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A Heme Periplasmic-Binding Protein hHBP Mediates Heme Transport in *Haemophilus Ducreyi*

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A HEME PERIPLASMIC-BINDING PROTEIN hHBP MEDIATES

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A thesis submitted to the School of Graduate Studies

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By

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ABSTRACT

Haemophilus ducreyi, a Gram-negative and heme-dependent bacterium, is the causative agent of chancroid, a genital ulcer sexually transmitted infection. Although the precise molecular mechanism of heme acquisition in *H. ducreyi* is unclear, heme uptake likely proceeds via a receptor mediated process. The initial event involves binding to either of two outer membrane receptors, TdhA and HgbA. Once heme is deposited into the periplasmic space, a heme permease is postulated to transport heme across the periplasmic space to the inner membrane. In prior experiments, using protein expression profiling of the *H. ducreyi* periplasmic proteome, we identified a periplasmic-binding protein hHBP that we propose is a component of a heme trafficking operon. Biochemical and genetic approaches were used to functionally characterize hHBP. First, purified hHBP was incubated with increasing concentrations of heme and the mixtures were resolved by non-denaturing polyacrylamide gel electrophoresis. Separated proteins were transferred onto PVDF membranes and heme-protein complexes were detected by enhanced chemiluminescence (ECL). Second, the *hhbp* gene was cloned in the *E. coli* recombinant mutant *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) which expresses the *Serratia marcescens* HasR heme receptor allowing heme translocation into the periplasm, but denies heme entry into the cytoplasm because of the presence of the double mutation (*dppA::Km mppA::Cm*) resulting in a mutant lacking the two periplasmic proteins DppA and MppA. We found that heme binding to hHBP was saturable as determined by ECL. Genetic complementation *in trans* with *hhbp* repaired the growth defect of the mutant *E. coli* for heme utilization as an iron source. The growth restoration was comparable to that seen with the *E. coli*

mutant complemented with the intact Dpp permease. Additionally, growth of the mutant was not rescued with the empty plasmid vector. We concluded that *H. ducreyi* hHBP functionally binds heme. Complementation of the *E. coli* mutant for heme competency supports the proposal that hHBP participates in the transit of heme in *H. ducreyi*.

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CHAPTER 1: LITERATURE REVIEW

1.1 *Haemophilus ducreyi* and Chancroid

The fastidious Gram-negative anaerobic coccobacillus *Haemophilus ducreyi* is the causative agent for the sexually transmitted infection, chancroid (Rosen et al., 2009; Trees et al, 1995). Because of growth requirements, biochemical properties and antigenic relatedness of the organism, *H. ducreyi* was originally classified as a *Haemophilus* species. However, rRNA analysis now suggests that *H. ducreyi* is only remotely related to the genus *Haemophilus*, e.g *Haemophilus influenzae*, and it is now classified in the *Actinobacillus* cluster (4B) of the Pasteurellaceae (Spinola et al., 2002).

H. ducreyi is a strict human pathogen. Because patients usually present at the ulcerative stage of infection, experience with natural disease can only provide information about the clinical features associated with later stages of natural chancroid. As a result, little is known about the initial stages of natural infection. Therefore, the human model of infection was developed in order to provide information about the initial stages of infection. In the human model, subjects are inoculated with an allergy-testing device at multiple sites on the skin of the upper arms and observed for papule and pustule formation during the first two weeks of infection (Bong et al., 2002). Our understanding of *H. ducreyi* infection in humans can therefore be derived from patients who are naturally infected and from the experimental infection of human volunteers (Bong et al., 2002).

H. ducreyi naturally infects genital and non-genital skin, mucosal surfaces, stratified squamous epithelium as well as regional lymph nodes (Bong et al., 2002). It is believed that *H. ducreyi* initiates an infection within the genital skin after the formation of epidermal microabrasions during intercourse (Lewis, 2003). *H. ducreyi* does not disseminate systemically but “kissing lesions” (multiple opposing ulcerations) and extra-genital lesions, although rare, occur and are thought to be due to autoinoculation (Bong et

al., 2002; Rosen et al., 2009; Lewis, 2003). Erythematous papules form at each entry site within several hours to days after infection and eventually evolve into pustules after 2 or 3 days. After several weeks, the pustules ulcerate. Patients usually develop 1 to 4 ulcers with central necrosis. These ulcers are often very painful, are tender to palpation and have the characteristic granulomatous base and purulent exudates. The edges are usually soft, ragged and undermined (Rosen et al., 2009; Lewis, 2003; Spinola et al., 2002). In both men and women, ulcers left untreated may persist for up to 3 months. In addition to the aforementioned symptoms, painful, tender inguinal lymphadenitis occurs in up to 50% of cases. The lymph nodes may develop into buboes and burst through the skin. If not aspirated or drained, fluctuant buboes can rupture spontaneously forming suppurative tracts (Schneede et al., 2003; Rosen et al., 2009; Lewis, 2003).

Chancroid occurs sporadically in developed countries such as the USA and Canada, but it is a major cause of genital ulceration syndrome within resource poor endemic areas such as sub-Saharan Africa, the Caribbean basin and southern Asia (Rosen et al., 2009; Lewis, 2003). Due to the inadequacies in data collection within resource poor endemic regions, the WHO (World Health Organization) does not include chancroid in their estimates of global STDs. However, both UNAIDS (the joint United Nations Program on HIV/AIDS) and WHO estimate that the annual global prevalence of Chancroid is 7 million cases, based primarily on the prevalence of syphilis and the relative estimates of the percentage of syphilis and Chancroid in genital ulcer disease (GUD) cases (Bong et al., 2002). Due to this poor understanding of the epidemiology, natural history of the disease and absence of good diagnostic tests, it is difficult to perform prevalence studies, and to estimate prevalence and duration of infection within endemic regions (Bong et al., 2002; Schneede et al., 2003). In spite of this, several trends in Chancroid epidemiology have been observed. A high proportion of infected men reported intercourse with commercial sex workers, thus implicating commercial sex workers as vectors of transmission. Other risk factors have been identified include crack

cocaine use, exchange of drugs for sex, and having a partner who uses or used cocaine (Bong et al., 2002; Rosen et al., 2009). With respect to race and gender, *H. ducreyi* infection has been more commonly reported in African-American and Hispanic men than those of other ethnic groups. In addition, men are infected more often than women with a male to female ratio ranging from 3:1 to 25:1 (Bong et al., 2002). The reasons for this gender difference in infection are unknown. However, it is likely that female sex hormones may have immune suppressing effects that would ironically result in less skin damage than that observed in men. In addition, the increase in basal body temperature that occurs around the time of ovulation could suppress bacterial growth as *H. ducreyi* does not grow well at temperatures above 35 degrees Celsius (Bong et al., 2002).

Several laboratory techniques for diagnosing Chancroid exist. None of these techniques are perfect in accuracy nor uniformly available. The first of these would be a Gram stain of the ulcer debris showing the classic “school of fish” pattern of the pleomorphic Gram-negative coccobacilli in parallel chains or clusters (Rosen et al., 2009). Secondly, diagnosis would require isolation of the bacteria on chocolate-based culture media under conditions of high humidity and low oxygen tension. However, due to the difficulty of this organism to be cultured in the hands of inexperienced laboratory staff, conventional laboratory culture facilities are often not available in STD clinics or simply not affordable in resource poor countries (Rosen et al., 2009; Lewis, 2003). A new diagnostic method called multiplex PCR (M-PCR) was developed by Orlelet. et al. in the 1990’s. This method was designed to simultaneously amplify DNA targets from the three most common aetiological agents of GUD, namely *H. ducreyi*, *Treponema pallidum* and *Herpes simplex* virus 1 and 2, with a resolved sensitivity and specificity for *H. ducreyi* of 98.4% and 99.6% , respectively. Unfortunately, M-PCR requires special training and the equipment is not commercially available for routine diagnosis (Bong et al., 2002; Lewis, 2003). Consequently, the practical diagnosis of Chancroid is largely based on clinical features. According to the CDC (Centres for Disease Control and Prevention), a diagnosis

of Chancroid can be made if all of the following criteria are met: i) the patient must have one or more painful genital ulcers, ii) dark-field examination of ulcer exudates or serological test for syphilis performed at least 7 days after the onset of ulcers must show no evidence of *T. palladium* infection, iii) the clinical presentation, appearance of genital ulcers and regional lymphadenopathy (if present) must be typical for Chancroid, and iv) a test (either viral culture or PCR) for *Herpes simplex* virus performed on the ulcer exudates must be negative (Rosen et al., 2009).

Current CDC recommendations for the treatment of Chancroid include the following antibiotic regimens: a single oral dose of 1g azithromycin, a single 250 mg intramuscular dose of ceftriaxone, 500 mg ciprofloxacin orally twice daily for three consecutive days or 500 mg erythromycin 3 times daily for 7 consecutive days (Rosen et al., 2009). However, many problems may arise during antibiotic treatment. In patients with underlying immunosuppression due to HIV infection, single dose therapy may fail. There also appears to be an increase likelihood of treatment failure in uncircumcised men. Single doses of either intramuscular ceftriaxone or oral ciprofloxacin have also not been successful in some individuals (Rosen et al., 2009; Lewis, 2003). This failure in antibiotic treatment may be a result of plasmid-mediated antimicrobial resistance, which has been documented for other antibiotics including penicillins, tetracyclines, chloramphenicol, sulfonamides and aminoglycosides (Lewis, 2003). Due to the difficulties observed in antibiotic therapy, discovery of an effective vaccine would play an important role in eradicating *H. ducreyi* infection. Preliminary research has indicated that a vaccine targeting the *H. ducreyi* hemoglobin receptor may be efficacious in preventing chancroid. Passive immunization using antibodies raised against this receptor was able to prevent infection in the porcine model of chancroid (Rosen et al., 2009).

The GUD Chancroid is of increasing concern because *H. ducreyi* infection facilitates the acquisition of HIV (Bong et al., 2002). In endemic areas, the risk of acquiring HIV infection in patients with GUD ranged from 3 to 18.2. It is estimated that

GUDs enhance HIV transmission 10-100 fold per individual sexual act (Spinola et al., 2002). *H. ducreyi* can enhance HIV infection by establishing an accessible portal of viral entry, promoting viral shedding from the ulcer, increasing the viral load in the blood and semen, and recruiting CD4+ macrophages into the skin. Therefore, effective diagnosis and treatment of Chancroid may make it possible to slow down the HIV-I endemic in those regions where both diseases are prevalent (Lewis, 2003; Spinola et al., 2002).

1.2 Iron

Iron is a first row transition metal that exists in one of two readily invertible redox states under physiological conditions, the reduced Fe²⁺ ferrous form, and the oxidized Fe³⁺ ferric form. In both forms, it may also adopt different spin states depending on the ligand environment. These properties make iron a versatile prosthetic component for incorporation into proteins as a biocatalyst or an electron carrier (Andrews et al., 2003). Iron is an essential co-factor for many metabolic pathways, including the respiratory chain of both host and microorganism. The metal is an essential catalytic site of many of the enzymes and gas transporting proteins in cells (Barasch et al., 2004; Schaible et al., 2004). Iron participates in many major biological processes such as photosynthesis, nitrogen fixation, methanogenesis, hydrogen production and consumption, respiration, the trichloroacetic acid cycle, oxygen transport, gene regulation and DNA biosynthesis. However, iron is in short supply within many ecological niches. In addition, when oxygen is present, ferrous iron can generate toxic hydroxyl radicals. Ferric iron is not as toxic but it is insoluble in water and, at neutral pH, produces ferrihydroxide precipitates (Barasch et al., 2004; Andrews et al., 2003). As a result, organisms must balance their biological need for free iron with the management of cellular free iron levels in order to protect themselves against iron-induced toxicity (Andrews et al., 2003).

1.3 Iron and Bacteria

Bacteria need iron in concentrations ranging from 10^{-6} to 10^{-7} M in order to perform various essential metabolic processes. The ability of a bacterial pathogen to acquire iron is related to the ability of the organism to cause disease (Schaible et al., 2004; Lee, 1991).

The level of free iron in the host is very scarce and insufficient to support microbial growth. Mammalian blood has 10^{-26} M of free iron (Barasch et al., 2004). Iron is sequestered in heme containing proteins such as hemoglobin, myoglobin, and ferritin or it is complexed to host iron binding glycoproteins such as lactoferrin (Lf) and transferrin (Tf) (Schaible et al., 2004; Lee, 1991). In addition to the scarce levels of iron within the host, bacteria compete for iron within mixed microbial communities. As a result, bacteria have developed specialized iron-uptake systems (Schaible et al., 2004). One of these systems is the production of siderophores. These are low molecular weight iron chelators that solubilise and bind iron prior to internalization (Lee, 1991). After scavenging the iron, the iron-siderophore complexes return to the cell surface where they are internalized via specific siderophore outer membrane receptors (Andrews et al., 2003). In eukaryotes, a highly conserved siderophore -like protein called Tf or the related Lf is used to bind and move extracellular iron into the host cell, thus reducing the amount of extracellular iron available to bacteria (Barasch et al., 2004; Andrews et al., 2003). Bacteria, however, have evolved mechanisms to scavenge iron from Tf and other iron-binding host proteins. They can either remove iron from host iron protein complexes using siderophores as in the case of the mycobacterial siderophore carboxymycobactin, or they can confiscate these host iron-binding proteins by producing specific outer membrane receptors that bind these host iron binding proteins as in the case of microorganisms such as *H. influenzae* and *Neisseria spp.* (Barasch et al., 2004; Schaible et al., 2004; Lee, 1991). In addition to stealing iron from the host, bacteria can also steal iron from other microorganisms by producing outer membrane receptors specific for

siderophores produced by other microorganisms (exogenous siderophores) as in the case of *E. coli* K12 which possesses at least six outer membrane receptors that enable the acquisition of eight iron-chelate complexes, four of which are produced exogenously (Andrews et al., 2003). Another iron assimilation pathway would be the use of iron (II) or iron (III) cytoplasmic pumps which transport iron with the expenditure of ATP (Stojiljkovic et al., 2002). At the outer membrane, receptors internalize intact iron-loaded siderophores, whereas the iron from other iron-containing proteins are unloaded at the cell surface and only the iron is transported into the cell (Wandersman et al., 2000). This process is driven by the electrochemical charge gradient of the cytosolic membrane (CM) potential and is delivered by the energy-transducing TonB-ExbB-ExbD protein complex system (Andrews et al., 2003). Exactly how the proton motive force of the cytoplasmic membrane is translated into iron transport remains elusive. After internalization, transport proteins within the periplasm of Gram-negative bacteria shuttle iron or ferri-siderophores to the CM ATP-binding cassette (ABC) transporters. These transporters deliver the complex to the cytosol via a process that is driven by the hydrolysis of ATP. It is unclear whether the periplasmic binding proteins collect the iron or ferri- siderophore complexes directly from the outer membrane or whether the protein picks up the molecules from the periplasm (Andrews et al., 2003). Once in the cytosol, the ferri- siderophore complex dissociates in order to liberate the complexed iron for its incorporation into other proteins. This likely involves a ferric reductase that reduces the siderophore -associated iron resulting in the dissociation of iron from the complex. Bacteria may also deposit iron within storage proteins that can be used to enhance growth during a shortage of external iron supplies (Andrews et al., 2003).

