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GRADE / DEGREE

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Identification and Molecular Characterization of a Putative Heme ABC Transporter in *Neisseria Meningitidis*

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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF A
PUTATIVE HEME ABC TRANSPORTER IN NEISSERIA MENINGITIDIS

A Thesis Submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
Department of Microbiology and Immunology
School of Medicine

By
Keely Ingrey



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Your file *Votre référence*
ISBN: 978-0-494-66259-5
Our file *Notre référence*
ISBN: 978-0-494-66259-5

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ABSTRACT

A detailed mechanism for heme uptake in pathogenic *Neisseriae* has not yet been elucidated. Once heme is deposited in the periplasmic space, a heme dedicated ABC transporter has been hypothesized to convey heme into the cytoplasm. To address this hypothesis, we used several molecular techniques to identify and characterize a putative heme ABC transporter in *Neisseria meningitidis*. Using 2-D gel electrophoresis, we identified six proteins with increasing expression under heme-limiting conditions. Although these proteins appear to be iron-regulated, the genes encoding these proteins are not associated with periplasmic components of ABC transporters. Using DNA microarray analysis, we identified eleven ABC transporter components. A mutation in one of these genes, the ATPase *NMB1993*, was constructed. No difference in the ability of various iron sources to support the growth of the mutant and wild-type strains was observed under iron-restrictive conditions. No difference in growth rates between the two strains was discerned, suggesting no role in heme metabolism. Another uncharacterized putative periplasmic component, *NMB0586*, exhibited 27% amino acid identity to a recently characterized periplasmic heme-binding protein in the *Haemophilus ducreyi* transporter. To address its role in heme metabolism, *NMB0586* was disrupted by insertional inactivation. The ability of various iron sources to support the growth of the mutant and wild-type strains was compared under iron-restrictive conditions. The mutant failed to display a heme-deficient phenotype. However, in view of the recognized degenerate binding specificity of the permease component of some ABC

transporters, the heme-binding property of NMB0586 was studied. Purified NMB0586 bound to heme at a concentration of 10^{-4} M, using enhanced chemiluminescence. Furthermore, the binding of purified NMB0586 protein to hemin-agarose demonstrated concentration dependent binding. However, increasing concentrations of competing heme ligand did not result in a decrease in NMB0586 protein binding to hemin-agarose. NMB0586 was also unable to functionally complement a heme-deficient mutant of *Escherichia coli*. These findings suggest that NMB0586 is unlikely involved in heme acquisition in meningococci. In conclusion, this study describes the difficulties in identifying a meningococcal heme ABC transporter, but highlights several techniques for the future identification and characterization of a heme ABC transporter in *Neisseriae*.

ACKNOWLEDGEMENTS

I would like to thank Dr. Craig Lee for his unwavering support, encouragement, and patience during my time in his laboratory. He has given me every opportunity to grow and develop in my academic endeavors, he has pushed me to better myself, and his approach to supervision has allowed me to become a confident and independent scientist. These are attributes that I am truly grateful to have gained during my time in his lab, and which I know will serve me well throughout my career.

I would like to acknowledge my thesis advisory committee members, Dr. Ken Dimock, Dr. Sandra Ramirez, and Dr. Alain Stintzi for a great deal of assistance and insight into this project. I would like to thank Dr. Marc Desjardins for reviewing my thesis. Furthermore, I would like to thank Dr. William Cameron for being a member of my thesis advisory committee, as well as providing the funding for the final years of this research project.

I am grateful to all the members of Dr. Lee's laboratory, past and present, for sharing ideas and making coming to work every day enjoyable. I would especially like to thank Cathy Breau, Shahed Ardalan and Jeff Sulpher, who made me laugh when times got challenging.

I would like to thank Dr. Fraser Scott for use of his laboratory equipment, as well as the members of his lab who provided guidance in the technique of 2-D gel electrophoresis. I would like to especially thank Dr. Alexander Strom who pushed me through the final year of research and stood by me through the difficult times.

A very special thank you is owed to the ``PhD girls``. I cannot imagine the long days in the lab without you all by my side throughout this graduate student experience. To Fiona Frappier, and Nicole Arbour, there will come a day when we will play euchre without talking about university life. To Christopher McLaughlin, who joined the ride along the way and always made me smile. To Angela Yeung, the most understanding lab mate I could ever ask for, I am proud to have spent the past seven years with you by my side. And to Charlene Young, who was there at the beginning of our scientific journey at Heritage College in 1996 and still stands by me today, we persevered through it all.

Finally, I would like to thank my family for their confidence and enthusiasm, without which I would not have accomplished what I have. I would especially like to thank my mother, Faye Ingrey, for always believing I could do anything, and for not letting me give up in the face of what seemed like insurmountable challenges. To John Lunde, who gave me the flexibility of time to be both a PhD student and a mother. And to Sydney, who showed me unconditional love and made it all worthwhile.

DEDICATION

This thesis is dedicated to the memory of Sidney Ingrey, who encouraged me to pursue a career in science and inspired me to be the best that I can be.

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LIST OF ABBREVIATIONS

1D	1-dimensional
2,2-Dip	2,2'dipyridyl
2DGE	2-dimensional gel electrophoresis
AA	alanine-alanine dipeptide
ABC	ATPase binding cassette
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CFU	colony forming units
CHAPS	cholamidopropyltrimethylammoniumpropanesulfonate
dCTP	deoxycytosine
ddH₂O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FA	formaldehyde agarose
FeII	ferrous iron
Fur	ferric uptake regulator
g	gravitational force

Ga-PPIX	gallium protoporphyrin
h	hour(s)
Hb	hemoglobin
His	histidine
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IEF	isoelectric focusing
IPTG	Isopropylthio-B-galactosidase
Kbp	kilobase pair
LB	Luria Bertani
LOS	lipooligosaccharide
M	molarity (moles per liter)
min	minute(s)
ml	milliliter(s)
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
MP	metalloporphyrin
mV	millivolts
ng	nanogram(s)
Ni-NTA	nickel-nitrilotriacetic acid
OD₆₀₀	optical density at wavelength 600 nm

ORF	open reading frame
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
PMSF	phenylmethanesulfonyl fluoride
PPIX	protoporphyrin
RNA	ribonucleic acid
rpm	rotations per minute
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
sec	second(s)
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TES	Tris-EDTA-sodium chloride
μg	microgram(s)
μl	microliter(s)
μM	micromolar
μV	ultraviolet
V	amperage in volts
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1: LITERATURE REVIEW

1.1. Pathogenic *Neisseriae*

Neisseria gonorrhoeae and *N. meningitidis* are the two pathogenic members of the *Neisseriae* genus of gram-negative bacteria. *Neisseria* spp. cells assume a coccoid shape and usually occur as diploids. *N. gonorrhoeae* and *N. meningitidis* only infect humans, initiating infection at the human mucosal epithelia. *N. meningitidis* is a facultative commensal bacterium, colonizing the nasopharynx of 3-30% of healthy individuals (Merz and So, 2000). *N. meningitidis* can cause disease when it crosses the mucosal barrier and enters the bloodstream causing septicemia. It can also cause meningitis when it crosses the blood brain barrier to enter the cerebrospinal fluid. The annual incidence of invasive meningococcal disease in Canada has averaged 1 per 100 000 population, however this number fails to convey the full impact of the disease. Most cases involve people less than 19 years old, with the highest incidence found among infants less than one year old (12.9 and 6.5 cases per 100 000 population in 1997 and 1998, respectively) (reviewed by Weir, 2002). The highest burden of meningococcal disease occurs in sub-Saharan Africa. In major African epidemics, attack rates range from 100 to 800 per 100 000 population, but individual communities have reported rates as high as 1000 per 100 000 population (WHO, 2001). Unlike *N. meningitidis*, *N. gonorrhoeae* is not a human commensal organism but instead a parasite. *N. gonorrhoeae* is one of the most frequently reported causes of sexually transmitted disease in the USA; however the incidence of gonococcal infections from 1986 to 2006 has been steadily declining to a target rate predicted by the CDC of 19 cases per 100 000 by 2010. *N. gonorrhoeae* has been

implicated in the increased spread of human immunodeficiency virus (HIV) infections, especially in third world countries (Cohen, 1998; Fleming and Wasserheit, 1999; Grosskurth et al., 1995). This arises from the recruitment of HIV-infected inflammatory cells to the mucosal surfaces infected with the gonococcus, thereby increasing the infective inoculum of HIV transmitted by up to 8 times. *N. gonorrhoeae* can cause infection of the cervix or urethra, and can develop into pelvic inflammatory disease (PID) which, if left untreated, can cause infertility and sterility (Haggerty and Ness, 2006). Therefore *Neisseria* spp. infections are a significant expense on health care costs for a government, and pathogenic *Neisseriae* are microorganisms that are of particular interest to study in order to find new treatments or vaccines.

The primary treatment of pathogenic *Neisseriae* infections is antibiotics, including penicillin and ampicillin, as well as fluoroquinolones, both interrupting transmission chains and greatly reducing long-term infections and mortality (CDC, 1993). However, there has been a reported increase in non- β -lactamase-producing penicillin-resistant meningococcal strains worldwide (Antignac et al., 2003; Canica et al., 2004; Jackson et al., 1994; Thulin et al., 2006). Resistance of *N. gonorrhoeae* to antibiotics, including quinolones, has also increased in recent years and has reduced the options for treatment (Tapsall, 2009). Many developed countries, including Canada, now recommend the use of third-generation cephalosporins instead of quinolones for the treatment of gonorrhea. But gonococcal resistance to third-generation cephalosporins, including cefixime, given orally has emerged in Japan (Akasaka et al., 2001; Ito et al., 2004; Muratani et al., 2001). As a result,

patients infected with antibiotic resistant gonococcal strains have more severe clinical courses and require prolonged hospital treatments. Since there has been little development of new antibiotics, with no new class of antibiotics discovered in the past four decades (Finlay and Cossart, 1997), discovery of an effective vaccine would be an important part of eradicating neisserial infections.

There is currently no vaccine for *N. gonorrhoeae*, but there are capsular polysaccharide vaccines available for *N. meningitidis* serogroups A, C, Y and W-135. However, the polysaccharide vaccines are ineffective in young children (<1 year) and the duration of the protection is limited in children between ages 1 to 4 years of age. A monovalent conjugate vaccine against serogroup C has recently been licensed in developed countries for use in children and adolescents, and has the benefit of being immunogenic, particularly for children under 2 years of age. Serogroup B polysaccharide, a homopolymer of sialic acid, has poor immunogenicity and a structural similarity to polysialic acid on neuronal tissue that may elicit autoantibodies, so there is currently no vaccine for this serogroup (Finne et al., 1983; Finne et al., 1987; Nedelec et al., 1990).

The pattern for meningococcal serogroup B disease is sporadic but a major cause of invasive disease in Europe and the United States (CDC, 2001). Vaccine candidates are difficult to identify because of the antigenic variation of surface proteins, with more than 1 million possible pilus antigens. A group B meningococcal vaccine consisting of outer membrane protein antigens has recently been developed, but it is not licensed in the United States. It is also hard to find a vaccine for *N. gonorrhoeae* because there is not an effective animal model to complete the study.

Therefore it is important to study the bacterial genetics of this organism and better understand its virulence so that new vaccine candidates can be developed.

Due to the high prevalence of disease caused by pathogenic *Neisseriae* species, these bacteria have been relatively well characterized. Several virulence genes have been identified in *N. meningitidis* that have a counterpart in *N. gonorrhoeae* (reviewed in Merz and So, 2000), encoding: proteases, lipooligosaccharide (LOS) (several antigenic types), RMP (protein III) (forms a complex with Por and LOS), Type IV pili (promote adhesion to host cells), and several proteins involved in iron and iron-containing compounds uptake, as discussed in detail below. The complete genomes of *N. meningitidis* serogroup B strain MC58 (Tettelin et al., 2000), serogroup A strain Z2491 (Parkhill et al., 2000) and serogroup C strain FAM18, were sequenced in 2000, as well as serogroup C strain 053442 (sequenced in 2007), which aided in identifying other putative virulence genes, as well as showing the similarities and differences between the serogroups to study disease patterns. The complete genome of two gonococcal strains, *N. gonorrhoeae* FA 1090 and *N. gonorrhoeae* NCCP11945, were completed in 2000 and 2008, respectively.

1.2. Bacteria and Iron

An essential component of bacterial growth is the requirement for iron, or compounds that contain iron (reviewed in Finkelstein et al., 1983; and Ratledge and Dover, 2000). Iron has an oxidation-reduction potential that ranges anywhere from +300 mV to -490 mV, and is thus an extremely useful atom in the reactive center of a variety of enzymes, including enzymes essential for the synthesis of DNA

(ribonucleotide reductase), electron transport and oxygen/energy metabolism. Comparison of published transcriptome data for *N. meningitidis* serogroup B indicated that expression of up to 20% of all meningococcal genes can be subject to regulation in function by iron availability (Basler et al., 2006). These include genes that encode outer membrane receptors, inner membrane components involved in uptake of iron-containing compounds, alternative sigma factor, and cell division regulators. The alternative sigma factor is involved in regulating expression of other genes, such as genes that encode proteins secreted from the bacteria to scavenge iron, or exotoxins. Iron is involved in regulation of these genes because the iron (FeII) forms a complex with ferric uptake regulator (Fur) protein, and the complex then binds to a 19 bp DNA sequence (Fur box) which overlaps promoters of iron regulated genes. Bacteria can also depend on iron for virulence since some virulence genes are regulated directly (ie. by Fur) or indirectly (ie. by Fur regulated proteins), such as diphtheria toxin being expressed in iron deficient conditions.

The shift from high to low iron environment is also an important signal that the bacteria has entered the host, since humans have a low concentration of available free iron. Humans sequester iron in many different compounds, including transferrin, lactoferrin, ferritin, and heme and heme-containing compounds such as hemoglobin, haemopexin, albumin and lipoproteins (reviewed by Weinberg, 1993). Bacteria have thus adapted two different mechanisms to acquire this iron: by producing siderophores that scavenge iron and having surface-expressed receptors specifically for iron containing compounds that bind these compounds with high affinity (Braun and Killmann, 1999).

1.3. Iron and pathogenic *Neisseriae*

Iron is a determinant of virulence in *N. meningitidis*, since the addition of iron-containing compounds such as iron dextran (Holbein, 1980), transferrin iron (Holbein, 1981) or haemoglobin (Brodeur et al., 1985) enhances the lethal effect of meningococci in a murine model of infection. *Neisseria* spp. do not produce siderophores, and thus rely on specific receptor-mediated iron uptake systems to survive in the human host, reviewed by Perkins-Balding and colleagues (2004). It should be noted that some studies have shown that exogenous siderophores can support the growth of pathogenic *Neisseriae* (West and Sparling, 1985) and ORFs that share homology with siderophore receptors have been identified in this microorganism (Turner et al., 1998), suggesting that they possess pathways for the uptake of iron-siderophore complexes. Pathogenic *Neisseriae* produce surface receptors for transferrin (TbpA and TbpB) and for lactoferrin (LbpA and LbpB) (Cornelissen, 2003; Schryvers et al., 1998). They are also capable of using hemoglobin, haptoglobin-haemoglobin and heme but not heme-haemopexin or haem-albumin as a source of iron for growth (Dyer et al., 1987). Two outer membrane receptors for heme (and heme-containing compounds) have been identified in *Neisseria* spp., HmbR (Stojiljkovic et al., 1995) and HpuAB (Lewis and Dyer, 1995; Lewis et al., 1997). HmbR is a hemoglobin receptor only, but HpuAB is also capable of binding hemoglobin-haptoglobin and apo-haptoglobin as well as hemoglobin. It has been proposed that HmbR and HpuAB receptors may remove heme from hemoglobin. However, *Neisseriae* mutants *hmbR*⁻ and *hpuAB*⁻, which are unable to use Hb and Hb-Hp as an iron source, are capable of growing on heme as an

iron source. Thus heme may diffuse passively across the outer membrane, as a result of its hydrophobic nature, or possibly through one or more porins, although the specific mechanism remains to be elucidated.

Analogous to the TonB-dependent siderophore acquisition systems found in enteric bacteria, *N. meningitidis* requires a *ton* system for the utilization of transferrin, lactoferrin, hemoglobin, haptoglobin-hemoglobin (Stojiljkovic and Srinivasan, 1997). The TonB complex, which consists of TonB, ExbB, and ExbD, is located in the inner membrane and functions to transduce the energy of the proton motive force into conformational changes in TonB-dependent outer membrane transporters (Postle and Kadner, 2003). The intracellular replication of *N. meningitidis* within epithelial cells has been shown to be a TonB dependent process, implicating TonB in acquisition of intracellular host iron (Larson et al., 2002). In the infant rat model of *N. meningitidis* septicemic infection, TonB, ExbB and ExbD were found to be essential (Sun et al., 2000).

1.4. ABC transporters

ABC transporters are usually involved in the transport of iron or iron-containing compounds across the inner membrane of gram-negative bacteria or cytoplasmic membrane of gram-positive bacteria (reviewed in Nikaido and Hall, 1998). ABC transporters are so named because they are adenosine 5'-triphosphate (ATP)-binding cassettes that either export or import solutes across the membranes of both prokaryotic and eukaryotic cells, a process driven by the hydrolysis of ATP. Their importance is demonstrated by a number of inherited disorders in humans related to mutations in specific ABC transporters, such as cystic fibrosis (Zielenski

and Tsui, 1995) and Stargardt macular dystrophy (Allikmets et al., 1997). ABC transporters possess a common minimum structure consisting of four domains: two hydrophobic intergral membrane domains and two ATP-binding domains (Higgins, 1992; Holland and Blight, 1999). The genes are usually organized in an operon and transcribed as polycistronic mRNA. A subfamily of ABC transporters that is responsible for the uptake of solutes is found exclusively in prokaryotes (Saurin et al., 1999). The members of this family can be distinguished from other ABC transporters by the presence of a solute binding protein, in addition to the other binding components. The solute binding protein is located in the periplasm of gram-negative bacteria and attached to the cell membrane of gram-positive bacteria. Members of this subfamily of transporters are required for the uptake of a variety of small molecules (including amino acids, metal ions and sugars), with each transporter having high affinity and specificity for an individual solute. It should be noted that the solute binding protein is not necessarily located in the same operon as the rest of the transporter genes, but could be located elsewhere in the bacterial genome. The ATP-binding proteins (ATPases) share extensive homology in a stretch of amino acids of 200 or more that spans the region between, and including, two short ATP-binding motifs (Walker motifs), the “signature sequence” (LSGGQQ/R/KQR) that is unique to the ABC transporter family, a helical domain and a linker domain, and forms the basis for the grouping of ABC transporters (Schneider and Hunke, 1998; Walker et al., 1982). The Walker motifs A (amino acid sequence GxxGxGKS/T where x can be any amino acid) and B (amino acid sequence LLLDEPXXXLD, or more generally hhhD where h is a hydrophobic

amino acid) also occur on transporters other than the ABC transporters, such as F-type and P-type ATPases, and therefore the motifs do not definitely identify an ABC transporter.

ABC transporters are of particular interest because they are important virulence factors in bacterial pathogens, comprising 5% of the *E. coli* and *B. subtilis* genomes (Linton and Higgins, 1998; Young and Holland, 1999), and can be used for the development of antibacterial vaccines and therapies. Since ABC transporters can be immunogenic, they can be exploited as candidate subunits for vaccination against pathogenic bacteria. Although no studies have been done to show the protective efficacy of ABC transporter components as vaccine candidates for gram-negative bacteria, studies have been done to show the efficacy of using components of ABC transporters in gram-positive bacteria. Tanghe and colleagues (1999) used plasmids containing *Mycobacterium tuberculosis* DNA of putative phosphate transport receptors and found that mice demonstrated a significant and sustained (3 months) reduction in bacterial CFU numbers in spleen and lungs compared to CFU counts in mice vaccinated with control DNA. Brown and colleagues (2001) successfully demonstrated protection against systemic challenge of *Streptococcus pneumoniae* infection in mice using the lipoprotein components PiuA and PiaA of the iron uptake ABC transporter, to a degree similar to those immunized with another existing candidate. These results are reasonable given the fact that in gram-positive bacteria, the ABC transporter is expressed on the cell surface and thus exposed to the host's immune system. It would be interesting to see if gram-negative bacteria ABC transporters can have the same immune response in the host. An alternative approach

would be to exploit ABC transporters as a system for the delivery of antimicrobials into the bacterial cell. Since mammalian host cells lack ABC import systems, an additional level of specificity would be introduced by exploiting these bacterial import systems. Artificially created drugs, in which the antibacterial compound is linked to the natural substrate for the transporter, have been studied on peptide ABC transporters, such as the OppA or DppA systems (Claesson et al., 1987; Fickel and Gilvarg, 1973; Higgins, 1987; Smith and Payne, 1990; Tame et al., 1994). For example, Smith and Payne (1990) demonstrated that synthetic peptide prodrugs could be transported into *E. coli* through peptide permeases. Once inside the bacteria, these peptide prodrugs were activated by intracellular peptidase action to release their intrinsically impermeant, antibacterial moieties.

1.5. Neisserial ABC transporters

The sequencing of the complete genome of three different *N. meningitidis* serogroups identified novel putative ABC transporters, as well as confirmed the presence of ones that had already been characterized. Based on DNA sequence homology to motifs in the ATPase component of the transporter, our collaborators, Tettelin and colleagues, (2000) identified 24 ATP-binding subunits of ABC transporters. Four of these transporters have been partially characterized and their substrates identified (such as spermidine/putrescine; *potA-potD*), and 14 are not located within a potential operon, therefore there are five ABC transporters that are possibly located in an operon and have no known function. These five transporters are of particular interest in this study since they can be potential ABC transporters

for heme, and include ORF01663, ORF01770, ORF03052, ORF03178 and ORF01072.

1.6. Heme

Heme (ferroprotoporphyrin IX) plays an important role as a prosthetic group of many proteins such as oxygen-carrier proteins, proteins of the electron transport system, mixed function oxidases, and peroxidases. In addition to being directly involved in biological reactions as a cofactor, heme also appears to serve as a regulatory molecule in such processes as the initiation of protein synthesis, ATP/ubiquitin-dependent protein degradation, and inhibition of DNA polymerase. Several pathogenic bacteria have been shown to be able to use heme as a sole iron source, and have adapted specific mechanisms for heme uptake and degradation, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Shigella* sp., and *Yersinia* sp., as described below.

