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A NEW CLASS OF HAEMOPHILUS DUCREYI? MOLECULAR CHARACTERIZATION OF SAMOA STRAINS SB 5755, SB 5756, SB 5757, AND BE 3145

by

Nichol S. Murray

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Department: Biology
Major: Biology
Major Professor: Dr. Catherine D. White

North Carolina A&T State University
Greensboro, North Carolina
2011

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Greensboro, North Carolina
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DEDICATION

This thesis is dedicated to my Mom and Dad for their constant support, love, prayers, and encouragement; to my brothers and sisters for their encouragement and motivation; to my grandparents for their love and support; and to my friends for their support, understanding, guidance and encouragement along the way. Finally, this thesis is dedicated to those who may have road blocks along the way; may this thesis be an example that with hard work, dedication, and faith, nothing is impossible.

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LIST OF ABBREVIATIONS

APs	Antimicrobial peptides
CAP	Chocolate agar plates
CDT	Cytolethal distending toxin
CI	Class I
CII	Class II
DltA	Ducreyi Lectin A
DsrA	Ducreyi Serum Resistance protein A
ECMs	Extracellular matrix
FN	Fibronectin
GUD	Genital ulcer disease
HIV	Human Immunodeficiency Virus
LOS	Lipooligosaccaride
NcaA	Necessary for Collagen Adhesion protein
Oca	Oligomeric Coiled proteins
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PMNs	Polymorphonuclear leukocyte
SRBCs	Sheep red blood cells
STDs	Sexually transmitted diseases
TAA	Trimeric autotransporter adhesion

ABSTRACT

Murray, Nichol S. A NEW CLASS OF HAEMOPHILUS DUCREYI? MOLECULAR CHARACTERIZATION OF SAMOA STRAINS SB 5755, SB 5756, SB 5757, AND BE 3145. **(Advisor: Dr. Catherine D. White)**, North Carolina Agricultural and Technical State University.

Haemophilus ducreyi is a Gram negative, strict human pathogen that causes the genital ulcer disease chancroid. Chancroid is a sexually transmitted infection that may aid in transmission of the Human Immunodeficiency Virus. *Haemophilus ducreyi* can be categorized into two strain classes, class I (CI) and class II (CII), based on variations in DNA sequence and protein expression of several virulence determinants including Ducreyi Serum Resistance protein A (DsrA) and the Necessary for Collagen Adhesion protein (NcaA). Recently, the first description of non-sexually associated chancroid transmission in children with chronic lower limb ulcerations was reported. Bacteria isolated from the children were sequenced and determined to possess a 16 S rDNA sequence that was 100% identical to *Haemophilus ducreyi* CII strain 33921. The purpose of this study was to categorize four strains of *Haemophilus ducreyi* isolated from children in Samoa as CI or CII based on DsrA and NcaA nucleotide and amino acid sequence. Based on the presence of a 16S rDNA that was 100% identical to CII strain 33921, we hypothesized that these strains were CII. To examine DsrA and NcaA, strains SB 5755, SB 5756, SB 5757, and BE 3135 were subjected to Polymerase Chain Reaction and DNA sequencing. SB 5755, SB 5756, and BE 3145 was 90%, 96% and 97% identical to *dsrA* in the CI parent strain 35000 HP, respectively. Moreover, alignment of DsrA the

predicted protein sequences demonstrated that SB 5755, SB 5756, and BE3145 were 71%, 87%, and 92% identical to 35000 HP. However, comparison with HMC112 *dsrA*$_{II}$ revealed a high degree of variability between the N-terminal regions. SB 5755 had 7% identites and 17% similarities, SB 5756 had 7% identites and 21% similarites, and BE 3145 had 5% identies and 17% similarites to *dsrA*$_{II}$ These data indicate that SB 5755, SB 5756, and BE 3145 are not CII, but are indeed CI strains.

CHAPTER 1

INTRODUCTION

Haemophilus ducreyi is a Gram negative bacterium that causes the genital ulcer disease chancroid. This strict human pathogen is transmitted through skin to skin contact and has no other known natural reservoir. Chancroid is not commonly detected in the United States, but is endemic in areas of Africa, Asia, Canada and Latin America (Trees and Morse, 1995; Bong et al., 2002; Spinola et al., 2002; White et al., 2005; Ussher and Wilson, 2007). The epidemiology of chancroid is not well defined likely due to a lack of diagnostic tests for *Haemophilus ducreyi*. However, there is an emphasis on syndromic management of this genital ulcer disease (GUD).

Early studies reported a high occurrence of *Haemophilus ducreyi* infection in areas where HIV is endemic (Dickerson et al., 1996; Steen and Dallabetta, 2003). Therefore, a possible relationship between *Haemophilus ducreyi* infection and the acquisition of HIV was examined. These studies revealed that the transmission of HIV and *Haemophilus ducreyi* infection commonly occurs in men who are not circumcised and who are often exposed to commercial sex workers (Dickerson et al., 1996; Steen and Dallabetta, 2003). It was also determined that the presence of an ulcer may increase susceptibility to HIV infection by inducing a cell mediated response that attracts HIV susceptible cells to the infection site (Cunningham A.L. et al., 1985; Steen and Dallabetta, 2003). In women with genital ulcers, a break down of the normal mucosal barrier in the skin resulted in vaginal shedding of HIV (Gadkari et al., 1998; Kreiss et al.,

1989; Brunham et al., 1990; Schacker et al., 1998). Men who are HIV positive and positive for chancroid ulcers are determined to shed 5 to 7 fold higher concentration of HIV in semen than HIV negative men (Wasserheit, 1991)(Dyer et al., 1998)

The majority of chancroid cases occur in low-socioeconomic areas where commercial sex is a common occupation. In 2003, Steen and Dallabetta demonstrated that HIV and ulcerative GUD had a high rate of co-transmission in mining communities in South Africa. Rates of HIV transmission were higher in cases of GUD than non-ulcerative sexually transmitted infections. This study also determined that effective GUD interventions, such as clinical service, pertaining to regular screening coupled with prevention, could reduce genital ulcer rates among women and men, which in turn could also have an impact on community HIV transmission and prevalence (Steen and Dallabetta, 2003).

The well studied type strain, *Haemophilus ducreyi* 35000 HP, was previously sequenced and annotated by Robert Munson and colleagues

(http://www.stdgen.lanl.gov/stdgen/bacteria/Haemophilus ducreyi/). In 2005, White et al. studied a panel of *Haemophilus ducreyi* strains to determine whether these strains expressed common virulence determinants. A western blot was performed to examine expression of the known virulence factor Ducreyi Serum Resistance protein A (DsrA) using a monoclonal (MAB) anti-DsrA antibody to 35000 HP. The MAB failed to bind *Haemophilus ducreyi* strains CIP 542 ATCC and HMC 112; however, a polyclonal antiserum made to 35000 HP DsrA antigen was reactive to these strains. Further studies

of five additional virulence factors expressed by the panel of 19 *Haemophilus ducreyi* strains revealed that some strains expressed virulence factors nearly homologous to 35000 HP and others nearly identical to CIP542 ATCC and HMC112. Therefore, strains like 35000 HP were classified as class I (CI) and those like CIP542 ATCC and HMC112 were classified class II (CII).

1.1 Statement of Problem

The sexually transmitted disease chancroid is a common cofactor for the transmission of HIV, specifically in HIV endemic areas. In 2007, Ussher *et al*. reported the first description of non-sexually associated chancroid transmission in children. Four children who had recently visited the island chain of Samoa located in the South Pacific Ocean, presented with chronic lower limb ulcerations. The cause of the ulcers was determined to be *Haemophilus ducreyi* based on isolation of the bacteria by growth on *Haemophilus ducreyi* medium and microscopic examination. Although previous studies had shown that auto-inoculation can occur by skin-to-skin contact with infected genitalia resulting in nongenital skin lesions, no other reports have demonstrated non-genital transmission. According to Ussher et al. (2007) a thorough examination of the children's sexual history and physical condition, revealed that the infections were not acquired by sexual contact. However, lower-limb ulceration was described in other household members, suggesting that close contact between the children may have contributed to disease transmission. To verify that the ulcers were in fact caused by *Haemophilus ducreyi*, three strains isolated from the children were sequenced and

determined to possess a 16 S rDNA sequence that was 100% identical to the *Haemophilus ducreyi* CII strain 33921. This high percentage of sequence identity lead the researchers to conclude that these strains were indeed *Haemophilus ducreyi*. Furthermore, these findings suggest that the three strains may also be CII.

1.2 Statement of Purpose and Hypothesis Tested

Recently, four *Haemophilus ducreyi* isolates were obtained from the lower limbs of children suggesting that *Haemophilus ducreyi* may function as a causative agent of chronic skin ulcers in tropical regions (Ussher et al., 2007). In 2005, White et al. described CII strains based on variations detected in DNA sequences and protein expression of several virulence determinants including DsrA and the Necessary for Collagen Adhesion protein (NcaA). According to Ussher et al. (2007), a notable observation was that the children infected with *Haemophilus ducreyi* had no history of urogenital symptoms or genital ulceration, suggesting that *Haemophilus ducreyi* may not be strictly transmitted person-to-person via sexual contact. These strains were suggested to be CII based solely on 16S rDNA sequence comparison. Therefore, the *goal* of this project was to characterize the four Somoa strains SB 5755, SB 5756, SB 5757, and BE 3145 as CI or CII based on the nucleotide and protein sequences of virulence-associated determinants *dsrA* and *ncaA*. We hypothesized that all four strains would be CII. The *objectives* were as follows:

1. To amplify *dsrA* and *ncaA* by polymerase chain reaction.

2. To obtain the DNA sequence of *dsrA* and *ncaA* in the Samoa strains.

3. To determine similarities between these strains at the nucleotide and amino acid levels through sequence comparison.

CHAPTER 2

LITERATURE REVIEW

2.1 *Haemophilus ducreyi* and Chancroid Infection

2.1.1 Haemophilus ducreyi

Haemophilus ducreyi is a fastidious, gram negative coccobacillus and the etiological agent of chancroid. Gram stain of chancroid secretions reveal pleomorphic coccobacilli in clusters or parallel chains, often described as a "school of fish" (Schmid, 1990; Wu and Huang, 2004). The average bacillus has a length of 1.2 to 1.5 µm and is approximately 0.5 µm in width with rounded ends (Albritton, 1989). The colony morphology of *Haemophilus ducreyi* on solid media is characterized as small, nonmucoid, yellow-grey, semi opaque, adherent colonies (Albritton, 1989). A unique feature of the bacterium is that the colonies can be pushed across the agar surface while remaining intact. *Haemophilus ducreyi* does not cause dissemination infection in immunocompromised or healthy individuals, but it appears to be well adapted to survive in the skin of its obligate human host (Fulcher and Cole, 2006)(Trees and Morse, 1995).

2.1.2 Etiology of Chancroid

Haemophilus ducreyi is a strict human pathogen. Natural infection has not been detected in other animal or environmental reservoirs (Bong, 2002). The bacterium is usually transmitted by sexual contact; however autoinoculation has been reported (Bong, 2002). Chancroid is commonly associated with commercial sex workers and crack cocaine users. Uncircumcised men are at a greater risk than circumcised men for

contracting chancroid (Hand, 1949; Asin 1952; Hammond et al., 1980; Van Howe, 1999; Weiss et al., 2006). It is common that individuals diagnosed with chancroid have visited areas where the disease occurs frequently, such as Africa, Asia, Latin America, and Canada, (Trees and Morse, 1995) . Outbreaks that have occurred in the inner cities in the United States are among those who have been associated with commercial sex workers (Hammond and Slutchuk, 1980).