Pathogens often use low environmental iron levels to signal the induction of iron uptake genes. The expression of these genes is controlled at the level of transcription. An example is the iron-dependent regulator IdeR found in mycobacteria. During high iron concentrations, iron binds IdeR and represses genes that encode iron acquisition

molecules while activating the expression of iron storage proteins (Schaible et al., 2004; Andrews et al., 2003). Another example is the ferric-uptake regulator protein (Fur). Fur is a homodimer composed of 17KDa subunits (Andrews et al., 2003). Acting as a positive repressor, this protein represses the transcription of iron acquisition genes upon interaction with its co-repressor iron and causes de-repression when iron is absent. In the absence of Fur, free iron levels become excessive (Andrews et al., 2003). The Fur protein controls the iron dependent expression of many genes in bacteria. For example, the Fur protein controls the expression of over 90 genes in *E. coli* (Andrews et al., 2003). The Fur protein can also control the expression of genes not directly related to iron metabolism. For example, Fur controls the expression of a sigma factor P_{vds}, which affects the expression of other iron-regulated genes (Clarke et al., 2001). After binding to iron, the iron-Fur complex normally binds between -35 and -10 sites on the promoters of the genes to be repressed by Fur (Andrews et al., 2003). Originally, Fur binding sites were thought to be a 19-bp palindromic consensus sequence known as the “fur box”. However, recent DNase I footprinting studies have shown that the binding site is larger than the original fur box (Andrews et al., 2003). Lavrrar et al. (2002) postulated that the Fur binding sites are overlapping 13bp “6-1-6” motifs that allow two Fur dimers to bind at each box on opposite faces of the double helix, allowing a further Fur dimer to bind, lengthening the fur-binding site, thus accounting for the extended binding site observed from DNase I footprinting (Andrews et al., 2003).

1.4 Heme

Porphyryns are enzyme co-factors that exist widely in nature. They participate in a variety of different metabolic pathways. Some porphyryns include chlorophyll and Vitamin B12. All porphyryns share a common delta- aminolevulinic acid precursor, but differ in the central metal (Stojiljkovic et al., 2002). Heme is a porphyryn consisting of a central iron atom bound by four nitrogen atoms. When in basic water solutions or on the

bacterial surface, two heme molecules may become bridged together through an oxygen atom forming oxo-dimers (Stojiljkovic et al., 2002). Although often used to describe both forms, heme describes the reduced ferrous (Fe(II)) iron protoporphyrin IX, whereas hemin describes the oxidized ferric (Fe(III)) form (Genco et al., 2001).

Heme is a biological catalyst ubiquitous in nature. It is a rich source of iron for both bacteria and humans. Because of the low solubility of iron(III) salts at physiological pH when oxygen is present, organisms can use heme as their sole iron source thus bypassing this problem (Stojiljkovic et al., 2002).

In human plasma, heme is normally bound to proteins such as hemoglobin, hemopexin, albumin and lipoproteins. Hemoglobin, however, is the major source of circulating heme. Hemoglobin is usually complexed to the circulating heterotetrameric glycoprotein haptoglobin (Hp). Small amounts of free hemoglobin can be found in normal human plasma as a result of spontaneous hemolysis (Stojiljkovic et al., 2002; Genco et al., 2001).

1.5 Heme and Bacteria

Heme is the most abundant source of iron in the body. Consequently, bacteria can satisfy all their iron requirements by directly utilizing free heme or heme associated with proteins (Andrews et al., 2003; Stojiljkovic et al., 2002). In fact, bacteria seem to prefer heme or heme-containing compounds as an iron source. Skaar et al. (2004) showed that *S. aureus* preferentially imports heme-iron over transferrin-iron. In addition, a heme transport system required for *S. aureus* pathogenesis was also discovered (Skaar et al., 2004). Furthermore, Lee (1991) showed that heme and various heme-containing compounds are the sole source of iron in *H. ducreyi*. Extracellular pathogens need to access host heme by freeing intracellular heme and hemoglobin from red blood cells via hemolysins and proteases. Once freed, heme may be rapidly bound to host heme binding proteins such as hemopexin and albumin. Conversely, bacteria may directly bind and

transport heme (Andrews et al., 2003). Analogous to iron up-take systems, heme up-take systems can be classified into two groups. The first system relies on the secretion of hemophores (siderophore -like proteins), which scavenge the extracellular environment for a heme source, bind it and present it to specific outer membrane receptors. A second method involves the direct binding of heme or heme- associated host proteins to specific outer membrane receptors (Wandersman et al., 2000).

Hemophores are secreted by ABC transporters via a process that involves a carboxy-terminal secretion signal. Some examples of hemophores are the 19kDa HasA protein from *Serratia marcescens* and the 100kDa HxuA protein of *H. influenzae* (Wandersman et al., 2000; Stojiljkovic et al., 2002). The *S. marcescens* HasA protein scavenges the extracellular medium for free heme and a variety of heme-proteins inclusive of hemoglobin, hemopexin and myoglobin (Stojiljkovic et al., 2002) (Wandersman et al., 2000). Once it comes into contact with these heme-proteins, it removes the heme component and delivers it to the *S. marcescens* outer membrane receptor HasR. While HasR is essential for heme acquisition in *S. marcescens*, HasA is not. However, it increases the efficiency of the system for free or hemoglobin bound iron by 100-fold (Stojiljkovic et al., 2002) (Wandersman et al., 2000). HasA is however essential for heme acquisition from hemopexin and myoglobin (Wandersman et al., 2000). Unlike the *S. marcescens* HasA hemophore, the HxuA hemophore of *H. influenzae* only has one substrate, hemopexin (Stojiljkovic et al., 2002). Once secreted by the protein, it binds hemopexin and delivers it to an outer membrane receptor. HxuA is required for the utilization of the heme-hemopexin complexes (Stojiljkovic et al., 2002). Some proteases might also function as hemophores, such as the *P. gingivalis* lysine-specific protease Kgp which binds both hemin and hemoglobin in the extracellular space and delivers it to the tonB-dependent receptor of HmuR (Genco et al., 2001). Because hemophores interact with a variety of hemoproteins, they enhance the capacity of the

bacterial heme-uptake system and broaden the spectrum of potential heme sources (Wandersman et al., 2000).

Once at the cell surface, heme is internalized and transported across the outer membrane into the bacterial periplasm by outer membrane receptors. However, heme from heme-associated proteins is unloaded at the cell surface and only heme is transported into the periplasm. On the other hand, iron from heme may be removed at this stage and transported across the outer membrane via separate iron-acquisition pathways (Stojiljkovic et al., 2002; Genco et al., 2001; Wandersman et al., 2000). Similar to iron outer membrane receptors, the transport of heme across the outer membrane is an active process that uses the energy provided by TonB in association with ExbB and ExbD proteins (Stojiljkovic et al., 2002; Genco et al., 2001). Once in the periplasm, heme transport is facilitated by ABC transporters, consisting of a periplasmic binding protein, permeases and ATPases (Stojiljkovic et al., 2002; Genco et al., 2001). The periplasmic binding protein would either pick up the heme molecule from the outer membrane receptor or bind the heme molecule in the periplasm after which it would deliver the heme to the permease for transport across the cytoplasmic membrane and into the cytoplasm via a process driven by ATP hydrolysis. The details of this process remains elusive. Once in the cytoplasm, if not degraded on the cell surface or in the periplasm, heme is degraded to release iron. This process may occur via a simple enzymatic iron removal mechanism such as the reverse ferrochelatase mechanism or via an enzymatic ligand destruction with concomitant release of iron, such as the heme oxygenase mechanism (Genco et al., 2001). Genetic or biological evidence for a reverse ferrochelatase is lacking, however, recent studies have shown that at least for the Gram-positive organism *C. diphtheriae*, iron removal from heme occurs via a bacterial heme oxygenase. The usage of heme oxygenases by eukaryotes has been well characterized. The process involves the oxidative degradation of heme through cleavage of the porphyrin ring with the production of carbon monoxide, iron and biliverdin (Genco et al.,

2001). Some bacteria are able to use heme, but lack a heme oxygenase. It is therefore possible that some bacteria do not use a heme degradation system. Stojiljkovic and Perkins-Balding (2002) postulated that bacteria may channel externally supplied heme to respiratory components and other heme-requiring enzymes, while the iron needed for metabolic processes would come from the iron-acquisition pathway or storage.

The regulation of heme utilization systems are mostly controlled by that of the iron assimilation strategies, i.e., Fur proteins or Fur-like proteins, whereas iron acts as the co-repressor and represses transcription of heme uptake genes. However, this is not always the case. Although *H. ducreyi* has a functional Fur homologue, the expression of the HgbA receptor of *H. ducreyi* is regulated by heme availability instead of free iron levels (Stojiljkovic et al., 2002; Carson et al., 1996). In addition, the *H. influenzae* hemoglobin receptors are also regulated by heme and not iron (Stojiljkovic et al., 2002). Expression of the *hmuO* gene of *C. diphtheriae* encoding the heme oxygenase protein is repressed by excess iron, but activated by heme or hemoglobin (Stojiljkovic et al., 2002).

1.6 ABC Transporters

ABC transport systems are ATP-dependent transport systems that transport various substrates across the membranes of both Gram-negative and Gram-positive bacteria using energy derived from the hydrolysis of adenosine 5'-triphosphate (ATP). Gram-negative ABC transporters transport solutes across the inner membrane while Gram-positive ABC transporters transport solutes across the cytoplasmic membranes of the cell. These systems are used for the uptake of many ligands inclusive of amino acids, peptides, iron and iron complexes (Clarke et al., 2001). In Gram-negative bacteria, they normally consist of soluble periplasmic proteins, transmembrane permeases and ATP-binding proteins. The stoichiometry of these proteins, however, may vary (Clarke et al., 2001). In Gram-positive bacteria, the protein analogous to the periplasmic protein found in gram negative bacteria is anchored to the cell membrane through the attachment of an

N-terminal lipid anchor, as observed in the ferrichrome uptake system in *Bacillus subtilis* (Clarke et al., 2001). Otherwise, the ABC transport systems of the Gram-positive bacteria are similar to that of Gram-negative bacteria (Clarke et al., 2001). The genes encoding for these proteins are normally organized together in an operon. However, sometimes the genes encoding for the periplasmic component may not necessarily belong to the operon. The periplasmic components of transport systems seem to differ greatly in sequence homology (Clarke et al., 2001). The permease and ATPase components, however, share some similarity in sequence. The permease component is 65% hydrophobic and has many transmembrane segments (Clarke et al., 2001). There is also a highly conserved motif “EAA(3X)G(9X)I(X)LP” located approximately 100 residues from the C terminus. This motif is slightly different in heme permeases where residues “E(X)A(3X)G” are present (Stojiljkovic et al., 2002). The ATP-binding proteins contain a homologous domain of about 200 amino acids flanked by Walker A: GPNGAGKSTLL and Walker B: hhhD consensus sequences (Lee, 1991; Clarke et al., 2001). It is postulated that the 200 amino acid consensus sequence is important for ATP hydrolysis since changes in the consensus sequence of *E. coli* FhuC abolished the hydrolase activity (Clarke et al., 2001). The Walker A and Walker B domains have been shown to bind ATP and Mg²⁺ ion, respectively (Stojiljkovic et al., 2002). In addition, in all ATPases, a linker motif “LSGGE(2X)R” is found preceding the Walker B sequence. In heme transport systems, this linker motif is believed to be responsible for the interaction with the permease via the “EAA(3X)G” motif (Stojiljkovic et al., 2002).

1.7 Heme and *H. ducreyi*

Very little is known about how *H. ducreyi* acquires heme. However, over the past few years, scientists have begun to unravel the mystery of its heme acquisition pathway.

H. ducreyi is a heme-obligate organism and needs to consume large amounts of heme in order to ensure its survival. Early studies suggest that 10 ug of heme/ml is

required to initiate growth, whereas 200 to 500 ug of heme/ml was required for optimal growth (Trees et. al, 1995). However, the organism lacks the enzyme ferrochelatase, which catalyzes the synthesis of heme by inserting ferrous iron into protoporphyrin IX, thus making it unable to synthesize its own heme (Trees et. al, 1995; Lee, 1991). The compounds bovine hemoglobin, human hemoglobin and bovine catalase have been shown to support the growth of *H. ducreyi* and therefore were capable of serving as the sole sources of exogenous iron. In addition, heme bound to human serum albumin and human hemoglobin bound to human haptoglobin also served as suitable sources of iron. Conversely, FeCl₃, human transferrin and human lactoferrin did not support in-vitro growth. This is not surprising as *H. ducreyi* lacks receptors for transferrin and lactoferrin, and also does not produce siderophores (Trees et. al, 1995; Lee, 1991). This evidence indicates that *H. ducreyi* can only utilize heme or the aforementioned heme-containing compounds as an iron source. Furthermore, hemoglobin seems to be the most likely source of heme-iron, as an *H. ducreyi* mutant unable to use hemoglobin as a heme-iron source was completely attenuated in virulence (Al-Tawfiq et al., 2000). These heme sources are primarily intracellular and it is possible that *H. ducreyi* gains access to these sources via the use of hemolysins and/or cytotoxins (Trees et al, 1995; Elkins et al., 1998). The mechanism by which *H. ducreyi* internalizes heme are not clear. From observations of other bacteria heme-uptake systems, it is postulated that the heme from heme-containing molecules may be internalized via Ton B dependent outer membrane receptors (Trees et al, 1995).

Recently, three Ton B dependent outer membrane receptors have been identified in *H. ducreyi*, the hemoglobin A receptor (HgbA), the TdhA receptor and an uncharacterized conserved hypothetical protein TdX (Nepluev et al., 2009; Leduc et al., 2008). Elkins (1995) observed that the expression of the conserved 100 KDa HgbA receptor was upregulated in conditions of low heme concentrations. In addition to this, an *H. ducreyi* mutant lacking the expression of *hgbA* did not bind hemoglobin or grow on

hemoglobin blood plates, indicating that the HgbA receptor is essential for hemoglobin utilization. On the other hand, the mutant was still able to use heme as an iron source indicating that the HgbA receptor is not required for heme utilization, and that another receptor is responsible for heme utilization (Genco et al., 2001; Elkins et al., 1995; Elkins et al., 1998; Thomas et al., 1998). The 75kDa TdhA protein was shown by Elkins et al. (1998) to be expressed only under conditions of heme limitation. They also showed that an *E. coli* mutant expressing the TdhA receptor and an intact *H. ducreyi* Ton system was able to use heme as its sole iron source. Furthermore, an isogenic *H. ducreyi* mutant of TdhA was still able to use heme as an iron source, thus indicating that TdhA is not the only heme receptor in *H. ducreyi*, or that there is another mechanism by which the organism obtains heme (Thomas et al., 1998). Another receptor Tdx, postulated to bind heme was discovered in *H. ducreyi*. This receptor, however, has not yet been properly characterized (Leduc et al., 2008).

An isogenic *hgbA* mutant was attenuated in the human challenge model of *H. ducreyi* infection. However, a double *tdx/tdhA* mutant was not. This indicated that while HgbA is important for *H. ducreyi* virulence, Tdx and TdhA are not (Genco et al., 2001; Leduc et al., 2008). The mechanism by which heme is transported through the periplasm and cytoplasmic membrane of *H. ducreyi* remains elusive. No clear candidates for heme-specific ABC transporters have emerged until now.

