2015

Characterization of the Regulation and Function of Neisseria Gonorrhoeae TonB-dependent Transporters: TdfG, TdfH and TdfJ

Sophonie Jean

Virginia Commonwealth University

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CHARACTERIZATION OF THE REGULATION AND FUNCTION OF *NEISSERIA GONORRHOEAE* TONB-DEPENDENT TRANSPORTERS: TDFG, TDFH AND TDFJ

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

SOPHONIE JEAN
Bachelor of Science, University of Richmond, 2009

Director: CYNTHIA NAU CORNELISSEN, PH.D.
PROFESSOR OF MICROBIOLOGY AND IMMUNOLOGY

Virginia Commonwealth University
Richmond, Virginia
July 2015
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Ultimately, I give all praise and glory to God from whom all blessings flow.
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<td>approximately</td>
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<td>&lt;</td>
<td>less than</td>
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<td>less than or equal to</td>
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<td>2D</td>
<td>two-dimensional</td>
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<td>bp</td>
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<td>BSA</td>
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<td>C-</td>
<td>carboxy</td>
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<td>CAT</td>
<td>chloramphenicol acetyltransferase cassette</td>
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<td>Cd$^{2+}$</td>
<td>cadmium ion</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>CDM</td>
<td>chemically defined media (chelexed)</td>
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<tr>
<td>CEACAM</td>
<td>carcinoembryonic related cell adhesion molecule</td>
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<td>CFU</td>
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<td>CP</td>
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<td>ECF</td>
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<td>EDTA</td>
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<td>Ent</td>
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<td>Erm(\textsuperscript{r})</td>
<td>erythromycin (resistance)</td>
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<td>iron</td>
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Fe(NO$_3$)$_3$ ferric nitrate
Fe$^{2+}$ ferrous
Fe$^{3+}$ ferric
fHbp Factor H binding protein
g gram
g gravity
G. vaginalis Gardnerella vaginalis
GCB GC medium base
GGI gonococcal genetic island
GISP Gonococcal Surveillance Isolate Project
GW Wade-Gaver media
$H. ducreyi$ Haemophilus ducreyi
$H. influenzae$ Haemophilus influenzae
H$_2$O$_2$ hydrogen peroxide
Hg hemoglobin
Hg-Hp hemoglobin-haptoglobin
His$_6$ 6X-histidine
HIV Human Immunodeficiency Virus
HmBP heme binding protein
Hp haptoglobin
HSPG heparin sulfate proteoglycan
Hx hemopexin
ICP-OES inductively coupled plasma optical emission spectrometry
IgG immunoglobulin G
IL-6 interleukin 6
IM inner membrane
IPTG isopropyl β-D-thiogalactopyranoside
Kan(') kanamycin (resistance)
NGAL  neutrophil gelatinase associated lipocalin
Nhba  Neisseria heparin binding antigen
Ni    nickel
Ni^{2+} nickel ion
Ni-NTA nickel-nitrilotriacetic acid
nM    nanometer
nM    nanomolar
nM    nanomolar
OD    optical density
OH^- hydroxide
OH•   hydroxyl radical
OM    outer membrane
Opa   Opacity protein
P. aeruginosa Pseudomonas aeruginosa
P. gingivalis Porphyromonas gingivalis
P. fluorescens Pseudomonas fluorescens
PBP   periplasmic binding protein
PBS   phosphate-buffered saline
PCR   polymerase chain reaction
PEA   phosphoethanolamine
PID   pelvic inflammatory disease
PMA   phorbol myristate acetate
polyC poly-Cytosine
polyG poly-Guanine
RBS   ribosome binding site
RNA   ribonucleic acid
rpm   revolutions per minute
ROS   reactive oxygen species
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<td>vancomycin, colistin, nystatin inhibitor</td>
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Abstract

CHARACTERIZATION OF THE REGULATION AND FUNCTION OF NEISSERIA GONORRHOEAE TONB-DEPENDENT TRANSPORTERS: TDFG, TDFH AND TDFJ

By Sophonie Jean, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015

Major Director: Cynthia Nau Cornelissen, Ph.D.
Professor, Department of Microbiology and Immunology

The obligate human pathogen Neisseria gonorrhoeae successfully overcomes host strategies to limit essential nutrients, termed “nutritional immunity” by expression of TonB-dependent transporters (TdTs): outer membrane receptors that facilitate nutrient transport in an energy-dependent manner. N. gonorrhoeae encodes eight TdTs, five of which facilitate utilization of iron or iron-chelates from host derived proteins including transferrin, lactoferrin and hemoglobin, in addition to siderophores from neighboring bacteria. The transferrin utilization system was previously shown to be critical for establishing infection in human males; demonstrating the possible contributions of TdTs
to gonococcal pathogenesis. As such, studies describing the biological function and contribution to pathogenesis of the remaining three uncharacterized TdTs (TdfG, TdfH and TdfJ) are needed. In this study we report that neither TdfG, TdfH nor TdfJ are heme receptors as gonococcal heme utilization occurs passively, independent of energy derived from the TonB system. We also report that TdfH and TdfJ are zinc (Zn) regulated and identify virulence associated regulators that modulate expression of these TdTs, which is in some cases strain-specific. We report that both TdfH and TdfJ contribute to Zn acquisition in *N. gonorrhoeae* and we characterize TdfH as a calprotectin receptor. Calprotectin, an immune effector protein highly expressed in neutrophils, has antimicrobial activity due to its ability to sequester Zn and Mn. We present evidence that TdfH confers resistance to calprotectin and that TdfH facilitates gonococcal calprotectin binding and Zn accumulation in the presence or absence of calprotectin. Finally, we demonstrate that TdfH expression enhances *N. gonorrhoeae* NET survival. These studies identify for the first time a novel gonococcal defense strategy to host-mediated nutritional immunity, in which *N. gonorrhoeae*, via the TdT TdfH, utilizes calprotectin as a Zn source neutralizing its antimicrobial activity. These studies have yielded novel insights into the function and regulation of TdfG, TdfH and TdfJ in *N. gonorrhoeae* and have laid the framework for future investigation of TdT-mediated Zn acquisition and its role in bacterial pathogenesis.
Chapter 1: Introduction

I. Neisseria

The genus Neisseria belongs to the family Neisseriaceae, which is also comprised of 3 other genera: Moraxella, Acinetobacter and Kingella [121]. Members of the Neisseriaceae family can be differentiated from one another by cell morphology and biochemical properties such as the ability to produce acid from glucose and reduce nitrate as well as the presence of oxidase, catalase, carbonic anhydrase and thymidine kinase [25]. The Neisseria genus is comprised of a number of species which can occur as normal flora or pathogens in humans. The Neisseria species are non-motile, Gram-negative diplococci with flattened adjacent sides that are aerobic or facultatively anaerobic and grow optimally at temperatures between 35-37°C [25]. Human associated Neisseria spp. can be identified by their acid production from carbohydrates, their ability to reduce nitrite and produce polysaccharide from sucrose [121].

II. Pathogenic Neisseria species

The only Neisseria species that is always considered pathogenic is Neisseria gonorrhoeae. It infects the mucosal surfaces of urogenital sites and the oronasopharynx, causing symptomatic or asymptomatic infections. In some cases,
Neisseria meningitidis can be pathogenic causing sporadic cases or epidemics of meningitis. Strains of *N. meningitidis* can be carried as normal flora in the nasopharynx of healthy individuals. Most *Neisseria* species are commensal and colonize the oropharynx without causing disease. Some commensal species have been sporadically isolated from disseminated sites, blood, and cerebrospinal fluid, although they are not normally associated with invasive infections. Commensal species appear to be opportunistic pathogens, causing infection in people with deficient immune systems [121].

### III. Meningococcal disease

#### A. Epidemiology

*Neisseria meningitidis* colonizes the nasopharynx where it exists as a commensal bacterium. Though carriage rates can vary among human populations, as many as 10% of adolescents and adults are asymptomatic carriers [37]. Transmission of *N. meningitidis* can occur directly via person-to-person contact or through respiratory droplets from an infected to a susceptible individual. As such, transmission and carriage rates increase dramatically in closed settings such as residential schools and military camps [37]. Carriage can last as long as months and is least prevalent in the early years of life and most prevalent in young adults [218]. Persons with immune deficiencies such as deficiencies in terminal complement components have increased susceptibility to meningococcal disease as well as people with exposure to tobacco smoke and concurrent viral infection of the upper respiratory tract [199].
Despite high carriage rates, meningococcal disease is rare, occurring at annual incidence rates of 1-1000 per 100,000 population throughout the world [37]. *N. meningitidis* strains can be separated into thirteen different serogroups on the basis of the polysaccharide capsule, of which six (A, B, C, W135, X and Y) are responsible for the majority of invasive meningococcal disease [218]. The highest incidence of meningococcal disease occurs in sub-Saharan Africa in the 'meningitis belt' where incidence can approach up to 1000 cases per 100,000 population during epidemics [89]. Serogroup A is most important in this region, though Serogroups C and X can also cause disease [89]. In the Americas, Serogroups B, C, and Y are predominant meningococcal disease causing strains and result in historically low incidence of diseases at 0.3-4 cases per 100,000. Serogroups B and C predominate in Europe, Australia, and New Zealand where incidence varies between countries from 0.2-14 cases per 100,000. In Asia, the burden of meningococcal disease is less well defined, though serogroups A and C are thought to be responsible for the infrequent outbreaks and low levels of endemic disease in the region [89].

**B. Infection**

In less than 1% of colonized individuals, meningococci penetrate the mucosal epithelia and enter the bloodstream to cause meningococcemia [41]. In half of the cases of meningococcal septicemia, the bacteria penetrate the blood brain barrier causing meningitis, the most common presentation of invasive meningococcal disease, which presents clinically as sudden onset of headache, fever, and neck stiffness and is sometimes accompanied by nausea, vomiting, sensitivity to light and altered mental status [199]. Meningococcemia or bloodstream infection is characterized by sudden
fever and rash and is often associated with hypotension and multi-organ failure [199]. The overall case fatality rate of invasive meningococcal disease is 9 -12%, with a rate of up to 40% among patients with meningococcal sepsis [199]. A significant number of meningococcal survivors have sequelae such as hearing loss, neurologic disability, or loss of a limb [199].

C. Treatment

*N. meningitidis* remains susceptible to many antibiotics including penicillin [199]. In the US, where few penicillin resistant strains have been reported, penicillin remains the first line of therapy recommended after *N. meningitidis* infections has been confirmed [41]. To prevent secondary infection of individuals with close contact to patients with meningococcal disease, prophylactic treatment of household members is often used as a method of control [199].

Several meningococcal vaccines are licensed for use in the US. MPSV4, the first meningococcal polysaccharide vaccine was approved in 1974. The current polysaccharide vaccine (*Menomune*; Sanofi Pasteur) confers protection against Serogroups A, C, Y and W-135 [32]. However, it is poorly immunogenic in infants, doesn’t confer long lasting immunity and does not reduce nasopharyngeal carriage of *N. meningitidis* to elicit herd immunity [153]. As such it is only recommended in individuals older than 55 years of age when other conjugate vaccines are not available [41]. Three meningococcal conjugate vaccines are licensed in the US: two single component vaccines—Menactra also known as MCV4 (Sanofi Pasteur) and Menevo (Novartis) and a two-component vaccine MenHibrix (GlaxoSmithKline). MCV4 was approved in 2005 and has meningococcal A, C, W and Y polysaccharides conjugated to diphtheria toxoid
protein carrier and is approved for use in persons from 9 months to 55 years of age [153]. Menevo, licensed for use in 2010 in persons 2-55 years of age, contains the meningococcal A, C, W and Y polysaccharides conjugated to CRM197 (MenA). MenHibrix, was licensed for use in 2012 and contains \textit{N. meningitidis} serogroups C capsular polysaccharide conjugated to tetanus toxoid, \textit{N. meningitidis} serogroup Y capsular polysaccharide conjugated to tetanus toxoid and \textit{Haemophilus influenzae} serogroup B capsular polysaccharide conjugated to tetanus toxoid. It is recommended as a 4 dose series in children between 2-18 months of age [41].

Until recently, there was no \textit{N. meningitidis} serogroup B vaccine licensed for use in the US, which causes 50\% of invasive disease worldwide and 30\% in the US [32]. Vaccine development strategies were hindered due to the fact that the polysaccharide of \textit{N. meningitidis} serogroup B contains polysialic structures identical to those expressed in fetal neural tissue, and does not induce a protective IgG response [217]. As such, novel vaccine development strategies have focused on non-capsular antigens [32, 199, 218]. Bexsero\textsuperscript{®} also known as 4CMenB (Novartis), licensed for use in Europe and Australia in 2013 and in the US in 2015, is the first broad-spectrum vaccine for \textit{N. meningitidis} serogroup B [130]. 4CMenB is comprised of 4 components against meningococcal serogroup B: factor H binding protein (fHbp) fused to accessory protein GNA 2091, \textit{Neisseria} heparin binding antigen (NHba) fused to GNA 1030 and \textit{Neisseria} adhesin A (NadA) all added to an outer membrane vesicle from the serogroup B New Zealand strain (strain NZ98/254) [130]. A second group B vaccine, Trumemba\textsuperscript{®} (Pfizer), which is composed of two fHbp variants (A05 and B01), was licensed for use in 2014 [130]. Despite the leaps on the development of vaccine strategies, comprehensive post-
implementation surveillance plans will be required to monitor the inevitable loss of immunity and the potential harmful effects of vaccination.

IV. Gonococcal disease

A. Epidemiology

*Neisseria gonorrhoeae* is the etiological agent of the sexually transmitted infection, gonorrhea. Gonorrhea is a significant global health concern causing an estimated 106 million new cases each year [267]. In the US, gonorrhea is the second most commonly reported communicable disease with 820,000 estimated new cases each year, less than half of which are detected and/or reported to the CDC. The key risk factor for infection is new or multiple sex partners. In the US, incidence rates are generally highest among adolescents and young adults, women and African Americans, for whom the rate of reported cases was 12.4 times greater than that of whites [40]. For the first time since 2003 the number of reported cases in men was greater than in women in 2013 [40].

B. Infection

*Neisseria gonorrhoeae* is transmitted via close sexual contact of infected individuals. It can be transmitted perinatally from mother to child during child birth. Thus, newborn infants are treated with an ocular prophylactic agent (erythromycin) to prevent gonococcal ophthal mia [266]. *Neisseria gonorrhoeae* causes infection in the urogenital, anorectal or pharyngeal mucosa. In men, infection is asymptomatic in a minority of cases. When present, symptoms occur within 14 days of infection and present as dysuria and purulent urethral discharge. Most women are asymptomatic. Symptoms
such as dysuria, increased vaginal discharge and vaginal bleeding can sometimes present but are non-specific and often mistaken for a bladder or vaginal infection [159]. Rectal infection in both men and women can present as soreness, itchiness, discharge and bleeding but can also be asymptomatic [120]. Most pharyngeal infection is asymptomatic but can also cause sore throat [264].

Untreated infection can lead to complications and severe morbidity is associated with downstream sequelae. In women, gonococcal infection can ascend to the upper reproductive tract to cause Pelvic Inflammatory Disease (PID), which can increase the risk of ectopic pregnancy and infertility. In men, complicated infection can lead to epidymitis, which can rarely lead to infertility. Though rare, *N. gonorrhoeae* can also enter the blood and cause Disseminated Gonococcal Infection (DGI), characterized by arthritis, tenosynovitis and dermatitis [99, 196]. Gonococcal infection is often associated with other infections including *Chlamydia trachomatis* and can increase the risk of acquiring other STI infection and the transmission of HIV [108].

### C. Treatment

Antimicrobials have been successfully used to treat gonococcal infection for the last 70-80 years [242]. However, the ability to treat gonococcal infection has become increasingly difficult due to the development of antibiotic resistance. In the 1990-2000 decade, fluoroquinolone resistance spread throughout the US. As such, in 2007 the CDC no longer recommended fluoroquinolones for treatment of uncomplicated gonorrhea leaving 3rd generation cephalosporins as the last line of defense to treat gonococcal infection [38]. In 2010 the STD Treatment Guidelines recommended dual therapy with a cephalosporin—Cefixime (oral) or Ceftriaxone (intramuscular) plus
azithromycin or doxycycline for the treatment of co-occurring pathogen *Chlamydia trachomatis*. However, treatment failures with cefixime and ceftriaxone and declining cefixime susceptibility to collected urethral isolates reported by the CDCs Gonococcal Surveillance Isolate Project (GISP) led to a 2012 revision, which now only recommends ceftriaxone plus azithromycin or doxycycline as the only reliable treatment of uncomplicated urogenital, anorectal, and pharyngeal gonorrhea [39].

Though the current recommendations appear to be effective, decreasing global susceptibility to ceftriaxone and the prevalence of azithromycin resistance in many settings as well as the prohibitive cost of dual-therapy in many resource poor settings indicate that dual therapy regimens are more likely short-term solutions. Additionally, multidrug resistant and extensively drug resistant strains have been reported throughout the world [242], further validating fears of untreatable gonorrhea. Importantly, gonococcal infection elicits no protective immunity and there is no vaccine for preventative measures [277]. Thus, strategies to treat and combat the spread of antimicrobial resistant gonorrhea are a top national and international priority.

V. *N. gonorrhoeae* virulence factors

*N. gonorrhoeae* expresses an array of surface molecules that help mediate its interaction with the human host and successfully cause infection. These structures contribute to adherence and invasion of mucosal sites as well as immune evasion and so are briefly discussed below.
A. Type IV Pilus

Type IV pili are filamentous polymers six nanometers in diameter and several micrometers in length that extrude from the cell surface [162]. Several genes are involved in the biogenesis, assembly and disassembly of pili. The pilus fiber is mainly comprised of individual subunits of the major pilin protein, PilE, arranged in a helical configuration. Minor pilin proteins such PilC, PilV and PilX are also incorporated into the pilus structure and modulate function [250]. PilD a peptidase/transmethylase, processes the prepilin precursor and facilitates the incorporation of pilin subunits into the growing fiber. PilQ forms an outer membrane secretin through which the fiber is extruded and PilF and PilT contribute to fiber assembly and disassembly, respectively [265]. Pili have multiple roles in gonococcal pathogenesis inducing bacterial self-agglutination [231], attachment to eukaryotic cells [162] and twitching motility [163]. Type IV pili also facilitate DNA uptake transformation and thereby contribute to the genetic variation [75].

Type IV pili undergo both phase and antigenic variation. Phase variation occurs as a result of slipped strand mis-pairing event that occurs at the polyC-tract within the pilE structural gene during DNA replication. The variable length of the polyC region results in frame-shift mutation and reversible on/off variation [13, 123]. Antigenic variation occurs as a result of high frequency gene conversion between the PilE expression locus and one or more antigenically distinct pil genes in silent pil loci (pilS). This process is RecA-dependent and uni-directional [95, 123]. The pilus fiber can also undergo post-translation modification such as glycosylation [220] as well as phosphoethanolamine (PEA) and phosphocholine modification, which can modulate its function [250]. Phase and antigenic variation and post-translational modification of pilus
fibers are thought to inhibit host immune recognition. Antigenic variation of pili is thought to be the biggest reason for the failure of a pili based vaccine to elicit protection [24, 250].

B. Porin

Neisserial porins are the most abundant outer membrane protein in *Neisseria*. Like other members of the Gram-negative porin superfamily, neisserial porins are comprised of three polypeptide subunits that form β-barrels and trimerize to form the native porin structure, which facilitates the passage of small nutrients and waste products across the outer membrane. *Neisseria* porins are anion selective and voltage-gated [272]. *N. meningitidis* expresses two porins: class I (PorA), which is phase variable [245] and either class 2 or class 3 (PorB). The limited antigenic variability of porins form the basis of the neisserial serotyping system [74]. In *N. gonorrhoeae*, PorA exists as a pseudogene due to mutations within the promoter and coding regions [69]. Thus, the gonococcus only expresses 1 porin that is homologous to the meningococcal PorB. PorB is further classified as protein IA (PorB1A) or protein IB (PorB1B) [81].

In addition to their essential role in cell viability, porins also have roles in gonococcal pathogenesis. Porins can spontaneously transfer as a functioning channel into model membranes [151, 158] and eukaryotic cell membranes where the resulting transient changes in voltage interfere with cell signaling [84, 114, 202]. Neisserial porins have also been shown to interact with mitochondria, where it is thought to induce calcium influx and apoptosis [167-168]; however, the role of porins in apoptosis is controversial since some studies indicate that porin interaction with mitochondria protects from apoptosis [156-157].
Additionally, neisserial porins have been described to have immuno-modulatory functions. Neisserial porins alter neutrophil signaling and inhibit neutrophil degranulation and phagocytosis [21, 84]. Neisserial porins also contribute to B-cell activation through induction of proliferation and enhancement of antibody secretion [213, 262]. As such, PorB has been incorporated into vaccine formulations as an adjuvant [260].

Porins have also been described to have a role in invasion of epithelial cells. PorBIA, which is the expressed por allele in gonococcal strains associated with disseminated gonococcal infection, facilitates invasion of epithelial cells [247, 255]. The PorBIA serotype is specifically associated with serum resistance [35] and was shown to mediate resistance to the alternative and classical complement pathway through binding of Factor H and C4b-binding proteins, respectively [189-190]. The PorBIB serotype is also able to bind C4b-binding protein indicating that neisserial porins mediate stable resistance to complement pathways [188].

C. Lipooligosaccharide (LOS)

Like most other Gram-negative bacteria, Neisseria express lipopolysaccharide (LPS), which is the main component of the outer leaflet of the outer membrane forming an essential barrier to the external environment. The structure of LPS is comprised of Lipid A, a lipid moiety which anchors the molecule to the outer membrane, a short oligosaccharide core, and a variable length repeating polysaccharide unit or O-antigen. The LPS structure in pathogenic Neisseria, lacks the repeating O-antigen and so is more accurately termed, lipooligosaccharide (LOS). Though the LOS structure is stably expressed, glycosyl transferases, which mediate the addition of terminal sugars to the LOS structure and are encoded by the lgt genes, are phase variable [80]. Phase
variation of the glycosyl transferases occurs as result of slipped strand mis-pairing during DNA replication due to a polyG tract within the coding region of lgt genes [270]. The combination of these enzymes results in the antigenic diversity and variable expression of LOS terminal structures.

The oligosaccharide groups on LOS terminal structures are identical to the oligosaccharides on host glycosphingolipids [92]. Specifically, the lacto-N-neotetraose (LNnT) moiety mimics human paragloboside. In N. gonorrhoeae, the LNnT moiety of LOS structures bind to the host asialoglycoprotein receptor to facilitate adherence to human sperm [91] and invasion of urethral epithelial cells [90]. This example of ‘molecular mimicry’ indicates that neisserial LOS contributes to hijacking of host receptor systems and immune evasion [92, 248].