2.1.3 Clinical Symptoms of Chancroid Infection

Chancroid is a Genital Ulcer Disease characterized by the formation of ulcers that bleed readily. A break in the integrity of the epithelium is the portal of entry for *Haemophilus ducreyi*. To initiate infection, *Haemophilus ducreyi* enters the skin through breaks in the epithelium that occur during intercourse (Sullivan, 1940; Morse, 1989; Spinola et al., 2002). The incubation period of chancroid ranges from 4 to 7 days (Ronald and Albritton, 1984)(Morse, 1989). When this abrasion occurs, the first pathological changes in the form of edematous and swollen epithelial cells are observed (Sullivan, 1940)(Morse, 1989). After an incubation period of 2 to 7 days, chancroid begins with the appearance of Erythematous papules (small, pimple-like bumps) at the entry site, which becomes painful and sharply defined. Within 2 to 3 days of the papual formation, a pustule forms that soon ruptures causing patients to develop 1 to 4 painful ulcers. The ulcers are sharply circumscribed with ragged edges and without indurations. The base of the ulcer has a granular appearance, irregular with many projections and depressions (Morse, 1989). Ulcers that form during natural chancroid infection are

described as having ragged edges and are well circumscribed. The base of the ulcer appears yellow or grey in color and frequently bleeds when scraped (Bong et al., 2002)(Lagergard, 1995). Typically, patients do not seek medical attention until they have had ulcers for 1 to 3 weeks (Hammond and Slutchuk, 1980; Morse, 1989; Chen et al., 1997; Spinola et al., 2002). The actual skin lesions are the likely site of HIV entry (Hammond and Slutchuk, 1980; Morse, 1989; Chen et al., 1997; Spinola et al., 2002).

In 50% of cases, regional lymphadenopathy and swelling of the lymph nodes, accompanies the ulcer-active stage of disease. Lesions are localized to mucosal surfaces or to stratified squamous epithelium. In males, the most common sites of involvement are the distal prepuce, the mucosal surface of the prepuce on the frenulum, or on the coronal sulcus. Lesions are seen less frequently on the shaft of the penis, the glands, or the anus (Gaisin and Heaton, 1975)(Morse, 1989). Fewer symptoms appear in infected women, but the entrance of the vagina is where the majority of lesions are seen. The lesions also include areas of the labia, fourchette, clitoris, and vestibule. Some less painful lesions are seen internally, such as the cervix and vaginal wall (Hammond and Slutchuk, 1980; Ronald and Albritton, 1984; Morse, 1989). Chancroid ulcers are very vascular and the base of the ulcer bleeds easily upon scrapping. Little inflammation is reported on the areas surrounding the infection. However, some ulcers are more painful than others. Because of the appearance of chancroid ulcers, they may often be confused with other causes of GUD such as syphilis and genital herpes, which may result in under diagnosing of chancroid (Morse, 1989).

Haemophilus ducreyi does not disseminate systematically, but due to autoinoculation, extragenital lesions do occur (Bong et al., 2002; Trees and Morse, 1995). The reason for the lack of disseminated disease is unclear. However, the narrow temperature growth of 33°- 35°C, which is the optimal growth of the organism *in vitro*, suggest that temperature sensitivity may affect its ability to spread systematically (Bong et al., 2002)(Trees and Morse, 1995).

2.1.4 Treatment of Chancroid Infection

There are four antibiotic therapies for treatment of chancroid infection. These therapies include azithromycin in a single dose; ceftriaxone intramuscular in a single dose; ciprofloxin orally twice for 3 days; or erythromycin orally three times a day for 7 days. Ulcers that heal with scars can still develop despite successful treatment with antibiotics. Patients with both chancroid and HIV are more likely to fail treatment and/or require longer periods of treatment (Wu and Huang, 2004). HIV infection was found to increase the probability of treatment failure following a single dose of either fleroxacin or ceftriaxone (Wu and Huang, 2004; CDC, 1998).

Most isolates of *Haemophilus ducreyi* worldwide are susceptible to erythromycin and ceftriaxone (Trees and Morse, 1995). However, antimicrobial resistance is extensive and caution is recommended when advocating widespread use of antibiotic for the treatment of chancroid due to the prevalence and spectrum of antimicrobial resistance (Trees and Morse, 1995). Resistance due to plasmid-mediated β-lacatmase is well

documented (Ussher and Wilson, 2007) and *Haemophilus ducreyi* is highly resistant to both tetracycline and penicillin (Ussher and Wilson, 2007).

2.1.5 Chancroid and HIV Infection

Chancroid is one of a number of genital ulcer diseases that is a cofactor for HIV (CDC 1998; Filiatrault et al., 2001). Two mechanisms have been described to explain how the presence of genital ulcers may enhance the transmission of HIV. One mechanism is by increasing the shedding of the virus through ulcers. The other theory is that the presence of the ulcer may increase the susceptibility to HIV infection (Cunningham, A. L. et al., 1985). This increased susceptibility may be due to the interruption of the epithelial barrier and an increase in the number of HIV susceptible cells at the point of entry (Trees and Morse, 1995). The control of GUD has become a major strategy for reading HIV transmission in the HIV and AIDS community. Since chancroid is most common in Africa, effective short course antimicrobial therapy for chancroid is a priority (Tyndall et al., 1994). HIV-infected men tend to have a greater number of ulcers than those who are not infected with HIV (Tyndall et al., 1994). Several recent reports have suggested that prior HIV infection can modify the appearance and clinical course of chancroid. The transmission of HIV is enhanced by common sexually transmitted diseases (STDs). Furthermore, concurrent HIV infection appears to modify the patient's response to therapy.

2.1.6 The Role of The Immune System in Chancroid Infection

In natural chancroid infection, *Haemophilus ducreyi* enters the host through microabrasions during intercourse and primarily stays localized to the site of infection. *Haemophilus ducreyi* naturally infects genital and nongenital skin, regional lymph nodes, and mucosa surfaces. Additionally, *Haemophilus ducreyi* infects mucosal epithelium as well as keratinized stratified squamous epithelium (Hammond and Slutchuk, 1980; Morse, 1989; Spinola et al., 2002).

Histologically, chancroidal ulcers have demonstrated the presence of polymorphonuclear leukocyte (PMN) infiltrate along with fibrin, necrotic material, and erythrocytes in the base of the ulcer. In addition, perivascular and interstitial mononuclear cell infiltrate and PMNs have been located in the dermis of ulcers (King et al., 1998; Margo et al., 1996; Bauer et al., 2006). Macrophages and T cells are the predominate mononuclear cells found; however few B cells and many T cells are infiltrated deep within the dermis (King et al., 1998; King and Gough, 1996; Bauer et al., 2006). Due to the fact that most patients seek medical treatment only late in the ulcerative stage of disease, localization of the bacteria in natural ulcers is limited (Choudhary et al., 1982; Zamzachin et al., 1986; Bauer, 2006).

The development of human and animal chancroid models have shed light on the factors involved in disease development. In the human model of chancroid infection, *Haemophilus ducreyi* colocalizes with PMNs, macrophages, and fibrin within micropustules and with collagen in the dermis at the papular and pustular stages of

disease (Bauer and Spinola, 2000; Bauer et al., 2001; Bauer et al., 2006). The organism remains extracellular in the pustule and dermis and does not colocalize with keratinocytes (Bauer and Spinola, 2000; Bauer et al., 2001; Bauer et al., 2006). Since *Haemophilus ducreyi* localization in chancroid ulcers is similar at the papular and pustular stages of disease in the human model, this suggests that after initiating infection, the disease process does not change from the papular through the ulcerative stages of disease (Bauer and Spinola, 2000; Bauer et al., 2001; Bauer et al., 2006).

Studies conducted to characterize the cellular make up of chancroid ulcers revealed that the ulcers contain fibroblast, epithelial cells, inflammatory cells such as polymorphonuclear leukocytes, macrophages, lymphocytes, and *Haemophilus ducreyi* (King and Gough, 1996; Dutro and Wood, 1999). A study by Bauer et al. (2001) revealed that *Haemophilus ducreyi* enters the skin through microabrasions and colonizes extracellularly. Phagocytes initiate the immune response and surround the bacteria in micropustules but are not able to clear the organism (Vakevainen et al., 2003; Bauer et al., 2006). The presence of the bacteria causes the micropustules to mature into PMN-filled, fibrinous ulcers, which allow *Haemophilus ducreyi* to replicate and prevent clearance of the bacteria. A lack of colocalization with collagen in the dermis was determined to be the main difference between naturally acquired ulcers, papules, and pustules in experimental infection. This finding suggests that the interactions with collagen are important for initiating infection because collagen adhesion is required for pustule formation in the human model of infection (Fulcher, 2006; Bauer et al., 2006).

Palmer et al. (1995) revealed that tissue destruction and the inability of inflammatory cells to survive within the ulcers was consistent with production of toxins by *H. ducreyi*. *Haemophilus ducreyi* produces two toxins including a cell associated hemolysin (Palmer and Munson, 1995; Totten and Norn, 1995) and a secreted cytotoxin (Purven and Lagergard, 1992; Cope and Lumbley, 1997). Lipooligosaccharide (LOS) produced by the organism may also contribute to ulcer formation which enhances the migration of inflammatory cells to the lesion site (Campagnari and Wild, 1991; Elkins and Chen, 1995; Stevens and Porcella, 1996).

2.2. *Haemophilus ducreyi* Virulence Factors

2.2.1 Bacteria Resistance to Immune Killing

In order to initiate infection in a host, pathogenic bacteria must use mechanisms to over come the host's immune system. At least five mechanisms have been identified: (i) resistance to phagocytosis, (ii) resistance to killing by antimicrobial peptides, (iii) the ability to acquire nutrition from the host, survival in the presence of innate and adaptive immunity, and (v) attachment to host tissue and proteins (Sullivan, 1940).

Haemophilus ducreyi, like other bacteria pathogens, has evolved specific mechanisms to avoid host defenses. For example, *Haemophilus ducreyi* survives in the presence of macrophages, both in regional lymph nodes and in chancroid lesions. Wood *et al* (2001) studied the interactions of *Haemophilus ducreyi* with the human macrophage-like cell line, U-937 to understand the mechanism by which *Haemophilus*

ducreyi survives and persists in the presence of phagocytes (Sundstrom and Nilsson, 1976)(Wood et al., 2001). Studies showed that *in vitro*, *Haemophilus ducreyi* strain 35000 adhered to U-937 cells efficiently, but resisted phagocytosis. Within 24 hours, the few *Haemophilus ducreyi* bacteria that were phagocytosed were killed by U-937 (Wood et al., 2001). The study also showed that by using a secondary target, opsonized sheep red blood cells, *Haemophilus ducreyi* inhibited Fc-mediated phagocytosis by U-937 macropahges (Wood et al., 2001). This suggests that antiphagocytic activity may also contribute to the establishment of secondary infections often found in chancroid lesions, since macrophages were unable to phagocytose other pathogens that were impaired. Furthermore, antiphagocytosis might also be a mechanism for survival of *Haemophilus ducreyi* in inguinal lymph nodes (Wood et al., 2001).

Haemophilus ducreyi also expresses two toxins and two large secreted proteins that function as cytotoxins. The cytolethal distending toxin CDT is composed of the CdtA, CdtB, and CdtC proteins. This toxin adheres to host cells and translocates the CdtB protein into the cell which functions as a DNase and cuases cell death. A hemolytic cytotoxin (*hhdA* and *hhdB*) is also produced by *Haemophilus ducreyi* and has been determined to cause cytopathic effects to human foreskin fibroblasts and epithelial cells (Bozue et al., 1999). The large supernatant proteins (LspA and LspB) have also been shown to be cytoxic to immune cells and protect *Haemophilus ducreyi* from phagocytosis (Hansen, 2006).

Secondly, in addition to resisting killing by phagocytes, many bacteria have developed resistance against other components of the immune system. The innate immune system responds to infection by deploying PMNs, epithelial cells, and macrophages which secrete antimicrobial peptides (APs) (Mount et al., 2010). APs are mostly small, cationic peptides that are secreted in the extracellular milieu and have both bactericidal and chemotatic properties (Jenssen and Hamill, 2006)(Mount et al., 2010). Cationic APs are attracted to the anionic bacterial cell membrane where they lyse the bacteria cell membrane. Most APs recruit PMNs, T cells, macrophages, and immature dendritic cells to the site of bacterial infection, serving as a bridge between innate and adaptive immunity (Niyonsaba and Ogawa, 2004) (Mount et al., 2010). Bacterial pathogens have evolved many mechanisms to resist killing by APs such as enzymatic inactivation of APs, electrostatic repulsion by the addition of positively charged residues on the surface, and expression of transporters that remove APs before they can attack the cell membrane (Kraus and Peschel, 2006)(Mount et al., 2010).

