Characterization of TonB-Dependent Metal Transporters within Neisseria gonorrhoeae

Mary Kathryne Dickinson

Virginia Commonwealth University

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CHARACTERIZATION OF TON-B DEPENDENT METAL TRANSPORTERS WITHIN
NEISSERIA GONORRHOEAE

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

By

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<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CDM</td>
<td>chemically defined media</td>
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<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>Fe</td>
<td>iron</td>
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<tr>
<td>GCB</td>
<td>gonococcal growth media</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HS-TBS</td>
<td>high salt Tris-buffered saline</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase pairs</td>
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kD  kilodalton
Ku  klett unit
LB  Luria Broth, *E. coli* growth media
LS-TBS  low salt Tris-buffered saline
M  molar
mA  milliamp
mM  millimolar
mL  milliliter
NaOH  sodium hydroxide
NBT  nitro blue tetrazolium
OD  optical density
OMP  outer membrane preparation
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PID  pelvic inflammatory disease
RNA  ribonucleic acid
rpm  revolutions per minute

RT-PCR  reverse transcriptase polymerase chain reaction

SDS-PAGE  sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SS1  start site 1

SS2  start site 2

Tbp  transferrin binding protein

TBS  tris-buffered saline

TBDT  TonB-Dependent transporter

Tf  transferrin

Tween 20  polyoxyethylene sorbitan monolaurate

µl  microliter

WT  wild-type
Abstract

CHARACTERIZATION OF TON-B DEPENDENT METAL TRANSPORTERS WITHIN *NEISERIA GONORRHOEAE*

By Mary Kathryne Dickinson  
B.S., James Madison University, 2012

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science  
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Virginia Commonwealth University, 2014

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Professor, Department of Microbiology and Immunology

*Neisseria gonorrhoeae*, the etiologic agent of gonorrhea, utilizes TonB-dependent  
transporters to import essential nutrients such as iron. Study of TonB-dependent transporters is  
extremely important due to the fact that they make excellent vaccine targets. In order to learn  
more about the structure, function, expression, and regulation of selected TonB-dependent  
transporters, three goals were established for this study. The first goal was to examine the role of  
two highly conserved regions of TbpB in lipidation. One of the conserved regions of TbpB, the  
LSAC motif, was shown to be critical for lipidation. The second goal was to determine whether  
MisR/MisS regulates expression of TbpA and TbpB. MisR/MisS was shown to regulate the
expression of TbpA and TbpB. The third goal was to assess the ability of recombinant TdfJ to bind hemin when expressed in *E. coli*. Recombinant TdfJ was shown to specifically bind hemin when expressed in *E. coli*.
Introduction

I. Genus *Neisseria*

The genus *Neisseria* lies within the family *Neisseriaceae*. The family *Neisseriaceae* also contains the genera *Moraxella*, *Acinetobacter*, and *Kingella* (49). The genus *Neisseria* is comprised of Gram-negative diplococci, ranging from 0.6-1.5 μm in size, occurring in pairs with flattened adjacent sides. They are non-motile, except for the twitching mobility caused by pilus retraction. Most *Neisseria* are catalase positive and all *Neisseria* are oxidase positive. There are 12 species within the genus *Neisseria* and the vast majority are commensals that are found as part of normal human flora (6). However, *Neisseria meningitidis* and *Neisseria gonorrhoeae* are obligate human pathogens that cause significant morbidity globally. The two species are very similar, although *N. meningitidis* is encapsulated, whereas *N. gonorrhoeae* is not. Although *N. meningitidis* causes disease in humans, it is also commonly carried by healthy individuals in the nasopharynx (81). However, it can disseminate and cause serious diseases such as meningococcaemia and meningococcal meningitis, which have very high mortality rates if untreated. The World Health Organization reports that there are 12 serogroups, and of these only six are the cause of frequent disease globally. These serogroups are A, B, C, W135, Y and X. Currently vaccines are on the market that protect against serogroups A, C, Y, and W-135 and
they have been successful in reducing the burden of disease when implemented on a large scale. However, since the introduction of the various vaccines the incidence of meningococcus B has been on the rise in many parts of the world (72). However, very recently a multicomponent meningococcal group B vaccine was released and has been approved for use in many countries (36). *Neisseria gonorrhoeae* is the etiologic agent of the sexually transmitted disease gonorrhea. Infection with gonorrhea is transmitted via intimate sexual contact, however it can also be transmitted prenatally (16). There is currently no vaccine available to prevent infection with *N. gonorrhoeae*. The subsequent sections of this thesis will focus exclusively on *N. gonorrhoeae*.

**II. Neisseria gonorrhoeae**

**A. Infection**

*N. gonorrhoeae* infects the urogenital or anorectal mucosa of both males and females. The gonococcal pilus allows for initial contact with the host cell, and facilitates adhesion, invasion, and traversal of monolayers (57). Following initial attachment, pilus retraction allows Opa adhesins to create a tight connection with host cell receptors. Attachment then causes cytoskeletal rearrangement of the host cell, followed by engulfment of the bacteria (37). After invasion, bacteria can persist intracellularly and undergo transcytosis through the epithelia, allowing entrance into the subepithelial compartment. This process leads to the establishment of localized infection, although disseminated infection can also follow (55).

**B. Disease and Treatment**

*Neisseria gonorrhoeae* is the causative agent of the sexually transmitted infection gonorrhea. Men generally begin to show signs of infection after an incubation period of 2 to 5 days. Early symptoms include acute urethritis, purulent discharge, and dysuria (15). Disease in
men is generally uncomplicated because the early symptoms cause them to seek medical attention. Ascension of the disease in men can lead to epididymitis and prostatitis. In women, symptomatic uncomplicated disease presents within 10 days of infection, manifesting as urethritis and/or cervicitis. However, it is estimated that up to 80% of infections in women are asymptomatic or cause minor symptoms that are ignored (56). Untreated gonococcal infections have serious and permanent downstream sequelae, including internal abscesses, chronic pelvic pain, and pelvic inflammatory disease (PID) (16). PID is a very serious consequence of untreated infection that increases the risk of ectopic pregnancy and leads to infertility in 10-15% of women that acquire the disease (17). Although it is rare, life-threatening disseminated infection is also possible in both men and women if the infection is left untreated. Symptoms of disseminated infection include fever, tenosynovitis, and dermatitis. Perinatal infection can occur if an infected mother delivers her child vaginally (16).

In contrast to *N. meningitidis*, there is no vaccine available to prevent infection with *N. gonorrhoeae*. In addition, natural infection with *N. gonorrhoeae* does not elicit protective immunity. The lack of an effective vaccine and protective immunity following infection is due to the highly variable cell surface of the gonococci.

**C. Epidemiology**

In 2012 there were 334,826 reported cases in the U.S., making it the second most commonly reported notifiable infectious disease in the United States. This number is likely inaccurate due to inefficient reporting practices and the CDC estimates that the number of actual infections per year could be more than double the number of reported infections (15). Within the U.S., infection is most common among young adults and African Americans. Gonococcal infections are also prevalent outside of U.S. The World Health Organization estimated that there
were 106.1 million new cases of *N. gonorrhoeae* in adults worldwide in 2008 (99). Although common amongst both men and women, gonococcal infections are slightly more common amongst women and cause significantly more morbidity in women due to the high frequency of asymptomatic infections.

**D. Antibiotic Resistance**

In 1935 the first curative treatment for gonorrhea was introduced in the form of sulphanilamide. However, shortly after its introduction *N. gonorrhoeae* quickly became resistant and the first line treatment recommendation was altered (47). Since then, penicillins, tetracyclines, macrolides, fluoroquinolones, and cephalosporins have experienced the same fate. In 2012 the CDC released a statement saying that they no longer recommended oral antibiotic cefixime as a first-line treatment option (18). Currently, the only recommended first-line treatment for gonococcal infection is a single dose of intramuscular ceftriaxone, a third-generation cephalosporin, along with oral azithromycin (18). In 2011, a ceftriaxone-resistant strain of *N. gonorrhoeae* was isolated from a pharyngeal specimen taken from a Japanese commercial sex worker (69). In 2013, the CDC published a report entitled “Antibiotic Resistance Threats in the United States.” They categorized pathogens based on the level of threat they posed to public health, and drug-resistant *N. gonorrhoeae* was placed into the “urgent” hazard level category, the most serious of the three categories (19). They state that *N. gonorrhoeae* is “an immediate public health threat that requires urgent and aggressive action.” The threat of untreatable gonorrhea infection a real possibility, therefore research into possible vaccine targets and novel treatments is warranted.
E. Vaccine

The development of an effective vaccine is imperative due to the aforementioned health impacts and increasing antibiotic resistance associated with gonococcal infections. The surface of the gonococcus is littered with virulence factors, such as lipoooligosaccharide (LOS), pilin, and opacity proteins (Opas), which would make logical vaccine targets. However, these surface exposed proteins undergo frequent variation that makes this impractical. The gonococcus constantly alters its surface exposed components through phase variation and antigenic variation. Antigenic variation is the frequent alteration of immunodominant epitopes. Phase variation is a reversible “on or off” switch for expression of certain proteins (35). Because of this variation gonococcal infection does not impart protective immunity and makes the creation of an effective vaccine very difficult. However, there are some surface-exposed proteins that do not undergo such significant variation and are consistently on the outer membrane due to their importance to the organism. The TonB-dependent transporters (TdTs) are nutrient acquisition systems that are ideal candidates for a vaccine due to their consistent expression and well-conserved structures. Further research into their possible role in the development of a vaccine is warranted.

F. Virulence Factors

*N. gonorrhoeae* possesses many important virulence factors that aid in adherence, invasion, and immune evasion. Important virulence factors include pili, Opas, LOS, and porin. Pili are long filamentous structures that project out of the bacterial outer membrane. Pili are composed of a pilin protein subunit, known as PilE, and are capped off by the PilC protein (35). This structure is depicted below in Figure 1. Pili are crucial for adherence, colonization of host tissue, and DNA uptake (34, 94). Nonpiliated cells are not competent for DNA uptake (10). Pili expression is phase variable due to the poly-cytosine tract in *pilE* (50). Pili expression is also
subject to antigenic variation due to nonreciprocal transfer of DNA sequences from one of the many silent pilS cassettes into the expressed pilE locus (40).

Opas are integral outer membrane proteins, and a single gonococcal strain can possess up to 11 different opa alleles. Opa proteins were named because their expression effects colony color and opacity (84). Opa proteins have been shown to bind to cell surface heparan sulfate proteoglycans (HSPG) or carcinoembryonic antigen-related cell adhesion molecules (CEACAM) (55, 78, 92). Opas also play a role in the downregulation of the host immune response towards the pathogen (11). Opas are also subject to phase variation due to slipped-strand mispairing. Slipped-strand mispairing causes changes in the CTCTT repeats, resulting in a frameshift mutation (63). Opas are thought to be important for infection based on the fact that bacteria recovered subsequent to successful human infection are usually Opa positive (46).

LPS is a common component of gram-negative bacterial outer membranes. N. gonorrhoeae produces a shorter form of LPS known as lipooligosaccharide (LOS). LOS is anchored to the membrane by the endotoxic lipid A moiety. LOS is phase variable and can undergo sialylation. Low levels of LOS sialylation allow for highly efficient entry into human mucosal epithelial cells. High levels of LOS sialylation disable the bacteria from entering the host cells as efficiently, but enable them to resist killing by complement and antibodies. Thus, LOS plays a role in both bacterial entry and immune evasion (37, 98).

Porin is the major outer membrane protein expressed by N. gonorrhoeae. Porin is essential for bacterial viability, and is therefore not subject to phase variation. All gonococcal strains contain the porB gene, and can possess either the porB1a or porB1b allele. porB1a encodes the P.1A protein, while porB1b encodes the P.1B protein. Expression of the PIA protein has been implicated in invasive disease (38). PorB forms a trimeric, hydrophilic pore across
Figure 1. Major virulence factors and morphology of *N. gonorrhoeae*. The figure depicts the diplococcus, containing the pair of cells with adjacent flattened sides. Depictions of the major virulence factors, including pili, Opas, LOS, and porin, are also included.
Figure 1. Major virulence factors and morphology of *N. gonorrhoeae*. 
the gonococcal membrane, which allows for the diffusion of small hydrophilic molecules (27).
Porin has been shown to be involved in invasion and regulation of apoptosis (38). Factor H has been shown to bind to a surface exposed region of P.1A, therefore aiding in serum resistance (77).

**III. Iron Acquisition**

Iron is a nutrient that is absolutely essential to survival for gonococci, as evidenced by its many dedicated iron uptake systems. Iron is vital for many cellular functions within bacteria, such as growth, RNA synthesis, and DNA synthesis. Iron is also important for bacterial metabolism, as it is used in the tricarboxylic acid cycle and oxidative phosphorylation. Iron plays an important role in protecting cells against oxidative stress. Catalases and peroxidases, which destroy harmful free radicals, contain iron. Many other important proteins and enzymes also require iron for proper function (58). Excess free iron is toxic due to the oxidative stress caused by hydroxyl radicals that are produced by the Fenton reaction: \( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH} \). Therefore, almost all of the iron present in the host is contained within various high affinity iron-binding proteins.

Some important iron sources available within the host include: transferrin, lactoferrin, ferritin, heme, hemoglobin, and siderophores. Transferrin is an 80 kD glycoprotein that is capable of binding two atoms of iron (93). Transferrin is present in the serum and helps to sequester and transport iron. Lactoferrin is an 80 kD glycoprotein involved in protective iron chelation. Lactoferrin is found in lymph and mucosal secretions. Both transferrin and lactoferrin have an exceptionally high affinity for ferric iron. *N. gonorrhoeae* has been shown to use transferrin and lactoferrin as iron sources (59, 60). Ferritin is a cytoplasmic protein consisting of 24 subunits (93). Ferritin is important for intracellular iron storage and is capable of binding
several thousand iron ions. Heme is a protoporphyrin that can bind one ferrous iron ion in its center. Solitary heme is very cytotoxic and is therefore not usually found free within the host (93). Iron bound to heme is the most abundant form of iron found in vertebrates, and the most of the heme is bound to hemoglobin (5). Heme serves as an iron source and is also essential for several aerobic processes in bacteria. Most bacteria have a dedicated transport system for heme uptake, however one such system has not been identified within *N. gonorrhoeae*. However, utilization of heme as an iron source and as a heme source has been demonstrated *in vitro* (88).

