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2011

The Regulation of TonB-dependent Transporters in Neisseria gnorrhoeae

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THE REGULATION OF TONB-DEPENDENT TRANSPORTERS IN *NEISSERIA GONORRHOEAE*

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

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September, 2011

Acknowledgements

First and foremost I would like to thank my graduate advisor Dr. Cornelissen. I am grateful for her patience and professionalism. She exemplifies excellence in both teaching and research. She also gave me the opportunity to develop myself professionally by presenting my data at conferences and attend the Kadner Institute.

I would like to express my sincere gratitude towards my graduate committee. Dr. Todd Kitten, Dr. Kimberly Jefferson, Dr. Joyce Lloyd, Dr. Munro and Dr. Joseph Ritter, who have been a source of support and knowledge throughout my graduate school career. Each of you has made yourselves available to me to discuss science and my professional development.

I would also like to acknowledge Dr. Gail Christie who has been immensely helpful in this project. She instilled my knowledge of prokaryotic genetics which was the basis for this research and has written countless letters of recommendation on my behalf.

I would like to thank our collaborators, Dr. Bill Shafer and Alexandra Mercante who have been a pleasure to work with. Their enthusiasm for this research was infectious and they were incredibly helpful with the EMSAS and Footprinting portion of this research.

I would also like to thank past and present members of the Cornelissen lab: Dr. Tracey Zola, Dr. Amanda Derocco, Dr. Jennifer McMillan Noto, Dr. Gregory Price, Ms. Heather Strange, Dr. Karen Ostberg, Dr. Rosuany Velez, Ms. Shreni Mistry, Ms. Aminat Oki, Ms. Meredith Weck, and Ms. Abena Watson-Siriboe. You all have been wonderful friends and colleagues over the years and I look forward to continuing our friendships. I would like to give a special acknowledgement to Dr. Rosuany Velez, who has provided an incredible amount of support throughout the writing of this dissertation.

I am grateful for the wonderful friends who have supported me throughout my doctoral education. Thank you to my amazing friends who started their professional training in Richmond with me: Ms. Kristen Pereira, Kara Plauger PharmD, Jennifer Shannon PharmD, Nitika Mittal DDS, Ms. Sarah Maybach and Dr. Amy Hawkins. You all have been there for me through professional and personal ups and downs and I am grateful for your friendship. I would also like to thank my closest friends including Mrs. Erin Spaulding and my sorority sisters who have been my cheerleaders since well before graduate school. I would like to thank the terrific group of friends I have made during my time in Richmond, VA.

I would like to thank my parents Ruth and Robert Hollander. They gave me the opportunity to explore my interest throughout my life and were always prepared with matzo ball soup and hugs. I would also like to thank my anchor Lieutenant Andrew Czarniak who has loved me unconditionally during my last years in graduate school. Thank you for all your support and patience throughout this experience.

Finally, I would like to dedicate this work to my grandmother Mrs. Louise Hollander who always told me to do what makes me happy and to enjoy life to the fullest.

Table of Contents

CHAPTERS

1 INTRODUCTION…………………………………………………...…………...1

List of Tables

List of Figures

List of Abbreviations

°C	degrees Celsius
Δ	delta, deletion
Ω	omega
α	alpha, anti
^{32}P	radiolabeled phosphorous
ABC	ATP binding cassette
Amp^r	ampicillin resistance
AP	alkaline phosphatase
ATP	adenosine triphosphate
β	beta
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
C	carboxy
cAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention
CDM	chelexed defined media
CEACAM	carcinoembryogenic antigen-related cell associated molecule
CFU	colony forming units
CM	cytoplasmic membrane
CMP-NANA	cytidine 5'-mono-phospho-*N*-acetylneuraminic acid
CO_2	carbon dioxide
Crp	cAMP receptor protein

C_T	cycle threshold
D1	dihydroxybenzoylserine monomer
D2	dihydroxybenzoylserine dimer
D3	dihydroxybenzoylserine trimer
DHBA	dihydroxybenzoic acid
DHBS	dihydroxybenzoylserine
DGI	disseminated gonococcal infection
DNA	deoxyribonucleic acid
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
Ent	enterobactin
Erm^r	erythromycin resistance
Fbp	ferric binding protein
FBS	fetal bovine serum
Fe	iron
$FeCl_3$	ferric chloride
$Fe(NO_3)_3$	ferric nitrate
FNR	fumarate and nitrate reduction regulator
Fur	ferric iron uptake regulator
g	gravity
GCB	gonococcal growth media
GGI	gonococcal genetic island

H_2O_2	hydrogen peroxide
Hb	hemoglobin
HEPES	4-(2-hydroxyethyle)-1 piper azine-ethanesulfonic acid
HIV	human immunodeficiency virus
Hpu	hemoglobin binding protein
hrs	hours
HS TBS	high salt Tris-buffered saline
HSPG	heparin sulfate proteoglycans
IgA	immunoglobulin A
IPTG	isopropyl-β-D-thiogalactopyranoside
Km^r	kanamycin resistance
kDa	kiloDalton
KU	klett unit
L	liter
LB	Luria Bertani *E. coli* growth media
Lbp	lactoferrin binding protein
LC-MS	liquid chromatography-mass spectrometry
Lf	lactoferrrin
LOS	lipooligosaccharide
LPS	lipopolysaccahride
LS TBS	low salt Tris-buffered saline
M	molar
MBP	maltose binding protein

min	minutes
mg	milligram
MgCl$_2$	magnesium chloride
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
N	amino
N.	*Neisseria*
NaCl	sodium chloride
NBT	nitro blue tetrazolium
NGAL	Neutrophil gelatinase-associated lipcalin
nM	nanometer
NSP	nonspecific
OD	optical density
OM	outer membrane
OMP	outer membrane protein
Opa	opacity protein
PBP	periplasmic binding protein
PBS	phosphate saline buffer
PID	pelvic inflammatory disease
PMNs	polymorphonuclear leukocytes
PCR	polymerase chain reaction

RMP	reduction-modifiable protein
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
S2	Salmochelin S2
S4	Salmochelin S4
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacyrlamide gel electrophoresis
SP	specific
Spe^r	Spectinomycin resistance
Strp^r	Streptomycin resistance
sRNA	small regulatory RNA
TBDT	TonB-dependent transport
Tbp	transferrin binding protein
Tf	transferrin
Tween 20	polyoxythylene sorbitan monolaurate
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
VR	variable region

w/v   weight per volume

WHO   World Health Organization

WT   wild-type

Abstract

THE REGULATION OF TONB-DEPENDENT TRANSPORTERS IN NEISSERIA GONORRHOEAE

By Aimee M Hollander

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

Virginia Commonwealth University, 2011

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Neisseria gonorrhoeae is an obligate human pathogen that causes the common sexually-transmitted infection, gonorrhea. Gonococcal infections cause significant morbidity, particularly among women, as the organism ascends to the upper reproductive tract, resulting in pelvic inflammatory disease, ectopic pregnancy and infertility. Antibiotic resistance rates have risen dramatically, leading to severe restriction of treatment options for gonococcal disease. Gonococcal infections do not elicit protective immunity nor is there an effective vaccine to prevent the disease. Thus, further characterization of expression, function and regulation of surface antigens could lead to better treatment and prevention modalities in the future. *N. gonorrhoeae* express a repertoire of TonB-dependent transporters for the acquisition of iron. All of these transporters are under the transcriptional regulation of Fur. We investigated putative intracellular iron sources utilized by gonococci and the role that the TonB-dependent transporter, TdfF, played in this acquisition. We determined that ascorbate which could prevent ferritin degradation or withhold iron from gonococci, inhibited intracellular survival. The utilization of iron

from the iron binding moiety 2, 5-DHBA of the putative mammalian siderophore was also examined. In this study we continued to investigate the regulation of TdfF and further investigate potential host-specific inducing molecules for TdfF expression. We investigated the regulation of *tdfF* expression and the role of MpeR, an AraC-like regulator, in *tdfF* expression. We determined that MpeR, interacted specifically with the DNA sequence upstream of *fetA* and activated FetA expression. We confirmed that the outer membrane transporter, FetA, allows gonococcal strain FA1090 to utilize the xenosiderophore, ferric-enterobactin, as an iron source. However, we further demonstrated that FetA has an extended range of substrates that encompasses other catecholate xenosiderophores, including ferric-salmochelin and the dimers and trimers of dihydroxybenzoylserine. We demonstrated that *fetA* is encoded as part of an iron-repressed, MpeR-activated operon, which putatively encodes other iron transport proteins. These iron transport proteins also play a role in xenosiderophore acquisition. We also identified genetic differences that may explain why some gonococcal strains are capable of xenosiderophore internalization in a TonB-dependent pathway and other strains are restricted to TonB-independent pathways.

Interestingly, the chromosomal locus that codes for *mpeR* and *tdfF* is pathogen specific. Thus understanding more about the TonB-dependent transporter and AraC-like regulator may further elucidate the pathogenicity of *N gonorrhoeae*.

CHAPTER 1 INTRODUCTION

I. *Neisseria*

The family *Neisseriaceae* contains the genera *Neisseria*, *Moraxella*, *Acinetobacter*, and *Kingella* which differ from each other by cell morphology (155). The members of the genus *Neisseria* are Gram-negative diplococci with adjacent flattened sides.

Neisseria species are both pathogens and normal flora in humans. They are differentiated based on their varying abilities to produce acid, as well as their DNase and catalase activities. Species are also differentiated based on their ability produce acids from carbohydrates through oxidation (not through fermentation) and their ability to reduce nitrate and nitrite as well as oxidize fatty acids (133) (Table 1).

Table 1. Biochemical Tests for the Identification of *N. gonorrhoeae* and Related Species

Species	Acid Production					Nitrate Reduction	Polysaccharide from Sucrose	Superoxide
	G	M	S	F	L			
N. gonorrhoeae	+	-	-	-	-	-	-	++++
N. meningitidis	+	+	-	-	-	-	-	+++
N. lactamica	+	+	-	-	+	-	-	++
N. polysaccharea	+	+	-	-	-	+	+	+
N. mucosa	+	+	+	+	-	+	+	+

Table adapted from Center of Disease Control and Prevention website.
Abbreviations: G, glucose; M, maltose; S, sucrose; F, fructose; L, lactose; (+) most
strains positive; (-) most strains negative. ++++ indicates strong positive; ++/+ indicates
weak positive.

II. Pathogenic *Neisseria* Species

Of all *Neisseria* species, only *N. gonorrhoeae* strains are always pathogenic whereas *N. meningitidis* are carried as normal flora of the nasopharynx. Under some circumstances *N. meningitidis* can become invasive, causing sporadic cases of meningitis and meningococcemia (155). These microorganisms are closely related yet highly adapted to their respective host niches and cause entirely different clinical diseases.

III. *Neisseria meningitidis*

A. Epidemiology

N. meningitidis colonizes the nasopharnyx mainly as a commensal and is carried by 5-10% of the healthy population in non-endemic times (269). Meningococci cause symptomatic disease if they disseminate from the nasopharnyx and cause meningococcemia or meningitis. Acquisition of *N. meningitidis* requires person-to-person transmission via direct contact or through dispersion of respiratory droplets from an infected individual to a susceptible individual. The frequency of meningococcal disease varies according to the demographic and geographical location of the population. The rate of nasopharyngeal carriage of *N. meningitidis* is lowest in young children, and highest among adolescents and young adults (269). Meningococcal disease in Europe and North America usually occurs as sporadic cases and the highest incidence rates are seen in children less than 5 years of age (46). In North America, meningococcal disease occurs at a rate of 1 case per 100,000 persons per year in the USA. The majority of these cases occur in the winter season and in young children (223), with a case fatality rate of

approximately 10% (223). Cases can also occur as clusters and localized outbreaks. *N. meningitidis* is a major public health concern in areas where the bacteria are responsible for pandemics and country-wide epidemics. Country-wide epidemics frequently occur in the Sub-Saharan Africa, which is known as the "meningitis belt" (46).

B. Infection

Meningococcal meningitis starts with the colonization of the nasopharnyx, followed by invasion and survival of bacteria in the bloodstream causing meningococcemia. Then the bacteria can cross the blood–brain barrier, causing infection in the central nervous system. Symptomatic meningococcemia may present as fever lethargy, shock, coma, intravascular coagulation and skin rash (40, 140). Symptoms of meningococcal meningitis include high fever, headache and stiff neck, which occur several hours to two days after infection.

C. Treatment

The Centers for Disease Control and Prevention recommends that meningitis treatment begin as early as possible in the course of the disease. An initial treatment with antibiotics is recommend if bacterial meningitis is diagnosed (47). Appropriate antibiotic treatment for bacterial meningitis reduces the risk of mortality to below 15%, although the risk is higher among the elderly (47). About 11–19% of survivors have complications due to infection which include neurologic disability, limb loss, and hearing loss (250). There are two kinds of vaccines that protect against *Neisseria meningitidis* available in the United States: meningococcal polysaccharide vaccine (Menomune®), and meningococcal conjugate vaccine (Menactra® and Menveo®). These vaccines protect

against meningococcal serogroups A, C, W135 and Y. There is currently no vaccine against meningococcal serogroup B.

IV. *Neisseria gonorrhoeae*

A. Epidemiology

N. gonorrhoeae primarily infects the urogenital and anorectal mucosa following intimate contact. Ejaculation does not have to occur for *N. gonorrhoeae* to be transmitted or acquired and it can be spread from mother to baby during delivery. *N. gonorrhoeae* causes the sexually transmitted infection gonorrhea which is responsible for an estimated 62.3 million infections worldwide (306). Over 300,000 infections are reported in the United States per year and it is the second most common reportable infectious disease in the US (49).

B. Infection

In men, gonorrhea can cause a symptomatic or asymptomatic infection. In symptomatic infections, symptoms arise 1-14 days after contact with an infected partner and include acute urethritis with purulent discharge and dysuria. Acute epdidymitis is the most common complication of untreated gonococcal infection in males; however, very rarely, disseminated gonococcal infection can occur.

It is estimated that up to 80% of women infected with *N. gonorrhoeae* are asymptomatic or present with minor symptoms (190). The primary site of infection is the endocervix and symptoms occur within the first 10 days of contact with an infected partner. Symptoms can include urethritis with increased vaginal discharge, or vaginal bleeding between periods. The spread of the microorganism into the upper genital tract

can result in the development of complications from infection (99). The ascending infection can lead to pelvic inflammatory disease which may cause ectopic pregnancy or infertility (203, 267). Symptoms of rectal infection in both men and women can present as asymptomatic or symptomatic infections. Symptoms may include discharge, anal itching, soreness, bleeding, or painful bowel movements.

Other areas that may be infected by *N. gonorrhoeae* include the throat and eye. Infections in the throat may cause a sore throat, but usually there are no symptoms. Gonococcal conjunctivitis in adults is a localized infection that can lead to corneal scarring and perforations leading to vision loss (165, 200, 287). In 0.5-3% of those infected, disseminated gonococcal infections (DGI) can develop. Classic symptoms include dermatitis, tenosynovitis, and fever. Besides serious secondary complications of DGI, gonococcal infections have also been correlated with increased transmission of HIV (60, 188).

C. Treatment

A variety of antimicrobial agents have been used for the treatment of gonorrhea over the years. However, the introduction of new drugs to treat gonorrhea has repeatedly led to the emergence and spread of *N. gonorrhoeae* with resistance to these new drugs (308). In 1935, sulphamides were recommended for the treatment of gonorrhea; however, by 1944 there was widespread resistance against this therapy (146, 167). Penicillin was then prescribed for treatment until 1976. The gonococcus became resistant to penicillin due to the plasmid-mediated resistance as well as other genetic mutations, which led to the end of penicillin being recommended as a therapeutic agent for gonorrhea in many areas of the world (216). Fluoroquinolones, such as Ciprofloxacin

were widely used to treat gonorrhea from the mid-1980s until 2007 when the Centers for Disease Control and Prevention no longer recommended this antibiotic for treatment of infection (50, 279, 308). Consequently, only one class of antimicrobials, the extended spectrum cephalosporins, is recommended and available for the treatment of gonorrhea in the United States; however, resistance against this particular therapy is on the rise (299). Most recently a gonococcal strain has been identified that is resistant to all known antibiotic therapies (30).

In contrast to *N. meningitidis*, there is no vaccine to prevent infection with *N. gonorrhoeae*. Gonococcal infections do not elicit protective immunity and individuals can be infected by the same strain over and over again because the gonococcal cell surface is extremely variable. Given the major morbidity from untreated gonococcal infections, along with the rise of antibiotic resistance and lack of vaccine, it is important to understand the genetic regulation of outer membrane proteins as well as the nutrient acquisition systems employed by the bacteria. These proteins could serve as vaccine antigens as well as targets of alternative treatments for the sexually transmitted infection caused by *N. gonorrhoeae*.

V. *N. gonorrhoeae* Virulence Factors

N. gonorrhoeae expresses a wide range of virulence factors that contribute to successful infection of the human host. These virulence factors include those that contribute to mucosal adherence, cellular invasion, and immune evasion.

A. Pilus

Gonococcal pili play an important role in colonization of host tissue and piliated gonococci have been recovered from primary cultures (150, 151). Pili are long (<6μm)

filamentous structures that extend from the bacterial surface. The pili allow the gonococcus to overcome the electrostatic barrier between the bacterium and the eukaryotic host cell (125). Gonococcal pili are not only associated with colonization but are important virulence factors because they play a role in the uptake of DNA. Piliated gonococci are 1000-fold more competent for transformation by DNA than non-piliated bacteria (24, 95, 268). Pili also play a role in twitching motility in which the pilus fibers retract causing the bacteria to move along most surfaces (35).

The pilus fiber is primarily composed of a single pilin protein subunit, PilE, which is assembled in a helical arrangement into a filament with a diameter of 6nm (214). Located on the tip of the pilus fiber is the PilC protein (247) which has also been identified as a surface component of *N. gonorrhoeae* (236, 246). Neisserial pili interact with many cell types including epithelial cells, endothelial cells, granulocytes, macrophages, and erythrocytes (248, 262, 294, 295). There are at least two different host cell binding epitopes on the pilus. One epitope is the PilE subunit along the pilus fiber and the other epitope is on the tip of the pilus mediated by PilC (257). Very little is known about the receptors that are recognized by the two binding functions of the pilus or about the cellular response to pilus-mediated adherence. Biochemical studies performed to elucidate the cellular receptors for pilin dependent adhesion were inconclusive with different studies concluding that receptors were either carbohydrate or proteinaceous in composition (110, 153).

Since pili are surface exposed and are major virulence factors of the gonococcus; attempts to produce an anti-gonococcal vaccine was focused on this surface antigen (33, 187, 285). However, pilus expression is phase variable due to the poly-cytosine tract in

pilE (156). This phase variation leads to reversible on and off switching of pilin. Along with phase variable expression, pili also undergo antigenic variation. Antigenic variation results from a nonreciprocal transfer of DNA sequences from one of many silent *pilS* cassettes into the expressed *pilE* locus (18, 112, 195, 276). As the *pilS* locus remains unchanged during the recombination reaction, the process resembles a classic gene conversion event (314). In addition to phase and antigenic variation, pilin also undergo post-translational modifications. These modifications include phosphorylation as well as glycosylation (126, 272). The high degree of PilE antigenic variation, phase variation and post-translational modifications likely contribute to both the failure of gonococcal infection to elicit protective immunity in the human host and the lack of efficacy associated with *N. gonorrhoeae* pili vaccines (33, 261).

B. Opas

Neisserial Opa proteins were originally identified because changes in their expression led to altered colony opacity and color due to changes in bacterial aggregation (29, 274). The Opa proteins constitute a family of closely related but size-variable integral outer membrane proteins that are predicted to span the membrane eight times with four surface exposed loops (124, 182). A single gonococcal strain can possess up to 11 unlinked chromosomal alleles that encode distinct Opa variants. It is thought that Opa expression is important for gonococcal infection because gonococci recovered after urogenital, cervical or rectal infection are typically Opa$^+$, as are bacteria recovered after the inoculation of human volunteers with transparent (Opa$^-$) bacteria (143, 275). The only exception to this Opa dependence is that Opa- bacteria predominate in the cervix early in the menstrual cycle (274). Opas are thought to play a role in intimate interactions with the

host cell that leads to trancytosis into sub-epithelial tissues. It has been demonstrated that a subset of Opa protein variants bind to host cell surface-associated heparin sulfate proteoglycans (HSPG). Many groups have also determined that Opas specifically bind to CEACAM receptors that are differentially expressed on multiple tissues in the human host (55, 292). Most Opa proteins bind to either CEACAM or HSPG glycoproteins however some interact with both cellular receptors. The Opa variants that do bind to both cellular receptors are only able to mediate cellular invasion by CEACAM (157). It has been demonstrated by Wang et al, that gonococci expressing CEACAM-specific Opa proteins are capable of passing from apical to the basolateral surface of polarized epithelial cell line monolayer (298). Opas have also been shown to down regulate the host immune response through the induction of B cell apoptosis, suppression of T-cell activation (34), and inhibition of an antibody response (213). Similar to pilin, the expression of each Opa allele is phase variable making it a poor vaccine antigen. Opa phase variation is due to slipped strand mispairing in which a change in the number of CTCTT repeats in the structural gene resulting in differential expression of Opas (204). Additionally, gonococcal strains can express zero or multiple Opas at one time (270).

C. Porin

Porin is the predominant outer membrane protein of *N. gonorrhoeae*. It is encoded by the *porB* gene. Gonococci possess one of two *porB* alleles, *porB1a* or *porB1b*, which encode the PIA or the PIB protein, respectively (74, 106) Gonococcal strains are separated into two serotypes based on the two different porin proteins. PorB assembles in the membrane as a trimeric complex in which each PIA or PIB monomer forms a β barrel structure with eight predicted surface-exposed loops (68, 142). PorB serves as an ion

channel and is essential for cell survival. Porin has several functions associated with gonococcal pathogenesis including effects on host cell apoptosis (201). Porin has been shown to either induce or inhibit apoptosis depending on the cell line that is infected (91, 201). Porin can aid in cell invasion (103) and can also translocate from bacterial membranes into the membranes of target cells. Porin has been identified in cytoplasmic and phagosomal membranes of host cells (300). In addition, PorB has been associated with antibiotic resistance (100). PorB aids in immune evasion in multiple ways as well. Purified gonococcal porins can inhibit phagocytosis and degranulation by PMNs (26, 114). The gonococcal porin PorB1A is critical in modulating stable serum resistance by binding to factor H (239). Porin can also down regulate the classical complement pathway by binding to the C4b binding protein (238). Unlike the majority of gonococcal surface structures, PorB is antigenically stable during infection. Porin diversity does exist among strains but primarily within the surface-exposed loops. Porin diversity as well as its ability to modulate immune responses makes it a poor vaccine candidate. However the diversity of porin is exploited by the conventional *N. gonorrhoeae* serotyping method, which uses a panel of monoclonal antibodies to type strains (277). Sequence-based methods, such as multiple antigen sequence typing, *porB* sequencing (288), and *porB* variable region (188) typing (282) are also used for serotyping strains.

D. Lipooligosaccharide

Lipopolysaccharide (LPS) is a component of the gram-negative outer membrane. It is the main component of the outer leaflet of the outer membrane, being anchored to the membrane by the lipid A moiety which is endotoxic. Extended outward from the membrane is a core oligosaccharide followed by the O antigen which is a polymer of

short oligosaccharide repeats. *N. gonorrhoeae* produce a short type of LPS known as lipooligosaccharide (LOS) because the core oligosaccharide structure is highly branched and it lacks repetitive O-antigen side chains. It has recently been observed that LOS may play a role in the resistance to antimicrobial peptides (173). LOS also induces the production of pro-inflammatory cytokines and binds to the TREM-2 receptor, which is expressed on genitourinary epithelial cells and by the fallopian tube epithelium in the host (231, 233).

LOS varies amongst gonococcal strains and this variation is due to phase-variable expression of the glycosyl transferases, which are responsible for the biosynthesis of variable α-chain carbohydrates of LOS. The phase variable expression is from slipped strand mispairing due to poly-guanosine tract within the coding sequence of the gene (70). LOS can also undergo post-translational modification in which a terminal galactose residue is modified by the membrane-associated bacterial sialyltransferase using host-derived cytidine 5'-mono-phospho-*N*-acetylneuraminic acid (CMP-NANA) as the sialyl donor (183). The sialylation of LOS prevents complement dependent killing of gonococci. (9, 305).

E. Reduction-modifiable protein

Reduction-modifiable protein (RMP) is a gonococcal outer membrane protein whose name refers to the observation that its molecular weight shifts following SDS-PAGE in reducing conditions (181). RMP is conserved amongst gonococcal strains and is thought to be a homolog of OmpA in *Enterobacteria* (104, 105). Antibodies produced during gonococcal infection are specific to RMP (191, 242). When these RMP specific antibodies are depleted from human sera, gonococci are then susceptible to serum-

dependent killing. It has been demonstrated that RMP specific antibodies block the binding of IgG, resulting in protection of the bacteria from the host immune response (242). It is also thought that RMP antibodies increase host susceptibility to infection (222).

F. IgA1 protease

Cleavage of IgA antibodies by the gonococcal protease leads to the separation of the $F_{(ab)}$ fragment, which is involved in the antigen binding, from the F_C domain, which is involved in effector function (221). This results in the pathogen becoming decorated in $F_{(ab)}$ fragments, masking gonococcal epitopes from the host immune system. It has also been demonstrated that IgA1 protease cleaves human lysosomal/late endosome associated membrane protein 1 (h-lamp-1) which may lead to survival during infection (123, 177). Additionally, it has been demonstrated that IgA protease plays a role in the transcytosis of gonococci across polarized cells (134). There are serologically distinct IgA1 proteases of *N. gonorrhoeae* and this is due to the horizontal exchange of *iga* (116). Thus IgA protease not only plays a role in immune evasion but may also play a role in gonococcal intracellular survival of epithelial cells.

VI. Host Iron Sources

Iron is an essential element for all living organisms and plays a crucial role in a variety of cellular functions in metabolism, cellular growth and differentiation as well as oxygen transport, DNA synthesis and energy production (37). However, excess free iron is toxic and donates electrons to produce hydroxyl radicals via the Fenton reaction:

$Fe^{2+} + H_20_2 \rightarrow Fe^{3+} + OH^- + OH^.$ These free hydroxyl radicals cause oxidative stress-induced damage of DNA, lipids and proteins leading to cell damage and death. Thus 99.9% of host iron is held in intracellular storage proteins or bound to host iron binding proteins for extracellular transport (189). The following are iron sources that are available within the human host and could provide this necessary nutrient to gonococci.

A. Ferritin

Ferritin is an iron-binding protein that is ubiquitous within the human host. Ferritin is a 24-mer globular protein with an iron core. Ferritin is made up of heavy and light chain proteins with molecular masses of 21 kDa and 19 kDa respectively (12, 93, 280). Naturally-occurring ferritin does not contain more than 3000 iron atoms/molecule, although in principle it can accommodate up to 4500 (120, 280). The sequestration of iron by ferritin serves two purposes. First, ferritin sequesters iron and segregates the iron in a non-toxic form. Second, ferritin serves as a cellular iron storage unit. In normal human sera, ferritin circulates at a relatively low concentration of 1μM therefore ferritin serves as an intracellular iron source to the host (1).

B. Transferrin

Transferrin is a single-chain glycoprotein with the molecular weight of about 80 kDa and has two structurally similar but functionally distinct lobes. Each lobe consists of two dissimilar domains enclosing a deep hydrophilic cleft bearing an iron binding site. Normally, all non-heme iron in circulation is bound to transferrin in human sera and only 30% of transferrin binding sites are occupied with iron. Transferrin has one of the highest metal binding affinities recorded, with a binding constant for ferric iron of 10^{-23} M (160) and can retain iron at a pH as low as 5 (185). The principal physiological role of serum

transferrin is to transport iron through the circulatory system and release the iron to iron-depleted cells in a receptor mediated endocytosis-dependent manner (4).

C. Lactoferrin

Similar to transferrin, lactoferrin is an 80 kDa glycosylated protein that is comprised of a polypeptide chain folded into two symmetrical lobes. Lactoferrin is secreted by mucosal epithelial cells and found in mucosal secretions including tears, saliva, vaginal fluids, semen, nasal, bronchial secretions, bile, gastrointestinal fluids, milk and urine (85). Lactoferrin has a binding constant for ferric iron of about 10^{-20} M and can retain iron at a pH as low as 3 (14). Lactoferrin is a multifunctional protein that is involved in the absorption of iron, but also in immune responses (168, 169), antimicrobial activities (310) and has both anticarcinogenic (21) and anti-inflammatory properties (61).

D. Heme

Heme is a porphyrin containing an iron atom in its center. Heme is produced in virtually all mammalian tissues. Its synthesis is most pronounced in the bone marrow and liver because of the requirements for incorporation into hemoglobin and cellular cytochromes (218, 271). Isolate heme is very toxic is toxic to cells but required for hemoproteins such as hemoglobin and hemopexin (271).

E. Hemoglobin

Hemoglobin, found in red blood cells, transports O_2 in the circulatory system and facilitates reactive oxygen and nitrogen species detoxification. It is tetrameric in structure and each subunit contains a heme group (217).

F. Haptoglobin

Haptoglobin is a plasma hemoglobin scavenger that binds to hemoglobin with a binding affinity of 10^{-12} M (148). Release of hemoglobin into plasma is the result of senescent erythrocytes being degraded. In plasma, stable hemoglobin-haptoglobin

complexes are formed and are delivered to the reticulo-endothelial system by receptor-mediated endocytosis (148).

G. Siderophores

Free iron concentrations in the human host are reduced to about 10^{-24}M in human serum due to host iron binding proteins such as transferrin, and hemoglobin (240). To overcome the lack of free iron availability in the human host, commensal and pathogenic bacteria produce siderophores. Siderophores are low molecular weight iron chelators that can strip iron from host iron binding proteins. They are also produced and secreted by yeast, fungi and many plants and have dissociation constants in the range of 10^{-20}M (57). Most siderophores are biosynthetically produced by large multienzyme synthases that resemble the eukaryotic fatty acid synthases (15, 69). They often have a peptide backbone, with modified amino acid side chains creating the iron-coordinating ligands. Siderophores are classified by the iron coordinating ligands into three groups including the catecholate, hydroxamate and hydroxycarboxylate groups (130). Once released into the environment or human host, the siderophore will sequester iron from iron binding proteins such as transferrin or will chelate free iron in the environment. The ferrated siderophore will then return to the bacteria and deliver the sequestered iron.

H. Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) also known as lcn2 or siderocalin is constitutively expressed in myelocytes and stored in specific granules of neutrophils as well as expressed in epithelial cells during inflammation. NGAL which is part of the lipocalin family, has a tertiary structure determined by highly conserved segments of the individual lipocalin proteins, termed the lipocalin folds. These folds form

eight anti-parallel β-sheets that surround a hydrophobic pocket. Lipocalins in general act as transport or carrier proteins (90) by binding substrates to the hydrophobic pocket. NGAL functions as a siderophore binding protein. Therefore, NGAL is like a counterstrike of the host against the bacteria scavenging for iron. Interestingly, certain bacteria are able to glucosylate the catecholate siderophore enterobactin to form salmochelin. Salmochelin, is not recognized by NGAL and can continue to strip host iron binding proteins of their iron (88).

I. 2,5 DHBA

It has been recently determined that mammalian cells may secrete a siderophore-like molecule. When distinguishing if NGAL binds to human derived iron coordinating molecules, 2,5 Dihydroxybenozic acid (DHBA) was identified. 2,5-DHBA is related to 2,3-DHBA which is the iron-binding moiety of the bacterial siderophore enterobactin (75). A mammalian homolog to *entA* was identified. EntA plays a critical role in synthesis of the bacterial siderophore enterobactin but was identified in murine cells to play a role in the synthesis of the siderophore with the 2,5 DHBA moiety (75). When this *entA* homolog was knocked down with RNA interference, there was a deregulation of cellular iron homeostasis within the murine cells. The composition and structure of the intact mammalian siderophore remain unknown (75).

VII. Iron Acquisition Systems of *N. gonorrhoeae*

Because *N. gonorrhoeae* is an obligate human pathogen it must rely on human iron sources for its nutritional needs. Unlike most bacteria, *N. gonorrhoeae* do not produce siderophores (304). Rather, gonococci employ 1- and 2- component systems to

acquire iron from host iron binding proteins. Gonococci acquire iron from multiple host
iron binding proteins (Figure 1).

Figure 1. Iron Acquisition Systems of *N. gonorrhoeae*
The outer membrane receptors for each iron source are depicted as barrels traversing the outer membrane (OM). In the 2-component iron acquisition systems, the associated lipidated proteins are shown tethered to the outer membrane. The periplasmic iron-binding protein (PBP) for each system is depicted as a brown semi-circle. The PBP is responsible for shuttling the iron across the periplasmic space. Transferrin and Lactoferrin acquisition systems utilize the PBP FbpA, the Fet system utilizes the protein FetB and it is hypothesized that FetB2 is associated with TdfF. TonB, ExbB, and ExbD are responsible for supplying energy to the iron acquisition systems and are light blue, attached to or imbedded within the cytoplasmic membrane (CM). The ABC transport system which is responsible for transporting iron across the cytoplasmic membrane is depicted in bright green and traversing the cytoplasmic membrane. The transferrin and lactoferrin transport system utilize FbpBC for transport of iron across the CM. It is hypothesized that the Fet system utilizes an ABC transport system encoded by genes ng2091-2089. There has not been a PBP or ABC transport system identified for hemoglobin transport. An ABC transport system has not been identified for TdfF-dependent intracellular iron acquisition system.
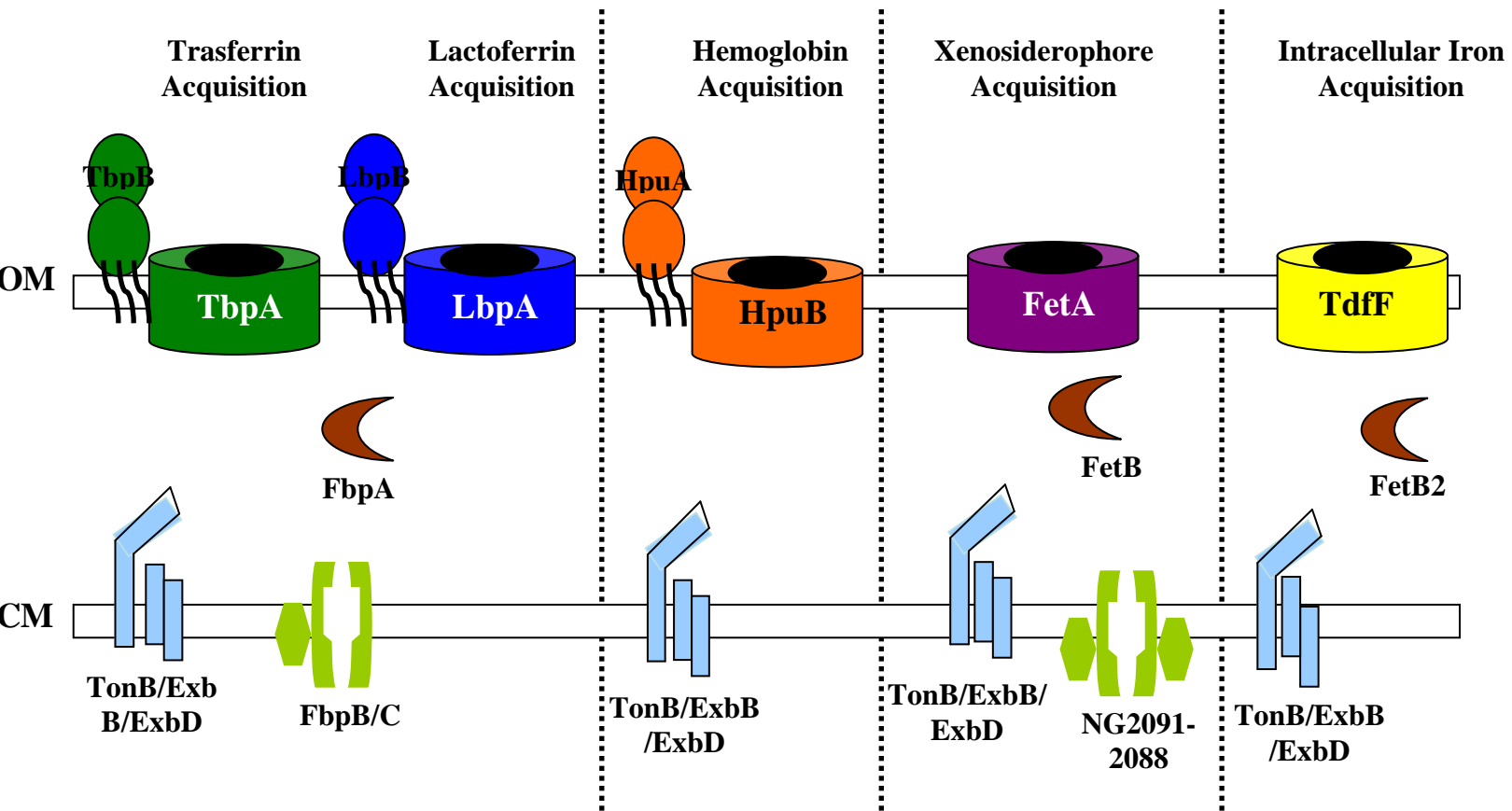
A. Transferrin iron acquisition system

The gonococcal transferrin-iron uptake system is a TonB-dependent system composed of two transferrin binding proteins, TbpA and TbpB (Figure 1). TbpA is an outer membrane barrel made up of 22 transmembrane β-strands (62, 64). It has 11 surface exposed loops and an amino terminal plug domain (62). TbpA is necessary for the internalization of iron from transferrin. TbpB is a surface-tethered lipoprotein that is important for increased efficiency of iron acquisition (6). The precise mechanism of iron removal and transport into the bacteria's periplasm on ferrated transferring has bound to TbpA/TbpB has not been completely elucidated. However, it has been proposed that once iron is extracted from transferrin it interacts with the plug domain of TbpA (207, 312) and once the plug domain interacts with energized TonB the plug unfolds resulting in the presentation of the iron to the periplasm (111). In the periplasm, FbpA binds to the iron (54) and shuttles it to the ABC transport system which is composed of FbpB and FbpC (Figure 1). The ABC transport system transports the iron across the bacterial cytoplasmic membrane.