Recent studies have shown that the *Haemophilus ducreyi* genome includes homologs of previously described transporter genes in nontypeable *Haemophilus influenza* that are sensitive to antimicrobial peptides. (Altschul et al., 1997)(Mount et al., 2010). The sensitive to antimicrobial peptides (*sap*) genes encode the Sap influx pump, which confers resistance to APs in several Gram-negative pathogens, such as *Salmonella entercia*, nontypeable *Haemophilus influenzae*, and *Proteus mirabilis* (Lopez-Solanilla and Garcia-Olmedo 1998)(Mason and Munson 2005)(Mount et al., 2010). Mount et al. (2010) demonstrated that the *sapA* open reading frame is present in both class I (CI) and

class II (CII) *Haemophilus ducreyi* strains (strain classes are discussed in section 2.3) Minor variations exist in the ORFs of CI strains but relatively greater variations exist between *sapA* ORFs of CI and CII strains (Bauer et al., 2009; Mount et al., 2010). The conservation of SapA among CI and CII clinical isolates of *Haemophilus ducreyi*, coupled with the conserved resistance may be a conserved mechanism in *Haemophilus ducreyi*. PCR amplification of other genes in the *sapABCD* operon further suggest that the operon structure may be conserved among CI and CII strains (Mount et al., 2010).

Thirdly, many pathogenic bacteria are unable to produce important nutritional requirements for survival. Therefore, another important mechanism developed by these bacteria is the ability to obtain nutritional supplements, such as iron, from their host. Invading bacteria must gain access to these sources to survive and initiate disease (Albritton, 1989; Lee. B.C., 1991; Elkins et al., 1998). Host iron is sequestered by several mechanisms. *Haemophilus ducreyi* requires X factor hemin for its growth; however, it is unable to synthesize hemin. Therefore, *Haemophilus ducreyi* acquires heme from its only known host, humans. Hemin is found in several forms such as hemoglobin, free hemin, or bound to carrier proteins such as albumin. Host heme is comprised of compounds such as transferrin, lactoferrin, heme, and hemoglobin that represent an important source of iron (Otto et al., 1992; Elkins et al., 1998). *Haemophilus ducreyi* expresses the outer membrane hemoglobin receptor, HgbA, which is required for virulence. Human and animal models of chancroid infections revealed that HgbA deficient mutants were unable to initiate infection (Elkins and Chen, 1995).

Additionally, pathogenic bacteria use multiple strategies to resist killing by serum complement. In 1995, Lagergard et al. (Lagergard, 1995) suggested that the classical pathway of the immune system plays an important role in the killing of *H. ducreyi.* Further studies demonstrated that *in vivo* survival of *Haemophilus ducreyi* is dependent upon the bacteria's ability to resist killing by complement in Normal Human Serum. *Haemophilus ducreyi* expresses two outer membrane proteins, DsrA and Ducreyi lectin A (DltA) that function to resist serum killing. DsrA is a multifunctional protein that mediates serum resistance (Elkins and Morrow, 2000) and is necessary and sufficient for virulence in the human experimental model of chancroid infection (Bong and Throme, 2000). Abdulah et al. (2005) found that DsrA may confer resistance by inhibiting attachment of the IgM antibody to the bacteria surface, thereby preventing initiation of the complement cascade. Leduc et al. (2004) found that DltA also influences serum susceptibility by interfering with the C' of the complement cascade (Leduc et al., 2004). Studies with a DltA deficient mutant in the human model of chancroid revealed that although the formation of papules and pustules was not completely halted, the DltA mutant was attenuated in its ability to cause infection (Leduc et al., 2004).

Finally, the mechanism by which *Haemophilus ducreyi* colonizes the skin is a major focus of study. *Haemophilus ducreyi* must have the ability to adhere to tissues, cells, and extracellular matrix (ECM) proteins to initiate infection. Three factors have been identified that aid in attachment. Lipooligosaccaride (LOS), a lipid containing polysaccharide expressed on the surface of *Haemophilus ducreyi*, has been shown to play a role in adherence of *Haemophilus ducreyi* to human foreskin fibroblast and

kerotinocytes (Rietschel et al., 1996; Xu et al., 2004; Zughaier, 2004; Lundqvist, 2009). Another membrane bound protein, DsrA, has been shown to be necessary for adhesion to host cells and ECMs and is necessary and sufficient for ulcer formation. Finally, NcaA was shown to bind Type I collagen in the dermis and is required for infection in the pig and human models of chancroid infection (Rietschel et al., 1996; Xu et al., 2004 ; Zughaier, 2004; Lundqvist, 2009)

2.2.2 Oligomeric Coiled Adhesion Proteins

The oligomeric coiled adhesion (Oca) protein family consists of a group of surface-exposed multifunctional proteins. This family of proteins includes the *Yersinia* adhesion YadA, UspA proteins of *Moraxella catarrhalis,* DsrA and necessary for collagen adhesion A (NcaA). YadA and UspA proteins function as eukaryotic cell adhesins (Cole and Kawula, 2002). Hoiczyk et al. (2000) proposed that these proteins are capable of forming oligomers, 'lollipop' shaped structures on the cell surface. These structures are composed of an N-terminal head domain, and a C-terminal anchoring domain. The C-terminal domain of YadA has been shown to be serum resistant (Roggenkamp and Ackermann, 2003), and the N-terminal domain confers binding to extra cellular matrix (ECM) such as fibronectin (Tertti and Skurnik, 1992).

2.2.3 Ducreyi Serum Resistance Protein A

The homology of DsrA and UspA suggested that DsrA might function as a possible *Haemophilus ducreyi* adhesion. Cole et al. (2002) demonstrated that DsrA is responsible for *Haemophilus ducreyi* binding of vitronectin and attachment to

karatinocytes (Cole et al., 2002). Furthermore, DsrA confers binding to the ECM proteins fibronectin and vitronectin and to HaCat keratinocytes (Cole et al., 2002; Leduc et al., 2009). DsrA mediates protection from the activity of complement by preventing deposition of serum bactericidal immunoglobulin M (IgM) at the surface of *Haemophilus ducreyi* (Abdullah et al., 2005; Leduc et al., 2009).

Further studies have determined that the DsrA protein is a member of the trimeric autotransporter adhesion (TAA) family of proteins. Autotransporters have three domains: an N-terminal signal peptide, a passenger or effector domain, and a C-terminal translocator or β domain (Cotter et al., 2006; Leduce et al., 2009). In TAAs the translocator domain is formed by the interactions between the C-terminal domains of three monomers, each monomer contributes 4 strands to the 12-strand β barrel of the TAA homotrimer (Leduce et al., 2009).

2.2.4 Necessary for Collagen Adhesion Protein A

NcaA is an outer membrane protein with an amino acid sequence similar to Oca protein in *Haemophilus ducreyi*, DsrA*, Yersinia enterocolitica*, YadA, *Eshericha coli*, Eib, and *Moraxella catarrhalis*, UspA proteins (Fulcher and Cole, 2006). Fulcher et al. (2006) determined the importance of NcaA in *Haemophilus ducreyi* pathogenesis using the swine and human experimental models of chancroid. In the *in vitro* experiments conducted by Fulcher et al. (2006), NcaA did not mediate adherence to cultured skin cells nor did it confer resistance to complement-mediated killing. *E. coli* expressing NcaA from *Haemophilus ducreyi* was tested to determine if NcaA was sufficient to confer

adherence to type I collagen (Fulcher and Cole, 2006). The results of the fluorescently labeled and incubated microplated wells coated with type I collagen indicated that NcaA is both necessary and sufficient to elicit specific association with type I collagen (Fulcher and Cole, 2006). In the experimental model using swine, the *ncaA* mutant was recovered from fewer lesions than the wild type. In the human volunteer model, pustules were unable to form suggesting that NcaA is required for pustual formation. Thus, making NcaA the seventh *Haemophilus ducreyi* protein shown to be essential for virulence in the native human host (Al-Tawfig et al., 2000; Fortney et al., 2000; Bong et al., 2001; Spinola et al., 2003; Janowicz et al., 2004; Fulcher and Cole, 2006).

2.3 Two Class Strains of *Haemophilus ducreyi*

Comparison of the N-termini of DsrA from these strains revealed little sequence homology, while the C-terminal domains were nearly identical. This finding suggested that the DsrA protein from strains CIP 542 ATCC and HMC 112 was antigenically different than that of strain 35000 HP. Based on these data, 35000 HP was termed class I (CI) and CIP 542 ATCC and HMC 112 were termed class II (CII). Likewise, DsrA from 35000 HP was designated $DsrA_I$, and DsrA from CIP 542 ATCC and HMC 112 was designated $DsrA_{II}$ (White et al., 2005).

To determine the prevalence of NcaA among $DsrA_I$ and $DsrA_{II}$ expressing strains, a panel of 19 *Haemophilus ducreyi* strains was examined in a Western blot with an anti-rNt-$NcaA_I$ antiserum. The antiserum of NcaA recognized the oligomeric form

(approximately 80 kDa) of the NcaA protein from CI but did not recognize either the oligomeric or monomeric (approximately 33-kDa) from CII strains. Sequence comparison of NcaA from 35000HP (CI) and CIP 542 ATCC (CII) revealed strongly conserved C-termini, with little conservation within the N-termini. A comparison of the NcaA sequence from CIP 542 ATCC strains showed 100% identity with in CII. This difference explained the lack of reactivity to CII NcaA with anti-rNt-$NcaA_I$, since this N-terminal region was the source of immunogen used for antibody production of anti-r-$NcaA_I$. Therefore strains expressing $DsrA_I$ also expressed $NcaA_I$, and those expressing $DsrA_{II}$ also expressed $NcaA_{II}$ (White et al., 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Strains and Media

Haemophilus ducreyi strains used in this study are listed in Table 3.1. All strains were maintained by subculture on chocolate agar plates (CAP) containing 1X GGC (0.1% glucose, 0.01% glutamine, and 0.026% cysteine) (Totten and Stamm, 1994), and 5% fetal bovine serum (Fetal Plex, Gemini Scientific, West Sacramento, CA). Strains were grown at 34.5°C in 5% CO_2 for 18-24 hours (CI strains) or 36-48 hours (CII strains). Frozen stocks were prepared by streaking bacteria for isolation on CAP and growing under conditions described above. A single colony was selected and streaked for lawn growth on CAP. The bacteria were then transferred to *Haemophilus ducreyi* freeze media (3g Trypticase, 25 ml glycerol and 75 ml of H_2O) with 10% glycerol and stored at -80°C.

Table 3.1. *Haemophilus ducreyi* Strains Used in This Study

Strain Name	Strain Class	Location/Year of Isolation	Source (Reference)
35000HP	Class I	Winnipeg (1975)	S. Spinola
HMC112	Class II	CDC (1984)	P. Totten (Totten, 1994)
SB5755	Unknown	Somoa Island, South Pacific (2007)	Ussher (Ussher, 2007)
SB5756	Unknown	Somoa Island, South Pacific (2007)	Ussher (Ussher, 2007)
SB5757	Unknown	Somoa Island, South Pacific (2007)	Ussher (Ussher, 2007)
BE3145	Unknown	Somoa Island, South Pacific (2007)	Ussher (Ussher, 2007)

3.2 Amplification and Sequencing of *dsrA* in *Haemophilus ducreyi* Strains

To determine whether *Haemophilus ducreyi* strains obtained from Samoa possessed *dsrA* identical to that in *Haemophilus ducreyi* CI or CII strains, primers designed by White et al. (2005) were used to amplify the *dsrA* open reading frame. Briefly, primer 42 (approximately 280 bp upstream) and primer 43 (approximately 285 bp downstream) were designed using the genome sequence of strain 35000HP (www. stdgen. lanl. gov). PCR was performed using primer 42 and primer 43 (Figure 3.1 and Table 3.2) at 100 pmol each, Master Mix (1X Go-taq DNA polymerase, dNTPs [200 µM each], 1.5 mM $MgCl_2$; Promega, Madison, WI) and whole *Haemophilus ducreyi* cells as DNA template according to the manufacturer's instruction. The PCR was performed under the following conditions: a single denaturation at 95°C for 5 min and 30 cycles, each consisting of 1 min denaturation at 95°C, annealing at 50°C for 2 min, and extension at 72°C for 2 min. Equal volumes of each sample was loaded onto an 8% agarose gel and subjected to electrophoresis. A 1,343 bp fragment of DNA was amplified from several colonies of strains SB5755, SB 5756, SB 5757, and BE3145, HMC112, and 35K.

