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Role of SodC in Heme Acquisition in *Haemophilus ducreyi*

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Role of SodC in Heme Acquisition in *Haemophilus ducreyi*

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ABSTRACT

Haemophilus ducreyi expresses outer-membrane receptors for both heme (*TdhA*) and hemoglobin (*HgbA*), but no mechanism yet has been identified for the transport of heme from the periplasm to the cytoplasm.

SodC protein in *H. ducreyi* binds to heme in addition to exhibiting superoxide dismutase activity. We hypothesized that SodC is involved in heme uptake in *H. ducreyi*. An isogenic *H. ducreyi sodC* mutant was constructed. The generation time of the *sodC* mutant was increased compared to the wild type strain (35000). The mutant was complemented in *trans* with either wild type *sodC* (sod+heme+), *sodCH86E* with wild type dismutase activity but less heme binding activity (sod+heme-) or with *sodCL81FH82QD83G* with diminished dismutase activity but wild type heme binding activity (sod-heme+). The generation times of the complemented strains showed no significant difference when grown under limited heme concentration. We conclude that SodC is unlikely to be involved in heme uptake in *H. ducreyi*.

DEDICATION

To those who made BIHE a reality beyond our dreams

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
ANOVA	Analysis of Variances
Anti-SodC	Rabbit Polyclonal Antibody Against *H. ducreyi 35000* SodC
BP	Binding Protein
BPD	Binding Protein Dependent
CA	Chocolate Agar
CFU	Colony Forming Units
HBP	Heme Binding Protein
HCM	Human Challenge Model
HIV	Human Immunodeficiency Virus
Hr	Hour
i.m.	Intramuscular
MCS	Multiple Cloning Site
Min	Minute
OD	Optical Density
OM	Outer Membrane
OMP	Outer Membrane Protein
OMR	Outer Membrane Receptor
ORF	Open Reading Frame
p.o.	Per Oral
PAGE	PolyAcrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHBP	Periplasmic Heme Binding Protein
psod	*H. ducreyi sodC* promoter
SBP	Solute Binding Protein
SDM	Semi Defined Media
SDS	Sodium Dodecyl Sulfate

Sec	Second
SEM	Standard Error Mean
SodC	Cu, Zn Super-Oxide Dismutase
SSD	Statistically Significant Difference
STD	Sexually Transmitted Disease
TD	Transmembrane Domain
TDRM	Temperature Dependent Rabbit Model
TEF	Translation Elongation Factor
TM	Melting Temperature
UV	Ultra Violet
WHO	World Health Organization
Wks	Weeks

CHAPTER ONE

INTRODUCTION

1.1 Chancroid

Chancroid (reviewed in [1-4]) is a sexually transmitted genital ulcerative infection caused by the Gram-negative coccobacillus, *Haemophilus ducreyi*, which is a strict human pathogen. Chancroid was initially described by Ricord in 1838 and was first differentiated from syphilis by Bassereau in 1852 [5].

Transmission occurs by direct person to person contact [2] through small breaks in the epithelium created during sexual intercourse [6]. Based on studies on human volunteers, the inoculum size required for infection is about 50 to 100 Colony Forming Units (CFU) with a 70% chance of pustule formation [7]. Natural infection occurs in genital and nongenital skin, mucosal surfaces and regional lymph nodes [8]. The incubation period is not well characterized in women, but ranges from 2 to 35 days with a mean of 7 days in men [9]. The first lesion is a small tender papule surrounded by a zone of erythema. The papule progress to a pustule and within 2 to 3 days erodes to a shallow ulcer as a result of friction [10, 11]. An ulcer, typically, has ragged, nonindurated margins with an erythematous edge and a granulomatous base covered by a necrotic purulent exudate, which bleeds when scraped [3, 12]. The ulcer is usually quite painful in males, but frequently is not noticed by females [1]. Within 1 week of ulceration, painful inguinal lymphadenopathy (chancroid buboes) appears in about 40% of men and

25% of women [5, 12]. The lymph nodes may rupture through the overlaying skin after fluctuation [10].

H. ducreyi has not been shown to cause systemic or opportunistic infection, or to become more invasive in immuno-compromised hosts [1, 4]. Both cell and humoral mediated immunity occur in response to infection with *H. ducreyi* [1]; however re-infection and serial autoinfection can occur [2].

1.1.1 Epidemiology

Chancroid has been endemic in most parts of the world well into the 20th century [13]. However, several decades before discovery of sulfa drugs and penicillin, chancroid started to decline steadily in Europe and North America [13]. Reported rates of chancroid in United States have decreased by more than 80-fold between 1947 and 1997 [13]. Three factors have played major roles in this decline: 1) improvement of social and public health conditions, 2) introduction of antibiotics, and 3) shifting patterns of prostitution [13].

Today, chancroid is uncommon in North America and Europe, but sporadic outbreaks do occur [14]. These epidemics have usually been associated with prostitution, cocaine use (exchange of drug for sex) and importation [4, 5, 9, 15]. Chancroid is endemic in tropical climates [2]. In developing countries with low socioeconomic standards, particularly in Africa, Asia and Latin America, chancroid is one of the main causes of genital ulcer disease [2, 4, 5, 15]. Southern, central and eastern Africa have the highest prevalence and prostitutes appear to be the reservoir [2, 4, 5, 15], because in

females, chancroid can be asymptomatic, allowing sexual activity to continue [1].

The probability of transmission in a single sexual exposure is about 0.35 (infected male to uninfected female) to 0.30 (infected female to uninfected male) [1, 4]. The incidence of chancroid is up to 25 times higher in males than females and the presence of foreskin appears to render males more susceptible to infection [11].

1.1.2 Chancroid and HIV

Chancroid experienced renewed attention in the late 1980's because of its role, along with the other genital ulcer diseases, in facilitating Human Immunodeficiency Virus (HIV) transmission. In all 18 countries in Africa, Asia and Caribbean where adult prevalence of HIV is higher than 8%, chancroid is common [13]. It has been shown that chancroid plays a role in increasing the incidence of HIV infection [16]. Factors that lead to this phenomenon include: 1) chancroid ulcers act as ports of entry facilitating HIV transmission about 10-300 times [1, 17], 2) chancroid increases susceptibility of epithelial cells to HIV infection via epithelial cell damage [11], 3) chancroid increases the number of cells receptive to HIV in genital secretions (in women with Sexually Transmitted Diseases (STD)) [18], and 4) increasing dissemination of HIV in chancroid positive patients [19, 20]. Thus chancroid increases both HIV susceptibility and HIV infectiousness [21].

In addition, HIV patients have shown an increased number of ulcers with a slower healing rate, though the characteristics of the ulcers were similar to HIV negative patients [22]. Support for the role of chancroid in facilitating HIV transmission has also been

derived from a number of population based therapeutic intervention studies. A general STD treatment program in Tanzania reduced the incidence of HIV infection by 40% [23, 24]. Other studies also confirmed these results by showing a 27%-70% decrease in HIV infection after a general STD treatment [25, 26], supporting the role of chancroid as a cofactor to the increased risk of HIV transmission and infection [17], and thus establishing that the control of chancroid and other STDs would be an effective strategy against HIV spread. The best and probably the first example of this occurred in Thailand which established a 100% condom policy in commercial sex workers in 1989. Within five years, Thailand succeeded in controlling common curable STDs (chancroid declined by 95%) and slowing HIV transmission [13].

1.1.3 Prevention and Control

Hygiene is effective in reducing *H. ducreyi* transmission. During the First World War, simple washing with soap and water within a few hours of sexual exposure was effective in reducing the risk of chancroid [13]. Wide spread use of condoms, male circumcision and post-exposure male hygiene are also important factors [13]. Currently there is no vaccine available for the prevention and control of *H. ducreyi* infection or chancroid [2]. Developing such a vaccine would be highly effective in both prevention and control [2].

Based on biological and epidemiological factors chancroid can be controlled and even eradicated because it is identifiable, both syndromically and etiologically. This helps with the treatment of chancroid and enables more accurate surveillance of the

disease. The ulcers are usually very painful and patients seek medical help; chancroid can be easily cured with a single dose of antibiotics. *H. ducreyi* does not have any non-human reservoir and does not sustain itself outside of the most active human sexual networks [7, 13]. Therefore, a highly focused effort to eliminate *H. ducreyi* infection from those networks can eliminate chancroid in endemic areas [13].

1.1.4 Treatment

Circumcision and saline soaks were the standard therapy for chancroid before the advent of antibiotics [1]. Thereafter, sulfonamides were the first antibiotic agents used with successful treatment [27], followed by β-lactams, tetracycline, streptomycin, kanamycin and chloramphenicol [1, 5]. However, emerging antibiotic resistance has changed these regimens. The current recommended treatment regimen from The World Health Organization (WHO) is erythromycin, 500 mg p.o. (oral), 3 times a day for 7 days (with no confirmed resistance [1], 91% efficacy [28]). The alternate regimens are the following: a single dose of either Ciprofloxacin, 500 mg p.o. (62%-92% efficacy [28]), or Ceftriaxone, 250 mg i.m. (intramuscular) (88%-100% efficacy [28]), or Spectinomycin, 2 g i.m.; and a combination of Trimethoprim (80 mg) and Sulfamethoxazole (400 mg), 2 tablets p.o. twice a day for 7 days (45%-81% efficacy [28]).

The Center for Disease Control and Prevention currently recommends a single dose of Azithromycin, 1 g p.o. (88%-100% efficacy [28]) or Ceftriaxone, 250 mg i.m., as a single dose or Erythromycin, 500 mg p.o., 4 times daily for 7 days; and as an alternate choice Ciprofloxacin, 500 mg p.o., twice a day for 3 days (reviewed in [11]).

1.2 *Haemophilus ducreyi*

Auguste Ducreyi was the first to describe *H. ducreyi* as the etiologic agent of chancroid in 1889 [5]. *H. ducreyi* (reviewed in [1, 4, 29]) is a pleomorphic, Gram-negative bacillus, approximately 1.2-1.5 μm in length and 0.5 μm in diameter [29]. It is non-motile and non-spore forming [29]. In liquid culture *H. ducreyi* colonies are observed as streptobacillary forming parallel chains described as "railroad tracks" [29]. Colonies on solid media are small, nonmucoid, yellow-gray, semiopaque, adherent and can be pushed across the surface intact. On Gram-stain, colonies show typical streptobacillary chaining described as "school of fish" or "fingerprints" [29].

Lenglet was the first to successfully culture *H. ducreyi in vitro* [11]. *H. ducreyi* is a facultative anaerobe and requires hemin (X factor) for growth; however, unlike most of the other species of *Haemophilus*, it does not need nicotinamide adenine dinucleotide (NAD or V factor) [30] due to the presence of a plasmid encoded gene (*nadV*) [31]. It grows well on nutritionally enriched media like chocolatized blood agar or hemoglobin agar with complex supplements such as IsoVitaleX (BBL) or CVA (Gibco). Growth on more defined agar medium containing peptone, glucose, glutamine, cysteine and starch has also been successful [32]. The best condition for growth is at 30 to 33°C in a water saturated environment with the addition of 5% CO_2 and in media with a pH of 6.5 to 7.0 [29]. The doubling time is between 1.8 to 4 hours (hr), which is consistent with the 48-72 hr incubation time required for producing visible colonies [4].

1.2.1 Animal Models

The virulence factors of *H. ducreyi* have been studied extensively both *in vitro* and *in vivo*. *In vivo* models for *H. ducreyi* infection include the Human Challenge Model (HCM) [33, 34], the mouse model [35], the temperature-dependent rabbit model (TDRM) [36], the primate model [37, 38], and the swine model [39], 42].

There are certain biosafety regulations for the HCM and infection is terminated at early stages (pustular) before ulceration [33, 34]. Thus, results from the *in vivo* studies based on the HCM should be interpreted carefully and are not necessarily conclusive.

Considering factors like 1) histopathology of the ulcers, 2) recovery of *H. ducreyi* from the ulcers, and 3) duration of the ulcers and the inoculum size needed to establish ulcers, the TDRM, the primate and the swine models resemble the natural infection better than the mouse model [35-43]. Adding to the above factors the cost, and ease of handling, the TDRM would be the most appropriate model both qualitatively and quantitatively for study of chancroid [35-43]. Rabbits are housed at 15-17 °C to lower their skin temperature, which is thought to mimic the lower temperature of human external genitalia [36]. The only disadvantage of the TDRM is that the infection protects the animal from subsequent homologous infection which is contrary to the natural infection in humans [36].

1.2.2 Virulence Factors

Several studies have been conducted to identify the virulence factors of *H.*

ducreyi. However, these are far from complete and even the roles of the suggested virulence factors are not completely clear. Some of the potential virulence factors of *H. ducreyi* are listed below:

Hemoglobin-binding outer membrane protein (HgbA) is a 100 kDa protein involved in heme uptake from hemoglobin. Its expression is regulated by the level of heme (not iron) in the environment and it has homology to the TonB-dependent family of Outer Membrane (OM) receptors [44-47]. An HgbA isogenic mutant was less virulent than the wild type strain in the TDRM [46] and was attenuated in the HCM [48].

Heme-binding outer membrane protein (TdhA) is a Ton-B dependent OM receptor for heme with homology to five other heme receptors in Gram-negative bacteria [49]. An isogenic mutant of *tdhA* required the same heme concentration as the parent strain *in vitro* [49], which may be due to the presence of HgbA compensating for the absence of TdhA.

Cytotoxins or **Cytolethal Distending Toxins (CTDs)** are soluble proteins (26, 33 and 22 kDa) encoded by a cluster of genes with homology to *cdtABC* in *Escherechia coli* [50-52]. Although, they induce apoptosis and cell cycle arrest *in vitro* [53-58], *in vivo* studies in the TDRM and the HCM have not been conclusive in demonstrating their role in virulence [55, 59, 60].

Hemolysins are two immunogenic proteins of 125 and 61 KDa (HhdA and HhdB) with hemolytic activity [61-63]. Though cytopathicity of the hemolysin has been shown *in vitro* [64, 65], expression of *HhdB* was not essential for virulence in the TDRM and the

HCM [60, 66, 67].

Hemagglutinin is a secreted 260 KDa protein. It is encoded by *lspA1* and has N-terminal homology with *Bordetella pertussis* filamentous hemagglutinin [68]. This gene was transcribed both *in vivo* and *in vitro*, but was not expressed in four avirulent strains [68], thus suggesting its importance in survival of virulent strains.

Lipooligosaccharide (LOS) is a heat stable [69], highly sialylated [70] and immunogenic [71] lipopolysaccharide which lacks the repeating *O*-antigenic side chains, and is therefore called LOS [72, 73]. *H. ducreyi* LOS is able to create dermal lesions in the mouse model [69]. It is involved in the attachment of *H. ducreyi* to the host tissues and cells *in vitro*. It has potent inflammatory activity in the mouse model and in the TDRM [69, 74-80]. Mutant strains were less virulent in the TDRM than the parent strain [36, 77, 78]. In contrast, a LOS mutant was as virulent as the wild type in the early stages of infection in the HCM [80].

Serum resistance factor (DsrA) is an Outer Membrane Protein (OMP) (28-35 kDa) conferring resistance to high levels (up to 50%) of normal human serum [81]. Compared to the wild type, an isogenic *dsrA* mutant was less virulent than the wild type strain in the HCM [82].

Peptidoglycan associated lipoprotein (PAL) is an 18 kDa OMP with homology to peptidoglycan associated lipoprotein in other Gram-negative bacteria [83, 84]. An isogenic PAL mutant was less virulent than the parental strain in the HCM [85].

Major outer membrane proteins (MOMP, OmpA2) are two OMPs (37 to 39

kDa, respectively), with homology to OmpA of *E. coli* [86, 87]. An isogenic MOMP mutant showed the same virulence as the parent strain in the HCM [88], which may have been due to OmpA2 compensating for the absence of MOMP or MOMP may have a role in the later stages of the infection [88].

Pili in *H. ducreyi* are fine and tangled, and the surface located pili are composed mainly of a 24 kDa protein (FtpA), with no homology to other known pilin proteins [89-91]. Despite *in vitro* and *in vivo* studies (TDRM and HCM), the role of pili in virulence has not yet been elucidated [40, 92-94].

Heat shock proteins (HSPs) are four proteins, GroEL (58 kDa), GroES (10-14 kDa), DnaJ (78 kDa) and DnaK (74 kDa), with high homology to other HSPs in Gram-negative bacteria [95-97]. These proteins increase thermo-tolerance and survival in the presence of heat and oxidative stress in the host environment and also have a role in binding of *H. ducreyi* to eukaryotic cells [98, 99].

Cu,Zn Superoxide Dismutase C (SodC) is a 21 kDa periplasmic protein which has been proposed to protect *H. ducreyi* from exogenous superoxide [100-102]. Unlike SodC in other bacteria, *H. ducreyi* SodC binds to heme [103]. The isogenic mutant, though impaired in virulence in the swine model, was as virulent as the parent strain in the HCM [104, 105].

1.2.3 Taxonomic Position

The taxonomic position of *H. ducreyi* has been questioned for a number of years

[4, 6]. It was originally placed in the genus *Haemophilus* because of both its requirement for hemin and guanine-plus-cytosine content that was within the accepted range for *Haemophilus* spp. [4]. Based on DNA hybridization and S1 nuclease treatment, *H. ducreyi* is distantly related to true *Haemophili* such as *H. influenzae* [4]. However based on DNA-RNA hybridization, it is classified in the family *Pasteurellaceae* but a member of cluster 1 instead of 4 which is for true *Haemophili* [4].

The genome of *H. ducreyi* strain 35000 is composed of a single 1.76 Mb chromosome and a total of 1,693 open reading frames (ORFs) have been identified [106]. In a collaboration among The Institute for Systems Biology (http://www.systemsbiology.org) in Seattle, WA; the laboratory of Dr. Robert Munson at the Children's Research Institute (http://www.columbuschildrens.com/cri/munson.shtml), Columbus, OH; and The Ohio State College of Medicine and Public Health, the complete genome sequence of *H. ducreyi 35000* has been determined (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/eub_u.html). The sequence was completed in September 2001, and the final-pass annotation checking is underway at this time (http://www.microbial-pathogenesis.org/H.ducreyi/). Based on BLAST analysis, *H. influenzae* and *Pasteurella multocida* have the closest homologues of 66% of *H. ducreyi* genes [8].

1.2.4 Heme Requirement

As a member of the genus *Haemophilus, H. ducreyi* requires heme (X factor) for growth [30]. Heme and hemin both have the same chemical composition. The difference

in names is related to the oxidative state of the prosthetic group, iron, which is in the reduced state (Fe^{2+}) in heme and in the oxidized state (Fe^{3+}) in hemin [94]. In this document both names have been used interchangeably. The heme requirement of *H. ducreyi* was further substantiated by the porphyrin test showing lack of enzymatic activity needed for conversion of δ-aminolevulinic acid to protoporphyrin which is essential in the hemin biosynthesis pathway [107]. Moreover, *H. ducreyi* also lacks ferrochelatase or heme synthetase, so the organism is unable to catalyze the insertion of iron into the porphyrin ring [32].

The heme requirements of *H. ducreyi* are higher than those of other hemin-requiring bacterial species, with most *H. ducreyi* strains requiring 25-50 μg/ml for growth compared with 1 to 10 μg/ml for *H. haemoglobinophilus* or *H. influenzae* [1].

H. ducreyi may also obtain heme from intracellular sources by invading or destroying host cells [1]. Interestingly, six of the twelve *H. ducreyi* studied virulence factors (Hemoglobin-binding OMP, Heme-binding OMP, Hemolysins, Hemagglutinin, Cytotoxins, CuZn-SodC) are directly or indirectly involved either in heme acquisition or in heme interaction. This indicates the importance of heme metabolism in *H. ducreyi*.

1.3 Importance of Iron for Pathogenic Bacteria

Iron is a necessary growth factor for virtually all bacteria and each bacterial cell contains about 10^5 to 10^6 iron ions [108, 109]. Acquisition of iron can be considered as the major factor that determines whether or not bacteria can survive within the animal

host [110]. Without this ability, bacteria will not be able to grow and would either starve to death or be eliminated by the host immune system [110]. Additionally, the process of withholding iron from bacteria is a known key host defense mechanism [110].

The injection of an iron compound into the animal host is known to enhance the virulence of many pathogenic bacteria [110] including: *Aeromonas* spp., *Clostridium* spp., enterobacteriaceae (*E. coli*, *Klebsiella* spp., *Salmonella* spp.), *Listeria* spp., *Neisseria* spp., *Pasteurella* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Vibrio* spp., *Yersinia* spp., *Mycobacterium avium* and *M. tuberculosis* [110]. This fact is emphasized by the variety of roles iron containing proteins play in bacterial metabolism, such as respiration and electron transport (cytochromes, ferredoxins and other iron-sulfur proteins), activation of oxygen (cytochrome oxidase), degradation of H_2O_2 and O^{2-} (heme-catalase, iron-superoxide dismutase, peroxidase), amino acid and pyrimidine biosynthesis (glutamate synthase, dihydro-orotate dehydrogenase), the citric acid cycle (fumarase, aconitase, succinate dehydrogenase), DNA synthesis (ribonucleotide reductase), nitrogen fixation (nitrogenase), carbon fixation metabolism (methane monooxygenase), oxygen binding (globins), degradation of aromatic compounds (toluate 1,2-dioxygenase), and gene regulation (FNR, SoxR, CooA) [111, 112].

The vast and different roles of iron containing proteins may be explained by their ability to form complexes with oxygen, sulfur and nitrogen ligands. These complexes can readily carry on acid-base and electron transfer reactions. Depending on the protein complexed with iron, a wide range of Fe(III)/Fe(II) redox potentials (+330 to -490 mV) is provided which makes iron an extremely useful redox mediator in biology [108, 111]. In

correlation with the importance of iron in metabolism, the process of withholding iron from invading bacteria has long been known to be of key importance in host defense. Thus bacterial iron uptake mechanisms have been considered a target for the design of new chemotherapeutic agents [110].

Although iron is the fourth most abundant element in nature, it is mostly present in insoluble iron-oxide minerals and is not a freely available nutrient for bacteria. In aerobic environments, iron exists primarily in the oxidized ferric form and, at pH 7, has a solubility of 1.4×10^{-9} M, which is very low [110].

Free uncomplexed ferric ions at physiological conditions precipitate spontaneously as insoluble hydroxides and, in the process, catalyze the formation of damaging free radical species. The propensity of iron to catalytically promote the production of hydroxyl radicals is illuminated by an appreciation of the Haber-Weiss-Fenton reaction:

$$O_2 + Fe^{2+} \leftrightarrow O_2^{-} + Fe^{3+}$$

$$2O_2^{-} + 2H^{+} \leftrightarrow H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \leftrightarrow Fe^{3+} + OH^{-} + OH$$

These superoxide (O_2^{-}) and hydroxyl (OH^{-}) species that are derived from the partial reduction of molecular oxygen have the propensity to oxidize lipid in the biological membranes causing the loss of integrity of the membrane bilayer. Therefore, the amount of free iron circulating within a host is tightly regulated. About 99.9% of the 4 g total human body iron is intracellular and not readily accessible [108]. The trace extra cellular iron is tightly bound to transferrin and lactoferrin and the small amount of

free hemoglobin is bound to the heterotetrameric glycoprotein, haptoglobin (Hpt) [108]. Therefore, a bacterial pathogen must compete for obtaining iron from the host to be able to survive and establish an infection.

1.3.1 Role of Heme in Bacterial Metabolism

Heme is a tetrapyrrole which comprises a family of compounds involved in many aspects of oxidative metabolism including: oxygen transport, electron-transport-dependent phosphorylation, oxidative stress responses, oxygen-sensing, oxygenation reactions and detoxifications [113]. They are also involved in the synthesis or sensing of diatomic gases like carbon monoxide and nitric oxide [114]. Quantitatively in most prokaryotes, cytochromes are the major heme compounds which are essential for aerobic respiration [113]. In some prokaryotes, oxygen limitation increases heme synthesis, so heme also contributes to metabolic adaptation [113]. Since uptake and transport of heme to the bacterial cell needs less energy than its biosynthesis [115], some bacteria acquire heme from the environment [113]. However, in most cases the bacterial requirement for heme is met by the biosynthesis of heme.

The crucial involvment of iron and heme in bacterial metabolism explains why the ability to acquire these two factors is considered a major virulence determinant for pathogenic bacteria.

1.3.2 Iron and Heme Acquisition in Pathogenic Bacteria

Bacteria have developed various and different systems for iron assimilation. The

explanations underlying this necessity may be understood better from an evolutionary point of view as it can be assumed iron uptake was not a problem until cyanobacteria appeared and began carrying out oxygenic photosynthesis [112]. As the atmosphere become oxidizing, Fe(II) was converted to Fe(III) and insoluble hydrated ferric oxides were formed by hydrolytic polymerization reactions [112]. So, perhaps about a billion years after the origin of life on earth, organisms were forced to develop efficient systems for iron assimilation after the basic biochemical pathway had arisen and it is, therefore not surprising that these pathways show great diversity at the biochemical level [112].

Iron obtaining mechanisms can be categorized as (Figure 1): 1) siderophore-mediated iron uptake, 2) receptor mediated iron uptake from host iron-glycoproteins and 3) uptake of heme-compounds as iron source [110].

In fact, the above mentioned systems reflect the iron resources found in the human body. Most of the iron in the human body is located intracellularly as ferritin or as heme-compounds and the trace amount of extracellular iron is attached to the iron-binding/transport glycoproteins, transferrin and lactoferrin [109].

1.3.2.1 Siderophore Mediated Iron Uptake

Siderophores are low molecular weight (generally less than 1000 Da) high affinity iron (Fe(III)) chelators which compete with lactoferrin (Lf) and transferrin (Tf) for iron [109, 110, 116]. Their iron dissociation constants (*Ks*) range from 10^{22} to 10^{50} and are regarded as strong enough to remove iron from Lf, Tf and ferritin but not from heme proteins [109, 110]. To date, more than 200 microbial siderophores have been identified

Figure 1. Schematic representation of iron uptake routes in bacteria (adapted from [109] with some modification).