B. Lactoferrin iron acquisition system

Similar to the transferrin acquisition system, the lactoferrin iron acquisition system is made up of two components and is TonB-dependent (22, 25). LbpA is similar to TbpA in that it is a TonB-dependent outer membrane transporter; LbpB is an outer membrane lipoprotein. Interestingly about 50% of gonococcal isolates have lost the ability to express the Lbps due to a deletion removing the *lbpB* gene and a portion of the *lbpA* gene (5). Also, *lbpB*, when present, is subject to phase variation due to a poly-cytosine tract within the gene's coding region. Details of how the Lbps bind lactoferrin, extract iron,

and are energized by TonB have not been elucidated. However, it has been determined that the Fbp system used by the Tbp system also transports the iron extracted by the Lbp system across the periplasm and into the cytoplasm (54) (Figure 1).

C. Hemoglobin iron acquisition system

Gonococci are capable of utilizing both free heme and heme bound to hemoglobin as an iron source (52, 53). There has not been a specific receptor identified for heme acquisition but hemoglobin acquisition is employed by a TonB-dependent 2-component system consisting of HpuA and HpuB (Figure 1). HpuB is the outer membrane β-barrel whereas HpuA is the associated lipoprotein. Both proteins must be present for iron acquisition from hemoglobin to occur. It is hypothesized that the HpuA/B proteins form a heteromultimer in order to constitute the obligate hemoglobin binding pocket (52). Similar to Lbp, the HpuA/B system is phase variable due to a poly-guanosine tract within the *hpuA* gene (52). Interestingly, HpuA and HupB do not exclusively interact with human hemoglobin and can recognize hemoglobin from non-human sources. The subsequent transport of iron from the HpuA/B system is presumed to involve a periplasmic binding protein and ABC transport system; however neither has been defined for this iron acquisition system (Figure1) .

D. Siderophore iron acquisition

As stated previously *N. gonorrhoeae* does not produce siderophores. However the bacteria can utilize iron from siderophores produced by other bacteria, or xenosiderophores. *N. gonorrhoeae* can acquire iron from the catecholate xenosiderophores aerobactin, enterobactin, salmochelin, and dihydroxybenzoylserine (43, 273, 303). The *fetA* gene formerly named *frpB* (20) encodes the TonB-dependent ferric

enterobactin transporter, FetA. It was demonstrated that FetA is an important component for the binding and acquisition of the xenosiderophore ferric enterobactin (43), which is a catecholate-type siderophore produced by enterics. Distinct from the Lbps, Tbps, and Hpu, FetA is a single component transporter (Figure 1). Downstream of *fetA* are genes that encode a putative periplasmic binding protein, *fetB*, and ABC transport system, which is thought to be involved in the transport of iron from FetA into the gonococcal cytoplasm (Figure 1). Similar to the majority of surface exposed proteins, *fetA* is phase variable due to a poly-cytosine track in the promoter of the gene (44). Our laboratory has also determined that gonococcal strain FA19 acquires enterobactin, salmochelin, and dihydroxybenzoylserine in a TonB-independent manner (273). Strain FA19 requires the FbpABC transport system for utilization of these xenosiderophores (273) rather than the Fet system. These findings indicate that there are Ton-dependent and independent pathways for the utilization of iron from xenosiderophores.

E. Intracellular iron acquisition

Another single component transporter, the TonB-dependent transporter, TdfF has been identified to be important for intracellular survival in gonococcal strain FA1090 (113). The *tdfF* mutant was defective in intracellular survival; however, excess iron overcame this survival defect indicating that TdfF is important for intracellular iron acquisition (113). Interestingly, *tdfF* expression only occurs in the presence of epithelial cells or cell culture media and not in the presence of bacterial growth media. This finding indicates that a cell or serum specific molecule is involved in inducing *tdfF* expression (113). Additionally, *tdfF* has only been identified in pathogenic *Neisseria* and not in commensals. Upstream of *tdfF* is a gene that codes for a putative periplasmic binding

protein that has homology to the periplasmic binding protein FetB and is annotated as FetB2. It is hypothesized that FetB2 is part of the intracellular iron acquisition system; however, the rest of the transport system remains unidentified (Figure 1). The ligand involved in TdfF-dependent iron acquisition has not been identified.

It has also been determined that TonB-dependent intracellular iron acquisition is strain specific. Gonococcal strains FA19 and MS11 do not depend upon the expression of TonB or TdfF for survival (317). Both of these strains differ from gonococcal strain FA1090 in that they both posses a gonococcal genetic island (178) which encodes a type IV secretion system (117). It is hypothesized that this GGI is involved in a TonB bypass mechanism (317). *Neisseria meningitidis* can trigger rapid redistribution and degradation of cytosolic ferritin within infected epithelial cells suggesting that *N. meningitidis* can tap into ferritin as an iron source (162). Both gonococci and meningococci can interfere with transferrin uptake by infected epithelial cells (31) implying the microorganisms affect iron transport within the host cell. Ferritin acquisition by *N. gonorrhoeae* has not been investigated.

F. TonB-dependent transport

The presence of an outer membrane in Gram negative bacteria limits access to many nutrients. Any molecules below the threshold size of about 600 kDa can cross the outer membrane by diffusion through porin channels (205). In contrast most nutrients and iron sources are either too big or too scarce to be acquired by diffusion through porins. Thus, high affinity receptors are employed and the energy necessary for the delivery of substrates to the periplasm is derived from a complex of proteins that include TonB, ExbB and ExbD. Almost all iron sources utilized by gonococci are taken up in a

high affinity, receptor-mediated, TonB-dependent manner. The exceptions are ferric citrate, heme, and some xenosiderophores, which are taken in a TonB-independent manner by gonococci (23, 273). TonB interacts with components in both the cytoplasmic and outer membranes whereas ExbB and ExbD are anchored in the cytoplasmic membrane. ExbB and ExbD harness energy from the proton motive force of the cytoplasmic membrane and transfer this energy to TonB (128). Once TonB is energized, it takes on a different conformation compared to when it is in an energy-deficient state. TonB then transfers its energy to the outer membrane transporters. There are currently two models for how TonB transduces energy from the cytoplasmic membrane to the outer membrane. In the shuttle model, TonB which dimerizes, transfers energy from the cytoplasmic membrane to the TonB-dependent transporter in four steps. In step one, the potential energy of the cytoplasmic membrane proton gradient is harnessed by the ExbB/D energy-harvesting complex. In step two, the energy is stored via a conformational change in the associated TonB dimer. In the third step, TonB shuttles through the periplasm, where the stored potential energy is transferred to a ligand bearing TonB-dependent transporter. The energization of the transporter leads to ligand release into the periplasmic space. In step four the de-energized TonB is then returned to the cytoplasmic membrane (225, 226). Another model of TonB-dependent energization is the propeller model. In the propeller model, TonB remains associated with the cytoplasmic membrane at all times. Once energized by the proton motive force through ExbB/D the C-terminal 'propeller' of TonB becomes associated with the plug portion of the outer membrane transport and causes the release of the bound ligand into the periplasm (45).

VIII. Genetic regulation of iron acquisition

Excess iron is toxic due to its ability to catalyze Fenton reactions which leads to the formation of reactive oxygen species. Iron uptake has to be well regulated to maintain the optimum concentration of intracellular iron and prevent intracellular iron overload.

A. Fur regulation

The Fur protein of *E. coli* is a 17 kDa polypeptide (253) which acts as a transcriptional regulator of iron-regulated promoters by virtue of its Fe^{2+}-dependent DNA binding activity (13, 73). Regardless of the presence or absence of Fe^{2+} the Fur protein has been isolated as a dimer (197). Under iron-replete conditions, Fur binds to the iron and acquires a conformation the enables it to bind to a target DNA sequence called a Fur box. When Fur is bound to the Fur box, transcription is inhibited and the genes are repressed. (Figure 2) When iron is scarce, Fur cannot complex with Fe^{2+} or bind to the Fur box, allowing the RNA polymerase to access the iron regulated genes (109, 154) (Figure 2). A Fur homolog has been identified in *N. gonorrhoeae* (19). Using DNA footprinting, it was established, that in *E. coli*, the strongest natural Fur binding site comprises two hexameric GATAAT direct repeats followed by a 6 base pair inverted repeat, ATTATC (82). The gonococcal Fur binding sequence is similar to that of *E. coli* with two hexameric direct repeats consisting of ATAAT followed by ATTAT (139). Early studies of *N. gonorrhoeae* using a missense mutant confirmed that Fur regulated not only known iron acquisition genes but also a broad range of other genes (78, 281). It was recently determined that roughly 10% of the genes in the *N. gonorrhoeae* genome are responsive to iron, with 30% of those open reading frames (ORFs) regulated directly by Fur (139).

Figure 2. Fur Regulation

Fur is a dimeric protein depicted in purple. Under iron-replete conditions, Fur binds to iron (red circles) and acquires a confirmation the enables it to bind to a target DNA sequence called a Fur box, usually located within the promoter of an iron regulated gene. The Fur box sequence, as depicted in the yellow rectangle is comprised of two hexameric GATAAT direct repeats followed by a 6 base pair inverted repeat, ATTATC. When Fur is bound to the Fur box, transcription (green arrow) is inhibited (red "X") and the gene is repressed. When iron is scarce, Fur cannot complex with Fe^{2+} or bind to the Fur box, allowing the RNA polymerase to access the promoter and the gene is transcribed (green arrow).

+Fe

ATAAT ATAAT ATTAT C
Promoter

Fur Regulated Gene

-Fe

ATAAT ATAAT ATTAT C
Promoter
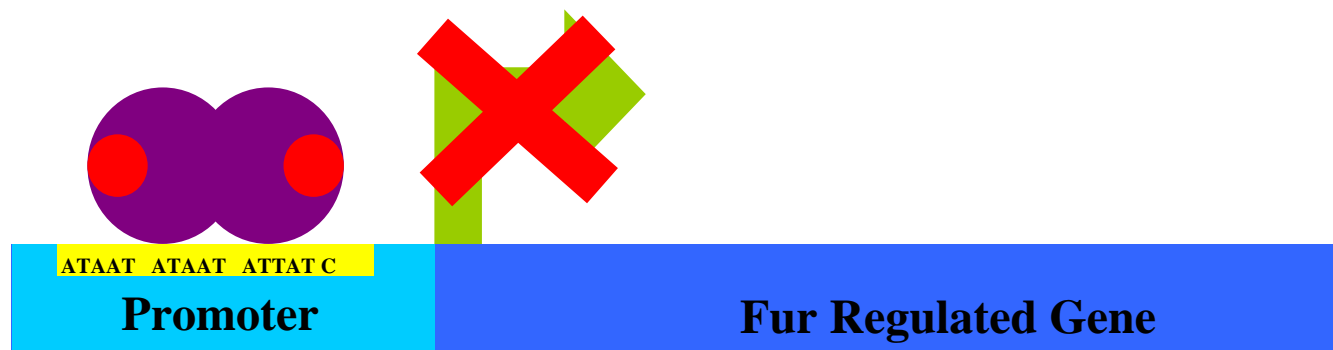
Fur Regulated Gene
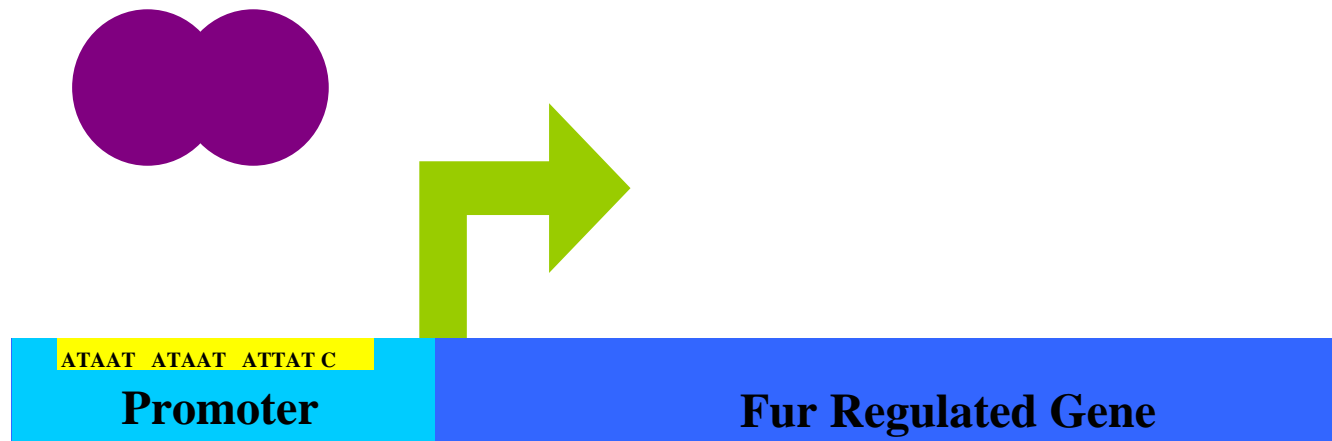
B. AraC-like regulation

The AraC family of transcriptional regulators constitutes one of the largest groups of regulatory proteins in bacteria (311). These regulators are involved in the transcriptional regulation of a variety of cellular processes in Gram-negative and Gram-positive bacteria, including carbon metabolism, stress responses and virulence (97). AraC, the regulator of the L-arabinose operon in *Escherichia coli*, was the first member to be identified, purified, and characterized biochemically (108, 258-260). In general, these regulators are between 200 and 300 amino acids in length arranged in two domains: a conserved helix-turn-helix DNA-binding domain at the C-terminus and variable N-terminal domain. In many cases the variable N-terminal domain is responsible for both protein dimerization and ligand binding. In the context of iron acquisition, AraC-like transcriptional regulators have been employed by a variety of bacteria including *Pseudomonas aeruginosa*, *Bordetella* species, and *Yersenia pestis* (16, 87, 127). In all three of these organisms the AraC-like transcriptional regulator is involved in the regulation of siderophore biosynthesis and acquisition.

The mechanism of AraC-like regulation of siderophore genes involves the cognate siderophore functioning as a co-inducer binding to the N-terminal domain of the AraC-like regulator. Generally, both the AraC-like regulator, and the siderophore biosynthesis/acquisition genes are repressed by Fur under iron-replete conditions (Figure 3). Once the Fur repression is relieved under iron-deplete conditions, the AraC-like regulator is expressed, as well as very low levels of siderophore biosynthesis/acquisition genes (Figure 3). Once the AraC-like regulator binds to its inducing agent, usually the

siderophore, it will then activate the transcription of the siderophore/biosynthesis genes (Figure 3). The gonococcal genome does encode AraC-like regulators and it has been recently demonstrated that one of these regulators, MpeR, (92) is Fur regulated (139).

Figure 3. AraC-like Regulation

The AraC-like regulator and siderophore biosynthesis/acquisition genes are repressed by Fur (purple) under iron-replete conditions. Once Fur repression is relieved under iron-deplete conditions, the AraC-like regulator gene is expressed (large green arrow), as well as very low levels of siderophore biosynthesis/acquisition genes (small green arrow). The AraC-like regulator protein (large orange half moon) then binds to an inducing molecule (blue circles). This inducing agent is usually a siderophore. After the AraC-like protein binds to its specific inducer, it positively regulates the transcription of the siderophore/biosynthesis genes (large green arrows).
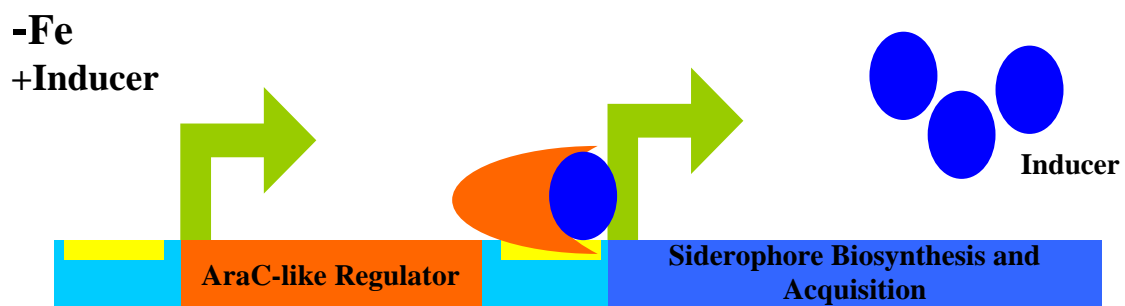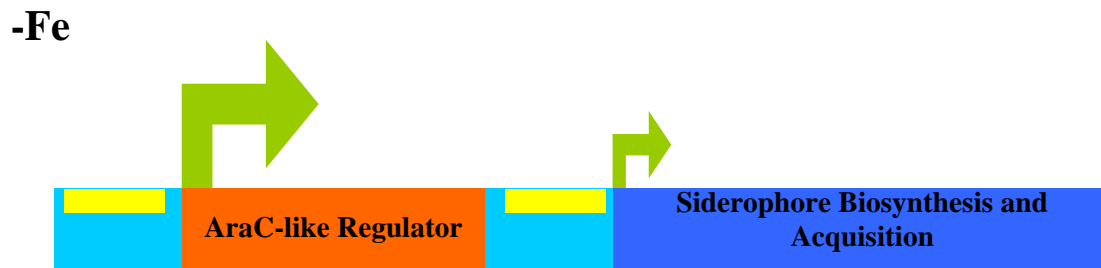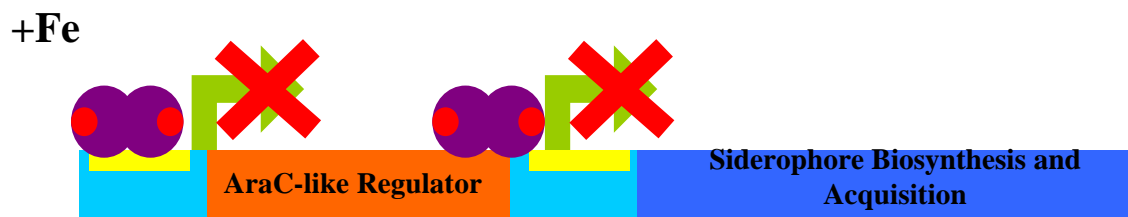
C. Slipped-strand mispairing

Slipped-strand mispairing occurs during DNA replication, when DNA polymerase 'slips' on a string of repeated nucleotides in the template DNA, either adding or deleting one repeat unit in one of the daughter strands (171). The string of repeated nucleotides can consist of a simple homopolymeric tracts or a single nucleotide (291). Slipped-strand mispairing can result in phase variation due to repeated nucleotides located within the open reading frame of a gene, which causes a frameshift mutation. This kind of phase variation due to slipped strand mispairing occurs in gonococcal genes that encode enzymes involved in LOS synthesis, as well as Opas, and HpuB (17, 52, 204). If the repeated nucleotides exist in the promoter region of a gene, slipped strand mispairing can result in differences in promoter-strength during transcription. Phase variation due to changes in promoter strength occurs in gonococcal gene *fetA* which encodes the xenosiderophore receptor, FetA (44).

IX. Objectives

The goal of the research described in this thesis was to characterize TonB-dependent transporters involved in intracellular and extracellular iron acquisition and the transcriptional regulation involved in the expression of these transport systems. This was accomplished through three major objectives. The first involved the analysis of TdfF expression in the presence of host derived inducing signals *in vitro* which could lead to potential ligands for TdfF-specific iron acquisition. The second objective was to further our understanding of potential intracellular iron sources acquired by *Neisseria*

gonorrhoeae in a TonB-dependent manner. The third objective was to elucidate the iron acquisition system responsible for xenosiderophore uptake and the regulation behind this acquisition system. In this objective we also further characterized specific xenosiderophores utilized by gonococci. These studies have not only elucidated the mechanism of two different TonB-dependent iron acquisition systems but also identified genetic differences between gonococcal strains responsible for variations in iron acquisition. Understanding the mechanism of intracellular and extracellular iron acquisition and the regulation behind these systems can lead to a better understanding of the pathogenesis by the microorganism.

Chapter 2 Material and Methods

I. Bacterial growth conditions

Gonococcal strains (Table 2) were maintained on GC medium base (GCB; Difco) containing Kellogg's supplement 1 (151) and 12 µM ferric nitrate. For mutant selection, strains were grown on media supplemented with 50µg/ml kanamycin or 1 µg/ml erythromycin. For iron-depleted growth conditions, gonococci were grown in a defined medium that was treated with Chelex 100 (BioRad) to render it iron free (CDM) (304). For iron-replete conditions, gonococcal strains were grown in CDM for one mass doubling before the addition 12 µM ferric nitrate. For growth in the presence of siderophores, gonococci were grown in liquid CDM for one mass doubling, and then ferric siderophores were added at a concentration of 10 µM. All liquid cultures were grown at 37°C with 5% CO_2. For large scale preparation of gonococcal membrane proteins, GCB broth containing Kellogg's supplement 1 was inoculated with plate-grown, non-piliated gonococci as described previously (64). For iron-deplete conditions, the iron chelator Desferal (deferroxamine mesylate; Sigma Aldrich) was added to a final concentration of 50 µM; for iron-replete conditions, 12 µM ferric nitrate was added. *E. coli* strains were maintained on Luria Bertani agar plates and grown in Luria Bertani media containing 200 µg/ml of carbenicllin at 37°C shaking at 225 rpm.

Table 2. Strains and Plasmids used in this study

Strains and plasmids	Genotype [a] and/or relevant characteristics	Reference or Source
N. gonorrhoeae		
FA1090	Wild type (Δ*lbpA*, HpuAB off)	(28)
MS11	Wild type strain	(196)
FA19	Wild type strain	(198)
UU108	Wild type strain	
FA6959	FA1090 *fetA*::Ω (Str^r Spc^r)	(43)
FA7241	FA1090 *ng*2090:: Ω (Str^r Spc^r)	Sparling Lab (unpublished)
FA7029	FA1090 *ng*2088 :: Ω (Str^r Spc^r)	Sparling Lab (unpublished)
MCV656	FA1090 *tonB*::Ω (Str^r Spc^r)	(113)
MCV659	FA1090 tdfF::Ω (Str^r Spc^r)	(113)
MCV304	FA1090 *mpeR*::*aphA-3* (Str^r Spc^r Km^r)	This study
MCV305	FA1090 *mpeR*^{C}(Str^r Spc^r Km^r Erm^r)	This study
MCV306	*fetA*::Ω, *mpeR*::*aphA-3* (Str^r Spc^r km^r)	This study
E. coli		
BL21 (DE3)	F- *ompT hsdS$_B$* (r$_B$-m$_B$-) *gal dcm* (DE3)	Novagen
C41 (DE3)	F- *ompT hsdS$_B$* (r$_B$-m$_B$-) *gal dcm* (DE3) derivative	Avidis
Top10F'	F´{*lac*Iq, Tn*10*(TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	InVitrogen
Rosetta	F$^-$ *ompT hsdS$_B$*(r$_B^-$ m$_B^-$) *gal dcm* (DE3) pLysSRARE2 (Cam^R)	Novagen
Plasmids		
pCR2.1 TOPO	Kan^R Amp^R	InVitrogen
peT22b(+)	Amp^R	Novagen
pVCU353	PCR 2.1 TOPO containing *tdfF* full length amplified with oVCU365 and oVCU367	This study

pVCU358	PCR 2.1 TOPO containing *tdfF* plug amplified with oVCU412 and oVCU413	This study
pVCU359	PCR 2.1 TOPO containing *tdfF* loops 1-5 amplified with oVCU414 and oVCU415	This study
pVCU364	PCR 2.1 TOPO containing ng2090 amplified with oVCU 629 and oVCU630	This study
pVCU354	pET22b(+) containing full length *tdfF*	This study
pVCU355	pET22b(+)containing *tdfF* plug	This study
pVCU357	pET22b(+)containing *tdfF* loops 1-5	This study
pGCC3-mpeR	pGCC3 containing the full-length *mpeR* gene and 250 bp of upstream sequence	This study
pVCU366	pCR 2.1 containing 800bp of *fetA* through *fetB* amplified by oVCU498 and oVCU499	This study

II. Cell culture Media and incubation conditions

The ME180 endocervical epithelial cell line (HTB 33; American Type Culture Collection) was maintained in McCoy's 5A medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco). Cells were maintained at 37°C in a 5% CO_2 atmosphere. Infection media was McCoy's 5A supplemented with 10% FBS and 24μM $Fe(NO_3)_3$ and replication media consisted of McCoy's 5A supplemented with 10% FBS and no additional iron. For detection of *tdfF*, gonococci were incubated in the following media: McCoy's 5A with 10% FBS (replication media), replication media supplemented with 50μM epinephrine (Sigma), replication media supplemented with 50μM norepinephrineenephrine (Sigma), replication media supplemented with 50μM human liver ferritin (Calbiochem), or McCoy's 5A media alone for 4 hours before RNA isolation.

III. Construction of gonococcal mutants

All strains and plasmids used in this study are listed in Table 2. Gonococcal strains FA1090, MS11, FA19 and UU1008 have been described previously (28, 196, 198). To create gonococcal strain MCV304, the *mpeR* gene was inactivated by the insertion of a kanamycin resistance cassette (*aphA-3*) as previously described (92). All oligonucleotide primers used in this study are described in Table 3. Primers 5'mpeR and 3'mpeR were used to PCR amplify chromosomal DNA from FA19 *mpeR::aphA-3* strain (92) . The amplicon was then used to transform gonococcal strain FA1090. To select for allelic exchange, FA1090 transformants were plated on GCB agar containing kanamycin. Strain FA6959 was previously constructed (43) by insertional inactivation of the FA1090 *fetA* gene with a polar Ω cassette (228). For the construction of the complemented strain,

mpeR^C (MCV305), the *mpeR* coding sequence from gonococcal strain FA19 was

amplified using primers 5' pMpeR pac and 3' GC4 MpeR (Table 3). The resulting

amplicon contains the *mpeR* gene and 250 bp upstream of the *mpeR* start codon. The

amplicon was inserted between the PacI and PmeI sites of pGCC3 (265). The resulting

plasmid, pGCC3-*mpeR*, was then digested with ClaI and the fragment containing *mpeR*,

lctP, *aspC*, and the erythromycin resistance cassette was purified and used to transform

MCV304. Transformants were selected on GCB agar supplemented with erythromycin.

The resulting complemented strain, MCV305, contains the original *mpeR* mutation and

an ectopically-inserted copy of the wild-type *mpeR* gene, preceded by 250 base pairs

including upstream regulatory signals. The *fetA*, *mpeR* double mutant strain, MCV306,

was constructed by transforming FA6959 with the *mpeR*::*aphA-3* amplicon as described

in the construction of MCV304. GCB agar supplemented with kanamycin was used for

selection. Strains MCV656 was created by inserting a polar Ω cassette into *tonB* and

strain MCV659 was created by inserting a polar Ω cassette into *tdfF* as previously

described (113).

TABLE 3. Oligonucleotides used in this study.

Oligonucleotide	Amplicon	Sequence (5'-3')
5'mpeR	*mpeR*::*aphA-3*	ATGAACACCGCCGCCATCT
3'mpeR	*mpeR*::*aphA-3*	GCACTTTTTCACATCCGAAGG
5'pMpeR pac	*mpeR*^{C} construct	GGTTAATTAACGAAACAACCTGCAGAAACC
3' GC4 MpeR	*mpeR*^{C} construct	GGTTTAAACTCAGCACTTTTTCACATCCGA
malEmpeRF	*mpeR* coding region	CACTGGGGATCCATGAATACCGCCGCCATCT
malEmpeRR	*mpeR* coding region	CACTGGCTGCAGTCAGCACTTTTTCACATCCGA
5'fetAup	500bp upstream *fetA* probe	GCCCGAACGGTTCGGACAAATT
3'fetAup	500bp upstream *fetA* probe	TTGTTCGTCCTTTTGAGTGT
3'fetAupint	*fetA*1 upstream probe	TCACTTGGTGCTTCAGCACC
5'fetAupint	*fetA*2 upstream probe	GGTGCTGAAGCACCAAGTGA
rnpB1F	*rnpB*	CGGGACGGGCAGACAGTCGC
rnpB1R	*rnpB*	GGACAGGCGGTAAGCCGGGTTC
oVCU 332	*tdfF* RT-PCR Rev	CGCCGTATTTGTTGCTGGCGTATTTGTAGC
oVCU333	*tdfF* RT-PCR Fwd	TTTGGAATACGACATCGCACCGCAAACC
oVCU365	full length *NdeI -tdfF* Fwd	CATATGACACGCTTCAAATACTCCCTGCTT
oVCU374	full length *tdfF-xho1* Rev	CTCGAGTTTAAACCGATAGGTAACGC
oVCU412	*NdeI-tdfF* plug domain Fwd	CATATGCCGACCATCACCGTTACCGCC
oVCU413	*tdfF-xho1* plug domain Rev	CTCGAGCTTGCGGGTCGGGTGTTTGCG
oVCU414	*tdfF-NdeI* loops 1-5 Fwd	CATATGCCATTGTTTGAAGTCCGCGCC
oVCU415	*tdfF-xho1* loops 1-5 Rev	CTCGAGGAGTATCAGCGAAACGTTGTC
oVCU484	*mpeR* RT-PCR Fwd	GCGTTTCCCACCGAAATCCACAAT
oVCU485	*mpeR* RT-PCR Rev	AGCGTAATAATCGGGCGGAGAGTT
oVCU486	*fetA* RT-PCR Fwd	AAAGATTACGAAGCCGGCAAAGGC
oVCU487	*fetA* RT-PCR Rev	TTCAACAGGGTTTGTTCGGCAAGG
oVCU498	*fetA-fetB* Fwd	CCAACGCTGCACCAATACCCTGC
oVCU499	*fetA-fetB* Rev	CCTTCAGCTTGTCGGCTTCCGCCTG
oVCU500	*fetB*-NG2091 Fwd	CAGGCGGAAGCCGACAAGCTGAACG
oVCU501	*fetB*-NG2091 Rev	GCACAGGCTGACGGCAAACAATACC
oVCU513	NG2091-NG2090 Fwd	GGTATTGTTTGCCGTCAGCCTGTCG
oVCU516	NG2091-NG2090 Rev	GCTCGCTCGGGACGGTATTGAATC
oVCU515	NG2090-NG2089 Fwd	GACTTTGTCTTGCACCTGCGCCTG
oVCU518	NG2090-NG2089 Rev	CAGTTCGCGGACGATGACGAACTG
oVCU517	NG2089-NG2088 Fwd	CTGTGCGGACATTCCTGCCCGAC
oVCU553	NG2089-NG2088 Rev	TGCTTTGGCAGAACACCATCGCAA
oVCU110	*16S* rRNA Fwd	TATCGGAACGTACCGGGTAGC
oVCU111	*16S* rRNA Rev	GTATTACCGCGGCTGCTGGCA
oVCU529	*fetA* qRT-PCR Fwd	ATCCAACACCAATTTGGCGTA
oVCU530	*fetA* qRT-PCR Rev	CATCGGCGGAATAGCGTTT
oVCU461	*mpeR* qRT-PCR Fwd	TCTACCGCCAGTACCAAACC
oVCU462	*mpeR* qRT-PCR Rev	GGCTGAAATTGTGGATTTCG
oVCU457	*rmpM* qRT-PCR Fwd	GGAGCAGGCTCCTCAATATG
oVCU458	*rmpM* qRT-PCR Rev	TAAAGTCGGTATGGCCTTCG
oVCU576	NG2091 qRT-PCR Fwd	GTGAGCGTGAATTTGGGTTT
oVCU577	NG2091 qRT-PCR Rev	TATTGCCGACCGTTACAATG

oVCU629	NG2090 sequencing Fwd	AAACGCTGACCAACAACCCGATTC
oVCU630	NG2090 sequencing Rev	GCTTAATACCGCCTTCATGCCCAA
oVCU676	*fetB* primer extension	CAG GGT CGG AAT TTT GCG GCG AAC AC

IV. Construction of expression plasmids

The full length *tdfF* expression plasmid was constructed by PCR amplification of the *tdfF* gene from gonococcal strain FA1090 genomic DNA. The forward primer oVCU365 (**CATATG**ACACGCTTCAAATACTCCCTGCTT) contained the *NdeI* site (bold) (Table 3) and amplified the *tdfF* native signal sequence. The reverse primer, oVCU357 (**CTCGAG**TTTAAACCGATAGGTAACGC) contained the *XhoI* restriction site (bold) (Table 3) and encoded the terminal phenylalanine of *tdfF* from gonococcal strain FA1090. The resultant PCR product was ligated into the TOPO pCR2.1 vector (InVitrogen) and transformed into competent *E. coli* strain TOP10 (InVitrogen). The transformants containing the correct plasmid, pVCU35,3 was screened for incorporation of the *tdfF* gene by restriction digest followed by agarose gel electrophoresis. After digestion with *NdeI,* and *XhoI*, the *tdfF* gene was purified from the agarose gel (Qiagen) and ligated into the pET-22b(+) expression vector (Novagen). The resultant plasmid, pVCU354, contained the full length *tdfF* gene under control of a T7 promoter, as well as a region encoding a 6X histidine tag immediately 3' of *tdfF*. For expression of recombinant TdfF, the plasmids were transformed into the *E. coli* expression strain BL21(DE3) (Novagen) which expresses T7 under the control of the lac promoter.

The *tdfF* plug expression plasmid, pVCU355, was constructed by PCR amplification of the plug domain and excluded the signal sequence. Forward primer oVCU412 (**CATATG**CCGACCATCACCGTTACCGCC) contained the *NdeI* site (bold) (Table 3) and amplified the FA1090 *tdfF* gene from the sequence that encodes amino acid P_{14} of the mature protein. The reverse primer oVCU413

(**CTCGAG**CTTGCGGGTCGGGTGTTTGCG) contained an *Xho1* site (bold) (Table 3) and amplified the plug domain from the sequence that encodes R_{166} of the mature sequence. The PCR product was ligated into the TOPO pCR2.1 vector (InVitrogen) and transformed into competent *E. coli* strain TOP10 (InVitrogen). The transformants of the resulting plasmid pVCU358 were screened for incorporation of the *tdfF* gene by restriction digest followed by agarose gel electrophoresis. After digestion with *NdeI* and *XhoI*, the plug encoding portion of the gene was purified from the agarose gel (Qiagen) and ligated into the pET-22b(+) expression vector (Novagen). The resultant plasmid pVCU355, encoded recombinant TdfF plug in which a C-terminal histidine tag was fused to R_{166} of the mature protein.

Similarly, the *tdfF* loops expression plasmid was constructed by amplifying the sequence that encodes the first half of the TdfF loop domain. The forward primer oVCU414 (**CATATG**CCATTGTTTGAAGTCCGCGCC) encodes the *NdeI* site (bold) (Table 3) and amplified the FA1090 *tdfF* gene from the sequence that encodes P_{181} of the mature sequence. The reverse primer oVCU415 (**CTCGAG**CTTGCGGGTCGGGTGTTTGCG) encodes an *Xho1* site (bold) (Table 3) and amplified the sequence that encodes L_{680} of the mature sequence. This PCR product was ligated into pCR2.1 vector (InVitrogen) and resulted in pVCU359. This plasmid was digested with *Xho1* and *NdeI*, separated on an agarose gel by electrophoresis and the *tdfF* loops gene was purified from the agarose gel (Qiagen). The subsequent purified gene product was ligated into the pET22b(+) expression vector (Novagen) and resulted in pVCU357 in which *tdfF* loops were expressed with a 5' histidine tag.

Both pVCU355 and pVCU 357 were transformed into the *E. coli* expression strain C41 (DE3) (Avidis).

V. Recombinant protein expression

For small scale induction of pVCU354, pVCU355, and pVCU357, starter cultures of *E. coli* (5 mls) were grown for approximately 6 hours at 37°C in Luria Bertani broth (LB) containing 1% glucose and 200μg/ml of carbenicillin before being stored overnight at 4°C. 1ml of the starter culture was used to inoculate 5 ml of LB broth containing 1% glucose and 200μg/ml carbenicillin. Cultures were shaken at 225rmp at 37°C until they reached the OD_{600} .04-1.0, about 2 hours. For cultures that were induced, 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce recombinant protein expression. The induced and not induced cultures were grown for 4 hours. Following the 4 hour growth, 1 ml of culture was pelleted and solubilized with 2x laemelli solubilizing buffers for subsequent SDS-PAGE analysis.

For large scale recombinant protein induction from plasmids pVCU354, pVCU355, and pVCU357 starter cultures of *E. coli* (5mls) were grown for approximately 6 hours at 37°C in LB medium containing 1% glucose and 200 μg/ml of carbenicllin and stored at 4°C until used for large-scale growth and induction. Prior to the large scale production, starter cultures were pelleted at 3200 X *g* for 5 minutes, and the supernants were decanted. Fresh LB was added at original volume and the pellets were resuspended. The resuspended starter cultures were inoculated into 1L of LB broth at pH 7.5 containing 1% glucose and 500 μg/ml carbenicillin. The cultures were placed at 37°C with shaking at 225rpm until the OD_{600} reached 0.4-0.6. Cultures were then subjected to centrifugation (4°C for 15 min at 6000 X *g*) to pellet the bacteria. Supernants were

decanted and the pellet was resuspended in 1L of fresh LB containing 1% glucose and 500 µg/ml carbenicillin. 0.5 mM IPTG (isopropyl-B-D-thiogalactopyranoside) was added to induce recombinant protein expression. Cultures were grown overnight at 27°C (about 16hrs). Following inductions, cultures were pelleted at 6,000 X g for 15min and stored at -80°C.

