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DRUG AND VACCINE DEVELOPMENT FOR NEISSERIA GONORRHOEAEA

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DRUG AND VACCINE DEVELOPMENT FOR *NEISSERIA GONORRHOEAEA*

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

By

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List of Symbols & Abbreviations

~	approximately
<	less than
%	percent
°C	degrees Celsius
α	alpha
β	beta
Δ	deletion
Ω	Omega
μg	microgram
μL	microliter
μM	micromolar
2D	two-dimensional
ABC	ATP-binding cassette
AP	alkaline phosphatase
ASPG-R	asialoglyco-protein receptor
ATP	adenosine tri-phosphate
Az	azithromycin
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate
bp	base pair
BSA	bovine serum albumin

bTf	bovine transferrin
C-	carboxy
CaCl ₂	calcium chloride
CDC	Centers for Disease Control and Prevention
CDM	chemically defined medium
CEACAM	carcinoembryonic related cell adhesion molecule
Cef	ceftriaxone
Cip	ciprofloxacin
Cm	chloramphenicol
CMP-NANA	cytidine-5'-monophospho-N-acetylneuraminic acid
CNS	central nervous system
CO ₂	carbon dioxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Fe	iron
⁵⁵ Fe	radioactive iron isotope
Fe ²⁺	ferrous
Fe ³⁺	ferric
Fe(NO ₃) ₃	ferric nitrate
fHbp	factor H binding protein
<i>g</i>	gravity
GCB	GC base medium
H ₂ O ₂	hydrogen peroxide
Hb	hemoglobin

HINT	hydropathic interactions
HIV	Human Immunodeficiency Virus
Hp	haptoglobin
HRP	horseradish peroxidase
hTf	human transferrin
IL-10	interleukin 10
IPTG	isopropyl β -D-thiogalactopyranoside
Kd	dissociation constant
<i>Kd</i>	<i>Kingella dentrificans</i>
KCN	potassium cyanide
kDa	kiloDalton
KU	Klett unit
L3H Δ	loop 3 helix deletion
L3HA	loop 3 hemagglutinin insertion
LB	Luria Bertani E. coli growth media
Lf	Lactoferrin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	molar
MgCl ₂	magnesium chloride
MIC	minimum inhibitory concentration
mL	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid

MSM	men who have sex with men
N-	amino
NaCl	sodium chloride
NadA	<i>Neisseria</i> adhesin A
NBT	nitroblue tetrazolium
Ng	<i>Neisseria gonorrhoeae</i>
NHBA	<i>Neisseria</i> heparin binding antigen
nM	nanomolar
Nm	<i>Neisseria meningitidis</i>
OD	optical density
OH-	hydroxide
OH·	hydroxyl radical
Opa	opacity protein
OSU	OSU-03012
PBP	periplasmic binding protein
PBP	penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PID	pelvic inflammatory disease
polyC	poly-Cytosine
polyG	poly-Guanine
RBS	ribosome binding site
RNA	ribonucleic acid

rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
Sil	sildenafil
STI	sexually transmitted infection
Tad	tadalafil
TBS	tris-buffered saline
TdT	TonB-dependent transporter
TGF- β	transforming growth factor beta
Th1	cell-mediated immunity
Th2	humoral immunity
Th17	innate immunity
Tf	transferrin
TfR1	human transferrin receptor
V	vehicle (DMSO)
WT	wild-type

Abstract

DRUG AND VACCINE DEVELOPMENT FOR *NEISSERIA GONORRHOEAEA*

By Devin Cash, B.S.

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

Virginia Commonwealth University, 2016

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Neisseria gonorrhoeae, the causative agent of the STI gonorrhea, is not preventable by vaccination and is rapidly developing resistance to antibiotics. One important strategy for gonococcal survival in the host is iron acquisition in the face of nutritional immunity. To overcome iron limitation, the gonococcus expresses TonB dependent transporters (TdT), outer membrane proteins that facilitate nutrient acquisition. Of the TdT, the transferrin (Tf), lactoferrin (Lf), and hemoglobin (Hb) receptors hijack iron directly from host proteins, and studies have already shown that the Tf receptor is essential for the initiation of human infection. Given that the TdT are virulence factors, they are widely conserved across strains, and are not subject to antigenic variation, they are ideal targets for novel therapeutics and

vaccine development. As such, studies exploring these proteins and their potential as vaccine candidates and antimicrobial targets are needed. In this study we report that loops of the Tf receptor protein TbpA are not strongly immunogenic, and the antibodies raised against them are incapable of inhibiting TbpA-Tf interactions on the gonococcal cell surface. We also report that the loop 3 helix motif of TbpA is a critical functional domain for Tf-binding and iron uptake; however, no single residue was identified that was essential for these functions. In addition, we report the development of a platform for the structure-function analysis of HpuA, a member of the poorly studied Hb receptor. We also present evidence that novel small molecules may be able to inhibit TbpA-Tf interaction, presenting the Tf receptor as a novel, species-specific antimicrobial target. Finally, we demonstrated that a novel drug, OSU-03012, has antimicrobial activity against the gonococcus through down-regulation of DnaK, a protein chaperone. These findings suggest that DnaK, a widely conserved protein, may be a universal target for antimicrobial development. These studies provide insight into the structure function relationship of TbpA, the drug potential of DnaK, and lay the framework for future investigations of the TdTs for use in a multi-antigen vaccine.

Chapter 1: Introduction

I. Neisseriaceae

The family *Neisseriaceae*, first proposed in 1933, included a group of Gram-negative, oxidase- and catalase-positive, aerobic or facultatively anaerobic, non-spore-forming, rod- or coccoid-shaped organisms and embraced four genera: *Neisseria*, *Kingella*, *Acinetobacter* and *Moraxella* (1). Over time, this family has undergone several revisions, including the transfer of genera *Moraxella* and *Acinetobacter* into the new family *Moraxellaceae* based on rRNA analyses (2). Additionally, the genera *Eikenella*, *Simonsiella* (3), and *Alysiella* (4) were added to the family *Neisseriaceae*, which comprises a major branch of the β -Proteobacteria.

Within the Neisseriaceae, the *Neisseria* species are non-motile, Gram-negative diplococci with flattened sides that grow optimally at temperatures between 35-37°C (1). Within this genus there are several species considered to be commensal in humans, and two species that are pathogenic. Human associated *Neisseria* spp. can be identified by their patterns of acid production from carbohydrates, their ability to reduce nitrate, and their ability to produce polysaccharide from sucrose (5).

II. Pathogenic *Neisseria* species

Neisseria gonorrhoeae and *Neisseria meningitidis* are both considered human specific, pathogenic *Neisseria* species; however, *N. meningitidis* can be carried asymptotically. The meningococcus frequently colonizes the nasopharynx of health individuals, and occasionally goes on to cause disease, usually in small outbreaks. In contrast, the gonococcus is always

considered pathogenic, and most often infects the mucosal surfaces of the urogenital system, the oronasopharynx, and the rectum. Most other *Neisseria* species are commensal, and can colonize the oropharynx without causing disease (6). However, these species have been associated with cases of endocarditis, meningitis, septicemia, otitis, bronchopneumonia, and possibly genital tract disease (7). Due to these rare clinical presentations, these species are considered opportunistic pathogens that only infect individuals with weakened immune systems (5). Indeed, there is evidence that colonization with commensal *Neisseria* may be protective against the pathogenic species (8).

III. Meningococcal Disease

A. Epidemiology

Neisseria meningitidis colonizes the nasopharynx in approximately 10% of people, where it predominately exists as a member of the normal flora (9, 10). Among carriers, colonization lasts months in 25% of individuals, is intermittent in about a third of colonized individuals, and the remaining 40% are transiently colonized (11). Age is a large determinant of meningococcal carriage. In North America and Europe, carriage rates are very low in the first years of life, sharply increase in teenagers, peak in those aged between 20 and 24 years, and decline afterwards (12). Meningococci are transmitted person to person by direct contact with nasal or oral secretions or through inhalation of large droplet nuclei (13). Susceptibility to meningococcal carriage can be dramatically altered by environmental factors. Exposure to tobacco smoke is correlated with meningococcal carriage and can increase risk of disease by 20% (14). In closed settings, such as residential schools or military barracks, transmission

increases dramatically and carriage rates may approach 100% (15, 16). Additionally, individuals with defects in the terminal complement pathway or complement regulation are at greater risk of recurrent and more serious meningococcal infections (17, 18).

Despite high rates of meningococcal carriage, disease incidence is rare with rates that vary from 1 to 1000 cases per 100,000 individuals, depending on location (19). Indeed, it is thought that nasopharyngeal colonization is an important immunizing process, which may protect against future illness (13). Meningococcal infection may also be influenced by the season. In the only area of the world with epidemic disease, the meningitis belt of Africa (defined as the area between Ethiopia in the East to Senegal in the West), epidemics arise at the end of the dry season (20, 21). *N. meningitidis* strains can be separated into 13 serogroups based on the chemical structure of the polysaccharide capsule. Of the 13 serogroups, A, B, C, W135, X and Y are responsible for greater than 90% of disease worldwide (19). Serogroup A causes the highest incidence of disease, with pandemic outbreaks every 5-10 years since 1905 in the meningitis belt of Africa. During these pandemics, infection rate can reach 1% of the population (22). The bulk of infections in the Americas and Europe are caused by serogroup B, and to a lesser extent serogroup C, with rates typically less than 10 per 100,000 population (22).

B. Disease

During colonization, meningococcal pilin and opacity proteins promote binding to epithelial cells. The meningococcal adhesins stimulate epithelial cells to engulf meningococci into phagocytic vacuoles, which may transverse the mucosal epithelium, granting the bacteria access to subepithelial tissues (23). In a small percentage of individuals, *N. meningitidis* penetrates the mucosa and gains access to the bloodstream, causing systemic disease (24).

Meningococcal bacteremia can result in the seeding of the meninges, pericardium and large joints. Up to 50% of patients with meningococcal disease present with meningitis, with the classic presentation of a sudden onset of headache, fever, and stiffness of the neck (25, 26). *N. meningitidis* can be isolated from the bloodstream in up to 75% of patients with meningococcal disease, but meningococcal sepsis, also called meningococemia, occurs in only 5 to 20% of patients (25). Meningococemia is characterized by an abrupt onset of fever and a petechial or purpuric rash, and is often associated with the rapid onset of hypotension, acute adrenal hemorrhage, and multiorgan failure (26). Invasive meningococcal infection can also result in pneumonia in 5 to 15% of patients; however, these infections are difficult to detect because isolation of *N. meningitidis* from sputum does not differentiate between carriage and active infection (26).

C. Treatment and Prevention

Until a century ago, meningococcal infection was fatal in up to 70% of infected individuals (27). However, the development of several antimicrobial therapies has dramatically reduced rates of mortality. Over the last two decades, mortality rates have remained stable at 9 to 12% for all infections, but up to 40% for those with meningococemia (26). Although mortality has sharply declined, 11 to 19% of survivors of meningococcal disease will have serious sequelae, including hearing loss, neurologic disability, or loss of a limb (28, 29).

Due to the serious nature of this infection, containment and prevention are key strategies for combating illness. People in close contact with infected individuals are at elevated risk for contracting the disease, and most secondary cases occur within 5 to 10 days of exposure to the infected person (13). To limit spread of disease, rapid antimicrobial chemoprophylaxis is

implemented to close contacts of the patient. Optimal prophylaxis is achieved using systemic antibiotics that effectively eliminate nasopharyngeal carriage of *N. meningitidis*, including rifampin, ciprofloxacin, and ceftriaxone. Antimicrobial resistance is rare among *N. meningitidis* isolates (30), but may be beginning to emerge (31).

Fortunately, great strides have been made in developing vaccines for meningococcal infection. A polysaccharide vaccine against serogroups A, C, Y, and W-135 (MPSV4) has been available since 1981, and has 85% clinical effectiveness in adults (32). Since it is a polysaccharide only vaccine, it stimulates B-cells but not T-cells, and thus does not stimulate immunologic memory or have any effect on meningococcal carriage (33). More recently, protein conjugate vaccines have been developed that induce a T-cell dependent response, which results in strong primary and anamnestic responses (34). In the 2000s, quadravalent conjugate meningococcal vaccines were introduced. Menactra (Sanofi Pasteur) and Menveo (Novartis) are composed of serogroup A, C, Y, and W-135 capsular polysaccharides conjugated to diphtheria toxoid and CRM197, respectively. GlaxoSmithKline released a trivalent vaccine, Menhibrix, which contains *N. meningitidis* serogroup C and Y capsular polysaccharides, as well as *Haemophilus influenzae* type b capsular polysaccharide, conjugated to tetanus toxoid. While these vaccines have been successful for the strains they cover, a vaccine was still needed for serogroup B, which causes 50% of invasive meningococcal disease globally and 30% in the United States (34).

Vaccine development for serogroup B was hindered due to the structure of the polysialic acid in its capsule, which has a specific linkage structure found in many human tissues, especially in the central nervous systems of fetuses and children (35). This molecular mimicry

was likely to reduce the immunogenicity of the capsule due to immunologic tolerance developed in humans, and would have the potential to trigger an autoimmune disorder if antibodies were generated to a self-antigen (36). Through the process of reverse vaccinology (36) and subsequent testing, factor H binding protein (fHBP), *Neisseria* adhesion A (NadA), and *Neisseria* heparin-binding antigen (NHBA) were identified as vaccine candidates (37). These three proteins were combined with an outer membrane vesicle derived from a serogroup B New Zealand strain (strain NZ98/254) to create the formulation for the 4CMenB vaccine (Novartis), which was licensed in the United States in 2013. *N. meningitidis* strains are considered sensitive to 4CMenB vaccine if they express the P1.4 subtype of PorA protein or if 1 of the 3 antigens has a sufficient level of expression (38). Studies have shown that if a strain exceeds the positive threshold for only 1 of the 3 vaccine antigens, the probability of being killed by the host antibodies is 80%, and the probability climbs to 96% if 2 out of 3 proteins exceed the positive threshold (39). In 2014, another serogroup B meningococcal vaccine, Trumemba (Pfizer), was licensed in the United States. This vaccine consists of two fHBP variants, which are immunologically distinct (A05 and B01). Although these new vaccines have generated promising results, continuing surveillance will be needed to determine immunity duration and vaccine safety profiles.

D. Animal Models of Infection

Several animal models have been developed to characterize meningococcal infection. Intraperitoneal models of infections have been developed in both mice and infant rats (40). Although intraperitoneal inoculation isn't the natural route of infection, these models are best used to characterize virulence and protection profiles from immunization. The intraperitoneal

mouse model is better for determining active resistance to infection, whereas the rat model can only study passive resistance. However, the intraperitoneal mouse model requires supplementation with exogenous iron sources, while the rat model does not. In contrast to the intraperitoneal models, an intranasal mouse model of infection has been developed to assist with characterizing mechanisms of meningococcal pathogenesis and disease progression (41).

Although these models allow for different aspects of meningococcal disease investigation, they each have specific limitations, and are collectively limited by the fact that *N. meningitidis* is a human specific pathogen. Accordingly, several human host proteins are absent from these models that may contribute to meningococcal pathogenesis. To address this concern, humanized transgenic mice are being developed to more closely mimic human infection. Thus far, mouse lines have separately been developed that express human CD46 (42), CEACAM1 (43), transferrin (44), and factor H protein (45). These newer models will allow for more detailed investigation of specific components of meningococcal-host interactions that contribute to infection.

IV. Gonococcal Disease

A. Epidemiology

Gonococcal infection is one of the oldest diseases known to man, dating back to at least biblical times with references made in Leviticus 15: 1-3 (46). *Neisseria gonorrhoeae*, the causative agent of the sexually-transmitted infection gonorrhea (47), is still a relevant medical issue today as it is the second most commonly reported sexually transmitted infection (STI) in the United States. Gonococcal disease affects approximately 106 million people worldwide

according to WHO estimates (48), with >300,000 cases of gonorrhea reported each year in the United States alone (49). A troubling contributor to these statistics is that infection with this bacterium does not result in any protective immunity (50). The gonococcus is spread predominately through sexual contact, which contributes to the correlation between age and gonococcal infection. Adolescents and young adults (age 15 to 24) account for majority of new cases every year. Additionally, ethnic minority groups, including African-Americans, Latinos, and Native Americans, are disproportionately represented among those infected (51). Infection is on the rise among populations of men who have sex with men (MSM), and it has been shown to be more prevalent HIV positive individuals (52). The association between gonococcal and HIV infections is well established (53, 54), and recent studies have demonstrated that gonococcal infection increases HIV gene expression and viral replication (55). In other populations, women often serve as reservoirs for the disease. Indeed, approximately 50% of women infected with the gonococcus are asymptomatic, resulting in increased spread and more severe clinical outcomes following infection (56). Although gonococcal infection is not as immediately serious as meningococcal infection, the disease still creates a substantial financial burden on the health system, with reports of spending approaching \$1 billion per year in the US alone (57). Individuals with gonococcal infection are frequently co-infected with *Chlamydia trachomatis*, and therefore testing should always be performed for both infections (58).

B. Disease

The most well known form of gonococcal infection is gonorrhea, a disease of the genital tract. *N. gonorrhoeae* invades mucosal and glandular surfaces of the genito-urinary system, resulting in a local inflammatory response that involves recruitment of neutrophils and

macrophages. Typical infections in males initially cause urethritis, which can ascend to cause prostatitis and epididymitis. Infections in men are symptomatic 90% of the time, and symptoms typically begin 2 to 5 days after exposure (59). Infection in females begins with cervicitis and urethritis, and then it ascends to cause pelvic inflammatory disease, salpingitis, and even perihepatitis. In the minority of women that have symptoms, the incubation period is longer than for men, at approximately 5 to 10 days after exposure (59). Although genital infections are the most common, gonococcal disease can also manifest in other organ systems, including the mouth, throat, eyes, and rectum. Because women are more likely to carry asymptomatic infection, they are at greater risk for experiencing secondary complications compared to men due to delay in treatment. Secondary complications for gonococcal infection include disseminated gonococcal infection, septic arthritis, and ascension of the genital tract which causes scarring that over time can cause ectopic pregnancy and infertility.

C. Treatment

The gonococcus has become increasingly drug resistant, with mounting evidence to suggest that current pharmacotherapies may be soon rendered obsolete (60, 61). To date, the characteristics of at least three multi-drug resistant isolates have been published, all of which are fully resistant to ceftriaxone, the previously recommended single agent therapy for treatment of gonorrhea (61-65). The World Health Organization and Centers for Disease Control have recommended that the treatment options for *N. gonorrhoeae* should be easily accessible, cost effective, and have more than a 95% cure rate as a single dose (66, 67). Due to mounting antimicrobial resistance, the CDC updated the treatment guideline for the management of gonococcal infections in 2015 (68). For all uncomplicated gonococcal infections

of the cervix, urethra, pharynx, and rectum, the recommended therapy is a combination of one dose of intramuscular ceftriaxone 250 mg plus one dose of oral azithromycin 1000 mg. Coincidentally, this therapy would also cover *Chlamydia* infection, if present. Alternative drug combinations, using single doses of azithromycin 2000 mg plus either oral gemifloxacin 320 mg or intramuscular gentamicin 240 mg, have also been tested (69). Gonococcal eradication was observed in 100% of the gentamicin/azithromycin group (202/202) and in 99.5% of gemifloxacin/azithromycin group (198/199). No serious adverse events were reported; however, gastrointestinal events were common in both groups, with over 25% of patients experiencing nausea and approximately 20% experiencing diarrhea. Due to the success of this trial, these drug combinations are now listed as alternative therapies for individuals with β -lactam sensitivity (68). Although a few therapeutic options remain, increasing levels of antimicrobial resistance, dwindling drug options, and lack of protective immunity post-infection dictate the need for further drug and vaccine development research to prevent an era of untreatable gonococcal infection.

D. Animal Models of Infection

The gonococcus is a human specific pathogen, and this host limitation has made animal modeling of gonococcal infection difficult. At first, genital tract infection was only accomplished in chimpanzees; however, their use is too costly to be a practical model for gonococcal infection (70). Discoveries that the murine estrous cycle affected gonococcal colonization eventually allowed for the development of a 17 β -estradiol treated germ-free BALB/c mouse model of infection that allowed for long-term colonization (71-73). Murine infection in this model typically last from 12-14 days, but can last as long as 40 days depending on the method of

estrogen administration (73). It is not precisely known how estrogen allows for gonococcal colonization, but it hypothesized that it temporarily suppresses the mouse immune system (73). This model succeeds in that the localization of infection closely mimics that of humans, and like humans, mice do not develop a humoral memory response to gonococcal infection.

Although this model allows for some *in vivo* characterization of gonococcal infection, it also has several limitations. During the course of infection, there are culture negative windows of dramatically reduced recovery of bacteria, followed by returns to bacterial carriage (72). Accordingly, multiple consecutive days of negative cultures are required to confirm that the infection has cleared. In terms of genital tract physiology, vaginal pH in mice is substantially higher than in humans (74), and mice do not undergo a period of menstrual bleeding (73). Both of these factors may be relevant to the survival of the gonococcus in its niche, but are not able to be tested with this model. Finally, as a host specific pathogen, there are many host-pathogen interactions that are specific to human proteins that cannot be tested in this model, including cell surface receptors, iron binding glycoproteins, and complement regulatory proteins. In order to overcome these limitations, several lines of transgenic mice are needed. To date, several of these transgenic mice have been developed, with mouse colonies that can express human transferrin (44), human CEACAM1 (43), or human factor H protein (45). Although these single transgene mice are helpful, the ultimate goal is to develop mice that can express multiple human proteins to more closely model human infection.

V. *N. gonorrhoeae* virulence factors

N. gonorrhoeae possesses an array of surface-associated virulence factors that contribute to interaction with host cells and development of infection (Fig. 1.1). These proteins contribute to

Figure 1.1. Overview of gonococcal virulence factors.

Image of gonococcal cell with several surface-exposed virulence factors and brief descriptions of their roles, adapted from (75).

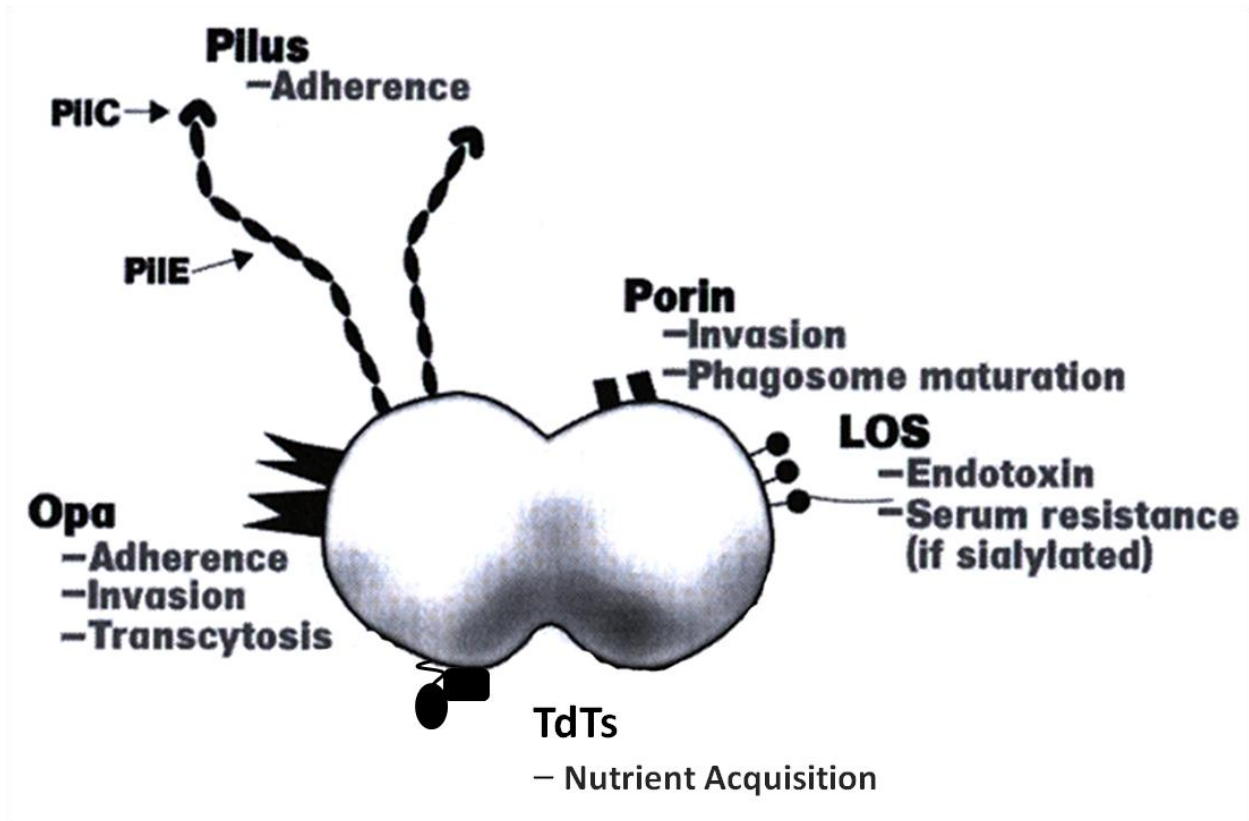


Figure 1.1. Overview of gonococcal virulence factors

Adapted from (75)

adherence, invasion, and immune avoidance. In addition, many of these genes are subject to one of two types of genetic variation: phase and antigenic. Phase variation can be considered a reversible on/off switch mechanism for gene expression. Antigenic variation is the expression of various alternative forms of an antigen on the cell surface. High frequency phase and antigenic variation are major contributors to gonococcal immune avoidance.

A. Type IV Pilus

Like other gram-negative bacteria, *N. gonorrhoeae* produces a type IV pilus, which is a filamentous polymer 6 nm in diameter and several micrometers in length that protrudes from the cell surface (76). Systematic genetic analyses have identified 15 proteins (known as Pil proteins) that are involved in the biogenesis, assembly and disassembly of pili (77). The pilus fiber primarily consists of numerous subunits of the major pilin, PilE, arranged in a helical configuration. In addition, several minor pilins, PilC, PilV, and PilX, can be incorporated in the fiber to modulate its function (78). The crystal structure of gonococcal pilus was resolved in 1995 (79), and the structure has since been updated based on additional images generated with cryo-electron microscopy (80).

The gonococcal pilus plays multiple roles in pathogenesis. It serves as a major adhesin to eukaryotic cells (76), and also plays a role in self-agglutination (78). The pilus can also assemble and disassemble rapidly, facilitated by the coordinate action of PilC and the ATPase PilT, resulting in “twitching motility” (81, 82). Notably, the pilus is responsible for uptake of foreign DNA from the extracellular milieu, thereby increasing the transformation frequency of bacteria and maintaining the genetic diversity that is characteristic of *Neisseria* spp (83, 84).

The pilus itself is subject to genetic variation, as it undergoes high frequency phase and antigenic variation. Phase variation impacts the *pilE* gene, which contains a polyC-tract within the structural gene. Changes in length of the polyC-tract can result in frameshift mutations, which can turn the gene off by creation of a premature stop codon. Phase and antigenic variation also occur from genetic recombination between the *pilE* expression locus and the one of several distinct *pil* genes in the silent *pilS* locus (85, 86). This process is RecA dependent, non-reciprocal, and happens at high frequency during human infection (87). In addition to genetic variation, the gonococcal pilus can undergo several distinct post-translational modifications, including glycosylation, which can indirectly have an effect on cellular interactions (88).

