2008

Analysis of the mechanism of transferrin-iron acquisition by Neisseria gonorrhoeae

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September 4th, 2008

ANALYSIS OF THE MECHANISM OF TRANSFERRIN-IRON ACQUISITION BY *NEISSERIA GONORRHOEAE*

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

by

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September 2008

Acknowledgements

I would like to sincerely thank my advisor, Dr. Cynthia Nau Cornelissen, for the opportunity to conduct my doctoral research in her laboratory. She has been a pioneer in the study of *Neisseria* iron acquisition and has provided the foundation for continued research in this field. Dr. Cornelissen has been a true role model for me, exhibiting the highest degree of scientific integrity and dedication to the teaching and development of future scientists. She has been a truly great mentor and her enthusiasm for research is contagious. I would like to express my sincere gratitude to her for always supporting and encouraging me throughout my graduate career.

I would also like to sincerely thank my committee members – Dr. Matthew Beckman, Dr. Francine Marciano-Cabral, Dr. Todd Kitten, and Dr. Joe Ritter. They have been an integral part of my success as a graduate student. They have provided invaluable advice and suggestions that have enhanced my research substantially. Their support and encouragement over the years have been constant and is very much appreciated.

I would also like to thank the Microbiology and Immunology administrative staff – Connie Babcock, Nancy Fogg, Bobbie Palumbo, and Martha VanMeter. They have always been such a great help with anything and everything I have needed along the way.

I cannot thank my friends and fellow lab members enough. The members of the Cornelissen lab – Dr. Amanda DeRocco, Dr. Greg Price, Dr. Tracey Zola, Aimee Hollander, Heather Strange, and Rose Velez – have truly defined the meaning of

friendship for me. They have all provided tremendous intellectual and emotional support over the years. We have shared many great times and have overcome many obstacles together – these memories will last forever.

I would also like the thank the friends that I have made during my time at Virginia Commonwealth University – Dr. Beth Rogers, Dr. Kelley Hovis, Krissy Sundy, Megan Price, and Dr. Paige Fox – these are friendships that will last a lifetime.

Above all, I express my deepest gratitude to my family, without whom I would have never made it this far. My parents, Pam & Bill Smith and Bob McMillan & Becky Gordos, have provided me with loving homes and constant love and support. They have always encouraged me to pursue my dreams and have provided me with every opportunity for success. They have taught me the most important lessons in life and for that I am eternally grateful.

Last, but certainly not least, I would like to thank my husband, Mike Noto. He has been a true inspiration to me and I cannot thank him enough for the joy he brings to my life everyday. He has always been understanding and has supported me through everything along the way. Most of all, I want to thank him for loving me and always believing in me even when I did not believe in myself.

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List of Abbreviations

^{o}C	degrees Celsius
Δ	delta, deletion
Ω	omega
^{55}Fe	radiolabeled iron
^{125}I	radiolabeled iodine
α	alpha or anti
ABC	ATP binding cassette
AP	alkaline phosphatase
ATP	adenosine triphosphate
β	beta
bp	base pair
BCA	bicinchoninic acid
BSA	bovine serum albumin
CDC	Centers for Disease Control and Prevention
CDM	chelexed defined media
CEACAM	carcinoembryonic antigen-related cell associated molecule
CFU	colony forming unit
CMP-NANA	cytidine 5'-mono-phospho-N-acetylneuraminic acid
CO_2	carbon dioxide
CPM	counts per minute

DFO	desferal
DGI	disseminated gonococcal infection
DNA	deoxyribonucleic acid
E.	*Escherichia*
ECL	enhanced chemiluminescence
Fbp	ferric binding protein
Fe	iron
$FeCl_3$	ferric chloride
$Fe(NO_3)_3$	ferric nitrate
Fur	ferric uptake regulator
GCB	gonococcal growth media
GCU	gonococcal uptake sequence
H.	*Haemophilus*
HA	hemagluttinin
Hb	hemoglobin
Hg	haptoglobin
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSPG	heparan sulfate proteoglycan
HS TBS	high salt Tris-buffered saline
IgA	immunoglobulin A
IgG	immunoglobulin G

IPTG	isopropyl-beta-D-thiogalactopyranoside
K.	*Klebsiella*
KCN	potassium cyanide
kDa	kiloDalton
K_d	affinity constant
L	liter
LAMP	lysosome/late endosome associated membrane protein
LB	lysis broth, *E. coli* growth media
Lbp	lactoferrin binding protein
Lf	lactoferrin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LS TBS	low salt Tris-buffered saline
M	molar
M.	*Moraxella*
mAmp	milliampere
mCi	millicuri
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	millliliter
mM	millimolar

mRNA	messenger ribonucleic acid
MLST	multilocus sequence typing
N.	*Neisseria*
$NaHCO_3$	sodium bicarbonate
$Na_3C_6H_5O_7$	sodium citrate
NBT/BCIP	nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate
ng	nanogram
nm	nanometer
nM	nanomolar
Opa	opacity protein
PBP	periplasmic binding protein
PBS	phosphate-buffered saline
PID	pelvic inflammatory disease
PMN	polymorphonuclear leukocyte, neutrophil
pmol	picomole
PCR	polymerase chain reaction
RCF	relative centrifugal force (x g)
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOE	splicing by overlap extension
T4SS	type IV secretion system
Tbp	transferrin binding protein

TCP	total cellular protein
TMP	total membrane preparation
Tf	transferrin
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
WT	wild-type

Abstract

ANALYSIS OF THE MECHANISM OF TRANSFERRIN-IRON ACQUISITION BY *NEISSERIA GONORRHOEAE*

By Jennifer McMillan Noto

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

Virginia Commonwealth University, 2008

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Neisseria gonorrhoeae is an obligate human pathogen that requires iron for its survival within the host. *N. gonorrhoeae* expresses high-affinity iron acquisition systems to acquire iron from host iron binding proteins. The gonococcal transferrin-iron uptake system is composed of two transferrin binding proteins, TbpA and TbpB. TbpA is a TonB-dependent, outer membrane transporter, while TbpB is a surface-exposed lipoprotein. Unlike TbpA, TbpB is not required for transferrin utilization, but makes the process more efficient. The precise mechanism by which TbpA and TbpB function to mediate transferrin-iron uptake has not been fully characterized. However, the

mechanism of iron acquisition from transferrin is distinct from characterized TonB-dependent ferric-siderophore uptake systems. The transferrin-iron uptake system is unique in two ways: the involvement of the TbpB lipoprotein component and the process of iron acquisition and internalization. Unlike siderophore transporters, the transferrin-iron uptake system requires the removal of iron from transferrin for its subsequent internalization. Based on analogy with characterized TonB-dependent transporters, TbpA is proposed to consist of two distinct domains: a β-barrel and plug domain. Previous studies suggest that the plug domain has a specific role in iron internalization and this study addresses the role of the plug domain in transferrin-iron acquisition. It is thought that the TbpA plug domain facilitates iron removal from transferrin and subsequent iron binding and transport. To analyze this, iron binding by the TbpA plug domain was performed and site-directed substitution mutagenesis of putative iron-coordinating residues was carried out. From these analyses, it can be concluded that the plug domain binds iron and likely plays an active role in the process of iron internalization. Mutagenesis revealed specific residues of the plug domain critical for transferrin-iron uptake, but defects imparted by these mutations were compensated for by TbpB. Thus, this study also attempts to characterize the compensatory function provided by TbpB. Through mutagenesis, critical domains involved in the efficiency of transferrin-iron acquisition were identified. One additional study describes and characterizes a novel mechanism of TonB-independent transferrin-iron acquisition. Overall, these studies further elucidate mechanisms utilized by *Neisseria gonorrhoeae* in the process of iron acquisition from human transferrin.

CHAPTER 1 – INTRODUCTION

Neisseria gonorrhoeae and *Neisseria meningitidis* are both human pathogens in the *Neisseriaceae* family. *N. gonorrhoeae* is the causative agent of the sexually transmitted disease gonorrhea. The first documented observations of gonorrhea were made by Hippocrates (460 – 355 BC) (146), and the bacterium *N. gonorrhoeae* was first discovered in 1879 by Albert Neisser. Although *N. gonorrhoeae* was not recognized until the nineteenth century, gonorrhea has been described since antiquity, with references made in biblical and other ancient texts (146). Until the advent of antibiotics in the 1900s, gonorrhea was very difficult to treat and control. To this day, gonorrhea has still proven difficult to control because of the increasing antibiotic resistance in isolates throughout the world (33, 34). In addition to the high incidence of antibiotic resistance (33), the lack of protective immunity and increase in HIV transmission correlated with gonococcal infection (44, 128) point to the need for development of an effective vaccine against *N. gonorrhoeae.*

Neisseria meningitidis is closely related to *N. gonorrhoeae* and was first described by Anton Weichselbaum in 1887. *N. meningitidis* is the causative agent of bacterial meningitis, which may lead to life threatening bacteremia. Despite the differences in disease manifestations, the two pathogenic *Neisseria* share a close evolutionary relationship (217) and have a very similar repertoire of virulence factors. Therefore,

experimental observations made in studying *N. gonorrhoeae* may be applied to *N. meningitidis*, and furthermore, the development of an effective vaccine against gonococcal infections may also provide protection against meningococcal disease.

I. Genus *Neisseria*

The genus *Neisseria* belongs to the family *Neisseriaceae*, which is comprised of many genera, including *Moraxella*, *Acinetobacter*, and *Kingella* (83, 100). Species belonging to the genus *Neisseria* include both obligate human pathogens and normal human flora. The most well studied of the *Neisseria* species are the pathogens *N. gonorrhoeae* and *N. meningitidis*. *N. gonorrhoeae* is the only true pathogen because it is always associated with the disease state (32), whereas *N. meningitidis* can be found among normal human flora.

Neisseria species are Gram-negative diplococci that have adjacent, flattened sides and range in size from 0.6 to 1.5 μm. *Neisseria* are classified as either aerobes or facultative aerobes, with optimal growth conditions between 35^{o}C and 37^{o}C in the presence 5% CO_2. *Neisseria* species are fastidious microorganisms that require complex growth media with glucose provided as a carbon source. Consistent with the fact that most *Neisseria* species rely on the human host for survival, all species have limited metabolic capabilities. This limited metabolic potential provides a basis for species differentiation based on abilities to produce acid from carbohydrates, polysaccharide from sucrose, and the ability to reduce nitrate. In addition, *Neisseria* produce a number of enzymes that allow for species differentiation (83, 100) (Table1).

II. *Neisseria* Infection

Both pathogenic *Neisseria* species are associated with human epithelial mucosa (136), and despite their close evolutionary relationship (217), meningococci colonize the nasopharynx, while gonococci primarily infect the urogenital tract. The differences in disease states and the few differences in virulence factors are likely linked to the different modes of transmission utilized by *N. gonorrhoeae* and *N. meningitidis*.

A. Meningococcal infection

Neisseria meningitidis is a commensal that is spread by respiratory droplets and colonizes the oro- or nasopharynx of approximately 5 – 30% of the human population (71). *N. meningitidis*, in some cases, can be classified as a member of the normal flora or, in others, can be associated with invasive meningococcal disease. It has been shown through multilocus sequence typing (MLST) that disease is caused by hypervirulent lineages of *N. meningitidis* (123) and that these lineages are vastly underrepresented in asymptomatic meningococcal carriers (41). Invasive meningococcal disease develops when meningococci cross the nasopharyngeal epithelium and enter the bloodstream, resulting in bacteremia. In turn, meningitis results when meningococci cross the blood-brain barrier, which can occur as a consequence of high level bacteremia (154). Invasive meningococcal disease usually develops rapidly and has a high rate of fatality between 5 – 15% (86). The rapid disease progression makes prompt diagnosis and intervention critical for survival of meningococcemia and meningitis.

Neisseria meningitidis can be classified into thirteen different serogroups, based on the polysaccharide capsule. Serogroups A, B, C, Y, and W-135 are most commonly associated with invasive meningococcal disease, with A, B, and C accounting for approximately 90% of cases worldwide (168). Currently there is an effective tetravalent vaccine against serogroups A, C, Y, and W-135; however, there is no vaccine against serogroup B because this capsular polysaccharide lacks immunogenicity. The lack of immunogenicity occurs through the decoration of serogroup B capsule with host sialic acid, which is considered a form of molecular mimicry (203). The need for an effective vaccine against all capsular serotypes is critical in eliminating meningococcal disease throughout the world.

B. Meningococcal epidemiology

The rate of meningococcal disease in the United States is relatively low, with approximately 0.9 – 1.5 cases per 100,000 people (189). The rates of meningococcal disease are highest among infants, but rates drop following infancy and then rise again during adolescence and early adulthood (190). In addition to fluctuations in disease rate among different age groups, fluctuations in rates are also observed during seasonal changes, with rates highest during the winter and early spring in the United States (190). Meningococcal disease is a much more serious problem in other parts of the world. Europe has slightly higher rates of disease than the United States, with approximately 0.3 – 7.1 cases per 100,000 people (159). Meningococcal disease occurs in major epidemics within the African meningitis belt, which includes many sub-Saharan countries. The rates of disease during these epidemics are 500 – 1000 times the rates seen in the United

States, with approximately 500 – 1000 cases per 100,000 people (237). Overall, meningococcal disease is serious and widespread throughout the world. The various factors that contribute to meningococcal disease and epidemics are complex and not well understood.

C. Gonococcal infection

Neisseria gonorrhoeae is the causative agent of the sexually transmitted disease gonorrhea. Gonorrhea is transmitted through sexual contact and causes a localized infection of the male and female lower urogenital mucosa. In men, gonorrhea typically presents as an acute urethritis, and epididymitis is a common complication of untreated urethritis. Although rare in males, other complications result from ascension of gonococci to the upper genital tract and include prostatitis, posterior urethritis, and seminal vesiculitis (68). In women, gonococcal infection causes cervicitis and/or urethritis, but up to 80% of women are asymptomatic and do not seek proper medical attention (8, 129, 167). It has been shown that 40% of women with localized gonococcal infection are also anorectally colonized with *N. gonorrhoeae* (99). Ascension of gonococci to the upper genital tract can result in pelvic inflammatory disease (PID), which is seen in 10 – 20% of female infections (82, 236). If left untreated, PID can cause fallopian tube scarring and can ultimately result in infertility and/or ectopic pregnancy (150, 202). Disseminated gonococcal infection (DGI) can also occur, but at a relatively low frequency (0.5 – 3%) and most commonly in young women (68). Classic symptoms of DGI include dermatitis, tenosynovitis, migratory polyarthritis, and even less common perihepatitis (120) and endocarditis (92). Additional primary sites of gonococcal

infection include the rectum, pharynx, and conjunctiva. Gonococcal conjunctivitis is a localized infection of the adult eye that can result in corneal scarring and without immediate treatment can lead to loss of vision (143, 221). Gonococcal infection can be transferred from mother to neonate during vaginal childbirth, causing neonatal conjunctivitis. Although not a significant problem in the United States, this infection remains a very common cause of blindness in developing countries (108). The serious sequelae resulting from gonococcal infection, the lack of protective immunity, and the correlation with HIV transmission (44, 128), all point to the need for an effective vaccine against *N. gonorrhoeae*.

D. Gonococcal epidemiology

Infection by *Neisseria gonorrhoeae* is a major health concern throughout the world. In 1998, the World Health Organization reported that there were 62.2 million cases of gonorrhea worldwide (238). The estimated number of cases in the United States reached approximately 360,000 cases in 2006, making gonorrhea the second most frequently reported communicable disease following chlamydial infection (33). Although the rates of gonorrhea declined 74% from 1975 to 1997, the rates steadily increased again, with the number of cases reaching 121 per 100,000 people in 1998 (32). Reporting of gonococcal cases is typically lower than the actual incidence of disease particularly due to underreporting and asymptomatic gonococcal infection in women.

The high rates of gonococcal infection seen throughout the world are a serious cause for concern. Gonorrhea has huge health implications in regard to PID and the role gonococcal infection plays in facilitating HIV infection (44, 128). The continuing

emergence of antibiotic resistant *N. gonorrhoeae* is of increasing concern for the control and prevention of gonorrhea. Recent reports of antibiotic resistance in *N. gonorrhoeae* show that there is still a high prevalence of isolates resistant to both penicillin and tetracycline, presence of multi-drug resistant strains, and an emergence of fluoroquinolone resistance (33). In addition, the Center for Disease Control and Prevention recommended in 2007 that fluoroquinolones should no longer be used for treatment of gonococcal infection (34). Currently, third generation cephalosporins are the treatment of choice against gonorrhea, but decreased susceptibility has been observed (33), which suggests that it is only a matter of time before resistance develops to all currently available antibiotic therapies. In addition to the health implications resulting from gonococcal infection, the increasing rates of resistance seen in *N. gonorrhoeae* again point to the need for the development of an effective vaccine.

III. *Neisseria* Virulence Factors

Pathogenic *Neisseria* express a wide range of virulence factors that contribute to pathogenesis within the human host. A number of these factors contribute to initial adherence to mucosal epithelium, while other adhesins facilitate more intimate adherence with specific host cell receptors. These interactions facilitate invasion and transcytosis across epithelial cells, which allows for interaction with endothelial and various immune cells. These interactions can ultimately lead to entry into the bloodstream and dissemination to distal sites. During this process there are many virulence factors involved in immune evasion, extracellular and intracellular survival, and disease

progression. These virulence determinants and their roles in gonococcal and meningococcal pathogenesis are discussed below.

A. Capsule

Polysaccharide capsule is one well-characterized virulence factor that is found in *N. meningitidis*, but absent in *N. gonorrhoeae* (68). Thus, capsule is likely one of the main virulence determinant that facilitates the different mode of transmission, disease progression, and disease outcome observed with meningococcal infection. *N. meningitidis* expresses 13 different capsule types, which differ in the polysaccharide composition. Meningococcal capsule is subject to high-frequency phase variation and various sialic acid modifications, which have both been shown to be important in resistance to phagocytosis and complement-mediated killing (68). The diversity of capsular polysaccharides in addition to the variations within each capsular serogroup make capsular polysaccharide a dynamic virulence factor in the pathogenesis of *N. meningitidis*.

B. Pilus

To initiate infection, pathogenic *Neisseria* must adhere to the mucosal epithelium in a step known as initial adherence. Type IV pilus is critical for the colonization of the mucosal nasopharynx and urogenital tract. In the absence of pili, gonococci are unable to initiate infection upon human challenge (95, 96). Type IV pili are long filamentous structures that extend from the gonococcal and meningococcal cell surface and facilitate binding to host mucosal epithelium. The long pili allow gonococci and meningococci to

overcome the negative electrostatic barrier that exists between bacterial and host cells to allow for initial attachment (80).

The type IV pilus is composed of two subunits: monomeric pilin subunits (PilE) that form the pilus fiber and PilC, the pilus tip adhesin (191). Pili have been shown to mediate many different cellular interactions with epithelial cells, endothelial cells, granulocytes, macrophages, and erythrocytes (101, 193, 196, 224, 227). PilC has been shown to be an important factor in host cell interactions and facilitates interaction with both epithelial and endothelial cells (153). PilC mutants are unable to interact with these cells, and purified PilC prevented piliated gonococci from interacting with epithelial cells in vitro (195).

Type IV pili of pathogenic *Neisseria* are antigenic and produce a local antibody response that blocks attachment of bacteria to epithelial cells (90, 127, 219); however, expression of pilus is subject to both phase and antigenic variation, which makes it a poor vaccine candidate. Phase variation of pilus results from RecA-independent frame shifting known as slipped-strand mispairing, while antigenic variation results from RecA-dependent, non-reciprocal gene conversion (103). Slipped-strand mispairing occurs during DNA replication, when DNA polymerase skips or adds bases in the poly cysteine tract of *pilE*, causing a frame shift and gene expression to be turned off (103). Phase variation occurs at a relatively high frequency of 10^{-4} per cell generation (103, 245). Antigenic variation of pilus results from the non-reciprocal exchange of DNA sequences from one of several silent *pilS* loci into the *pilE* expression locus (73, 134, 135, 199). In addition to phase and antigenic variation, pilin is also subject to posttranslational

modifications through phosphorylation and glycosylation (205, 227). The receptor responsible for pilus interactions with host cells has not been identified and the only receptor (93) that has been implicated in this interaction is subject to much controversy (125).

C. Opacity proteins

The neisserial opacity proteins (Opa) were identified and named for the color and opacity that they impart on gonococcal colonies (87, 212). Opacity proteins are integral outer membrane proteins that have been associated with *Neisseria* virulence and host cell interaction. Opas are linked to *N. gonorrhoeae* virulence because gonococci recovered from urogenital, cervical, and rectal infections always express at least one Opa protein (213). In addition, gonococcal strains, not expressing Opas, used in volunteer infection studies are always recovered following infection expressing these proteins (89). These studies point to the fact that opacity proteins are required for survival and virulence in vivo. Any single gonococcal strain can possess up to eleven distinct *opa* alleles that encode antigenically distinct Opa variants, while meningococcal strains only possess up to four distinct *opa* alleles (13, 106). Opacity proteins from both *N. meningitidis* and *N. gonorrhoeae* are subject to high-frequency phase variation that in *N. gonorrhoeae* results in the expression of zero to four Opas at any one time (17). This phase variation occurs via slipped-strand mispairing in which the number of CTCTT repeats in a poly CTCTT tract vary and cause frame shifts that result in differential expression of none, one, or multiple Opas at one time (152, 204).

Opacity proteins have also been characterized as factors involved in intimate attachment to host cells. Opas have been shown to facilitate binding and invasion of host cells through interactions with surface heparan sulfate proteoglycans (HSPG) as well as carcinoembryonic antigen-related cell adhesion molecules (CEACAM) on various cell and tissue types (68). Several of the eleven different Opas expressed by *N. gonorrhoeae* bind to HSPGs (39, 106, 223) and one in particular has been shown to mediate intimate adherence that results in receptor-mediated endocytosis into epithelial cells (106, 230, 231). This Opa protein has also been shown to mediate binding and promote invasion of endothelial cells and fibroblasts, although in a less efficient manner than seen with epithelial cells (67).

CEACAMs are differentially expressed on a variety of cell types (9, 173, 216) and Opas have been shown to interact with a number of CEACAMs (19, 69). Opa-mediated binding and invasion has been observed in many cells, including epithelial, endothelial, and phagocytic cells and these events ultimately leads to CEACAM-dependent intracellular signaling. Although Opa-CEACAM interactions are important for gonococcal cell tropisms, this interaction is also important in the down regulation of the host immune response through the suppression of T-cell activation and proliferation, inhibition of antibody production, and induction of B-cell apoptosis (21, 164)

D. Opc

Opc is a neisserial outer membrane adhesin that is similar in size to the opacity proteins, but structurally and antigenically distinct. Opc was originally identified as a meningococcal-specific protein, but is also found in *N. gonorrhoeae* (132). The majority

of work done on Opc has been in the meningococcus, but its role in intimate adherence and invasion is important to consider for *N. gonorrhoeae*. Opc has been shown to interact with epithelial and endothelial cells (54, 133, 225). Opc interaction with endothelial cells occurs via $\alpha_v\beta_3$ integrin in a vitronectin-dependent fashion (226). Opc likely serves as another adhesin and invasin important for *Neisseria* pathogenesis.

E. Porins

Porins are the most abundant proteins on the outer membrane of *Neisseria*. They form hydrophilic pores that allow for the passive diffusion of small nutrients (molecular weight ≤ 600 daltons) across the outer membrane (158). Porins have a variety of different cellular functions that contribute to the pathogenesis of *Neisseria*. Porin has been shown to translocate into eukaryotic cytoplasmic and phagosomal membranes (230). Once inserted in the eukaryotic membranes, porins are regulated by the host cell (192), which suggests that they have characteristics of voltage-dependent anion channels. Once porins form pores in eukaryotic membranes, they cause rapid calcium influx and ultimately induce cellular apoptosis (148, 149). Porins also have effects on the host cell phagocytic response, by interference with PMN signaling, inhibition of degranulation, down regulation of opsonin receptor-mediated phagocytosis, and modulation of phagosomal maturation (16, 75, 147).

N. meningitidis expresses two types of porin, PorA and PorB, while *N. gonorrhoeae* only expresses one porin, PorB. PorB of both pathogenic *Neisseria* have two antigenically distinct serotypes, designated PIA and PIB. Expression of PorBIA (PIA) is known to cause increased gonococcal serum resistance and is correlated with

disseminated gonococcal infection (142). Serum resistance mediated by PIA is attributable to its ability to bind complement factors, Factor H and C4 binding protein, and thereby prevent complement-mediated killing (178, 179). Thus, porins are important in basic nutrient acquisition as well as immune modulation and evasion in the pathogenic *Neisseria*.

F. Lipooligosaccharide

Lipopolysaccharide or LPS is a major component of the Gram-negative outer membrane. LPS is comprised of three parts: lipid A, which is embedded in the outer membrane, the core polysaccharide, and the O-antigen side chains. Neisserial LPS can be distinguished from enteric LPS because of its highly-branched core oligosaccharide structure and lack of repetitive O-antigen side chains. Thus, LPS from *Neisseria* species is called lipooligosaccharide or LOS. LOS is an endotoxin and a major virulence factor involved in the inflammatory response seen during *Neisseria* infection. LOS, like many other virulence factors of pathogenic *Neisseria*, is subject to high-frequency variation. Variation in LOS occurs at the α side chains within the core polysaccharide, where sugar residues are added by various glycosyl transferases. These enzymes are subject to slipped-strand mispairing at poly glycine tracts, which causes variations in expression patterns. Differential expression of the glycosyl transferases, leads to overall changes in LOS size, structure, and carbohydrate composition (52, 241). In addition, LOS is subject to post-translational modification in vivo, which is known to enhance *Neisseria* serum resistance. Sialylation of LOS occurs in vivo by gonococcal and meningococcal sialyltransferases that utilize host derived cytidine 5'-mono-phospho-N-acetylneuraminic

acid (CMP-NANA) as a sialyl donor (124). This modification enhances gonococcal and meningococcal survival in vivo through inhibition of complement deposition (180) and phagocytic killing (141). Sialylation of LOS with host CMP-NANA represents another example of molecular mimicry that aids in survival of *Neisseria* in the human host. Additionally, LOS sialylation protects *Neisseria* against PorB-specific bactericidal antibodies (59).

G. Reduction-modifiable protein

Reduction-modifiable protein (Rmp) is a gonococcal outer membrane protein found in all gonococcal isolates and is named based on the observed molecular weight shift following reduction in SDS-PAGE (65). Rmp, an OmpA homolog (66), is often found in association with LOS and PorB in the outer membrane (81, 130) and is highly conserved between strains (91, 239). Rmp-specific antibodies serve as blocking antibodies that protect gonococci from LOS- and PorB-specific bactericidal antibodies (151, 185). In fact, Rmp antibodies actually increase host susceptibility to gonococcal infection (171).

H. IgA protease

Pathogenic *Neisseria* secrete a serine IgA protease that has a high specificity for human IgAI antibodies. IgAI is a specific subtype of IgA and is the most prominent IgA antibody found in mucosal secretions (194). Therefore, gonococci and meningococci come in contact with IgAI antibodies during localized infection and are able to cleave the F_{ab} antigen binding fragment from the F_c effector domain. This cleavage event essentially decorates *Neisseria* with F_{ab}, which aids in masking the immunodominant,

surface epitopes from host bactericidal antibodies (68). Neisserial IgA protease also cleaves human lysosome/late endosome associated membrane protein 1 (LAMP-1) and this cleavage event is important for gonococcal survival and growth within epithelial cells (79, 116). Not only does IgA protease aid in colonization at mucosal surfaces, but it also appears to have a significant role in intracellular survival.

IV. Iron and Pathogenesis

A. Iron

Iron is one of the most abundant elements on the earth and is essential for the survival of virtually all organisms (25). Iron has a versatile redox potential, and can exist in the reduced ferrous (Fe^{2+}) form or the oxidized ferric (Fe^{3+}) state. Under aerobic conditions, ferrous iron (Fe^{2+}) is highly unstable and through the Fenton reaction is converted to ferric iron (Fe^{3+}) with the production of toxic, reactive oxygen species:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

The reactivity and toxicity of iron creates an environment in which iron sequestration is critical within the human host. Although iron is very abundant in nature, it exists in an inaccessible form due to its extreme insolubility. This property of insolubility creates an iron-limiting environment in the human host, with a concentration of free, ferric iron (Fe^{3+}) of 10^{-18} M (29), which is far below the nutrient requirement for survival of microorganisms (232). Within the human host, this concentration is even lower as a result of host iron binding proteins sequestering free iron from the environment (182). All microorganisms require iron, with the exception of only two known bacterial genera,

Lactobacillus (5) and *Borrelia* (172). Iron is critically important as an electron carrier in microbial oxidative metabolism and as the catalytic center of a variety of essential enzymes. As a result, pathogenic microorganisms have evolved mechanisms to acquire iron from host iron binding proteins to survive and persist within the human host.

B. Pathogenic mechanisms for iron acquisition

There are many mechanisms by which microorganisms acquire iron from the human host. Many microorganisms produce and secrete siderophores, low-molecular-weight iron-chelating molecules, during iron stress to scavenge iron from their environments (155, 156). Siderophores function to specifically bind, solubilize, and deliver iron via bacterial ferric-siderophore uptake systems. Some bacteria can obtain iron through the expression and secretion of ferric reductases, which reduce ferric iron (Fe^{3+}) to the more soluble ferrous (Fe^{2+}) form for utilization (51, 197). These reductases have also been shown to remove iron from host iron binding proteins through this reduction event (51, 197). Other bacteria have receptor-mediated mechanisms to scavenge and utilize iron from heme, hemoproteins, and other host serum proteins, such as lactoferrin and transferrin. The human host goes to great lengths to sequester iron in an attempt to make free iron inaccessible, and pathogenic microorganisms have developed a variety of different mechanisms to bypass this iron sequestration for survival. These mechanisms of host iron sequestration and the pathogenic mechanisms in which bacteria acquire iron from the human host are discussed in the next two sections.

V. Iron Sources in the Human Host

A. Ferric and ferrous iron

Ferric iron (Fe^{3+}) is the major form available in aerobic environments, but is highly insoluble in the form of ferric hydroxides. Ferrous iron (Fe^{2+}), however, is the predominant form under anaerobic or reducing conditions. Although not typically present in high concentrations within the human host, soluble, ferrous iron (Fe^{2+}) can diffuse freely across Gram-negative outer membranes via porins. Subsequently, iron is transported through the cytoplasmic membrane by ABC ferrous iron (Fe^{2+}) transporters, which are conserved among many bacterial species (94).

B. Ferritin

Ferritin is the major cytoplasmic iron storage protein within the human host. Ferritins function intracellularly to provide iron during iron shortages and also protect the host from the toxic effects of iron accumulation. Ferritin consists of 24 subunits that form a icosahedron structure (30) that has a capacity for harboring more than 4000 ferric iron (Fe^{3+}) atoms (1, 2, 181). Although ferritins have not been shown to be a substantial iron source for bacterial pathogens, they constitute a major intracellular iron source.

C. Heme & heme binding proteins

a. Heme

Heme is an iron-containing porphyrin ring that is highly toxic to cells and as a result, is scarcely found free in the human host. Despite its scarcity in the host environment, heme does serve as an iron source for a variety of bacterial pathogens. These microorganisms express specific, high-affinity uptake systems to acquire heme-

iron from the human host. However due to its toxicity, free heme exists at very low concentrations because it is sequestered by the host hemoproteins: hemoglobin, haptoglobin, and hemopexin.

b. Hemoglobin

Hemoglobin is found in red blood cells and functions as an oxygen carrier in the human host. It is tetrameric in structure and each subunit binds heme. Hemoglobin can exist in one of three states: (1) methemoglobin, which is oxidized and contains ferric iron (Fe^{3+}); (2) oxyhemoglobin, which is the oxygen carrying form; or (3) carboxyhemoglobin, which is reduced and contains ferrous iron (Fe^{2+}). Many bacterial species express hemoglobin-specific heme uptake systems for the acquisition of heme-iron complexes.

c. Haptoglobin

Haptoglobin is a host serum glycoprotein that binds hemoglobin when it is released into the serum following hemolysis. The hemoglobin-haptoglobin interaction occurs at such a high affinity in the host that dissociation only occurs through degradation of the protein complex in the liver. It has been shown that pathogenic *Neisseria* (112, 115) and *Haemophilus influenzae* (122, 145) are able to utilize the hemoglobin-haptoglobin complex as a heme-iron source through receptor-mediated events.

d. Hemopexin

Hemopexin is another host serum glycoprotein that binds heme with a high affinity (165). Hemopexin functions to remove free heme from the serum and transport it to the liver, where apopexin is recycled following release of heme intracellularly. To

date, *Haemophilus influenzae* has been the only bacterium shown to specifically bind and utilize hemopexin as a source of heme (78).