Hemoglobin is a tetramer, and each subunit can bind a heme molecule. Hemoglobin is an oxygen transporter found in red blood cells. The gonococcus has been shown to use hemoglobin as an iron source (31). Siderophores are low molecular weight compounds secreted by many different microorganisms for the purpose of iron acquisition. These chelating agents bind ferric iron with high affinity and can strip iron from host iron binding proteins (65). After iron is bound, the ferrated siderophore can be utilized as an iron source by the organism. *N. gonorrhoeae* has specific iron transport systems that facilitate the use transferrin (24), lactoferrin (9), hemoglobin (21), and some heterologous siderophores (97). *N. gonorrhoeae* does not produce its own siderophores, and therefore can only utilize those produced by neighboring bacteria (96). *N. gonorrhoeae* has been shown to utilize aerobactin, enterobactin, and other catecholate siderophores including dimers and trimers of dihydroxybenzoylserine and salmochelin S2 (14, 43, 96).

**A. TonB-Dependent Transport**

TonB-Dependent transporters (TBDTs) are utilized by Gram-negative bacteria in order to access many necessary nutrients. Porins allow for concentration-dependent, and non-specific diffusion of solutes smaller than 600 daltons; however, many nutrients required by the gonococci
are larger than this (66). The majority of the substrates for TBDT are iron complexes, however nickel chelates, carbohydrates, and vitamin B\textsubscript{12} are transported in this fashion (67). All known TBDTs contain a beta-barrel composed of 22 amphipathic beta-strands and a globular plug domain. The globular plug domain is folded up inside of the beta-barrel and functions to bind specific substrates (67). TBDTs interact with a cytoplasmic membrane protein complex composed of TonB, ExbB, and ExbD. ExbB an ExbD derive energy from the proton motive force of the cytoplasmic membrane and transfer it to TonB, which in turn transfers the energy to the outer membrane transporter (70, 75). TonB interacts with components in both the outer and cytoplasmic membrane, while ExbB and ExbD are anchored to the cytoplasmic membrane. Both ExbB and ExbD are necessary for TonB to respond to proton motive force and are both necessary for TonB to associate with the cytoplasmic membrane (42). After the substrate binds the receptors and is transported through the channel, the substrate rapidly binds a specific periplasmic binding protein. This periplasmic binding protein then shuttles the substrate to ABC transporters that are embedded in the cytoplasmic membrane. Finally, the ABC transporter transports the substrate into the cytoplasm (32).

**B. Two Component TonB-Dependent Transporters**

Two component TBDTs are composed of the beta-barrel transmembrane channel and a surface exposed companion lipoprotein. Gonococcal iron acquisition from transferrin, lactoferrin, and hemoglobin involve two component TonB-dependent transporters, depicted in Figure 2.
**Figure 2. Two component TonB-dependent transporters.** The figure depicts the 3 iron acquisition systems that are composed of two component TonB-dependent transporters. These systems are responsible for iron acquisition from transferrin, lactoferrin, and hemoglobin. The outer membrane transporters are shown as cylinders that span the outer membrane (OM). The companion lipoproteins are shown tethered to the outer membrane, adjacent to their respective transporters. The TonB, ExB, and ExD complex (purple) is depicted as imbedded within the cytoplasmic membrane (CM), with TonB spanning the periplasm. The periplasmic binding protein, FbpA, is shown in yellow. The arrows pointing from the transmembrane channels to the periplasmic binding protein shown the path of the iron as it is transported. Lastly, the cytoplasmic membrane permease FbpB, and its associated ATPase FbpC, are shown in red within the cytoplasmic membrane. The hemoglobin receptors utilizes the TonB, ExbB, and ExbD complex, but a periplasmic binding protein or an ABC transporter has not been identified for the system.
Figure 2. Two component TonB-dependent transporters.
1. Transferrin Iron Acquisition

Transferrin iron acquisition by the gonococcus has been studied intensely and much is known about the process. The system contains two outer membrane proteins known as transferrin binding protein A (TbpA) and transferrin binding protein B (TbpB). All gonococcal strains studied to date have both \textit{tbpA} and \textit{tbpB} genes. The \textit{tbpA} and \textit{tbpB} genes have been shown to be expressed during natural mucosal infection with \textit{N. gonorrhoeae} (1). A gonococcal transferrin receptor mutant was unable to establish infection in human male volunteers, making the transferrin receptor both a nutrient acquisition system and an essential virulence factor (26). The immunogenicity of the transferrin binding proteins during natural infection has been examined, and although antibodies to recombinant TbpA and TbpB were detected, the levels were not significantly different from the control population (76). This indicates that despite the fact that the transferrin binding proteins are surface exposed, well conserved, and needed for successful infection, their immunogenicity would need to be boosted in order to serve as an effective vaccine candidate.

TbpA is a classical TBDT, composed of a transmembrane beta-barrel and a globular plug domain. TbpA contains 11 surface exposed loops, and loops 4 and 5 were shown to be critical for transferrin binding (12). TbpB is a surface tethered lipoprotein that contains two distinct domains. TbpB is completely surface exposed and contains two transferrin-binding-competent lobes (28). \textit{E. coli} expressing recombinant TbpA were able to specifically bind human transferrin (23). Mutants unable to express TbpA are incapable of internalizing iron from transferrin (24), however mutants unable to express TbpB were still able to internalize iron from transferrin at approximately 50\% wild-type levels (4). Based on this information, it is understood that while TbpA expression is essential for iron acquisition from transferrin, the presence of
TbpB makes the process more efficient (25). The current model for iron uptake from transferrin is as follows: first, ferrated transferrin binds to TbpA and TbpB. TbpA and TbpB then extract the iron from transferrin, and the iron then interacts with the plug domain of TbpA for transport. TonB then initiates transport by pulling on the plug domain. Following transport, iron is released from the plug and subsequently captured by the periplasmic binding protein FbpA. FbpA resembles a single lobe of transferrin. Apo-FbpA has been shown to bind specifically and directly to TbpA (80). This interaction allows for apo-FbpA to be in the correct place and ready to receive iron from TbpA efficiently. After the transfer of iron, ferrated FbpA loses its affinity for TbpA and is allowed to pass through the periplasm. Holo-FbpA then associates with FbpB, a cytoplasmic permease. FbpB then accomplishes transport of the iron into the cytoplasm, using energy derived from FbpC, an ATPase (25).

2. Lactoferrin Iron Acquisition

Less is known about lactoferrin iron acquisition, although the process is thought to be similar to transferrin iron acquisition. Lactoferrin iron acquisition is accomplished by LbpA and LbpB. Similarly to TbpA and TbpB, LbpA is a TBDT and LbpB is a surface tethered lipoprotein. LbpA is required for human lactoferrin utilization, but LbpB is not (8). Roughly 50% of gonococcal isolates contain an identical deletion of the lbpB gene and a portion of lbpA gene, leaving them unable to express the Lbps (2). In addition, if the genes are present, lbpB is subject to phase variation. While all isolates containing the lbpA and lbpB genes express LbpA, 70% do not express LbpB (2).
3. Hemoglobin Iron Acquisition

The hemoglobin uptake system is composed of HpuA and HpuB. HpuB is the TBDT spanning the outer membrane, and HpuA is the surface tethered lipoprotein. HpuA is roughly half the size of the other lipoproteins involved in two component TonB-dependent transport. Both proteins are absolutely required for iron acquisition from hemoglobin (20). Expression of hemoglobin utilization system is phase variable due to slipped-strand mispairing, caused by frameshifting that results from variable-length poly G tract in the hpuA gene (20). Most lab strains are in the hemoglobin receptor “off” phase (21). However, the hemoglobin receptor is often found in the “on” phase in women during the first half of the menstrual cycle (3). Hemoglobin binding to HpuA/B is not species specific, unlike the transferrin and lactoferrin binding proteins.

C. Single Component TonB-Dependent Transporters

Single component TBDTs are composed of the beta-barrel transmembrane channel but do not utilize a surface exposed companion lipoprotein. Gonococcal iron acquisition from siderophores involves single component TonB-dependent transporters, depicted in Figure 3. The other TBDTs are largely uncharacterized and were identified via bioinformatics.

1. Enterobactin Iron Acquisition

Enterobactin iron acquisition is accomplished by the single component TBDT known as ferric enterobactin transporter (FetA). Expression of FetA is iron repressed and subject to phase variation. Enterobactin is produced by various bacteria, including E. coli, Klebsiella species, and Salmonella species (68, 73, 74). Enterobactin is a catecholate-type siderophore. The fetA gene is upstream of genes that putatively encode a periplasmic binding protein, FetB, and an ABC
Figure 3. Single component TonB-dependent transporters. The figure depicts the 5 TBDT systems that are composed of a single transmembrane channel. These systems are largely uncharacterized, although FetA is known to transport certain xenosiderophores. The outer membrane transporters are shown as cylinders that span the outer membrane (OM). The TonB, ExB, and ExD complex (purple) is depicted as imbedded within the cytoplasmic membrane (CM), with TonB spanning the periplasm. The periplasmic binding protein FetB (aqua) is involved in siderophore transport. The periplasmic binding protein FetB2 (pink) is encoded near TdfF. It is hypothesized that the Fet system utilizes the FetC/D/E/F ABC transport system (plum).
Figure 3. Single component TonB-dependent transporters.
transport system, FetC/D/E/F (Figure 3). Mutants lacking FetB expression were deficient in the utilization of enterobactin (14). More recently, FetA was also shown to transport other catecholate siderophores including dimers and trimers of dihydroxybenzoylserine and salmochelin S2 (43).

2. Uncharacterized Single Component Transporters

The rest of the single component TBDTs are mostly uncharacterized and were identified via bioinformatics based on their similarity to other TBDTs. They were named TonB-dependent function (Tdf) F, G, H and J. TdfF is a 78 kD iron regulated protein that has been shown to be important for intracellular survival (41), perhaps suggesting that TdfF plays a role in intracellular iron acquisition after invasion of the host cell. There is a gene for a putative periplasmic binding protein upstream of TdfF, and it has been named fetB2 based on its similarity to fetB. TdfF is only detected in *N. gonorrhoeae* grown in cell culture medium in the presence of serum (41).

TdfG is iron repressed and is very large at 136 kD. Expression of TdfG is only detected in 17% of gonococcal strains, despite the fact that all gonococcal genomes contain tdfG. This is because the *tdfG* genes fall into 2 clades, and the antibodies used to assess TdfG expression only recognized some of the TdfG proteins. Based on its sequence, TdfG is most similar to heme transporters (89). However, a mutant lacking TdfG was still able to utilize heme as a sole iron source. TdfH is also large at 104 kD and also has sequence similarity to heme transport systems (89). Again, TdfH mutants were able to grow on heme as a sole iron source. Based on this finding, it is unlikely that TdfH or TdfG transport free heme (89).

TdfJ is an 86 kD TBDT that is present in all *Neisseriae*. Again, TdfJ shares sequence similarity with heme and siderophore transporters. TdfJ has been shown to be iron induced (30).
The meningococcal homologue of TdfJ is known as ZnuD, and it has been demonstrated to facilitate zinc acquisition (83). ZnuD is regulated by zinc uptake regulator, Zur, and ferric uptake regulator, Fur (51). ZnuD has also been shown to bind heme when expressed in *E. coli*, however there is no defect in heme utilization in a meningococcal *znuD* mutant (51). The *znuD* mutant was however defective for survival within epithelial cells and in adherence to and invasion of epithelial cells (51). ZnuD has been proposed as a potential candidate for inclusion in a meningococcal group B vaccine (44). It is said to be a good candidate because it is expressed in almost all meningococcal group B strains tested, is expressed during infection, and antibodies against it were able to trigger complement mediated killing of meningococcal group B strains under conditions of zinc limitation.

**D. Regulation**

Iron uptake is highly regulated in order to maintain an optimum concentration within the cell and avoid the toxic effects of excess iron. Ferric uptake regulator, or Fur, controls the transcription of iron acquisition genes and a broad range of other genes (30, 85). Under iron-replete conditions dimerized Fur binds iron, and the conformational change that occurs allows it to bind a DNA sequence known as the Fur box. The gonococcal Fur binding sequence is two hexameric direct repeats composed of ATAAT followed by ATTAT (45). When Fur is bound to the DNA, transcription is inhibited and genes are repressed. Under iron-deplete conditions, no iron is bound to Fur, and the protein is therefore unable to bind the Fur box and inhibit transcription (87).

Another form of regulation occurs via the two component regulatory system known as MisR/MisS. The MisR/MisS system is analogous to the PhoP/PhoQ system in *Salmonella enterica*. The systems are composed of a response regulator and an environmental sensor kinase.
In the case of *S. enterica*, PhoP is the response regulator and PhoQ is the sensor kinase (39). In the case of *N. gonorrhoeae*, MisR is the response regulator and MisS is the sensor kinase that is imbedded in the cytoplasmic membrane. The PhoP/PhoQ system directs virulence, regulates cellular activities, and mediates the adaption to Mg$^{2+}$ limiting environments within *S. enterica* (39). Little is known about the gonococcal MisR/MisS system, except that it responds to antimicrobial peptides like LL-37. This indicates that MisR/MisS may play a role in responding to the host environment.
III. Research Objectives

In order to further investigate the structure, function, regulation, and expression of certain TonB-dependent transporters in *N. gonorrhoeae*, three goals were established for this study:

1. **Examine the role of two highly conserved regions of TbpB in lipidation.**

   It was hypothesized that mutation of the LSAC motif would prevent lipidation of TbpB. This hypothesis was addressed by examining expression and lipidation of TbpB in two mutants: MCV839 (TbpB LSAC) and MCV43 (TbpB ΔGly).

2. **Determine whether MisR/MisS regulates expression of TbpA and TbpB.**

   It was hypothesized that the MisR/MisS system played a role in iron-dependent regulation of TbpA and TbpB. This hypothesis was addressed by examining protein and RNA expression of TbpA and TbpB in three strains: FA19 (wild type), misR, and misRc.

