The Role of Oxidative Stress Tolerance and Transformation Competence in the Acquisition of Antibiotic Resistance in *Haemophilus ducreyi*

By

Austin W. Becker

April, 2013



Allegheny College

Department of Biology

The Role of Oxidative Stress Tolerance and Transformation Competence in the Acquisition of Antibiotic Resistance in *Haemophilus ducreyi*

By

Austin W. Becker

April, 2009

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.

_____

Pledge

Approval by the Senior Thesis Committee:

_____

Dr. Ann Kleinschmidt

_____

Dr. Tricia Humphreys

Table of Contents

Acknowledgements:	**Pg. 4**
Abstract:	**Pg. 5**
Introduction:	**Pg. 6**
Materials and Methods:	**Pg. 17**
Results:	**Pg. 23**
Discussion:	**Pg. 30**
Appendix	**Pg. 40**
Work Cited	**Pg. 44**

Acknowledgements

I want to say thank you to Dr. Ann Kleinschmidt for your unparalleled inspiration and guidance.

I would like to say thank you to Dr. Tricia Humphreys, for being awesome, opening up your lab to me and always being willing to help.

I would like to say thank you to Dana D'amico and Allison Nettnin for the critical help with the writing process, my writing has grown exponentially thanks to you both.

I would like to say thank you to my friends and comp group.

Thanks to the Allegheny College Department of Biology for the funding necessary for this project

Abstract

Understanding the factors that contribute to the acquisition of antibiotic resistance in pathogenic bacteria is essential for maintaining and increasing the efficacy of current antibiotic therapies. This study explored the source of beta-lactam resistance and contributing factors allowing acquisition of antibiotic resistance in *Haemophilus ducreyi* strain HD85-023233 (HD85). The antioxidant protein Cu,Zn superoxide dismutase (SOD) was analyzed because it may promote tolerance to neutrophil generated oxidative burst and oxidative induced damage from antibiotics. Tolerance to both these factors may have aided in the ability of the HD85 strain to persist for a long enough time to acquire antibiotic resistance genes. Expression of *sodC* mRNA was present and equal in HD85 and 35000HP (35K) during exponential phase suggesting a potential role of Cu,Zn superoxide dismutase in oxidative tolerance through constitutive expression. To understand the origin of antibiotic resistance in HD85, the antibiotic resistance genes of HD85 were studied, as well as its competence genes to assess transformation as a potential mechanism of antibiotic resistance acquisition. This study shows the resistance of strain HD85 is due to a plasmid carrying a TEM-1 beta-lactamase gene and that HD85 has premature stop codons in 3 competence genes. Thus, transformation was an unlikely contributor to the acquisition of plasmid DNA encoding resistance genes in HD85.

Introduction

An obligate human bacterial pathogen must respond to and survive a variety of selective pressures while invading their hosts. During an infection, pathogenic bacteria are subjected to a range of selective pressures from the host immune system in the form of cellular responses and molecular components. One of the most effective and abundant parts of the immune system are neutrophil cells. Neutrophils, which account for roughly 50-70% of the white blood cells in the body phagocytose pathogens and then use oxidative burst to induce cellular killing (Edwards, 11; Edwards, 2). Oxidative burst occurs through the consumption of excess O_2 after which neutrophils utilize NADPH Oxidase to generate superoxide (Clancy et al., 1992). This superoxide can lead to the generation of harmful reactive oxygen species (ROS) and lead to bacterial oxidative stress, structural damage for the bacteria, or bacterial death (Clark et al., 1987; Segal, 1988; Lock et al., 1990).

If pathogenic bacteria persist, despite the presence of the host immune response, antibiotic treatment may be utilized. Bactericidal antibiotics target and disrupt specific cellular structures to induce bacterial death (Kinato and Tomasz, 1979). Bactericidal antibiotics can also lead to the production of ROS which, in combination with the primary mechanism of antibiotic action, may induce cellular death (Kohanski et al., 2007). Treatment with bactericidal antibiotics leads to ROS formation via rapid consumption of NADH, which then overloads the electron transport chain and causes increased electron flow, which leads to the production of the

superoxide through the reaction of O_2 with an electron (Kohanski et al., 2007). Similar to oxidative burst, superoxide created through antibiotic action can then contribute to cellular death.

Superoxide is a highly reactive molecule that can lead to the production of the extremely toxic and damaging hydroxyl radical (Chiang and Schellhorn, 2012). High levels of superoxide can destabilize iron-sulfur cluster proteins and lead to the release of free iron (Kohanski et al., 2007; Liochev and Fridovich, 1999). Superoxide also spontaneously decays to form hydrogen peroxide. This hydrogen peroxide may then interact with the free iron through the Fenton reaction (Figure 1). Hydrogen peroxide that interacts with free iron leads to the production of hydroxide and a hydroxyl radical (Liochev and Fridovich, 1999). This hydroxyl radical is extremely reactive and can lead to significant alteration and damage of cellular structures.

$$\mathbf{O_2 + e^- \xrightarrow{FAD} O_2^{\cdot -}} \quad (1)$$

$$\mathrm{Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^{\bullet}OH} \quad (2)$$

Figure 1: Reaction of hydrogen peroxide with ferric iron to form hydroxyl radicals and hydroxide. *Adapted from Chiang and Schellhorn, 2012

Superoxide, hydrogen peroxide and hydroxyl radicals are all considered reactive oxygen species (ROS), which in bacterial cells can cause severe damage to lipids, protein, and DNA structures (Scandolias, 2005). ROS can interact with and break down the lipid bilayer, which is necessary for separating the bacteria from the external environment. ROS can also react with proteins to alter the electrical charge, change the shape of the proteins, and/or mark proteins for degradation. These modifications occur by the oxidation of specific structures on the amino acids that make up proteins. For instance, ROS can oxidize the thiol groups of cysteine, the imidazole ring of histidine, and the carbon rings of phenylalanine, tyrosine, and tryptophan (Luschack,

2001). The oxidation of these structures in certain amino acids can alter, neutralize, and/or lead to the deterioration of the protein function. Oxidative damage can also lead to the severing of the DNA phosphate backbone and prevent effective transcription and replication (Scandalios, 2005). The severe oxidative damage caused by ROS to specific cellular targets can lead to bacterial death.

Despite the fact that both antibiotics and neutrophil activity utilize oxidative stress to mediate bacterial death, ROS formation is a common and inevitable byproduct of aerobic life for many bacteria. ROS are produced in all cells in both times of stress and of homeostasis alike (Alscher et al., 2002). Oxidation of flavins, hydroquinones, catercholamines, and many other molecules necessary for basic metabolism create ROS as byproducts of their reactions. Even aerobic respiration, one of the most fundamental bacterial methods of synthesizing ATP, leads to the creation of superoxide and hydrogen peroxide. (Alscher et al., 2002; Scandalios, 2005). Here, the electron transport utilizes oxygen as an acceptor of electrons and the charged oxygen species are normally converted to water, but electrons can diffuse away from the pathway and may interact with oxygen to form superoxide (Scandolias 2). Hydrogen peroxide can also originate from several different enzymatic reactions involved in metabolism (Scandolias 3). Because ROS have damaging potential, bacteria have oxidative defense systems that keep the creation of ROS at balance with the reduction of these compounds to less harmful forms. It is only when these antioxidant defenses are overwhelmed does the organism enter a state of oxidative stress (Scandalios, 2005).

To prevent oxidative stress, bacteria have a variety of oxidative defense proteins. Currently there is no known molecule that prevents damage from hydroxyl radicals once they have formed; instead many of the oxidative defenses focus on converting the precursors of

hydroxyl radicals to less reactive forms or circumventing the damage the ROS causes. Multiple types of enzymes including reductases, catalases and peroxidases can interact with hydrogen peroxide to convert it to more stable forms and prevent it from undergoing the Fenton reaction. For instance, the catalase in *Escherichia coli* converts hydrogen peroxide to water and oxygen (Figure 2, Chiang and Schellhorn, 2012). Catalases and peroxidase type enzymes often work in tandem with superoxide dismutase (SOD) proteins to detoxify ROS (Scandolios 2005). SOD proteins are the first and primary response to superoxide in multiple organisms. Due to the prevalence of ROS in all types of organisms, orthologs of SOD are present in both prokaryotes and eukaryotes (Culotta et al., 2006). Thermal dismutation of superoxide to non-charged oxygen and hydrogen peroxide occurs at $5x10^{-5}$ moles per second. SOD enzymes can perform this conversion 3200 times faster (Culotta et al., 2006). The substrate of SOD proteins, superoxide, interacts within the active site of the protein to form hydrogen peroxide and oxygen (Figure 2, Chiang 2012). Though hydroxyl radicals can still result from the interaction of hydrogen peroxide and metal ions, the transformation by SOD proteins can lessen the damage by preventing the release of free iron (Liochev, 1996). With less free iron released, less hydrogen peroxide can undergo Fenton chemistry to be converted to hydroxide and hydroxyl radicals (Liochev, 1996). Because SOD is the first responder to ROS, specifically superoxide, it makes it a valuable candidate for studying bacterial defense and tolerance of neutrophil oxidative burst and bactericidal antibiotic action.

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} \mathbf{H_2O_2} + O_2$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

Figure 2: Reactions of Superoxide dismutase and catalase enzymes
*Adapted from Chiang and Schellhorn, 2012

SOD is a critical component of the antioxidant system that promotes tolerance for many bacteria to both the host immune system and antibiotic treatment. This protein may be an important factor in the virulence of pathogens that infect humans. SOD activity is essential for survival of *Shigella, Nocoardia,* and *Campylobacter* to oxidative burst exposure (Beaman et al., 1985; Franzon et al.,1990; Pesci et al.,1994). Recent studies also point to antioxidant defenses as a critical part of antibiotic tolerance. For *Bacillus anthracis, Pseudomonas aerruginosa, Staphyloccoccus aureus,* and *Escherichia coli* antibiotic tolerance can be explained by the induction of hydrogen sulfide gas, which increased the activities of catalase and SOD by 1.5 times and thus contributed to reduced antibiotic stress (Shatalin et al., 2011). For *P. aeruginosa* antibiotic tolerance is increased while they are in biofilms due to the contribution of the stringent response (SR), which utilizes iron chelation and antioxidant enzymes, including SOD (Nguyen et al., 2011). The inactivation of the *P. aerginosa* SR leads to greater antibiotic sensitivity during infection of murine models (Nguyen et al., 2011). These studies suggest that SOD is potential candidate for bacterial survival and tolerance against neutrophil oxidative burst and bactericidal antibiotic action.

Altered SOD expression in bacteria could promote tolerance to antibiotics or oxidative burst, which may in turn allow bacteria to acquire antibiotic resistance. Bactericidal antibiotics are concentration dependent, and treatment with sublethal concentrations of antibiotics can lead to reduction in antibiotic sensitivity of the population (Kohanski et al. 2010). Treatment with sub lethal concentrations of antibiotic results in selection for tolerant cells, based upon the natural variability of the population. Surviving tolerant cells can then restore the population and this new population has a higher overall tolerance to that antibiotic than the original population. In *E. coli,* sub lethal antibiotic treatment of bacteria leads to the hydroxyl radical induced mutagenesis,

which can lead to the development of multidrug tolerance. Treatments with kanamycin led to the mutation of oxidative stress regulator, *arcA*, and led to a reduction in sensitivity in kanamycin's killing action of *E. coli*. More specifically, treatment with sub-lethal concentrations antibiotics can potentially lead to the development or changed expression of certain oxidative stress enzymes. This may promote survival by changing sensitivity to oxidative stress of antibiotics and oxidative burst. The development of oxidative stress tolerance can then lead to the acquisition of antibiotic resistance by allowing the bacteria to survive long enough in the presence of oxidative stressors to acquire antibiotic resistance (Kohanski et al., 2010).