B. Porin

Porins, the most abundant outer membrane proteins in *Neisseria spp.* (89), function as ion and nutrient transport channels that are essential for neisserial viability (76, 90). *N. meningitidis* encodes two porins: class 1 (PorA), which is phase variable (91), and either class 2 or class 3 (PorB). However, the PorA gene exists as a pseudogene in *N. gonorrhoeae* (92), and therefore the gonococcus only expresses one porin, which is homologous to meningococcal PorB. PorB is subclassified as either protein 1A (PorB1A) or protein 1B (PorB1B) (93).

In addition to their role in cell viability, these proteins also contribute to gonococcal pathogenesis. Porins have been reported to translocate from the outer membrane of gonococci into artificial membranes (94) as well as into that of the host cell membrane (95, 96). Epidemiologic observations indicate a strong association of PorB1A with disseminated gonococcal infections, whereas PorB1B-expressing strains have mostly been isolated from patients with localized infections (97). This association has been supported by data

demonstrating that PorB1A, but not PorB1B, expressing strains can invade epithelial cells, even in the absence of opacity proteins (98). PorB1A has also been associated with serum resistance, and mediates resistance to the alternative and classical complement pathways through association with Factor H and C4b-binding protein, respectively (99-101). PorB1B is also capable of binding C4b-binding protein, demonstrating that porins mediate serum resistance among gonococcal isolates (99).

Porins also possess immunomodulatory properties. Translocation of PorB into the membrane of activated neutrophils initiates a series of events that include a transient change in the membrane potential (96), inhibition degranulation without affecting the NADPH oxidase activity (102), and inhibition of actin polymerization and subsequent phagocytosis (103). Further studies have shown that porin is also capable of arresting phagosome maturation within macrophages, which may contribute to intracellular survival (104).

C. Lipooligosaccharide (LOS)

Lipopolysaccharide (LPS) is the major glycolipid expressed on the outer membrane of Gram-negative bacteria. LPS contains three structural regions: Lipid A, which anchors the LPS to the bacterial membrane, a short oligosaccharide core, and a variable length repeating O-antigen polysaccharide. In the pathogenic *Neisseria*, however, the LPS lacks O-antigen and is therefore termed a lipooligosaccharide (LOS) (105). Although the LOS structure is stably expressed, there are a variety of glycosyl transferases that modify the terminal sugars of the LOS (106). These glycosyl transferases, encoded by *lpt* genes, are subject to phase variation due to polyG tracts within their coding regions (107). Due to this phase variable expression, LOS undergoes high frequency variation (10^{-2} per generation), resulting in substantial antigenic diversity.

LOS, in addition to being an endotoxin, plays several roles in bacterial virulence. Gonococcal LOS mimics the human glycosphingolipid paragloboside (108), and can bind to the human asialoglyco-protein receptor (ASGP-R), which is present on human sperm (109). The LOS-ASGP-R interactions also increases invasion of urethral epithelial cells (110). Some LOS variants can also serve as acceptors for sialic acid deposition (111). The gonococcus is not able to synthesize sialic acid, but it does encode a sialyltransferase (112, 113). During the course of infection, the gonococcus can use host derived cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NANA) as a sialyl donor (114, 115). The presence of sialic acid on gonococcal LOS confers serum resistance (116), partially through binding of Factor H (117); however, it also impairs Opa-mediated invasion of some cell lines (118). This observation has led to the suggestion that LOS antigenic phase variation may allow the gonococcus to fluctuate between invasive and serum-resistant phenotypes, enhancing bacterial survival. Gonococcal LOS has also been implicated in manipulation of host dendritic cells, directing the immune response to favor bacterial survival (119). Although not directly linked to bacterial survival, it has also been discovered that heptose shedding from gonococcal LOS leads to increased HIV viral replication, which has substantial public health implications (55).

D. Opacity (Opa) proteins

Opacity (*opa*) proteins are a family of related transmembrane proteins that form eight-stranded β -barrel structures in the outer membrane of the bacterium with four surface-exposed loops (76). Gonococcal strains typically have up to 11 *opa* loci, whereas meningococcal strains typically have 4 to 5 *opa* loci (120). Opas were originally named for their ability to impart an opaque phenotype in gonococcal colonies (121). More recently, these proteins are

considered a family of invasins that mediate invasion of epithelial cells and leukocytes (122-125). The importance of these proteins for infection is demonstrated by their expression in both natural and experimental infections (121, 126, 127).

Like the other proteins listed here, Opa proteins are subject to phase and antigenic variation. All *opa* genes contain tandem repeats [CTCTT]_n that cause high-frequency phase variable expression (128, 129). As a result, the gonococcus can reversibly express none, one, or multiple Opa proteins at the same time, although no strain has been isolated with more than 4 Opa proteins expressed (130). In addition, horizontal gene transfer promotes the formation of hybrid recombinant *opa* loci (131).

Opa proteins can be categorized based on their receptor tropisms. Opa proteins have been demonstrated to bind to two sets of host receptors: heparin sulfate proteoglycans (132, 133) and extracellular matrix proteins vitronectin and fibronectin (134, 135), and the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family of receptors (136, 137). In addition to being invasins, Opa proteins can possess immune-modulatory capabilities. Interactions between gonococcal Opa proteins and CEACAM1 on B cells result in cell death, which lowers antibody production (138). Among T cells, CEACAM1 interaction with gonococcal Opa proteins arrests activation of T cells and prevents proliferation (139). Further studies have shown that Opa proteins are involved in directing the host immune response away from Th1/Th2 responses, and towards the more favorable Th-17 response through both TGF- β (140, 141) and IL-10 dependent mechanisms (140, 141).

VI. Iron sources in humans

Iron is an essential nutrient for microorganisms, and acquisition of iron is recognized as one of the key steps in the survival of a pathogen within its host (142). Iron, a transition metal, exists in two readily inter-convertible redox states: ferrous, Fe^{2+} or ferric, Fe^{3+} . Iron's properties make it an extremely versatile prosthetic component for incorporation into proteins as a biocatalyst or electron carrier, and it is involved in many major biological processes including respiration and DNA biosynthesis (143). Although iron has many beneficial attributes, it also possesses significant potential for toxicity. In the presence of radical oxygen species, which are natural products of aerobic metabolism (144), iron can cause the creation highly damaging hydroxyl radicals through the Fenton reaction [$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} \text{OH}^- + \text{HO}\cdot$] (145).

Though the human body has significant amounts of iron, its availability is tightly regulated to prevent against toxicity. Indeed, iron is distinct from other important nutrients because it is not a freely available in the host. In aerobic environments, iron exists primarily in the oxidized, ferric form, which at pH 7, has a solubility of 1.4×10^{-9} M (146). Iron withholding, known as nutritional immunity, is a mechanism of innate defense against invading microorganisms (147). During microbial invasion, humans can suppress the amount of iron circulating on carrier proteins in the blood and also decrease dietary absorption of iron to further minimize its presence (148). Therefore, the ability of microorganisms to acquire iron in a hostile host environment is considered a virulence determinant. In the absence of free iron, microbes can often hijack host proteins to meet their iron demands. Several human iron sources are described below.

A. Transferrin

Transferrin (Tf) is an 80 KDa glycoprotein found in the blood that transports iron throughout the body. It has a bi-lobed structure (N and C lobes), with each lobe containing a high affinity binding site for a single Fe^{3+} atom (149). The N- and C-lobes are ~40% identical and likely arose from gene duplication followed by fusion (150). Each lobe folds into two subdomains, and the Fe^{3+} binds in a deep cleft formed between the two subdomains. In human serum, where Tf concentrations are ~25 to 50 μM , approximately 30% of Tf is iron bound (151, 152). Of the monoferric Tf, the iron is not evenly distributed; there is a slight bias towards N-lobe iron binding (153). At pH 7.4, the human transferrin receptor (TFR1), which is present on all iron requiring cells (152), binds Tf with nanomolar affinities (K_d ~4 to 26 nM depending on iron bound state) (154). Once the ligand is bound, the TFR1/Tf complex undergoes clathrin-dependent endocytosis (155, 156). The endosome is acidified to ~pH 5.6 and the combination of low pH and salt induce iron release from Tf. Critically, apo-Tf still has high affinity for TFR1 at endosomal pH so it remains bound. This allows the complex to be recycled to the cell surface instead of being degraded, and the apo-Tf is released back into the serum (152).

B. Lactoferrin

Lactoferrin (Lf) is an 80 KDa glycoprotein of the transferrin family. Lf is found in mucosal secretions, including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids, urine, and colostrums (157). LF is the second most abundant protein in milk (158), after caseins, and it can also be found in blood plasma and amniotic fluid. Although Lf is present at low levels in serum (3.8–8.8 nM), it is a significant component of mucosal secretions (6–13 μM) (159). Similar to Tf, Lf has a bilobed structure where the lobes are 37%

identical, and each lobe possess a high affinity Fe^{3+} binding site (160). Unlike Tf, Lf can retain iron binding over a wide pH range, including extremely acidic pH (161). Lf also serves a role in innate immunity. Lf is a significant component of secondary neutrophil granules (155, 162), and at sites of inflammation, Lf concentration can rise to 200 $\mu\text{g}/\text{mL}$ (163, 163, 163). Lf has antimicrobial activity attributed to two distinct mechanisms. The first is its iron scavenging ability which deprives microorganisms of this nutrient. The second is that the N terminus of Lf can undergo proteolysis to produce antimicrobial compounds known as lactoferricins (164-166). These positively charged molecules interact with negatively charged bacterial surface components, including LPS and lipoteichoic acid, and cause membrane destabilization, increasing the effects from other innate effectors like lysozyme, which ultimately results in cell death (157, 167).

C. Ferritin

When intracellular iron exceeds cellular requirements, the redox potential of the excess is controlled by sequestration of iron in ferritin. Mammalian ferritins are heteropolymers of 24 subunits of two types, designated H for heavy, and L for light, where both types of chains are required for normal function of ferritin. The mature protein shell has a molecular weight near 450 kDa, with subunits arranged to form a nearly spherical structure enclosing a cavity capable of accommodating up to 4500 oxygen- and hydroxyl-bridged iron atoms (168). During inflammation and infection, serum iron decreases and cell associated iron increases, leading to increases in the amount of ferritin in macrophages, and greater amounts of iron stored per ferritin molecule (169, 170). Hemosiderin, a water-insoluble degradation product of ferritin, can result from incomplete lysosomal processing (171). The iron core of hemosiderins is more

heterogeneous than ferritin, and slower to release iron (172); however, iron release from hemosiderin does occur under acidic conditions (173).

D. Heme/Hemoproteins

Approximately 70% of the iron in the human body is contained within heme, a porphyrin ring that coordinates one ferrous iron atom (174). Heme solubilizes iron and enhances its catalytic activity by at least 5 orders of magnitude (175). Due to this high catalytic activity, heme is critical for functions involved in oxygenation reactions, oxidative stress responses, electron transport, oxygen transport, oxygen sensing, and oxygen storage (176). Although heme is an essential biomolecule, excess free heme is toxic to cells due to its lipophilic nature, lipid peroxidation capacity, and ability to catalyze the production of reactive oxygen species (176). Accordingly, over 95% of heme is bound to proteins (hemoproteins), 67% of which is bound to hemoglobin within erythrocytes. Hemoglobin is a tetramer composed of two α - and two β -subunits, each of which binds a heme molecule. Although the majority of heme is intracellular, hemolysis results in extracellular hemoglobin in the blood at concentrations of 80 to 800 nM (159). Upon hemolysis, Hb disintegrates into $\alpha\beta$ dimers, which are rapidly bound by excess serum haptoglobin (177). Haptoglobin stabilizes and protects against the toxic effects of Hb, and chaperones Hb until its clearance through receptor mediated endocytosis in monocytes (178). Damaged hemoproteins can also result in free heme in the blood, which is bound by hemopexin, and to a lesser degree albumin. Similar to haptoglobin, hemopexin protects against the toxic effects of heme, and shuttles it to the liver for clearance (177).

E. Siderophores

Siderophores are secreted, low molecular mass (1 kDa) iron chelating compounds used by many microorganisms to overcome the iron limitations of the human host (142). Siderophores are divided into three categories based on the structure of their iron ligation groups: catecholate, hydroxamate, and hydroxycarboxylate (179). The binding power of the siderophore for iron, for which values of the dissociation constant (K_d) range from 10^{22} to 10^{50} (142), is sufficiently strong for the siderophore to remove iron attached to ferritin, Tf, and Lf, but not to remove iron from hemoproteins (142). Once secreted, siderophores chelate free or protein-bound iron in the environment. After acquiring iron, the siderophore can be imported whole, and the iron harvested, by any bacterium that possesses the siderophore specific receptor (180). Many species of bacteria can utilize xenosiderophores, which are siderophores that a bacterium does not produce, but for which it does have the receptor (181).

In response to the use of siderophores by bacteria to steal iron from host iron-sequestering proteins, humans produce proteins that sequester ferric siderophore complexes away from bacterial siderophore receptors (182). These proteins belong to the lipocalin family of binding proteins, and are termed siderocalins for siderophore-binding lipocalins. Scn, the best studied of these proteins, possess a highly polyspecific recognition mechanism to sequester a wide range of enterobactin-like siderophores and the chemically distinct carboxymycobactins (183). The importance of these proteins is demonstrated in Scn knock-out mice, which are significantly more susceptible to infections with siderophore dependent bacteria (184). In a step further in the evolutionary battle for iron access, some bacteria have evolved mechanisms

to glycosylate their siderophores, making them no longer susceptible to siderocalin binding (185).

Similar to siderophores, proteins called hemophores have been discovered that can directly bind heme or acquire heme from hemoproteins (186). These proteins are structurally diverse and can be surface-associated or secreted (187). Hemophores can deliver heme to cells that express the hemophore specific receptor; however, unlike siderophores, only the heme is internalized (187).

VII. Iron acquisition systems of *N. gonorrhoeae*

As the gonococcus has evolved to be a human-specific pathogen, it has developed an array of iron acquisition systems to survive within its host. Unlike many enteric bacteria, the gonococcus does not secrete siderophores (188). Instead, it possesses several surface receptors designed to hijack iron directly from host iron and heme transporting proteins.

A. Two component systems

The gonococcus encodes three distinct two-component systems for iron acquisition, the best studied of which is the transferrin receptor (Fig. 1.1). The transferrin receptor is composed of an integral, outer-membrane, TonB dependent transporter (TdT), TbpA, and a surface exposed lipoprotein, TbpB. The structure of TbpA is similar to other TonB dependent transporters: a transmembrane β -barrel comprised of 22 amphipathic β -strands, surface exposed loops, and a folded plug domain inside the barrel (189, 190). TbpB has a bilobed structure, with homologous N and C lobes that both contribute to N-lobe transferrin binding (191, 192). Both TbpA and TbpB have nM affinities for Tf; however, only TbpA is required for Tf-

iron internalization (193-195). Although TbpB is not essential for uptake of Tf-iron, it does increase the efficiency of Tf iron acquisition due to its preferential recruitment of iron laden Tf (196). The transferrin receptor is not subject to phase or antigenic variation. The *tbpA* and *tbpB* genes are encoded by an iron repressed operon, and have been found in all sequenced gonococcal strains (197). The importance of the Tf receptor for gonococcal pathogenesis has been demonstrated in human infection trials, in which the absence of the Tf receptor resulted in attenuated virulence (198).

The lactoferrin receptor is analogous to the Tf receptor, where there is a TdT, LbpA, and a surface exposed lipoprotein, LbpB (199, 200). Like the *tbpAB* operon, the *lbpAB* genes are encoded on an iron repressed operon where the lipoprotein is encoded before the TdT (201). Unlike the Tf receptor, the Lf receptor is subject to phase variation due to a polyC-tract in the coding region of *lbpB* (199). While the Lf receptor is present in all meningococcal strains, it is absent in approximately 50% of gonococcal isolates due to a large deletion encompassing parts of *lbpB* and *lbpA* (201). The Lbps are not required for human infection (198), but can serve as a means of acquiring iron in the absence of the Tf receptor (201).

The hemoglobin receptor is the last of the two component iron acquisition systems encoded by the gonococcus. The Hb receptor is also comprised of a TdT, HpuB, and a lipoprotein, HpuA. Although the naming convention is reversed, in all three cases the lipoprotein is encoded before the TdT in an iron repressed operon (202). The Hb receptor, like the Lf receptor, is phase variable due to a polynucleotide repeat in the lipoprotein gene, *hpuA* (203). The hemoglobin receptor differs from the other two component systems in three distinct ways. Unlike the Tf and Lf receptors, the Hpus extract heme instead of atomic iron (204). The

Hb receptor can obtain heme from hemoglobin alone, or hemoglobin complexed to haptoglobin (202, 205). The heme acquired through this receptor is sufficient as a sole iron source to support gonococcal growth (206). This receptor is also unique in that it may not be specific to human hemoglobin, as binding has been demonstrated to an array of other species' hemoglobin (207). Lastly, the Hb receptor differs from the other two component systems in that both proteins are required for heme internalization (206).

Figure 1.2. Overview of two-component iron acquisition systems.

Schematic representation of the gonococcal two-component iron acquisition systems, representing the transferrin, lactoferrin, and hemoglobin receptors. The TonB-dependent transporter (TdT) is shown as a barrel traversing the outer-membrane (OM), with several surface exposed loops and a plug domain occluding the barrel. The companion lipoprotein is shown on the outer leaflet of the OM. The TonB/ExbB/ExbD system is shown embedded in the inner membrane (IM) and extending into the periplasm. TonB interacts with the plug of the TdT to facilitate iron transport. Iron that reaches the periplasm is bound by the periplasmic binding protein FbpA, which shuttles the iron to the inner membrane permease FbpB. Upon hydrolysis of ATP by the ATPase FbpC, iron is transported through FbpB into the cytoplasm.

Two Component System

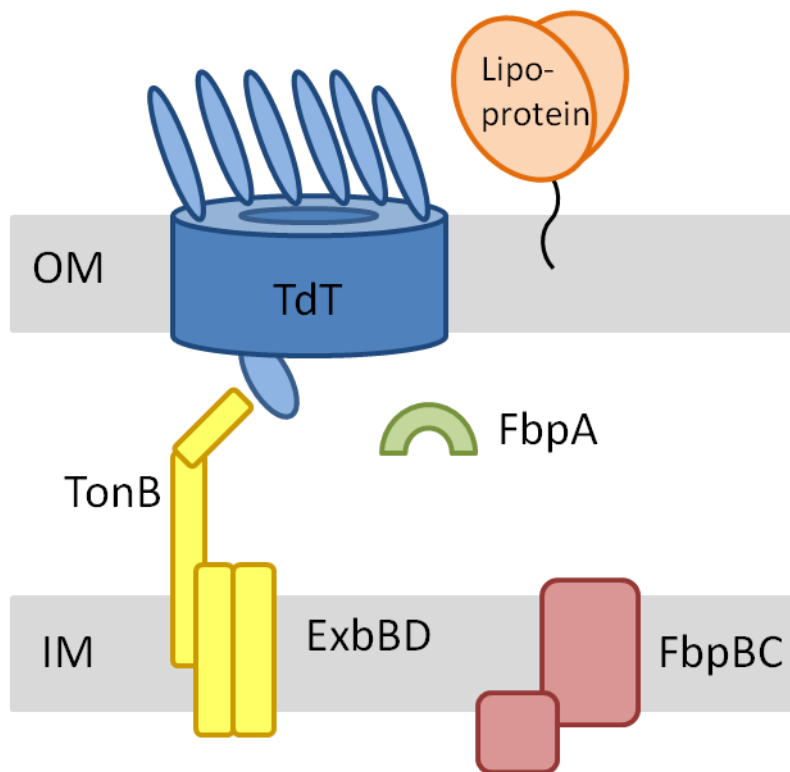


Figure 1.2. Overview of two-component iron acquisition systems

B. Single component systems

In addition to the two component iron acquisition systems, *Neisseria* species also encode single component systems in which a TdT is an independent receptor. The HmbR Hb receptor in *N. meningitidis* is a single component receptor, and is subject to phase variation (208, 209). However, HmbR is not expressed by the gonococcus due to a premature stop codon in the coding sequence (210). FetA, a receptor for catechol-type siderophores, is another single component receptor (211). Like many other surface proteins, FetA is subject to phase variation due to a polynucleotide repeat in the gene promoter (212). Lastly, there is some evidence that TdfF, a TonB dependent protein of unknown function, may also be a single component iron transporter (213). TdfF is specific to the pathogenic *Neisseria*, and can only be detected in iron deplete conditions in the presence of serum (197, 214). A *tdfF* mutant was demonstrated to be defective for intracellular survival, and this defect could be reversed with addition of iron. These data suggest that TdfF may participate in intracellular iron acquisition, which would enhance survival; however, the ligand has not yet been identified.

C. TonB dependent transport

All of the previously described transporters are TonB dependent, meaning that subsequent to the ligand binding to its receptor, the transmembrane transport of the iron or iron chelate is dependent on the Ton system for metabolic energy (215, 216). The Ton system is composed of TonB, ExbB, and ExbD, and their respective genes are encoded in an iron repressed operon (217, 218). TonB has 3 functional domains: an N-terminal transmembrane domain, a proline-rich spacer located in the periplasm, and a C terminal domain that interacts with TonB-dependent receptors (219). Although the mechanism by which TonB transduces energy has not

been fully defined, it has been demonstrated that the energy that drives the Ton system is harnessed from the proton motive force (220). Once energized, TonB interacts with the TonB Box, a conserved region within the plug domain of TdTs, and facilitates transfer of the substrate across the membrane and into the periplasm (Fig. 1.1).

D. Cytoplasmic transport

Once the iron or iron chelate arrives in the periplasm, the previously described iron uptake pathways utilize a periplasmic binding protein (PBP) and an inner membrane ATP-binding cassette (ABC) transporter to deliver the substrate to the cytoplasm (221). The periplasmic transfer of ferric iron derived from Tf and Lf requires FbpA, a PBP that in its apo form has been hypothesized to dock to the periplasmic surface of TbpA (222). Once ferrated, holo-FbpA traverses the periplasm and associates with the cytoplasmic permease protein, FbpB. FbpB in cooperation with FbpC and ATP hydrolysis, facilitates iron transport across the cytoplasmic membrane (223). An analogous ABC transport system is hypothesized to exist for heme transport, but to date it has not been identified. Siderophore utilization through FetA requires the PBP FetB, and the putative FetCDEF ABC transporter (211). Both transport systems, FbpABC and FetBCDEF, are iron repressed (212, 217).

E. Regulation

All of the TdTs involved in iron acquisition are subject to iron-dependent repression, which is mediated by the *ferric uptake regulator* (Fur) protein. In high iron conditions, Fur complexes with ferrous iron, dimerizes, and binds to a DNA sequence called a “Fur box,” which generally overlaps with the promoter region of iron-repressed genes (218, 224). Fur binding inhibits RNA production through the inaccessibility of the promoter to the RNA polymerase or the inability of

the RNA polymerase to transcribe the entire gene (213). When iron is a limiting nutrient, apo-Fur dissociates from the Fur box, alleviating repression and allowing gene transcription. There are no known direct activators of iron uptake proteins in *Neisseria* species; however, Fur-repressed, AraC-like regulator MpeR activates expression of *fetA* (225).

VIII. Vaccine Efforts

Gonococcal infection does not elicit protective immunity, and with mounting drug resistance, the development of a preventative vaccine is urgently needed. Unfortunately, many of the gonococcal surface proteins and polysaccharides are subject to high-frequency variation (as described above), limiting their potential as vaccine candidates. The gonococcus is also capable of blocking antibody deposition on its surface. Decoration of LOS with sialic acid has been demonstrated to prevent antibody deposition on gonococcal porin (226). Similarly, antibodies developed against a conserved protein, RmpM, can also prevent porin antibody deposition (227). These distinct mechanisms of antibody blocking present difficulties even if an appropriate vaccine antigen is found. Another general barrier to successful vaccine development is that the genital tract is devoid of specialized lymphatic tissue, and naturally needs to be tolerant of foreign antigens (228, 229). Fortunately, progress has been made in generating genital tract immune responses through intranasal immunization (230-232).

In addition to these general limitations, there are additional antigen specific concerns for vaccine candidacy. Compounding gonococcal pilin variation, it has been demonstrated that the conserved pilin domains are immunosilent, virtually eliminating the capacity for cross-protective pilin based immunity (233). Although Opa proteins are selected for during human

infection, evidence shows that Opa proteins alone (139) or in outer-membrane vesicle (234) can suppress host T cell activity. These findings suggest that any gonococcal vaccine would need to omit Opa proteins in order to optimize the host immune response. Despite the protections afforded to porin described above, specific, cross-reactive, bactericidal antibodies have been elicited against PorB in the serum and mucosal secretions (235). However, these studies relied on outer-membrane vesicles preparations that contained LOS, which is toxic.

In order to expand the scope of antigens for vaccine development, the TdTs have been considered as immunogens (236). It is thought that using vaccines to target iron receptors could work through a two pronged approach: limiting access to an essential nutrient and also complement and cell mediated killing. Of the TdTs, the Tf receptor stands out as an ideal candidate, based on its consistent expression and requirement for initiation of infection (198). Preliminary work in a mouse model of infection began by conjugating TbpA and TbpB to the B subunit of cholera toxin, which resulted in antibodies in the serum and vaginal secretions. It was also determined that although TbpB was more immunogenic, TbpA antibodies elicited better cross protection among strains (232). In a second attempt to use the Tbps as immunogens, a chimeras composed of peptide fragments of TbpA loop 2 combined the N lobe of TbpB were conjugated to the A2 subunit of cholera toxin. These immunizations yielded bactericidal and cross protective antibodies, and the vaginal secretions from immunized mice were capable of interfering with *in vitro* gonococcal Tf-dependent growth (237). These results suggest that TdTs are credible vaccine targets, and warrant further studies.

IX. Research Objectives

The goal of the research described in this thesis is to characterize the structure-function relationships of select gonococcal TdTs, and to investigate novel approaches to gonococcal antimicrobial development. Within this framework, four main objectives were pursued. First, TbpA surface exposed loops were evaluated as immunogens, and the TbpA loop 3 helix was probed for its role in Tf-iron acquisition. Second, a platform was developed to characterize the structure-function relationship of the Hb receptor protein HpuA. Third, novel small molecules were developed and tested for inhibition of TbpA function. Lastly, an investigational cancer drug, OSU-01032, was tested for antimicrobial properties in gonococcal culture. These studies have more fully described the function and vaccine capacity of TbpA, while also finding preliminary data that it could serve as a novel drug target. Additionally, these studies describe DnaK, a prokaryotic protein folding chaperone, as a novel target for antimicrobial therapy. These studies provide additional insight into the TdTs and other drug targets, which may advance the development of novel protective and/or therapeutic strategies against the gonococcus.

Chapter 2: Materials and Methods

I. Bacterial strains and maintenance

Strains and plasmids used in these studies are listed in Tables 1 and 2. Plasmids were propagated in either Top10 (Invitrogen), XL-10 Gold (Agilent Technologies), or Stellar (Clontech) *E. coli* cells. The expression strains for pUNCH412 and pVCU757 were BL21(DE3) (New England Biolabs) and C41(DE3) (Lucigen), respectively. *E. coli* was cultured in Luria-Bertani broth in the presence of 34 µg/ml chloramphenicol (Sigma), 200 µg/ml ampicillin (Sigma), or 50 µg/ml kanamycin (Sigma). Gonococcal cells were propagated on GC medium base (GCB; Difco) agar with Kellogg's Supplement 1 (238) and 12 µM Fe(NO₃)₃ at 37°C with 5% atmospheric CO₂. When necessary, chloramphenicol was added to GCB agar plates at a concentration of 1 µg/ml for selection of the resistance phenotype. Growth conditions for specific assays are listed below.

