2013

Characterization of potential interactions between transferrin binding proteins in Neisseria gonorrhoeae

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CHARACTERIZATION OF POTENTIAL INTERACTIONS BETWEEN TRANSFERRIN BINDING PROTEINS IN *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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February 2013

Acknowledgements

I would sincerely like to thank my advisor, Dr. Cynthia Nau Cornelissen. Her guidance, patience and professionalism have helped me throughout my time in her laboratory. She has truly led by example with her enthusiasm for science and outstanding work ethic towards the teaching and development of future scientists. Thank you Dr. C for the opportunity to train under your supervision, for supporting me and providing a strong foundation for my future career.

I thank my committee members: Dr. Peterson, Dr. Kitten, Dr. Barbour, and Dr. Jefferson. Their advice, suggestions and discussions enhanced my research and enabled me to view my research through different perspectives. They all have been a constant source of support and encouragement on my committee.

The members of the Cornelissen lab: Aminat Oki, Abena Watson-Siriboe, Sophonie Jean, Devin Cash, Angie Claxton, and Jeremy Cornelissen, a big thank you to all of you. Thanks for adding cheer in lab, for the tremendous encouragement and for always being there with a helping hand. I would like to thank past lab members: Dr. Amanda DeRocco and Dr. Jennifer Noto for their help towards my scientific projects; Heather Strange, Dr. Rose Velez, Dr. Aimee Hollander, Dr. Karen Ostberg, Tyler Thomas and Meredith Weck for their intellectual support and friendships. Life in lab would not be same without you all and I will cherish the friendship I shared with each one of you.

To all my friends-in the lab, across the lab, in the department, in Richmond, around the world and back home: thank you for being with me and providing laughter and fun times. Your presence in my life has made it a lot easier to travel through the challenges and difficult times.

I dedicate this accomplishment to my dad and mom, Dilip and Bharati Mistry and my brother Anuj Mistry. To my parents, thank you for encouraging me to pursue my dreams and allowing me to fly to new horizons. I am deeply grateful for the trust you put in me and the love you shower on me. To my brother, Anuj Mistry, thank you for believing in me more than I believe in myself, for being my best friend and my pillar of support. I hope that I have made you all proud.

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List of Abbreviations

^{o}C	degrees Celsius
Δ	delta, deletion
Ω	omega
A	alpha or anti
ABC	ATP binding cassette
AP	alkaline phosphatase
ATP	adenosine triphosphate
B	beta
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
CO_2	carbon dioxide

DGI	disseminated gonococcal infection
DNA	deoxyribonucleic acid
E.	*Escherichia*
ECL	enhanced chemiluminescence
Fbp	ferric binding protein
Fe	iron
$FeCl_3$	ferric chloride
$Fe(NO_3)$	ferric nitrate
Fur	ferric uptake regulator
GCB	gonococcal growth media
GCU	gonococcal uptake sequence
H.	*Haemophilus*
HA	hemagglutinin
Hb	hemoglobin
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSPG	heparin sulfate proteoglycan

HS TBS	high salt Tris-buffered saline
IgA	immunoglobulin A
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl-beta-D-thiogalactopyranoside
K.	*Klebsiella*
kDa	kiloDalton
L	liter
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

mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimolar
MS	Mass Spectrometry
N.	*Neisseria*
$NaHCO_3$	sodium bicarbonate
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 lymphocyte, neutrophil

PCR	polymerase chain reaction
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T4SS	type IV secretion system
Tbp	transferrin binding protein
TCP	total cellular protein
TMP	total membrane preparation
Tf	transferrin
Tween 20	polyoxythylene sorbitan monolaurate
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
WT	wild-type

Abstract

CHARACTERIZATION OF POTENTIAL INTERACTIONS BETWEEN TRANSFERRIN BINDING PROTEINS IN *NEISSERIA GONORRHOEAE*

By Shreni Dilipkumar Mistry

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2013

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Neisseria gonorrhoeae requires iron for survival and establishment of infection in the human host. Pathogenic *Neisseriae* have evolved a repertoire of high-affinity iron acquisition systems to facilitate iron uptake in the human host. This requires specific outer membrane receptors and energy-harnessing cytoplasmic membrane proteins. The transferrin receptor proteins of *Neisseria gonorrhoeae* are necessary for iron uptake from transferrin in the host. The iron uptake system consists of two transferrin binding proteins, (Tbp) A and B. TbpA is an

integral outer membrane, TonB-dependent transporter that forms the pore for iron internalization. TbpB is a surface exposed lipoprotein that makes the iron internalization process more efficient.

TbpA is proposed to consist of two distinct domains: a β barrel and an N-terminal plug domain. Previous studies have shown that the EIEYE sequence in the TbpA plug domain plays an important role in iron internalization. We undertook a collaborative project to test the hypothesis that the conserved EIEYE sequence in the wild-type TbpA plug binds Fe^{3+} during the outer membrane iron transport process. CD spectra analysis and fluorescence emission titration spectra of purified recombinant wildtype and mutated plug proteins revealed that Fe^{3+} is sequestered by the wildtype TbpA plug protein, unlike the mutated plug protein. Modeling data with the wild-type plug predicts the EIEYE sequence is part of a flexible loop structure and acts as an Fe^{3+} binding site.

Characterization of the Tbps constituting the gonococcal receptor is important to understanding how the gonococcus survives within its host. TbpA and TbpB act together to acquire iron from human transferrin. We hypothesize that the presence of TbpA impacts the exposure or conformation of TbpB. In this study, we have utilized photoactivable cross-linkers to assess the effect of TbpA on TbpB in live gonococcal cells and studied it in presence of ligand and TonB derived energy. We employed insertion mutants, in which TbpA and TbpB contained the hemagglutinin (HA) epitope tag, to probe for impact of TbpA on TbpB. Our results demonstrate that photo-cross-linking altered TbpB size and migration and was dependent on the presence of TbpA. HA epitope insertion mutants in surface exposed loops of TbpA and TbpB did not impact the mobility of cross-linked TbpB. Addition of human transferrin to the de-energized

mutant caused a change in TbpB migration after cross-linking. This result indicates that when ligand is bound tightly and irreversibly to de-energized TbpA, the surface accessibility and perhaps conformation of TbpB is altered and TbpA does not interact with TbpB. Our findings were confirmed with recent structural studies of TbpA-TbpB-ligand triple complex, which illustrate that Tbps bind ligand through unique, non-overlapping binding sites such that TbpA and TbpB do not interact.

TbpB is not an essential member of transferrin-iron acquisition pathway. It is surface exposed and tethered to the outer membrane by a lipid moiety. The role of TbpB has not been clearly outlined in the transferrin iron acquisition system. The last objective of this study was to look at the significance of two specific conserved regions of TbpB and its importance in transferrin iron utilization and TbpA-TbpB interaction. Using site-directed mutagenesis we created two mutants, in the first mutant the conserved lipobox of TbpB was replaced with a signal I peptidase cleavage site, and the second mutant contained a deletion of the conserved poly glycine residue stretch, immediately downstream of the lipobox. Our results indicate that lipobox is required for lipidation of TbpB, both the mutants were impaired for transferrin-iron utilization, and neither of the mutations altered TbpA-TbpB interaction.

Overall, these studies help elucidate the functional importance of the specific regions in TbpA and TbpB in *Neisseria gonorrhoeae*, thereby adding to our understanding of the process of iron acquisition through the transferrin binding proteins**.**

CHAPTER 1: INTRODUCTION

I. The family *Neisseriaceae*

Historically, the family *Neisseriaceae* is comprised of four genera *Neisseria*, *Moraxella*, *Kingella*, and *Acinetobacter* (21, 101, 219, 220). These four genera are classified as Gram-negative and differentiated from each other based on cell morphology, presence of oxidase, catalase, thymidine kinase, carbonic anhydrase, acid production from glucose, and nitrite reduction (Table 1). The microorganisms included in these four genera are medically significant to humans as pathogens and or commensals.

II. The Genus *Neisseria*

Albert Neisser discovered *Neisseria gonorrhoeae* in 1897 (219) and the genus *Neisseria* was named after him (219). Following this discovery, *N. meningitidis* was isolated by Weischelbaum in 1887, *N. sicca*, *N. mucosa*, and *N. subflava* were isolated by Ligenlsheim in 1906, and *N. flavescens* was discovered by Branhman in 1930 (219).

The *Neisseria* species are Gram-negative, non-motile, non-spore forming cocci, found in pairs. Depending on the species, age of cultures, and source of the isolate, individual cells of *Neisseria* may range from 0.6 to 1.5 μm in size. These are fastidious microorganisms, requiring complex growth media. They are aerobic or facultative aerobic and grow best between 35-37^{o}C in a humidified atmosphere of 5-10% CO_2. *Neisseria* species are differentiated on the biochemical basis of production

Table 1. Characteristics of the genera included in the Family *Neisseriaceae*

Characteristics	Genus			
	Neisseria	*Kingella*	*Moraxella*	*Acinetobacter*
Cell Morphology	Diplococci*	Cocobacilli	Rods	Rods
Oxidase	+	+	+	-
Catalase	+	-	+	-
Acid production from Glucose	+	+	-	+/-
Nitrite reduction	+	+	-	-

*exception is *N. elongata*, it is rod-shaped

This table has been adapted from CDC (38)

of acid from carbohydrates and nitrite reduction. This genus includes species that are commensals or human pathogens, as illustrated in Table 2. Most of these *Neisseria* species are found in the mucous membranes of the respiratory and urogenital tracts.

The *Neisseria* species can be divided into two broad groups; the pathogenic and the non-pathogenic. The two important human pathogens are *Neisseria gonorrhoeae* and *Neisseria meningitidis*. These two pathogens are closely related genetically (209), despite expressing some of the same virulence factors, the diseases caused by them are different. *Neisseria meningitidis* is the causative agent of bacterial meningitis and severe sepsis, and *Neisseria gonorrhoeae* is the principle agent of the sexually transmitted infection, gonorrhea.

III. Diseases caused by *Neisseria* species

A. Meningococcal Disease- Infection and Epidemiology

Meningococcal disease can occur as epidemic or endemic cases. *N. meningitidis* serogroup A has caused major epidemics of meningitis in Africa with periodic epidemics occurring in the Sub-Saharan African region, especially in the 'meningitis belt' (200). *Neisseria meningitidis* is a common commensal, isolated from the nasopharynx of 8–20% of healthy individuals (92, 200). The duration of carriage is variable (approximately 4 months), with differences being observed between different *N. meningitidis* serogroups (108). The carriage strains are more widespread in nature than disease-causing strains and *N. meningitidis* is an occasional pathogen, as sometimes during the bacteria-host interaction meningococcal disease is

Table 2: Characteristics of Human-Associated *Neisseria* Species

Species	Acid production from					Polysaccharide from Glucose	Reduction of Nitrate	Association with Humans
	Glucose	Maltose	Sucrose	Fructose	Lactose			
N. gonorrhoeae	Y	N	N	N	N	N	N	Pathogen
N. meningitidis	Y	Y	N	N	N	N	N	I
N. lactamica	Y	Y	N	N	Y	N	N	Commensal
N. cinerea	N	N	N	N	N	N	N	Commensal
N. polysaccharea	Y	Y	N	N	N	Y	N	Commensal
N. subflava	Y	Y	N	N	N	N	N	Commensal
N. sicca	Y	Y	Y	Y	N	Y	N	Commensal
N. mucosa	Y	Y	Y	Y	N	Y	Y	Commensal
N. flavescens	N	N	N	N	N	Y	N	II
N. elongata	N	N	N	N	N	N	N	Commensal

I) *N. meningitidis* occurs in carrier state, with some serogroups causing epidemics/pandemics
II) *N. flavescens* was isolated from an outbreak of meningitis. There have been no reliable descriptions of isolation of this species, apart from the original description.

Table adapted from CDC (38)

an accidental outcome (208). Meningococcal disease incidence varies in human populations from rare to over 1000/100,000 population/year. Meningococcal disease occurs as sporadic cases, localized outbreaks and case clusters, and epidemic and pandemic disease (144).

Structural differences in capsular polysaccharide, lipo-oligoscaachride and outer membrane proteins help in characterizing and classifying the meningococci into different serogroups and serotypes. There are 13 diverse polysaccharide capsules but only A, B, C, W-135, and Y commonly cause invasive infections (78). The highest incidence of meningococcal disease occurs in the meningitis belt of sub-Saharan Africa. During epidemics, the incidence can approach 1000 cases per 100,000 population, or 1% of the population. Serogroup A has been the most important serogroup in this region. However, serogroup C and X mediated disease has also occurred (76, 78, 233).

In the Americas, the reported incidence of disease is in the range of 0.3-4 cases per 100,000 population, which is much lower than in the meningitis belt. The bulk of the disease in the Americas is caused by serogroups C and B, although serogroup Y causes a substantial proportion of infections in some countries and W-135 is becoming increasingly problematic as well (26, 40). The majority of meningococcal disease in European countries, which ranges in incidence from 0.2 to 14 cases per 100,000, is caused by serogroup B strains (201).

N. meningitidis is a frequent asymptomatic colonizer of the human upper respiratory tract, and is carried in the nasopharynx by 5-15% of healthy adults and

children and it may be acquired through the inhalation of respiratory droplets (26, 35, 213). The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells briefly before migrating back to the apical surfaces of the cells for transmission to a new host.

In susceptible individuals, once in the blood, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body and into the CSF (26, 146). Meningitis occurs when bacteria interact with the endothelial cells of the brain microvessels and cross the blood-brain barrier. Symptoms develop within 1–14 days of acquisition and include fever, nausea, headache, purpura, petechiae, photophobia, stiff neck, and change in mental status (221). Less common manifestations of meningococcal disease include septic arthritis, pneumonia, purulent pericarditis and conjunctivitis, among others (33, 202). Serious infections are usually manifested as meningitis and septicaemia (33, 237) Meningococcal sepsis, which is also called meningococcemia, occurs in only 5 to 20 percent of patients. Meningococcemia is characterized by an abrupt onset of fever and purpuric rash. (179). The overall case fatality rates over the past 20 years have been at 9 to 12 percent, with a rate of up to 40 percent among patients with meningococcal sepsis. Eleven to 19 percent of survivors sustain permanent sequelae including deafness and mental retardation (179).

The outcomes of meningococcal infection may be devastating and, in the absence of timely intervention, can lead to neurological disorders and death. For management of the disease the recommended treatment includes administration of antibiotics like penicillin or ceftriaxone (35, 179). Due to the high rates of morbidity and mortality

associated with meningococcal disease and increasing antibiotic resistance, for prevention control, two vaccines are available in the United States. They are (i) MCV4-a meningococcal conjugate vaccine, where cell surface polysaccharides from serogroups A, C, Y and W-135 are conjugated to diphtheria toxoid; and (ii) MPSV4-a meningococcal polysaccharide vaccine against serogroups A, C, Y, and W-135 (26). The ultimate control of meningococcal disease requires widespread surveillance, the expanded use of polysaccharide conjugate vaccines, and the development of broadly effective serogroup B vaccines to prevent this microorganism from posing as a major threat to human health in industrialized countries.

B. Gonococcal Disease- Infection and Epidemiology

Gonorrhoea is a global health concern and cause of serious reproductive complications in women. After chlamydia, gonorrhea is the second most commonly reported notifiable disease in the United States (36, 37). In 2011, a total of 321,849 cases of gonorrhea were reported in the United States, yielding a rate of 104.2 cases per 100,000 population, increasing the rate by 4.0% since 2010 (36). In reality these numbers are thought to be under-represented due to under-reporting and asymptomatic infection. In 2011, persons aged 15–44 years accounted for 94.6% of reported gonorrhea cases, with the highest rates observed among women and men aged 20-24 years. Gonorrhea rates were higher in women than in men and the African American community showed gonorrhea infection rates that were 4-5 times the national average with a rate of 427.3 cases per 100,000 population (33, 36).

N. gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhoea. *N. gonorrhoeae* is transmitted through close sexual contact with infected individuals, and vertical transmission occurs when the gonococcal infection is passed from mother to her newborn during childbirth. *N. gonorrhoeae* primarily infects the urethral epithelium in males (2, 46) and the ectocervial and endocervical epithelium in females (65). Secondary infections can occur in the rectum, conjunctiva and the pharynx (66). In men, symptoms typically begin 2-5 days after infection, characterized by purulent urethral discharge and dysuria. Secondary infections occur as bacteria ascend the upper genital tract to cause epididymitis, prostatitis, and urethritis. Approximately 10 days after infection, women infected with *N. gonorrhoeae*, experience cervicitis/urethritis, causing increased vaginal discharge, dysuria and menstrual abnormalities. (17, 87, 102, 196). It is estimated that as many as 80% of infected females are asymptomatic (197). This makes diagnosis difficult and leads to almost half of the infected females developing ascending gonococcal infections. If left untreated this can lead to serious downstream sequelae such as pelvic inflammatory disease (PID), resulting in scarring of the fallopian tubes, ectopic pregnancies, and infertility (149, 207). It is estimated that 10% of women suffer from PID (3, 66). Dissemination of the gonococci into the bloodstream results in disseminated gonococcal infection (DGI), though it is less common and occurs in 0.5-3% of infected individuals. DGI can cause arthritis-dermatitis syndrome, endocarditis, sepsis or meningitis in rare cases (66, 174, 197).

Gonorrhea treatment has been complicated by the ability of *N. gonorrhoeae* to develop resistance to antimicrobials used for treatment. Gonorrhea has progressively

developed resistance to the antibiotic drugs prescribed to treat it. In the past gonorrhoea was treatable with single-dose treatment with penicillin or streptomycin; however by 1958 resistance had been reported (156). By 1986, the CDC reported that over 22% of all isolates collected by gonococcal isolate surveillance project (GISP) were resistant to tetracycline and or penicillin (103). In 1993, fluoroquinolones and cephalosporins (ceftriaxone and cefixime) were the recommended treatments for gonorrhea. In the early 2000s ciprofloxacin resistance was detected among men who have sex with men (MSM) with gonorrhea. By 2006, 13.8% of isolates exhibited resistance to ciprofloxacin. On April 13, 2007, CDC stopped recommending fluoroquinolones as treatment for gonococcal infections for all persons in the United States (33, 34, 36). In 2011, 30.4% of isolates collected in GISP were resistant to penicillin, tetracycline, ciprofloxacin, or a combination of these antimicrobials. The effectiveness of cefixime for treating gonorrhea is declining, which has made CDC update its gonorrhea treatment guidelines. CDC no longer recommends the routine use of cefixime. CDC now recommends using ceftriaxone to treat gonorrhea (61). The first known failure of ceftriaxone and cefixime to successfully treat a case of gonorrhea reportedly occurred in Japan in 2003.The World Health Organization recently reported that a major public health crisis emerging, in the form of gonorrhea that doesn't respond to antibiotics. In 2009, the so-called gonorrhea "superbug" was found in Japan as well. It has been subsequently found at least once in Spain and France, but not yet in the U.S. (147, 214). Another cause for concern is the observation that gonococcal infection leads to increased transmission of the human immunodeficiency virus (HIV) (57, 72, 110). HIV transmission increases due

to the stimulation of viral HIV-LTR-dependent transcription by exposure to *Neisseria gonorrhoeae* (41). It has also been reported that in HIV infected individuals, concomitant infection with gonococci, leads to increased HIV shedding in genital secretions (45). These findings coupled with major rise in antibiotic resistance among *Neisseria gonorrhoeae* isolates make it critical to continuously monitor antibiotic resistance in *Neisseria gonorrhoeae* and encourage research, development and identification of alternative effective treatment regimens for gonococcal infections.

IV. Gonococcal Virulence Factors

For successful survival in the human host, gonococci express several cellular surface proteins that help in colonization and evasion of the host immune response. These surface proteins or virulence factors are subject to high frequency phase or antigenic variation, such that it offers several benefits to the bacterium. These virulence factors are discussed, to gain a greater understanding of the contribution of these virulence factors to successful gonococcal pathogenesis. A repertoire of virulence factors has been identified, which allow this bacterium to successfully adapt to variable microenvironments within its human host. This human adaptability and the repeated phase and antigenic change displayed by the gonococcus have hampered vaccine development. Many of the major virulence factors undergo phase and/or antigenic variation.

A. Porin

Porins are virulence proteins present in the outer membrane of the pathogenic *Neisseria* species, and are characterized by a trimeric β-barrel structure. Neisserial porins function by forming aqueous transmembrane

channels and they modulate the exchange of ions between the bacteria and the surrounding environment (66, 124). *N. meningitidis* expresses two types of porins, PorA and PorB. *N. gonorrhoeae* expresses only a PorB homolog; this is classified into two serotypes termed PIA and PIB (83). Gonococcal PorB is not subject to high frequency phase or antigenic variation (198), whereas meningococcal PorA is phase variable (216).

Gonococcal porin can associate with G-actin, affect the kinetics of actin filament formation, and cause remodeling of preformed actin filaments, suggesting a role for this protein in the rearrangement of the actin cytoskeleton that occurs during the early stages of the infective process (228). It has been reported that gonococcal PorB has the ability to translocate into the eukaryotic cell membranes and form voltage-gated channels (modulated by ATP and GTP); this promotes invasion of host cells (14, 66, 118, 226). The neutrophil expression of immunoglobulin G (IgG) F_c receptors II and III, as well as the activation-dependent down regulation of F_c gamma RIII, were reduced by the meningococcal and gonococcal porins; they also inhibited the phagocytic capacity of neutrophils (12, 139).

Gonococcal porins play an important role in enabling the bacteria to evade complement-mediated killing. The gonococcal porin PorB1A is critical in modulating stable serum resistance, which is not mediated by lipooligosaccharide (LOS) sialylation. Binding of factor H to PorB1A downregulates the alternative pathway of complement (124). It has been demonstrated that the *N. gonorrhoeae* porin PorB1B interacts with

mitochondria of HeLa cells and induces calcium efflux and caspase activity along with apoptosis (141, 142).

B. Lipooligosaccharide (LOS)

Most Gram-negative bacteria possess lipopolysaccharide (LPS) on their outer membrane. LPS of pathogenic *Neisseria* strains is comprised of a lipid A moiety, which anchors the molecule in the outer membrane, and a relatively short variable polysaccharide unit that lacks the repeating 0-antigens that are characteristic of the LPS of Gram-negative enteric bacteria (121, 218). The LOS undergoes high frequency antigenic variation. Glycosyl transferases add sugar residues to the core polysaccharide during LOS synthesis. These transferases contain poly G tracts within their genes, which enables them to undergo slip-strand mispairing, and resulting in the turning on or off of these genes. Altering the expression of these transferases leads to changes in LOS size and structure, due to variations in carbohydrate composition (27, 38, 58, 66, 90). LOS can also be modified using host derived cytidine 5'-mono-phospho-*N*-acetyleneuraminic acid (CMP-NANA) found in human sera and secretions. CMP-NANA is used as the sialyl donor and membrane bound sialyltransferase mediate the process (121, 122).

Neisseria are able to exhibit molecular mimicry through LOS, as they evade recognition as foreign by the host cells and affect cell function or even gain access to the cell interior (121). LOS sialylation makes it bind factor H, allowing the gonococcus to acquire serum resistance since complement

mediated killing via the alternate pathway is inhibited (167). Phase variation of LOS controls both bacterial entry into human mucosal cells, and bacterial susceptibility to killing by antibodies and complement (217). This reversible switch between cell-evasive to an immune-invasive phenotype helps the bacteria adapt to rapidly changing host environment. LOS oligosaccharide side chains terminate in epitopes that mimic sugar moieties of mammalian glycosphingolipids. This form of molecular mimicry not only provides the bacterium with a method of immune avoidance but also allows the bacterium to use host-derived molecules that normally associate with the mimicked structure.

C. Type IV Pilus

The *N. gonorrhoeae* pilus protein is one of the major antigenic determinants on the gonococcal cellular surface. Gonococcal pili are surface appendages composed of identical polypeptide subunits termed pilin, which polymerize to form linear structures, about 1,000-4,000 nm in length. The major structural subunit, the pilin or PilE protein, assembles into a helical pilus fiber and tip of the pilus is decorated with the adhesin, PilC (83).

The pilus is a highly variable structure on the gonococcal surface; the pilin protein undergoes both antigenic and phase variation. Phase variation occurs during DNA replication in a RecA-independent manner. The *pilE* gene has a poly C tract located at the beginning of the gene; during replication DNA polymerase can add/delete a C, thus causing frame-shifting and the gene to be

turned on or off (105, 240). Multiple *pil* loci exist within the genome, antigenic variation occurs when high frequency, enabling recombination to occur between expressed *pilE* gene with many silent *pilS* gene copies (129, 130). The *pilE* recombination event is non-reciprocal and RecA-dependent (77, 105, 189). Posttranslational modifications provide additional sources for PilE structural and functional diversity. Three distinct posttranslational modifications have been described; they include glycosylation (222), phosphoethanolamine modification (85), and phosphorylcholine modification (85, 227).

Several studies have suggested a role for PilE in receptor recognition, as different antigenic forms of PilE influence the adherence of *Neisseria* to epithelial cells and tissue tropism. Type IV pili of *Neisseria gonorrhoeae* and *Neisseria meningitidis* mediate the first contact to human mucosal epithelial cells, an interaction which is also critical for the interaction with vascular endothelial cells. Pili bind epithelial cell receptor molecules and thereby promote mucosal colonization. They also interact with polymorphonuclear leukocytes and macrophages (104, 181, 184). An important factor in the pathogenesis of gonorrhea may be the ability of pili to facilitate attachment of *N. gonorrhoeae* by overcoming the initial electrostatic repulsive barrier that exists between the bacterial cell and the host cell (82). Type IV pili (Tfp) mediate the movement of bacteria over surfaces without the use of flagella. These movements are known as twitching motility (224).

Human volunteer infection studies have suggested a possible correlation between maintenance of virulence in association with piliation status of *N. gonorrhoeae* (95). Previous studies have suggested the cell surface protein CD46 as a receptor for neisserial pili on human epithelial cells (93).

However, recent studies point out pilus-mediated gonococcal infection of epithelial cells can occur in a CD46-independent manner (98, 99), thus questioning the function of CD46 as an essential pilus receptor for pathogenic *Neisseriae.*

D. Opacity (Opa) proteins

The Opa (opacity) proteins are a family of antigenic- and phase-variable integral outer membrane proteins, found in the pathogenic *Neisseria*. According to the predicted protein structure, the proteins are comprised of eight membrane spanning domains with four surface- exposed loops (120). Opa proteins were originally identified by their ability to confer opacity and color changes to colonies of *N. gonorrhoeae* grown on translucent agar when they are viewed under diffused light with a stereomicroscope (66). It was demonstrated that the intravaginal inoculation of female mice with gonococci of an Opa^{-} phenotype results in their conversion to an Opa^{+} phenotype; these proteins are important for gonococcal survival and pathogenesis (91, 104).