LOS can be further modified by the addition of sialic acid moieties to the terminal lactosamine of LOS. Neisseria cannot synthesize sialic acid, instead it utilizes host derived cytidine 5′-monophospho-N-acetyl neuraminic acid (CMP-NANA) as a sialyl donor and requires a sialyl transferase for the modification [212]. Because sialic acid acceptor sites only exist on certain variants of LOS terminal structures, which are regulated by the phase variable expression of glycosyl transferases, sialic acid modification is also variable. Sialic acid modification mediates serum resistance and specifically, the sialic acid moiety of LOS binds to Factor H, mediating phase variable resistance to complement mediated killing [191, 261]. While sialic acid modification contributes to serum resistance, it also inhibits invasion of epithelial cells indicating that variation of LOS terminal structures can contribute to conversion between invasive and serum resistant phenotypes [246]. The phase variation and modification of LOS terminal
structures implicate a much larger role in gonococcal pathogenesis beyond stabilization of the outer membrane, essential for viability.

**D. Opacity (Opa) proteins**

Opacity (Opa) proteins are a family of *Neisseria* outer membrane proteins that mediate invasion of epithelial cells and leukocytes [127, 154, 195, 256]. Opa proteins were originally described for their capacity to impart opacity to the colonies that express them [229]. While *N. meningitidis* encodes 4 opa loci, *N. gonorrhoeae* have 11 that are dispersed throughout the genome [16, 219]. The selection of Opa+ gonococci during natural and experimental infection [109, 230-231] is indicative of their important role in gonococcal infection.

Expression of Opa proteins is subject to phase variation at the translation level as a result of slipped-strand mis-pairing of a pentameric repeat sequence (5′-CTCTT-3′) within the signal peptide of the coding region of the gene [219]. Thus, a gonococcus can express one or multiple Opas at a time; however, expression of no more than 4 Opas have simultaneously been detected in an individual gonococcus [22]. Opa proteins also undergo antigenic variation due to recombination events between opa loci [97].

Opa proteins are ~30kDa in size and form eight stranded β-barrel structures with four surface-exposed loops [250]. Loops 1, 2 and 3 are variable and the sequence diversity in these regions confer receptor specificity. Opa proteins can be categorized based on their receptor tropism. A small group of Opas bind to heparin sulfate proteoglycans (HSPGs) and extracellular matrix proteins vitronectin and fibronectin, while most Opas bind to the human carcinoembryonic related cell adhesion molecule (CEACAM) family of receptors [60]. The combined phase and antigenic variation of Opa
proteins provides important functional diversity via their recognition of specific cellular receptors, which mediates tissue tropism and influences interactions with epithelial and phagocytic cells.

**VI. Iron in the human host**

Iron is an essential element for most living organisms. Iron can exist in two readily reversible redox states: ferrous, Fe$^{2+}$ or ferric, Fe$^{3+}$. Thus, it is incorporated as a co-factor or electron carrier of a wide range of proteins, where it can adopt a redox potential from -300 to +700mV depending on its local protein environment [9]. Iron-containing proteins participate in essential physiological functions such as respiration and DNA biosynthesis [9]. While the chemical properties of iron contribute to its biological versatility, they also potentiate toxicity. In the presence of oxygen, the reduced ferrous iron form is predominate and can activate the Fenton Reaction [Fe$^{2+}$ + H$_2$O$_2$ = OH$^-$ + OH$^-$ + Fe$^{3+}$], generating highly reactive free hydroxyl radicals that are detrimental to most macromolecules [244].

Though the human body contains 3-4g total iron, iron is tightly regulated to mitigate its potential toxicity. Storage proteins such as ferritin sequester iron intracellularly while extracellular iron is carefully chaperoned through the body by carrier protein transferrin. The presence of transferrin in plasma, lymph and cerebrospinal fluid and lactoferrin in exocrine secretions purge the human host of free iron [258]. In normal human plasma, free iron levels are ~ 10$^{-18}$ M [177]. Thus, the human host is a hostile environment for invading microorganisms that require micro molar amounts of iron for growth. Additionally, host iron withholding defenses, also termed “nutritional immunity”,

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in response to infection further limit iron availability by reducing dietary iron absorption and plasma iron levels and increasing intracellular iron storage [257]. The ability of pathogenic microbes to acquire iron from host sources can therefore be considered a critical virulence determinant. In the absence of free iron, host-derived iron binding proteins can be biological iron sinks to invading microbes that are capable of utilizing them. Some potential iron sources within the human host are briefly described below.

A. Transferrin

Transferrin (Tf) is an 80kDa glycoprotein whose primary role is to transport iron throughout the body. Tf structure is bi-lobal (C- and N-lobe) and each lobe can bind an iron atom, though the N-lobe appears to be predominately occupied by iron in vivo [150]. Tf binds to Fe$^{3+}$ with high affinity ($K_d \sim 10^{-22}$ M). In serum, Tf is \(~30\% saturated with iron and is present at substantial concentrations (25-44µM); however, only 0.2-1.3µM is present in mucosal secretions [209]. To facilitate iron trafficking, iron-laden Tf binds to the human transferrin receptor (TfR1) expressed on the surface of all iron requiring cells [2]. The Tf-TfR1 complex is internalized via receptor mediated endocytosis and the endosome is subsequently acidified to release Fe$^{3+}$. Apo-Tf remains associated with the TfR1 and the complex is recycled back to the cell surface where Tf is released into serum [150].

B. Lactoferrin

Lactoferrin (Lf) is iron binding glycoprotein within the transferrin family. It is found in highest abundance in milk, but it also present in other biological fluids including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids, urine [79]. In serum, Lf is present at low levels (3.8-8.8nM), but is a significant
component of mucosal secretions at 6-13µM [209]. Like Tf, Lf is ~ 80kDa, bi-lobal and can bind Fe$^{3+}$; however, unlike Tf, Lf retains bound iron in acidic conditions [68]. In addition to roles in iron-sequestration, Lf has been attributed a number of other physiological roles, one of which is innate immunity [79]. A significant amount of Lf is found in the specific granules of polymorphonuclear leukocytes and has demonstrated iron-independent antibacterial activity. Proteolytic processing of Lf results in the cationic peptide lactoferricin, which is thought to interact with negatively charged structures on the surface of bacteria such as LPS and contribute to disorganization of the outer membrane resulting in cell lysis [68].

C. Ferritin

Excess iron is stored in the intracellular storage protein, ferritin. Ferritin is a hetero-polymer comprised of 24 heavy and light polypeptide subunits that assemble to form an almost perfectly spherical nano-cage [2]. The mature protein shell is ~450kDa with a diameter of 8-12nm and can hold up to 4500 oxygen bound iron atoms [2, 96]. Iron is stored in the Fe$^{3+}$ form in ferritin. Hemosiderin, a degradation product of ferritin contains a heterogeneous iron mineralization products and can release iron in acidic conditions [179].

D. Heme/ hemoproteins

Approximately 70% of the iron content in the human body is within heme, a heterocyclic organic ring called porphyrin covalently bound to Fe$^{2+}$ (heme) or Fe$^{3+}$ (hemin) [203]. Heme solubilizes iron and enhances its catalytic activity; as such it is incorporated as a prosthetic group of hemoproteins involved in oxygenation, oxidative stress-response, electron transport, oxygen transport, oxygen sensing, and oxygen
storage [10]. Over 95% of heme is in hemoproteins, the majority of which is in hemoglobin found mainly within erythrocytes [203]. Hemoglobin (Hg) is a tetramer comprised of two α- and two β- chains. Each subunit binds to a heme molecule, of which the heme-iron can bind to oxygen to facilitate oxygen transport. Though intracellular, Hg can be released via spontaneous hemolysis resulting in 80-800nM of Hg in serum [209]. However, excess quantities of the tetrameric glycoprotein, haptoglobin (Hp) rapidly complexes Hg and removes it from circulation by delivering it the endocytic system via receptor-mediated endocytosis [173]. Likewise, any heme dissociated from Hg during hemolysis is bound to the glycoprotein hemopexin (Hx) and removed from circulation in a receptor-dependent manner [173].

**E. Siderophores**

In response to iron limitation many microorganisms (bacteria, yeast and fungi) synthesize and secrete, small (<1kDa) iron chelating molecules termed siderophores [193]. These siderophores specifically bind Fe$^{3+}$ with $K_d$ $\approx$ 10$^{-22}$-$10^{-50}$ M and can thus remove iron from host sources such as ferritin, Tf, Lf, but not from heme or hemoproteins [193]. Most siderophores are biosynthetically produced by large multi-enzyme synthases and have a backbone of modified amino acid side chains creating the iron-coordinating ligand [47]. They can be classified into one of three groups: catecholate, hydroxamate and hydroxycarboxylate [252]. Upon secretion, siderophores scavenge free or host protein bound iron in the extracellular environment. The iron-laden siderophores can then deliver the sequestered iron to microorganisms that express siderophore specific receptors.
Mammals fight back against bacterial siderophores by producing the lipocalin family of proteins termed siderocalins, for siderophore binding lipocalins. Siderocalins include lipocalin 1 (Lcn1) or tear lipocalin and neutrophil gelatinase associated lipocalin (NGAL or Lcn2, 24p3, Scn). These proteins bind enterobactin-like siderophores, preventing bacteria from scavenging iron [54]. Some bacteria go further to modify their siderophores via glycosylation, which prevents siderocalin binding [70].

Another variation of the siderophore theme are hemophores. Hemophores are bacterial proteins that bind heme or host hemoproteins with high affinity. They are structurally diverse and have different mechanisms of heme coordination. Like siderophores, hemophores deliver heme back to the bacteria that secrete them via cell surface receptors; however, only the heme moiety is internalized while the hemophore is recycled at the cell surface [253].

VII. Iron acquisition systems of *Neisseria gonorrhoeae*

As an obligate human pathogen *N. gonorrhoeae* has evolved a diverse catalogue of systems to assimilate iron from the hostile host. Unlike other bacteria *N. gonorrhoeae*, does not secrete any siderophores [259]. Instead it relies on single and two-component systems to acquire iron from host derived iron or heme binding proteins.

A. Two-component systems

A schematic of two-component systems for iron acquisition in *N. gonorrhoeae* are depicted in Figure 1. The Tf-iron acquisition system is comprised of a TonB-dependent transporter (TdT), TbpA and a surface exposed lipoprotein, TbpB. The TbpA/B system is found in most *Neisseria*; notably it is found in all *N. meningitidis* and
Figure 1. Two-component iron acquisition systems of *N. gonorrhoeae*.

Host iron sources transferrin (hTf), lactoferrin (hLf), hemoglobin (Hg), and hemoglobin bound to haptoglobin (Hg-Hp) are shown in the extracellular space with their gonococcal receptor systems. The TonB-dependent transporters (TbpA, LbpA and HpuB) are depicted as barrels traversing the outer membrane (OM) with a plug domain occluding the barrel. The companion lipoproteins (TbpB, LbpB and HpuA) are shown attached to the outer leaflet of the OM. The TonB/ExbB/ExbD system is shown attached to or imbedded within the inner membrane (IM). TonB interacts with the plug domain of TdTs to facilitate iron transport through the TdT barrel. The PBP, FpbA is shown localized to the periplasm where it binds ferric iron from TbpA and LbpA and shuttles it to the inner membrane permease, FbpB which transports iron into the cytosol upon ATP hydrolysis by ATPase, FbpC.
Figure 1. Two-component iron acquisition systems of *N. gonorrhoeae*. 
N. gonorrhoeae strains [155]. Additionally, the absence of the Tf acquisition system attenuated virulence in a human male model of infection indicating the critical role of Tf utilization to pathogenesis [52]. TbpA and TbpB are both encoded in an iron repressed operon in which \( tbpB \) precedes \( tbpA \). The structure of TbpA is consistent with the common topology described for all TonB-dependent transporters: a \( \beta \)-barrel comprised of 22 amphipathic \( \beta \)-strands, surface exposed loops and a globular plug domain inside the barrel [175]. TbpB has a bi-lobal structure; the N- and C-terminal lobes can both can bind Tf [62]. Both TbpB and TbpA can bind Tf with nanomolar affinities and remove its iron [53, 211]. Only TbpA is required for uptake of iron from Tf, but the presence of TbpB makes the process more efficient since TbpB preferentially binds holoTf [6, 50, 63].

The Lf acquisition system is analogous to the Tf acquisition system comprising a TdT, LbpA, and a surface exposed lipoprotein, LbpB [18, 20, 208]. Like the Tf acquisition system the \( lbpA/B \) genes are encoded on an iron repressed operon where the lipoprotein precedes the TdT [4]; however, LbpB is subject to phase variation due to a polyC-tract within the coding region [18]. The LbpA/B system is present in most Neisseria [155]. While present in all \( N. meningitidis \) strains, the Lf acquisition system is absent in half of gonococcal isolates due to a large deletion of \( lbpB \) and the 5' end of \( lbpA \) [4]. The Lf acquisition system is not required for virulence, as \( N. gonorrhoeae \) lacking the LbpA/B are still able to cause infection in men [52].

The hemoglobin/ haptoglobin (Hg-Hp) acquisition system is another two-component acquisition system comprised of a TdT, HpuB, and a lipoprotein, HpuA present in most Neisseria. Unlike the Tf and Lf systems, which specifically recognize
human Tf or Lf, the HpuB/A system recognizes Hg, Hp or Hg-Hp complexes [42-43, 138, 140]. The system internalizes the heme moiety from Hg which can support use of Hg or Hg-Hp as a sole source of iron or heme [141]. Both HpuB and HpuA are required for Hg binding and growth in the presence of Hg as the sole iron source [42]. The operon encoding hpuB and hpuA is iron repressed and phase varies by virtue of a polyG tract within the coding sequence of hpuA [42, 139-140].

B. Single component systems

In addition to the two component systems described, Neisseria also encode single component systems in which a TdT is the sole surface exposed component. The HmbR receptor in N. meningitidis facilitates utilization of Hg and is subject to phase variation by virtue of a polyG tract within its coding region [139, 197]. HmbR however, is present in N. gonorrhoeae as a pseudogene due to a premature stop codon [197, 222]. FetA, another single component iron acquisition system, facilitates the utilization of catechol-type siderophores and undergoes phase variation due to a polyC tract in its promoter [36, 98]. TdfF is a TdT specific to pathogenic Neisseria [155, 214]. Its expression has only been detected in iron repressed conditions in the presence of serum [83, 238]. TdfF was found to be important for intracellular survival and is hypothesized to facilitate utilization of an intracellular iron source though the ligand has not been identified [83]. Gonococcal single component systems for iron acquisition are depicted in Figure 2.
Figure 2. Single component systems for iron transport in *N. gonorrhoeae*.

The TonB-dependent transporters (FetA and TdfF) are depicted as barrels traversing the outer membrane (OM) with a plug domain occluding the barrel. FetA facilitates utilization of catechol-type siderophores secreted by neighboring bacteria such as enterobactin (Ent, pictured in the extracellular space). The ligand to which TdfF binds to support gonococcal survival in cervical epithelial cells is not known. The TonB/ExbB/ExbD system is shown attached to or imbedded within the inner membrane (IM). TonB interacts with the plug domain of TdT to facilitate substrate transport through the TdT barrel. The PBP, FetB is shown localized to the periplasm where it is expected to bind ferric-siderophores and shuttle them to the putative inner membrane permease encoded by *fetCDEF*. The putative PBP, FetB2 encoded near TdfF is expected to behave similarly to FetB.
Figure 2. Single component systems for iron transport in *N. gonorrhoeae*.
C. TonB-dependent transport

Subsequent to binding of host iron sources at the cell surface, transport of iron or iron-chelate through TdTs requires metabolic energy provided by the TonB/ExbB/ExbD complex localized to the inner membrane. In Neisseria, tonB, exbB and exbD are within an operon and are iron-repressed [19, 64, 106, 224]. TonB has an N-terminal transmembrane domain, a proline-rich spacer region that allows it to span the periplasmic space and a C-terminal domain essential for interaction with TdTs [125]. The mechanism by which energy is transduced to the TdT on the outer membrane is not fully resolved. TonB in complex with ExbB and ExbD harnesses energy from the proton motive force [184] and is thought to transduce energy to TdTs via interaction with the TonB box found on the plug domain of TdTs, resulting in movement of the substrate through the channel and into the periplasm [125].

D. Cytoplasmic transport

Once in the periplasm, transport of iron or iron-chelates across the cytoplasmic membrane requires an ATP-binding cassette (ABC) family transporter comprised of a periplasmic binding protein (PBP), and inner membrane permease and ATP binding domains [124]. The transport of iron from Tf and Lf into the cytoplasm requires FbpA, the PBP of the FbpABC system that binds Fe$^{3+}$ with a $K_d \sim 10^{-18}$M [45, 211]. The FbpABC operon is also iron repressed [64, 72, 106]. A heme ABC transport system is hypothesized to exist in Neisseria but has not been identified. Enterobacteria utilization also requires the PBP, FetB of the putative ABC transporter FetCDEF, which are all transcriptionally linked to FetA [36, 98].
E. Regulation

All of the TdTs involved in iron/iron-chelate acquisition are regulated by Fur. Generally, Fur acts a repressor. In high iron concentrations Fe$^{2+}$ binds to Fur, increasing its affinity for the specific DNA binding motif, termed Fur box, within promoter of target genes [28]. Fe/Fur binding to the Fur box blocks RNA polymerase recruitment inhibiting transcription. Fur can also act positively on gene expression through direct and indirect mechanisms [274]. FetA, for example is activated by MpeR [98], an AraC-like regulator that is Fur repressed [106].

F. Uncharacterized TdTs in N. gonorrhoeae

Additional TdTs in N. gonorrhoeae whose functions have not yet been characterized are listed in Table 1. These include TdfG, which is present only in N. gonorrhoeae; TdfH, which found in most Neisseria and TdfJ, which is present in all Neisseria [51, 155]. Of the TdTs characterized, the majority have roles in iron acquisition and are iron repressed [206]. The uncharacterized TdTs of N. gonorrhoeae are differentially regulated by iron. TdfG is iron repressed supportive of a role in transport of iron or an iron chelate [64, 106, 238]. However, TdfH is not regulated by iron, which may imply a function outside of iron acquisition [64, 238]. Transcriptional studies indicate that tdfJ is iron-induced, which is also inconsistent with a role in iron acquisition [64, 273]. The function of these receptors is further occluded by the fact that their closest homologs are heme or siderophores receptors, though their contribution to gonococcal heme utilization has not been demonstrated [51, 238]. Furthermore studies in N. meningitidis have implicated TdfH and TdfJ in Zn and/or heme acquisition [126, 225-226], but these phenotypes have not been explored in N. gonorrhoeae.
Table 1. Uncharacterized TdT in *N. gonorrhoeae*

<table>
<thead>
<tr>
<th>Name</th>
<th>Predicted size</th>
<th>Closest homologs</th>
<th>Distribution</th>
<th>Iron repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TdfG</td>
<td>136kDa</td>
<td>Heme transporters</td>
<td><em>N. gonorrhoeae</em> only</td>
<td>Iron repressed</td>
</tr>
<tr>
<td>TdfH</td>
<td>104kDa</td>
<td>HasR and heme transporters</td>
<td>Most <em>Neisseria</em></td>
<td>Not iron regulated</td>
</tr>
<tr>
<td>TdfJ</td>
<td>86kDa</td>
<td>Siderophore and heme transporters</td>
<td>All <em>Neisseria</em></td>
<td>Iron induced</td>
</tr>
</tbody>
</table>

1Predicted molecular weight  
2Distribution indicates whether the gene is possessed by some or all *Neisseria* including commensal species
VIII. Vaccine Development

In light of the rising threat of untreatable gonorrhea, the need for novel therapies and a protective vaccine is even more urgent. Gonococcal vaccine development strategies have stalled due to the complications of antigenic variation of past vaccine targets such as pili and porin during natural infection as well as the immunosuppressive effects of natural infection [277]. However, recent insights have revealed that the active suppression of adaptive immunity by N. gonorrhoeae can be reversed in vivo by adding blocking antibodies against innate immunity-inducing cytokines, resulting in an anti-gonococcal immune response and protective immunity in a mouse model of female genital tract infection [146-147]. These novel insights may propel forward vaccine development strategies. As such, the identity of new potential vaccine targets remains relevant.

The surface exposure, limited sequence variation and wide-spread distribution among gonococcal strains indicate that TonB-dependent transporters may be good potential vaccine targets. Past work has demonstrated that intranasal vaccination of TbpA and TbpB conjugated to the B subunit of cholera toxin elicits mucosal and serum antibodies that are bactericidal in the presence of human complement in the mouse model of female genital tract infection [186]. Given the promising results with TbpA and TbpB, the use of other TdTs as vaccine antigens may also be successful. For example, ZnuD, the meningococcal TdfJ homolog, was shown to elicit cross reactive bactericidal antibodies against genetically distinct meningococcal strains when used to immunize mice and guinea pigs in an outer membrane vesicle vaccine formulation [103].
IX. Research Objectives

The goal of the research described in this thesis is to characterize the regulation and function of the gonococcal TonB-dependent transporters TdfG, TdfH and TdfJ. Three main objectives were pursued to achieve this goal. First, the role of these receptors in gonococcal heme utilization was assessed. Next, the regulation of these receptors in response to iron and zinc was explored. Additionally, the regulation of these TdTs by known virulence regulators was evaluated. Finally, the contribution of TdfH and TdfJ to gonococcal Zn acquisition was determined. These studies have ascribed functions to TdfH and TdfJ and characterized a novel gonococcal defense strategy against host mediated nutritional immunity. Additionally, these studies have identified a novel regulator of TdfG and revealed strain specific regulation of TdfH. Together the work described herein will provide insight into the contribution of these receptors to gonococcal pathogenesis, which may be useful for the development of novel protective and/or therapeutic strategies.
Chapter 2: Materials and Methods

I. Bacterial strains and routine maintenance

Strains and plasmids used in this study are listed in Table 2. All plasmids were propagated in *E. coli* TOP10 cells (Invitrogen). *E. coli* was cultured in Luria-Bertani (LB) broth media [14-15] with antibiotic selection at 100µg/mL for ampicillin (Sigma), 34 µg/mL for chloramphenicol (Sigma), or 50µg/mL for kanamycin (Sigma). *N. gonorrhoeae* was routinely maintained on GC medium base (GCB; Difco) agar with Kellogg’s supplement I [118] and 12µM Fe(NO$_3$)$_3$ at 37ºC in 5% CO$_2$ atmospheric conditions. *N. gonorrhoeae* growth conditions for specific assays are described below.