3. **Assess the ability of recombinant TdfJ to bind hemin when expressed in *E. coli*.**

   It was hypothesized that TdfJ would bind hemin when expressed in *E. coli*. It was also hypothesized that TdfJ overexpressed in its native organism would bind hemin. These hypotheses were addressed by performing hemin sedimentation assays with *E. coli* strains expressing TdfJ and various other TBDTs, and performing hemin sedimentation assays with gonococci overexpressing TdfJ. Hemin binding was also assessed with hemin binding blots.
Materials and Methods

I. Bacterial Strains and Growth Conditions

The *N. gonorrhoeae* and *E. coli* strains used throughout this study are listed in Table 1. Gonococcal strains were maintained on GC medium base (Difco) containing Kellogg’s supplement 1 (48) and 12 μM ferric nitrate at 37°C with the addition of 5% CO₂. Before growth in liquid media, gonococcal strains were passed twice on GC agar. Liquid cultures of gonococcal strains used in the palmitate labeling experiments were grown in chemically defined media (CDM) in acid-washed glassware. The CDM was rendered iron free by treatment with Chelex 100 (BioRad). Liquid cultures of gonococcal strains used in the MisR/MisS experiments were grown in gonococcal growth media (GCB) in acid-washed glassware. These strains were provided by the Shafer lab. Strains were grown in iron-replete and iron-deplete conditions for 2 or 4 hours after doubling. Iron-replete conditions were accomplished by the addition of ferric nitrate at the beginning of growth and at doubling, and iron-deplete conditions were accomplished by the addition of desferrioxamine B (desferal) at doubling. 1mM IPTG was added to the MisR complement strain. All liquid cultures of gonococcal strains were grown at 37°C with the addition of 5% CO₂ and shaking at 225 rpm. *E. coli* strains were cultured in Luria Broth (LB) containing 100 μg/ml carbenicillin, 50 μg/ml kanamycin, or 34 μg/ml chloramphenicol at 37°C with shaking at 225 rpm. Expression strains were induced using 0.02% L-arabinose or 3 mM IPTG added at an OD₆₀₀ = 0.5.
### Table 1. Strains and Plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Description</th>
<th>Source or Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>F- <em>ompT hsdSB</em> (rB-mB-) <em>gal dcm</em> (DE3)</td>
<td>Novagen</td>
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<tr>
<td>TOP10</td>
<td>F- <em>mcrA Δ(mrr-hsdRMS-mcrBC)</em> Φ 80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu) 7697 <em>galU galK rpsL</em> (StrR) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
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<td><strong>Gonococcal Strains</strong></td>
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<td></td>
</tr>
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<td>Wild type</td>
<td>Lab strain</td>
</tr>
<tr>
<td>FA1090</td>
<td>Wild type</td>
<td>Lab strain</td>
</tr>
<tr>
<td>FA6905</td>
<td>TbpB&lt;sup&gt;−&lt;/sup&gt; (ΔtbpB)</td>
<td>Lab strain</td>
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<tr>
<td>MCV407</td>
<td>FA1090 ΔopaKEB <em>tdfJ</em>&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Lab strain</td>
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<tr>
<td>MCV411</td>
<td>FA1090 ΔopaKEB <em>nspA</em>&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Lab strain</td>
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<tr>
<td>MCV412</td>
<td>FA1090 ΔopaKEB <em>tdfJ</em> (with foreign RBS)</td>
<td>Lab strain</td>
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<td>MCV839</td>
<td>TbpB LSAC (<em>lbpB::Ω</em>)</td>
<td>(71)</td>
</tr>
<tr>
<td>MCV843</td>
<td>TbpB ΔGly (<em>lbpB::Ω</em>)</td>
<td>(71)</td>
</tr>
<tr>
<td>misR</td>
<td></td>
<td>Shafer</td>
</tr>
<tr>
<td>misR&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td>Shafer</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pBAD-TOPO</td>
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<td>Invitrogen</td>
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<tr>
<td>pUNCH412</td>
<td>BL21(DE3)pLysE containing pUNCH412</td>
<td>(23)</td>
</tr>
<tr>
<td>pUNCH1313</td>
<td>pET30a containing <em>tdfH</em></td>
<td>(89)</td>
</tr>
<tr>
<td>pUNCH1316</td>
<td>pET30a containing <em>tdfG</em></td>
<td>(89)</td>
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<tr>
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<td>pET30a containing <em>tdfF</em></td>
<td>(89)</td>
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<td>This study</td>
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<td>This study</td>
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<tr>
<td>pVCU314</td>
<td>pBAD-TOPO containing <em>tdfJ</em> SS2</td>
<td>This study</td>
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</table>
II. Construction of Expression Plasmids

The full length *tdfJ* expression plasmid pVCU313 (Table 1) was constructed by first amplifying the *tdfJ* gene from wild-type gonococcal strain FA1090 genomic DNA. PCR was performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and primers (Table 2) were synthesized by Integrated DNA Technologies. The forward primer, oVCU764 (CCATGGCAGGAAATGTCACAAACATCACA), contained an NcoI site (bold) (Table 2) and amplified the *tdfJ* native signal sequence. The NcoI site was engineered onto the 5’ end of forward primer so that the N-terminal leader sequence provided by the pBAD-TOPO vector could be removed. The reverse primer, oVCU763 (TTAAAACTTCACGTTTACGCCGCGGTAAAGCTG), encoded the terminal phenylalanine of *tdfJ*, thus excluding the V5 epitope and polyhistidine region provided by the pBAD-TOPO vector (Table 2). The pVCU313 expression plasmid yielded what will be referred to as the start site 1 (SS1) expression strain.

A second *tdfJ* expression plasmid, pVCU314 (Table 2), was constructed in which the *tdfJ* gene was amplified from the second methionine, which is located 5 amino acids downstream of the first methionine in the protein sequence. This second expression plasmid was created because the true start site for *tdfJ* is unknown. The second forward primer, oVCU765 (CCATGGCACAAATCA CACTCAACCCATTGGTTTTA), also contained an NcoI site (bold) (Table 2). The pVCU314 expression plasmid yielded what will be referred to as the start site 2 (SS2) expression strain.

PCR products were then cloned directly into the pBAD-TOPO vector (Invitrogen) according to manufacturer’s instructions. Transformants were screened via restriction enzyme digest, followed by gel electrophoresis. The restriction enzymes used in screening and in the removal of the pBAD-TOPO leader sequence were obtained from New England Biolabs. During screening, expression plasmid pVCU312 (Table 1), containing the empty pBAD-TOPO vector with no
Table 2. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Amplicon</th>
<th>Sequence (5’-3’)</th>
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</thead>
<tbody>
<tr>
<td>oVCU68</td>
<td>tbpA RT-PCR Fwd</td>
<td>GGGGGTCACGAAAAGTGTAAAGCGA</td>
</tr>
<tr>
<td>oVCU72</td>
<td>tbpA RT-PCR Rev</td>
<td>CGACGCTTAGGGGAACCCTTTCAG</td>
</tr>
<tr>
<td>oVCU70</td>
<td>tbpB RT-PCR Fwd</td>
<td>TAAACAGTCCCGCCGTATTTGTGTT</td>
</tr>
<tr>
<td>oVCU73</td>
<td>tbpB RT-PCR Rev</td>
<td>GTGGGTCAGGTGCTCGTAAAGTTGTC</td>
</tr>
<tr>
<td>oVCU763</td>
<td>full length tdfJ Reverse</td>
<td>TTAAAACCTTCACGTGTTACGCGCCGTAAGGCTG</td>
</tr>
<tr>
<td>oVCU764</td>
<td>full length Nco1-tdfJ Fwd SS1</td>
<td>CCATGGCAGAGGCAAATGGCACAATCACA</td>
</tr>
<tr>
<td>oVCU765</td>
<td>full length Nco1-tdfJ Rev SS2</td>
<td>CCATGGCACAATACAATCGACAACCCATGTTTA</td>
</tr>
</tbody>
</table>
insert, was identified. After the restriction enzyme digest, transformants that resulted in bands corresponding to the correct size insert were then verified via DNA sequencing at the VCU Nucleic Acids Core. The resulting expression strains contained \textit{tdfJ} from start site 1 and start site 2, under control of the \textit{araBAD} promoter (\textit{P}_{\text{BAD}}), which is tightly regulated by the product of the \textit{araC} gene. L-arabinose forms a complex with AraC which allows transcription to begin.

**III. Recombinant Protein Expression**

For induction of \textit{TdfJ} expression from pVCU313 and pVCU314, overnight cultures of the \textit{E. coli} were grown in LB containing 100 μg/ml carbenicillin at 37°C with shaking at 225 rpm. For induction of \textit{TbpA} expression from pUNCH412, overnight cultures of the \textit{E. coli} were grown in LB containing 100 μg/ml carbenicillin at 37°C with shaking at 225 rpm. For induction of \textit{TdfF} expression from pUNCH1321 and \textit{TdfG} expression from pUNCH1316, overnight cultures of the \textit{E. coli} were grown in LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37°C with shaking at 225 rpm. For induction of \textit{TdfH} expression from pUNCH1313, overnight cultures of the \textit{E. coli} were grown in LB containing 50 μg/ml kanamycin at 37°C with shaking at 225 rpm. 100 μl of overnight culture was then used to inoculate 10 mL LB containing the appropriate antibiotics. Cultures were grown for approximately 2 hours until they reached an OD$_{600}$=0.5. Once the appropriate density was achieved either L-arabinose (Sigma Aldrich) or isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the cultures to induce protein expression. To induce \textit{TdfJ} expression from start site 1 and start site 2, L-arabinose was added to the cultures at a final concentration of 0.02%. The empty vector control was treated in the same manner. To induce \textit{TdfF}, \textit{TdfG}, and \textit{TdfH} expression, 3 mM IPTG was added to the cultures. To induce \textit{TbpA} expression, 1 mM IPTG was added to the cultures. For each strain an induced and uninduced condition was performed. No L-
arabinose or IPTG was added to the uninduced cultures to serve as a negative control. The cultures were then allowed to grow for an additional 4 hours at 37°C with shaking at 225 rpm. Following the four hour growth, whole cell lysates were made by pelleting 1 mL of culture. The pellets were solubilized in 2x laemmlı solubilizing buffer for subsequent SDS-PAGE analysis.

IV. Whole Cell Dot Blots

Whole cell dot blots were performed in order to test for surface exposure of gonococcal proteins expressed within the various *E. coli* expression strains. *E. coli* cultures were grown as described above. First, nitrocellulose was wetted in LS-TBS and placed in the S&S Minifold I Dot Blot system (Whatman) on top of gel blot paper (Whatman). After a 4 hour induction, cultures were standardized to 10,000 Ku (Klett unit) and added to wells in duplicate or triplicate. The membrane was allowed to dry for one hour and it was removed and stored overnight. The membrane was blocked, probed, and developed in the same manner as a western blot. The empty vector control (pVCU312) was included on the dot blots and was probed with every protein specific antibody to ensure antibody specificity.

A) Detection of TbpA (using HRP-Tf)

In order to detect TbpA, nitrocellulose membranes were first blocked with HS-TBS containing 5% BSA for one hour with gentle shaking. The membrane was probed with primary HRP-Tf at a dilution of 1:10,000 for 1 hour. The membrane was washed 3 times for 10 minutes in HS-TBS containing 0.05% Tween 20. The membrane was developed using the Opti-4CN system (Biorad).
B) Detection of TdfF, TdfG, and TdfH

In order to detect TdfF, TdfG, and TdfH, nitrocellulose membranes were first blocked with HS-TBS containing 5% BSA for one hour with gentle shaking. The membrane was probed with primary anti-TdfF, TdfG, and TdfH antibodies at a dilution of 1:5,000 for 1 hour. The membrane was washed 3 times for 10 minutes in HS-TBS. The primary antibody was detected with secondary goat anti-rabbit IgG-AP (Southern Biotech) at a dilution of 1:5,000 for 1 hour. The membrane was washed again, and then developed using the NBT/BCIP system.

D) Detection of TdfJ

TdfJ was detected using anti-TdfJ (guinea pig 117) as described below.

V. Hemin Sedimentation Assays

Hemin sedimentation assays were performed in order to assess the ability of each gonococcal protein, expressed on the surface of E. coli, to bind hemin. For this experiment the following proteins were assessed: TbpA (pUNCH412), TdfF (pUNCH1321), TdfG (pUNCH1316), TdfH (pUNCH1313), TdfJ from SS1 (pVCU313), and TdfJ from SS2 (pVCU314). The TdfF, TdfG, and TdfH expression strains were provided by the Sparling lab (89). The TbpA expression strain was created by Dr. Cornelissen (23). The strain containing the empty vector (pVCU312) served as the negative control. Cultures were grown and protein expression was induced for four hours. After the four hour induction, 1 mL of culture was removed in order to prepare whole cell lysates for subsequent SDS-PAGE analysis. The remainder of the culture was moved to a 14 mL conical tube and was centrifuged at 3,000 rpm for 15 minutes. The supernatant was removed and the pellet was resuspended in 1 mL sterile PBS containing 5% dimethyl sulfoxide (DMSO). The DMSO was included in order to prevent
non-specific hemin binding. The cells were centrifuged at 3,000 rpm for 15 minutes. The supernatant was removed and the pellet was again resuspended in 1 mL sterile PBS containing 5% DMSO. The cultures were standardized to and OD$_{600}$=1. Hemin (Sigma Aldrich), dissolved in 0.1 N sodium hydroxide (NaOH) and PBS containing 5% DMSO at a concentration of 15 µM and 20 µM, was added to the appropriate wells of a v-bottom 96-well plate. 150 µl of the standardized cultures was added to the appropriate wells containing hemin. Each condition was performed in quadruplicate for both induced and uninduced conditions and in the presence of: no hemin, 15 µM hemin, or 20 µM hemin. Wells containing only hemin and PBS containing 5% DMSO at 15 µM and 20 µM (no cells) served as the 100% saturated, or 100% unbound, control. These wells were used as the “100% unbound” control because they represent the condition in which no hemin binds the cells, leaving all of the hemin unbound. The plate was allowed to incubate at room temperature for one hour with gentle rocking. After incubation, the plate was centrifuged at 3,000 rpm for 15 minutes in order to pellet the cells and any hemin that they precipitated. 100 µl of the supernatant in each well was transferred to a flat-bottom 96-well plate and the OD$_{405}$ of each sample was read with a plate reader. The OD$_{405}$ of each well was compared to the corresponding 100% saturated control well and the percent hemin bound was calculated. This was done by multiplying the OD$_{405}$ value by 100 and then dividing the product by the OD$_{405}$ value of the 100% saturated control well. This resulted in the percent hemin unbound, indicating the amount of hemin remaining in the supernatant that did not bind to the cells. This value was then subtracted from 100, yielding the percent hemin bound for each condition. This assay was also done with gonococcal strains in order to assess TdfJ's ability to bind hemin in its native organism. MCV407, the tdfJ knockout, and MCV412, the tdfJ complement strain which overexpresses TdfJ, were grown in GCB for 5 hours in the shaker.
incubator at 37°C with shaking at 225 rpm with the addition of 5% CO₂ in acid-washed flasks. MCV411, the nspA complement strain, was used as a negative control. IPTG was added to the induced complement strain flasks at the beginning of growth. Uninduced MCV412 was also cultured. After growth, the assay was performed in the way described above with one exception. During the one hour incubation step during which the hemin is allowed to bind to the cells, the 96-well plate was kept in the shaker incubator at 37°C with gentle shaking and the addition of 5% CO₂. Protocol for hemin sedimentation assay was adapted from the one used to assess heme binding ability of meningococcal ZnuD (51).

VI. Multiple Sequence Alignment

TdfJ, FetA, and TbpA were aligned with other representative heme receptors using ClustalW, a multiple sequence alignment program (52). The slow/accurate setting was used to align the given protein sequences. For the pairwise alignment parameters, the gap open penalty was reduced to 5. For the multiple alignment parameters, the gap open penalty was reduced to 5. The multiple sequence alignment was then uploaded to Jalview (95), and the presence of the FRAP motif, the conserved histidine, and the NPNL motif was assessed.