1.7. Bacteria and heme

Bacteria have been shown to devote up to 10% of the total cell iron content and a significant amount of cell energy to the biosynthesis of heme (Beale and Yeh, 1996; Matzanke et al., 1991). Due to the high energy costs of biosynthesis of heme and the availability of heme and hemoproteins in the host environment, bacteria have evolved and developed heme-scavenging systems. A large number of bacteria use heme compounds as a source of iron including *Haemophilus ducreyi* (Lee, 1991), *Vibrio cholera* (Stoebner and Payne, 1988), *N. gonorrhoeae* (Dyer et al., 1987), *Yersinia pestis* and *Y. enterocolitica* (Perry and Brubaker, 1979). The key steps involved in the acquisition of external heme by bacteria are the following: binding a

heme-containing compound, releasing heme from the compound, and transporting heme into the cell.

Although little is known about the fate of the heme after it enters the cytoplasm of the cell, transport studies with *Y. enterocolitica* and ¹⁴C-labeled hemin have shown that the entire heme molecule is transported into the cell (Stojiljkovic and Hantke, 1994). There is strong experimental evidence to suggest that the iron is removed from the heme molecule with a heme oxygenase. Heme oxygenase mutants of both *Corynebacterium diphtheriae* and *C. ulcerans* fail to use heme as an iron source (Schmitt 1997; Wilks and Schmitt, 1998; Wyckoff et al., 2004). *N. meningitidis*, *P. aeruginosa*, and *S. aureus* all contain heme oxygenases, and the enzyme is required for the efficient use of heme as a source of iron (Ratliff et al., 2001; Schuller et al., 2001; Skaar et al., 2004; Zhu et al., 2000).

Although heme and hemoglobin are preferred substrates for the majority of bacterial heme acquisition systems (Lee, 1995), bacterial pathogens have also adapted mechanisms to acquire heme from heme-protein complexes like hemopexin-heme, haptoglobin-Hb, heme-albumin, and myoglobin. *Serratia marcescens* produces a small protein (hemophore) that extracts heme from heme-protein complexes and delivers it to a heme-specific outer membrane receptor (Ghigo et al., 1997; Letoffe et al., 1994). *Porphyromonas gingivalis* and *Vibrio vulnificus* secrete proteases to degrade hemoglobin and other heme-protein complexes (Fujimara et al., 1998; Nishina et al., 1992). There are also heme-utilizing bacterial pathogens that have multiple outer membrane receptors specific for heme-protein complexes. For example, *Haemophilus influenzae* expresses several distinct hemopexin and

haptoglobin-hemoglobin receptors (Cope et al., 1995; Jin et al., 1996; Maciver et al., 1996; Morton et al., 1998). More than twenty two different gram-negative outer membrane proteins involved in the utilization of heme compounds are listed in the GeneBank data base. All share a limited sequence identity (17% to 23%) with the outer membrane receptors for transferrin and lactoferrin (Wandersman and Stojiljkovic, 2000). Transport of heme across the outer membrane of gram-positive bacteria and the inner membrane of gram-negative bacteria occurs via a heme ABC transporter. Several heme ABC transporters have been identified in bacteria, including *Y. pestis* (Rossi et al., 2001), *Bradyrhizobium japonicum* (Neinaber et al., 2001), *P. aeruginosa* (Ochsner et al., 2000) and *C. diphtheriae* (Drazek et al., 2000)

1.8. Properties of bacterial heme ABC transporters

The protein domains by which heme receptors bind heme compounds are not fully elucidated, although key histidiny residues are required for the assembly of c-type cytochromes binding to heme (Goldman et al., 1998). In 1999, Bracken and colleagues identified key receptor residues in the heme/hemoglobin outer membrane receptor (HemR) in *Y. enterocolitica* that specifically recognize the heme molecule. Histidines are common axial ligands of heme-iron in proteins, and it was assumed that histidine residues in HemR play an important role in heme binding (Dickerson and Geis, 1983; O'Halloran, 1993). Using site-directed mutagenesis and phenotypic characterization, Bracken and colleagues (1999) were able to identify two histidine residues of HemR that confirmed the involvement of His128 and His461 in heme utilization. These residues were found to be conserved in heme/hemoglobin receptors of other pathogenic bacteria, such as HmuR (*Y. pestis*), ChuA (*E. coli*), ShuA (*S.*

dysenteriae), and HxuC (*H. influenzae*), and are located in the substrate translocation channel. However, binding assays of the His-mutants to hemin- or hemoglobin-agarose demonstrated that the HemR single mutants were still able to bind the hemin- or hemoglobin-agarose, suggesting that several histidine sites as well as other amino acid motifs may function together in heme/hemoglobin binding at the cell surface. Heme-binding periplasmic components of ABC transporters have also been shown to contain histidine residues involved in heme binding, such as SiaA of *Shigella dysenteriae* and IsdE of *S. aureus* (Grigg et al., 2007; Sook et al., 2008). In contrast, ShuT, the heme-periplasmic binding protein from *S. dysenteriae*, has a single tyrosine as an axial ligand (Eakanunkul et al., 2005).

Burkhard and Wilks (2008) incorporated the heme ABC transporter, ShuUV, of *S. dysenteriae* into proteoliposomes to study the in vitro translocation of heme from the periplasmic heme-binding protein, ShuT, through the transporter, ShuUV, to the cytoplasmic heme-binding protein ShuS. This work provides the first look into the mechanism of heme transport in bacterial pathogens.

1.9. Methods of identification of heme ABC transporters

One of the difficulties with identifying heme ABC transporters is that there is little DNA sequence homology between ABC transporter genes in different bacteria. However, heme ABC transporter genes have been shown to be located in close proximity to *tonB*, *exbB* and *exbD* genes, as is the case in *V. cholerae* in which the genes involved in heme periplasmic transport (*hutBCD*) are located as part of an operon including *tonB1*, *exbB1*, and *exbD1* genes (Occhino et al., 1998). Heme ABC transporter genes have also been shown to be located in close proximity to outer

membrane heme receptor genes in the bacterial genome, as in the case of *S. dysenteriae* (Wyckoff et al., 1998). Therefore, genome sequence analysis can sometimes be a useful method to identify heme ABC transporters.

Researchers have adapted different molecular and biochemical techniques to identify heme ABC transporters in bacteria. Drazek and colleagues (2000) complemented a mutant strain of *C. ulcerans*, that was unable to utilize hemin and hemoglobin as iron sources, with a plasmid library of *C. diphtheriae* chromosomal DNA. By screening the *C. diphtheriae* library for clones capable of restoring the heme-deficient phenotype, they identified a ABC transporter composed of three genes (*hmuTUV*) essential for heme utilization. A similar approach was used by Slakeski and colleagues (2000), by screening a genomic library of *P. gingivalis* with an oligonucleotide probe directed to a TonB box motif to identify a TonB-linked adhesin involved in hemin uptake. Due to the location of this adhesin immediately downstream of a previously uncharacterized four gene ABC transporter, they identified the heme ABC transporter (*htrABCD*) for heme.

Another difficulty in identifying bacterial heme ABC transporters is the degenerate binding specificity exhibited by the periplasmic component. Letoffe and colleagues (2006) studied a recombinant *E. coli* K12 strain expressing a foreign outer membrane heme receptor (HasR), allowing use of exogenous heme as an iron source. Using transposon mutagenesis and isolating mutants that were unable to use heme as an iron source, they identified a heme ABC transporter DppABCDF. This transporter had been previously characterized as a dipeptide inner membrane transporter for Ala-Ala (AA). Using in vivo competition experiments, the presence of AA in the media

blocked the ability of the periplasmic component to utilize heme as an iron source. This finding suggests that there may be more than one substrate for periplasmic components of heme ABC transporters. Letoffe and colleagues also identified a second periplasmic component, MppA, that interacts with the DppBCDF transporter and is involved in heme uptake. A mutation in either *dppA* or *mppA* does not abolish the heme phenotype, but a double *mppA dppA* mutant is unable to use heme as an iron source (Letoffe et al., 2008). This finding suggests that the periplasmic component of the heme ABC transporter need not be in the same operon as the rest of the heme ABC transporter components. However, the heme ABC transporter of *S. marcescens* (HemTUV) exhibits a narrower substrate specificity, restricted to heme only (Eakanunkul et al., 2005; Letoffe et al., 2008).

Thompson and colleagues (1999) constructed a *hmuP'RSTUV* mutant, the hemin uptake locus of *Y. pestis*, and demonstrated in *in vitro* studies that the mutant was still able to grow in iron-depleted medium containing hemoglobin, suggesting two separate mechanisms for heme and hemoglobin uptake. They also demonstrated that infection of the *hmuP'RSTUV Y. pestis* mutant strain in mice had no effect on the ability of the bacteria to grow *in vivo* compared to wildtype. The redundancy in heme, and more specifically iron, transport systems has made it more difficult to determine the role of individual systems *in vivo* and *in vitro*, but it may reflect the overall importance of iron in growth and survival of *N. meningitidis*.

1.10. *Neisseriae* and heme

A hypothetical model of utilization of heme-containing compounds by *N. meningitidis* was first described by Stojiljkovic and colleagues (1995) and updated

by Perkins-Balding and colleagues (2004) but not confirmed by experimental analysis. This model proposes that an ABC transporter is used to transport heme across the cytoplasmic membrane. An ABC transporter for iron (FbpABC) was identified in *Neisseriae* sp., however, a study done by Khun and colleagues (1998) found that mutants in *fbpA* can continue to use heme as an iron source indicating that heme uses a different pathway for cell entry in *Neisseriae* spp.

1.11. Heme ABC transporters as antibacterial targets

Stojiljkovic and colleagues (1999) discovered a group of chemical compounds, non-iron metalloporphyrins (MPs), which possess a strong and broad antibacterial activity. Specifically, they are effective against growth of heme- and hemoglobin-utilizing bacteria, and only bacteria expressing heme/hemoglobin utilization pathways were sensitive to the MPs. Gallium-protoporphyrin IX (Ga-PPIX), the MP with the strongest antibacterial activity, did not cause cytotoxicity when applied *in vitro* or *in vivo*. These results are promising for treatment of clinical bacterial isolates that have resistance mechanisms against currently available treatments, and support the importance of identifying and characterizing a heme ABC transporter in pathogenic *Neisseriae*.

1.12. Summary

In summary, pathogenic *Neisseriae* are a cause of significant infections in both the developing and developed worlds and are becoming an increasing problem due to an increase in antibiotic resistance and the lack of development of new vaccines. Understanding the heme uptake mechanism of these bacteria would aid in the development of new vaccine candidates.

HYPOTHESIS

A dedicated ABC transporter is an essential component of the heme uptake pathway in pathogenic *Neisseriae*.

OBJECTIVES

To address the hypothesis, the following objectives were pursued:

- 1) Identification of the heme ABC transporter in *N. meningitidis* using genomic, proteomic and biochemical approaches;
- 2) Construction of isogenic heme ABC transporter mutants by insertional inactivation;
- 3) Genetic characterization of the isogenic heme ABC transporter mutants; and
- 4) Functional characterization of the heme ABC transporter mutants.

CHAPTER 2: MATERIALS AND METHODS

2.1. Bacterial strains

The wildtype clinical isolate *N. meningitidis* serogroup B. Barden, a strain recovered from a patient with meningitis, was used in this study and all *N. meningitidis* serogroup B. Barden gene knock-outs are described in Table 1. *E. coli* Top10 cells and BL21 StarTM (DE3) cells were purchased from Invitrogen (Carlsbad, CA, USA) and were used for cloning and expression of the *NMB0586* gene, as well as cloning of plasmid constructs used for construction of *N. meningitidis* gene knock-outs. All bacterial strains were stored at -80°C in LB broth containing 15% v/v glycerol.

2.2. Media and Culture Conditions:

2.2.1. Mueller-Hinton Agar Plates or Broth

All *N. meningitidis* strains were grown in Mueller-Hinton broth or on Mueller-Hinton agar (Mueller-Hinton broth containing 1.5% w/v BactoAgar (Difco/Becton Dickinson, Sparks, MD, USA)). Strains were grown at 37°C with 5% CO₂ on agar plates, and at 37°C in ambient air in broth. For growth curves of *N. meningitidis* strains using a single iron source, desferroxamine (desferal; Ciba Pharmaceutical Co., Summit, NJ, USA) was added to the Mueller-Hinton broth at a final concentration of 100 µM. Desferroxamine stocks (10 mM) were made by dissolving in ddH₂O and were filter sterilized using 0.75 µM filters (Nalge Nunc International, Rochester, NY, USA).

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

Bacteria Strain	Properties	Reference
<i>E. coli</i> FB827 <i>dppA::Km</i> <i>mppA::Cm</i> pAM238- HasR	Heme-deficient <i>E. coli</i> strain with antibiotic resistance to kanamycin and chloramphenicol	Provided by C. Wandersman
<i>E. coli</i> FB827 <i>dppA::Km</i> <i>mppA::Cm</i> pAM238- HasR pET101- NMB0586	Heme-deficient <i>E. coli</i> strain with antibiotic resistance to kanamycin and chloramphenicol, complemented with NMB0586	This study
<i>E. coli</i> pCR::catorifdDUS	<i>E. coli</i> strain containing a pCR2 plasmid with a <i>cat</i> cassette	Provided by Bonnah and Haas
<i>Neisseria</i> <i>meningitidis</i> B. Barden	Wildtype <i>N. meningitidis</i> serogroup B isolate involved in invasive meningococcal disease	Clinical isolate
<i>Neisseria</i> <i>meningitidis</i> NMB1993-CAT	<i>N. meningitidis</i> B. Barden mutant with a <i>cat</i> cassette inserted into the NMB1993 gene	This study
<i>Neisseria</i> <i>meningitidis</i> NMB0586-CAT	<i>N. meningitidis</i> B. Barden mutant with a <i>cat</i> cassette inserted into the NMB0586 gene	This study

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

E. coli FB827 *dppA::Km mppA::Cm* pAM238-HasR and *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR pET101-NMB0586 were grown at 37°C on M63 or M63* agar plates, or in M63 or M63* broth. M63 agar is prepared by the addition of 1.5% w/v BactoAgar (Difco/Becton Dickinson, Sparks, MD, USA) to M63 media (100 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, FeSO₄·7H₂O, pH to 7.0 with KOH, 0.4% (v/v) glucose). M63* broth and agar lacks FeSO₄·7H₂O. For growth promotion assays, various supplements were added to the broth or agar. When necessary, antibiotics were added at a final concentration as follows: ampicillin at 100 µg/ml, chloramphenicol at 15 µg/ml, kanamycin at 25 µg/ml, tetracycline at 10 µg/ml, and spectinomycin at 50 µg/ml. For the growth promotion assays, media was supplemented with various concentrations of heme (4 to 80 µM) and hemoglobin (5 to 50 µM). Heme stock solutions were made by dissolving bovine hemin chloride (Sigma, St. Louis, MO, USA) in 0.1N NaOH and were used without further sterilization. Hemoglobin stock solutions were made by dissolving bovine hemoglobin in sterile ddH₂O, and filter-sterilized using 0.2 µm filters. To chelate any free iron in the broth or agar, 2,2'-dipyridyl (2,2-Dip) was added at a final concentration of 150 µM. Isopropylthio-B-galactoside (IPTG) was also added to the agar plates at a concentration of 1 mM, to induce the expression of NMB0586 on the pET101-NMB0586 vector.

2.2.3. 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 200 rpm using the appropriate growth conditions.

2.3. Chromosomal DNA Isolation

Chromosomal DNA from *N. meningitidis* isolates was isolated using the protocol of Caparon and Scott (1991). Overnight cultures of *N. meningitidis* were prepared in Mueller-Hinton broth. A 1.5 ml aliquot of each culture was centrifuged for five minutes at 5,000 x g at room temperature and the supernatant removed. The pellet was resuspended in 400 µl of TES buffer. One hundred µl of a 100 mg/ml lysozyme solution was added to the suspension. Following incubation for 1 h at 37°C, 100 µl of a 6% w/v SDS solution in TE buffer was added and the microfuge tube incubated for 20 min at 37°C. The mixture was vortexed followed by the addition of 67 µl of 5M NaCl and the tube was then placed on ice for 1 h. The solution was centrifuged at 12 000 x g for 10 min and the supernatant transferred to a new tube. An equal volume of a 50:50 v/v phenol-chloroform mixture was added to the supernatant and mixed by inversion. The tube was then centrifuged for five min at 12 000 x g. The aqueous phase was removed and the phenol-chloroform extraction was repeated. A sodium acetate solution (pH 5.5) was added to the resulting aqueous phase to give a final concentration of 0.3 M. Two volumes of 95% v/v ethanol were added to the tube and the contents of the tube were mixed well. Following incubation on ice for 1 h, the solution was centrifuged at 12 000 x g for 10 min and the supernatant removed. The pellet was dried for 1 h at 37°C and then resuspended in 50 µl of TE buffer. To confirm isolation of the chromosomal DNA, the resuspended DNA was electrophoresed on a 0.8% w/v agarose gel.

2.4. Agarose Gel Electrophoresis

DNA samples, whether PCR products, plasmid constructs or genomic DNA, were electrophoresed on 0.8% (w/v) agarose gels 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) 0.5 M EDTA, pH 8.0)). Prior to gel loading, 10X DNA gel loading buffer (Eppendorf, Westbury, NY, USA) was added to the samples to a final concentration of 1X. Gels were electrophoresed at 80-120 V and DNA bands were visualized by adding 0.005% (v/v) ethidium bromide (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). Three μ l of the GeneRuler™ 1 kb DNA ready-to-use ladder (Fermentas, Burlington, ON) was loaded on each gel to determine the size of the plasmid or PCR product.

2.5. Polymerase Chain Reaction

Primers were designed using the program Primer 3 Design (<http://frodo.wi.mit.edu/>), and Net Primer (www.premierbiosoft.com/netprimer/index.html) was used to verify the rating of the primer sets, based on possible stem-loop structures and primer dimers. A rating of ≥ 75 was deemed acceptable. Primers were ordered from Sigma-Genosys Canada (Oakville, ON) and are listed in Table 2.

Table 2. Primers used in this study. ORF and NMB represent the open reading frame and the GenBank accession numbers for

N. meningitidis serogroup B.

Name	ORF	NMB	Sequence
98-right	01770	0098	5'-TCAGAAATTTTATCTGCCGATTG-3'
98-left	01770	0098	5'-TTATTTATCAAGACTGTCCTTTGGA-3'
1122-left	03052	1122	5'-GCAAAACCGAGCTTATTCCA-3'
1122-right	03052	1122	5'-CCAATTCGTCCAAAAGCTG-3'
1880-right	01072	1880	5'-GCTCTCTAGAAAAAACTGTATCCGCCGCAIC-3'
1880-left	01072	1880	5'-GCTAAAAGCTTCGCAACGGGTTCTGCCTTTTA-3'
1993-right	01289	1993	5'-TACTTAAAGCTTCTGCTGCCCTGGTTGATGTCG-3'
1993-left	01289	1993	5'-CACATATCTAGAAGCGCAAGCGCAGAAAGAACTC-3'
1989-right	01282	1989	5'-GCTTCTTAGACAAGAACAACAGCGGTTTCC-3'
1989-left	01282	1989	5'-ACGAAAAGCTTCCGGCTGCCAAAATAAGTTT-3'
1990-right	01284	1990	5'-TACTTAAAGCTTCCGGTCCGCAATCAGATAGG-3'
1990-left	01284	1990	5'-CTGCTAICTAGAGGTTGCCGACGCTTTTAAACG-3'
0586-right	03613	0586	5'-GCGTTAAAGCTTCCCTCAAACCTCACCCCTATTGTC-3'
0586-left	03613	0586	5'-GATCGGCTAGATGGTCAAAGGCTTTGATGTTG-3'
0588-right	03611	0588	5'-GATTGAAGCTTGCATGAGCATCATGTCGAA-3'
0588-left	03611	0588	5'-GGCTCTCTAGATTTCCCTGTTGCTGCAATTTTG-3'
0586-Rstop	03613	0586	5'-TTGCTTCATCGCGTTGGT-3'
0586-Lstop	03613	0586	5'-CACCATGAAAACACCTCAAAC-3'
CAT-right	n/a	n/a	5'-TCGATCCCTTTAGGGTTCCCGATT-3'
CAT-left	n/a	n/a	5'-AGTGAATTTCCGCTGCCGGGT-3'

A 100 μ l PCR Master Mix was prepared with 2 units of Biotools DNA polymerase (Biotools, Madrid, Spain), 0.2 mM dNTP solution, 0.5 μ M solution of each primer, 10 μ l of 10X Biotools PCR buffer. DNA template and sterile ddH₂O were then added to a final volume of 100 μ l. DNA template concentrations were 2-3 μ g/25 μ l PCR reaction for genomic DNA and 50ng/25 μ l PCR reaction for plasmid DNA.

PCR conditions varied depending on the DNA template used, and the melting temperature of the primers. For plasmid or genomic DNA, the initial denaturation was 3 min at 94°C, followed by 30 cycles of the following three steps; denaturation for 30 sec at 94°C, annealing for 45 sec at 50°C to 70°C (depending on the melting temperature of the primers and determined using a gradient PCR program), followed by elongation of the PCR product at 72°C. The length of the elongation step was dependent on the size of the expected PCR product (1min/kbp). A final elongation step for 10 min at 72°C ensured that all PCR products were complete. For colony PCR, a single colony was resuspended in 25 μ l of PCR Master Mix and the PCR conditions were the same as those used for the plasmid or genomic DNA templates, except the initial denaturation step was increased to 10 min.

2.6. DNA Quantification

The quantity of genomic, plasmid or PCR DNA was estimated by running a known volume of DNA sample with a high or low mass ladder (both from Invitrogen, Carlsbad, CA, USA) on a 0.8% v/v agarose gel. The DNA samples were also quantified spectrophotometrically using a RNA/DNA calculator GeneQuant II (Pharmacia Biotech, Cambridge, England).