D. Lactoferrin

Lactoferrin is an iron binding glycoprotein found in mucosal secretions among many other places. It is found in highest concentration in breast milk, but is also present in secondary granules of neutrophils and various other secretions (26). The lactoferrin structure consists of two highly homologous lobes that both have deep binding clefts that function in ferric iron (Fe^{3+}) coordination (3). Lactoferrin is similar to transferrin, but has two features that make it distinct. Those features include its higher affinity for iron and its higher isoelectric point (pI) (26). Lactoferrin functions primarily in iron chelation to protect the host from the toxic effects of iron accumulation. However, lactoferrin has also been attributed to a large number other functions unrelated to its iron binding capacity (26, 27). One of importance is its antimicrobial properties mediated by both full-length lactoferrin as well as small, cleaved lactoferrin peptide fragments (26, 240). Lactoferrin plays an important role in the host through both its immunoregulatory functions (26) and iron sequestering ability. Many bacterial pathogens have evolved specific and efficient mechanisms to acquire iron from lactoferrin within the human host.

E. Transferrin

Transferrin is the most abundant iron transport protein found in the human host. It is an 80 kDa glycoprotein found in high concentrations in serum, lymph, and seminal fluid. Transferrin has two major functions in the host: (1) iron binding for transport throughout the human body and (2) protecting the host from iron-mediated toxicity.

Similar to lactoferrin, transferrin has a high affinity for iron, with an association constant that has been reported as high as 10^{31} M (2). The structure of transferrin shows that it is bi-lobed and both lobes function in high-affinity iron coordination (88). However, in vivo, transferrin is only about 30% saturated with iron (2). Ferric iron (Fe^{3+}) is hexacoordinated in the lobes of transferrin by two tyrosines, a histidine, an aspartic acid, a carbonate, coordinating anion, and a hydroxyl group from water (88). Despite the high-affinity coordination of iron, many bacterial pathogens are able to acquire iron from transferrin within the human host.

VI. TonB-Dependent Iron Acquisition

The majority of pathogenic mechanisms to obtain iron from the human host are classified as TonB-dependent iron acquisition systems. The components of TonB-dependent iron uptake include, but are not limited to, an integral, outer membrane protein that transports iron/iron complexes through the outer membrane, a TonB protein complex that harnesses energy for the process of iron internalization, and a periplasmic iron binding protein (PBP) that shuttles iron/iron complexes to an ABC transporter within the cytoplasmic membrane. Various mechanisms of TonB-dependent iron acquisition have been identified and these systems are discussed (Figure 1).

A. Siderophore-mediated iron acquisition

Siderophores are low-molecular-weight, high-affinity iron chelators of which more than 500 have been identified (181). They are divided into three major classes based on the structure and chemical nature of iron coordination. Siderophores are

classified as catecholates, hydroxamates, and carboxylates; however, this classification scheme is becoming more complex with new information on siderophores that have structural and chemical features from more than one class (182). These molecules specifically bind ferric iron (Fe^{3+}) with a high affinity and rarely bind the ferrous (Fe^{2+}) form. Ferric iron is complexed in a 1:1 ratio with each siderophore and is typically found in a hexacoordinated state, which forms an octahedral structure in aqueous solution (182). Interestingly, ferric-siderophores have a wide range of affinities for iron, with dissociation constants ranging from 10^{22} to 10^{50} (181), over 30 orders of magnitude differences between some (139). These relative affinities for iron are sufficient for siderophore-mediated removal of iron from human ferritin, lactoferrin, and transferrin, but not the various hemoproteins (181).

Siderophores are synthesized and secreted by microorganisms to scavenge iron from the environment (228). Once ferric iron (Fe^{3+}) is bound and mobilized by siderophores, it becomes accessible by one of two ways: (1) iron is released from the siderophore complex through a reduction at the bacterial cell surface, whereby iron is taken up by the cell (51) or (2) the entire ferric-siderophore complex is internalized by a TonB-dependent receptor-mediated event (139). However, bacterial systems of reduction-mediated iron removal from siderophores are less well known and the receptor-mediated events are considered the major route of siderophore-mediated iron acquisition (139).

Ferric-siderophore uptake (Figure 1), like all TonB-dependent transporters, requires TonB energization and an outer membrane transporter to facilitate uptake

through the outer membrane. The TonB complex is composed of three proteins: TonB, which is embedded in the cytoplasmic membrane and spans the periplasmic space and ExbB and ExbD, which are both embedded in the cytoplasmic membrane. These proteins work together to harness the proton motive force of the cytoplasmic membrane to energize TonB, which is thought to promote its interaction with specific outer membrane transporters and facilitates ferric-siderophore uptake (139). It also requires a periplasmic iron binding protein (PBP) and an ABC transporter for movement across the periplasm and through the cytoplasmic membrane, respectively.

Many of these TonB-dependent, outer membrane transporters have been crystallized and their overall structures, shown in Figure 2, are very similar (28, 40, 42, 43, 119, 244). These structures show the presence of two distinct domains: (1) a C-terminal β-barrel consisting of 22 β-strand transmembrane-spanning domains and (2) an N-terminal plug domain that folds up with the β-barrel. Mutagenesis (35, 36) and disulfide tethering (58) of the plug domain, have revealed that the plug domain undergoes conformational rearrangement that allows for ferric-siderophore transport through the outer membrane. This conformational change is thought to occur following interaction with TonB and results in siderophore translocation through the outer membrane (166, 200).

Following ABC-mediated transport through the cytoplasmic membrane, the ferric-siderophore gains access to the bacterial cytoplasm. Within the cytoplasm, there are two known mechanisms that result in iron release from siderophore complexes. The first involves the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by cytoplasmic

ferric-siderophore reductases (139). The second involves specialized enzymes that promote hydrolysis, resulting in destabilization of the siderophore and release of iron (139). Overall, these mechanisms of TonB-dependent siderophore-mediated iron acquisition are utilized by many pathogenic microorganisms to meet their iron requirements in the midst of severe iron starvation within the human host.

B. Non-siderophore-mediated iron acquisition

a. Heme and hemoproteins

Many pathogenic bacteria utilize heme as an iron source within the human host. Similar to iron, heme is cytotoxic and thus sequestered by host hemoproteins, hemoglobin, haptoglobin, and hemopexin. One mechanism pathogens utilize to gain access to heme within the host is the secretion of exotoxins that cause cell lysis (109, 162, 170, 201, 206), which results in the release of intracellular heme and hemoproteins. Once heme and/or hemoproteins are accessible, pathogens utilize two mechanisms for acquiring heme (reviewed in (229)). The first involves direct uptake of heme from host hemoproteins through TonB-dependent outer membrane transporters, as shown in Figure 1 (169). This process resembles the mechanism described for siderophore-mediated iron acquisition, requiring an outer membrane transporter, periplasmic binding protein (PBP), and cytoplasmic membrane-associated ABC transporter. However, unlike ferric-siderophore transporters these hemoprotein receptors consist of two proteins that are both required for heme acquisition and uptake. Briefly, the hemoprotein is bound to the outer membrane receptor, heme is removed, and transported through the outer membrane in a TonB-dependent step. Subsequently, heme is received and bound by a periplasmic heme

binding protein (PBP) and shuttled to the ABC transporter, which facilitates uptake into the bacterial cytoplasm. Once heme reaches the cytoplasm, it is degraded and iron is released and utilized by the cell.

The second mechanism by which bacteria utilize heme is through the secretion of hemophores, which scavenge heme from the environment and deliver it back to specific TonB-dependent receptors for internalization (reviewed in (55)). Once the heme-hemophore complex binds the receptor, the mechanism of heme uptake is essentially identical to that for the process involved in hemoprotein-heme acquisition described previously.

b. Lactoferrin and transferrin

Many pathogenic bacteria express high-affinity receptors for human lactoferrin and transferrin binding (70). These receptors not only function in lactoferrin/transferrin binding, but also TonB-dependent iron transport. Thus, both of these receptors have two ligand specificities, one for lactoferrin/transferrin and another for iron. In contrast to the ferric-siderophore transporters, both lactoferrin- and transferrin-iron acquisition systems involve two protein components: an integral, outer membrane protein and a surface-exposed lipoprotein. In both systems, the outer membrane transporter (LbpA/TbpA) functions in lactoferrin or transferrin binding as well as iron transport, while the lipidated protein (LbpB/TbpB) functions only in ligand binding. The process of lactoferrin- and transferrin-mediated iron acquisition is thought to be similar to the process described in siderophore-mediated iron acquisition (Figure 1). However, there are two major differences in the lactoferrin- and transferrin-iron acquisition systems that make them

unique from characterized TonB-dependent siderophore transporters. Firstly, the lipoprotein components are not present in siderophore-mediated iron acquisition systems. Secondly, the mechanism of iron internalization likely differs in that both lactoferrin and transferrin must be stripped of iron in the process of transport, whereas, ferric-siderophore complexes are transported without this iron removal step. Although the crystal structures of these integral, outer membrane proteins and the lipoproteins have not been solved, the outer membrane transporters are thought to look very similar to crystallized siderophore transporters (Figure 2), consisting of two distinct domains.

VII. Iron Acquisition Systems in Pathogenic *Neisseria*

A. Siderophore utilization

Neisseria do not produce any known siderophores (6, 131, 235), but have been shown to utilize siderophores produced by other microorganisms (31, 234). The receptors for utilization of these xenosiderophores have not been identified; however, a number of putative receptors have been, which have significant homology to characterized *E. coli* TonB-dependent siderophore transporters (220). The possible role of these putative transporters in xenosiderophore utilization has yet to be determined. To date, there has been only one siderophore receptor, FrpB/FetA, in *N. gonorrhoeae* linked to ferric-enterobactin binding, but its specific role in ferric-enterobactin uptake has not been shown (31). Although ferric-siderophore utilization is a major mechanism of iron acquisition for many bacterial pathogens, it does not appear to play a significant role in

Neisseria iron acquisition. As a result, pathogenic *Neisseria* express high-affinity iron acquisition systems to acquire iron from host iron binding proteins.

B. Hemoprotein utilization

Pathogenic *Neisseria* are not able to use hemopexin, myoglobin, or other heme sources, such as albumin, catalase, or cytochromes (23), but can utilize free heme in a TonB-independent mechanism (209). Through TonB-dependent mechanisms, pathogenic *Neisseria* are able to utilize hemoglobin and haptoglobin as heme-iron sources through the expression of HpuA and HpuB (38, 112, 114, 115). Similar to the lactoferrin- and transferrin-iron acquisition systems, the hemoglobin and haptoglobin utilization system has two components. HpuB is the TonB-dependent, outer membrane heme transporter, while HpuA is the surface-exposed lipoprotein (114). Unlike the lactoferrin and transferrin systems, both components are required for hemoglobin- and haptoglobin-mediated heme uptake (115). Also unlike the components of the lactoferrin and transferrin utilization systems, the hemoglobin and haptoglobin utilization components, HpuA and HpuB, are subject to high-frequency phase variation, which occurs through slipped-strand mispairing at a poly glycine tract within the *hpuAB* locus (113).

N. meningitidis, but not *N. gonorrhoeae*, also expresses another TonB-dependent receptor, HmbR, which functions in only hemoglobin utilization (110, 207, 208). This hemoglobin utilization system is comprised of only one outer membrane receptor, HmbR, unlike the two component system for hemoglobin and haptoglobin utilization (110, 207, 208). Similar to the components of the hemoglobin and haptoglobin utilization system,

HmbR is also subject to high-frequency phase variation though slipped-strand mispairing at a poly glycine tract in the *hmbR* locus (113, 186).

C. Lactoferrin utilization

Neisseria species are able to use lactoferrin as an iron source in the human host (6, 18, 111, 131, 137). However, only about 50% of gonococcal isolates are able to utilize this iron source, which indicates that lactoferrin may not be required for survival of *N. gonorrhoeae* at mucosal surfaces or in disseminated infection (137). Lactoferrin utilization is mediated by the lactoferrin binding proteins, LbpA and LbpB (14, 15, 198). LbpA is a TonB-dependent integral, outer membrane transporter, required for lactoferrin-mediated iron acquisition. However, LbpB is a lipid-modified, surface-exposed protein that is not essential for lactoferrin utilization. This system is thought to function similarly to other TonB-dependent iron acquisition systems and is shown in Figure 1.

D. Transferrin utilization

Among the many iron acquisition systems of pathogenic *Neisseria*, the transferrin-iron acquisition system is likely important during most stages of *Neisseria* pathogenesis. Although the transferrin- and lactoferrin-iron acquisition systems are very similar, they only function in specifically binding transferrin and lactoferrin, respectively. Unlike the lactoferrin utilization system, the transferrin-iron acquisition system is expressed by all clinical isolates (138), which implicates its importance in virulence. In addition, the transferrin-iron uptake system is not subject to high-frequency phase variation as is seen in the hemoglobin, haptoglobin, and lactoferrin utilization system.

Similar to the other iron acquisition systems in *Neisseria*, the transferrin-iron acquisition system is composed of two transferrin binding proteins (TbpA and TbpB).

The transferrin binding proteins are encoded by a bicistronic operon with *tbpB* upstream of *tbpA* (188). The two genes are separated by an 86 base pair intergenic region that is thought to form a putative, secondary stem loop structure in the mRNA (188). This region is thought to be involved in the 2:1 ratio of *tbpB* and *tbpA* transcripts (188). Although there are likely many regulatory mechanisms involved, it has been shown that expression of the transferrin binding proteins is regulated by the ferric uptake regulator (Fur), whereby expression is repressed during iron replete conditions and derepressed under iron deplete conditions (188).

TbpA and TbpB are maximally expressed under iron stress and can function specifically and independently in high-affinity transferrin binding. TbpA is a TonB-dependent, integral, outer membrane protein that is critical for transferrin-mediated iron acquisition. TbpB, on the other hand, is a surface-exposed lipoprotein that is not essential for iron uptake. TbpB not only binds transferrin but also discriminates between the apo and holo forms of transferrin, with a preference for holo-transferrin (20, 50, 183, 184). Although not required for transferrin-mediated iron acquisition, TbpB is known to make the process more efficient.

Although the crystal structure has not been solved, TbpA has significant homology to characterized TonB-dependent siderophore transporters (48), of which the structures are known (Figure 2). Based on these crystal structures, a TbpA two-dimensional, topological model has been predicted (Figure 3), in which TbpA is thought

to look very similar to TonB-dependent transporters. TbpA is proposed to have two distinct domains: (1) a C-terminal β-barrel pore comprised of 22 β-strand transmembrane-spanning domains and (2) an N-terminal globular plug that is predicted to fold up and occlude the β-barrel. As mentioned, TbpA is required for transferrin-mediated iron acquisition and functions not only in transferrin binding, but also iron transport. Following TbpA-transferrin binding, it is hypothesized that TbpA removes iron from transferrin and transports iron through the outer membrane in a TonB-dependent step (Figure 1). It is known that TonB associates with TbpA via a TonB box of the plug domain (97). This interaction is thought to promote a conformational change in TbpA that allows for iron removal from transferrin and transport through the TbpA β-barrel. Although TbpB is not required for this iron internalization event, it does make the process more efficient, for in its absence approximately 50% of wild-type is observed (4).

There is much known about the mechanisms of TonB-dependent siderophore transport, but the mechanism of TonB-dependent transferrin-iron uptake is less well known. This system represents a novel TonB-dependent transporter because the unique mechanism of iron acquisition and the involvement of a secondary, lipoprotein component. Characterized siderophore transporters are known to bind and internalize ferric-siderophore complexes, whereas the transferrin-iron acquisition system must bind holo-transferrin, remove and transport iron through the outer membrane, and ultimately release apo-transferrin at the surface. These two features make the mechanism of transferrin-mediated iron acquisition unique and thus important for investigation.

VIII. Objectives

The goal of the work described here was to further characterize the mechanism of transferrin-mediated iron acquisition by *N. gonorrhoeae*. This was accomplished by three major objectives. The first involved analysis of the TbpA plug domain in TonB-dependent transferrin-iron acquisition and its overall contribution to iron binding and transport through the TbpA β-barrel. The second objective included analysis of both TbpA and TbpB, with the goal of identifying specific regions of both proteins required for transferrin-mediated iron acquisition. The third objective, although more distantly related to the first two, involved analysis of a TonB-independent mechanism of transferrin-mediated iron acquisition that required TbpA and TbpB. Although different, each study addressed features of TbpA and TbpB and their specific involvement in iron acquisition from human transferrin and provided insight into the transferrin-iron acquisition system of *Neisseria gonorrhoeae*. These studies are important in fully dissecting the mechanisms of transferrin utilization used by the pathogenic *Neisseria* and also shed light on the vaccine potential of the transferrin binding proteins, TbpA and TbpB.

Table 1. Biochemical tests for differentiation of *Neisseriaceae*

Species	Acid from					Polysaccharide from S	Nitrate reduction	Oxidase	Catalase
	G	M	S	F	L				
N. gonorrhoeae	+	–	–	–	–	–	–	+	+
N. meningitidis	+	+	–	–	–	–	–	+	+
N. lactamica	+	+	–	–	+	–	–	+	+
N. polysaccharea	+	+	–	–	–	+	–	+	+
N. cinerea	–	–	–	–	–	–	–	+	+
N. flavescens	–	–	–	–	–	+	–	+	+
N. mucosa	+	+	+	+	–	+	+	+	+
N. subflava	+	+	+	+	–	+	–	+	+
			–	–		–			
N. sicca	+	+	+	+	–	+	–	+	+
N. elongata	–	–	–	–	–	+	–	+	–
M. catarrhalis	–	–	–	–	–	–	+	+	+
K. denitrificans	+	–	–	–	–	–	+	+	–

Table adapted from Center for Disease Control and Prevention website
Abbreviations: G, glucose; M, maltose; S, sucrose; F, fructose; L, lactose

Figure 1. Models of TonB-dependent transport in Gram-negative bacteria. Various mechanisms of TonB-dependent transport in pathogenic microorganism are shown and distinguished by color. The model of siderophore-mediated iron uptake is shown in yellow. The model of lactoferrin (Lf)- and transferrin (Tf)-mediated iron acquisition is shown in blue. The third system, shown in red, illustrates the mechanisms of heme-iron uptake from hemoglobin (Hb) and haptoglobin (Hg) in *Neisseria*. Components of each system are labeled: integral, outer membrane transporters in the outer membrane, periplasmic binding proteins (PBP) in the periplasm, cytoplasmic membrane-associated TonB/ExbB/ExbD complexes and ABC transporters.

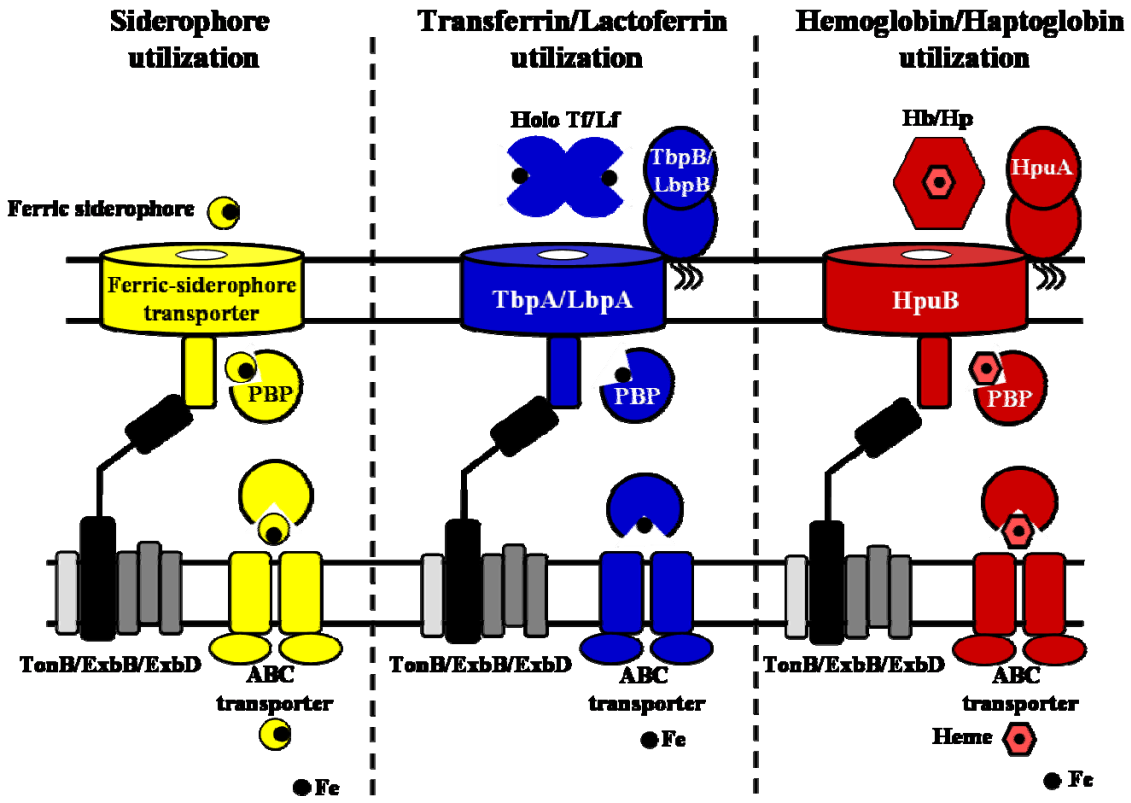

Siderophore utilization
Transferrin/Lactoferrin utilization
Hemoglobin/Haptoglobin utilization
Holo Tf/Lf
TbpB/ LbpB
Hb/Hp
HpuA
Ferric siderophore
Ferric-siderophore transporter
TbpA/LbpA
HpuB
PBP
PBP
PBP
TonB/ExbB/ExbD
ABC transporter
TonB/ExbB/ExbD
ABC transporter
TonB/ExbB/ExbD
ABC transporter
Fe
Fe
Heme
Fe

Figure 2. Characterized TonB-dependent transporters. Figure modified from K. D. Krewulak and H. J. Vogel review (104). TonB-dependent transporters are represented as ribbon diagrams: (1) *E. coli* BtuB (40), vitamin B_{12} transporter; (2) *E. coli* FecA (61), ferric-citrate transporter; (3) *E. coli* FepA (28), ferric-enterobactin transporter; (4) *E. coli* FhuA (62, 119), ferrichrome transporter; (5) *P. aeruginosa* FptA (43), pyochelin transporter; and (6) *P. aeruginosa* FpvA (42), pyoverdine transporter. The characteristic β-barrel domains, shown in blue, are each comprised of 22 β-stand transmembrane-spanning domains. The globular plug domains, shown in green, are folded up within the β-barrel domain and occlude the pore.

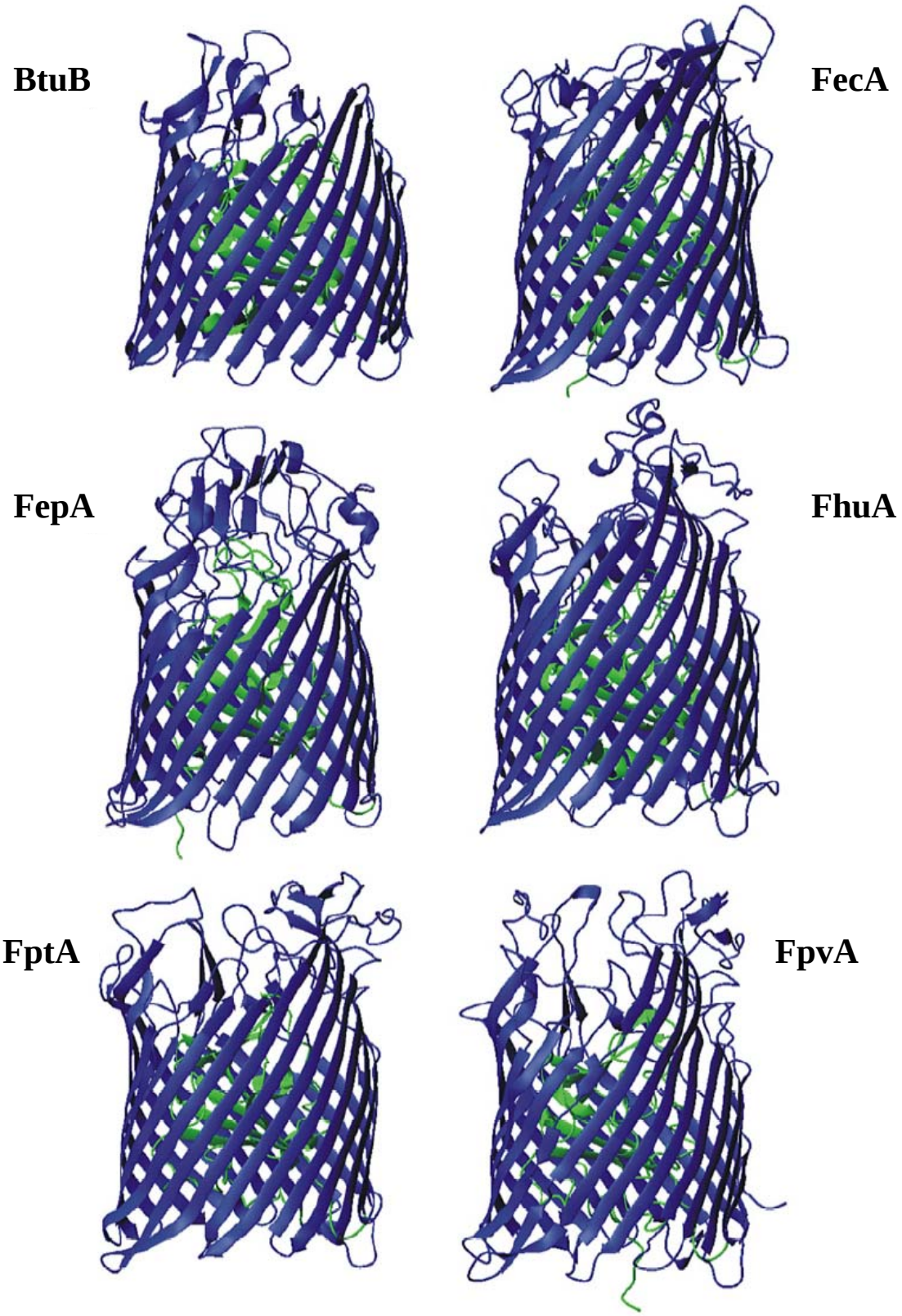
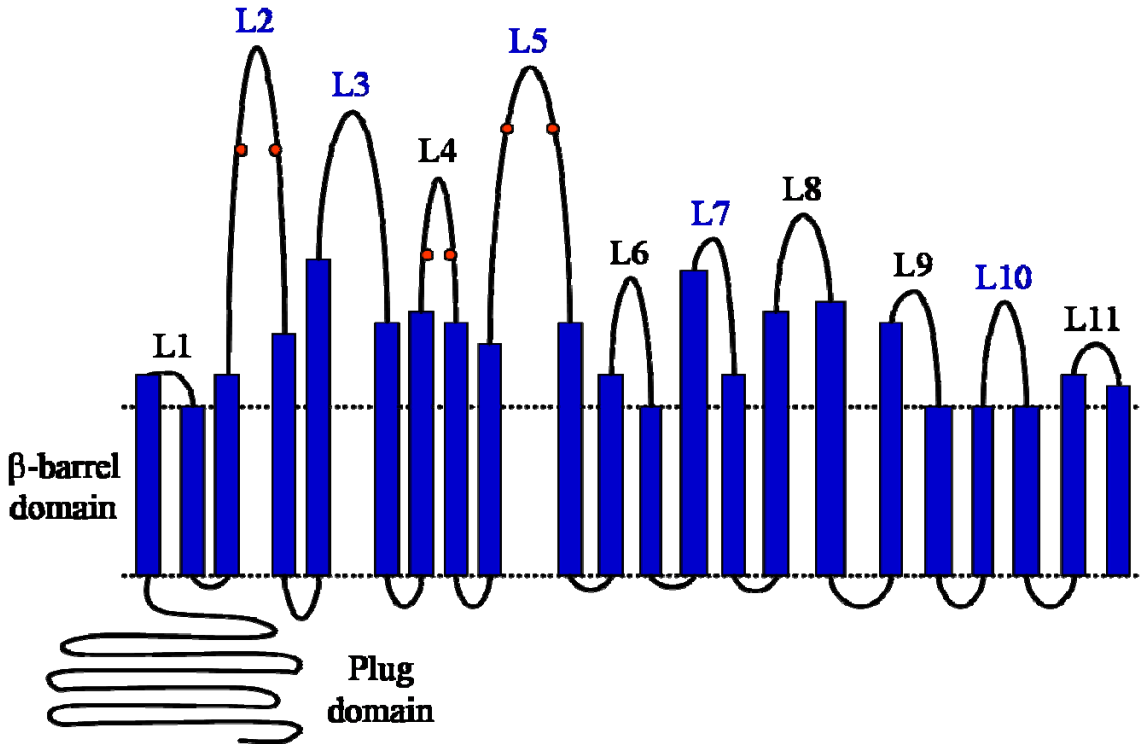

BtuB
FecA
FepA
FhuA
FptA
FpvA

Figure 3. *Neisseria* TbpA topology model. This TbpA two-dimensional, topology model was generated based on the structures of characterized *E. coli* TonB-dependent siderophore transporters. From these structures, it is predicted that TbpA has two distinct domains. The C-terminal β-barrel domain is shown in blue with 22 putative β-strand transmembrane domains and eleven putative extracellular loops. The loops labeled in blue indicate those that are known to be surface exposed (242). The red circles indicate cysteine residues that likely participate in disulfide bond formation within the loops. The N-terminal plug domain is shown in the periplasm, but is predicted to fold up within the β-barrel to occlude the pore.


L2
L3
L5
L4
L8
L7
L6
L9
L10
L11
L1
β-barrel
domain
Plug
domain

CHAPTER 2 – MATERIALS AND METHODS

I. Bacterial Growth Conditions

Gonococcal strains were routinely propagated on GC medium base (Difco) with Kellogg's supplement 1 (96) and 12 μM $Fe(NO_3)_3$. For selection of gonococcal transformants, gonococci were grown on GC agar plates supplemented with 100 μg/ml of streptomycin (Sigma) for selection of *lbpB*::Ω, *tonB*::Ω, and *pilQ*::Ω mutations, 1 μg/ml of chloramphenicol (Sigma) for selection of the *tbpA*::mTn3cat mutation, or 1 μg/ml of erythromycin (Sigma) for selection of *dsbC*::*ermC*, *traC*::*ermC*, *traH*::*ermC*, and *traN*::*ermC* mutations. For growth under iron-stressed conditions, which promotes maximal expression of the transferrin binding proteins, gonococci were cultured from GC agar plates into acid-washed glassware containing liquid, chelexed defined medium (CDM) (144, 234). All gonococcal strains were cultivated at 37°C with 5% CO_2. CDM agarose plates were supplemented with 5%, 10%, 30%, 50%, or 80% iron-saturated human transferrin (Sigma) as needed to assess the ability of gonococcal mutants to utilize transferrin-bound iron (48). Gonococcal growth on CDM-transferrin plates was monitored over 24 – 72 hours. Plasmids were routinely propagated in TOP10 (Invitrogen) or NovaBlue (Novagen) *E. coli* strains, grown at 37°C in LB media (10, 11)

supplemented with 50 μg/ml of kanamycin (Sigma) or 100 μg/ml of ampicillin (Sigma). Strains and plasmids described in this study are listed in Tables 2 and 3, respectively.

II. *Neisseria gonorrhoeae* Mutagenesis

A. Mutagenic oligonucleotide design

According to the method of Horton et al. (84), both gene specific and mutagenic oligonucleotides were designed to overlap for gene splicing by overlap extension or gene SOEing (Figure 4). Oligonucleotides were designed to carry out site-directed alanine substitution mutagenesis of the *tbpA* plug-encoding domain and to incorporate unique restriction sites for subsequent screening of gonococcal mutants. Mutagenic and gene specific oligonucleotides were synthesized by Integrated DNA Technologies and are listed in Tables 4 and 5, respectively.