A) Siderophore mediated iron uptake

B) Receptor mediated iron uptake from iron-glycoproteins

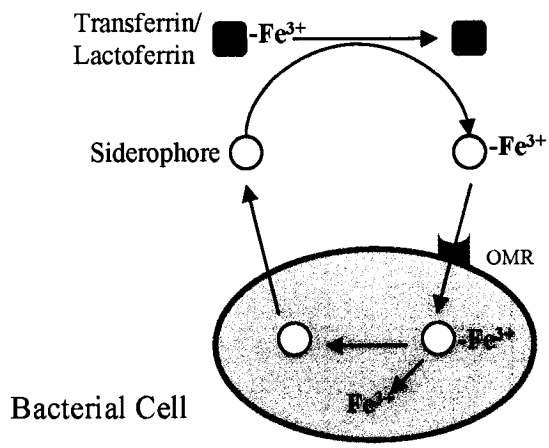
C) Heme-compounds as iron source:

I) Heme-specific OMR (Outer Membrane Receptor). Heme could be released from hemoproteins by bacterial proteinase

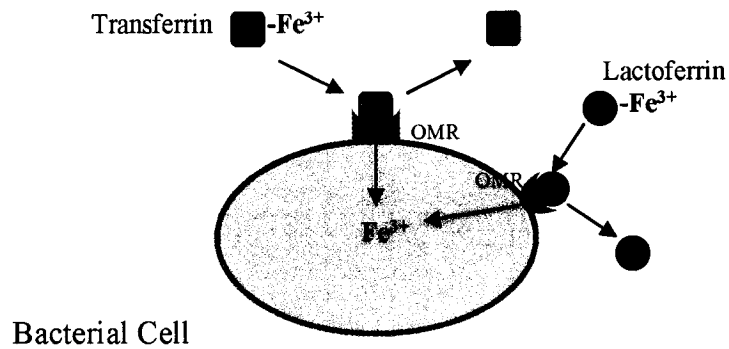
II) Hemophore/proteinase mediated removal of heme from heme-compounds (e.g. hemoglobin) and Hemophore specific OMR

III) Heme-compound specific OMR (e.g. Hemoglobin OMR)

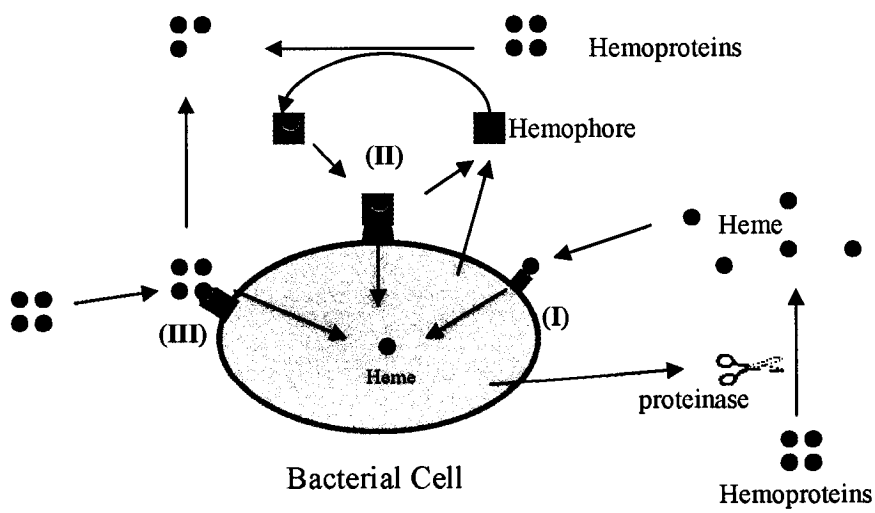
A)


Transferrin/
Lactoferrin
-Fe3+
Siderophore
-Fe3+
OMR
-Fe3+
Fe3+
Bacterial Cell

B)


Transferrin
-Fe3+
Lactoferrin
-Fe3+
OMR
OMR
Fe3+
Bacterial Cell

C)


Hemoproteins
Hemophore
(II)
Heme
(III)
(I)
Heme
proteinase
Bacterial Cell
Hemoproteins

[111]. Aerobactin and enterobactin from *E. coli* are the best characterized [112]. Siderophores can be divided into two major chemical groups: phenolates and hydroxamates [109]. In Gram-negative bacteria, the iron-siderophore complex binds to specific high-affinity outer membrane receptors (in *E. coli* FepA for enterobactin and IutA for aerobactin [112]) and is subsequently internalized where the iron is removed and the siderophore is recycled [109]. Since the expression of these receptors is dependent on the iron concentration in the environment, they are called IROMPs (iron repressible Outer Membrane Proteins) [109]. The translocation of these molecules has been shown to be an active process and is powered by the proton motive force requiring a functional Ton system [109, 110, 112]. From the periplasm to the cytoplasm, transport is via an adenosine triphosphate (ATP)-binding-cassette transporter (ABC-transporter) [112].

The **Ton system** consists of three proteins TonB, ExbB and ExbD with direct physical interactions [110, 117, 118]. TonB is considered the energy transducer component of the proton motive force generated at the cytoplasmic membrane to the outer membrane receptor for the physical translocation of the ligand [117]. It has been suggested that binding of a ligand induces a conformational change in the outer membrane receptor which facilitates the interaction of the outer membrane receptor with TonB in a highly conserved region of the receptor close to its N-terminal region called the TonB-box [117]. This interaction would allow the opening of a small channel in the receptor in which the siderophore moves to the periplasm [110, 117]. The subsequent transfer of the siderophore into the cytoplasm is facilitated by periplasmic binding protein-dependent transport (PBT) systems which are a subclass of the ABC superfamily

of transporters [110, 117].

1.3.2.2 Receptor Mediated Iron Uptake From Host Iron-Glycoproteins

Lactoferrin, transferrin and ovotransferrin comprise a family of iron-binding glycoproteins of approximately 75-80 kDa, referred to as transferrins [109, 110, 116]. A number of bacteria use these glycoproteins as an iron source. Such bacterial pathogens have developed highly specific outer membrane receptors which are able to bind transferrin, lactoferrin or ovotransferrin and remove iron from them without involving siderophores [110]. These receptors exhibit TonB dependency and function as channels through which iron can cross the outer membrane to the periplasm following its removal from the receptor in Gram-negative bacteria [110]. Similar to siderophores, the transport of iron from the periplasm to the cytoplasm is performed by ABC transporters, and a Fe(III)-binding protein [109]. *Neisseria meningitidis* and *N. gonorrhoeae* are two examples of bacteria using such an iron uptake system, as these two organisms do not secrete siderophores [109]. In the meningococcus and gonococcus, TbpA and TbpB are TonB-dependent, OM receptors for transferrin which serve as channels through which ferric iron crosses the outer membrane after its release from transferrin bound to the OM receptor [109]. Transferrin OM receptors have also been identified in some *Moraxella* and *Haemophilus* spp [109].

An ABC-transporter including an iron-binding protein transfers the released iron from the periplasm to the cytoplasm. Such a system has been characterized in many bacteria including *Serratia marcescens (sfuABC)* [119], *H. influenzae (hitABC)* [120], *N. meningitidis (fbpABC)* [121], *Staphylococcus epidermidis (sitABC)*

[122], *Salmonella typhimurium (sitABCD)* [123], *Brachyspira hyodysenteriae (bidABCD)* [124], *Yersinia pestis (yfuABC)* [125], *Streptococcus pneumoniae (pit1, pit2)* [126], *Corynebacterium diphtheriae (irp6ABC)* [127], and *Actinobacillus pleuropneumoniae (fhuABCD)* [128].

1.3.2.3 Uptake of Heme Compounds as an Iron Source

Bacterial pathogens are also able to use heme compounds as an iron source. In the host, these compounds are primarily intracellular and only small amounts of hemoproteins are released by either spontaneous hemolysis or from damaged tissues. The latter may result from the effect of bacterial toxins released to gain access to the intracellular sources of nutrients including heme-compounds [109, 110].

Different mechanisms and systems have been described for heme uptake which include receptor-mediated, hemophore-mediated, and protease-mediated uptake [115]:

A) In receptor mediated uptake, heme or hemoproteins (like hemoglobin and hemopexin) are bound to outer membrane receptors and are then transferred to the periplasm. Examples include HemR in *Y. enterocolitica* [129], HmuR in *Y. pestis* [130], HbpA, HxuC and HxuB in *H. influenzae* [131, 132], HgbA and TdhA in *H. ducreyi* [45, 49].

B) In haemophore-mediated systems, bacteria produce extracellular heme/hemoprotein-binding proteins that capture and shuttle the ligand to the outer membrane receptor. This system has been described in *S. marcescens*, [133, 134], *Y.*

pestis [135] and *Ps. aeruginosa* [136].

C) In protease mediated systems, bacteria produce extracellular proteases that degrade host hemoproteins, such as hemoglobin protease in *Porphyromonas gingivalis* [137, 138].

It is interesting that all of the above mentioned systems involve TonB dependent outer membrane receptors for transfer of the heme or hemoprotein to the periplasm. It has been suggested that histidine residues are essential for the transport of heme through the receptor pore [139]. From the periplasm, heme is transferred to the cytoplasm presumably by an ABC transporter system using a periplasmic binding protein [110]. The potent and destructive oxidative effects of heme and also its tendency to aggregate at physiological pH [140, 141] further explain the need for such a transport system. Although iron may be removed from heme/hemoprotein and be transported into the cell, from an energetic point of view, transport of heme into the cell requires less energy than heme biosynthesis [115]. Moreover, some bacteria like *Haemophilus spp* depend on external sources for the heme, since these organisms lack the ability to synthesize heme.

1.4 ABC Transporters

The ABC transporter superfamily is one of the most widespread, and ubiquitous of all gene families [142]. This superfamily currently has in excess of 2000 members in bacteria, archaea and eukarya including humans and thus, may be considered as an ancient proteinaceous device for transporting solutes across a lipid bilayer against a concentration gradient [142-144]. ATP-binding cassette transporters are

so named because one of the domains or component proteins of the members of this family contains a homologous ATP-binding sequence [143, 145]. This domain couples the energy of ATP hydrolysis to the translocation of solutes across biological membranes [143, 145]. These transporters are also involved in a variety of processes like signal transduction, protein secretion, drug and antibiotic resistance, antigen presentation and bacterial pathogenesis and sporulation [143].

Typically, a bacterial ABC transporter is composed of four parts, including two hydrophobic transmembrane domains (each containing six transmembrane helices) and two hydrophilic ATP-binding domains (Figure 2-B) [145, 146]. The sequence of ABC or ATPase domains always shares homology in a region of about 200 amino acids [147]. This region contains two motifs called Walker A and Walker B [147]. It also contains a signature motif or linker peptide which differentiates ABC-transporters from other ATPases (Figure 2-A) [147]. Though there are two ATP binding sites (one on each domain), only one ATP molecule binds to a catalytic pocket incorporating both ATP-binding domains and these domains hydrolyze ATP alternately [148].

1.4.1 Binding protein dependent (BPD) transport system

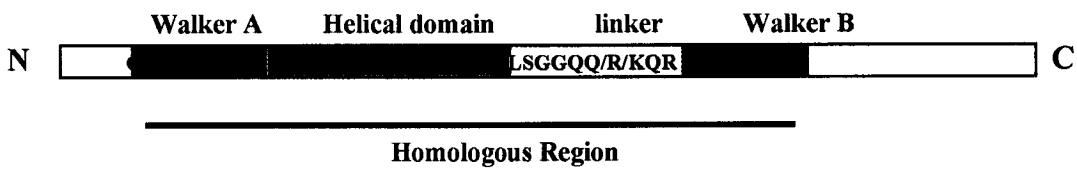
Bacterial binding protein-dependent transport systems are a subclass (Class 3) [142] of the ABC transporter superfamily and carry out uptake of a wide range of nutrients like sugars, amino acids, peptides, phosphate esters, inorganic phosphates, sulfates, phosphonates, metal cations, iron-chelator complexes, vitamins and polyamines [142, 143, 145]. Investigations regarding these transporters started approximately two

Figure 2. Linear representation of ATP-binding Domain of a Typical ABC-Transporter.

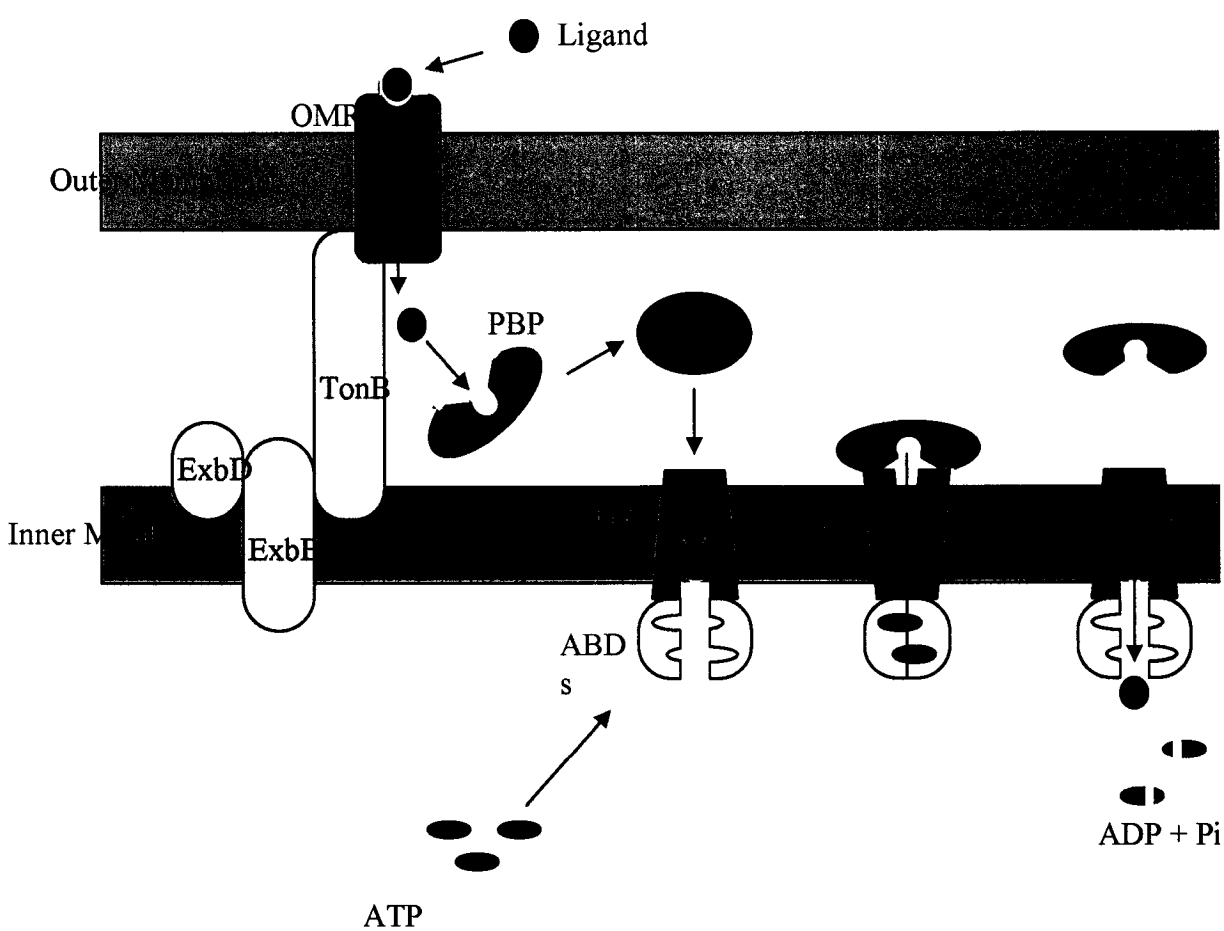
A) Bold line shows the homologous region between ATP-binding domains of ABC-Transporters. x: variable amino acids, h: hydrophobic amino acids. (Adapted from [143, 145] with some modifications).

B) A typical TonB-dependent Outer Membrane Receptor (OMR) involved in uptake of Siderophore or heme and the typical related ABC-Transporter system for transport of the ligand from the periplasm to the cytoplasm. General mechanism of action is based on the maltose transporter in *E. coli* (Adapted from [112, 141, 149] with some modifications). PBP: Periplasmic Binding Protein; TDs: Hydrophobic TransMembrane Domains; ABDs: ATP Binding Domains.

A)


Walker A
Helical domain
linker
Walker B
N
LSGGQQ/R/KQR
C
Homologous Region

B)


Ligand
OMR
Out
PBP
TonB
ExbD
Inner M
ExbB
ABD
s
ATP
ADP + Pi

decades ago [150]. This subclass is uniquely equipped with a ligand binding protein component which is located in the periplasm of Gram-negative bacteria and on the surface of Gram-positive bacteria [143]. In the later case it is anchored to the membrane through a lipid extension at the N-terminus [145]. All binding proteins appear to consist of two globular domains connected by a hinge, providing a cleft for the ligand to bind [146]. It has been suggested that the main functions of the binding proteins are to confer high affinity to the transport process, to act as a scavenger or original recognizer for the ligand, and in some cases as a chemoreceptor [143, 145]. It has also been proposed that the binding proteins confer directionality to the transport process as only import systems have binding proteins and exporter systems do not [151]. In addition, it has been suggested that binding proteins act as a trap for the substrate, preventing it from leaving through the outer membrane [147]. Thus, BPD transport systems can be seen as recapture systems which prevent the leakage of the substrate from the cell after it has been lost from the cytoplasm [147]. The high amount of binding proteins relative to that of the membrane components likely reflects this consideration [147].

1.4.2 BPD Transporters and the TonB System

Class 3 of ABC transporters includes all known BPD transporters [142]. The ISVH family belongs to this class and contains transporters specific for iron-siderophores, hemin and vitamin B-12 [142]. In Gram-negative bacteria, each of these transporters is associated with a high affinity outer membrane receptor for its cognate ligand [142]. Through this receptor, the ligand is translocated from the surrounding environment to the periplasm. The energy for this translocation is provided by the TonB

system [118, 142]. Together, the TonB energized receptor and BPD transporter provide a complete system for uptake of iron, heme or Vitamin B-12 from the environment to the cytoplasm [118, 142].

1.4.3 Mechanism of Action of BPD Transporters

Though the mechanism of the translocation of ligand from periplasm to cytoplasm is unclear, a model has been proposed based on the maltose importer of *E. coli* and the histidine importer of *Salmonella typhimurium* which includes several conformational changes in the components of the transporter (Figure 2-B) [145, 146, 151]. The process starts upon binding of ligand to the binding protein (BP), which induces a conformational change in the BP, increasing its affinity for the transmembrane domains (TDs). After the BP-ligand complex binds to the TDs, the ligand is released to interact with the TDs. This interaction induces a conformational change in the TDs which is transmitted to the ATP-binding domains causing ATP hydrolysis. ATP hydrolysis induces further conformational changes in ATP-binding domains leading to reorientation of the TDs and release of the ligand to the cytoplasm [145, 146, 151]. It has been proposed that the TDs binding site for the ligand can face either the periplasm or the cytoplasm. The periplasmic face of the TDs has high affinity for the ligand while the cytoplasmic face has low affinity. Thus, reorientation of TDs can control binding or release of the ligand [146, 151].

1.5 Heme Uptake and ABC transporters in Gram-negative Bacteria

The heme uptake system comprising both the TonB-dependent outer membrane receptor and BPD transport across the cytoplasmic membrane was first reported in *Y. enterocolitica* [129, 152]. The system comprises the 78 kDa HemR, the OMR for heme, which exhibits a well conserved TonB box at its amino terminus [129]. HemR is located within a cluster of genes, designated *hemT*, *hemU* and *hemV* [129]. Based on homology studies, HemU has been assigned as the putative heme-specific permease and HemV has been assigned as the putative ATPase [152]. Further amino acid sequence comparison of these proteins with the periplasmic BPD transport system indicated that these proteins are components of such a system [152]. HemT was considered to be the putative periplasmic heme-binding protein [152], which presented the first evidence of the involvement of ABC-transporters in heme uptake. This report was followed by similar observations for other bacteria including: *Shigella dysenteriae* [153], *Y. pestis* [130], *C. diphtheriae* [154], *Porphyromonas gingivalis* [155, 156], *Ps. aeruginosa* [157], *Bradyrhizobium japonicum* [158] and *S. pyogenes* [159]. These observations suggested a pattern for heme uptake systems in bacterial spp. comprising a TonB dependent outer membrane receptor and a BPD transport system. It should be noted that most of the above systems have been identified solely on amino acid homology to existing ABC transporters and none of the ATPases, permeases or periplasmic binding-proteins for heme transport have been

biochemically characterized. A few have been functionally characterized using mutation analysis. No periplasmic binding protein has been purified as yet or characterized functionally with heme binding activity.

1.5.1 Mechanism of Iron and Heme Acquisition in *H. ducreyi*

H. ducreyi has an absolute need for heme mirroring the fact that about 50% of the described virulence factors are related to heme acquisition or heme metabolism. However, no siderophore production has been detected in *H. ducreyi* [32]. As *H. ducreyi* is a heme obligate organism, it has been difficult to determine if human transferrin or lactoferrin can serve as a source of iron. The organism can use a variety of heme-compounds as a source of both iron and heme. The identification of TonB-dependent OMRs for heme and hemoglobin in *H. ducreyi* suggests that a heme dedicated ABC transporter is involved in the transport of heme across the periplasmic space to the cytoplasm.

1.6 Superoxide Dismutase

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of free radical (superoxide anion) to oxygen and hydrogen peroxide by alternate reduction and oxidation of their metal ion in their catalytic site [104, 160]. SODs are one of the most studied enzymes since their discovery in 1969 [161]. They have been found in both prokaryotes and eukaroytes (including human) and are apparently ubiquitous in all organisms [162].

There are three major types of bacterial SOD (A, B and C) depending on the ion in their catalytic center. This ion can be manganese (SodA or MnSOD), iron (SodB or FeSOD) and copper (SodC or Cu-ZnSOD). Both SodA and SodB are located in the cytoplasm while SodC is located extracellular or in the periplasm in the case of Gram-negative bacteria [162]. The primary function of SodA and SodB is to protect the cell against endogenously generated reactive oxygen species (ROS) produced during the respiratory events of aerobic and facultative organisms [162]. SodC protects the cell from extracellular ROS [162].

1.6.1 Bacterial SodC

Although bacterial SodC was first reported in 1974 in *Photobacterium leiognathi* [163], it was not identified in other bacteria until the 1990s. The repression of SodC expression during the log-phase of growth may have accounted for its delayed recognition [162]. Periplasmic located SodC has been identified in diverse pathogenic Gram-negative bacteria [164]. Considering that periplasmic SOD is not able to cross the cytoplasmic membrane [165], it has been proposed that SodC could play a role in the pathogenicity and survival of bacteria in the animal host and would probably protect the bacteria against extracellular ROS produced by host monocytes, macrophages and polymorphonuclear cells [101, 105, 160, 162]. Prokaryotic SODs are often in dimer form with each unit having its own metal ion and active catalytic site [162]. Interestingly *H. ducreyi* SodC, unlike all the other known SodCs from bacteria and eukaryotic species, has the unique ability of binding to heme [103]. Cloning this enzyme in *E. coli* resulted in the accumulation of heme in the *E. coli* periplasm compared to the control

strain [103]. Further investigation showed that histidine-82 covalently binds to heme. The heme binding is stable at 100 °C in the presence of detergents (2% SDS) [103]. These observations led to the proposed role of SodC in the metabolism of heme and possibly in transport or detoxification in *H. ducreyi* [103].

1.7 Hypothesis

The hypothesis of this research proposal is that a BPD transport system is involved in transporting heme from the periplasmic space to the cytoplasm in *H. ducreyi*. A corollary to the central hypothesis suggests that interruption of this system would significantly impair the growth of the organism.

1.8 Objectives

To address this hypothesis, we have pursued the following objectives:

1- Cloning of the heme-specific PBP of *H. ducreyi*.

2- Characterization of an isogenic *H. ducreyi* heme ABC transporter mutant

3- Phenotypic characterization of the heme ABC transporter mutant.

4- Genetic and functional complementation of the heme ABC transporter mutant

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

2.1.1 *H. ducreyi* Strains

H. ducreyi 35000 was isolated during an outbreak in Winnipeg, Canada in 1975 [166]. Stocks from a sample provided kindly by Dr. D. W. Cameron (University of Ottawa, Ottawa, ON, Canada) were made in Tryptic Soy Broth (Difco/Becton Dickinson, Sparks, MD, USA) with 15% (w/v) glycerol (BDH, Toronto, ON, Canada) and maintained at -70°C. Other *H. ducreyi* strains used in this study (Table 1) were clinical isolates stored in 2% (w/v) skim milk (Difco, Detroit, USA) and 20% (w/v) glycerol at –70 °C [167] and were kindly provided by Dr. D. W. Cameron.