Eleven *opa* genes are possessed by *Neisseria gonorrhoeae,* while *Neisseria meningitidis* only has four *opa* genes. The Opa proteins are subject to high frequency phase variation and express up to 0-4 Opas at a time (9, 13). The

changes in the number of poly CTCTT coding repeats (located in the signal-encoding region of the genes) leads to phase variation. Slipped-strand mispairing affects changes in the CTCTT repeats, resulting in translational frame shifts, so that multiple or one Opas are expressed at a time (91, 143).

The Opas mediate a tight secondary attachment with the epithelial cells of the urogenital mucosa; they also have two host cell receptors: heparin-sulfate proteoglycans (HSPG) and carcinoembryonic antigen-like molecules (CEACAMS) (44, 60, 75, 223). The interaction of Opa proteins with CEACAM, on host neutrophils leads to down regulation or death of host immune cells (44). When Opas interact with phagocytes, they trigger signaling cascade such that Src-family tyrosine kinases and small G-protein Rac1 are activated, thereby facilitating the intracellular accommodation and survival of gonococci in professional phagocytes (80, 195).

E. IgA Protease

IgA1 is the most abundant IgA subtype on human mucosal surfaces and is generated by mucosal lymphocytes. All pathogenic *Neisseria* secrete one of two closely related types of IgA1 proteases. The secreted enzyme cleaves human IgA1, but not IgA2. The IgA1 proteases are useful in cleaving IgA1 molecules to yield intact F_c and $F_{(ab)}$ fragments (140). Cleavage of intact IgA1 results in the loss of F_c-mediated secondary effector functions, such as inhibition of adherence and the retention of antigen-binding activity by the $F_{(ab)}$ fragments. It has been hypothesized that $F_{(ab)}$ fragments may simultaneously block the binding of intact

(functional) antibodies of IgA or other isotypes to bacterial epitopes and thereby inhibit complement activation and bacteriolysis (83).

The potential role(s) of the IgA1 proteases may extend beyond cleavage of IgA1. Human LAMP1 (hLAMP1), a major integral membrane glycoprotein of lysosomes, can be cleaved by IgA1 protease (4, 79) and thereby help in gonococcal transepithelial trafficking (88, 115) and inhibition of TNF-α-mediated apoptosis (6). Since IgA1 is thought to be the predominant antibody subclass protecting mucosal tissues against microorganisms, the cleavage of IgA1 by the neisserial enzyme might impair the local mucosal immune function (212).

F. Reduction Modifiable Protein (Rmp)

Reduction modifiable protein is a surface exposed outer membrane protein expressed by *Neisseria* species. This protein shows homology to OmpA of *E. coli* and after reduction and SDS-PAGE analysis the protein shows a shift in molecular weight, thereby earning its name (74). It is unique among the major outer membrane antigens of the gonococcus, as it differs from the other surface-exposed gonococcal outer membrane proteins by its high degree of intrastrain and interstrain homology in molecular weight, structure, and immunology (119). Rmp appears to be stably associated with porin and LOS, even after being subjected to harsh dissociating conditions (86, 125). It was shown that antibodies produced against Rmp inhibit the binding by anti-porin

and anti-LOS antibodies, thus reducing bactericidal activity by the serum. Rmp may act to promote infection in the host by *Neisseria* (15).

V. Iron availability in the human host

Iron is an essential element for most organisms, including bacteria, with the exception of the bacterial genera *Lactobacillus* and *Borrelia*. It is contained in many redox enzymes of the intermediary metabolism and in membrane-bound electron transport chains of respiratory systems and photosynthesis. Iron is perfectly suited for its role in electron transport chains because of the wide range of Fe^{2+}/Fe^{3+} redox potentials from −300 to +700 mV, depending on the iron ligands and the protein environment (25).

However, despite its abundance on earth, and the micromolar concentrations required for cell growth, it is biologically unavailable in most environments. In aerobic inorganic environments, iron is present essentially in the oxidized ferric form Fe^{3+}, which aggregates into insoluble oxy-hydroxide polymers. When reduced, the Fe^{2+} activates the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$), leading to the partial reduction of oxygen into hydroxyl radicals that are deleterious for most macromolecules as they damage DNA, lipids, and proteins (222). Protection against iron is achieved by iron sequestration into carrier proteins. To avoid Fenton chemistry, iron must be carefully chaperoned through the body by human serum transferrin (hTf) where it is solubilized and stabilized as Fe^{3+} or stored in ferritin. The various iron carrier proteins in the host are:

A. Transferrin

Transferrin (Tf), an iron transport protein, is found in serum, lymph and seminal fluids. It has an approximate molecular weight of 80kDa and is a bilobed glycoprotein (225, 134). Each lobe of Tf (N- and C- lobe) is capable of binding of two atoms of iron, and each lobe contains two domains. X-ray crystal structures reveal that the two similarly folded globular lobes are divided into two domains by a large cleft that carries the iron binding site (225). Tf is present in substantial levels (25–44 μM) in serum, but is only a minor component (0.2–1.3 μM) of mucosal secretions (185). In serum, the iron saturation of Tf is usually 30%, with iron primarily occupying the N-lobe, and the level of iron saturation can be reduced substantially during infection (239). Tf has both an iron transport function and a protective function against iron toxicity. Iron-bearing hTF in the blood binds tightly to the specific transferrin receptor (TFR). After undergoing endocytosis, acidification of the endosome initiates the release of $Fe^{(3+)}$. Iron-free hTF is recycled back to the cell surface and it is released to sequester more iron (117).

B. Lactoferrin

Lactoferrin (Lf) is a non-haem iron-binding protein that is found in mucosal secretions, including tears, saliva, vaginal fluids, semen and released by leucocytes at sites of inflammation (73). Lf exhibits an extremely high affinity constant for the FeIII ion (K_a -10^{20} M^{-1}) compared to affinity constant for FeII (10^3 M^{-1}). Lf is structurally very similar to Tf and is an 80 kDa

glycosylated protein, but differs in that it is capable of retaining iron under acidic conditions (pH < 6) (225). Lf is present at very low levels in serum (3.8–8.8 nM) but is a significant component of mucosal secretions (6–13 μM) (185). Lf plays a key role in maintaining cellular iron levels in the body and in Fe^{3+} homeostasis. The antibacterial activity of Lf is due to its ability to take up the Fe^{3+} ion, limiting use of this nutrient by bacteria at the infection site and inhibiting the growth of these microorganisms as well as the expression of their virulence factors. Lactoferrin is a modulator of the innate and acquired immune systems. Depending on the immune status of an individual, Lf can have anti-inflammatory properties that down regulate the immune response and prevent septic shock and damage to tissues (73).

C. Ferritin

In the human host, ferritin is the major iron storage protein. Ferritins constitute a broad superfamily of spherical, shell-like iron-storage proteins and are composed of 24 protein subunits that form a hollow sphere. The majority of iron in the body is stored intracellularly in ferritin (up to 4,500 Fe^{3+} atoms per ferritin molecule). Ferritin protects eukaryotic cells from the toxic effects of iron accumulation. It also stores iron, making it available in case of iron shortage. Following cell lysis, ferritins are also found in plasma (169, 225).

D. Heme binding proteins

Heme bound to hemoproteins constitutes the major iron reservoir in mammalian hosts and is the preferred iron source of bacterial pathogens. Hemoglobin is located in red blood cells, where it functions as the oxygen transporter. It is a tetramer with two α- and two β-chains. Each subunit binds a heme molecule. Haptoglobin is a serum tetrameric glycoprotein. In serum, haptoglobin binds the hemoglobin, released by hemolysis, with a high affinity. The haptoglobin-hemoglobin complex is an iron source for several bacteria like *Neisseria meningitidis*, and *Haemophilus influenzae* (225). Heme, the iron protoporphyrin IX molecule, is a prosthetic group for many enzymes. It is a protoporphyrin source for several species, including *Enterococcus faecalis*, *Lactococcus lactis*, and *Haemophilus influenzae* (225).

VI. Iron acquisition Systems

Iron acquisition is accomplished by microorganisms via two general mechanisms: iron acquisition by cognate receptors using low molecular weight iron chelators termed siderophores and receptor-mediated iron acquisition from host proteins. The first mechanism relies on siderophores, synthesized and released by bacteria into the extracellular medium. The second involves direct contact between the bacterium and the exogenous iron sources using specific host-iron binding protein receptors. The main difference between these two mechanisms is that siderophores and heme can be taken up by the bacterial cell as intact molecules whereas iron must be extracted from

host carrier proteins such as transferrin or lactoferrin prior to being transported into the bacterial cell.

The uptake of iron from transferrin, lactoferrin, hemoglobin, and siderophores has been identified in both Gram-negative and Gram-positive bacteria. Transferrin, lactoferrin, hemoglobin, and most ferric-siderophore complexes exceed the molecular weight cut off of porins and thus require specific outer membrane receptors for uptake into the periplasmic space. These high-affinity outer membrane receptors share some common characteristics. They have a low level of primary sequence homology that is most pronounced in their amino termini, and their transport function depends on a complex of three proteins localized in the cytoplasmic membrane (TonB, ExbB, and ExbD) that we call the TonB complex (225). Gram-negative pathogens use a variety of transporters to access the host iron sources. For example in *V. cholerae*, iron bound to haem/hemoglobin is actively transported into the cell via ligand-specific outer membrane receptors (128, 203), in *E. coli* iron bound to organic acids like citrate is utilized by FecA transporter (24), *Haemophilus influenzae* and *H. haemolyticus* acquire iron bound to human transferrin through specific transporters (138).

Siderophores (from the Greek: “iron carriers”) are defined as relatively low molecular weight, ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress. The role of these compounds is to scavenge iron from the environment and to make the mineral available to the microbial cell (148). More than 500 different siderophores, mostly produced by Gram-positive and Gram-

negative bacteria, have been described. Despite this great variety, most have a peptide backbone with several nonprotein amino acid analogs. Based on their iron ligation groups, siderophores have been tentatively classified into three main chemical types: hydroxamates (e.g. aerobactin), catecholates (e.g. enterobactin), and hydroxyacids (e.g., pyochelin) (169, 225).

A common pattern of bacterial siderophore transport systems in Gram-negative bacteria has been identified. An outer membrane transporter binds the Fe^{3+}-siderophore complex. The barrel of the transporter, consisting of 22 β-strands and an inside plug, binds the iron complex in the barrel entrance. Internalization of this complex is an energy-dependent process, with the TonB-ExbB-ExbD complex providing the energy (25). Many bacteria are able to utilize siderophores produced by other organisms, so-called xenosiderophores. For example, *E. coli* K-12 can utilize the fungal siderophores ferrichrome and coprogen and citrate in addition to it own siderophore enterobactin. In *E. coli* multiple receptors have been identified and their crystal structures have been solved. Some of them are FepA (enterobactin) and FhuA (ferrichrome) (23, 39).

VII. TonB-Dependent Transporters

In Gram-negative bacteria, transport of siderophores into the periplasm is mediated by TonB-dependent receptors (68, 225). TonB-dependent transporters have also been well characterized in iron acquisition pathways mediated by host-iron binding proteins (52, 188, 194).

TonB-dependent transporters share a common topology. They all are composed of two characteristic domains: (i) a C-terminal beta-barrel (spanning the outer membrane), comprised of 22 amphipathic, antiparallel β-strands, and a globular plug domain, located at the N-terminal position and is folded up inside the barrel (55, 68, 107). The plug domain of the outer membrane receptor contains a conserved, 7 amino acid sequence: the TonB-box. The TonB box is found at the N terminus of the plug domain and in some structures protrudes into the periplasm. In other structures, the TonB box is tucked up into the plug domain within the barrel or is disordered and not visible in the structures (22, 30, 225). This conserved region adopts a variety of conformations, ranging from folded to unfolded, and is believed to participate in interactions between TonB and the TonB-dependent transporter and is the primary signature of TonB dependence (152, 162).

The energy that is required for the active transport of iron-laden substrates through the TonB-dependent outer membrane receptor is provided by the TonB/ExbB/ExbD complex, which is located in the cytoplasmic membrane. The TonB protein complex harnesses energy generated by the proton motive force in the inner membrane, and then transduces it to the outer membrane (100, 107, 161). The binding of iron-bound ligand to the extracellular pockets of outer membrane receptors causes a TonB-independent allosteric transition that is propagated through the outer membrane. This results in formation of a transmembrane channel within the receptor, through which the ligand permeates into the periplasm. Upon arrival in the periplasm, siderophores are rapidly bound by specific periplasmic binding proteins, and shuttled

to distinct ABC transporters embedded within the cytoplasmic membrane for transport into the cytoplasm (68, 106, 135).

TonB-dependent uptake systems permit bacterial growth in a wide range of iron-limited environments. These systems are also considered targets for novel antibiotics, because they contribute to the specific permeability of the Gram-negative cell envelope (135).

VIII. Iron Acquisition in Pathogenic *Neisseria*

Two general mechanisms of iron acquisition in bacteria have been described: siderophore-mediated iron acquisition by cognate receptors and receptor-mediated iron acquisition from host iron-binding proteins. Many enteric bacteria use siderophore-mediated iron uptake. In *Neisseria*, however, there is no known production of siderophores (185, 231). Still enterobactin and aerobactin have been shown to support the growth of pathogenic *Neisseria*. To overcome the iron limited host environment, these highly adapted obligate human pathogens can efficiently utilize multiple host iron (Fe) sources, allowing replication on mucosal surfaces, in the bloodstream, and intracellularly. Pathogenic *Neisseria* express multiple high-affinity iron transporters to acquire iron from transferrin, lactoferrin and hemoglobin (113, 132, 187). Although these receptors may vary in complexity and composition, all three systems are structurally similar in consisting of an outer membrane TonB-dependent transporter and an accessory lipoprotein.

The transferrin binding proteins and the receptor complex are discussed in detail in the next chapter. The lactoferrin iron utilization system is made up of lactoferrin

binding proteins A and B (LbpA and LbpB). LbpA is the TonB-dependent transporter and essential for iron internalization, LbpB is a lipoprotein and not required for lactoferrin iron utilization. The HpuAB receptor is responsible for utilization of iron from hemoglobin and hemoglobin-haptoglobin. The receptor is made up of HpuB and HpuA; both of which are required for iron internalization. HpuB is an 85 kDa TonB-dependent outer membrane transporters and HpuA, is a 34.8 kDa lipoprotein (156). To utilize iron bound to hemoglobin, only *Neisseria meningitidis* expresses the HmbR hemoglobin receptor. HmbR is an iron-regulated 89.5 kDa outer membrane protein that mediates iron acquisition from hemoglobin but not from hemoglobin-haptoglobin (204).

IX. Gonococcal Transferrin Receptor

The neisserial transferrin–iron import system consists of two dissimilar transferrin binding proteins, TbpA and TbpB. The neisserial receptor involved in iron acquisition from human Tf is preferentially expressed under conditions of iron deprivation (49, 189). The *tbpA* and *tbpB* genes are arranged in a bicistronic operon, with the *tbpB* gene located upstream of *tbpA* (52, 112, 178). The two genes are separated by an 86 base pair region, potentially capable of forming a stem-loop structure in the mRNA. Using *lacZ* fusion and RT-PCR approaches, under iron-stressed conditions, it was shown that there are twice as many *tbpB*-specific transcripts as *tbpA*-specific transcripts, yielding an apparent *tbpB*/*tbpA* ratios of approximately 2:1 (178). This implies that the secondary structure of the intergenic region might function in maintaining transcript stoichiometry (178). The *tbpAB* promoter contains a Fur binding site and is under the control of the Fur protein. Fur (ferric uptake regulator) is

a global regulator, which in the presence of iron represses gene transcription. This regulatory mechanism leads to preferential expression of Tbps under iron-limited conditions.

TbpA is an integral outer membrane protein that provides the pore through which iron is transported across the outer membrane of gonococci (52). Recently, the crystal structures of components of the meningococcal transferrin–iron uptake system were reported (31, 151). TbpA is conserved in pathogenic *Neisseria* and shares similarity with other TonB-dependent outer membrane proteins (166, 171). Similar to the solved crystal structures of many such transporters, the TbpA structure is characterized by a C-terminal beta-barrel domain comprised of 22 amphipathic beta-strands and an N-terminal globular plug domain that is folded up inside the beta-barrel (150). The TonB-dependent transporter is energized by TonB and ExbB and ExbD (106, 150). Gonococcal mutants not expressing TbpA were capable of binding some transferrin but incapable of growth on transferrin as the sole iron source (48, 52), making TbpA essential for transferrin-iron uptake.

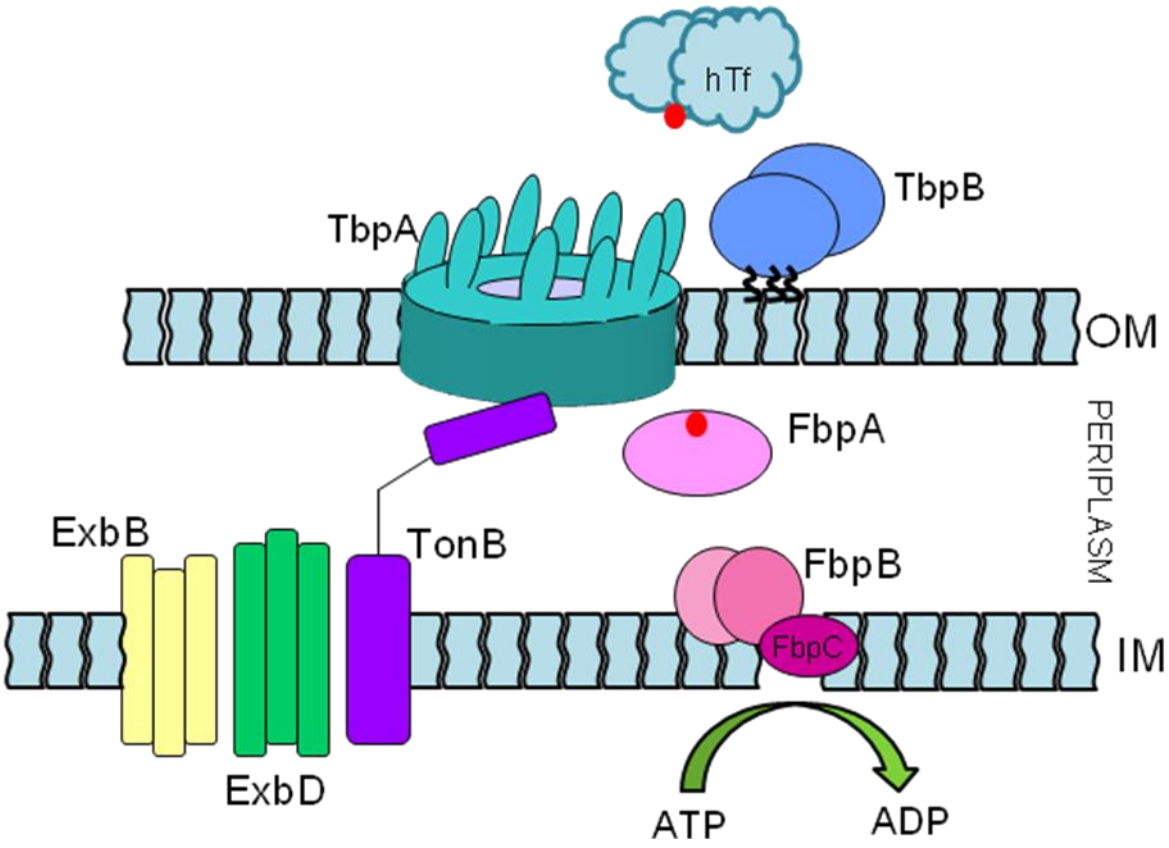
The second member of the transferrin-iron uptake system is TbpB, which is a lipid-anchored protein, presented on the outer leaflet of the outer membrane. TbpB is capable of binding transferrin (1, 112). TbpB is not essential for iron acquisition but makes the process more efficient (1). TbpB is capable of discriminating between apo- and holo-transferrin and preferentially binds holo-transferrin. Gonococcal TbpB mutants showed decreased iron-uptake from transferrin but grew with transferrin as a source of iron (1). TbpB has the ability to affect rapid association and dissociation of

transferrin on the cell surface, thereby allowing optimum ligand to be delivered to TbpA and once the iron has been removed from the ligand, to aid in the release of apo-Tf (63). TbpB may also participate in deferrating transferrin (191) and work in concert with TbpA to make the transferrin-iron acquisition process more efficient.

In the gonococcal iron internalization process (figure 1), transferrin binds to TbpA and TbpB on the outer membrane. The cytoplasmic membrane TonB-ExbB-ExbA protein complex interacts with the TonB box sequence in the TbpA plug domain. Iron is stripped from transferrin, in an energy dependent process, and translocated through the TbpA barrel into the cell periplasm. Iron is then bound to apo-FbpA, a periplasmic iron binding protein, which shuttles the iron across the periplasm to the cytoplasmic membrane. The inner membrane FbpB-FbpC permeases transport iron to the cytoplasm in an ATP dependent manner (figure 1).

Several studies have demonstrated that TbpA and TbpB function together for extracting iron form holo-transferrin. Evidence of complex formation between TbpA and TbpB has been described in the pathogenic *Neisseria*. Using radiolabelled transferrin binding assays, it was demonstrated in *Neisseria meningitidis,* that TbpA and TbpB did form a complex (160). Studies utilizing photon correlation spectroscopy (19) to analyze complex formation in *N. meningitidis* have concluded that affinity purified TbpA and TbpB physically associate with each other. Results of transferrin-binding affinity purification, and protease accessibility experiments

Figure 1: Schematic model of the transferrin iron acquisition system in *Neisseria gonorrhoeae*. The transferrin iron acquisition system is composed of two transferrin binding proteins, the surface exposed TbpA and TbpB. TbpA is a TonB-dependent transporter, serving as a channel for iron internalization. TbpB is an accessory protein, tethered on the outer leaflet of the outer membrane through its lipid moiety. Ferrated transferrin binds to the Tbps and iron is removed and internalized in an energy dependent manner, the energy is supplied by the TonB-ExbD-ExbB complex by TbpA-TonB interaction. Iron is internalized and in the periplasm binds the periplasmic binding protein, FbpA; it is then shuttled across the periplasm to the FbpB-FbpC permease complex in the inner membrane, which then brings the iron into the cytoplasm in an ATP-dependent step.


hTf
TbpB
TbpA
OM
FbpA
PERIPLASM
ExbB
TonB
FbpB
FbpC
IM
ExbD
ATP
ADP

showed that both TbpA and TbpB function together in the wild type gonococcal strain and both of the Tbps together show binding conformations that are distinct than those expressed by either of the Tbp alone in the mutant. The protease exposure of TbpB was affected by the energization of TbpA, consistent with an interaction between these proteins (48, 54). Co-immunoprecipitation assays have suggested that TbpA and TbpB may interact with one another on the cell surface. TbpA-specific antibodies were able to co-immunoprecipitate TbpB from outer membrane fractions, even in the absence of transferrin (96), suggesting that even in the absence of transferrin the two proteins associate with each other. Recent reports of complex crystal structures and electron microscopy analysis, have proposed the Tbps functioning together to bind and extract iron from transferrin (150).

X. Gonococcal Vaccine Development

The new superbug *Neisseria gonorrhoeae* has maintained resistance to previously described antibiotics and developed resistance to ceftriaxone, the last remaining option for treating gonorrhea (214). The rise in untreatable gonorrhea poses an exceedingly serious public health problem. No vaccine to protect against gonorrhea is available. Progress in developing a vaccine has largely been stymied due to antigenic variation displayed by the organism, coupled with the host's inability to generate a protective immune response (84). Gonococci are well-adapted pathogens since many of its surface antigens like pilin and opacity proteins undergo high frequency phase or antigenic variation, making the surface of the gonococcus difficult to target for a

protective immune response. Much vaccine work has focused on surface exposed antigens that clustered in the outer membrane, like porins, pili, and Rmp. A trial of a purified gonococcal pilus vaccine composed of a single pilus type was tested in male volunteers. This gonococcal pilus vaccine composed of a single pilus type failed to protect men against gonococcal urethritis (18) possibly due to antigenic variation. Porin was evaluated as a vaccine candidate as it does not undergo phase variation, is a major outer membrane protein and is important for invasion. The results were unsuccessful as sialylated LOS partially masks adjacent porins and reduces antibody binding to porins. Rmp elicits blocking antibodies that subverts anti-porin antibodies (175, 232, 241).

Utilizing proteins involved in iron acquisition as vaccine candidates seems rational as iron is critical for *Neisseria* survival. The Tbps are attractive vaccine candidates because they are not subject to high frequency phase or antigenic variation, are surface exposed, and are expressed by all clinical strains (132). In a study with human male volunteers, intraurethral inoculation with Tbp^- mutant strain was incapable of initiating urethritis, indicating that Tbps are necessary for establishment of infection in human male infection model (53). Analysis of intranasal immunization with recombinant Tbp proteins conjugated to the cholera toxin B subunit showed that these antigens elicited antibody responses in the serum and genital tract of female mice (164). The results reported strengthen the proposal to include components of the neisserial transferrin receptor in vaccine candidates.

XI. Research Objectives

A number of reports have together contributed to the study of iron utilization from human transferrin in the *Neisseria* species. Studies, in the past, have attempted to decipher the gonococcal transferrin receptor and provided several indirect lines of evidence demonstrating complex formation between the Tbps. Previous studies in our lab have generated hemagglutinin (HA) epitope insertion mutants in TbpA and TbpB, to investigate the surface-exposed regions in the Tbps. The Hemagglutinin (HA) epitope tag was inserted in a number of conserved and variable regions of TbpA and TbpB, with the intention to study structure-function relationships between the Tbps. The overall goal of the proposed studies here was to probe the structure-function relationships in both the Tbps in order to determine the important domains for interaction between the two Tbps, thereby yielding important information for a rational design of a vaccine against gonococcal infections. First we examined the iron binding properties of the conserved EIEYE sequence in the TbpA plug domain, with the intention of extending our knowledge about transferrin-iron internalization through TbpA. Secondly, we utilized photoactivable cross-linkers to assess the effect of TbpA on TbpB in the gonococci and studied it in presence of ligand and TonB derived energy. We employed insertion mutants, in which TbpA contained the hemagglutinin (HA) epitope tag, to probe for impact of TbpA on TbpB. And lastly, we sought to characterize the importance of TbpB regions in the functional and structural relationships of TbpB .

This study will help to elucidate additional characteristics of the interaction between the gonococcal Tbps *in vivo*. By looking at the structural and functional properties of the two members of the gonococcal transferrin receptor, we will have a better understanding of mechanisms of iron acquisition through the Tbps. The Tbps have a potential to be vaccine candidates and knowledge derived from our study might be important for the rational design of a vaccine comprised of one or both of the transferrin binding proteins.

CHAPTER 2: Materials and Methods

I. Bacterial Growth Conditions

A. Strains and media

The *N. gonorrhoeae* strains used in this study are listed in Table 3. Gonococci were routinely maintained on GC medium base (Difco) containing Kellogg's supplement 1 (94) and 12 μM ferric nitrate (Supplement II) at 37^{o}C in a 5% CO_2 atmosphere. For growth under iron-stressed conditions, which allows for maximal expression of transferrin binding proteins, liquid cultures of gonococcal strains were grown in acid-washed glassware containing chemically defined media (CDM) (137, 230). The CDM was rendered iron free by treatment with Chelex 100 (BioRad). Plasmids were propagated in TOP10 (Invitrogen) *E.coli* strains, grown at 37^{o}C in Luria-Betani broth (7,8), supplemented with 50μg/ml of kanamycin (Sigma).