2.7. Plasmid Clone DNA Sequencing

Plasmid DNA was extracted as described above using the Qiagen mini-prep kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. The sample was diluted with sterile ddH₂O to a final concentration of 12.5 ng in 10 µl and sent to the OGIC for fluorescent DNA sequencing with the Applied Biosystems 3730 DNA Analyzer using either the T7 promoter standard primer (5'-TAA TAC GAC TCA CTA TAG GG) or the primer corresponding to the cloned gene (see Table 1).

2.8. Microarrays

Transcription profiling was performed by our collaborators at The Institute of Genomic Research (TIGR) (Rockville, Maryland) using microarray analysis to identify putative ABC transporter genes that were either up- or down-regulated when *N. meningitidis* B. Barden was grown in iron limited conditions supplemented with either high or low heme concentrations.

2.8.1. Growth Conditions

Wild-type *Neisseria meningitidis* B. Barden was grown in supplemented Mueller-Hinton broth to mid-logarithmic phase (OD₆₀₀ = 0.25-0.3). To test for gene up-regulation in the presence of heme as a sole iron source, the bacteria was grown in the presence of 10 µM heme or 100 µM heme, and 100 µM desferroxamine was added to chelate any free iron in the media.

2.8.2. RNA Isolation

RNA was isolated from *N. meningitidis* using the RiboPureTM-Bacteria Kit (Applied Biosystems, Streetsville, ON). Briefly, the bacterial cells were resuspended

in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), incubated with 0.4 $\mu\text{g}/\mu\text{l}$ lysozyme at room temperature for 5 min and then lysed by the addition of glass beads with 0.1 mm diameter to the sample and use of a bead beater at 5000 rpm for 1 min. DNase I (10 $\mu\text{g}/\mu\text{l}$) was added to the lysed cells and incubated at 37°C for 30 min. mRNA was isolated from the *N. meningitidis* samples using the RNeasy RNA isolation kit (Qiagen, Mississauga, ON) as per the manufacturer's instructions.

2.8.3. Formaldehyde Agarose Gel

To check the quality of mRNA isolated, the RNA sample was run on a 1.2 % v/v formaldehyde agarose (FA) gel (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0, 0.2214 M formaldehyde, 10 $\mu\text{g}/100\text{ml}$ ethidium bromide, 1.2% w/v agarose). The RNA sample was diluted in 5X RNA loading buffer (0.16% (v/v) saturated bromophenol blue solution, 1 mM EDTA pH 8.0, 20mM MOPS, 5 mM sodium acetate, 0.2214 M formaldehyde, 20% (v/v) glycerol, 30% (v/v) formamide), incubated for 5 min at 65°C, chilled on ice and loaded onto the FA gel. The gel was electrophoresed at 5-7 V/cm of gel length in 1X running buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0, 0.2214 M formaldehyde). Agarose gel images were visualized and photographed with UV light, as described above.

2.8.4. Microarray Experiments

The microarray experiments were performed and analyzed by Julie Dunning Hotopp at TIGR. Briefly, reverse transcription of the isolated RNA was prepared with either Cy3 or Cy5 dCTP. Gene expression was analyzed by hybridization of the reverse-transcribed RNA to a *N. meningitidis* DNA microarray comprising 2194 open reading frames from *N. meningitidis* MC58 (Tettelin et al, 2000). A Neisserial

housekeeping gene, *dnaK*, whose expression was unchanged in both RNA samples, was used as a control. The data generated was normalized using total intensity, and was analyzed using the Significance Analysis of Microarrays (SAM) software package (Tusher et al., 2001). SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate. Such a method allows for determining the significance of changes in expression of genes between different biological states while accounting for the large number of genes.

2.8.5. Analysis of Microarray Results

We focused our study of the microarray data on genes with no homology to known sequences or domains but that were located within a known (or putative) ABC transporter locus; on genes that had homology to ABC transporter genes; and on genes that had iron-binding properties. Candidate genes were further analyzed to determine if more than one gene in an ABC transporter operon had increased expression levels. We postulated that the increased expression level of genes was in response to the decreased heme concentration in the growth media, and therefore these genes may be involved in heme uptake.

2.9. Neisserial Gene Knock-outs

To address the potential role of several ABC transporter loci as periplasmic heme transporters, we constructed meningococcal mutants by insertional inactivation

using an antibiotic cassette, and subsequently determined the phenotype of the mutant with respect to heme acquisition.

2.9.1. Construction of the pDrive constructs

The wildtype gene of interest was cloned by PCR amplification using the chromosomal DNA as a template. Site-specific primers were designed to encompass the entire coding region of the gene, including the native Shine-Dalgarno region and the stop codon. The primers also included added restriction sites at the 3' and 5' ends compatible for enzymes (*Xba*I and *Bam*HI respectively) with restriction sites not present within the amplified gene or pDrive cloning vector. The PCR-amplified gene was ligated into the pDrive cloning vector (Qiagen, Mississauga, ON) which subsequently underwent double digestion to confirm the presence of the gene of interest. A clone that possessed the gene of interest was then selected, based on its ability to grow on LB agar plates supplemented with 100 µg/ml ampicillin. The meningococcal gene was then digested with the restriction enzyme *Bmg*BI with no corresponding cleavage site within the pDrive backbone that produced a single blunt end fragment with a minimum of 200 bp flanking the restriction site. This resulted in a linear vector with blunt ends that was used to construct the isogenic meningococcal mutant.

The strategy for insertional inactivation sequence used a *cat* cassette located on the plasmid pCR2CmOFDU, kindly provided by R. Bannah (University of Calgary, Calgary, Alberta, Canada). The *cat* cassette is composed of a promoterless Tn9 *cat* gene placed under the transcriptional control of the opacity gene promoter of gonococcal strain MS11, and an fd-terminator sequence. The antibiotic cassette is

flanked by an *ori_{fd}* origin of replication at the 5' end and by a 60 bp sequence carrying the 10 bp gonococcal DNA uptake signal sequence immediately downstream of the transcriptional terminator. The *cat* cassette was excised from pCR2CmOFDU with BmgBI that also generated a blunt end fragment.

The *cat* cassette was then ligated into the linear pDrive vector encoding the gene of interest, as prepared above. Briefly, T4 DNA ligase and T4 DNA ligase buffer was incubated with 50 ng of the pDrive vector and the *cat* cassette at a vector:insert ratio of 1:3, as per the manufacturer's instructions (New England Biolabs, Ipswich, MA). To optimize blunt end ligation efficiency, the ligation was incubated at 4°C, 16°C or 37°C for 16 hours. Furthermore, to stimulate blunt end ligation, either or both of 150 mM NaCl and 5% v/v polyethylene glycol was added to the reaction. The resulting construct was transformed into *E. coli* Top10 cells, and a clone which possessed the gene of interest was then selected, based on its ability to grow on LB agar plates supplemented with 25 µg/ml chloramphenicol. The pDrive-gene-*cat* construct was isolated and the insert sequenced, as described above.

2.9.2. Construction of the chromosomal gene knock-outs by transformation

The pDrive-gene-*cat* construct was linearized by restriction digestion using an enzyme targeting a single restriction site on the backbone of the plasmid, but with no restriction sites within the cloned DNA sequence. Wildtype *N. meningitidis* B. Barden was grown overnight in Mueller-Hinton broth to mid-logarithmic phase (OD₆₀₀ 0.25-0.3), and then plated on Mueller-Hinton agar. 1 µg/µl of the linearized pDrive-gene-*cat* was spotted on top of the newly plated *N. meningitidis* culture, and the plates were then incubated overnight at 37°C with 5% CO₂. The resulting lawn of

bacterial growth was replated onto Mueller-Hinton plates supplemented with 15 $\mu\text{g/ml}$ chloramphenicol and incubated for up to 7 days at 37°C with 5% CO₂, until at least one colony was observed on the agar plate.

2.9.3. Screening of the Neisserial Gene Knock-outs

Two methods were used to confirm that the proper allelic exchange had occurred in each mutant strain. First, PCR analysis was performed using a combination of ABC transporter gene and *cat* specific primers, and chromosomal DNA preparations from the wildtype *Neisseria* strain B. Barden and from the mutant strains as templates. Primers designed to anneal to sequences bracketing the *cat* cassette would be predicted to generate a larger product from the mutant than from the wildtype strain. Primers designed to amplify the *cat* cassette alone would confirm that the *cat* cassette had been inserted intact. A negative control using primers to another gene in the Neisserial genome was also performed.

The second method used to verify that the appropriate gene replacement had occurred consisted of sequencing the PCR product from the PCR reaction using a primer set for the gene containing the inserted *cat* cassette, to confirm the presence and location of the *cat* cassette.

2.9.4. Growth curves for phenotype analysis

Growth assays were conducted to determine the ability of the ABC transporter mutants to use various iron compounds as the sole exogenous source of iron. The Neisserial mutants were grown overnight in Mueller-Hinton broth, and then 1 ml of this culture was added to 100 ml of Mueller-Hinton broth supplemented with desferroxamine at a final concentration of 100 μM , to chelate any free iron in the

media. Heme, haemoglobin and ferric chloride were added at the indicated concentrations to serve as the sole exogenous iron source. The cultures were incubated at 37°C/225 rpm for 10 h, until stationary phase was attained. An aliquot was removed from the cultures every hour, and the OD₆₀₀ was measured. Wildtype *N. meningitidis* B. Barden was used as a positive control, and was prepared and analyzed as described for the mutants.

2.10. Cell Fractionation Procedures:

2.10.1. 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 on Mueller-Hinton agar plates containing 100 µM desferroxamine under heme-limiting (10 µM) or heme-replete (50 µM) conditions for 24 h. After 24 h of incubation, bacterial lawns were harvested by recovering cells with a sterile loop. Cells were suspended in sterile PBS and pelleted by centrifugation at 4000 x g for 10 min at room temperature. The pellet was washed twice with sterile PBS prior to suspension in an equal volume of sonication buffer (10mM Tris-HCl, 5 mM EDTA) and chloroform (EMD, Gibbstown, NJ, USA) with the addition of phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 3 mM. 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.

2.10.2. Whole Cell Lysates extraction

Bacteria were grown on Mueller-Hinton agar plates containing 100 μM desferroxamine under heme-limiting (10 μM) or heme-replete (50 μM) conditions for 24 h. After 24 h of incubation, bacterial lawns were harvested by recovering cells with a sterile loop. Cells were suspended in sterile PBS and pelleted by centrifugation at 4000 x g for 10 min at room temperature. The pellet was washed twice with sterile PBS prior to suspension in an equal volume of sonication buffer. Sonication was performed six times using the sonifier/disrupter at 15-20 W output (15 sec sonication followed by 10 sec interruption on ice, repeated six times). The mixture was centrifuged for 60 min at 100 000 x g at 4°C. The supernatant was collected and stored at -20°C.

2.11. Two-Dimensional Gel Electrophoresis:

The ZOOM[®] IPGRunner[™] System from Invitrogen (Carlsbad, CA, USA) was used for two-dimensional gel electrophoresis (2DGE) and was performed in the laboratory of Dr. Fraser Scott (Ottawa Health Research Institute, Ottawa, ON, Canada).

2.11.1. Periplasmic Protein Desalting

Protein desalting spin columns (Pierce, Rockfort, IL, USA) were used to desalt the periplasmic extracts according to the manufacturer's instructions prior to the rehydration step described below. The desalted periplasmic extracts were quantified and stored at -20°C.

2.11.2. Step One – Rehydration

Desalted *N. meningitidis* B. Barden periplasmic proteins at a concentration of 12.5 µg/10 µl were suspended in 155 µl Sample Rehydration Buffer (8 M Urea, 2% CHAPS, 0.5% (v/v) ZOOM[®] Carrier Ampholytes, 0.002% Bromophenol Blue, 20 mM dithiothreitol). After mixing by inversion and briefly vortexing, the rehydration buffer periplasmic protein solution was added to a well in the ZOOM[®] IPGRunner[™] cassette, to which the ZOOM[®] Strip was then gently inserted, ensuring no trapped air bubbles. ZOOM[®] strips of pH 3-10 (linear), pH 4-7 and pH 9-12 were used, with the corresponding ampholyte used in the rehydration buffer. The strips were rehydrated at room temperature for 12-16 h.

2.11.3. Step Two – Isoelectric Focusing (IEF)

The ZOOM[®] IPGRunner[™] cassette was inserted in the ZOOM[®] IPGRunner[™] chamber and electrophoresed following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, the strips were run at 175 V for 15 min, ramped from 175 V to 2000 V for 60 min, and then 2000 V for 60 min.

2.11.4. Step Three – Equilibration of Strips

Following the IEF separation, the strips were equilibrated for 15 min in equilibration buffer (1X NuPAGE[®] Sample Reducing Agent in 1X NuPAGE[®] LDS Sample Buffer), and then in alkylation buffer (125 mM iodoacetamide in 1X NuPAGE[®] LDS Sample Buffer) for 15 min, with gentle agitation at room temperature.

2.11.5. Step Four – Second Dimension Protein Separation

The equilibrated strip was carefully applied to the NuPAGE[®] Novex 4-12% Bis-Tris ZOOM[®] Gel and covered with overlay agarose (BioRad, Hercules, CA, USA). The gel was then placed in a gel electrophoresis chamber containing 1X MES buffer and run at 150 V for 45 min. Five µl of the Unstained Page Ruler Protein Ladder (Fermentas, Burlington, ON) was loaded in a well of each gel, to confirm the size of protein bands.

2.12. One-Dimensional Gel Electrophoresis

All one-dimensional (1D) SDS-PAGE gels were performed with a 12% 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% stacking gel (0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 4.5% (w/v) acrylamide) using a discontinuous buffer system. Protein samples were diluted to the desired concentration in ddH₂O in an equal volume of SDS-PAGE 2X sample buffer (125 mM 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 150 V in the Mini-Protean II electrophoresis cell system (BioRad, Richmond, CA, USA). Five µl of the Unstained Page Ruler Protein Ladder (Fermentas, Burlington, ON) was loaded in a well of each gel, to confirm the size of protein bands.

2.13. Staining Methods for SDS-PAGE:

2.13.1. Coomassie and Rapid Staining

1D SDS-PAGE gels were stained with Coomassie [0.1% (w/v) Coomassie Brilliant Blue R-250 (EM Science, Darmstadt, Germany, Bismark brown R (Sigma, St. Louis, MO, USA), 40% (v/v) ethanol (Commercial Alcohols Inc., Brampton, ON, Canada), 7% (v/v) glacial acetic acid] followed by destaining with 40% (v/v) ethanol and 7% (v/v) glacial acid until protein bands were visible and the background was clear. Alternatively, 1D SDS-PAGE gels 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.

2.13.2. Sypro Ruby Staining

2D gels were washed twice for 30 min each in fixer solution (50% v/v ethanol, 5% v/v acetic acid). The gels were then immersed in Sypro Ruby Stain (Sigma, St. Louis, MO, USA) and stained overnight with gentle agitation at room temperature. The gels were washed twice for 15 min each in ddH₂O. The gels were visualized and photographed with UV light using the MultiImage Light Cabinet. 2D gels were also analyzed for changes in net intensities using 2D analysis software (PDQuest; Bio-Rad, Hercules, CA, USA).

2.14. Mass Spectrometry of Periplasmic Binding Protein Candidates Identified by 2DGE

Desired protein spots resulting from 2DGE were excised from the SDS-PAGE gel and sent to the Queen's University Protein Function Discovery Group (Kingston, ON, Canada) for identification by trypsin digest and MALDI-TOF. The

results were searched using the Mascot search engine and MSDB database at 50ppm mass accuracy.

2.15. Western Immunoblotting

Protein samples were separated by SDS-PAGE in duplicate in order to transfer one gel onto Immobilon-FL (Millipore, Billerica, MA, USA). The second gel was stained with Coomassie or Rapid Stain as described above to ensure proper protein separation. The SDS-PAGE gel for transfer was first equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol (Fisher, Fair Lawn, NJ, USA)) for 30 min. The semi-dry Mini-Protean II blotting apparatus was then assembled according to the manufacturer's instructions. The transfer conditions comprised 30 min at 20 V with a constant amperage of 0.5A. After transfer was complete, the blot was stained with 10% (v/v) Ponceau S (Sigma, St. Louis, MO, USA) to confirm the transfer of protein bands. The stain was removed by washing the blot several times in ddH₂O, followed by overnight incubation in blocking solution (5% (w/v) skim milk (EM Science, Gibbstown, NJ, USA) in PBS). The blot was then probed with anti-V5-HRP (Horse Radish Peroxidase) antibody, anti-His-HRP antibody or anti-hHbp antibody for 1.5 h at room temperature in blocking solution, with gentle agitation. Anti-V5-HRP and Anti-His-HRP antibodies (Invitrogen, Carlsbad, CA, USA) were used at a dilution of 1:5000 in immunoblot experiments to detect recombinant protein containing the V5 epitope or the C-terminal histidine tag, respectively. The rabbit polyclonal anti-hHbp was produced at Cedarlane laboratories (Hornby, ON, Canada) and was used in immunoblot experiments at a dilution of 1:8000. For the anti-hHbp immunoblots, the blots were

incubated with a 1:10000 dilution of goat anti-rabbit immunoglobulin horse radish peroxidase conjugated secondary antibody (Biosource, Camarillo, CA, USA) solution in blocking solution for 30 min at room temperature. Since both anti-V5 and anti-His antisera were conjugated to HRP, no secondary antibody was necessary. After three washes of five min 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.

2.16. Champion™ pET Directional TOPO® Expression System

The pET101/D-TOPO kit (Invitrogen, Carlsbad, CA, USA) was used to express a recombinant NMB0586 protein with a C-terminal fusion tag containing the V5 epitope and 6xHis region using the manufacturer's instructions.

2.16.1. TOPO® Cloning Reaction and Transformation

A molar ratio of 1:4 of PCR product to TOPO vector was used in the ligation of the *NMB0586* gene into pET101/D-TOPO vector, and the ligation mixture was incubated at room temperature for 5 min. The resulting plasmid constructs were used to transform chemically competent OneShot TOP10 *E. coli* cells for 30 min on ice according to the manufacturer's instructions. The transformed cells were plated onto pre-warmed LB agar plates containing ampicillin at 100 µg/ml. After overnight incubation at 37°C, transformants were selected for further analysis.

2.16.2. Analysis of Positive TOP10 Transformants

Transformants selected from LB agar plates containing ampicillin were analyzed by colony PCR using the PCR amplification conditions listed above for the RightPET-0586-NoStop/LeftPET-0586 primers with the exception that initial

denaturation time was increased to 10 min. Vector size was determined by agarose gel electrophoresis of plasmid extracted using the Qiagen Mini-Prep kit. Transformants exhibiting the appropriate size amplicon were frozen in LB broth containing 100 µg/ml ampicillin with 15% v/v glycerol and stored at -80°C.

2.16.3. Expression of Recombinant Fusion Protein

To express the recombinant NMB0586 protein, plasmid isolated from a TOP10 transformant was used to transform BL21 StarTM (DE3) One Shot *E. coli* cells according to the manufacturer's instructions. Expression of the recombinant fusion protein was achieved by induction with IPTG at a final concentration of 1 mM added at mid-log phase of growth (OD₆₀₀ of 0.5-0.8).

2.16.4. Ni-NTA Protein Purification

The Ni-NTA purification system (Invitrogen, Carlsbad, CA, USA) was used to purify the poly-histidine-containing recombinant protein from IPTG induced BL21 StarTM (DE3) *E. coli* cells containing the pET101-NM0586 vector. Briefly, cells were resuspended in sonication buffer and the bacteria were disrupted by sonication. rNMB0586 was purified with Ni-NTA agarose under hybrid conditions. In the final elution step, the bound rNMB0586 was eluted in native elution buffer with sequentially increasing imidazole concentrations (50 mM to 250 mM). The rNMB0586 eluted optimally in the 100 mM and 150 mM imidazole fractions as verified by SDS-PAGE. These fractions were then concentrated using Amicon Ultra centrifugal filter devices (Millipore, Carrigtwohill, Cork, Ireland). Five percent glycerol (v/v) and PMSF (at a final concentration of 3 mM) were added to concentrated protein fractions, which were stored at -80°C.

2.16.5. 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.

2.17. Direct Heme Agarose Binding

Twenty microlitres of bovine hemin-agarose suspension ($\geq 1 \mu\text{mol hemin/ml}$; Sigma, St. Louis, MO, USA) was washed three times with 1 ml binding buffer (100 mM NaCl, 25 mM Tris-HCl, pH 8.0) and the affinity matrix was pelleted by centrifugation at 10 300 rpm for 5 min at room temperature. The hemin-agarose was suspended in 500 μl of binding buffer to which 10 μg of affinity purified rNMB0586 was added. In the specificity assay, the affinity gel was incubated with increasing concentrations of affinity purified rNMB0586 ranging from 500 ng to 10 μg . The mixture was gently agitated on a Labquake Shaker Rotisserie (Barnstead/Thermolyne, Dubuque, Iowa, USA) for 2 h at 37°C followed by three washes with 500 μl of binding buffer. Bound proteins were eluted by the addition of 50 μl 2X sample buffer and the sample was incubated at 100°C for 5 min. After centrifugation at 10 300 rpm for 5 min at room temperature, 40 μl of the supernatant was used for SDS-PAGE and Western blot analysis.

2.18. Competitive Inhibition Heme Agarose Binding

Competitive binding experiments were performed in which 10 μg of the affinity purified rNMB0586 was pre-incubated with increasing concentrations of competing heme (0.5 nM to 800 μM) in a final volume of 30 μl of binding buffer. The heme was dissolved in 0.1N NaOH, and the pH adjusted to 8.0 with the addition

of concentrated HCl. Following incubation for 60 min at 37°C without shaking, the samples were added to the heme affinity gel and suspended in 470 µl of binding buffer. Heme affinity chromatography was conducted as described above.