II. Gonococcal mutant construction

A. TdT double mutants, MCV935 & MCV936

To generate double mutants incapable of expressing both TdfG and TdfJ or TdfH and TdfJ in the FA1090 background, the *XbaI* and *SacI* flanked *tdfJ* fragment from pVCU703 was first sub-cloned into pVCU403, which contains gonococcal DNA uptake sequence [176]. The resulting plasmid, pVCU937, was subjected to random transposon mutagenesis using the Ez-Tn5™<Kan-2> Kit (Epicentre). Kanamycin resistant clones were screened via restriction mapping for Tn5<Kan-2> insertion within the *tdfJ* gene fragment; the insertionally mutagenized plasmid employed subsequently was named
pVCU938. Gonococcal strains MCV660 and MCV661 contain an Omega insertion within the coding region of genes \textit{tdfG} and \textit{tdfH}, respectively in an FA1090 background and have been described elsewhere [83]. MCV660 and MCV661 were transformed with pVCU938 and transformants were selected on GCB agar supplemented with 50\(\mu\)g/mL kanamycin. Chromosomal mutation was confirmed via PCR with MyTaq polymerase (Bioline) according to manufacturer’s instructions. Primers used in this study are listed in Table 3. The following pairs of gene specific primers were used: oVCU199/200, \textit{tdfG}; oVCU201/202, \textit{tdfH}; oVCU203/204, \textit{tdfJ}. The resulting strains were designated MCV935 and MCV936.

\textbf{B. Heme auxotrophic mutants}

To generate heme auxotrophs in FA1090, \textit{tonB}, and TdT mutant backgrounds we obtained pUNCH1304, which contains the \textit{hemH} gene disrupted with a chloramphenicol acetyltransferase cassette (\textit{CAT}) [237]. Gonococcal strains FA1090, FA6916, MCV656, MCV660, MCV661, MCV662 and MCV935, which have been described previously [52, 83, 169], were transformed with pUNCH1304 and transformants were selected on GCB agar plates supplemented with 16\(\mu\)M hemin (Sigma) and 1\(\mu\)g/mL chloramphenicol. Mutations were confirmed via PCR as previously described with \textit{hemH} specific primers oVCU793 and oVCU794 (Table 3). The resulting strains, MCV937, MCV939, MCV943, MCV944, MCV945, MCV946 and MCV947 and their genotypes are listed and Table 2.
C. FA19 and FA1090 zur mutants, MCV963 & MCV964

To construct the gonococcal zur mutant, we obtained zur::kan from Dr. Alastair G. McEwan at University of Queensland, which contains the full length zur gene in the Smal site of pUC19 interrupted by a kanamycin resistance cassette. This plasmid was linearized with Scal and used to transform gonococcal strains FA19 and FA1090. Transformants were selected on GCB agar plates supplemented with kanamycin at 50μg/mL and the chromosomal mutation confirmed via PCR as previously described with zur specific primers oVCU801 and oVCU802. The resulting strains were named MCV963 (FA19 zur) and MCV964 (FA1090 zur).

D. tdfH complimented derivative, MCV956

Gonococcal strain MCV927 contains an Omega insertion within the coding region of the tdfH gene in an FA19 background and has been described elsewhere [227]. To generate a complemented derivative of MCV927, the full length tdfH gene including its native ribosome binding site (RBS) was PCR amplified from FA1090 chromosomal DNA using the following primers: oVCU807 and oVCU798. The amplicon was cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen) according to manufacturer’s instructions to generate pVCU944. The tdfH sequence was confirmed by sequencing and pVCU944 was digested with restriction endonucleases Scal and Pmel to isolate the tdfH fragment, which was then purified and inserted at the Pmel site of pGCC4 [160]. The proper orientation of tdfH in the resulting plasmid, pVCU945, was verified by restriction mapping with Scal and Kpnl. For gonococcal transformation, pVCU945 was digested with NotI and the fragment containing tdfH, lctP, aspC and the erythromycin resistance cassette was purified and used to transform MCV927. Transformants were
selected on GCB agar plates supplemented with 1µg/mL erythromycin. The resulting strain, MCV956, contains the chromosomal \textit{tdfH} mutation and an ectopically inserted copy of the wild type \textit{tdfH} gene preceded by its native RBS under the control of a \textit{lac} promoter.

E. Opaless \textit{tdfH} mutant, MCV955

A \textit{tdfH} mutant in the FA1090 Opaless background [12] was constructed for NET survival studies. To generate this mutant, the \textit{XbaI} and \textit{SacI} flanked \textit{tdfH} fragment from pVCU702 was sub-cloned into pVCU403. The resulting plasmid, pVCU947, was also subjected to Ez-Tn5<\textit{Kan-2}> random transposon mutagenesis, restriction mapped for Tn5<\textit{Kan-2}> insertion within the \textit{tdfH} gene fragment and the insertionally mutagenized plasmid was named pVCU948. FA1090 Opaless was then transformed with pVCU948 and transformants were selected on GCB agar supplemented with 30µg/mL kanamycin, resulting in strain MCV955.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and/or relevant characteristics</th>
<th>Ref. or source</th>
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<td>Wild type</td>
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<tr>
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<td>Wild type (ΔlbpA, HpuAB off)</td>
<td>[169]</td>
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<td>[12]</td>
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<tr>
<td>Δnuc</td>
<td>Opaless Δnuc (Kan')</td>
<td>[115]</td>
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<td>Dr. William M. Shafer</td>
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<td>[98]</td>
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<td>FA1090 hemH:: CAT (Cm')</td>
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</tr>
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<td>FA1090 tdfH::Ω hemH::CAT (Cm', Str', Spc')</td>
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MCV946  FA1090  tonB::Ω hemH::CAT (Cm', Str', Spc')  This Study
MCV947  FA1090  Δtbp hemH::CAT (Cm')  This Study
MCV955  FA1090  ΔopaA-K tdfH::Tn5  This Study
MCV956  FA1090  tdfH::Ω tdfJ (Erm', Str', Spc')  This Study
MCV963  FA19  zur::kan (Kan')  This Study
MCV964  FA1090  zur::kan (Kan')  This Study

E. coli

TOP10  F− mcrA Δ(mrr-‐hsdRMS-mcrBC) φ80lacZΔM15  Invitrogen
ΔlacX74 recA1 araD139 Δ(ara-‐leu)7697galU galK
rpsL (Str') endA1 nupG

Rosetta™ (DE3)  F−ompThsdSB(rB− mB−) galdcm (DE3) pRARE2 (Cm')  Novagen

Plasmids

pCR2.1 TOPO  Cloning plasmid (Kan' Amp')  Invitrogen
pUC18  Cloning plasmid (Amp')  Invitrogen
pGCC4  Complementation plasmid (Kan' Erm')  [160]
pUC19zur::kan  pUC19 containing full length zur disrupted by Kan cassette  Dr. Alastair G. McEwan
pUNCH1304  pCRII derivative containing hemH::CAT  [237]
pETR7  pET24d(+) w/ rHusA (excl. signal peptide) + C-term.
thrombin cleavage site and 6xHis(Kan')  [77]
pVCU403  pUC18 with gonococcal uptake sequence (Amp')  [176]
pVCU702  pCR2.1 containing tdfH fragment  [83]
pVCU703  pCR2.1 containing tdfJ fragment  [83]
pVCU937  pVCU403 containing tdfJ fragment from pVCU703  This study
pVCU938  pVCU937 with tdfJ disrupted by Ez-Tn5<Kan-2>  This study
pVCU944  pCR2.1 containing full length tdfH + native RBS  This study
pVCU945  pGCC4 containing tdfH allele from pVCU944  This study
pVCU947  pVCU403 containing tdfH fragment from pVCU702  This study
<table>
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<tr>
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<th>pVCU947 with <em>tdfH</em> disrupted by Ez-Tn5&lt;Kan-2&gt;</th>
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<tr>
<td></td>
<td>Amp, ampicillin; Cm, chloramphenicol; Erm, erythromycin; Kan, kanamycin; Spc, spectinomycin; Str, streptomycin</td>
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<tr>
<td>Primer</td>
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<td>oVCU202</td>
<td>tdfH</td>
<td>5′-GCG CCA GGT TTT CCC GCC AGC TTT TAT CAT-3′</td>
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<td>5′-CCC CTC CTC GCC CAA GCG CAT GAA ACT GAG-3′</td>
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<td>tdfJ</td>
<td>5′-GGC GTG GCA ATC ATC GTA TTC GTG GCT GTG-3′</td>
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<td>oVCU793</td>
<td>hemH</td>
<td>5′-CGC AAA CCG CAT TAC CTG AT-3′</td>
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<td>hemH</td>
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<td>5′-CAC AGT TTA AAC AAA CGC GGG CTG-3′</td>
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<td>oVCU801</td>
<td>zur</td>
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<td>oVCU807</td>
<td>tdfH</td>
<td>5′-GGG CAG TAC TGA GGA AAA TAT GAG ATC T-3′</td>
</tr>
</tbody>
</table>
III. Limited heme growth assays

For limited heme growth in Wade-Graver (GW) media, GW media was prepared as previously described [251] except that Supplement A was treated with Chelex-100 (50g per 500mL) before being added at a 1:2 v/v ratio to normal strength M199 (Sigma Aldrich). Gonococcal strains were first grown in the partially chelexed GW media until log-phase growth was achieved. At log phase, gonococci were diluted to OD$_{600}$ of 0.02 in fresh partially chelexed GW media and transferred to a 96-well micro titer plate. Each well was supplemented with iron specific chelator, Desferal (deferoxamine mesylate; Sigma Aldrich) and hemin to a final concentration of 10µM and 5µM, respectively and contained a total volume of 100µL. Hemin stock solutions of 5mg/mL were made in 0.1M NaOH, filter sterilized (0.22µm) and stored at 4°C in the dark. The micro titer plate was incubated at 37°C and 5%CO$_2$ with vigorous shaking and OD$_{600}$ readings were collected every 2 hours for 6 hours. For determination of viable bacteria, 20µL of culture were recovered from wells, serially diluted, and spot plated onto GCB agar plates. After incubation overnight at 37°C and 5%CO$_2$, CFU of recovered gonococci were enumerated.

Individual colonies of heme auxotroph mutants from GCB agar plates supplemented with 16µM hemin were grown in chemically defined media treated with Chelex 100 (Bio-Rad), CDM, [259] supplemented with 1µM heme until log-phase growth was achieved. At log phase, cultures were diluted to OD$_{600}$ of 0.02 in fresh CDM and transferred to a micro titer plate. Each well was supplemented with hemin at a final concentration of 5µM. The micro titer plate was incubated at 37°C, 5% CO$_2$ and growth as monitored via OD$_{600}$ readings every 2 hours for 6 hours.
IV. Expression and purification of *P. gingivalis* hemophore, HusA

The plasmid pETR7, which contains recombinant HusA, *P. gingivalis* hemophore like protein was obtained from Dr. Neil Hunter at Westmead Centre for Oral Health and has been described previously [77]. Starter cultures of *E. coli* expression strain Rosetta™ (DE3) containing pETR7 were grown in LB broth media with kanamycin (LB-Kan) overnight at 37°C with vigorous shaking. The following day, starter cultures were centrifuged at 4,000 x g for 10 minutes and the bacterial cell pellet was re-suspended in fresh LB-Kan. The entire starter culture was used to inoculate 1L of LB-Kan, which was grown in at 37°C with vigorous shaking until an OD$_{600}$ of 0.6 was achieved. IPTG was then added at a final concentration of 0.5mM to induce protein expression for 3 hours. After induction, bacterial cells were harvested by centrifugation at 5,000 rpm for 30 minutes at 4°C. Cell pellets were washed with sterile 1X PBS, pH 7.2 and centrifuged at 5,000 rpm for 15 minutes. Supernatants were discarded and cell pellets were dried, weighed and stored at -80°C overnight.

For protein purification, bacterial pellets were thawed on ice and re-suspended in cold native lysis buffer (50mM NaH$_2$PO$_4$, 300mM NaCl, 10mM Imidazole [pH 8.0]) at 5mL buffer/1g pellet. 1X Protease Inhibitor Cocktail (Roche) and 1mg/mL lysozyme was added before cultures were incubated for 30 minutes on ice. The cellular mixture was sonicated (5x, 30 sec pulse, 1 minute pause between pulses) and the lysate centrifuged at 10,000 rpm for 20 minutes at 4°C to remove debris. The cleared lysate was then incubated with pre-washed 50% Ni-NTA agarose resin (Qiagen) overnight at 4°C. On following day, the loaded Ni-NTA resin was packed into a polyprep column (Bio-Rad)
and the flow through collected. The column was washed twice with 5mL wash buffer (50mM NaH$_2$PO$_4$, 300mM NaCl, 20mM Imidazole [pH 8.0]) that was collected. The recombinant protein was eluted from the column with elution buffer (50mM NaH$_2$PO$_4$, 300mM NaCl, 250mM Imidazole [pH 8.0]). The column was rinsed with 1X PBS before all sample fractions were stored at 4°C until SDS-PAGE analysis. Protein concentrations of eluted samples were determined using the BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Before use in growth assays, purified rHusA protein, was loaded with heme (2:1 molar ratio) and un-bound heme was removed by dialysis (250mM NaCl, 100mM Tris [pH8.0]).

V. Hemophore utilization assay

CDM agarose plates treated with 10µM Desferal to chelate excess iron were inoculated with gonococcal strains using a sterile Darcon swab (Puritan). A sterile pipette was used to bore wells into the solidified media. Subsequently, 20µL of various iron solutions including heme loaded HusA at indicated concentrations were added to each well before incubation at 37°C in 5%CO$_2$ atmosphere overnight. The following day, plates were recovered and evaluated for growth around each iron source.

VI. Bacterial growth conditions for regulation studies

A. Iron replete and depleted conditions

Gonococcal strains were grown in GCB broth containing Supplement I until log-phase growth was achieved. At log-phase, 24µM Fe (NO$_3$)$_3$ or 100µM Desferal were added for iron replete and depleted conditions, respectively. All liquid cultures were
grown at 37°C with 5%CO₂. Cultures were grown for 2 hours at the described condition before whole cell lysates were harvested.

**B. Varied Zn concentrations**

Gonococcal strains were grown in CDM until log phase growth was achieved. At log-phase cultures were before back diluted and Fe(NO₃)₃, ZnSO₄ and/or Zn specific chelator N,N,N’N’-Tetrakis-(2pyridylmethyl)-ethylenediamine (TPEN; Sigma) were added at the stated concentrations and grown for 4 hours before whole cell lysates were harvested. Stock solutions of TPEN were dissolved in 100% ethanol and stored at -20°C. Subsequent working solutions were diluted in deionized water, filter sterilized (0.22µm) and stored short-term at 4°C.

**C. Zn replete and depleted conditions**

Gonococcal strains were grown in GCB broth media containing Supplement I until log-phase growth was achieved. At log phase, ZnSO₄ or TPEN were added at a final concentration of 25µM for Zn replete and depleted conditions, respectively. Cultures were grown for 4 hours at the described conditions before whole cell lysates were harvested.

**VII. Protein analysis methods**

**A. Whole cell lysate preparation**

Gonococcal whole cell lysates were collected by centrifuging cultures of standardized density at 21, 000 x g for 10 minutes; gonococcal cell pellets were then re-
suspended in Laemmli solubilizing buffer [128] and stored at -20°C until use. Immediately prior to SDS-PAGE, whole cell lysates were treated with 5% β-mercaptoethanol, drawn through a 28-gauge syringe to reduce viscosity and boiled for 3 minutes.

**B. SDS-PAGE and Coomassie stain**

Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% or 15% polyacrylamide gels using a Mini-Protean III apparatus (Bio-Rad). To detect purified proteins, SDS-PAGE gels of separated proteins were stained with Coomassie blue (0.25% Coomassie R-250, 50% methanol, 10% glacial acetic acid) for 1 hour and de-stained in 20% methanol and 5% acetic acid overnight at room temperature to minimize background staining.

**C. Western blot analysis**

Proteins separated on SDS-PAGE gels were transferred to nitrocellulose membranes (GE Healthcare Life sciences) in 20mM Tris base, 150mM glycine and 20% methanol [234] in a submerged transfer apparatus (Bio-Rad) at constant current of 28mA for 16 hours. Membranes were stained with Ponceau S solution (0.1% w/v Ponceau S, 5% acetic acid) for 10-12 minutes to confirm equal protein loading of lanes before immunodetection of specific proteins. For TdfG detection, membranes were blocked with 5% bovine serum albumin (BSA) in a high salt Tris-buffered saline (TBS) (20mM Tris, 500mM NaCl, [pH 7.5] 0.05% Tween 20), probed with polyclonal antiserum against TdfG [238] and washed with high salt TBS followed by incubation with secondary antibody conjugated to alkaline-phosphatase (AP) (Bio-Rad). Blots were developed with the nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-
indolylphosphate (BCIP) system (Sigma). For TdfH detection, membranes were blocked with 5% BSA in a high salt TBS, probed with polyclonal antiserum against TdfH [238] and washed with high salt TBS followed by incubation with AP conjugated secondary antibody. Blots were developed with NBT/BCIP. For TdfJ detection, we generated polyclonal anti-peptide antibodies (New England Peptides) against regions predicted to correspond to Loop 2 (sera 116, 117) and Loop 5 (sera 118, 119) after alignment of TdfJ to a 2D-topology model of TbpA. Membranes were blocked with 5% skim milk in a low salt TBS (50mM Tris, 150mM NaCl, [pH 7.5]), probed with serum 117 and washed with low salt TBS before being incubated with secondary antibody conjugated to horseradish peroxidase (Southern Biotech). Blots were developed with Pierce-ECL-2 kit (Thermo Scientific). For TbpB detection, rabbit anti-TbpB primary antibody [185] was used after blocking with 5% skim milk. Blots were then washed with low salt TBS before incubation with secondary antibody conjugated to AP and developed with NBT/BCIP.

VIII. Enumeration of bacterial growth in Zn replete and depleted conditions

Gonococci were grown until log-phase growth was achieved in Zn restricted rich media, GCB broth containing Supplement I, 12μM Fe(NO₃)₃ and 12.5μM TPEN (GCB + supplement I, Fe, TPEN). At log phase, cultures were back-diluted and were not further treated to represent Zn depleted conditions or were treated with ZnSO₄ (18.75μM) for Zn replete conditions. After growth for 6 hours, bacterial cells were harvested by centrifugation at 4,000 x g for 15 minutes at 4°C, washed twice with cold PBS, 1mM EDTA and re-suspended in PBS before being serially diluted and spot plated on GCB
IX. Calprotectin growth assay

Purified recombinant human calprotectin (CP) was obtained from Dr. Walter Chazin at Vanderbilt University. CP was stored in 20mM Tris pH 8.0 100mM NaCl 10mM β-mercaptoethanol buffer solution and kept at -80°C. Aliquots were thawed on ice immediately prior to use and stored short-term at 4°C to prevent multiple freeze-thaw cycles. For CP growth assays, gonococci were first grown in CDM until log phase growth was achieved. At log phase, cultures were diluted to OD$_{600}$ of 0.02 and transferred to a 96-well micro titer plate. Each well contained 7.5µM 30% saturated human transferrin (hTf) as the sole iron source, 2.5µM apo-bovine transferrin (bTf) to sequester residual iron and 1µM TPEN for Zn chelation. In some cases, 2mM IPTG was also added to induce TdfH expression in MCV956. Some wells were further supplemented with either CP+ Zn (10µM CP, 5µM ZnSO$_4$) or 5µM ZnSO$_4$ alone. The micro titer plate was incubated at 37°C and 5% CO$_2$ with vigorous shaking and OD$_{600}$ readings were collected every 2 hours for 6-8 hours.

X. Whole cell calprotectin binding assay

Gonococcal strains were grown in CDM treated with 24µM Fe(NO$_3$)$_3$ until log-phase growth was achieved. At log phase, cultures were back-diluted and 1µM TPEN was added. 2mM IPTG was also added when indicated to induce TdfH expression in
MCV956. Cultures were grown in the described conditions for 4 hours before being applied to nitrocellulose membranes using a dot-blot apparatus (Bio-Rad). Dried membranes were blocked for 1 hour with 5% skim milk in low salt TBS before being incubated with CP + Zn (0.17µM CP, 0.01µM ZnSO₄) in blocker. After washing with low salt TBS, blots were probed for CP using rabbit anti-s100A9 polyclonal antibody (Thermo-Scientific) and secondary antibody conjugated to AP. Dot blots were then developed with NBT/BCIP. For densitometry, replicate blots were scanned and intensities of individual dots were quantitated using NIH ImageJ [207]. Quantitated intensities were normalized to wild type and expressed as % CP binding.

XI. Zn accumulation assay

Gonococcal strains were first grown in Zn restricted rich media (GCB + supplement I, Fe, TPEN) until log-phase growth was achieved, at which point cultures were back diluted and either treated with 18.75µM ZnSO₄ (Zn replete) or no further addition (Zn deplete). For Zn accumulation in the presence of CP, cultures were treated with CP + Zn (2µM CP, 1µM ZnSO₄) after back dilution. Cultures were grown for 6 hours in the described conditions before cells were harvested by centrifugation at 4°C for 15 minutes at 4,000 x g and washed twice with cold, chelexed PBS supplemented with 1mM EDTA. Bacterial cells were then re-suspended in undiluted Trace Metal Grade Nitric Acid, heated at 95°C for 2 hours, and cooled at room temperature overnight. For analysis, samples were diluted 12.5 or 22-fold in chelexed deionized water. Metal composition was determined using an MPX Vista Inductively Coupled Plasma-Optical Emission Spectrometer (Varian, Inc.). To determine the concentration of
metals associated with the cells, a standard curve was generated with a 10μg/mL multi-element standard (CMS-5; Inorganic Ventures) diluted in chelexed deionized H₂O and then serially diluted twofold in 1% nitric acid to generate dilutions ranging from 0.640μg/mL to 0.020μg/mL. Cell pellets from parallel cultures were re-suspended in PBS, treated with 5% SDS and vortexed for 30 sec for lysis. Protein concentrations were determined using BCA kit.

**XII. NET survival assay, performed in the laboratory of Dr. Alison Criss at University of Virginia**

Neutrophils were purified from venous blood collected from healthy donors according to a protocol approved for use in the laboratory of Dr. Alison Criss by the University of Virginia Institutional Review Board for Health Science Research. Viable exponential-phase gonococci were obtained by sequential dilution in rich medium as previously described [55] except at the final dilution, cultures were re-suspended in phenol red-free Roswell Park Memorial Institute (RPMI) with 1μM TPEN and grown to an OD₅₅₀ of 0.4. Bacterial suspensions were then centrifuged at 10,000 x g for 3 minutes and washed once with RPMI before exposure to neutrophils. Survival of gonococcal strains upon exposure to PMA stimulated neutrophils treated with cytochalasin D only or cytochalasin D plus DNase I, was determined as previously described [115]. Briefly, neutrophils were seeded in 24-well plates and treated with 20nM PMA for 15 minutes at 37°C in 5% CO₂. One set of wells was treated with 10μg/mL cytochalasin D (Sigma), while another set was treated with cytochalasin D and 1 U of DNase I for, each for 15 minutes. Neutrophils were then exposed to Opaless
parent, MCV955 (tdfH) or Δnuc gonococci grown as described above at a multiplicity of infection (MOI) of 1 for 1 hour. After infection, well contents were serially diluted and plated for CFU enumeration. Bacterial survival is expressed as a percentage of the initial inocula per well.
Chapter 3: The role of TonB-dependent transporters: TdfG, TdfH and TdfJ in heme acquisition

I. Introduction

As an essential co-factor for many enzymes functioning in crucial cellular processes within all kingdoms of life, iron is an indispensable nutrient for most organisms. In aerobic conditions, iron is present in the ferric (Fe³⁺) oxidized form but is poorly soluble. The reduced ferrous (Fe²⁺) form is much more soluble but can catalyze the production of free oxygen radicals which are highly toxic to macromolecules through the Fenton reaction. As such, iron levels are tightly controlled to maintain iron homeostasis in vertebrates and free iron is scarce. Instead, iron is sequestered intracellularly by the iron storage protein ferritin and heme bound to hemoproteins. Extracellular iron is in the ferric form and is tightly bound to iron transport protein transferrin as well as lactoferrin present in mucosal secretions [209]. For a bacterial pathogen to colonize a mammalian host, it must be able to assimilate iron from one or more these host iron sources [254].