VI. Recombinant protein purification

For rTdfF plug and rTdfF loop domain purification, bacterial cell pellets were thawed on ice and cells were lysed under denaturing conditions with 8 mM urea buffer pH 6.3 (10mM NaH_2PO_4, 10 mM Tris-Cl, 8 mM urea). The solubalized rTdfF plug and rTdfF loops domain extracts were bound with nickel-nitriloacetic affinity resin (Qiagen) for at least 1 hour while shaking at room temperature. Following batch binding, the resin was loaded onto a disposable column and washed with 8mM urea buffer pH 6.3 (10 mM NaH_2PO_4, 10 mM Tris-Cl, 8 mM urea) and eluted 4 times with 8mM urea buffer pH 5.9 and 8 mM urea buffer pH4.5. The elutions were pooled together for each recombinant protein and dialyzed 2 times, overnight at 4°C, in 6 mM urea at pH 4.5 (NaH_2PO_4, 10 mM Tris-Cl, 6 mM).

VII. Coomassie blue protein staining

SDS-PAGE gels were stained with Coomassie blue (0.25% Coomassie R-250, 50% methanol, 10% glacial acetic acid) following electrophoresis. Gel were incubated in Coomassie blue stain overnight at room temperature and then destained in 20% methanol and 5% glacial acetic acid at room temperature until background staining was minimized. Gels were dried in gel drying buffer (40% methanol, 10% glycerol, and 7.5% glacial acetic acid) over night.

VIII. Production of TdfF specific polyclonal rabbit sera

Purified rTdfF plug and rTdfF loops as described above were used to immunize rabbits. Each antigen was used to immunize two separate female New Zealand –SPF rabbits. Two rabbits designated F2522 and F2523 were immunized with rTdfF plug and two rabbits designated F2388 and F2389 were immunized with rTdfF loops domain. The rabbit immunization and bleed schedule was as follows, Day 0 pre-immune sera was collected and rabbits were initially immunized with rTdfF in Freund's complete adjuvant. Day 14 and 28 rabbits received rTdfF boost and on day 35 and day 40 the rabbits were bled. Serum samples were screened by Western blot for specificity against rTdfF plug or rTdfF loops domain. Immunizations and sera collections were done on a fee for serum basis by New England peptide.

IX. Sequence Analysis

To determine the sequence of FA19 and FA1090 *ng*2090, chromosomal DNA from each strain was used in a PCR amplification reaction in which the following primers were used to amplify the full length gene, oVCU629 and oVCU630. The resultant PCR product was ligated into the TOPO pCR2.1 vector (InVitrogen) competent *E. coli* strain TOP10 (InVitrogen) were transformed with this construct resulting in plasmid oVCU364. The resultant plasmid was sequenced through the VCU Nucleic Acid Research Facilities using commercially available primers specific to TOPO pCR2.1.

X. **Gentamicin protection assay**

Epithelial cells were seeded into 12-well plates and grown to 70-80% confluence in McCoy's 5A media supplemented with 10% FBS. Prior to infection, gonococcal strains were maintained on GC agar plates supplemented with Kellogg's Supplement 1 (151) and 12 μM $FeNO_3$ at 37°C in a 5% CO2 atmosphere. Piliated, gonococcal strains were harvested from GC plates and suspended in CDM (304). The infection inocula were prepared by diluting each strain in infection media containing McCoy's 5A with 10% FBS and 24 μM $FeNO_3$. Supplemental iron in the media promotes efficient growth and invasion. A multiplicity of infection (MOI) of 10 was maintained throughout the experiments. Epithelial cell monolayers were infected for 4 hours followed by a phosphate buffer saline (PBS) wash to remove any extracellular, non-adherent bacteria. Infected cells were then incubated in McCoy's 5A and 10% FBS supplemented with 25μg/ml gentamicin for 1 hour. Gonococci are susceptible to gentamicin but the drug cannot be absorbed by the epithelial cells.   After 1 hour incubation, gentamicin was removed and cultures were washed twice with PBS. The time 0 cells were immediately lysed by treatment with saponin mix (PBS, 2 mM EDTA, 0.5% saponin) for 1 minute and then gonococci were diluted and plated for viable counts on GC agar plate.   The 24 hour time point cells were incubated in replication media which consists of McCoy's 5A and 10% FBS. One hour prior to the 24 hour time point, cells were washed with PBS, and treated a second time with gentamicin for 1 hour before being washed and plated as described above. For assays in which the cells were serum starved, after the initial 4 hour incubation and gentamicin treatment, the replication media added back for the subsequent 24 hour incubation contained McCoy's 5A only. When we tested the addition of 40 uM

apo-bovine transferrin, we added the apo-bovine transferrin to a replication media consisting of either McCoy's 5A alone or McCoy's 5A and 10% FBS. When we incubated infected cells in the presence of ferritin degradation inhibitors, we incubated the cells in one of the following replication media: McCoy's 5A and 10% FBS with 200 µM ascorbate (Sigma), McCoy's 5A and 10% FBS with 10µM leupeptin (Sigma), McCoy's 5A and 200 µM ascorbate (Sigma), or McCoy's 5A and 10 µM leupeptin (Sigma). Each assay was conducted independently at least 3 times and data presented are the mean of 3 separate experiments.

XI. Isolation of cell fractions containing membrane proteins

As described previously (64), gonococci were pelleted after large scale growth under iron-replete or iron-deplete conditions. Gonococcal cells were then resuspended in 10 mM HEPES [4-(2-hydroxyethyl)-1-piper azine-ethanesulfonic acid] and passed through a French pressure cell once at 20,000 lb/in^2. Intact gonococcal cells were removed by centrifugation at 8,500 X g and membrane proteins were pelleted by centrifugation at 140,000 X g for 1h. Total membrane protein fractions were resuspended in 10mM HEPES and protein concentrations were determined by bicinchoninic acid assay (Pierce).

XII. Separation of total membrane protein fractions and mass spectrometry analysis

Membrane protein fractions, isolated as described above, were solubilized and proteins were separated on a 7.5% polyacrylamide gel. Proteins were visualized by Coomassie blue staining. One band of interest was extracted from the stained gel and submitted to the VCU Mass Spectrometry Resource Center for identification. The sample was digested overnight with trypsin and the resulting peptides were extracted. The

peptides were analyzed on a LC-MS system that consisted of a Thermo Electron Deca XP Plus mass spectrometer with a nanospray ion source interfaced with a reversed-phase capillary column.

XIII. Immunodetection of FetA, TbpA and TdfF

For detection of FetA in total membrane protein preparations, aliquots containing 20μg of protein were resolved using SDS-PAGE. For detection of FetA in whole cell lysates, gonococcal strains were grown in the presence of iron-containing catechols for 6 hours; every 2 hours, aliquots were removed and standardized to culture density. Cells were pelleted and lysed with Laemmli solubilizing buffer (159) and stored at –20°C. Before use, 5% β-mercaptoethanol was added to all preparations prior to heating at 95°C for 3 minutes. After SDS-PAGE, proteins were electroblotted to nitrocellulose membranes in 20 mM Tris base, 150 mM glycine, and 20% methanol (284) within a submerged transfer apparatus (BioRad). For detection of FetA, membranes were blocked with 5% skim milk in low-salt TBS (LS-TBS). FetA blots were then probed with a FetA-specific monoclonal antibody (44) and washed with LS-TBS, followed by a secondary goat anti-mouse antibody conjugated to alkaline phosphatase (BioRad). Blots were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

For detection of TbpA in whole cell lysates, proteins were separated by SDS-PAGE as described above, followed by electroblotting to nitrocellulose. Membranes were blocked with 5% bovine serum albumin (Roche) in high-salt Tris-buffered saline (TBS) plus 0.05% Tween 20 (Sigma). TbpA blots were then probed with a polyclonal, TbpA-specific antiserum (67) and washed with high-salt TBS plus 0.05% Tween, followed by a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (BioRad). Blots

were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

To evaluate TdfF expression during infection, ME180 cells were inoculated with gonococcal strain FA1090 at an MOI 20. After 4 hours, supernants were collected and pooled. Monolayers containing ME180 cells and gonococci were washed with PBS twice and treated with Saponin (PBS, 2 mM EDTA, 0.5% saponin); lysates were pooled and vortexted. Bacteria from the supernatant or cell-associated populations were pelleted by centrifugation at 5,000 x g for 10min at 4°C. The pellets were solubalized with Laemmli solubilizing buffer (159) and stored at –20°C. Before use, 5% β-mercaptoethanol was added to all preparations prior to heating at 95°C for 3 minutes.

For gonococci incubated in different cell culture conditions, non-pilliated bacteria were resuspended to 100 klett unit in 1XCDM and added to the following media conditions: McCoy's 5A with 10% FBS (replication media), replication media supplemented with 50μM epinephrine (Sigma), replication media supplemented with 50 μM norepinephrineenephrine (Sigma), replication media supplemented with 50 μM human liver ferritin (Calbiochem), or McCoy's 5A media alone for 4 hours before pelleted by centrifugation at 50000 X g. The pelleted bacteria were lysed with Laemmli solubilizing buffer (159) and stored at –20°C. Before use, 5% β-mercaptoethanol was added to all preparations prior to heating at 95°C for 3 minutes. For detection of TdfF in whole cell lysates, proteins were separated by SDS-PAGE as described above, followed by electroblotting on nitrocellulose. Membranes were blocked with 5% bovine serum albumin (Roche) in high-salt Tris-buffered saline (TBS) plus 0.05% Tween 20 (Sigma). TdfF blots were then probed with either polyclonal, TdfF plug or TdfF loop domain

specific antiserum (this study) and washed with high-salt TBS plus 0.05% Tween, followed by a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (BioRad). Blots were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

XIV. RNA isolation

 For gonococcal strains grown in CDM under iron-deplete and iron-replete conditions as described above in bacterial growth conditions, after one mass doubling, 12 μM $FeNO_3$ (iron replete), or no additional iron (iron-deplete) was added and the cultures were grown for an additional 2 h.

For gonococcal strains incubated in different cell culture media for *tdfF* detection, gonococci were incubated in the following media conditions: McCoy's 5A with 10% FBS (replication media), replication media supplemented with 50 μM epinephrine (Sigma), replication media supplemented with and 50 μM norepinephrineenephrine (Sigma), replication media supplemented with 50μM human liver ferritin (Calbiochem), or McCoy's 5A media alone for 4 hours before pelleted by centrifugation at 50000 X *g*

Total RNA was isolated from cultures using the RNeasy mini kit as directed by the manufacturer (Qiagen). Purified RNA was treated twice with RNase-free DNase as directed by the manufacturer (Qiagen). SUPERase-In (Ambion) was added before storage at −80°C.

XV. Qualitative reverse transcriptase PCR (RT-PCR)

Portions of *16S rRNA, tdfF*, *fetA, mpeR,* and intergenic regions between the *fet* genes were amplified using the Thermoscript RT-PCR system (InVitrogen) as described previously (113). A portion of this reaction was used as template for PCR amplification with Platinum *Taq* Polymerase (Invitrogen) according to the manufacturer's protocol.

Oligonucleotide sequences of the primers used in this analysis are listed in Table 3. After an initial denaturation step at 94°C for 3 min, DNA was amplified for 30 cycles. Each cycle consisted of 1 min at 94°C, 30 s at 60°C and 1.5 min at 72°C, followed by a final extension step of 10 min at 72°C. To detect any DNA contamination of RNA preparations, parallel RT-PCR reactions were conducted in the absence of reverse transcriptase. Amplicons resulting from the RT-PCR reactions were detected by ethidium bromide staining of agarose gels.

XVI. Real time RT-PCR

cDNA was generated by reverse transcription of 100 ng of total RNA using the Accuscript High Fidelity 1st strand cDNA synthesis Kit (Stratagene) according to the manufacturer's protocol. Synthesized cDNA was used as PCR template. The SensiMix SYBR No-ROX Kit (Bioline) and CFX96 Real Time System (BioRad) was employed for the real time RT-PCR reactions. Oligonucleotide sequences of the primers used in this analysis are listed in Table 2. The polar Ω insertion in *fetA* was located between the binding sites for the primers used to detect *fetA* expression; the *aphA-3* cassette was located downstream of the primer binding sites for *mpeR* expression analysis. The cDNA/SensiMix mixture was initially heated to 94°C for 10 min and subjected to 40 cycles conducted under the following conditions: 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Transcription of *rmpM* was employed as an internal control. For each experimental condition, *fetA*, *mpeR*, and *ng2091* transcripts were normalized to *rmpM* levels. The relative C_T method (179) was employed to compare normalized expression levels under different conditions. Three biological replicates were analyzed, each conducted in triplicate.

XVII. Identification of *fetB* transcriptional start site by primer extension analysis performed in the laboratory of Dr. William Shafer

In order to identify the *fetB* transcriptional start site, RNA was harvested from FA1090 grown under iron-replete or iron-deplete conditions as described above. The reverse primer, oVCU676 (Table 3), was radiolabeled with [γ-^{32}P] using T4 polynucleotide kinase; 5µg of RNA was incubated with the radiolabeled primer and reverse transcriptase to generate the primer extension product. In order to generate reference sequence products, plasmid pVCU366 (Table 2) was sequenced using the reverse primer, oVCU676, and the SequiTherm EXCEL II DNA Sequencing kit (Epicenter) as described previously (245). The sequencing template plasmid, pVCU366, was generated by amplification of wild-type FA1090 genomic DNA with the primers oVCU498 and oVCU499 (Table 3). The resulting amplicon contained the *fetB* promoter region and was cloned into pCR 2.1 (InVitrogen). Both the primer extension product and the reference sequence were subjected to electrophoresis on a 6% acrylamide sequencing gel, which was dried and subjected to autoradiography for visualization.

XVIII. Electrophoretic mobility shift assay (EMSA) performed in the laboratory of Dr. William Shafer

MpeR was fused in-frame at its amino-terminus to the maltose-binding protein (MBP) using the pMal-c2x fusion vector (New England Biolabs). For this purpose, the *mpeR* coding region was PCR-amplified from FA19 chromosomal DNA, isolated as described (186) using primers 5' malEmpeRF and 3' malEmpeRR (Table 3). The resulting amplicon was purified using the QIAquick PCR purification kit (Qiagen). Both the vector and PCR product were digested with PstI and BamHI (New England Biolabs) and ligated using T4 DNA Ligase (New England Biolabs). *E. coli* transformants

harboring the construct were selected on LB agar (Difco) plates containing 100µg/ml of ampicillin. Both strands of the cloned insert were sequenced to ensure fidelity of the PCR amplification reaction and in-frame fusion with *malE*. Growth of the *E. coli* transformant bearing the plasmid construct, induction of expression and purification of MBP-MpeR was performed as described previously for an MBP-MtrR fusion (164).

For the EMSA studies, the 500 bp, intergenic region immediately upstream of the FetA start codon was PCR-amplified from FA1090 chromosomal DNA using primers 5'fetAup and 3'fetAup (Table 2). This upstream region was further divided into two smaller products by PCR amplification. The 5'fetAup and 3'fetAupint primers (Table 3) resulted in *fetA*1. The 5'fetAupint and 3'fetAup primers (Table 3) were used to amplify *fetA*2 region. These three PCR products was purified using the QIAquick PCR purification kit (Qiagen) and end-labeled with [γ- ^{32}P] (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). The radiolabeled PCR products were purified by excising the DNA from nondenaturing polyacrylamide gels, and recovered by crush-soak elution overnight at 37°C into 750 µl of PB buffer from the QIAquick PCR purification kit (Qiagen). The radiolabeled DNA/buffer mixture was centrifuged at 15,800 X g for 10min and the resulting supernatant was removed and added to a QIAquick PCR purification kit (Qiagen) column, which was then washed with 750 µl PE buffer. The DNA was eluted in 100 µl of nuclease-free water (Ambion). Five nanograms of each radiolabeled probe was incubated with 10 µg of MBP-MpeR for 30 minutes at room temperature.

The specificity of the MpeR-*fetA* promoter interaction was evaluated by adding either specific or non-specific, unlabeled competitor DNA to the binding reactions. The specific, unlabeled competitor DNA was generated by PCR amplification of the *fetA*1 promoter region as described above. The *fetA*2 sequence was also amplified as described above and used as an unlabeled competitor. The non-specific competitor DNA was generated by PCR amplification of a portion of the *rnpB* gene using the primers rnpB1F and rnpB1R (Table 3). All binding reactions were incubated in DNA binding buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 1 µg/ml Poly dIdC) for 30 min. at room temperature. All samples were subjected to electrophoresis on a 5% (wt/vol) polyacrylamide gel at 4°C. After electrophoresis, the gel was dried onto Whatman filter paper and exposed to X-ray film for autoradiography.

XIX. Preparation of ferric-siderophores

The siderophores used in this study were purchased from EMC Microcollections (Tübingen, Germany). Siderophores were resuspended to a final concentration of 1mg/ml in sterile deionized water and ferrated to 80% saturation using $FeCl_3$ (273). In some experiments, the siderophores were purchased pre-ferrated and the lyophilized ferric-siderophores were dissolved in methanol (enterobactin) or water prior to final dilution in water. The results of the siderophore utilization assays were the same regardless of whether the siderophores were purchased in the ferrated state or ferrated immediately before use. The following is a list of the siderophores tested for growth support of gonococcal strain FA1090: ornibactin, aerobactin, ferrichrysin, ferrirubin, coprogen, neocoprogen, enterobactin, dihydroxybenzoylserine (DHBS) monomer, DHBS dimer, DHBS trimer, salmochelin S4, and salmochelin S2. Both 2,3-DHBA (Sigma) and

2,5-DHBA (Sigma) were resuspended to a final concentration of 1mg/ml in sterile deionized water and ferrated to 80% saturation using $FeCl_3$(273).

XX. Xenosiderophore utilization assays

Plate bioassays to evaluate xenosiderophore utilization were performed using CDM plates supplemented with 2.5 µM apo-bovine transferrin to chelate excess iron. As described previously (273) strains were inoculated onto plates using a sterile Dacron swab (Puritan). A sterile pipette was used to bore a well into the agar. Subsequently, 10µl of the diluted ferric-siderophore solution (100 µg/ml) was added to each well. Ten microliters of apo-bovine transferrin at a concentration of 10mg/ml was used as a negative control and 10µl of ferric citrate (10µM) was used as a positive control. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours and then evaluated for ferric-siderophore dependent growth.

XXI. Statistical analyses

Statistical significance of xenosiderophore-dependent growth data was determined by using a two-tailed, unpaired Student's *t* test. *P* values for specific comparisons are reported in the figure legend. For real time RT-PCR data (Table 4), the relative C_T method (179) was utilized to calculate the fold change for each comparison. The range for each value is shown in parentheses. The average fold change values were calculated from three independently-conducted RT-PCR reactions and are representative of results generated from three independent RNA preparations (biological replicates). Statistical significance of intracellular survival was assessed by a Student's T-test in which a P value ≤ .01 and ≤.05 were considered significant.

Chapter 3 DETECTION OF THE EXPRESSION OF TONB -DEPENDENT TRANSPORTER, TdfF

I. Introduction

Neisseria gonorrhoeae is a Gram negative diplococcous and the causative agent of the sexually transmitted infection, gonorrhea*.* To establish infection, gonococci need to acquire iron from the human host. *N. gonorrhoeae* employs multiple TonB-dependent acquisition systems for the utilization of various host iron sources. All gonococcal strains express the transferrin iron acquisition system composed of TbpA/TbpB (6, 62, 64) and 50% of gonococcal isolates are capable of acquiring lactoferrin, a major iron source of mucosal secretions, through components of the LbpA/B system (5). Gonococci acquire iron from hemoglobin as well; however, expression of the hemoglobin transporter system HpuA/B is subject to high frequency variation and most clinical and laboratory isolates do not express the receptor (52). All of these iron sources are found outside the host epithelial cell. Iron is necessary for *N. gonorrhoeae* to establish its intracellular localization *in vitro* (113). TonB is also necessary for intracellular localization (113). TonB-dependent iron acquisition systems are necessary for intracellular survival of other microorganisms including *Legionella pneumophila* (102) and *Shigella dysenteriae* (241).

Transport of iron into Gram negative microorganisms is initiated by the passage of iron across the outer membrane and into the periplasmic space. Once in the periplasm, the iron is translocated into the cytoplasm. TonB-dependent transporters are responsible for the transport of iron across the outer membrane. These transporters have a high affinity and specificity for their iron binding ligands. TonB-dependent transport requires energy which is derived from the inner membrane proton motive force. TonB-dependent transporters (TBDTs) interact with an inner membrane complex consisting of TonB/ExbB/ExbD for energization (128). The exact energy transduction pathway from TonB/ExbB/ExbD to the TBDT remains undefined (226). The first crystal structures of two *Escherichia coli* TBDTs, the ferrichrome transporter (FhuA) (86, 180) and the ferric enterobactin transporter (FepA) (39), revealed that these TDBTs consist of 2 structural domains, a 22-stranded β-barrel that spans the outer membrane and a plug domain folded into the barrel interior. The plug domain functions to occlude the barrel pore and to bind ligand at the extracellular side of the membrane as well as interact with the TonB/ExbB/ExbD complex at the periplasmic side of the outer membrane (290).

Since the sequencing of the gonococcal genome, multiple putative TonB-dependent transporters have been identified. Three putative non-contiguous TonB-dependent receptor genes *tdfF*, *tdfG* and *tdfH* were identified in strain FA1090 genome database using BLAST programs comparing amino acid sequences from a panel of characterized TonB-dependent receptors (286). Detailed comparisons of these putative transporters to known TonB-dependent transporters revealed extensive areas of homology within previously described conserved domains of TonB-dependent proteins (64, 145). Such domains include the presence of a signal sequence which is necessary for

the transporter to be exported out of the cytoplasm. Once the protein is exported to the cytoplasm this signal sequence is cleaved. TonB-dependent transporters also contain a terminal phenylalanine residue which is important for outer membrane localization. The N-terminal region of TonB-dependent transporters makes up the plug domain and the C-terminus consists of membrane spanning domains which make up the β-barrel (64, 145, 286).

Previously, our laboratory investigated the role of the TonB system as well as TonB-dependent transporters in survival within cervical epithelial cells. The Ton system is necessary for intracellular survival of gonococci within epithelial cells and has been demonstrated for *N. meningitidis* as well (161). None of the previously characterized TonB-dependent transporters are necessary for intracellular survival; however, TdfF is required for survival (113). The attenuated survival of the *tdfF* mutant is rescued with the addition of iron indicating that *tdfF* is important for intracellular iron acquisition (113). Additionally, *tdfF* expression is only detected when gonococci are incubated in the presence of cervical epithelial cells or cell culture media (McCoy's 5A and 10% heat-inactivated fetal bovine serum (FBS)) and not in bacterial growth media (113). Expression of *tdfF* is also detected during non-complicated infections of the female urogenital tracts (2) implying that *tdfF* expression is host/intracellular niche- specific.

Understanding the regulation and inducing signal for *tdfF* expression could lead to the identification of the iron source utilized by intracellular gonococci. In numerous bacteria, gene expression for TBDTs involved in iron uptake, was regulated by the ferric uptake regulator (Fur) (166). In the presence of iron, Fur binds to a DNA sequence called the Fur box using Fe^{2+} as a cofactor and thereby represses expression of iron regulated

genes (13). When iron is limiting, Fur cannot bind DNA, leading to derepression of genes that encode iron transporters (13). *tdfF* contains a putative Fur box (113). Iron levels may not be the only signal for *tdfF* expression as *tdfF* expression is not detected in bacterial growth media under iron-deplete conditions and is only detected when gonococci are incubated in epithelial cell culture conditions (113). These results indicate that the inducing signal for *tdfF* expression could also be a ligand responsible for a regulatory cascade that signaled that the bacteria were associated with or intracellular to epithelial cells.

In this study we investigated potential proteins and molecules that could up-regulate *tdfF* expression. We detected *tdfF* expression in the presence of heat-inactivated fetal bovine serum (FBS) but did not determine the specific component of serum responsible for induction of *tdfF* expression. We identified *tdfF* in all laboratory isolates of *N. gonorrhoeae* and determined that the gene sequence was identical amongst all gonococcal strains sequenced. Additionally, we expressed and purified rTdfF plug and rTdfF loop domains from *E. coli* successfully. These recombinant proteins were used to synthesize rabbit polyclonal anti-TdfF sera for detection assays.

II. Results

A. TdfF sequence is identical in gonococcal laboratory strains

Since TdfF is crucial for intracellular survival in wild type gonococcal strain FA1090 we wanted to determine if other laboratory strains contained this gene. We were also interested in the nucleotide sequence of this transporter because the gene may be present in all strains but inactivated. For example, not all gonococcal strains express the

genes that encode the TonB-dependent lactoferrin acquisition system LbpA/LbpB due to a deletion removing the *lbpB* gene and a portion of the *lbpA* gene (5). To determine the presence and nucleotide sequence of *tdfF* from multiple strains, we proceeded to sequence the portion of *tdfF* that encodes the plug and half of the β-barrel domain that we will proceed to call loop domain for the rest of this study from a variety of gonococcal strains. Using FA1090-specific *tdfF* primers we amplified the plug and loop domains from strains FA19, MS11, UU108 and compared these sequences to strain FA1090. We aligned the nucleotide sequence from each gonococcal isolate and determined they were identical in all of the gonococcal strains investigated. We also translated the nucleotide sequence and observed identical amino acid sequences (Figure 4). Since this study, other gonococcal and meningococcal strains have been sequenced, and *tdfF* has been identified as pathogen-specific gene (184, 266) indicating it is important in pathogenicity. We have since aligned TdfF from all sequenced gonococcal strains and observed that they shared 100% sequence identity. The identical sequences imply that the substrate acquired by *tdfF* is only expressed in the intracellular environment away from any immune pressures that would select for antigenic diversity.

Figure 4. Sequence alignments of the plug and loop domain of TdfF

Panel A) Amino acid sequence alignment of loops 1-5 from FA1090, FA19, MS11, and UU108.

Panel B) Amino acid sequence alignment of the plug domain of TdfF from FA1090, FA19, MS11, and UU108. The MS11, FA19, and UU108 sequences, determined as part of this study. Gray shading with black font indicates 100% identical sequence. Primer sequence excluded from this analysis.

A

```
        1                                                    50
FA1090  HFGLGADVSGSLNAEGTLRGRLVSTFGRGDSWRQLERSRDAELYGILEYD
FA19    HFGLGADVSGSLNAEGTLRGRLVSTFGRGDSWRQLERSRDAELYGILEYD
MS11    HFGLGADVSGSLNAEGTLRGRLVSTFGRGDSWRQLERSRDAELYGILEYD
UU108   HFGLGADVSGSLNAEGTLRGRLVSTFGRGDSWRQLERSRDAELYGILEYD

        51                                                   100
FA1090  IAPQTRVHAGMDYQQAKETADAPLSYAVYDSQGYATAFGPKDNPATNWSN
FA19    IAPQTRVHAGMDYQQAKETADAPLSYAVYDSQGYATAFGPKDNPATNWSN
MS11    IAPQTRVHAGMDYQQAKETADAPLSYAVYDSQGYATAFGPKDNPATNWSN
UU108   IAPQTRVHAGMDYQQAKETADAPLSYAVYDSQGYATAFGPKDNPATNWSN

        101                                                  150
FA1090  SRNRALNLFAGIEHRFNQDWKLKAEYDYTRSRFRQPYGVAGVLSIDHSTA
FA19    SRNRALNLFAGIEHRFNQDWKLKAEYDYTRSRFRQPYGVAGVLSIDHSTA
MS11    SRNRALNLFAGIEHRFNQDWKLKAEYDYTRSRFRQPYGVAGVLSIDHSTA
UU108   SRNRALNLFAGIEHRFNQDWKLKAEYDYTRSRFRQPYGVAGVLSIDHSTA

        151                                                  200
FA1090  ATDLIPGYWHADPRTHSASMSLTGKYRLFGREHDLIAGINGYKYASNKYG
FA19    ATDLIPGYWHADPRTHSASMSLTGKYRLFGREHDLIAGINGYKYASNKYG
MS11    ATDLIPGYWHADPRTHSASMSLTGKYRLFGREHDLIAGINGYKYASNKYG
UU108   ATDLIPGYWHADPRTHSASMSLTGKYRLFGREHDLIAGINGYKYASNKYG

        201                                          246
FA1090  ERSIIPNAIPNAYEFSRTGAYPQPSSFAQTIPQYDTRRQIGGYLAT
FA19    ERSIIPNAIPNAYEFSRTGAYPQPSSFAQTIPQYDTRRQIGGYLAT
MS11    ERSIIPNAIPNAYEFSRTGAYPQPSSFAQTIPQYDTRRQIGGYLAT
UU108   ERSIIPNAIPNAYEFSRTGAYPQPSSFAQTIPQYDTRRQIGGYLAT
```

B

```
        1                                                    50
FA1090  DGYTVSGTHTPFGLPMTLREIPQSVSVITSQQMRDQNIKTLDRALLQATG
FA19    DGYTVSGTHTPFGLPMTLREIPQSVSVITSQQMRDQNIKTLDRALLQATG
MS11    DGYTVSGTHTPFGLPMTLREIPQSVSVITSQQMRDQNIKTLDRALLQATG
UU108   DGYTVSGTHTPFGLPMTLREIPQSVSVITSQQMRDQNIKTLDRALLQATG

        51                                                   100
FA1090  TSRQIYGSDRAGYNYLFARGSRIANYQINGIPVADALADTGNANTAAYER
FA19    TSRQIYGSDRAGYNYLFARGSRIANYQINGIPVADALADTGNANTAAYER
MS11    TSRQIYGSDRAGYNYLFARGSRIANYQINGIPVADALADTGNANTAAYER
UU108   TSRQIYGSDRAGYNYLFARGSRIANYQINGIPVADALADTGNANTAAYER

        101        116
FA1090  VEVVRGVAGLPDGTGE
FA19    VEVVRGVAGLPDGTGE
MS11    VEVVRGVAGLPDGTGE
UU108   VEVVRGVAGLPDGTGE
```

B. TdfF topology model

Nearly all Gram-negative bacteria have TonB-dependent transporters involved in the uptake of nutrients such as iron, vitamin B$_{12}$, nickel, carbohydrates, and probably other substrates (255). TonB-dependent transporters share a common topology based on twelve different TonB-dependent transporters that have been crystallized to date (206). They all share two characteristic domains: a β-barrel comprised of 22 amphipathic β-strands, and a globular plug domain that is folded up inside the barrel (56, 206). The topology of TdfF was determined with the online protein homology analog recognition engine (PHYRE) (149). We analyzed the TdfF amino acid sequence from gonococcal strain FA1090. FpvA, was selected as the biological template structure for the TdfF topology model by PHYRE. FpvA is the outer membrane transporter required for iron acquisition via the siderophore pyoverdine in *Pseudomonas aeruginosa*. FpvA, like other ferrisiderophore transporters, consisted of a membrane-spanning β-barrel occluded by a plug domain (254). FpvA had 29.15% sequence identity to TdfF. The predicted structure of TdfF was consistent with the structure of characterized TonB-dependent transporters (Figure 5A). Consistent with the previously crystallized TonB-dependent transporters (206) TdfF contained a β-barrel and plug domain (Figure 5A). It was predicted that 11 outer membrane loops extend from the β-barrel and could be surface exposed (Figure 5B).

Figure 5. TdfF topology model
A) Ribbon diagram of TdfF generated using the online protein homology analog recognition engine (PHYRE) (149). The three dimensional model was synthesized using FpvA as the biological template structure and shows a lateral view in which the characteristic β-barrel structure can be observed. A bird's eye view from the top of TdfF looking into the β-barrel shows the globular plug domain. The protein secondary structures are indicated by color: yellow indicates β-sheets, magenta indicates α-helices, and blue represents turns.

B) TdfF two-dimensional topology generated based on characteristic structures of TonB-dependent transporters. TdfF has two distinct domains: the C-terminal β-barrel domain which is shown in yellow with 22 putative β-strand transmembrane domains and eleven putative extracellular loops. The loops are labeled 1-11. The N-terminal plug domain is show in the periplasm but is predicted to fold up into the β-barrel. The plug and loops 1-5 were sequenced from all available gonococcal laboratory strains. OM indicates outer membrane.

A
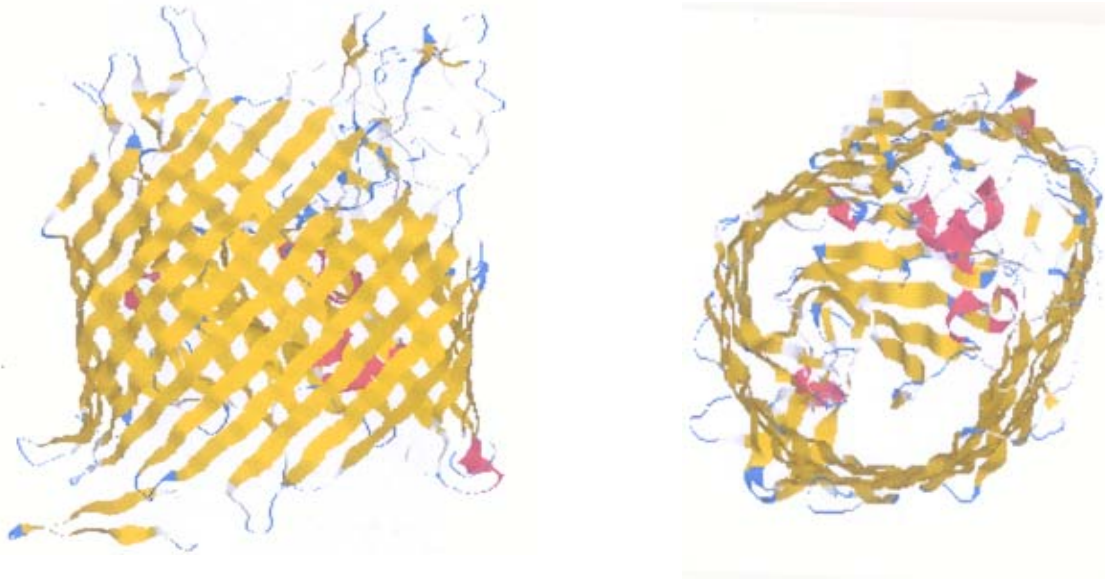
B
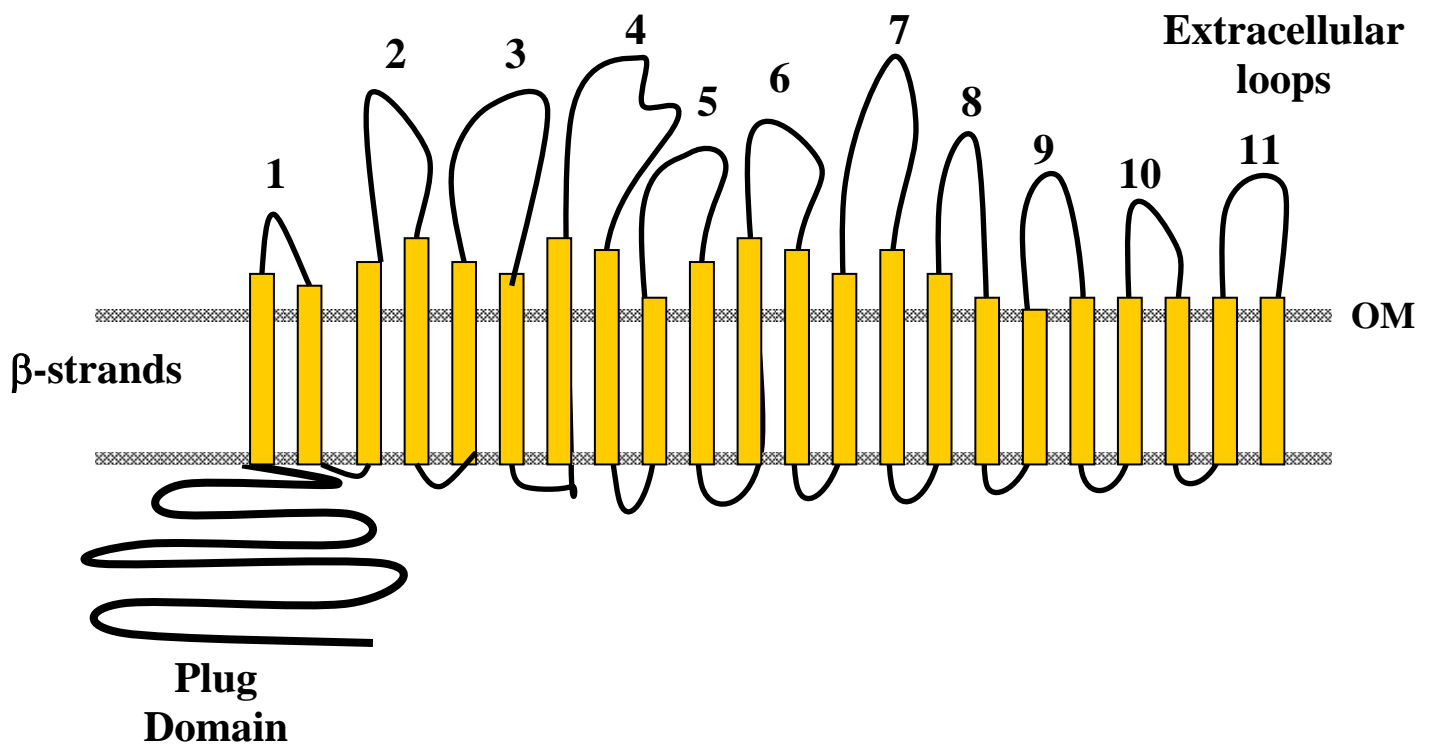
C. Fetal bovine serum induces *tdfF* expression

Gene regulation is essential for microorganisms as it increases versatility and adaptability by allowing the cell to express proteins when appropriate. When initially identified, *tdfF* expression was not detected from gonococci grown in bacterial growth media (286). Our laboratory demonstrated that *tdfF* transcription is detected only when gonococci are incubated in the presence of cervical epithelial cells (113). Bacteria that are adhered to or within cervical epithelial cells have increased *tdfF* transcript levels compared to gonococci isolated from the infection supernatant (113). Similar to previous work we demonstrated that *tdfF* expression is not detected in the presence of bacterial growth media (113). Additionally, microarray analysis of gonococci isolated from women with uncomplicated infections indicated an increase in *tdfF* expression (2) suggesting that the gene is expressed more highly within the host.