2.1.2 *E. coli* strains

E. coli 71/18 harboring plasmids *pPHdSOD*, *pPHdSOD64E* and *pPHdSODL59FH60QD61G* were provided kindly by Dr. A. Battistoni (University of Rome, Rome, Italy). *E. coli Top10* (One Shot® Top10) competent cells were purchased

Table 1. Bacterial Strains used in this study

Bacterial strains	Origin of isolation or relevant genetic description	Reference
***H. ducreyi* strains**		
35000	Clinical isolate, Winnipeg, Canada	[167]
AX557	Clinical isolate, Nairobi, Kenya (1981)	[167]
BG411	Clinical isolate, Nairobi, Kenya (1981)	
C148	Clinical isolate, Nairobi, Kenya (1981)	
V1157	Clinical isolate, Seattle, U.S.A.	[167]
J1159,	Clinical isolate, Nairobi, Kenya, 1986-1987	[167]
PPC358/1315	Clinical isolate, Nairobi, Kenya, 1986-1987	
36-F-2	Pasteur Institute, Paris, France	[167]
sodC	*H. ducreyi35000* with insertional inactivation of SodC with Kanamycin cassette	This Study
***E. coli* strains**		
71/18	*F' leuB6 supE44 hsdS20($r_B^-m_B^-$) recA13 ara14 proA2 galK2 lacY1 rpsL20 xyl-5 mtl-1*	[103]
Top10 (One Shot® Top10)	*F' mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galK rpsL (Str^R) endA1 nupG*	Invitrogen; Burlington, Ontario, Canada

Table 2. Plasmids used in this study

Plasmid	Relevant genetic description	Reference
pPHduSOD	Vector expressing *H. ducreyi* wild-type SodC	[103]
pPHduSODE64	Vector expressing mutant *H. ducreyi* SodCH86E	[103]
pPHduSODL59FH60QD61G	Vector expressing mutant *H. ducreyi* SodC L81FH82QD83G	[103]
pHDMK4	5.2 kbp *Eco*RV fragment of *H. ducreyi 35000* chromosome with the insertion of kanamycin resistance cassette into *Bam*HI site of *sodC* cloned into *pIT4*. Suicide vector for insertion of kanamycin resistance cassette into *sodC*	[101]
pFP10	*ori from pJD5 + cat from pACYC184*	[168]
pFP12	Shuttle vector, p*FP10* + *plac-MCS-GFP-MCS from pEGFP*	[168]
pSW	*pFP12* containing wild-type *H. ducreyi sodC*	This study
pSE	*pFP12* containing *H. ducreyi sodC H86E*	This study
pSQ	*pFP12* containing *H. ducreyi sodC L81FH82QD83G*	This study
pPSW	*pFP12* containing wild-type *H. ducreyi sodC* under control of *psod*	This study
pPSE	*pFP12* containing *H. ducreyi sodC H86E* under control of *psod*	This study
pPSQ	*pFP12* containing *H. ducreyi sodC L81FH82QD83G* under control of *psod*	This study

from Invitrogen (Burlington, ON, Canada). Transformed colonies were subcultured in LB broth for 12-16 hrs before adding 15% (v/v) sterile glycerol and stored at –70 °C.

2.2 Media and Culture Conditions

2.2.1 Chocolate Agar (CA) Plates

H. ducreyi strains were routinely grown on CA plates [GC Medium Base (Difco/Becton Dickinson, Sparks, MD, USA) with 1% (w/v) hemoglobin (BBL/Becton Dickinson, Cockeysville, MD, USA), supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS; Gibco/Invitrogen, Burlington, ON, Canada) and 1% (v/v) IsoVitaleX (BBL/Becton Dickinson, Cockeysville, MD, USA)] for 18-24 hrs at 33 °C in an atmosphere of 5% CO_2 and saturated humidity. Where necessary, antibiotics were added to the following concentration: kanamycin (Kan) (Sigma, St. Louis, MO, USA), 35 μg/ml and chloramphenicol (Cm) (Sigma, St. Louis, MO, USA), 2 μg/ml.

2.2.2 Semi-Defined Media (SDM) Plates

For determination of minimum heme requirement, *H. ducreyi* strains were grown on SDM plates [32] [GC Medium Base, supplemented with 0.1% (w/v) D(+)glucose (Sigma-Aldrich, Oakville, ON, Canada), 0.025% (w/v) L-cysteine hydrochloride (Fisher Scientific, Ottawa, ON, Canada), 0.01% (w/v) L-glutamine (BDH, Toronto, ON, Canada), 200μM ethylenediamine di-o-hydroxyphenyl acetic acid (EDDHA) (Sigma; St. Louis, MO, USA) and defined concentrations (2-100 μg/ml) of bovine hemin chloride

(Sigma-Aldrich, Oakville, ON, Canada) dissolved in 40μl of 0.1N NaOH (Sigma, St. Louis, MO, USA) per μg hemin chloride] for 18-24 hrs at 33 °C in an atmosphere of 5% CO_2 and saturated humidity.

2.2.3 HD Broth

After introduction of DNA into *H. ducreyi* strains by electroporation, bacteria were inoculated into HD Broth [1.5% (w/v) Proteose Peptone No. 3 (Difco/Becton Dickinson, Sparks, MD, USA), 0.1% (w/v) corn starch (BDH, Toronto, ON, Canada), 0.4% (w/v) dibasic potassium phosphate (Fisher Scientific, Ottawa, ON, Canada) and 0.1% (w/v) monobasic potassium phosphate (Fisher Scientific, Ottawa, ON, Canada), 0.5% (w/v) sodium chloride (BDH, Toronto, ON, Canada), supplemented with 10% (v/v) heat inactivated fetal calf serum, 25 μg/ml hemin chloride, and 1% (v/v) IsoVitaleX]. The mixture was incubated for 6 hrs at 33 °C in an atmosphere of 5% CO_2.

2.2.4 Charcoal Chocolate Agar (CCA) Plates

To select mutant colonies, *H. ducreyi* strains, after introduction of DNA and incubation in HD broth, were inoculated onto CCA plates [GC medium base with 1% (w/v) hemoglobin and 0.25% (w/v) activated charcoal (Sigma-Aldrich, Oakville, ON, Canada), supplemented with 5% (v/v) heat inactivated FBS, 1% (v/v) IsoVitaleX and 35 μg/ml Kan] and were incubated for 48 hrs at 33 °C in at atmosphere of 5% CO_2 with saturated humidity.

2.2.5 Gonococcal (GC) Broth

For growth assays, *H. ducreyi* strains were inoculated into GC broth [1.5% (w/v) Proteose Peptone No. 3, 0.1% (w/v) corn starch, 0.4% (w/v) dibasic potassium phosphate and 0.1% (w/v) monobasic potassium phosphate, 0.5% (w/v) sodium chloride, supplemented with 0.1% (w/v) D-(+)-glucose (Sigma, St. Louis, MO, USA), 0.025% (w/v) L-cysteine hydrochloride (Fisher Scientific, Ottawa, ON, Canada), 0.01% (w/v) L-glutamine (BDH, Toronto, ON, Canada) and the desired amount of hemin chloride] in a 200 ml flask. Flasks were incubated at 33 °C in a shaking incubator at 175 rpm and an atmosphere of 5% CO_2 for 24 hrs.

2.2.6 Luria Bertani (LB) Broth and Plates

E. coli strains used in this study (Table 2) were routinely grown on Luria Bertani (LB) agar plates (LB broth [Difco/Becton Dickinson, Sparks, MD, USA] with 1% (w/v) Bacto-Agar [Difco/Becton Dickinson, Sparks, MD, USA]) overnight at 37 °C, or in LB broth at 37 °C, in a shaking incubator at 175 rpm for 12 to 16 hrs. Where necessary, antibiotics were added to the following concentration: ampicillin (Amp) (Sigma, St. Louis, MO, USA), 100 μg/ml and Cm, 50 μg/ml.

2.3 Growth Assays

2.3.1 Determination of the Minimum Heme Requirement

To determine the minimum heme requirement on agar based plates, heme was

supplied as both the sole exogenous source of iron and heme. *H. ducreyi 35000* was grown on CA plates for 18 hrs, scraped off the plates with a cotton swab and resuspended in sterile PBS (Phosphate buffered saline, [169]) to an A_{600} of 1.0. One hundred µl of this suspension was inoculated onto SDM plates. Plates were incubated for 24-36 hrs and bacteria were subcultured onto fresh SDM plates containing the same concentration of heme. After 24 hrs incubation, the plates were inspected visually for growth.

2.3.2 Phenotypic Characterization of *H. ducreyi sodC* Mutant and Complemented Strains

Growth assays were conducted to determine the ability of the wild-type *H. ducreyi 35000*, the *H. ducreyi sodC* mutant and the *H. ducreyi sodC* mutant complemented strains to grow at low (5 µg/ml) and high (50 µg/ml) heme concentrations. *H. ducreyi* strains were grown overnight on CA plates with the addition of the appropriate antibiotic (2 µg/ml Cm or 35 µg/ml Kan) as needed. Bacteria were collected from plates and resuspended in sterile PBS to an A_{600} of 1.0 (DU 640B, Beckman, Fullerton, CA, USA). One ml of this suspension was delivered to 100 ml of prewarmed (33°C) GC broth containing the desired amount of heme in a 200 ml flask. Flasks were incubated at 33 °C in an atmosphere of 5% CO_2 in a shaking incubator at 175 rpm. Growth was monitored every 2 hrs for 16 hrs and at 24 hrs using a Beckman DU 640 spectrophotometer at A_{600}. Serial dilutions of the culture (10^{-3} to 10^{-7}) at each time point were plated in duplicate to obtain the colony forming units (CFU). Plates were incubated for 24-36 hrs before colonies were enumerated.

2.4 Bacterial Cell Fractionation

Periplasmic extraction was performed using chloroform or osmotic shock methods [170, 171].

2.4.1 Chloroform Method

Briefly, bacteria were grown for 24 hrs on CA or SDM plates, and collected by scraping the plates with a cotton swab, or were grown for 12-16 hrs in LB broth followed by pelleting the broth culture at 4000 ×g for 10 min at 22 °C. Bacteria were washed twice with filter sterilized 1× phosphate buffered saline (PBS), then resuspended in an equal volume of filter sterilized 0.1M Tris-HCl (BDH, Toronto, ON, Canada), pH 8.0, 5mM EDTA (Sigma-Aldrich, Oakville, ON, Canada) and an equal volume of chloroform (BDH, Toronto, ON, Canada), followed by a brief vortex. The mixture was incubated for 20 min at 22 °C and then centrifuged for 60 min at 100,000 ×g at 4 °C. The supernatant was collected as the periplasmic fraction and stored at -70°C.

2.4.2 Osmotic Shock Method

Bacteria were grown for 24 hrs on CA or SDM plates, and collected by scraping the plates with a cotton swab, or were grown for 12-16 hrs in LB broth followed by pelleting the broth culture at 4000 ×g for 10 min at 22 °C. Bacteria were washed twice with filter sterilized PBS, then resuspended in 200 µl per gram of wet weight of the cells of sterilized 0.1M Tris-HCl, pH 8.0, 5mM EDTA, 20% (w/v) sucrose (Fisher). To the

suspension, 60 µl per gram of wet weight of cells of a 10 mg/ml solution of lysozyme (Amersham Bioscience Biotech, Piscataway, NJ, USA) was added and the reaction was incubated on ice for 40 min. To the reaction, 160 µl per gram of wet weight of cells of 0.5M $MgCl_2$ (Sigma- Aldrich, Oakville, ON, Canada) was added and the mixture was gently shaken. The mixture was centrifuged at 100,000 ×g for 60 min at 4 °C. The supernatant was collected as periplasm and stored at –70 °C.

2.4.3 Cytoplasmic Fraction

To obtain the cytoplasmic fraction of cells, the pellet remaining after removal of periplasm in each of the above periplasmic extraction methods, was resuspended in 1ml per gram of wet weight of 0.1M Tris-HCl, pH 8.0, 5mM EDTA and sonicated for 60 sec with an output of 15-20 watts (Model 60 Sonic Dismembrator, Fisher Scientific, Ottawa, ON, Canada) at 4 °C. The mixture was then centrifuged at 100,000 ×g for 60 min at 4 °C and the supernatant was collected as the cytoplasmic fraction and stored at –70 °C.

2.4.4 Total Cell Lysates

Bacteria were grown for 24 hrs on CA or SDM plates, and collected by scraping the plates with a cotton swab, or were grown for 12-16 hrs in LB broth followed by pelleting the broth culture at 4000 ×g for 10 min at 22 °C. Bacteria were washed twice in PBS, resuspended in an equal volume of filter sterilized 0.1M Tris-HCl, pH 8.0, 5mM EDTA and sonicated for 60 sec with an output of 15-20 watts at 4 °C. The lysates were then centrifuged at 100,000 ×g for 60 min at 4 °C and the supernatant was collected as

total cell lysate and stored at –70 °C.

2.5 Protein Biochemistry

2.5.1 Heme Affinity Chromatography

Heme-binding proteins were purified by batch hemin affinity chromatography [172]. Briefly, 100 to 1000 µl of periplasmic extract or total cell lysate was incubated with 20 µl (6.7 µmol hemin/ml) of hemin-agarose suspension (Sigma-Aldrich, Oakville, ON, Canada) for 1 hr at 37 °C with gentle rocking. The suspension was centrifuged at 1500 ×g for 5 min at 22 °C and the supernatant discarded. The pelleted beads were washed three times at 22 °C each for 1 hr with 10 ml of low-salt wash solution (50mM Tris-HCl, 100 mM sodium chloride, pH 8.0, 5mM EDTA and 0.5% (w/v) N-lauroyl-sarcosine [Sigma-Aldrich, Oakville, ON, Canada]), followed by a final wash at 22 °C for 1 hr with 10 ml of low-salt solution without EDTA and N-lauroyl-sarcosine. The suspension was pelleted and 100 to 250 µl of Laemmli SDS-PAGE loading buffer (10% (v/v) glycerol, 0.76% (w/v) Tris base [BDH, Toronto, ON, Canada], 1% (w/v) sodium dodecyl sulfate [SDS; ICN Biomedicals; Aurora, Ohio, USA], 0.001% (w/v) bromophenol blue [BDH; Poole, UK], pH 6.8) [169, 173] was added. The mixture was incubated at 95 °C for 5 min to remove proteins bound to the hemin affinity resin and then centrifuged at 1500 ×g for 3 min at 22 °C to pellet the beads. The final supernatant was collected and analyzed by SDS-PAGE.

2.5.2 Polyacrylamide Gel Electrophoresis

Proteins were separated by one dimensional discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), ranging from 7.5% to 15% w/v acrylamide (fixed or gradient) in the separating gel and the Tris-HCl/glycine buffer system [169]. Prior to loading, protein preparations were suspended in an equal volume of Laemmli SDS-PAGE loading buffer and denatured for 5 min at 95 °C. The resolving gels consisted of 0.375 M Tris-HCl pH 8.8, 0.1% (w/v) SDS and 7.5% to 15% (w/v) acrylamide [Protogel, 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, National Diagnostics, Atlanta, GA, USA]. Stacking gels consisted of 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, and 4.5% acrylamide. Electrophoresis was carried out at 100-150v in electrophoresis buffer (25mM Tris, 192 mM glycine and 0.1% (w/v) SDS) using either the Mini-Protean II electrophoresis cell system (BioRad, Mississauga, ON, Canada) for 1 hr or using the ProteanII electrophoresis cell for 8 to 14 hrs. Following electrophoresis, the protein bands were visualized by soaking the gels in Coomassie blue/Bismarck brown [174] [0.1% (w/v) Coomassie brilliant blue R-250 (EM Science, Darmstadt, Germany), 0.02% (w/v) Bismarck brown R (Sigma, St. Louis, IL, USA), 40% (v/v) ethanol (Commercial Alcohols Inc., Brampton, ON, Canada) and 7% (v/v) glacial acetic acid (EM Science, Darmstadt, Germany)] followed by a destaining step in 40% (v/v) methanol (Fisher Scientific, Fair lawn, NJ, USA) and 7% (v/v) glacial acetic acid until the background cleared. The gels were then washed with distilled water and dried overnight between two cellophane sheets (Research Products International Corp., Mount Prospect, IL, USA).

2.5.3 Western Immunoblotting

For Western immunoblot analysis, proteins separated by SDS-PAGE were electro-transferred onto Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the Mini-Protean II blotting apparatus (BioRad laboratories, Richmond, CA, USA). Prior to transfer, the membrane and gel were equilibrated in fresh transfer buffer [25 mM Tris (BDH), 192 mM glycine (EM Science), 20% (v/v) methanol (Fisher)] for 15 min. The transblotting cassette was assembled according to the instructions provided by BioRad [175] and transfer was performed at 150 v for 2 hrs at 4 °C. The nitrocellulose membrane was stained with 10% (v/v) Ponceau S (Sigma, St. Louis, MO, USA) to mark the molecular weight marker bands prior to incubation in blocking solution (0.1% (v/v) Tween 20 [BioRad, Mississauga ON, Canada], 1% (w/v) skimmed milk in PBS) for 1 hr at 37°C. After blocking, the membrane was incubated with a 1:2000 dilution of rabbit polyclonal antibody raised against *H. ducreyi35000* SodC (anti-SodC) (produced in this study or kindly provided by Dr. T. Kawula, University of North Carolina, NC, USA) or against total *H. ducreyi* OMPs (kindly provided by Dr. D. W. Cameron, University of Ottawa) in blocking solution for 30 min at 22 °C. The membrane was then quickly rinsed with PBS before incubation with 1:10,000 dilution of goat anti-rabbit immunoglobulin (IgG) horse radish peroxidase conjugate (BioSource, Camarillo, CA, USA) in 0.1% (w/v) Tween 20 in PBS for 30 min at 22 °C. The membrane was washed 3 times, 3 min each with PBS and developed with 3-amino-9-ethyl carbazole (3AEC) (Sigma, Oakville, ON, Canada) (prepared by mixing 45 ml of N,N-dimethylformamide [BDH, Toronto, ON, Canada]

containing 180 mg of AEC and 105 ml of 0.05 M sodium acetate (Fisher Scientific, Toronto, ON, Canada) pH 5.5, filtered through a 0.22 μm filter) containing 1 μl/ml of 30% H_2O_2 (Sigma, Oakville, ON, Canada) until the desired intensity was reached. The membrane was washed 3 times, for 5 min each in distilled water. Alternately, blots were developed with 1ml of TMB membrane peroxidase substrate (KPL, Gaithersburg, Maryland, USA) per cm^2 of membrane for 1-5 min until the desired intensity was reached.

2.5.4 Protein Sequencing

Peptides were sequenced by the Protein Chemistry Laboratory, University of Texas Medical Branch, Galveston, Texas (http://www2.utmb.edu/proch) using an Applied Biosystems 477A pulsed-liquid sequencer on-line with a 120A PTH amino acid analyzer with a 610 Data Analysis System or an Applied Biosystems 494/HT PROCISE Sequencing System controlled by PROCISE Control Software and a 610/A Data Analysis System. A small portion of each sample protein was applied to gas mass spectrophotometry before sequencing and compared to the prior records in major data banks.

Protein samples were prepared as directed by the Protein Chemistry Laboratory (http://www2.utmb.edu/proch/seqtip.htm). Briefly, proteins were separated by 10% SDS-PAGE and electro-blotted onto modified polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was then stained with Ponceau S for 10 min at 22 °C to visualize the bands. The band of interest was excised

and submitted for N-Terminal sequencing. For internal sequencing, proteins were separated by 10% SDS-PAGE. The gels were then stained with Coomassie blue [0.1% (w/v) Coomassie brilliant blue R-250, 40% (v/v) methanol and 1% (v/v) glacial acetic acid] and destained in 50% (v/v) methanol until the background cleared. The band of interest was excised and vacuum dried. Proteins were digested with trypsin and two of the peptides were sequenced.

2.6 Molecular Biology Techniques

2.6.1 Bacterial DNA Isolation and Quantification

Overnight growth (12-16 hrs) of *E. coli* strains in LB broth, or 18-24 hrs growth of *H. ducreyi* strains on CA plates were used for plasmid DNA isolation by the QIAprep Spin Miniprep kit (Qiagen Inc, Mississauga, ON, Canada) or SNAP Midiprep kit (Invitrogen, Burlington, ON, Canada) as directed by the manufacturers' protocol.

H. ducreyi strains grown for 18-24 hrs on CA plates were used for genomic DNA isolation by a genomic DNA extraction kit (Qiagen Inc, Mississauga, ON, Canada) as directed by the provider's protocol.

The DNA concentration in samples was determined using a GeneQuant II spectrophotometer (Pharmacia Biotech, Buckinghamshire, UK).

2.6.2 Polymerase Chain Reaction (PCR) Amplification

PCR amplification using a template of either chromosomal or plasmid DNA was performed in a 25-100 µl reaction volume, containing 1× *Taq* reaction buffer (Biotools, B&M Labs, Madrid, Spain), 1 unit *Taq* polymerase (Biotools), 0.8 mM dNTPs (Invitrogen) at 0.2 mM each, 1 mM primers mix at 0.5 mM each and 0.01-0.1 ng genomic DNA or plasmid DNA as template. Thirty cycles of amplification were performed using a Touchgene Gradient Thermocycler (Techne; Cambridge, UK). The reaction profile consisted of a 30 sec denaturation step at 94 °C, a 30 sec primer annealing step at 52 °C and a 1 min extension/elongation step at 72 °C. The profile for specific PCR amplification reactions was established by varying the annealing temperature between 45 °C and 60 °C and duration of primer extension which was adjusted to about 30 sec per kbp of template DNA to be amplified.

2.6.2.1 Colony PCR

A small fraction of a single colony from an agar plate was suspended in the PCR amplification reaction as template. A 10 min denaturation step at 94°C was added to the beginning of each specific profile to release the genomic or plasmid DNA. Colony PCR using primers CF and RF was performed to investigate chromosomal integration of the kanamycin resistance cassette.

2.6.2.2 Oligonucleotide Primers for PCR

Primers used in this study are listed in Table 3. Primers SR and SF for

amplification of *sodC*, PF and PR for amplification of *sodC* native promoter (*psod*), CF and CR for colony PCR, and SF and SP for amplification of DIG-labeled *sodC* probe were designed according the sequence of *sodC* in the NCBI GenBank (accession # U47664) with the addition of appropriate endonuclease restriction sites when required. Primers KF and KR for amplification of DIG-labeled kanamycin resistance cassette (*kan*) were designed according to the sequence available in the NCBI GenBank (accession # X06404). All primers were synthesized by the University of Ottawa Biotechnology Institute (http://www.medicine.uottawa.ca/microbio/bmi/uobri.html).

2.6.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed as described previously [169] using Hoefer HE 33 Mini Submarine or Hoefer HE 99X Max Submarine units (Amersham Biosciences; Piscataway, NJ, USA) as directed by the manufacturer's instructions. Electrophoresis grade agarose (GibcoBRL; Grand Island, NY, USA) was added to a final concentration of 0.7% to 1.5% (w/v) in 1× TBE buffer [169] (0.55% (w/v) Boric Acid [EM Science, Darmstadt, Germany], 1.08% (w/v) Tris base , 0.4% (v/v) of 0.5M EDTA, pH 8.0). Prior to loading, DNA preparations were mixed with 10× agarose gel loading buffer [169] (0.1M Na_2EDTA [BDH, Toronto, ON, Canada], 20% (w/v) Ficol 400 [EM Science, Darmstadt, Germany], 1.0% (w/v) SDS, 0.25% (w/v) Bromophenol Blue [BDH; Poole, UK]). The electrophoresis was conducted under constant voltage ranging between 30 v to 250 v and the DNA bands were visualized by the addition of 0.005% (v/v) ethidium bromide solution (EM Science, Darmstadt, Germany) to the melted agarose and

Table 3 . Oligonucleotide primers used in this study

Primer	Relevant characteristics
CF	5' AGGATTTTATCTGCAGATGCAAATAGATTTC 3'; forward primer for *sodC* colony PCR, based on bps 85 to 116 of the GenBank accession # U47664.
CR	5' ATGGCCATGGAATTATTTAATTCACCG 3'; reverse primer for *sodC* colony PCR, based on bps 813 to 786 of the GenBank accession # U47664.
KF	5' TGAAGAAGGTGTTGCTGACTCATACC 3'; forward primer for amplification of *kan* probe; based on bps 451 to 476 of the GenBank accession # X06404
KR	5' AAAGCCACGTTGTGTCTCAAAATC 3'; reverse primer for amplification of *kan* probe; based on bps 1645 to 1622 of the GenBank accession # X06404
PF	5'GATTTTATATTAATATGCAAATAGATTTCTGGTC 3'; forward primer for amplification of *psod*; based on bps 88 to 121 of the GenBank accession # U47664; *Ase*I restriction site underlined.
PR	5' AATTTTACTTTATCTCCTGGTACCATAAA 3'; reverse primer for amplification of *psod*; based on bps 209 to 181 of the GenBank accession # U47664; *Kpn*I restriction site underlined.
QL	5' TGTGGAATTGTGAGCGGATA 3'; left primer for sequencing *sodC*, based on bps 204 to 223 of the GenBank accession # U47664.
QR	5' TTGCACAGGCACTACAATTT 3'; right primer for sequencing *psod*, based on bps 357 to 337 of the GenBank accession # U47664.
SF	5' ATGGTACCAGGAGATAAAATGAAATTAACG 3'; forward primer for amplification of *sodC*; based on bps 184 to 213 of the GenBank accession # U47664; *Kpn*I restriction site underlined
SP	5' CCTAAACCTGCTACTAATTTACCGTCTTTTTC 3'; reverse primer for amplification of *sodC*; based on bps 540 to 508 of the GenBank accession # U47664.
SR	5' GTTATGCGGCCGCAATTATTTAATTAC 3'; reverse primer for amplification of *sodC*; based on bps 816 to 790 of the GenBank accession # U47664; *Not*I restriction site underlined

observing and photographing the gels under UV light using a MultiImage Light Cabinet (Alpha Innotech Corp.; San Leandro, CA, USA).

2.6.4 Purification of DNA Fragments

DNA fragments obtained from either PCR amplification or from endonuclease digestion reactions were separated by 0.7% to 1.5% (w/v) agarose gel electrophoresis. The fragment of interest was recovered from the agarose gel using the QIAquick gel extraction kit (Qiagen Inc, Mississauga, ON, Canada) according to the manufacturer's instructions. Alternatively, DNA fragments obtained from PCR amplification were purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions.

2.6.5 DNA Digestion and Ligation

Genomic or plasmid DNA and DNA fragments obtained from PCR amplification were digested when needed with the appropriate restriction endonucleases (New England bolas, Mississauga, ON, Canada) as directed by the manufacturer. Digested DNA fragments were dephosphorylated when required using the shrimp alkaline phosphatase (MBI Fermentas, Burlington, ON, Canada). DNA fragments of interest were purified by agarose gel electrophoresis and were ligated using T4 DNA Ligase (Invitrogen, Burlington, ON, Canada) as directed by the provider.