One of the most common forms of acquired antibiotic resistance comes from antibiotic altering or degrading enzymes. These enzymes break down antibiotic function by altering or degrading their key components. An extremely ubiquitous and medically problematic class of antibiotic altering enzyme is the beta-lactamase group, which prevent the function of beta-lactams. Beta-lactam antibiotics function by irreversibly binding penicillin binding proteins (PBP), which cross link the bacterial cell wall (Kinato and Tomsaz, 1979). As a result of treatment with beta-lactams, the bacterial cell wall loses its integrity and hydroxyl radicals form (Kinato and Tomasz 1979; Kohanski et al., 2010). Beta-lactamases protect bacteria by degrading beta-lactam molecules which prevents their interaction with the PBPs to induce cellular stress.

Antibiotic resistance genes, like beta-lactamases, can be transferred to other bacteria either vertically or horizontally. Vertical transfer is the passing of genetic material from mother to daughter cells through the replication of DNA and binary fission (Furuya and Lowy, 2006). Horizontal transfer between bacteria occurs through three means: transduction, conjugation, and transformation. Transduction is the passage of genetic material through phage; conjugation is the

transfer of genetic material through direct contact between bacteria; transformation is the uptake of genetic material from the environment (Furuya and lowry, 2006).

For a bacterium to be able to acquire genetic material from the environment it must enter a specialized physiological state. This physiological state is known as competence in which the bacteria has assembled the proper machinery to acquire the DNA from the environment (Claverys and Martin, 2003). The proteins, and their organization, required for the state of competence can vary between species, as seen in figure 3 (Claverys and Martin, 2003). Once the bacterium is in the state of competence, the bacterium may take up the DNA. The first step in transformation requires the binding of exogenous DNA to the cellular surface and then the DNA is pulled into the cytoplasm, often by pili complexes (Claverys and Martin, 2003). The DNA can then be degraded, incorporated into the genome as single stranded DNA or take on a plasmid form (Redfield et al. 2006). Once the DNA is inside the cell, if it codes for antibiotic resistance it may be utilized to aid in the resistance to antibiotics.

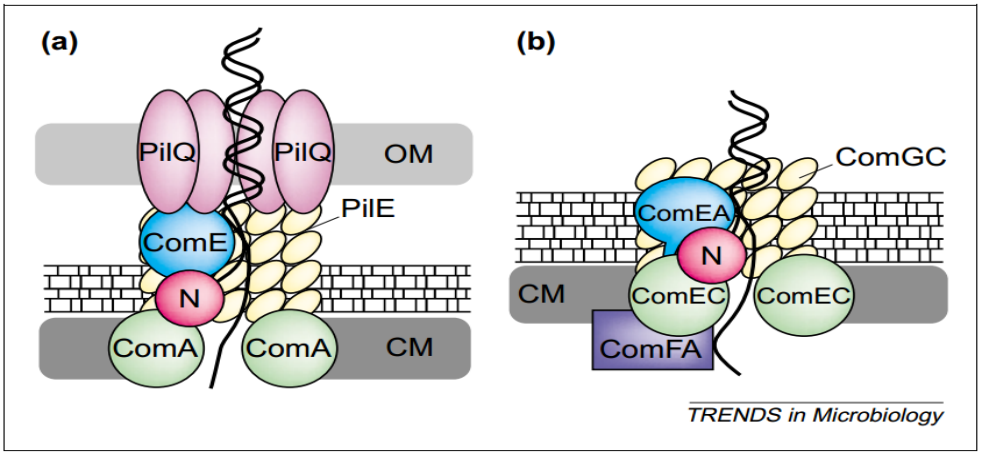


Figure 3: DNA uptake machinery in *Neisseria gonorrhoeae* (a) and *Bacillus subtilis* (b). Homolog proteins in both species are identified with similar colors. In *N. gonorrhoeae* PilQ binds exogenous DNA and pulls it through the membrane. PilE and ComE work in tandem to pull this bound DNA into the cytoplasm. In *B. subtilis,* comEA binds the DNA with the aid of comGC and pulls the DNA through the membrane. After nuclease, N, degrades a strand of DNA

and comFA pulls the DNA into cytoplasm. *Figure and description adapted from Claverys and Martin, 2003.

For an obligate human pathogen to obtain antibiotic resistance through transformation, it must survive long enough in the presence of the immune system, including neutrophils, and may have even had to tolerate antibiotic exposure. SOD may allow the organism to tolerate the oxidative stress long enough to obtain antibiotic resistance and survive the neutrophil action. Due to several unique characteristics, the species of *Haemophilus ducreyi* allows for a unique look at the role of SOD and the ways in which obligate human pathogens acquire antibiotic resistance genes. *H. ducreyi* contains a variant of SOD that contains copper and zinc metal ions (Cu,Zn SOD), which may positively contribute to the pathogen's survival and its ability to acquire antibiotic resistance genes.

Haemophilus ducreyi as a model for the acquisition of antibiotic resistance through ROS tolerance and transformation.

H. ducreyi is a fastidious gram negative bacterium that causes the genital ulcer disease known as chancroid. The pustules and ulcers this disease causes facilitate the spread of HIV (Wasserheit, 1992). *H. ducreyi* spreads to new hosts through breaks in the integrity of the epidermal layer (Sullivan, 1940). Endemic to many developing countries, *H. ducreyi* infects 7 million people annually, but the number of infections is hard to estimate based on the medical care of these regions (Steen, 2001). Antibiotics have significantly reduced the amount of outbreaks of bacterial diseases, like chancroid, in the last 50 years, particularly in developed countries (Mayaud and McCormick, 2001; Steen, 2001). Traditionally the suggested treatment for chancroid includes bacteriostatic, and bactericidal antibiotics in multiple doses or more powerful drugs in single doses (Steen, 2001). In cases where treatment requires multiple doses

the costs or availability of these drugs in these developing countries may interfere with proper treatment, thus promoting an environment that can lead to the development of antibiotic resistant strains of bacteria (Mayaud and McCormick, 2001).

There are multiple strains of antibiotic resistant *H. ducreyi* and frequently the antibiotic resistance in *H. ducreyi* is associated with plasmid DNA (Dangor et al., 1988). The origin of these plasmids and mechanisms of acquisition are less understood. As a species *H. ducreyi* is considered not capable of transformation. *H. ducreyi* belongs to the order of Pasteurellaceae. The Pasteurellaceae order has 12 common ancestral competence genes that are all considered essential for transformation (Redfield et al. 2006). The only studies on competene in *H. ducreyi* focus on one strain the 35K strain which does not have a plasmid. Based on the sequence of the 35K genome, *H. ducreyi* has truncating defects in 3 of these 12 competence genes: *comA, comB,* and *comM*. These defects presumably would make them not able to acquire DNA from the environment through transformation (Redfield et al. 2006). Yet, many strains have plasmids. Understanding whether or not the defects in the competence genes of 35K are present in plasmid containing strains would help elucidate the potential mechanisms by which *H. ducreyi* obtains plasmid DNA that code for antibiotic resistance genes.

H. ducreyi also presents a novel opportunity to observe how Cu,Zn SOD contributes to survival before the acquisition of antibiotic resistance. *H. ducreyi* has Cu,Zn SOD which helps protect it against exogenous superoxide similar to the neutrophil oxidative burst (San Mateo et al., 1998). In swine infection models, a Cu,Zn SOD deficient strain was still able to induce lesions but was recovered from significantly fewer inoculated sites than the wild type. When the Cu,Zn SOD deficient mutant was infected in neutrophil deficient, neutropenia, swine models the Cu,Zn SOD mutant was recovered from a similar number of inoculation sites as the isogenic

parent (San Mateo et al., 1999). This difference suggests that Cu,Zn SOD helps contribute to the overall survival of the bacteria *in vivo*. When this Cu,Zn SOD deficient mutant strain was inoculated in humans it led to lesion development, but there was no significant difference in the number of bacteria recovered from the lesions (Bong et al., 2002). Though Cu,Zn SOD is not considered essential for the virulence of *H. ducreyi*, it may still aid the bacteria in the defense against the immune system. Additionally the Cu,Zn SOD could aid in the defense against bactericidal antibiotics due to its ability to counter act superoxide.

Traditionally SOD enzymes are paired with catalase-like enzymes to detoxify oxidative stress, but *H. ducreyi* is not known to have a catalase-like gene. In the *H. ducreyi* genome there are no known genes that code for catalase like proteins. The presence of a catalase activity in *H. ducreyi* is highly debatable and there are many conflicting reports (Morse, 1989) This suggests that *H. ducreyi* does not have an enzyme to detoxify hydrogen peroxide and instead *H. ducreyi* likely may rely upon SOD to detoxify and the prevent damage from ROS. Even though Cu,Zn SOD converts superoxide to hydrogen peroxide, which may ultimately result in hydroxyl radicals, Cu,Zn SOD can still play a positive role. Cu,Zn SOD can prevent superoxide from interacting with the iron-sulfur clusters of metalloproteins in the area, reducing the production of hydroxyl radicals (Liochev,1996). As an additional protective role, the Cu,Zn SOD of *H. ducreyi* has the unique ability bind heme and potentially sequester it, removing the amount of avaible iron available for Fenton chemistry. The conversion by Cu,Zn SOD is also useful because superoxide is not able to cross the cell membrane, but hydrogen peroxide may exit the cell. The hydrogen peroxide diffusion can make the damage to the bacteria less localized (Alscher et al., 2002).

Preliminary work suggests that Cu,Zn SOD may have played a contributing role in the acquisition of ampicillin resistance for a particular strain known as HD85-0230233 (HD85). Previous work with HD85 assessed the ampicillin resistance and SOD expression relative to a non-resistant strain through SOD activity assays and qPCR analysis. This study found a positive correlation between ampicillin resistance and CuZnSOD expression. (Saylor, 2011) Further studies have analyzed the resistance of HD85 to a variety of bactericidal antibiotics (Appendix figure A1 and figure A2). These found that the strongest resistance/tolerance to be associated with certain beta-lactam antibiotics (Figure A1). Further studies found that the HD85 strain was positive for the presence of a beta-lactamase and a plasmid (Appendix and Figure A3). While the beta-lactamase is the likely source of the antibiotic resistance, this does not rule out the possibility that elevated Cu,Zn SOD levels may have influenced the ability of HD85 to resist initial host defense and resist the effect of increased ROS. High levels of SOD in HD85 may contribute to ROS tolerance to antibiotic treatment and the neutrophil oxidative burst. Increased tolerance to elevated ROS could let the bacterium live longer until it gains antibiotic resistance genes.