A putative Fur box has been identified in the hypothetical -10 region of the *tdfF* promoter (113). When gonococci are incubated in bacterial growth media under iron-deplete conditions, *tdfF* transcript is not detected (113) suggesting that *tdfF* expression is not only regulated by Fur, but that another regulator and inducing signal from eukaryotic cells and/or the cell culture media supplemented with FBS is necessary for gene expression. To determine if the inducing signal for *tdfF* expression is derived from epithelial cells or cell culture media we separated the components involved in cell culture and investigated *tdfF* transcript levels when gonococci were incubated in these components.

Cell culture media consists of McCoy's 5A media and 10% heat-inactivated fetal bovine serum. McCoy's 5A media was originally synthesized to support the amino acid

requirements for *in vitro* cell cultivation. Hsu and Kellogg employed this medium to support the growth of primary cultures derived from multiple tissue samples (135) It was purchased as a sterile liquid media and in addition to amino acids contained sodium bicarbonate and L-glutamine. Fetal bovine serum (FBS) is the most widely used growth supplement for cell culture media because of its high content of embryonic growth promoting factors. When used at appropriate concentrations it supplies many defined and undefined components that satisfy specific metabolic requirements for the culture of cells. Some of the defined components of serum included albumin, alpha-, beta-, and gamma-globulins (210) as well as bovine transferrin, ferritin, hormones, lipids, and growth factors.

We assessed *tdfF* expression through qualitative RT-PCR in which we incubated gonococcal strain FA1090 in either McCoy's 5A media alone or McCoy's 5A and 10% FBS for 4 hours. After the incubation, RNA was isolated from the bacteria and RT-PCR was performed. We probed for *tdfF* expression and used *16S* rRNA expression as a control because it was constitutively expressed under all conditions tested. We detected increased *tdfF* specific transcript when the bacteria were incubated in the presence of McCoy's 5A and 10% FBS compared to gonococci incubated McCoy's 5A alone, (Figure 6). There are two possible explanations for these results. One is that a serum derived component was responsible for inducing *tdfF* expression. If a serum component was responsible for the induction of *tdfF* expression then we should be able to define the serum component responsible for increased transcript. We incubated gonococci in different serum components such as the iron binding protein ferritin, and the hormones norepinephrine and epinephrine which have been shown to induce the expression of a

TonB-dependent transporter in *B. pertsussis* (7). After 4 hour incubation in McCoy's 5A supplemented with these different serum components *tdfF* expression was examined by RT-PCR. We did not detect *tdfF* when gonococci were incubated in these conditions (data not shown). We also isolated RNA from gonococci incubated in defined serum (Hyclone) and performed RT-PCR. Defined serum is commercially available and all the components in this serum have been identified. Transcript of *tdfF* was not detected using qualitative RT-PCR when gonococci were incubated in the defined serum (data not shown). These results indicate an undefined serum component is responsible for *tdfF* expression.

Another possible explanation from these results is that the *tdfF* expression observed was modulated by iron availability. The presence of FBS chelates any excess iron in the McCoy's 5A, and this iron chelation was derepressing *tdfF* transcript through Fur. *N. gonorrhoeae* cannot utilize bovine transferrin, (263) which is the major iron source in FBS and it is also demonstrated that gonococci cannot multiply in cell culture media (McCoy's 5A and 10% FBS) (113). McCoy's 5A is not an iron chelated media and any available iron would be utilized by gonococci and cause Fur to repress *tdfF*. In the presence of FBS, the bovine transferrin would chelate any iron away from gonococci making the media more iron deplete in which Fur would no longer repress *tdfF*. The addition of norepinephrine or epinephrine in McCoy's 5A alone would not decrease free iron from the media hence the lack of *tdfF* transcript detected. It was previously demonstrated that when gonococci are incubated in cell culture media supplemented with iron, *tdfF* transcript is not detected; however, *tdfF* is expressed in cell culture media in

the absence of the supplemented iron (113). Therefore *tdfF* expression could be

modulated by both iron availability and a serum specific molecule or molecules.

Figure 6. *tdfF* **transcript levels detected in the presence of fetal bovine serum**
Wild type strain (FA1090) was incubated in the presence of McCoy's 5A + 10% heat-
inactivated FBS (+) or in McCoy's 5A alone. *tdfF* specific transcripts were assayed by
RT-PCR. *16S* rRNA was used as a positive control because it was constitutively
expressed under all growth conditions. Expression of *16S rRNA* in the absence of reverse
transcriptase, (*16S* rRNA-RT was used as a negative control.
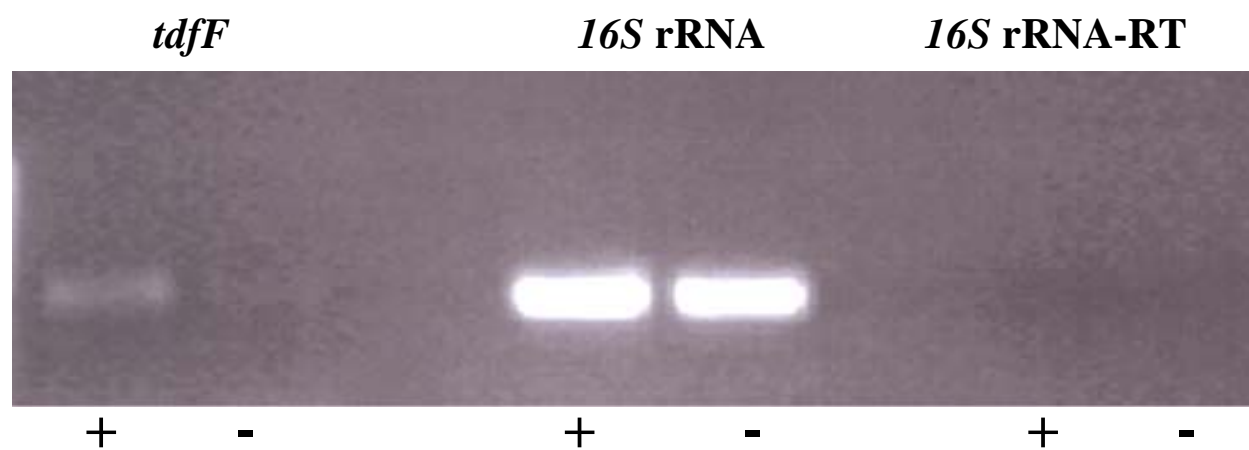
D. Expression and Isolation of rTdfF

TdfF is predicted to be a 78kDa outer membrane protein that is only expressed when gonococci are incubated in cell culture conditions in the absence of additional iron (Hagen, 2006 #143; Turner, 2001 #221). As another means for *tdfF* detection, we chose to generate a polyclonal TdfF specific antisera, which necessitated purification of rTdfF for subsequent immunoblot assays.

We utilized the pET vector (Novagen) which is a bacterial plasmid designed to enable the production of a large quantity of any desired protein. The *tdfF* gene was cloned into this vector and was expressed under the control of the IPTG inducible T7 promoter. We chose to clone full length *tdfF* into the pET vector pET-22b(+) (Novagen) which encoded ampicillin resistance, a C-terminal histidine tag and a T7*lac* promoter. A *lac* operator sequence was just downstream of the T7 promoter within the plasmid, and this plasmid also carried the lac repressor (*lacI*) which repressed the expression of the T7 RNA polymerase in the host cell. The addition of isopropyl-β-D-thiogalactopyranoside (IPTG) induced T7 RNA polymerase production within the host cell, which in turn transcribed the target DNA in the pET plasmid.

pVCU354 (pET-22b(+)*tdfF*) was transformed into *E. coli* strain BL21(DE3) because this strain was the most widely used host for target gene expression. A single colony from the transformation was grown overnight and this culture was split and used as a stock to grow two parallel cultures. Once the cultures grew to an OD_{600} of 2.5, IPTG was added to one culture and nothing was added to the other culture which was used as a control. The cultures were grown for an additional 4 hours. We observed an initial increase and then decrease in OD_{600} readings in our induced culture throughout the 4 hour

induction. Whereas, the uninduced culture continued to increase in optical density over the 4 hour growth period. Protein production was monitored in the two cultures with the use of Coomassie stained SDS-PAGE analysis, we expected that if rTdfF was produced then a dark band around 80kDa would be observed in whole cell lysates from the induced culture compared to the uninduced culture. There was no difference in protein banding pattern between the two cultures. Western blot analysis was also performed on whole cell lysates isolated from both cultures and the blots were probed with anti-histidine tag antibody. If rTdfF was expressed we would expect the C-terminal histidine tag to interact with the anti-histidine antibody. rTdfF was not detected from either culture on these blots by the histidine specific antibody. All together, these data indicate that the expression of full length *tdfF* in *E. coli* could be toxic to the bacteria or expression is not occurring due to insufficient tRNA pools.

Most amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons. When the mRNA of heterologous target genes is over expressed in *E. coli*, differences in codon usage can impede translation due to the demand for one tRNA that may be rare or lacking in the population. Insufficient tRNA pools can lead to translational stalling, premature translation termination or frame shifting (147, 158). The amino acid content of TdfF consisted of 27 prolines encoded by the sequence CCC which was considered a rarely used codon by *E. coli*. These codons were found in the N-terminus of TdfF and could lead to premature termination of translation. To enhance rTdfF expression in *E. coli* we transformed pVCU354 plasmid into Rosetta strain (Novagen) which expressed rare codons. IPTG induction of TdfF in the Rosetta cultures resulted in a very slight increase

in OD_{600} compared to the uninduced cultures over a 4 hour growth period. When proteins from whole cell lysates isolated from the two cultures were separated by SDS-PAGE, Coomassie staining of this gel indicated no difference in protein banding pattern between the two cultures. Also, western blot analysis in which anti-histidine antibodies were used to probe for rTdfF expression did not detect an 80kDa protein. The lack of growth and rTdfF expression in this system indicated that full length TdfF was toxic to the *E. coli*.

Since it was difficult to express full length rTdfF in the pET expression system we attempted to express portions of the transporter. The signal sequence was removed from the N-terminal sequence of *tdfF* which would prevent transport of the protein out of the cytoplasm and could improve protein yield and *E. coli* viability. The sequences encoding the plug domain or portion of the β-barrel, now called loop domain, were cloned into pET-22b(+) (Figure 4B) to generate the subsequent plasmids pVCU355 and pVCU357 respectively. The plasmid constructs were transformed into *E. coli* strain C41. The strain C41 was derived from BL21 (DE3) and had at least one uncharacterized mutation, which prevented cell death associated with the expression of toxic recombinant proteins (199). This *E. coli* strain was effective in over expressing toxic proteins from all classes of organisms, eubacteria, archaea, yeast, plant and mammals and therefore was expected to successfully produce portions of TdfF (199). The *E. coli* strain C41 containing either pVCU355 or pVCU357 were grown to an OD_{600} of 2.5 and then one culture was induced with IPTG and the other remained uninduced for an additional 4 hours. Similar levels of increased optical density were observed for the induced cultures, compared to the uninduced strains indicating that the induced cultures were viable. Whole cell lysates were isolated from the induced and non-induced *E. coli* strains and separated by SDS-

PAGE. A protein of the predicted size of 15kDa was identified by Coomassie stained SDS-PAGE from the IPTG induced plug expressing strain (Figure 7A). Additionally, histidine tag specific antibodies detected a protein of 15kDa expressed by the IPTG induced culture via Western blot analysis (Figure 7B).   Similarly, the loop domain which was predicted to be 27kDa was detected in the induced cultures through Coomassie stained SDS-PAGE analysis (Figure 7A) and anti-histidine antibodies detected a 27 kDa protein by Western blot analysis. A smaller species between 15kD and 20kD was detected by the anti-histidine antibody in lysates expressing rTdfF loops. This could be a break down product or smaller protein expressed by the *E. coli* (Figure 7B). Both recombinant proteins were insoluble and purified using denaturing conditions and a histidine column. A stepwise dialysis was conducted to increase the pH of the solution in which the proteins were eluted.

Figure 7. Expression of rTdfFplug and rTdfF loops

A) Coomassie blue stained SDS-PAGE containing proteins from *E. coli* expressing rTdfF protein. Lanes contain solubalized *E. coli* lysates that express either pVCU355 or pVCU357 induced with IPTG (I) or uninduced (NI) for 4 hours. Proteins were separated on a 15% acrylamide gel. The arrows on the left indicate the band of rTdfF expression. The position of molecular weight markers is indicated on the left.

B) Western blot analysis of the solubalized *E. coli* lyates that express either rTdfF plug or rTdfF loops grown in the presence of IPTG (I) or no IPTG (NI) for 4 hours. Proteins were separated on a 15% acrylamide gel. Blots were probed with an anti-histidine antibody.

A

rTdfF plug   rTdfF loops

B

rTdfF plug   rTdfF loops
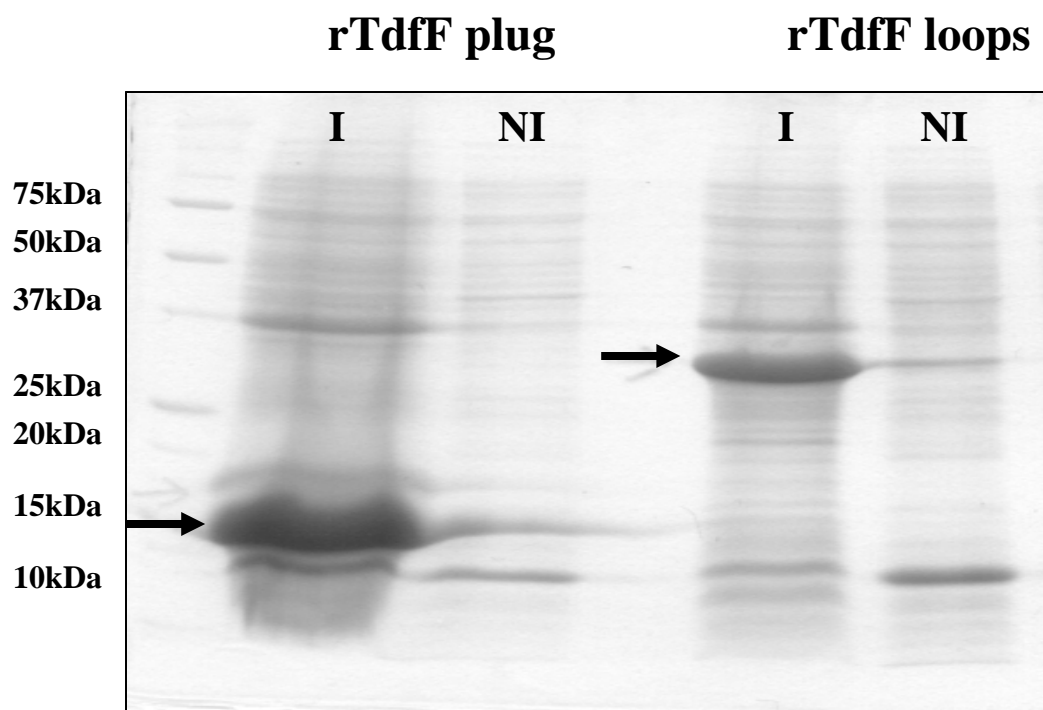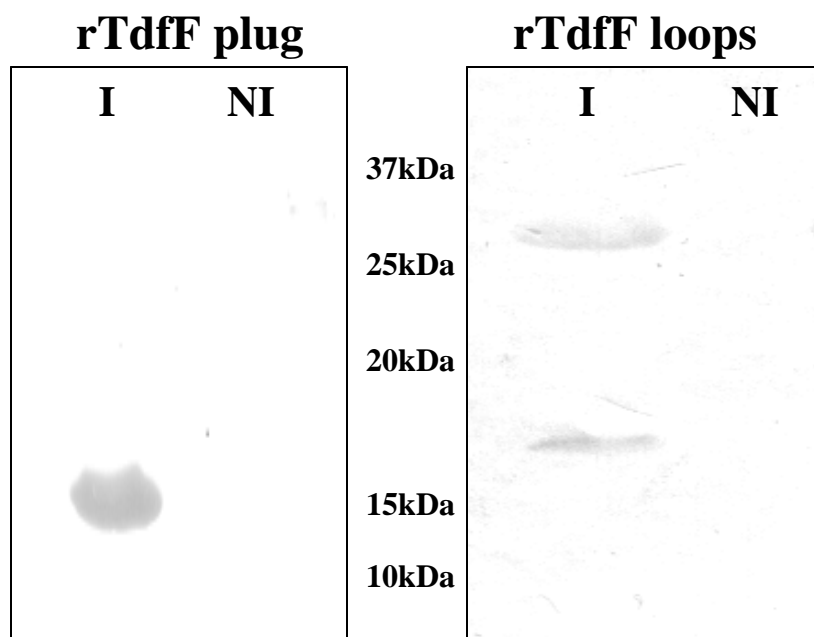
E. Anti-TdfF rabbit polyclonal serum detects rTdfF

Antibodies recognize their target with high specificity and generally bind with a very high affinity, within the nano/picomolar range to their target (249)**.** Polyclonal anti-TdfF antibodies were generated in rabbits against the rTdfF plug and rTdfF loops for analysis of TdfF expression. Recombinant proteins were sent to New England Peptide antibody program where female New Zealand Rabbits were immunized with rTdfF plug or rTdfF loops. Sera were collected 35 and 40 days after the primary boost. We determined the specificity of rabbit sera against the recombinant proteins and optimal dilutions for the sera for future detection assays. The anti-TdfF sera detected rTdfF from whole cell lysates isolated from induced *E. coli* cultures (Figure 8). The anti-TdfF plug sera detected rTdfF plug from induced *E. coli* cultures and anti-rTdfF loops detected rTdfF loops from induced *E. coli* strains (Figure 8). As expected, the anti-TdfF plug and anti-TdfF loops sera also detected their corresponding purified rTdfF proteins (Figure 9).

Figure 8. Polyclonal α-TdfF detects rTdfF
Western blot analysis of the solubalized *E. coli* lyates that express rTdfF. Blot 1
expresses rTdfF plug and blot 2 expresses rTdfF loops induced with IPTG (I) or
uninduced (NI) for 4 hours. Proteins were separated on a 15% acrylamide gel. Blot 1 was
probed with rabbit polyclonal α-TdfF plug and blot 2 was probed with rabbit polyclonal
α-TdfF loops sera as indicated below each blot. An arrow designates detection rTdfF
protein by α-TdfF sera

1
NI   I

2
NI   I

75kDa

37kDa

25kDa

20kDa
15kDa

10kDa

α-rTdfF plug   α-rTdfF loops
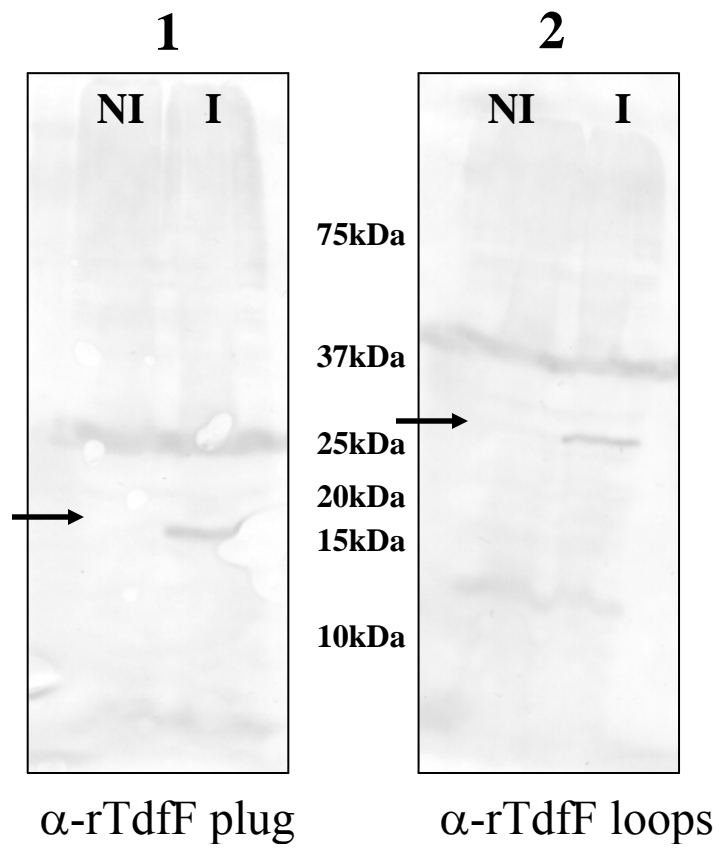
Both plug and loop specific anti-TdfF sera were utilized for analysis of TdfF expression in gonococcal strain FA1090. We first performed assays in which *tdfF* expression was observed, in which gonococci were incubated in the presence of cervical epithelial cells. We used gonococcal strain FA1090 and *tdfF* mutant strain (MCV659) as our negative control in these TdfF detection assays. Gonococci were incubated with cervical epithelial cells for 4 hours. After the 4 hour incubation the cell associated bacteria and supernant associated bacteria were separated and solubilized. Gonococcal protein expression was analyzed by immunoblotting and the blots were probed with anti-TdfF sera. TdfF expression was not detected in the wild type strain (FA1090) (Figure 9) under these incubation conditions. As expected, TdfF was not detected in proteins isolated from the *tdfF* mutant strain (MCV659). We also incubated wild type gonococcal strain FA1090 and *tdfF* mutant strain (MCV659) in various serum components for 4 hours. After the 4 hour incubation gonococci were solubilized and immunoblot analysis was used to determine TdfF expression. Ferritin, a potential intracellular iron source, and hormones such as norepinephrineeniphrine and epinephrine were added to the McCoy's 5A media individually. None of these components induced detectable TdfF expression (data not shown). In all our assays the anti-TdfF sera detected purified rTdfF loop domain, rTdfF plug domain (Figure 9) or whole cell lysates from C41 expressing the rTdfF proteins which were employed as positive controls (Figure 8). There could be many reasons why we do not detect TdfF expression from these assays. One possible explanation for the lack of TdfF detection by α-TdfF sera was that proteins from cell culture media or epithelial cell were blocking antibody detection. TdfF detection was expected to be between 70 kDa and 100 kDa based on the predicted molecular weight.

There was a lack of protein detection in this area and that could be due to steric hindrance of antibody binding by the serum specific protein (Figure 9). One such protein could be bovine albumin which is abundant in bovine serum and has a molecular weight around 70 kDa. This phenomenon occurred in both the wild type and *tdfF* mutant strain (MCV659) indicating we were observing a non-specific interaction.

The lack of TdfF detection could also be due to a lack of TdfF expression by gonococci. Based on our previous observations TdfF expression is greatest in the presence of cell culture media in the absence of additional iron (113). We observed very low levels of TdfF transcript when gonococci were incubated in cell culture media alone. The FBS may not be chelating all the iron in McCoy's 5 media making it iron-replete. Then gonococci would be able to obtain this iron and *tdfF* expression would be repressed by Fur. Also, lots of FBS vary, and the particular lot used for these experiments could lack a co-inducing signal necessary for TdfF expression hence the lack of TdfF detected in these assays.

Finally, western blot analysis is not as sensitive as qualitative RT-PCR. TdfF could be expressed but in very low levels under these conditions. We attempted to detect TdfF from solubilized whole cell lysates which contain proteins from the outer membrane, inner membrane, and cytoplasm. With all these proteins separated by SDS-PAGE other TonB-dependent transporters as well as other proteins with a similar molecular weight to TdfF could migrate to the same part of the gel as TdfF and prevent TdfF detection. One way to enrich for TdfF would be to isolate outer membrane proteins from gonococci incubated under cell culture conditions.

Figure 9. Western blot analysis of TdfF expression
Western blot analysis of whole cell lysates probed with α-TdfF plug or α-TdfF loops as
indicated above each blot. Lane 1 contains whole cell lysates isolated from gonococcal
wild type strain (FA1090) adherent/intracellular with ME180 cells. Lane 2 contains
whole cell lysates isolated from *tdfF* mutant strain (MCV659) adherent/intracellular with
ME180 cells. Lane 3 contains purified rTdfF protein that corresponds with the α-rTdfF
sera that was used to probe the blot. Poncceau stain below each blot indicates equal
loading of protein for each lane.
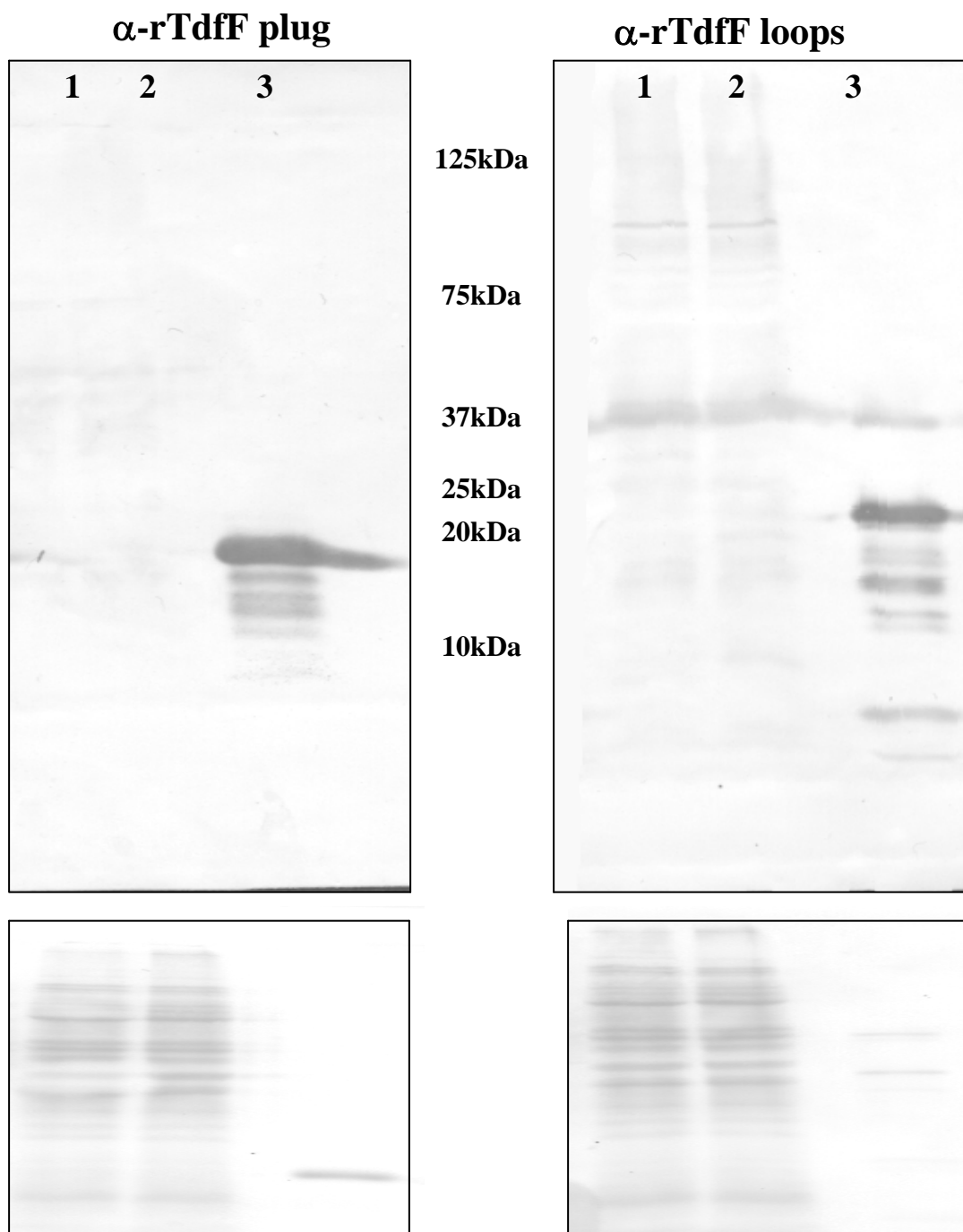
III. Discussion

Gonococcal infections require that the bacteria circumvent the innate iron-withholding mechanisms employed by the human host. Epithelial cells obtain iron from ferrated transferrin from the serum via receptor-mediated endocytosis (224). The low pH of transferrin-containing endosomes facilitates the release of iron from transferrin and is then transported out of the endosome to the cytosol (224). Epithelial cells respond to *Neisseria* infection by reducing transferrin receptor gene expression and transferrin receptor cycling thus further limiting host iron availability (32). It was hypothesized that gonococcal strain FA1090 utilized iron sources within the epithelial cell through the TonB-dependent transporter TdfF (113). Understanding how *tdfF* gene expression is induced may lead to the identification of the specific ligand for TdfF as well as elucidate new mechanisms of intracellular pathogenesis.

We first wanted to determine if other gonococcal genomes contained the *tdfF* gene. This TonB-dependent transporter is crucial for intracellular survival in gonococcal strain FA1090 (113); however, other gonococcal strains have exhibited Ton-independent survival in the presence of a strain specific gonococcal genetic island (317). Gonococcal strain FA1090 does not possess this gonococcal genetic island indicating that TdfF-dependent survival could be FA1090 specific and other strains may not encode for this TonB-dependent transporter. We analyzed common gonococcal laboratory strains for *tdfF* through PCR and sequence analysis. Using FA1090 specific *tdfF* primers, we amplified the plug and loop domains from strains FA19, MS11 and UU108 and compared these sequences to strain FA1090. Gonococcal strains FA1090 and FA19 were isolated from patients with disseminated gonococcal infections (DGI) as well as localized

infection. Strain MS11 was isolated from a localized infection and both FA19 and MS11 posses the before mentioned genetic island. All four laboratory isolates contain full length *tdfF*. Since our investigation the Broad Institute has sequenced many more gonococcal isolates and recently, *tdfF* was identified as a pathogen specific gene (184, 266).

All gonococcal strains sequenced to date have *tdfF* sequences that are 100% identical. Since strains that contain a GGI also contain full length *tdfF*, then perhaps in the presence of a functional Ton system TdfF is employed for intracellular survival and the GGI is employed only if the Ton system is no longer functional. Also, the lack of sequence diversity between the eleven putative outer membrane loops may indicate a lack of immune pressure and support data that *tdfF* expression only occurs in the intracellular environment. The transferrin receptor, TbpA isresponsible for acquisition of iron from the serum, human iron binding protein, transferrin (63). In TonB-dependent transporters that acquire iron from the extracellular environment it is common to observe sequence diversity amongst surface exposed loops. When comparing TbpA sequence amongst gonococcal isolates, both antigenic and sequence diversity was identified in hypervariable regions (62). These regions are surface exposed loops on the TonB-dependent transporter (312). It is possible that the intracellular niche would protect gonococci from the immune system and thus sequence diversity of the loops domain would be unnecessary. The lack of sequence diversity could also indicate a lack of immunogenicity by TdfF. This could explain the difficulty in detecting TdfF by immunoblot.

We attempted to express full length rTdfF in *E. coli* for purification and subsequent immunization to generate rTdfF anti-sera. We could not express full length rTdfF due to multiple complications. It seems that full length TdfF was toxic to the *E. coli* induced to express this protein as we observed a lack of growth in these cultures. Even after addressing the possibility that TdfF employed a rarely used codon to encode 27 proline residues we still could not express full length rTdfF. This was not the first report in which expression of a Neisserial outer membrane protein (OMP) was found to be lethal to *E. coli* (106).

OMPs are synthesized with a signal sequence and are translocated through the inner membrane by the SecA/Y/E/G export machinery in an unfolded form and in a process that requires energy in the form of ATP (96, 278). Once the protein has arrived in the periplasmic space, the signal sequence is removed by a signal peptidase (96, 278). The later stages in OMP biogenesis such as transport through the periplasm and assembly into the OM, are much less well understood. It has been demonstrated that the acquisition of tertiary structure proceeds, at least partially, the insertion of the proteins into the OM (81). Recent studies have led to the identification of Omp85 in *Neisseria* which is involved in the insertion and assembly of OMPs. Omp85 homolog has been identified in all Gram-negative bacteria studied (297) and the *E. coli* homolog, which is encoded by the *yaeT* gene, was demonstrated to be essential and required for OMP biogenesis (77, 302). Interestingly, the outer membrane porin, PorA, from *N. meningitidis* does not stimulate the assembly or transport activity of *E. coli* Omp85 indicating that, even though the process of OMP assembly and transport by Omp85- related machinery is evolutionarily conserved, species-specific adaptations appear to have occurred (243).

Furthermore, in *E. coli*, Omp85 was recently renamed BamA, and is associated with at least 4 lipoproteins: BamB, BamC, and BamD to make up the beta-barrel assembly machinery necessary for the transport of beta-barrels to the outer membrane (309) like that of TdfF (Figure 4). *Neisseria* have homologs to all components of the Bam system in *E. coli* except BamB (296). Mutations in the Bam system that are tolerated by *E. coli* are not in *Neisseria* (296). Thus, there are fundamental differences between *Neisseria* and *E. coli* in outer membrane beta-barrel biogenesis that could prevent expression and export inhibit high levels of full length TdfF protein from being expressed and could explain why we could express plug and loop domains of TdfF.

We also observed two loop species when expressing and purifying rTdfF loops (Figure 7). One species was assumed to be full length due to its size of 27 kDa whereas the other species was smaller and identified at a molecular weight between 15 kDa and 20 kDa. It was unclear if the truncated species resulted from premature translational termination or from proteolytic cleavage, both of which were artifacts of over expression in *E. coli*.

The polyclonal rabbit anti-rTdfF sera produced from rTdfF plug and rTdfF loops detected heterologous TdfF plug and TdfF loop domains from *E. coli*, but we were not able to detect full length TdfF from *N. gonorrhoeae*. A possible explanation for this phenomenon was that gonococci were not expressing *tdfF* under the conditions we tested. Expression of *tdfF* has only been observed in the presence of or adhered/within epithelial cells or cell culture media in the absence of additional iron (113). In this study *tdfF* expression was only detected with the use of qualitative RT-PCR when gonococci were incubated in cell culture media containing 10% heat-inactivated fetal bovine serum

(Figure 6). We concluded that this expression could be due to increased iron stress by the FBS as well as a serum specific inducing molecule. When we investigated TdfF expression under these same conditions using western blot analysis the detection of TdfF was not possible. We hypothesized that a serum derived protein of similar molecular weight of TdfF was blocking anti-TdfF sera. This was a non-specific TdfF interaction because TdfF was not detectable in either the wild type (FA1090) or *tdfF* mutant strain (MCV659) under the same incubation conditions. We also concluded that the lack of TdfF detection could be due to lack of sensitivity by Western blot analysis. The qualitative RT-PCR assay showed low levels of *tdfF* specific transcript and thus low levels of TdfF could be expressed on the surface of gonococci. To detect low levels of TdfF, we suggested western blot analysis of outer membrane proteins only. Finally, TdfF may not have been expressed in these assays. Due to differences in cell culture media based on FBS lots, the FBS used in these assays could contain excess iron that repressed *tdfF* expression or contain a decreased amount of a co-inducing molecule required to produce TdfF.

In numerous bacteria, gene expression for TonB-dependent transporters involved in iron uptake was regulated by Fur. Since TdfF played a role in intracellular iron acquisition and contained a putative Fur box it was assumed to be regulated by Fur. However when gonococci are incubated in bacterial growth media under iron-deplete conditions, *tdfF* expression is not detected and additional iron in cell culture media diminished expression (113) indicating that *tdfF* may be under a more complex regulatory circuit involving iron. Within close proximity of *tdfF* on the gonococcal chromosome was a gene that encoded an AraC-like regulator, *mpeR* (see chapter 4).

MpeR was first identified due to its role in regulating hydrophobic efflux pump activity (92). MpeR was also iron regulated and had a Fur box in its putative promoter (78, 139). Since MpeR was iron regulated and within close proximity of *tdfF* we hypothesized that it could be regulating gene expression. AraC-like regulators are characterized by an N-terminal ligand binding domain and a C-terminal DNA binding domain. When an AraC-like regulator binds to it ligand, it regulates the gene that corresponds to its DNA binding domain. AraC-like regulators have been involved in invasion by other bacteria (144). Since MpeR also regulated efflux pumps in gonococci it was conceivable that it was a global regulator and could also regulate *tdfF* expression in the presence of a host inducing signal. MpeR could also be regulated by other global regulators. Some genes that encode TonB-dependent transporters rely on a signal transduction cascade to lead to the subsequent transcription of the receptor. For example, the TonB-dependent transporter FecE, in *E. coli* relies on sigma factors for expression. Other TonB-dependent transporters are under the control of Fur as well as global regulators such as Crp (cAMP receptor protein) (315) or regulatory small RNAs (sRNAs). sRNAs are short RNA molecules that are synthesized as discrete transcripts and act by base-pairing with target mRNAs over short regions of complementarity. Base-pairing between an sRNA and an mRNA can lead to repression of mRNA translation (193, 289, Neisseria posses a Fur and iron regulated sRNA, NrrF (Mellin, 2007 #283). sRNA-mediated regulation requires a cofactor RNA-binding protein (Hfq) for proper gene regulation and stabilization which was also identified in *Neisseria* (76). NrrF and Hfq have been shown to be involved in *Neisseria* gene regulation in iron-depleted conditions (76, 193). Therefore the lack of *tdfF* expression may be due to a missing inducing molecule that signals that gonococci are

within or in contact with epithelial cells. This signal could be necessary for MpeR to activate expression of *tdfF* or activate a complex regulatory cascade that may involve MpeR, sigma factors and/or small RNAs.