2.6.6 Plasmid Construction

The wild type *sodC* (*SW*) gene was cloned by PCR amplification from *H. ducreyi 35000* genomic DNA. The two *sodC* mutants *sodCH86E* (*SE*) and *sodCL81FH82QD83G* (*SQ*) were cloned by PCR amplification from the plasmids *pPHDSodH64E* and *pPHDSodL59FH60QD61G*, respectively. Site specific primers (SF and SR) were designed to encompass the entire coding region of the gene (bps 183 to 816 in NCBI GenBank, accession # U47664). Primers had unique restriction sites of *Kpn*I and *Not*I upstream of the start codon and downstream of the stop codon respectively. PCR amplified *SW*, *SE* and *SQ* were double digested with the restriction endonucleases *Kpn*I and *Not*I and cloned into *pFP12* after excision of *EGFP* (a red-shifted variant of wild type green fluorescent protein (GFP)) by digestion with *Kpn*I and *Not*I and dephosphorylation (Figure 15). The resultant plasmids were named *pSW*, *pSE* and *pSQ*, respectively.

The *H. ducreyi* native promoter, *psod*, was cloned by PCR amplification from *H. ducreyi 35000* genomic DNA. Site specific primers (PF and PR) were designed to encompass the entire coding region of the *psod* (bps 88 to 209 of NCBI GenBank, accession # U47664). The unique restriction sites *Ase*I and *Kpn*I were designed in the forward and reverse primers, respectively. The amplified DNA fragment was double digested and cloned into *pSW*, *pSE* and *pSQ* after excision of the *lacZ* promoter by digestion with *Ase*I and *Kpn*I and dephosphorylation. The resultant plasmids were named *pPSW*, *pPSE* and *pPSQ*, respectively.

2.6.7 DNA Sequencing

Plasmid DNA or DNA fragments obtained from PCR amplification were purified as described above and suspended to a final concentration of 70 μg/ml in sterile double distilled water before submission for automated DNA sequencing. Automated DNA sequencing was performed by the University of Ottawa Biotechnology Institute (http://www.medicine.uottawa.ca/microbio/bmi/uobri.html) with an Applied Biosystems 373 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye™ Terminator V3.0 Cycle Sequencing Ready Reaction DNA sequencing kit (Applied Biosystems).

2.6.7.1 Oligonucleotide Primers for Sequencing

For sequencing the region of *sodC* containing point mutations (bps 444, 445, 447, 457 and 459 accession # U 47664 in the NCBI GenBank), left (forward) primer SeqL was used. The sequence of nucleotides in this primer conforms to bps 174 to 193 of pEGFP and bps 5 to 24 of pFP12 [168]. For sequencing the junction of the *psod* and *sodC* in *pPSW*, *pPSE* and *pPSQ* right (reverse) primer SeqR was used. The sequence of this primer conforms to the reverse strand of bps 338 to 357 (accession # U47664 in the NCBI GenBank, bps 137 to 156 of *sodC*). Both primers were synthesized by the University of Ottawa Biotechnology Institute.

2.6.8 Southern blot

Southern Blotting was performed as previously described [169] with some

modifications as follows.

2.6.8.1 Labeling of DNA Probes

Digoxigenin (DIG)-labeled DNA probes were PCR amplified using the PCR DIG-probe synthesis kit (Roche Diagnostics; Mannheim, Germany) with gene specific oligonucleotide primers. Primers SF and SP were used to amplify the *sodC* probe and primers KF and KR were used to amplify the *kan* probe.

2.6.8.2 Preparation of Genomic DNA for Hybridization

H. ducreyi genomic DNA was digested with *Eco*RV restriction endonuclease. The digested DNA fragments were separated through a 0.7% (w/v) agarose gel at 35 v for 14 hrs. DNA bands within the agarose gel were photographed and prepared for the transfer onto nylon membrane. The gel was submerged in 500 ml of depurination solution (0.2 M HCl [Fisher Scientific, Ottawa, ON, Canada]) with gentle agitation for 10 min and rinsed with water 3 times. The gel was then incubated twice in 500 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH [Sigma, St. Louis, MO, USA]) for 15 min. Subsequently, the gel was submerged in 500 ml of neutralizing solution (1 M Tris base pH 7.4, 1.5 M NaCl) for 30 min followed by immersion in 0.5× TBE for 5 min with gentle shaking. The treated DNA fragments were subsequently electrotransferred onto a positively charged nylon membrane (Boehringer Mannheim; Mannheim, Germany) using a Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad Laboratories; Richmond, CA, USA) for 30 min at 3.5 mA/cm^2. The blot was rinsed for 5 min in 2× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate [BDH, Toronto, ON, Canada], pH 7.0). Then the

DNA was fixed or covalently linked to the membrane by exposing the DNA side of the membrane to an UV exposure at 1500 kj/cm^2 for 60 seconds (Spectrolinker XL-1000 UV Crosslinker; Spectronics corporation/Fisher Scientific; Ottawa, ON, Canada).

2.6.8.3 Hybridization with DIG-Labeled Probes

The membrane was submerged in 30 ml of prehybridization buffer (6× SSC, 5× Denhardt's reagent (100× Denhardt's reagent: 1% (w/v) Ficol 400 [EM science, Gibbstown, NJ, USA], 1% (w/v) polyvinylpyrolidine [ICN Biomedicals, Aurora, OH, USA], 1% BSA [Sigma, St. Louis, MO, USA]), 0.5% (w/v) SDS, 100µg/ml salmon sperm DNA [Invitrogen, Burlington, ON, Canada]) for 6 hrs at 44.5 °C for probing *sodC* and at 42.5 °C for probing *kan* in a micro-hybridization incubator (Robbins Scientific, Sunnyvale, CA, USA). After 6 hrs, the membrane was then immersed in 30 ml of hybridization solution (pre-hybridization solution containing 100 ng/ml of the probe of interest) and the incubation continued for a further 6 hrs. The membrane was then washed twice in 250 ml of low stringency wash solution (2× SSC, 0.1% (w.v) SDS) at 22°C for 5 min and twice in 250 ml of high stringency wash solution (0.5% SSC, 0.1% (w/v) SDS) at 65 °C for 15 min.

2.6.8.4 Detection

The DNA fragments hybridized with the DIG-labeled probes were detected by enzyme immunoassay with luminescence using CSPD® provided in the DIG luminescent detection kit (Roche Diagnostics; Mannheim, Germany) as directed by the provider's manual. The membrane was washed with 100 ml of wash buffer (maleic

acid buffer (0.1 M maleic acid [BDH, Toronto, ON, Canada], 0.15 M NaCl, pH 7.5) with 0.3% (v/v) Tween 20 [BioRad, Richmond, CA, USA]) for 5 min at 22 °C followed by incubation in 100 ml of blocking solution (maleic acid buffer with 1% blocking reagent (provided in the kit)) for 30 min at 22 °C. The membrane was then incubated in 30 ml of antibody solution (Anti-DIG-AP (sheep anti-digoxigenin conjugated to alkaline phosphatase) diluted 1:10,000 in blocking solution) for 30 min at 22 °C. The membrane was washed twice in 100 ml of wash buffer (maleic acid buffer with 0.3% (v/v) Tween 20) for 15 min at 22 °C. The membrane was then placed in a hybridization bag (Boehringer Mannheim; Mannheim, Germany) and 2 ml of 1:100 CSPD in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) was added to the bag and incubated for 5 min at 22 °C before the excess liquid was drained and the bag was sealed. The bag containing the membrane was incubated for 10 min at 37 °C to enhance luminescence. To visualize the results, Kodak BioMax films (NEN Life Science Products; Boston, MA, USA) were exposed to the membranes between 1 min to 12 hrs, depending upon the intensity of the autoradiograghy band as compared to the background level. The exposed films were developed using a Kodak X-OMAT 2000-A Processor (Eastman Kodak Co.; Rochester, NY. USA).

2.6.9 Transformation

2.6.9.1 Transformation of *E. coli*

E. coli Top10 competent cells (Invitrogen; Burlington, ON, Canada) were transformed using the calcium chloride method [176] as instructed by the

manufacturer. For each transformation, one vial (50 µl) of competent cells was removed from -70 °C storage and thawed on ice. An appropriate amount of the plasmid DNA (1-100 pg) or ligation reaction (1-5 µl) of interest was added to the cell suspension and mixed by tapping gently. Then 250 µl of SOC medium (provided in the kit) was added to the cell mixture and the mixture was incubated for 1 hr at 37 °C in a shaking incubator at 225 rpm. Twenty to 200 µl of the mixture containing the transformed cells were plated onto LB agar containing the appropriate antibiotic(s).

2.6.9.2 Electroporation of *H. ducreyi*

DNA was introduced into *H. ducreyi* by electroporation as described previously [63]. Briefly, overnight cultures of *H. ducreyi* grown on two CA plates were collected, and washed 3 times at 4 °C with 3 ml of 10% (v/v) glycerol. The cells were pelleted by centrifugation at 3000 ×g for 2 min at 4 °C. The pellet was resuspended in an equal volume of wash solution. One µg of plasmid DNA suspended in 2-20 µl of double distilled sterile water was added to the cell suspension and mixed. Forty µl of this suspension was transferred to a 0.1 cm gap gene pulser electroporation cuvette (BioRad Laboratories; Mississauga, ON, Canada) and exposed to a current of 2.5 kV, 3µF capacitance and 200Ω resistance with a Gene Pulser System (BioRad laboratories; Hercules, CA, USA). One ml of pre-warmed 33 °C HD Broth was added to the cell suspension. The mixture was transferred to a 12 × 75 mm tube and incubated for 6 hrs at 33 °C in an atmosphere of 5% CO_2 and saturated humidity. Thereafter, the cells were pelleted by centrifugation at 3000 ×g for 2 min at 22°C and the pellet was inoculated onto

a CCA plate containing the appropriate antibiotic(s) and incubated for 48 hrs at 33 °C in an atmosphere of 5% CO_2 with saturated humidity.

2.7 Molecular Biology Data Bases, Analytical Tools and Software Programs

2.7.1 Data Bases and Search Engines

Access to the *H. ducreyi35000* unfinished genome, search for oligonuclotides in the *H. ducreyi35000* unfinished genome and in other bacterial genomes, was performed by the National Center for Biotechnology Information (NCBI) BLAST program at http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi.

Kanamycin resistance cassette and *sodC* sequences were obtained through the NCBI sequence database (GenBank) at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide with accession numbers U47664 and X06404, respectively.

The open reading frame (ORF) containing the sequences generated by protein sequencing was located in the *H. ducreyi 35000* unfinished genome by the NCBI ORF finder at http://www.ncbi.nlm.nih.gov/gorf/gorf.html.

Analysis of proteins for presence of conserved domains was performed by the NCBI Conserved Domain Search at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

The sequence of nucleotides, location of the *lacZ* promoter and endonuclease restriction sites of *pFP12* were obtained from BD Bioscience, Clontech vector information at http://www.clontech.com/techinfo/index.shtml by the protocol # PT3078-5.

2.7.2 Protein and DNA Translation

The amino acid sequences generated by peptide sequencing were back-translated to nucleotide sequences using the standard codes by Entelechon back-translate tool available at http://www.entelechon.com/eng/backtranslation.html.

Translation of the DNA sequence to amino acid sequence was performed by the Expert Protein Analysis System (ExPASy) translate tool at http://us.expasy.org/tools/dna.html and the mass of predicted peptide was calculated by ExPASY PeptideMass program at http://us.expasy.org/cgi-bin/peptide-mass.pl.

2.7.3 Promoter Prediction

The sequence of *psod* was predicted by the Berkeley Drosophila Genome Project (BDGP) promoter prediction program at http://www.fruitfly.org/seq_tools/promoter.html as 5'AGAGTAAATCTTTGCATAAATCGACCGCTATTAGTAAAATAATGCTAGCC3' with a score of 0.98 between 0 and 1 at –30 to –75 corresponding to the bps 127 to172 at GenBank accession # U47664.

2.7.4 Oligonuclotide Primer Prediction and Analysis

Oligonucleotide primers for PCR amplification of DNA fragments were designed to have a melting temperature (TM) less than 70 °C, absence of any secondary structure and lack of primer dimer formation using the Sigma Genosys program at http://www.genosys.com/cgi-win/oligo_calconly.exe.

The optimal sequences of the Oligonucleotide primers for the automated sequencing were predicted by the Whitehead Institute/MIT Center for Genome Research Primer3 program at

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/results_from_primer3.

2.8 Antibody Production

2.8.1 SodC Purification

Total cell lysates of *E. coli71/18* harboring plasmid *pPHDSod* expressing wild type *H. ducreyi* SodC were separated by SDS-PAGE. The band of interest containing SodC was excised. SodC was recovered from the gel pieces using an Electro-Eluter (Model 422, BioRad Laboratories, Hercules, CA, USA) as described in the product literature using volatile buffer (0.4% (w/v) ammonium bicarbonate [BDH, Toronto, ON, Canada] and 0.1% (w/v) SDS). The protein concentration of the eluate was determined spectrophotometrically using the BCA protein assay kit (Pierce; Rockford, IL, USA) adapted from bicinchoninic acid assay [177].

2.8.2 Rabbit Immunization

Rabbit immunization was performed as described by Bryan *et al.* [178] with some modifications. All injections and blood collections were performed by the Animal Care staff of the University of Ottawa. Briefly, one 4-6 month old, female New Zealand white rabbit (Charles River Co.; St. Constant, QC, Canada) was immunized by injection with an immunization suspension consisting of 800 μl SodC solution (containing 100 μg SodC) and 200 μl (20 μg) of Gebru adjuvant 100 (Gebru Biotechnik, Gaiberg, Germany), to potentiate the humoral immune response. Active ingredients of Gebru adjuvant 100 consist of GMDP (N-acetylglucosaminyl-N-acetylmuramyl-L-alanyl-D-isoglutamine, a cell-wall glycopeptide from *Lactobacillus bulgarious*, at 100 μg/ml) and ultrafilterable particles of lipid DDA (dimethyldioctadecylammoniumchloride, 40 μg/ml) (http://www.gerbu.de). Each round of immunization consisted of four 200 μl subcutaneous injections in widely separated sites on the back of the rabbit and two 100 μl i.m. injections in the biceps femoris of the two hind limbs of the animal. Two boosters were administered at 3 and at 6 wks after the first immunization.

2.8.3 Collecting and Testing the Antisera

Before each series of injections and at 9 wks after the first immunization, a 3 ml sample of blood was collected from the rabbit dorsal ear veins. The serum was tested for specificity (strong reaction to SodC and lack of cross-reaction with other proteins) and for the titer of antibody by Western blot. After reaching the desired titer in nine weeks after the first immunization, the rabbit was terminally anesthetized and the total blood

volume of the animal was collected by cardiac puncture. Serum was prepared by incubating the blood at 4 °C for 12 hrs followed by centrifugation at 1500 ×g for 30 min at 4 °C. The serum was collected and aliquots were stored at –70 °C.

2.9 Data Analysis

2.9.1 Growth Curves

Data collected by monitoring growth of *H. ducreyi* strains were plotted versus time using Microsoft® Excel 97 SR-2 (Microsoft Corp. 1997). Standard error of the mean (SEM) for each time point was calculated by the same program.

2.9.2 Generation time

Equal CFU for each A_{600} was extrapolated by plotting the CFU versus the A_{600}. The equation of the plot relating A_{600} to CFU was obtained by Microsoft® Excel 97 SR-2. Using this equation, A_{600} obtained in other growth assays were converted to A_{600}. Generation time was calculated using the formula: $G = t / 3.3 \log (b/B)$ where the G is the generation time or doubling time (time for the cells to divide), t is the time interval, B is the number of bacteria at the beginning of a time interval and b is the number of bacteria at the end of the time interval.

2.9.3 Statistical Analysis

Data were analyzed statistically using the SigmaStat Wilcoxon signed rank test

(SigmaStat statistical software, SPSS Inc. San Rafael, CA, USA).

CHAPTER 3

RESULTS

3.1 Genetic Approach

The putative ABC transporters involved in bacterial heme uptake have been identified because the genes encoding them have been located adjacent to the genes encoding OMRs for heme, hemoglobin and other heme-compounds. Examples include the *hemRSTUV* gene cluster in *Y. enterocolitica* where hemR is OMR for heme, hemT is the PHBP, hemU is the permease protein and hemV is the ATP binding protein [129] or *hasRADEB* in *Y. pestis* [135], *shuASTUVWYX* in *S. dysenteriae* [153] and *phuSTUVW* in *Ps. aeruginosa* [157]. In each case, the putative ABC transporter is located immediately downstream of the OMR. Since *H. ducreyi* possesses an OMR for each of heme and hemoglobin (*tdhA* [49] and *hgbA* [45] respectively), a genetic analysis was performed to find possible ABC transporters adjacent to these genes.

3.1.1 ORFs Adjacent to *tdhA* and *hgbA*

Using NCBI BLAST, DNA sequences 5 Kbp upstream and 5 Kbp downstream of *tdhA* and *hgbA* were obtained. With the NCBI ORF finder, all the ORFs within the obtained sequences were determined. These ORFs were further analyzed with the NCBI Conserved Domain Search for the presence of any conserved domain related to ABC transporters. None of the ORFs adjacent to these two genes was related to ABC

transporters.

3.1.2 Periplasmic Heme Binding Proteins (PHBPs)

Since the specificity of an ABC transporter is determined by the PBP, a series of NCBI BLAST searches were performed to find any significant similarity with other putative PHBPs including *Y. enterocolitica hemT* (GenBank accession # X77867), *S. dysenteriae shuT* (GenBank accession # U64516), *Ps. aeroginosa phuT* (GenBank accession # AF055999) or *V. cholerae hutB* (GenBank accession # AF016580) in the *H. ducreyi 35000* unfinished genome. No significant similarities were found.

3.2 Biochemical Approach

We then turned to a biochemical method for the identification of a *H. ducreyi* periplasmic heme binding protein. Heme affinity chromatography has previously been used to identify several bacterial OMRs for heme and heme compounds [179-183] including HgbA and TdhA of *H. ducreyi* [45, 49]. We, therefore, applied this technique in an attempt to isolate a heme dedicated PBP from periplasmic preparations of *H. ducreyi.*

Isolation of a PHBP rather than the membrane permease or ATB binding domain has several advantages. Since the PBP is the key determinant in specificity in an ABC transporter [143, 145], this approach would facilitate detection of the heme-specific ABC transporter. Affinity purification of the permease or ATP binding components of an ABC transporter has not been demonstrated. Moreover, the copy number of the PBP usually

exceeds that of the membrane components [147]. In addition, the PBP is soluble and therefore easier to purify compared to the membrane components. Finally, cloning and expression of a soluble protein is easier compared to a membrane protein.

3.3 Minimum Heme-Requirement

Limiting the extracellular concentration of a ligand enhances the expression of the related ABC transporter [123]. Therefore, growing *H. ducreyi* in a heme and iron limited environment would be expected to enhance PHBP expression, facilitating its detection.

A knowledge of the minimum heme concentration necessary to support the growth of *H. ducreyi* is a required first step to establishing the growth conditions for optimal expression of the PHBP.

The minimum heme requirement of *H. ducreyi* was determined by plating *H. ducreyi* on SDM plates [32] with defined concentrations of heme (2 to 120 μg/ml). The advantage of this media was the absence of serum or IsoVitaleX which could serve as a heme/iron source. The inclusion of the iron chelator, EDDHA, also would limit the availability of inorganic iron. In the absence of iron, heme would serve as the only exogenous source of both heme and iron. No difference in growth was seen on plates with heme content higher than 20 μg/ml heme (data not shown). The lowest concentration of heme that allowed *H. ducreyi 35000* to grow was 3 μg/ml (data not shown). However, as the amount of growth at this concentration was insufficient to support large scale protein extraction, 5 μg/ml heme was arbitrarily selected as the

practical minimum heme concentration for growing *H. ducreyi 35000.*

3.4 Isolation of *H. ducreyi* Periplasmic Fractions

Since the PHBP is putatively located in the periplasm, these cell fractions of *H. ducreyi* were subjected to biochemical studies.

Periplasmic fractions of *H. ducreyi* were obtained by either the osmotic shock method or the chloroform method. The osmotic shock method has been previously used for the extraction of *H. ducreyi* periplasm and this method for periplasmic isolation has been previously validated [102, 171] using assays for periplasmic located enzymes, such as Alkaline Phosphatase and Glucose-6-Phosphate Dehydrogenase [102].

3.4.1 SDS-PAGE and Immunoblot Analysis

Periplasmic extracts and cytoplasmic fractions obtained by each method were applied to SDS-PAGE (Figure 3). Differences between the protein profiles of periplasmic fractions isolated by the chloroform and osmotic shock methods were evident (Figure 3). The profile of periplasmic proteins isolated by either method was distinctly different from that of the cytoplasmic fractions (Figure 3).

To verify that the osmotic shock method resulted in cell fractions enriched for the presence of periplasmic proteins, Western immunoblots were performed using antibody to *H. ducreyi* SodC, a previously identified periplasmic protein and antibody to *H. ducreyi* OMPs (Figure 4). Results confirmed the presence of SodC in periplasmic extracts

Figure 3. Electrophoretic patterns of *H. ducreyi 35000* periplasmic and cytoplasmic extracts obtained by chloroform and osmotic shock methods.

Overnight growth of *H. ducreyi 35000* on and SDM plates containing 5 μg/ml heme was subjected to periplasmic and cytoplasmic extraction with the osmotic shock or the chloroform method. Samples were applied to 10% SDS-PAGE. Sizes of the molecular mass markers (in kDa) are shown on the left.

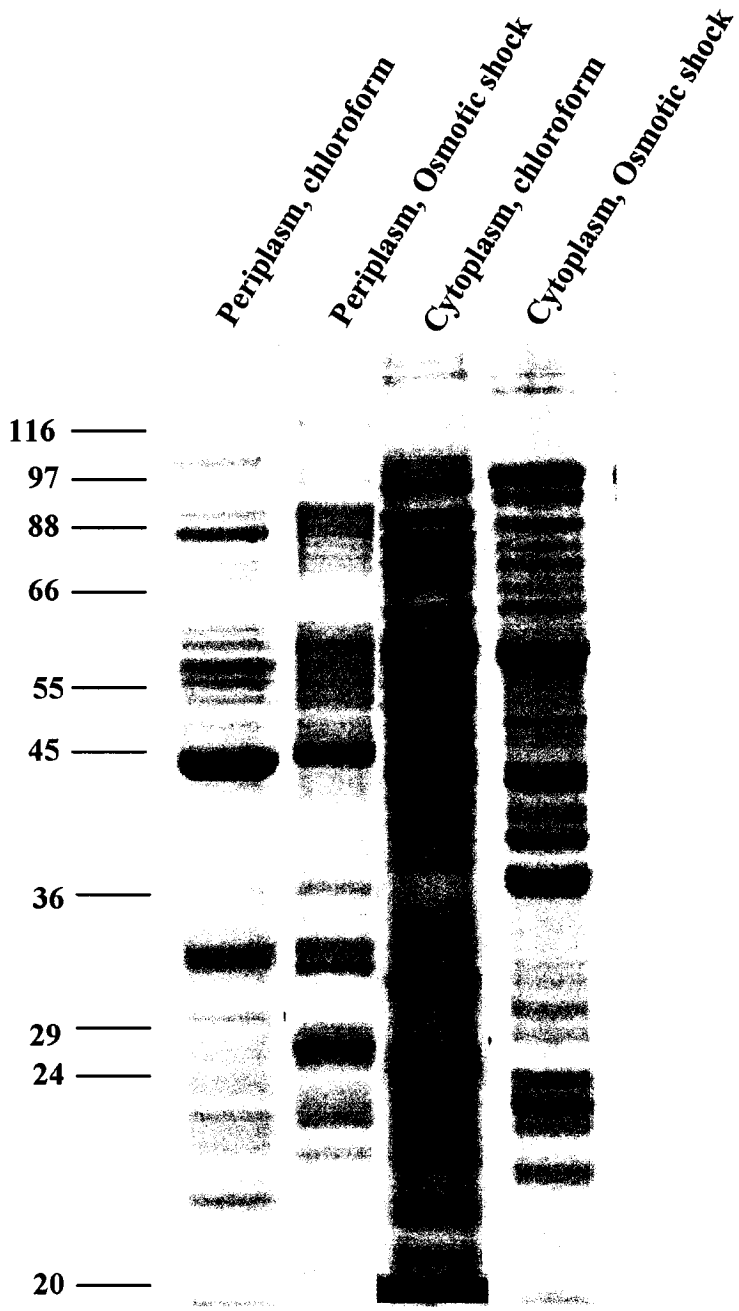
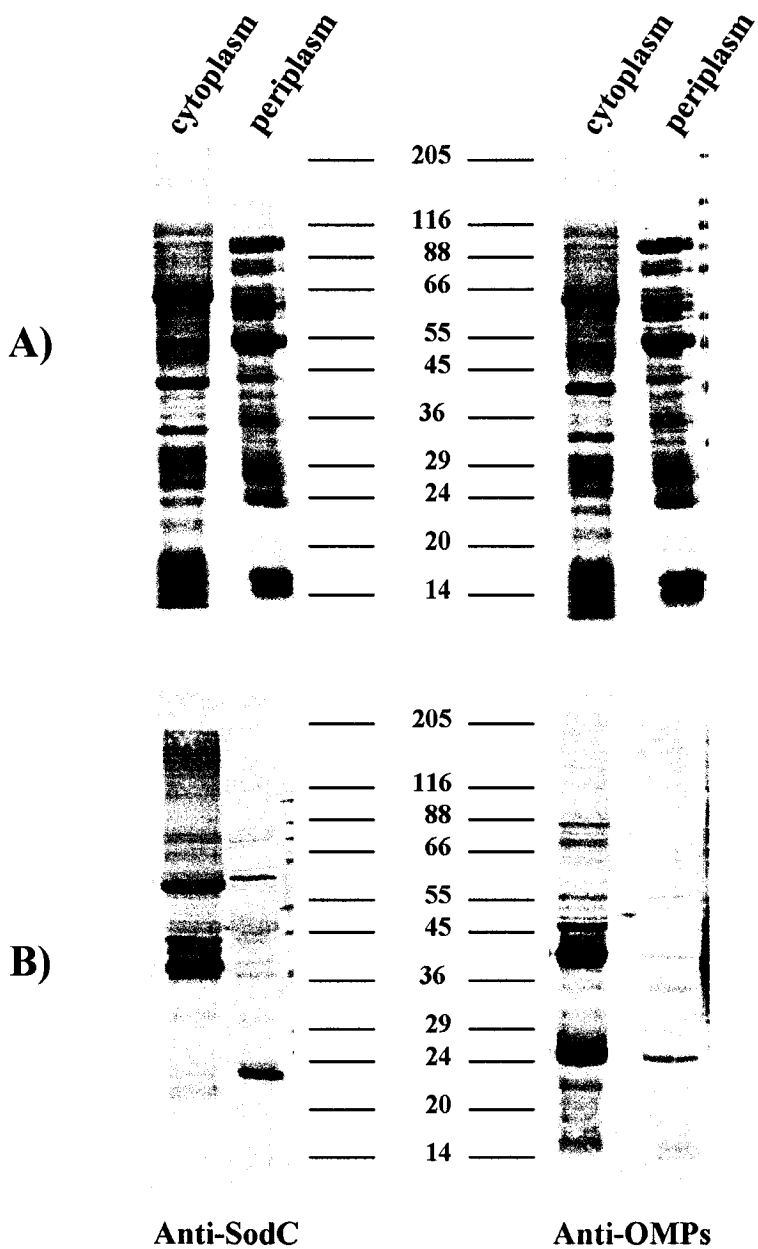

Periplasm, chloroform
Periplasm, Osmotic shock
Cytoplasm, chloroform
Cytoplasm, Osmotic shock
116
97
88
66
55
45
36
29
24
20

Figure 4. Immuno-detection of SodC and OMPs in periplasmic and cytoplasmic fractions of *H. ducreyi 35000* prepared by osmotic shock.