My goal was to analyze the mechanism by which HD85 is able to resist certain beta-lactam antibiotics and the factors that led to its acquisition. This project aimed to first establish that HD85 has elevated levels of Cu,Zn SOD expression. The work of Saylor 2011 analyzed the Cu,ZnSOD expression of HD85 at an unknown point in the growth cycle. Recent considerations suggest this may have interfered with the data (Saylor, 2011. In many pathogenic bacteria, Cu,ZnSOD expression is dependent on the phase of the growth cycle due to the expression of the RpoS sigma factor (Lacour and Landini., 2004; Lynch and Kuramitsu, 2000). Considering the position of HD85 in the growth cycle, is necessary for accurate assessment of the CuZnSOD

expression in HD85. Additionally the ampicillin resistance due to the presence of a beta-lactamase needs to be associated with the plasmid in HD85. Competence genes were also analyzed for functionality, relative to a non-competent strain, to elucidate the possible mechanism of acquisition of resistance genes for HD85.

Methods

Primer design:

Using the NCBI Entrez gene database, the primers were designed from the 35000HP genome (Accession NC_002940.2) and PJD4 plasmid from *Neisseria gonorrhoeae* (Acession NC_002098.1). For the competence genes (*com*), the primers were designed to bind to the upstream and downstream regions from the gene. The *bla* primers were designed from the ends of the BLA gene on the PJD4 plasmid. Primer lengths and GC content were adjusted to obtain a Tm of 56-60°C using the equation Tm = 60.3+(.41*%GC) -500/n, where n is the number of nucleotides in the primer. All primers were ordered from MWG Eurofins (Table 2).

Table 1: Primers used for PCR. *comA, comB, comB* primers designed from 35000HP genome. A Forward slash indicates a separate primer. *bla* primer designed from PJD4 plasmid of *Neisseria gonorheae.* Sequences used for primer design were obtain from www.ncbi.nlm.nih.gov.

Gene	Sequence 3'-5' (F/R)	Expected amplicon size
comM	CAGCTAAACGGCTAGCCAAC/ GCGATCACGATCGTGAAAGTG	787

comB	GTGGTGTAAATTTAACCGGGCAG/ GTTTCTCCATAAACTCTGCCATGC	585
comA	ACGGCAGATTGGTCTTGCCG/ CTCAATCTCTTTGCCTGAAAAGCG	506
Bla	CGTGTCGCCCTTATTCCC/ GCACCTATCTCAGCGATCTG	821

PCR Amplification of *com* and *bla* genes for sequencing:

Using sterile wooden dowels isolated colonies were selected from solid media and added to 20µl of GeneReleaser® (Bioventures), and subjected to thrermocycler conditions of 65°C for 30 seconds, 8°C for 30 seconds, 65°C for 90 seconds, 8°C for 1 minute, 65°C for 3 minutes, 97°C for 1 minute, and held at 80°C. For PCR amplification, the reaction contained 12.5µl of Mastermix (Promega), 2.5 µM of each primer was layered on the gene releaser and the volume of the reaction was raised to 25µl. The reaction mixture was then incubated in a thermocycler at 95°C for 2 minutes, 30 cycles of (95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds), 72°C for 2 minutes and held at 4°C. The products were analyzed by electrophoresis in a 1.2% agarose gel in TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.2) and size determined by comparison to a 2 log ladder (Phenix).

Preparation of Genomic PCR products for sequencing:

Following the PCR reaction, products were purified using the Wizard SV Gel and PCR clean up system (Promega). The PCR reaction was added to a cartridge, incubated for 1 min, and centrifuged at 16,000xg for 1 min. The flow through was discarded, 700µl of a wash solution

containing ethanol was added, the sample was then centrifuged at 16,000xg for 1 min and the flow through was discarded. The membrane was treated with 500µl of the membrane wash solution, centrifuged for 1 min at 16,000xg and the flow-through was discarded. The membrane was then dried by centrifuging the column for 1 min at 16,000xg with the microcentrifuge lid open. The column was transferred to a collection tube and 50µl of Nuclease free water was added to the column. After one minute incubation at room temperature the column was centrifuged for 1 min at 16,000xg. The column was discarded. The concentration of DNA was determined using Nanodrop spectroscopy. The DNA was stored at 4˚C until it was sent to Eurofins Operon MWG for sequencing.

Sequence analysis

To determine the sequence similarity of the *comB, comA,* and *comM* genes in HD85 to the 35K's genes the PCR products were sequenced by MWG Eurofins Operon. Additionally the *bla* PCR product was sent to MWG Eurofins Operon to determine the type of *bla* gene. Electrophoretograms received from MWG Eurofins Operon were analyzed and contigs were aligned using the FINCHTV alignment software. Using, the sequence data of the *com* genes in HD85 was compared to the genome of 35K (Accession NC_002940.2) and the *bla* gene was compared to the PJD4 plasmid sequence (Acession NC_002098.1).

Plasmid extraction:

Haemophilus ducreyi cells were harvested from solid chocolate agar and suspended in PBS buffer to A_{660} of 1.0-1.5 or $1x10^9$ cells per ml. Using 4 ml of this solution, a bacterial pellet was formed by centrifugation at 14,000xg for 2 min and the supernatant was discarded. Using

the Mini prep kit from Origene, cells were suspended with 250µl of Cell suspension buffer with RNase A. Lysozyme , 2.7 mg, was added to the solution to bring the final concentration of lysozyme in the solution to 1mg/ml. After a 5 minute incubation at 37°C, 250µl of cell lysis buffer was added. This mixture was inverted and incubated for 5 min at ambient temperature. Following incubation 350µl of neutralization buffer was added and mixed by inversion until it was homogenous. The mixture was centrifuged for 10 min at 12,000XG and the supernatant was loaded into a spin cartridge, centrifuged for 1 min at 12,000XG and the flow through was discarded. Optional wash buffer was added to the column and then centrifuged for 1 min at 12,000XG and the flow through was discarded. The cartridge was washed twice, by adding 700 µl of a 70% ethanol buffer, centrifuging for 1 min at 12,000XG and discarding the flow through. The cartridge was placed in a recovery tube, 75µl of preheated TE, 65°C, was added to the cartridge. The cartridge with warmed TE was incubated for 1 min at ambient temperature and centrifuged the sample at 12,000XG for 1 min. Analysis of samples was conducted on 1.2% agarose gel in TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.2) and compared to a 2 log ladder (NEB). Nanodrop analysis was used to determine concentration. Samples were stored at 4°C.

Transformation:

Chemically competent DH5α *Escherichia coli* cells, obtained from Dr. Brad Hersh at Allegheny College, 50 µl, were thawed on ice. The volume of cells were split equally into 3 tubes, one received 50ng of a PGEM 3Z plasmid as a positive control because it contained a beta-lactamase gene, one received 300ng of plasmid isolated from HD85-023233, and one received 1 µl of TE as a negative control. The cells were incubated for 5-10 min on ice, then incubated 42 ^{o}C for 60 seconds and allowed to recover on ice for 2 minutes. LB broth at ambient

temperature was added and cells were incubated for 10 min at 37 ^{o}C. Samples were plated on ampicillin enriched LB agar (50µg/ml), Carbenicillin enriched agar (100µg/ml) and LB agar. Plates were incubated overnight at 37 ^{o}C.

Cell harvesting and RNA isolation of *Haemophilus ducreyi*:

Cultures were grown in brain-heart infusion broth with 5% Hemin, 5% fetal bovine serum, and 1% IsoVitalex. Overnight cultures were incubated at 33 ^{o}C in a waterbath, and at 155 rpm. Absorbance of all samples was calculated using a spectrometer (Milton Roy). Media from the overnight culture was transferred to 30ml of fresh BHI until the A_{660} of the new culture reached .06-.08. These cultures were subjected to the same growth conditions as the overnight. Absorbance was recorded every hour. When the A_{660} reached 0.6, 1 mL of the bacterial cells were collected, centrifuged at 12,000XG for 1 min, the supernatant was removed and the process was repeated. The supernatant was removed and the tube containing the pellet was quickly frozen by placing in ethanol at -80 ^{o}C. Samples were stored at -80 ^{o}C.

RNA isolation:

The bacterial pellets were suspended in a 100µl of TE solution with 1mg/ml lysozyme and incubated at ambient temperature for 5 min. The RNA isolation reagents were from RNAqueous kit (Ambion). The samples were combined with 300µl of lysis/ binding solution, and vortexed until homogenous and centrifuged at 12,000XG for 5 min. In a clean tube, 350 µl of supernatant was combined with an equal volume of 64% ethanol. The samples were vortexed, the mixtures were added onto a column, centrifuged at 12,000XG and the flow-through was discarded. The column was washed with 700µl of wash solution 1 by centrifugation for 1 min at

12,000XG and the flow through was discarded. The column was then washed twice with wash solution 2/3 centrufuging the sample for 1 min at 12,000XG and discarding the flow through each time. To dry the column, it was centrifuged again at 12,000xg and flow through was discarded. To collect the RNA product, the column was then transferred to a collection tube and 40µl of 70 ^{o}C-80 ^{o}C TE was added and centrifuged for 1 min. Nanodrop analysis determined concentration of RNA and samples were analyzed by electrophoresis in a 0.7% agarose gel in TBE along with a 2 log ladder (NEB). Samples were stored at -80 ^{o}C.

Reverse Transciptase -PCR:

For cDNA synthesis, 500ng of isolated RNA as combined with .83 µM of random decamer primers (Ambion) in a final volume of 6µl. The sample was incubated at 85˚C for three minutes, chilled to 4˚C and 1µl 10X RT Buffer (500mM Tris-HCl, pH8.3, 750mM KCl, 30mM $MgCl_2$, 50mM DTT), 2µl 2.5mM dNTP, 0.5µL 10u/µl RNase inhibitor, 0.5µL 100u/µl reverse transcriptase (Ambion) were added. Reactions were incubated at 44˚C for 1 hour, and chilled to 4˚C. Samples were stored at -20˚C.

qPCR analysis:

The volume of each qPCR sample was 20µl and contained: 10µl of 2X Power SYBR green(Applied Biosystem), 0.5 µl RT reaction, 0.4 µM of each primer. Analyses of samples were conducted in triplicate using STEP-ONE software and a thermocylcer (Applied Biosystem). The samples were incubated at 95˚C for 10 min, 40 cycles of 95˚C for .15 min and 60˚C for 1 min, followed by a melt curve analysis (Applied Biosystems, StepOne). Results were analyzed using StepOne software. Single Ct values of a triplicate set that were 1 Ct value different than the other two were excluded, and if all three values varied the variability was retained. To determine

relative levels of Cu,Zn SOD, the Ct values for GyrB were subtracted from those for Cu,Zn SOD and then the $2^{-\Delta Ct}$ value was calculated.

Statistical analysis of relative mRNA expression was conducted with Microsoft Excel 2010. Quantitative PCR results were analyzed using an individual 2 tail t-test.