B. Bacterial growth in photoreactive CDM Media

Photoreactive amino acids L-Photo-Leucine and L-Photo-Methionine (Thermo Scientific) were used as cross-linking reagents. To the liquid chelexed defined medium (CDM) photoreactive amino acids were added at a final concentration of 0.7 mM photo-leucine and 0.1mM photo-methionine. Bacterial cultures were grown in a shaking incubator at 37°C, in an atmosphere supplemented with 5% CO_2. After four hours of growth, cultures were UV irradiated and standardized to culture density.

Table 3: *Neisseria gonorrhoeae* strains

Strain	Phenotype (genotype)	Reference/Source
	***Neisseria gonorrhoeae* strains**	
FA19	Wild type ($TbpA^+$ $TbpB^+$)	(131)
FA6747	$TbpA^-$ (*tbpA*::mTn3cm)	(52)
FA6905	$TbpB^-$ (Δ*tbpB*)	(48)
MCV515	$L9HA_{(750)}$ TbpA Lbp^- (*tbpA*▽HA *lbpB*::Ω)	(238)
MCV519	$L11HA_{(843)}$ ΔAla847 TbpA Lbp^- (*tbpA*▽HA *lbpB*::Ω)	(238)
MCV523	$β16HA_{(713)}$ TbpA Lbp^- (*tbpA*▽HA *lbpB*::Ω)	(238)
MCV527	$L2HA_{(229)}$ TbpA Lbp^- (*tbpA*▽HA *lbpB*::Ω)	(238)
MCV260	$TbpAE_{120}AY_{121}AE_{122}ATbpB^+$	(153)
MCV814	$HA4_{(293)}$ TbpB Lbp^- (*tbpA*▽HA *lbpB*::Ω)	(62)
MCV822	$HA8_{(607)}$ TbpB Lbp^- (*tbpA*▽HA *lbpB*::Ω)	(62)
MCV839	TbpB LSAC (*lbpB*::Ω)	(157)
MCV843	TbpB Δgly (*lbpB*::Ω)	(157)

II. Recombinant Protein Expression and Purification

A. Recombinant *tbpA* (WT) and *tbpA* ($EYE_{120}AAA$) plug protein expression

Starter cultures of *E. coli* expression strain BL21 (DE3) (containing the wildtype and mutagenized *tbpA* plug-encoding domains) were grown in LB media containing 100 μg/ml Ampicillin, for approximately 4 hours at 37°C. After growth, the starter cultures were centrifuged at 5000 x g for ten minutes and the bacterial cell pellet was resuspended into fresh LB media with 100 μg/ml ampicillin. The freshly resuspended starter cultures were then used to inoculate 1L of LB broth with 100 μg/ml ampicillin. The cultures were incubated at 37°C with a constant shaking at 225 rpm. When the cultures reached an O.D. between 0.4-0.8, protein expression was induced with the addition of 1mM IPTG (isopropyl-B-D-thiogalactopyranoside) (Sigma). The cultures were allowed to induce for four hours at 37°C with a constant shaking at 225 rpm. After the induction, the cultures were allowed to centrifuge at 8000 x g for 25 minutes. The supernatant was discarded and the pellets were resuspended in sterile 1X PBS (pH 7.2) and centrifugation was performed at 9000 rpm for 20 minutes. The supernatants were removed and the bacterial pellets were dried and stored at -20°C overnight.

B. *tbpA* (WT) and *tbpA* ($EYE_{120}AAA$) plug protein purification

The bacterial pellets were thawed on ice and resuspended in Lysis buffer (50mM NaH_2PO_4, 300 mM NaCl, 10mM Imidazole, at pH 8.0). Lysozyme and His-tagged

Protease Cocktail inhibitor (Biorad) was added at a concentration of 1mg/ml and 1ml/20gms respectively. The cell pellet was resuspended and allowed to incubate for 30 minutes on ice. The cellular mixture was sonicated and the lysate separated from the debris by centrifugation at 10000 x g for 30 minutes at 4^{o}C. The supernatant was then incubated with 50% Ni-NTA slurry (Qiagen) overnight at 4^{o}C. Then, the next day the mixture (containing the Ni-NTA resin) was added to a column and the first flow-through fraction was collected in a fresh tube. The resin in the column was washed twice with 5ml of Wash Buffer (50mM NaH_2PO_4, 300 mM NaCl, 20mM Imidazole, at pH 8.0) and collected in two separate tubes. The recombinant proteins were eluted from the resin-column with the elution buffer (50mM NaH_2PO_4, 300 mM NaCl, 250mM Imidazole, at pH 8.0). The purified proteins were dialyzed against 2000 fold 1X PBS (pH 7.2).

C. SDS-PAGE, protein transfer and detection of purified protein

The various purified protein fractions were solubilized in 2X Laemmli solubilizing buffer (109). The samples were then heated at 100^{o}C for 2 minutes after the addition of 5% β-mercaptoethanol. After allowing the heated samples to cool, they were loaded on to a 15% gel and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (109). The separated proteins were then transferred to a nitrocellulose membrane at 28mAmp for 16 hours in 20mM Tris base, 150mM glycine and 20% methanol (211).

For detection of His-tagged purified recombinant proteins, the nitrocellulose membrane was blocked with 5% skim milk in low-salt Tris-buffered saline (LS-TBS).

Blots were probed with anti-His-tag monoclonal antibodies (Calbiochem) at a dilution of 1:1000, as primary antibody the blots were then washed with LS-TBS containing Tween-20 and then probed with goat-anti-mouse IgG AP (BioRad) at a 1:5000 dilution NBT/BCIP (Sigma) was used for developing and visualizing the protein bands on the membranes.

D. Coomassie blue staining

To detect purified protein bands, the SDS-PAGE gels (with the separated proteins) were stained with Coomassie blue (0.25% Coomassie R-250, 50% methanol, 10% glacial acetic acid) the gels were left in the blue stain solution overnight at room temperature. The gels were destained in 20% methanol and 5% acetic acid at room temperature till the background was destained and the protein bands visualized.

III. Immunoblotting and Detection

A. Preparation of whole cell lysates

To evaluate the expression of Tbps, gonococcal cultures were grown in CDM under iron-depleted conditions. After four hours of growth, cultures were standardized to constant cell density. Samples were centrifuged at 14000rpm for 10mins. Cell pellets consisting of approximately 1 x 10^{12} cfu were resuspended in 100 µl of 2X Laemmli solubilizing buffer (109).

B. SDS-PAGE and protein transfer

Prior to gel analysis 2µl of benzonase nuclease (Sigma) was added to the whole cell lysates and incubated for 10 minutes at room temperature, to decrease the viscosity of the prepared cellular lysates. After the incubation 5% β-mercaptoethanol (Sigma) was added to the whole cell lysates and they were heated at 95°C for 2 minutes. Samples were allowed to cool down to room temperature and centrifuged for 30 seconds at 14000 rpm. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% polyacrylamide gel using a Mini-Protean III apparatus (Biorad). Separated proteins on the gels were transferred to nitrocellulose membrane (Schleicher and Schull) in 20mM Tris base, 150mM glycine and 20% methanol in a submerged transfer apparatus (Biorad) at constant current of 28mAmp for 16 hours. Before western blotting, to confirm equal protein loading in each lane, the nitrocellulose membranes were stained with Ponceau S solution (0.1% w/v Ponceau S, 5% acetic acid) for 10-12 minutes and excess stain rinsed with distilled water.

i) TbpA detection

To detect TbpA nitrocellulose membranes were blocked with 5% BSA in high salt Tris-buffered saline (HS-TBS: 20mM Tris base, 500mM NaCl). The blocked membrane was probed with 1:1000 dilution of primary anti-TbpA polyclonal antibodies for 1 hour as described (48). The primary antibody was

detected with a goat anti-rabbit antibody conjugated to alkaline phosphatase (Biorad) at a dilution of 1:5000 for 1 hour. The membrane was washed in HS-TBA with 0.05% Tween-20 (Sigma). Blots were developed using NBT/BCIP (Sigma) in buffer containing 100 mM Tris (pH 9.5), 50 mM $MgCl_2$, and 100 mM NaCl.

ii) TbpB detection (using anti-TbpB antibodies)

To detect TbpB, the nitrocellulose membranes were blocked with low-salt Trus buffered saline (LS-TBS) containing 5% non-fat dry skim milk (Sigma) for an hour. Membranes were probed with 1:1000 diluted primary anti-TbpB polyclonal antibodies (163), washed in low salt-TBS and 0.05% Tween-20. Next the membranes were probed with goat anti-rabbit alkaline phosphatase conjugated (Biorad) secondary antibody at a 1:5000 dilution. The membranes were developed with NBT/BCIP developing system.

iii) TbpB detection (using transferrin)

As an alternative method to detect TbpB, we blocked the nitrocellulose membranes in 5% non-fat dry skim milk (Sigma) and LS-TBS. The membranes were washed with low-salt TBS and 0.05% Tween-20. To detect transferrin-binding by TbpB, the membranes were probed with horseradish peroxidase conjugated Tf (HRP-Tf) at a concentration of 1µg/ml for an hour. HRP was detected using Chemiluminesence (ECL) 2 Western Blotting Substrate kit (Pierce) (48). Blots were exposed to film to detect TbpB bands bound to transferrin.

IV. Image J analysis

The photo-crosslinked samples were analyzed by SDS-PAGE and separated samples were transferred to the nitrocellulose membranes. Subsequent to immunoblotting, the blot images were captured and the band intensities were quantitated using the Image J 1.46r (168) imaging system and associated software.

CHAPTER 3- DEMONSTRATION OF IRON BINDING BY THE EIEYE SEQUENCE IN THE TbpA PLUG OF *Neisseria gonorrhoeae*

I. INTRODUCTION

Neisseria do not produce any known siderophores, however they can utilize siderophores produced by other microorganisms (28, 67, 182, 183, 231). In the human host iron is sequestered by a variety of soluble iron-carrying glycoproteins or heme compounds. Gonococci have evolved mechanisms for obtaining iron from these host iron binding proteins. To utilize the bound iron, they express high-affinity receptors to utilize iron bound to human transferrin (16, 67, 111, 126, 131), lactoferrin (16, 67, 111, 126, 133) and hemoglobin (1,10). The lactoferrin-iron acquisition pathway and the hemoglobin-iron acquisition pathway are similar to the transferrin-iron acquisition system; all of them comprised of a TonB-dependent transporter and an outer membrane-tethered lipoprotein (10, 11, 42, 43, 52, 55). Both of these components work together, in a sequential manner, to allow the iron-binding glycoproteins to interact with the bacterial cell surface, extract iron from the bound form and internalize it across the membranes.

Gonococci are capable of utilizing human transferrin as a sole source of iron (131), through the expression of iron regulated transferrin-iron acquisition system. The transferrin iron uptake system consists of Tf- binding protein A (TbpA) and B (TbpB). TbpA is a 100-kDa integral outer membrane protein, belonging to the family of TonB-dependent transporters. Tf-binding protein B is an ~80-kDa co-receptor, attached to the outer membrane by a lipid anchor. TbpA is essential for transferrin-iron acquisition but TbpB is not required for the same process, instead its presence makes the process more efficient (1). TbpB serves

as an accessory protein in the iron acquisition process, with a capability of differentiating between apo- and holo- transferrin (19, 48, 170, 173). Figure 1 depicts the iron acquisition process in *Neisseria gonorrhoeae* (27). The process involves the following steps: TbpA/TbpB recognize and bind holo-transferrin, remove apo-transferrin after stripping iron (using TonB derived energy), periplasmic FbpA binds iron after it travels through the outer membrane, FbpA shuttles the iron across the periplasm to the inner membrane permeases FbpB/FbpC, later transporting it to the cytosol (54, 97, 190)

Recently the crystallization of *Neisseria meningitidis* TbpA, helped in furthering the structural study of the neisserial iron import machinery (151). The crystal structures of several of these transporters have been reported by various groups (31, 150, 155). These structures are all TonB-dependent transporters, characterized by an N-terminal plug domain of ~ 160 residues (plug domain) folded inside a C-terminal 22-stranded beta-barrel domain (β-barrel domain). The plug domain acts as a ligand sensor, by preventing entry of substances into the periplasm until the appropriate ligand is bound.

TbpA is significantly larger than other TonB-dependent transporters, the structure consists of 22 transmembrane β-strands, arranged as a barrel, with long surface exposed loops, and an N-terminal plug domain. The TbpA barrel is occluded by the N terminal plug domain. The plug domain does not allow the passage of small molecules through the TbpA β-barrel. The plug could undergo a conformational change that leads to the ejection of the plug domain into the periplasm, thereby forming an entry pathway for the iron cargo directly through the outer membrane transporter.

Previous studies have shown the significance of the TbpA plug domain in the iron internalization process (238), however the precise mechanism is unknown. In the plug

domain of TbpA the sequence EIEYE (118-122) is highly conserved (figure 2). The triple alanine substitution of the Glu120, Tyr121 and Glu122 in a TbpA mutant (EIAAA 118-122) showed an 80% reduction in transferrin bound iron utilization (153). This demonstrated the importance of the EIEYE sequence in the TbpA plug in transferrin iron utilization. The EIEYE motif is well conserved and is suitable for coordinate-covalent bonding to Fe and capable of scavenging available iron in the iron deprived host milieu. We hypothesize, that at the TbpA/TbpB surface, iron released from transferrin, is bound by the TbpA plug sequence (EIEYE) and is transported through β-barrel of TbpA across the outer membrane. In this study *in vitro* and *in silico* experiments were conducted, utilizing wild-type and triple alanine mutated (EIEYE-EIAAA) recombinant TbpA plug samples, and three small peptides (S1, S2, S3); each peptide containing the putative iron-coordinating site and specific portions of the wild-type TbpA plug domain.

II. RESULTS

To study the protein characteristics of the recombinant TbpA plug proteins, we first undertook large scale growth of the *E. coli* strains expressing the recombinant plug proteins (5), and then purified them using the His-tag purification protocol. Protein concentration for the purified wild type recombinant TbpA plug protein was 18.18 mg/ml and for the mutated recombinant TbpA plug protein was 42.02 mg/ml. After characterizing them for quality and protein concentration (figure 3), we examined the secondary structure of the plug (without the barrel). First, CD spectra analysis of the wild-type and mutated recombinant TbpA plug protein was carried out. The CD spectra for the wild-type and mutated recombinant TbpA

Figure 2: Iron coordinating residues in the TbpA plug. In the sequence alignment of TbpA plug domains from bacterial pathogens, the first two letters preceding the amino acid sequences represent the genus and species of the bacterial pathogen: Ng, *Neisseria gonorrhoeae*; Nm, *Neisseria meningitidis*; Hi, *Haemophilus pleuropneumoniae*; Ap, *Actinobacillus pleuropneumoniae*; and Mc, Moraxella catarrhalis. The sequences represent mature TbpA plug domains. Dots indicate identical amino acids, letters indicate specific amino acids and dashes indicate positions in which gaps were introduced. The inverted triangle represents the site of HA insertion and 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).

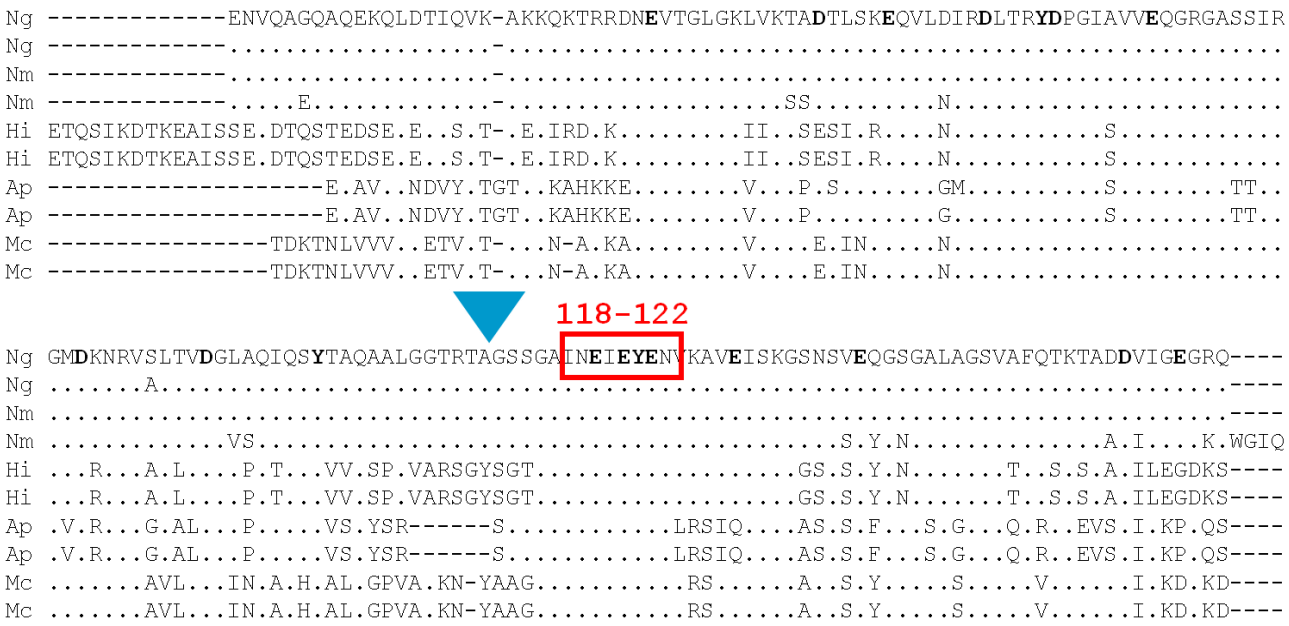
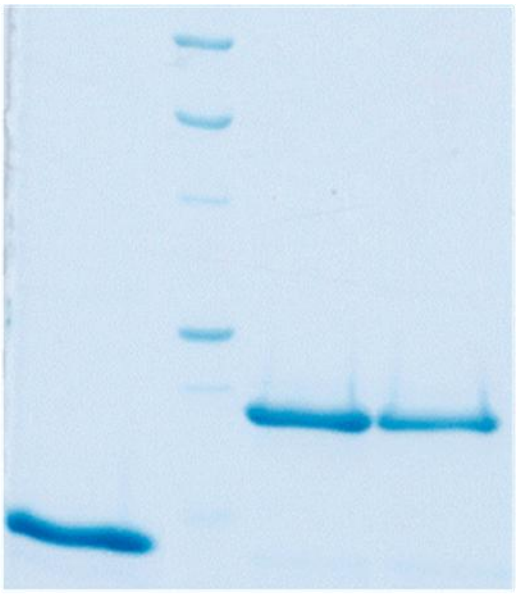

118-122

Figure 3: Characterization of purified wild-type and mutated recombinant TbpA plug proteins. Proteins were separated on a SDS-PAGE gel and stained with coomassie blue. The contents of the lanes are: lane1- lysozyme, lane 2-protein molecular weight marker, lane 3- wild-type plug protein, lane 4- mutant plug protein.



Lanes: 1 2 3 4

plug protein was centered around 200nm, in the absence of Fe^{3+}. This indicates unfolded structures for the wildtype and mutated recombinant plug proteins. This was expected because when the proteins are expressed without the β-barrels, there are no stabilizing hydrogen bonds formed to induce native secondary structure. This kind of CD spectra has been previously reported for the recombinant wildtype FbpA plug and the above observation holds true to that study (191). To investigate an interaction between Fe^{3+} and the wildtype and mutated recombinant plug protein, and its consequential conformational change in the plug, CD spectra studies were carried in presence of Fe^{3+}. The interaction between recombinant plug domain and its respective cargo has been demonstrated in *E. coli* (20, 69, 70, 215). The CD spectra for wildtype and mutated plug protein in presence of Fe^{3+}, did not show any significant change for either of the protein signals. This observation tells us that no apparent conformational change occurs when (or rather if) binding of Fe^{3+} happens at the plug domain.

Fluorescence emission titrations help us to study the binding interactions. In the system we are studying, we looked at the binding (if any) between the TbpA plug domain and the peptides with Fe^{3+}. In the fluorescence emission titrations, the 310nm peak was used as a probe. % quenching (Q%) at 310nm as a function of increasing Fe^{3+}, was measured for the wild-type recombinant TbpA plug. The conditional K_d for wildtype recombinant TbpA plug is 10^{-7}M and the K_d value for S1 (peptide) is 10^{-4}M. Upon binding of Fe^{3+}, to the wildtype recombinant TbpA plug, other amino acids in the TbpA plug could participate to stabilize the protein- Fe^{3+} interaction and therefore we probably observed the higher binding affinity for the wildtype recombinant TbpA plug compared to the peptide S1. The TbpA wildtype recombinant plug contains four tyrosine residues; the EIEYE sequence in the plug,

contains one of the tyrosine residues. The S1 peptide under study here contains one tyrosine residue. Fluorescence emission properties of the S1peptide and the recombinant TbpA plug depends on these tyrosine residues, with the surface exposed tyrosine residues emitting fluorescence at 310nm.

The fluorescence emission experiments aid in studying interaction between Fe^{3+} and the TbpA plug/peptide, using 310nm peak as a probe, because the binding between the tyrosine and Fe^{3+} leads to decrease in free tyrosine in solution and resultant quenching of the 310nm band. In fluorescence emission titration spectra for the wildtype recombinant TbpA plug, it was seen that the addition of increasing amount of Fe^{3+}, led to the quenching of the 310nm band, indicating a binding event. Similar quenching behavior for the peptide S1was observed. The Q_{max} values at the theoretical endpoint of titration, differ for the wildtype recombinant TbpA plug (Q_{max} value is approx 40%) and the S1 peptide (Q_{max} value is 100%). For the S1 peptide at the end point of titration, Q_{max} value of approx 100% indicates that all (in this case 1) tyrosine residues are bound to added Fe^{3+}. The wildtype recombinant TbpA plug has a Q_{max} value of approx 40%, indicates that all the tyrosine residues (wildtype recombinant TbpA plug contains four tyrosine residues) do not participate in Fe^{3+} binding. We can speculate that there are two different tyrosine populations, one that participates in Fe^{3+} binding (shown by the 310nm band present for quenching), and the other population that doesn't show quenching due to non-binding to Fe^{3+}. The S1 peptide contains the EIEYE sequence and shows 100% binding. The wildtype TbpA recombinant plug containing the EIEYE sequence, shows a Q_{max} value of 40%, thereby allowing us to say that EIEYE sequence is responsible for binding Fe^{3+} and subsequent quenching of the wildtype TbpA recombinant plug.

The predicted structures of the wildtype and mutated TbpA plug of *Neisseria gonorrhoeae* were predicted as models, under native conditions through I-TASSER. The top of the molecule shows the presence of a loop, called the top hat region, which is formed by the presence of a highly conserved sequence, found prior to the EIEYE motif (figure 4). This top hat region contains the Ala110 residue, and this residue by HA-insertion tag analysis has been shown to lie in the surface exposed region of TbpA (238). This tells us that the top hat region is surface exposed and located at the apex of the molecule. The EIEYE motif is in a long loop, anchored by beta strands. Prior to binding Fe^{3+}, reorganization of atoms takes place to maximize binding in the loop; this requires the binding domain to be flexible so as to utilize minimum energy. The present model, predicts this sequence to be in a flexible loop and allowing ligand binding. The predicted models of the wildtype and mutated TbpA plug proteins, show that the EIEYE-EIAAA mutation had the most drastic effect in the surface exposed top hat region.

III. DISCUSSION

In *Neisseria gonorrhoeae,* iron bound to human transferrin is acquired in the following manner: holo transferrin binds to TbpA/TbpB, apo transferrin is recycled from the TbpA/TbpB surface after stripping iron, iron passes through the β-barrel of TbpA, iron binds to apo FbpA in the periplasmic side and is transported through the periplasm, it is then transferred to the cytoplasm with the help of FbpB/FbpC. TbpA, in this iron acquisition process, must help in removing iron from transferrin and then transporting the iron through its β-barrel across the outer membrane. A previous study in our lab (154) investigated the role of TbpA, specifically the TbpA plug in iron acquisition and iron internalization. In vitro

studies have indicated that the TbpA plug domain functions in iron internalization in the gonococcal system. Single, double, and triple alanine substitution mutations of the putative iron-coordinating residues (EYE- residues 120-122) in the TbpA plug domain resulted in mutants that expressed wildtype levels of TbpA on cell surface and bound transferrin with wildtype affinity (154). When the double and triple alanine substitution mutants were analyzed through transferrin-iron uptake assays they showed a significant defect in internalizing iron. The triple alanine substitution mutant was unable to utilize transferrin-bound iron during transferrin-iron utilization growth assays, in absence of TbpB (154). This demonstrates that the TbpA plug domain and residues EYE are critical for transferrin-iron acquisition.

The mutagenesis of the TbpA plug domain could have altered iron binding sites, which may be involved in iron removal from transferrin, and subsequently iron binding by the plug, and transport across the outer membrane. The substitution mutations in the TbpA plug domain could have resulted in indirect impacts within the plug binding domain, resulting in the mutated plug's inability to bind or transport iron.

TbpA, in addition to being a transferrin receptor, is also an iron transporter. FepA (TonB-dependent transporter) of *E. coli*, in the absence of the β-barrel domain, binds directly to its ligand (ferric-enterobactin) (215). This suggests that the plug is important for ligand binding and transport. It is possible that in addition to residues 120-122 of the plug domain, there could be the involvement of other residues/domain in the transferrin-mediated iron acquisition process. Upon TonB interaction with the TbpA plug, the iron binding site or sites unravel and facilitate traversal of iron through the outer membrane. This could mean that iron is coordinated at multiple sites, with movement of iron from one coordination site to the next

one till it reaches FbpA in the periplasm. FbpA has one iron coordination site. In vitro recombinant iron binding data shows that TbpA plug domain binds more iron than FbpA, suggesting that the TbpA plug could bind iron at more than one site.