Following PCR amplification of *dsrA*, DNA sequencing was performed. To prepare samples for sequencing, the PCR products were purified using the DNA Wizard Genomic Preparation Kit (Promega, Madison, WI). Next, approximately 30 ng of the purified DNA was mixed with 100 pmol of either primer 42 or primer 43 in a fresh tube. Samples were submitted to Eurofins MWG Operon (www.operon.com/products/sequencing/) for sequence determination.

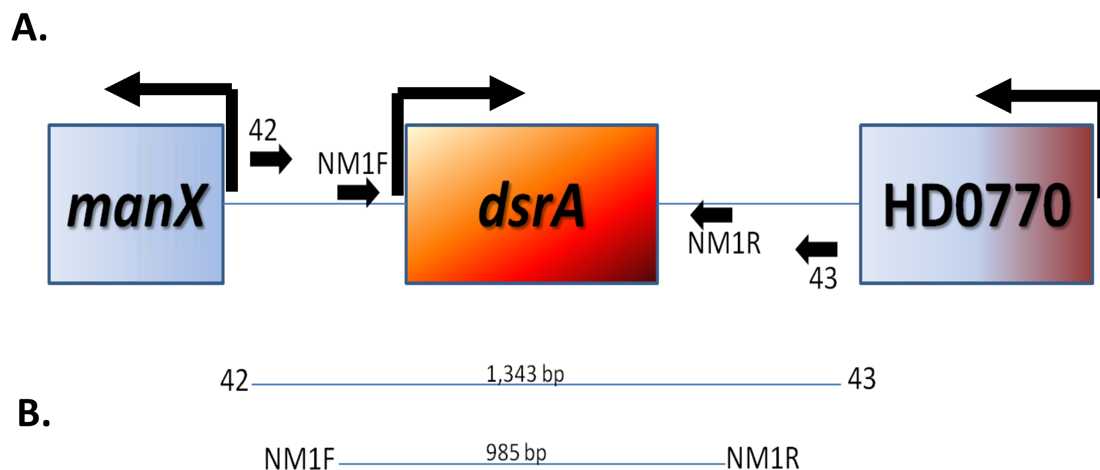

Figure 3.1. Diagram of *dsrA* and Expected PCR Products
(A) The *dsrA* open reading frame is represented by an orange shaded box. The partial *manX* open reading frame is represented by a light blue box upstream of *dsrA*; the partial HD0770 is represented by a blue shaded box downstream of *dsrA*. Curved black arrows indicate the polarity of each gene. Numbered black arrows indicate the direction and location of primers used for PCR and sequencing. (B) The sizes of the expected PCR products are represented by solid blue lines. Open reading frames are not drawn to scale.

Table 3.2. List of *dsrA* Specific Primers Used in This Study

Name	Class	Oligo Sequence	Source (Reference)
dsrA 42	I	CTAGGTCTAGATGCCTTGCTCTTAATGACG	(White, 2005)
dsrA 43	I	GCCCAAGCTTTAAAAGCACATAAACAAGCG	(White, 2005)
NM1F	I	GGAGTGGACCAGGACAGCATTCAGTGAAT	This study
NM1R	I	CAGCAAAAGCTATAACAAAGATAAATAAG	This study

Although primers 42 and 43 worked to amplify *dsrA* in a PCR reaction, only poor quality sequence data was obtained with these primers.

Since high quality sequence data was not obtained using primers 42 and 43, an alternate set of PCR primers flanking *dsrA* was designed. Primer NM1F (approximately 175 bp upstream) and primer NM1R (approximately 102 bp downstream) (Figure 3.1 and Table 3.2) were designed using the genome sequence of strain 35000HP (www.stdgen.lanl.gov). PCR was performed according to the manufacturer's instruction. Reactions consisted of primers NM1F and NM1R at 100 pmol each, Master Mix (1X Go-taq DNA polymerase, dNTPs [200 µM each], 1.5 mM $MgCl_2$; Promega, Madison, WI) and whole *Haemophilus ducreyi* cells as DNA template. PCR was performed under the following conditions: a single denaturation at 95°C for 5 min and 30 cycles, each consisting of 1 min denaturation at 95°C, annealing at 54°C for 2 min, and extension at 72°C for 2 min; and a final cycle of 72°C for 10 minutes. A 985 bp fragment of DNA was amplified from several colonies of strains SB5755, SB 5756, SB 5757, and BE3145, HMC112, and 35000 HP.

3.3 Amplification and Sequencing of *ncaA* in *Haemophilus ducreyi* Strains

To determine whether *Haemophilus ducreyi* strains obtained from Samoa possess *ncaA* identical to that in *Haemophilus ducreyi* CI or CII strains, primers were designed 273 bp upstream (*ncaA*1; GCGATGGCAGTTCCTACTCC) and 201 bp downstream

(*ncaA*2; GGCTAATCACCTGAGCATCA) of the *ncaA* open reading frame (Figure 3.2). PCR was performed according to the manufacturer's instruction using primer *ncaA*1 and

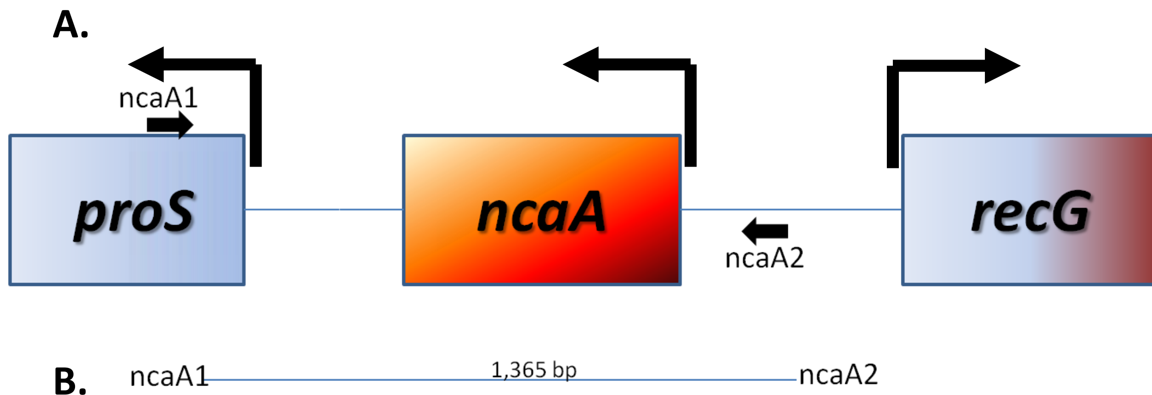

Figure 3.2. Diagram of *ncaA* and Expected PCR Product
(A) The *ncaA* open reading frame is represented by an orange shaded box. Two house-keeping genes, *pros* represented by a light blue box and *recG* represented by a blue shaded box, are located upstream and downstream of the *ncaA* open reading frame, respectively. Curved black arrows indicate the polarity of each gene. Numbered black arrows specify the direction and location of primers used for PCR and sequencing. (B) The size of the expected PCR product is represented by a solid blue line. Open reading frames are not drawn to scale.

primer *ncaA*2 at 100 pMol each, Master Mix (1X Go-taq DNA polymerase, dNTPs [200 µM each], 1.5 mM $MgCl_2$) (Promega, Madison, WI) and whole *Haemophilus ducreyi* cells as template DNA. The PCR was performed under the following conditions: a single denaturation at 94°C for 1 min, and 30 cycles each consisting of 1minute denaturation at 94°C, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes; and a final cycle of 72°C for 10 minutes. A 1,365 bp fragment of DNA was

amplified from several colonies of strains SB 5755, SB 5756, SB 5757, and BE 3145, HMC112, and 35000 HP.

Following PCR amplification of *ncaA*, DNA purification and sequencing was performed as described above for *dsrA*, except that approximately 30 ng of the purified DNA was mixed with 10 pmol of either primer *ncaA*1 or primer *ncaA*2. Samples were submitted to Eurofins MWG for sequence determination.

3.4 Analysis and Alignment of DsrA nucleotide and Protein Sequences

DNA sequences were obtained electronically from Eurofins MWG in an ABI format. To view, edit and perform BLAST (Basic Local Alignment Search Tool) searches with the nucleotide sequences, FinchTV (www.geospiza.com/Products/finchtv/), a free web-based DNA sequencing chromatogram trace viewer was used. Sequence alignments and phylogenetic trees were constructed with the free web-based Molecular Evolutionary Genetics Analysis (MEGA4) program (http://www.megasoftware.net/). Neighbour-joing and bootstrap were used to calculate the confidence intervals for branch lengths. Finally, BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and BoxShade (http://www.boxshade.sourceforge.net) were used to generate protein lineups and determine sequence identity and similarity.

CHAPTER 4

RESULTS

In 2005, White et al. (2005) reported the characterization of two classes of *Haemophilus ducreyi* based on amino acid sequence comparison of virulence determinants, including *dsrA* and *ncaA*. *Haemophilus ducreyi* strains categorized as CII expressed DsrA and NcaA proteins that were nearly 100% identical within the strain class. Conversely, comparison of CII DsrA and NcaA to CI strains revealed a high degree of variation at the N-terminal ends of the proteins; while the C-terminal ends were nearly identical. In this study, to characterize strains SB 5755, SB 5756, SB 5757, and BE 3145 as CI or CII, the N-terminal variable regions of *dsrA* and *ncaA* were examined.

4.1 Amplification of *dsrA* in *Haemophilus ducreyi* Samoa Strains

Previous studies by White et al. (2005) demonstrated that *dsrA* in CI and CII strains could be amplified using PCR primers *dsrA* 42 and *dsrA* 43, designed to CI strain 35000 HP (Figure 3.1). Therefore, to amplify *dsrA* from *Haemophilus ducreyi* strains 35000 HP, HMC112, SB 5755, SB 5756, SB 5755 and BE 3145, whole cells were subjected to PCR and agarose gel electrophoresis. As predicted, an approximately 1.3 kb DNA product containing *dsrA* was successfully amplified (Figure 4.1A). Interestingly, noticeable differences in the DNA yield (band intensity) was observed. The HMC112 DNA fragment was approximately four to five times brighter than the PCR product in the other strains tested when stained with Ethidium Bromide. Furthermore analysis by agarose gel electrophoresis revealed that SB 5757, BE 3145, and HMC 112

migrated slightly higher than SB 5755 and SB 5756, suggesting sequence variation between these strains.

A second set of PCR primers, NM1F and NM1R, was designed to amplify *dsrA* in *Haemophilus ducreyi* strains (Table 3.2). In comparison to primer 42 and primer 43, these primers were designed 105 bp and 183 bp closer to the *dsrA* start and stop codon, respectively. As expected, a 985 bp product was obtained as shown in figure 4.1B. Similar to results obtained with *dsrA42* and *dsrA43*, variation in DNA yield between the strains was observed. The BE 3145 DNA fragment was approximately three times more intense than the other strains tested. Furthermore, variation in DNA fragment migration on the agarose gel was observed. The *dsrA* products from SB 5756 and SB 5757 migrated more slowly than SB 5755, BE 3145 and 35000 HP, suggesting sequence variation between these strains.