Approximately 70% of the iron within the human body is bound to heme [203]. Heme solubilizes iron and increases its catalytic ability: a property harnessed as the prosthetic group of intracellular hemoproteins such as hemoglobin (Hg), myoglobin and
cytochromes to function in electron transport as well as oxygen transport, sensing and storage [10]. Heme itself can be toxic due to its chemical properties and ability to partition into lipid membranes [221]. Thus, free heme is rarely available in vertebrate hosts. Small amounts of extracellular heme can be found in blood Hg from lysed erythrocytes [173]. However, Hg is rapidly bound by serum protein haptoglobin (Hp) [105]. The Hg-Hp complex is cleared from serum in a receptor mediated mechanism by monocytes that express receptor CD163 [173]. The globin components of the Hg-Hp complex are degraded in the lysosome and the heme detoxified while CD163 is recycled to the plasma membrane to continue to function in Hg clearance and thus mitigation of heme-mediated oxidative damage [173]. Likewise, free heme dissociated from hemoglobin or other hemoproteins is tightly bound by various serum heme binding proteins including high and low density lipoprotein (HDL and LDL, respectively), serum albumin, hemopexin (Hx) and α1-microglobulin [11]. Similar to Hg-Hp clearance, Hx facilities free heme uptake and subsequent degradation upon interaction with receptor CD91, which triggers receptor mediated endocytosis in macrophages and hepatocytes [11].

Despite the rapid recycling of heme and Hg from serum, 80-800nM of Hg can be found in plasma [209] and can be a significant source of iron, albeit protein bound and heme associated. Thus, pathogenic bacteria have developed strategies to assimilate iron from heme/ hemoproteins. Bacterial heme acquisition systems can be classified into two categories. The first category relies on the secretion of hemophores, bacterial hemoproteins that scavenge heme from the extracellular environment that is then
relayed back to the bacteria through specific receptors. The second category involves receptors that directly bind to heme or host heme-containing proteins [254].

The secretion of hemophores is mostly limited to Gram-negative bacteria but has been described in one Gram-positive pathogen, *B. anthracis* [10, 66]. Hemophores are bacteria-derived proteins that are secreted into the extracellular milieu where they bind free heme or host hemoproteins with high affinity. There are two types of hemophores described for Gram-negative bacteria: the HasA-type, which has been described in pathogens, *S. marcescens, P. aeruginosa, P. fluorescens*, and *Y. pestis* [10], or HxuA, which is only found in *H. influenzae*. HasA-type hemophores capture free heme or heme from host hemoproteins. Since HasA has higher affinity for heme than host hemoproteins, heme is thought to passively transfer from host hemoproteins to HasA [253]. Upon interaction with its specific outer-membrane receptor, HasA delivers heme to the bacterial cell surface and only the heme molecule is internalized [253]. HxuA binds to the serum heme recycling protein, hemopexin (Hx) to provide heme from the heme-hemopexin complex. Unlike HasA, HxuA does not display high affinity heme binding [73]. Instead, it liberates heme from Hx without heme capture inactivating the biological function of Hx [253]. The released heme is then accessible to the nearby receptor that facilitates its transport across the outer-membrane. Both HusA and HxuA-type hemophores require outer membrane receptors for heme internalization. The receptors are TonB-dependent transporters consisting of a transmembrane β-barrel with 22 antiparallel β-strands, extracellular loops that interact with heme/ hemoprotein/ hemophore ligand and an N-terminal plug domain that closes the β-barrel pore. The transport of heme across the outer membrane requires energy from the
TonB/ExbB/ExbD system, so all receptors also contain a “TonB box” motif in the plug domain, through which TonB and the receptor interact.

In direct heme acquisition systems, the TonB-dependent transporter itself directly binds to heme and/or host hemoproteins. These TdTs can be substrate specific, recognizing only one type of host hemoprotein like the Hg-Hp utilization proteins of *H. influenzae*, *P. gingivalis*, and pathogenic *Neisseria* [233]. A subset of these substrate specific TdTs are bipartite systems that include a lipoprotein in addition to the TdT for utilization of the host hemoprotein as is described for pathogenic *Neisseria* and *P. gingivalis* [253]. Other direct heme acquisition systems are non-specific and thought to directly interact with heme to support heme assimilation from free heme as well as a variety of host hemoproteins as is the case for *HemR* of *Y. enterolitica* and its homologues in *B. pertussis*, *S. dysenteriae*, *V. cholerae*, *C. jejuni*, *B. quintana*, and *E. coli* O157:H7 [10]. Over 30 heme receptors have been well-characterized in Gram-negative bacteria that share a range of sequence similarity anywhere from 20-90% [210] indicating a wide range of sequence diversity. Most share a highly conserved amino acid motif “FRAP(10X)H(XX)NPNL(2X)E”, which contains an invariable His residue thought to coordinate heme [27, 223]. However, some heme TdTs lack this motif indicating that additional mechanisms of heme coordination exist [203, 233]. To fully optimize heme utilization from a variety of host heme sources bacteria can adopt either of two strategies: expression of a heme acquisition system that has broad specificity to a variety of heme sources or expression of multiple receptors for different hemoproteins.
After transport across the outer membrane of Gram negative-bacteria via TdTs, heme is localized in the periplasmic space. ATP-binding cassette (ABC) transporters facilitate the transport of heme from the periplasm to the cytosol. These transporters are usually comprised of a high affinity periplasmic binding protein (PBP) that shuttles heme through the periplasm to an inner membrane permease, through which heme is transported to the cytoplasm upon ATP-hydrolysis [203]. The ABC transport system is usually encoded within the same operon of the TonB-dependent transporter. Additional components of the operon may encode cytoplasmic heme binding proteins which can sequester heme and prevent it from catalyzing reactive oxygen species like ShuS of S. dysenteriae or shuttle heme to degradative enzyme HemO like PhuS of P. aeruginosa [203]. Heme oxygenase, encoded by hemO, which is ubiquitous in nature, catalyzes the oxidation of heme into biliverdin, carbon monoxide, and free iron and is critical for iron assimilation from heme and for mitigating heme toxicity [10, 233].

Pathogenic Neisseria express receptors that directly utilize heme from host hemoproteins. The HpuB/A system is expressed in both N. meningitidis and N. gonorrhoeae. It is comprised of a TonB-dependent transporter, HpuB, and a lipoprotein, HpuA, analogous to the bipartite neisserial transferrin acquisition system [138, 140]. These genes are co-transcribed in a bi-cistronic operon and are subject to phase variation [43, 140]. The HpuB/A system facilitates utilization of Hg or the Hg-Hp complex and requires expression of both the lipoprotein and the TdT for Hg utilization [42]. Pathogenic Neisseria also encode HmbR, another phase-variable TonB-dependent transporter that utilizes only Hg as an iron source; however, it is only expressed in N. meningitidis due to a pre-mature stop codon in N. gonorrhoeae [197, 222]. Pathogenic
*Neisseria* cannot utilize heme from Hx or albumin though it is capable of robust growth in presence of free heme [65]. A heme ABC transport system has long been postulated in *Neisseria* but has yet to be identified. Genome searches have not revealed an obvious heme PBP or ABC transport system [198]. FbpA, a PBP, which is critical for the use of iron from transferrin (Tf), lactoferrin (Lf), and Hg is not required for heme utilization in *Neisseria* [119, 237]. In pathogenic *Neisseria*, the *hemO* gene, encoding heme oxygenase, is upstream of the HmbR receptor and their expression is transcriptionally linked. HemO was found to be critical for the use of all heme compounds and for the mediation of heme toxicity [278].

Neither HmbR, HpuB/A, nor TonB are required for the utilization of free heme [19, 43, 222, 224]. TonB-independent heme utilization is suggestive of heme passively diffusing across the outer-membrane, possibly through the use of porins; but TonB-independent heme uptake may not be relevant *in vivo* as large amounts of heme are rare in mucosal environments [209]. Thus, the existence of a free heme receptor has been speculated in pathogenic *Neisseria* for some time. Heme-binding proteins (HmBPs) isolated from both *N. gonorrhoeae* and *N. meningitidis* were suggested to be heme specific receptors given the ability of a monoclonal antibody directed against the 97kDa HmBP to inhibit growth in the presence of heme [131-132, 134]. The genes encoding the 97 and 44kDa HmBPs proteins have never been identified, though these proteins may be the HpuB/A proteins given their similar size and similar heme binding and inhibition profiles [44, 132].

There is no evidence that *N. gonorrhoeae* produces a hemophore, but it may still be able to utilize bacterial derived hemophores as it capable of utilizing bacterial derived
siderophores via the TdT, FetA [36, 98]. Gonococcal infection occurs at mucosal epithelia, primarily of the urogenital tract a host niche rich in both normal and sometimes pathogenic bacteria. The female genitourinary tract, for example, contains commensal bacteria which help to protect against pathogenic [142]. Bacterial vaginosis (BV), a condition in which there is an imbalance between normal ‘good’ and abnormal bacteria, is characterized by an increase in vaginal pH and BV-associated bacteria including *Mycoplasma hominis*, *Gardnerella vaginalis*, *Prevotella spp.*, *Mobiluncus spp.*, and *Leptotichia/Sneathia* [144]. BV has been demonstrated to be a risk factor for *N. gonorrhoeae* infection [263] but the molecular mechanisms underlying the increased risk have not been identified. Some BV-associated bacteria are cytotoxic and so can help to liberate intracellular nutrients like heme bound to hemoproteins and provide these to invading microbes. *G. vaginalis*, for example, has been shown to secrete a hemolysin, termed vaginolysin with lytic activity specific to human erythrocytes, neutrophils, and endothelial cells [271]. *Sneathia amnii* was also reported to have weak hemolytic activity and to encode 2 putative hemolysins [93]. Bacteroidetes species including *Prevotella* and *Porphyromonas* are also heme auxotrophs and thus encode hemolysins as well as heme acquisition systems to fulfill this nutritional requirement [137, 275]. Thus, it is possible that the cytotoxic and hemolytic activity of BV-associated bacteria can provide an environment favorable to gonococcal infection by either increasing the abundance of host hemoproteins or even providing bacterial derived hemoproteins.

The role of heme iron utilization in gonococcal pathogenesis is difficult to ascertain since expression of known hemoglobin receptors is phase variable and the
gonococcus can assimilate iron from other host derived sources. Menstruation is however a major risk factor for complicated gonococcal infection as half the number of affected women reported DGI symptoms within seven days of the onset of menses [196]. It is likely that strategies that contribute to the utilization of heme and hemoproteins during menses, when the levels of host heme sources are presumably elevated in the female genital tract, could contribute to pathogenesis. Indeed, the expression of the HpuB/A system and utilization of Hg and Hg-Hp was found to be correlated to the onset of menses in a study examining naturally occurring gonococcal infection [5]. These results suggest a selective advantage for gonococci expressing HpuB/A in the female genital tract during menses and point to the need to characterize any additional receptors that may contribute to heme utilization.

Given their reported homology to known heme receptors *N. gonorrhoeae* TdT (TdfG, TdfH and TdfJ) have all been suggested to participate in heme utilization since their identification in genome annotations [126, 238]. Table 4 summarizes the data reported in the literature on their potential role in heme acquisition. Turner et al. assessed the role of TdfG and TdfH in heme acquisition and reported that isogeneic mutants unable to express TdfG or TdfH demonstrated no defect in their ability to grow with heme as the sole iron source compared to parent strains [238]. Additionally, expression of TdfH in an *E. coli hemA* mutant did not restore heme utilization [238]. *E. coli hemA* is a heme auxotroph incapable of *de novo* heme synthesis and therefore requires uptake of exogenous heme through a heme receptor to support growth in the presence of heme. The ability of putative heme receptors to restore growth in the presence of heme upon expression in *E. coli hemA* has served as positive indication of
Table 4. Summary of reported TdT heme phenotypes

<table>
<thead>
<tr>
<th>Gene, species</th>
<th>Growth with heme&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Restores heme utilization in &lt;i&gt;E. coli hemA&lt;/i&gt;</th>
<th>Binds heme&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Ref.</th>
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</thead>
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<tr>
<td>&lt;i&gt;tdfG&lt;/i&gt;, Ng</td>
<td>+</td>
<td>NR</td>
<td>NR</td>
<td>[238]</td>
</tr>
<tr>
<td>&lt;i&gt;tdfH&lt;/i&gt;, Ng</td>
<td>+</td>
<td>-</td>
<td>NR</td>
<td>[238]</td>
</tr>
<tr>
<td>&lt;i&gt;znuD&lt;/i&gt;, Nm</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[126]</td>
</tr>
</tbody>
</table>

Ng, <i>N. gonorrhoeae</i>; Nm, <i>N. meningitidis</i>; NR, not reported

<sup>1</sup>Isogenic mutants were tested for their growth with heme as the sole iron source.

<sup>2</sup>Recombinant ZnuD was tested for its ability to bind hemin <i>in vitro</i>. 
function as a heme receptor [27, 224]. TdfH was also thought to be one of the hemin affinity purified HmBPs, but failure of monoclonal antibody directed against the 97kDa HmBP [134] to specifically detect TdfH, conclusively ruled out that possibility [238]. TdfJ, identified later as another gonococcal TdT [83], has been investigated for its contributions to heme utilization. Though uncharacterized in the gonococcus, the meningococcal TdfJ homologue, ZnuD was not required for growth in the presence of heme, but could restore heme utilization and growth when expressed in E. coli hemA [126].

In light of inconsistent findings implicating TdfJ in heme acquisition, we revisited the contributions of TdfG, TdfH and TdfJ to gonococcal heme utilization. The studies presented herein address three main points in an attempt to resolve gaps in the literature with regards to the role of these TdT's in gonococcal heme utilization. First, we address the possibility that N. gonorrhoeae may express functionally redundant receptors for heme utilization. Since, the TdfJ homologue in N. meningitidis has an observable heme phenotype we generated double mutants unable to express TdfJ and TdfG or TdfH and assessed their ability to grow in the presence of heme. We expected that if these receptors contribute to heme utilization, double mutants would be defective for growth in limited heme conditions. Next, we restricted gonococcal growth to exogenous heme uptake in an attempt to maximize TonB-dependent heme uptake mechanisms and resolve the potential contribution of the aforementioned TdTs. To do this, we generated tonB and TdT mutants in a gonococcal heme auxotrophic background. We expected that growth under limited heme conditions would require TonB and TdT-dependent mechanisms. Finally, we address the possibility that TdfG,
TdfH, or TdfJ may facilitate the utilization of a “xeno-hemophore”. We identified a potential candidate hemophore and tested its ability to support gonococcal growth as the sole iron/ heme source. We expected that gonococcal growth in the presence of the hemophore would be indicative of the presence of a gonococcal hemophore receptor. This study thus presents a thorough examination of the roles of TdfG, TdfH and TdfJ in gonococcal heme utilization.
II. Results

A. TonB and TdT double mutants are not defective for growth in limited heme conditions

Since the meningococcal TdfJ homologue, ZnuD exhibited some positive indicators of a role in heme acquisition (see Table 4) we sought to evaluate the contribution of TdfG and TdfH to growth in heme limited conditions in double mutants unable to express TdfJ as well as TdfG or TdfH. Double mutants were made by first generating a construct, pVCU938, in which the \textit{tdfJ} gene fragment was inactivated by a transposon encoding a Kan resistance cassette (Tn5<Kan-2>). This construct was then used to transform gonococcal strains FA1090 \textit{tdfG} (MCV660) and FA1090 \textit{tdfH} (MCV661) \cite{83}. A schematic of the approach to mutant construction is presented Figure 3. Kanamycin resistant (Kan\textsuperscript{r}) gonococci were colony purified and subjected to a PCR screen for confirmation of the chromosomal mutation. In Figure 4, the genes \textit{tdfG}, \textit{tdfH} and \textit{tdfJ} were PCR amplified with gene specific primers from DNA preparations of Kan\textsuperscript{r} gonococcal transformants. The presence of a larger PCR product corresponding to the size of the insertion in gonococcal transformants compared to wild type is shown in Figure 4, confirming the successful generation of the \textit{tdfG} \textit{tdfJ} and \textit{tdfH} \textit{tdfJ} double mutants designated MCV935 and MCV936, respectively.

To determine if TdfG, TdfH and TdfJ contribute to heme utilization, the growth of wild type, \textit{tonB} and TdT isogenic and double mutant strains were evaluated in a liquid growth assay with limited heme as the sole iron source. We hypothesized that under these conditions, TonB and TdT-dependent mechanisms of heme utilization
Figure 3. Schematic of double mutant construction

Diagram of strategy for double mutant construction. (A) The plasmid, pVCU938, which contains the gonococcal uptake sequence (GCU) as well as a fragment of \textit{tdfJ} disrupted by Tn5<Kan-2> used to transform MCV660 (\textit{tdfG}) and MCV661 (\textit{tdfH}) is depicted. (B) Cartoon of homologous recombination event resulting in chromosomal integration of mutagenized \textit{tdfJ} to generate double mutants. Genetic elements are not drawn to scale.
Figure 3. Schematic of double mutant construction

pVCU938, tdfJ::Tn5<kan-2>

MCV660 (tdfG) or MCV661 (tdfH)
Figure 4. PCR screen of TdT double mutants.

Boilate DNA preparations from Kan\textsuperscript{r} MCV660 (tdfG) and MCV661 (tdfH) transformed with pVCU938 were PCR amplified with primers specific for \textit{tdfG}, \textit{tdfH} and \textit{tdfJ}. PCR products were separated on a 1% agarose gel. (A) PCR screen of MCV935, \textit{tdfG tdfJ} double mutant. Gene targets are listed above. Template DNA from the following strains were used for PCR: lane 1—wild type, FA1090; lane 2—MCV660 (\textit{tdfG}); lane 3—MCV661 (tdfH); lane 4—Kan\textsuperscript{r} MCV660 clone; lane 5—No template DNA. (B) PCR screen of MCV936, \textit{tdfH tdfJ} double mutant. Template DNA from the following strains were used for PCR: lane 1—MCV660 (\textit{tdfG}); lane 2—MCV661 (\textit{tdfH}); lane 3—Kan\textsuperscript{r} MCV661 clone 1; lane 4—Kan\textsuperscript{r} MCV661 clone 2; lane 5—No template DNA.
Figure 4. PCR screen of TdT double mutants.
would be observed by a growth defect in mutants lacking putative heme receptors. Gonococci were grown in partially chelexed GW media, a defined media permitting growth of gonococci from low inocula [251], in the presence of limited amounts of hemin and Desferal to chelate excess iron. Bacterial growth was measured via optical density (OD) readings every 2 hours for 8 hours, the results of which are presented in Figure 5. We found that tonB and TdT isogenic and double mutants were not defective for growth in heme limited conditions compared to wild-type strain FA1090 (Figure 5A). In fact, the tdfG isogenic and tdfG tdfJ double mutants grew significantly better than all of the strains tested. To ensure that optical density readings adequately represented any observable differences in growth, viable bacteria recovered from wild type and tonB after heme limited growth were enumerated. There was no significant difference in the amount of viable bacteria recovered indicating that TonB and the aforementioned TdTs do not contribute to gonococcal growth in these conditions (Figure 5B).
Figure 5. Heme-limited growth of *tonB* and TdT isogenic and double mutants.

Gonococcal strains were grown in partially chelexed GW media supplemented with limited amounts of heme, 5μM. (A) Bacterial growth was measured via OD$_{600}$ readings every 2 hours for 8 hours. Means and standard error of n=5 independent experiments are shown. (B) Viable bacteria recovered from wild type and *tonB* gonococci after growth for 8 hours in limited heme conditions were enumerated. Mean and standard error for n=4 independent experiments are shown. An un-paired Student’s *t*-test was used to determine significant differences in means; nd, no difference.
Figure 5. Heme-limited growth of \textit{tonB} and TdT isogenic and double mutants.
B. Heme utilization in gonococcal heme auxotroph is TonB-independent

Many bacterial pathogens that express heme receptors have incomplete heme biosynthetic pathways and are thus natural heme auxotrophs. As such, these pathogens require uptake of exogenous heme to support growth. *N. gonorrhoeae* has a complete heme biosynthetic pathway and does not require heme if it is provided exogenously along with an alternate iron source. It is possible that in the conditions of the previous assay, the gonococcal strains tested were not restricted to uptake of exogenous heme. To optimize the heme-dependent growth assay conditions and better resolve any TonB and/or TdT-dependent heme utilization mechanisms, we generated a *N. gonorrhoeae* heme auxotroph as well as *tonB* and TdT mutants in this background and evaluated heme-dependent growth. We hypothesized that growth of the gonococcal heme auxotroph in limited heme conditions would require energy dependent uptake of exogenous heme and thus require TonB and a heme receptor.

To generate gonococcal heme auxotrophs, we obtained pUNCH1304, a construct in which gonococcal *hemH* is disrupted by a chloramphenicol acetyltransferase cassette, *CAT* [237]. The enzyme ferrochelatase, encoded by *hemH*, catalyzes the addition of iron to protoporphyrin IX to form protoheme. The *hemH::CAT* construct was then used to transform wild type, *tonB*, and TdT isogenic and double mutants. Chloramphenicol resistant (Cm') gonococci were screened for chromosomal *hemH* mutation via PCR amplification with gene specific primers. As shown in Figure 6, a wild-type copy of *hemH* results in a PCR product of 1.3Kb. The mutant copy or insertionally inactivated *hemH* was detected in transformed gonococci as indicated by
PCR products of 2.3Kb, corresponding to the 1Kb CAT (Lanes 2, 7, 9-12). Table 5 lists all of the heme auxotrophic mutants generated.

The growth of wild type, *hemH*, and *tonB hemH* were evaluated in a liquid growth assay with limited heme. While both *hemH* and *tonB hemH* strains grew less well than wild type, there was no difference in growth observed between *hemH* and *tonB hemH* (Figure 7). These data indicate that TonB is not required for gonococcal growth restricted to exogenous heme uptake. Since heme uptake and subsequent growth was found to be TonB-independent in the gonococcal heme auxotrophic background, the remaining TdT *hemH* mutant strains were not further evaluated.
Figure 6. PCR screen of *N. gonorrhoeae* hemH mutants.