We attempted to induce *tdfF* expression with the use of hormones and iron sources identified in serum. We tested the hormones norepinephrineinephrine and epinephrine. These neurotransmitters induce the expression of the *B. pertussis* TonB-dependent iron transporter BfeA in the presence of serum (7). We also tried an intracellular iron binding protein, ferritin, which gonococci cannot utilize as an iron source but may sense in the intracellular environment. Finally, we tried to detect *tdfF* expression in the presence of defined serum which contains specific serum components as a way to exclude potential inducing molecules. We were not successful in detecting *tdfF* expression in these sera using either qualitative RT-PCR or western blot approaches. The lack of induction may be due to excess iron in the McCoy's 5A media causing a repression of *tdfF* which would inhibit any induction from these molecules.

To conclude, we did determine that fetal bovine serum in the cell culture media does induce *tdfF* expression and this could be due to iron availability and the presence of an inducing signal. We also determined that *tdfF* was identical amongst all gonococcal isolates sequenced to date indicating there was little immune pressure for sequence variability. We successfully expressed rTdfF plug and loop domains and polyclonal rabbit anti-TdfF sera from these recombinant proteins. The sera detected rTdfF by western blot but could not be employed to detect TdfF in gonococcus whole cell lysates. Elucidating the mechanism of *tdfF* expression may provide clues to the intracellular pathogenesis of *N. gonorrhoeae* including the identity of the ligand required for TdfF-

dependent intracellular survival.

Chapter 4 INTRACELLULAR IRON ACQUISITION BY *Neisseria gonorrhoeae*

I. Introduction

Neisseria meningitidis and *Neisseria gonorrhoeae* are closely related obligate human pathogens. Both microorganisms possess an array of virulence factors that contribute to their ability to adhere and invade mucosal epithelial cells (for a review see (107, 194)). The first stages of infection involve adherence and invasion of epithelial cells, mediated by bacterial cell surface components such as pili, opacity-associated proteins (Opa) and lipooligosaccharide (LOS). Several gonococcal-specific studies indicate that pili play a major role in the initial adherence of *N. gonorrhoeae* (192) and that the Opa proteins are involved in both adherence and invasion of epithelial cells (157). LOS aids in adherence and uptake of gonococcal cells, in addition to evoking a strong pro-inflammatory response in urethral epithelial cells (122).

Another important virulence determinant for the bacteria is the ability to acquire iron from the human host. Free iron exists in extremely low concentrations within the human body. This presents a nutritional barrier to invading microbes called nutritional immunity (301). In order to colonize humans, bacteria must acquire iron from human iron binding proteins. Meningococci and gonococci employ multiple TonB-dependent transport systems for the acquisition of iron from extracellular human iron binding proteins

including, transferrin, lactoferrin and hemoglobin (22, 25, 53, 64, 174, 175, 212, 220, 307). Iron is necessary for intracellular survival and this survival is TonB-dependent for both *N. meningitidis* and gonococcal strain FA1090 (113, 161). Furthermore, the TonB-dependent transporter TdfF is important for gonococcal survival in an iron-dependent manner, (113) however its intracellular iron source remains undefined. TdfF is pathogen specific and has been identified in both *N. meningitidis* and *N. gonorrhoeae* (184, 266). It is important to note that TonB-independent intracellular survival has been observed in gonococcal strain MS11 (317). MS11 possesses the gonococcal genetic island (178) (178) and TonB-independent intracellular survival is directly linked to type IV secretion system structural components encoded by the GGI (317).

Iron is also essential for basic physiological processes within the epithelial cell. Epithelial cells take up ferrated transferrin from the serum via receptor-mediated endocytosis. The low pH of transferrin-containing endosomes facilitates the release of iron from transferrin and the apo-transferrin is recycled to the cell surface (224). Iron is then transported out of the endosome to the cytosol. Biochemical data suggests that additional iron uptake mechanisms may exist through putative receptors for serum iron-binding proteins and molecules but these have not been characterized at a molecular level or *in vivo* (129). One such molecule could be the recently identified mammalian siderophore. Siderophores are low molecular weight iron binding compounds secreted by many microorganisms. Mammalian cells express a homolog of *entA* from *E. coli*, which encodes a bacterial protein that has a critical role in synthesis of the bacterial siderophore enterobactin (75). The iron binding moiety of the mammalian siderophore consists of 2,5-

DHBA, however the composition and structure of the intact siderophore remains to be determined (75).

Once iron has been acquired by the epithelial cell, any excess iron is stored in the cytosolic iron storage protein ferritin. Ferritin is a 24-mer proteinacious cage made up of two subunits, H-ferritin and L-ferritin (280). Iron is taken up by the two subunits cooperatively and H-ferritin possesses ferroxidase activity that converts soluble ferrous ions into ferric hydroxides (170). L-ferritin lacks ferroxidase activity but is more efficient in inducing iron nucleation and mineralization within the protein cage (12, 251). When the cell needs to utilize iron from the ferritin, the ferritin monomers within the cytoplasm of the cell form clusters, by an as yet-undefined mechanism, that are preferentially taken up by lysosomes (234). Once the lysosome acidifies, ferritin releases the iron atoms which can then be transported and utilized by the cell in a yet to be determined manner. The degradation of ferritin can be blocked *in vitro* with the addition of leupeptin or ascorbate. Leupeptin is a lysosomal protease inhibitor that has been used to prevent ferritin degradation in multiple cell lines (244). Ascorbate prevents autophagy of ferritin and its subsequent degradation within epithelial cells (38, 131, 211).

Host-pathogen interactions have been investigated in the context of *Neisseria* induced alteration of epithelial cell iron homeostasis. During gonococcal and meningococcal infections epithelial cells experience an alteration in transferrin receptor expression and iron metabolism (31). *Neisseria*-infected human epithelial cells have reduced levels of transferrin receptor messenger RNA and exhibit a reduction in transferrin receptor cycling (31), which could promote the host cells to deplete intracellular iron stores. Microarray data also establishes that cells infected with

meningococci demonstrate an iron starvation gene expression profile (32). It was hypothesized that depleting intracellular iron stores would prevent the proliferation of pathogenic bacteria that were generally more invasive than commensal species. It was demonstrated that during meningococcal infections, ferritin is redistributed from the host cell cytosol into aggregates that associated closely with the intracellular bacteria (162) and induced a rapid degradation of host cell ferritin. Thus meningococcal infection of epithelial cells causes a reduction of transferrin uptake, which triggers an iron starvation response (162). As a consequence, the host cell degrades cytosolic ferritin, releasing iron in order to meet its own metabolic needs. The meningococci, in turn, hijack the ferritin-derived iron in order to replicate within the epithelial cells (31, 32, 162).

Iron is required for enzymatic functions; however, excess free iron can be detrimental by increasing oxidative damage. Therefore, iron homeostasis within the bacterial cell must be tightly regulated (37). The ferric uptake regulator (Fur) acts as a transcriptional regulator for iron acquisition genes in *N. gonorrhoeae*. Under iron-replete conditions, Fur binds to a specific DNA sequence called the Fur box in the promoter of iron regulated genes and blocks transcription (13). Under iron-deplete conditions Fur disassociates from the Fur box and allows the RNA polymerase to bind to the promoter and transcribe the iron regulated gene (13). Fur may not be the only transcriptional regulator of iron acquisition genes in *N. gonorrhoeae*. An AraC-like regulator, MpeR, was first identified as a regulator of a hydrophobic agent efflux pump (92). AraC-like regulators have been shown to regulate the acquisition of iron in multiple microorganisms including *Yersenia pestis* and *Bordetella pertussis* (16, 87). Interestingly, *mpeR* is iron regulated (78, 139) indicating it could in turn regulate iron acquisition systems and it was

within close proximity to *tdfF* on the gonococcal chromosome (Figure 10). In other microorganisms in which AraC-like regulation occurs on iron transport genes, the AraC-like regulator is encoded within close proximity of the transport system that it regulates (16, 87). Therefore we hypothesize that MpeR may play a regulatory role in the expression of *tdfF*.

Figure 10. Chromosomal locus of *tdfF*
The gene, *tdfF* is shaded in yellow and encodes a TonB-dependent transporter important
for intracellular survival. Upstream of *tdfF* is a gene that encodes a conserved
hypothetical protein (CHP), a periplasmic binding protein (PBP) and another conserved
hypothetical protein where are all shaded in light blue. Encoded in the opposite direction
within close proximity to *tdfF* is *mpeR*, shaded in orange.
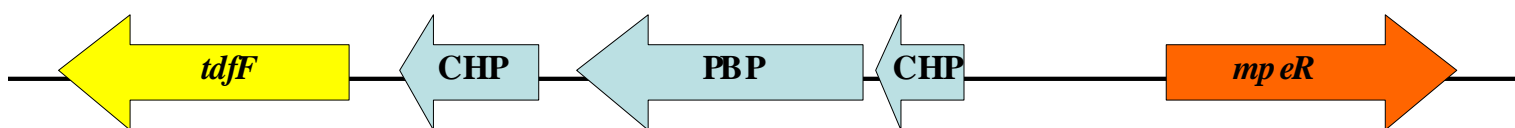
In this study, we investigated the role of intracellular iron in the survival of *N. gonorrhoeae*. We examined the role of MpeR during intracellular survival as a means to determine if MpeR regulated TdfF. Utilizing gentamicin protection assays we determined that MpeR did not play a regulatory role in intracellular survival under these *in vitro* conditions. We explored gonococcal survival in ME180 epithelial cells incubated in varying serum and iron conditions and demonstrated that similar to *N. meningitidis*, gonococci have increased survival when epithelial cells were serum starved however, the survival in the absence of serum was not due to increased intracellular ferritin degradation but due to increased iron in the serum-deplete media. Additionally, we investigated gonococcal utilization of 2,5- DHBA with plate bioassays and demonstrated that gonococcal strain FA1090 utilized 2,5- DHBA at intermediate levels compared to iron sources known to be utilized by gonococci and that iron acquisition by 2,5-DHBA was TonB-independent.

II. Results

A. MpeR does not have an affect on intracellular survival

ME180 cervical epithelial cells support gonococcal invasion (137, 141) and within 4 hours gonococci adhere to and invade the epithelial cells (113). Therefore, to determine the number of bacteria that survive within the intracellular environment we employed gentamicin protection assays with ME180 cells. In these assays, pilliated gonococci were suspended in infection media and added to two separate epithelial cell monolayer at an MOI of 10. This infection media consisted of McCoy's 5A cell culture media (Gibco), 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and iron supplementation to enhance invasion. Fetal bovine serum contains bovine transferrin

which is not an iron source for gonococci because the transferrin binding proteins (TbpA/TbpB) are species specific for human transferrin (63, 263). After the monolayers were incubated with the infection media consisting of gonococci for 4 hours, the monolayers were washed twice with PBS and gentamicin was added to kill any extracellular bacteria. The extracellular gonococci were susceptible to gentamicin killing but intracellular bacteria were protected because the drug was not able to penetrate eukaryotic cells.   Both monolayers were then washed again to remove any remaining gentamicin. One monolayer was lysed and plated for intracellular bacteria enumeration. This was time point 0. Replication media, consisting of McCoy's 5A cell culture media and 10% heat-inactivated FBS was added to the other monolayer. After 24 hours of incubation, the monolayer was washed, treated with gentamicin, lysed and plated for the enumeration of intracellular bacteria which was the 24 hour time point.

The *mpeR* gene was originally identified as encoding an AraC-like regulator that plays a role in maximizing the function of hydrophobic agent efflux pumps (92). Members of the AraC/XylS transcriptional regulator family have been characterized from a wide variety of prokaryotes including both Gram-negative and Gram-positive bacteria. The characteristic features used to identify MpeR as an AraC-like regulator are a conserved stretch of approximately 100 amino acids at the C-terminal end that serves as the DNA-binding domain and an N-terminal ligand binding domain (97). The main regulatory roles of AraC-like regulators are in carbon metabolism, stress response, and virulence and/or pathogenesis (97). In many microorganisms AraC-like transcriptional regulators play a role in intracellular survival. For example, an AraC-like transcriptional regulator PerA contributes to *Enterococcus faecalis* intracellular survival within

macrophages (59). In *Salmonella enterica*, two AraC-like regulators play a role in intracellular survival within epithelial cells (79, 237).

We initially determined that *mpeR* was iron regulated, (data not shown) which was recently confirmed by microarray assays, FURTA assays, and the presence of a Fur box in its promoter (78, 139). We hypothesized that since MpeR itself was iron regulated, it could also regulate iron acquisition systems in gonococci, particularly the one putatively encoded by the *tdfF* locus thus impacting intracellular survival. To determine if MpeR played a role in intracellular survival, we performed a gentamicin protection assay comparing the wild type strain to the *mpeR* mutant strain (MCV304) and the *mpeR*^{C} (MCV305) complemented strain. The *mpeR* mutant strain (MCV304) consisted of the *mpeR* gene interrupted by a kanamycin resistance cassette. We used the *tonB* (MCV656) and *tdfF* (MCV659) mutant strain as controls in these protection assays. As expected both the *tonB* (MCV656) and *tdfF* (MCV659) had attenuated survival with reduction in viable counts by 4 log and 1 log respectively, compared to the wild type strain (FA1090) which had been previously observed (113) (Figure 11A). Both, the *mpeR* (MCV304) and *mpeR*^{C} (MCV305) strains survived similarly to the wild type strain (FA1090) (Figure 11A). It appears that under these *in vitro* conditions, MpeR does not have an effect on intracellular survival indicating it may not regulate *tdfF* expression which has shown to be important for intracellular survival.

Figure 11. Intracellular survival is not MpeR-dependent and increases in serum starved epithelial cells

A) Intracellular survival in the presence of replication media containing McCoy's5A and 10% heat-inactivated FBS. The bars represent intracellular survival in CFU/ml (log10) for the following gonococcal strains: Wild type (FA109) (black), *tonB* mutant (MCV656) (checkered), *tdfF* mutant (MCV659) (horizontal black lines) *mpeR* mutant (MCV304) (vertical black lines) and *mpeR*^{C} strain (MCV304) (93). Time points in hours indicated along the x-axis. Intracellular survival assays were performed in triplicate and standard deviation is indicated above each bar. A P value of $\leq$ .01 was considered statistically significant. An asterisk (*) denotes a significant decrease in intracellular bacteria counts as compared to the wild type is indicated by an asterisk (*).

B) Intracellular survival in the presence of replication media containing McCoy's 5A alone. The bars represent intracellular survival in CFU/ml (log10) for the following gonococcal strains: Wild type (FA1090) (black), *tonB* mutant (MCV 656) (checkered), *tdfF* mutant (MCV659) (horizontal black lines) *mpeR* mutant (MCV304) (vertical black lines) and *mpeR*^{C} strain (MCV305) (93). Time points are indicated in hours along the x-axis. Intracellular survival assays were performed in triplicate and standard deviation is indicated above each bar. A P value of $\leq$ .01 was considered statistically significant. An asterisk (*) denotes a significant decrease in intracellular bacteria counts as compared to the wild type is indicated by an asterisk (*).

A

B
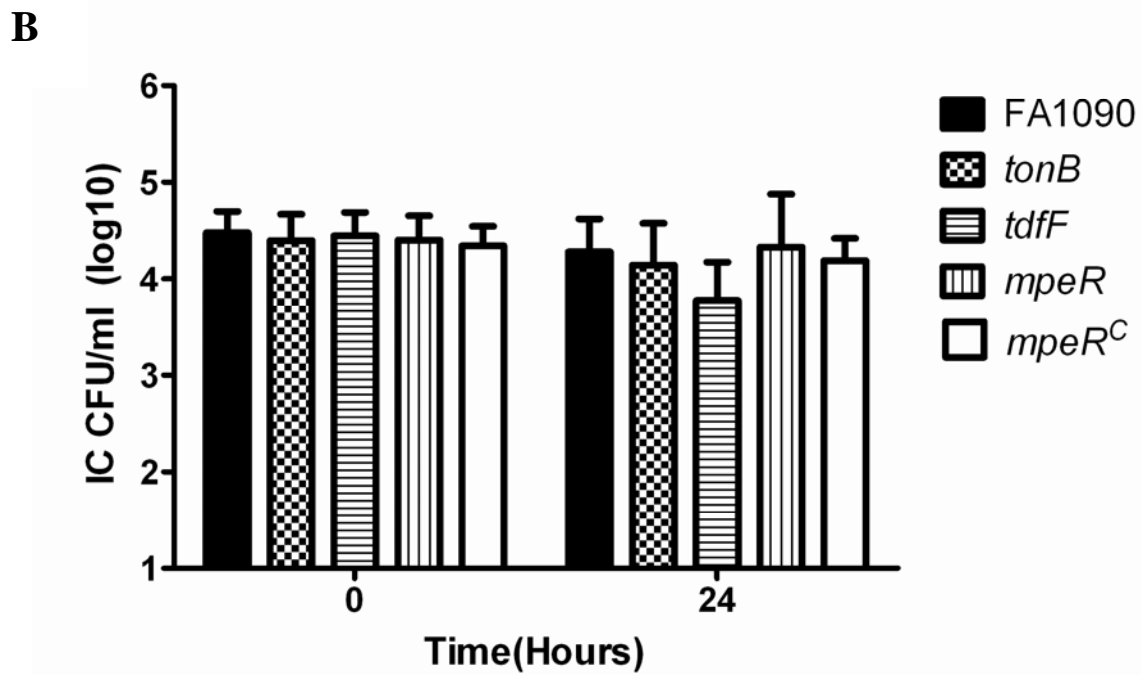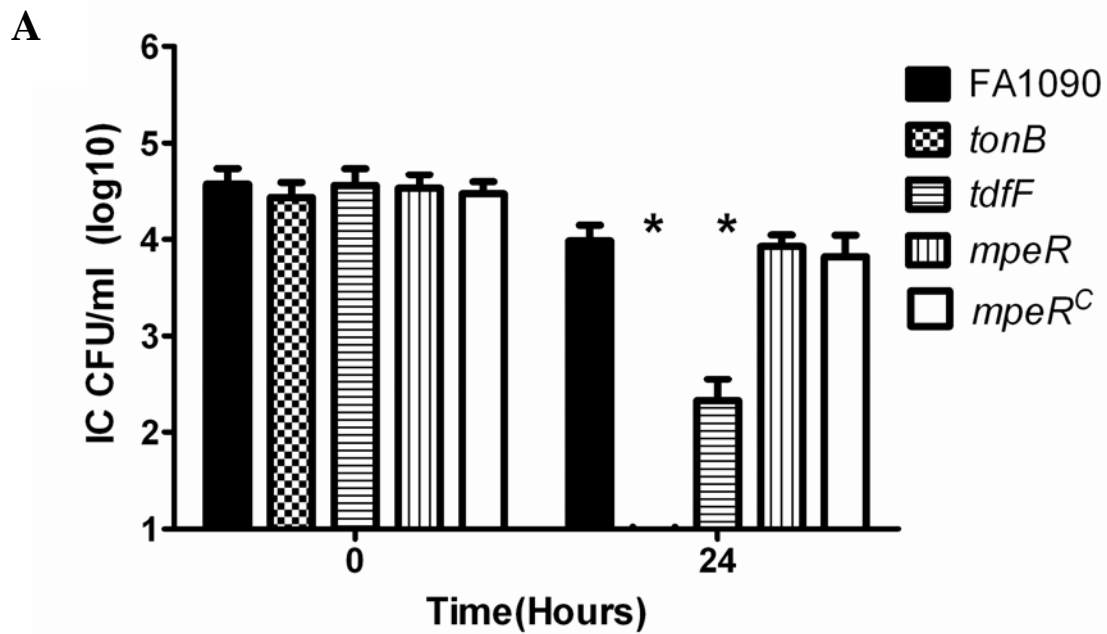
B. Serum starvation increases gonococcal intracellular survival

In *N. meningitidis*, intracellular survival increases in serum starved epithelial cells (162). Serum starvation increases the desferal-chelatable iron pool in cultured hepatocytes and this increase in desferal-chelatable pools is derived from degraded ferritin (211). Therefore, bacteria that survive in serum-starved epithelial cells could be acquiring iron from degraded ferritin. We hypothesized that if *N. gonorrhoeae* were utilizing iron from degraded ferritin then they would survive in serum starved epithelial cells similar to *N. meningitidis*. Gentamicin protection assays were performed as described above, but after the initial 4 hour incubation and gentamicin treatment, the cells were incubated in McCoy's 5A (Gibco) alone for 24 hours to induce serum starvation. We investigated intracellular survival under serum-deplete conditions with the following gonococcal strains: the wild type strain (FA1090), the *tonB* mutant strain (MCV659), the *tdfF* mutant strain (MCV659) and the *mpeR* mutant strain (MCV305). The *tonB* (MCV656) and *tdfF* (MCV659) mutant strains survived as well as the wild type strain (FA1090) (Figure 11B) both mutant strains survived within 0.5 log of the wild type strain. The *mpe*R (MCV304) and *mpeR*^{C} (MCV305) strains survived as well as wild type which was observed previously in the presence of serum (Figure 11A). These results indicate that gonococci could be utilizing iron from degraded ferritin in a TdfF- and/or a TonB-independent manner.

C. Intracellular survival is slightly attenuated in the presence of apo-bovine transferrin

Replication media which consisted of McCoy's 5A and 10% FBS contains bovine specific iron binding proteins including bovine transferrin making the media iron-deplete

for gonococci. Previous assays demonstrated that gonococci could not replicate in this media (113). The attenuated survival of *tonB* and *tdfF* mutant strains could be rescued with the addition of iron to the replication media (113) (Figure 12). McCoy's 5A alone is not iron free and could contain significant amounts of iron to rescue the mutants, similar to what we observed when additional iron ($FeNO_3$) was added to replication media (Figure 12) (113).

Figure 12. Gonococcal mutants are rescued with the addition of excess iron
Intracellular survival in the presence of McCoy's 5A +10% heat-inactivated FBS
supplemented with additional iron. The bars represent gonococcal survival in CFU/ml
(log10) for the following gonococcal strains: Wild type (FA1090) (black), *tonB* mutant
(MCV656) (checkered), *tdfF* mutant (MCV659) (horizontal black lines) *mpeR* mutant
(MCV304) (vertical black lines). Time points in hours indicated along the x-axis.
Intracellular survival assays were performed in triplicate and standard deviation is
indicated above each bar.
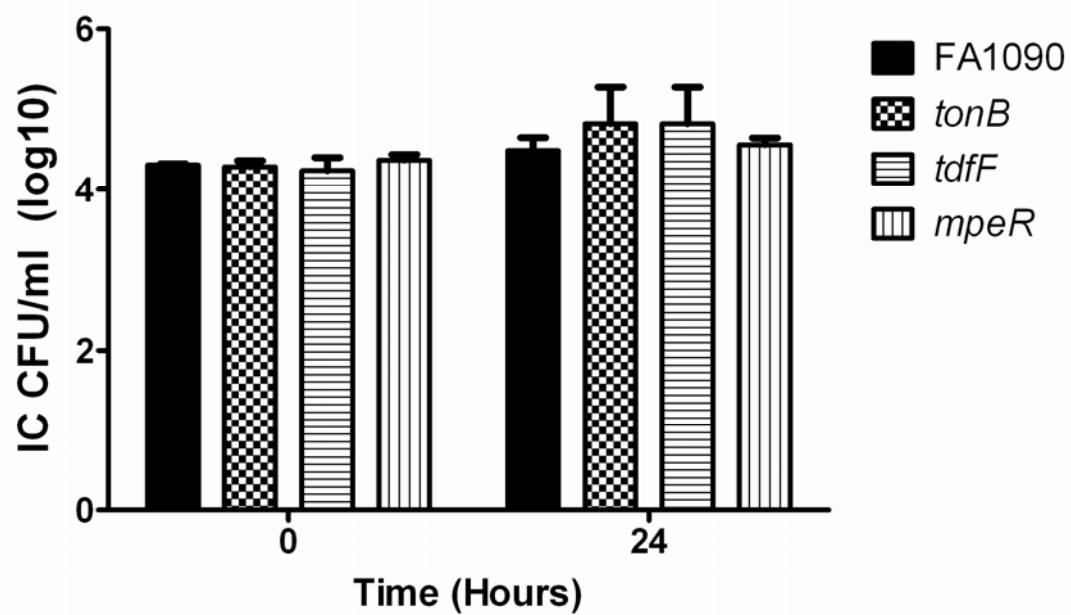
To ensure that the TonB- TdfF-independent survival phenomenon that was observed in the absence of serum was not due to extracellular iron, we performed intracellular survival assays in which the replication media was supplemented with 40 µg/ml of apo-bovine transferrin. After the 4 hour infection and initial gentamicin treatment, cells were incubated with replication media consisting of McCoy's 5A and apo-bovine transferrin or McCoy's 5A, 10% FBS, and additional apo-bovine transferrin for 24 hours. The bovine transferrin would chelate any available iron away from gonococci. The following gonococcal strains were tested for intracellular survival in the presence of excess apo-bovine transferrin: the wild type strain (FA1090), the *tonB* mutant strain (MCV656) and the *tdfF* mutant strain (MCV659). In the presence of FBS and 40 µg/ml apo-bovine transferrin we observed similar survival phenotypes as was observed in the presence of McCoy's 5A and FBS (Figure 13A). Both the *tonB* (MCV656) and *tdfF* (MCV659) strains were significantly attenuated in survival compared to the wild type strain (FA1090) with viable counts 4 log and 1 log lower, respectively (Figure 13A). When McCoy's 5A only replication media was supplemented with 40 µg/ml of apo-bovine transferrin, the *tonB* mutant strain still survived but was slightly attenuated compared to wild type. We also observed a slight attenuation in survival of the *tdfF* mutant strain compared to the wild type in the absence of FBS. The decreased survival of the *ton* and *tdfF* mutant strains was not significant in the McCoy's 5A + apo-bovine transferrin (Figure 13B) compared to the observed attenuation in the presence of FBS with additional apo-bovine transferrin (Figure 13A). One possible explanation for this phenomenon is that the addition of 40 µg/ml of apo-bovine transferrin was not enough to chelate all the excess iron in the McCoy's 5A replication media and therefore the mutants

were being rescued by any remaining unbound iron. Another possible interpretation of these results is that the bovine-transferrin was delivering any iron in McCoy's 5A to the ME180 epithelial cells. This would decrease ferritin degradation and increase intracellular stores within the ferritin. Gonococci can not acquire iron directly from ferritin (10, 41).   If gonococci were tapping into iron from degraded ferritin then a decrease in ferritin degradation could contribute to the lower level of survival in the presence of apo-bovine transferrin in McCoy's 5A alone.

Figure 13. Intracellular survival in the presence of apo-bovine transferrin

A) Intracellular survival in the presence of McCoy's 5A +10% heat-inactivated FBS with an additional 40μg/ml apo-bovine transferrin. The bars represent gonococcal survival in CFU/ml (log10) for the following gonococcal strains: wild type (FA1090) (black), *tonB* mutant (MCV656) (checkered) and *tdfF* mutant (MCV659) (horizontal black lines). Time points in hours indicated along the x-axis. Intracellular survival assays were performed in triplicate and standard deviation is indicated above each bar. A P value of ≤ .01 and ≤ .05 was considered statistically significant. A significant decrease in intracellular bacteria compared to wild type by P≤.01 is indicated by an asterisk (*) and a significant decrease in survival compared to wild type at P≤.05 is indicated by (@).

B) Intracellular survival in the presence of McCoy's 5A with an additional 40μg/ml apo-bovine transferrin. The bars represent intracellular survival in CFU/ml (log10) for the following gonococcal strains: FA1090 (black), *tonB* mutant (MCV656) (checkered) and *tdfF* mutant (MCV659) (horizontal black lines). Time points in hours indicated along the x-axis. Intracellular survival assays were performed in triplicate and standard deviation is indicated above each bar. A P value of ≤ .01 and ≤ .05 was considered statistically significant. A significant decrease in intracellular bacteria compared to wild type by P≤.01 is indicated by an asterisk (*) and a significant decrease in survival compared to wild type at P≤.05 is indicated by (@).
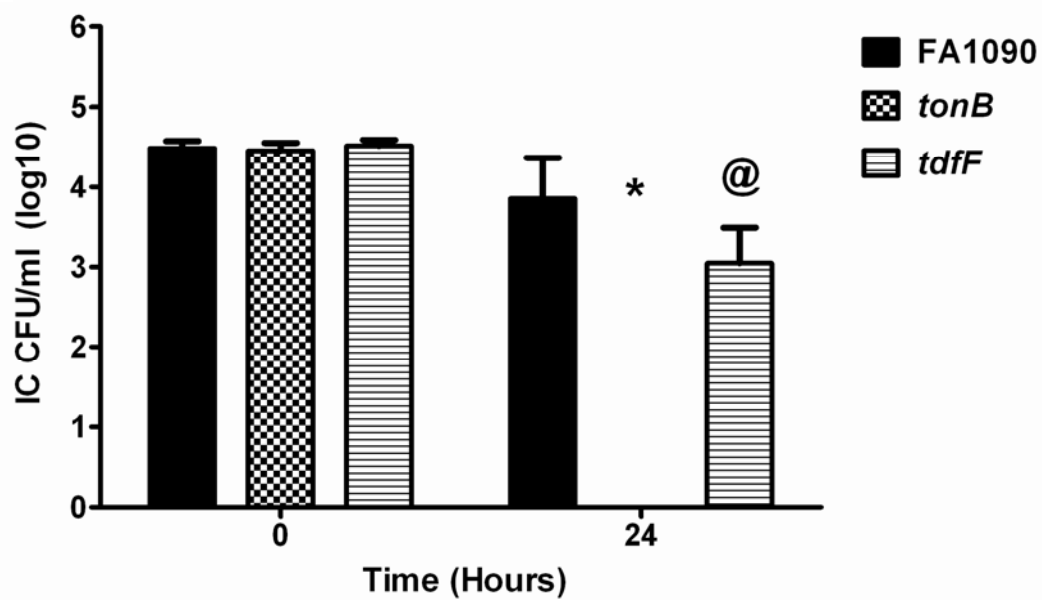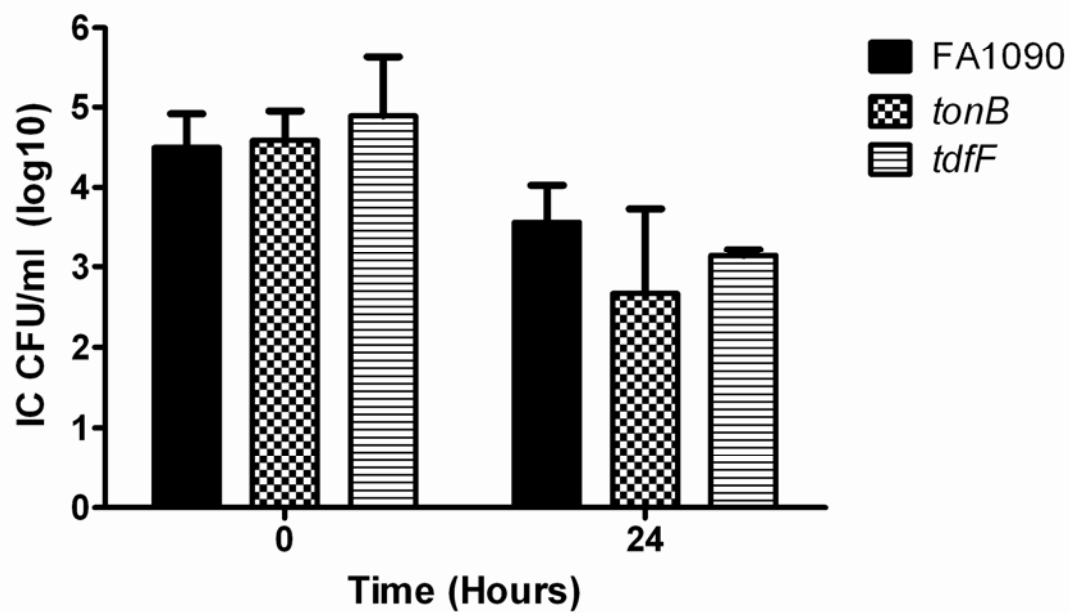
D. **Addition of ascorbate inhibits gonococcal intracellular survival in the presence of FBS**

Ferritin cannot be a direct iron source for *Neisseria* (10, 41) however if the ferritin were degraded, the subsequently released iron could be acquired by the bacteria. Meningococcal infections cause ferritin to cluster (162) within the cytoplasm similar to what is observed when the epithelial cell needs to utilize iron from ferritin (234). *N. meningitidis* also induce ferritin degradation within host epithelial cells (162). Ferritin degradation can be pharmacologically blocked with the addition of ascorbate (vitamin C) or leupeptin. The biosynthesis of ferritin and the rate of iron taken up by transferrin are not affected by the presence of ascorbate. However, ascorbate does prevent autophagy of ferritin and its subsequent degradation in several cell types (38, 131, 211). During *N. meningitidis* infection, cells treated with ascorbate exhibit a decrease in intracellular meningococci and increased levels of ferritin compared to untreated epithelial cells (162).

We hypothesized that if gonococci were accessing iron from degraded ferritin, then survival would be attenuated in the presence of ascorbate similarly to what was observed in *N. meningitidis*. We performed gentamicin protection assays in which the replication media was supplemented with 200 µM ascorbate. This concentration of ascorbate inhibited intracellular ferritin degradation (38, 131) but did not affect gonococcal survival in the replication media alone (data not shown). Using the wild type strain (FA1090) we performed parallel intracellular survival assays in which the replication media was one of the following: McCoy's 5A + 10% FBS, McCoy's 5A + 10% FBS supplemented with 200 µM ascorbate or McCoy's 5A + 10% FBS

supplemented with 10 µM leupeptin. When ascorbate was added to replication media containing McCoy's 5A and FBS there was a significant decrease in gonococcal survival with a decrease of 1.2 log in viable counts (Figure 14A) indicating that gonococcal survival could be dependent on iron from ferritin degradation. We were also interested in the intracellular survival of the wild type gonococci in the presence of leupeptin, a lysosomal protease inhibitor. Interestingly there was only a slight decrease in intracellular survival in the presence of 10µM leupeptin with a decrease by 0.2 logs (Figure 14A). In *N. meningitidis*, intracellular survival was attenuated in the presence of leupeptin, but leupeptin only partially prevented ferritin degradation (162). Therefore, in our experiments, in the presence of leupeptin, ferritin could be partially degraded and still acting as a potential iron source for the intracellular bacteria.

We also investigated the effect of ascorbate and leupeptin when gentamicin protection assays were performed with replication media containing McCoy's 5A in the absence of FBS. We performed gentamicin protection assays using the wild type strain (FA1090) under the following conditions: McCoy's 5A + 10% FBS, McCoy's 5A alone, McCoy's 5A supplemented with 200µM ascorbate, or McCoy's 5A supplemented with 10µM leupeptin. We previously observed that gonococci survived in a Ton and TdfF-independent manner in the absence of FBS. We correlated increased survival phenotype in these mutants with the rationale that serum starved cells had degraded their ferritin and gonococci were utilizing the subsequent stored iron in a receptor and TonB-independent manner. We expected a decrease in survival in the presence of both ascorbate and leupeptin since both have been proven to decrease ferritin degradation in epithelial cells (38, 244). Surprisingly, gonococci survived very well in the presence of both ferritin

degradation inhibitors (Figure 14B). Neither ascorbate nor leupeptin decreased gonococcal wild type (FA1090) intracellular survival indicating that the TonB and TdfF-independent survival we had previously observed was not due to acquisition of iron from degraded ferritin.

Figure 14. The effect of ascorbate and leupeptin on gonococcal strain FA1090 intracellular survival

A) Intracellular survival assay for gonococcal strain FA1090. The bars represent intracellular survival in CFU/ml (log10) in which the replication media contains: McCoy's 5A+10% FBS (black bar with white hatch mark pattern) McCoy's 5A+10%FBS + 200μM ascorbate (checkered pattern) or McCoy's 5A+ 10% FBS + 10μM leupeptin (black and white horizontal stripes). Time points in hours indicated along the x-axis. All intracellular survival assays were performed in triplicate and the standard deviation is indicated above each bar. A P value of $\leq$ .05was considered statistically significant. A significant decrease in intracellular bacteria compared to wild type in cell culture media alone by $P\leq.05$ is indicated by (@).

B) Intracellular survival assays for gonococcal strain FA1090.The bars represent intracellular survival in CFU/ml (log10) in which the replication media contains: McCoy's 5A + 10% FBS (black bars with white hatch mark pattern), McCoy's 5A alone (solid black), McCoy's 5A + 200uM ascorbate (checkered pattern) or McCoy's 5A+ 10μM leupeptin (black and white horizontal stripes). Time points in hours indicated along the x-axis. All intracellular survival assays were performed in triplicate and the standard deviation is indicated above each bar. A P value of $\leq$ .05 was considered statistically significant. A significant decrease in intracellular bacteria compared to wild type in cell culture media alone by $P\leq.05$ is indicated by (@).