Our laboratory has identified a putative heme-specific transport operon in *H. ducreyi* consisting of four genes. A lone putative promoter is located immediately upstream of the first gene in the operon and a possible site for transcriptional termination occurs after the final gene in the operon. The first gene is believed to encode for an inner membrane protein permease. The second gene is postulated to encode for a dissimilatory desulfovridin gamma subunit due to homology to *dsvC* encoding for the dissimilatory desulfovridin gamma subunit (DSG) in *Desulfovibrio vulgaris*. The *D. vulgaris* DSG is a component of the dissimilatory sulfite reductase, desulfovridin. Within sulfate-reducing

bacteria, desulfovirdin catalyzes the six-electron reduction of sulfite to sulfide and functions as the terminal oxidase in the sulfate-reduction pathway (Laue, et al., 2001; Karkhoff-Schweizer, et al., 1993). The role of the gamma subunit in sulfate-reducing bacteria is not clear. Some biochemical studies suggest that the gamma subunit confers the thiosulfate reductase activity of the enzyme within *Desulfovibrio desulfuricans* (Cort et al., 2001). Moreover, the gamma subunit is suggested to function together with the *dsvD* gene product, the delta subunit as an independent thiosulfate reductase (Cort et al., 2001; Skaar et al., 2004). The presence of the *dsvC* gene product in non-sulfate reducing bacteria is rare, however, it is postulated to be involved in the stabilization of siroheme cofactors (Karkhoff-Schweizer, et al., 1993; Molitor et al., 1998; Cort et al., 2001). We therefore believe that the DSG protein encoded by *dsvC*, though not typical of an ABC transporter, may play a role in stabilizing the interaction between the heme molecule and the periplasmic component hHBP (encoded by the third gene in the operon). The fourth gene in the proposed operon is believed to encode for an ATPase.

Work in our laboratory has provided experimental support for the role of hHBP in heme uptake. First, hHBP is homologous to iron or metal iron transport proteins. Secondly, hHBP was consistently upregulated under heme-limiting conditions, whereas expression was not affected by the addition of exogenous iron in vitro. hHBP also bound to hemin-agarose in a concentration dependent manner and was also shown to recognize the porphyrin ring of the heme molecule, regardless of the presence of the internal metal moiety. Lastly, hHBP was ubiquitous and structurally conserved among *H. ducreyi* clinical isolates. The focus of this study will be to further characterize the function of hHBP in vitro and in vivo.

HYPOTHESIS

The periplasmic binding protein (hHBP) mediates the transport of heme across the periplasmic space in *H. ducreyi*.

OBJECTIVES

In order to address the hypothesis, the following objectives were pursued:

1. Functionally characterize hHBP by providing in-vitro evidence that hHBP binds heme using non-denaturing PAGE (polyacrylamide gel electrophoresis) and detection of heme binding by chemiluminescence (ECL).
2. Show via heterologous complementation of an *Escherichia coli* heme uptake mutant that hHBP binds heme in-vivo, and aids in heme transportation across the periplasm into the cell.

CHAPTER 2: MATERIALS AND METHODS

2.1. Champion™ pET Directional TOPO® Expression System

The *E. coli* strain BL21 Star™ (DE3) pET151-hHBP was constructed by a former master's student in our laboratory, Melissa St. Denis. The pET151/D-TOPO® kit (Invitrogen, Carlsbad, CA, USA) was used to express a recombinant hHBP protein with an N-terminal fusion tag containing the V5 epitope and 6xHis region using the manufacturer's instructions with the modifications described below.

2.1.1. Expression of Recombinant Fusion Protein

Expression of the recombinant hHBP fusion protein (rhHBP) was achieved by induction with isopropylthio-B-galactoside (IPTG; Invitrogen, Carlsbad, CA, USA) for four hours, at a final concentration of 1mM added at mid-log phase of growth (OD₆₀₀ of 0.5-0.8).

2.1.2. Ni-NTA Protein Purification (Hybrid Method)

The Ni-NTA purification system (Invitrogen, Carlsbad, CA, USA) was used to purify the poly-histidine-containing recombinant protein from IPTG induced BL21 Star™(DE3) *E. coli* cells containing the pET151-hHBP vector. Briefly, bacterial cell lysates were prepared under denaturing conditions (i.e. washes with denaturing binding buffers [8M Urea, 20mM Sodium Phosphate pH 7.8, 500mM NaCl] and denaturing wash buffers [8M Urea, 20mM Sodium Phosphate pH 6.0, 500mM NaCl]), according to the manufacturer's instructions. rhHBP was purified with Ni-NTA agarose under hybrid conditions (i.e., the entire process of washing with denaturing binding and wash buffers and subsequent washes in native wash buffers [1X Native Purification Buffer,

20mM imidazole] and then eluting in native elution buffers) which is described as follows. In the elution step, the bound rhHBP was eluted in native elution buffer (1 X Native Purification Buffer with the addition of various concentrations of imidazole) with sequentially increasing imidazole concentrations (50mM to 250mM). The rhHBP eluted optimally in the 100mM, 150mM, 200mM, 250mM imidazole fractions as verified by SDS-PAGE. These fractions were then concentrated using Amicon Ultra centrifugal filter devices (Millipore, Carrigtwohill, Cork, Ireland). The affinity purified rhHBP was then stored at 4°C.

2.1.3. Protein Quantification

Protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockfort, IL, USA). A standard curve using bovine serum albumin (BSA) served as a protein standard.

2.2 Polyacrylamide Gel Electrophoresis

2.2.1. One-Dimensional Gel Electrophoresis

All one-dimensional (1D) SDS-PAGE gels were performed with a 12% (w/v) resolving gel [0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 12% (w/v) acrylamide (Protogel; 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, National Diagnostics, Atlanta, GA, USA)] and a 4.5% (w/v) stacking gel [0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 4.5% (w/v) acrylamide] using the Laemmli discontinuous buffer system. Protein samples were diluted to the desired concentration in equal volumes of ddH₂O and SDS-PAGE 2X sample buffer [125mM Tris, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol (BDH, Toronto, ON, Canada), 0.002% (w/v) Bromophenol Blue

(Fisher, Fair Lawn, NJ, USA)]. Samples were heated at 100°C for 5 min prior to electrophoresis at 100 V in the Mini-Protean II electrophoresis cell system (Bio-Rad, Richmond, CA, USA).

2.3 Staining Methods for SDS-PAGE (Rapid Stain)

SDS-PAGE gels (1D) were washed in double distilled water (ddH₂O) three times for 5 min each, after which they were stained with RapidStain (G Bioscience, St. Louis, MO, USA) overnight followed by several washes in ddH₂O until the protein bands became visible and the background cleared.

2.4. Heme Binding Detection via Chemiluminescence

The technique of detection of c-type cytochromes using enhanced chemiluminescence (ECL) was first developed by Vargas and colleagues (1993), and then adapted to detect binding of bacterial proteins to heme by Letoffe and colleagues (2006). A total of 10 µg of each purified protein was incubated at room temperature for 30 min with either increasing concentrations of heme from 10⁻⁷ M to 10⁻⁴ M or with binding buffer alone. Heme stock solutions were made by dissolving bovine hemin (Sigma, St. Louis, MO, USA) in 0.1N NaOH and were used without further sterilization. The mixtures were then separated by PAGE, using the Native PAGE Ready Gel System (Bio-Rad, Hercules, CA, USA), as per the manufacturer's instructions. 10X Native Running buffer (30.3g Tris Base, 144g Glycine to 1L ddH₂O) was diluted to 1X and used as the running buffer. The resulting proteins on the gel were transferred onto nitrocellulose membranes, as per the protocol for Western blot analysis

described in section 2.8. Heme complexed with protein bands on the gel retains intrinsic peroxidase activity, and was detected by chemiluminescence via the addition of equal amounts of Lumigen TMA-6 solutions A and B to the membrane (ECL+; Amersham, Pharmacia). The signal intensity was measured using the Storm Imager Analysis System (GE Healthcare Bio-Sciences, Baie d'Urfe, Que.).

2.5. Bacterial strains and Growth Conditions

E. coli strain FB827 *dppA::Km mppA::Cm* (pAM238-HasR), kindly provided by C. Wandersman (Institut Pasteur, Paris, France) was used in this study as a negative control for the growth promotion experiments, and as a background strain in which pET151-hHBP and pET151 were transformed, creating the experimental strain FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151-hHBP) and the negative control FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151), respectively. *E. coli* strain FB827 *dppF::Km* (pAM238-HasR)(pTRC99-dppBCDF), also provided by C. Wandersman, was used in this study as a positive control for the growth promotion experiments. *E. coli* Top10 cells and BL21 StarTM (DE3) cells were purchased from Invitrogen (Carlsbad, CA, USA) and were used for transformation of the pET151 and pET151-hHBP plasmid constructs as well as expression of the *hhbp* gene. All bacterial strains were grown at 37°C in either LB broth or M63 Minimal Media and were stored at -80°C in LB broth containing 15% (v/v) glycerol. All bacterial strains used are further described in Table 1.

2.6. Media and Culture Conditions

2.6.1. Luria Bertani Agar Plates or Broth with or without Antibiotics

E. coli strains were grown on Luria Bertani (LB) plates [LB broth (Difco/Becton Dickinson, Sparks, MD, USA), 1.5% (w/v) Bacto agar (Difco/Becton Dickinson, Sparks, MD, USA)] or in LB broth with vigorous shaking at 225 rpm using the appropriate growth conditions. To induce expression of the *hhbp* gene in BL21 Star™ (DE3) *E. coli* cells, isopropylthio-B-galactoside (IPTG; Invitrogen, Carlsbad, CA, USA) was added to a final concentration of 1mM at the mid-log phase (OD₆₀₀ 0.5-0.8) of growth. For positive transformant selection, ampicillin (AMP; Sigma, St. Louis, MO, USA) was also added to the media to a final concentration of 100 µg/ml.

2.6.2. M63 and M63* Agar Plates or Broth with or without Supplements

E. coli FB827 *dppA::Km mppA::Cm* (pAM238-HasR), *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151-hHBP), *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151) and *E. coli* strain FB827 *dppF::Km* (pAM238-HasR)(pTRC99-dppBCDF) were grown at 37°C on M63 or M63* agar plates, or in M63 broth. M63 agar was prepared by the addition of 1.5% (w/v) BactoAgar (Difco/Becton Dickinson, Sparks, MD, USA) to M63 media [100mM KH₂PO₄, 15mM (NH₄)₂SO₄, 1.798x10⁻⁶M FeSO₄·7H₂O, pH to 7.0 with KOH, 0.4% (v/v) glucose, MgSO₄·7H₂O]. M63* broth and agar lack FeSO₄·7H₂O. For growth promotion assays, various supplements were added to the broth or agar. When necessary, antibiotics were added at the following concentrations: ampicillin at 100 µg/ml, chloramphenicol at 15

Table 1. Bacterial strains used in this study and their specific properties.

Bacteria Strain	Properties	Reference
<i>E. coli</i> FB827 <i>dppA::Km mppA::Cm</i> (pAM238-HasR)	Siderophore deficient <i>E. coli</i> strain (carrying the <i>entF::Tn10</i> mutation to suppress enterobactin production) with antibiotic resistance to kanamycin and chloramphenicol	Provided by C. Wandersman
<i>E. coli</i> FB827 <i>dppA::Km mppA::Cm</i> (pAM238-HasR) (pET151-hHBP)	Siderophore deficient <i>E. coli</i> strain (carrying the <i>entF::Tn10</i> mutation to suppress enterobactin production) with antibiotic resistance to kanamycin and chloramphenicol, complimented with hHBP	This study
<i>E. coli</i> FB827 <i>dppA::Km mppA::Cm</i> (pAM238-HasR) (pET151)	Siderophore deficient <i>E. coli</i> strain (carrying the <i>entF::Tn10</i> mutation to suppress enterobactin production) with antibiotic resistance to kanamycin and chloramphenicol carrying the pET151 empty vector	This study
<i>E. coli</i> FB827	Siderophore deficient	Provided by C.

<p><i>dppF::Km</i> (pAM238-HasR)(pTRC99-dppABCDF)</p>	<p>(carrying the <i>entF::Tn10</i> mutation to suppress enterobactin production). Heme-sufficient <i>E. coli</i> strain (complimented with the heme/dipeptide permease) with antibiotic resistance to kanamycin</p>	<p>Wandersman</p>
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µg/ml, kanamycin at 25 µg/ml, tetracycline at 10 µg/ml, and spectinomycin at 50 µg/ml. For the growth promotion assays, media were supplemented with various concentrations of bovine hemoglobin (1 to 50 µM). Hemoglobin stock solutions were made by dissolving bovine hemoglobin S (Sigma, St. Louis, MO, USA) in sterile ddH₂O, and were filter-sterilized using 0.2 µM filters. To sequester free iron in the broth or agar, the iron chelator, 2,2'-dipyridyl (2,2-Dip), was added at a final concentration of 100 µM. IPTG was also added to the agar plates at a concentration of 1 mM, to induce the expression of hHBP from the pET151-hHBP vector.

2.7. Cell Fractionation Procedures

2.7.1. Whole Cell Lysates

Bacteria were grown in LB broth at the appropriate growth conditions. At 4 h post addition of IPTG, the broth was pelleted by centrifugation at 4000 x g for 15 min at room temperature. The pellet was then washed three times with 1ml sterile 1X PBS and subsequently suspended in an equal volume of sonication buffer (10mM Tris-HCl, 5mM EDTA). Sonication was performed four times using the sonifier/disrupter at 15-20W output (Branson Sonic Power Co., Danbury, CT, USA) with each round comprising 15 seconds of sonication followed by 10 seconds interruption on ice. The mixture was centrifuged for 60 min at 100 000 x g at 4°C. The supernatant was collected and stored at -20°C.

2.7.2. Periplasmic Extraction (Chloroform Method)

The chloroform method of protein extraction was adapted from a previously described technique (Ames et al., 1984; Judd and Porcella, 1993). Bacteria were grown in LB broth at the appropriate growth conditions. At 4 h post addition of IPTG, the broth was pelleted by centrifugation at 4000 x g for 15 min at room temperature. The pellet was then washed three times with sterile 1ml 1X PBS prior to suspension in an equal volume of sonication buffer and chloroform (EMD, Gibbstown, NJ, USA) with the addition of PMSF to a final concentration of 1mM. Following incubation for 20 min at room temperature, the mixture was centrifuged for 60 min at 100 000 x g at 4°C. The supernatant from the supernatant-chloroform interface was collected and frozen at -20°C. The pellet was used for the extraction of the cytoplasmic fraction as described below.

2.7.3. Cytoplasmic Extraction

After removal of the periplasmic protein fraction contained in the supernatant, the chloroform interface was discarded to avoid periplasmic protein contamination during the cytoplasmic protein extraction. The pellet was resuspended in sonication buffer (1mL per gram wet weight) followed by four short bursts of sonication using the sonifier/disrupter at 15-20W output, with each round consisting of 15 seconds sonication followed by 10 seconds interruption on ice. The sonicated mixture was then centrifuged at 100 000 x g for 60 min at 4°C. The supernatant was collected and frozen at -20°C.

2.8. Western Immunoblotting

The protein samples underwent SDS-PAGE in duplicate in order to transfer one gel onto Immobilon-FL (Millipore, Billerica, MA, USA) and to stain the second gel with

Rapid stain as described above to ensure correct loading. The transfer gel was first equilibrated in fresh Towbin transfer buffer [25mM Tris, 192mM glycine, 20% (v/v) methanol (Fisher, Fair Lawn, NJ, USA)] for approximately 30 min. The semi-dry Mini-Protean II blotting apparatus (BioRad, Richmond, CA, USA) was then assembled according to the manufacturer's instructions. The transfer conditions were as follows: 30 min at 20V with a constant amperage of 0.5A. After the transfer was complete, the blot was stained with 10% (v/v) Ponceau S (Sigma, St. Louis, MO, USA) to identify the molecular weight markers. The stain was removed by washing the blot several times in ddH₂O followed by overnight incubation in a 2% skim milk blocking solution [2% (w/v) skim milk (EM Science, Gibbstown, NJ, USA) in PBS]. The blot was rinsed with ddH₂O followed by incubation for 30 min in a 1% skim milk solution [1% (w/v) skim milk, 0.1% (v/v) Tween 20 (Sigma, St. Louis, MO, USA) in PBS]. After rinsing with ddH₂O, the blot was probed for 30 min at room temperature with the primary antibody solution [0.1% (v/v) Tween 20 in PBS]. After three washes of 5 min each in PBS, the blot was incubated with a 1:10 000 dilution of goat anti-rabbit immunoglobulin horse radish peroxidase conjugated secondary antibody (BioSource, Camarillo, CA, USA) solution [0.1% (v/v) Tween 20 in PBS] for 30 min at room temperature. After three washes of 5 minutes each in PBS, the membrane was developed with TMB peroxidase substrate (KPL, Gaithersburg, Maryland, USA) for 1-5 min. The reaction was stopped by rinsing the blot with ddH₂O.