VII. Ribonuclease Leakage Assays

Ribonuclease (RNase) leakage assays were performed in order to see which E. coli expression strains had leaky outer membranes. It has previously been shown that the TbpA E. coli expression strain (pUNCH412) leaks RNase. The E. coli strains used in the assay were grown on LB agar plates containing the appropriate antibiotics (without RNA) overnight at 37°C. LB agar plates containing carbenicillin, kanamycin, or kanamycin and chloramphenicol were made that also contained RNA. Type VI ribonucleic acid from torula yeast (Sigma) was
added to the media at a final concentration of 1.5%. The media, antibiotic, and RNA mixture was brought to a pH of 7.0 with 10 N NaOH before adding the agar, autoclaving, and pouring the plates. After the plates cooled, either L-arabinose or IPTG was spread on top of the plates to induce protein expression. A final concentration of 0.02% L-arabinose was spread onto plates containing RNA and carbenicillin in order to induce expression of TdfJ from SS1 and SS2. A final concentration of 3mM IPTG was spread onto plates containing RNA and carbenicillin, kanamycin, or kanamycin and chloramphenicol to induce TbpA, TdfF, TdfH, and TdfG expression. The strains were re-streaked onto these plates and grown overnight at 37°C. The following day, the plates were gently flooded with 0.1 N HCl and photographed. The E. coli strain expressing the empty vector was used as a negative control and the E. coli strain expressing TbpA was used as a positive control. If the bacteria were leaking RNase, the addition of HCl would cause a dark halo around the bacterial growth. If the bacteria were not leaking RNase, the agar would remain opaque and off-white in color.

VIII. Palmitate Labeling of Lipidated Proteins

In order to assess the importance of the LSAC motif to the lipidation of TbpB, the Click-iT labeling system (Invitrogen) was employed. The following strains were used for this experiment: FA19 (wild type), FA6905 (TbpB⁻), MCV839 (TbpB LSAC), and MCV843 (TbpB ΔGly). FA6905 does not produce TbpB, and therefore served as a negative control. In MCV839, the LSAC sequence was mutated to an LAAA. In MCV843, a conserved stretch of four glycine residues downstream of the LSAC sequence was deleted. The strains were grown in CDM as described above. After doubling, azide-labeled palmitic acid (Invitrogen) was added to a final concentration of 50µM. Cultures were then allowed to grow for an additional 4 hours. Bacteria were then centrifuged, washed twice with sterile PBS, and resuspended in lysis buffer (50 mM
Tris [pH 8.0], 0.1% SDS). Benzonase nuclease was added and the bacteria were allowed to incubate for 1 hour at 4° to ensure complete lysis. Samples were centrifuged at a low speed in order to get rid of intact bacteria and cellular debris. The lysate was transferred to a clean tube and solubilized proteins were coupled to biotin alkyne according to the manufacturer’s instructions. Samples were resuspended in Laemmli solubilizing buffer and stored at -20°C. Samples were heated at 100°C for 2 minutes and loaded onto a 7.5% polyacrylamide gel (without the addition of β-mercaptoethanol). After electrophoresis, the proteins were transferred to nitrocellulose and stained as detailed below. The same blot was then probed with avidin-HRP (Biorad) to detect palmitoylated proteins, and then again with anti-TbpB antibody to detect full length TbpB, as described below.

IX. Detection of Lipidated Proteins

In order to detect lipidated proteins, nitrocellulose membranes were first blocked with high salt Tris-buffered saline (HS-TBS) (20mM Tris base, 500mM NaCl) containing 1% bovine serum albumin (BSA) for one hour with gentle shaking. The membrane was then probed with avidin-HRP (Biorad) at a dilution of 1:2,000 for 1 hour. The blot was washed 3 times for 10 minutes in HS-TBS containing 0.05% Tween 20, and developed using enhanced chemiluminescence (ECL) (Pierce) according to the manufacturer’s instructions. Bands were visualized by exposing X-ray film to the blots after the addition of the ECL reagents. The same blot was then re-blocked and probed for full length TbpB as detailed below.

X. Western Analysis

Samples of *N. gonorrhoeae* or *E. coli* were grown as described above. For the MisR/MisS experiments, gonococcal cultures were grown under iron-deplete and iron-replete
conditions, standardized to cell density, and pelleted. For analysis of protein expression within the \textit{E. coli} expression strains used in the hemin sedimentation assays, cultures were induced for four hours and 1 mL was removed and centrifuged at 14,000 rpm for 1 minute. All cell pellets were resuspended in 100 µl 2X laemmli solubilizing buffer and stored at -20°C. When needed, 1 µl of benzonase buclease (Sigma) was added to whole cell lysates in order to reduce viscosity. Whole cell lysates were incubated with the benzonase at room temperature for 30 minutes. Before loading, 5% β-mercaptoethanol (Sigma) was added and samples were heated at 95°C for 2 minutes. Proteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated on 7.5% polyacrylamide gels using a Mini-Protean III apparatus (Biorad). Separated proteins were subsequently transferred to a nitrocellulose membrane (86) in 20mM Tris base, 150mM glycine and 20% methanol in a submerged transfer apparatus (Biorad) for 16 hours at a constant current of 28mA. After the transfer, membranes were washed quickly with deionized water and then stained with Ponceau S solution (0.1% w/v Ponceau S, 5% acetic acid) for roughly 10 minutes to ensure equal loading. Excess dye was removed with deionized water and the stained gels were scanned.

\textbf{A) Detection of TbpA (with anti-TbpA antibody, Rabbit 11581)}

In order to detect TbpA, nitrocellulose membranes were first blocked with HS-TBS containing 5% BSA for one hour with gentle shaking. The membrane was probed with primary anti-TbpA antibodies at a dilution of 1:1,000 for 1 hour. The membrane was washed 3 times for 10 minutes in HS-TBS containing 0.05% Tween 20. The primary antibody was detected with secondary goat anti-rabbit IgG-AP (goat anti-rabbit IgG conjugated to alkaline phosphatase) (Southern Biotech) at a dilution of 1:5,000 for 1 hour. The membrane was washed again, and
developed using the nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) development system (Sigma).

**B) Detection of TbpB (with anti-TbpB antibody, Rabbit 162-3)**

In order to detect TbpB, nitrocellulose membranes were first blocked with low salt Tris-buffered saline (LS-TBS) (50 mM Tris, 150 mM NaCl) containing 5% non-fat skim milk (Sigma) for one hour with gentle shaking. The membrane was probed with primary anti-TbpB antibodies at a dilution of 1:5,000 for 1 hour. The membrane was washed 3 times for 10 minutes in LS-TBS containing 0.05% Tween 20. The primary antibody was detected with secondary goat anti-rabbit IgG-AP (Southern Biotech) at a dilution of 1:3,000 (for palmitate labeling experiment) or 1:5,000 (for the MisR/MisS experiments) for 1 hour. The membrane was washed again, and developed using the NBT/BCIP system.

**C) Detection of TdfJ (with anti-TdfJ antibody, Guinea Pigs 116, 117, 118, and 119)**

In order to detect TdfJ, nitrocellulose membranes were first blocked with low salt Tris-buffered saline (LS-TBS) containing 5% non-fat skim milk (Sigma) for one hour with gentle shaking. The membrane was probed with primary anti-TdfJ antibody at 1:200 (guinea pigs 116 and 117), or at 1:100 (guinea pigs 118 and 119) for one hour. Once anti-TdfJ antibodies were characterized antibody 117 was used at 1:200 dilution for the remaining experiments. The membrane was washed 3 times for 10 minutes in LS-TBS containing 0.05% Tween 20. The primary antibody was detected with secondary rabbit anti-guinea pig IgG-AP (Thermo Scientific) at a dilution of 1:3,000 for 1 hour. The membrane was washed again, and developed using the NBT/BCIP system.
Alternatively, the primary anti-TdfJ antibody was detected with goat anti-guinea pig IgG-HRP (Southern Biotech) and developed using ECL (Pierce) according to the manufacturer’s instructions. Bands were visualized by exposing autoradiographic film to the blots after the addition of the ECL reagents.

XI. Coomassie Blue Protein Staining

Following electrophoresis, SDS-PAGE gels were stained with Coomassie blue (0.25% Coomassie R-250, 50% methanol, 10% glacial acetic acid) overnight at room temperature. The following day the gels were then destained in 20% methanol and 5% glacial acetic acid at room temperature with gentle shaking for 3-6 hours until background staining was diminished.

XII. Hemin Binding Blots

Hemin binding blots were performed in order to identify hemin-binding gonococcal proteins. FA1090 sarkosyl insoluble outer membrane preparations (OMP), provided by a fellow lab member, were used for the hemin binding blots. The FA1090 outer membrane preparations were made from cultures grown in iron-replete and iron-deplete conditions. The proteins were separated on 7.5% and 15% polyacrylamide gels by subjecting them to electrophoresis. Duplicate gels were made for both the 7.5% and 15% gels. After electrophoresis, one of each of percentage gel was transferred onto nitrocellulose according to the method described above. The remaining gels were stained with Coomassie blue as detailed above. The gels that were transferred onto nitrocellulose were subsequently rinsed with TBS containing Tween 20 (10 mM pH 8.0 Tris-HCl containing 150 mM NaCl and 0.1% Tween 20) 3 times for 10 minutes. The blots were probed with TBS containing $10^{-6}$ M hemin (Sigma Aldrich) for 1.5 hours at room temperature with gentle shaking. The blots were washed 3 times for 10 minutes. Bound hemin
was visualized via its inherent peroxidase activity using ECL. The blots were exposed to autoradiographic film for approximately 20 seconds. Bands on the Coomassie stained gels that corresponded to hemin binding bands on the film were excised and sent for protein identification at the VCU Mass Spectrometry Core.

XIII. RNA Isolation

Gonococcal cultures were grown in GCB in iron-replete and iron-deplete conditions as detailed above. At 2 hours and 4 hours after doubling, 5 mL of culture was mixed with 10 mL RNAProtect (Qiagen), vortexed for 5 seconds, and incubated at room temperature for 5 minutes. The culture was centrifuged for 15 minutes at 3,000 rpm. The supernatant was decanted and the pellets were stored at -20°C. Total RNA was isolated using the RNeasy Mini Kit according to manufacturer’s instructions (Qiagen). Purified RNA was cleaned up and treated with RNase-free DNase twice as directed by the manufacturer. Lastly, 1 µl of Superase-In (Ambion) was added prior to storage at -80°C.

XIV. Qualitative Reverse Transcriptase PCR

RNA isolated from FA19 (wild type), misR (MisR−), and misRc (complement) strains grown under iron-replete and iron-deplete conditions was reverse transcribed using the ThermoScript RT-PCR system (Invitrogen). In order to create the cDNA, random hexamers, RNA, and dNTPs were mixed and incubated at 70°C for 10 minutes in order to denature the RNA. The cDNA synthesis mix was made according to manufacturer’s instructions and added to each reaction tube while on ice. The mixture was incubated at 25°C for 10 minutes, followed by a 1 hour incubation at 60°C. The reaction was terminated by incubating the reaction tubes at 85°C for 5 minutes. Finally, 1 µl of RNase H was added and the tubes were incubated at 37°C
for 20 minutes. In order to rule out DNA contamination, parallel reactions were carried out in the absence of reverse transcriptase.

Using the resultant cDNA, portions of TbpA and TbpB were amplified from each condition using Platinum Taq DNA Polymerase (Invitrogen) according to the manufacturer’s instructions. The primers used for PCR amplification are located in Table 2. In order to amplify part of TbpA, oVCU68 (GGGGGTCACGAAAAGTGTAAAGCGA) and oVCU72 (GGGGGTCACGAAAAGTGTAAAGCGA) were used. In order to amplify part of TbpB, oVCU70 (CGACGCTTAGGGGAAACCGCTTCAG) and oVCU73 (GTCGGGTAGCGTTGCTGAAGTTGTC) were used. TbpA and TbpB were also amplified from FA19 chromosomal DNA using the same primers for size comparison. After the initial denaturation step at 95°C for 5 minutes, DNA was amplified for a total of 30 cycles. Each cycle began with 1 min at 94°C to denature, followed by 30 seconds at 59°C to anneal, and 1 minute and 20 seconds at 72°C to extend the DNA. Lastly, there was a final extension step of 10 minutes at 72°C. Amplicons were detected by ethidium bromide staining of 2.5% agarose gels.
Results

I. Role of LSAC Motif in the Lipidation of TbpB

As mentioned previously, TbpB is the surface tethered lipoprotein that works in conjunction with TbpA, a TBDT, to transport iron from human transferrin. TbpA expression is essential for iron acquisition from transferrin, however the presence of TbpB is not required but makes the process more efficient. The \textit{tbpB} gene is present in all gonococcal isolates characterized to date, and has been shown to be expressed during infection (1). Due to its potential as a vaccine candidate, further study into the presentation of TbpB on the gonococcal cell surface is warranted.

In order to learn more about the presentation of the TbpB protein on the bacterial surface, the role of two highly conserved regions within TbpB were assessed. The first conserved region of interest was the LSAC motif. This motif is representative of the classical lipobox, and is located at the amino terminus of TbpB. This motif consists of four residues, and it is the recognition site for signal II peptidase. The cysteine residue within the LSAC sequence serves as the first amino acid in the mature protein after cleavage. It was hypothesized that this cysteine is also the site of lipidation, which anchors TbpB to the outer membrane. TbpB was previously demonstrated to be lipidated (4), but the role of the LSAC motif in this process has not yet been
elucidated. Lipidation is the covalent modification of a protein with a variety of lipids. Many outer membrane proteins undergo lipidation, and these lipid modifications play important roles in protein function and localization (64). The second conserved region, consisting of four glycine residues in tandem, is two amino acid residues downstream of the LSAC motif.