A
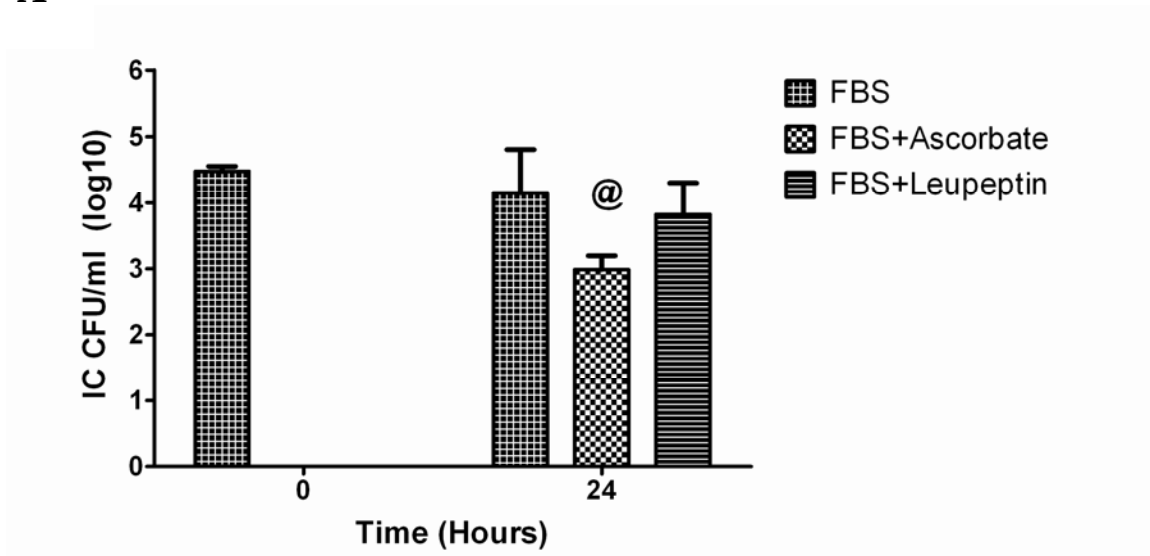
B
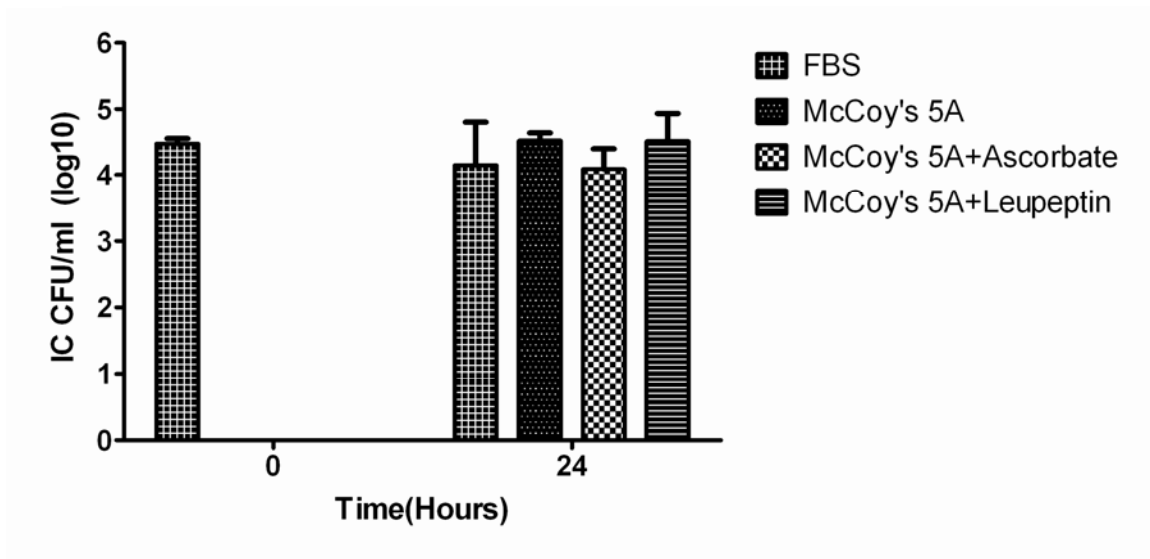
E. Intermediate levels of Ton-independent growth in the presence of 2,5- and 2,3-DHBA

TdfF was originally identified as a TonB-dependent transporter due to its homology to FhuE (286) which is a TonB-dependent siderophore transporter in *E. coli*, (252). FhuE is required for the uptake of iron from the hydroxamate siderophores coprogen, ferrioxamine B and rhodotorulic acid by *E. coli* (119, 152). *N. gonorrhoeae* do not secrete siderophores; however, the bacteria can utilize siderophores produced by other bacteria, known as xenosiderphore utilization. Enterobactin, a catecholate siderophore produced by *E. coli* is utilized by *N. gonorrhoeae* in a TonB-dependent receptor mediated way (43). Based on the homology that TdfF shares with the siderophore receptor of *E. coli*, we hypothesized that gonococci could utilize a host derived siderophore-like molecule in a TonB- or TdfF-dependent manner. Recently a gene encoding a protein important for mammalian siderophore biosynthesis was identified (75). This siderophore-like molecule has a 2, 5-dihydroxybenzoic acid (DHBA) binding moiety which is similar to the bacteria derived siderophore, enterobactin which has a 2, 3-DHBA binding moiety (240). Therefore we investigated the utilization of ferrated 2, 3- and 2, 5-DHBA with plate bioassays. In these assays, gonococci were streaked out onto chemically defined, iron chelated growth media. Wells were bored into the media and each iron source tested was added to these wells. Growth in millimeters around each iron source was measured and an indication that gonococci utilize the iron sources. Growth in the presence of ferrated 2, 3- and 2, 5-DHBA was

analyzed in the following strains: the wild type strain (FA1090), the *tonB* mutant strain

(MCV656), the *tdfF* mutant strain (MCV659), the *fetA* mutant strain (FA6959), and

mpeR mutant strain (MCV304). FetA was identified as a TonB-dependent

xenosiderphore receptor important for enterobactin utilization (43). Ferric citrate was

used as a positive control since gonococci utilize it as an iron source in a Ton-

independent manner. Bovine transferrin was used as a negative control because

gonococci do not use it as an iron source (63, 263). Dihydroxybenozic serine dimer (D2)

is an enterobactin derivative and was used as a TonB-dependent control since gonococci

could only utilize this xenosiderphore in a FetA, TonB-dependent manner (see chapter 4).

Figure 15 shows growth zones in millimeters detected around each iron source indicating

iron source utilization. As expected, D2 utilization was TonB and FetA dependent. When

comparing growth of the wild type in the presence of D2, a growth zone of 30 mm was

observed whereas growth zones of 19mm and 16mm were observed for 2,3-DHBA and

2,5-DHBA (Figure 15). There seems to be a slight increase in growth zones around 2,3-

DHBA compared to 2,5-between the gonococcal strains tested (Figure 15). The wild

type and mutant gonococcal strains exhibited similar intermediate growth in the presence

of ferrated 2, 3-DHBA and 2,5-DHBA compared to iron sources known to be utilized.

These results imply that *N. gonorrhoeae* could utilize iron from these compounds

however less efficiently compared to known iron sources and in a TonB- receptor-

independent manner.

Figure 15. Utilization of 2, 3-DHBA and 2, 5-DHBA by gonococcal strain FA1090
CDM plates were supplemented with apo-bovine transferrin to chelate any iron in the
growth media. Wells within the plates were inoculated with 10µl of the following iron
sources: D2: dimer form of dihydroxybenzoylserine, 2, 3-DHBA, Ferric citrate (+), 2, 5-
DHBA, apo-bovine transferrin. Each bar indicates growth in millimeters around each iron
source. Bars represent the growth zone of the following strains: wild type (FA1090)
(black), *fetA* mutant strain (FA6959) (checkered), *tonB* mutant strain (MCV656) (gray),
mpeR mutant strain (MCV304) (93) or *tdfF* mutant strain (MCV659) (horizontal stripes).
Horizontal dotted line indicates the limit of detection because it is the diameter of the
well containing each iron source. Plate bioassay was only performed once in triplicate.
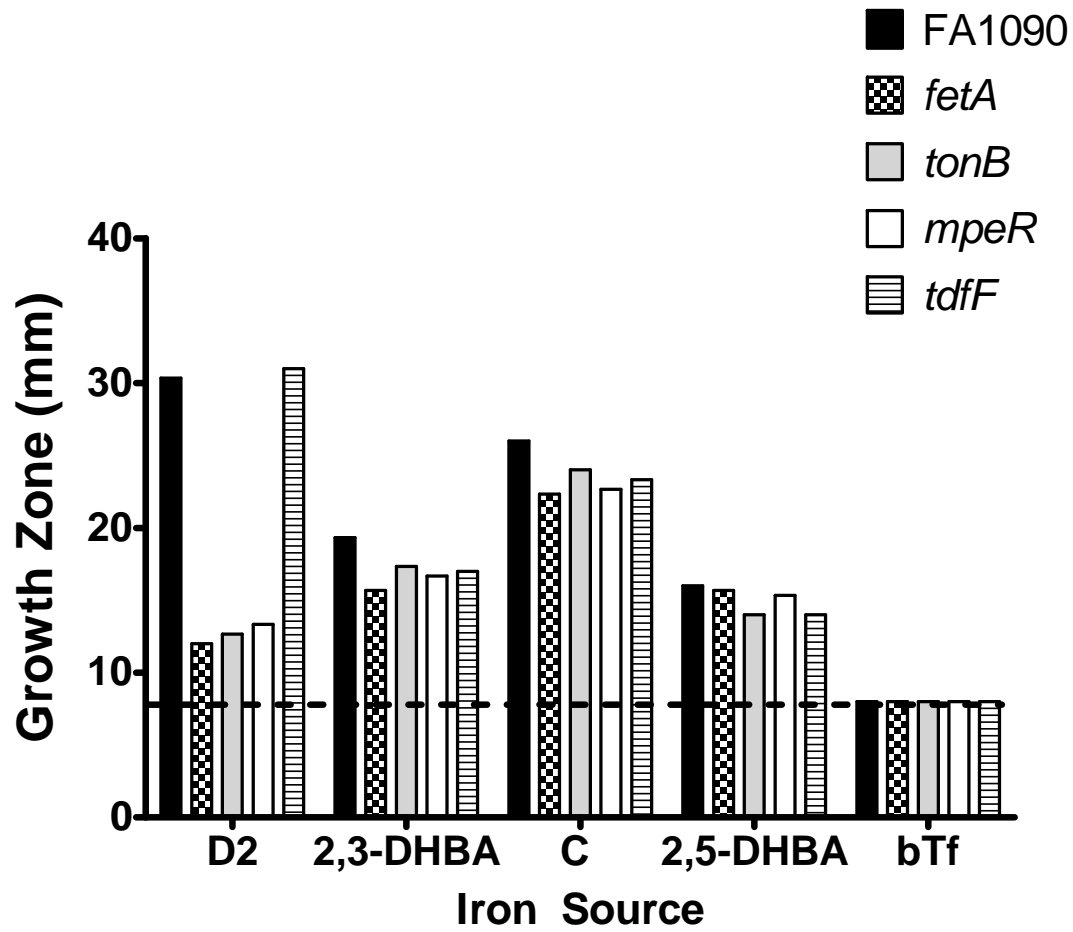
III. Discussion

N. gonorrhoeae, an obligate human pathogen, has a diverse array of iron acquisition systems for utilization of host extracellular iron sources. For a review see (65). Gonococci also possess a pathogen specific gene that codes for a TonB-dependent transporter, TdfF (184, 266). Attenuated intracellular survival was demonstrated by the *tdfF* mutant strain and was rescued with the addition of iron indicating this transporter was important for intracellular iron acquisition (Figure 11) (113).

AraC-like regulators have been shown to be important for host cell invasion. In *Salmonella enterica* serovar Typhimurium, which is a major cause of gastroenteritis, multiple transcriptional regulators have been identified that activate the expression of invasion genes in response to both environmental and genetic regulatory factors (144). Two of these transcriptional regulators, HilC and HilD are predicted to be members of the AraC/XylS family of transcriptional activators, based on homology within their C-terminal domains that contain a characteristic helix-turn-helix DNA binding motif (79, 237, 256). A mutation in *hilD* results in 53-fold decrease in invasion of cultured epithelial cells and a mutation in *hilC* also results in attenuated invasion of host epithelial cells (256). The host specific signals sensed by these AraC-like regulators have not been identified (144). AraC-like regulators also play a role in iron acquisition in other microorganisms including *Y. pestis* and *B. pertussis* (16, 87). In these systems the AraC-like regulator is Fur regulated and once the inducing molecule is sensed under iron-deplete conditions, the AraC-like regulator activates the iron acquisition system. Within close proximity to *tdfF* on the gonococcal chromosome is the gene *mpeR* which encodes an AraC-like transcriptional regulator (Figure 10). MpeR plays a role in the regulation of

hydrophobic agent efflux pumps mediated by the Mtr system (92). MpeR is iron regulated and contains a confirmed Fur box (78, 139). This suggests that MpeR could modulate iron acquisition systems such as TdfF. If MpeR did regulate *tdfF* expression we would expect a change in intracellular survival phenotype in the *mpeR* mutant strain (MCV304). For example, if MpeR acted as an activator for *tdfF* expression then the *mpeR* mutant strain (MCV304) should exhibit decreased intracellular survival. However, if MpeR repressed *tdfF* expression then we would expect the opposite in which the *mpeR* mutant strain (MCV304) would exhibit increased intracellular survival. During our *in vitro* intracellular survival assays the *mpeR* mutant (MCV304) survived as well as the wild type strain suggesting that it does not regulate *tdfF* (Figure 11). One possible explanation for this outcome is that MpeR may be under the control of another regulator. AraC-like regulators are often part of complex regulatory cascades involving hierarchal induction of sequential regulators in response to multiple signals. Additionally, it has been reported that the intergenic region between *mpeR* and *tdfF* contained two Fur boxes and that this intergenic region could serve as two divergent promoters (84). If the bacteria were under iron-replete conditions within the intracellular environment, then the expression of *mpeR* would be repressed and thus the wild type (FA1090) would not express MpeR similar to the *mpeR* mutant strain (MCV304). Recently, an *in vitro* study using S1 nuclease protection assays showed no differential regulation of the genes co-transcribed with *tdfF* in meningococcal mutant strains either lacking or over expressing *mpeR* (84). This study concluded that MpeR was not involved in the regulation of the putative *tdfF* operon under *in vitro* conditions (84).

Since a ligand is expected bind to the N-terminal portion of an AraC-like regulator before it can act as a regulator, the signal specific for MpeR regulation could be missing in our *in vitro* assays as well. In the absence of this inducing ligand, MpeR would not repress or activate *tdfF* expression and a change in intracellular survival between wild type and *mpeR* mutant strains would not be observed.

TdfF was first identified in the *N. gonorrhoeae* FA1090 genome database by comparing its amino acid sequence to a panel of characterized TonB-dependent receptors (286). TdfF has an amino acid identity of 30% to the *E. coli* siderophore receptor FhuE (252, 286). Since *tdfF* expression is host specific and encodes a receptor similar to a siderophore receptor in *E. coli*, we hypothesized that the ligand for TdfF was host derived and possibly a mammalian siderophore-like molecule. Mammalian cells have homologous genes to those that encode siderophores in *E. coli*. A small molecule identified in mammalian cells through gas chromatography-mass spectrometry contains a 2, 5-dihydroxybenzoic acid (DHBA) moiety (75). Dihydroxybenozic acid is incorporated into various bacterial derived siderophores. 2,3-DHBA is the iron-binding moiety of bacterial enterobactin (240) and similar to 2,3-DHBA, 2,5-DHBA can chelate iron (75). As previously stated, DHBA is a binding moiety that contains a carboxylic acid group by which the ring attaches to various scaffolds via amide linkages. Enterobactin consists of three dihydroxybenzoic acid subunits (2,3-DHBA) linked together with serine backbone (208). Gonococcal strain FA1090 utilized the xenosiderophore enterobactin through a FetA, TonB-dependent mechanism (43). TonB-independent siderophore utilization has also been observed for xenosiderophores in other gonococcal strains (273). Therefore we investigated whether gonococcal strain FA1090 could utilize 2, 5-DHBA and if

utilization was TonB- and/or TdfF-dependent, potentially identifying the ligand for TdfF. Gonococci utilized both ferrated 2,5- and 2,3-DHBA in a Ton- FetA- and TdfF-independent manner (Figure 15). For wild type strain FA1090, DHBA derivatives were utilized at intermediate levels in comparison to the positive control or DHBS dimer (D2) (Figure 15). It is possible that the intermediate growth observed in the presence of DHBA was because the TonB-dependent receptors did not recognize the iron binding moieties alone. Perhaps, the xenosiderophore receptor recognized the amino acid side chains associated with the DHBA binding moiety. In *E. coli* the TonB-dependent receptor for enterobactin, FepA, is an outer membrane transporter with eleven surface exposed loops at the entrance of the membrane channel. FepA transports the catecholate enterobactin, but no other catecholates, nor any non-catecholate compounds (8, 42). Studies in which the 11 surface loops are mutagenized indicate that the receptor's loops enwrap the ferric siderophore at binding equilibrium and this interaction is specific to the siderophore amino acid backbone (8, 42). The gonococcal TonB-dependent xenosiderophore receptors may have a similar discrimination for specific catecholate iron sources and may still recognize and utilize iron from the intact putative mammalian siderophore even if it does not utilize iron from the binding moiety within the siderophore. The TonB-independent intermediate levels of growth observed through the plate bioassays may be due to an alternative uptake pathway that involves the periplasmic ABC transport system, FbpABC and porin acting as the outer membrane portal for the ferrated DHBA derivatives as well as free iron. This Ton-independent pathway has been identified in gonococcal strain FA19 (273) and we hypothesized that gonococcal strain FA1090 uses

both a TonB-, receptor dependent pathway as well as a Ton-independent FbpABC pathway for iron utilization from xenosiderophores.

Another potential iron source for intracellular gonococci could be degraded ferritin. Ferritin cannot directly act as an iron source for *Neisseria* (10, 41). However, iron from degraded ferritin could be utilized by gonococci when bound to a protein or transport molecule that was involved in the trafficking from degraded ferritin to the cellular cytosol. During meningococcal infections, ferritin is redistributed within the epithelial cells to where the meningococci are located (162). Ferritin is also degraded during meningococcal infections and iron previously contained within ferritin is released as a low-molecular-weight species during infection (162). Preventing ferritin degradation with the use of ascorbate or leupeptin reduces meningococcal intracellular replication (162). Thus during infection, meningococci could possibly access the iron stored by ferritin bound to a low molecular weight iron transporter. Epithelial cells infected with *N. gonorrhoeae* exhibited an altered distribution of surface and cycling transferrin receptors as well as reduced internalization of ferrated transferrin (31). A reduction in transferrin cycling by the epithelial cells would lead to iron-deplete conditions for the cell and ferritin degradation. Therefore we hypothesized that *N. gonorrhoeae* intracellular survival was dependent on iron stored within the cytosolic ferritin. We first investigated intracellular survival in serum starved cells.

 Serum starvation increased desferal-chelatable pools derived from degraded ferritin within the epithelial cell (211). We observed an increase in intracellular survival in the absence of FBS amongst all gonococcal strains tested indicating that gonococci could access iron from desferal-chelatable pools in a TonB- TdfF-independent manner

(Figure 11B). Excess apo-bovine transferrin was added to the replication media to chelate any excess iron in the McCoy's 5A cell culture media and resulted in a TonB-TdfF- independent survival (Figure 13B). We hypothesized that the bovine transferrin was not chelating all the excess iron in the McCoy's 5A media and we were observing an iron induced invasion (Figure 12). An alternative hypothesis was that the excess bovine transferrin was chelating any excess iron away from gonococci and delivering it to the epithelial cells, preventing ferritin degradation due to the influx of iron. To elucidate the mechanism of increased survival in the absence of FBS we performed intracellular survival assays in the presence of the ferritin degradation inhibitors ascorbate and leupeptin. Neither inhibitor affected intracellular survival indicating that the survival observed in the presence of McCoy's 5A was not dependent on iron originating from ferritin (Figure 14B). We suspect that the TonB- TdfF- independent survival observed in the presence of McCoy's 5A alone is due to excess iron within the media. McCoy's 5A medium was originally developed for the growth of Novikoff hepatoma cells. It is a general purpose medium for both primary and established cell lines (293). McCoy's 5A contains inorganic salts, amino acids, vitamins, peptone, glutamine and glucose. Iron is not a component of McCoy's 5A; however, iron is not chelated and iron could be in any of the media's ingredients. The addition of fetal bovine serum to McCoy's 5A chelated any free iron due to bovine specific serum iron binding proteins. We concluded that McCoy's 5A media supplemented with 10% heat-inactivated FBS has the lowest amount of free iron for gonococci to utilize, McCoy's 5A media supplemented with 40ug/ml apo-bovine transferrin has an intermediate amount of free iron, and McCoy's 5A media has the freest iron when comparing all three media. Hence, the difference in intracellular

survival phenotypes in the various media could be due to gonococci acquiring free iron from these media.

When investigating intracellular survival in the presence of FBS, we confirmed previous findings that survival is both TonB- and TdfF- dependent (113, 161) (Figure 10A) and it was dependent on iron acquisition (Figure 11). Interestingly, in the presence of FBS we did observe attenuated gonococcal growth in the presence of ascorbate (Figure 14A). One conclusion we can draw from this result was that when intracellular iron was the only source of iron available to gonococci, the host cell must first degrade ferritin before gonococci could use the iron contained within the ferritin core. However, if ascorbate prevented intracellular survival, then why did we not observe a difference in survival in the presence of Leupeptin? Leupeptin inhibits lysomal protease and also prevents ferritin degradation. We may have been observing an indirect effect by ascorbate that did not involve ferritin degradation. Ascorbate can also complex with iron and could be sequestering iron from the gonococci during the infection. Then the observed effect that ascorbate had on gonococcal infection would not be due to lack of iron from degraded ferritin but rather survival under iron deplete conditions.

If gonococci were acquiring iron originating from degraded ferritin then it would still be assumed that ferritin degradation leads to the production of another iron-binding protein that was used by the bacteria. As investigations into cellular iron metabolism continue it will be important to determine if these iron transport protein/molecules could be hijacked by N. *gonorrhoeae*.

In conclusion we determined that gonococci could utilize iron from iron binding moieties commonly found in bacterial siderophores and a mammalian siderophore in a

Ton and receptor-independent manner. We also determined that ascorbate inhibits intracellular gonococcal survival which could be due to the inhibition of ferritin degradation or iron sequestration by the ascorbate. Finally, MpeR did not play a role in intracellular survival in these *in vitro* assays. Future studies on understanding gonococcal intracellular iron acquisition would provide a clear picture of intracellular pathogenesis caused by *N. gonorrhoeae*.

**Chapter 5 The IRON-REPRESSED, AraC-LIKE REGULATOR, MpeR,
ACTIVATES EXPRESSION OF *fetA* IN *Neisseria gonorrhoeae***

I. Introduction

Neisseria gonorrhoeae is an obligate human pathogen that primarily infects the

urogenital or anorectal mucosa following intimate contact. *N. gonorrhoeae* is the

etiological agent of gonorrhea, which is the second most commonly-reported, notifiable

infectious disease in the United States. In 2009, the Centers for Disease Control and

Prevention reported a total of 301,174 cases of gonorrhea in the United States(48);

however, this is thought to be a conservative estimate due to underreporting. In men, a

gonococcal infection is characterized by acute urethritis with symptoms that include

purulent discharge and dysuria. It is estimated that up to 80% of women infected with *N.*

gonorrhoeae are asymptomatic or present with very minor symptoms (190). Women

with symptomatic disease experience cervicitis and vaginal discharge. When left

untreated, due to the asymptomatic nature of the infection in women, the bacteria can

ascend to the upper female genital tract. This ascending infection can result in pelvic

inflammatory disease, which may lead to ectopic pregnancy or infertility (203, 267). The

Centers for Disease Control currently only recommends extended-spectrum

cephalosporins for treatment due to increased antimicrobial resistance to all previously-

recommended therapies(50, 138, 172). Unfortunately, resistance to this class of antimicrobial agent has already emerged (48). *N. gonorrhoeae* infections do not elicit protective immunity and there is evidence that gonococcal infections increase the spread of HIV(60, 188). Since gonococcal disease poses a significant public health challenge, it is important to understand the pathogenesis of *N. gonorrhoeae* in order to identify new therapies.

Iron is an essential macronutrient for most microorganisms including the *Neisseriae* (37). Many microorganisms acquire iron from the human host by synthesizing and secreting siderophores. Siderophores are low-molecular weight iron-chelating molecules that scavenge iron from the environment or host iron binding proteins. *N. gonorrhoeae* does not synthesize siderophores but instead obtains iron directly from human iron binding proteins including transferrin, lactoferrin, and hemoglobin in a receptor-mediated mechanism (25, 53, 64, 163). Expression of either the transferrin or lactoferrin receptor by *N. gonorrhoeae* is necessary to establish infection in human male volunteers (5, 66). Gonococci can also hijack siderophores produced by other bacteria, which is known as xenosiderophore utilization. It has been previously demonstrated that strains of gonococci can obtain iron from the xenosiderophores enterobactin, aerobactin, and salmochelin, which are all synthesized by enteric bacteria (43, 273, 303).

Iron acquisition is tightly regulated since excess iron can promote Haber-Weiss-Fenton chemistry, creating highly reactive, toxic hydroxyl radicals within the cell (115). In many bacteria including *N. gonorrhoeae*, the ferric uptake regulator (94) acts as a transcriptional regulator for iron acquisition genes. Under iron-replete (+Fe) conditions, a dimer of Fur binds to its co-repressor, ferrous iron, and assumes a DNA binding

conformation. The Fur-Fe^{2+} complex binds to a specific DNA sequence called the Fur box found in the promoter regions of iron regulated genes (13). Once Fur is bound to the Fur box it blocks gene transcription. As intracellular iron stores become depleted, apo-Fur dissociates from the Fur box allowing RNA polymerase to bind to the promoter and transcribe the Fur regulated gene (83). Gonococcal Fur not only regulates iron acquisition genes but also impacts the expression of a broad range of genes including those that encode Opa proteins, NADH dehydrogenase, sodium pumps and other transcriptional regulators (139). The regulation of genes involved in iron acquisition, adhesion and metabolism establishes Fur as a global regulator (139, 264).

Fur may not be the sole transcriptional regulator of iron acquisition systems in *N. gonorrhoeae*. AraC-like regulators operate as both positive and negative regulators of iron acquisition systems in other microorganisms including *Pseudomonas aeruginosa*, *Yersinia pestis*, and *Bordetella* (16, 87, 127, 227). In these microorganisms, the AraC-like regulator functions as a transcriptional regulator of siderophore biosynthesis and acquisition genes. The mechanism of AraC-like regulation of siderophore genes involves the cognate siderophore functioning as a co-inducer. The AraC-like regulator is under the transcriptional control of Fur and therefore AraC-like regulation occurs under iron deplete conditions. The gonococcal genome encodes multiple AraC-like regulators and it has been recently demonstrated that one of these regulators, MpeR (92) is Fur regulated (139).

In this study, we demonstrate that MpeR, an iron-regulated AraC-like regulator is required for up-regulated expression of the outer membrane transporter, FetA, in gonococcal strain FA1090. We also establish that *fetA* is part of an iron regulated operon

that encodes a periplasmic binding protein and components of a putative ABC transport system; however, only *fetA* but not the downstream genes require MpeR for detectable expression. Additionally, we determined that gonococcal strain FA1090 acquires iron from enterobactin, enterobactin derivatives and salmochelin S2 in a FetA- and TonB-dependent manner and identified genetic differences between strains that could clarify the Ton-dependent and Ton-independent pathway for xenosiderophore utilization. Expression of MpeR was necessary to achieve maximal growth on these siderophores, but none of the utilizable iron sources appear to act as a co-inducer for MpeR-dependent activation of *fetA*.

II. Results

A. The *mpeR* mutant displays differential protein expression under iron-deplete conditions The *mpeR* gene was originally identified by Folster *et al.* as encoding an AraC-like regulator that plays a role in the coordinate expression of hydrophobic agent efflux pumps (92). We began investigating whether MpeR plays other regulatory roles in gonococci due to its proximity to a gene that encodes an uncharacterized TonB-dependent transporter, TdfF(113). We initially determined that *mpeR* was iron regulated, which was recently confirmed by Jackson *et al.* via microarray, FURTA assays, and the presence of a Fur box in its promoter (139). We hypothesized that since MpeR is iron regulated, it could also regulate iron acquisition systems in gonococci, particularly the one putatively encoded by the *tdfF* locus. To test this hypothesis, the wild type (FA1090) and *mpeR* mutant (MCV304) were grown under iron-deplete (-Fe) and iron-replete (+Fe) conditions and total membrane protein fractions were isolated. Using SDS-PAGE analysis, we determined that an 80 kDa protein was expressed by the wild-type strain only under iron-deplete conditions, but this protein was not detectable in the *mpeR* mutant strain (Figure 16A). The 80 kDa band was excised from the gel and analyzed by mass spectrometry, which unambiguously identified the protein as FetA. Western blot analysis of total membrane proteins isolated from the wild type (FA1090), *mpeR* mutant (MCV304), *fetA* mutant (FA6959), and *mpeR*[C] (MCV305) grown under iron-deplete (-) and iron-replete (+) conditions were used to confirm the mass spectrometry results. The wild-type and complemented strains grown under iron-deplete conditions expressed detectable levels of FetA, whereas neither the *fetA* mutant nor the *mpeR* mutant expressed

this protein (Figure 16B). From this analysis, we concluded that the iron-regulated AraC-

like regulator, MpeR, controls the expression of FetA in gonococcal strain FA1090.

Figure 16. FetA expression is regulated by MpeR

A) SDS-PAGE analysis of protein expression. Total membrane proteins were isolated from WT (FA1090) and *mpeR* mutant (MCV304) strains grown under iron-deplete (-) and iron-replete (+) conditions for 4 hours. Proteins were separated on a 7.5% acrylamide gel. The arrow on the left indicates the band that was excised and identified as FetA by mass spectrometry analysis. The position of molecular weight markers is indicated on the right.

B) Western blot analysis of FetA expression. The WT (FA1090), *mpeR* mutant (MCV304), *fetA* mutant (FA6959) and the complemented *mpeR*^{C} strain (MCV305) were grown under iron-deplete (-) and iron-replete (+) conditions. Total membrane proteins from each strain were isolated and standardized before being separated by SDS-PAGE and then transferred to nitrocellulose. Blots were probed with an anti-FetA monoclonal antibody.
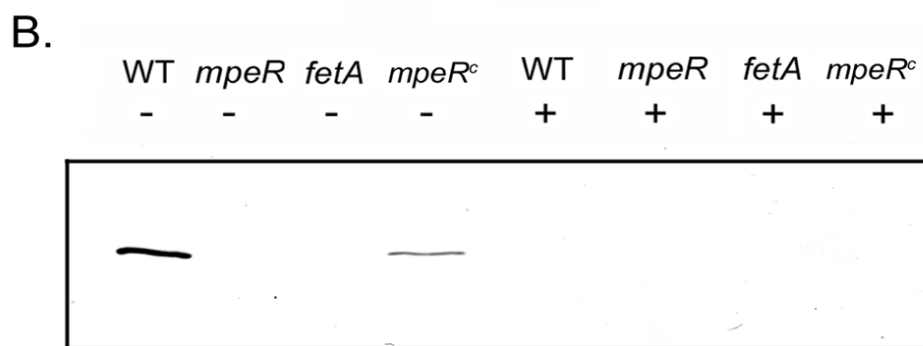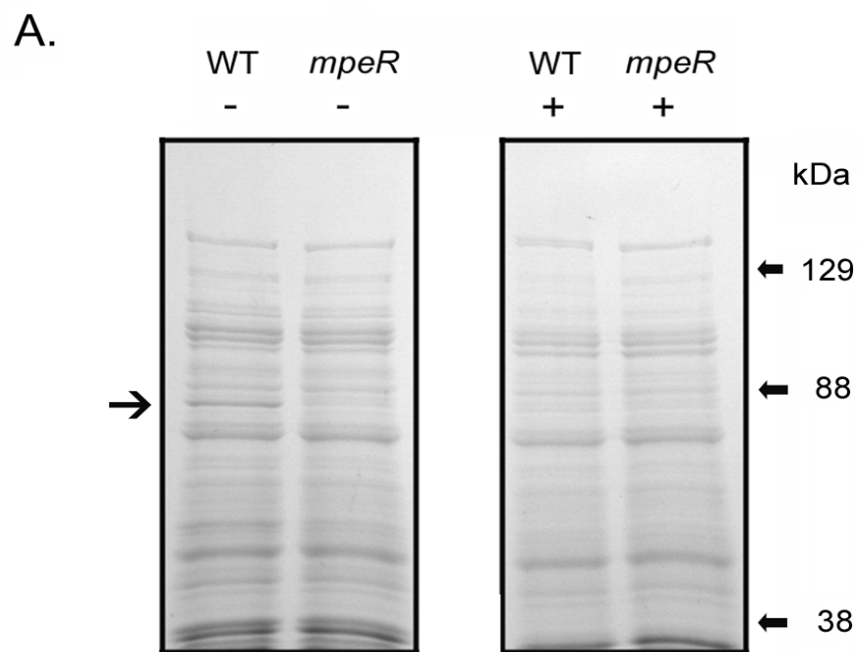
B. *fetA* transcription requires MpeR expression and iron-deplete growth conditions

fetA, previously called *frpB*, encodes a TonB-dependent outer membrane transporter (FetA) that is immunogenic, and subject to Fur regulation (20, 43, 219, 283). FetA was renamed by Carson *et al.* when they discovered that this transporter was necessary for efficient ferric-enterobactin transport (43). To determine whether MpeR transcriptionally regulates *fetA*, end-point relative RT-PCR was utilized to detect *fetA* and *mpeR* transcripts. RNA was isolated from gonococcal strains grown under iron-deplete (-) and iron-replete (+) conditions. Transcripts from *fetA* and *mpeR* were detected in the wild-type strain preferentially under iron-deplete conditions (Figure 17), consistent with previous published studies (44, 139). The *fetA* transcript was not detected in the *mpeR* mutant (MCV304). In the *mpeR*^{C} strain (MCV305), both *mpeR* and *fetA* transcripts were detected (Figure 17) indicating that the MpeR effect on *fetA* expression was restored by complementation. Expression of *mpeR* in the *mpeR*^{C} strain (MCV305) was iron regulated (Figure 17), consistent with the presence of iron-sensitive regulatory signals in the sequence upstream of the MpeR start codon, which were included in the complementation construct. Overall, these data allow us to conclude that MpeR is necessary for *fetA* expression under iron-deplete conditions.

Figure 17. MpeR activates *fetA* **transcription under iron-deplete conditions** The WT (FA1090), *fetA* mutant (FA6959), *mpeR* mutant (MCV304), and the complemented *mpeR*[C] strain (MCV305) were grown under iron-deplete (-) and iron-replete (+) conditions. RNA samples isolated from each gonococcal strain were analyzed for expression of *fetA* and *mpeR* by RT-PCR. *16S rRNA* (*16S*) was used as a positive control because it was constitutively expressed under all growth conditions. Expression of *16S rRNA* in the absence of reverse transcriptase, (*16S* (-)) was used as a negative control.
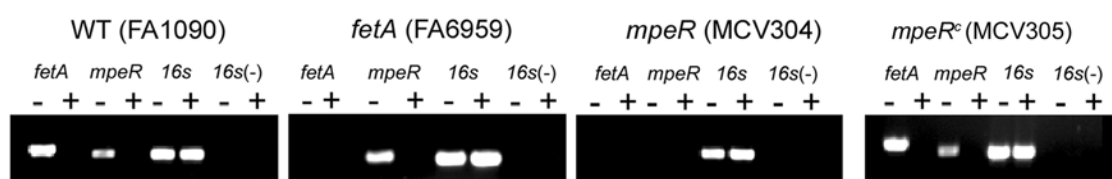
Real time RT-PCR was employed to confirm the end-point RT-PCR results and to quantify the level of gene expression. All C_T values were normalized to *rmpM* gene expression and then fold change was determined using the relative C_T method (179). The *rmpM* gene encodes an outer membrane protein that is constitutively expressed under all growth conditions tested in this study. In Table 4, the average fold change is shown, representing the comparison between expression under iron-deplete and iron-replete conditions or expression by wild-type and mutant under iron deplete conditions. As expected, the fold change in expression under iron deplete vs. iron replete conditions was large for both *fetA* and *mpeR* (97-fold for *fetA* and 198-fold for *mpeR*; Table4). When the wild type and its isogenic *fetA* mutant were compared, there was 194-fold more *fetA* expression in the wild type (Figure 18A). *mpeR* expression was unaffected in the *fetA* mutantm (Figure 18). When the wild type and the *mpeR* mutant were compared, there was a 117-fold change in *fetA* expression (Table 4), which is consistent with the inability to detect *fetA* transcripts in the *mpeR* mutant as observed by qualitative RT-PCR (Figure 17). As shown in Table 4 and Figure 18B, the restoration of the *mpeR* gene in the *mpeR*^C strain resulted in *mpeR* expression levels that approached that of the wild-type (1.9 fold difference between wild-type and the *mpeR*^C strain). Commensurate with the return of *mpeR* expression, we detected an increase in FetA expression in the *mpeR*^C strain relative to the *mpeR* mutant (Table 4), again supporting the relative RT-PCR data (Figure 17). Together, the expression data support the previous observations that both *fetA* and *mpeR* are iron regulated (20, 139) and furthermore demonstrate that *mpeR* expression was not impacted by the *fetA* mutation. Results from the relative and real-time expression studies establish that when *mpeR* was absent, *fetA* expression decreased and when *mpeR*

expression was restored by complementation with a wild-type copy, *fetA* levels returned to near wild-type levels under iron-deplete conditions. Cumulatively, these data indicate that MpeR is necessary for *fetA* transcription under iron-deplete conditions.

Figure 18. Real time RT-PCR analysis for MpeR activation of *fetA* expression under iron-deplete conditions

A. Normalized *fetA* gene expression ratio. Average fold change of *fetA* expression for each comparison is shown. Bars represent the median fold change for the following comparisons: WT-Fe/WT+Fe (gray bars) WT-Fe/ FA6959-Fe (checkered bars), WT-Fe/MCV304-Fe (horizontal striped bar), WT-Fe/MCV305-Fe (vertical striped bar) and WT-Fe/MCV306-Fe (white bar). The median fold change is detected from three independently-conducted real time RT-PCR assays. Numerical values from all three assays indicated in Table 4.

B. Normalized *mpeR* gene expression ratio. Average fold change of *mpeR* expression for each comparison is shown. Bars represent the median fold change for the following comparisons: WT-Fe/WT+Fe (gray bars) WT-Fe/ FA6959-Fe (checkered bars), WT-Fe/MCV304-Fe (horizontal striped bar), WT-Fe/MCV305-Fe (vertical striped bar) and WT-Fe/MCV306-Fe (white bar). The median fold change is detected from three independently-conducted real time RT-PCR assays. Numerical values from all three assays indicated in Table 4.