Results

Analysis of Cu,Zn SOD mRNA levels in *Haemophilus ducreyi*

To understand the role of Cu,Zn SOD in ROS tolerance in *Haemophilus ducreyi,* the relative mRNA expression of the *sodC* gene in 35K and HD85 was investigated. Past studies have evaluated the relative level of *sodC* mRNA expression in 35K and HD85, and found that HD85 had 1.5 fold expression of *sodC* relative to 35K but did not account for the phase of the growth cycle the bacteria were in during the RNA extraction (Saylor, 2011). To account for the potential role of growth phase on *sodC* expression, cells were harvested during mid-log phase , and RNA from HD85 and 35K was extracted. To investigate the quality of the RNA, 500ng each RNA isolation was inspected by electrophoretic analysis on a 1.2% agarose gel (Figure 4A). Each isolation had bands of high concentration which suggests that the RNA was not degraded and the presence of ribosomal RNA. The RNA isolation extract was converted to cDNA and quantitative PCR analysis was conducted revealing that the relative mRNA expression of *sodC* in HD85 and 35K was not significantly different (Figure 4B). Though, *sodC* is expressed in both HD85 and 35K during mid-log, which suggests a capacity for constitutive expression in both strains.

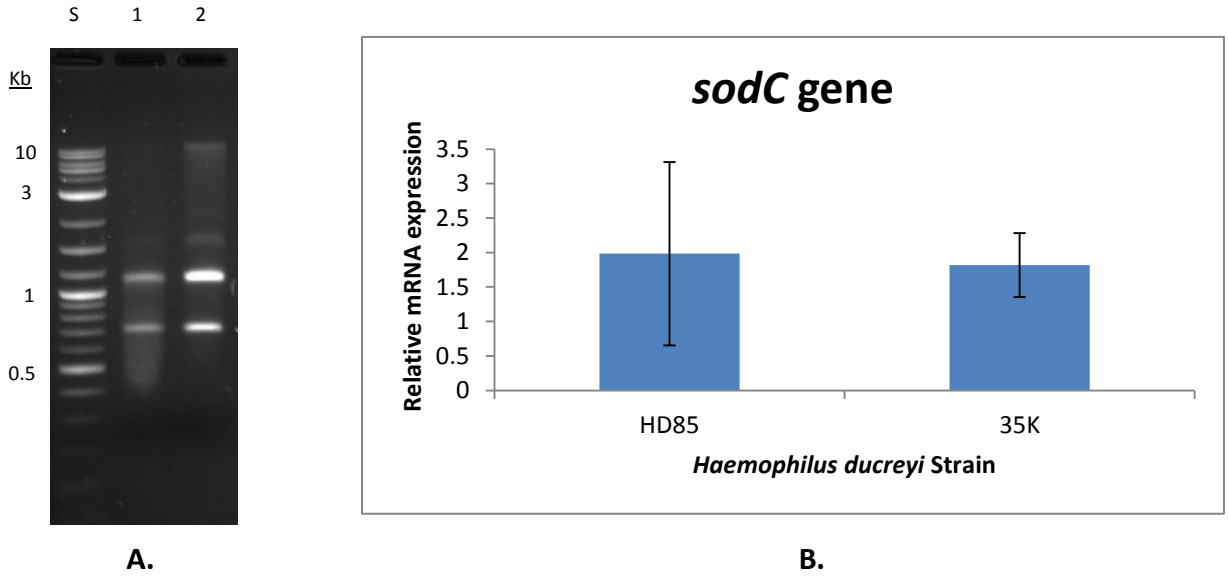
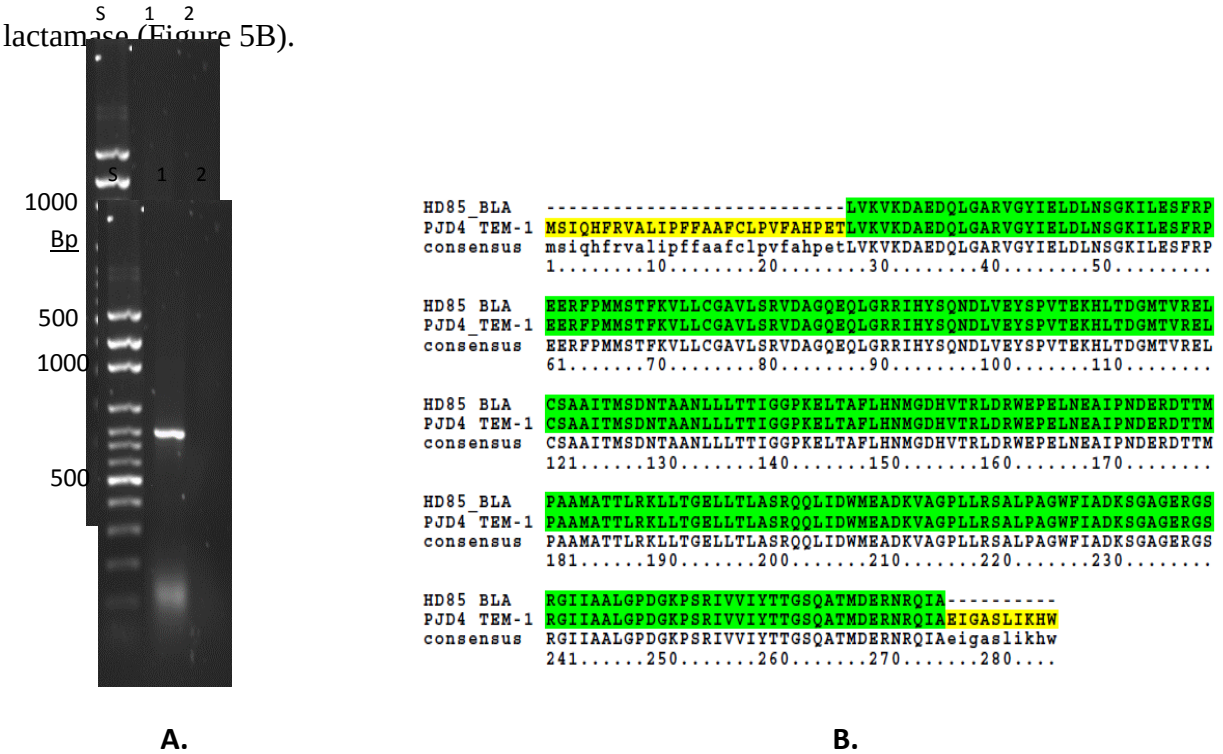


Figure 4: Analysis of Cu,Zn SOD expression during logarithmic growth phase
(A) RNA extracts from HD85 and 35K during mid-logarithimic growth phase and 500ng of each extract was loaded onto agarose gel. HD85 RNA extract in lane 1 and 35K RNA extract in lane 2. Lane S contains an NEB 2 log ladder. **(B)** Relative mRNA expression of Cu,Zn SOD of *H. ducreyi* strains, HD85 and 35K. Real time PCR conducted with Cu,Zn SOD primers compared against control (gyrB) using StepOnesoftware and Power SYBR Green master mix. Relative mRNA expression of Cu,Zn SOD expression during exponential phase was compared to between HD85 and 35K. Error bars represent standard error of three trials (n=3).

Analysis of antibiotic resistance in HD85

In preliminary studies, HD85 was found to be resistant to ampicillin (Figure A1, Figure A2) and other beta-lactam antibiotics (data not shown). A nitrocefin assay was conducted, which suggested HD85 contained a beta-lactamase enzyme to counteract the ampicillin. PCR analysis of total DNA extracted from HD85 and 35K using *bla* gene primers, designed from the PJD4 plasmid of *Neisseria gonorrhoeae*, revealed the presence of a *bla* gene in HD85 and not in 35K (Figure 5A). Amplification of the *bla* gene from HD85 is consistent with the observation that this strain is resistant to certain beta-lactams. The product size of 820 from HD85 is similar to the expected product size of 821 from the PJD4 plasmid, suggesting similar regions in both. To determine the amino acid composition and type of beta-lactamase, the HD85 *bla* PCR product from total DNA extract was sequenced. The 754 bp sequence of the HD85 *bla* gene was blasted against the NCBI database and the *bla* gene was 100% similar to a TEM-1 type beta-lactamase. All amino acids of the sequenced HD85 *bla* gene aligned to the PJD4 TEM-1 type beta-lactamase (Figure 5B).

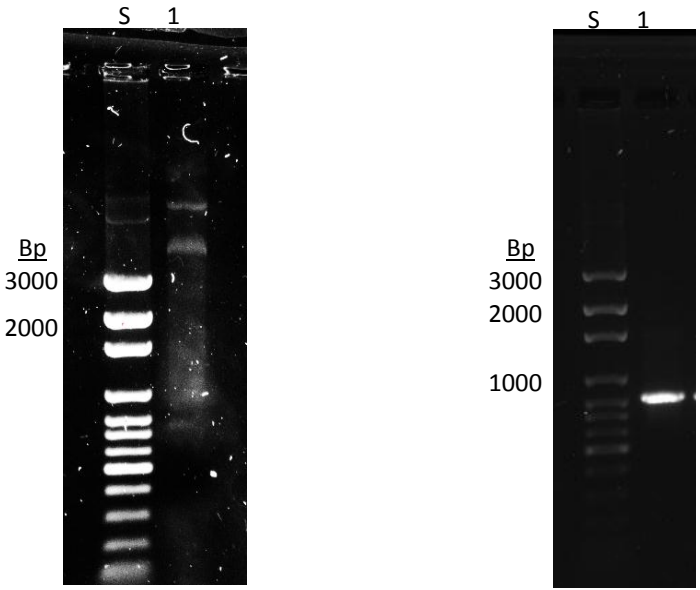


A. **B.**

Figure 5: Analysis of *bla* gene from total DNA extract of HD85 and 35K.

(A)PCR was conducted on total DNA extracted from HD85 and 35K, results shown on a 1.2% agarose gel *bla* gene PCR products for HD85 (Lane 1) and 35K (Lane 2). Lane S contains a 100bp ladder (Phenix). Size of PCR product was determined by migration pattern and standard curve analysis. **(B)** An alignment of the HD85 *bla* gene amino acid sequence with the *Neisseria gonorrhoeae* PJD4 plasmid Tem-1 type *bla* amino acid sequence. Alignments are to be read vertically. Identical residues are shown in green. Dashes and yellow highlights represent residues that were not sequenced HD85 but present in the PJD4 amino acid sequence. Numbers indicate position in the amino acid sequence.

To identify the location of the *bla* gene in HD85, plasmid DNA was needed. Previous isolations of plasmid DNA in HD85 were successful but insufficient for further testing. Another plasmid mini-prep of HD85 was performed and it yielded a sufficient amount of plasmid DNA extract for further anaysis. To assess the plasmid extract quality, the plasmid was run on a 0.7% agarose gel. During the electrophoretic analysis the plasmid extract produced two distinct bands with greater molecular weight than 3000 bp (Figure 6A). To determine if HD85 plasmid DNA contains a *bla* gene, a PCR amplification using *bla* gene primers was conducted from the HD85 plasmid DNA extract. According to electrophoretic analysis the the pcr amplification of the *bla* gene from plasmid extract yielded a product of 818 bp (Figure 6B). The *bla* pcr product size from the plasmid DNA was similar to the region amplified using the *bla* gene primers from total DNA extract suggesting the same gene was amplified and that the plasmid contains a TEM-1 type *bla* gene.