B. Site-directed alanine substitution mutagenesis

Site-specific, alanine substitution mutagenesis of the *tbpA* plug-encoding domain was performed using gene splicing by overlap extension shown in Figure 4 (84). Briefly, in the primary PCR step, two reactions were performed to amplify the upstream and downstream portions of the *tbpA* plug-encoding domain. Mutagenic and non-mutagenic, gene specific oligonucleotides used in these reactions are listed in Table 4 and 5, respectively. Each mutagenic oligonucleotide was designed to encode one or more alanines as well as novel restriction sites for subsequent screening of gonococcal mutants. The template used in these reactions was pUNCH411, which contains the entire coding region of *tbpA* (47) (Table 3). For the secondary PCR, the two primary amplification

products were used as template with non-mutagenic oligonucleotides (Table 5). In this reaction, the alanine-encoding sequences annealed to one another and served to prime the next polymerization step. The final PCR product was gel extracted (Qiagen), purified, and cloned into pCR2.1 TOPO (Invitrogen). The plasmids containing the various mutagenized *tbpA* fragments (pVCU250 – pVCU260) are listed in Table 3. Mutagenized *tbpA* fragments were sequenced by the Nucleic Acids Research Facility at Virginia Commonwealth University to verify the expected sequences.

C. *E. coli* transformation

PCR products generated by gene splicing by overlap extension (84) were inserted into pCR2.1 TOPO (Invitrogen) (Table 3) and these constructs were used to transform TOP10 (Invitrogen) *E. coli* (Table 2). These plasmids were propagated in *E. coli* then isolated and purified using the Qiagen QIAprep Spin Miniprep Kit. These plasmid constructs were sequenced to ensure proper gene sequence as well as the preservation of the reading frame by the Nucleic Acids Research Facilities of Virginia Commonwealth University.

For subcloning into pHSS6-GCU (60) (Table 3), pCR2.1 TOPO constructs were digested with EcoRI (New England BioLabs) to remove the PCR product inserts. EcoRI-linearized pHSS6-GCU and inserts were purified (Qiagen) and pHSS6-GCU plasmids were treated with shrimp alkaline phosphatase (SAP) (Roche Molecular Biosciences). Linearized pHSS6-GCU and inserts were incubated with DNA ligase (Invitrogen) for one hour at 25^{o}C. Chemically competent TOP10 *E. coli* cells (Invitrogen) were transformed with pHSS6-GCU constructs (pVCU251 – pVCU261), containing *tbpA* encoding alanine

substitutions (Table 3). The resulting *E. coli* strains (RIC250 – RIC261) are listed in Table 2.

D. *N. gonorrhoeae* spot transformation

Alanine-encoding *tbpA* fragments were subcloned into pHSS6-GCU (60) (Table 3) to incorporate the gonococcal uptake (GCU) sequence necessary for transformation. Resulting plasmids (pVCU251 – pVCU261) were then used to transform gonococcal strains FA19 (TbpB+) and FA6905 (TbpB–) (Table 2). Congression, described below, was used to provide a selectable marker for the transformation event. Chromosomal DNA from MCV601 (14) (Table 2), which contains an Ω cassette inserted into *lbpB* (encoding lactoferrin binding protein B), was used in conjunction with the linearized pHSS6-GCU contructs. These donor DNAs were combined and used to transform piliated FA19 (TbpB+) or FA6905 (TbpB–). Approximately 10 CFU were spotted on a GC agar plate and both donor DNAs were applied on top of the piliated gonococci. Transformation mixtures were incubated on non-selective media for 24 hours at 37^{o}C with 5% CO_2. Transformants were then selected on 100 μg/ml of streptomycin, resistance to which was encoded by the Ω cassette. A subsequent PCR and restriction digest identified streptomycin-resistant transformants with the introduced restriction sites. This process yielded strains MCV250 – MCV261 (Table 2), which contained single, double, or triple alanine-encoding mutations within the *tbpA* plug-encoding domain.

As previously described (57), when two mutations were combined, linearized plasmids containing each independent mutation were simultaneously added to piliated gonococci along with the streptomycin-resistant MCV601 chromosomal DNA. These

combined mutants (MCV262 – MCV266) contain mutations in both *tbpA* and *tbpB* and are listed in Table 2. Gonococcal *tbpA* and *tbpB* were sequenced by the Nucleic Acids Research Facility at Virginia Commonwealth University to verify mutations in both genes.

E. *N. gonorrhoeae* liquid transformation

For revertant analysis, various mutations were made in FA19, MCV511 (L3HA), and MCV267 (L3HA revertant) backgrounds. Plasmids pUNCH290 (*pilQ*::Ω), pHH17 (*dsbC*::*ermC*), pKS83 (*traC*::*ermC*), pJD1175 (*traH*::*ermC*), and pKS72 (*traN*::*ermC*) (Table 3) were used to generate PilQ and T4SS deletion mutants. pUNCH290 and pHH17 were linearized to select for a double crossover insertion of the Ω cassette and *ermC* into *pilQ* and *dsbC*, respectively. pSK83, pJD1175, and pKS72 were not linearized to select for a single crossover insertion of *ermC* into *traC*, *traH*, and *traN*, respectively. Gonococcal strains, suspended in GCB/$MgCl_2$ media, were mixed with the plasmid DNAs and allowed to incubate for 30 minutes at 37°C with 5% CO_2. Following incubation, fresh GCB/$MgCl_2$ media was added and gonococcal-plasmid DNA mixture was incubated at 37°C with 5% CO_2 for five hours. After five hour incubation, strains were plated on selective media (100 μg/ml streptomycin for Ω insertion and 1 μg/ml erythromycin for *ermC* insertions) and resulting transformants were selected (MCV270 – MCV286) and are listed in Table 2.

III. Western Blot Analysis and Solid-Phase HA Binding

A. SDS-PAGE and protein transfer

Gonococcal strains were grown in liquid CDM to induce iron stress (234). After four hours of growth, aliquots were removed and standardized to culture cell density. Cells were pelleted and lysed with Laemmli solubilizing buffer (107) and stored at –20°C. Before use, 5% β-mercaptoethanol (Fisher Scientific) was added to the whole cell lysates; lysates were heated at 95°C for three minutes, and then drawn through a 28-gauge syringe (Beckton Dickinson) to decrease viscosity of samples. Whole cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5%, 12%, or 15% gels. Proteins were subsequently transferred to nitrocellulose membranes (Schleicher and Schuell) in 20 mM Tris base, 150 mM glycine, and 20% methanol (218) within a submerged transfer apparatus (Bio-Rad) at 28 mAmp for 18 – 20 hours. Prior to immunoblotting, nitrocellulose membranes were incubated in Ponceau S (Fisher) stain (0.1% w/v Ponceau S and 5% acetic acid) to ensure equal protein loading.

B. TbpA, TbpB, and TonB detection

For detection of TbpA, TbpB, and TonB, membranes were blocked with 5% bovine serum albumin (BSA) (Roche) in high salt Tris-buffered saline (HS TBS) plus 0.05% Tween-20 (Sigma). TbpA blots were then probed with a 1:1,000 dilution of primary anti-TbpA polyclonal antibodies (50), while TbpB and TonB blots were probed with a 1:5,000 dilution of primary anti-TbpB (215) or anti-TonB (97) polyclonal antibodies. Blots were then washed with HS TBS plus 0.05% Tween-20, and probed with a 1:10,000 dilution of secondary goat anti-rabbit AP (alkaline phosphatase) antibody (Bio-Rad). Western blots were developed using NBT/BCIP developing system (Sigma).

C. Hemagglutinin (HA) epitope detection

For detection of the hemagglutinin (HA) epitope, membranes were blocked in 5% Western blocking reagent (Roche) in low salt Tris-buffered saline (LS TBS). HA blots were then probed with a 1:500 dilution of anti-HA horseradish peroxidase high-affinity monoclonal antibody (HRP-HA) (Roche) and subsequently washed with LS TBS. Blots were developed using Perkin Elmer Chemiluminescence (ECL) Plus and reactive bands were detected by exposure to film (Kodak).

D. Solid-phase anti-HA binding assay

Gonococcal strains were grown for four hours in liquid CDM to induce iron stress (234). Aliquots were standardized to culture cell density and then spotted onto nitrocellulose membranes (Schleicher and Schuell). Membranes were dried and then blocked with 5% Western blocking reagent (Roche) in LS TBS. Dot blots were then probed with a 1:500 dilution of anti-HA horseradish peroxidase-conjugated monoclonal antibody (HRP-HA) (Roche) and subsequently washed with LS TBS. Dot blots were developed using Opti-4CN (BioRad).

IV. Surface Exposure and Transferrin Binding

A. Solid-phase transferrin binding assay

Gonococcal strains were grown in liquid CDM for four hours to induce iron stress (234). Aliquots were standardized to culture cell density and then spotted onto nitrocellulose membranes (Schleicher and Schuell). Membranes were dried and blocked with 5% skim milk (BioRad) in LS TBS. To assess solid-phase transferrin binding by

gonococcal cells, membranes were incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated transferrin (HRP-Tf) (Jackson ImmunoResearch), washed with LS TBS, and then developed with Opti-4CN (BioRad).

B. Solid-phase transferrin discrimination assay

Gonococcal strains were grown in liquid CDM for four hours to induce iron stress (234). Aliquots were standardized to culture cell density and then spotted onto nitrocellulose membranes (Schleicher and Schuell). Membranes were dried and then blocked with 5% skim milk in LS TBS. Ligand discrimination was determined by probing membranes with a mixture of unlabeled competitor (100% saturated human transferrin or apo-transferrin) (Sigma) and HRP-Tf. Two-fold serial dilutions of unlabeled competitor, ranging from 0.05 – 6 μM, were mixed with 0.33 μg/ml of HRP-Tf. Following incubation with labeled and unlabeled transferrin, dot blots were washed with LS TBS and developed with Opti-4CN (BioRad).

V. Quantitative Transferrin Binding

A. Iodination of human transferrin

Approximately 200 μl of 100% saturated human transferrin (Calbiochem) was mixed with 10 μl (1 mCi) of $Na^{125}I$ (GE Biosciences) in an Iodogen-coated tube (Pierce). The iodination reaction was carried out for 15 minutes at room temperature. The mixture was added to 200 μl of column loading dye (10% glycerol, 0.1% bromophenol blue, 0.3% blue dextran) and transferred to a dextran desalting column (Pierce). This column was equilibrated with 50 ml of PBS before the addition of ^{125}I-transferrin. The column

separated free ^{125}I from the ^{125}I-transferrin, which was collected in the flow through. The concentration of ^{125}I-transferrin was then determined by BCA assay (Pierce).

B. Equilibrium-phase transferrin binding assay

Equilibrium-phase transferrin binding assays were performed as previously described (50) to determine TbpA or TbpB transferrin binding affinity and capacity. Briefly, human transferrin (Calbiochem) was iodinated with ^{125}I (GE Biosciences) and specific activity was determined by gamma counting. Both iodinated transferrin and unlabeled competitor transferrin were quantitated using a BCA assay (Pierce). Gonococcal strains were grown in liquid CDM for three hours to induce iron stress (234). Following growth, 100 µl of each culture was added to a Millipore Multiscreen microtiter plate and incubated with various concentrations of ^{125}I-labeled transferrin (0 – 100 nM) to determine total transferrin binding. In addition, cultures were incubated with ^{125}I-transferrin and excess unlabeled transferrin to determine non-specific transferrin binding. Specific transferrin binding was determined by subtracting non-specific binding from total binding. Specific transferrin bound (ng Tf) was standardized to micrograms of total cellular protein (µg TCP) in 100 µl of culture, as determined by BCA assays (Pierce). Each graph represents averages and standard deviations from at least three separate assays, each of which was performed in quadruplicate. K_d (affinity) and capacity values as well as standard errors were calculated using Grafit software (Erithacus Software).

VI. Transferrin-Iron Internalization

A. Iron-saturation of human transferrin

Apo-human transferrin (Calbiochem) was resuspended in ferration buffer, pH 8.4 (100 mM Tris, 150 mM NaCl, 20 mM $NaHCO_3$) to a concentration of 10 mg/ml. Iron solution, pH 8.6 (100 mM $Na_3C_6H_5O_7$, 100 mM $NaHCO_3$, 5 mM $FeCl_3$ or $^{55}FeCl_3$) was added to achieve the desired saturation level and saturation was allowed to proceed for one hour at room temperature. Ferrated transferrin was then dialyzed overnight at 4°C in a 400-fold volume of dialysis buffer, pH 7.4 (400 mM Tris, 150 mM NaCl, 20 mM $NaHCO_3$). The buffer was then replaced with fresh dialysis buffer and dialysis was allowed to proceed for an additional four hours at room temperature. Dialysis of ^{55}Fe-transferrin was performed similarly against a 1000-fold volume of dialysis buffer for four hours at room temperature without buffer exchange.

To verify the iron saturation level of the transferrin, ferrozine assays were performed. A standard curve of iron concentrations was created in the range of 0.05 –1.0 μg/ml and the transferrin-iron saturation was determined from this curve. Acid reagent [0.01 M ferrozine (3-(2-puridyl)-5, 6 bis (4-phenlysulphonic acid) 1, 2, 3 triamine) in approximately 50% hydrochloric acid] was added to each sample and then allowed to boil for ten minutes. Buffer reagent (5.2 M ammonium acetate in 35% acetic acid) was then added to each sample after cooling to room temperature. Absorbance readings were taken at 562 nm. The concentration of transferrin was determined using a BCA assay (Pierce) and the percent saturation was calculated based on the ability of transferrin to bind two atoms of iron.

B. Transferrin-iron uptake assay

Transferrin-iron uptake assays were performed as previously described (4, 12, 46). Briefly, human transferrin (Calbiochem) was ferrated with $^{55}FeCl_3$ (Perkin Elmer) to achieve 20% iron saturation. Gonococcal strains were grown in liquid CDM for three hours to induce iron stress (234). Following growth, 100 μl of each culture was added in quadruplicate sets to two Millipore multiscreen microtiter plates. To one set of cultures 40 μM potassium cyanide (KCN) (Sigma) was added to determine non-specific iron binding. Approximately 0.9 μM 20% iron-saturated transferrin was added to each well and incubated at 37°C with 5% CO_2 for 30 minutes to allow for iron internalization. Following incubation, plates were filtered to remove transferrin, washed with citrate buffer (100 mM $Na_3C_6H_5O_7$, 1.0 mM $MgCl_2$, 0.25 mM $CaCl_2$), dried, and counted using a Beckman LS6500 beta scintillation counter. All counts were averaged and non-specific counts (KCN plate) were subtracted from total counts to obtain specific iron internalization. Specific iron uptake, reported in picomoles, for each strain was standardized to micrograms of total cellular protein (TCP) in 100 μl of culture, as determined by BCA assay (Pierce). Each graph represents the means and standard deviations from at least six separate assays, each of which was performed in quadruplicate.

C. Modified transferrin-iron uptake assay with desferal

Modified transferrin-iron uptake assays were performed similarly to what was previously described (4, 12, 46) with some modifications. Briefly, human transferrin (Calbiochem) was ferrated with $^{55}FeCl_3$ (Perkin Elmer) to achieve 20% iron saturation. Gonococcal strains were grown in liquid CDM for three hours to induce iron stress (234).

Following growth, 100 μl of each culture was added in quadruplicate sets to two Millipore multiscreen microtiter plates. To one set of cultures 40 μM potassium cyanide (KCN) (Sigma) was added to determine non-specific iron binding. Approximately 0.9 μM 20% iron-saturated transferrin and 50 mM desferal (Sigma) were added to wells for 0 – 40 minutes of incubation at room temperature. Following incubation, plates were filtered to remove transferrin and desferal, washed with citrate buffer (100 mM $Na_3C_6H_5O_7$, 1.0 mM $MgCl_2$, 0.25 mM $CaCl_2$), dried, and counted using a Beckman LS6500 beta scintillation counter. Counts were averaged at each time point and non-specific counts (KCN plate) were subtracted from total counts to obtain specific iron internalization. Specific iron uptake, reported in picomoles, for each strain was standardized to micrograms of total cellular protein (TCP) in 100 μl of culture, as determined by BCA assay (Pierce). Each graph represents averages and standard deviations from three separate experiments, each of which was performed in quadruplicate.

VII. Recombinant Protein Expression and Purification

A. Construction of S-tag and His-tag protein expression plasmids

For S-tagged protein constructs, *tbpA* plug, *fetA* plug, *fbpA*, and *tonB* expression plasmids were constructed by PCR amplification of *tbpA*, *fetA*, *fbpA*, and *tonB* from gonococcal strain FA19 chromosomal DNA. Primers were designed to amplify these genes and engineered with BglII and XhoI restriction sites at the 5' and 3' ends,

respectively (Table 4). These restriction sites allowed for directional cloning into the pET-29b(+) expression vector (Novagen) (Table 3).

For His-tagged protein constructs, *tbpA* plug, *fbpA*, and *exbB* expression plasmids were constructed by PCR amplification of *tbpA*, *fbpA*, and *exbB* from FA19 chromosomal DNA. Primers were designed to amplify these genes and engineered with NdeI and XhoI restriction sites at the 5' and 3' ends, respectively (Table 4). These restriction sites allowed for directional cloning into the pET-22b(+) expression vector (Novagen) (Table 3).

The resultant PCR products were ligated into pCR2.1 TOPO (Invitrogen) (pVCU262 – pVCU270) and propagated in *E. coli* TOP10 cells (Invitrogen) (RIC262 – RIC274) (Table 2). Plasmids were then sequenced by the Nucleic Acids Research Facility at Virginia Commonwealth University to verify the expected sequences. Plasmids were digested with BglII or NdeI and XhoI enzymes (New England BioLabs) to excise inserts. Following digestion, the gene products were isolated and purified using the Qiagen QIAprep Spin Miniprep Kit and ligated into linearized pET-29b(+) or pET-22b(+) expression vectors (Novagen) (pVCU263 – pVCU271) (Table 3). The resultant plasmids were propagated in *E. coli* NovaBlue cells (Novagen) (RIC263 – RIC275) (Table 2). These plasmid constructs contained the gene of interest under the control of a T7 promoter, as well as a 5' region encoding an S-tag or a 3' region encoding a 6x His-tag for purification. For expression of these gene products, the *E. coli* expression strain BL21(DE3) (Novagen), which expressed T7 polymerase under the control of the *lac*

promoter, was transformed with these constructs. These strains, RIC264 – RIC276, are described in Table 2.

B. Recombinant protein expression

For expression of recombinant proteins, starter cultures of BL21(DE3) expression strains were grown for approximately six hours at 37^{o}C, shaking at 225 rpm, in LB media containing 50 μg/ml of kanamycin (pET29b(+)) or 100 μg/ml of ampicillin (pET22b(+)). Following growth, cultures were stored at 4^{o}C until use in large-scale growth and induction. Prior to large-scale induction, starter cultures were subject to centrifugation at 5000 x g for ten minutes and the supernatants were removed. Fresh LB media with 50 μg/ml of kanamycin or 100 μg/ml of ampicillin was added to resuspend the bacterial cell pellets. The starter cultures were then used to inoculate 1L of LB broth containing 50 μg/ml of kanamycin or 100 μg/ml of ampicillin. The cultures were incubated at 37^{o}C with shaking at 225 rpm until the OD_{600} reached 0.4 – 0.8. After cultures reached the optimal cell density, 1 mM IPTG (isopropyl-B-D-thiogalactopyranoside) (Sigma) was added to induce protein expression. Induction was allowed to proceed for four hours at 37^{o}C with shaking at 225 rpm. Following four hour protein induction, cultures were subject to centrifugation at 10,000 x g for ten minutes and then supernatants were removed. Pellets were dried and stored at –20^{o}C overnight.

C. Recombinant protein purification

For purification of S-tagged recombinant proteins, bacterial cell pellets were thawed and resuspended in BugBuster reagent (Novagen) according to manufacturer's instructions. After cells were completely resuspended, benzonase nuclease (Novagen),

protease inhibitors (Sigma), and lysozyme (Sigma) were added to the cultures and incubated for 30 minutes at room temperature. Solubilized preparations were then subject to centrifugation at 16,000 x g at 4^{o}C for 30 minutes to remove insoluble cellular debris. Cleared lysates were then transferred to sterile tubes and incubated with S-protein agarose (Novagen) at room temperature for 30 minutes on an orbital shaker. Samples were then subjected to centrifugation at 500 x g for ten minutes and supernatants were carefully removed. S-protein agarose resin was then resuspended in 5 ml of 1x Bind/Wash Buffer (200 mM Tris-HCl pH 7.5, 1.5 M NaCl, and 1% Triton X-100), gently mixed, and pelleted at 500 x g three times. Washed resin was then resuspended in 3 M $MgCl_2$ elution buffer at a volume 1.5 times that of the settled resin and incubated for ten minutes at room temperature. Resin was then applied to a disposable column and S-tagged recombinant proteins were eluted.

For purification of His-tagged recombinant proteins, Qiagen protocols were followed. Bacterial cell pellets were thawed on ice and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, final pH 8.0), benzonase nuclease (Novagen), protease inhibitors (Sigma) and lysozyme (Sigma) mixture. Resuspended cells were incubated for 30 minutes on ice and then subjected to sonication. Cellular debris was removed by centrifugation at 16,000 x g for 30 minutes at 4^{o}C. Cleared lysates were transferred to sterile tubes and incubated with 50% Ni-NTA slurry (Qiagen) at 4^{o}C for one hour. Lysate-Ni-NTA mixtures were then added to disposable columns and flow-through fractions were collected. Resin was washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, final pH 8.0) and washed fractions

were collected. His-tagged recombinant proteins were eluted from the column with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, final pH 8.0). Purified proteins were dialyzed against a 1000-fold excess PBS.

D. SDS-PAGE, protein transfer, and detection

Protein fractions were diluted with Laemmli solubilizing buffer (107) and stored at –20°C. Before use, 5% β-mercaptoethanol (Fisher Scientific) was added to the samples and they were heated at 95°C for three minutes. After boiling, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel. Proteins were subsequently transferred to nitrocellulose membranes (Schleicher and Schuell) in 20 mM Tris base, 150 mM glycine, and 20% methanol (218) within a submerged transfer apparatus (Bio-Rad) at 28 mAmp for 18-20 hours.

For detection of S-tagged and His-tagged recombinant proteins, membranes were blocked with 5% skim milk in low salt Tris-buffered saline (LS TBS). Blots were then probed with a 1:5,000 dilution of anti-S-tag monoclonal antibodies (Novagen) or a 1:1,000 dilution of anti-His-tag monoclonal antibodies (Calbiochem) , washed with LS TBS, and then probed with a 1:10,000 (S-tag) or 1:5,000 (His-tag) dilution of a secondary goat anti-mouse IgG AP (BioRad). Blots were developed using NBT/BCIP developing system (Sigma).

E. 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. Gels were incubated with 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. Prior to drying, gels were incubated in gel drying buffer (40% methanol, 10% glycerol, and 7.5% glacial acetic acid) for one hour.

VIII. Recombinant Protein-Iron Binding Assay

Recombinant iron binding assays were performed in Millipore Multiscreen microtiter plates. To assess total iron binding, 50 μg of each S-tagged or His-tagged recombinant protein was incubated with 5% BSA in PBS, $NaHCO_3$, $^{55}FeCl_3$, and Ni-NTA resin. Phosphate and carbonate served as coordinating anions for iron binding in this assay. To assess non-specific iron binding by recombinant proteins, proteins were treated the same as above, but incubated in the presence of 1000-fold excess cold $FeCl_3$ or $Fe(NO_3)_3$ competitor. As an additional control for non-specific binding to the Ni-NTA resin, 5% BSA, $NaHCO_3$, $^{55}FeCl_3$, and Ni-NTA resin were incubated in the absence of recombinant protein. Microtiter plates were incubated at room temperature for 30 minutes on a platform shaker at 200 rpm. Following incubation, wells were filtered and washed with citrate buffer (100 mM $Na_3C_6H_5O_7$, 1.0 mM $MgCl_2$, 0.25 mM $CaCl_2$). Once dried, filters were removed and counted using a Beckman LS6500 beta scintillation counter. Counts were averaged and specific iron binding was determined by subtracting both sets of non-specific counts from the total iron binding counts. Each graph represents averages and standard deviations from three separate experiments, each performed in quadruplicate sets.

IX. Statistical Analysis

Statistical significance of equilibrium-phase transferrin binding data, modified and standard transferrin-iron uptake data, and iron binding data was determined using a two-tailed equal variance Student's t test. Statistical significance is noted when $P \leq 0.05$ and specific values are shown with each data set.

Figure 4. Schematic representation of mutagenesis by gene SOEing. Non-mutagenic primers 1 and 4 were used to amplify the upstream (black) and downstream (gray) regions of the plug-encoding region of *tbpA* in primary PCR reactions. Mutagenic primers 2 and 3 were also used to introduce sequences encoding altered amino acid residues and novel restriction sites (red) in the primary PCR reactions. The primary PCR products were amplified with non-mutagenic primers 1 and 4 in the secondary PCR. The primary PCR products served as template in this reaction and overlapping sequences served to prime the secondary PCR.

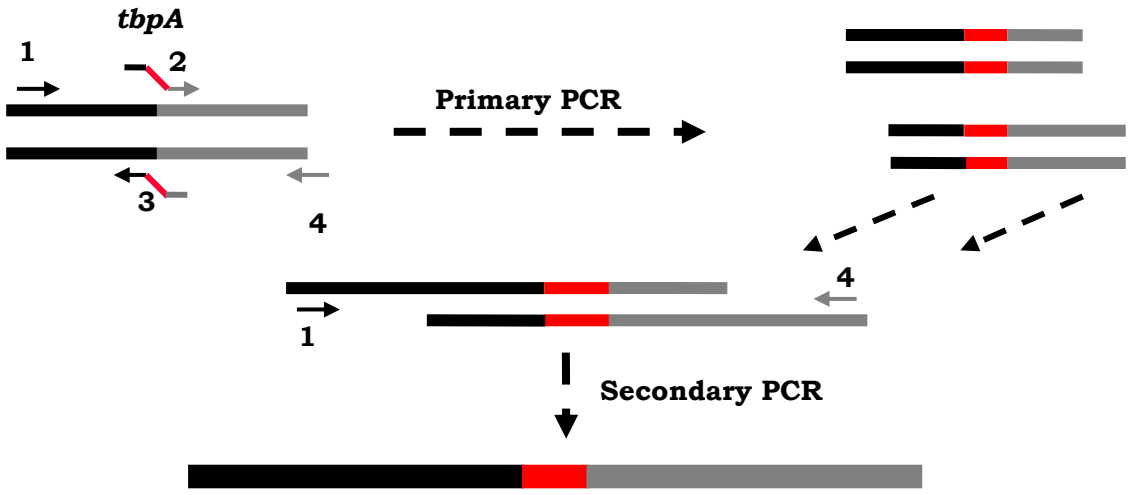

tbpA
1
2
3
4
Primary PCR
4
1
Secondary PCR

Table 2: *Escherichia coli* and *Neisseria gonorrhoeae* strains

Strain	Phenotype (genotype)	Reference
	E. coli	
TOP10	F– *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 alU galK rpsL*(Str^R) *endA1 nupG*	Invitrogen
NovaBlue	*endA1 hsdR17* (r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′[*proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*::Tn*10*] (Tet^R)	Novagen
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
RIC250	TOP10 transformed with pVCU250 (*tbpA* $E_{118}A$)	This study (160)
RIC251	TOP10 transformed with pVCU251 (*tbpA* $E_{118}A$)	This study (160)
RIC252	TOP10 transformed with pVCU252 (*tbpA* $E_{120}A$)	This study (160)
RIC253	TOP10 transformed with pVCU253 (*tbpA* $E_{120}A$)	This study (160)
RIC254	TOP10 transformed with pVCU254 (*tbpA* $Y_{121}A$)	This study (160)
RIC255	TOP10 transformed with pVCU255 (*tbpA* $Y_{121}A$)	This study (160)
RIC256	TOP10 transformed with pVCU256 (*tbpA* $E_{122}A$)	This study (160)
RIC257	TOP10 transformed with pVCU257 (*tbpA* $E_{122}A$)	This study (160)
RIC258	TOP10 transformed with pVCU258 (*tbpA* $EY_{120}AA$)	This study (160)
RIC259	TOP10 transformed with pVCU259 (*tbpA* $EY_{120}AA$)	This study (160)
RIC260	TOP10 transformed with pVCU260 (*tbpA* $EYE_{120}AAA$)	This study (160)
RIC261	TOP10 transformed with pVCU261 (*tbpA* $EYE_{120}AAA$)	This study (160)
RIC262	TOP10 transformed with pVCU262 (*tbpA* plug)	This study
RIC263	NovaBlue transformed with pVCU263 (*tbpA* plug)	This study
RIC264	BL21(DE3) transformed with pVCU263 (*tbpA* plug)	This study

RIC265	TOP10 transformed with pVCU264 (*tbpA* plug)	This study
RIC266	NovaBlue transformed with pVCU265 (*tbpA* plug)	This study
RIC267	BL21(DE3) transformed with pVCU265 (*tbpA* plug)	This study
RIC268	TOP10 transformed with pVCU266 (*fetA* plug)	This study
RIC269	NovaBlue transformed with pVCU267 (*fetA* plug)	This study
RIC270	BL21(DE3) transformed with pVCU267 (*fetA* plug)	This study
RIC271	TOP10 transformed with pVCU268 (*fbpA*)	This study
RIC272	NovaBlue transformed with pVCU269 (*fbpA*)	This study
RIC273	BL21(DE3) transformed with pVCU269 (*fbpA*)	This study
RIC274	TOP10 transformed with pVCU270 (*tonB*)	This study
RIC275	NovaBlue transformed with pVCU271 (*tonB*)	This study
RIC276	BL21(DE3) transformed with pVCU271 (*tonB*)	This study
RIC277	TOP10 transformed with pVCU272 (*tbpA* plug $EYE_{120}AAA$)	This study
RIC278	NovaBlue transformed with pVCU273 (*tbpA* plug $EYE_{120}AAA$)	This study
RIC279	BL21(DE3) transformed with pVCU273 (*tbpA* plug $EYE_{120}AAA$)	This study

	N. gonorrhoeae	
FA19	TbpA+, TbpB+	(138)
FA6905	TbpA+, TbpB– (Δ*tbpB*)	(50)
FA6747	TbpA– (*tbpA*::mTn3cat), TbpB+	(48)
FA6815	TbpA–, TbpB– (*tbpB*::Ω)	(4)
MCV601	FA19 derivative, LbpB– (*lbpB*::Ω)	(14)
MCV250	TbpA $E_{118}A$, TbpB+	This study (160)
MCV251	TbpA $E_{118}A$, TbpB– (Δ*tbpB*)	This study (160)
MCV252	TbpA $E_{120}A$, TbpB+	This study (160)
MCV253	TbpA $E_{120}A$, TbpB– (Δ*tbpB*)	This study (160)
MCV254	TbpA $Y_{121}A$, TbpB+	This study (160)
MCV255	TbpA $Y_{121}A$, TbpB– (Δ*tbpB*)	This study (160)
MCV256	TbpA $E_{122}A$, TbpB+	This study (160)
MCV257	TbpA $E_{122}A$, TbpB– (Δ*tbpB*)	This study (160)