Overnight growth of H. ducreyi 35000 on CA and SDM plates containing 25 μg/ml heme was subjected to periplasmic and cytoplasmic extraction with osmotic shock method. Samples of the extracts were applied to 10% SDS-PAGE (A), followed by Western blotting for detection of SodC and OMPs (B). Sizes of the molecular mass markers (in kDa) are shown on the middle.


A)
B)
cytoplasm
periplasm
cytoplasm
periplasm
205
116
88
66
55
45
36
29
24
20
14
205
116
88
66
55
45
36
29
24
20
14
Anti-SodC
Anti-OMPs

(Figure 4B). Low levels of contamination of the periplasmic fraction with OMPs were observed (Figure 4B).

3.5 Effect of Heme Restriction on Protein Expression

The expression of ABC transporters is regulated by ligand concentration in the environment. Thus, growing *H. ducreyi* in a heme limited environment would be expected to enhance expression of the putative PHBP. Periplasmic fractions prepared from *H. ducreyi 35000*, grown on SDM plates containing 120 μg/ml or 5 μg/ml heme, were analyzed by SDS-PAGE. Several candidate proteins with increased expression under low heme concentration compared to high heme concentration were identified, most notably proteins of 95 kDa, 58kDa, 32 kDa and 29 kDa (Figure 5).

3.6 Periplasmic Heme Binding Protein

To determine whether periplasmic proteins whose expression increased under low heme conditions bound to heme, periplasmic fractions of *H. ducreyi 35000* grown on SDM plates with 5 μg/ml heme were subjected to heme affinity chromatography. The eluted proteins were separated by 10% SDS-PAGE. A band of approximately 45 kDa was consistently identified in eluted preparations (Figure 6).

3.7 Protein Sequencing

To identify this protein obtained from heme affinity chromatography, N-terminal

Figure 5. Change in electrophoretic pattern and expression levels of *H. ducreyi 35000* periplasmic fractions induced by heme limitation.

Overnight growth of *H. ducreyi 35000* on SDM plates containing 120 μg/ml or 5 μg/ml heme were subjected to periplasmic extraction. Samples of the extracts were applied to 10% SDS-PAGE. Sizes of the molecular mass markers (in kDa) are shown on the left.

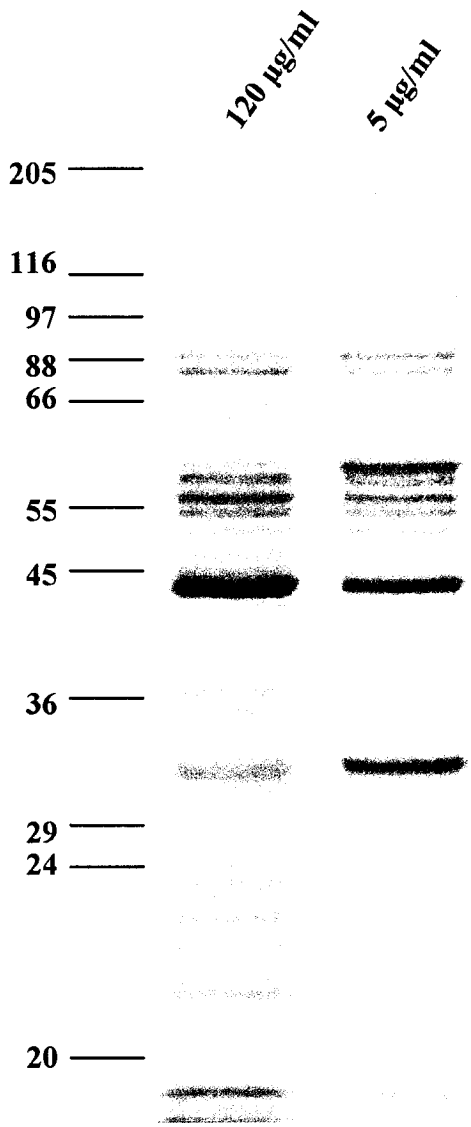
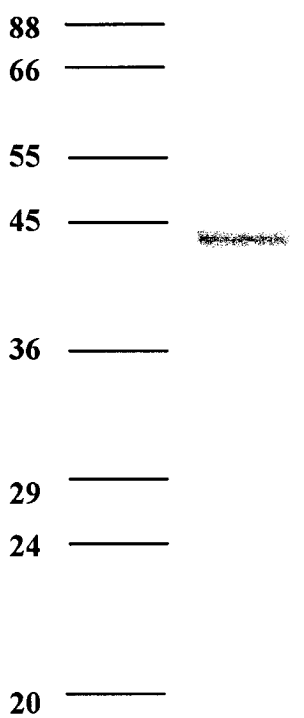

120 µg/ml
5 µg/ml
205
116
97
88
66
55
45
36
29
24
20

Figure 6. Heme binding proteins of *H. ducreyi 35000* periplasm purified by heme affinity chromatography.

Overnight growth of *H. ducreyi 35000* on SDM plates containing 5 μg/ml heme was subjected to periplasmic extraction. A sample of the extract was applied to heme affinity column. Elutes of the column were applied to 10% SDS-PAGE. Sizes of the molecular mass markers (in kDa) are shown on the left.


88
66
55
45
36
29
24
20

sequencing was performed. A sample examined with Mass Spectrophotometry showed that this protein was unique to major protein data bases. However, no sequence information could be obtained from N-terminal sequencing (possibly because of N-terminal blockage). Therefore, internal sequencing, following trypsin digestion, was performed. Two peptide fragments were sequenced and produced the following results: Thr-Thr-Asp-Val-Thr-Gly-Thr-Ile-Glu-Leu-Pro-Glu-Gly-Val-Glu (TTDVTGTIELPEGVE) and Thr-Thr-Leu-Thr-Ala-Ala-Ile-Thr-Thr-Val-Leu-Ala-Lys (TTLTAAITTVLAK).

3.8 Translation Elongation Factor (TEF)

The amino acid sequences obtained from protein sequencing were back-translated to the nucleotide sequences resulting in the following: 5' ACTACAGATGTAACCGGAACGATAGAATTACCAGAAGGAGTAGAG 3' and 5' ACAACATTAACGGCAGCAATAACCACTGTATTAGCAAAA 3'. The NCBI BLAST search of the *H. ducreyi* unfinished genome identified an ORF of 1236 bps (Figure 7). Translation of this ORF produced a protein of 45.5 kDa (Figure 7).

To define the putative function of this protein, an NCBI conserved domain search was performed. Three highly conserved domains present in the *H. ducreyi* protein matched those present in a protein identified as translation elongation factor (TEF) of *E. coli*. In another independent NCBI BLAST search of 220 bacterial genomes, numerous homologous proteins were found with homology approximately 98%. All these

Figure 7. Identification of the prime *H. ducreyi* periplasmic protein obtained from heme affinity column.

Sequences obtained from protein sequencing (printed in red) were located in *H. ducreyi 35000* unfinished genome using NCBI BLAST in an ORF of 1236 bps (A) (ATG: start codon; TAA: stop codon). Translation of the above ORF to amino acids resulted in a 411 amino acid peptide with a mass of 45.5 kDa (B).

A)

```
   1 atgtggttat gtaccatttg taaccgtgtt tatttaagag gctttctagc aatgtctaaa
  61 gaaaaatttg aacgtacaaa accgcacgtt aacgtgggta caatcggcca cgttgaccat
 121 ggtaaaacaa ctctaactgc agcaattact accgtattag caaaacactt cggtggtgct
 181 gcgcgtgcat ttgaccaaat cgataacgcg cctgaagaaa aagcgcgcgg tatcaccatc
 241 aatacttctc acgttgagta cgacactgaa actcgtcact atgctcacgt tgactgtcca
 301 ggacacgccg actatgttaa aaacatgatt actggtgcgg cacaaatgga cggcgctatc
 361 ttagttgtag cagcaactga tggtcctatg cctcaaactc gtgaacacat cttattaggt
 421 cgccaagttg gtgttcctta catcatcgta ttcttaaata aatgcgatat ggtagatgat
 481 gaagaattat tagaattagt tgaaatggaa gttcgtgaac ttctttctca atatgatttc
 541 ccaggtgacg atactcctat cgttcgtggt tcagcattac aagcattaaa tggtgtgcct
 601 gagtgggaag aaaaaatcat tgaattagca caacacttag attcttatat ccctgagcct
 661 gagcgtgcga ttgataaacc tttcttatta ccaatcgaag acgtattctc aatttcaggt
 721 cgtggtacag tagtaaccgg tcgtgttgag cgtggtatca tcaaatcagg tgaagaagtt
 781 gaaatcgtag ggattaaaga aacgacaaaa acaacagtaa ccggtgttga gatgttccgt
 841 aaactattag acgaaggtcg tgcgggtgaa aacgtaggtg ccttattacg tggtactaaa
 901 cgtgaagaaa tcgaacgtgg tcaagtatta gcgaaaccag gtacaattac accacacact
 961 gattttgaat cagaagttta tgtattatca aaagaagaag gtggccgtca tactccattc
1021 ttcaaaggct atcgtcctca gttctacttc cgtacaacgg acgtaacagg aacgattgaa
1081 ttacctgaag gtgttgagat ggtaatgcct ggtgataata tcaagatgac agtaagctta
1141 attcacccta tcgcgatgga cgaaggtctt cgtttcgcta ttcgtgaagg cggtcgtact
1201 gtaggtgccg gcgttgttgc gaaaatcatt aaataa
```

B)

```
  1 MWLCTICNRV YLRGFLAMSK EKFERTKPHV NVGTIGHVDH GKTTLTAAIT TVLAKHFGGA
 61 ARAFDQIDNA PEEKARGITI NTSHVEYDTE TRHYAHVDCP GHADYVKNMI TGAAQMDGAI
121 LVVAATDGPM PQTREHILLG RQVGVPYIIV FLNKCDMVDD EELLELVEME VRELLSQYDF
181 PGDDTPIVRG SALQALNGVP EWEEKIIELA QHLDSYIPEP ERAIDKPFLL PIEDVFSISG
241 RGTVVTGRVE RGIIKSGEEV EIVGIKETTK TTVTGVEMFR KLLDEGRAGE NVGALLRGTK
301 REEIERGQVL AKPGTITPHT DFESEVYVLS KEEGGRHTPF FKGYRPQFYF RTTDVTGTIE
361 LPEGVEMVMP GDNIKMTVSL IHPIAMDEGL RFAIREGGRT VGAGVVAKII K
```

homologues were TEFs. Thus, sequencing analysis indicated that the protein was likely the *H. ducreyi* TEF. The closest homologues were found in other *Haemophilus* species including *P. multocida* and *H. influenzae*. Some of the other bacteria holding homologues were *S. typhimurium, S. enterica, E. coli, S. flexneri* and *Y. pestis*.

Several considerations suggested that TEF was unlikely to be a component of a heme dedicated ABC transporter in *H. ducreyi*. First, TEF is a cytoplasmic located protein [184]. Second, TEF has been implicated in RNA synthesis [185]. Third, there have been no reports indicating that TEF exhibits heme binding activity. Therefore, we concluded that *H. ducreyi* TEF was unlikely to be involved in heme uptake.

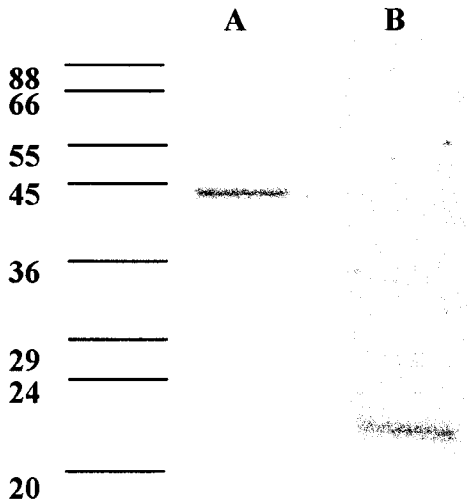
3.9 *H. ducreyi* SodC

During the course of our investigations, Pacello et al reported that *H. ducreyi* SodC demonstrated heme binding activity [103]. This property, coupled with the periplasmic location of SodC, led us to hypothesize that SodC plays a role in heme transport by shuttling heme across the periplasm to the cytoplasmic membrane. Upon further review of our own data, a minor protein corresponding to the published molecular weight of SodC was consistently present in the heme affinity purified preparations (Figure 6). Western blot analysis of the hemin affinity preparations using antisera against SodC provided by Dr. T. Kawula confirmed the identity of this protein as SodC (Figure 8A, lane B). When the total cell lysates of *H. ducreyi* were applied to heme affinity chormatography, SodC appeared to be the main protein affinity purified from the column

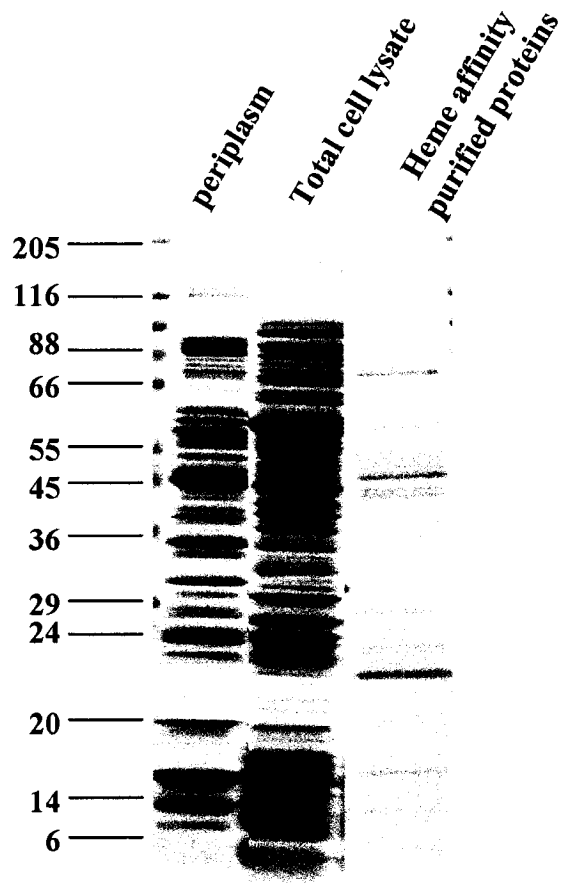
Figure 8. Electrophoretic pattern of *H. ducreyi 35000* total cell lysates and affinity purified heme binding proteins.

Overnight growth of *H. ducreyi 35000* on SDM plates containing 5 μg/ml heme was subjected to periplasmic extraction. A sample of the extract was applied to heme affinity column. Elutes of the column were applied to 10% SDS-PAGE (Panel A, lane A), followed by Western blotting for immuno-detection of SodC (Panel A, lane B). Sizes of the molecular mass markers (in kDa) are shown on the left.

A)


A
B
88
66
55
45
36
29
24
20

B)


periplasm
Total cell lysate
Heme affinity purified proteins
205
116
88
66
55
45
36
29
24
20
14
6

(Figure 8B). This finding was confirmed by Western blot analysis using *H. ducreyi* anti-SodC polyclonal antibody (data not shown).

3.10 Rabbit Polyclonal Antibody Against *H. ducryei* SodC

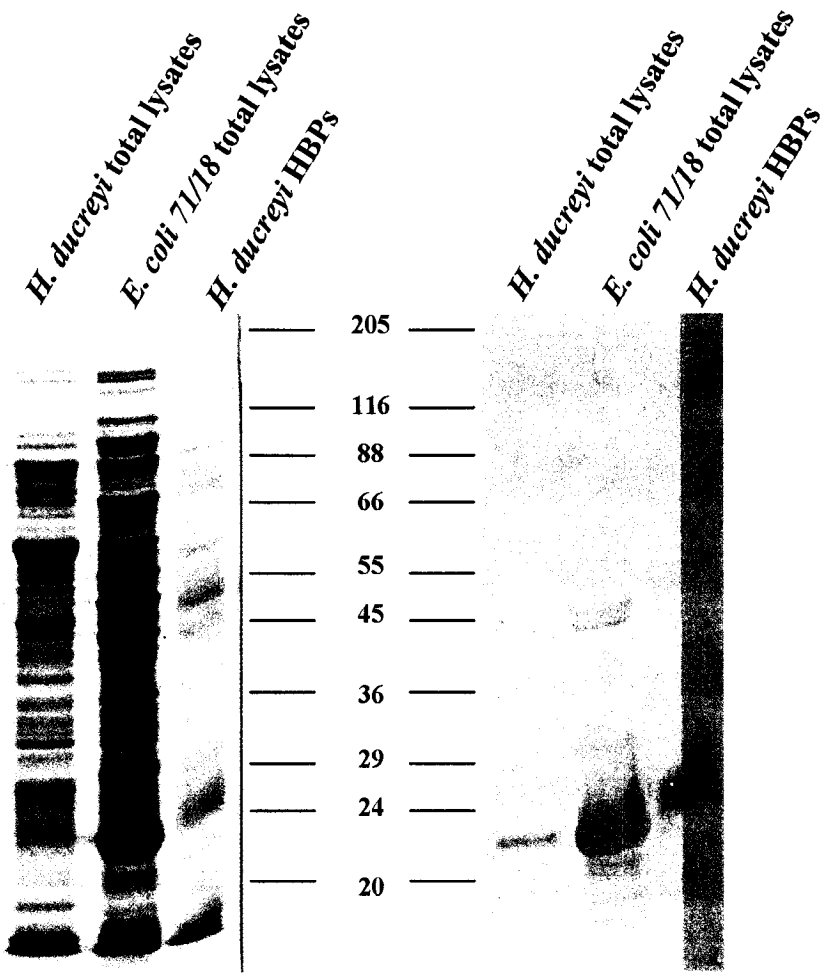
As the anti-SodC antisera obtained from the laboratory of Dr. T. Kawula lacked the required specificity (Figure 4), we raised rabbit polyclonal antisera against *H. ducreyi* SodC. Antibody production required about 1 mg of purified protein. As SodC is expressed at low levels in *H. ducreyi*, *E. coli 71/18* containing the plasmid expression vector *pPHduSod* (Figure 9) was obtained from Dr. A. Battistoni (University of Rome, Rome, Italy). This construct is a derivative of *pUC119* in which expression of *H. ducreyi* SodC is under the transcriptional control of the *lacZ* promoter [103]. SodC was purified from total cell lysates prepared from *E. coli 71/18* by electroelution. Using the purified SodC, a rabbit was immunized and the resulted polyclonal immune sera was collected and designated as anti-SodC. Using the collected antisera, a 22 kDa band was detected in the Western blots of total cell lysates from *H. ducreyi*, *E. coli 71/18* expressing SodC from *pPHduSod* and from heme affinity purifications of periplasmic fractions of *H. ducreyi* cells grown under low heme concentrations (Figure 9). An additional immunoreactive band of approximately 45 kDa was seen in the *E. coli* lysates, likely representing the dimeric form of SodC (Figure 9). The dimeric form of *H. ducreyi* SodC has previously been reported [103].

The concentration of antibody in the antisera was not determined. However,

Figure 9. Specificity of antiserum raised against SodC of *H. ducreyi 35000*.

Specificity of the raised polyclonal antibody against SodC was tested by Western blotting. Samples of *H. ducreyi 35000* total lysates, HBPs and total lysates of *E. coli 71/18* bearing *pPHduSOD* were applied to SDS-PAGE (A) and Western blotting using the raised antibody (B). Sizes of the molecular mass markers (in kDa) are shown in the middle.

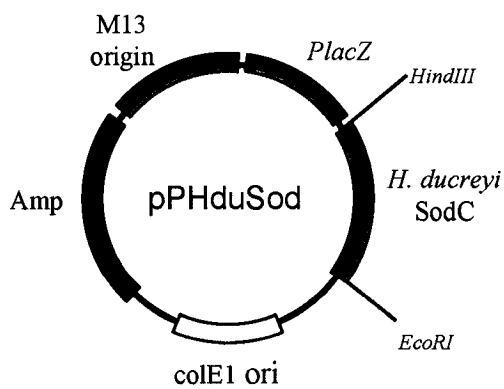
Schematic representation of the plasmid expression vector *pPHduSod* (C).


H. ducreyi total lysates
E. coli 71/18 total lysates
H. ducreyi HBPs
205
116
88
66
55
45
36
29
24
20
H. ducreyi total lysates
E. coli 71/18 total lysates
H. ducreyi HBPs

A)

B)

C)


M13
origin
PlacZ
HindIII
H. ducreyi
SodC
Amp
pPHduSod
EcoRI
colE1 ori

1:2000 to 1:5000 dilution of antiserum reacted with only a protein of approximately 22 kDa in total lysates of *H. ducreyi* (data not shown). Preimmune sera did not detect any protein in *H. ducreyi 35000* or *E. coli 71/18* lysates (data not shown).

3.10 SodC in Different *H. ducreyi* Strains

To determine whether SodC was present in heterologous *H. ducreyi* strains, a Western blot was performed. Total cell lysates from *H. ducreyi* strains *35000*, *C148*, *BG411*, *AX557*, *V1157*, *J1159*, *PPC358/1315* and *36-F-2* were applied to SDS-PAGE followed by Western blot (Figure 10). These strains have been collected previously from different geographical areas (Table 1). Immuno-reactive bands were detected in strains *C148*, *AX557*, *V1157*, *PPC358/1315* and *36-F-2*. No bands were observed in total cell lysates probed from *BG411* and *J1159*.

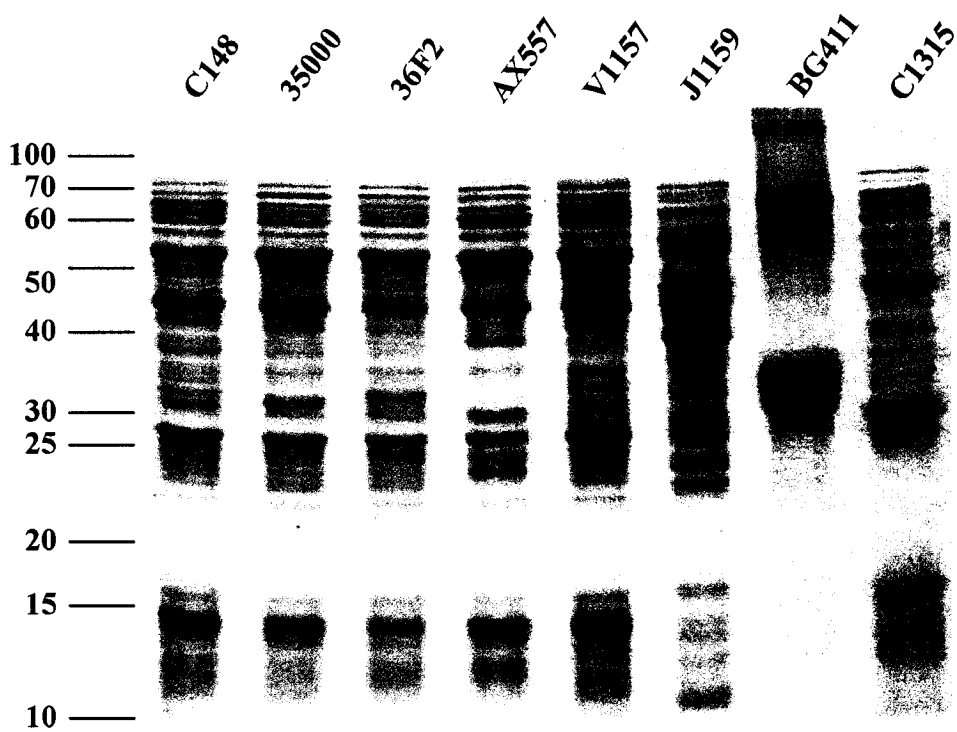
3.11 Construction and Characterization of the *H. ducreyi sodC* Mutant

To investigate the role of SodC in heme transport, a *sodC* mutant of *H. ducreyi* was generated by introducing plasmid *pHDMK4* into *H. ducreyi 35000* [101]. This plasmid, kindly provided by Dr. Simon Kroll (Imperial College of Science, Technology and Medicine, London, UK), and has been constructed by cloning of a 5.2 kb *Eco*RV fragment of *H. ducreyi* chromosome which includes *sodC* into *pIT4* [101]. Then a 1264 bp gene cassette encoding aminoglycoside phosphotransferase conferring

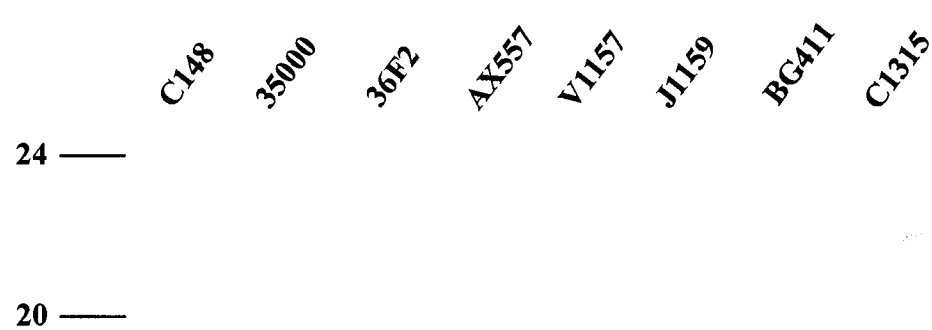
Figure 10. Immuno-reactivity of SodC in different *H. ducreyi* strains with the antiserum against *H. ducreyi 35000* SodC.