A. LSAC Motif is Necessary for the Lipidation of TbpB

In order to assess the roles of these two conserved regions on the lipidation of TbpB, two mutant strains were tested: MCV839 and MCV843 (Table 1). MCV839 has the LSAC sequence mutated to LAAA. MCV843 contains a deletion of the four conserved glycines. FA19 was used as the wild type control, while FA6905 was used as the negative control because it is unable to express TbpB. These four strains were grown under iron-depleted conditions in chemically defined media in the presence of azide labeled palmitic acid. Biotin alkyne was then conjugated to the proteins containing the palmitic acid. Whole cell lysates of the cultures were prepared and subjected to SDS-PAGE, and subsequently transferred to nitrocellose. Palmitoylated proteins were then detected using avidin-HRP and developed using ECL (Figure 4A). TbpB was lipidated in FA19 (wild type) and in MCV843 (TbpB ΔGly mutant), but not in MCV839 (TbpB LSAC mutant). There is a lipidated protein slightly larger than TbpB which is apparent in all lanes; however, the black arrow denotes the band of interest whose size corresponds to the size of TbpB. The same blot was also probed with anti-TbpB antibodies and developed using the NBT/BCIP system (Figure 4B). Full length TbpB was detected in FA19, MCV839, and MCV843, but not in FA6905 (TbpB⁻). Based on these results, we concluded that although full length TbpB was produced by MCV839, it was not lipidated. On the other hand, lipidated, full length TbpB was expressed in MCV843. Therefore, the LSAC mutation had an effect on TbpB lipidation and the glycine deletion did not.
Figure 4. TbpB produced by MCV839 is not lipidated. (A) Wild type, \textit{tbpB} \textsuperscript{-}, LSAC mutant, and glycine deletion mutant were grown under iron-depleted conditions in the presence of azide-labeled palmitic acid. Biotin alkyne was then conjugated to any proteins containing the palmitic acid. Whole cell lysates were prepared and subjected to SDS-PAGE. Following transfer to nitrocellulose, proteins containing biotin-azide labeled palmitic acid were detected using avidin-HRP and developed with ECL. (B) Full length TbpB was detected on the same blot using anti-TbpB antibody 162-4 and developed using the NBT/BCIP system. Approximate positions of molecular mass markers are shown on the right.
Figure 4. TbpB produced by MCV839 is not lipidated.
II. Regulation of the Transferrin Binding Proteins by MisR/MisS

The transferrin binding proteins, TbpA and TbpB, are critical to the uptake of iron from human transferrin. Since they are involved in the uptake of iron, they are heavily regulated in order to maintain an optimal intracellular iron concentration. Expression of both proteins is repressed under iron-replete conditions (24). The system is also regulated by Fur, and the Fur box lies upstream of the TbpB start site.

RNA-Seq studies performed by the Shafer lab indicated that *tbpA* and *tbpB*, among many other genes, were effected by MisR expression. The Shafer lab also showed that MisR was able to bind upstream of the *tbpBA* operon via DNA footprinting. Based on these preliminary results it was hypothesized that gonococcal MisR/MisS may also play a role in regulating TbpA and TbpB.

MisR/MisS is analogous to the PhoP/PhoQ system in Salmonella. The PhoP/PhoQ system responds to various antimicrobial peptides (79), and this seems to hold true for the MisR/MisS system in *N. gonorrhoeae*, as it has been shown to respond the antimicrobial peptide LL-37. The MisR/MisS system regulates and influences numerous biological functions within *N. meningitidis*. Genes regulated by MisR/MisS in *N. meningitidis* are necessary for transcriptional regulation, iron assimilation, metabolism, type I protein transport, and protein folding (91). MisR/MisS also influences the immunotype and inner core structure of LOS in the meningococcus (90). Of the TBDTs expressed by *N. meningitidis*, only *tdfH* and *hmbR* are affected by MisR/MisS (91). HmbR is the meningococcus-specific hemoglobin outer membrane receptor (82).
A. Protein Expression of TbpA and TbpB is regulated by MisR/MisS

In order to examine the effect that MisR has on expression of TbpA and TbpB, protein expression in the following strains was examined via western blot: FA19 (wild type), misR (MisRˉ), and misRc (MisR complement). The strains were grown in GCB under iron-replete and iron-depleted conditions for two or four hours after doubling. In order to achieve iron-replete conditions, ferric nitrate was added to the media at the beginning of growth and at doubling. In order to achieve iron-deplete conditions, no ferric nitrate was added to the media, and desferal was added at doubling. 1mM IPTG was added to the misRc strain at the beginning of growth in order to induce the expression of ectopically provided misR. Whole cell lysates were made from the cultures at 2 hours and 4 hours post doubling. The whole cell lysates were then subjected to SDS-PAGE and the separated proteins were subsequently transferred onto nitrocellulose membranes. The membranes were then probed for TbpA or TbpB using protein specific antibodies and developed using the NBT/BCIP system.

At 2 hours post doubling, expression of TbpA looks roughly equivalent across all strains under iron-deplete conditions. However, expression of TbpA in the misR strain is much greater than in the wild type or complement strains in iron-replete conditions (Figure 5A). This indicates that MisR down-regulates expression of TbpA under iron-replete conditions, and this effect is lost when MisR is not present. At 4 hours post doubling, the same trend is not seen (Figure 5B). At this time point it appears that expression of TbpA in the wild type strain is greater than expression of TbpA in both the misR and misRc strain under both iron-deplete and iron-replete conditions. It is also apparent at both time points that the expression of TbpA in misRc strain does not appear to fully complement wild type expression levels. Expression levels in misRc strain
Figure 5. MisR regulated TbpA protein expression. Strains were grown for 2 hours (A) and 4 hours (B) after doubling in iron-replete and iron-depleted conditions. Standardized whole cell lysates were made and subjected to SDS-PAGE. After transfer to nitrocellulose, the blots were probed with anti-TbpA antibody 11581 in order to examine the effects on TbpA expression. The arrows on the right side indicate the band of TbpA expression. Molecular mass markers are shown on the left. The non-specific band detected at approximately 45 kD is used to show equivalent loading.
Figure 5. MisR regulated TbpA protein expression.
appear to fall somewhere between the wild type strain and the mutant strain. One possible explanation for this phenomena is that insertionally inactivating MisR results in decreased MisS expression. It is not a polar mutation, but is likely causing differences in protein expression between wild type and complement. Another possible explanation for this phenomena is that differences in expression are caused by the fact that misR is ectopically provided in the complement strain. The ectopically provided misR has an IPTG inducible promoter, and is therefore expressed in a different way than in the wild type strain.

At 2 hours post doubling, TbpB expression also looks roughly equivalent across all strains under iron-depleted conditions. However, expression of TbpB in the misR strain is greater than in the wild type or complement strains in iron-replete conditions (Figure 6A). This indicates that MisR is also down-regulating the expression of TbpB under iron-replete conditions, and this effect is lost when MisR is not present. At 4 hours post doubling, the trend is mostly lost (Figure 6B). However, expression of TbpB in the misR strain under iron-replete conditions looks to be slightly greater than TbpB expression in the wild type or complement strains under iron-replete conditions. Interestingly, there is a marked increase in the detection of TbpB breakdown products in the misR strain under iron-deplete conditions, despite the fact that expression of full length TbpB looks approximately equivalent across all strains (Figure 6B). Again, the misRstrain does not appear to fully complement wild type levels of TbpB expression.

B. *tbpA and tbpB* transcripts are regulated by MisR/MisS

In order to examine the affect that MisR has on the expression of TbpA and TbpB, transcripts from the following strains were detected via RT-PCR: FA19 (wild type), misR (MisRˉ), and misRˉ (MisR complement). The strains were grown in GCB under iron-replete and iron-depleted conditions for four hours after doubling. In order to achieve iron-replete
Figure 6. **MisR regulated TbpB protein expression.** Strains were grown for 2 hours (A) and 4 hours (B) after doubling in iron-replete and iron-depleted conditions. Standardized whole cell lysates were made and subjected to SDS-PAGE. After transfer to nitrocellulose, the blots were probed with anti-TbpB antibody 162-4 in order to examine the effects on TbpB expression. The arrows on the right side indicate the band of TbpB expression. A section of the ponceau stain is included below the blot to show even loading. Molecular mass markers are shown on the left.
Figure 6. MisR regulated TbpB protein expression.
conditions, ferric nitrate was added to the media at the beginning of growth and at doubling. In order to achieve iron-deplete conditions, no ferric nitrate was added to the media, and desferal was added at doubling. 1mM IPTG was added to the misR<sup>c</sup> strain at the beginning of growth in order to induce the expression of ectopically provided misR. At 2 hours and 4 hours post doubling, culture was removed, treated with RNAprotect (Qiagen), pelleted, and stored before total RNA was isolated using the RNeasy Mini Kit (Qiagen). Total RNA from each strain was reverse transcribed using the ThermoScript RT-PCR system (Invitrogen). Using the resultant cDNA, portions of TbpA and TbpB were amplified from each condition using Platinum Taq DNA Polymerase (Invitrogen). Amplicons were then detected using ethidium bromide staining of 2.5% agarose gels.

The trend seen above in the analysis of TbpA protein expression is very similar to the one seen here for TbpA RNA expression (Figure 7A). The amount of TbpA-specific RT-PCR product looks roughly equivalent across all strains under iron-deplete conditions. However, the amount of TbpA-specific RT-PCR product in the misR strain was much greater than in the wild type or complement strains in iron-replete conditions (Figure 7A). This indicates that MisR is down-regulating the expression of TbpA under iron-replete conditions, and this effect is lost when MisR is not present. The same trend is seen at the 4 hour time point (Figure 7B). The trend seen above in the analysis of TbpB protein expression is very similar to the one seen here for TbpB-specific transcript levels (Figure 8A). The amount of TbpB-specific RT-PCR product looks roughly equivalent across all strains under iron-depleted conditions. However, the amount of TbpB-specific RT-PCR product in the misR strain was much greater than in the wild type or complement strains in iron-replete conditions (Figure 8A). This indicates that MisR is down-regulating the expression of TbpA under iron-replete conditions, and this effect is lost when
Figure 7. MisR regulated TbpA RNA expression. Strains were grown for 2 hours (A) and 4 hours (B) after doubling in iron-replete and iron-depleted conditions. Total RNA was isolated and reverse transcribed. Part of TbpA was amplified from the cDNA and detected using ethidium bromide staining of 2.5% agarose gels. The expected product size of the TbpA amplicon was 343 bp. The same part of TbpA was amplified from chromosomal DNA and loaded on the far right for size comparison. Molecular mass markers are shown on the left and right.
Figure 7. MisR regulated TbpA RNA expression.
Figure 8. MisR regulated TbpB RNA expression. Strains were grown for 2 hours (A) and 4 hours (B) after doubling in iron-replete and iron-depleted conditions. Total RNA was isolated and reverse transcribed. Part of TbpB was amplified from the cDNA and detected using ethidium bromide staining of 2.5% agarose gels. The expected product size of the TbpB amplicon was 360 bp. The same part of TbpA was amplified from chromosomal DNA and loaded on the far right for size comparison. Molecular mass markers are shown on the left and right.
Figure 8. MisR regulated TbpB RNA expression.
MisR is not present. At 4 hours post doubling, the same trend is seen (Figure 8B). At both 2 and 4 hours post doubling, the difference in the amount of TbpB-specific RT-PCR product present under iron-replete conditions in each strain is much more pronounced than in the analysis of TbpA. The band representing TbpB-specific RT-PCR product in the wild type and complement strains is very faint, while the band representing TbpB DNA in the misR mutant strain is much darker. The difference between these bands is much more pronounced for TbpB, especially at the 4 hour time point. This could indicate that MisR plays a larger role in regulating TbpB than TbpA. Also, the trend seen for protein expression at the 2 hour time point for both TbpA and TbpB is lost at 4 hours, while the trend seen for the analysis of RNA expression holds for both time points.

III. Characterization of TdfJ

TdfJ is a largely uncharacterized TBDT expressed by N. gonorrhoeae. TdfJ is iron induced (30) and is present in all commensal and pathogenic Neisseriae. The meningococcal homologue of TdfJ is known as ZnuD, and it has been demonstrated to facilitate zinc acquisition (83). Bactericidal antibodies were induced in mice immunized with outer membrane vesicles from a strain overexpressing ZnuD. Also, ZnuD-specific antibodies were detected in the sera of patients recovering from meningococcal infection (83). TdfJ is a potential vaccine target because it is surface exposed, well conserved, immunogenic, and is present in all strains of N. gonorrhoeae to date. Therefore, research on the function of gonococcal TdfJ is warranted. Based on sequence similarity to other heme transporters, it was hypothesized that TdfJ would bind hemin when expressed in E. coli and when overexpressed in its native organism.
A. Characterization of TdfJ antisera

TdfJ antisera was produced for use in future studies. Amino acid sequences in putatively surface exposed loops of TdfJ were identified and used make peptides. New England Peptide generated the peptides, conjugated the peptides to keyhole limpet hemocyanin (KLH), and subsequently immunized four guinea pigs. Peptide 1 (Ac-KRLPDHSADTSQTGSIC-amide) is located in putative loop 2, and peptide 2 (Ac-VRVEKQKASIRYDKAC-amide) is located in putative loop 5 of TdfJ. Guinea pigs 116 and 117 were immunized with peptide 1, yielding serum 116 and serum 117. Guinea pigs 118 and 119 were immunized with peptide 2, yielding serum 118 and serum 119. After immunization, pre-immunization and post-immunization samples from each guinea pig were sent from New England Peptide. Western blots were performed with each of the samples in order to assess whether or not the sera reacted specifically with TdfJ. Whole cell lysates, provided by another lab member, were prepared from the following strains grown in iron-replete and iron-depleted conditions, or in the presence or absence of IPTG: FA1090 (wild type), MCV407 (tdfJ<sup>−</sup>), MCV412 (tdf<sup>J</sup>C), and FA1090 sarkosyl insoluble outer membrane preparations (OMP). MCV407 cannot express TdfJ and was therefore used as a negative control. The whole cell lysates and OMPs were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. Following transfer, one membrane was probed with the each pre-immunization serum, and another membrane was probed with each post-immunization serum. The blots were then developed using the NBT/BCIP system.

Pre-immunization serum from guinea pig 116 used at a dilution of 1:50 did not detect anything on the membrane (Figure 9A). However, post-immunization serum used at the same dilution detected TdfJ in the induced and uninduced complement, as well as in the OMP under iron-replete conditions (Figure 9B). TdfJ is approximately 80 kD, so the bands are the
Figure 9. Serum 116 reacted specifically with TdfJ. Strains grown in iron-replete or iron-depleted conditions, or in the presence (IN) or absence (UN) of IPTG, were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were probed with pre-immunization serum 116 (A) or post-immunization serum 116 (B). TdfJ is approximately 80 kD in size. Molecular mass markers are shown on the left.
Figure 9. Serum 116 reacted specifically with TdfJ.
appropriate size. TdfJ was not detected in the \( tdf/J^- \) strain, and surprisingly, was also not detected in the wild type strain under iron-replete or iron-depleted conditions. These results indicate that serum 116 reacts specifically with TdfJ. The intensity of the bands, coupled with the fact that TdfJ was not detected in the wild type strain, indicate that the complemented strain (MCV412) overexpressed TdfJ.