4.2 Strains SB 5755, SB 5756, SB 5757 and BE 3145 Possess Class I DsrA

White et al. (2005) showed that the complete sequence of the *dsrA* open reading frame in classes I and II could be obtained using primers *dsrA* 42 and *dsrA* 43. Therefore, these primers were used for PCR and sequencing. Examination of the deduced nucleotide sequences with FinchTV revealed that poor sequence data was acquired using these primers. Specifically, the length of a successful sequence read is

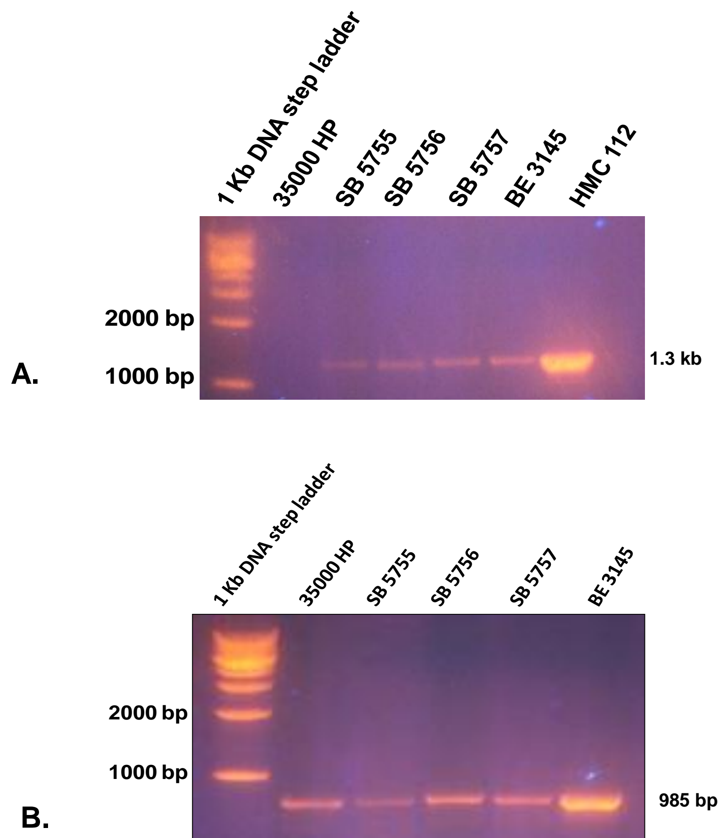

Figure 4.1. Amplification of *dsrA* in SB 5755, SB 5756, SB 5757 and BE 3145
(A) PCR reactions performed using primers *dsrA* 42 and *dsrA* 43 produced the expected 1.3 kb PCR product in all strains tested, except 35000 HP. Previous PCR reactions indicate a positive result for 35000 HP (data not shown here). (B) An approximately 985 bp DNA fragment was obtained from all strains using primers NM1F and NM1R. All PCR samples were subjected to electrophoresis using a 0.8 % agarose gel. Strain names are listed above. Molecular weight standards are indicated to the left and amplified DNA fragment lengths are indicated to the right.

expected to result in the determination of 600-700 nucleotides. The lengths of the sequence reads obtained with primers *dsrA* 42 and *dsrA* 43 were 100-200 nucleotides (data not shown).

Since high quality sequence data could not be obtained using primers *dsrA* 42 and *dsrA* 43, primers NM1F and NM1R were designed and used in subsequent PCR and sequencing reactions. Approximately 600 nucleotides were obtained from all strains except SB 5757. Several attempts to repeat the sequencing reaction with strain SB 5757 resulted in poor quality sequence data (data not shown); therefore this strain was omitted from subsequent analyses.

To characterize *dsrA* in *Haemophilus ducreyi* stains SB 5755, SB 5756, and BE 3145, nucleotide and protein alignments were constructed using MEGA4 and visualized using BoxShade (Figure 4.2 and 4.3). The DNA sequence lineup consisted of 521 nucleotides of the N-terminus variable region, beginning with the ATG start of the *dsrA* coding sequence. As demonstrated in figure 4.2, the N-terminus of *dsrA* in the Samoa strains was similar to the N-terminus of *dsrA* in CI strain 35000 HP.

```
35000dsrA     1   ATGAAAATTAAATGTTTAGTTGCCGTAGTGGGATTAGCTTGTTCTACTATTACAACAATG
SB56dsrA      1   ATGAAAATTAAATGTTTAGTTGCCGTAGTGGGATTAGCTTGTTCTACTATTACAACAATG
SB55dsrA      1   ATGAAAATTAAATGTTTAGTTGCCGTAGTGGGATTAGCTTGTTCTACTATTACAACAATG
BE3145dsrA    1   ATGAAAATTAAATGTTTAGTTGCCGTAGTGGGATTAGCTTGTTCTACTATTACAACAATG
consensus     1   ATGAAAATTAAATGTTTAGTTGCCGTAGTGGGATTAGCTTGTTCTACTATTACAACAATG

35000dsrA    61   GCTCAGCAGCCGCCAAAGTTTGCTGGAGTATCTTCTTTGTATAGCTATGAGTATGACTAT
SB56dsrA     61   GCTCAGCAGCCGCCAAAGTTTGCTGGAGTATCTTCTTTGTATAGCTATGACTATGACTAT
SB55dsrA     61   GCTCAGCAGCCGCCAAAGGTTGCTGGAGTATCTTCTTTGTTTAGCTATGACTATGACTAT
BE3145dsrA   61   GCTCAGCAGCCGCCAAAGTTTGCTGGAGTATCTTCTTTGTATAGCTATGAGTATGACTAT
consensus    61   GCTCAGCAGCCGCCAAAGtTTGCTGGAGTATCTTCTTTGTaTAGCTATGA TATGACTAT

35000dsrA   121   GGTAAGGGTAAATGGACTTGGTCTAATGAAGGCGGTTTCGATATTAAAGTGCCAGGGATT
SB56dsrA    121   GGTAAGGGTAAATGGACTTGGTCTAATGAAGACGGTTTCGATATTAAAGCGCCAAGGATT
SB55dsrA    121   GGTAAGGGTAAGATGACATGGTCTACTGGACACGGTTTCGATTTTAACCCGCCAAGGATT
BE3145dsrA  121   GGTAAGGGTAAATGGACTTGGTCTAATGAAGACGGTTTCGATATTAAAGCGCCAAGGATT
consensus   121   GGTAAGGGTAAatgGACtTGGTCTAaTGaAgaCGGTTTCGATaTTAAagcGCCAaGGATT

35000dsrA   181   AAAATGAAGCCAAAAGAATGGATTTCTAAACAGGCTACTTATCTTGAATTACAGCATTAT
SB56dsrA    181   AAAATGAAGCCAAAAGAATGGATTTCTAAACAGGTTACTTATCTTAAATTACAGCATTAT
SB55dsrA    181   AAAACGACGACATAAGAATGGATTTCTAC-CAGGCTACTTATCTAAAACCACAGCATTTT
BE3145dsrA  181   AAAATGAAGCCAAAAGAATGGATTTCTAAACAGGTTACTTATCTTAAATTACAGCATTAT
consensus   181   AAAAtGAaGcCAaAAGAATGGATTTCTAaaCAGG TACTTATCTtaAAttACAGCATTaT

35000dsrA   241   ATGCCTTATACTCCTGTTCTCGTGACAT---ATGCTCCTGGCGTTTCTCCTAGCCCTATA
SB56dsrA    241   ATGCCTTATACTCCTGTTCTCNNGACATCTGACGTTCCTCCTCCTCCTCCTAGCTCTATA
SB55dsrA    240   ATGCCTCATACTCCTGTTCT-AAGACAT------CTAACGTTCCTCCTCCTAGCTCTATA
BE3145dsrA  241   ATGCCTTATACTCCTGTTCTCGTGACAT---CTGACGTTCCTCCTCCTCCTAGCTCTATA
consensus   241   ATGCCTtATACTCCTGTTCTcgtGACAT   atgctcct ctccTcCTCCTAGCtCTATA

35000dsrA   298   CTGTTATATCCGATGTCTGATCCTGATCAACTTGGAATAAATCGGCAGCAGCTGAAATTG
SB56dsrA    301   CTGTTATATCCGATGTCTGATCCTGATCAACTTGGAATAAATCGGCAGCAGCTGAAATTG
SB55dsrA    293   CCGTTATATCCCATGTCTGATCCTGAACACCATGGAATATTTCCCCAGCCGCTGAAATAC
BE3145dsrA  298   CTGTTATATCCGATGTCTGATCCTGATCAACTTGGAATAAATCGGCAGCAGCTGAAATTG
consensus   301   CtGTTATATCCgATGTCTGATCCTGAtCAaCtTGGAATAaaTCggCAGCaGCTGAAATtg

35000dsrA   358   AATTTGTATAGTTATTTTAACGATTTAAGACACGATTTTAAATTAAAAGTTCTTGATGCA
SB56dsrA    361   AATTTGTATAGTTATTTTAACGATTTAAGACACTATTTTAAATTAAATGTTCTTGATGCA
SB55dsrA    353   AATTTGTATAATTATTTTAACAATTTAAGACACTTTTTTAAATTAAATGTTCTTGATGCA
BE3145dsrA  358   AATTTGTATAGTTATTTTAACGATTTAAGACACGATTTTAAATTAAATGTTCTTGATGCA
consensus   361   AATTTGTATAgTTATTTTAACgATTTAAGACAC aTTTTAAATTAAAtGTTCTTGATGCA

35000dsrA   418   CGTATTTCCAAAAATAAACAAAA
SB56dsrA    421   CGTATTTCCAAAAATAAACAAAA
SB55dsrA    413   CGTATTTCCAAAAATAAACAAAA
BE3145dsrA  418   CGTATTTCCAAAAATAAACAAAA
consensus   421   CGTATTTCCAAAAATAAACAAAA
```

Figure 4.2. Alignment of *dsrA* nucleotide sequences from CI and SB 5755, SB 5756 and BE 3145. The nucleotide sequences of *dsrA* are compared. Strain names are listed to the left. Black, shaded regions indicate identity. Gray boxes indicate residues that are similar to the column consensus. Numbers to the left of the figure indicate the nucleotide number within the strains.

```
35000dsrA     1  MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYEYDYGKGKWTWSNEGGFDIKVPGI
SB5755dsrA    1  MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYEYDYGKGKMTWSTGHGFDFNPPGI
SB5756dsrA    1  MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYDYDYGKGKWTWSNEDGFDIKAPRI
BE3145dsrA    1  MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYEYDYGKGKWTWSNEDGFDIKAPRI
consensus     1  MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYeYDYGKGKwTWSnedGFDikaP I

35000dsrA    61  KMKPKEWISKQATYLELQHYMPYTPVLVTYAPGVSP-SPILLYPMSDPDQLGINRQQLKL
SB5755dsrA   61  KTKTKEWISKQATYHKITAFYASYSCSRDICTFLLS-SSIPLYPMSDPEHHGIFPQPLKY
SB5756dsrA   61  KMKPKEWISKQVTYLKLQHYMPYTPVLVTSDVPPPPPSSILLYPMSDPDQLGINRQQLKL
BE3145dsrA   61  KMKPKEWISKQVTYLKLQHYMPYTPVLVTSDVPPPP-SSILLYPMSDPDQLGINRQQLKL
consensus    61  KmKpKEWISKQ TYlklqhympytpvlvtsdvpppp SsilLYPMSDPdqlGInrQqLKl

35000dsrA   120  NLYSYFNDLRHDFKLKVLDARISKNKCNIDTISKYLLELGTYLDDSYRMVEQNT
SB5755dsrA  120  NLYNYFNNLRHYFKLYVIDARISQNKHTIDTISKHLLQLGTYLDGSYRMVEQNT
SB5756dsrA  121  NLYSYFNDLRHYFKLNVLDARISKNKCNIDTISKHLLKLGTYLDGSYRMVEQI-
BE3145dsrA  120  NLYSYFNDLRHDFKLNVLDARISKNKCNIDTISKHLLELGTYLDGSYRMVEQNT
consensus   121  NLYsYFNdLRH FKLnVlDARISkNKqnIDTISKhLLeLGTYLDgSYRMVEQnt
```

Figure 4.3. The DsrA in *Haemophilus ducreyi* Strains Isolated from Samoa Is Similar to DsrA$_{I}$. The DsrA amino acid sequences from 35000 HP and SB 5755, SB 5756 and BE 3145 are compared. Strain names are listed on the left. Black, shaded regions indicate identity. Gray boxes indicate residues that are similar to the column consensus. Numbers to the left of the figure indicate the amino acid number within the strains.