Boilate DNA preparations from Cm<sup>f</sup> FA1090 (wild type) and MCV656 (tonB) gonococci transformed with pUNCH1304 (*hemH*::CAT) were PCR amplified with *hemH* specific primers. PCR products were separated on a 1% agarose gel. Lanes 1 and 6 contain DNA ladder. Lanes 2 and 3 are clones from Cm<sup>f</sup> FA1090. Lane 4 is a positive control with wild type template DNA. Lane 5 is a negative control with no template DNA. Lanes 7-12 are clones from Cm<sup>f</sup> MCV656.
Figure 6. PCR screen of *N. gonorrhoeae* hemH mutants.
Table 5. *N. gonorrhoeae* heme auxotrophic mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>PCR confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV937</td>
<td>$tdfG\Omega, tdfJ::Tn5, hemH::cat$</td>
<td>+</td>
</tr>
<tr>
<td>MCV939</td>
<td>$tdfJ\Omega, hemH::cat$</td>
<td>+</td>
</tr>
<tr>
<td>MCV943</td>
<td>$tdfG\Omega, hemH::cat$</td>
<td>+</td>
</tr>
<tr>
<td>MCV944</td>
<td>$hemH::cat$</td>
<td>+</td>
</tr>
<tr>
<td>MCV945</td>
<td>$tdfH\Omega, hemH::cat$</td>
<td>+</td>
</tr>
<tr>
<td>MCV946</td>
<td>$tonB\Omega, hemH::cat$</td>
<td>+</td>
</tr>
<tr>
<td>MCV947</td>
<td>$\Delta tbp, hemH::cat$</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 7. Heme-dependent growth of \textit{N. gonorrhoeae} wild type and heme auxotrophic strains.

Wild-type strain FA1090, \textit{hemH}, and \textit{tonB} \textit{hemH} were grown in CDM supplemented with 5µM heme. Bacterial growth was measured via OD readings every 2 hours for 6 hours. Means and standard error of a representative of \textit{n}=3 independent experiments is shown. Differences in means at \textit{T}=6 hours were determined by a Bonferroni Post-test in GraphPad Prism. \textit{P}-values < 0.05 were considered significant and denoted with **.
Figure 7. Heme-dependent growth of \( N. \) gonorrhoeae wild type and heme auxotrophic strains.
C. *N. gonorrhoeae* cannot utilize *P. gingivalis* hemophore, HusA as a sole iron source

The data reported thus far demonstrate that neither TonB nor TdfG, TdfH and TdfJ contribute to growth under limited heme conditions indicating that these TdTs are not free heme receptors. The possibility remains however that these TdTs may not function in uptake of free heme but rather assimilate heme from a heme-containing protein. *N. gonorrhoeae* has only been demonstrated to assimilate iron and heme from the host hemoprotein, Hg. It cannot utilize heme-Hx, serum albumin, myoglobin, or catalase as has been shown for other pathogenic bacteria [27, 65, 237]. However, another hemoprotein may be present within the context of infection: bacterial derived hemophores. The urogenital tract, the primary site of gonococcal infection, is rich in both normal and potentially harmful bacteria, which may produce proteins and metabolites facilitating iron/heme acquisition. Despite being unable to produce a siderophore [259], *N. gonorrhoeae* is capable of utilizing siderophores secreted by other bacteria [36, 98]. Similarly, *N. gonorrhoeae* may be able to utilize hemophores potentially secreted by bacteria within the same ecological niche.

To identify potential candidate hemophores, we conducting a literature review to identify BV-associated bacteria with iron acquisition systems that *N. gonorrhoeae* could potentially hijack. Table 6 lists reported iron acquisition systems in BV-associated bacteria. Of the BV-associated bacteria considered, recent genome sequences revealed that *Fusobacterium spp.*, *Prevotella spp.*, and *Porphyromonas gingivalis*
<table>
<thead>
<tr>
<th>BV-associated bacteria</th>
<th>Normal site of infection</th>
<th>Cytotoxin/hemolysin</th>
<th>Siderophore/hemophore</th>
<th>TonB-dependent transporters (TdT)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G. vaginalis</strong></td>
<td>UGT</td>
<td>Vaginolysin, 1 putative hemolysin</td>
<td>siderophore biosynthesis genes</td>
<td>NR</td>
<td>[271]</td>
</tr>
<tr>
<td><strong>Fusobacterium spp.</strong></td>
<td>ORAL/UGT</td>
<td>NR</td>
<td>NR</td>
<td>5 putative TdTs</td>
<td>[116]</td>
</tr>
<tr>
<td><strong>P. intermedia 17</strong></td>
<td>ORAL</td>
<td>Several hemolysins</td>
<td>NR</td>
<td>8 putative heme TdTs</td>
<td>[201, 275]</td>
</tr>
<tr>
<td><strong>P. bivia</strong></td>
<td>UGT</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>[104]</td>
</tr>
<tr>
<td><strong>P. gingivalis</strong></td>
<td>ORAL/UGT</td>
<td>several gingipains</td>
<td>HusA</td>
<td>3 heme receptors 5 additional putative TdT</td>
<td>[77, 137]</td>
</tr>
<tr>
<td><strong>Sneathia amnii</strong></td>
<td>UGT</td>
<td>2 putative hemolysins</td>
<td>NR</td>
<td>NR</td>
<td>[93]</td>
</tr>
</tbody>
</table>

UGT, urogenital tract; ORAL, oral cavity; NR, not reported
encode genes annotated as TonB-dependent transporters with putative or described functions in heme uptake [116, 170, 201]. Proteomic analysis of the outer membrane of *Prevotella intermedia* 17 also revealed the expression of TonB-dependent transporters involved in heme uptake in iron limited conditions [275]. Interestingly, the *hmu* locus of *P. gingivalis* encoding the bipartite heme receptors HmuR/Y was also found in *Prevotella intermedia* 17. *P. gingivalis* and *Prevotella* spp. are within the Bacteroidetes phylum. A hemophore-like protein, HusA from *P. gingivalis* was recently identified [77]. This 21kDa protein was found to be both extracellular and cell-surface associated, demonstrated high affinity heme binding, and was required for heme utilization [77]. Given the relatedness of *P. gingivalis* and *Prevotella* spp., their expression of some homologous heme utilization systems, and their potential role in BV [180, 194, 275], the hemophore HusA was tested for its ability to support gonococcal growth as a sole iron/heme source.

Recombinant HusA was expressed and purified from *E.coli* BL21 strain expressing pETR7, a pET24d (+) derivative expressing HusA minus the first 23 amino acid residues of a putative signal peptide sequence followed by a C-terminal His⁶-tag [77]. The purified recombinant protein at approx. 21kDa is shown in elution samples in Figure 8. To test the ability of HusA to support gonococcal growth, wild type, *tonB*, *hemH*, and *tonB hemH* strains were spread onto CDM plates supplemented with Desferal to chelate contaminating iron. Various iron sources were then added to wells bored into the solidified media. HusA, saturated with 50% heme, was one of the iron sources tested to support gonococcal growth. Control iron sources included apo bovine
Samples collected during purification of recombinant *P. gingivalis* hemophore, HusA were separated on a 15% SDS-PAGE gel and Coomassie stained. Lanes are labeled as follows: M, molecular weight marker; FT, flow through; W1-2, wash samples; E1-5, elution samples; R, final rinse.
Figure 8. Purified recombinant *P. gingivalis* hemophore, HusA.
transferrin (apo-bTf), 30% saturated human transferrin (hTf), and free hemin. Plates were incubated overnight at 37°C with 5% CO₂ and growth around each iron source was recorded. The growth of wild-type strain FA1090 around various iron sources is shown in Figure 9. As expected, apo-bTf did not support growth of wild type while 30% sat. hTf and free hemin in the presence or absence of excess Desferal all supported growth. HusA; however, at 50% heme saturation, was not able to supplement growth of wild-type *N. gonorrhoeae* or any of the strains tested. The growth phenotypes of *tonB*, *hemH*, and *tonB hemH* strains are listed in Table 7. As expected *tonB* did not grow when supplemented with apo-bTf or 30% saturated (sat.) hTf as utilization of hTf requires energy derived from TonB. The *tonB* mutant was still capable of heme utilization consistent with our findings and previous reports that gonococcal heme utilization is TonB-independent [19]. Also, the *hemH* and *tonB hemH* strains behaved as expected in that neither strain grew without heme supplementation despite the presence of an alternative iron source.
Figure 9. Growth of *N. gonorrhoeae* wild-type strain FA1090 with hemophore HusA and various iron sources.

Gonococcal strains were spread onto CDM agarose plates supplemented with 10µM Desferal. Wells were bored into the solidified media and 20µL aliquots of various iron sources were placed into each well before incubation at 37°C with 5% CO₂ overnight. Growth of wild-type strain, FA1090 is pictured. Iron sources, labeled 1-7 counter-clockwise, are as follows: 1—7.5µM apo-bTf; 2—7.5µM 30% sat. hTf; 3—10µM hemin; 4—10µM 50% sat. HusA; 5—10µM hemin + 10µM Desferal; 6—7.5µM 30% sat. hTf + 10µM Desferal; 7—7.5µM 30% sat. hTf + 10µM hemin + 10µM Desferal.
Figure 9. Growth of *N. gonorrhoeae* wild-type strain FA1090 with hemophore HusA and various iron sources.
Table 7. Growth of *N. gonorrhoeae* strains with hemophore HusA and various iron sources

<table>
<thead>
<tr>
<th>Strain</th>
<th>apo-bTf</th>
<th>30% sat. hTf</th>
<th>10µM Heme</th>
<th>50% sat. HusA</th>
<th>10µM Heme + DFO</th>
<th>30% sat. hTf + DFO</th>
<th>30% sat. hTf + 10µM Heme + DFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA1090, wild type</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MCV656, <em>tonB</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MCV944 <em>hemH</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MCV946 <em>tonB hemH</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

DFO, Desferal; +, growth; ++, robust growth; -, no growth; sat., saturated
III. Discussion

*Neisseria gonorrhoeae* is quite adept at utilizing host derived proteins to entirely suffice for its nutritional requirement for iron. As such it encodes several TonB-dependent receptors, four of which have been characterized to facilitate iron assimilation from host proteins Tf, Lf and Hg as well as bacteria derived siderophores [198]. In this study, we evaluated three uncharacterized gonococcal TdT's (TdfG, TdfH and TdfJ) for their role in heme utilization. While some evidence of their potential contribution to heme utilization has been reported (see Table 4), we re-examined them as candidate heme receptors by focusing on three aims not yet considered in the body of literature reported.

First, we examined the possibility of redundant heme receptors and constructed double mutants unable to express TdfJ and TdfG or TdfH. When *tonB* as well as TdT isogenic and double TdT mutants where grown in limited heme conditions we found that neither TonB nor the TdTs alone or in combination contributed to growth (see Figure 5). We next considered the possibility that our system was not stringent enough to require gonococcal heme uptake and constructed gonococcal heme auxotrophs to require that growth was restricted to uptake of exogenous heme. While we expected heme uptake and growth of gonococcal heme auxotrophs in limited heme conditions to require energy, we found that TonB and by definition TdTs were not required (see Figure 7). These data lead us to conclude that under limited heme conditions, *N. gonorrhoeae* utilization of free heme occurs independently of TonB.

It is possible that there are un-identified TonB systems which support gonococcal heme utilization despite lack of obvious gonococcal *tonB* paralogs in genome
annotations. Most bacteria with multiple TdTs for the uptake of various nutrients in different conditions encode more than one *tonB* paralog (anywhere from 2-9 have been reported) with partial redundancy and distinct specificity [48]. *S. marcescens*, for example, encodes six *tonB* paralogs [178]. HasB, a *tonB* paralog is encoded by the *has* operon and provides energy to the TdT HasR, to internalize free heme or heme from hemoproteins. Either TonB or HasB of *S. marcescens* can energize HasR, but HasB does so more efficiently [178]. Additionally, the structural diversity of *tonB* paralogs and limited sequence similarity may further obscure the presence of non-obvious *tonB* paralogs [59]. However, the observation that disruption of the one known TonB system is sufficient to abrogate transferrin, lactoferrin, and hemoglobin utilization [224] suggests that *N. gonorrhoeae* probably encodes a single TonB system, which is sufficient to support the function of all gonococcal TdTs.

TonB-independent mechanisms of heme utilization have been dismissed as irrelevant because of the assumption that in the absence of energy, passive diffusion of nutrients across the outer membrane would require high heme concentrations, a scenario rare to mucosal environments where pathogenic *Neisseria* cause infection [209]. Our data suggest that even in very limited heme conditions, heme uptake occurs independently of TonB indicating that TonB-independent heme utilization may be physiologically relevant. As such, mechanisms of TonB-independent heme utilization may be of interest. Chen *et al.*, reported that spontaneous mutants in PilQ increased the entry of heme, TX-100 and antibiotics as demonstrated by a hyper-sensitivity to heme and hydrophobic agents [44]. The effects of the pilQ mutations were decreased by loss of pilT, indicating that PilT helps to increase entry of small molecules through PilQ. PilQ
forms an exit pore for the assembled pilus fibril and PilT is responsible for twitching motility mediated by pilus retraction. Additionally, PilT is required for DNA transport into the cell and facilitates pilus degradation in the absence of PilQ [265]. As such, Chen et al., argued that entry of heme and hydrophobic agents may be mechanically linked to pilus retraction [44]. PilQ however cannot be the only mechanism of TonB-independent heme entry as strains lacking PilQ are still able to grow on free heme.

Porins have been suggested as a non-specific heme entry mechanism [209]. However, lipophilicity in solute molecules has been shown to retard diffusion through the porin channel [174]. Additionally, heme tends to form hydrophobic aggregates which may be too large to diffuse through porins [133]. Diffusion of heme across the outer membrane may not require a protein channel, as the Neisseria outer membrane is generally highly susceptible to lipophilic agents [174]. This may be due to modification or masking of the phospholipid head groups as mutants with increased sensitivity to hydrophobic agents also demonstrated increased sensitivity to phospholipase C compared to wild type and mtr containing strains which were only sensitive to phospholipase C upon treatment with exoglycosidase [152]. The structure of neisserial LOS may also contribute to increased outer-membrane permeability to hydrophobic agents. LPS with short or no O-side chains was found to have increased fluidity compared to LPS with long O-side chains [174]. Additionally, faster lateral diffusion of an outer membrane protein was found in mutants unable to make LPS O-chain [174]. Increased membrane permeability to lipophilic molecules due to short O-side chains may also explain why heme uptake in Neisseria is TonB-independent, but TonB-dependent and receptor mediated in other bacteria that would produce LPS with long O-
side chains and thus potentially have membranes less permeable to lipophilic molecules. In *N. meningitidis*, hyper-sensitivity to heme has been attributed to mutations in *ght*, named gene of hydrophobic agent tolerance [192]. The *ght* gene product was originally thought to be a periplasmic protein but recent studies have shown that Ght is an N-terminally anchored inner membrane protein with a cytoplasmic domain that interacts directly with LpxC to help regulate LPS biosynthesis [187]. Meningococcal studies have shown that mutations in *ght* result in decreased production of LPS and increased sensitivity to heme and other hydrophobic agents further supporting the role of LPS structure in outer-membrane permeability and potentially non-specific heme entry in *Neisseria*. Interestingly, attempts to construct *ght* mutant in *N. gonorrhoeae* have failed suggesting that *ght* is an essential gene in the gonococcus [192].

Our studies also examined the possibility that the TdT, TdfG TdfH and TdfJ were not receptors for free heme, but rather other hemoproteins. Besides Hg and Hg-Hp complexes, pathogenic *Neisseria* cannot utilize other host derived hemoproteins like heme-Hx and heme-albumin as has been demonstrated for other heme receptors [65, 209, 254]. The utilization of a bacterial derived hemophore is not inconsistent with *Neisseria* biology, as *N. gonorrhoeae* has been shown to be capable of utilizing siderophores synthesized by other bacteria [36, 98, 227]. As far as we are aware, this is the first study to examine the ability of *N. gonorrhoeae* to utilize a bacterial hemophore. We found that hemophore-like protein, HusA from *P. gingivalis* was not able to support gonococcal growth as the sole iron/heme source suggesting that *N. gonorrhoeae* does not encode a hemophore receptor for this protein. We hypothesized that HusA would be the best candidate hemophore to test since it may likely be encountered by *N.*
* gonorrhoeae* given its expression by *P. gingivalis* and the association of *Porphyromonas* and *Prevotella* species with BV, a known risk factor for gonococcal infection [263]. However, the hemophores described in the literature thus far (HusA, HasA-type, and HxuA) have no sequence similarity and demonstrate different mechanisms of heme coordination, and so it is also possible that *Neisseria gonorrhoeae* may be able to utilize another type of hemophore. The ability of HasA and HxuA to support gonococcal growth would need to be tested to conclusively state that *N. gonorrhoeae* does not encode a hemophore receptor. Since HxuA does not actually bind heme, but rather inactivates the biological function of Hx by inducing release of heme upon interacting with Hx [253], a gonococcal receptor for utilization of HxuA or HxuA-hemopexin is unlikely. The expression of the HasA-type hemophore in multiple bacteria may indicate an increased likelihood to be a ligand of any putative receptor-mediated hemophore utilization in *N. gonorrhoeae*.

These studies and others reported in the literature have attempted to identify additional TdTs contributing to heme utilization in *Neisseria gonorrhoeae*, but the lack of identification of novel specific mediators of gonococcal heme utilization despite reported and unreported efforts, may indicate that the currently described mechanisms of heme utilization represent the sum total of specific receptor-mediated strategies. Additionally, the collection of mutants in either *pilQ, mtrCDE* or *ght* demonstrating phenotypes of hyper-sensitivity further support findings that heme entry in *Neisseria* is non-specific [26, 44, 192]. By demonstrating that in low heme conditions gonococcal heme utilization occurs independently of TonB even when exogenous heme uptake is required for growth and that *N. gonorrhoeae* cannot assimilate heme from a candidate hemophore,
our findings support the conclusion that heme utilization in *Neisseria gonorrhoeae* is non-specific and independent of TonB and TdTs.
I. Introduction

In order to readily adapt to changing environments, bacteria regulate expression of genes that optimize growth in specific nutritional conditions. TonB-dependent transporters, as mediators of active uptake of essential nutrients that are either poorly permeable though porins or present at low concentrations, are highly regulated to optimize their expression in conditions where they are required. Regulation of the TdTTs described thus far occurs at the transcriptional and post-transcriptional level through the activity of transcriptional regulators, riboswitches, and regulatory small RNAs (sRNAs) [175].

All TdTTs that function in the transport of iron or iron-chelates are regulated by the transcriptional regulator, Fur. Fur, for ferric uptake regulator, generally acts as a repressor. In the presence of high intracellular levels of Fe\(^{2+}\), the Fur homodimer binds co-repressor Fe\(^{2+}\) and the holo-protein binds to a DNA sequence motif called the Fur box in the promoter of regulated genes thereby repressing their transcription. In low cellular Fe\(^{2+}\) levels, apo-Fur does not bind the Fur box, resulting in de-repression of regulated genes. Other transcriptional regulators within the Fur family of
metalloregulatory proteins have also been shown to repress TonB-dependent transporters; for example NikR, for which Ni\textsuperscript{2+} acts as a co-repressor, represses the Ni transporter FrpB4 in *Helicobacter pylori* [205]. Additionally, Fur can indirectly regulate iron transport genes by regulating other types of regulators including two-component regulatory systems (TCS), AraC-like activators, and extra cytoplasmic function (ECF) sigma factors [87].

A sub-class of TonB-dependent transporters, termed transducers, function as both transporters and signal transducers that facilitate transcriptional activation of genes involved in transport of the TdT ligand. In addition to the plug and β-barrel domains of TonB-dependent transporters, these transducers are characterized by the presence of a unique N-terminal extension that functions as the signaling domain [122]. Perhaps the best characterized of these transducers is FecA, the TonB-dependent receptor for ferric citrate in *E. coli* K12. Signal transduction requires additional components; FecR, an inner membrane protein that functions as an anti-σ factor and FecI, an ECF σ factor that recruits RNA polymerase upon DNA binding to activate transcription [31]. Upon binding of the cognate ligand, ferric citrate, to the receptor FecA, conformational changes in FecA promote interaction of the N-terminal signaling domain of FecA with the periplasmic C-terminal end of FecR. The signal is transduced through the transmembrane segment of FecR to the N-terminal cytoplasmic end that activates FecI. FecI in turn binds to the promoter of the *fecABCD* operon and recruits RNA polymerase to induce transcription [29]. The FecI/R genes and the *fecABCD* operon are also Fe\textsuperscript{2+}/Fur repressed so the *fecABCD* operon is doubly controlled guaranteeing rapid response to the cell’s iron status [31]. TonB-dependent transducers represent the
minority of TonB-dependent receptors; however, Fecl/R type regulation has been identified in the homologous Fec systems of *Klebsiella pneumoniae*, *Aerobacter aerogenes*, and *Shigella flexneri* as well as heme and hemophore acquisition systems in *Bordetella* spp. and *Serratia marcescens*, respectively [30]. While the genomes of pseudomonads and *Bacteroides* spp. are highly representative of transducer-type TdTs, genera of several important human pathogens including *Hemophilus*, *Salmonella*, and *Neisseria* seem to encode none [122].

At the post transcriptional level some TdTs are regulated by riboswitches, RNA elements located at the 5’ end of mRNAs that change conformation upon ligand binding and modulate transcription or translation of the downstream gene(s) [175]. Expression of BtuB, the TdT for vitamin B$_{12}$ (cobalamin) is controlled by a riboswitch at the 5’ end of the *btuB* mRNA. Vitamin B$_{12}$ and adenosylcobalamin, a downstream product of vitamin B$_{12}$ metabolism, bind to the riboswitch and induce the conformational change responsible for *btuB* repression [175]. The cobalamin and thiamine pyrophosphate responsive riboswitches may also regulate putative Co$^{2+}$ and thiamine transport TdTs in addition to the vitamin B$_{12}$ receptor [206].

The small regulatory RNAs, OmrA and OmrB have also been shown to negatively regulate expression of TonB-dependent transporters: CirA, FecA, and FepA, the colicin I, ferric citrate, and ferric enterochelin receptors, respectively. The sRNAs, OmrA/B bind to the RNA chaperone, Hfq, which promotes RNA-RNA interactions [175]. OmrA/B are thought to repress CirA, FecA and FepA by directly base-pairing to early translation regions of the TdT mRNAs, this sRNA/mRNA binding inhibits translation and leads to subsequent degradation of the RNA complex [175]. OmrA/B are themselves
regulated by the TCS EnvZ/OmpR, osmolarity regulators further demonstrating a relationship between environmental conditions and TdT expression. OmrA/B are present in some other enterobacteria such as *Salmonella*, *Klebsiella*, and *Yersinia spp.* and while the regions of OmrA/B base pairing are conserved in *cirA*, the regulation of CirA or FecA and FepA by OmrA/B has not yet been demonstrated in the latter species [175].