A. **B.**

Figure 6: Analysis of *bla* gene in HD85 plasmid extract.
(A) An electrophoretic analysis, using .7% agarose gel, of plasmid DNA extracted from HD85 in lane 1. Lane S shows a 100bp ladder (Phenix). **(B)** Gel electrophoresis of PCR product of *bla* gene from HD85 plasmid DNA extract. PCR using *bla* gene primers using 50 ng of plasmid extract was conducted. (Lane 1) Lane S represents a 100bp ladder (phenix). Size of PCR product was determined by migration pattern and standard curve analysis.

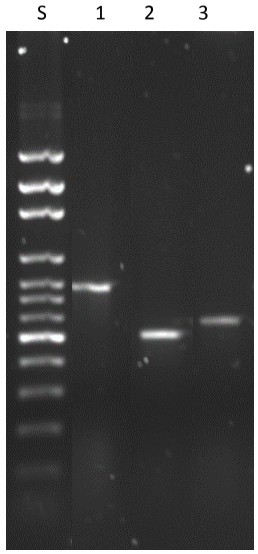
To determine if the beta-lactam resistance phenotype was associated with the HD85 plasmid, isolated HD85 plasmid was transformed into carbenicillin sensitive DH5α cells. As a positive control, pGEM-3Z plasmid, which harbors a beta-lactamase gene, was transformed into DH5α cells. Cells transformed with the HD85 plasmid, as well as those with the positive control, formed colonies on carbenicillin rich agar, suggesting that the HD85 plasmid harbors a beta-lactam resistance gene. To account for the possibility of chromosomal contamination in the HD85 plasmid extract, plasmid DNA was extracted from the 1˚ DH5α cells and used for an additional transformation into new DH5α cells. pGEM-3Z plasmid was used as a positive control again. The 2˚ transformants were also able to grow on carbenicillin, suggesting the plasmid in HD85 is associated with a beta-lactam resistance phenotype.

Analysis of competence genes

According to the analyses of 35K strain, the *comM, comB,* and *comA* genes of *H. ducreyi* contain critical defects and internal stop codons. *H. ducreyi* should not be able to acquire DNA through transformation due to these defects. To understand the role of competence in antibiotic

resistance acquisiton in *H. ducreyi,* the *comM, comB* and *comA* genes of the HD85 strain were amplified via the PCR. Each gene primer set was designed to amplify the defective regions/stop codons of the *comM, comB,* and *comA* genes, to determine if the defects were conserved in HD85. The PCR amplification of each gene was successful for 35K (Figure 7A) and HD85 (Figure 7B). Product sizes were determined by migration patterns and standard curve analysis from agarose gels (Figure 7A, figure 7B). In 35K, the product size for *comM, comB,* and *comA* were 785, 586, 510 bp (Figure 7A) and for HD85 778, 590, 501 bp respectively (Figure 7B). These products roughy match the expected product size of 787, 585, 506 bp respectively. The similar sizes of the PCR products between strains suggest similar regions of amplification.

To analyze the competence genes in HD85, the products of *comM, comB,* and *comA* were sequenced. The PCR products yielded interpretable sequence data 744, 526, 517 bp long and according to BLAST analysis, these sequences all matched the 35K genome with 100% similarity. Amino acid alignment of the HD85 sequence data to the 35K genome showed that the critical defects and stop codons in *comM, comB* and *comA* were conserved (Figure 7C, Figure 7D, Figure 7E).



B.

genes in HD85 and 35K.
CR of competence genes
5k and **(B)** HD85. PCR
ose gel with PCR products
, *comA* in lane 3, and a
zes of PCR products was
and standard curve
e sequenced HD85

```
HD85_comM MSLAIVYSRASMGVEAPLVTIEVHLSNGKPGLTIVGLPETTVKEAGDRVRSALMNANFLY
35K_comM  MSLAIVYSRASMGVEAPLVTIEVHLSNGKPGLTIVGLPETTVKEAGDRVRSALMNANFLY
consensus MSLAIVYSRASMGVEAPLVTIEVHLSNGKPGLTIVGLPETTVKEAGDRVRSALMNANFLY
          1........10........20........30........40........50.........

HD85_comM PPQRITINLAPADLPKEGGRFDLPIAIGILAASGQMDSEKLKEFELLGELALTGGLRAVH
35K_comM  PPQRITINLAPADLPKEGGRFDLPIAIGILAASGQMDSEKLKEFELLGELALTGGLRAVH
consensus PPQRITINLAPADLPKEGGRFDLPIAIGILAASGQMDSEKLKEFELLGELALTGGLRAVH
          61.......70........80........90........100.......110........

HD85_comM GVIPAVIAADQAHRQMIIAAPNANEASLVSNVETYFAHSLLEVVNFMNKRENLPIAQQVN
35K_comM  GVIPAVIAADQAHRQMIIAAPNANEASLVSNVETYFAHSLLEVVNFMNKRENLPIAQQVN
consensus GVIPAVIAADQAHRQMIIAAPNANEASLVSNVETYFAHSLLEVVNFMNKRENLPIAQQVN
          121......130.......140.......150.......160.......170........

HD85_comM QQSEQNRPLIKRDLTDIIGQQHAKGINDCSSRTAXFIAFR--------------------
35K_comM  QQSEQNRPLIKRDLTDIIGQQHAKGINDCSSRTAXFIAFRSTRHRKNNVGXPFSXFITHY
consensus QQSEQNRPLIKRDLTDIIGQQHAKGINDCSSRTA-FIAFRstrhrknnvg-pfs-fithy
          181......190.......200.......210.......220.......230........
```

C.

```
HD85_comB ---------IFDFNNAVIEFIYCNIVFYSNLANXXTKXTACYAKSTGXKXNKXADAKNXF
35K_comB  MAKNNTQKYIFDFNNAVIEFIYCNIVFYSNLANXXTKXTACYAKSTGXKXNKXADAKNXF
consensus maknntqkyIFDFNNAVIEFIYCNIVFYSNLAN--TK-TACYAKSTG-K-NK-ADAKN-F
          1........10........20........30........40........50.........

HD85_comB VKAXWXSSQTVLFKNKRNTVFYTINNLPLRQGGIEWVHLENKNKPILKMTGKLAQQQAFG
35K_comB  VKAXWXSSQTVLFKNKRNTVFYTINNLPLRQGGIEWVHLENKNKPILKMTGKLAQQQAFG
consensus VKA-W-SSQTVLFKNKRNTVFYTINNLPLRQGGIEWVHLENKNKPILKMTGKLAQQQAFG
          61.......70........80........90........100.......110........

HD85_comB ALENYLAGQVNLILTIDDFHINENKQVEFSLNVEWQEXRNELVNXILFISXXLCFXHGRV
35K_comB  ALENYLAGQVNLILTIDDFHINENKQVEFSLNVEWQEXRNELVNXILFISXXLCFXHGRV
consensus ALENYLAGQVNLILTIDDFHINENKQVEFSLNVEWQE-RNELVN-ILFIS--LCF-HGRV
          121......130.......140.......150.......160.......170........

HD85_comB YGET--------
35K_comB  YGETFEMDLNFX
consensus YGETfemdlnf-
          181......190
```

D.

```
HD85_comA --------------RQIGLAENAEYYCVVYQNSDQIIADWHIKPFNVQAWLTQTLPFDEV
35K_comA  MNIFSKICKKETKKRQIGLAENAEYYCVVYQNSDQIIADWHIKPFNVQAWLTQTLPFDEV
consensus mnifskickketkkRQIGLAENAEYYCVVYQNSDQIIADWHIKPFNVQAWLTQTLPFDEV
          1........10........20........30........40........50.........

HD85_comA ATFIRPIPFSYIWRKYLFFPLNLDKNVLYRQLLHILRQELPLNLEEIYFDYQIFPLTDDR
35K_comA  ATFIRPIPFSYIWRKYLFFPLNLDKNVLYRQLLHILRQELPLNLEEIYFDYQIFPLTDDR
consensus ATFIRPIPFSYIWRKYLFFPLNLDKNVLYRQLLHILRQELPLNLEEIYFDYQIFPLTDDR
          61.......70........80........90........100.......110........

HD85_comA LQKVIVYAVRKHYADSLICMPNTILDCELLCFAXGMDYLMADNDTKTHVDYVMNDTLLRF
35K_comA  LQKVIVYAVRKHYADSLICMPNTILDCELLCFAXGMDYLMADNDTKTHVDYVMNDTLLRF
consensus LQKVIVYAVRKHYADSLICMPNTILDCELLCFA-GMDYLMADNDTKTHVDYVMNDTLLRF
          121......130.......140.......150.......160.......170........

HD85_comA SGKEIE-
35K_comA  SGKEIEV
consensus SGKEIEv
          181....
```

E.

Discussion

To better understand factors that contribute to antibiotic resistance acquisition in *H. ducreyi*, the antibiotic resistance and tolerance characteristics of the strains HD85 and 35K were assessed. Cu,Zn SOD was analyzed as a potential ROS tolerance factor in *H. ducrei* by qPCR analysis. qPCR analysis revealed that the HD85 and 35k strains had *sodC* mRNA expression at mid-log but the levels were not significantly different (Figure 4). The ampicillin resistance and susceptibility of HD85 and 35K were also assessed. Sensi disc assays with a variety bactericidal antibiotics revealed HD85 is significantly more resistant to ampicillin than 35k strain (Figure A1, Figure A2). Nitrocefin disc assays show HD85 contains a beta-lactamase enzyme (Appendix). Utilizing a plasmid miniprep kit, a plasmid was extracted from HD85 (Figure A3). A beta-lactamase gene (*bla*) was amplified using total DNA extract of HD85 but not 35K (Figure 5A). The sequence of this product matched a TEM-1 type *bla* gene and the amino acid sequence was aligned with the PJD4 plasmid *bla* gene revealing 100% similarity (Figure 5B). When PCR was conducted using *bla* gene primers on HD85 plasmid DNA extract a similarly sized product was amplified (Figure 6). Transformation of HD85 plasmid DNA extract into DH5α cells allowed

them to grow on agar plates containing carbenicillin, indicating that the plasmid is associated with the resistance phenotype.

To determine if transformation was a possible mechanism of resistance acquisition in HD85, competence genes were studied. The genome of 35K suggests is not capable of transformation due to critical defects and stop codons in the *comM, comB,* and *comA* genes (Redfield et al. 2006), yet HD85 has a plasmid which can be acquired through transformation. To identify if the defects were conserved in HD85, the *comM, comB,* and *comA* genes in HD85 and 35K were amplified, and found to contain similarly sized regions for each gene (Figure 7A, Figure 7B). Sequence alignments of the PCR products to the 35K geneome show that the *comM, comB,* and *comA* genes in HD85 contained the same critical defects and stop codons (Figure 7C, Figure 7D, Figure 7E).