A N-terminal 6xHis tagged purified wheat storage globulin (Glb1), expressed in SF21 insect cells using the Baculovirus expression system, was kindly provided by Alexander Strom (University of Ottawa, Ottawa, ON, Canada), and the N-terminal 6xHis tagged purified outer membrane lipoprotein from *Leptospira* (rLipL32), expressed in *E. coli* BL21 StarTM (DE3), was kindly provided by Hanhong Dan (Canadian Food Inspection Agency, Ottawa, ON, Canada). Both of these His-tagged proteins served as negative controls in the heme-agarose binding experiments. Ten µg of each recombinant protein was used in both the direct heme binding assay and the competition binding assay.

2.19. Heme Binding Detection by 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). Briefly, 20 µl of each purified protein at a concentration of 4×10^{-5} M was incubated at room temperature for 30 min with either heme at concentrations of 10^{-7} M to 10^{-4} M or with binding buffer alone. The mixtures were then separated by PAGE, using the NativePAGE Novex Bis-Tris Gel System (Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. The resulting proteins on the gel were transferred onto nitrocellulose membranes, as per the protocol for Western blot analysis described above. Heme complexed with

protein bands on the gel retains intrinsic peroxidase activity, and was detected by chemiluminescence (ECL+; Amersham, Pharmacia). The signal was measured using the Storm Imager Analysis System (GE Healthcare Bio-Sciences, Baie d'Urfe, Que.).

2.20. Complementation Assays:

E. coli strain FB827 *dppA::Km mppA::Cm* pAM238-HasR, kindly provided by C. Wandersman (Institut Pasteur, Paris, France), 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 *dppABCD*. We tested whether the expression of NMB0586 in the *E. coli* double knock-out mutant strain had the ability to restore the wildtype heme phenotype.

2.20.1. Preparation of Competent *E. coli*

E. coli FB827 *dppA::Km mppA::Cm* pAM238-HasR was inoculated into 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). Cultures were centrifuged at 4000 rpm for 10 min at 4°C, and the pelleted cells were resuspended in 10 ml of ice-cold 0.1 M CaCl₂. The cells were again pelleted by centrifugation, resuspended in 4 ml 0.1 M CaCl₂ and stored at 4°C for up to 48 h prior to transformation.

2.20.2. Transformation

pET101-NMB0586 (50 ng in 10 µl 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. Eight hundred µl of Super Optimal Broth with catabolite repression (SOC) medium (2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, pH 7.0) was added to the cells and the cell suspension was incubated for 45 min at 37°C without shaking. *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR pET101-NMB0586 transformants were selected on LB agar plates containing kanamycin (25 µg/ml), chloramphenicol (15 µg/ml) and ampicillin (100 µg/ml).

2.20.3. Confirmation of Transformation and NMB0586 Expression

Transformation of *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR with pET101-NMB0586 was confirmed using several methods. Transformants were tested for growth on M63 plates containing 100 µg/ml ampicillin. An ampicillin resistant phenotype would provide presumptive evidence for the presence of pET101 as this vector harbors the b-lactamase gene cassette. Isolation of the pET101-NMB0586 vector from transformed cells was confirmed by PCR, using the primer sets RightPET-0586-NoStop/LeftPET-0586, and RightNMB0586/LeftNMB0586, and by DNA sequencing of the isolated plasmid using the same two primer sets. Transformants were assessed for expression of rNMB0586 by IPTG induction followed by separation of the cell lysates by SDS-PAGE. Western immunoblots of the cell lysates were probed with anti-V5 antibody. The cellular location of rNMB0586 in *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR was determined by cellular fractionation and Western blot analysis (both described above).

2.20.4. Growth Promotion Assays

The ability of NMB0586 to restore the heme phenotype in *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR pET101-NMB0586 was determined by growth on M63* plates containing various heme and hemoglobin concentrations. Following overnight growth of a 50 ml culture of *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasRpET101-NMB0586 in M63 broth, the cells were centrifuged at 4000 x g, resuspended in M63* broth to an OD₆₀₀ of 0.8 and incubated at 37°C/225 rpm for 2 h. An aliquot of 10⁵ cells was streaked onto M63 agar plates and M63* agar plates containing various iron sources, as described above. Transformants were also inoculated onto M63 (with no added supplements) agar plates in order to ensure cell viability. *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR pTRCC-MppA, the double knock-out *E. coli* strain complemented with the heme binding periplasmic component MppA, was used as a positive control in the growth promotion assays. *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR pET101, the double knock-out *E. coli* strain transformed with the empty vector served as a negative control. Growth was measured by abundance of colonies on the M63* plates in comparison to the control M63 plate for each *E. coli* strain.

CHAPTER 3: RESULTS

To identify the heme periplasmic binding protein in *N. meningitidis*, several approaches were used that capitalized on the observation that the expression of an ABC transporter is enhanced under ligand-restrictive conditions.

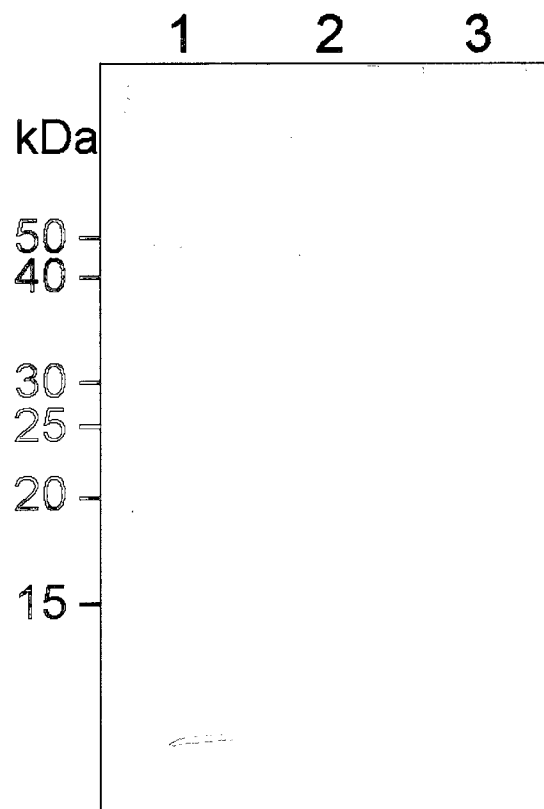
3.1 Determination of heme limiting conditions in *N. meningitidis*

The minimum concentration of ferric chloride, heme and hemoglobin required to support the growth of the wild-type meningococcal strain B. Barden in iron-limited broth was determined in a series of titration experiments in which growth of *N. meningitidis* in iron-limited Mueller-Hinton broth was assessed in response to increasing amounts of iron salt. The minimum concentrations of ferric chloride, heme and hemoglobin required to support growth are 5 μM , 2 μM and 0.5 μM , respectively. These concentrations are supported by similar growth study experiments determined by Khun et al. for *Neisseria meningitidis* (1998).

3.1.1. Periplasmic protein extraction

Periplasmic and cytoplasmic extracts were isolated from *N. meningitidis* B. Barden, and the protein profiles were compared using SDS-PAGE (Figure 1). Over a size range of 10-120 kDa, the protein banding pattern of both extracts is significantly different, with bands present in one sample and not the other, as well as bands of the same size with brighter intensity in one sample over the other. We relied on a well recognized periplasmic protein isolation technique, and the obvious difference in the banding profiles of the protein extracts with SDS-PAGE, to prepare periplasmic protein extracts to do 2DGE. Judd and Porcella (1993) had previously demonstrated that the chloroform-Tris extraction procedure we used selectively released

Figure 1. SDS-PAGE of periplasmic and cytoplasmic protein extracts from *Neisseria meningitidis*. Lane 1: molecular weight marker, Lane 2: periplasmic protein extract, Lane 3: cytoplasmic protein extract. 10 µg of each protein extract was loaded per well. Results were reproducible (n=3).



periplasmic components in several strains of both *N. meningitidis* and *N. gonorrhoeae*, a technique experimentally verified by others (Weiman and Gortz, 1991; Monsoroi et al. 2001). They stained SDS-PAGE gels with either Coomassie brilliant blue or silver stain to characterize periplasmic extract in comparison to cytoplasmic extract and whole cell lysate, and demonstrated a significant difference in banding profiles of the three protein samples. Our periplasmic profile was similar to that seen in *N. gonorrhoeae*, with three significant bands of 44 kDa, 34 kDa and 14 kDa in both periplasmic *Neisseria* samples that were not observed in either of the cytoplasmic extracts (Figure 1). Judd and Porcella (1993) further characterized the periplasmic samples by Western blot analysis using monoclonal antibodies directed to the outer membrane protein PorA, and lipopolysaccharide (LPS), and to the 70S ribosome, to ensure that the periplasmic protein sample was not contaminated with either outer membrane or cytoplasmic components. Unfortunately, these antibody reagents were not available to us to exclude the presence of outer membrane and cytoplasmic protein contamination of our periplasmic extract.

3.1.3. Identification of periplasmic proteins with up-regulated expression under heme-limiting conditions by 2-dimensional electrophoresis

Each of three periplasmic extraction samples collected from *N. meningitidis* under heme-limiting conditions was analyzed by 2-D gel electrophoresis in conjunction with corresponding periplasmic extractions collected under heme-replete conditions, resulting in a total of six pairwise comparisons. Six proteins consistently exhibited differential expression under heme-limiting conditions compared to heme-replete conditions (Figure 3). Visually identified spots were further analyzed using

Figure 2. *Neisseria meningitidis* periplasmic protein extracts electrophoresed using 2DGE and stained with Sypro Ruby. The isoelectric range of the gel was pH 9 to pH 12. *N. meningitidis* was grown using either high or low concentrations of heme as a sole iron source in the growth media; (a) 10 μ M heme and (b) 50 μ M heme. M = molecular weight marker. Results were reproducible (n=3).

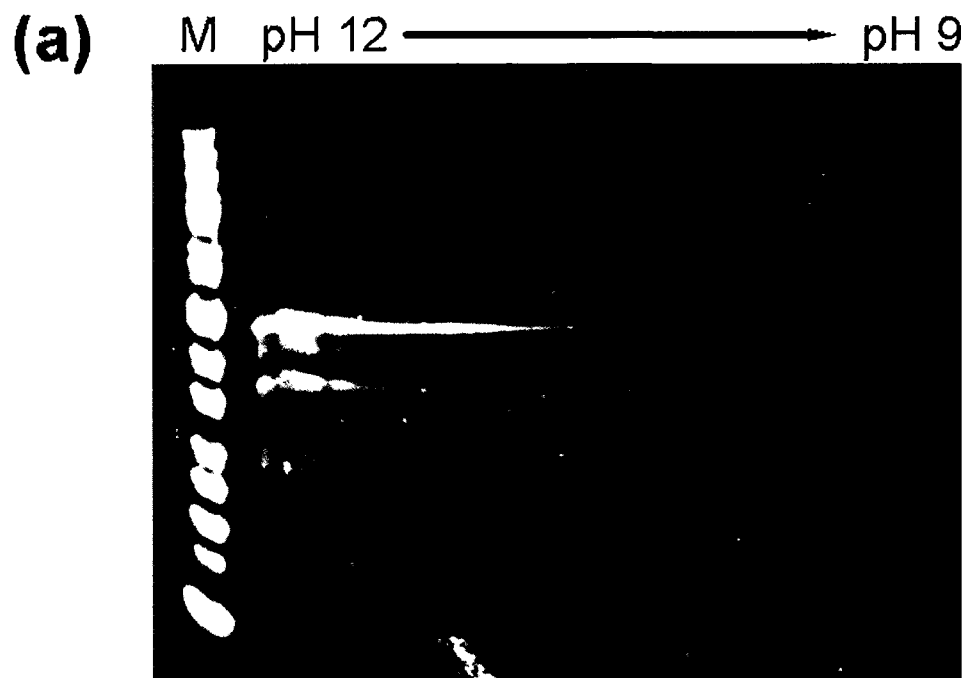
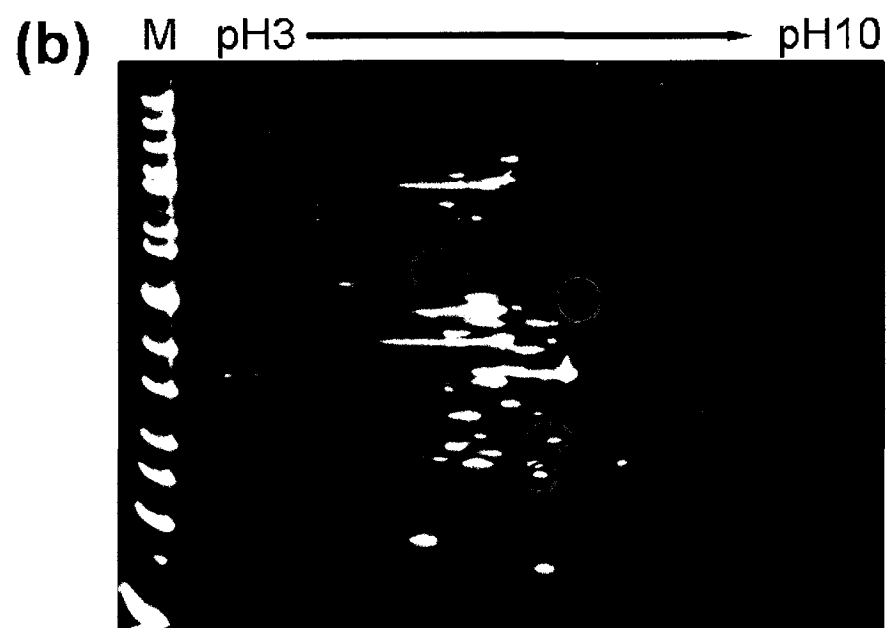
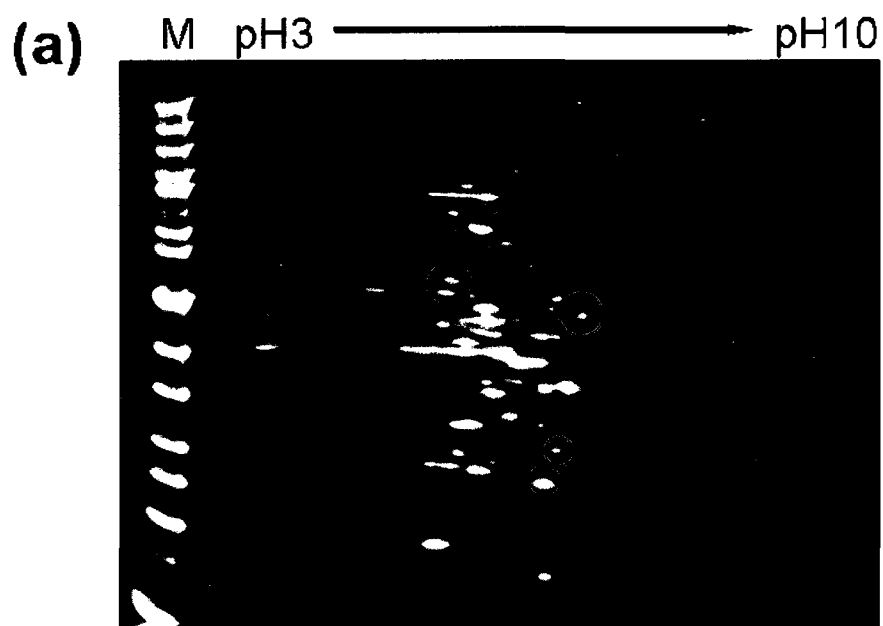


Figure 3. *Neisseria meningitidis* periplasmic protein extracts electrophoresed using 2DGE and stained with Sypro Ruby. The isoelectric range of the gel was pH 3 to pH 10. *N. meningitidis* was grown using either high or low concentrations of heme as a sole iron source in the growth media; (a) 10 μ M heme and (b) 50 μ M heme. M = molecular weight marker. The grey circles around spots represent proteins that have increased expression (ie. brighter spot) when grown using a low heme concentration in the growth media. Results were reproducible (n=3).



scanning densitometry to quantitate the difference in spot intensity in low heme conditions compared to high heme, using a 50% increase in intensity as a measure of significance. These six spots underwent trypsin digestion and the candidate protein spots were sent to the Queen's University Protein Function Discovery Group (Kingston, ON, Canada) for identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The peptide mass maps were analyzed by Mascot (Matrix Science) to identify the protein spots. A 95% confidence level threshold was used for Mascot scores. The following is a summary of the results from Table 3:

- 1) The dihydrolipoamide dehydrogenase is the E3' component of the 2-oxoglutarate dehydrogenase complex and is involved in catalyzing the oxidation of dihydrolipoamide to lipoamide in *Neisseria* sp. It has been identified as an immunodominant antigen, and has been of particular interest as a vaccine candidate, as well as a diagnostic agent. In *Neisseria* sp., p64k is partially found associated with the cell envelope (Alejandro et al., 2003) or localized to the cytoplasm of the bacterial cell as part of a multienzyme complex.
- 2) The serine hydroxymethyltransferase is a homotetramer involved in the conversion of serine to glycine. It is located in the cytoplasm of bacteria, as reviewed by Devor and Dill-Devor (1997). The *Neisseria* homologue, GlyA, is uncharacterized to date, but contains homologous motifs to known serine hydroxymethyltransferases.
- 3) The putative oxidoreductase catalyzes the oxidation of one component with the reduction of another. In *N. gonorrhoeae*, some oxidoreductase proteins are

Table 3. MALDI-TOF N-terminal sequencing results for protein spots located on the 2DGE results shown in Figure 2. The sequence identity with the highest homology to the unidentified spot is listed, as well as the size of the corresponding protein and the putative or known biological function in the bacteria.

Protein spot	Relative density of spot*	Protein identification	Size (kDa)	Function
1	1.71 X	Dihydroliipoamide dehydrogenase (also known as p64k)	51.1	Pyridine nucleotide disulphide oxidoreductase (in clinical trials)
2	1.62 X	Serine hydroxymethyltransferase	45.3	Amino acid transport and metabolism
3	2.50 X	Probable oxidoreductase	20.7	Predicted flavoprotein (general function only)
4	1.75 X	Probable phosphate acyltransferase	52.4	Energy production and conversion
5	2.25 X	Trigger factor	48.2	Molecular chaperone
6	1.58 X	Phosphoglycerate mutase	26.0	Carbohydrate transport and metabolism

* Resulting from a comparison of the density of each protein spot ("10 μM heme" density/"50 μM heme" density), where X represents how much more intense the protein spot is in the "10 μM heme" compared to "50 μM heme".

considered major outer membrane proteins. However in *N. meningitidis*, these enzymes can either be polytopic membrane proteins that span the cytoplasmic membrane or periplasmic proteins (Vivian et al. 2008).

- 4) The putative phosphate acetyltransferase is involved in the catalysis of the transfer of an acetyl group to an acceptor molecule. In *Neisseria* sp. it is usually a cytoplasmic protein, but can be transferred to the periplasmic space (Mendun et al., 2009; Vaughan et al., 2006).
- 5) The trigger factor found in *N. meningitidis* (Parkhill et al., 2000) is a homologue of trigger factors in other bacteria that are involved in protein export, and act as chaperones by maintaining newly synthesized proteins in an open conformation (Hoffmann et al., 2006). The specific activity of the protein is as a peptidyl-prolyl cis-trans isomerase, and the protein is localized in the cytoplasm of the cell.
- 6) The phosphoglycerate mutase (termed GpmA in *E. coli*) is a 2,3-biphosphoglycerate dependent phosphoglycerate mutase and catalyzes the conversion of 2-phosphoglycerate and 3-phosphoglycerate in the bacterial cytoplasm of the bacteria. The *Neisseria* homologue, NMB1604, is currently uncharacterized but contains homologous motifs to other phosphoglycerate mutases.

Database searches for amino acid sequence homology to known bacterial proteins revealed that none of the above proteins are involved in heme/iron transport or uptake, and none of the genes encoding these proteins are located near either ABC

transporter components or heme/iron binding proteins in the genome. Furthermore, none of the proteins we identified appear to have a readily recognizable Fur-binding site in the promoter proximal region.

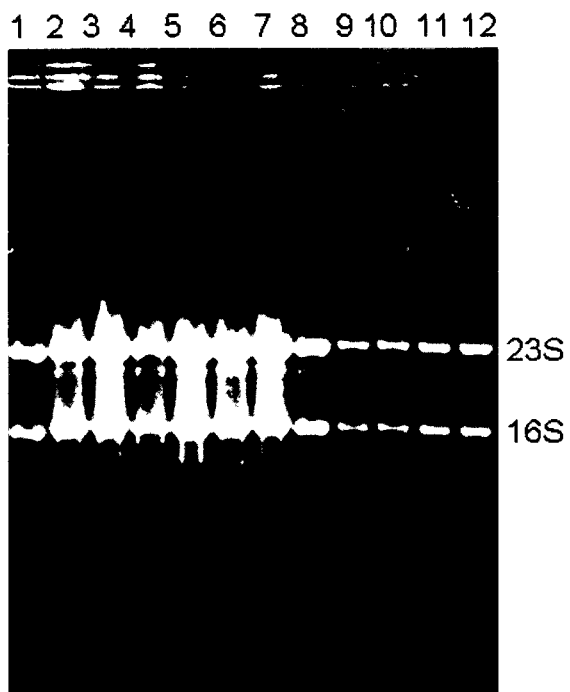
3.2. Microarray analysis of *N. meningitidis* B. Barden

The second approach used in this study to identify a heme periplasmic binding protein was the analysis of the transcriptional profiles of *N. meningitidis* when the organism was grown under heme-limiting and heme-replete conditions.

3.2.1. Purity of RNA extracts

RNA was isolated from *N. meningitidis* grown in the presence of low (10 μ M) and high (100 μ M) heme as the sole iron source. The absorbance ratio of A260/A280 of the isolated RNA was 2.1, indicating that the RNA sample was not contaminated by protein. The resulting formaldehyde agarose gel confirmed the purity and integrity of the RNA, with two intense bands representing the 16S and 23S RNA, and a smear throughout each lane representing the mRNA (Figure 4). The microarray experiments were performed by Julie Dunning Hotopp, a graduate student collaborator at TIGR, who performed inter-slide and intra-slide comparisons to obtain an average value with standard deviations for the level of expression of each *N. meningitidis* ORF when the bacteria had been grown in the presence of low heme (10 μ M) compared to high heme (100 μ M). The mean values for each ORF (2005 ORFs in total) were then analyzed by us for genes of particular interest to the present study. To further support the growth conditions of heme deprivation used in this study, the Neisserial heme oxygenase gene, *hemO*, demonstrated increased expression under heme-limiting growth conditions in our DNA microarray results.