MCV258	TbpA $EY_{120}AA$, TbpB+	This study (160)
MCV259	TbpA $EY_{120}AA$, TbpB– (Δ*tbpB*)	This study (160)
MCV260	TbpA $EYE_{120}AAA$, TbpB+	This study (160)
MCV261	TbpA $EYE_{120}AAA$, TbpB– (Δ*tbpB*)	This study (160)
MCV845	TbpA+, TbpB $CC_{481}AA$	This study (56)
MCV846	TbpA– (*tbpA*::mTn3cat), TbpB $CC_{481}AA$	This study (56)
MCV812	TbpA+, TbpB $HA3_{175}$ (*tbpB*∇HA)	(57)
MCV816	TbpA+, TbpB $HA5_{327}$ (*tbpB*∇HA)	(57)
MCV824	TbpA+, TbpB $HA9_{660}$ (*tbpB*∇HA)	(57)
MCV515	TbpA $L9HA_{750}$ (*tbpA*∇HA), TbpB+	(243)
MCV516	TbpA $L9HA_{750}$ (*tbpA*∇HA), TbpB– (Δ*tbpB*)	(243)
MCV519	TbpA $L11HA_{843}$ (*tbpA*∇HA), TbpB+	(243)
MCV520	TbpA $L11HA_{843}$ (*tbpA*∇HA), TbpB– (Δ*tbpB*)	(243)
MCV527	TbpA $L2HA_{229}$ (*tbpA*∇HA), TbpB+	(243)
MCV528	TbpA $L2HA_{229}$ (*tbpA*∇HA), TbpB– (Δ*tbpB*)	(243)
MCV262	TbpA $EYE_{120}AAA$, TbpB $CC_{481}AA$	This study (DeRocco unpublished)
MCV263	TbpA $EYE_{120}AAA$, TbpB $HA3_{175}$	This study (DeRocco unpublished)
MCV264	TbpA $EYE_{120}AAA$, TbpB $HA5_{327}$	This study (DeRocco unpublished)
MCV265	TbpA $EYE_{120}AAA$, TbpB $HA9_{660}$	This study (DeRocco unpublished)
MCV266	TbpA $L9HA_{750}$, TbpB $CC_{481}AA$	This study (DeRocco unpublished)
MCV828	TbpA $L9HA_{750}$, TbpB $HA3_{175}$	(57)
MCV830	TbpA $L9HA_{750}$, TbpB $HA5_{327}$	(57)
MCV834	TbpA $L9HA_{750}$, TbpB $HA9_{660}$	(57)
	Pseudo-revertant gonococcal studies	
MCV511	TbpA $L3HA_{343}$, TbpB+	(243)

MCV512	TbpA L3HA$_{343,}$ TbpB– (Δ*tbpB*)	(243)
MCV523	TbpA β16HA$_{713}$, TbpB+	(243)
MCV524	TbpA β16HA$_{713}$, TbpB– (Δ*tbpB*)	(243)
MCV267	TbpA L3HA$_{343}$, TbpB+ revertant	This study (Yost-Daljev unpublished)
MCV268	TbpA β16HA$_{713}$, TbpB+ revertant	This study (Yost-Daljev unpublished)
	FA19 derived strains	
MCV269	TbpA+, TbpB+, TonB– (*tonB*::Ω)	This study
MCV270	TbpA+, TbpB+, PilQ– (*pilQ*::Ω)	This study
MCV271	TbpA+, TbpB+, DsbC– (*dsbC*::*ermC*)	This study
MCV272	TbpA+, TbpB+, TraC– (*traC*::*ermC*)	This study
MCV273	TbpA+, TbpB+, TraH– (*traH*::*ermC*)	This study
MCV274	TbpA+, TbpB+, TraN– (*traN*::*ermC*)	This study
MCV904	TbpA+, TbpB+, FbpA– (*fbpA*::*ermClacIP*) –RBS	(211)
MCV906	TbpA+, TbpB+, FbpA– (*fbpA*::*ermClacIP*)	(211)
	MCV511 (L3HA) derived strains	
MCV275	TbpA L3HA$_{343}$, TbpB+, PilQ– (*pilQ*::Ω)	This study
MCV276	TbpA L3HA$_{343}$, TbpB+, DsbC– (*dsbC*::*ermC*)	This study
MCV277	TbpA L3HA$_{343}$, TbpB+, TraC– (*traC*::*ermC*)	This study
MCV278	TbpA L3HA$_{343}$, TbpB+, TraH– (*traH*::*ermC*)	This study
MCV279	TbpA L3HA$_{343}$, TbpB+, TraN– (*traN*::*ermC*)	This study
	MCV267 (pseudo-revertant) derived strains	
MCV280	TbpA L3HA$_{343}$, TbpA– (*tbpA*::mTn3cat), TbpB+	This study
MCV281	TbpA L3HA$_{343}$, TonB– (*tonB*::Ω)	This study
MCV282	TbpA L3HA$_{343}$, TbpB+, PilQ– (*pilQ*::Ω)	This study
MCV283	TbpA L3HA$_{343}$, TbpB+, DsbC– (*dsbC*::*ermC*)	This study
MCV284	TbpA L3HA$_{343}$, TbpB+, TraC– (*traC*::*ermC*)	This study
MCV285	TbpA L3HA$_{343}$, TbpB+, TraH– (*traH*::*ermC*)	This study
MCV286	TbpA L3HA$_{343}$, TbpB+, TraN– (*traN*::*ermC*)	This study
MCV287	TbpA L3HA$_{343}$, TbpB+, FbpA– (*fbpA*::*ermClacIP*) –RBS	This study
MCV288	TbpA L3HA$_{343}$, TbpB+, FbpA– (*fbpA*::*ermClacIP*)	This study

Table 3: Plasmids

Plasmid	Description	Reference
	Constructs for generation of *N. gonorrhoeae* mutants	
pCR2.1 TOPO	Kan^R Amp^R	Invitrogen
pHSS6-GCU	Vector containing gonococcal uptake sequence (Kan^R)	(60)
pUNCH411	pBS-SK(+) containing entire *tbpA* gene	(47)
pUNCH403	Vector containing *tbpA*::mTn3cat	(48)
pVCU693	pCR2.1 TOPO containing *tonB*::Ω	(74)
pUNCH290	Vector containing *pilQ*::Ω	(37)
pHH17	pHSS6-GCU derivative containing *dsbC*::*ermC*	(Hamilton unpublished)
pKS83	pHSS6-GCU derivative containing *traC*::*ermC*	(76)
pJD1175	pHSS6-GCU derivative containing *traH*::*ermC*	(77)
pKS72	pHSS6-GCU derivative containing *traN*::*ermC*	(76)
pVCU912	pHSS6-GCU containing *fbpA*::*ermClacIP* (–RBS)	(211)
pVCU913	pHSS6-GCU containing *fbpA*::*ermClacIP*	(211)
pVCU250	pCR2.1 TOPO containing *tbpA* gene amplified with oVCU334 and oVCU335; $E_{118}A$, PvuI restriction site	This study (160)
pVCU251	pHSS6-GCU containing EcoRI fragment from pVCU250	This study (160)
pVCU252	pCR2.1 TOPO containing *tbpA* gene amplified with oVCU334 and oVCU335; $E_{120}A$, NdeI restriction site	This study (160)
pVCU253	pHSS6-GCU containing EcoRI fragment from pVCU252	This study (160)
pVCU254	pCR2.1 TOPO containing *tbpA* gene amplified with oVCU334 and oVCU335; $Y_{121}A$, HaeIII restriction site	This study (160)
pVCU255	pHSS6-GCU containing EcoRI fragment from pVCU254	This study (160)
pVCU256	pCR2.1 TOPO containing *tbpA* gene amplified with oVCU334 and oVCU335; $E_{122}A$, HpyCH4V restriction site	This study (160)
pVCU257	pHSS6-GCU containing EcoRI fragment from pVCU256	This study (160)
pVCU258	pCR2.1 TOPO containing *tbpA* gene amplified with oVCU334 and oVCU335; $EY_{120}AA$, PstI restriction site	This study (160)
pVCU259	pHSS6-GCU containing EcoRI fragment from pVCU258	This study (160)
pVCU260	pCR2.1 TOPO containing *tbpA* gene amplified with oVCU334 and oVCU335; $EYE_{120}AAA$, PstI and AluI	This study (160)

	restriction sites	
pVCU261	pHSS6-GCU containing EcoRI fragment from pVCU260	This study (160)
pVCU834	pCR2.1 TOPO containing *tbpB* encoding $CC_{481}AA$	This study (56)
pCVU835	pHSS6-GCU containing *tbpB* encoding $CC_{481}AA$	This study (56)
pVCU813	pCR2.1 TOPO containing *tbpB* encoding $HA3_{175}$	This study (57)
pVCU814	pHSS6-GCU containing *tbpB* encoding $HA3_{175}$	This study (57)
pVCU817	pCR2.1 TOPO containing *tbpB* encoding $HA5_{327}$	This study (57)
pVCU818	pHSS6-GCU containing *tbpB* encoding $HA5_{327}$	This study (57)
pVCU825	pCR2.1 TOPO containing *tbpB* encoding $HA9_{660}$	This study (57)
pVCU826	pHSS6-GCU containing *tbpB* encoding $HA9_{660}$	This study (57)
pVCU521	pCR2.1 TOPO containing *tbpA* encoding $L9HA_{750}$	This study (243)
	Constructs for expression of His-tag proteins	
pET-22b(+)	Amp^R	Novagen
pVCU911	pET22b(+) containing *fbpA* from gonococcal strain FA19	(175)
pVCU914	pET22b(+) containing *exbB* from gonococcal strain FA19	This study (Strange unpublished)
pVCU262	pCR2.1 TOPO containing *tbpA* plug-encoding region amplified with oVCU289 and oVCU290	This study
pVCU263	pET-22b(+) containing NdeI and XhoI fragment from pVCU262	This study
pVCU272	pCR2.1 TOPO containing *tbpA* plug-encoding region ($EYE_{120}AAA$) amplified with oVCU289 and oVCU290	This study
pVCU273	pET-22b(+) containing NdeI and XhoI fragment from pVCU272	This study
	Constructs for expression of S-tag proteins	
pET-29b(+)	Kan^R	Novagen
pVCU264	pCR2.1 TOPO containing *tbpA* plug-encoding region amplified with oVCU375 and oVCU376	This study

pVCU265	pET-29b(+) containing BglII and XhoI fragment from pVCU264	This study
pVCU266	pCR2.1 TOPO containing *fetA* plug-encoding region amplified with oVCU377 and oVCU378	This study
pVCU267	pET-29b(+) containing BglII and XhoI fragment from pVCU266	This study
pVCU268	pCR2.1 TOPO containing *fbpA* gene amplified with oVCU379 and oVCU380	This study
pVCU269	pET-29b(+) containing BglII and XhoI fragment from pVCU268	This study
pVCU270	pCR2.1 TOPO containing *tonB* gene amplified with oVCU381 and oVCU382	This study
pVCU271	pET-29b(+) containing BglII and XhoI fragment from pVCU270	This study

Table 4. Mutagenic oligonucleotides

Primer	Oligonucleotide sequence (5'-3')	Target
	Alanine-encoding *tbpA* oligonucleotides[a]	
oVCU336	GGCGCAATCAAT**GCG**ATCGAGTATGAG (PvuI)	*tbpA* $E_{118}A$
oVCU337	CTCATACTCGAT**CGC**ATTGATTGCGCC (PvuI)	*tbpA* $E_{118}A$
oVCU338	ATCAATGAAATC**GCA**TATGAGAACGTC (NdeI)	*tbpA* $E_{120}A$
oVCU339	GACGTTCTCATA**TGC**GATTTCATTGAT (NdeI)	*tbpA* $E_{120}A$
oVCU340	AATGAAATCGAG**GCC**GAGAACGTCAAG (HaeIII)	*tbpA* $Y_{121}A$
oVCU341	CTTGACGTTCTC**GGC**CTCGATTTCATT (HaeIII)	*tbpA* $Y_{121}A$
oVCU342	GAAATCGAGTAT**GCA**AACGTCAAGGCT (HpyCH4V)	*tbpA* $E_{122}A$
oVCU343	AGCCTTGACGTT**TGC**ATACTCGATTTC (HpyCH4V)	*tbpA* $E_{122}A$
oVCU344	ATCAATGAAATC**GCTGCA**GAGAACGTCAAG (PstI)	*tbpA* $E_{120}A$ $Y_{121}A$
oVCU345	CTTGACGTTCTC**TGCAGC**GATTTCATTGAT (PstI)	*tbpA* $E_{120}A$ $Y_{121}A$
oVCU346	GCAATCAATGAAATC**GCTGCAGCT**AACGTCAAGGCTGTC (PstI, AluI)	*tbpA* $E_{120}A$ $Y_{121}A$ $E_{122}A$
oVCU347	GACAGCCTTGACGTT**AGCTGCAGC**GATTTCATTGATTGC (PstI, AluI)	*tbpA* $E_{120}A$ $Y_{121}A$ $E_{122}A$
	Restriction site incorporating oligonucleotides[b]	
oVCU289	**CATATG**GAAAATGTGCAAGCCGGACAAGC (NdeI)	*tbpA* plug

oVCU290	**CTCGAG**CCTGCCTTCCCCGATAACATCGTCG (XhoI)	*tbpA* plug
oVCU375	**AGATCTG**GAAAATGTGCAAGCCGGACAAGCA (BglII)	*tbpA* plug
oVCU376	**CTCGAGTTA**CCTGCCTTCCCCGATAACATCGT (XhoI)	*tbpA* plug
oVCU377	**AGATCTG**GCAGAAAATAATGCCAATGTCGCA (BglII)	*fetA* plug
oVCU378	**CTCGAGTTA**CCAGTTTTTATCCAAGCCTTTGAG (XhoI)	*fetA* plug
oVCU379	**AGATCTG**GACATTACCGTGTACAACGGCCAA (BglII)	*fbpA*
oVCU380	**CTCGAGTTA**TTTCATACCGGCTTGCTCAAGCAG (XhoI)	*fbpA*
oVCU381	**AGATCTG**GTGATAGAGTCAGGAAATGTTATCGA (BglII)	*tonB*
oVCU382	**CTCGAGTTA**ATTCAATTCAAACTTGACGGGGAC (XhoI)	*tonB*

[a] Underlined nucleotides indicate alanine-encoding sequences and novel restriction sites
[b] Underlined nucleotides represent engineered restriction sites for directional subcloning

Table 5. Gene specific oligonucleotides

Primer	Oligonucleotide sequence (5'-3')	Target
	Non-mutagenic *tbpA* plug-specific primers	
oVCU334	AAGCTTGTGAAATAAGCACGGCTGCCG	*tbpA*
oVCU335	GAAGCGGTTGGGGCCCGTGTAGTCTCG	*tbpA*
	Revertant analysis – *tbpB* specific primers	
oVCU136	ATGAACAATCCATTGGTGAATC	*tbpB* upstream
oVCU141	GCCGCCGCTCAAAGAAGAC	*tbpB* upstream
oVCU265	GAATTGGGTTTCCGCTTTTTG	*tbpB* middle
oVCU266	GGAAGCCTTGCCGCTCCAGCT	*tbpB* middle
GC556	CACCGCAAATGGCAATG	*tbpB* downsteam
oVCU267	TCCATTGCCGGATTCAACTGTTGC	*tbpB* downsteam
	Revertant analysis – *tbpA* specific primers	
oVCU272	GATTAGGGAAACACTATGCA	*tbpA* upstream
TfBP21	CCGATGTAGTGCCGTTTGTT	*tbpA* upstream
TfBP13	GGTCGTGGCTGTTCCGCCCG	*tbpA* middle
TfBP28	AAATCCAGCCAGTCGGCAGG	*tbpA* middle
TfBP16	GTACGGCTGGCGGTCGGGCG	*tbpA* downstream
oVCU291	CGCCGTCTGAAGCCTTTCAG	*tbpA* downstream

CHAPTER 3 – ANALYSIS OF TBPA PLUG IRON BINDING AND IDENTIFICATION OF RESIDUES REQUIRED FOR TRANSFERRIN-IRON ACQUISITION BY *NEISSERIA GONORRHOEAE*

I. Introduction

Neisseria species do not produce or secrete siderophores (6, 131, 235). Instead they have the ability to utilize human transferrin (6, 18, 111, 131, 138), lactoferrin (6, 18, 111, 131), and hemoglobin (6, 138) through the expression of high-affinity receptors for these host iron-binding proteins. Models of iron acquisition mediated by these high-affinity receptors are based on the well-characterized TonB-dependent siderophore transporters (Figure 1) (28, 40, 42, 43, 61, 62, 119).

All gonococcal clinical isolates are able to utilize human transferrin (138) through expression of the transferrin-iron uptake system, composed of TbpA and TbpB. TbpA is a TonB-dependent outer membrane transporter that is essential for iron uptake, while TbpB is a surface-exposed lipoprotein that discriminates between apo- and holo-transferrin (20, 50, 183, 184). Unlike TbpA, TbpB is not required for transferrin-iron acquisition but makes the process more efficient (4). Although TbpA has not yet been crystallized, by analogy with characterized TonB-dependent transporters (Figure 2), TbpA is predicted to form two distinct domains (Figure 3). The C-terminal β-barrel domain, comprised of 22 transmembrane-spanning β-strands, likely functions as a

channel for iron transport. The N-terminal plug domain is predicted to fold up within the β-barrel domain to occlude the pore.

Characterized TonB-dependent transporters (Figure 2) serve as a paradigm for the transferrin-iron uptake system, but the TbpB lipoprotein component and the requirement for iron removal from transferrin are unique to this system. The precise mechanism by which TbpA mediates iron internalization has not been elucidated. However, the mechanism of iron acquisition through TbpA is unique in that the transferrin receptor must bind holo-transferrin, remove iron from transferrin, transport iron across the outer membrane, and release apo-transferrin at the cell surface. Thus, elucidation of the detailed mechanism of transferrin-iron acquisition through TbpA would represent a significant step towards characterization of a novel system for the acquisition of iron in *Neisseria gonorrhoeae*.

Previous studies suggested that the TbpA plug domain plays a role in iron internalization (243); however, the precise mechanism was not defined. We hypothesize that the plug domain of TbpA functions in multiple steps of transferrin-iron uptake. Specifically, we hypothesize that iron coordination by the TbpA plug domain is critical for iron removal from transferrin, iron binding, and iron transport through the outer membrane. The overall goal of this work was to demonstrate iron binding by the TbpA plug domain and determine the mechanism of iron acquisition through TbpA, specifically with respect to the contribution of the plug.

II. Results

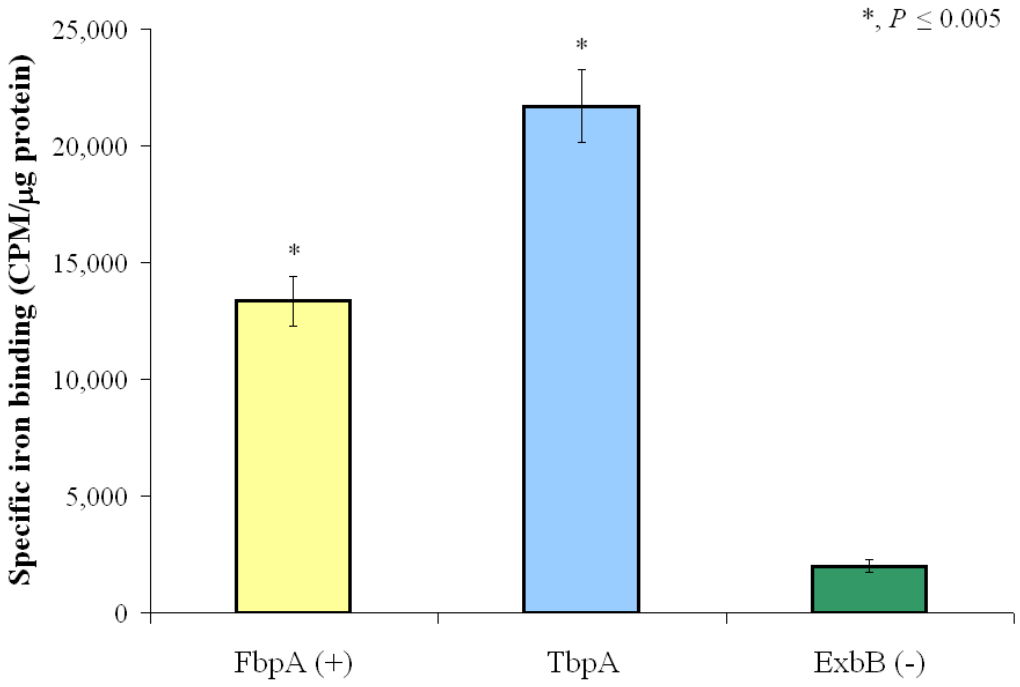
A. Gonococcal TbpA plug binds iron in vitro

To determine whether the neisserial TbpA plug domain binds iron, in vitro iron binding assays were carried out (Figure 5). Recombinant, gonococcal FbpA, the characterized periplasmic iron binding protein, serves as the positive control for iron binding, while gonococcal ExbB, the cytoplasmic membrane protein of TonB complex, serves as the negative control for iron binding. Recombinant gonococcal TbpA plug bound significant levels of iron compared to the ExbB negative control, as was observed for the FbpA positive control. Interestingly, the TbpA plug domain bound significantly higher levels of iron than FbpA ($P \leq 0.02$). These data indicate that the gonococcal TbpA plug domain binds iron in vitro and likely plays a significant role in iron coordination and transport in vivo.

B. Site-directed alanine substitution mutagenesis

Since the TbpA plug bound iron, putative iron-coordinating residues were identified throughout this domain. The TbpA plug domain is highly conserved among bacteria expressing the transferrin-iron acquisition system (45). An amino acid sequence alignment of the TbpA plug from a variety of bacterial pathogens is shown in Figure 6. The plug domain of TbpA contains several conserved sequence motifs, some of which have potential iron-coordinating residues similar to those that coordinate iron in human transferrin (88) and bacterial ferric binding protein A (FbpA) (214). Having shown iron coordination by the plug, we hypothesize that potential iron-coordinating residues function to remove iron from transferrin and transport iron through the outer membrane. To test this hypothesis, site-directed, alanine substitution mutagenesis of conserved,

Figure 5. Recombinant gonococcal TbpA plug binds iron in vitro. His-tagged, recombinant FbpA (+), TbpA plug, and ExbB (–) proteins were expressed and purified. Standardized levels of recombinant protein were bound to Ni-NTA agarose resin, incubated with $^{55}FeCl_3$ to determine total iron binding. Non-specific counts were determined in two ways: (1) incubation in the presence excess cold $Fe(NO_3)_3$ competitor and (2) incubation in the absence of recombinant protein. Levels of non-specific iron binding were subtracted from total iron binding to determine specific iron binding by recombinant proteins. These data are reported as specific counts per minute (CPM) standardized to μg of recombinant proteins. Each bar represents the average of at least three independent experiments, each performed in quadruplicate. Standard deviation is represented by error bars. *, $P \leq 0.005$ indicates significance difference from recombinant ExbB(–) protein.


*, $P \leq 0.005$
Specific iron binding (CPM/μg protein)
25,000
20,000
15,000
10,000
5,000
0
*
*
FbpA (+)
TbpA
ExbB (-)

potential iron-coordinating residues within the TbpA plug domain was performed. We selected the **E**I**EYE** conserved sequence motif, shown in Figure 6, for analysis because of the abundance of potential iron-coordinating residues, but more importantly, because the region immediately upstream was shown to be critical for transferrin utilization (243). We successfully generated six alanine substitution mutants: four single mutants at amino acids 118 (E), 120 (E), 121 (Y), and 122 (E), one double mutant at amino acids 120 (E) and 121 (Y), and one triple mutant at amino acids 120–122 (EYE). These alanine substitution mutants are identified by their strain names (MCV250 – MCV261), but for clarity, are also identified by the amino acid substitutions (Table 2).

C. Alanine substitution mutants express full-length TbpA and TbpB and bind transferrin to the cell surface

To determine if alanine mutagenesis of the TbpA plug domain disrupted TbpA protein expression and to ensure that TbpB expression was similar to the parental strain, Western blot analyses were carried out (Figure 7). In panel A, expression of TbpA and TbpB by the alanine substitution mutants in the FA19 (TbpB+) background was evaluated. All alanine substitution mutants in this background expressed wild-type levels of both TbpA and TbpB. In panel B, expression of TbpA and TbpB by the alanine mutants in the FA6905 (TbpB–) background was evaluated. Alanine substitution mutants in this background expressed wild-type levels of TbpA and did not express TbpB, as was detected in the FA6905 parental control. We conclude that site-directed, alanine substitution mutagenesis does not disrupt TbpA protein expression in any of the mutants.

Figure 6. Sequence alignment of TbpA plug domains from bacterial pathogens. The first two letters preceding the amino acid sequences represent the genus and species name of the bacterial pathogen: Ng, *Neisseria gonorrhoeae*; Nm, *Neisseria meningitidis*; Hi, *Haemophilus influenzae*; Ap, *Actinobacillus pleuropneumoniae*; and Mc, *Moraxella catarrhalis*. The subsequent numbers or letters represent the bacterial strain selected for analysis. The sequences represent mature TbpA plug domains and the amino acids are numbered accordingly to the right of the sequence. Dots indicate identical amino acids, dashes represent positions in which gaps were introduced in the alignment, and letters indicate the specific amino acid changes. Amino acids, in bold type and underlined, represent potential iron-coordinating residues. These amino acids coordinate iron in human transferrin (YHD) (88) and bacterial ferric binding protein A (YHE) (214). The box indicates the conserved sequence motif selected for site-directed, alanine substitution mutagenesis at amino acids 118 (E), 120 (E), 121 (Y), and 122 (E).

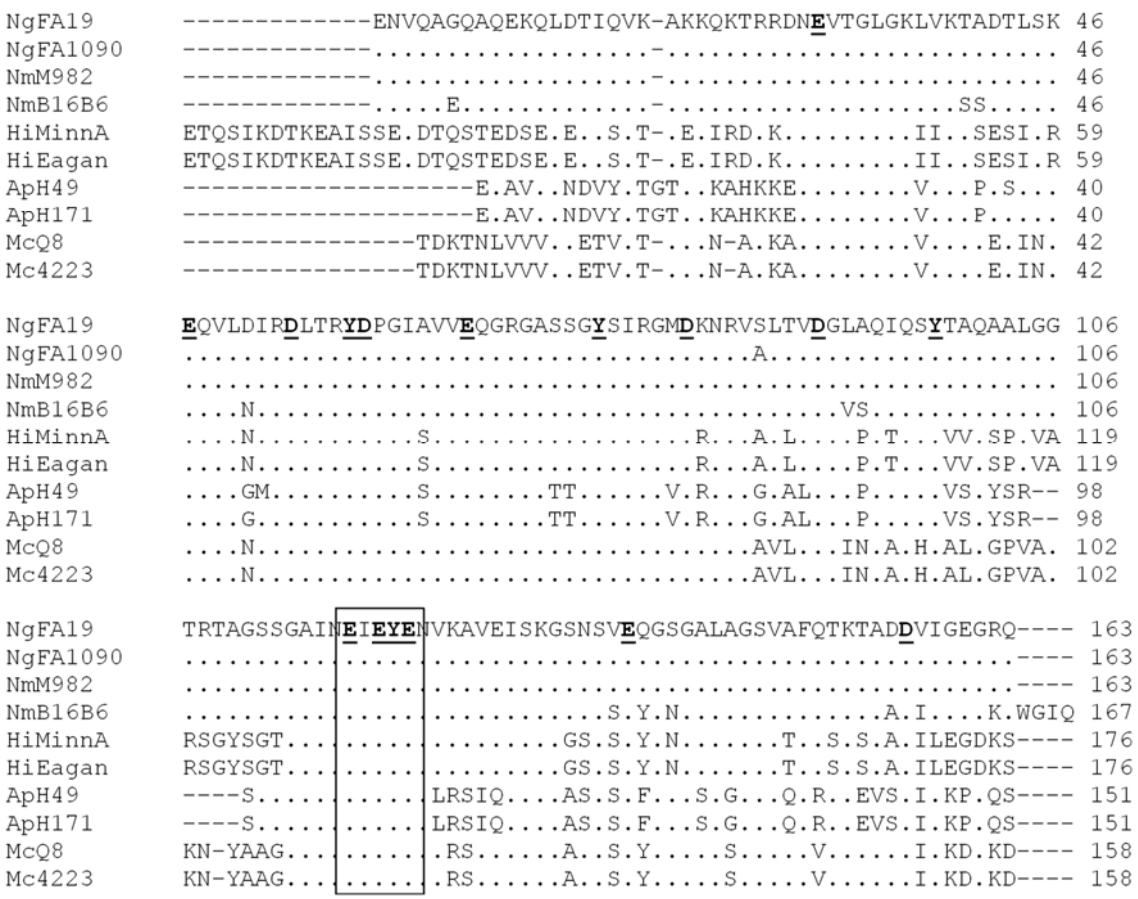
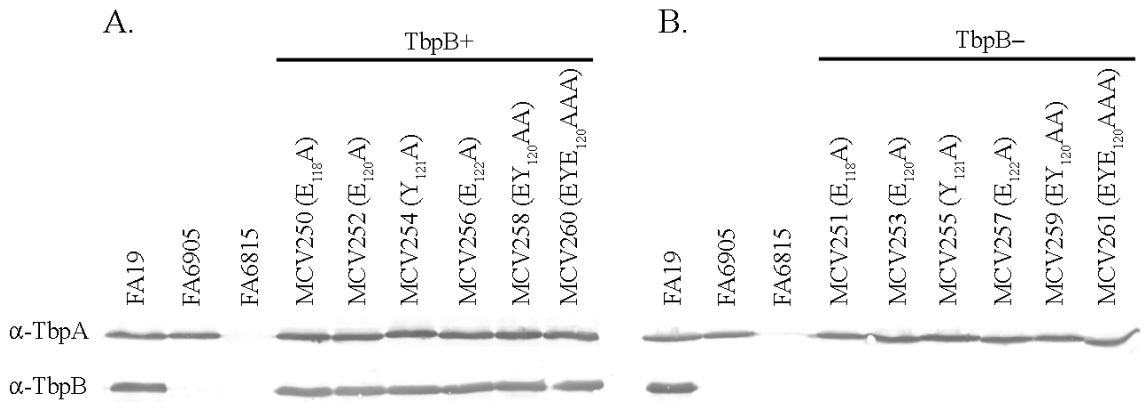

NgFA19 ----------ENVQAGQAQEKQLDTIQVK-AKKQKTRRDNEVTGLGKLVKTADTLSK 46
NgFA1090 ----------.....-..... 46
NmM982 ----------.....-..... 46
NmB16B6 ----------.....E.....-.....SS..... 46
HiMinnA ETQSIKDTKEAISSE.DTQSTEDSE.E..S.T-.E.IRD.K.....II..SESI.R 59
HiEagan ETQSIKDTKEAISSE.DTQSTEDSE.E..S.T-.E.IRD.K.....II..SESI.R 59
ApH49 ----------E.AV..NDVY.TGT..KAHKKE.....V...P.S... 40
ApH171 ----------E.AV..NDVY.TGT..KAHKKE.....V...P..... 40
McQ8 ----------TDKTNLVVV..ETV.T-...N-A.KA.....V....E.IN. 42
Mc4223 ----------TDKTNLVVV..ETV.T-...N-A.KA.....V....E.IN. 42
NgFA19 EQVLDIRDLTRYDPGIAVVEQGRGASSGYSIRGMDKNRVSLTVDGLAQIQSYTAQAALGG 106
NgFA1090 .....A..... 106
NmM982 106
NmB16B6 ....N.....VS..... 106
HiMinnA ....N.....S.....R...A.L....P.T...VV.SP.VA 119
HiEagan ....N.....S.....R...A.L....P.T...VV.SP.VA 119
ApH49 ....GM.....S.....TT.....V.R...G.AL...P.....VS.YSR-- 98
ApH171 ....G.....S.....TT.....V.R...G.AL...P.....VS.YSR-- 98
McQ8 ....N.....AVL...IN.A.H.AL.GPVA. 102
Mc4223 ....N.....AVL...IN.A.H.AL.GPVA. 102
NgFA19 TRTAGSSGAINEIEYENVKAVEISKGSNSVEQGSGALAGSVAFQTKTADDVIGEGRQ---- 163
NgFA1090 .....|.....|.....---- 163
NmM982 .....|.....|.....---- 163
NmB16B6 .....|.....|.....S.Y.N.....A.I....K.WGIQ 167
HiMinnA RSGYSGT...|.....|.....GS.S.Y.N.....T..S.S.A.ILEGDKS---- 176
HiEagan RSGYSGT...|.....|.....GS.S.Y.N.....T..S.S.A.ILEGDKS---- 176
ApH49 ----S.....|.....|LRSIQ....AS.S.F...S.G...Q.R..EVS.I.KP.QS---- 151
ApH171 ----S.....|.....|LRSIQ....AS.S.F...S.G...Q.R..EVS.I.KP.QS---- 151
McQ8 KN-YAAG...|.....|.RS.....A..S.Y.....S.....V.....I.KD.KD---- 158
Mc4223 KN-YAAG...|.....|.RS.....A..S.Y.....S.....V.....I.KD.KD---- 158

Figure 7. Alanine substitution mutants express wild-type levels of TbpA and TbpB by Western blot. Iron-stressed gonococci were lysed and standardized to a constant cell density. Whole-cell lysates were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Blots were probed with anti-TbpA (α-TbpA) or anti-TbpB (α-TbpB) polyclonal antibodies. Each lane is labeled according to the strain name with amino acid substitutions in parentheses. Panel A shows alanine substitution mutants in the FA19 (TbpB+) background, while panel B shows alanine substitution mutants in the FA6905 (TbpB–) background. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–).