II. Bacterial iron deplete growth conditions

For growth under iron-stressed conditions on solid media, gonococci were either grown overnight on GCB agar plates with the addition of 5 µM Desferal (Sigma), or on chemically define medium (CDM) (239) agar plates. For transferrin-iron dependent solid phase growth, CDM agar plates were supplemented with 2.5 µM 10% iron-saturated human Tf (Sigma). To achieve iron stress in liquid media, gonococcal strains were cultured from GCB agar plates into liquid CDM, which had been pretreated with Chelex-100 (Bio-Rad). Typical liquid culture growth in CDM involved starting a culture at approximately 20 Klett units (KU) and growing until

approximately 40 KU, at which point the cultures were diluted back to 15 KU and allowed to grow for 4 additional hours.

III. Gonococcal mutant construction

Mutants made in this study were made with a combination of traditional digestion and ligation cloning, Quikchange cloning (Agilent), and In-fusion cloning (Clontech). For specific cloning strategies, see individual chapters. Primers used in this study are listed in Table 3. After mutant construction, plasmids were linearized in preparation for gonococcal transformation. Piliated gonococci were grown on GCB agar plates. Cells were then transferred to GC medium plus Kellogg's supplement 1 and 10mM MgCl₂. Bacteria were incubated with linearized plasmid DNA in liquid suspension, and then plated on GCB agar plates containing appropriate antibiotics, either 1 µg/ml chloramphenicol or 50 µg/ml kanamycin.

IV. Solid phase ligand-blocking assay

Solid phase ligand blocking assays were performed as previously described (194, 240, 241). Briefly, gonococcal strains were iron-stressed in liquid CDM for 4 hours and *E. coli* strains were grown in LB broth with 1 mM IPTG to induce TbpA expression. Bacteria were standardized to culture density and applied to a nitrocellulose membrane (GE Health Life Sciences). Blots were blocked with 5% skim milk (BioRad) in low-salt Tris-buffered saline (LS-TBS) (50 mM Tris, 150 mM NaCl [pH 7.5]) for 1 hour, washed 5 times with LS-TBS, and subsequently incubated with HRP-Tf (Jackson ImmunoResearch) at 200 ng/mL plus either TbpA loop-specific mouse antisera (190), polyclonal TbpA rabbit antisera (242), unlabeled human Tf (hTf) (Sigma), or unlabeled

bovine Tf (bTf) (Sigma) for 1 hour. Blots were washed 5 more times with LS-TBS, then they were developed with the Opti-4CN (Bio-Rad) development system.

V. Whole cell ligand blocking ELISA

Maxisorp microtiter dishes (Nunc) were coated with 0.01% poly-L-lysine (Sigma) in PBS overnight at 4°C. Gonococci were iron-stressed by overnight growth on GCB agar plates containing 5 µM Desferal. Gonococcal cells were harvested from the agar plates and standardized to an OD₆₀₀ of 1.0 in phosphate buffered saline (PBS). One hundred microliters of the cell suspension was applied in triplicate for each strain and allowed to incubate on the plate for 1 hour. The microtiter plate was washed 5 times with PBS, and then 200 µl of 3% bovine serum albumin (BSA; Pierce) in PBS was added for 1 hour. After blocker was removed, anti-peptide or anti-holo TbpA serum was diluted 1:50 in 3% BSA, applied to the cells for 1 hour, and then washed 5 times with PBS. Then, 1 µg/ml HRP-Tf in 3% BSA was applied for 1 hour, followed by 5 washes with PBS. Subsequently, 100 µl of 1-Step Slow-TMB (Thermo) was added to colorimetrically detect the amount of HRP-Tf bound to cells in each well. After 10 minutes, 100 µl of 2 M sulfuric acid was added to each well to stop the reaction. The microtiter plate was then read at OD₄₂₀. Antibody blocking specificity was determined by performing these assays on bacterial strains without transferrin receptor proteins (gonococcal strain FA6815 and *E. coli* expressing empty vector) and subtracting these values from the experimental strains prior to normalization to the positive control.

VI. Transferrin binding assay

Assays were performed in a manner similar to that described above for the whole cell ELISA except that the step in which anti-peptide and anti-holo TbpA antisera were added was omitted. HRP-Tf was used at concentrations ranging from 2 nM to 50 nM. A standard curve of HRP-Tf, diluted in PBS, was prepared with concentrations ranging from 10 ng/mL to 1 µg/mL. Cell-containing wells were compared to the standard curve to determine the amount of Tf bound. Data was then normalized to the positive control.

VII. Radiolabeled iron uptake assay

Tf-iron uptake assays were performed as described previously (192, 196, 243, 244). Briefly, apo-human Tf (Sigma) was saturated to 20% with ⁵⁵Fe (Perkin-Elmer). Gonococci were iron-stressed in liquid CDM for 3 hours, and then 100 µl of the culture was applied in triplicate to two multiscreen microtiter dishes (Millipore). Each microtiter well contained 1.5% BSA as a non-specific protein blocker. One dish received 215 µM potassium cyanide (KCN) to determine counts bound but not internalized. Both plates were incubated for 10 minutes at 37°C and 5% CO₂. Subsequently, 3 µM 20% ⁵⁵Fe-saturated human Tf was added to each well, and plates were again incubated for 30 minutes to allow iron internalization. Following incubation, each plate was filtered, washed with citrate buffer (100 mM NaCitate, 1 mM MgCl₂, 0.25 mM CaCl₂ [pH 7.0]), dried, and individual filters from each well were removed. Radioactive iron was detected using a Beckman LS6500 beta counter. All counts were averaged, and surface associated counts (KCN condition) were subtracted from total counts to determine the specific amount of iron internalized in 30 minutes. Internalized iron was standardized to micrograms of total cellular

protein in 100 μ l of culture, as determined by bicinchoninic acid assay (BCA; Pierce). Final data is presented as values normalized to the positive control.

VIII. Protein analysis methods

A. Whole cell lysates preparation

To analyze iron repressed proteins, gonococcal strains were grown in liquid CDM as described above. After growth, cultures were standardized, and centrifuged at 16,000 x *g* for 2 minutes. Then, supernatants were removed, the pellet was re-suspended in 100 μ L of Laemmli solubilizing buffer (245), and the samples were frozen at -20° C until use. Immediately prior to SDS-PAGE, lysates were supplemented with 5% β -mercaptoethanol, passed through a 28 gauge syringe several times to reduce viscosity, and boiled for 3 minutes.

B. SDS-PAGE

Lysate proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% Tris-HCl Criterion precast gels (BioRad) using a Criterion vertical electrophoresis cell (BioRad).

C. Western blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes submerged in 16 mM Tris base, 120 mM glycine, and 20% methanol (246) in a Criterion blotter (BioRad) at 28 mAmps for 16 hours. Membranes were stained with Ponceau S solution (0.1% w/v Ponceau S, 5% acetic acid) to confirm equal protein loading in each lane prior to immunodetection, and then de-stained with in distilled water. For detection of TbpA, proteins were blocked with 5% BSA in high-salt TBS (20 mM Tris, 500 mM NaCl [pH 7.5], 0.02% NaN₃, 0.05% Tween 20).

Blocked membranes were probed with polyclonal rabbit serum against full-length TbpA (194). Goat anti-rabbit IgG conjugated to alkaline phosphatase (AP; BioRad) was used as the secondary antibody. AP conjugates were detected using the nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) development system (Sigma).

IX. Protease accessibility assay

Protease accessibility experiments were performed as described previously (193). Briefly, whole iron-stressed gonococci were exposed to trypsin for 0, 10, 20, or 30 minutes, the trypsin was quenched with aprotinin (Sigma), then cultures were pelleted, lysed, and subjected to SDS-PAGE and western blotting.

X. OSU and PDE-5 inhibitor growth assays

OSU-03012 (OSU) and the PDE-5 inhibitors sildenafil (Sil), tadalafil (Tad), and vardenafil (Var) were dissolved in 100% dimethyl sulfoxide (DMSO) to concentrations up to 4000 fold higher than the desired final concentration due to the toxic effects of DMSO on *N. gonorrhoeae* growth. The drug was then diluted to twofold the desired final concentration in CDM, and 100 μ L was applied in triplicate to an untreated 96-well flat-bottom microtiter dish (Corning). Gonococcal strains were grown in liquid CDM until doubling as described above. Instead of diluting the culture to 15 KU, cultures were diluted to OD₆₀₀ 0.2 with fresh CDM, and 100 μ L standardized culture was applied to the drug-loaded microtiter dish in triplicate. The initial OD₆₀₀ of each well was measured using a Vmax kinetic plate reader (Molecular Devices), and

then the microtiter plates were placed on a platform shaker at 200 rpm inside a standing incubator at 37 °C with 5% CO₂. The OD₆₀₀ was measured hourly until assay termination.

XI. Small molecule inhibitor assays

These assays were carried out similarly to the whole cell ligand blocking ELISAs described above with one exception. The inhibitory molecules are applied before the blocking step, due to possible inhibition of the albumin, at 50 mM concentration dissolved in PBS. For this assay, the negative control is unlabeled transferrin, which acts as a competitive inhibitor of HRP-Tf. The positive control is a vehicle only condition, which is most often DMSO. Final data is presented as values normalized to the positive control.

XII. Statistics

Comparison of results between the positive control and mutant strains was performed after analysis of variance using the Student's *t* test. Pairwise comparisons with a *P*-value of <0.05 were considered statistically significant. ELISAs and radiolabeled iron uptake assays shown are the means of multiple individual concentration points from studies performed at least in triplicate (\pm SEM).

*Some methods adapted from: Copyright © American Society for Microbiology, *Infection and Immunity*, 83, 2015, 4438-4449, doi:10.1128/IAI.00762-15

Table 1. Bacterial strains used in this study

Strain	Description	Ref. or Source
E. coli		
BL21(DE3)	<i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS</i> <i>λ DE3 = λ sBamH1o ΔEcoRI-B int:::(lacI::PlacUV5::T7 gene1) i21</i> <i>Δnin5</i>	New England Biolabs
C41(DE3)	F ⁻ ompT hsdS _B (rB ₋ mB ₋)(rB ₋ mB ₋)gal dcm (DE3) uncharacterized derivative of BL21 (DE3)	Lucigen
Top10	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL(Str ^r) endA1 nupG	Invitrogen
Stellar	F ⁻ , endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ (lacZYA-argF) U169, Δ (mrr-hsdRMS-mcrBC), ΔmcrA, λ ⁻	Clontech
XL-10 Gold	endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tet ^R F'[proAB lacI ^q ZΔM15 Tn10(Tet ^R Amy Cm ^R)]	Agilent Technologies
<i>N. gonorrhoeae</i>		
FA19	Wild type	(247)

FA6747	TbpA- (<i>tbpA::mTn3cat</i>)	(194)
FA6905	TbpB- (Δ <i>tbpB</i>)	(193)
FA6815	TbpAB- (<i>tbpB::Ω</i>)	(196)
FA1090	Wild type (Δ <i>lbpA</i> , HpuAB off)	(249)
H041	Multi-drug resistant isolate	(61)
F89	Multi-drug resistant isolate	(63)
MCV511	L3HA ₍₃₄₃₎ TbpA Lbp ⁻ (<i>tbpA∇HA lbpB::Ω</i>)	(250)
MCV512	L3HA ₍₃₄₃₎ TbpA TbpB ⁻ Lbp ⁻ (<i>tbpA∇HA lbpB::Ω Δ<i>tbpB</i>)</i>	(250)
MCV161	TbpA K351A point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV162	TbpA D355A point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV163	TbpA D355K point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV164	TbpA N357A point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV165	TbpA Q358A point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV166	TbpA K359A point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV167	TbpA K359E point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV168	TbpA K359R point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV169	TbpA Q360A point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV170	TbpA Q360E point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV171	TbpA Q360K point mutation TbpB- (Δ <i>tbpB</i>)	(242)
MCV172	TbpA Loop 3 Helix Deletion (T350-A361) TbpB- (Δ <i>tbpB</i>)	(242)
MCV181	TbpA K351A point mutation	(242)
MCV182	TbpA D355A point mutation	(242)

MCV183	TbpA D355K point mutation	(242)
MCV184	TbpA N357A point mutation	(242)
MCV185	TbpA Q358A point mutation	(242)
MCV186	TbpA K359A point mutation	(242)
MCV187	TbpA K359E point mutation	(242)
MCV188	TbpA K359R point mutation	(242)
MCV189	TbpA Q360A point mutation	(242)
MCV190	TbpA Q360E point mutation	(242)
MCV191	TbpA Q360K point mutation	(242)
MCV192	TbpA Loop 3 Helix Deletion (T350-A361)	(242)
MCV140	HpuA- (hpuA::aphA-3)	This study

Table 2. Plasmids used in this study

Plasmid	Description	Ref. or Source
pHSS6-GCU	Vector containing gonococcal uptake sequence (Kan ^r)	(248)
pUNCH412	FA19 <i>tbpA</i> in pET-11	(251)
pUNCH755	Vector containing truncated and nonfunctional <i>tbpB</i> gene, full length <i>tbpA</i> gene, and <i>tbpA</i> downstream region with an mTn3Cm insertion	(196)
pKH37	<i>Neisseria lctP-aspC</i> complementation construct	(252)
pUC18K	pUC-18 with <i>aphA-3</i> kanamycin resistance gene	(253)
pVCU150	pHSS6-GCU containing <i>tbpA</i> gene with K351A mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU151	pHSS6-GCU containing <i>tbpA</i> gene with D355A mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU152	pHSS6-GCU containing <i>tbpA</i> gene with D355K mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU153	pHSS6-GCU containing <i>tbpA</i> gene with N357A mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU154	pHSS6-GCU containing <i>tbpA</i> gene with Q358A mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU155	pHSS6-GCU containing <i>tbpA</i> gene with K359A mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)

pVCU156	pHSS6-GCU containing <i>tbpA</i> gene with K359E mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU157	pHSS6-GCU containing <i>tbpA</i> gene with K359R mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU158	pHSS6-GCU containing <i>tbpA</i> gene with Q360A mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU159	pHSS6-GCU containing <i>tbpA</i> gene with Q360E mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU160	pHSS6-GCU containing <i>tbpA</i> gene with Q360K mutation, novel silent BamHI site at <i>tbpA</i> bp 1488-1493, and no stop codon	(242)
pVCU161	pUNCH755 containing <i>tbpA</i> gene from pVCU150	(242)
pVCU162	pUNCH755 containing <i>tbpA</i> gene from pVCU151	(242)
pVCU163	pUNCH755 containing <i>tbpA</i> gene from pVCU152	(242)
pVCU164	pUNCH755 containing <i>tbpA</i> gene from pVCU153	(242)
pVCU165	pUNCH755 containing <i>tbpA</i> gene from pVCU154	(242)
pVCU166	pUNCH755 containing <i>tbpA</i> gene from pVCU155	(242)
pVCU167	pUNCH755 containing <i>tbpA</i> gene from pVCU156	(242)
pVCU168	pUNCH755 containing <i>tbpA</i> gene from pVCU157	(242)
pVCU169	pUNCH755 containing <i>tbpA</i> gene from pVCU158	(242)
pVCU170	pUNCH755 containing <i>tbpA</i> gene from pVCU159	(242)
pVCU171	pUNCH755 containing <i>tbpA</i> gene from pVCU160	(242)

pVCU172	pUNCH755 containing <i>tbpA</i> gene with deletion from bp 1453-1488 (loop 3 helix region)	(242)
pVCU173	<i>hpuA</i> wt in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU174	<i>hpuA</i> Y78A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU175	<i>hpuA</i> F79A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU176	<i>hpuA</i> Y81A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU177	<i>hpuA</i> Y111A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU178	<i>hpuA</i> D115A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU179	<i>hpuA</i> F116A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU180	<i>hpuA</i> S140R in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU181	<i>hpuA</i> G141R in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU182	<i>hpuA</i> Y226A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU183	<i>hpuA</i> Y227A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU184	<i>hpuA</i> F295A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU185	<i>hpuA</i> R299A in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU186	<i>hpuA</i> CC136/137SS in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU187	<i>hpuA</i> Δ77-82 in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU188	<i>hpuA</i> Δ111-116 in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU189	<i>hpuA</i> Δ225-31 in pCVD005, locked phase-on	Dr. Stephen Hare
pVCU234	pKH37 with RBS added between PacI and SmaI	This study
pVCU235	pVCU234 with <i>hpuA</i> from pVCU173 at SmaI site	This study
pVCU236	pVCU234 with <i>hpuA</i> from pVCU174 between SmaI and XhoI	This study

pVCU237	pVCU234 with <i>hpuA</i> from pVCU175 between SmaI and XhoI	This study
pVCU238	pVCU234 with <i>hpuA</i> from pVCU176 between SmaI and XhoI	This study
pVCU247	pVCU234 with <i>hpuA</i> from pVCU185 between SmaI and XhoI	This study
pVCU249	pVCU234 with <i>hpuA</i> from pVCU187 between SmaI and XhoI	This study
pVCU250	pVCU234 with <i>hpuA</i> from pVCU188 between SmaI and XhoI	This study
pVCU251	pVCU234 with <i>hpuA</i> from pVCU189 between SmaI and XhoI	This study
pVCU252	pVCU403 with <i>hpuA</i> R299A inserted at the SmaI site	This study
pVCU253	pVCU252 with <i>aphA-3</i> Kn ^R cassette in PpuMI site	This study
pVCU403	pUC18 with the gonococcal uptake sequence	(254)
pVCU757	FA1090 <i>tbpA</i> in pET22b	This study

Table 3. Primers used in this study

Primer	Use	Sequence (5'-3')
oVCU745	Fwd: <i>tbpA</i> loop 3 sequencing	CGGTCGTGGCTGTTCCGCCC
oVCU747	Fwd: <i>tbpA</i> loop 3 amplification	CGGGGGAAATCCGCGCCC
oVCU748	Rev: <i>tbpA</i> loop 3 amplification	CCGATGCCCTGCCGGTCGTAAGAG
oVCU750	Fwd: <i>tbpB</i> deletion screening	TCGGGCGATGAAGGCGAAACAACCTCCA
oVCU752	Rev: <i>tbpB</i> deletion screening	CTGCATAAGCGGGCAGCGCAGTCAT
oVCU785	S: <i>tbpA</i> loop 3 helix deletion	GTTCCGGCATTCTGGGATCCTTGCGCGGC
oVCU786	AS: <i>tbpA</i> loop 3 helix deletion	GCCGCGCAAGGATCCCAGAAATGCCGGAAC
oVCU790	Fwd: <i>tbpA</i> Apal-RsrII sequencing	GCAAAAACGGGGGTCACGAAAAGTGTAAGCG
oVCU791	Fwd: <i>tbpA</i> Apal-RsrII sequencing	GCACACCAAAGCCGCTACGGTTTGGAATATGTC
oVCU814	S: RBS inserted into pKH37	TAAAAGGAGCCC
oVCU815	AS: RBS inserted into pKH37	GGGCTCCTTTAAT
oVCU816	Fwd: pKH37 sequencing	CTAGGCACCCCAGGCTTTACAC
oVCU817	Fwd: <i>hpuA</i> sequencing	TCGCCGACGGCAGCCGCATCTTTA
oVCU818	Fwd: <i>hpuA</i> amplification	CAAAGCCCTGCCCTTACTGCCGCTT
oVCU819	Rev: <i>hpuA</i> amplification	GGGAGCGGTGCGCGTCAAAGG
oVCU821	Fwd: In-fusion <i>hpuA</i> into pVCU234 SmaI site	AATTAAGGAGCCCAATTCATG- AAATACAAAGCCCTGCCCTTACTG

oVCU822	Rev: In-fusion <i>hpuA</i> into	GAATTCCTGCAGCCCTTATTGTTT-
	pVCU234 <i>SmaI</i> site	AGTAAGGTGGTTGGTATCTTGACTGGTG
oVCU823	Fwd: In-fusion <i>hpuA</i> into	CTCTAGAGGATCCCCATGAAATA-
	pVCU403 <i>SmaI</i> site	CAAAGCCCTGCCCTTACTGCCG
oVCU824	Rev: In-fusion <i>hpuA</i> into	TCGAGCTCGGTACCCTTATTGTTTAGTAA-
	pVCU403 <i>SmaI</i> site	GGTGGTTGGTATCTTGACTGGTGTCATC
oVCU825	Fwd: In-fusion <i>aphA-3</i> into	GGGCGTGGATAGGTCCCCGGGT-
	pVCU252 <i>PpuMI</i> site	GACTAACTAGGAGGAATAAATGG
oVCU826	Rev: In-fusion <i>aphA-3</i> into	TTTCGCCGTTGGGACCCCGGGTC-
	pVCU252 <i>PpuMI</i> site	ATTATTCCTCCAGGT
oVCU841	Rev: <i>hpuA</i> slipstrand sequencing	TCTGCTGCGGCTCGGCGCGTC

Fwd, Forward; Rev, Reverse; S, Sense; AS, anti-sense

Chapter 3: The Function and Vaccine Potential of Gonococcal TbpA Extracellular Loops

I. Introduction

One approach to development of therapeutics has been to target how the gonococcus acquires iron, an essential nutrient for nearly all microorganisms (255). During human infection, microorganisms are confronted with the challenge of obtaining iron in an environment that has evolved to specifically restrict its availability. In humans, iron is circulated throughout the body bound to several transport proteins, including lactoferrin and transferrin (Tf). These iron binding proteins minimize free iron concentrations, which helps to reduce free radical generation as well as to starve any invading microbes. In response to this iron-limitation, many bacteria produce siderophores to compete with these human proteins for iron (256). However, *N. gonorrhoeae* has evolved the ability to acquire iron directly from these human proteins.

The gonococcus expresses receptors that can extract iron or heme from Tf, lactoferrin and hemoglobin, but the receptors are not universally expressed (213, 236). The lactoferrin receptor proteins, LbpA and LbpB, are only expressed in approximately 50% of strains (215, 247). The hemoglobin receptor proteins, HpuA and HpuB, are expressed only in isolates from women in the first half of the menstrual cycle due to phase variation (257). By contrast, the Tf receptor system composed of an integral, outer-membrane, TonB dependent transporter, TbpA, and a surface exposed lipoprotein, TbpB, is expressed in 100% of clinical isolates (258). This set of human adapted receptors has made the gonococcus well suited to surviving in the iron-limited environment of the human host.

Iron acquisition systems are potential targets for novel drug therapies as well as for vaccine development. Because the gonococcal Tf receptors are not subject to phase or antigenic variation, are present in all clinical isolates, and are necessary for initiation of infection in humans (198), they stand out as ideal candidates for further investigation. Much work has already been done to probe this receptor complex for structure-function relationships and vaccine potential (213, 236, 258). Recently, the Tf receptors, TbpA and TbpB, were crystallized from the closely-related pathogen *Neisseria meningitidis*, which share 94% and 69% identity with the respective proteins in *N. gonorrhoeae* strain FA19 (190, 191). The structural studies provided the molecular details for how these receptors interact with Tf and will significantly contribute to the study of these proteins for therapeutic development. Based on the structure of TbpA, experiments were performed to test various surface exposed epitopes as immunogens and to determine if antibodies raised against these regions could interrupt protein function (190). The results of these studies were promising; however, they were performed using recombinant *N. meningitidis* TbpA in an *in vitro* assay. Here, we follow up these studies to test the degree to which these antibodies inhibit ligand binding to TbpA in the native bacterium *N. gonorrhoeae*. Further, we developed our own loop specific antibodies in an attempt to improve their inhibitory efficiency. To probe the structure-function relationships of this receptor, we built on previous work to target the TbpA loop 3 helix, a motif located in close proximity to the iron-chelation center within the C-lobe cleft of Tf (Fig. 3.1). Based on the TbpA crystal structure and previous evidence that interruption of loop 3 led to a loss of protein function (250), this motif is predicted to be a key to protein function. To assess the contribution of the loop 3 helix to protein function, site specific mutagenesis of polar helix residues and a total helix deletion

were employed, followed by ligand binding and radiolabeled iron uptake assays. Mutants created were also tested for the ability to grow on Tf as a sole iron source.

The studies described here demonstrate that the original loop antibodies had only modest abilities to block ligand binding to TbpA. Similarly, newly-generated loop-specific antibodies led to modest inhibition of ligand binding to TbpA. However, unlike the original antibodies, the newer antibodies had a greater effect on the gonococcus than in recombinant *E. coli*. With regards to the loop 3 helix, reduction of TbpA function could be achieved with single amino acid mutations; however, the co-receptor, TbpB, could largely compensate for these defects in TbpA function. All singly mutated strains were capable of growth on poorly saturated Tf; however, a total helix deletion strain could not grow under these conditions. This work provides new insights into improved antibody development against TbpA loop peptides, which will enhance vaccine efforts going forward. The current study also demonstrates that the entire loop 3 helix significantly contributes to TbpA function, but that there is no single amino acid that is crucial. This information contributes to our expanding understanding of key ligand-interacting domains, which will likely be needed in order to optimize immunogen interaction with the host immune system during vaccination (259)

Figure 3.1. Homology model for TbpA from gonococcal strain FA19.

A) A sequence alignment of TbpA from *N. meningitidis* strain K454 (NmTbpA) and from *N. gonorrhoeae* strain FA19 (NgTbpA), which are 94% identical in sequence. This alignment served as the basis for homology modeling of TbpA from strain FA19. B) Based on the complex crystal structure with NmTbpA, human Tf (hTf-C/hTf-N) (shown in gold) was modeled interacting with NgTbpA (shown in light green), with the plug domain shown in red and the loop 3 (L3) helix shown in dark green. The C1 and C2 domain of the C-lobe of hTf, which directly interact with TbpA are also indicated. C) Loops 3, 7, 11 and part of the plug were selected for initial antibody blocking studies against TbpA from strain K454. Highlighted in magenta are the conserved regions of the NgTbpA model, to which those antibodies were developed. Pairwise comparisons of the peptide sequences are also shown.