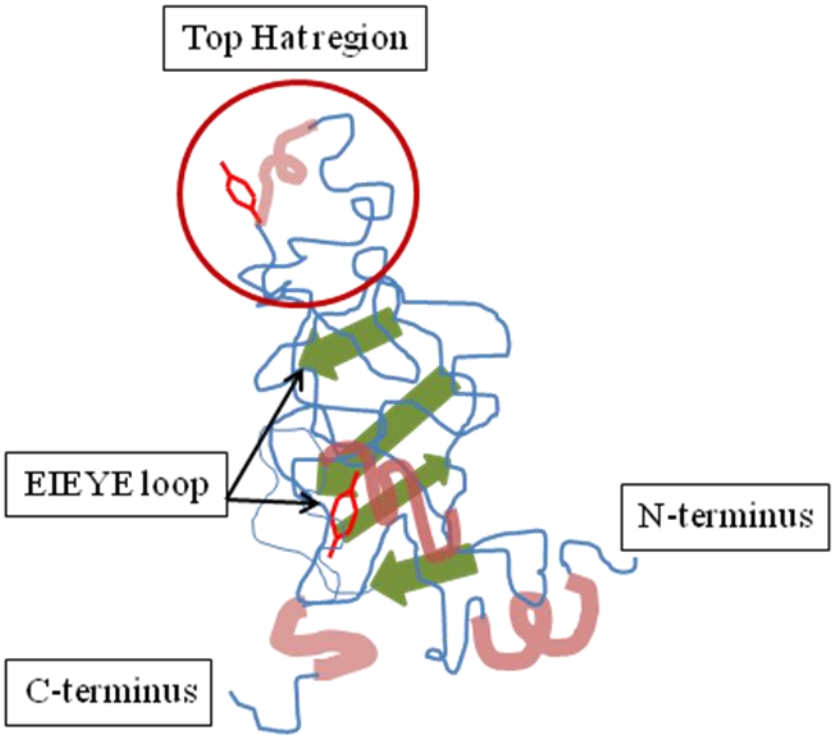
In this study we set out to test our hypothesis that iron that is released from transferrin at the TbpA surface binds with the plug sequence (EIEYE) and is transported through β-barrel. We utilized wildtype and triple alanine mutated recombinant TbpA plugs to conduct *in vitro* experiments to prove the hypothesis. We started the study with large scale expression and purification of wildtype and triple alanine substituted recombinant TbpA plug proteins from *E. coli* expression strains. Three small peptides (S1,S2,S3) containing the EIEYE sequence of the wildtype TbpA plug were also synthesized to be used in this study. CD spectroscopy was used to study the secondary structures of the wildtype and mutant recombinant plug proteins; the possible change in secondary structure (if any at all) due to addition of Fe^{3+} was also studied. The CD data suggested an unfolded structure for both the wildtype and recombinant plug proteins which could be because the plugs were expressed without the β-barrel and did not fold into their native conformation. Fluorescence emission titrations were performed to determine the conditional binding constants of the wildtype and mutant recombinant TbpA plug proteins. The conditional K_d value for the wildtype recombinant TbpA plug is 10^{-7}M and for the S1 model peptide is 10^{-4}M. Upon the addition of Fe^{3+} both the wildtype recombinant TbpA plug and the S1 peptide showed similar quenching of the 310nm band, indicating a binding event. No Fe^{3+} binding was detected for the mutated (EIEYE-EIAAA) plug (5). This supports our hypothesis that in the TbpA plug domain the conserved EIEYE sequence is responsible for binding Fe^{3+}. Previous studies by our lab (153) have shown that *in vivo* that there is an 80% reduction in transferrin bound iron utilization in

the triple alanine substituted TbpA plug, compared to the wildtype. This observation further supports our hypothesis for this study.

The in-silico modeling data helped in creating a model for the wildtype and mutant gonococcal TbpA plug domain. The EIEYE sequence is a long loop, with β-strands anchoring the EIEYE motif. This loop could be flexible in nature due to the need for coordination event occurring during the binding of Fe^{3+}. The CD spectra and folding studies showed that both the wildtype and mutated recombinant plug proteins did not show any change in folding characteristics or stability properties in presence of Fe^{3+}, this suggests that the plug containing the EIEYE sequence must be a flexible loop, which allows for a fluid reorganization during the ligand binding and transferring of Fe^{3+}.

Upstream of the EIEYE sequence is another highly conserved region which showed conformational differences between the wildtype and mutated TbpA plug structure. This region is surface exposed, located at the apex of the molecule and called the top hat region (figure 4). In the predicted models of the wildtype and mutated TbpA plug proteins, the EIEYE-EIAAA mutation had the most drastic effect in the surface exposed top hat region. A previous study from our lab worked with the triple alanine TbpA mutant, to look at the ability of this triple mutant to grow on transferrin as the sole source of iron. The study showed that the triple alanine mutant bound transferrin with wildtype affinity but showed 80% reduction in iron uptake and was unable to grow on transferrin, in a $TbpB^-$ background. These data indicate that the EIEYE motif of the TbpA plug domain is important for transferrin-iron internalization and plays an important role in iron acquisition.

Figure 4: Schematic representation of the predicted structure of *Neisseria gonorrhoeae* wild type TbpA plug domain. The highlighted EIEYE sequence is postulated to be important for iron transport functions through TbpA. The red circle denotes the top hat region. Its importance has been discussed in the test.


Top Hat region
EIEYE loop
N-terminus
C-terminus

This study is one of the first reports of the expression and isolation of the recombinant wildtype TbpA plug. Our studies and results have provided information about the iron coordination by the plug domain of TbpA in *Neisseria gonorrhoeae*. These results pave the way for future studies to determine how the tightly-bound iron is released from transferrin via the membrane-bound receptor complex, the importance of TbpA/TbpB in the stripping of iron from transferrin, how iron is transferred to FbpA through the β-barrel architecture and the role of TbpB in this process of transferrin mediated iron acquisition. These studies will help us further our understanding to clarify the role(s) that each protein plays in the multi-step transferrin-mediated iron transport mechanism in *Neisseria gonorrhoeae*.

CHAPTER 4: CONTRIBUTIONS OF TbpA DOMAINS, ENERGY AND LIGAND TO INTERACTION BETWEEN GONOCOCCAL TRANSFERRIN BINDING PROTEINS

I. INTRODUCTION

In the past, many studies have described indirect lines of evidence pointing towards the formation of a gonococcal receptor complex between the transferrin binding proteins. Gonococcal mutants expressing only TbpA showed reduced binding of transferrin, decreased iron uptake from transferrin, but normal growth on transferrin plates. Mutants expressing TbpB and not TbpA bound less transferrin, did not grow on transferrin plates, and did not take up iron from transferrin (1). TbpB apparently facilitates binding of transferrin but is not essential for acquisition of iron from transferrin; both the transferrin binding proteins need to be expressed and work together to accomplish iron utilization from transferrin (1).

Protease accessibility assays showed that TbpA was more susceptible to proteolytic attack in the absence of TbpB. TbpB can be efficiently cleaved by trypsin. All of the TbpB expressed in the absence of TbpA was readily accessible to trypsin; however in the wild type receptor there is some TbpB that is resistant to cleavage by trypsin (48). The presence of TbpA partially protected TbpB from trypsin proteolysis, and TbpB also protected TbpA from trypsin exposure. These observations indicate that TbpA and TbpB function together in the wild-type strain, to evoke binding conformations that are distinct from those expressed by the mutants lacking either protein. Protease accessibility experiments suggested that the conformation or exposure of TbpB was dependent upon the presence of TbpA, consistent with the hypothesis that the two proteins interact with each other (48).

Like its meningococcal homolog (89), gonococcal Sarkosyl-solubilized TbpB could be affinity purified (by transferrin) in the presence of TbpA, but not in absence of TbpA, suggesting that TbpB might have bound to transferrin through TbpA under these conditions (96). Alternatively, TbpB in the absence of TbpA may have adopted a different conformation so that it no longer bound to transferrin in Sarkosyl. Under the same conditions, when TbpB does not bind transferrin, affinity chromatography could help in the co-purification and increase in the yield of TbpA-TbpB (96, 97). The altered properties shown by the proteins in the affinity purification assay suggest that TbpA and TbpB appear to interact with one another in wildtype gonococci.

Binding assays clearly depicted that the binding affinities of individual Tbps are different from the intact receptor comprising of both of the Tbps (48). It was shown that the binding characteristics of the wildtype receptor were described better using a two-site model. In contrast, mutant gonococcal strains expressing only TbpA or mutant strain expressing only TbpB exhibited binding affinities which were described by a single binding site model (48). These results indicate that TbpA and TbpB when expressed independently in the mutants bound transferrin with relatively high affinity in the liquid-phase binding experiments. Analysis of the mutants indicated that neither protein expressed individually bound transferrin with binding parameters that approximated those of the wild-type strain. This suggested that the wild-type binding phenomena resulted from the combination of TbpA and TbpB at the cell surface (48). This might allow us to say that TbpA and TbpB interact with one another on the cell surface such that it leads to unique conformation and transferrin interaction of the combined receptor complex.

Studies with TbpA mutants in the TonB box of TbpA showed that these mutations had an effect on the conformation of TbpB, implying that TbpA-TbpB interact on the gonococcal cell surface (54, 158). To help define the individual roles of these receptors in the process of transferrin-iron acquisition, the kinetics of the receptor proteins in regards to ligand association and dissociation were evaluated. Both TbpA and TbpB demonstrated a two-phase release pattern; however, TbpA required both TonB and TbpB for efficient transferrin dissociation from the cell surface. Looking further into the roles of TbpA and TbpB in transferrin dissociation it was proposed that TbpA and TbpB function synergistically during the process of transferrin iron acquisition and that TbpB makes the process of transferrin-iron acquisition more efficient at least in part by affecting association and dissociation of transferrin from the cell surface (64).

All the above observations provide indirect evidence of interaction between TbpA and TbpB. There are a number of different techniques to show direct interaction between corresponding protein partners. In order to directly demonstrate an interaction event between TbpA and TbpB we chose to utilize crosslinking assays. We used photo-activable crosslinkers to probe the impact that TbpA has on the migration and conformation of TbpB.

Crosslinking reagents provide the means for capturing in-vivo protein-protein complexes by covalently binding them together as they interact. The rapid action of the common functional groups on crosslinkers allows even transient interactions to be frozen in place or weakly interacting molecules to be seized in a complex stable enough for isolation and characterization. Cross-linking experiments (in conjunction with immunoprecipitation) have been utilized to identify major proteins of the gonococcal outer membrane that are

responsible for imparting serotypic specificity (125), and yield information about the antigenicity of surface-exposed, outer membrane gonococcal proteins (205, 206).

Sophisticated crosslinker designs have been created that incorporate photoreactive groups, which react only in response to irradiation by UV light. We utilized L-photo-Leucine and L-Photo-Methionine. These are photoreactive derivatives of amino acids and during bacterial growth, proteins get metabolically labeled. Upon UV-irradiation, at specific time points, the proteins get covalently crosslinked within protein-protein interaction domains in their native environment in live cells. This method enables both stable and transient protein interactions in cells to be studied and characterized without the use of completely foreign chemical crosslinkers and associated reagent solvents during the interaction experiment that can adversely aspects of the cell biology being studied. These photo-activable amino acids have several advantages:

- *In vivo* labeling of proteins using normal cellular machinery of metabolically active cells.
- *In vivo* crosslinking of interacting proteins in the native cellular environment.
- Increased specificity as proteins correctly positioned at their interfaces within protein interaction domains are crosslinked.
- Efficient recovery after crosslinking.
- Compatible and easy to use as they are photo-stable.

When used in combination with specially formulated limiting media that is devoid of leucine and methionine, the photo-activable derivatives are treated like naturally occurring amino acids by the protein synthesis machinery within the cell. As a result, they can be substituted for leucine or methionine in the primary sequence of proteins

during catabolism and growth. Photo-Leucine and Photo-Methionine derivatives contain diazirine rings that activate when exposed to UV light to become reactive intermediates that form covalent bonds with nearby protein side chains and backbones. Naturally associating binding partners within the cell can be instantly trapped by photoactivation of the diazirine-containing proteins in the cultured cells. Crosslinked protein complexes can be detected by decreased mobility on SDS-PAGE followed by Western blot detection.

Based on earlier work, using computer predictions and similarity with other TonB-dependent transporters, for which crystal structures have been determined, a hypothetical model was proposed for gonococcal TbpA (20, 129). To investigate the surface-exposed regions in TbpA, the Hemagglutinin (HA) epitope tag was inserted to create site-directed mutants and the functional impacts of this mutagenesis were investigated. Of these mutants, insertions into putative loops 2, 9, and 11 resulted in variants that bound transferrin with wild-type affinity but could complete the iron acquisition process only in the presence of a functional TbpB protein (20), which supports the contention that TbpA and TbpB interact on the cell surface and could implicate putative loops 2, 9, and 11 as contributors to a TbpA-TbpB interaction. Another TbpA mutant was created by targeting the conserved motif in the plug domain. The triple alanine substitution mutant in TbpA maintained wild type transferrin binding affinity (153). The mutant was unable to grow on transferrin as a sole source of iron but TbpB expression compensated for this defect.

We are interested in investigating the characteristics of these mutants in terms of an interaction between TbpA and TbpB, using photo-activable crosslinkers. Table 4 lists the selected mutants to be studied, and Table 5 depicts the transferrin binding and growth capabilities of these mutants.

We hypothesize that TbpA and TbpB interact on the gonococcal cell surface. We believe that certain residues of TbpA and TbpB are important for the interaction between the two proteins. We would like to demonstrate the interaction between TbpA and TbpB and assess the factors which play a role in the interactive abilities between these two proteins, namely the presence of ligand and TonB derived energy.

This study will aid in better visualization of key structure-function relationships in the gonococcal transferrin binding proteins. Knowledge about the transferrin receptor complex, will give us a better understanding as to how these proteins function and the characteristics of the receptor that are expressed in the bacteria.

Table 4: HA-insertion mutants in TbpA selected for this study

Strains	Relevant characteristics	References
MCV515	L9HA$_{(750)}$ TbpA Lbp^{-} (*tbpA*▽HA *lbpB*::Ω)	238
MCV519	L11HA$_{(843)}$ ΔAla847 TbpA Lbp^{-} (*tbpA*▽HA *lbpB*::Ω)	238
MCV527	L2HA$_{(229)}$ TbpA Lbp^{-} (*tbpA*▽HA *lbpB*::Ω)	238
MCV260	TbpAE$_{120}$AY$_{121}$AE$_{122}$ATbpB^{+}	153
MCV511	L3HA$_{(343)}$ TbpA Lbp^{-} (*tbpA*∇HA *lbpB*::Ω)	238
MCV505	L5HA$_{(509)}$ TbpA Lbp^{-} (*tbpA*∇HA *lbpB*::Ω)	238

Table 5: Transferrin binding and growth capabilities of the selected site specific mutants in transferrin receptor components.

Mutant	Transferrin binding in presence of TbpB	Growth on transferrin in presence of TbpB	Transferrin-iron uptake in presence of TbpB
MCV515	+	+	Not Determined
MCV519	+	+	Not Determined
MCV527	+	+	Not Determined
MCV260	+	+	+
MCV511	-	-	Not determined

II. RESULTS:

A. Identification of interaction between TbpA and TbpB

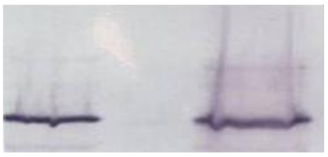
To study the effect of photo crosslinking on TbpA-TbpB interaction, gonococci were grown with photo-activable cross-linkers under iron depleted conditions. Interacting proteins, including the Tbps which are hypothesized to be situated extremely close to each other on the gonococcal cell surface would then be cross-linked by UV exposure. The shift or change in protein molecular mass was visualized by running protein samples on SDS-PAGE gels and transferred to nitrocellulose membrane. TbpA was detected using antibodies against TbpA and wild-type sized TbpB was detected by ligand-binding blot. As an evidence of cross-linking of TbpA-TbpB, we measured the loss of full-length TbpB. In the wild type strain we observed loss of TbpB under photo cross-linking conditions (figure 5 and 6); however we didn't detect specific higher molecular weight (>200 kDa, cross-linked) protein complexes due to their inability to migrate through the SDS-PAGE gel. In FA19 (the wild-type strain) photoactivation resulted in the formation of multiple cross-linked protein species containing TbpA (seen in lane 2 of figure 5A) and in the ligand binding blot it resulted in the loss of wild-type sized TbpB. We measured loss of full-length TbpB as an indication of TbpA-TbpB interaction. The loss of TbpB after photo crosslinking is shown graphically in figure 6. To check for specificity in terms of a potential interaction between Tbps, we used a *tbpA*$^{-}$ mutant gonococcal strain for analysis. In the *tbpA* mutant (FA6747), the wild-type TbpB protein was

detectable to the same degree with and without photoactivation (figure 5B and figure 6), indicating that the change in migration of TbpB was dependent upon the presence of TbpA. Photo-crosslinking altered TbpB migration, only in the presence of TbpA.

Figure 5: TbpA and TbpB detection in crosslinked samples of wildtype and TbpA mutant. Gonococcal strains FA19 (wild type), and FA6747 (TbpA$^-$ mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

(A)

FA19-TbpA^{+}TbpB^{+} (wild type)



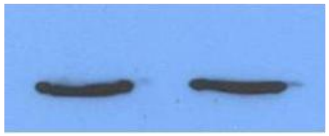
Photoactivation - +

(B)

FA19-TbpA^{+}TbpB^{+} (wild type)

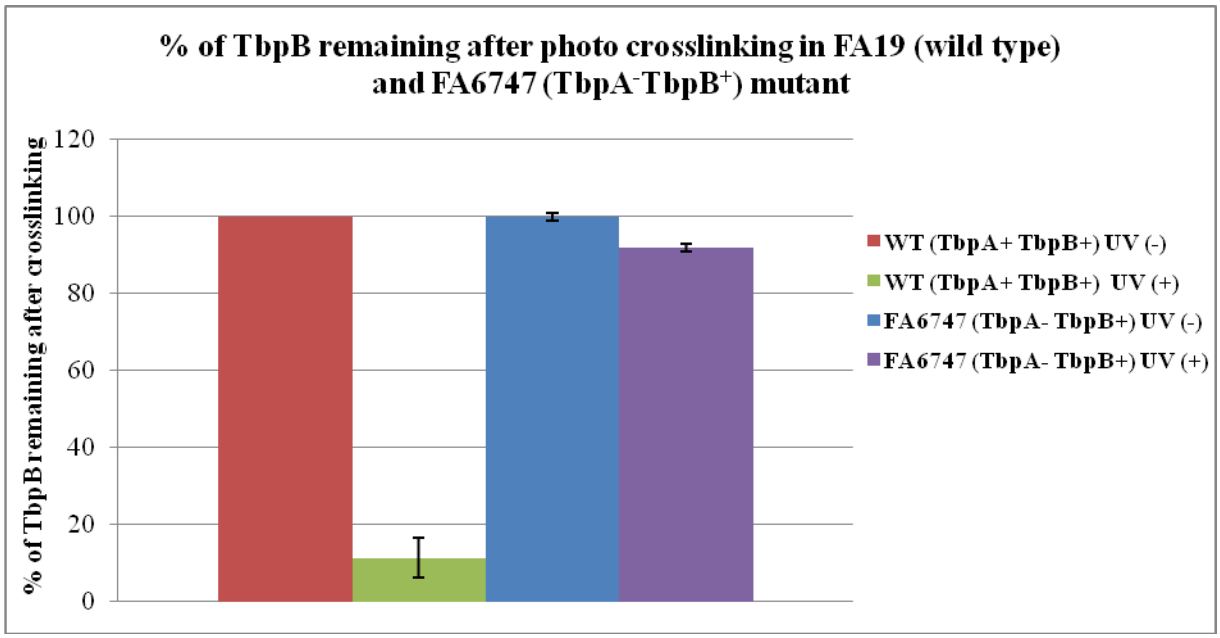
Photoactivation - +

FA6747-TbpA^{-}TbpB^{+} mutant



- +

Figure 5: Quantification of TbpB in the wildtype and TbpA mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV (-).


% of TbpB remaining after photo crosslinking in FA19 (wild type) and FA6747 (TbpA-TbpB+) mutant
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
FA6747 (TbpA- TbpB+) UV (-)
FA6747 (TbpA- TbpB+) UV (+)

B. Investigation of TbpA-TbpB interaction in HA-TbpA mutant

We tested selected HA insertion mutants (in the surface exposed loops of TbpA), to investigate whether they were competent for interaction between TbpA and TbpB. We selected loop mutants in positions L2, L3, L5, L9, L11 to be tested for studying the effect of crosslinking on interaction between TbpA and TbpB. Insertions into putative loops 2, 9, and 11 resulted in mutants that bound transferrin with wild-type affinity but could complete the iron acquisition process only in the presence of a functional TbpB protein (238). TbpB compensates for a surface exposed mutation in TbpA; this implicates loops 2, 9, and 11 as contributors to a TbpA-TbpB interaction. When we conducted ligand blot analysis, the results showed that the TbpA-TbpB interactions between the wild type strain and the loop 2 mutant (depicted in figures 7), loop 9 mutant (figures 9) and the loop 11 mutants (figures 11), were similar. Graphical representation of % of TbpB remaining after photo crosslinking gave similar results between the wild type and loop 2 (figure 8), loop 9 (figure 10) and loop 11 (figure 12) mutants.

Previous deletion data indicated that L5 contains residues essential for transferrin binding and utilization (20). The L5 HA-TbpA was unaffected in ligand binding or transferrin-iron utilization. We chose to look at the TbpB interaction abilities of the TbpA loop 5 HA mutant.

Figure 7: TbpA and TbpB detection in crosslinked samples of wildtype and L2 HA-TbpA mutant. Gonococcal strains FA19 (wild type), and MCV527 (L2 HA-TbpA mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

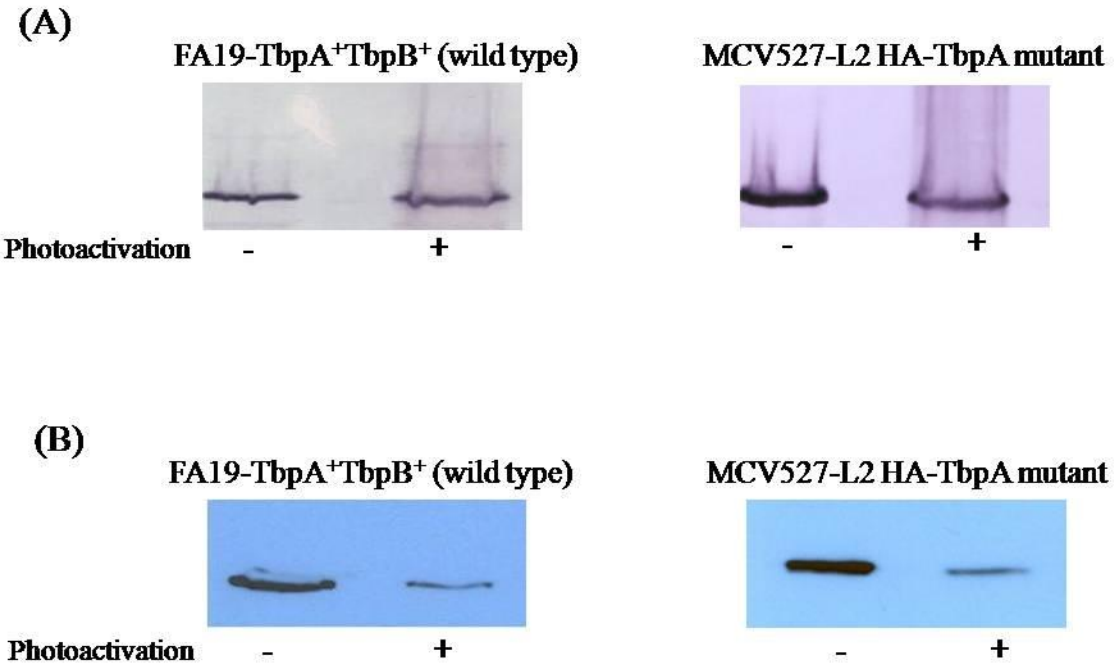

(A)
FA19-TbpA+TbpB+ (wild type)
MCV527-L2 HA-TbpA mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV527-L2 HA-TbpA mutant
Photoactivation
-
+
-
+

Figure 8: Quantification of TbpB in the wildtype and L2 HA-TbpA mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).

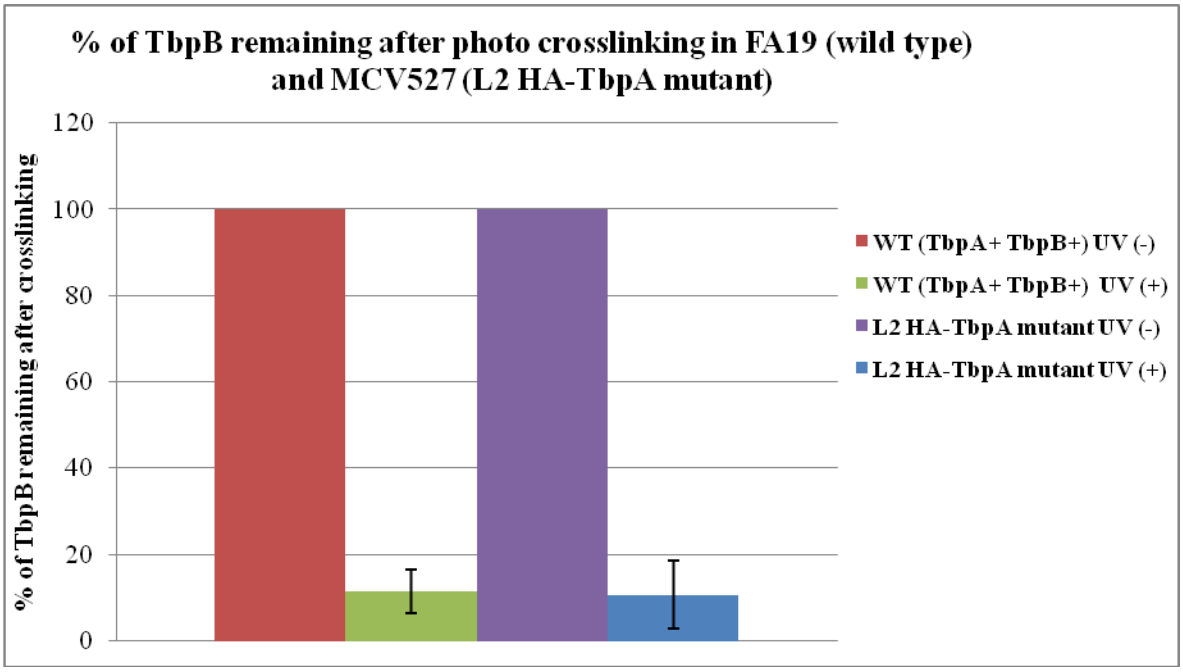

% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV527 (L2 HA-TbpA mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
L2 HA-TbpA mutant UV (-)
L2 HA-TbpA mutant UV (+)

Figure 9: TbpA and TbpB detection in crosslinked samples of wildtype and L9 HA-TbpA mutant. Gonococcal strains FA19 (wild type), and MCV515 (L9 HA-TbpA mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

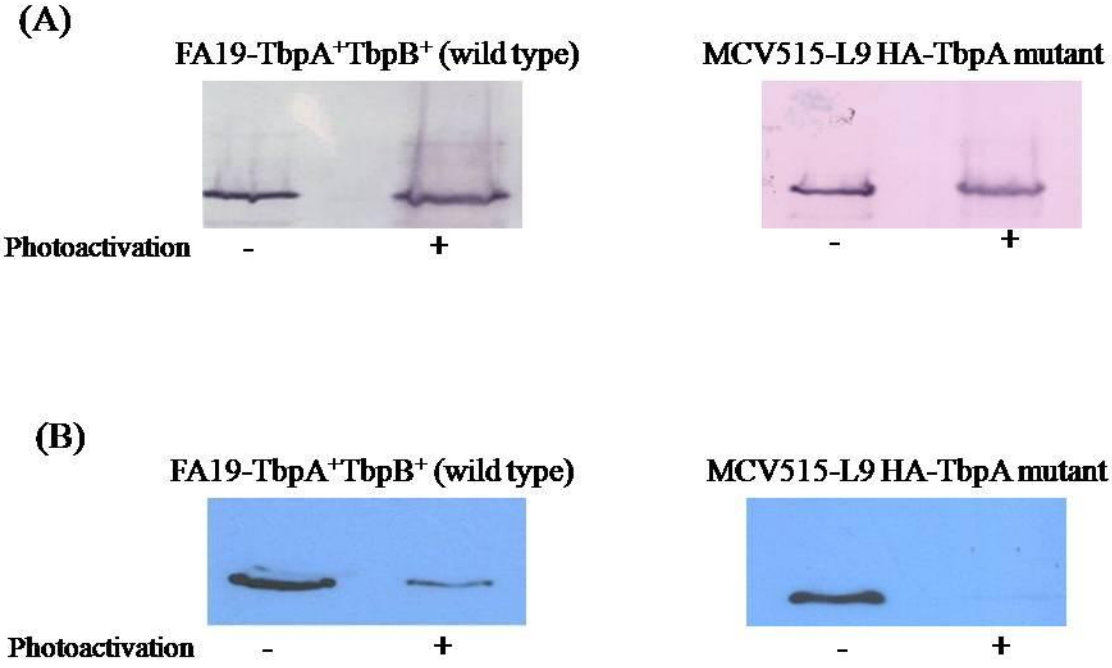

(A)
FA19-TbpA+TbpB+ (wild type)
MCV515-L9 HA-TbpA mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV515-L9 HA-TbpA mutant
Photoactivation
-
+
-
+

Figure 10: Quantification of TbpB in the wildtype and L9 HA-TbpA mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).