Pre-immunization serum from guinea pig 117 used at a dilution of 1:50 did not detect anything on the membrane (Figure 10A). There are very faint bands present in all lanes, but they are not same size as TdfJ, and are therefore non-specific. Post-immunization serum used at the same dilution detected TdfJ in the induced and uninduced complement, as well as in the OMP under iron-replete and iron-depleted conditions (Figure 10B). The band is more intense in the +Fe OMP than the –Fe OMP lane, demonstrating that TdfJ is iron induced. Again, TdfJ was not detected in the \( tdf/J^- \) strain or in the wild type strain under iron-replete or iron-deplete conditions. These results indicate that serum 117 reacts specifically with TdfJ.

Pre-immunization serum from guinea pig 118 used at a dilution of 1:50 did detect bands in every lane, but they are not same size as TdfJ, and are therefore non-specific. (Figure 11A). Post-immunization serum used at the same dilution detected TdfJ in the induced and uninduced complement, as well as in the OMP under iron-replete and iron-depleted conditions (Figure 11B). The band is more intense in the +Fe OMP than the –Fe OMP lane, demonstrating that TdfJ is iron induced. Again, TdfJ was not detected in the \( tdf/J^- \) strain or in the wild type strain under iron-replete or iron-depleted conditions. These results indicate that serum 118 reacts specifically with TdfJ.
Figure 10. Serum 117 reacted specifically with TdfJ. Strains grown in iron-replete or iron-depleted conditions, or in the presence (IN) or absence (UN) of IPTG, were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were probed with pre-immunization serum 117 (A) or post-immunization serum 117 (B). TdfJ is approximately 80 kD in size. Molecular mass markers are shown on the left.
Figure 10. Serum 117 reacted specifically with TdfJ.
Figure 11. Serum 118 reacted specifically with TdfJ. Strains grown in iron-replete or iron-depleted conditions, or in the presence (IN) or absence (UN) of IPTG, were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were probed with pre-immunization serum 118 (A) or post-immunization serum 118 (B). TdfJ is approximately 80 kD in size. Molecular mass markers are shown on the left.
Figure 11. Serum 118 reacted specifically with TdfJ.
Before analyzing the pre and post-immunization sera from guinea pig 119, the TdfJ complement strain was regrown in order to ascertain why TdfJ was being detected in both the induced and uninduced MCV412 samples. The newly prepared MCV412 whole cell lysates were used in the analysis of pre and post-immunization sera from guinea pig 119. Pre-immunization serum from guinea pig 119 used at a dilution of 1:50 did not detect anything on the membrane (Figure 12A). Post-immunization serum used at the same dilution detected TdfJ in the induced complement, as well as in the OMP under iron-replete and iron-deplete conditions (Figure 12B). The band is more intense in the +Fe OMP than the –Fe OMP lane, demonstrating that TdfJ is iron induced. Again, TdfJ was not detected in the \( tdfJ^- \) strain or in the wild type strain under iron-replete or iron-deplete conditions. TdfJ was also not detected in the uninduced complement strain for the first time. Based on this result it is assumed that the MCV412 whole cell lysates provided initially were mislabeled. As a whole, these results indicate that serum 119 reacts specifically with TdfJ.

Overall, the initial analysis revealed specificity to the given peptide for each sera. However, the sera were unable to detect TdfJ in the wild type strains grown under iron-replete or iron-depleted conditions. For this reason, all four guinea pigs were given a booster with a different carrier protein. Following the booster, final serum 116 and serum 118 were used to probe both +Fe wild type and induced MCV412 \( tdfJ^+ \) whole cell lysates. Both sera were tested using a range of different dilutions: 1:5, 1:10, 1:50, 1:100, 1:500, 1:1,000, and 1:5,000. Serum 116 and serum 118 did not efficiently detect TdfJ in wild type whole cell lysates, even after the booster (Figure 13). However, a very faint band was detected using a 1:5 dilution of serum 118. This could simply indicate that expression of TdfJ under wild type conditions is low. Serum 116 and serum 118 did detect TdfJ in the \( tdfJ^- \) whole cell lysate, though serum 116 did so much more
Figure 12. Serum 119 reacted specifically with TdfJ. Strains grown in iron-replete or iron-depleted conditions, or in the presence (IN) or absence (UN) of IPTG, were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were probed with pre-immunization serum 119 (A) or post-immunization serum 119 (B). TdfJ is approximately 80 kD in size. Molecular mass markers are shown on the left.
Figure 12. Serum 119 reacted specifically with TdfJ.
Figure 13. Serum 116 and 118 did not efficiently detect TdfJ in wild type whole cell lysate. Wild type FA1090 whole cell lysate, grown under iron-replete conditions, was subjected to SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. The membrane was probed with serum 116 and serum 118 and developed with the NBT/BCIP system. The black arrow indicates the presence of a faint band at the highest concentration of serum 118.
Figure 13. Serum 116 and 118 did not efficiently detect TdfJ in wild type whole cell lysate.
efficiently (Figure 14). Serum 116 was capable of detecting TdfJ at a dilution as low as 1:5,000. On the other hand, serum 118 was capable of detecting TdfJ at a dilution as low as 1:500. Both sera worked, but serum 116 was much more sensitive.

Based on the information gained from testing the final sera 116 and 118 at many dilutions, one last test was run in order to establish a working concentration for each serum to be used in future experiments. Again, induced and uninduced MCV412 whole cell lysates, along with OMPs prepared under iron-replete and iron-deplete conditions, were subjected to SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Following the transfer, the membranes were probed with the final sera. Final serum 116 and serum 117 were used at a 1:200 dilution, while final serum 118 and serum 119 were used at a 1:100 dilution. A 1:3,000 dilution of the secondary rabbit anti-guinea pig AP (Southern Biotech) was used in order to increase sensitivity. Final serum 116 detected TdfJ in the induced complement strain, and in both the +Fe and –Fe OMPs (Figure 15A). Final serum 117 also detected TdfJ in the induced complement strain, and in both the +Fe and –Fe OMPs (Figure 15B). Final serum 118 and serum 119 appear to detect TdfJ at a similar level of sensitivity. Serum 118 (Figure 15C) and serum 119 (Figure 15D) both detected TdfJ in the induced complement strain and in the +Fe OMP, however both failed to detect TdfJ in the –Fe OMP at this dilution. Based on these results, serum 117 appears to be the best and most sensitive of the four sera.

In an effort to detect TdfJ in wild type whole cell lysate, FA1090 (wild type) and MCV407 (tdfJ) whole cell lysates, grown under iron-replete and iron-depleted conditions, were subjected to SDS-PAGE and subsequently transferred to a nitrocellulose membranes. Following transfer, the membrane was probed with primary serum 117 at a dilution of 1:200. Primary serum 117 was detected using goat anti-guinea pig HRP (Southern Biotech) and developed using
Figure 14. Serum 116 and 118 detected TdfJ in MCV412 whole cell lysate. MCV412 (tdfJ<sup>F</sup>) whole cell lysate, induced with IPTG, was subjected to SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. The membrane was probed with serum 116 and serum 118 and developed with the NBT/BCIP system. The black arrows indicate TdfJ specific bands.
Figure 14. Serum 116 and 118 detected TdfJ in MCV412 whole cell lysate.
Figure 15. Final sera detected TdfJ in induced MCV412 and OMPs. Strains grown in iron-replete or iron-deplete conditions, or in the presence (IN) or absence (UN) of IPTG, were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were then probed with serum 116 (A), serum 117 (B), serum 118 (C), or serum 119 (D). TdfJ is approximately 80 kD in size. Molecular mass markers are shown on the left.
Figure 15. Final sera detected TdfJ in induced MCV412 and OMPs.
ECL. Through the use of a more sensitive detection system, detection of TdfJ with serum 117 was visualized (Figure 16). TdfJ was not detected in MCV407, which served as the negative control. Based on this results, blots would need to be developed using ECL in order to detect TdfJ in wild type whole cell lysate.

**B. Expression of TdfJ in E. coli**

TdfJ was expressed in *E. coli* in order to assess its possible role in heme binding. TdfJ, from start site 1 (SS1) and start site 2 (SS2), was cloned into the pBAD-TOPO vector (Invitrogen). TOP10 *E. coli* cells (Invitrogen) were transformed and subsequently screened. After transformants were verified, a pilot expression experiment was performed using various concentrations of L-arabinose (Sigma Aldrich) to induce expression. The empty vector served as the negative control, as it does not contain the *tdfJ* sequence. Overnight cultures were used to inoculate LB containing the appropriate antibiotics, and the cultures were grown to an OD$_{600}$ =0.5. Cultures were induced with 0%, 0.002%, or 0.02% arabinose. After a four hour induction, whole cell lysates were made. Whole cell lysates were subjected to SDS-PAGE and subsequently transferred to a nitrocellulose membrane. Induced MCV412 (*tdfJ*) whole cell lysate was used as a positive control. The membrane was probed with anti-TdfJ serum 117 and developed using the NBT/BCIP system. Expression of TdfJ was almost completely absent at 0% arabinose, although there is a very faint band present for both SS1 and SS2 (Figure 17). This indicates fairly tight control of TdfJ expression by the P$_{BAD}$ promoter. Expression of TdfJ from SS1 and SS2 was present at both 0.002% and 0.02% arabinose, but expression was optimal when induced with 0.02% arabinose (Figure 17). There was no TdfJ expressed by the empty vector control.
Figure 16. TdfJ detected in wild type whole cell lysate when developed with ECL.
FA1090 (wild type) and MCV407 (tdfJ⁻) whole cell lysates, grown under iron-replete and iron-deplete conditions, were subjected to SDS-PAGE and subsequently transferred to a nitrocellulose membranes. Following transfer, the membrane was probed with serum 117 at a dilution of 1:200 and developed using ECL. The black arrow indicates TdfJ specific bands. Approximate positions of the molecular mass markers are shown on the left.
Figure 16. TdfJ detected in wild type whole cell lysate when developed with ECL.
Figure 17. L-arabinose Induction of TdfJ expression in *E. coli*. TdfJ, from SS1 and SS2, was cloned into the pBAD-TOPO vector and TOP10 *E. coli* cells were subsequently transformed. After transformants were verified, expression of TdfJ was induced by various levels of L-arabinose. Whole cell lysates were made, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-TdfJ serum 117. The empty vector served as the negative control, while induced MCV412 (*tdfJ*) served as the positive control. Approximate position of the molecular mass marker is shown on the left.
Figure 17. L-arabinose induction of TdfJ expression in *E. coli*.
Whole cell dot blots were performed in order to assess surface exposure of TdfJ as expressed in *E. coli*. Overnight cultures were used to inoculate LB containing the appropriate antibiotics, and the cultures were grown to an OD$_{600} = 0.5$. At that point cultures were induced with 0%, 0.00002%, 0.0002%, 0.2%, or 0.02% arabinose. After a four hour induction, cultures were standardized and added in duplicate to the S&S Minifold I Dot Blotter (Whatman) containing a nitrocellulose membrane. The membrane was allowed to dry for one hour and stored overnight. The membrane was probed with anti-TdfJ serum 117 at a 1:200 dilution and developed using the NBT/BCIP system. FA1090 (wild type), induced MCV412 (*tdfJ*), and MCV407 (*tdfJ*-) were also grown and used as controls.

TdfJ was surface exposed when expressed from SS1 in *E. coli* (Figure 18A). Expression of TdfJ increased as the concentration of arabinose increased. At 0.2% arabinose the signal is the most intense, however at this concentration of arabinose the bacteria were lysing. Lysis products could be seen in the culture and the final density is much lower than in the other conditions. This indicates that expression of TdfJ at this level is toxic to the bacteria. There was signal in the 0% arabinose condition, indicating leaky expression from the inducible promoter. TdfJ was also surface exposed when expressed from SS2 in *E. coli* (Figure 18B). Again, expression of TdfJ increased as the concentration of arabinose increased, with the most intense signal seen in the 0.2% arabinose condition. There was an even more intense signal in the 0% arabinose condition, indicating that expression from the inducible promoter is leakier when expressing TdfJ from SS2. There was a small amount of signal in the wild type condition (Figure 18C). TdfJ appeared to be surface exposed in the MCV412 complement strain (Figure 18C). Lastly, there was no signal in the MCV407 (*tdfJ*-) negative control.
Figure 18. TdfJ expressed in *E. coli* was surface exposed. *E. coli* TdfJ SS1 cultures (A) and TdfJ SS2 cultures (B) were induced with 0%, 0.00002%, 0.0002%, 0.002%, 0.2%, or 0.02% arabinose. After a four hour induction, cultures were standardized and added to the dot blotter containing a nitrocellulose membrane. The membrane was allowed to dry for one hour, stored overnight, and probed with anti-TdfJ serum 117 at a 1:200 dilution. The membrane was developed using the NBT/BCIP system. Gonococcal strains FA1090 (wild type), induced MCV412 (*tdfJ*), and MCV407 (*tdfJ*) were used as controls (C).
Figure 18. TdfJ expressed in *E. coli* was surface exposed.
After confirming that TdfJ was surface exposed when expressed by *E. coli*, the rest of the *E. coli* expression strains were tested. *E. coli* expressing TbpA, TdfF, TdfG, and TdFH were also used in subsequent experiments, therefore surface exposure of these proteins was assessed in the same manner. Overnight cultures were used to inoculate LB containing the appropriate antibiotics, and the cultures were grown to an $\text{OD}_{600} = 0.5$. At that point cultures were induced with arabinose or IPTG. After a two or four hour induction, cultures were standardized and added in triplicate to the S&S Minifold I Dot Blotter (Whatman) containing a nitrocellulose membrane. The membrane was allowed to dry for one hour and stored overnight. To detect TdfF, TdfG, TdfH and TdfJ, the membrane was probed with protein specific antibodies and developed using the NBT/BCIP system. To detect TbpA, the membrane was probed with HRP labeled transferrin and developed using the Opti-4CN system (Biorad). The empty vector control in both the TOP10 and BL21 background was included and probed with each antibody (Figure 19).