Overnight growth of *H. ducreyi* strains *C148, 35000, 36F2, AX557, V1157, J1159, BG411* and *1315* on CA plates were used to obtain total cell lysates. A sample of each extract was applied to SDS-PAGE (A) followed by Western blotting with the antiserum against *H. ducreyi 35000* SodC (B). Sizes of the molecular mass markers (in kDa) are shown on the left.

A)


C148
35000
36F2
AX557
V1157
J1159
BG411
C1315
100
70
60
50
40
30
25
20
15
10

B)


C148
35000
36F2
AX557
V1157
J1159
BG411
C1315
24
20

kanamycin resistance was inserted into the central *Bam*HI site of *sodC* [101].

Three methods were used to confirm that the proper allelic exchange had occurred in the mutant strain.

3.11.1 Genotypic Analysis

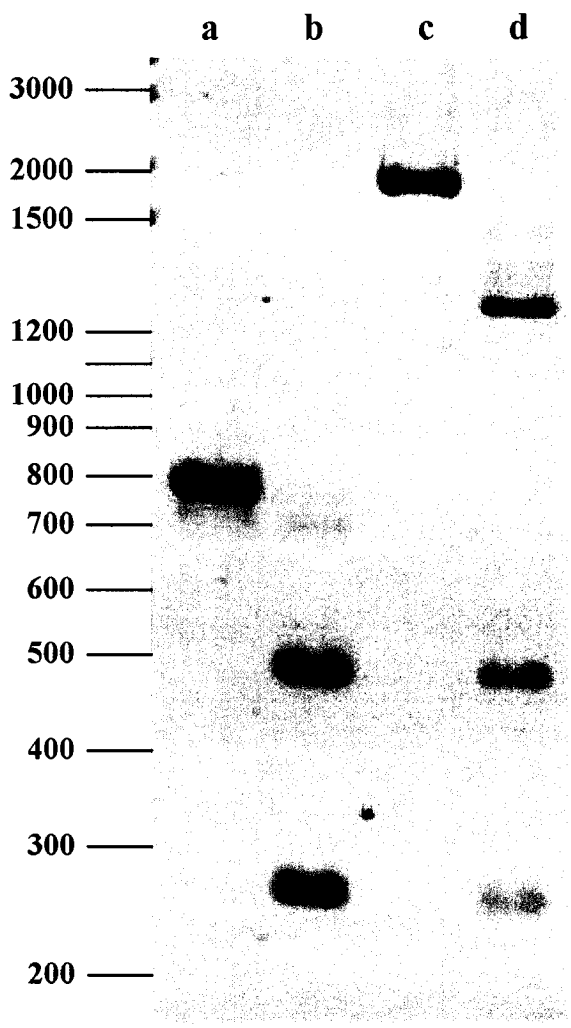
The first method of verifying that the appropriate gene replacement had occurred consisted of colony PCR amplification and restriction digestion analysis of the amplicon. Primers designed to anneal to *sodC* sequences bracketing the kanamycin cartridge would be predicted to generate a larger product (1993 bps) from the *sodC* mutant than from the wild type strain (729 bps). Using oligonucleotides CF and CR, products of the expected size were amplified from the *sodC* mutant and parental strain (Figure 11, lanes a and c).

Restriction digestion of the amplicon derived from the wild type isolate with *Bam*HI would be expected to yield two fragments of 259 and 470 bps. In contrast, in addition to these two fragments, a third product of 1264 bps, arising from the presence of the kanamycin cassette, would result from *Bam*HI digestion of the amplicon from the *sodC* mutant. Restriction analysis of the two PCR amplification products generated the expected fragments (Figure 11, lanes b and a)

In the second method, genomic DNAs purified from the wild type strain *H. ducreyi 35000* and from the *sodC* mutant strain were digested to completion with the restriction enzyme *Eco*RV. The DNA fragments were hybridized against either DIG-labeled kanamycin- resistance cassette or *sodC*- specific gene probes by Southern blot

Figure 11. Colony PCR results for identification of *H. ducreyi SodC.*

sodC was amplified by colony PCR from *H. ducreyi 35000* and a kanamycin resistant colony. Amplified DNA was digested with *Bam*HI. Samples of amplified DNA before and after digestion were applied to agarose gel electrophoresis. Lanes a and c represent PCR product amplified respectively from *H. ducreyi 35000* and *H. ducreyi sodC* mutant. Lane b and d represent *sodC* amplicons respectively from *H. ducreyi 35000* and from *H. ducreyi sodC* mutant digested with *Bam*HI. Sizes of the molecular mass markers (in kbps) are shown on the left.


a
b
c
d
3000
2000
1500
1200
1000
900
800
700
600
500
400
300
200

analysis.

The *sodC* gene probe, a 357 bps fragment internal to the complete *sodC* coding sequence, hybridized to an approximately 5.2 kb fragment derived from the wild-type strain, whereas the mutant strain displayed a hybridizing fragment of approximately 6.5 kb (Figure 12A). The increase in size of the 6.5 kb fragment corresponds to the presence of the kanamycin cassette in the *sodC* locus of the mutant strain, as indicated by hybridization of this same fragment to the kanamycin-specific probe (Figure 12B). As expected, the DNA preparation from the wild type strain did not bind to this probe (Figure 12B). These results indicate that a single copy of the mutated *sodC* gene was present in the *sodC* mutant.

3.11.2 Phenotypic Analysis

To confirm the absence of *sodC* in the mutant strain, whole cell lysates were reacted with *sodC*-specific polyclonal antiserum in Western immunoblots. The antibody recognized a 22 kDa band corresponding to SodC in the wild-type strain (Figure 13B). In contrast, no immuno-reactive band was observed in the mutant strain (Figure 13B). The results indicated that SodC expression either in full length or truncated form was abolished in the mutant.

3.12 Growth Characteristics of the Mutant

Growth assays were conducted to determine the ability of the *sodC* mutant to use

Figure 12. Southern blot analysis of *H. ducreyi sodC*.

Genomic DNA of *H. ducreyi 35000* and of *H. ducreyi sodC* were digested with *Eco*RV and were applied to agarose gel electrophoresis followed by electro-transfer to nylon membranes. Membranes were hybridized with DIG-labeled probes specific for *sodC* (A) and the kanamycin resistance cassette (B).

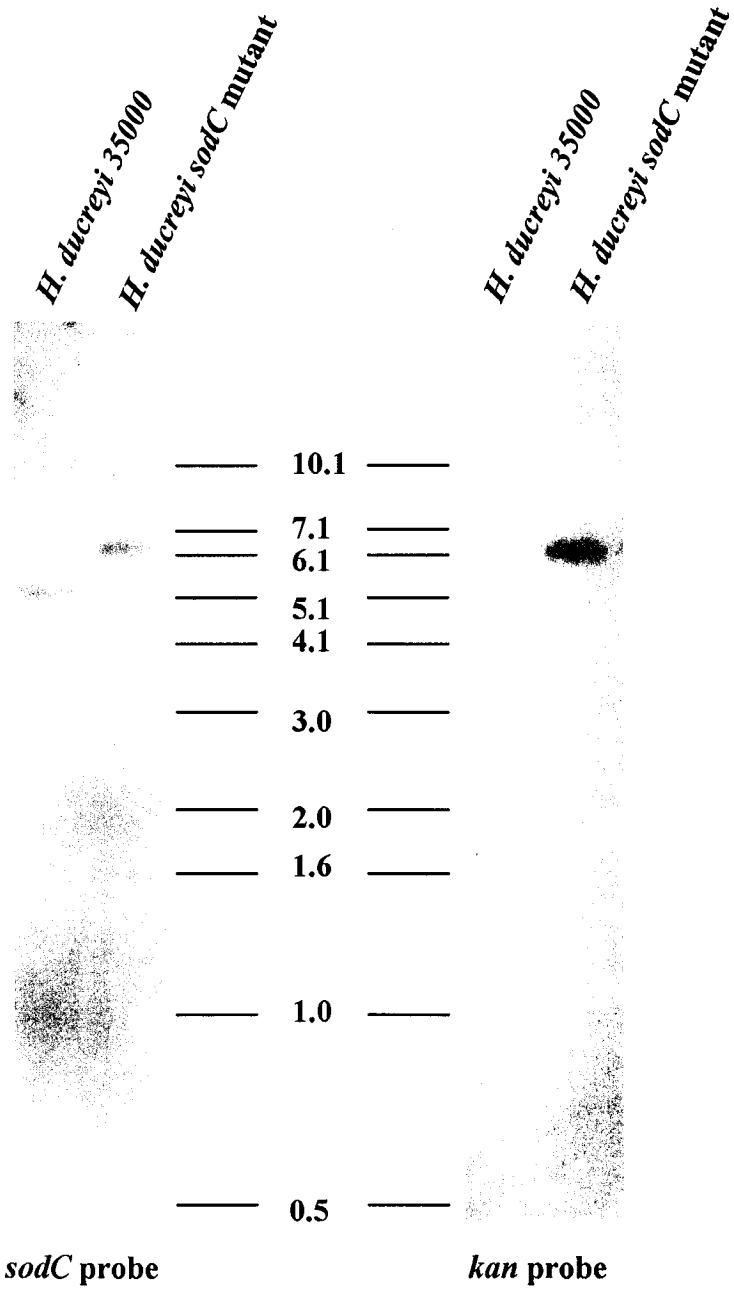
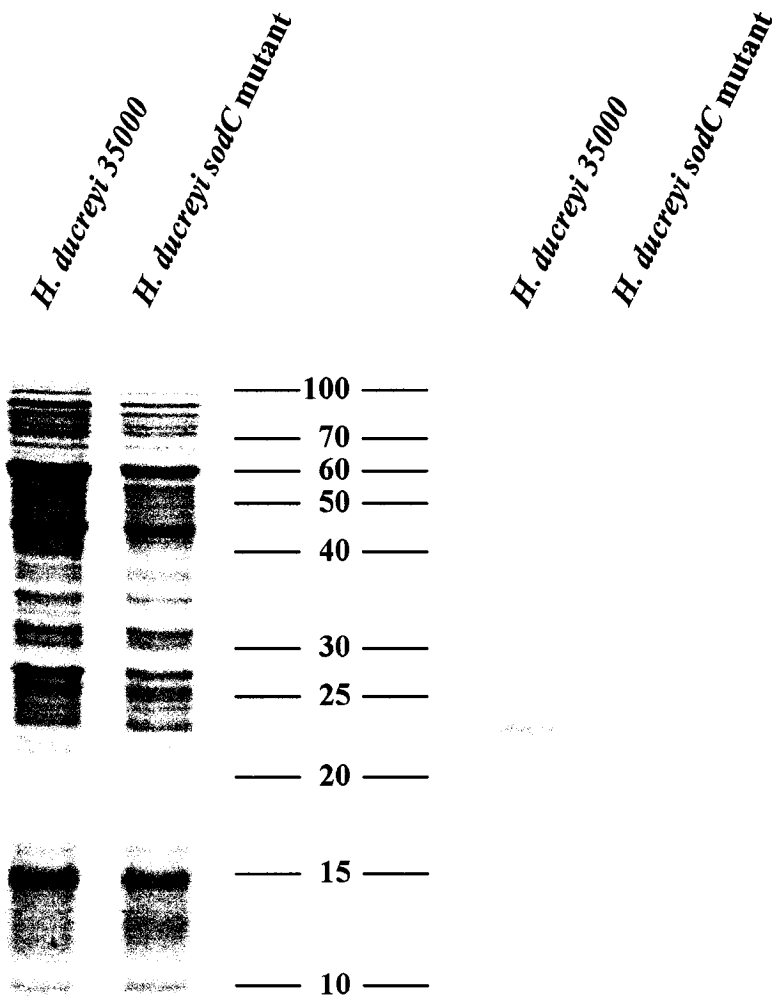

H. ducreyi 35000
H. ducreyi sodC mutant
H. ducreyi 35000
H. ducreyi sodC mutant
10.1
7.1
6.1
5.1
4.1
3.0
2.0
1.6
1.0
0.5
sodC probe
kan probe
A
B

Figure 13. Lack of SodC expression in *H. ducreyi sodC* mutant.

Total cell lysates of *H. ducreyi 35000* and *H. ducreyi sodC* mutant were separated by 10% SDS-PAGE (A) followed by Western blotting with antiserum against SodC (B).


H. ducreyi 35000
H. ducreyi sodC mutant
100
70
60
50
40
30
25
20
15
10
A)
H. ducreyi 35000
H. ducreyi sodC mutant
B)

bovine heme as the sole exogenous source of heme and iron.

The growth of the *sodC* mutant as monitored by both A_{600} (Figure 14A) and CFU (Figure 14B) appeared impaired compared to that of the parental strain when heme was provided at a concentration of 50 μg/ml. The overlap in data points is in part due to the intrinsic property of *H. ducreyi* to aggregate in broth cultures. This characteristic is most pronounced when growth is assessed by numeration of CFUs. Therefore, generation (or doubling) times were compared. Average of generation times were calculated for each time interval up to the 12 hrs. A statistical difference ($P = 0.031$) in the generation times between the *sodC* mutant and the wild type-strain was observed.

These results indicated that SodC contributed to the growth of *H. ducreyi* when heme was supplied as the sole source of heme and iron at a concentration of 50 μg/ml.

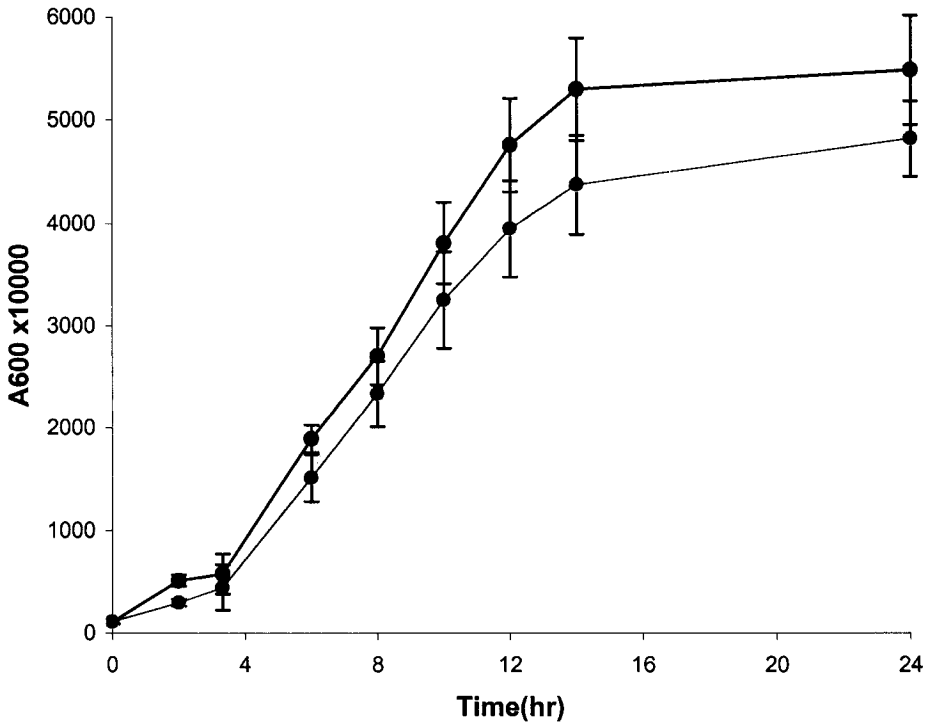
To explore this role further, growth assays were performed under low heme conditions (5 μg/ml). Such a heme limited environment would be predicted to exaggerate the difference in growth kinetics between the *sodC* mutant and the wild-type strain if SodC participated in heme-iron acquisition. However, no difference in growth between the two strains, as measured by both A_{600} and generation times, was observed (Figure 23).

Figure 14. Monitoring *H. ducreyi 35000* and *H. ducreyi sodC* growth in 50 μg/ml heme.

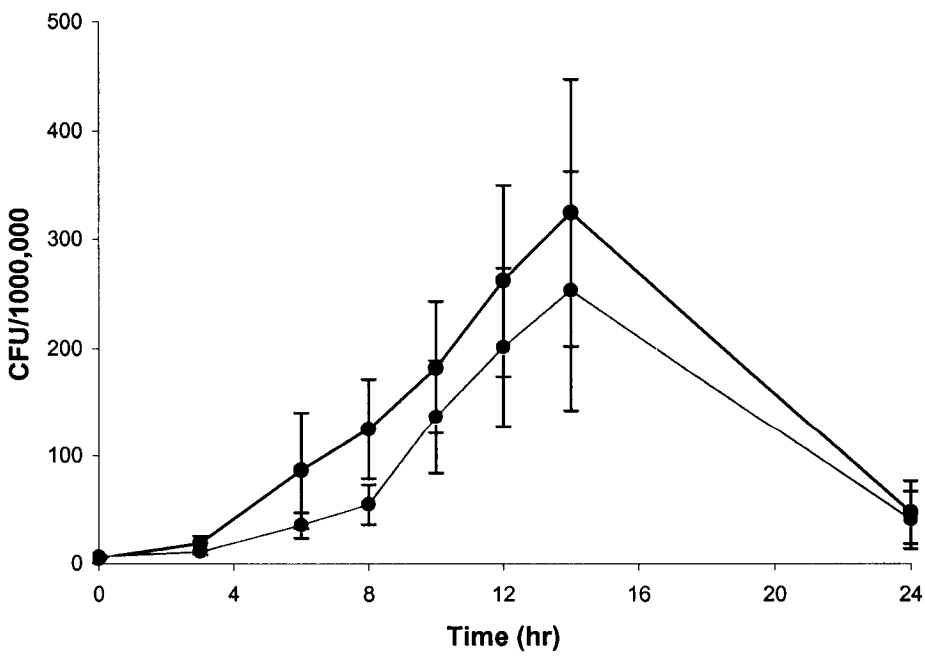
H. ducreyi35000 and *H. ducreyi sodC* mutant were cultured for 24 hrs in GC broth containing 50 μg/ml heme. Growth was monitored by measuring A_{600} (A) and plating serial dilutions for obtaining the CFU (B). Error bars show SEM. Each data point represents the mean of 6 separate experiments measuring A_{600} or 3 experiments measuring CFU.

Legend. Black line = *H. ducreyi35000*, Red line = *H. ducreyi sodC* mutant.

A)


A600 x10000
6000
5000
4000
3000
2000
1000
0
0
4
8
12
16
20
24
Time(hr)

B)


CFU/1000,000
500
400
300
200
100
0
0
4
8
12
16
20
24
Time (hr)

3.13 Functional and Genetic Complementation of the *H. ducreyi sodC* mutant

As SodC in *H. ducreyi* possesses both SOD and heme binding activities, separating these properties would be essential to clarify the role of SodC in heme uptake or transport. Therefore, complementing the *H. ducreyi sodC* mutant with versions of *sodC* displaying either heme binding or superoxide dismutase activity was necessary. These SodC versions were kindly provided by Dr. A. Battistoni [103]. *sodCH86E* (*SE*) shows less affinity for heme but normal superoxide dismutase activity (sod+heme-) as a result of substitution of nucleotides C457 and C459 with G and A respectively (NCBI GenBank, accession # U47664) [103]. *sodCL81FH82QD83G* (*SQ*) has normal heme affinity but reduced superoxide dismutase activity (sod-heme+) as a result of substitution of nucleotides A444, C447 and A449 with C, A and G respectively (regarding the NCBI GenBank, accession # U47664). The heme binding and SOD activity of the mutants were assessed by the absorption spectra (Resonance Raman and electronic absorption spectra), acid/acetone extraction and by the staining of SDS-PAGE gels for heme-dependent activity [103].

3.13.1 Vector Construction

The *E. coli-H. ducreyi* shuttle vector *pFP12* [168] was used for transformation and complementation of the *H. ducreyi sodC* mutant. This vector has been shown to replicate stably in both *E. coli* and *H. ducreyi*. Moreover, the plasmid has two MCSs and

a chloramphenicol resistance cassette for selection purposes. The relatively small size of this vector (6.6 kbp) compared to the other available shuttle vectors for *H. ducreyi* was another advantage. Finally, this vector replicates in *H. ducreyi* only in very low number (less than 10), which decreases the gene dosage effect.

The mutated versions of *sodC*, along with the wild type *sodC*, were cloned into *pFP12*, creating vectors *pSE*, *pSQ* and *pSW*, respectively (Figure 15). The newly constructed vectors were then cloned into *E. coli Top 10* creating *E. coli SW*, *E. coli SE* and *E. coli SQ*. These plasmids were then isolated from transformed *E. coli* and subjected to DNA sequencing. The results confirmed the identity of each plasmid regarding the mutations (Figure 16). Expression of wild type or mutated *sodC* from these plasmids in *E. coli* was confirmed by Western blot analysis (Figure 17).

3.13.2 Promoter Replacement

In plasmids *pSW*, *pSE* and *pSQ*, the cloned genes were expressed from the *lacZ* promoter (*Plac*). Although pFP12 is a low copy number plasmid, the possibility of overexpression of these various *sodC* alleles existed. Such an occurrence would potentially mask the effects of functional complementation. Therefore, the *lacZ* promoter was replaced by the native *sodC* promoter resulting in the plasmid constructs *pPSW*, *pPSE* and *pPSQ* (Figure 15). The replacement was performed by excising the *lacZ* promoter as an *AseI-Kpn*I (the most suitable available restriction sites) fragment from *pSW*, *pSE* and *pSQ* and cloning the PCR amplified *sodC* promoter into the vector. The location of native *sodC* promoter in the above mentioned plasmids was the same as in the

Figure 15. Construction of new plasmids used in this study

pSW, pSE, pSQ were created by replacing *EGFP* in *pFP12* respectively with *sod, sod86E* and *sod81F82Q83G* between *Kpn*I and *Not*I sites. Replacement of *plac* with *psod* between *Ase*I and *Kpn*I sites in the resultant plasmids created, respectively, *pPSW*, *pPSE* and *pPSQ*.

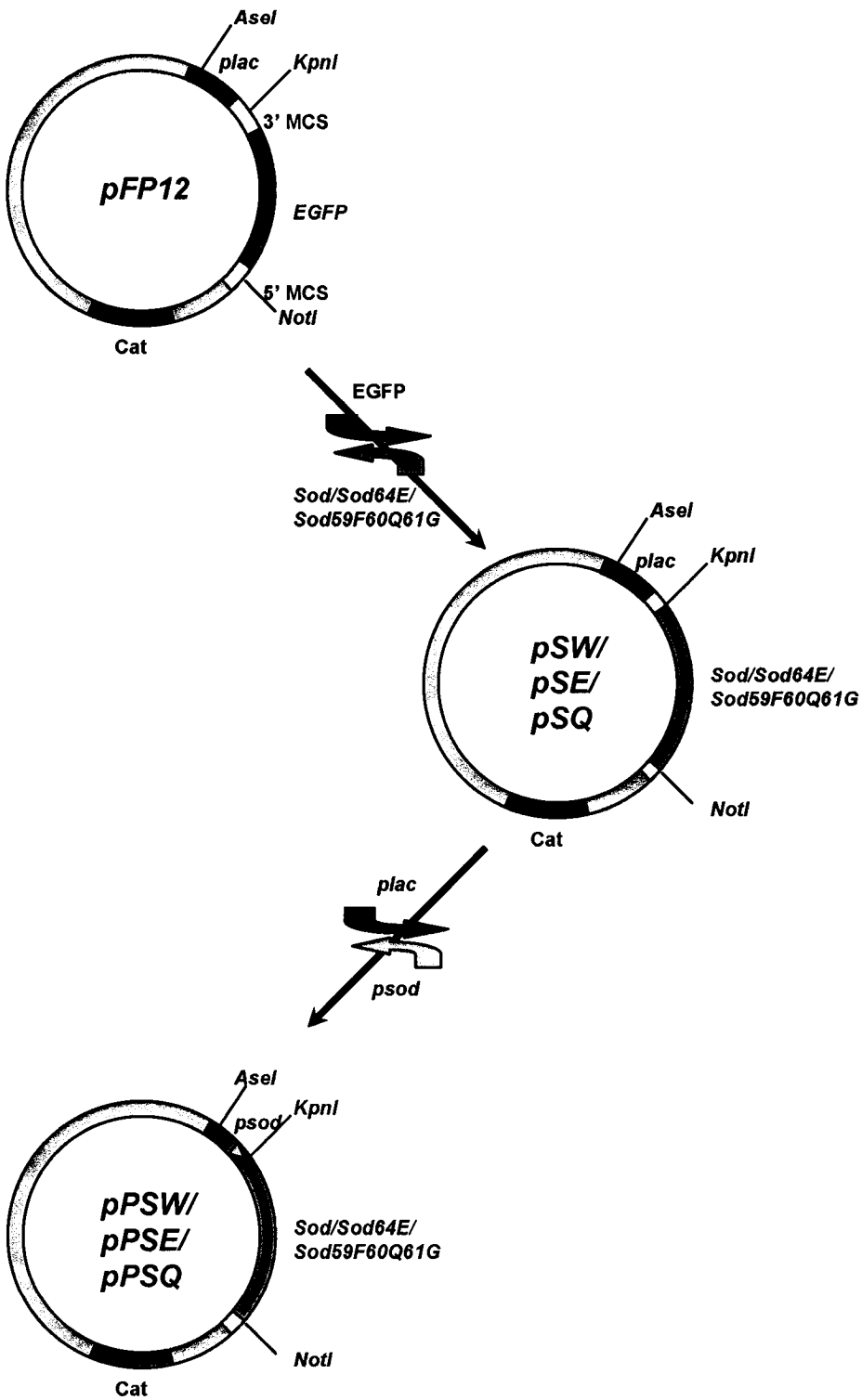

AseI
plac
KpnI
3' MCS
pFP12
EGFP
5' MCS
NotI
Cat
EGFP
Sod/Sod64E/
Sod59F60Q61G
AseI
plac
KpnI
pSW/
pSE/
pSQ
Sod/Sod64E/
Sod59F60Q61G
NotI
Cat
plac
psod
AseI
psod
KpnI
pPSW/
pPSE/
pPSQ
Sod/Sod64E/
Sod59F60Q61G
NotI
Cat

Figure 16. Point mutations in *sodCL59FH60QD61G* and *sodH64E*.