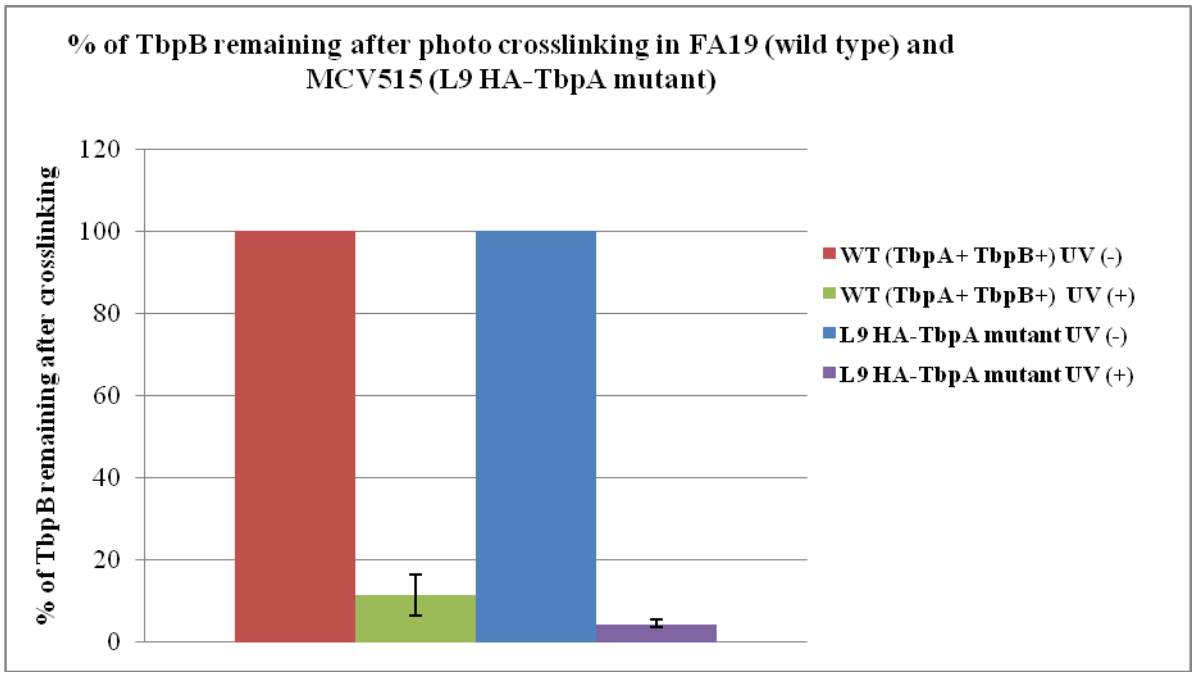

% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV515 (L9 HA-TbpA mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
L9 HA-TbpA mutant UV (-)
L9 HA-TbpA mutant UV (+)

Figure 11: TbpA and TbpB detection in crosslinked samples of wildtype and L11 HA-TbpA mutant. Gonococcal strains FA19 (wild type), and MCV519 (L11 HA-TbpA mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

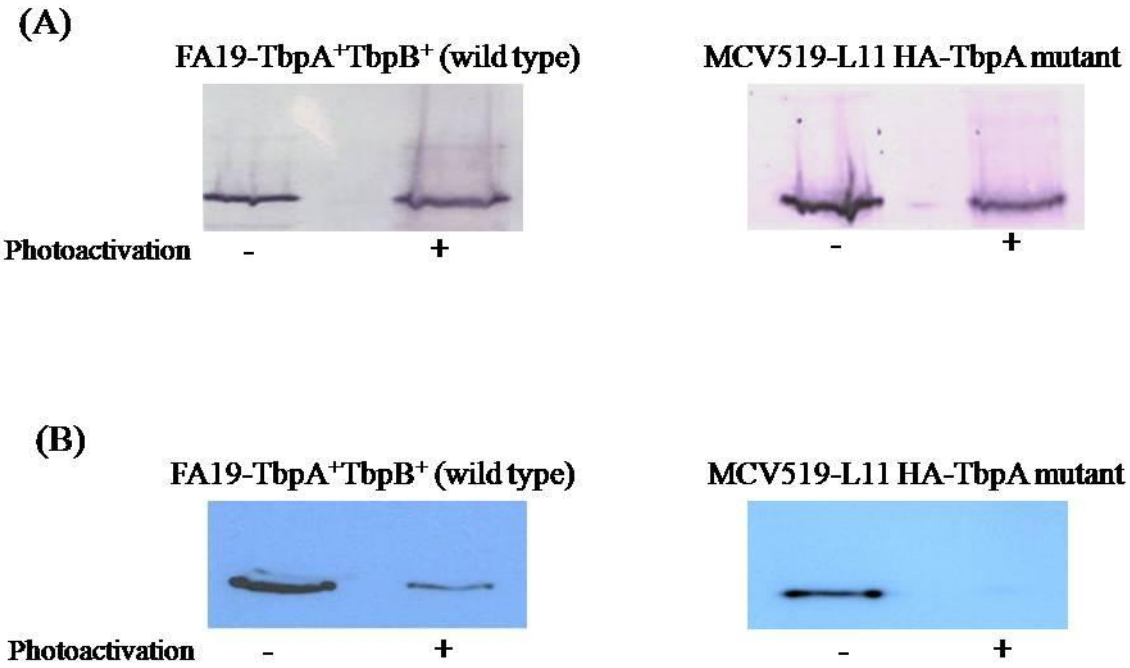
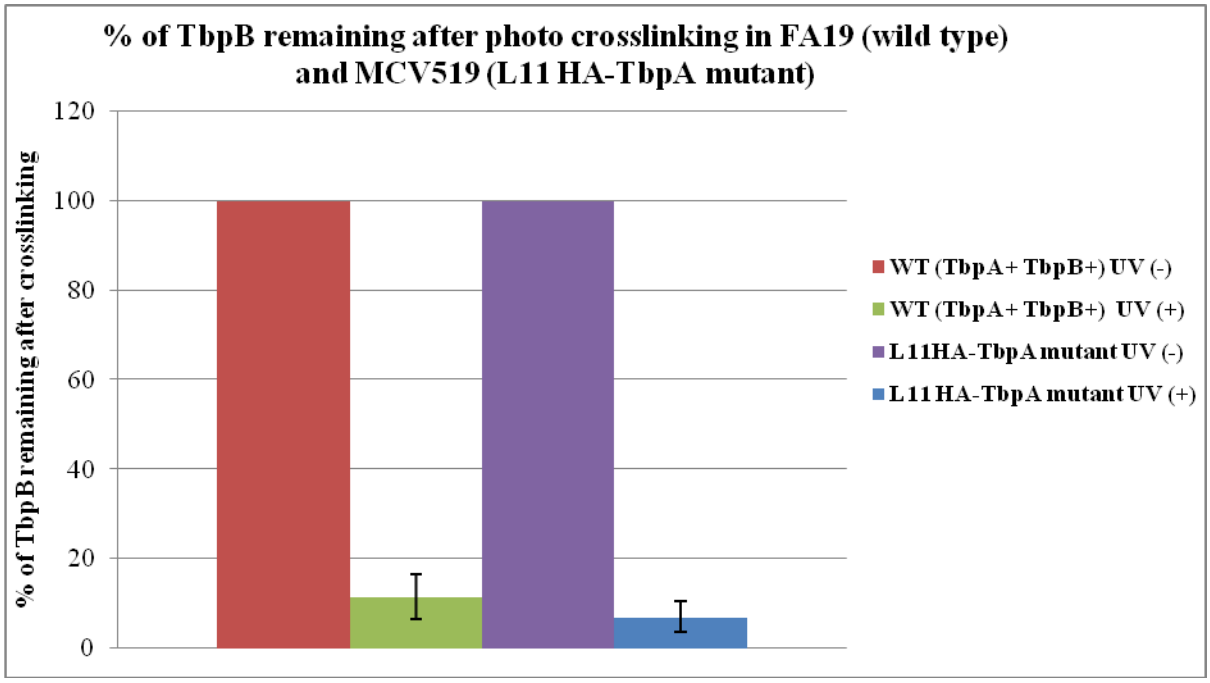

(A)
FA19-TbpA+TbpB+ (wild type)
MCV519-L11 HA-TbpA mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV519-L11 HA-TbpA mutant
Photoactivation
-
+
-
+

Figure 12: Quantification of TbpB in the wildtype and L11 HA-TbpA mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).


% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV519 (L11 HA-TbpA mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
L11HA-TbpA mutant UV (-)
L11HA-TbpA mutant UV (+)

Upon photo crosslinking, we observed that the L5 HA-TbpA mutant behaved in a similar manner to the wild type strain, with the TbpB migration changed after UV irradiation being similar between the wild type and the mutant (figures 12 and 13).

The L3HA mutant was not capable of transferrin binding; it was also unable to mediate the transferrin-iron uptake process. That makes the mutant interesting to study, since the inability to utilize transferrin-iron underlines the importance of this region. We found that the migration of TbpB after crosslinking in the L3 HA-TbpA mutant was similar to the wildtype (figures 15 and 16).

Previously in our lab, we created a TbpA triple alanine substitution mutant in a conserved motif of TbpA plug, with the intention of finding residues in TbpA important for iron utilization. This TbpA triple alanine mutant was defective in transferrin-iron uptake and utilization (153). To test the capability of this mutated TbpA plug to interact with wild type TbpB, we conducted photo crosslinking assays with this mutant. The results showed similarity between the TbpB migration after photo crosslinking between the wild type and the TbpA triple alanine mutant (figures 17 and 18).

Figure 13: TbpA and TbpB detection in crosslinked samples of wildtype and L5 HA-TbpA mutant. Gonococcal strains FA19 (wild type), and MCV505 (L5 HA-TbpA mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

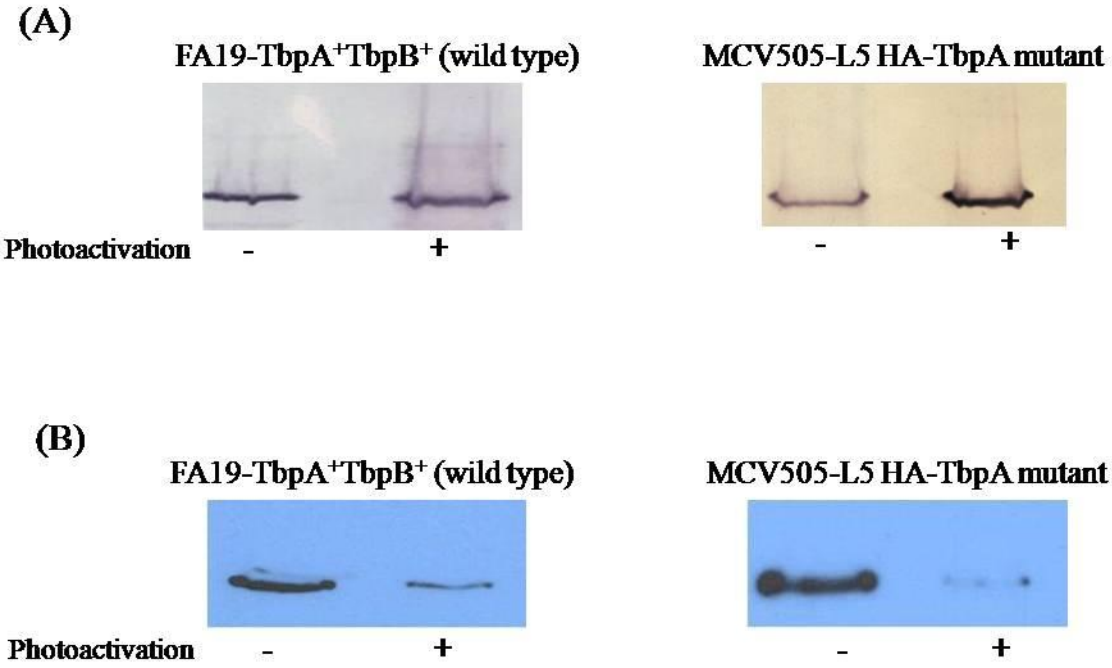

(A)
FA19-TbpA+TbpB+ (wild type)
MCV505-L5 HA-TbpA mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV505-L5 HA-TbpA mutant
Photoactivation
-
+
-
+

Figure 14: Quantification of TbpB in the wildtype and L5 HA-TbpA mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).

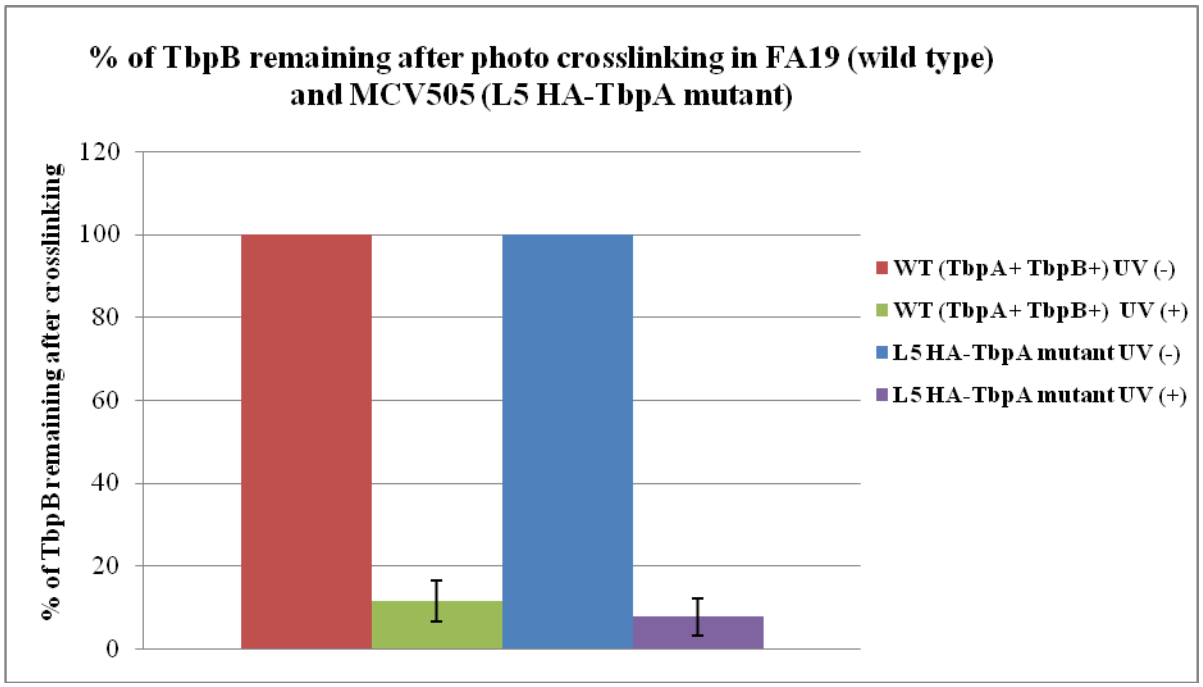

% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV505 (L5 HA-TbpA mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
L5 HA-TbpA mutant UV (-)
L5 HA-TbpA mutant UV (+)

Figure 15: TbpA and TbpB detection in crosslinked samples of wildtype and L3 HA-TbpA mutant. Gonococcal strains FA19 (wild type), and MCV511 (L3 HA-TbpA mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

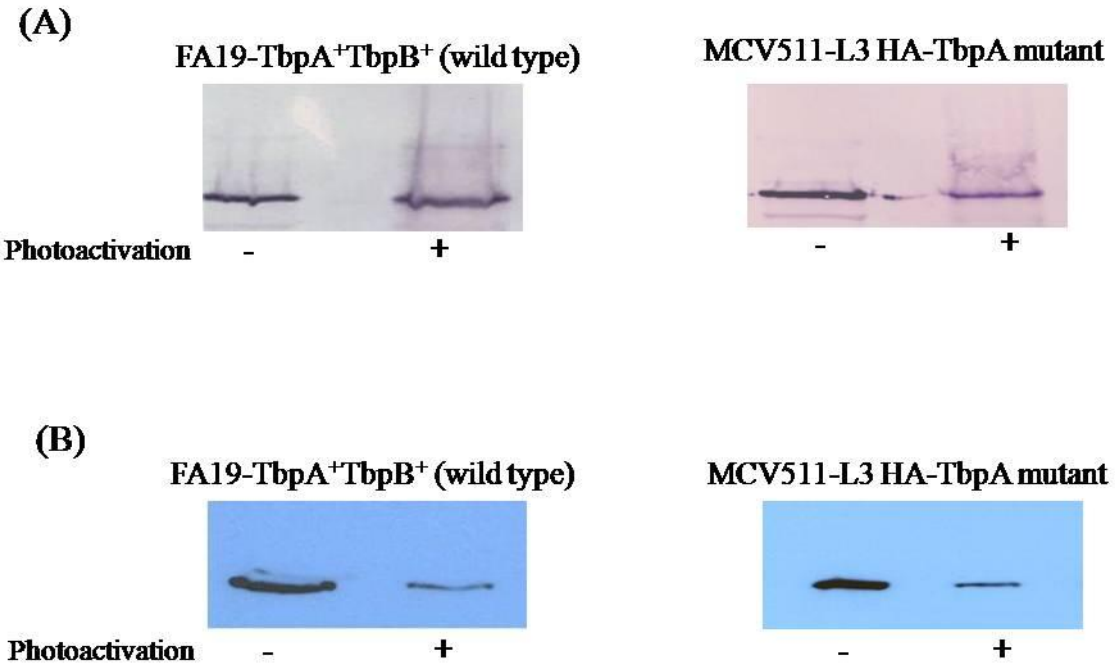

(A)
FA19-TbpA+TbpB+ (wild type)
MCV511-L3 HA-TbpA mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV511-L3 HA-TbpA mutant
Photoactivation
-
+
-
+

Figure 16: Quantification of TbpB in the wildtype and L3 HA-TbpA mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).

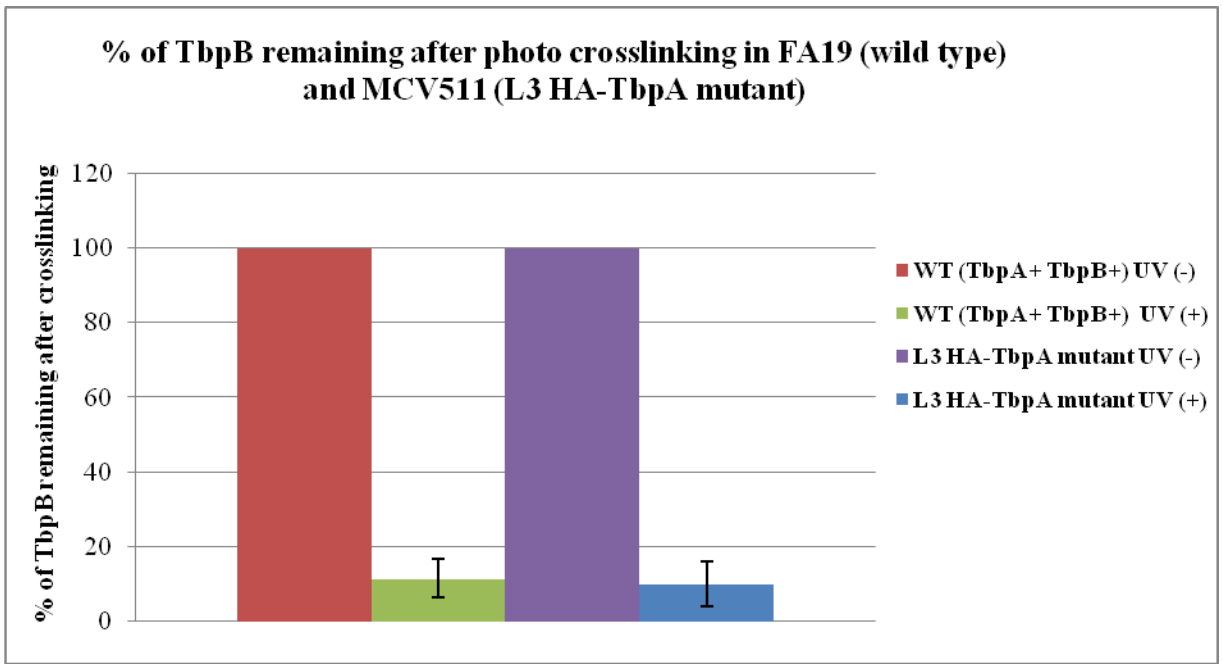

% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV511 (L3 HA-TbpA mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
L3 HA-TbpA mutant UV (-)
L3 HA-TbpA mutant UV (+)

Figure 17: TbpA and TbpB detection in crosslinked samples of wildtype and TbpA plug mutant. Gonococcal strains FA19 (wild type), and MCV260 (TbpA plug mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

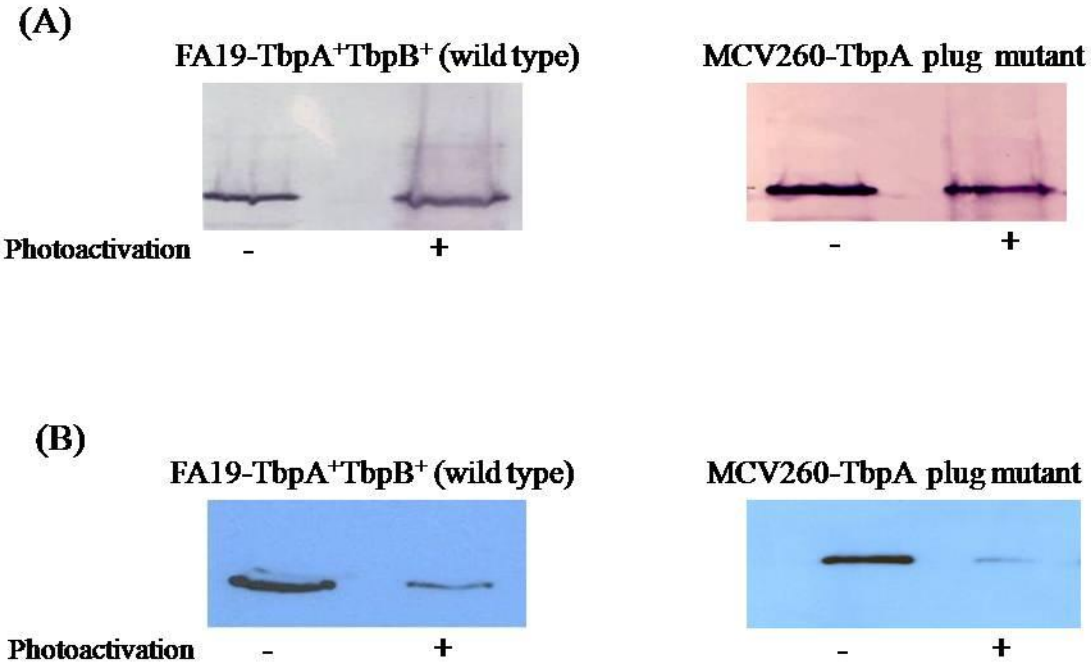
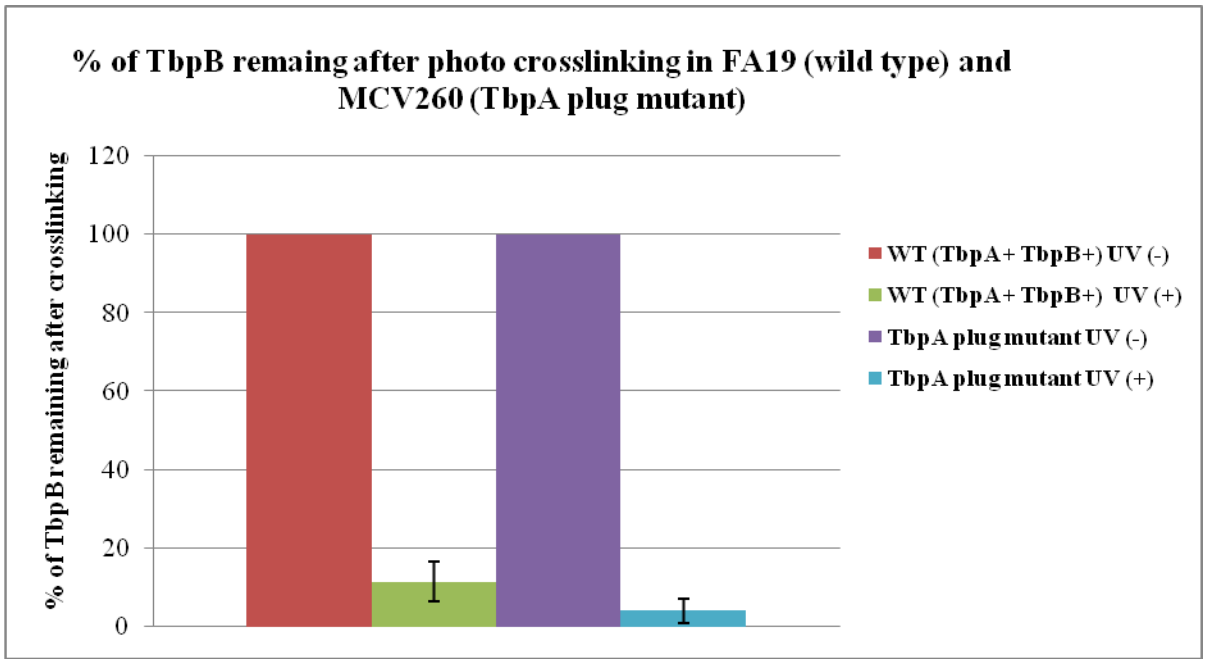

(A)
FA19-TbpA+TbpB+ (wild type)
MCV260-TbpA plug mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV260-TbpA plug mutant
Photoactivation
-
+
-
+

Figure 18: Quantification of TbpB in the wildtype and TbpA plug mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).