Role of Cu,Zn SOD in ROS tolerance in H. ducreyi

As an obligate human pathogen, *H. ducreyi,* is likely to encounter oxidative stress during infection. The ability of an obligate human pathogen to withstand superoxide from bactericidal antibiotics and oxidative burst from neutrophils, and resist antibiotic treatment could provide a critical selective advantage. The HD85 strain of *H. ducreyi* was previously known to be antibiotic resistant but this study sought to explore the elements that contributed to its resistance and the potential factors that may have allowed it to tolerate the selective pressures prior to its resistance acquisition. To understand the role of tolerance in antibiotic resistance acquisition this study examined the role of Cu,Zn SOD as a potential ROS tolerance factor by analyzing relative *sodC* mRNA expression between a resistant strain, HD85, and a susceptible strain, 35K.

According to qPCR analysis, *sodC* mRNA expression in the HD85 and 35K strains at mid-log is not significantly different. These results contradict a previous study which suggests that HD85 had elevated levels of Cu,Zn SOD expression relative to 35K (Saylor, 2011). In that study, relative *sodC* mRNA expression was analyzed but it was not known what growth phase the cells were in when the RNA was extracted. The difference in *sodC* expression between strains may have been due to samples possibly being extracted at different points in the growth cycle, because for many bacteria Cu,Zn SOD is expressed differently depending on growth phase (Lynch and Kuramitsu, 2000). Variability in the data set could have also been another explanation. Contrary to my hypothesis that increased levels of Cu,Zn SOD expression in HD85 aided in acquisition of ampicillin resistane, there was not a statistically significant difference in *sodC* mRNA expression relative to 35K. But the presence of *sodC* mRNA in both strains during mid-log suggests that *sodC* may be constitutively expressed. For *H. ducreyi*, constitutive expression of Cu,Zn SOD could provide a mechanism of tolerance to neutrophil killing and antibiotic stress.

For many bacteria Cu,Zn SOD is expressed mainly in stationary phase, but our results suggest that Cu,Zn SOD in *H. ducreyi* may constitutively expressed. One study in *H. ducreyi* states, according to unpublished data, that Cu,Zn SOD expression is independent of growth phase and may be consititutively expressed (San Mateo, 1998). This was tested using a Cu,Zn SOD activity assay on whole cell lysates. Our results support these claims by suggesting that *H. ducreyi* expresses *sodC* mRNA at mid-log. For most pathogenic bacteria that contain Cu,Zn SOD, Cu,Zn SOD is not constitutively expressed or is expressed most strongly in stationary phase (Lynch and Kuramitsu, 2000). In *E. coli*, Cu,Zn SOD prevents the damage caused by ROS and decreases hydrogen peroxide sensitivity in stationary phase (Gort, 1999). Cu,Zn SOD

expression in many bacteria is facilitated by the alternative sigma factor, RpoS, which is expressed in stationary phase (Lynch and Kuramitsua, 2000). For *E. coli, rpoS* expression is only present in stationary phase (Lacour and Lindini, 2004). My results suggest *H. ducreyi* mRNA expression of *sodC* occurs in mid-log phase, which in *E. coli* is not even transcribed until stationary phase (Figure 1B, Imlay and Imlay, 1996). This suggests the regulation of Cu,Zn SOD is not like that of *E. coli* and many other bacteria. Some other organisms, like *Legionella pneumophila*, can express Cu,Zn SOD during mid-log but this is due to RpoS expression (Lynch and Kuramitsua, 2000). According to the 35K genome, *H. ducreyi* lacks an *rpoS* gene suggesting Cu,Zn SOD expression is regulated through alternative means. If Cu,Zn SOD is constitutitively expressed or its expression is independent of growth phase this may suggest the importance of this protein for the organism's survival *in vivo*.

Constitutive expression of Cu,Zn SOD may allow *H. ducreyi* increased capacity to respond to the oxidative stress from neutrophil action during infection of its host. It has been shown in previous studies that Cu,Zn SOD expression aids in preventing damage from exogenous superoxide (San Mateo, 1998). Inducing exogenous superoxide damage by pyrogallol in an Cu,Zn SOD deficient mutant was found to decrease survival relative to its isogenic parent (San Mateo, 1998). This suggests a role of Cu,Zn SOD in the defense against superoxide created during neutrophil oxidative burst. Infection of swine models reinforced this idea by utilizing Cu,Zn SOD knockouts in swine models. In normal swine models the knockout has attenuated growth but neutropenia in the swine models restored the growth, suggesting that Cu,Zn SOD played a critical role in the defense against neutrophil (San Mateo et al., 1999). If Cu,Zn SOD is constitutively expressed it could allow *H. ducreyi* to tolerate oxidative burst of the host immune response. As an obligate human pathogen, *H. ducreyi* will likely come into interaction with

neutrophils and expressing Cu,Zn SOD at all times may allow *H. ducreyi* an increased capacity to respond to this selective pressure.

The expression of Cu,Zn mRNA at mid-log suggests that Cu,Zn may have a capacity to respond to oxidative stress induced by antibiotics in exponential phase. When bacteria are treated with bactericidal antibiotics, ROS is produced via the interaction of superoxide with the iron sulfur clusters (Kohanski et al., 2007). This production of ROS can lead to damages of the bacterial membrane and allow superoxide to leak into the periplasmic space, where Cu,Zn SOD is located (San Mateo, 1998). Superoxide that leaks into the periplasmic space can encounter Cu,Zn SOD and if Cu,Zn SOD is constitutively expressed it may be able to dismutate the superoxide to less harmful forms immediately. Constitutive expression of Cu,Zn SOD in *H. ducreyi* could promote survival during bactericidal antibiotic treatment by dismutating the superoxide released into the periplasmic space to prevent the damage to the iron-sulfur clusters, to prevent the release of free iron, and to prevent the generation of ROS from superoxide.

Constitutive expression of Cu,Zn SOD could have aided HD85 in obtaining antibiotic resistance. In order for an organism to acquire antibiotic resistance, it must first survive long enough in the host. Constitutive Cu,Zn SOD expression in HD85 could have aided by protecting the bacteria against exogenous superoxide from neutrophils and potentially reduce the oxidative damage caused by antibiotic treatment. By aiding in the bacteria's ability to survive in the host this would allow the bacteria more time to acquire its antibiotic resistance plasmid. Future research would need to be done to determine if Cu,Zn SOD has a potential capability to help aid in antibiotic resistance acquisition.

Further research is required to understand the role of Cu,Zn SOD in ROS tolerance from antibiotics and neutrophil action in *H. ducreyi*. Understanding the expression pattern of Cu,Zn

SOD in *H. ducreyi* would be the first step. To understand how Cu,Zn SOD is expressed in *H. ducreyi*, time course analyses of normal mRNA expression and Cu,Zn SOD activity, could be utilized. Though previous work suggests Cu,Zn SOD activity in *H. ducreyi* is similar at each growth phase, data supporting this has not been published (San Mateo, 1998). *H. ducreyi* also could have differential capacity to express Cu,Zn SOD in each phase of growth. If differential expression, capacity and/or activity was found, sensitivity to ROS and/or antibiotic induced ROS could be assessed at these different time points to understand how *H. ducreyi* responds to oxidative stress at these time points. Knowledge of the expression pattern and capacity in *H. ducreyi* can help elucidate the function of Cu,Zn SOD *in vivo* and how Cu,Zn SOD expression affects survival and tolerance to ROS.

Studies altering the expression of Cu,Zn SOD in *H. ducreyi* could be very informative in understanding the role of Cu,Zn SOD in response to the oxidative stress from neutrophil action and antibiotics. Over expressing or nullifying Cu,Zn SOD expression in strains of *H. ducreyi* could allow for analyses into the effect of Cu,Zn SOD expression on oxidative stress tolerance. To study the role of Cu,Zn SOD in tolerance to antibiotics, Cu,Zn SOD over-expression and null expression mutants could be assessed for their sensitivity to antibiotics and compared to their isogenic parents. Similar studies could be done to test for the effect of Cu,Zn SOD in defense against oxidative burst. Previous studies with Cu,Zn SOD null expression mutants suggest a role of Cu,Zn SOD in defense against the host immune response but over-expression mutants have not been reported (San Mateo, 1998; San Mateo et al., 1999, Bong et al., 2002). If over-expressing Cu,Zn SOD in *H. ducreyi* led to increased capacity to tolerate exogenous superoxide, this would further suggest an effective role of Cu,Zn SOD in tolerating oxidative stress from neutrophil oxidative burst.

Plasmid mediated beta lactamase resistance in HD85

The potential protective effect of Cu,Zn SOD expression could have allowed the HD85 strain to survive long enough to gain antibiotic resistance. To understand more about the antibiotic resistance of HD85 and its origins, the antibiotic resistance characteristics were studied. Previously the source of antibiotic resistance in HD85 had not been clearly identified. Prior work had identified the presence of beta-lactamase protein via nitrocefin assays (Appendix) and the presence of plasmid in HD85 (Figure A3). These results suggest that the two are linked. Transformations of the HD85 plasmid into DH5α cells led to the transfer of a beta-lactam resistance phenotype, suggesting the plasmid carried an expressible antibiotic resistance gene. PCR amplification of a *bla* gene from plasmid extract further supports this (Figure 6). PCR amplification and sequencing using *bla* gene primers from total DNA extract indicated that this was a TEM-1 *bla* gene (Figure 5). In conjunction the results of the pcr amplification of the *bla* gene from plasmid extract, the identification of the TEM-1 type *bla* from total DNA extract, and the transformation leading to the development of antibiotic resistance all suggest that the *bla* gene on the plasmid is a TEM-1 type beta-lactamase and is responsible for an beta-lactam resistance phenotype.

Association of plasmids coding for beta-lactam resistance due to the presence of the TEM-1 *bla* gene on the plasmid is observed frequently in other studies in *H. ducreyi* and other antibiotic resistance bacteria. In many *H. ducreyi* antibiotic resistance characterization studies, resistance is most often associated with the presence of a plasmid (Dangor et al. 1988; Haydock et al., 1999) According to one estimate, 70% of antibiotic resistance cases in *H. ducreyi* are the result of plasmids carrying antibiotic resistance genes. Additionally, the presence of TEM type

BLA on HD85's plasmid is not unexpected. TEM type beta-lactamases are very common for antibiotic resistant bacteria. In a related species, *H. influenza,* 94.6% beta-lactamases are of the TEM variant. In *H. ducreyi*, TEM-1 variants are very common and have been found in multiple isolates (Maclean et al., 1980; Brunton, 1982). Despite the prevalence of TEM type *blas* in *H. ducreyi*, *rob* and *pCb* type beta-lactamases have also been isolated (Maclean et al., 1992; Prachayitskittul et al., 2000). But like many other *H. ducreyi* isolates, the beta-lactam resistance of HD85 appears to originate from the TEM-1 type *bla* on the plasmid.

The role of competence genes in the acquisition of plasmid coding resistance

The presence of plasmid containing antibiotic resistance in HD85 required investigation because *H. ducreyi* as a species supposedly is not capable of one of the major mechanisms to acquire resistance genes, transformation. Traditionally transformation requires multiple competence proteins that allow the protein to enter a transformable state (Claverys and Martin, 2003) . *H. ducreyi* belongs to the order of the Pasteurellaceae and the species in this order requires 12 competence genes to be able to acquire DNA from the environment (Redfield et al., 2006). According to the analysis of the 35K genome, *H. ducreyi*'s competence genes are defective because *comM* and *comB* have internal frame shifts and *comB* and *comA* have premature stop codons (Redfield et al. 2006). The truncations and frame shifts in these genes suggest their protein products are non-functional and *H. ducreyi* should not be able to acquire DNA from the environment (Redfield et al. 2006).