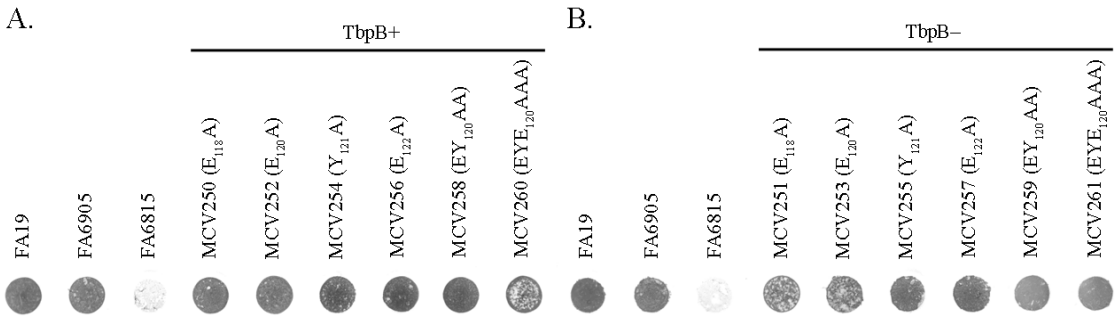

A.
TbpB+
FA19
FA6905
FA6815
MCV250 (E_{118}A)
MCV252 (E_{120}A)
MCV254 (Y_{121}A)
MCV256 (E_{122}A)
MCV258 (EY_{120}AA)
MCV260 (EYE_{120}AAA)
α-TbpA
α-TbpB
B.
TbpB–
FA19
FA6905
FA6815
MCV251 (E_{118}A)
MCV253 (E_{120}A)
MCV255 (Y_{121}A)
MCV257 (E_{122}A)
MCV259 (EY_{120}AA)
MCV261 (EYE_{120}AAA)

In addition to protein expression, TbpA surface exposure and transferrin binding were also evaluated. To determine surface exposure and transferrin binding, solid-phase transferrin binding assays, in which whole gonococcal cells were spotted to a nitrocellulose membrane and probed with HRP-labeled transferrin, were performed (Figure 8). In panel A, cell surface transferrin binding was evaluated for alanine substitution mutants in the FA19 (TbpB+) background. Although not quantitative, it is clear that all alanine substitution mutants bound transferrin to the cell surface when compared to the positive and negative controls. Similarly, panel B shows cell surface transferrin binding by alanine substitution mutants in the FA6905 (TbpB−) background. Again, all alanine substitution mutants in the TbpB− background maintained the ability to bind transferrin to the cell surface compared to the positive and negative controls. Overall, these results show that site-directed, alanine substitution mutagenesis does not affect TbpA expression levels, surface exposure, or the ability of TbpA to bind human transferrin to the cell surface.

D. Alanine substitution mutants bind transferrin with wild-type affinity and capacity

Solid-phase transferrin binding assays are not quantitative; therefore, to determine the transferrin binding affinity of the mutated TbpA proteins, whole-cell, equilibrium-phase transferrin binding assays were carried out (Figure 9). Since both TbpA and TbpB function in high-affinity transferrin binding, these assays were performed with only alanine substitution mutants in the FA6905 (TbpB−) background. In this manner, transferrin binding by TbpA, alone, could be assessed and quantitated. Panel A shows

Figure 8. Alanine substitution mutants bind transferrin to the cell surface in solid-phase transferrin binding assays. Whole, iron-stressed gonococci, standardized to a constant cell density, were applied to nitrocellulose membranes and probed with HRP-labeled transferrin. Each lane is labeled according to the strain name with amino acid substitutions in parentheses. Panel A shows the alanine substitution mutants in the FA19 (TbpB+) background, while panel B shows the alanine substitution mutants in the FA6905 (TbpB–) background. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–).


A.
TbpB+
FA19
FA6905
FA6815
MCV250 ($E_{118}A$)
MCV252 ($E_{120}A$)
MCV254 ($Y_{121}A$)
MCV256 ($E_{122}A$)
MCV258 ($EY_{120}AA$)
MCV260 ($EYE_{120}AAA$)
B.
TbpB–
FA19
FA6905
FA6815
MCV251 ($E_{118}A$)
MCV253 ($E_{120}A$)
MCV255 ($Y_{121}A$)
MCV257 ($E_{122}A$)
MCV259 ($EY_{120}AA$)
MCV261 ($EYE_{120}AAA$)

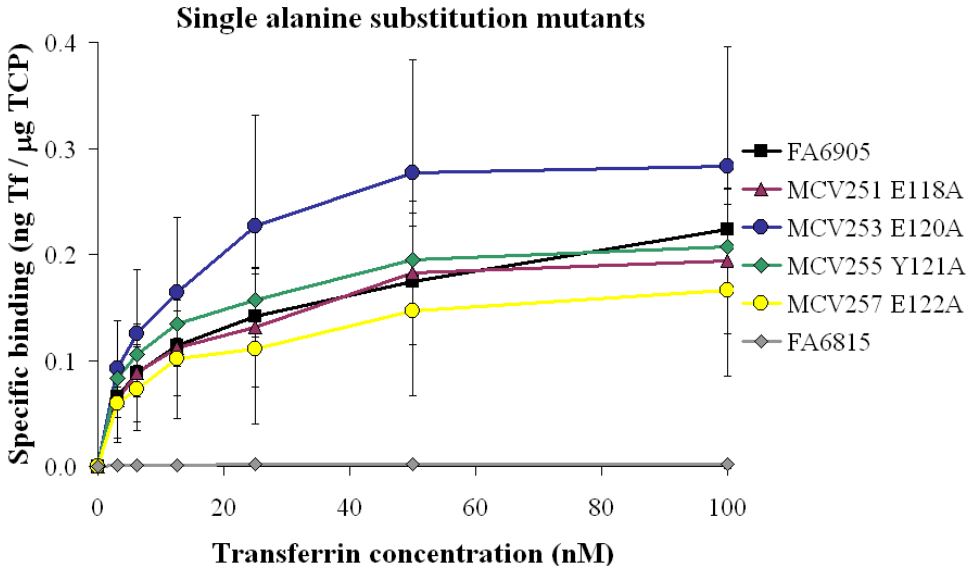
transferrin binding by the single alanine substitution mutants in the FA6905 (TbpB−) background, while panel B shows binding by the double and triple alanine substitution mutants in the FA6905 (TbpB−) background. All the alanine substitution mutants bound similar levels of transferrin compared to the parental control FA6905. Affinity and capacity measurements were calculated from the equilibrium transferrin binding data. These values, shown in Table 6, revealed that all alanine substitution mutants have similar transferrin binding affinities and capacities to the parental control FA6905. Overall these data support the solid-phase transferrin binding data, but specifically show that mutagenesis does not have a quantitative effect on transferrin binding affinity or on receptor capacity.

E. Double and triple alanine substitution mutants demonstrate decreased transferrin-iron internalization

TbpA plays two major roles in the process of iron acquisition by the gonococcal cell: transferrin binding and iron internalization. Transferrin binding was evaluated in the alanine substitution mutants and found to be at wild-type levels. Iron acquisition, the other major step in transferrin-mediated iron uptake, was also evaluated. This assay was performed by incubating gonococci with 20% iron-saturated human transferrin and specific iron uptake was measured (Figure 10). Strain FA6815 (TbpA− TbpB−) was unable to internalize iron due to the absence of TbpA and therefore served as the negative control in this assay. Although TbpB is not required for transferrin-iron uptake, it has been shown to make the process of iron internalization more efficient (4), and these data show that FA6905 (TbpB−) internalized iron around 50% of wild-type levels. Alanine

Figure 9. Alanine substitution mutants bind transferrin at wild-type levels in equilibrium-phase transferrin binding assays. Whole, iron-stressed gonococci were mixed with various concentrations of ^{125}I-labeled human transferrin (0-100 nM). Specific transferrin binding was determined by subtracting nonspecific binding (with excess competing unlabeled human transferrin) from total binding. Specific transferrin binding is reported on the y-axis as nanograms of transferrin bound per microgram of total cell protein (ng Tf/ μg TCP). Only strains in the FA6905 (TbpB–) background are shown in order to evaluate specific transferrin binding attributable to TbpA. Each curve is labeled according to the strain name with amino acid substitutions shown. Each point represents an average and standard deviation of at least three independent experiments. Panel A shows the single alanine substitution mutants, while panel B shows the double and triple alanine substitution mutants. Controls include FA6905 (positive control, TbpA+ TbpB–) and FA6815 (negative control, TbpA– TbpB–).

A.


Single alanine substitution mutants
Specific binding (ng Tf / µg TCP)
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
Transferrin concentration (nM)
FA6905
MCV251 E118A
MCV253 E120A
MCV255 Y121A
MCV257 E122A
FA6815

B.

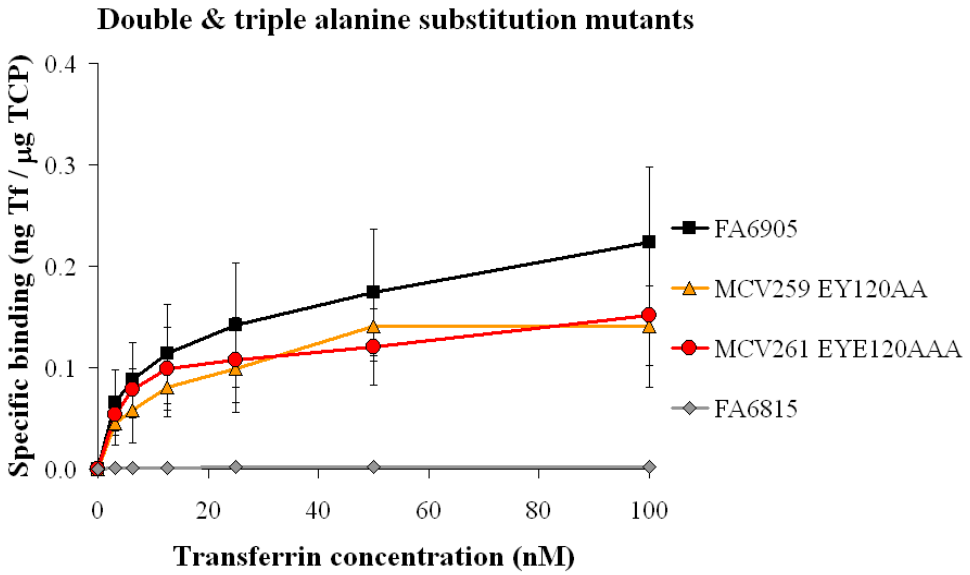

Double & triple alanine substitution mutants
Specific binding (ng Tf / µg TCP)
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
Transferrin concentration (nM)
FA6905
MCV259 EY120AA
MCV261 EYE120AAA
FA6815

Table 6. Affinity and capacity measurements for alanine substitution mutants generated from equilibrium-phase transferrin binding assays

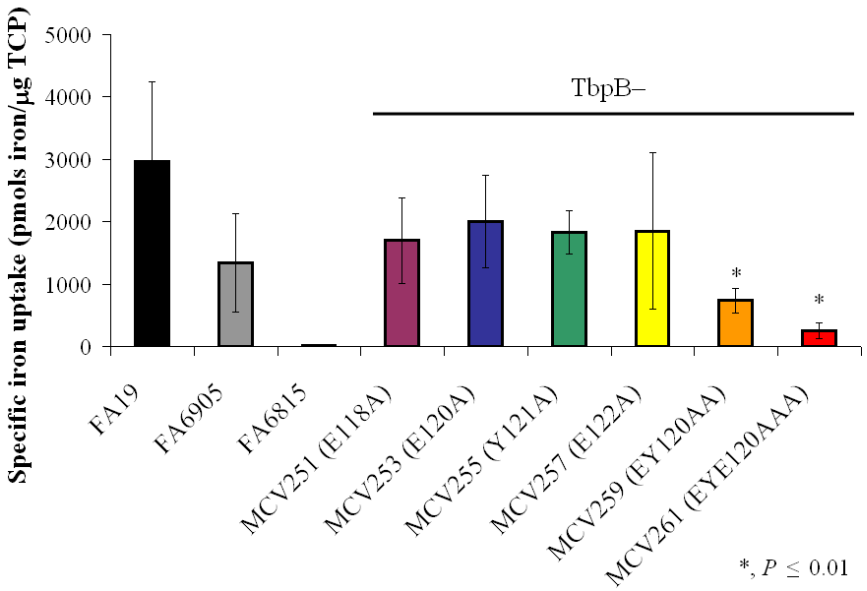
Strains	Phenotype	K_d[a] (nM)	Capacity (# receptors/μg TCP)[b]
FA6905	TbpA+, TbpB–	10.7 ± 2.9	1.67×10^8
MCV251	TbpA E_{118}A, TbpB–	9.2 ± 1.8	1.53×10^8
MCV253	TbpA E_{120}A, TbpB–	9.4 ± 1.4	2.37×10^8
MCV255	TbpA E_{121}A, TbpB–	6.8 ± 1.1	1.65×10^8
MCV257	TbpA E_{122}A, TbpB–	9.2 ± 2.1	1.32×10^8
MCV259	TbpA EY_{120}AA, TbpB–	11.2 ± 1.9	1.19×10^8
MCV261	TbpA EYE_{120}AAA, TbpB–	6.0 ± 1.2	1.10×10^8

[a] K_d, capacity, and standard error calculated with Grafit software
[b] TCP, total cell protein was determined by bicinchoninic acid (BCA) assay

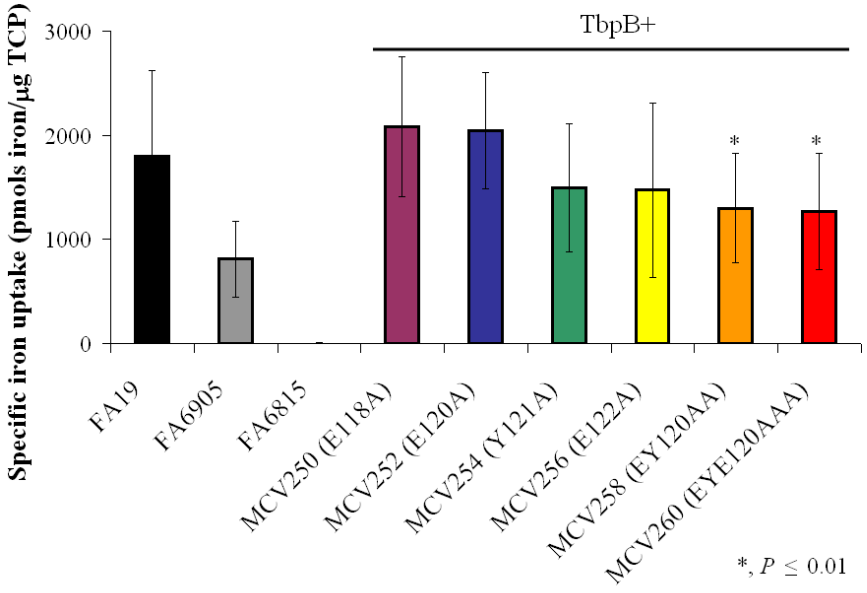
substitution mutants in the FA6905 (TbpB–) background were tested to determine the specific contribution of TbpA in the process of iron internalization (Figure 10, panel A). All single alanine substitution mutants in the FA6905 (TbpB–) background internalized similar amounts of iron compared to the FA6905 parental control. However, the double and triple alanine substitution mutants had significantly decreased iron internalization ($P \leq 0.01$) when compared to the FA6905 parental control. Specifically, the double alanine substitution mutant (MCV259) internalized approximately 50% less iron than FA6905, while the triple alanine substitution mutant (MCV261) internalized approximately 80% less iron than FA6905. These data show that the single alanine substitution mutants bind transferrin with a wild-type affinity and internalize iron at a similar rate as wild-type. While the double and triple alanine substitution mutants had wild-type transferrin binding affinities, they demonstrated a significant decrease in iron internalization. Alanine substitution mutants in the FA19 (TbpB+) background were evaluated in panel B. All single alanine substitution mutants in the FA19 (TbpB+) background internalized similar amounts of iron compared to the FA19 parental control. However, the double and triple alanine substitution mutants still demonstrated a significant decrease in iron internalization ($P \leq 0.01$) when compared to the FA19 parental control, but the presence of TbpB resulted in increased iron uptake by about 50% in both mutants. These results suggest that the wild-type sequence at residues 120–122 (EYE) facilitates efficient iron internalization.

Figure 10. Double and triple alanine substitution mutants demonstrate decreased iron internalization in transferrin-iron uptake assays. Iron-stressed gonococci were incubated with ^{55}Fe-labeled human transferrin. Specific iron uptake was measured in picomoles (pmols) of iron internalized after 30 minutes. Each bar represents the mean of at least six independent experiments and is labeled according to the strain name with amino acid substitutions in parentheses. Panel A shows the alanine substitution mutants in the FA6905 (TbpB–) background, while panel B shows the alanine substitution mutants in the FA19 (TbpB+) background. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–). Standard deviations are represented by error bars. *, $P \leq 0.01$ indicates significance from both FA19 and FA6905.

A.


Specific iron uptake (pmols iron/µg TCP)
5000
4000
3000
2000
1000
0
TbpB–
*
*
FA19
FA6905
FA6815
MCV251 (E118A)
MCV253 (E120A)
MCV255 (Y121A)
MCV257 (E122A)
MCV259 (EY120AA)
MCV261 (EYE120AAA)
*, $P \leq 0.01$

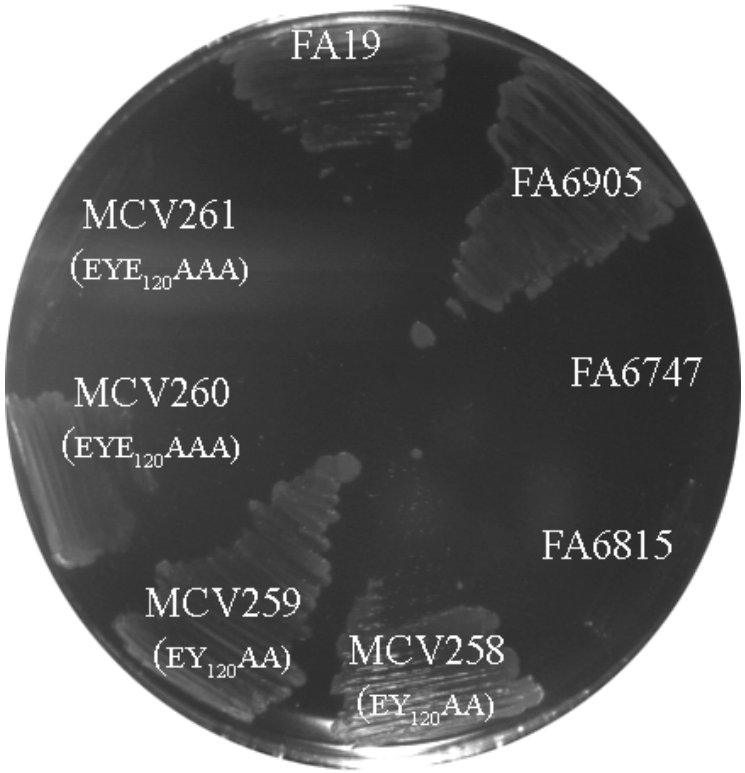
B.


Specific iron uptake (pmols iron/µg TCP)
3000
2000
1000
0
TbpB+
*
*
FA19
FA6905
FA6815
MCV250 (E118A)
MCV252 (E120A)
MCV254 (Y121A)
MCV256 (E122A)
MCV258 (EY120AA)
MCV260 (EYE120AAA)
*, $P \leq 0.01$

F. Triple alanine substitution mutant only utilizes transferrin as a sole iron source in the presence of TbpB

Since the double and triple alanine substitution mutants had a significant decrease in iron internalization, the ability of these mutants to utilize human transferrin as a sole iron source was evaluated. To test this, transferrin-iron utilization growth assays, in which gonococci were grown on CDM supplemented with 30% iron-saturated human transferrin as the sole iron source, were carried out (Figure 11). Alanine substitution mutants in both the FA19 (TbpB+) and FA6905 (TbpB–) backgrounds were tested. The single alanine substitution mutants in both backgrounds maintained the ability to utilize transferrin as a sole source of iron. In addition, although the double alanine substitution mutants (MCV258 and MCV259) had significantly decreased iron internalization, these mutants were able to utilize transferrin-bound iron for growth in both the presence and absence of TbpB. The triple alanine substitution mutant in the FA19 (TbpB+) background (MCV260), despite its decreased iron internalization, maintained the ability to utilize transferrin as a sole iron source. However, consistent with the significant decrease in iron internalization, the triple alanine substitution mutant in the FA6905 (TbpB–) background (MCV261) was unable to utilize transferrin, as shown in Figure 11. These results suggest that TbpB has the ability to compensate for the defect observed in the triple alanine substitution mutant. Overall, these data suggest that residues 120–122 (EYE) of the TbpA plug domain are critical for optimal iron internalization from transferrin, but the function provided by this conserved plug domain motif can be compensated for by the presence of TbpB.

Figure 11. Triple alanine substitution mutant utilizes transferrin as a sole iron source in transferrin utilization growth assays only in the TbpB-positive background. Gonococcal strains were grown on CDM agarose plates containing 30% iron-saturated human transferrin as a sole iron source. The ability of mutants to utilize transferrin as a sole source of iron was evaluated by growth at 37^{o}C with 5% CO_2 for 24 hours. Strains are labeled according to the strain name with specific amino acid substitutions in parentheses. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), FA6747 (negative control, TbpA– TbpB+), and FA6815 (negative control, TbpA– TbpB–).


FA19
FA6905
MCV261
(EYE120AAA)
FA6747
MCV260
(EYE120AAA)
FA6815
MCV259
(EY120AA)
MCV258
(EY120AA)

Since these assays were carried out using 30% iron-saturated transferrin, the growth phenotypes of the alanine substitution mutants in the presence of a variety of iron saturations ranging from 5% – 80% were determined (Table 7). The single and double alanine substitution mutants in both backgrounds maintained the ability to utilize transferrin at all the transferrin-iron saturations tested. Similar to the results in Figure 11, the triple alanine substitution mutant in the FA19 (TbpB+) background (MCV260) maintained the ability to utilize transferrin at all the transferrin-iron saturation levels. However, the triple alanine substitution mutant in the FA6905 (TbpB–) background (MCV261) was unable to utilize transferrin regardless of the level of iron saturation. These data suggest that TbpA with alanine substitutions at residues 120–122 (EYE) is completely unable to utilize transferrin and that the defect in transferrin utilization is not due to inefficiency at low iron saturation levels. Furthermore, these data imply that the compensatory function provided by TbpB is not due solely to the ability of TbpB to specifically bind the ferrated form of transferrin.

G. MCV261 is unable to utilize transferrin in the presence of exogenous TbpB

In an attempt to characterize the compensatory function provided by TbpB, transferrin-iron uptake assays were carried out in the presence of recombinant, full-length TbpB (Figure 12) and FA6747 (TbpA– TbpB+) total membrane preparations (TMP) (data not shown). We hypothesized that if the TbpA triple alanine substitution mutant had a defect in iron removal from transferrin that TbpB was able to compensate for, then perhaps exogenous TbpB could provide the stripping function thereby allowing MCV261

Table 7. Phenotypes of alanine substitution mutants in transferrin-iron utilization growth assays

Strain	Amino acid substitution	Iron-saturated transferrin*				
		5%	10%	30%	50%	80%
FA19	TbpA+, TbpB+	+	+	+	+	+
FA6905	TbpA+, TbpB– (Δ*tbpB*)	+	+	+	+	+
FA6747	TbpA– (*tbpA*::mTn3cat), TbpB+	–	–	–	–	–
FA6815	TbpA–, TbpB– (*tbpB*::Ω)	–	–	–	–	–
MCV250	TbpA E_{118}A, TbpB+	+	+	+	+	+
MCV251	TbpA E_{118}A, TbpB– (Δ*tbpB*)	+	+	+	+	+
MCV252	TbpA E_{120}A, TbpB+	+	+	+	+	+
MCV253	TbpA E_{120}A, TbpB– (Δ*tbpB*)	+	+	+	+	+
MCV254	TbpA Y_{121}A, TbpB+	+	+	+	+	+
MCV255	TbpA Y_{121}A, TbpB– (Δ*tbpB*)	+	+	+	+	+
MCV256	TbpA E_{122}A, TbpB+	+	+	+	+	+
MCV257	TbpA E_{122}A, TbpB– (Δ*tbpB*)	+	+	+	+	+
MCV258	TbpA EY_{120}AA, TbpB+	+	+	+	+	+
MCV259	TbpA EY_{120}AA, TbpB– (Δ*tbpB*)	+	+	+	+	+
MCV260	TbpA EYE_{120}AAA, TbpB+	+	+	+	+	+
MCV261	TbpA EYE_{120}AAA, TbpB– (Δ*tbpB*)	–	–	–	–	–

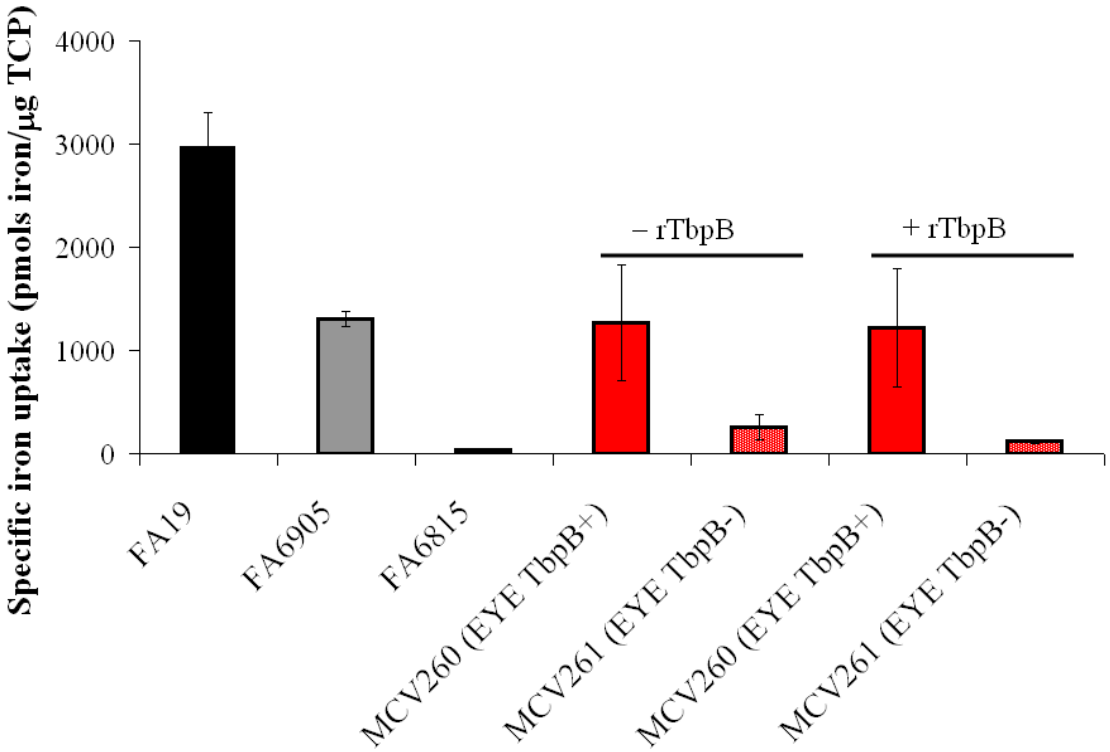
*, Transferrin-iron utilization growth assays were carried out with CDM plates supplemented with 5%, 10%, 30%, 50%, or 80% iron-saturated human transferrin

(TbpB–) to function in transferrin-iron utilization. However, if the triple alanine substitution mutant had a transport defect and maintained the ability to remove iron from transferrin, then exogenous TbpB would not facilitate transferrin-iron utilization by this mutant. Transferrin-iron internalization assays were carried out with MCV261 (TbpB–) as described previously, but in the presence of recombinant TbpB or FA6747 (TbpA–, TbpB+) total membrane preparations (TMP) at concentrations equivalent to the amount of transferrin present. Results from both assays showed that neither recombinant TbpB nor TbpB from total membrane preparations (TMP) were able to compensate for the TbpA plug domain mutant in transferrin-iron uptake. It is likely that the function provided by TbpB requires expression of TbpB within the same membrane as TbpA. Thus, these data suggest that TbpA and TbpB function together to facilitate the removal of iron from transferrin and transport of iron through the outer membrane.

III. Discussion

In order for gonococci to utilize transferrin-bound iron, TbpA must carry out two major functions: iron removal from transferrin and iron transport through the outer membrane. This study was designed to analyze the functional role of the TbpA plug domain in this process and to test the hypothesis that the plug functions in iron-coordination and subsequent iron internalization. In vitro studies indicated that the plug domain of TbpA functions in iron coordination. Single, double, and triple alanine substitution mutagenesis of putative iron-coordinating residues within the TbpA plug

Figure 12. Exogenous TbpB is unable to compensate for MCV261 ($EYE_{120}AAA$) in transferrin-iron uptake assays. Iron-stressed gonococci were incubated with ^{55}Fe-labeled human transferrin in the presence (+ rTbpB) or absence (– rTbpB) of equal molar amounts of recombinant TbpB (125μM). Specific iron uptake was measured in picomoles (pmols) of iron internalized after 30 minutes. Each bar represents the mean of three independent experiments and is labeled according to the strain name with amino acid substitutions in parentheses. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–). Standard deviations are represented by error bars.


Specific iron uptake (pmols iron/µg TCP)
4000
3000
2000
1000
0
– rTbpB
+ rTbpB
FA19
FA6905
FA6815
MCV260 (EYE TbpB+)
MCV261 (EYE TbpB-)
MCV260 (EYE TbpB+)
MCV261 (EYE TbpB-)

domain resulted in mutants that expressed wild-type levels of TbpA on the gonococcal cell surface and bound transferrin with wild-type affinity and capacity. Furthermore, no growth abnormalities were observed with any of the alanine substitution mutants suggesting that the alanine mutagenesis had relatively little impact on the structure or function of gonococcal TbpA. Previous studies, in which deletion and insertion mutagenesis of TbpA were performed (22, 243), are in agreement with these data and support the theory that gonococcal TbpA, like other TonB-dependent receptors (7, 24, 98, 102, 117, 140, 157), is resilient to various types of mutagenesis.