A

fetA gene expression

B

mpeR gene expression
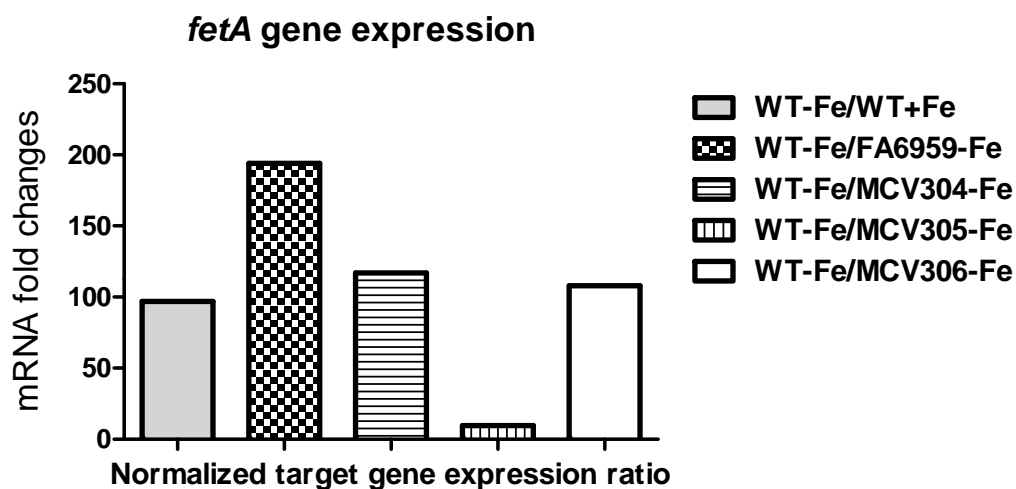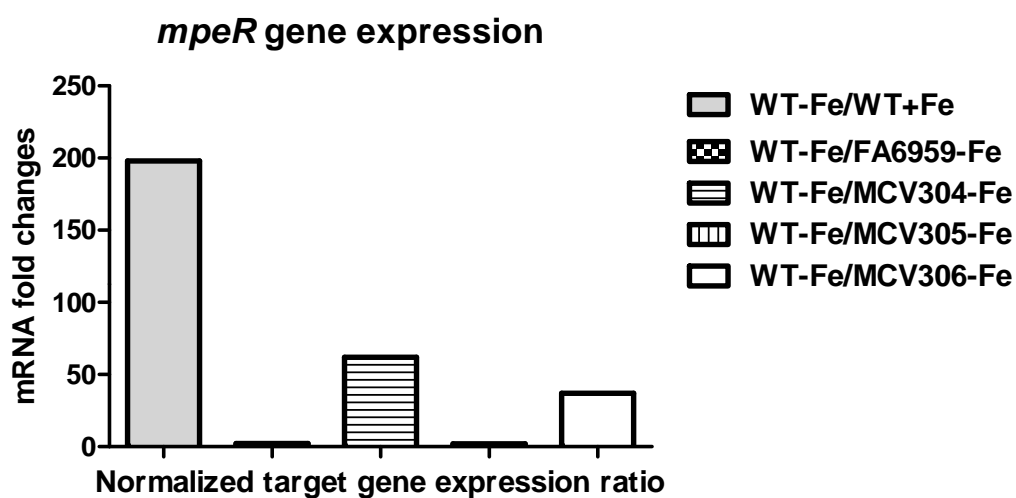
TABLE 4. Effects on *fetA* and *mpeR* expression measured by real time RT-PCR.

Comparison	Normalized target gene expression ratio	
	fetA	*mpeR*
WT-Fe / WT+Fe	97 (103-83)[1]	198 (301-106)
WT-Fe / FA6959-Fe	194 (209-167)	2.2 (3.1-.6)
WT-Fe / MCV304-Fe	117 (185-33)	62 (123-46)
WT-Fe / MCV305-Fe	9.6 (15-5)	1.9 (2.4-1.3)
WT-Fe / MCV306-Fe	108 (154-70)	37 (45-26)

[1] Average fold change for each comparison is shown, with the range detected among three independently-conducted real time RT-PCR assays shown in parentheses.

C. Specific binding of MpeR to DNA upstream of *fetA*

In order to determine whether MpeR-dependent *fetA* regulation occurred as a result of direct interaction, we used EMSA to test whether MpeR bound to target DNA sequences upstream of the FetA start codon. Results from preliminary EMSA experiments using increasing amounts of MBP-MpeR (1-10 µg) incubated with the radiolabeled *fetA* probe showed shifting of a DNA fragment of 500 bp in length (data not shown). This upstream region was further divided into two smaller DNA fragments of 250 bp in length (Figure 18A), both of which were tested for MpeR interaction by EMSA. The probe fragment corresponding to the most upstream portion of the intergenic region (*fetA*1, Figure 19A) was shifted by addition of MpeR protein (Figure 19B). However, the probe fragment corresponding to the sequence immediately preceding the *fetA* start codon (*fetA*2; Figure 19B) was not shifted in the presence of MpeR (data not shown). In order to determine whether MpeR binding upstream of *fetA* was specific, a competitive EMSA experiment was performed in which increasing amounts (2-20X) of excess unlabeled specific (*fetA*) or non-specific (*rnpB*) DNA fragments were added to the binding reaction. The results demonstrate that the specific competitor, but not the non-specific competitor, reduced binding of MpeR to the labeled *fetA* probe (Figure 19B). The unlabeled *fetA*2 sequence modestly competed with the binding of MpeR to the *fetA*1 probe, suggesting that MpeR may bind preferentially to *fetA*1 sequence and with a lower affinity to the *fetA*2 sequence. Cumulatively, these results allow us to conclude that binding of MpeR to the DNA sequence upstream of *fetA* is specific and that its capacity to activate *fetA* expression is likely by a direct mechanism.

Figure 19. MpeR binds upstream of *fetA* in a specific manner

A) Sequence of the 500-base pair, intergenic region immediately upstream of *fetA*. The sequence highlighted in teal is contained within the *fetA*1 probe employed for the EMSA shown in panel B. The sequence highlighted in yellow is contained within the *fetA*2 competitor DNA. The sequence highlighted in blue is contained in both *fet*A1 and *fet*A2 amplicons. The Fur-binding site (139) is highlighted in gray. The previously-mapped (14) promoter elements (underlined) and transcriptional start site (asterisk below nucleotide) are identified. The start codon for FetA is shown in red.

B) 5 nanograms of the 250-base pair, labeled *fetA*1 probe (lane 1) was incubated with 10 μg of MBP-MpeR in the absence of unlabeled competitor (lane 2) or in the presence of 2, 10 or 20X excess unlabeled competitor DNA. Lanes 3-5 contain reactions including increasing concentrations of the specific competitor (*fetA*1); lanes 6-8 contain reactions including increasing concentrations of *fetA*2; and lanes 9-11 contain reactions including increasing concentrations of the non-specific competitor, *rnpB*.

A.

CTCCGATAAATGGTTTGAAAACAATCATCTGCCCGAACGGTTCG
GACAAATTGAAGTGGAAACCGGGCTGCCGCCTAACGGCCTGCCC
GTACAAGTTGGCAAAATTAGGGTGTGTGCGGGAAAATTCAAGTG
AGGCGGAAAAAAATTTATTTCCGCCGTTTTTTATAGTGGACTAA
ATTTAAACCGGTACGGCGTTGCCTCGCCTTAGCTCAAAGAGAAC
GATTCTCTAAGGTGCTGAAGCACCAAGTGAATCGGTTCCGTACT
ATCTGTACTGCCTGCGGCTTCGCCGCCTTGTCCTGATTTTTGTT
AATCCACTATACATTTCCGACAAAACCTGTCAACAAAAAACAAC
GCTTCGCAAATAAAAACGATAATCAGCTTTACACAACCCCCCCCC
CCCCGCTAATATAAACAAAAATAATTATTATTATTTTTTCTTA
TCCTGCCAAACCTTAACGGTTTGGCTTAACTTCCCTTCATACAC
TCAAAAGGACGAACAAATG

B.

1 2 3 4 5 6 7 8 9 10 11
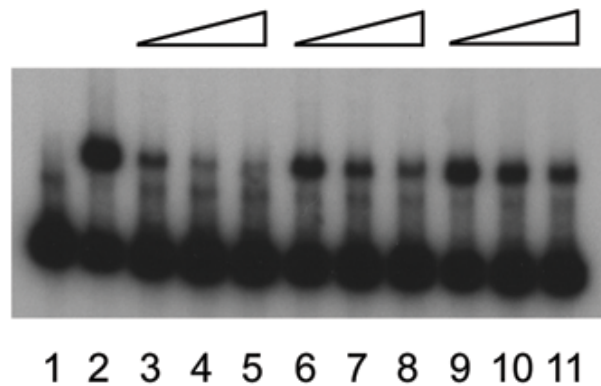
D. *fetA is* **part of an iron responsive operon that is differentially affected by MpeR expression**

The DNA region downstream of *fetA* encodes putative xenosiderophore acquisition genes (Figure 20A). The genes downstream of *fetA* include: *fetB*, which encodes a putative periplasmic binding protein; *ng2091* and *ng2090*, which encode two predicted permease proteins; and *ng2088*, encoding an ATP binding protein. The latter three genes are therefore predicted to form an ABC transport complex. *ng2089* encodes an uncharacterized protein. Most siderophore acquisition systems are co-transcribed and all genes involved in the system are coordinately regulated. Therefore, end point RT-PCR was utilized to determine if *fetA* and the downstream genes were co-transcribed and also activated by MpeR. Primers were designed to amplify the intergenic regions between each gene in the hypothetical operon (Figure 20A). RNA was isolated from gonococcal strains grown under iron-deplete (-) and iron-replete (+) conditions. In the wild-type strain (FA1090) all amplicons were detected under iron-deplete conditions (Fig. 19B) indicating that *fetA* and downstream genes are part of an iron regulated operon. Interestingly, the *fetA* mutant, which contains a polar Ω (228) insertion in the *fetA* gene, maintained the ability to express all of the downstream putative genes (Figure 20B). Similar results were observed in the *mpeR* mutant, in which genes *fetB*-NG2088 was co-transcribed under iron-deplete conditions (Figure 20B). A *fetA mpeR* double mutant strain was also employed to investigate co-transcription of the ABC transport genes. Again, the downstream putative transport genes, in the absence of both *fetA* and *mpeR* were all transcribed under iron-deplete conditions (Figure 20B). Finally, when the *mpeR* mutant was complemented with the wild-type *mpeR* gene, *fetA* and the

downstream co-transcripts were detected under iron-deplete conditions, similar to the wild-type strain. Based on these RT-PCR results, we propose that the wild-type strain produces two different transcripts under iron-deplete conditions (Figure 20A). One transcript encodes *fetA-ng2088* whereas the other transcript encodes *fetB-ng2088*, expression of which is iron regulated but not qualitatively affected by MpeR (Figure 20A).

Figure 20. FetA is encoded as part of a multi-gene operon
 A) Genetic locus including *fetA* and downstream genes. Genes are depicted by boxes. Hatched regions 5' of *fetA* and *fetB* indicate approximate locations of Fur boxes (139). Below the chromosomal locus are small dark arrows indicating primer locations. Numbered black bars denote the amplicons generated from each primer set. Long, dark gray arrows indicate length and start positions of two proposed transcripts.

B) RT-PCR analysis of the *fet* operon. RNA was isolated from the indicated gonococcal strains which were grown under iron deplete (-) and iron replete (+) conditions. Amplicon numbers correspond to the diagram in panel A. *16S rRNA* (*16S*) was used as a positive control because it is constitutively expressed under all conditions test. Expression of *16S rRNA* in the absence of reverse transcriptase, (*16S* (-)) was used as a negative control.
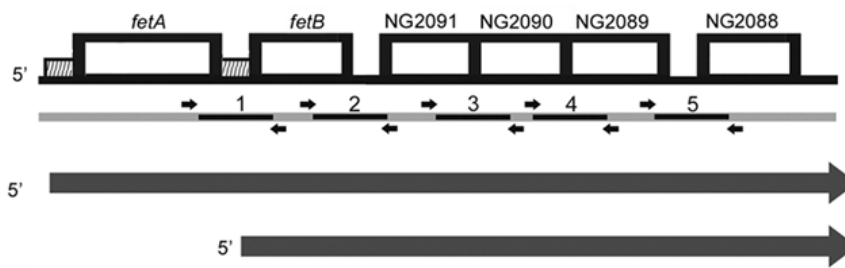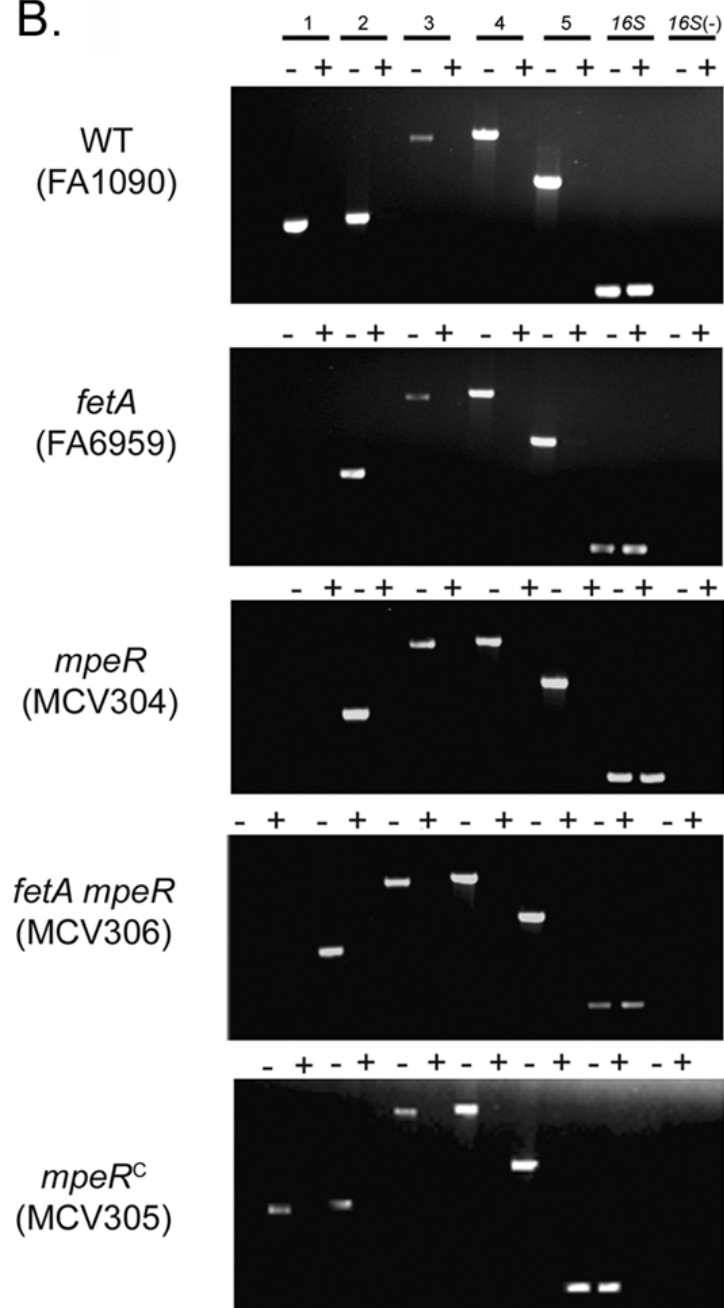
The *fetA* transcriptional start site was previously mapped (44) and is identified in Figure 18. To confirm the presence of a second transcriptional start site upstream of *fetB*, we employed primer extension analysis. As shown in Figure 21, we identified a transcriptional start site downstream of several possible -10 sequences, within the previously-identified Fur binding site (139). The transcript starting with *fetB* was modestly iron regulated and the initiation site is located 72 nucleotides upstream of the FetB start codon.

Figure 21. Identification of the *fetB* transcription start site

A) Sequence of the intergenic region immediately upstream of *fetB*. The ATG at the end of the sequence represents the FetB start codon. Several overlapping, potential -10 promoter elements are underlined. The Fur-binding site (139) is highlighted in gray. The transcriptional start site identified in this analysis is identified by the asterisk.

B) Primer extension products generated from RNA samples harvested from wild-type gonococcal strain FA1090 grown under iron replete (+Fe) and iron deplete (-Fe) conditions. Equivalency of the amount of RNA template in each sample was confirmed by ethidium bromide staining of RNA separated on an agarose gel. For comparison, the sequencing reaction using the same primer as was used for the primer extension reaction, is shown on the left. The T residue highlighted by the asterisk marks the point of transcript initiation on the non-coding strand.

A.

```
TAAAACGCACATCCCGAAAAAATGCCGTCTGAAAGCCTTTCAGA
CGGCATCTGTCCTGATAATTTGATATAGATTATCATTTATCCTT
TCTAAAGCCGTTCCGGTTTGTCCGACCGGCGGCTTTGCCCCAAT
ATCCCCATTTTGGAGACACCCTATG
```

*

B.

C   T   A   G   -Fe   +Fe
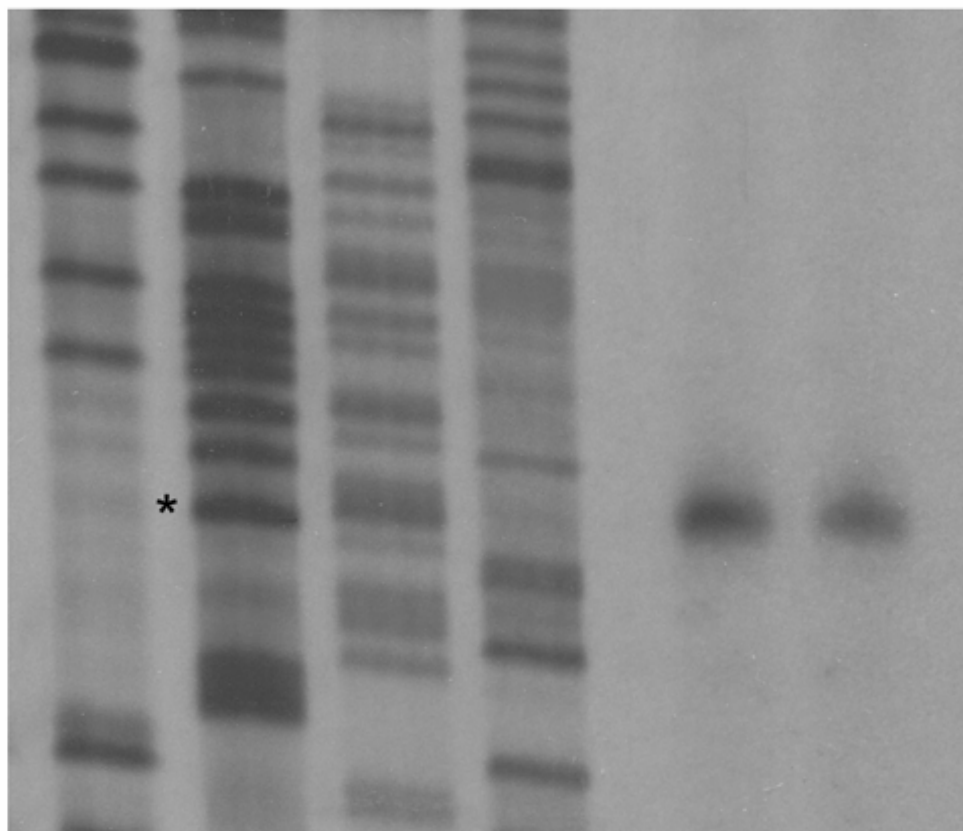
To establish whether *mpeR* or *fetA* interruption quantitatively impact expression of the downstream ABC transport genes, real-time RT-PCR was employed. *ng2091* is the first gene transcribed downstream of the gene encoding the putative periplasmic binding protein (*fetB*) (Figure 19A). The same RNA samples isolated for qualitative RT-PCR (Figure 18B) were used for real-time RT-PCR analysis. All C_T values were normalized to *rmpM* gene expression. In Table 5 and Figure 22, the average fold change is shown, representing the comparison between expression under iron-deplete and iron-replete conditions or expression by wild-type and mutant under iron deplete conditions. As shown in Table 5, *ng2091* gene expression was iron repressed; 16.3 fold more transcript was detected under iron deplete conditions. When the wild-type and mutant strains were compared, we observed that *ng2091* gene expression levels were 23-fold higher in the wild type relative to the *fetA* mutant (Figure 22). Similarly, *ng2091* expression was 22.3-fold higher in the wild type relative to the *mpeR* mutant. The wild-type strain expressed 11.5-fold more *ng2091* transcript than the *fetA mpeR* double mutant (Table 5). The *mpeR*^C strain expressed more *ng2091* transcript than did the *mpeR* mutant, as expected (Figure 22). Cumulatively, these results indicate that *fetA* is part of an iron-regulated operon encoding a putative periplasmic binding protein and ABC transport system. The quantitative expression studies suggest that the ABC transport system is not as tightly iron regulated as is either *fetA* or *mpeR*, which is consistent with the transcriptional start site mapping data presented in Figure 21. Furthermore, the real-time RT-PCR data indicate that expression of *ng2091* is activated by MpeR, but that the extent of this activation is not as great as was detected for *fetA*.

Figure 22. Real time RT-PCR of *ng*2091

Normalized *ng*2091 gene expression ratio. Average fold change of *ng*2091 expression for each comparison is shown. Bars represent the median fold change for the following comparisons: WT-Fe/WT+Fe (gray bars) WT-Fe/ FA6959-Fe (checkered bars), WT-Fe/MCV304-Fe (horizontal striped bar), WT-Fe/MCV305-Fe (vertical striped bar) and WT-Fe/MCV306-Fe (white bar).   The median fold change is detected from three independently-conducted real time RT-PCR assays. Numerical values from all three assays indicated in Table 5.

*ng*2091 gene expression

TABLE 5. Effects on gene expression measured by real time RT-PCR.

Comparison	Normalized target gene expression ratio
	ng2091
WT-Fe / WT+Fe	16.3 (17-16)
WT-Fe / FA6959-Fe	23 (28-16)
WT-Fe / MCV304-Fe	22.3 (24.22)
WT-Fe / MCV305-Fe	7.1 (9.7-5)
WT-Fe / MCV306-Fe	11.5 (16-9)

[1] Average fold change for each comparison is shown, with the range detected among three independently-conducted real time RT-PCR assays shown in parentheses.

E. Xenosiderophore-iron acquisition by gonococcal strain FA1090

N. gonorrhoeae does not produce siderophores; however, the pathogen is capable of utilizing siderophores synthesized by other microorganisms (43, 273, 303). In gonococcal strain FA1090, Carson et al. demonstrated that FetA functions as a receptor for the xenosiderophore enterobactin (43). Enterobactin is a cyclic catecholate siderophore composed of three 2,3-dihydroxybenzoylserine (DHBS) subunits and was first characterized in E. coli. Enterobactin derivatives including the DHBS monomer (D1), the DHBS dimer (D2), and the DHBS trimer (D3) have also been identified as siderophores secreted by E. coli (118, 209). Carson et al. determined that enterobactin is utilized by gonococcal strain FA1090 in a FetA- and TonB-dependent manner (43). We tested whether strain FA1090 could also utilize other xenosiderophores in a similar manner. Since MpeR activates fetA expression, MpeR was predicted to affect catecholate utilization in gonococcal strain FA1090 as well.

Plate bioassays were employed to measure siderophore-dependent growth of gonococcal strain FA1090. We tested a variety of siderophores including: ornibactin, aerobactin, ferrichrysin, ferrirubin, coprogen, neocoprogen, enterobactin, DHBS and salmochelin for growth support. We found that ferrichrysin, ferrirubin, coprogen and neocoprogen did not support growth at all. Ornibactin and aerobactin supported growth but only in a TonB-independent fashion (data not shown). Thus we focused on the catecholate-type siderophores including: enterobactin, DHBS monomer (D1), DHBS dimer (D2), DHBS trimer (D3), salmochelin S4 (a cyclic, diglucosylated form of enterobactin), and the linear derivative of salmochelin (S2) (202). We evaluated xenosiderophore utilization in the wild type, the tonB mutant, the fetA mutant, the mpeR

mutant, the *mpeR*[C] strain, and the double *fetA mpeR* mutant. Ferric citrate was used as a positive control and bovine transferrin was used as a negative control since gonococci cannot utilize iron bound to non-human transferrin (10, 163). Figure 23 shows the average growth zone in millimeters detected around each iron source with standard deviation reflecting the variability within four or five independently-conducted experiments, each of which was performed in triplicate. As previously shown by Carson *et al.* (43), gonococcal strain FA1090 grows in the presence of enterobactin (Figure 23). Enterobactin-dependent growth by the *fetA* and *tonB* mutants was reduced to just above background levels (dotted line, Figure 23). Enterobactin-dependent growth by the *mpeR* mutant was also decreased relative to the wild-type strain, although growth inhibition was not as great as was observed with the *fetA* and *tonB* mutant strains. Enterobactin-dependent growth in the *mpeR*[C] strain was near wild-type amounts, consistent with the recovery of MpeR regulatory function in this strain. The double *fetA mpeR* mutant was severely restricted in enterobactin-dependent growth. These findings are consistent with those of Carson *et al.* (43), but further extends our observation that MpeR activates FetA expression, and leads to an enhanced ability to utilize the xenosiderophore enterobactin.

Figure 23. Xenosiderophore utilization by gonococcal strain FA1090
CDM plates were supplemented with apo-bovine transferrin and wells within the plates were inoculated with 10µl of the following siderophores: ENT: enterobactin; D1: dihydroxybenzoylserine (DHBS); D2: the dimer form of DHBS; D3: the trimer form of DHBS; S2: the linear derivative of salmochelin; and S4: the cyclized form of salmochelin.   Ferric citrate (+) was used as the positive control and apo-bovine transferrin (-) was used as the negative control as indicated along the x-axis of the graph. Each bar indicates the average growth in millimeters around each siderophore source; the average and standard deviations were determined from seven independent experiments, each conducted in triplicate. Bars represent average growth zone for the following strains: wild type FA1090 (black bars), *fetA* mutant strain FA6959 (checkered bars), *tonB* mutant strain MCV656 (gray bars*), mpeR* mutant strain (white bars), and *mpeR*[C] complement strains (striped bars). The horizontal dotted line indicates the diameter of the well containing each iron source.   Pairwise comparisons between the wild-type and mutant strains resulted in the following *P*-values: * $\leq$ 0.001; # = 0.0124; ^ = 0.0197.
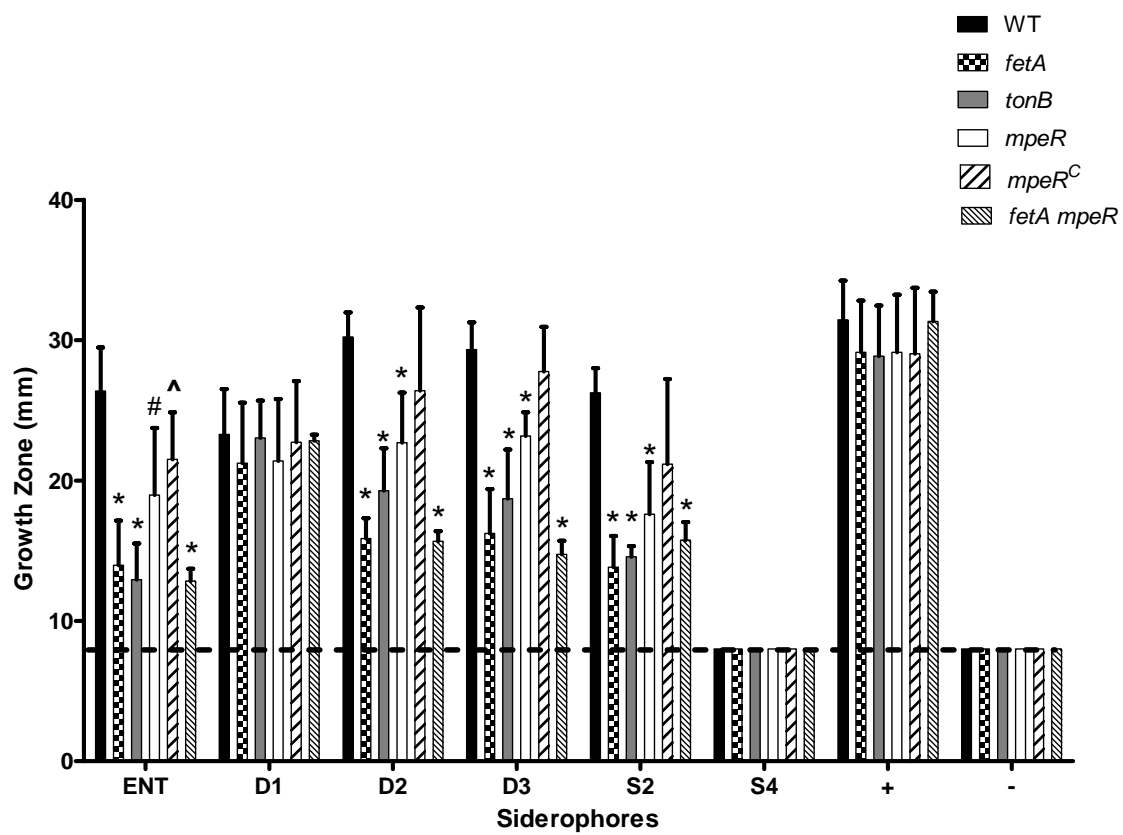
As shown in Figure 23, our analysis additionally demonstrates that gonococcal strain FA1090 can employ the enterobactin derivatives D2 and D3 in a FetA- and TonB-dependent manner. As was detected with enterobactin, use of these enterobactin derivatives was also maximized when MpeR was expressed. The *mpeR* mutant was capable of less growth around D2 and D3 (Figure 23) relative to the wild-type strain and complementation of the *mpeR* mutation led to an increase in D2- and D3-dependent growth. Interestingly, there was also a significant decrease in salmochelin S2-dependent growth by both the *fetA* mutant and *tonB* mutant, indicating that FA1090 utilized salmochelin S2 in a FetA-and TonB-dependent manner as well. These results demonstrate that gonococcal strain FA1090 is capable of employing enterobactin, the DHBS dimer (D2) and trimer (D3) derivatives and salmochelin S2 as iron sources. FetA, TonB and MpeR are critical for this process, consistent with the ability of MpeR to induce expression of FetA. This is the first demonstration that gonococcal strain FA1090 utilizes DHBS dimers and trimers and salmochelin S2 as iron sources and that this uptake pathway depends upon expression of FetA, TonB, and MpeR activation. While salmochelin S4 was not utilized by any FA1090 variants tested in this analysis, the DHBS monomer was employed by all strains, both mutant and wild-type. These results imply that FA1090 does not have the capacity to internalize salmochelin S4 but can import DHBS in a TonB- and FetA-independent pathway.

F. ABC transport genes NG2088 and NG2090 are necessary for xenosiderophore acquisition

The ABC transporters are ubiquitous membrane proteins that couple adenosine triphosphate (ATP) hydrolysis to the translocation of diverse substrates across cell membranes. In a classical transport reaction, two highly conserved ATP-binding domains or subunits couple the binding/hydrolysis of ATP to the translocation of particular substrates across the membrane, through interactions with membrane-spanning domains of the transporter (72). In siderophore acquisition, once the siderophore is bound to the periplasmic binding protein, it must be transported into the cytoplasm. This is accomplished by an ABC transporter protein complex. Bacterial ABC transporters commonly consist of four structural domains: two transmembrane domains that form a channel through which the ferric-siderophore passes through and two nucleotide binding domains that hydrolyze ATP. The Fet operon encodes the periplasmic binding protein *fetB* as well as a putative ABC transport system encoded by *ng2091-ng2088* (Figure 20A). We were interested in the impact these ABC transport genes have on siderophore acquisition. Employing the plate bioassay we investigated the growth of two ABC transport mutant strains and compared growth to the wild type strain. A polar omega cassette was cloned into *ng2088* which encodes an ATP binding protein to construct a mutant *ng2088* strain (FA7029). The same omega cassette was also cloned into *ng2090* which encoded a transmembrane permease to construct the *ng2090* mutant strain (FA7241). We tested the acquisition of the following catecholate-type siderophores including: enterobactin, DHBS monomer (D1), DHBS dimer (D2), DHBS trimer (D3), salmochelin S4 (a cyclic, diglucosylated form of enterobactin), and the linear derivative

of salmochelin (S2) (202). Growth zones indicated utilization of the iron source tested with the limit of detection of 8mm indicated by a dotted line (Figure 24). Figure 24 shows the mean and standard deviation from four independent experiments that were each performed in triplicate. We observed an attenuated growth pattern in the presence of the catecholates with the exception of DBHS monomer (D1) for both mutants when comparing growth zones to the wild type strain (FA1090). This is not surprising, since the omega cassette is polar, the *ng2090* mutant is not expected to express any of the genes downstream including *ng2088*. Both ABC transport mutants exhibit growth similar to the *tonB* and *fetA* mutants (Figure 23) in which growth was significantly attenuated in the presence of enterobactin, DHBS dimer (D2), DHBS trimer (D3), and salmochelin S2. Interestingly, growth in the presence of DHBS monomer (D1) was attenuated but this difference did not reach a level of statistical significance. Growth above the threshold of detection was observed in these ABC transport mutants similar to the *tonB* and *fetA* mutant strains implying that an alternative pathway may be utilized for low levels of xenosiderophore acquisition in these mutant strains. Furthermore, these results indicate that the entire ABC transport system operon must be expressed for optimal levels of xenosiderophore utilization and that as in all ABC transport systems, the ATP binding protein plays a crucial role in transport. When the ATP binding protein encoded by *ng2088* was mutangenized, the mutant strain (FA7021), *only* supported very low levels of growth compared to the wild type strain (FA1090). The ATP-binding cassettes power the transporter by binding and hydrolyzing ATP (71). It is presumed that in the absence of *ng2088*, ABC transport through this system cannot occur, due to a lack of energy.

Figure 24. Xenosiderophore utilization by gonococcal strain FA1090 ABC transport mutants

CDM plates were supplemented with apo-bovine transferrin and wells within the plates were inoculated with 10µl of the following siderophores: ENT: enterobactin; D1: dihydroxybenzoylserine (DHBS); D2: the dimer form of DHBS; D3: the trimer form of DHBS; S2: the linear derivative of salmochelin; and S4: the cyclized form of salmochelin.   Ferric citrate (+) was used as the positive control and apo-bovine transferrin (-) was used as the negative control as indicated along the x-axis of the graph. Each bar indicates the average growth in millimeters around each siderophore source; the average and standard deviations were determined from four independent experiments, each conducted in triplicate. Bars represent average growth zone for the following strains: wild type FA1090 (black bars), *ng2088* strain (FA7029) (checkered bars), *ng2090* mutant strain (FA7241) (white bars with black dots). The horizontal dotted line indicates the diameter of the well containing each iron source.   Asterisks (*) indicate significant values from pairwise comparisons between the wild-type and mutant strains at *P*-values: ≤ 0.001
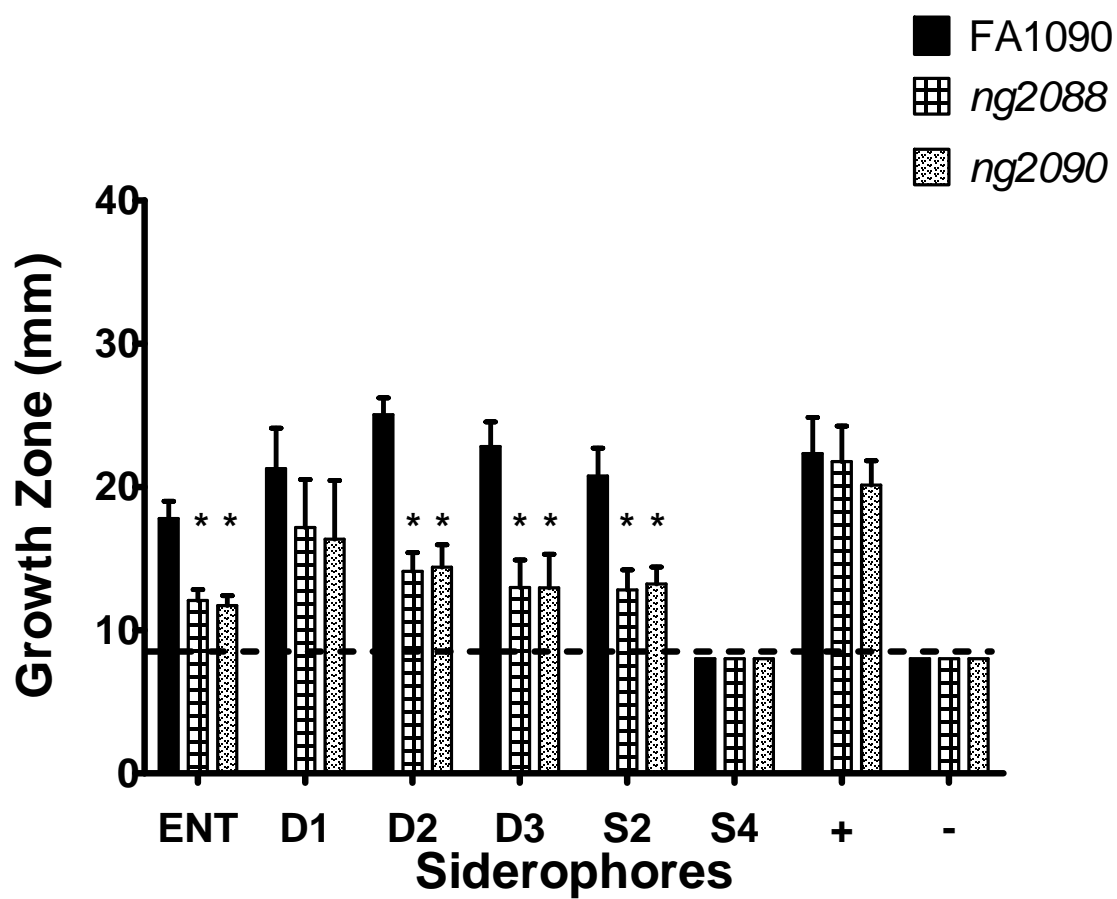
G. Xenosiderophores that support growth do not serve as co-inducers for MpeR-dependent *fetA* activation

AraC-like transcriptional regulators are distinguished by a C-terminal helix-turn-helix motif, which is responsible for DNA binding. These regulators also contain an N-terminal binding site to which activator molecules bind conferring specificity on regulation (97). MpeR, an AraC-like regulator, enhances *fetA* transcription (Figures 17 and 18) and MpeR binds to the region upstream of the *fetA* gene (Figure 19). Gonococcal strain FA1090 can utilize enterobactin, DHBS, and salmochelin S2 as iron sources (Figure 22). Given these findings, we tested whether these xenosiderophores could serve as co-inducers for MpeR-dependent FetA activation. Western blot analysis was utilized to investigate FetA expression when gonococci were grown in the presence of ferrated catecholates. The wild-type strain was grown in the presence of the ferrated forms of enterobactin, DHBS monomer (D1), DHBS dimer (D2), DHBS trimer (D3), salmochelin S4, and salmochelin (S2). Every two hours, aliquots were removed from the cultures, and standardized to cell density. FetA expression was analyzed by SDS-PAGE and western blot analysis. FA1090 grown in the presence of all tested siderophores except salmochelin S4 exhibited FetA expression levels similar to that detected under iron-replete conditions (Figure 25A). The wild-type strain, grown in the presence of S4, exhibited similar FetA expression levels to those expressed when grown in iron-deplete conditions (Figure 25A). To determine whether the decrease in FetA expression was siderophore specific or due to iron status, we analyzed TbpA expression from the same cultures. TbpA is a Fur regulated, outer membrane transporter of iron from human transferrin (64). TbpA expression mirrored that of FetA as a function of growth on

xenosiderophores (Figure 25A). As observed for FetA, TbpA expression was greatest in the presence of S4 and when strains were grown under iron-deplete conditions (Figure 24 A). As shown in Figure 23, salmochelin S4 did not support the growth of FA1090. Thus the increase in FetA expression in the presence of salmochelin S4 is due to iron stress rather than S4 serving as a co-inducer for MpeR in the activation of FetA. Furthermore, the other siderophores and derivatives that supported growth resulted in high internal iron pools and consequently resulted in repression of FetA. We repeated this experiment, replacing the ferrated siderophores with the iron-free forms during gonococcal growth (Figure 25B). As seen with the ferrated-xenosiderophores, there was no evidence of siderophore-dependent induction as all strains expressed FetA at levels similar to those detected in iron depleted growth conditions. Cumulatively, these results suggest that FetA expression is sensitive to the iron status of the cell and that the presence of the xenosiderophores, either in ferrated or iron-free form, did not further influence FetA expression.