2.8.1 Primary Antibodies for Western Immunoblotting

The rabbit polyclonal antibody raised against the *H. ducreyi* hHBP protein (anti-hHBP) was produced by Melissa St. Denis, a former Master's student in our laboratory. Anti-hHBP was used at a dilution of 1: 8000.

2.9. Plasmid DNA Isolation

pET151-hHBP was extracted from BL21 cells using the Qiagen Plasmid Midi Kit (Qiagen, Mississauga, ON, Canada) according to the instructions supplied by the manufacturer. A total of 50 ml of overnight inoculum in LB were harvested by centrifugation at 4100 rpm for 15 mins at 4° C. The pellet was resuspended in 10 ml Buffer P1 (Qiagen, Mississauga, ON, Canada), after which 10 ml of Buffer P2 (Qiagen, Mississauga, ON, Canada) was added and mixed by inversion up to 6 times. The incubation was left at room temperature for 5 min. A total of 10 ml of buffer P3 (Qiagen, Mississauga, ON, Canada) was added and mixed by inversion up to 6 times. Lysate was poured into the barrel of the QIAfilter cartridge, and left to incubate for 10 min without agitation. The lysate was then filtered into the Hi-Speed tip and allowed to pass through the resin via gravity flow. The tip was then washed with 60 ml of buffer QC (Qiagen, Mississauga, ON, Canada). The DNA was eluted with 15 ml of Buffer QF (Qiagen, Mississauga, ON, Canada). The DNA was precipitated via the addition of 10.5 ml isopropanol at room temperature. The mixture was left to incubate for 5 mins. The elutate/ isopropanol mixture was then filtered through the QIA precipitator under constant pressure. The DNA bound to membrane was then washed by pressing 2 ml 70% ethanol through the QIA precipitator. The membrane was dried twice by pressing air

through the QIA precipitator. The DNA was eluted by pressing 1 ml of ddH₂O through the QIA precipitator.

2.10 Agarose Gel Electrophoresis

Gels composed of either 0.7 or 1.0% (w/v) agarose were electrophoresed using the Hoefer HE 33 Mini Submarine (Amersham Biosciences, Piscataway, NJ, USA) as per the manufacturer's instructions. Briefly, UltraPure™ agarose (Invitrogen, Carlsbad, CA, USA) was diluted in 1x Tris-Borate-EDTA buffer [TBE; 0.55% (w/v) boric acid (BioShop, Burlington, ON, Canada), 1.1% (w/v) Tris base, 0.4% (v/v) 0.5M EDTA, pH 8.0)]. Prior to gel loading, 1µl of 10x DNA gel loading buffer (Eppendorf, Westbury, NY, USA) was added to each sample. Gels were electrophoresed at 100-120V and DNA bands were visualized by adding 0.005% (v/v) ethidium bromide (EtBr; Invitrogen, Carlsbad, CA, USA) to the molten agarose prior to casting. Agarose gel images were observed and photographed with UV light using the MultiImage Light Cabinet (Alpha Innotech Corp., San Leandro, CA, USA).

2.11. DNA Quantification

The quantity of plasmid DNA was quantified spectrophotometrically using a RNA/DNA calculator GeneQuant II (Pharmacia Biotech, Cambridge, England).

2.12. Complementation Assays:

E. coli strain FB827 *dppA::Km mppA::Cm* pAM238-HasR, is unable to use heme as an iron source due to mutations in the two periplasmic components, *dppA* and

mppA, of the *E. coli* heme ABC transporter *dppABCDF*. We tested whether the expression of hHBP in this mutant strain had the ability to restore the wild type heme phenotype.

2.12.1. Preparation of Competent *E. coli*

A single colony of *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) grown overnight on LB agar was used to inoculate 100 ml of LB broth containing 25 µg/ml kanamycin and 15 µg/ml chloramphenicol, and grown at 37°C with vigorous shaking (300 rpm) until a cell density of 10⁸ cells/ml was attained (OD₆₀₀ of 0.8). Cells were cooled for 10 mins on ice, after which they were centrifuged at 4000 rpm for 10 min at 4°C. The pelleted cells were resuspended in 10 ml of ice-cold 0.1 M CaCl₂. The cells were again pelleted by centrifugation, resuspended in 2 ml of ice-cold 0.1 M CaCl₂ and stored at 4°C for up to 48 h prior to transformation. Competent cells that were not used within 48 h were stored at -80 °C.

2.12.2. Transformation

pET151-hHBP (50 ng in 10 µl or less of ddH₂O) was added to a 200 µl suspension of competent *E. coli* FB827 *dppA::Km mppA::Cm*(pAM238-HasR) cells and the mixture was incubated on ice for 30 min. The cells were heat-shocked at 42°C for 90 sec and then placed directly on ice. A total of 800 µl of Super Optimal Broth with catabolite repression (SOC) medium [2% (w/v) bacto-tryptone (Becton Dickinson and Company, Sparks, MD, USA), 0.5% (w/v) bacto-yeast extract (Becton Dickinson and Company, Sparks, MD, USA), 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 20mM glucose, pH 7.0] was added to the cells and the cell suspension was incubated for 45 min in a 37°C water bath. *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-

HasR)(pET151-hHBP) transformants were selected on LB agar plates containing tetracycline(10 µg/ml), spectinomycin (50 µg/ml), kanamycin (25 µg/ml), chloramphenicol (15 µg/ml), and ampicillin (100 µg/ml). Transformation using pET151, isolated from *E. coli* Top10 cells via plasmid DNA isolation procedures described in section 2.9, into competent *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) was done using the same procedure.

2.12.3. Confirmation of Transformation

Transformation of *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) with pET151-hHBP and pET151 was confirmed using several methods. Transformants were tested for growth in LB plates containing 100 µg/ml ampicillin, 50 µg/ml spectinomycin and 10 µg/ml tetracycline. An ampicillin-resistant phenotype would provide presumptive evidence for the presence of pET151, as this vector harbors the β -lactamase gene cassette. In addition, a spectinomycin and tetracycline resistant phenotype would ensure that the transformants retained pAM238-HasR. Vectors pET151-hHBP and pET151 were isolated as described in section 2.9, and the size of the plasmids were verified by agarose gel electrophoresis. Additionally, the presence of the two vectors was confirmed by PCR.

2.12.3.1. Polymerase Chain Reaction (PCR)

Colonies picked from LB agar plates containing AMP were analyzed by colony PCR using pET151-hHBPFOR: 5'-CACCATGAATCTTTCCTTTCTAA and pET151-hHBPREV: 5'-GGATGCTGTAGCTTGTGTTATATTG primers (Invitrogen, San Diego, CA, USA). A 100 µl PCR Master Mix was prepared with 2 units of Biotools Platimun Taq DNA polymerase (Biotools, Madrid, Spain), 0.2mM dNTP solution, 0.5

μM solution of each primer, 10 μl of 10X Biotools PCR buffer. DNA template and sterile ddH₂O were then added to a final volume of 100 μl. DNA template concentrations were between 10- 50 ng/25 μl PCR reaction for plasmid DNA. The insert was amplified using the Touchgene Gradient Thermocycler (Techne; Cambridge, UK) with the following conditions: an initial denaturation of 94°C for 3 min; 25 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; and a final extension of 72°C for 10 min. Insert size was determined by agarose gel electrophoresis of the PCR product. Transformants exhibiting the appropriate size amplicon were frozen in LB broth containing AMP with 15% (v/v) glycerol and stored at -80°C. Confirmation of pET151 using PCR analysis was done using the same conditions with the exception that T7 Forward and Reverse primers (Invitrogen, Carlsbad, CA, USA) were used.

2.12.4. hHBP Expression in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151-hHBP)

Transformants were grown in LB broth and were assessed hourly for 8 h for expression of hHBP following IPTG induction at mid-log phase followed by the cellular fractionation procedures, described in section 2.7, to determine the cellular location of hHBP in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151-hHBP). The cell lysates were separated by SDS-PAGE. Western immunoblots of the cell lysates were probed with anti-hHBP antibody (described above).

2.12.5. Growth Promotion Assays

The ability of hHBP to restore the heme phenotype in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151-hHBP) was determined by growth on M63*

plates containing various hemoglobin concentrations. *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151-hHBP) was grown in 10 ml of M63 broth, with appropriate antibiotics, until the cells reached an OD₆₀₀ of 1.0. The cells were then inoculated onto M63* agar plates supplemented with hemoglobin concentrations varying from 1 to 50 μ M, 1mM IPTG, 100 μ M 2,2-Dip, and the appropriate antibiotics (using a steers replicator). Each culture was placed into 4 or 7 wells. Pins dipping into the wells were used to inoculate plates in sets of 4 or 7, with each pin delivering an equal volume of inoculum to the agar plates. Cells were inoculated onto M63 agar plates (with no added supplements) in order to ensure cell viability. In addition, cells were inoculated onto M63* agar plates containing no iron source as a negative control. *E. coli* FB827 *dppF::Km* (pAM238-HasR)(pTRC99-dppBCDF), the double knock-out *E. coli* strain complemented with the heme/dipeptide permease, was used as a positive control in the growth promotion assays. *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR), the double knock-out *E. coli* strain; and *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)(pET151), the double knock-out *E. coli* strain transformed with the empty pET151 vector, served as negative controls. The difference in growth was visualized after 72 h incubation at 37 °C.

CHAPTER 3: RESULTS

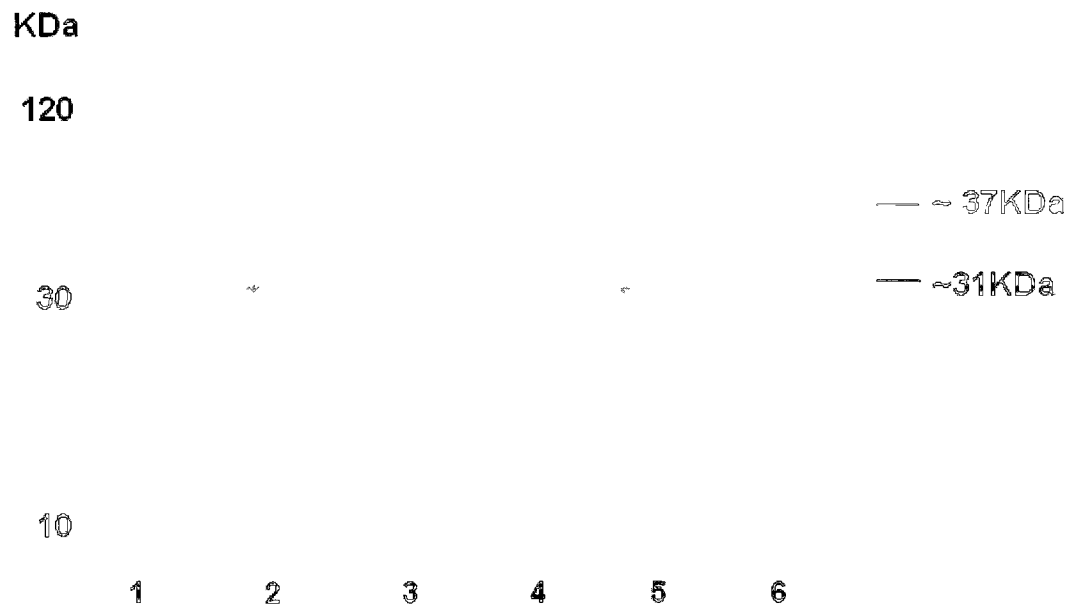
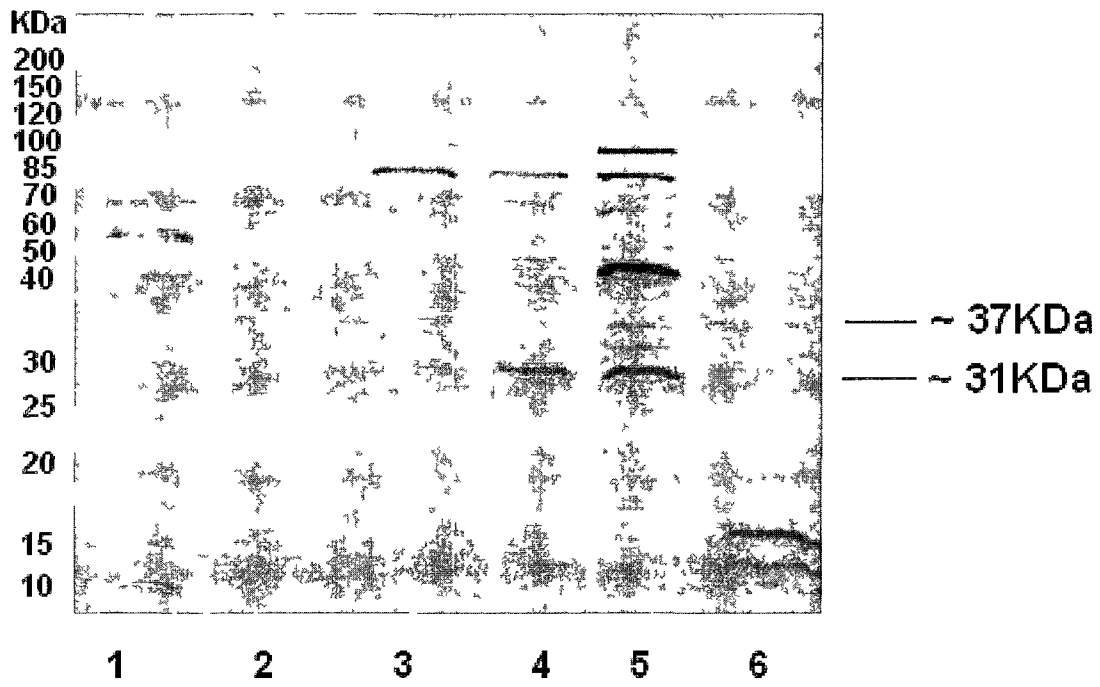
Our laboratory has previously uncovered a heme dedicated periplasmic protein in *Haemophilus ducreyi*, designated hHBP. This protein was shown to be consistently upregulated under heme-limiting conditions in comparison to conditions that were heme replete (St. Denis, unpublished). In addition, recombinant hHBP (rhHBP) bound heme agarose in a concentration dependent manner. Competitive binding experiments demonstrated that hHBP bound heme specifically. In this study, several biochemical and genetic approaches were used to further functionally characterize hHBP both in vitro and in vivo.

3.1. Expression of the Recombinant Fusion Protein

Our laboratory has previously cloned the gene encoding the hHBP protein into the pET151/D-TOPO[®] vector, and the plasmid was transformed into *E. coli* BL21 cells. Upon IPTG induction, the mature protein was found to be predominantly in the periplasmic fraction (Figs 1A and 1B lane 5). The mass of the mature protein was 31kDa as demonstrated by SDS-PAGE. The hHBP protein was also detected in the whole cell lysate (WCL) fractions prior to IPTG induction (figure 1A and 1B, lane 2), an observation likely due to the recognized ability of the T7 promoter found on pET151 to drive the expression of the cloned gene in the absence of the inducer IPTG. However, a 4h IPTG induction time-course experiment demonstrated an increase in hHBP protein expression in the presence of IPTG as compared to the absence of IPTG (Fig 1A and 1B lanes 3 and 4). A total of 6.87 μ g of protein was loaded in each lane.