Analysis of the double and triple alanine substitution mutants in transferrin-iron uptake assays revealed a significant defect in iron internalization. Furthermore, analysis of the triple alanine substitution mutant in transferrin-iron utilization growth assays revealed that this mutant was unable to utilize transferrin-bound iron in the absence of TbpB. This suggests that the defect in the TbpA plug domain can be overcome by the presence of TbpB. This phenomenon has been previously observed with insertion mutations in putative loops 2, 9, and 11 of TbpA (243), suggesting that TbpA and TbpB have redundant function(s). Similar to the triple alanine substitution mutant, the loop 2, 9, and 11 TbpA insertion mutants showed no defect in transferrin binding, but a defect in transferrin-iron utilization in the absence of TbpB (243). Therefore, the function of TbpB is not limited to transferrin binding and holo/apo-transferrin discrimination (20, 50, 183, 184), but also appears to play an additional role in the mechanism of transferrin-mediated iron uptake. The compensatory function provided by TbpB suggests that TbpA and TbpB work together to accomplish transferrin-iron uptake. Although not much is known

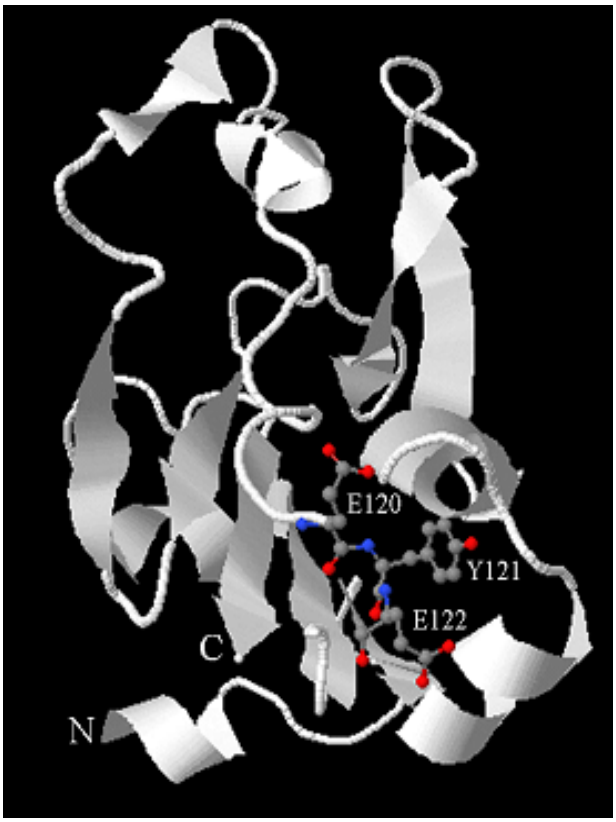
about the specific interactions between TbpA and TbpB, it has been shown that both gonococcal and meningococcal TbpB associate with TbpA (63, 64, 97, 210). The defect in transferrin-iron uptake and utilization seen in the TbpA triple alanine substitution mutant could be attributed to either a defect in iron removal from transferrin, iron binding by the plug, and/or iron transport through the β-barrel. Therefore TbpB may provide one or more of these functions in wild-type gonococci. Since TbpB is a surface-exposed lipoprotein, it is not likely to compensate for iron transport through the TbpA β-barrel, but it may function in iron removal from transferrin by an unknown mechanism.

As mentioned previously, gonococcal TbpB is not typically required for transferrin utilization, but in the case of the TbpA triple alanine substitution mutant, TbpB is required for iron acquisition from transferrin. This dependence on TbpB function is also observed in *Neisseria meningitidis* strain B16B6, which expresses a low molecular weight class of the transferrin binding proteins. In B16B6, iron acquisition from human transferrin requires TbpB (85), which is most likely due to the lack of critical regions in the TbpA from this strain. Meningococcal TbpA from B16B6 shares only 75% identity to gonococcal TbpAs (45) and shows sequence and length diversity within the exposed loops (45, 163). These data suggest that there are multiple domains within TbpA and TbpB that are important in transferrin-mediated iron acquisition. Cumulatively, our data (160, 243) demonstrate that disruption of one or more epitopes prevents TbpA function in the absence of TbpB. These domains may similarly be lacking or nonfunctional in meningococcal strain B16B6.

Our data demonstrate that the TbpA plug domain binds iron and residues 120–122 (EYE) of the TbpA plug domain are critical for transferrin-iron acquisition. This may be due to alteration of an iron binding site, which may promote iron removal from transferrin, subsequent iron binding by the plug, and transport through the outer membrane. It has been shown that the plug domain of the *E. coli* TonB-dependent transporter FepA binds directly to its ligand, ferric-enterobactin, in the absence of the β-barrel domain (222), which suggests the importance of the plug in ligand binding and possibly transport. Oke et al. found that the neisserial TbpA plug domain was unable to bind human transferrin (161). However, the TbpA plug domain most likely has a ligand specificity for iron rather than transferrin. Although TbpA is a characterized transferrin receptor, it is also an iron transporter. Therefore, TbpA has two ligand specificities during the process of transferrin-mediated iron internalization. It is important to note that, although residues 120–122 (EYE) of the TbpA plug domain are clearly involved in transferrin-mediated iron acquisition, others may be involved as well.

Figure 13 shows a homology model for the gonococcal TbpA plug domain, which was derived from comparison with the known crystal structure of the homologous plug domain of the ferric-enterobactin transporter, FepA. The positions of amino acids 120 (E), 121 (Y), and 122 (E), mutated in the current study, are shown as ball and stick representations. Interestingly, this conserved motif is located near the bottom of the plug in this model. The position of this critical domain relative to the surface-exposed opening of the barrel (top) is consistent with the hypothesis that the plug coordinates iron via ligands from distant amino acid residues within the plug. Alternatively, iron may be

Figure 13. Predicted structural model of *N. gonorrhoeae* TbpA plug domain. The mature *N. gonorrhoeae* TbpA plug domain (amino acids 1-162) was aligned with the homologous sequence of the *E. coli* FepA plug domain using the 3D-Jigsaw comparative modeling program. The modeled region of TbpA spans from Thr_{25} (N-terminus) to Thr_{151} (C-terminus). The resulting output file was visualized with First Glance in Jmol. The image shown was captured and imported into Adobe Photoshop. The amino acids 120 (E), 121 (Y), and 122 (E) are represented by balls and sticks. Red indicates hydrophobic atoms, while blue indicates hydrophilic atoms. Alpha helices and beta strands are shown as ribbons, with arrowheads pointing toward the C-terminus. Random coils are shown as smooth backbone traces.


E120
Y121
E122
C
N

coordinated at multiple, different sites within the plug domain during transport through the β-barrel. Our recombinant iron binding data may support this hypothesis in that the TbpA plug domain binds more iron than FbpA, which may indicate more than one site of iron coordination. It is also possible that these substitution mutants resulted in indirect impacts within the plug domain leading to the plug's inability to bind or transport iron. Thus, the results of this study support the hypothesis that the plug domain coordinates iron. This iron coordination is likely critical for transferrin-mediated iron internalization, for mutations in possible coordination sites result in decreased iron uptake and losses in TbpA-mediated transferrin utilization.

Overall, these studies provide insight into the mechanism of TbpA-mediated iron acquisition. Further studies are necessary to determine the function of the TbpA plug domain in respect to the multiple steps of transferrin-iron uptake: iron removal from transferrin, iron binding and coordination, and iron transport through the outer membrane. Further studies are also needed to address the function of TbpB in this process of iron acquisition from transferrin. A better understanding of this mechanism may reveal new sites within TbpA to target for vaccine development or to exploit for treatment of gonococcal infection.

IV. Acknowledgements

Funding of this work was provided by Public Health Service grant ROI AI047141 from the National Institutes of Allergy and Infectious Diseases, National Institutes of

Health. I was supported by the Training in Molecular Pathogenesis grant (T32 AI07617) from the National Institutes of Health while carrying out this work.

I would like to acknowledge Bill Barton at Virginia Commonwealth University for his assistance with TbpA homology modeling and Susan Buchanan at NIH for critical reading of this chapter. I also would like to thank Cara Olsen at Uniformed Services University for useful discussions regarding appropriate statistical analysis. Finally, I would also like to acknowledge Lori Walsh for her assistance in construction of some mutant strains in this study.

CHAPTER 4 – ANALYSIS OF TBPA AND TBPB COOPERATION IN TRANSFERRIN-IRON ACQUISITION BY *NEISSERIA GONORRHOEAE*

I. Introduction

The gonococcal transferrin-iron acquisition system is composed of two dissimilar proteins, TbpA and TbpB. TbpA is the TonB-dependent outer membrane iron transporter required for transferrin-iron acquisition. However, the TbpB lipoprotein component is not required for transferrin-iron internalization, but has been shown to make the process more efficient (4). There have been few studies to address the mechanism by which TbpB increases the efficiency of iron uptake; however, it has been shown that ligand discrimination and holo-transferrin binding are not sufficient for this TbpB-dependent increase in efficiency (56, 57).

TbpB is a bi-lobed structure with a C-terminal and N-terminal domain that both function in transferrin binding (57). This was recently determined in a structure and function analysis of mutants with hemagglutinin (HA) epitope insertions throughout the length of TbpB (57). This study confirmed that TbpB was entirely surface-exposed and that there were two distinct domains that function in high-affinity transferrin binding (57). These HA epitope insertion mutants in TbpB have been fully characterized and vary in their transferrin binding abilities (57).

Although TbpB is not required for transferrin-iron acquisition in the wild-type receptor, there have been a number of characterized TbpA mutants that require TbpB for function. These include one site-directed substitution mutant in the TbpA plug domain (MCV260) (160) and HA epitope insertion mutants within the surface-exposed loops 2, 9, and 11 of TbpA (MCV527, MCV515, MCV519) (242). All of these mutants exhibited identical phenotypes, in which the mutagenized TbpA maintained wild-type transferrin binding, but was unable to utilize transferrin as a sole iron source in the absence of TbpB. To date, TbpB has been shown to only function in transferrin binding and discrimination (20, 50, 183, 184). Therefore, the ability of TbpB to compensate for defects in iron internalization suggests that TbpB provides a novel function that does not directly pertain to transferrin binding and discrimination. Thus, we hypothesize that specific domains of TbpB may function in iron removal from transferrin, allowing for iron uptake and thus restored function in transferrin utilization.

To address which domains in TbpB were required for the compensatory function, TbpB was mutagenized in two gonococcal strains that require it for transferrin utilization (MCV260 – EYE and MCV515 – L9HA). We hypothesized that these gonococcal strains that require TbpB for function, despite having similar phenotypes, might exhibit different defects in transferrin-iron acquisition. In addition, we hypothesized that TbpB has various domains that are important for the observed compensatory functions. Therefore, by combining characterized TbpA and TbpB mutations, specific regions of TbpB required for TbpA-mediated transferrin utilization may be identified. Also, using this approach, it may be possible to distinguish between phenotypically similar TbpA

mutants, in an effort to elucidate the specific TbpA defects in transferrin-mediated iron acquisition.

II. Results

A. Site-directed alanine substitution mutagenesis

TbpB has an N-terminal and C-terminal lobe, which both contain high-affinity transferrin binding domains (57). Other regions within TbpB are highly conserved at the amino acid sequence level (56, 57). One unique region of conservation is in the C-terminus and this region consists of two, adjacent cysteine residues (56, 57). To investigate the role these residues play in transferrin-mediated iron acquisition, site-directed alanine substitution mutagenesis was utilized to change the double cysteines to double alanines (56). This mutation was generated in both FA19 (TbpA+) and FA6747 (TbpA–) backgrounds for analysis and resulting strains MCV845 and MCV846 are listed in Table 2. It has been shown that cysteine residues can coordinate metal ions (105), and therefore, these residues may play a role in the removal of iron from transferrin. Additionally, these residues may be important for the compensatory function provided by TbpB in either MCV260 ($EYE_{120}AAA$) or MCV515 (L9HA). Therefore, in addition to the previously characterized TbpB HA epitope insertion mutants (57), this cysteine mutant was used for TbpA and TbpB combined mutagenesis, discussed below.

B. MCV845 expresses full-length TbpB that is capable of binding transferrin

To determine if site-directed mutagenesis of the TbpB conserved cysteine residues disrupted TbpB protein expression levels or transferrin binding ability, Western

blot analyses were carried out (Figure 14). Expression of TbpB and transferrin binding ability were assessed by immunoblotting with anti-TbpB antibodies and HRP-labeled transferrin, respectively. MCV845 ($CC_{481}AA$, TbpA+) expressed wild-type levels of TbpB and bound transferrin by Western blot. Therefore, site-directed alanine substitution mutagenesis of cysteine residues does not disrupt TbpB protein expression. In addition, TbpB is not subject to proteolytic degradation as a result of mutagenesis.

C. MCV846 binds transferrin with wild-type affinity and capacity

MCV845 ($CC_{481}AA$, TbpA+) expressed wild-type levels of TbpB, but it was also necessary to determine if site-directed mutagenesis affected TbpB surface exposure and whole-cell transferrin binding ability. Thus, solid-phase transferrin binding assays were used to assess surface expression of TbpB and transferrin binding to the gonococcal cell surface (Figure 15). Whole-cell, iron-stressed gonococci were spotted to a nitrocellulose membrane and probed with HRP-labeled transferrin to assess transferrin-binding competent surface proteins. Although not quantitative it was clear that MCV845 ($CC_{481}AA$, TbpA+) and MCV846 ($CC_{481}AA$, TbpA–) expressed surface exposed and transferrin binding competent TbpA and TbpB. Therefore, site-directed mutagenesis does not disrupt TbpB overall protein expression levels, protein expression on the gonococcal cell surface, or transferrin binding potential.

Since solid-phase transferrin binding assays are not quantitative, equilibrium-phase transferrin binding assays were carried out to quantitate specific transferrin binding affinity and capacity (Figure 16, Table 8). Since both TbpA and TbpB function in transferrin binding, equilibrium-phase transferrin binding was assessed in the FA6747

Figure 14. MCV845 expresses wild-type levels of TbpB and binds transferrin by Western blot. Iron-stressed gonococci were lysed and standardized to a constant cell density. Whole-cell lysates were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Blots were probed with anti-TbpB (α-TbpB) polyclonal antibody (panel A) or HRP-transferrin (panel B). Each lane is labeled according to the strain name with amino acid substitutions in parentheses. Controls include FA19 (positive control, TbpA+ TbpB+) and FA6815 (negative control, TbpA– TbpB–).

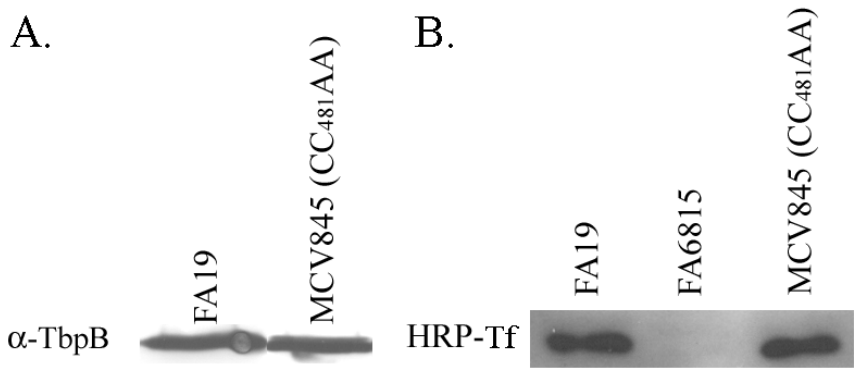
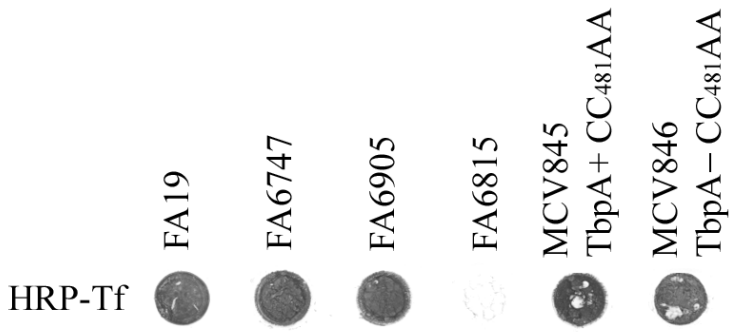

A.
FA19
MCV845 (CC481AA)
α-TbpB
B.
FA19
FA6815
MCV845 (CC481AA)
HRP-Tf

Figure 15. Double cysteine mutants bind transferrin to the cell surface in solid-phase transferrin binding assays. Whole, iron-stressed gonococci, standardized to a constant cell density, were applied to nitrocellulose membranes and probed with HRP-labeled transferrin. Each lane is labeled according to the strain name with amino acid substitutions shown below. Controls include FA19 (positive control, TbpA+ TbpB+), FA6747 (TbpA– TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–).


HRP-Tf
FA19
FA6747
FA6905
FA6815
MCV845
TbpA+ CC481AA
MCV846
TbpA– CC481AA

(TbpA–) background (MCV846) to determine the transferrin binding ability of TbpB only. From these studies it is clear that MCV846 ($CC_{481}AA$, TbpA–) bound wild-type levels of transferrin as shown in Figure 16. Affinity and capacity measurements calculated from these equilibrium-phase transferrin binding data show that MCV846 exhibited wild-type transferrin binding affinity and capacity (Table 8).

D. MCV845 demonstrates decreased transferrin-iron internalization

Although TbpB is not required for transferrin-iron internalization, it does increase the efficiency of iron uptake (4). Since the TbpB double cysteines may have a role in iron removal from transferrin, this mutant may exhibit decreased efficiency in iron internalization when compared to the wild-type. Thus, transferrin-iron internalization assays were carried out in the FA19 (TbpA+) background (MCV845). Iron-stressed gonococci were incubated with 20% iron-saturated human transferrin, and specific iron uptake is shown in Figure 17. As mentioned previously, TbpB is known to increase the efficiency of iron internalization and that was apparent with the FA6905 (TbpB–) control, which internalized about 50% of wild-type FA19. In support of our hypothesis, MCV845 ($CC_{481}AA$, TbpA+) showed a significant decrease in iron uptake, approximately 60% of wild-type FA19. Iron internalization by MCV845 was also significantly greater than that of FA6905 (TbpB–) control. Thus, double cysteine residues in the C-terminus of TbpB play an important role in increasing the efficiency of transferrin-iron uptake, but replacement of the cysteine residues does not completely abolish TbpB function. To further analyze the contribution these double cysteines play in transferrin-mediated iron uptake, MCV845 ($CC_{481}AA$, TbpA+) as well as other

Figure 16. MCV846 binds transferrin at wild-type levels in equilibrium-phase transferrin binding assays. Whole, iron-stressed gonococci were mixed with various concentrations of ^{125}I-labeled human transferrin (0-100 nM). Specific transferrin binding was determined by subtracting nonspecific binding (with excess competing unlabeled human transferrin) from total binding. Specific transferrin binding is reported on the y-axis as nanograms of transferrin bound per microgram of total cell protein (ng Tf/μg TCP). Only MCV846, the TbpA– strain, is shown in order to evaluate specific transferrin binding attributable to TbpB. Each curve is labeled according to the strain name with amino acid substitutions shown. Each point represents the average and standard deviation of at least three independent experiments. Controls include FA6747 (positive control, TbpA– TbpB+) and FA6815 (negative control, TbpA– TbpB–).

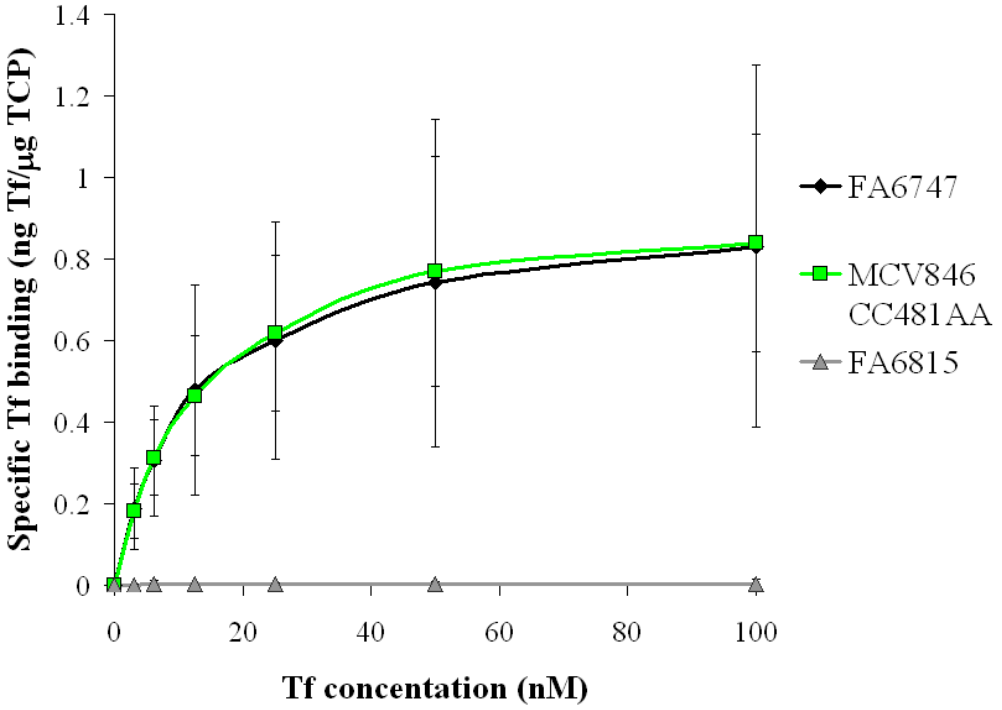
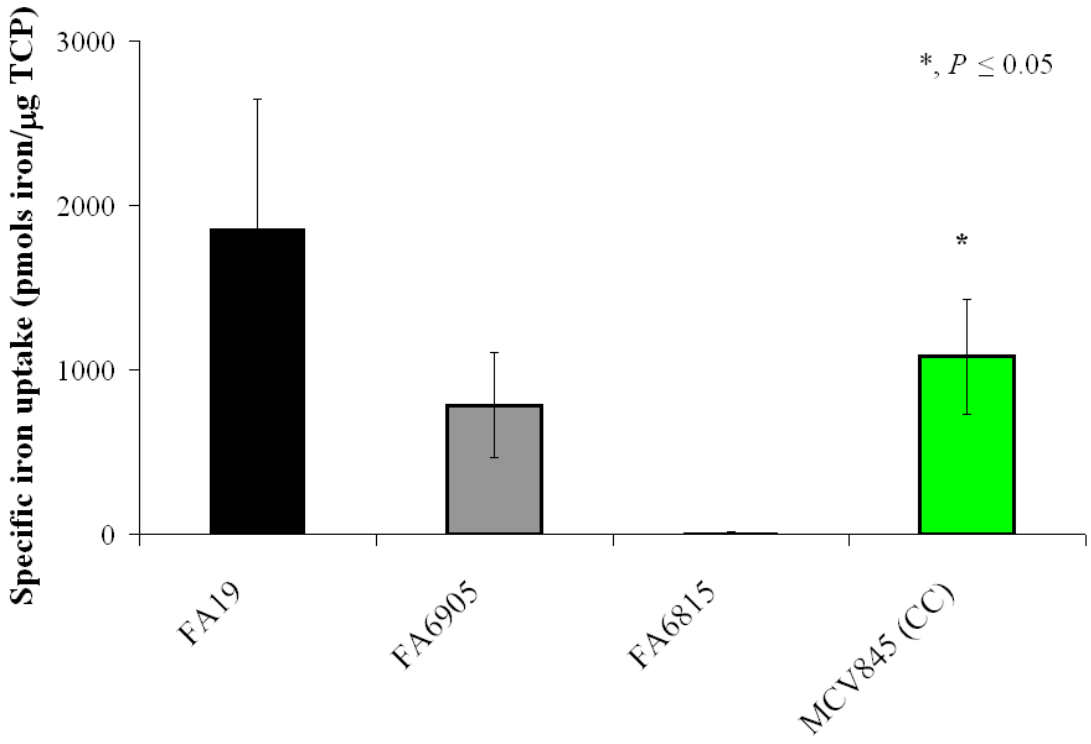

Specific Tf binding (ng Tf/μg TCP)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Tf concentation (nM)
FA6747
MCV846
CC481AA
FA6815

Table 8. Affinity and capacity measurements for MCV846 generated from equilibrium-phase transferrin binding assays

Stain	Phenotype	*Kd*[a] (nM)	Capacity[b] (# receptors/μg TCP)
FA6747	TbpA–, TbpB+	12.6 ± 0.6	7.00×10^8
MCV846	TbpA–, TbpB $CC_{481}AA$	13.1 ± 0.4	7.19×10^8

[a] K_d, capacity, and standard error calculated with Grafit software
[b] TCP, total cell protein was determined by bicinchoninic acid (BCA) assay

Figure 17. MCV845 demonstrates decreased iron internalization in transferrin-iron uptake assays. Iron-stressed gonococci were incubated with ^{55}Fe-labeled human transferrin. Specific iron uptake was measured in picomoles (pmols) of iron internalized after 30 minutes. Each bar represents the mean of at least six independent experiments and is labeled according to the strain name with amino acid substitution in parentheses. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–). Standard deviations are represented by error bars. *, $P \leq 0.05$ indicates significance from both FA19 and FA6905.

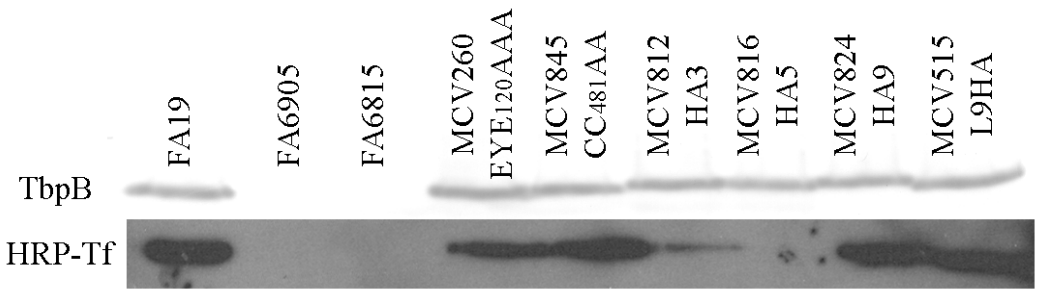

Specific iron uptake (pmols iron/μg TCP)
3000
2000
1000
0
*, $P \leq 0.05$
*
FA19
FA6905
FA6815
MCV845 (CC)

previously characterized TbpB HA-epitope insertion mutants were used for TbpA and TbpB combined mutagenesis and are discussed below. All the single TbpA and TbpB mutations utilized for combined mutagenesis are described below.

E. TbpA and TbpB single mutants express transferrin binding competent, full-length TbpB

To confirm previously described phenotypes, all single TbpA and TbpB mutants used in combined mutagenesis were subjected to Western blot analysis to evaluate TbpB protein expression and HRP-transferrin binding (Figure 18). HRP-transferrin binding by Western blot assesses TbpB protein expression as well as transferrin binding ability. Following SDS-PAGE the N-terminal high-affinity transferrin binding domain of TbpB retains its transferrin binding ability. Therefore, HRP-transferrin probes for transferrin binding by the N-terminus of TbpB. As previously observed (57, 160, 243), this study confirmed that all single mutants expressed wild-type levels of TbpB. In addition, transferrin binding phenotypes were also consistent with previous results (57, 243). Mutations in the TbpA plug domain (MCV260 – $EYE_{120}AAA$) and loop 9 of TbpA (MCV515 – L9HA) were able to bind transferrin by Western blot, indicating the presence of a wild-type, transferrin-binding competent TbpB. Similarly, MCV845 ($CC_{481}AA$, TbpA+) bound wild-type levels of transferrin by Western blot. TbpB HA epitope insertion mutants showed varying levels of transferrin binding. HA epitope insertion into position 3 of TbpB (MCV812 – HA3) caused a decrease in transferrin binding, while HA epitope insertion into position 5 (MCV816 – HA5) abolished transferrin binding by Western blot. These findings are consistent with previous studies and are not surprising

Figure 18. Mutations in TbpA and TbpB do not affect TbpB protein expression or transferrin binding by Western blot. Iron-stressed gonococci were lysed and standardized to a constant cell density. Whole-cell lysates were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Blots were probed with anti-TbpB (TbpB) polyclonal antibodies (top panel) or HRP-labeled transferrin (bottom panel). Each lane is labeled according to the strain name and mutation. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–).

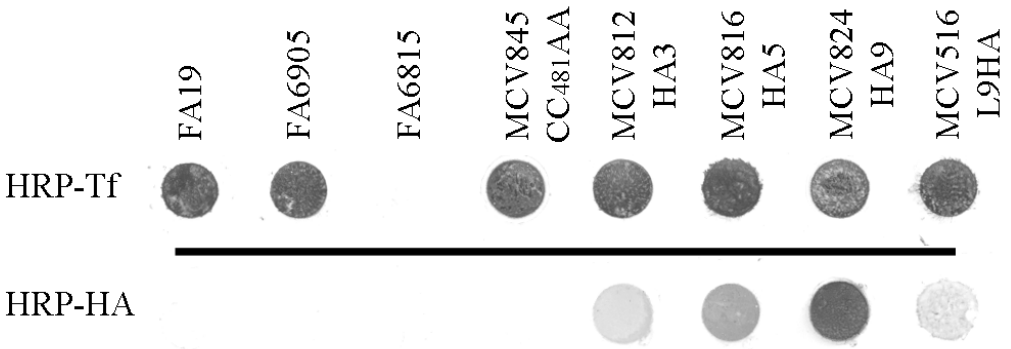

FA19
FA6905
FA6815
MCV260
EYE120AAA
MCV845
CC481AA
MCV812
HA3
MCV816
HA5
MCV824
HA9
MCV515
L9HA
TbpB
HRP-Tf

because positions 3 and 5 are both within the N-terminal high-affinity transferrin binding domain of TbpB. As previously shown, HA epitope insertion into position 9 of TbpB (MCV824 – HA9) caused no defect and wild-type levels of transferrin binding were observed.

F. TbpA and TbpB single mutants bind transferrin to the cell surface

Transferrin binding and surface exposure were assessed by solid-phase transferrin binding assays (Figure 19). HA epitope surface expression was also assessed by solid-phase anti-HA antibody binding. Whole-cell, iron-stressed gonococci were spotted to nitrocellulose membranes and probed with HRP-transferrin or an HRP-HA antibody. Dot blot analysis confirmed HA epitope surface exposure; although, as previously shown, HA epitope insertions were not equally accessible by HA antibody (57, 243). Unlike transferrin binding by Western, cell-surface transferrin binding detects both TbpA- and TbpB-mediated transferrin binding as well as transferrin binding by both domains of TbpB. Thus, phenotypes observed by Western blot are masked in solid-phase assays by the ability of both TbpB N-terminal and C-terminal domains ability to bind transferrin to the cell surface. These data show that all single mutants express TbpA and TbpB on the gonococcal cell surface and maintain the ability to bind transferrin. These studies show that mutagenesis of TbpA or TbpB does not disrupt protein expression or transferrin binding ability to the gonococcal cell surface.

Figure 19. TbpA and TbpB single mutants bind transferrin and express HA epitopes on the cell surface in solid-phase transferrin binding assays. Whole, iron-stressed gonococci, standardized to a constant cell density, were applied to nitrocellulose membranes and probed with HRP-labeled transferrin (top panel) or HRP-HA antibody (bottom panel). Each lane is labeled according to the strain name with mutations shown below. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–).


FA19
FA6905
FA6815
MCV845
CC481AA
MCV812
HA3
MCV816
HA5
MCV824
HA9
MCV516
L9HA
HRP-Tf
HRP-HA

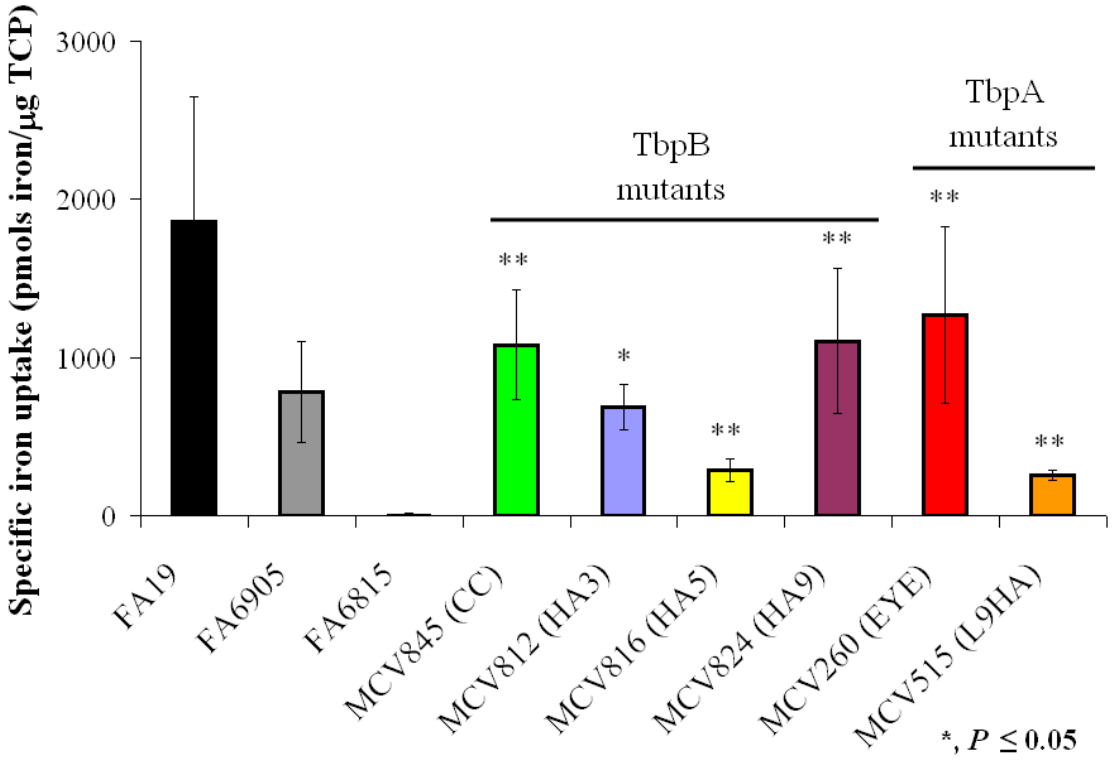
G. TbpA and TbpB single mutants vary in ability to internalize iron from transferrin

To analyze the ability of each mutant to internalize iron, transferrin-iron uptake assays were carried out (Figure 20). For this assay, iron-stressed gonococci were incubated with 20% iron-saturated human transferrin and specific iron uptake was measured. As previously observed, FA6905 (TbpB–) was less efficient in iron uptake and internalized about 42% of wild-type FA19. All of the mutants tested in iron uptake assays had significant defects in iron internalization. As previously shown, MCV260 ($EYE_{120}AAA$) internalized iron at 60% of wild-type FA19. The other TbpA mutant tested in this assay was the HA-encoding epitope insertion into loop 9 of TbpA (MCV515 – L9HA). Although MCV515 (L9HA) has been shown to have the same phenotype as the TbpA plug mutant, it is clear from the iron uptake data, that MCV515 (L9HA) has a much greater defect in transferrin-iron internalization, only internalizing 15% of wild-type FA19. Although these mutants have similar growth phenotypes, they clearly have different defects in transferrin-iron acquisition.