Figure 4. Ethidium bromide stained formaldehyde agarose gel of RNA isolated from *N. meningitidis* B. Barden. Lanes 1-6 and lanes 7-12 are from *N. meningitidis* cultures grown in the presence of 10 μ M and 100 μ M heme, respectively, as a sole source of iron. The RNA samples in lanes 5 and 7 were used for microarrays analysis.



3.2.2. Identification of genes differentially-regulated under heme-limiting conditions by DNA microarray analysis

Our analysis of the microarray data focused on genes that were either up-regulated by a factor of 50% or higher when *N. meningitidis* was grown in the presence of low heme compared to high heme as a sole iron source. Over 350 genes were thus identified. The search was then restricted to genes that encoded: genes with no homology to known sequences or domains but were located within a known (or putative) ABC transporter locus; genes that had homology to ABC transporter genes; or genes that encoded proteins displaying iron-binding properties. The designation of an ABC transporter was based on the presence of a putative ATPase gene with Walker A and B motifs within a putative operon. Table 3 lists the putative ABC transporter components that were differentially-regulated when *N. meningitidis* was grown under iron-deficient conditions using 10 μ M heme as a sole iron source. Particular attention was placed on ORF1282, 1284, 1287 and 1289, since they were located in an uncharacterized putative ABC transporter operon.

A BLAST search of these ORFs disclosed no sequence homologies to characterized heme binding or iron binding proteins. At the time that this BLAST search was performed, the *N. meningitidis* ORF1282 was putatively identified as a gene encoding a periplasmic protein with iron binding properties. No Fur-binding motif was identified in upstream sequences to these four ORFs. Nonetheless, all the proteins in Table 4 were further studied to determine their potential involvement in heme uptake.

Table 4. Summary of the identified open reading frames (ORF), encoding putative components of ABC transporters, that were up- or down-regulated when *N. meningitidis* was grown in the presence of a low heme concentration compared to high heme as a sole source of iron in the growth media. Also included are the putative function of the ORF based on both sequence homology to known proteins and genome location of the ORF with respect to other genes. The ORFs are located in seven separate putative ABC transporter operons, whereby the operons are characterized by a single promoter upstream of the genes in each operon.

ORF	Putative protein designation	Up- or down-regulation	Factor difference with control
01770	ATP-binding component of an ABC transporter	Up	5X
03052	ATP-binding component of an ABC transporter	Up	5X
01072	Periplasmic solute-binding component of an ABC transporter	Up	3X
03178	ATP-binding component of an ABC transporter	Up	3X
01287 *	Permease component of an ABC transporter for iron (III)	Up	3X
01289 *	ATP-binding component of an ABC transporter for iron (III)	Up	2X
01282 *	Periplasmic solute-binding component of an ABC transporter for iron (III)	Up	2X
01284 *	Permease component of an ABC transporter for iron (III)	Up	1.7X
00218	ATP-binding component of an ABC transporter	Up	1.4X
01663	Periplasmic solute-binding component of an ABC transporter	Down	2.8X

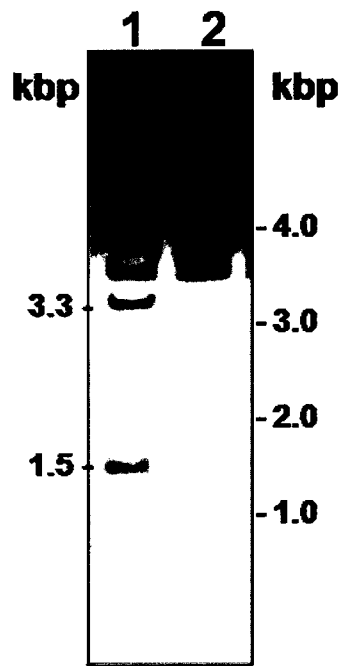
* These four ORFs are all located in the same putative ABC transporter operon.

3.2.3. Construction of *N. meningitidis* mutants

To assist in the functional characterization of these genes, individual mutants were constructed by insertional inactivation, using a chloramphenicol (CAT) cassette that contained a gonococcal DNA uptake signal sequence at the 3' end of the antibiotic cassette. The *cat* cassette was released from plasmid pCR2CmOFDU following BmgBI digestion, generating the expected 1.5 kbp fragment (Figure 5, lane 1) that was confirmed by nucleotide sequencing.

Primers were designed to flank the 5' and 3' ends of all the genes listed in Table 4 to allow for PCR amplification. However, due to difficulties in designing ideal primers and optimization of the PCR conditions, four ORFs were unable to be PCR amplified (ORF1770, ORF3052, ORF3178 and ORF0218). We then encountered further difficulties with ligation of the *cat* cassette within the amplicon for 2 of the genes (ORF1072 and ORF1663). To overcome the difficulty of blunt-end ligation, the linear pDrive constructs were dephosphorylated using shrimp alkaline phosphatase to remove the phosphate groups at the 5' ends of the DNA strands on both ends of the digested vectors in order to prevent the vectors from self-ligating. Polyethylene glycol was also added to the ligation reactions, since T4 DNA ligase has higher ligation efficiency in the presence of high concentrations of a variety of non-specific polymers. However, even with the use of these optimization steps, we were unable to ligate the *cat* cassette within ORF1072 and ORF1663. Recombinant plasmids were produced for ORFs 1282, 1284, 1287 and 1289 (Table 4). However, no meningococcal transformants were obtained for three of these ORFs (ORF1284,

Figure 5. Ethidium bromide stained agarose gel of pCR2CmOFDU plasmid with BmgBI to isolated the *cat* cassette. Lane 1: BmgBI digest of pCR2CmOFDU; Lane 2: undigested pCR2CmOFDU.



ORF1287 and ORF1289), suggesting that the resulting mutations were likely lethal to the bacteria.

3.2.4. Construction of a meningococcal mutant in ORF1282

We were successful in constructing a mutation in ORF1282. For clarity, ORF1282 will be referred to as NMB1993, corresponding to the current annotation in PubMed. *NMB1993* was PCR amplified from *N. meningitidis* chromosomal DNA using site-specific primers flanking the gene, producing an 800 bp product (Figure 6, lane 2) that was verified by nucleotide sequencing. The gel-purified product was ligated into the PCR cloning vector pDrive. A recombinant plasmid isolated from an ampicillin resistant transformant yielded the expected construct of 4.65 kbp (Figure 7, lane 1)

The *cat* cassette was ligated into the BmgB1 site of pDrive-NMB1993 resulting in a plasmid of the expected 6.55 kbp size. An XbaI/BamHI double digest resulted in the expected three DNA fragments of 3.85 kbp, 1.5 kbp and 800 bp (Figure 8, lane 1). Sequencing of the *NMB1993-cat* insert in pDrive confirmed the insertion of the *cat* cassette within the *NMB1993* gene. Following EcoRI digestion, the linearized recombinant plasmid was introduced into *N. meningitidis* B. Barden resulting in the appearance of a single chloramphenicol transformant after 5 days of incubation.

Two methods were used to verify that the proper allelic exchange had occurred. First, primers designed to anneal to the 3' and 5' ends of the *NMB1993* gene would be expected to generate a 1.4 kb larger product from the mutant than from the wild-type strain. The mutant yielded a 2.4 kb PCR product compared to a

Figure 6. Ethidium bromide stained agarose gel of the PCR result for genomic DNA isolated from *N. meningitidis* B. Barden, using site-specific primers for the NMB1993 gene. Lane 1: PCR master mix without genomic DNA template; Lane 2: Wild-type *N. meningitidis* genomic DNA with PCR master mix. M = Linear DNA molecular weight marker

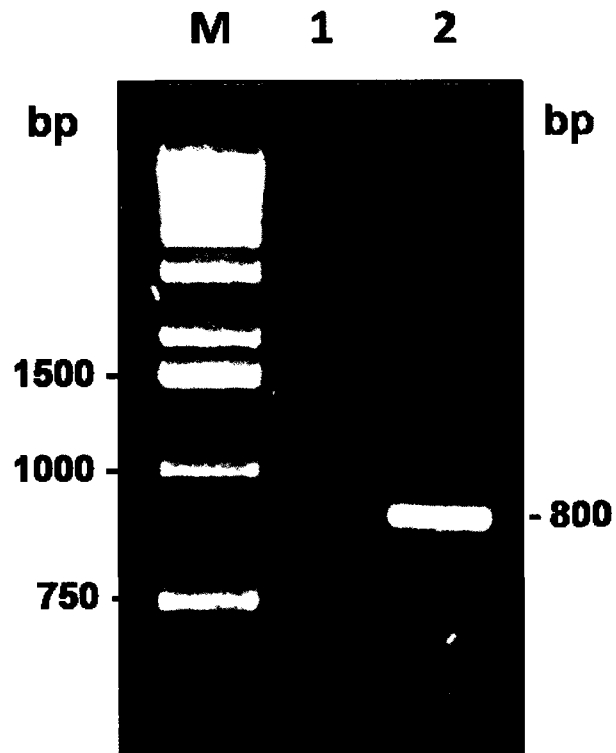


Figure 23. Western blot of whole cell lysate (WCL) of *E. coli* strain FB827 *dppA::Km mppA::Cm* pAM238-HasR complemented with pET101-NMB0586, probed with anti-V5 antibody. Lanes 1, 3, 5 and 7 represent WCL from uninduced cultures, lanes 2, 4, 6 and 8 represent WCL from IPTG-induced cultures. Lanes 1 and 2 are $t = 0$, lanes 3 and 4 are $t = 1$ hour, lanes 5 and 6 are $t = 2$ hours, and lanes 7 and 8 are $t = 3$ hours.

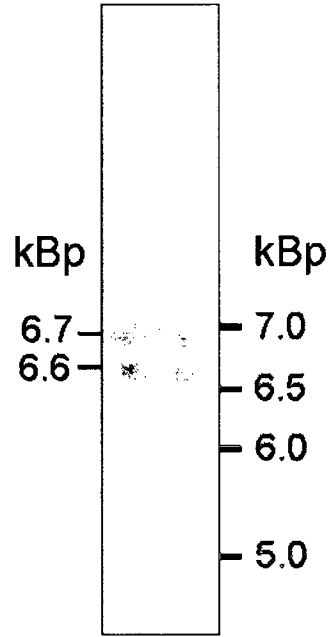


Figure 22. Ethidium bromide stained agarose gel of undigested isolated DNA (both plasmid and genomic) from *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR complemented with pET101-NMB0586.

the results of the hemin-agarose binding assays do not clearly demonstrate that the protein interacts specifically with heme.

3.3.9 Complementation Assays

To further understand the role of NMB0586 in heme binding, we performed functional complementation experiments. NMB0586 was expressed in an *E. coli* K12 mutant lacking the ability to use heme as a sole iron source. Wild-type *E. coli* K-12 lacks a heme outer membrane receptor and is unable to use exogenously added heme as an iron source. However, expression of a foreign heme outer membrane receptor (HasR from *Serratia marcescens*) in *E. coli* K-12 allows the organism to use heme as an iron source. Based on this finding, Letoffe et al. (2006 and 2008) identified a dipeptide ABC transporter (DppBCDF) with two periplasmic components (MppA and DppA) involved in heme metabolism. A double mutant with insertional inactivation in both MppA and DppA (*E. coli* strain FB287 *dppA::Km mppA::Cm* pAM238 HasR) resulted in a heme deficient phenotype.

To determine the ability of NMB0586 to restore the wild-type heme phenotype of a heme deficient mutant of *E. coli*, *E. coli* strain FB287 *dppA::Km mppA::Cm* pAM238 HasR was transformed with pET101-NMB0586. DNA isolation from the transformed *E. coli* strain resulted in three bands, representing the genomic DNA, the plasmid pAM238 HasR (6.7 kbp) and pET101-NMB0586 (6.6 kbp) (Figure 22). Western blot analysis of IPTG-induced cultures of *E. coli* FB287 *dppA::Km mppA::Cm* pAM238 HasR pET101-NMB0586 confirmed the expression of NMB0586, with increasing expression of the protein over four hours (Figure 23). Western blot analysis performed on subcellular fractions of the transformed *E. coli*

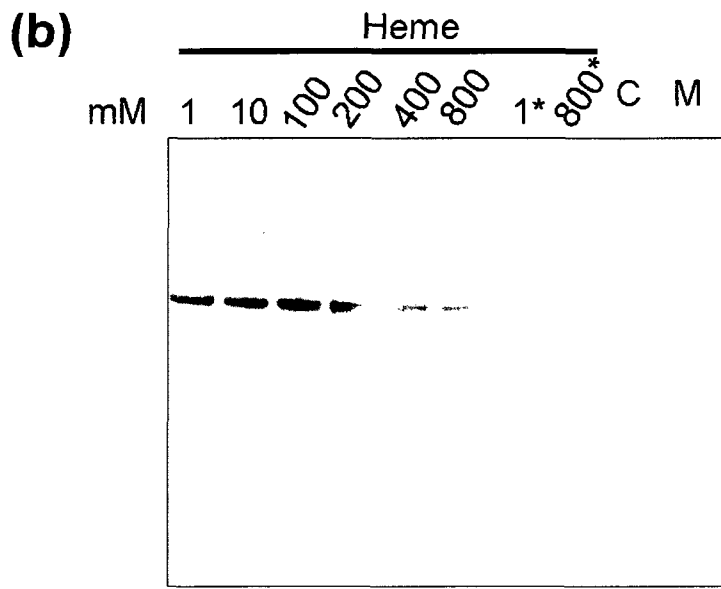
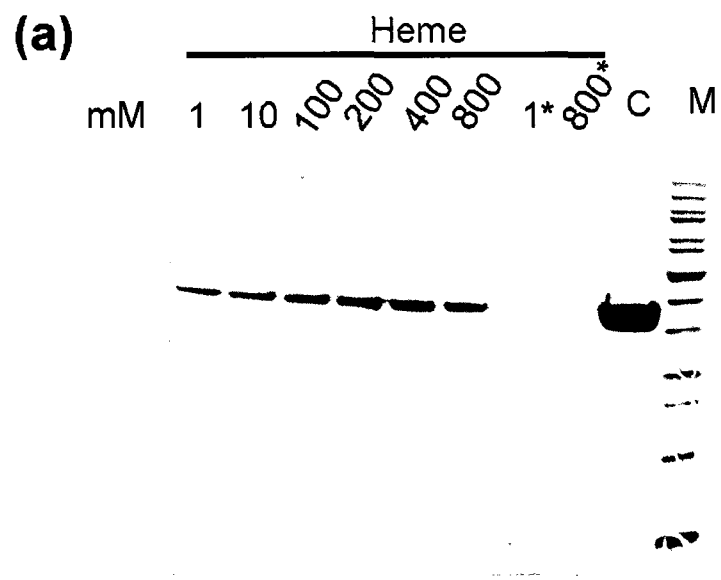


Figure 21. SDS-PAGE (a) and corresponding Western Blot (b) of haemin competitive binding of NMB0586 affinity purified using haemin agarose. Increasing amounts (1-800 mM) of heme was incubated with 10 μ g of NMB0586, and NMB0586 was subsequently eluted. 1 and 800 mM of heme (1* and 800*) was incubated with haemin-agarose in the absence of protein as negative controls. 10 μ g of pure NMB0586 protein (C) was added as a control for comparative band intensity. An anti-V5 epitope antibody was used for the Western Blot analysis. M = molecular weight marker.

from hemin-agarose (lane 1), indicating that the binding of NMB0586 for heme is quite low. The 10 μg NMB0586 protein sample represents a protein concentration of 5 μM that was incubated with hemin-agarose at a hemin concentration of 800 μM . Assuming a binding stoichiometry of 1:1 for NMB0586 binding to hemin, these results do not demonstrate saturation of NMB0586 to the hemin-agarose affinity column, and further confirm only a weak binding affinity of NMB0586 for heme.

A 65 kDa band specific for Glb-1 was present when 10 μg of Glb-1 was incubated with hemin-agarose (Figure 20a). The presence of the histidine tag fused to Glb-1 may be responsible for this binding, as discussed previously.

3.3.8. Heme competitive binding assay

To confirm that the binding of the protein was specific for heme, competitive binding assays were performed using heme as a competitive ligand to the hemin-agarose. Increasing concentrations of competing heme ligand did not result in a decrease in the amount of NMB0586 eluted from the hemin agarose as would have been expected (Figure 21). In fact, it appears that the amount of NMB0586 slightly increases as the competing heme concentration increases. This may be due to heme aggregation resulting from the hydrophobic nature of heme. Heme aggregation could potentially decrease the effective concentration of free heme available to bind to NMB0586, such that at higher free heme concentrations there would be less competitive ligand and more NMB0586 would be bound to the hemin-agarose. However, this seems unlikely since the concentration of free heme used in this study was 800 μM , and thus well in excess of the concentration of protein (5 μM). Overall,

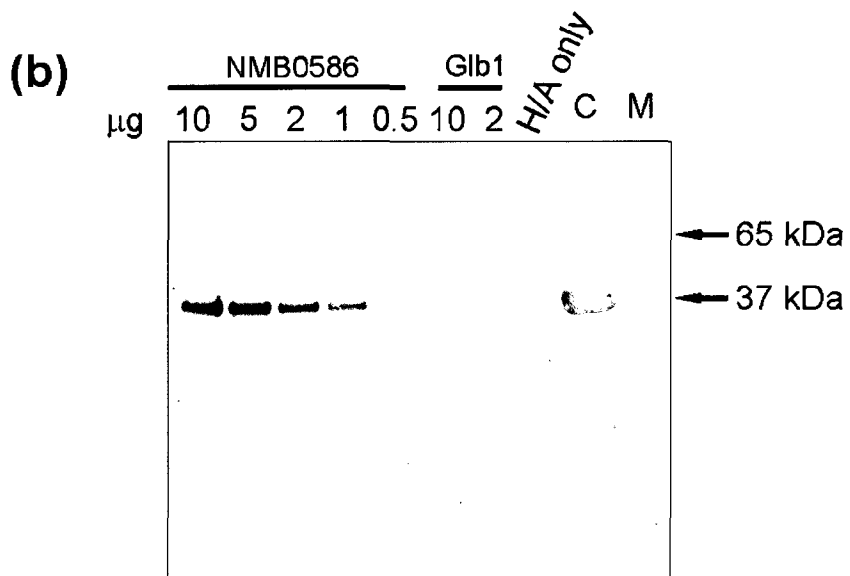
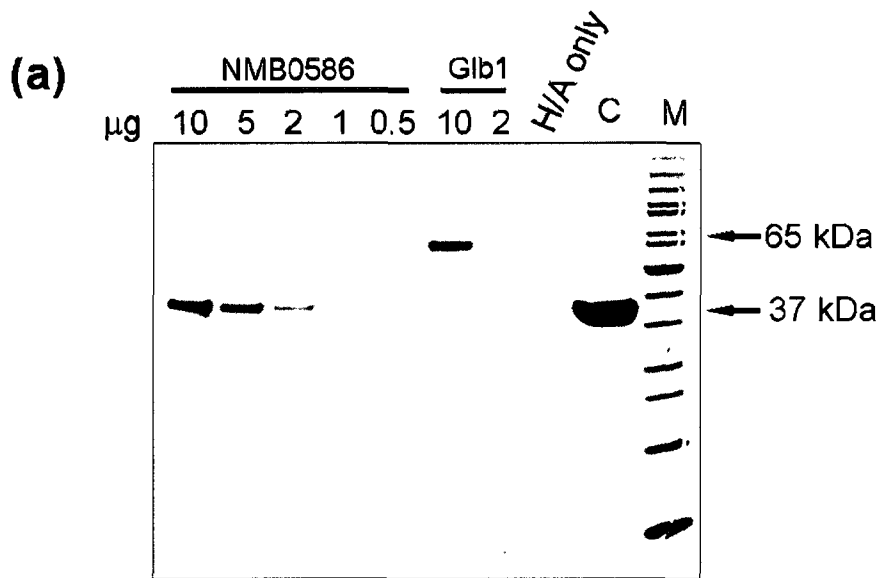


Figure 20. SDS-PAGE (a) and corresponding Western Blot (b) of haemin specificity binding of NMB0586 affinity purified using haemin agarose. Increasing amounts (0.5 μg to 10 μg) of NMB0586 were affinity purified. Glb1 (2 and 10 μg), and heamin agarose alone (H/A only) were used as negative controls. 10 μg of pure NMB0586 protein (C) was added as a control for comparative band intensity. An anti-V5 epitope antibody was used for the Western Blot analysis. M = molecular weight marker.

concentration of 10^{-5} M, a faint band was seen when the protein was incubated with heme at a concentration of 10^{-4} M. A possible explanation for this result is the presence of the polyhistidyl tag used to purify Glb-1. As histidine residues are involved in the binding of heme to the polypeptide chain in heme-protein interactions (Goldman et al., 1998, Brakcen et al., 1999, Sook et al., 2008), Glb-1 may be binding to heme via the N-terminal histidine tag. NMB0586 is also fused to a histidine tag, but since the band intensity of NMB0586 bound to 10^{-4} M heme is so much stronger than that for Glb1 it is unlikely that it is the presence of the histidine tag that is solely responsible for the binding to heme. These results suggest that NMB0586 binds heme only at relatively high concentrations and has a low affinity for heme. To further confirm this finding, a second method was used to determine the heme binding property of NMB0586.