Plasmids *pSW/pPSW* (bearing wild type *sodC*), *pSE/pPSE* (bearing *sodCH86E*) and *pSQ/pPSQ* (bearing *sodCL81FH82QD83G*) were subjected to DNA sequencing (B, C and D, respectively). The wild-type *sodC* sequence is shown in A (NCBI GenBank, accession # U47664).

Mutated nucleotides have been underlined and shaded. Each three nucleotides coding the same amino acid have been shown in a different color (green/blue/red). Location of mutations have been shown with bold letters, same as start and stop codon (ATG, TAA).

A)

```
   1 CATTTTGCAT TTGTGTTGCT CCTAATATGA AGATGAATAG ATTACGTTCT AATTTGCGCC
  61 ATTGCAAATA GGGCTGATAA ATACAGGATT TTATATGAAA ATGCAAATAG ATTTCTGGTC
 121 GAGAGTAAAT CTTTGCATAA ATCGACCGCT ATTAGTAAAA TAATGCTAGC CGATCATGGC
 181 TTTATTTAAT AAGGAGATAA AATGAAATTA ACGAAAGTCG CGTTATTCTC ACTTGGTTTA
 241 TTTGGTTTTT CATCAATGGC TTTGGCACAT GGTGATCATA TGCATAATCA TGACACCAAA
 301 ATGGATACCA TGTCCAAAGA TATGATGTCA ATGGAAAAAA TTGTAGTGCC TGTGCAACAA
 361 TTAGATCCAC AAAATGGTAA TAAAGATGTT GGTACAGTAG AAATTACTGA ATCTGCATAT
 421 GGTTTAGTAT TTACACCAAA ATTACACGAT TTAGCCCACG GATTACATGG CTTCCATATT
 481 CACGAAAAAC CAAGCTGTGA ACCAAAAGAA AAAGACGGTA AATTAGTAGC AGGTTTAGGT
 541 GCCGGTGGCC ATTGGGATCC AAAGCAAACT CAAAAACATG GCTATCCTTG GTCAGATGAT
 601 GCGCATATGG GCGATTTACC AGCATTATTT GTGATGCATG ATGGTTCAGC AACAACGCCT
 661 GTATTGGCAC CACGCCTTAA AAAATTAGCA GAAGTTAAAG GCCACTCTTT AATGATTCAC
 721 GCAGGTGGTG ATAACCACTC AGATCACCCA GCACCACTTG GCGGTGGCGG TCCTCGTATG
 781 GCATGCGGTG TAATTAAATA ATTGATTTGC CATAACGAAA AAGGGGCGTT GAGCGTCCCT
 841 TTTATGTTTT TAATTTACCC TCTTTAAGAT GAAATAATTT GTCTTGCGCA TATTCTTGCC
 901 AATGCATTTG ATTAAGTTGT TCAGCAGTGA TTGCAGTCAC AAATACTTGT GAACCGGTTT
 961 CACGTAAACG ATGTGCAAGT AATTCGCGCT TCATTGGATC AAGCTCTGAG GCGAAATCAT
1021 CAATTAAGAA TAAACATTGA CGTT
```

B)

```
361 TTAGATCCAC AAAATGGTAA TAAAGATGTT GGTACAGTAG AAATTACTGA ATCTGCATAT
421 GGTTTAGTAT TTACACCAAA ATTACACGAT TTAGCCCACG_GATTACATGG CTTCCATATT
481 CACGAAAAAC CAAGCTGTGA ACCAAAAGAA AAAGACGGTA AATTAGTAGC AGGTTTAGGT
```

C)

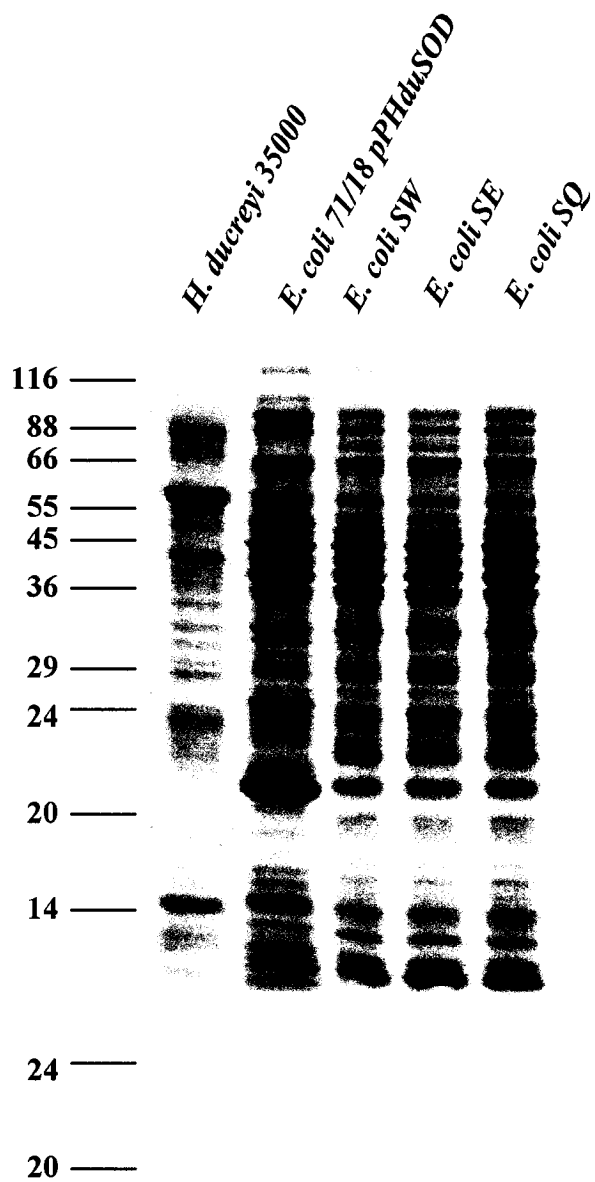
```
361 TTAGATCCAC AAAATGGTAA TAAAGATGTT GGTACAGTAG AAATTACTGA ATCTGCATAT
421 GGTTTAGTAT TTACACCAAA ATTACACGAT TTAGCCGAAG GATTACATGG CTTCCATATT
481 CACGAAAAAC CAAGCTGTGA ACCAAAAGAA AAAGACGGTA AATTAGTAGC AGGTTTAGGT
```

D)

```
361 TTAGATCCAC AAAATGGTAA TAAAGATGTT GGTACAGTAG AAATTACTGA ATCTGCATAT
421 GGTTTAGTAT TTACACCAAA ATTCCAAGGT TTAGCCCACG GATTACATGG CTTCCATATT
481 CACGAAAAAC CAAGCTGTGA ACCAAAAGAA AAAGACGGTA AATTAGTAGC AGGTTTAGGT
```

Figure 17. Immuno-detection of *sodC* expression in transformed *E. coli* with *pSW*, *pSE* and *pSQ*.

Overnight growth of *E. coli* strains *SW*, *SE* and *SQ*; *H. ducreyi 35000*; and *E. coli 71/18* bearing *pPHduSOD* was used to prepare total cell lysates. Samples of the extracts were applied to SDS-PAGE (A) and a Western blot analysis was performed with antiserum raised against SodC (B). Numbers represent the molecular weight in kDa.


H. ducreyi 35000
E. coli 71/18 pPHdsSOD
E. coli SW
E. coli SE
E. coli SQ
116
88
66
55
45
36
29
24
20
14
24
20

H. ducreyi chromosome relative to the start codon. This strategy would also partially address titration effects in which a high copy number vector interferes with proper gene regulation. *E. coli Top10* cells were transformed with these plasmid constructs to generate plasmids *pPSW*, *pPSE*, *pPSQ*. The plasmids were subjected to DNA sequencing. Results confirmed the replacement of the *plac* with the *psod* in these plasmids (Figure 18). Western blotting also, confirmed the expression of wild type and the mutated *sodC* genes from these vectors (Figure 19)

3.13.3 Construction of the Backbone Plasmid Control

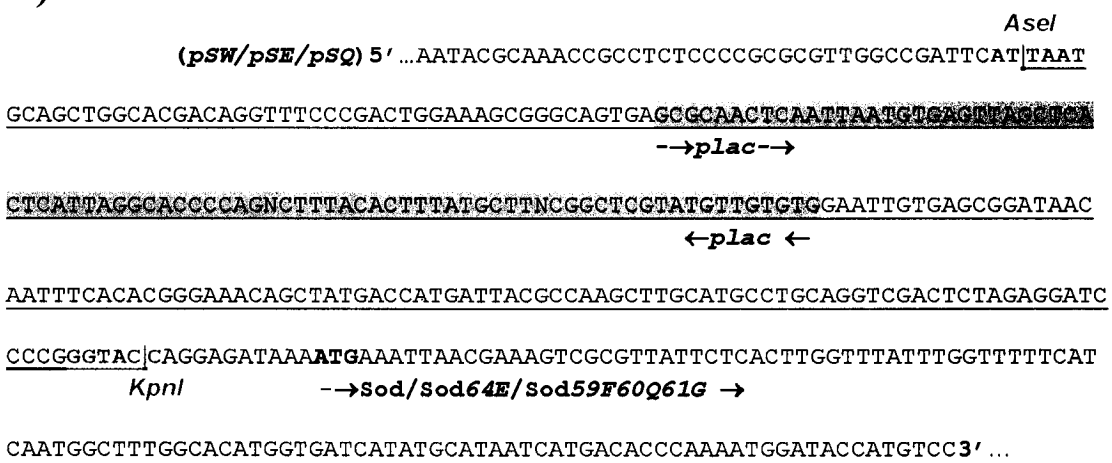
A control vector for the complementation studies was needed. This vector would share the same backbone DNA sequence and structure as the plasmids *pPSW*, *pPSE* or *pPSQ*, but without *psod* and the various versions of *sodC*. Since there were no restriction sites upstream of the promoter compatible with restriction sites in the 5'MCS, the only available restriction site, *AseI*, along with the *Not*I in the 5'MCS were used for excision of the fragment of interest. Blunt end ligation was performed for religation of the vector backbone. Several attempts to religate the above plasmid after the excision of the *psod-sodC* as an *AseI-Not*I fragment were unsuccessful. Therefore, *pFP10* [168] (provided kindly by Dr. J. R. Dillon; University of Ottawa) was considered as the required negative control.

pFP10 is the parent vector of *pFP12* [168] (Figure 21). The difference of *pFP10* with the ideal control vector is the loss of 161 bps, including 40 bps upstream of the 5' MCS and 121 bps down stream of the 3' MCS (Figure 18). These sequences flank the

Figure 18. Replacement of *plac* with *psod*.

Plasmids *pPSW*, *pPSE* and *pPSQ* were sequenced (B) (all three resulted in the same sequence as shown) and compared to sequence of *pSW/pSE/pSQ* (A). Both promoters (*plac* and *psod*) have been shaded and the excised and replaced sequences have been underlined. *Ase*I and *Kpn*I are the restriction sites used for replacement of *plac* with *psod*. ATG: Start codon.

A)


AseI
(pSW/pSE/pSQ) 5′ …AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAT|TAAT
GCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACTCAATTAATGTGAGTTAGCTCA
-→plac-→
CTCATTAGGCACCCCAGNCTTTACACTTTATGCTTNCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAAC
←plac ←
AATTTCACACGGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATC
CCCGGGTAC|CAGGAGATAAAATGAAATTAACGAAAGTCGCGTTATTCTCACTTGGTTTATTTGGTTTTTCAT
KpnI -→Sod/Sod64E/Sod59F60Q61G →
CAATGGCTTTGGCACATGGTGATCATATGCATAATCATGACACCCAAAATGGATACCATGTCC3′…

B)

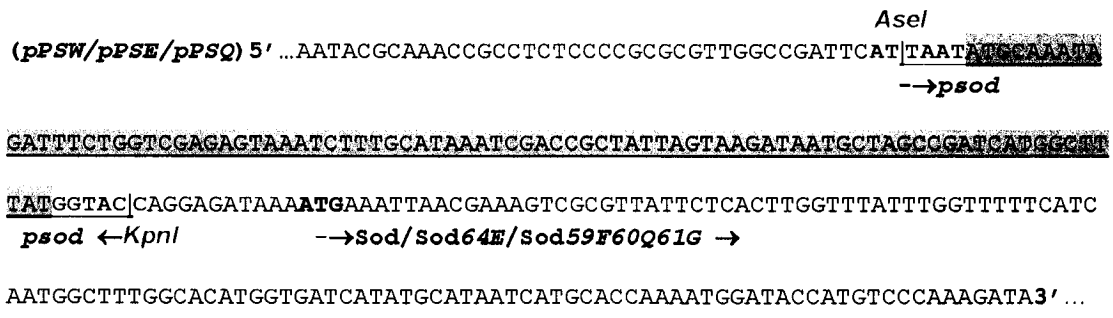
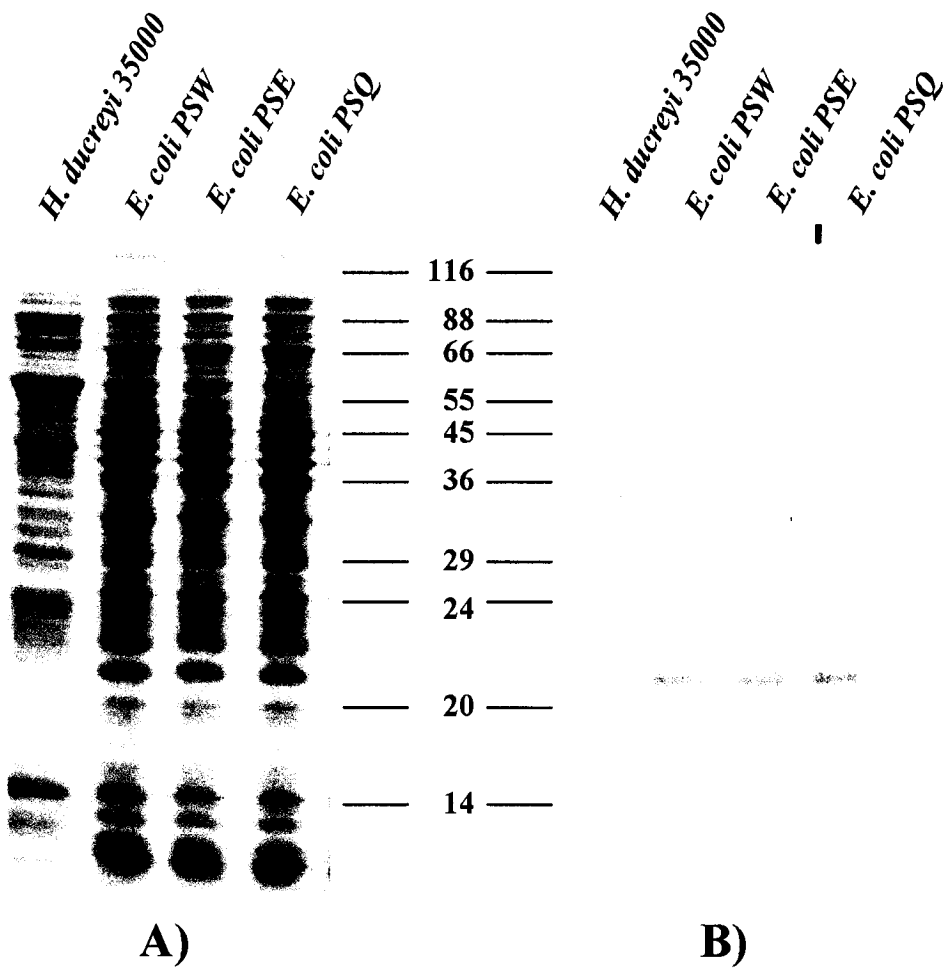

AseI
(pPSW/pPSE/pPSQ) 5′ …AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAT|TAATATGCAAATA
-→psod
GATTTCTGGTCGAGAGTAAATCTTTGCATAAATCGACCGCTATTAGTAAGATAATGCTAGCCGATCATGGCTT
TATGGTAC|CAGGAGATAAAATGAAATTAACGAAAGTCGCGTTATTCTCACTTGGTTTATTTGGTTTTTCATC
psod ←KpnI -→Sod/Sod64E/Sod59F60Q61G →
AATGGCTTTGGCACATGGTGATCATATGCATAATCATGCACCAAAATGGATACCATGTCCCAAAGATA3′…

Figure 19. Immuno-detection of *sodC* expression in transformed *E. coli* with *pPSW*, *pPSE* and *pPSQ*.

Overnight growth of *E. coli* strains *PSW*, *PSE* and *PSQ*; and *H. ducreyi 35000* was used to prepare total cell lysates. Extracts were applied to SDS-PAGE (A) and Western blotting was performed with antiserum raised against SodC (B). Sizes of the molecular mass markers (in kDa) are shown between panel A and panel B.


H. ducreyi 35000
E. coli PSW
E. coli PSE
E. coli PSQ
116
88
66
55
45
36
29
24
20
14
H. ducreyi 35000
E. coli PSW
E. coli PSE
E. coli PSQ
A)
B)

EGFP in *pEGFP* gene and were incorporated into *pFP10* to create *pFP12*. Since no function has been assigned to these sequences (BD Biosciences Clontech; Basingtoke, Hampshire, UK), *pFP10* was considered as an acceptable plasmid control and was electroporated into *H. ducreyi sodC* to generate *H. ducreyi BB*. Western blots confirmed absence of SodC in lysates of the *H. ducreyi BB* (figure 20).

3.14 Complementation of the *H. ducreyi sodC* Mutant

pPSW, *pPSE* and *pPSQ* were electroporated into *H. ducreyi sodC* to generate respectively *H. ducreyi PSW*, *H. ducreyi PSE* and *H. ducreyi PSQ*. The nucleotide sequence of these plasmid constructs, isolated from the transformed *H. ducreyi* strains verified the presence of the appropriate point mutations in *sodC* gene (Figure 16). The complemented *H. ducreyi* strains expressed SodC, as evidenced by Western blot analysis (Figure 20). No immunoreactive bands were present in lysates obtained from the *sodC* mutant and *H. ducreyi sodC* mutant bearing the plasmid backbone pFP10.

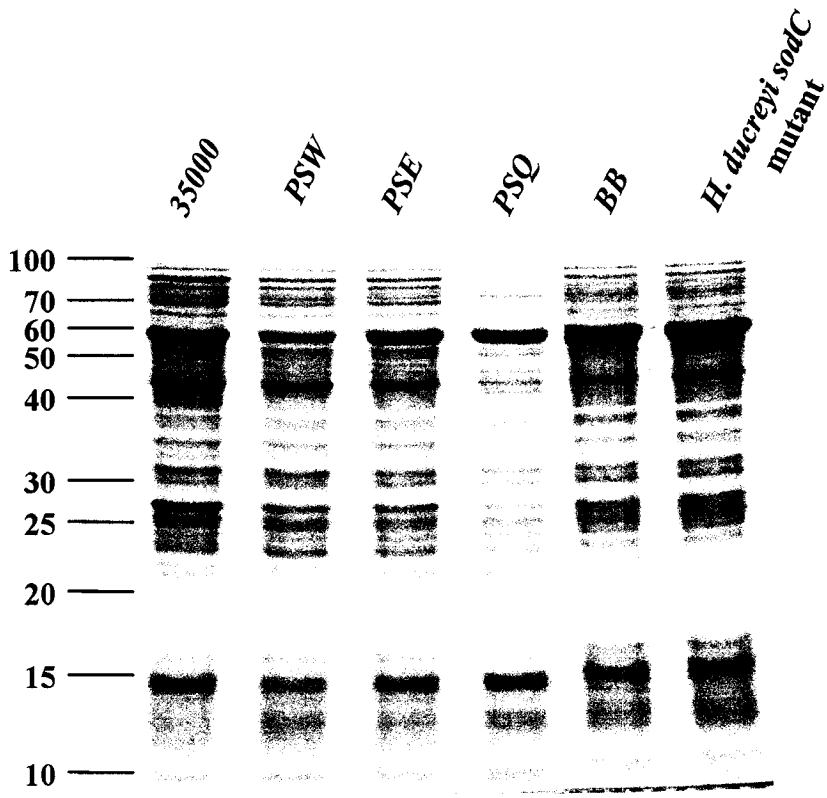
3.15 Functional Complementation of the *H. ducreyi sodC* Mutant

Growth assays were conducted to determine the ability of the *H. ducreyi sodC* mutant complemented by the various versions of *sodC* to use heme as the sole exogenous source of heme and iron. It was expected that the *H. ducreyi sodC* mutant complemented with the wild-type *sodC* (*pPSW*), to show enhanced growth compared to the other

Figure 20. SodC Expression in *H. ducreyi* strains *PSW, PSE, PSQ* and *BB*.

Total lysates of *H. ducreyi 35000*, *H. ducreyi PSW*, *H. ducreyi PSE*, *H. ducreyi PSQ*, *H. ducreyi BB.* and *H. ducreyi sodC* obtained from overnight growth on chocolate agar plates were applied to SDS-PAGE (A) followed by Western blotting with antiserum against SodC (B). Numbers represent molecular weight in kDa.

A)


35000
PSW
PSE
PSQ
BB
H. ducreyi sodC mutant
100
70
60
50
40
30
25
20
15
10

B)

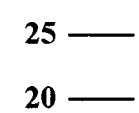
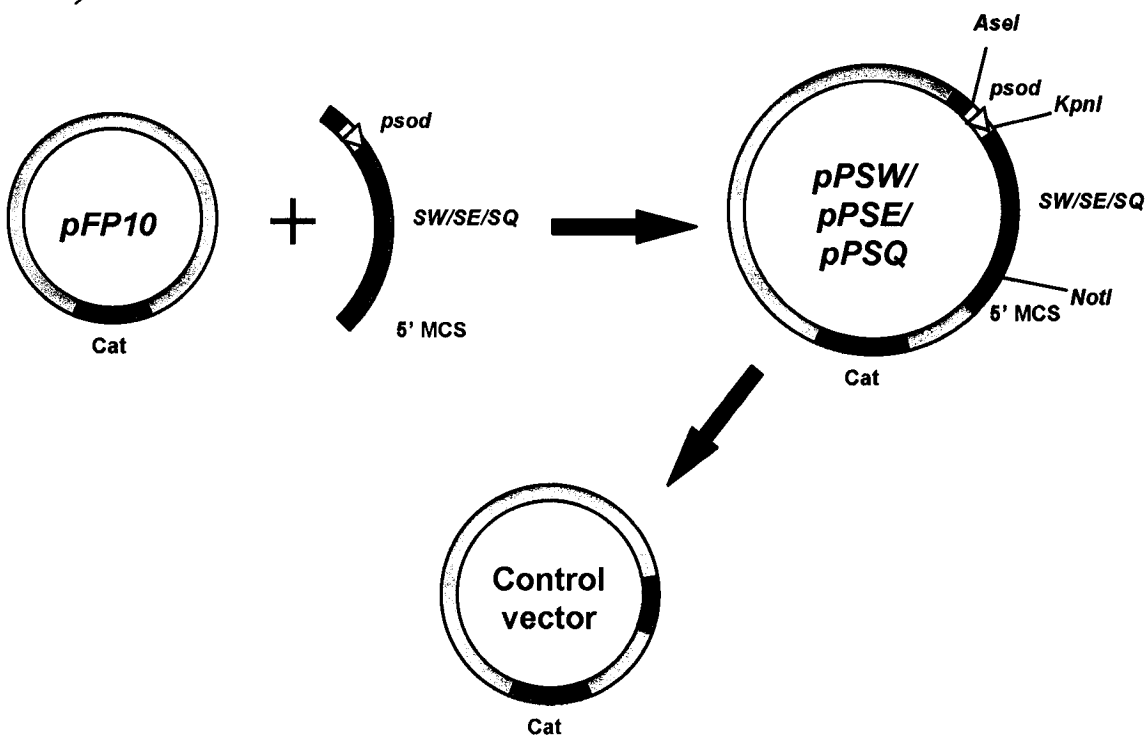

25
20

Figure 21. Comparison of the vectors *pFP10* and *pPSW/pPSE/pPSQ*.