In pathogenic *Neisseria*, the TdTs that facilitate iron/iron-chelate utilization from transferrin (TbpA), lactoferrin (LbpA), hemoglobin/haptoglobin (HmbR and HpuB), and foreign siderophores (FetA) are all repressed by Fe/Fur [64, 106, 183]. Additionally, the TonB system and the *fbpABC* system, which transports ferric iron across the cytoplasmic membrane, are all Fe/Fur repressed. *Neisseria spp.* do not encode any TonB-dependent transducers and so do not display regulation by ECF σ-factors [122].

Recently, our laboratory reported that the bi-cistronic operon encoding the transferrin binding proteins TbpB and TbpA were found to be regulated by a long non-coding RNA located in the un-translated region upstream of *tbpB* [249]. In contrast to the described sRNAs, this RNA species is large at approximately 1.8Kb, does not overlap with the 5’ end of target gene *tbpB* and demonstrates limited regions of sequence complementarity [249]. This is the only instance of a putative regulatory RNA to regulate a neisserial TdT and so may represent a novel mechanism of TdT regulation in *Neisseria*; however, activity on other gonococcal genes and expression of this RNA species in *N. meningitidis* remains to be determined. In addition to Fur some neisserial TdTs have also been identified as targets in regulons of the two-component regulatory system (TCS) MisR/S and the AraC-like regulator, MpeR as well as the Fur family regulator.
PerR/Zur. As such the reported activity of these transcriptional regulators in pathogenic *Neisseria spp.* is summarized below.

The TCS, MisR/S is also referred to as PhoP/Q due to its homology to the PhoP/Q system of *Salmonella* and other enteric bacteria that regulate expression of virulence genes in response to Mg levels and pH [111]. Inactivation of PhoP/Q in other bacteria results in dramatic attenuation of virulence [164, 232]. Like other described TCS, MisR/S is comprised of a sensory histidine kinase, MisR, which is an inner membrane protein with periplasmic and cytosolic domains and response regulator, MisR, which is localized to the cytoplasm. Upon activation by an environmental stimulus, MisS undergoes auto-phosphorylation. MisS interacts with MisR via its cytoplasmic domain and activates MisR by transferring the phosphoryl group to MisR. The phosphorylation state of MisR modulates its DNA binding affinity resulting in differential regulation of target genes, which includes the *misR/S* operon itself demonstrating auto-induction [241]. Mutations of *misR/phoP* in *N. meningitidis* results in a variety of phenotypes including strain dependent sensitivity to Mg levels [111, 239] increased sensitivity to antimicrobial peptides and environmental conditions [111, 171-172] as well as loss of PEA decorations from the inner core structure of LOS leading to the designation of MisR for *meningococcal inner core structure* [239]. Mutation of *misR/phoP* also resulted in increased sensitivity to mouse serum and reduced ability transverse a human epithelial cell monolayer [111] and attenuated virulence in the mouse model of infection indicating the role of this regulator in meningococcal virulence [171]. More recently, the MisR/S was found to be induced in contact with host cells and modulate genes involved in the adaptation of the bacterium to host cells suggesting that
the inducing signal for the system is host cell derived [107]. Two MisR/PhoP regulons have been described in \textit{N. meningitidis} with approximately 30% overlap in regulated genes possibly due to difference in strains and growth conditions used [172, 240]. Both regulons identified TonB-dependent receptor TdfH as targets and TdfJ and HmbR were also each identified by one of the two studies [172, 240]. These data suggest that the MisR/S TCS may regulate some neisserial TdT.

The transcriptional regulator MpeR is an AraC-like regulator expressed specifically in pathogenic compared to be commensal \textit{Neisseria} suggesting a potential role in virulence [155, 214]. More than 10,000 AraC homologs have been identified in protein databases throughout Gram-positive and Gram-negative bacteria thus representing one of the largest families of bacterial regulatory proteins [269]. Generally, AraC-like regulators have a conserved C-terminal DNA-binding domain and a variable N-terminal domain that is responsible for both protein dimerization and ligand binding. The paradigm for AraC-like regulators is the “light-switch” model of activation, wherein the dimeric regulator binds to distal operator sites of target genes resulting in DNA-looping and repression of gene expression. Upon binding of the inducing ligand, the regulator undergoes allosteric change resulting in binding to proximal sites in the promoter of target genes resulting in opening of the DNA loop and transcriptional activation [269]. In \textit{Neisseria}, MpeR is one of three AraC-like regulators [67]. MpeR is repressed by Fe/Fur and so can also act as a secondary regulator for indirect Fur activity [61, 64, 106]. The TdT, TdfF, which was found to be important for intracellular survival [83], is oriented in the opposite direction within the same genetic locus encoding MpeR. Thus, MpeR is hypothesized to regulate expression of TdfF [51],
although this has not been directly demonstrated. MpeR was however found to bind to the promoter of a putative iron related periplasmic binding protein (PBP) located upstream and in the same orientation of TdfF; thus its promoter is thought to drive the activity of the \textit{tdfF} containing operon [67]. MpeR was also demonstrated to repress expression of MtrF, an inner membrane protein that enhances \textit{mtrCDE} mediated induction of resistance to hydrophobic agents [71]. Additionally, MpeR was shown to activate FetA, the TdT that mediates utilization of catechol-like siderophores [36, 98]. Microarray studies of the MpeR regulon in \textit{N. gonorrhoeae} revealed that TdfH was repressed late in the growth phase of \textit{mpeR} mutant suggesting that TdfH is an MpeR target [161].

The Fur family transcriptional regulator, PerR was originally identified in \textit{N. gonorrhoeae} as a peroxide sensing, Mn-dependent repressor that negatively regulates MntC, the PBP of the Mn-binding ABC transporter encoded by \textit{mntABC} responsible for Mn-dependent resistance to oxidative stress [236, 268]. MntABC was later found to bind both Mn and Zn with equal affinity and PerR was concluded to behave like Zur, repressing target genes when bound to co-repressor Zn$^{2+}$ in the presence of high intracellular Zn levels analogous to the Fe/Fur repression [135, 143]. Microarray studies of the PerR/Zur regulon in \textit{N. gonorrhoeae} and the zinc responsive regulon of \textit{N. meningitidis} share significant overlap identifying genes involved in Zn transport and maintenance of Zn homeostasis [181, 268]. Both regulons identified the TdTs TdfH and TdfJ as Zn/Zur targets [181, 268]. Interestingly, a gonococcal \textit{perR} mutant demonstrated reduced invasion and intracellular survival in primary cervical epithelial cells [268] indicating that this regulator contributes to host cell interactions.
In the absence of described regulatory mechanisms beyond Fe/Fur described for neisserial TdTs, the transcriptional regulators MisR and MpeR represent the only other known modulators of TdT expression. Therefore, we sought to determine whether Fur, MisR and MpeR regulate the uncharacterized TdT: TdfG, TdfH and TdfJ in \textit{N. gonorrhoeae}. The identification of the regulatory networks to which these TdTs belong may reveal pertinent information about their function and contributions to the pathogenesis and fitness of \textit{N. gonorrhoeae} in which little to no regulatory data on these TdTs has been reported. In addition to the aforementioned transcriptional regulators, we also assessed the expression of these TdTs in response to Zn and the Fur family Zn regulator, PerR/Zur. Together these studies present a comprehensive analysis of potential regulators of \textit{N. gonorrhoeae} TdT: TdfH TdfJ and TdfJ.
II. Results

A. TdfG and TdfJ are Fur regulated

To determine whether TdfG and TdfJ were regulated by Fur, we evaluated the expression of the gonococcal TdTs TdfG and TdfJ in a fur mutant. Wild type and fur mutant strains were grown in GC broth in the presence of Fe$^{3+}$-specific chelator, Desferal to represent iron depleted conditions or in the presence of Fe(NO$_3$)$_3$ to represent iron replete conditions. Whole cell lysates standardized by culture density were analyzed via Western blot analysis with specific antiserum to detect TdfG and TdfJ. We found that TdfG expression was repressed in the presence of iron but elevated in iron depleted conditions in the wild type (Figure 10A) as has been previously reported [238]. There was no significant difference in TdfG levels detected in iron replete versus iron-depleted conditions in the fur mutant strain due to de-repression of TdfG in iron replete conditions (Figure 10A). These results indicate that the TdfG iron-repression is Fur-mediated. Interestingly, there was still a trend of increased TdfG expression in iron depleted conditions in the fur mutant suggesting that other factors may contribute to TdfG expression in the absence of Fur. Overall TdfJ expression was reduced in the fur mutant compared to wild type. This difference was significant in iron depleted conditions indicating that Fur contributes to TdfJ activation in low iron conditions (Figure 10B). TdfJ expression is reported to be iron induced [64, 273]; however, densitometry analysis did not find significant differences in TdfJ levels detected in iron replete versus depleted conditions in either wild type or the fur mutant (Figure 10B).
Figure 10. Fur regulation of TdfG and TdfJ.

Wild-type, FA1090 and fur mutant strains were grown for 2 hours in GC broth media treated with Desferal or Fe(NO₃)₃ to represent iron depleted or iron replete conditions, respectively. Whole cell lysates were standardized by culture density and analyzed via Western blot analysis. Densitometry was performed on a single immunoblot with three sets of replicate samples to determine arbitrary units (AU) of protein levels using NIH ImageJ. An un-paired Student’s t-test was used to compare means of protein levels detected. *P*-value < 0.05 was considered significant and denoted with *. (A) Left, a representative immunoblot probed for TdfG is shown. Right, densitometry results of TdfG levels detected (B) Left, a representative immunoblot probed for TdfJ is shown. Right, densitometry results of TdfJ levels detected.
Figure 10. Fur regulation of TdfG and TdfJ.
**B. TdfG is misR activated**

We next assessed the expression of TdfG, TdfH and TdfJ in wild-type strain FA1090 and the misR mutant to determine if MisR regulated any of these TdTs. Western blots of whole cell lysates from wild type and misR strains grown in iron replete and depleted conditions probed for each TdT are shown in Figure 11. Contrary to microarray studies identifying TdfH and TdfJ as MisR targets in *N. meningitidis* [172, 240], we found that expression profiles of neither TdfH nor TdfJ were modulated in misR mutant strains compared to wild type (Figure 11A). Surprisingly, we found that TdfG levels were significantly reduced in the misR mutant in iron depleted conditions ($P$-value = 0.0130) suggesting that MisR activates TdfG expression (Figure 11B). Additionally, there was no difference in TdfG levels in the misR mutant grown in iron replete versus depleted conditions further indicating that MisR activation contributes to the iron regulation of TdfG (Figure 11B).

**C. MpeR does not regulate TdfG, TdfH or TdfJ**

TdfH was predicted to be an MpeR target in a gonococcal microarray study [161]. To determine if MpeR contributes to regulation of TdfH or any other TdTs, whole cell lysates from wild-type FA1090 and the mpeR mutant grown in iron replete and depleted conditions and analyzed via Western blot with specific antiserum. In Figure 12, we demonstrated that none of the TdTs had altered expression profiles in the mpeR mutant compared to wild-type strain FA1090.
Figure 11. MisR regulation of TdfG, TdfH and TdfJ.

Wild-type, FA1090 and misR mutant strains were grown for 2 hours in GC broth media treated with Desferal or Fe(NO₃)₃ to represent iron depleted or iron replete conditions, respectively. Whole cell lysates were standardized by culture density and analyzed by Western blot with antisera against TdfG, TdfH or TdfJ as indicated. Densitometry was performed on a single immunoblot with three sets of replicate samples to determine arbitrary units (AU) of protein levels using NIH ImageJ. An un-paired Student’s t-test was used to compare means of protein levels detected. P-value < 0.05 was considered significant and denoted with *. (A) Left, representative immunoblot probed for TdfH is shown. Right, representative immunoblot probed for TdfJ is shown. (B) Top, a representative immunoblot probed for TdfG is shown. Bottom, densitometry results of TdfG levels detected.
Figure 11. MisR regulation of TdfG, TdfH and TdfJ.
Figure 12. MpeR regulation of TdfG, TdfH and TdfJ.

Wild-type strain FA1090 and mpeR mutant were grown for 2 hours in GC broth media treated with Desferal or Fe(NO$_3$)$_3$ to represent iron depleted or iron replete conditions, respectively. Whole cell lysates were standardized by culture density and analyzed by Western blot with antisera against TdfG, TdfH or TdfJ as indicated.
Figure 12. MpeR regulation of TdfG, TdfH and TdfJ.
D. TdfH and TdfJ are Zn repressed

We did not observe any changes in TdfH expression in response to iron as has been previously reported [238]; thus, we sought to determine whether expression of TdfH and TdfJ were responsive to Zn levels since they are predicted to be targets of the Zn/Zur regulon in *N. meningitidis* and *N. gonorrhoeae* [181, 268]. Gonococcal wild-type strain FA1090 was grown in CDM in the presence of the zinc specific chelator, TPEN, ZnSO$_4$ and/or Fe(NO$_3$)$_3$ at the indicated concentrations. We found that TdfH and TdfJ levels were repressed in presence of Zn (Figure 13). Conversely, expression of both proteins was up-regulated in the presence of increasing concentrations of TPEN. TdfH expression levels did not change in response to iron; however, TdfJ expression was further elevated when supplemented with iron, even at TPEN concentrations shown to de-repress TdfJ expression (Figure 13) suggesting that TdfJ is iron-induced and zinc repressed. To verify that TPEN treatment only altered Zn levels, we assessed expression of transferrin binding protein B (TbpB), which is known to be iron regulated. TbpB levels did not change at varying TPEN concentrations, indicating that TPEN treatment in these conditions is Zn specific.

E. Zur mediates TdfH and TdfJ Zn repression

To assess whether the observed zinc repression of TdfH and TdfJ was Zur-mediated, we generated *zur* isogenic mutants by transforming gonococcal strains FA19 and FA1090 with a *zur::kan* construct in which the *zur* gene is disrupted with a kanamycin resistance cassette. Kan$^r$ gonococci were selected and screened for chromosomal mutation via PCR with gene specific primers. In Figure 14 PCR products from Kan$^r$ gonococci show an increase in size corresponding to the size of the
**Figure 13. Zn regulation of TdfH and TdfJ expression.**

Whole cell lysates standardized by culture density were analyzed by Western blot with antisera specific to TdfH, TdfJ or TbpB as indicated. Wild-type strain FA1090 was grown for 4 hours in CDM supplemented with Fe(NO$_3$)$_3$, ZnSO$_4$ or Zn specific chelator, TPEN at the given concentrations.
Figure 13. Zn regulation of TdfH and TdfJ expression.
Boilate DNA preparations from Kanr FA1090 and FA19 gonococci transformed with zur::kan were PCR amplified with zur specific primers. PCR products were separated on a 1% agarose gel. (A) Lane 1—DNA ladder, lane 2—wild type template, lane 3—no DNA template, lanes 4-9—clones from Kanr FA19. Positive clones are denoted with an * and were designated MCV963. (B) Lane 1—DNA ladder, lane 2—wild type template, lane 3—no DNA template, lanes 4-10—clones from Kanr FA1090. Positive clones are denoted with * and were designated MCV964.
Figure 14. PCR screen of FA19 and FA1090 zur mutants.
kanamycin cassette confirming mutation. *Zur* mutants in FA19 and FA1090 background were designated MCV963 and MCV964, respectively. FA1090, FA19 and their respective *zur* mutant derivatives were then grown in GCB treated with ZnSO$_4$ or TPEN to represent Zn replete or Zn depleted conditions, respectively and whole cell lysates were analyzed by Western blot. We found that expression of both TdfH and TdfJ was up-regulated under Zn depleted conditions in the wild type backgrounds and was significantly increased in *zur* mutant derivatives in both backgrounds (Figure 15). Additionally, TdfH and TdfJ were not differentially expressed under Zn replete versus Zn depleted conditions in *zur* mutants (Figure 15). These data indicate that Zn repression of TdfH and TdfJ is Zur mediated. We also observed that TdfH expression was noticeably higher in strain FA1090 compared to FA19 regardless of Zn levels while TdfJ expression was similar in both backgrounds (Figure 15). Interestingly, the increased TdfH expression in FA1090 versus FA19 was observed in the respective *zur* mutants. This suggests that the observed increase of TdfH expression in FA1090 occurs independently of Zur.

**F. TdfH is MisR activated in gonococcal strain FA19**

In an attempt to identify factors contributing to decreased TdfH expression in strain FA19, we re-examined the role of MisR in the FA19 background since the MisR regulatory network is distinct in different gonococcal strains (William M. Shafer, personal communication). We found that the *misR* mutant derivative in the FA1090 background showed no difference in TdfH expression compared to its parent when grown in Zn replete and Zn depleted conditions (Figure 16). By contrast, in FA19 the *misR* mutant derivative expressed less TdfH compared to its parent in both Zn replete and depleted
conditions (Figure 16). MisR was not found to alter the expression of TdfJ in either FA1090 or FA19. These data indicate that TdfH expression is controlled by additional players beyond Zn/Zur such as MisR, and that this regulatory pathway is distinct in different gonococcal strains.
Figure 15. Zur regulation of TdfH and TdfJ.

Parental strains FA1090 and FA19 and respective zur mutant derivatives were grown in Zn replete and depleted conditions. Whole cell lysates standardized by culture density were analyzed by Western blot with antisera to TdfH or TdfJ as indicated.
Figure 15. Zur regulation of TdfH and TdfJ.
Figure 16. TdfH is MisR regulated in gonococcal strain FA19.

Parental strains FA1090 and FA19 and respective misR mutant derivatives were grown under Zn replete and depleted conditions. Whole cell lysates standardized by culture density were analyzed by Western blot with antisera specific to TdfH or TdfJ as indicated.
Figure 16. TdfH is MisR regulated in gonococcal strain FA19.
III. Discussion

In this study we analyzed the expression of TdTs TdfG, TdfH and TdfJ in response to Fe and Zn as well as transcriptional regulators Fur, MisR, MpeR and Zur. Given the abundance and at times conflicting reports of the TdTs expression in *N. meningitidis* and *N. gonorrhoeae* this study condenses the predicted regulators and examines their contribution to TdT regulation in *N. gonorrhoeae* to provide a comprehensive assessment of their expression and yield novel information about their potential function.

Perhaps the least information is known about the gonococcal TdT, TdfG. It is the largest TdT in the neisserial genome, with an apparent molecular weight of 130kDa. Interestingly, it is absent in all meningococcal genomes as well as commensal *Neisseria* with the exception of *N. elongata*, the most ancestral of the *Neisseria* species evaluated in a comparative genomic study of commensal and pathogenic *Neisseria* [155]. This indicates that TdfG may represent a product of niche adaptation and be of particular importance for survival in the urogenital tract. TdfG was previously reported to be up-regulated under conditions of iron limitation [64, 238] and the presence of a Fur box in the TdfG promotor was confirmed by Fur Titration Assay [106]. As such, we were not surprised to find that TdfG iron repression was Fur mediated. However, we were surprised to observe that TdfG expression was also MisR regulated specifically in iron limited conditions. MisR is not regulated by Fe/Fur indicating that the observed MisR activation occurs independently of Fur. This is the first report of an additional regulator modulating TdfG expression. Given the reported role of MisR/S in virulence and its potential role as regulator mediating adaptation to host cells [107, 171], the apparent
presence of TdfG in the MisR/S and Fur regulons may be indicative of a role in facilitating the utilization of a host-derived iron chelate. TdfG however, was not found to contribute to intracellular survival of *N. gonorrhoeae* in ME180 cervical epithelial cells [83]. Nonetheless, this novel finding in regards to TdfG expression represents an interesting development. Further studies to determine whether the observed MisR effect occurs directly or indirectly should be pursued.

In stark contrast to TdfG, perhaps the most data, for an uncharacterized TdT has been reported for TdfJ, which is found throughout human *Neisseria spp.* TdfJ was reported to be up-regulated under iron replete conditions in *N. gonorrhoeae* [64, 273]. In the absence of TdfJ specific antibodies, changes in protein expression were not previously examined. However, our lab has found that TdfJ is indeed induced in the presence of iron [176]. We also report here for the first time in the gonococcus that TdfJ is Zn/Zur repressed. A Fur box was previously detected in the promoter of *tdfJ* (NGO1205) in *N. gonorrhoeae* and a mechanism of direct Fur-mediated activation has been proposed since iron induction of *tdfJ* transcript was not observed in a *fur* mutant and Fur was found to specifically interact with the *tdfJ* promoter [273]. However, a Zur box adjacent to and slightly overlapping the putative Fur box was also detected in the promoter of meningococcal homologue *znuD* [126, 225]. Zur specifically co-factored to Zn²⁺ was demonstrated to directly interact with the *znuD* promoter in *N. meningitidis* [181]. In *N. meningitidis*, *znuD* promoter activity was up-regulated in the presence of iron even in a *zur* mutant and Fur was also found to specifically interact with the *znuD* promoter [126]. Additionally, in the presence of both zinc and iron *znuD* promoter activity was repressed in wild type but induced in the *zur* mutant indicating that Zur
repression out competes the Fur-mediated activation when both Fur and Zur are expressed [126]. Together these data lead to proposed model whereby Fur binding can further augment ZnuD expression levels in zinc limiting conditions wherein *znuD* is already de-repressed. However, in zinc replete conditions, Zn/Zur binds to the Zur box and blocks Fe/Fur binding repressing *znuD* transcript and preventing Fur mediated activation in the presence of iron [126]. Our findings that TdfJ is both Fur activated and Zn/Zur repressed in the gonococcus support this model.

It remains unclear why TdfJ would be simultaneously iron induced and zinc repressed. If TdfJ is in indeed a Zn transporter, it may be preferable to actively take in more Zn in high iron conditions to protect against the potential toxicity of high intracellular iron levels, although this would require simultaneously low Zn conditions to de-repress *tdfJ*. We did not observe any changes in TdfJ expression in the *misR* mutant, despite its identification as a MisR target in microarray studies [172] and its reported contribution to adhesion, invasion, and survival in A549, human lung epithelial, cells in *N. meningitidis* [126].

In addition to TdfJ, we also report that in *N. gonorrhoeae*, TdfH is Zn/Zur repressed in support of microarray studies of zinc-responsive and Zur regulons in *N. meningitidis* and *N. gonorrhoeae*, respectively [181, 268]. We also report that while TdfH expression was unaffected by the presence of MisR in *N. gonorrhoeae* strain FA1090, it was reduced in the *misR* mutant in gonococcal strain FA19 indicating that TdfH is MisR activated in FA19 and differentially regulated in these two gonococcal strains. The overall increase in TdfH expression in strain FA1090 versus FA19 that remains evident in both *zur* and *misR* mutants suggest that other
factor(s)/mechanism(s) independent of Zur and MisR also contribute to the difference in TdfH levels detected between strains. This is the first study to report that TdfH expression is distinct in different gonococcal strains and may also be controlled by additional factors beyond transcriptional regulators Zur and MisR.