3.3.7. Binding of NMB0586-his to hemin-agarose

The second method to test binding of NMB0586 to heme was using direct heme agarose binding. The binding of NMB0586 to heme demonstrated concentration dependent binding, a characteristic that is generally accepted as part of the functional definition of a receptor-ligand interaction. In Figure 20, as the concentration of NMB0586 is increased, the amount of protein bound to the hemin-agarose also increased. However, the amount of NMB0586 bound to the hemin-agarose is relatively low compared to the amount of total protein (10 μ g) that was initially incubated with the hemin-agarose. This can be seen by the strong band intensity of the control lane (C) containing 10 μ g of NMB0586 added directly to the SDS-PAGE gel in comparison to the weaker band observed for NMB0586 eluted

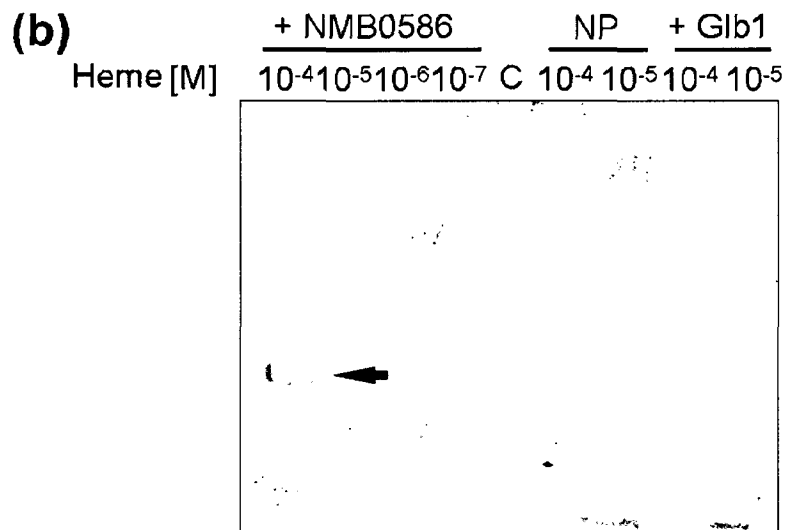
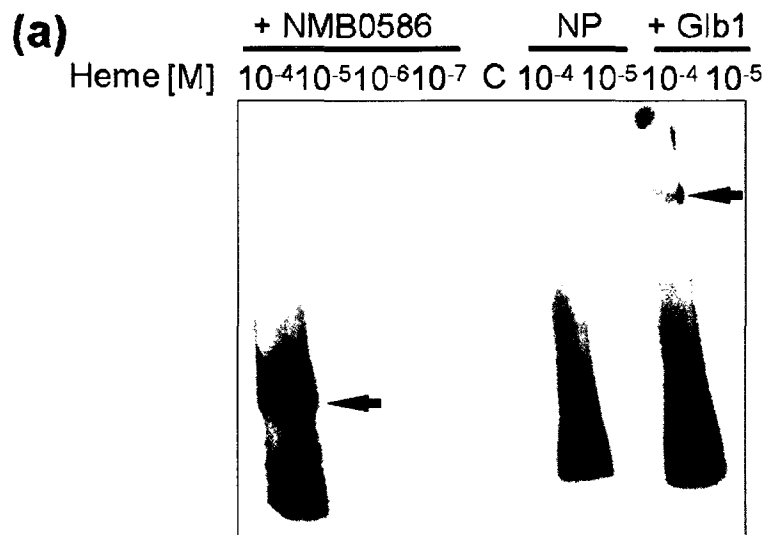


Figure 19. Enhanced chemiluminescence detection (a) after nondenaturing PAGE and transfer onto a nitrocellulose membrane, and corresponding Western Blot analysis (b). The molar concentrations of heme were 10^{-7} to 10^{-4} M. The final concentration of NMB0586 and Glb1 was 4×10^{-5} M. A heme sample without protein (NP), as well as Glb1 incubated with heme, were used as negative controls. 4×10^{-5} M of NMB0586 not incubated with heme but prepared the same (C) was also added as a control. An anti-V5 epitope antibody was used for the Western Blot analysis.

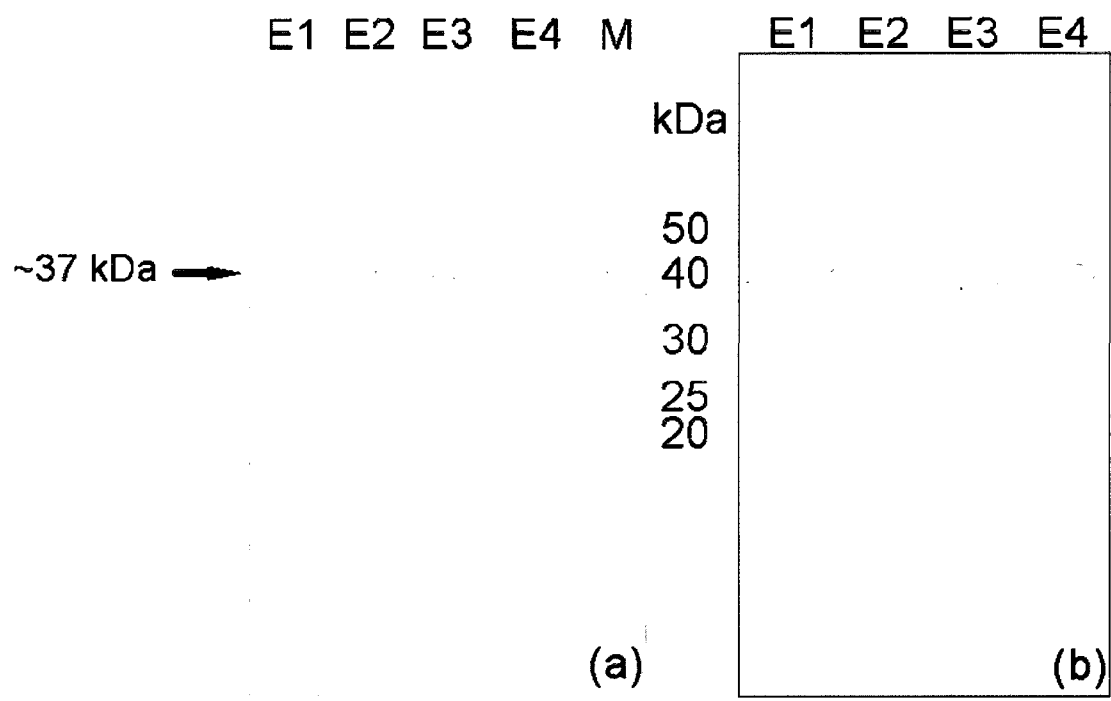


Figure 18. The SDS-PAGE and Western Blot analysis of purified NMB0586-His protein eluted using Ni-NTA purification. Lanes E1-E4 represent elution steps 1-4, using increasing concentrations of imidazole (50mM, 100 mM, 200 mM and 250 mM). M = molecular weight marker. An anti-V5 epitope antibody was used for the Western Blot analysis. (a) represents the SDS-PAGE; (b) represents the corresponding Western Blot.

blot analysis following imidazole elution (Figure 18). The lowest three imidazole concentrations used for elution (50mM, 100 mM and 200 mM) were contaminated with other proteins (Lanes E1-E3 in Figure 18). These contaminating proteins likely represented polypeptides containing stretches of histidine amino acids that were loosely bound to the column, or residual proteins that were bound non-specifically to the column before elution with imidazole. The highest imidazole concentration used (250 mM) resulted in a pure sample of NMB0586-his, as detected by a single 37 kDa band in both the SDS-PAGE and Western blot. The identity of the protein was confirmed by N-terminal sequencing.

3.3.6. Functional characterization of the NMB0586 protein by enhanced chemiluminescence

The first method to detect whether the NMB0586 protein bound heme used enhanced chemiluminescence. This staining procedure capitalizes on the intrinsic peroxidase activity of heme which allows the identification of hemoproteins. NMB0586-his bound to heme at a concentration of 10^{-4} M, but no binding was observed at lower heme concentrations (Figure 19). To confirm that the detected spot was in fact the NMB0586 protein, the corresponding Western Blot was performed and the anti-V5 epitope antibody specifically bound to the NMB0586 when it was bound to heme at a concentration of 10^{-4} M. The smear seen at the bottom of the gel represents aggregated heme detected by the peroxidase substrate.

The negative control protein used for the enhanced chemiluminescence assay was Glb-1, a wheat storage globulin protein that does not bind heme, or heme-containing compounds. However, although Glb-1 did not bind to heme at a

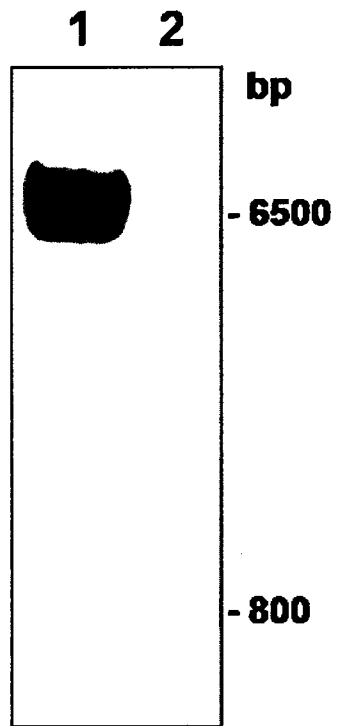


Figure 17. Confirmation of cloning NMB0586 into pET101. Lane 1: pET101-NMB0586; Lane 2: PCR results for plasmid pET101-NMB0586, using site specific primers for NMB0586 (0586-right and 0586-left).

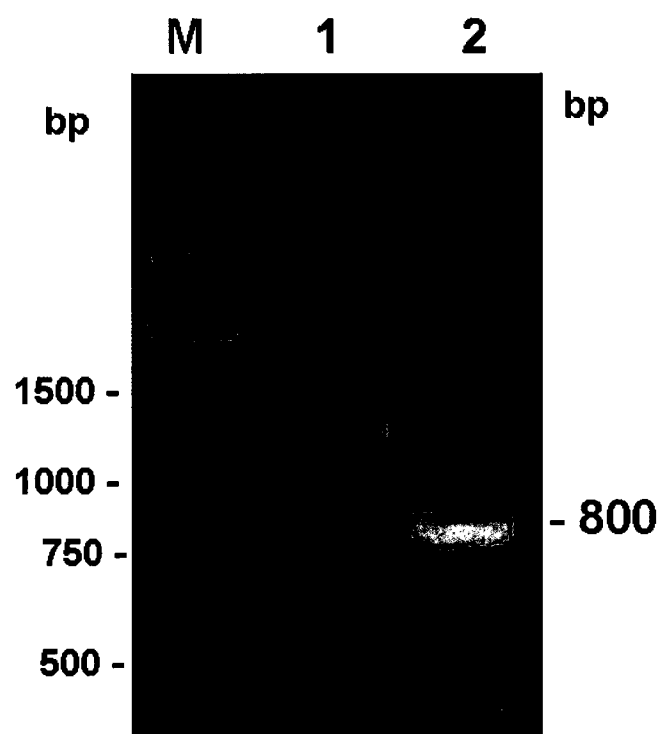


Figure 16. Ethidium bromide stained agarose gel of the PCR result for genomic DNA isolated from *N. meningitidis* B. Barden, using site-specific primers for the NMB0586 gene. Lane 1: PCR master mix without genomic DNA template; Lane 2: Wild-type *N. meningitidis* genomic DNA with PCR master mix. M = Linear DNA molecular weight marker

3.3.4 Cloning *N. meningitidis* NMB0586 into the pET101/D-Topo Vector

We PCR amplified the *NMB0586* gene using NMB0586 site-specific primers annealing to the 3' and 5' ends of the gene and confirmed the purity of the PCR product by agarose gel electrophoresis (Figure 16, lane 1). The resulting 800 bp band was excised from the agarose gel, purified, and cloned it into the pET101/D-TOPO vector in *E. coli* BL21 StarTM. Plasmid was recovered from an ampicillin-resistant *E. coli* transformant was analysed by agarose gel electrophoresis. To confirm transformation, we performed plasmid isolation on an *E. coli* clone that grew on agar plates containing ampicillin and ran the resulting plasmid on an agarose gel. A single band was observed at 6.5 kbp (Figure 17, lane 1), the size expected for pET101-NMB0586 since pET101 is 5735 bp and NMB0586 is 800 bp. To further confirm that *NMB0586* had inserted into the multiple cloning site of pET101, PCR was performed on the isolated plasmid using *NMB0586* site-specific primers. A single band of 800 bp was observed on the agarose gel (Figure 17, lane 2). Sequencing of this 800 bp PCR product confirmed the presence of the NMB0586 gene into pET101.

3.3.5. Purification of the protein encoded by NMB0586

Expression of NMB0586-his was induced by addition of IPTG to the growth media at mid-log phase. Following cell lysis, the protein was purified using the Ni-NTA purification system, wherein the polyhistidine tag attached to the NMB0586 protein binds to the nickel column. The nickel bound proteins was eluted from the column using increasing concentrations of imidazole and the fractions were collected. The purity of the samples were confirmed by SDS-PAGE and Western

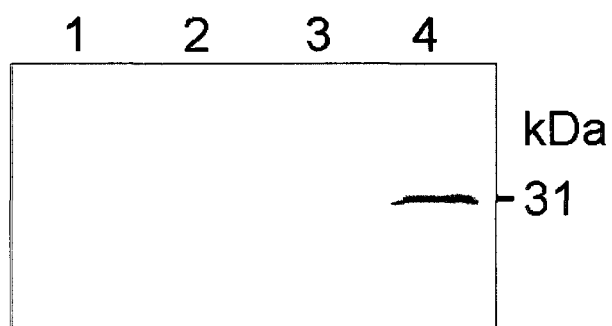


Figure 15. Western Blot analysis using polyclonal anti-hHbp antibody. All bacteria were grown using heme as a sole iron source in the media. Lane 1: *N. meningitidis* whole cell lysate, 10 μ M heme; Lane 2: *N. meningitidis* periplasmic extract, 10 μ M heme; Lane 3: *N. meningitidis* periplasmic extract, 50 μ M heme; *H. ducreyi* whole cell lysate, 10 μ M heme.

conclusively exclude the involvement of this operon in heme transport since ABC transporters that can accommodate two periplasmic-binding components have been shown in other bacteria, such as *E. coli* and *Salmonella typhimurium* (Letoffe et al., 2006; Higgins and Ames, 1981). This provides a potential explanation as to why the *NMB0586* mutant was still able to use heme as a sole iron source. As a result, functional characterization of the protein encoded by *NMB0586* was explored.

3.3.3. Isolation and purification of the protein encoded by *NMB0586*

To further study the putative role of the NMB0588-NMB0586 operon in heme uptake, experiments to determine the heme-binding properties of the protein encoded by *NMB0586* were performed. To facilitate purification of the protein, meningococcal whole cell lysates were probed with hHbp polyclonal antibody in order to identify a meningococcal homologue to hHbp. In Western blot analysis, no immunoreactive bands were detected when meningococcal whole cell lysates were reacted with hHbp antibody raised against the *H. ducreyi* protein (lanes 1-3, Figure 15). The antibody recognized the 31 kDa hHbp in the corresponding *H. ducreyi* whole cell lysate (lane 4, Figure 15). The hHbp epitopes recognized by the polyclonal hHbp antibody are thus not present in NMB0586. This is not surprising since the amino acid identity is only 27% between the two proteins, and the proteins are from different bacterial genera. This result does not exclude the possibility that both proteins have a similar biological function. Therefore, the NMB0586 protein was isolated by metal affinity-purification.

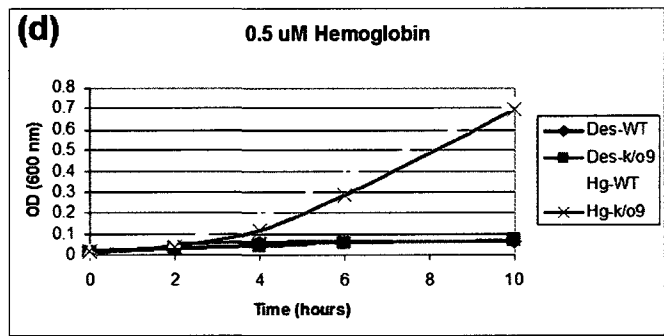
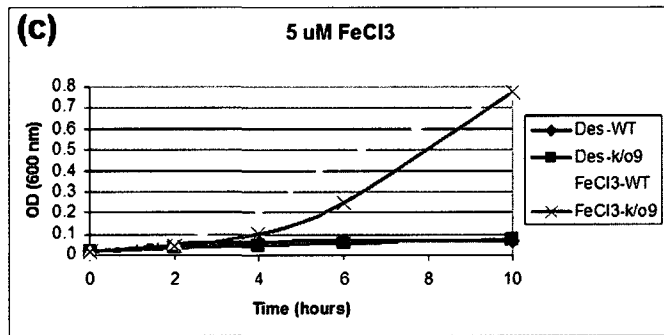
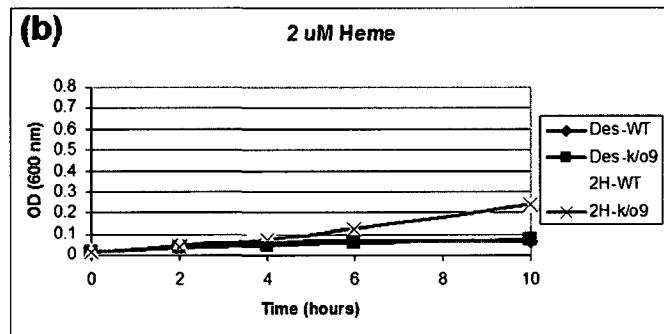
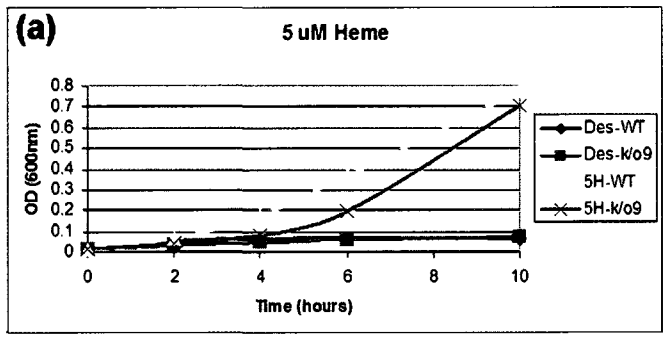


Figure 14. Growth of the wild-type (WT) and NMB0586 mutant strain of *N. meningitidis* B. Barden (k/o9) in iron-limited Mueller-Hinton broth (Des) supplemented with various iron compounds. The parental isolated B. Barden and the NMB0586 mutant strain were grown in the presence of 5 μ M heme (5H) (a), 2 μ M heme (2H) (b), 5 μ M FeCl₃ (FeCl₃) (c), and 0.5 μ M hemoglobin (Hg) (d). Both the mutant and parental strain were also grown in iron-limited Mueller-Hinton broth (Des) as baseline controls. These are representative growth curves (n=3 for each growth condition).

product from the *NMB0586* mutant strain (data not shown). No such PCR product was obtained when wild-type chromosomal DNA was used as a template.

Finally, sequencing of the PCR product generated by using primers annealing to the 3' and 5' ends of the *NMB0586* gene confirmed the presence of the *cat* cassette within the mutated *NMB0586* gene. These results indicate that the *NMB0586-cat* construct had recombined appropriately within the chromosome of *N. meningitidis* strain B. Barden. Furthermore, a single insertion of the *cat* cassette into *NMB0586* had occurred, and no gross rearrangements or deletions had occurred.

3.3.2. Growth assay of the mutant and parental strain

Growth assays were conducted to determine the ability of the *N. meningitidis* *NMB0586* mutant to use various iron-containing compounds as the sole exogenous source of iron, as described in detail above for the *NMB1993* mutant. Samples were taken at regular intervals for 10 hours (Figure 14), and stationary phase was attained in all cultures at approximately 10 hours.

The ability of the *NMB0586* mutant to assimilate iron from heme (Figure 14a) and hemoglobin (Figure 14d) was unimpaired when compared to that of the wild-type strain B. Barden. In addition, there were no differences in the ability of heme, hemoglobin or FeCl_3 to serve as a source of iron for the mutant. These results indicate that the inactivation of *NMB0586* does not affect the growth of *N. meningitidis* B. Barden when heme, haemoglobin or FeCl_3 is provided as the iron source.

From the results of these growth studies, it appears that *NMB0586* is not involved in heme uptake in *N. meningitidis* B. Barden. However, this result does not

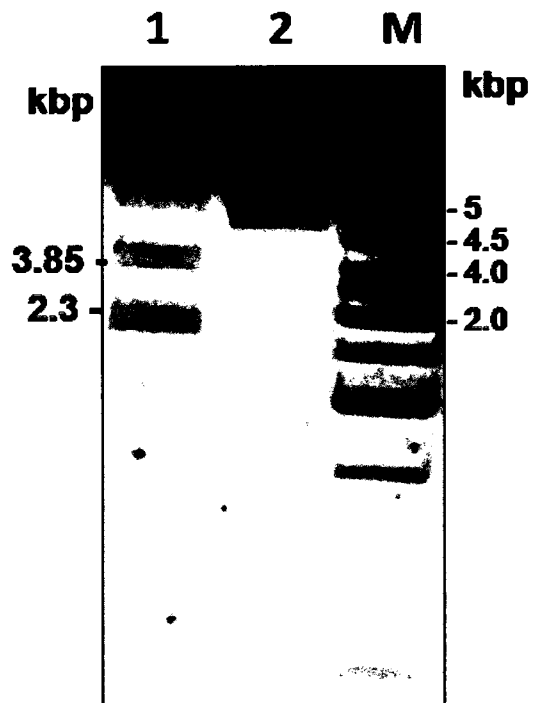


Figure 13. Ethidium bromide stained agarose gel of XbaI/BamHI double digested pDrive-NMB0586-CAT. Lane 1: XbaI/BamHI digested pDrive-NMB0586-CAT; Lane 2: Undigested pDrive NMB0586-CAT. M= Linear DNA molecular weight marker.