Fig1A: Rapid stain illustrating the protein band pattern observed in the WCL, periplasmic and cytoplasmic extractions. Lane 1 represents the PageRuler unstained protein ladder from Fermentes (Burlington On, Canada); lane 2 represents WCL at T=0 (right before IPTG addition); lane 3 represents WCL 4 h into the induction experiment without the addition of IPTG; lane 4 represents WCL 4 h after IPTG induction; lane 5 represents the periplasmic extraction after 4 h of IPTG induction; lane 6 represents the cytoplasmic extraction after 4 h of IPTG induction.

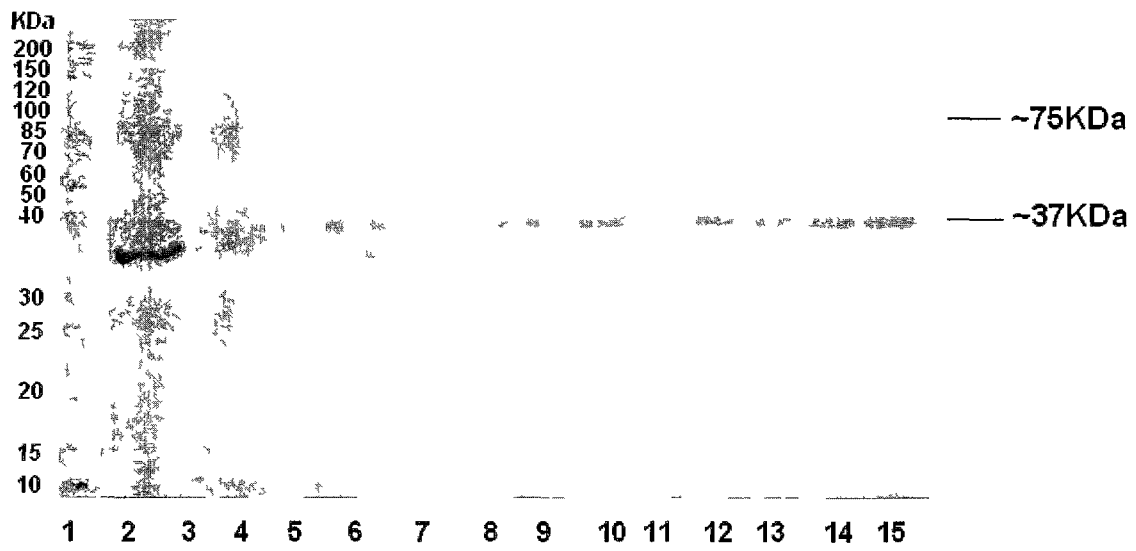
Fig 1B: Immunoblot with anti-hHBP antibody illustrating the presence of hHBP observed in the WCL, periplasmic and cytoplasmic extractions. Lane 1 represents the PageRuler unstained protein ladder from Fermentes; lane 2 represents WCL at T=0 (right before IPTG addition); lane 3 represents WCL 4 hours into the induction experiment without the addition of IPTG; lane 4 represents WCL 4 hours after IPTG induction; lane 5 represents the periplasmic extraction after 4 hours of IPTG induction; lane 6 represents the cytoplasmic extraction after 4 hours of IPTG induction.



3.2. Purification of the Recombinant Fusion Protein

Metal affinity chromatography was used to purify rhHBP. Mature hHBP was shown to reside in the periplasmic space (Fig 1B lane 5) when the protein was expressed in BL21 *E. coli*. In BL21 *E. coli*, the 6x histidine fusion tag required for metal affinity chromatography is located upstream of the signal sequence of the protein. As cleavage of the signal sequence would occur once hHBP reached its destination in the periplasmic space, the mature hHBP protein located within the soluble periplasmic fraction would lack the fusion tag, rendering metal affinity chromatography impossible. However, the rhHBP expressed within the insoluble fraction of *E. coli* (visible in the cytoplasmic extraction and WCL as the higher band of ~37KDa in fig 1A and 1B) retains the 6x his fusion tag (4KDa) and the native signal sequence (2KDa). Consequently, the insoluble fraction was used to purify the rhHBP. The Ni-NTA purification hybrid method of protein purification was used to maintain protein activity for functional characterization of the protein. Cell lysates of the remaining pellet protein fractions were prepared under denaturing conditions. Elution was performed using native buffers to refold the proteins in order to restore function. An excess of imidazole was added to the fusion-tagged proteins bound to the Ni ions of the chromatography column to free the fusion-tagged proteins. rhHBP eluted mainly in fractions containing 100, 150, 200 and 250mM imidazole (Fig 2). When the eluted fractions were applied to a 1D SDS-PAGE gel, two protein bands representative of the rhHBP monomer (37.24KDa) and the rhHBP homodimer (~75KDa) were observed.

Fig 2: Rapid stain of the protein fractions eluted using the Ni-NTA hybrid method of protein purification. Lane 1 represents the PageRuler unstained protein ladder from Fermenter; lane 2 represents the flow through, lanes 3 and 4 represents fractions eluted from the column using the denaturing binding buffer twice; lanes 5 and 6 represents fractions eluted from the column using the denaturing wash buffer twice, lanes 7- 10 represents fractions eluted from the column four times using the native wash buffers consisting of 20mM Imidazole, lanes 11- 15 represents fractions eluted from the column using native elution buffers with 50, 100, 150, 200, 250mM imidazole respectively.

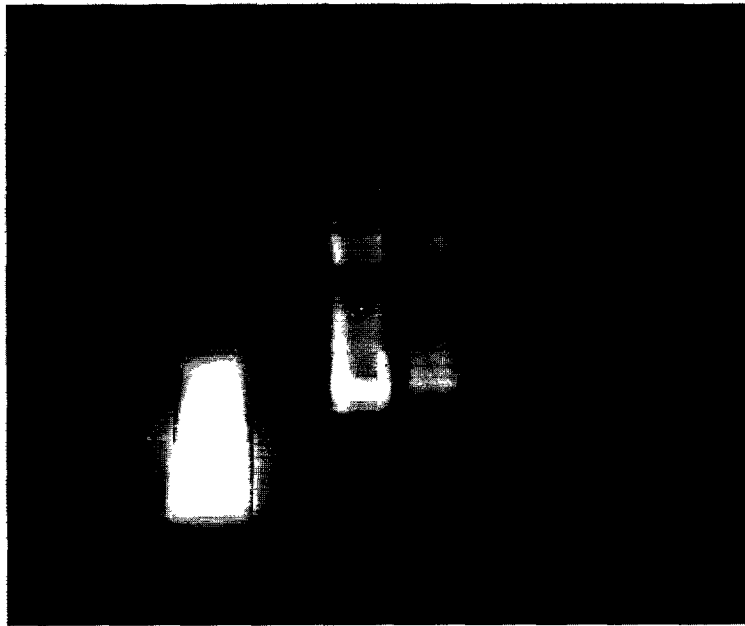


3.3. Functional characterization of the hHBP Protein by enhanced chemiluminescence.

Enhanced chemiluminescence was used to detect whether rhHBP bound heme. A fixed amount of affinity-purified rhHBP (10 µg) was pre-incubated with increasing concentrations of heme. This method capitalizes on the intrinsic peroxidase activity of heme, which catalyses the oxidation of the substrate “luminol”, which in the presence of an enhancer produces light emission that can be detected on a photographic film/ storam imager allowing for the identification of hemoproteins (Vargas, 1993). As the heme concentration increased ($10^{-7}\text{M} - 10^{-4}\text{M}$), rhHBP binding to heme also increased (Fig 3A). To confirm that the detected bands comprised hHBP-heme complexes, the same membrane used for chemiluminescence was probed with anti-sera directed against hHBP. An immunoreactive band was present corresponding to the band seen on the chemiluminescent assay, verifying the identity as hHBP. The Western blot also showed an increase in band intensity as the heme concentration decreased (Fig 3B). As the anti-hHBP antibody was raised against the unbound form of hHBP, binding of hHBP to heme would be expected to produce a conformational change in the protein. This structural alteration of hHBP would reduce the number of epitopes recognized by the antiserum. As a consequence, the intensity of the immunoreactive band would diminish in the face of increasing heme concentrations, reflecting an increase in the amount of hHBP binding to heme.

As a negative control, a ~31KDa recombinant 5' 6x his-tagged outer membrane lipoprotein from *Leptospira*, rLipL32, was examined for it's ability to

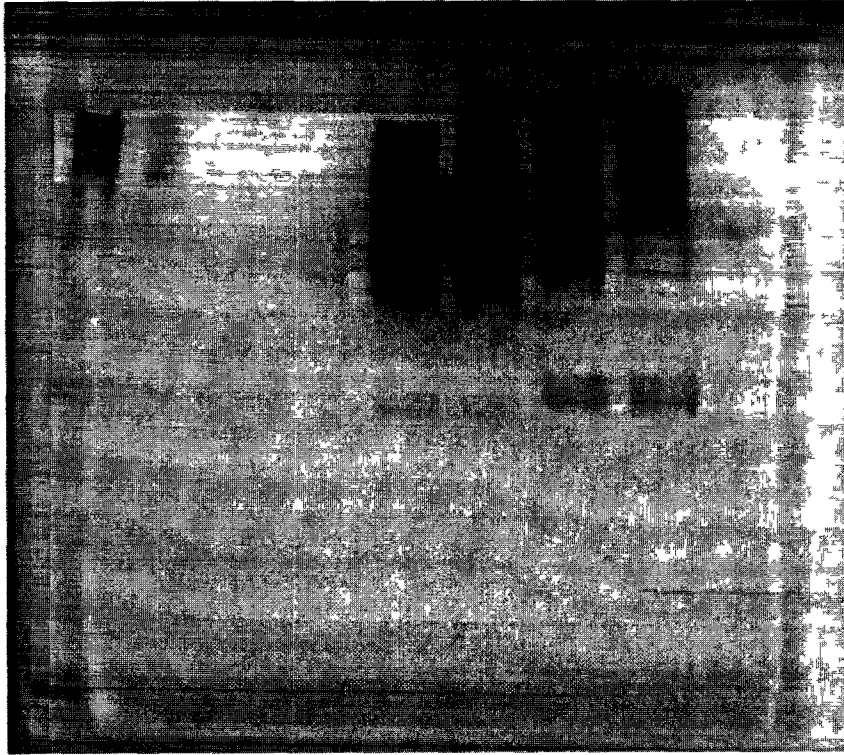
Fig 3A: ECL detection after non-denaturing PAGE and transfer onto nitrocellulose membrane. Lane 1 represents hHBP alone; lane 2 represents heme alone at a concentration of 10^{-4} M, lanes 3-6 represent 10 μ g of hHBP incubated at room temperature for 30 min with concentrations of heme ranging from 10^{-4} M – 10^{-7} M, respectively.



— hHBP

hHBP	heme	hHBP	hHBP	hHBP	hHBP
(10ug)	(10-4M)	(10ug)	(10ug)	(10ug)	(10ug)
		+	+	+	+
		heme	heme	heme	heme
		(10-4M)	(10-5M)	(10-6M)	(10-7M)

Fig 3B: Immunoblot with anti-hHBP antibody of the same membrane used for chemiluminescence. Lane 1 represents hHBP alone; lane 2 represents heme alone at a concentration of 10^{-4} M, lanes 3-6 represent 10 μ g of hHBP incubated at room temperature for 30 min with concentrations of heme ranging from 10^{-4} M – 10^{-7} M, respectively.



— hHBP

**hHBP
(10ug) heme
(10-4M)**

**hHBP hHBP hHBP hHBP
(10ug) (10ug) (10ug) (10ug)
+ + + +
heme heme heme heme
(10-4M) (10-5M) (10-6M) (10-7M)**

bind heme in the chemiluminescence assay (Fig 4). rLipL32 did not bind heme in a concentration dependent manner, as it would appear that there is no band in the ECL assay that corresponds to rLipL32. All ECL experiments were done in triplicate. These results indicate that hHBP binds heme in a concentration-dependent manner.

3.4. Transformation of pET151-hHBP into *E. coli* strain FB827

***dppA::Km mppA::Cm* (pAM238-HasR).**

To further investigate the role of hHBP in heme binding, we performed functional complementation experiments. hHBP was expressed in an *E. coli* K12 mutant lacking the ability to use heme as an iron source. Wild type *E. coli* K12 lacks a heme outer membrane receptor and is unable to use exogenous heme as an iron source (Letoffe et al., 2006). However, the expression of a foreign heme outer membrane receptor (the *Serratia marcescens* HasR outer membrane receptor) in *E. coli* allows the organism to use exogenously added heme as an iron source (Letoffe et al., 2006). Letoffe et al. (2006, 2008) identified two periplasmic-binding protein (MppA and DppA) components of a dipeptide ABC transporter (DppABCDF) that were also engaged in heme transport. Creation of a double mutant with insertional inactivation in both *mppa* and *dppa* genes by these investigators resulted in a heme deficient phenotype, *E. coli* strain FB827 *dppA::Km mppA::Cm* (pAM238-HasR).

To determine the ability of hHBP to restore the wild-type heme phenotype of *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR), pET151-hHBP was isolated from *E.*

Fig 4: ECL detection after non-denaturing PAGE and transfer onto nitrocellulose membrane. Lane 1 represents heme alone at a concentration of 10^{-5} M; lane 2 represents 10 μ g of hHBP incubated at room temperature for 30mins with heme at a concentration of 10^{-5} M; lanes 3-5 represents 10 μ g of rLipL32 incubated at room temperature for 30mins with concentrations of heme ranging from 10^{-5} M – 10^{-7} M respectively.

Fig 5: Miniprep of *E. coli* BL21 (pET151-hHBP) showing the presence of the pET151-hHBP vector construct at 6.7 kbp. Lane 1 represents the invitrogen supercoiled DNA ladder; lane 2 represents the miniprep.