The various TbpB single mutants were also tested to evaluate how the efficiency of iron uptake was affected. Although some of these mutants had been previously tested and characterized, the studies presented here were performed and analyzed differently than what was previously reported (57). Therefore, any differences observed in iron uptake phenotypes from this study in comparison to the previous study (57) are attributed to the way in which these assays were performed and these data were analyzed. The

MCV845 ($CC_{481}AA$, TbpA+) as previously shown only internalized 60% of iron compared to wild-type FA19. HA epitope insertion mutants in the N-terminus of TbpB at positions 3 and 5 (HA3 and HA5) resulted in significantly decreased iron uptake. MCV812 (HA3) internalized about 40% of wild-type FA19, similar to the TbpB− (FA6905) control. Overall, MCV812 (HA3) exhibited decreased transferrin binding and an iron uptake efficiency similar to that of a TbpB− strain (FA6905). In contrast, MCV816 (HA5), which had abolished transferrin binding by Western blot, demonstrated iron uptake below that of a TbpB− (FA6905) strain, about 15% of wild-type FA19. Iron uptake levels below 50% of wild-type were not expected as they suggest a defect in TbpA. This mutant may express lower levels of TbpA, which could account for significant decrease in iron uptake below TbpB− (FA6905) levels. The final TbpB HA epitope insertion mutant, MCV824 (HA9), had an identical phenotype to MCV845 ($CC_{481}AA$). MCV824 (HA9) exhibited wild-type transferrin binding and demonstrated a decreased efficiency in transferrin-iron uptake, about 60% of wild-type FA19. Thus, both mutations in the C-terminus of TbpB, $CC_{481}AA$ and HA9, caused similar defects whereby TbpB still functioned in transferrin binding and contributes to the efficiency of transferrin-iron uptake. Overall, these data illustrate the phenotypic diversity that these mutations have on TbpA and TbpB function. Combined TbpA and TbpB mutagenesis, discussed below, will allow us to determine specific domains in TbpB that are required for compensatory functions and will help elucidate the mechanism of transferrin-iron acquisition utilized by these mutants.

Figure 20. TbpA and TbpB single mutants internalize varying levels of iron in transferrin-iron uptake assays. Iron-stressed gonococci were incubated with ^{55}Fe-labeled human transferrin. Specific iron uptake was measured in picomoles (pmols) of iron internalized after 30 minutes. Each bar represents the mean of at least six independent experiments and is labeled according to the strain name with mutations in parentheses. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–). Standard deviations are represented by error bars. *, $P \leq 0.05$ indicates significance from FA19, while ** indicates significance from both FA19 and FA6905.


Specific iron uptake (pmols iron/μg TCP)
3000
2000
1000
0
TbpB mutants
TbpA mutants
**
*
**
**
**
**
FA19
FA6905
FA6815
MCV845 (CC)
MCV812 (HA3)
MCV816 (HA5)
MCV824 (HA9)
MCV260 (EYE)
MCV515 (L9HA)
*, P ≤ 0.05

H. Combined TbpA and TbpB mutagenesis

Combined TbpA and TbpB mutants were generated for the purpose of determining regions of TbpA and TbpB critical for iron acquisition from transferrin. By utilizing specific TbpA mutants that require TbpB for function, our goal was to determine the functions required by TbpB for TbpA compensation. TbpA mutants, MCV260 ($EYE_{120}AAA$) and MCV515 (L9HA), were both combined with MCV845 ($CC_{481}AA$) to generate strains MCV262 (EYE/CC) and MCV266 (L9/CC), respectively. Previous mutagenesis and analyses were carried out in which MCV515 (L9HA) was combined with various TbpB HA epitope insertion mutants (57). In a similar fashion, MCV260 ($EYE_{120}AAA$) was combined with the various TbpB HA epitope insertion mutants: MCV812 (HA3), MCV816 (HA5), and MCV824 (HA9). It was hypothesized that MCV816 (HA5), which phenotypically resembles the TbpB− strain (FA6905) should not compensate for either TbpA mutation. However, the TbpB mutants that exhibit an intermediate phenotype, MCV845 ($CC_{481}AA$), MCV812 (HA3), and MCV824 (HA9) may or may not compensate for TbpA, depending on the role these regions play in transferrin-mediated iron uptake.

I. Combined mutants express full-length TbpA and TbpB

Before determining the effects of mutagenesis on transferrin-iron acquisition, these mutants were characterized to ensure that they expressed wild-type levels of TbpA and TbpB. Therefore, these strains were subjected to Western blot analyses, for detection of TbpA, TbpB, and HRP-transferrin binding (Figure 21). All combined mutants

expressed wild-type levels of full-length TbpA and TbpB; therefore, combined mutagenesis does not disrupt the expression of either transferrin binding protein. Western blotting with HRP-transferrin, as mentioned previously, detects only transferrin binding by the N-terminus of TbpB. Thus, these results only reflect transferrin binding by TbpB present in each strain. These data show that combined mutagenesis does not alter the transferrin binding phenotypes observed for the single TbpB mutants in Western blot analysis.

J. Combined mutants bind transferrin to the cell surface

To ensure that these combined mutants expressed TbpA and TbpB on the gonococcal cell surface, solid-phase transferrin binding assays were carried out (Figure 22). Iron-stressed, whole-cell gonococci were spotted to nitrocellulose membrane and probed with HRP-labeled transferrin for detection of transferrin binding to the gonococcal cell surface. Unlike Western blot analysis with HRP-transferrin, solid-phase dot blots represent transferrin binding by TbpA and both binding domains of TbpB. From this analysis, it was concluded that both TbpA and TbpB are surface exposed and transferrin binding competent.

K. Combined mutants vary in ability to internalize iron from transferrin

To determine the effects of combined mutagenesis on iron internalization, transferrin-iron uptake assays were performed, whereby gonococcal strains were incubated with 20% iron-saturated human transferrin and specific iron uptake was measured (Figure 23). Each combined mutant exhibited significantly lower levels of iron internalization as compared to the parental control (FA19). Many of the mutants

Figure 21. TbpA and TbpB combined mutants express wild-type levels of TbpA and TbpB and bind transferrin by Western blot. Iron-stressed gonococci were lysed and standardized to a constant cell density. Whole-cell lysates were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Blots were probed with anti-TbpA (TbpA) (top panel), anti-TbpB (TbpB) (middle panel), or HRP-labeled transferrin (HRP-Tf) (bottom panel). Each lane is labeled according to the strain name with mutation shown below. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–).

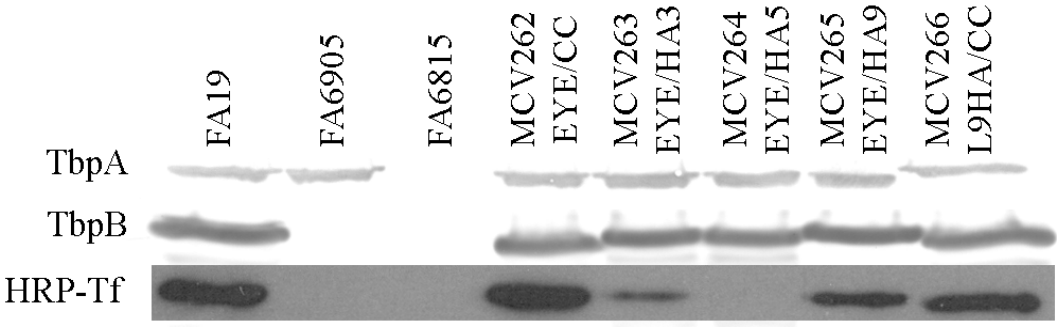
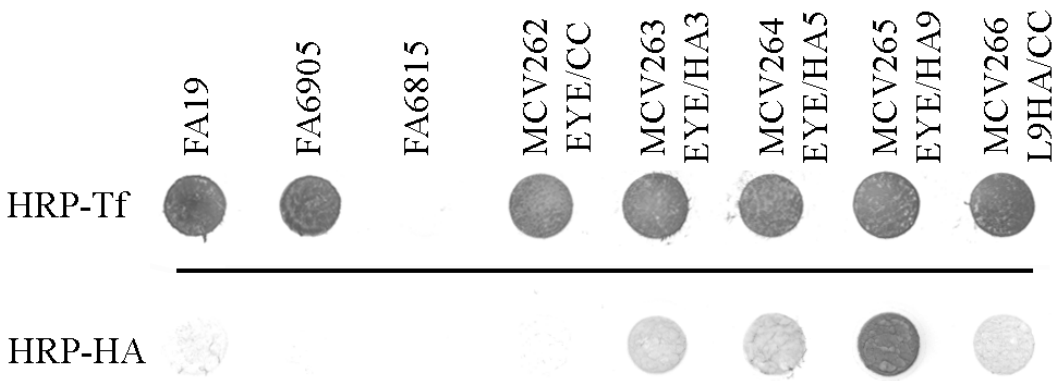

FA19
FA6905
FA6815
MCV262 EYE/CC
MCV263 EYE/HA3
MCV264 EYE/HA5
MCV265 EYE/HA9
MCV266 L9HA/CC
TbpA
TbpB
HRP-Tf

Figure 22. TbpA and TbpB combined mutants bind transferrin and express HA epitope on the cell surface in solid-phase transferrin binding assays. Whole, iron-stressed gonococci, standardized to a constant cell density, were applied to nitrocellulose membranes and probed with HRP-labeled transferrin (top panel) or HRP-HA antibody (bottom panel). Each lane is labeled according to the strain name with mutations shown below. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB−), and FA6815 (negative control, TbpA− TbpB−).


FA19
FA6905
FA6815
MCV262 EYE/CC
MCV263 EYE/HA3
MCV264 EYE/HA5
MCV265 EYE/HA9
MCV266 L9HA/CC
HRP-Tf
HRP-HA

exhibited even lower levels of iron uptake than the TbpB– control (FA6905). Therefore, wild-type TbpA and TbpB are required for efficient iron uptake and the different TbpB defects cause variations in the levels of transferrin-iron internalization.

L. Combined mutants utilize transferrin only when TbpB is transferrin-binding competent

Transferrin-iron utilization growth assays were carried out to determine if these decreases in iron internalization were substantial enough to prevent utilization of transferrin as a sole iron source. For these assays, gonococcal strains were grown on CDM supplemented with 30% iron-saturated transferrin as the sole iron source (Table 9). Growth phenotypes of the single and combined mutants are summarized in Table 9. The TbpA single mutants, as shown previously (160, 243), were only able to utilize transferrin in the FA19 (TbpB+) background. Thus, MCV260 ($EYE_{120}AAA$) and MCV515 (L9HA) are dependent on TbpB for transferrin utilization. The TbpB single mutants were able to utilize transferrin, as expected, because they express wild-type TbpA. Despite the significantly low levels of iron internalization, all of the combined mutants, with the exception of MCV264 (EYE/HA5), were able to utilize transferrin as a sole iron source. These data show that in order for TbpB to compensate for these TbpA defects, the function of the N-terminal transferrin binding domains of TbpB is required.

Compensation, up to this point, has been defined as the ability of mutant gonococcal strains to grow, in the presence of TbpB, with transferrin provided as the sole iron source. If, however, we look at the ability of TbpB to compensate in terms of iron

Figure 23. TbpA and TbpB combined mutants internalize varying levels of iron in transferrin-iron uptake assays. Iron-stressed gonococci were incubated with ^{55}Fe-labeled human transferrin. Specific iron uptake was measured in picomoles (pmols) of iron internalized after 30 minutes. Each bar represents the mean of at least six independent experiments and is labeled according to the strain name with mutations in parentheses. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–). Standard deviations are represented by error bars. *, $P \leq 0.05$ indicates significance from FA19, while ** indicates significance from both FA19 and FA6905.

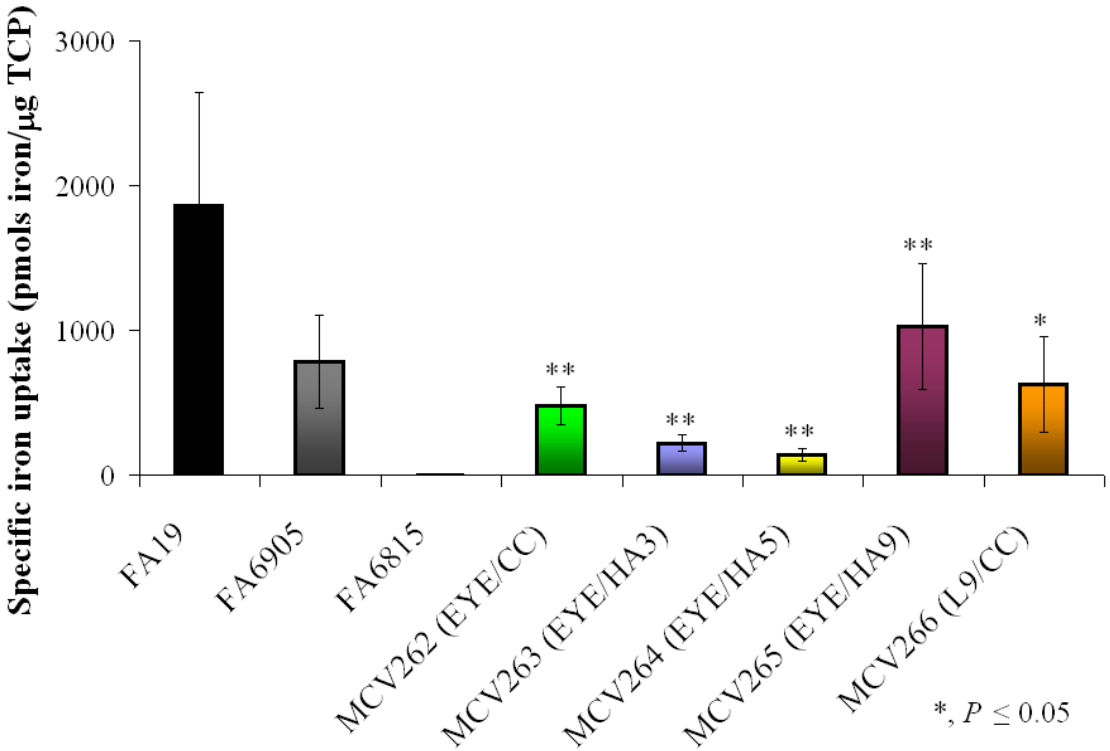

Specific iron uptake (pmols iron/μg TCP)
3000
2000
1000
0
**
**
**
**
*
FA19
FA6905
FA6815
MCV262 (EYE/CC)
MCV263 (EYE/HA3)
MCV264 (EYE/HA5)
MCV265 (EYE/HA9)
MCV266 (L9/CC)
*, $P \leq 0.05$

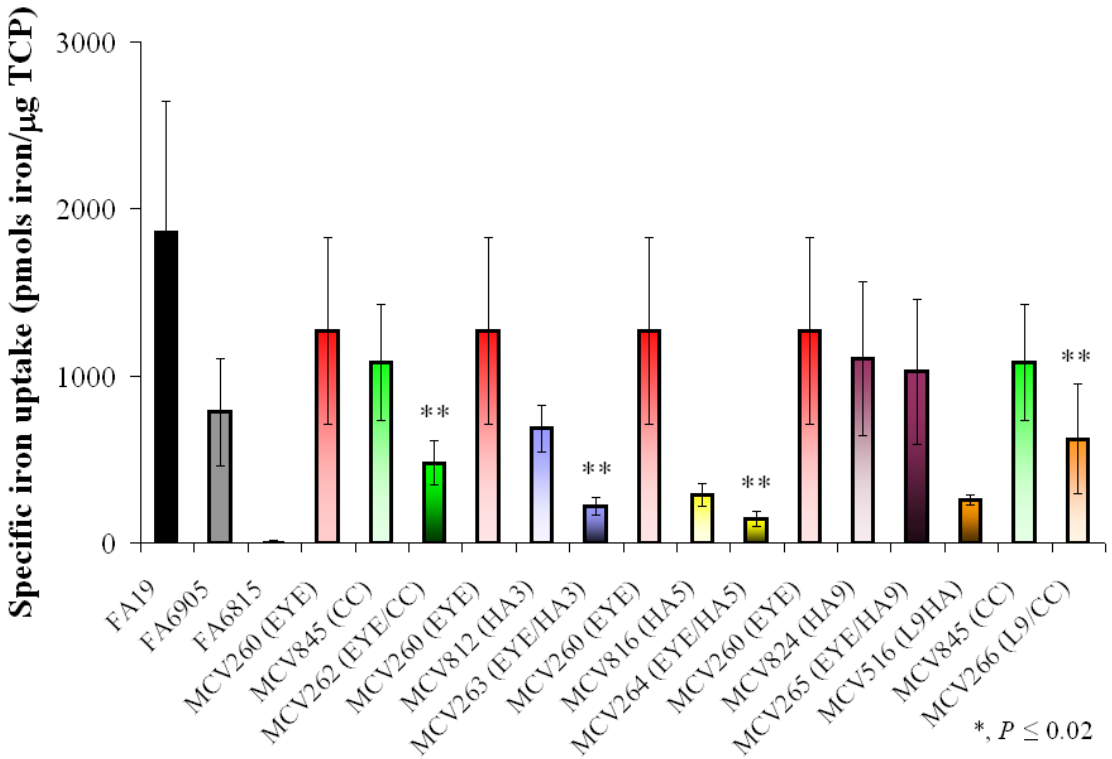
Table 9. Phenotypes of TbpA and TbpB combined mutants in transferrin-iron utilization growth assays

Strain	Phenotype	Growth phenotype	Reference
	Controls		
FA19	TbpA+, TbpB+	+	This study
FA6905	TbpA+, TbpB–	+	This study
FA6747	TbpA–, TbpB+	–	This study
FA6815	TbpA–, TbpB–	–	This study
	Single mutations		
MCV260	TbpA $EYE_{120}AAA$, TbpB+	+	This study (160)
MCV261	TbpA $EYE_{120}AAA$, TbpB–	–	This study (160)
MCV845	TbpA+, TbpB $CC_{481}AA$	+	This study (56)
MCV812	TbpA+, TbpB $HA3_{175}$	+	This study (57)
MCV816	TbpA+, TbpB $HA5_{327}$	+	This study (57)
MCV824	TbpA+, TbpB $HA9_{660}$	+	This study (57)
MCV515	TbpA $L9HA_{750}$, TbpB+	+	This study (243)
MCV516	TbpA $L9HA_{750}$, TbpB–	–	This study (243)
MCV519	TbpA $L11HA_{843}$, TbpB+	+	(243)
MCV520	TbpA $L11HA_{843}$, TbpB–	–	(243)
MCV527	TbpA $L2HA_{229}$, TbpB+	+	(243)
MCV528	TbpA $L2HA_{229}$, TbpB–	–	(243)
	Combined mutations		
MCV262	TbpA $EYE_{120}AAA$, TbpB $CC_{481}AA$	+	This study (DeRocco unpublished)
MCV263	TbpA $EYE_{120}AAA$, TbpB $HA3_{175}$	+	This study

			(DeRocco unpublished)
MCV264	TbpA $EYE_{120}AAA$, TbpB $HA5_{327}$	–	This study
			(DeRocco unpublished)
MCV265	TbpA $EYE_{120}AAA$, TbpB $HA9_{660}$	+	This study
			(DeRocco unpublished)
MCV266	TbpA $L9HA_{750}$, TbpB $CC_{481}AA$	+	This study
			(DeRocco unpublished)
MCV828	TbpA $L9HA_{750}$, TbpB $HA3_{175}$	+	(57)
MCV830	TbpA $L9HA_{750}$, TbpB $HA5_{327}$	–	(57)
MCV833	TbpA $L9HA_{750}$, TbpB $HA8_{607}$	–	(57)
MCV834	TbpA $L9HA_{750}$, TbpB $HA9_{660}$	+	(57)

internalization, we observed that specific domains of TbpB were required for compensation of some TbpA mutants, but not others. Figure 24 shows the relative levels of iron uptake by all the single and combined mutants. Both TbpA mutations ($EYE_{120}AAA$ and L9HA) were combined with MCV845 ($CC_{481}AA$). Interestingly, MCV262 (EYE/CC) resulted in a decrease in iron internalization that was significantly greater than the additive effects of either single mutation alone ($P \leq 0.02$). However, MCV266 (L9/CC) did not exhibit a significant decrease in the efficiency of iron uptake when compared to each single mutation in isolation. In contrast, the TbpB $CC_{481}AA$ mutation actually increased TbpA (L9HA)-mediated iron internalization (MCV266 – L9/CC). Therefore, the double cysteine residues in TbpB are required for compensatory function in MCV260 ($EYE_{120}AAA$), whereas, they play no role in compensation for the MCV515 (L9HA). These data support the hypothesis that these TbpA mutants have different defects and specific domains of TbpB are required for different compensatory functions. This is also supported by the data seen in comparing MCV262 (EYE/CC) and MCV265 (EYE/HA9). Single mutants MCV845 ($CC_{481}AA$) and MCV824 (HA9) have identical phenotypes; however, when these mutations are combined with the MCV260 ($EYE_{120}AAA$), they exhibit different levels of TbpB-mediated compensation. Despite the fact that these mutations in TbpB cause identical defects, the compensatory functions differ, such that the $CC_{481}AA$ mutation in TbpB does not allow for compensation of the $EYE_{120}AAA$ mutation in TbpA (MCV262 – EYE/CC), but the HA9 mutation in TbpB does (MCV265 – L9/CC). Although the specific mechanism by which TbpB compensates for TbpA defects has not been determined, we have identified specific

Figure 24. Comparative analysis of transferrin-iron uptake by single and combined TbpA and TbpB mutants. Iron-stressed gonococci were incubated with ^{55}Fe-labeled human transferrin. Specific iron uptake was measured in picomoles (pmols) of iron internalized after 30 minutes. Each bar represents the mean of at least six independent experiments and is labeled according to the strain name with mutations in parentheses. Controls include FA19 (positive control, TbpA+ TbpB+), FA6905 (TbpA+ TbpB–), and FA6815 (negative control, TbpA– TbpB–). Standard deviations are represented by error bars. *, $P \leq 0.02$ indicates significance from both single mutants.


Specific iron uptake (pmols iron/μg TCP)
3000
2000
1000
0
**
**
**
**
FA19
FA6905
FA6815
MCV260 (EYE)
MCV845 (CC)
MCV262 (EYE/CC)
MCV260 (EYE)
MCV812 (HA3)
MCV263 (EYE/HA3)
MCV260 (EYE)
MCV816 (HA5)
MCV264 (EYE/HA5)
MCV260 (EYE)
MCV824 (HA9)
MCV265 (EYE/HA9)
MCV516 (L9HA)
MCV845 (CC)
MCV266 (L9/CC)
*, $P \leq 0.02$

regions of TbpB that are critical for TbpA compensation.

III. Discussion

Transferrin-iron acquisition by *Neisseria gonorrhoeae* is mediated by the transferrin binding proteins, TbpA and TbpB. In the wild-type gonococcus, TbpA is required for transferrin-iron acquisition, while TbpB is not essential (4). However, there have been a number of TbpA mutants that require TbpB for transferrin utilization (160, 243), suggesting that TbpB can compensate for selected TbpA defects and plays an active role in the process of transferrin-iron acquisition. This study was designed to analyze the compensatory role of TbpB in transferrin-iron acquisition and to identify regions critical for its compensatory functions. Our approach involved the combination of various TbpB mutations with TbpA mutations, which resulted in TbpA that required TbpB for transferrin utilization. The results presented here suggest that combined mutagenesis does not significantly impact the structure or surface localization of the transferrin binding proteins. This study is in agreement with other studies in which gonococcal TbpA and TbpB were subjected to substitution (160), insertion (57, 243), deletion (22), and combined mutagenesis (57) and shows that the transferrin binding proteins are resilient to many types of mutagenesis.

All TbpA and TbpB combined mutations caused a significant decrease in transferrin-iron internalization by gonococcal mutant strains. Despite the decreased iron internalization, most combined mutants maintained the ability to utilize transferrin as a sole source of iron. Although unexpected, these data suggest that wild-type gonococci

acquire iron from transferrin at levels that exceed their need for growth under laboratory conditions, and mutants capable of acquiring iron at 12% of wild-type strains are still capable of growth in vitro. Analysis and quantitation of specific iron uptake by each mutant revealed subtle differences in phenotypes and compensatory functions attributable to TbpB. Full compensation of TbpA-transferrin utilization required the function of both high-affinity transferrin binding domains of TbpB. However, iron uptake data revealed different domains of TbpB that compensate for specific defects in TbpA. Specifically, the TbpB C-terminal double cysteines play a role in compensation for TbpA plug domain defects (MCV262 – EYE/CC), while they have no role in compensating for TbpA L9HA defects (MCV266 – L9/CC). Although MCV527 (L2HA) and MCV519 (L11HA) were not tested in these combined mutagenesis studies, we would hypothesize that they would be phenotypically similar to MCV515 (L9HA) in regard to TbpB compensation.

These findings support the hypothesis that the TbpA and TbpB interaction is complex and multiple domains of these proteins cooperate in transferrin-iron acquisition. Although the specific interaction between TbpA and TbpB is difficult to address in these studies, these proteins have been shown to interact in various other studies (63, 64, 97, 210). The major function provided by TbpB that is critical for TbpA compensation is the ability of the N-terminal and C-terminal domains to cooperate in high-affinity transferrin binding. If the N-terminal high-affinity binding domain is not functional as observed in MCV264 (EYE/HA5) and MCV830 (L9/HA5) (57), TbpB cannot compensate for TbpA. The same is true if C-terminal transferrin binding is disrupted (MCV833 – L9/HA8) (57). From these studies, another region of TbpB identified as important for compensation of

TbpA-defective mutants is the two highly conserved C-terminal cysteine residues. These double cysteines are conserved within TbpB among many bacterial pathogens (57), suggesting their importance in transferrin-mediated iron acquisition. It has been shown that cysteines in close proximity can coordinate metal ions (105) and even solubilize insoluble metal complexes in solution (118). In wild-type TbpB, it is possible that the double cysteines function in iron coordination or even in solubilizing iron, which may contribute to the compensatory function observed with MCV260 ($EYE_{120}AAA$). Therefore, these double cysteines in TbpB may also contribute to the increased efficiency provided by TbpB in the wild-type transferrin-iron acquisition system. Furthermore, if the TbpA plug domain mutation ($EYE_{120}AAA$) prevents iron coordination and therefore iron removal and internalization, the double cysteines of TbpB may compensate for either of these functions. Although the specific function that the TbpB double cysteines play in the compensation of TbpA defects has not been determined, it is clear that they do contribute to the efficiency of transferrin-iron acquisition as well as TbpB compensatory function.

Overall these studies provide insight into the cooperation of TbpA and TbpB in the mechanism of transferrin-iron acquisition. However, future studies are necessary to determine the specific mechanism by which TbpB compensates for TbpA. This study has identified regions in TbpB important in the efficient functioning of the wild-type receptor, but also in TbpA compensation in defective receptors. Understanding the mechanism of transferrin utilization and the critical components of the transferrin binding

proteins could provide greater insight into development of novel antimicrobials as well as identification of new vaccine targets.

IV. Acknowledgements

Funding of this work was provided by Public Health Service grant ROI AI047141 from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health. I was supported by the Training in Molecular Pathogenesis grant (T32 AI07617) from the National Institutes of Health while carrying out this work. I would like to acknowledge and thank Amanda DeRocco for assistance in generation of combined TbpA and TbpB gonococcal mutants and initial characterization of strains MCV845 and MCV846.

CHAPTER 5 – ANALYSIS OF A TONB-INDEPENDENT MECHANISM OF TRANSFERRIN-IRON UTILIZATION BY *NEISSERIA GONORRHOEAE*

I. Introduction

The transferrin binding proteins (TbpA and TbpB) of *Neisseria gonorrhoeae* promote the efficient acquisition of iron from human transferrin. Characterization of various site-directed and insertion mutations in these receptors have aided the understanding of the mechanisms of transferrin-iron acquisition (22, 57, 126, 160, 243). TbpA mutants that require TbpB for transferrin utilization have been discussed in the previous chapters. These phenotypes highlight the importance of TbpB in the process of transferrin-iron acquisition. Additionally, there are a number of TbpA mutants that are unable to utilize transferrin even in the presence of TbpB (243). A subset of these mutants regain the ability to utilize transferrin after 72 hours of incubation with human transferrin provided as the sole iron source. The two mutants that exhibited this phenotype contained HA epitope insertions within β-strand 16 (MCV523 – β16HA, TbpB+) and loop 3 (MCV511 – L3HA, TbpB+) of TbpA (242, 243). Both MCV511 (L3HA) and MCV523 (β16HA) expressed wild-type levels of TbpA (243). MCV511 (L3HA) but not MCV523 (β16HA) expressed the HA epitope on the gonococcal cell surface, indicating that loop 3 was surface exposed, while β-strand 16 was not (243).

TbpA of MCV511 (L3HA) was unable to bind human transferrin, while MCV523 (β16HA) bound transferrin with wild-type affinity and capacity (243). Both of these mutants were unable to utilize transferrin in either FA19 (TbpB+) (MCV511 and MCV523) or FA6905 (TbpB–) (MCV512 and MCV524) backgrounds (243). However, upon 72 hours of incubation, both of these mutants in the FA19 background regained the ability to utilize transferrin as a sole iron source. This reversion was specific to MCV511 (L3HA) and MCV523 (β16HA) as it did not occur in other mutants that were unable to utilize transferrin. Furthermore, this phenotype was not transient, but allowed for continued use of transferrin as the sole iron source over multiple generations.

The objective of this study was to determine the mechanism by which these mutants reverted to a transferrin-positive growth phenotype. It is important to consider this reversion mechanism in looking at TbpA and TbpB as vaccine candidates. It has been shown that TbpA and TbpB are expressed by all clinical isolates of *N. gonorrhoeae* (138), which implicates their importance in virulence. In addition, the gonococcal transferrin-iron acquisition system is required to initiate infection in human challenge experiments (49). Thus far, the components of the transferrin utilization system, TbpA and TbpB, of *N. gonorrhoeae* have been identified as possible vaccine candidates. However, if *Neisseria* have the ability to utilize transferrin through an alternative mechanism this has negative implications for the vaccine potential of TbpA and TbpB.