Figure 25. FetA expression is not induced by the presence of xenosiderophores

A) WT (FA1090) was grown in CDM with the indicated ferrated-xenosiderophores
(final concentration of 10 μM) as the sole iron source. The following ferric-
xenosiderophores were tested: ENT: enterobactin; D1: dihydroxybenzoylserine (DHBS);
D2: the dimer form of DHBS; D3: the trimer form of DHBS; S2: the linear derivative of
salmochelin; and S4: the cyclized form of salmochelin. As controls the WT strain was
grown in the absence of iron (-) or with ferric nitrate (+) but without the addition of
siderophores. Aliquots collected at 2, 4 and 6 hours (indicated above the blots) were lysed
and subjected to SDS-PAGE. After separation, proteins were transferred to nitrocellulose.
Blots were probed with anti-FetA (top) or anti-TbpA antibodies (bottom).

B) As in panel A, except the WT (FA1090) was grown in CDM with the indicated
xenosiderophores in the iron-free or apo form.

A.

B.
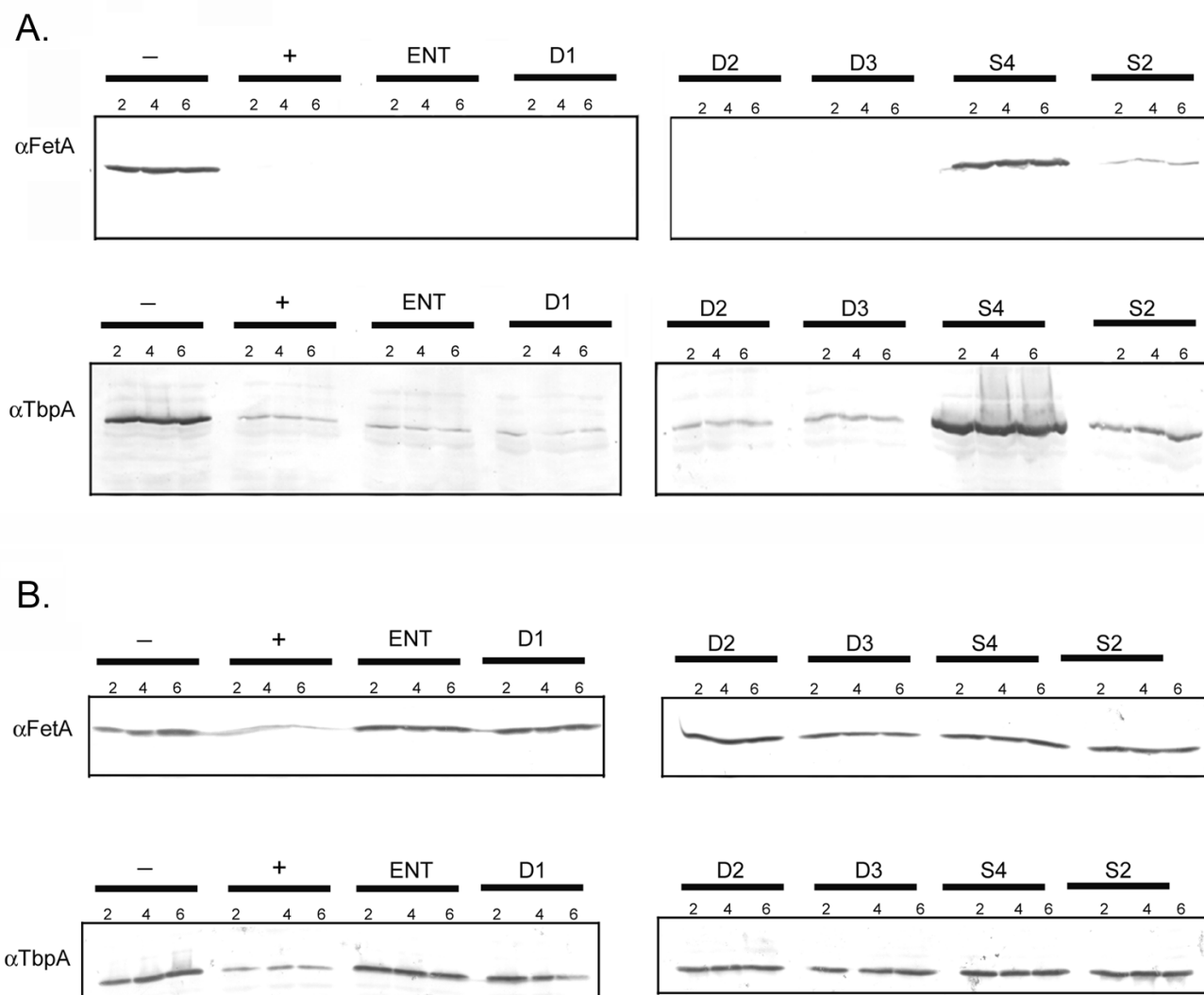
III. Discussion

MpeR was first identified by Folster and Shafer as a homolog of other AraC-like regulators (92). MpeR was originally described as a transcriptional regulator of the *mtrF* gene, which encodes a protein that modulates antimicrobial efflux pump activity in gonococcal strain FA19. In the present study, we determined that in contrast to its repressive action on *mtrF*, MpeR activates *fetA* transcription under iron-deplete conditions. While MpeR clearly plays a role, other regulators or co-factors may also be involved in controlling *fetA* expression. Importantly, this is the first example of an AraC-like regulator that is involved in the regulation of an outer membrane xenosiderophore transporter in *N. gonorrhoeae*. Thus, the transcriptional regulatory activities of MpeR impact at least two important properties needed for survival of gonococci during infection: efflux of host-derived antimicrobials by the Mtr system (92) and xenosiderophore-iron acquisition via FetA. In the context of the present work, we suggest that MpeR regulation of *fetA* may aid in gonococcal immune evasion. Anti-FetA antibodies are present in sera from patients convalescing from meningococcal disease and these antibodies are cross-reactive against gonococcal strains (3, 27). In addition, monoclonal antibodies against FetA are bactericidal in the presence of human complement (219). Therefore, continuous, unregulated FetA expression during the entirety of an infection is expected to elicit a host response that would inhibit the bacteria from thriving *in vivo*. Thus *fetA* expression is expected to be tightly controlled.

In the current study, we determined that enterobactin and DHBS dimers (D2) and trimers (D3) were utilized by strain FA1090 in a FetA- and TonB-dependent manner. MpeR-dependent activation of FetA enhanced the ability of FA1090 to employ these

xenosiderophores as sole iron sources. We also observed that the ABC transport system co-transcribed with FetA is important for xenosiderophore utilization as mutations in *ng2090* and *ng2088* resulted in reduced growth in the presence of the siderophores. While the ability of *N. gonorrhoeae* to utilize enterobactin had been previously recognized (43), this is the first demonstration that gonococci can use DHBS dimers (D2) and trimers (D3) as iron sources. Like enterobactin, these enterobactin derivatives were employed by wild-type FA1090 in a FetA-, TonB-, and MpeR-dependent mechanism. This is somewhat surprising since these siderophores are internalized via distinct TonB-dependent transporters in other Gram-negative bacteria (98, 316) Moreover, strain FA1090 can also employ the xenosiderophore salmochelin S2 in the same FetA-dependent pathway. The broad specificity of FetA for all four xenosiderophores is remarkable, as their import into *E. coli* is facilitated by four distinct transporters, including FepA, Cir, FiuA and IroN (98, 316). Carson *et al.* (43) noted that the sequence of FetA from gonococcal strain FA1090 retained those residues known to be important for enterobactin binding to FepA (51); however, the spacing between the conserved residues was distinct. In addition, the binding affinity of FetA for enterobactin was found to be much weaker (43) than that described for *E. coli* FepA (42). Thus we propose that FetA and the downstream ABC transport system has evolved as a gonococcal transporter capable of importing a broad spectrum of catecholate-type xenosiderophores, perhaps at the expense of high affinity interactions with any single siderophore.

Salmochelin is known as a "stealth siderophore" and is derived by glucosylation of enterobactin via the products of the *iroA* locus (202). Virulent pathogens, including uropathogenic *E. coli*, *Salmonella enterica*, and *Shigella dysenteriae* harbor the *iroA*

locus, sometimes on pathogenicity islands (215). Modification of the enterobactin molecule by addition of two glucose molecules leads to increased hydrophilicity and to the ability of salmochelin to evade the host's innate immune response. In the human host, enterobactin is sequestered and made ineffectual by the innate immunity protein, siderocalin (also known as lipocalin 2), whereas salmochelin is not (89, 101). Thus, salmochelin production by pathogens allows for efficient iron acquisition in the presence of siderocalin (235), which is found in lymphocytes. Salmochelin S4 is a cyclic form of the siderophore and salmochelin S2 is the linear derivative of S4; both forms coordinate iron and can be employed as "stealth siderophores". Gonococcal strain FA1090 did not utilize iron from salmochelin S4 but did obtain iron from S2. As microbial producers of enterobactin, DHBS and salmochelin inhabit the same niche as the gonococcus (80, 230), it seems likely that the ability to hijack these siderophores, in the presence of neutrophil-derived siderocalin, enhances the survival of *N. gonorrhoeae* in vivo.

Interestingly, our laboratory has observed differences in xenosiderophore utilization among gonococcal strains. Strain FA19 utilizes enterobactin, D1, and S2 in a TonB- and FetA-independent mechanism that requires expression of the FbpABC system (273). In the current study, we demonstrated that FA1090 utilizes D1 in a TonB- and FetA-independent mechanism. However, FA1090 utilizes enterobactin, D2, D3, and salmochelin S2 in a FetA- and TonB-dependent manner. Our hypothesis is that the differences in xenosiderophore utilization phenotypes between strains are due to two different pathways by which iron from xenosiderophores can be transported into the gonococcus. One pathway is TonB- and FetA-dependent while the other pathway is TonB-independent and requires the FbpABC system. In gonococcal strain FA1090, the

fetA and *tonB* mutants were capable of significantly less growth with enterobactin, D2 D3, and S2 relative to the wild-type strain; however, both mutants exhibit xenosiderophore-dependent growth above background (dotted line in Figure 23). Growth was diminished but not abolished in the *tonB* and *fetA* mutant strains; therefore we hypothesize that the FbpABC-dependent, Ton-independent pathway is also employed by these mutants in the FA1090 background. In further support of this hypothesis, Carson *et al*. demonstrated that both *fetA* and *fetB* mutants of FA1090 exhibited a decrease in growth in the presence of enterobactin but growth was not abolished(43). Similar results were also observed with the *tonB* mutant in the previous study (43). Thus, some iron was transported into the *fet* and *tonB* mutants in an energy- and *fet* operon-independent pathway. The difference in xenosiderophore-dependent growth between FA19 and FA1090 is likely due to differential use of these two distinct pathways. Gonococcal strain FA19 seems to be limited to use of the TonB-independent pathway that employs the FbpABC system for iron transit through the periplasm and cytoplasmic membrane. A possible explanation for the exclusive use of Ton-independent pathway in strain FA19 could be related to the observation that the genome sequence of gonococcal strain FA19 contains a frame-shift mutation within *ng2090*. We have confirmed by direct sequencing of a PCR product amplified from FA19 chromosomal DNA that this lesion represents a genuine mutation and is not a genome sequencing error (Figure 26). This mutation would result in a truncated Ng2090 protein and may prevent expression of downstream genes as well (65). Similar to the attenuated growth we observed in gonococcal mutant *ng2090* strain (FA7041) we detect attenuated growth of FA19 on the xenosiderophores. Therefore, we hypothesize that this genetic difference between strains FA1090 and FA19

results in a defective Fet system and a reliance on the TonB-independent uptake pathway

for uptake of iron from xenosiderophores in strain FA19 (65).

Figure 26. Amino Acid Sequence alignment of Ng2090
Amino acid sequence alignment of Ng2090 from gonococcal strain FA1090 and FA19.
Amino acid position indicated above each line, gray shading with black font indicates
100% identical sequence. Black font with white background indicates not identical and
dash marks indicate lack of sequence in strain FA19.

```
          50
FA1090  MPSEKNIGFMAGSSRPLRVAFALLLVSCILFMTLNVKGDWDFVLHLRLTK
FA19    MPSEKNIGFMAGSSRPLRVAFALLLVSCILFMTLNVKGDWDFVLHLRLTK

          100
FA1090  LAALLMVAYAVGVSTQLFQTLTNNPILTPSILGFDSLYVFLQTLLVFTFG
FA19    LAALLMVAYAVGVSTQLFQTLTNNPILTPSILGFDSLYVFLQTLLVFTFG

           101                                             150
FA1090  GVGYTSLPLTGKFGFELVVMMGGSLLLFYTLIRQGGRDLPHMILIGVIFG
FA19    GVGYTSLPLTGKFGFELVVMMGGSLLLFYTLIRQGGRDLPHMILIGVIFG

          151                                              200
FA1090  ILFRSLSSLLSRMIDPEEFTAAQANMFAGFNTVRSELLGIGALVLLVSAA
FA19    ILFRSLSSLLSRMIDPEEFTAAQANMFAGFNTVRSELLGIGALVLLVSAA

          201                                              250
FA1090  VVWHERYRSDVHLLGRDQAVNLGISYTRNTLWILLWIAALVATATAVVGP
FA19    VVWRERYRSDVHLLGRDQAVNLGISYTRNTLWILLWIAALVATATAVVGP

          251                                              300
FA1090  VSFFGLLAASLANHFSPSVRHSVRLPMTVCVGGILLVGGQTVFEHFLGMK
FA19    VSFFGLLAASLANHFSRPCAIPSACR------------------------------------------

          301                   324
FA1090  AVLSVVVEFAGGLVFLYLVLKHKK
FA19    ------------------------------------------------
```

In *N. gonorrhoeae*, the *fetA* gene is the only TonB-dependent transport system that is also encoded with a periplasmic binding protein and a complete set of ABC transport genes (*fetB-ng2088*). Data presented in the current study suggest that the putative periplasmic binding protein and ABC transport system are co-transcribed with *fetA*, but also independent of FetA and MpeR. Expression of the shorter transcript encoding *fetB-ng2088* was detected only in gonococci grown in iron-deplete conditions. A Fur box upstream of *fetB* was identified by FURTA and EMSA assays (139), consistent with our finding of a separate iron-regulated transcript including the *fetB-ng2088* genes. The FetA-independent but iron regulated transcription of the ABC transport genes suggests that the ABC transport system could be utilized by other TonB-dependent transporters, in addition to FetA.

Enterobactin, D2, D3, and salmochelin S2 were acquired by gonococcal strain FA1090, which led us to consider the possibility that one or all of these xenosiderophores could serve as a co-inducer of MpeR for the activation of *fetA* expression. However, contrary to our hypothesis, FetA expression was not altered by the presence of any of the siderophores tested, regardless of their iron status. We are currently entertaining three possible mechanisms to explain these results. First, MpeR may not require a co-inducing molecule in order to activate FetA expression. Second, because MpeR is involved in modulating the expression of proteins involved in antimicrobial efflux, the co-inducing agent for MpeR-dependent regulation could be related to efflux. In this context, since the Mtr system is necessary for efflux of host-derived antimicrobials, MpeR might sense an efflux substrate as a signal of location within the host. And third, other catechols might

serve as co-inducers with MpeR. Given the broad specificity of FetA, it is possible that an as yet unrecognized catecholate molecule could serve as an inducer and also provide iron in a FetA-dependent manner.

The *mpeR* gene has only been identified in the genomes of the pathogenic *Neisseria* and is absent from the genomes of commensal *Neisseria* (184, 266). The MpeR proteins are highly conserved, sharing 97-100% sequence identity among the pathogenic *Neisseria* species (data not shown). This conserved, pathogen-specific regulator controls the expression of FetA, which is a pan-*Neisseria* transporter (184). In *Staphylococcus aureus*, different community-acquired strains vary in their virulence. The difference amongst strains in pathogenic potential is linked to increased expression of core genome-encoded virulence genes (176). It was hypothesized by Li *et al.* (176) that global regulators of virulence genes are responsible for variable virulence amongst strains. Similarly, commensal *Neisseria* have an extensive repertoire of virulence alleles that are also expressed by pathogenic *Neisseria* and other bacterial genera (184). Thus differential regulation of these alleles, rather than their presence or absence, may contribute to increased virulence in the pathogenic *Neisseria*. Perhaps MpeR-dependent activation of *fetA* and other potential transporters enhances virulence in pathogenic *Neisseriae* relative to the commensals.

In conclusion, in this study we demonstrated that MpeR, a pathogen-specific regulator in *N. gonorrhoeae* enhances expression of the siderophore receptor FetA under iron-deplete conditions. Up-regulation of *fetA* by MpeR was by a direct mechanism. A second, internal transcriptional start site was identified upstream of *fetB*, positioned near several potential -10 promoter elements, overlapped by the Fur-binding site. Additional studies

will be required, however, to fully define the mechanisms that control *fetB* transcription and whether other regulatory proteins are involved. We also determined that enterobactin, D2, D3 as well as salmochelin S2 were utilized in a FetA- and TonB-dependent mechanism in strain FA1090. Expression of MpeR enhanced the ability of strain FA1090 to utilize enterobactin and salmochelin, consistent with MpeR-dependent activation of FetA. Gonococcal strain FA1090 was also capable of utilization of the DHBS monomer, but did so in a TonB- FetA-independent manner. We identified one possible mechanism that may elucidate why gonococcal strain FA19 exhibits Ton-independent xenosiderophore acquisition. None of the catecholates that supported growth in this study appeared to act as a co-inducer for MpeR-dependent activation of *fetA*. To our knowledge, this study represents the first description of a pathogen-specific regulator in *N. gonorrhoeae* that activates expression of a pan-*Neisseria* TonB-dependent transporter. In addition, the current study is the first to link regulation of iron transport and antimicrobial efflux systems through the action of a pathogenic *Neisseria*-specific regulator.

CHAPTER 6 DISCUSSION

Neisseria gonorrhoeae is a well adapted human pathogen. Multiple TonB-dependent and TonB-independent iron acquisition systems have been characterized including those involved in iron acquisition from transferrin (63, 64), lactoferrin (22, 25), hemoglobin (53), xenosiderophores (43, 273) and intracellular iron (113, 317). All of the TonB-dependent receptors are regulated by the ferric uptake regulator Fur to prevent iron overload within the bacteria.

The overall goal of this study was to identify additional iron sources utilized by *Neisseria gonorrhoeae* and characterize the regulation of the receptors specific to these iron sources. This would provide valuable insight into the pathogenesis of the microorganism and could also lead to new therapeutic targets. Understanding the mechanism of iron acquisition within the intracellular environment gives us insight into gonococcal intracellular survival. Currently, the precise intracellular localization of *Neisseria gonorrhoeae* is unknown. Investigating potential intracellular iron sources such as iron derived from degraded ferritin or a putative mammalian siderophore could further our understanding of gonococcal localization within the cervical epithelial cells. Ferritin is an iron binding protein ubiquitous in the human host. It is found intracellular, in the cytosol, as well as in the nucleus, in endo-lysosomal compartments and in the

mitochondria (120) of the epithelial cells. Extracellular ferritin is found in fluids such as serum and synovial and cerebrospinal fluids (120). In our study we determined that the addition of ascorbate to cells incubated in cell culture media inhibited intracellular survival. Ascorbate inhibits ferritin degradation which could be the major source of iron for intracellular gonococci. TdfF, a TonB-dependent transporter is crucial for intracellular survival, and the *tdfF* mutant strain was rescued during gentamicin protection assays with the addition of iron (113). This indicates that TdfF is important for intracellular iron acquisition (113). One hypothesis is that TdfF is a receptor for an iron carrying molecule involved in the transport of iron from degraded ferritin to other parts of the epithelial cells. Ascorbate also binds iron and the attenuated intracellular survival observed could be due to the ascorbate sequestering any iron that gonococci acquire within the epithelial cell. We cannot conclude that *N. gonorrhoeae* utilize iron directly from degraded ferritin until we investigate ferritin levels within an infected epithelial cell. We also investigated gonococcal growth in the presence of 2,5-DHBA which is the binding moiety identified for a putative mammalian siderophore (75). Enterobactin, aerobactin and dihydroxybenzoylserine are xenosiderophores utilized by *N. gonorrhoeae* (43, 273, 303). When we performed plate bioassays in which ferrated 2,5-DHBA was the only iron source available, we observed intermediate growth of gonococci compared to growth in the presence of utilizable iron sources. The intermediate growth observed occurred in a TonB-, TdfF- independent manner. These results do not rule out the structurally complete mammalian siderophore as a possible iron source for gonococcal strain FA1090 nor that utilization of this siderophore could be TonB- and/or TdfF-dependent. Once the structure

of the siderophore is elucidated we could test gonococcal iron utilization from this mammalian siderophore.

Furthermore, understanding how *tdfF* expression is induced could lead to an understanding of the regulation of virulence genes specific to the intracellular environment. From previous studies we understand that *tdfF* expression was only observed in the presence of epithelial cells or within cell culture media in the absence of additional iron (113). In this study we determined that the heat-inactivated fetal bovine serum component of cell culture media was an iron-depleted media that could contain an inducing molecule responsible for *tdfF* expression. These observations indicate that *tdfF* expression only occurs in the presence of a host specific signal under iron-deplete conditions. We also determined that *tdfF* is identical in all gonococcal strains sequenced to date. This leads us to the conclusion that TdfF is either not surface exposed or the protein is only expressed inside the epithelial cell and away from the pressures of the immune system. TdfF could also not be very immunogenic which could explain our difficulty in detecting TdfF expression through western blot analysis. We hypothesize that MpeR, an AraC-like regulator involved in the regulation of hydrophobic agent efflux pumps (92) and the xenosiderophore receptor *fetA* could also play a role in *tdfF* expression. The gene encoding *mpeR* is in very close proximity to *tdfF* on the gonococcal chromosome and the locus of the gonococcal chromosome from *tdfF* to *mpeR* is conserved only in pathogenic *Neisseria* species (184, 266). Additionally, *mpeR* itself is iron regulated and directly activates the xenosiderophore acquisition system by FetA as demonstrated in this study. AraC-like regulators are identified due to their homology to AraC, which regulates arabinose metabolism and transport in *E. coli*. In *E. coli*, AraC

plays a dual role as both an activator and a repressor in this system based on the availability of the co-inducing molecule, arabinose. MpeR could also be a dual regulator which activates or represses based on the availability of a co-inducing molecule. For example, a specific inducing signal for intracellular invasion could make MpeR, activate or derepresses *tdfF* expression. When gonococci are in the extracellular environment, MpeR might no longer bind to this intracellular environment specific co-inducer and activates the extracellular iron acquisition system, FetA while repressing TdfF. The opposite could be true such that in the extracellular environment an unknown co-inducing molecule could bind to MpeR, activating *fetA* and repressing *tdfF* expression. The co-inducing molecule for MpeR activation of iron acquisition or efflux pumps has not been elucidated and we tested various xenosiderophores, as well as human specific molecules such as norepinephrine, epinephrine and ferritin. Since we have determined that MpeR is involved in the regulation of xenosiderophore uptake and it has already been demonstrated to regulate efflux pumps, MpeR could be a global regulator. If MpeR is a global regulator then the inducing signal for MpeR activation could be anything.

FetA and TdfF could also be connected by the ABC transport system encoded by the *fet* operon. The FetA-independent but iron regulated transcription of the ABC transport genes *ng*2091-2088 suggests that the ABC transport system could be utilized by other TonB-dependent transporters, such as TdfF. We defined the transcriptional start site for the ABC transport operon; however, the -35 region could not be identified. This suggests that this transport system could also be regulated by a transcriptional regulator other than Fur. This alternative regulation could be important for the transport of other iron sources besides xenosiderophores. TdfF does not have an associated ABC transport

system; however, the iron source utilized by TdfF would need to cross the cytoplasmic membrane. Perhaps, both TdfF and this ABC transport system are regulated by the same intracellular regulatory cascade.   Both TdfF and FetA have associated periplasmic binding proteins (PBPs), and it is possible that the ABC transport system encoded by the *fet* operon recognizes both PBPs. PBPs play an integral role in mediating the transport of carbohydrates, amino acids, peptides, metal ions, metal chelate complexes and other nutrients across the periplasm from the outer to the inner membrane (58). It has been demonstrated that ABC transport systems are inactive until a holo-PBP interacts with the transporter (178). We aligned both FetB from the *fet* operon and the periplasmic binding protein encoded downstream of *tdfF*, NG0023 also annotated FetB2 and found that they were 44% identical. We also used the SWISS-MODEL program (11) which is an automated protein structure homology-modeling server to determine structural similarities between FetB and NG0023. We used the amino acid sequence for the PBPs from gonococcal strain FA1090. The SWISS-MODEL program selected Yclq, a *Bacillus subtilis* PBP as the biological template structure for the topology model for both gonococcal PBPs. Yclq is the PBP responsible for the transport of the siderophore petrobactin in *B. subtilis* (313). FetB had a sequence identity of 35.4 % to Yclq and NG0023 had a sequence identity of 32.5% to Yclq. The results of the models reveal that the PBP structures were almost identical (Figure 26).

Figure 27. PBP topology models Ribbon diagrams of PBPs generated using the SWISS-MODEL program (11) which is an automated protein structure homology-modeling server. The amino acid sequence from gonococcal strain FA1090 was used to generate the topology models. Yclq, a *B. subtilis* PBP was used as the biological template for both FetB and NG0023. FbpA has been crystallized in *Neisseria.* Protein secondary structures are indicated by color: β-sheets in yellow, α-helices in magenta and turns in blue. Three dimensional models of FetB (A), NG0023 (B) and FbpA (C) were synthesized.
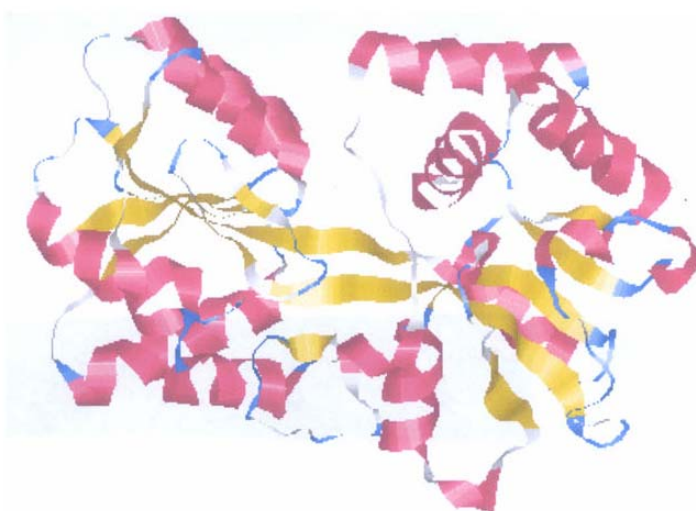
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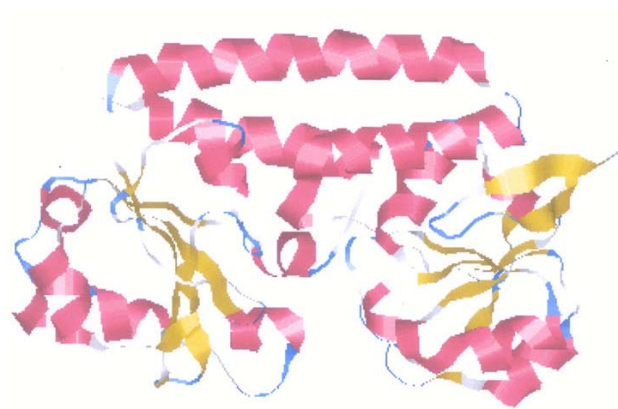
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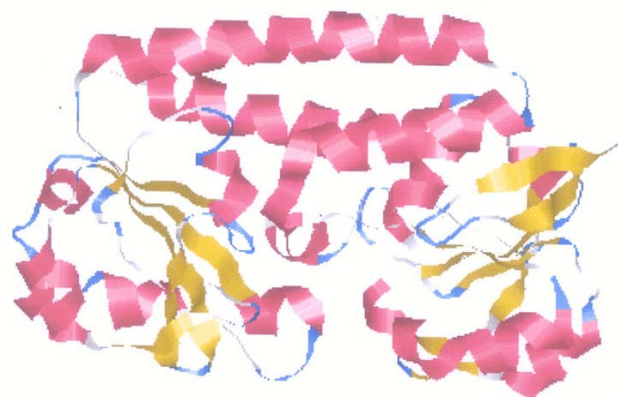
C

FbpA, the PBP associated with the transferrin and lactoferrin iron acquisition system (54), has a very different structure compared to NG0023 and FetB (Figure 25). FbpA shuttles iron across the periplasm to the ABC transport system, FbpBC. The interaction between the PBP and its ABC transporter is mediated by charge-charge interactions formed by surface exposed acidic residues on the PBP and basic residues found on the periplasmic face of the transmembrane domains of the ABC transporter (132, 136). Since FetB and NG0023 are nearly identical in structure, the acidic residues on the surface of both PBPs could interact with the ABC system coded by the *fet* operon in a similar manner. Both FetB and NG0023 have structures that would classify them in the class III of PBPS. Class III is a large family of proteins with low sequence homology but similar overall 3D structures. Other PBPS that are in this class include PBPs responsible for transport of catecholate siderophopres (58). FbpA belongs to Class II which binds to a large variety of substrates including ferric and ferrous iron. Therefore it is plausible that both FetB and NG0023 periplasmic binding proteins deliver their specific ligand to the same ABC transport system. The nearly identical structure amongst PBPs could also signify that both bind to a similar ligand. The ABC system coded by the *fet* operon is responsible for xenosiderophore acquisition from enterobactin, enterobactin derivatives and salmochelin. If the structural similarity between the PBPs indicates similar ligands, then NG0023 would be responsible for the transport of a catecholate siderophore-like molecule like the recently identified mammalian siderophore.

Prevention as well as treatment of gonococcal disease is a critical global health concern. Antibiotic resistance prevails and gonococcal infection increases the transmission of HIV. Presented in these studies is the investigation of potential

intracellular iron sources utilized by *N. gonorrhoeae* strain FA1090 as well as extracellular iron sources produced by commensal bacteria in the female genital tract. Understanding xenosiderophore utilization can lead to alternative therapeutic strategies for the treatment of gonorrhea. In this study, we determined that gonococcal strain FA1090 utilizes enterobactin and its breakdown product derivatives as well as salmochelin in a FetA and Ton-dependent and independent manner. Previous studies have indicated the xenosiderophore utilization occurs in Ton-independent, Fbp-dependent manner for strain FA19 (273). We have determined that the difference in xenosiderophore utilization between these strains is likely due to a mutation in the ABC transport system encoded by the *fet* operon. However, despite the mechanism of xenosiderophore utilization, understanding which xenosiderophores are utilized by gonococci can allow us to exploit the nutrient acquisition pathway for new therapeutic strategies.

Siderophores or analogs can be used as iron transport–mediated drug delivery or "Trojan Horse" antibiotics. Siderophore-mediated drug transport in bacteria and fungi have been demonstrated in both the laboratory and in nature. Sideromycins are naturally occurring Fe^{3+}-siderophores that are covalently linked to an antibiotic moiety. Only a few naturally occurring sideromycins have been found, among them albomycin and salmycin (121). A wide spectrum of siderophore–antibiotic conjugates has been chemically synthesized. Although the antibiotic moieties account for a substantial part of the sideromycins and drastically change the shape of the siderophore from which they are derived, the sideromycins are nevertheless recognized and transported by the Fe^{3+}-siderophore transport proteins (232).

For many antibiotics, the diffusion rate across the cell membranes into the cytoplasm is poor. This is frequently a problem in antibiotic therapy for Gram negative bacteria since the outer membrane reduces permeation to such an extent that the minimal inhibitory concentration required reaches toxic levels (36). In contrast, for sideromycins, the outer membranes and the cytoplasmic membranes do not serve as permeability barriers, but rather actively contribute to the entry of the antibiotics to their targets in the cytoplasm. Active transport reduces the minimal inhibitory concentrations more than a 100-fold (36). Thus, understanding how xenosiderophores are acquired could help in the design of siderophore-antibiotic conjugates that could be applied for the specific gonococcal infection. The challenge with using a siderophore-antibiotic conjugate is that the siderophore used needs to be specific to the pathogen and not affect commensal bacteria of the genital tract.   We could also use this technology for the ligand specific to TdfF. If this specific ligand is permeable across the epithelial cells, such as the mammalian siderophore, then we could specifically target intracellular gonococci. Therefore, identifying specific iron sources within the host and how they are acquired by gonococci can lead to innovative therapies and a better understanding of the pathogenesis of the microorganism.

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VITA

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Publications:

Hollander, A.M., Mercante, A., Shafer W.M., and Corneliessen, C.N.: MpeR, an AraC-like regulator activates FetA expression in strain FA1090. Infection and Immunity (Accepted)

Cornelissen, C.N, and **Hollander, A.M**: TonB-dependent transporters expressed by *Neisseria gonorrhoeae*, Frontiers in Microbiology, 2011 http://www.frontiersin.org/Journal/Abstract.aspx?s=149&name=cellular and infection microbiology&ART_DOI=10.3389/fmicb.2011.00117

Price, G.A., Masri H.P., **Hollander**, **A.M.,** Russell, M.W., and Cornelissen, C.N.: Gonoccocal transferrin binding protein-cholera toxin B chimeras induce serum bactericidal and vaginal transferrin utilizations blocking antibodies in mice, Vaccine, 25 (41): 7247-7260, 2007.

Delboy, M.G., Patterson, J.L., **Hollander, A.M.**, Nicola A.V.: Nectin-2-mediated entry of a syncytial strain of herpes simplex virus via pH-independent fusion with the plasma membrane of Chinese hamster ovary cells, Virology Journal, 2006 Dec 27;3:105**(229)**

Abstracts and Presentations:

Aimee Hollander[1], Alexandra Mercante[2], William M. Shafer[2], Cynthia Nau Cornelissen[1] (2010) Gonococcal FetA Expression is Regulated by MpeR and Enables Strain FA1090 to Utilize Enterobactin and its Linear Derivatives. 17th International Pathogenic Neisseria Conference September 11th-16th 2010, Banff, Alberta Canada (Poster)

Aimee Hollander[1], Alexandra Mercante[2], Lydgia Jackson[3], Cynthia Nau Cornelissen[1], William M. Shafer[2], David Dyer[3], (2010) The AraC-like Transcriptional Regulator,

MpeR, Activates Expression of FetA in *Neisseria gonorrhoeae.* American Society of Microbiology 110th General Meeting May 23rd-27th, San Diego, CA (Poster)

Aimee Hollander[1], Cynthia Nau Cornelissen[1], (2009) The role of Mper, an AraC-like Regulator and Iron on the Expression of Outer Membrane Proteins in *Neisseria gonorrhoeae.* American Society of Microbiology, Virginia Branch Meeting, November 13th-14th, Richmond, VA. (Presentation)

Awards

1st Place Best Student Presentation November 2009
Virginia ASM Branch Meeting, Virginia Commonwealth University, Richmond VA