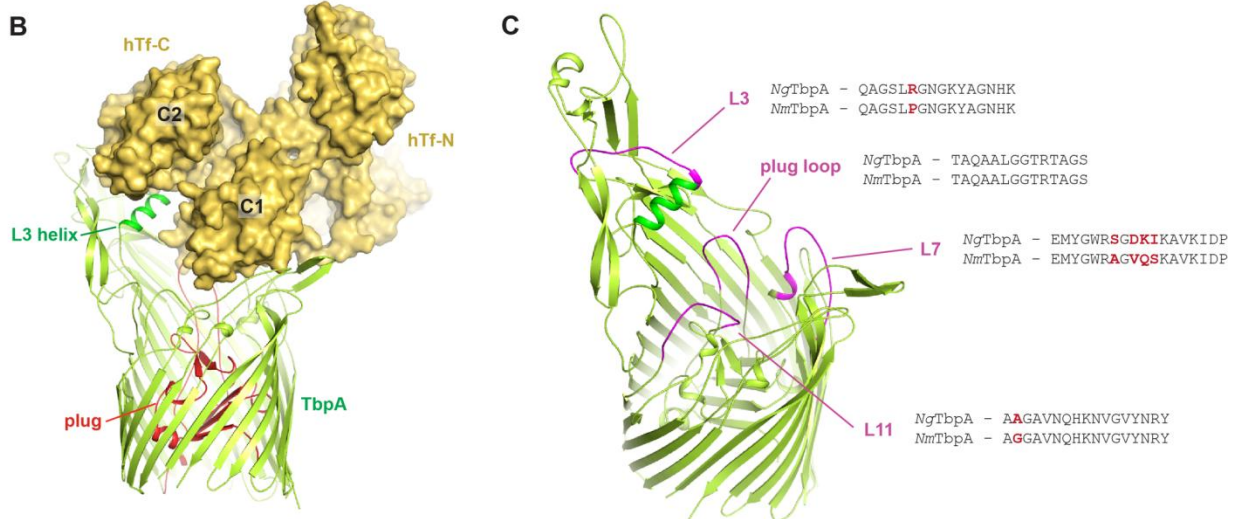
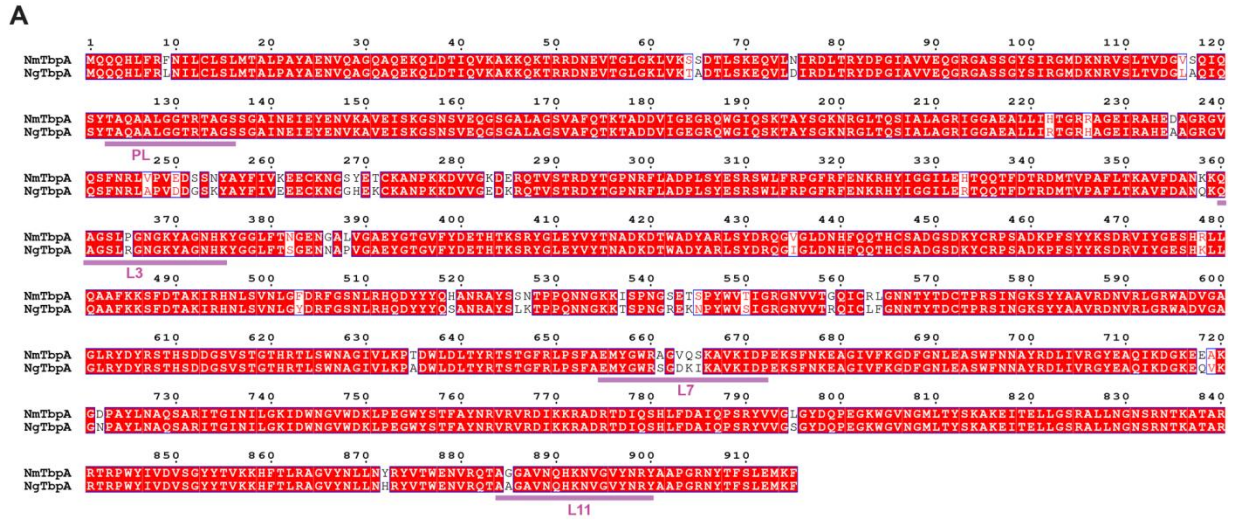


Figure 3.1. Homology model for TbpA from gonococcal strain FA19

II. Results

A. Antibodies raised against linear TbpA loop peptides minimally inhibit ligand binding to gonococcal whole cells

Recent studies (190) reported the TbpA crystal structure from *N. meningitidis* and identified surface exposed loops that facilitated substantial interaction with human Tf (Fig. 3.1). Linear peptide domains from several interactive loops were used as antigens in mice, and the subsequently produced sera were applied to *E. coli* strains expressing recombinant meningococcal TbpA. In recombinant *E. coli*, these antibodies were individually capable of blocking human Tf binding by approximately 50%; however the previous study did not address whether the antibodies were capable of direct interaction with TbpA in whole *Neisseria* cells (190). To investigate this in *N. gonorrhoeae*, we first used the structure of TbpA from *N. meningitidis* strain K454 to create a homology model of TbpA (Fig. 3.1). Because the selected loops have highly conserved sequences (Fig. 3.1A), we tested whether the antibodies generated against the meningococcal antigens could block ligand binding in the gonococcus. We implemented a solid-phase binding assay using whole iron-stressed gonococci to address this question. We first demonstrated the specificity of this assay by showing that unlabeled hTf, but not bovine Tf (bTf), was capable of blocking deposition of horseradish peroxidase (HRP) tagged Tf (Fig. 3.2A). We then determined that the loop antibodies alone and in combination were

Figure 3.2. Solid phase antibody-mediated ligand blocking assays.

Whole iron-stressed gonococci or *E. coli* expressing recombinant TbpA were applied to a nitrocellulose membrane and allowed to dry. A) Blots were blocked with either 20 µg/mL hTf or bovine Tf (negative control), followed by the application of HRP-Tf. After washing, HRP was detected using Opti4CN (BioRad). Positive control contains no blocking agent (-) and therefore represents maximal hTf binding. B) Blots were blocked with antibodies, followed by the application of HRP-Tf. Peroxidase activity was detected using Opti4CN. Also tested was an antibody raised against full-length TbpA (anti-TbpA). Negative controls are strains in which TbpA is absent (A-/B-). C) A similar assay was performed using whole cells in a microtiter dish for an ELISA (n=3). TMB (Thermo) was used as the peroxidase substrate, and absorbance was measured at OD₄₂₀. The positive control is the condition without antibody (No Ab), and the negative control is incubation with unlabeled Tf. Data represents specific binding by subtracting values obtained from strains without transferrin receptors from the experimental strains. Significant differences are noted, where * represents $P < .01$, and # represents $P < .05$. Statistics were calculated using the Student's *t* test.

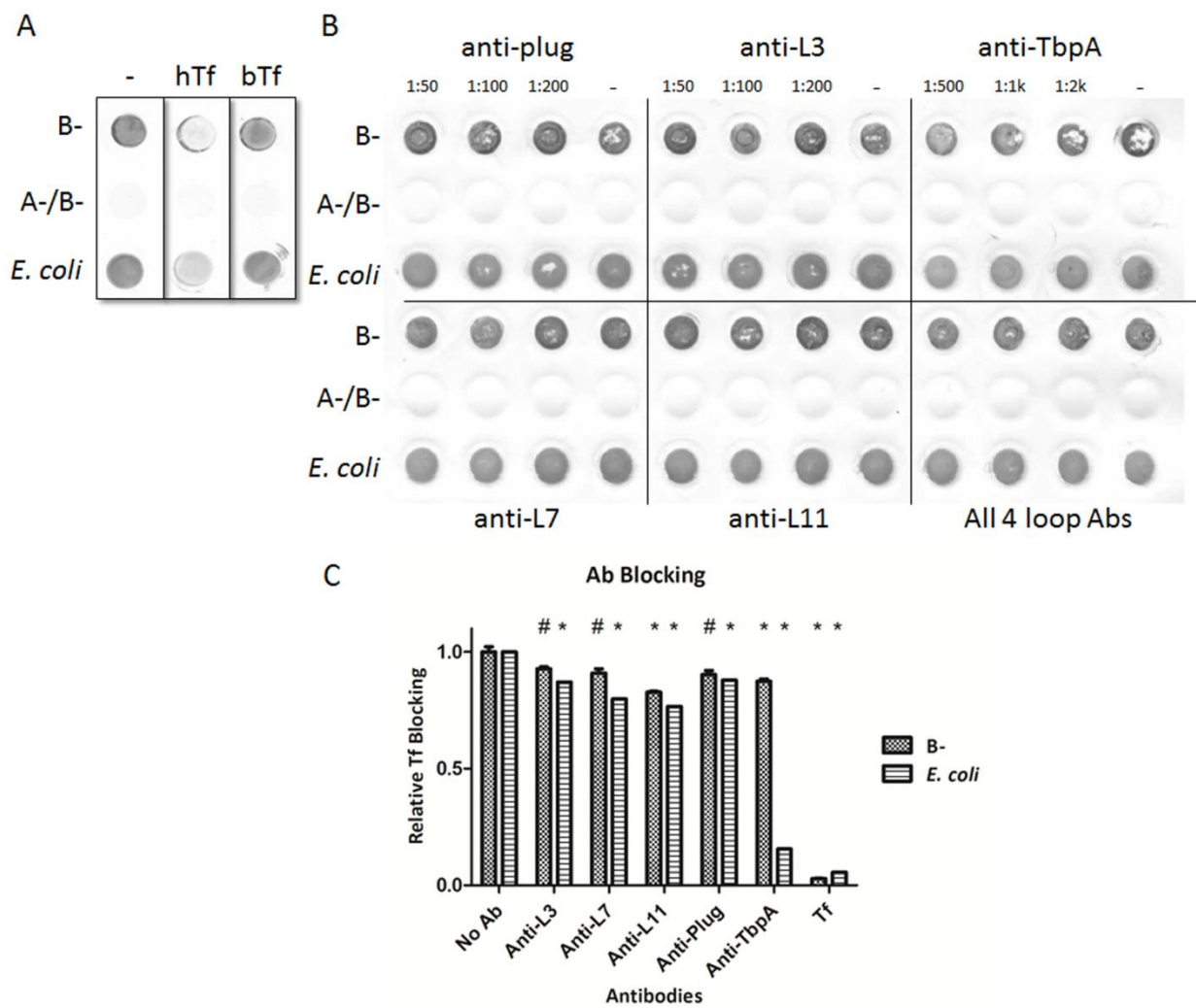


Figure 3.2. Solid phase antibody-mediated ligand blocking assays

unable to substantially block ligand binding (Fig. 3.2B). An antibody developed against full-length TbpA was capable of modest blocking. These conclusions were further supported by antibody mediated ligand blocking ELISAs (Fig. 3.2C). When quantified, none of the antibodies was capable of accomplishing greater than 20% inhibition of ligand binding on the gonococcal surface. Interestingly, the antibodies had a larger inhibitory effect on the *E. coli* strain over-expressing TbpA. This phenomenon is more pronounced when evaluating the effects of the holo-TbpA antibody, which only inhibited 13% of ligand binding in the gonococcus, but resulted in an 85% reduction in ligand binding in *E. coli*. With the evidence that these loop antibodies were insufficient to significantly abrogate ligand binding, TbpA loop antibodies were regenerated using a different approach in an effort to optimize their inhibitory characteristics.

B. Antibodies raised against cyclized TbpA loop peptides demonstrate modest Tf blocking on whole cells

In reviewing the way the original TbpA loop antibodies were designed, several areas for possible improvement were discovered. The first was that the linear epitopes may not have taken on the appropriate 3D conformation required to target surface exposed, folded TbpA. The second was that the antibodies were developed against peptides with *N. meningitidis* sequence, which is not identical to the gonococcal sequence (Fig. 3.1), particularly in loop 7. Lastly, the peptide fragments were short, often less than half the length of the entire surface exposed loop. In the redesign of the loop antibodies, all three of these concerns were addressed. The peptides were designed to contain gonococcal sequence, were substantially extended in length, and were cyclized by adding cysteine residues to both ends (Table 4). Mice were immunized subcutaneously using Titermax as the adjuvant, with boosters given at days 21

Table 4. Peptide sequences for second generation immunizations

Loop	Peptide Sequence
Loop 3	CTKAVFDANQKQAGSLRGNGKYAGNHKC
Loop 7	CRLPSFAEMYGWRSGDKIKAVKIDPC
Loop 11	CRYVTWENVRQTAAGAVNQHKNVGVYNRYAAPGRC

and 42. On first attempt, titers for loop 7 and loop 11 derived antibodies were robust, but loop 3 was found to be poorly immunogenic. After analyzing the peptide sequence for MHC class II epitopes, the loop 3 peptide had a weak score compared to the other loop peptides. A second attempt at loop 3 peptide immunization was performed, adding ovalbumin to the peptide and Titermax adjuvant, which resulted in improved titers. With the new sera (second generation), the solid phase ligand blocking assay described above was repeated. Despite the additional considerations applied to their design, there was no detectable ligand blocking capacity for the second generation antibodies (Fig. 3.3A). Again, these results were supported by antibody mediated ligand blocking ELISAs (Fig. 3.3B). The second generation antibodies had comparable effects to the original antibodies (Fig. 3.2C) on the gonococcal surface, with reductions in ligand binding of 11- 17%. However, the redesigned antibodies had less inhibitory effect on the recombinant TbpA over-expressing *E. coli* strain. Although no greater inhibitory effect was obtained with the redesigned antibodies, these data suggest that there are differences in the presentation of TbpA between *E. coli* and the gonococcus. This is most prominently shown with our holo-TbpA control antibody, which demonstrated substantial Tf blocking in *E. coli*, but very little blocking in the gonococcus. Accordingly, differences in bacterial surfaces should be taken into consideration in future studies. Having addressed the interaction of loop-specific antibodies with the native bacterium, focus was shifted to probing the structure-function properties of the loop 3 helix of TbpA, which was hypothesized to be a key functional domain.

Figure 3.3. Antibody-mediated ligand blocking assays for second generation loop-specific antibodies.

Whole, iron-stressed gonococci or *E. coli* expressing recombinant TbpA were applied to a nitrocellulose membrane and allowed to dry. A) To determine loop-antibody blocking capability, blots were blocked with antibodies, followed by the application of HRP-Tf. For both blots, HRP was detected using Opti4CN. The condition labeled (–) contained no antibody and therefore represents the maximal amount of hTf bound. B) A similar assay was performed using whole cells in a microtiter dish for an ELISA (n=3). TMB (Thermo) was used as the peroxidase substrate, and absorbance was measured at OD₄₂₀. The positive control is the condition without antibody (No Ab), and the negative control is incubation with unlabeled hTf (Tf). Data represents specific binding by subtracting values obtained from strains without transferrin receptors from the experimental strains. Significant differences are noted, where * represents $P < .01$, and # represents $P < .05$. Statistics were calculated using the Student's *t* test.

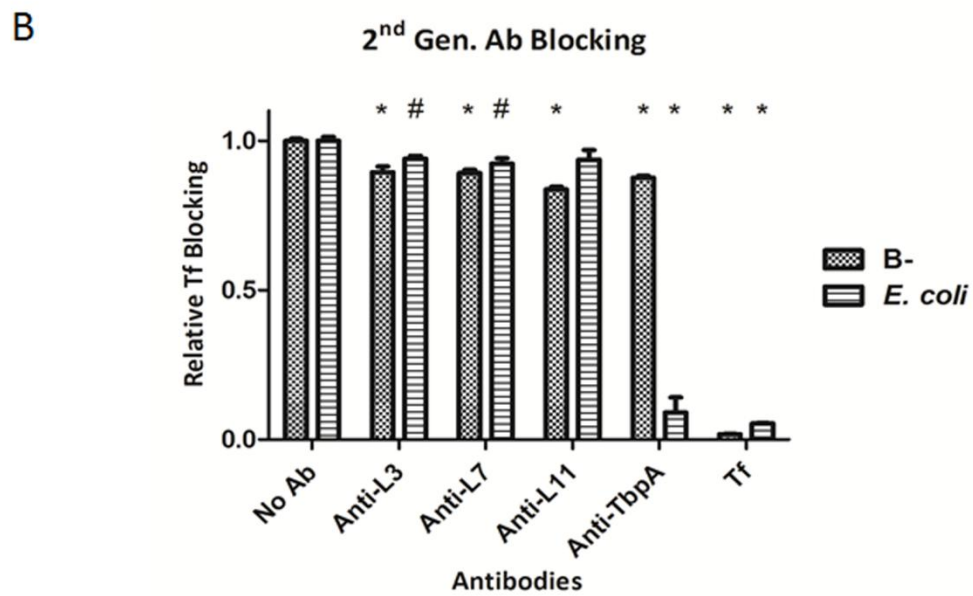
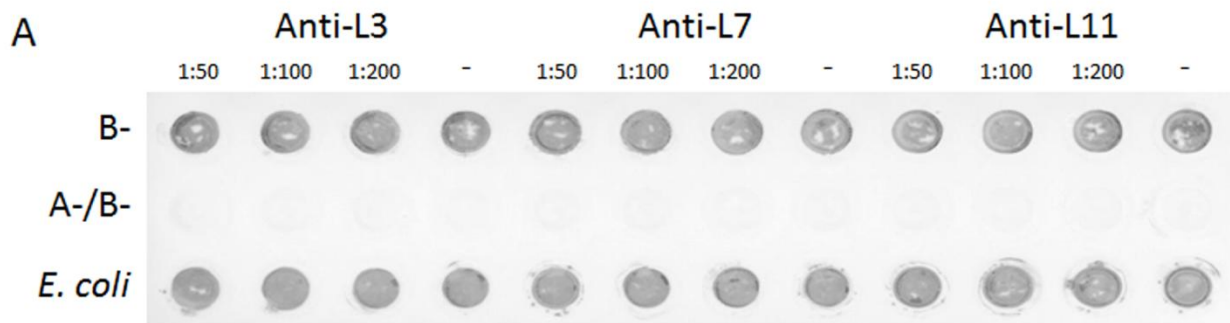


Figure 3.3. Antibody-mediated ligand blocking assays for second generation loop-specific antibodies

C. Creation of TbpA loop 3 mutants

Within the iron chelation center in Tf, a triad of pH sensing residues has been shown previously to control iron binding affinity (258, 260). The crystal structure of TbpA suggests that a polar residue on the loop 3 helix might be able to destabilize the triad's charge balance in Tf, leading to iron release. To test this hypothesis, substitution mutations for all of the polar residues on the loop 3 helix were created (Fig. 3.4, Table 3). Site-directed point mutations and deletions were constructed using the Quikchange system (Agilent). Briefly, the *tbpA* gene was amplified from strain FA19 and inserted into pHSS6-GCU (248) at the EcoRI restriction site. Subsequently, a silent BamHI restriction site was added adjacent to the DNA encoding the loop 3 helix. Mutagenic primers were used for creating point mutants resulting in new plasmids with point mutations in *tbpA*. The resulting plasmids (pVCU150-160) were then digested with Apal and RsrII to obtain an approximately 870 bp region of *tbpA* containing the mutations. The pUNCH755 plasmid (Fig. 3.5), which contains a truncated *tbpB* gene, the full *tbpA* gene, and the *tbpA* downstream region containing a chloramphenicol resistance gene, was equivalently digested. The wild-type Apal-RsrII *tbpA* region of pUNCH755 was replaced by the mutated Apal-RsrII regions from plasmids pVCU150-160, resulting in plasmids pVCU161-171. Top10 *E. coli* cells were then transformed with the newly constructed plasmids, with selection for chloramphenicol resistance as described. Because the new plasmids with mutated *tbpA* also contained a truncated *tbpB* gene, a single gonococcal transformation yielded colonies that had mutated TbpA with or without functional TbpB, depending on the distance between crossovers (Fig. 3.5). Transformants were analyzed for the presence or absence of the complete *tbpB* gene by PCR. PCR amplification of *tbpA* was followed by restriction with BamHI to confirm that the

mutagenized region was incorporated into the chromosome. The *tbpA* PCR products were also sequenced to confirm that the desired mutations were successfully incorporated into the gonococcal chromosome.

Figure 3.4. TbpA loop 3 helix polar residues.

Loop 3 helix with all polar residues displayed as stick models. All residues displayed were mutagenized for this study as listed in Table 3.

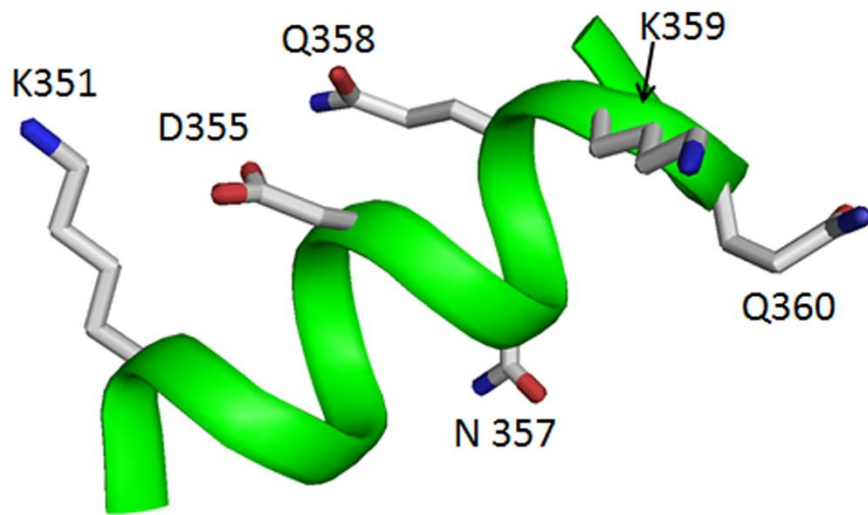


Figure 3.4. TbpA loop 3 helix polar residues

Table 5. Mutagenesis of TbpA Loop 3 helix residues

Pos.	Wild Type	Mutations
351	Lysine	Alanine
355	Asp Acid	Alanine, Lysine
357	Asparagine	Alanine
358	Glutamine	Alanine
359	Lysine	Alanine, Glutamic Acid, Arginine
360	Glutamine	Alanine, Glutamic Acid, Lysine

Figure 3.5. Gonococcal transformation with pUNCH755 derived plasmids pVCU161-171.

Image demonstrating how transformation with a single plasmid construct can result in two distinct genotypes following allelic exchange. The downstream crossover location is constrained by selection with chloramphenicol, whereas the upstream crossover can occur anywhere in *tbpA* or the truncated *tbpB*. If the crossover occurs upstream of the *tbpB* deletion, as seen on the left, the result is a TbpB⁻ phenotype. Conversely, if the crossover occurs downstream of the deletion location, as shown on the right, the result is a TbpB⁺ phenotype.

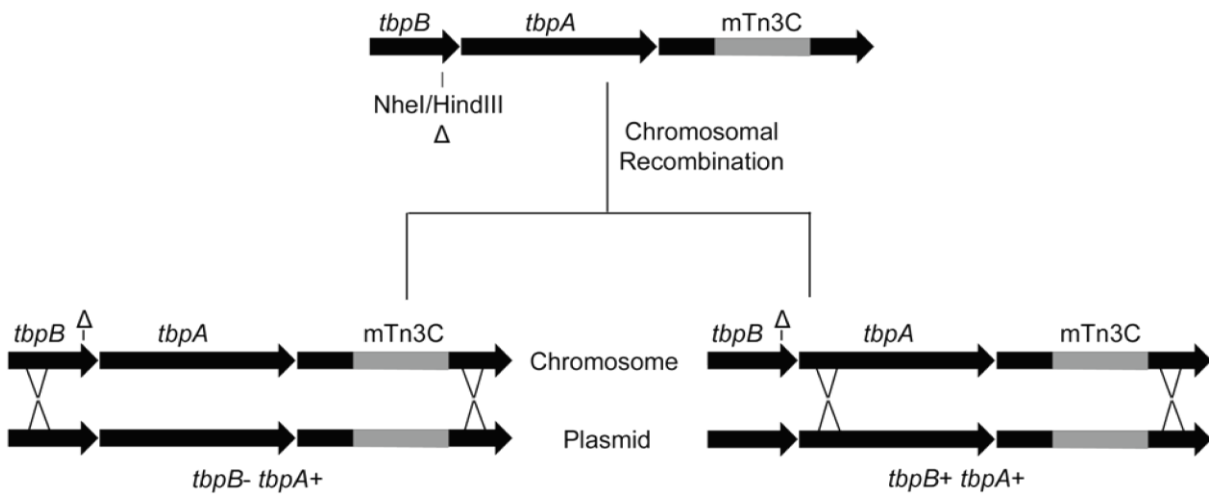


Figure 3.5. Gonococcal transformation with pUNCH755 derived plasmids pVCU161-171

D. Loop 3 helix mutant TbpA proteins are impaired for Tf binding and iron uptake in the absence of TbpB

For all newly created mutants, TbpA expression was determined to be equal to wild-type by western blot (Fig. 3.6). As the first metric of protein function, we assessed the ability of mutagenized TbpA to bind to its ligand, hTf. In order to detect a binding defect specific for TbpA, whole cell ELISAs with TbpB deficient strains were implemented. In these assays there were two negative controls. The “Comp” condition (Fig. 3.7), or competitive inhibition, contained excess unlabeled hTf applied with HRP-labeled Tf. The 6905 L3HA condition contains a *tbpB* deletion strain that simultaneously has an HA epitope insertion in TbpA loop 3. This strain has been previously shown to be incapable of binding hTf (250). In the current study, we determined that the singly substituted TbpA proteins were able to bind Tf between 40 and 80% of wild-type levels, while the loop 3 helix deletion (L3HΔ) mutant bound Tf at approximately 9% wild-type levels (Fig. 3.7). Next, the ability of each mutated TbpA to mediate iron internalization was quantified using a radiolabeled iron uptake assay. This assay employs the same 6905 L3HA strain as a negative control. In these experiments we determined that the singly substituted TbpA proteins were able to internalize iron between 14 and 47% of wild-type levels, while the loop 3 helix deletion mutant internalized iron at less than 1% of wild-type levels (Fig. 3.8). These data demonstrate that all of the single residue mutations substantially impacted TbpA function.

Figure 3.6. TbpA expression determination by western blot.

WT and mutant gonococci were iron stressed in liquid CDM for 4 hours, standardized to cell density, pelleted, and lysed. Lysates were subjected to a bicinchoninic acid assay (Thermo) to assess protein levels, and then evenly loaded for SDS-PAGE and subsequent transfer to nitrocellulose. Equivalent protein loading was confirmed by ponceau staining. The western blot was probed with polyclonal TbpA antibody, and developed with the NBT/BCIP development system (Sigma). TbpA bands are present at 100 kDa.

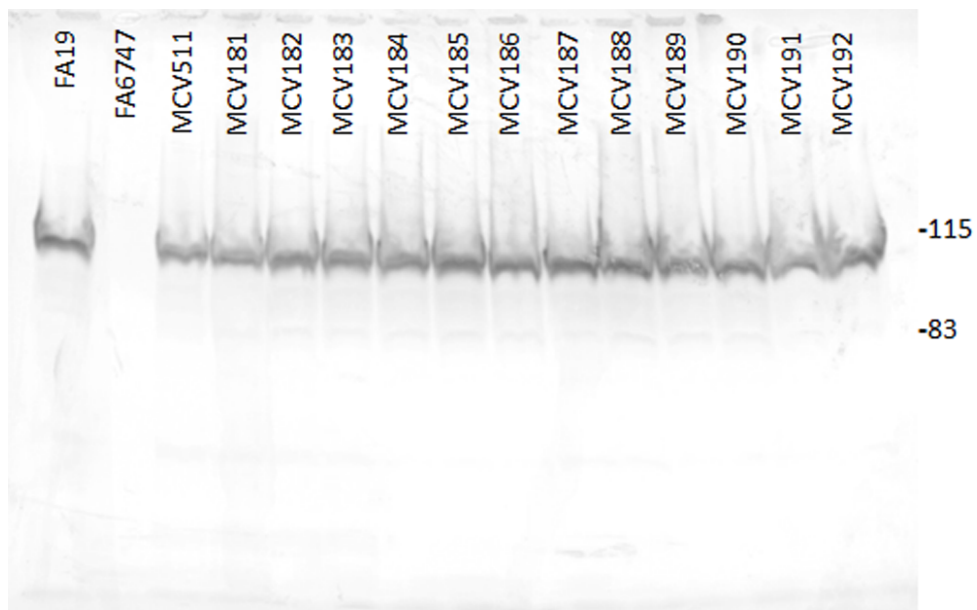


Figure 3.6. TbpA expression determination by western blot

Figure 3.7. TbpA-Tf binding ELISAs in TbpB- strains.