The sequence analysis of HD85's competence genes supports the assumption that *H. ducreyi* as a species has defective competence genes. PCR amplification and sequencing was used to determine the similarity of the *comA, comB* and *comM* genes between HD85 and 35K.

Primers were designed to amplify the regions of each gene that caused the critical defects. The critical defects of the *comM, comB* and *comA* genes in 35K were all conserved in HD85 and the sequenced portions of these genes in HD85 had 100% sequence similarity to their 35K gene counterparts(Figure 4). These data suggest that transformation mechanism of HD85 is not functional. It is unlikely that an ancestor of HD85 gained the plasmid and later developed the exact mutations as 35K for the three competence genes. A common ancestor of 35K and HD85 could have had the plasmid and lost it, but this is also not likely. Instead it seems more likely that HD85 gained the plasmid through means other than transformation.

There are two other major methods in which the ancestral strains of HD85 could have obtained the plasmid: transduction and conjugation. Transduction does not appear to be common in *H. ducreyi*. Transduction requires infection by phage but transduction and infection by phage has not been reported in *H. ducreyi*. Conjugation is the best studied form of antibiotic resistance acquisition for *H. ducreyi*. Multiple isolates of *H. ducreyi* have been found to be capable of conjugation (Trees and Morse, 1995). Conjugation in *H. ducreyi* is facilitated by the presence of conjugative plasmids that allow *H. ducreyi* to transfer genetic material from other strains and even other species (Deneer et al., 1981). It is believed that the presence of conjugative plasmids and plasmid exhange is a major cause of the similar plasmids in *H. ducreyi* and *Neisseria gonorrhoeae* (Deneer et al. 1981).

The plasmid extraction results suggest that HD85 may have a conjugative plasmid (Figure 6). The plasmid electrophoresis revealed to distinct bands greater than 3kb. This information may be interpreted in two ways. First, the banding patterns may represent two forms of a single plasmid, nicked and supercoiled. Alternatively, the two bands may be interpreted as two different plasmid sets. If one of these plasmids were a conjugative plasmid it could clarify

the mechanism by which HD85 obtained antibiotic resistance. Most conjugative plasmids are 23.5 MDa and contain a 1.3 Mda region which codes for its conjugative capability (Mcnicol and Ronald, 1984) To determine if HD85 contains a conjugative plasmid, the molecular weight and genetic composition would need to be determined. Identification of the 1.3 mDal region could be useful for determining if HD85 has a conjugative plasmid. This would need to be investigated in order to understand the origin of the TEM-1 *bla* resistance genes in HD85.

To prevent the spread of antibiotic resistance genes, like those of HD85, it is necessary to understand the mechanisms and contributing factors that lead to the development of antibiotic resistance, not only in *H. ducreyi* but many other bacteria. It is not enough to simply study the mechanisms of antibiotic resistance. To combat the spread of antibiotic resistance, the mechanisms of tolerance, resistance, and resistance acquisition must be studied. This study has attempted to explore these critical factors in *H. ducreyi* and the HD85 strain. Antibiotic tolerance is considered a key contributor to the development of antibiotic resistance. It appears that through constititutive expression Cu,Zn SOD in *H. ducreyi* may be a mechanism to tolerate antibiotic induced oxidative stress and neutrophil killing action, which would be critical in allowing *H. ducreyi* to survive long enough to gain antibiotic resistance. By further researching the anti-oxidant system and its role in *H. ducreyi* in ROS tolerance from antibiotics and neutrophil action, we can learn more about how strains like HD85 gained its TEM-1 type *bla* gene. Further assessing the role of conjugation in *H. ducreyi* could help uncover the mechanism of antibiotic resistance acquisition for HD85 and *H. ducreyi*. By combining the studies of tolerance factors, with studies of antibiotic resistance gene acquisition mechanisms we can truly move towards preventing the spread of antibiotic resistance and increasing the efficacy of medical treatment.

Appendix

Material and Methods specifically for appendices:

Kirby Bauer Assay:

Bacterial samples were suspended in a saline solution to a McFarland standard of .5. The suspension was spread, using swabs, over the entire surface of chocolate agar. Antibiotic sensi discs (BD Diagnostic Systems)were placed in the center of the agar plates. Samples were incubated for 24 hours at 35˚ C. Zones of inhibition were measured after a 24 hour incubation at 35˚ C and 10% CO_2.

Minimum inhibition assay

In a 96 well plate antibiotics were diluted in saline via 2 fold serial dilutions in a concentration ranging from 250 - .625. Bacterial samples were suspended in an A_{660} solution of .400-.450 of Brain heart Infusion (EMD millipore) broth containing, Fetal Calf Serum (Thermoscientific) and Isovitalex. The suspension, 100µl, was added to the 96 well plate. Samples were incubated for 24 hour incubation at 35˚ C and 10% CO_2. Absorbencies were determined with a 96 well plate reader for prior and following incubation to determine the total growth of the samples.

Assay for Beta-Lactamase activity

Nitrocefin assays (Sigma-Aldrich) were conducted with appropriate strains to determine the presence of beta-lactamase proteins. Discs were moistened with distilled water to facilitate reaction. Single colonies were selected from solid media and spread on the nitrocefin disc. Colormetric changes of the discs confirm the presence of beta-lactamase activity

Appendix A:

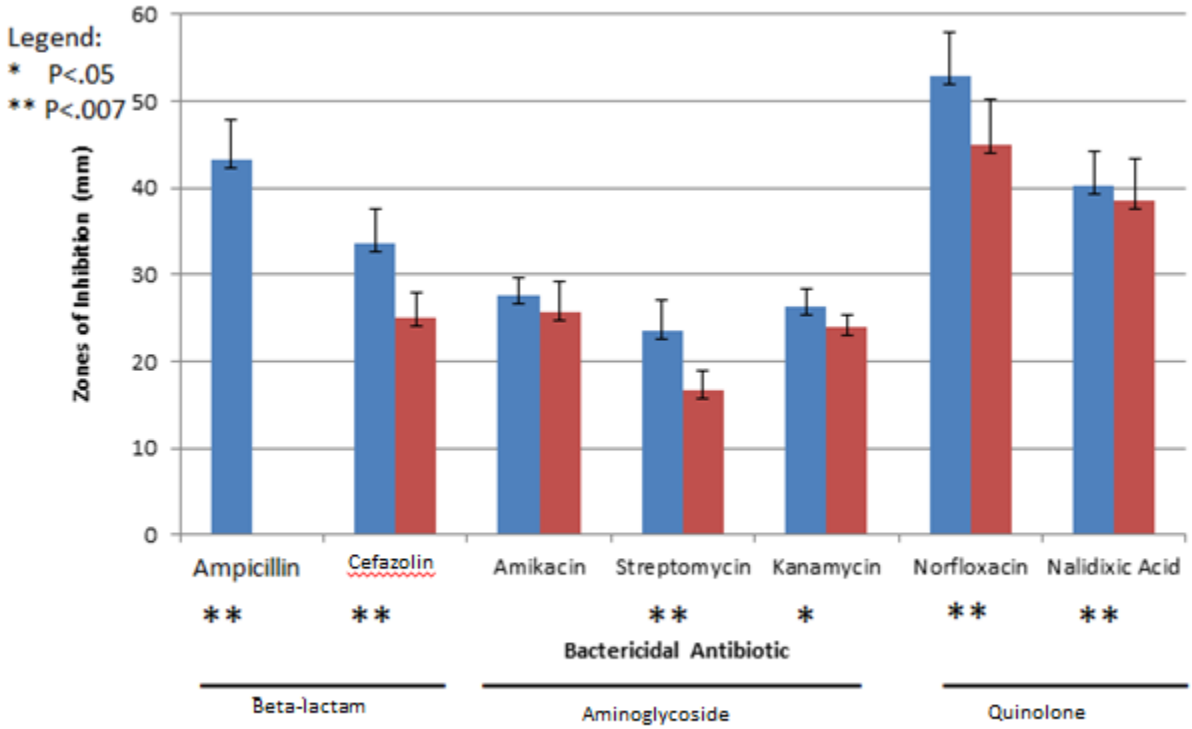


Figure A1. Mean of Zones of Inhibition for multiple bactericidal antiobiotics for the strains 35000HP(Blue) and HD85 (Red). The Zones of Inhibition were measured from an antibiotic disk diffusion assay after 48 hours of incubation. Ampicillan, streptomycin, Norfloxacin were 10 micrograms. Cefazolin, Amikacin,Kanamycin, and Nalidixic acid were 30 micrograms. Error bars represent standard deviation. (n=9)

Previous work suggested that HD85 had elevated levels of Cu,Zn SOD expression than 35K, and that differential expression of Cu,Zn SOD may have a capacity to aid in the tolerance against antibiotic induced oxidative stress. Therefore, using sensi disc assays I tested the antibiotic resistance of HD85 and 35K to various bactericidal anitbiotics which are known to induce oxidative stress (Kohanski et al., 2007). It was found that HD85 had significantly smaller zones of inhibition than 35K for Ampicillin, cefazolin, streptomycin, kanamycin, norfloxacin, and nalidixic acid. This suggests that HD85 was broadly less susceptible to all of these antibiotics. The zone of inhibition for amikacin was not significantly different for HD85 and 35K, suggesting equal sensitivity to the antibiotic. The largest difference in antibiotic sensitivity between HD85 and 35K was to ampicillin. HD85 did not have a zone of inhibition for ampicillin suggesting it was completely resistant.

Appendix B:

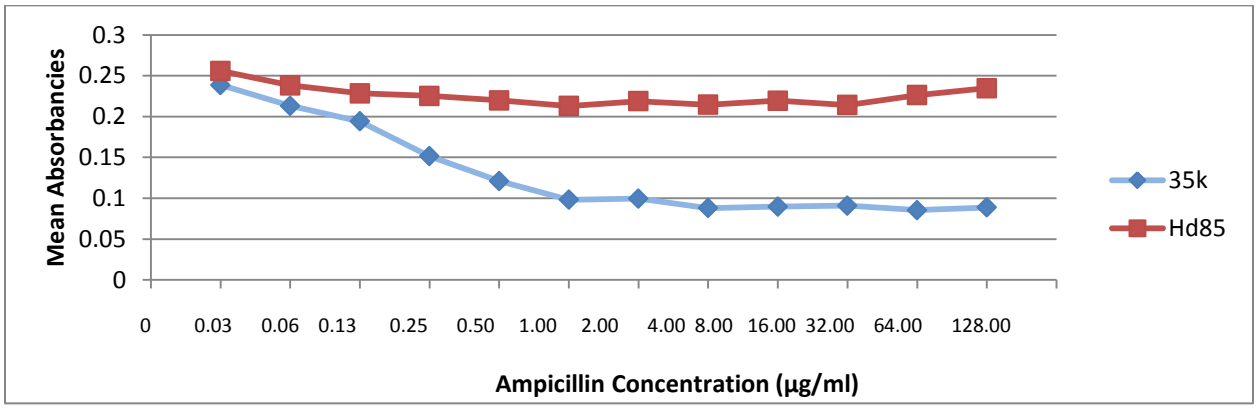


Figure A2: Determination of minimum inhibitory. Minimum inhibitory concentrations were determined by diluting ampicillin 2-fold in BHI and inoculating with HD85 or 35K. Bacterial growth in various concentrations of ampicillin as determined by A_{660} after 24 hours of growth (N=3).