% of TbpB remaing after photo crosslinking in FA19 (wild type) and MCV260 (TbpA plug mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
TbpA plug mutant UV (-)
TbpA plug mutant UV (+)

C. Effect of ligand addition on TbpA-TbpB interaction

To assess the effect of addition of ligand to the interaction between the Tbps, we added 30% iron-saturated human transferrin to our photo crosslinkers containing iron depleted media and allowed for the wildtype gonococcal strain to grow in presence of ligand. TbpA presence was detected by using polyclonal antibodies against TbpA. The banding pattern in presence of ligand did not change much from what was observed without it. The reduction of TbpB after photo crosslinking was similar for both the conditions (figures 19 and 20). Binding of ligand does not alter the interaction between the Tbps in the wildtype.

D. Effect of TonB derived energy on the TbpA-TbpB interaction

We next tested the TonB mutant, by growing the mutant gonococcal strain with photo crosslinkers. We found that the TbpA immunoblots were similar between the wildtype and the TonB mutant in terms of detecting TbpA under non crosslinked and crosslinked conditions (figure 21-A). The amount of TbpB remaining after crosslinking is also similar between the wildtype and the TonB mutant (figure 21-B and 22). This indicates that there is interaction occurring between the Tbps, in the TonB mutant strain, implying that the interaction was demonstrated in the absence of TonB derived energy.

Figure 19: TbpA and TbpB detection in crosslinked samples of wildtype strain in absence and presence of transferrin. Gonococcal strain FA19 (wild type) was grown under iron-depleted conditions in the presence of photo-activable cross-linkers, with or without the addition of human transferrin. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

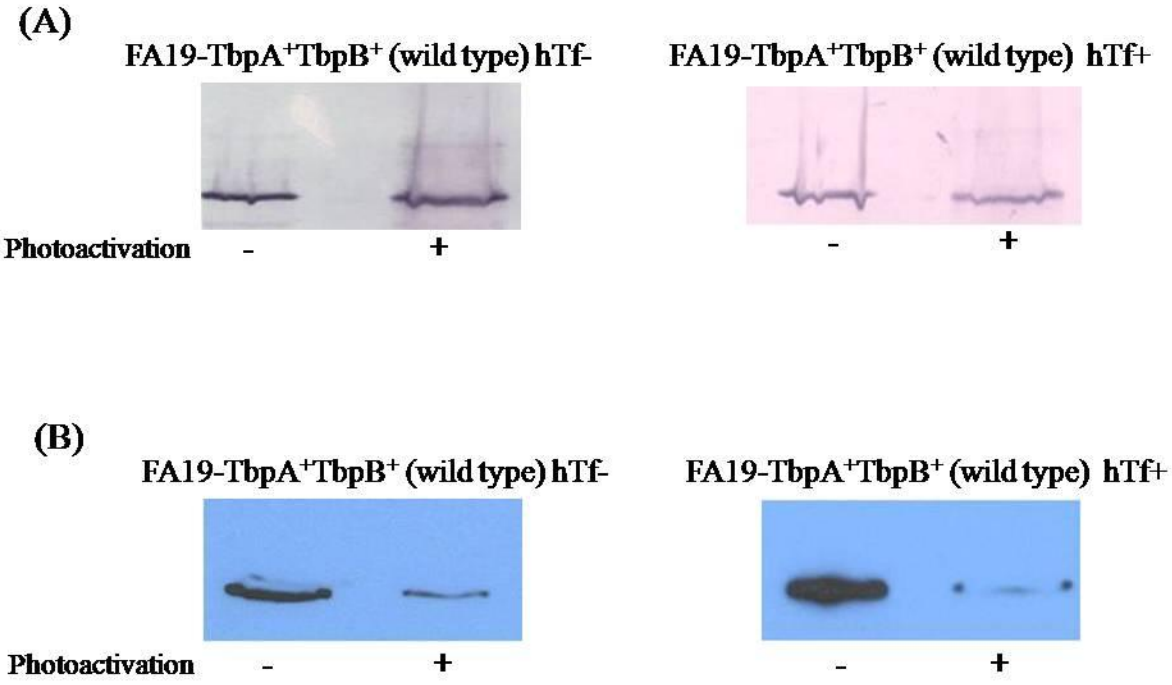

(A)
FA19-TbpA+TbpB+ (wild type) hTf-
FA19-TbpA+TbpB+ (wild type) hTf+
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type) hTf-
FA19-TbpA+TbpB+ (wild type) hTf+
Photoactivation
-
+
-
+

Figure 20: Quantification of TbpB in the wildtype strain in absence and presence of transferrin. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).

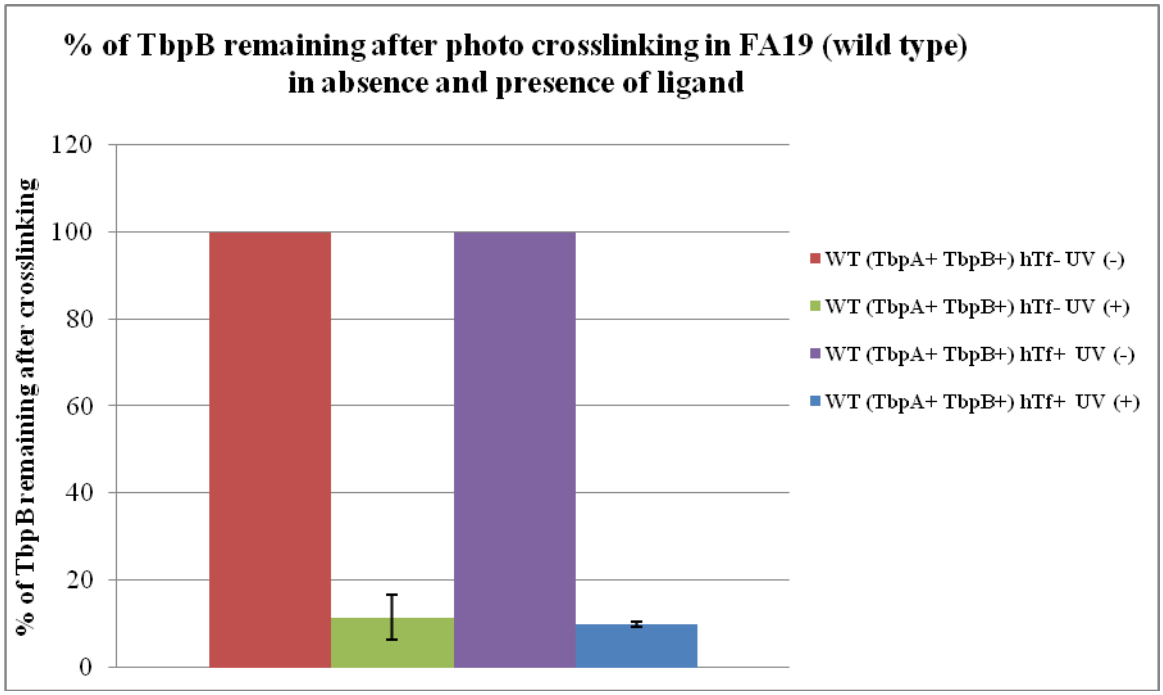

% of TbpB remaining after photo crosslinking in FA19 (wild type) in absence and presence of ligand
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) hTf- UV (-)
WT (TbpA+ TbpB+) hTf- UV (+)
WT (TbpA+ TbpB+) hTf+ UV (-)
WT (TbpA+ TbpB+) hTf+ UV (+)

Figure 21: TbpA and TbpB detection in crosslinked samples of wildtype and TonB mutant. Gonococcal strains FA19 (wild type), and MCV650 (TonB mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

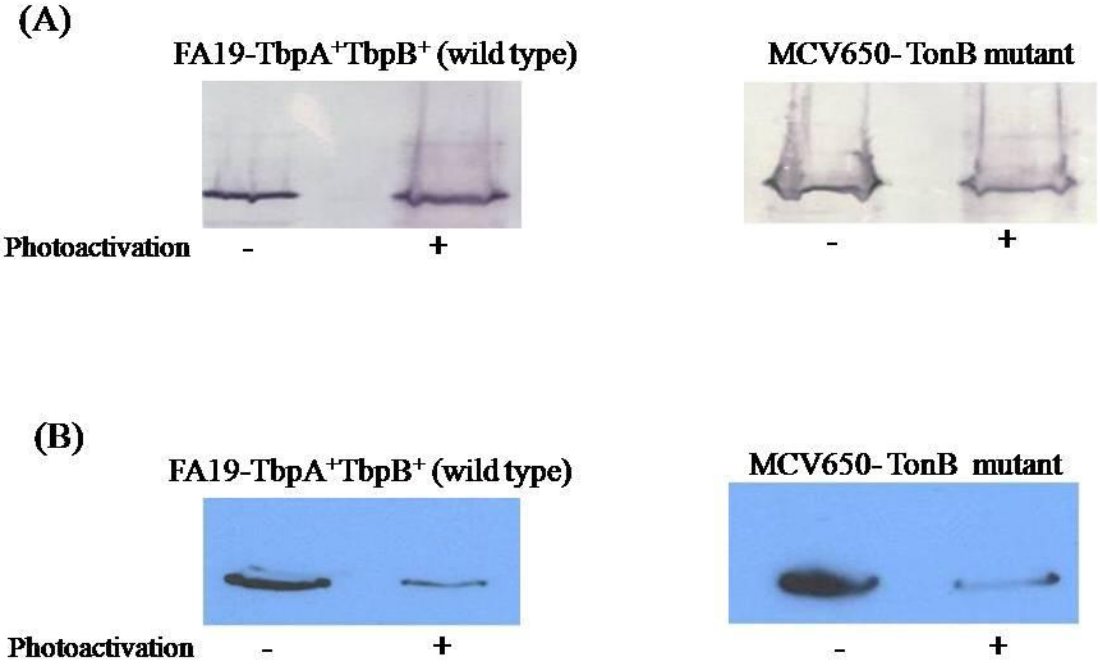
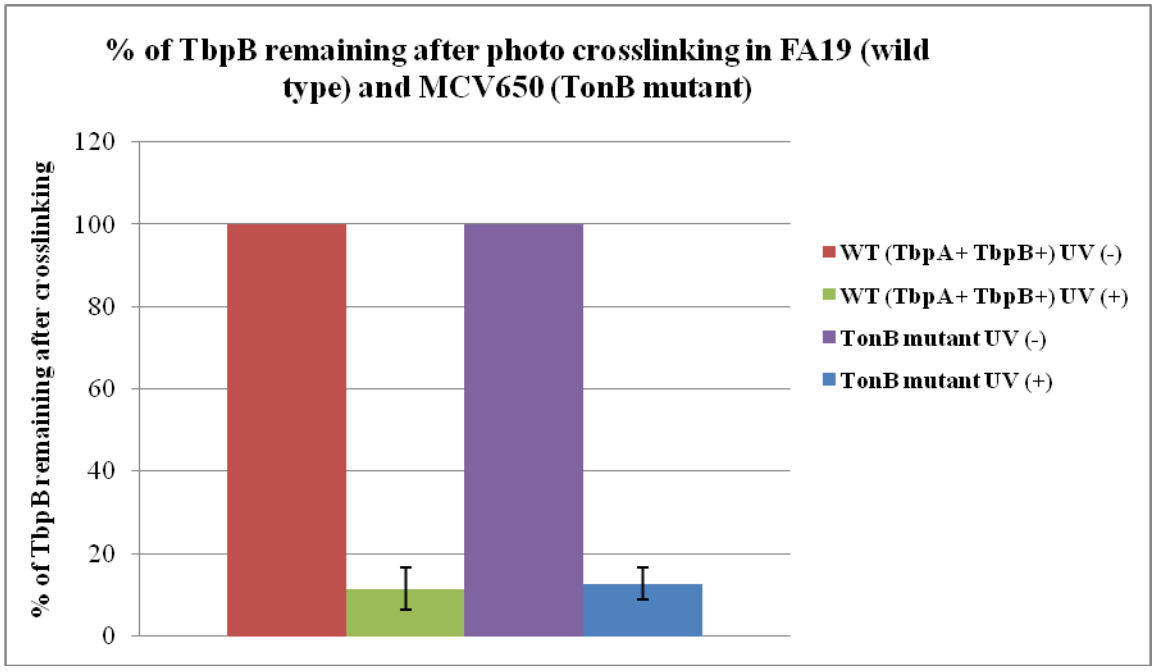

(A)
FA19-TbpA+TbpB+ (wild type)
MCV650- TonB mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV650- TonB mutant
Photoactivation
-
+
-
+

Figure 22: Quantification of TbpB in the wildtype and TonB mutant strains. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).


% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV650 (TonB mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
TonB mutant UV (-)
TonB mutant UV (+)

When we added ligand (30% iron-saturated transferrin) to the TonB mutant strain while growing with photo crosslinkers, we found that the amount of TbpB detected in the TonB mutant, in crosslinked and non-crosslinked conditions remains the same (figures 23 and 24). This is in sharp contrast to what we observed for the wildtype strain where under photo crosslinking condition there was loss of wildtype sized TbpB. The loss of TbpB due to crosslinking indicates an interaction between TbpA-TbpB in the wildtype and the above results indicate that in the TonB mutants addition of transferrin prevents the interaction between TbpA-TbpB.

Overall the data suggests that photo-crosslinking altered TbpB migration, only in the presence of TbpA. Upon addition of transferrin, we observed no significant difference in the TbpA-TbpB interaction. The HA epitope insertions in TbpA do not seem to cause disruption in TbpA-TbpB interaction. Binding of ligand does not alter the interaction between the Tbps in the wildtype, similar to the result when a TonB mutant was used for analysis, indicating that the TbpA-TbpB interaction is independent of TonB derived energy. However in the de-energized system, addition of ligand prevented TbpA-TbpB interaction.

Figure 23: TbpA and TbpB detection in crosslinked samples of wildtype and TonB mutant strains in presence of transferrin. Gonococcal TonB mutant strain was grown under iron-depleted conditions in the presence of photo-activable cross-linkers, with addition of human transferrin. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with α-TbpA serum (A) to detect TbpA, and hTf-HRP (B) to detect TbpB. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

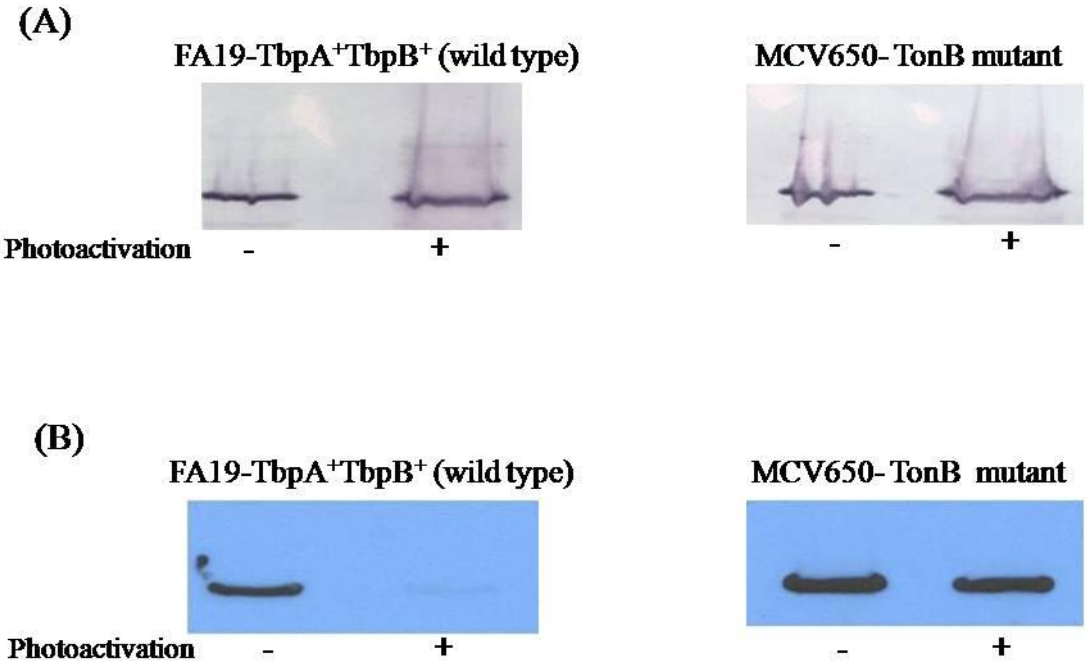
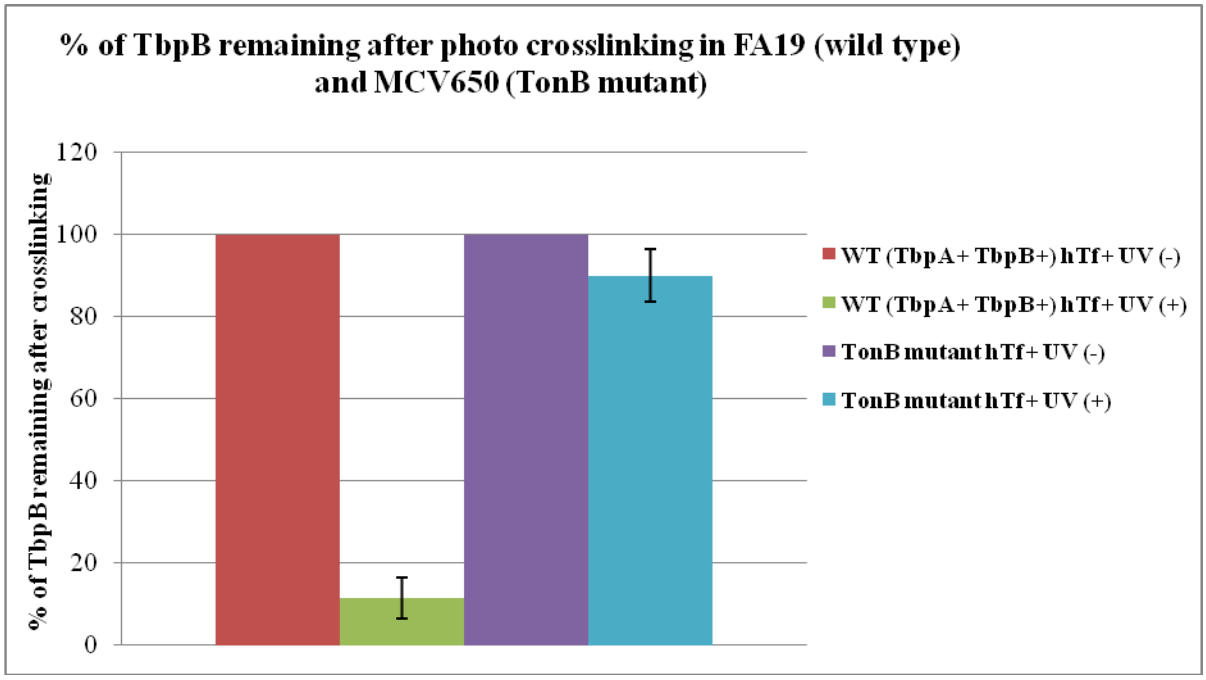

(A)
FA19-TbpA+TbpB+ (wild type)
MCV650- TonB mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV650- TonB mutant
Photoactivation
-
+
-
+

Figure 24: Quantification of TbpB in the wildtype and TonB mutant strains in presence of transferrin. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV(-).


% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV650 (TonB mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) hTf+ UV (-)
WT (TbpA+ TbpB+) hTf+ UV (+)
TonB mutant hTf+ UV (-)
TonB mutant hTf+ UV (+)

III. DISCUSSION

Several studies aimed at elucidating the structural and functional characteristics of the transferrin binding proteins have shown that the presence or absence of TbpA has a profound effect on the functionality of TbpB. A TonB-box mutation in TbpA showed that the energization state of TbpA had an effect on the protease accessibility of TbpB (48, 54). These observations suggested that both Tbps are in close proximity of one another on the cellular surface, and the TbpA energy-induced conformational change in TbpB could imply that TbpA and TbpB interact on the gonococcal surface. Co-immunoprecipitation assays conducted with the outer membrane fractions of gonococcal strains, led to the co-immunoprecipitation of TbpB using TbpA-specific antibodies (96). This suggests that there is some population of TbpB that interacts with TbpA on the cell surface.

We hypothesized that the presence of TbpA impacts the exposure or conformation of TbpB. In this study, we have utilized photoactivable cross-linkers to assess the effect of TbpA on TbpB in live gonococcal cells and studied it in the presence of ligand and TonB derived energy. We employed insertion mutants, in which TbpA contained the hemagglutinin (HA) epitope tag, to probe for the impact of TbpA mutagenesis on TbpB. The crosslinking approach has been successfully used before to study the interaction and recognition residues between *E. coli* periplasmic protein partners, CusF and CusB (127). These two proteins are part of the CusCFBA efflux system, which is involved in the metal extrusion from the periplasm to the extracellular space. The structural details of the interactions

between CusF and CusB were mapped through chemical cross-linking, coupled with high-resolution mass spectrometry (127).

In our study, photo crosslinking experiments demonstrated that in the wildtype gonococcal strain there was loss of wildtype size TbpB; this was absent in the TbpA mutant strain. Our results demonstrate that photo-cross-linking altered TbpB size and migration and was dependent on the presence of TbpA. HA epitope insertion mutants in surface exposed loops of TbpA did not impact the mobility of cross-linked TbpB. It was demonstrated that insertions into putative loops 2, 9, and 11 resulted in mutants that bound transferrin with wild-type affinity but could complete the iron acquisition process only in the presence of a functional TbpB protein (238). This suggests that TbpB can compensate for a mutation in TbpA, resulting in a functional receptor. Because TbpB is exposed on the outer membrane, the compensatory function of TbpB for overcoming the defect is bound to be on the cellular surface. This implicates putative loops 2, 9, and 11 as contributors to a TbpA-TbpB interaction. Our results indicate that the these loop 2, 9 ,11 mutants showed no hampering of the TbpA-TbpB interaction. These loops might not contribute to the interaction between the Tbps in the gonococci. The L3HA mutant was incapable of transferrin binding, (this mutant was stuck at the first step of transferrin-iron utilization process), and it was unable to mediate the iron uptake process, which is not surprising since the initial step of the process could not be accomplished (238). This indicates that L3 region could be important for transferrin binding and transferrin-iron acquisition process. This observation helped us choose the L3 HA mutant for analysis of the TbpA-TbpB interaction. Photo crosslinking experiments showed that the TbpA-TbpB interaction was not affected in this mutant and indicates that L3 might not be involved in the TbpA-TbpB interaction. In the L5HA mutant the insertion

region for the HA epitope tag was a variable region of TbpA. This mutant was unaffected in ligand binding or transferrin-iron utilization, and as per our crosslinking analysis it was unaffected in the TbpA-TbpB interaction. This might indicate that variable, surface-exposed residues are not critical for the function of the receptor but may be important for immune evasion and camouflage of the underlying conserved binding epitopes.

The triple alanine substitution mutants demonstrated decreased transferrin-iron internalization and maintained the ability to utilize transferrin as a sole iron source, only in the presence of TbpB (153), suggesting that for TbpB to compensate for TbpA mutation they may have to interact for this process. However this mutant also demonstrated no disruption of the TbpA-TbpB interaction in our study. This region of the plug might not play an important role in the interaction between the Tbps; however the plug does bind iron during the iron acquisition process (chapter 1).

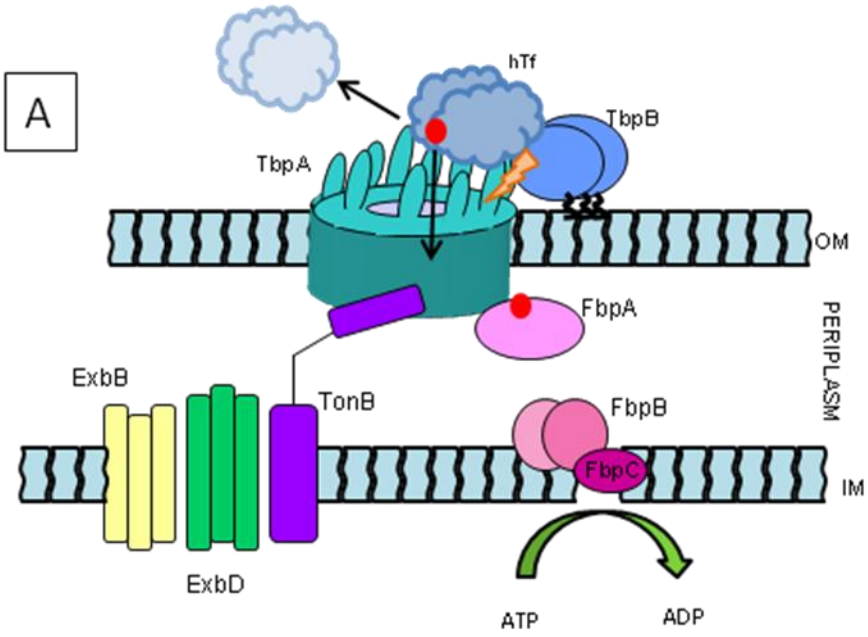
Creation of a TonB mutant (54) allowed us to investigate the effect of energy status on TbpA-TbpB interaction. Performing the crosslinking assay with the TonB mutants (both in absence and presence of ligand) enabled us to make key observations with the TbpA-TbpB interactions. In the TonB mutants, we observed that the de-energized state resulted in wildtype TbpA and TbpB migration after cross-linking. Addition of ligand to the wild type strain did not hamper the Tbps' interaction. However the addition of human transferrin to the de-energized mutant caused a change in TbpB migration after cross-linking. This result indicates that when ligand is bound tightly and irreversibly to TbpA, the accessibility and perhaps conformation of TbpB is altered.

