IE  T  G VK VD V++TL EG+I GLVGESGSGKSLIAK ICGV KD
Sbjct  1    MALLDIRHLSIEIDTPTGRVKMVDNVNLTLDEGQICGLVGESGSGKSLIAKVICGVMKDE  60

Query  61   WRVTADRMRFDDIDllrlssrerrklVGHNVSMIFQEPQSCLDPSERVGRQLMQNIPAWT  120
            W VTADR RF+DI+LL+LS  +RRKLVG  ++MIFQEP+S LDPS+ +G+QL+Q I
Sbjct  61   WIVTADRFRFNDIELLKLSPIQRRKLVGEQIAMIFQEPRSSLDPSKNIGQQLIQTI---N  117

Query  121  YKGRWWQRLGWRKRRAIELLHRVGIKDHKEPMRSFPYELTDGECQKVMIAIALANQPRLL  180
            +KG+WWQ  GW+K++AIELLHRVGI+DHK  M+S+P ++T+GE QKV+IA+A+ANQPRLL
Sbjct  118  FKGKWWQWFGWKKKKAIELLHRVGIRDHKAMMQSYPVDITEGEAQKVIIAMAVANQPRLL  177

Query  181  IADEPTNSMEPTTQAQIFRLLTRLNQNSNTTILLISHDLQMLSQWADKINVLYCGQTVET  240
            IADEPT+++EPTTQ QI+RLL  +N+N  T+ILL+++D++   +W D  NVLYCGQT E
Sbjct  178  IADEPTHTLEPTTQLQIYRLLASMNKNLGTSILLVANDIRSTHKWVDSFNVLYCGQTAEA  237

Query  241  APSKDLVTMPHHPYTQALIRAIPDFGSAMPHKSRLNTLPGAIPLLEQLPIGCRLGPRCPY  300
               + ++  P HPYT  L+  IPDF   +  KS LNTL G+IP+ + +PIGCRLGPRCP+
Sbjct  238  GSKEVIMEEPFHPYTAVLLNGIPDFSQPLVFKSSLNTLKGSIPMFQHIPIGCRLGPRCPF  297

Query  301  AQRECIITPRLTGAKNHLYACHFPLNM  327
            AQ++C+  P L   K H +ACHFP+N
Sbjct  298  AQKKCVQKPPLRKYKQHEFACHFPINF  324
```

Figure B11: Sequence homology from comparison of *Salmonella enterica* subsp. Serovar typhimurium transport protein gene SapD with *H. ducreyi* strain 35000HP whole genome

SapF GENE ID: 1490808 sapF | peptide transport system ATP-binding protein SapF [Haemophilus ducreyi 35000HP]

```
 Score =  295 bits (754),  Expect = 1e-81, Method: Compositional matrix adjust.
 Identities = 138/262 (53%), Positives = 190/262 (73%), Gaps = 0/262 (0%)

Query  5    LLEVRNLSKTFRYRTGWFRRQTVDAVKPLSFTLRERQTLAIIGENGSGKSTLAKMLAGMI  64
            LLEV NL+K F      F +Q   AVK +SF L E +T+AIIGENG+GKSTLAKM+AG+
```

```
Sbjct  3    LLEVHNLNKRFTDHISLFHKQRFYAVKNISFNLNEGETIAIIGENGAGKSTLAKMIAGIT  62

Query  65   EPTSGELLIDDHPLHYGDYSFRSQRIRMIFQDPSTSLNPRQRISQILDFPLRLNTDLEPE  124
            +PTSG++      L YGDY FR++ IRM+FQDP+ + NP   I QILD PLRL TD+  +
Sbjct  63   KPTSGDMFFRYKKLTYGDYQFRTKHIRMMFQDPNHTFNPNHNIGQILDAPLRLATDMNEQ  122

Query  125  QRRKQIVETMRMVGLLPDHVSYYPHMLAPGQKQRLGLARALILRPKVIIADEALASLDMS  184
            +R + I  T+++VG+ P++        +  QKQR+ LARALIL P++IIAD+ L++LD S
Sbjct  123  ERNECIFNTLKLVGMYPEYALIPIKETSTSQKQRIALARALILSPEIIIADDTLSTLDFS  182

Query  185  MRSQLINLMLELQEKQGISYIYVTQHIGMMKHISDQVLVMHQGEVVERGSTADVLASPLH  244
            +++QL NLML LQEK GI++IYV QH+G++KHI+D++++MHQGE +E G T +VL +P +
Sbjct  183  VKTQLTNLMLNLQEKLGIAFIYVGQHLGIIKHIADKLIIMHQGEAIEYGDTKEVLLNPQN  242

Query  245  ELTRRLIAGHFGEALTADAWRK  266
             +T RLI  HFG+ LT DAW K
Sbjct  243  PITARLIESHFGKMLTEDAWAK  264
```

Figure B12: Sequence homology from comparison of *Salmonella enterica* subsp. Serovar typhimurium transport protein gene SapF with *H. ducreyi* strain 35000HP whole genome

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