Whole, iron-stressed, TbpB deficient gonococcal cells were applied to microtiter dishes for ELISAs using HRP-labeled Tf as the ligand. FA6905 (WT) serves as the positive control, and FA6905 L3HA (L3HA) and excess competitor hTf condition (Comp) serve as negative controls. All data was normalized to FA6905. Data shown represents the means +/- standard errors for at least three independent binding experiments. For all mutants when compared to the positive control, $P < .001$, with the exception of Q360K, where $P < .01$. Statistics were calculated using the Student's *t* test.

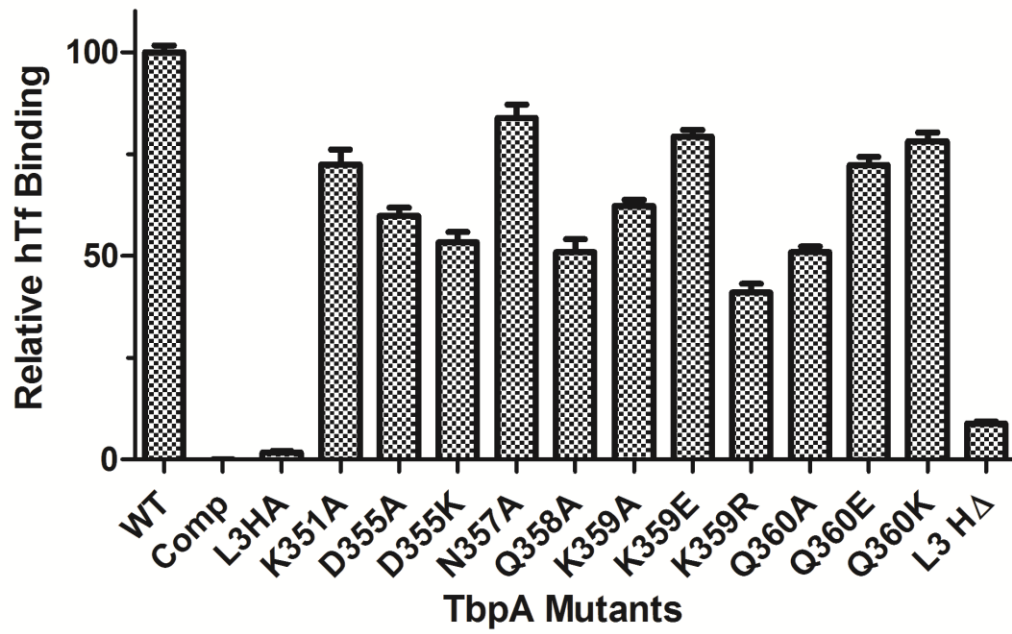


Figure 3.7. TbpA-Tf binding ELISAs in TbpB⁻ strains

Figure 3.8. Iron internalization by TbpB deficient strains.

Whole, iron-stressed, TbpB deficient gonococcal cells were applied to microtiter dishes for radiolabeled iron uptake assays. Iron uptake was calculated as counts/ μg protein. Specific uptake was calculated by subtracting +KCN counts from those count generated from metabolically active cells. Specifically internalized iron counts were then normalized to that from FA6905 (WT TbpA). FA6905 serves as the positive control, and FA6905 L3HA (L3HA) and excess competitor hTf condition (Comp) serve as negative controls. Data shown represents the means \pm standard errors for at least three independent binding experiments. For all mutants when compared to the positive control, $P < .001$. Statistics were calculated using the Student's *t* test.

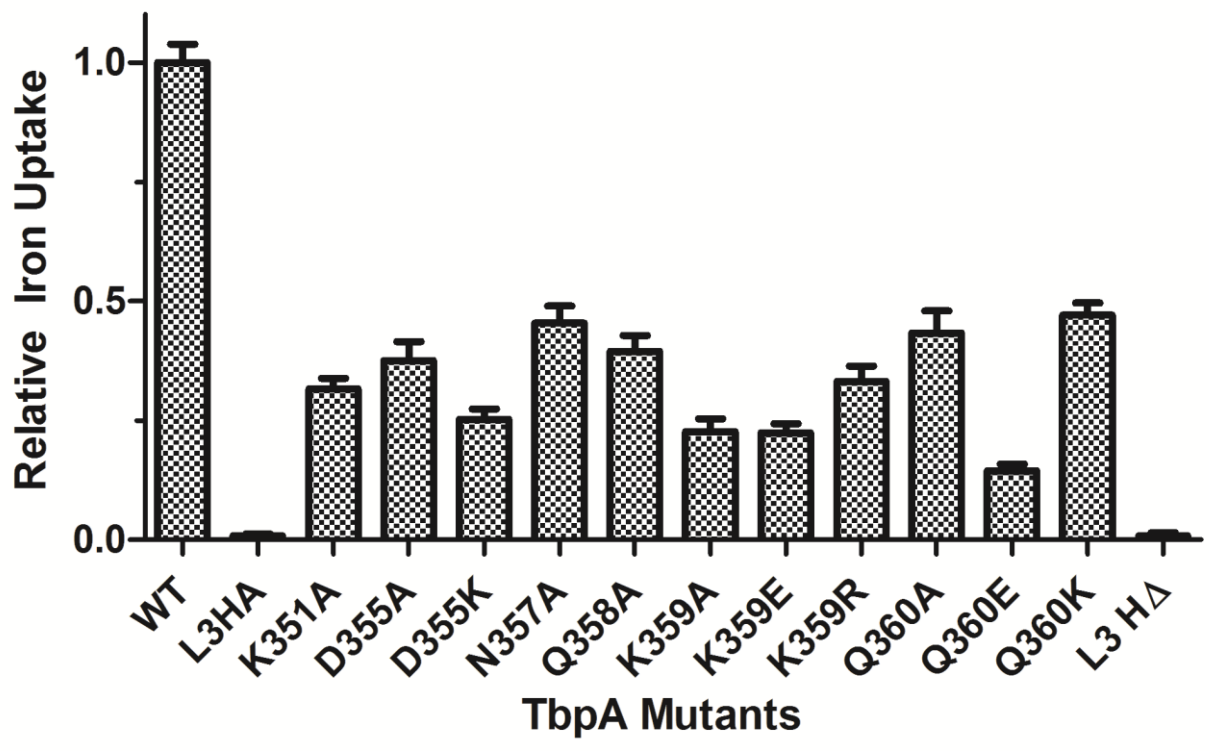


Figure 3.8. Iron internalization by TbpB deficient strains

E. TbpB expression enables iron internalization in the loop 3 helix mutant TbpA proteins

We next assessed whether TbpB was able to restore iron uptake function in strains with a defective- TbpA transport protein, as this phenomenon has been observed in our previous studies (250). This hypothesis was tested with a subset of the isogenic TbpA mutants, co-expressing a functional TbpB, in the radiolabeled iron uptake assay described above. In the presence of TbpB, all point mutants demonstrated greatly increased iron uptake, to levels 70-98% of WT (Fig. 3.9). The helix deletion strain, however, continued to demonstrate a severe defect, with iron uptake rates below 5% of wild-type. The range of defects seen in the Tf-binding ELISA and the iron uptake assays were quite varied for single residue substitutions, but no information about the ability to survive on Tf as a sole iron source can be derived from these experiments. We therefore set out to determine if any of the TbpA mutants, in the presence or absence of TbpB, were capable of growing on iron depleted media supplemented with partially ferrated Tf.

Figure 3.9. Iron internalization by TbpB expressing strains.

Whole, iron-stressed, gonococcal cells were applied to microtiter dishes for radiolabeled iron uptake assays. Iron uptake was calculated as counts/ μg protein. The amount of iron internalized was determined by subtracting +KCN counts from the counts generated with metabolically active cells. Internalized counts were then normalized to that from FA19 (WT TbpA). FA19 (WT) serves as the positive control, and FA6905 L3HA (L3HA, B-) and FA19 L3HA (L3HA, B+) serve as negative controls. Data shown represents the means \pm standard errors for at least three independent binding experiments. Significant differences are noted, where * represents $P < .001$, and # represents $P < .01$. Statistics were calculated using the Student's *t* test.

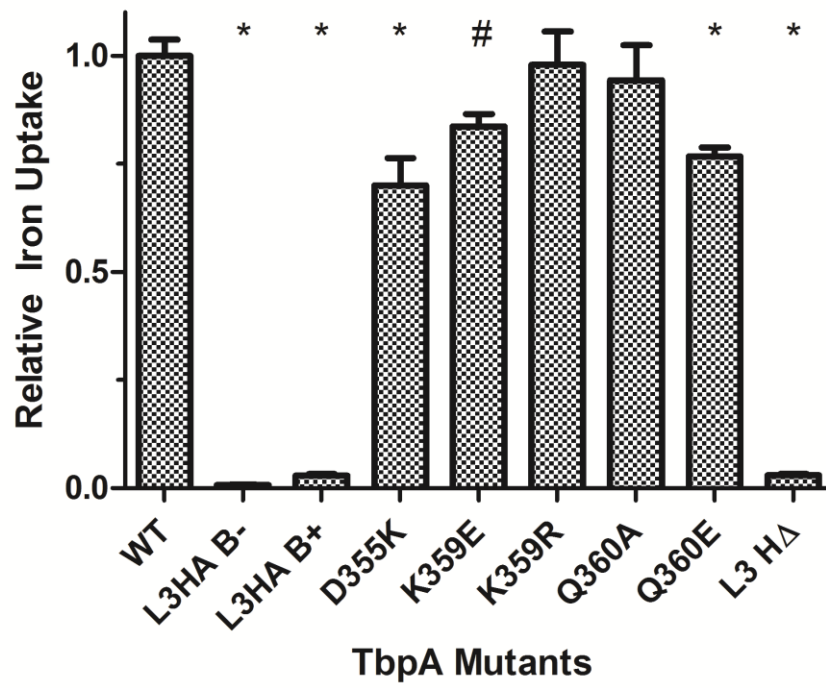


Figure 3.9. Iron internalization by TbpB expressing strains

F. Strains with TbpA loop 3 helix mutations are capable of growth on Tf as a sole iron source

In order to assess the abilities of the TbpA mutants to grow on Tf as a sole iron source, we grew all 12 *tbpA* mutants, with and without a functional TbpB, on GCB agar plates. The next day, single colonies were patched onto CDM agar plates containing 10% ferrated Tf, and grown for 48 hours at 37°C with 5% CO₂. Since Tf in circulation in the human body is 30% ferrated, this condition represents a saturation level below that which is physiologically achieved. At 48 hours, all strains except the negative control and the helix deletion strains were capable of growth on Tf as a sole iron source (Fig. 3.10).

Figure 3.10. Growth of *tbpA* mutants on hTf as a sole iron source.

A) CDM + 10% saturated hTf agar plates with 6 strains grown per plate. Each plate has FA6905 as a positive control and FA6747 as a negative control in addition to several mutants with point mutations. B) Plate layout diagram describing the phenotypes of the strains streaked on each sector of the above plates.

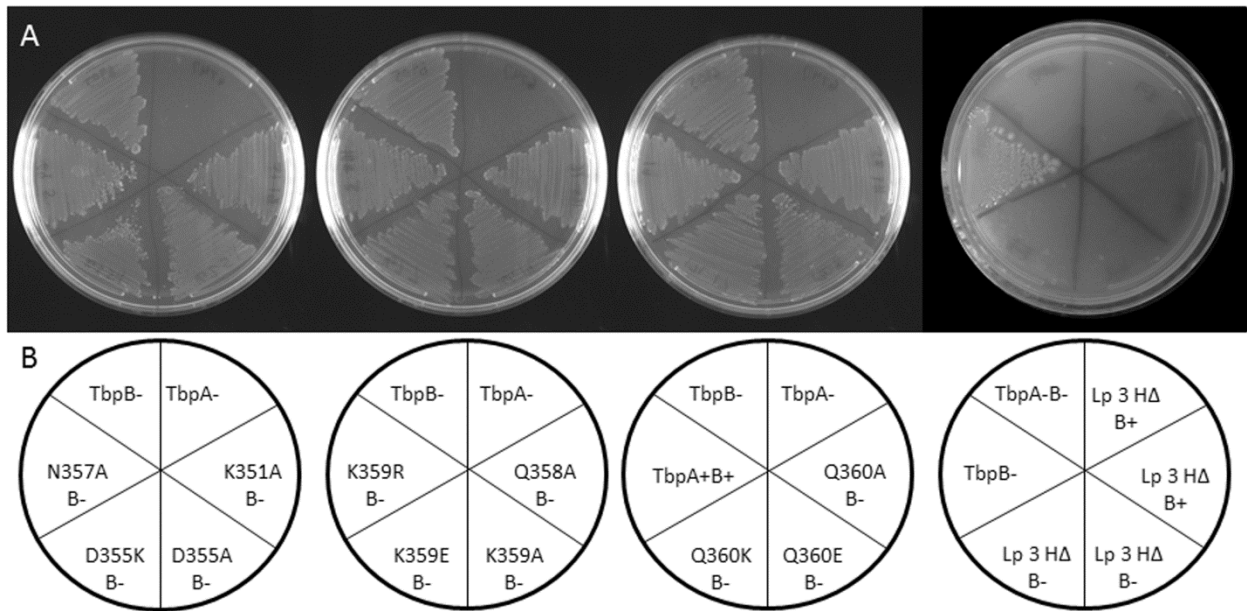


Figure 3.10. Growth of *tbpA* mutants on hTf as a sole iron source

G. TbpA in the loop 3 helix mutants is surface exposed

To confirm the surface exposure of the mutated TbpA proteins, we used a proteolytic cleavage assay on whole iron-stressed gonococci. Cells were subjected to a time course of trypsin digestion and then processed for preparation of whole cell lysates. Whole cell proteins were separated by SDS-PAGE and then transferred to nitrocellulose for Western blotting. The presence of TbpB did not affect the digest pattern (Fig. 3.11), and all single residue mutants had the same digest pattern as wild type (some mutants not shown). The loop 3 helix deletion strain had a similar digest pattern, but the relative intensities of the bands were slightly affected. The digest patterns of the single residue mutants, combined with their retention of iron uptake functions, confirm that the proteins are surface exposed and in the proper conformation. The digest pattern of the loop 3 deletion is similar to the pattern displayed by the loop 3 HA epitope insertion strain described previously (250). Although the digest pattern suggests that the TbpA conformation may be slightly altered in these latter two mutants, no new digestion products were visualized, and proteolysis demonstrates the TbpA proteins are indeed surface exposed.

Figure 3.11. TbpA mutations do not prevent surface exposure.

Whole, iron-stressed gonococcal cells were exposed to trypsin for 0, 10, 20, and 30 minutes after which the reaction was stopped by addition of aprotinin. Lanes labeled "X" were not treated with trypsin. Bacteria were pelleted, and subjected to SDS-PAGE followed by transfer to nitrocellulose. Western blots were probed with polyclonal TbpA antibody. Full length TbpA is 100kDa. Trypsin cleavage resulted in TbpA fragments of approximately 95 and 55 kDa.

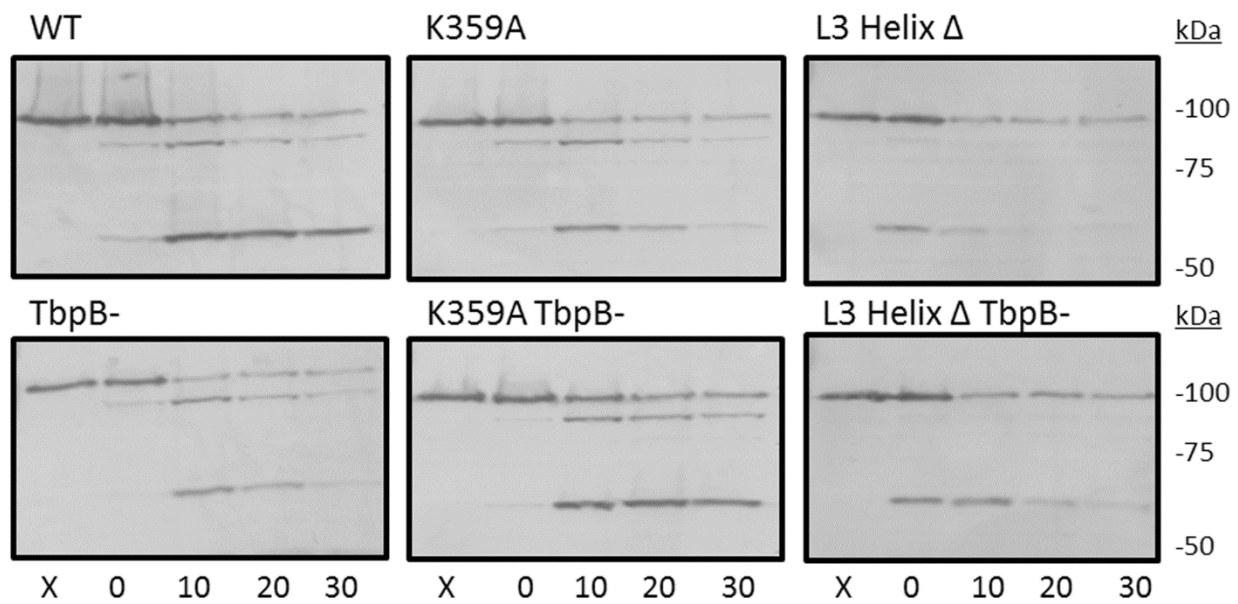


Figure 3.11. TbpA mutations do not prevent surface exposure

III. Discussion

TbpA is considered a viable vaccine candidate for prevention of gonococcal infection, due to its lack of antigenic and phase variation (261), but there have been complications in developing a successful vaccine. One issue is that, when used as an immunogen, TbpA alone results in a relatively weak immune response (232, 262). In addition, although TbpA is required for initiation of human infection (198), it is not required for survival in the estradiol-treated female mouse model used for vaccine development (73, 263), complicating efforts to target TbpA alone (for review see (236)). Some challenges to vaccine development were to be expected when the primary resource was a 2D topology map of TbpA that took over a decade of work to refine (264). However, there were hopes that once a 3D structure was developed, it would greatly accelerate the ability to target key regions of TbpA for vaccine development. With the resolution of the TbpA crystal structure from *N. meningitidis* in 2012, which largely confirmed the 2D topology model in the gonococcus, it was anticipated that this data would be invaluable for gonococcal studies due to the high sequence homology between the related pathogens. After solving the structure, Noinaj et al. developed antibodies against four small, predicted functional domains of the protein (190). In recombinant *E. coli*, these antibodies showed some promise, although the sera had to be used at high concentrations (1:20) to demonstrate any inhibitory effect. These crystal structure derived epitopes showed potential, but they were not tested in the native bacterium.

We set out to bridge the gap between characterizing antibody interactions with recombinant *E. coli* versus *N. gonorrhoeae*. Using the antibodies from the previously mentioned study, we determined that when applied to the gonococcus, these antibodies did not interact

with TbpA in the same manner that they did for recombinant *E. coli*. In fact, the anti-peptide antibodies were twice as effective against *E. coli* in some cases, and even then demonstrated only modest ability to block TbpA-ligand interactions. It is possible these antibodies did not block ligand well because the immunogens were too small to elicit robust titers. We have determined previously that antibodies raised against larger loop antigens for loops 2, 4, and 5 can bind to the gonococcal surface (265). However, even these longer loop-specific antibodies were unable to block Tf binding (265). Another possible explanation for why the original loop antibodies did not block TbpA-ligand interactions is that the linear presentation of the peptide immunogens may not have represented the structural complexity of the loops in their native context, resulting in decreased binding to folded TbpA. These unanticipated findings demonstrate the complexity of specifically developing immunogens from the crystal structure alone. However, with modification to length, composition, and conformation, we were able to make second generation TbpA loop specific antibodies that we predicted could overcome some of the limitations of the original loop antibodies. Although similar levels of ligand blocking were demonstrated with these new antibodies on the gonococcal surface, there was an observed decrease in efficacy against *E. coli*. This finding presents new questions about effective antibody development. Further work needs to be done to investigate the roles of various loops, explore how peptide presentation translates to antibody efficacy, and determine why some antibodies, like the holo-TbpA antibodies, are effective against *E. coli*, but not the gonococcus. Despite these lingering questions, this work's innovative approach to development of peptide-specific antibodies will provide helpful insights for future work targeting the Tbps for vaccine development.

After addressing antibody function on the gonococcal surface, attention was shifted to further exploring the structure-function relationships predicted for TbpA. Based upon our previous studies, we concluded that an HA tag insertion into loop 3 completely inactivated TbpA function (250). The TbpA crystal structure also shows that the loop 3 helix seems to fit inside the C-lobe cleft of Tf, in close proximity to the iron chelation center. Within this chelation center, there is a triad of pH sensing residues (K534, R632, and D634) that are predicted to control iron binding and release. TbpA residue K359 is adjacent to this triad, and was hypothesized to disrupt the charge balance enough to release the iron from the coordinating residues. To test this hypothesis, mutations were made to change each of the polar residues on the helix. A complete helix deletion was also generated. Gonococcal strains that had these TbpA mutations with and without the co-receptor, TbpB, were created so that effects on TbpA could specifically be evaluated. Finally, the phenotypes of the mutants were determined in the two steps of the TbpA-Tf interaction: ligand binding and iron extraction/internalization.

We assessed mutant TbpA-Tf binding using ELISAs, and determined that in the absence of the co-receptor there was a moderate reduction in ligand binding. The helix deletion strain, however, suffered a severe reduction in binding, suggesting the entire helix domain is important for ligand interaction. Although single residue mutations had only modest effects, there are 81 predicted TbpA residues that interact with Tf, so it was unlikely that any single residue mutation would completely abrogate TbpA-Tf binding. Although interference with ligand binding was a promising outcome, assessing iron uptake was the primary objective, and more likely to be affected by the selected mutations.

We proceeded to analyze the abilities of the mutant TbpAs to utilize Tf bound iron using a radiolabeled iron uptake assay. As predicted, the iron uptake function was impacted more than ligand binding, supporting our hypothesis that Loop 3 helix polar interactions are involved in iron release from its chelation center in Tf. Mutating the polar residues all along the helix had substantial negative effects, but certain residues stood out as more important than others. As mentioned previously, it was predicted that the lysine residue at TbpA position 359 was the key residue in contributing to iron release. Indeed, when this residue was mutated to residues of opposite or no charge, some of the most substantial decreases in iron uptake occurred. Surprisingly, the greatest decrease in protein function came from an additional negative charge at residue 360. Together, these data would suggest that a positive charge presence is required in the Tf C-lobe cleft to cause iron release and utilization. Additionally, changing the identity of all tested residues along the length of the helix caused greater than 50% reduction in protein function. This implies that all of these residues are required to position the helix properly in the cleft for optimal functioning. This concept is further supported by the loop 3 helix deletion strain, which was incapable of iron internalization.

Although it is easiest to see defects when evaluating TbpA alone, this is not a realistic view of how the Tf iron acquisition system functions in the gonococcus. Indeed, no TbpB deficient gonococcal strains have ever been identified. Therefore, we repeated the radiolabeled iron uptake assays in the presence of the co-receptor TbpB with some of the best and worst performers from the TbpA-only iron uptake study. In each case, a dramatic recovery of iron uptake function was observed, although uptake function in some mutants was still significantly lower than wild-type. This might have been anticipated for several reasons. First, previous

studies have demonstrated that the Tf receptor complex is relatively insensitive to point mutations (190, 258). Second, it has been hypothesized that TbpB causes a closure of the chamber formed between Tf and TbpA, so that free iron cannot diffuse away. And third, it is predicted that TbpB is largely responsible for the release of deferrated Tf. Together, this suggests that even if the Tf-iron transporter is greatly handicapped, TbpA can still accomplish its goal of sufficient nutrient acquisition if the co-receptor is present. The exception to this, again, is the helix deletion strain. This TbpA mutant has almost no capacity to internalize iron, even in the presence of TbpB. Because this mutant binds Tf at less than 10% of WT levels, it is difficult to assess whether TbpB truly cannot compensate for the defect, or if the 3-part complex never forms. In sum, these findings highlight the importance of the loop 3 helix motif in iron acquisition, and demonstrate the likely importance of simultaneously targeting both Tf receptors to achieve maximal inhibition.

We were able to partially inactivate the Tf receptor complex with mutations, therefore we next tested whether these mutations similarly inhibited gonococcal growth on hTf as a sole iron source. When the mutant strains were grown on CDM-Tf agar plates, we found that all single residue mutants with and without TbpB could grow. The only strains unable to grow were the helix deletion strains. These findings suggest that even at TbpA function as low as 15% of WT, the gonococcus is able to survive on poorly saturated Tf as a sole iron source. The helix deletion strains demonstrate that there is a level at which growth cannot be sustained, and the level exists somewhere between 3-15% TbpA function. These data are novel in that they are the first to report the degree to which TbpA needs to be impaired in order to prevent growth on hTf. These data also provide new insights into the function of the loop 3 helix and further indicate

that while residue K359 is important for TbpA function, other helix residues apparently play equally important roles.

We confirmed that the mutants constructed in this study were still surface exposed and folded properly. Using a proteolytic accessibility assay, it was demonstrated that all the single residue mutations, both in the presence or absence of TbpB, displayed the same cleavage patterns as the parental strain. The helix deletion strain was protease accessible, demonstrating that it was surface exposed, but the relative intensities of the cleavage products were altered. This suggests that the presentation might be slightly different compared to the wild-type protein. Indeed, previous insertions into loop 3 demonstrated the same protease-sensitivity pattern, showing that moderate perturbation to the loop potentially alters its conformation slightly.

This study demonstrates that, in the case of TbpA, structure-driven epitope selection for vaccine development does not necessarily translate into successful binding to the surface of the native bacterium. We found that loop 3 was poorly immunogenic, supporting the hypothesis that the gonococcus has evolved to keep functional domains from being targeted by the immune system (233). We also clearly demonstrated that the loop 3 helix plays a critical role in protein function, both in binding and in iron extraction/internalization. Of note, the function of the loop 3 helix was not solely dependent on K359 as predicted. Instead, all of the residues targeted for mutagenesis play important roles in TbpA function. We determined that the co-receptor TbpB can largely compensate for mutations in TbpA, and that provision of a functional TbpB is sufficient to allow growth even at greatly reduced TbpA functionality.

The studies described in the current report represent the first gonococcal structure-function characterization of the Tf receptor system since it was crystallized from *N. meningitidis* in 2012. The resolution of these crystal structures provides critical information to the field and serves as a foundation for studies, like those described herein, and represent the start of a new era in the pursuit of vaccine development and therapeutic intervention efforts targeting these proteins.

Chapter 4: Development of a Platform to Characterize Gonococcal HpuA Structure- Function Relationships

I. Introduction

Nearly two thirds of iron in the human body is sequestered in hemoglobin (Hb) inside of human erythrocytes (266). Upon hemolysis, tetrameric Hb dissociates into dimers that are quickly bound by haptoglobin (Hp), which serves to protect the body from Hb induced oxidative stress, and to facilitate Hb clearance by the reticuloendothelial system (178, 267, 268). In order to gain access to this vast iron reservoir, the *Neisseriaceae* family expresses two hemoglobin receptor systems, HpuAB, and HmbR. Interestingly, while both receptors can extract heme from Hb for use as an iron source, only HpuAB can extract heme from the Hb:Hp complex (202, 205). HpuAB is also the only functional Hb receptor in *N. gonorrhoeae*, as all published gonococcal chromosomes contain a premature stop codon in HmbR (204, 210, 269). This is in contrast to *N. meningitidis*, where 99% of disease associated meningococcal strains possess at least one of the Hb receptor systems, and strains responsible for the highest rates of disease encode both (270).