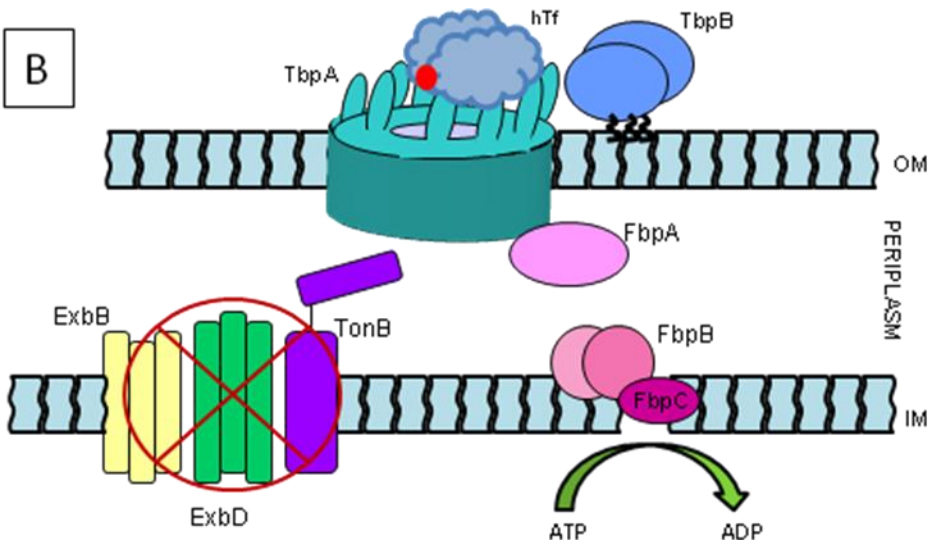
In the TonB box mutants, TbpB seemed to show a difference in protease susceptibility assays indicating an energy-induced conformational change in TbpB (54). This observation

allowed the conclusion that the energization state of TbpA resulted in a conformational change in TbpB, consistent with a physical interaction between the two outer membrane proteins. It was speculated that the transient TbpA-TbpB interaction occurs only when TbpA is energized. To illustrate our findings in context of the gonococcal transferrin mediated iron acquisition system, we present two models (figure 25-A and 25-B) to put forth a possible topology model for the difference in TbpB mobility and interaction with TbpA in a de-energized mutant. In presence of ligand, when TonB energizes TbpA**,** TbpA interacts with TbpB, impacting its migration following crosslinking. When ligand is locked on TbpA (occurs when TbpA is de-energized in a *tonB* mutant), TbpA cannot interact with TbpB. Our *in vivo* studies suggest that in presence of ligand, de-energized TbpA does not interact with TbpB.

Our conclusions from the crosslinking assays and its effects on TbpA-TbpB interactions have been confirmed by the recent crystal structures of the homologous meningococcal proteins (150, 151). These structural studies of TbpA-TbpB-ligand triple complex illustrate that Tbps bind ligand through unique, non-over lapping binding sites such that TbpA and TbpB do not interact. The X-ray, SAXS and EM structures support a consistent arrangement for the TbpA–TbpB–(holo)hTF complex, where TbpA and TbpB bind through unique, non-overlapping sites to assemble a triple complex. This assembled tri-complex encloses a chamber at the union of the three protein components, and the TbpA and TbpB do not seem to interact even though the walls of this chamber are comprised of TbpA-TbpB-hTf.

Figure 25: Model of conformational change in the gonococcal transferrin binding proteins. The gonococcal envelope consisting of outer membrane (OM), periplasm and inner membrane (IM) is shown, along with the proteins constituting the transferrin mediated iron acquisition pathway. Part (A) shows the model for conformation of the Tbps in energized state, after crosslinking, and in part (B) the conformations during the de-energized conditions are shown. In part (A), complex of TonB, ExbB, ExbD in the inner membrane transmits energy to TonB-dependent TbpA. When TbpA is energized and ligand is present, then upon crosslinking, TbpA interacts with TbpB and after iron extraction, apo-transferrin is recycled from the cell surface; this affects the migration pattern of TbpB. When TbpA is de-energized (figure 21 part B), the ligand gets locked onto TbpA, and this does not allow TbpA and TbpB to interact


A
hTf
TbpB
TbpA
OM
FbpA
PERIPLASM
ExbB
TonB
FbpB
FbpC
IM
ExbD
ATP
ADP


B
hTf
TbpB
TbpA
OM
FbpA
PERIPLASM
ExbB
TonB
FbpB
FbpC
IM
ExbD
ATP
ADP

CHAPTER 5: CONTRIBUTION OF TbpB DOMAINS TO INTERACTION BETWEEN GONOCOCCAL TRANSFERRIN BINDING PROTEINS

I. INTRODUCTION

Pathogenic Gram-negative bacteria rely on the surface-exposed transferrin receptor complex to acquire iron from host transferrin. TbpB, the second protein component of the gonococcal transferrin receptor is a surface exposed lipoprotein. TbpB is not required for iron internalization in the process of Tf-iron acquisition; it makes the process more efficient. The exact mechanism by which efficiency is enhanced is not known but a number of key studies have pointed out the importance of TbpB in transferrin mediated iron acquisition process. TbpB increases the efficiency by its ability to differentiate between apo- and holo-transferrin. Utilizing the relative transferrin-binding properties of both Tbps, it was showed that TbpB discriminated between ferrated transferrin and apo-transferrin, while TbpA did not (48). In solid-phase binding assays with isolated membranes, TbpA was shown to bind both apo and iron-loaded transferrin, however TbpB discriminated between apo and holo transferrin. In binding assays with TbpB and recombinant fusion proteins, it was observed that both intact TbpB and the N-terminal half of TbpB, preferentially bound iron-loaded transferrin (173). A mutant with deletion of *tbpB*, but expressing wild-type levels of TbpA, showed reduced binding and iron uptake from transferrin, but normal growth on transferrin plates. Mutants expressing only TbpB bound less transferrin, and did not take up iron from transferrin nor did they grow on transferrin plates. These results suggest that TbpB facilitates binding of transferrin but is not essential for acquisition of iron from transferrin (1).

To help understand the role of TbpB in transferrin iron utilization, a previous study in our lab evaluated the kinetics of the receptor proteins with regards to ligand association and dissociation (64). Transferrin association with TbpB was rapid as compared to TbpA. TbpA required both TonB and TbpB for efficient transferrin dissociation from the cell surface. It was proposed that TbpB makes the process of Tf-iron acquisition more efficient at least in part by affecting association and dissociation of transferrin from the cell surface (64). Using the SUPREX technique, for assessing the thermodynamic stability of protein –ligand complexes, it was reported that TbpB (individually or along with TbpA) can deferrate transferrin without energy supplied from TonB resulting in sequestration by apo-FbpA (191).

Recently X-ray crystallography, small-angle X-ray scattering and electron microscopy methods were used to look at the TbpB receptor from two *N. meningitidis* strains in its apo form and in complex with human transferrin (31, 151). This analysis was undertaken to elucidate how TbpA and TbpB function in binding human transferrin selectively and extract its tightly bound iron at physiological pH. TbpB is a bilobed protein; the two lobes are structurally similar. The N-terminal and C-terminal lobe are each subdivided into a 8-stranded β-barrel domain and an adjacent β-rich handle domain. The TbpB structures aligned closely with three TbpB structures from porcine pathogens (32, 136) and N-lobe of TbpB showed the major sequence and conformational variations. Residues affecting transferrin binding were situated on the distal surface of the N lobe (32, 50, 136, 192) and confer much of the transferrin species specificity. TbpB primarily binds the C lobe of human transferrin through its N lobe (151). It was also demonstrated

that the major site of interaction lies in the N lobe. TbpB stabilizes holo-human transferrin C-lobe by recruiting and sequestering holo- transferrin and maintains the iron-loaded status of transferrin until its delivery to TbpA.

To look at regions important in TbpB for transferrin binding and transferrin-iron uptake, HA epitope tagging was used. Nine insertion mutants were created, placing the HA epitope downstream of the signal peptidase II cleavage site and the impact of each insertion on the function of TbpB and the transferrin acquisition process was examined. The results showed that both the TbpB lobes (N-terminal and C-terminal lobes) are critical for efficient iron uptake from human transferrin (62, 150). These conclusions were further strengthened by studies with TbpB from *Neisseria meningitidis*, which showed that both N- and C-terminal domains together interact with human transferrin (171). These findings point out one of TbpB's contribution in the efficient binding of TbpB to human transferrin and thereby increasing the efficiency of the transferrin-iron acquisition pathway.

TbpB proteins are heterogeneous, with 69 to 84% sequence identity among *Neisseria gonorrhoeae* strains and 64 to 75% identity when gonococcal TbpB proteins are compared to those of *Neisseria meningitidis* (50). Regions of conservation have also been demonstrated between TbpB proteins of *N. gonorrhoeae*, *Actinobacillus pleuropneumoniae*, and *Moraxella catarrhalis*. Sequence analysis revealed conserved regions interspersed within hypervariable domains of TbpB (172, 176). It is unclear as to how or what portions of TbpB are involved in the protein's function.

One conserved region in TbpB is the LSAC region, located at the N-terminal domain of TbpB. This is a consensus sequence of lipoprotein modification/processing site of Leu-(Ala, Ser)-(Gly, Ala)-Cys at -3 to +1 positions, representing the cleavage region of 75% of lipoprotein signals in bacteria. Unmodified prolipoprotein with the consensus sequence undergoes sequential modification and processing, the steps involved are: prior to cleavage diacylglycerol group is transferred to the available sulfhydryl group of the cysteine, after cleavage of the prolipoprotein the free amine group of cysteine is acylated by N-acyl transferase to form mature lipoprotein (81, 235). The LSAC region located in the N-terminus of TbpB, contains the signal II peptidase cleavage site. This signal sequence is responsible for targeting TbpB through the inner membrane into the periplasm. The LSAC lipobox with the cysteine residue serves as the first amino acid in the mature lipoprotein and the site of lipidation.

Located just two amino acids downstream of the LSAC site is the second region of interest, consisting of four glycine residues adjacent to each other. The importance of this conserved poly-glycine stretch has still not been determined. This study aims to determine the importance of the two, above mentioned conserved regions of TbpB (the LSAC motif and the poly-glycine stretch), and the effect of mutations (in these two regions) on the protein translocation, transferrin mediated iron utilization and TbpA-TbpB interactions. HA epitope insertions mutants in homologous regions in the N-terminal and C-terminal of TbpB were also used for investigating the interaction between the transferrin binding proteins, thereby enabling us to study the importance of these regions in the interaction between the transferrin binding proteins.

II. RESULTS

Using alanine substitution mutagenesis, in gonococcal TbpB, the prototypical lipobox with the sequence LSAC was replaced with LAAA. The lipobox was replaced with a consensus signal I peptidase cleavage site. This would allow the cleavage and export of the lipoprotein but not the lipidation through the LSAC motif. The poly-glycine region in TbpB was deleted to create a glycine deletion mutant (TbpB Δgly). Both of these mutants were evaluated for lipidation and the translocation of the mature lipoprotein through the cytoplasmic membrane. Figure 26 depicts a linear model of TbpB, showing the LSAC and the glycine rich regions. This figure also shows the sites of HA4 and HA8 insertions in TbpB.

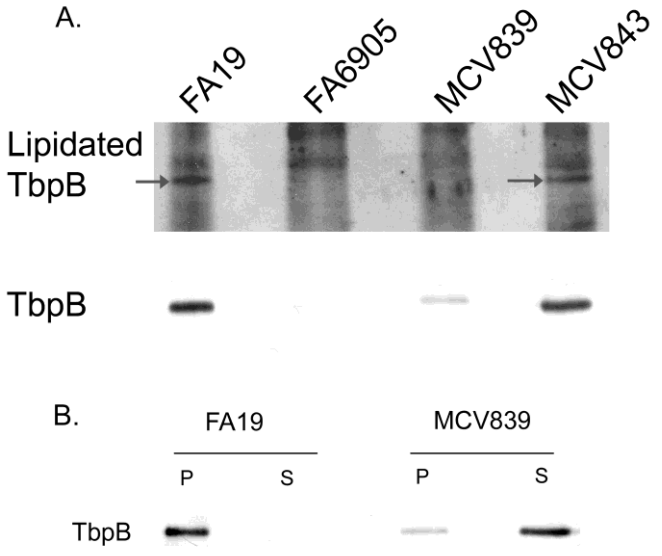
A. LSAC mutation abolishes lipidation in TbpB and decreases the amount of detectable, cell-associated TbpB

We determined the state of lipidation of TbpB. It was predicted that mutating LSAC (lipidation site) could lead to absence of lipidation in the LSAC mutant. Lipidation was prevented in the alanine substituted LSAC (LSAC-LAAA) mutant, whereas in the Δglycine mutant the lipidation that was observed was similar to what was observed in the wild type strain (figure 27, part A). These observations clearly point out that deletion of the poly-glycine stretch (2 amino acids downstream of the LSAC site) had no effect on lipidation, but the LSAC region is necessary for lipidation of TbpB.

Figure 26: Linear model of TbpB: the model depicts the N-lobe and C-lobe of the TbpB protein, with the LSAC site and the downstream glycine rich regions highlighted.

Linear model of TbpB

Figure 27: Effect of the LSAC mutation on TbpB lipidation and cellular localization. (A) Gonococci were grown under iron-depleted conditions in the presence of azide-labeled palmitic acid. Bacteria were lysed and azide-labeled proteins were conjugated to biotin-alkyne. Following separation by SDS-PAGE and transfer to nitrocellulose, proteins were probed with either anti-TbpB antiserum or avidin-HRP. Control strains included FA19 (wild type) and FA6905 ($TbpB^{-}$). **(B)** Gonococci were grown under iron-deplete conditions and harvested by centrifugation. The culture supernatant was treated with TCA to precipitate proteins. Following separation by SDS-PAGE and transfer to nitrocellulose, membranes were probed using anti-TbpB antiserum. Lanes labeled P contain proteins from the pelleted, whole cell fraction. Lanes labeled S contain proteins from the supernatant fraction.

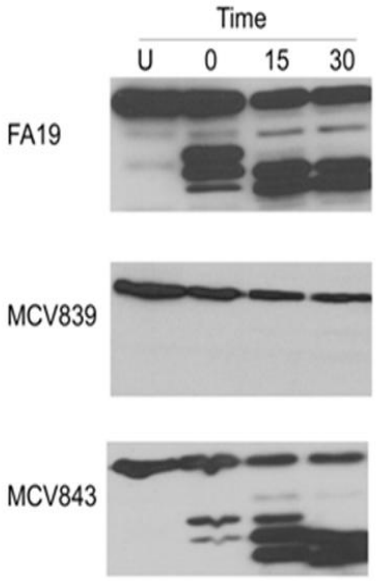

A.
FA19
FA6905
MCV839
MCV843
Lipidated
TbpB
TbpB
B.
FA19
MCV839
P
S
P
S
TbpB

With the absence of lipidation of the non-processed lipoprotein, it would seem difficult for the protein to be anchored to the outer membrane, due to the absence of a lipid moiety. To detect proteins released from the cell surface, TCA precipitation was conducted with the cell-free culture supernatant. Presence or absence of TbpB was investigated in the cellular pellet and the supernatant. In the wildtype strain most of the TbpB was present in the cell pellet, with less TbpB detected in the supernatant. The LSAC mutant showed some interesting results; majority of the TbpB was detected in the soluble fraction of the supernatant, lesser quantities of TbpB being detected in the cell pellet (figure 27, part B). This suggests that the absence of lipidation had no effect on the transport of TbpB across the outer membrane, but led to the absence of tethered-TbpB on the bacterial cell surface.

B. TbpB is surface accessible in the Δgly mutant but less so in the LSAC mutant

If the LSAC motif and the poly-glycine region played a role in the cellular transport to the surface, it would play a role in the surface exposure of the TbpB. To look at the surface accessibility of TbpB, protease accessibility assays were performed. The LSAC mutant upon exposure to trypsin, showed an absence of proteolytically digested (cell associated) products (figure 28). These results tell us that in the lipobox mutant, TbpB is not presented on the cellular surface and absence of cell-tethered TbpB peptides implies that the TbpB is not anchored to the gonococcal cell surface. In the Δglycine mutant, trypsin addition gave rise to

Figure 28: Surface exposure of TbpB. Bacteria were grown under iron depleted conditions prior to exposure to trypsin. WCLs were prepared following 0, 15, or 30 minutes of trypsin digestion. Undigested samples (U) were also included as a control. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with hTf-HRP. Strain FA19 was included as a wild-type control.

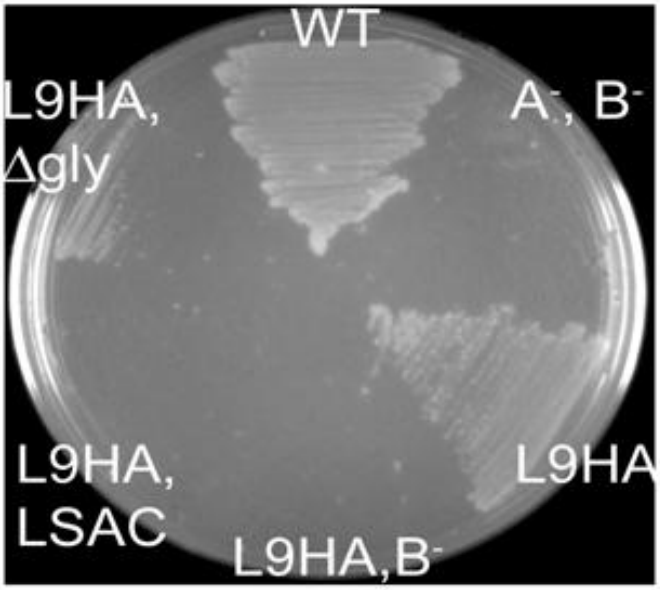

Time
U
0
15
30
FA19
MCV839
MCV843

protease-digested, cell-associated, small peptide fragments; this observation was similar to the wild type (figure 28), indicating that the TbpB was functional and tethered to the outer membrane.

C. Impact of TbpB mutagenesis on transferrin-iron transporter function

To test transferrin-iron internalization by the LSAC and the Δglycine mutated TbpB, double mutants were created by moving each of these mutations separately into a TbpA L9HA background, and then testing these double mutants for growth on transferrin as the sole source of iron. Gonococcal strain MCV515 is unique in the sense that it is capable of growth on transferrin only when TbpB is expressed. The insertion of HA epitope tag in the loop 9 of TbpA somehow disrupts epitopes in TbpA such that iron internalization occurs only when wild type TbpB is present in the strain. The MCV515 strain was able to grow on transferrin, the *tbpB* knockout strain showed no growth; this was a similar trend in the TbpB LSAC mutant, which showed no growth in presence of transferrin (figure 29). The TbpB Δglycine mutant showed highly reduced growth, more easily deciphered at lower saturation levels of transferrin (figure 29). These data inform us that the LSAC and the Δglycine mutations in TbpB affect the iron-transferrin transporter function of TbpB.

Figure 29: Effect of TbpB mutations on transferrin-iron utilization. Gonococci were plated on CDM agarose plates containing human transferrin. Strains tested include (clockwise, beginning at the top): (1) FA19 (WT), (2) FA6815 (A^-B^-), (3)MCV515 (L9HA), (4)MCV516 (L9HA, B^-), (5) MCV858 (L9HA, LSAC), and (6) MCV860 (L9HA, Δgly)



25%

D. Mutations of the conserved amino-terminal regions of TbpB does not impair interaction with TbpA

We utilized the cross-linking assay to evaluate the effects of LSAC and Δglycine mutations on the TbpA-TbpB interaction. We incorporated photo-activable methionine and leucine, into iron-depleted growth medium; and allowed the bacterial strains to grow in presence of these cross-linkers. The cultures were UV exposed to allow for cross-linking of interacting proteins, and then we identified full-length TbpB using a ligand binding blot. As an evidence of cross-linking of TbpA-TbpB, we measured the loss of full-length TbpB. In the wild type strain we observed loss of TbpB under photo cross-linking conditions; however we didn't detect any higher molecular weight (cross-linked) protein complexes due to their inability to traverse the SDS-PAGE gel. In the TbpA$^-$ strain, we observed that the intensity of TbpB being detected was same both in non-cross-linked and cross-linked conditions, which indicated to us that TbpA expression was important for the change in migration of TbpB (figure 30). The LSAC and Δglycine mutants showed similar migration patterns for TbpB, when compared to each other and the wildtype strain. When we graphically measured the percentage of TbpB remaining after photo cross linking, we observed that the decrease in TbpB in both the mutants was similar to the wild type strain (figures 31 and 32). Even though the LSAC mutated TbpB was not anchored on the outer membrane, it still possessed the capability of interacting with TbpA.

Figure 30: TbpB detection in crosslinked samples of TbpB mutants. Gonococcal strains FA19 (wild type), FA6747 ($TbpA^-$ mutant), MCV839 (LSAC mutant), and MCV843 (Δgly mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. TbpB was detected by probing the blots with HRP labeled Tf. Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

FA19-TbpA^{+}TbpB^{+} (wild type)

Photoactivation - +

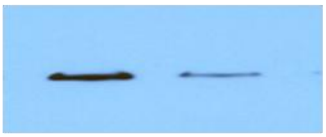
FA6747-TbpA^{-}TbpB^{+} mutant

- +

MCV839-TbpB LSAC mutant

Photoactivation - +

MCV843-TbpB Δgly mutant



- +

Figure 31: Quantification of TbpB in the wildtype and LSAC mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV (-).

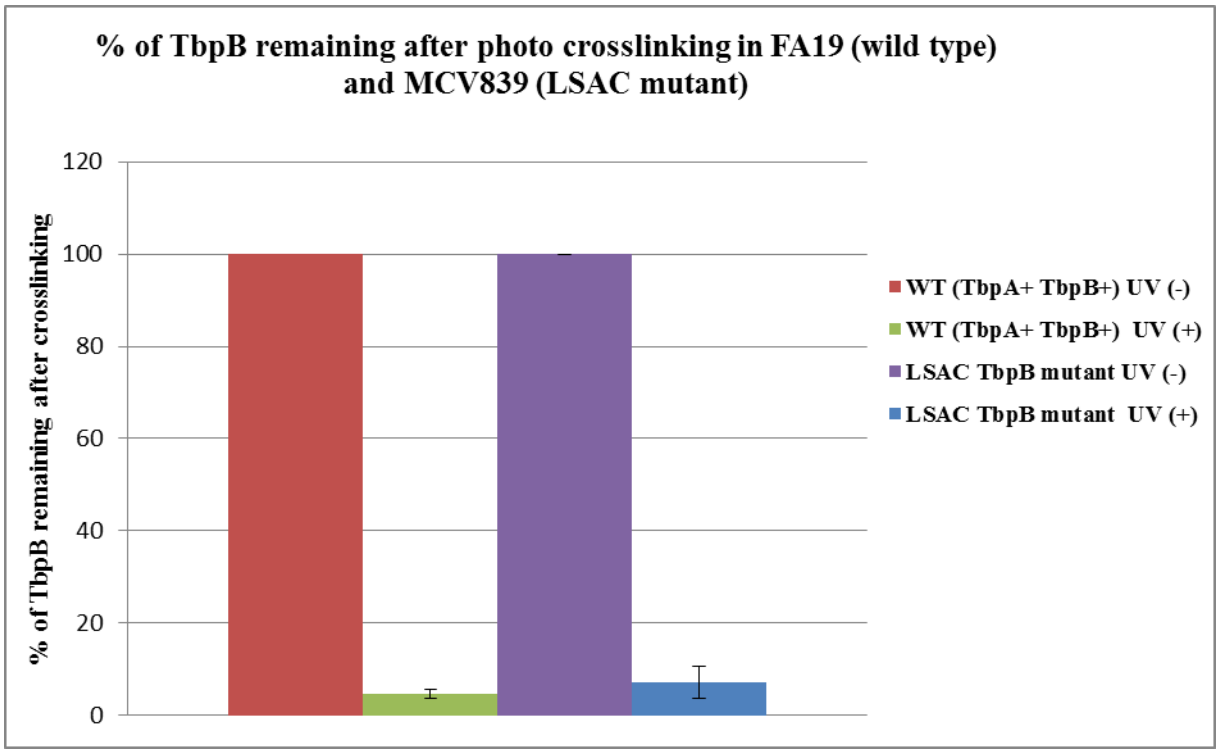
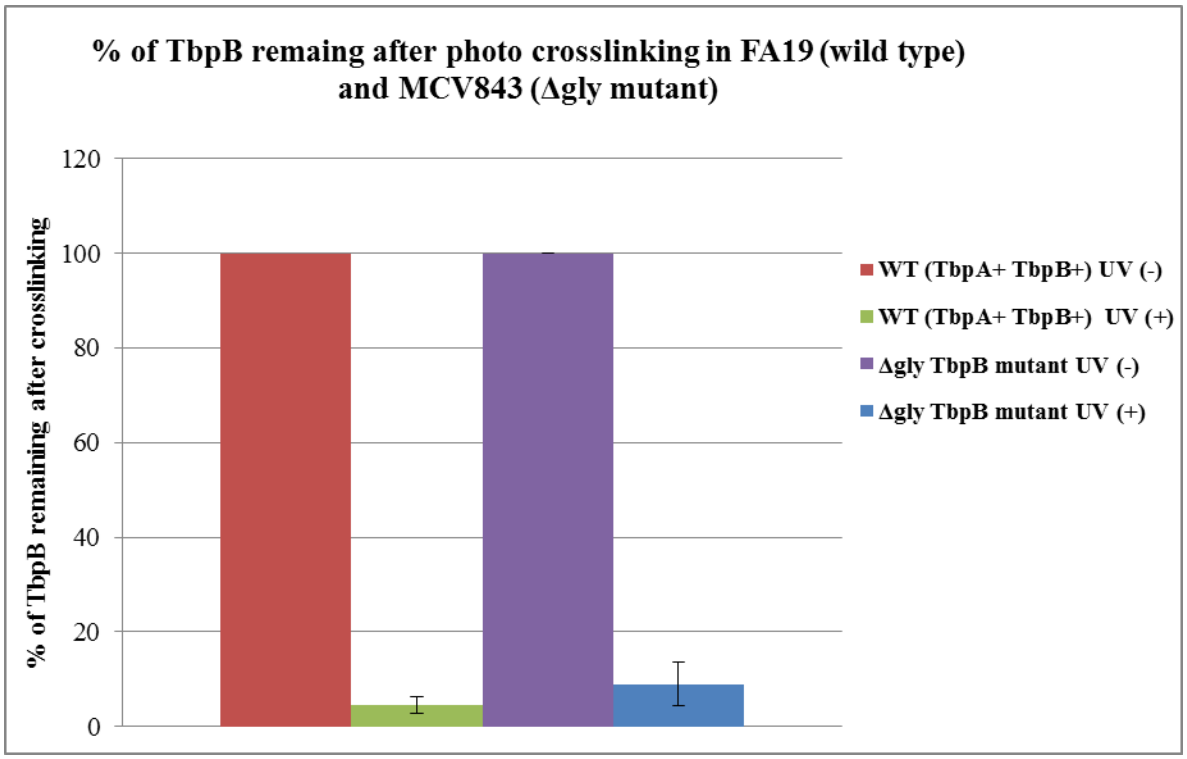

% of TbpB remaining after photo crosslinking in FA19 (wild type) and MCV839 (LSAC mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
LSAC TbpB mutant UV (-)
LSAC TbpB mutant UV (+)

Figure 32: Quantification of TbpB in the wildtype and Δgly mutant. TbpB was detected by ligand-binding blot, and expressed as percent of TbpB that remains at the wild-type molecular mass after cross-linking. Samples that are UV exposed are signified as UV (+) and that are non-UV exposed are noted as UV (-).