SB 5755, SB 5756, and BE 3145 were 90%, 96% and 97% identical to *dsrA* in 35000 HP, respectively. Moreover, alignment of the predicted protein sequences demonstrated that SB 5755, SB 5756, and BE3145 were 71%, 87%, and 92% identical to 35000 HP (Figure 4.3). However, comparison with HMC112 $dsrA_{II}$ revealed a high degree of variability between the N-terminal regions. SB 5755, SB 5756, and BE 3145 were 28%, 29% and 28% identical to *dsrA* in HMC 112, respectively (Figure 4.4). SB 5755 was 7% identical and 17% similar, SB 5756 was 7% identical and 21% similar, and BE 3145 was 5% identical and 17% similar to $dsrA_{II}$ (Figure 4.5). Taken together, these results indicate that *dsrA* in strains SB 5755, SB 5756 and BE 3145 is CI.

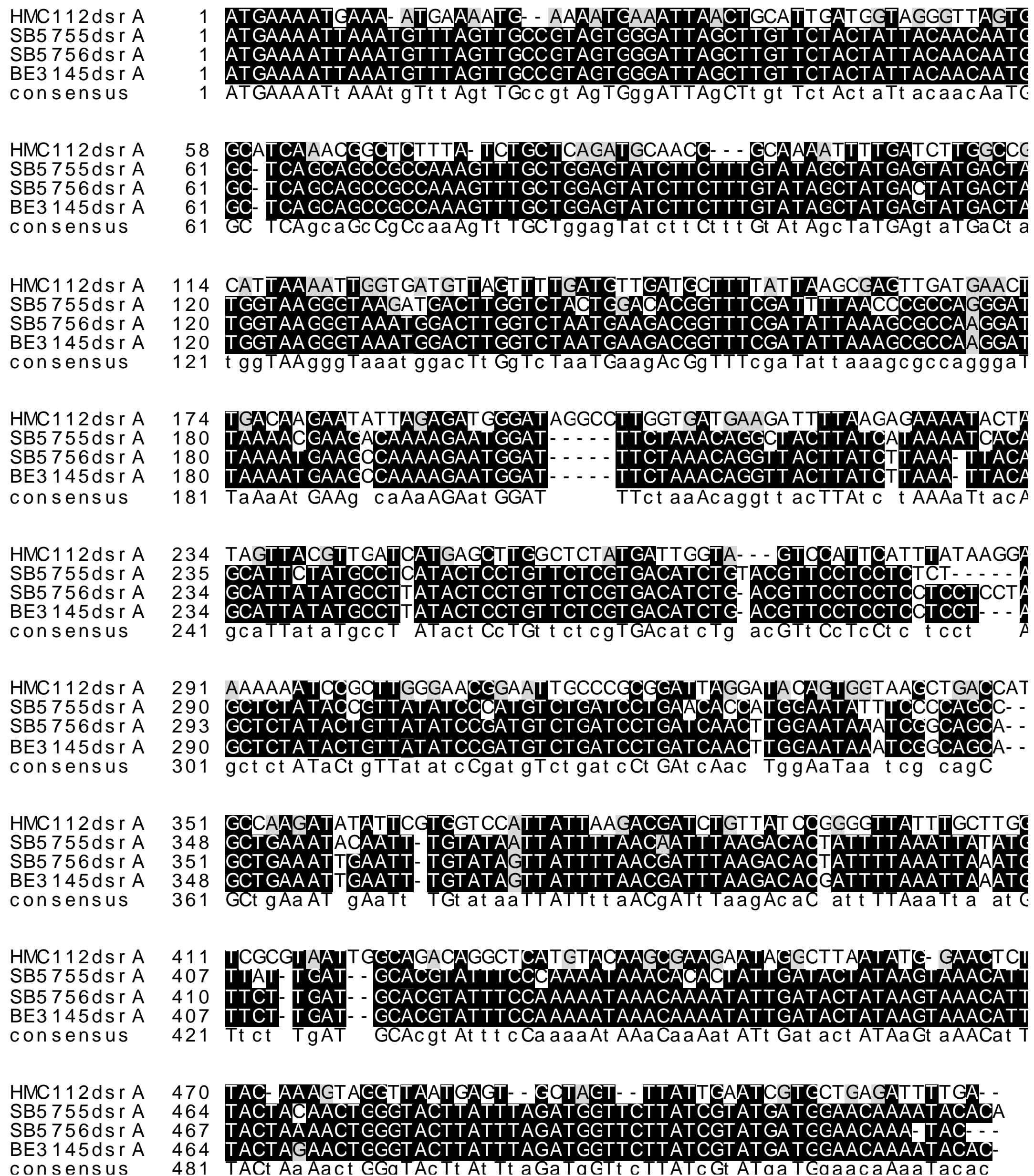

Figure 4.4. ***dsrA*** **in** ***Haemophilus ducreyi*** **Strains Isolated from Samoa Is Not** $\boldsymbol{dsrA_{II}}$**.** The nucleotide sequences of *dsrA* from 35000 HP and SB 5755, SB 5756 and BE 3145 are compared. Strain names are listed on the left. Black, shaded regions indicate identity. Gray boxes indicate residues that are similar to the column consensus. Numbers to the left of the figure indicate the nucleotide number within the strains.

```
HMC112dsrA    1  MKMKMKMKMKLTALMVGLVASNGSLSAQMQPQNFDLGRIKIGDVSFDVDAFIKRVDELDK
SB5755dsrA    1  ------MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYEYDYGKGKMTWSTGHGFD
SB5756dsrA    1  ------MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYDYDYGKGKWTWSNEDGFD
BE3145dsrA    1  ------MKIKCLVAVVGLACSTITTMAQQPPKFAGVSSLYSYEYDYGKGKWTWSNEDGFD
consensus     1        MKiKclvavVGLacStittmAQqpPkfagvsslysydydygkgkwtwsnedgfd

HMC112dsrA   61  NIRDGIGLGDEDFKRKYYSYVDHELGSMIGSPFIYKEK-----------NPLGNGIARGL
SB5755dsrA   55  FNPPGIKTKTKEWISKQATYHKITAF--YASYSCSRDICTFLLSSSIPLYPMSDPEHHGI
SB5756dsrA   55  IKAPRIKMKPKEWISKQVTYLKLQHYMPYTPVLVTSDVPPPPP-SSILLYPMSDPDQLGI
BE3145dsrA   55  IKAPRIKMKPKEWISKQVTYLKLQHYMPYTPVLVTSDVPPPP--SSILLYPMSDPDQLGI
consensus    61  ikap IkmkpkewisKqvtYlklqhympyt vlvtsdvpppp  ssillyPmsdpdqlGi

HMC112dsrA  110  GYSGKLTMPRYIRGPLLRRSVIRGYLLGRVIGRQAHVQAKNRLNMELLQSRLMSASLLNF
SB5755dsrA  113  FPQ-PLKYNLYNYFNNLRHYFKLYVIDARISQNKHTIDTISKHLLQLGTYLDGSYRMMEQ
SB5756dsrA  114  NRQ-QLKLNLYSYFNDLRHYFKLNVLDARISKNKQNIDTISKHLLKLGTYLDGSYRMMEQ
BE3145dsrA  113  NRQ-QLKLNLYSYFNDLRHDFKLNVLDARISKNKQNIDTISKHLLELGTYLDGSYRMMEQ
consensus   121  nrq qLklnlYsyfndLRhyfklnvldaRisknkqnidtiskhlleLgtyldgSyrmmeq

HMC112dsrA  170  AEIL
SB5755dsrA  172  NT--
SB5756dsrA  173  I---
BE3145dsrA  172  NT--
consensus   181  nt
```

Figure 4.5. DsrA in *Haemophilus ducreyi* Strains Isolated from Samoa Are Not Similar to Class II DsrA. Protein alignment of DsrA amino acid sequences from 35000 HP and SB 5755, SB 5756 and BE 3145 are compared to DsrA from HMC 112. Shaded regions indicate the similarities between the strains. Numbers to the left of the figure indicate the amino acid number within the strains.

Phylogenetic analysis of CI, CII, SB 5755, SB 5756 and BE 3145 *dsrA* was performed using neighbour-joing and bootstrap in Mega4. As shown in figure 4.6, 521 nucleotides of SB 5755, SB 5756 and BE 3145 *dsrA* was compared to $dsrA_I$ from 35000 HP and $dsrA_{II}$ from HMC 112 by constructing a phylogenic tree using Mega4 (Figure 4.6). The phylogenic tree showed that the samoa strains are more closely related to 35000 HP than HMC 112.

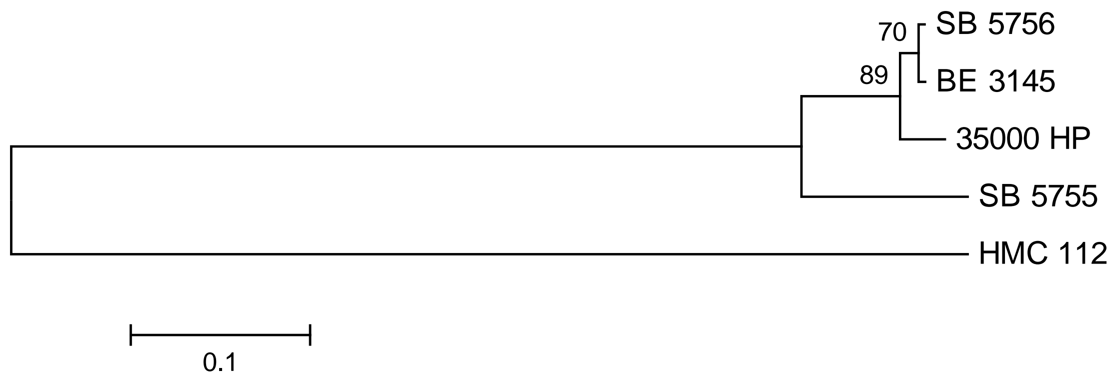

Figure 4.6. Phylogenetic Analysis of *dsrA* in *Haemophilus ducreyi* Samoa Strains. Phylogeny was determined using the neighbor-joining method from an analysis of *dsrA* sequences in *H. ducreyi* strains 35000 HP, HMC 112, SB 5755, SB 5756, and BE 3145. The value on each branch is the estimated confidence limit (expressed as a percentage) for the position of the branch as determined by bootstrap analysis. The scale bar represents differences in nucleotide sequences.

4.3 Amplification of ncaA in *Haemophilus ducreyi* strains

In order to determine whether *ncaA* in strains SB 5755, SB 5756, SB 5755 and BE 3145 was CI or CII, primers *ncaA1* and *ncaA2*, were designed to CI strain 35000 HP (Figure 3.2). Therefore, whole cells were subjected to PCR as described in materials and methods. As predicted, a 1.3kb DNA product containing *ncaA* was successfully amplified in all strains (Figure 4.7). A noticeable difference in DNA yield between the strains was detected. The BE3145 DNA fragment was approximately three times more intense than the other strains tested, indicating a greater DNA yield.

4.4 Analysis of ncaA in Samoa strains

Previous studies determined that the C termini of $NcaA_I$ (35000 HP) and $NcaA_{II}$ (CIP 542 ATCC) were highly conserved; however little conservation within the N termini was detected. Conversely, a comparison of the $NcaA_{II}$ from strains CIP 542 ATCC, DMC64, DMC111, SSMC71, and HMC112 showed 100% identity between CII strains (White et al., 2005). In this study, several attempts to determine the sequence of *ncaA* from Samoa strains SB 5755, SB 5756, SB 5757 and BE 3145 were unsuccessful. Due to poor quality of sequence data, characterization of *ncaA* could not be determined.

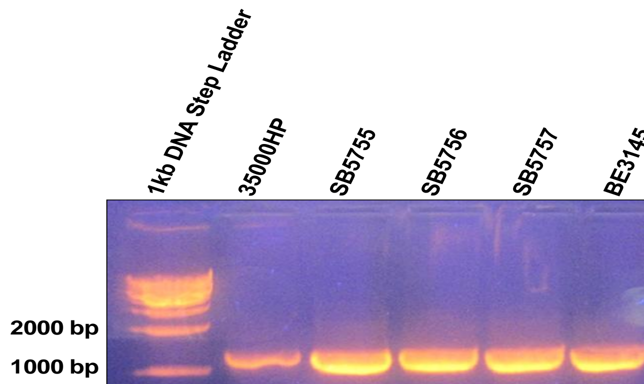

Figure 4.7. Amplification of *ncaA* in *Haemophilus ducreyi* Samoa Strains. PCR reactions performed using primers *ncaA*1and *ncaA*2 produced the expected 1.3 kb PCR product in all strains tested, except HMC 112. Previous PCR reactions indicate a positive result for HMC 112 (data not shown here). All PCR samples were subjected to electrophoresis using a 0.8 % agarose gel. Strain names are listed above each gel. Molecular weight standards are indicated to the left of the gels and amplified DNA fragment lengths are indicated to the right of each gel.