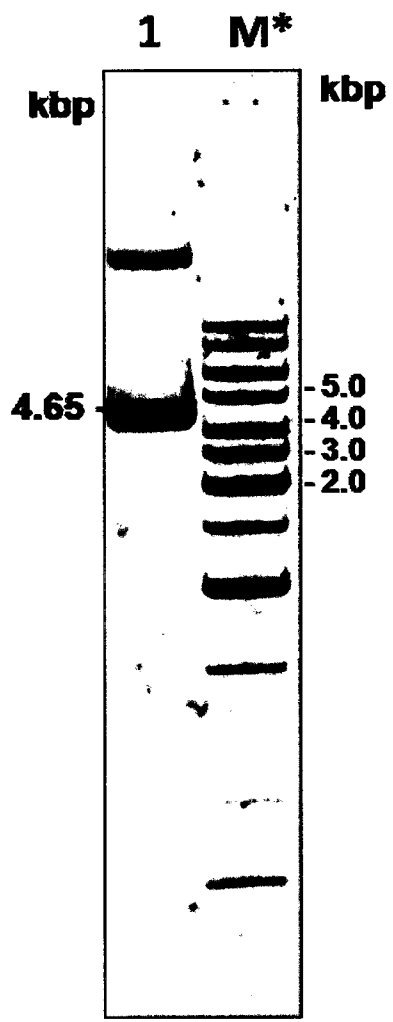


Figure 12. Ethidium bromide stained agarose gel of isolated plasmid from *E. coli* Top10 cells transformed with pDrive-NMB0586. Lane 1: plasmid pDrive-NMB0586. M* = Supercoiled DNA molecular weight marker

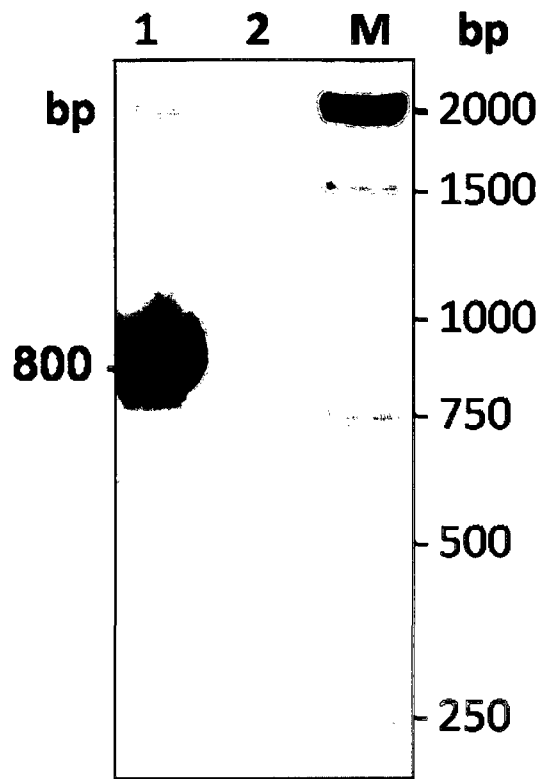


Figure 11. Ethidium bromide stained agarose gel of the PCR result for genomic DNA isolated from *N. meningitidis* B. Barden, using site-specific primers for the NMB0586 gene. Lane 1: Wild-type *N. meningitidis* genomic DNA with PCR master mix; Lane 2: PCR master mix without genomic DNA template. M = Linear DNA molecular weight marker

3.3.1. Construction of a meningococcal NMB0586 mutant

A *NMB0586* mutant was constructed by insertional inactivation using the aforementioned *cat* cassette. The 800 bp gene was PCR amplified from chromosomal DNA from strain Barden using primers flanking the 3' and 5' ends of the gene (Figure 11, lane 1). The identity of the amplicon was verified by DNA sequencing. The gel-purified product was ligated into the PCR cloning vector pDrive, and a plasmid isolated from an ampicillin resistant *E. coli* transformant was subjected to agarose gel electrophoresis. A single band of 4.65 kbp (Figure 12, lane 1) conforming to the expected size of the plasmid construct was seen.

The *cat* cassette was ligated into the BmgB1 site of pDrive-NMB0586 resulting in a plasmid of the expected 6.55 kbp size. An XbaI/BamHI double digest resulted in the expected two DNA fragments of 3.85 kbp and 2.3 kbp (Figure 13, lane 1). Sequencing of the *NMB0586-cat* insert in pDrive confirmed the insertion of the *cat* cassette within the *NMB0586* gene. Following EcoRI digestion, the linearized recombinant plasmid was introduced into *N. meningitidis* B. Barden resulting in the appearance of a single chloramphenicol transformant after 5 days of incubation.

Two methods were used to verify that the proper allelic exchange had occurred. First, primers designed to anneal to the 3' and 5' ends of the *NMB0586* gene would be expected to generate a 1.4 kbp larger product from the mutant than from the wild-type strain. The mutant yielded a 2.4 kbp PCR product compared to an 800 bp fragments from the wild-type isolate (lanes 5 and 3, respectively, in Figure 9). Oligonucleotides bracketing the *cat* cassette generated the expected 1.4 kbp

meningitidis B. Barden when heme, haemoglobin or FeCl₃ is provided as the iron source.

3.3. Identification of a meningococcal homologue to a *H. ducreyi* periplasmic heme-binding protein

During the course of this study, a heme dedicated periplasmic protein in *Haemophilus ducreyi*, designated hHbp, was uncovered in our laboratory by another graduate student. We performed a sequence alignment search to identify a meningococcal homologue. No sequences with nucleic acid homology to *hhbp* were found. However, a gene encoding a protein displaying a 27% amino acid identity and 47% amino acid similarity to the *H. ducreyi* protein was detected. This ORF encoded a periplasmic component (NMB0586) of an uncharacterized ABC transporter that was present in three strains of *N. meningitidis* (MC58, FAM18 and Z2491). Furthermore, *NMB0586* was found to be up-regulated 1.7 fold under iron-restricted conditions in *N. meningitidis* serogroup C in a microarray study and present only in gels in which *N. meningitidis* serogroup A was grown in iron-limiting media (Basler et al., 2006). A re-examination of our prior microarray analysis revealed that *NMB0586* was up-regulated 8.6 fold when *N. meningitidis* serogroup B was grown under iron restrictive conditions in which heme was supplied as the sole iron source. This gene was ignored in the prior search as it was assigned a function as a putative adhesin and displayed no similarity to an ABC transporter involved in heme acquisition. Therefore, based on this evidence, the *NMB0588-NMB0586* operon was further analyzed for its involvement in heme uptake in *N. meningitidis*.

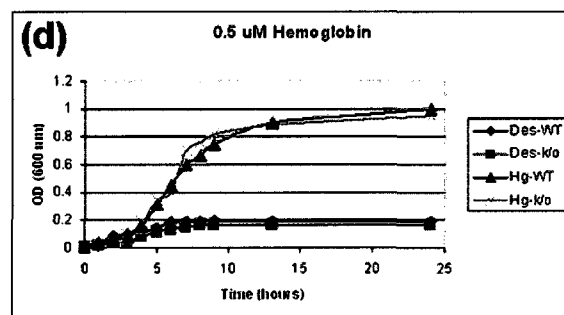
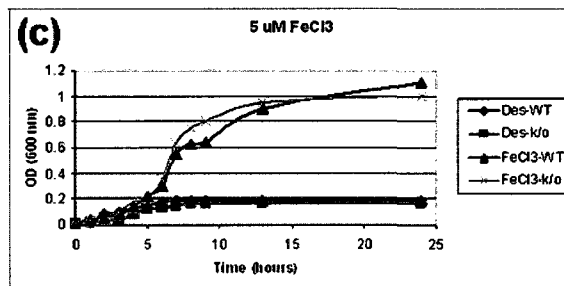
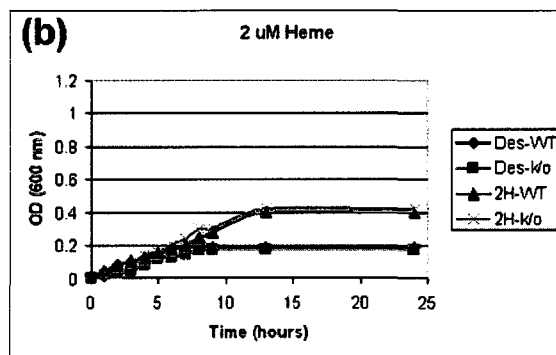
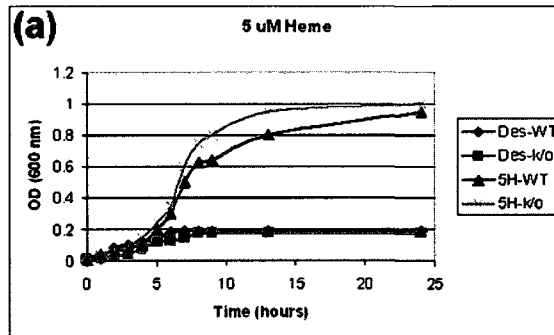


Figure 10. Growth of the wild-type (WT) and NMB1993 mutant strain of *N. meningitidis* B. Barden in iron-limited Mueller-Hinton broth (Des) supplemented with various iron compounds. The parental isolated B. Barden and the NMB1993 mutant strain were grown in the presence of 5 μ M heme (5H) (a), 2 μ M heme (2H) (b), 5 μ M FeCl₃ (FeCl₃) (c), and 0.5 μ M hemoglobin (Hg) (d). Both the mutant and parental strain were also grown in iron-limited Mueller-Hinton broth (Des) as baseline controls. These are representative growth curves (n=3 for each growth condition).

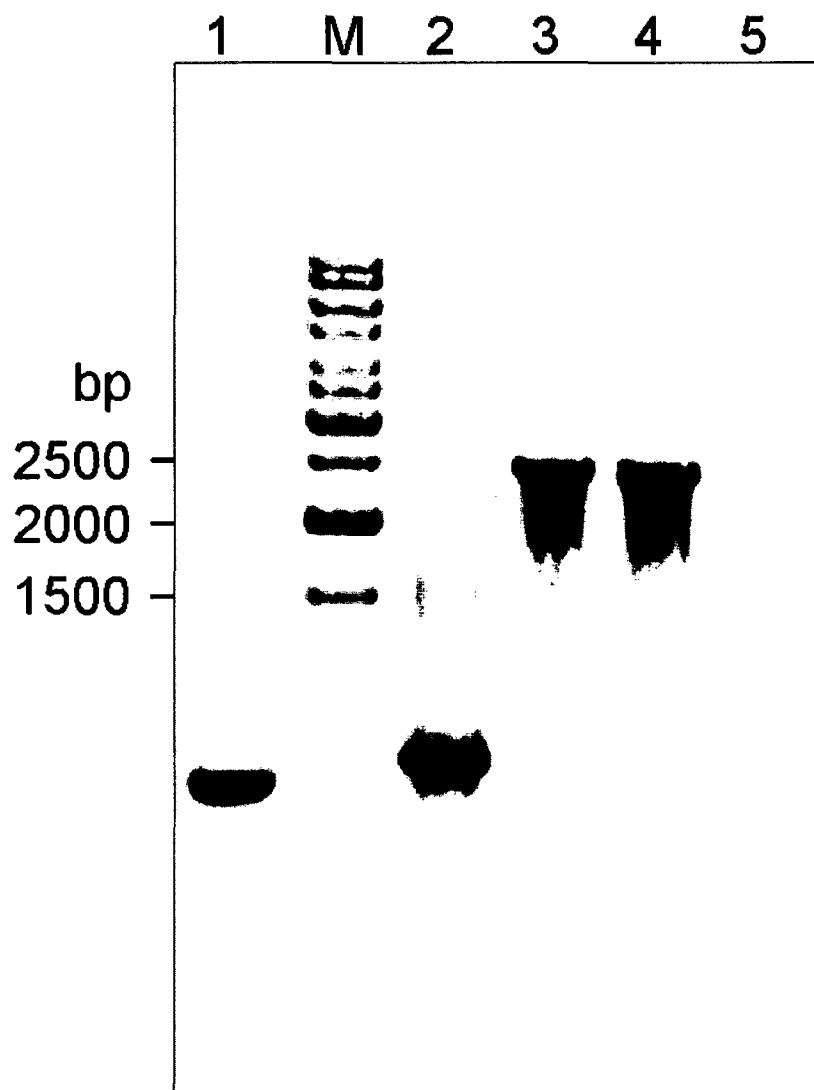


Figure 9. Ethidium bromide stained agarose gel of PCR results for genomic DNA isolated from *N. meningitidis* B. Barden mutants, using specific primers for the *NMB0586* and *NMB1993* genes. Lane 1: Wild-type *N. meningitidis* using NMB1993 primers; Lane 2: molecular weight marker; Lane 3: Wild-type *N. meningitidis* using NMB0586 primers; Lane 4: NMB0586 mutant using NMB0586 primers; Lane 5: NMB1993 mutant using NMB1993 primers; Lane 6: PCR reaction mixture with no DNA template, containing NMB0586 primers.

800 bp fragments from the wild-type isolate (lanes 5 and 1, respectively, in Figure 9). Oligonucleotides bracketing the *cat* cassette generated the expected 1.4 kb product from the *NMB1993* mutant strain (data not shown). No such PCR product was obtained when wild-type chromosomal DNA was used as a template.

Finally, sequencing of the PCR product generated by using primers annealing to the 3' and 5' ends of the *NMB1993* gene confirmed the presence of the *cat* cassette within the genomic *NMB1993* gene. These results indicate that the *NMB1993-cat* construct had recombined appropriately within the chromosome of *N. meningitidis* strain B. Barden. Furthermore, a single insertion of the *cat* cassette into *NMB1993* had occurred, with no gross rearrangements or deletions.

3.2.5. Growth curve of the *N. meningitidis* mutant

Growth assays were conducted to determine the ability of the *N. meningitidis* *NMB1993* mutant to use various iron-containing compounds as the sole exogenous source of iron (Figure 10). The 5 μ M FeCl₃ (Figure 10) represents the concentration of the iron compound in excess of the concentration of the chelator desferroxamine. Samples were taken at regular intervals for 24 hours, and stationary phase was attained in all cultures at approximately 10 hours.

The ability of the *NMB1993* mutant to assimilate iron from heme (Figure 10a) and hemoglobin (Figure 10d) was unimpaired when compared to that of the wild-type strain B. Barden. In addition, there were no differences in the ability of heme, hemoglobin or FeCl₃ to serve as a source of iron for the mutant. These results indicate that the inactivation of *NMB1993* does not affect the growth of *N.*

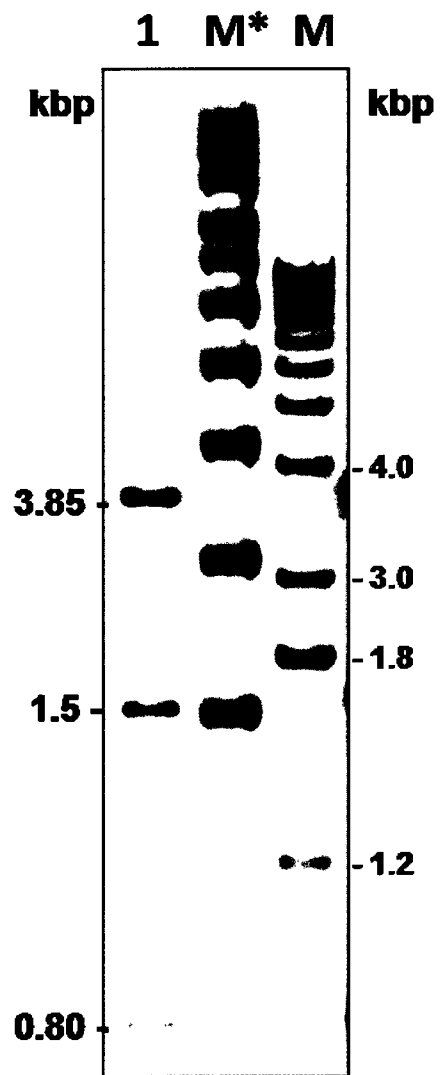


Figure 8. Ethidium bromide stained agarose gel of XbaI/BamHI double digested pDrive-NMB1993-CAT. Lane 1: XbaI/BamHI digested pDrive-NMB1993-CAT. M* = Supercoiled DNA molecular weight marker. M= Linear DNA molecular weight marker.

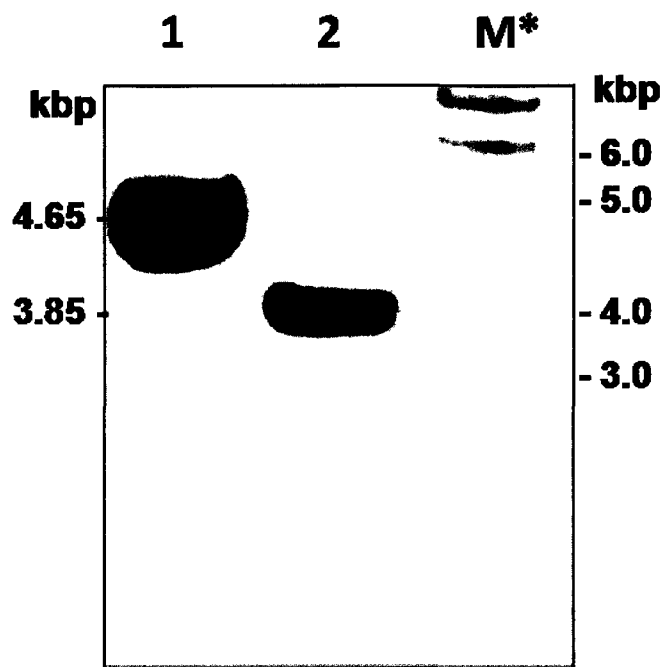
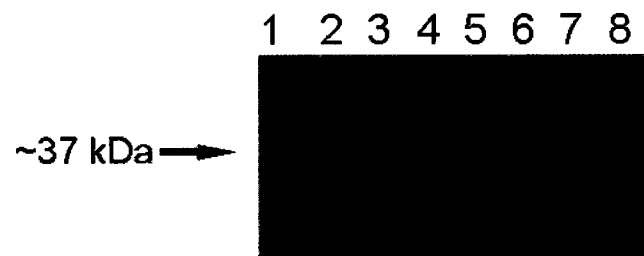


Figure 7. Ethidium bromide stained agarose gel of isolated plasmid from *E. coli* Top10 cells transformed with pDrive-NMB1993. Lane 1: pDrive-NMB1993; Lane 2: pDrive (without insert). M* = Supercoiled DNA molecular weight marker



strain confirmed the expression of NMB0586 in the periplasm of the bacteria (data not shown).

To determine if the presence of the NMB0586 would restore the heme phenotype, the transformed strain was grown on agar with varying concentrations of iron sources. The pET101-NMB0586 transformed *E. coli* was unable to grow on agar containing various concentrations of heme or hemoglobin, but was able to grow on agar with FeSO₄ as an iron source (Table 5). These results are the same as were observed for *E. coli* strain FB287 *dppA::Km mppA::Cm* pAM238 HasR transformed with the backbone plasmid pET101 as a negative control, indicating that the presence of NMB0586 is unable to restore the heme phenotype of the heme deficient *E. coli* strain. The positive control, *E. coli* strain FB287 *dppA::Km mppA::Cm* pAM238 HasR complemented with pTRCC-MppA, demonstrated a restored ability to grow on media containing either heme or hemoglobin as sole iron sources, with concentration-dependent growth. None of the recombinant strains were able to grow on media with no iron source.

Table 5. Growth results for *E. coli* (strain FB827 *dppA::Km mppA::Cm* pAM238-HasR) complimented with pET101-NMB0586 grown on M63 agar (FeSO₄), M63* agar (No iron source), or M63* agar supplemented with heme sources at various concentrations, to determine the ability of NMB0586 to restore the heme phenotype of the *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR strain. *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR complimented with pTRCC-MppA was used as a positive control. *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR complimented with pET101, as well as *E. coli* FB827 *dppA::Km mppA::Cm* pAM238-HasR, were used negative controls.

Iron source	E. coli + pTRCC-MppA	E. coli	E. coli + pET101NM B0586	E. Coli + pET101
No iron source	-	-	-	-
FeSO ₄	++	++	++	++
5 μM Hg	+/-	-	-	-
10 μM Hg	+/-	-	-	-
40 μM Hg	++	-	-	-
50 μM Hg	+	-	-	-
4 μM heme	+/-	-	-	-
10 μM heme	+/-	-	-	-
40 μM heme	+	-	-	-
80 μM heme	+	-	-	-

++ represents maximum growth, + represents mediocre growth levels, +/- represents minimal growth, and - represents no growth, observed on the agar after 48 hours at 37°C.

CHAPTER 4: DISCUSSION

Success, in terms of bacterial survival inside the host, depends on an organism's ability to scavenge essential nutrients. Acquisition of iron and iron complexes has long been recognized as a major determinant in the pathogenesis of *N. meningitidis* and *N. gonorrhoeae*. The ability to acquire iron under iron-restricted conditions has undoubtedly contributed to the success of these pathogenic *Neisseria* in causing human disease. Understanding the mechanisms by which pathogenic *Neisseriae* acquire iron is of utmost importance to eradicating diseases caused by these bacteria, and is the driving force behind this study.

4.1. *Neisseria* and heme

Since Mickelsen and Sparling (1981) first demonstrated the ability of *N. meningitidis*, *N. gonorrhoeae* and commensal *Neisseria* species to use heme as a sole iron source, researchers have been trying to elucidate the mechanisms by which this occurs. The overall hypothesis that directed the course of this study was that pathogenic *Neisseriae* have an ABC transporter dedicated to shuttle heme across the periplasmic membrane of the bacteria. The first proposal of such a system existing in *Neisseria* for heme transport was suggested by Stoljikovic et al. (1995), and since then several groups of researchers have been trying to find a heme ABC transporter in *Neisseria*. Even as recently as 2008, Cornelissen reviewed the likelihood of a dedicated ABC transporter for heme in *Neisseria*.

The sequencing of the complete genomes of three *N. meningitidis* and one *N. gonorrhoeae* serogroups was thought to provide further insight to identify such a transporter. However, based on sequence analysis alone, this is a difficult task.

Periplasmic heme binding proteins have minimal or no sequence homology between bacterial genera, therefore BLAST analysis of known heme ABC transporters against the genome sequences of pathogenic *Neisseriae* have proven to be unsuccessful. The *N. meningitidis* serogroup B genome encodes 24 putative ABC transporters, 12 of which have been shown to be experimentally devoid of heme-specificity binding properties (Tettelin et al. 2000). However, we suspect that one of the other 12 uncharacterized ABC transporters is likely involved in heme uptake across the inner membrane in *N. meningitidis*. In order to address this hypothesis, we used several parallel molecular approaches that have been successful in identifying heme ABC transporters in other bacteria.