A minimum inhibitory concentration assay was used to test the degree to which HD85 is resistant to ampicillin treatment. The 35K strain was used for a comparison as it was ampicillin sensitive. It was found that for all concentrations of ampicillin tested, that growth of HD85 remained unaffected but as concentration of ampicillin concentration increased the growth of 35K decreased. When treating

35K with 8 µg/ml or greater the absorbance of did not change over the 24 hour period suggesting this concentration effectively killed the bacteria.

Appendix C:

Due to the resistance of HD85 to ampicillin, HD85 was tested for the presence of beta-lactamase activity by a nitrocefin assay. These assays test for beta-lactamase activity by using a dye that causes a colormetric change in the presence of beta-lactamase. HD85 tested positive and 35K tested negative for this colormetric change, suggesting that HD85 has beta-lactamase activity and 35K does not. This is logical considering 35K was sensitive to ampicillin treatment and HD85 was not (Figure A1 and Figure A2).

Appendix D:

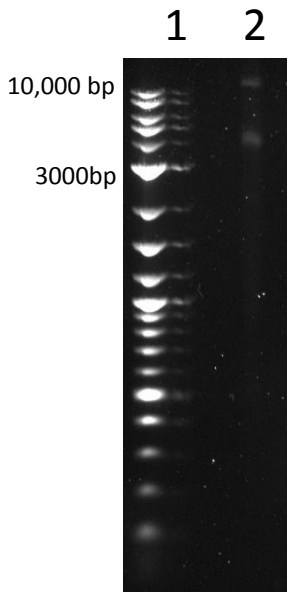


Figure A3: Extraction of plasmid DNA from HD85-023233
An .7% agarose gel showing plasmid extraction product from HD85-023233 in lane 2. Lane 1 shows the NEB 2 log ladder.

For many strains of *H. ducreyi,* antibiotic resistance is due to the presence of a plasmid (Deneer et al.,

1981). To test to see if HD85 contained a plasmid a plasmid extraction was conducted and HD85 does contain a plasmid. The plasmid extract was run on a .7% agarose gel. During the electrophoretic analysis the plasmid extract produced two distinct bands one with greater molecular weight than 3000 bp and one with greater molecular.

Literature Cited

Alscher, R.G., Erturk, N., and Heath, L.S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. *53,* 1331-1341.

Beaman, B.L., Black, C.M., Doughty, F., and Beaman, L. (1985). Role of superoxide dismutase and catalase as determinants of pathogenicity of *Nocardia asteroides*: importance in resistance to microbicidal activities of human polymorphonuclear neutrophils. Infect. Immun. *47,* 135-141.

Bong, C.T., Fortney, K.R., Katz, B.P., Hood, A.F., San Mateo, L.R., Kawula, T.H., and Spinola, S.M. (2002). A superoxide dismutase C mutant of Haemophilus ducreyi is virulent in human volunteers. Infect. Immun.*70,* 1367-1371.

Brunton, J., Meier, M., Ehrman, N., Maclean, I., Slaney, L., and Albritton, W.L. (1982). Molecular epidemiology of beta-lactamase-specifying plasmids of Haemophilus ducreyi. Antimicrob. Agents Chemother. *21,* 857-863.

Clancy, R.M., Leszczynska-Piziak, J., and Abramson, S.B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. J. Clin. Invest. *90,* 1116-1121.

Clark, R.A., Leidal, K.G., Pearson, D.W., and Nauseef, W.M. (1987). NADPH oxidase of human neutrophils. Subcellular localization and characterization of an arachidonate-activatable superoxide-generating system. J. Biol. Chem. *262,* 4065-4074.

Chiang, S.M., and Schellhorn, H.E. (2012). Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria. Arch. Biochem. Biophys. *525,* 161-169.

Claverys, J.P., and Martin, B. (2003). Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol. *11,* 161-165.

Culotta, V.C., Yang, M., and O'Halloran, T.V. (2006). Activation of superoxide dismutases: putting the metal to the pedal. Biochim. Biophys. Acta *1763,* 747-758.

Deneer, H. G., Slaney, L., MacLean, I. W., Albritton, W. L. 1981. Mobilization of nonconjunctive antibiotic resistance plasmids in *Haemophilus ducreyi*. Journal of Bacteriology. 149: 726-732.

Dangor, Y., Miller, S. D., Exposto, F. L., Koornhof, H. J. 1988. Antimicrobial susceptibilities of Southern African isolates of *Haemophilus ducreyi*. Antimicrobial Agents and Chemotherapy. 32: 1458-1460.

Edwards, S. W., 1994, *Biochemistry and physiology of the neutrophil,* Cambridge University Press, New York City, 295 p.

Franzon, V.L., Arondel, J., and Sansonetti, P.J. (1990). Contribution of superoxide dismutase and catalase activities to Shigella flexneri pathogenesis. Infect. Immun. *58,* 529-535.

Furuya, E.Y., and Lowy, F.D. (2006). Antimicrobial-resistant bacteria in the community setting. Nat. Rev. Microbiol. *4,* 36-45.

Gort, A.S., Ferber, D.M., and Imlay, J.A. (1999). The regulation and role of the periplasmic copper, zinc superoxide dismutase of Escherichia coli. Mol. Microbiol. *32,* 179-191.

Haydock, A.K., Martin, D.H., Morse, S.A., Cammarata, C., Mertz, K.J., and Totten, P.A. (1999). Molecular characterization of Haemophilus ducreyi strains from Jackson, Mississippi, and New Orleans, Louisiana. J. Infect. Dis. *179,* 1423-1432.

Imlay, K.R., and Imlay, J.A. (1996). Cloning and analysis of sodC, encoding the copper-zinc superoxide dismutase of Escherichia coli. J. Bacteriol. *178,* 2564-2571.

Kitano, K., and Tomasz, A. (1979). Triggering of autolytic cell wall degradation in Escherichia coli by beta-lactam antibiotics. Antimicrob. Agents Chemother. *16,* 838-848.

Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A., and Collins, J.J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. Cell *130,* 797-810.

Kohanski, M.A., DePristo, M.A., and Collins, J.J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol. Cell *37,* 311-320.

Kohanski, M.A., Dwyer, D.J., and Collins, J.J. (2010). How antibiotics kill bacteria: from targets to networks. Nat. Rev. Microbiol. *8,* 423-435.

Lacour, S., and Landini, P. (2004). ÏƒS-Dependent Gene Expression at the Onset of Stationary Phase in Escherichia coli: Function of ÏƒS-Dependent Genes and Identification of Their Promoter Sequencesâ€ . J. Bacteriol. *186,* 7186-7195.

Liochev, S.L. (1996). The role of iron-sulfur clusters in in vivo hydroxyl radical production. Free Radic. Res. *25,* 369-384.

Liochev, S.I., and Fridovich, I. (1999). Superoxide and iron: partners in crime. IUBMB Life *48,* 157-161.

Lock, R., Dahlgren, C., Linden, M., Stendahl, O., Svensbergh, A., and Ohman, L. (1990). Neutrophil killing of two type 1 fimbria-bearing Escherichia coli strains: dependence on respiratory burst activation. Infect. Immun. *58,* 37-42.

Lushchak, V.I. (2001). Oxidative stress and mechanisms of protection against it in bacteria. Biochemistry (Mosc) *66,* 476-489.

Lynch, M., and Kuramitsu, H. (2000). Expression and role of superoxide dismutases (SOD) in pathogenic bacteria. Microbes Infect. *2,* 1245-1255.

Mayaud, P., and McCormick, D. (2001). Interventions against sexually transmitted infections (STI) to prevent HIV infection. Br. Med. Bull. *58,* 129-153.

MacLean, I.W., Bowden, G.H., and Albritton, W.L. (1980). TEM-type beta-lactamase production in Haemophilus ducreyi. Antimicrob. Agents Chemother. *17,* 897-900.

Maclean, I.W., Slaney, L., Juteau, J.M., Levesque, R.C., Albritton, W.L., and Ronald, A.R. (1992). Identification of a ROB-1 beta-lactamase in Haemophilus ducreyi. Antimicrob. Agents Chemother. *36,* 467-469.

Mcnicol, P.J., Ronald, A.R. (1984) The plasmid of *Haemophilus ducreyi*. Journal of Antimicrobial Chemptherapy, 14, 561-573

Morse, S. A. 1989. Chancroid and *Haemophilus ducreyi*. Clinical Microbiology Reviews. 2: 137-157.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B.E., and Singh, P.K. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science *334,* 982-986.

Pesci, E.C., Cottle, D.L., and Pickett, C.L. (1994). Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of Campylobacter jejuni. Infect. Immun. *62,* 2687-2694.

Prachayasittikul, V., Lawung, R., and Bulow, L. (2000). Episome profiles and mobilizable beta-lactamase plasmid in Haemophilus ducreyi. Southeast Asian J. Trop. Med. Public Health *31,* 80-84.

Redfield, R.J., Findlay, W.A., Bosse, J., Kroll, J.S., Cameron, A.D., and Nash, J.H. (2006). Evolution of competence and DNA uptake specificity in the Pasteurellaceae. BMC Evol. Biol. *6,* 82.

San Mateo, L.R., Hobbs, M.M., and Kawula, T.H. (1998). Periplasmic copper-zinc superoxide dismutase protects Haemophilus ducreyi from exogenous superoxide. Mol. Microbiol. *27,* 391-404.

San Mateo, L.R., Toffer, K.L., Orndorff, P.E., and Kawula, T.H. (1999). Neutropenia restores virulence to an attenuated Cu,Zn superoxide dismutase-deficient Haemophilus ducreyi strain in the swine model of chancroid. Infect. Immun. *67,* 5345-5351.

Saylor, Z. (2011). Allegheny College Senior Comprehensive Project

Scandalios, J.G. (2005). Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz. J. Med. Biol. Res. *38,* 995-1014.

Scandalios, J.G., 1997, Oxidative Stress and the molecular biology of antioxidant enzymes, Cold Spring Harbor Laboratory Press, Plainwview, New York, 890 p.

Segal, A.W. (1988). The molecular and cellular pathology of chronic granulomatous disease. Eur. J. Clin. Invest. *18,* 433-443.

Shatalin, K., Shatalina, E., Mironov, A., and Nudler, E. (2011). H2S: a universal defense against antibiotics in bacteria. Science *334,* 986-990.

Steen, R. (2001). Eradicating chancroid. Bull. World Health Organ. *79,* 818-826.

Sullivan, M. (1940). Chancroid. American Journal of Syphillus, Gonorrhea, and Veneral Diseases *24,* 482-521.

Trees, D.L., and Morse, S.A. (1995). Chancroid and Haemophilus ducreyi: an update. Clin. Microbiol. Rev. *8,* 357-375.

Wasserheit, J.N. (1992). Epidemiological synergy. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. Sex. Transm. Dis. *19,* 61-77.