% of TbpB remaing after photo crosslinking in FA19 (wild type) and MCV843 (Δgly mutant)
% of TbpB remaining after crosslinking
120
100
80
60
40
20
0
WT (TbpA+ TbpB+) UV (-)
WT (TbpA+ TbpB+) UV (+)
Δgly TbpB mutant UV (-)
Δgly TbpB mutant UV (+)

We also used the HA epitope insertion mutants in TbpB, in the cross-linking assays. The TbpA and TbpB blots, with the non-crosslinked and crosslinked samples, showed no difference between the wildtype strain and the HA4 mutant (part A and B in figure 33). In contrast, the HA8 mutant showed no reduction of wild type sized TbpA and TbpB (part A and B in figure 34) after crosslinking, unlike the wild type strain.

Findings from this section suggest that the lipid-tether deficient TbpB and glycine-region deleted TbpB have not lost their capability of interacting with TbpA, despite mutations in conserved regions of TbpB. We observed that the HA insertions in positions 4 and 8 (depicted in figure 26) of TbpB protein, did not disrupt the interaction between the transferrin binding proteins.

III. DISCUSSION

Surface-exposed lipoproteins have been described in many Gram-negative bacteria, but the signals and machinery involved in their localization remain to be characterized (112, 114, 159). In *Neisseria*, the cellular surface shows the presence of the lipoproteins (including TbpB), which are tethered to the outer leaflet of the outer membrane by a lipid moiety (62). Lipoproteins are also present in other gram-negative bacteria, like *Klebsiella* (145), *Bordetella* (56), and *E. coli* (166); the mechanism for the transport of these lipoproteins across the cellular membranes is largely unknown. In *E. coli* the lipoproteins are associated

Figure 33: TbpB detection in crosslinked samples of TbpB mutants. Gonococcal strains FA19 (wild type) and MCV814 (HA4-TbpB mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. TbpA was detected by probing the blots with α-TbpA serum (A) and TbpB was detected by probing the blots with α-TbpB serum (B). Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).

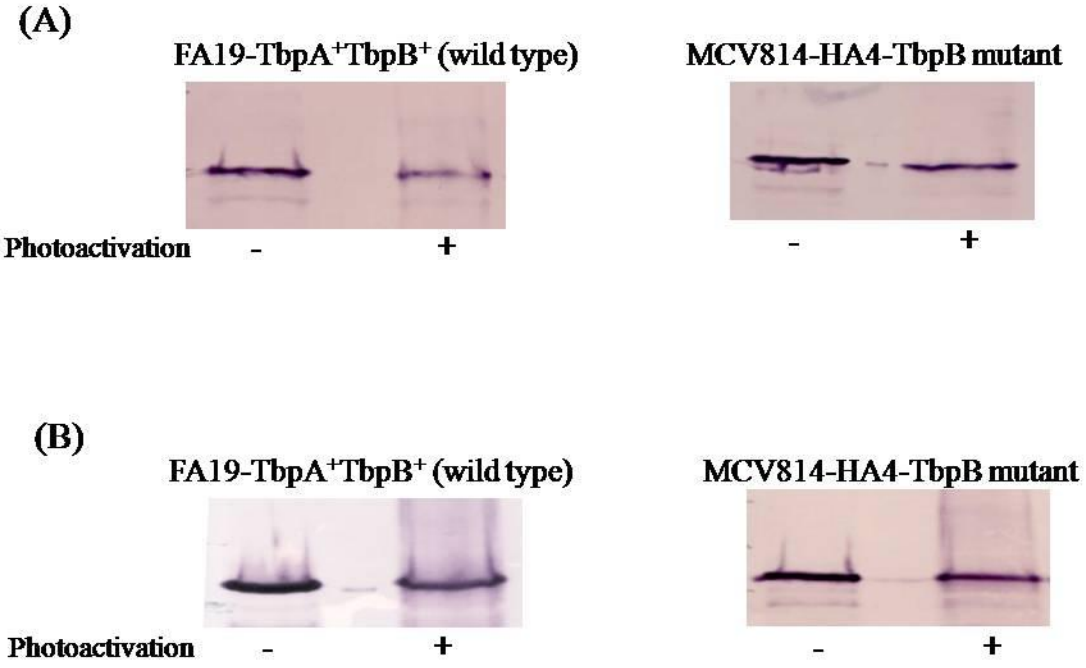
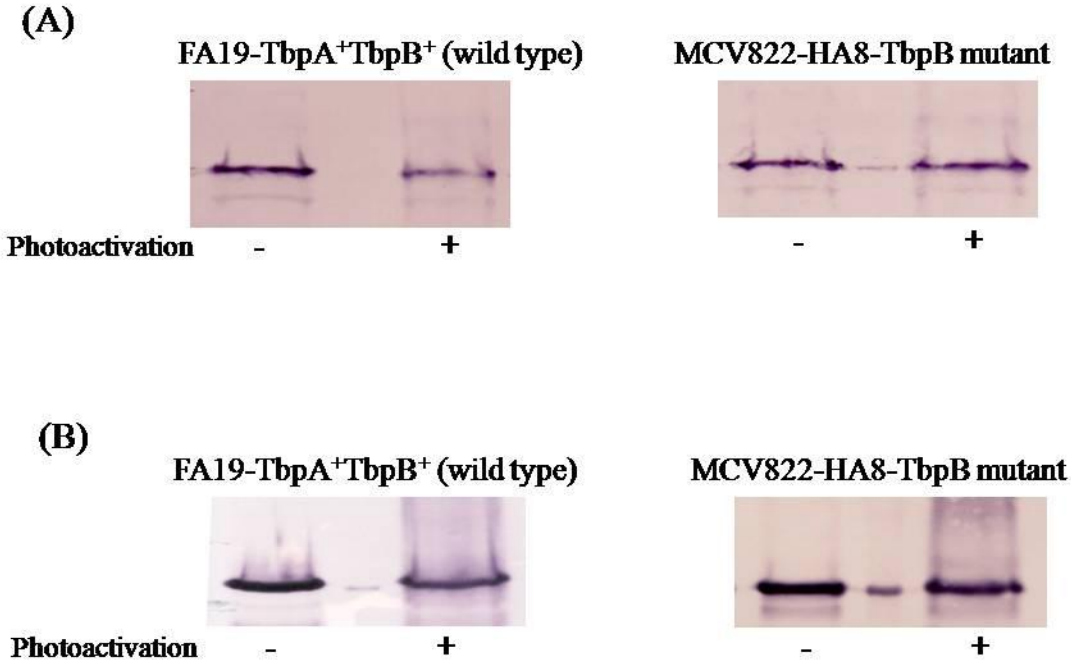

(A)
FA19-TbpA+TbpB+ (wild type)
MCV814-HA4-TbpB mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV814-HA4-TbpB mutant
Photoactivation
-
+
-
+

Figure 34: TbpB detection in crosslinked samples of TbpB mutants. Gonococcal strains FA19 (wild type) and MCV822 (HA8-TbpB mutant) were grown under iron-depleted conditions in the presence of photo-activable cross-linkers. Cultures were UV irradiated and whole cell lysates were prepared. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. TbpA was detected by probing the blots with α-TbpA serum (A) and TbpB was detected by probing the blots with α-TbpB serum (B). Samples that were UV exposed are labeled as (+) and non-UV exposed are labeled as (-).


(A)
FA19-TbpA+TbpB+ (wild type)
MCV822-HA8-TbpB mutant
Photoactivation
-
+
-
+
(B)
FA19-TbpA+TbpB+ (wild type)
MCV822-HA8-TbpB mutant
Photoactivation
-
+
-
+

with the cell surface and majority of the *E. coli* lipoproteins are localized to the inner leaflet of the outer membrane, on the periplasmic side (166). Many of these proteins are transported through the inner membrane with the help of the general secretory pathway. The Sec pathway that transports unfolded proteins into the periplasm consists of multiple subunits. Proteins that are targeted to the Sec system have an N-terminal signal sequence, with the lipoprotein signal II (lipoprotein peptidase cleavage site) being unique due to the cysteine residue. This signal sequence is responsible for targeting lipoproteins across the inner membrane. Unmodified prolipoprotein is processed in a sequential manner to form mature lipoprotein. (81, 235). The ABC transporter LolCDE binds these lipoproteins in the inner membrane, releases them to LolA in the periplasm. LolA binds at the lipid moiety of the protein, transports it through the periplasm and passes it onto to LolB. LolB, situated at the periplasmic side of the outer membrane, incorporates the lipoprotein into the outer membrane (210).

In the Gram-negative bacteria, the Lol system is well conserved (234). However, *Neisseria* possesses only some of the Lol protein homologues. Since lipoproteins do localize to the outer membrane in *Neisseria*, it is not known how the lipoproteins are transported from the inner membrane to the outer membrane and then flipped over to the outer leaflet of the outer membrane, unlike in *E. coli*.

In this study, we found that the TbpB mutant lacking the lipobox was released and present extracellularly after transport across the outer membrane; with the non-lipidated TbpB being retained less on the cellular surface. This indicates that the lipid moiety is essential for TbpB to be tethered to the outer leaflet of the outer membrane and lipid

modification was not necessary for transportation of TbpB into the extracellular matrix. However, the LSAC motif (lipobox) of TbpB is necessary and sufficient for lipidation as suggested by the palmitate labeling experiments. The cross-linking data showed the presence of interaction between TbpA and mutated TbpB (from the LSAC mutant). The presence of an interaction of TbpA with non-lipidated TbpB, suggests that this interaction is not dependent on lipidation of TbpB and the small amount of mutated TbpB present on the cellular surface or in the extracellular mileu, is still capable of interacting with TbpA, by the virtue of its affinity for TbpA. The surface-exposure of TbpB was important since lipid-modified TbpB, in the TbpA L9HA background, could not grow on transferrin as a sole source of iron. In the transferrin mediated iron utilization the non-lipidated TbpB is not able to assist TbpA for acquiring iron from transferrin; this could be due to its reduced presence on the cellular surface or the non-tethering of the lipoprotein on the outer membrane.

Though the poly-glycine stretch is only 4 amino-acids long, the deletion mutation in this conserved region brought up some interesting findings. The protease accessibility assay and solid-phase transferrin binding assay confirmed that the glycine deleted TbpB remained surface exposed. The Δgly mutated TbpB was lipidated as confirmed by the palmitate-labeling experiments. The TbpA-TbpB interaction was not affected by the deletion of the poly glycine stretch in TbpB, however the same mutation when expressed in the TbpA$^-$ background showed reduced growth in presence of transferrin. This indicates that the mutated TbpB is deficient in transferrin-iron utilization. These findings imply an important role of this poly-glycine region in TbpB and its function.

This sort of glycine-rich domain is also found in surface exposed lipoprotein (also a subtilisin-autotransporter) SphB1, in *Bordetella pertussis*. The N-terminal located glycine-rich and proline-rich sequences facilitate the surface presentation of the lipoproteins and the 14 residue long Glycine stretch is also hypothesized to be important in targeting the lipoprotein to the outer membrane. Several other surface lipoproteins, including *Neisseria meningitidis* TbpB and LbpB, hemoglobin (HpuA) receptor contain Gly-rich motifs following the modified Cys residue that are reminiscent of the Gly-rich stretch of SphB1(112, 114, 159). It is probable that such motifs are involved in the surface presentation of these lipoproteins. Therefore, the short glycine-rich region could function as an outer membrane localization signal, and may contribute to the surface presentation and/or stability of gonococcal TbpB.

Data presented in a previous study indicated that both the N- and C-terminal halves of gonococcal TbpB have the capacity to bind Tf, consistent with internal sequence conservation of TbpB (62). The analysis identified internally homologous regions within N- and C-terminal halves of TbpB. Homologous domains in N-terminal and C-terminal halves of TbpB were targeted for HA insertion mutagenesis (figure 1). The TbpB mutants with HA4 and HA8 insertions were similar with regard to surface binding and iron internalization. Interruption of either region in TbpB reduced transferrin binding and completely eliminated all known biological functions of gonococcal TbpB (62). These mutants showed a dissimilar phenotype when they were analyzed through crosslinking assays. The HA4 mutant showed similar interaction pattern to the wildtype strain. It seems that the regions of TbpB, targeted for HA insertions, are less accessible

and located within domains that could be partially hidden by other parts of the protein *in vivo*. Therefore the insertion mutations did not disrupt the interaction between the transferrin binding proteins. The HA8 TbpB mutant showed no diminishing of wild type TbpA and TbpB being detected after crosslinking when compared to the crosslinked condition and the wild type strain. This result indicates that *in vivo* the C-lobe of TbpB may participate in interacting with TbpA and iron import. These results highlight the critical contributions that each of these regions provides in the wild-type functions of gonococcal TbpB.

Recent structural characterization of the neisserial iron import machinery has helped refine our understanding of the manner in which TbpA and TbpB interact with human transferrin during transferrin mediated iron acquisition. Human transferrin is a bilobed glycoprotein (80 kDa) with each lobe consisting of two subdomains which form the cleft: N1, N2, C1 and C2. Human transferrin interacts with TbpA and TbpB solely through the C lobe. Whereas TbpA binds to both ferrated and deferrated forms of human transferrin, TbpB has a strong preference for human transferrin with iron bound in the C lobe (151). TbpB, which is tethered to the cell surface by an unstructured polypeptide chain, is able to preferentially interact with iron-loaded human transferrin. TbpB functions in selecting and capturing only those human transferrin entities, which are able to provide iron on the cell surface of the bacteria. Recent X-ray, SAXS and EM structures enabled in the study the TbpA-TbpB-(holo)hTf complex (150, 151). This triple complex showed presence of an enclosed chamber, formed at the union of the three protein partners such that TbpA and TbpB associate with different regions of the human transferrin C lobe; to allow for iron transport (151). Therefore TbpB is important

functionally to identify the iron-loaded and usable forms of human transferrin and to efficiently sequester iron released from human transferrin and directionally transport it to the TbpA barrel (through the formation of a potential iron chamber).

It has been shown in meningococcal TbpB, there is a presence of relatively unstructured N-terminal anchor peptide (composed of amino acids 2-40), which is associated with the C-terminal lobe of TbpB (236). It has been demonstrated that the presence of the N-terminal anchor peptide region on TbpB is required for interacting with TbpA in presence of transferrin. When the N-terminal lobe of TbpB binds transferrin, it displaces the anchor peptide from its interaction with the C-terminal lobe. This brings conformational changes in the anchor peptide, such that it moves the TbpB-Tf complex closer on the outer membrane, so that it is able to interact with "activated" TbpA (bound to TonB) (236). The anchor peptide region could extend TbpB sufficiently from the outer membrane surface to facilitate capture of iron-loaded transferrin in the presence of surface materials (193).

These observations when extrapolated to our study indicate that the poly glycine region could function as an anchor region in gonococcal TbpB. The presence of the 4 glycine residues could be important in modulating the position of the TbpB (relative to the outer membrane surface) and the formation of secondary structural features. These secondary conformations could potentiate an interaction with TbpA. The glycine deletion TbpB mutant was deficient in growth in presence of transferrin as a sole source of iron, because the TbpB was impaired in its ability to interact with TbpA subsequent to human

transferrin binding; thereby not allowing the iron internalization through the TbpA β-barrel conduit.

Through our study we have highlighted the functional importance of the two conserved regions (LSAC motif and the poly-glycine region) of gonococcal TbpB. The evidence presented here helps us to better understand the interactions occurring between TbpA-TbpB-human transferrin. This data provides a deeper understanding of the structural and functional aspects of two conserved regions in gonococcal TbpB.

CHAPTER 6: SUMMARY AND PERSPECTIVES

Pathogenic bacteria, such as *Neisseria,* have specific outer membrane receptors that bind to the host's glycoprotein transferrin. Transferrin is 80 kDa in molecular weight and large enough that it cannot pass through the bacterial outer membrane. Therefore, additional steps are required to remove iron from transferrin at the surface of the outer membrane. Extraction of the iron from transferrin is brought about by TbpA-TbpB, the two members of the transferrin-iron acquisition pathway. The mechanism of transferrin–iron acquisition by *N. gonorrhoeae* has been the subject of a great deal of study. The transferrin-iron acquisition system is composed of TbpA and TbpB protein members. TbpA, the TonB-dependent transporter, is the essential member and the necessary portal for iron travel through the outer membrane (52). TbpB, the surface-tethered lipoprotein, is important for increased efficiency of the iron acquisition system (1, 62). The gonococcal Tbp proteins are expected to interact through their interaction domains/residues. The precise mechanism and nature of interaction between the transferrin binding proteins is still poorly understood. The studies presented here helped in understanding the interaction phenomenon between the transferrin binding proteins and the importance of specific conserved regions in TbpA and TbpB in the overall process of transferrin mediated iron acquisition.

The iron acquisition process in *N. gonorrhoeae* can conceptually be divided into the following steps: recognition and binding of holo-transferrin at the TbpA/TbpB surface; release of iron from transferrin and removal of apo-transferrin from TbpA surface using TonB derived energy; passage of “free iron” through the β-barrel of TbpA;

binding of the iron at the periplasm by FbpA (which is a part of an ABC transport system); and finally the passage of iron to the cytosol with the help of FbpB and FbpC. One of the aims of our study was to investigate the iron binding and extraction properties of TbpA, specifically the conserved EIEYE motif in the TbpA plug domain. We also investigated the interactions between TbpA and TbpB and study the importance of TonB derived energy and ligand on these interactions. TbpB shares similarity with other surface-exposed lipoproteins and the presence of an accessory lipoprotein is one of the properties that makes the transferrin-iron acquisition system different from the siderophore mediated iron acquisition. We studied the contribution of two conserved regions in the TbpB protein (the LSAC and poly-glycine region) and their contribution to interaction between the transferrin binding proteins.

Recently, the crystal structures of components of the meningococcal transferrin–iron uptake system were reported (151, 189). The published reports indicate that TbpA is one of the largest TonB-dependent transporters. TbpA consists of an N-terminal globular plug domain loop that occluded the barrel pore and C-terminal β-barrel domain, comprised of 22 β-strand transmembrane-spanning domains, with surface exposed loops. Previous studies have suggested the importance of TbpA in transferrin utilization (238), with the plug domain being critical for transferrin-iron acquisition and specifically functioning in iron binding and uptake (154). Binding of Fe^{3+}-siderophore complexes to similar plug domains in outer membrane transporters during iron internalization has been reported for *E. coli* receptors FepA and FhuA, which indicates an important role for these plug domains in iron transport (69, 116, 215). Through sequence analysis it was shown that in the plug domain of TbpA, sequence EIEYE (118–122) is highly conserved. In a

triple-alanine substitution mutant of *tbpA* (EIAAA 118–122) there was an 80% reduction in transferrin-bound iron utilization (although the mutant binds transferrin with wild type affinity) (154). This shows the importance of the TbpA-plug sequence EIEYE in transferrin-iron utilization by gonococci. We hypothesized that iron released from transferrin at the TbpA surface interacts and binds with this plug sequence (EIEYE) and is subsequently transported through the β-barrel of TbpA. Sequestration of Fe^{3+} by the TbpA-plug supports the notion that the ferric iron must always remain chelated and controlled throughout the transport process. Observations from the first part of this study confirm that the EIEYE sequence in the wild-type TbpA plug binds Fe^{3+} during the outer membrane transport process. This work has provided in-depth knowledge about transferrin-iron internalization and is the first report of the expression/isolation of the recombinant wild-type TbpA plug. The binding event between the sequence EIEYE and Fe^{3+} is also supported by *in silico* modeling, depicting this sequence to be a part of a flexible loop that can reorganize more easily. The models also predict another important conserved sequence, which is surface exposed, as an important region for transferrin-iron utilization and shows a considerable conformational change between the wild type and recombinant plug. This 'top hat' region could act as a secondary recognition site for transferrin interaction during iron internalization process, and this observation needs further studies to be confirmed.

The gonococcal Tbp proteins are expected to work together during the transferrin-iron acquisition process, particularly in the presence of transferrin. The transferrin binding proteins function together as a unit to acquire iron from transferrin but the nature

in which they interact with transferrin is different for both of the proteins. In the second part of this study we studied the interaction between the transferrin binding proteins by utilizing crosslinking assays that included the use of photo crosslinkers. Our results demonstrate that photo-crosslinking altered TbpB size and migration and was dependent on the presence of TbpA. Previous studies using an HA epitope tagging approach had been reported to probe the regions of TbpB and TbpA that are important for transferrin binding and transferrin–iron uptake in gonococci (62, 123, 154). None of the HA epitope insertion mutants in surface exposed loops of TbpA and TbpB deviated from the interaction pattern that was seen in the wild type strain and the mutations did not impact the mobility of cross-linked TbpB. The addition of transferrin to wildtype strain did not change the interaction pattern of the transferrin binding proteins. Addition of transferrin to the de-energized mutant caused a change in TbpB migration after cross-linking. This result indicates that when ligand is bound tightly and irreversibly to TbpA, the surface accessibility and perhaps conformation of TbpB is altered. Recent structural studies of TbpA-TbpB-ligand triple complex (151) illustrate that transferrin binding proteins bind ligand through unique, non-over lapping binding sites such that TbpA and TbpB do not interact. Our *in vivo* studies suggest that in presence of ligand, de-energized TbpA does not interact with TbpB. While the structures have significantly advanced our understanding of the role of TbpA and TbpB in transferrin-iron acquisition pathway, more studies are needed to fully elucidate the role of TbpB and its interactions with TbpA.

The last aim of the current study was to determine the contribution of two conserved regions (the LSAC lipobox and the poly-glycine region), and the region with the HA4 and HA8 epitope insertions within TbpB (figure 1) to the interactions between the transferrin binding proteins. In a previous study, homologous domains in N-terminal and C-terminal halves of TbpB were targeted for HA insertion mutagenesis (figure 1) (62). The HA4 and HA8 TbpB insertion mutants were similar in surface binding and iron internalization. Both of these mutants showed reduced transferrin binding and completely eliminated all known biological functions of gonococcal TbpB (62). These mutants were dissimilar in their interaction analysis through crosslinking assays. The HA4 mutant showed similar interaction pattern to the wildtype strain, with no reduction in wildtype sized TbpA and TbpB. It seems that the region of TbpB, targeted for HA4 insertion, is located within a domain that could be partially hidden by other parts of the protein *in vivo* and the insertion did not disrupt the interaction between the transferrin binding proteins. The HA8 TbpB mutant showed no diminishing of wild type TbpA and TbpB being detected after crosslinking when compared to the crosslinked condition and the wild type strain. This result indicates that *in vivo* the C-lobe of TbpB may participate in interacting with TbpA and subsequently iron import. These results highlight the critical contributions that each of these regions provides in the wild-type functions of gonococcal TbpB.

We demonstrated that the lipobox, but not the glycine-rich domain, is required for lipidation of TbpB and tethering to the outer membrane. These results indicate that TbpB transport is lipoprotein-specific mechanism. When these mutant TbpB proteins were expressed in a strain simultaneously expressing a mutant form of TbpA that requires TbpB for iron acquisition, growth on transferrin was either completely abrogated or

dramatically diminished. We conclude that surface localization of TbpB is required for optimal performance of the transferrin-iron acquisition system, while the presence of the poly-glycine stretch near the amino-terminus of TbpB contributes significantly to the function of the transporter. These results provide important insights into the functional roles of two conserved regions of TbpB, furthering our understanding of this critical iron uptake system. Furthermore these observations may be broadly applied to other lipid-modified receptor proteins of the pathogenic *Neisseria.*

In search of a cross-protective gonococcal vaccine, focus is on receptors that are necessary for nutrient acquisition. One such nutrient is iron. Iron acquisition is critical for the bacterial survival *in vivo* and in tissue culture systems, and bacterial components involved in the iron acquisition process could represent conserved targets for vaccine development. Members of the transferrin-iron acquisition pathway, TbpA and TbpB are prospective vaccine candidates because TbpA and TbpB are not subject to high frequency phase or antigenic variation like other virulence factors of *Neisseria gonorrhoeae*, they are expressed by all gonococcal isolates (132), and they are required for initiation of infection in human male volunteers (53). The transferrin binding proteins of *Neisseria gonorrhoeae* and *Neisseria meningitidis* are similar in sequence (1, 47), suggesting that a vaccine comprised of these antigens could potentially protect against both gonococcal and meningococcal infections. In *Neisseria meningitidis* it has been shown that antibodies against transferrin binding proteins are bactericidal, cross-reactive and block the binding of transferrin (59, 177, 229). In *Neisseria gonorrhoeae* antibodies against recombinant gonococcal transferrin binding proteins are bactericidal and cross-reactive (164, 165). These observations highlight the potential that the transferrin binding proteins possess to

be developed as vaccine candidates. Identification of novel and important targets in TbpA and TbpB will aid in the development of vaccines against *Neisseria* infection.

Our results presented here, help in elucidating the involvement of both the transferrin binding proteins in the transferrin mediated iron acquisition pathway. With this knowledge we have gained more in-depth understanding of the requirements for a functional interaction between gonococcal TbpA and TbpB. By looking at the structural and functional properties of the gonococcal transferrin receptor, we have a better understanding of mechanisms by which the gonococcus acquires iron from human transferrin. Knowledge about the transferrin receptor proteins will give us a better understanding as to how these proteins function and the characteristics of the receptor that are expressed in the bacteria. This knowledge is essential for the rational design of a vaccine comprised of one or both of the transferrin binding proteins. Information derived about this receptor may be extended to other transferrin receptors such as those expressed in *N. meningitidis, Moraxella catarrhalis,* and *Haemophilus influenzae*.

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VITA

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PUBLICATIONS

Karen L. Ostberg, Amanda J. DeRocco, **Shreni D. Mistry**, and Cynthia N. Cornelissen. Conserved regions of Gonococcal TbpB that are critical for surface exposure and transferrin iron utilization. (in preparation)

Banerjee S, Parker Siburt CJ, **Mistry S**, Noto JM, Dearmond P, Fitzgerald MC, Lambert LA, Cornelissen CN, Crumbliss AL. Evidence of Fe(3+) interaction with the plug domain of the outer membrane transferrin receptor protein of *Neisseria gonorrhoeae*: implications for Fe transport. Metallomics. 2012 Apr;4(4):361-72

Rajendran G, **Mistry S**, Desai AJ, Archana G. Functional expression of *Escherichia coli fhuA* gene in *Rhizobium* spp. of *Cajanus cajan* provides growth advantage in presence of $Fe3^{+}$: ferrichrome as iron source. Arch Microbiol. 2007 Apr: 187(4):257-64

ABSTRACTS AND PRESENTATIONS

Shreni Mistry and Cynthia Nau Cornelissen. 2012. Characterization of potential interactions between transferrin binding proteins in *Neisseria gonorrhoeae*. 18th International Pathogenic Neisseria Conference (IPNC), Wurzburg, Germany

HONORS AND AWARDS

Graduate Student Travel Grant October 2012
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