In gonococcal clinical isolates, HpuAB is most often phase off, but expression has been demonstrated to increase during early stages of menstruation in women, when Hb is in greater supply (203, 257). Meningococcal isolates are far more likely to express Hb receptors, with 91% of disease isolates and 71% of carriage isolates found to express at one or both receptors (270-272). These findings seem to suggest that there may be an association between Hb utilization and disease, but other studies have demonstrated that transferrin still remains the main source of iron during meningococcal growth in human blood (273). Regardless of which iron source is

most used, an HmbR knockout strain of *N. meningitidis* displayed attenuated infectivity in an infant rat model of infection, demonstrating the importance of Hb utilization (269).

Due to the importance of hemoglobin for meningococcal infection, and its selected use in gonococcal infection, the HpuAB receptor appeared to have potential to be included in a multivalent vaccine. Further investigation, however, demonstrated that the sequences of both proteins across *Neisseria* species are under significant immune selection due to their highly exposed localization (274), resulting in rapid evolution through genetic change to thwart host defenses (84). This finding was further supported by a study that demonstrated antibodies directed against HmbR and HpuA were reactive to the cell surface, but incapable of initiating a bactericidal response (273). Together, these findings would suggest that Hb receptors have low vaccine potential due to non-productive responses and concerns about cross protectiveness.

The hemoglobin receptors of *Neisseria gonorrhoeae* have been the subject of far less investigation than the other TonB dependent iron uptake systems. Despite the limited vaccine potential of HpuAB, there are still many questions about Hb receptor structure-function relationships and mechanisms of heme uptake. To help address these questions, the crystal structure for HpuA from *Kingella dentrificans* (*Kd*), a member of the *Neisseriaceae* family, was recently published (275). A model of HpuA from *N. gonorrhoeae* (*Ng*) based on the *Kd* crystal and a partial crystal of the C-terminus of *Ng* HpuA was also constructed, allowing for comparisons to be made across species. This study provided several new pieces of information essential to understanding this receptor system. For the first time it was demonstrated the HpuA alone can interact with human Hb, whereas it was already known the HpuB could interact with Hb and Hb:Hp (276). It has also been demonstrated that HpuA binds the Hb dimer in a

different region from the Hp binding site (277), suggesting that HpuA could also bind Hb:Hp, but would not directly bind apo-Hp(275). Most notably, mutations were constructed to probe deletions and point mutations in predicted regions of *Kd* HpuA-Hb interaction. Although the assays were qualitative, clear decreases in ligand binding were demonstrated in several deletions and mutations. These data set the foundation to specifically probe similar interactions between *Ng* HpuA and Hb in the native bacterium.

In collaboration with Dr. Stephen Hare, studies were initiated to test similar HpuA mutations in the gonococcus. Mutations were designed based on findings in Dr. Hare's previous work (275), and he shared plasmids with our lab that contained mutated copies of *hpuA* (pVCU173-189). In total, 13 point mutations and 3 deletions were constructed in homologous regions to mutations in *Kd* HpuA to further evaluate the *Ng* HpuA structure function relationships (Fig. 4.1). In order to initiate these studies, a platform needed to be developed to characterize these mutants, which require three preliminary steps. First, an *hpuA*⁻ strain needed to be created in order to test mutant copies of the gene. Second, a complementation vector needed to be generated that had all the necessary components for proper *hpuA* expression. And lastly, the mutant gene copies needed to be cloned into the complementation vector. These steps have been completed, as described below, and the platform is now available to phenotypically characterize the HpuA mutants.

Figure 4.1. Structural models of HpuA.

Crystal structure of HpuA in complex with hemoglobin (Hb) from closely related *Kingella dentrificans* (*Kd*) and superimposed model of HpuA-Hb from *Neisseria gonorrhoeae* (*Ng*). HpuA residues predicted to be sites of Hb interaction are highlighted in blue.

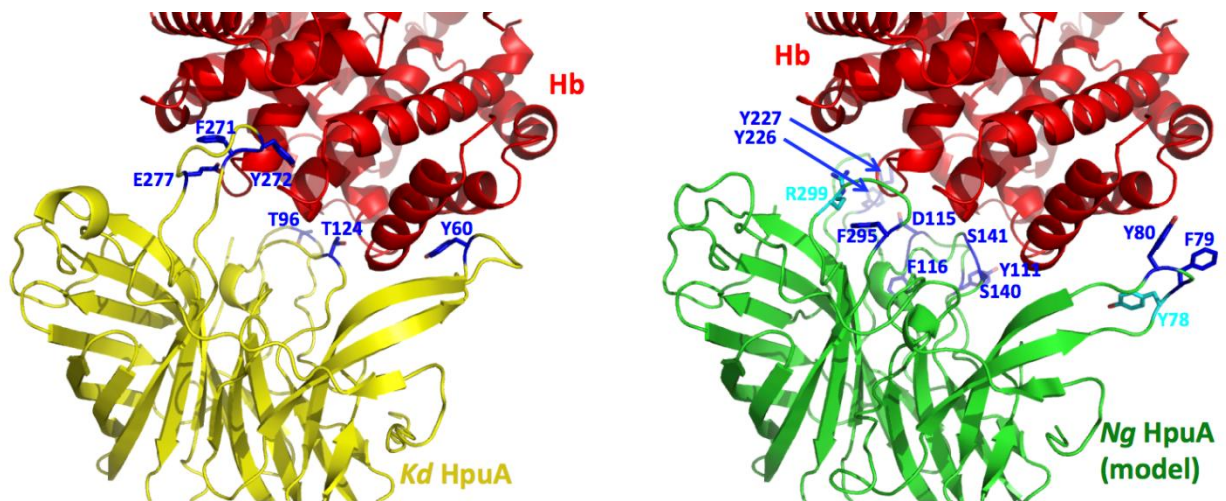


Figure 4.1. Structural models of HpuA

II. Results

A. Construction of HpuA⁻ strain

i. Insertional inactivation of *hpuA*

The inactivated *hpuA* plasmid (pVCU253) was constructed in two steps with the In-fusion HD cloning kit (Clontech), which uses proprietary enzymes to recombine PCR products with linearized plasmid DNA when they share 15 bp of homology at their ends. First, the phase-on locked, mutant *hpuA* R299A from pVCU185 was amplified with primers oVCU823 and oVCU824 (Fig. 4.2). Using the In-fusion kit, this gene was inserted into *Sma*I digested pVCU403, creating plasmid pVCU252. Then, the *aphA-3* kanamycin resistance cassette was amplified from pUC-18K using primers oVCU825 and oVCU826. The *aphA-3* PCR product was inserted into the PpuMI site on pVCU252, creating pVCU253.

ii. Gonococcal transformation

To create the *hpuA*⁻ strain (MCV140), pVCU253 was digested with *Sca*I-HF, and an FA19 Hb⁺ strain was transformed with the linearized DNA. Gonococcal transformants were screened by PCR amplification of *hpuA* to determine that only the *aphA-3* inactivated form of the gene was present. In addition, the *hpuA* PCR products were digested with *Bsa*II, which digests the wild-type but not the mutated, locked phase-on slip-strand region. This screening step was necessary to ensure the downstream gene, *hpuB*, would always be transcribed during phenotypic characterization.

Figure 4.2. Plasmid construction of inactivated *hpuA* gene.

The inactivated *hpuA* was constructed in two steps. First, HpuA R299A from pVCU185 was inserted into SmaI digested pVCU403, creating pVCU252. Subsequently, the *aphA3* cassette from pUC18K was inserted into PpuMI digested pVCU252, creating pVCU253.

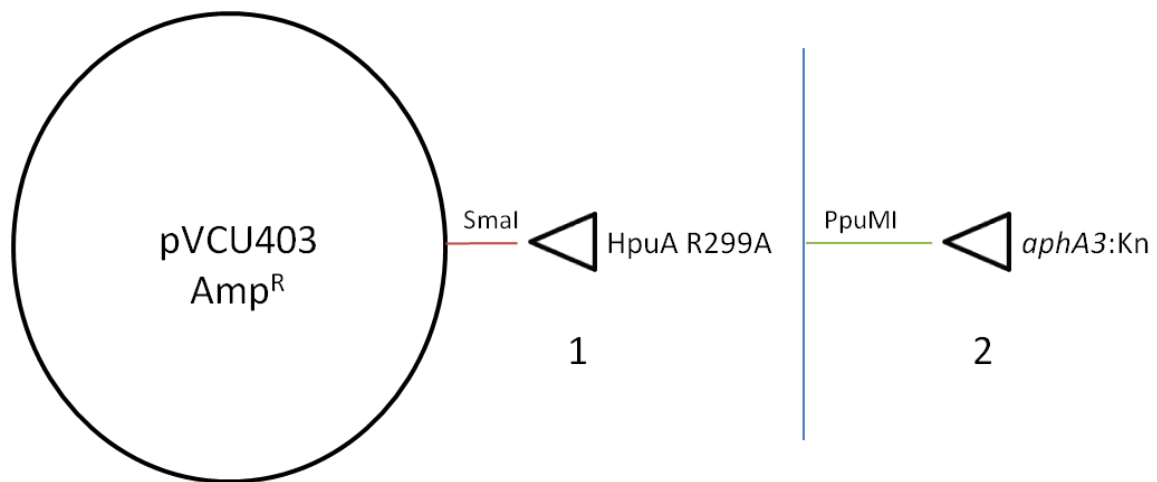


Figure 4.2. Plasmid construction of inactivated *hpuA* gene

B. Complementation vector construction

To construct a complementation vector to ectopically express mutant copies of *hpuA*, a ribosomal binding site (RBS) was added to plasmid pKH37 (252). To do this, annealed DNA oligomers (oVCU814 and 815) coding for an 8 base pair (bp) RBS were inserted into *PacI* and *SmaI* digested pKH37, creating pVCU234 (Fig. 4.3).

Figure 4.3. Construction of complementation vector.

A ribosomal binding site was added to pKH37 by digesting the plasmid with *PacI* and *SmaI*, and inserting annealed oligomers oVCU814 and oVCU815 between the restriction sites. The RBS region of the oligomers is underlined.

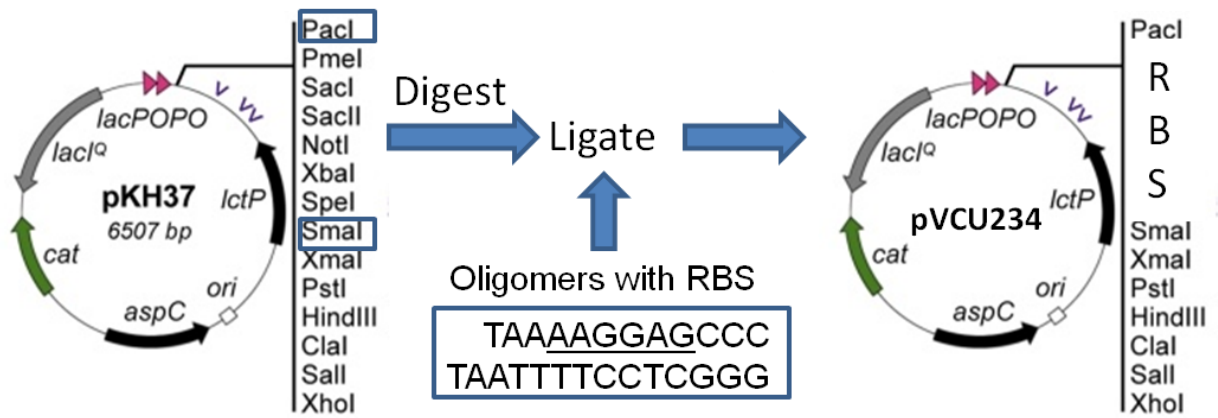


Figure 4.3. Construction of complementation vector

Image adapted from (278)

C. Mutant *hpuA* insertion into complementation vector

Initially, *hpuA* was cloned into pVCU234 using traditional digestion and ligation methods. *hpuA* containing plasmids pVCU174-6 and pVCU187-9 were digested with EcoRI, blunt-ended with DNA Polymerase I Large (Klenow) Fragment, and subsequently digested with XhoI. The complementation vector pVCU234 was digested with SmaI and XhoI, and the *hpuA* PCR fragment was ligated in between those restriction sites. Blunt-end ligation resulted in low cloning efficiency, so In-fusion HD cloning was adopted to optimize the cloning process. In this approach, pVCU234 was digested with SmaI only. Mutant copies of *hpuA* were amplified from plasmids pVCU173 and pVCU185 with primers oVCU821 and oVCU822. The digested vector and PCR products were mixed together with the proprietary In-fusion enzymes per kit instructions, and the DNA overlap regions were recombined to create a complete plasmid with one directional PCR product insertion (Fig. 4.4).

Figure 4.4. Cloning mutant *hpuA* into pVCU234.

In order to clone mutant copies of *hpuA* into the complementation vector (pVCU234), In-fusion primers oVCU821 and oVCU822 were used to PCR amplify the mutant *hpuA* genes, and the genes were inserted into SmaI digested pVCU234.

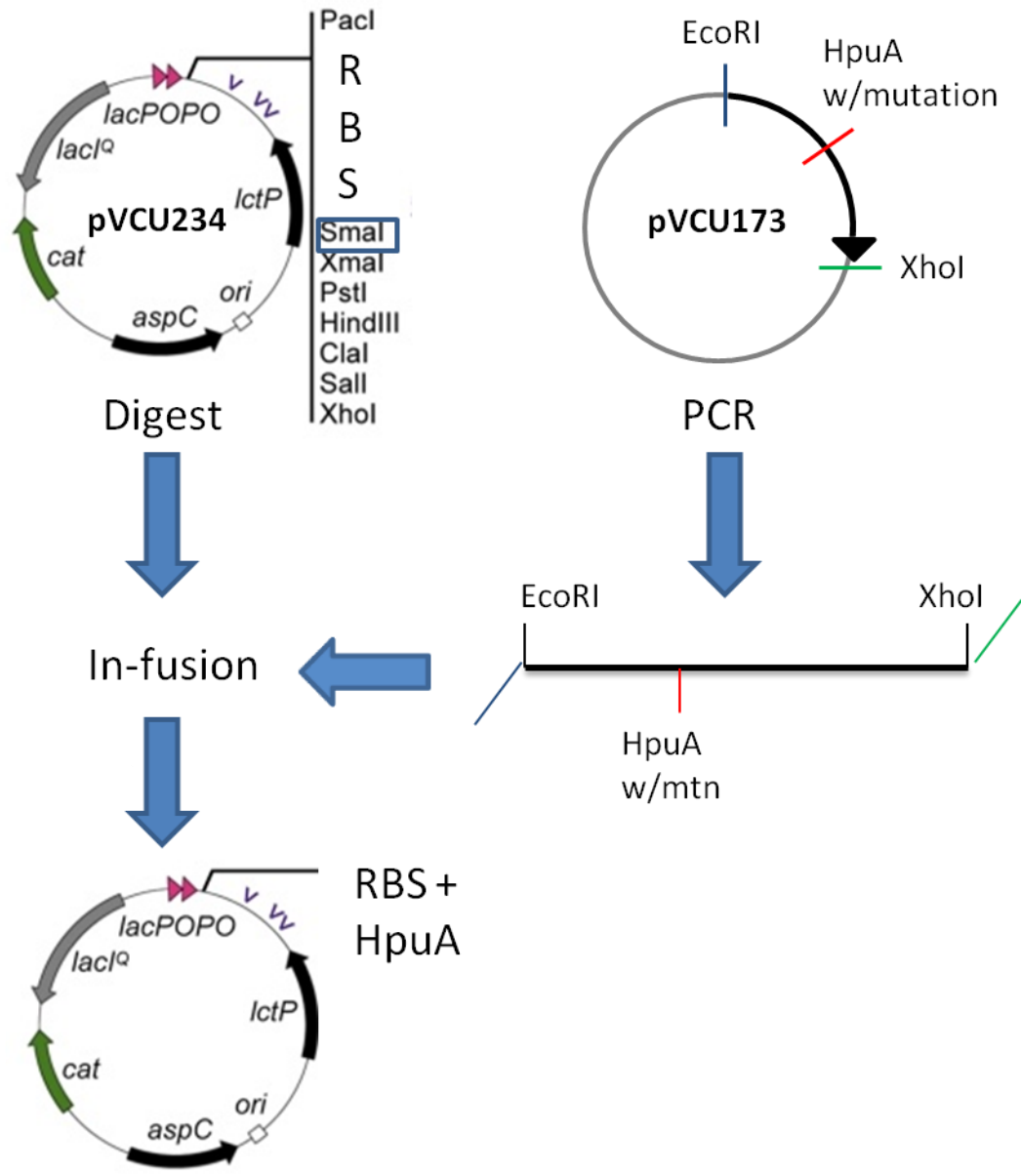


Figure 4.4. Cloning mutant *hpuA* into pVCU234

Image adapted from (278)

III. Discussion

With the creation of MCV140 and the complementation plasmids made from pVCU234, the platform to test mutations in *hpuA* is fully developed. After MCV140 is transformed with the complementation plasmids, mutant copies of *hpuA* will insert ectopically between the *lctP* and *aspC* genes, and will be under control of the *lac* promoter. Since the *aphA3* insertion in wild-type *hpuA* locus is non-polar and the native slipstrand region of *hpuA* is locked in the on position, native levels of HpuB will be produced. Because the ectopic locus of *hpuA* will be overexpressed, a wild-type copy of *hpuA* will also be ectopically inserted to serve as the positive control during experimentation. Conditions that are un-induced should serve as the negative controls. If there is basal expression from the ectopic site without the addition of IPTG, MCV140 could serve as an additional negative control.

With regards to characterizing the mutant HpuA proteins, ligand binding and growth on hemoglobin should be assessed. Hemoglobin binding assays could be performed similar to the Tf binding ELISAs described previously, with the exception that bound hemoglobin will need to be detected by antibodies, which could then be detected colorimetrically. Hemoglobin dependent growth should be characterized with hemoglobin as a sole iron source both in solid media and in liquid culture. Solid phase growth could be assessed on varying concentrations of hemoglobin as described for the solid phase transferrin dependent growth. Liquid culture analysis should evaluate OD₆₀₀ readings of gonococcal cultures in microtiter dishes over several hours to determine if *hpuA* mutants have growth handicaps relative to the positive control.

Chapter 5: Small Molecule Inhibition of TbpA

I. Introduction

In addition to pursuing the transferrin receptors as vaccine targets, they are also considered potential drug targets. Given that iron is an essential micronutrient, depriving the gonococcus access to host derived iron sources could serve as either a standalone antimicrobial modality or an adjunctive mechanism to enhance the antimicrobial effects of other drugs. Since transferrin is considered the primary iron source for the gonococcus *in vivo*, and the Tbps are the best studied of the gonococcal iron acquisition systems, it was hypothesized that novel drugs could be developed to inhibit the use of transferrin as an iron source.

This project required complex *in silico* analysis of the TbpA crystal structure to select possible regions for drug targeting, as well as selection of small molecules to use as the inhibitors. Once compounds were selected, preliminary analysis of TbpA-Tf inhibition would be carried out through whole cell transferrin binding ELISAs.

II. Results

In order to identify potential binding sites on TbpA, and to screen molecules that could bind those sites, collaboration was established with Dr. Glen Kellogg in VCU's department of Medicinal Chemistry. Using the co-crystal structure of TbpA in complex with human Tf, at least two binding sites were identified for small molecule inhibitor studies (Fig. 5.1-5.4). In order to select molecules that would bind those pockets, small molecule databases were screened for compounds that share structural similarity with the Tf amino acids that bind those locations

(Fig. 5.2). Compounds were selected based on their hydrophobic interactions (HINT) scores, which describe and quantify all interactions in the biological environment. The HINT score factors in all types of interactions, including the coulombic, hydrogen bond, and hydrophobic interactions which are expected to be found between molecules. The HINT score also encodes a free energy force field, and thus includes entropy and solvation/desolvation, besides the other enthalpic terms. Higher HINT scores indicate more favorable interactions between the molecule and TbpA. An example of a small molecule with high HINT score docked to TbpA is shown in Fig. 5.4.

After screening small molecule databases, 15 compounds were selected for preliminary evaluation. Due to lack of solubility information, the compounds were dissolved in DMSO at a concentration of 10 mM. Compounds were applied to whole cell ELISAs as described above at 50 μ M concentration prior to application of HRP-labeled transferrin. PBS and DMSO conditions served as the negative controls, and excess unlabeled transferrin (Comp), a competitive inhibitor of HRP-Tf, served as the positive control. Preliminary results are represented in Fig. 5.5, where compound 7 demonstrated approximately 20% inhibition of Tf binding. Compound 7 had reproducible, modest inhibition of the TbpA-Tf interaction; however, the other 12 tested compounds failed to demonstrate substantial, reproducible inhibition. Indeed, several compounds seemingly increased the binding of HRP-Tf. Optimization of this project is ongoing.

Figure 5.1. Location of binding site I.

TbpA is shown in cartoon representation colored by secondary structure (α -helices in red, β -sheets in yellow and loops in green), Tf is shown in stick representation in grey and binding site I location is shown in pink. A) side view and B) top view (PDB ID 3V8X).

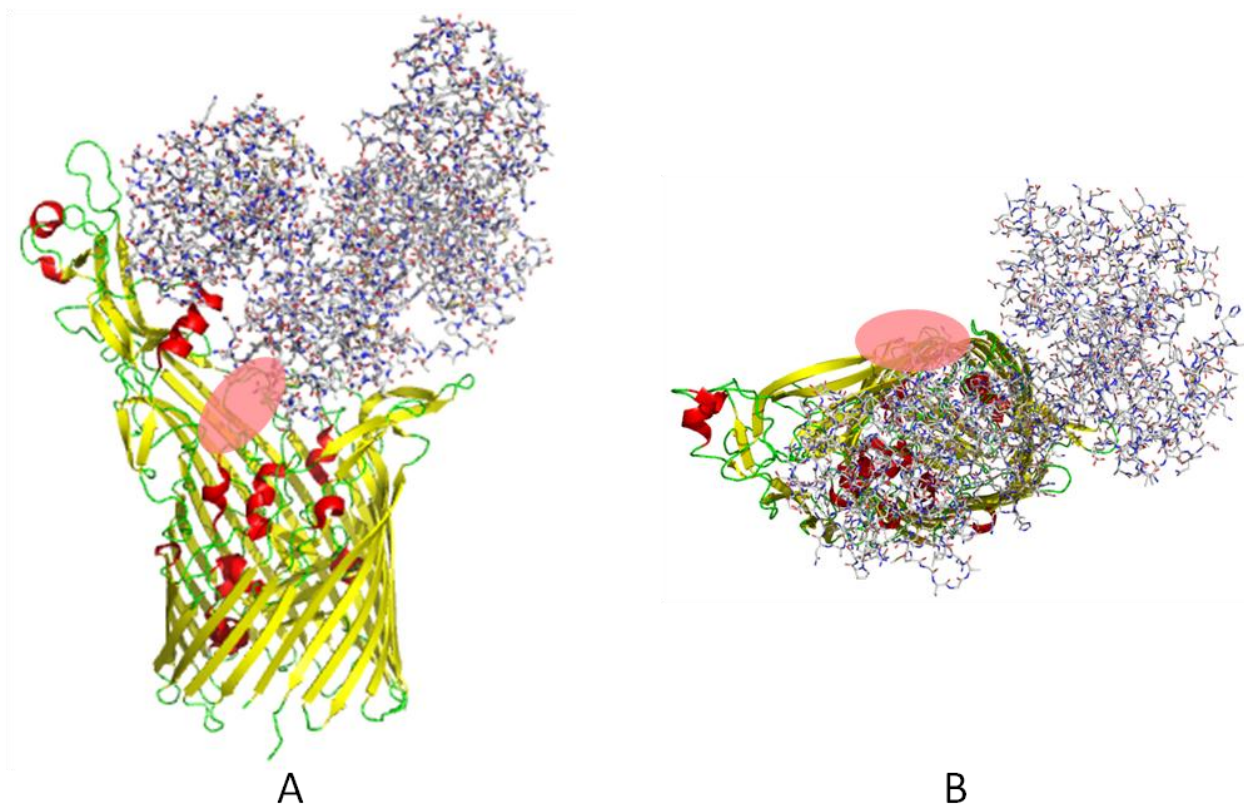


Figure 5.1. Location of binding site I

Figure 5.2. Binding site I residues.

TbpA residues forming binding site I in grey, with colored secondary structure (α -helices in red, β -sheets in yellow, and loops in green). ASP416 and ASN417 from Tf are shown in magenta (PDB ID 3V8X).

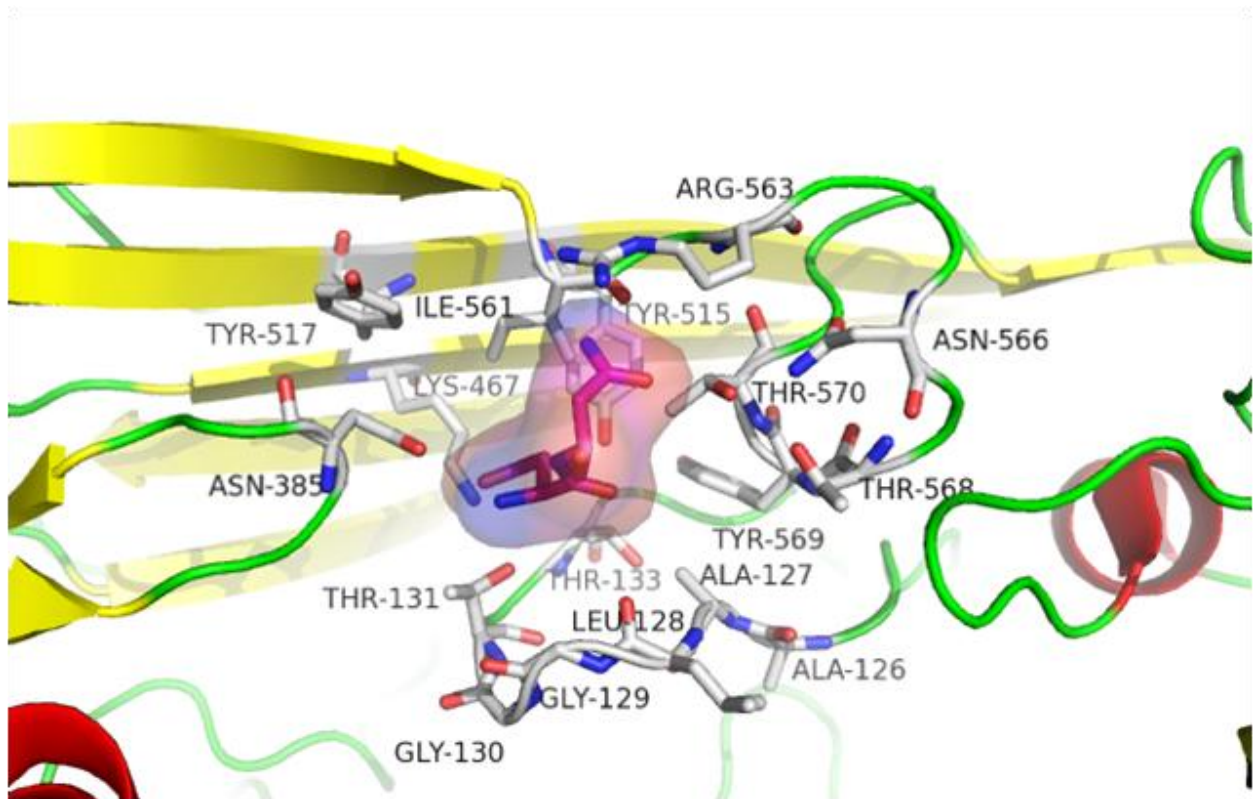


Figure 5.2. Binding site I residues

Figure 5.3. Location of binding site II.