4.2. 2-D Gel Electrophoresis

Comparative 2DGE is a technique that has been widely used to identify bacterial proteins whose expression is substrate regulated. For example, Friedman and colleagues (2006) used comparative 2DGE to identify a novel heme-regulated transport system (HrtAB) in *Staphylococcus aureus*, which plays a critical role in staphylococcal heme metabolism. A former graduate student in our laboratory, Melissa St. Denis (manuscript submitted) also used this technique to successfully identify a heme-binding periplasmic protein in *H. ducreyi* (hHbp). Specifically to pathogenic *Neisseriae*, Basler et al. (2006) used 2DGE to study the iron-regulated proteome of *N. meningitidis* serogroup C and identified 41 proteins that were up-regulated when the bacteria was grown under iron-limiting conditions. However, to the best of our knowledge, our study is the first study to date that has investigated

comparative periplasmic protein profiles of *N. meningitidis* when the bacteria was grown under heme-limiting and heme-replete conditions.

Six proteins consistently exhibited increased expression under heme-limiting compared to heme-replete conditions. Two of these proteins, a putative oxidoreductase and a putative phosphate acetyltransferase, are typically located in the cytoplasm of the cell but can be transferred to the periplasmic space. The other four proteins identified are cytoplasmic proteins, and include dihydrolipoamide dehydrogenase, serine hydroxymethyltransferase, trigger factor and phosphoglycerate mutase. An explanation for increased expression of these proteins derives from a study done by Basler et al. (2006) that found that 20% of the Neisserial genome (400 genes) can be subject to regulation in function by iron availability, and *N. meningitidis* was grown under heme-limiting culture conditions, and therefore low iron availability. Furthermore, two of the proteins were found by Basler and colleagues to be iron-regulated in *N. meningitidis* serogroup A, serine hydroxymethyltransferase and putative oxidoreductase. It is thus likely that these six proteins are regulated by iron.

Analysis of the proteome using 2DGE suffers mainly from poor gel separation, poor protein staining sensitivity and reproducibility. However, our results were both reproducible and demonstrated excellent protein separation. Furthermore, Sypro Ruby stain used in this study offers high sensitivity for protein detection (0.25 to 1 ng for most proteins), plus a much wider range of proteins classes detected, in comparison to the traditional silver staining method. Therefore, the challenges encountered in this study are centered on the preparation of the protein sample.

Future experiments should focus on the isolation of a pure periplasmic protein sample, to be confirmed by Western blot analysis using monoclonal antibodies to *Neisseria* periplasmic markers (such as FbpA). Western blot analysis of the periplasmic sample using antibodies to both cytoplasmic and membrane proteins would also be ideal to further ensure a pure periplasmic protein sample. Furthermore, our lack of success in identifying a periplasmic binding protein using 2DGE could be the result of using a heme concentration that was not stringent enough to permit the over-expression of the periplasmic binding protein under heme-limiting conditions. Desai and colleagues (1995) demonstrated binding and accumulation of heme in *N. gonorrhoeae* at a heme concentration of 5 μ M. Stojiljkovic and colleagues (1996) used the same heme concentration to demonstrate increased expression of the outer membrane HmbR receptor in *N. meningitidis*. More stringent heme-limiting growth conditions and purification of the periplasmic protein sample were not pursued during the course of this study, since our collaborators at The Institute of Genomic Research provided us with heme-binding protein candidates using another molecular technique, DNA microarrays.

Although 2DGE did not identify any candidate heme binding proteins for future study, there are several advantages to using this technique to evaluate differential expression of periplasmic components of transporters. First, the periplasmic component of ABC transporters is typically expressed in excess of its corresponding membrane components, resulting in an abundance of the protein in periplasmic extracts. Second, ABC transporter components are differentially expressed, by up-regulation under substrate deficient growth conditions. Therefore,

periplasmic extracts from bacteria grown under heme-limiting conditions would yield a higher concentration of the periplasmic component of a heme specific ABC transporter in comparison to growth under heme-replete conditions. Third, use of the subcellular periplasmic protein preparation would result in a more defined protein set in comparison to the use of whole cell lysate. This would overcome the inherent caveat of 2DGE wherein many protein spots can be comprised of multiple proteins. Furthermore, the technique of 2DGE suffers from poor spot resolution at higher pI values and a difficulty in electrophoresing large and hydrophobic proteins in the first dimension, as well as poor representation of extreme acidic and basic proteins. Since periplasmic heme binding proteins are typically small (30-50 kDa) and have a pI range between 9 and 10, separation and identification of periplasmic heme binding proteins using 2DGE would be ideal. In conclusion, 2DGE is a valid molecular approach to identify periplasmic heme binding proteins and is recommended for future study.

4.3. Microarray Analysis

The first paper describing the use of microarrays for monitoring expression of a broad spectrum of genes simultaneously appeared in 1995 (Schena et al., 1995). DNA microarrays are a well recognized technique for the identification of genes that are differentially expressed under two separate growth conditions. In one of the first publications of meningococcal transcriptome analyses, Grifantini et al. (2002) used DNA microarrays based on the meningococcal genome sequence of strain MC58 to study the transcriptome of serogroup B meningococci, by measuring induced changes in the expression of genes when *N. meningitidis* was cultured with human

bronchial epithelial cells. Since then researchers have used DNA-based microarrays to study differential gene expression in *Neisseria*, as reviewed by Claus et al. (2007), and the resulting data obtained is an impressive example of how microarray analysis can foster consecutive functional studies assigning function to previously hypothetical genes. DNA-based microarray studies were previously done to study iron responsive genes in both *N. meningitidis* and *N. gonorrhoeae* (Basler et al. 2006, Ducey et al. 2005, Delany et al. 2006, Grifantini et al. 2003), but our investigation is the first study done to analyze differential gene expression in *Neisseria* when grown under heme-limiting and heme-replete conditions.

Our DNA microarray analysis identified 350 genes that were up- or down-regulated when *N. meningitidis* was grown in the presence of low heme as a sole iron source. This number is a reflection of the 20% of the Neisserial genome subject to regulation in function by iron availability (Basler et al., 2006). To further refine this number to a more manageable number of genes, we limited our analysis to only those genes that encoded putative ABC transporter components or to genes that were located within putative ABC transporter operons. Ten genes were identified that were either components of or affiliated with seven ABC transporters whose substrates had not been characterized at the time of this study.

Challenges were encountered in the construction of mutations in some of these genes, including: the inability to PCR amplify select genes; blunt-end ligation of the antibiotic cassette within select gene constructs; and transformation of wild-type *N. meningitidis* B. Barden. Technical problems are accountable for PCR amplification and ligation, although optimization of each technique was attempted.

Furthermore, *Neisseriae* are naturally transformable, with a transformation efficiency using linear plasmid of 6.6×10^{-7} per μg of DNA, resulting in a single transformant when 1 μg DNA plasmid is incubated with 10^7 bacterial cells. However, using linear plasmid constructs in the range of 1 μg to 80 μg per 10^7 bacterial cells under ideal transformation conditions, we were unable to construct mutants in three of the putative transporter genes. The difficulty in obtaining mutants in pathogenic *Neisseria* is well known and has limited the ability to genetically define determinants responsible for virulence (Kathariou et al., 1990; Nassif et al., 1991). This is reflected in the length of time between the discovery of FbpA (Berish et al., 1990), the periplasmic component of the iron transporter in *N. meningitidis*, and the construction of an *fbpA* mutant (Khun et al., 1998). A Neisserial DNA uptake sequence located in close proximity to the antibiotic cassette was present to increase transformation efficiency, however we remained unable to construct mutants in some of the ABC transporter genes identified by DNA microarray analysis.

It is also possible that a resulting mutant in some of the genes was lethal to *Neisseria*, as has been shown for other bacterial ABC transporters. For example, mutations in the *lolCDE* complex that is involved in release of outer membrane-specific lipoproteins from the inner membrane of *E. coli* is lethal for the bacteria and thus is an essential ABC transporter for the bacteria (Narita et al., 2002). However, mutations in ABC transporter genes involved in heme/iron uptake generally result in bacteria with decreased virulence, and are not lethal to bacteria unless they are heme-obligates. Since *Neisseriae* are not heme-obligate bacteria, it is unlikely that the resulting mutations were lethal to *N. meningitidis*.

The ability of the NBM1993 ATPase mutant to assimilate iron from heme and hemoglobin was unimpaired when compared to that of the wild-type strain. Since the use of the ATPase component from another transporter is unlikely, we would have expected that if this operon was involved in heme uptake then a mutation in NMB1993 would have abolished the ability of the mutant *Neisseria* strain to use heme as a sole iron source. However, mutants in heme-specific ABC transport systems do not always result in abrogation of the bacteria to use heme as an iron source. For example, mutations in the *Yersinia pestis* heme-specific ABC transporter system genes (*hmuTUV*) abrogated iron utilization from myoglobin, heme and heme-albumin but did not affect the use of hemoglobin and hemopexin. Another potential explanation could be that there is more than one transporter for heme in *N. meningitidis* and thus a double knock-out of both transporters would be required for a heme deficient phenotype. However, this seems unlikely based on a review of the available scientific literature to date. For example, mutations in the heme ABC transporter operons of *Pseudomonas aeruginosa* (Ochsner et al., 2000) and *Sinorhizobium meliloti* (Cuiv et al., 2008) demonstrated a reduced ability to acquire heme as a sole iron source, suggesting the presence of a second heme acquisition system in these bacteria. Therefore, even if the genome of *Neisseria* encodes two separate ABC transporters for heme, we would have at least expected a reduction in heme metabolism for the NMB1993 mutant if it was involved in heme uptake. However, due to experimental support provided by the characterization of other bacterial heme transporters, mutational analysis of ABC transporter candidates remains an ideal avenue to pursue for future study to identify a heme ABC

transporter in *Neisseria*. Future studies should focus on construction of mutations in the other six ABC transporters identified by microarray analysis to determine their role in heme uptake.

Analysis of the six proteins identified from 2DGE using the microarray analysis data demonstrated that the genes that encoded these six proteins had increased transcription levels when the bacteria was grown under heme-limiting conditions by a factor of 1.8 times or higher compared to heme-replete conditions. This further supports the finding from the 2DGE that these proteins are likely iron-regulated. Candidates identified in future studies by 2DGE should be compared to the results obtained from our DNA microarray results. Furthermore, a search of novel proteins involved in heme uptake identified in the upcoming literature should also be evaluated against our microarray data.

There are limitations in using DNA microarrays to study differential gene expression profiles. These include limitations in sensitivity of transcript detection and a dynamic range that is usually not wide enough. A further disadvantage specific to bacterial samples is the rapid turnover and very short half-life of certain mRNA. Furthermore, higher mRNA levels do not necessarily mean higher amounts of protein, due to post-transcriptional regulation of the gene expression, protein turnover or modification levels. However, these limitations are unlikely to affect the expression profile of periplasmic components of ABC transporters since periplasmic genes are often small, and increasing levels of mRNA degradation is directly proportional to length of mRNA. Furthermore, since iron/heme regulated genes are primarily transcriptionally regulated in bacteria, differential expression of Neisserial

genes under heme-replete and heme-limiting conditions are likely to be detected by transcriptome analysis using DNA microarrays. DNA microarray analysis has been used in the successful identification of ABC transporters that are differentially expressed in other bacteria, including *Corynebacterium glutamicum*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* (Ishige et al., 2003; Bunai et al., 2004; Domenech-Sanchez et al., 2006; Song et al., 2008). Overall, DNA microarray analysis is a strong approach to study differential expression of ABC transporter genes in response to heme availability in *Neisseria*. To confirm DNA microarray analysis results, quantitative real-time PCR analysis should also be performed.

4.4. The role of NMB0586 in heme-binding

A significant part of this study focused on the putative role of the periplasmic protein NMB0586 in heme-binding, as a component of a heme ABC transporter. The *N. meningitidis* NMB0586 was unaffected by the bacteria's ability to use heme as a sole iron source. Therefore, it appears that NMB0586 is not involved in heme uptake in *N. meningitidis*. However, mutants in heme-specific ABC transport systems do not always result in abrogation of the bacteria to use heme as an iron source, as discussed previously. Furthermore, it has been shown in previous studies that more than one periplasmic protein can interact with the same membrane-bound components of an ABC transporter. For example, the periplasmic heme-binding proteins MppA and DppA both interact with the same membrane component in *E. coli* (Letoffe et al., 2006), and in *Salmonella typhimurium*, two periplasmic binding proteins (HisJ and ArgT) interact with the same site on the permease component of a

membrane transporter (Higgins and Ames, 1981). Since NMB0586 is the periplasmic component of the ABC transporter, this is a potential explanation as to why the heme phenotype was unaffected by the NMB0586 mutation. However, it should be noted that periplasmic protein redundancy is not a common phenomenon for bacterial ABC transporters, as observed by only a minimal number of scientific publications on the topic. Therefore this is likely not a variable for significant consideration in this study.

In future studies, mutational analysis to identify a new heme ABC transporter candidates in *Neisseria* should incorporate the technique of random insertional inactivation. Genetic systems that allow bacterial genomes to be mutagenized in a random fashion have provided the means for developing new tools for the diagnosis, prevention and treatment of bacterial diseases by allowing potential targets to be identified and validated. Specifically, this technique has been used to identify heme binding proteins in bacteria. Random transposon mutagenesis of *Leptospira biflexa* and the screening of the resulting mutants onto media with and without hemin resulted in the identification of five hemin-requiring mutants and the putative genes responsible for the heme-deficient phenotype were identified (Louvel et al., 2005). This method was also used by Letoffe and colleagues (2006) to identify a heme ABC transporter in *E. coli* K12. Use of transposons to generate a library of mutants encompassing the whole bacterial genome has been studied in *Neisseria* sp. to identify the roles of previously uncharacterized genes (Kathariou et al., 1990; Nassif et al., 1991). For example, Mehr and Seifert (1997) constructed mutants in *N. gonorrhoeae* by random shuttle mutagenesis, and studied the ability of the mutants to

undergo pilin antigenic variation. Of over 8000 mutants screened, the novel role of 22 Neisserial genes in pilin antigenic variation was discovered (Mehr and Seifert, 1997). Therefore, this is a promising technique to apply to *N. meningitidis* to identify novel genes involved in heme metabolism.

Direct binding of NMB0586 to heme, detected using enhanced chemiluminescence, demonstrated that NMB0586 binds heme at a heme concentration of 10^{-4} M. For comparison, the periplasmic heme-binding protein from *Y. enterocolitica*, HemT, binds heme at 10^{-6} M using the same experimental conditions (Letoffe et al., 2008). Thus confirming the heme-binding ability of NMB0586, albeit with low affinity. This finding is further supported by the affinity binding assays, demonstrating non-saturated binding to hemin agarose, as well as no effect in binding of NMB0586 to hemin-agarose when in the presence of competing ligand. A summary of the heme-binding results for NMB0586 suggest that this protein is not a high affinity periplasmic heme-binding protein.

Since histidine residues are often located in heme-binding pocket of heme-binding proteins and are responsible for heme-binding, it is possible that the presence of the histidine tag at the C-terminal of the purified NMB0586 could be responsible for this low binding affinity for heme. This would further explain why the negative control protein, Glb1, also bound to the heme in both the enhanced chemiluminescence and hemin-agarose binding assays. However, Sook and colleagues (2008) demonstrated that NMR results of the heme methyl resonances of SiaA, a periplasmic heme-binding protein of *Streptococcus pyogenes*, was unaffected by the presence or absence of the 6-histidine-Xpress epitope, indicating that the

histidines in the His-tag do not interact with any heme in solution and the presence of the His-tag does not alter the heme binding site. St. Denis (unpublished) also demonstrated a similar finding in which the presence of a His-tag fused to the N-terminal of the *H. ducreyi* heme-binding protein hHbp did not affect the heme-binding properties of the protein. Therefore this lends further support to the possibility of low affinity binding of NMB0586 to heme, regardless of the presence of the histidine tag.

4.5 Functional complementation

Functional complementation is a technique that has been widely used to study the ability of bacterial genes to restore mutant strains to wild-type phenotypes. For example, to identify motifs involved in the protein/protein interactions through which TolA acts, Weitzel and Larsen (2008) expressed TolA, a protein that spans the periplasmic space to communicate with proteins in both the cytoplasmic and periplasmic membranes, from *Y. enterocolitica* in *E. coli* to test the ability of a phylogenetically distinct TolA protein from *Y. enterocolitica* to function in the Tol system of *E. coli*. Specifically in *Neisseria*, Stohl and colleagues (2002) expressed *N. gonorrhoeae* RecA, a protein involved in pilin antigenic variation, in an *E. coli recA* mutant under the control of *lac* regulatory sequences in *E. coli*. The gonococcal RecA protein was shown to fully complement the ability of the *E. coli* mutant for pilin antigenic variation.

NMB0586 was unable to functionally complement an *E. coli* K-12 heme-deficient mutant. Since NMB0586 and MppA/DppA, the two periplasmic heme-binding proteins in *E. coli*, do not have any significant amino acid sequence

homology, it is possible that NMB0586 was unable to complement the *E. coli* K-12 heme deficient phenotype due to the lack of recognition of the permease component of the *E. coli* K-12 transporter. It is also possible that NMB0586 was unable to restore the heme phenotype, based on the low affinity of NMB0586 for heme demonstrated in our study.

Although we were unable to demonstrate functional complementation of *E. coli* K-12 with NMB0586, this remains an invaluable tool for further identification of a Neisserial heme ABC transporter. Many Neisserial genes have been shown to be expressed in *E. coli* strains, including ABC transporters. For example, Adhikari et al. (1996) expressed the *fbpABC* locus into *E. coli* to study the iron binding properties of the operon. It would be interesting to study whether other candidate heme ABC transporters identified in *Neisseria* could complement the heme-deficient phenotype of the *E. coli* K-12 strain.

Future directions for identification of a heme ABC transporter should also include expression cloning of a selection of specific polypeptides, generated from a cDNA or genomic DNA library of *Neisseria*. This could be done by construction of a genomic, random shear lambda expression library for restoring the heme phenotype in *E. coli* K-12. This approach has been used successfully by others in the discovery of antigenic components of diagnostics and vaccines for several infectious agents, including *Mycobacterium tuberculosis* and *Chlamydia* spp., as reviewed by Lodes et al. (2004).

During the latter part of our investigation into the heme-binding function of NMB0586, a BLAST search disclosed that the NMB0586 homologue in *N.*

gonorrhoeae, MntC, was characterized as a Neisserial manganese transporter. Prior repeated BLAST searches performed in our laboratory during the course of this study consistently showed functional assignment of NMB0586 as a putative adhesin (Tseng et al., 2001). It should also be noted that no Neisserial nucleotide sequence information was provided in this earlier publication by Tseng and colleagues. Functional annotation of the NMB0586 homologue in *N. gonorrhoeae* was determined by Lim and colleagues (2008), at which time our studies of NMB0586 were near completion.

Manganese plays an important role in defense against oxidative stress. Anjem et al. (2009) demonstrated that manganese uptake mechanisms in *E. coli* are switched on when iron levels are low, suggesting that the role of manganese is to compensate for low availability of iron in the cell. This provides an explanation as to why increased expression of NMB0586 was observed in our DNA microarray analysis results when *N. meningitidis* was grown under heme-limiting conditions.

Lim et al. (2008) and Wu et al. (2009) further disclosed that MntC has specificity for two substrates, manganese and zinc, with almost equal affinity, and that the binding specificity to zinc is suggested to be due to the histidine-rich loop in MntC. Although it would be interesting to hypothesize that this transporter has more than two substrates, one being heme, there is no evidence in the literature to suggest that a periplasmic binding protein can have affinity for more than two substrates. Furthermore, although manganese could take the place of ferrous iron in some mononuclear metalloenzymes, it is unable to use heme and iron sulphur containing prosthetic groups. Therefore, based on the current knowledge about this transporter

to date, no further examination of the heme binding properties of NMB0586 is required.

4.5. The importance of this study

The importance for the continuation of the study to identify a heme ABC transporter, and to understand the pathogenesis of *Neisseria* further, is the lack of a wide-range vaccine for the prevention of *N. meningitidis* and *N. gonorrhoeae*, as well as increasing antibiotic resistance to infections caused by pathogenic *Neisseria*. Since *Neisseria* can use heme as a sole iron source for growth, the heme uptake mechanisms are considered important virulence determinants. To date, vaccine study efforts in *Neisseria* have been focused on surface-exposed antigens, which are likely to be involved in ligand binding and accessible to immune factors. Since the ABC transporter components in gram-negative bacteria are not surface-exposed, they will likely not be ideal vaccine candidates. However, they could be targets for antimicrobial treatment of diseases caused by pathogenic *Neisseria*. Early studies by Kaneko et al. (1999) focused on the susceptibility of *Pseudomonas aeruginosa* to porphyrin-based compounds, and their antimicrobial potential to kill microbial pathogens. Kaneko et al. found that the metalloporphyrin Ga-PPIX exploit the natural heme uptake system in *P. aeruginosa*. Bozja et al. (2004) further studied Ga-PPIX as a topical microbicide to treat infections caused by *N. gonorrhoeae* and *H. ducreyi*, and found that both STI pathogens were susceptible to the bactericidal action. Although the mechanism by which Ga-PPIX kills *N. gonorrhoeae* do not use the hemoglobin transport system HpuAB, this does not discount the fact that these compounds could be entering the bacteria by another heme transport pathway.

Understanding the heme uptake mechanisms in pathogenic *Neisseria* is pivotal to identifying mechanisms by which Ga-PPIX or other novel antimicrobial compounds can enter the bacteria.

4.7. Conclusion

Several molecular techniques were used in this study in an attempt to identify a heme ABC transporter. Although the results of this study did not conclusively identify a dedicated heme ABC transporter in *N. meningitidis*, we are still confident that such a transporter does exist but remains to be elucidated. Identification of a heme ABC transporter is important for increased understanding of the virulence mechanisms of pathogenic *Neisseriae*, as well as the development of therapeutic interventions exploiting such a transporter as a high affinity drug delivery system. Future studies should focus on the techniques we have used in this study, taking advantage of any advancements or improvements to these techniques in the past few years.

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