There was background binding in all uninduced conditions, but the signal is comparable to the signal in the empty vector controls (Figure 19). The signal from each expression strain was the most intense in the induced condition, indicating that each protein was surface exposed when expressed from its respective *E. coli* expression strain (Figure 19).
Figure 19. TbpA, TdfF, TdfG, TdfH, and TdfJ were surface exposed when expressed in *E. coli*. *E. coli* cultures were induced with IPTG or arabinose. After a two or four hour induction, cultures were standardized and added to the dot blotter containing a nitrocellulose membrane. The membrane was allowed to dry for one hour, and stored overnight. The membrane was probed with protein specific antibodies to detect TdfF, TdfG, TdfH, and TdfJ and developed with the NBT/BCIP system. To detect TbpA, the membrane was probed with HRP labeled transferrin and developed with the Opti-4CN system. The empty vector control in both the TOP10 and BL21 background was included and probed with each antibody. The “+” symbol indicates induced cultures, while the “−” symbol indicates uninduced cultures.
Figure 19. TbpA, TdfF, TdfG, TdfH, and TdfJ were surface exposed when expressed in *E. coli*. 
C. Hemin Sedimentation Assays

Heme sedimentation assays were performed in order to assess TdfJ’s ability to bind hemin when expressed by *E. coli*. *E. coli* expressing TbpA, TdfF, TdfG, and TdfH were included as controls. Briefly, cultures were induced by the addition of arabinose or IPTG and allowed to grow for two or four hours. After induction of protein expression, aliquots were washed once with PBS containing 5% DMSO, centrifuged, resuspended in 1 mL of PBS containing 5% DMSO, and standardized to an OD$_{600}$ = 1.0. Cultures were then allowed to incubate with 15 µM or 20 µM hemin for one hour at room temperature in a v-bottom 96-well plate. After one hour the 96-well plate was centrifuged, and the supernatant from each well was transferred to a flat-bottom 96-well plate. The OD$_{405}$ of the supernatants were read and recorded by a plate reader. Wells containing hemin and no cells were used as the 100% saturated controls, meaning that 100% of the hemin was present in the supernatant and was not bound to cells. Percent hemin bound was determined by the decrease in absorbance of the supernatant as compared to the 100% saturated or unbound control.

1. Alignment of TdfJ, TbpA, and FetA with Representative Heme Receptors

The FRAP and NPNL motifs have been shown to be characteristic of receptors known to bind heme or hemoproteins (13). The conserved histidine residue that falls in between the two motifs has been shown to be critical for heme utilization by HmuR in *Porphyromonas gingivalis* (54) and HemR in *Yersinia enterocolitica* (13). The gonococcal TBDTs were aligned with other representative heme receptors using ClustalW. The multiple sequence alignment was then uploaded to Jalview, and the presence of the FRAP motif, the conserved histidine, and the NPNL motif was assessed. Of the gonococcal TBDTs analyzed, only TbpA, FetA, and TdfJ contained one or more of these characteristics (Figure 20). TdfJ contains the FRAP motif, the conserved
Figure 20. Alignment of TbpA, FetA, and TdfJ with representative heme receptors. The gonococcal TBDTs were aligned with representative heme receptors using ClustalW. The conserved FRAP and NPNL motifs are marked by black lines. The identical residues within those motifs are marked with an asterisk. The highly conserved histidine residue is marked with a pound sign. The numbers following the identifier are the residue numbers within each gene. Sd, *Shigella dysenteriae*; Ye, *Yersinia enterocolitica*; Hi, *Haemophilus influenzae*; Hd, *Haemophilus ducreyi*; Nm, *Neisseria meningitidis*; Ng, *Neisseria gonorrhoeae*; Mc, *Moraxella catarrhalis*; Sm, *Serratia marcescens*; Pg, *Porphyromonas gingivalis*. 
Figure 20. Alignment of TbpA, FetA, and TdfJ with representative heme receptors.
histidine residue, and the NPNL motif. FetA contains the FRAP motif and the conserved histidine residue. TbpA only contains the FRAP motif. However, there are two highly conserved histidine residues in surface exposed loops 2 and 11 that could participate in hemin binding (Figure 21).

2. TdfJ and TbpA Bind Hemin when Expressed in E. coli

TdfJ, expressed from SS1 and SS2, bound hemin in the 15 µM hemin condition (Figure 22B). In the uninduced 15 µM hemin condition, none of the proteins exhibited hemin binding above background levels (Figure 22A). However, when TdfJ expression was induced from SS1, ~11% of total hemin was bound (Figure 22B). When TdfJ expression was induced from SS2, ~12% of total hemin was bound (Figure 22B). Surprisingly, TbpA also bound hemin in the 15 µM hemin condition (Figure 22B). When TbpA expression is induced, ~7% of total hemin is bound (Figure 22B). Hemin binding to TdfJ (SS1 and SS2) and TbpA is statistically significant as compared to the empty vector control.

TdfJ, expressed from SS1 and SS2, also bound hemin in the 20 µM hemin condition (Figure 23B). In the uninduced 20 µM hemin condition, none of the proteins exhibited hemin binding above background levels (Figure 23A). However, when TdfJ expression was induced from SS1, ~23% of total hemin was bound (Figure 23B). When TdfJ expression was induced from SS2, ~18% of total hemin was bound (Figure 23B). Again, TbpA also bound hemin in the 20 µM hemin condition. When TbpA expression was induced, ~20% of total hemin is bound (Figure 23B). Hemin binding to TdfJ (SS1 and SS2) and TbpA was statistically significant as compared to the empty vector control.
Figure 21. **2-D Topology Model of TbpA.** The figure depicts the putative 2-D topology of TbpA. The grey dotted lines indicate the outer membrane and the cytoplasmic membrane. The red rectangle indicates the location of the FRAP motif. The red arrows indicate the location of the conserved histidine residues. L = loop.
Figure 21. 2-D Topology Model of TbpA.
Figure 22. TdfJ and TbpA bound hemin in the 15 µM hemin condition when expressed in *E. coli*. Cultures were induced by the addition of arabinose or IPTG and allowed to grow for two or four hours. After induction of protein expression, cultures were washed once with PBS containing 5% DMSO and standardized to an OD$_{600}$ = 1.0. Cultures were incubated with 15 µM hemin for one hour at room temperature in a v-bottom 96-well plate. After one hour the 96-well plate was centrifuged, and the supernatant from each well was transferred to a flat-bottom 96-well plate. The OD$_{405}$ of the supernatants were read and recorded by a plate reader. Results from both uninduced bacteria (A) and induced bacteria (B) are shown. Wells containing hemin and no cells were used as the 100% saturated controls. Percent hemin bound was determined by the decrease in absorbance of the supernatant as compared to the 100% saturated or unbound control. The data represents the means and standard deviations of one experiment performed in quadruplicate. Statistically significant differences were determined by paired Student’s t test, with two tailed hypotheses. Statistically significant differences are marked with asterisks (*, P<0.05).
Figure 22. TdfJ and TbpA bound hemin in the 15 µM hemin condition when expressed in *E. coli.*
Figure 23. TdfJ and TbpA bound hemin in the 20 μM hemin condition when expressed in *E. coli*. Cultures were induced by the addition of arabinose or IPTG and allowed to grow for two or four hours. After induction of protein expression, cultures were washed once with PBS containing 5% DMSO and standardized to an OD<sub>600</sub> = 1.0. Cultures were incubated with 20 μM hemin for one hour at room temperature in a v-bottom 96-well plate. After one hour the 96-well plate was centrifuged, and the supernatant from each well was transferred to a flat-bottom 96-well plate. The OD<sub>405</sub> of the supernatants were read and recorded by a plate reader. Wells containing hemin and no cells were used as the 100% saturated controls. Percent hemin bound was determined by the decrease in absorbance of the supernatant as compared to the 100% saturated or unbound control. The data represents the means and standard deviations of one experiment performed in quadruplicate. Statistically significant differences were determined by paired Student’s t test, with two tailed hypotheses. Statistically significant differences are marked with asterisks (*, P<0.05).
Figure 23. TdfJ and TbpA bound hemin in the 20 µM hemin condition when expressed in *E. coli.*
In both the 15 µM and 20 µM hemin conditions there were negative results when the strains were uninduced, or when the induced strains were unable to bind hemin. The data shown represents the inverse of the percent hemin unbound, which is representative of the amount of hemin left in the supernatant as compared to the 100% unbound control, which contains no cells. Therefore, the negative data indicates an increased amount of hemin present in the supernatant as compared to the 100% unbound control. This phenomena is likely occurring due to the release of hemin from the cells as a result of small scale lysis. This manifests as negative data when the cells are unable to bind hemin, thus causing an overall increase in absorbance. This is also likely occurring in the induced expression strains shown to bind hemin, but the increase is not seen due to their ability to bind hemin and decrease the overall amount of hemin left in the supernatant.

3. Hemin Binding Phenotype of TdfJ Inconclusive when Overexpressed in *N. gonorrhoeae*.

The ability of TdfJ to bind hemin was assessed in its native organism. The assay was performed in the same way, except the 96-well plate was kept at 37°C with the addition of 5% CO₂ during the hour long incubation step. The following strains were used: induced and uninduced MCV412 (*tdfJ*) and MCV411 (*nspA*). MCV411 was included as a negative control. MCV411 was also a useful control because it is overexpressing a surface exposed outer membrane protein. In the presence of 15 µM hemin, all of the conditions resulted in a relatively high amount of hemin binding, but none of the conditions were significantly different from the others (Figure 24A). Induced MCV412, which overexpresses TdfJ, bound hemin at a similar level to its uninduced counterpart. MCV411 bound hemin at approximately the same level as induced and uninduced MCV412 (Figure 24A). There were no statistically significant differences in the percent hemin bound between the three conditions. In the presence of 20 µM hemin, the same was true (Figure 24B). In the 20 µM hemin condition, hemin binding was
Figure 24. Hemin binding phenotype of TdfJ inconclusive when overexpressed in *N. gonorrhoeae*. Cultures were induced by the addition of arabinose or IPTG and allowed to grow for two or four hours. After induction of protein expression, cultures were washed once with PBS containing 5% DMSO and standardized to an OD$_{600}$ = 1.0. Cultures were incubated with 15 µM (A) 20 µM (B) hemin for one hour at room temperature in a v-bottom 96-well plate. After one hour the 96-well plate was centrifuged, and the supernatant from each well was transferred to a flat-bottom 96-well plate. The OD$_{405}$ of the supernatants were read and recorded by a plate reader. Wells containing hemin and no cells were used as the 100% saturated controls. Percent hemin bound was determined by the decrease in absorbance of the supernatant as compared to the 100% saturated or unbound control. The data represents the means and standard deviations of one experiment performed in quadruplicate.
Figure 24. Hemin binding phenotype of TdfJ inconclusive when overexpressed in *N. gonorrhoeae.*
present in all strains, but there were no statistically significant differences in the percent hemin bound between the three conditions. The amount of hemin binding to gonococci was generally higher than hemin binding to the *E. coli* strains, despite protein expression. This indicates a high level of background hemin binding due to some surface characteristic of the gonococcus, or the presence of other hemin binding surface molecules.

**D. Ribonuclease Leakage Assay**

As an additional control, an RNase leakage assay was performed to ensure that hemin binding was not an artifact of protein overexpression. Although we hypothesized that TdfJ would bind hemin, the results that indicated that TbpA bound hemin were completely unexpected. The Ribonuclease (RNase) leakage assay was performed in order to rule out the possibility that membrane permeabilization was responsible for hemin binding. The RNase leakage assay had been performed using the pUNCH412 TbpA *E. coli* expression strain and the strain was shown to leak RNase from its outer membrane. Since the TbpA expression strain had been shown to leak RNase, we tested all of the other expression strains. Although the assay is in no way quantitative, the qualitative results would help establish confidence in the hemin sedimentation assay results. If all strains appeared to leak RNase this would suggest that membrane permeabilization is not responsible for the hemin binding and that the results are indicative of specific hemin binding. However, if only the TdfJ and TbpA expression strains leaked RNA, this might indicate that hemin binding is a nonspecific phenomenon caused by the expression of a large, foreign protein on the outer membrane.

Leaking RNase was detected by plating each *E. coli* expression strain on LB plates containing RNA and inducing protein expression with arabinose or IPTG. After 24 hours of growth, the plates were flooded with 0.1 N HCl and photographed. The HCl caused any RNA
remaining in the agar to precipitate, causing the agar to look opaque or light. Areas in the agar where the RNA has been degraded by RNases appear clear or dark. The TbpA expression strain was used as the positive control because it is known to leak RNase. The empty vector control was used as the negative control. This experiment was performed twice with the same outcome, and the results from one replicate were photographed (Figure 25). All strains, except for the empty vector control, leaked RNase to various degrees. Dark or clear areas indicating RNase leakage are marked with a red arrow. The phenomena is best viewed at the edge of dense colony growth.

E. Hemin Binding Blots

Hemin binding blots were performed in order identify any gonococcal outer membrane proteins with the ability to bind hemin via a different method. FA1090 sarkosyl insoluble OMPs, grown under iron-replete or iron-deplete conditions, were subjected to SDS-PAGE on a 7.5% gel, and subsequently transferred to a nitrocellulose membrane. The membrane was probed with TBS containing 10⁻⁶ M hemin for 1.5 hours. Bound hemin was visualized via its inherent peroxidase activity using ECL. The blot was exposed to autoradiographic film for approximately 20 seconds. Bands representing TdfJ and TbpA are not predominate, however there appears to be a faint band in the –Fe OMP lane around 100 kD, which is likely TbpA (Figure 26). There is also a faint band around 75 or 80 kD in the +Fe OMP, which is likely TdfJ (Figure 26). Surprisingly, a much more distinct band appeared at approximately 70 kD.

Based on these unexpected results, the experiment was performed again, except this time the same OMP samples were run out on duplicate 7.5% and 15% polyacrylamide gels. After electrophoresis, one of each percentage gel was transferred onto nitrocellulose, probed with
Figure 25. Ribonuclease Leakage Assay. Leaking RNase was detected by plating the TbpA expression strain (A), the TdfF expression strain (B), the TdfG expression strain (C), the TdfH expression strain (D), the TdfJ (SS1) expression strain (E), the TdfJ (SS2) expression strain (F), and the empty vector control strain (G), on LB plates containing RNA and inducing protein expression with arabinose or IPTG. After 24 hours of growth, the plates were flooded with 0.1 N HCl and photographed. Areas where RNases have degraded the RNA in the agar appear clear or dark, and are indicated by the red arrows.
Figure 25. Ribonuclease Leakage Assay.
Figure 26. A ~70 kD protein in the –Fe OMP bound hemin. Sarkosyl insoluble outer membrane preparations were subjected to SDS-PAGE and subsequently transferred to a nitrocellulose membrane. The membrane was probed with hemin for 1.5 hours, developed with ECL, and exposed to autoradiographic film. Molecular mass markers are shown on the left.
Figure 26. A ~70 kD protein in the –Fe OMP bound hemin.
hemin, developed with ECL, and exposed to autoradiographic film. The remaining gels were stained with Coomassie blue overnight, and destained the following day until background staining was minimized.

After transfer and probing, the 7.5% gel yielded the same results. Faint bands likely representing TdfJ and TbpA were seen at 100 kD in the –OMP and 80 kD in the +OMP, respectively. Again, a distinct band approximately 70 kD in size was seen (Figure 27A). This band was missing in the +Fe OMP lane. The corresponding band on the Coomassie stained gel, shown by the arrow, was excised and sent for protein identification at the VCU Mass Spectrometry Core (Figure 27B). The 15% gel, after transfer and probing, revealed another distinct band approximately 35 kD in size in the –Fe OMP (Figure 28A). This band was markedly absent in the +Fe OMP lane. The corresponding band on the Coomassie stained gel, shown by the arrow, was also excised and sent for protein identification at the VCU Mass Spectrometry Core (Figure 28B).