TbpA is shown in cartoon representation colored by secondary structure (α -helices in red, β -sheets in yellow and loops in green), Tf is shown in stick representation in grey and binding site II location is shown in pink (PDB ID 3V8X).

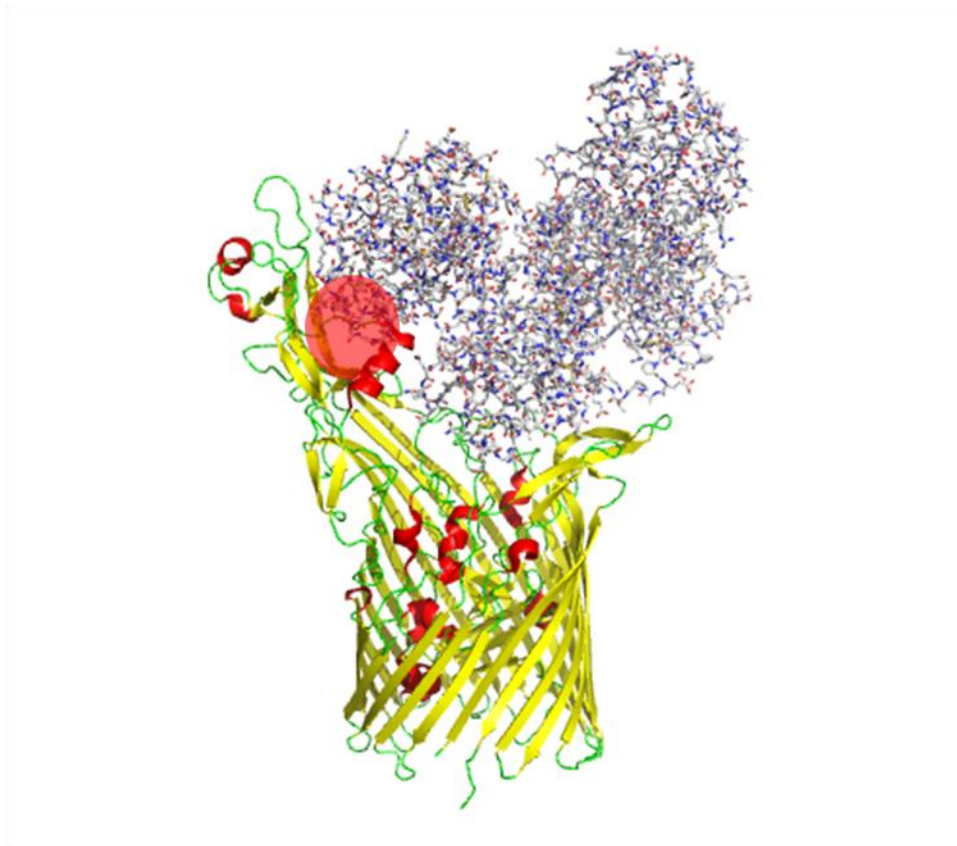


Figure 5.3. Location of binding site II

Figure 5.4. Binding site II residues.

TbpA Residues forming binding site II colored by secondary structure (α -helices in red, β -sheets in yellow and loops in green). Candidate compound ZINC04896026 shown in grey (PDB ID 3V8X).

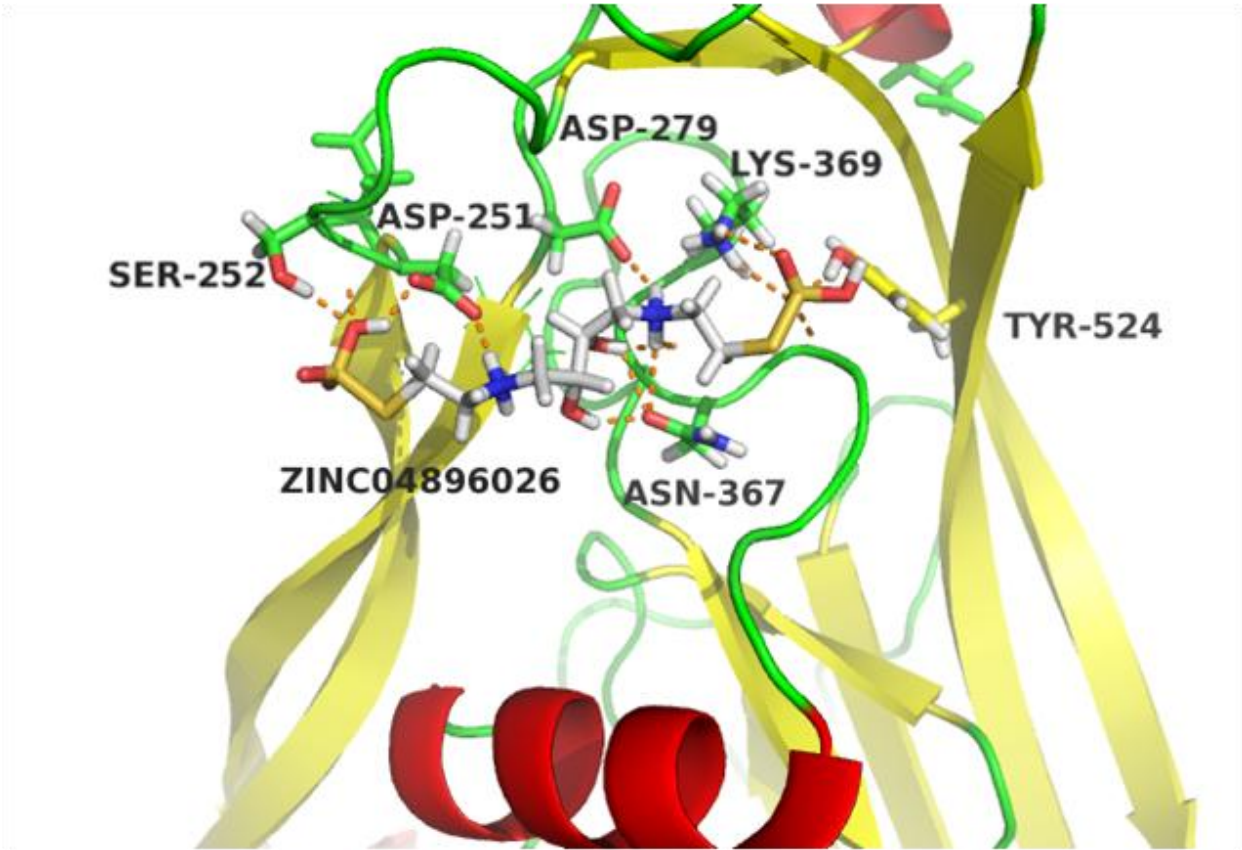


Figure 5.4. Binding site II residues

Figure 5.5. Preliminary inhibitory data.

Small molecules were applied to whole cell ELISAs prior to the application of HRP-Tf. Tf bound to the gonococcal surface was detected colorimetrically, and data was standardized to the DMSO positive control. All conditions were performed in triplicate. This is a representative image of preliminary inhibitory data.

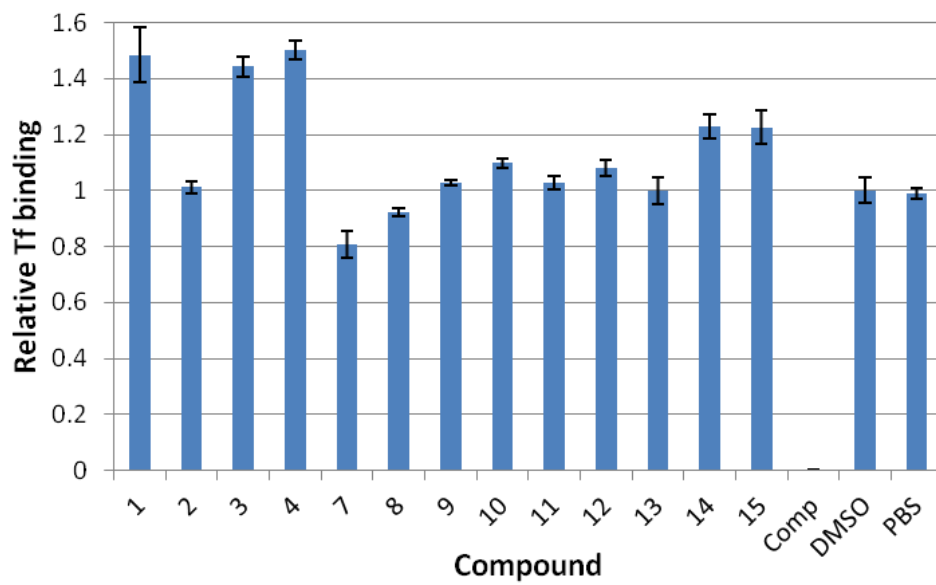


Figure 5.5. Preliminary inhibitory data

III. Discussion

Although these results are preliminary, there is evidence that some of the drugs in the first set have the capacity to limit TbpA-Tf interactions, even if the effect is modest. Once initial compounds that have inhibitory effects are identified, the molecular structures can be subtly changed and retested for improved or altered activity. It should also be considered, however, that transferrin is not the only usable iron source available to the gonococcus during the course of human infection. Accordingly, similar efforts should be directed at other acquisition systems, namely the lactoferrin receptors, given lactoferrin is released by neutrophils as part of the innate immune response (155, 279). Since LbpA and TbpA have approximately 41% sequence identity and 67% sequence similarity, it is predicted that these proteins adopt similar structure and interact with their ligands in similar ways (280). This suggests there is a possibility that one drug may be able to inhibit both receptor systems, which warrants further investigation. If one drug is not found that inhibits both receptors, combinations of drugs should be evaluated against gonococcal growth to determine whether inhibiting multiple iron acquisition pathways is advantageous for attenuating gonococcal growth.

Compounds designed to inhibit iron acquisition represent a novel mechanism for antimicrobial agents, and they could also be species specific. These drugs are designed against specific proteins, gonococcal TbpA in this case, so it is predicted that there should be little effect on other species. Even if these drugs are cross reactive, only members of the families Neisseriaceae, Pasteurellaceae, and Moraxellaceae are capable of acquiring iron from host iron-binding proteins (281). Tf binding inhibitors, therefore, could have activity against a range of

pathogens, without impacting the native gut flora, which predominately use siderophores for iron acquisition.

Antimicrobial resistance is often accelerated through misuse and overuse of current antibiotics (282, 283). The mechanism of action and protein specific nature of iron uptake inhibition drugs would, by default, reduce the spectrum of their activity. Although this presents some limitation in their clinical use, it could also contribute to slower resistance development. This is especially important given that commensal species often develop resistance genes, and through the natural competence of gonococci, rapidly transfer these genes to pathogenic strains (284-286). With no single agent therapy remaining to tackle gonococcal infection, and growing resistance to many classes of current antimicrobials, iron acquisition inhibitors represents a new mechanism for the treatment of gonococcal infections that warrants further investigation.

Chapter 6: OSU-03012 and PDE-5 Inhibitor Antimicrobial Activity

I. Introduction

OSU-03012 (OSU), a derivative of the COX2 inhibitor celecoxib, was initially designed as a cancer therapy, since COX2 inhibition had been shown to decrease growth and increase tumor specific cell death (287-290). After its development, it was determined that OSU lacks COX2 inhibitory activity (291, 292), but was initially thought to inhibit PDK-1 in the PI3K pathway (291). However, later studies demonstrated that OSU toxicity was due to enhanced endoplasmic reticulum (ER) stress signaling and down regulation/reduced half life of ER and plasma membrane localized HSP70 family chaperone GRP78/BiP/HSPA5 (293, 294). It was already known that prokaryotic cells have a GRP78 homologue, DNA K, and that this protein plays an essential role as a chaperone for proteins like RecA, which is essential for DNA maintenance and repair (295). Therefore, it was hypothesized that OSU may have antibacterial activity, especially during times of rapid cellular division when protein production is high. This hypothesis was supported by the discoveries that GRP78 is essential for viral replication during infection with human immunodeficiency virus (296), respiratory syncytial virus (297), measles virus (298), rotavirus (299), influenza virus (300), and Ebola virus (301, 302), and that OSU treatment can be down-regulate receptors for these viruses and also inhibit viral reproduction (303).

It was hypothesized that OSU has antimicrobial properties, and that combining OSU with other drugs might amplify its effects. Since bacterial cells have phosphodiesterases (304), and PDE inhibitors have been shown to increase chemotherapy effects in cancer (305, 306), PDE-5

inhibitors sildenafil and tadalafil were applied to gonococcal cultures with OSU. In order to test synergy with other known antibiotics, OSU was also applied to gonococcal cultures in combination with ceftriaxone, azithromycin, or ciprofloxacin.

II. Results

A. OSU and PDE-5 inhibitors' anti-microbial properties for lab strains

Lab strains FA19 (Fig. 6.1) and FA1090 (data not shown) were grown in the presence of OSU and PDE-5 inhibitors alone and in combination. OSU was applied at concentrations ranging from 2-8 μM when applied alone, while sildenafil (SIL) and tadalafil (Tad) were used at 4 μM . When used in combination, OSU was applied at 2 μM with either 4 μM SIL or TAD. Chloramphenicol (Cm), a conventional bacteriostatic antibiotic, was also applied at 34 μM in order to provide a reference for the effects of the investigational drugs. The investigational drugs were solubilized with DMSO, so vehicle (V) controls were added to determine the effects of solvent in drug conditions. CDM only growth was used as the positive control.

OSU at 2 μM had no effect compared to vehicle controls, however, 4 and 8 μM OSU caused rapid gonococcal cell death within the first hour. Compared to the Cm control, these results suggest that OSU alone has bactericidal properties. Sildenafil and Tadalafil had no effect compared to the vehicle control when used alone. However, when non lethal doses of OSU (2 μM) and SIL/TAD (4 μM) were combined, gonococcal growth was attenuated. These results suggest that these drugs have a synergistic effect, and that the addition of a PDE-5 inhibitor can effectively lower the minimum inhibitory concentration (MIC) of OSU. Of note, when 2 μM OSU

was added to the Cm control, there was no change compared to the Cm alone condition. This suggests that these two drugs did not have synergistic effects.

Figure 6.1 FA19 growth in the presence of OSU and PDE-5 inhibitors.

These data are from a single assay performed in triplicate, which is a representative image of multiple assays in which gonococcal lab strain FA19 was grown in CDM in a microtiter dish in the presence of OSU (O), Sildenafil (SIL, S), Tadalafil (TAD, T), Chloramphenicol (Cm), DMSO (V), or combinations thereof. OD₆₀₀ measurements were taken at the time of drug application and then hourly for four hours. Concentrations in μM units are represented by the numbers in the figure legend adjacent to the drug abbreviation. DMSO percentage represents the volume/volume ratio with CDM. Each condition was performed in triplicate, and data points represent the mean \pm the SEM.

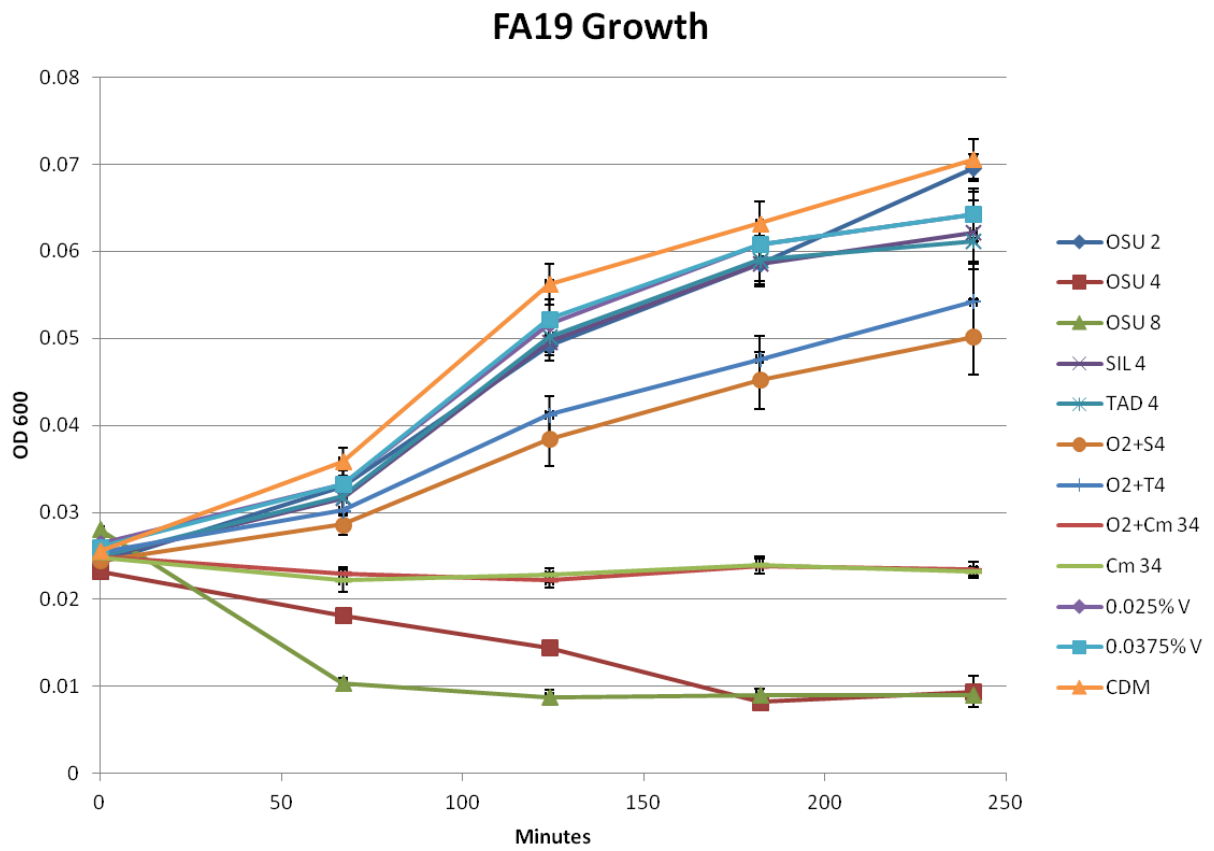


Figure 6.1. FA19 growth in the presence of OSU and PDE-5 inhibitors

B. OSU effects on drug resistant gonococcal strains H041 and F89

After demonstrating that OSU alone is able to attenuate growth in lab strains, and that this effects is enhanced by PDE-5 inhibitors, the same drug assay was repeated to determine if recent, multi-drug resistant “super bug” strains H041 and F89 (62, 63) are also susceptible to the investigational drugs. It was quickly determined that PDE-5 inhibitors, as with the lab strains, had no effect on superbug growth. In addition, the PDE-5 inhibitors were no longer synergistic with OSU when applied to the superbug strains (data not shown), leading to the decision to discontinue there use in future assays. Despite the observation that Sildenafil and Tadalafil could not impact growth of drug resistant strains, OSU continued to demonstrate its bactericidal effects in these strains (Fig. 6.2). As observed with the lab strains, 2 μM OSU had no effect on gonococcal growth, but at doses of 3 μM and above, the drug was rapidly lethal. This observation was important, because it demonstrated the approximate MIC for OSU. These assays were also carried out with higher concentration stocks of OSU, limiting the amount of DMSO in the assay. Vehicle controls (not shown), were identical to the CDM growth condition.

Figure 6.2. Superbug growth in the presence of OSU.

These are representative images of multiple assays in which gonococcal strains H041 and F89 were grown in the presence of 2 and 3 μM OSU in microtiter dishes. OD_{600} measurements were taken at the time of drug application and then hourly for four hours. Each condition was performed in triplicate, and data points represent the mean \pm the SEM.

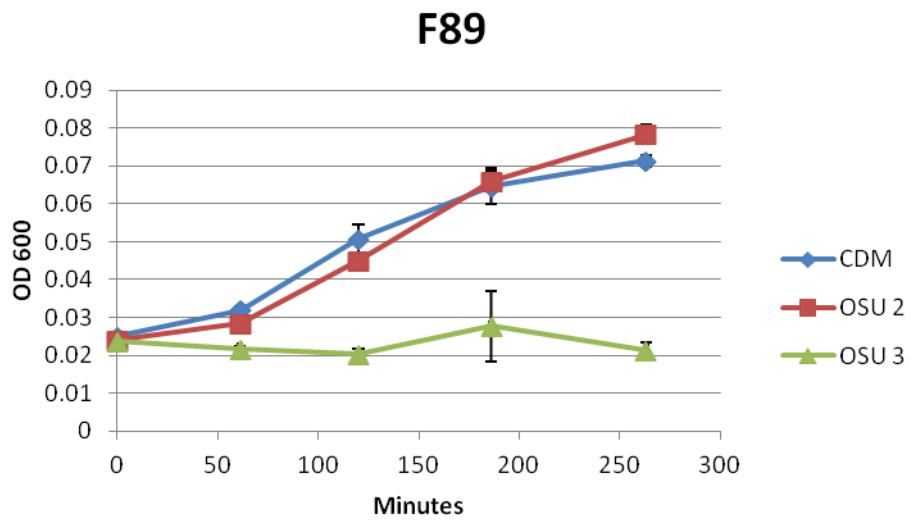
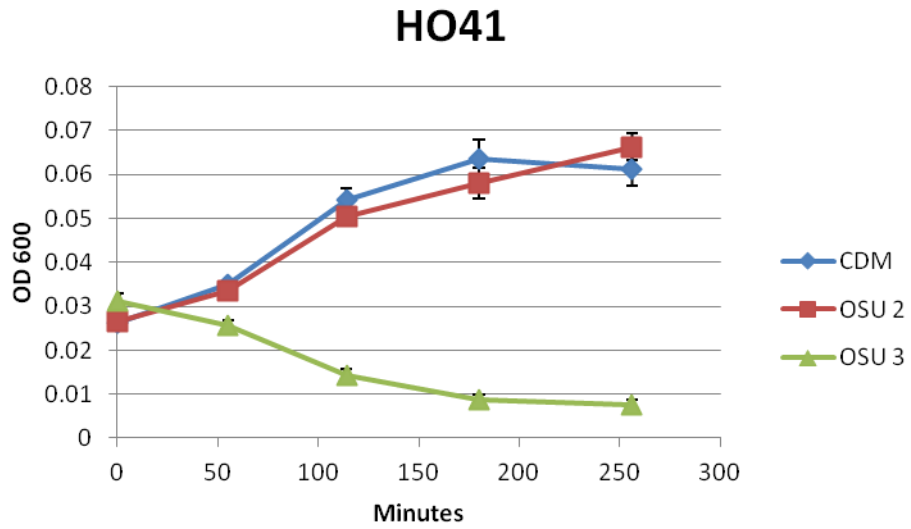


Figure 6.2. Superbug growth in the presence of OSU

C. OSU has synergy with conventional antibiotics

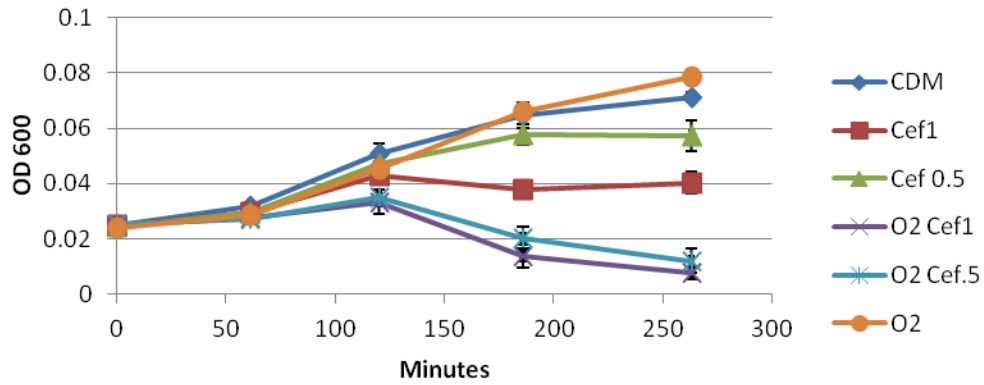
Since synergy was observed between OSU and the PDE-5 inhibitors in lab strains, but not in superbug strains, it was hypothesized that OSU might enhance the effects of conventional antibiotics, even those whose clinical use has been discontinued due to gonococcal resistance. To test this hypothesis, drug resistant gonococcal strains were grown in the presence of MIC and sub-MIC levels of antibiotics alone and in combination with sub-MIC levels of OSU. F89 and H041 have approximately equivalent MICs for ciprofloxacin (Cip) and azithromycin (Az), however the ceftriaxone (Cef) MIC for H041 is double that of F89 (61, 63). Interestingly, the reported MICs for Cef and Cip were valid in our assay, but the strains seemed more sensitive to Az than expected. Accordingly, the Az dose was titrated down until a level that was not inhibitory on its own was reached, approximately 10 fold below the reported MIC.

These assays, carried out similarly to those above, demonstrated that the combination of Cef and OSU has a robust synergistic effect in both superbug strains, and resulted in rapid cell death starting at approximately two hours (Figs. 6.3, 6.4). A similar synergistic effect starting at two hours was observed for OSU and Az, but in contrast to Cef, the effect was not bactericidal. Lastly, the combination of OSU and Cip demonstrated modest synergy, which was more pronounced with H041 than with F89. This phenomenon may be the result of higher Az sensitivity in F89, as the Cipro alone condition were highly effective at attenuating growth. In sum, these assays demonstrate that OSU has synergy with three independent classes of antibiotics within two of the most drug resistant isolates of *N. gonorrhoeae* known to date.

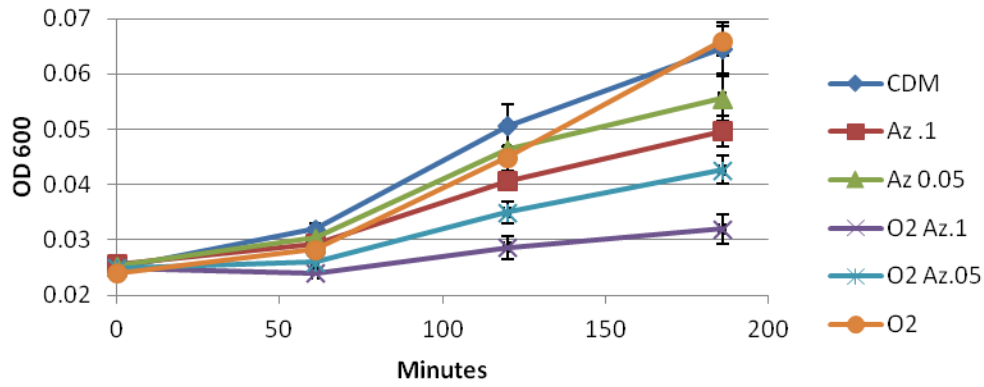
Figure 6.3. F89 Growth in the presence of OSU and conventional antibiotics.