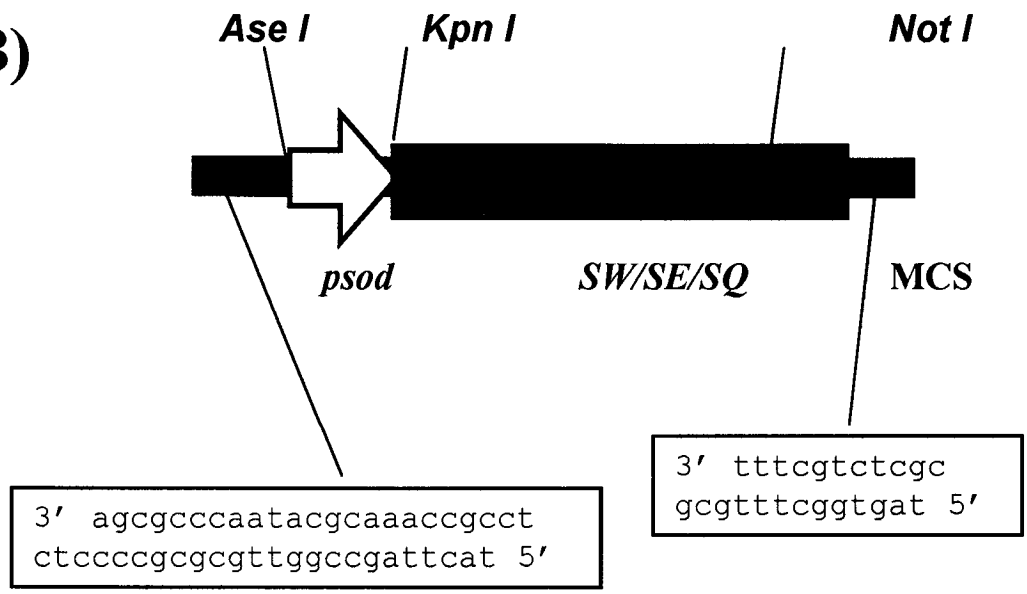
A) Schematic difference between vectors *pFP10* and *pPSW/pPSE/pPSQ* and the ideal control vector. Insertion of the *psod* and either of the genes *SW*, *SE* or *SQ* has converted *pFP10* to either of *pPSW*, *pPSE* or *pPSQ*. Excision of the *AseI-Not*I fragment would create the ideal control vector

B) The DNA Sequence other than present in the *psod*, *SW/SE/SQ* and 5' MCS, which is absent in *pFP10* has been shown in the boxes.

A)


pFP10
Cat
psod
SW/SE/SQ
5' MCS
AseI
psod
KpnI
pPSW/
pPSE/
pPSQ
SW/SE/SQ
NotI
5' MCS
Cat
Control
vector
Cat

B)


Ase I
Kpn I
Not I
psod
SW/SE/SQ
MCS
3′ agcgcccaatacgcaaaccgcct
ctccccgcgcgttggccgattcat 5′
3′ tttcgtctcgc
gcgtttcggtgat 5′

complements. However, the growth of the *H. ducreyi sodC* mutant containing vectors *pPSQ* (heme+sod-) and *pPSE* (heme-sod+) as monitored by A_{600} appeared enhanced compared to that of the *PSW* (heme+sod+) and *BB* (heme-sod-) when heme was provided at a concentration of 50 μg/ml heme (Figure 22). When generation times for the first 12 hrs of growth were compared, a statistical difference ($P = 0.031$) between the strains *BB* and *PSQ* was observed. No differences were observed when growth was assayed by CFUs (Figure 22).

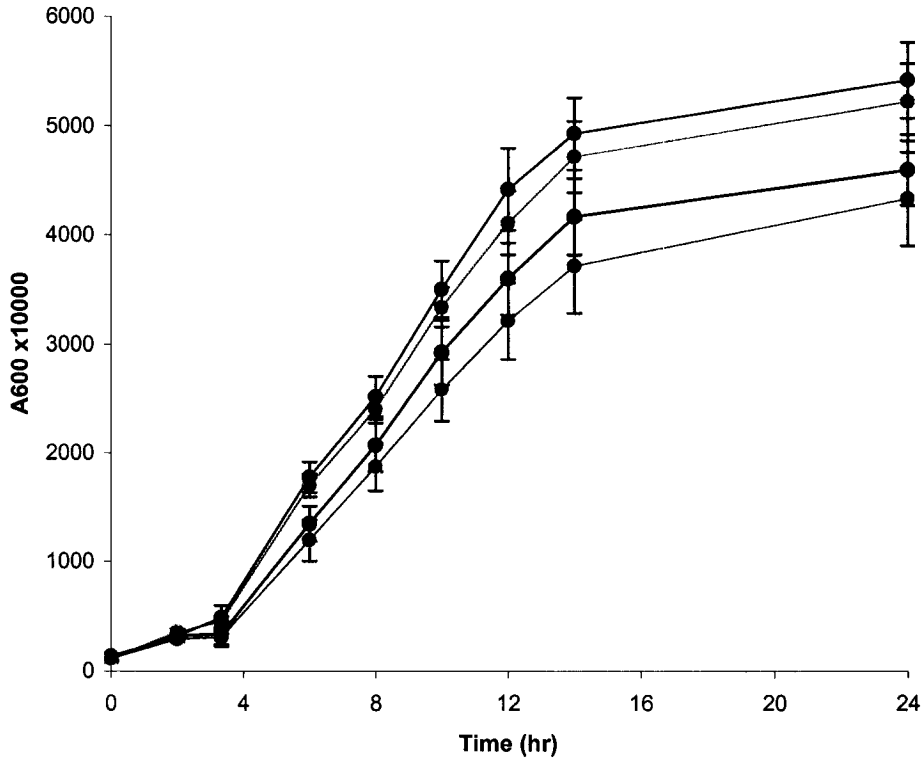
Growth assays were performed under low heme conditions (5 μg/ml) (Figure 23). This condition would be expected to exaggerate any difference in growth kinetics among the strains if sodC was involved in heme-iron acquisition. Both strains *PSQ* (heme+sod-) and *PSE* (heme-sod+) appeared enhanced compared to the strains *PSW* (bearing the wild-type *sodC*) and *BB* (containing the backbone plasmid *pFP10*). However, the difference was less than observed in 50 μg/ml heme and none of the differences in growth between these strains, as measured by A_{600} and generation time, was statistically significant ($P \leq 0.05$).

Figure 22. Monitoring growth of *H. ducreyi* complemented strains in 50 μg/ml heme using A_{600}.

H. ducreyi PSW, *H. ducreyi PSE*, *H. ducreyi PSQ* and *H. ducreyi BB* were grown in GC broth containing 50 μg/ml heme for 24 hours. Growth was monitored by measuring A_{600} (A) and plating serial dilutions for obtaining CFU (B). Error bars show the SEM. Each data point represents the mean of 6 separate experiments measuring A_{600} or 3 experiments measuring CFU.

Legend. Black line = *H. ducreyi PSW*, Red line = *H. ducreyi BB*, Green line = *H. ducreyi PSE*, Blue line = *H. ducreyi PSQ*.

A)


6000
5000
4000
3000
2000
1000
0
A600 x10000
0
4
8
12
16
20
24
Time (hr)

B)

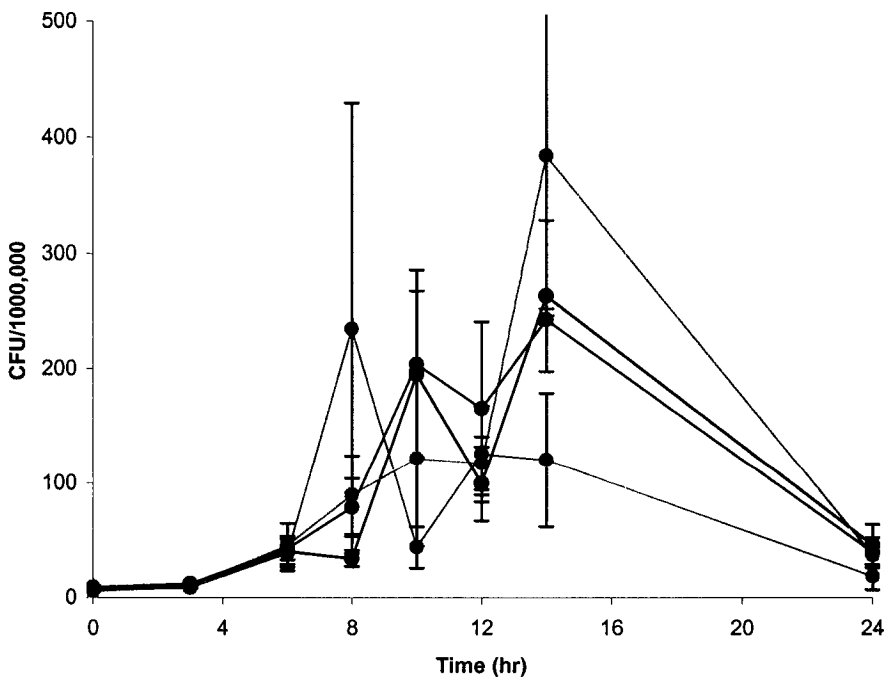

500
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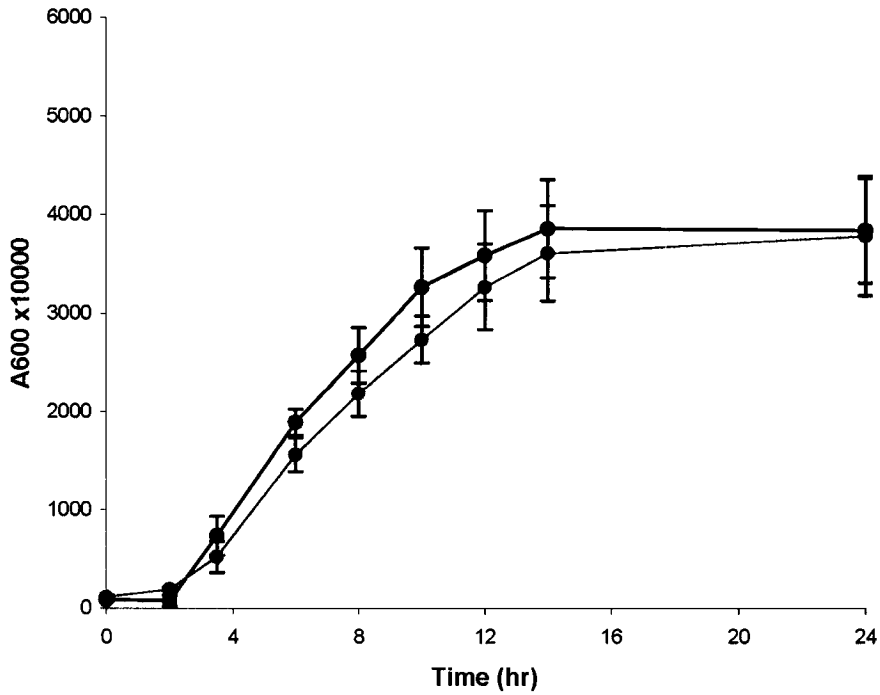
Figure 23. Growth curve of *H. ducreyi 35000*, *H. ducreyi sodC* and the complemented strains in 5 μg/ml heme.

H. ducreyi 35000, H. ducreyi sodC (A), *H. ducreyi PSW*, *H. ducreyi PSE*, *H. ducreyi PSQ* and *H. ducreyi BB* (B) were grown in GC broth containing 5 μg/ml heme for 24 hours. Growth was monitored by measuring A_{600} at time points. Error bars show the SEM. Each data point represents the mean of 6 separate experiments measuring A_{600}.

Legend A. Black line = *H. ducreyi 35000*, Red line = *H. ducreyi sodC.*

Legend B. Black line = *H. ducreyi PSW*, Red line = *H. ducreyi BB*, Green line = *H. ducreyi PSE*, Blue line = *H. ducreyi PSQ.*

A)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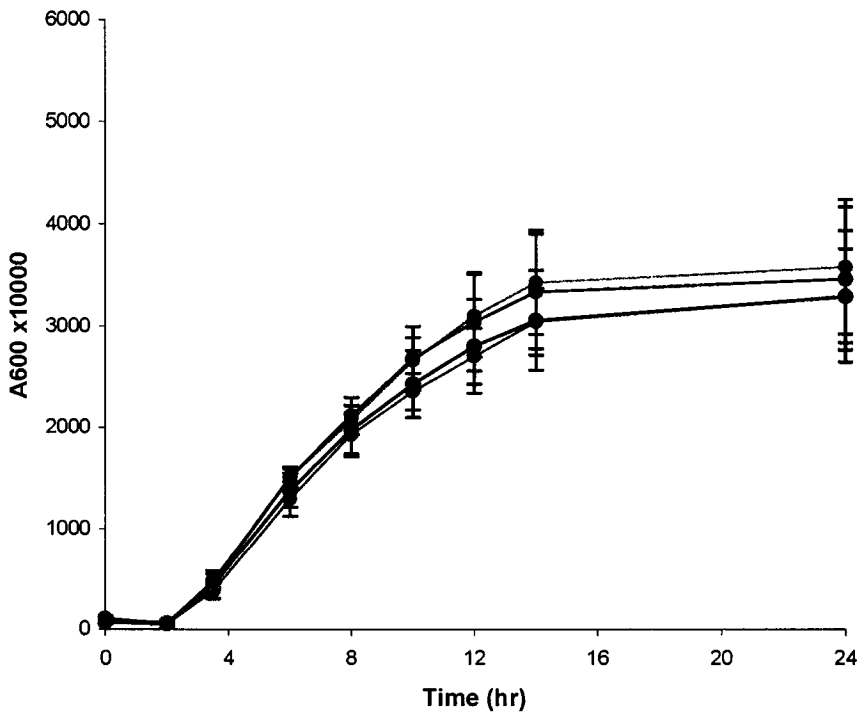
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Time (hr)

CHAPTER 4

DISCUSSION

Heme is one of the essential requirements of bacterial metabolism as it is involved in a wide variety of metabolic activities including oxygen transport, electron-transport dependent phosphorylation, oxidative stress responses, oxygen sensing, detoxifications, carbon monoxide and nitric oxide synthesis. Most bacteria are able to synthesize heme; however, as member of the genus *Haemophilus*, *H. ducreyi* is dependent on exogenous heme for survival, as *H. ducreyi* lacks the enzymatic activity required for biosynthesis of heme [107]. No siderophores have been detected in *H. ducreyi*. Heme has also been proposed to be a source of iron for *H. ducreyi* [32]. Therefore, it is expected that *H. ducreyi* possesses one or more sophisticated systems for heme acquisition. Such a system should have a high specific affinity for heme and be able to actively transport heme across the cell membranes. A transport system with the above mentioned characteristics has been detected in several other bacteria and includes a TonB-dependent OMR and an ABC transporter. In such a typical system, heme or heme containing compounds are bound to the OMR. Heme is then deposited in the periplasm where a heme-specific ABC transporter transfers heme to the cytoplasm. Since the TonB-dependent heme and hemoglobin receptors in *H.* ducreyi have been characterized, we proposed that *H. ducreyi* possesses a heme-specific ABC transporter which transfers the heme across the cytoplasmic membrane. Bacterial ABC transporters have long been recognized for their role in importation of vital molecules including sugars, vitamins and trace elements like

iron [142, 143, 145]. This family of transporters is able to couple ATP-hydrolysis with the import of molecules and maintains the specificity of the transport by the solute binding protein. Therefore an ABC transporter would be a favorable model for the transport of heme in *H. ducreyi* in view of the importance of this molecule for the survival of *H. ducreyi.*

4.1 Genetic Approach

All known bacterial ABC transporters involved in heme uptake have been detected by a genetic approach. In 1992 when the *Y. enterocolitica* DNA sequences upstream and downstream of the OMR for heme were studied, the first ABC transporter involved in heme uptake was identified. Shortly after, studies conducted in other bacteria including *S. dysenteriae* [153], *Y. pestis* [130], *C. diphtheriae* [154], *Porphyromonas gingivalis* [155, 156], *Ps. aeruginosa* [157], *Bradyrhizobium japonicum* [158], *S. pyogenes* [159] resulted in similar findings. In all these bacteria, genes coding a heme-uptake system are located within the same gene cluster. The cluster would normally include genes for a TonB dependent OMR and an ABC transporter system. As the OMRs HgbA and TdhA in *H. ducreyi* had been previously characterized, we chose the genetic approach as the initial method for detecting such a system in *H. ducreyi*,

Our inability to identify a heme-specific ABC transporter in *H. ducreyi* by DNA interrogation does not eliminate the existence of such a system because the genes involved in a transport system may not be organized in an operon, or the periplasmic component may not be contiguous to the ABC transporter [186]. Moreover, the

homology among various PHBPs is low [147, 187].

4.2 Heme Affinity Chromatography

Heme affinity chromatography has previously been used for detection of OMRs specific for heme and hemoglobin, including TdhA and HgbA in *H. ducreyi* [45, 49]. This technique has the following advantages: 1) it is a functional assay and identifies the proposed HBP based on its heme binding activity, 2) it has been successfully used to isolate OM heme binding proteins in both *H. ducreyi* and other bacterial genera, 3) required reagent is available commercially in low cost. However, it should be noted that in previous applications only membrane proteins and not soluble proteins have been detected with this technique.

Thus, it seemed reasonable to attempt purification of the proposed *H. ducreyi* PHBP from periplasmic fractions using heme affinity chromatography. However, TEF was the predominant protein that was detected using this method although SodC was also affinity purified in the same preparations, but in lower concentrations compared to TEF. The isolation of cytoplasmic located TEF may have resulted from the following considerations. First, periplasmic extraction methods are not very precise and contamination of the periplasmic preparations by cytoplasmic proteins, especially low molecular weight proteins is common during the procedure. The osmotic shock method in particular allows for the selective release of cytoplasmic proteins regardless of their size [184]. Up to 50% of the total amount of cellular TEF can pass through the cytoplasmic membrane [184] during osmotic shock. Second, SodC is expressed under

very low concentration and can not be detected in periplasmic extracts analyzed by SDS-PAGE. As the periplasmic contents are extracted in a very dilute protein solution, the concentration of SodC in starting preparations used for affinity chromatography is very low. Therefore, the amount of SodC present in periplasmic extracts would be insufficient to saturate the binding sites in the matrix-bound hemin. The unbound ligand could then be occupied by proteins binding nonspecifically to the column. As TEF represents one of the most abundant proteins present in the periplasmic extracts, its isolation would be favored. This consideration also likely accounts for isolation of SodC from total cell lysates. Unlike the periplasmic preparations, total cell lysates can be prepared as a concentrated solution of proteins. In such a starting material, a sufficient amount of SodC is able to bind to the column and appear as the principal affinity-purified protein. Third, using a more stringent washing condition could allow the removal of proteins binding nonspecifically to the affinity column such as TEF and facilitate the isolation of an authentic periplasmic hemin-binding protein.

4.3 SodC Heme Binding Activity

Unlike SodC of other bacteria, *H. ducreyi* SodC binds to heme [103]. The results of this study show that wild-type and *sodC* mutant *H. ducreyi* strains display no significant difference in growth under low heme concentrations. When the *sodC* mutant was complemented with the wild-type *sodC* or the backbone vector, no difference in growth under low heme concentrations was observed. Growth impairment would be expected if SodC participated in heme iron acquisition. Therefore, SodC is unlikely to be

involved in heme transport or uptake in *H. ducreyi*. Furthermore, there is no genetic evidence suggesting that bacterial SodC is related to an ABC transporter. Therefore, the role and importance of SodC heme binding activity in survival or pathogenesis of infection due to *H. ducreyi* remains unclear. However, two potential inter-related roles for the heme binding activity exhibited by *H. ducreyi* SodC are possible.

Heme has been shown to mediate oxidative degradation of proteins by the free radical production via the Haber-Weiss reaction [188]. In addition, heme can damage cells by inhibition of ATPase and superoxide dismutase can block heme-mediated ATPase inhibition [189]. Artemisinin, an antimalarial compound, enhances heme-mediated inhibition of ATPase and has antimalarial effects [189]. The antimalarial effects of this agent have been related to the high concentration of heme in malarial parasites [189]. Therefore, for an organism like *H. ducreyi* that requires unusually high concentrations of heme, superoxide dismutase would be expected to have an important protective role. Thus, *H. ducreyi* SodC would provide a dual protective role through the superoxide dismutase activity and heme binding property. The demonstration that the enzymatic activity of *H. ducreyi* SodC is independent of its heme binding activity (Battistoni 2003, unpublished data) supports this contention.

H. ducreyi SodC is a homodimer and the heme binding pocket is located at the dimer interface (Battistoni 2003, unpublished data). Alternatively, in view of the high heme requirements of *H. ducreyi*, SodC may function as a heme storage protein, similar to bacterioferritin [111]. Thus SodC may serve as a heme reservoir.

Although, there is no evidence of *H. ducreyi* exhibiting an

intracellular life style, the heme binding activity of SodC may permit survival in such an environment. The results from our growth assays in which the growth of the *sodC* mutant was impaired compared to the wild-type strain at high but not low heme concentration lends credence to the proposed heme protective role of SodC.

Unexpectedly, the growth of the *sodC* mutant complemented by either version of *sodC* containing nucleotide substitutions exceeded that of the *sodC* mutant complemented with the wild-type *sodC* gene. Two potential explanations may account for this observation, arising from either an artifact of monitoring growth or from a gene dose effect.

Since *H. ducreyi* tends to aggregate, measurements by A_{600} or CFU only reflect the number of particles or aggregates rather than the actual number of bacterial cells. This leads to an underestimation of the growth of *H. ducreyi.* It is not known why *H. ducreyi* forms clumps or aggregates. It is possible that point mutations in *sodC* may cause alterations in surface exposed proteins decreasing the hydrophobicity of the surface proteins, which would diminish the ability of *H. ducreyi* to aggregate. As a result, without shortening *H. ducreyi* generation time, both A_{600} and CFU measurements of the *sodC* mutant would show a higher number of particles in the growth media while the actual number of bacterial cells would be lower than the comparable wild-type strain. It is also possible that the *H. ducreyi sodC* mutant strain and the strains complemented with versions of *sodC* with point mutations, form smaller aggregates compared to the wild type strain, thus inflating the values obtained by either A_{600} or CFU monitoring.

Another explanation for the difference in growth of the

complemented strains could be a gene dosage effect arising from plasmid copy number or from the titration of transcriptional regulators. *H. ducreyi* has one copy of wild type *sodC* in the genome. Although *pFP12* is a low copy number plasmid, each cell is provided with 2 to 10 copies of the gene, leading to overexpression of SodC, which may have toxic effects. In addition gene regulatory elements controlling the transcription and expression of *sodC* located upstream of the *sodC* promoter may be absent from the plasmid constructs. Titration of regulatory proteins would be insufficient to control the multiple copies of the wild-type *sodC*. Therefore, plasmid copy number and lack of gene regulatory elements, may have led to overexpression of SodC with resultant deleterious effects. The *sodC* mutant strains that were complemented with a mutated version of SodC were preserved from this adverse effect as the expressed SodC protein exhibited only heme-binding or superoxide dismutase activity.

The other possibility that should be considered is the function of the expressed SodC from the plasmid vectors used to complement the *sodC* mutant. Though SodC has been previously shown to have both superoxide dismutase and heme binding activities when expressed from *pPHduSOD* there is the possibility that a qualitative or quantitative difference in SodC exists as expressed by these constructs.

4.4 Future Works

Heme uptake is of crucial importance for *H. ducreyi*. Therefore, it is likely that a high-affinity acquisition system for heme exists for *H. ducreyi*. The presence of a heme dedicated ABC transporter would satisfy this mechanistic requirement. The proposed

ABC transporter could be identified using proteomic, genetic or biochemical studies.

A proteomic approach is advantageous because of the increased sensitivity of this method. Periplasmic extracts of *H. ducreyi* grown under low heme conditions would be subjected to 2-D gel analysis. Proteins whose expression are increased under low heme concentration would represent candidates to be sequenced. The identity of these proteins would be obtained by computational analysis, facilitating cloning of the relevant genes and subsequent mutational analysis.

Since this study has started, tools and soft wares for genetic analysis have been improved to a higher degree of precision and reliability. At the same time, *H. ducreyi* genome project is in the final stages. Therefore a careful genetic analysis of *H. ducreyi* inventory of ABC transporters for finding similarities with heme-specific ABC transporters in other bacteria may identify the candidate transporter.

Heme specific transport system in *H. ducreyi* or the proposed PHBP can also be detected by using radioisotope-labeled heme. Growing *H. ducreyi* in an environment containing labeled heme, followed by detection of the protein(s) bound to heme in a non-denaturing gel electrophoresis, is likely to detect the proposed PHBP.

Further experiments can be undertaken to investigate the heme-binding role of SodC. Such experiments would involve the study of SodC and heme interaction in different heme concentrations to clarify if the heme-binding activity is dependent on heme concentration. A second investigation would involve a comparison of the growth between the *sodC* mutant and wild-type *H. ducreyi 35000* under a higher heme

concentration such as 75 or 100 μg/ml. If SodC subserves a heme detoxifying role, growth of the *sodC* mutant would be expected to be further impaired relative to the wild-type strain. Also, it would be of interest to investigate whether complementing a *sodC* mutant *E. coli* with different versions of SodC would affect its growth or survival in different heme concentrations. If this complementation allows the organism to survive in a higher heme concentration than is possible for the wild-type, then it is likely that SodC may possess a heme detoxification role.

4.5 CONCLUSIONS

Our study has resulted in the following conclusions:

- The minimum heme concentration for growth of *H. ducreyi 35000* on SDM plates is 3 μg/ml.
- The predominant heme binding protein in the periplasm of *H. ducreyi 35000* isolated by heme affinity chormatography is SodC.
- SodC is unlikely to be involved in heme uptake or transport in *H. ducreyi 35000.*
- *H. ducreyi* SodC may serve to detoxify the cellular deleterious effects of heme.
- The results of this study do not exclude the possibility for the presence of an ABC transporter for heme uptake in *H. ducreyi 35000.*

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