CHAPTER 5

DISCUSSION AND CONCLUSION

Haemophilus ducreyi is a fastidious, gram negative coccobacillus and the etiological agent of chancroid. In nature, this strict human pathogen has not been detected in other animal or environmental reservoirs (Trees and Morse, 1995; Bong, Bauer et al., 2002; Spinola, et al., 2002). *Haemophilus du*creyi is most commonly transmitted by sexual contact; however autoinoculation has been reported. Individuals diagnosed with chancroid have commonly visited areas where the disease occurs frequently and HIV is endemic.

In a 2005 report by White et al., it was suggested that *Haemophilus ducreyi* strains could be characterized into two groups based on amino acid sequence and expression of known virulence factors. Strains possessing proteins similar to parent strain 35000 HP were termed CI, while strains possessing proteins similar to strains CIP 542 ATCC and HMC112 were termed CII. Two virulence determinants, *dsrA* and *ncaA*, previously determined to be required for infection in the human and animal models of chancroid, were thoroughly examined. Comparison of the N-termini of $DsrA_I$ and $DsrA_{II}$ revealed little homology, while the C-terminal domains were nearly identical. In addition, $NcaA_I$ and $NcaA_{II}$ were composed of divergent N-termini, but highly conserved C-terminal domains.

In 2007 three children who had recently visited the island chain of Samoa, located in the South Pacific Ocean, presented with chronic lower limb ulcerations. The cause of the ulcers was determined to be *Haemophilus ducreyi* based on isolation of the bacteria

by growth on *Haemophilus ducreyi* media and microscopic examination. These strains were determined to possess a 16S rDNA sequence that was 100% similar to 16S rDNA in a CII strain. We hypothesized that these strains, SB 5755, SB 5756, SB 5757, and BE 3145, would be CII based on the report by Ussher et al. (2007). Our objectives were to amplify *dsrA* and *ncaA* by polymerase chain reaction, obtain the nucleotide and amino acid sequences, and determine the percent identity in comparison to the CI parent strain 35000 HP, and the CII parent strain HMC112.

In this report, we demonstrate for the first time that *dsrA* in strains SB 5755, SB 5756, and BE 3145 should be categorized as $dsrA_I$. Amino acid sequence analysis of SB 5755, SB 5756, and BE 3145 revealed that these strains were closely related to 35000 HP *dsrAI*, 71%, 87%, and 92%, respectively, as determined by pairwise alignment (Figure 4.2). Additionally, nucleotide sequence alignment of *dsrA* obtained with primer NM1F revealed that identity was highly conserved in the N- terminus in Samoa strains SB 5755 (90%), SB 5556 (96%), and BE 3145 (97%) when compared to CI 35000 HP and not CII HMC 112. As demonstrated in figure 4.6, phylogenetic analysis of 35000 HP $DsrA_I$ and HMC 112 $DsrA_{II}$, in relation to the DsrA in Samoa strains SB 5755, SB 5756, and BE 3145, supports the idea that the Samoa strains were more closely related to 35000 HP than HMC 112.

While $DsrA_{II}$ is nearly identical among CII strains, immunoblot analysis of DsrAI revealed some variation among CI strains White et al. (2005). In this study, PCR DNA fragments amplified with primers NM1F and NM1R revealed a noticeable difference in migration on a 0.8% agarose gel (Figure 4). This finding suggested that some variability

may exist in the *dsrA* open reading frame, the DNA flanking *dsrA*, or both. Comparison of the DsrA N-terminus among the Samoa strains revealed that SB 5755, SB 5756 and BE 3145 were 90-98% identical to one another.

Examination of *ncaA* in SB 5755, SB 5756, SB 5757 and BE 3145 proved challenging. Although PCR was successful using primers *ncaA*1 and *ncaA*2 (Figure 4.5), DNA sequencing results were very poor for all strains except BE 3145 and could not be used for analysis. Interestingly, preliminary data for BE 3145 resulted in a lengthy sequence read; however the sequence chromatogram revealed multiple areas of *ncaA* that appeared to be a 'mixture' of CI and CII sequences (data not shown). Studies of NcaA in these strains are ongoing.

Although Ussher et al. (2007) reported that strains isolated from the children were sequenced and determined to possess a 16 S rDNA sequence that was 100% identical to the *Haemophilus ducreyi* CII strain 33921, the data reported in this study does not support the hypothesis that theses strains are CII. Sequence analysis of the DsrA N-termini revealed the strains to be identical to that of the CI strain 35000 HP. In conclusion, this study demonstrates that Samoa strains SB 5755, SB 5756, and BE 3145 belong to CI based on the *dsrA* nucleotide and protein sequences.

BIBLIOGRAPHY

Abdullah, M. , I. Nepluev, and Afonina, G. (2005). "Killing of Dsra Mutants of *Haemophilus Ducreyi* by Normal Human Serum Occurs Via the Classical Complement Pathway and Its Initiated by Immunoglobulin M Binding " Infect. Immun. **Vol. 73**: 3431-3439.

Al-Tawfig, J. A. , A. C. Thornton, and Katz, B.P. (2000). "An Isogenic Hemoglobin Receptor-Deficient Mutant of *Haemophilus Ducreyi* is Attenuated in the Human Model of Experimental Infection. " J. Infect. Dis **Vol. 181**: 1049-1054.

Albritton, W. L. (1989). "Biology of *Haemophilus Ducreyi* " Microbiol **Vol. 53**: 377-389.

Altschul, S. , T. Madden, and Schaffer, A. (1997). "Gapped BLAST And PSI-BLAST ; A New Generation of Protein Database Search Programs. " Nucleic Acids Res. **Vol. 25**: 3389-3402.

Asin, J. (1952). "Cahncroid : A Report of 1402 Cases " Am J Syph Gonorrhea Vener Dis **Vol. 36**: 483-487.

Bauer, M. , C. Townsend, and Doster, R. (2009). "A Fibrinogen- Binding Lipoprotein Contributes to the Virulence of *Haemophilus Ducreyi* in Humans " J. Infect. Dis. **Vol. 199**: 684-692.

Bauer, M. E. , M. P. Goheen, and Townsend, C. (2001). "*Haemophilus Ducreyi* Associates With Phagocytes, Collagen, and Fibrin and Remains Extracellular Throughout Infection of Human Volunteers. " Infect. And Immunity **Vol. 69**: 2549-2557.

Bauer, M. E. And S. M. Spinola (1999). "Binding of *Haemophilus Ducreyi* to Extracellular Matrix Proteins. " Infection And Immunity **Vol. 67**: 2649-2652.

Bauer, M. E. and S. M. Spinola (2000). "Localization of *Haemophilus Ducreyi* at the Pustular Stage of Disease in the Human Model of Infection " Infect. And Immunity **Vol. 68**: 2309-2314.

Bauer, M. E. , C. A. Townsend, and Ronald, A.R. (2006). "Localization of *Haemophilus Ducreyi* in Naturally Acquired Chancroidal Ulcers. " Microbes Infect **Vol. 8**: 2465-2468.

Bong, C. T. , R. E. Throme, and Fourtney, B.P. (2001). "Dsaa-Deficient Mutant of *Haemophilus Ducreyi* is Impaired in Its Ability to Infect Human Volunteers " Infect. Immun. **Vol. 69**: 1488- 1491.

Bong, C. T. , M. E. Bauer, and Spinola, S.M. (2002). "*Haemophilus Ducreyi*: Clinical Features, Epidemiology, and Prospects for Disease " Microbes Infect **Vol. 4**: 1141-1148.

Bozue, J. , M. Tullius, and Wang, J. (1999). "*Haemophilus Ducreyi* Produces a Novel Sialyltransferase. " Journal Of Biological Chemistry **Vol. 274**: 4106-4114.

Brunham, R.C. and F.A. Plummer. (1990). "A General Model of Sexually Transmitted Disease Epidemiology and its Impilication for Control." Med. Clin. North. Am **Vol. 74**: 1339-1352.

Campagnari, A. A. and L. M. Wild (1991). "Role of Lipooligosaccharides in Experimental Dermal Lesions Caused by *Haemophilus Ducreyi*. " Infection And Immunity **Vol. 59**: 2601-2608.

CDC (1998). "HIV Prevention Through Early Detection and Treatment of Other Sexually Transmitted Diseases- United States. Recommendations of the Advisory Committee for HIV and STD Prevention." Centers For Disease Control And Prevention, Atlanta, GA.

Chen, C. , K. J. Mertz, and Spinola, S.M. (1997). "Comparison of Enzyme Immunoassays for Antibodies to *Haemophilus Ducreyi* in a Community Outbreak of Chancroid in the United States. " J. Infect. Dis **Vol. 175**: 1390-1395.

Choudhary, B. P. , S. Kumari, and Bhatia, R. (1982). "Bacteriological Study of Chancroid " Indian J. Med. Res **Vol. 76**: 379-385.

Cole, L. E. and T. H. Kawula (2002). "The *Haemophilus Ducreyi* Serum Resistance Antigen DsrA Confers Attachment to Human Keratinocytes " Infection And Immunity **Vol. 70**: 6158-6165.

Cope, L. D. and S. Lumbley (1997). "A Diffusible Cytotoxin of *Haemophilus Ducreyi*. " Proc. Natl. Acad. Sci. **Vol. 94**: 4056-4061.

Cotter, S. E. and N. K. Surana,. (2006). "Trimeric Autotransporters Require Trimerization of the Passenger Domain for Stability and Adhesion Activity " J. Bacteriol **Vol. 188**: 5400-5407.

Cunningham. A. L. , R. R. Turner, and Miller, A.C. (1985). "Evolution of Recurrent Herpes Simplex Lesions: an Immunohistologic Study. " J. Clin. Invest **Vol. 75**: 226-233.

Dickerson, M. D. , J. Johnston, and Delea, T.E. (1996). "The Casual Role for Genital Ulcer Disease as a Risk for Transmission of Human Immunodeficiency Virus: an Application of the Bradford Hill Criteria. " Sex Trans Dis **Vol. 23**: 429-440.

Dutro, S. M. and G. E. Wood (1999). "Prevalence of, Antibody Response to, and Immunity Induced by *Haemophilus Ducreyi* Hemolysin. " Infection And Immunity **Vol. 67**: 3317-3328.

Dyer, J. R. , P. Kazembe, and Vernazza, P.L. (1998). "High Levels of Human Immunodeficiency Virus Type 1 in Blood and Semen of Seropositive Men in Sub-Saharan Africa. " Journal Of Infectious Disease **Vol. 177:** 742-1746.

Elkins, C. And C. Chen (1995). "Characterization of the Hgba Locus Encoding a Hemoglobin Receptor from *Haemophilus Ducreyi*. " Infection And Immunity **Vol. 63**: 2194-2200.

Elkins, C. and J. Morrow (2000). "Serum Resistance in *Haemophilus Ducreyi* Requires Outer Membrance Protein DsrA. " Infection And Immunity **Vol. 37**: 192-206.

Elkins, C. , P. A. Totten, and Olsen, B. (1998). "Role of the *Haemophilus Ducreyi* Ton System in Internalization of Heme from Hemoglobin " Infect. And Immunity **Vol. 66**: 151-160.

Filiatrault, M. , J. R. S. Munson, and Campagnari, A.A. (2001). "Genetic Analysis of a Pyocin-Resistant Lipooligosaccharide (LOS) Mutant of *Haemophilus Ducreyi*: Restoration of Full-Length LOS Restores Pyocin Sensitivity. " Journal Of Bacteriology **Vol. 183**: 5756-5761.