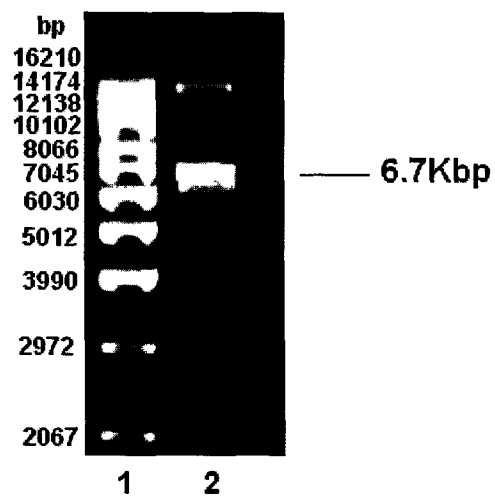
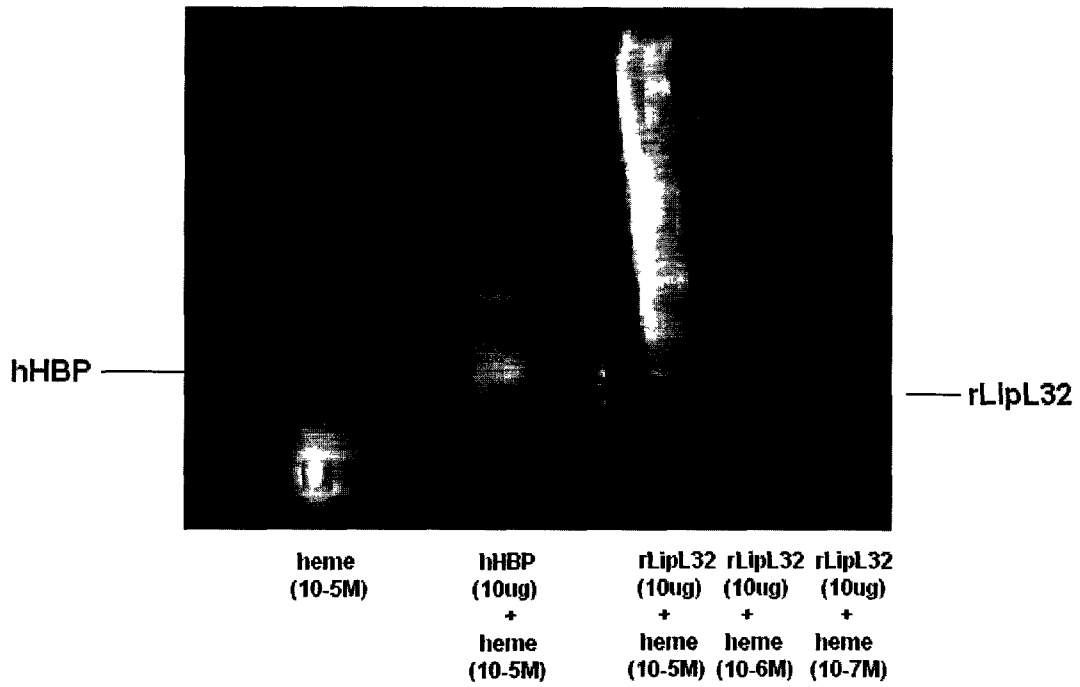


Fig 6A: Miniprep of *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP) showing the presence of the transformed pET151-hHBP vector construct at 6.7 kbp. Lane 1 represents a miniprep of a positive ampicillin transformant; lane 2 represents a miniprep of the *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) lacking the pET151-hHBP vector; lane 3 represents the invitrogen supercoiled DNA ladder.

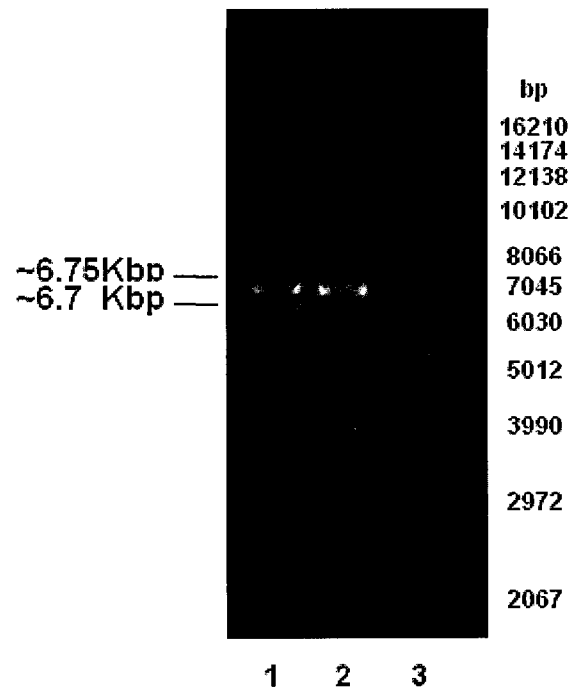
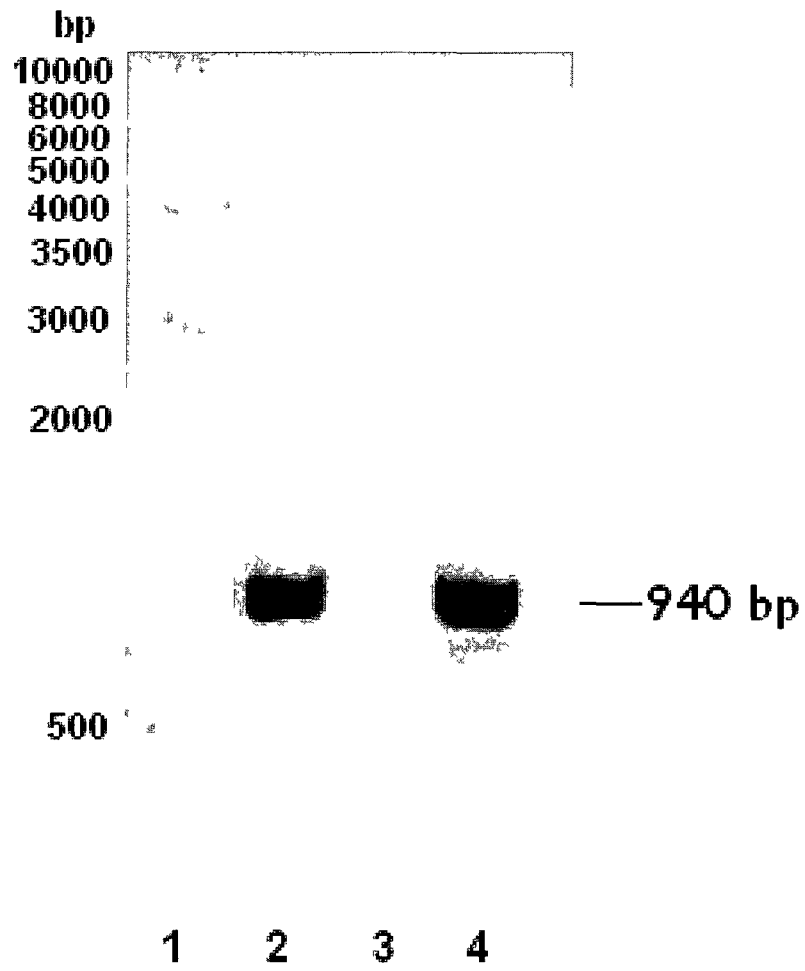


Fig 6B: PCR of the *E. coli* strain FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP) miniprep revealing the presence of the 940bp *hhbp* insert. Lane 1 represents the GeneRuler 1 kb DNA ladder; lane 2 represents pET151-hHBP serving as a positive control; lane 3 represents water serving as a negative control; lane 4 represents the miniprep of the positive ampicillin transformant.



coli BL21 cells (as shown in Fig 5 by the presence of the 6.7 kb band) and was introduced by transformation into competent *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) cells. A miniprep prepared from one antibiotic resistant (chloramphenicol, kanamycin, tetracycline, spectinomycin and ampicillin) transformant (Fig 6A) revealed two bands of 6.75 kb and 6.7 kb. The higher band of 6.75 kb represents the pAM238-HasR vector present in the *E. coli* strain FB827 *dppA::Km mppA::Cm* (pAM238-HasR), while the lower 6.7 kb band represents the pET151-hHBP vector, composed of the 5760bp plasmid backbone and the 940bp *hhbp* insert. To confirm the identity of the transformed pET151-hHBP vector construct, PCR amplification using pET151 forward and reverse primers, that flanked the pET151 cloning site, was performed. A PCR product of 940 bp which represents the size of the *hhbp* insert, was amplified (Fig 6B).

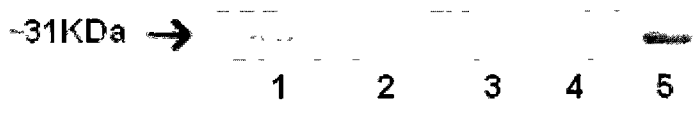
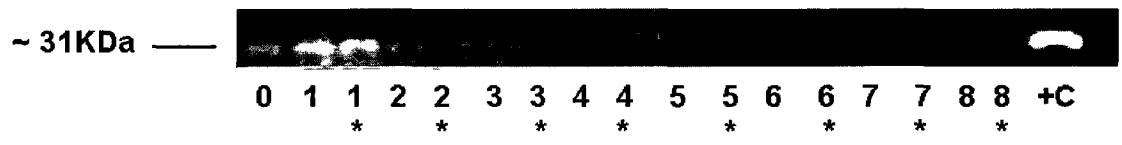
These results confirm that pET151-hHBP had been successfully transformed into *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) cells.

3.5. Expression of hHBP in *E. coli* strain FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP).

The expression of hHBP in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP) was induced by IPTG. Whole cell aliquots were removed hourly for 8 h. An immunoblot of whole cell lysates (WCL) prepared from each sample revealed the presence of hHBP for up to 3 h without the addition of IPTG, as well as 3 h following the addition of IPTG (Fig 7A). A periplasmic extract previously prepared from

Fig 7A: Immunoblot with anti-hHBP antibody illustrating the presence of hHBP in the WCL. Aliquots were removed hourly for 8 h. The asterisk represents samples in which IPTG was added whereas lanes lacking the asterisk represent samples in which IPTG was not added. Lane 0 represents aliquots removed prior to IPTG addition. 7 μ g of protein were loaded in each lane.

Fig 7B: Immunoblot with anti-hHBP antibody illustrating the presence of hHBP in the periplasmic fractions. Lanes 1 and 2 represent the periplasmic extractions 1 and 2 hours after IPTG induction; lanes 3 and 4 represent the cytoplasmic extractions 1 and 2 hours after IPTG induction; lane 5 represents the periplasmic extraction from *E. coli* BL21. Lanes were loaded with 40 μ l of the cell extract.



E. coli BL21 showing the expression of hHBP in large amounts was used as a positive control. Little difference in the expression of hHBP was observed between the induced and uninduced fractions. In addition, hHBP expression in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP) was lower than that seen in *E. coli* BL21. This result can be explained by the observation that in a T7 promoter-based expression system such as that present in pET151, the RNA polymerases synthesize mRNA at a rapid rate, thus uncoupling the transcription and translation processes in the bacterium (Invitrogen, Carlsbad, CA, USA). The nascent mRNA transcripts are left unprotected by ribosomes, exposing the transcripts to enzymatic degradation by endogenous RNases. The reduced level of mRNA transcripts in turn leads to a reduction in the protein yield. *E. coli* BL21, however, contains a mutation in the gene *rne131* encoding RNase E, which is a major enzyme for mRNA degradation (Invitrogen, Carlsbad, CA, USA) (Kido et al., 1996; Lopez et al., 1999). This mutation enables transcripts in this strain to be more stable than those in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP), leading to a higher protein yield.

Another explanation for the low protein yield observed would be that the T7 promoter is highly selective for the enzyme T7 RNA polymerase, which is introduced into the cell either via a lambda bacteriophage carrying the gene encoding for the T7 RNA polymerase (like in *E. coli* BL21 which carries the DE3 bacteriophage lambda lysogen carrying the gene encoding for the T7 RNA polymerase) or the gene encoding for T7 RNA polymerase is inserted into the host chromosome (Invitrogen, Carlsbad, CA, USA). A lambda bacteriophage carrying the gene encoding for the T7 RNA polymerase was not introduced into in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR)

(pET151-hHBP); therefore expression of hHBP via the pET151 vector was unlikely. It is however possible that *E. coli* FB827 contained endogenous RNA polymerases that slightly mimicked the T7 RNA polymerase thus leading to hHBP being expressed in low amounts.

Periplasmic and cytoplasmic fractions prepared from samples taken at 1 and 2 h after IPTG induction revealed the presence of hHBP in the periplasmic space, but not in the cytoplasm (Fig 7B).

These results indicate that hHBP was expressed in low amounts in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP) and that the protein was translocated into the periplasmic space.

3.6. Construction of the negative control *E. coli* strain FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151)

To perform the functional complementation growth promotion assays, an *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) strain that carried the empty pET151 cloning vector was constructed to serve as a negative control. However, direct transformation of the linear pET151/D-TOPO[®] into competent *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) proved to be very difficult. The two topoisomerase enzymes flanking both ends of the linear vector prevented re-ligation of the plasmid upon introduction into this *E. coli* strain. However, transformation of pET151 into *E. coli* TOP10 cells proved successful, as demonstrated by the presence of a 5760 bp band seen from a miniprep prepared from an ampicillin-resistant transformant (Fig 8A). This may have occurred because *E. coli* TOP10 cells are genetically modified to have high

transformation efficiency (Invitrogen, Carlsbad, CA, USA). The higher band observed in Fig 8A represents a higher order derivative of the plasmid. PCR amplification of the pET151-cloning site using T7 forward and reverse primers that annealed to the regions flanking the T7 promoter revealed the expected 265 bp amplicon (Fig 8B).

Following the successful transformation into *E. coli* TOP10 cells, the re-ligated plasmid was extracted and introduced by transformation into *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR). An analysis of plasmids isolated from an ampicillin resistant transformant disclosed two bands representing the 5760bp pET151 and the 6.75 kbp pAM238-HasR (FIG. 9A). Higher faint bands represent the genomic DNA or higher order derivatives of the plasmid. PCR amplification of the transformant plasmid DNA as template, using T7 forward and reverse primers that bracketed the T7 promoter site, resulted in the expected 265 bp PCR product (Fig 9B).

Fig 8A: Miniprep of an ampicillin resistant *E. coli* TOP 10 transformant. Lane 1 represents the 1 kb invitrogen supercoiled DNA ladder. Lane 2 represents pET151 recovered from the *E. coli* TOP 10 transformant.

Fig 8B: PCR amplification of the pET151-cloning site within the positive transformant, using T7 forward and reverse primers that annealed to the regions flanking the T7 promoter. Lane 1 represents the invitrogen ready-load 1 kb plus DNA ladder; lane 2 represents the 265bp amplicon from the *E. coli* transformant; lane 3 represents water used as a negative control; lane 4 represents a 265bp amplicon from pET151, which was used as a positive control.

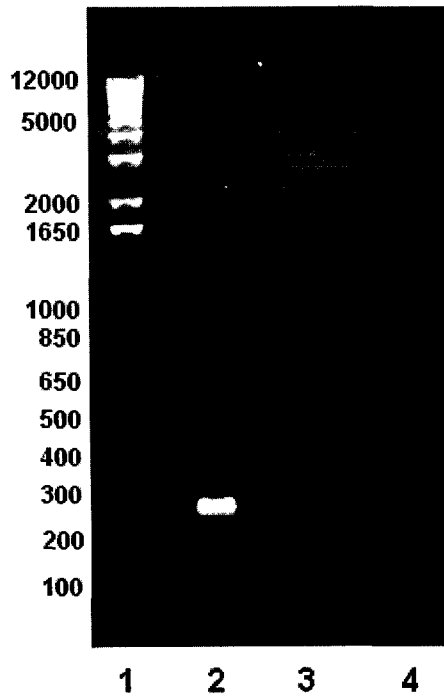
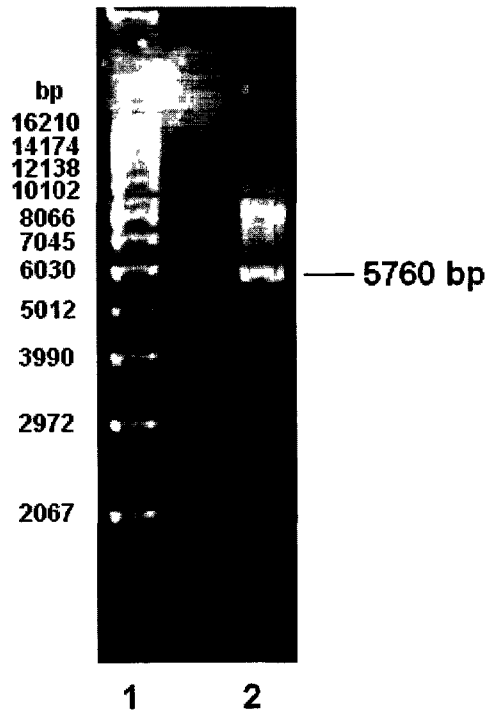


Fig 9A: Miniprep of an ampicillin resistant *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151) transformant. Lane 1 represents *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) lacking the transformed plasmid; lane 2 represents pET151 recovered from *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151); lane 3 represents the 1 kb invitrogen supercoiled DNA ladder.