II. Results

A. MCV511 and MCV523 revert to a transferrin-positive growth phenotype at a high frequency and only in the presence of TbpB

The frequency of spontaneous base pair changes that result in a reversion event is approximately 10^{-7} to 10^{-8} (174). Quantifying the frequency with which MCV511 (L3HA) and MCV523 (β16HA) reverted to a transferrin-positive growth phenotype was complicated by the fact that colonies continued to arise with increasing incubation time. However, the rate of reversion for these strains, which was calculated to be around 10^{-5}, was considerably higher than the rate of spontaneous reversion. These isolates were designated "pseudo-revertants" because the frequency with which they occur was higher than that expected from a typical mutational reversion. However, the rate with which MCV523 (β16HA) reverted to a transferrin-positive growth phenotype was slower than that of MCV511 (L3HA), suggesting that these events occur through different mechanisms. Interestingly, pseudo-reversion to a transferrin-positive growth phenotype only occurred in the FA19 (TbpB+) background, as MCV512 (L3HA, TbpB–) and MCV524 (β16HA, TbpB–) were never able to utilize transferrin as a sole iron source. Based on these findings, we conclude that reversion does not occur through spontaneous base pair changes and occurs in a TbpB-dependent manner. Since MCV511 (L3HA) and MCV523 (β16HA) likely revert via different mechanisms, the mechanism by which MCV511 (L3HA) reverted to a transferrin-positive growth phenotype was investigated as described below.

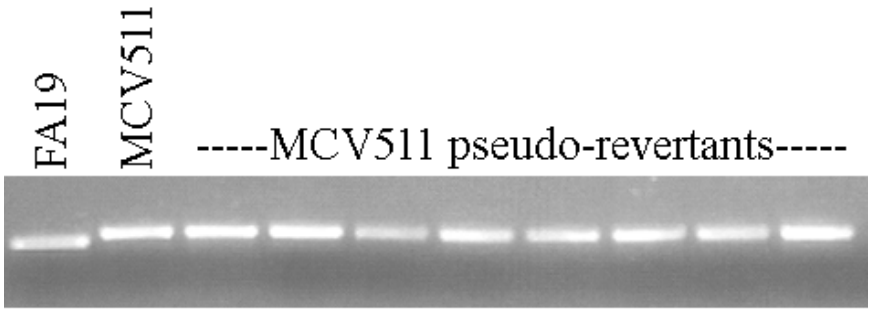
B. Pseudo-revertants retain HA-encoding epitope in *tbpA*

To ensure that MCV511 (L3HA) pseudo-reversion to a transferrin-positive growth phenotype did not occur through the loss of the HA-encoding epitope, PCR amplification of *tbpA* was carried out on a number of individually isolated pseudo-revertants (Figure 25). FA19 served as the negative control for it contains no HA-encoding epitope insertion. However, MCV511 (L3HA) and the various pseudo-revertants clearly retained the HA-encoding epitope as indicated by the increased DNA fragment size. Sequence analysis of *tbpA* from two individually isolated pseudo-revertants was also performed. Both PCR amplification and sequence analysis confirmed the presence of the HA-encoding epitope in these pseudo-revertant isolates. Therefore, the MCV511 (L3HA) pseudo-reversion to a transferrin-positive growth phenotype is not due to the loss of the HA-insertion and may occur through some other change.

C. Pseudo-revertants have no sequence changes within the *tbp* locus

Although the frequency of reversion was much higher than that expected for a spontaneous base pair change, sequence analysis of *tbpA* and *tbpB* was performed with MCV511 (L3HA) to determine if a sequence change caused the reversion to a transferrin-positive growth phenotype. Sequencing of the *tbpA* and *tbpB* coding regions was performed using a number of *tbpA*- and *tbpB*-specific primers listed in Table 4. Sequencing of two pseudo-revertants revealed no intragenic (*tbpA*) or intergenic (*tbpB*) sequence changes. Therefore, MCV511 (L3HA) pseudo-reversion to a transferrin-positive growth phenotype does not involve spontaneous sequence changes in the coding regions of either *tbpA* or *tbpB*.

Figure 25. HA-encoding epitope is present in *tbpA* of pseudo-revertants. *tbpA* was PCR amplified using *tbpA* specific oligonucleotides for detection of HA-encoding epitopes. The shift in size indicates the presence of the HA-encoding epitope in *tbpA*. Controls include MCV511 (positive control, L3HA) and FA19 (negative control).


FA19
MCV511
-----MCV511 pseudo-revertants-----

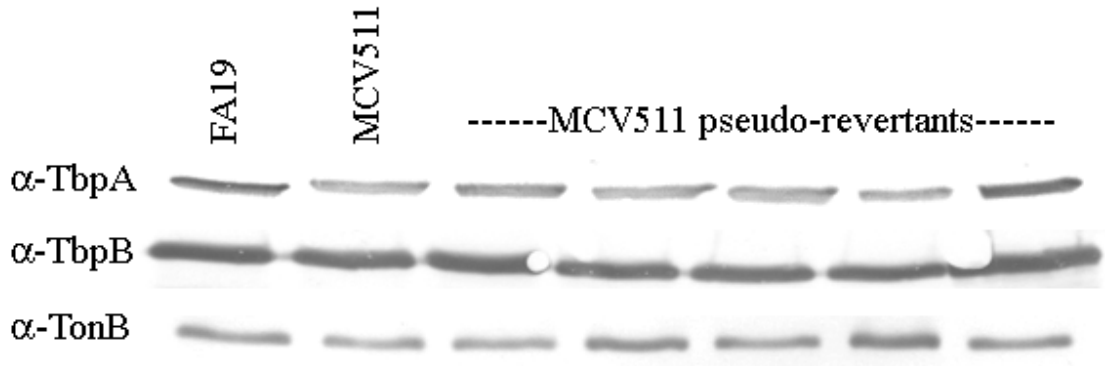
D. Pseudo-revertants express wild-type levels of TbpA, TbpB, and TonB

To determine if MCV511 (L3HA) reverted to a transferrin-positive growth phenotype through changes in protein expression levels, Western blot analyses were carried out. To assess changes in the transferrin-iron uptake system, antibodies were used for the detection of TbpA, TbpB, and TonB (Figure 26). FA19 and MCV511 (L3HA) were used as positive controls for wild-type expression levels of TbpA, TbpB, and TonB proteins. A number of MCV511 (L3HA) pseudo-revertants were analyzed by Western blot and all expressed wild-type levels of TbpA, TbpB, and TonB proteins. Therefore, MCV511 (L3HA) pseudo-reversion does not occur through sequence changes or any changes in the expression of TonB or the transferrin binding proteins. In addition, these data suggest that regulatory events that control protein expression of TbpA, TbpB, and TonB are not involved in the mechanism of MCV511 (L3HA) pseudo-reversion.

E. Pseudo-revertant growth is TbpA-dependent and TonB-independent

Since MCV511 (L3HA) pseudo-reversion occurred in a TbpB-dependent manner, we sought to determine if this mechanism was also dependent on TbpA and TonB. Therefore, TbpA and TonB mutants were constructed in a L3HA pseudo-revertant strain, MCV267. The TbpA knockout strain, MCV280, was generated through insertion of a mini transposon, encoding chloramphenicol resistance, into *tbpA* (*tbpA*::mTn3cat) (Table 2 and 3). Similarly, the TonB knockout strain, MCV281, was generated through the insertion of an omega cassette, encoding streptomycin resistance, into *tonB* (*tonB*::Ω) (Table 2 and 3). To assess if either TbpA or TonB were required for reversion to a

Figure 26. Pseudo-revertants express wild-type levels of TbpA, TbpB, and TonB by Western blot. Iron-stressed gonococci were lysed and standardized to a constant cell density. Whole-cell lysates were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Blots were probed with anti-TbpA (α-TbpA) polyclonal antibodies (top panel), anti-TbpB (α-TbpB) polyclonal antibodies (middle panel), or anti-TonB (α-TonB) polyclonal antibodies (bottom panel). Each lane is labeled according to the strain name. Controls include FA19 (positive control, TbpA+ TbpB+) and MCV511 (positive control, L3HA, TbpB+).


FA19
MCV511
------MCV511 pseudo-revertants------
α-TbpA
α-TbpB
α-TonB

transferrin-positive growth phenotype, MCV280 (TbpA–) and MCV281 (TonB–) were tested in transferrin-iron utilization growth assays and the phenotypes are listed in Table 10. This assay measures the ability of gonococcal strains to grow on CDM supplemented with 30% saturated human transferrin as the sole iron source. The ability to grow indicates an ability to utilize transferrin as a sole iron source and for this analysis measures whether pseudo-revertant growth was dependent on TbpA or TonB. Since MCV511 (L3HA) pseudo-reversion was dependent on TbpB, it was not surprising that pseudo-revertant growth was also dependent on TbpA. However, it was surprising that pseudo-revertant growth was TonB-independent (Table 10). These data suggest that MCV511 (L3HA) reverts to a transferrin-positive growth phenotype via a TbpB- and TbpA-dependent mechanism that bypasses the need for TonB-derived energy. This represents the first account of a novel TonB-independent mechanism of transferrin-mediated iron acquisition and suggests that *N. gonorrhoeae* has the ability to bypass TonB-dependent transferrin utilization.

F. Pseudo-revertants may release a soluble factor involved in transferrin utilization

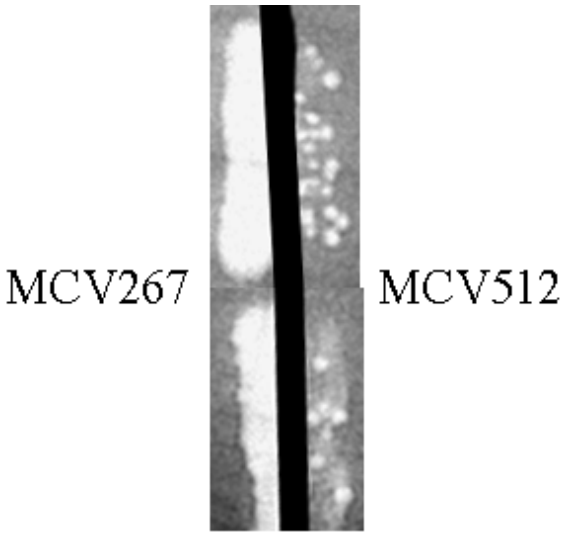
It has been previously shown that MCV267 (L3HA pseudo-revertant) has the ability to promote the growth of MCV512 (L3HA, TbpB–) (Yost-Daljev, unpublished data) (Figure 27). MCV512 (L3HA, TbpB–) was incapable of growth on CDM supplemented with 30% iron-saturated human transferrin as the sole source of iron (CDM-Tf) and was unable to revert, presumably due to the lack of TbpB. However, when MCV512 (L3HA, TbpB–) was plated on CDM-Tf in close proximity to MCV267

(L3HA pseudo-revertant), as shown in Figure 27, numerous colonies arose within 24 hours. The ability of MCV512 (L3HA, TbpB–) to utilize transferrin increased over time and colonies that arose were not pseudo-revertants in that the phenotype was not maintained following passage. Consequently, when MCV512 (L3HA, TbpB–) colonies were patched to CDM-Tf in the absence of MCV267 (L3HA pseudo-revertant), MCV512 was no longer able to grow, indicating that this strain did not retain the ability to utilize transferrin as a sole iron source. Therefore, the ability of MCV512 (L3HA, TbpB–) to utilize human transferrin was a transient phenotype, dependent upon the presence of MCV267 (L3HA pseudo-revertant) for growth. From these data, we conclude that MCV267 (L3HA pseudo-revertant) may secrete a soluble factor that allows MCV512 (L3HA, TbpB–) to transiently utilize human transferrin as a sole iron source.

G. Pseudo-revertant growth is PilQ- and T4SS-independent

Since the previous results suggest that MCV267 (L3HA pseudo-revertant) utilizes transferrin through a TonB-independent mechanism and may secrete a soluble iron chelating factor, we sought to determine the mechanism by which these events may occur. The mechanism of TbpA-dependent, TonB-independent transferrin utilization suggests that transferrin-derived iron is not transported through TbpA because TonB-derived energy would be required. Therefore, TbpA and TbpB are likely required for only transferrin binding, while another unidentified component participates in TonB-independent iron internalization. PilQ, the pore for pilus extrusion, or the type IV secretion system may be involved in this mechanism of TonB-independent transferrin-iron acquisition. It was previously shown in *N. gonorrhoeae* that PilQ served as a pore

Figure 27. Pseudo-revertants promote growth of MCV512 (L3HA, TbpB–) in transferrin-iron utilization growth assays. Gonococcal strains were patched on CDM agarose plates containing 30% iron-saturated human transferrin as a sole iron source. Strains are labeled according to the strain name. The thick black line indicates the boundary that separates MCV267 (L3HA pseudo-revertant) and MCV512 (L3HA, TbpB–). The bottom and top panels are representative of the results observed using different isolates of the L3HA pseudo-revertant, MCV267.


MCV267
MCV512

through which heme could be internalized (37); therefore, it is possible that PilQ may allow for transferrin-mediated iron uptake as well. In addition, the type IV secretion system (T4SS) of many pathogens has been shown to be important for the secretion of protein effectors and various other factors. Therefore, the neisserial T4SS may be involved in secretion of an iron chelating molecule or iron binding protein to allow for TonB-independent transferrin utilization. PilQ and various T4SS mutants were generated in wild-type FA19, MCV511 (L3HA), and MCV267 (L3HA pseudo-revertant) to assess if either the reversion event or pseudo-revertant growth were dependent on these systems for transferrin utilization (Table 10). In transferrin-iron utilization growth assays, MCV511 (L3HA) pseudo-reversion and MCV267 (L3HA pseudo-revertant) transferrin utilization were not dependent on either PilQ or the T4SS components. Therefore the mechanism of TbpA- and TbpB-dependent, TonB-independent transferrin-iron acquisition does not require the PilQ pore for iron internalization or the T4SS for release or uptake of possible soluble iron-chelating factors.

III. Discussion

N. gonorrhoeae does not produce any known siderophores and therefore utilizes high-affinity, TonB-dependent iron acquisition systems to acquire iron from host iron-binding proteins. The gonococcal transferrin-iron acquisition system is important for in vivo iron acquisition and has been linked to virulence, as it is required to initiate infection in the human host (49). This study represents the first account of a means by which *N. gonorrhoeae* bypasses TonB-dependent transferrin-iron acquisition. Interestingly, this

Table 10. Growth phenotypes of MCV511 (L3HA) and MCV267 (L3HA pseudo-revertant) derived mutants in transferrin-iron utilization assays

Strain	Phenotype	Growth phenotype
	Controls	
FA19	TbpA+, TbpB+	+
FA6905	TbpA+, TbpB– (Δ*tbpB*)	+
FA6815	TbpA–, TbpB– (*tbpB*::Ω)	–
	FA19 derived strains	
MCV269	TbpA+, TbpB+, TonB– (*tonB*::Ω)	–
MCV270	TbpA+, TbpB+, PilQ– (*pilQ*::Ω)	+
MCV271	TbpA+, TbpB+, DsbC– (*dsbC*::*ermC*)	+
MCV272	TbpA+, TbpB+, TraC– (*traC*::*ermC*)	+
MCV273	TbpA+, TbpB+, TraH– (*traH*::*ermC*)	+
MCV274	TbpA+, TbpB+, TraN– (*traN*::*ermC*)	+
MCV904	TbpA+, TbpB+, FbpA– (*fbpA*::*ermClacIP*) –RBS	–
MCV906	TbpA+, TbpB+, FbpA– (*fbpA*::*ermClacIP*)	+
	MCV511 (L3HA) derived strains	
MCV511	TbpA $L3HA_{343,}$ TbpB+	–
MCV512	TbpA $L3HA_{343,}$ TbpB– (Δ*tbpB*)	–
MCV275	TbpA $L3HA_{343,}$ TbpB+, PilQ– (*pilQ*::Ω)	+
MCV276	TbpA $L3HA_{343,}$ TbpB+, DsbC– (*dsbC*::*ermC*)	+
MCV277	TbpA $L3HA_{343,}$ TbpB+, TraC– (*traC*::*ermC*)	+
MCV278	TbpA $L3HA_{343,}$ TbpB+, TraH– (*traH*::*ermC*)	+
MCV279	TbpA $L3HA_{343,}$ TbpB+, TraN– (*traN*::*ermC*)	+
	MCV267 (L3HA revertant) derived strains	
MCV267	TbpA $L3HA_{343}$, TbpB+ revertant	+
MCV280	$L3HA_{343}$, TbpB+, TbpA– (*tbpA*::mTn3cat)	–
MCV281	TbpA $L3HA_{343}$, TonB– (*tonB*::Ω)	+
MCV282	TbpA $L3HA_{343}$, TbpB+, PilQ– (*pilQ*::Ω)	+
MCV283	TbpA $L3HA_{343}$, TbpB+, DsbC– (*dsbC*::*ermC*)	+
MCV284	TbpA $L3HA_{343}$, TbpB+, TraC– (*traC*::*ermC*)	+

MCV285	TbpA L3HA$_{343}$, TbpB+, TraH– (*traH*::*ermC*)	+
MCV286	TbpA L3HA$_{343}$, TbpB+, TraN– (*traN*::*ermC*)	+
MCV287	TbpA L3HA$_{343}$, TbpB+, FbpA– (*fbpA*::*ermClacIP*) –RBS	–
MCV288	TbpA L3HA$_{343}$, TbpB+, FbpA– (*fbpA*::*ermClacIP*)	+

TonB-independent mechanism of transferrin-iron utilization requires TbpA and TbpB. The wild-type transferrin-iron acquisition system functions in two major steps: transferrin binding and iron internalization. TonB provides the energy for iron internalization but is not necessary for transferrin binding. Therefore, in MCV267 (L3HA pseudo-revertant), TbpA and TbpB are likely only required for transferrin binding and are unlikely to play a role in iron uptake, for this process requires TonB-derived energy. However, HA epitope insertion into loop 3 of TbpA prevented transferrin binding (243). Therefore, it is likely that TbpA expressed by MCV267 (L3HA pseudo-revertant) is also unable to bind human transferrin. Thus, these data suggest that TbpA and TbpB form a complex that is required for transferrin utilization in a TonB-independent mechanism. As addressed in previous chapters, wild-type TbpA and TbpB association have been demonstrated (63, 64, 97, 210), but the extent of this complex interaction has not been characterized. It appears that even this TonB-independent mechanism requires the formation or association of the TbpA and TbpB complex.

Although most mechanisms of iron acquisition are dependent on TonB, there have been various TonB-independent mechanisms described. *N. gonorrhoeae* has been shown to bypass TonB-dependent, HpuAB-mediated heme acquisition from hemoglobin through a TonB-independent, PilQ-dependent mechanism (37). In addition, *N. gonorrhoeae* can utilize heme in a TonB-independent process (209). Furthermore, many mechanisms of xenosiderophore utilization have been shown to occur in a TonB-independent manner (74, 211). The TonB-independent mechanism of xenosiderophore utilization depended on gonococcal ferric binding protein A (FbpA) (211). FbpA has been characterized as a

periplasmic high-affinity iron binding protein, but has also been identified in gonococcal culture supernatants (211), which suggests that it may play a role in iron binding at the gonococcal cell surface. Thus, TonB-independent transferrin-iron utilization observed by MCV267 (L3HA pseudo-revertants) may involve an FbpA-dependent mechanism of acquiring iron from human transferrin. Furthermore, although the type IV secretion system (T4SS) does not appear to be involved, it is possible that TbpB may be released from the gonococcal cell surface and promote growth of MCV512 (L3HA, TbpB–) or TbpB itself may promote iron release from transferrin. In vitro, TbpB has been shown to promote the transfer of iron from human transferrin to FbpA, which suggests that TbpB has the ability to remove iron from transferrin, thus making it accessible (Crumbliss unpublished data). Additionally, iron removal from transferrin may occur independent of TbpB through an unidentified extracellular reductase. Although the specific mechanism by which FbpA or TbpB may promote transferrin-utilization has not been determined, it is of great importance to understand this bypass mechanism in the context of pathogenic mechanisms of iron acquisition.

IV. Acknowledgements

Funding of this work was provided by Public Health Service grant ROI AI047141 from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health. I was supported by the Training in Molecular Pathogenesis grant (T32 AI07617) from the National Institutes of Health while carrying out this work.

I would like to acknowledge Rob Nicholas at the University of North Carolina for kindly providing pUNCH290. Additionally, I would like to thank Holly Hamilton and Joe Dillard at the University of Wisconsin-Madison for kindly providing pHH17, pKS83, pJD1175, and pKS72 plasmids. Finally, I would also like thank Mary Kate Yost-Daljev and Cynthia Nau Cornelissen for preliminary work and assistance with pseudo-revertant isolation and characterization.

CHAPTER 6 – SUMMARY & PERSPECTIVES

The precise mechanism by which *Neisseria gonorrhoeae* acquires iron from human transferrin has not been elucidated, but these studies have provided greater insight into this process. It has been shown that transferrin-iron acquisition system is composed of two transferrin binding proteins, TbpA and TbpB. It has also been shown that the TonB-dependent, outer-membrane iron transporter, TbpA, is required for transferrin utilization, while the lipoprotein TbpB is not (4). Furthermore, it has been shown that TbpB makes the process of iron acquisition from human transferrin more efficient (4). TbpB is known to function in transferrin binding and discrimination (20, 50, 183, 184), but these roles are not solely responsible for increasing the efficiency of iron uptake through TbpA (57). The specific mechanism by which TbpA mediates iron internalization and the involvement of TbpB in this process has not yet been determined. The studies presented here were designed to address many of these questions and have aided in the understanding of this complex mechanism of transferrin-iron utilization by *N. gonorrhoeae*.

Models of TonB-dependent, transferrin-iron acquisition system have been proposed and resemble TonB-dependent siderophore transporters (28, 40, 42, 43, 119, 244). However, TonB-dependent transferrin-iron acquisition differs from that of characterized siderophore-iron acquisition for two reasons. Firstly, the siderophore

systems transport ferric-siderophore complexes, whereas the transferrin system transports only iron. Therefore the transferrin-iron acquisition system requires multiple steps in the process of iron internalization: (1) transferrin binding; (2) iron removal from transferrin; (3) iron transport across the outer membrane; and (4) apo-transferrin release. Therefore one objective of the studies presented here was to elucidate the mechanism by which the TbpA plug facilitates iron binding and uptake. The second major difference between siderophore transporters and the transferrin-iron acquisition system is the involvement of the transferrin binding lipoprotein, TbpB. Ferric-siderophore transporters do not possess a second component for iron acquisition. The studies presented here attempt to elucidate the involvement of TbpB in the process of transferrin-iron utilization. Taken together, these studies have allowed us to further characterize TbpA and TbpB in the mechanism of transferrin-iron utilization and have identified specific regions in both proteins that are required for efficient transferrin-mediated iron uptake by *Neisseria gonorrhoeae*. Additionally, a novel TonB-independent mechanism of transferrin-iron acquisition has been described.

Analogous to TonB-dependent transporters, TbpA consists of two distinct domains: a C-terminal β-barrel domain and an N-terminal, globular plug domain. Previous studies have suggested that the TbpA plug domain is critical in transferrin utilization (243); however, the specific contribution of the plug domain had not been shown up to this point. The analyses presented here confirm that the plug domain is critical for transferrin-iron acquisition and specifically functions in iron binding and uptake. Mutagenesis of specific iron-coordinating residues suggests that iron is

coordinated by the plug domain, and disruption of iron binding sites results in the inability of gonococci to internalize iron from human transferrin. Although these residues are critical for iron acquisition, we hypothesize that there are additional residues involved in this process of iron internalization. Similar to the mechanism described for TonB-dependent, BtuB-mediated vitamin B_{12} uptake (72), we hypothesize that TonB interaction with the TbpA plug domain facilitates unraveling of this domain. This partial denaturation of the globular, plug domain opens the iron binding site or sites and facilitates traversal of iron through the outer membrane. Through this process we propose that iron is coordinated at multiple sites and the sequential unraveling of the plug promotes the movement of iron from one coordination site to another until iron is passed to ferric binding protein A (FbpA) in the periplasm. This hypothesis is supported by the in vitro recombinant protein iron binding data, whereby the TbpA plug domain binds more iron than FbpA, which only has one iron coordination site, suggesting that the plug binds iron in more than one site. Furthermore, the TbpA plug structural model predicts that residues 120–122 (EYE) are positioned at the base of the plug, which supports the idea that iron is coordinated at multiple sites and unraveling of the plug domain allows for the traversal of iron through the β-barrel of TbpA.

Mutagenesis studies in siderophore transporters have shown that the N-terminal plug domain is expelled from the β-barrel during siderophore transport (121), while others have shown that it merely undergoes a rearrangement (36). If the plug domain is tethered to the β-barrel domain and therefore unable to undergo rearrangement, siderophore transport cannot occur (58). In the case of transferrin-iron acquisition, the

conformational change in the TbpA plug domain is critically important and we propose this allows for the sequential coordination of iron at multiple sites within the TbpA plug domain. In these studies we show that disruption of one potential iron coordination site within the TbpA plug domain prevents iron internalization from transferrin. This work has provided further insight into the mechanism of transferrin-iron internalization and represents the first study to demonstrate the involvement of the plug domain in iron binding. Further mutagenesis studies are necessary to identify additional iron coordination sites with the TbpA plug domain. In addition, iron binding studies will be performed with recombinant TbpA plug proteins encoding mutagenized iron-coordination sites, particularly at residues 120–122 (EYE). The controversy regarding conformational changes that occur within the plug domain needs to be addressed in the *Neisseria* transferrin-iron acquisition system. Furthermore, a crystal structure of full-length TbpA or the TbpA plug domain would greatly assist in characterizing the mechanism of transferrin-iron acquisition mediated by TbpA.

The data presented in this work show that TbpB is also important in the mechanism of iron acquisition from transferrin and new functional domains within this protein have been identified. It has been shown that TbpB specifically and independently binds human transferrin. This lipoprotein also has the ability to discriminate between apo- and holo-transferrin, with a preference for the iron-loaded form. TbpB increases the efficiency of iron acquisition, but the mechanism by which it does so has not been characterized. The studies presented here have identified a specific domain in TbpB that contributes to increasing the efficiency of transferrin-mediated iron acquisition. This C-

terminal double cysteine region may be important in destabilization and subsequent removal of iron from transferrin. It has been demonstrated that wild-type TbpB, in the absence of TbpA, can promote in vitro destabilization of human transferrin that results in iron release (Crumbliss unpublished data) and the double cysteines may play a role in this process. The ability of TbpB to preferentially bind holo-transferrin and the proposed ability to remove iron from transferrin may be the qualities that allow for increased efficiency in transferrin-mediated iron internalization. Further studies are necessary to identify the specific function of these residues and their contributions in transferrin-mediated iron acquisition by the wild-type, neisserial transferrin receptor of *N. gonorrhoeae*.

Combined mutagenesis of the neisserial transferrin binding proteins has revealed the complexity of interactions and cooperation between TbpA and TbpB in the process of transferrin-iron internalization. From these studies, it is clear that specific domains of TbpA and TbpB provide different functions in the internalization of iron from transferrin. The ability of TbpB to compensate for TbpA-defective mutants has allowed us the opportunity to investigate the domains of TbpB that share some functional redundancy with TbpA. The TbpA and TbpB combined mutagenesis presented here has provided insight into the compensatory functions provided by TbpB, and further investigation may reveal additional functional properties of TbpB.

Many studies, including those presented here, have revolved around defining the mechanism of TonB-dependent transport in Gram-negative bacteria. As described in the introduction, bacterial pathogens depend on a large number of TonB-dependent systems

for nutrient and iron acquisition during infection. TbpA and TbpB are the characterized neisserial transferrin binding proteins and mediate TonB-dependent iron acquisition from human transferrin. The studies presented here are the first to describe a TonB-independent mechanism of transferrin-iron uptake mediated by gonococcal TbpA and TbpB. The ability of gonococci to bypass the need for TonB-derived energy in transferrin-mediated iron transport is important in the context of all mechanisms of TonB-dependent transport utilized by *N. gonorrhoeae*. Furthermore, the ability of pathogenic bacteria to bypass TonB-dependent systems of iron acquisition is significant in understanding the evolution of bacterial pathogens as they continue to compete for iron within the human host.

Overall, investigation of the mechanism of transferrin-iron acquisition mediated by TbpA and TbpB is important because it represents a novel mechanism of TonB-dependent iron acquisition. Further investigation is necessary to fully characterize each step in this process of TonB-dependent transferrin-iron acquisition. Additionally, the novel mechanism of TonB-independent transferrin utilization needs to be further characterized and is currently being evaluated in regard to the contribution of FbpA in this process.

TbpA and TbpB are prospective vaccine candidates because these proteins are expressed by all gonococcal isolates (138). In addition TbpA and TbpB are not subject to phase or antigenic variation like many of the other virulence factors of *N. gonorrhoeae*. Most importantly, TbpA and TbpB are required to initiate infection in male volunteers (49). Therefore, characterization of TbpA and TbpB is important in looking at these

proteins as potential vaccine candidates. It has been shown in the closely related *N. meningitidis*, antibodies raised against the transferrin binding proteins are cross-reactive, bactericidal, and have the ability to block transferrin binding (53, 187, 233). Furthermore, it was recently shown that antibodies raised against recombinant gonococcal transferrin binding proteins are also cross-reactive and bactericidal (176, 177). Taken together these data suggest that the components of the neisserial transferrin-iron uptake system are promising candidates for an effective vaccine against *Neisseria* infections. Therefore identification of novel targets within TbpA and TbpB could aid in the future development of vaccines and new antimicrobials.

The plug domain of TbpA is of particular interest due to the important role it plays in transferrin-iron acquisition. This domain has been shown to be surface exposed (243) and antigenic. Additionally, it is highly conserved among bacterial pathogens, expressing the transferrin binding proteins. Thus, the plug domain of TbpA could serve as a new target for antimicrobial or vaccine development, and due to the high conservation of the plug domain, treatments may be cross protective for a number of bacterial pathogens.

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VITA

Jennifer McMillan Noto was born on August 12th, 1981 in Cincinnati, Ohio. She graduated from Midlothian High School in Richmond, Virginia in 1999. In 2003, she graduated from Mary Washington College, now called the University of Mary Washington with a Bachelors of Science in Biology. Starting in 2003, she began working on her graduate degree at Virginia Commonwealth University in the Department of Microbiology and Immunology. Publications and accomplishments are listed below.

PUBLICATIONS

Noto, J. M. and C. N. Cornelissen. 2008. Identification of TbpA residues required for transferrin-iron utilization in *Neisseria gonorrhoeae*. Infect. Immun. **76**: 1960-1969.

ABSTRACTS AND PRESENTATIONS

Jennifer L. McMillan and Cynthia N. Cornelissen. 2008. Identification of TbpA residues required for transferrin-iron utilization by *Neisseria gonorrhoeae*. 108th General ASM Meeting, Boston, Massachusetts.

Jennifer L. McMillan and Cynthia N. Cornelissen. 2007. Identification of TbpA residues required for transferrin-iron utilization by *Neisseria gonorrhoeae*. Virginia ASM, Richmond, Virginia. University of Richmond.

Jennifer L. McMillan and Cynthia N. Cornelissen. 2007. Analysis of the TbpA plug domain of *Neisseria gonorrhoeae*. ASM Kadner Institute, Boulder, Colorado. University of Colorado at Boulder.

Jennifer L. McMillan and Cynthia N. Cornelissen. 2007. Analysis of the TbpA plug domain of *Neisseria gonorrhoeae*. 35th Annual John C. Forbes Graduate Student Honors Colloquium, Richmond, Virginia. Virginia Commonwealth University.

Jennifer L. McMillan and Cynthia N. Cornelissen. 2007. Analysis of the TbpA plug domain of *Neisseria gonorrhoeae*. 3rd Annual Women's Health Research Day, Richmond, Virginia. Virginia Commonwealth University.

Jennifer L. McMillan and Cynthia N. Cornelissen. 2007. Analysis of the TbpA plug domain of *Neisseria gonorrhoeae*. 10th Annual Graduate Student Association Research Symposium, Richmond, Virginia. Virginia Commonwealth University.

Jennifer L. McMillan and Cynthia N. Cornelissen. 2007. Analysis of the TbpA plug domain of *Neisseria gonorrhoeae*. 4th Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, Virginia.

AWARDS AND HONORS

Preparing Future Faculty Program Certificate December 2008
Virginia Commonwealth University School of Graduate Studies, Richmond, Virginia

Virginia ASM Best Student Presentation November 9-10th, 2007
University of Richmond, Richmond, Virginia

Graduate Student Association Travel Grant Award April 2007
Virginia Commonwealth University School of Graduate Studies, Richmond, Virginia

Travel Grant February 2007
4th Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, Virginia

Public Health Services Training Grant September 2006 – 2008
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