Fortney, K. R. , R. S. Young, and Bauer, M.E. (2000). "Expression of Peptidoglycan-Associated Lipoprotein is Required for Virulence in the Human Model of *Haemophilus Ducreyi* Infection. " Infect. Immun. **Vol. 68**: 6441-6448.

Fulcher, R. A. and L. E. Cole (2006). "Expression of *Haemophilus Ducreyi* Collagen Binding Outer Membrane Protein NcaA is Required for Virulence in Swine and Human Challenge Models of Chancroid. " Infection And Immunity **Vol. 74:** 2651-2658.

Gadkari, D. A. and T.C. Quinn (1998). " HIV-1 DNA Shedding in Genital Ulcers and Its Associated Risk Factors in Pune, India." Acquir Immune Defic Syndr. **Vol. 18:** 277-281.

Gaisin, A. and C. L. Heaton (1975). "Chancroid: Alias the Soft Chancre. " Int. J. Dermatol **Vol. 14**: 188-197.

Hammond, G. W., M. Slutchuk, and Scatliff, J. (1980). "Epidemiologic, Clinical, Laboratory, and Therapeutic Features of an Urban Outbreak of Chancroid in North America. " Rev. Infect. Dis. **Vol. 2**: 867- 879.

Hand, E. A. (1949). "Circumcision and Venereal Disease " Arch Dermatol Syphilol **Vol. 36**: 483- 487.

Hoiczyk, E. and A. Roggenkamp (2000). "Structure and Sequence Analysis of Yersinia Yada and Moraxella Uspa Reveal a Novel Class of Adhesins. " EMBO **Vol. 19**: 5989-5999.

Janowicz, D. , K. R. Fortney, and Katz, B.P. (2004). "Expression of the Lspa1 and Lspa2 Proteins By *Haemophilus Ducreyi* is Required for Virulence in Human Volunteers " Infect. Immun. **Vol. 72**: 4528-4533.

Jenssen, H. and P. Hamill (2006). "Peptide Antimicrobial Agents " Clinical Microbiol **Vol. 19**: 491- 511.

King, R. , S. H. Choudhri, and Nasio, J. (1998). "Clinical and in situ Cellular Response to *Haemophilus Ducreyi* in the Presence or Absence of HIV Infection. " Int. J. STD AIDS **Vol. 9:** 531-536.

King, R. and J. Gough (1996). "An Immunohistochemical Analysis of Naturally Occuring Chancroid. " Infect. Dis. **Vol. 174**: P: 427-430.

Kraus, D. and A. Peschel (2006). "Molecular Mechanisms of Bacterial Resistance to Antimicrbial Peptides " Curr. Top. Microbiol. Immunol. **Vol. 306**: 231-250.

Kreiss, J.K., R. Coombs, and Plummer, F. (1989). "Isolation of Human Immunodeficiency Virus from Genital Ulcers in Nairobi Prostitues." Infect. Dis. **Vol. 160:** 380-384.

Lagergard, T. (1995). "*Haemophilus Ducreyi*: Pathogenesis and Protective Immunity. " Microbiol **Vol. 3**: 87-92.

Leduc, I. , P. Richards, and Davis, C. (2004). "A Novel Lectin, Dlta, is Required for Expresssion of a Full Serum Resistance Phenotype in *Haemophilus Ducreyi* " Infection And Immunity **Vol. 72**: 3418- 3428.

Leduce, I. , B. Olsen, and Elkins, C. (2009). "Localization of the Domains of the *Haemophilus Ducreyi* Trimeric Autotransporters DsrA Involved in Serum Resistance and Binding to the Extracellular Matrix Proteins Fibronectin and Vitronectin. " Infection And Immunity **Vol. 77**: 657-666.

Lee. B. C. (1991). "Iron Sources For *Haemophilus Ducreyi* " Journal Of Microbiol **Vol. 34**: 317-322.

Lopez-Solanilla, E. and F. Garcia-Olmedo (1998). "Inactivation of the Sap A to SapF Locus of Erwinia Chrysanthemi Reveals Common Features in Plant and Animal Bacterial Pathogensis " Plant Cell **Vol. 10**: 917-924.

Lundqvist, A., J. Klubler-Kielb, Teneberg, S., Ahlman, K., and Lagergard, T. (2009). "Immunogenic and Adjuvant Properties of *Haemophilus Ducreyi* Lipooligosaccharides." Microbes Infect **Vol. 11:** 352-360.

Margo, C. M. , A. N. Crowson, Alfa, M. (1996). "A Morphological Study of Penile Chancroid Lesions in Human Immunodeficiency Virus (HIV)-Positive and Negative African Men with a Hypothesis Concering the Role of Chancroid in HIV Transmission " Hum. Pathol\ **Vol. 27**: 1066-1070.

Mason, K. and J. Munson, R. S. (2005). "A Mutation in the Sap Operon Attenuates Survival of Nontypeable Haemophilus Influenzae in a Chinchilla Model of Otitis Media " Infection And Immunity **Vol. 73**: 599-608.

Morse, S. A. (1989). "Chancroid and *Haemophilus Ducreyi*. " Clin. Microbiol **Vol. 2**: 137-157.

Mount, K. , C. Townsend, and Rinker, S. (2010). "*Haemophilus Ducreyi* SapA Contributes to Cathelicidin Resistance and Virulence in Humans " Infection And Immunity **Vol. 78**: 1176- 1184.

Niyonsaba, F. and H. Ogawa (2004). "Human B-Defensin-2 Functions as a Chemotatic Agent for Tumor Necrosis Factor -X-Treated Human Neutrophils " Immunology **Vol. 111**: 1776-1784.

Otto, B. R. , V. V. Vaght, and MacLaren, D.M. (1992). "Transferrins and Heme-Compounds as Iron Sources Pathogenic Bacteria " Crit. Rev. Microbiol **Vol. 18**: 217-233.

Palmer, K. L. and J. R. S. Munson (1995). "Cloning and Characterization of the Genes Encoding the Haemolysin of *Haemophilus Ducreyi*. " Mol. Microbiol. **Vol 21:** 821-830.

Purven, M. And T. Lagergard (1992). "*Haemophilus Ducreyi*, a Cytotoxin-Producing Bacterium. " Infect. Immun. **60:** 1156-1162.

Rietschel, E. T. , H. Brade, and Holst, O. (1996). "Bacterial Endotoxin: Chemical Constitution, Biological Recognition, Host Response, and Immunological Detoxification " Curr. Top. Microbiol Immunol **Vol. 216**: 39-81.

Roggenkamp, A. and N. Ackermann (2003). "Molecular Analysis of Transport and Oligomerization of the Yersinia Enterocolitica Adhesin Yada. " J. Bacteriol **Vol. 185**: 3735-3744.

Ronald, A. R. and W. L. Albritton (1984). "Chancroid and *Haemophilus Ducreyi* ": 385-393.

Schacker, T., H. Hu, and Ryncarz, A.J. (1998). "Famciclovir for the Suppression of Symptomatic and Asymptomatic Herpes Simplex Virus Reaction in HIV- Infected Persons: A Double-Blind, Placebo Control Trail." Ann Intern Med **Vol. 128**: 21-28.

Schmid, G. P. (1990). "Approach the Patient with Genital Ulcer Disease. " Med Clin North Am **Vol. 74**: 1559-1572.

Spinola, S. M. , M. E. Bauer, and Munson, J.R.S. (2002). "Immunopathogenesis of *Haemophilus Ducreyi* Infection (Chancroid) " Infect. Immun. **Vol. 70**: 1667-1676.

Spinola, S. M. , K. R. Fortney, and Katz, B.P. (2003). "*Haemophilus Ducreyi* Requires an Intact Flp Gene Cluster for Virulence in Humans " J. Infect. Dis. **Vol. 71**: 7178-7182.

Steen, R. and G. Dallabetta (2003). "Genital Ulcer Disease Control and HIV Prevention " Journal Of Clin. Virology **Vol. 29:** 143-151.

Stevens, M. K. and S. Porcella (1996). "A Hemoglobin-Binding Outer Membrane Protein is Involved in Virulence Expression by *Haemophilus Ducreyi* in an Animal Model." Infect. Immun. **64:** 1724-1735.

Sullivan, M. (1940). "Chancroid. " Am. J. Syph. Gon. **Vol. 24**: 482-521.

Sundstrom, C. and K. Nilsson (1976). "Establishment and Characterization of Human Histiocytic Lymphoma Cell Line (U-937) " Int. J. Cancer **Vol. 17**: 565-577

Tertti, R. and T. Skurnik (1992). "Adhesion Protein Yada of Yersinia Species Mediates Binding of Bacteria to Fibronectin. " Infection And Immunity **Vol. 60**: 3021-3024.

Totten, P. A. and D. V. Norn (1995). "Characterization of the Hemolytic Activity of *Haemophilus Ducreyi*. " Infection And Immunity **Vol. 63:** 4409-4416.

Totten, P. A. and W. E. Stamm (1994). "Clear Broth and Plate Media for the Culture of *Haemophilus Ducreyi*. " J. Clin. Microbiol. **Vol. 32**: 2019-2023.

Trees, D. L. and S. A. Morse (1995). "Chacroid and *Haemophilus Ducreyi*: an Update. " Clin. Microbiology Reviews **Vol. 8:** 357-375

Tyndall, M. , E. Agoki, and Plummer, F. (1994). "Single Dose Azithromycin for the Treatment of Cnacroid: a Randomized Comparison With Erythromycin. " Sexually Transmitted Diseases **Vol. 21**: 231-234.

Ussher, J. E. and E. Wilson (2007). "*Haemophilus Ducreyi* Causing Chronic Skin Ulceration in Children Visiting Samoa. " Clinical Infectious Diseases **Vol 44**: 85-87.

Vakevainen, M. , S. Greenberg, and Hansen, E.J. (2003). "Inhibition of Phagocytosis by *Haemophilus Ducreyi* Requires Expression of the Lspa1 and Lspa2 Proteins. " Infect. And Immunity **Vol. 71**: 5994-6003.

Van Howe, R. S. (1999). "Circumcision and HIV Infection: Review of the Literature and Meta-Analysis " International Journal Of STD And AIDS **Vol. 10**: 8-16.

Wasserheit, J. N. (1991). "Interrelationships Between Human Immunodeficiency Virus Infection and Other Sexually Transmitted Diseases. " Sex Trans Dis **Vol. 19**: 61-77.

Weiss, H. A. , S. L. Thomas, Munabi, S.K., and Hayes, R.J. (2006). "Male Circumcision and Risk of Syphili, Chancroid, and Genital Herpes: a Systematic and Meta-Analysis " Sex Transm Infect **Vol. 82**: 101- 110.

White, C. D. And I. Leduce, Olsen, B., Jeter, C., Harris, C., Elkins, C. (2005). "*Haemophilus Ducreyi* Outer Membrane Determinants, Including DsrA, Define Two Clonal Populations. " Infection And Immunity **Vol. 73:** 2387- 2398.

Wood, G. , S. Dutro, and Totten, P.A. (2001). "*Haemophilus Ducreyi* Inhibits Phagocytosis By U-937 Cells, a Human Macrophage-Like Cell Line " Infection And Immunity **Vol. 69**: 4726-4733.

Wu, J. J. and D. B. Huang (2004). "Selected Sexually Transmitted Diseaes and Their Relationship to HIV " Clinics In Dermatology **Vol. 22**: 499-508

Xu, T. , A. Lundqvist, and Ahmed, H.J. (2004). "Interactions of *Haemophilus Ducreyi*: and Purified Cytolethal Distending Toxin with Human Monocyte-Derived Dendritic Cells, Macrophages and CD+4 T Cells. " Microbes Infect **Vol. 6**: 1171-1181.

Zamzachin, G. , U. Gupta, and Singh, O.P. (1986). "A Clinico-Bacteriological Study of "Chancroidal" Ulcer, Treated at AIIMS Hospital, New Delhi. " Indian J. Med. Res **Vol. 76**: 277-283.

Zughaier, S. M., Y. L. Tzeng, and Zimmer, S.M. (2004). "Neisseria Meninigitis Lipooliogosaccharide Structure -Dependent Pathway " Infection And Immunity **Vol. 72**: 371-380.