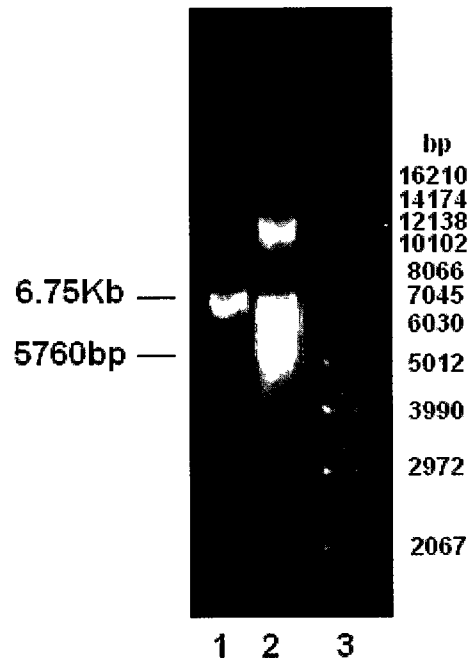
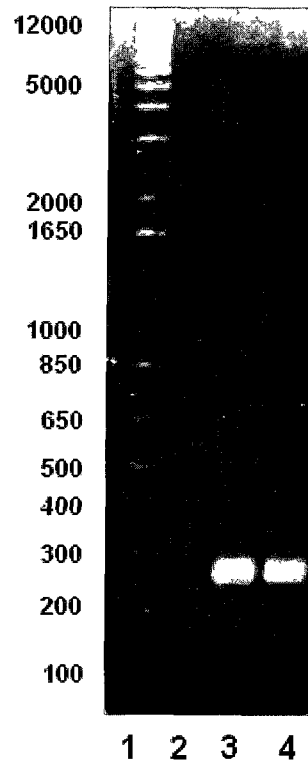


Fig 9B: PCR amplification of the pET151-cloning site within the positive transformant, using T7 forward and reverse primers that annealed to the regions flanking the T7 promoter. Lane 1 represents invitrogen ready-load 1 kb plus DNA ladder; lane 2 represents water used as a negative control; lane 3 represents the amplicon from the transformant; lane 4 represents a 265 bp amplicon from pET151, which was used as a positive control.



3.7. Complementation growth promotion Assays

To determine if the presence of hHBP would restore the heme phenotype of the *E. coli* strain containing the mutations in the *mppA* and *dppA* genes, the transformed strains were grown onto M63* agar supplemented with hemoglobin ranging from 1-10 μ M. Bovine hemoglobin was used as the heme source in these experiments because this hemoprotein was more soluble than heme. Inoculated M63* agar plates without added hemoglobin served as a negative controls. Two strains, *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151) containing the empty vector, and *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) were used as the negative controls. *E. coli* FB827 *dppF::Km* (pAM238-HasR)(pTRC99-dppABCDF) carrying the pTRC99-dppABCDF vector construct with genes encoding for the dipeptide ABC transporter that restored the wild type heme phenotype, was used as a positive control. *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP) grew on plates supplemented with 1, 5 and 10 μ MHb (Fig 10A) in a concentration-dependent manner. Growth of the positive control strain was slightly more confluent on the same plates, but most noticeable on plates supplemented with 1 μ g hemoglobin. Two explanations may have accounted for this observation. First, the low amount of hHBP expression shown in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP) may have been insufficient to fully restore the heme competency of the complemented strain. A second more plausible reason for the difference in growth arises from the use of a heterologous protein to rescue the heme deficiency in the double mutant. A lack of stability of the *H. ducreyi* protein in the *E. coli* recipient or subtle structural differences between hHBP and the *E. coli* authentic periplasmic binding proteins DppA and MppA may have compromised the

ability of hHBP to completely functionally complement the mutant strain. Neither the mutant containing the *mppA* and *dppA* double mutation, nor the host strain complemented with the empty vector grew on any of the plates supplemented with hemoglobin (Fig. 10A and 10B). No growth was seen on M63* agar that was devoid of hemoglobin (Fig. 10A, data not shown). All strains grew on M63 agar plates in which $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was supplied as an iron source. This was done as a control in order to ensure that all the strains were still viable.

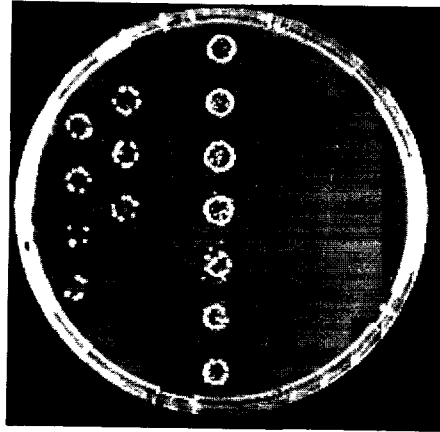
These results indicate that the *H. ducreyi hhbP* gene complements *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) for iron-heme utilization.

Fig 10A: Complementation of the *dppA mppA* double mutant for heme iron utilization. Bacteria were grown on M63* agar plates supplemented with 1, 5 and 10 μ MHb, 100 μ M 2,2-Dip to chelate any free iron in the media, 1mM IPTG to induce the expression of the hHBP on the pET151-hHBP vector. Lanes marked hHBP represent *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP); + represents *E. coli* FB827 *dppF::Km* (pAM238-HasR)(pTRC99-dppABCDF); - represents *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR). Strains were inoculated onto agar with Steers replicator in replicates of 7. All complementation experiments were performed in triplicate.

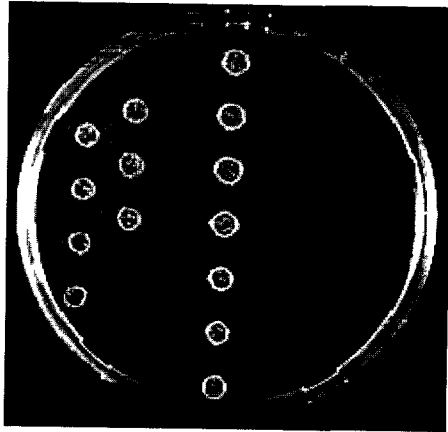
0uM Hb



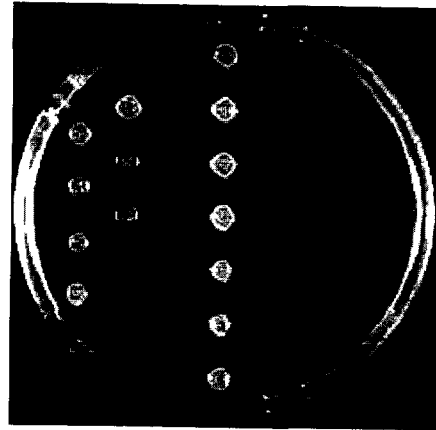
1uM Hb



5uM Hb



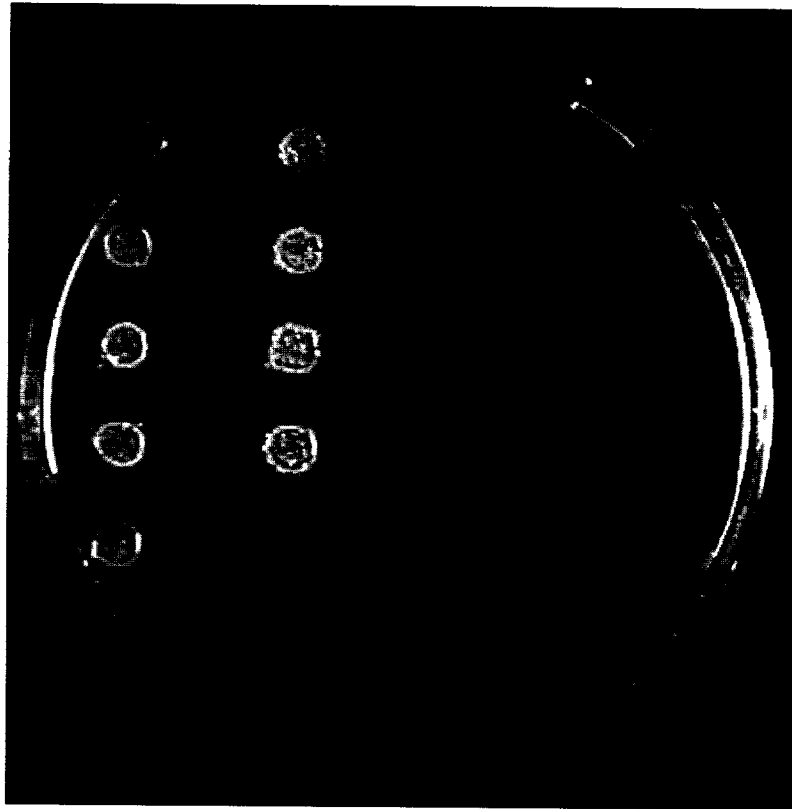
10uM Hb



hHBP + -

hHBP + -

Fig 10B: Complementation of the *dppA mppA* double mutant for heme iron utilization. Bacteria were grown on M63* agar plates supplemented with 10 μ M Hb, 100 μ M 2,2-Dip to chelate any free iron in the media, 1mM IPTG to induce the expression of the hHBP on the pET151-hHBP vector. Lanes marked hHBP represent *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-hHBP); + C represents *E. coli* FB827 *dppF::Km* (pAM238-HasR)(pTRC99-dppABCDF); -C1 represents *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR); -C2 represents *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151). Strains were inoculated onto agar with a Steers replicator in replicates of 4. All complementation experiments were performed in triplicate.



hHBP

+C

-C1

-C2

CHAPTER 4: DISCUSSION

4.1 Our experimental Approach

The current study functionally characterizes a heme dedicated periplasmic binding protein in *H. ducreyi*. Taking advantage of biochemical and genetic techniques used by other investigators, we explored the function of hHBP in vitro and in vivo. This study is the first to identify the function of hHBP in vivo.

4.1.1 ECL

Using the biochemical technique, enhanced chemiluminescence (ECL), we detected a heme-protein interaction in-vitro. This method was primarily devised as a rapid, highly sensitive immunodetection method used to identify specific antigens immobilized on filters using species-specific secondary antibodies conjugated with horseradish peroxidase. The peroxidase catalyzes the oxidation of the substrate “luminol”, which in the presence of a chemical enhancer emits a sustained light emission (Vargas et al., 1993). Based on the peroxidase activity of the heme group, any hemoprotein should be detected using this procedure (Vargas et al., 1993). Vargas et al. (1993) first used this technique to detect C-Type cytochromes (Vargas et al., 1993). Letoffe et al. (2006, 2008) also used this technique to detect heme binding to the *E. coli* periplasmic binding proteins MppA and DppA, and the *Serratia marcescens* periplasmic binding protein HemT. Using this technique, direct binding of a fixed amount of rhHBP to increasing amounts of heme showed that binding of protein to heme increased proportionally to the concentration of heme, with the highest heme binding being observed at heme concentrations of 10^{-4} M, thus confirming that rhHBP interacts

specifically and in a concentration-dependent manner with heme (Fig 3A). This finding corroborates previous in-vitro affinity binding assays, demonstrating an increased binding of rhHBP to heme agarose, as well as a decreased binding of rhHBP to heme agarose in the presence of a competing ligand (St. Denis, unpublished). Additionally, Western blot results from this study also confirmed findings from the ECL assays. As antibody raised against the unbound form of hHBP would recognize apo-hHBP more readily than holo-hHBP, the Western blot results showed an increase in antibody binding to the immobilized protein preparations that bound less heme (Fig 3B).

Although ECL is the most sensitive test available for detecting heme bound to protein and also facilitates re-probing of the Western blot, problems may arise when using 6X histidine tagged fusion proteins (Vargas et al., 1993; Letoffe et al., 2006). Histidine residues are often located within the heme-binding pockets of heme binding proteins (Arnoux et al., 1999). It is therefore possible that the histidine tag located on the N-terminal of the recombinant protein was responsible for mediating heme binding to rhHBP. To exclude this possibility, rLip 32, a *Leptospira* outer membrane protein containing a poly-histidine tag was pre-incubated with heme using the same experimental conditions as the hHBP-protein assays. Heme binding did not increase as the concentration of heme increased, indicating that the histidine tag was not responsible for the interaction of rhHBP with heme (Fig 4). This is further supported by previous competition binding assays where dose dependent binding of rLip 32 to heme was not observed (St. Denis, unpublished).

4.1.2 Functional Complementation

Investigating the function of hHBP in vivo is important to truly understand the role of hHBP in heme transport in *H. ducreyi*. Construction of an hHBP isogenic mutant by insertional inactivation would be a method by which one could elucidate the in vivo function of hHBP. However, this mutation could be lethal in view of the proposed central role of the transporter in *H. ducreyi* heme acquisition. Conversely, mutations in the periplasmic components of heme transport permeases may not always abrogate heme-binding, leading to difficulty in examining the role of the periplasmic protein encoded by the inactivated gene. Periplasmic protein redundancy exists in many systems. An example of this are the two periplasmic proteins MppA and DppA, both of which bind heme and interact with the same membrane components of the *E. coli* Dpp ABCDF permease (Letoffe et al., 2006). Letoffe et al. (2006) discovered that a mutation in *dppA* (the first gene of the *dppABCDF* operon encoding for the periplasmic component of the Dpp ABCDF transport system) was still able to bind heme because DppA was replaced by MppA (a periplasmic protein encoded by an isolated gene unlinked to the genes encoding the aforementioned ABC transporter) (Letoffe et al., 2006). Other examples in which two proteins can interact with the same membrane component of an ABC transporter would be the two peptide-binding proteins DppA and DppP of the Dpp ABCDF transporter in some strains of the Gram-positive bacterium *Lactococcus lactis* (Doeven et al., 2005). Both DppA and DppP participate in the acquisition of di/tripeptides, thus leading to degenerate substrate specificity within this ABC transporter. DppP also binds oligopeptides thus enabling the Dpp transporter to transport oligopeptides in addition to di/tripeptides, widening the specificity of the ABC

transporter (Doeven et al., 2005). This indicates that the periplasmic component and not the inner membrane permease component of ABC transporters within Gram-negative bacteria, determines their uptake selectivity. An example of this would be the two periplasmic binding proteins J and LAO (lysine-arginine-ornithine-binding protein) of the Gram-negative bacterium *Salmonella Typhimurium*. Both proteins interact with the same inner-membrane protein P of the same transporter. However, the two periplasmic proteins interact with different substrates (J binding histidine and LAO binding arginine), thus using the same transporter to transport two different substrates, depending on the periplasmic protein it comes into contact with (Higgins et al., 1981).