The protein identification results for the smaller, ~35 kD band, were fairly straightforward. The protein was putatively identified as ferric binding protein A. FbpA is the 37 kD periplasmic binding protein responsible for shuttling the iron released by the transferrin and lactoferrin acquisition systems across the periplasm. This periplasmic protein was likely included in the sarkosyl insoluble outer membrane preparation because apo-FbpA binds directly and specifically TbpA, an outer membrane protein (80). Also, the gonococci express a very large amount of FbpA.
Figure 27. A ~70 kD protein in the μ-Fe OMP bound hemin. Sarkosyl insoluble outer membrane preperations were subjected to SDS-PAGE in duplicate. Separated proteins was subsequently transferred to a nitrocellulose membrane or stained with Coomassie blue. The nitrocellulose membrane was probed with hemin for 1.5 hours, developed with ECL, and exposed to autoradiographic film. The band in the Coomassie stained gel that corresponded to the hemin-binding band was excised and sent for protein identification. This band is indicated by a black arrow. Molecular mass markers are shown on the left.
Figure 27. A ~70 kD protein in the –Fe OMP bound hemin.
Figure 28. A ~35 kD protein in the –Fe OMP bound hemin. Sarkosyl insoluble outer membrane preparations were subjected to SDS-PAGE in duplicate. Separated proteins was subsequently transferred to a nitrocellulose membrane or stained with Coomassie blue. The nitrocellulose membrane was probed with hemin for 1.5 hours, developed with ECL, and exposed to autoradiographic film. The band in the Coomassie stained gel that corresponded to the hemin-binding band was excised and sent for protein identification. This band is indicated by a black arrow. Molecular mass markers are shown on the left.
Figure 28. A ~35 kD protein in the –Fe OMP bound hemin.
The protein identification results for the larger, ~70 kD band, were less clear. Many proteins were detected in the band, including TbpA and porin, however these proteins are the incorrect size. This is likely due to the fact that a 1-D gel analysis was used. The outer membrane preparations used were heavily processed and proteins that were the incorrect size were likely identified due to the presence of protein fragments in the bands. In order to avoid this contamination issue a 2-D gel analysis could be performed.

However, there were two matches that were a bit more reasonable: PilQ and FetA. PilQ is a ~78 kD protein that functions in type IV pilus organelle biogenesis, which is essential for DNA transformation competence in the gonococcus (29). FetA is a ~78 kD protein and is the TBDT responsible for the import of ferric enterobactin and other catecholate-type siderophores (14, 43). Also, FetA is iron repressed like the ~70 kD band seen in the hemin binding blot.
Discussion

This study was a multifaceted investigation into the structure, regulation, expression, and function of selected TonB-dependent transporters in *N. gonorrhoeae*. The study had three main objectives, the first of which was to investigate the role of two highly conserved regions of TbpB in lipidation. It was hypothesized that mutation of one of those conserved regions, the LSAC motif, would prevent lipidation of TbpB. The hypothesis was confirmed, as our data indicates that the LSAC motif is necessary for lipidation of TbpB. The TbpB LSAC mutant was able to produce full length TbpB, however the protein was not lipidated. The second focus was on the regulation of TbpA and TbpB by the two component regulatory system MisR/MisS. It was hypothesized that the MisR/MisS system played a role in iron-dependent regulation of TbpA and TbpB. This hypothesis was confirmed, as our data shows that a MisR⁻ strain demonstrates dysregulated expression of TbpA and TbpB. The third focus of this study was on the expression and function of the largely uncharacterized TBDT TdfJ. Based on sequence similarity to other heme transporters, it was hypothesized that TdfJ would bind hemin when expressed in *E. coli*. This hypothesis was also confirmed, as our data shows that *E. coli* expressing TdfJ are able to specifically bind hemin.

The first focus of this study was to examine the role of two highly conserved regions of TbpB, and examine whether or not they play a role in lipidation. TbpA and TbpB are potential
vaccine candidates, so it was important to investigate the roles of the conserved regions of TbpB as they might influence lipidation, and thus attachment of the protein to the cell surface. TbpB was previously shown to be surface exposed (4) and lipidated (28), however the site of lipidation had yet to be elucidated. Also, the roles of two highly conserved amino-terminal regions of TbpB had not yet been established. It was hypothesized that the LSAC motif was the site of lipidation in TbpB, and our results confirmed this hypothesis. Full length TbpB was expressed and lipidated by MCV843 (TbpB ΔGly), showing that the conserved stretch of four glycine residues was not important for lipidation. Full length TbpB was produced by MCV839 (TbpB LSAC), however it was not lipidated, thus demonstrating the importance of the LSAC motif to the lipidation of the protein.

Although the conserved glycine residues were not shown to be involved in the lipidation of TbpB, MCV843 (TbpB ΔGly) had a significant defect in growth when transferrin was provided as the sole iron source (71). This indicates that the glycine residues play an important role in transferrin iron acquisition. These results help to shed light on the role of two conserved regions within TbpB. These results give us a more detailed understanding of TbpB and how it associates with the cell surface, which could aid in the development of new therapies or vaccines targeted against infection with N. gonorrhoeae. Also, lipidated proteins are particularly immunogenic, so understanding the mechanism of lipidation could help improve immunogenicity of a potential vaccine utilizing TbpB (100).

The second goal of the study was to assess whether or not the MisR/MisS two component regulatory system played a role in regulating TbpA and TbpB. Unpublished RNA-Seq data performed by the Shafer lab showed that MisR plays a role in the regulation of tbpA and tbpB expression. Under iron replete conditions tbpA expression was repressed 3 fold, while tbpB
expression was repressed 5 fold. Also, phosphorylated MisR was shown to bind specifically to the region upstream of the \textit{tbpBA} operon by electrophoretic mobility shift assay. Following this result DNA footprinting was performed, and MisR was shown to bind to five distinct sequences upstream of the \textit{tbpBA} operon. Meningococcal MisR/MisS does not regulate TbpA and TbpB (91), so it is noteworthy that the system has been demonstrated to bind to and regulate the gonococcal transferrin binding proteins. The regions upstream of the Fur box are distinct between the two species, and this could be responsible for the difference. The discrepancy in the regulation of the well conserved transferrin acquisition between the two species may shed light on how the species have evolved to survive optimally in their individual niches.

Analysis of both protein and RNA expression revealed that MisR plays a role in regulating TbpA and TbpB, especially under iron-replete conditions. Little is known about the gonococcal MisR/MisS system, except that it responds to antimicrobial peptides like LL-37. This indicates that the two component regulatory system is sensing host signals and regulating important virulence factors, like TbpA and TbpB, accordingly. These results also suggest that TbpA and TbpB are under the control of two forms of regulation, Fur and MisR/MisS, both of which respond to iron availability. The finding that MisR differentially regulates TbpA and TbpB according to iron availability exemplifies how important iron and the corresponding regulation of iron acquisition proteins is to the gonococcus. Complete knowledge of the complex regulatory elements that control the expression of the transferrin binding proteins is essential and could shed light on how other important nutrient acquisition systems in \textit{N. gonorrhoeae} may function. If TbpA and TbpB are to be used as vaccine targets, understanding their regulation is essential.
MisR/MisS is especially important concerning vaccine and therapeutic development because this form of regulation responds to extracellular signals in the host environment. The sensor, MisS, resides in the cytoplasmic membrane, and upon exposure to host factors like antimicrobial peptides, phosphorylates the response regulator MisR. MisR is then able to bind DNA targets upstream of the \textit{tbpBA} operon. MisR is able to bind to five different sites, and this may allow for the formation of a secondary structure which negatively impacts transcription, and therefore expression, of these important proteins. This two component regulatory system senses the host environment and assists the gonococcus in choosing the appropriate expression of different iron acquisition systems. The environment within the host, and therefore the iron sources available in the host, changes throughout the many stages of infection, and therefore the MisR/MisS system likely plays a role in responding to changes in iron availability in the host. Knowledge of how TbpA and TbpB are regulated and expressed during infection in the host is of paramount importance. Also, the MisR/MisS system may serve as an immune evasion strategy. The repression of the transferrin binding proteins in the presence of LL-37 may be an attempt to hide these important, well conserved virulence factors from the immune system, and thus halt production of antibodies against the proteins. If these proteins are not optimally expressed during certain stages of infection it may impact and direct the development of a vaccine. Perhaps a subunit vaccine, including many different TBDTs, would be more effective.

The third focus of this study was to better characterize TdfJ. Based on sequence similarity to other heme transporters, it was hypothesized that TdfJ would bind hemin when expressed in \textit{E. coli} and when overexpressed in its native organism. Research into heme uptake in \textit{N. gonorrhoeae} is extremely important. Iron is critical to the survival of many bacteria, and heme is an abundant potential iron source in the human host. Gonococci can utilize heme in the
absence of the hemoglobin receptor (31) and also in a TonB-independent manner (9, 88). Many organisms have dedicated heme receptors, such as Moraxella catarrhalis (33), Shigella dysenteriae (61), and Bordetella pertussis (62). It has been proposed that N. gonorrhoeae also has a dedicated heme receptor (53), however one has never been clearly identified. The meningococcal homologue of TdfJ, ZnuD, has been implicated in heme utilization (51). The meningococcal homologue has also been proposed as a vaccine target (44). Therefore, investigation into TdfJ’s role in heme uptake was warranted because little is known about this TBDT that has the potential to be an excellent vaccine target.

The first of the two hypotheses was confirmed, as TdfJ did bind hemin when expressed in E. coli from SS1 and SS2. However, when the hemin binding assay was performed in the gonococcal background the results were inconclusive. There are many potential explanations for the lack of a conclusive hemin binding phenotype when the assay was performed in the native organism. As aforementioned, N. gonorrhoeae have been shown to utilize heme in the absence of the hemoglobin receptor and in the absence of tonB. This indicates that there are redundancies in the organism’s ability to import heme. Without removing the other heme uptake pathways it would be difficult to see a heme binding phenotype because there are obviously other proteins binding heme in addition to TdfJ. This is likely why the levels of background hemin binding were so much higher when the assay was performed with N. gonorrhoeae rather than E. coli.

Surprisingly, TbpA was also able to bind hemin when expressed in E. coli. Although TbpA only contained the FRAP motif, and did not contain the conserved histidine residue or NPNL motif, inspection of a TbpA 2-D topology model revealed that there are 2 highly conserved histidine residues in surface exposed loops 2 and 11. These histidine residues may be involved in hemin binding. This unexpected result suggests the possibility that TbpA is a multi-
factorial transporter. Interestingly, it has been suggested that TbpA participates in hemin utilization previous to this finding. In a gonococcal mutant unable to express TbpA, heme uptake was reduced by greater than 50% as compared to wild type heme uptake (7). In order to ensure that hemin binding was not an artifact of recombinant protein overexpression, a ribonuclease leakage assay was performed. The assay revealed that all of the E. coli expression strains used in the hemin sedimentation assay leaked RNase at various levels. This finding further supported the specificity of hemin binding to TbpA and TdfJ, and helped to rule out the possibility that membrane disruption was causing nonspecific sticking of hemin to the cells.

After recombinant TbpA and TdfJ were shown to specifically bind hemin, hemin binding blots were performed in order to assess their ability to bind hemin via a different method. The results were surprising because bands representing TdfJ and TbpA were not predominant. We expected that there would be bands corresponding to TbpA and TdfJ, but instead a hemin-binding band of a different size resulted. Bands that could correspond to TdfJ and TbpA are present, however they are very faint. This was not discouraging because after SDS-PAGE, TbpA is unable to bind transferrin due to the loss of protein conformation. Also, the low intensity of the signals representing TdfJ and TbpA could simply correspond to lower levels of expression in general. The two dark hemin-binding bands, approximately ~35 and ~70 kD in size, were sent for protein identification. Protein identification from a 1D gel can be tough because parts and pieces of many different proteins can be found in one band. However, the results for the identification of the smaller hemin-binding band were fairly clear. The ~35 kD protein was putatively identified as FbpA. FbpA is the periplasmic binding protein responsible for shuttling the iron released by the transferrin and lactoferrin acquisition systems across the periplasm. As previously mentioned, this periplasmic protein was likely included in the sarkosyl insoluble outer
membrane preparation because apo-FbpA binds directly and specifically TbpA (80). This is interesting because it is the second protein involved in the transferrin acquisition system that was implicated in heme binding. This could indicate that the pathway used to import iron from transferrin could also be used to import heme, thus suggesting that the transferrin acquisition system has broad specificity. Until this point, the transferrin acquisition system was only known to have one substrate.

The protein identification results for the larger, ~70 kD band, were less clear. Many things were detected in the band, including TbpA and Porin, however the best matches were to PilQ and FetA. PilQ functions in type IV pilus organelle biogenesis (29), and FetA is the TBDT responsible for the import of ferric enterobactin and other catecholate-type siderophores (14, 43). Interestingly, it has also been suggested that FetA participates in hemin utilization previous to this study. In a gonococcal mutant unable to express FetA, heme uptake was reduced by greater than 50% as compared to wild type heme uptake (7). PilQ has also been previously implicated in heme uptake. PilQ forms a channel that allows for the entry of heme and antimicrobial compounds (22). A mutation within PilQ resulted in increased heme entry. However, a strain lacking both PilQ and the hemoglobin receptor were still able to grow on free heme, so there is obviously another mechanism for entry (22). These results indicate that there may be many players involved in heme binding and uptake. The hypothesis that there is one discrete heme uptake system in N. gonorrhoeae was disproved by these studies. There are likely additional proteins involved in hemin binding and uptake that have yet to be identified. Information provided by this study will possibly help to elucidate other heme uptake pathways.

Together, the results from all of the aspects of this study increase the knowledge base associated with gonococcal TBDTs. The data provided in this study offers valuable insight into
the pathogenesis of *N. gonorrhoeae* and could aid in the development of a vaccine or new therapeutic agents. The development of effective new therapeutics or a vaccine would greatly benefit public health. Although gonorrhea has long been a treatable disease, the ability to easily fight this pathogen is waning. Antibiotic resistance is on the rise and will continue to be a problem in the future. If the issue is not dealt with, gonorrhea could become untreatable. This would be very problematic for human health, and especially for women’s health. Downstream sequelae resulting from untreated gonococcal infection are numerous, including detrimental effects for reproductive health. Infections and their resulting symptoms are costly to the economy and burden the healthcare system. These costs will only increase as the infection becomes more and more difficult to treat. As previously mentioned, TBDTs make excellent vaccine targets because they are surface exposed, well-conserved, and provide nutrients that are essential for bacterial survival. This is why knowledge pertaining to their structure, function, expression, and regulation is important to attain.
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