These are representative images of multiple assays performed in triplicate in which gonococcal strain F89 was grown in the presence of MIC and sub-MIC levels of Cef, Az, and Cip, with or without 2 μ M OSU in microtiter dishes. OD₆₀₀ measurements were taken at the time of drug application and then hourly for four hours. Each condition was performed in triplicate, and data points represent the mean \pm the SEM.

F89 Ceftriaxone Dose Curve



F89 Azithromycin Dose Curve



F89 Ciprofloxacin Dose Curve

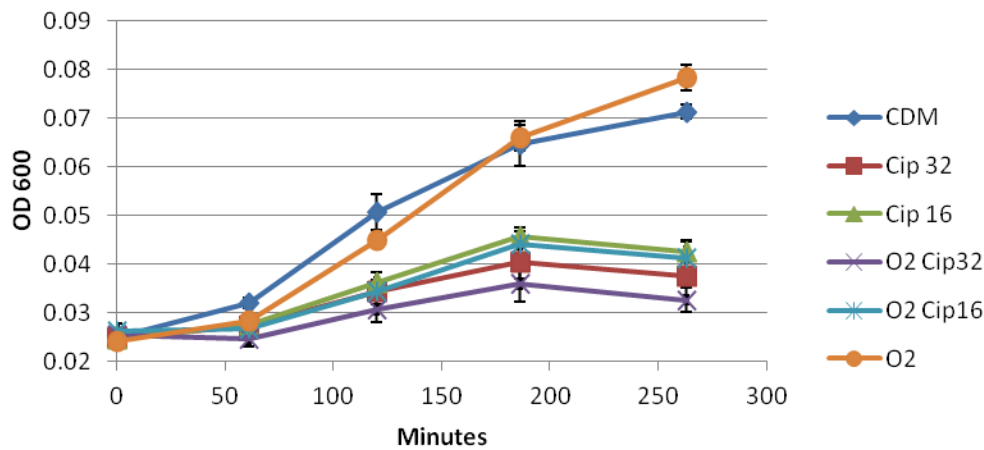


Figure 6.3. F89 Growth in the presence of OSU and conventional antibiotics

Figure 6.4. H041 Growth in the presence of OSU and conventional antibiotics.

These are representative images of multiple assays in which gonococcal strain H041 was grown in the presence of MIC and sub-MIC levels of Cef, Az, and Cip, with or without 2 μ M OSU in microtiter dishes. OD₆₀₀ measurements were taken at the time of drug application and then hourly for four hours. Each condition was performed in triplicate, and data points represent the mean \pm the SEM.

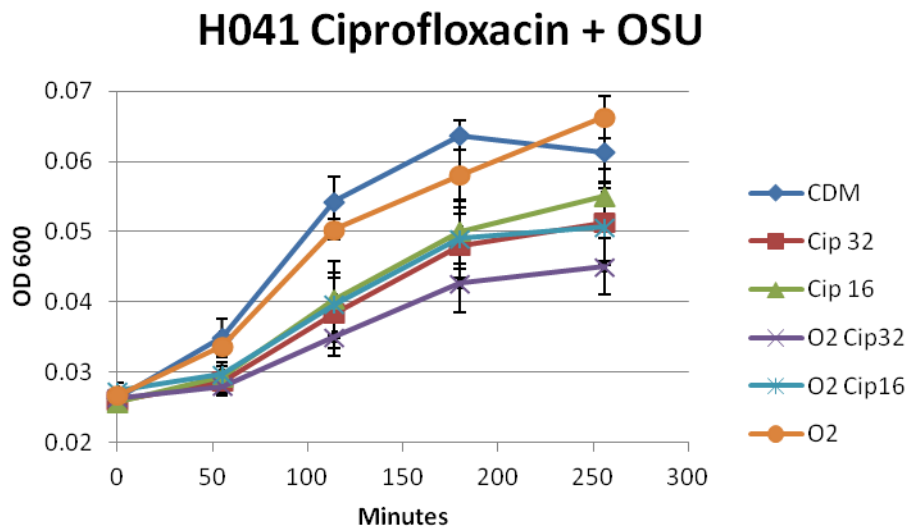
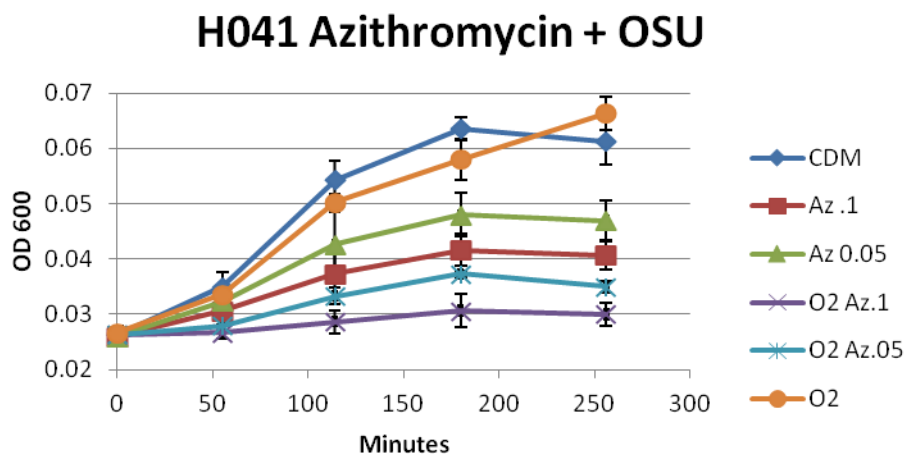
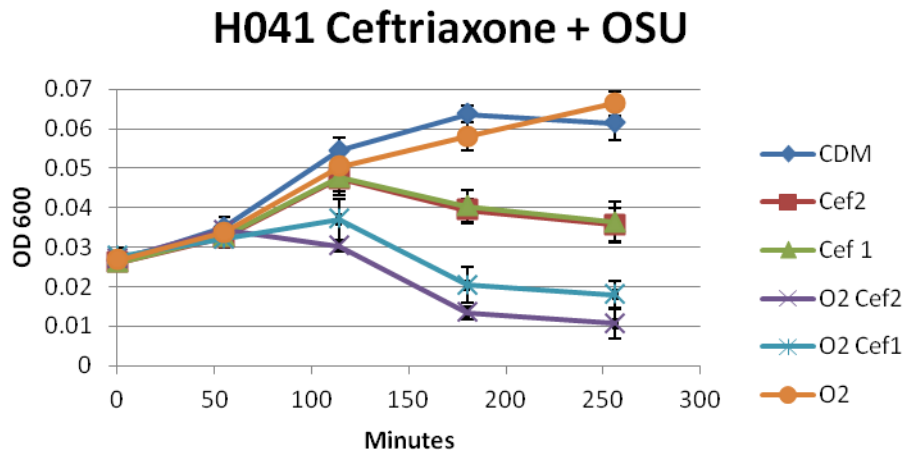


Figure 6.4. H041 Growth in the presence of OSU and conventional antibiotics

III. Discussion

The data presented here demonstrate that OSU alone has antimicrobial effects against lab strains and drug resistant clinical isolates of *N. gonorrhoeae*. These data support the hypothesis that OSU has antimicrobial effects at concentrations that are physiologically achievable in humans; however, the therapeutic window is narrow. OSU rarely achieved serum concentrations above 4 μM in human clinical trials (294), suggesting that microbial resistance to OSU would render the drug ineffective almost immediately. Several pairings were tested between OSU and currently used drugs to determine if synergistic effects exist that could decrease the amount of OSU necessary for antimicrobial effects, thus extending its therapeutic window. This line of inquiry had the potential to discover antimicrobial effects in drugs designed for other purposes, as well as resurrect the utility of certain antimicrobials that are no longer capable of eliminating gonococcal infection when used alone.

PDE-5 inhibitors were chosen for use with OSU based on observations that they enhanced effects of standard of care chemotherapy drugs in CNS, gastrointestinal, and genitourinary cancers (305, 306), and the observation that prokaryotic cells have cyclic di-GMP (307), which is regulated by phosphodiesterases (308). Indeed, cyclic di-GMP has been shown to be a regulator of bacterial virulence (309, 310), enhancing interest in it as an antimicrobial target. There does not appear to be any data in the literature that directly shows that PDE inhibitors affect bacterial phosphodiesterases, therefore, the potential of these drugs potential was tested.

Although PDE-5 inhibitors moderately attenuated gonococcal growth in the presence of OSU when applied to lab strains, they did not display the same effect in drug resistant strains. This suggests that PDE-5 inhibitors have very modest effects in bacteria. This result is not

surprising, given that PDE-5 inhibitors have mixed effects in the literature when applied to bacteria. One study found that non-specific phosphodiesterase inhibitors could increase sensitivity to gentamicin in several bacteria *in vitro*, but this effect was specifically not attributable to the PDE-5 inhibitor sildenafil, and could not be attributed to any PDE-specific inhibition that was tested (311). Another study in a mouse model of tuberculosis found that PDE-3 and 5 inhibition resulted in quicker clearance of infection, but PDE-4 inhibition led to accelerated time to death (312). Yet another study suggests that PDE-5 inhibitors actually reduced the activity of ciprofloxacin against a wide array of bacteria during disc diffusion assays (313). Given these varying results, it is hard to know what effects PDE inhibitors have on prokaryotic cells. There are possible confounders in that *in vitro* assays may yield different results than *in vivo* experiments based on host-pathogen interactions. Accordingly, further studies are warranted to fully investigate the role PDE inhibitors may have in antimicrobial therapy.

Although the combination of OSU and the PDE-5 inhibitors was not effective in drug resistant strains of *N. gonorrhoeae*, there was still potential for OSU to enhance the effects of conventional antimicrobial drugs. For these assays, drugs with three different mechanisms of action were chosen. Ceftriaxone, a 3rd generation cephalosporin, produces bactericidal effects by irreversibly inactivating bacterial transpeptidases responsible for cross-linking peptidoglycan in the cell wall (314). Without this repair mechanism, cells eventually lyse from osmotic pressure. Ciprofloxacin, a fluoroquinolone derivative, inhibits bacterial topoisomerases and promotes DNA cleavage, normally resulting in rapid cell killing (315, 316). Azithromycin, a macrolide antibiotic, and chloramphenicol possess different mechanisms of action; however

both inhibit ribosome function (317). Without the ability to synthesize new proteins, these drugs arrest cell function, leading to bacteriostatic effects. Combining these drugs, with their varying mechanisms, with OSU allowed for the determination of which, if any, mechanisms of cell disruption were able to produce synergistic effects.

The cell killing demonstrated with the combination of OSU and ceftriaxone was robust. Adding two separate bactericidal drugs together was likely to produce bactericidal effects. However, the ability for this combination to be lethal when used at individually sublethal doses showed that the two drugs had more than additive effects. One could speculate that because ceftriaxone inactivates transpeptidases, the resulting cell stress causes an increase in transpeptidase production. However, OSU likely prevents the proper folding of those additional proteins, allowing for a lower dose of ceftriaxone to successfully inhibit cell wall repair. In other words, OSU likely reduces the MIC for ceftriaxone, which until recently was the only remaining single agent therapy for gonococcal infection. Although ceftriaxone is still a part of the current gold standard therapy, this finding raised hope that combining OSU with discontinued antimicrobials might make them therapeutically relevant again.

Azithromycin, the other component of the current gold standard of gonococcal therapy, also had synergistic effects with OSU. However, the combination resulted in more attenuated growth, as opposed to cell death. These results suggest that OSU improves the effects of azithromycin treatment, effectively decreasing its MIC. However, the azithromycin assays left some questions about the effects of using two drugs that function in the same pathway, protein synthesis. Fortunately, some additional information can be gleaned from the chloramphenicol and OSU trials in the lab strain FA19. Because chloramphenicol was used as a bacteriostatic

control, it was applied at a concentration above the MIC. When OSU was applied in combination with chloramphenicol, there was no change in cell growth, it remained bacteriostatic. Given that OSU's effects lie in both the inability to form functional proteins, and in the cell stress derived from unfolded proteins, this result is not surprising. Completely shutting down protein synthesis leaves nothing for chaperones to fold, which completely abrogates the negative effects of OSU. Taken together, these data suggest that OSU can decrease the MIC for protein synthesis inhibitor antibiotics, but as protein synthesis is more inhibited, OSU's effects decrease. Although this combination could be used to extend the therapeutic window for protein synthesis inhibitors, the bactericidal effects of OSU are lost, suggesting that other combinations may be more effective.

Lastly, OSU was applied to gonococci in combination with ciprofloxacin, resulting in minimal synergy. This is unlike the synergy observed with ceftriaxone, which is also bactericidal. However, there is no mechanistic reason to assume these drugs would enhance each other, and H041 and F89 are particularly resistant to fluoroquinolones. It might have been possible for OSU to cause misfolded protein accumulation during ciprofloxacin induced growth attenuation, but that does not appear to have been the case. Alternatively, it could be possible that in addition to preventing DNA replication, ciprofloxacin inhibition of DNA gyrase may reduce rRNA and possibly mRNA production, resulting in decreases in protein synthesis (318-321). Were this to occur, OSU effects would be limited in the same way discussed with protein synthesis inhibitors above. Given these data, it is likely that ciprofloxacin treatment of gonococcal infection would not significantly be enhanced by the addition of OSU.

In summary, OSU has bactericidal activity against the gonococcus, and it also enhances the bactericidal effects of ceftriaxone and the bacteriostatic effects of azithromycin. However, OSU does not substantially affect the use of ciprofloxacin in killing gonococci. Future studies should test the effects of the addition of OSU to the combination of ceftriaxone and azithromycin. It is possible that OSU could enhance antimicrobial activity, but also possible that OSU's effects could be limited and azithromycin. Although OSU has initially shown promise in the treatment of *N. gonorrhoeae*, as well as other bacteria, parasites, and viruses, its narrow therapeutic window and issues with drug delivery (303) limit its potential to make it to pharmaceutical market. However, these data serve as an excellent proof of principal for targeting protein chaperones as novel drug targets, which certainly warrants further investigation.

Chapter 7: Summary and Perspectives

With an estimated 800,000 new cases and a financial burden approaching \$1 billion per year in the United States, coupled with the fact that the gonococcus does not elicit protective immunity, gonococcal infection is a serious public health concern (57, 322). Recent reports of increasingly drug resistant clinical isolates from around the world have heightened the severity of this public health issue, as the number of drugs we have left to treat this infection are dwindling (61-63). The mechanisms the gonococcus employs to resist the effects of antimicrobial drugs are summarized in Table 5. Indeed, there are legitimate concerns that we may be entering an era of untreatable gonococcal infection. This concern is reflected by the Centers for Disease Control and Preventions listing *Neisseria gonorrhoeae* as an urgent threat for anti-microbial resistance, making it one of three pathogens in this highest level category of threat.

In response to the rise of drug resistant *N. gonorrhoeae*, several new drugs have been evaluated in clinical trials for the treatment of gonococcal infection. The farthest along in testing is solithromycin, a fluoroketolide, which is currently in phase III trials. Solithromycin inhibits protein synthesis by binding the 50S ribosomal subunit, similar to macrolides (323). During *in vitro* testing against over 200 gonococcal isolates, there was no documented resistance to solithromycin, even in strains that are resistant to extended spectrum cephalosporins (ESCs) (324). The MIC required to inhibit 90% (MIC₉₀) of gonococcal strains was 0.25 µg/mL, similar to ceftriaxone, but MICs were reported up to 32 µg/mL.

Table 6. Summary of gonococcal drug resistance

Drug Class	Mechanisms of resistance (325)
Penicillin	Alteration of the penicillin binding proteins (PBPs) Plasmid mediated production of penicillinases Alterations in drug influx through porin Alterations in MtrCDE efflux pump expression
Cephalosporins	Mutations in PBPs Alterations in porin, mediating influx Alterations in MtrCDE efflux pump expression
Macrolides	Alterations in the 23S ribosomal subunit Alterations in the efflux pump expression
Fluoroquinolones	Amino acid mutations in topoisomerases II and IV
Tetracyclines	TetM, which removes tetracycline from the ribosome Alteration in target structure Alterations in porin, mediating influx Alterations in MtrCDE efflux pump expression

GSK2140944, a novel topoisomerase II inhibitor, is currently in phase II trials. This drug has a mode of action distinct from fluoroquinolones. GSK2140944 stabilizes the topoisomerase-DNA complex prior to DNA cleavage and generates single strand breaks, whereas fluoroquinolones stabilize double strand breaks after cleavage (326). In vitro data for this drug is not currently available. AZD0914, a spiropyrimidinetrione, is also in phase II trials for gonococcal infection. AZD0914 possesses a novel mechanism of action; it inhibits DNA biosynthesis and enhances accumulation of double-strand breaks. In a study of 250 gonococcal isolates, the MIC₉₀ for AZD0914 was 0.25 µg/mL (327). Strains with higher resistances were mapped to alterations in *gyrB*, further distinguishing this drug from fluoroquinolones, to which resistance emerges by alterations to *gyrA* (328). A fourth drug, delafloxacin, a fluoroquinolone, was until recently also in phase III trials. Unfortunately, that trial was halted due to an interim review that suggested that delafloxacin may not be sufficient to adequately treat patients with gonococcal infection (325). The results of these clinical trials will be eagerly anticipated, but it is important to note that one of the selection criteria for patients is uncomplicated gonorrhoea. It will take time to determine how these drugs perform in more complex cases, and if they will be active against highly drug resistant strains *in vivo*.

In addition to these drugs which have entered clinical trials, there are several more with *in vitro* data suggesting they may be viable therapies. However, most of the drugs are from classes to which the gonococcus has already started to develop resistance (325). New drugs from old drug classes could have the potential for the development of rapid resistance to these drugs, suggesting they will not be clinically useful for a long span of time. Although there is value in extending our treatment options in the short term, there is still a need for the discovery of

novel drug targets, and ultimately, a preventative vaccine. The studies described here were performed in an effort to probe new targets for antimicrobial and vaccine development.

TonB dependent transporters have several criteria that make them ideal drug and vaccine targets: they are virulence factors known to contribute to gonococcal pathogenesis, they are not subject to antigenic variation, and they are essential for iron and possibly other metal acquisition. Given their therapeutic potential, we wanted to further investigate TbpA. This study began with attempts to reproduce preliminary evidence that antibodies developed against TbpA surface exposed loops were capable of substantially inhibiting TbpA-Tf interaction (190). We obtained the antibodies from the preliminary study, and also attempted to make optimized loop antibodies. Despite promising results in recombinantly expressed TbpA, we were unable to produce similar antibody mediated inhibition of the TbpA-Tf interaction on the surface of the gonococcus. These data suggest that small peptide fragments, at least from TbpA, are unlikely to be successful vaccine candidates. We also probed the structure function relationship of the TbpA loop 3 helix, in efforts to validate the hypothesis that it was a key motif involved in iron release from Tf. Although there was no single residue responsible for iron release from Tf, our data suggests that the loop 3 helix, as a whole, is critical for Tf binding and iron uptake.

Our studies demonstrate that more complex approaches will be needed in order to develop an efficacious, protective vaccine against the Tbps. Despite the fact that the Tbps elicit minimal antibodies during natural infection (262), studies have shown that when applied as immunogens in animals, the Tbps are capable of eliciting immune responses that are bactericidal and cross protective (232, 237). Future steps towards developing a Tbp vaccine

formulation may require inactivation of ligand binding functions of TbpA, as recent work has shown that decreasing host ligand binding by bacterial proteins increases the effectiveness of the immune response. The proposed mechanism is that naked bacterial proteins expose more epitopes to immune cells than ligand bound proteins, allowing antibodies to be more effective at blocking epitopes that would exist between the ligand and receptor. In support of this hypothesis, Beernink et al. demonstrated that ligand-binding deficient fHbp from *N. meningitidis* was as immunogenic as the wild-type protein but produced higher serum bactericidal activity (SBA) titers when introduced to transgenic mice expressing human fH (45). Rossi et al. went on to investigate sub-family A fHbp, which has two distinct point mutations that decrease fH binding. Immunization with this protein led to significantly higher IgG titers and SBA responses in human fH transgenic mice (329). The phenomenon of ligand binding resulting in decreased immunogenicity was also seen to a lesser degree in rhesus macaques (330). Recently, similar results were described for TbpB in *Haemophilus parasuis* (259), suggesting this approach may be applicable for the development of a gonococcal vaccine. These findings suggest that ongoing structure-function analyses will be critical, in order to determine how to minimally alter protein residues to reduce binding while still maintaining a native overall protein structure.

In order to further the study of TdTs and their potential use in a cocktail vaccine, we developed a platform to characterize the structure function relationship of the Hb receptor protein, HpuA. The rationale for this project is similar to the TbpA structure function project: to characterize and exploit iron uptake mechanisms that allow for gonococcal survival during exposure to host nutritional immunity. HpuA and HpuB are not as well studied as some of the

other TdTs, so there are unanswered questions about how the gonococcus utilizes Hb as an iron source. In addition, although the Hb receptor is phase variable, HpuA and HpuB may still be helpful additions to a potential cocktail vaccine. Further structure-function analyses, based on the recent report of the HpuA crystal structure (275), would aid the approach of trying to develop non-ligand binding versions of the proteins, which may improve their immunogenicity. It is also possible that expression of the Hb receptor could undergo a compensatory increase in instances where other iron acquisition systems are impaired, as could be the case if the Tf receptor is effectively inhibited by antibodies. Beyond gonococcal infection, studies of the HpuAB system would have implications for other pathogens known to express the Hpus to use Hb as an iron source during infection. *Neisseria meningitidis*, along with *Kingella* and *Eikenella* species (members of the HACEK group of endocarditis causing organisms) also express HpuA and HpuB for acquisition of Hb-iron in the blood (275). Although there are some differences in sequence and loop structures among species, investigation of the HpuAB receptor of *N. gonorrhoeae* is likely to yield information that will have meaningful overlap across species.

In order to impact gonococcal growth in the acute setting, we developed and tested novel small molecules that could interrupt TbpA Tf-iron acquisition. Given that iron is an essential nutrient, Tf is the primary source of iron *in vivo*, and that we now have the crystal structure of TbpA (190) we hypothesized that these molecules could have antimicrobial effects on gonococcal growth. The molecules were designed to mimic the structure of human Tf in specific binding pockets of TbpA, with the hope that they could compete for TbpA binding. During whole cell Tf binding assays, we have observed modest preliminary inhibition of TbpA-Tf binding. In future studies, the compounds with modest effects could be structurally refined in

attempts to increase TbpA-Tf binding inhibition. These assays constitute a novel approach to antimicrobial development for the gonococcus. Because there are redundant iron acquisition systems in the gonococcus, this model can and should be applied to multiple receptors systems in order to maximize iron starvation.

In our last project, we departed from the TdTs to test a novel antimicrobial target, DnaK. The novel chemotherapy agent OSU-03012, a derivative of celecoxib, had promising results in initial cancer studies (287, 294). When it was determined that its mechanism of action was inhibition of protein folding by Hsp70 family chaperones (293, 294), it was hypothesized that this drug might have antimicrobial properties through inhibition of the homologous prokaryotic protein, DnaK. We determined that OSU was bactericidal in gonococcal culture, even against drug resistant gonococcal strains, at physiologically achievable doses. We were unable to demonstrate synergy between OSU and PDE-5 inhibitors; however, OSU was synergistic with cephalosporin and macrolide class antibiotics. Although the therapeutic window for OSU is small, these data provide proof of principle that DnaK is a novel antimicrobial target. Further studies are needed to determine if OSU-03012 can be structurally modified to improve its pharmacodynamic properties. OSU's activity extends beyond the gonococcus, as it has activity against many other classes of bacteria, and also possesses antiviral properties (303). Additional studies are needed to explore DnaK as a drug target, the spectrum of coverage that DnaK inhibition would provide, and whether OSU specificity could be enhanced to affect prokaryotic chaperones without affecting human proteins. Despite lingering questions, these preliminary findings suggest DnaK may be a novel target for broad spectrum antimicrobial therapy, which could have enormous impact on clinical practice.

In sum, the data presented in this study has yielded new information for structure function understanding of TdTs, for the development of a TdT based vaccine, and for novel gonococcal drug targets. Understanding of the structure-function relationships in the TdTs will assist in determining which domains are critical for protein function, and which residues could be altered to optimize these proteins for vaccine development. The TdTs continue to stand out as ideal targets for vaccine and drug development, as they are surface exposed, widely conserved across gonococcal strains, and are not subject to high frequency phase or antigenic variation. Additionally, these studies have provided new insights into a novel antimicrobial target, DnaK, which is widely conserved across bacterial species. These findings have opened new avenues of investigation to further develop protective and therapeutic treatment options for gonococcal infection, which has and will continue to be a major public health concern.

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Vita

Devin Cash was born on June 30, 1987 in Lynchburg, VA. He graduated from Holy Cross Regional Catholic School, Lynchburg, VA in 2005. In 2009, Devin received his Bachelor of Science in Biological Chemistry from the University of Virginia, Charlottesville, VA. He matriculated in the Virginia Commonwealth University School of Medicine in the fall of 2010, and into the VCU M.D.-Ph.D. program in the fall of 2011. His accomplishments and publications are listed below.

FELLOWSHIPS

F30 Fellowship Award #1F30AI112199-01, 2014-2019

National Institute for Allergy and Infectious Disease (NIAID)

AWARDS

Charles C. Clayton Award, 2015

Virginia Commonwealth University; Dept. of Microbiology and Immunology

Travel Award, 2015

Mid-Atlantic Microbial Pathogenesis Meeting (MAMPM)

Aesculapian Scholarship, 2010

Virginia Commonwealth University School of Medicine

PRESENTATIONS

Poster:

Contribution of the TbpA loop 3 helix to transferrin-iron acquisition by *Neisseria gonorrhoeae*.

Cash D, Noinaj N, Buchanan S, Cornelissen CN. Presented at:

- XIXth International Conference for Pathogenic Neisseria. Asheville, NC. October 13, 2014
- Mid-Atlantic Microbial Pathogenesis Meeting. Wintergreen, VA. January, 2015

Role of the 5' untranslated region of the algD gene in alginate gene regulation in *Pseudomonas aeruginosa*. **Cash D**, Ohman D. VCU MIDPH poster presentation. Richmond, VA. July 29, 2011

PUBLICATIONS

1. **Cash, DR**, Noinaj, N, Buchanan, SK, Cornelissen, CN. 2015. Beyond the Crystal Structure: Insight into the Function and Vaccine Potential of TbpA Expressed by *Neisseria gonorrhoeae*. *Infection and Immunity*. 83:4438-4449.
2. Booth, L, Roberts, JL, **Cash, DR**, Tavallai, S, Jean, S, Fidanza, A, Cruz-Luna, T, Siembiba, P, Cycon, KA, Cornelissen, CN, Dent, P. 2015. GRP78/BiP/HSPA5/DnaK is a universal therapeutic target for human disease. *J. Cell. Physiol*. 230:1661-1676.
3. Leslie DC, Melnikoff BA, Marchiarullo DJ, **Cash DR**, Ferrance JP, Landers JP. A simple method for the evaluation of microfluidic architecture using flow quantitation via a multiplexed fluidic resistance measurement. *Lab Chip*. 2010 Aug 7;10(15):1960-6.
4. Kanbar R, Stornetta RL, **Cash DR**, Lewis SJ, Guyenet PG. Photostimulation of Phox2b medullary neurons activates cardiorespiratory function in conscious rats. *Am J Respir Crit Care Med*. 2010 Nov 1;182(9):1184-94.