As *H. ducreyi* is a heme-obligate organism, and relies on large amounts of heme for survival, periplasmic protein redundancy within this bacterium would be expected. In addition, a second heme dedicated ABC transporter has been functionally assigned (<http://stdgen.northwestern.edu>). However, the function of this putative secondary heme ABC transporter has not yet been experimentally examined. Therefore, an hHBP mutant may not be practical, as another unrecognized periplasmic-binding protein and/or this putative secondary heme ABC transporter may be able to compensate for the loss of hHBP, thus making the functional investigation of hHBP difficult. Therefore, an alternate more effective method of investigating the role of a periplasmic-binding protein in vivo would be functional complementation. This technique is based upon the ability of bacterial genes to restore mutant strains to wild-type phenotypes. For example, in an experimental model of ascending urinary tract infection, Lloyd et al. (2009) suppressed the hyper inflammatory phenotype of an *E. coli* delta SisA delta SisB double deletion mutant (lacking the ShiA-like inflammation suppressor genes A and B) by

complementing the mutant with either the SisA or Sis B gene in trans, restoring the wild type immunosuppression phenotype (Lloyd et al., 2009). In another example, Zhang et al., complemented an *E. coli* Cys Q mutant (lacking the Cys Q protein responsible for the dephosphorylation of 3'-phosphoadenosine-5'-phosphosulfate to AMP) with SMMU.1297 (novel protein in *Streptococcus mutants*) restoring the phenotype of the mutant to that of the wild type (Zhang and Biswas, 2009). In addition to the aforementioned examples, Doeven et al. (2005) functionally complemented a *L. lactis* mutant lacking the OppA periplasmic component of the oligopeptide ABC transporter OppBCDF with another putative peptide binding protein OppA2, restoring the ability of the mutant to use oligopeptides. Furthermore, Letoffe et al. (1994) performed heterologous complementation of HasA (originally secreted by *S. marcescens*) secretion by the *E. chrysanthemi* ABC transporter in order to observe whether the foreign HasA protein could be secreted by the specific metalloprotease transporter (Letoffe et al., 1994). Problems may arise when complementing a mutant from one bacterial species with a foreign protein from another species. The foreign protein may not be as stable as the native proteins in the host bacteria. Additionally, structural differences between the foreign protein and the authentic proteins may prevent full restoration of the phenotype of the complemented mutant to that of the wild type. In any event, we adopted this method to investigate the in-vivo function of hHBP, and found that hHBP was able to functionally complement an *E. coli* K12 heme-deficient mutant lacking the periplasmic heme binding proteins MppA and DppA, thus restoring the heme uptake phenotype.

4.2 Model of heme uptake in *H. ducreyi*

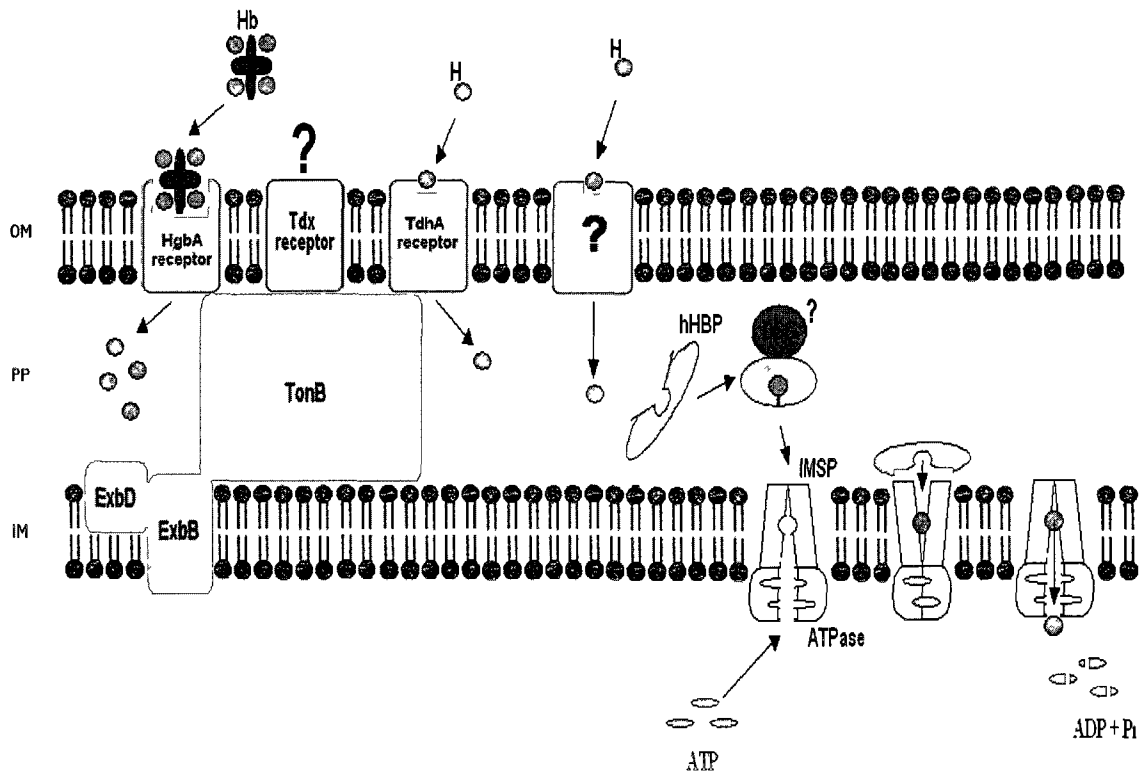
In Gram-negative bacteria, heme is usually transported into the cell via a functional ABC transporter that consists of a periplasmic binding protein, an inner transmembrane spanning permease, and an ATPase (Stojiljkovic et al., 2002). An example of this is the *Y. enterocolitica* Hem TUV system, where Hem T functions as the periplasmic component; Hem U, the transmembrane permease and Hem V, the ATPase (Letoffe et al., 2008). Heme specific outer membrane receptors are generally present on the cell surface for active transport and delivery of heme to the periplasm (Wandersman et al., 2000). For *Y. enterocolitica*, this would be Hem R (Stojiljkovic et al., 1999). In *H. ducreyi*, these receptors have so far been identified as the tdhA receptor, which binds heme, the HgbA receptor, which binds hemoglobin, and the uncharacterized Tdx receptor. All three receptors require the energy provided by TonB in association with ExbB and ExbD proteins for substrate transport (Thomas et al., 1998; Leduc et al., 2008). It is interesting to note that a TonB deficient *H. ducreyi* mutant does not use hemoglobin, but is still able to use hemin at the same levels as the wild type to fulfill its heme requirement (Elkins et al., 1998). This suggests that *H. ducreyi* possesses a separate Ton B independent heme uptake mechanism to deposit heme into the periplasmic space.

In *H. ducreyi*, a putative heme transport operon consisting of four genes has been identified from experiments performed in our laboratory. The four genes encode for an inner transmembrane spanning permease, a dissimilatory desulfovridin gamma subunit (DSG), a periplasmic heme-binding protein hHBP and an ATPase, respectively. The gene encoding for the DSG, *dsvC* seems to be unique to this operon, and is not a typical component of a heme dedicated ABC transporter. The role of this protein in non-sulfate

reducing bacteria like *H. ducreyi* remains elusive, but it has been proposed to be involved in the formation, maintenance or stabilization of the siroheme cofactors in bacteria lacking the sulfate reductase enzyme (Karkhoff-Schweizer et al., 1993; Molitor et al., 1998; Cort et al., 2001). It is therefore possible that DSG plays a role in the formation and stabilization of the hHBP-heme complex in *H. ducreyi*, as a highly efficient heme transport system may be necessary to fulfill the heme requirements for a bacteria with such high demands for heme.

Combining prior knowledge of heme transport ABC transporters with the knowledge obtained from this study and previous studies, we propose the following model for heme acquisition in *H. ducreyi*. Heme or hemoglobin binds the TdhA and HgbA receptors, respectively. Hemoglobin is dissociated into heme molecules at the cell surface at the site of the HgbA receptor and is transported into the periplasm using energy provided by the Ton system. Heme is also actively transported into the periplasm via TdhA using energy provided by the Ton system. Heme however, is also transported into the periplasm via an unknown receptor using a Ton B-independent mechanism. In the periplasm, heme is then bound by hHBP. This interaction is stabilized by the DSG protein for efficient transport through the periplasm. hHBP subsequently engages the inner membrane permease for transport of the heme molecule into the cytosol with the expenditure of ATP (see Fig 11). Another possible scenario for the fate of periplasmic deposited heme is the removal of iron from the porphyrin ring with the subsequent transport of iron into the cytoplasm, presumably via an iron specific ABC transporter.

Fig 11: Diagram illustrating the model of heme uptake in *Haemophilus ducreyi*. H represents heme; Hb represents hemoglobin; IMSP represents innermembrane spanning permease; IM represents the inner membrane; PP represents the periplasmic space; OM represents the outermembrane.



4.3 Importance of Study

Not only does *H. ducreyi* use heme as an iron source, but heme is necessary for the organisms survival (Trees et al., 1995; Lee, 1991). Therefore, *H. ducreyi* heme transport systems are considered important for virulence. In addition, Al-Tawfiq et al. (2000) showed that an isogenic *hgbA* mutant was completely attenuated in virulence in the human challenge model of infection, thus suggesting an importance in hemoglobin utilization for the pathogenesis of *H. ducreyi*. Consequently, the understanding of these heme acquisition systems could provide clues to the prevention and/or eradication of the bacteria and therefore the disease it causes. Antimicrobial strategies could include the exploitation of this heme transport system as portals of entry into the cell. An example of this would be the use of non-iron metalloporphyrins. Stojiljkovic et al. (1999) showed that non-iron metalloporphyrins possessed strong and broad antibacterial activity against Gram-negative bacteria. In addition to out competing heme for transport into the cell via the heme transport mechanism, these porphyrin compounds were shown to stimulate the bacterial production of reactive oxygen radicals in addition to targeting the bacterial respiratory pathway. Anaerobically grown bacteria and microbes that did not respire and/or express heme uptake systems were resistant to non-iron metalloporphyrins (non-iron MPs) (Stojiljkovic et al., 1999). Bozja et al. (2004) showed that porphyrin-based compounds, with or without an internal metal ion, had potent bactericidal action against *Neisseria gonorrhoeae* and *H. ducreyi* in-vitro. It is interesting to note that non-iron MPs and porphyrins were most potent when bacteria were grown in low-iron conditions (Stojiljkovic et al., 1999). This is because, during iron limiting conditions (such as that within the human host), bacterial heme uptake systems are induced to maximum

expression (Stojiljkovic et al., 1999). Stojiljkovic et al. (1999) found that the most potent of the non-iron MPs was gallium- protoporphyrin IX (Ga-PPIX). It inhibited the growth of the Gram-negative bacteria *Y. enterocolitica*, *S. aureus* and *M. smegmatis* in vitro, and had the capacity to block a gonococcal infection in a murine vaginal model (Stojiljkovic et al., 1999; Bozja et al., 2004). The high potency of this metalloporphyrin may be due to the similarities between the gallium ion and the iron ion, thus making Ga-PPIX indistinguishable from Fe-PPIX or heme. The gallium ion has an ionic radius (0.62Å) that is almost identical to Fe (0.64Å) (Stojiljkovic et al., 1999; Kaneko et al., 2007). In addition, some gallium and iron porphyrins are isomorphic (Stojiljkovic et al., 1999). It is possible that upon uptake into the cell, enzymes or proteins that use heme as a co-factor may insert Ga-PPIX into their catalytic centers instead of heme thus rendering enzymes inactive, resulting in the disruption of major biological processes, as gallium is not suitable for biological reactions (Stojiljkovic et al., 1999). Gallium has only one valence state and therefore cannot be oxidized or reduced, and sequential oxidation and reduction are critical for many of iron's biological functions (Kaneko et al., 2007). Previous studies in our laboratory have shown that hHBP only recognizes the tetrapyrrole ring of Fe-PPIX, as PPIX competitively inhibited the binding of rhHBP to heme in-vitro (St. Denis, unpublished). In addition, Zn-PPIX eliminated the interaction between rhHBP and heme in vitro (St. Denis, unpublished). This suggests that non-iron metalloporphyrins and porphyrins may enter the bacterium via the hHBP ABC transporter and this could explain the in vivo findings of Bozja et al. (2004) where non-metal PPIX and Ga-PPIX had potent bactericidal action against *H. ducreyi*.

One may assume that hHBP as a vaccine candidate may not be likely because it is not a surface protein and thus not easily assessable to immune factors. However, Raulston et al. (2007) showed that patients infected with *Chlamydia trachomatis* generated antibodies against a *C. trachomatis* iron-binding periplasmic protein, YtgA. Therefore, the possibility that hHBP could be immunogenic cannot be ruled out based on location within the cell alone.

An important advantage of MPs and non-metal protoporphyrins is that they enter the bacteria using high affinity heme-transport systems, thus ensuring that their journey into the cells is not affected by the barrier functions of bacterial membranes (Stojiljkovic et al., 1999). In conclusion, understanding the heme transport mechanisms of *H. ducreyi* is pivotal in identifying ideal pathways for novel targeted drug delivery.

4.4 FUTURE WORK

In the future, it would be interesting to confirm via RT-PCR, that the four genes of the proposed operon are transcribed as a single transcriptional unit. Additionally, in-vivo experimental investigation of the functions of the proteins encoded by ORFs 1,2 and 4 of the operon can be achieved by insertional inactivation in each gene using a *cat* cassette (because of the stability of this cassette enabling prolonged laboratory passage of the strains in the absence of antibiotic selection) derived from a mini-Tn3 and observing the phenotype of the mutant on media supplemented with hemoglobin. In light of the functional redundancy of a putative secondary heme dedicated ABC transporter, functional complementation of each gene in a mutant lacking the proposed functions of each gene may also be performed to elucidate the in-vivo role of each protein in the

putative heme transport system. Furthermore, in light of the similarities between non iron-metalloporphyrins (non-Fe MPs) and heme and the fact that hHBP binds non-Fe MPs and porphyrins (PPs) in vitro, it would be interesting to investigate the mechanism of entry of these compounds in vivo. One could compare the sensitivity of *H. ducreyi* mutants lacking key heme/ hemoglobin receptors and key components of the proposed heme transporter to non-Fe MPs and PPs, with that of the wild type. Based on the hypothesis that porphyrins enter the cell through the heme uptake pathway, a decrease in sensitivity of the knock out mutants would suggest that non-Fe MPs and PPs exploit this heme uptake pathway within the bacterium. Conversely, one could over express key components of the heme uptake system in *H. ducreyi* and compare the sensitivity of the over expression mutants to non-Fe MPs and PPs to that of the wild type. An increase in sensitivity of the mutants would suggest that the non-Fe MPs and PPs exploit the heme uptake pathway of the bacterium. However, because *H. ducreyi* is a heme obligate organism, and due to the fact that non-Fe MPs and PPs may outcompete heme for access to the heme uptake pathway, these methods may result in lethality of the cells. An alternative method would be to perform in vitro binding assays of the various heme/ hemoglobin receptors and the proposed permease with non-Fe MPs and PPs.

Additionally, it would be of interest to investigate the possible existence of a second heme periplasmic binding protein that may interact with the hHBP heme permease or a parallel ABC transporter. One could do so by first performing insertional inactivation in *hhbp*. If the mutant is still able to use heme as an iron source, one would then search the genome for genes encoding periplasmic proteins similar to hHBP, after which one could test whether any combination of the *hhbp* mutant and mutations in the

genes encoding for the newly discovered periplasmic binding proteins leads to loss of heme utilization. Another experimental approach would be to construct a plasmid chromosomal *H. ducreyi* gene bank and screen the ability of this plasmid library to complement the *E. coli mppa dppa double* mutant. This may also shed light on the function of the components of the uncharacterized secondary putative heme dedicated ABC transporter.

Finally, it would be interesting to investigate the antigenicity of hHBP in order to determine if it would be a possible vaccine candidate. This could be done by immobilizing hHBP on nitrocellulose membranes and performing immunoblots with sera taken from patients infected with *H. ducreyi*.

4.5 CONCLUSIONS

In our study, we have characterized the function of hHBP in vivo and further characterized its function in vitro. hHBP had a high affinity for heme and the binding of hHBP to heme was directly proportional to the increase in concentration of heme. In addition, hHBP was able to restore the heme transport mechanism of an *E. coli* K12 heme-deficient mutant. Therefore, these results support the hypothesis that hHBP participates in the heme acquisition pathway in *H. ducreyi*.

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