We did not detect any changes in the expression of any of the TdTs tested in an mpeR mutant despite the report that tdfH transcript was reduced late in the log-growth phase of an mpeR mutant [161]. We should note that the expression studies reported here were conducted in the FA1090 background, while the MpeR regulon was reported for gonococcal strain FA19. This may be of significance since we detected dramatic changes in TdfH expression in the two backgrounds. As such, MpeR regulation should also be assessed in FA19.

FA1090 and FA19 are both experimental strains of N. gonorrhoeae routinely used in the laboratory. FA1090 was isolated from the cervix of an individual with DGI, while FA19 was isolated from a patient with uncomplicated gonorrhea [113]. Distinct regulatory mechanisms have been previously reported in FA19 and FA1090. For example, MtrA, another AraC-like transcriptional activator that has been shown to activate expression of the multiple transferable resistance efflux pump (mtrCDE), is fully expressed in strain FA19 but contains an 11-bp deletion within the coding region in strain FA1090. Thus, FA19 but not FA1090 can induce expression of the Mtr efflux pump to induce resistance to hydrophobic agents [200]. In addition to activating mtrCDE, MtrA also represses MpeR [242]. It is possible that the misR effect in gonococcal strain FA19 reported here may be indirect as MisR/S, MpeR, and MtrA are connected in a regulatory network [161, 242], although MisR was found to directly
interact with the \textit{tdfH} promoter in \textit{N. meningitidis} [240]. Further investigation of the direct or indirect role of these regulators on TdfH expression will help to identify the novel players and/or the mechanism of any strain specific regulation.

In previous studies, TdfH expression was detected in all meningococcal strains tested [226, 238] and 81\% of gonococcal strains [238]. Of note, TdfH expression was not detected in any species of commensal \textit{Neisseria} except for one out of six \textit{Neisseria lactamica} strains [238] despite the presence of the gene in some commensal species [155]. In their comparison of genome sequences among human neisserial species, Marri \textit{et al.} note that \textit{Neisseria lactamica} has the most virulence associated alleles of all the commensals and can occasionally cause infection [155]. As previously noted, TdfH expression may be regulated by transcriptional regulator MpeR, which is found on a genetic locus specific to pathogenic \textit{Neisseria} [155, 214]. It is possible that despite the presence of \textit{tdfH} in select commensal species, its expression may be limited to pathogenic bacteria, in which TdfH expression can be activated. Thus, distinct regulatory profiles of TdfH may contribute to varying levels of virulence between strains.

The data presented in this study resolves conflicting reports of regulation of TdfG, TdfH and TdfJ in \textit{N. gonorrhoeae}. We report that TdfG is activated by MisR specifically in iron depleted conditions identifying for the first time a novel regulator of TdfG expression that may act independently of Fur. Additionally, we demonstrate for the first time in \textit{N. gonorrhoeae} that both TdfH and TdfJ are Zn/Zur repressed implying roles in Zn acquisition in the gonococcus. Finally, we also report for the first time that TdfH is differentially regulated between gonococcal strains. We identify MisR to be an activator of TdfH in strain FA19, but also note that other factors contributing to strain-specific
TdfH expression may exist. Together these data presents new lines of investigation to pursue such as the contribution of TdfH and TdfJ to gonococcal Zn acquisition, which will be discussed in the following chapter.
Chapter 5: Gonococcal TdT, TdfH, facilitates calprotectin utilization and contributes to survival in neutrophil extracellular traps

I. Introduction

Transition metals have many functional roles in crucial biological processes that make them a necessary requirement for all living organisms. Zn, in particular, is an important catalytic and structural component of proteins. As such Zn-binding proteins are present in all kingdoms of life and represent approximately 8% of eukaryotic and 6% of archaeal and bacterial proteomes [7]. Zn containing proteins are involved in protein synthesis and degradation, DNA metabolism and repair, and neurotransmission [8]. The chemical properties of Zn, that are important for its biological activity also potentiate its toxicity, thus Zn homeostasis is tightly controlled across all domains of life.

Bacteria require 1nM-10µM Zn for optimal growth [228]. To maintain Zn homeostasis, bacteria tightly regulate Zn transporters that facilitate Zn uptake and Zn efflux systems that mediate Zn tolerance. Zn acquisition in prokaryotes primarily occurs via an ABC transporter, ZnuABC. ZnuABC is comprised of PBP that exhibits high affinity Zn-binding, an inner membrane permease and an ATP binding domain that hydrolyzes ATP to provide the energy required to actively transport the substrate into the cytoplasm. ZnuABC systems are tightly regulated by Zur, a member of the Fur family of metal response regulators, which can sense sub-femtomolar concentrations of
cytosolic Zn [23]. In excess Zn conditions, the Zur dimer is associated with Zn and binds to a specific DNA motif, termed the Zur box, in the promoter of target genes and inhibits RNA polymerase binding and activity [23]. In low Zn conditions, Zn disassociates from Zur; apo-Zur has reduced affinity for the Zur box resulting in de-repression of the target genes. In addition to ZnuABC, low affinity Zn transporters such as ZupT in *E. coli*, which is homologous to eukaryotic ZIP family transporters, have also been described [88]. Zn can also enter bacteria non-specifically through broad specificity metal ion transporters such as MntH, which is a proton-dependent divalent cation importer and an Nramp (natural resistance-associated macrophage protein) homologue, as well as MgtA, a P-type ATPase that may transport Zn as well as Mg. Both MntH and MgtA have been identified in *Salmonella typhimurium* and *E. coli* [23]. In conditions of excess Zn, Zn-efflux systems such as ZntA of *E. coli* facilitate energy dependent efflux of Zn$^{2+}$ and Cd$^{2+}$ from the cytosol to mediate Zn tolerance [23, 86].

Under normal physiological conditions, serum Zn levels are reported to be about 13-20µM and is probably sufficient to support microbial growth [228]. However, the reduction of serum Zn levels along with the concomitant increase of Zn binding proteins and Zn uptake in the liver in association with infectious and non-infectious disease has long been noted [228]. More recently, a variety of mechanisms for host-mediated Zn limitation have been described. In a mouse model of acute inflammation and infection, LPS treated mice developed hypozincemia, which was mediated by a cytokine (primarily IL-6) signaling cascade that stimulates increased synthesis of metallothioein, a host Zn storage protein, and liver specific Zn importer, ZIP14 [148]. The host strategy of limiting essential nutrients from invading pathogens has been designated “nutritional
immunity”. While traditionally characterized as the sequestration of iron from pathogens, roles for other transition metals such as zinc and manganese are now being examined [101, 117, 257]. The host protein calprotectin has been identified as a key mediator of nutritional immunity at sites of infection.

Calprotectin (CP) is a heterodimeric protein complex comprised of S100A8/A9, members of the calgranulin subgroup of the S100 family proteins, which are low molecular weight (10-14kDa) and characterized by two EF-hand calcium-binding domains separated by a hinge region. These proteins are expressed in monocytes, and can be induced in epithelial cells, endothelial cells, keratinocytes and activated macrophages [102, 276]. Members of the calgranulin super family including S100A7 and S100A12 in addition to S100A8 and S100A9 have been implicated in inflammatory diseases such as rheumatoid arthritis, psoriasis, inflammatory bowel disease and cancer because of their immuno-modulatory roles that lead to overly active immune responses and pro-tumor environments [78, 102, 182, 276]. S100A8 and S100A9 have the highest expression in neutrophils where they represent 40-60% of neutrophil cytosolic proteins. All of the S100 family proteins form homodimers; S100A8 and S100A9 preferentially form heterodimers. The S100A8/S100A9 heterodimeric complex is called calprotectin because of its antimicrobial activity attributed to its ability to chelate transitions metals Zn and Mn [49, 216]. Higher order oligomers, which are responsible for some biological activities, can also form in a calcium-dependent manner [136].

Calprotectin has two transition metal ion binding sites along the dimer interface. One site (Site I) binds both Zn and Mn at high affinity, while the other (Site II) only binds Zn at high affinity [58]. Calcium binding increases the affinity of Zn-binding sites [34].
Given the calcium ion gradient across the plasma membrane, calcium is thought to act a molecular switch for Zn-chelating activity whereby cytoplasmic CP primarily exists in a lower Zn affinity form, extracellular CP is predominately calcium bound and thus adopts a higher Zn affinity form [34]. CP may also act as an intracellular calcium sensor since conformational changes that occur as a result of calcium binding during calcium signaling cascades can modulate calprotectin’s interaction with target proteins [276].

CP can be secreted into the extracellular environment actively via intact microtubule networks or released by activated neutrophils during neutrophil extracellular trap (NET) formation or cell necrosis [276]. CP concentrations can reach >500µg/mL at sites of infection [110]. \textit{In vivo}, CP accumulates in tissue abscesses upon neutrophil influx and has been shown to have potent anti-microbial activity against \textit{Staphylococcus aureus} [49], \textit{Candida albicans} [243], \textit{Klebsiella pneumonia} [1], \textit{Acinetobacter baumannii} [100] and \textit{Helicobacter pylori} [76]. \textit{In vitro}, the recombinant protein also has antimicrobial activity against a wide number of pathogens including: \textit{Candida albicans} [215], \textit{Aspergillus fumigatus} [3], \textit{P. aeruginosa} [57], \textit{Staphylococcus epidermis}, \textit{Staphylococcus lugdunensis}, \textit{Enterococcus faecalis}, \textit{E. coli}, and \textit{S. flexneri} [58], which is reversed upon zinc supplementation. Given the effective host strategy of Zn limitation, microbial Zn acquisition systems have been shown to be important for pathogenesis. Zn uptake systems in \textit{C. jejuni}, \textit{Salmonella enterica}, \textit{H. ducreyi}, uropathogenic \textit{E. coli}, \textit{Brucella abortus}, and \textit{Streptococcus pyogenes} are required for virulence or colonization [117].

Pathogenic \textit{Neisseria} encode the ZnuABC system with homology to the \textit{E. coli} ZnuABC system for Zn accumulation [46, 225]. Interestingly, ZnuABC, which has also
been reported as MntABC, binds both Zn and Mn at equimolar affinities; other bacteria maintain separate systems with unique binding affinities for each metal [143, 236]. ZupT was also identified in *N. meningitidis* as a Fe/Fur repressed gene but is absent in *N. gonorrhoeae* [61]. PerR, also known as Zur, is a Fur homolog that represses Zn/MntABC in the presence of Zn [143, 268]. Microarray analysis of the PerR/Zur regulon in both *N. meningitidis* and *N. gonorrhoeae* revealed that two TonB-dependent transporters: TdfH and TdfJ were PerR/Zur regulated, suggestive of their roles in Zn acquisition [181, 268]. In *N. meningitidis*, the TdfJ homologue, ZnuD, was identified as the first bacterial outer membrane transporter to facilitate Zn acquisition as it was found to contribute to meningococcal growth in low Zn conditions [225]. More recently, CbpA, the TdfH homologue, was found to facilitate meningococcal growth in presence of CP in a TonB-dependent manner [226]. The functions of the meningococcal homologues of ZnuD and CbpA have not been tested in *N. gonorrhoeae*.

In the following studies we examine the role of TonB-dependent transporters, TdfH and TdfJ in Zn acquisition and CP utilization in *N. gonorrhoeae*. We also examined the ability of TdfH and TdfJ to bind CP specifically in the context of whole gonococcal cells. The studies described herein go further to evaluate the contributions of these TdTs to Zn accumulation in the presence and absence of CP. Additionally, we evaluated the contribution of TdfH to gonococcal survival in neutrophil extracellular traps (NETs). Together with the data reported for *N. meningitidis*, the results presented in this study characterize the function of these TdTs in pathogenic *Neisseria* and report for the first time the contribution of TdfH to *N. gonorrhoeae* survival in NETs.
II. Results

A. TdfH, TdfJ and TonB contribute to growth under Zn restricted conditions

Given the regulation of these TonB-dependent receptors by Zn/Zur reported in Chapter 4, we sought to determine whether these receptors contribute to gonococcal Zn acquisition. We hypothesized that those gonococcal strains lacking a Zn receptor would be deficient for growth under Zn restricted conditions. Likewise, we expected that upon supplementation with Zn, growth of wild-type gonococci would recover, whereas strains lacking receptors contributing to Zn utilization would remain suppressed for growth. To test this hypothesis, wild type, *tdfH*, *tdfJ*, and *tonB* mutant strains (FA19, MCV927, MCV928 and MCV650) [227] were grown in GCB treated with Zn-specific chelator, TPEN in the presence or absence of additional Zn. After growth for 6 hours viable bacteria were enumerated. As expected, fewer viable wild-type bacteria were recovered when grown in Zn restricted compared to Zn replete conditions (Figure 17). We found that significantly fewer viable bacteria were recovered from the *tdfH* mutant than wild type after growth in the presence of Zn. Additionally, there was no difference in the number of bacteria recovered from the *tdfH* mutant grown with or without Zn, consistent with a function for TdfH in Zn acquisition. The *tdfJ* mutant was the most impaired for growth in Zn replete or depleted conditions; however, growth was partially restored by addition of Zn. These data suggest that TdfJ is important for growth under Zn restriction but also imply additional mechanisms of Zn acquisition in the absence of TdfJ expression. Significantly fewer bacteria were also recovered from the *tonB* mutant.
Figure 17. Gonococcal growth in Zn replete and Zn restricted conditions.

Wild type (FA19), tdfH (MCV927), tdfJ (MCV928) and tonB (MCV650) gonococcal strains were grown in GCB treated with Kellogg's Supplement I, 12μM Fe(NO$_3$)$_3$ and 12.5μM TPEN in the presence or absence of ZnSO$_4$ (18.75μM) for 6 hours before being serial diluted and spot plated on GCB-VCN plates for CFU determination. Mean and standard error from a representative of n=3 independent experiments are plotted above. An un-paired Student's $t$-test was used to determine significance. *, $P \leq 0.05$; **, $P \leq 0.005$, n.d., no difference.
Figure 17. Gonococcal growth in Zn replete and Zn restricted conditions.
than wild type, indicating that TonB also contributes to growth under Zn restricted conditions.

**B. TdfH is required for growth in the presence of calprotectin**

Since both *tdfH* and *tdfJ* were found to contribute to growth under Zn restricted conditions, we tested the possibility that these transporters may be required for the assimilation of Zn from host derived Zn-binding protein, calprotectin (CP). CP is a hetero-oligomeric protein complex that constitutes 45% of the cytosolic protein content of neutrophils [94]. It has been demonstrated to have antimicrobial activity against a wide range of pathogens, which has been attributed to its Zn$^{2+}$ and Mn$^{2+}$ chelating ability [49, 216]. We therefore tested the ability of wild type, *tdfH* and *tdfJ* mutant strains to grow in the presence of CP supplemented with Zn under Zn restricted conditions. Contrary to the antimicrobial activity reported against other pathogens [49, 58], we found that the growth of wild-type gonococci was enhanced in the presence of Zn–laden CP (Figure 18). Addition of CP also specifically suppressed growth of the *tdfH* mutant, as growth of the *tdfJ* mutant was similar to that of wild type. These data suggest that *N. gonorrhoeae* is capable of overcoming the antimicrobial effects of CP via a mechanism that requires TdfH. To confirm that TdfH was indeed responsible for the observed CP resistance, we generated a complemented derivative of the *tdfH* mutant strain, MCV956 (*tdfH*). Expression of TdfH upon treatment with IPTG was confirmed (Figure 19). Growth in the presence of CP was restored in *tdfH* (Figure 20A), demonstrating that growth in the presence of CP specifically requires TdfH expression. Additionally, the *tdfH* growth defect was specific to CP treatment as neither *tdfH* nor
Figure 18. *tdfH* is defective for growth in the presence of CP

Gonococcal strains were grown under Zn-limited conditions (CDM, 1μM TPEN). Some cultures were further supplemented with a CP + Zn mixture (10μM CP, 5μM ZnSO₄). Growth was monitored by OD₆₀₀ readings every 2 hours for 6 hours. Plotted are the mean and standard error of the mean of n=2 independent experiments.
Figure 18. *tdfH* is defective for growth in the presence of CP
Figure 19. Expression of TdfH in complimented derivative strain, MCV956 (tdfH<sup>C</sup>)

Gonococcal strains FA19, MCV927 (tdfH), and MCV956 (tdfH<sup>C</sup>) were grown in CDM supplemented with 24μM Fe(NO<sub>3</sub>)<sub>3</sub> +/- 2mM IPTG. Whole cell lysates standardized by culture density were subjected to SDS-PAGE analysis followed by immunoblotting with antiserum specific to TdfH.
Figure 19. Expression of TdfH in complimented derivative strain, MCV956 (tdfH*)
Figure 20. Growth in the presence of CP is restored in MCV956 (tdfH<sup>C</sup>).

Gonococcal strains were grown under Zn-limited conditions (CDM, 1μM TPEN). (A) Cultures were supplemented with a CP + Zn mixture (10μM CP, 5μM ZnSO<sub>4</sub>) (B) Cultures were supplemented 5μM Zn alone or not further treated. Growth was monitored by OD<sub>600</sub> readings every 2 hours for 8 hours. Plotted are the mean and standard error of the mean of n=3 independent experiments.
Figure 20. Growth in the presence of CP is restored in MCV956 ($tdfH^C$).
$tdfH^C$ were more defective for growth compared to wild type when supplemented with Zn alone (Figure 20B).

C. TdfH enables binding of CP by *N. gonorrhoeae*

Given the ability of *N. gonorrhoeae* to grow in the presence of CP, we next sought to determine whether gonococci were capable of CP binding at the cell surface. Whole gonococcal cells, grown in CDM with Fe(NO$_3$)$_3$ and TPEN, were applied to nitrocellulose paper, presenting cell surface proteins in their native conformation within an intact bacterial outer membrane. After blocking, membranes were incubated with Zn-laden CP, before being washed and probed for CP. CP was specifically detected associated with wild-type gonococci indicating that *N. gonorrhoeae* is indeed capable of binding CP (Figure 21A). Additionally, CP binding was reduced by 80% in both $tdfH$ isogenic and $tdfH$ $tdfJ$ double mutants in FA1090 indicating that TdfH mediates the gonococcal/CP interaction. CP binding by wild-type strain FA19 was much reduced compared to FA1090 consistent with our previous findings that less TdfH was detected in whole cell lysates of strain FA19 compared to FA1090 (see Figure 15). However, CP binding was restored in $tdfH^C$ upon IPTG induction (Figure 21B) demonstrating that TdfH specifically facilitates the gonococcal/CP interaction in both FA1090 and FA19.

D. TdfH and TonB are important for Zn accumulation in *N. gonorrhoeae*

We suspected that TdfH, as a TonB-dependent transporter, and TonB would contribute to Zn internalization. To test this, we grew wild type, $tdfH$ and $tonB$ mutants under Zn replete and depleted conditions and assessed the level of internalized Zn using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). All of the
Figure 21. CP binding to gonococcal whole cells.

Gonococcal strains were grown in CDM supplemented with 24μM Fe(NO3)3 for 4 hours before equal culture densities of bacteria were applied to nitrocellulose paper. Membranes were first blocked before incubation with CP. After washing, blots were then subjected to immunoblotting using CP specific antiserum. Densitometry was performed on replicate blots using NIH ImageJ. Signal intensities were normalized to that of wild type and expressed as % CP binding. A paired Student’s t-test was used to compare % CP binding; $P \leq 0.05$ was considered significant and is designated by an *. (A) A representative dot blot with gonococcal strains FA1090 (wild type), MCV661 (tdfH), MCV662 (tdfJ), MCV936 (tdfH tdfJ) applied to nitrocellulose before CP incubation and detection. Below, densitometry results of n=3 replicate blots are shown. (B) A representative dot blot with gonococcal strains FA19 (Wild type), MCV927 (tdfH), and MCV956 (tdfH2) applied to nitrocellulose. TdfH expression in tdfH2 was un-induced (UI) or induced (I) by the addition of IPTG (2mM). Densitometry results of n=3 replicate blots are shown below the dot blot.
Figure 21. CP binding to gonococcal whole cells.
gonococcal strains tested accumulated significantly more Zn when grown under Zn replete versus Zn-depleted conditions ($P$-value $< 0.05$) indicating that the growth conditions adequately represented Zn replete and depleted conditions (Figure 22). Additionally, we found that $tdfH$ and $tonB$ mutants internalized significantly less Zn when grown in both Zn replete and depleted conditions compared to wild type ($P$-value $< 0.05$). These data suggest that TdfH and TonB contribute to Zn accumulation.

Given the demonstrated interaction between TdfH and CP and the role of TdfH and TonB in Zn accumulation (Figure 22A), we determined whether TdfH contributed to Zn accumulation in the presence of CP. Wild type and $tdfH$ mutants where grown under Zn depleted conditions in the presence of Zn-laden CP and the levels of internal Zn was assessed via ICP-OES. We found that the $tdfH$ mutant accumulated less Zn than wild type in the presence of CP (Figure 22B). These data indicate that TdfH facilitates Zn assimilation from CP.

**E. TdfH contributes to gonococcal NET survival**

Gonococcal infection is characterized by a robust recruitment of neutrophils, which migrate to the site of infection and attempt to eliminate the invading microbes via phagocytosis and/or NET formation [165]. As a major component of neutrophil cytosolic proteins, CP is released during NET formation and remains associated with NETs where it has been demonstrated to be the active component in NET-mediated killing of *Candida albicans* [243]. Thus, we sought to determine if gonococcal CP utilization, as mediated by TdfH, conferred a bacterial survival advantage in the presence of NETs. Gonococcal opacity proteins (Opa), which are subject to phase and antigenic variation,
Figure 22. TdfH and TonB contribute to gonococcal Zn accumulation.

Gonococcal strains were grown in Zn replete or depleted media before bacterial cells were harvested and analyzed for metal levels via ICP-OES. Means and standard error from a representative of n=3 independent experiments are plotted. An un-paired Student’s t-test was used to determine significance. *P ≤ 0.05, **P ≤ 0.005. Trends in statistical significance were consistent between biological replicates. (A) Strains FA19 (WT), MCV927 (tdfH), and MCV650 (tonB) were analyzed for Zn levels. (B) Strains FA19 and MCV927 (tdfH) were grown in Zn depleted media supplemented with CP + Zn (2μM CP, 1μM Zn) before bacterial cells were harvested for metal analysis via ICP-OES. Means and standard error from a representative of n=3 independent experiments are plotted.
Figure 22. TdfH and TonB contribute to gonococcal Zn accumulation.
have been shown to differentially stimulate and survive exposure to neutrophils [12]. To eliminate this variability, we constructed a \textit{tdfH} mutant (MCV955) in the FA1090 Opaless background (Figure 23), and assessed its survival in NETs compared to the Opaless parent.

Primary human neutrophils were treated with phorbol myristate acetate (PMA) to activate neutrophils and stimulate NET formation [33]. Activated neutrophils were then treated with cytochalasin D to inhibit phagocytosis and select for only extracellular mechanisms of neutrophil killing. DNase I was added to some NETs to assess the role of intact DNA fibers on NET-mediated killing. Neutrophils were then infected with the Opaless parent, MCV955 (\textit{tdfH}) or \textit{Δnuc}. The \textit{Δnuc} strain was used as a positive control for DNase I-reversible sensitivity to NET-mediated killing [115]. We found that while the Opaless parental strain demonstrated little to no survival defect in the presence of NETs with or without DNase I, only 40% of the \textit{tdfH} mutant survived exposure to neutrophils producing NETs (Figure 24). Additionally, we found that treatment with DNase I significantly increased survival of the \textit{tdfH} mutant indicating that intact DNA fibers contribute to the observed NET sensitivity of the \textit{tdfH} mutant. These results demonstrate that TdfH is important for gonococcal resistance to NET-mediated killing.
Figure 23. Confirmation of *tdfH* mutant in FA1090 Opaless background.

Gonococcal strains Opaless parent, MCV661 (*tdfH*) and MCV955 (Opaless *tdfH*) were grown in CDM supplemented with 24μM Fe(NO₃)₃. Whole cell lysates standardized by culture density were subjected to SDS-PAGE analysis followed by immunoblotting with antiserum specific to TdfH.
Figure 23. Confirmation of \textit{tdfH} mutant in FA1090 Opaless background.
Figure 24. TdfH enhances bacterial survival in NETs.

Human neutrophils were stimulated to induce NET formation and infected with Opaless, MCV955 (tdfH) or Δnuc at an MOI= 1 in the presence of Cytochalasin D +/- DNase I for 1 hour. Bacterial survival is expressed as percentage of initial inocula. Mean and standard error of n=6 independent experiments are shown. Significance was determined by an un-paired Student’s t-test. *, P-value < 0.05; **, P-value ≤ 0.005.
Figure 24. TdfH enhances bacterial survival in NETs.
III. Discussion

The data presented in these studies demonstrate that *N. gonorrhoeae* can resist the host derived Zn-chelating immune effector protein CP. We find that CP resistance is mediated by the TdT, TdfH, which facilitates CP binding and growth in the presence of CP. These data demonstrate for the first time that *N. gonorrhoeae* deploys an additional strategy to evade nutritional immunity by TdT-dependent utilization of the host nutrient binding protein, in this case CP. While TdfH was originally suggested to contribute to heme acquisition due to its similarity to heme receptors, previous studies found that TdfH was not able to restore heme utilization in an *E. coli* hemA mutant [238] nor was it required for gonococcal growth in the presence of heme. The data reported here demonstrate that gonococcal TdfH is instead a receptor for CP as we have found that it is required for gonococcal binding of CP and is required for growth in the presence of CP. These findings are consistent with the report that CbpA, the meningococcal TdfH homologue, is required for growth of *N. meningitidis* in the presence of CP and for CP binding [226]. The current report goes further to demonstrate that TdfH facilitates Zn accumulation both in the presence and absence of CP in *N. gonorrhoeae* in support of the model that TdfH facilitates utilization of Zn from CP to overcome its antimicrobial effects.

CP has largely been described as having inhibitory activity on the growth of numerous microbial pathogens [49, 58, 276]. Bacterial Zn acquisition systems in *Aspergillus fumigatus* [3], *Acinetobacter baumannii* [100], *Pseudomonas aeruginosa* [57] and *Salmonella typhimurium* [145] have been reported to facilitate resistance to CP. Specifically in Gram-negative bacteria, the bacterial factors contributing to CP
Resistance have been limited to the high affinity zinc uptake system, ZnuABC [57, 100, 145]. The report that meningococcal CbpA, binds to CP [226] and our findings that the gonococcal homologue, TdfH not only binds CP but is also required for Zn accumulation from CP identifies an outer membrane receptor that can directly interact with CP to overcome CP mediated Zn sequestration.

At the mucosal epithelia, neutrophils are the first line of defense, localizing to the site of infection and killing invading microbes by phagocytosis and subsequent degranulation or via NET formation. *Neisseria gonorrhoeae* can survive neutrophil influx through a variety of mechanisms including arresting maturation of phagosomes containing gonococci, suppressing oxidative burst, and expression of a nuclease in NETs [55, 112, 115].

NETs are mainly comprised of DNA, histones as well as neutrophil granule and cytosolic proteins. CP has been detected as major component of NETs [243]. NET-mediated killing is thought to occur by ‘trapping’ of microbes by chromatin fibers and high levels of antimicrobial proteins and molecules like CP and cathepsin G in the micro-environment of the traps [33]. DNase treatment degrades NETs and destroys the NET micro-environment and can thus abrogate NET-mediated killing as has been shown for other bacteria [33]. CP, through its Zn-chelating activity, was found to be the major antimicrobial component in NETs against *Candida albicans*, *Klebsiella pneumoniae*, and *Aspergillus nidulans* [1, 17, 243]. DNA was also found to have antimicrobial activity though metal chelation in NETs [85]. As such, a mechanism to out-compete or overcome host nutrient sequestration strategies would provide a significant survival advantage. Indeed in *N. meningitidis*, the Zn-outer membrane transporter,
ZnuD was found to contribute to survival within NETs [129]. While we did not evaluate the contribution of gonococcal ZnuD homologue, TdfJ to NET survival, the data reported in this study demonstrate for the first time that TdfH facilitates gonococcal resistance to CP and that TdfH expression contributes to survival in NETs. CP has been shown to be the key antimicrobial component of NETs; thus, these findings represent a novel gonococcal neutrophil resistance strategy wherein TdfH assimilates Zn from CP, neutralizing its antimicrobial activity, to contribute to survival in NETs.

We also report that TdfJ contributes to Zn acquisition in *N. gonorrhoeae* as fewer viable bacteria were recovered from the *tdfJ* mutant compared to wild type when grown in Zn replete and depleted conditions. The meningococcal TdfJ homologue, ZnuD was previously reported to have functions in both Zn and heme acquisition [126, 225]. However, our findings, reported in Chapter 3, did not support a role for TdfJ or any other TdT in gonococcal heme acquisition. ZnuD was also reported to contribute to adherence, invasion and intracellular survival of *N. meningitidis* in lung epithelial cells [126]. Previous studies from our laboratory have found that TdfJ does not contribute to invasion or intracellular survival of *N. gonorrhoeae* in cervical epithelial cells [83, 176]. Thus, it appears that while sharing 96% identity with TdfJ, meningococcal ZnuD has additional functions beyond Zn acquisition that are not observed in *N. gonorrhoeae*.

Little is known about the Zn concentration at mucosal sites and how Zn is transported across the outer-membrane of Gram-negative bacteria. It is thought that transport of the hydrophilic divalent Zn cation occurs passively via porins [86]. Our findings that TdfH, TdfJ and TonB contribute to Zn acquisition in *N. gonorrhoeae* as well as similar findings for the homologous proteins in *N. meningitidis* [225-226] indicate that
efficient Zn acquisition in *Neisseria* is TdT and TonB-mediated. ZnuD homologs with 50-60% similarity have also been identified in other Gram-negative pathogenic bacteria residing primarily in mucosal epithelia [225]. This may indicate that Zn concentrations at mucosal sites are too low for passive Zn diffusion; as such an outer-membrane Zn transporter and metabolic energy may be required for active Zn uptake and growth in those environments [225].

In sum, the data presented in this study indicates that TdfH and TdfJ contribute to Zn acquisition in *N. gonorrhoeae* and that TdfH is a calprotectin receptor. These findings are consistent with functions reported for TdfH and TdfJ homologues in *N. meningitidis* [225-226]. The data reported here, goes further to demonstrate that TdfH also assimilates Zn from CP and that direct utilization of CP enhances gonococcal NET survival. Our data presents a novel mechanism of gonococcal NET survival and highlights that many ways in which *N. gonorrhoeae* have successfully manipulated host-pathogen interactions to its advantage.
CHAPTER 6: Summary and Perspectives

The host strategy of limiting essential nutrients from invading pathogens, known as “nutritional immunity” is a prevalent theme in microbial defense. While traditionally characterized as the sequestration of iron from pathogens [257], roles for other transition metals such as zinc and manganese are now being examined [101, 117]. TonB-dependent transporters, as key mediators of nutrient acquisition in Gram-negative bacteria, are at the forefront of this fight for essential nutrients. Neisseria gonorrhoeae is particularly adept at overcoming host nutritional immunity strategies. Despite being unable to produce any siderophores to scavenge iron or iron chelates from the iron-limited host environment, N. gonorrhoeae can express eight TonB-dependent transporters, five of which enable utilization of the following as iron sources: siderophores produced by other bacteria [36, 98], host iron binding proteins, transferrin [50] and lactoferrin [20] and the heme binding protein, hemoglobin [43]. Given the role of the transferrin and lactoferrin receptors in gonococcal pathogenesis [52], it is likely that the remaining uncharacterized TdTTs also have important roles in the ability of N. gonorrhoeae to cause disease. These transporters also have the potential to be manipulated for the development of protective and anti-infective therapies. Accordingly, studies focusing on the function of the uncharacterized gonococcal TdTTs, TdfG, TdfH and TdfJ, are well-justified.
In this study, we sought to characterize the function and regulation of TdfG, TdfH, and TdfJ in *Neisseria gonorrhoeae*. To achieve this goal we first sought to determine the contribution of these receptors to gonococcal heme utilization. TonB-independent heme utilization has been demonstrated in *N. gonorrhoeae* [19]; although it is not expected to be biologically relevant *in vivo*, where low heme levels would necessitate receptors that enable active and efficient heme utilization [19, 133, 209]. Given their similarity to heme receptors, TdfG, TdfH and TdfJ were naturally considered as candidates for gonococcal heme TdTs. However, neither TdfG nor TdfH were found to contribute to heme utilization in *N. gonorrhoeae* [238]. ZnuD, the meningococcal TdfJ homolog, binds heme and restores heme utilization in an *E. coli* hemA mutant but it is not required for heme utilization in *N. meningitidis* [126] indicating that alternative mechanisms of heme utilization outside of ZnuD may exist.

In order to reconcile these reports we addressed the possibility that these receptors may be functionally redundant and generated double mutants incapable of expressing either TdfG and TdfJ or TdfH and TdfJ. When growth of these strains was evaluated, neither the TdTs nor TonB was found to contribute to growth. Additionally, TonB was not required for growth in a gonococcal heme auxotroph, dependent on exogenous heme uptake. Together these results indicate that TonB and by definition TdTs are not required for heme uptake in *N. gonorrhoeae*. Contrary to previous suppositions, we find that gonococcal heme utilization occurs independently of TonB and is thus passive even at low heme concentrations. These findings are in stark contrast to reports in other bacteria, which indicate that free heme utilization requires high affinity heme receptors for active TonB-dependent transport of heme [133]. It is not
clear why heme utilization in *N. gonorrhoeae* is TdT and TonB-independent. We postulate that the increased permeability of *N. gonorrhoeae* outer membranes to hydrophobic agents compared to other Gram-negative bacteria may allow for passive non-specific heme entry.

We also addressed the possibility that the TdTs may not be required for free heme uptake but may facilitate utilization of a bacteria derived hemophore as a heme source analogous to *N. gonorrhoeae* xenosiderophore utilization [36, 98]. We identified a candidate hemophore and tested its ability to support gonococcal growth as a sole iron and/or heme source. We found that *P. gingivalis* hemophore-like protein, HusA, could not support gonococcal growth. While our results appear to suggest that *N. gonorrhoeae* does not encode a “xeno-hemophore” utilization system, further studies with different types of hemophores will be needed to confirm that conclusion since the bacterial hemophores described thus far are structurally and functionally diverse [253].

In the next objective of this study, the regulation of these TdTs was evaluated. All TdTs that facilitate the utilization of iron or iron-chelates are iron repressed. These TdTs are differentially regulated by iron; TdfG is iron-repressed, TdfH is not iron responsive and TdfJ is iron-induced, suggesting that some of these receptors may have functions outside of iron uptake. To better understand the context in which these receptors may be expressed and ascertain some information about their function, we assessed their expression in the absence of Fur, MisR and MpeR, which are all regulators of *Neisseria* virulence factors. We found that both Fur and MisR contribute to the iron regulation of TdfG. While Fur repressed TdfG expression in iron replete conditions, MisR activated TdfG expression in iron depleted conditions. Given the specificity of TdfG to *Neisseria*
*gonorrhoaeae* and its regulation by MisR, which may be important for *Neisseria* adaptation to host cells [107], we predict that TdfG contributes to gonococcal pathogenesis by facilitating iron acquisition from a host specific source. TdfG is the largest gonococcal TdT; its larger size may indicate that it binds a protein ligand as opposed to a siderophore [226].

We also found that TdfH and TdfJ are Zn-repressed and Zur regulated and that TdfH expression is distinct in different gonococcal strains. In *N. gonorrhoeae* strain FA1090, TdfH is not MisR regulated. However, overall levels of TdfH detected are reduced in strain FA19 compared to FA1090 and TdfH is MisR activated. TdfH expression is almost always detected in *Neisseria meningitidis* and *Neisseria gonorrhoeae* strains but is rarely detected in commensal *Neisseria* species despite the presence of *tdfH* in most *Neisseria* genomes [155, 226, 238] suggesting that TdfH expression is differentially regulated in pathogenic versus commensal *Neisseria*. Our studies did not find TdfH to be regulated by pathogenic *Neisseria* specific regulator MpeR in gonococcal strain FA1090, but this may be not be the case in different gonococcal strains. As such further studies to determine the direct or indirect contribution of any potential regulators should be conducted in multiple strains.

Our findings emphasize the importance of characterizing gene products in various strains given the evidence of strain specific differences. FA19 and FA1090 are both routinely used lab strains but have reported genetic and phenotypic differences. In FA19, expression of the *mtrCDE* encoded efflux pump is inducible but is not in FA1090 due to a deletion in MtrA, an AraC-like activator of the efflux pump [200]. Also, FA19 encodes the gonococcal genetic island (GGI) while FA1090 does not. Components of a
GGI encoded Type IV secretion system confer a TonB and TdfF-independent mechanism of intracellular survival in FA19 [279]. Additionally, xerosiderophore utilization in FA19 is TonB and TdT-independent but is FetA dependent in FA1090 [98, 227]. FA19 was originally isolated from a patient with uncomplicated infection and FA1090 isolated from the cervix from a patient with disseminated infection. Characterizing regulatory differences between strains will help to better understanding the variety of potential expression patterns of gene products during natural gonorrhea infections.

In the final objective of this study, the contribution of TdfJ and TdfH to gonococcal Zn utilization was evaluated given their observed Zn/Zur repression. We found that TdfH, TdfJ and TonB were all important for growth in Zn depleted conditions indicating that Zn-utilization is TonB and TdT-dependent in *N. gonorrhoeae*. Zn passage across the outer-membrane of Gram-negative bacteria is thought to occur passively, potentially through porins [86]. The identification of two TdTs that facilitate Zn acquisition and are expressed in naturally occurring isolates of *N. gonorrhoeae* and *N. meningitidis* [225-226, 238] indicate that *Neisseria* must encounter low Zn concentrations *in vivo* and that efficient Zn utilization may in some conditions require TdT. ZnuD homologs have been identified in pathogenic bacteria of the respiratory tract including *Moraxella catarrhalis*, *Acinetobacter baumannii*, and *Bordetella pertussis* [225] suggesting that active, TdT-mediated Zn acquisition may be a wide-spread theme in mucosal pathogens. Studies investigating the function of these homologs and their contribution to pathogenesis will be needed to better understand the importance of TdT-mediated Zn acquisition.
This study also reports that TdfH is a calprotectin receptor. We demonstrate that TdfH is required for growth in the presence of CP, specifically binds CP and accumulates Zn from CP. These findings along with those reported for CbpA, the meningococcal TdfH homolog [226] identify a novel host protein that pathogenic Neisseria hijack to acquire the essential nutrient Zn.

Many studies have focused on the antimicrobial effects of CP in vitro and in vivo [49, 58]. Several studies have found that Zn transport systems, specifically ABC transporters in Gram-negative bacteria enhance survival in the presence of CP [3, 57, 100, 145]. This study along with that reported in N. meningitidis, represent the first reports of a surface receptor that binds to CP and is critical for CP resistance [226]. TdfH homologs are present in other bacteria, thus CP receptors may represent a new theme in microbial resistance to nutritional immunity. Apart from Neisseria spp. where TdfH homologs share over 90% identity, we have detected TdfH homologs in mostly opportunistic pathogens that normally colonize the human respiratory tract or mouth (Table 8). These homologs show 50-60% identity to TdfH and are often annotated as heme utilization proteins. One of such proteins, Hup of H. influenzae, shares 52% identity with TdfH and has been shown to contribute to heme utilization [166, 238], though our findings reported in Chapter 3 as well as previous reports [238] indicate that TdfH does not contribute to heme utilization in N. gonorrhoeae. As such, studies investigating the function of homologous TdTbs will be needed to determine whether they are actually heme or CP receptors. Additional studies identifying epitopes involved in CP binding and Zn coordination will also help to better annotate homologs and further characterize this novel class of TdTbs.
Calprotectin is highly expressed in neutrophils and is an active antimicrobial component of NETs [243]. Thus, we also evaluated the contribution of TdfH to *N. gonorrhoeae* NET survival and found that TdfH enhances gonococcal NET survival. Neutrophil recruitment is a hallmark of gonococcal infection. Despite the role of neutrophils in innate immunity, the ability to recover viable *N. gonorrhoeae* from neutrophil rich urethral exudates indicate that neutrophils are ineffective at clearing gonococcal infection [56]. *Neisseria gonorrhoeae* have a plethora of mechanisms to resist neutrophil mediated killing including resistance to oxidative stress, modulation of neutrophil granule release and efflux of antimicrobial components [56]. *N. gonorrhoeae* also induce NET formation independently of Opas and Pili, though these virulence factors can modulate NET formation [82].

Surprisingly, *N. gonorrhoeae* induced NETs do not stimulate ROS nor do they kill the bacteria [82] indicating that *N. gonorrhoeae* has NET resistance strategies. Recently *N. gonorrhoeae* was found to secrete a nuclease that facilitates escape from NETs [115]. Our findings that TdfH enhances gonococcal NET survival define a novel strategy of gonococcal NET resistance: direct utilization of CP as a Zn source, which neutralizes CP antimicrobial activity. In *N. meningitidis*, ZnuD was also found to be important for NET survival [129]. We did not evaluate the contribution of gonococcal ZnuD homolog, TdfJ to NET survival, though we suspect that it may also contribute to NET survival given its potential to mediate Zn acquisition. The evidence that TdTs involved in Zn
Table 8. TdfH homologs in opportunistic pathogens

<table>
<thead>
<tr>
<th>Organism</th>
<th>Family</th>
<th>Disease</th>
<th>Accession number</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acintobacillus ureae</em></td>
<td>Pasteurellaceae</td>
<td>Pneumonia, chronic respiratory disease</td>
<td>WP_005621380.1</td>
<td>52</td>
</tr>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
<td>Pasteurellaceae</td>
<td>Periodontis, bacterial endocarditis</td>
<td>WP_005578440</td>
<td>60</td>
</tr>
<tr>
<td><em>Haemophilus haemolyticus</em></td>
<td>Pasteurellaceae</td>
<td>Bacteremia, pneumonia, endocarditis</td>
<td>WP_005630735.1</td>
<td>51</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>Pasteurellaceae</td>
<td>Bacteremia, pneumonia, otitis media, epiglottitis, meningitis</td>
<td>AAS75800.1</td>
<td>51</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>Moraxellaceae</td>
<td>Sinusitis, otitis media, bronchitis, pneumonia</td>
<td>WP_013107855.1</td>
<td>54</td>
</tr>
<tr>
<td><em>Morococcus cerebrosus</em></td>
<td>Neisseriaceae</td>
<td>†Cerebellar abscess, Endophthalmitis,</td>
<td>WP_039407935.1</td>
<td>65</td>
</tr>
</tbody>
</table>

†Only two instances of disease by *M. cerebrosus* have been reported; see refs. [149, 235]
acquisition contribute to NET survival indicate that nutritional immunity via CP-mediated Zn sequestration is a major component of NET-mediated killing.

In all, the data presented in this study has yielded new information into the function and regulation of these TdTs in *N. gonorrhoeae*. Our initial characterization of TdfH as a CP receptor has laid the foundation for the description of a new class of TdTs that utilize CP as a nutrient source conferring a novel neutrophil/NET resistance strategy. Understanding gonococcal mechanisms that subvert neutrophil defenses will help to expose pathways that can be manipulated for successful clearance of gonococcal infection. Furthermore, these TdTs may be good targets for novel and specific anti-gonorrhea therapies since they are surface-exposed, widely conserved in *Neisseria gonorrhoeae* strains and are not phase variable. Additionally, this study has revealed new insights into the conditions in which these TdTs may be expressed and strain specific differences in their regulation. These findings have opened new lines of investigation to further ascertain the functional mechanisms governing these TdTs as well as their potential for use as targets of protective and therapeutic strategies against gonococcal infection.
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Literature Cited


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Vita

Sophonie Jean was born on November 21, 1987 in Stamford, CT. She graduated from Trinity Catholic High School, Stamford, CT in 2005. In 2009, Sophonie received her Bachelor of Science in Biochemistry & Molecular Biology from University of Richmond, Richmond, VA. She matriculated to VCU Integrative Life Sciences Ph.D. program in the Fall of 2009. Her accomplishments and publications are listed below.

**FELLOWSHIPS**

Robert D. Watkins Graduate Research Fellowship, 2012-2015  
American Society for Microbiology (ASM)

Summer Research Fellowship, 2006  
University of Richmond, School of Arts & Sciences

**AWARDS**

1st Place Surface Structures Poster Award, 2014  
XIXth International Pathogenic Neisseria Conference (IPNC) Asheville, NC

Black History in the Making Award, 2011  
Virginia Commonwealth University; Dept. of African American Studies

Travel Award, 2010 & 2011  
American Society of Parasitologists (ASP)

Travel Award, 2007  
American Society for Biochemistry & Molecular Biology (ASBMB)

ASBMB Sophomore Research Award, 2007  
University of Richmond; Dept. of Chemistry

Undergraduate Speaker Award, 2006  
Protein Society
PRESENTATIONS

Poster:
The role of gonococcal TonB-dependent Transporters, TdfH and TdfJ in heme and zinc acquisition. S. Jean and CN Cornelissen. Presented at:
- ILS Research Showcase Richmond, VA November 2014
- XIXth IPNC Asheville, NC October 2014

*Neisseria gonorrhoeae* outer-membrane protein TdfJ is regulated by iron and zinc and binds hemin. S Jean, MK Dickinson and CN Cornelissen. American Society for Microbiology 114th General Meeting Boston, MA May 2014.

Regulation of TonB-dependent transporters, TdfG and TdfH, and their role in heme acquisition in *Neisseria gonorrhoeae*. S Jean and CN Cornelissen. American Society for Microbiology 113th General Meeting Denver, CO May 2013.

Oral:


PUBLICATIONS

